

Amino Acid Metabolism

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Third Edition

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Preface

When Antoine Lavoisier discovered nitrogen in 1787, he named it *azote*, meaning without life, because of its lack of chemical reactivity and its inability to support life when provided as the atmosphere for experimental animals. However, the metabolism of nitrogenous compounds is central to the metabolic processes of all living organisms. On one level, understanding of the pathways of amino acid metabolism and their regulation is fascinating ‘because they are there’, and they present an intellectual challenge to biochemists, molecular biologists and other biological scientists.

We can also justify research to further our knowledge and understanding of the pathways and their regulation for their importance in human nutrition, both in human and animal health and disease, and also commercially. Several hundred tonnes of each amino acid are manufactured each year by bacterial biosynthesis for use in pharmaceuticals, foodstuffs and nutritional supplements. Selective breeding and genetic modification of plants permits the development of food crops with higher yields of essential amino acids (and especially methionine and lysine, which are limiting in most food crops). Enzymes in the pathways in microorganisms for the biosynthesis of amino acids that are dietary essentials for mammals provide targets for antibacterial, antifungal and antiparasite medication. In plants, enzymes in these pathways provide targets for herbicides that will have little or no effect on human beings and other mammals.

It is more than a quarter of a century since the last edition of this book was published. In that time, there have been major advances in the molecular biosciences that have increased our knowledge and understanding of amino acid metabolism considerably. Structural biology has advanced to the extent that, in many cases, we can effectively sit at the catalytic site of an enzyme and watch the stages in the reaction as different amino acid side-chains in the enzyme donate or remove electrons or form free radicals to catalyze the reaction. We can now visualize the conformational and other changes

associated with binding of inhibitors and activators of the enzymes, and also the movement of intermediates through intra-molecular tunnels between one catalytic site of an enzyme and another.

Molecular biology has given us complete genome sequences of many organisms, allowing genes that are homologues of known enzymes to be identified in other organisms. Gene cloning and over-expression, as well as genetic knockout techniques, have allowed us to study the function and regulation of enzymes and pathways. Metabolomic techniques have permitted us to investigate the effects of changes in the activity of individual enzymes on a wide range of metabolites – a far cry from the days when we measured only a limited number of compounds by (often laborious) manual analytical techniques.

The pace and excitement of research on amino acid metabolism is reflected in the many specialist conferences and workshops that are now held. Some concentrate on a single amino acid; others have a broader remit. My students have frequently been surprised, and even amused, by my attendance at the meetings of the International Society for Tryptophan Research. They wonder how it is that a hundred or more apparently sane people can talk about just one amino acid for three or four days at a time, every third or fourth year. The answer is that, for all we know, there remain many areas of amino acid metabolism that are not yet clear. Indeed, three apparently simple questions remain unanswered and cause considerable debate: how much protein does a human being need, to what extent is dietary protein digested, and how much of each essential amino acid is required in the diet? An international symposium on *Dietary Protein for Human Health*, followed by a United Nations expert consultation held in New Zealand in April 2011, failed to answer these fundamental questions.

This book is on a specialized area of biochemistry, and I have assumed that the reader will have an understanding of the principles of enzymology, metabolism and cell, molecular and structural biology equivalent to that achieved at the end of the second year of a UK BSc course in biochemistry, nutrition or medical bioscience. There are many excellent text books on general biochemistry, and a number of excellent dictionaries of biochemistry and molecular biology. A very useful online dictionary is published by the Biochemical Society at <http://www.portlandpress.com/pp/books/online/glick/default.htm>.

An advance since the last edition of this book was published that is more to the benefit of the author than the reader is the advent of the online library. No longer do I have to delve among the library stacks to find relevant papers and carry round weighty (and often dusty) volumes. They are all available to me electronically, from the comfort of my desk. I have cited more than a thousand references in the bibliography, and I have probably read five times that many papers in preparing this book – and without physically setting foot in the library! In general, I have cited reviews rather than primary research

papers, because these are more likely to be useful to students and will, in turn, lead them into the primary research literature. To those colleagues whose papers I have not cited, I apologize for any unintended insult. I may well have read your papers and found them helpful to my thinking, but perhaps less potentially useful to readers than those papers that I have cited.

December 2011

David A Bender

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.

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.

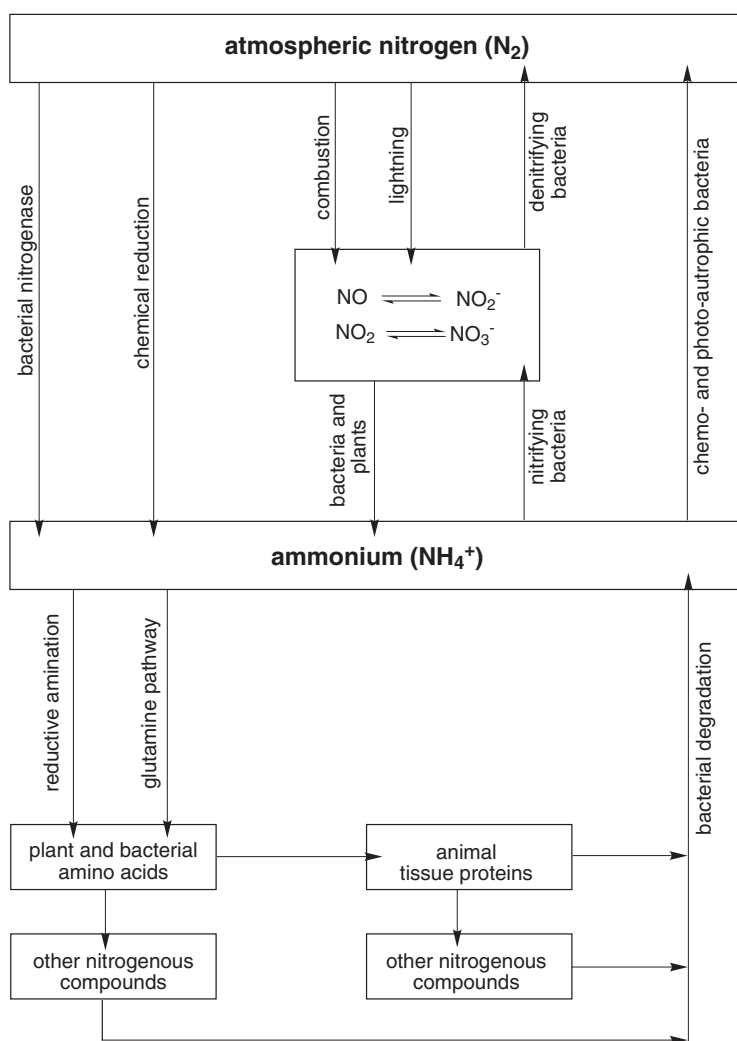


Figure 1.1 The nitrogen cycle.

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.

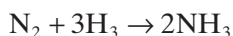
The burning of fossil fuels and biomass accounts for release into the atmosphere of some 20×10^6 tonnes of nitrogen oxides each year, and lightning probably produces about half as much. It is estimated that terrestrial ecosystems produced $90\text{--}140 \times 10^6$ tonnes of fixed nitrogen a year prior to human activity and that widespread cultivation of legume crops has added $32\text{--}55 \times 10^6$ tonnes of fixed nitrogen per year. Marine ecosystems are estimated to fix $30\text{--}300 \times 10^6$ 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 $\text{N} \equiv \text{N}$ 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 $300\text{--}550^\circ\text{C}$ and pressures of 15–25 MPa (150–250 atm), with an iron catalyst, to reduce nitrogen with hydrogen gas to form ammonia:



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

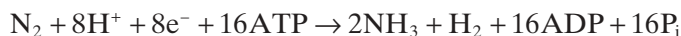
Some wood-eating insects (e.g. termites) and molluscs (e.g. the shipworm, *Teredo* spp.) have symbiotic diazotrophic bacteria that may make a significant contribution to the host's nitrogen nutrition. Commensal bacteria in ruminants fix nitrogen, but there is no evidence that non-ruminant mammals (including 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 $16 \times$ 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:



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

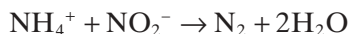
A variety of nitrite-oxidizing microorganisms oxidize nitrite to nitrate (NO_3^-), which is then a substrate for denitrification. Many microorganisms and fungi use nitrate and nitrite as terminal electron acceptors, forming nitric oxide, nitrous oxide and then nitrogen:



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:



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 $10\mu\text{mol/l}$ to 100mmol/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 $1 \times \text{ATP}$ 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).

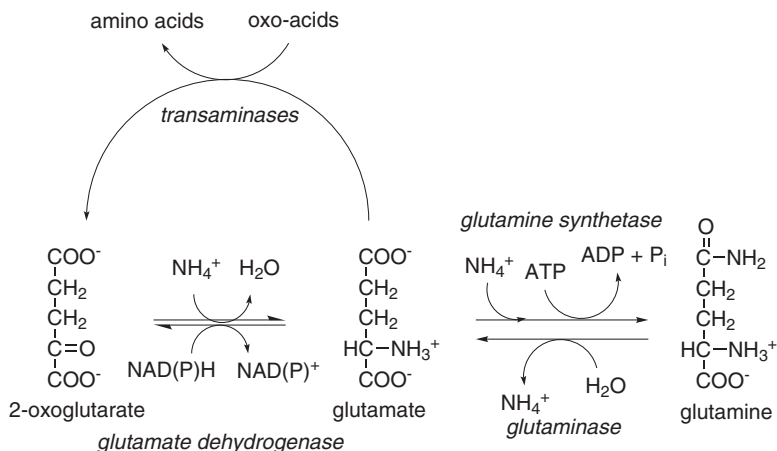


Figure 1.2 Incorporation of ammonia into glutamate and glutamine. Glutamate dehydrogenase EC 1.4.1.2 (NAD-linked), EC 1.4.1.4 (NADP-linked), EC 1.4.1.3 (linked to either NAD or NADP), glutamine synthetase EC 6.3.1.2, glutaminase EC 3.5.1.2.

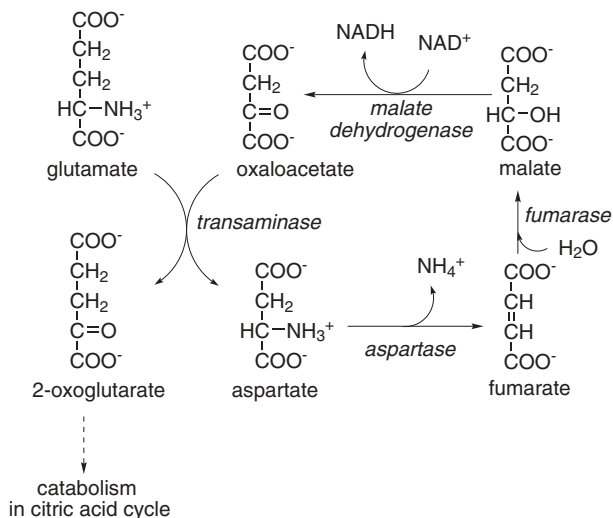


Figure 1.3 The catabolism of glutamate. Glutamate-oxaloacetate transaminase EC 2.6.1.1, aspartase (aspartate ammonia lyase) EC 4.3.1.1, fumarase EC 4.2.1.2, malate dehydrogenase EC 1.1.1.37.

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 deamination 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 $\mu\text{mol/l}$ up to 80–100 $\mu\text{mol/l}$, 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 $\mu\text{mol/l}$, 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

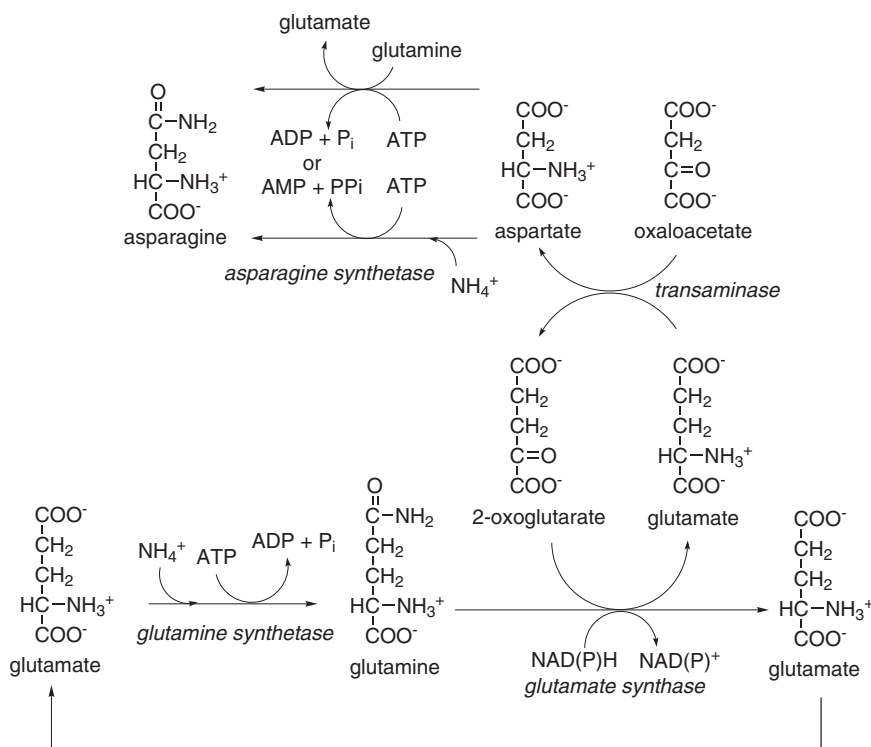


Figure 1.4 The synthesis of glutamine and asparagine.

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 utilising).

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 sulphhydryl 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).

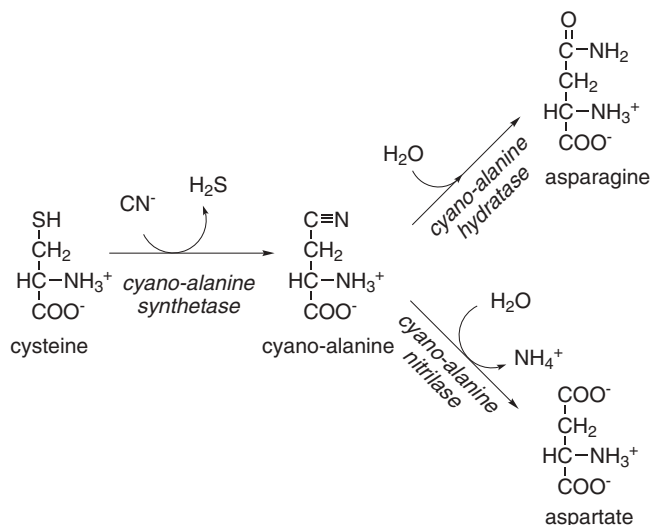


Figure 1.5 The role of cyanide in nitrogen incorporation.

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 anti-viral 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).

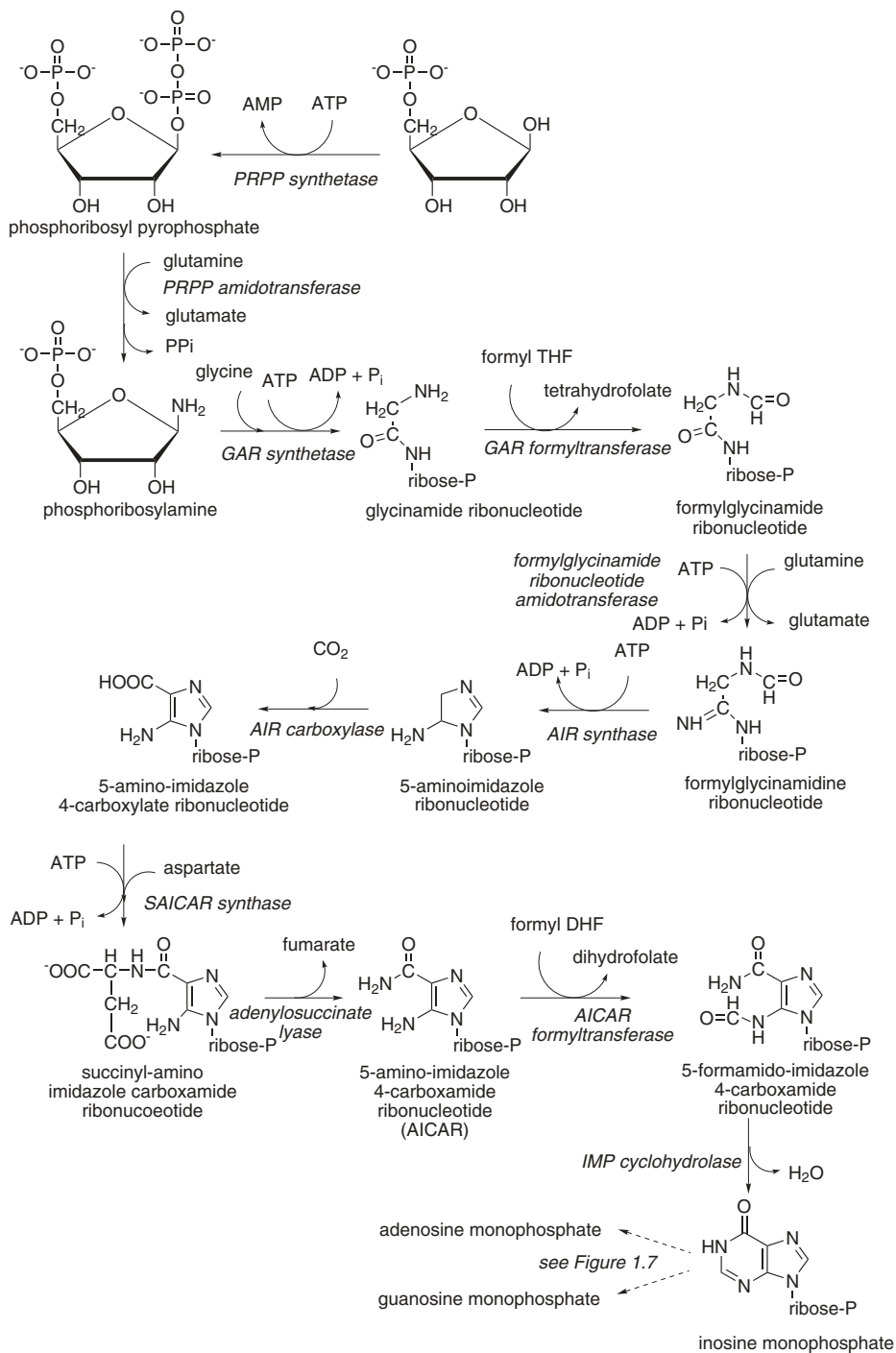
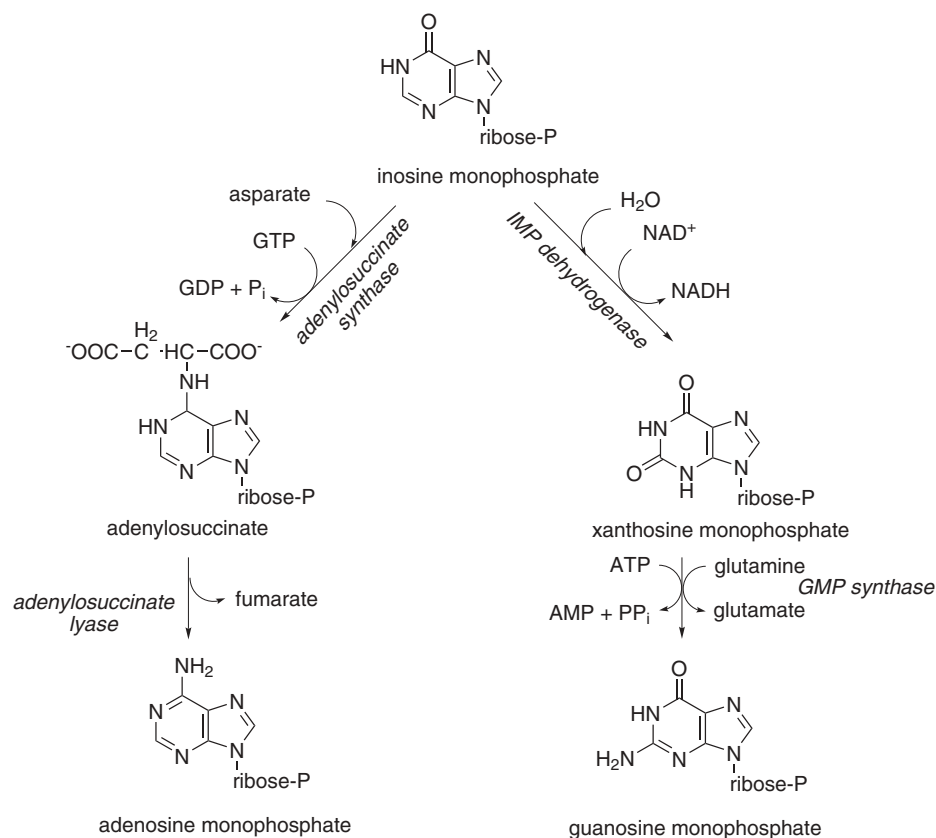


Figure 1.6 Purine synthesis.

AICAR = aminoimidazole carboxamide ribonucleotide, AIR = aminoimidazole ribonucleotide, GAR = glycineamide ribonucleotide, THF = tetrahydrofolate; phosphoribosylpyrophosphate (PRPP) synthetase EC 2.7.6.1, PRPP amidotransferase EC 2.4.2.14, glycineamide 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, N⁵-carboxyaminoimidazole ribonucleotide synthetase EC 6.3.4.18, N⁵-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, diaz-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 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	<i>de novo</i> 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 B₁). 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 (Koenigsnecht *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 $\mu\text{mol/l}$, compared with a normal intracellular concentration of 2–30 $\mu\text{mol/l}$.

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 $\mu\text{mol/l}$. 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 $5'\text{AMP}$ 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.

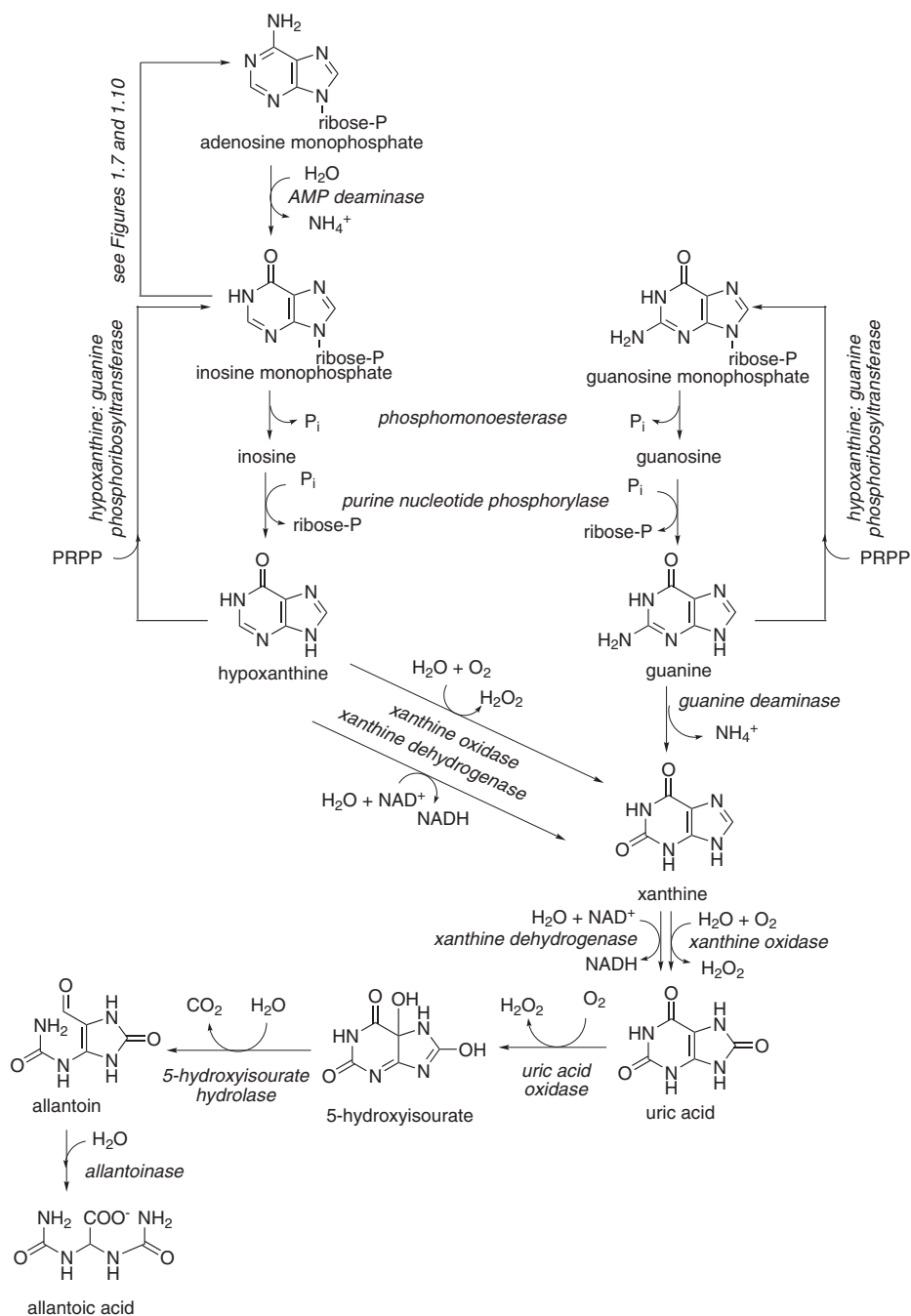
When lysosomes are disrupted during tissue homogenization, almost all of the enzyme is recovered as the oxidase, with little or no dehydrogenase activity detectable. The dehydrogenase form of the enzyme reacts rapidly with NAD^+ and only slowly with oxygen, while the oxidase form reacts rapidly with 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

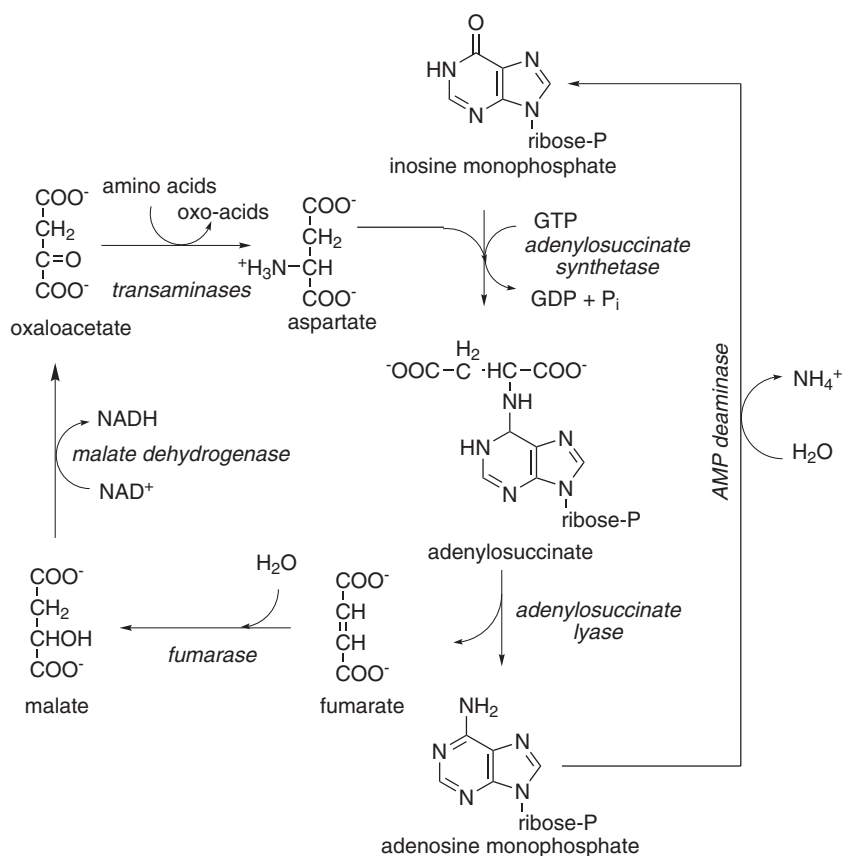


Figure 1.9 AMP deaminase as a source of ammonia.

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

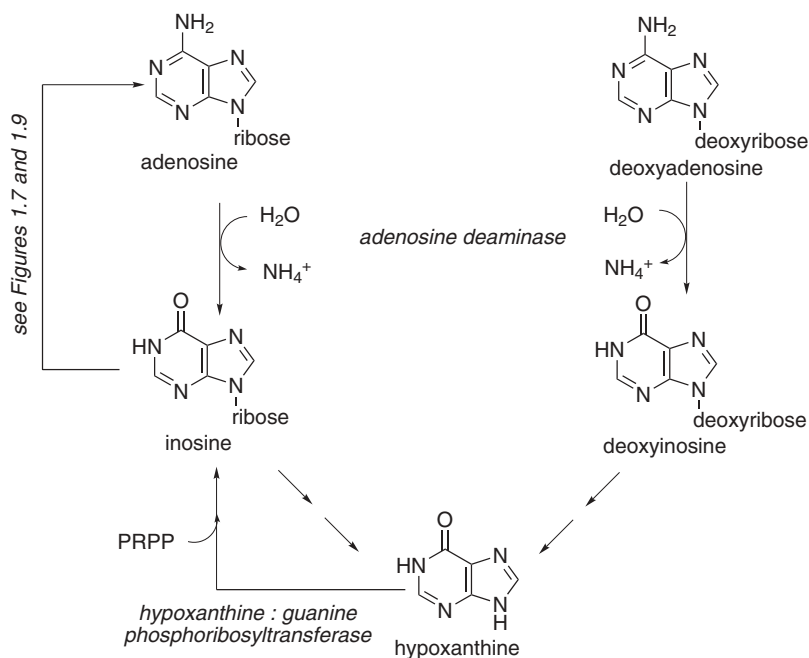


Figure 1.10 Purine salvage.

Adenosine deaminase EC 3.5.4.4, hypoxanthine : guanine phosphoribosyltransferase EC 2.4.2.8.

crystallization. Alternative 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).

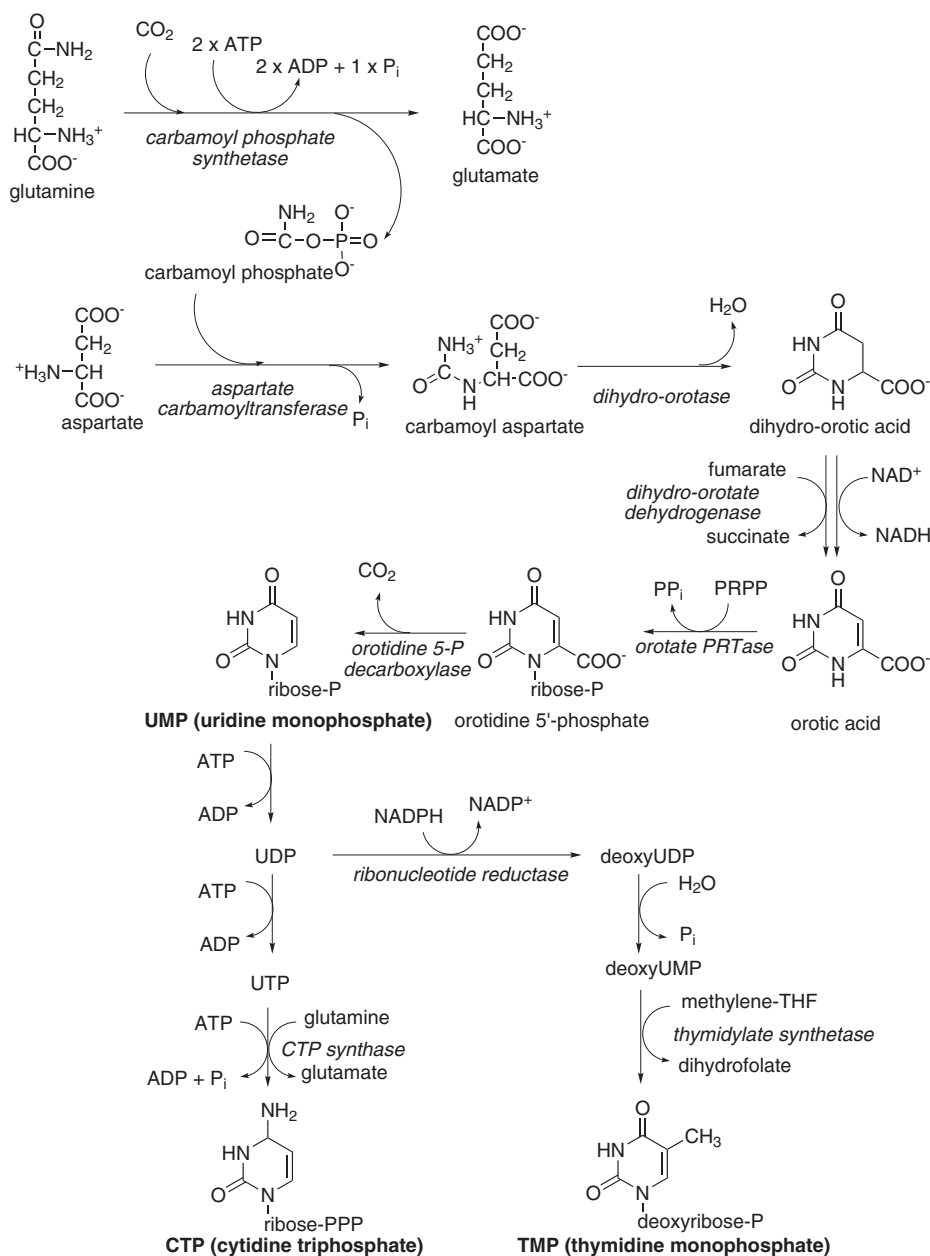


Figure 1.11 Pyrimidine synthesis.

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 carbamoyltransferase 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 serine¹⁰⁴⁶ decreases sensitivity to activation by PRPP, while the result of MAP kinase phosphorylation on threonine⁴⁵⁶ 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 B₁₂, 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 acidemia 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 B₁₂-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).

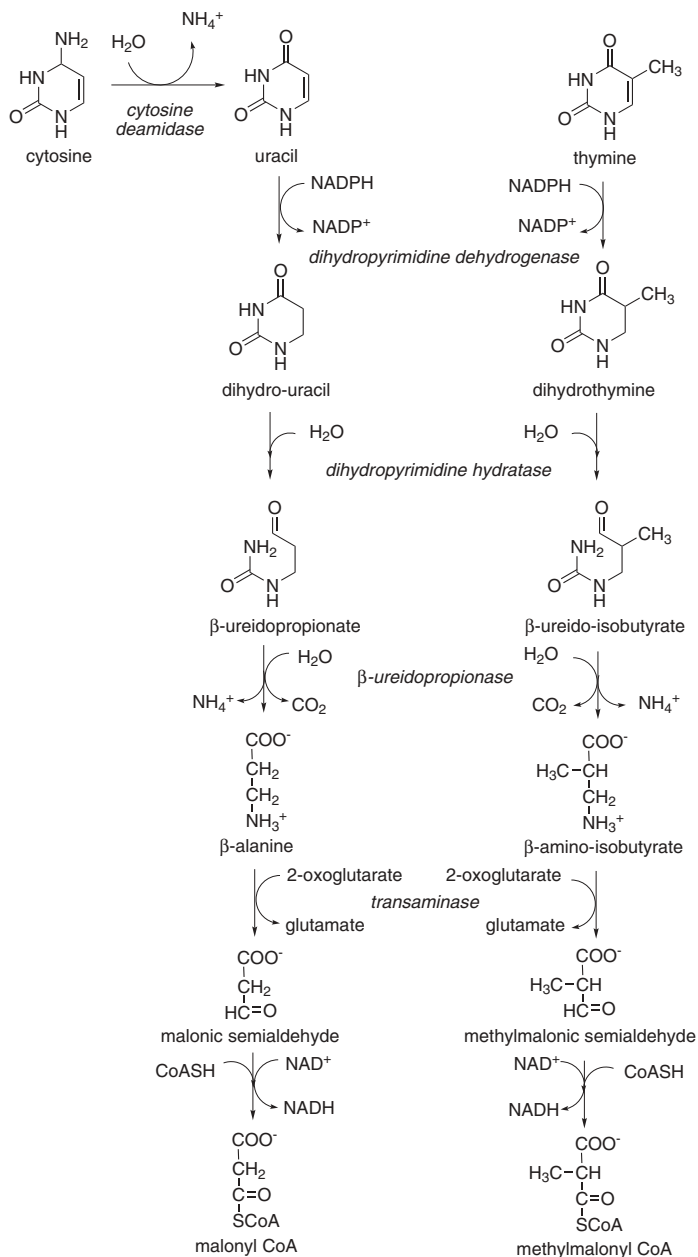


Figure 1.12 Pyrimidine catabolism.

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 $\approx 2.5 \times \text{ATP}$ 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 $\approx 1.5 \times \text{ATP}$; there is a gain of NADH (equivalent to $\approx 2.5 \times \text{ATP}$ 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 $1 \times \text{ATP}$ 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

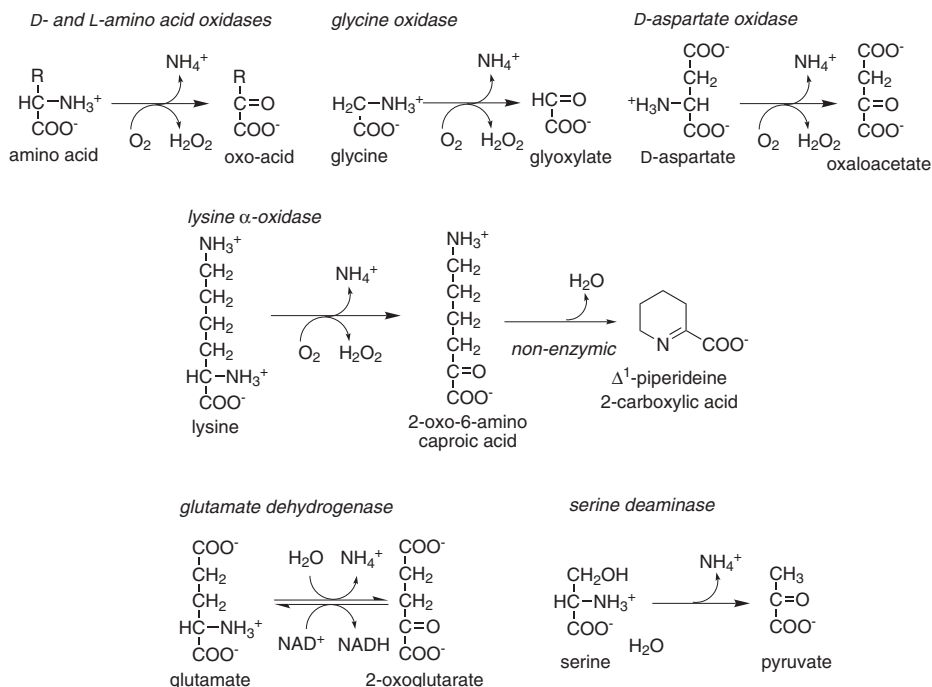


Figure 1.13 Deamination of amino acids.

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, H₂. 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 B₆ 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

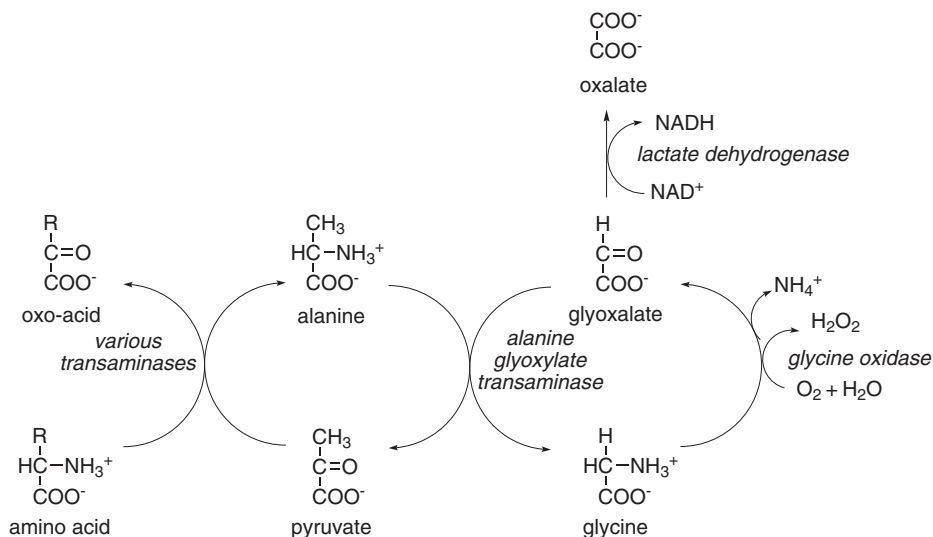


Figure 1.14 Transdeamination – transamination linked to glycine oxidase. Glycine oxidase EC 1.4.3.19, lactate dehydrogenase EC 1.1.1.27.

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

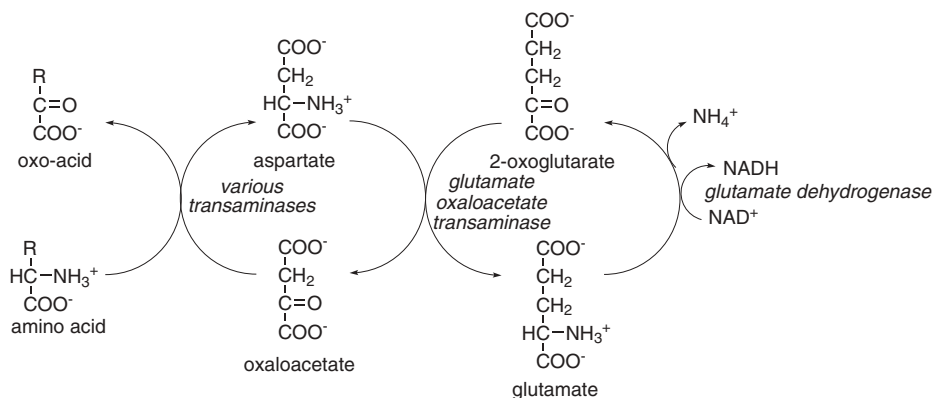


Figure 1.15 Transdeamination – transamination linked to glutamate dehydrogenase. Glutamate dehydrogenase EC 1.4.1.2, glutamate-oxaloacetate transaminase EC 2.6.1.1.

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

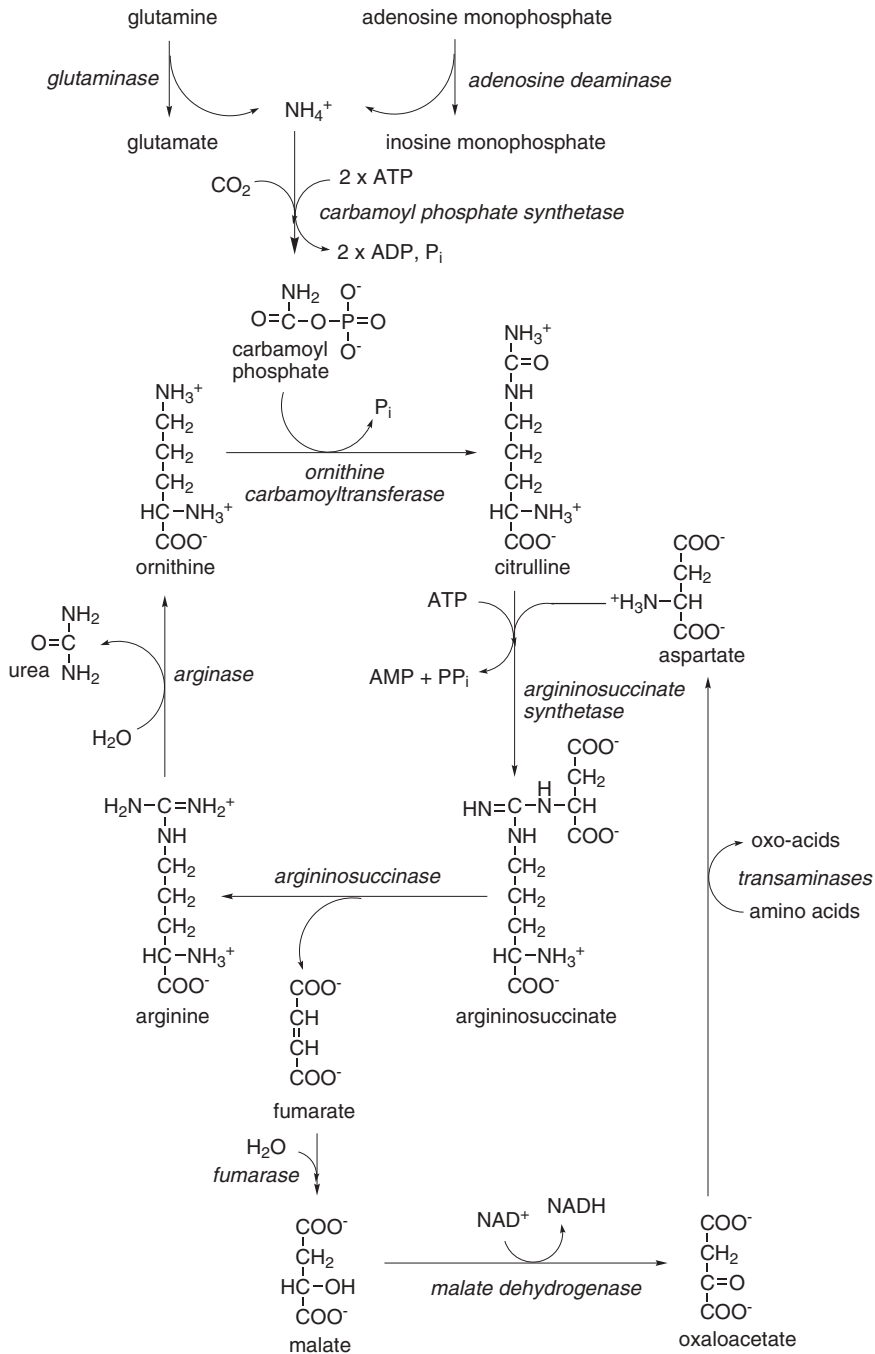


Figure 1.16 The urea synthesis cycle.

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 $^{14}\text{CO}_2$ 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 than 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 immuno-reactive 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 $1 \times \text{NADH}$ 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 metallo-enzymes. 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 acidemia, 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 (Regoezci *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

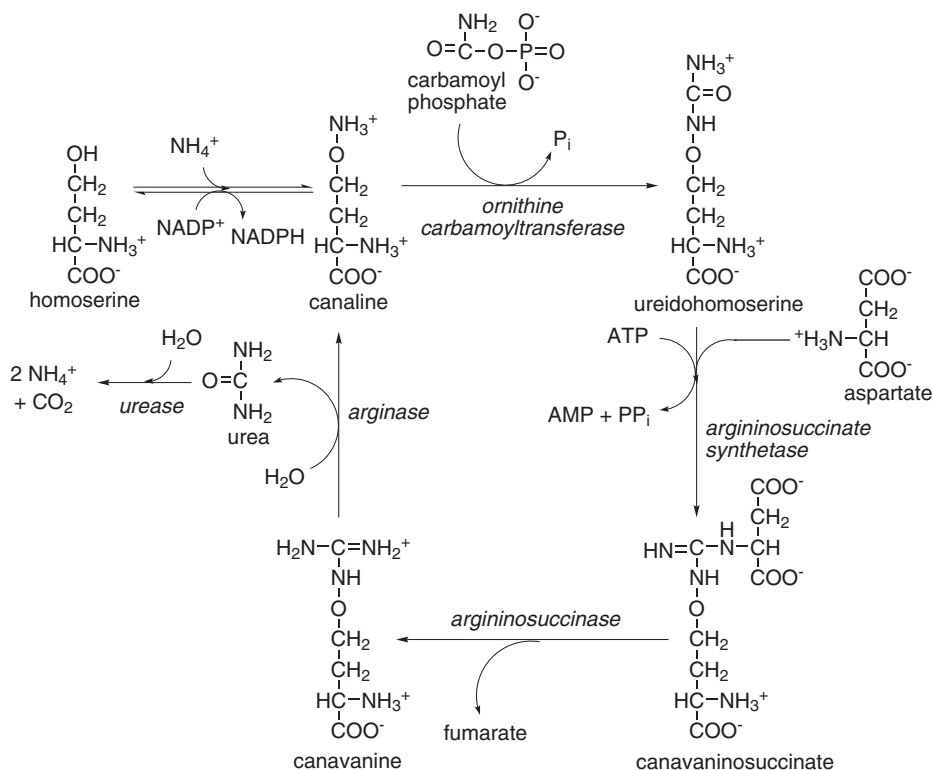


Figure 1.17 The metabolism of canavanine.

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	$\frac{1}{3}$ as free amino acids $\frac{1}{3}$ as small peptides $\frac{1}{3}$ 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 μ mol

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 μ mol/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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2

Nitrogen Balance and Protein Turnover – Protein and Amino Acids in Human Nutrition

2.1 Nitrogen balance and protein requirements

The method that is still used today for measurement of total nitrogenous compounds in biological samples was developed in the 1883 by Johan Kjeldahl. The sample is 'digested' with concentrated sulphuric acid, in the presence of a mercury or selenium catalyst, causing almost all nitrogenous compounds within it to be reduced to ammonium salts. The sample is then made strongly alkaline and the ammonia is steam-distilled into an acid solution and measured by back-titration with an alkali. For most foods, $\text{mg nitrogen} \times 6.25$ gives the protein content, since on average proteins contain 16 per cent nitrogen, although there are different conversion factors for some foods. Nitrogen balance is measured as the difference between dietary intake of nitrogenous compounds (mainly protein, but also nucleic acids and small amounts of other compounds) and the losses of nitrogen from the body in urine and faeces (and sometimes also sweat, shed skin cells and hair growth).

For a healthy adult animal or human being, the intake and excretion of nitrogenous compounds should be equal – the state of nitrogen balance or equilibrium. There is no gain or loss of the total body protein. In young animals, the output of nitrogenous compounds is less than the intake. There is retention of nitrogenous compounds in the body, mainly as an increase in total body protein. This is positive nitrogen balance, which is normal in growth, pregnancy and recovery from tissue protein loss.

By contrast, negative nitrogen balance, where the losses of nitrogenous compounds from the body are greater than the dietary intake, so that there

is a net loss of body protein, is never normal. It indicates either a pathological process or the fact that the intake of protein is inadequate to meet requirements. Even when animals or humans are fed on a protein-free diet, there is still loss of nitrogenous compounds in urine and faeces; this is the so-called obligatory endogenous loss of nitrogen, equivalent to some 35 g of protein per day in an adult human being.

Measurement of nitrogen balance thus provides a way of determining protein requirements. Groups of people are maintained on varying intakes of protein (with time to adapt to each level of intake) until the intake at which they can just maintain nitrogen balance is found. At any intake below this, they are unable to maintain nitrogen balance and they lose more nitrogen than their intake. However, if they are subsequently fed more protein than they require, then, once any losses from a period of negative nitrogen balance have been replaced (a period during which they are in positive nitrogen balance), they maintain nitrogen equilibrium with an increase in the excretion of nitrogenous compounds to match the increased intake. Obviously, children and pregnant and lactating women have a higher requirement to meet the need for net deposition of tissue protein and the secretion of milk proteins.

Table 2.1 shows the sources of nitrogen losses from the body. While most is lost as a variety of metabolites in urine (Table 1.5), faecal losses of undigested dietary protein, mucus and other gastro-intestinal secretions and bacterial biomass account for about 12 per cent of total losses.

Combining the results of published studies of nitrogen balance in which three or more levels of protein intake were used gives an estimated average requirement for healthy adults of 105 mg nitrogen (equivalent to 0.65 g of protein)/kg body weight/day to achieve nitrogen equilibrium. The 97.5th centile of the observed requirements to maintain nitrogen equilibrium is 12 mg nitrogen (equivalent to 0.83 g protein)/kg body weight/day. This is set as the reference or recommended intake, also known as the safe level of intake, meaning one that is safe and adequate to prevent deficiency – it does not imply that higher levels of intake are unsafe.

Table 2.1 Nitrogen losses from the body.

	mg N/day	% of total
Urine	10,000	84.4
Faeces	1,500	12.7
Skin cells and sweat	200	1.7
Hair and nails	30	0.25
Saliva	30	0.25
Breath	50	0.4
Miscellaneous	<30	<0.25

Source: from data reported by Calloway & Margen (1971).

This is a level of intake greater than the requirement of almost everyone in the population. For a 70 kg adult, this safe level of intake would translate to some 58 g of protein per day, compared with average intakes in developed countries of the order of 80–100 g/day (Rand *et al.*, 2003). This safe level of protein intake was adopted in the most recent WHO report on protein requirements (WHO, 2007). It represents a 10 per cent increase in the previously accepted safe level and it suggests that, in developing countries, more people have a marginal protein intake than was thought previously.

An alternative way of looking at protein requirements is to calculate the percentage of dietary energy coming from protein. If total energy intake is too low, amino acids will be used as metabolic fuel and for gluconeogenesis. A number of studies have shown that increased dietary energy intake (and especially carbohydrate) has a protein-sparing effect and permits increased nitrogen retention at levels of protein intake that would otherwise seem inadequate to permit nitrogen balance.

Protein provides 4 kcal (17 kJ)/g. Assuming a 'standard' 2,000 kcal (8.5 MJ) diet, the safe level of protein intake represents about 12 per cent of energy intake, while typical Western diets provide 14–15 per cent of energy from protein. In developing countries, it is likely that the poorest diets will provide about 11–12 per cent of energy from protein, which is marginally adequate. The main problem is not specifically a lack of dietary protein, but a lack of total food.

Although simple in concept, the interpretation of nitrogen balance studies to estimate protein requirements raises a number of problems. There is adaptation to habitual protein intake, so the time allowed for the experimental subjects to adapt to each level of protein intake is important. Short-term studies suggest a higher requirement than those in which subjects were fed on each diet for more than two weeks, and full adaptation may require more time than is feasible in most studies. It is easy to measure nitrogen losses in urine and faeces, but determination of losses in sweat and other body fluids, shed skin cells and hair growth (Table 2.1) is less easy. Underestimating these losses will give a falsely low estimate of protein requirements.

Finally, there is the problem of determining the precise level of intake at which nitrogen balance is just achieved; this may be by extrapolation from, or interpolation into, experimental data, but it is difficult to know whether this extrapolation or interpolation should be on the basis of a linear or a curvilinear response to changing intake (WHO, 2007).

2.1.1 Protein digestion and absorption

The first stage in protein digestion is denaturation; folded native proteins are relatively resistant to enzymic hydrolysis. Denaturation is achieved by heat, as when foods are cooked, and also by the strong acid of gastric juice. Table 2.2 shows the major human proteolytic enzymes, as well as a number of plant,

Table 2.2 Proteolytic enzymes.

	EC number	Source	Specificity
Digestive endopeptidases			
chymosin (rennin)	3.4.33.4	neonatal gastric juice	hydrophobic, preferably aromatic; Phe ¹⁰⁵ -Met in κ -casein
pepsin A	3.4.23.2	gastric juice	hydrophobic, preferably aromatic
pepsin B (parapepsin)	3.4.23.3	gastric juice	hydrolyzes gelatine
gastricsin (pepsin C)	3.4.23.4	gastric juice	Tyr-
trypsin	3.4.21.4	pancreatic juice	Arg-, Lys-
chymotrypsin	3.4.21.1	pancreatic juice	Tyr-, Trp-, Phe-, Leu-
elastase	3.4.21.36	pancreatic juice	Ala-
pancreatic elastase II	3.4.21.71	pancreatic juice	Leu-, Met-, Phe-
enteropeptidase	3.4.21.9	crypts of Lieberkühn	Lys ⁶ -Ile in trypsinogen
Digestive exopeptidases			
aminopeptidase	3.4.11.x	crypts of Lieberkühn	N-terminal except Arg or Lys.
carboxypeptidase A	3.4.16.x	pancreatic juice	C-terminal except -Asp, -Glu, -Arg, -Lys, -Pro
carboxypeptidase B	3.4.16.x	pancreatic juice	C-terminal Lys or Arg
dipeptidases	3.4.13.x	mucosal brush border	various
tripeptide aminopeptidase	3.4.14.x	mucosal brush border	N-terminal of a tripeptide
Plant, bacterial and fungal proteases			
ananain	3.4.22.31, 3.4.22.32	pineapple, <i>Ananas comosus</i>	broad
actinidain	3.4.22.14	kiwi fruit, <i>Actinidia chinensis</i>	broad, preferably hydrophobic
caricain	3.4.22.30	pawpaw, <i>Carica papaya</i>	broad, preferably hydrophobic
chymopapain	3.4.22.6	pawpaw, <i>Carica papaya</i>	broad, preferably hydrophobic
clostripain	3.4.22.8	<i>Clostridium histolyticum</i>	Arg-
collagenase	3.4.24.3	<i>Clostridium histolyticum</i>	-Gly in collagen
cerevisin	3.4.21.48	<i>Saccharomyces cerevisiae</i>	broad
endopeptidase K	3.4.21.64	<i>Tritirachium album</i>	broad, hydrolyzes keratin
ficin (ficain)	3.4.22.3	fig, <i>Ficus glabrata</i>	broad, preferably hydrophobic

Table 2.2 *Continued*

	EC number	Source	Specificity
fruit bromelain	3.4.22.32	pineapple, <i>Ananas comosus</i>	broad
mucorpepsin	3.4.23.23	<i>Mucor pusillus</i> , <i>M. miehei</i>	hydrophobic, clots milk
oryzin	3.4.21.63	<i>Aspergillus oryzae</i>	broad
papain	3.4.22.2	pawpaw, <i>Carica papaya</i>	broad, preferably hydrophobic
stem bromelain	3.4.22.32	pineapple, <i>Ananas comosus</i>	broad
subtilisin	3.4.21.62	<i>Bacillus subtilis</i>	broad
thermolysin	3.4.24.27	<i>Bacillus thermoproteolyticus</i>	-Leu, -Phe

bacterial and fungal enzymes that are important in food technology and laboratory studies; Table 2.3 shows the Enzyme Commission classification of proteolytic enzymes, based on their mechanisms of action.

The first stage of enzymic digestion of proteins occurs in the stomach, catalyzed by pepsin. Pepsin is secreted as an inactive zymogen, pepsinogen. This is activated either by acid hydrolysis (which removes the peptide fragment that overlies and blocks the catalytic site in the zymogen) or by enzymic hydrolysis (to remove the blocking peptide, catalyzed by pepsin that has already been activated). Pepsin is an endopeptidase that catalyzes hydrolysis of peptide bonds adjacent to large neutral amino acids (the aromatic and branched-chain amino acids and methionine), so the result of pepsin action is the production of a relatively large number of peptide fragments. Gastric digestion may result in up to half of the ingested protein being hydrolyzed to peptides smaller than ten amino acids.

Table 2.3 The Enzyme Commission (EC) classification of peptidases.

3.4.11.x	aminopeptidases
3.4.13.x	dipeptidases
3.4.14.x	dipeptidyl peptidases and tripeptidyl peptidases
3.4.15.x	peptidyl dipeptidases
3.4.16.x	serine-type carboxypeptidases (Ser in catalytic site)
3.4.17.x	metallocarboxypeptidases (zinc or another divalent ion in the catalytic site)
3.4.18.x	cysteine-type carboxypeptidases (Cys in the catalytic site)
3.4.19.x	omega peptidases (peptide bonds other than α -carboxyl- α -amino)
3.4.21.x	serine endopeptidases (Ser in the catalytic site)
3.4.22.x	cysteine endopeptidases (Cys in the catalytic site)
3.4.23.x	aspartic endopeptidases (Asp in the catalytic site)
3.4.24.x	metalloendopeptidases (zinc or another divalent ion in the catalytic site)
3.4.99.x	endopeptidases of (as yet) unknown mechanism

Protein digestion continues in the small intestine, where three more inactive precursors of endopeptidases are secreted in the pancreatic juice: trypsinogen, chymotrypsinogen and pro-elastase. Trypsinogen is activated to trypsin by the enzyme enteropeptidase (sometimes called enterokinase, although it is not a kinase), secreted by the crypts of Lieberkühn in the duodenum. Once activated, trypsin catalyzes the activation of chymotrypsinogen to chymotrypsin, and of pro-elastase to elastase. Like pepsinogen, these three zymogens are inactive because of a terminal peptide region that overlies and blocks the active site, and activation results from cleavage of this blocking peptide.

Trypsin, chymotrypsin and elastase are so-called serine proteases, with a serine residue at the catalytic site. These three enzymes show very considerable sequence homology, and the catalytic site is made up by histidine 57, aspartate 102 and serine 195. In all three enzymes, the peptide substrate sits in a groove in the surface of the enzyme, with the amino acid that provides the carboxyl group of the bond to be cleaved sitting in a pocket under the groove. This explains the different substrate specificities of the enzymes:

- In trypsin, which catalyzes hydrolysis of esters of lysine and arginine, there are two glycine residues on the sides of the substrate-binding pocket and an aspartate residue at the base, so providing a negative charge to attract the positive charge of the substrate.
- In chymotrypsin, which catalyzes hydrolysis of the esters of aromatic amino acids, the amino acid at the base of the pocket is serine.
- In elastase, which catalyzes hydrolysis of esters of small neutral amino acids, the substrate-binding pocket is blocked by a valine and a threonine residue on the sides.

The end result of the actions of these endopeptidases is the liberation of a large number of small peptides that are substrates for aminopeptidases and carboxypeptidases, which catalyze hydrolysis of the amino- and carboxy-terminal amino acids of peptides respectively. Procarboxypeptidases are secreted in the pancreatic juice and pro-aminopeptidases by the crypts of Lieberkühn, and these are activated by partial proteolysis catalyzed by trypsin. Neither amino- nor carboxypeptidases acts on di- and tripeptides, which are hydrolyzed by di- and tripeptidases in the brush border of intestinal mucosal cells.

As shown in Figure 2.1, in addition to the dietary intake of protein of some 90 g/day, a considerably larger amount of endogenous protein and other nitrogenous compounds enters the intestinal tract. Most of the protein is

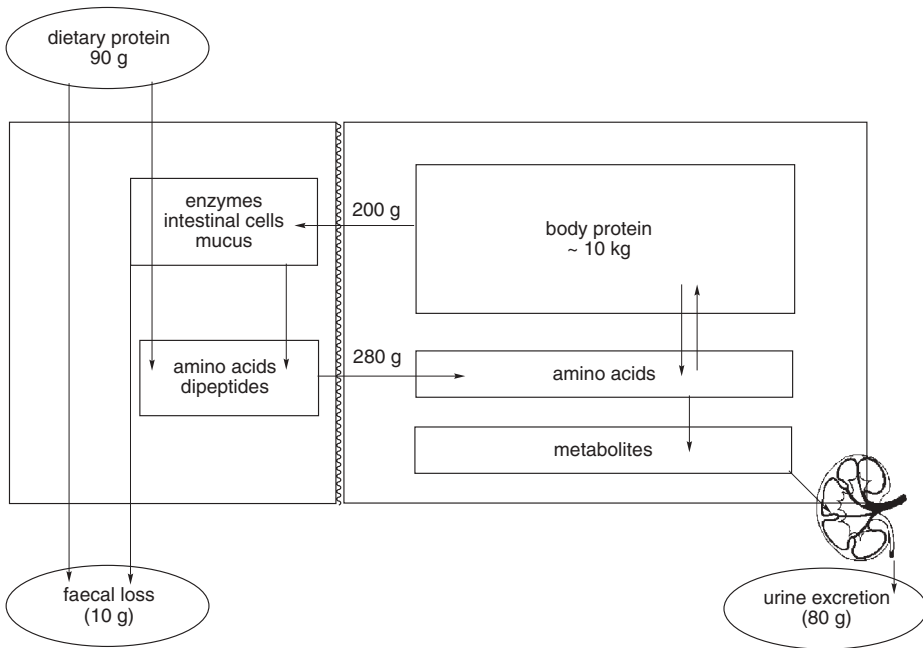


Figure 2.1 Nitrogen balance – protein flux through the gastro-intestinal tract.

hydrolyzed and the resultant amino acids are absorbed. Much of the endogenous protein consists of digestive enzymes secreted into the gastro-intestinal tract and proteins in mucus, which is secreted in order to protect the intestinal mucosa from attack by digestive enzymes, together with shed mucosal cells. In addition, some of the proteins synthesized by intestinal bacteria are hydrolyzed, and the resultant amino acids are absorbed in the large intestine. Much of the faecal loss of protein is mucin, which is very resistant to enzymic hydrolysis (Bergen & Wu, 2009; Fuller & Reeds, 1998).

As will be discussed in section 2.5, amino acids are absorbed from the intestine by a number of different transport proteins, depending on the chemistry of their side-chains. In most cases, amino acid uptake is by sodium-dependent secondary active transport. Di- and tripeptides are taken up into intestinal mucosal cells by a separate set of transporters from those that are involved in the absorption of free amino acids, and are hydrolyzed intracellularly.

There is also significant absorption of intact proteins and relatively large peptides from the intestinal lumen. This is especially important in neonates, who acquire passive immunity from the maternal antibodies in milk (especially colostrum), but it also continues throughout life. The uptake of intact proteins and peptides may be either paracellular, by diffusion through the

tight junctions between epithelial cells, or transcellular, by endocytosis into the intestinal M cells in Peyer's patches, followed by transfer of (potential) antigens to sub-epithelial lymphocytes. Such absorption of intact proteins and relatively large peptides is a major factor in food allergy.

In addition, a number of smaller peptides are also absorbed intact. Many of these resemble the neuro-active endorphins and, since they have some endorphin-like activity, they have been termed exorphins (Gardner, 1988).

2.1.2 Protein digestibility and unavailable amino acids in dietary proteins

The digestibility of a protein is a measure of the extent to which its constituent amino acids are available for absorption from the gastro-intestinal tract in a form that can be used for tissue protein synthesis. It can be determined by measurement of faecal nitrogen losses in the same person or experimental animal (pigs are frequently used as a good model for human digestion) after consuming the test protein and after a period on a protein-free diet to estimate obligatory faecal losses (see section 2.1.3). Faecal nitrogen loss on a protein-free diet almost certainly underestimates true obligatory nitrogen loss. This is because of reduced secretion of proteolytic enzymes and a reduction in the rate of tissue protein turnover and cell proliferation, leading to less secretion of protein into the gastro-intestinal tract and a lower rate of desquamation of intestinal epithelial cells.

Studies in pigs show that there is a considerable difference between the pattern of amino acids in faeces and what enters the colon from the ileum. What leaves the ileum is a mixture of undigested dietary protein and peptides and amino acids that have not been absorbed, as well as endogenous proteins, including digestive enzymes, mucus, desquamated mucosal cells, albumin and urea (see section 1.6.2.4).

About 80 per cent of faecal nitrogen is inside bacterial cells, so the pattern of amino acids in faeces reflects bacterial interconversion and synthesis of amino acids more than that of undigested dietary proteins. Ileal digestibility of dietary proteins is always lower than faecal digestibility. A small number of studies have been conducted in which the same proteins have been fed to patients with ileostomies (to determine ileal digestibility) and control subjects (to determine faecal digestibility). As in the pig studies, ileal digestibility is lower than faecal digestibility, and especially so for proteins that have a low digestibility (Darragh & Hodgkinson, 2000; Moughan, 2003; Rutherfurd & Moughan, 2007).

Not all of the amino acids in dietary proteins are liberated in digestion, because the digestive proteases (see Table 2.2) only hydrolyze α -peptide bonds. Covalent cross-links between peptide chains formed by isopeptide

bonds between the ϵ -amino group of lysine and the side-chain carboxyl group of aspartate or glutamate will not be hydrolyzed. If isopeptides between the ϵ -amino group of lysine and the γ -carboxyl group of glutamate are absorbed, then γ -glutamyl cyclotransferase (section 5.4.5) catalyzes hydrolysis to form oxoproline and lysine, so that the availability of lysine may be greater than would be predicted (Yasumoto & Suzuki, 1990).

A significant amount of lysine in dietary proteins is unavailable because the ϵ -amino group has reacted with a reducing sugar in processing or storage (the Maillard reaction – see Figure 6.6), in the same way as proteins are glycosylated non-enzymically in diabetics with poor glycaemic control. The glycation products of lysine are not hydrolyzed by digestive enzymes, so that the lysine that is apparently present in the protein when measured after acid hydrolysis is greater than that which is available for absorption. Lysine may also be involved in formation of cross-links to dihydroxyphenylalanine, and tyrosine may be unavailable because of formation of di- and tri-tyrosine cross-links (Bailey, 1991).

It is not only the lysine or tyrosine itself that is unavailable; side-chain peptide bonds, glycation products and other cross-links inhibit proteases acting on nearby α -peptide bonds, so further reducing the digestibility of the protein (Moughan, 2003). Various methods have been developed to react free ϵ -amino groups of lysine in proteins (e.g. with fluorodinitrobenzene or methylisourea), so permitting estimation of the proportion of lysine that is free and therefore available for absorption. Proteins that are cross-linked by disulphide bridges are also resistant to digestion.

2.1.3 Obligatory nitrogen losses

Even on a protein-free diet, there is excretion of nitrogenous compounds in urine and faeces. Part of this is due to the metabolic demand for products of amino acid metabolism, such as nucleotide bases (see section 1.4), neurotransmitters and hormones. Urea synthesis and excretion continue as a result of the catabolism of amino acids released in protein turnover, and especially use of their carbon skeletons for gluconeogenesis in the fasting state. In addition, there are relatively small amounts of proteins that are lost from external parts of the body (e.g. in hair, nails and shed skin cells) and so are not available for catabolism and re-utilization of their constituent amino acids.

Obligatory losses on a protein-free diet are 36 mg nitrogen per kg body weight/day (equivalent to some 15.75 g of protein for a 70 kg person) in urine, 12 mg nitrogen (equivalent to 5.25 g protein) in faeces and 8 mg nitrogen/kg body weight/day (equivalent to 2.75 g of protein) in sweat, shed skin cells, hair and nails (Tomé & Bos, 2000).

Obligatory faecal nitrogen losses include residues of digestive enzymes, bacterial proteins and nucleic acids, and protein and nucleic acids from shed

intestinal mucosal cells, as well as mucin, the main protein in the protective mucus that is secreted throughout the gastro-intestinal tract. Mucin is particularly resistant to enzymic hydrolysis, as befits the function of mucus in protecting the gastro-intestinal epithelium from attack by digestive enzymes.

2.1.4 Dynamic equilibrium and tissue protein turnover

Although studies of nitrogen balance in the late 19th and early 20th centuries led to estimates of requirements for dietary protein, it was assumed that there were separate pools of endogenous and exogenous (dietary) protein, with the nitrogen output in urine and faeces arising from the exogenous pool. Animal studies using [^{15}N]amino acids conducted by Schoenheimer in the 1940s demonstrated that this was not so. Less than half the label in dietary amino acids was recovered in urine and faeces, and the remainder was found to have been incorporated into tissue proteins. Schoenheimer (1946) coined the term ‘dynamic equilibrium’ to explain this – a state in which although the total protein content of the adult animal does not change, protein is nevertheless being catabolized and replaced.

Interestingly, Schoenheimer interpreted his results in terms of individual amino acids being excised from tissue proteins and replaced. At that time, it was assumed that once proteins had been synthesized, they remained in the cell. The French physiologist Magendie had predicted our present understanding of continual turnover of tissue proteins in 1829, when he said that: ‘all parts of the body of man experience an intimate movement that has the double effect of expelling molecules that can or ought no longer to compose the organs, and replacing them with new molecules’. Not only is this still a very succinct definition of dynamic equilibrium, it also has the correct driving force – the catabolism of aged proteins, leading to a need for their replacement.

Individual proteins turn over at very different rates. This can be demonstrated by administering labelled amino acids to experimental animals and measuring the specific radioactivity or isotope enrichment in the protein in question over a period of time. The time taken for the label to fall from the peak value after administration of the labelled amino acid to half the peak is the biological half-life of the protein. Table 2.4 shows the half-lives of a number of proteins. Those with half-lives of hours to a few days are regulatory enzymes; changes in the rate of their catabolism and synthesis in response to hormones and dietary factors are important in metabolic regulation. Proteins with half-lives of several days are ‘house-keeping’ proteins, whose activity does not vary much from day to day. Structural proteins such as collagen turn over only very slowly and, indeed, the crystallin in the lens of the eye apparently does not turn over at all.

Even in times of rapid growth, or recovery from losses, when there is a significant positive nitrogen balance and an increase in total body protein,

Table 2.4 Half-lives of some tissue proteins.

Enzyme	EC number	Half-life
ornithine decarboxylase	4.1.1.17	11 minutes
5-aminolaevulinic synthase	2.3.1.37	20 minutes
lipoprotein lipase	3.1.1.34	1 hour
tyrosine transaminase	2.6.1.5	1.5 hours
tryptophan dioxygenase	1.13.11.52	2 hours
phosphoenolpyruvate carboxykinase	4.1.1.32 and 4.1.1.33	2 hours
thymidine kinase	2.7.1.21	3 hours
hydroxymethylglutaryl CoA (HMG CoA) reductase	2.7.11.3	3 hours
phosphoenolpyruvate carboxylase	4.1.1.31	5 hours
dihydro-orotase	3.5.2.3	12 hours
glucokinase	2.7.1.2	12 hours
glucose 6-phosphate dehydrogenase	1.1.1.49	15 hours
phosphoglycerate dehydrogenase	1.1.1.95	15 hours
alanine aminotransferase	2.6.1.2	17–24 hours
serine deaminase	4.3.1.17	20 hours
ferritin	n/a	1.3 days
peroxisomal catalase	1.11.1.6	1.4 days
acetyl CoA carboxylase	6.3.4.15	2.0 days
aspartate carbamoyltransferase	2.1.3.2	2.5 days
histidine ammonia lyase	4.3.1.3	2.5 days
histidine-pyruvate transaminase	2.6.1.58	2.8 days
aspartate transaminase	2.6.1.1	3.0 days
fatty acid synthase	2.3.1.85	3.0 days
glyceraldehyde 3-phosphate dehydrogenase	1.2.1.12	3.1 days
alanine transaminase	2.6.1.2	3.5 days
pyruvate kinase	2.7.1.40	3.5 days
urocanase	4.2.1.49	3.5 days
serum albumin	n/a	3.5 days
cytochrome c reductase	1.6.99.3	3–4 days
xanthine oxidase	1.17.3.2	4.0 days
arginase	3.5.3.1	4–5 days
cytochrome b ₃	n/a	5–6 days
lactate dehydrogenase	1.1.1.27	16 days
infant collagen	n/a	1–2 days and 150 days
adult collagen	n/a	300 days

Source: from data reported by Waterlow *et al.*, 1978.

there is still catabolism of tissue proteins. It has been estimated that some 1.4 g of protein are synthesized for each 1 g net gain in total body protein.

2.1.5 Tissue protein catabolism

There are two main routes for the normal catabolism of tissue proteins: a more or less random process involving uptake into lysosomes (section 2.1.5.1),

which is the main mechanism for catabolism of proteins with a relatively long half-life; and targeting of proteins with a short half-life for catabolism by either the proteasome (section 2.1.5.2) or lysosomes.

Caspases are proteases with cysteine at the catalytic site that cleave peptide bonds to the carboxyl side of aspartate residues. In response to signals for apoptosis, the synthesis of caspases is increased and inactive caspase zymogens are activated. When activated, they cleave a wide variety of structural and other proteins in the cell, so initiating the programmed events of apoptosis (Earnshaw *et al.*, 1999).

The calcium-activated neutral cysteine proteases, the calpains, are non-lysosomal proteases that are normally inhibited by the associated inhibitory protein calpastatin, and they are activated by an increase in intracellular (or cytoplasmic) calcium ion concentration. Their physiological function is unclear, but they produce relatively large, sometimes catalytically active, peptide fragments from their target proteins, suggesting that they may have a role in cell signalling.

In muscle cells, calpains are associated with myofibrils, and they cleave tropomyosin and myosin, suggesting that they may have a role in muscle atrophy in response to trauma and prolonged bed rest. In non-muscle cells, they are associated with the cell membrane (again suggesting a role in signal transduction) and the cytoskeleton, suggesting a role in remodelling of the cytoskeleton (Croall & DeMartino, 1991; Goll *et al.*, 2003).

2.1.5.1 Lysosomal autophagy A primary response to nutrient limitation in the cell is the formation of autophagosomes, whereby cytoplasm and organelles are non-specifically sequestered in a double membrane vesicle that then fuses with a lysosome. The various cathepsins (proteases) and other hydrolytic enzymes in the lysosome then hydrolyze the macromolecules in the autophagosome and return the products to the cytosol for re-utilization. This autophagy is thus the intra-cellular equivalent of phagocytosis. Because the capture of proteins in autophagosomes is a random process, it is likely that most of the proteins catabolized in this way will be those with a relatively long half-life.

The formation of autophagosomes is regulated by insulin and glucagon. In response to insulin, mTOR kinase is activated, and phosphorylated mTOR (mammalian target of rapamycin) inhibits autophagosome formation. In response to nutrient depletion, there is dephosphorylation and, hence, down-regulation of mTOR, activating the formation of autophagosomes. Intracellular free amino acids also activate the mTOR kinase pathway, so inhibiting autophagy (Kilberg *et al.*, 2005; Mizushima & Klionsky, 2007).

Some proteins are transported into lysosomes for catabolism, either because they have a lysosomal targeting sequence (Lys-Phe-Glu-Arg-Gly), or because they have been ubiquitinated (section 2.1.5.2). Proteins with the

lysosomal targeting sequence are transported into lysosomes by a chaperone protein.

2.1.5.2 Ubiquitin and the proteasome Ubiquitin is a small (76 amino acid), highly conserved protein that is present in all eukaryotic cells. It is a globular protein with a protruding carboxy-terminal region, and it is highly resistant to proteolysis. It forms a peptide bond from its carboxy-terminal glycine to the ϵ -amino group of a lysine residue in the target protein. Like lysosomal autophagy (section 2.1.5.1), ubiquitination of proteins occurs in response to nutritional deprivation and stress, or the need for repair. However, ubiquitination is additionally important in controlling the intracellular concentrations of regulatory enzymes with a short half-life, and targeting abnormal proteins or proteins that have undergone oxidative or other damage for catabolism. In addition to this role in intracellular protein catabolism, ubiquitination is involved in the DNA repair mechanism, remodelling of chromatin, antigen processing and regulation of the cell cycle and endocytosis (Reyes-Turcu *et al.*, 2009).

Three genes code for ubiquitin. Classes I and II encode a fusion protein between ubiquitin and a zinc-finger protein that is required for the formation of ribosomes. These genes are expressed constitutively and, since one gene product is involved in protein catabolism and the other in protein synthesis, they presumably act mainly to balance the rates of protein catabolism and synthesis under normal conditions. The class III ubiquitin gene encodes head-to-tail repeats of ubiquitin, which yield free ubiquitin after post-synthetic cleavage. Expression of this gene is induced in response to metabolic and other stress.

Three enzymes are involved in the ubiquitination of target proteins:

- Ubiquitin activating enzyme catalyzes adenylation of ubiquitin and the formation of a thio-ester bond between the carboxy-terminal glycine of ubiquitin adenylate and a cysteine residue in the enzyme.
- The next stage is the transfer of ubiquitin adenylate onto a cysteine residue in one of the ubiquitin carrier proteins. Most of these are small proteins that require ubiquitin ligase to transfer ubiquitin onto the target protein, but some have amino- and carboxy-terminal extensions and catalyze transfer of the ubiquitin onto the target protein themselves.
- Ubiquitin ligase transfers ubiquitin adenylate from the thio-ester on the carrier protein onto the ϵ -amino group of a lysine residue on the target protein. There are at least 600 ubiquitin ligase genes in the human genome, and this stage obviously provides the main specificity for the proteins to

be targeted for catabolism. At least four ubiquitin molecules have to be attached to a target protein (as a poly-ubiquitin chain with each successive ubiquitin forming a peptide bond between its carboxy-terminal glycine and lysine-48 of that already attached) in order for proteins to be targeted for catabolism (Deshaies & Joazeiro, 2009; Hershko, 1991).

It is the amino-terminal amino acid of the target protein that confers specificity for binding to, and ubiquitination by, ubiquitin ligase. In a number of cases, the target protein has undergone post-synthetic modification of an acidic amino-terminal amino acid by addition of arginine from arginyl tRNA^{Arg}. The arginine tRNA synthase involved in this reaction is distinct from that involved in charging tRNA for ribosomal protein synthesis (Ciechanover & Schwartz, 1989; Morris, 2009; Tasaki & Kwon, 2007).

After the degradation of poly-ubiquitinated proteins in either the proteasome or lysosomes, poly-ubiquitin is released, and this is cleaved to free ubiquitin by stepwise removal of ubiquitin molecules by a ubiquitin-specific protease that hydrolyzes the glycine- ϵ -amino lysine peptide bond.

There are at least one hundred genes for ubiquitin-specific proteases in the human genome, suggesting that there is a considerable degree of specificity for the de-ubiquitination of proteins. It is likely that proteins are mono-ubiquitinated more or less at random. Only if the target protein is damaged, sensed by exposure of a hydrophobic region that causes a conformational change in ubiquitin, is the side-chain of lysine-48 of ubiquitin exposed for further ubiquitination. Otherwise, the single ubiquitin molecule is removed. Up to 40 per cent of newly synthesized protein is catabolized within minutes because of errors in translation or folding. Some of the peptides formed by partial proteolysis in the proteasome can bind to MHC (major histocompatibility complex) class I molecules and establish cell immunogenicity (Kloetzel, 2001; Reyes-Turcu *et al.*, 2009; Wilkinson, 1995).

The eukaryotic proteasome is a 2.5MDa multi-enzyme complex with 33 subunits, 28 of which form a hollow barrel-like core. Proteasomes occur free in the cytosol, attached to the endoplasmic reticulum and in the nucleus. Similar multi-enzyme complexes are found in archaea and prokaryotes. Poly-ubiquitinated proteins bind to the regulatory subunit, which catalyzes the ATP-dependent removal of ubiquitin and ATP-dependent unfolding of the protein. When a ubiquitinated protein is bound, a conformational change in the regulatory subunit causes the opening of a channel into the core protein. This permits the unfolded target protein to enter the central chamber of the core protein. This central chamber is lined with proteases that have a catalytic threonine residue at the amino-terminal. There are three proteases, one each with specificity for esters of acidic, basic and aromatic amino acids, and the end product is a mixture of small (8–10 amino acid) peptides (Finley, 2009).

A number of cell surface receptors, including the GABA receptor, are mono-ubiquitinated, internalized and either sequestered in intracellular vesicles for recycling to the cell surface, or catabolized in lysosomes. Mono-ubiquitination is also involved in endocytosis, histone regulation and the budding of retroviruses from the cell membrane (Barriere *et al.*, 2007; Weissman, 2001).

The proteasome normally requires ubiquitination of target proteins, but the protein antizyme targets ornithine decarboxylase to the proteasome without ubiquitination. As will be discussed in section 5.8.1, ornithine decarboxylase is the regulatory enzyme of polyamine synthesis; it has a very short half-life, of the order of 11 minutes.

2.1.5.3 Active site proteolysis of apo-enzymes In most pyridoxal phosphate-dependent enzymes there is a highly conserved sequence of amino acids around the lysine that forms the Schiff base with the cofactor (Figure 3.2). There is a specific protease in small intestine and skeletal muscle that targets this sequence and initiates the hydrolysis of the apo-enzymes of pyridoxal phosphate-dependent enzymes. It does not hydrolyze other proteins and also does not hydrolyze the holo-enzymes, in which this conserved sequence is hidden by the coenzyme. In experimental animals, the activity of this enzyme increases 10–20 fold in vitamin B₆ deficiency, so reducing the competition between different apo-enzymes for the scarce cofactor.

A similar, but distinct, protease has been isolated from tissues of niacin-deficient rats that catalyzes hydrolysis of the conserved NAD binding site of a number of NAD-dependent enzymes when it is not occupied (Katunuma *et al.*, 1971a, 1971b).

2.1.6 Whole body protein turnover

Studies of nitrogen balance measure only the difference between intake and excretion, and hence they reflect only the net difference between protein synthesis and catabolism. Isotopic studies of whole body protein turnover permit determination of the overall rates of protein synthesis and catabolism.

Early studies used a single dose of a ¹⁵N labelled amino acid and estimated the turnover rate and size of the metabolic pool by determination of the isotopic enrichment of urinary urea or the plasma free amino acid during the first few hours. This approach makes a number of assumptions that are not valid, and more recent studies depend on a constant infusion of labelled amino acid and measurement of the isotopic abundance in either the precursor amino acid or a metabolic end product.

2.1.6.1 The constant infusion, labelled precursor method If a labelled amino acid is infused at a constant rate over a period of 12–30 hours, rather than as a single dose, there will be an initial exponential increase in the isotopic enrichment of the amino acid in plasma, followed by a period over which it will remain constant. The rate of entry of label from the infusion equals the rate of efflux from the free amino acid pool into either protein synthesis or catabolism. Later, the isotopic enrichment in plasma will increase again as amino acids enter the pool from protein catabolism. When the isotopic enrichment of the amino acid is constant:

influx (from infusion) = efflux (to protein and metabolites)

hence: rate of infusion $\times$ isotopic enrichment of infused amino acid
= total efflux from the pool $\times$ isotopic enrichment at plateau

Rearranging gives: total efflux into protein + amino acids
= (rate of infusion $\times$ isotopic enrichment of infused
amino acid)/isotopic enrichment at plateau

Knowing the amount of amino acid infused, it is possible to calculate the amount entering protein rather than being metabolized (Waterlow & Stephen, 1967).

2.1.6.2 The constant infusion, labelled end product method A modification of the constant infusion technique involves measuring the isotopic enrichment of end products of metabolism rather than the plasma free amino acid precursor (Picou & Taylor-Roberts, 1969). Such end products may be exhaled $^{13}\text{CO}_2$, or urinary ^{15}N urea and $^{15}\text{NH}_4^+$. This has two advantages: no assumptions are made about the isotopic enrichment in plasma being equal to that in tissues; and it is a less invasive procedure, since it does not require plasma samples.

At the plateau of labelling in the end product:

total flux (turnover) of nitrogen = (measured) dietary intake
+ entry of amino acid from infusion
= efflux of N to protein synthesis + efflux to metabolites

Percentage of administered isotope being excreted
= rate of excretion of labelled metabolite/
rate of infusion of labelled amino acid
= efflux of N to metabolites/total flux of nitrogen

Hence, protein synthesis = total N flux – excretion.

2.1.6.3 Rates of whole-body protein turnover Studies of protein turnover by constant infusion methods have shown that the rate of protein turnover is considerably greater than would be predicted from levels of protein intake required to maintain nitrogen balance. Even in growing children, there is a considerably greater rate of total protein synthesis than the net increase in body protein shown by positive nitrogen balance. Rapidly growing pigs synthesize 2 g of protein for each gram of net synthesis and, in recovery from protein-energy malnutrition, children can synthesize 2.5 g protein/g net synthesis.

These results imply that there must be considerable re-utilization of the amino acids released by tissue protein catabolism. Indeed, the observation that the total rate of protein synthesis is some six-fold higher than the dietary intake required to maintain nitrogen balance raises the question of why an adult has any need for a dietary intake of protein at all, other than to replace the (relatively small) amounts lost irreversibly in skin, hair, sweat and mucus, and amino acid metabolites such as purines and pyrimidines (Young & Pellett, 1987).

2.1.6.4 The catabolic drive and amino acid oxidation The main factor determining protein requirements is a catabolic drive – irreversible catabolism of amino acids that are not immediately required for protein synthesis. In the fed state, amino acids in excess of immediate requirements for protein synthesis are not stored to any significant extent, but are catabolized. The rate of oxidation of amino acids is determined by habitual protein intake, and it changes only relatively slowly as the intake is changed. In the fasting state, the carbon skeletons of amino acids released by protein catabolism are used as far as possible for gluconeogenesis, and otherwise as metabolic fuel (section 2.3; Table 2.9; Millward, 2003; Millward & Rivers, 1988).

The aminoacyl tRNA synthetases have values of K_m such that they are saturated at normal concentrations of substrate, and they therefore act at a more or less constant rate to direct amino acids into protein synthesis. It is only when an amino acid is relatively deficient that there is a significant amount of uncharged tRNA in the cell and, as discussed in section 2.2.3, uncharged tRNA regulates overall protein synthesis. By contrast, many of the enzymes that initiate the catabolism of amino acids (and especially lysine, phenylalanine and the branched-chain amino acids) have relatively high values of K_m and, hence, show increasing activity as the concentration of substrate rises (Young & Marchini, 1990). Withdrawal of these amino acids leaves others that cannot be used for protein synthesis, and will therefore be catabolized.

The pattern of amino acids arising from catabolism of tissue proteins will presumably match the pattern required for re-synthesis of tissue proteins reasonably well, apart from those amino acid formed by post-synthetic

modification, for which there are no codons, and which therefore cannot be re-utilized (see Table 2.7). However, as discussed in section 2.1.6.6, protein synthesis slows in the fasting state, so that catabolism exceeds synthesis, and most of the amino acids released will be used for gluconeogenesis.

2.1.6.5 The energy cost of protein turnover A minimal estimate of the energy cost of protein synthesis can be derived from the biochemistry of protein synthesis. On the ribosome, there is utilization of 4 mol of (ATP plus GTP) per peptide bond formed, which is equivalent to 2.80 kJ/g protein synthesized. Allowing for the ATP cost of amino acid active transport into the cell (section 2.5) gives a cost of 3.59 kJ/g protein synthesized, and allowing for the cost of synthesizing mRNA gives a cost of 4.19 kJ/g protein synthesized. On this basis, a 70 kg adult synthesizing 5 g protein/kg body weight/day would expend 1.5 MJ, or almost 20 per cent of basal metabolic rate, on protein synthesis (Robinson *et al.*, 1990).

There is an increase both in resting energy expenditure and in protein synthesis after a meal, and, as shown in Table 2.5, this is higher after a high-protein meal than after a high-carbohydrate meal. After a meal, between 5–10 per cent of the energy yield of the meal may be expended in protein synthesis. Overall nitrogen balance depends not only on an adequate intake of protein (and individual amino acids – see section 2.2.3) but also on an adequate intake of energy. Subjects fed only the safe level of protein intake are unable to maintain nitrogen balance when provided with only their calculated minimum energy intake, but they can do so at higher levels of energy intake (Garza *et al.*, 1977; Robinson *et al.*, 1990).

A similar effect of energy intake on amino acid and protein metabolism is seen with intestinal bacteria. If these are provided with only small amounts of carbohydrate substrate, they catabolize amino acids, using their carbon skeletons as energy sources as far as possible, and they form a variety of products, including hydrogen sulphide from the sulphur amino acids (methionine and cysteine – see section 6.3.6), indole and skatole from tryptophan

Table 2.5 Protein synthesis and energy expenditure after feeding.

	Increased energy expenditure as percentage of energy intake	Protein turnover (g in 9-hour study)
Fasting	–	17.6 ± 2.2
High-carbohydrate meal (0.9 g protein/kg body weight)	5.7	27.4 ± 1.4
High-protein meal (4.2 g protein/kg body weight)	9.6	58.2 ± 5.3

Source: from data reported by Robinson *et al.*, 1990.

(section 9.4.2) and relatively large amounts of ammonium from deamination of amino acids. However, if they are provided with an adequate carbohydrate source (e.g. resistant starch or fermentable non-starch polysaccharides that are not digested in the small intestine), they are able to utilize this for energy-yielding metabolism, and to use the amino acids for protein synthesis, leading to reduced formation of sulphides, indole and ammonium (Blachier *et al.*, 2007).

There is also a significant energy cost of the catabolic phase of protein turnover. As discussed in section 2.1.5.2 below, there is a requirement for at least six ATP for the unfolding of each molecule of protein catabolized in the proteasome, as well as the ATP cost of synthesizing ubiquitin and of ubiquitinating target proteins. Formation of the autophagosome (section 2.1.5.1) must also carry some ATP cost.

2.1.6.6 Diurnal variation in protein turnover The effect of energy nutrition on protein synthesis is seen during the normal daily cycle of feeding and fasting. In the fed state, the rate of protein synthesis exceeds that of catabolism, resulting in positive nitrogen balance. In the fasting state, protein synthesis is reduced by about 25 per cent, while catabolism is reduced by only about 3 per cent, resulting in negative nitrogen balance. This means that, while overall an individual is in nitrogen balance, there is cycling between negative nitrogen balance in the fasting state and positive balance in the fed state. The amplitude of this cycling between positive and negative balance increases with increasing protein intake (Clugston & Garlick, 1982; Millward *et al.*, 1991).

It is unclear to what extent this diurnal variation is simply a reflection of the availability of energy and amino acids, since there is also hormonal control of protein turnover. In muscle, insulin-like growth factor and testosterone stimulate protein synthesis. Insulin decreases protein catabolism and stimulates the uptake of glucose and amino acids, so permitting an increase in protein synthesis. When insulin is given with an amino acid infusion, there is an increase in whole body protein synthesis of about 8 per cent and a decrease in catabolism of about 20 per cent. In muscle, but not in other tissues, insulin leads to reduced synthesis of ubiquitin mRNA (Kimball *et al.*, 1994; Waterlow, 1999).

Growth hormone also stimulates protein synthesis, partly as a result of decreased amino acid oxidation and partly by stimulating the release of insulin-like growth factor. Glucagon, catecholamines and glucocorticoids all cause muscle protein catabolism, but this is mainly due to increased gluconeogenesis in the liver, and consequent depletion of amino acid pools, rather than direct effects on muscle itself. Glucocorticoids stimulate net muscle protein catabolism indirectly, by inducing the synthesis of two enzymes catalyzing amino acid catabolism in the liver – tyrosine transaminase (see section

9.2) and tryptophan dioxygenase (section 9.4.4.1). This depletes the body pool of two amino acids, leaving an incomplete mixture that cannot be used for protein synthesis and thus slowing the rate of protein synthesis. Glucocorticoids also induce the key enzymes of gluconeogenesis, so further depleting amino acid pools. In response to trauma and sepsis (and, indeed, prolonged bed rest) there is a considerable loss of muscle protein. This is partly due to the actions of catabolic hormones and partly due to insulin resistance as a result of cytokine secretion (Rooyackers & Nair, 1997).

2.2 Requirements for individual amino acids

Early studies showed that different amounts of different proteins were required for the maintenance of nitrogen balance and that the requirement is not just for total protein intake, but for individual amino acids. Amino acids can be divided into two groups: those that cannot be synthesized in the body, but must be provided in the diet (the essential or indispensable amino acids); and those that can be synthesized from more or less common metabolic intermediates, provided that there is an adequate amount of total fixed nitrogen available for transamination of their carbon skeletons (see section 3.3). These are the non-essential or dispensable amino acids (Table 2.6).

The nine indispensable amino acids are: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. In addition, two amino acids are synthesized from indispensable amino acid precursors: tyrosine from phenylalanine (see section 9.2) and cysteine from methionine (section 6.3.3). Dietary provision of these two amino acids spares the requirement for the precursor. Commonly, the limiting amino acid in foods (section 2.2.4) is methionine, and because of the importance of methionine as a source of cysteine it is common to refer to the sum of methionine plus cysteine in foods, or simply total sulphur amino acids.

Table 2.6 Essential and non-essential amino acids.

Essential	Essential precursor	Non-essential	Conditionally essential
histidine		alanine	
isoleucine		aspartate	asparagine
leucine		glutamate	glutamine
lysine			arginine
methionine	cysteine		glycine
phenylalanine	tyrosine		proline
threonine			serine
tryptophan			
valine			

Only three amino acids can be regarded as completely dispensable, in that their carbon skeletons are common metabolic intermediates that are unlikely to be scarce under any metabolic conditions: alanine (synthesized from pyruvate), aspartate (synthesized from oxaloacetate) and glutamate (synthesized from 2-oxoglutarate). While the other amino acids can normally be synthesized in adequate amounts, there are conditions under which the requirement may outstrip the capacity to synthesize them.

Table 2.7 shows the amino acids that are found in proteins. In addition to the 20 'conventional' protein amino acids, selenocysteine (section 4.9) and pyrrolysine (section 6.2.4.3) are incorporated during ribosomal protein synthesis by context-sensitive reading of STOP codons. A number of other amino acids are formed by post-synthetic modification of precursor proteins. Apart from reversible phosphorylation of serine, threonine and tyrosine, when these modified proteins are catabolized, the modified amino acids cannot be used for new protein synthesis but are either metabolized or excreted unchanged, mainly as small peptides.

Glutamine is a major fuel for enterocytes and other rapidly proliferating cells, providing both nitrogen for nucleotide synthesis (see section 1.4) and also carbon for energy-yielding metabolism. Glutamine also stimulates protein synthesis and reduces the post-operative loss of muscle protein. Muscle maintains a high concentration of free glutamine (60 per cent of the total free amino acid pool), which provides a reservoir of fixed nitrogen for synthesis of amino acids and other nitrogenous compounds, as well as a source of ammonium for maintenance of acid-base balance. Muscle glutamine falls to half in the hypermetabolic and catabolic states that occur after surgery, burns and in sepsis, and a number of studies have shown enhanced healing and recovery with glutamine supplements. There is evidence that in patients on total parenteral nutrition, the supply of glutamine is inadequate to meet the needs of intestinal mucosal cells (Furst & Stehle, 2004; Rennie *et al.*, 1994).

At times of rapid growth, arginine requirements are greater than the capacity for its synthesis, and it is considered at least partially essential for young infants (section 5.9.1).

Collagen is especially rich in proline (section 5.7.2) and, in response to severe trauma, when there is increased collagen and connective tissue synthesis, the demand for proline may outstrip the ability to synthesize it (Jaksic *et al.*, 1991).

Many xenobiotics are excreted as glycine conjugates, and high intakes of compounds such as benzoic acid can outstrip the capacity to synthesize glycine, leading to excretion of 5-oxoproline, an intermediate in glutathione synthesis (section 5.4.4), which has been attributed to glycine deficiency (Jackson *et al.*, 1987). At times of rapid growth and in response to severe trauma, there is a need for glycine for collagen synthesis (glycine is one-third of the amino acids in collagen), and in infants recovering from protein-energy

Table 2.7 The protein amino acids.

		Single letter code	M _r	Side chain (and pK _a where appropriate)	Codon(s)
Amino acids incorporated in ribosomal protein synthesis					
alanine	Ala	A	89.1	methyl	GCNu ⁽¹⁾
arginine	Arg	R	174.2	ureido (12.48)	CGNu, AGPu ⁽¹⁾
asparagine	Asn ⁽²⁾	N	132.1	amide	AAPy ⁽¹⁾
aspartate	Asp	D	133.1	carboxylate (3.90)	GAPy
cysteine	Cys	C	121.1	sulphydryl (10.46)	UGPy
glutamate	Glu ⁽²⁾	E	147.1	carboxylate (4.07)	GAPu
glutamine	Gln	Q	146.1	amide	CAPu
glycine	Gly	G	75.1	H	CGNu
histidine	His	H	155.2	imidazole (6.04)	CAPy
isoleucine	Ile	I	131.2	branched aliphatic	AUA, AUPu
leucine	Leu	L	131.2	branched aliphatic	UUPu, CUNu
lysine	Lys	K	146.2	amino (10.79)	AAPu
methionine	Met	M	149.2	-SCH ₃	AUG
phenylalanine	Phe	F	165.2	benzene ring (aromatic)	UUPy
proline	Pro	P	115.1	–	CCNu
pyrrolysine	Pyl	O	255.3	methylpyrroline carboxylate amide to ε-amino of lysine	UAG ⁽³⁾
selenocysteine	Sec	–	168.0	-SeH (5.7)	UGA ⁽³⁾
serine	Ser	S	105.1	hydroxyl	UCNu, AGPy
threonine	Thr	T	119.1	hydroxyl	ACNu
tryptophan	Trp	W	204.2	indole (aromatic)	UGG
tyrosine	Tyr	Y	181.2	phenol (aromatic) (10.13)	UAPy
valine	Val	V	117.1	branched aliphatic	GUNu
Amino acids formed by post-synthetic modification of proteins					
γ-carboxyglutamate	Gla	–	191.1	bis-carboxylate (2.03 and 4.37)	as Glu
cystine	(Cys) ₂	–	240.2	disulphide bridge, no side chain	as Cys
hydroxylysine	Hyl	–	162.2	amino (9.67)	as Lys
hydroxyproline	Hyp	–	131.1	–	as Pro
hypusine	–	–	233.3	–	as Lys
3-methylhistidine	–	–	169.2	methyl-imidazole-	as His
5-oxoproline (pyroglutamate)	–	–	129.1	–	–
phosphoserine	–	–	185.1	O-phosphate	as Ser
phosphotyrosine	–	–	261.2	O-phosphate	as Tyr

1 Nu is used to indicate any nucleotide, Pu either purine (A or G), and Py either pyrimidine (C or U).

2 Where it is not known from protein sequencing whether the amino acid is aspartate or asparagine, the abbreviation Asx (and the single letter B) is used. Similarly, for either glutamate or glutamine the symbol is Glx and the single letter code is Z.

3 UAG and UGA are normally stop codons; they are read in a context-sensitive manner as coding for pyrrolysine and selenocysteine respectively in a very limited number of proteins.

malnutrition there is increased excretion of 5-oxoproline, which is prevented by providing supplements of glycine (Persaud *et al.*, 1996). In healthy adults on a low-protein diet, the endogenous synthesis of glycine may not be adequate to meet the needs for synthesis of nucleotides (section 1.4), haem (section 4.8), creatine (section 5.9.7), glutathione (section 5.4) and bile salts (Yu *et al.*, 1985).

In patients with impaired renal function, serum concentrations of serine are low, and there is evidence that the capacity for serine synthesis from glycine (section 4.2) is inadequate to meet requirements (Furst & Stehle, 2004).

Tyrosine is synthesized from phenylalanine (section 9.3). Phenylalanine hydroxylase activity does not develop until some eight days after birth, so tyrosine is an essential amino acid for premature and new-born infants. Cysteine is synthesized from methionine (section 6.3.3) and cystathionine synthetase activity develops after birth, so that cysteine is also an essential amino acid for premature and new-born infants.

2.2.1 Nitrogen balance studies

Requirements for eight of the nine essential amino acids were established in a series of studies of nitrogen balance in subjects consuming mixtures of amino acids in the 1950s (Rose, 1957). As shown in Table 2.8, these studies gave values that are lower than more recent estimates based on isotope tracer studies. The two methods are, in fact, estimating different requirements. Nitrogen balance studies measure the minimum amount of amino acid required to maintain nitrogen balance, after a period of adaptation to low

Table 2.8 Reference patterns of essential amino acids.

	From N balance studies		From stable isotope studies	
	mg/kg bw/d	mg/g protein	mg/kg bw/d	mg/g protein
histidine	–	–	10	15
isoleucine	10	15	20	30
leucine	14	21	39	59
lysine	12	18	30	45
methionine + cysteine	13	20	15	22
methionine	–	–	10	16
cysteine	–	–	4	6
phenylalanine + tyrosine	14	21	25	38
threonine	7	11	15	23
tryptophan	3.5	5	4	6
valine	10	15	26	39
total essential amino acids	93.5	141	184	277

Source: WHO, 2007.

intake, while isotope tracer studies measure the metabolic loss of an amino acid and, hence, the need for a dietary intake to replace loss under normal conditions rather than at a minimal level of intake.

The problem of interpreting nitrogen balance studies is that the efficiency of nitrogen utilization decreases as the intake nears the requirement. In order to determine the point at which requirements have been met and nitrogen balance has been achieved, it is necessary to use non-linear regression analysis. Furthermore, nitrogen balance requires several days of adaptation at each level of intake in order to establish a new equilibrium. The isotope tracer methods show adaptation to a new level of intake much more rapidly (Pencharz & Ball, 2003).

A further problem is that nitrogen balance studies to determine the requirement for any one amino acid may mask deficiency because of a change in the pattern of proteins being synthesized, and thus in the pattern of amino acids required (Fuller & Garlick, 1994). In addition, part of the discrepancy between nitrogen balance and isotope oxidation studies may be explained by intestinal bacterial synthesis of amino acids. Intestinal bacteria may provide as much as 11–68 mg of lysine per kg body weight. This lysine, and other amino acids, will mainly be from bacteria in the small intestine, where there are amino acid transporters, but there may also be absorption from the large intestine, where there is a considerably larger bacterial population (Jackson, 1983; Metges & Barth, 2000).

In nitrogen balance studies, depriving the subjects of histidine for several days has no effect on nitrogen balance, so it was concluded that, although histidine was an essential amino acid for other species that had been examined, it was not essential for human beings (Rose *et al.*, 1951a, 1951b). It was not until 1975 that the essentiality of histidine in human nutrition was established, when it was observed that patients maintained on long-term total parenteral nutrition without histidine in the amino acid mixture were unable to maintain nitrogen balance (Kopple & Swendseid, 1975).

It is not clear why deprivation of histidine does not affect nitrogen balance, but two reasons have been advanced. There may be adequate amounts of histidine released by the catabolism of haemoglobin that is not replaced in the short term. Also, there is a relatively high concentration of the dipeptide carnosine (β -alanyl-histidine – see section 8.5) in muscle, and this may serve as a buffer store of histidine in times of depletion. Deprivation of histidine for up to 48 days leads to a fall in whole body protein turnover and a reduction in plasma histidine, as well as a reduction in haemoglobin, but it does not affect nitrogen balance (Kriengsinyos *et al.*, 2002).

2.2.2 Isotope tracer studies

The requirement for each amino acid can be considered to be the dietary intake that balances all losses from the body. To first approximation, losses

can be estimated by measuring exhaled $^{13}\text{CO}_2$ while the subject is receiving a constant infusion of ^{13}C amino acid, as an index of oxidation of the amino acid. There are two problems with such studies. First, not all of the $^{13}\text{CO}_2$ formed from amino acid oxidation is exhaled; much is sequestered in the body pool of bicarbonate. This can be overcome by parallel studies of $^{13}\text{CO}_2$ exhalation after infusion of ^{13}C bicarbonate. Perhaps more importantly, however, the sensitivity for determination of $^{13}\text{CO}_2$ is relatively low, and more of the ^{13}C amino acid has to be infused than is simply a tracer dose. In many cases, the infusion significantly increases the plasma and tissue concentrations of the amino acid. Both the plasma concentration of the amino acid under consideration and its rate of oxidation remain low at intakes below requirement, then increase as the intake exceeds the requirement (Pencharz & Ball, 2003; WHO, 2007).

An alternative approach is to measure the oxidation of an amino acid other than the one under test – the so-called indicator amino acid method. This is based on the assumption that if one essential amino acid is present in amounts that are not adequate for protein synthesis, then the surplus of the other amino acids will be oxidized. As the intake of the limiting amino acid increases, so the oxidation of the indicator amino acid decreases, because it is now being used for protein synthesis. There is an inflection in the curve of indicator amino acid oxidation against intake of the test amino acid when the intake of the test amino acid is equal to the requirement.

This method overcomes the problem of the infused labelled amino acid not being a tracer dose, since any that is not required for protein synthesis will be oxidized (Pencharz & Ball, 2003; WHO, 2007).

2.2.3 Control of protein synthesis by the availability of amino acids

Muscle contains the main pool of body protein, and studies of muscle protein turnover have shown that the availability of amino acids (and especially of the essential amino acids) regulates the rate of protein synthesis. In response to infusion of a mixture of essential amino acids, there is a linear dose response of increased protein synthesis over the range of normal diurnal variation in plasma amino acid concentrations – a two-fold difference between the fed and fasting states. Providing carbohydrate together with amino acids gives a greater increase in protein synthesis. This is not simply an effect of an increased source of metabolic fuel; insulin secreted in response to the carbohydrate load also stimulates protein synthesis when amino acids are available.

Although short-term studies show that feeding a relatively high protein intake to [elderly] people who have a habitually low intake leads to an increase in total body protein, it is also associated with an increase in protein

catabolism and amino acid oxidation (section 2.1.6.4), so that nitrogen balance is restored after a short time. There is little or no evidence that high intakes of protein are beneficial for increasing muscle mass in athletes and sports players; protein supplements give no increase in muscle anabolism beyond that seen in response to exercise and training (Metges & Barth, 2000; Rennie & Tipton, 2000; Tomé & Bos, 2000).

Experimental animals will reject a diet that is deficient in an essential amino acid and will forage for a diet that is complete and will support protein synthesis. This is an adaptive response, and the animals can become conditioned to avoid signals associated with an incomplete diet. Rats stop eating an amino acid deficient diet before they are satiated, but not so rapidly that it is a simple taste aversion response. Rather, there is a chemosensor in the anterior piriform cortex of the brain that depends on detection of uncharged tRNA (Gietzen *et al.*, 2007).

Uncharged tRNA activates the amino acid response pathway by activating a protein kinase, leading to phosphorylation of translation initiation factor eIF-2 β , resulting in a general reduction in protein synthesis. Selected mRNA species, including those for amino acid transport proteins, show increased translation. In addition, uncharged tRNA inhibits phosphofructokinase, the rate-limiting enzyme of glycolysis, so slowing the rate of glycolysis. Fructose 1,6-bisphosphate, the product of phosphofructokinase, stimulates protein synthesis by releasing inhibitory GDP from translation factor eIF-2 β , so permitting the initiation of translation of mRNA. Fructose 1,6-bisphosphate presumably acts as a feed-forward signal of an adequate supply of ATP (Kilberg *et al.*, 2005; Rabinowicz, 1996).

In intestinal mucosal cells, arginine and glutamine activate signalling pathways to increase protein synthesis and decrease autophagy-induced proteolysis (section 2.1.5.1). Glutamine activates the MAP kinase pathway and arginine the mTOR (mammalian target of rapamycin) kinase pathway, both of which lead to increased protein synthesis and cell proliferation, and reduced apoptosis. Activation of the mTOR kinase pathway leads to increased translation of the mRNA for ribosomal proteins, so permitting increased protein synthesis. Glutamine deprivation activates stress signalling pathways, leading to increased apoptosis (Kilberg *et al.*, 2005; Rhoads & Wu, 2009).

2.2.4 Protein quality (protein nutritional value)

The body's requirement is for amino acids in amounts that match the amounts being incorporated into newly synthesized proteins. A dietary protein that is relatively deficient in one or more of the essential amino acids will be less useful for tissue protein synthesis than one that provides (more than) enough of all the essential amino acids – i.e. it will be of lower nutritional value or quality. This is obviously important when dietary protein intakes are

marginal, although it is of less importance when intakes are higher than requirements.

The limiting amino acid of a protein is that essential amino acid present in the least amount relative to the requirement for complete utilization of the protein for tissue protein synthesis. Generally, cereal proteins are limited by their content of lysine, while legume and animal proteins are limited by their content of the sulphur amino acids (methionine + cysteine). The result of this is that there is complementation between different proteins eaten in the same meal, and judicious mixtures of proteins may have very much higher nutritional value, as determined experimentally, than would appear likely from the values of the individual proteins. Overall, mixed diets, regardless of the major protein sources, have a Biological Value (see next section) between 0.7–0.8.

2.2.4.1 Biological assays of protein quality Biological assays of protein quality are based on the ability of the test protein to support nitrogen balance or growth; in experimental animals, this may include carcase analysis to measure nitrogen retention in the body. Regardless of the method used, all biological methods of protein quality determination are a function solely of the limiting amino acid. Unless experimental diets are supplemented with the limiting amino acid, and the experiments repeated, such assays give no information about the other amino acids present in the test protein. Furthermore, biological assays give no information about relative surpluses of essential amino acids in the test protein, which are important for complementation in mixed diets (Bender, 1982).

- The Biological Value (BV) of a protein is the proportion of the absorbed protein that is retained in the body, i.e. it takes no account of the digestibility of the protein. It is determined by studies of nitrogen balance; endogenous urinary and metabolic faecal nitrogen losses are estimated from a group of control animals fed a protein-free diet. Proteins that are completely useable for tissue protein synthesis have $BV = 0.95\text{--}1.0$.
- Protein Efficiency Ratio (PER) is the gain in weight of growing animals per gram of dietary protein consumed. It therefore does not require determination of nitrogen balance, so has the advantage of simplicity. However, apparent PER varies with the amount of food consumed, the stage of growth of the test animal and the duration of the experiment. Furthermore, the weight gain is assumed to be protein, but it can include large and variable amounts of fat, which can give erratic and misleading results.
- Net Protein Ratio (NPR) was developed in order to overcome some of the problems associated with PER; a control group of animals is fed a

protein-free diet and the experiment lasts a standardized 10–14 days. NPR is the weight gain of animals fed on the test protein, plus the weight loss of the protein-free controls, divided by the protein consumed.

- Net Protein Utilization (NPU) is the proportion of dietary protein that is retained in the body, and therefore takes account of the digestibility of the protein: $NPU = BV \times \text{digestibility}$. By convention, NPU is measured at 10 per cent dietary protein (NPU_{10}), a level of intake at which all of the dietary protein can be used for tissue protein synthesis as long as the balance of essential amino acids is appropriate. When fed at 4 per cent of the diet, the result is termed NPU standardized. If the food or diet is fed as it is normally eaten, the result is NPU operative (NPU_{op}). Determination of NPU may be by nitrogen balance or carcass analysis.

2.2.4.2 Chemical analysis and protein quality Analysis of the essential amino acid content of a protein permits prediction of its nutritional value, subject to the problem of unavailable amino acids that are present in the protein but which are not released on digestion (section 2.1.2). The original such method compared the amino acid content of the test protein with that of a reference protein such as egg protein, which has a high BV. The amino acid present in the least amount relative to the content in the reference protein is the limiting amino acid, and the ratio of the amount in the test protein : that in the reference protein is the Chemical Score.

The Amino Acid Score compares the essential amino acid content of the test protein with a reference pattern of essential amino acid requirements (see Table 2.8) rather than the arbitrary comparison with the amino acid content of a protein of high BV. The Protein Digestibility Corrected Amino Acid Score (PDCAAS) is the Amino Acid Score $\times$ digestibility, and this is now the most widely used way of expressing protein quality. The main problems are in determining the appropriate amino acid reference pattern, and the use of faecal digestibility, which overestimates ileal or true digestibility because of the contribution of intestinal bacteria to faecal nitrogen (section 2.1.2; Schaafsma, 2000).

2.3 The fate of amino acid carbon skeletons and the thermic effect of protein

There is an increase in resting metabolic rate after a meal. This diet-induced thermogenesis is mainly the cost of synthesizing tissue reserves of glycogen, triacylglycerol and protein, although there is also the energy cost of absorbing the products of digestion. The diet-induced thermogenesis associated with

protein is considerably higher than that associated with carbohydrate or fat. Some 20–30 per cent of the energy consumed as protein is dissipated in diet-induced thermogenesis, compared with 5–10 per cent of that from carbohydrate and 0–3 per cent of that from fat (Jequier, 2002; Tappy, 1996). A number of mechanisms have been proposed to explain this thermic effect of protein: increased protein turnover (section 2.1.6.5); the energy cost of urea synthesis (section 1.6.2.1) and gluconeogenesis from amino acids; and the relative inefficiency of the catabolism of amino acid carbon skeletons.

Table 2.9 shows the metabolic fates of the carbon skeletons of amino acids. Most give rise to intermediates that can be used for gluconeogenesis, either as pyruvate or one of the intermediates of the citric acid cycle that lead to an increase in the intracellular concentration of oxaloacetate that can then be withdrawn for gluconeogenesis. Isoleucine, phenylalanine, tyrosine and tryptophan also give rise to ketogenic intermediates (acetyl CoA or acetoacetate), and leucine and lysine give rise only to ketogenic intermediates.

Acetoacetate and acetyl CoA can never be substrates for gluconeogenesis because, for each two carbons added to form citrate, two are lost as carbon dioxide in the citric acid cycle; thus, there is no gain of oxaloacetate that can be withdrawn for gluconeogenesis. They can be used in the fasting state for synthesis of ketone bodies, or directly as metabolic fuels, and in the fed state for fatty acid synthesis.

Figure 2.2 shows the entry points of amino acid carbon skeletons into the citric acid cycle. Pyruvate arising from glycine, serine, methionine, cysteine, tryptophan and alanine can be carboxylated to oxaloacetate by pyruvate

Table 2.9 Metabolic fates of the carbon skeletons of amino acids.

	Glucogenic intermediates	Ketogenic intermediates
alanine	pyruvate	–
arginine → ornithine	2-oxoglutarate	–
asparagine → aspartate	oxaloacetate	–
aspartate	oxaloacetate <i>or</i> fumarate	–
cysteine	pyruvate	–
glutamine → glutamate	2-oxoglutarate	–
glycine → serine	pyruvate	–
histidine → glutamate	2-oxoglutarate	–
isoleucine	propionyl CoA	acetyl CoA
leucine	–	acetoacetate <i>and</i> acetyl CoA
lysine	–	acetyl CoA
methionine	propionyl CoA	–
phenylalanine → tyrosine	fumarate	acetoacetate
proline → glutamate	2-oxoglutarate	–
tryptophan	pyruvate	acetyl CoA
valine	succinyl CoA	–

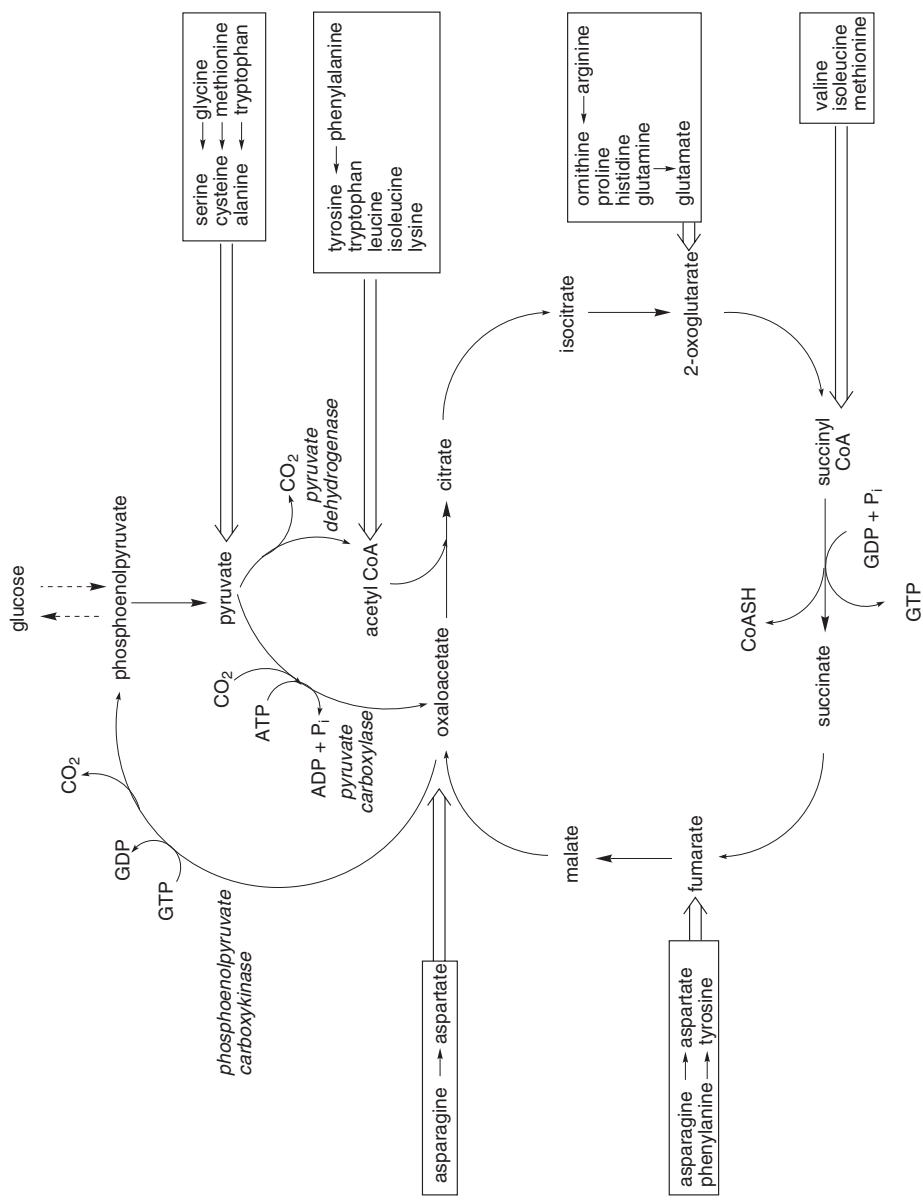


Figure 2.2 The entry of amino acid carbon skeletons into the citric acid cycle. Pyruvate dehydrogenase multi-enzyme complex EC 1.2.4.1 + 2.3.1.12 + 1.8.1.4, pyruvate carboxylase EC 6.4.1.1, phosphoenolpyruvate carboxykinase 4.1.1.38.

carboxylase. Oxaloacetate in excess of the amount required to maintain citric acid cycle activity can be withdrawn for gluconeogenesis by conversion to phosphoenolpyruvate, catalyzed by phosphoenolpyruvate carboxykinase.

Gluconeogenesis is an ATP-expensive process. For those amino acids that give rise to citric acid cycle intermediates, the cost of synthesizing glucose is $6 \times \text{ATP}$ equivalents per mol of glucose formed. The NADH needed in the cytosol for gluconeogenesis comes from the indirect export of oxaloacetate from the mitochondrion. In the mitochondrion, oxaloacetate is reduced to malate, which is exported and oxidized to oxaloacetate in the cytosol. This formation and utilization of NADH can be considered to add $\approx 2.5 \times \text{ATP}$ equivalents of non-productive thermogenesis.

For those amino acids that give rise to pyruvate, there is an additional $1 \times \text{ATP}$ per mol of pyruvate ($2 \times \text{ATP}$ per mol of glucose formed) for the carboxylation of pyruvate to oxaloacetate. In the fed state, if the resultant glucose 6-phosphate is isomerized to glucose 1-phosphate and used for glycogen synthesis in the liver, there is an additional requirement of $1 \times \text{UTP}$ (equivalent to ATP) per mol. If glucose is released from the liver and used for glycogen synthesis in muscle, then there is an additional requirement of $2 \times \text{ATP}$ equivalents per mol of glucose incorporated.

The citric acid cycle is generally considered to provide a pathway for the complete oxidation of amino acid carbon skeletons. This is clearly so for amino acids that yield pyruvate, acetyl CoA or acetoacetate (Table 2.9 and Figure 2.2). However, for amino acids that provide intermediates of the citric acid cycle, the cycle does not, *per se*, permit complete oxidation. This is because increasing the amount of any one of the intermediates of the cycle will, unless intermediates are withdrawn for synthesis of other metabolites, result in an increase in the amount of oxaloacetate over and above that which is required to maintain cycle activity. For complete oxidation, this excess oxaloacetate must be reduced to malate, exported from the mitochondrion into the cytosol, oxidized to oxaloacetate and then undergo phosphorylation (at the expense of GTP) and decarboxylation to phosphoenolpyruvate, followed by the pyruvate kinase reaction to yield pyruvate and ATP. This sequence of reactions is therefore thermogenic, with consumption of GTP and formation of ATP.

Tracing the pathways for catabolism and complete oxidation to carbon dioxide and water of the carbon skeletons of the amino acids permits calculation of the total ATP yield and the ATP and reduced coenzyme cost. Table 2.10 shows the total ATP yield and the ATP (or reduced coenzyme) cost of amino acid catabolism (as appropriate, with different values for alternative pathways). The difference between the total ATP yield and the ATP plus reduced coenzyme requirement for catabolism is the net yield of ATP per mol of amino acid catabolized. The ATP- or NADPH-utilizing steps can be considered as contributing to thermogenesis, and hence the thermic effect of

Table 2.10 ATP yield and thermogenesis from the oxidation of amino acid carbon skeletons.

	Gain				Consumed		Net gain	Thermo- genesis ATP equivalents
	ATP	NAD(P)H	FADH	ATP eq	ATP	NAD(P) H	ATP eq	
alanine	1	4	1	12.5	0	0	12.5	0
arginine	3	8	2	26	1	0	25	1
asparagine	2	4	1	13.5	1	0	12.5	1
aspartate	2	4	1	13.5	1	0	12.5	1
cysteine via pyruvate	1	4	1	12.5	0	0	12.5	0
cysteine via taurine	0	0	2	3	0	0	3	0
glutamate	3	7	2	23.5	1	0	22.5	1
glutamine	3	7	2	23.5	1	0	22.5	1
glycine via glyoxylate	0	0	0	0	0	1	-2.5	2.5
2 x glycine via serine	1	4	1	12.5	0	0	12.5	0
histidine	3	7	2	23.5	1	0	22.5	1
isoleucine	4	11	3	36	2	0	34	2
leucine	3	11	3	35	2	0	33	2
lysine via pipecolic acid	1	7	4	24.5	2	0	22.5	2
lysine via saccharopine	1	7	2	21.5	2	1	17	4.5
methionine, Cys via Tau	3	6	3	22.5	2	0	20.5	2
methionine, Cys via pyruvate	4	10	3	33.5	2	0	31.5	2
phenylalanine	4	11	3	36	2	2	29	7
proline	3	9	2	28.5	1	0	27.5	1
serine	1	4	1	12.5	0	0	12.5	0
threonine	1	6	2	19	0	0	19	0
tryptophan	3	12	3	37.5	0	0	37.5	0
tyrosine	4	11	3	36	2	1	31.5	4.5
valine	3	9	2	28.5	1	0	27.5	1

Source: Bender (2012).

protein. The consumption of ATP or reduced coenzymes can be considered to be equivalent to ‘unproductive’ or wasteful thermogenesis.

There is no such unproductive thermogenesis for alanine, cysteine, serine (or glycine metabolized via glyoxylate), threonine and tryptophan. For most other amino acids, there is unproductive thermogenesis of 1 or 2 × ATP.

Catabolism of lysine metabolized by way of saccharopine and of tyrosine involves $4.5 \times \text{ATP}$ equivalents of thermogenesis, and catabolism of phenylalanine $7 \times \text{ATP}$ equivalents (Bender, 2012).

2.4 Inter-organ metabolism of amino acids

Most amino acids are deaminated in the liver, and their carbon skeletons are used for either gluconeogenesis and ketogenesis (Table 2.9 and Figure 2.2) or to meet the liver's energy requirements. However, the branched-chain amino acids leucine, isoleucine and valine are mainly transaminated in skeletal muscle, where they provide a major source of amino groups for synthesis of alanine in the fasting state. The resultant branched-chain oxo-acids are either used for energy-yielding metabolism in muscle or are returned to the liver, where they are transaminated back to the corresponding amino acids, at the expense of alanine.

In the fasting state, there is a net output from skeletal muscle of all except the branched-chain amino acids, but there is considerably more alanine and glutamine than can be accounted for by the alanine and glutamine content of muscle proteins. Much of the glutamine comes from the large pool of free glutamine in muscle (section 2.2.3). Glutamine is taken up by the kidney and small intestine, where it is a substrate for gluconeogenesis and also for the synthesis of alanine, which is exported to the liver. The liver takes up alanine and transaminates it, either using the amino group to reaminate the branched-chain oxo-acids exported by muscle, or using transaminases linked to amination of glyoxylate to glycine or oxo-glutarate to glutamate (trans-deamination reactions, Figures 1.15 and 1.16). The resultant pyruvate is a substrate for gluconeogenesis.

This inter-organ glucose-alanine cycle, shown in Figure 2.3, provides an indirect way in which muscle glycogen can be used to maintain the plasma concentration of glucose in the fasting state. Muscle cannot liberate glucose from its glycogen reserves, because it lacks glucose 6-phosphatase. In the fasting state, muscle glycogen yields glucose 1-phosphate, which undergoes glycolysis as far as pyruvate, which is then transaminated to alanine at the expense of either branched-chain amino acids taken up from the circulation or other amino acids arising from the catabolism of muscle protein (Felig & Wahren, 1974).

As discussed in section 1.6.2.3, a significant amount of urea diffuses into the large intestine, where it is hydrolyzed by bacterial urease, yielding ammonium that is incorporated into bacterial proteins. Label from $[^{15}\text{N}]$ urea is found in both non-essential and essential amino acids in human tissues. To some extent this is the result of hydrolysis of bacterial proteins in the colon and absorption of the resultant amino acids. However, there is also absorption

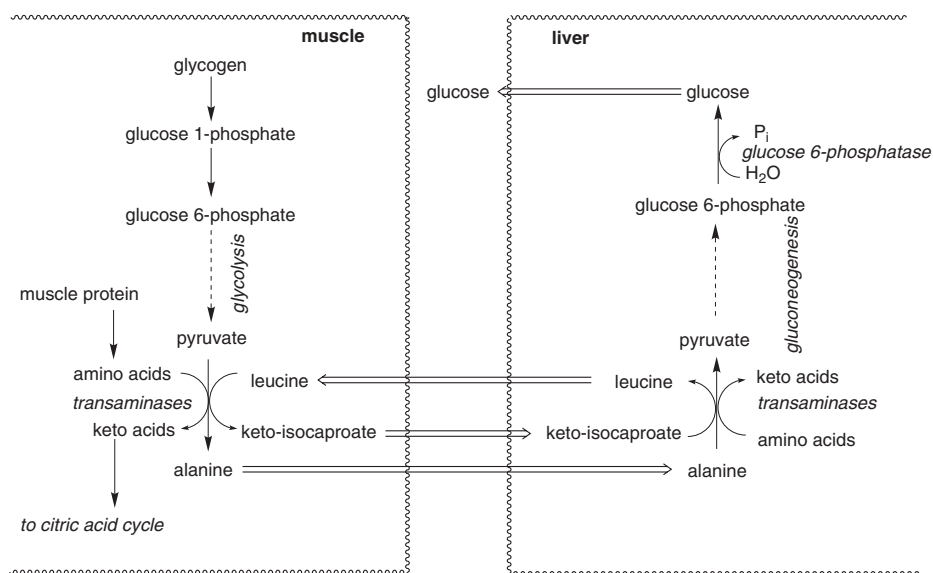


Figure 2.3 The inter-organ glucose-alanine cycle.
Glucose 6-phosphatase EC 3.1.3.9.

of ammonium from the gut. This can be incorporated into glutamate and glutamine in the liver (section 1.3.2), and then into other amino acids by transamination. Although there is no net synthesis of essential amino acids, the oxo-acids arising from transamination of essential amino acids, if not catabolized, will be available for transamination back to amino acids, so incorporating label from ammonium via glutamate (Jackson, 1995).

2.5 Transport of amino acids across membranes

Early studies of amino acid transport used hepatoma cells in culture, everted gut sacs and perfused kidney tubules, as well as observations in patients with genetic defects leading to aminoaciduria (see section 1.7.1). These studies showed that amino acids with chemically related side-chains compete with each other for transport. More recent studies have depended on cloning the genes for transport proteins into *Xenopus laevis* oocytes, and sometimes also inserting the gene products into synthetic lipid membranes in order to study them. Using the gene products, it is possible to raise antibodies and, therefore, to localize different transport proteins to the luminal and systemic sides of epithelial cells in the small intestine and kidney tubule.

In addition to intestinal uptake of dietary amino acids and renal reuptake of filtered amino acids, uptake of amino acids into tissues may be rate-limiting for their metabolism. As will be discussed in section 9.4.3, this is certainly so

for the synthesis of the neurotransmitter serotonin; changes in the rate of tryptophan uptake into the brain have a considerable effect on the rate of serotonin synthesis.

2.5.1 Families of amino acid transporters

The classification of amino acid transporters is on the basis of their specificity for the amino acids transported, and also whether or not they are sodium- or proton-dependent. The amino acid transporters contain 10–14 transmembrane domains, but there are a number of associated proteins with a single transmembrane domain that activate the transporters. Collectrin is one such single transmembrane protein that is essential for activity of large neutral amino acid transport. Knockout mice excrete not only the large neutral amino acids but also proline and glycine, suggesting that collectrin is essential for the function of other transporters as well.

Collectrin is not expressed in the intestinal mucosa, but the angiotensin-converting enzyme ACE2 (a carboxypeptidase) serves the same function. Collectrin and ACE2 show considerable sequence homology, and both are expressed in the kidney, but here ACE2 does not activate amino acid transport (Verrey *et al.*, 2009).

In general, the basolateral transporters in the intestinal mucosal cells are the same as those found in other organs, including the blood-brain barrier, and they permit either efflux of dietary amino acids into the circulation, or uptake of amino acids from the circulation in the fasting state. Several of the apical transporters are unique to the intestinal epithelial cell membrane (Brandsch, 2006; Collarini & Oxender, 1987; Kilberg *et al.*, 1993). The γ -glutamyl cycle of glutathione metabolism (see section 5.4.5) may also be involved in membrane transport of amino acids, and 5-oxoproline formed from glutathione stimulates the activity of sodium-dependent amino acid transporters (Goldberg, 1980; Hawkins *et al.*, 2006).

System A preferentially transports amino acids with short, polar or linear side-chains. It is sodium-dependent. While system A transports glycine, there is also a glycine-specific sodium-dependent transporter – system Gly. There is *de novo* synthesis of system A protein when cells are incubated in an amino acid-free medium, and it is subject to trans-inhibition – the presence of the substrates inside the cell inhibits further uptake. However, the synthesis of the system A protein, or its translocation to the cell surface, increases in response to insulin in most tissues, permitting increased amino acid uptake for protein synthesis.

In hepatocytes, system A synthesis and translocation to the cell surface also increase in response to glucagon, permitting increased uptake of the small neutral amino acids that are major substrates for gluconeogenesis. This response to glucagon is in two phases. Initially, there is an increase of 20–40

per cent that is not inhibited by cycloheximide, and is mainly due to translocation of existing transporter protein to the cell surface. After a time lag, there is a 5–10 fold increase in activity due to new protein synthesis. It is not clear whether this is *de novo* synthesis of the transport protein itself, or a single transmembrane domain-activating protein.

System ASC transports alanine, serine, glycine and proline, and it is subject to trans-stimulation – the presence of the substrates inside the cell increases the rate of uptake from the incubation medium. Again, it is sodium-dependent.

System N has been identified in hepatocytes, muscle and placenta. It transports glutamine, asparagine and histidine, and it is sodium-dependent. There is an intercellular glutamine cycle in the liver; the periportal hepatocytes that receive blood from the hepatic portal vein take up glutamine using the system N transporter and hydrolyze it, releasing ammonium. The perivenous hepatocytes (some 10 per cent of the total, localized as a ring of 1–3 cells around the terminal central venules) synthesize glutamine from any ammonium remaining, so preventing the entry of ammonium into the peripheral circulation (Haussinger, 1990; Haussinger *et al.*, 1992). Glutamine efflux from these cells is sodium-independent.

In the kidney, there is basolateral uptake of glutamine from the circulation in the distal part of the proximal tubule for the production of ammonium to regulate urine pH. In response to acidosis, the activity of this transporter is increased, and it is also localized in the earlier part of the proximal tubule.

The system L neutral amino acid transporter carries the aromatic and branched-chain amino acids. It is sodium-independent, but it may be proton-dependent; there is some evidence of leucine-proton co-transport. Like the system ASC transporter, the system L transporter is subject to trans-stimulation. There are two system L transporters in hepatocytes: a low-affinity high-capacity transporter and one with high affinity but low capacity. This latter transporter is presumably important for hepatic uptake of amino acids to meet the liver's needs for protein synthesis.

The system T transporter was originally described in erythrocytes; it is sodium-independent, and it transports aromatic amino acids, with a preference for tryptophan. It is also present in hepatocytes and in the kidney.

In several tissues, including the brain, intestine and kidney, proline, glycine and alanine are carried by the PAT1 proton-dependent transporter, which has a low affinity and high capacity. It co-transportes one proton and one amino acid, and it also transports short-chain fatty acids (acetate, propionate and butyrate – Metzner *et al.*, 2006).

Positively charged (basic) amino acids are transported by the sodium-independent cationic amino acid transporter (sometimes also known as the system y^+ transporter). Like system A, this transporter is repressed when the intracellular concentrations of lysine and arginine rise; unlike system A, it is

de-repressed when the concentration of any of the essential amino acids (not just its substrates) falls. It is unaffected by limitation of non-essential amino acids. This amino acid responsiveness is regulation by the amino acid-sensing mechanism associated with eIF2 (eukaryotic initiation factor, section 2.2.3).

Although this protein catalyzes bidirectional transport, it permits amino acid accumulation against a concentration gradient because of the membrane potential across the plasma membrane. There is very low activity of this transporter in hepatocytes. As noted in section 5.9.1, net synthesis of arginine occurs in the kidney; in the liver, arginine is an intermediate in the synthesis of urea (section 1.6.2.1), and lack of the y^+ transporter protects extra-hepatic arginine from the action of liver arginase (Hatzoglou *et al.*, 2004).

The main transporter for negatively charged (acidic) amino acids is the sodium-dependent x^-ag carrier that transports both aspartate and glutamate. In foetal cells, there are two separate transporters: the x^-a protein preferentially transports aspartate and is sodium-dependent, while the x^-c protein is a sodium-independent transporter for glutamate and cysteine. Mature hepatocytes have no x^-a carrier and low activity of the x^-c carrier, although this latter is induced in response to insulin.

System $b^{0,+}$ is a sodium-independent transporter that carries both neutral and cationic amino acids, so that its specificity overlaps those of systems L and y^+ . It shows preference for amino acids with bulky unbranched side-chains. System $B^{0,+}$ is a sodium-dependent carrier with specificity similar to that of system $b^{0,+}$, but it also transports branched-chain amino acids. It is found in fibroblasts and endothelial cells, but not hepatocytes.

There are also heterodimeric amino acid transporters that are essentially dimers of two different transport proteins that act in opposite directions, so transporting one amino acid into a cell and another out (Wagner *et al.*, 2001). These include:

- two system L heterodimers that carry out sodium-independent exchange of large or small neutral amino acids;
- two system y^+L heterodimers that exchange dibasic amino acids and large or small neutral amino acids;
- an X^-c dimer that exchanges glutamine and cystine;
- a system asc dimer that exchanges small neutral amino acids and also transports D-serine;
- a system $b^{0,+}$ dimer that exchanges neutral and dibasic amino acids and is also involved in reabsorption of cysteine, arginine, lysine and ornithine in the kidney.

Mitochondrial uptake of amino acids is indirect. Amino acids are transaminated on the cytosolic side of the inner mitochondrial membrane, generally linked to pyruvate or 2-oxoglutarate as the amino acceptor. The oxo-acids enter the mitochondrion and are reaminated by glutamine-dependent transaminases. Net influx of oxo-acids and, hence, concentrative uptake of amino acids, is ensured because the product of glutamine transamination – 2-oxoglutarate – undergoes non-enzymic cyclization if it is not deamidated by ω -amidase (see section 5.3.2), so that the substrate for the back reaction is not available (Cooper & Anders, 1990).

2.5.1.1 Dipeptide transport There are at least two di- and tripeptide transporters: PEPT1 in the gut, liver, bile duct, kidney and pancreas; and PEPT2 in kidney, lung, brain, mammary gland and eye. Both are proton symporters and are examples of tertiary active transport. The proton gradient for peptide transport is generated by an Na^+/K^+ ATPase at the basolateral membrane and an Na^+/H^+ exchanger at the apical membrane. These peptide transporters also carry β -lactam antibiotics, ACE inhibitors and other peptidomimetic drugs, but not free amino acids or tetrapeptides. PEPT2 may be involved in glutathione metabolism (see section 5.4.4) by providing cysteinyl-glycine from extracellular glutathione (Biegel *et al.*, 2006).

Further reading

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

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3

The Role of Vitamin B₆ in Amino Acid Metabolism

The central importance of vitamin B₆ in amino acid metabolism is demonstrated by the lactic acid bacteria. Vitamin B₆ is a growth factor for these organisms. When they are grown without it, all of the amino acids are required for growth. However, when they are grown in the presence of vitamin B₆, none of the amino acids is essential – all can be synthesized. Some 4 per cent of the enzyme-catalyzed reactions in the IUPAC-IUB Enzyme Commission list (more than 140 enzymes) are vitamin B₆ dependent, and some 1.5 per cent of prokaryotic genes (but a smaller percentage of eukaryotic genes) code for vitamin B₆-dependent reactions (Amadasi *et al.*, 2007). As discussed later in this chapter, in section 3.5, a small number of enzymes catalyze reactions similar to those catalyzed by vitamin B₆-dependent enzymes but use pyruvate as the carbonyl catalyst.

The metabolically active form of vitamin B₆ is pyridoxal 5'-phosphate. As shown in Figure 3.1, the alcohol (pyridoxine), the aldehyde (pyridoxal) and the amine (pyridoxamine) can all be phosphorylated, and pyridoxine and pyridoxamine phosphates can be oxidized to pyridoxal phosphate. As will be seen in section 3.3, pyridoxamine phosphate is also formed in the half-reaction of transamination, and transaminases can catalyze the interconversion of pyridoxamine and pyridoxal phosphates.

Although the name 'pyridoxine' now applies specifically to the alcohol form of the vitamin, at one time it was in use as a generic descriptor for all six biologically active vitamers, so some of the older literature is confusing; 'vitamin B₆' is now the accepted generic descriptor. 4-Pyridoxic acid is the main excretory metabolite of vitamin B₆; it has no biological activity and the oxidation of pyridoxal to the acid is irreversible.

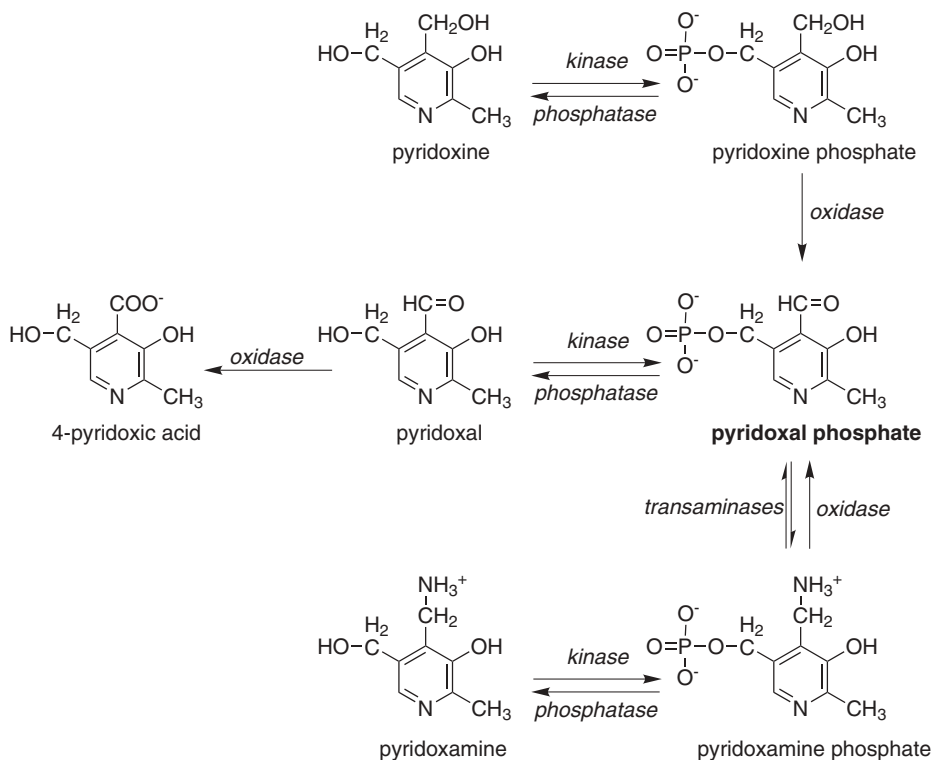


Figure 3.1 Interconversion of the vitamin B₆ vitamers.

Pyridoxal kinase EC 2.7.1.38, pyridoxine oxidase EC 1.1.1.65, pyridoxamine phosphate oxidase EC 1.4.3.5, pyridoxal oxidase EC 1.1.3.12.

3.1 Pyridoxal phosphate-dependent reactions

As shown in Figure 3.2, in the absence of substrate, pyridoxal phosphate is covalently bound to the enzyme, forming an aldimine (Schiff base) to a lysyl residue. The substrate amino acid displaces the lysyl residue, forming a substrate aldimine (see Figure 3.3 for the steps involved in this transaldimination reaction). This means that it is difficult to resolve pyridoxal phosphate-dependent enzymes to yield the apoenzyme. The coenzyme cannot be removed by dialysis except in the presence of substrate, when the substrate aldimine has been formed, or, in the case of transaminases (section 3.3), after the half-reaction in which pyridoxamine phosphate has been formed. Pyridoxamine phosphate is not covalently bound to the enzyme, and it can be removed by dialysis.

In decarboxylation (see section 3.4), the α -carbon-carboxyl bond of the substrate is cleaved, with release of an amine and carbon dioxide. Some

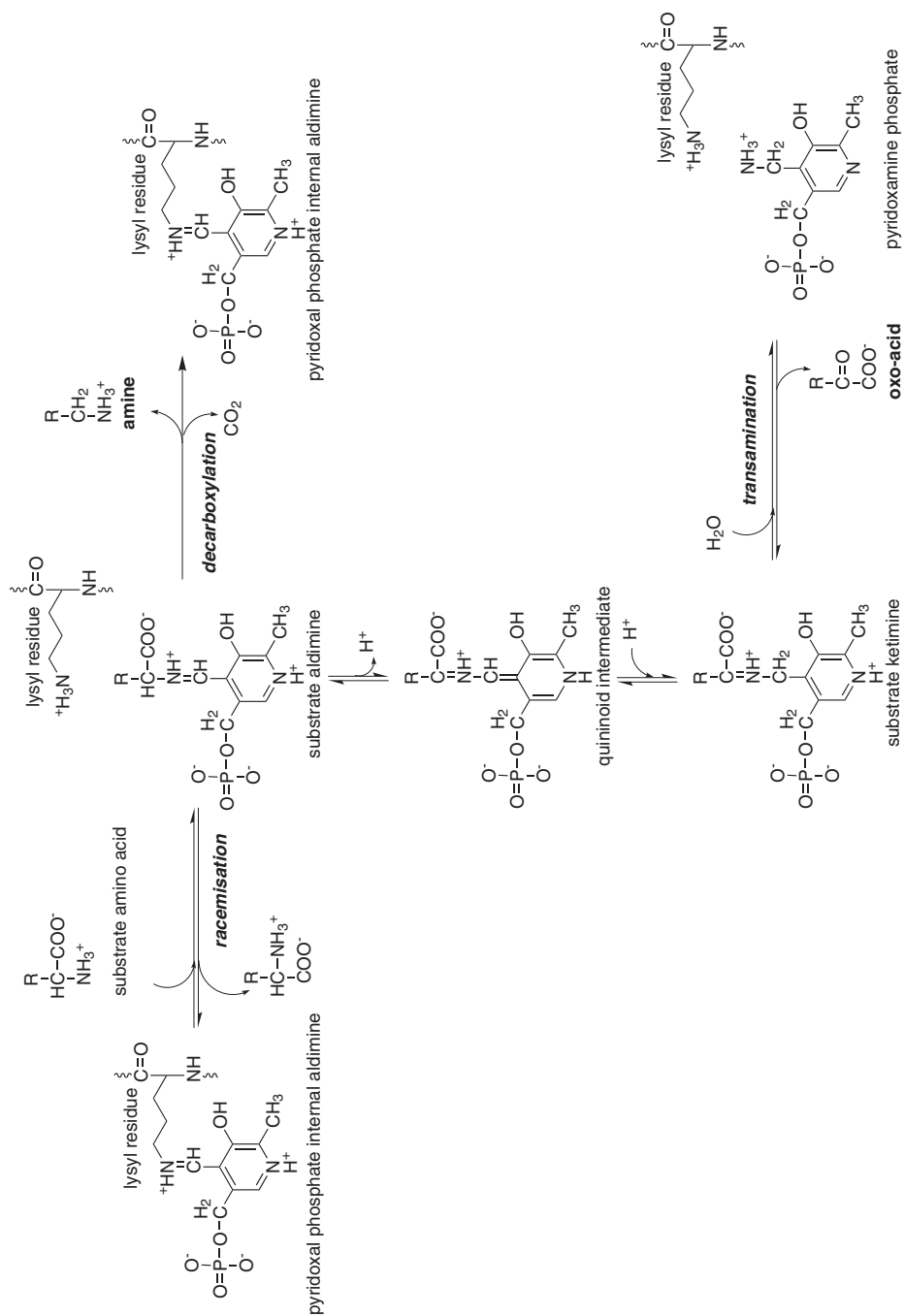


Figure 3.2 Pyridoxal phosphate-catalyzed reactions of amino acids.

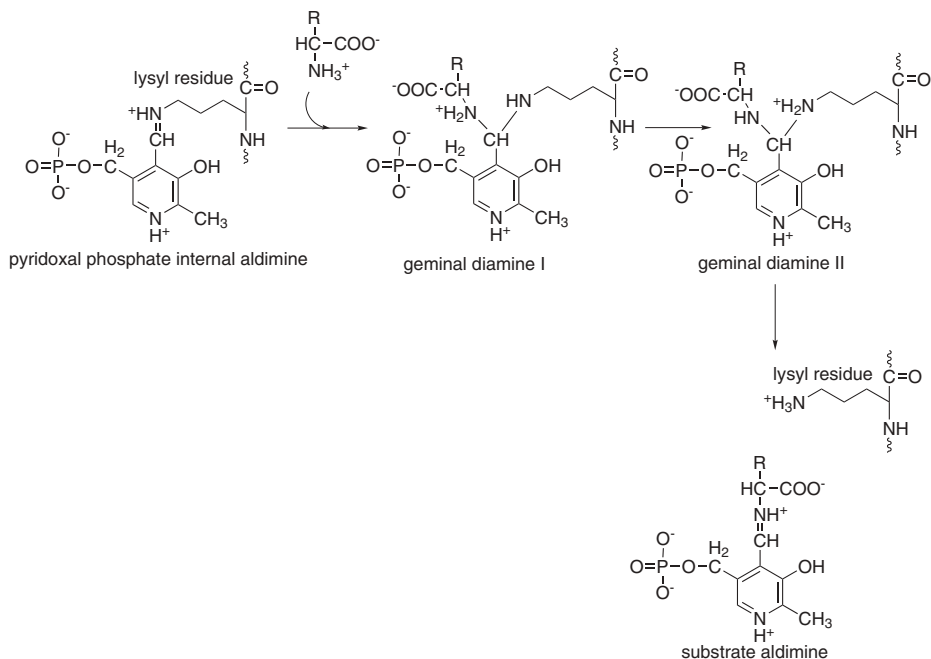


Figure 3.3 The transaldimination reaction in pyridoxal phosphate-catalyzed reactions.

enzymes catalyze cleavage of bonds at the β - or γ -carbon of the substrate aldimine, leading to side-chain elimination and replacement reactions (Table 3.1).

In transaminases, the substrate aldimine is deprotonated to a quinonoid intermediate, then reprotonated to yield a ketimine, followed by cleavage of the α -carbon-amino bond, forming the oxo-acid and leaving pyridoxamine phosphate at the active site of the enzyme. An acceptor oxo-acid then undergoes the reverse sequence of reactions to form its corresponding amino acid and pyridoxal phosphate at the active site, which then reforms the internal aldimine with the reactive lysine residue.

In racemases (section 3.2), the quinonoid intermediate is reprotonated on the opposite side from which the proton was abstracted from the original substrate aldimine, yielding a substrate aldimine with the opposite stereochemistry to that of the original. Displacement of the substrate by the reactive lysyl residue reforms the internal aldimine and releases the isomerized amino acid (Tunncliffe & Ngo, 1998).

Deprotonation and reprotonation of the intermediate could occur stereospecifically, at either the *si* or *re* face of the planar aldimine (i.e. either above or below the plane of the ring), or non-stereospecifically. As would be expected, racemases show no stereospecificity of deprotonation and reprotonation, but they can act at either face of the aldimine. By contrast, most

Table 3.1 Pyridoxal phosphate-catalyzed enzymic reactions.

Reaction	Bond cleaved	Reaction	
Reactions at α -carbon			
racemization	$\alpha\text{C-H}$	L-amino acid $\rightleftharpoons$ D-amino acid	(section 3.2)
transamination	$\alpha\text{C-NH}_3^+$	amino acid $\rightleftharpoons$ oxo-acid + PMP	(section 3.3)
decarboxylation	$\alpha\text{C-COO}^-$	amino acid $\rightarrow$ amino + CO_2	(section 3.4)
oxidative deamination	$\alpha\text{C-NH}_3^+$, $\alpha\text{C-H}$	amine $\rightarrow$ aldehyde + NH_4^+	
loss of side-chain	$\alpha\text{C-}\beta\text{C}$	threonine aldolase serine, hydroxymethyltransferase	(section 6.1.5) (section 4.2.1)
Reactions at β -carbon			
elimination	$\beta\text{C-X}$	tryptophanase, kynureninase	(section 9.4.2) (section 9.4.4.2)
elimination	$\alpha\text{C-H}$	serine deaminase aspartate, β -decarboxylase	(section 4.6.2), (Figure 3.7)
replacement	$\beta\text{C-X}$, $\alpha\text{C-H}$	tryptophan synthase	(section 9.1.3)
Reactions at γ -carbon			
elimination	$\gamma\text{C-X}$, $\alpha\text{C-H}$	homocysteine desulphydrylase	
replacement	$\gamma\text{C-X}$, $\alpha\text{C-H}$	cystathionine γ -synthetase	(section 6.3.1.1)

transaminases, and other enzymes that undergo transamination as a side-reaction (section 3.4.1), catalyze deprotonation and reprotonation specifically at the *si* face. D-amino acid transaminase and branched-chain L-amino acid transaminase catalyze deprotonation and reprotonation at the *re* face, and the arrangement of amino acid side-chains at the catalytic site is opposite to that of those in the transaminases that catalyze reactions at the *si* face (Yoshimura *et al.*, 1996).

Although most pyridoxal phosphate-dependent reactions involve proton abstraction and the formation of a carbanion intermediate that is stabilized by the coenzyme ring and the formation of the Schiff base, 1,2-aminomutases use pyridoxal phosphate to stabilize a free radical intermediate (Toney, 2005).

In non-enzymic model systems, both pyridoxal and pyridoxal phosphate will form aldimines with a variety of amino acids and, in the presence of catalysts such as Cu^{2+} , Fe^{3+} or Al^{3+} to stabilize the planar arrangement of the π -bond system of the aldimine, these can undergo the various reactions catalyzed by pyridoxal phosphate-dependent enzymes shown in Table 3.1.

In enzyme-catalyzed reactions, pyridoxal phosphate is required, not free pyridoxal. The 5'-phosphate group and the 2-methyl group are required for binding to the enzyme in the correct alignment to permit rotation of the

substrate aldimine to bring the appropriate bonds close to the various reactive amino acid residues that make up the catalytic site. Thus, in these reactions there is, as might be expected, a high degree of specificity for the reaction catalyzed.

Nevertheless, as discussed in section 3.4.1, the aldimine at the active site of enzymes other than transaminases can occasionally undergo tautomerization to the ketimine, leading to cleavage of the α -carbon-amino bond, leaving pyridoxamine phosphate at the active site. Since the enzyme does not have a binding site for an acceptor oxo-acid, this leads to inactivation of the enzyme, and the pyridoxamine phosphate can only be displaced when the concentration of pyridoxal phosphate is relatively high.

The main way in which enzymes control the reaction specificity is that the bond around the α -carbon that is perpendicular to the plane of the pyridoxal ring is most readily broken, because it has the largest overlap between its electron cloud and the extended π -electron cloud of the imine and the ring. In transaminases, racemases and other enzymes in which the α -hydrogen is removed (forming the quinonoid intermediate), it is the α -carbon-hydrogen bond that is perpendicular to the plane of the ring. In decarboxylases, it is the α -carbon-carboxyl bond that is perpendicular to the plane of the ring.

Subsequent stages in the various reactions are catalyzed by electron withdrawing or by donating groups of amino acid residues that make up the catalytic site. In many pyridoxal phosphate-dependent enzymes, there is a considerable conformational change on binding the substrate. This does not affect the substrate specificity significantly, but it does affect the reaction specificity, bringing reactive groups adjacent to the bond(s) to be broken (Eliot & Kirsch, 2004; Hayashi, 1995; Toney, 2005).

In addition to reactions with amino acids and amino acid derivatives, pyridoxal phosphate is the coenzyme for glycogen phosphorylase. Here it is the phosphate group that is the reactive moiety, rather than the carbonyl group. The coenzyme is bound to the enzyme by an internal aldimine to a lysyl residue, but reduction of the aldimine with sodium borohydride does not affect enzyme activity (Helmreich, 1992; Palm *et al.*, 1990).

Pyridoxal phosphate is the coenzyme for Δ^6 desaturase in polyunsaturated fatty acid metabolism (Cabrini *et al.*, 2005). It also has a role in modulating tissue responsiveness to steroid hormones, forming an aldimine with a lysyl residue in the hormone receptor. This results in release of the hormone-receptor complex from DNA binding, so terminating the action of the hormone (Bender, 1987).

As well as pyridoxal phosphate, some enzymes contain additional coenzymes (section 9.5; Amadasi *et al.*, 2007; Hayashi *et al.*, 1990):

- Mammalian cystathionine β -synthetase (see section 6.3.3) contains a haem group.

- Mammalian GABA transaminase (section 5.5.1) has a non-haem iron-sulphur cluster.
- Lysine 2,3-aminomutase (section 6.3.5) contains *S*-adenosylmethionine.
- Glutamate decarboxylase from *E. coli* contains both pyridoxal phosphate and also pyrroloquinoline quinone.

3.1.1 Families of pyridoxal phosphate-dependent enzymes

Pyridoxal phosphate-dependent enzymes can be grouped into five families on the basis of their structure and fold type. These separate families of proteins seem to have arisen very early in evolution, before the divergence of the three kingdoms, and within each fold type (except type V) there are examples of enzymes that catalyze all of the main reactions of pyridoxal phosphate-dependent enzymes shown in Table 3.1. Thus, transaminases are found in fold types I and IV, and racemases are found in fold types I, II and III. Fold type V includes only glycogen and starch phosphorylases, where it is the phosphate, rather than the carbonyl group, that is the catalytic moiety of the coenzyme (Amadasi *et al.*, 2007; Christen & Mehta, 2001; Eliot & Kirsch, 2004; Yoshimura & Goto, 2008).

- Fold type I enzymes (the aspartate transaminase family) are homodimers with two active sites at the interface of the two monomers, and each monomer contributes amino acid side-chains to both active sites.
- Fold type II enzymes (the tryptophan synthase family) are again homodimers, but each active site is formed from amino acids from only one monomer unit. These enzymes frequently have additional regulatory binding sites. Threonine synthase (section 6.1.4) and cystathionine β -synthase (section 6.3.3) are both allosterically regulated by *S*-adenosylmethionine; the biosynthetic isoenzyme of threonine deaminase (section 6.1.5) is allosterically regulated by isoleucine and valine.
- Fold type III enzymes (the alanine racemase family) are structurally very different from types I, II and IV, but again they are obligatory homodimers, because both monomers contribute amino acid side-chains to both active sites.
- Fold type IV enzymes (the D-amino acid transaminase family) are structurally similar to types I and II, but the pyridoxal phosphate is bound in

a mirror image to that in types I and II, so that it is the *re* rather than *si* face that is exposed to the solvent.

3.2 Amino acid racemases

D-amino acids have long been known to be important constituents of bacterial peptidoglycans and some antibiotics which are synthesized enzymically, rather than ribosomally. It was originally believed that the main function of mammalian D-amino acid oxidase and D-aspartate oxidase (see section 1.5.1) was to detoxify the small amounts of D-amino acids that may be absorbed from intestinal bacteria. More recently, free, endogenously synthesized D-amino acids have been identified in mammalian tissues, including the central nervous system, and D-amino acids have been identified in a number of proteins and peptides that are synthesized on ribosomes. These may arise as a result of post-synthetic modification of the protein or they may arise as a result of accidental damage to (long-lived) proteins. In aquatic crustaceans and some bivalve molluscs, D-alanine accumulates as a major osmolyte when the salinity of the environment increases.

Most of the amino acid racemases are pyridoxal phosphate-dependent, but cofactor-independent aspartate, glutamate and proline racemases have been identified in archaea, bacteria and fungi, and also proline racemase in *Trypanosoma cruzi*, the parasite that causes Chaga's disease. Diaminopimelate epimerase (see section 6.2.1) is also a cofactor-independent isomerase. These enzymes have a reactive cysteine residue at the active site.

Pyridoxal phosphate-dependent enzymes in bacteria include alanine racemase (which has a low substrate specificity and will catalyze the racemization of other amino acids as well), arginine and serine racemases. In eukaryotes, there are serine, aspartate and alanine racemases. Bacterial alanine racemase is a fold type III enzyme, while eukaryotic serine racemase is fold type II (Yoshimura & Goto, 2008).

In addition to amino acid racemases, bacterial D-amino acid transaminase is a significant source of D-amino acids for synthesis of proteoglycan and other D-amino acid-containing peptides. D-Amino acid transaminase is also found in germinating pea seedlings (Hayashi *et al.*, 1990). D-Glutamate may be formed in two different ways. Some bacteria have glutamate racemase, while it is formed in *Bacillus* spp. and higher plants by a transamination reaction between D-alanine and 2-oxoglutarate. In *Staphylococcus* spp., both pathways are used (Amadasi *et al.*, 2007).

3.2.1 Bacterial alanine racemase

Bacterial alanine racemase has been much studied, because of the possibility of developing specific inhibitors that would be clinically useful antibacterial

agents. D-amino acids are required for the synthesis of bacterial peptidoglycan; eukaryotes do not synthesize peptidoglycan. Most bacteria have two isoenzymes of alanine racemase: one is induced by D-alanine and is presumably mainly important in catabolism of excess D-alanine; the other is constitutive and is required for the production of D-alanine for peptidoglycan synthesis (Hayashi *et al.*, 1990).

The reaction of bacterial alanine racemase involves deprotonation of the α -carbon to yield a symmetrical anionic intermediate that is stabilized by formation of the pyridoxal quinonoid intermediate. This intermediate then undergoes reprotonation on the opposite side. Two separate bases are involved in the deprotonation/reprotonation reactions. They lie on opposite sides of the substrate aldimine. Tyrosine-265 acts to deprotonate the substrate aldimine formed by L-alanine, or to reprotonate the quinonoid intermediate to yield L-alanine. Lysine-39 acts to deprotonate the substrate aldimine formed by D-alanine, or to reprotonate the quinonoid intermediate to yield D-alanine. It is unclear how the proton is transferred between the lysyl and tyrosyl residues; it may occur via the carboxyl group of the quinonoid intermediate or via the phosphate group of pyridoxal phosphate (Yoshimura & Goto, 2008).

3.2.2 Eukaryotic serine racemase

The concentration of D-serine in the mammalian brain is about one-third of that of L-serine, and higher than that of most essential amino acids. It has a clear role as a neurotransmitter or neuromodulator and is synthesized in both neurons and glia. It binds to the so-called glycine binding site of the NMDA-type glutamate receptor (so called because it was originally thought that the endogenous ligand for this site was glycine) and potentiates the response to glutamate.

Serine racemase knockout mice have very low brain concentrations of D-serine and much reduced NMDA neurotransmission (Wolosker *et al.*, 2008). The NMDA receptor only opens to allow calcium and sodium influx when both the glutamate binding site (which binds glutamate or N-methyl-D-aspartate) and the glycine site (which binds glycine or D-serine) are occupied (Schell, 2004). In the silkworm (*Bombyx mori*), the activity of serine racemase and the tissue concentration of D-serine both increase considerably during metamorphosis, suggesting a morphogenic signalling role for D-serine. Silkworm serine racemase is distinct from alanine racemase and it has only very slight activity towards alanine (Uo *et al.*, 1998).

Serine racemase has been identified in fungi, plants, invertebrates and mammals, and in all species studied it also catalyzes an α - β elimination reaction from either L- or D-serine, yielding pyruvate, ammonium and water – the same reaction as mammalian liver L-serine deaminase (which does not have

any racemase activity and does not act on D-serine – see section 4.6.2). The α - β elimination and racemase activities of the enzyme are more or less equal.

In addition to pyridoxal phosphate, the enzyme also requires a divalent cation and ATP (which is an allosteric activator) for activity. There is little D-amino acid oxidase activity in the brain regions that are rich in D-serine, and the α - β elimination reaction seems to be the main way in which D-serine is catabolized in the central nervous system (Baumgart & Rodriguez-Crespo, 2008; De Miranda *et al.*, 2002; Foltyn *et al.*, 2005; Neidle & Dunlop, 2002).

Although serine racemase (which is a fold type II enzyme) has no structural similarity to bacterial alanine racemase (which is a fold type III enzyme), it has a similar mechanism of action, and two bases are involved in the deprotonation/reprotonation reactions. In the enzyme from *Saccharomyces pombe*, these are lysine-57 and serine-82, which lie on opposite sides of the substrate aldimine from each other. Lysine-57 is the residue that forms the internal aldimine with pyridoxal phosphate. Mutation of serine-82 leads to loss of racemase and D-serine deaminase activity, but it does not affect L-serine deaminase activity. This suggests that serine-82 is involved in the deprotonation of D-serine and the reprotonation of the quinonoid intermediate to form D-serine, while lysine-57 is involved in the deprotonation of L-serine and the reprotonation of the quinonoid intermediate to form L-serine (Yoshimura & Goto, 2008).

In rat liver serine deaminase (which does not have racemase activity) the proton that is required for the protonation of the hydroxyl group to catalyze α - β elimination is donated by the phosphate group of pyridoxal phosphate. Elimination of the α -hydrogen and β -hydroxyl group leads to the formation of the Schiff base of pyridoxal phosphate aminoacrylate. The internal aldimine is then re-formed by displacement of aminoacrylate by the lysine that forms the internal aldimine. The aminoacrylate is then hydrolyzed to pyruvate and ammonium.

In serine racemase, it is not clear whether the proton for α - β elimination comes from the phosphate group of pyridoxal phosphate, as in serine deaminase, or whether it is the proton that has been abstracted from the α -carbon by one of the catalytic bases, lysine-57 or serine-82, depending on the stereochemistry of the substrate (Yoshimura & Goto, 2008).

3.2.3 D-Aspartate in eukaryotes

Just as D-serine has a role in signalling during morphogenesis in the silkworm (see previous section), D-aspartate has a role in brain development in birds and mammals. In embryonic birds and early post-natal mammals, there are transient peaks of D-aspartate that coincide with specific periods of development. There is an aspartate racemase in the hypothalamus, and possibly other

brain regions, and D-aspartate oxidase is widely distributed in bacteria, fungi and animals.

D-aspartate is also a neurotransmitter or neuromodulator. It is released from synaptic vesicles in response to chemical or electrical stimulation, causes an increase in neuronal concentration of cAMP and is taken up into presynaptic neurons from the synaptic cleft by a specific transporter. In the hypothalamus, it stimulates the secretion of gonadotrophin releasing hormone and induces the synthesis of oxytocin and vasopressin mRNA. In the pituitary, it stimulates the secretion of prolactin, luteinizing hormone and growth hormone (D'Aniello *et al.*, 1993; Homma, 2007).

D-aspartate is also the precursor for endogenous synthesis of *N*-methyl-D-aspartate (NMDA), which is a potent agonist of the NMDA-type of glutamate receptor.

3.2.4 D-Amino acids in aquatic invertebrates

Aquatic crustaceans and some bivalve molluscs accumulate large amounts of D-alanine in their tissues, and the amount increases as the salinity of their environment increases, suggesting that D-alanine has a role in osmoregulation. They have an alanine racemase that both synthesizes D-alanine as salinity increases and also isomerizes it to L-alanine when salinity falls. Some species also accumulate relatively large amounts of L-glutamine, proline and glycine as osmolytes when salinity increases (Abe *et al.*, 2005).

3.2.5 D-Amino acids in gene-encoded peptides and proteins

The dermorphins and deltorphins in amphibian skin secretions are potent opioid hepta-peptides that have D-alanine as the second amino acid, and the deltorphins also contain D-methionine. Dermorphins are the most potent μ -opioid agonists known, with a potency some 1,000-fold higher than morphine on a molar basis. D-alanine is essential for their biological activity. Deltorphins are δ -opioid receptor agonists.

The gene sequences show that D-alanine is coded for by the normal L-alanine codon (GCG) and D-methionine by the normal codon for L-methionine (AUG). The L-amino acid is incorporated on the ribosome, then, before the signal peptide sequence has been cleaved, the amino acid undergoes isomerization. This most likely occurs by deprotonation at the α -carbon, followed by reprotonation, as occurs in amino acid racemases.

There are two neuropeptides in the African giant land snail that contain D-amino acids: D-asparagine in fullicin and D-phenylalanine in achatin. Again, it is likely that these peptides are synthesized ribosomally, incorporating the

L-amino acids, followed by post-synthetic modification to yield the D-amino acids (Kreil, 1997).

The aga toxins from the venom of the funnel web spider *Agelenopsis aperta* are 48 amino acid peptides that differ from each other only in that amino acid 46 may be L- or D-serine. The L-serine is slowly racemized to D-serine by a peptide isomerase that has considerable sequence homology with serine proteases (section 2.1.1). There is no evidence that pyridoxal phosphate is involved in the reaction, but it is likely that the reaction proceeds by way of α -carbon deprotonation and reprotonation, as in the pyridoxal phosphate-dependent amino acid racemases (Kreil, 1997).

Some D-amino acids in eukaryotic proteins accumulate as a result of age-related non-enzymic isomerization. D-aspartate accumulates in crystallin in the lens of the eye (a protein that apparently does not turn over throughout life) at a rate of 0.14 per cent per year. However, it can be re-isomerized to L-aspartate by way of methylation catalyzed by a protein carboxymethyltransferase that recognizes D-aspartyl residues, followed by ester hydrolysis that involves isomerization back to L-aspartate. Two aspartyl residues in crystallin are especially sensitive to isomerization and, indeed, the ratio of D- to L-aspartyl residues may exceed 1. D-aspartyl residues also occur in amyloid plaque in the brain in Alzheimer's disease, and in the aorta in atherosclerosis (Fujii, 2005).

There are two groups of protein carboxymethyltransferases, both of which use *S*-adenosylmethionine (section 6.3.2) as the methyl donor (Clarke, 1985; Shimizu *et al.*, 2005). Type I carboxymethyltransferases catalyze the methylation of specific aspartyl and glutamyl residues in target proteins, with a clear and consistent stoichiometry. This is a reversible post-synthetic modification of target proteins that alters their function. The methylation is reversed by simple hydrolysis. In bacteria, carboxymethylation regulates the activity of chemoreceptors that control flagellar motor activity. In the mammalian central nervous system, protein carboxymethylation is involved in the regulation of heterotrimeric G-proteins and protein phosphatase A, by methylating the carboxy-terminal amino acids. There is some evidence that, both in bacteria and in mammalian central nervous systems, this methylation is important in memory (Li & Stock, 2009).

Type II carboxymethyltransferases catalyze the methylation of modified acidic amino acid residues in proteins (D-aspartate, D-glutamate and L-iso-aspartate), with no clear stoichiometry and no specificity either for the site of methylation or for the proteins methylated. These enzymes are not involved in modifying the activity of their targets, but rather with labelling proteins that have undergone non-enzymic modification, either as a repair mechanism or to target them for intracellular proteolysis (see section 2.1.5).

In *iso*-aspartyl residues in proteins, the α -carboxyl group is free and the peptide bond is to the β -carboxyl group. This may occur as a result of mis-

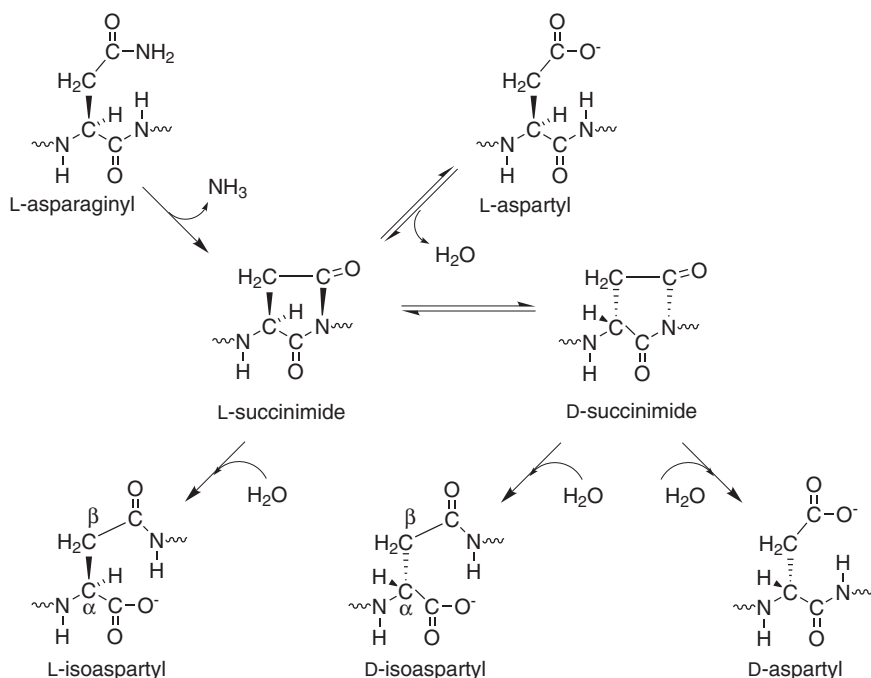


Figure 3.4 Non-enzymic reactions leading to the formation of *iso*-aspartyl and D-aspartyl residues in proteins.

alignment of aspartate on tRNA, resulting in formation of a β -peptide bond on the ribosome, or as a result of non-enzymic reactions of aspartyl and asparaginyl residues in the protein, as shown in Figure 3.4. Demethylation of methyl D-aspartyl and D- or L-*iso*-aspartyl residues involves dehydration to the succinimide intermediates and either hydration of L-succinimide to an L-aspartyl residue or amidation to yield an L-asparaginyl residue (Clarke, 1985; Shimizu *et al.*, 2005).

3.3 Transamination

As shown in Figure 3.5, transaminases (aminotransferases) catalyze a bisubstrate ‘ping-pong’ reaction. In the first half-reaction, the substrate amino acid displaces the lysine residue that binds the pyridoxal phosphate in the internal aldimine. The substrate aldimine then undergoes deprotonation to yield the quinonoid intermediate and reprotonation to the ketimine (see Figure 3.2). Cleavage of the α -carbon-amino bond releases the oxo-acid, leaving pyridoxamine phosphate at the active site.

In the second half-reaction, the oxo-acid substrate forms a ketimine with pyridoxamine phosphate, then undergoes the reverse sequence of reactions;

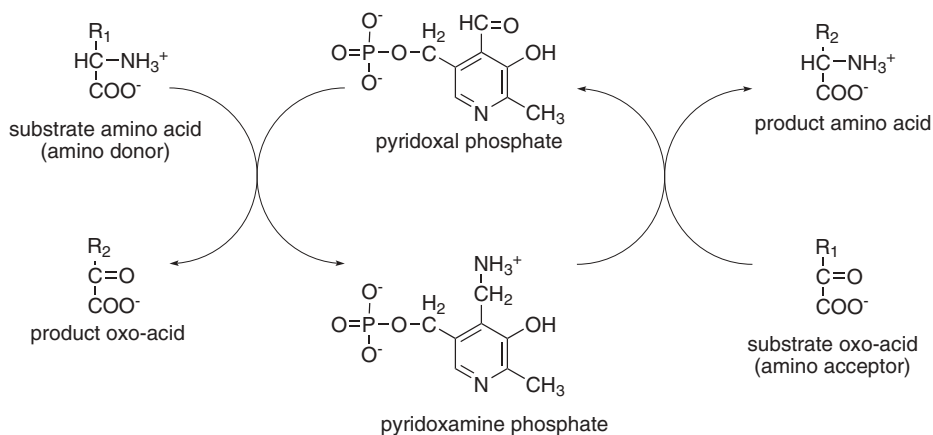


Figure 3.5 The reaction of transamination.

the internal aldimine to pyridoxal phosphate is reformed, liberating the amino acid product.

Most of the transaminases are linked to one of three common metabolic intermediates as acceptor oxo-acids: pyruvate (forming alanine); oxaloacetate (forming aspartate); and 2-oxoglutarate (forming glutamate). Refer to Figures 1.9, 1.14 and 1.15 for the roles of these transaminases in overall deamination of amino acids linked to adenosine deaminase, glutamate dehydrogenase and glycine oxidase.

The transamination products of the amino acids are shown in Table 3.2. Apart from lysine, all of the amino acids can undergo reversible transamination; sometimes it is transamination of the oxo-acid to synthesize the amino acid, while in other cases transamination is a major route of catabolism, with the oxo-acid providing metabolic fuel or substrates for gluconeogenesis. Table 2.9 shows the metabolic fates (gluconeogenesis or ketogenesis) of amino acids in mammals, and Figure 2.2 the entry points of amino acid carbon skeletons into the citric acid cycle. Transamination of lysine is irreversible because the oxo-acid, α -oxo- ϵ -aminocaproate, undergoes spontaneous dehydration and cyclization to pipercolic acid (see section 6.2.3.1).

The most studied transaminase is mammalian aspartate transaminase, which catalyzes transfer of the amino group between aspartate (forming oxaloacetate) and 2-oxoglutarate (forming glutamate). The crystallized enzyme will form enzyme-substrate and enzyme-product complexes, and if the enzyme is crystallized with its substrate it undergoes a very slow reaction, permitting X-ray crystallography of the enzyme-substrate, enzyme-intermediate and enzyme-product complexes.

Each monomer of aspartate transaminase has two domains: the coenzyme is bound to the larger domain in a pocket near the interface of the two subu-

Table 3.2 Transamination products of the amino acids.

Amino acid	Oxo-acid
alanine	pyruvate
arginine	α -keto- β -guanidoacetate
aspartic acid	oxaloacetate
cysteine	γ -mercaptopyruvate
glutamic acid	2-oxoglutarate
glutamine	2-oxo-glutaramic acid
glycine	glyoxylate
histidine	imidazolepyruvate
isoleucine	α -keto- β -methylvalerate
leucine	α -keto-isocaproate
[lysine ⁽¹⁾]	α -keto- ϵ -aminocaproate $\rightarrow$ pipecolic acid]
methionine	S-methyl- β -thiol α -oxopropionate
ornithine	glutamic- γ -semialdehyde
phenylalanine	phenylpyruvate
proline	γ -hydroxypyruvate
serine	hydroxypyruvate
threonine	α -keto- β -hydroxybutyrate
tryptophan	indolepyruvate
tyrosine	<i>p</i> -hydroxyphenylpyruvate
valine	α -keto-isovalerate

1 Lysine does not normally undergo transamination; if it did, the product would undergo non-enzymic cyclization to pipecolic acid.

nits; and the carboxyl groups of aspartate bind to arginine residues, one of which comes from each of the subunits. When the substrate binds, there is a conformational change in the protein. The small domain closes the active site crevice and moves one of the arginine residues, to which the substrate is bound, closer to the coenzyme in the internal aldimine. The substrate now displaces lysine-258, which formed the internal aldimine, to form the substrate aldimine.

At this stage, the attachment of the coenzyme to the enzyme through the 2'-methyl group and the 5'-phosphate group is important in maintaining rigid geometry, as the coenzyme ring rotates some 30° about its C2–C5 axis to bring the α -carbon of the amino acid close enough to lysine-258 to permit this residue to catalyze the deprotonation and reprotonation to yield the ketimine. Then, or after hydrolysis to release the oxo-acid, the coenzyme rotates back to an orientation intermediate between that in the substrate aldimine and that in the internal aldimine. The small domain then moves back to its original position, permitting release of the oxo-acid product and binding of the oxo-acid substrate for the second half-reaction.

In branched-chain amino acid transaminase, which is a fold type IV enzyme, the coenzyme ring rotates 180° about its C2–C5 axis (Ivanov & Karpeisky, 1969; Karpeisky & Ivanov, 1966; Taylor *et al.*, 1990; Torchinsky *et al.*, 1990).

3.3.1 Dual substrate recognition in transaminases

Transaminases have two amino acid substrates. These frequently have very different side-chains, yet the enzymes show considerable specificity for the amino acids they bind. The two amino acid substrates have to bind to the same active site, with the α -carboxyl group in the same place (Hirotsu *et al.*, 2005).

Branched-chain amino acid transaminase (a fold type IV enzyme) has a classic 'lock and key' substrate binding mechanism. The side-chain of the amino acid is accommodated in a mainly hydrophobic pocket that will bind the hydrophobic branched side-chain, but the pocket also has three hydrophilic sites, so placed that they will recognise the γ -carboxyl group of glutamate. The large and small domains of the enzyme do not move when substrate binds, but a small inter-domain loop moves to shield the active site from the solvent.

Aromatic amino acid transaminase (a fold type I enzyme) can bind either an aromatic amino acid or glutamate, and it has a classic induced-fit substrate binding mechanism. There is a considerable conformational change in fold type I transaminases when the substrate binds, and the small domain moves to shield the active site from the solvent.

Both the α -carboxyl group and the side-chain bind in exactly the same places, whether the substrate is an aromatic amino acid or glutamate. However, very different active site residues surround the side chain of the substrate, depending on which substrate binds. When glutamate approaches the active site, an arginine residue from the other subunit moves to form a salt bridge with the γ -carboxyl group, and aspartate, tryptophan, serine and a water molecule are also involved in binding the side-chain. By contrast, when an aromatic amino acid approaches the active site, the conformation changes so that leucine, tryptophan and tyrosine residues form the side-chain binding site.

3.3.2 Aspartate transaminase and the malate-aspartate shuttle

There are two isoenzymes of aspartate transaminase in mammalian cells: a cytosolic form and a mitochondrial form. These are involved in the malate-aspartate shuttle to transfer reducing equivalents from cytosolic NADH into the mitochondrion. As shown in Figure 3.6, the shuttle involves reduction of oxaloacetate to malate in the cytosol (with the oxidation of cytosolic NADH to NAD⁺). Malate enters the mitochondria and is reduced back to oxaloacetate, with the reduction of intra-mitochondrial NAD⁺ to NADH. Oxaloacetate cannot cross the mitochondrial inner membrane, but undergoes transamination to aspartate with glutamate acting as amino donor, yielding

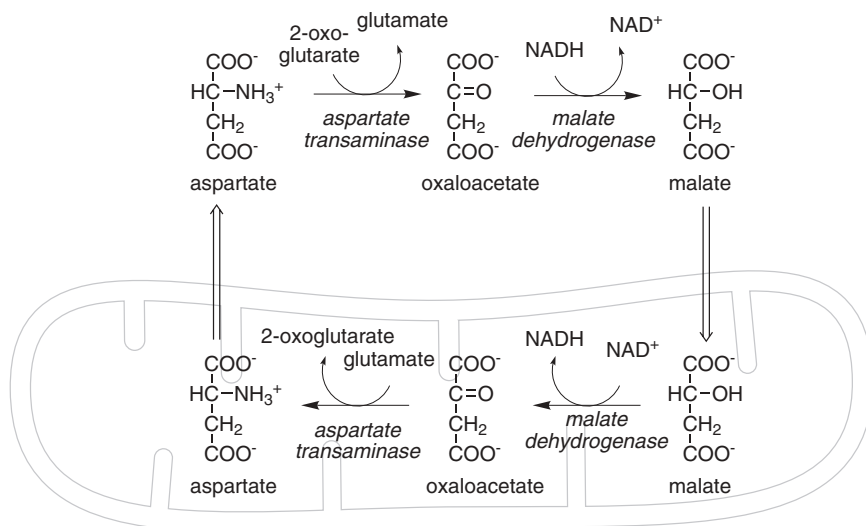


Figure 3.6 The mitochondrial malate-aspartate shuttle.
Malate dehydrogenase EC 1.1.1.37, aspartate transaminase EC 2.6.1.1.

2-oxoglutarate, which then leaves the mitochondrion using an antiporter that transports malate inwards. Aspartate leaves the mitochondrion in exchange for glutamate entering. In the cytosol, the reverse transamination reaction occurs, forming oxaloacetate (for reduction to malate) from aspartate, and glutamate (for transport back into the mitochondrion) from 2-oxoglutarate.

Mitochondrial aspartate transaminase is encoded by a nuclear gene; it was the first such enzyme to be studied in order to determine how proteins enter the mitochondrion. Martinez-Carrion *et al.* (1990) cloned the gene for mitochondrial aspartate transaminase into an expression vector and showed that the gene product was catalytically active, but differed from the purified mitochondrial enzyme in electrophoretic mobility, molecular mass and UV absorbance. Partial proteolysis with trypsin gave a protein that was identical to the mitochondrial enzyme, and the precursor protein was imported into isolated mitochondria. They showed that the newly synthesized protein folded into the active form, but with a hydrophobic and basic helical region exposed. This is the mitochondrial targeting sequence that is cleaved after the precursor protein has been imported into the mitochondrion.

Mitochondrial aspartate transaminase has another, quite unrelated, role in energy metabolism. The plasma membrane fatty acid binding protein that is responsible for tissue uptake of non-esterified fatty acids appears to be identical to mitochondrial aspartate transaminase. Mono-specific antisera against the proteins cross-react with each other, and the two proteins have identical electrophoretic mobility (Berk *et al.*, 1990).

3.4 Decarboxylation and side-chain elimination and replacement reactions

Decarboxylation of amino acids is important in the synthesis of the diamines and polyamines (section 5.8), and of a number of hormones and neurotransmitters, including γ -aminobutyrate (GABA) from glutamate (see section 5.5.1), histamine from histidine (section 8.4), dopamine (and then noradrenaline and adrenaline) from dihydroxyphenylalanine (section 9.2.4), and 5-hydroxytryptamine (serotonin) from 5-hydroxytryptopan (section 9.4.3).

When the α -carbon-carboxyl bond of the substrate lies perpendicular to the plane of the substrate aldimine, it is this bond that is cleaved, by deprotonation at the α -carbon, leading to the release of carbon dioxide. Reprotonation leads to displacement of the resultant amine and reformation of the internal aldimine.

When the bond that is perpendicular to the plane of the substrate aldimine is the α -carbon-side-chain bond, the substrate may undergo an α - β or β - γ elimination or replacement reaction. Such reactions include serine hydroxymethyltransferase (see section 4.2.1), kynureninase (section 9.4.4.2), serine deaminase (section 4.6.2), threonine deaminase (section 6.1.5), tryptophanase (section 9.4.2), and tryptophan synthase, which catalyzes the reverse reaction, addition of a side-chain (section 9.1.3).

Dialkylglycine decarboxylase is unusual in that it has a transaminase-like reaction mechanism and forms pyridoxamine phosphate as an intermediate. When the substrate is decarboxylated, the carbanion is protonated on C-4' of the coenzyme rather than C- α of the substrate. This leads to formation of an oxo-group, leaving pyridoxamine phosphate at the active site. The second half-reaction is a typical transamination, binding pyruvate and releasing alanine, thus restoring pyridoxal phosphate at the active site. The enzyme has two separate carboxyl binding sites, which bind the substrates with different orientations. The dialkylglycine binding site will accommodate the bulky side-chain, and it binds the substrate so that decarboxylation at C- α of the substrate and protonation of C-4' of the coenzyme occur simultaneously; there is therefore no possibility of protonation occurring at C- α , as occurs in most decarboxylases (Toney, 2005).

3.4.1 Transamination of decarboxylases and enzymes catalyzing side-chain elimination reactions

Although it was noted above that the enzyme confers both reaction and substrate specificity compared with non-enzymic pyridoxal-catalyzed reactions, decarboxylases and enzymes that catalyze side-chain elimination reactions can undergo reaction-dependent inactivation as a result of catalyzing

the half-reaction of transamination of the substrate, leaving pyridoxamine phosphate at the active site. Because these enzymes, unlike transaminases, do not have a binding site for an oxo-acid substrate to undergo the second half of the transamination reaction, the enzyme is inactive unless the pyridoxamine phosphate is displaced by a relatively high concentration of pyridoxal phosphate. Transamination of decarboxylases occurs when the quinonoid intermediate is protonated at carbon-4' rather than the α -carbon (John, 1995; Meister, 1990).

The extent to which individual enzymes catalyze transamination varies. In aspartate β -decarboxylase, the ratio of decarboxylation:transamination is 500–3,000:1, while in glutamate decarboxylase it is 10,000:1. α -Methyl derivatives of the amino acids are effective inhibitors of decarboxylases because the bulky α -methyl group distorts the active site, increasing the probability of the transamination reaction occurring.

Bacterial aspartate β -decarboxylase catalyzes two β -elimination reactions: the β -decarboxylation of aspartate to alanine and the desulphination of cysteine sulphinat to yield alanine and sulphur dioxide. The carbanion formed by decarboxylation or desulphination can undergo either transaldimination to release alanine and reform the internal aldimine, or hydrolysis to release pyruvate, leaving pyridoxamine phosphate at the active site (Figure 3.7). Initially the rate of decarboxylation is high, but this gradually falls as pyridoxamine phosphate accumulates at the active site. However, because pyruvate is a product of the half-reaction of transamination, it can undergo the reverse half-reaction as it accumulates, forming alanine and reactivating the enzyme (Meister, 1990).

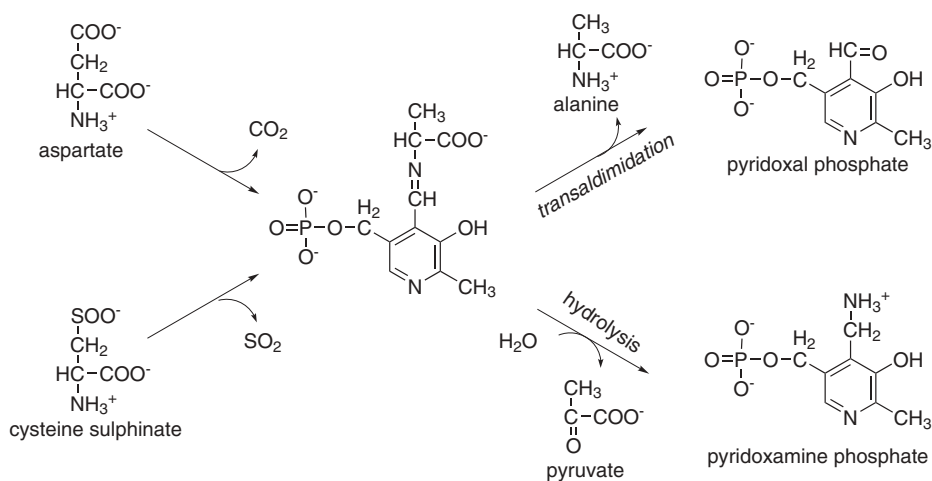


Figure 3.7 Reactions of bacterial aspartate β -decarboxylase.

Aspartate β -decarboxylase EC 4.1.1.12, also catalyzes the desulphination of cysteine sulphinat.

This accidental inactivation of the enzyme, by catalyzing the half-reaction of transamination, explains why the decarboxylases that lead to the formation of neurotransmitter amines are so sensitive to marginal vitamin B₆ status, leading to impaired synthesis of the neurotransmitters. When pyridoxal phosphate availability is low, there is not enough to replace the non-covalently bound pyridoxamine phosphate that diffuses off the active site, and so reactivate the enzyme (John, 1995). As will be discussed in section 9.4.4.3, kynureninase is especially sensitive to the availability of pyridoxal phosphate, because it undergoes transamination very readily, and the ability to metabolize a test dose of tryptophan provides an index of vitamin B₆ nutritional status.

Aspartate is a poor substrate for decarboxylation by glutamate decarboxylase (which catalyzes the synthesis of the neurotransmitter GABA from glutamate – see section 5.5.1), but it readily undergoes transamination, inactivating the enzyme. It is likely that aspartate has a role in regulating the formation of GABA and in maintaining the balance between glutamate and GABA as neurotransmitters in the central nervous system.

Threonine and serine deaminases (see sections 4.6.2 and 6.1.5) undergo the half-reaction of transamination accidentally, but the pyridoxamine phosphate form of the enzyme can be reactivated by catalyzing transamination of the product oxo-acid: pyruvate from serine, forming alanine; or 2-oxobutyrate from threonine, forming aminobutyrate. The enzymes do not catalyze transamination of alanine or aminobutyrate (Leoncini *et al.*, 1998).

Transamination and inactivation of the enzyme may provide a mechanism for metabolic regulation. The apoenzyme formed when pyridoxamine phosphate diffuses off the active site is more susceptible to proteolysis (section 2.1.5.3), thus permitting regulation of the amount of enzyme protein in the cell. The pyridoxamine phosphate can then be oxidized back to pyridoxal phosphate (see Figure 3.1).

Some transaminases also catalyze β -elimination reactions as side-reactions. Cysteine conjugate β -lyase catalyzes the cleavage of cysteine *S*-conjugates formed by reaction of halogenated alkanes with glutathione (see section 5.4) to yield pyruvate, ammonium and a vinyl-thiol. In the liver, this reaction is catalyzed by kynureninase (section 9.4.4.2) but, in the kidney, it is a side-reaction of glutamine transaminase. Similarly, aspartate transaminase catalyzes the elimination of chloride from β -chloroglutamate and β -chloro-alanine, and of sulphate from serine-*O*-sulphate (Cooper & Anders, 1990; Cooper *et al.*, 2002).

3.5 Pyruvate-containing enzymes

A number of enzymes that catalyze decarboxylation of amino acids and some side-chain cleavage reactions do not contain pyridoxal phosphate, but

rather have a carbonyl group provided by a pyruvoyl group at the active site. Histidine decarboxylase (section 8.4) in Gram-positive bacteria is a pyruvoyl enzyme, while in Gram-negative bacteria and eukaryotes it contains pyridoxal phosphate. In both prokaryotes and eukaryotes (including mammals), *S*-adenosylmethionine decarboxylase (see section 5.8.2) is a pyruvoyl enzyme, as are phosphopantotheinyl cysteine decarboxylase, an enzyme involved in the synthesis of coenzyme A from pantothenic acid, and phosphatidylserine decarboxylase, the enzyme that catalyzes the synthesis of phosphatidylethanolamine.

Unlike pyridoxal phosphate, the reactive pyruvoyl residue is not bound in an internal aldimine in the absence of substrate. Substrate binding thus involves condensation with pyruvate to form a substrate aldimine, which then undergoes decarboxylation and protonation of the carbanion, followed by hydrolysis to release the amine.

S-adenosylmethionine decarboxylase undergoes inactivation during the reaction because, like pyridoxal phosphate-dependent decarboxylases, it catalyzes the half-reaction of transamination (section 3.4.1), leading to formation of alanine in place of the catalytic pyruvate. Unlike the pyridoxal phosphate-dependent decarboxylases, this inactivation is irreversible, because the reactive pyruvate and the alanine formed from it in transamination are covalently bound at the amino-terminal of the α -chain of the enzyme.

Pyruvate-dependent enzymes are hetero-dimers coded for by a single gene. The reactive pyruvate is formed by post-synthetic modification of a serine residue in the precursor protein. The protein undergoes an autocatalytic, non-hydrolytic cleavage, in which there is initially formation of an internal ester from the side-chain of serine to the carboxyl group of the adjacent amino acid. This is followed by transfer of oxygen to form the carboxyl group of what is now the carboxy-terminal amino acid of the β -chain of the protein. The serine residue at the amino-terminal of what is now the α -chain of the enzyme loses ammonium to leave a pyruvoyl residue (Pegg, 2009; van Poelje & Snell, 1990).

3.6 Vitamin B₆ deficiency and dependency

It is obvious from the discussion above that vitamin B₆ deficiency will have a very considerable impact on amino acid metabolism, and the changes in the metabolism of test doses of tryptophan (section 9.4.4.3) and methionine (section 6.3.3) have been used as indices of vitamin B₆ nutritional status and to determine vitamin B₆ requirements in depletion/repletion studies.

Erythrocyte aspartate and alanine transaminases also provide a means of assessing vitamin B₆ nutritional status. There is normally a small proportion of the enzyme present as catalytically inactive apoenzyme, and this increases

in vitamin B₆ deficiency at the expense of (catalytically active) holoenzyme. The activity of the transaminase is measured in a sample of washed erythrocytes, with and without added pyridoxal phosphate. The incubation without added coenzyme measures what was present as holoenzyme, while that with added coenzyme measures both what was present as holoenzyme and also that which was present as apoenzyme. The ratio of activity with pyridoxal phosphate to activity without added pyridoxal phosphate is the activation coefficient of the enzyme, and this serves as an indicator of the adequacy (or otherwise) of body vitamin B₆ reserves (Bender, 1989b).

As discussed in section 3.4.1, enzymes that undergo inactivation as a result of transamination will be especially sensitive even to moderate vitamin B₆ depletion. There is a specific protease in the rat that hydrolyzes the apoenzymes of a number of pyridoxal phosphate-dependent enzymes. It has no effect on other proteins or on the holoenzymes (section 2.1.5.3), but it attacks the conserved amino acid sequence around the active lysine residue which forms the internal aldimine.

The activity of the enzyme is increased some 10–20 fold in vitamin B₆ deficiency, suggesting that its function is to degrade those enzymes that lose their coenzyme more readily, thus making more pyridoxal phosphate available for use by other enzymes. There is also evidence that some pyridoxal phosphate-dependent apoenzymes undergo modification in vitamin B₆ deficiency. These thus become incapable of activation by pyridoxal phosphate, though retaining immunological cross-reactivity with the normal form of the enzyme (Katunuma *et al.*, 1971a; Nagata & Okada, 1985).

In addition to dietary deficiency of vitamin B₆, rare genetic conditions lead to functional deficiency of pyridoxal phosphate (Clayton, 2006). Lack of pyridoxine phosphate oxidase (Figure 3.1) results in neonatal epileptic encephalopathy (as does severe vitamin B₆ deficiency). These infants are unresponsive to pyridoxine, although there is a dramatic cessation of seizures when they are given pyridoxal phosphate.

Although the main transport form of vitamin B₆ in the bloodstream is pyridoxal phosphate bound to serum albumin, this has to be dephosphorylated to pyridoxine for tissue uptake. Lack of tissue non-specific alkaline phosphatase leads not only to clinical rickets, but also to epileptiform seizures. Alkaline phosphatase knockout mice have elevated serum pyridoxal phosphate, with low central nervous system GABA (section 5.5), and they suffer fatal seizures, which can be prevented by administration of pyridoxal.

Lack of Δ^1 -pyrroline 5-carboxylate dehydrogenase (hyperprolinaemia type II, section 5.7.1.1) leads to accumulation of pyrroline 5-carboxylate, which forms an adduct between the aldehyde group of pyridoxal phosphate and carbon-4 of the pyrroline ring. Again, the affected infants suffer epileptiform seizures and have low plasma pyridoxal phosphate and high urinary excretion

Table 3.3 Vitamin B₆ responsive inborn errors of metabolism.

	Enzyme affected	EC number	
Convulsions of the newborn	unknown	–	
Cystathioninuria	cystathionase	EC 4.4.1.1	Figure 6.15
Gyrate atrophy with ornithinuria	ornithine δ -transaminase	EC 2.6.1.13	Figure 5.14
Homocystinuria	cystathionine synthase	EC 4.2.1.22	Figure 6.15
Primary hyperoxaluria, type I	peroxisomal alanine-glyoxylate transaminase	EC 2.6.1.44	section 4.3.1
Sideroblastic anaemia	5-aminolevulinate synthase (reduced haem synthesis)	EC 2.3.1.37	section 4.8
Xanthurenic aciduria	kynureninase	EC 3.7.1.3	Figure 9.16

of xanthurenic and kynurenic acids, reflecting impaired tryptophan metabolism (section 9.4.4.3).

In pyridoxine-responsive epilepsy, lack of amino-adipic acid/piperidine 6-carboxylate dehydrogenase leads to accumulation of piperidine 6-carboxylate, which again can condense with pyridoxal phosphate. Seizures can be controlled with doses of 50–100 mg pyridoxine, and the infants can be maintained seizure-free on about 10 mg of pyridoxine per day (compared with the reference intake of less than 1 mg/day for infants). The seizures recur if the supplements cease.

A small number of patients show one or other of the biochemical signs associated with vitamin B₆ deficiency, despite apparently adequate status, and such patients require high intakes of the vitamin to normalize the abnormal metabolic marker. These are genetic diseases and have been termed vitamin B₆ dependency syndromes (Clayton, 2006; Frimpter *et al.*, 1969; Mudd, 1971).

As shown in Table 3.3, vitamin B₆ dependency has been reported in cases of type I primary hyperoxaluria, xanthurenic aciduria, homocystinuria, X-linked sideroblastic anaemia, gyrate atrophy with ornithinaemia and vitamin B₆ responsive infantile convulsions. In this last condition, the underlying defect has not been identified, but it is almost certainly not simply impaired activity of glutamate decarboxylase.

The molecular basis of the vitamin B₆ dependency syndromes is a severely reduced affinity of the defective enzyme for its cofactor, and patients respond well to doses of 50–1,000 mg of vitamin B₆/day. Apart from the affected enzyme, other biochemical indices of vitamin B₆ nutritional status are normal in these patients.

In gyrate atrophy with ornithinaemia, the K_m of ornithine δ -transaminase is 80–310 $\mu\text{mol/L}$, compared with a normal value of 20 $\mu\text{mol/L}$. However, in this and other cases, it is possible that not only is the affinity of the mutant enzyme for pyridoxal phosphate as a coenzyme abnormally high, but there may also be a need for pyridoxal phosphate as a chaperone to ensure correct folding of the nascent enzyme during synthesis, and the abnormally folded enzyme will be a target for ubiquitinylation and proteolysis (section 2.1.5.2).

Further reading

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

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4

Glycine, Serine and the One-Carbon Pool

Glycine is a semi-essential amino acid for mammals, because, as discussed in section 2.2, under some conditions the capacity for its synthesis may be inadequate to meet requirements. Apart from its role in protein synthesis, it is important as a precursor for synthesis of creatine (see section 5.9.7), glutathione (section 5.4), porphyrins (section 4.9) and purines (section 1.4.1). As discussed in section 1.5.1, glycine oxidase provides a route for the net deamination of a wide variety of amino acids whose transamination can be linked (directly or indirectly) to the transamination of glyoxylate to glycine. In mammalian liver, glycine is important for the conjugation and detoxication of a variety of compounds. Benzoic acid derivatives are conjugated with glycine to yield hippuric acids that are excreted in the urine, and a high intake of benzoates can deplete the metabolic pool of glycine and impair nitrogen balance.

Serine, like threonine and tyrosine, has a hydroxyl side-chain, and thus contributes to the hydrophilicity of proteins. In many proteins, the hydroxyl group can be phosphorylated. This may be a means of metabolic regulation, when protein kinases or phosphatases are activated in response to hormone action, thereby increasing or decreasing the activity of key regulatory enzymes. In these cases, it is specific serine (and tyrosine and threonine) residues that are phosphorylated. Casein, the major protein of milk, is heavily phosphorylated, serving as a vehicle for export of phosphate and providing most of the phosphate that is required by the infant for bone mineralization.

Serine also has a role in the catalytic sites of many proteases and other hydrolases, the so-called serine hydrolases (section 2.1.1). Inactivation of these enzymes by diisopropyl fluorophosphate (DFP) is the result of reaction

with the hydroxyl group of serine to form a diisopropyl phosphate ester. DFP is a potent neurotoxin, because choline esterase, the enzyme that hydrolyzes acetylcholine, is a serine hydrolase.

Serine is also important in the biosynthesis of phospholipids. Phosphatidylserine may be synthesized by reaction between CDP-serine and diacylglycerol or by base exchange of serine for ethanolamine or choline in a phospholipid. In turn, phosphatidylethanolamine may be formed by reaction of CDP-ethanolamine with diacylglycerol, base exchange of ethanolamine for serine or choline in a phospholipid, or decarboxylation of phosphatidylserine. Phosphatidylserine decarboxylase is a pyruvoyl enzyme (section 3.5).

D-Serine (section 3.2.2) is a neurotransmitter and, as will be discussed in section 6.3.1.1, serine is the substrate for transsulphuration for the synthesis of cysteine from homocysteine. Serine, esterified to tRNA^{Sec}, is the precursor for the synthesis of selenocysteine, which is incorporated into a number of proteins during ribosomal synthesis (section 4.9)

Two pathways of threonine catabolism provide a minor source of glycine (see section 6.1.5). Threonine aldolase cleaves threonine to yield glycine and acetaldehyde (Figure 4.1). Threonine dehydrogenase forms 2-amino-oxybutyrate, which is a substrate for 2-amino-oxybutyrate CoA ligase to yield glycine and acetyl CoA. 2-Amino-oxybutyrate is unstable, and rapidly undergoes non-enzymic decarboxylation to aminoacetone. The reverse reaction of 2-amino-oxybutyrate CoA ligase thus provides an alternative, if minor, pathway of glycine catabolism, yielding aminoacetone, and then methylglyoxal after oxidation by plasma amine oxidase.

4.1 Sources of glycine

As shown in Figure 4.1, glycine is formed by the oxidation and demethylation of choline arising from phosphatidylcholine, and by the action of serine hydroxymethyltransferase. In plants and bacteria that have the glyoxylate anaplerotic pathway, the main source is transamination of glyoxylate synthesized from isocitrate.

4.1.1 Choline as a source of glycine

Choline oxidase is a flavoprotein that catalyzes two successive reactions: oxidation of choline to betaine aldehyde and onwards oxidation of betaine aldehyde to betaine, using molecular oxygen and forming hydrogen peroxide in both reactions. In bacteria and mammals, there is also a choline dehydrogenase, which is a quinoprotein (see section 9.5) forming betaine aldehyde, linked to the reduction of cytochrome c. Plants have a ferredoxin-dependent choline monooxygenase that catalyzes the oxidation of choline to betaine

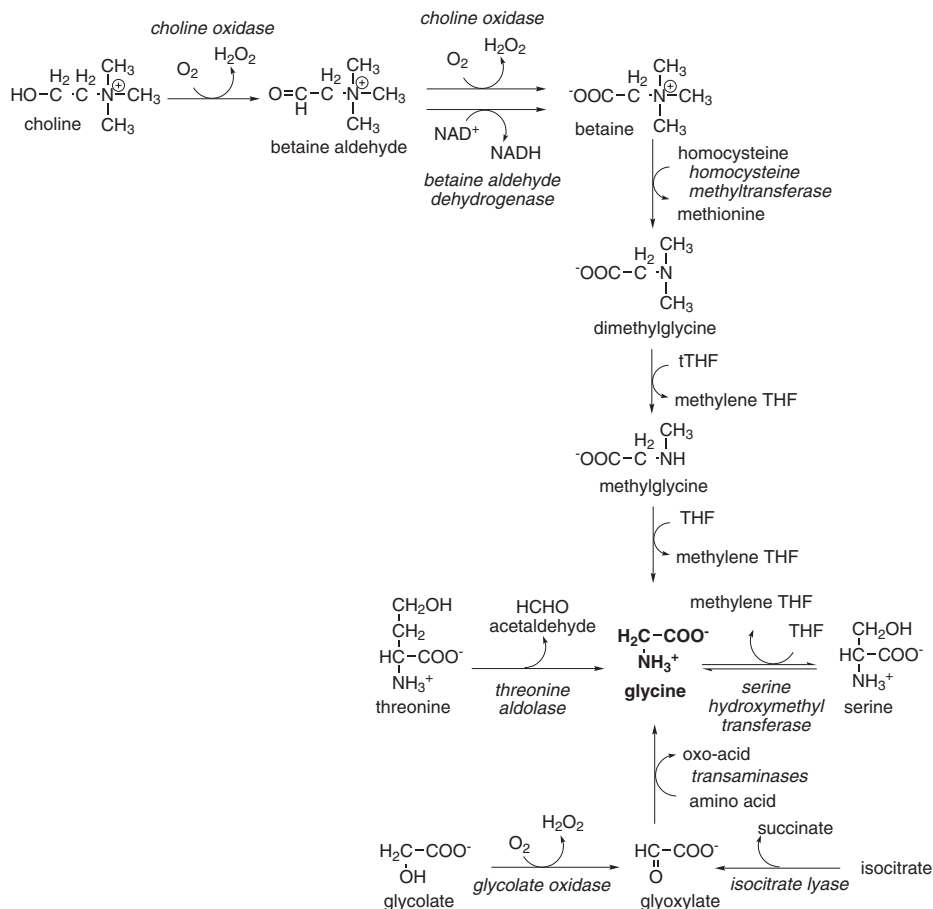


Figure 4.1 Metabolic sources of glycine.

THF = tetrahydrofolate; choline dehydrogenase EC 1.1.99.1 (membrane-bound quinoprotein in bacteria and animals), EC 1.1.3.17 (soluble choline oxidase in bacteria and animals), EC 1.14.15.7 (choline mono-oxygenase in plants), betaine aldehyde dehydrogenase EC 1.2.1.8, betaine homocysteine methyltransferase EC 2.1.1.5, glycine oxidase EC 1.4.3.19, isocitrate lyase EC 4.1.3.1, serine hydroxymethyltransferase EC 2.1.2.1, threonine aldolase EC 4.1.2.5, hydroxy-acid oxidase (glycolate oxidase) EC 1.1.3.15.

aldehyde and water. Bacteria, plants and animals also have an NAD-dependent betaine aldehyde dehydrogenase that catalyzes the oxidation of betaine aldehyde to betaine.

Betaine is a major source of methyl groups. Betaine homocysteine methyltransferase provides an alternative pathway for methylation of homocysteine to methionine in all tissues except the central nervous system (which is wholly reliant on the vitamin B_{12} -dependent methionine synthase – see section 6.3.2). Two further demethylation reactions, linked to the formation

of methylene tetrahydrofolate, yield glycine. Betaine also has a role in plants and microorganisms as an osmolyte, and there is increased synthesis of betaine in response to salt or water stress.

Transgenic plants over-expressing bacterial choline oxidase have increased tolerance to salt and water stress (Chen & Murata, 2011). In plants, betaine aldehyde dehydrogenase also acts on 4-aminobutyraldehyde, forming γ -aminobutyrate (GABA – see section 5.5), and diverting it away from the synthesis of 2-acetyl-1-pyrroline, which is the compound responsible for the aroma of fragrant rice (e.g. Indian basmati and Thai jasmine rice) and other crops. Mutations leading to reduced activity of betaine aldehyde dehydrogenase lead to increased accumulation of the 2-acetyl-1-pyrroline and increased fragrance of the crop (Sakthivel *et al.*, 2009).

4.1.2 Glycine transaminase

Glycine can also be synthesized by transamination of glyoxylate, which can arise from the pentose phosphate pathway of carbohydrate metabolism, from the oxidation of ascorbate (vitamin C) or glycolic acid and, in organisms that have the enzymes of the glyoxylate shunt pathway, from the cleavage of isocitrate catalyzed by isocitrate lyase. The glyoxylate shunt is an anaplerotic sequence for the citric acid cycle that avoids the two carbon dioxide-generating steps of the cycle. Isocitrate lyase catalyzes the cleavage of isocitrate to glyoxylate and succinate. Glyoxylate may either undergo transamination to glycine or be reacted with acetyl CoA to yield malate, in a reaction catalyzed by malate synthase. This permits net accumulation of oxaloacetate that can be used for gluconeogenesis from acetate (a pathway that is impossible in mammalian tissues), and it is especially important in germinating seeds, when fatty acids form the main metabolic fuel.

The activity of isocitrate lyase and malate synthase is especially important in the pathogenicity of bacteria, yeasts and fungi in plants and animals (Dunn *et al.*, 2009; Kornberg & Krebs, 1957).

There are two glycine transaminases in mammalian tissues, one linked to glutamate/2-oxoglutarate and the other to alanine/pyruvate. Glycine is a poor substrate for both enzymes, and it is likely that they act mainly in the direction of glycine synthesis from any glyoxylate that is available. As shown in Figure 1.14, alanine-glyoxylate transaminase serves to reaminate glyoxylate formed by glycine oxidase, thus providing a pathway for trans-deamination of a wide variety of amino acids.

4.2 The interconversion of glycine and serine

As shown in Figure 4.2, glycine and serine are readily interconvertible; glycine can provide serine as a precursor for gluconeogenesis, and serine can be

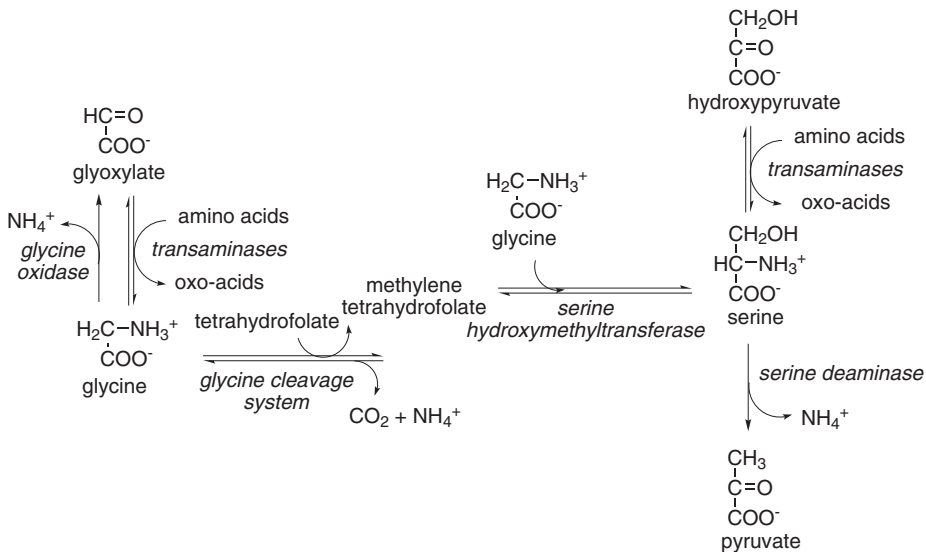


Figure 4.2 The interconversion of glycine and serine.

Glycine cleavage system (Figure 4.3) consists of: glycine dehydrogenase EC 1.4.4.2, aminomethyltransferase EC 2.1.2.10 and lipoamide dehydrogenase EC 1.8.1.4; serine hydroxymethyltransferase EC 2.1.2.1, serine deaminase EC 4.3.1.17.

cleaved to yield glycine and a single carbon fragment, the methylene group of methylene tetrahydrofolate. Glycine and serine thus provide the main source of one-carbon fragments for methyl transfer reactions (section 4.4). Because of its central role in providing one-carbon units, serine hydroxymethyltransferase is a potential target for drugs for use in cancer chemotherapy.

4.2.1 Serine hydroxymethyltransferase

In animals and most microorganisms, serine hydroxymethyltransferase is a pyridoxal phosphate-dependent aldolase that catalyzes the cleavage of serine to yield glycine and 5,10-methylene-tetrahydrofolate. The mammalian enzyme is a tetramer, while the prokaryotic enzyme is a homodimer. In plants, some studies suggest that the enzyme has a reactive pyruvoyl residue at the active site (section 3.5), and others that it is pyridoxal phosphate-dependent, like the animal enzyme (Douce *et al.*, 2001).

In animals, there are both mitochondrial and cytoplasmic isoenzymes of serine hydroxymethyltransferase, encoded by separate genes. In rat liver, about 70 per cent of the activity is mitochondrial. Mammalian MCF7 cells cultured in a vitamin B₆-deficient medium have lower activity of serine hydroxymethyltransferase and a lower concentration of *S*-adenosylmethionine

(see section 6.3.2) than when cultured in a medium containing adequate amounts of vitamin B₆. The cytosolic enzyme is more affected than the mitochondrial one and, while there is a decrease in the cytosolic enzyme protein, there is no decrease in mRNA, suggesting that the apo-enzyme is unstable and is catabolized more rapidly than usual (Katunuma *et al.*, 1971a; Perry *et al.*, 2007).

Mammalian cell lines lacking the mitochondrial isoenzyme are auxotrophic for glycine, suggesting that the primary function of the mitochondrial enzyme is synthesis of glycine from serine, while the cytosolic isoenzyme is important for the synthesis of serine as a substrate for gluconeogenesis (Narkewicz *et al.*, 1996). Mice lacking cytosolic serine hydroxymethyltransferase are viable and fertile, but have uracil in their DNA and altered concentrations of *S*-adenosylmethionine in the liver. This suggests that, while the mitochondrial enzyme is the main source of one-carbon units, the cytosolic enzyme is important in partitioning one-carbon units between thymidylate synthesis and remethylation of homocysteine (MacFarlane *et al.*, 2008).

Mimosine is a plant non-protein amino acid that arrests the cell cycle in late G₁ phase. Part of its action is to inhibit transcription of cytosolic serine hydroxymethyltransferase by chelating zinc. There is a zinc-sensing response element upstream of the cytosolic serine hydroxymethyltransferase gene, so the availability of zinc controls one-carbon metabolism (Amadasi *et al.*, 2007; Perry *et al.*, 2005).

When serine is the substrate, the reaction proceeds by way of nucleophilic attack on carbon-3 of the serine-pyridoxal phosphate aldimine by tetrahydrofolate, cleaving the carbon-2-carbon-3 bond to form *N*⁵-hydroxymethylene tetrahydrofolate and leaving a glycine anion bound to pyridoxal phosphate. Glycine is released by protonation of the anion, and the hydroxymethylene-tetrahydrofolate is dehydrated to yield *N*⁵⁻¹⁰-methylene tetrahydrofolate (Schirch & Szebenyi, 2005; Szebenyi *et al.*, 2004).

Early studies suggested that mammalian serine hydroxymethyltransferase also catalyzes aldol cleavage of threonine and allothreonine to yield acetaldehyde and glycine. However, there are no plant or animal genes that are homologous with any of the bacterial or yeast threonine aldolases (section 6.1.5). Bacterial mutants lacking serine hydroxymethyltransferase are auxotrophic for glycine and grow only poorly on media providing threonine, but no glycine or serine. *E. coli* mutants lacking serine hydroxymethyltransferase can grow normally on media providing glucose as the sole carbon source. Thus, threonine is not a source of serine and glycine, and threonine aldolase is more likely to provide a pathway for disposal of excess threonine.

Yeast mutants lacking serine hydroxymethyltransferase do not show any requirement for glycine but a mutant lacking threonine aldolase does, suggesting that threonine is a source of glycine in yeasts, and that serine

hydroxymethyltransferase does not have significant threonine aldolase activity (Ogawa *et al.*, 2000).

It is likely that the apparent threonine aldolase activity that has been reported in preparations from mammalian tissues is an artefact of the assay method, which utilizes alcohol dehydrogenase to measure the acetaldehyde released by threonine aldolase, measuring the disappearance of NADH. If lactate dehydrogenase is present in the tissue sample, it will oxidize the 2-oxobutyrate that is formed by threonine deaminase (which is a mammalian enzyme – see section 6.1.5), so leading to oxidation of NADH. Antibodies against threonine deaminase abolish the apparent aldolase activity (Bird & Nunn, 1983; Yeung, 1986).

4.2.2 The glycine cleavage system

The glycine cleavage system is a mitochondrial multi-enzyme complex that catalyzes the oxidative decarboxylation and deamination of glycine to yield carbon dioxide, ammonium and N⁵⁻¹⁰-methylene tetrahydrofolate (Figure 4.3). The reaction is fully reversible and, at least *in vitro*, the enzyme complex catalyzes the synthesis of glycine from serine, ammonium, carbon dioxide and methylene tetrahydrofolate. The glycine cleavage system consists of four separate proteins (Bauwe & Kolukisaoglu, 2003; Douce *et al.*, 2001):

- The P-protein is a pyridoxal phosphate-containing decarboxylase that releases carbon dioxide, leaving an amino-methyl moiety bound to pyridoxal phosphate.

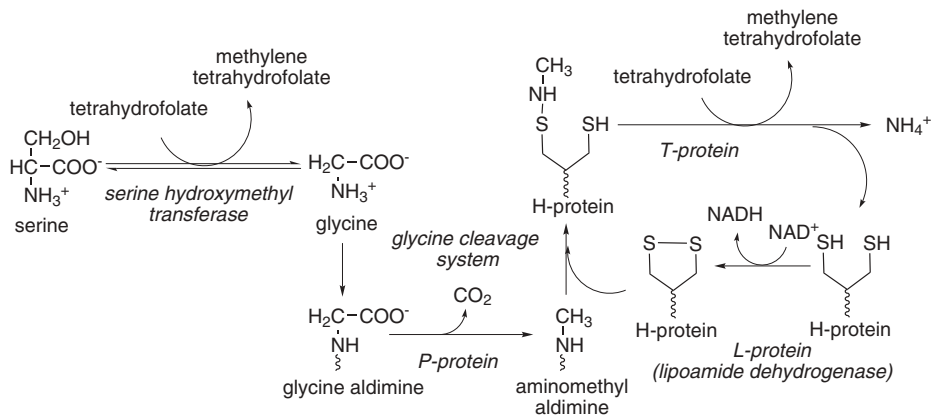


Figure 4.3 The glycine cleavage system.

Serine hydroxymethyltransferase EC 2.1.2.1, P-protein is glycine decarboxylase EC 1.4.4.2, T-protein is aminomethyltransferase EC 2.1.2.10, L-protein is lipoamide dehydrogenase EC 1.8.1.4.

- The H-protein is a lipoamide carrier protein that has no catalytic activity but collects the amino-methyl moiety from the P-protein, bound to the H-protein as a thioester to oxidized lipoamide.
- The T-protein catalyzes nucleophilic attack of the aminomethyl lipoamide by tetrahydrofolate, forming N⁵⁻¹⁰-methylene tetrahydrofolate and releasing ammonium, leaving fully reduced lipoamide attached to the H-protein.
- The L-protein is the same lipoamide dehydrogenase as the E3 subunit of pyruvate, 2-oxoglutarate and branched chain oxo-acid dehydrogenases (section 7.2.2). It contains tightly bound FAD and catalyzes oxidation of reduced lipoamide linked to reduction of NAD⁺ to NADH.

The activity of the glycine cleavage system in renal cortical cells increases in response to metabolic acidosis, although the enzyme is unresponsive to changes in pH on incubation *in vitro*, suggesting that this is an adaptive response involving induction of the enzyme to produce ammonia to buffer excess acidity. Kidneys from acidotic rats take up twice as much glycine as do those from control animals, providing about 5 per cent of renal ammonia synthesis; the remainder comes from glutamine, as will be discussed in section 5.3.2 (Lowry *et al.*, 1985).

4.2.3 Serine hydroxymethyltransferase and the glycine cleavage system in photosynthetic tissue

The glycine cleavage system is present in relatively low amounts in mitochondria from non-photosynthetic tissues in plants, and in etiolated seedlings. On exposure to light, the activity of the multi-enzyme complex increases considerably (Bourguignon *et al.*, 1988).

Plants that lack serine hydroxymethyltransferase or the glycine cleavage system are unable to carry out oxygenic photosynthesis. A side-reaction of oxygenic photosynthesis is the formation of 2-phosphoglycerate, which is converted to glycine in peroxisomes, then transported to mitochondria for catabolism of the glycine, yielding ammonium and carbon dioxide (section 1.3.2.4). The methylene tetrahydrofolate formed in the reaction of the glycine cleavage system is used by serine hydroxymethyltransferase to synthesize serine from another mol of glycine, and the serine is rapidly transported out of the mitochondria in exchange for glycine entering.

In plants, the mitochondrial pool of tetrahydrofolate and methylene tetrahydrofolate does not equilibrate with that in the cytosol. Unlike non-photosynthetic organisms, in photosynthetic tissues the mitochondrial enzymes are concerned only with the catabolism of glycine, not the provision

of one-carbon fragments for biosynthetic reactions (Bauwe & Kolukisaoglu, 2003).

The maximum activity of the glycine cleavage system in plant leaves is some 2.5 times higher than that of serine hydroxymethyltransferase, driving the reaction in the unfavoured direction of serine synthesis. The continuous action of the glycine cleavage system is driven by reoxidation of the NADH via the mitochondrial electron transport chain and the removal of methylene tetrahydrofolate by serine hydroxymethyltransferase (Rebeille *et al.*, 1994). Plant glycine decarboxylase is inhibited by *S*-nitrosylation by nitric oxide (section 5.9.3), a post-synthetic modification with a short half-life. This leads to mitochondrial depolarization, hydrogen peroxide production and, in cultured cells, cell death in response to stress (Palmieri *et al.*, 2010).

Metabolic stress to plants, including heat, dehydration and salinity, leads to closing of stomata to reduce water loss, and hence a decrease in the availability of carbon dioxide for fixation and slowing of the rate of photosynthesis. However, light absorption and photo-excitation continue, which can cause bleaching of chlorophyll. Photorespiration increases to provide carbon dioxide, and the glyoxylate that is produced is reduced to glycolate (so minimizing damage to proteins and nucleic acids from the reactive aldehyde). The reduction of glyoxylate to glycolate provides a sink for the excess reducing equivalents being produced by photosynthesis.

There is also an increase in the activity of the GABA shunt (see section 5.5), which generates succinic semialdehyde. Reduction of succinic semialdehyde to γ -hydroxybutyrate similarly provides a sink for excess reducing equivalents (Allan *et al.*, 2009).

4.2.4 Non-ketotic and ketotic hyperglycinaemia

Non-ketotic hyperglycinaemia (also known as glycine encephalopathy) is due to a defect of the glycine cleavage system; indeed, the glycine cleavage system as a pathway for glycine catabolism was discovered in studies of children with non-ketotic hyperglycinaemia. All of the affected infants have elevated serum glycine. The severity of the condition depends on the extent to which the glycine cleavage system is impaired and the extent to which glycine accumulates in the central nervous system, over-activating the NMDA receptor by binding to the allosteric activator site. In most patients (85 per cent), the defect is in the glycine decarboxylase P protein (Hayasaka *et al.*, 1982, 1987; Tada & Hayasaka, 1987; Tada & Kure, 1993).

In the neonatal variant, the new-born infant is lethargic, with poor muscle tone and myoclonic jerks, progressing to apnoea, which is commonly fatal. Those who survive suffer intractable seizures and profound mental retardation. In the infantile variant, the infants develop normally until aged about six months, then present with seizures and varying degrees of mental

retardation. When there is still a significant residual activity of the glycine cleavage system, the condition is episodic, and affected children show mild mental retardation with episodes of delirium, chorea and vertical gaze palsy during febrile illnesses.

Standard treatment for the condition is the administration of benzoate to lower serum glycine by forming hippuric acid, and dextromethorphan as an antagonist of glycine at the NMDA receptors in the central nervous system.

In ketotic hyperglycinaemia, the failure of glycine metabolism is secondary to propionic acidemia (due to propionyl CoA carboxylase deficiency) or methylmalonic aciduria (due to methylmalonyl CoA mutase deficiency). When the patient's condition is well controlled by a low protein diet, the serum concentration of glycine is normal and liver biopsies show normal activity of the glycine cleavage system. However, *post mortem* measurement of the glycine cleavage system in livers from two patients who died in metabolic acidosis showed very low activity, especially of the H-protein, which is the lipoamide carrier protein in the multi-enzyme complex. This suggests that there is repression of the synthesis of the protein by one or other of the abnormal metabolites that accumulate when the condition is poorly controlled (Hayasaka *et al.*, 1982).

4.3 Glycine oxidase and glyoxylate metabolism

The cycle of transamination of glyoxylate to glycine, followed by oxidative deamination of glycine to glyoxylate, catalyzed by glycine oxidase, provides a pathway for overall deamination of most amino acids (Figure 1.14). In addition to transamination, glyoxylate can undergo five possible metabolic fates, as shown in Figure 4.4.

Glyoxylate is a substrate for lactate dehydrogenase, which catalyzes its oxidation to oxalate. Accumulation of glyoxylate and its subsequent oxidation to oxalate is the cause of primary hyperoxaluria (section 4.3.1). Glyoxylate can also be reduced to glycolate by glyoxylate reductase, the enzyme that is deficient in type II primary hyperoxaluria. Glyoxylate reductase is the same enzyme as hydroxypyruvate reductase (see Figure 4.8), which catalyzes the reduction of hydroxypyruvate arising from serine transamination to D-glycerate (Behnam *et al.*, 2006).

In bacteria, condensation of two molecules of glyoxylate, eliminating carbon dioxide and forming tartronic semialdehyde, is catalyzed by the thiamin diphosphate-dependent enzyme glyoxylate carboxylase. Tartronic semialdehyde can be reduced to D-glyceric acid, so providing a direct gluconeogenic pathway from glycine, as an alternative to the intermediate synthesis of serine (see section 4.2.1). This pathway is not found in mammals.

2-Hydroxy-3-oxoadipic acid synthase catalyzes the thiamin diphosphate-dependent condensation of glyoxylate with 2-oxoglutarate, eliminating

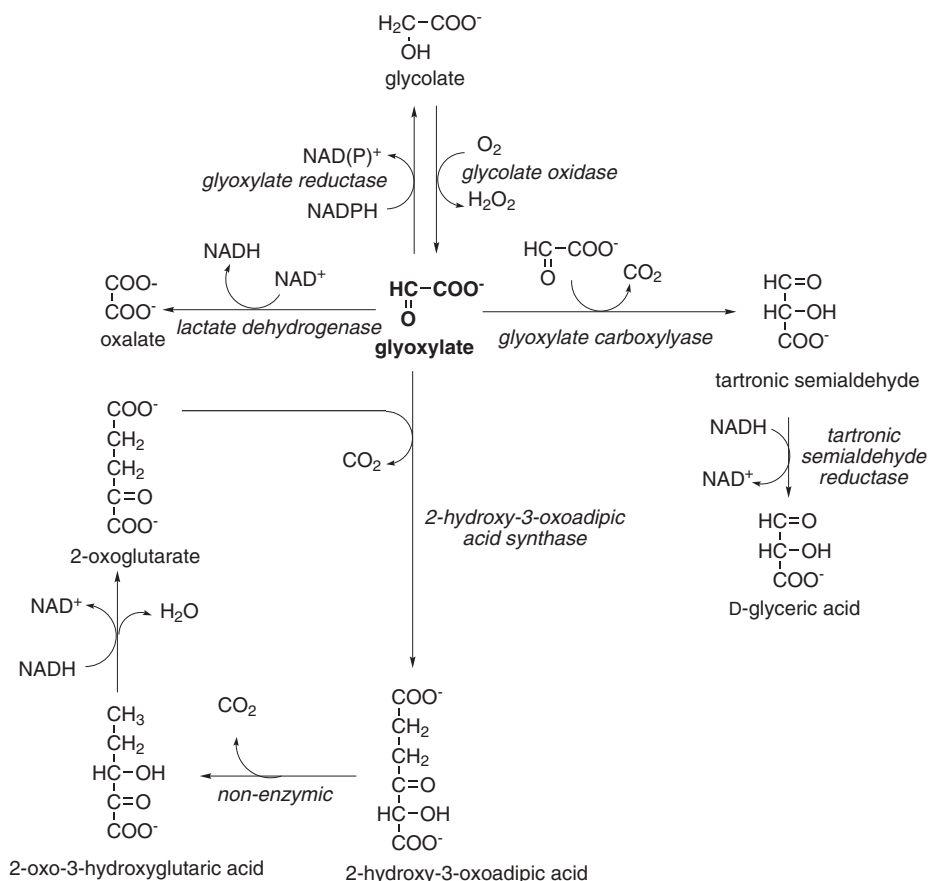


Figure 4.4 Metabolic fates of glyoxylate.

Lactate dehydrogenase EC 1.1.1.27, glyoxylate reductase EC 1.1.1.26, glyoxylate carboxylase EC 4.1.1.47, tartronic semialdehyde reductase EC 1.1.1.60, 2-hydroxy-3-oxo-adipic acid synthase EC 2.2.1.5.

carbon dioxide and forming 2-oxo-3-hydroxyadipate. This can undergo further decarboxylation to 2-oxo-hydroxyglutarate and reduction to yield 2-oxoglutarate, thus providing a cyclic pathway for complete catabolism of glyoxylate. The importance of this pathway is shown by observations that, in rats, both magnesium deficiency and thiamin deficiency lead to a decrease in glyoxylate oxidation and accumulation of glyoxylate and oxalate, although the enzymes involved in the conversion of 2-oxo-3-hydroxyadipate to oxoglutarate have not been characterized (Bais *et al.*, 1991; Rattan *et al.*, 1993; Sidhu *et al.*, 1987).

In plants and bacteria, pyruvate decarboxylase catalyzes the decarboxylation of glyoxylate to formaldehyde. Glyoxylate inhibits the decarboxylation of pyruvate to acetaldehyde, but pyruvate and acetaldehyde accelerate the

decarboxylation of glyoxylate, because acetaldehyde forms a condensation product with formaldehyde bound to thiamin at the active site and enhances its removal.

4.3.1 Primary hyperoxaluria

Any glyoxylate that accumulates in the cell and is not metabolized by one of the pathways discussed in section 4.3 is a substrate for lactate dehydrogenase, forming oxalate. Oxalate has a low solubility, and excessive excretion of oxalate leads to crystallization of calcium oxalate in the kidney and urinary tract. As renal function is progressively impaired, so systemic oxalosis develops, with crystallization of oxalate throughout the body.

Type I primary hyperoxaluria is due to defects in alanine-glyoxylate transaminase. Some 150 different mutations in the alanine-glyoxylate transaminase gene have been reported. In some cases, the effect of the mutation is that the enzyme has little or no activity (either a very low $V_{\max}$ or an abnormally high K_m). In other cases, there is no detectable enzyme protein. Sometimes the defect is in the coenzyme binding site, and doses of vitamin B₆ of the order of 250–500 mg/day (as compared with reference intakes of the order of 15–20 mg/day) permit maintenance of more or less normal enzyme activity (see section 3.6 for a discussion of other vitamin B₆ dependency syndromes).

However, the commonest cause of primary hyperoxaluria is mis-targeting of the enzyme, so that it is found in mitochondria rather than peroxisomes. Human alanine-glyoxylate transaminase is a peroxisomal enzyme; however, in carnivores the enzyme is mainly mitochondrial, and in rodents it is equally distributed between mitochondria and peroxisomes. There is a common polymorphism of human alanine-glyoxylate transaminase, with proline rather than leucine at position 11. About 95 per cent of the Pro11Leu protein folds and dimerizes normally, and is imported into the peroxisomes, but 5 per cent is imported into mitochondria before it can fold and dimerize. It then folds, and is active, inside the mitochondria.

A second mutation, Gly170Arg, delays protein folding and dimerization and, when this is superimposed on the Pro11Leu polymorphism, it results in the unfolded protein being transported into mitochondria, where it folds and dimerizes, with only about 10 per cent of the enzyme being in the peroxisomes where glyoxylate is formed and needs to be metabolized. Mitochondria can only import unfolded or loosely folded proteins, whereas peroxisomes can import the folded and dimerized enzyme with its pyridoxal phosphate cofactor bound (Danpure, 1993, 2004, 2005).

Type II primary hyperoxaluria is very much rarer than type I, and it is caused by defects in glyoxylate reductase, which also catalyzes the interconversion of D-glycerate and hydroxypyruvate (see Figure 4.8). In this condi-

tion, not only oxalate but also glycerate is excreted in the urine. D-Glycerate reductase is less severely affected than glyoxylate reductase, which is virtually undetectable, suggesting that there are other enzymes that can catalyze the reduction of D-glycerate, but not of glyoxylate (Cramer *et al.*, 1999; Giafi & Rumsby, 1998; Kemper *et al.*, 1997).

4.4 One-carbon metabolism

Quantitatively, glycine and serine are the main sources of one-carbon units used for biosynthetic reactions, forming methylene tetrahydrofolate through the actions of serine hydroxymethyltransferase and the glycine cleavage system (see Figure 4.3). As shown in Figure 4.1, the catabolism of choline is also a source of methylene tetrahydrofolate and, as discussed later in section 8.2.1, the catabolism of histidine leads to formation of methenyl tetrahydrofolate and formate yields formyl tetrahydrofolate. Methylene, methenyl and formyl tetrahydrofolates are interconvertible.

When there is a greater supply of one-carbon units from catabolic reactions than can be used in biosynthetic reactions, the formyl group of formyl tetrahydrofolate can be oxidized to carbon dioxide, so maintaining a supply of free tetrahydrofolate. Formyl tetrahydrofolate is used in purine synthesis (Figure 1.6), while methylene tetrahydrofolate is used in synthesis of serine and TMP (Figure 1.11). However, as will be seen in section 6.3.2, S-adenosylmethionine is the donor in most methylation reactions. In the methylation cycle (Figure 6.15), the methyl group is provided by methyl tetrahydrofolate. The reduction of methylene tetrahydrofolate to methyl tetrahydrofolate is irreversible. The one-carbon substituted folates are shown in Figure 4.5 and their interconversions in Figure 4.6.

4.5 Serine biosynthesis

Serine does not cross the placenta well and, in the foetal liver, there is net synthesis of serine from glycine that is synthesized by the foetal placenta (little glycine crosses the placenta from the maternal circulation). Shortly after birth there is a change and, while other tissues continue to synthesize serine, the liver of the neonate now becomes a net consumer of serine for gluconeogenesis (Narkewicz *et al.*, 2002). The cytosolic isoenzyme of serine hydroxymethyltransferase (section 4.2.1) is an important source of serine for gluconeogenesis; synthesis of serine in non-gluconeogenic tissues is mainly from the glycolytic intermediate 3-phosphoglycerate, as shown in Figure 4.7 below.

In the brain, *de novo* synthesis of serine from 3-phosphoglycerate is essential for the maintenance of normal levels of both D- and L-serine (see section

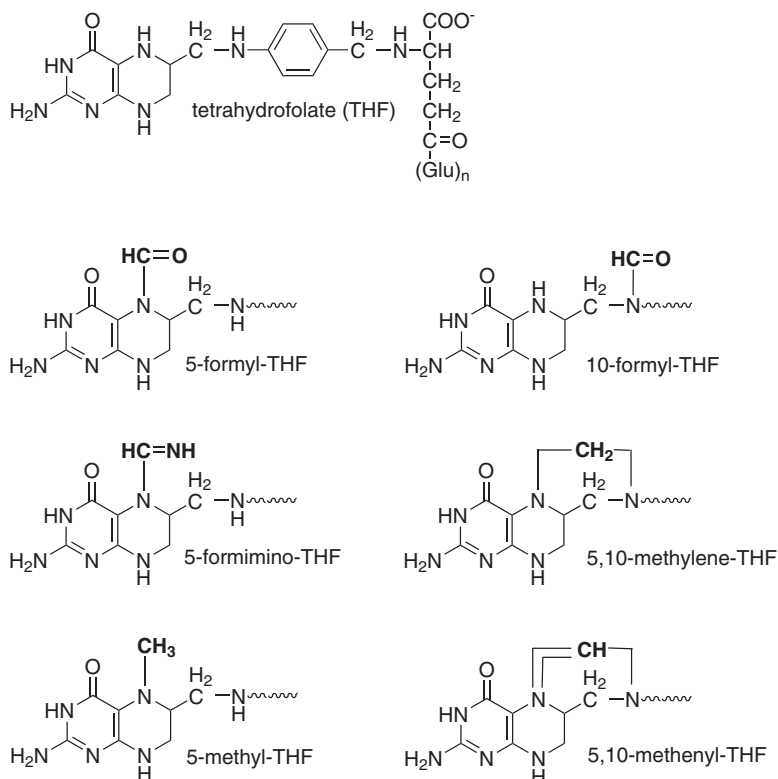


Figure 4.5 One-carbon substituted folate derivatives.

THF = tetrahydrofolate.

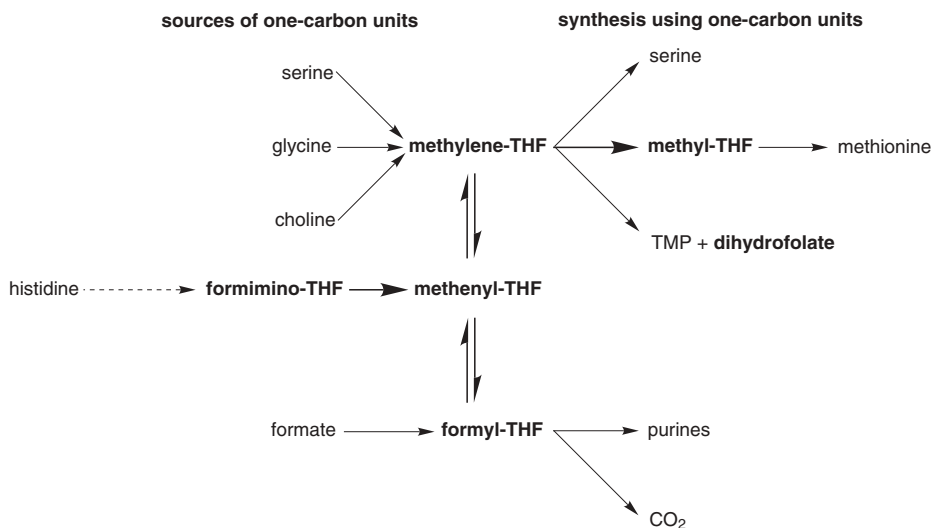


Figure 4.6 Overview of one-carbon metabolism.

THF = tetrahydrofolate.

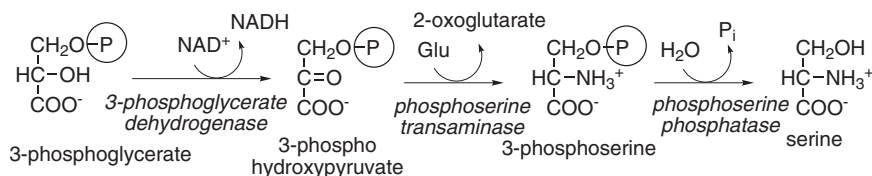


Figure 4.7 Serine synthesis.

3-Phosphoglycerate dehydrogenase EC 1.1.1.95, phosphoserine transaminase EC 2.6.1.52, phosphoserine phosphatase EC 3.1.3.3.

3.2.2 for a discussion of the role of D-serine in the central nervous system). Rare deficiency of 3-phosphoglycerate dehydrogenase or phosphoserine phosphatase leads to serine deficiency with severe neurological consequences (Yang *et al.*, 2010). The 3-phosphoglycerate pathway is also important for serine synthesis in non-photosynthetic tissues of plants.

Bacterial and plant 3-phosphoglycerate dehydrogenase is allosterically inhibited by serine. Each regulatory domain of the tetrameric enzyme in *E. coli* contains two serine binding sites, and serine forms hydrogen bonds between the regulatory domains of two subunits, leading to a decrease in the $V_{\max}$ of the enzyme with little or no effect on the K_m (Al-Rabee *et al.*, 1996).

The main regulatory step in serine synthesis in mammals is not 3-phosphoglycerate dehydrogenase, as in plants and bacteria, but phosphoserine phosphatase, which is inhibited by serine. In mice maintained on a low serine diet, phosphoserine phosphatase is upregulated (and serine deaminase and serine racemase in liver are downregulated), so both increasing serine synthesis and reducing its catabolism. Unlike most phosphatases, in which there is intermediate formation of a phosphorylated enzyme in which the phosphate is esterified to serine (or sometimes histidine or cysteine), in phosphoserine phosphatase, the enzyme is transiently phosphorylated on an aspartate residue. Aspartate phosphorylation is also seen in the P-type ATPases that are involved in ion transport across membranes (Collet *et al.*, 1999; Fell & Snell, 1988).

In theory, serine could also be synthesized by the non-phosphorylated pathway from 2-phosphoglycerate, via D-glycerate and the formation of hydroxypyruvate – reversal of the pathway for serine catabolism (Figure 4.8). However, the enzymes of the non-phosphorylated pathway are found only in gluconeogenic tissues (liver and kidney), while the enzymes of the phosphorylated pathway (Figure 4.7) are found in all tissues. Reversal of the phosphorylated pathway cannot be a source of substrate for gluconeogenesis, because the final step – dephosphorylation of phosphoserine – is irreversible (de Koning & Klomp, 2004; Tabatabaie *et al.*, 2010).

Further evidence that the phosphorylated pathway is the only one for serine synthesis in non-gluconeogenic tissues comes from rare cases of infants

lacking one or other of the enzymes of the pathway. They have very low serum and cerebrospinal fluid concentrations of serine and glycine, and they also have severe neurological problems, including congenital microcephaly and intractable seizures. The latter, however, can be prevented in some cases by supplementation of the mother with serine during pregnancy, despite the poor placental transport of serine. In most cases, the seizures can be controlled by serine supplementation after birth. The congenital problems reflect the importance of serine as a precursor for phospholipid and sphingolipid synthesis, as well as the roles of D-serine and glycine as neurotransmitters. In the central nervous system, serine is synthesized in astrocytes, not neurons, and neurons are dependent on astrocytes for a supply of the amino acid (Tabatabaie *et al.*, 2010).

4.6 Serine catabolism

As shown in Figure 4.8, serine can undergo one of three metabolic fates: the reaction of serine hydroxymethyltransferase to yield glycine and 5,10-methylene-tetrahydrofolate (section 4.2.1); transamination to hydroxypyruvate; or deamination catalyzed by serine deaminase to yield pyruvate, which is a substrate for gluconeogenesis in the fasting state. Except under gluconeogenic conditions, serine hydroxymethyltransferase probably provides the major route of serine catabolism. The $V_{\max}$ of serine transaminase is less than 10 per cent of that of serine hydroxymethyltransferase or deaminase (Snell, 1984).

4.6.1 Serine transamination

The transamination product of serine, hydroxypyruvate, can undergo either of two fates: reduction to D-glycerate, followed by phosphorylation to 3-phosphoglycerate, which is a glycolytic intermediate, and hence a substrate for gluconeogenesis in the fasting state; or decarboxylation and reduction to glyoxylate (hence a precursor of glycine).

Hydroxypyruvate reductase is the same protein as glyoxylate reductase, the enzyme that is defective in primary hyperoxaluria type II, and serine-pyruvate transaminase is the same protein as alanine-glyoxylate transaminase, the enzyme that is defective in primary hyperoxaluria type I (section 4.3.1).

The main route for gluconeogenesis from serine in the rat is via serine deaminase. Flux through transamination increases in response to glucagon, but still accounts for only 10–20 per cent of total gluconeogenesis from serine. In dogs (where the transaminase is mitochondrial), rabbits and human beings (where the transaminase is peroxisomal), the main flux for gluconeogenesis

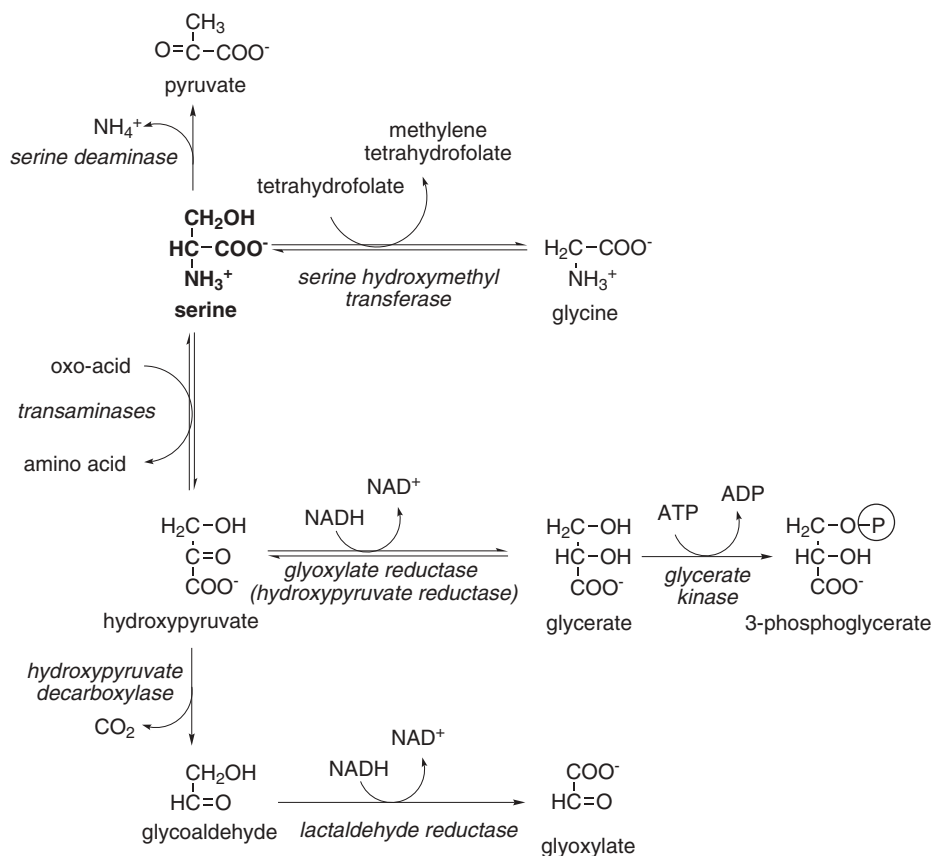


Figure 4.8 Metabolic fates of serine.

Hydroxypyruvate reductase (glycerate dehydrogenase) EC 1.1.1.29 and EC 1.1.1.81, glyoxylate reductase (EC 1.1.1.79) also acts on hydroxypyruvate, glycerate kinase EC 2.7.1.31, serine deaminase EC 4.3.1.7, serine hydroxymethyltransferase EC 2.1.2.1, lactaldehyde reductase EC 1.1.1.77.

is through transamination, with a negligible contribution from serine deaminase (Xue *et al.*, 1999a, 1999b).

4.6.2 Serine deaminase

The deamination of serine is catalyzed by serine deaminase (which is sometimes known as serine dehydratase). The reaction involves an initial dehydration, followed by isomerization and hydrolysis to release ammonium and pyruvate. In mammals, serine deaminase is the same enzyme as threonine deaminase (section 6.1.5), although serine racemase (section 3.2.2) also catalyzes the deamination reaction, as does bacterial tryptophanase (section 9.4.2; Griffiths & DeMoss, 1970).

Stimulation of gluconeogenesis in cultured hepatocytes by glucagon or glucocorticoid hormones leads to induction of serine deaminase, while insulin represses its synthesis. In the liver, it is also induced in response to feeding a high-protein diet. Leucine seems to be the signal for increased gluconeogenesis, and feeding excess leucine to rats leads to induction of serine deaminase mRNA synthesis (Mak & Pitot, 1981; Zhong *et al.*, 2007).

There is an inverse correlation between body size and hepatic serine deaminase activity among mammals, suggesting that the need for gluconeogenesis is greater in smaller animals that have smaller reserves of glycogen. Serine deaminase also seems to be important in thermogenesis, which again is a greater requirement for smaller mammals that have a larger surface area to volume ratio than larger animals. In rats, there is a 3–4 fold increase in activity on exposure to cold, while cold-blooded animals (e.g. amphibia and fishes) have very low or undetectable serine deaminase activity (Rowse *et al.*, 1979).

Bacteria have separate serine and threonine deaminases, neither of which acts on the other amino acid and, as discussed in section 6.1.5, microorganisms capable of isoleucine synthesis have separate biosynthetic and catabolic isoenzymes of threonine deaminase. While bacterial threonine deaminases are pyridoxal phosphate-dependent enzymes, like the mammalian deaminase, in Gram-positive bacteria there is an oxygen-sensitive serine deaminase that does not contain pyridoxal phosphate but has a catalytic iron-sulphur cluster (Grabowski *et al.*, 1993; Hofmeister *et al.*, 1993).

4.7 Peptidyl glycine hydroxylase (peptide α -amidase)

Half or more of the peptide hormones in both animals and plants undergo post-synthetic modification to form a carboxy-terminal amide, which is essential for biological activity. One function of this amidation is to render the peptides more hydrophobic, thus enhancing receptor binding. The amide group is derived from a glycine residue that is to the carboxyl side of the amino acid that will become the amidated terminal of the mature peptide.

As shown in Figure 4.9, the first step in the reaction is hydroxylation of the glycine residue to yield hydroxyglycine. Peptidyl glycine hydroxylase is a copper-containing oxygenase that uses ascorbate to reduce the two copper ions to Cu^+ ; these then activate oxygen, with incorporation of one atom into hydroxyglycine and reduction of the other to water. This is followed by cleavage of the peptide bond, with amidation of the amino acid to the amino side of the hydroxyglycine residue and the release of glyoxylate. In mammals, these two activities occur in a single bifunctional protein, although in invertebrates there are separate hydroxylase and lyase proteins.

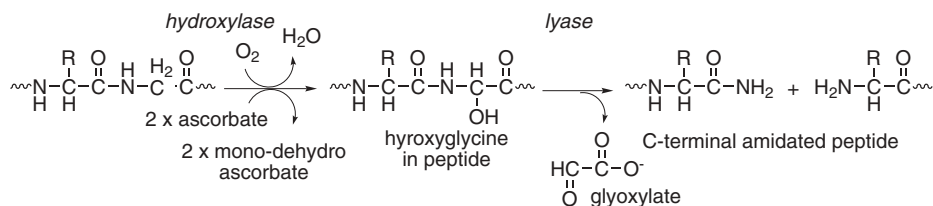


Figure 4.9 Synthesis of peptide C-terminal amides.

Peptidyl glycine hydroxylase EC 1.14.17.3, peptidylaminoglycolate lyase EC 4.3.2.5.

Although the carboxy-terminal amino acid amides are different in different hormones, the same enzyme seems to be involved in all post-synthetic amidation reactions, although there is some evidence that it undergoes differential splicing in different tissues. The enzyme is secreted together with the amidated peptides, because it is present in the secretory vesicles that develop from the Golgi where the post-synthetic modification occurs, and it is possible that some amidation of precursor proteins occurs in the circulation. Peptidyl glycine hydroxylase shows considerable sequence homology with dopamine β -hydroxylase (see section 9.2.4), which is also an ascorbate-dependent copper-containing enzyme (Bradbury & Smyth, 1991; Prigge *et al.*, 2000).

4.8 5-Aminolevulinic acid and porphyrin synthesis

The porphyrins include haem (in haemoglobin and cytochromes), chlorophyll in plants, photosynthetic algae and bacteria, sirohaem and phytychromobilin in plants, and the corrinoids (including vitamin B_{12}) in bacteria and algae. Sirohaem is the prosthetic group in nitrite reductase (see section 1.2) and sulphite reductase, which catalyzes the six-electron reduction of sulphite to sulphide. Phytychromobilin is the cofactor for phytyochrome, the light sensor and photoreceptor in plants.

The precursor for porphyrin synthesis is 5-aminolevulinic acid. In animals, yeasts and some bacteria, this is synthesized from glycine and succinyl CoA, as shown in Figure 4.10. In plants, algae and other bacteria, glutamate is the precursor for 5-aminolevulinic acid synthesis (section 5.1). Some microorganisms use both pathways for synthesis of 5-aminolevulinic acid (Kannangara *et al.*, 1988).

The condensation and decarboxylation of glycine and succinyl CoA to yield 5-aminolevulinic acid is catalyzed by 5-aminolevulinic acid synthase, a mitochondrial pyridoxal phosphate-dependent enzyme. There are different isoenzymes in haematopoietic and non-haematopoietic tissues, coded for by

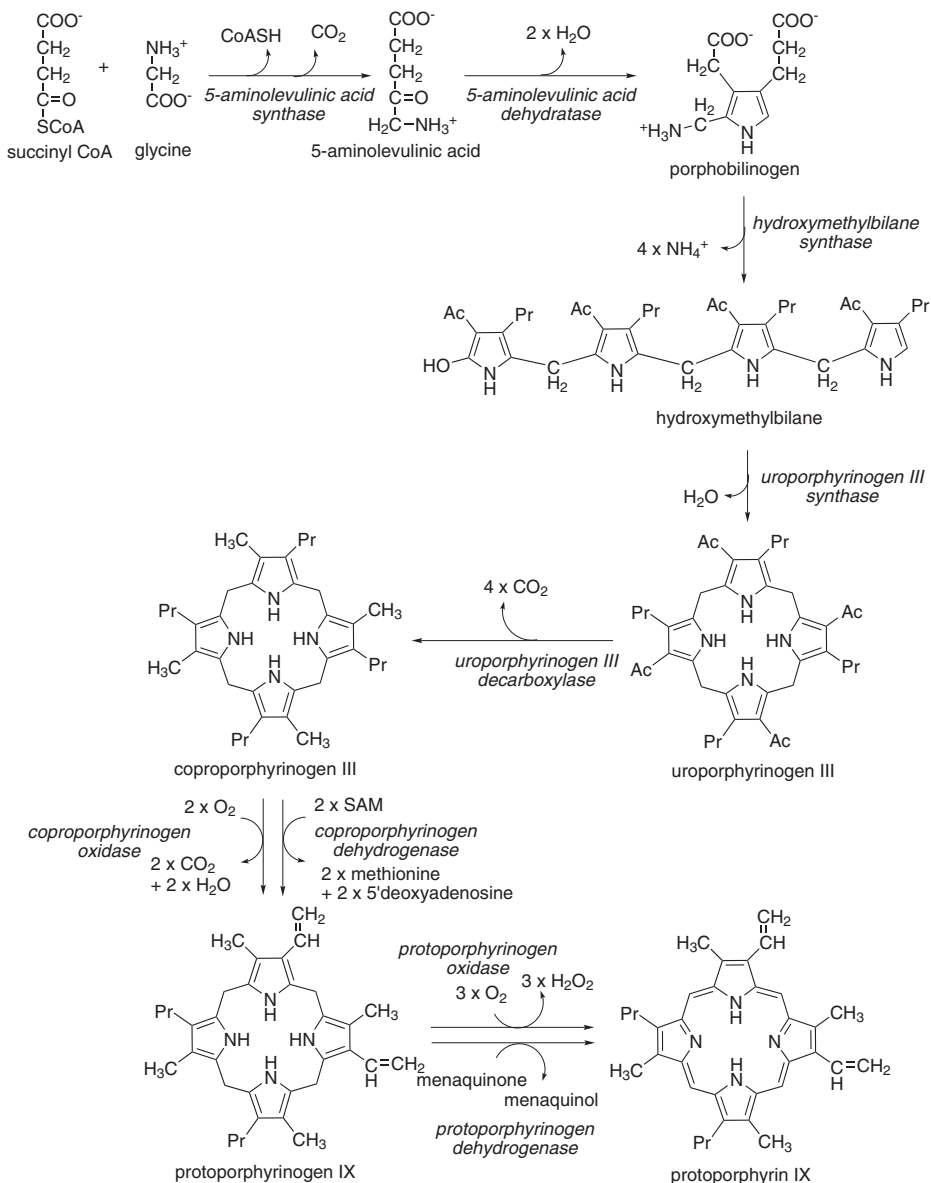


Figure 4.10 Synthesis of porphyrins.

Ac = acetyl, CH_2COO^- ; Pr = propionyl, $\text{CH}_3\text{-CH}_2\text{-COO}^-$; SAM = S-adenosylmethionine; 5-aminolevulinic acid synthase EC 2.3.1.37, 5-aminolevulinic acid dehydratase EC 4.2.1.24, hydroxymethylbilane synthase (porphobilinogen deaminase) EC 2.5.1.61, uroporphyrinogen III synthase EC 4.2.1.75, uroporphyrinogen III decarboxylase EC 4.1.1.37, coproporphyrinogen oxidase EC 1.3.3.3, coproporphyrinogen dehydrogenase EC 1.3.99.22, protoporphyrinogen oxidase EC 1.3.3.4, protoporphyrinogen dehydrogenase EC 1.3.5.3.

different genes. The erythroid isoenzyme is only expressed in developing erythrocytes, and is encoded on the X chromosome. This isoenzyme is responsible for 90 per cent of haem synthesis in human beings. Genetic defects lead to X-linked sideroblastic anaemia, which is characterized by inadequate haem synthesis (hence hypochromic anaemia) and excessive accumulation of iron in erythroblast mitochondria (Hunter & Ferreira, 2009; May & Bishop, 1998). Some cases of X-linked sideroblastic anaemia are vitamin B₆ responsive (see section 3.6).

Expression of the erythroid 5-aminolevulinic acid synthase gene is upregulated by erythropoietin. Haem, the end-product of the pathway, induces the synthesis of globin, so that neither haem nor free globin accumulates. Translation of 5-aminolevulinic acid synthase mRNA is dependent on an adequate supply of iron – the 5′-untranslated region of the mRNA contains an iron-response element. In non-erythroid cells, the expression of 5-aminolevulinic acid synthase I is constitutive and the enzyme is inhibited by haem. In erythroid cells, 5-aminolevulinic acid synthase II is not inhibited by haem, but haem regulates cellular uptake of iron from transferrin. Erythroid cells also express 5-aminolevulinic acid synthase I constitutively and, as in other tissues, this isoenzyme is inhibited by haem (Ponka, 1999; Sadlon *et al.*, 1999).

5-Aminolevulinic acid synthase shows considerable structural similarity to glutamic semialdehyde transaminase, which is responsible for 5-aminolevulinic acid synthesis in plants, and it probably evolved from this enzyme. The active site is at the bottom of a deep cleft. Glycine binds first, then succinyl CoA binds and occludes the cleft. The reaction proceeds with the decarboxylation of glycine. When succinyl CoA is bound to the enzyme, the rate of formation of the glycine aldimine to pyridoxal phosphate is two orders of magnitude faster than when succinyl CoA is not bound. The reaction is reversible, and the enzyme will catalyze the formation of labelled glycine from 5-aminolevulinic acid and labelled carbon dioxide (Hunter & Ferreira, 2009).

5-Aminolevulinic acid dehydratase (porphobilinogen synthase) in the cytosol catalyzes the asymmetric condensation of two molecules of 5-aminolevulinic acid to yield porphobilinogen. There are two adjacent lysine residues in the active site, each of which forms a Schiff base with a molecule of 5-aminolevulinic acid. The human enzyme exists as an equilibrium of functionally distinct quaternary structures (morpheesins) – an octomer that has high activity and a hexamer that has low activity.

Like 5-aminolevulinic acid synthetase, the deaminase is induced in bone marrow erythroid cells by erythropoietin. 5-Aminolevulinic acid deaminase is a zinc-dependent enzyme but, in some bacteria and plants, one or both of the zinc atoms can be replaced by magnesium. The enzyme is inhibited by lead, and polymorphisms in the gene are associated with differences in sensitivity to lead poisoning. Urinary excretion of 5-aminolevulinic acid is an early marker of lead intoxication (Jaffe, 2004; Jaffe & Stith, 2007).

Hydroxymethylbilane synthase (porphobilinogen deaminase) catalyzes the stepwise addition of four mol of porphobilinogen to yield hydroxymethylbilane, which is unstable, and in the absence of uroporphyrinogen III synthase undergoes non-enzymic condensation to yield uroporphyrinogen I. In *Plasmodium* spp., a single enzyme catalyzes the reactions of porphobilinogen deaminase and uroporphyrinogen III synthase, while in animals there are two separate enzymes (Nagaraj *et al.*, 2008). There are different isoenzymes of the deaminase in haematopoietic and non-haematopoietic tissues, but these result from differential splicing of the RNA transcript from the same gene. However, different promoter regions are involved in the transcription of the gene in haematopoietic and non-haematopoietic tissues (Grandchamp *et al.*, 1987; Sassa, 1990).

Hydroxymethylbilane synthase has an unusual prosthetic group – dipyrromethane bound to a cysteine residue at the active site. This is formed autocatalytically in the apoenzyme by head-to-tail condensation of two molecules of porphobilinogen, and experimentally the holoenzyme can be resolved to yield the apoenzyme, which is then reactivated by incubation with porphobilinogen. The enzymic reaction proceeds by addition of porphobilinogen to the free α -position of the prosthetic group, with loss of ammonium, followed by repeated addition of porphobilinogen until a hexapyrrole is formed. This is then cleaved to release the tetrapyrrole, hydroxymethylbilane, leaving the dipyrrole prosthetic group to undergo another reaction cycle (Warren & Scott, 1990).

The final cytosolic step in the pathway is catalyzed by uroporphyrinogen decarboxylase, which catalyzes the removal of four carboxyl groups from the acetate side-chains, yielding coproporphyrinogen. The remaining steps are again mitochondrial.

Coproporphyrinogen oxidase (in eukaryotes) and coproporphyrinogen dehydrogenase (in bacteria) catalyze the oxidation and decarboxylation of propionyl side-chains to yield vinyl groups. Coproporphyrinogen oxidase catalyzes the introduction of molecular oxygen into the propionyl side-chain to form a hydroperoxide, followed by elimination of water and decarboxylation to form the vinyl side-chain. Bacterial coproporphyrinogen dehydrogenase is oxygen-independent and uses *S*-adenosylmethionine as the oxidant – it is a radical SAM enzyme (section 6.3.5). The reaction starts with the transfer of an electron from a reduced iron-sulphur cluster onto *S*-adenosylmethionine, cleaving it to methionine and the 5-deoxyadenosine radical. The radical then initiates attack on the propionyl side-chains, leading to decarboxylation and formation of the vinyl side-chains (Layer *et al.*, 2003; Silva & Ramos, 2008).

In eukaryotes and Gram-positive bacteria, protoporphyrinogen IX is oxidized to protoporphyrin IX in a six electron reaction catalyzed by protoporphyrinogen oxidase, a flavoprotein. In mammals, the oxidase is

membrane-bound, while the bacterial enzyme is soluble but otherwise very similar to the eukaryotic enzyme. In anaerobic Gram-negative bacteria, this reaction is catalyzed by protoporphyrinogen dehydrogenase, a flavoprotein that reduces menaquinone to menadiol (Boynton *et al.*, 2009).

The final step in haem synthesis is the insertion of iron, catalyzed by ferrochelatase. Protoporphyrinogen oxidase is located on the outer face of the inner mitochondrial membrane, while ferrochelatase is on the matrix face of the membrane. There is evidence of direct channelling of protoporphyrin across the membrane between the two enzymes (Ferreira *et al.*, 1988; Ferreira & Zhang, 2002). In plants, magnesium is incorporated into protoporphyrin IX as the first committed step in chlorophyll synthesis.

Sirohaem is synthesized from uroporphyrinogen III by methylation and insertion of iron. Phytychromobilin is an open-chain porphyrin synthesized by oxidation of haem to biliverdin, the same reaction as occurs in the catabolism of haem in mammals.

Vitamin B₁₂ is synthesized only by bacteria, although some algae synthesize other corrinoids that are analogues of vitamin B₁₂, but which do not have vitamin activity for mammals. Uroporphobilinogen III undergoes successive methylation reactions, in which *S*-adenosylmethionine is the methyl donor, followed by excision of C-20 to give the direct fused link between the A and D pyrrole rings. This is followed by insertion of the central cobalt atom, attachment of 5'-deoxyadenosine to the cobalt atom (yielding cobyrinic acid), amidation of the acidic side-chains to yield cobinamide and, finally, attachment of the dimethylbenzimidazole nucleotide, which is either formed from riboflavin or is synthesized by a similar pathway to that of riboflavin synthesis, yielding adenosylcobalamin (Raux *et al.*, 2000; Roessner *et al.*, 2001).

4.8.1 Porphyrrias – diseases of porphyrin synthesis

Genetic defects of various of the enzymes of porphyrin synthesis lead to porphyria – accumulation of 5-aminolevulinic acid and the substrate of the affected enzyme in plasma and urine (Bloomer, 1981). Lead inhibits both 5-aminolevulinic acid synthetase and ferrochelatase, leading to porphyria and the formation of zinc protoporphyrin instead of haem, and hence to the development of anaemia.

The very rare condition of acute hepatic porphyria is due to lack of 5-aminolevulinic acid deaminase activity. Eight variants of the 5-aminolevulinic acid deaminase gene that are associated with porphyria have been expressed in *E. coli*, and all show an increased tendency to form the low-activity hexamer rather than the high-activity octomer (Jaffe & Stith, 2007).

Acute intermittent porphyria is due to lack of porphobilinogen deaminase. It is characterized by acute attacks of abdominal pain, neuritis and psychosis. The condition is precipitated by drugs such as barbiturates and

sulphonamides, which induce haem synthesis. Alcohol, infection and starvation can also precipitate attacks. Symptoms rarely occur before puberty, and only 10–20 per cent of heterozygotes are symptomatic.

Congenital porphyria is due to lack of uroporphyrinogen III synthase. It may be erythropoietic or hepatic, depending on which of the isoenzymes is affected. It results in over-production of type I porphyrins, which are accumulated in tissues, especially the skin, leading to photosensitivity and severe skin damage.

Porphyria cutanea tarda is due to lack of uroporphyrin decarboxylase. It is characterized by photosensitive dermatitis and urinary excretion of uroporphyrin, but not neuritis or psychotic episodes. The onset is associated with alcoholism and, sometimes, oestrogens. Most cases are sporadic – the result of new mutations in the gene – although familial cases have been reported.

Porphyria variegata is due to lack of protoporphyrin oxidase, and it is associated with variable skin signs, including hyperpigmentation. Affected people have elevated faecal protoporphyrin and coproporphyrin at all times, and they suffer acute attacks of abdominal pain, neuritis and psychosis, with elevated urinary excretion of aminolevulinic acid and porphobilinogen.

4.9 Selenocysteine

A number of proteins in archaea, prokaryotes and eukaryotes (including mammals, but excluding fungi and higher plants) contain selenocysteine, the selenium analogue of cysteine. This is not the result of a post-synthetic modification of the protein but, like pyrrolysine, (see section 6.2.4.3), involves incorporation of the unconventional amino acid during ribosomal protein synthesis. The codon for selenocysteine is the opal stop codon, UGA, which is read in a context-sensitive manner as coding for selenocysteinyl tRNA^{Sec}. Selenocysteinyl tRNA^{Sec} is synthesized from seryl tRNA^{Sec}.

Most of the selenoproteins whose function is known catalyze redox reactions. There are 25 known human selenoproteins, which include glutathione peroxidases (section 5.4.1), thioredoxin reductases, thyroxine deiodinases (section 9.2.6) and selenophosphate synthetase, as well as a number of selenoproteins whose metabolic function is not known (Brigelius-Flohe, 1999).

As shown in Figure 4.11, the first step in incorporation of selenium is the synthesis of selenophosphate by reaction between selenium and ATP. While it might be assumed that selenide would be the substrate for this reaction, the K_m for selenide is close to its toxic concentration in the cell, so it is more likely that the true substrate is elemental selenium, Se⁰. It has long been known that organic selenium (selenocysteine and selenomethionine) is considerably more effective as a source of selenium for experimental animals and in nutritional supplements than are inorganic selenium salts.

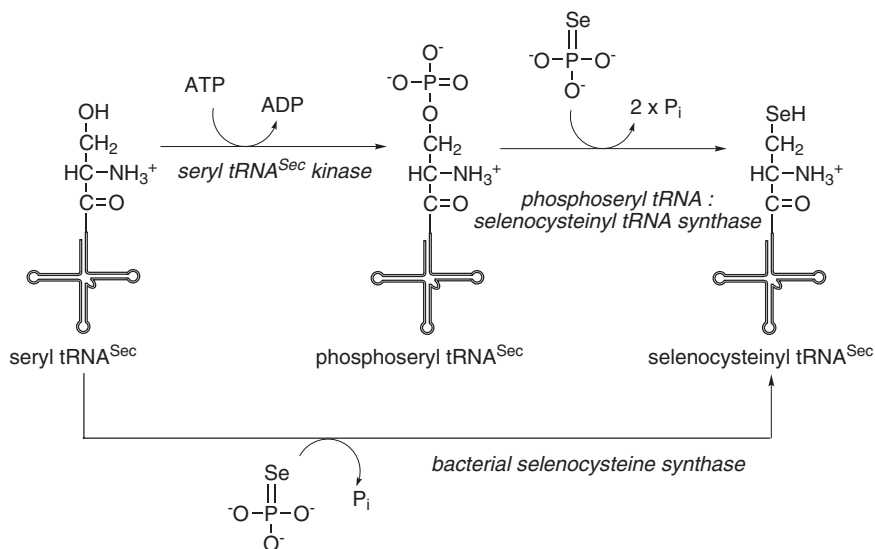


Figure 4.11 Synthesis of selenocysteine.

Seryl tRNA^{Sec} kinase EC 2.7.1.164, phosphoseryl tRNA^{Sec}:selenocysteinyl tRNA synthase EC 2.9.1.2, bacteria selenocysteine synthase EC 2.9.1.1.

Selenocysteine lyase catalyzes the cleavage of selenocysteine to alanine and elemental selenium. It is only in *in vitro* incubations containing a reducing agent such as dithiothreitol that selenium is reduced to selenide. Selenocysteine lyase permits both the salvage of selenium arising from the catabolism of selenoproteins and the utilization of dietary selenocysteine (Lacourciere & Stadtman, 2001).

Selenocysteine can also be formed by the same sequence of reactions as the synthesis of cysteine from methionine (see section 6.3.2) from selenohomocysteine, which arises from the demethylation of selenomethionine, followed by reactions catalyzed by cystathionine β -synthase and cystathione γ -lyase (Soda *et al.*, 1999).

Selenocysteine lyase is a pyridoxal phosphate-dependent enzyme, catalyzing a β -elimination reaction (see section 3.4). It acts on selenocysteine, which is present in very low concentrations in the cell, but not on cysteine, which is present in considerably higher concentrations. However, cysteine is a competitive inhibitor of the reaction with selenocysteine.

The reaction proceeds by formation of a selenium-persulphide transition state intermediate to a cysteine residue in a flexible loop of the enzyme, which directs the selenium adduct, but not the cysteine adduct, to the catalytic site in the correct orientation to undergo reaction. Like other pyridoxal phosphate-dependent enzymes catalyzing β -elimination reactions (section 3.4.1), selenocysteine lyase undergoes gradual inactivation as a result of

accidental transamination, leaving pyridoxamine phosphate at the active site (Esaki *et al.*, 1985; Omi *et al.*, 2010).

The incorporation of selenium occurs with serine esterified to tRNA^{Sec}, a tRNA species that differs from the normal tRNA^{Ser}, and indeed other tRNA species, in having a six base-pair D stem, rather than the usual 3–4 base-pairs, and a longer amino acid acceptor arm than normal. These features target seryl tRNA^{Sec} for incorporation of selenium, and also the binding of specialized transcription factors that permit ribosomal incorporation of selenocysteine rather than binding of termination factors to the UGA codon. There is no evidence of a separate amino acyl synthetase for tRNA^{Sec}, and it is assumed that the same enzyme catalyzes the esterification of serine to both tRNA^{Sec} and tRNA^{Ser} (Allmang & Krol, 2006).

There are two separate pathways for incorporation of selenium into seryl tRNA^{Sec}. In bacteria, seryl tRNA^{Sec} is the substrate for reaction with selenophosphate, yielding selenocysteinyl tRNA^{Sec}, while in both archaea and eukaryotes there is intermediate formation of phosphoseryl tRNA^{Sec}. Bacterial selenocysteine synthase is a pyridoxal phosphate-dependent enzyme which catalyzes an initial β -elimination (dehydration) reaction to form an amino-acryl intermediate that is the acceptor for selenium from selenophosphate. The mammalian enzyme, phosphoseryl tRNA:selenocysteinyl tRNA synthase, is also pyridoxal phosphate-dependent, and it catalyzes dephosphorylation and dehydration of the phosphoseryl tRNA substrate (Sheppard *et al.*, 2008; Xu *et al.*, 2007a, 2007b, 2007c).

Incorporation of selenocysteine during protein synthesis depends on decoding the UGA codon to mean 'selenocysteine' rather than 'stop'. This is achieved by a stem-loop sequence in the mRNA. In prokaryotes, this sequence embeds the UGA codon; in eukaryotes and archaea, it is in the 3' untranslated region. In prokaryotes, the decision between termination and insertion of selenocysteine depends to a considerable extent also on the next base after the UGA codon, because the release factors for termination bind not only to the UGA codon but also to the next base. UGAA and UGAC bind the termination factors poorly, thus permitting binding of selenocysteine tRNA^{Sec}. Selenocysteine tRNA^{Sec} is not recognized by the standard elongation factors because of the structural differences from the usual tRNA species; there are unique trans-acting proteins that bind to the stem-loop sequence and deliver it to the ribosome (Driscoll & Copeland, 2003; Yuan *et al.*, 2010).

Further reading

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

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5

Amino Acids Synthesized from Glutamate: Glutamine, Proline, Ornithine, Citrulline and Arginine

Like aspartate (see Chapter 6), glutamate and its oxo-acid 2-oxoglutarate, which is an intermediate in the citric acid cycle, provide a metabolic cross-roads between amino acid and carbohydrate metabolism. As discussed in section 1.3.2, the synthesis of glutamate and glutamine provides the main route for incorporation of ammonium into amino acids, and the activities of glutaminase and glutamate dehydrogenase provide a major route for disposal of fixed nitrogen as ammonium.

In *E. coli*, in addition to the approximately 250 $\mu\text{mol/g}$ of glutamate in proteins, an additional 810 μmol is required for the synthesis of glutamine, arginine, proline, polyamines and peptidoglycans, and 7,100 μmol is needed to provide the amino groups of other amino acids, giving a total requirement for synthesis of some 8,170 μmol of glutamate per gram (Reitzer, 2003). In mammals, little dietary glutamate reaches the bloodstream; most is metabolized in the intestinal mucosa, where it provides the major metabolic fuel for mucosal cells in the fed state (Reeds *et al.*, 2000a, 2000b).

Glutamate is an excitatory neurotransmitter, acting through both ionotropic receptors (calcium selective channels) and metabotropic receptors (G-protein-coupled receptors acting through either phospholipase C or adenylate cyclase). There are distinct metabotropic and ionotropic glutamate receptors on the tongue, which detect glutamate synergistically with ribonucleotides as the so-called umami or savoury flavour. Glutamate also has a

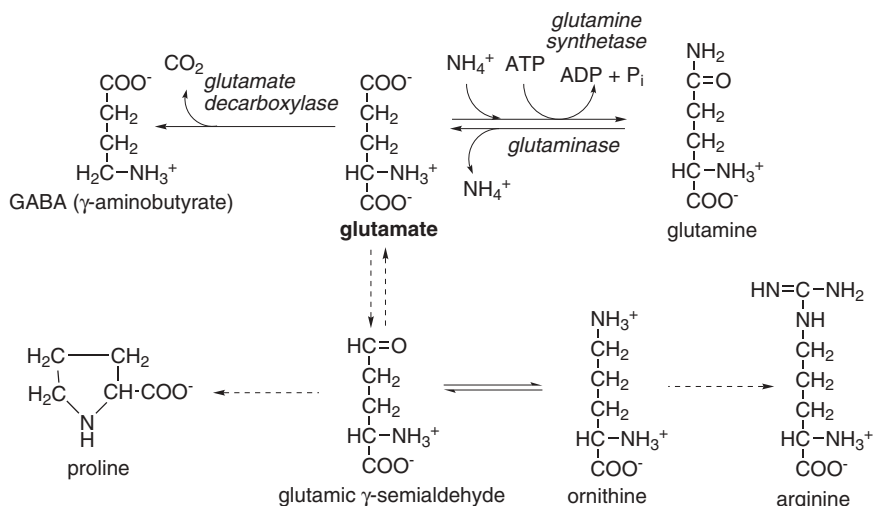


Figure 5.1 Amino acids synthesized from glutamate.

Glutamate decarboxylase EC 4.1.1.15, glutamine synthetase EC 6.3.1.2, glutaminase EC 3.5.1.2.

long-term neuromodulatory role in the brain by activation of transcription factors and changes in protein synthesis (Brand, 2000; Kaczmarek, 1994; Kurihara & Kashiwayanagi, 2000). Primitive organisms such as sponges and slime moulds respond to extracellular glutamate, and to agonists and antagonists of the mammalian metabotropic glutamate receptors, and have genes homologous with the mammalian metabotropic glutamate and GABA_B receptors. In *Arabidopsis*, there are at least 20 genes that have significant homology with the mammalian ionotropic glutamate receptors (Forde & Lea, 2007).

The decarboxylation product of glutamate, γ -aminobutyrate (GABA – see section 5.5) is an inhibitory neurotransmitter, and synthesis and catabolism of GABA provides an alternative pathway to 2-oxoglutarate dehydrogenase in the citric acid cycle, especially under conditions of thiamin deficiency.

As shown in Figure 5.1, glutamate is the precursor for the synthesis of proline (section 5.7.1) and arginine (section 5.9.1), and glutamine provides the glutamate residues for formation of isopeptide bonds with the ϵ -amino group of lysine residues in cross-linking of proteins (section 5.3.3). Arginine is the precursor for synthesis of nitric oxide (section 5.9.3), agmatine (section 5.9.4) and the muscle phosphagen creatine (section 5.9.7).

Post-synthetic modification of arginine residues in proteins leads to the formation of citrulline, an amino acid that is found in many proteins but which is not incorporated during ribosomal protein synthesis (section 5.9.5). Vitamin K-dependent post-synthetic modification of glutamate residues in proteins leads to the formation of γ -carboxyglutamate (section 5.6). Gluta-

mate is also the precursor (by reaction with ethylamine) for synthesis of the non-protein amino acid theanine, which accounts for half the total free amino acids in tea and is responsible for much of the flavour.

Ornithine is the precursor for synthesis of the diamine putrescine, and hence the polyamines spermidine and spermine (see section 5.8).

Together with leucine, glutamine and arginine activate signalling pathways to promote protein synthesis, and possibly also to inhibit autophagy-mediated protein catabolism in intestinal epithelial cells. Glutamine stimulates MAP kinase, and arginine the mTOR pathway, to enhance cell proliferation and migration. Both amino acids have cytoprotective actions in response to nutrient deprivation, oxidative injury, stress and immunological challenge (Marc Rhoads & Wu, 2009).

A number of proteins contain an amino-terminal 5-oxoproline residue that arises as the result of post-synthetic modification – either deamidation of a glutamine residue or phosphorylation and subsequent dephosphorylation and dehydration of a glutamate residue.

5.1 Synthesis of 5-aminolevulinic acid from glutamate in plants

The precursor for porphyrin synthesis is 5-aminolevulinic acid. In animals, yeasts and some bacteria, this is synthesized from glycine and succinyl CoA (see section 4.8). In plants, algae and other bacteria, glutamate is the precursor for 5-aminolevulinic acid synthesis. Some microorganisms use both pathways for the synthesis of 5-aminolevulinic acid (Kannangara *et al.*, 1988).

As shown in Figure 5.2, the first step in the glutamate pathway is esterification of glutamate to tRNA, followed by NADPH-dependent reduction to glutamate semialdehyde, catalyzed by glutamyl tRNA reductase. The

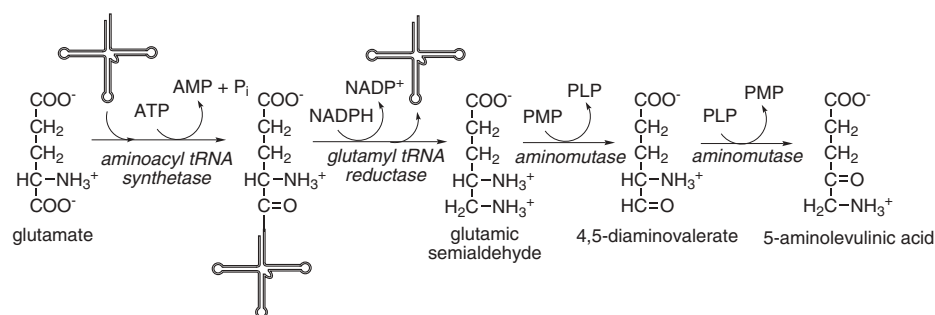


Figure 5.2 Synthesis of 5-aminolevulinic acid from glutamate in plants.

Glutamate aminoacyl tRNA synthetase EC 6.1.1.17, glutamyl tRNA reductase EC 1.2.1.70, glutamic semialdehyde aminomutase EC 5.4.3.8.

reductase is subject to feedback inhibition by haem. Etiolated seedlings synthesize chlorophyll and turn green, even in the dark, if they are provided with 5-aminolevulinic acid, suggesting that the light-controlled step of chlorophyll synthesis is the formation of 5-aminolevulinic acid from glutamate, presumably at the level of glutamyl tRNA reductase. Glutamic semialdehyde is very unstable under physiological conditions, and the glutamyl tRNA reductase forms a complex with glutamic semialdehyde aminomutase to channel the semialdehyde from one enzyme to the next (Luer *et al.*, 2005; Nogaj & Beale, 2005).

The final step is an unusual internal transamination reaction yielding 5-aminolevulinic acid, catalyzed by glutamic semialdehyde aminomutase. The reaction involves an intramolecular exchange of the oxo and amino groups, with intermediate formation of diaminovalerate. This reaction does not require any additional amino donor and, at the beginning and end of the catalytic cycle, the enzyme contains pyridoxamine phosphate at the catalytic site.

Diaminovalerate is formed by aminotransfer from pyridoxamine phosphate, transiently leaving pyridoxal phosphate at the active site. The enzyme is a dimer with two catalytic sites. There is an active site loop that undergoes a large conformational change during catalysis, so that one active site is open and the other is closed. In most organisms, the enzyme is asymmetric; the two active sites act alternately and, at the steady state, one catalytic site contains pyridoxal phosphate and the other pyridoxamine. In some organisms, however, the enzyme is symmetrical and there is no cooperativity between the two active sites.

Glutamic semialdehyde aminomutase shows considerable structural homology with 5-aminolevulinic acid synthase (section 4.8), suggesting that animal 5-aminolevulinic acid synthase has evolved from bacterial or plant glutamic semialdehyde aminomutase (Amadasi *et al.*, 2007; Jahn *et al.*, 1992; Kannangara *et al.*, 1988; Schulze *et al.*, 2006; Stetefeld *et al.*, 2006).

5.2 The catabolism of glutamate

Deamination or transamination of glutamate leads to the production of 2-oxoglutarate, an intermediate in the citric acid cycle. However, the cycle does not provide a pathway for the total oxidation of 2-oxoglutarate, since more oxaloacetate is formed than is required to maintain cycle activity. As shown in Figure 5.3, oxaloacetate is decarboxylated to phosphoenolpyruvate, which may then either be used as a substrate for gluconeogenesis or be dephosphorylated to pyruvate and enter the citric acid cycle as acetyl CoA. This is a thermogenic reaction sequence, adding to the thermogenesis associated with protein intake (section 2.3).

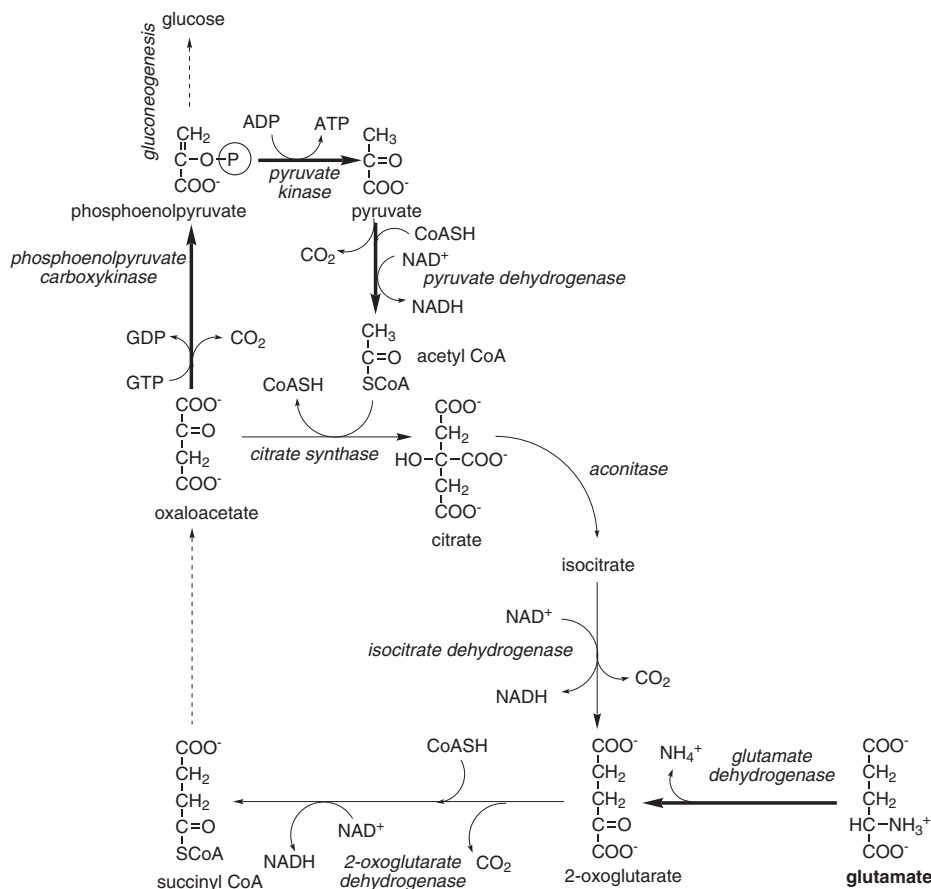


Figure 5.3 Catabolism of glutamate.

Glutamate dehydrogenase EC 1.4.1.2, 2-oxoglutarate dehydrogenase multi-enzyme complex EC 1.2.4.2 + 2.3.1.61 + 1.8.1.4, phosphoenolpyruvate carboxykinase EC 4.1.1.32, pyruvate kinase EC 2.7.1.40, pyruvate dehydrogenase multi-enzyme complex EC 1.2.4.1 + 2.3.1.12 + 1.8.1.4, citrate synthase EC 2.3.3.1, aconitase EC 4.2.1.13, isocitrate dehydrogenase EC 1.1.1.41.

5.3 Glutamine

Glutamine accounts for about 5–6 per cent of the amino acids in tissue proteins and some 60 per cent of the total body pool of free amino acids, of which 95 per cent is in skeletal muscle. It accounts for some 20 per cent of the plasma pool of free amino acids, with a turnover of around 80g per day. In the fasting state, via the formation of glutamate and 2-oxoglutarate, it provides about half the substrate for gluconeogenesis from amino acids – twice as much of the carbon for gluconeogenesis as alanine. Infusion of glutamine leads to increased gluconeogenesis with no change in the insulin:glucagon ratio,

suggesting that either glutamine activates gluconeogenesis or, more likely, that the only fate for excess glutamine is gluconeogenesis (Roth, 2008).

Although it is generally regarded as a non-essential amino acid, glutamine is an essential nutrient and a major metabolic fuel for enterocytes and immune system cells. Almost all of the dietary glutamine is metabolized in intestinal mucosal cells, with little entering the portal circulation, so that almost all of the body's glutamine is synthesized *de novo*. In pancreatic β -islet cells, glutamine suppresses the secretion of insulin and increases that of glucagon (Marc Rhoads & Wu, 2009; Souba, 1991; Watford, 2008).

Glutamine is synthesized mainly in skeletal muscle, lungs and adipose tissue, as well as other tissues, as a means of transporting ammonia nitrogen to the liver for urea synthesis (section 1.6.2.1) and to the kidney for ammoniogenesis (section 1.5.5). It is a substrate for gluconeogenesis in both liver and kidney. Neurons take up glutamine as the precursor for the synthesis of glutamate and GABA (section 5.5). Glial cells take up GABA and glutamate as precursors for re-synthesis of glutamine.

In the liver, glutamine synthetase is only expressed in a 1–3 cell thick layer of perivenous hepatocytes, where it acts to remove any ammonium that would otherwise enter the peripheral circulation (Haussinger *et al.*, 1992). Cells cultured in the presence of glutamine show reduced activity of glutamine synthetase, with no change in the content of mRNA. This seems to be due to increased catabolism of the enzyme protein, apparently a direct effect of glutamine on the enzyme, since it is mimicked by the non-metabolizable analogue diaza-norleucine (Watford, 2008).

Although glutamine is used in all tissues for the synthesis of purines (see section 1.4.1), pyrimidines (section 1.4.3) and amino sugars, as well as other amino acids, this represents only a small fraction of total body turnover. It is a major metabolic fuel for enterocytes, immune system cells (thymocytes, lymphocytes and macrophages), the foetus and many tumours.

Skeletal muscle releases a considerable amount of glutamine in response to stress and in hypercatabolic states (metabolic acidosis, sepsis, trauma and cachexia) and, while the muscle concentration of free glutamine falls somewhat, the rate of synthesis is increased to maintain output. Glutamine synthesis and output from the lungs also increases in response to surgical trauma and sepsis. Immune system cells in culture metabolize glutamine at a very high rate, and glutamine is required to support lymphocyte proliferation and the production of cytokines by lymphocytes and macrophages, as well as macrophage phagocytosis. Sepsis, burns, surgery, endurance exercise and excessive athletic training all lead to a fall in plasma and skeletal muscle glutamine, and this reduced availability of glutamine contributes to the immunosuppression seen in these conditions.

Only about 25 per cent of the glutamine consumed by immune system cells is completely oxidized; these cells export significant amounts of glutamate,

aspartate, lactate and ammonium, as well as some alanine, lactate and pyruvate. Administration of glutamine improves immune function in patients under severely catabolic conditions (Calder & Yaqoob, 1999; Roth, 2008). In addition, glutamine activates mTOR signalling and the expression of ornithine decarboxylase, the rate-limiting enzyme for the synthesis of the polyamines that are essential for cell proliferation and differentiation (see section 5.8).

Like immune system cells, intestinal mucosal cells catalyze only partial oxidation of glutamine, exporting lactate, alanine, citrulline and proline as well as ammonium. The ammonium, lactate and alanine are taken up by the liver, while citrulline is mainly taken up by the kidneys for synthesis of arginine (section 5.9.1). In the fasting state, intestinal mucosal cells take up glutamine from the circulation (Curthoys & Watford, 1995; Souba, 1991).

5.3.1 Indirect formation of glutamine-tRNA

Most bacteria, all known archaea, and also chloroplasts and mitochondria, lack glutaminyl-tRNA synthetase and form glutaminyl-tRNA indirectly, from glutamyl-tRNA^{Gln}. The glutamyl-tRNA^{Gln} is channelled directly from the aminoacyl-tRNA synthetase to an amidase; the CCA tail of the aminoacyl-tRNA flips from the aminoacyl-tRNA synthetase onto the amidase. This is presumably essential in order to avoid incorporation of glutamate instead of glutamine on the ribosome; there is no free glutamyl-tRNA^{Gln} to be taken up onto the ribosome (Sheppard *et al.*, 2008).

The amidase catalyzes three reactions:

- The kinase site catalyzes the phosphorylation of glutamine to γ -glutamyl phosphate esterified to tRNA, as occurs in the synthesis of glutamine from glutamate.
- The glutaminase site catalyzes the hydrolysis of glutamine to glutamate and ammonia.
- The ammonia is channelled through a molecular tunnel in the enzyme to the kinase site, where it amidates the amino acid by displacing the phosphate, again as occurs in the synthesis of free glutamine from glutamate.

Most prokaryotes synthesize asparaginyI-tRNA^{Asn} in a similar indirect manner, because they lack asparaginyI-tRNA synthetase, and many methanogenic archaea lack cysteinyl-tRNA synthetase and synthesize cysteinyl-tRNA^{Cys} by modification of seryl-tRNA^{Cys}. No organisms have an aminoacyl tRNA synthetase for selenocysteine; all synthesize it by modification of seryl-tRNA^{Sec} (section 4.9).

5.3.2 Glutaminases

A number of enzymes catalyze overall deamidation of glutamine to glutamate, but only two – the liver and kidney isoenzymes of glutaminase – are true glutaminases, catalyzing simple hydrolysis of the amide group.

The liver isoenzyme of glutaminase is expressed only in periportal hepatocytes that have urea cycle activity (section 1.6.2.1). Perivenous cells have a high activity of glutamine synthetase to prevent entry of ammonium into the circulation (Haussinger *et al.*, 1992). The expression of glutaminase is increased in response to starvation, diabetes and feeding a high protein diet – conditions under which there is an increased burden of nitrogen arising from amino acid catabolism. The enzyme is activated by its product (ammonium), and hepatocytes in culture lose glutaminase activity unless they are cultured in a medium containing ammonium. Presumably, *in vivo*, this ammonium is provided via the hepatic portal vein from enterocyte metabolism of glutamine.

The activity of glutaminase in the proximal renal tubule is increased in response to metabolic acidosis by a post-transcriptional mechanism. There is no change in the rate of transcription, but the mRNA is more stable than usual. The resultant glutamate is deaminated by glutamate dehydrogenase, providing more ammonium for secretion into the urine, and the carbon dioxide produced by oxidation of 2-oxoglutarate (Figure 5.3) provides bicarbonate to enter the bloodstream as additional compensation for acidosis.

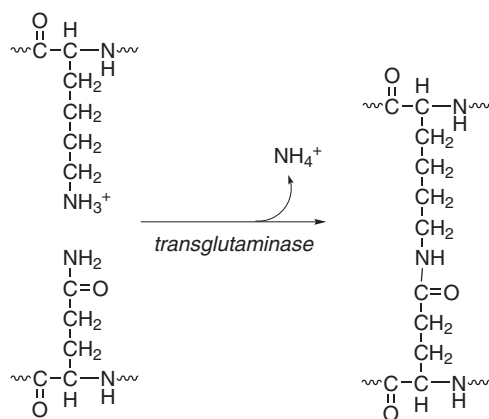
What has been called glutaminase II is, in fact, a combination of a variety of transaminases that act on glutamine, coupled with ω -amidase, which hydrolyzes the amide group. The oxo-acid substrates for glutamine transaminases are those arising from phenylalanine, methionine, glycine, serine, cysteine and asparagine. All of these oxo-acids are normally present in low concentrations. It is thus more likely that glutamine provides a source of nitrogen for transamination of these oxo-acids back to their corresponding amino acids, than that this represents a pathway for catabolism of glutamine (Curthoys & Watford, 1995).

5.3.2.1 Glutamine-dependent amidotransferases In many, if not all, of the reactions in which glutamine acts as a nitrogen donor (section 1.3.2.1), the reaction proceeds in two stages: one catalytic site catalyzes the hydrolysis of glutamine to glutamate and ammonium, while another catalyzes the (commonly ATP-dependent) incorporation of ammonium. There is an ammonium tunnel through the enzyme connecting the two catalytic sites. While some amidotransferases can utilize exogenous ammonium as nitrogen donor at the amidation site, others cannot, and these are reliant on forming the ammonium from glutamine at the glutaminase site (Raushel *et al.*, 2003).

Table 5.1 lists amidotransferases in which ammonium tunnels have been identified.

Table 5.1 Glutamine-dependent amidotransferases.

anthranilate synthase	EC 4.1.3.27	Figure 9.3
asparagine synthase	EC 6.3.5.4	Section 1.3.2.5
carbamoyl phosphate synthase	EC 6.3.5.5	Figures 1.11 and 1.16
CTP synthase	EC 6.3.4.2	Figure 1.11
formylglycinamide amidotransferase	EC 6.3.5.3	Figure 1.6
glutamate synthase	EC 1.4.1.13	Figure 1.4
GMP synthase	EC 6.3.5.2	Figure 1.7
imidazole glycerol phosphate synthase	no EC number	Figure 8.1
iminodeoxychorismate synthase	no EC number	in folic acid biosynthesis
PRPP synthetase	EC 2.7.6.1	Figure 1.6

**Figure 5.4** Formation of isopeptide bonds by transglutaminase.

Transglutaminase EC 2.3.2.13, also known as clotting factor XIIIa or fibrin stabilizing factor.

5.3.3 Transglutaminases

As discussed in section 2.1.2, a number of proteins contain isopeptide bonds between the ϵ -amino group of a lysine residue and the γ -carboxyl group of a glutamate or aspartate residue, which reduce the digestibility of the protein and the availability of lysine. The availability of lysine in these isopeptide links is higher than might be expected, because some of the isopeptides are absorbed from the small intestine and are substrates for γ -glutamyl cyclotransferase (Yasumoto & Suzuki, 1990; see section 5.4.5).

The ϵ -amino lysine- γ -carboxyl glutamate bonds are formed by post-synthetic modification of the protein, catalyzed by transglutaminases. The carboxyl group of the isopeptide bond is derived from a glutamine residue in the precursor protein, as shown in Figure 5.4. The various mammalian transglutaminases show significant sequence homology; they are members of the papain-like superfamily of cysteine proteases and have a conserved

Tyr-Gly-Gln-Cys-Trp sequence at the catalytic site. The cysteine residue forms a thioester with the glutamine residue in the substrate, displacing ammonium (Beninati & Piacentini, 2004).

Transglutaminases (either from animal blood or from bacterial sources) are used in food manufacture to bind proteins in products such as surimi, fish balls and mechanically recovered meat products, where they are commonly known as 'protein glue'. They are also used to cross-link pectin and chitosan to proteins in order to produce edible films for food packaging and coating, and they are important in tissue engineering and textile and leather processing (Porta *et al.*, 2011).

Transglutaminase 1 is a membrane-bound enzyme in the plasma membrane of epidermal cells. It catalyzes the formation of a highly cross-linked protein layer, the cornified or cross-linked cell envelope, inside the cell. It also catalyzes the formation of ester bonds between glutamine residues in the precursor protein to ω -hydroxyceramides to form the lipid envelope of surface epidermal keratinocytes. Keratinocytes migrate to the surface of the skin as an interlocking layer of dead stratum corneum cells.

Genetic lack of transglutaminase 1 leads to the condition of lamellar ichthyosis, which is characterized by defective epidermal barrier function. There is reduced activity of transglutaminase 1 in psoriasis and basal cell epithelioma (Peterson *et al.*, 1983).

Recessive X-linked lamellar ichthyosis is due to the accumulation of cholesterol 3-sulphate in the epidermis, which inhibits the cross-linking and esterification actions of transglutaminase 1. The enzyme now catalyzes deamidation of glutamine residues in the precursor protein instead of transglutamination, because cholesterol 3-sulphate distorts the active site of the enzyme, permitting the access of water and hydrolysis (Nemes *et al.*, 2000).

Transglutaminase 2 is the so-called tissue transglutaminase, and it has a dual action; in addition to its transglutaminase activity, the carboxy-terminal functions as a G-protein. The activity of transglutaminase 2 increases markedly in apoptosis, when it has a role in forming an intracellular scaffold of insoluble protein, stabilizing intracellular membranes and preventing the release of lysosomal enzymes. Over-expression of transglutaminase 2 in cells in culture leads to increased susceptibility to apoptosis, and knocking out the enzyme with antisense cDNA leads to a decrease in both spontaneous and induced apoptosis.

Transglutaminase 2 is involved in the pathogenesis of neurodegenerative, auto-immune and inflammatory diseases, including coeliac disease. Inhibitors or antibody targeting have therapeutic potential in these conditions. Transglutaminase 2 is a member of the α h family of G-proteins, involved in the activity of the α 1 adrenoceptor, leading to the activation of phospholipase C. Adrenergic stimulation leads to inhibition of transglutaminase activity and an increase in G-protein signalling. It is also involved in receptor-mediated

endocytosis (Autuori *et al.*, 1998; Caccamo *et al.*, 2010b; Levitzki *et al.*, 1980; Nakaoka *et al.*, 1994; Park *et al.*, 2010; Piacentini *et al.*, 1991).

Transglutaminase 3 is a pro-enzyme in terminally differentiating epidermal and hair keratinocytes that requires activation by partial proteolysis. Like transglutaminase 1, it acts in the formation of the cornified cell envelope of epidermis and hair follicles. It is the major auto-antigen in coeliac disease and dermatitis herpetiformis, both of which are due to sensitivity to the gliadin fraction of gluten, the major protein of wheat and other cereals. Transglutaminase is involved in the deamidation of glutamine residues in gliadin, but then catalyzes the formation of highly immunogenic aggregates (Beninati & Piacentini, 2004; Sardy *et al.*, 2002).

Transglutaminase 4 is the prostate-specific transglutaminase, although it is also expressed in a number of other tumour cell lines. In rodents, it is responsible for formation of the copulatory plug in the female genital tract after coitus, and it may mask the immunogenicity of the male gametes. The mRNA for the human enzyme undergoes differential splicing in the development of benign prostate hyperplasia and prostate cancer, and it has a role in the adhesion of prostate cells to epithelial cells (Cho *et al.*, 2010; Davies *et al.*, 2007; Jiang *et al.*, 2009; Jiang & Ablin, 2011).

Blood clotting Factor XIII (section 5.6.1) is the zymogen of a transglutaminase (fibrologase) that catalyzes stabilization of the fibrin clot by forming inter-chain isopeptide bonds. It also protects the newly formed fibrin against fibrinolysis by binding plasmin inhibitor to the fibrin network. It consists of two A-chains (that have the active site) and two B-chains (with no catalytic activity). The zymogen is activated by partial proteolysis, releasing a 36-amino acid peptide, followed by a conformational change to expose the catalytic site cysteine. This partial proteolysis is catalyzed by thrombin, but only occurs if uncross-linked fibrin is also present. The A chain of Factor XIII is also expressed in platelets, monocytes, macrophages and the placenta, and it is activated non-proteolytically in response to an increase in the intracellular concentration of calcium ions, which leads to a conformational change, exposing the catalytic site (Bereczky & Muszbek, 2011; Board *et al.*, 1993; Lewis *et al.*, 1985; Muszbek *et al.*, 1996, 2011).

Transglutaminases also catalyze the incorporation of amines into proteins, forming γ -glutamyl amides by displacement of the amide group of glutamine residues. Putrescine, spermine and spermidine (see section 5.8) are good substrates for incorporation, and either or both of the primary amino groups can be linked to glutamate. Reaction of the free amino group of a γ -glutamyl amide with a second glutamine residue leads to formation of inter- and intra-chain cross-links in the target protein. There is a great deal of specificity for the environment of the glutamine residues that are amidated, but the transglutaminases generally have a broad specificity for the polyamines that they incorporate (Lentini *et al.*, 2004).

Excessive stimulation of glutamate receptors in the central nervous system leads to over-stimulation of calcium-dependent kinases and other downstream effectors, as a result of a massive influx of calcium ions via the ionotropic glutamate receptors, leading to neurodegeneration. At least part of the pathogenesis of this excitotoxicity is due to the activation of calcium-dependent transglutaminases, leading to cross-linking of proteins.

There is some evidence that transglutaminases are also involved in cross-linking neuronal proteins in Alzheimer's and Huntington's diseases, so measurement of ϵ -(γ -glutamyl)lysine isopeptides in plasma may be a useful marker of tissue damage in these and other conditions. Alzheimer's, Huntington's and Parkinson's diseases are all associated with proteolytic stress due to incorrectly folded and isopeptide cross-linked proteins that overload the ubiquitin-proteasome system (section 2.1.5.2). Tau protein, amyloid-beta, α -synuclein and huntingtin are all substrates for transglutaminase (Caccamo *et al.*, 2004, 2010a).

5.4 Glutathione and the γ -glutamyl cycle

Glutathione is a tripeptide, γ -glutamyl-cysteinyl-glycine (GSH) that acts as a major redox buffer in cells, being oxidized to the disulphide-linked hexapeptide (glutathione disulphide, GSSG) by glutathione peroxidases, which are seleno-enzymes (see section 4.9). As shown in Figure 5.5, GSSG is reduced back to GSH by glutathione reductase. The synthesis of glutathione increases in response to signals for cell proliferation before there is an increase in DNA synthesis; the role of glutathione here is to provide the reductant to maintain an adequate pool of reduced glutaredoxin for ribonucleotide reductase. Although thioredoxin can also act as reductant for ribonucleotide reductase, inhibition of glutathione synthesis after partial hepatectomy impairs the ability of the liver to regenerate (Lu, 2009).

In addition to its redox role, and *S*-conjugation of various compounds (section 5.4.3), glutathione and its derivative, γ -glutamyl glutathione can chelate heavy metals, forming complexes that are transported across cell membranes.

A major role of glutathione is the storage of cysteine, which is unstable extracellularly because of oxidation to the disulphide cystine. Cysteine is also cytotoxic at high concentrations, so formation of glutathione provides a safe tissue reservoir of cysteine. Cystine is one of the best acceptors for γ -glutamyl transpeptidase (section 5.4.5), forming γ -glutamyl-cystine; this is taken up into cells, hydrolyzed and the resultant cystine is reduced to cysteine (Lu, 2009; Stipanuk *et al.*, 2006).

The concentration of glutathione in most cells is 1–2 mmol/l, but it may be as high as 10 mmol/l in hepatocytes. Almost all of this is as GSH, with only

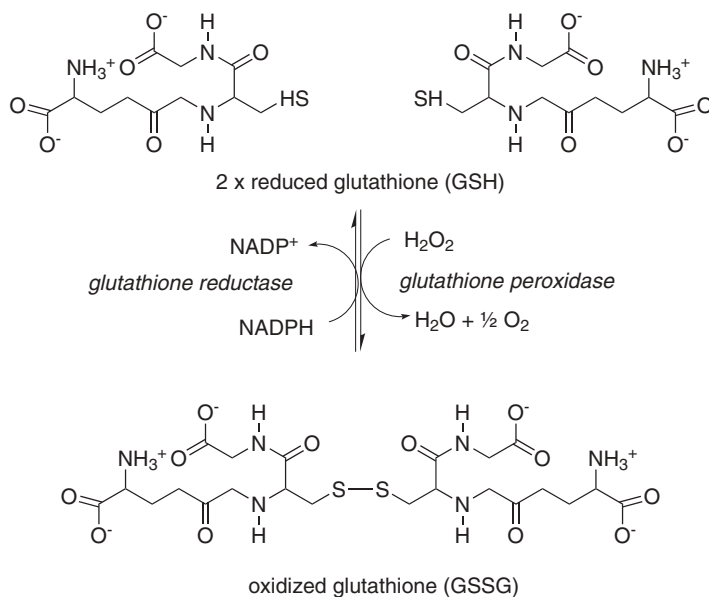


Figure 5.5 Glutathione.

Glutathione reductase EC 1.8.1.7, glutathione peroxidase EC 1.11.1.9, phospholipid hydroperoxide glutathione peroxidase EC 1.11.1.12.

about 2 per cent normally present as GSSG. The liver exports glutathione, which is concentrated in extracellular spaces, such as the fluid lining of the lungs, as an antioxidant. In addition, exported glutathione is a source of cysteine for extra-hepatic tissues. About half of the glutathione synthesized in the liver is exported to the plasma and half to the bile, where it provides the main (osmotic) stimulus for bile secretion. However, much is catabolized in the biliary spaces, with the resultant amino acids being re-absorbed into the liver. The efflux of glutathione and conjugates from cells is by active transport, catalyzed by ATP-cassette binding proteins (Ballatori *et al.*, 2009).

Glutathione regulates apoptosis; increasing the intracellular concentration of glutathione reduces apoptosis, while depleting cells of glutathione increases it. Cells undergoing apoptosis release glutathione into the incubation medium.

Glutathionylation of proteins leads to activation of a number of regulatory enzymes (e.g. glucose 6-phosphatase and fructose 1,6-bisphosphatase) and inactivation of others (e.g. glycogen synthetase, pyruvate kinase, adenylate cyclase, phosphofructokinase and fatty acid synthetase). It is also important in the regulation of signalling proteins, transcription factors, histones, Ras proteins, ion channels, cytoskeletal proteins and DNA methyltransferases, giving glutathione roles in metabolic regulation, cell proliferation and differentiation. Glutathionylation of nuclear proteins during the first phase of

the cell cycle leads to the opening of the structure of chromatin to permit DNA replication (Ballatori *et al.*, 2009; Pallardo *et al.*, 2009).

Glutathione has further roles in cell signalling, by forming thio-ethers with leukotrienes, prostaglandins, nitric oxide (section 5.9.3), dopa and dopamine (section 9.2.4), and also by forming amides between the carboxyl group of glycine and spermidine (section 5.8).

5.4.1 Glutathione peroxidases

There are five mammalian glutathione peroxidases, all of which are selenoproteins (section 4.9; Brigelius-Flohe, 1999, 2006). The 'classical' enzyme (glutathione peroxidase 1) is found in all cells. Its activity falls in selenium deficiency, and is at its highest in tissues that have a high rate of peroxide production (erythrocytes, liver, kidney and lungs). Its activity in the lungs increases markedly post-natally as the lungs are exposed to oxygen. Glutathione peroxidase 6 is expressed only in the olfactory system; its function is unknown.

The gastro-intestinal glutathione peroxidase 2 provides a barrier against the absorption of ingested hydroperoxides and those formed by the metabolism of xenobiotics. In rats, it is only expressed in the gastro-intestinal epithelium, but in human beings it is also expressed in the liver. It is remarkably stable under conditions of selenium deficiency, suggesting that it is critically important.

The plasma glutathione peroxidase 3 is expressed by various tissues. Like the classical enzyme, its activity falls in selenium deficiency. This enzyme also acts to silence lipoxygenase and cyclo-oxygenase, both of which require hydrogen peroxide, and thus it limits the synthesis of prostaglandins and leukotrienes in response to macrophage activation, as well as acting to limit inflammatory responses.

Phospholipid hydroperoxide glutathione peroxidase (glutathione peroxidase 4) was originally considered to provide protection against phospholipid hydroperoxides resulting from oxidative damage to cell membranes. It shows little sequence homology with other glutathione peroxidases and is a monomer, whereas the others are homotetrameric proteins. It can use reductants other than glutathione, including protein thiols. It is now known to have a role in cell signalling by silencing lipoxygenases and cyclo-oxygenase (as does the plasma enzyme).

Lipid hydroperoxides have a role in cell signalling through the TNF- and interleukin-1-mediated activation of the NF κ B transcription factor. They are also involved in triggering apoptosis, and have roles in cell proliferation and differentiation. This gives phospholipid hydroperoxide glutathione peroxidase a role in controlling the tissue content of intracellular signalling molecules, in addition to its antioxidant role. Like the gastro-intestinal enzyme, it responds slowly to selenium depletion and recovers rapidly on repletion. It

also has a role as an enzymically inactive structural protein in the mitochondrial membrane of spermatozoa. About half of the keratin-like capsule protein in mature sperm is inactive, oxidatively cross-linked glutathione peroxidase, explaining the importance of selenium nutrition for male fertility (Conrad *et al.*, 2007).

5.4.2 Glutathione reductase

Glutathione reductase is a flavoprotein that uses NADPH to reduce GSSG to GSH (Figure 5.5). Genetic lack of the enzyme results in haemolytic anaemia, as is seen in favism (lack of glucose 6-phosphate dehydrogenase, leading to impaired synthesis of NADPH in erythrocytes). In favism, haemolytic crises are precipitated by ingestion of broad beans (*Vicia faba*) and a variety of drugs that undergo redox cycling to generate reactive oxygen species. In the absence of adequate amounts of reduced glutathione, peroxidation of erythrocyte membrane lipids leads to haemolysis.

Erythrocyte glutathione reductase provides a means of assessing riboflavin (vitamin B₂) nutritional status. There is normally a small proportion of the enzyme present as catalytically inactive apoenzyme, and this increases in riboflavin deficiency at the expense of (catalytically active) holoenzyme. The activity of glutathione reductase in a sample of washed erythrocytes is measured with and without added flavin adenine dinucleotide. The incubation without added FAD measures what was present as holoenzyme, while that with added FAD measures both what was present as holoenzyme and also that which was present as apoenzyme. The ratio of activity with FAD to activity without FAD is the activation coefficient of the enzyme, and this serves as an indicator of the adequacy (or otherwise) of body riboflavin reserves (Tillotson & Baker, 1972).

Trypanosomes and *Leishmania* spp. have a parasite-specific dithiol compound, trypanothione (*bis*-(glutathionyl)-spermidine), rather than glutathione. It is used as the reductant for DNA synthesis and detoxication of hydroperoxides. Oxidized trypanothione is reduced by an NADPH-dependent flavoprotein, trypanothione reductase, in the same way a glutathione reductase reduces GSSG to GSH (Krauth-Siegel & Comini, 2008). In *Arabidopsis* (and possibly other plants) the NADPH-dependent thioredoxin system provides an alternative route for reduction of GSSG to GSH, although there is no evidence that mammalian thioredoxin can maintain the pool of reduced glutathione (Marty *et al.*, 2009).

5.4.3 Glutathione S-transferases

Glutathione is important in the phase II detoxication of hydrophobic and electrophilic compounds, forming glutathione S-conjugates that are excreted in urine and bile. The reaction is catalyzed by glutathione S-transferases.

There are four classes of cytosolic glutathione *S*-transferase and two classes of microsomal membrane-bound enzyme, as well as a structurally distinct kappa class that is found in mitochondria and peroxisomes. Glutathione *S*-transferases are homo- or heterodimers of at least seven different types of subunit, and specific subunits are induced by different xenobiotics (Pickett & Lu, 1989). Because glutathione *S*-transferases also bind a number of ligands that are not substrates – including bilirubin, steroid hormones and some carcinogens and their metabolites – they are sometimes known as ligandin.

Glutathione *S*-transferase binds bilirubin at a site distinct from the catalytic site, transporting it from the bloodstream into the liver, then to the endoplasmic reticulum for conjugation with glucuronic acid, and excretion in the bile. There is a second, low-affinity bilirubin binding site that overlaps the catalytic site, and high concentrations of bilirubin inhibit the enzyme (Bhargava *et al.*, 1978).

The liver has a very high activity of glutathione *S*-transferase, and *in vitro* the entire pool of glutathione can be depleted within minutes on exposure to xenobiotic substrates (Rinaldi *et al.*, 2002). The activity of glutathione *S*-transferase is upregulated in many tumours, leading to problems of resistance to chemotherapy. Similarly, in insects, high activity of glutathione *S*-transferase is associated with insecticide resistance.

In plants, herbicides and organic pollutants are detoxified by glutathione *S*-transferases. The conjugates are initially compartmentalized in vacuoles, then undergo long-range transport to the roots for excretion through the root tips. There seems to be a specific transport system for glutathione conjugates, since they travel faster than cysteine conjugates despite their higher molecular weight (Schroder *et al.*, 2007).

Glutathione *S*-transferases also have glutathione peroxidase activity, acting on phospholipid hydroperoxides. This selenium-independent reaction may account for about half of the glutathione peroxidase activity in liver, but a considerably smaller proportion in other tissues (Yang *et al.*, 2002). Some glutathione *S*-transferases catalyze dehydrochlorination reactions, using glutathione as a cofactor, rather than forming conjugates. In this reaction, glutathione forms an enzyme-bound conjugate, releasing chloride, but this is followed by release of the glutathione and the formation of a carbon-carbon double bond in the substrate (Enayati *et al.*, 2005).

Glutathione conjugates may be transported out of the liver, where they are substrates for extracellular γ -glutamyltranspeptidase and dipeptidases (Figure 5.6). The resultant cysteine *S*-conjugates are taken up by other tissues (especially the kidney) and *N*-acetylated to yield mercapturic acids (*N*-acetyl cysteine *S*-conjugates), which are excreted by the kidneys. Some hepatic glutathione *S*-conjugates enter the bile canaliculi, where they are broken down to cysteine *S*-conjugates that are then taken up into the liver for *N*-acetylation, and then re-excreted in the bile (Hinchman & Ballatori, 1994).

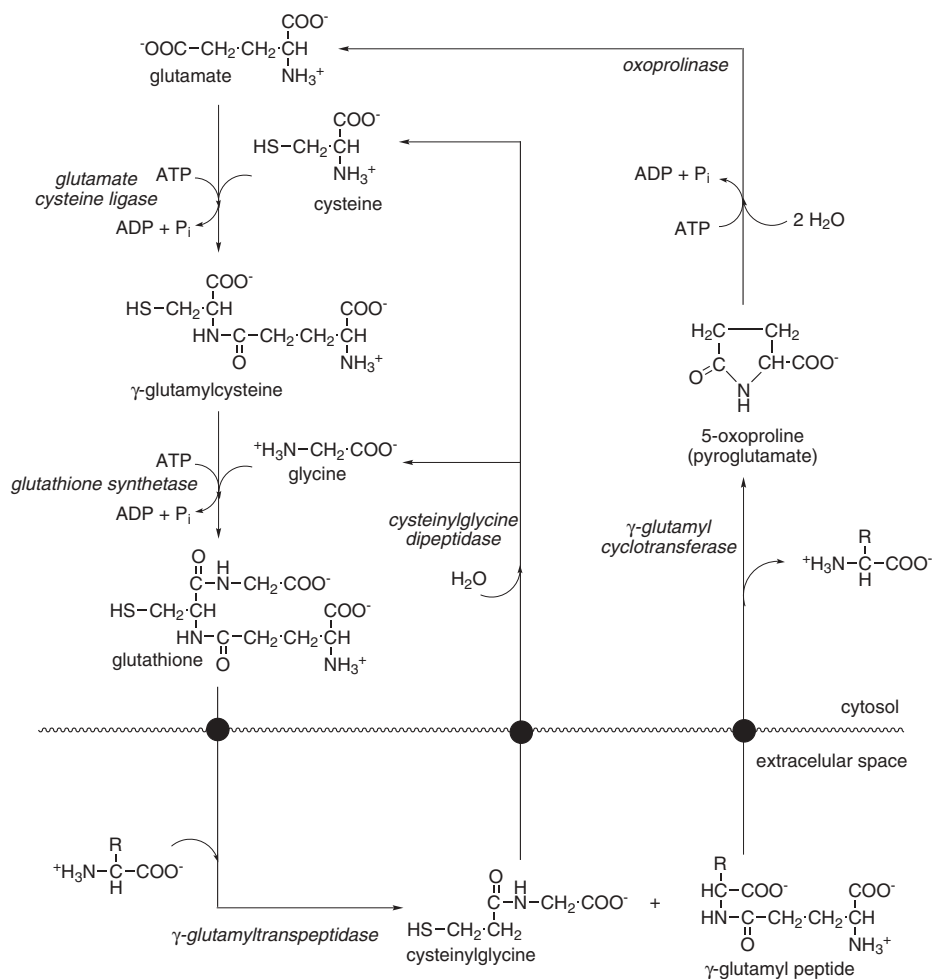


Figure 5.6 The γ -glutamyl cycle.

Glutamate cysteine ligase EC 6.3.2.2, glutathione synthetase EC 6.3.2.3, γ -glutamyltranspeptidase EC 2.3.2.2, γ -glutamyl cyclotransferase EC 2.3.2.4, oxoprolinase EC 3.5.2.9, cysteinylglycine dipeptidase 3.4.11.2.

Glutathione *S*-transferase is an ectozyme on sperm, and is essential for fertilization to occur. Antibodies against the enzyme that do not inhibit its activity nevertheless prevent fertilization. There are two isoenzymes of glutathione *S*-transferase on sperm and both bind, via their amino-terminal regions, to the same site on the zona pellucida of the ovum, leading to signaling for intracellular events in the sperm, initiating the acrosome reaction and then penetration of the sperm into the ovum (Hemachand *et al.*, 2002).

5.4.4 Glutathione synthesis

Both enzymes involved in the synthesis of glutathione, glutamate cysteine ligase (also known as γ -glutamyl-cysteine synthetase – see Figure 5.6) and glutathione synthetase are upregulated in response to oxidative stress via the antioxidant stress response element. Deficiency of either enzyme leads to haemolytic anaemia because of the lack of glutathione in erythrocytes.

In some Gram-positive bacteria, there is a single bifunctional enzyme that catalyzes both the glutamate cysteine ligase and glutathione synthetase reactions. The enzyme is not subject to feedback inhibition by glutathione, and these organisms maintain a higher intracellular concentration of glutathione than do bacteria in which glutamate cysteine ligase is subject to feedback inhibition (Janowiak & Griffith, 2005).

5.4.4.1 Glutamate cysteine ligase Glutamate cysteine ligase is the rate-limiting enzyme of glutathione synthesis and is subject to feedback inhibition by glutathione at normal intracellular concentrations. This is non-allosteric inhibition; glutathione binds both to the glutamate binding site of the enzyme and to a sulphhydryl group elsewhere on the enzyme. The availability of cysteine is a potentially limiting factor in synthesis of γ -glutamyl-cysteine, and hence glutathione, because the K_m of the enzyme for cysteine is approximately equal to the normal intracellular concentration of cysteine (Richman & Meister, 1975).

4-Hydroxynonenal is an end-product of lipid peroxidation. It is *S*-conjugated with glutathione, causing depletion of glutathione, followed by induction of glutamate cysteine ligase. The ligase is also induced by flavonoids (see section 9.2.2), suggesting that dietary flavonoids may have antioxidant actions not only in their own right but also by increasing glutathione synthesis (Iles & Liu, 2005; Moskaug *et al.*, 2005).

Mammalian glutamate cysteine ligase has two subunits, encoded by separate genes: a catalytic protein and a small modifier protein. In the presence of the modifier subunit, the catalytic subunit has a six-fold lower K_m for ATP, a lower K_m for glutamate, an increased K_i for glutathione and overall four-fold increase in k_{cat} . In most tissues, the modifier subunit is present in lower amounts than the catalytic subunit, so that there is some protein with a lower activity than the holoenzyme. Insulin and glucocorticoid hormones have a permissive effect on the synthesis of the catalytic subunit of the enzyme. Insulin-deficient and adrenalectomized animals have low hepatic glutathione and low activity of glutamate cysteine ligase, which are restored to normal – but not increased above normal – by hormone replacement (Chen *et al.*, 2005; Lu, 2009).

Cysteine residues in both subunits of the mammalian enzyme are involved in formation of the holoenzyme, forming an inter-subunit disulphide bridge.

In response to oxidative stress, there is an increase in the activity of glutamate cysteine ligase due to increased formation of the active holoenzyme. Under reducing conditions, there is increased dissociation of the holoenzyme into separate catalytic and modifier proteins, and hence a reduction in catalytic activity (Franklin *et al.*, 2009).

In plants, yeasts and bacteria, there is only a single catalytic protein, and no modifier protein. The plant enzyme consists of homodimers of the catalytic protein. It is regulated by a post-translational redox switch involving oxidation to form, or reduction to remove, two inter-subunit disulphide bridges. Reduction of one leads to a conformational change that blocks the active site; reduction of the other causes dissociation of the dimer into monomers that have very low activity (Franklin *et al.*, 2009).

The fall in intracellular glutathione in apoptosis is partly due to increased export of glutathione from the cell, and partly due to caspase-dependent cleavage of glutamate cysteine ligase. The enzyme also responds to a number of signals; it can be phosphorylated, leading to a reduction in $V_{\max}$, by protein kinase A, protein kinase C and calcium-calmodulin-dependent protein kinase. It also undergoes autophosphorylation, leading to reduced activity, but this is blocked by the binding of glutamate at the active site. The first step in the latter reaction is phosphorylation of glutamate bound at the active site to form γ -glutamyl phosphate (Franklin *et al.*, 2009; Lu, 2009).

Very rare genetic lack of glutamate cysteine ligase leads to very low erythrocyte concentrations of glutathione and haemolytic anaemia. Mild impairment of the enzyme activity is common in Tasmanian merino sheep and is associated with low tissue concentrations of glutathione, but no significant pathology (Beutler, 1989).

5.4.4.2 Glutathione synthetase Glutathione synthetase catalyzes the condensation of the amino group of glycine to the carboxyl group of cysteine in γ -glutamyl cysteine, forming glutathione.

There are three forms of glutathione synthetase deficiency (Njalsson & Norgren, 2005; Ristoff & Larsson, 2007). The mild form is due to low stability of the mutant enzyme. Because mature erythrocytes cannot synthesize proteins, this means that there is haemolysis of older erythrocytes in response to oxidative stress. The condition is characterized by haemolytic anaemia.

In the moderately severe form of the disease, the haemolytic anaemia is accompanied by metabolic acidosis and oxoprolinuria. The low concentration of glutathione means that there is little feedback inhibition of glutamate cysteine ligase. Thus, a considerable amount of γ -glutamyl cysteine accumulates, and this is a substrate for γ -glutamyl cyclotransferase (see section 5.4.5), forming oxoproline in excess of the capacity of oxoprolinase to convert it to glutamate. Severely affected patients also suffer from recurrent bacterial

infections and progressive central nervous system dysfunction, because of the oxidation of nervous system proteins by oxoproline.

Oxoprolinuria may develop in previously malnourished children during rapid catch-up growth, because of the limited availability of glycine for rapid growth and glutathione synthesis (Persaud *et al.*, 1996).

Glutathione *S*-transferase deficiency (section 5.4.3) may develop secondarily to severe glutathione synthetase deficiency, because the enzyme is unstable in the absence of an adequate concentration of glutathione.

5.4.5 The γ -glutamyl cycle

There is a rapid turnover of glutathione. As shown in Figure 5.6, glutathione is exported from cells and is a substrate for extracellular γ -glutamyltranspeptidase, yielding cysteinylglycine and the γ -glutamyl peptide of the substrate amino acid. Inside the cell, cysteinylglycine is a substrate for a dipeptidase, yielding cysteine and glycine that can be used for re-synthesis of glutathione, and the γ -glutamyl peptide is a substrate for γ -glutamyl cyclotransferase, liberating the amino acid that has thus been imported into the cell and yielding 5-oxoproline (pyroglutamate). Oxoprolinase catalyzes the ATP-dependent hydrolysis of oxoproline to glutamate, which can be used for re-synthesis of glutathione.

Meister (1973) originally proposed that this γ -glutamyl cycle provides a pathway for ATP-dependent active transport of amino acids into cells. Its importance in amino acid transport, compared with the sodium-dependent and independent amino acid transporters, is unclear. Although all of the protein amino acids (except proline) are substrates for γ -glutamyltranspeptidase, this cycle does not explain the group specificity of amino acid transport (section 2.5). In addition, the γ -glutamyl cycle entails a cost of three mol of ATP for each mol of amino acid transported, which is considerably higher than the ATP cost of sodium-linked active transport. Nevertheless, there is high activity of γ -glutamyltranspeptidase in the blood-brain barrier and other tissues that transport relatively large amounts of amino acids, such as the proximal convoluted tubule of the kidney (facing the luminal side of the membrane), the lactating mammary gland and the apical region of intestinal epithelial cells.

Quite apart from the possible direct role of γ -glutamyltranspeptidase and the γ -glutamyl cycle in amino acid transport, there is evidence that 5-oxoproline stimulates the activity of sodium-dependent amino acid transporters (Goldberg, 1980; Hawkins *et al.*, 2006).

γ -Glutamyltranspeptidase is a plasma membrane-bound glycoprotein. It is synthesized as an inactive precursor that undergoes cleavage in the endoplasmic reticulum to expose a new amino-terminal amino acid that is the nucleophile for the catalytic reaction and also catalyzes the activation of further

molecules of the precursor protein. A variety of tissue-specific isoenzymes are formed by alternative splicing affecting both the coding region of the mRNA and also the 5'-untranslated region (Ikeda & Taniguchi, 2005; Joyce-Brady *et al.*, 2001; Kinlough *et al.*, 2005; Zhang & Forman, 2009). In addition to the reaction shown in Figure 5.6, γ -glutamyltranspeptidase also catalyzes five further reactions:

- transpeptidation of two mol of γ -glutamyl amino acid to liberate a free amino acid and form γ -glutamyl- γ -glutamyl amino acid;
- hydrolysis of γ -glutamyl amino acids to liberate glutamate + amino acid;
- hydrolysis of glutamine to glutamate and ammonium (and so can be considered to be a glutaminase – see section 5.3.2);
- reaction of two molecules of glutathione to form γ -glutamyl-glutathione and cysteinyl-glycine;
- cleavage of glutathione *S*-conjugates to yield cysteinylglycine conjugates that are cleaved by peptidases, followed by onward metabolism to mercapturic acids (section 5.4.3).

γ -Glutamyl-glutathione is not a substrate for glutathione *S*-transferases (section 5.4.3), and it seems to have limited redox activity. It is found in blood, urine and bile, but it is not clear whether it has a function or is simply the product of an accidental reaction of γ -glutamyltranspeptidase. Because of the additional charge, it is a more potent chelator of metal ions than is glutathione. It is a substrate for γ -glutamyl cyclotransferase, yielding oxoproline and glutathione (Abbott *et al.*, 1986).

Serum activity of γ -glutamyltranspeptidase is measured in routine clinical chemistry as a marker of liver disease and pancreatitis. However, the serum activity is also increased in cancer, because of high activity of the enzyme on the surface of many tumour cells. Isolated elevation of γ -glutamyltranspeptidase, with no other markers of tissue damage (i.e. no other abnormally increased enzyme activities), reflects induction of the enzyme in liver by alcohol, barbiturates or other drugs, and in response to oxidative stress, rather than liver damage (Whitfield, 2001).

γ -Glutamyl cyclotransferase catalyzes the cleavage of γ -glutamyl isopeptides to yield the free amino acid and oxoproline (pyroglutamate). In plants, γ -glutamyl cyclotransferase acts directly on glutathione, yielding oxoproline and cysteinylglycine, so that glutathione turnover does not require γ -glutamyltranspeptidase. γ -Glutamyltranspeptidase knockout *Arabidopsis*

shows normal turnover of glutathione and formation of oxoproline (Ohkama-Ohtsu *et al.*, 2008).

γ -Glutamylamine cyclotransferase catalyzes a similar reaction to that of γ -glutamyl cyclotransferase, acting on γ -glutamyl amines to liberate the free amine and oxoproline. It has high activity towards γ -glutamyl- ϵ -lysine liberated by the hydrolysis of fibrin and other proteins that have isopeptide cross-links formed by the action of transglutaminases (section 5.3.3). Dietary proteins with γ -glutamyl- ϵ -lysine isopeptide links have higher availability of lysine than is predicted from *in vitro* digestibility (section 2.1.2). This may be due partly to isopeptidases in intestinal bacteria, and partly also to absorption of the isopeptides, followed by the action of γ -glutamylamine and γ -glutamyl cyclotransferases liberating lysine and forming 5-oxoproline (Oakley *et al.*, 2010; Yasumoto & Suzuki, 1990).

Patients with glutathione synthetase deficiency (section 5.4.4.2) excrete large amounts of oxoproline. They synthesize abnormally large amounts of γ -glutamylcysteine because of lack of the normal feedback inhibition of glutamate cysteine ligase by glutathione. γ -Glutamylcysteine is a substrate for γ -glutamyl cyclotransferase, producing oxoproline in excess of the capacity of oxoprolinase to convert it to glutamate. Patients who lack oxoprolinase excrete large amounts of oxoproline but have normal tissue concentrations of glutathione, reflecting the complete dispensability of glutamate (Richman & Meister, 1975).

5.5 Glutamate decarboxylase and the GABA shunt

γ -Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system; almost one-third of central nervous system neurons are sensitive to GABA to mediate inhibitory signalling. GABA has roles in locomotor activity, learning, reproduction and the control of circadian rhythms. GABA_A and GABA_C receptors are ligand-gated chloride channels, while GABA_B receptors are G-protein-linked metabotropic receptors.

GABA metabolism and transmission are disordered in epilepsy, Huntington's disease, Parkinson's disease and general anxiety disorders. GABA and the enzymes of the GABA shunt occur in a variety of non-neuronal tissues, including liver, kidney, pancreas, testis, adrenal gland, the gastro-intestinal tract and red blood cells (Tillakaratne *et al.*, 1995). GABA also has a signalling role in plants; there is a gradient of GABA concentration along the pistil that acts as a chemo-attractant for formation of the pollen tube (Bouche & Fromm, 2004).

In addition to decarboxylation of glutamate, GABA may arise from the metabolism of putrescine, spermine and spermidine (section 5.8.3) and orni-

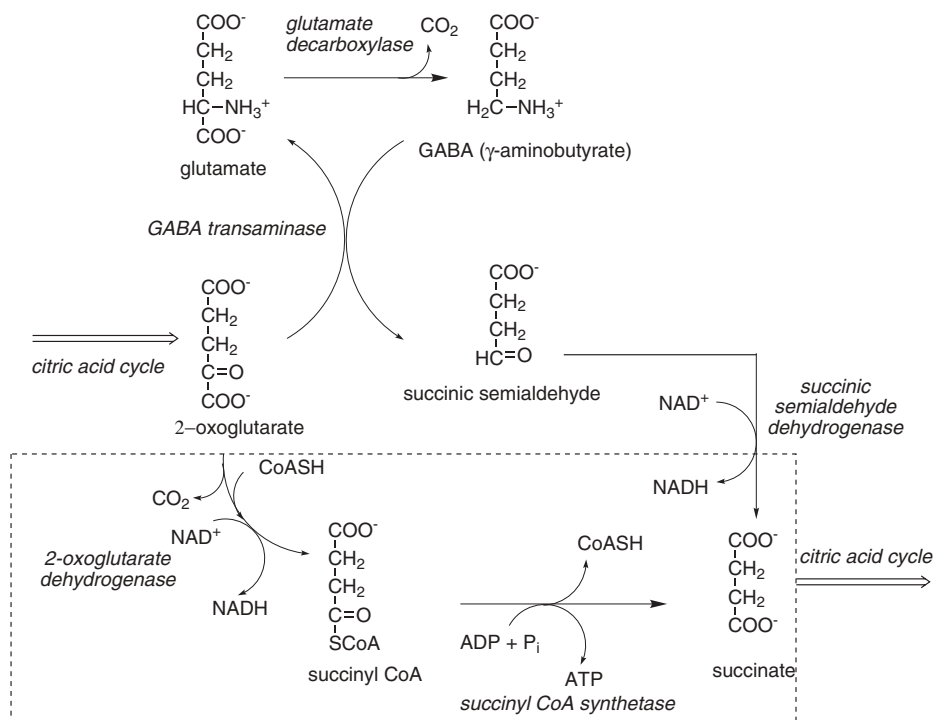


Figure 5.7 The GABA shunt as an alternative to the citric acid cycle.

Glutamate decarboxylase EC 4.1.1.15, GABA transaminase EC 2.6.1.19, succinic semialdehyde dehydrogenase EC 1.2.1.24, 2-oxoglutarate dehydrogenase multi-enzyme complex EC 1.2.4.2 + 2.3.1.61 + 1.8.1.4, succinyl CoA synthetase EC 6.2.1.5 (ADP-linked), EC 6.2.1.4 (GDP-linked, in gluconeogenic tissues).

thine (section 5.9), although in most tissues these are minor sources of GABA. In the central nervous system, small amounts of GABA are condensed with histidine to form homocarnosine (section 8.5).

As shown in Figure 5.7, GABA is synthesized by decarboxylation of glutamate. In the central nervous system, inactivation of GABA is by uptake into either pre-synaptic neurons (where it is taken up into vesicles for re-secretion) or into astrocytes, where it is catabolized by transamination with 2-oxoglutarate to yield succinic semialdehyde, which is oxidized to succinate. This sequence of reactions thus provides an alternative pathway to that catalyzed by 2-oxoglutarate dehydrogenase and succinyl CoA synthetase in the citric acid cycle – the GABA shunt. Increased flux through the GABA shunt may be important in maintaining ATP production in thiamin deficiency, where the activity of 2-oxoglutarate dehydrogenase is impaired. The GABA shunt may also protect the central nervous system from the excitotoxic effects of excess glutamate that is formed from 2-oxoglutarate when the activity of

2-oxoglutarate dehydrogenase is impaired by thiamin deficiency. It may also serve to remove excess glutamate formed in ammoniagenesis from glutamine in the kidney (McEntee, 1997; Page *et al.*, 1989; Tillakaratne *et al.*, 1995).

There is a relatively high concentration of GABA in the β -islet cells of the pancreas, but not other islet cells, and there is only a very low concentration in endocrine (acinar) pancreatic cells. Most is contained in synaptic-like vesicles (but not in the insulin secretory vesicles), suggesting that it may be secreted from β -cells. However, there is no evidence that it has any effects on other pancreatic cells, nor that it affects the secretion of insulin or glucagon, and the function of β -cell GABA is unclear. It is likely that its main role is in energy-yielding metabolism. Incubation of isolated islets with [^{14}C]glucose leads to rapid labelling of GABA, and glucose also stimulates the synthesis of glutamate decarboxylase (Lernmark, 1996; Michalik & Erecinska, 1992).

Auto-antibodies against glutamate decarboxylase (the GAD-65 isoenzyme, not the structurally very similar GAD-67 form – see section 5.5.1), as well as other β -islet cell proteins, are characteristic of type 1 diabetes. They can be detected before the development of overt signs and symptoms of diabetes, and so may be useful in detection of people at risk, as well as in monitoring the progression of β -islet cell destruction. There is some evidence that auto-antigen specific treatment with glutamate decarboxylase may reduce the loss of β -cell function after the clinical onset of diabetes (Bingley, 2010; Larsson & Lernmark, 2011).

In plants, GABA synthesis is increased in response to hypoxia, temperature shock and water stress, as a result of activation of glutamate decarboxylase by calcium-calmodulin. Transgenic plants with a mutant glutamate decarboxylase lacking the calmodulin binding domain show a variety of morphological abnormalities, suggesting that regulation of GABA formation is important for normal plant development as well as stress responses. Reduction of succinic semialdehyde to γ -hydroxybutyrate provides a sink for excess reducing equivalents when carbon dioxide for carbohydrate synthesis is limiting because stomata have closed to preserve water, but photo-excitation of chlorophyll continues (Baum *et al.*, 1996; Bouche & Fromm, 2004).

5.5.1 Glutamate decarboxylase

There are two mammalian isoenzymes of glutamate decarboxylase – GAD-65 and GAD-67 – named for their molecular size. They are the products of separate genes, but have very high sequence homology and the same fold structure. Knockout mice lacking GAD-65 survive, but suffer from seizures and anxiety; by contrast, GAD-67 knockout is lethal (Buddhala *et al.*, 2009; Wei & Wu, 2008).

GAD-65 is concentrated in nerve terminals, where it forms a complex with the vesicular GABA transporter and other proteins that are required

for packaging GABA into synaptic vesicles. Its function is the production of GABA as a neurotransmitter. It undergoes post-synthetic palmitoylation, forming thioesters to two cysteine residues in the protein. This is essential for post-Golgi trafficking to synaptic vesicles, and the enzyme is recycled back to the Golgi by depalmitoylation, catalyzed by protein palmitoyl thioesterase.

GAD-67 is found throughout the neuronal cytoplasm, and also in non-neuronal tissues. Its function is to synthesize GABA other than as a neurotransmitter – as a trophic factor in synaptogenesis, for the GABA shunt as an alternative to part of the citric acid cycle, and as a regulator of redox potential during oxidative stress.

During neuronal development, there are two splice variants of GAD-67. GAD-44 is enzymically active, but GAD-25, which is synthesized in early development, is not. This suggests that it has some other, as yet unknown, function.

GAD-67 is expressed early in embryonic development, but GAD-65 is only expressed later, when there is a need for GABAergic neurotransmission. The two enzymes are regulated in opposite directions by phosphorylation; GAD-65 is activated by phosphorylation catalyzed by protein kinase C, while GAD-67 is inhibited by phosphorylation catalyzed by protein kinase A.

Both enzymes are also regulated by shuttling between the apoenzyme and the holoenzyme with pyridoxal phosphate bound at the active site. About 73 per cent of GAD-67 is normally present as the holoenzyme, but only about 7 per cent of GAD-65. In response to the need for synthesis of GABA as a neurotransmitter, there is a transient increase in the amount of GAD-65 holoenzyme. Accumulation of GABA leads to dissociation of the holoenzyme to the apoenzyme. As in other pyridoxal phosphate-dependent enzymes (Chapter 3), the coenzyme is tightly bound to holo-glutamate decarboxylase, and formation of the apoenzyme involves transamination of GABA to succinic semialdehyde, leaving pyridoxamine phosphate at the active site; this is not bound in an aldimine, and so leaves the enzyme.

ATP also promotes conversion of the holoenzyme to the apoenzyme by an allosteric effect, again suggesting the importance of the GABA shunt in energy-yielding metabolism. Binding of ATP to the apoenzyme stabilizes it; in the absence of ATP, the apoenzyme rapidly undergoes denaturation at 37°C. Inorganic phosphate antagonizes the effect of ATP and accelerates the formation of the holoenzyme (Buddhala *et al.*, 2009; Martin & Rinvall, 1993; Petroff, 2002).

5.5.2 Alternative pathways of GABA synthesis

Arginine can be a source of GABA; in organisms that lack arginase (section 5.9.2), this provides the major route for arginine catabolism, with onward

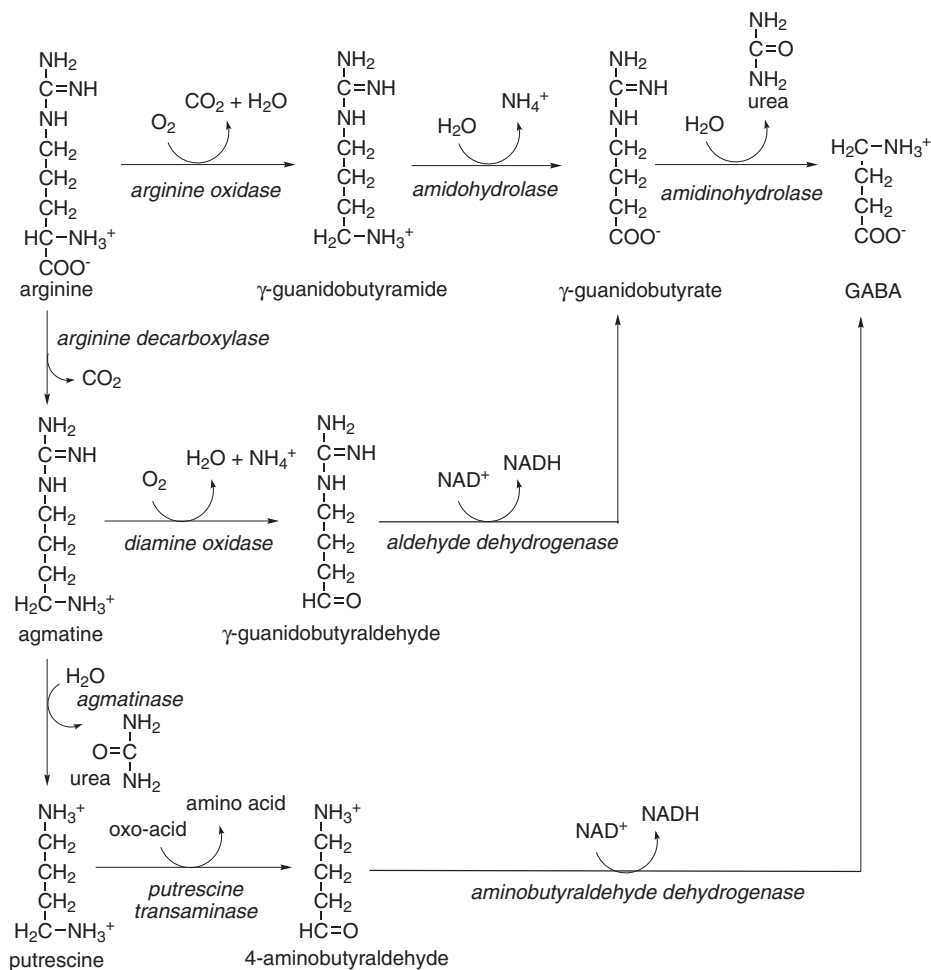


Figure 5.8 Synthesis of GABA from arginine.

Arginine oxidase EC 1.13.12.1, amidohydrolase (guanidobutyramidase) EC 3.5.1.4, amidinohydrolase (guanidobutyrase) EC 3.5.3.7, arginine decarboxylase EC 4.1.1.19, agmatinase EC 3.5.3.11, putrescine transaminase EC 2.6.1.82, aminobutyraldehyde dehydrogenase EC 1.2.1.19, diamine oxidase EC 1.4.3.21 and 1.4.3.22, aldehyde dehydrogenase EC 1.2.1.3.

metabolism of GABA to succinate (Figure 5.7). As shown in Figure 5.8, arginine oxidase catalyzes the oxidative decarboxylation of arginine to γ -guanidobutyramide, which is then deaminated to γ -guanidobutyrate, which is hydrolyzed to yield urea and GABA. Although this reaction is the same as that catalyzed by arginase, it is a distinct enzyme that has little or no activity towards arginine. In organisms that have arginase, this pathway is not a significant route of either arginine catabolism or GABA formation. Alternatively, arginine may be decarboxylated to agmatine, which may be a substrate

for putrescine synthesis (see section 5.9.4), or it may be oxidized by diamine oxidase to form γ -guanidobutyraldehyde and then by aldehyde dehydrogenase to yield γ -guanidobutyrate (Grillo & Colombatto, 2004a, 2004b).

Decarboxylation of ornithine leads to the formation of the diamine putrescine (section 5.8.1). Putrescine is oxidized by diamine oxidase to GABA aldehyde, which can be oxidized to GABA. Ornithine decarboxylase is stabilized (and hence its activity is increased) in the presence of moderately high concentrations of GABA, suggesting that GABA modulates the synthesis of putrescine rather than that putrescine is a source of GABA as a neurotransmitter (Lapinjoki *et al.*, 1983).

Ornithine undergoes δ -transamination to yield glutamic- γ -semialdehyde, which can be oxidized to glutamate. The brain takes up ornithine from the bloodstream, and ornithine δ -transaminase is subject to feedback inhibition by GABA, suggesting that ornithine may be the source of much of the glutamate that is required for GABA synthesis.

Genetic lack of ornithine δ -transaminase leads to the recessive eye disease gyrate atrophy of the choroid and retina – a cause of progressive loss of vision. In some cases, the condition is responsive to supplements of vitamin B₆ (section 3.6). In others, the mutation results in failure of mitochondrial targeting of the enzyme, although most cases involve more or less complete loss of enzyme activity (Mitchell *et al.*, 1988a, 1988b; Seiler & Daune-Anglard, 1993; Yoneda *et al.*, 1982).

5.5.3 GABA catabolism

As shown in Figure 5.7, GABA is catabolized by transamination to succinic semialdehyde, linked to the amination of 2-oxoglutarate to form glutamate (although transaminases linked to the amination of other oxo-acids will also act on GABA). In plants, there are two specific GABA transaminases – one linked to 2-oxoglutarate as the acceptor, and the other to pyruvate (Bouche & Fromm, 2004). Most succinic semialdehyde is then dehydrogenated to succinate by succinic semialdehyde dehydrogenase, but some is reduced to 4-hydroxybutyrate. There are two NADPH-dependent enzymes that catalyze this reaction, as well as an NADPH-independent transhydrogenase that catalyzes a reaction between succinic semialdehyde and hydroxyglutarate to yield 4-hydroxybutyrate and 2-oxoglutarate (Tillakaratne *et al.*, 1995).

4-Hydroxybutyrate was originally developed as an anaesthetic agent, because it readily crosses the blood-brain barrier and, as shown in Figure 5.7 is converted to GABA. However, this was abandoned because of adverse effects at pharmacological levels in experimental animals. It was also used by bodybuilders, for the presumed growth hormone releasing action of the GABA formed. It is now a controlled substance because of its abuse in ‘date rape’, when it causes loss of short-term memory (Malaspina *et al.*, 2009).

Patients with genetic defects of succinic semialdehyde dehydrogenase have elevated concentrations of GABA in the cerebrospinal fluid and urine, and excrete 4-hydroxybutyrate. They suffer from seizures, ataxia, psychomotor retardation and hypotonia, suggesting that 4-hydroxybutyrate has a neuromodulatory or neurotransmitter role. It is a weak agonist of GABA_B receptors, but there is also evidence for specific 4-hydroxybutyrate pre-synaptic G-protein-coupled receptors. Succinic semialdehyde dehydrogenase knockout mice show a 20–60-fold increase in brain GABA and a 2–3-fold increase in 4-hydroxybutyrate, associated with ataxia and seizures (Wong *et al.*, 2003).

5.6 Glutamate carboxylase and vitamin K-dependent post-synthetic modification of proteins

It has long been known that vitamin K is essential for the synthesis of prothrombin and other blood clotting factors. In vitamin K deficiency, or after the administration of coumarin derivatives that have anti-vitamin activity, such as the anticoagulant Warfarin, blood clotting is impaired and an abnormal form of prothrombin is found in the blood stream. Stenflo *et al.* (1974) demonstrated the presence of a hitherto unknown amino acid, γ -carboxyglutamate (Gla), in prothrombin. In the abnormal protein that circulates in vitamin K deficiency, glutamate is found where γ -carboxyglutamate occurs in active prothrombin, and it is glutamate that is incorporated into what is now known as pre-prothrombin.

Pre-prothrombin is sometimes known as PIVKA – the **p**rotein **i**nduced by **v**itamin **K** **a**bsence. γ -Carboxyglutamate is formed by post-synthetic modification of the precursor protein, catalyzed by the vitamin K-dependent carboxylase.

In addition to prothrombin and other proteins of the blood clotting cascade (section 5.6.1 and Figure 5.10), a number of other mammalian proteins also contain γ -carboxyglutamate, including osteocalcin and the bone matrix Gla protein (section 5.6.2), the anticoagulant proteins S and Z, the product of the *Gas-6* gene (section 5.6.3) and a number of transmembrane proteins of (as yet) unknown function, in which the γ -carboxyglutamate domain is at the outer face of the plasma membrane.

Glutamate carboxylase itself also requires carboxylation for activity (Berkner, 2005, 2008). γ -Carboxyglutamate-containing proteins are also found in species that do not have a blood coagulation system like that found in mammals, including the sea squirt, *Drosophila* and marine snails. Marine cone snails express a number of γ -carboxylated peptides that modulate the activity of ion channels and paralyze their prey by inhibiting neurotransmis-

sion. Some snake venoms, and some of the connexin proteins, also have γ -carboxyglutamate domains (Bandyopadhyay, 2008; Benzakour, 2008).

All of the mammalian vitamin K-dependent proteins have a carboxylase recognition propeptide sequence of about 18 basic amino acids adjacent to the γ -carboxyglutamate domain, which is cleaved after carboxylation. The γ -carboxyglutamate domain consists of about 45 amino acids, with 9–13 γ -carboxyglutamate residues. Binding of calcium to the γ -carboxyglutamate domain maintains the protein in the appropriate conformation to bind to phospholipids, and especially those that contain phosphatidylserine. Phosphatidylserine is normally in the inner leaflet of the plasma membrane, but it is exposed at the cell surface in response to intracellular mobilization of calcium, as occurs in apoptosis and platelet activation. In apoptotic cells, the exposure of phosphatidylserine marks the cells for phagocytosis. In platelet activation, the exposure of phosphatidylserine provides a surface for the binding and activation of clotting factors (Kulman *et al.*, 2007).

The vitamin K-dependent carboxylase is an integral membrane protein. Most of the proteins that contain γ -carboxyglutamate are extra-cellular proteins, and the main activity of the carboxylase is at the luminal face of the rough endoplasmic reticulum. In human tissues, all of the vitamin K-dependent proteins are carboxylated by the same enzyme.

As shown in Figure 5.9, the initial reaction is oxidation of vitamin K hydroquinone to the epoxide, linked to γ -deprotonation of the glutamate residue to yield a carbanion, catalyzed by glutamate carboxylase. The glutamate carbanion reacts with carbon dioxide to form γ -carboxyglutamate. At saturating concentrations of carbon dioxide, there is equimolar formation of γ -carboxyglutamate and vitamin K epoxide (Silva & Ramos, 2007).

The peptide substrate binds to the amino-terminal region of the carboxylase, while the carboxy-terminal region has the vitamin K epoxidase activity. The epoxidase activity is regulated by the binding of the peptide substrate and in the absence of the peptide, epoxidation does not occur. During a single peptide binding event, the enzyme catalyzes multiple carboxylations of glutamate residues, each associated with epoxidation of vitamin K hydroquinone. No partially carboxylated peptide is released from the enzyme because the dissociation of the product is slower than the carboxylation reaction. In vitamin K deficiency, the rate of carboxylation is slower, and dissociation of partially carboxylated proteins occurs.

Detection of undercarboxylated prothrombin and matrix Gla protein provides a sensitive index of vitamin K nutritional status. Sequencing of the partially carboxylated preprothrombins formed in the presence of Warfarin shows that the carboxylation is not random, but proceeds in an orderly fashion from the amino-terminal of the substrate. In preprothrombin containing 80 per cent of the normal amount of γ -carboxyglutamate, it is the last two glutamate residues before the carboxy-terminal that are not carboxylated. In

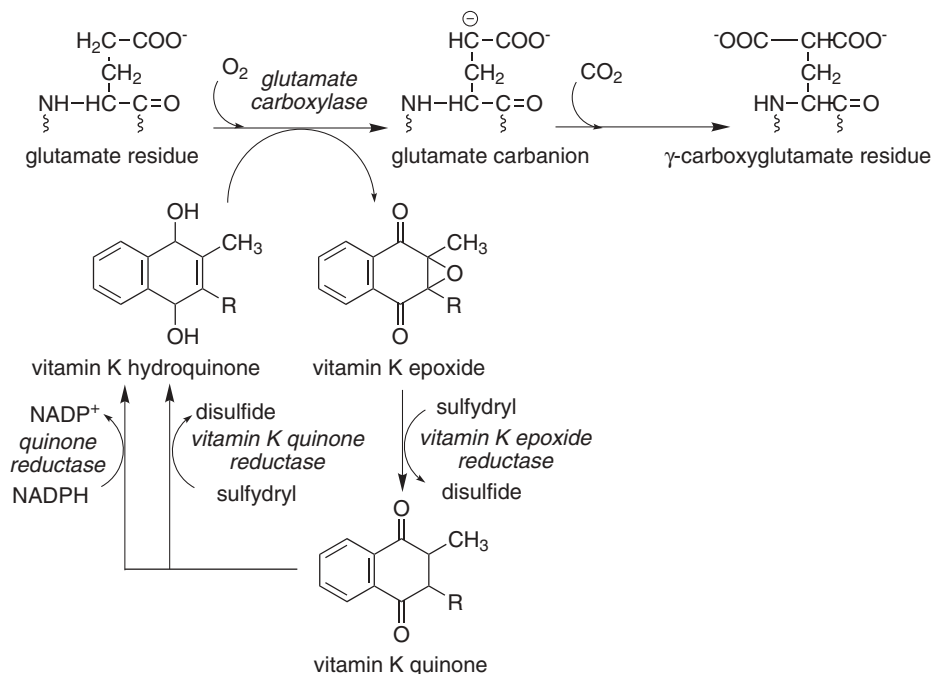


Figure 5.9 The reaction of glutamate carboxylase.

Glutamate carboxylase EC 4.1.1.90, vitamin K epoxide and quinone reductase (Warfarin-sensitive) EC 1.1.4.1, vitamin K epoxide reductase (Warfarin insensitive) EC 1.1.4.2, quinone reductase EC 1.6.5.2.

60 per cent undercarboxylated preprothrombin, it is the last four glutamate residues that are not carboxylated (Berkner, 2005, 2008; Furie & Furie, 1997; Liska & Suttie, 1988; Morris *et al.*, 1995; Sugiura *et al.*, 1997).

Vitamin K epoxide is reduced to the quinone in a reaction involving oxidation of a dithiol to the disulphide, catalyzed by epoxide reductase. Like other dithiol-linked flavoprotein reductases, the enzyme initially undergoes an internal dithiol-disulphide reaction, followed by reaction with the dithiol substrate. The initial step in the reaction is an attack on the epoxide ring by an enzyme-bound sulphhydryl group, with intermediate formation of a thioether adduct. Thiol reagents such as iodoacetamide and *N*-ethylmaleimide inhibit the enzyme irreversibly, but the addition of vitamin K epoxide protects the enzyme against this inactivation. The physiological dithiol substrate has not been unequivocally identified, but is assumed to be thioredoxin. There are two epoxide reductases; one is inhibited by Warfarin, the other is not (Mukharji & Silverman, 1985).

Vitamin K quinone is reduced to the active hydroquinone substrate for the epoxidase reaction by either a dithiol-linked reductase that is almost certainly

the same enzyme as the epoxide reductase, or by NADPH-dependent quinone reductase. Like the epoxide reductase, the dithiol-linked reductase is inhibited by Warfarin. In Warfarin-resistant rats, there is a Warfarin-insensitive epoxide reductase, which also has quinone reductase activity (Gardill & Suttie, 1990; Hildebrandt *et al.*, 1984).

The NADPH-dependent reduction of vitamin K quinone to the hydroquinone is not inhibited by Warfarin. In the presence of adequate amounts of vitamin K, the carboxylation of glutamate residues can proceed normally, despite the presence of Warfarin, with the stoichiometric formation of vitamin K epoxide, which cannot be re-utilized. Small amounts of vitamin K epoxide, plus hydroxides formed by its reduction by other enzymes, are normally found in plasma; in Warfarin-treated animals and patients, there is a significant increase in the plasma concentration of both. There is also an increase in the urinary excretion of the products of side-chain oxidation of the epoxide and hydroxides.

5.6.1 Vitamin K-dependent proteins in blood clotting

The formation of blood clots is due to the conversion of the soluble protein fibrinogen into fibrin, an insoluble network of fibres. This is achieved by specific proteolysis of fibrinogen at two arginine-glycine junctions, removing two pairs of small peptides (fibrinopeptides) from fibrinogen, catalyzed by thrombin. The resultant fibrin monomer aggregates into the insoluble fibrin polymer, which undergoes further covalent cross-linkage, catalyzed by a transglutaminase (section 5.3.3), which is the fibrin stabilizing factor or Factor XIII. Fibrin stabilizing factor is normally present as an inactive dimeric precursor which is activated by thrombin. Bleeding is rapidly stopped by the formation of an insoluble clot.

Thrombin, which catalyzes the proteolysis of fibrinogen, circulates as an inactive precursor – prothrombin – which in turn is activated by partial proteolysis to remove a peptide sequence which masks the catalytic site. There are two distinct pathways leading to the activation of prothrombin to thrombin (Figure 5.10). The extrinsic pathway is initiated by thromboplastin released from injured tissues, plus the protease proconvertin (Factor VII). The intrinsic pathway is initiated by the activation of Factor XII as a result of adsorption onto collagen, platelet membranes or (under laboratory conditions) glass. The intrinsic pathway is involved in the clotting of blood in glass tubes, and in the undesirable intravascular clotting which results in thrombosis. Control of the clotting mechanism is thus central to haemostasis, to avoid both haemorrhage and thrombosis.

Both the extrinsic and intrinsic pathways for the activation of prothrombin – and hence initiation of blood clotting – involve a number of intermediate factors. The nomenclature of the factors is based on the history of their

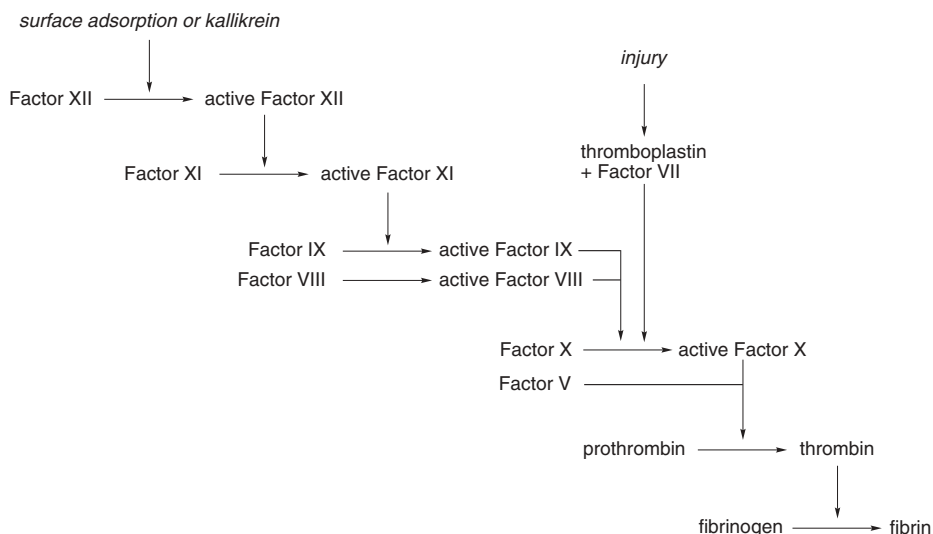


Figure 5.10 The intrinsic and extrinsic blood clotting cascades.

discovery, which was largely as a result of studies in patients with various congenital defects of blood clotting. Most of the blood clotting factors are serine proteases, which circulate as inactive zymogens. Each factor is activated by partial proteolysis, and then in turn activates the next factor – a cascade that results in considerable amplification of the original stimulus for clotting.

The cascade is not a simple linear one. The concerted action of activated Factors VIII and IX is required in the intrinsic pathway for the activation of Factor X. The rate of prothrombin activation by activated Factor X alone is inadequate to meet physiological needs; an additional protein – proaccelerin or Factor V – is also required. Factor X can also be activated by kallikrein; in turn, prekallikrein is activated to kallikrein by activated Factor XII, thus prolonging the initial contact activation of Factor XII.

In addition to prothrombin, Factors VII, IX and X contain γ -carboxy-glutamate and, hence, are vitamin K-dependent, as are three further proteins – proteins C, S and Z, which are anticoagulants. Proteins C and S are proteases that hydrolyze activated Factors V and VIII, and activate clot lysis. They circulate as zymogens and are activated by thrombin. Protein Z serves as the cofactor for the inhibition of activated Factor X by a protease inhibitor. In turn, the protease inhibitor-protein Z complex is hydrolyzed by activated factors X and XI (Broze, 2001; Castoldi & Hackeng, 2008; Furie & Furie, 1988; Vasse, 2008).

In addition to their role in blood clotting, thrombin and other vitamin K-dependent clotting factors bind to, and activate, G-protein coupled

protease-activated receptors that are involved in platelet activation. They may also have roles in neurodegeneration and sepsis (Benzakour, 2008).

Apart from prothrombin, all of the vitamin K-dependent proteins involved in blood clotting have multiple domains that resemble endothelium-derived growth factor and contain β -hydroxyaspartate, which is synthesized by post-synthetic hydroxylation of aspartyl residues catalyzed by the iron and 2-oxoglutarate-dependent aspartyl hydroxylase (section 5.7.2). This hydroxylation does not require the presence of γ -carboxyglutamate; it can occur in the uncarboxylated precursor proteins, and β -hydroxyaspartate is also found in non-Gla proteins (Stenflo *et al.*, 1987).

5.6.2 Osteocalcin and matrix Gla protein

Treatment with Warfarin or other anticoagulants during pregnancy can lead to bone abnormalities in the foetus – the so-called foetal Warfarin syndrome. It is due in part to impaired synthesis of osteocalcin, a small calcium binding protein containing three γ -carboxyglutamate residues, which is found in bone matrix and dentine. Osteocalcin also contains a hydroxyproline residue, and thus undergoes both vitamin K- and vitamin C-dependent post-translational modifications (see section 5.7.2). It is the most abundant of the non-collagen proteins of bone matrix, accounting for some 1–2 per cent of total bone protein, or 15 per cent of non-collagen bone protein. Osteocalcin synthesis is induced by physiological concentrations of calcitriol (the active metabolite of vitamin D), and the release of osteocalcin into the circulation provides a sensitive marker of vitamin D action and metabolic bone disease.

Osteocalcin is synthesized in the osteoblasts as a precursor protein which then undergoes γ -carboxylation before secretion into the extracellular space, where it binds to hydroxyapatite. It modifies the crystallization of calcium phosphates, retarding the conversion of brushite [$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$] to hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] and inhibiting the mineralization of bone. Osteocalcin knockout mice have a higher bone mineral density than normal; the absence of osteocalcin leads to increased bone formation without impairing resorption (Ducy *et al.*, 1996).

The matrix Gla protein (also known as atherocalcin) was originally described in bone, where it binds to both organic and mineral components, and it is now known to occur in the circulation and a number of tissues. It acts to prevent mineralization of cartilage and other connective tissues. Matrix Gla protein knockout mice develop a variety of abnormalities within two weeks of birth and die within two months from rupture of the aorta, associated with extensive calcification of blood vessels. It is the major inhibitor of vascular calcification associated with atherosclerotic plaque (Benzakour, 2008; Luo *et al.*, 1997).

Nephrocalcin in the kidney has considerable homology with matrix Gla protein. It is probably involved in renal re-absorption of calcium, but it also acts to solubilize calcium salts in the urine, and it is found in calcium oxalate renal stones.

5.6.3 Vitamin K-dependent proteins in cell signalling – Gas-6 and protein S

The product of the growth arrest specific gene 6 (*gas-6*) is a γ -carboxyglutamate-containing growth factor. It has significant sequence homology with the anticoagulant protein S, but has no role in blood coagulation. However, protein S also has activity as a growth factor, and both have a major role in controlling phagocytosis of apoptotic cells.

Both *gas-6* and protein S are anchored to the plasma membrane by binding to exposed phosphatidylserine by their amino-terminal γ -carboxyglutamate domains. They bind to, and activate, the TAM (Tyro3, Axl and Mer) family of receptor tyrosine kinases, leading to the induction of mitogen-activated protein kinase (MAP kinase). The *gas-6*-Axl system regulates cell survival, proliferation, migration, adhesion and phagocytosis (Fernandez-Fernandez *et al.*, 2008; Hafizi & Dahlback, 2006a, 2006b; Saxena *et al.*, 2001). Both *gas-6* and protein S stimulate osteoclasts in bone. *Gas-6* is also implicated in the formation of foam cells from macrophages and cell proliferation in blood vessel intima in response to vascular injury, so it has roles in atherosclerosis and thrombosis (Maree *et al.*, 2007).

5.7 Proline

Proline is considered to be a conditionally essential amino acid, in that, while it can be synthesized in the body, demand may outstrip supply when there is an increased need for proline for collagen synthesis. Provision of supplements of proline does not, however, increase collagen synthesis. As shown in Figure 5.11, proline may be synthesized from either glutamate or ornithine and, while the major route under normal conditions is probably from glutamate, administration of supplementary glutamine (as a source of glutamate) similarly does not increase collagen synthesis. However, supplements of ornithine or arginine (as a source of ornithine, see section 5.9) do, and, in response to wound healing, arginine becomes limiting for maintenance of nitrogen balance (section 2.1).

True deficiency of proline *per se* probably only occurs in rare cases of deficiency of prolylase, the final enzyme of collagen catabolism, which liberates C-terminal proline (and hydroxyproline) from imino di- and tripeptides (Barbul, 2008).

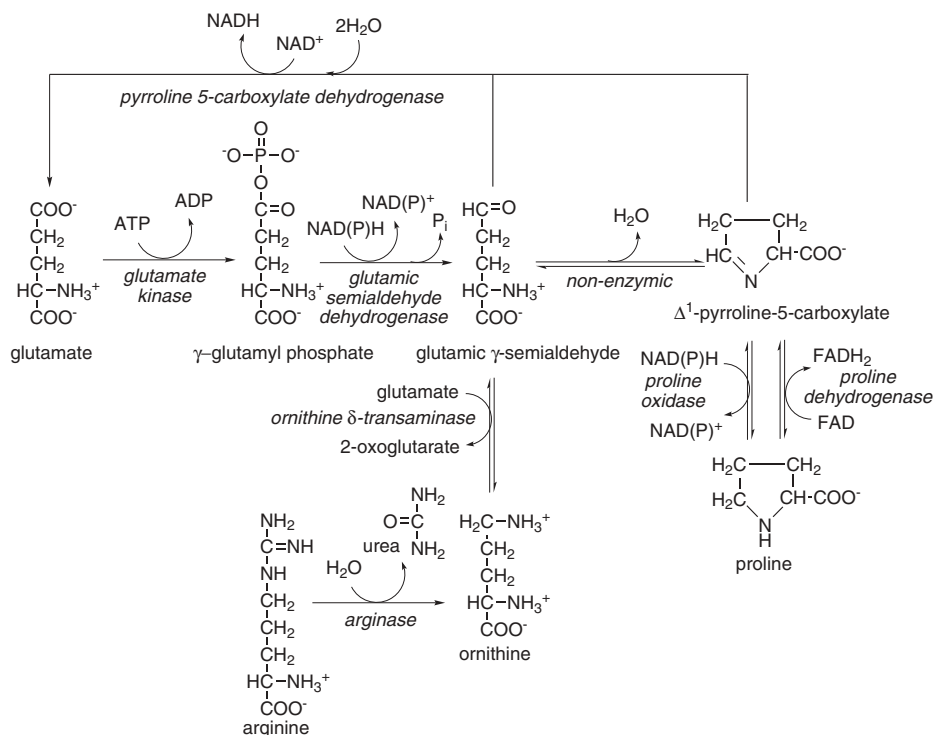


Figure 5.11 Synthesis of proline from glutamate and ornithine.

Glutamate kinase EC 2.7.2.11, glutamic semialdehyde dehydrogenase EC 1.2.1.41, pyrroline-5-carboxylate dehydrogenase EC 1.5.1.12, ornithine δ -transaminase EC 2.6.1.13, proline oxidase EC 1.5.1.2, proline dehydrogenase EC 1.5.99.8, arginase EC 3.5.3.1.

The milk of many mammals is relatively deficient in arginine, and most of the Δ^1 -pyrroline-5-carboxylate formed from glutamine in the infant's intestinal mucosa is used for synthesis of ornithine and then arginine. Targeted disruption of ornithine transaminase in the gut is lethal in neonatal mice, because of the lack of arginine (Hu *et al.*, 2008a).

In addition to its role in protein synthesis, proline:

- is a major metabolic fuel for insect flight muscle (section 5.7.1);
- is an important osmolyte in prokaryotes and plants;
- is involved in transporting reducing equivalents from cytosolic NADPH into the mitochondrion in both mammals and insects (section 5.7.1.1);
- is involved in regulation of apoptosis; and
- has a probable role in modulating neurotransmission.

In plants, proline provides a major transport form for nitrogen in xylem and phloem, and plants also accumulate proline in response to drought stress, salinity and extremes of temperature, when there is an increase in proline synthesis and also a reduction in the rate of its catabolism (Verbruggen & Hermans, 2008; Yoshida *et al.*, 1997).

5.7.1 Proline synthesis and catabolism

In eukaryotes, the first two reactions of proline synthesis from glutamate are catalyzed by a single bifunctional enzyme, Δ^1 -pyrroline-5-carboxylate synthetase. The glutamate kinase site catalyzes the phosphorylation of glutamate to γ -glutamyl phosphate, and the glutamate semialdehyde dehydrogenase site catalyzes reduction to glutamic γ -semialdehyde. The first reaction, phosphorylation of glutamate, is subject to feedback inhibition by proline (Forde & Lea, 2007). Glutamic γ -semialdehyde is a key intermediate for the synthesis of proline and ornithine (Figure 5.11) and, from ornithine, also arginine (section 5.9.1). This bifunctional enzyme has evolved from two separate bacterial enzymes that are encoded in a single operon (Hu *et al.*, 1992).

There are two splice variants of Δ^1 -pyrroline-5-carboxylate synthetase. A short form is expressed mainly in the intestinal mucosa, and this is inhibited by ornithine, suggesting that it is important in the synthesis of arginine. A longer form is expressed ubiquitously and is insensitive to ornithine. Synthesis of the long form of the enzyme is repressed in response to glucocorticoid hormones, but is induced by oestrogens and as an early response to p53-stimulated apoptosis. The apoptotic effect seems to be due to the induction of proline oxidase (section 5.7.1.1) rather than induction of Δ^1 -pyrroline-5-carboxylate synthetase *per se*. Over-expression of Δ^1 -pyrroline-5-carboxylate synthetase in various cell types has no effect on cell survival, but it will provide additional proline for cycling via proline oxidase to generate reactive oxygen species (Hu *et al.*, 2008b; Perez-Arellano *et al.*, 2010).

5.7.1.1 Δ^1 -pyrroline-5-carboxylate reductase and proline oxidase The final reaction in proline synthesis is the NAD(P)H-dependent reduction of Δ^1 -pyrroline-5-carboxylate to proline. The reductase is a cytosolic enzyme and preferentially uses NADPH as the electron donor. In proline catabolism, this reaction is reversed by mitochondrial proline oxidase, which has an FAD prosthetic group and directly reduces cytochrome c in the electron transport chain.

Proline and Δ^1 -pyrroline-5-carboxylate can form a shuttle system to transport cytosolic reducing equivalent from NADPH into the mitochondrion, with reduction of Δ^1 -pyrroline-5-carboxylate in the cytosol and oxidation of proline in the mitochondrial matrix. This is energetically less efficient than the malate-aspartate shuttle (Figure 3.6), since NADPH yields $\approx 2.5 \times$ ATP

in the mitochondrial electron transport chain, while FADH yields only $\approx 1.5 \times \text{ATP}$. However, unlike the malate-aspartate shuttle, the proline shuttle is not sensitive to the mitochondrial NAD:NADH ratio.

The accumulation of proline in plants under osmotic stress may also serve to re-oxidize NADPH generated by photosynthesis, when the availability of carbon dioxide is reduced because stomata have been closed to minimize water loss (Verbruggen & Hermans, 2008).

Erythrocytes have a high activity of Δ^1 -pyrroline-5-carboxylate reductase; this cannot be for synthesis of proline for protein synthesis, because erythrocytes do not synthesize protein. There is an inter-organ cycle, with oxidation of proline in the liver, and export of Δ^1 -pyrroline-5-carboxylate that is taken up by erythrocytes and reduced at the expense of NADPH. This seems to be a way of re-oxidizing excess NADPH. The addition of Δ^1 -pyrroline-5-carboxylate to erythrocyte incubations leads to an increase in the activity of the pentose phosphate pathway to generate NADPH (Phang *et al.*, 1982).

Proline oxidase seems to have two separate roles. Under basal conditions, it catalyzes the catabolism of proline for use as a metabolic fuel, and it is induced in nutrient stress via the mTOR signalling pathway. Additional proline is made available under these conditions by increased catabolism of collagen in the extra-cellular matrix by metalloproteinases and prolidase, the enzyme that catalyzes the removal of carboxy-terminal proline and hydroxyproline from imino di- and tripeptides formed by the action of metalloproteinases on collagen (Phang *et al.*, 2008a, 2008b).

When the FAD at the active site is exposed to oxygen in the solvent, proline oxidase catalyzes the reduction of oxygen to superoxide, rather than passing the electrons on to the electron transport chain. A flexible α -helix domain of the enzyme can either move to block solvent access to the prosthetic group (in which case the enzyme transfers electrons to the electron transport chain) or permit access (in which case it catalyzes superoxide formation). The enzyme is induced in response to activation of the p53 tumour suppressor gene and activation of the PPAR γ receptor, both of which induce apoptosis, and presumably the production of superoxide by proline oxidase is important in this apoptotic response (Phang *et al.*, 2008a, 2008b).

In some bacteria, proline oxidase and Δ^1 -pyrroline-5-carboxylate dehydrogenase, the two enzymes that catalyze the conversion of proline to glutamate, are fused into a single bifunctional enzyme; in other species, however, there are two separate enzymes, as in eukaryotes. In some organisms, this bifunctional enzyme has a third function, as a transcriptional repressor of the high affinity sodium-dependent proline transporter (Tanner, 2008).

In insects, proline is a significant metabolic fuel. Fruit beetles use proline exclusively during pre-flight warm-up, and again in prolonged flight after carbohydrate reserves have been depleted; otherwise they use proline and carbohydrate more or less equally in flight. However, dung beetles have

negligible carbohydrate reserves and are more or less completely reliant on proline as a metabolic fuel. The adipokinetic hormones in beetles that stimulate the mobilization of glycogen reserves by activation of glycogen phosphorylase also stimulate synthesis of proline in the fat body (the insect equivalent of the liver), for export to muscle (Gade & Auerswald, 2002). In flight muscle, proline is oxidized to provide a source of 2-oxoglutarate, then on to malate and pyruvate. There is an active NAD-linked malic enzyme in flight muscle that catalyzes the synthesis of pyruvate, which is the amino acceptor for the transamination of glutamate to 2-oxoglutarate. The alanine is exported from the flight muscle to the fat body, where it provides substrate for the synthesis of proline.

Flying insects have the ability to taste proline, and plants that require insect pollination produce nectar that is rich in proline. The nectar of ornamental nicotiana flowers contains about 2 mmol/l proline, and studies with synthetic nectars show that honey bees have a preference for proline-rich nectar (Carter *et al.*, 2006).

Some studies have demonstrated a link between mutations of proline oxidase and the risk of schizophrenia. A number of missense mutations have been detected both in hyperprolinaemia and schizophrenia, and ten of these are present at sufficient frequency in the population as to be considered to be polymorphisms (Bender *et al.*, 2005; Willis *et al.*, 2008). The mutant hyperprolinaemic mouse has defective proline oxidase and shows a defect in sensorimotor gating and regional changes in brain neurochemistry, suggesting that proline may have a role as a modulator of neurotransmission (Gogos *et al.*, 1999).

Hyperprolinaemia may arise as a result of genetic defects of either proline oxidase or Δ^1 -pyrroline-5-carboxylate dehydrogenase. In proline oxidase deficiency, there is high urinary excretion of proline and also glycine and hydroxyproline, which share the same renal transport mechanism. In Δ^1 -pyrroline-5-carboxylate dehydrogenase, the plasma concentration and urinary excretion of Δ^1 -pyrroline-5-carboxylate are also elevated. In most cases, there are few, if any, clear clinical effects, although in some cases the condition is associated with mental retardation.

5.7.1.2 Hydroxyproline catabolism While much of the proline released by catabolism of collagen can be re-used for protein synthesis, the hydroxyproline cannot; as discussed in section 5.7.2, hydroxyproline is formed by post-synthetic modification of proline residues in the precursor protein and is not incorporated on the ribosome. Hydroxyproline is the substrate for proline oxidase, and the resultant Δ^1 -pyrroline-3-hydroxy-5-carboxylate is a substrate for the same enzymes that are involved in proline catabolism, leading to the formation of 4-hydroxy-2-oxoglutarate. As shown in Figure 5.12, 4-hydroxy-2-oxoglutarate undergoes aldolase cleavage to glyoxylate and pyruvate and,

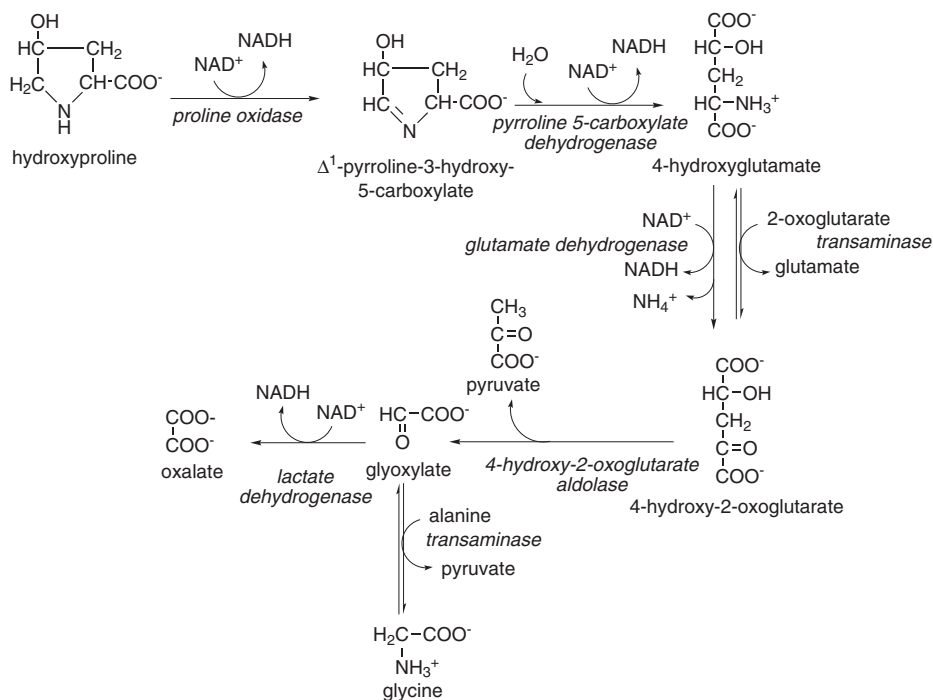


Figure 5.12 Catabolism of hydroxyproline.

Proline oxidase EC 1.5.1.2, pyrroline-5-carboxylate dehydrogenase EC 1.5.1.12, glutamate dehydrogenase EC 1.4.1.2 (NAD-linked), EC 1.4.1.4 (linked to either NAD or ANDP), EC 1.4.1.3 (NAD(P)-linked), hydroxyglutamate transaminase EC 2.6.1.23, 4-hydroxy-2-oxoglutarate aldolase EC 4.1.3.16, lactate dehydrogenase EC 1.1.1.27.

as discussed in section 4.3, glyoxylate is a substrate for either lactate dehydrogenase (leading to oxalate formation) or transamination to glycine.

Total daily turnover of collagen in an adult human being is 2–3 g, releasing 240–420 mg of hydroxyproline and leading to the formation of some 140–240 mg of oxalate – 5–20 per cent of the total amount of oxalate normally excreted. Feeding gelatine, a source of collagen-derived peptides and hence of hydroxyproline, leads to a significant increase in urinary excretion of oxalate (Knight *et al.*, 2006).

Genetic defects of hydroxyproline oxidase lead to a 5–10 fold increase in the plasma concentration of hydroxyproline and a 100-fold increase in its excretion, but with no clinical symptoms. This seems to be a metabolic abnormality with no associated disease. Mutations in 4-hydroxy-2-oxoglutarate aldolase that lead to increased activity are associated with the rare type III primary hyperoxaluria because of the increased formation of glyoxylate and oxalate (Belostotsky *et al.*, 2010). See section 4.3.1 for a discussion of primary hyperoxaluria types I and II.

5.7.2 Peptide prolyl hydroxylase

A number of iron-containing hydroxylases share an unusual reaction mechanism, in which hydroxylation of the substrate is linked to decarboxylation of 2-oxoglutarate. Prolyl and lysyl hydroxylases are required for the post-synthetic modification of collagen, and prolyl hydroxylase is also needed for the post-synthetic modification of osteocalcin (section 5.6.2) and the C1q component of complement. A separate prolyl hydroxylase catalyzes post-synthetic modification of the hypoxia-inducible factor (section 5.7.2.1).

Aspartate β -hydroxylase is required for the post-synthetic modification of a variety of proteins that have an endothelium-derived growth factor (EGF)-like domain, including all of the vitamin K-dependent clotting proteins except prothrombin (section 5.6.1). It catalyzes hydroxylation of both aspartate and asparagine residues. Trimethyllysine and γ -butyrobetaine hydroxylases are required for the synthesis of carnitine (section 6.2.5; Kivirikko & Pihlajaniemi, 1998).

A number of bacterial antibiotic peptides that are synthesized enzymically, rather than on the ribosome, contain 4-hydroxyproline, which is synthesized as a free amino acid by proline hydroxylase rather than prolyl hydroxylase. Like prolyl hydroxylase, bacterial proline hydroxylase is an iron-containing, 2-oxoglutarate-dependent enzyme.

Procollagen prolyl 4-hydroxylase is the best studied of this class of enzymes. While 3-hydroxyproline is found only in collagen, 4-hydroxyproline and hydroxylysine are found in a variety of other proteins, including the C1q component of complement, the macrophage receptor proteins and a variety of transmembrane and inter-cellular proteins and proteins of the cytoskeleton, as well as some enzymes. 4-Hydroxyproline, but not hydroxylysine, also occurs in elastin.

In collagen, hydroxyproline stabilizes the triple helix structure by forming hydrogen bonds via water between adjacent chains or between regions of the same chain. Hydroxylysine provides sites for glycosylation of proteins, and it is essential for the stabilization of inter-molecular cross-links formed by reaction between lysine aldehyde or hydroxylysine aldehyde and the ϵ -amino group of lysine or hydroxylysine. Type I collagen contains 338 Xaa-Yaa-Gly triplets; proline is most commonly found as the X-amino acid, and hydroxyproline as the Y-amino acid. Pro-Hyp-Gly occurs in 10.5 per cent of these Xaa-Yaa-Gly triplets (Ramshaw *et al.*, 1998).

Prolyl and lysyl hydroxylases are found in the lumen of rough endoplasmic reticulum, and hydroxylation of the peptide substrate occurs both cotranslationally and later as a post-synthetic modification. The enzymes act only on peptides, not on free amino acids, and only on procollagen chains, not the triple helix. Unlike glutamate carboxylase (see section 5.6), which acts sequentially along the peptide chain of the substrate, prolyl hydroxylase does not,

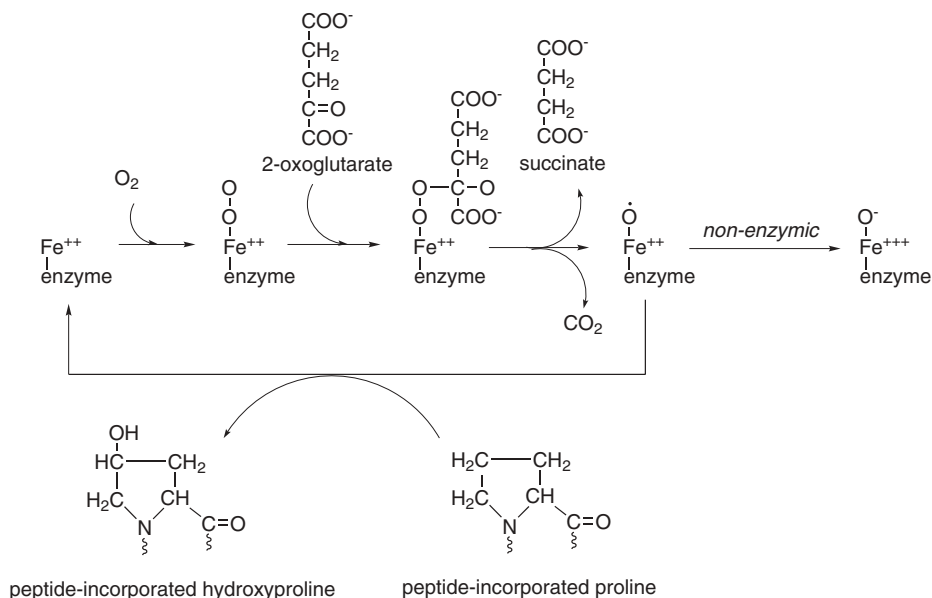


Figure 5.13 The reaction of peptide prolyl hydroxylase.

Collagen prolyl hydroxylase EC 1.14.11.2, hypoxia-inducible factor hydroxylase EC 1.14.11.29, prolyl 3-hydroxylase EC 1.14.11.7.

so not all Pro-Pro-Gly sites are hydroxylated. 3-Hydroxyproline is found mainly in type IV collagen – less commonly than 4-hydroxyproline – and it is synthesized by procollagen prolyl 3-hydroxylase, acting mainly at the first proline in Pro-Pro-Gly triplets.

As shown in Figure 5.13, the first step in the prolyl hydroxylase reaction is the binding of oxygen to the enzyme-bound iron, followed by attack on the 2-oxoglutarate substrate, resulting in decarboxylation to succinate and leaving a ferryl radical at the active site of the enzyme. This catalyzes the hydroxylation of proline, restoring the free iron to undergo further reaction with oxygen.

It has long been known that ascorbate is oxidized during the reaction, but not stoichiometrically with hydroxylation of proline and decarboxylation of 2-oxoglutarate. The purified enzyme is active in the absence of ascorbate but, after some 5–10 seconds (about 15–30 cycles of enzyme action), the rate of reaction begins to fall. This loss of activity is due to a side-reaction of the highly reactive ferryl radical, in which the iron is oxidized to Fe^{3+} , which is catalytically inactive. This is uncoupled decarboxylation of 2-oxoglutarate without hydroxylation of proline. Activity is only restored by ascorbate, which reduces the iron back to Fe^{2+} (Gorres & Raines, 2010; Kivirikko & Pihlajaniemi, 1998).

5.7.2.1 The hypoxia-inducible factor The cellular response to low availability of oxygen is mediated by the hypoxia-inducible factor, HIF, a transcription factor that induces synthesis of key enzymes involved in the response to hypoxia, including those of glycolysis, erythropoiesis and the key enzymes involved in intestinal iron absorption – the divalent metal transporter, ferrereductase and ferroportin. HIF also stimulates angiogenesis (the development of blood vessels) and the maintenance of vascular tone.

There are two subunits of HIF: HIF β is stable, while HIF α is highly unstable. In the presence of oxygen, HIF α is hydroxylated on either of two proline residues by an intracellular prolyl hydroxylase that is distinct from the extracellular hydroxylase involved in collagen synthesis. The hydroxylation sites in HIF α have no homology with those in procollagen. The K_m of this hydroxylase for oxygen is such that it is active at normal partial pressures of oxygen, but becomes inactive as hypoxia develops; it is thus the ultimate sensor of oxygen availability to tissues.

Hydroxylation of either of the susceptible proline residues targets HIF α for poly-ubiquitination and catabolism by the proteasome (section 2.1.5.2). Under conditions of hypoxia, HIF α is stable and translocates to the nucleus, where it dimerizes with HIF β , leading to upregulation of gene expression (Kaelin, 2005).

HIF α is also susceptible to hydroxylation of an asparagine residue, catalyzed by a 2-oxoglutarate-dependent hydroxylase that is distinct from that involved in hydroxylation of aspartate and asparagine residues in blood clotting and other proteins. Hydroxylation of the asparagine residue does not affect the stability of HIF α , but blocks its interaction with transcriptional co-activators and thus inhibits its activity. Like the proline hydroxylase, the K_m of this asparagine hydroxylase is such that it acts as a sensor of tissue oxygen availability (Lancaster *et al.*, 2004a, 2004b).

5.8 The polyamines

The diamine putrescine and the polyamines spermidine and spermine are essential for cell growth and proliferation. Depletion of polyamines leads to cessation of cell proliferation, which is restored when polyamines are provided again. Because of their multiple amino groups, which are protonated at physiological pH, they can interact with the negative charges of nucleic acids, acidic proteins and phospholipids, giving them roles in gene expression and chromatin regulation.

Polyamines accumulate in cancerous tissue, and the plasma concentration rises in patients with cancer. Thus, measurement of blood and urine concentrations of polyamines provides a way of monitoring cancer therapy. Ornithine decarboxylase, the first enzyme of polyamine synthesis, is induced during carcinogenesis. Several of the proto-oncogenic pathways control the

transcription and translation of the ornithine decarboxylase gene and lead to constitutive over-expression of the enzyme. Inhibitors of polyamine synthesis are potential drugs for cancer chemotherapy and as anti-parasite agents (Paz *et al.*, 2011; Shantz & Levin, 2007).

Putrescine is the product of decarboxylation of ornithine. Spermidine, then spermine, are synthesized by addition of an aminopropyl group from *S*-adenosyl-1-(methylthio)-3-propylamine, the decarboxylation product of *S*-adenosyl methionine (section 5.8.2). Spermidine has a role in control of translation of mRNA. It provides the aminobutyl moiety for post-synthetic modification of a lysine residue in eukaryotic initiation factor 5A, an essential step in activation of this initiation factor (section 6.2.4; Agostinelli *et al.*, 2010).

As shown in Figure 5.14, ornithine can be formed from glutamate or proline by way of glutamic- γ -semialdehyde (Figure 5.11), or from arginine by hydrolysis of the guanido group to liberate urea, catalyzed by arginase (Figure 1.16). Putrescine can also be synthesized from arginine by decarboxylation to agmatine (section 5.9.4), followed by hydrolysis of the guanido group to liberate urea, catalyzed by agmatinase. This is the main source of putrescine in plants.

5.8.1 Ornithine decarboxylase

The decarboxylation of ornithine to putrescine is the rate-limiting step of polyamine synthesis. Ornithine decarboxylase has a very rapid turnover, with a half-life of 11 minutes. Degradation of the enzyme protein is regulated by polyamines, which stimulate the synthesis of antizyme, a protein that both inactivates ornithine decarboxylase and targets it for catabolism by the proteasome. Unlike most proteins that are targeted to the proteasome for catabolism (section 2.1.5.1), targeting of ornithine decarboxylase does not require ubiquitination (Kahana, 2009b; Persson, 2009).

Ornithine decarboxylase is a dimer, with two symmetrical catalytic sites at the interface between the monomer subunits. Amino acids from both subunits contribute to each active site, so that the monomer is catalytically inactive. The association between the subunits is weak and there is rapid association and dissociation of the active dimer. This is important for regulation of enzyme activity by antizyme, which binds to ornithine decarboxylase monomers with a very much greater affinity than the monomers have for each other. This results in trapping of the enzyme as inactive monomers bound to antizyme.

Binding of antizyme also leads to a conformational change that exposes a 37 amino acid carboxy-terminal region that is a proteasome recognition signal. Antizyme is not catabolized when the antizyme-ornithine decarboxylase complex arrives at the proteasome. How the antizyme escapes proteolysis is unclear, but it is subsequently ubiquitinated and catabolized by the

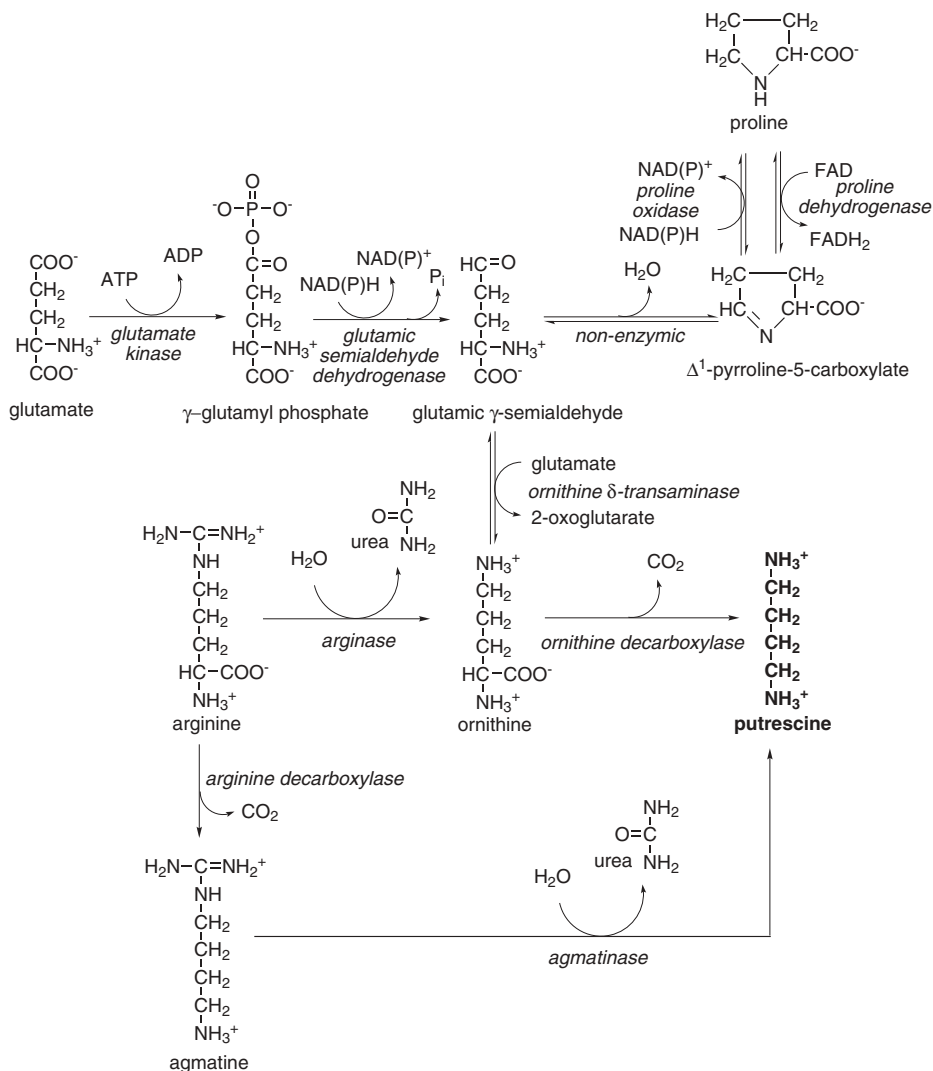


Figure 5.14 Synthesis of putrescine.

Glutamate kinase EC 2.7.2.11, glutamic semialdehyde dehydrogenase EC 1.2.1.41, pyrroline-5-carboxylate dehydrogenase EC 1.5.1.12, proline oxidase EC 1.5.1.2, proline dehydrogenase EC 1.5.99.8, ornithine δ -transaminase EC 2.6.1.13, ornithine decarboxylase EC 4.1.1.17, arginase EC 3.5.3.1, arginine decarboxylase EC 4.1.1.19, agmatinase EC 3.5.3.11.

proteasome. In addition to inhibiting ornithine decarboxylase, antizyme also inhibits the uptake of polyamines into the cell, and stimulates their excretion.

Antizyme was originally called the polyamine-induced ornithine decarboxylase inhibitory activity. The antizyme gene has two open reading frames, the first of which leads to a short inactive protein because of a stop codon.

The ribosome has to be subverted to skip this stop codon and continue with the second open reading frame in order to synthesize full-length active antizyme. Polyamines enhance the efficiency of this frame shift and so increase translation of active antizyme.

Antizyme itself is regulated by the antizyme inhibitor, which increases cell proliferation because it binds to antizyme and so permits ornithine decarboxylase to dimerize to the active form. There are two antizyme inhibitor proteins: azin-1 is expressed ubiquitously, while azin-2 is restricted to the testes and some brain regions. Azin-2 is more stable to proteosomal catabolism than azin-1 or ornithine decarboxylase. Unlike ornithine decarboxylase, both antizyme inhibitors require ubiquitination for catabolism (Lopez-Contreras *et al.*, 2010).

The antizyme inhibitors are highly homologous with ornithine decarboxylase, but lack catalytic activity. They have a higher affinity for antizyme than does ornithine decarboxylase, so they compete with the enzyme for available antizyme. The antizyme inhibitors form a dimer, but the interactions at the monomer interface are considerably weaker than those of ornithine decarboxylase, so that they are mainly present as monomer units that can interact with antizyme (Kahana, 2009a, 2009b; Persson, 2009).

5.8.2 *S*-Adenosylmethionine decarboxylase and polyamine synthesis

As shown in Figure 5.15, the decarboxylation product of *S*-adenosylmethionine, *S*-adenosyl-1-(methylthio)-3-propylamine, is the source of the aminopropyl group that is added to putrescine to yield spermidine, and to spermidine to yield spermine. The methylthioadenosine arising from polyamine synthesis is recycled via the methionine salvage pathway (the Yang cycle – see section 6.3.4).

All *S*-adenosylmethionine decarboxylases are pyruvoyl enzymes, with a catalytic pyruvate residue formed by post-synthetic modification of a serine residue in the precursor protein (see section 3.5). *S*-adenosylmethionine decarboxylase is synthesized as a pro-enzyme that undergoes autocatalytic cleavage to leave a serine residue at the amino-terminal of the α -subunit, which is then deaminated to pyruvate.

The availability of *S*-adenosyl-1-(methylthio)-3-propylamine is rate-limiting for the synthesis of spermidine and spermine. Putrescine increases the amount of active enzyme protein in the cell by activating the autocatalytic modification of the precursor protein to active decarboxylase. It is also an allosteric activator of the decarboxylase, stabilizing the active conformation of the enzyme. Indeed, the enzyme from protozoa, fungi and animals requires putrescine for activity, whereas that from bacteria and plants does not (Bale & Ealick, 2010; Pegg, 2009; Wada & Shirahata, 2010).

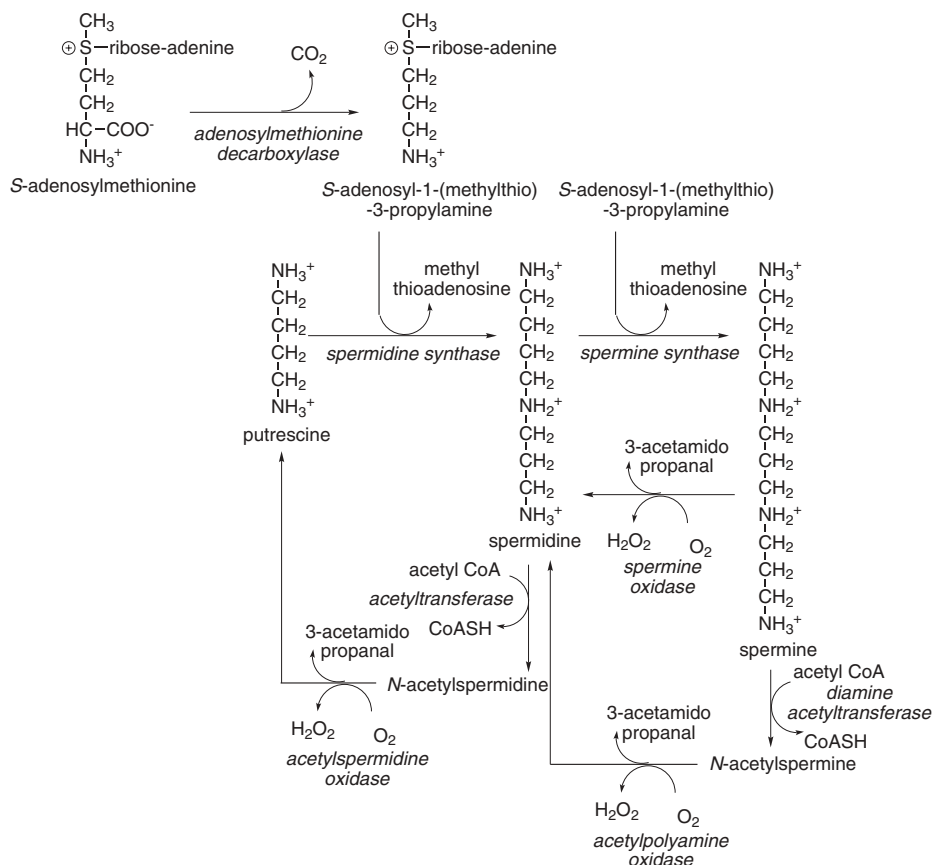


Figure 5.15 Synthesis and catabolism of the polyamines.

Adenosylmethionine decarboxylase EC 4.1.1.50, spermidine synthase EC 2.5.1.16, spermine synthase EC 2.5.1.22, spermine oxidase EC 1.5.3.16, diamine acetyltransferase EC 2.3.1.57, acetylpolyamine oxidase EC 1.5.3.13 (acts on *N*-acetylspermine and *N*-acetylspermidine), acetylspermidine oxidase EC 1.5.3.15, polyamine oxidase EC 1.5.3.14 catalyses oxidation of spermidine to propane-1,3-diamine and 4-aminobutanal, non-specific polyamine oxidase catalyses oxidation of acetylspermine, spermine, acetylspermidine and spermidine.

S-Adenosylmethionine decarboxylase is more rapidly ubiquitinated and catabolized in the presence of high concentrations of *S*-adenosylmethionine. This is probably because there is accidental transamination in the presence of a high concentration of substrate, leaving alanine in place of pyruvate at the active site, and the inactivated enzyme is a target for ubiquitination. Spermidine and spermine protect the enzyme against catabolism, but both also decrease synthesis of the enzyme by downregulating both transcription and translation of the mRNA (Anton & Kutny, 1988; Pegg, 2009; Persson, 2009).

Two separate propylamine transferases catalyze the transfer of the propylamine moiety of *S*-adenosyl-1-(methylthio)-3-propylamine onto putrescine to form spermidine (spermidine synthase) and onto spermidine to form spermine (spermine synthetase). Bacteria lack spermine synthase and form only spermidine, not spermine (Ikeguchi *et al.*, 2006).

Human spermidine and spermine synthases are highly specific for the amine substrates, while those from many bacteria (and especially extreme thermophiles) have a relatively broad specificity for the amine substrate. In most species, the distribution of spermine is more limited than that of spermidine, and it is only in animal tissues that the concentration of spermine is greater than that of spermidine. Human spermine synthase is a dimer, linked by an amino-terminal domain that does not contribute to the active site and has considerable structural homology with the precursor protein of *S*-adenosylmethionine decarboxylase. The catalytic domain is in the carboxy-terminal region, and there is a linking region that forms a lid over the active site. Spermidine synthase is very similar, but the amine binding pocket of spermine synthase is larger, to allow spermidine, rather than putrescine, to bind (Pegg & Michael, 2010).

Many bacteria lack *S*-adenosylmethionine decarboxylase and spermidine synthase but synthesize spermidine. They use aspartic semialdehyde as the donor, forming carboxyspermidine, which is then decarboxylated to spermidine.

5.8.3 Polyamine catabolism and the interconversion pathway

There is a rapid turnover of polyamines in the cell, but the spermidine formed by the catabolism of spermine and the putrescine formed by the catabolism of spermidine can be recycled back to spermine and spermidine respectively.

As shown in Figure 5.15, spermine can be oxidized to spermidine by spermine oxidase, or may be acetylated and then oxidized to spermidine by acetyl polyamine oxidase, a peroxisomal enzyme, liberating acetamidopropanal. Spermidine also undergoes acetylation and oxidation to putrescine, again liberating acetamidopropanal. The acetyltransferase catalyzes the transfer of an acetyl group from acetyl CoA onto N-1 of spermine or spermidine (the aminopropionyl amino group), but there is also an N-8 acetyltransferase that catalyzes reaction at the aminobutyl amino group, and this will therefore acetylate putrescine and spermidine. Acetylputrescine and N⁸-acetylspermidine are excreted in the urine; they are not substrates for acetyl polyamine oxidase. The N-1 acetyltransferase has a rapid turnover and is induced by polyamines and a variety of hormones.

By contrast, acetylpolyamine oxidase and diamine oxidase are relatively stable enzymes, and the main regulation of polyamine turnover is by regulation of the acetyltransferase. Diamine oxidase is also known as histaminase (section 8.3.3); it is a quinoprotein, with topaquinone (see section 9.5) at the active site (Persson, 2009; Seiler, 2004).

The 3-aminopropanal released by the action of acetylpolyamine oxidase is oxidized to *N*-acetyl- β -alanine, which is deacetylated to β -alanine. β -alanine is a substrate for transamination catalyzed by GABA transaminase to yield malonic semialdehyde, which undergoes oxidative decarboxylation to yield acetyl CoA.

Putrescine is a substrate for diamine oxidase, yielding γ -guanidobutyraldehyde. This is oxidized by aldehyde dehydrogenase to yield γ -guanidobutyrate, which is hydrolyzed to GABA (see section 5.5.2).

5.8.4 Hypusine

Hypusine is an amino acid formed by post-synthetic modification of a lysine residue in eukaryotic initiation factor 5A (eIF-5A), and apparently in no other proteins. eIF-5A is highly conserved and is essential for all organisms – from archaea to mammals – except the eubacteria. The formation of hypusine is essential to its function. As shown in Figure 5.16, it is formed by transfer of the aminobutyl moiety of spermidine onto the ϵ -amino group of a lysine

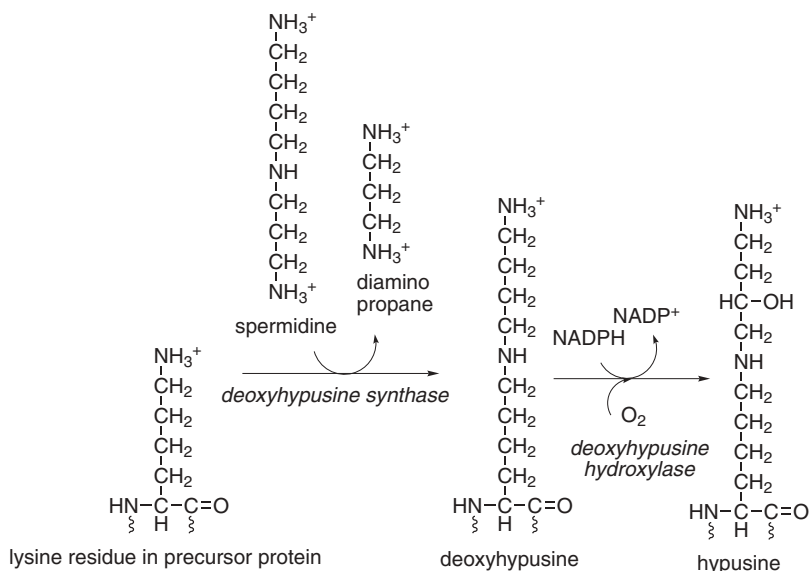


Figure 5.16 Synthesis of hypusine.

Deoxyhypusine synthase EC 2.5.1.46, deoxyhypusine hydroxylase EC 1.14.99.29.

residue in the precursor protein to yield deoxyhypusine, followed by hydroxylation to hypusine (Wolff *et al.*, 2007).

The reaction of deoxyhypusine synthase involves dehydrogenation of spermidine to dehydrospermidine, followed by cleavage of the 4-aminobutyl moiety initially onto the ϵ -amino group of a lysine residue at the active site of the enzyme, then onto the lysine residue in eIF-5A. This forms an imine that is reduced to a deoxyhypusine residue by the enzyme-bound NADH (Park, 2006).

5.9 Arginine, citrulline and ornithine

Arginine is a conditionally essential amino acid for most mammals, and absolutely essential for carnivores, birds and some aquatic animals. Catabolic conditions such as sepsis, injury and cancer cachexia lead to arginine depletion, so that the requirement is greater than the capacity for its synthesis, because of the synthesis of nitric oxide in response to inflammatory cytokines (section 5.9.3).

Almost half of the dietary arginine is hydrolyzed in the small intestinal mucosa to ornithine, which is converted to citrulline and exported to the kidneys for arginine synthesis. This serves to protect dietary arginine against uptake into the liver, where it would be a substrate for arginase and would serve only to increase urea synthesis (section 1.6.2.1). There is no net synthesis of arginine in the liver because of the high activity of arginase, and the hepatic concentration of arginine is lower than that of other amino acids. Young infants have a limited capacity for the synthesis of citrulline as a precursor for arginine synthesis, so that a dietary source of arginine is essential.

In addition to its role in protein synthesis and the urea cycle (section 1.6.2.1), arginine is the precursor for the synthesis of nitric oxide (section 5.9.3), ornithine for the synthesis of putrescine and the polyamines (section 5.8) and creatinine (section 5.9.7). It can also undergo decarboxylation to agmatine (section 5.9.4). Arginine is a secretagogue for growth hormone, insulin, insulin-like growth factor and prolactin. It also stimulates the immune system by increasing the activity of T-cells and natural killer cells.

Arginyl-tRNA has a role in protein catabolism. Arginyltransferase catalyzes the transfer of arginine from arginyl-tRNA onto the amino-terminal aspartate, glutamate or cysteine of a protein. This targets the protein for ubiquitination and catabolism by the proteasome (see section 2.1.5.2). The arginyl-tRNA synthetase involved in this reaction is distinct from that involved in charging tRNA^{Arg} for ribosomal protein synthesis (Ciechanover & Schwartz, 1989; Morris, 2009; Tasaki & Kwon, 2007).

Arginine is the major storage form of nitrogen in plants, and seed proteins are especially rich in arginine. Conifer roots are able to take up not only

inorganic nitrogen but also organic nitrogen, as arginine and glutamate. There is evidence that arginine is synthesized by mycorrhizal fungi, then taken up into roots, where it catabolized to yield ammonium, which is then fixed by formation of glutamine and glutamate (section 1.3.2).

Similarly, in seeds, where arginine accounts for half of the stored nitrogen, it is catabolized on germination by the actions of arginase to yield ornithine and urea (section 1.6.2.1), then urease to yield ammonium, which is again fixed by formation of glutamine and glutamate (Canovas *et al.*, 2007).

5.9.1 Arginine biosynthesis

In microorganisms, plants and non-ureotelic animals, ornithine, as the precursor for arginine synthesis, is synthesized from *N*-acetylglutamate, as shown in Figure 5.17. In ureotelic animals, arginine is synthesized from citrulline (section 5.9.1.1).

In prokaryotes, there are two routes for synthesis of *N*-acetylglutamate: the acetyl group may be transferred from acetyl CoA, catalyzed by *N*-acetylglutamate synthase; or from *N*-acetylornithine, an intermediate in the pathway, catalyzed by ornithine acetyltransferase. Most organisms use mainly ornithine acetyltransferase, with *N*-acetylglutamate synthase fulfilling an anaplerotic role to replace *N*-acetylglutamate that is lost by catabolism. Other organisms lack ornithine acetyltransferase and are reliant on the energetically less efficient pathway of synthesis from acetyl CoA. In these organisms, *N*-acetylglutamate synthase is subject to feedback inhibition by arginine.

In organisms that use mainly the ornithine acetyltransferase pathway, both *N*-acetylglutamate synthase and *N*-acetylglutamate kinase are inhibited by arginine. In plants, the main regulatory point in arginine synthesis is *N*-acetyl glutamate kinase, which is inhibited by arginine and activated by *N*-acetylglutamate. In some microorganisms, *N*-acetylornithine is not deacetylated, but it reacts with carbamoyl phosphate to form *N*-acetylcitrulline, which is then deacetylated (Caldovic & Tuchman, 2003; Forde & Lea, 2007; Xu, Y *et al.*, 2007).

When fixed nitrogen is abundant, photosynthetic microorganisms and plants store nitrogen as arginine. Under these conditions, the feedback inhibition of *N*-acetylglutamate kinase by arginine is relieved. This is signalled by the PII proteins that are widely distributed in bacteria and plants, which act as sensors of energy, carbon and nitrogen availability via ATP, ADP and 2-oxoglutarate. The effectors bind to the PII protein cooperatively, causing a conformational change that allows the protein to bind to the target enzymes, transcription factors and transport proteins, and regulate their activity (Llacer *et al.*, 2008; Radchenko & Merrick, 2011).

Under conditions of drought and strong light, the wild watermelon (*Citrullus lanatus*, a desert plant) accumulates a high concentration of citrulline (up

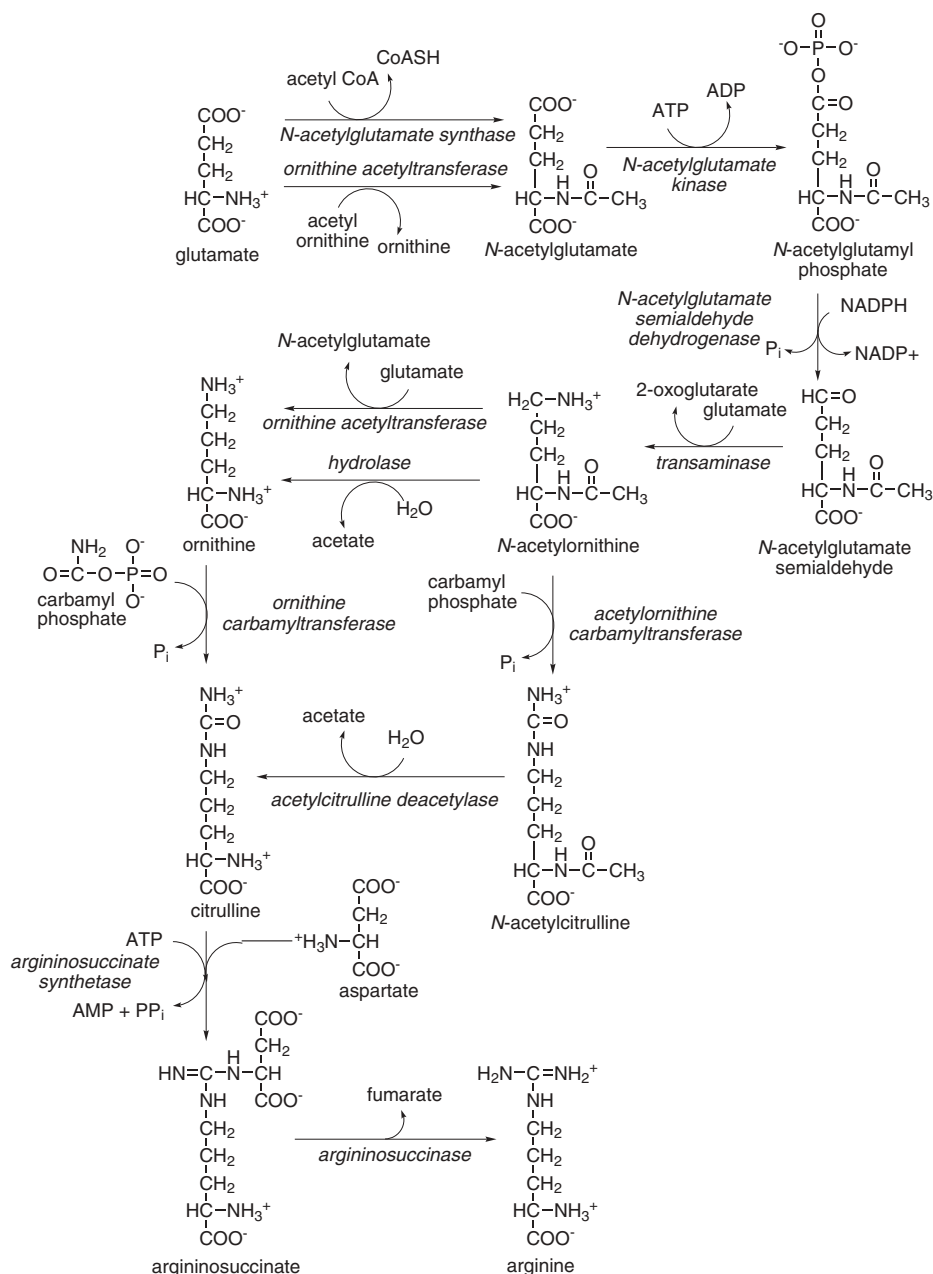


Figure 5.17 Arginine synthesis from glutamate.

N-acetylglutamate synthase EC 2.3.1.1, ornithine acetyltransferase EC 2.3.1.35, *N*-acetylglutamate kinase EC 2.7.2.8, *N*-acetylglutamate semialdehyde dehydrogenase (*N*-acetyl γ -glutamyl reductase) EC 1.2.1.38, *N*-acetylglutamate semialdehyde transaminase EC 2.6.1.11, acetyl ornithine deacetylase EC 3.5.1.16, ornithine carbamoyltransferase EC 2.1.3.3, argininosuccinate synthetase EC 6.3.4.5, argininosuccinase EC 4.3.2.1, acetylornithine carbamoyltransferase EC 2.1.3.9, acetylcitrulline deacetylase EC 3.5.1.16.

to 30 mmol/l) and also arginine (up to 7 mmol/l) in the leaves, synthesized via the *N*-acetylglutamate pathway. Ornithine acts as a hydroxyl radical scavenger. Ornithine acetyltransferase in this plant is unusually thermostable and retains activity up to about 70°C. Unlike the enzyme in most plants and microorganisms, it is not inhibited by arginine or citrulline (Takahara *et al.*, 2005).

In mammals, *N*-acetylglutamate is not a precursor for arginine synthesis but is an essential cofactor for the activity of mitochondrial carbamoyl phosphate synthetase, which is more or less completely inactive in the absence of *N*-acetylglutamate (section 1.6.2.1).

5.9.1.1 The role of citrulline in arginine biosynthesis in mammals In mammals, citrulline can arise from three sources: as a result of the synthesis of nitric oxide from arginine (Figure 5.19); by the action of ornithine carbamoyltransferase (Figure 5.17); or the catabolism of citrullinated proteins (section 5.9.6.). Most tissues that synthesize nitric oxide have sufficient activity of argininosuccinate synthetase and argininosuccinate lyase to allow them to recycle the citrulline formed in the nitric oxide synthase reaction back to arginine, although they do not take up citrulline from the circulation. The liver synthesizes citrulline as an intermediate in the urea synthesis cycle (section 1.6.2.1; Curis *et al.*, 2005).

The small intestinal mucosa synthesizes citrulline from dietary arginine and from both dietary glutamine (in the fed state) and plasma glutamine (in the fasting state). The citrulline is then exported and taken up by the kidney, which synthesizes arginine in the proximal renal tubules, releasing it into the circulation. Argininosuccinate synthase and argininosuccinase are concentrated mainly in the proximal convoluted tubule, which is also the site for re-absorption of most of the amino acids in the glomerular filtrate, and it is likely that citrulline is delivered to the kidneys for arginine synthesis by way of glomerular filtration and re-absorption in the renal tubules. The rate of arginine synthesis in the kidney is regulated by the delivery of citrulline rather than the dietary intake of arginine. Renal synthesis is some 2 g/day, compared with a dietary intake of 4–5 g (Brosnan & Brosnan, 2004; Curis *et al.*, 2005; Wu *et al.*, 2009).

In adults, the renal synthesis of arginine is sufficient to meet the need for nitrogen balance, but this is not so in infants, for whom arginine is at least partially a dietary essential amino acid. Other tissues that can synthesize arginine from intracellular citrulline do not have a citrulline transporter and cannot take up citrulline from the circulation.

The inter-organ cycle of arginine catabolism in the intestinal mucosa and synthesis of citrulline, which is exported to the kidneys for arginine synthesis (Figure 5.17) serves to protect dietary arginine against uptake into the liver, where it would be a substrate for arginase action and would increase overall

urea synthesis (section 1.6.2.1). The liver does not take up citrulline from the portal blood. The synthesis of citrulline in the small intestine is limiting for renal synthesis of arginine, and there is increased intestinal synthesis of citrulline in response to a low-protein diet (Curis *et al.*, 2005; Moinard & Cynober, 2007).

5.9.2 Arginine catabolism in microorganisms

There are four pathways of arginine catabolism in microorganisms (Abdelal, 1979). Arginase catalyzes hydrolysis, liberating urea and yielding ornithine, which undergoes transamination to Δ^1 -pyrroline-5-carboxylate, a substrate for synthesis of polyamines, glutamate and proline (Figure 5.14). The urea may either be hydrolyzed by urease to yield carbon dioxide and ammonium, or it may be carboxylated to allophanate, which is then hydrolyzed to yield carbon dioxide and ammonium. Ornithine can also be formed by transamidination, in which the guanido group is transferred to an acceptor.

The arginine deiminase pathway (Figure 5.18) catalyzes the hydrolysis of arginine to ornithine with the release of carbon dioxide and ammonium, coupled to the phosphorylation of ADP to ATP. Three enzymes are involved. Arginine deiminase catalyzes hydrolysis to citrulline and ammonium. Ornithine carbamoyltransferase catalyzes the reverse of the reaction in arginine synthesis (Figure 5.17), yielding ornithine and carbamoyl phosphate. Carbamate kinase catalyzes the reverse of the carbamoyl phosphate synthase reaction, phosphorylation of ADP to ATP and release of carbon dioxide and ammonium.

Carbamoyl phosphate synthase is essentially irreversible; by contrast, the reaction of carbamate kinase is reversible. While its main role is in the

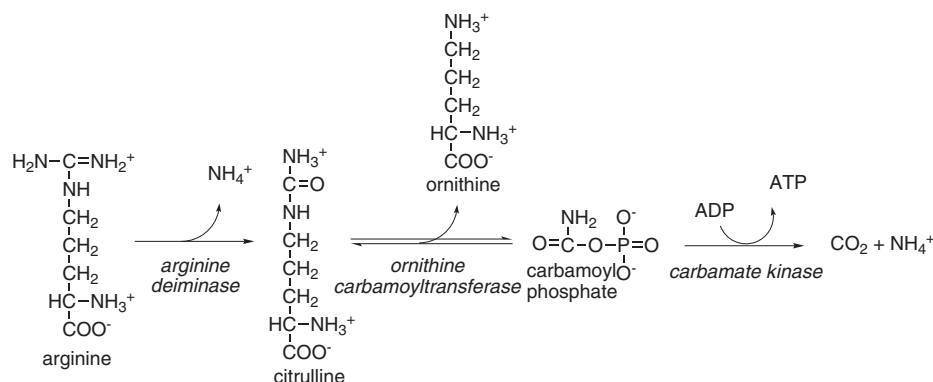


Figure 5.18 Arginine catabolism through arginine deiminase.

Arginine deiminase EC 3.5.3.6, ornithine carbamoyltransferase EC 2.1.3.3, carbamate kinase EC 2.7.2.2.

arginine deiminase pathway, linked to phosphorylation of ADP to ATP, it can be the source of carbamoyl phosphate for synthesis of arginine and pyrimidines in some bacteria (Rubio & Cervera, 1995).

The dominant lactic acid bacterium in many fermented meat products is *Lactobacillus sakei*; there is little or no carbohydrate in meat, and the arginine deiminase pathway provides a major pathway for carbon and nitrogen metabolism and ATP formation.

The reaction of arginine deiminase is essentially irreversible, and the equilibrium of the catabolic carbamate kinase is strongly towards ATP synthesis. Most bacteria excrete ornithine into the medium in exchange for uptake of arginine, while some metabolize ornithine further. The production of ammonia in this pathway provides some degree of acid tolerance to the bacteria, which is important in oral *Streptococcus* spp. involved in dental plaque and caries. Some have proton pump ATPases to resist acid, while others use the arginine deiminase pathway to produce ammonia (Quivey *et al.*, 2001).

Depletion of arginine by use of recombinant arginine deiminase is a potential therapy for cancer, especially melanomas and hepatocellular carcinomas, which have a low capacity for arginine synthesis (Ni *et al.*, 2008).

Arginine may also be decarboxylated to agmatine (see section 5.9.4), which may be hydrolyzed to urea and putrescine, or may undergo deimination and follow the same reaction sequence as described above for the arginine deiminase pathway.

5.9.3 Nitric oxide

Nitric oxide, which is synthesized from arginine, was first identified as the endothelium-derived relaxation factor. Nitric oxide synthesized in, and released from, vascular endothelium inhibits smooth muscle contraction and platelet aggregation, thus regulating blood flow. It has subsequently been identified as a signalling molecule in the central nervous system and elsewhere, and also as a key cytotoxic agent in the immune system (Jacklet, 1997). Nitrosylation of proteins is a short-term post-synthetic modification regulating enzyme activity.

Nitric oxide is also synthesized by invertebrates and bacteria. Bacterial nitric oxide synthases show many similarities to the mammalian enzymes (section 5.9.3.1), albeit with different cofactors. In bacteria, nitric oxide has roles in toxin synthesis, protection against oxidative stress and regulation of recovery from radiation damage (Crane *et al.*, 2010; Gkaliagkousi & Ferro, 2011; Moroz & Kohn, 2011).

The highest concentration of nitric oxide in the mammalian body is found in neurons. It has a role in the central nervous system as a neuromodulator, and in the autonomic nervous system as a neurotransmitter. In the central nervous system, it is involved in control of synaptogenesis, neuronal plasticity,

neuroendocrine secretion and memory function. Excessive release of nitric oxide in response to over-excitation of glutamatergic neurons accounts for excitotoxic neuronal death. It is involved in the neural control of smooth muscle, including peristalsis and penile erection.

In skeletal muscle, nitric oxide is involved in the control of contractility, exercise-induced glucose uptake and the vascular perfusion of skeletal muscle. Inhibition of nitric oxide synthesis blunts the exercise-induced increase in glucose uptake into skeletal muscle, but it does not affect insulin-stimulated glucose uptake. The expression of nitric oxide synthase in muscle is induced by exercise training, suggesting a possible role for nitric oxide in the improvement in glucose tolerance seen in response to exercise (Balon, 1998; Bredt, 1999). In endocrine glands, nitric oxide regulates the synthesis of steroid hormones (Ducsay & Myers, 2011).

Nitric oxide also has a role in whole body energy balance. It triggers mitochondriogenesis in brown adipose tissue and other cells, and cold-induced mitochondriogenesis is impaired in nitric oxide synthase knockout mice, which have a reduced metabolic rate and accelerated weight gain. It stimulates glucose uptake and the oxidation of glucose and fatty acids in skeletal muscle, heart, liver and adipose tissue, inhibits the synthesis of glucose, glycogen and fat and increases lipolysis in adipose tissue. Inhibition of nitric oxide synthesis in rats leads to hyperlipidaemia and fat accretion, and provision of dietary supplements of arginine results in reduced fat mass in diabetic obese rats (Jobgen *et al.*, 2006; Nisoli *et al.*, 2003; Otasevic *et al.*, 2011).

Nitric oxide is synthesized in activated macrophages as a cytotoxic agent against engulfed microorganisms and parasites, and nitric oxide synthesis is a prerequisite for cytokine signalling and function. In the absence of nitric oxide, interleukin-12 does not stimulate natural killer cells for cytotoxicity or interferon- γ secretion (Diefenbach *et al.*, 1999).

Nitric oxide has three mechanisms of action. Extracellularly it activates G-protein coupled receptors. Intracellularly, it activates guanyl cyclase by binding to haem at the active site, so increasing the production of cGMP, and it also *S*-nitrosylates cysteine residues in target proteins.

5.9.3.1 Nitric oxide synthase As shown in Figure 5.19, nitric oxide synthesis involves NADPH-dependent oxidation of arginine to yield *N*-hydroxyarginine. *N*-Hydroxyarginine only dissociates from the enzyme slowly, and it normally remains enzyme-bound and undergoes a second oxidation to citrulline, releasing nitric oxide. Any *N*-hydroxyarginine that does dissociate from the enzyme inhibits arginase, so making more arginine available for nitric oxide synthesis.

Nitric oxide synthase is a homodimer, and the two subunits are tightly associated and have tightly bound FAD, FMN, haem and tetrahydrobiopterin. The enzyme has two catalytic domains: a reductase domain that reacts with

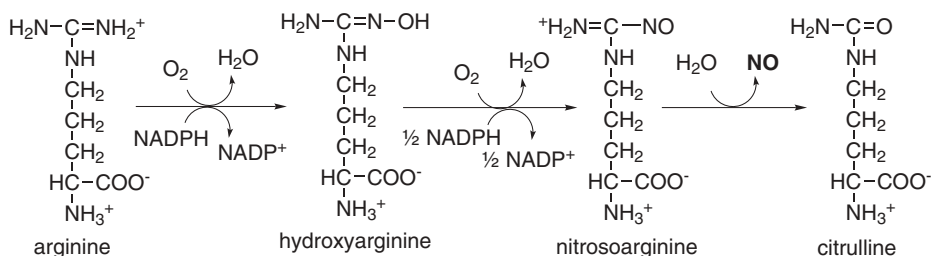


Figure 5.19 Synthesis of nitric oxide.

Nitric oxide synthase EC 1.14.13.39.

NADPH, and an oxygenase domain. The role of the flavins is to couple the two-electron reaction of NADPH with the one-electron reduction of haem. The electron flow in the reductase domain is from NADPH to FAD to FMN to haem in the oxygenase domain in the opposite subunit, permitting the binding and activation of oxygen to the haem.

Tetrahydrobiopterin is essential for the first hydroxylation reaction, but its role is unclear. There is no evidence that it undergoes oxidation to dihydrobiopterin, as occurs in aromatic amino acid hydroxylases (section 9.2.3), but there is evidence for the formation of a biopterin radical. It may function in electron transfer between FMN and haem (Alderton *et al.*, 2001; Luo & Zhu, 2011; Santolini, 2011; Stuehr, 2004; Stuehr *et al.*, 2004).

At the interface between the monomers there is a zinc atom that is tetrahedrally coordinated by two cysteine residues from each subunit. Tetrahydrobiopterin, haem and arginine all promote the formation of the active dimer, but there is a considerable surface for interaction between the monomer subunits. There is no evidence that once the dimer has been formed, it dissociates.

There are three separate mammalian nitric oxide synthases, each encoded by a different gene and with some 50 per cent homology between their sequences. Between species, there is 80–90 per cent homology of the same type of nitric oxide synthase (Alderton *et al.*, 2001):

- Neuronal nitric oxide synthase (nNOS) was originally described in neurons, but splice variants are expressed in a wide variety of cells and tissues. It is a soluble enzyme (Brenman *et al.*, 1997; Zhou & Zhu, 2009).
- Endothelial nitric oxide synthase (eNOS) is mainly expressed in endothelial cells. It is important for maintaining blood flow through organs by regulating vascular tone, modulating the interactions of platelets and leukocytes with the vascular endothelium and limiting neointimal hyperplasia by inducing apoptosis of vascular smooth muscle cells. It is a

membrane-bound enzyme. Although it was originally considered to be expressed constitutively, expression is actually regulated both transcriptionally and post-transcriptionally (Rafikov *et al.*, 2011).

- Inducible nitric oxide synthase (iNOS) was originally described in macrophages, but it is also inducible in a variety of other tissues. It is a soluble enzyme, important in inflammation and as a source of cytotoxic nitric oxide. In the central nervous system, it is expressed constitutively in glial cells and its expression is upregulated in response to immunological challenge. The expression of iNOS is regulated both transcriptionally and post-transcriptionally, with different patterns of regulation in different tissues. In all cases, activation of transcription factors NF κ B and STAT-1 α is essential for activation of the iNOS promoter. Post-transcriptional regulation is through a variety of RNA-binding proteins and non-coding RNA species (Amitai, 2010; Pautz *et al.*, 2010).

eNOS and nNOS are low-output enzymes concerned with cell signalling, whereas iNOS is a high-output enzyme, producing cytotoxic amounts of nitric oxide. eNOS and nNOS are generally regarded as being constitutive enzymes, although their expression is modulated by a variety of hormonal, physical and other stimuli. iNOS is absent from quiescent macrophages, but there is high activity in macrophages, liver and spleen a few hours after exposure to bacterial endotoxin, lipopolysaccharide or inflammatory cytokines. Argininosuccinate synthetase and argininosuccinase are co-induced with iNOS, so permitting recycling of citrulline to arginine. A later response is induction of arginase to regulate the supply of arginine for nitric oxide synthesis (Mori & Gotoh, 2004; Mori *et al.*, 1998; Wu & Meininger, 2002).

eNOS and nNOS are dependent on calcium-calmodulin for activity. These two isoenzymes have a 40–50 amino acid insertion, known as the auto-inhibitory loop, in the FMN binding domain that is absent from iNOS. At low concentrations of calcium, this region destabilizes calmodulin binding and, in the absence of calmodulin, electron flow from FMN to haem is inhibited. iNOS binds calmodulin regardless of the concentration of calcium (Alderton *et al.*, 2001).

Monomethylarginine and, more potently, asymmetrical dimethylarginine, arising from the catabolism of proteins containing methylarginine (section 5.9.5), inhibit nitric oxide synthase. Symmetrical dimethylarginine does not inhibit nitric oxide synthase, but it does compete with arginine for tissue uptake.

Inhibition of nitric oxide synthase by asymmetrical dimethylarginine explains the ‘arginine paradox’. While plasma and tissue concentrations of arginine are high enough to saturate the enzyme (the plasma concentration of arginine is 90–110 μ mol/l, while the K_m of nitric oxide synthase is 3 μ mol/l),

supplements of arginine increase nitric oxide production and function. The additional arginine out-competes asymmetrical dimethylarginine, both for transport into the cell and also as an inhibitor of the enzyme. In kidney and liver failure, there is an accumulation of asymmetrical dimethylarginine, leading to decreased nitric oxide synthesis. This is a factor in multiple organ failure and mortality in patients in intensive care (Arrigoni *et al.*, 2010; Nijveldt *et al.*, 2004; Siroen *et al.*, 2006).

eNOS is phosphorylated in response to fluid shear stress in the blood vessel, and this results in an increase in calcium-independent activity, with increased electron flow through the reductase domain. By contrast, nNOS is phosphorylated by a calcium-calmodulin-dependent kinase, resulting in decreased activity, so that calcium has two opposite effects on nNOS activity. The phosphorylation serves to modulate the activation caused by binding of calcium-calmodulin to the enzyme. The enzyme is inhibited by nitrosylation under basal conditions and, in response to stimulation by agonists, there is rapid denitrosylation and increased activity. This is followed by re-nitrosylation as nitric oxide accumulates. eNOS also undergoes translocation between the plasma membrane (where it is active) and intracellular membranes (where it is inactive) as a result of reversible palmitoylation and depalmitoylation of cysteine residues (Alderton *et al.*, 2001; Erwin *et al.*, 2006).

iNOS and cyclo-oxygenase 2 are co-induced by inflammatory cytokines, with the same time course, and they immunoprecipitate together in response to antibodies against either enzyme. The enzyme-bound iNOS nitrosylates – and so activates – cyclo-oxygenase 2, increasing the synthesis of eicosanoids, but free nitric oxide does not nitrosylate cyclo-oxygenase 2. There is no interaction between iNOS and cyclo-oxygenase 1. Cytosolic phospholipase A2 α is rate-limiting for the provision of arachidonic acid for the synthesis of eicosanoids, including prostaglandin E2, which is a key mediator of inflammation. The enzyme is activated by *S*-nitrosylation by nitric oxide, so that the induction of iNOS leads to increased phospholipase activity, increased release of arachidonic acid and, hence, increased availability of substrate for cyclo-oxygenase 2.

5.9.3.2 Arginase and the control of arginine availability for nitric oxide synthesis or polyamine synthesis The citrulline formed by nitric oxide synthase is recycled back to arginine by the actions of argininosuccinate synthase and argininosuccinase, and these two enzymes are co-induced with iNOS in some cells. Under some conditions, arginase is co-induced with iNOS to regulate the availability of arginine for nitric oxide synthesis. In colon epithelial cells, polyamines (section 5.8) are essential for cell proliferation, and nitric oxide has an antiproliferative action. Imbalance between nitric oxide and polyamine synthesis is a factor in colon cancer (Blachier *et al.*, 2011; Mori, 2007).

Arginase is a trimer of three identical subunits, each of which has a catalytic site containing two Mn^{++} ions, which are essential for activity. There are two mammalian arginases, encoded by different genes:

- Type I is expressed mainly in the liver; it is a cytosolic enzyme and its role in the liver is in the urea cycle (section 1.6.2.1).
- Type II is expressed in many tissues; it is a mitochondrial enzyme and its main role is in regulating the availability of arginine for synthesis of nitric oxide.

Macrophages express type II arginase constitutively, but type I is induced by cytokines and is involved in regulation of the availability of ornithine for polyamine and proline synthesis (see sections 5.7.1 and 5.8). In blood vessels, arginase in the endothelium regulates nitric oxide synthesis, but in vascular smooth muscle it acts to provide a source of ornithine for both polyamine synthesis for cell proliferation and proline for collagen synthesis (Ash, 2004; Grillo & Colombatto, 2004b; Mori, 2007; Morris, 2009; Wu *et al.*, 2009).

In the lactating mammary gland, there is an increase in the activity of arginase to provide proline in the milk. The activity of arginase in the myometrium increases some 25-fold during pregnancy to supply the rapidly growing foetus with polyamines, and arginase is also increased in many tumours.

In the presence of high concentrations of nitric oxide, type I arginase is nitrosylated on a specific cysteine residue, leading to a six-fold decrease in K_m , so that less arginine is available for nitric oxide synthesis. By contrast, *N*-hydroxyarginine released from nitric oxide synthase is an inhibitor of arginase, and thus serves to increase the availability of arginine for nitric oxide synthesis. Type II arginase does not undergo nitrosylation, since it lacks the relevant cysteine residue.

Knockout mice lacking type I arginase die in the perinatal period from hyperammonaemia, since they lack a functional urea cycle (section 1.6.2.1). Those lacking type II arginase are viable but have elevated plasma concentrations of arginine, increased polyamine synthesis and changes in vasorelaxation as a result of the increased availability of arginine for polyamine and nitric oxide synthesis.

Patients with sickle cell anaemia have a low plasma concentration of arginine, a low ratio of arginine to ornithine and a high activity of arginase in plasma, as a result of haemolysis of red blood cells. This results in low availability of arginine for nitric oxide synthesis. At the same time, free haemoglobin in plasma acts as a trap for nitric oxide, so that there is a considerable loss of nitric oxide function, and these patients are at considerably increased risk of pulmonary hypertension (Morris, 2007).

The early response to infection or an inflammatory insult is induction of nitric oxide synthase, which has an antibacterial and cytostatic action. High concentrations of nitric oxide lead to nitrosylation and, hence, inhibition of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, reducing polyamine synthesis. Aldehyde dehydrogenase is also inhibited by nitrosylation. A later response is induction of arginase, so reducing nitric oxide synthesis and providing ornithine for polyamine synthesis.

This switch is regulated by both inflammatory cytokines (which have opposite effects on the expression of nitric oxide synthase and arginase) and arginine metabolites (especially agmatine and its oxidation product, γ -guanidobutyraldehyde – see section 5.9.4 below). Agmatine induces antizyme and spermidine/spermine *N*-acetyltransferase, so reducing polyamine synthesis and increasing catabolism (section 5.8). The aldehyde product of agmatine metabolism, γ -guanidobutyraldehyde, is an inhibitor of nitric oxide synthase, and the initiation of the switch is the induction of amine oxidase, which catalyzes the oxidation of agmatine to γ -guanidobutyraldehyde. This relieves the inhibition of polyamine synthesis by agmatine and provides γ -guanidobutyraldehyde to inhibit nitric oxide synthase. Nitric oxide also inhibits the aldehyde dehydrogenase that would otherwise catalyze oxidation of γ -guanidobutyraldehyde (Satriano, 2004).

5.9.4 Agmatine

Decarboxylation to agmatine has long been known to be a significant fate of arginine in plants and bacteria, and also as an alternative source of putrescine for polyamine synthesis (section 5.8). Arginine decarboxylase is a mitochondrial enzyme and is inhibited by calcium ions and polyamines. Low concentrations of agmatine also occur in animal tissues and, while agmatine may arise from intestinal bacterial decarboxylation of arginine, it is also synthesized endogenously. As discussed in section 5.9.3.2, agmatine has a role in controlling the direction of arginine metabolism towards either nitric oxide or polyamine synthesis. In the central nervous system, it is packaged into synaptic vesicles and is released on neuronal depolarization, having a neuromodulatory action (Mella *et al.*, 2010).

Agmatine, like nitric oxide, causes vasodilatation, and increases the glomerular filtration rate. Infusion of arginine leads to increased glomerular filtration even when nitric oxide synthase is inhibited, while inhibition of arginine decarboxylase abolishes the effect. Infusion of agmatine also increases the glomerular filtration rate. These actions of agmatine may be mediated either by activation of the imidazoline receptor or by an increase in intracellular calcium concentration. Agmatine increases intracellular calcium by stimulating the ryanodine receptor/cyclic ADP-ribose system.

Agmatine acts synergistically with constitutive nitric oxide synthase to increase vasodilatation. Following cytokine simulation, agmatine aldehyde, the result of oxidation of agmatine by diamine oxidase, suppresses inducible nitric oxide synthase, indicating a protective role of agmatine in septic shock and acting to down-regulate inflammatory pathways (Blantz *et al.*, 2000; Satriano *et al.*, 2001).

There are two possible metabolic fates for agmatine: hydrolysis catalyzed by agmatinase to yield putrescine as a substrate for polyamine synthesis; or oxidation by diamine oxidase to yield γ -butyramide (agmatine aldehyde), then γ -guanidobutyraldehyde (Figure 5.8), and finally γ -guanidobutyrate. In hepatocytes, the major route of agmatine metabolism is oxidation but, in the central nervous system, the major fate is hydrolysis catalyzed by agmatinase (Grillo & Colombatto, 2004a; Halaris & Plietz, 2007; Reis & Regunathan, 2000).

5.9.5 Post-synthetic methylation of arginine in proteins

Methylation of arginine is an apparently irreversible post-synthetic modification of a number of proteins, including histones and transcription factors. Protein arginine methylation is involved in protein-protein interactions, signal transduction and histone function. It is dysregulated in chronic renal, vascular and pulmonary disease, and measurement of methylarginines released by proteolysis of modified proteins may provide a biomarker of disease.

There are two families of peptidyl arginine methyltransferases, both of which use *S*-adenosylmethionine as a methyl donor (section 6.3.2). Type I enzymes catalyze synthesis of mono-methylarginine, then asymmetrical dimethylarginine, in which both methyl groups are attached to the same nitrogen. Type II enzymes catalyze synthesis of mono-methylarginine then symmetrical dimethylarginine, in which one methyl group is attached to each nitrogen (Figure 5.20).

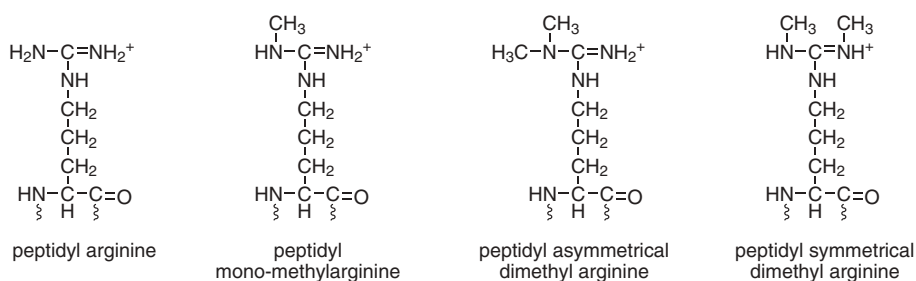


Figure 5.20 Methylarginine.

There is no evidence that free arginine is methylated, so the only source of free monomethyl, and symmetrical and asymmetrical dimethylarginine, is the catabolism of methylated proteins. Much methylarginine is excreted in the urine. Monomethyl arginine and asymmetrical dimethylarginine, but not symmetrical dimethylarginine, are substrates for dimethylarginine dimethylaminohydrolase, leading to the formation of citrulline and either methylamine or dimethylamine. Asymmetrical dimethylarginine is also a substrate for transamination, catalyzed by alanine-glyoxylate transaminase, leading to the formation of 2-oxovaleric acid.

As discussed in section 5.9.3.1, asymmetrical dimethylarginine is a potent physiological inhibitor of nitric oxide synthase, and cells take it up from the bloodstream using cationic amino acid transporters. There are two isoenzymes of dimethylarginine dimethylaminohydrolase: type 1 is found in tissues that express nNOS; type 2 is found in tissues that express eNOS (Nijveldt *et al.*, 2004; Zakrzewicz & Eickelberg, 2009).

5.9.6 Post-synthetic formation of citrulline in proteins

A number of proteins, including histones, keratin-related proteins and myelin basic protein, contain citrulline, which is formed by hydrolysis of arginine residues, catalyzed by peptidyl arginine deimidases and liberating ammonium. Deimination of arginine in histones prevents methylation. Dimethylation of arginine prevents the action of the deimidase, but there is some evidence that monomethyl arginine residues are substrates for the deimidases, releasing methylamine and leaving a citrulline residue in the protein – although this activity has been challenged (Raijmakers *et al.*, 2007). Obviously, since deimination leaves citrulline rather than arginine in the protein, deamidation of monomethyl arginine does not really reverse the methylation, but it does prevent onwards methylation to dimethylarginine, so serving to control histone methylation (Cuthbert *et al.*, 2004; Denman, 2005; Wang *et al.*, 2004).

Citrullinated proteins are regarded as non-self, and they stimulate antibody production. Citrullinated myelin basic protein is probably the auto-antigen in multiple sclerosis, and autoantibodies against citrullinated peptides from filaggrin and fibrin are markers of rheumatoid arthritis (Thompson & Fast, 2006).

There are a number of enzymes that are structurally and functionally related to the peptidyl arginine deimidases. Arginine deimidase in prokaryotes acts on free arginine, the first step in the arginine deimidase pathway (section 5.9.2). Dimethylarginine dimethylaminohydrolase in bacteria and animals acts on free, but not peptide-incorporated, dimethylarginine, liberating methylamine and citrulline. While this does not reverse the methylation

of arginine in proteins, it does permit recycling of dimethylarginine after catabolism of methylated proteins (Thompson & Fast, 2006).

5.9.7 Creatine

Creatine phosphate is a phosphagen. It acts to transfer high-energy phosphate groups from the site of ATP synthesis in the mitochondrial matrix to the sites of ATP utilization in the cytosol, in tissues where simple exchange of ADP and ATP across the mitochondrial membrane is inadequate to meet changing demands for cytosolic ATP.

Although, overall, the energy consumption of the central nervous system is relatively constant, individual rapidly firing neurons show a large fluctuation in their energy needs. In fast-twitch skeletal muscle, brain and other tissues where there is a high and fluctuating demand for ATP, creatine phosphate has a second function, acting as a reserve of high-energy phosphate that can be transferred onto ADP to maintain muscle contraction and other activities during the first few seconds of intense activity, before there is a significant increase in glycolysis.

The buffer pool of creatine phosphate in muscle can maintain a more or less constant ATP:ADP ratio for several seconds of intense activity (Bessman & Carpenter, 1985; Wallimann *et al.*, 2011). Because of this, supplements of creatine have become popular as an ergogenic aid, to improve athletic performance. However, it is obviously likely only to be useful in short periods of intense activity. The majority of (mainly short-term) studies of creatine supplementation show that there is indeed, an increase in muscle creatine and creatine phosphate, with improved performance in short-term high-intensity exercise (less than 30 seconds), but there is little or no evidence that it is beneficial for performance in exercise lasting more than 150 seconds. The effects on short-term intense exercise are more marked in vegetarians, who have little or no dietary creatine and who have lower muscle concentrations of creatine than have omnivores (Brosnan & Brosnan, 2007; Kreider, 2003; Paddon-Jones *et al.*, 2004).

Creatine and creatine kinase are widely distributed not only in vertebrates but in a large number of invertebrates, including such primitive organisms as marine sponges. However, most invertebrates lack creatine kinase and utilize other guanido compounds, including guanidine, guanido-acetate (an intermediate in the synthesis of creatine – see Figure 5.22) and arginine as phosphagens (Wyss & Kaddurah-Daouk, 2000).

As shown in Figure 5.21, there are therefore two sites where creatine kinase acts: in the mitochondrion and in the cytosol (or the sarcolemma in muscle).

Four genes code for mammalian creatine kinase. There is a ubiquitous mitochondrial enzyme, separate brain-type and muscle-type cytosolic enzymes, and

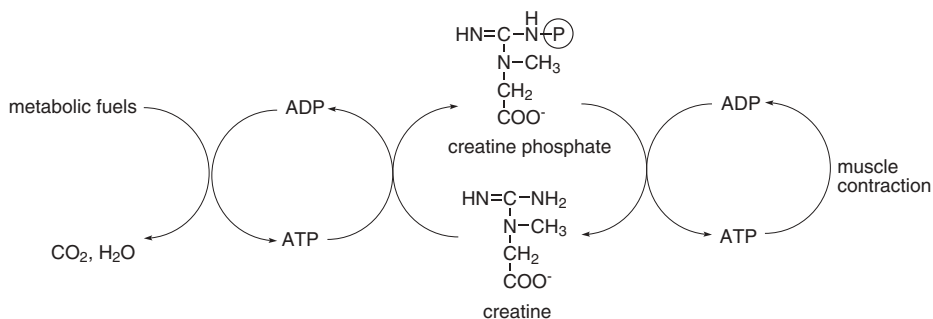


Figure 5.21 The role of creatine as a phosphagen.

a sarcomere-specific enzyme in muscle. The cytosolic enzyme is a dimer, consisting of two brain-type monomers in brain and smooth muscle, two muscle-type monomers in skeletal muscle and a heterodimer in heart muscle and other tissues.

The mitochondrial creatine kinase forms an octamer that spans the inter-membrane space and transports creatine phosphate into the cytosol and creatine into the mitochondrion. There is relatively little mitochondrial creatine kinase in fast twitch skeletal muscle but, in heart muscle, some 35 per cent of total creatine kinase activity is the mitochondrial enzyme (Wyss & Kaddurah-Daouk, 2000).

The mammalian creatine kinases have random reaction kinetics. Either creatine or ATP may bind first (or ADP and creatine phosphate in the reverse direction), and there is no evidence of the formation of a phosphorylated enzyme intermediate. Some of the crustacean arginine kinases similarly have random kinetics, while others have ordered binding and form a phosphorylated enzyme intermediate.

The pathway of creatine synthesis and degradation is shown in Figure 5.22. Some ten per cent of the total metabolic flux of arginine is for the synthesis of creatine (compared with about one per cent for nitric oxide synthesis – see section 5.9.3). Guanidoacetate is synthesized mainly in the kidneys, and to some extent also the pancreas, and is transported to the liver for methylation to creatine. Glycine amidinotransferase occurs in the liver of some species (e.g. cows, pigs, primates) but not in others (e.g. rat, mouse, cat, dog, rabbit). However, even in those species that have amidinotransferase activity in the liver, it is unlikely that there is any significant hepatic synthesis of guanidoacetate because, as discussed in section 5.9.1, there is little or no net synthesis of arginine in the liver.

The expression of glycine amidinotransferase is down-regulated by creatine so that, when supplements are taken or there is a high dietary intake, there will be less endogenous synthesis of creatine. There is also some evi-

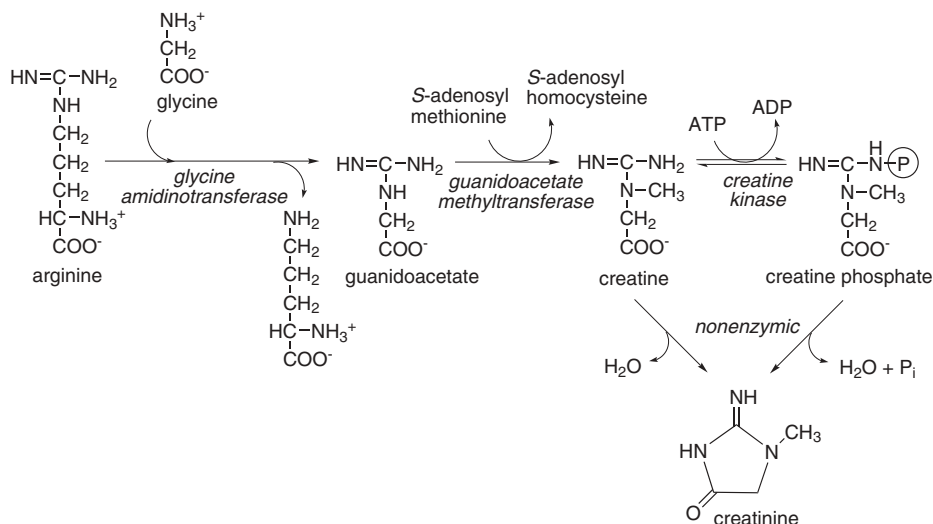


Figure 5.22 Synthesis and catabolism of creatine.

Glycine amidinotransferase (glycine guanidotransferase) EC 2.1.4.1, guanidoacetate methyltransferase EC 2.1.1.2, creatine kinase EC 2.7.3.2.

dence that expression of the enzyme is increased in folate deficiency, when the availability of methyl groups is limiting. The reaction of glycine amidinotransferase involves a large conformational change, with formation of amidinocysteine at the active site of the enzyme (Brosnan & Brosnan, 2007; Humm *et al.*, 1997a, 1997b; Wyss & Kaddurah-Daouk, 2000).

Methylation of guanidoacetate occurs mainly in the liver, with *S*-adenosylmethionine acting as the methyl donor (section 6.3.2), but other tissues also express the methyltransferase and may be capable of synthesizing creatine from guanidoacetate. Synthesis of creatine accounts for about three-quarters of all *S*-adenosylmethionine-dependent methylation reactions.

There is no enzyme-catalyzed catabolism of creatine or creatine phosphate. Both undergo non-enzymic dehydration and cyclization to creatinine, which is metabolically useless and is excreted in the urine. This means that there is a considerable turnover of creatine – about 1.7 per cent of the total body pool is lost each day. A 70 kg man excretes some 16 mmol of creatinine per day, and a 70 kg woman some 10 mmol. By contrast, there is very little excretion of creatine, except under conditions of muscle wasting disease or the normal post-menstrual breakdown of the myometrium in women. Growing children normally show moderate creatinuria as a result of the plasticity of muscle tissue during growth.

Because the formation of creatinine is non-enzymic, the rate of formation depends on the total amount of creatine plus creatine phosphate in the body.

The gender difference in creatinine excretion simply reflects the differences in body composition; women have more adipose tissue and less muscle than men of the same body weight. For any one individual, urinary excretion of creatinine is relatively constant from day to day, and it is common to express urinary excretion of various metabolites per mol of creatinine when a 24 hour urine sample is not available. Creatinine excretion – or, more correctly, creatinine clearance (the amount excreted over 24 hours divided by the plasma concentration) – is used as an index of kidney function, and specifically glomerular filtration rate. Creatinine is actively secreted by the renal tubules, and therefore measurement of creatinine clearance overestimates glomerular filtration rate compared with measurement of inulin clearance, because there is no active secretion of inulin in the kidneys.

There are three inborn errors of metabolism affecting creatine metabolism: deficiency of glycine amidinotransferase and guanidoacetate methyltransferase (both of which are autosomal recessive conditions) and deficiency of the cell membrane creatine transporter (which is an X-linked condition). All three conditions are associated with developmental delay and mental retardation as a result of low concentrations of creatine and creatine phosphate in the central nervous system. Methyltransferase deficiency is also associated with intractable seizures, as a result of accumulation of guanidoacetate in the central nervous system. Administration of creatine supplements is successful in controlling the enzyme defects but not the transporter defect, since creatine cannot enter tissues (Schulze, 2003).

Further reading

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

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6

Amino Acids Synthesized from Aspartate: Lysine, Methionine (and Cysteine), Threonine and Isoleucine

Like glutamate (see Chapter 5), aspartate provides an interface between amino acid and carbohydrate metabolism. It is synthesized by transamination of oxaloacetate, the citric acid cycle intermediate that is the main substrate for gluconeogenesis in the fasting state. In mammals, aspartate is one of the three amino acids that can be considered to be completely non-essential (section 2.2).

Oxaloacetate can arise from metabolism of amino acids to citric acid cycle intermediates in the mitochondrion (Figure 2.2) or, in the cytosol, the reaction of pyruvate carboxylase. Aspartate is important in the transfer of reducing equivalents from the cytosol into the mitochondrion – the malate-aspartate shuttle shown in Figure 3.6. As discussed in section 3.2.3, D-aspartate is a neurotransmitter.

In plants, asparagine, synthesized by amidotransfer from glutamine, is the main transport and storage form of fixed nitrogen, and the synthesis of asparagine competes with the synthesis of lysine, methionine, threonine and isoleucine for available aspartate. Asparagine synthase has a rapid turnover, and plant tissues also contain a high activity of asparaginase as a means of liberating ammonium from this storage compound. In response to various stresses, there is increased synthesis and accumulation of asparagine.

Mammalian asparagine synthase (section 1.3.2.5) requires ATP, and the reaction involves the intermediate formation of β -aspartyl-AMP, releasing

AMP and pyrophosphate. *In vitro*, the enzyme can use ammonia as the nitrogen donor as well as glutamine. There are two catalytic domains: one is a glutaminase and the other catalyzes the formation of β -aspartyl-AMP and then displacement of the AMP by ammonia. As in other glutamine-dependent amidotransferases (section 5.3.2.1), the two catalytic sites are linked by an intramolecular tunnel that permits ammonia to move from one site to the other.

Lymphocytes lack asparagine synthase and are reliant on a supply of asparagine from the bloodstream. Bacterial asparaginase is effective in the treatment of acute lymphoblastic leukaemia, leading to near complete remission in 40–60 per cent of cases, and nearer 95 per cent when combined with vincristine (Richards & Kilberg, 2006).

The amino acids synthesized from aspartate, lysine, methionine, threonine and isoleucine are all dietary essential amino acids for mammals. Because most plant proteins are limited by either methionine (in legumes) or lysine (in cereals), there is interest in the regulation of the pathway of amino acid synthesis from aspartate as a means of increasing the nutritional quality of plant proteins (section 2.2.4) and providing targets for herbicides.

The opaque-2 high-lysine variety of maize has a high content of lysine, partly because its storage proteins are rich in lysine and partly because there is a high concentration of free lysine in the seeds. This is because of low activity of lysine 2-oxoglutarate reductase and saccharopine dehydrogenase, two key enzymes of lysine catabolism (section 6.2.3). Regulation of the bacterial pathway is of interest because of the use of bacteria (especially *Corynebacterium* and *Brevibacterium* spp.) for the industrial synthesis of lysine, methionine and threonine. Specific inhibitors of any of the enzymes involved may be valuable as antibacterial and antifungal agents, since none of the enzymes of the pathway (apart from aspartate kinase) occurs in mammalian tissues.

Lysine and hydroxylysine are important in mammals for formation of the cross-links in collagen and elastin (section 6.2.4.1), as well as synthesis of carnitine (section 6.2.5), which transports fatty acids into the mitochondrion for β -oxidation. In sporulating bacteria, dipicolinic and diaminopimelic acids (intermediates in the pathway of lysine biosynthesis) are essential for spore formation (section 6.2.1.1). Lysine is also the precursor for synthesis of pyrrolysine, an amino acid that is incorporated into some bacterial proteins (section 6.2.4.3).

In plants and microorganisms, inorganic sulphide is incorporated into cysteine, and methionine is synthesized by transsulphuration from cysteine – the reactions of cystathionine γ -synthase and β -cystathionase (section 6.3.1.1). This incorporation of inorganic sulphur in plants provides the sole source of organic sulphur for animals. In mammals, it is cysteine that is synthesized from methionine by transsulphuration – the reactions of cystathionine β -synthase and γ -cystathionase (section 6.3.3). Methionine, as *S*-

adenosylmethionine, is the main methyl group donor in biosynthetic reactions, and decarboxylation of *S*-adenosylmethionine provides the substrate for polyamine synthesis from putrescine (section 5.8.2). Methionine is also the precursor for synthesis of ethylene in plants and microorganisms (section 6.3.4).

Taurine, synthesized by oxidation and decarboxylation of cysteine, is a major osmolyte in many tissues and it (as well as glycine) is also conjugated with the bile acids to increase their hydrophilicity (see section 6.3.7). The disulphide bond of cystine, formed by oxidation of cysteine residues in proteins, is important in the stabilization of protein tertiary and quaternary structures, forming intra- and inter-chain covalent bonds.

The role of cysteine in glutathione is discussed in section 5.4. Because cysteine is synthesized from methionine in animals, it is conventional to consider total sulphur amino acids (methionine + cysteine) in protein quality, since providing cysteine spares methionine (Ball *et al.*, 2006).

In leguminous plants that synthesize canavanine (section 1.6.2.4) as a nitrogen storage compound in seeds and as a potent insecticidal compound, homoserine is the likely precursor of canaline, the canavanine precursor. Catabolism of canavanine and canaline in the germinating seed leads to production of ammonium, as well as homoserine that can be used for the synthesis of lysine, methionine, threonine and isoleucine.

Threonine residues in proteins, like serine and tyrosine, are sites for reversible phosphorylation in regulation of enzyme activity, as are aspartate residues.

Mucin, the main protein in the mucus that is secreted to protect the gastrointestinal tract from proteolysis by digestive enzymes (see section 2.1.1) is especially rich in threonine and cysteine. It has been estimated that more than half of the dietary requirement for threonine can be accounted for by faecal loss of undigested mucin.

6.1 Regulation of the pathway of amino acid synthesis from aspartate

The branched pathway for synthesis of lysine, methionine, threonine and isoleucine from aspartate is shown in Figure 6.1. The first enzyme, aspartate kinase, is subject to feedback regulation by lysine, methionine and threonine, and homoserine dehydrogenase is subject to feedback regulation by methionine and threonine. The first enzyme unique to each of the branch points is subject to feedback regulation by the end-product of that branch of the pathway. The branch point for the synthesis of methionine differs. In bacteria, homoserine is the precursor for methionine synthesis, whereas in plants it is homoserine phosphate.

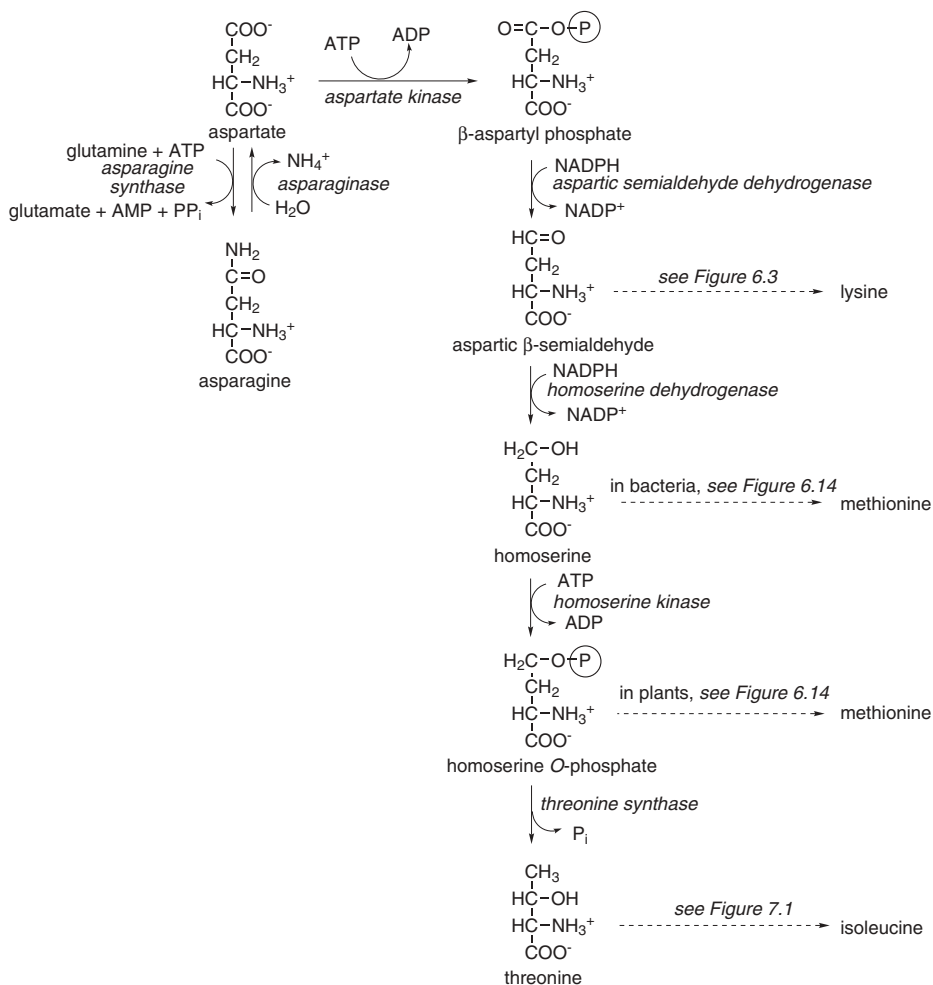


Figure 6.1 Amino acids synthesized from aspartate.

Asparagine synthase EC 6.3.5.4 (glutamine as amide donor), EC 6.3.1.1 (ammonium as amide donor), asparaginase EC 3.5.1.1, aspartate kinase EC 2.7.2.4, aspartic semialdehyde dehydrogenase EC 1.2.1.11, homoserine dehydrogenase EC 1.1.1.3, homoserine kinase EC 2.7.1.39, threonine synthase EC 4.2.3.1.

6.1.1 Aspartate kinase

In addition to its role as the source of β-aspartyl phosphate for amino acid synthesis, aspartate kinase also acts on aspartate residues in proteins. The reversible phosphorylation of aspartate residues is part of the response to extracellular signals in both bacteria and mammals.

As the first enzyme in the pathway, aspartate kinase is subject to feedback regulation by threonine, lysine and methionine, with different patterns of

regulation in different organisms. Most organisms have both a monofunctional aspartate kinase that is subject to regulation by lysine, and a bifunctional enzyme (aspartate kinase with homoserine dehydrogenase) that is subject to regulation by methionine and/or threonine. It is relatively unusual for two non-sequential steps in a pathway to form a bifunctional enzyme encoded by a single gene. There is no possibility of channelling the substrate of one active site to the other, since the product of aspartate kinase must leave the enzyme to undergo the reaction of aspartic semialdehyde dehydrogenase, and aspartic semialdehyde must then bind to the homoserine dehydrogenase active site.

The threonine-sensitive bifunctional enzyme from *E. coli* is a single polypeptide chain that can be cleaved by partial proteolysis into a carboxy-terminal fragment with homoserine dehydrogenase activity that is insensitive to threonine, and an amino-terminal fragment that has aspartate kinase activity and is inhibited by threonine. In the intact bifunctional enzyme, binding of threonine to the amino-terminal region inhibits both activities of the enzyme (Veron *et al.*, 1972, 1973).

Cleavage of the gene for the bifunctional enzyme and expression of the two separate enzymes produces a threonine-sensitive homoserine dehydrogenase and a threonine-insensitive aspartate kinase, the opposite of the results obtained by partial proteolysis of the bifunctional protein (Viola, 2001). Obviously, the gene cleavage and the partial proteolysis act at different points, and the threonine-sensitive region must lie at the interface of the two catalytic domains of the protein.

In *E. coli*, there are three isoenzymes of aspartate kinase: a monofunctional enzyme that is inhibited by lysine; a bifunctional aspartate kinase with homoserine dehydrogenase that is inhibited by threonine; and a bifunctional enzyme that is repressed by methionine (Azevedo *et al.*, 1997).

In *Bacillus brevis*, there are two isoenzymes of aspartate kinase. One is inhibited and repressed by lysine, and also repressed by diaminopimelate (see Figure 6.3); the other is inhibited by lysine and threonine, and repressed by threonine. Neither methionine nor isoleucine has any effect in this organism (Hitchcock *et al.*, 1980).

In *Streptococcus bovis*, there are two aspartate kinase isoenzymes: one that is inhibited by *meso*-diaminopimelate (Figure 6.3); and one that is both inhibited and repressed by lysine (Kalcheva *et al.*, 1994).

In plants, there are five isoenzymes of aspartate kinase, compared with one to three in bacteria. These comprise three monofunctional enzymes and two bifunctional enzymes – aspartate kinase with homoserine dehydrogenase. The monofunctional enzymes are inhibited by lysine, and isoenzyme I is inhibited synergistically by lysine and *S*-adenosylmethionine. In the absence of *S*-adenosylmethionine, this enzyme has a very low affinity for lysine, and neither end-product alone is a significant inhibitor of the enzyme.

The bifunctional enzymes are regulated by threonine, and one isoform also by isoleucine.

There are two threonine binding sites on the bifunctional enzyme. Binding of threonine to one inhibits the aspartate kinase domain and also enhances binding of threonine to the second site, which inhibits homoserine dehydrogenase. A number of amino acids, including alanine, serine and cysteine, compete with threonine and activate the enzymes (Azevedo *et al.*, 2006; Curien *et al.*, 2007; Jander & Joshi, 2010). In plants, transcription of all of the aspartate kinase genes is down-regulated in the dark and under conditions of low sugar availability (Ufaz *et al.*, 2011).

In higher fungi, lysine is synthesized from 2-oxoglutarate, not aspartic β -semialdehyde (section 6.2.2), and they have a single monofunctional aspartate kinase that is sensitive to threonine.

6.1.1.1 Aspartate kinase in post-synthetic modification of proteins

Although mammals do not synthesize any of the amino acids discussed here from aspartate, antibodies against bacterial aspartate kinase nevertheless demonstrate the presence of a cross-reacting protein in both rat cerebellar cortex and human liver. Aspartate kinase activity can be measured in human liver, where it acts to phosphorylate aspartate residues in proteins (Arenas-Diaz & Marshall, 1990; Arenas-Diaz & Martinez-Rodriguez, 1990).

In bacteria, a cell surface receptor undergoes autophosphorylation on a histidine residue when the ligand binds, then transfers the phosphate onto an aspartate residue of a regulatory protein that undergoes a conformational change and interacts with a genomic promoter region. There may also be a phosphorylation cascade, with transfer of phosphate onto further effector proteins. Among other actions, phosphorylation of aspartate residues is important in controlling sporulation of bacteria such as *B. subtilis* (see section 6.2.1.1).

6.1.1.2 Aspartic semialdehyde dehydrogenase In most bacteria, aspartic semialdehyde dehydrogenase is not subject to regulation, but the enzyme is repressed by lysine in *E. coli* (Cahyanto *et al.*, 2006). The enzyme shows ordered bi-bi kinetics; NADPH binds first, and this causes a conformational change that creates the binding site for β -aspartyl phosphate (Hadfield *et al.*, 2001).

6.1.2 Homoserine dehydrogenase

In Gram-negative organisms such as *E. coli*, homoserine dehydrogenase occurs as part of the bifunctional enzyme with aspartate kinase, as discussed in section 6.1.1. In Gram-positive bacteria such as *Bacillus subtilis* and *Corynebacterium glutamicum*, there is a monofunctional homoserine dehy-

drogenase that is sensitive to threonine inhibition. This enzyme has a carboxy-terminal threonine-binding domain that is absent from the *E. coli* enzyme. Yeasts have a monofunctional homoserine dehydrogenase that lacks this carboxy-terminal domain and is insensitive to threonine (Viola, 2001).

The enzyme from *B. subtilis* is also repressed by methionine and isoleucine. Its activity is highest during the exponential phase of growth and declines steadily through the static phase of growth, so that there is increased availability of aspartic semialdehyde for the lysine branch of the pathway (Figure 6.3) to provide the dipicolinic and diaminopimelic acids that are required for sporulation (Yeggy & Stahly, 1980).

In addition to the threonine-sensitive bifunctional aspartate kinase with homoserine dehydrogenase in chloroplasts and plastids, plants also have a threonine-insensitive cytoplasmic homoserine dehydrogenase. The importance of this enzyme is unclear, since amino acid synthesis from aspartate occurs in chloroplasts and, in non-photosynthetic tissues, in plastids (Azevedo *et al.*, 2006).

Soya beans have two bifunctional aspartate kinase + homoserine dehydrogenase enzymes that are sensitive to threonine, plus a monofunctional enzyme that is not sensitive to any of the end-products of the pathway (Schroeder *et al.*, 2010). Maize has a threonine-sensitive monofunctional homoserine dehydrogenase which can exist in four distinct states:

- The T state is a dimer that is formed in the presence of threonine.
- The K state a dimer that is formed in the presence of potassium chloride.
- In the absence of both threonine and KCl (or in the presence of both at effective concentrations), the enzyme forms a tetramer (the B state) that is distinct from the tetramer that is formed in the presence of aspartic β -semialdehyde and NADPH.
- Both threonine and KCl cause dissociation of the active tetramer to an inactive dimer. This is the opposite of the bacterial enzymes, where threonine causes association of the active dimer to an inactive tetramer (Krishnaswamy & Bryan, 1983).

6.1.3 Homoserine kinase

In bacteria, homoserine kinase is inhibited by threonine, competitively with respect to homoserine and non-competitively with respect to ATP. Although threonine also inhibits aspartate kinase and homoserine dehydrogenase, this

is weaker inhibition than that of homoserine kinase. There is no evidence that bacterial homoserine kinase is subject to allosteric inhibition; threonine binds at the active site as a substrate analogue (Miyajima & Shiio, 1972).

The activities of homoserine kinase and the aspartate kinase and homoserine dehydrogenase bifunctional enzyme in *E. coli* are coordinately induced and repressed under various conditions. The genes are in the same region of the *E. coli* genome and probably form part of the same operon.

6.1.4 Threonine synthase

Threonine synthase is a pyridoxal phosphate-dependent enzyme. It catalyzes the dephosphorylation of homoserine *O*-phosphate to threonine. The dephosphorylation of the substrate leads to the formation of aminocrotonate at the active site, followed by addition of water to form the threonine Schiff base, then displacement of threonine by the lysine residue at the active site to reform the internal aldimine (see section 3.1). Aminocrotonate is also the immediate product of threonine deaminase (section 6.1.5); after leaving the enzyme, it undergoes non-enzymic hydrolysis to 2-oxobutyrate and ammonium. Aminocrotonate may also leave the active site of threonine synthase and similarly undergo non-enzymic hydrolysis.

In some bacteria, there is a threonine synthase that catalyzes the dephosphorylation and deamination of homoserine phosphate to 2-oxobutyrate for isoleucine synthesis without forming threonine (Schildkraut & Greer, 1973).

In plants, threonine synthase represents the final branch point, and the reaction is in competition with cystathionine γ -synthase, the first reaction unique to methionine synthesis. The plant enzyme is allosterically activated by *S*-adenosylmethionine so that, when tissue concentrations are high and there is no need for further methionine synthesis, homoserine *O*-phosphate is diverted to synthesis of threonine and isoleucine. The plant enzyme is not inhibited by threonine or isoleucine, but cysteine inhibits threonine synthase, so that more homoserine phosphate is available for methionine synthesis. The bacterial enzyme does not represent a branch point and is not subject to allosteric regulation (Azevedo *et al.*, 1997, 2006; Curien *et al.*, 2008).

6.1.5 Threonine catabolism

As shown in Figure 6.2, there are three pathways for threonine catabolism. Threonine deaminase (section 6.1.5.1) catalyzes deamination to 2-oxobutyrate. This may either be metabolized onwards via propionyl CoA and succinyl CoA, or it may be the precursor for isoleucine synthesis (section 7.1).

In bacteria, fungi and plants, threonine aldolase catalyzes cleavage to acetaldehyde and glycine. The apparent threonine aldolase activity in mammalian liver is an artefact of the assay procedure (section 4.2.1) and is abol-

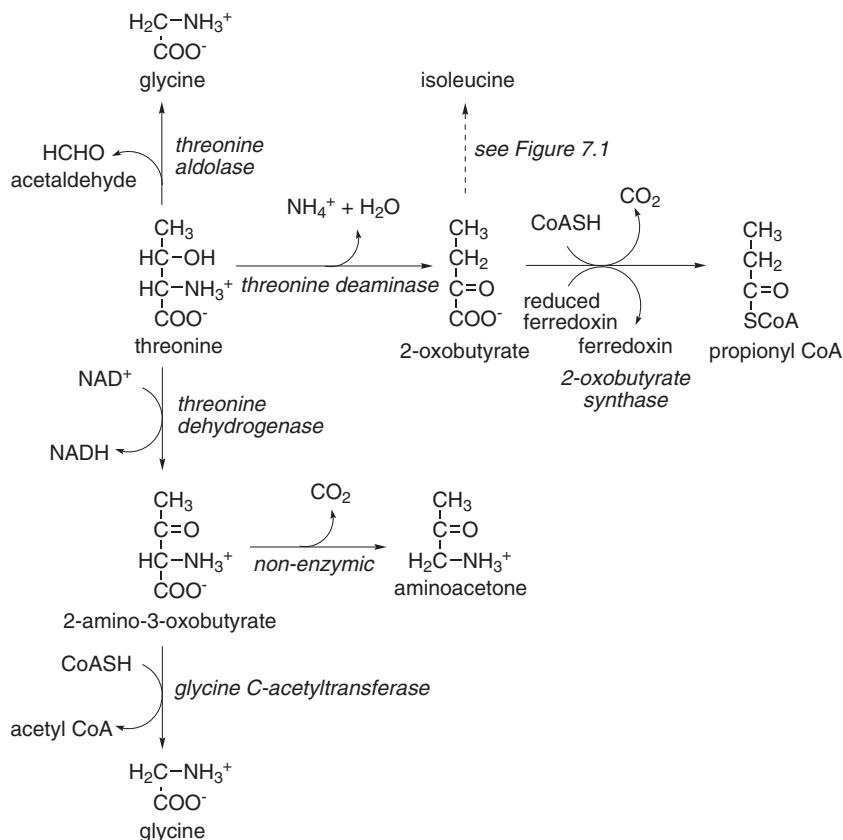


Figure 6.2 Pathways of threonine catabolism.

Threonine aldolase EC 4.1.2.5, threonine deaminase EC 4.3.1.19, 2-oxobutyrate synthase EC 1.2.7.2, threonine dehydrogenase EC 1.1.1.103, glycine C-acetyltransferase EC 2.3.1.29.

ished by specific antibodies against threonine deaminase (Yeung, 1986). Threonine aldolase deficiency in *Arabidopsis* is lethal. This may be the result of inhibition of the other aspartate-derived amino acids by accumulation of threonine. Alternatively, threonine aldolase may represent a significant source of glycine for photorespiration (section 4.2.3), and the lethality of lack of threonine aldolase may be due to glycine deficiency (Joshi, V *et al.*, 2006).

Threonine dehydrogenase catalyzes oxidation to 2-amino-3-oxobutyrate, which is cleaved to glycine and acetyl CoA by glycine C-acetyltransferase. The dehydrogenase and acetyltransferase form a complex that channels 2-amino-3-oxobutyrate from one active site to the other, so avoiding non-enzymic decarboxylation to aminoacetone. The acetyltransferase is a pyridoxal phosphate-dependent enzyme. It forms a Schiff base between the

substrate and the coenzyme that is then attacked by CoA, forming acetyl CoA and leaving the glycine Schiff base at the active site.

Bacterial glycine C-acetyltransferase and threonine dehydrogenase also catalyze the synthesis of threonine from glycine and acetyl CoA. The beef liver glycine C-acetyl transferase does not form a complex with threonine dehydrogenase. It catalyzes the formation of aminoacetone from glycine and acetyl CoA, so demonstrating the importance of substrate channelling in the plant and bacterial enzymes. This also explains why mammals are unable to synthesize threonine, despite having both glycine C-acetyltransferase and threonine dehydrogenase (Fubara *et al.*, 1986; Marcus & Dekker, 1993; Schmidt *et al.*, 2001).

Threonine dehydrogenase provides the main pathway of threonine catabolism in rat liver in the fed state, although, in fasting, threonine deaminase is more important. Animal studies show that threonine dehydrogenase is induced in response to feeding a high threonine diet (Bird & Nunn, 1983; Darling *et al.*, 2000). Threonine dehydrogenase is not involved in human catabolism of threonine. As a result of mutations, the human threonine dehydrogenase gene is expressed as two truncated proteins that lack catalytic activity and do not have either the NAD binding site or the carboxy-terminal domain that binds threonine (Edgar, 2002).

6.1.5.1 Threonine deaminase Threonine deaminase catalyzes an initial dehydration reaction, followed by isomerization and hydrolysis of the C-N bond. Because of this, it was formerly known as threonine dehydratase, although overall the reaction does not lead to dehydration. There is some overlap of specificity between threonine and serine deaminases, and threonine deaminase from most sources will also catalyze the deamination of serine (section 4.6.2).

In organisms that synthesize isoleucine from threonine, there are separate catabolic and biosynthetic isoenzymes of threonine deaminase. The biosynthetic threonine deaminase is constitutive, and it is inhibited by isoleucine and activated by valine (so ensuring balance between the parallel pathways of isoleucine and valine synthesis, section 7.1).

In *E. coli*, binding of isoleucine to the regulatory domain of the enzyme lowers the affinity for threonine and increases cooperativity of the subunits. Binding of valine has the opposite effect. In *Arabidopsis*, there are two isoleucine binding sites in the regulatory domain. Binding of isoleucine to the first site leads to a conformational change that (paradoxically) activates the enzyme but also increases the affinity of the second site for isoleucine. The binding of a second mol of leucine leads to inhibition of the enzyme. Binding of valine to the first site also activates the enzyme, but it also lowers the affinity of the second site for isoleucine. Inhibition by isoleucine is associ-

ated with formation of a less active dimer of the enzyme; activation by valine is due to the formation of a tetramer (Wessel *et al.*, 2000).

Catabolic threonine deaminase is induced in response to threonine and is insensitive to isoleucine and valine; it lacks the C-terminal regulatory domain that binds isoleucine in the biosynthetic enzyme. The catabolic enzyme requires 5'AMP for activity (or, in some bacteria, ADP), suggesting that its activity is regulated by the energy balance of the cell. Binding of 5'AMP (or ADP) leads to formation of a more active tetramer of the enzyme that has a lower K_m for threonine. The catabolic deaminase is also inhibited by a variety of oxo-acids and intermediates of glycolysis and the citric acid cycle (Sawers, 1998; Simanshu *et al.*, 2007).

In most organisms, threonine deaminase is a pyridoxal phosphate dependent enzyme. However, in *Pseudomonas putida* it does not contain pyridoxal phosphate, but has another carbonyl group as catalyst that is assumed to be pyruvate (section 3.5). In *Ps. putida*, threonine deaminase acts to deaminate threonine attached to tRNA, yielding 2-oxobutyrate tRNA^{Thr}, which is incorporated at the amino-terminal of urocanase (section 8.2.1) to provide a catalytic prosthetic group (Cohn & Phillips, 1974; Daumy *et al.*, 1974).

Tomato and related plants have a second gene for catabolic threonine deaminase that is co-expressed with a variety of genes associated with resistance to attack by caterpillars and other herbivorous insects. The deaminase is synthesized as an inactive pro-enzyme, and it is activated in the insect mid-gut by cleavage of the regulatory carboxy-terminal domain by a chymotrypsin-like enzyme. It accumulates in the insect mid-gut and deaminates threonine, thus starving the insects of an essential amino acid (Chen *et al.*, 2007; Gonzales-Vigil *et al.*, 2011).

6.2 Lysine

In plants, most bacteria and lower fungi, lysine is synthesized via the diaminopimelate pathway (section 6.2.1), while in some bacteria (especially extreme thermophiles), yeasts and higher fungi, it is synthesized via the α -amino adipic acid pathway (see section 6.2.2). In plants and many bacteria, lysine is decarboxylated to the diamine cadaverine, which has a role in the induction of cell division in plants and as a precursor for the synthesis of a variety of alkaloids. Little is known about the relationship of cadaverine to the polyamines derived from ornithine and arginine (section 5.8).

In addition to its other roles, lysine provides the cross-links of the cell wall peptidoglycan of Gram-positive bacteria; in Gram-negative bacteria, it is *meso*-diaminopimelic acid, an intermediate in lysine synthesis, that is used. Both diaminopimelate (an intermediate in the pathway) and dipicolinic acid

(a side-product of the pathway) are important in spore-forming bacteria (section 6.2.1.1)

6.2.1 Lysine biosynthesis in bacteria and plants – the diaminopimelate pathway

Dihydrodipicolinate synthase is the first enzyme unique to lysine synthesis. As shown in Figure 6.3, it catalyzes the condensation of aspartic β -semialdehyde and pyruvate to yield dihydrodipicolinate. The plant enzymes are inhibited by lysine, as are those from Gram-negative bacteria, but the enzymes from Gram-positive bacteria are insensitive to lysine inhibition. Pyruvate binds to the enzyme first, forming a Schiff base to a lysine residue at the active site. After the first water molecule has been lost, aspartic semi-aldehyde binds and undergoes condensation to yield dihydrodipicolinate (Hutton *et al.*, 2007; Laber *et al.*, 1992).

Expression of lysine-insensitive bacterial aspartate kinase and dihydrodipicolinic acid synthase in plants does not lead to as great an accumulation of lysine as might be expected, because there is increased expression of 2-oxoglutarate reductase (the first enzyme of lysine catabolism, section 6.2.3.1). There is thus coordinated regulation of lysine synthesis and catabolism to maintain appropriate tissue concentrations. The high concentration of free lysine in Opaque-2 maize is due to low activity of 2-oxoglutarate reductase (Karchi *et al.*, 1994).

Dihydrodipicolinate is reduced to tetrahydrodipicolinate by an NADPH-dependent reductase. In different organisms, there are three possible fates for tetrahydrodipicolinate: reductive amination to *meso*-diaminopimelic acid, transamination to L,L, α , ϵ -diaminopimelic acid or an indirect pathway involving succinylation or acetylation, transamination and deacylation to yield L,L, α , ϵ -diaminopimelic acid. All Gram-negative, and many Gram-positive bacteria use the succinylation pathway; *Bacillus* spp. use the acetylation pathway. Some bacteria have both the succinylation and acetylation pathways and also the diaminopimelate dehydrogenase-catalyzed reductive amination of tetrahydrodipicolinate. In all plants and some prokaryotes, including *Chlamydia* spp. and cyanobacteria, there is direct transamination of tetrahydrodipicolinic acid to L,L, α , ϵ -diaminopimelic acid (Born & Blanchard, 1999; Hudson *et al.*, 2006, 2011; McCoy *et al.*, 2006; Scapin & Blanchard, 1998).

Tetrahydrodipicolinate undergoes ring opening when it binds to the succinyltransferase, before succinyl CoA binds to the enzyme; succinylation then blocks the α -amino group, preventing cyclization. Succinyl diaminopimelate transaminase is the same enzyme as the *N*-acetylornithine transaminase that is involved in arginine synthesis (section 5.5.1; Ledwidge & Blanchard, 1999). The direct transamination of tetrahydrodipicolinate to LL-diaminopimelate

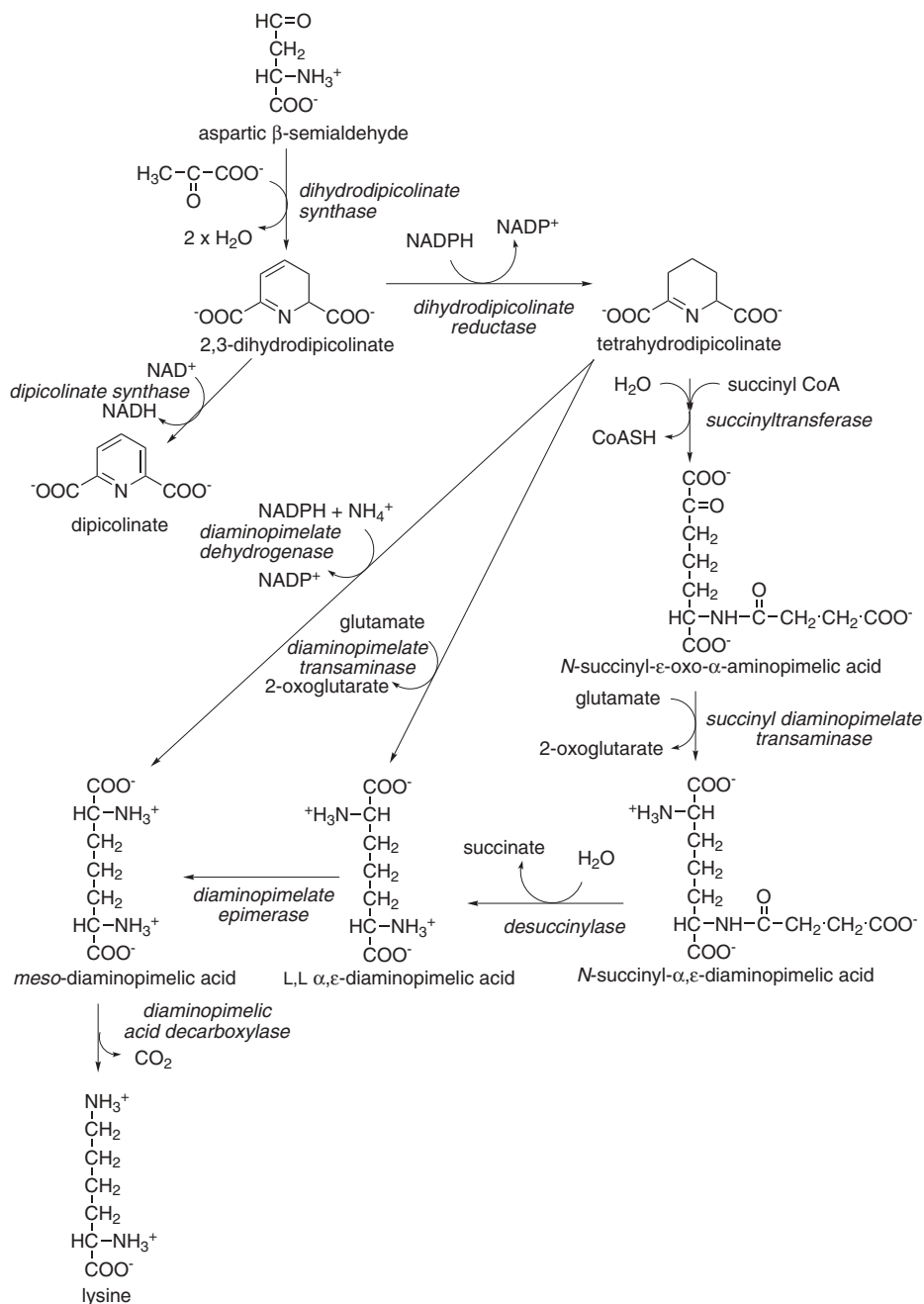


Figure 6.3 Lysine biosynthesis in bacteria and plants – the diaminopimelate pathway. Dihydrodipicolinate synthase EC 4.2.1.52, dihydrodipicolinate reductase EC 1.3.1.26, tetrahydrodipicolinate succinyltransferase EC 2.3.1.117, succinyl diaminopimelate transaminase EC 2.6.1.17, succinyl diaminopimelate desuccinylase EC 3.5.1.18, diaminopimelate epimerase EC 5.1.1.7, diaminopimelic acid decarboxylase EC 4.1.1.20, diaminopimelate transaminase EC 2.6.1.83, meso-diaminopimelate dehydrogenase EC 1.4.1.16, dipicolinate synthase has no EC number.

in plants, and the reductive amination to *meso*-diaminopimelate in some bacteria, suggest that ring opening can occur without the need to block the α -amino group by acylation.

Diaminopimelic acid epimerase is unusual for an amino acid racemase in that (like proline and glutamate racemases) it is not a pyridoxal phosphate-dependent enzyme (section 3.2), and it requires a reducing agent for activity. Two sulphhydryl groups catalyze the deprotonation and reprotonation reactions required for isomerization of the substrate, which binds exactly midway between the two sulphhydryl groups (Born & Blanchard, 1999; Brunetti *et al.*, 2008).

The final step in lysine synthesis is decarboxylation of *meso*-diaminopimelic acid, catalyzed by a pyridoxal phosphate-dependent decarboxylase that seems to be the only known D-amino acid decarboxylase. It shows significant sequence homology with mammalian ornithine decarboxylase (section 5.8.1), but little homology with other bacterial amino acid decarboxylases (Martin *et al.*, 1988; Selli *et al.*, 1994).

6.2.1.1 Diaminopimelate and dipicolinate in sporulating bacteria Spore formation is the response of some bacteria to nutritional and environmental stress. Two cells cooperate, leading to the packaging of the contents of one into a resistant coat, while the other cell contributes most of its resources to the process and then undergoes lysis.

Diaminopimelate is important in the peptidoglycan of the cortex of bacterial spores, and calcium dipicolinate accumulates in the spore, accounting for up to 10–15 per cent of the dry weight. As shown in Figure 6.3, dipicolinate arises by oxidation of dihydrodipicolinate, as opposed to the reduction to tetrahydrodipicolinate that occurs in lysine and diaminopimelate synthesis. Spores that are deficient in calcium dipicolinate, as a result of either mutation in the dipicolinate synthase gene or calcium deficiency, show decreased resistance to heat and impaired germination. In response to environmental signals for germination acting on spore surface receptors, calcium dipicolinate moves from the core of the spore and activates one or more of the key enzymes that lyse the spore coat (Forman & Aronson, 1972; Moir, 2006).

In *Bacillus subtilis*, there are two separate isoenzymes of aspartokinase – one that is sensitive to lysine (as in other organisms) and one that is sensitive to diaminopimelate. Thus, even when there is an adequate supply of lysine, β -aspartyl phosphate can be available for synthesis of diaminopimelic and dipicolinic acids. During spore formation, there is little net protein synthesis, and the lysine and other amino acids that are required for protein synthesis are derived mainly from the catabolism of existing proteins. The first three enzymes of the diaminopimelate pathway, aspartokinase, aspartate semialdehyde dehydrogenase and dihydropicolinate synthase, as well as dipicolinate synthase (Figure 6.3), are clustered as a single diaminopimelate sensitive

operon. By contrast, in *E. coli*, where diaminopimelate and dipicolinate are not important, the genes for the first three enzymes of the pathway are scattered through the genome (Chen *et al.*, 1993).

In *B. cereus*, there is a single form of aspartokinase that is normally repressed by lysine. At the onset of spore formation the enzyme loses its sensitivity to lysine. During growth, the activity of diaminopimelate decarboxylase varies inversely with the intracellular concentration of lysine. The activity of the enzyme falls at the end of exponential growth, and it is undetectable by the time dipicolinate synthesis begins, although the intracellular pool of lysine is below the level at which there would normally be de-repression of enzyme synthesis (Forman & Aronson, 1972; Grandgenett & Stahly, 1971).

6.2.2 Lysine biosynthesis in yeasts and fungi – the α -amino adipic acid pathway

The first part of the lysine biosynthesis pathway in yeasts, higher fungi, some green algae and some extreme thermophilic bacteria is mitochondrial – synthesis of 2-oxoadipic acid from 2-oxoglutarate and acetyl CoA via homocitrate (Figure 6.4). The sequence of reactions is analogous to the synthesis of 2-oxoglutarate from oxaloacetate and acetyl CoA in the citric acid cycle. Although the reaction of homocitrate synthase is analogous to that of citrate synthase, there is little or no sequence homology between the enzymes. Subsequent reactions occur in the cytosol, and most are readily reversible (Bhattacharjee, 1985; Xu *et al.*, 2006; Zabriskie & Jackson, 2000).

In yeasts, all enzymes of the pathway are repressed by lysine; as 2-amino adipic acid accumulates, it binds to a repressor protein that affects the expression of all the genes of the pathway. Homocitrate synthase is also inhibited by CoASH binding to a regulatory site. Isopropylmalate synthase in leucine biosynthesis (see section 7.1.3) is similarly inhibited by CoASH, while citrate synthase is not. This suggests that when the ratio of mitochondrial CoASH to acetyl CoA is high, such acetyl CoA as is available will be used for energy-yielding metabolism via the citric acid cycle rather than amino acid synthesis (Kohlhaw, 2003).

Homocitrate synthase is a homodimer with two catalytic sites. A lid domain from the carboxy-terminal of each subunit occludes the entrance to its active site. Binding of 2-oxoglutarate to one subunit leads to a conformational change to reveal the active site of the other subunit. In some organisms, lysine binds to an allosteric regulatory site; in others, it competes directly with 2-oxoglutarate. The active site of the enzyme contains both basic residues that will interact with the C-5 carboxylate of 2-oxoglutarate, and also acidic residues that will bind the ϵ -amino group of lysine (Bulfer *et al.*, 2009, 2010).

There are two isoenzymes of homocitrate synthase in yeasts – the products of the *lys-20* and *lys-21* genes. The product of the *lys-21* gene is responsible

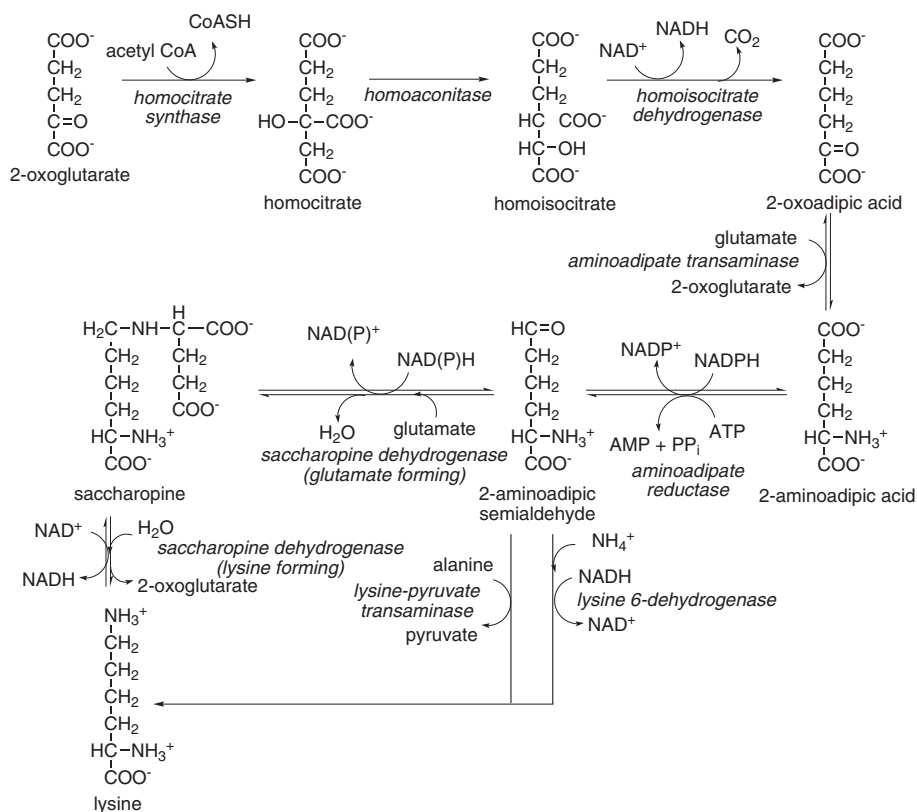


Figure 6.4 Lysine biosynthesis in yeasts and fungi – the α -aminoadipate pathway.

Homocitrate synthase EC 2.3.3.14, homoaconitase EC 4.2.1.36, homoisocitrate dehydrogenase EC 1.1.1.87, aminoadipic acid transaminase EC 2.6.1.39, aminoadipic semialdehyde dehydrogenase EC 1.2.1.32, saccharopine dehydrogenase (glutamate forming = saccharopine reductase) EC 1.5.1.9 (NAD-linked), EC 1.5.1.10 (NADP-linked), saccharopine dehydrogenase (lysine forming) EC 1.5.1.7 (NAD-linked), EC 1.5.1.8 (NADP-linked), lysine pyruvate transaminase EC 2.6.1.71, lysine 6-dehydrogenase EC 1.4.1.18.

for lysine biosynthesis under aerobic conditions, while both are active during anaerobic fermentation. Regulation of these two enzymes provides control over the use of 2-oxoglutarate and partitioning between lysine synthesis and citric acid activity under aerobic conditions. The product of the *lys-21* gene is strongly inhibited by lysine, although lysine binding also leads to cooperativity in the binding of 2-oxoglutarate. It is essential for growth under aerobic conditions. The product of the *lys-20* gene has a greater capacity for lysine synthesis, is less sensitive to inhibition by lysine, and its synthesis is down-regulated under aerobic conditions (Quezada *et al.*, 2008).

The genes for the yeast enzymes have considerable sequence homology with the *NifV* gene in nitrogen fixing organisms and 90 per cent sequence

identity with each other, suggesting that they have evolved by gene duplication. In nitrogen-fixing organisms, homocitrate forms part of the iron-molybdenum cofactor of nitrogenase (section 1.1.1); in yeast, the same enzyme has become the first enzyme of lysine synthesis. Although the synthesis of homocitrate occurs in the mitochondria, the genes for the enzymes do not have a mitochondrial targeting sequence and there seem to be significant amounts of the enzymes in the cytosol. Immunocytochemistry shows that the enzyme is also located in the nucleus. In addition to its role in lysine synthesis, homocitrate synthase has a specific nuclear role in chromatin function and DNA damage repair via histone acetyltransferases (Scott & Pillus, 2010).

The active sites of homoaconitase, aconitase and isopropylmalate dehydratase (section 7.1.3) are highly conserved, although otherwise these three iron-sulphur proteins show little homology. Homoaconitase catalyzes a homologous reaction to aconitase, catalyzing the isomerization of homocitrate to homoisocitrate by dehydration to form enzyme-bound homoaconitate as an intermediate (Irvin & Bhattacharjee, 1998).

Oxidative decarboxylation of homoisocitrate leads to the formation of 2-oxoadipic acid, which is transaminated to 2-aminoadipic acid. There are two isoenzymes of 2-oxoadipic acid transaminase in yeast. One is mitochondrial and can use either glutamate or aspartate as the amino donor, while the other is cytosolic and is specific for glutamate. In addition, one isoenzyme of kynurenine transaminase (see section 9.4.4.2) has a broad specificity and catalyzes the transamination of 2-oxoadipic acid (Han *et al.*, 2008).

Aminoadipate reductase catalyzes the ATP-dependent reduction of aminoadipate to 2-aminoadipic semialdehyde. The reaction proceeds by way of a δ -carboxy-adenylated intermediate, releasing pyrophosphate, then AMP. This is analogous to the reaction involved in non-ribosomal peptide synthesis in bacteria, in which an α -carboxy-adenylated intermediate is formed to activate the amino acid for peptide synthesis. Two separate proteins are involved in this reaction: the reductase itself and a separate protein that catalyzes a post-synthetic modification of the reductase – transfer of phosphopantetheine from CoASH onto a serine residue. This activates the enzyme.

The first step in the reductase reaction is adenylation of the δ -carboxyl group of aminoadipate, releasing pyrophosphate. This is followed by acyl-transfer of the aminoadipoyl moiety to the sulphhydryl group of the enzyme-bound phosphopantetheine, then NADPH-dependent reduction to regenerate phosphopantetheine and release the semialdehyde product (Ehmann *et al.*, 1999; Guo *et al.*, 2001).

In free solution, aminoadipic semialdehyde rapidly undergoes dehydration and cyclization to piperidine 6-carboxylate. There is no evidence of channelling of aminoadipic semialdehyde between the active sites of aminoadipate reductase and saccharopine reductase, but there is evidence that aminoadipic semialdehyde is protein bound in the cell, to the product of the

lys-14 gene. When this protein has aminoadipic semialdehyde bound, it acts as a transcriptional activator for several of the genes involved in lysine synthesis. Repression or inhibition of homocitrate synthase by high concentrations of lysine will result in a reduced amount of aminoadipic semialdehyde, and hence less activation of the transcriptional activator and reduced expression of the genes coding for the enzymes of lysine synthesis.

This positive regulation of gene expression by an intermediate in lysine synthesis is in contrast to the repression of enzyme synthesis by end-products binding to *trans*-acting repressor proteins seen in bacteria. Similar positive regulation of genes for amino acid biosynthesis in yeast occurs in the regulation of leucine synthesis (section 7.1.3) and methionine synthesis (section 6.3.1; Feller *et al.*, 1999; Ramos *et al.*, 1988).

In β -lactam antibiotic-producing filamentous fungi, there is competition for aminoadipic semialdehyde between aminoadipic semialdehyde reductase for lysine synthesis and aminoadipyl-cysteinyI-valine synthetase for antibiotic synthesis. Activity of the dehydrogenase is high during the growth phase before the onset on antibiotic production, then it falls.

Saccharopine reductase is also known as saccharopine dehydrogenase (glutamate forming). It catalyzes the NADPH-dependent reductive condensation of aminoadipic semialdehyde with glutamate to form saccharopine. When saccharopine is the substrate, the enzyme catalyzes NADP⁺-dependent oxidative hydrolysis of saccharopine to glutamate and succinic semialdehyde. The final step in lysine synthesis is NAD⁺-dependent oxidative hydrolysis to yield lysine and 2-oxoglutarate, catalyzed by saccharopine dehydrogenase (lysine forming).

The pathway between aminoadipic acid and lysine is reversible. Lysine-requiring mutants of *Penicillium* spp. in which the defect is before the formation of aminoadipate are able to synthesize penicillin (for which aminoadipate is the precursor) when adequate amounts of lysine are provided (Esmahan *et al.*, 1994).

In some organisms, aminoadipic semialdehyde can be transaminated directly to lysine at the expense of alanine; in others, it may undergo reductive amination to lysine catalyzed by lysine 6-dehydrogenase. Some organisms have a pipercolic acid oxidase and can use pipercolic acid, a product of lysine catabolism (section 6.2.3.1), as a substrate for lysine synthesis, oxidizing it to piperideine 6-carboxylate, followed by hydrolysis to aminoadipic semialdehyde. Some bacteria have a lysine synthetic pathway in which aminoadipate is *N*-acetylated, then phosphorylated on the δ -carboxyl group, followed by reductive dephosphorylation to yield *N*-acetyl lysine.

6.2.3 Lysine catabolism

The main pathway for lysine catabolism in plants and animals is formally the reverse of the α -amino adipic acid pathway via saccharopine for lysine syn-

thesis in yeasts and fungi (section 6.2.2). There is a distinct pathway for lysine catabolism in the mammalian central nervous system, via pipecolic acid. This pathway is also found in some plants and bacteria, and it becomes the major route of lysine catabolism in patients with inborn errors of the saccharopine pathway.

Bacteria and yeasts are capable of catabolizing lysine through a wide variety of pathways, including transamination reactions leading to formation of 2-aminoadipic semialdehyde (which is the common intermediate of the saccharopine and pipecolic acid pathways – see Figure 6.5), decarboxylation to cadaverine, oxidation to 6-amino 2-oxocaproic acid or 5-aminovaleramide, ϵ -N acetylation or hydroxylation, racemization to D-lysine and isomerization to β -lysine, catalyzed by lysine 2,3-aminomutase. Lysine 2,3-aminomutase is vitamin B₁₂-dependent in some organisms, and it utilizes S-adenosylmethionine and pyridoxal phosphate rather than adenosylcobalamin in others (Broquist, 1991; Frey, 1993; Zabriskie & Jackson, 2000).

6.2.3.1 The saccharopine pathway of lysine catabolism As shown in the lower part of Figure 6.5, the pathway of lysine catabolism via saccharopine involves the same intermediates as the α -amino adipic acid pathway for lysine synthesis from oxoadipic acid (Figure 6.4). The first two steps – the reductive synthesis of saccharopine and the oxidative cleavage to 2-aminoadipic semialdehyde – are catalyzed by a bifunctional enzyme that has two separate catalytic sites: lysine-oxoglutarate reductase and saccharopine dehydrogenase. The amino-terminal lysine-oxoglutarate reductase and the carboxy-terminal saccharopine dehydrogenase regions can be separated by partial proteolysis to yield monofunctional enzymes.

In plants, the same gene codes for both the bifunctional enzyme and separate monofunctional enzymes, and in mice there is also a monofunctional saccharopine dehydrogenase derived from the same gene by alternative splicing (Papes *et al.*, 1999; Sacksteder *et al.*, 2000; Stepansky *et al.*, 2006).

Both the synthesis and catabolism of lysine are highly regulated in plants. Transgenic plants over-expressing the bacterial lysine-insensitive dihydrodipicolinate synthase over-produce lysine, but they also show a ten-fold induction of lysine-oxoglutarate reductase and saccharopine dehydrogenase. The high-lysine Opaque-2 cultivar of maize not only has a different pattern of storage proteins (with less of the lysine-deficient zein), but also has low activity of lysine-oxoglutarate reductase and saccharopine dehydrogenase, so that it accumulates free lysine to a greater extent than other varieties (Azevedo *et al.*, 1997; Stepansky *et al.*, 2006).

2-Aminoadipic semialdehyde arising from both oxidative cleavage of saccharopine and also hydrolysis of Δ^1 -piperidine 6-carboxylic acid (section 6.2.3.2) undergoes oxidation and transamination to 2-oxoadipic acid, followed by oxidative decarboxylation to glutaryl CoA and onward oxidation

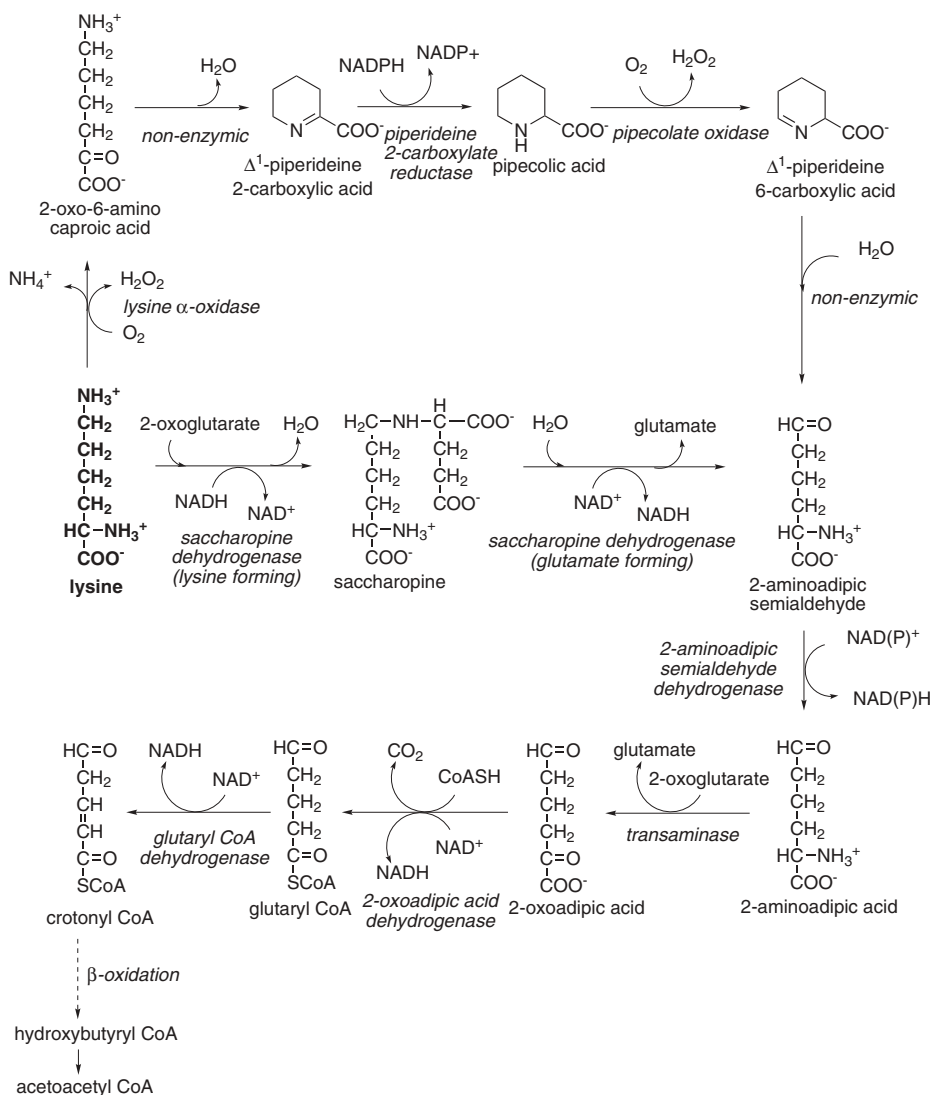


Figure 6.5 Pathways of lysine catabolism.

Lysine α -oxidase EC 1.4.3.14, piperideine 2-carboxylate reductase EC 1.4.1.21 (reaction also catalysed by pyrroline 2-carboxylate reductase, EC 1.5.1.1), pipecolate oxidase EC 1.5.3.7, 2-aminoadipic semialdehyde dehydrogenase EC 1.2.1.31, 2-aminoadipate transaminase EC 2.6.1.39, 2-oxoadipic acid dehydrogenase is catalysed by the 2-oxoglutarate dehydrogenase multi-enzyme complex EC 1.2.4.2 + 2.3.1.61 + 1.8.1.4, saccharopine dehydrogenase (lysine forming) EC 1.5.1.7 (NAD-linked), EC 1.5.1.8 (NADP-linked)saccharopine dehydrogenase (glutamate forming) EC 1.5.1.9 (NAD-linked), EC 1.5.1.10 (NADP-linked).

to acetoacetyl CoA. Oxoadipic acid is also an intermediate in tryptophan catabolism (section 9.4.4).

6.2.3.2 The pipecolic acid pathway of lysine catabolism In the mammalian central nervous system, lysine is catabolized by a separate pathway, via pipecolic acid, as shown in the upper part of Figure 6.5. The enzymes of the saccharopine pathway are not expressed in the brain. Lysine α -oxidase is a flavoprotein that catalyzes oxidative deamination of lysine to 2-oxo-6-aminocaproic acid, a reaction similar to the amino acid oxidases discussed in section 1.5.1. 2-Oxo-6-aminocaproic acid undergoes non-enzymic dehydration to Δ^1 -piperidine 2-carboxylic acid, followed by NADPH-dependent reduction to pipecolic acid and FAD-dependent oxidation to Δ^1 -piperidine 6-carboxylic acid, which is hydrolyzed to aminoadipic semialdehyde. Aminoadipic semialdehyde then undergoes the same sequence of reactions leading to acetoacetyl CoA, as described in section 6.2.3.1.

In human beings and other primates, pipecolate oxidase is a peroxisomal enzyme, and pipecolic acid accumulates in patients with a variety of peroxisomal diseases. In other mammals, the enzyme is mitochondrial (Mihalik & Rhead, 1991). In addition to being an intermediate in lysine catabolism in the brain, pipecolic acid may have a role in potentiating GABAergic neurotransmission (section 5.5; Zabriskie, 1996).

Streptomyces spp. synthesize rapamycin and other macrolactones that are potent antifungal and immunosuppressive agents containing pipecolic acid. In these organisms, pipecolic acid is synthesized by a single enzyme – lysine cyclodeaminase – which is not a flavoprotein but uses NAD. The reaction sequence is essentially the same as that shown for the mammalian synthesis of pipecolic acid in Figure 6.5, but the initial oxidative deamination of lysine uses NAD^+ , and the 2-oxo-6-aminocaproic acid cyclizes at the active site of the enzyme. Δ^1 -piperidine 2-carboxylic acid is then reduced to pipecolic acid by the NADH that remains tightly bound to the enzyme (Gatto *et al.*, 2006).

6.2.4 Post-synthetic modification of lysine in proteins

Lysine in proteins can react non-enzymically with glucose and other reducing sugars – the Maillard reaction (Figure 6.6). Much, if not all, of this lysine is biologically unavailable, so reducing the nutritional quality of proteins (section 2.2.4). However, Maillard reaction products that form during the cooking of food provide important flavours. Many of the adverse effects of poor glycaemic control in diabetes mellitus are the result of non-enzymic glycation of amino-terminal amino acids and lysine ϵ -amino groups in tissue and plasma proteins.

The ϵ -amino group of lysine residues in proteins can form isopeptide bonds with carboxyl groups of glutamate and aspartate residues, forming inter- and

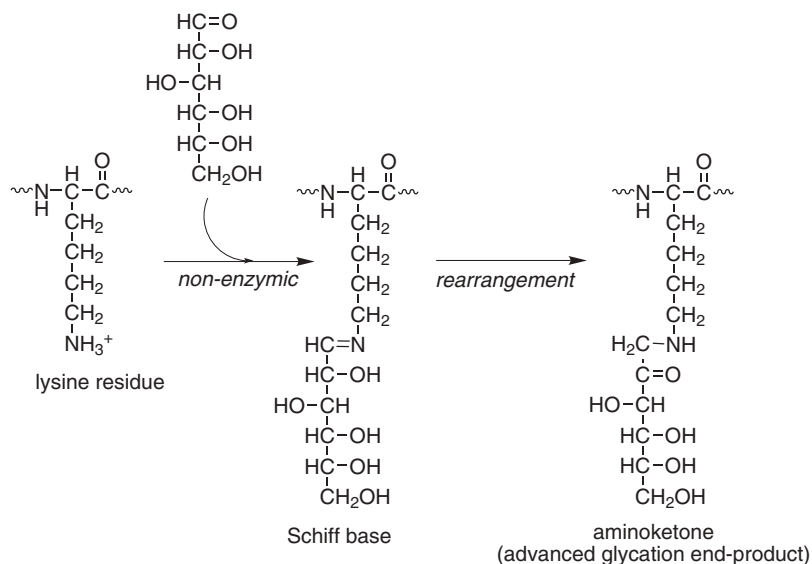


Figure 6.6 The Maillard reaction.

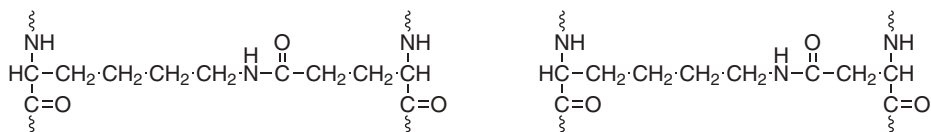


Figure 6.7 Isopeptide links in proteins.

intra-chain covalent links (Figure 6.7). As discussed in section 2.1.1, mammalian digestive enzymes do not hydrolyze isopeptide bonds and, as a result, not only is the lysine itself unavailable, but the isopeptide bond inhibits hydrolysis of adjacent α -peptide bonds by digestive enzymes, thus reducing the digestibility of the protein. If γ -glutamyl- ϵ -lysine di-isopeptides are absorbed, they are substrates for γ -glutamyl cyclotransferase (section 5.4.5), so that some of the isopeptide-linked lysine is biologically available and the nutritional quality of the protein is higher than might be expected.

Lysine and hydroxylysine form the cross-links in collagen and elastin (section 6.2.4.1), and lysine undergoes reversible methylation that is important in regulating histone function and, therefore, gene expression and epigenetic regulation (section 6.2.4.2). As discussed in section 5.8.4, specific lysine residues in eukaryotic initiation factor 5A (eIF-5A) are modified by transfer of the aminobutyl moiety of spermidine onto the ϵ -amino group in the precursor protein to yield deoxyhypusine, followed by hydroxylation to hypusine.

6.2.4.1 Hydroxylysine, lysine aldehyde (allysine) and cross-links in collagen and elastin

Peptide lysyl hydroxylase, like prolyl hydroxylase (section 5.7.2), is an oxoglutarate-dependent oxygenase that requires ascorbate to reduce the active site iron back to Fe^{2+} when it has undergone accidental oxidation to Fe^{3+} by the oxygen it binds. Hydroxylation of lysine residues is both a co- and post-translational event in collagen and elastin synthesis. Some of the hydroxylysine in collagen and elastin is glycosylated with galactose or glucosyl-galactose, and one of the isoenzymes of lysyl hydroxylase (LH3) has three catalytic activities – first lysine hydroxylase, then followed sequentially by galactosyl and glucosyl transferase activities. Unlike the other isoenzymes, which are found only in the endoplasmic reticulum, this isoenzyme is located in both the endoplasmic reticulum and the extracellular matrix in some tissues (Maki, 2009; Myllyla *et al.*, 2007).

Lysyl oxidase and the lysyl oxidase-like proteins catalyze oxidative deamination of lysine and hydroxylysine residues to the ϵ -aldehyde, aminoadipic semialdehyde (allysine) and hydroxyaminoadipic semialdehyde (hydroxyallysine). They are copper-containing quinoproteins, with lysyl tyrosylquinone (section 9.5) as the redox cofactor. The aldehydes then undergo non-enzymic condensation with either other lysine aldehyde residues or unmodified lysine residues to form inter- and intra-chain cross-links in proteins, as shown in Figure 6.8.

Lysyl oxidase is secreted as an inactive precursor protein that undergoes two post-synthetic modifications in the extracellular matrix – hydrolysis of the propeptide to reveal the catalytic site, and autocatalytic modification to form the lysyl tyrosylquinone prosthetic group (see section 9.5). The propeptide region is essential for association of the enzyme with collagen fibrils, and the same protease catalyzes cleavage of both the propeptide of the enzyme and also the telopeptides of the collagen fibrils. The ϵ -amino group of lysine in the substrate protein forms a Schiff base to the quinone prosthetic group, followed by oxidation of the lysine residue and reduction to the quinol, release of the aldehyde, and finally oxidation of the quinol using molecular oxygen, forming hydrogen peroxide and hydrolysis of the quinone imine to release ammonium. The hydrogen peroxide produced by the reaction acts as a chemo-attractant for peripheral mononuclear leukocytes and vascular smooth muscle cells (Knott & Bailey, 1998; Lucero & Kagan, 2006; Uzel *et al.*, 2000).

Mature collagen contains relatively fewer reducible cross-links between two peptide chains and a number of cross-links between three peptide chains, as shown in Figure 6.9. Lysyl pyridinolone is formed by reaction between lysinonorleucine and a hydroxyallysine residue. Hydroxylysine pyridinolone and the pyrrole cross-link are formed by reaction between hydroxylysine-oxonorleucine (Figure 6.8) and a hydroxyallysine residue (Knott & Bailey, 1998).

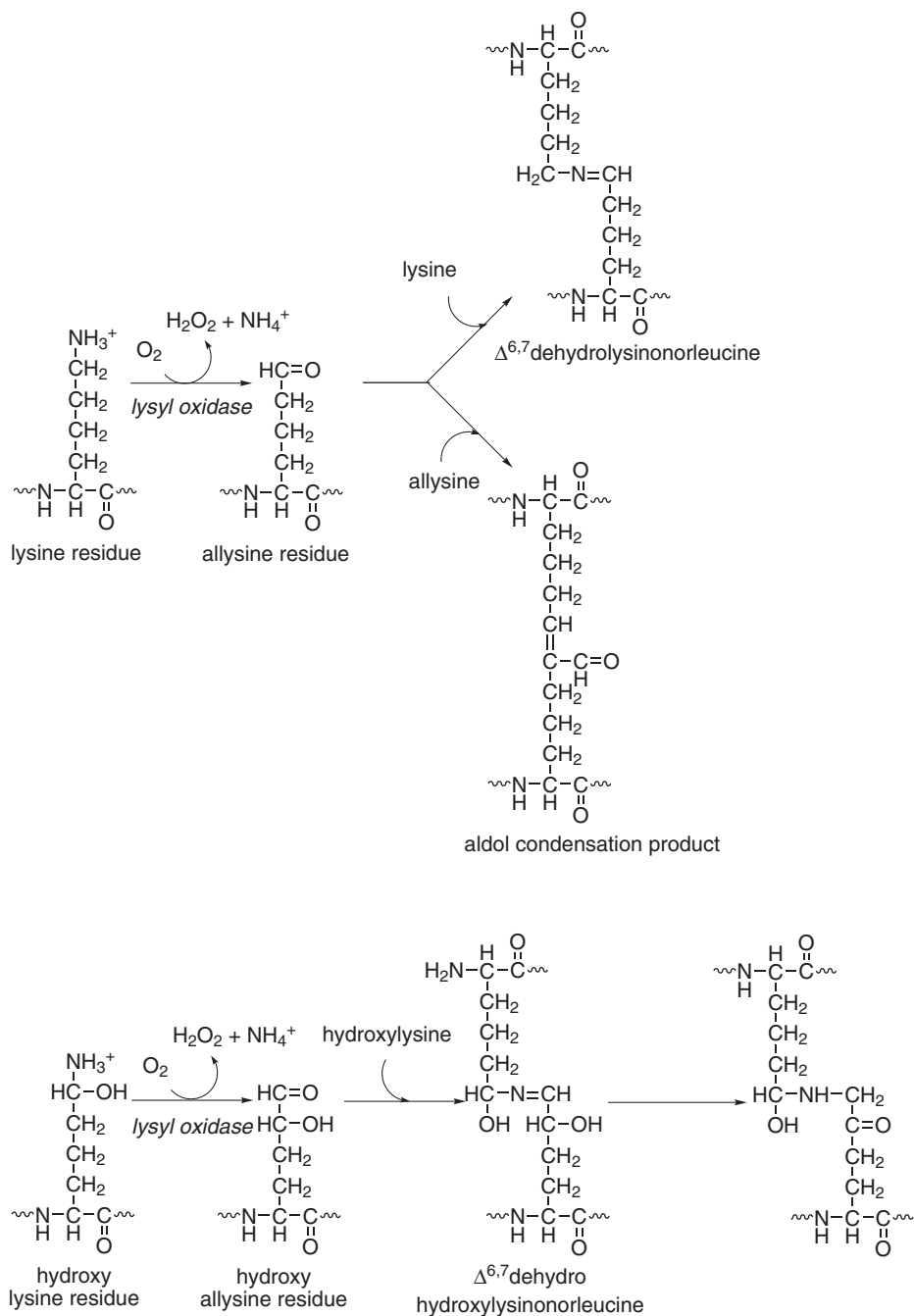


Figure 6.8 Lysine-derived cross-links in collagen.

Lysyl oxidase EC 1.4.3.13.

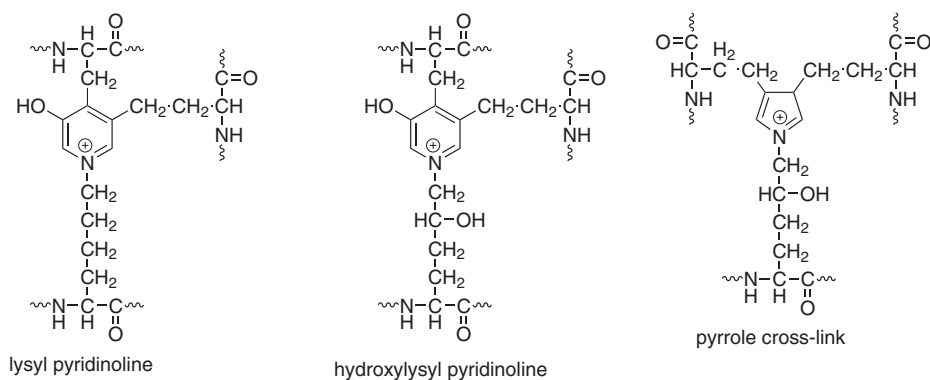


Figure 6.9 Three-way lysine-derived cross-links.

Elastin is considerably more cross-linked than collagen, with some 5–16 lysine residues per 100 amino acids involved in the formation of inter- and intra-chain cross-links. In addition to dehydrolysinonorleucine cross-links, elastin has more complex cross-links involving the formation of desmosine and isodesmosine by reaction between four lysine or allysine residues on separate peptide chains (Figure 6.10).

Lathyrism is a condition in which the cross-linking of collagen and elastin is inhibited, so that collagen is more soluble than normal and elastin less elastic. It is commonly fatal, because inelasticity of the elastin-rich region of the aorta near the heart leads to aortic rupture. A number of compounds that inhibit lysyl oxidase cause lathyrism, including β -aminopropionitrile, the toxin in the sweet pea (*Lathyrus* spp.), from which the condition derives its name. *Lathyrus* spp. are especially resistant to drought, and it is not uncommon to encounter outbreaks of lathyrism in drought-stricken regions, since these peas provide more or less the sole source of food.

In experimental animals, copper deficiency also leads to lathyrism, and aortic rupture is the cause of death in Menkes disease, which is a functional copper deficiency due to a genetic defect of the copper transport protein. In both copper deficiency and Menkes disease, bones are also abnormally fragile as a result of impaired cross-linking of collagen (Barrow *et al.*, 1974).

A number of proteins other than collagen and elastin are also substrates for lysyl oxidase, and the active enzyme can re-enter cells from the extracellular matrix. It is located in the nucleus and oxidizes a small number of lysine residues in histones, suggesting a role in the regulation of gene expression.

6.2.4.2 Methyl lysine Post-synthetic methylation of lysine residues in the amino-terminal tail of histones is a major epigenetic modification. Six different lysine residues in histones H3 and H4 are known to undergo methylation,

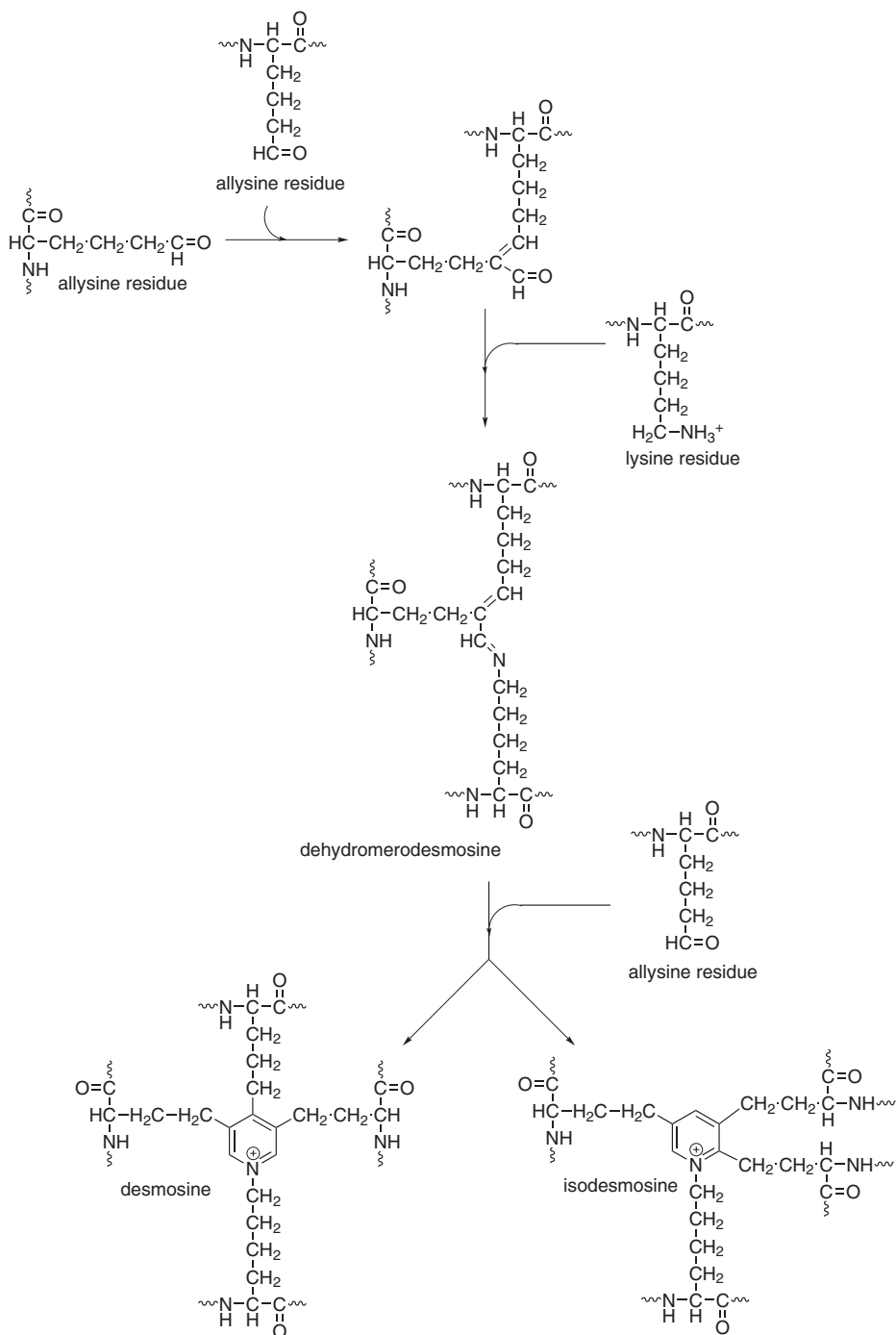


Figure 6.10 Formation of desmosine and isodesmosine in elastin.

yielding mono- di- and trimethyllysine. Unlike histone acetylation, which generally leads to transcriptional activation, methylation may result in increased or decreased transcription depending on both the site and the extent of methylation. A number of other proteins also undergo post-synthetic trimethylation of lysine residues, including actin, myosin, cytochrome c and calmodulin (Hitchler & Domann, 2007; Martin & Zhang, 2005).

The methylation of histone lysine residues is reversible. Both mono- and dimethyllysine residues (but not trimethyllysine) are substrates for lysine demethylases, which are flavoproteins that are nuclear homologues of amine oxidases. The methyl group is oxidized to formaldehyde and, at least *in vitro*, the reaction is coupled to reduction of oxygen to hydrogen peroxide, although there may be an alternative electron acceptor *in vivo*.

There is a separate family of histone demethylases that also catalyze oxidation of the methyl group to formaldehyde, but linked to oxidation of 2-oxoglutarate to succinate, as in lysyl and prolyl hydroxylases (section 5.7.2). Some of these act on trimethyllysine as well as the mono- and dimethyl derivatives. All of these demethylases have considerable specificity for which methyllysine residues they act on (Forneris *et al.*, 2009; Mosammamaparast & Shi, 2010). Trimethyllysine arising from the catabolism of methylated proteins is the precursor for carnitine synthesis (section 6.2.5)

6.2.4.3 Pyrrolysine As shown in Figure 6.11, pyrrolysine (Pyl) is lysine linked by an ϵ -peptide bond to 4-methyl-pyrroline-5-carboxylate. It is considered to be the 22nd amino acid incorporated in ribosomal protein synthesis (the 21st is selenocysteine – see section 4.9). Pyrrolysine is found in

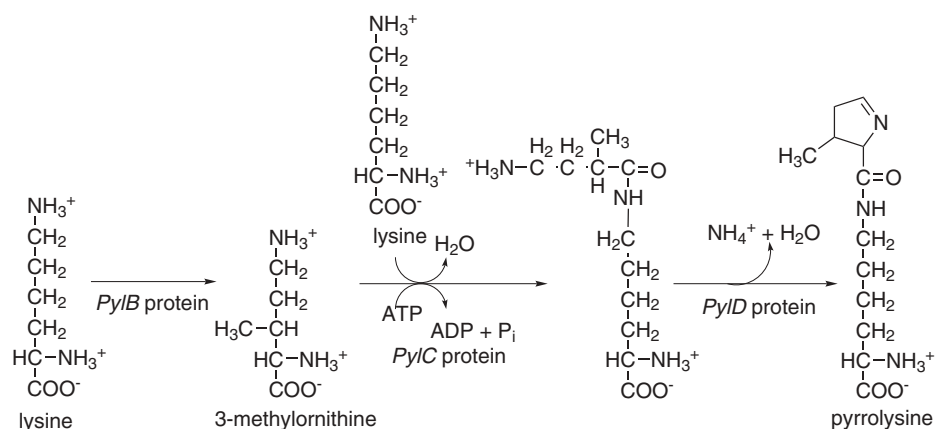


Figure 6.11 Pyrrolysine synthesis.

The enzymes involved have not been characterized or assigned EC numbers; they are the products of the *pyr-B*, *C* and *D* genes.

methylamine methyltransferases in methanogenic archaea and a small number of Gram-positive bacteria, where there is the amber (UAG) stop codon in the open reading frame.

Unlike selenocysteine, which is synthesized from serine esterified to tRNA^{Sec}, preformed pyrrolysine is esterified to tRNA^{Pyl}, and the charged tRNA acts as a suppressor of the stop codon, permitting incorporation of pyrrolysine and continued protein synthesis. The *pyl* operon has been cloned into *E. coli*, and pyrrolysine can be incorporated into *E. coli* proteins where a UAG stop codon has been inserted, because of the suppressor activity of Pyl-tRNA^{Pyl}. (Gaston *et al.*, 2011a; Hao *et al.*, 2002; Namy *et al.*, 2007; Srinivasan *et al.*, 2002).

Pyrrolysine is synthesized from two molecules of lysine. Three gene products have been identified in the reaction shown in Figure 6.11, although the enzymes have not been characterized or assigned EC classification numbers. The *pyl*-B gene product catalyzes a lysine mutase reaction to yield 3-methylornithine. Like lysine aminomutase, this is a radical SAM enzyme, using S-adenosylmethionine to generate a radical at the active site (section 6.3.5). The *pyl*-C gene product catalyzes the ATP-dependent condensation of 3-methylornithine with a second molecule of lysine. The resultant dipeptide undergoes dehydration and deamination catalyzed by the *pyl*-D gene product, forming pyrrolysine (Gaston *et al.*, 2011b).

6.2.5 Carnitine

The main function of carnitine is in the transport of long-chain fatty acids into the mitochondrion for β -oxidation. There are two isoenzymes of carnitine palmitoyl transferase: CPT I, embedded in the outer face of the outer mitochondrial membrane; and CPT II, which is less tightly associated with the matrix face of the inner mitochondrial membrane.

As shown in Figure 6.12, at the outer face of the mitochondrion, CPT I catalyzes the transfer of long-chain fatty acyl groups from CoA onto carnitine. The acyl carnitine translocase in the mitochondrial membranes only transports long-chain fatty acyl carnitine inwards in exchange for either free carnitine or short-chain acyl carnitine. At the inner face of the inner mitochondrial membrane, CPT II catalyzes the transfer of long-chain fatty acyl groups from carnitine onto CoA, ready to undergo β -oxidation.

The dependence of the translocases on counter-transport of long-chain acyl carnitine with free or short-chain acyl carnitine provides regulation of the uptake of long-chain fatty acids into the mitochondrion. If CoASH is not available to accept fatty acyl groups in the matrix, then carnitine cannot be freed to be transported out, so long-chain fatty acid carnitine cannot be transported inwards in excess of requirements for β -oxidation (Kerner & Hoppel, 2000; Strijbis *et al.*, 2010; Vaz & Wanders, 2002).

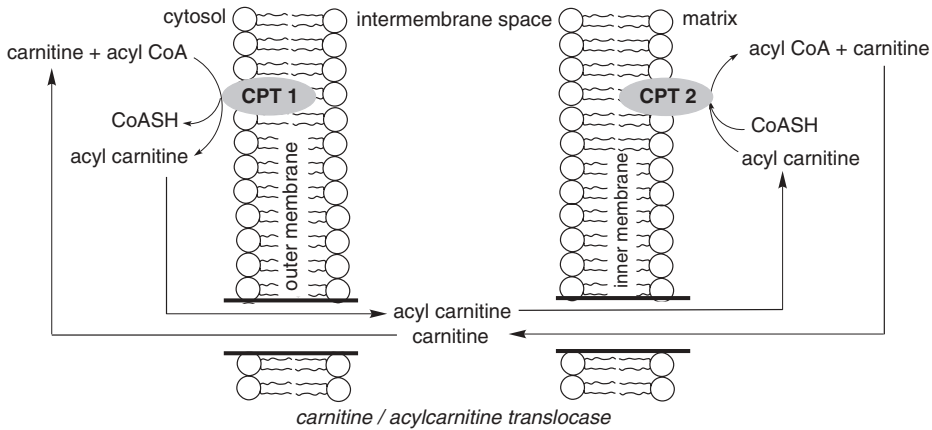


Figure 6.12 The role of carnitine and carnitine palmitoyltransferases in the mitochondrial uptake of fatty acids.

Carnitine palmitoyltransferase EC 2.3.1.21, medium chain fatty acids are transferred using carnitine acyltransferase, EC 2.3.1.137.

Carnitine palmitoyltransferase I is inhibited by malonyl CoA, the precursor for fatty acid synthesis. In tissues that synthesize fatty acids, this serves to prevent mitochondrial uptake and oxidation of fatty acids when they are being synthesized. Muscle, which does not synthesize fatty acids, also has active acetyl CoA carboxylase and malonyl CoA decarboxylase. These two enzymes are regulated reciprocally in response to 5'AMP, and they serve to regulate fatty acid uptake into mitochondria for oxidation (Saggerson, 2008).

In addition to this role in transporting long-chain fatty acids into the mitochondrion, carnitine is used to esterify short-chain fatty acids, especially abnormal acyl CoA metabolites such as propionyl CoA that accumulates in propionyl CoA carboxylase deficiency, and the metabolites of a number of drugs, including valproic acid. Esterification to carnitine serves to liberate CoASH, and the acyl carnitine esters are excreted in the urine. However, this leads to urinary excretion of abnormally large amounts of acyl carnitine esters, which can deplete tissue reserves of carnitine, leading to carnitine deficiency. Acetyl CoA produced in peroxisomes by β -oxidation of very long-chain fatty acids is transported to mitochondria as acetyl carnitine. In high intensity exercise, when the rate of formation of acetyl CoA in type I muscle fibres is greater than the rate of its oxidation, carnitine acts as a buffer for the excess acetyl moieties (Stephens *et al.*, 2007).

There are no known inborn errors of carnitine biosynthesis, but carnitine deficiency can occur as a result of defects in the carnitine transporter protein, leading to loss in the urine and failure of uptake into muscle and other tissues. In many cases, treatment with carnitine supplements, plus feeding a

high-carbohydrate, low-fat diet to minimize the need for fatty acid oxidation, is successful (Kerner & Hopfel, 1998).

The precursor for carnitine synthesis is trimethyllysine released from proteins when they undergo lysosomal or proteasomal catabolism (section 6.2.4.2). There is no specific precursor protein, and all the available evidence suggests that the availability of trimethyllysine from protein catabolism is limiting for carnitine synthesis.

The main dietary sources of carnitine are meat and dairy products. In omnivores, about 75 per cent of carnitine is from the diet and the remainder from endogenous synthesis; vegetarians are more reliant on endogenous synthesis. Between 30–50 per cent of the trimethyllysine released from protein catabolism is used for carnitine synthesis, with the remainder being excreted in the urine.

As shown in Figure 6.13, there are four steps in the synthesis of carnitine from trimethyllysine: hydroxylation to 3-hydroxy-trimethyllysine; aldol cleavage to γ -butyrobetaine aldehyde; dehydrogenation to γ -butyrobetaine; and a further hydroxylation to yield carnitine (Kaufman & Broquist, 1977; Strijbis *et al.*, 2010; Vaz & Wanders, 2002).

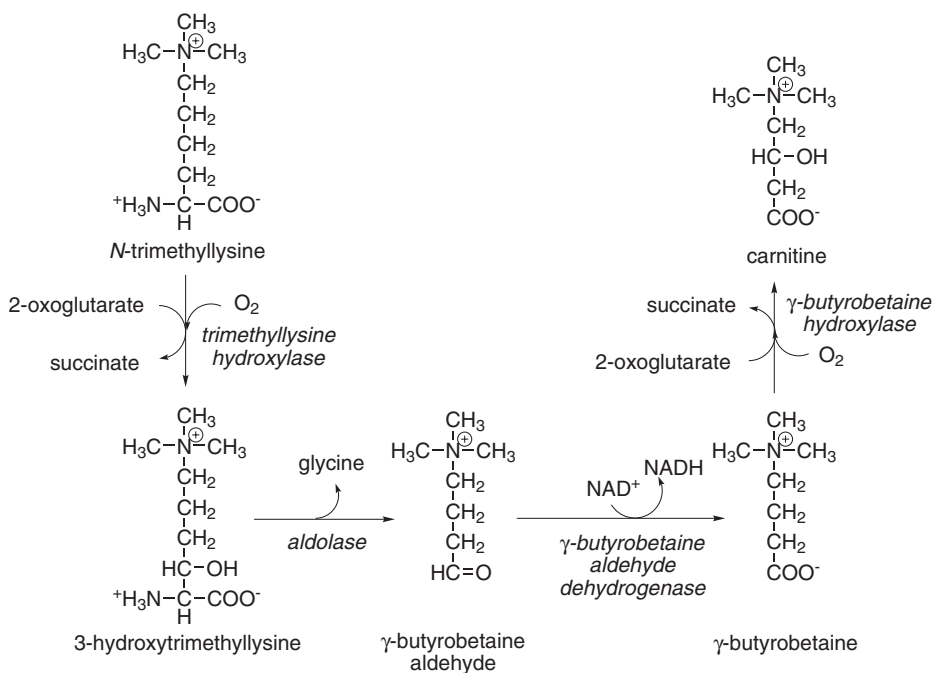


Figure 6.13 Carnitine biosynthesis.

Trimethyllysine hydroxylase EC 1.14.11.8, 3-hydroxytrimethyllysine aldolase has no EC number, γ -butyrobetaine aldehyde dehydrogenase EC 1.2.1.47, γ -butyrobetaine hydroxylase EC 1.14.11.1.

Trimethyllysine hydroxylase is a mitochondrial enzyme, and γ -butyrobetaine hydroxylase (like the other enzymes of carnitine biosynthesis) is cytosolic. Both enzymes are iron-containing mixed-function oxidases that, like prolyl and lysyl hydroxylases, also catalyze the oxidation of 2-oxoglutarate to succinate and require ascorbate to reduce the iron when it is accidentally oxidized by reaction with molecular oxygen (section 5.7.2). Trimethyllysine and γ -butyrobetaine hydroxylases have considerable sequence homology with each other, but little with the other 2-oxoglutarate-dependent hydroxylases, and they seem to represent a distinct family of enzymes.

Although liver contains all the enzymes for carnitine biosynthesis, the main site for synthesis of γ -butyrobetaine is the kidney, which exports γ -butyrobetaine for uptake by the liver and hydroxylation to carnitine (Vaz & Wanders, 2002).

It is unclear whether or not there is a specific aldolase to catalyze the cleavage of trimethyl hydroxylysine to γ -butyrobetaine aldehyde. Both threonine and serine aldolases, and serine hydroxymethyltransferase (section 4.2.1), also catalyze this reaction.

Both carnitine and acyl carnitine esters are excreted in the urine, although there is efficient re-absorption of free carnitine from the glomerular filtrate to ensure an adequate supply of carnitine for tissues. There is no mammalian catabolism of carnitine, but intestinal bacteria ferment it to trimethylamine, which is absorbed and oxidized; trimethylamine oxide is excreted in the urine. Rare children who lack trimethylamine oxidase excrete volatile trimethylamine. Trimethylamine is the major odorant in rotten fish, and the condition of trimethylaminuria is also known as ‘fish odour syndrome’ (Hernandez *et al.*, 2003; Zhou & Shephard, 2006).

6.3 Methionine and cysteine

In addition to its role in protein synthesis, methionine, as the *S*-adenosyl derivative, is the major source of methyl groups for one carbon transfer reactions. Decarboxylation of *S*-adenosylmethionine is important in synthesis of the polyamines (section 5.8), and *S*-adenosylmethionine is also the precursor for synthesis of the plant hormone ethylene (section 6.3.4) and provides a catalytic 5′deoxyadenosine in some enzymes (section 6.3.5). Cysteine is the limiting precursor for glutathione synthesis (section 5.4.4) and hence is important in maintaining redox homeostasis. It is also the precursor for taurine synthesis (section 6.3.7).

In plants, bacteria, yeasts and fungi, the sulphur for methionine synthesis comes from inorganic sulphide, and this is incorporated into serine to form cysteine via intermediate formation of acetyl serine or, in some organisms, phosphoserine. Cysteine synthase is a bifunctional hetero-oligomer of serine

acetyltransferase and acetylserine (thiol) lyase, a pyridoxal phosphate-dependent enzyme catalyzing a β -replacement reaction via an enzyme-bound aminoacrylate intermediate.

Formation of the cysteine synthase complex occurs via a two-step process: the rapid formation of an 'encounter complex' between the two enzymes is followed by a slow conformational change due to closure of the thiol lyase active site by the carboxy-terminal region of the acetyltransferase. Sulphide ions stabilize the complex, and cysteine is a feedback inhibitor of the acetyltransferase and destabilizes the complex. The rate-limiting step is the acetyltransferase; there is an excess of the thiol lyase in the complex (Salsi *et al.*, 2010; Wirtz & Hell, 2006).

As shown in Figure 6.14, plants, fungi and bacteria synthesize methionine by transsulphuration from cysteine. Cystathionine γ -synthase catalyzes the formation of cystathionine, which is then lysed to homocysteine and pyruvate by β -cystathionase. By contrast, animals synthesize cystathionine by reaction between homocysteine and serine (catalyzed by cystathionine β -synthase), followed by cleavage catalyzed by γ -cystathionase to yield cysteine (section 6.3.3). Cystathionine γ -synthase is a fold type I pyridoxal phosphate-dependent enzyme, while the β -synthase is a fold type II enzyme (section 3.1). Yeasts and some archaea have both cystathionine γ -synthase and β -synthase; they can either synthesize methionine *de novo* or use it for synthesis of cysteine, as in animals (Aitken & Kirsch, 2005).

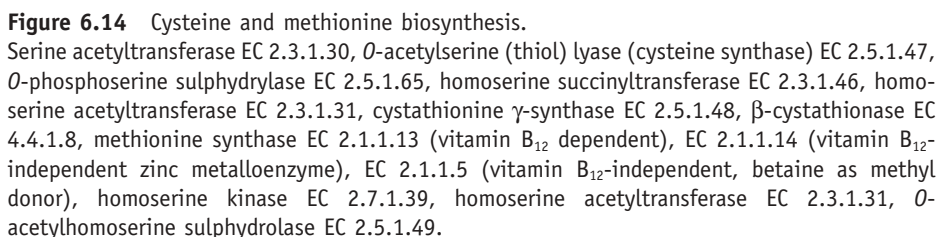
6.3.1 Methionine biosynthesis

The precursor for methionine synthesis in bacteria, yeasts and fungi is homoserine, which undergoes acylation to *O*-succinyl or *O*-acetyl homoserine. By contrast, in plants, the precursor for methionine synthesis is homoserine *O*-phosphate, which is also the immediate precursor for threonine synthesis.

The homoserine succinyl and acetyltransferases are different enzymes, with little or no sequence homology, and the succinyltransferase from *E. coli* does not use acetyl CoA as an acyl donor. However, *B. cereus* has an enzyme that is, by its sequence, a succinyltransferase, but uses acetyl CoA rather than succinyl CoA. The extremophile *Thermotoga maritima* also has an enzyme that is, by its sequence, a succinyltransferase, but it uses acetyl CoA with 30-fold greater efficiency than succinyl CoA.

Both succinyl and acetyltransferases have 'ping-pong' mechanisms, with the acyl CoA binding first and acylating an acceptor residue in the enzyme before the release of CoASH and binding of homoserine. Both acyltransferases are subject to feedback inhibition by methionine and *S*-adenosylmethionine. (Goudarzi & Born, 2006; Zubieta *et al.*, 2008).

In most microorganisms, homoserine succinyltransferase is inhibited synergistically by methionine and *S*-adenosylmethionine. By contrast, homoserine



acetyltransferase in organisms such as *C. glutamicum* is not inhibited by either. The yeast acetyltransferase is inhibited by *S*-adenosylmethionine, but there is no synergy with methionine. In *N. crassa*, the acetyltransferase is not subject to regulation. Unusually, in this organism it is the second enzyme of the pathway, cystathionine γ -synthase, that is subject to feedback inhibition by *S*-adenosylmethionine, and this inhibition is antagonized by methyltetrahydrofolate (see section 6.3.2). Homoserine succinyltransferase in *E. coli* is a short-lived enzyme. Like many mammalian regulatory enzymes (but unlike most bacterial regulatory enzymes), it is subject to rapid energy-dependent proteolysis (Biran *et al.*, 2000; Curien *et al.*, 2008).

In plants, it is the availability of homoserine, rather than the activity of homoserine kinase, that is rate-limiting for the synthesis of threonine and methionine. The flux of homoserine *O*-phosphate to either cystathionine (and hence methionine) or threonine is controlled by activation of threonine synthase by *S*-adenosylmethionine. Thus, at times of high methionine availability, homoserine phosphate is used for synthesis of threonine and then isoleucine. In the absence of *S*-adenosylmethionine, the pyridoxal phosphate of the plant enzyme is distant from the catalytic residues of the active site; binding of *S*-adenosylmethionine causes a large conformational change in the active site, and it also increases the affinity of threonine synthase for its substrate (Amir *et al.*, 2002; Mas-Droux *et al.*, 2006).

6.3.1.1 Cystathionine γ -synthase and cystathionine β -lyase Although cystathionine γ -synthase is the first enzyme unique to methionine synthesis in plants, it is not subject to feedback inhibition. However, in at least some plants, the enzyme is subject to regulation by *S*-adenosylmethionine at the level of mRNA stability. A highly conserved sequence of 11–13 amino acids in the amino-terminal region, coded for by the first exon, binds *S*-adenosylmethionine and then reduces the stability of the mRNA. This sequence is absent from bacterial cystathionine γ -synthase and is not necessary for activity of the plant enzyme. Transgenic plants expressing a truncated enzyme that lacks this *S*-adenosylmethionine-binding region over-synthesize methionine, but they do not accumulate it; rather, they produce large amounts of dimethyl sulphide and carbon disulphide. This suggests that, as well as regulation of synthesis, tissue methionine is controlled by increasing its catabolism (Amir *et al.*, 2002; Hesse & Hoefgen, 2003).

The main pathway for methionine synthesis from homoserine *O*-phosphate (in plants) and acetyl- or succinylhomoserine (in bacteria, yeasts and fungi) is synthesis of cystathionine, catalyzed by cystathionine γ -synthase. In some microorganisms, however, there is direct replacement of the acetyl group of acetylhomoserine by inorganic sulphide, catalyzed by *O*-acetylhomoserine sulphydrylase. In some organisms, both the cystathionine and sulphydrylase pathways are used for methionine synthesis, and both are subject to feedback

regulation by methionine and *S*-adenosylmethionine. In yeast (and possibly other organisms), the same sulphhydrylase catalyzes the incorporation of inorganic sulphide into *O*-acetylhomoserine to yield homocysteine, and also into *O*-acetylserine to yield cysteine. Plant cystathionine γ -synthase can use inorganic sulphide instead of cysteine to a limited extent (Hesse & Hoefgen, 2003; Yamagata, 1989).

Hydrolysis of cystathionine catalyzed by cystathionine β -lyase leads to the release of homocysteine, which is then methylated by methionine synthase, using methyl tetrahydrofolate as the methyl donor. Plants use triglutamyl folate, while bacteria, yeasts and fungi use the monoglutamate derivative (Hesse & Hoefgen, 2003).

6.3.1.2 Methionine synthase There are two families of methionine synthases. In animals, bacteria that synthesize vitamin B₁₂, and those (such as *E. coli*) that take it up from other (enteric) bacteria, the enzyme has zinc and methyl cobalamin at the active site. However, in plants and bacteria that neither synthesize nor take up vitamin B₁₂, the enzyme is a zinc metalloprotein that does not require any other cofactor and has little or no sequence homology with the cobalamin-dependent enzymes (Pejchal & Ludwig, 2005). Some microorganisms contain both cobalamin-dependent and independent methionine synthases.

In plants, homocysteine is synthesized in the chloroplasts, but methionine synthase is a cytosolic enzyme. Methylation to methionine is the only route for the removal of potentially cytotoxic homocysteine in plants, which lack the transsulphuration pathway to form cysteine from methionine (section 6.3.3).

The cobalamin-dependent methionine synthase binds homocysteine and methyl tetrahydrofolate, then catalyzes transfer of the methyl group from the enzyme-bound methylcobalamin onto homocysteine, followed by remethylation of the cobalamin cofactor at the expense of methyl tetrahydrofolate. The cobalamin cofactor sometimes undergoes oxidation to Co²⁺ cobalamin, so inactivating the enzyme, which is reactivated by reduction back to Co⁺ cobalamin.

In *E. coli*, this reactivation is catalyzed by methionine synthase itself; the coenzyme is reduced to Co⁺ cobalamin by reaction with flavodoxin, then the reduced cobalamin is trapped as the methyl derivative by methyl transfer from *S*-adenosylmethionine. The enzyme thus catalyzes two different methyl transfer reactions onto cobalamin: from methyl tetrahydrofolate during the normal reaction cycle; and from *S*-adenosylmethionine in reactivation of the oxidized enzyme (Jarrett *et al.*, 1998). Mammalian methionine synthase requires a separate enzyme for reactivation after accidental oxidation.

Methionine synthase reductase is a flavoprotein containing both FAD and FMN, and it uses NADPH as the electron donor (Olteanu & Banerjee, 2001).

Genetic defects of this enzyme are one of the causes of hereditary homocystinuria (section 6.3.3.1).

6.3.1.3 S-Methylmethionine in plants Plants synthesize *S*-methylmethionine by methyl transfer from *S*-adenosylmethionine onto methionine in leaves, and they transport it to developing seeds, where it is demethylated by homocysteine methyltransferase, yielding two mol of methionine. Methionine *S*-methyltransferase is found only in plants, but homocysteine methyltransferase using *S*-methylmethionine as the methyl donor is found in bacteria, yeasts and animals. All of these can therefore utilize *S*-methylmethionine from plants as a source of methionine and an alternative source of methyl groups for remethylation of homocysteine (section 6.3.2).

Human *S*-methylmethionine:homocysteine methyltransferase is highly homologous with betaine:homocysteine methyltransferase (section 6.3.2) and, indeed, the two genes are adjacent on chromosome 5, suggesting that gene duplication has occurred. However, despite the sequence homology, this enzyme uses *S*-methylmethionine as the methyl donor and has very low activity with betaine (Mudd & Datko, 1990; Szegedi *et al.*, 2008).

In plants, *S*-methylmethionine:homocysteine methyltransferase is found in seeds and cotyledons, but not in leaves. In leaves, adenosylmethionine hydrolase, which catalyzes hydrolysis of *S*-adenosylmethionine to homoserine and methylthioadenosine, also catalyzes hydrolysis of *S*-methylmethionine to dimethylsulphide and homoserine. This reaction thus provides a pathway for disposal of the sulphur (and methyl groups) of surplus methionine as volatile dimethylsulphide.

Methionine *S*-methyltransferase also catalyzes methylation of selenomethionine to *S*-methyl selenomethionine, which is a substrate for hydrolysis by adenosylmethionine hydrolase, liberating volatile dimethylselenide. This reaction sequence is important in the removal of excess selenium when plants are grown in selenium-rich soil (Mudd & Datko, 1990).

6.3.2 S-Adenosylmethionine and the methylation cycle

The major metabolic role of *S*-adenosylmethionine is as a source of methyl groups for a wide variety of methyltransferases, and synthesis of creatine (section 5.9.7) and phosphatidylcholine consumes the greater part of the methyl group pool. Most of the methyl groups incorporated into phosphatidylcholine are recovered in the catabolism of choline. As discussed in section 5.8.2, *S*-adenosylmethionine is the precursor for polyamine synthesis, which consumes some 10–30 per cent of the total amount synthesized, and it also has a role in regulation of hepatocyte growth, differentiation and death (Martinez-Lopez *et al.*, 2008; Mato & Lu, 2007).

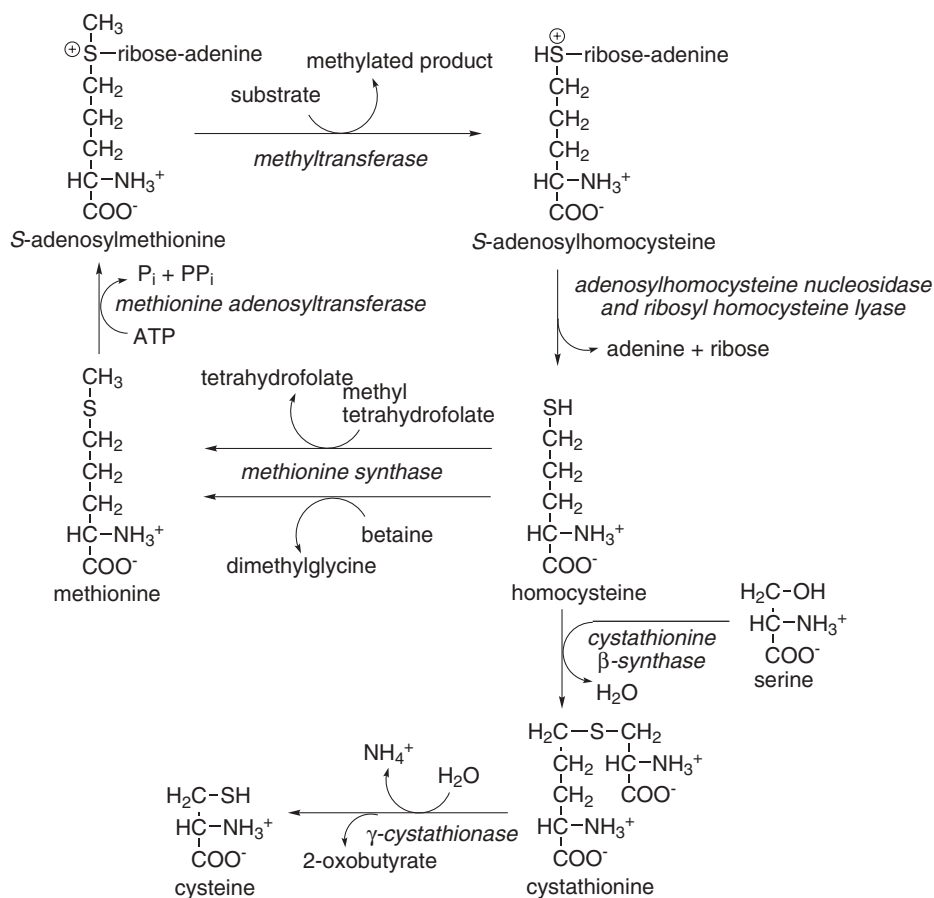


Figure 6.15 Methionine metabolism in mammals – the methionine cycle and the transsulfuration pathway for cysteine biosynthesis.

Methionine adenosyltransferase EC 2.5.1.6, methyltransferases EC 2.1.1.x, adenosylhomocysteine nucleosidase EC 3.2.2.9, ribosylhomocysteine lyase EC 4.4.1.21, methionine synthase EC 2.1.1.13 (vitamin B_{12} dependent), EC 2.1.1.5 (vitamin B_{12} -independent, betaine as methyl donor), cystathionine β -synthase EC 4.2.1.22, γ -cystathionase EC 4.4.1.1.

As shown in Figure 6.15, methionine is activated to *S*-adenosylmethionine by adenosyltransfer from ATP, with *S*-adenosylmethionine as the immediate methyl donor. The resultant *S*-adenosylhomocysteine is then hydrolyzed to homocysteine and adenosine, and homocysteine is remethylated to methionine by methionine synthase. In animals, homocysteine may also be used for the synthesis of cysteine via cystathionine – the transsulfuration pathway (section 6.3.3).

Methionine adenosyltransferase catalyzes attack on ATP by the sulphur of methionine, leading to formation of *S*-adenosylmethionine and triphospho-

phate, which is hydrolyzed by the adenosyltransferase to inorganic phosphate and pyrophosphate. Key sequences of methionine adenosyltransferase are highly conserved across all species, from bacteria to lower eukaryotes and mammals (Gil *et al.*, 1997; Markham & Pajares, 2009; Mato *et al.*, 1997; Mingorance *et al.*, 1997).

There are three mammalian isoenzymes of methionine adenosyltransferase, encoded by two genes. The liver-specific enzyme that can exist as a homodimer or as a homotetramer, with different regulatory and kinetic properties, and the product of a separate gene that is expressed in all tissues. The enzyme that is expressed in all tissues has a low K_m for methionine and is inhibited by *S*-adenosylmethionine. The tetramer of the liver isoenzyme has a moderate K_m for methionine and is also inhibited by *S*-adenosylmethionine. By contrast, the dimeric liver isoenzyme (which is the major form) has a high K_m for methionine, and its activity is increased in response to *S*-adenosylmethionine. This permits rapid clearance of excess methionine to form *S*-adenosylmethionine that will be used to methylate glycine (section 6.3.2.1), providing homocysteine that will be used for cysteine synthesis (section 6.3.3), and then transamination to pyruvate and sulphite (section 6.3.7; Stipanuk, 2004a; Stipanuk *et al.*, 2006).

The immediate product of methyltransferase reactions, *S*-adenosylhomocysteine, is a potent inhibitor of most methyltransferases, and it is rapidly hydrolyzed. The equilibrium of this reaction is in the direction of *S*-adenosylhomocysteine synthesis, and the products are rapidly removed. Adenosine is deaminated by adenosine deaminase (section 1.4.2) and, under normal conditions, homocysteine is either remethylated to methionine or it is used for synthesis of cystathionine.

Homocysteine arising from methyltransferase reactions can be remethylated back to methionine by three mammalian enzymes. Vitamin B₁₂-dependent methionine synthase uses methyl tetrahydrofolate as the methyl donor (section 6.3.1.2). Betaine:homocysteine methyltransferase uses betaine (trimethylglycine) arising from catabolism of choline as the methyl donor, and is vitamin B₁₂-independent. In the liver, this enzyme accounts for as much as 25 per cent of the remethylation of homocysteine. *S*-Methylmethionine:homocysteine methyltransferase (section 6.3.1.3) is only relevant when there is a dietary source of *S*-methylmethionine.

In vitamin B₁₂-deficiency, there is failure of methylation of arginine residues in myelin basic protein, leading to sub-acute combined degeneration of the spinal cord. The central nervous system is especially susceptible, because it only contains methionine synthase and no betaine homocysteine methyltransferase.

S-Adenosylmethionine is a positive effector of cystathionine β -synthase (section 6.3.3) and glycine *N*-methyltransferase (section 6.3.2.1) and a negative effector of methionine synthase and betaine:homocysteine methyltrans-

ferase, so controlling the fate of homocysteine and its intracellular concentration (Finkelstein, 2007).

6.3.2.1 Glycine *N*-methyltransferase At times of high methionine availability, the high- K_m isoenzyme of methionine adenosyltransferase becomes active; its function is to lower the tissue concentration of methionine. The resultant *S*-adenosylmethionine, in excess of requirements for methylation reactions, is used by glycine *N*-methyltransferase, forming sarcosine (*N*-methylglycine) and *S*-adenosylhomocysteine. This provides a sink for excess *S*-adenosylmethionine and permits regulation of the *S*-adenosylmethionine to *S*-adenosylhomocysteine ratio in tissues.

The enzyme is present in large amounts in liver (accounting for 1–3 per cent of cytosolic protein), and is also present in tissues such as proximal renal tubule, intestinal mucosa, exocrine pancreas and others that are active in secretion. Sarcosine has a role as an osmolyte, but otherwise its metabolic function is as a source of methylene groups to form methylene tetrahydrofolate. This may either be used for other folate-dependent reactions (section 4.4 and Figure 4.6), or it may be oxidized to formyl tetrahydrofolate, then to carbon dioxide and free tetrahydrofolate, so eliminating surplus methyl groups (Martinov *et al.*, 2010).

Glycine *N*-methyltransferase is inhibited by methyl tetrahydrofolate. The enzyme is a tetramer that binds two molecules of methyl tetrahydrofolate, each of which interacts with the amino-terminal regions of two subunits, impairing the entry of *S*-adenosylmethionine into the active site. When there is a high concentration of methionine and *S*-adenosylmethionine in the cell, there is no need for remethylation of *S*-adenosylhomocysteine. *S*-adenosylmethionine inhibits methylene tetrahydrofolate reductase, so that there is less methyl tetrahydrofolate available, reducing the inhibition of glycine *N*-methyltransferase and permitting disposal of excess *S*-adenosylmethionine to sarcosine and *S*-adenosylhomocysteine. By contrast, when the concentration of methionine is low, the inhibition of methylene tetrahydrofolate reductase is relieved, more methyl tetrahydrofolate is formed and glycine *N*-methyltransferase is inhibited, so conserving *S*-adenosylmethionine (Luka *et al.*, 2009; Mato *et al.*, 1997; Stipanuk, 2004b).

Rare patients with genetic defects of glycine *N*-methyltransferase develop hypermethioninaemia, with normal homocysteine, suggesting that this enzyme provides a route for catabolism of excess methionine. Knockout mice lacking glycine *N*-methyltransferase are similarly hypermethioninaemic, and they have a 100-fold increase in the ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine in tissues. They develop liver cancer as a result of activation of the Ras and JAK/STAT signalling pathways due to hypermethylation of inhibitors of the pathways. They also show abnormal methylation of histones (Luka *et al.*, 2009).

Further evidence that glycine *N*-methyltransferase provides a pathway for catabolism of surplus methionine comes from patients with genetic defects of sarcosine dehydrogenase. On a normal diet that provides adequate methionine but little in excess of requirements, they excrete little sarcosine but, if they are fed a high-methionine diet, their excretion of sarcosine increases.

6.3.2.2 Megaloblastic anaemia and the methyl folate trap Deficiency of either folate or vitamin B₁₂ leads to megaloblastic anaemia – the release of immature red cell precursors into the circulation. It is easy to see why this occurs in folate deficiency, given the importance of folate derivatives in purine and pyrimidine synthesis, and hence in DNA synthesis (see sections 1.4.1 and 1.4.3). It is less easy to explain the effect of vitamin B₁₂ deficiency on erythropoiesis, since no vitamin B₁₂-dependent enzyme is involved in nucleotide metabolism (the only mammalian vitamin B₁₂-dependent enzymes are methionine synthase and methylmalonyl CoA mutase).

The reduction of methylene tetrahydrofolate to methyl tetrahydrofolate is irreversible. The only pathway for utilization of methyl tetrahydrofolate, and hence the liberation of free folate, is the methionine synthase reaction. This means that in vitamin B₁₂ deficiency, there is secondary functional folate deficiency, because folate is trapped as methyl folate that cannot be used in the absence of vitamin B₁₂ (Chanarin *et al.*, 1985; Krebs *et al.*, 1976).

6.3.2.3 Methionine γ -lyase In plants, methionine γ -lyase is induced by methionine. It catalyzes the first step of methionine catabolism, forming methanethiol (methyl mercaptan), 2-oxobutyrate (the precursor for isoleucine synthesis – see section 7.1) and ammonium. Methanethiol can react with serine to form *S*-methylcysteine, which provides storage for both methyl groups and fixed sulphur. In seeds, the mRNA for methionine γ -lyase is highly expressed but not translated; when seeds imbibe water, the mRNA is translated and the protein is highly expressed, suggesting that it has a role in germination.

Methionine γ -lyase is a pyridoxal phosphate-dependent enzyme, catalyzing a β - γ elimination reaction. It is found in a wide variety of microorganisms, including oral bacteria that cause halitosis because of the volatile methanethiol. The bacteria and yeasts associated with cheese ripening also express methionine γ -lyase and form a variety of volatile sulphur compounds from methanethiol. In anaerobic bacteria, the 2-oxobutyrate formed by methionine γ -lyase is an important energy-yielding metabolite; methanethiol is involved in the pathogenicity of some bacteria, and it may provide plants with protection against herbivore attack.

Methionine γ -lyase is not a human enzyme, so it is a potential drug target, and the recombinant enzyme has potential value in cancer chemotherapy as a targeted enzyme to deplete tumour reserves of methionine. Trifluorome-

thionine (trifluoromethyl homocysteine) is an antibacterial and anti-parasite drug that is a substrate for methionine γ -lyase, forming trifluoromethanethiol. The latter is converted non-enzymically to carbonothionic difluoride, which cross-links amino groups of proteins and is therefore toxic to the organisms that metabolize the prodrug (Amadasi *et al.*, 2007; Rebeille *et al.*, 2006; Sato & Nozaki, 2009).

6.3.3 Transsulphuration and cysteine synthesis in animals

Other than remethylation to methionine, the fate of homocysteine in animals is the transsulphuration pathway shown in Figure 6.15: condensation of homocysteine with serine to form cystathionine, catalyzed by cystathionine β -synthase, followed by hydrolysis catalyzed by γ -cystathionase to yield cysteine and 2-oxobutyrates. This is the reverse of the reaction sequence for methionine synthesis, as shown in Figure 6.14. Both cystathionine β -synthase and γ -cystathionase are pyridoxal phosphate-dependent enzymes, and elevated plasma homocysteine and urinary excretion after a test dose of methionine has been used as a test of functional vitamin B₆ nutritional status (Linkswiler, 1981). Cystathionine β -synthase also catalyzes a β -elimination reaction, leading to the formation of hydrogen sulphide (see section 6.3.6).

The 2-oxobutyrates formed by γ -cystathionase undergoes oxidative decarboxylation to propionyl CoA and enters the citric acid cycle after carboxylation and isomerization to succinyl CoA. Both the pyruvate and 2-oxoglutarate decarboxylase complexes catalyze the oxidative decarboxylation of 2-oxobutyrates (Stipanuk, 2004b; Stipanuk *et al.*, 2006).

Human cystathionine β -synthase is possibly unique among pyridoxal phosphate-dependent enzymes, as it is also a haem protein. There are three clear domains in the enzyme: a carboxy-terminal regulatory region; a middle catalytic domain; and the haem-containing amino-terminal domain. The availability of *S*-adenosylmethionine regulates the activity of the enzyme so that, when there is little *S*-adenosylmethionine available, little or no homocysteine enters the transsulphuration pathway, but most is remethylated to methionine.

S-adenosylmethionine binds to the inhibitory carboxy-terminal domain of the enzyme, causing a conformational change that increases its activity. When cells in culture are exposed to TNF α , and when mice are treated with bacterial lipopolysaccharide (both of which are sources of chronic oxidative stress), a highly active truncated form of cystathionine synthase is expressed, lacking the carboxy-terminal regulatory region. Cystathionine β -synthase from yeast and protozoa does not have the haem-binding domain, and is not activated by *S*-adenosylmethionine (Banerjee *et al.*, 2003; Stipanuk, 2004b; Stipanuk *et al.*, 2006).

The haem domain does not resemble any other known haem binding protein, and it serves to modulate the activity of the enzyme, increasing its activity in response to oxidative stress, when the haem iron is oxidized. This leads to increased availability of cysteine for synthesis of glutathione (section 5.4.4) and, hence, increased antioxidant protection. Cells in culture exposed to oxidative stress show increased metabolic flux through the transsulphuration pathway, while those provided with antioxidants show reduced flux (Banerjee & Zou, 2005).

Cystathionine β -synthase expression is increased in response to glucagon (and other factors that increase intracellular cAMP), and expression is down-regulated in response to insulin. This allows conservation of methionine for protein synthesis in the fed state, and catabolism to provide succinyl CoA as a gluconeogenic substrate in fasting. The K_m of the enzyme is an order of magnitude higher than that of methionine synthase or homocysteine methyltransferase so that, at normal levels of methionine, transsulphuration is secondary to maintaining the intracellular pool of methionine by remethylation of homocysteine. High concentrations of *S*-adenosylmethionine both inhibit remethylation and also activate the transsulphuration pathway.

6.3.3.1 Homocystinuria, hyperhomocysteinaemia and cardiovascular disease

Rare genetic defects of cystathionine β -synthase, methionine synthase and methionine synthase reductase are all causes of homocystinuria. The commonest cause is a mutation in cystathionine β -synthase, and about half the cases are due to low affinity of the enzyme for its coenzyme, pyridoxal phosphate. Affected children can be treated successfully with supplements of vitamin B₆ of the order of 250–500 mg/day (compared with reference intakes of 1.5–2 mg/day). Children who are unresponsive to vitamin B₆ are treated with a diet that provides minimal methionine to meet requirements and supplements of cysteine, which is now an essential amino acid because they are unable to synthesize it by transsulphuration.

Children with homocystinuria suffer multiple thromboses and, if untreated, they commonly die in their teens. There are abnormalities of collagen cross-linking (section 6.2.4.1) because of chelation of copper by homocysteine (the oxidized dimer of homocysteine) and the cysteine-homocysteine dimer, leading to fragility of the long bones (Clayton, 2006; Finkelstein, 2006; Yap, 2003).

A number of epidemiological studies during the 1990s identified moderate elevation of plasma homocysteine (significantly lower than is seen in homocystinuric children) as an independent risk factor for cardiovascular disease, atherosclerosis and stroke (D'Angelo & Selhub, 1997; Herrmann, 2001; Selhub *et al.*, 2000). As shown in Table 6.1, homocysteine has a variety of potential atherogenic, hypertensive and procoagulant actions (Herrmann, 2001; Perla-Kajan *et al.*, 2007; Stamler & Slivka, 1996; Welch & Loscalzo, 1998).

Table 6.1 Adverse effects of hyperhomocysteinaemia.**Atherogenic**

Especially in the presence of transition metal ions, homocysteine can undergo redox cycling $\rightarrow \cdot\text{O}_2^-$ – hence possibility of oxidative damage to LDL.

May react with cysteine-SH groups and modify apo-lipoproteins – hence impaired uptake by LDL receptors.

Hypertensive

Reacts with $\cdot\text{NO} \rightarrow S$ -nitrosohomocysteine. This reacts with O_2
 $\rightarrow$ homocysteine + $\text{NO}_3^- + \cdot\text{O}_2^-$.

- generation of $\cdot\text{O}_2^-$ and loss of vasodilation action of $\cdot\text{NO}$.

Procoagulant

Inhibits or down-regulates anticoagulants:

- decreases prostacyclin synthesis;
- decreases activation of protein C;
- decreases thrombomodulin expression;
- suppresses the expression of heparan sulphate;
- decreases fibrinolysis.

Activates procoagulants:

- increased Factor V activity;
- increased tissue clotting factor activity.

Causes desquamation of vascular endothelium and impairs regeneration:

- endothelium has anti-coagulant activity.

Causes proliferation of vascular smooth muscle:

- vascular smooth muscle, when exposed, has potent pro-coagulant activity because of tissue clotting factor.

Increases platelet coagulability and so activates platelet aggregation.

Connective tissue abnormalities

Inhibition of lysyl oxidase

$\rightarrow$ disturbance of collagen and elastin cross-linking.

In the presence of homocysteine, fibroblasts produce excessively sulphated proteoglycans:

- the product is granular rather than fibrillar.

Excessively sulphated proteoglycans will attract and bind ϵ -amino groups of lysine in lipoproteins.

Deficiencies of vitamins B_6 , B_{12} or folate are all associated with elevated plasma homocysteine – vitamin B_6 deficiency as a result of impaired activity of cystathionine synthase, and folate and vitamin B_{12} deficiency as a result of impaired activity of methionine synthase. There is a genetic polymorphism affecting methylene tetrahydrofolate reductase, the enzyme that reduces methylene tetrahydrofolate to methyl tetrahydrofolate. People who are homozygous for the thermolabile variant of the enzyme (which is unstable

in vivo) have low activity of the enzyme, impaired ability to remethylate homocysteine to methionine and elevated plasma homocysteine. This can be overcome by increased folic acid intake, possibly because the enzyme is more stable in the presence of its substrate or, more likely, because most dietary folate is converted to methyl tetrahydrofolate in the intestinal mucosa. Intestinal mucosal cells turn over within about 48 hours, so an unstable enzyme is of little relevance and, with a high intake of folate, there is continuing provision of methyl tetrahydrofolate to tissues.

Meta-analysis of intervention trials with folate and vitamins B₆ and B₁₂ shows the expected 25–30 per cent reduction in plasma homocysteine and a 25 per cent reduction in stroke incidence, but no effect on cardiovascular or all-cause mortality (Wang *et al.*, 2007). Although there are plausible mechanisms to suggest that homocysteine is a causative factor in cardiovascular disease, it is possible that hyperhomocysteinaemia is a result of the renal damage that is an early event in cardiovascular disease, and hence a proxy marker of disease rather than a causative factor. Alternatively, it may be that the intervention trials were conducted in people who already had significant atherosclerosis, and that intervention needs to occur earlier in life.

6.3.4 Ethylene synthesis in plants

Ethylene is synthesized by plants as a hormone that acts through cell surface receptors. It regulates many aspects of the plant life cycle, including seed germination, root initiation, flower development, fruit ripening and senescence. It is also produced in response to mechanical wounding, viral infection and cold and drought stress.

In fruit ripening, ethylene coordinates the expression of genes that are responsible for the climacteric increase in respiration, degradation of chlorophyll, synthesis of carotenoids, the conversion of starch to sugars and increased activity of cellulases and other cell wall-degrading enzymes. As shown in Figure 6.16, it is synthesized from *S*-adenosylmethionine by the actions of aminocyclopropane carboxylate synthase and oxidase. The resultant methylthioadenosine is recycled back to methionine, as is that formed as a result of decarboxylation of *S*-adenosylmethionine in polyamine synthesis (section 5.8.2; Lin *et al.*, 2009).

The rate-limiting step of ethylene synthesis is catalyzed by aminocyclopropane carboxylate synthase. Preclimacteric fruit produces *S*-adenosylmethionine, but little ethylene or aminocyclopropane carboxylate and, even when provided with aminocyclopropane carboxylate, little ethylene is produced compared with ripening fruit. The climacteric involves increased expression of both aminocyclopropane carboxylate synthase and oxidase. There is positive feedback of ethylene synthesis; in response to the action of ethylene, ripening fruits produce more ethylene. Auxin (indolylacetic acid,

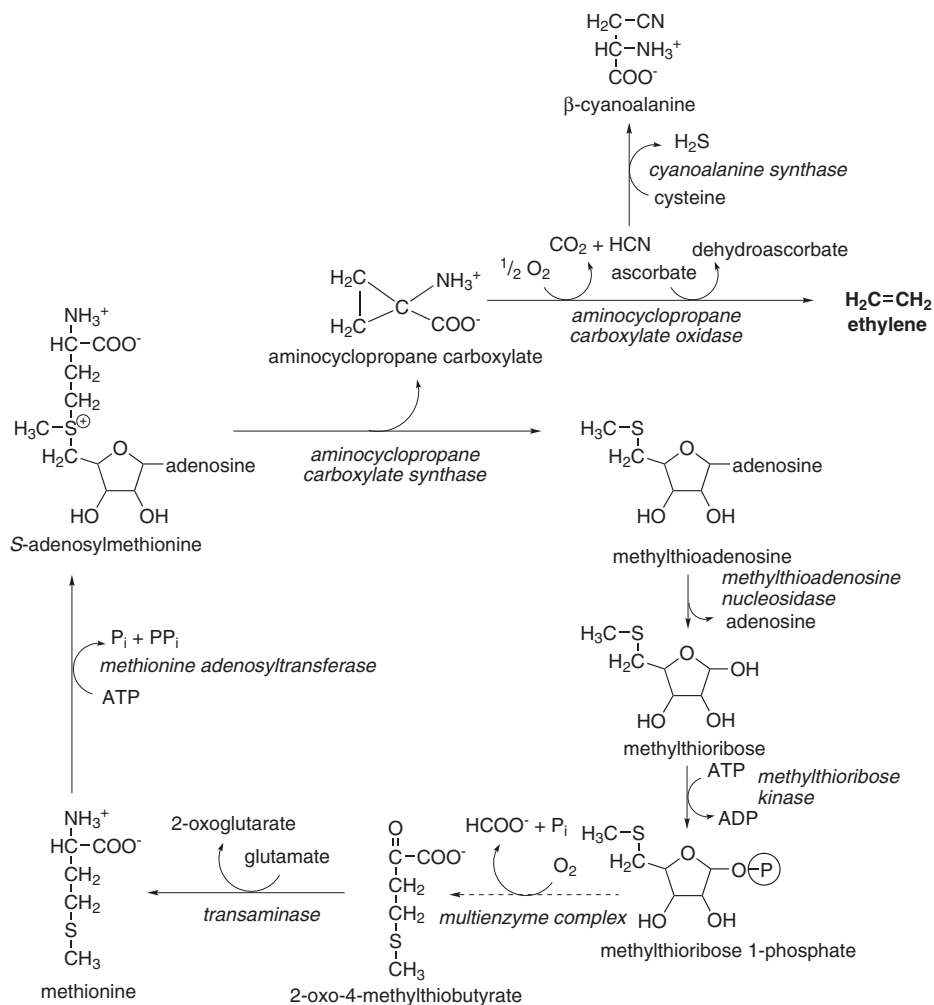


Figure 6.16 Ethylene biosynthesis and the methylthioadenosine cycle.

Methionine adenosyltransferase EC 2.5.1.6, aminocyclopropane carboxylate synthase EC 1.14.14.4, cyanoalanine synthase EC 4.4.1.9, methylthioadenosine nucleosidase EC 3.2.2.16, methylthioribose kinase EC 2.7.1.100, multi-enzyme complex (methylthioribose 1-phosphate isomerase EC 5.3.1.23, methylthioribulose 1-phosphate dehydratase EC 4.2.1.109, acireductone synthase EC 3.1.3.77, acireductone dioxygenase EC 1.13.11.54), methionine transaminase EC 2.6.1.5 and EC 2.6.1.57.

section 9.4.1) stimulates ethylene production by inducing synthesis of aminocyclopropane carboxylate synthase and oxidase, and many of the tissue responses to auxin are mediated by ethylene.

Ethylene controls its own formation by activation and stabilization of aminocyclopropane carboxylate synthase, as well as regulating expression of the gene. In vegetative tissues and preclimacteric fruit, ethylene down-regulates

the enzyme but, in climacteric fruit, it leads to increased activity and increased ethylene production. Transgenic tomatoes expressing aminocyclopropane carboxylate synthase antisense RNA do not ripen until they are provided with an exogenous source of ethylene.

Aminocyclopropane carboxylate synthase is a pyridoxal phosphate-dependent enzyme, related to transaminases. *S*-adenosylmethionine forms a Schiff base with the coenzyme, and the proton on the α -carbon is abstracted to form a carbanion that attacks the γ -carbon to expel methylthioadenosine and form the imine of aminocyclopropane carboxylate. The imine is released as aminocyclopropane carboxylate when the internal Schiff base to the reactive lysine residue is reformed. The enzyme undergoes mechanism-based inactivation approximately one in 30,000 reaction cycles, because of the formation of vinylglycine that alkylates and inactivates the enzyme (Jakubowicz, 2002).

There are multiple isoenzymes of aminocyclopropane carboxylate synthase in most plants. It has a short half-life (≈ 0.5 hours), so it is readily amenable to rapid changes in expression and, hence, ethylene synthesis. It is also phosphorylated by mitogen-activated protein kinases, which leads to increased stability of the protein, with an increased half-life. In addition, aminocyclopropane carboxylate in excess of requirements for ethylene synthesis is rapidly reacted with malonyl CoA, so preventing its oxidation to ethylene (Lin *et al.*, 2009; Yang & Hoffman, 1984).

Aminocyclopropane carboxylate oxidase is a mixed function oxygenase. It is a non-haem iron protein that utilizes ascorbate as a co-substrate (unlike the non-haem iron oxygenases that utilize 2-oxoglutarate as co-substrate and are dependent on ascorbate to reduce the iron when it has undergone accidental oxidation – see section 5.7.2). Like aminocyclopropane carboxylate synthase, there are different isoenzymes of the oxidase and different patterns of expression in vegetative tissues, preclimacteric and climacteric fruit (Binnie & McManus, 2009). The cyanide formed in the oxidase reaction is detoxified by reaction with cysteine to form β -cyanoalanine and hydrogen sulphide.

The pathway for recycling of methylthioadenosine to methionine shown in Figure 6.16 is variously known as the methionine salvage pathway, the methylthioadenosine cycle and, in plants, the Yang cycle. The formation of methylthioribose 1-phosphate is catalyzed by a single enzyme (methylthioadenosine phosphorylase) in animals, where methylthioadenosine is a product of polyamine synthesis (section 5.8.2). Bacteria, protozoa and plants have a separate nucleosidase and kinase.

In both cases, there is rapid removal of methylthioadenosine, which is a potent inhibitor of polyamine aminopropyltransferase (section 5.8.2), because the phosphorylase and nucleosidase have high affinity for their substrates. The multi-enzyme complex catalyzes synthesis of 2-oxo-4-methylthiobutyrate, the oxo-acid of methionine. It consists of an isomerase, a dehydratase, enolase,

phosphatase and dioxygenase. The enolase and phosphatase are found in a single polypeptide in eukaryotes and some Gram-positive bacteria, but in separate proteins in other organisms (Albers, 2009).

6.3.5 Radical SAM enzymes

A number of enzymes utilize *S*-adenosylmethionine to generate a 5'-deoxyadenosine radical at the catalytic site that then initiates a radical attack on the substrate. In some cases, the *S*-adenosylmethionine is regenerated at the end of the reaction cycle, so it acts as a coenzyme; in other cases, it is not regenerated, so it acts as a cosubstrate, leaving methionine and 5'-thioadenosine as the products. In these latter enzymes, the role of the 5'-deoxyadenosine radical is to abstract a proton from a glycine residue at the catalytic site, yielding a glycine radical that catalyzes the reaction (Booker & Grove, 2010; Fontecave *et al.*, 2004; Grillo & Colombaro, 2007, 2008; Wang & Frey, 2007).

These so-called radical SAM enzymes have a characteristic Cys-x-x-Cys-x-x-Cys motif. The three cysteine residues in the conserved sequence of the enzymes chelate three of the four iron atoms in an Fe₄S₄ iron-sulphur cluster. The fourth iron atom is chelated by the amino and carboxyl groups of *S*-adenosylmethionine, which undergoes reductive cleavage at the expense of the iron being oxidized, forming the 5'-deoxyadenosine radical. In some enzymes, there are two separate proteins, the iron-sulphur protein that binds *S*-adenosylmethionine and generates the 5'-deoxyadenosine radical, and a separate protein that utilizes the radical. In other cases, a single protein both generates and utilizes the radical.

The first of the radical SAM enzymes to be identified was bacterial lysine, 2,3-aminomutase, which is involved in lysine fermentation in *Clostridium* spp. and is the source of β-lysine for antibiotic synthesis in other organisms. It was originally thought to be a cobalamin (vitamin B₁₂) dependent enzyme, because it catalyzes an isomerization reaction that is formally the same as other cobalamin-dependent aminomutase reactions. However, it is activated not by cobalamin but by *S*-adenosylmethionine. In the cobalamin-dependent aminomutase, the reactive intermediate is a 5'-deoxyadenosine radical formed from adenosylcobalamin; in lysine 2,3-aminomutase, the 5'-deoxyadenosine radical is formed from *S*-adenosylmethionine.

Radical SAM enzymes are now known in all domains of life, catalyzing a variety of reactions, including unusual methylations, isomerization, ring formation, anaerobic oxidations and insertion of sulphur in the synthesis of thiamin (in bacteria and plants) and lipoic acid (in mammals as well as bacteria and plants). They are also involved in the synthesis of molybdopterin, the iron-molybdenum nitrogenase cofactor (section 1.1.1) and pyrroloquinoline quinone (section 9.5).

Viperin, the virus-inhibitory protein that is induced by interferon, is a radical SAM protein that is highly conserved across species from fish to mammals. The bacterial protein that confers resistance against antibiotics binding to the peptidyl transferase site of the bacterial ribosome is a radical SAM protein that methylates C-8 of adenosine in the 23S ribosomal RNA, which is at the centre of the peptidyl transferase site. This does not affect protein synthesis, but it does impair the binding of antibiotics.

6.3.6 Hydrogen sulphide

Like nitric oxide (section 5.9.3), hydrogen sulphide is a gasotransmitter, having a neuromodulatory role in the central nervous system. In the brain, it is produced in response to neuronal excitation and alters hippocampal long-term potentiation, suggesting a role in memory, as well as regulating the secretion of corticotrophin releasing-hormone from the hypothalamus. It stimulates cAMP release in neurons and increases the cytoplasmic calcium ion concentration in astrocytes. It also enhances the NMDA receptor-mediated current.

In blood vessel smooth muscle, hydrogen sulphide provides (together with nitric oxide) part of the endothelium-derived relaxation factor, causing smooth muscle relaxation by increasing the outwards flux of potassium. It has both inflammatory and anti-inflammatory actions, and protects neurons and cardiac muscle against oxidative stress.

Hydrogen sulphide acts by *S*-sulphydrating cysteine residues in proteins, parallel to the *S*-nitrosylation of proteins by nitric oxide, but while *S*-nitrosylation generally inhibits enzymes, *S*-sulphydration generally leads to activation (Gadalla & Snyder, 2010; Kimura, 2002, 2010; Yang *et al.*, 2008).

As shown in Figure 6.17, there are three pathways for hydrogen sulphide generation. The capacity of all three pathways is relatively low and is unaffected by sulphur amino acid intake, so their function is presumably provision of hydrogen sulphide, rather than disposal of surplus cysteine and methionine (Kamoun, 2004).

In the brain, hydrogen sulphide is formed by the action of cystathionine β -synthase, catalyzing a reaction between cysteine and homocysteine to yield cystathionine and hydrogen sulphide. In vascular endothelium, it is formed by the action of γ -cystathionase acting on cystine to yield thiocysteine, pyruvate and ammonium. Thiocysteine can then react with cysteine or another sulphhydryl compound to form a disulphide and hydrogen sulphide. Knockout mice lacking γ -cystathionase are hypertensive. In both the brain and vascular endothelium, as well as in the heart, hydrogen sulphide can be formed by transamination of cysteine (catalyzed by aspartate transaminase) to yield 3-mercaptopyruvate. Mercaptopyruvate sulphurtransferase catalyzes cleavage of mercaptopyruvate to pyruvate and hydrogen sulphide.

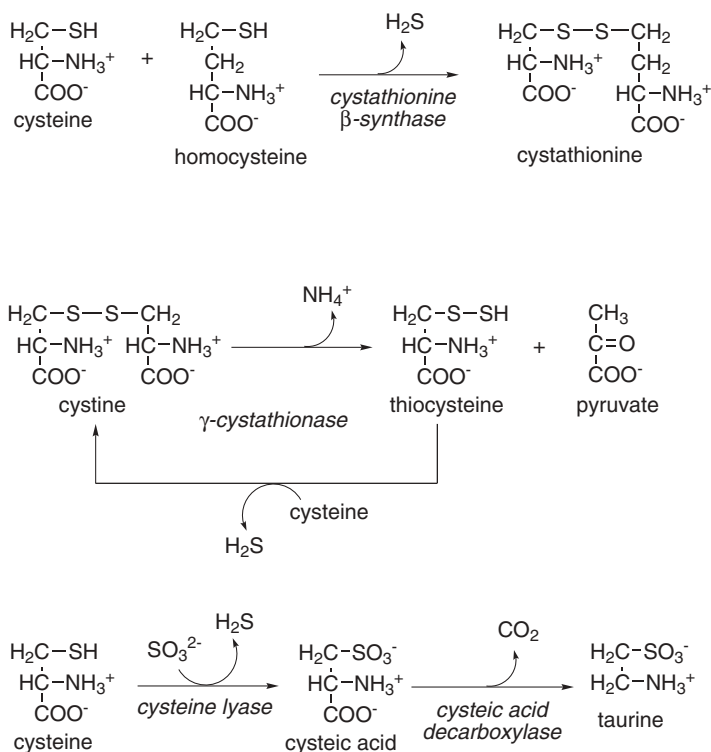


Figure 6.17 Pathways for hydrogen sulphide formation in mammals.

Cystathionine β -synthase EC 4.2.1.22, γ -cystathionase EC 4.4.1.11, cysteine lyase EC 4.4.1.10, cysteic acid decarboxylase EC 4.1.1.29.

Hydrogen sulphide is oxidized to thiosulphate and then to sulphate, which is either excreted in the urine or activated to 3'-phosphoadenosine 5'-sulphate for synthesis of sulphate esters.

6.3.7 Taurine and the catabolism of cysteine

Taurine was discovered in 1827 in ox bile, where it is conjugated with the bile acids. Until about 1976, it was assumed that it was a metabolic end-product whose only function was the conjugation of bile acids. It is present in most tissues at millimolar concentrations, and in many tissues it is the most abundant free amino acid. It is not incorporated into proteins. It is known to be a dietary essential for cats, which lack cysteine sulphinate decarboxylase, but is not known to be essential for human beings, nor is there any evidence that increased intakes have any beneficial effect. There are few plant sources of taurine, so strict vegetarians have a very low intake. Nevertheless, they maintain a plasma concentration of 40–50 $\mu\text{mol/l}$, compared with 55–70 $\mu\text{mol/l}$ in

omnivores. Although the milk of Vegan mothers has a very low concentration of taurine, there is no evidence of any signs of taurine deficiency in their breast-fed infants.

The major functions of taurine are as a tissue osmolyte (and hence a role in regulation of ion transport), and the conjugation of bile acids and some xenobiotics. It also forms water-soluble conjugates with vitamin A that may be important for tissue uptake of the vitamin and cycling of retinoids between photoreceptors and the retinal pigment epithelium in the eye. Other fat-soluble vitamins may also be absorbed, transported and excreted as taurine conjugates (Petrosian & Haroutounian, 2000).

The bile acids are conjugated with either taurine or glycine to increase their polarity. Increased availability of taurine results in decreased glycine conjugation and an increase in biliary taurocholic acid. Conversely, glycine overload results in an increase in the plasma concentration of taurine, as a result of increased glycocholic acid formation, and reduced utilization of taurine for bile acid conjugation. Although supplements of taurine alter the ratio of taurocholic to glycocholic acids, they have no effect on the total output of bile salts, nor on fat absorption in normal subjects.

There is some evidence that patients with cystic fibrosis have improved fat absorption if given taurine supplements. This may be because taurine-conjugated bile acids are generally re-absorbed lower down the small intestine than glycine conjugates. In patients whose intestinal absorption is compromised, this may give a beneficial increase in the total length of intestinal tract available for fat absorption.

There is a relatively high concentration of taurine in the central nervous system – higher than would be expected for a neurotransmitter, and without a specific anatomical localization. The main function of taurine in the central nervous system seems to be as an osmolyte. The concentration of taurine in the developing brain is some 3–4 fold higher than in the adult brain, and this falls rapidly between birth and weaning. Unlike other amino acids, taurine is transported within axons to a greater extent in young animals than in adults. The highest concentrations of taurine in the brain, and the greatest rates of axonal transport, occur prior to and during the process of synaptic development, suggesting that it may have a role in the development of the central nervous system and in the post-natal development of synaptic connections (Hussy *et al.*, 2000; Lima *et al.*, 2001; Saransaari & Oja, 2000).

Cardiomyopathy is a problem in taurine-deficient cats; after prolonged deficiency, there is a failure of contractility, leading to heart failure. Heart muscle concentrates taurine from the bloodstream, and the heart can synthesize taurine by oxidation of cysteamine, though not by the cysteine sulphinic acid decarboxylase pathway. Pharmacologically, taurine affects drug-induced cardiac arrhythmias by depressing the hyperirritability caused by loss of potassium – a digitalis-like action which suggests an effect on membrane

permeability and ion flux, and perhaps especially on the maintenance of stable intracellular concentrations of calcium (Militante *et al.*, 2000; Nittynen *et al.*, 1999).

Taurine also occurs in mitochondrial tRNA as tauromethyluridine and tauromethyl-thiouridine. There is a high concentration of taurine in leukocytes (up to 50 mmol/l), where it reacts with hypochlorous acid to form the more stable and less toxic taurine chloramine. The latter is a potent immune system regulator, downregulating the production of pro-inflammatory mediators by inhibiting the cytokine signalling pathway mediated by NFκB (Schuller-Levis & Park, 2004).

As shown in Figure 6.18, taurine is synthesized from cysteine by oxidation of the sulphhydryl group to yield cysteine sulphinic acid (sulphino-alanine), followed by decarboxylation to hypotaurine and oxidation to taurine.

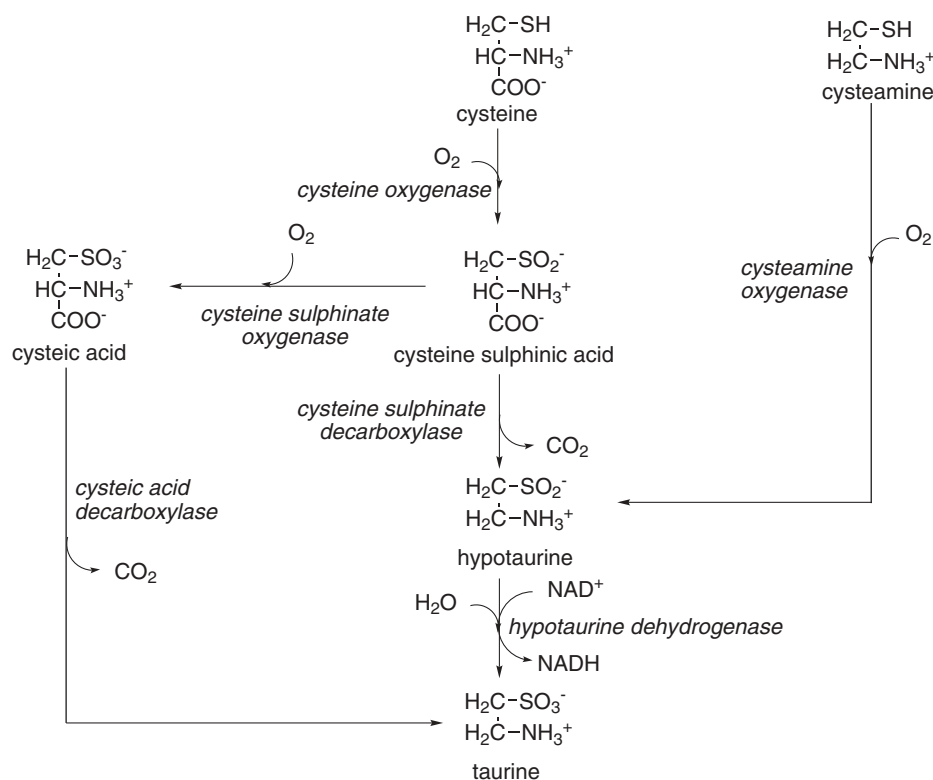


Figure 6.18 Taurine biosynthesis.

Cysteine oxygenase EC 1.13.11.20, cysteine sulphinate decarboxylase and cysteic acid decarboxylase EC 4.1.1.29, (glutamate decarboxylase EC 4.1.1.15 also acts on cysteine sulphinate and cysteic acid), hypotaurine dehydrogenase EC 1.8.1.3, cysteine sulphinate oxidase has no EC number.

Cysteine dioxygenase is a non-haem iron protein that uses NADPH. The enzyme concentration increases up to 45-fold in response to a three-fold increase in protein or sulphur amino acid intake, with no change in mRNA. This suggests that this reaction provides the major route for disposal of surplus cysteine and methionine. In animals on a low-protein diet, the enzyme is rapidly ubiquitinated and catabolized (section 2.1.5.2) but, in the presence of a relatively high concentration of cysteine, polyubiquitination of the enzyme is inhibited, allowing it to remain active for longer.

The enzyme is also activated by cysteine by an unusual feed-forward mechanism. The immature enzyme has low activity, and it undergoes an autocatalytic post-synthetic modification to form a tyrosyl-cysteine thio-ether cross-link that acts as the coenzyme for the reaction. Formation of this cross-link requires catalytic activity of the enzyme, and therefore requires the presence of cysteine (Stipanuk, 2004b; Stipanuk *et al.*, 2006, 2009).

The rate-limiting enzyme for taurine synthesis is cysteine sulphinate decarboxylase; cats, for which taurine is a dietary essential, have very low or no activity of the enzyme in the liver and other tissues. Hypotaurine, the product of the decarboxylase, is oxidized to taurine by hypotaurine dehydrogenase, a molybdenohaemoprotein that uses NAD^+ as the electron acceptor.

An alternative, and quantitatively less important, pathway for taurine synthesis is decarboxylation of cysteine to cysteamine, which is required for synthesis of CoA and acyl carrier protein from pantothenic acid, and which provides the reactive sulphhydryl group. Cysteamine, arising from the turnover of CoA and acyl carrier protein, or synthesized by decarboxylation of cysteine, is oxidized to hypotaurine by a non-haem iron dioxygenase that is distinct from cysteine dioxygenase, has no activity towards cysteine and does not change in activity with the intake of sulphur amino acids.

Only a relatively small proportion of total cysteine catabolism proceeds through taurine; cysteine sulphinic acid in excess of requirements for taurine synthesis is transaminated, yielding pyruvate and sulphite. The enzyme catalyzing this transamination is aspartate transaminase, linked to the amination of 2-oxoglutarate to glutamate. The immediate product of the reaction is enzyme-bound β -sulphinylpyruvate, which decomposes to pyruvate and sulphite. Sulphite is readily oxidized to sulphate by sulphite oxidase, which has a molybdenum cofactor. Sulphate is either excreted in the urine or activated to 3'-phosphoadenosine 5'-sulphate, for synthesis of sulphate esters.

Further reading

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

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7

The Branched-Chain Amino Acids: Leucine, Isoleucine and Valine

The three branched-chain amino acids – leucine, isoleucine and valine – are dietary essentials for animals and are required for protein synthesis. However, unlike other essential amino acids, they do not give rise to any unique metabolites; rather, they are metabolized to common metabolic intermediates. Leucine is ketogenic, forming acetyl CoA and acetoacetate; isoleucine is both ketogenic and glucogenic, giving rise to acetyl CoA and propionyl CoA; and valine is glucogenic, giving rise to succinyl CoA (or, in bacteria, propionyl CoA).

Because they are extremely hydrophobic, the branched-chain amino acids are important in the folding of globular proteins and in interactions of membrane proteins with lipids, as well as in the leucine zipper motif of DNA-binding proteins, which is a coiled coil of helices. Leucine forms mainly α -helices, while isoleucine and valine form mainly β -pleated sheet.

In bacteria, the branched-chain oxo-acids are precursors for synthesis of the branched-chain fatty acids that are important in cell membranes. Since there is branched-chain amino acid transaminase in skin, the branched-chain oxo-acids may also be the precursors for the branched-chain fatty acids that are found in (rat) skin surface lipids. In yeast, they are precursors for synthesis of branched-chain higher alcohols that contribute to the aroma of alcoholic beverages. In *Lactobacillus* spp. that are auxotrophic for branched-chain amino acids, there is a catabolic branched-chain amino acid transaminase that is especially active in cheese ripening, and this is important for flavour development in cheese.

Unlike most amino acids, which are transaminated and deaminated mainly in the liver in mammals, transamination of the branched-chain amino acids

occurs mainly in skeletal muscle. They provide amino groups for the transamination of pyruvate arising from glycolysis of muscle glycogen to alanine, which is exported to the liver for gluconeogenesis in the fasting state (section 2.4). The oxo-acids arising from the branched-chain amino acids may either be oxidized in muscle, where they provide an important source of metabolic fuel, or they may be exported to the liver, where they are transaminated back to the amino acids by reaction with alanine (Figure 2.3). In fasting and diabetes, the capacity of muscle to oxidize branched-chain amino acids increases some 3–5 fold, as a result of increased activity of the branched-chain oxo-acid dehydrogenase (section 7.2.2).

Leucine acts as a signal of increased amino availability after a meal. It stimulates muscle (and other tissue) protein synthesis, both alone and together with insulin. In response to an oral dose of leucine, there is a transient increase in insulin secretion, but insulin secretion alone (as after an oral dose of glucose) does not result in increased protein synthesis unless leucine is also present in relatively high concentrations. This stimulation of protein synthesis is mediated by increased translation of mRNA as a result of phosphorylation of proteins that are involved in the binding of mRNA to the 40S ribosomal subunit.

Some of these effects are inhibited by rapamycin, suggesting that leucine is acting via the mTOR pathway, while others are not inhibited by rapamycin and are therefore the result of signalling through other pathways. There is also evidence that leucine down-regulates tissue protein catabolism, but this is less clear than the stimulation of protein synthesis. The studies showing increased protein synthesis in response to leucine have been conducted in experimental animals, and there is less evidence for an effect of leucine on protein synthesis in human beings (Dennis *et al.*, 2011; Kimball & Jefferson, 2006a, 2006b; Matthews, 2005).

7.1 Synthesis of the branched-chain amino acids

As shown in Figure 7.1, the three branched-chain amino acids are synthesized by a parallel pathway leading to formation of oxomethylvaleric acid (the oxo-acid of isoleucine) and oxo-isovaleric acid, which is the oxo-acid of valine and precursor for the synthesis of leucine (section 7.1.3). In fungi, the enzymes are mitochondrial, while in plants they are found in the chloroplasts. In bacteria, they are membrane-bound. Although the activities of each of the individual enzymes of the pathway can be demonstrated with solubilized enzymes, synthesis of the amino acids from pyruvate and 2-oxobutyrates can only be demonstrated with membrane-associated assemblies of the enzymes, which

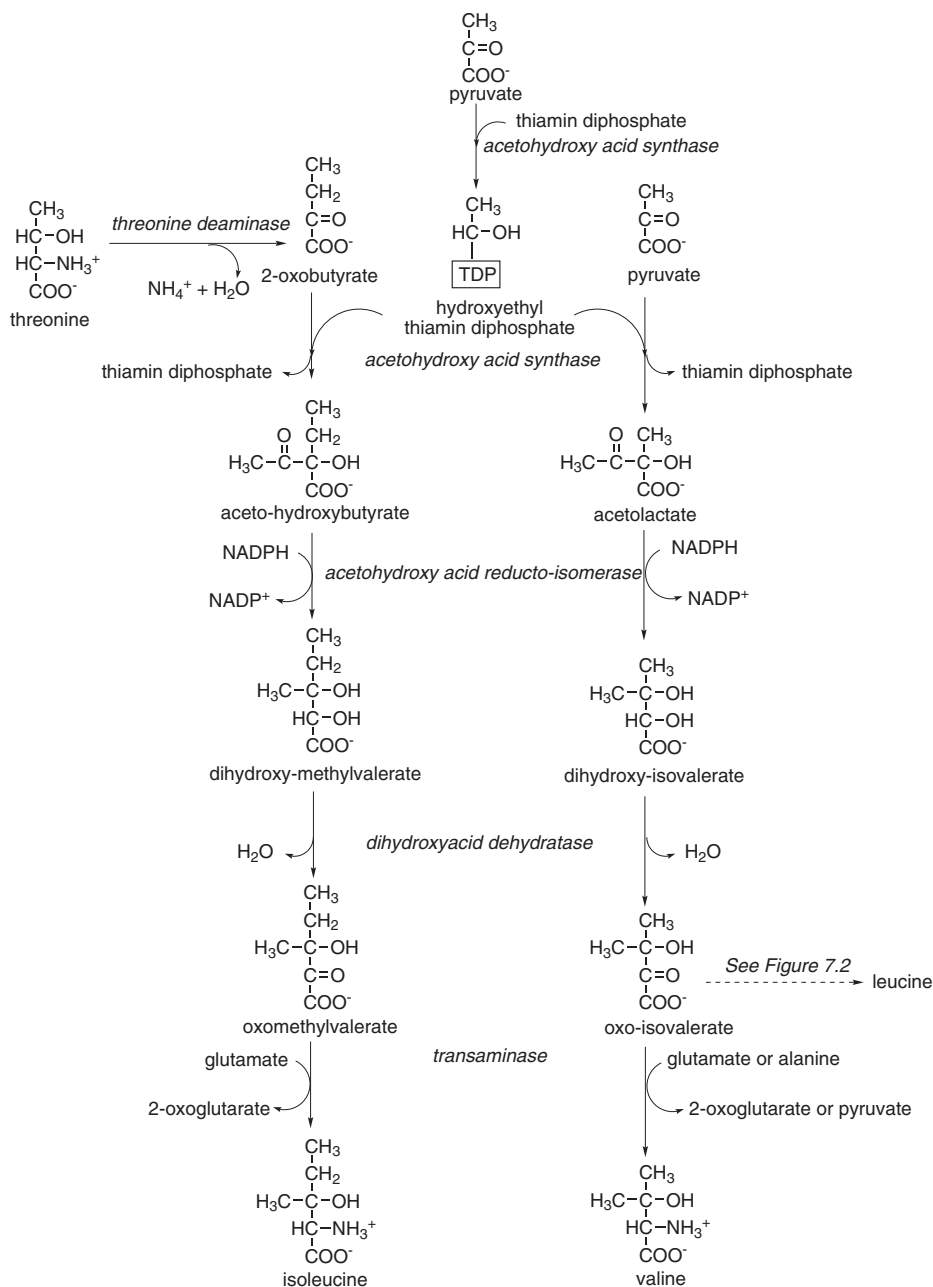


Figure 7.1 The common pathway of branched-chain amino acid synthesis.

Threonine deaminase EC 4.3.1.19, acetohydroxyacid synthase EC 2.2.1.6, acetohydroxyacid reducto-isomerase EC 1.1.1.86, dihydroxyacid dehydratase EC 4.2.1.9, branched chain amino acid aminotransferase (2-oxoglutarate-linked) EC 2.6.1.42, valine-pyruvate transaminase EC 2.6.1.66.

presumably form a metabolon, channelling the product of each enzyme onto the catalytic site of the next (Cronenwett & Wagner, 1965).

Isoleucine synthesis requires 2-oxobutyrate, which is formed by the biosynthetic isoenzyme of threonine deaminase (section 6.1.5.1). In addition, methionine may be a significant source of 2-oxobutyrate for isoleucine synthesis. Expression of methionine γ -lyase (section 6.2.3.2) is increased in threonine deaminase-deficient mutants of *Arabidopsis*, and is down-regulated in mutants that over-express threonine deaminase. Transcription of methionine γ -lyase is increased in response to salt and drought stress and, under these conditions, there is increased incorporation of label from methionine into isoleucine. However, the lethality of herbicides such as cyclohexenylglycine that inhibit threonine deaminase suggests that methionine γ -lyase cannot provide enough 2-oxobutyrate to meet isoleucine requirements, although it is possible that the herbicides inhibit the lyase as well as threonine deaminase (Jander & Joshi, 2010; Joshi & Jander, 2009).

7.1.1 Acetohydroxyacid synthase

Acetohydroxyacid synthase catalyzes the decarboxylation of pyruvate bound to thiamin diphosphate to form hydroxymethyl thiamin diphosphate, followed by condensation with either 2-oxobutyrate to form acetohydroxybutyrate (for isoleucine synthesis) or another mol of pyruvate to form acetolactate for valine and leucine synthesis. The enzyme has separate catalytic and regulatory subunits; the catalytic subunit has low activity on its own. It is activated by binding the regulatory subunit, which also confers sensitivity to feedback inhibition by the branched-chain amino acids.

In bacteria and fungi, valine provides the main feedback inhibition of the enzyme, while in plants, all three branched-chain amino acids are inhibitory. The plant regulatory subunit is considerably larger than the bacterial one and has two amino acid binding sites, thus permitting synergistic inhibition by leucine and either isoleucine or valine. Simple over-expression of the synthase gene does not lead to increased production of branched-chain amino acids because of feedback inhibition. Genetic manipulation of the regulatory subunit to retain the ability to activate the catalytic subunit while deleting the feedback inhibitory site provides a way of enhancing branched-chain amino acid synthesis in plants and microorganisms (Chen *et al.*, 2010; Chipman *et al.*, 2005; Duggleby *et al.*, 2008; Slutzker *et al.*, 2011).

The synthase also requires FAD, but this appears to have a structural role rather than any function in catalysis, and the requirement may simply reflect the evolutionary ancestry of thiamin diphosphate-dependent enzymes. Some bacteria have a separate acetolactate synthase that is not involved in amino acid synthesis, but which is part of the pathway of butanediol fermentation. It does not contain or require FAD. In this enzyme, the groove in the catalytic

subunit that is occupied by FAD in biosynthetic acetohydroxyacid synthase is filled by amino acid side-chains (Duggleby *et al.*, 2008; Lee & Duggleby, 2001).

In bacteria, there are three isoenzymes of acetohydroxyacid synthase, one of which is repressed by valine and leucine, one repressed by leucine and one by all three branched-chain amino acids. There is competition between pyruvate and 2-oxobutyrate, and the enzymes have considerably lower affinity for pyruvate than for 2-oxobutyrate. This means that *in vitro*, with equal concentrations of the two substrates, the reaction mainly leads to the formation of acetohydroxybutyrate and, hence, isoleucine. *In vivo*, however, the intracellular concentration of pyruvate is considerably higher than that of 2-oxobutyrate, and metabolic flux is equal through the parallel branches of the pathway (Barak *et al.*, 1987).

Acetohydroxyacid synthase is a dimer, with two substrate access tunnels on opposite sides, leading to the two catalytic sites. The tunnels are formed by regions of both monomer units. In the absence of the regulatory subunits, these tunnels are poorly defined and binding of the regulatory subunits leads to a conformational change that defines the substrate access tunnels. Sulphonylurea and imidazolone herbicides inhibit acetohydroxyacid synthase by binding to, and blocking, these tunnels.

Mutations in the enzyme lead to herbicide resistance. Some mutants are resistant to sulphonylurea herbicides, some to imidazolones and some to both, suggesting that the inhibitor binding sites are separate, but overlap. While spontaneously acquired herbicide resistance in weeds is undesirable, genetic engineering and conventional breeding techniques are used to produce crop plants that are resistant to herbicides (Duggleby *et al.*, 2008; McCourt & Duggleby, 2006; McCourt *et al.*, 2006; Singh & Shaner, 1995; Tan *et al.*, 2005).

Aspergillus nidulans synthesizes large amounts of branched-chain amino acids under hypoxic conditions and excretes them into the culture medium, as a result of increased transcription of the genes of the biosynthetic pathway. Acetohydroxyacid synthase knockout leads to a higher NAD(P)H:NAD(P)⁺ ratio in hypoxia. The increased synthesis of the amino acids provides an electron sink in hypoxia; if glucose is provided, to permit fermentation to lactate and ethanol, then there is less synthesis of the branched-chain amino acids (Shimizu *et al.*, 2010).

7.1.2 Acetohydroxyacid reducto-isomerase, dihydroxyacid dehydratase and transamination of the oxo-acids

Acetohydroxyacid reducto-isomerase has similar affinity for both substrates, but it has 5–8 fold higher activity towards acetohydroxybutyrate than

acetolactate, so it will favour synthesis of isoleucine. However, isoleucine acts to control the provision of acetohydroxybutyrate by feedback inhibition and repression of threonine deaminase (section 6.1.5.1). It is a bifunctional enzyme, catalyzing alkyl migration and reduction. The isomerization reaction is unfavoured and is driven in the forward direction by NADPH-dependent reduction of the intermediate oxo-acid product to the dihydroxy-acid. Alkyl migration reactions of this type are usually associated with cobalamin-dependent or radical SAM enzymes (section 6.3.5), but there is no requirement for cobalamin or *S*-adenosylmethionine in acetohydroxyacid reductoisomerase (Dumas *et al.*, 2001; Tyagi *et al.*, 2005).

Dihydroxyacid dehydratase is an iron-sulphur protein. The reaction proceeds by way of an enol intermediate. The bacteriostatic actions of nitric oxide (section 5.9.3) and hyperbaric oxygen are mainly due to inactivation of the iron-sulphur cluster in this enzyme, leading to growth inhibition as a result of deprivation of branched-chain amino acids (Duan *et al.*, 2009; Hyduke *et al.*, 2007).

The oxo-acid products are substrates for transamination of oxomethylvalerate to isoleucine and oxo-isovalerate to valine. In addition to the glutamate-linked branched-chain transaminase that acts on all three branched-chain oxo-acids, there is a glutamate-linked oxo-isocaproate transaminase for the synthesis of leucine and an alanine-linked oxo-isovalerate transaminase for the synthesis of valine. Leucine transaminase also acts on methionine (Amadasi *et al.*, 2007). Oxo-isovalerate is also the precursor for leucine synthesis (section 7.1.3).

7.1.3 Leucine synthesis

As shown in Figure 7.2, leucine is synthesized from oxo-isovalerate in a sequence of reactions that follows the same chemistry as the first three reactions of the citric acid cycle: condensation with acetyl CoA; an aconitase-like isomerization via intermediate dehydration and rehydration; and oxidative decarboxylation of the hydroxy-acid to yield 2-oxo-isocaproic acid, which is transaminated to leucine. It has been suggested that the enzymes of this pathway evolved in primitive organisms and later underwent mutation to become the first three enzymes of the citric acid cycle. In plants, the pathway is entirely within the chloroplasts. In yeast, however, the first enzyme – isopropylmalate synthase – is mitochondrial, and the remainder of the pathway occurs in the cytosol. In bacteria, the genes for the enzymes of leucine biosynthesis form an operon which is regulated by the availability of leucyl-tRNA but, in yeast, the genes are scattered throughout the genome and are not all on the same chromosome (Kohlhaw, 2003).

There are two genes for isopropylmalate synthase in yeast. Isoenzyme 1 (which accounts for 80 per cent of the total activity in the cell) can be tran-

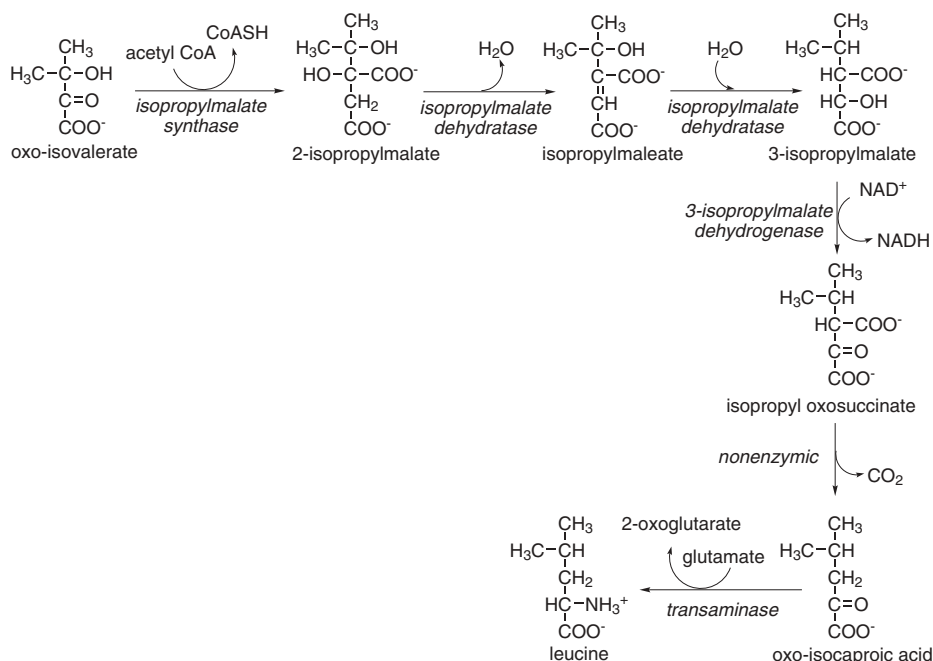


Figure 7.2 Leucine biosynthesis.

Isopropylmalate synthase EC 2.3.3.13, isopropylmalate dehydratase EC 4.2.1.33, isopropylmalate dehydrogenase EC 1.1.1.85, branched chain amino acid aminotransferase EC 2.6.1.42, leucine transaminase EC 2.6.1.6.

scribed with two different start sites: translation of the long form gives an amino-terminal mitochondrial targeting sequence, while the protein coded for by the short form mRNA does not, and it remains in the cytosol. The reaction involves initial synthesis of isopropylmalate CoA by deprotonation and enolization of acetyl CoA, followed by hydrolysis to release isopropylmalate and CoASH (de Carvalho & Blanchard, 2006).

Isopropylmalate synthase is subject to feedback inhibition by leucine, and it also undergoes zinc-dependent reversible inactivation by CoASH. This is not simple product inhibition, but involves CoASH binding at a separate site on the enzyme that only opens in the presence of zinc ions. The inactivation by CoASH is reversed by ATP, and physiological concentrations of ATP prevent inactivation by CoASH. The result of this is that when the ratio of CoASH to acetyl CoA is high, such acetyl CoA as is available will be used for energy-yielding metabolism rather than amino acid synthesis. Homocitrate synthase in lysine biosynthesis (see section 6.2.2) is similarly inhibited by CoASH.

The regulatory domain of isopropylmalate synthase is not only responsible for feedback inhibition by leucine but is also required for catalytic activity.

The truncated enzyme, lacking the regulatory domain, catalyzes hydrolysis of acetyl CoA, but not the condensation with 2-oxoisovalerate. The inhibition by leucine occurs in two stages: initially there is some inhibition as a result of the binding of leucine, but then there is a slow equilibration to a more inhibited form of the enzyme. The leucine binding site is some 50 Å from the catalytic site, and leucine binding leads to a slow conformational change that allows the linker region between the regulatory and catalytic domains of one subunit to interact with the active site of the other (de Carvalho *et al.*, 2009; Huisman *et al.*, 2010; Kohlhaw, 2003).

Isopropylmalate dehydratase catalyzes the dehydration of 2-isopropylmalate to isopropylmaleate, followed by rehydration to 3-isopropylmalate. The enzyme shows considerable sequence homology with aconitase, which catalyzes a similar reaction to form isocitrate from citrate, and homoaconitase in the fungal pathway of lysine biosynthesis (section 6.2.2). It is an iron-sulphur protein.

The fungal isomerase is a single polypeptide with two distinct domains; the bacterial and plant enzymes are heterodimers of a large protein containing the iron-sulphur cluster and a small subunit. The large subunit (or the large domain of the fungal enzyme, which contains the iron-sulphur cluster) is involved in leucine synthesis, the side-chain elongation of methionine for glucosinolate synthesis and the homoaconitase reaction in lysine biosynthesis (section 6.2.2). The small subunit (or smaller domain in the fungal enzyme) dictates the substrate specificity of the enzyme (He *et al.*, 2010; Irvin & Bhattarjee, 1998; Knill *et al.*, 2009).

3-Isopropylmalate dehydrogenase catalyzes the oxidation of isopropylmalate to isopropyl oxosuccinate, which undergoes non-enzymic decarboxylation to oxo-isocaproic acid, the substrate for transamination to leucine.

In addition to transamination to leucine, isopropylmalate is transported in to the nucleus, where it binds to the leu3p protein. With isopropylmalate bound, this protein is a transcriptional activator, up-regulating the transcription of the genes for isopropylmalate synthase 1, isopropylmalate isomerase and 3-isopropylmalate dehydrogenase, as well as the genes for enzymes of the common pathway for branched-chain amino acid synthesis (Figure 7.1). Without isopropylmalate bound, leu3p acts as a transcriptional inhibitor, completely stopping transcription of the genes for leucine synthesis (Kohlhaw, 2003).

7.1.3.1 The pyruvate pathway of isoleucine synthesis In a small number of microorganisms, there is an alternative pathway of isoleucine biosynthesis that follows the same sequence of reactions as the pathway for leucine biosynthesis to form 2-oxobutyrate, rather than deamination of threonine. The first reaction is condensation of pyruvate with acetyl CoA to form citramalate, catalyzed by citramalate synthase. This is followed by reactions catalyzed by

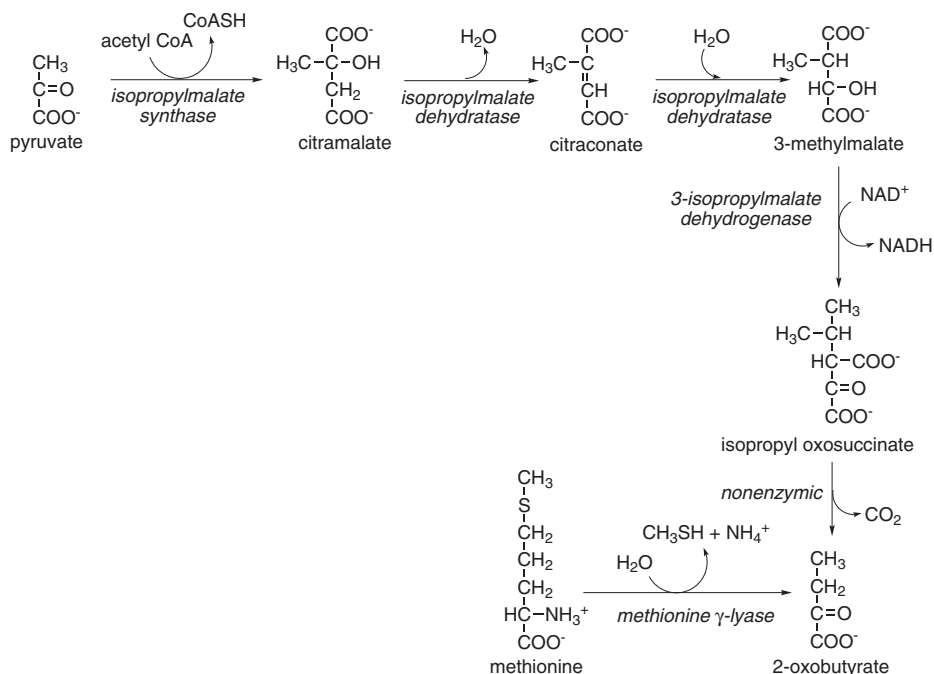


Figure 7.3 Alternative pathways for 2-oxobutyrate synthesis.

Isopropylmalate synthase EC 2.3.3.13, isopropylmalate dehydratase EC 4.2.1.33, isopropylmalate dehydrogenase EC 1.1.1.85, methionine γ -lyase EC 4.1.1.11.

the same enzymes that are involved in leucine biosynthesis from oxo-isovalerate: isomerization catalyzed by isopropylmalate isomerase, and oxidative decarboxylation catalyzed by 3-isopropylmalate dehydrogenase to yield 2-oxobutyrate. This pathway is shown in Figure 7.3 (Drevland *et al.*, 2007; Xu *et al.*, 2004). Methionine can also be a source of 2-oxobutyrate, through the action of methionine γ -lyase (section 6.3.2.3).

7.2 Mammalian catabolism of the branched-chain amino acids

Unlike most amino acids, the major sites of catabolism of the branched-chain amino acids are extra-hepatic. Leucine is mainly oxidized in muscle and adipose tissue; liver has little capacity for branched-chain oxo-acid oxidation. Isoleucine and valine are transaminated in muscle, but oxo-methylvaleric acid arising from isoleucine and 3-hydroxyisobutyrate arising from valine (Figure 7.4) are mainly exported to the liver for gluconeogenesis (though heart and skeletal muscle can oxidize them to some extent). Again, unlike the catabolism of most of the essential amino acids (which is controlled mainly by the

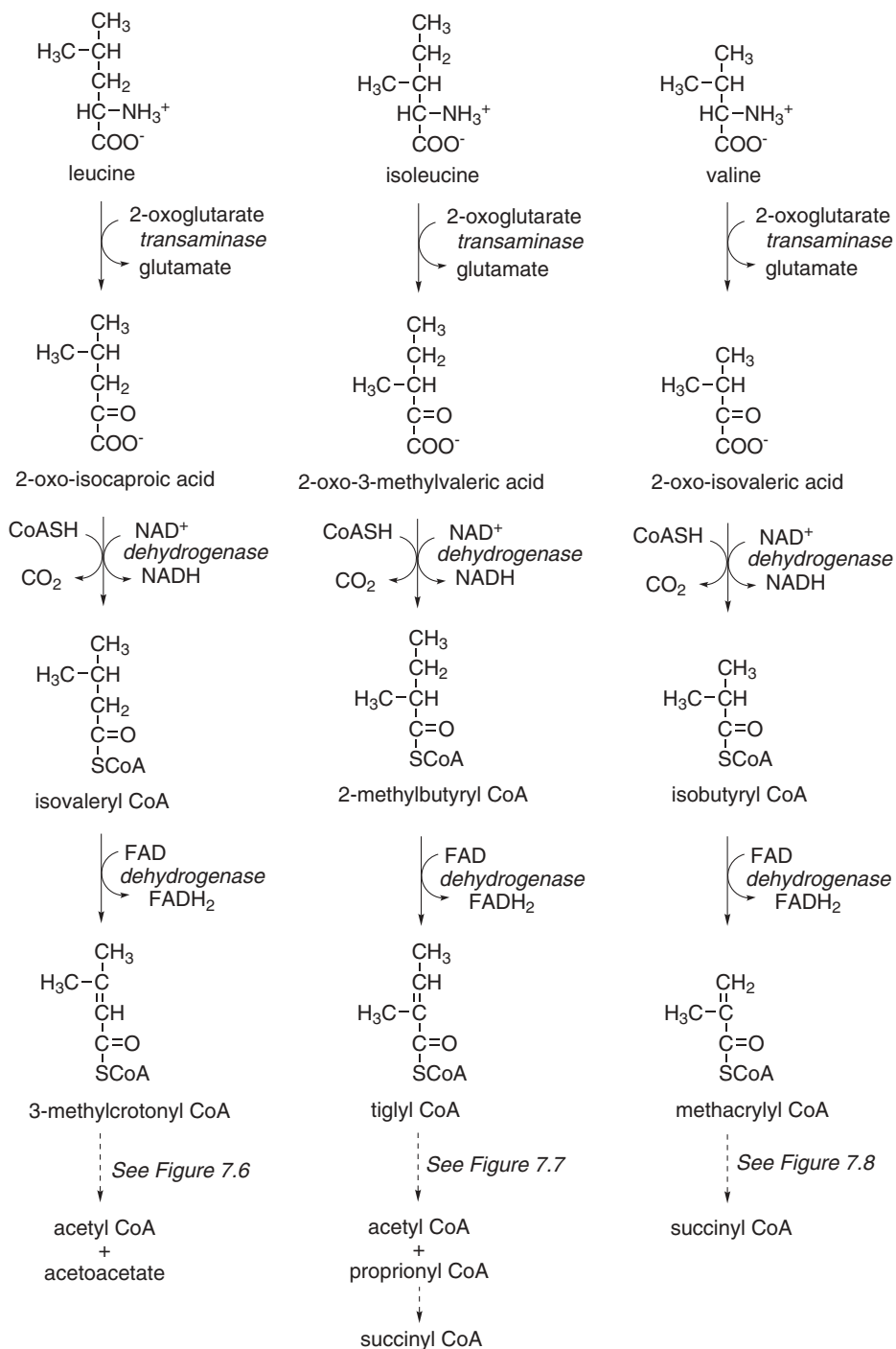


Figure 7.4 The common pathway of branched-chain amino acid catabolism.

Branched-chain amino acid transaminase EC 2.6.1.42, leucine transaminase EC 2.6.1.42, valine-pyruvate transaminase EC 2.6.1.66, branched-chain oxo-acid dehydrogenase multi-enzyme complex see Figure 7.5, isovaleryl CoA dehydrogenase EC 1.3.99.10, branched chain acyl CoA dehydrogenase EC 1.3.99.12.

availability of substrates), catabolism of the branched-chain amino acids is tightly regulated by phosphorylation and dephosphorylation of the second enzyme in the pathway, the branched-chain oxo-acid dehydrogenase complex (section 7.2.2).

As shown in Figure 7.4, the first three steps in catabolism are common to all three amino acids: transamination; oxidative decarboxylation to yield an acyl CoA derivative; and dehydrogenation to yield an α - β unsaturated acyl CoA derivative. In each case, the same enzymes act on all three substrates, although, as discussed in section 7.2.1, there is a separate leucine transaminase that does not act on isoleucine or valine (Brosnan & Brosnan, 2006; Harper *et al.*, 1984).

7.2.1 Branched-chain amino acid transaminases

There are three mammalian branched-chain transaminases. Unlike most transaminases, they are fold type IV enzymes (see section 3.1.1) – the only mammalian enzymes with this fold type. The main isoenzyme is mitochondrial and has specificity for all three branched-chain amino acids and a broad tissue distribution. This enzyme is associated with branched-chain oxo-acid dehydrogenase, and it channels the oxo-acid products directly to the dehydrogenase multi-enzyme complex (section 7.2.2). The metabolon formed between the transaminase and branched-chain oxo-acid dehydrogenase also includes glutamate dehydrogenase, which is activated allosterically by leucine but not isoleucine or valine (Islam *et al.*, 2010).

The cytosolic isoenzyme also shows specificity for all three branched chain amino acids, but has a more limited tissue distribution. It is found mainly in brain, uterus, placenta and other tissues that do not express significant amounts of branched-chain oxo-acid dehydrogenase. The onward metabolism of branched-chain oxo-acids is mitochondrial, and it is likely that much of the oxo-acids formed by the cytosolic transaminase in muscle and adipose tissue is exported to liver and kidney.

In the liver, the oxo-acids are mainly used for gluconeogenesis and ketogenesis, although some will be used in transamination of alanine and re-exported as the amino acids. The kidney has a net uptake of branched-chain oxo-acids and a net output of the corresponding amino acids (Brosnan & Brosnan, 2006; Harper *et al.*, 1984). In the brain, the cytosolic transaminase is involved in maintaining the neuronal pool of glutamate as a neurotransmitter, acting to shuttle amino groups from glial cells, where the branched-chain oxo-acids are transaminated to the amino acids, to the neuronal cells, where the amino acids are used for the synthesis of glutamate from 2-oxoglutarate (Yudkoff *et al.*, 2005).

Both the mitochondrial and cytoplasmic transaminases also have β -lyase activity towards cysteine *S*-conjugates. In the presence of a cysteine

S-conjugate, there is no transamination of 2-oxo-isocaproate; the β -lyase reaction takes precedence and leads to inactivation of the enzyme after 40–100 cycles of activity (Cooper *et al.*, 2003).

There is also a liver-specific leucine transaminase that does not act on isoleucine or valine but does have activity towards methionine. Its main function is probably in ketogenesis from leucine in prolonged fasting. The activity of this leucine-specific transaminase, as well as branched-chain amino acid transaminase in skeletal muscle (but not kidney or brain), is increased in response to a high protein diet (Torres *et al.*, 1998b).

The mitochondrial branched-chain transaminase has relatively high values of K_m – some two to six times higher than the normal intracellular concentration of leucine and isoleucine and 5–10 times that of valine, so that transamination will increase sharply as the concentration of substrate increases.

In the fasting state, muscle shows a net uptake of branched-chain amino acids and a net output of alanine, whose carbon skeleton is derived from pyruvate formed by glycolysis of glucose 1-phosphate arising from muscle glycogen. This is an indirect way in which muscle glycogen can contribute to maintenance of blood glucose in the fasting state, since the alanine is taken up by the liver and used as a substrate for gluconeogenesis. Branched-chain amino acid transaminase is linked to amination of 2-oxoglutarate to glutamate, and the formation of alanine from the amino groups of the branched-chain amino acids requires transamination of pyruvate by glutamate (Figure 2.3).

7.2.2 Branched-chain 2-oxo-acid dehydrogenase

Branched-chain 2-oxo-acid dehydrogenase is a large multi-enzyme complex with a molecular mass of 4 megadaltons, on the inner face of the inner mitochondrial membrane. It is similar to the pyruvate and 2-oxoglutarate dehydrogenase multi-enzyme complexes. As shown in Figure 7.5, it catalyzes decarboxylation of the oxo-acid attached to enzyme-bound thiamin diphosphate, reductive *S*-acylation of lipoic acid esterified to the side-chain amino group of lysine (lipoamide), followed by transfer of the acyl group onto CoA and NAD^+ -dependent re-oxidation of the reduced lipoamide. Thiamin deficiency in experimental animals leads to a considerable decrease in branched-chain amino acid catabolism in the central nervous system and increased concentrations of leucine in the brain; this suggests that, in addition to impaired energy-yielding metabolism, impaired catabolism of the branched-chain amino acids may be important in the neuronal dysfunction of thiamin deficiency (Navarro *et al.*, 2008).

The multi-enzyme complex consists of:

- 1 Twelve units of E1, the decarboxylase. E1 is an $\alpha_2\beta_2$ heterotetramer which catalyzes the decarboxylation of the substrate and binding to

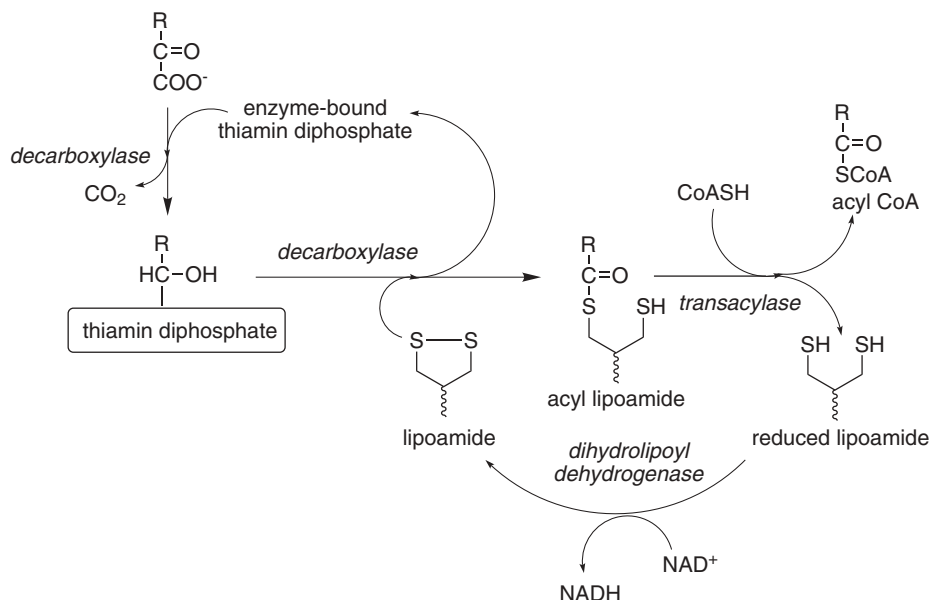


Figure 7.5 The reaction of branched-chain oxo-acid dehydrogenase.

Dehydrogenase (E1) EC 1.2.4.4, dihydrolipoyl transacylase (E2) EC 2.3.1.168, dihydrolipoyl dehydrogenase (E3) EC 1.8.1.4.

thiamin diphosphate, followed by reductive acylation of the lipoamide bound to E2.

- 2 Twenty-four units of E2, the transacylase. E2 both forms the core of the multi-enzyme complex, to which the other enzymes attach. It also catalyzes the transfer of the acyl group from lipoamide onto CoA. The lipoamide-binding domain of E2 is attached to the core domain by a flexible polypeptide chain, permitting lipoamide to move between the three active sites of the complex.
- 3 Six units of the homodimer of E3, which is dihydrolipoamide dehydrogenase – a flavoprotein that then reduces NAD^+ .

The E1 and E2 proteins are unique to branched-chain oxo-acid dehydrogenase; the E3 protein, dihydrolipoamide dehydrogenase, is the same protein that is found in pyruvate and 2-oxoglutarate dehydrogenases. Free lipoamide is a substrate for E2 and E3 but not for E1, which requires multiple interactions with the 80-amino acid lipoamide-binding domain at the amino-terminal of E2 (Perham *et al.*, 2002; Perham & Packman, 1989).

Transamination of the branched-chain amino acids is readily reversible, and branched-chain oxo-acid dehydrogenase is the first committed step in

their catabolism. It is subject to regulation by phosphorylation of the E1 subunit (which inactivates the enzyme) and reactivation by dephosphorylation. In the presence of excess concentrations of the branched-chain amino acids, the kinase is inhibited and the phosphorylase is activated.

Branched-chain oxo-acid dehydrogenase kinase is inhibited by 2-oxo-isocaproic acid arising from leucine transamination. Thus, when there is a relatively large amount of leucine undergoing transamination, branched-chain oxo-acid dehydrogenase is dephosphorylated and is maximally active. Feeding a low-protein diet leads to increased expression of the kinase, so that branched-chain amino acids are conserved. This is probably mediated through thyroid hormone, which also causes increased expression of the kinase. Glucocorticoids and ligands of the PPAR α receptor repress expression of the kinase. Starvation and experimental diabetes also result in decreased expression of the kinase and, hence, increased oxidation of the branched-chain oxo-acids as metabolic fuel and for gluconeogenesis (Harris *et al.*, 2001; Joshi *et al.*, 2006).

The kinase is inactive in free solution; for activity, it has to be bound to the lipoamide-binding domain of the transacylase. Inhibition of kinase activity by 2-oxo-isocaproic acid, and also in response to TNF α , is due to displacement of the kinase from the multi-enzyme complex. In muscle, the kinase is normally bound to the multi-enzyme complex, so that there is little oxidation of branched-chain oxo-acids. In the liver, by contrast, there is normally little kinase bound to the multi-enzyme complex; the liver catalyzes rapid oxidation of branched-chain oxo-acids coming from extra-hepatic tissues. In response to exercise, the activity of the kinase in muscle falls rapidly, as a result of displacement from the multi-enzyme complex. This leads to a ten-fold increase in oxo-acid dehydrogenase activity in skeletal muscle and a five-fold increase in heart muscle. Exercise training leads to an increase in kinase mRNA but a decrease in kinase protein, suggesting that there is post-transcriptional control of kinase synthesis (Chuang *et al.*, 2002; Harris *et al.*, 2001; Kasperek *et al.*, 1985; Shimomura *et al.*, 2001).

There are five mitochondrial protein kinases, four of which act on pyruvate dehydrogenase and one that is specific for branched-chain oxo-acid dehydrogenase. All of them act to phosphorylate specific serine residues in the target enzymes, but their primary structure is more closely related to prokaryotic histidine kinases than to eukaryotic serine kinases. It is likely that the (nuclear) genes for these mitochondrial kinases originated from endocytosed bacteria (Harris *et al.*, 2001, 2005).

A phosphoprotein phosphatase also co-purifies with the multi-enzyme complex, and this is responsible for activation of the phosphorylated enzyme. It is activated by calcium ions and, in addition to displacement of the kinase from the complex, this may account for the increase in branched-chain amino acid oxidation in muscle in response to exercise. Genetic deficiency of this

phosphatase may underlie some cases of maple syrup urine disease (section 7.2.2.1; Lu *et al.*, 2009). The phosphorylated multi-enzyme complex can also be activated without dephosphorylation by interacting with a poorly characterized activator protein that is present in liver and kidney mitochondria but not heart or skeletal muscle. The activator protein from liver will activate the phosphorylated enzyme from muscle (Randle, 1983).

Synthesis of branched-chain oxo-acid dehydrogenase is also regulated by a microRNA that targets the mRNA for the acyltransferase core protein of the multi-enzyme complex, reducing its stability so that less of the core protein, and hence less of the multi-enzyme complex, is synthesized (Mersey *et al.*, 2005).

7.2.2.1 Maple syrup urine disease Maple syrup urine disease is due to a genetic defect in branched-chain oxo-acid dehydrogenase; it is so called because the branched chain oxo-acids that are excreted in the urine have a smell reminiscent of maple syrup. In the classical form of the disease, there is only 1–2 per cent of the normal activity of the enzyme. The disease has an early onset, with ketoacidosis, encephalopathy and mental retardation if the child survives. The intermediate variant of the disease is milder because there is more residual activity of the enzyme, and there is an intermittent variant with late onset and only episodic acidosis. There is a thiamin-responsive variant and, most severe of all, a variant due to lack of dihydrolipoamide dehydrogenase. This last variant will affect pyruvate and 2-oxoglutarate dehydrogenases as well, leading to severe impairment of the energy-yielding metabolism.

The encephalopathy associated with high brain concentrations of branched-chain oxo-acids is due to decreased glutamate, glutamine and GABA in the central nervous system (see section 7.2.1) In addition, high plasma concentrations of the branched-chain amino acids will inhibit brain uptake of aromatic amino acids, leading to reduced synthesis of catecholamines (section 9.2.4) and serotonin (section 9.4.3), because they are carried by the same large neutral amino acid transporter. In thiamin-responsive maple syrup urine disease, the defect is not in E1, as might be expected, but in E2. Thiamin diphosphate is a potent inhibitor of the kinase that regulates the activity of E1, so thiamin supplements lead to increased activity of the dehydrogenase complex (Chuang *et al.*, 2006).

7.2.3 Branched-chain acyl CoA dehydrogenases

There are four enzymes that catalyze α - β -elimination reactions of the branched-chain acyl CoA derivatives. All four show significant sequence homology with the three acyl CoA dehydrogenases involved in fatty acid β -oxidation. They are flavoproteins that transfer hydrogen onto

electron-transfer flavoprotein and then reduce ubiquinone in the mitochondrial electron transport chain. Two hydrogen atoms are abstracted from the substrate onto the enzyme-bound flavin, forming a *trans* double bond. The enoyl CoA remains bound until the enzyme-bound flavin has been re-oxidized by reaction with the electron-transferring flavoprotein that reduces ubiquinone (Battaile *et al.*, 2004; Ikeda *et al.*, 1983; Ikeda & Tanaka, 1983; Thorpe & Kim, 1995).

Isovaleryl CoA dehydrogenase catalyzes the oxidation of isovaleryl CoA arising from leucine. Genetic defects of this enzyme lead to isovaleric acidemia. The acute neonatal variant is associated with very low activity of the enzyme, leading to profound metabolic acidosis from the first few days of life, and ultimately to early death. The chronic variant is associated with higher residual enzyme activity and is characterized by intermittent attacks of metabolic acidosis with asymptomatic intervals. The attacks are commonly precipitated by infection and fever.

The condition can be controlled by maintaining a low intake of leucine and providing supplements of glycine (so permitting conjugation of isovaleric acid and urinary excretion of isovaleryl-glycine) and carnitine (so permitting excretion of isovaleryl-carnitine). Isovaleric acid gives the urine a characteristic 'sweaty foot' odour. However, interestingly, up to ten per cent of people cannot smell isovaleric acid (Tanaka *et al.*, 1988; Vockley & Ensenauer, 2006).

Hypoglycin is a toxic amino acid (2-methylenecyclopropanylalanine) found in unripe ackee fruit (*Blighia sapida*). It inhibits acyl CoA dehydrogenases, thus inhibiting fatty acid metabolism in the fasting state and leading to profound hypoglycaemia – Jamaican vomiting sickness. It is a potent inhibitor of isovaleryl CoA dehydrogenase, and feeding it to experimental animals leads to isovaleric acidemia. In addition to hypoglycaemia, some of the signs and symptoms of Jamaican vomiting sickness may be due to isovaleric acidemia (Tanaka & Ikeda, 1990).

Methylbutyryl CoA dehydrogenase and short-chain/branched-chain acyl CoA dehydrogenases both catalyze oxidation of methylbutyryl CoA arising from isoleucine and isobutyryl CoA arising from valine, but not isovaleryl CoA. Unlike the rat enzyme, human short-chain/branched-chain acyl CoA dehydrogenase has little activity towards isobutyryl CoA, but there is a separate human isobutyryl CoA dehydrogenase. Short-chain/branched-chain acyl CoA dehydrogenase also catalyzes oxidation of butyrate, a reaction that is also catalyzed by the separate short-chain acyl CoA dehydrogenase (He *et al.*, 2003).

Genetic defects of all three of these dehydrogenases are known but, apart from urinary excretion of methylbutyrate and isobutyrate and their glycine and carnitine conjugates, these seem to be asymptomatic. This is presumably because, in the absence of one of the dehydrogenases, there is sufficient activity of the others to prevent the occurrence of metabolic acidosis.

The anticonvulsant drug valproic acid is metabolized by esterification with CoA, followed by dehydrogenation catalyzed by methylbutyryl CoA dehydrogenase. None of the other dehydrogenases acts on valproyl CoA, and the (sometimes fatal) liver damage that is caused by valproic acid may be associated with genetic defects of methylbutyryl CoA dehydrogenase (Ito *et al.*, 1990; Nguyen *et al.*, 2002).

7.2.4 Leucine catabolism

As shown in Figure 7.6, leucine is solely ketogenic, giving rise to hydroxymethylglutaryl CoA, which is cleaved to yield acetyl CoA and acetoacetate. Hydroxymethylglutaryl CoA is also potentially a precursor for cholesterol synthesis by reduction to mevalonate, catalyzed by hydroxymethylglutaryl CoA reductase. However, this is unlikely to be a significant fate of carbon arising from leucine. Rather, mevalonate formed by hydroxymethylglutaryl CoA reductase can be diverted away from cholesterol synthesis by conversion to methylcrotonyl CoA, leading to formation of acetyl CoA and acetoacetate (Kopito *et al.*, 1984; Weinstock *et al.*, 1984).

The first step unique to leucine catabolism is the carboxylation of 3-methylcrotonyl CoA to 3-methylglutaconyl CoA, catalyzed by methylcrotonyl CoA carboxylase. This is a hetero-multimeric protein with α - and β -subunits that are encoded by different genes. The α -subunit contains biotin and catalyzes the formation of carboxybiotin, while the β -subunit catalyzes the carboxylation of the substrate. Methylcrotonic aciduria occurs in experimental biotin deficiency and in multiple carboxylase deficiency due to lack of biotinidase or holocarboxylase synthetase (section 7.2.7.1). It also occurs as a result of isolated genetic defects of methylcrotonyl CoA carboxylase, with a variable phenotype ranging from neonatal onset with severe neurological involvement to more or less asymptomatic adults, depending on the residual activity of the enzyme (Baumgartner *et al.*, 2001).

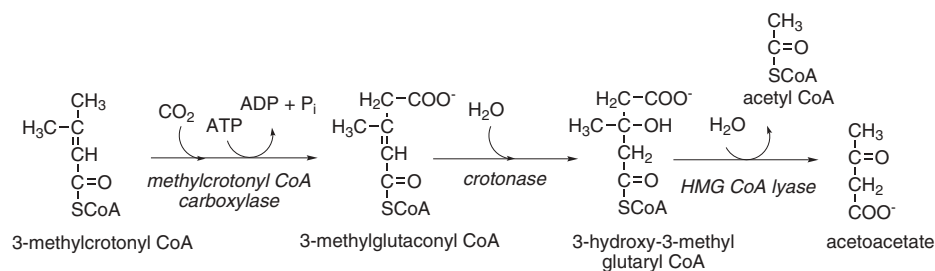


Figure 7.6 Leucine catabolism.

Methylcrotonyl CoA carboxylase EC 6.4.1.4, crotonase (enoyl CoA hydratase) EC 4.2.1.7 or 4.2.1.17, hydroxymethylglutaryl CoA lyase EC 4.1.3.4.

The second step in leucine catabolism is hydration of the double bond, catalyzed by crotonase, an enoyl CoA hydratase that does not act on methylcrotonyl CoA. It forms part of the mitochondrial trifunctional protein of fatty acid β -oxidation, together with hydroxyacyl CoA dehydrogenase and ketoacyl CoA thiolase (Agnihotri & Liu, 2003). The resultant hydroxymethylglutaryl CoA is cleaved by hydroxymethylglutaryl CoA lyase to yield acetyl CoA and acetoacetate, as occurs in the final step of ketogenesis in the fasting state.

7.2.5 Isoleucine catabolism

As shown in Figure 7.7, isoleucine is both ketogenic and glucogenic, yielding acetyl CoA and succinyl CoA, which is metabolized via the citric acid cycle to yield oxaloacetate that can be used for gluconeogenesis.

Tiglyl CoA, the product of methylbutyryl CoA dehydrogenase or short-chain/branched-chain acyl CoA dehydrogenase, undergoes hydration of the double bond catalyzed by crotonase (section 7.2.4). The resultant 2-methyl-3-hydroxybutyryl CoA is oxidized by a dehydrogenase that has a broad specificity for short-chain hydroxyacyl CoA derivatives, and it also acts as a 17β -hydroxysteroid dehydrogenase. β -Ketothiolase catalyzes the cleavage of methyl-acetoacetyl CoA to acetyl CoA and propionyl CoA; this is the same enzyme that is involved in the synthesis of acetoacetyl CoA for ketogenesis in the liver and the cleavage of acetoacetyl CoA in extrahepatic tissues.

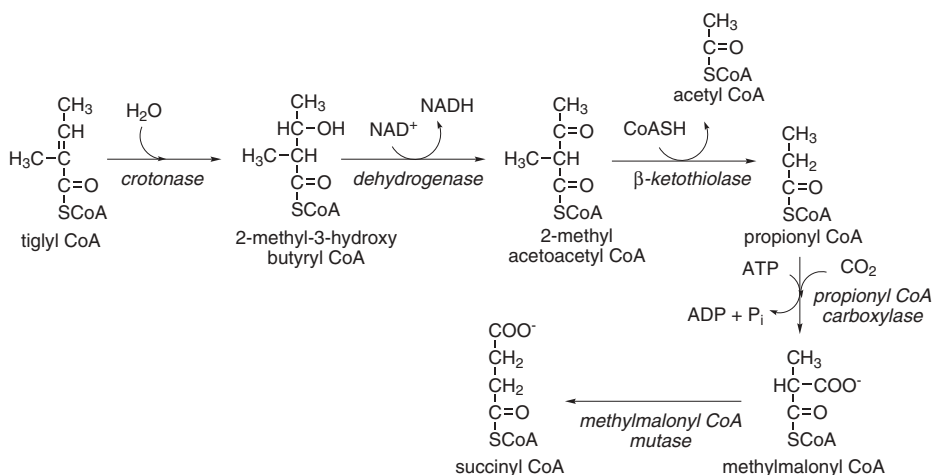


Figure 7.7 Isoleucine catabolism.

Crotonase (enoyl CoA hydratase) EC 4.2.1.7 or 4.2.1.17, methyl-acetoacetyl CoA dehydrogenase EC 1.1.1.178, β -ketothiolase EC 2.3.1.16, propionyl CoA carboxylase EC 6.4.1.3, methylmalonyl CoA epimerase EC 5.1.99.1, methylmalonyl CoA mutase EC 5.4.99.2.

Propionyl CoA undergoes biotin-dependent carboxylation to D-methylmalonyl CoA (section 7.2.7) followed by isomerization to L-methylmalonyl CoA, which is the substrate for isomerization to succinyl CoA, catalyzed by methylmalonyl CoA mutase.

Methylmalonyl CoA mutase is one of only two cobalamin (vitamin B₁₂) dependent mammalian enzymes, the other being methionine synthase (see section 6.3.1.2). In addition to isoleucine and valine catabolism, methylmalonyl CoA is also an intermediate in the catabolism of fatty acids with an odd number of carbon atoms, and the side-chain of cholesterol.

Methylmalonic aciduria may be the result of vitamin B₁₂ deficiency, so the excretion of methylmalonic acid provides a sensitive index of vitamin B₁₂ nutritional status. Methylmalonic aciduria also occurs as a result of mutations in the gene for methylmalonyl CoA mutase, one or other of the enzymes involved in the synthesis of adenosyl cobalamin as the coenzyme, or a transport protein for import of vitamin B₁₂ into the mitochondrion (Martinez *et al.*, 2005).

The synthesis of adenosyl cobalamin involves the transfer of the adenosyl moiety of ATP onto the central cobalt atom of cobalamin, with the release of triphosphosphate. Adenosyltransferase is a dual function enzyme that both synthesizes the coenzyme and also acts as a chaperone to deliver it to the mutase (Padovani & Banerjee, 2009; Yamanishi *et al.*, 2005).

The overall reaction of methylmalonyl CoA mutase is the exchange of a hydrogen atom and a methyl group on adjacent carbon atoms. Although this is the only mammalian adenosylcobalamin-dependent enzyme, there are a number of bacterial enzymes that catalyze similar reactions. The cofactor acts to initiate a free radical, followed by homolysis of the cobalt-carbon bond to yield a cobalt radical and the 5′deoxyadenosine radical. The 5′deoxyadenosine radical abstracts hydrogen from the substrate, forming the substrate radical that undergoes methyl group migration to form the product radical, which then abstracts hydrogen from 5′deoxyadenosine. The resultant 5′deoxyadenosine radical then reacts with the cobalt radical to reform the stable coenzyme (Banerjee, 2003; Mansoorabadi *et al.*, 2005).

7.2.6 Valine catabolism

As shown in Figure 7.8, valine is a purely glucogenic amino acid, yielding succinyl CoA, which is metabolized via the citric acid cycle to yield oxaloacetate that can be used for gluconeogenesis.

Methacrylyl CoA arising from the action of the dehydrogenases on isobutyryl CoA is toxic because it reacts with free thiol compounds, so the activities of crotonase and hydroxyisobutyryl CoA hydrolase are important to maintain very low intracellular concentrations. Both enzymes are present in human liver at considerably higher activity than the branched-chain oxo-acid

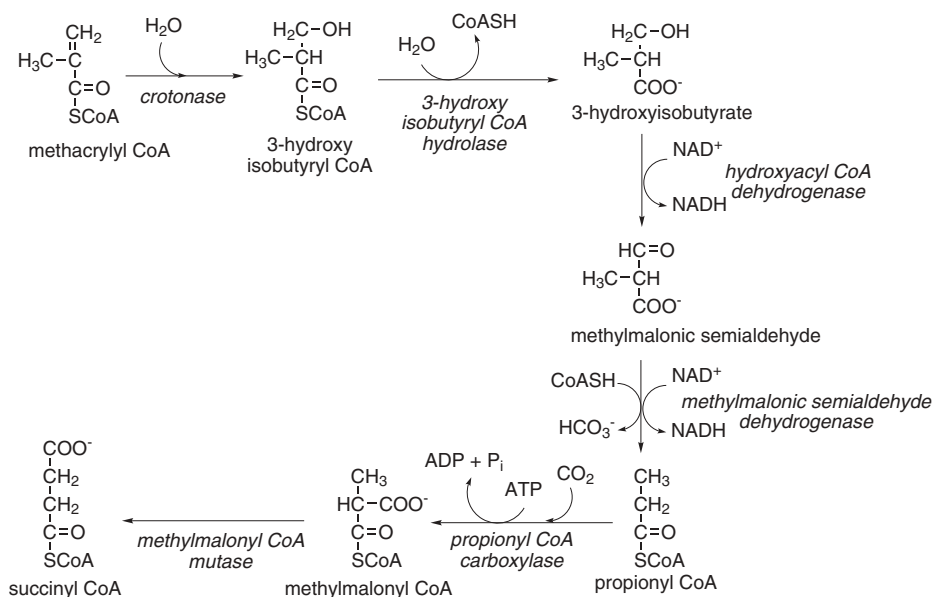


Figure 7.8 Valine catabolism.

Crotonase (enoyl CoA hydratase) 4.2.1.17, 3-hydroxyisobutyryl CoA hydrolase EC 3.1.2.4, hydroxyacyl CoA dehydrogenase EC 1.1.1.35, methylmalonic semialdehyde dehydrogenase EC 1.2.1.27, propionyl CoA carboxylase EC 6.4.1.3, methylmalonyl CoA epimerase EC 5.1.99.1, methylmalonyl CoA mutase EC 5.4.99.2.

dehydrogenase multi-enzyme complex, so methacrylyl CoA does not accumulate. However, the reaction catalyzed by crotonase is readily reversible, and a genetic defect of hydroxyisobutyryl CoA hydrolase leads to a variety of congenital malformations, as well as urinary excretion of cysteine and cysteamine conjugates of methacrylic acid (Brown *et al.*, 1982; Shimomura *et al.*, 2004; Taniguchi *et al.*, 1996).

Hydroxyisobutyrate undergoes oxidation catalyzed by hydroxyisobutyrate dehydrogenase, and the resultant methylmalonic semialdehyde is the substrate for oxidative decarboxylation to yield propionyl CoA, which is carboxylated to methylmalonyl CoA, ultimately yielding succinyl CoA (as discussed in section 7.2.7). Methylmalonic semialdehyde dehydrogenase is unusual among aldehyde dehydrogenases, in that it catalyzes the formation of a CoA ester rather than a free acid. It catalyzes decarboxylation to propionate, followed by esterification with CoASH. It also acts on malonic semialdehyde, and is therefore involved in the catabolism of both uracil and thymine (section 1.4.4; Goodwin *et al.*, 1989; Stines-Chaumeil *et al.*, 2006).

The heart and other muscle tissue releases hydroxyisobutyrate, which is taken up by the liver and kidneys for gluconeogenesis. Pre-adipocytes lack hydroxyisobutyrate dehydrogenase and methylmalonic semialdehyde dehy-

drogenase but, on differentiation into adipocytes, the activities of these two enzymes – and of branched-chain oxo-acid dehydrogenase – increase considerably. Thus, although it is glucogenic in the fasting state, valine can be used for lipogenesis in adipose tissue in the fed state (Kedishvili *et al.*, 1994).

7.2.7 Biotin-dependent carboxylation reactions

Two of the four mammalian biotin-dependent carboxylases, methylcrotonyl CoA carboxylase (Figure 7.6) and propionyl CoA carboxylase (Figures 7.7 and 7.8), are involved in the catabolism of branched-chain amino acids (the other two are acetyl CoA carboxylase in fatty acid synthesis and pyruvate carboxylase in gluconeogenesis and anaplerosis of the citric acid cycle). In all four enzymes, there is an α -subunit that contains biotin as ϵ -biotinyllysine (biocytin) and catalyzes the formation of carboxybiotin, and a β -subunit that catalyzes the carboxylation of the substrate. As shown in Figure 7.9, the

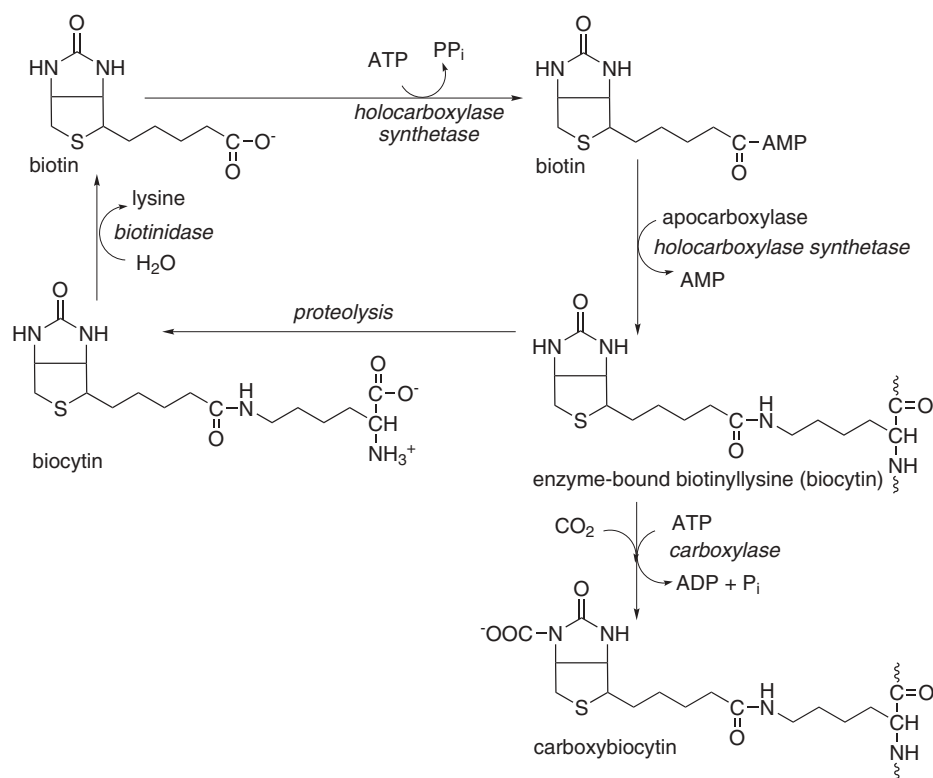


Figure 7.9 The role of biotin in carboxylation reactions.

Biotinidase EC 3.5.1.12, biotin (propionyl CoA carboxylase) synthetase EC 6.3.4.10, biotin (methylcrotonyl CoA carboxylase) synthetase EC 6.3.4.11, biotin (acetyl CoA carboxylase) synthetase EC 6.3.4.15.

attachment of biotin to the α -subunit of the enzyme is a two-step reaction catalyzed by holocarboxylase synthetase, with intermediate formation of biotinyl-AMP. Carboxylation of the biotinyllysine is ATP-dependent, and the carboxyl group is then transferred to the substrate by the β -subunit.

A single holocarboxylase synthetase acts on the apo-enzymes of all four biotin-dependent carboxylases. Holocarboxylase synthetase from a wide variety of species will act on all four apo-carboxylases from other species, and also on a variety of bacterial biotin-dependent apo-enzymes. In all of the biotin-dependent enzymes investigated to date, the reactive lysine residue is flanked by methionine residues on either side and there is a high degree of conservation of the amino sequence around this Met-Lys-Met sequence (Chapman-Smith & Cronan, 1999a, 1999b). Biotin has a role in regulation of the cell cycle, and holocarboxylase synthetase is targeted to the nucleus, where it catalyzes biotinylation of histones (Gravel & Narang, 2005; Zempleni & Mock, 2001).

Proteolysis of biotin-containing enzymes releases biocytin, either as free biotinyllysine or as a variety of small biocytin-containing peptides; the ϵ -amino lysine link of biocytin is not a substrate for peptidases. Biocytin is hydrolyzed by biotinidase, which acts on free or peptide-incorporated biocytin to release biotin but has no general peptidase or esterase activity. Biotinidase is most active towards free biocytin, but it will also release biotin from biocytin-containing peptides. The activity decreases as the size of the peptide increases, so it is likely that, *in vivo*, the catabolism of biotin-containing enzymes is by proteolysis, followed by biotinidase action rather than the release of biotin, leaving the apo-enzyme as a substrate for proteolysis. Biotinidase is found in all tissues, including the pancreatic juice and intestinal mucosa. Biotinidase thus functions both to release free biotin from biocytin in foods and to recycle and conserve biotin following the turnover of biotin-containing enzymes.

Biotinidase is also the major plasma-binding protein for biotin. It functions as a transport protein for biotin, preventing its urinary excretion. Children with biotinidase deficiency (section 7.2.7.1) excrete large amounts of both biocytin and free biotin. Biotin is covalently bound to biotinidase in plasma, as a thio-ester to a cysteine residue in the active site of the enzyme. This thio-ester is formed only from biocytin, not free biotin, and it is presumably the (normally transient) intermediate in the hydrolysis of biocytin. Free biotin can be released from biotinidase at low pH, and the enzyme can also catalyze a biotinyl transferase reaction, biotinylating histones and other nucleophilic acceptors (Hymes & Wolf, 1996, 1999; Wolf, 2005).

7.2.7.1 Multiple carboxylase deficiency Isolated deficiency of one or other of the carboxylases can occur. Lack of methylcrotonyl CoA carboxylase is relatively benign and can be controlled by a low protein diet to minimize the

amount of leucine to be catabolized; at high intakes of protein, affected infants become hypoglycaemic and comatose. Propionic acidaemia due to propionyl CoA carboxylase deficiency causes severe ketosis and acidosis, resulting in failure to thrive and mental retardation, and it is generally fatal in infancy.

Genetic lack of either holocarboxylase synthetase or biotinidase leads to defective activity of all four carboxylases. Affected infants have a scaly dermatitis and alopecia, as is seen in biotin deficiency, and they also develop potentially life-threatening keto-acidosis and sometimes also hyperammonaemia, with mental retardation, delayed development and acute neurological problems (Nyhan, 1988). They also have high blood and urine concentrations of a number of substrates of biotin-dependent enzymes. The metabolic precursors and alternative metabolites of these substrates are shown in Table 7.1.

Lack of holocarboxylase synthetase leads to the neonatal form of multiple carboxylase deficiency. Most infants present within the first six weeks of life, although some may not present until 15 months old. The affected infants have a normal plasma concentration of biotin, and they excrete normal amounts of biotin in the urine. Skin fibroblasts have extremely low activities of all four biotin-dependent carboxylases when they are cultured in media containing

Table 7.1 Abnormal urinary organic acids in biotin deficiency and multiple carboxylase deficiency due to lack of holocarboxylase synthetase or biotinidase.

Arising from impaired activity of acetyl CoA carboxylase

2-ethyl-3-hydroxyhexanoic acid
2-ethylhexanedioic acid

Arising from impaired activity of pyruvate carboxylase

lactate
pyruvate
alanine

Arising from impaired activity of propionyl CoA carboxylase

propionic acid
3-hydroxypropionic acid
propionylglycine
methylcitric acid
tiglic acid (from leucine catabolism)
tiglylglycine
2-methyl-3-hydroxybutyric acid
lactate

Arising from impaired activity of methylcrotonyl CoA carboxylase

3-methylcrotonic acid
3-hydroxy-isovaleric acid (hydration product of 3-methylcrotonic acid)
3-methylcrotonyl glycine

approximately physiological concentrations of biotin, but culture with considerably higher concentrations of biotin results in normal activity of all four carboxylases.

The defect is in the affinity of holocarboxylase synthetase for biotin (its K_m is 20–70 fold higher than normal). The condition is a biotin-responsive genetic disease, and patients can be maintained in good health with supplements of high doses of biotin. Doses of biotin of the order of 1 mg/day prevent clinical signs in most patients, but 10 mg/day or more is required to correct the organic aciduria (Baumgartner & Suormala, 1997, 1999).

Genetic lack of biotinidase results in the late-onset variant of multiple carboxylase deficiency. Patients generally present later in life than those with holocarboxylase synthetase deficiency, and they have a lower than normal blood concentration of biotin. Culture of fibroblasts in media containing low concentrations of biotin results in normal activities of carboxylases, and holocarboxylase synthetase activity is normal.

The problem is a functional deficiency of biotin, due both to an inability to release free biotin from dietary biocytin, and also to failure of the normal salvage of biotin by biotinidase action on the biocytin released by proteolysis of biotin-containing enzymes. Normal intakes of biotin are inadequate to meet the requirements of these patients; the provision of pharmacological doses of free biotin provides an adequate amount to meet requirements without the need for reutilization. The delayed development of clinical and biochemical abnormalities is a result of the accumulation of biotin by the foetus, so that the infant has adequate stores of the vitamin at birth.

Biotinidase-deficient patients have higher than normal amounts of biocytin in plasma and urine, and they excrete higher than normal amounts of biotin, reflecting the importance of protein binding of biotin to prevent urinary loss. Therapy with 10 mg of biotin/day prevents the development of most symptoms, although some patients develop neurosensory hearing loss and optic atrophy despite therapy with biotin. This reflects the role of biotinidase in tissue uptake of biotin (Baumgartner & Suormala, 1997, 1999; Wolf & Feldman, 1982; Wolf & Heard, 1991).

Further reading

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

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8

Histidine

The early studies of nitrogen balance to determine amino acid requirements (see section 2.2.1) suggested that histidine was not an essential amino acid for human beings, although it was known to be essential for other mammals. This was because people deprived of histidine for relatively short periods of time were able to maintain nitrogen balance. Because of its chemical reactivity, histidine was not originally included in amino acid mixtures used for total intravenous nutrition.

However, patients maintained for relatively prolonged periods of time on total intravenous nutrition without histidine are unable to maintain nitrogen balance. Kopple & Swendseid (1975) showed that feeding a histidine-free amino acid mixture for a month led to failure to maintain nitrogen balance and a fall in haematocrit, with an increase in serum iron. Five subjects also developed scaly, dry skin. Repletion with histidine reversed these changes and led to normal erythropoiesis. Therefore, histidine is now regarded as an essential amino acid, although it is not clear why histidine-deprived subjects can maintain nitrogen balance in short-term studies.

Histidine has an important role in enzymes because its pKa (between 6 and 7, depending on its environment in the protein) is such that it can act as a general acid-base catalyst at physiological pH. For the same reason, histidine contributes significantly to the buffering capacity of plasma and tissue proteins. In plants and microorganisms, free histidine has a role in preventing nickel and other metal toxicity, and organisms that over-produce histidine are nickel-tolerant or even, in some cases, over-accumulators of nickel from the soil. In contrast, mutant yeasts that are defective in histidine synthesis have poor tolerance of nickel, cobalt and cadmium in the culture medium.

In mammals, histidine is important as the precursor of the peripherally pharmacologically active amine and central neurotransmitter histamine (section 8.3). Urocanic acid, an intermediate in histidine catabolism (section 8.2.1), is the main ultraviolet absorbing compound in the skin. Changes in histidine metabolism may be important in the development of the photosensitive dermatitis seen in pellagra (section 9.4.5) and zinc deficiency. Histidine is also required for the synthesis of two dipeptides of unknown function – carnosine (β -alanylhistidine) and homocarnosine (γ -aminobutyrylhistidine – see section 8.5).

Histidinaemia, due to a defect in histidase (section 8.2.1) was originally identified in children with mental retardation and speech defects. However, as a result of neonatal screening programmes, it has now been recognized as a benign metabolic defect, with a frequency varying from 1:8,000 in Japan to 1:37,000 in Sweden. The prevalence of mental retardation and speech defects in children with histidinaemia is no higher than in the general population (Virmani & Widhalm, 1993).

8.1 Biosynthesis of histidine

The pathway for histidine biosynthesis is highly conserved, from archaea through bacteria to fungi and higher plants. With few exceptions, the genes and enzymes of the pathway show a high degree of sequence homology. In plants, the genes have an amino-terminal chloroplast-targeting sequence, and histidine is synthesized in the chloroplasts. In bacteria, the genes coding for the enzymes of the pathway are arranged as a single operon; indeed, the *his* operon, together with the *lac* operon and the *trp* operon (section 9.1.3.1), was responsible for the development of the operon theory. As shown in Table 8.1, the order of the genes in the operon is not the same as the order in which the enzymes catalyze the steps of the pathway. Prokaryotes do not have operons, and the genes for the histidine biosynthetic pathway in yeasts, fungi

Table 8.1 Genes of the *his* operon of *Salmonella typhimurium*.

Enzyme	Gene
ATP phosphoribosyltransferase	G
phosphoribosyl-ATP diphosphatase	I (carboxy-terminal)
phosphoribosyl-AMP cyclohydrolase	I (amino-terminal)
phosphoribulosyl formimino-AICRP isomerase	A
imidazole glycerol phosphate synthase	H + F
imidazole glycerol phosphate dehydratase	B (carboxy-terminal)
imidazole acetol phosphate transaminase	C
histidinol phosphate phosphatase	B (amino-terminal)
histidinol dehydrogenase	D

and plants are scattered throughout the genome, frequently on different chromosomes (Alifano *et al.*, 1996; Rees *et al.*, 2009; Stepansky & Leustek, 2006).

Histidinyl-tRNA acts as a repressor of the operon. Like the *trp* operon (section 9.1.3.1), the *his* operon in bacteria is also regulated by attenuation. There are transcription termination sites (attenuators) within the operon as well as at the end. The translation product of the early sequence of the operon is rich in histidine. When histidine is in short supply, translation is stalled for long enough for succeeding segments of the transcript to form secondary structures that allow RNA polymerase to proceed through the attenuator. In contrast, when translation proceeds at a normal rate because there is an adequate supply of histidinyl-tRNA, transcription is halted at the attenuator site (Yanofsky, 1981).

As can be seen from Figure 8.1, histidine biosynthesis is very energy-expensive, second only to the synthesis of aromatic amino acids (see section 9.1) and requiring $41 \times$ ATP per molecule of histidine formed. Because of the high energy cost of histidine synthesis, plants that over-produce histidine because they over-express ATP phosphoribosyltransferase, the first enzyme of the pathway, show poor growth and low biomass accumulation.

The first step in histidine synthesis is the formation of phosphoribosyl-ATP, by reaction between phosphoribosyl pyrophosphate and ATP, catalyzed by ATP phosphoribosyltransferase. This enzyme is the main control point for histidine synthesis and is subject to feedback inhibition by histidine. Binding of histidine or 5'AMP (an indicator of the energy state of the cell) leads to association of the active dimer into a less active hexamer, while binding of phosphoribosyl pyrophosphate leads to dissociation of the hexamer to the active dimer.

In some bacteria, there is a truncated ATP phosphoribosyltransferase that lacks the 80 amino acid carboxy-terminal regulatory domain. This enzyme forms a hetero-octamer with a regulatory protein that shows significant homology with histidinyl tRNA synthetase. The regulatory protein not only confers sensitivity to 5'AMP and histidine, it is also essential for activity of the catalytic subunit. The truncated ATP phosphoribosyltransferase has a four-fold higher K_m for ATP than the full-length enzyme found in most organisms, suggesting that it is more sensitive to the energy state of the cell (Champagne *et al.*, 2006; Lohkamp *et al.*, 2004).

There are two genes for ATP phosphoribosyltransferase in plants, and over-expression of either leads to a 40-fold increase in the concentration of free histidine in shoots, with no effect on other amino acids. Over-expression of any of the other genes of the pathway has no effect, confirming that the phosphoribosyltransferase is the main controlling step in the pathway (Alifano *et al.*, 1996; Rees *et al.*, 2009).

Phosphoribosyl-ATP diphosphatase catalyzes hydrolysis of phosphoribosyl-ATP to phosphoribosyl-AMP and pyrophosphate. This reaction is reversible,

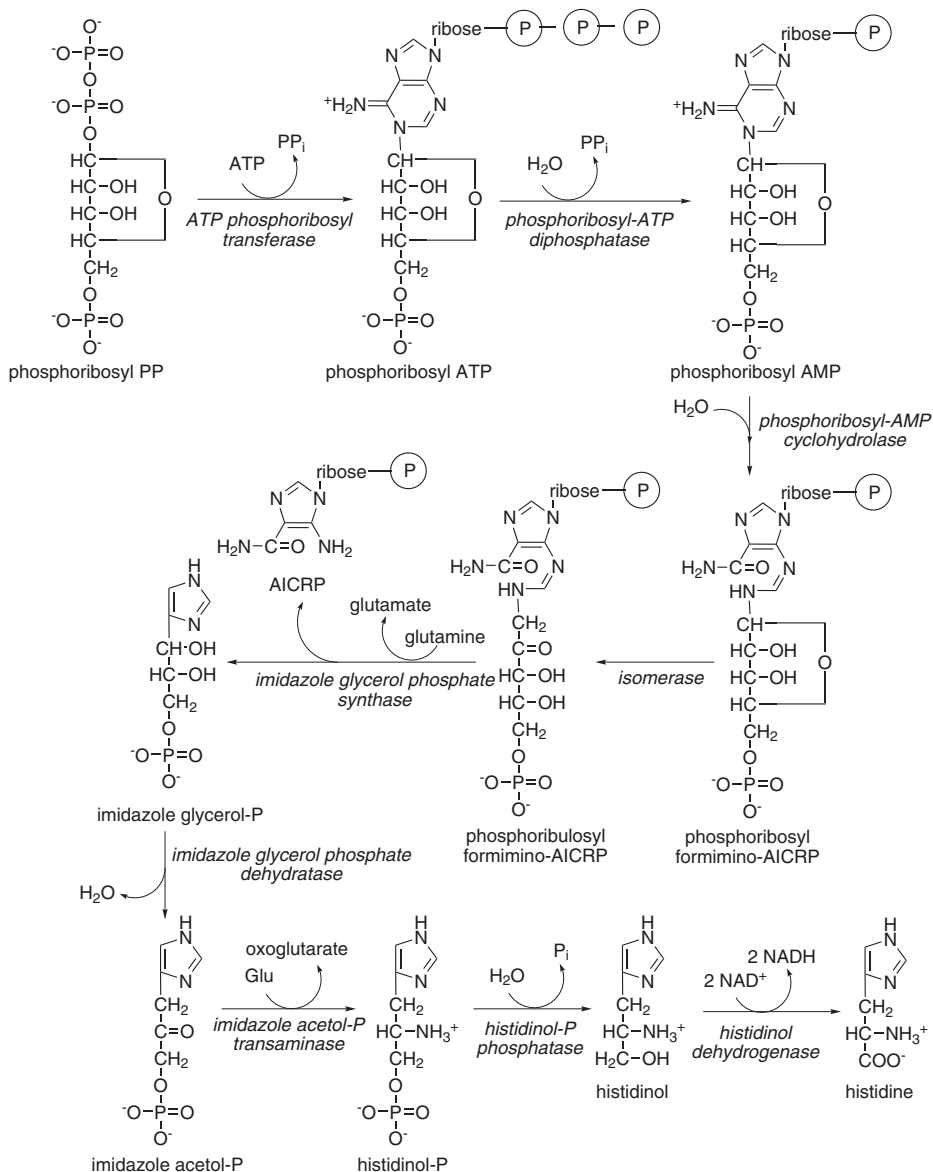


Figure 8.1 Histidine biosynthesis.

AICRP = aminoimidazole carboxamide ribonucleotide; ATP phosphoribosyltransferase EC 2.4.2.17, phosphoribosyl-ATP diphosphatase EC 3.6.1.31, phosphoribosyl-AMP cyclohydrolase EC 3.5.4.19, phosphoribosyl formimino-AICRP isomerase EC 5.3.1.16, imidazole glycerolphosphate synthase no EC number, imidazole glycerolphosphate dehydratase EC 4.2.1.19, histidinol phosphate transaminase EC 2.6.1.9, histidinol phosphate phosphatase EC 3.1.3.15, histidinol dehydrogenase EC 1.1.1.23. See Figure 1.7 for the role of AICRP in purine biosynthesis).

and it is maintained in the forward direction by the hydrolysis of pyrophosphate, catalyzed by pyrophosphatase. In most bacteria and plants, this activity is at the carboxy-terminal of a bifunctional enzyme. The amino-terminal of this is phosphoribosyl-AMP cyclohydrolase, which catalyzes the next step in the pathway.

In archaea, these two reactions are catalyzed by separate enzymes. Gene fusion to yield the bifunctional enzyme obviously occurred early in the evolution of the pathway. In yeasts and fungi, there is a trifunctional enzyme, catalyzing these two reactions and also the final reaction of the pathway, histidinol dehydrogenase. The cyclohydrolase is a zinc-dependent enzyme catalyzing cleavage of the N-1 – C-6 bond of the purine ring (Fujimori & Ohta, 1998).

The product of the cyclohydrolase reaction then undergoes isomerization of the sugar moiety to yield phosphoribulosyl formimino-amino-imidazole-carboxamide ribonucleotide (formimino-AICAR). This isomerase has a structure that is very similar to that of phosphoribosyl anthranilate isomerase in the tryptophan synthesis pathway (section 9.1.3). A single site-directed mutation in phosphoribosyl-AICRP isomerase allows it to act on phosphoribosyl anthranilate as well. In some bacteria, a single enzyme catalyzes both isomerization reactions, so that deletion of the phosphoribosyl-AICRP isomerase gene leads to an organism that is auxotrophic for both histidine and tryptophan (Barona-Gomez & Hodgson, 2003; Leopoldseder *et al.*, 2004).

Imidazole glycerol phosphate synthase catalyzes amide transfer from glutamine and cleavage of phosphoribulosyl formimino-AICRP to imidazole glycerol phosphate and amino-imidazole-carboxamide ribonucleotide. This provides an alternative source of AICRP for *de novo* purine synthesis (Figure 1.6) and can be considered to be a salvage pathway for the adenine ring of the ATP incorporated in the first step of the pathway. Only C-2 and N-6 of the purine ring remain in imidazole glycerol phosphate – the complete imidazole ring of the purine is not incorporated into histidine.

In most organisms, imidazole glycerol phosphate synthase is a dimer of two different proteins, derived from different genes. One subunit catalyzes the hydrolysis of glutamine to glutamate and ammonia, and the other catalyzes the ammonia-dependent cleavage of phosphoribulosyl formimino-AICRP. The glutaminase only has significant activity when it is complexed with the second protein, and only when the acceptor substrate is also bound. As with other glutamine-dependent amidotransferases (section 5.3.3), ammonia is channelled from the glutaminase active site to the acceptor site through a tunnel in the enzyme.

In plants and fungi, there is a single bifunctional enzyme, representing fusion of the two ancestral bacterial genes. Unlike the bacterial enzyme, the bifunctional enzyme can use ammonia directly and has a relatively high K_m for glutamine. However, the activity of the glutaminase domain increases 1,000-fold on binding of the acceptor substrate (Chittur *et al.*, 2000).

Imidazole glycerol phosphate dehydratase catalyzes an unusual dehydration reaction in that it involves removal of a non-acidic hydrogen atom (in most dehydratases, the hydrogen is relatively acidic because it is adjacent to a carbonyl or imine group). The dehydratase is a manganese-dependent enzyme; the apo-enzyme is an inactive trimer which associates to form the active 24-mer in the presence of Mn^{2+} ions. The triazole phosphonate herbicides act by inhibition of this enzyme. In yeast, plants and some bacteria, the dehydratase is a monofunctional enzyme, but in *E. coli* and other bacteria it is a bifunctional enzyme with histidinol phosphate phosphatase (Hawkes *et al.*, 1995; Sinha *et al.*, 2004).

Imidazole acetol phosphate undergoes transamination to histidinol phosphate, followed by hydrolysis to yield histidinol, catalyzed by histidinol phosphate phosphatase. In enterobacteria, the phosphatase is a bifunctional enzyme, with imidazole glycerol phosphate dehydratase activity at the carboxy-terminal and phosphatase activity at the amino-terminal. As might be expected for a bifunctional enzyme catalyzing non-adjacent steps in a pathway, the two activities are independent. In other bacteria and in yeast, the phosphatase is a monofunctional enzyme. The phosphatase has aspartate at the catalytic site and forms β -aspartyl phosphate as an intermediate in the reaction (Rangarajan *et al.*, 2006).

There is no plant gene with significant homology to either of these types of phosphatase. Plants have instead a broad-specificity phosphatase that also acts on galactose 1-phosphate; it resembles the myoinositol monophosphatase that acts on histidinol phosphate in some bacteria (Petersen *et al.*, 2010).

Histidinol dehydrogenase catalyzes the four-electron oxidation of histidinol to histidine via the intermediate formation of histidinal, which remains bound to the enzyme when the first NADH leaves and is replaced by the second NAD^+ . Both reactions occur at the same active site, and the overall reaction is irreversible although, *in vitro*, histidinal can undergo either oxidation to histidine or reduction to histidinol (Barbosa *et al.*, 2002).

Inhibitors of histidinol dehydrogenase are potential chemotherapeutic agents for treatment of brucellosis, which is globally one of the most common zoonotic infections. The causative agent, *Brucella suis*, can establish persistent infections that are resistant to antibiotics, because it enters macrophages through lipid raft domains rather than by phagocytosis, so avoiding bactericidal attack and phagolysosomal fusion. It can then replicate inside the macrophages. Inhibitors of histidinol dehydrogenase starve the organism of histidine and so prevent this replication (Joseph *et al.*, 2007).

8.2 Histidine catabolism

The main pathway of histidine catabolism, through urocanic acid (section 8.2.1), is remarkably conserved from bacteria that can utilize histidine as a

sole source of carbon and nitrogen through fungi and plants to mammals. Transamination (section 8.2.3) is a relatively minor pathway of histidine catabolism.

8.2.1 The urocanic acid pathway of histidine catabolism

As shown in Figure 8.2, the first step in histidine catabolism is a non-oxidative deamination, catalyzed by histidine ammonia lyase (histidase). Histidase is closely related to the aromatic amino acid ammonia lyases that occur in plants (section 9.3). These ammonia lyases all have an unusual cofactor – methylidene-imidazolone – which is formed autocatalytically by dehydration of three adjacent amino acids in the active site (alanine, serine and glycine),

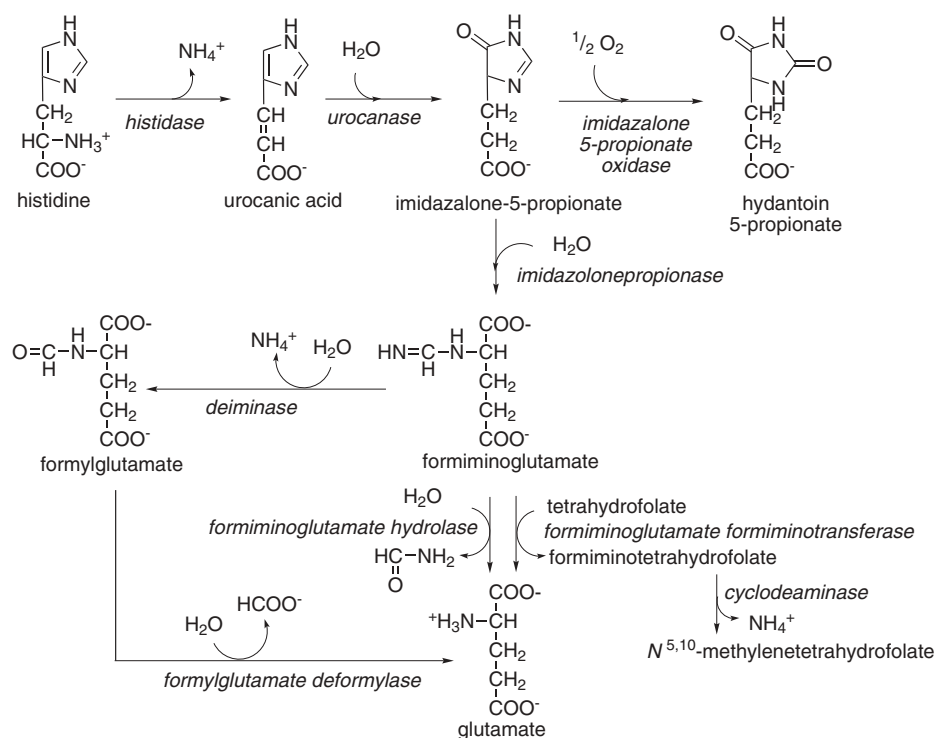


Figure 8.2 Histidine catabolism.

Histidase (histidine ammonia lyase) EC 4.3.1.3, urocanase EC 4.2.1.49, imidazolone propionate oxidase no EC number (xanthine oxidase, EC 1.17.3.2, also catalyzes this reaction), imidazolone propionase EC 3.5.2.7, formiminoglutamate deiminase EC 3.5.3.13, formylglutamate deformylase EC 3.5.1.68, formiminoglutamate hydrolase EC 3.5.3.8, formiminoglutamate formiminotransferase EC 2.2.2.5, cyclodeaminase EC 4.3.1.4.

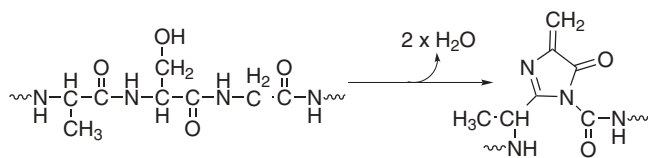


Figure 8.3 Formation of the methylidene-imidazole cofactor of histidase.

at a late stage in folding the nascent protein (Figure 8.3). The reaction of histidase involves removal of the non-acidic β -proton without affecting the more acidic protons on the amino group; methylidene-imidazolone provides the electrophile to catalyze the reaction (Poppe & Retey, 2005; Retey, 2003).

In mammals, histidase is expressed mainly in the liver (in periportal cells, as would be expected for an enzyme that generates ammonium) and the stratum granulosum of the skin. The liver enzyme (but not that in the skin) is induced by feeding a high-protein diet, but not by feeding histidine alone. After a test dose of histidine, however, there is increased catabolism, with no change in the activity of histidase, because of the relatively high K_m of histidase for its substrate. After puberty, the activity of histidase in the liver is twice as high in female rats than in males. It is induced in response to oestrogens and repressed in response to testosterone. It is also induced by glucocorticoid hormones and glucagon and repressed by all-*trans* retinoic acid (Lamartiniere, 1985; Sano *et al.*, 1997; Torres *et al.*, 1998a).

Although there is high activity of histidase in the stratum granulosum of the skin, none of the subsequent enzymes of histidine catabolism are found there, and urocanic acid accumulates in the stratum corneum, where it provides the main protection against ultraviolet irradiation. It is also important in maintaining an acidic pH in the stratum corneum, and it forms part of the so-called 'natural moisturizing factor' of the skin – a mixture of low molecular weight compounds that maintains hydration. Repression of histidase expression by all-*trans* retinoic acid may explain the photosensitization that occurs with use of therapeutic retinoids.

Exposure to ultraviolet light also leads to isomerization of *trans*-urocanic acid to *cis*-urocanic acid, which suppresses immune function, both locally and systemically, and also acts as a potent chemoattractant for the parasitic skin-penetrating nematode *Strongyloides stercoralis*. Histidinaemic mice, with impaired activity of histidase, accumulate only ten per cent of the normal amount of urocanic acid in the skin and show considerably reduced immune suppression after ultraviolet irradiation. Conversely, feeding relatively large amounts of histidine leads to increased immunosuppression after irradiation. *Cis*-urocanic acid interacts with target cells in the skin (possibly fibroblasts)

and stimulates production of systemic factors that alter the function of antigen-presenting cells so that suppressor T-cells are formed. *Trans*-urocanic acid is lost from the skin, either in sweat or in shed skin cells; *cis*-urocanic acid may also diffuse into the bloodstream (Eckhart *et al.*, 2008; Noonan & De Fabo, 1992; Norval *et al.*, 1995).

The second reaction of histidine catabolism is addition of water across the carbon-carbon double bond of urocanic acid, catalyzed by urocanase. The enzyme contains tightly bound NAD^+ , which acts as an electrophile rather than catalyzing a redox reaction. Urocanase seems to be the only enzyme in which NAD has this role. The enzyme catalyzes formation of an adduct between the nicotinamide ring of the coenzyme and the imidazole ring of the substrate, followed by release of hydroxy-imidazole propionate, which then spontaneously tautomerizes to imidazolone propionate. The two hydrogens that are added across the double bond are derived from water. The NAD^+ is tightly, but non-covalently, bound to the enzyme in a typical NAD^+ binding domain that sits like a lid over the substrate binding domain. The substrate, NAD^+ and five mol of water are completely sequestered in a cavity in the protein, and the NAD-binding domain has to move to permit access of the substrate and egress of the product. NAD cannot be incorporated in to the apo-enzyme *in vitro*; it has to be present during folding of the nascent protein (Kessler *et al.*, 2004; Retey, 1994).

Urocanase from *Pseudomonas putida* has an amino-terminal 2-oxobutyrate prosthetic group that is incorporated during ribosomal synthesis. It is formed by deamination of threonyl tRNA^{Thr} , catalyzed by threonine deaminase (Cohn & Phillips, 1974; Daumy *et al.*, 1974).

Imidazolone propionate undergoes cleavage of the imidazole ring catalyzed by a hydrolase, yielding formiminoglutamate. Alternatively, it may be oxidized to hydantoin propionate. Small amounts of hydantoin propionate are excreted by mammals and, in some bacteria, it is metabolized onwards to yield glutamate (section 8.2.2). Imidazolone propionate is unstable; it may undergo a non-enzymic reaction to formylisoglutamine then isoglutamine (glutamate α -amide) and formate, or non-enzymic decarboxylation and deamidation to 4-oxoglutaramic acid and further deamidation to oxoglutarate (see Figure 8.4).

There are three separate pathways for conversion of formiminoglutamate to glutamate. In most bacteria, formiminoglutamate hydrolase catalyzes the hydrolysis of formiminoglutamate to glutamate and formamide. This enzyme is closely related to arginase (see section 1.6.2.1) and agmatinase (section 5.9.4). In *Pseudomonas* spp. and some other bacteria, there is a two-step reaction: release of ammonium from formiminoglutamate, catalyzed by formiminoglutamate deaminase; followed by hydrolysis to release formate, catalyzed by formylglutamate deformylase (Marti-Arbona & Raushel, 2006; Marti-Arbona *et al.*, 2006).

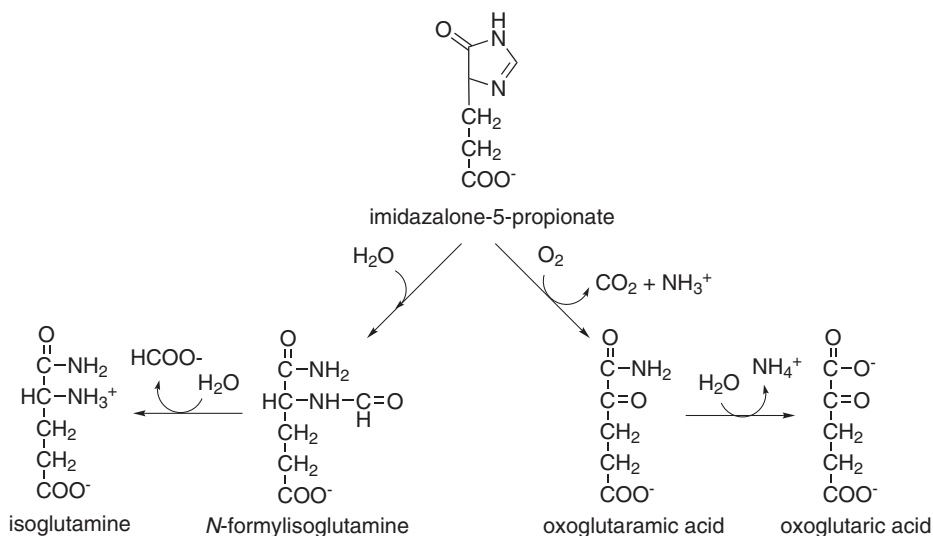


Figure 8.4 Non-enzymic products formed from imidazolone propionate.

In eukaryotes, formiminoglutamate is converted to glutamate by the tetrahydrofolate-dependent enzyme formiminoglutamate formiminotransferase, and the resultant formimino-tetrahydrofolate undergoes cyclodeamination to *N*^{5,10}-methylene-tetrahydrofolate at a second catalytic site in the enzyme. In bacteria, formiminoglutamate formiminotransferase and the cyclodeaminase are separate enzymes.

In addition to its catalytic role, the bifunctional eukaryotic enzyme has a role in maintaining the cytoskeleton. It is normally tightly associated with the Golgi apparatus, and over-expression leads to a dramatic rearrangement of the cytoskeleton. It is also the antigen recognized by anti-liver cytosol antibodies in type 2 auto-immune hepatitis. The two dominant antigenic determinants that interact with the antibodies have considerable sequence homology with the antigenic virion proteins from human herpes virus, suggesting that herpes virus infection may trigger auto-immune liver disease (Mao *et al.*, 2004).

8.2.1.1 The histidine load test (FIGLU test) for folate nutritional status

Urinary excretion of formiminoglutamate after a test dose of histidine is used as a functional test of folate nutritional status, since the activity of the formiminotransferase is impaired in folate deficiency. Although the catabolism of normal amounts of histidine is unaffected by moderate deficiency, providing a relatively large amount of histidine exceeds the limited capacity of the enzyme. Because vitamin B₁₂ deficiency leads to secondary, functional folate

deficiency (section 6.3.2.2), urinary excretion of formiminoglutamate after a histidine load is also increased in vitamin B₁₂ deficiency.

In experimental animals, and with isolated tissue preparations and organ cultures, the test can be refined by measuring the production of ¹⁴CO₂ from [¹⁴C]histidine in the presence and absence of added methionine. If the impairment of histidine metabolism is the result of primary folate deficiency, the addition of methionine will have no effect. By contrast, if the problem is trapping of folate as methyl tetrahydrofolate (section 6.3.2.2), then the addition of methionine will restore normal histidine oxidation; this is a result of restoring the inhibition of methylene tetrahydrofolate reductase by S-adenosylmethionine and restoring the activity of 10-formyl tetrahydrofolate dehydrogenase, so permitting normal folate metabolism.

8.2.2 The hydantoin propionate pathway

Imidazolone propionate arising from the action of urocanase (Figure 8.2) may be oxidized to hydantoin propionate by imidazolone propionate oxidase, a flavoprotein linked to the reduction of NAD⁺. Human beings and other mammals excrete modest amounts of hydantoin propionate after a relatively high intake of histidine. In many bacteria, hydantoin propionate can be further metabolized by hydrolysis of the hydantoin ring to yield *N*-carbamoylglutamate, followed by hydrolysis to yield glutamate and release carbon dioxide and ammonium, as shown in Figure 8.5 (Hassall & Greenberg, 1963).

Bacteria have two distinct carbamoyl amino acid hydrolases, one specific for L-enantiomers and the other for D-enantiomers. Despite the similarity of their reactions, the two enzymes have little sequence homology and appear to be unrelated. Both hydrolases have a broad specificity for the carbamoyl amino acid substrate, and they have been exploited for the synthesis of

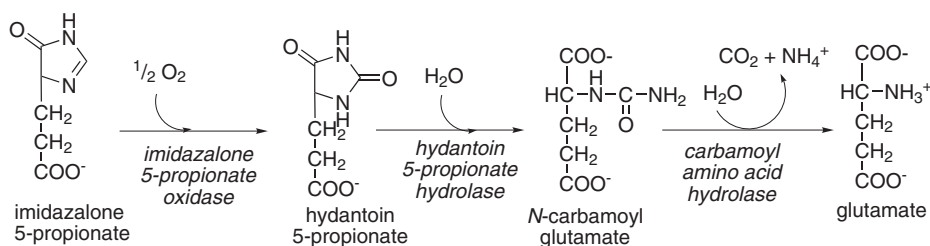


Figure 8.5 The hydantoin propionate pathway of histidine catabolism.

Imidazolone propionate oxidase no EC number (xanthine oxidase, EC 1.17.3.2, also catalyzes this reaction), hydantoin propionate hydrolase no EC number, carbamoyl L-amino acid hydrolase (L-carbamoylase) EC 3.5.1.87, carbamoyl D-amino acid hydrolase (D-carbamoylase) EC 3.5.1.77. See Figure 8.2 for the formation of imidazolone propionate from histidine.

enantiomer-specific amino acids from racemic hydantoin derivatives via the formation of carbamoyl derivatives. Overall, this is known as the ‘hydantoinase process’ for amino acid synthesis (Martinez-Rodriguez *et al.*, 2010).

8.2.3 The transaminase pathway of histidine catabolism

Transamination is a minor pathway of histidine catabolism, but it becomes more important in histidinaemia due to urocanase deficiency, although there is no increase in the amount of histidine transaminase in the liver. Animals have a histidine-pyruvate transaminase that can also act on phenylalanine and tyrosine, and a number of other transaminases may also act on histidine. There are separate cytosolic and mitochondrial isoenzymes of histidine-pyruvate transaminase, and these may function to permit mitochondrial uptake of histidine as imidazole pyruvate. As shown in Figure 8.6, the resultant imidazolepyruvate can be oxidized to imidazole lactate, either by a specific imidazole lactate dehydrogenase or by lactate dehydrogenase (Emes & Hassall, 1973).

8.3 Histamine

Histamine, formed by the decarboxylation of histidine, is, like adrenaline and noradrenaline (section 9.2.4), active both peripherally and as a neurotransmitter in the central nervous system. In the central nervous system, it is involved in the control of diurnal cycling, and it has an awakening role. Knockout mice lacking histidine decarboxylase (see section 8.3.2) show increased sleep, decreased wakefulness and decreased locomotor activity in the dark. Histamine is also involved in the appetite suppressant action of leptin (Ohtsu & Watanabe, 2003; Yoshimatsu, 2008).

Peripherally, histamine, mainly synthesized in and secreted by mast cells, mediates inflammatory reactions leading to the contraction of smooth muscle

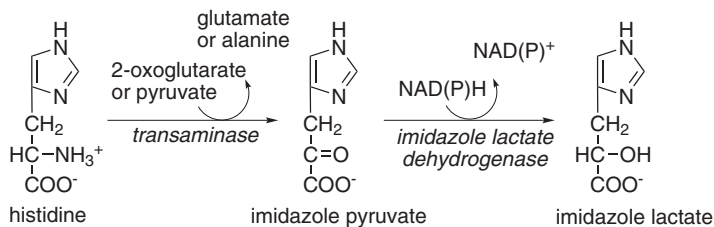


Figure 8.6 The transamination pathway of histidine catabolism. Histidine-2-oxoglutarate transaminase EC 2.6.1.38, histidine (phenylalanine)-pyruvate transaminase EC 2.6.1.58, imidazole lactate dehydrogenase EC 1.1.1.111.

and increase in vascular permeability. Mast cells and basophils synthesize histamine in the Golgi apparatus and store it in granules complexed with heparin and proteins. In sensitized people, degranulation of mast cells and basophils, leading to release of histamine, occurs following the binding of allergens to immunoglobulin IgE antibodies on the cell surface. The inflammatory actions of histamine are mediated by H₁ receptors on target cells, and specific antagonists of the H₁ receptor are widely used to treat allergic reactions, including allergic rhinitis (hay fever) (Ohtsu & Watanabe, 2003).

In the stomach, histamine is synthesized and released by enterochromaffin-like cells in the glandular region of the gastric fundus. It acts on parietal cells to stimulate acid secretion, via H₂ receptors. The enterochromaffin-like cells are stimulated to secrete histamine, either by gastrin or by acetylcholine released by the vagus nerve; ghrelin stimulates histamine secretion via the vagus nerve.

Somatostatin inhibits gastrin-induced secretion of histamine, and there is also negative feedback of histamine secretion by histamine itself, acting via H₃ receptors on the enterochromaffin-like cells. Specific H₂ receptor antagonists are used to reduce gastric acid secretion in the treatment of gastric and duodenal ulcers. Chronic secretion of gastrin leads to induction of histidine decarboxylase, followed by proliferation of enterochromaffin-like cells, possibly resulting in the development of carcinoid tumours (section 9.4.5.3; Hocker *et al.*, 1996).

Studies of knockout mice lacking either histidine decarboxylase or one of the four classes of histamine receptor have led to the discovery of additional roles for histamine, including signalling in tissue development and differentiation, angiogenesis mediated by vascular endothelium growth factor, and chemotaxis in neutrophils and macrophages. In inflammatory responses, histamine H₄ receptors mediate the recruitment of mast cells, leading to amplification of the signal and, eventually, chronic inflammation. Histamine also acts as an autocrine and paracrine regulator of cell proliferation in various cancers. Most tumour cell lines and experimental cancers both synthesize histamine and also have histamine H₄ receptors (Medina & Rivera, 2010; Ohtsu & Watanabe, 2003; Zampeli & Tiligada, 2009).

8.3.1 Bacterial histamine poisoning (scombroid poisoning)

Histamine consumption can lead to diarrhoea, hypotension, headache, pruritis and flushing, beginning within a few minutes of eating food containing relatively large amounts of histamine, and lasting for up to 24 hours. In people with low activity of amine oxidases in the intestinal mucosa, the reaction may be fatal.

The problem was first identified in people who had eaten scombroid fish (tuna, mackerel and bonito), and the condition is sometimes known as scombroid poisoning or the skipjack reaction (skipjack is a type of tuna). However, a wide variety of other fish can be involved, and bacterial histamine poisoning has also been associated with cheese and other fermented dairy products, sauerkraut and fermented meats, and also wines and beer. Especially with fish, the toxicity is greater than that of pure histamine taken orally, suggesting that other compounds, including diamines and polyamines (section 5.8) may potentiate the effects of histamine. Urocanic acid (section 8.2.1) may also be present, acting as a mast cell degranulator and so increasing the systemic effects of histamine (Calles-Enriquez *et al.*, 2010; Lehane & Olley, 2000).

The problem is due to the formation of histamine by bacteria, although there may be no sign that the food has been spoilt by bacterial action, and it appears to be fresh and wholesome. In fish it is commonly the result of bacterial contamination after catching, though in some rare cases the normal intestinal flora of the fish may be histamine producers. Many of the histamine-forming bacteria involved are tolerant to cold and will continue to produce histamine at temperatures as low as 2.5°C, while some can tolerate salt solutions as high as ten per cent. In dairy products and other fermented foods, the histamine-producing bacteria may be the result of accidental contamination, or may be mutant strains of the normal dairy starter culture lactic acid-producing bacteria.

In histamine-producing bacteria, histidine decarboxylase is expressed together with a histidine-histamine counter-transport membrane protein. The export of histamine in exchange for uptake of histidine results in a trans-membrane proton gradient that can be coupled to ATP synthesis. In lactic acid-producing bacteria, the export of histamine acts as a buffer for the exported lactic acid, maintaining the extracellular pH above 4, at which pH growth would cease (Landete *et al.*, 2008).

8.3.2 Histidine decarboxylase

In Gram-negative bacteria and eukaryotes, histidine decarboxylase is a pyridoxal phosphate-dependent enzyme (section 3.4); in Gram-positive bacteria, it has a catalytic pyruvate residue instead (section 3.5). The pyruvoyl enzyme is synthesized as an inactive pro-enzyme that undergoes autocatalytic cleavage between two adjacent serine residues with the elimination of ammonium, leading to a β -subunit with serine at the C terminal and the α -subunit with a pyruvate residue at the amino-terminal (Landete *et al.*, 2008).

Mammalian (pyridoxal phosphate-dependent) histidine decarboxylase is unstable, and it undergoes rapid proteolysis in the absence of substrate. It has a half-life of 50–100 minutes. The histidine decarboxylase gene encodes a 74kDa protein that has low catalytic activity, but may be important for the

synthesis of histamine in activated macrophages and immature mast cells. In most histamine-synthesizing cells, the main activity is represented by a series of proteolytic products of 53–55 kDa, with differing patterns of the proteolytic products in different tissues. The carboxy-terminal region that is cleaved to produce the fully active enzyme restricts access of the substrate to the catalytic site and also contains sequences that target the enzyme for catabolism (Fleming *et al.*, 2004; Furuta *et al.*, 2007).

Histidine decarboxylase in the stomach, but not elsewhere, is induced in response to stress (acting via increased secretion of glucocorticoid hormones) and drugs that cause gastric ulceration, such as aspirin, indomethacin and dexamethasone. This induction of histidine decarboxylase may be indirect, since all of these ulcerogenic treatments lead to decreased synthesis of protective prostaglandins. There may be a local sensor of prostaglandins in the gastric mucosa, which would explain the organ-specific induction of the enzyme. Treatments that reduce the secretion of gastric acid or buffer it, such as antacids, histamine H₂ receptor antagonists and proton pump inhibitors, also result in induction of histidine decarboxylase. This presumably acts via a feedback loop to attempt to maintain acid secretion (Ayada *et al.*, 2003).

There are two irreversible inhibitors of mammalian histidine decarboxylase. Histidine methyl ester binds to the enzyme and forms the substrate-coenzyme aldimine, but it then cannot undergo decarboxylation. α -Fluoromethyl histidine undergoes decarboxylation, but then forms inactive derivatives of the pyridoxal phosphate-product adduct that are slowly released from the active site. In the presence of adequate amounts of the coenzyme, this second inhibition will be reversible, since the apoenzyme can bind pyridoxal phosphate. N^ε-methyl histidine (section 8.4) is a substrate for histidine decarboxylase, with an activity about 15 per cent of that seen with histidine (Moya-Garcia *et al.*, 2005).

8.3.3 Histamine catabolism

In most tissues, histamine catabolism is by oxidation to imidazole acetaldehyde, catalyzed by histaminase, then onwards oxidation to imidazole acetate catalyzed by aldehyde dehydrogenase. In the central nervous system, bronchus and kidney, histamine catabolism is mainly by methylation, catalyzed by histamine N-methyltransferase. This is followed by oxidation to methylimidazole acetaldehyde, catalyzed by monoamine oxidase (section 9.2.4.2), and onwards oxidation to methylimidazole acetate, catalyzed by aldehyde dehydrogenase.

Histaminase is a cell surface enzyme, anchored on the plasma membrane by the amino-terminal domain, and is released into the circulation on stimulation. It therefore only acts on extracellular histamine. Histamine N-methyltransferase is a cytosolic enzyme and only acts on intracellular

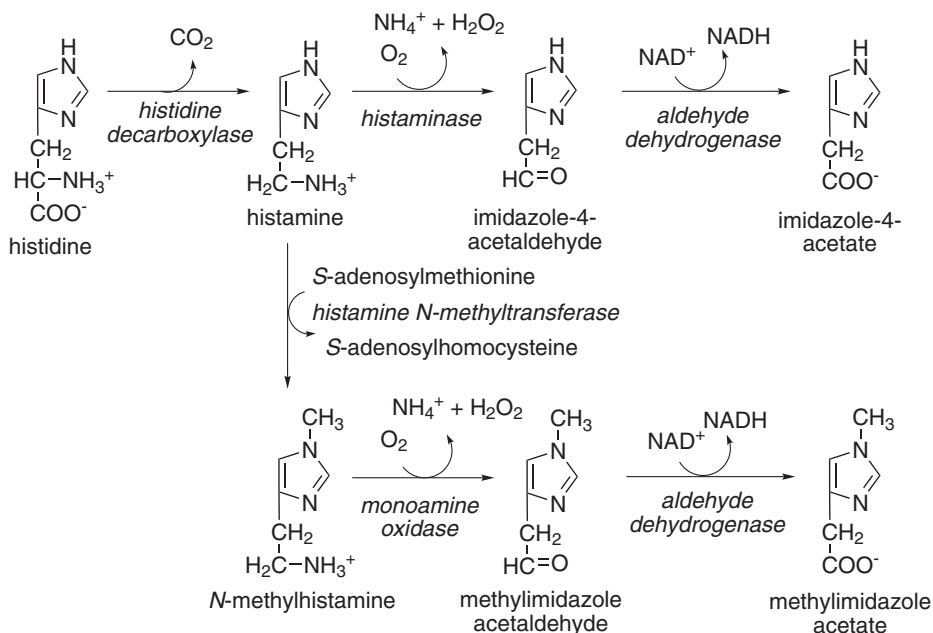


Figure 8.7 Histamine metabolism.

Histidine decarboxylase EC 4.1.1.22, diamine oxidase (histaminase) EC 1.4.3.22, histamine *N*-methyltransferase EC 2.1.1.8, monoamine oxidase EC 1.4.3.4, aldehyde dehydrogenase EC 1.2.1.3.

histamine in the central nervous system. Therefore, unlike catabolism of catecholamines (section 9.2.4.2), there is no competition between the oxidation and methyltransferase pathways for histamine catabolism shown in Figure 8.7 (Ogasawara *et al.*, 2006).

Histaminase (diamine oxidase) is a copper-containing quinoprotein, with topaquinone (trihydroxyphenylalanine quinone) at the active site (section 9.5). Because the quinoprotein amine oxidases are inhibited by semicarbazide and other carbonyl group reagents, they are sometimes known as semicarbazide-sensitive amine oxidases, to distinguish them from the flavo-protein monoamine oxidases, which are not inhibited by semicarbazide (section 9.2.4.2). The topaquinone is formed by autocatalytic oxygen-dependent oxidation of a tyrosine residue in the precursor protein; the copper atom is essential for this oxidation.

The first half-reaction of histaminase involves formation of a Schiff base between histamine and topaquinone, followed by α -deprotonation and hydrolysis to yield the aldehyde product and the aminoquinol. The second half of the reaction involves transfer of an electron from the aminoquinol to Cu^{2+} , forming the semiquinone radical and Cu^+ . Oxygen then binds to the copper ion, forming Cu^{2+} -superoxide, followed by transfer of a second elec-

tron and two protons from the semiquinone to form hydrogen peroxide and the oxidized iminoquinone that is hydrolyzed to release ammonium and reform the topaquinone (Mukherjee *et al.*, 2008; Welford *et al.*, 2007).

In addition to its action in oxidizing extracellular histamine, histaminase also functions as a vascular adhesion molecule. The enzyme is abundant in adipose tissue and, in the presence of histamine and vanadate, it acts to recruit glucose transporters to the cell surface, thus increasing glucose uptake and mimicking the action of insulin (Maintz & Novak, 2007; Zorzano *et al.*, 2003).

8.4 Methyhlhistidine

Both actin and myosin contain 3-methylhistidine (formed by post-synthetic modification of histidine residues), which is released on turnover of the proteins but can neither be re-utilized for protein synthesis nor demethylated. It has been suggested that measurement of urinary methylhistidine will give an index of muscle protein turnover. It increases in response to a high protein diet (when protein turnover is increased) and is decreased in fasting and protein deprivation in parallel with the loss of muscle tissue (Nishizawa *et al.*, 1977).

Skeletal muscle contains most of the body's methylhistidine – 3.3 $\mu\text{mol/kg}$ protein, compared with 2–3 $\mu\text{mol/kg}$ in smooth and cardiac muscle (which are a considerably smaller proportion of body weight) and less than 1 $\mu\text{mol/kg}$ protein in tissues such as liver and kidneys. However, studies with [^{14}C]S-adenosylmethionine show that the fractional turnover of methylhistidine is 1 per cent per day in skeletal muscle, 3.2 per cent per day in skin and 9 per cent in the intestinal mucosa, suggesting that less than 40 per cent of urinary methylhistidine arises from skeletal muscle (Millward & Bates, 1983).

A further problem in attempting to use urinary methylhistidine as an index of muscle protein turnover is that not all of it is excreted unchanged; a small proportion undergoes decarboxylation to methylimidazole acetate and other metabolites (Murray *et al.*, 1985).

8.5 Carnosine and related histidine-containing peptides

Carnosine is β -alanyl-histidine and homocarnosine is γ -aminobutyryl-histidine. Carnosine is found mainly in muscle, and homocarnosine in the central nervous system. In birds and some mammals, anserine (β -alanyl-1-methylhistidine) is formed in muscle rather than carnosine, while in marine mammals there is balenine (β -alanyl-3-methylhistidine). In all cases, these peptides are accumulated at relatively high concentrations.

The same enzyme, carnosine synthase, catalyzes synthesis of both carnosine and homocarnosine, linked to the hydrolysis of ATP to ADP and phosphate. The enzyme has greater activity for carnosine synthesis but, in the central nervous system, the greater availability of γ -aminobutyrate (GABA, section 5.5) than of β -alanine permits synthesis of homocarnosine rather than carnosine. β -Alanine arises mainly as an intermediate in cytosine and uracil catabolism (Figure 1.12).

The functions of carnosine and related peptides are unclear. There is some evidence that homocarnosine in the central nervous system may have a neurotransmitter or neuromodulatory action. It is synthesized in glial cells, and it may serve to store and recycle GABA released from neurons, as an alternative to catabolism via the GABA shunt (Figure 5.7). In muscle, carnosine (or anserine or balenine) may function mainly as a buffer against the lactic acid produced, especially in white muscle fibres, during maximum exertion. All four peptides can also chelate divalent metal ions, act as antioxidants and inhibit non-enzymic glycation of proteins; in cell culture, they have anti-senescence activity. However, all of these functions are speculative, and there is little clear evidence for any specific functions of the peptides (Drozak *et al.*, 2010; Hipkiss *et al.*, 2002; Quinn *et al.*, 1992).

Further reading

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

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9

The Aromatic Amino Acids: Phenylalanine, Tyrosine and Tryptophan

The aromatic amino acids, phenylalanine, tyrosine and tryptophan, are dietary essential amino acids for animals, although (as discussed in section 9.2.3) tyrosine can readily be synthesized from phenylalanine. In plants and micro-organisms, they are synthesized from phosphoenolpyruvate and erythrose 4-phosphate via a common pathway – the shikimate pathway – leading to formation of chorismic acid. This is the substrate for two separate pathways, one leading to phenylalanine and tyrosine and the other to tryptophan.

Because mammals lack the shikimate pathway, enzymes in this pathway are potential targets for herbicides (e.g. glyphosate) and antibacterial, anti-fungal and anti-parasite drugs. Inhibitors of the pathway deprive the organism not only of the aromatic amino acids, but also ubiquinone and *p*-aminobenzoate. Shikimic acid is the starting material for chemical synthesis of the antiviral drug Tamiflu, which is a neuraminidase inhibitor.

Synthesis of the aromatic amino acids is very energy expensive. Tryptophan synthesis requires $78 \times \text{ATP}$ per mol and phenylalanine and tyrosine 62–65 ATP per mol, depending on which branch of the pathway is followed – twice the ATP cost of synthesizing most other amino acids. In *E. coli* and other bacteria, all the enzymes of the tryptophan pathway from chorismate are encoded in a single operon. In eukaryotes, however, the enzymes for tryptophan synthesis are scattered throughout the genome and each requires a separate mechanism for regulation of synthesis – unlike the bacterial *trp* operon, where synthesis of all the enzymes of the pathway is regulated coordinately by a single step. In yeast, most of the genes for aromatic amino acid synthesis are regulated by the transcriptional activator GCN4, which is a

general control mechanism for synthesis of a number of amino acids. At least 30 genes are de-repressed by GCN4 (Braus, 1991).

In plants, phenylalanine is the precursor for synthesis of lignins (section 9.2.1), flavonoids and polyphenols (see section 9.2.2), as well as a number of volatile aromatic compounds in flowers. The latter include phenylethyl acetate, phenylethyl acetaldehyde and phenylethyl alcohol, benzyl and salicyl compounds, and also eugenol and isoeugenol.

In mammals, tyrosine is the precursor for synthesis of melanin (section 9.2.5) and the catecholamine neurotransmitters and hormones, dopamine, noradrenaline and adrenaline (section 9.2.4). Tyrosine residues in thyroglobulin are the precursor for synthesis of the thyroid hormones (section 9.2.6), and post-synthetic modification of tyrosine and tryptophan residues in proteins leads to the formation of the quinone cofactors of copper-dependent amine oxidases (section 9.5). The hydroxyl group of tyrosine is important in the catalytic sites of many enzymes, and it also provides a site for reversible phosphorylation catalyzed by protein kinases to regulate enzyme activity in response to hormones. Tyrosine also provides the cross-links of the elastic proteins of the insect exoskeleton – resilin and sclerotin.

Tryptophan is the precursor for synthesis of the neurotransmitters serotonin (5-hydroxytryptamine) and melatonin (section 9.4.3), as well as the nicotinamide ring of NAD and NADP. As will be discussed in section 9.4.4.4, synthesis from tryptophan is a more important source of nicotinamide than dietary preformed niacin, and disturbances of tryptophan metabolism lead to the development of the niacin deficiency disease pellagra (section 9.5). Tryptophan is also the precursor of the plant growth hormone auxin (indoleacetate – see section 9.4.1), and the indole nucleus is incorporated into the fungal toxic peptides phalloidin and aminitin. Hallucinogenic indole derivatives are found in many fungi.

Some of the earliest described and best characterized genetic diseases are inborn errors of phenylalanine and tyrosine metabolism, including phenylketonuria (section 9.2.3), alkaptonuria and albinism (section 9.2.5).

9.1 Biosynthesis of phenylalanine, tyrosine and tryptophan

There is a common pathway for the biosynthesis of all three of these aromatic amino acids from erythrose 4-phosphate and the glycolytic intermediate phosphoenolpyruvate to form chorismate via shikimate. The shikimate pathway is not the only pathway for synthesis of aromatic compounds in plants and microorganisms, but chorismate is the precursor for the synthesis of a number of aromatic compounds. These include *p*-aminobenzoate for folate synthesis (sulphonamide antibacterial drugs act by competing with *p*-

aminobenzoate), ubiquinone, vitamin K and other compounds, including salicylates, flavanones and naphthquinones. Chorismate is the branch point in aromatic amino acid biosynthesis, with separate pathways leading to the synthesis of phenylalanine, tyrosine and tryptophan.

9.1.1 The shikimate pathway

The shikimate pathway is shown in Figure 9.1. In yeasts and fungi, there is a multifunctional enzyme that catalyzes all five reactions from deoxyarabinoheptulosonic acid phosphate to chorismate. This pentafunctional enzyme is a mosaic of monofunctional domains with significant homology to the monofunctional enzymes of bacteria and plants (Braus, 1991).

The first step in the pathway is synthesis of deoxyarabinoheptulosonic acid phosphate (DAHP). This is an aldol condensation of phosphoenolpyruvate

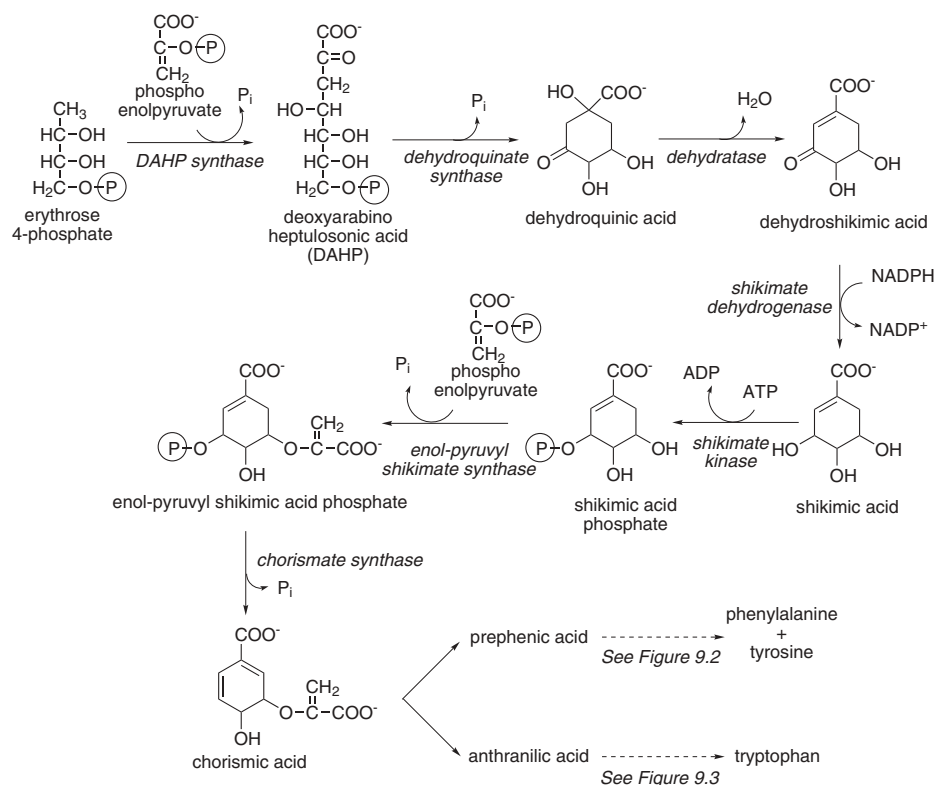


Figure 9.1 The shikimate (common) pathway of aromatic amino acid biosynthesis.

DAHP (deoxyarabino heptulosonic acid) synthase EC 2.5.1.54, dehydroquinase synthase EC 4.2.3.4, dehydroquinase (dehydroquinase dehydratase) EC 4.2.1.10, shikimate dehydrogenase EC 1.1.1.25, shikimate kinase EC 2.7.1.71, enol-pyruvyl shikimate synthase EC 2.5.1.19, chorismate synthase EC 4.2.3.5, phenylalanine hydroxylase EC 1.14.16.1 (see Figure 9.6).

with erythrose 4-phosphate, catalyzed by DAHP synthase, a zinc or manganese metallo-enzyme. Binding of phosphoenolpyruvate causes a conformational change in the enzyme that permits erythrose 4-phosphate to bind. The carbonyl oxygen of erythrose 4-phosphate is coordinated to the metal ion, permitting nucleophilic attack on the carbon-carbon double bond of phosphoenolpyruvate, in turn leading to the cleavage of the carbon-oxygen bond of phosphoenolpyruvate and the release of inorganic phosphate (Konig *et al.*, 2004).

In bacteria, DAHP synthase is regulated by both transcriptional regulation and feedback inhibition by aromatic amino acids. In *E. coli*, there are three separate isoenzymes of DAHP synthase, each inhibited and repressed by one of the three aromatic amino acids. The genes for these three isoenzymes are scattered through the *E. coli* genome.

Although physiological concentrations of phenylalanine and tyrosine inhibit their respective isoenzymes more or less completely, tryptophan leads to only partial inhibition of the tryptophan-sensitive enzyme. This means that, when all three aromatic amino acids are present in adequate concentrations, there will still be sufficient enzyme activity to provide chorismate and other intermediates for the synthesis of non-amino acid products.

In some bacteria, DAHP synthase is a bifunctional enzyme with chorismate mutase, the first enzyme of the phenylalanine and tyrosine pathway (Figure 9.2). Chorismate mutase may be fused to either the amino- or the carboxy-terminal of DAHP synthase in different bacteria (Herrmann & Weaver, 1999; Shumilin *et al.*, 2002).

In plants, DAHP synthase is induced by ozone and in response to wounding or insect attack, because of the need for chorismate for synthesis of lignin (section 9.2.1) and protective secondary metabolites including phytoalexins and flavonoids, but it is not inhibited by aromatic amino acids. Indeed, it is activated by tryptophan and, to a lesser extent, tyrosine, although it is inhibited by aroenate (pretyrosine) at concentrations that are unphysiologically high. When chorismate synthase is inhibited by the herbicide glyphosphate, there is induction of DAHP synthase in response to the lack of aromatic amino acids. DAHP synthase in plants is regulated by the electron flow through photosystem I; it requires reduced thioredoxin for activity (Entus *et al.*, 2002; Herrmann, 1995a, 1995b; Janzik *et al.*, 2005).

Dehydroquinate synthase catalyzes a complex multistep reaction involving alcohol oxidation, elimination of phosphate, carbonyl reduction, ring opening and an intramolecular aldol condensation. The enzyme is a zinc metalloprotein; it has tightly bound NAD^+ which accepts hydride from carbon-5 of the substrate in the first step, then catalyzes the reverse reaction in the carbonyl reduction step. NAD does not leave the enzyme during the reaction sequence (Bender *et al.*, 1989a, 1989b; Carpenter *et al.*, 1998; Widlanski *et al.*, 1989).

Dehydroquinate is then dehydrated to dehydroshikimic acid. There are two types of dehydroquinate dehydratase, with little sequence homology, although both have a reactive arginine residue in a conserved sequence that is presumed to be the substrate-binding domain. They catalyze the dehydration by different mechanisms, with different stereochemistry, and they are sensitive to different inhibitors.

Type I dehydratase is found in plants, fungi and many bacteria, and it is solely biosynthetic. It is a dimer and it catalyzes the dehydration by formation of a Schiff base between the carbonyl group of the substrate and the ϵ -amino group of a lysine residue in the enzyme.

Type II dehydratase is found in bacteria and fungi, and it is both biosynthetic and catabolic. It consists of 12 subunits and does not form a Schiff base with the substrate; rather, the reaction is catalyzed by conserved arginine and tyrosine residues in a flexible loop that closes over the active site when the substrate binds.

A variety of bromo- and fluoro-analogues of dehydroquinic acid are substrates for the type II enzyme, but are irreversible inhibitors of the type I enzyme because they form a Schiff base intermediate that cannot undergo the elimination reaction (Gonzalez-Bello & Castedo, 2007; Krell *et al.*, 1996).

In fungi, a monofunctional dehydroquinate dehydratase is important in the catabolism of quinate, which is a significant carbon source, comprising some ten per cent of the dry weight of decaying leaf litter. Quinate is reduced to dehydroquinate, followed by dehydration to dehydroshikimate and further dehydrogenation to pyrocatechuate, and on to yield succinate and acetyl CoA. The fungal biosynthetic dehydratase is a type I enzyme and is part of the pentafunctional enzyme that catalyzes the conversion of DAHP to chorismate. The pentafunctional enzyme permits channelling of intermediates in the biosynthetic pathway and separates it from the quinate catabolic pathway. This channelling is not perfect. *Aspergillus* with a mutated type II dehydratase cannot grow on quinate as the sole carbon source but, if the pentafunctional enzyme is over-expressed, it can. This shows that the dehydratase of the pentafunctional enzyme can act catabolically as well as anabolically (Bentley, 1990).

In some plants, dehydroquinate dehydratase is a bifunctional enzyme with the next enzyme in the pathway, shikimate dehydrogenase; it shows sequence homology with the monofunctional enzyme from bacteria. Shikimate dehydrogenases form a novel group of enzymes, showing little or no sequence homology with other dehydrogenases. The turnover number of the dehydroquinate dehydratase is only about ten per cent of that of shikimate dehydrogenase, so that there is no accumulation of dehydroshikimate, which would otherwise induce the enzymes of the catabolic pathway via pyrocatechuate (Deka *et al.*, 1994; Herrmann, 1995b).

The reaction of enolpyruvyl shikimate synthase, like that of DAHP synthase, involves cleavage of the carbon-oxygen bond of phosphoenolpyruvate to liberate inorganic phosphate. There are two forms of enolpyruvyl shikimate synthase:

- Type I, from plants, is sensitive to the herbicide glyphosate, which is a competitive inhibitor with respect to phosphoenolpyruvate, although other enzymes that act on phosphoenolpyruvate are not inhibited by glyphosate.
- Type II enolpyruvyl shikimate synthase, from *Agrobacterium* spp. and other Gram-positive bacteria, is insensitive to glyphosate because it has alanine in the catalytic site where the type I enzyme has glycine. This affects the binding of glyphosate, so that it is in a different orientation and does not inhibit. 'Round-up ready' genetically modified crop plants have the type II synthase from *Agrobacterium tumefaciens*.

A number of weeds have become resistant to glyphosate. In some cases, although their enolpyruvyl shikimate synthase is sensitive to the herbicide, they have undergone gene amplification and have up to 160 times more copies of the gene than wild type plants. These copies are distributed in all of their chromosomes, and gene expression reflects the number of copies of the gene (Funke *et al.*, 2006; Gaines *et al.*, 2010; Schonbrunn *et al.*, 2001).

Chorismate synthase catalyzes elimination of the 3-phosphate and hydrogen from carbon-6 of enolpyruvyl shikimate. The enzyme is a flavoprotein, and the reaction proceeds by formation of the flavin and chorismate radicals. In fungi and some bacteria, chorismate synthase is a bifunctional enzyme that also catalyzes the one-electron reduction of flavin mononucleotide to the semiquinone radical; in other bacteria, the synthase requires a separate flavin reductase to provide the flavin radical, and in plants the flavin may be reduced by photosystem I (Macheroux *et al.*, 1999).

9.1.2 Synthesis of phenylalanine and tyrosine

As shown in Figure 9.2, the first step in synthesis of phenylalanine and tyrosine is isomerization of chorismate to prephenate, catalyzed by chorismate mutase. There are then three parallel pathways leading to the formation of phenylalanine and tyrosine, namely via phenylpyruvate, *p*-hydroxyphenylpyruvate or arogenate (also known as pretyrosine). In some bacteria and other microorganisms, phenylalanine can also be converted to

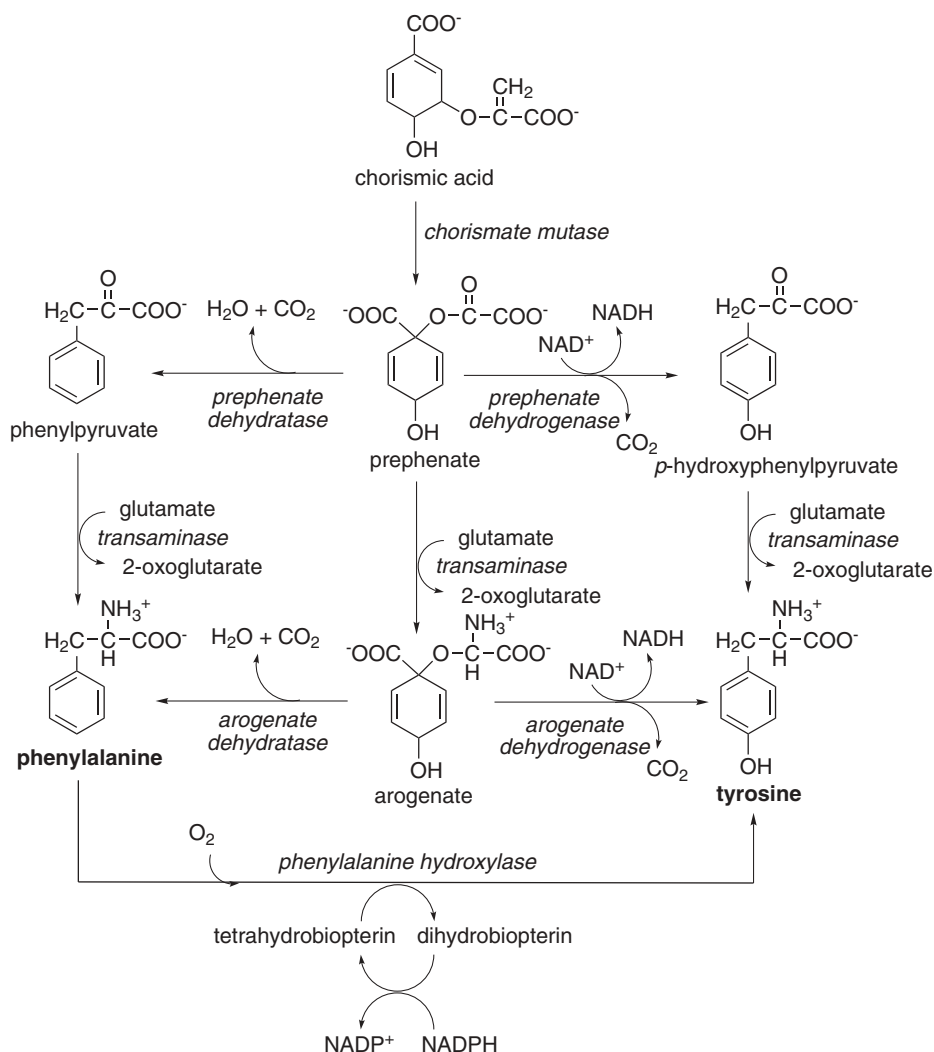


Figure 9.2 Biosynthesis of phenylalanine and tyrosine from chorismate.

Chorismate mutase EC 5.4.99.5, prephenate dehydratase EC 4.2.1.51, glutamine-phenylpyruvate transaminase (forming 2-oxoglutaramate) EC 2.6.1.64, phenylalanine (histidine) transaminase (pyruvate-linked) EC 2.6.1.58, tryptophan transaminase (also acts on phenylalanine) EC 2.6.1.27, aspartate-phenylpyruvate transaminase EC 2.6.1.70, tyrosine (phenylalanine) transaminase (2-oxoglutarate linked) EC 2.6.1.5, aspartate-prephenate transaminase EC 2.6.1.78, glutamate-prephenate transaminase EC 2.6.1.79, arogenate dehydratase EC 4.2.1.91, prephenate dehydrogenase EC 1.3.1.12, arogenate dehydrogenase (NAD-linked) EC 1.3.1.43, NADP-linked EC 1.3.1.78, NAD(P)-linked EC 1.3.1.79, phenylalanine hydroxylase EC 1.14.16.1. See Figure 9.6 for the reaction of phenylalanine hydroxylase.

tyrosine by hydroxylation. Phenylalanine hydroxylase is discussed in section 9.2.3.

In some organisms, chorismate mutase is a monofunctional enzyme. In others, it is a bifunctional enzyme with either prephenate dehydratase or prephenate dehydrogenase, so that the product is channelled either into the phenylalanine or the tyrosine branch of the pathway. In *Mycobacterium tuberculosis*, chorismate mutase forms a non-covalent association with DAHP synthase, the first enzyme of the shikimate pathway. In the absence of DAHP synthase, the activity of chorismate mutase is very low, and the catalytic efficiency increases 100-fold on forming the enzyme-substrate complex as a result of a conformational change in the active site of the mutase. In other organisms, DAHP synthase and chorismate mutase are covalently bound to form a bifunctional enzyme (Lutke-Eversloh & Stephanopoulos, 2005; Sasso *et al.*, 2009).

In yeast, chorismate mutase is a homodimer that is activated by tryptophan and inhibited by tyrosine. The two allosteric effectors bind at the same site. It is also activated by its substrate, chorismate. Tryptophan is also a feedback inhibitor of the first enzyme unique to its synthesis, anthranilate synthase (section 9.1.3). While most of the other enzymes of aromatic amino acid synthesis are regulated at the transcriptional level, chorismate mutase and anthranilate synthase are not (Braus 1991; Kong *et al.*, 2006; Schmidheini *et al.*, 1990).

In yeast and some bacteria, prephenate may be dehydrated and decarboxylated to phenylpyruvate, or it may be oxidized and decarboxylated to *p*-hydroxyphenylpyruvate. There are three types of dehydrogenase in different organisms:

- Prephenate dehydrogenase (mainly in Gram-positive bacteria) acts only on prephenate to yield *p*-hydroxyphenylpyruvate.
- Arogenate dehydrogenase (in higher plants and some bacteria) acts only on arogenate to yield tyrosine.
- Cyclohexenyl dehydrogenase (in most bacteria) acts on either prephenate or arogenate.

Phenylpyruvate and *p*-hydroxyphenylpyruvate are substrates for a variety of transaminases to yield phenylalanine and tyrosine respectively. In plants the main pathway is transamination of prephenate to arogenate, which may either be dehydrated and decarboxylated to phenylalanine or be oxidatively decarboxylated to tyrosine. In many microorganisms, the arogenate pathway

is used for tyrosine synthesis and the phenylpyruvate pathway for phenylalanine synthesis (Braus, 1991; Chiu *et al.*, 2010; Sun *et al.*, 2009).

9.1.3 Synthesis of tryptophan

As shown in Figure 9.3, the first step unique to tryptophan synthesis is elimination of the pyruvate side-chain of chorismate and the introduction of an amino group from glutamine, catalyzed by anthranilate synthase. In some organisms, anthranilate synthase is part of a multi-enzyme complex with the other enzymes of tryptophan synthesis, while in others it is an enzyme complex with two catalytic sites, a glutaminase site that forms ammonia and a separate synthase site that binds tryptophan as an allosteric inhibitor.

As in other enzymes that have glutamine amidotransferase activity, there is channelling of the ammonia from one active site to the other through a tunnel in the enzyme that is inaccessible to solvent, so it is ammonia (rather than ammonium) that is transferred between the active sites (see section 5.3.3). In the absence of chorismate, glutamine is bound to the glutaminase

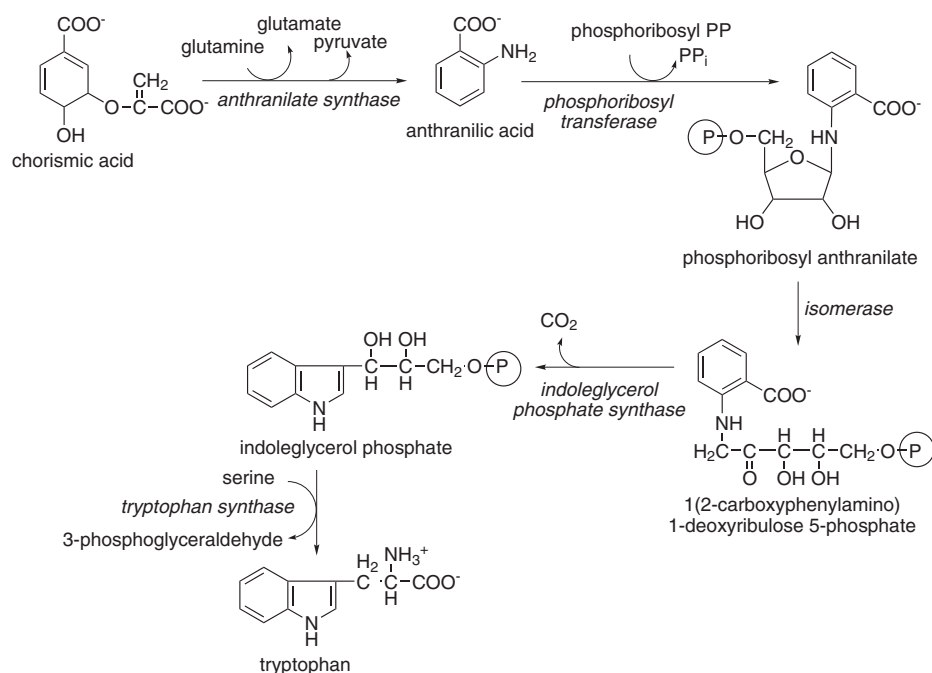


Figure 9.3 Biosynthesis of tryptophan from chorismate

Anthranilate synthase EC 4.1.3.27, anthranilate phosphoribosyltransferase EC 2.4.2.18, phosphoribosylanthranilate isomerase EC 5.3.1.24, indoleglycerol phosphate synthase EC 4.1.1.48, tryptophan synthase EC 4.2.1.20, indoleglycerol phosphate lyase EC 4.1.2.8.

site in a non-productive orientation. Binding of chorismate to the synthase site causes a conformational change that permits glutaminase activity and opens the ammonia channel (Mouilleron & Golinelli-Pimpaneau, 2007).

In some bacteria, anthranilate synthase is a tetrameric protein with two glutaminase monomers and two synthase monomers, which may or may not show cooperativity in inhibitory binding of tryptophan. In some organisms, the enzyme is a heterodimer that is sensitive to tryptophan inhibition. In others, the enzyme is unaffected by tryptophan (Morollo & Eck, 2001). In *B. subtilis*, the glutaminase gene is not in the *trp* operon, but rather is in the operon that encodes the enzymes for folic acid synthesis. The same glutaminase is involved in both pathways (Gollnick *et al.*, 2005). Some plants have both tryptophan-sensitive and tryptophan-insensitive isoenzymes of anthranilate synthase. The tryptophan-insensitive isoenzyme is involved in the synthesis of defence-related indole alkaloids, rather than tryptophan itself.

In various organisms, anthranilate synthase may be part of a bifunctional enzyme with anthranilate phosphoribosyltransferase or indoleglycerol phosphate synthase, or a trifunctional enzyme with phosphoribosyl anthranilate isomerase and indoleglycerol phosphate synthase. In many organisms, anthranilate phosphoribosyltransferase is a bifunctional enzyme with phosphoribosylanthranilate isomerase. Efficient channelling of phosphoribosyl anthranilate from the synthase to the isomerase prevents the otherwise relatively rapid non-enzymic hydrolysis of phosphoribosyl anthranilate to anthranilate and ribose 5-phosphate. In other organisms, where the phosphoribosyltransferase and isomerase are separate enzymes, the activity of the isomerase is several times higher than that of any of the other enzymes of the pathway, so that phosphoribosyl anthranilate is rapidly removed before it can be hydrolyzed (Braus *et al.*, 1988; Hommel *et al.*, 1989).

Indoleglycerol phosphate synthase catalyzes decarboxylation of 1-deoxyribulosyl 5-phosphate, and then condensation to form the indole ring. Tryptophan synthase consists of two subunits. The α -subunit catalyzes the cleavage of indoleglycerol phosphate to indole and glyceraldehyde 3-phosphate. The indole is then channelled to the β -subunit, where it is condensed with serine in a pyridoxal phosphate-dependent reaction to yield tryptophan. In some organisms, tryptophan synthase is a bifunctional enzyme with one or other of the enzymes of the pathway.

Although indole is channelled from the α -subunit to the β -subunit, the intact enzyme can also use exogenous indole as a substrate. This is inhibited by binding of glyceraldehyde 3-phosphate to the active site of the α -subunit, suggesting that exogenous indole enters through the α -subunit and is transferred to the β -subunit through the intra-molecular tunnel. Indole produced by the α -subunit is not released into the solvent.

Tryptophan synthase is a hetero-tetramer, with two β -subunits at the centre and one α -subunit at each end. Most of the intramolecular tunnel is within

the β -subunit, and it can accommodate up to four mol of indole in each β -subunit (Huang *et al.*, 2001).

Tryptophan synthase catalyzes a classical pyridoxal phosphate-dependent β -replacement reaction (section 3.4). The pyridoxal phosphate is initially bound to the ϵ -amino group of a lysine residue, and undergoes transaldimination onto serine. This is followed by deprotonation to yield a quinonoid intermediate, and then elimination of water to form an aminoacrylate intermediate that condenses with indole to form a second quinonoid intermediate. This is protonated to yield the second external aldimine, then the lysine residue of the enzyme displaces tryptophan to reform the internal aldimine.

In the absence of serine, there is only very slow cleavage of indoleglycerol phosphate to form indole at the catalytic site of the α -subunit. The binding of serine to the β -subunit leads to a conformational change that activates the α -subunit by decreasing the K_m and increasing the V_{max} . The signal for this conformational change is the conversion of the external aldimine between serine and the coenzyme to the quinonoid intermediate. Formation of the product aldimine is the signal for a reverse conformational change to reduce the activity of the α -subunit. This ensures that the activities of the two sites are synchronized, and indoleglycerol phosphate is not cleaved unless there is serine available for tryptophan synthesis.

The conformational change in the α -subunit is between an open conformation that has low activity and a closed conformation that has high activity. Conversion to the closed form of the enzyme prevents the release of indole to the solvent. This means that, when the enzyme uses exogenous indole, it must enter the tunnel from the α -subunit in the open state, and it is then trapped in the tunnel when the β -subunit forms the first quinonoid intermediate (Pan *et al.*, 1997).

9.1.3.1 The *trp* operon The *trp* operon of bacteria, which encodes all of the enzymes for synthesis of tryptophan from chorismate, is the model for repressible operons in the same way as the *lac* operon is for inducible operons. It is regulated by both repression and attenuation of transcription. The order of the genes in the operon differs in different bacteria. The ways in which transcription of the *trp* operon is attenuated in *E. coli* and *B. subtilis* (the two most studied organisms) are different.

In *B. subtilis*, the *trp* operon is embedded within the *aro* super-operon that encodes all of the enzymes of aromatic amino acid biosynthesis. There are two promoter regions – one at the beginning of the super-operon that controls transcription of genes for all the enzymes involved, and one at the beginning of the *trp* operon.

The tryptophan-activated repressor protein of the *trp* operon is a dimer that binds two mol of tryptophan. It cannot bind to DNA unless both monomers have tryptophan bound. When tryptophan is bound, there is a

conformational change, bringing the DNA-binding regions of the protein into the position required for binding to one or more of three operator sites in the promoter region of the operon. When the occupied repressor protein is bound to DNA, it blocks the binding of RNA polymerase to the promoter region, so preventing transcription. There are interactions between the separate molecules of repressor protein bound to adjacent operator sites, which increases the likelihood that each protein remains bound to DNA, so enhancing repression of transcription (Yanofsky, 2007).

In addition, transcription of the *trp* operon in *E. coli* is also controlled by the availability of charged tRNA^{Trp}. The transcription start of the *trp* operon is separated from the first structural gene (that for anthranilate synthase) by a 160-nucleotide leader region that is responsible for attenuation of transcription. A hairpin in regions 1 and 2 of the leader sequence causes a pause in transcription, which allows the ribosome to assemble on the leader sequence and begin translation. The moving ribosome then disrupts the pausing complex and allows transcription to continue.

The leader sequence contains two adjacent tryptophan codons and, if there is abundant charged tRNA^{Trp}, the first section of the leader is translated rapidly and the ribosome starts translation of the second region. This allows sections 2 and 3 of the leader sequence to form a hairpin that is recognized as a transcription termination signal. However, if the ribosome stalls at either of the tryptophan codons in the first segment of the leader (because there is inadequate charged tRNA^{Trp}), then an anti-terminator hairpin forms, allowing transcription to continue to the end of the operon (Santillan & Zeron, 2006; Yanofsky, 1981, 2007).

In *B. subtilis*, formation of the termination or anti-termination loop in the mRNA is controlled by an RNA-binding regulatory protein, TRAP. TRAP consists of 11 identical subunits, with a tryptophan binding site between each pair of subunits. Each bound tryptophan stabilizes the surface of the protein, enhancing its ability to bind to mRNA at XAG triplet codons. There are 11 such triplets in the transcript leader sequence. Eleven Lys-Lys-Arg motifs on the surface of the activated TRAP protein bind to these XAG triplets, wrapping the mRNA around the protein. This frees the sequence that forms the anti-terminator hairpin and permits formation of the terminator hairpin.

When tryptophan is growth limiting, TRAP is not activated and does not bind to mRNA, so that the anti-terminator structure is formed. TRAP is encoded by a gene upstream of the *trp* operon; its synthesis is not regulated by tryptophan, but is expressed constitutively. In 5–10 per cent of the TRAP-bound mRNA, transcription is not terminated, but a second hairpin is formed that prevents translation of the gene for anthranilate synthase (Gollnick *et al.*, 2005; Yanofsky, 1981, 2007).

The availability of charged tRNA^{Trp} also regulates expression of the *trp* operon in *B. subtilis*, but by a different mechanism from that used in *E. coli*.

An anti-TRAP protein binds to tryptophan-activated TRAP, preventing its interaction with mRNA and thus reducing the termination of transcription of the *trp* operon. Uncharged tRNA^{Trp} binds to a leader sequence in the mRNA for anti-TRAP, stabilizing an anti-terminator loop and preventing the formation of a terminator loop, so that the gene for anti-TRAP is transcribed. Charged tRNA^{Trp} acts during translation of the leader sequence (which contains three successive tryptophan codons) to permit the ribosome to pass the terminator loop and translate the mRNA for anti-TRAP. This is similar to the way in which the availability of charged tRNA^{Trp} is sensed in control of the transcription of the *trp* operon in *E. coli* (Yanofsky, 2007).

9.2 Metabolism of phenylalanine and tyrosine

In plants, phenylalanine is the precursor for synthesis of lignins (section 9.2.1) and polyphenols (section 9.2.2); in mammals, it is the precursor for synthesis of tyrosine (section 9.2.3) and the catecholamines, dopamine, noradrenaline and adrenaline (section 9.2.4). Tyrosine is the precursor for melanin synthesis in all organisms (section 9.2.5), and tyrosine residues in thyroglobulin are the precursor for synthesis of the thyroid hormones (section 9.2.6). Post-synthetic modification of tyrosine residues in precursor proteins leads to the formation of quinone cofactors in copper-dependent amine oxidases and other quino-proteins (section 9.5).

9.2.1 Phenylalanine ammonia lyase and lignin biosynthesis in plants

Lignin is an ill-defined complex heteropolymer of the monolignols (cinnamoyl alcohols). It is deposited in plant cell walls, together with cellulose and other non-starch polysaccharides. It strengthens and waterproofs cells and provides the major structural support for woody plants. The composition of lignin varies from one plant species to another. The monolignols are cross-linked to each other in lignin, and to cellulose in lignocellulose.

Flax fibres, used for linen manufacture, consist of 70 per cent cellulose, but they also contain lignin. This gives the fabric poor elasticity compared with cotton, which does not contain lignin. Transgenic flax, in which cinnamoyl alcohol dehydrogenase (the final step in the synthesis of the monolignols) has been down-regulated contains less lignin, so the fibres have better elasticity. The transgenic plants have lowered resistance to *Fusarium oxysporum* fungal attack. This may be beneficial, since this is the fungus used in the retting process to extract the fibre, so reduced resistance to the fungus will enhance the extraction of the fibre (Wrobel-Kwiatkowska *et al.*, 2007).

Modification of lignins in trees used for paper pulp manufacture is also commercially important. Some transgenic trees in which cinnamoyl alcohol dehydrogenase is knocked out grow normally (although others have impaired growth), but the modified lignins that are formed contain aldehyde rather than alcohol monolignans, and so are easier to extract, resulting in higher quality cellulose pulp with less use of chemicals and water (Pilate *et al.*, 2002).

As shown in Figure 9.4, the first step in the phenylpropanoid pathway for monolignol synthesis is deamination of phenylalanine to form *trans*-cinnamic acid, catalyzed by phenylalanine ammonia lyase. *Trans*-cinnamic acid is hydroxylated to 4-coumaric acid in a reaction catalyzed by the cytochrome P₄₅₀ enzyme cinnamate 4-hydroxylase. 4-Coumaric acid can also be formed from tyrosine, catalyzed by tyrosine ammonia lyase.

There are multiple isoenzymes of phenylalanine ammonia lyase in most plants; they are expressed at different stages of growth and development, and also in response to wounding. Some are involved in the synthesis of monolignols for lignification, while others are involved in the synthesis of flavonoids (section 9.2.2) and other secondary metabolites. Some plants also have tyrosine ammonia lyase, and some have an enzyme that has equal activity towards both phenylalanine and tyrosine. Like histidine ammonia lyase (section 8.2.1), phenylalanine and tyrosine ammonia lyases have a dihydromethylidene imidazolone cofactor that is formed autocatalytically by cyclization and dehydration of adjacent alanine, serine and glycine residues in the precursor protein.

Phenylalanine ammonia lyase also occurs in some bacteria and yeasts, where it has a catabolic role, permitting the use of phenylalanine (and sometimes also tyrosine) as a sole carbon and nitrogen source. The enzyme from *Rhodotorula* spp. has been used to measure plasma phenylalanine concentrations in patients with phenylketonuria, and also to prepare low phenylalanine diets for them, as well as the production of phenylalanine and phenylalanine methyl ester for the manufacture of the sweetener aspartame. It has additionally been used in experimental animals as an anti-tumour agent, in the form of an enzyme-antibody complex that has catalytic activity (MacDonald & D'Cunha, 2007).

An injectable polyethyleneglycolated enzyme can significantly reduce the plasma concentration of phenylalanine in patients with phenylketonuria, and it is undergoing clinical trials. Because of the enterohepatic circulation of phenylalanine, experiments are also under way to produce a genetically modified phenylalanine ammonia lyase that is resistant to chymotrypsin and can be bound to a silica gel matrix, to be used as an oral medication for reduction of whole-body phenylalanine burden in phenylketonuria (Kang *et al.*, 2010).

The further hydroxylation and methylation reactions forming ferulic and sinapic acids may occur either at the level of free acids or CoA esters. The catechol *O*-methyltransferases of different plants have different, and some-

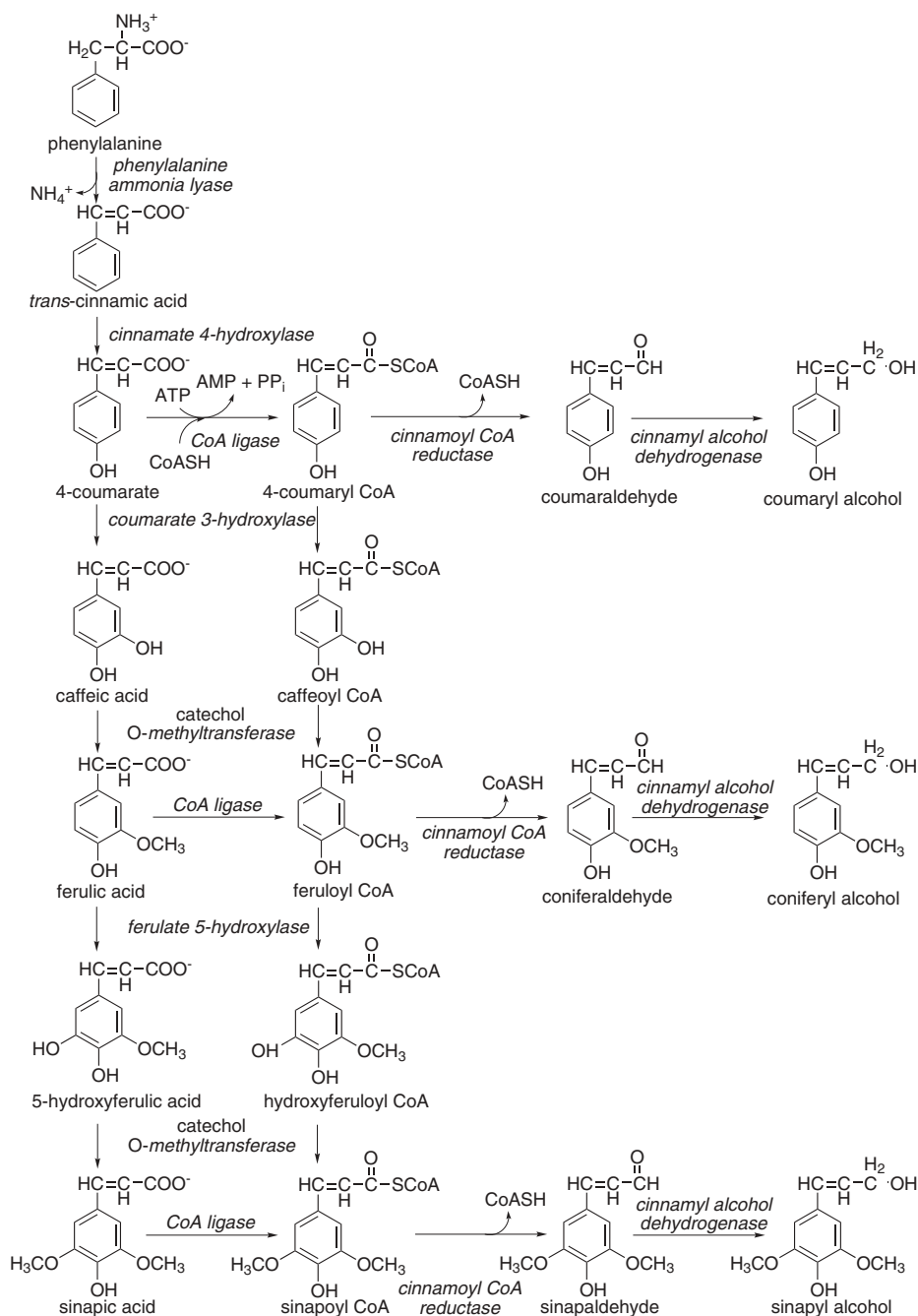


Figure 9.4 The phenylpropanoid pathway for lignin biosynthesis.

Phenylalanine ammonia lyase EC 4.3.1.5, cinnamate 4-hydroxylase EC 1.14.13.11, coumarate 3-hydroxylase EC 1.14.13.127, catechol O-methyltransferase EC 2.1.1.6, ferulate 5-hydroxylase no EC number, cinnamate CoA ligase (also acts on ferulic and sinapic acids) EC 6.2.1.12, cinnamoyl CoA reductase EC 1.2.1.44, cinnamyl alcohol dehydrogenase EC 1.1.1.195.

times overlapping, specificity. Cinnamoyl CoA reductase catalyzes the reduction of all three CoA esters to the aldehydes, with elimination of CoASH. It has equal affinity for coumaryl, feruloyl and sinapoyl CoA esters, but its activity is highest with feruloyl CoA. Reduction of the aldehydes to the alcohols yields the monomers for lignin synthesis. The polymerization to form lignin is by oxidation catalyzed by laccase, a copper-dependent oxygenase that catalyzes oxidation of diphenols and aminophenols to semiquinones, which may then undergo enzymic or non-enzymic reaction to form polymers. The resulting oligolignols are further oxidized by hydrogen peroxide formed by peroxidases. Laccase also occurs in fungi, where its function is catabolism of lignin rather than its synthesis. It is also found in insects, where it is involved in the formation of the external cuticle (Boudet *et al.*, 1995).

9.2.2 Polyphenol biosynthesis in plants

Coumaroyl CoA, formed as an intermediate in the phenylpropanoid pathway (Figure 9.4), is the precursor for the synthesis of more than 9,000 different polyphenols that act as phytoalexins (antimicrobial compounds synthesized by plants in response to pathogen infection), ultraviolet protectants, insect repellents and signalling molecules in plant-microbe interactions (including, in legumes, the induction of symbiotic root nodulation by *Rhizobium* spp. for nitrogen fixation – see section 1.1.1.7), as well as pigments in flowers.

Because they are capable of forming relatively stable radicals that persist long enough to undergo reaction to non-radical products, polyphenols in fruits and vegetables are considered to be beneficial antioxidants in human nutrition, quenching oxygen radical chain reactions. However, they are also capable of forming damaging oxygen radicals, and they are potentially genotoxic and mutagenic (Mennen *et al.*, 2005).

As shown in Figure 9.5, the first committed step in polyphenol synthesis is transfer of three acetyl groups from malonyl CoA onto 4-coumaryl CoA, catalyzed by chalcone synthase, followed by cyclization and aromatization to yield naringenin chalcone (also catalyzed by chalcone synthase). The reaction of chalcone synthase is parallel to that involved in fatty acid biosynthesis, an iterative decarboxylating condensation of malonyl CoA with a CoA-linked starter molecule. There is a super-family of chalcone synthases leading to the synthesis of the various polyphenols, and the different plant enzymes have significant sequence homology with each other, but little homology with bacterial enzymes. The specific activity of chalcone synthase is low, but in compensation there is a high level of expression of the mRNA and enzyme protein (Abe & Morita, 2010; Ferrer *et al.*, 2008; Hahlbrock & Scheel, 1989).

Onward metabolism of chalcone gives rise to flavanones, which are the precursors of flavanols, isoflavones and flavones; flavanols are the precursors of the anthocyanidins and anthocyanin pigments of plants.

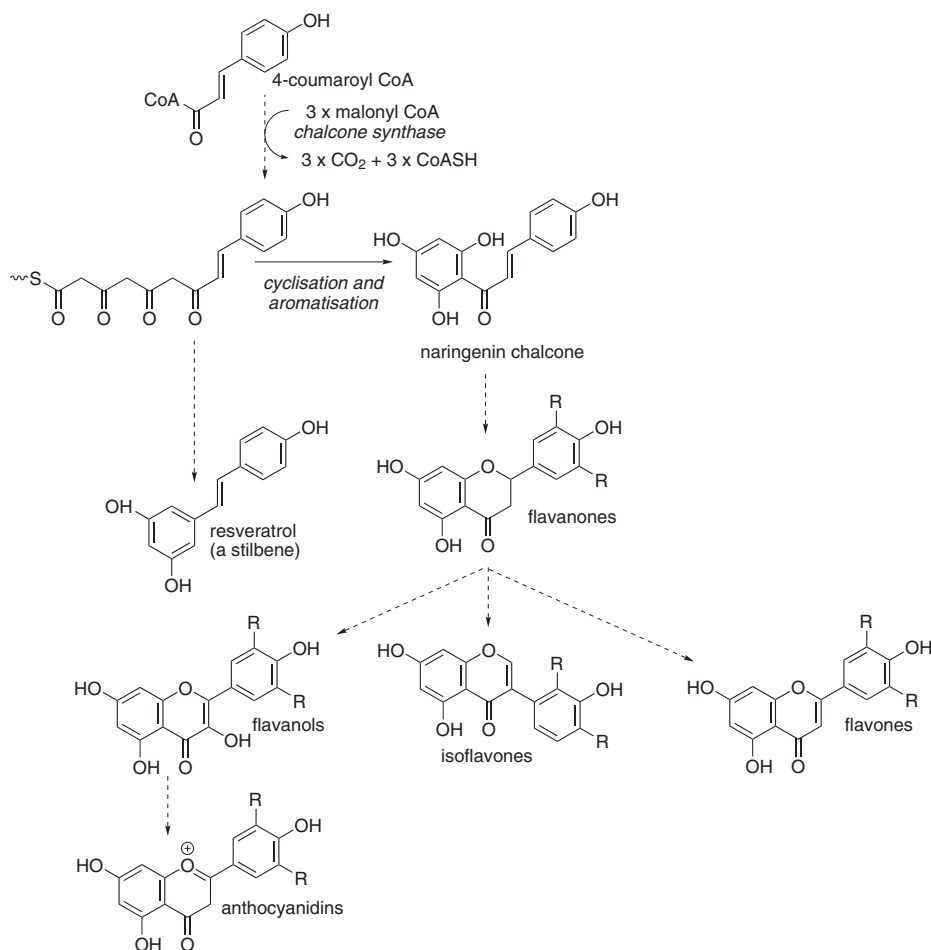


Figure 9.5 Polyphenols synthesized from coumaroyl CoA.
Chalcone synthase EC 2.3.1.74.

9.2.3 Phenylalanine hydroxylase and phenylketonuria

In mammals, phenylalanine hydroxylase is the enzyme required for both the synthesis of tyrosine and also the first enzyme of phenylalanine catabolism. As noted in section 9.1.2, some bacteria also synthesize tyrosine by hydroxylation of phenylalanine, and the catalytic regions of the bacterial hydroxylases are very similar to that of the mammalian enzyme. Although phenylalanine can undergo transamination to phenylpyruvate, this is normally a minor pathway because of the high K_m of the transaminase for phenylalanine.

Phenylketonuria is a genetic disease due to lack of phenylalanine hydroxylase, and it is characterized by severe mental retardation, very high blood

concentrations of phenylalanine and very low concentrations of tyrosine, as well as excretion of phenylpyruvate and its onward metabolites phenylacetate (which gives the urine a characteristic mouse-like odour) and phenyllactate. Collectively, these compounds are termed phenylketones, although only phenylpyruvate is chemically a ketone. Impaired activity of phenylalanine hydroxylase, but with some residual activity, leads to less severe hyperphenylalaninaemia. To date, some 500 different mutations of the phenylalanine hydroxylase gene have been identified (Scriver, 2007).

Phenylketonuria is diagnosed seven days after birth by measurement of blood phenylalanine (the hydroxylase is not normally fully developed until then). In most developed countries, all infants are routinely screened for the disease (as well as a number of other inborn errors of amino acid metabolism), using a spot of blood from a heel prick. The original method used was the Guthrie bacterial inhibition test, in which the growth of a phenylalanine-dependent strain of *B. subtilis* was measured in the presence of a competitive inhibitor of phenylalanine utilization, β -thienylalanine. Elevated concentrations of phenylalanine, as in samples from infants with phenylketonuria, are sufficient to overcome the inhibition and permit bacterial growth. The size of the bacterial colony on the agar plate is proportional to the phenylalanine concentration in the blood sample (Guthrie & Susi, 1963). The Guthrie test has now been largely superseded by liquid chromatographic or mass spectrometric measurement of phenylalanine, or sometimes enzymic measurement using bacterial phenylalanine ammonia lyase (section 9.2.1; Chace & Kalas, 2005).

Phenylketonuria is treated by feeding the affected child a diet that is very low in phenylalanine – just sufficient to permit normal growth – and containing additional tyrosine. Originally, these diets were based on hydrolyzed protein from which all three aromatic amino acids had been removed by treatment with charcoal, then small amounts of phenylalanine and adequate amounts of tyrosine and tryptophan were added back. More commonly, now, affected infants and children are fed a low-protein diet with supplements of crystalline amino acids. Bacterial phenylalanine ammonia lyase (section 9.2.1) has been used experimentally to catabolize phenylalanine formed during digestion of proteins, and so reduce phenylalanine absorption (Kang *et al.*, 2010; van Spronsen & Enns, 2010).

The mental retardation and brain damage of untreated phenylketonuria are due to defective myelination in the central nervous system, at least partly due to competition by high concentrations of phenylalanine for tRNA^{Met}. Once myelination is complete – by early adolescence – dietary control can be relaxed, but it has to be reinstated for phenylketonuric women who wish to become pregnant, in order to avoid severe damage to the foetus. Phenylketonurics who are diagnosed in early infancy and treated by dietary restriction develop normally, with no mental retardation (de Baulny *et al.*, 2007).

Some 1–2 per cent of infants with phenylketonuria do not respond to dietary restriction of phenylalanine. In these cases, the defect is not in phe-

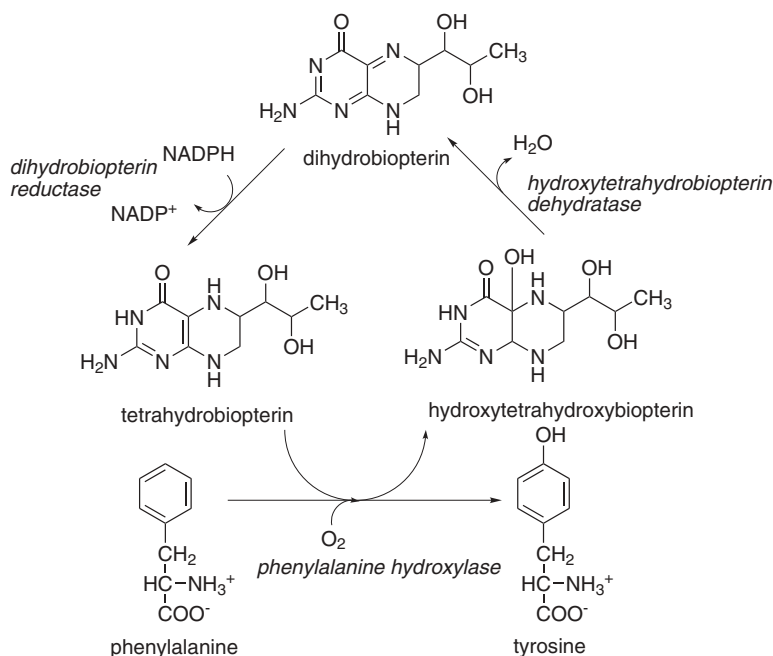


Figure 9.6 The reaction of phenylalanine hydroxylase.

Phenylalanine hydroxylase EC 1.14.16.1, hydroxytetrahydrobiopterin dehydratase EC 4.2.1.96, dihydrobiopterin reductase EC 1.5.1.34. Tyrosine hydroxylase (Figure 9.7) tryptophan hydroxylase (Figure 9.15) and anthranilic acid hydroxylase (Figure 9.16) also use tetrahydrobiopterin as the reductant.

nylalanine hydroxylase itself, but in either the synthesis of the tetrahydrobiopterin cofactor, or one of the two enzymes involved in recycling the cofactor after the hydroxylation reaction (Figure 9.6). Most infants who cannot synthesize tetrahydrobiopterin can be treated with supplements of the cofactor. Some infants with classical phenylketonuria also respond to supplements of tetrahydrobiopterin; presumably their defect is a reduced affinity of the hydroxylase for its cofactor (Blau *et al.*, 1996, 2010).

Defects of tetrahydrobiopterin synthesis and metabolism affect not only phenylalanine hydroxylase but also three other enzymes of aromatic amino acid metabolism that have a similar mechanism of action and show considerable sequence homology with phenylalanine hydroxylase: tyrosine hydroxylase (and hence impaired synthesis of catecholamines, section 9.2.4); tryptophan hydroxylase (and hence impaired synthesis of serotonin and melatonin, section 9.4.3); and anthranilic acid hydroxylase (and hence impaired catabolism of tryptophan and synthesis of NAD, section 9.4.4). Tetrahydrobiopterin is also the cofactor for nitric oxide synthase (section 5.9.3.1).

In plants, aromatic amino acid hydroxylases use 10-formyl tetrahydrofolate as the reductant rather than tetrahydrobiopterin – a novel role for a folate derivative. There is no evidence for tetrahydrobiopterin-dependent hydroxylases in plants (Pribat *et al.*, 2010).

Mammalian phenylalanine hydroxylase is a non-haem iron protein, but there is no evidence that the iron undergoes a redox change during the reaction. The enzyme is a homotetramer, in which each subunit has an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. In the absence of substrates, the regulatory domain blocks the entrance to the catalytic site.

Binding of phenylalanine to the regulatory domain leads to a conformational change that opens the catalytic site. The enzyme is activated by phosphorylation of a serine residue in the regulatory domain, leading to a conformational change that again opens the catalytic site. Binding of phenylalanine to the regulatory domain renders this serine residue more accessible for phosphorylation. By contrast, binding of tetrahydrobiopterin to the regulatory domain renders this serine residue less accessible and leads to inhibition of the enzyme when the concentration of phenylalanine is low.

Phenylalanine hydroxylase is inhibited by catecholamines, which chelate the catalytic iron, blocking the active site and oxidizing the iron. It can be reduced back to the active form by (relatively high) concentrations of tetrahydrobiopterin. Tyrosine hydroxylase (section 9.2.4) is regulated in the same way, except that phosphorylation reduces the inhibition by catecholamines (Fitzpatrick, 1998, 1999, 2003; Flatmark & Stevens, 1999; Li *et al.*, 2011).

Hydroxytetrahydrobiopterin dehydratase acts as a tetramer in the cytosol, recycling the cofactor of phenylalanine hydroxylase; in the nucleus, however, the dimer enhances the activity of the diabetes-associated transcription factor hepatic nuclear factor 1 α . There are two mammalian isoenzymes of the dehydratase. Knockout mice lacking one isoenzyme show both hyperphenylalaninaemia and also impaired glucose tolerance; hepatic nuclear factor 1 α regulates the expression of a number of genes in liver, kidney, pancreas and gut that are critical for glucose homeostasis. However, the effect is less severe than knockout of hepatic nuclear factor 1 α itself, which leads to frank diabetes (MODY type III), since the second isoenzyme can compensate to a limited extent (Bayle *et al.*, 2002; Citron *et al.*, 1992).

9.2.4 The catecholamines: dopamine, noradrenaline and adrenaline

The catecholamines dopamine, noradrenaline (norepinephrine in American usage) and adrenaline (epinephrine in American usage) are neurotransmitters. In addition, noradrenaline and adrenaline are hormones released by the

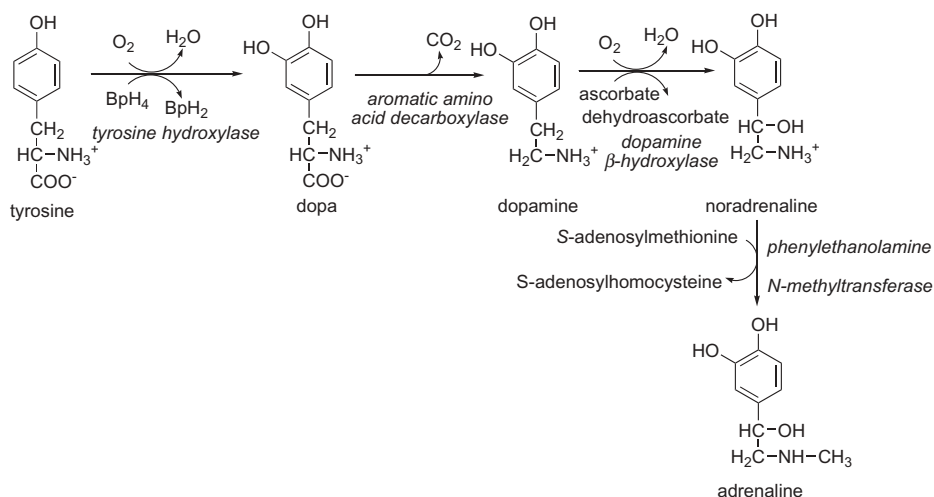


Figure 9.7 Catecholamine synthesis from tyrosine.

BpH₄ = tetrahydrobiopterin, BpH₂ = dihydrobiopterin. Tyrosine hydroxylase EC 1.14.16.2), aromatic amino acid decarboxylase EC 4.1.1.28, dopamine β-hydroxylase EC 1.14.17.1, phenylalanine *N*-methyltransferase EC 2.1.1.28. See Figure 9.6 for the role of tetrahydrobiopterin in aromatic amino acid hydroxylases.

chromaffin cells of the adrenal medulla that act on tissues through a series of G-protein coupled receptors. The α₁ adrenoceptor activates a G-protein leading to phospholipase C activation and increasing intracellular inositol trisphosphate and diacyl glycerol. The α₂-adrenoceptor activates an inhibitory G-protein, leading to decreased intracellular cAMP, while the β-adrenoceptor activates a stimulatory G-protein, leading to an increase in intracellular cAMP. As shown in Figure 9.7, the catecholamines are synthesized by hydroxylation of tyrosine to dopa (3,4-dihydroxyphenylalanine), followed by decarboxylation to dopamine. Noradrenaline is synthesized by β-hydroxylation of dopamine, and adrenaline by *N*-methylation of noradrenaline.

Like phenylalanine hydroxylase (section 9.2.3), tyrosine hydroxylase is a tetrahydrobiopterin-dependent enzyme. It is inhibited by catecholamines binding to the non-haem iron, and it is activated by phosphorylation of serine-40 in the regulatory domain, which increases the dissociation constant of inhibitory catecholamines by several orders of magnitude. At least seven different protein kinases can phosphorylate tyrosine hydroxylase. Phosphorylation of serine-19 accelerates the phosphorylation of serine-40. Although phosphorylation activates the enzyme, it also renders it more susceptible to proteolysis, and the phosphorylated enzyme has a shorter half-life than the non-phosphorylated form.

Catecholamines also compete with tetrahydrobiopterin for binding to the catalytic site, and most of the enzyme in the brain is inactive until it is phosphorylated by protein kinases. The binding of catecholamines also stabilizes the (inactive) enzyme against proteolysis. There are four isoforms of human tyrosine hydroxylase, formed by alternative splicing, leading to different regulatory domains with different affinity for catecholamines and different susceptibility to protein kinases. There are only two isoforms in other primates, and only one form in non-primates. A number of factors unrelated to catecholamine metabolism, including heparin, phospholipids and polyanions, also increase the activity of tyrosine hydroxylase by increasing its affinity for tetrahydrobiopterin (Daubner *et al.*, 2011; Fujisawa & Okuno, 2005; Kumer & Vrana, 1996).

Dopamine (3,4-dihydroxyphenylethylamine) is formed by the pyridoxal phosphate-dependent decarboxylation of dopa. In the mammalian nervous system, dopamine is a neurotransmitter in its own right, as well as the precursor for synthesis of noradrenaline and adrenaline. In insects, dopamine has a role in formation of the cuticle and hardening of the egg shell.

There has long been controversy as to whether a single pyridoxal phosphate-dependent aromatic amino acid decarboxylase catalyzes the decarboxylation of dopa in catecholamine synthesis and 5-hydroxytryptophan (5-HTP) in serotonin synthesis (section 9.4.3). Relative activity towards the two substrates differs in different tissues, and the sensitivity to some inhibitors differs for the two substrates. In experimental animals, vitamin B₆ deficiency leads to a fall in brain serotonin, with little effect on catecholamines.

It is now clear that a single gene codes for the enzyme, but it contains some 800 untranslated bases in introns, suggesting that there may well be differential splicing, leading to proteins with differing specificity and inhibitor sensitivity. The enzyme from the brain stem has a lower affinity for pyridoxal phosphate and different kinetics from the enzyme in other brain regions. The neurotoxin methylphenyltetrahydropyridine (MTPT) enhances the activity towards 5-hydroxytryptophan, but not towards dopa. Dopa (but not 5-HTP) decarboxylase activity is inhibited by partial denaturation with sodium dodecyl sulphate; 5-HTP (but not dopa) decarboxylase activity is inhibited by partial denaturation with urea. The purified enzyme shows activity towards both substrates, but the ratio of dopa to 5-HTP decarboxylation changes through purification (Bender & Coulson, 1972; Siow & Dakshinamurti, 1990).

The mammalian decarboxylase has negligible activity towards other amino acids, but plant enzymes and various bacterial decarboxylases catalyze the decarboxylation of phenylalanine, tyrosine and tryptophan, as well as dopa and 5-hydroxytryptophan. Although the activity of the decarboxylase is not limiting for catecholamine (or serotonin) synthesis, it is regulated in response to physiological and pharmacological stimuli. It is activated by phosphoryla-

tion, and it is also subject to long-term regulation by changes in transcription (Hadjiconstantinou & Neff, 2008).

Unlike mammals and insects, in plants there are separate aromatic amino acid decarboxylases that show specificity for either indolic or phenolic side chains. However, these enzymes have considerable sequence homology with the mammalian enzyme. They are involved in the synthesis of a variety of secondary metabolites, including alkaloids such as the anti-neoplastic agent vinblastine, ajmaline (which is used to treat heart arrhythmias), quinine and strychnine.

Decarboxylation of tryptophan provides a minor route for synthesis of indoleacetic acid (auxin, section 9.4.1). The dominant compound responsible for the scent of roses and some other flowers and fruits is 2-phenylethanol, formed by decarboxylation of phenylalanine, followed by oxidative deamination to phenylacetaldehyde and oxidation to the alcohol (Facchini *et al.*, 2000; Sakai *et al.*, 2007).

Aromatic amino acid decarboxylase also catalyzes the half-reaction of transamination under anaerobic conditions, leading to inactivation because of the formation of pyridoxamine phosphate at the active site (section 3.4.1). However, in the presence of oxygen (which is not involved in the decarboxylation reaction), it does not catalyze this reaction with L-amino acids, though it still catalyzes the half-reaction of transamination with D-amino acids. Although oxygen is not involved in the decarboxylation reaction, there is evidence that oxygen binds at the catalytic site. The enzyme also catalyzes the oxidative deamination of aromatic amines, forming dihydroxyphenylacetaldehyde and ammonium from dopamine, and 5-hydroxyindoleacetaldehyde and ammonium from serotonin, consuming $\frac{1}{2}\text{O}_2$ in the process (Bertoldi & Borri Voltattorni, 2000, 2003).

Dopamine β -hydroxylase is a copper-dependent oxygenase; one atom of oxygen is introduced into the substrate, forming noradrenaline, while the other is reduced to water at the expense of ascorbate (vitamin C) being oxidized to dehydroascorbate. It is in the same enzyme family as peptidyl glycine hydroxylase (section 4.7). The enzyme is released into the bloodstream, together with noradrenaline and adrenaline, when the adrenal vesicles fuse with the cell membrane to release the hormones, but there seems to be no function of the enzyme in the circulation. Early studies of the enzyme did not show the stoichiometric consumption of ascorbate because of rapid reduction of the small pool of intravesicular ascorbate by a vesicular cytochrome-dependent pathway linked to the larger cytosolic pool of ascorbate (Stewart & Klinman, 1988).

Polymorphisms of dopamine β -hydroxylase that lead to low activity are associated with low blood pressure; those that lead to increased expression are associated with hypertension (Chen *et al.*, 2010). In insects, there is a

similar tyramine β -hydroxylase that catalyzes the synthesis of octopamine as a neurotransmitter.

The final step in adrenaline synthesis is *N*-methylation of noradrenaline, catalyzed by phenylethanolamine *N*-methyl transferase, using *S*-adenosyl methionine as the methyl donor. Overall, the adrenal medulla secretes about four times as much adrenaline as noradrenaline. Any one chromaffin cell synthesizes and secretes either adrenaline (if it expresses phenylethanolamine *N*-methyltransferase) or noradrenaline (if it does not).

9.2.4.1 Parkinson's disease and inhibitors of dopa decarboxylase Parkinson's disease is a progressive degeneration of dopaminergic neurons in the substantia nigra of the brain, leading to difficulty in initiating movement, tremor or shaking, slowness of movement and rigidity. There may also be non-motor problems, including pain, depression, constipation and sweating. Progression of the disease can be slowed by giving supplements of dopa (dopamine cannot cross the blood-brain barrier), but decarboxylation of dopa in liver, kidney and gut leads to severe adverse side-effects. Inhibitors of dopa decarboxylase that do not cross the blood-brain barrier (and therefore inhibit only the extra-cerebral decarboxylase) are used together with dopa in treatment, as are dopamine agonists (Goldenberg, 2008).

α -Methyldopa is a centrally-acting antihypertensive drug. It is both a substrate and irreversible inhibitor of dopa decarboxylase. The enzyme catalyzes the intermediate formation of α -methyldopamine, followed by deamination to form 3,4-dihydroxyphenylacetone. This forms an aldol bond to the aldehyde of the pyridoxal phosphate coenzyme, so inactivating the enzyme (Amadasi *et al.*, 2007).

9.2.4.2 Catabolism of the catecholamines For neurotransmitters and very short-acting hormones like adrenaline and noradrenaline, there must be a rapid mechanism for their catabolism and inactivation or also (in the case of neurotransmitters) for their removal from the synaptic cleft and re-uptake into the pre-synaptic neuron for storage and re-release. Two enzymes are involved in the inactivation of catecholamines: catechol *O*-methyltransferase and monoamine oxidase. As shown in Figure 9.8, these may act in either order on dopamine and noradrenaline, leading to intermediate methylated or oxidized products. Adrenaline is not a substrate for monoamine oxidase, because the amino group is blocked by the methyl group.

Mammalian catechol *O*-methyltransferase acts more rapidly on catecholamines than catechols, but it does act on oestrogens and some flavonoids. Inhibitors are potential adjuncts to dopa therapy in Parkinsonism (section 9.2.4.1).

Monoamine oxidases (MAO) are mitochondrial outer-membrane flavo-proteins. MAO-A is on the cytosolic side of the membrane, while MAO-B is

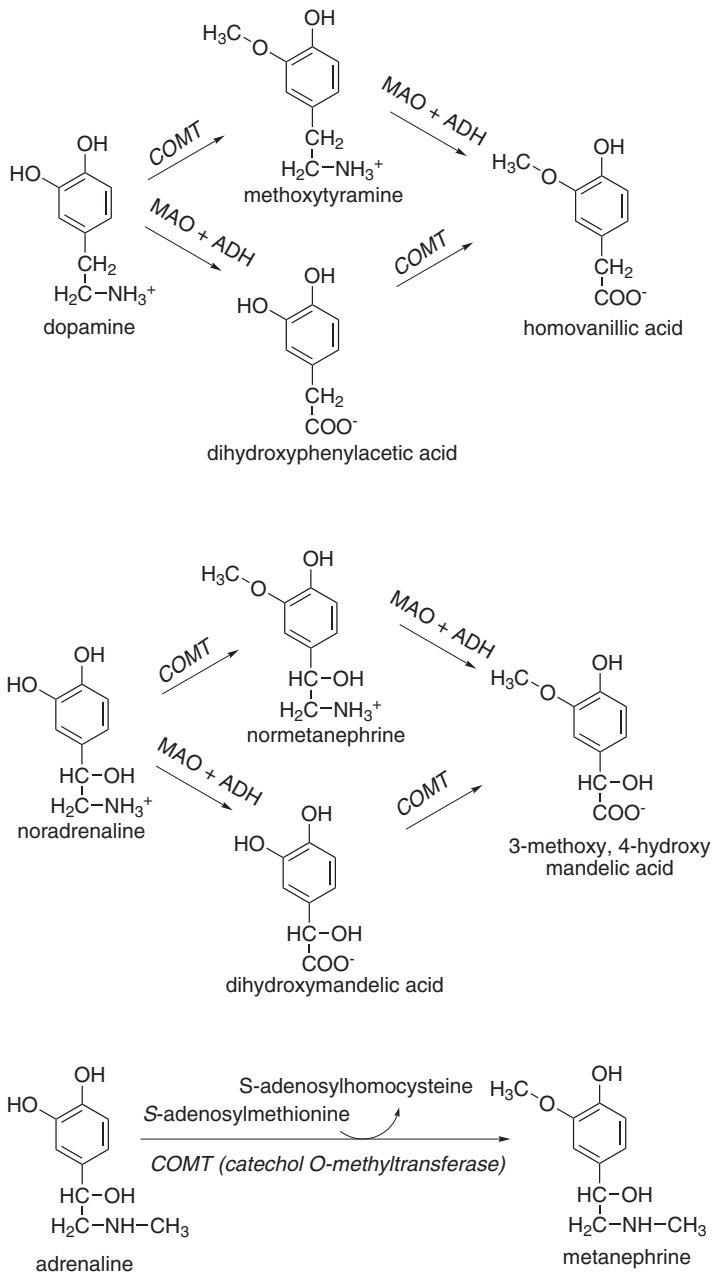


Figure 9.8 Catabolism of the catecholamines.

MAO = monoamine oxidase EC 1.4.3.4, ADH = aldehyde dehydrogenase EC 1.2.1.3, COMT = catechol O-methyltransferase EC 2.1.1.6. See Figure 9.9 for the reaction of monoamine oxidase and aldehyde dehydrogenase.

on the inner face. The two isoenzymes differ in substrate specificity and inhibitor sensitivity. The preferred substrates of MAO-A are serotonin (section 9.4.3) and noradrenaline, while MAO-B preferentially acts on phenylethylamine and benzylamine. Tyramine and dopamine are oxidized equally by both isoenzymes.

The genes for MAO-A and MAO-B show 70 per cent sequence identity and are located side by side on the X chromosome, with a head-to-tail orientation and identical intron-exon patterns, suggesting duplication of a common ancestral gene. Both isoenzymes are expressed in most tissues. MAO-A is prevalent in fibroblasts and placenta, but MAO-B is the only form in blood platelets (Fitzpatrick, 2010).

MAO-A is a drug target for treatment of depression, while MAO-B is a target for treatment of Parkinsonism and other neurodegenerative diseases, either alone or together with dopa and inhibitors of extra-cerebral dopa decarboxylase (section 9.2.4.1). Early MAO inhibitors used in the treatment of depression were non-selective, and the 'cheese reaction' was a hypertensive crisis caused by ingestion of tyramine and other amines in foods (especially cheese). Inhibition of the MAO in the intestinal mucosa and liver that would normally oxidize these amines led to concentrations of tyramine high enough to cause the release of inappropriate amounts of adrenaline and noradrenaline from the adrenal medulla.

This is still a problem with irreversible selective inhibitors of MAO-A, but not with the selective inhibitors of MAO-B at dosages that are used therapeutically. Reversible competitive inhibitors of MAO-A do not cause a 'cheese reaction' because, as the concentration of tyramine increases, it out-competes the inhibitor (Youdim & Weinstock, 2004).

As shown in Figure 9.9, the first step in the monoamine oxidase reaction is oxidation of the C–N bond to form a C=N double bond at the expense of reducing the flavin cofactor. This is followed by hydrolysis, releasing ammo-

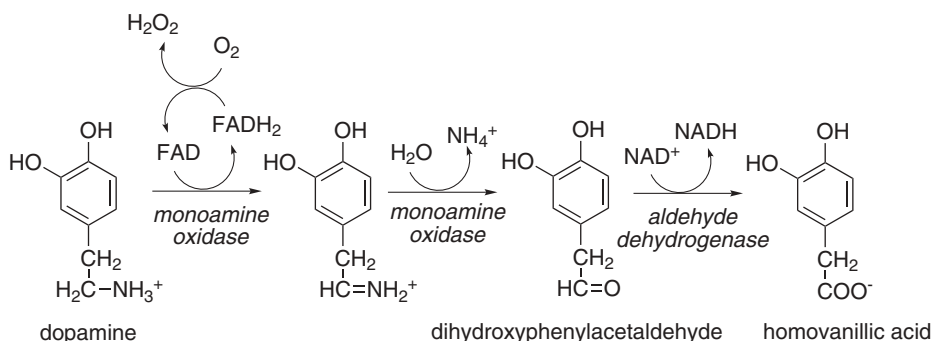


Figure 9.9 The reaction of monoamine oxidase and aldehyde dehydrogenase. Monoamine oxidase EC 1.4.3.4, aldehyde dehydrogenases EC 1.2.1.3–5.

nium. The resultant aldehyde is then oxidized to the acid by aldehyde dehydrogenase. A minor pathway is reduction of the aldehyde to the alcohol, catalyzed by aldehyde reductase.

Renalase is a protein secreted by the kidney and expressed in other tissues, including the central nervous system. It has some sequence homology with MAO-A and MAO-B, and has the same secondary and tertiary structures. It has been called MAO-C. It catalyzes the oxidation of catecholamines, but not serotonin, tyramine, benzylamine or diamines. It is not sensitive to the same inhibitors as MAO-A and MAO-B. Renalase secretion is down-regulated in hypertension, and renalase knockout mice are hypertensive. Administration of a single dose of renalase to the mice normalizes blood pressure for about 24 hours.

The function of the enzyme secreted by the kidney is presumed to be catabolism of catecholamines in the circulation, so terminating the action of adrenaline and noradrenaline secreted by the adrenal glands. Under basal conditions, plasma renalase has little activity but, in response to a brief surge of adrenaline, there is a 50-fold increase in activity as well as increased synthesis and secretion (Boomsma & Tipton, 2007; Li *et al.*, 2008).

There are two structural families of flavoprotein amine oxidases. The monoamine oxidase family includes monoamine oxidases A and B, renalase, L-amino acid oxidase (section 1.5.1), spermine and polyamine oxidases (section 5.8.3) and histone lysine demethylases (section 6.2.4.2). The D-amino acid oxidase family includes D-amino acid oxidase and glycine oxidase (section 1.5.1), as well as enzymes that oxidize *N*-methylated amino acids.

9.2.5 Tyrosinase and melanin synthesis

Melanins are brown and black pigments formed by polymerization of the oxidation products of tyrosine. Melanin synthesis is initiated by tyrosinase, a copper-dependent phenol oxidase that is found in almost all organisms. Tyrosinase activity and melanin formation are responsible for the blackening of fruits and vegetables when they are cut, and also the pigments of mammalian skin, hair and eyes. In the skin, hair follicles, and the iris and retinal pigment epithelium of the eye, melanin is synthesized and stored in melanocytes.

Eumelanin is formed by polymerization of onward metabolites of dopaquinone (Figure 9.10); it is insoluble and is brown or black in colour. In the presence of cysteine or glutathione, the more soluble yellow-red pheomelanin is formed by polymerization of cysteinyl-dopa. Allomelanins are formed by the polymerization of homogentisic acid (Figure 9.12) and a variety of other catechols.

In bacteria, melanins may act as the final electron acceptor in anaerobic metabolism; in *Azotobacter* spp., melanin synthesis may provide an additional sink for oxygen to maintain the low pO_2 needed for nitrogen fixation (section

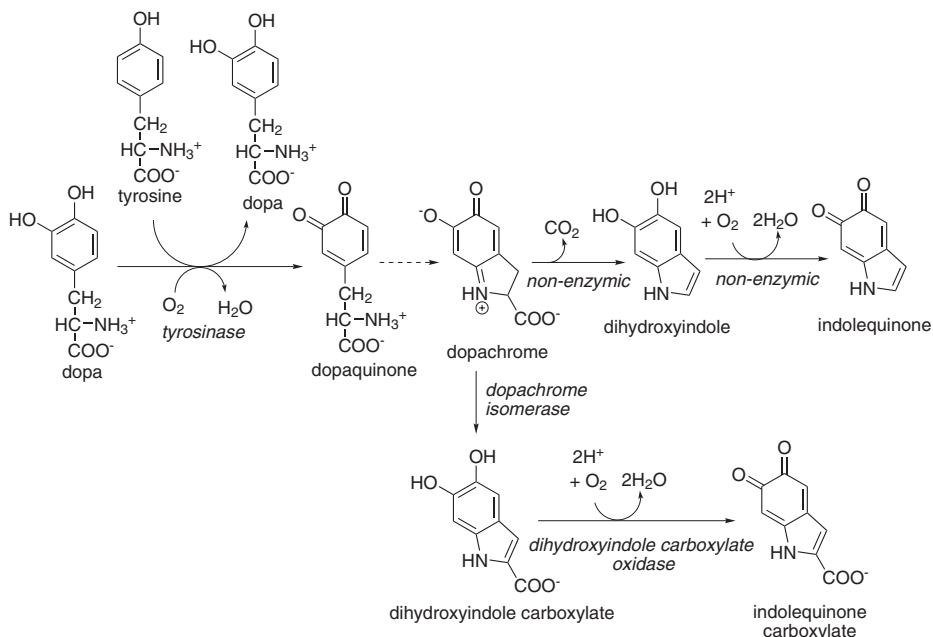


Figure 9.10 Tyrosinase and the synthesis of melanin

Tyrosinase EC 1.14.18.1, dopachrome isomerase EC 5.3.1.12, dihydroxyindole carboxylate oxidase no EC number.

1.1.1.3). In pathogenic bacteria, melanin synthesis is correlated with virulence, reflecting its ability to act as a sink for reactive oxygen species produced in response to infection. Before the development of DNA fingerprinting methods, the spectrum of melanins formed was important in the taxonomy of bacteria and fungi (Plonka & Grabacka, 2006).

In the substantia nigra of the brain, neuromelanin is formed by oxidation and polymerization of dopamine rather than tyrosine (section 9.2.4). In albino animals that lack tyrosinase, the substantia nigra is normally pigmented. However, in patients with Parkinson's disease (section 9.2.4.1), where there is failure of dopamine synthesis in the central nervous system, there is little or no pigmentation in the substantia nigra.

In insects, there is a proteolytic cascade in response to microbial infection leading to localized melanization as a result of activation of a pro-phenol oxidase. There is some evidence that melanin synthesis is involved in initiating the coagulation of the haemolymph, and melanin is involved in the hardening of clots (Eleftherianos & Revenis, 2011).

Albinism in mammals is the absence of melanin from skin, hair and eyes. In some animals, total albinism is common – white fur and no pigmentation in either the skin or eyes. Although total albinism (oculocutaneous albinism)

occurs in human beings, it is relatively uncommon. Partial albinism occurs quite frequently, with patches or streaks of unpigmented skin and hair. The tendency to partial albinism is inherited, as is the pattern of distribution of the albinotic patches – a white forelock is frequently a familial trait. This reflects genetic control over the developmental migration of melanoblasts from the neural crest to the dermis and epidermis to become melanocytes.

The genetic control of skin and hair colour is complex; in addition to the enzymes of melanin synthesis, more than 100 genes are involved in the differentiation of melanocytes and subcellular melanosomes, and in intracellular trafficking in melanocytes (Guyonneau *et al.*, 2004). In black cats with white paws, it is not uncommon for some pads of the paws to be black and others unpigmented (and therefore pink). Black cats have a higher requirement for tyrosine for melanin synthesis than for maximum growth. If they are fed minimally adequate phenylalanine and tyrosine for maximum growth, they have a reddish fur (Anderson *et al.*, 2002).

Vitiligo is an auto-immune disease characterized by loss of melanocytes and patchy depigmentation of the skin. It is genetically complex, with multiple susceptibility genes and environmental triggers, and it is associated with increased risk of other auto-immune diseases, including auto-immune thyroiditis (section 9.2.6), rheumatoid arthritis, psoriasis, adult-onset type I diabetes mellitus, pernicious anaemia, systemic lupus erythematosus and Addison's disease (Rezaei *et al.*, 2007).

There is interest in inhibitors of tyrosinase as skin lightening agents for cosmetic reasons and in the treatment of disorders involving hyperpigmentation of the skin. Experimental treatments for malignant melanoma are based on prodrugs that are activated by tyrosinase; they are effective because of the high activity of tyrosinase in melanomas, compared with normal tissue.

As shown in Figure 9.10, three enzymes are involved in the formation of melanin in mammals, although the dopaquinone formed by tyrosinase action can undergo non-enzymic oxidation to indolequinone, which polymerizes to yield melanin. Tyrosinase is a copper-dependent oxygenase that catalyzes the parallel hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone. There are two copper atoms in the enzyme, each coordinated by six histidine residues, and the copper pair binds oxygen to catalyze the hydroxylation of a tyrosine to dopa and the oxidation of a diphenol (dopa) to the quinone with the formation of water.

The other two enzymes in mammalian melanogenesis show considerable sequence homology with tyrosinase, and they have probably arisen as a result of gene duplication. Tyrosinase-related protein I is probably dihydroxyindole carboxylate oxidase; tyrosinase-related protein II is dopachrome isomerase, which catalyzes the keto-enol tautomerization of dopachrome to dihydroxyindole carboxylate. Unlike tyrosinase, dopachrome isomerase is a zinc metallo-enzyme. The activity of the isomerase controls the relative amounts

of indolequinone and indolequinone carboxylate incorporated into melanin, and hence the size and structure of the polymer. Melanin containing indolequinone carboxylate is more protective against ultraviolet irradiation and sunburn; knockout mice lacking the isomerase are more susceptible to ultraviolet-induced oxidative stress in the skin (Aroca *et al.*, 1992; Jiang *et al.*, 2010; Olivares & Solano, 2009).

9.2.6 The thyroid hormones, thyroxine and tri-iodothyronine

The thyroid hormones have general effects on growth, development and the control of basal metabolic rate. They regulate gene expression, acting via nuclear receptors that bind to hormone response-elements on DNA. The thyroid hormone receptor forms a heterodimer with the retinoid X receptor. Vitamin A nutritional status affects the availability of 9-*cis*-retinoic acid. In the presence of the retinoid ligand, the heterodimer activates gene transcription but, when the retinoid receptor is unoccupied, the heterodimer acts as a transcription inhibitor. However, excess vitamin A also leads to down-regulation of thyroid hormone function (and hence functional hypothyroidism) because the retinoid X receptor then forms homodimers, so that it is not available to form heterodimers with the thyroid hormone receptor.

The hormones are synthesized by iodination of a small number of the 134 tyrosine residues in thyroglobulin. Iodine deficiency leads to hypothyroidism and enlargement of the thyroid gland (goitre), because of stimulation of the gland by thyrotropin (the hypothalamic thyroid-stimulating hormone). Autoimmune disease (Hashimoto's auto-immune thyroiditis) also leads to hypothyroidism and goitre.

Hypothyroidism is characterized by weight gain, lethargy and sensation of cold, with a low metabolic rate. Severe adult hypothyroidism is known as myxoedema, and there is subcutaneous oedema, especially of the hands and face. Goitrous cretinism is the result of hypothyroidism *in utero* and in infancy, most commonly due to iodine deficiency; there is considerable stunting of growth, a low mental age and often also deafness.

Iodine deficiency goitre is a major problem of public health, especially in upland areas of the world, where there is a thin soil over limestone. In parts of the Himalayas and the Matto Grosso of Brazil, the incidence of iodine deficiency goitre is almost 100 per cent. Prevention is by enrichment of a dietary staple with iodide; salt and bread are used in many countries. Alternatively, intramuscular injection of iodized oil at six-monthly intervals provides adequate iodine.

One problem of iodine supplementation is that people whose thyroid glands have enlarged as a result of iodine deficiency now become transiently

hyperthyroid, since the enlarged gland has a greater capacity for hormone synthesis than is needed when adequate iodine is available. A number of plants, especially *Brassica* spp., contain goitrogens – compounds that inhibit iodide metabolism and lead to the development of hypothyroidism and goitre. It is unlikely that the amounts normally consumed will have any adverse effect, but there are reports of goitre in cattle fed on large amounts of brassica. Also, the former high incidence of iodine deficiency goitre in the Netherlands was apparently due to consumption of large amounts of sauerkraut (fermented cabbage); sufficient amounts were habitually eaten to prevent the development of the vitamin C deficiency disease scurvy among Dutch sailors in the 16th century.

Hyperthyroidism (excessive activity of the thyroid gland) may result from repletion of iodine-deficient goitrous subjects with iodine, but it is more commonly an autoimmune disease (Graves' disease), which may affect some two per cent of women (but fewer men) in developed countries. The autoantibodies mimic the action of thyrotropin and over-stimulate the thyroid. Hyperthyroidism is characterized by weight loss and a high metabolic rate, as well as exophthalmus (wide staring eyes). Synthetic goitrogens are commonly used to treat hyperthyroidism (Yen, 2001).

As shown in Figure 9.11, the first step in synthesis of the thyroid hormones is the oxidation of iodide to iodine, catalyzed by iodide peroxidase. This is a haem protein, and it is the antigen in auto-immune hypothyroidism. The peroxidase requires hydrogen peroxide, which is generated by NADPH oxidase, for the oxidation of iodide. As well as the production of iodine, the peroxidase also catalyzes the iodination of specific tyrosine residues in thyroglobulin and the coupling reaction in which the di-iodophenol of one di-iodotyrosine residue is transferred onto the hydroxyl group of another, leading to partial proteolysis of the protein. Some tri-iodothyronine (T_3) is formed by the transfer of mono-iodophenol in the coupling reaction. Thyroxine (T_4) and some tri-iodothyronine are released by proteolysis. In the thyroid gland, about five per cent of thyroxine is deiodinated to tri-iodothyronine, but most is released by the gland and is deiodinated to the active hormone in target tissues. Although T_4 is regarded as a prohormone for T_3 , it also has some non-genomic actions in its own right, acting via cell surface receptors to regulate ion pumps and protein trafficking (Taurog *et al.*, 1996).

There are three thyroxine deiodinases; all are selenoproteins with selenocysteine at the catalytic site (section 4.9), and selenium deficiency can contribute to hypothyroidism and goitre. Types I and II enzyme catalyze deiodination of the outer ring of T_4 to give active T_3 . Type III catalyzes deiodination of the inner ring of T_3 or T_4 to inactivate the hormone, then catalyzes onward deiodination so that iodide can be recycled.

The enzymes act by catalyzing a reductive deiodination of the aromatic ring to form selenocysteine iodide at the active site, followed by the release

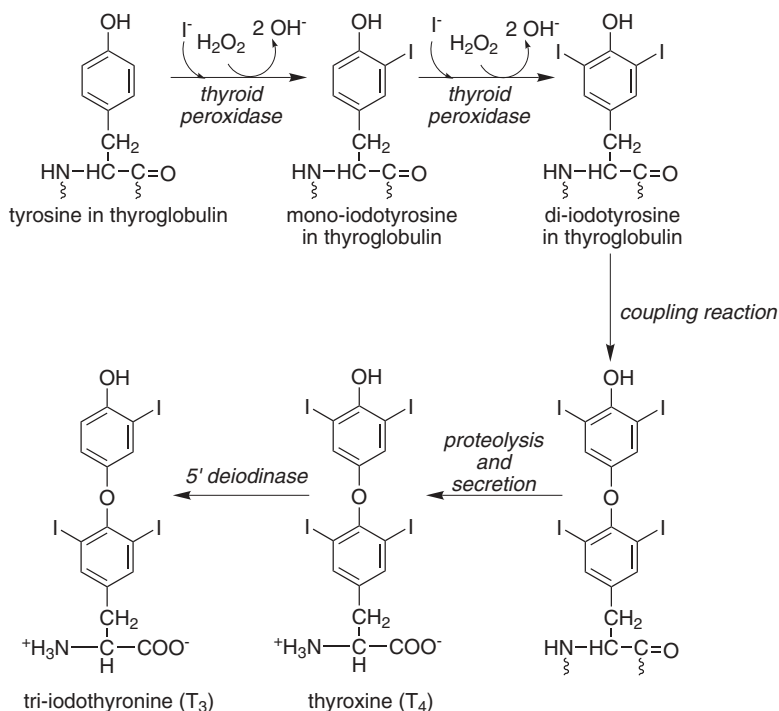


Figure 9.11 Biosynthesis of the thyroid hormones.

Iodide peroxidase EC 1.11.1.8, NADPH oxidase EC 1.6.3.1, 5'deiodinase EC 1.97.1.10.

of iodide. Type II deiodinase is the major activating enzyme in most target tissues. Knockout mice lacking type II deiodinase have impaired cold responsiveness, a low metabolic rate, and they gain weight because they metabolize more carbohydrate and less lipid. This deiodinase is up-regulated in hypothyroidism and down-regulated in hyperthyroidism (Dentice & Salvatore, 2011; Marsili *et al.*, 2011).

Selenium deficiency is a problem in several areas of the world where the soil selenium concentration is low, and it contributes to hypothyroidism. In some countries, selenium is added to fertilizer to overcome the problem of deficiency. The low availability of selenium from Dutch soil was probably a contributory factor in the former high incidence of iodine deficiency goitre in the Netherlands.

T₃ and T₄ can be decarboxylated to thyronamines that have short-term actions, via cell surface G-protein receptors, to oppose the classical actions of the thyroid hormones. There is some evidence that the decarboxylation is catalyzed by aromatic amino acid decarboxylase (section 9.2.4), but the recombinant human enzyme does not act on T₃ or T₄ (Piehl *et al.*, 2011).

9.3 Catabolism of phenylalanine and tyrosine

As discussed in section 9.2.3, the first step in mammalian catabolism of phenylalanine is hydroxylation to tyrosine. It is only when tissue concentrations of phenylalanine are extremely high, as in uncontrolled phenylketonuria, that transamination of phenylalanine occurs to any significant extent. Although phenylalanine hydroxylation is an important source of tyrosine, the regulation of the hydroxylase suggests that its main role is in catabolism of phenylalanine. In cultured hepatocytes, tyrosine has little effect on phenylalanine hydroxylation. Exogenous tyrosine that has been transported into the cells is in a separate metabolic pool from that newly synthesized from phenylalanine, which is preferentially channelled into the catabolic pathway unless the extracellular availability of tyrosine is too low to permit protein synthesis (Shiman & Gray, 1998).

As shown in Figure 9.12, catabolism of tyrosine begins with transamination to *p*-hydroxyphenylpyruvate. Although the transaminase reaction is readily reversible (phenylpyruvate hydroxylase represents the first essentially irreversible step of tyrosine catabolism), it is the main regulatory step in the pathway. Tyrosine transaminase is one of the enzymes of amino acid catabolism that are induced by cortisol and other glucocorticoid hormones, leading to the depletion of essential amino acids pools and, hence, decreased protein synthesis and increased gluconeogenesis from amino acids (the other enzyme is tryptophan dioxygenase, section 9.4.4.1).

p-Hydroxyphenylpyruvate hydroxylase is a non-haem iron protein that catalyzes a decarboxylation, substituent migration and hydroxylation in a single catalytic cycle. Structurally, it belongs to the family of 2-oxoglutarate-dependent oxygenases (section 5.7.2). However, it does not utilize 2-oxoglutarate as a second substrate, but rather incorporates both atoms of oxygen into homogentisic acid – one into the α -carbon of the pyruvate side-chain, and the other into carbon-1 of the aromatic ring.

In plants, homogentisic acid is important as the precursor for synthesis of plastoquinone and tocopherol, which are important in linking photosystems I and II, so inhibition of *p*-hydroxyphenylpyruvate hydroxylase uncouples photosynthesis. The enzyme is a target for selective benzocyclohexanedione herbicides; treated plants undergo bleaching and die within a few days (Moran, 2005).

Homogentisic acid oxidase is also a non-haem iron protein. It catalyzes oxidative cleavage of the aromatic ring to form maleylacetoacetate, which is then isomerized, and the resultant fumarylacetoacetate is hydrolyzed to fumarate and acetoacetate. Maleylacetoacetate isomerase catalyzes the breaking and reforming of the carbon-carbon double bond in a glutathione-catalyzed reaction. In the first half-reaction, the enzyme catalyzes a glutathione *S*-transferase reaction (section 5.4.3) to break the carbon-carbon double

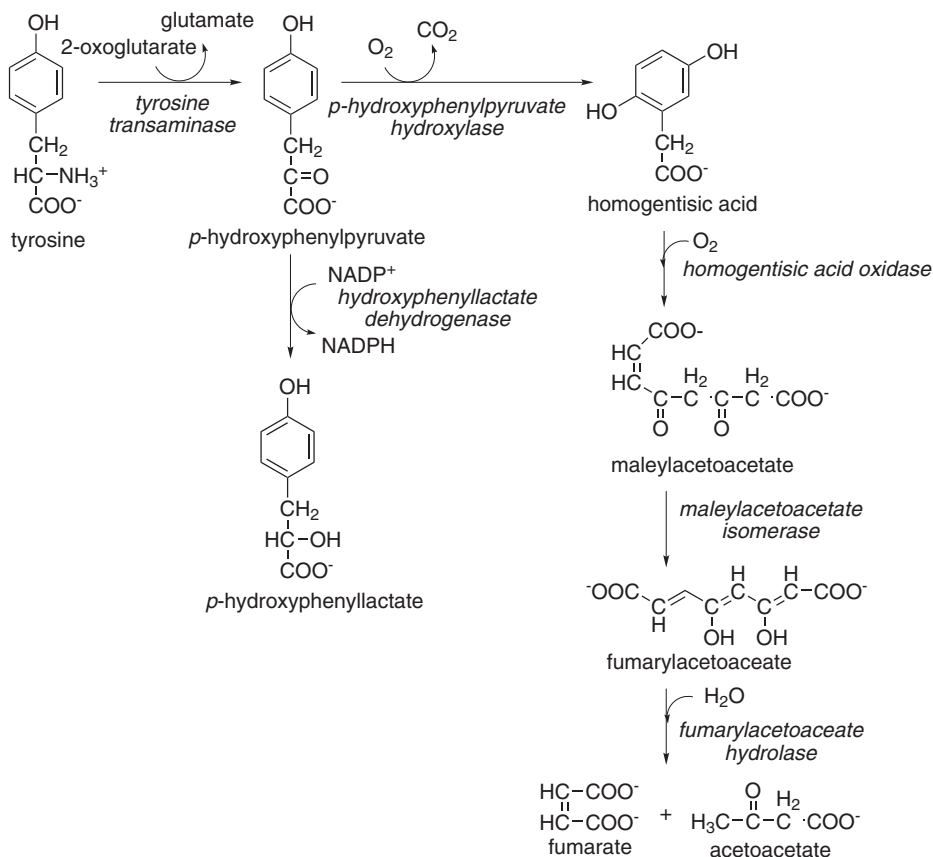


Figure 9.12 Tyrosine catabolism.

Tyrosine transaminase EC 2.6.1.5, *p*-hydroxyphenylpyruvate hydroxylase EC 1.13.11.27, homogentisic acid oxidase EC 1.13.11.5, maleylacetoacetate isomerase EC 5.2.1.2, fumarylacetoacetate hydrolase EC 3.7.1.2, *p*-hydroxyphenyllactate dehydrogenase EC 1.1.1.222.

bond, which is then reformed by removal of the glutathione. Glutathione thus acts as a coenzyme and is not consumed in the reaction. Knockout mice lacking the isomerase seem to be little affected; glutathione can catalyze the isomerization non-enzymically to a limited extent (Marsh *et al.*, 2008).

There are several genetic defects known in the tyrosine catabolic pathway:

- Lack of tyrosine transaminase, *p*-hydroxyphenylpyruvate oxidase or fumarylacetoacetate hydrolase all lead to tyrosinaemia, which is commonly fatal in early life unless treated by severe restriction of phenylalanine and tyrosine intake.

- Hawksinuria (type II tyrosinaemia) is due to a mutation in the amino-terminal region of *p*-hydroxyphenylpyruvate oxidase that leads to uncontrolled activity and the urinary excretion of homogentisic acid thiol conjugates. It can be controlled with the benzocyclohexanediones that are used as herbicides (Scott, 2006).
- Alkaptonuria is due to a lack of homogentisic acid oxidase, leading to accumulation of homogentisic hydroquinone. This undergoes oxidation and polymerization to the so-called ochromotic pigment that is excreted in the urine (giving it a very dark colour) but is also deposited in cartilage and collagen, leading to the development of arthritis.

In trypanosomes, the catabolism of phenylalanine, tyrosine and tryptophan is by simple transamination to oxo-acids that are then reduced to the corresponding lactate derivatives and excreted. This means that, unlike most organisms, there are no energy-yielding steps in the catabolism of the aromatic amino acids by trypanosomes (Nowicki & Cazzulo, 2008).

9.4 Metabolism of tryptophan

In bacteria, tryptophan is catabolized to indole (section 9.4.2) and skatole (methylindole). In plants, it is the precursor for synthesis of the growth hormone indoleacetic acid (auxin – see section 9.4.1 below). In mammals, tryptophan is the precursor for synthesis of the neurotransmitter serotonin (5-hydroxytryptamine, section 9.4.3) and the neurohormone melatonin (section 9.4.3.1). A branch of the mammalian pathway of tryptophan catabolism permits synthesis of the nicotinamide ring of NAD (section 9.4.4.4).

9.4.1 Auxin (indoleacetic acid)

Indoleacetic acid is a plant growth hormone (auxin). It is synthesized in the shoot tip region and is transported to other regions, where it stimulates growth. Thus, when a plant is exposed to unilateral illumination, it grows towards the source of the light (phototropism), either because more auxin is synthesized on the dark side, or less auxin is catabolized by photo-oxidation so that growth is stimulated on the dark side. Auxin also stimulates the development of roots from stem tissue, and preparations of auxin (or the synthetic hetero-auxins such as naphthylacetic acid) are widely used to promote the rooting of plant cuttings for vegetative propagation. More potent synthetic auxins are used as selective herbicides that have little effects on grasses but kill broad-leaved plants by over-stimulating their growth.

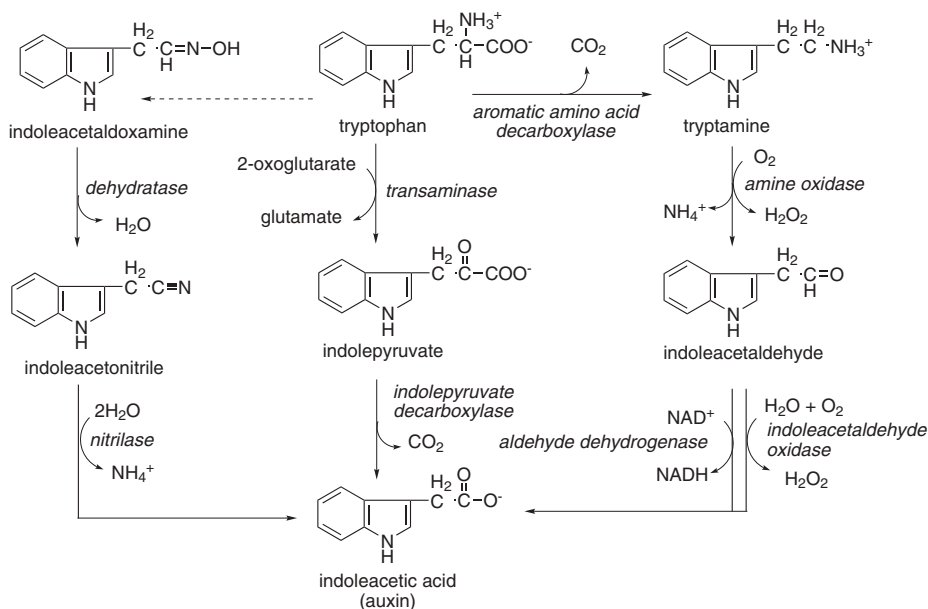


Figure 9.13 Auxin biosynthesis from tryptophan.

Tryptophan transaminase EC 2.6.1.27, indolepyruvate decarboxylase EC 4.1.1.74, aromatic amino acid decarboxylase EC 4.1.1.28, monoamine oxidase EC 1.4.3.4, indoleacetaldehyde oxidase EC 1.2.3.7, aldehyde dehydrogenases EC 1.2.1.3–5, indoleacetaldoxime dehydratase EC 4.99.1.6, nitrilase EC 3.5.5.1.

As shown in Figure 9.13, there are multiple pathways for auxin synthesis in different plants, from tryptophan via indolepyruvate or indoleacetaldoxamine, from tryptamine via indoleacetaldehyde or directly from indole or indoleglycerol phosphate. In addition to *de novo* synthesis, plants have reserves of auxin in a variety of conjugates that can be released for use. Some bacteria also synthesize auxin, and mammals excrete small amounts as the result of transamination of small amounts of tryptophan (Bartel, 1997; Normanly *et al.*, 1995).

9.4.2 Indole formation

In maize and other graminaceae, indole glycerol phosphate (an intermediate in tryptophan biosynthesis – see Figure 9.3) is the substrate for a lyase that seems to have evolved from the α -subunit of tryptophan synthase (section 9.1.3) and which releases free indole and 3-phosphoglyceralddehyde. This production of indole is important in defence against insect pests. Indole is volatile (and has a strong faecal odour), and it is released as an attractant to parasitic wasps that prey on the herbivorous insects. Application of oral secretions from herbivorous insects to wounds on plant tissue leads to induction of

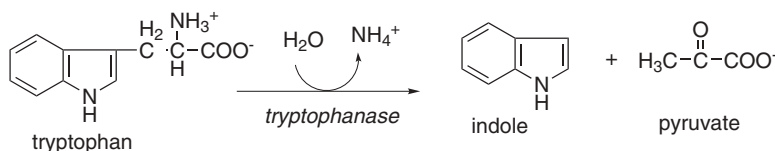


Figure 9.14 Indole formation from tryptophan.
Tryptophanase EC 4.1.99.1.

indoleglycerol phosphate lyase. Various fatty acid-amino acid conjugates have been identified as the inducers in different insects (Frey *et al.*, 2004; Gierl & Frey, 2001).

As shown in Figure 9.14, in bacteria, indole is synthesized from tryptophan by tryptophanase, a pyridoxal phosphate-dependent enzyme that catalyzes hydrolysis of tryptophan (a β -elimination reaction – see section 3.4) to indole, pyruvate and ammonium. The reaction is reversible and, in the presence of high concentrations of indole, it can provide a pathway for tryptophan synthesis. Before the development of DNA fingerprinting techniques, the ability to form indole provided a tool for bacterial taxonomy.

The enzyme is strongly activated by potassium, which is absolutely required for activity and seems to anchor the two subunits of the enzyme together. Purified tryptophanase has been used for measurement of plasma potassium concentrations (Kimura *et al.*, 1992). Despite the similarity of the reaction to that of tryptophan synthase (section 9.1.3), there is little or no structural similarity between the two enzymes (Isupov *et al.*, 1998).

In bacteria that produce indole, it acts as a signalling molecule to regulate the expression of factors that promote adhesion to surfaces and biofilm formation. Bacterial film formation is important both clinically and in terms of food hygiene. However, indole synthesized by intestinal bacteria may also have beneficial effects in signalling to intestinal mucosal cells to reduce the expression of inflammatory cytokines, increase the expression of anti-inflammatory cytokines and increase the synthesis of mucin to protect and strengthen the mucosal barrier. Indole synthesis is increased when intestinal bacteria have an inadequate supply of fermentable carbohydrate to permit utilization of tryptophan and other amino acids for protein synthesis, and the resulting pyruvate can be used as a carbon source. In *E. coli*, a small non-coding RNA binds to tryptophanase and increases its affinity for tryptophan, thus increasing indole formation, which leads to stabilization of plasmids and regulates the cell cycle (Bansal *et al.*, 2010; Chant & Summers, 2007; Martino *et al.*, 2003).

9.4.3 Serotonin and melatonin

Serotonin (5-hydroxytryptamine) is a neurotransmitter in the central nervous system. Although there are relatively few serotonergic neurons (only about

one in a million neurons synthesizes serotonin), it is involved in modulation of almost all behavioural processes. Serotonin is also synthesized in the enterochromaffin cells of the intestinal mucosa. Peripherally synthesized serotonin has roles in haemostasis, vasoconstriction and the control of immune responses, gut motility and bladder function.

Platelets do not synthesize serotonin, but take it up from the circulation and store it for release on activation. Serotonin released by platelets causes haemostasis by promoting platelet aggregation and vasoconstriction. Serotonin-specific reuptake inhibitors used as antidepressant medication can increase bleeding time by inhibiting platelet uptake of serotonin (Berger *et al.*, 2009). Carcinoid syndrome (section 9.4.5.3) is the result of excessive synthesis and release of serotonin by metastatic carcinoid tumours of enterochromaffin cells.

Impaired serotonin synthesis in the central nervous system is a factor in the aetiology of clinical depression. Depression can be treated with monoamine oxidase inhibitors to inhibit serotonin catabolism, or with specific inhibitors of serotonin reuptake to increase the amount remaining in the synaptic cleft. Supplements of tryptophan have shown some benefit, but there is a narrow therapeutic window, since tryptophan at relatively high concentrations stabilizes tryptophan dioxygenase, so increasing its catabolism through the kynurenine pathway (section 9.4.4.1). 5-hydroxytryptophan has also been shown to be beneficial in the treatment of depression.

Melatonin is synthesized from serotonin in the pineal gland. Its synthesis and secretion are dependent on light, and melatonin is responsible for the generation and maintenance of circadian rhythms and (in animals) seasonal breeding cycles. There is some evidence that supplements of melatonin can help people to adjust to different time zones and overcome jet lag.

As shown in Figure 9.15, the first step in serotonin and melatonin synthesis is hydroxylation of tryptophan to 5-hydroxytryptophan, followed by decarboxylation to serotonin. Like phenylalanine and tyrosine hydroxylases (section 9.2.3), tryptophan hydroxylase is a tetrahydrobiopterin-dependent oxygenase. In variant phenylketonuria, due to defects in tetrahydrobiopterin synthesis or metabolism, the synthesis of serotonin is severely impaired.

The availability of tryptophan in the central nervous system controls the synthesis of serotonin. The normal range of brain tryptophan concentration is 10–30 $\mu\text{mol/l}$ in different regions, while the K_m of the hydroxylase is around 50 $\mu\text{mol/l}$. Thus, the rate of hydroxylation and, hence, serotonin synthesis will vary with the availability of tryptophan. Tryptophan is transported into the brain by the same transporter as the other large neutral amino acids (phenylalanine, tyrosine, leucine, isoleucine and valine), and high plasma concentrations of these competing amino acids reduce tryptophan availability for serotonin synthesis.

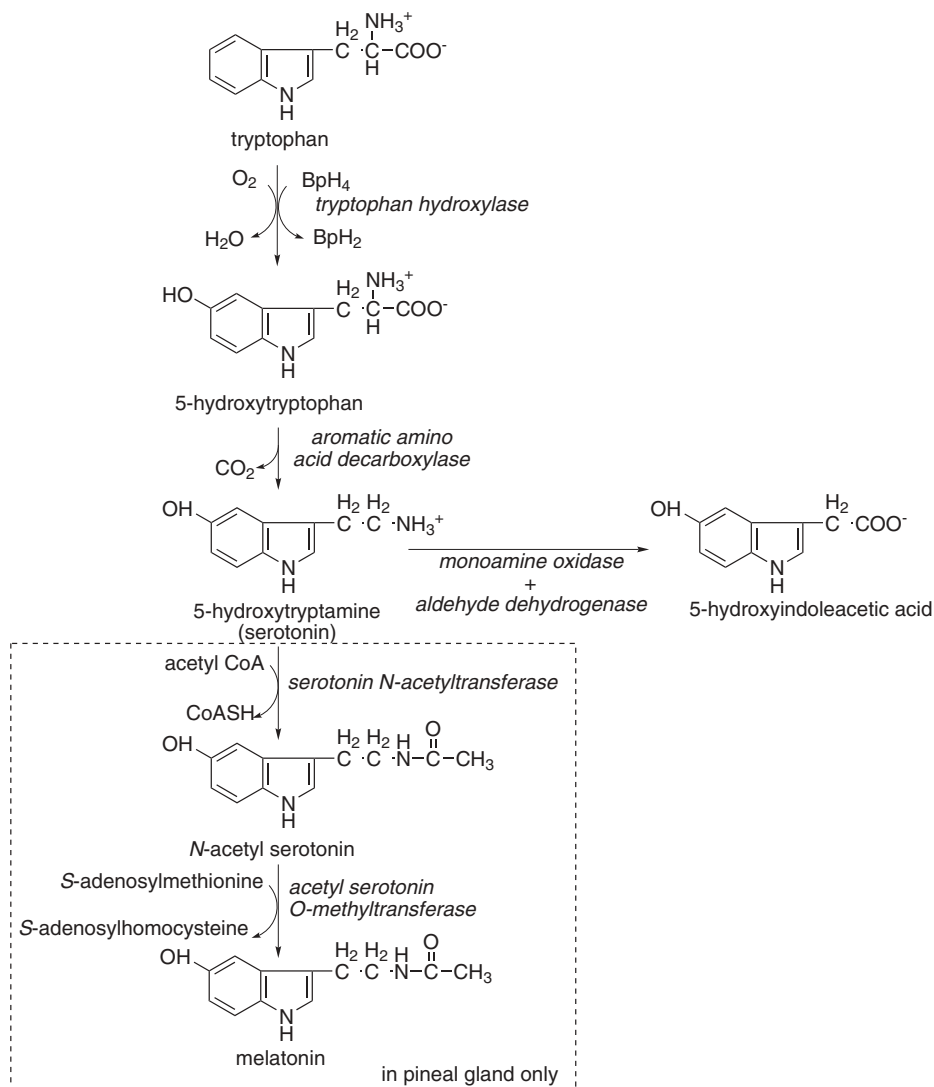


Figure 9.15 The biosynthesis of serotonin and melatonin.

Tryptophan hydroxylase EC 1.14.16.4, aromatic amino acid decarboxylase EC 4.1.1.28, monoamine oxidase EC 1.4.3.4, aldehyde dehydrogenases EC 1.2.1.3–5, serotonin acetyltransferase EC 2.3.1.87, acetylserotonin O-methyltransferase EC 2.1.1.4.

Tryptophan is transported in the bloodstream bound non-covalently to serum albumin. Non-esterified fatty acids and drugs that are transported bound to serum albumin (such as salicylates) displace tryptophan and increase its uptake into the central nervous system. In addition, unlike phenylalanine and tyrosine hydroxylases, tryptophan hydroxylase is subject to end-product

inhibition by serotonin (Boadle-Biber, 1993; Fernstrom, 1983; McMenamy & Oncley, 1958).

There are two genes for tryptophan hydroxylase. Type I is expressed in the peripheral tissues including the gut, spleen and thymus, as well as the central nervous system and pineal gland; type II is expressed only in the central nervous system and brain stem. Knockout mice lacking type I hydroxylase are still able to synthesize serotonin in the central nervous system, but not peripherally or in the pineal gland.

Like phenylalanine and tyrosine hydroxylases, tryptophan hydroxylase is activated by phosphorylation of the regulatory domain in response to hormonal and neurotransmitter signals. Phosphorylation of tryptophan hydroxylase I also increases its susceptibility to proteasomal degradation. The two tryptophan hydroxylases have considerable sequence homology and similar kinetics. The main difference, apart from their tissue distribution, is that the type II enzyme has a larger regulatory domain and is subject to different regulation (Matthes *et al.*, 2010; Walther & Bader, 2003).

Serotonin is formed from 5-hydroxytryptophan by decarboxylation, catalyzed by the same aromatic amino acid decarboxylase as is involved in catecholamine synthesis (section 9.2.4). It is inactivated by oxidation catalyzed by monoamine oxidase and aldehyde dehydrogenase (section 9.2.4.2), yielding 5-hydroxyindoleacetic acid.

In plants, serotonin has a signalling role in flowering, morphogenesis and senescence. It is also synthesized in response to fungal infection (e.g. of rice with *Bipolaris oryzae*) and is incorporated into the cell walls of lesion tissue, together with lignin (section 9.2.1). The brown spots of infected leaves are due to oxidative polymerization products of serotonin, and mutant plants defective in serotonin synthesis are more susceptible to fungal infection.

In plants, serotonin is synthesized by decarboxylation of tryptophan, followed by hydroxylation of the resultant tryptamine. Unlike tryptophan hydroxylase, plant tryptamine hydroxylase is a cytochrome P₄₅₀-dependent enzyme (Fujiwara *et al.*, 2010; Ishihara *et al.*, 2008).

9.4.3.1 Melatonin synthesis and catabolism Two reactions are involved in melatonin synthesis from serotonin: *N*-acetylation and methylation. Serotonin *N*-acetyltransferase is activated by a cAMP-dependent protein kinase, and its activity peaks at night. The phosphorylated enzyme is also more resistant to proteasomal degradation. In rodents, cAMP also leads to increased transcription of the *N*-acetyltransferase gene, although there is no change in mRNA in primates and other animals. Pineal tryptophan hydroxylase is also activated by cAMP-dependent phosphorylation, so that there is a night-time surge in serotonin production to provide a substrate for *N*-acetylation. Under these conditions the rate-limiting factor in melatonin synthesis is the activity of the methyltransferase and there is accumulation of *N*-acetylserotonin in

the pineal at night, although the activity of the methyltransferase also increases at night. The methyltransferase is rapidly inactivated after exposure to light, as a result of a fall in cAMP, a conformational change in the enzyme and proteasomal degradation (Chattoraj *et al.*, 2009; Iuvone *et al.*, 1997; Klein, 2007; Klein *et al.*, 1997).

About 15 per cent of melatonin is excreted unchanged, and 70 per cent as sulphate or glucuronate conjugates. The remainder is oxidized by myeloperoxidase or indoleamine dioxygenase (section 9.4.4) to yield a kynurenine analogue (Ferry *et al.*, 2005).

9.4.4 The kynurenine pathway of tryptophan metabolism

The main pathway of tryptophan catabolism in prokaryotes is the kynurenine pathway, leading to formation of acetyl CoA and acetoacetyl CoA. An intermediate in the pathway, aminomuconic semialdehyde is also the precursor for *de novo* synthesis of the nicotinamide ring of NAD and NADP (section 9.4.4.4).

Kynureninase is a pyridoxal phosphate-dependent enzyme that is exquisitely sensitive to vitamin B₆ depletion, and the ability to metabolize a test dose of tryptophan has been used as an index of vitamin B₆ nutritional status (section 9.4.4.3). In insects and crustaceans, kynurenine and 3-hydroxykynurenine are the precursors for synthesis of ommochrome pigments that determine eye colour.

As shown in Figure 9.16, the first step in tryptophan catabolism in mammals is oxidative cleavage of the indole ring to yield *N*-formylkynurenine. This reaction is catalyzed by two enzymes – tryptophan dioxygenase (also known as tryptophan pyrrolase) and indoleamine dioxygenase. Both are haem enzymes but, while tryptophan dioxygenase is specific for L-tryptophan and uses molecular oxygen as the oxidizing agent, indoleamine dioxygenase has a broader specificity for indole derivatives (including D-tryptophan, 5-hydroxytryptophan, tryptamine, serotonin and melatonin) and uses superoxide.

Tryptophan dioxygenase is mainly confined to the liver, although it is expressed in the central nervous system and some other tissues; indoleamine dioxygenase is expressed in all tissues except the liver. Tryptophan dioxygenase is a tetrameric enzyme that shows cooperative substrate binding, while indoleamine dioxygenase is a monomer with hyperbolic kinetics (Rafice *et al.*, 2009).

The function of tryptophan dioxygenase is hepatic catabolism of tryptophan and synthesis of the nicotinamide ring of NAD and NADP. The function of indoleamine dioxygenase is to regulate the availability of tryptophan in extrahepatic tissues; local depletion of tryptophan can have antimicrobial

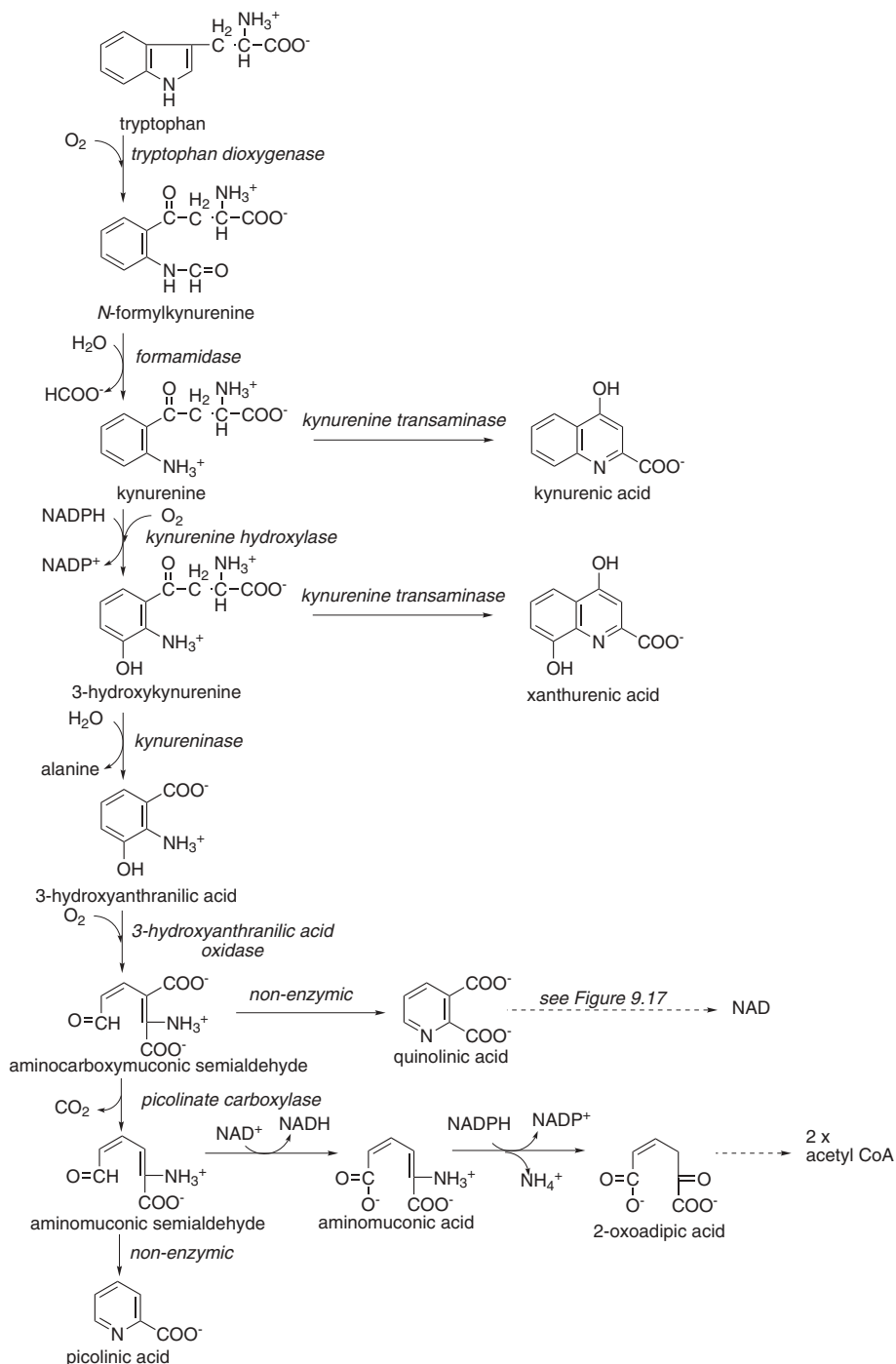


Figure 9.16 The kynurenine pathway of tryptophan catabolism.

Tryptophan dioxygenase EC 1.13.11.11 indoleamine dioxygenase EC 1.13.11.52, formylkynurenine formamidase EC 3.5.1.9, kynurenine hydroxylase EC 1.14.13.9, kynureninase EC 3.7.1.3, 3-hydroxyanthranilic acid oxidase EC 1.10.3.5, picolinate carboxylase EC 4.1.1.45, kynurenine-oxoglutarate transaminase EC 2.6.1.7, kynurenine glyoxylate transaminase EC 2.6.1.63.

actions. The kynurenine produced has paracrine actions on adjacent cells that lack the enzyme, including T-lymphocytes, where kynurenine inhibits proliferation and differentiation, leading to immunological tolerance. This immune tolerance leads to escape of cancer cells from immune surveillance, and inhibitors of indoleamine dioxygenase that do not inhibit tryptophan dioxygenase are used in cancer chemotherapy.

In pregnancy, placental indoleamine dioxygenase is involved in maternal immune tolerance of the foetus. In macrophages and dendritic cells, both indoleamine dioxygenase and tryptophanyl-tRNA synthase are induced by interferon- γ , so providing protection against tryptophan depletion in the cell (by enhancing tryptophan use for protein synthesis) while depleting surrounding cells of tryptophan (Boasso *et al.*, 2005; Capece *et al.*, 2010; Mellor & Munn, 2004).

There are two isoenzymes of indoleamine dioxygenase that differ in inhibitor sensitivity and tissue distribution. Indoleamine dioxygenase 1 is induced by interferon- γ . Indoleamine dioxygenase 2, is induced in response to some cytokines, but not interferon- γ (Ball *et al.*, 2009).

The product of tryptophan dioxygenase or indoleamine dioxygenase, *N*-formylkynurenine, is hydrolyzed to kynurenine and formate by formamidase, an enzyme that acts on a variety of amides and some esters. Its catalytic site is similar to that of enzymes in the esterase/lipase family rather than other amidases, and like esterases it is inhibited by organophosphorus insecticides.

Knockout mice lacking formamidase still have some activity towards *N*-formylkynurenine in the kidneys, suggesting that there is a second (renal) enzyme in addition to the established formamidase. Under normal conditions, the activity of formamidase is considerably greater than that of tryptophan dioxygenase; thus, unless highly purified preparations of the dioxygenase are used, the first product that can be measured is kynurenine (Dobrovolsky *et al.*, 2005; Pabarcus & Casida, 2002).

9.4.4.1 Regulation of tryptophan dioxygenase Tryptophan dioxygenase was the first mammalian enzyme to be shown to be inducible. The enzyme has a short half-life (of the order of two hours) and three distinct mechanisms are involved in its regulation: induction by glucocorticoid hormones; increased translation of mRNA in response to glucagon; and stabilization and activation of the enzyme protein by tryptophan. These three effects are additive.

Glucocorticoid hormones lead to an increase in both tryptophan dioxygenase mRNA and protein, and their action is inhibited by both actinomycin as an inhibitor of transcription and cycloheximide as an inhibitor of translation. However, the effect of glucagon (or dibutyryl cAMP) leads to no change in mRNA, and it is unaffected by cycloheximide. The actions of both hormones are down-regulated in the presence of insulin (Nakamura *et al.*, 1980).

Early studies, based largely on changes in tryptophan metabolism in response to high oestrogen oral contraceptive agents (section 9.4.4.3), suggested that tryptophan dioxygenase is also induced by oestrogens. However, although ovariectomy leads to a decrease in enzyme activity in the liver, administration of oestrogens does not lead to any increase (Bender *et al.*, 1983).

Tryptophan enhances the effects of glucocorticoids and glucagon on liver tryptophan dioxygenase, and the amount of the enzyme protein is increased in the presence of tryptophan. This is not induction, although early papers referred to the effect of tryptophan on the action of the hormones as 'super-induction'. Rather, it is the result of stabilization of the enzyme protein against degradation, as a result of binding to a site other than the catalytic site. While only the oxidation of tryptophan is catalyzed by the enzyme, α -methyltryptophan and a number of other analogues, which are not substrates, also act to stabilize the enzyme protein. Binding of tryptophan or analogues at the second site also leads to increased activity of the enzyme *in vitro*, changing the substrate/velocity curve from the sigmoid (allosteric) curve seen with tryptophan alone to a hyperbola (Feigelson & Maeno, 1967; Schimke *et al.*, 1965a, 1965b).

This stabilization of the enzyme by tryptophan means that when more tryptophan is available, there is more active enzyme to catalyze its catabolism. As a result, when tryptophan is used as a precursor for serotonin synthesis as antidepressant medication, there is a narrow therapeutic window. Too little tryptophan has no beneficial effect on serotonin synthesis, but too much leads to increased catabolism through the kynurenine pathway, so that less is available for serotonin synthesis. Conversely, part of the action of some antidepressant drugs is down-regulation of tryptophan dioxygenase, so that more tryptophan is available for serotonin synthesis (Bano *et al.*, 2010).

Under basal conditions, there is normally a relatively large amount of tryptophan dioxygenase in the liver as the inactive apo-enzyme, which can be activated by administration of haem or haem precursors *in vivo*, or by the addition of haematin to *in vitro* incubations. This may be important in the psychiatric problems associated with porphyria (section 4.8.1), since increased activity of tryptophan dioxygenase in response to the increased availability of haem will lead to depletion of body pools of tryptophan and, hence, decreased serotonin synthesis.

When adrenalectomized animals are treated with glucocorticoids, there is a considerably greater induction of 5-aminolevulinate synthase (the first enzyme in haem biosynthesis – see section 4.8) by allyl isopropylacetamide (a porphyrogenic drug) than when glucocorticoids are not given. It has been suggested that this is the result of induction of tryptophan dioxygenase by the glucocorticoid, providing a sink for newly synthesized haem and thus

reducing the feedback inhibition of 5-aminolevulinate synthase by its end-product (Badawy & Morgan, 1980; Morgan & Badawy, 1980).

9.4.4.2 Kynurenine metabolism Kynurenine may undergo hydroxylation to 3-hydroxykynurenine, which is a substrate for onward metabolism to yield 3-hydroxyanthranilic acid by hydrolysis to remove the side-chain as alanine. Kynurenine hydroxylase is a mitochondrial outer membrane flavoprotein that uses NADPH as reductant. Alternatively, kynurenine may be a substrate for kynureninase, yielding anthranilic acid. Although anthranilic acid may be a substrate for hydroxylation to 3-hydroxyanthranilic acid, at least in rats, anthranilic acid is not a precursor for NAD synthesis. However, in the brain, both 3-hydroxykynurenine and anthranilic acid are substrates for formation of 3-hydroxyanthranilic acid.

In eukaryotes, kynureninase acts primarily on 3-hydroxykynurenine, with 100-fold lower activity towards kynurenine. By contrast, in prokaryotes, the enzyme acts primarily on kynurenine, with little activity towards 3-hydroxykynurenine. Some organisms (especially fungi) have both types of kynureninase (Baran & Schwarcz, 1990; Bender & McCreanor, 1982).

Both kynurenine and 3-hydroxykynurenine are substrates for transamination to yield kynurenic and xanthurenic acids respectively. Under basal conditions, there is little transamination in the liver. It is only when there is increased flux through the pathway as a result of induction of tryptophan dioxygenase (section 9.4.4.1), or the activity of kynureninase is impaired by inhibition or in vitamin B₆ deficiency (section 9.4.4.3), that kynurenic and xanthurenic acids are formed to any significant extent.

At least four different enzymes catalyze the transamination of kynurenine and 3-hydroxykynurenine. The main two are kynurenine transaminases linked to amination of 2-oxoglutarate and glyoxylate respectively, but aminoadipate transaminase and cysteine conjugate β -lyase also have kynurenine transaminase activity (Han *et al.*, 2010a, 2010b; Rossi *et al.*, 2008).

Kynurenine transamination is important in the brain, where kynurenic acid acts as an antagonist of the NMDA-sensitive glutamate receptor. Quinolinic acid (Figure 9.16) is an excitotoxin, acting to over-stimulate the NMDA-sensitive glutamate receptor and leading to neuronal damage, and kynurenic acid is neuroprotective. The brain damage in strokes, Alzheimer's disease and Parkinsonism, as well as in response to trauma, is at least partly due to over-stimulation of the NMDA-sensitive glutamate receptor (Erhardt *et al.*, 2009; Nemeth *et al.*, 2005; Stone, 2000, 2001).

3-Hydroxyanthranilic acid is oxidized to aminocarboxymuconic semialdehyde. This may undergo enzymic decarboxylation to aminomuconic semialdehyde and onward metabolism to acetyl CoA, or non-enzymic cyclization to quinolinic acid, the precursor for NAD synthesis (section 9.4.4.4).

Aminomuconic semialdehyde may also undergo non-enzymic cyclization to picolinic acid, which has immunomodulatory actions, including activation of macrophages.

Aminocarboxymuconic semialdehyde decarboxylase (also known as picolinate carboxylase) is a zinc metalloenzyme, and it was the first example of a non-oxidative decarboxylation catalyzed by a metalloenzyme. Although, structurally, the enzyme belongs to the amidohydrolase super-family of enzymes, the reaction involves the non-hydrolytic cleavage of a carbon-carbon bond. The enzyme is up-regulated in response to a high-protein diet and in streptozotocin-diabetic animals (but down-regulated when insulin is given), and it is down-regulated by polyunsaturated fatty acids. The non-enzymic synthesis of quinolinic acid is decreased when the enzyme is upregulated, and is increased when the enzyme is down-regulated (Egashira *et al.*, 2007; Li, T *et al.*, 2007; Pucci *et al.*, 2007).

9.4.4.3 Kynureninase and the tryptophan load test for vitamin B₆ nutritional status In vitamin B₆ deficiency, the activity of kynureninase (a pyridoxal phosphate-dependent enzyme) is significantly impaired, and kynurenine and hydroxykynurenine accumulate in the liver and undergo transamination to kynurenic and xanthurenic acids. All four compounds are excreted in the urine in increased amounts, especially after a test dose of 2–5 g of tryptophan. This tryptophan load test was one of the first metabolic tests for functional vitamin nutritional status to be developed, and it has been widely used to estimate vitamin B₆ requirements in depletion/repletion studies. It has also been used to assess vitamin B₆ status in epidemiological studies but, as discussed below, it is not reliable in such studies.

Kynureninase is especially sensitive to vitamin B₆ depletion, because it undergoes the half-reaction of transamination, leaving pyridoxamine phosphate at the catalytic site (section 3.4.1). Since it does not have a substrate-binding site for an oxo-acid, this inactivates the enzyme. However, it can be reactivated by relatively high concentrations of pyridoxal phosphate since, unlike pyridoxal phosphate, pyridoxamine phosphate is not bound as the internal aldimine. In vitamin B₆ deficiency, the activity of kynureninase is lower than that of tryptophan dioxygenase, and this now becomes the rate-limiting step in tryptophan metabolism.

A number of studies have shown that, in patients suffering from a wide variety of different diseases, there is impaired metabolism of a test dose of tryptophan, with increased excretion of kynurenine metabolites suggestive of vitamin B₆ deficiency. Liver biopsy shows elevated tryptophan dioxygenase activity, presumably as a result of induction of the enzyme by glucocorticoid hormones secreted in response to the stress of illness. Under these conditions, the activity of tryptophan dioxygenase exceeds that of kynureninase, and kynurenine and hydroxykynurenine accumulate and undergo transamina-

tion. The apparent vitamin B₆ deficiency is thus an artefact resulting from induction of tryptophan dioxygenase (Altman & Greengard, 1966; Coon & Nagler, 1969).

Studies in the 1960s of women taking high-dose oestrogen oral contraceptive agents suggested that oestrogens cause vitamin B₆ deficiency. There was increased excretion of kynurenine metabolites after a test dose of tryptophan, which was only normalized by doses of about 25 mg of vitamin B₆, compared with a reference intake of 1.4–1.7 mg/day (Rose, 1966a, 1966b). However, when other indices of vitamin B₆ status were measured (plasma concentrations of the vitamin or the activation of plasma transaminases by pyridoxal phosphate added *in vitro* – see section 3.3), there was no evidence of vitamin B₆ depletion caused by oestrogens.

The abnormal tryptophan metabolism is not due to oestrogen-induced vitamin B₆ deficiency, but simple competitive inhibition of kynureninase by oestrogen conjugates. While modern low-dose oestrogen oral contraceptives do not impair tryptophan metabolism, the gender difference in the incidence of pellagra (section 9.4.5) again suggests that the tryptophan load test may not be appropriate for epidemiological studies (Bender, 1987; Bender & Wynick, 1981).

9.4.4.4 *De novo* synthesis of NAD *De novo* synthesis of NAD from tryptophan occurs only in liver and kidney; other tissues are reliant on nicotinamide released from the liver. Although pellagra (section 9.4.5) is generally regarded as a niacin deficiency disease, *de novo* synthesis from tryptophan is quantitatively more important than utilization of dietary nicotinic acid and nicotinamide (Bender, 1996).

The competition between the non-enzymic cyclization of aminocarboxymuconic semialdehyde to quinolinic acid and the enzyme-catalyzed decarboxylation to aminomuconic semialdehyde for complete oxidation (Figure 9.16) means that *de novo* synthesis of NAD from tryptophan depends on the rate of flux through the kynurenine pathway. At low flux, there is little aminocarboxymuconic semialdehyde available for the non-enzymic reaction, and most will be metabolized to acetyl CoA. At higher flux, aminocarboxymuconic semialdehyde decarboxylase approaches saturation and there is now more aminocarboxymuconic semialdehyde to undergo non-enzymic cyclization to quinolinic acid for NAD synthesis. In turn, this means that the availability of tryptophan, induction of tryptophan dioxygenase by hormones (section 9.4.4.1), inhibition of kynureninase (e.g. by oestrogen metabolites, section 9.4.4.3) or impairment of kynureninase in vitamin B₆ deficiency or of kynurenine hydroxylase in riboflavin deficiency, will all affect the amount of NAD synthesized from tryptophan, and hence the equivalence of dietary tryptophan and preformed niacin (nicotinic acid and nicotinamide).

Conventionally, it is assumed that 60 mg of tryptophan is equivalent to 1 mg of preformed dietary niacin, but this equivalence can vary widely depending on factors that affect the kynurenine pathway. Cats, which have a very high activity of aminocarboxymuconic semialdehyde decarboxylase (some 30–50 times more than other animals) form little or no NAD from tryptophan (Horwitt *et al.*, 1981; Ikeda *et al.*, 1965; Sanada & Miyazaki, 1980).

As shown in Figure 9.17, nicotinic acid mononucleotide can be synthesized from either quinolinic acid or nicotinic acid, in the reactions catalyzed by quinolinate phosphoribosyltransferase and nicotinic acid phosphoribosyltransferase. The immediate product of quinolinate phosphoribosyltransferase is quinolinic acid mononucleotide, which undergoes rapid non-enzymic decarboxylation to nicotinic acid mononucleotide. Nicotinic acid mononucleotide adenyllyltransferase acts on both nicotinic acid mononucleotide and nicotinamide mononucleotide. NAD synthase catalyzes the amidation of nicotinic acid adenine dinucleotide to NAD. There are two enzymes, one using ammonium as the amide donor and the other glutamine.

Like other glutamine-dependent amidotransferases (section 5.3.3), NAD synthase has separate glutaminase and amidotransferase sites, with an intramolecular tunnel between them for transfer of ammonia (Hara *et al.*, 2003; LaRonde-LeBlanc *et al.*, 2009; Magni *et al.*, 2004).

Nicotinamide may either be deamidated to nicotinic acid or be incorporated into NAD directly, by the actions of nicotinamide phosphoribosyltransferase and nicotinic acid mononucleotide adenyllyltransferase. Nicotinamide phosphoribosyltransferase is the same as the adipocytokine visfatin, which is secreted by adipocytes and has insulin-mimetic activity. It also enhances the maturation of pancreatic β -islet cell precursors (Sonoli *et al.*, 2011).

9.4.5 Pellagra

Pellagra is the tryptophan and niacin deficiency disease. It is characterized by a photosensitive dermatitis, like severe sunburn, typically with a butterfly-like pattern of distribution over the face, affecting all parts of the skin that are exposed to sunlight. Similar skin lesions may also occur in areas not exposed to sunlight but subject to pressure, such as the knees, elbows, wrists and ankles. Advanced pellagra is also accompanied by a dementia or depressive psychosis, and there may be diarrhoea.

Untreated pellagra is fatal. It was a major problem of public health in the early part of the 20th century, and continued to be a problem until the 1980s in some parts of the world. It is now rare, although occasional cases are reported in alcoholics in developed countries, as well as among people being treated with isoniazid and some other drugs.

Although the nutritional aetiology of pellagra is well established, and additional tryptophan or niacin will prevent or cure the disease, there are a

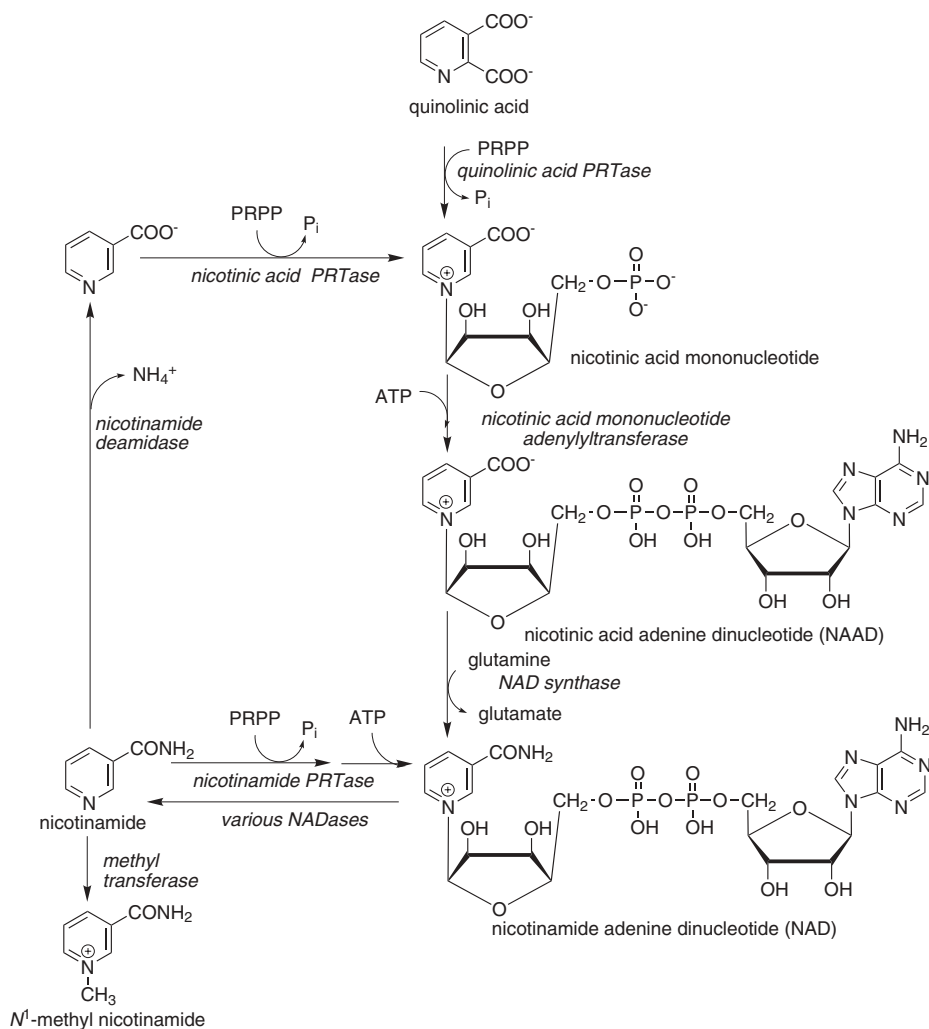


Figure 9.17 Biosynthesis of NAD.

Quinolinic acid phosphoribosyltransferase EC 2.4.2.19, nicotinic acid mononucleotide adenylyltransferase EC 2.7.7.18, NAD synthase EC 6.3.1.5 (utilizing ammonia) EC 6.3.5.1 (utilizing glutamine), nicotinamide phosphoribosyltransferase EC 2.4.2.12, nicotinamide mononucleotide adenylyltransferase EC 2.7.7.1, NAD glycohydrolase (NADase) EC 3.2.2.5, ADP-ribosyltransferase EC 2.4.2.31 and 2.4.2.36, poly(ADP-ribose)polymerase EC 2.4.2.30, nicotinamide deamidase EC 3.5.1.19, nicotinic acid phosphoribosyltransferase EC 2.4.2.11, nicotinamide N-methyltransferase EC 2.1.1.1.

number of reports that suggest that additional factors may be involved. Carpenter & Lewin (1985) re-examined the diets associated with the development of pellagra in the USA during the early part of the 20th century, and they showed that the total intake of tryptophan and niacin was apparently adequate as judged by current knowledge of requirements. They suggested

that deficiency of riboflavin (and hence impaired activity of kynurenine hydroxylase) or vitamin B₆ (and hence impaired activity of kynureninase) may have been significant factors in the aetiology of pellagra when intakes of tryptophan and niacin were only marginally adequate. Iron deficiency may also be a factor, since impairment of haem synthesis will both reduce the activity of tryptophan dioxygenase and also increase its susceptibility to proteolysis (Oduho *et al.*, 1994).

9.4.5.1 The pellagrigenic effect of excess dietary leucine Pellagra was a major problem in parts of India, where jowar (*Sorghum vulgare*) is the dietary staple, despite the fact that the tryptophan content of sorghum proteins is higher than that of maize, the cereal traditionally associated with endemic pellagra. The intake of tryptophan and niacin was as great among people whose dietary staple was jowar as that of rice eaters, yet pellagra was common among jowar eaters but not among rice-eating communities. It was suggested that the relatively high content of leucine in the proteins of jowar might be a contributory factor.

A number of studies have demonstrated a pellagrigenic effect of excess dietary leucine in experimental animals and in human beings (Gopalan & Rao, 1975), although other studies failed to show any effect (Manson & Carpenter, 1978a, 1978b). Magboul & Bender (1983) showed that when rats were fed on diets that were only marginally adequate with respect to tryptophan and niacin, the addition of 1.5 per cent of leucine led to a significant depletion of liver and blood nicotinamide nucleotides. This effect was only apparent when the niacin content of the diet was such that it provided less than half the minimum requirement, and it was most marked when the diets provided virtually no preformed niacin, suggesting that the effect of leucine is on the metabolism of tryptophan, and not on the utilization of niacin.

Studies with [¹⁴C]tryptophan in animals and isolated hepatocytes show that leucine does, indeed, inhibit the synthesis of NAD from tryptophan, inhibiting metabolism at the level of kynurenine hydroxylase and kynureninase and causing the accumulation of intermediates. In isolated hepatocytes, the more important effect seems to be at the level of uptake of tryptophan into the cells; leucine and tryptophan share a common transport mechanism with the other large neutral amino acids (Bender, 1983, 1989a; Salter *et al.*, 1985).

It is likely that leucine is only a factor in the aetiology of pellagra when the dietary intakes of both tryptophan and niacin are extremely low – a condition that may occur when sorghum is the dietary staple, especially at times of food shortage.

9.4.5.2 Inborn errors of tryptophan metabolism A number of inborn errors of metabolism of the tryptophan oxidative pathway (Figure 9.16) have

been reported, all of which result in the development of pellagra, which responds to high doses of niacin. These conditions include:

- vitamin B₆ responsive xanthurenic aciduria, due to a defect of kynureninase;
- hydroxykynureninuria, apparently due to a defect of kynureninase;
- tryptophanuria, apparently due to tryptophan dioxygenase deficiency;
- a hereditary pellagra-like condition, apparently due to increased activity of picolinate carboxylase; and
- Hartnup disease.

Hartnup disease is a rare genetic condition, in which there is a defect of the membrane transport mechanism for tryptophan and other large neutral amino acids. The result is that the intestinal absorption of free tryptophan is impaired, although dipeptide absorption is normal. There is a considerable urinary loss of tryptophan (and other amino acids) as a result of failure of the normal re-absorption mechanism in the renal tubules – renal aminoaciduria. In addition to neurological signs that can be attributed to a deficit of tryptophan for the synthesis of serotonin in the central nervous system, the patients show clinical signs of pellagra, which respond to the administration of niacin (Baron *et al.*, 1956; Broer, 2009).

9.4.5.3 Carcinoid syndrome Carcinoid is a tumour of the enterochromaffin cells that normally synthesize 5-hydroxytryptophan and 5-hydroxytryptamine. The carcinoid syndrome is seen when there are significant metastases of the primary tumour in the liver. It is characterized by much increased gastrointestinal motility and diarrhoea, as well as regular periodic flushing due to vasodilatation. These symptoms can be attributed to the systemic release of large amounts of serotonin, and they can be controlled with inhibitors of tryptophan hydroxylase such as *p*-chlorophenylalanine.

The synthesis of 5-hydroxytryptamine in advanced carcinoid syndrome may be so great that as much as 60 per cent of the body's tryptophan metabolism proceeds by this pathway, compared with about one per cent under normal conditions. A significant number of patients with advanced carcinoid syndrome develop clinical signs of pellagra because of this diversion of tryptophan away from the oxidative pathway of metabolism.

9.4.5.4 Drug-induced pellagra The anti-tuberculosis drug isoniazid (*isonicotinic acid hydrazide*) can cause pellagra by forming a biologically inactive

complex with pyridoxal phosphate, so reducing the activity of kynureninase. This isoniazid-induced pellagra responds to the administration of niacin supplements. However, isoniazid may also cause peripheral neuropathy, which responds to vitamin B₆ and not niacin, and therefore it became usual to give vitamin B₆ supplements together with isoniazid.

During the 1960s, the dose of isoniazid used in the treatment of tuberculosis was reduced considerably as a result of the introduction of other effective anti-mycobacterial agents in therapeutic cocktails, so it was no longer thought necessary to give patients supplements of vitamin B₆. There have been some reports of the development of pellagra in patients treated with relatively low doses of isoniazid. Most patients were of Indian origin, and it is likely that they were genetically slow acetylators of isoniazid, so that an apparently low dose of the drug was, in fact, high. Up to 60 per cent of Indians are slow acetylators of isoniazid.

9.5 Quinone cofactors in amine oxidases

Although monoamine oxidase (section 9.2.4.2) is a flavoprotein, the copper-dependent amine oxidases (including lysyl oxidase (section 6.2.4), plasma amine oxidase and diamine oxidase (histaminase – see section 8.3.3)) that are sensitive to inhibition by semicarbazide have a variety of quinone cofactors. They are formed by post-synthetic modification of tyrosine or tryptophan residues in the precursor proteins. The first quinone cofactor to be identified was pyrroloquinoline quinone (PQQ), in bacterial alcohol and glucose dehydrogenases. It is not a cofactor for any eukaryotic enzyme, and is bound to the enzymes non-covalently.

The other quinone cofactors (shown in Figure 9.18) are formed autocatalytically by copper-dependent oxidation of tyrosine (trihydroxyphenylalanine quinone, TPQ, and lysyltyrosyl quinone, LTQ) or tryptophan (tryptophan tryptophylquinone, TTQ, and cysteine tryptophylquinone, CTQ) residues in the precursor proteins. The copper-dependent amine oxidases have two separate activities: the precursor protein catalyzes a single turnover reaction for the oxygen-dependent formation of the quinone prosthetic group, then the active holoenzyme catalyzes oxidation of the amine substrate (Dubois & Klinman, 2005).

The first stage of the copper-dependent amine oxidase reaction is oxidative deamination of the substrate amine to the aldehyde, via intermediate formation of a Schiff base to the quinone, with reduction of the enzyme. The second reaction is re-oxidation of the enzyme by oxygen, generating hydrogen peroxide. The copper does not have a role in the reductive first half-reaction; it is unclear whether it has a redox role in the oxidative phase, or whether it binds and activates oxygen (Dooley, 1999; Klinman, 2003; Mure, 2004).

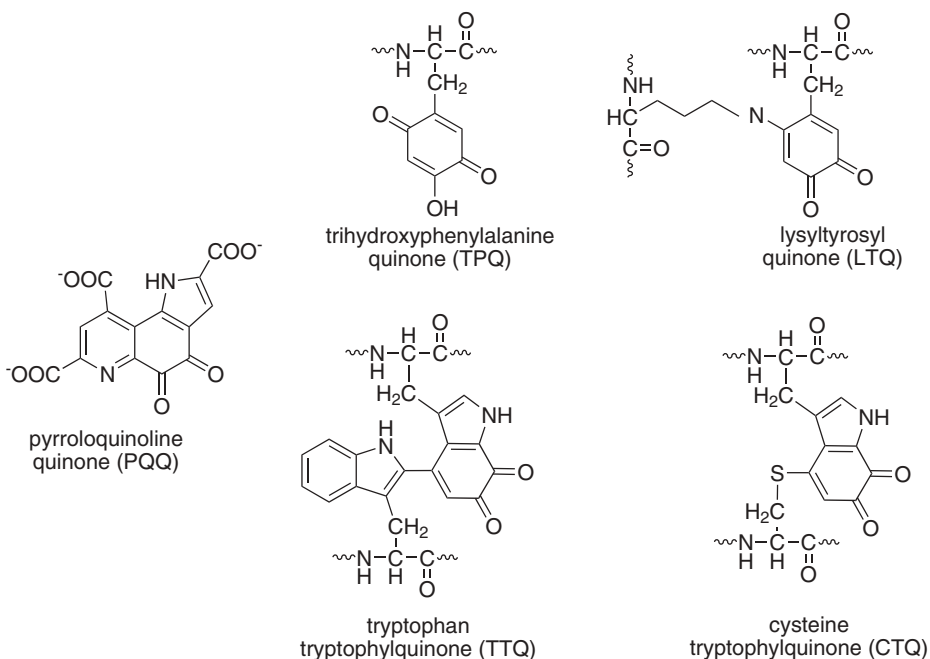


Figure 9.18 Quinone cofactors formed by post-synthetic modification of proteins.

Further reading

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

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