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THE BIOSYNTHESIS AND METABOLISM OF THE ASPARTATE DERIVED AMINO ACIDS IN HIGHER PLANTS

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Key Word Index—Aspartate pathway; isoleucine, lysine, methionine and threonine synthesis and catabolism; metabolic and gene regulation.

Abstract—The essential amino acids lysine, threonine, methionine and isoleucine are synthesised in higher plants via a common pathway starting with aspartate. The regulation of the pathway is discussed in detail, and the properties of the key enzymes described. Recent data obtained from studies of regulation at the gene level and information derived from mutant and transgenic plants are also discussed. The herbicide target enzyme acetohydroxyacid synthase involved in the synthesis of the branched chain amino acids is reviewed.
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INTRODUCTION

Of the 20 amino acids normally incorporated into protein, only 11 can be synthesised by adult animals. The remaining nine amino acids, which have been termed 'essential' have to be provided in the diet. Plants and the majority of bacteria and fungi have the capacity of synthesise all twenty amino acids provided they are supplied with an adequate carbon and inorganic nitrogen source. Four of the amino acids, lysine, threonine, methionine and isoleucine are synthesised via a branched pathway with aspartate as the precursor (Fig. 1).

The major plant sources of protein are cereal and legume seeds. It is perhaps unfortunate that cereals are deficient in lysine and threonine [1, 2] and legumes are deficient in methionine [3, 4]. Supplementation of plant seeds with essential amino acids has been shown to greatly increase their nutritive value [5]. Due to the deficiencies noted above, there has been considerable interest in studying the pathway of the conversion of aspartate to lysine, threonine and methionine. More recently the conversion of threonine to isoleucine has been the subject of a number of detailed investigations, when it became apparent that it was the target site of a number of extremely potent herbicides [6, 7].

In this review article, we will describe in detail the properties of the enzymes in the pathway and discuss the mechanism by which the synthesis of the end product amino acids is regulated. Particular emphasis will be placed on the use of mutant and transgenic plants.

The aspartate pathway outlined in Fig. 1 has been subject to a number of previous review articles [8–11]. However, confirmation of the pathway, in particular the route of methionine synthesis has come from the definitive studies of Giovanelli and his colleagues [12], that culminated with a paper that described the regulatory structure of the biosynthetic pathway of the aspartate family of amino acids in the water growing plant *Lemna paucicostata* Hegelm 6746 [13]. Giovanelli *et al.* [13] followed the metabolism of [^{14}C]-aspartate into both protein and soluble lysine, threonine, methionine and isoleucine in the presence of the individual amino acids and a number of different combinations. A number of important conclusions were obtained from this work:

1. Aspartate kinase (also known as aspartokinase) was not a major factor in the regulation of the flux through the pathway. The authors concluded from *in vitro* studies that the enzyme was present in a 42-fold excess of the *in vivo* requirements.

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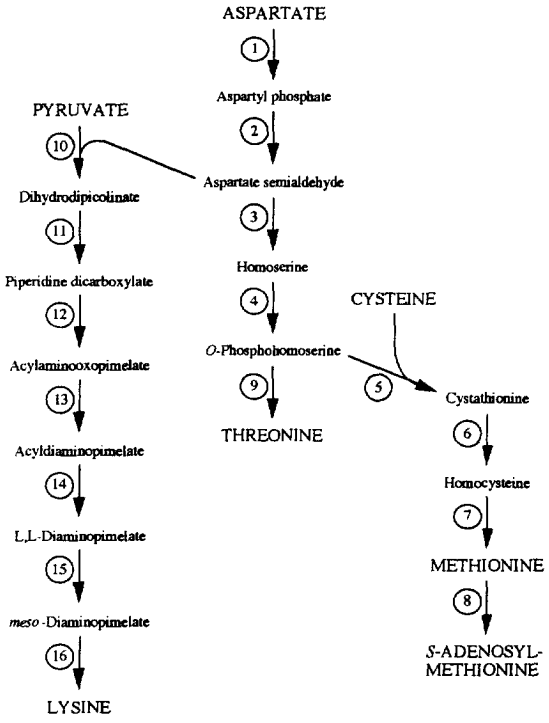

Fig. 1. The aspartate pathway: biosynthesis of lysine, threonine, methionine and *S*-adenosylmethionine. Enzymes: (1) aspartate kinase (EC 2.7.2.4); (2) aspartate-semialdehyde dehydrogenase (EC 1.2.1.11); (3) homoserine dehydrogenase (EC 1.1.1.3); (4) homoserine kinase (EC 2.7.1.39); (5) cystathionine γ-synthase (EC 4.2.99.9); (6) cystathionine β-lyase (EC 4.4.1.8); (7) methionine synthase (EC 2.1.1.13); (8) *S*-adenosylmethionine synthetase (EC 2.5.1.6); (9) threonine synthase (EC 4.2.99.2); (10) dihydrodipicolinate synthase (EC 4.2.1.52); (11) dihydrodipicolinate reductase (EC 1.3.1.26); (12) piperidine dicarboxylate acylase (EC 2.3.1.-); (13) acyldiaminopimelate aminotransferase (EC 2.6.1.17); (14) acyldiaminopimelate deacylase (EC 3.5.1.18); (15) diaminopimelate epimerase (EC 5.1.1.7); (16) diaminopimelate decarboxylase (EC 4.1.1.20).

2. Lysine synthesis was strongly regulated, without affecting the flux into the other branches of the pathway. As dihydrodipicolinate synthase is known to be subject to inhibition by low concentrations of lysine, it was proposed that this was the key regulatory step.
3. Under most conditions, there was no regulation of threonine synthesis *in vitro*. This is despite the fact that at least two enzymes in the pathway have been shown to be sensitive to feedback inhibition by threonine.
4. Evidence for the regulation of methionine synthesis was obtained. It was concluded that the key regulatory step was probably cystathionine-γ-synthase, although homoserine kinase may also be involved. No evidence was obtained that the stimulatory effect of *S*-adenosylmethionine on threonine synthase was a key regulatory factor.
5. Isoleucine was shown to inhibit the conversion of threonine to isoleucine, with an accompanying increase in the size of the soluble threonine pool. It is likely that the major regulatory enzyme is threonine deaminase (dehydratase).

The paper by Giovanelli *et al.* [13] was submitted on December 31st 1988. Since that time there have been major advances in our understanding of the molecular regulation of a small number of the key enzymes at the gene level [14]. In addition a number of transgenic plants [15] and even animal cells [16], have been constructed that contain elevated levels of these enzymes. In the following sections, the *in vitro* properties of the enzymes and their gene regulation will be described, and their possible role *in vitro* will be discussed using the conclusions from the data obtained by Giovanelli *et al.* [13] as a framework.

THREONINE SYNTHESIS

Aspartate kinase and homoserine dehydrogenase

Aspartate kinase (EC 2.7.2.4) is the first enzyme of the pathway catalysing the conversion of aspartate into β-aspartyl phosphate (Fig. 1). The identification, purification and characterization of aspartate kinase was first carried out in microorganisms and most of the information was obtained from experiments with *Escherichia coli*.

In plants, aspartate kinase has been studied for over 35 years and has been identified, isolated, partially purified and biochemically characterised from a wide range of economically important crops such as maize [17–23], barley [24–27], carrot [28–31], pea [32, 33] and soybean [34]. Various degrees of feedback inhibition have been detected using lysine, threonine and methionine. Distinct isoenzymes have been shown to be feedback inhibited by lysine alone, threonine alone or synergistically by lysine and *S*-adenosylmethionine (Table 1). By feedback inhibition of aspartate kinase activity, lysine and threonine can regulate their own synthesis since both are derived from the product of aspartate kinase activity, β-aspartyl phosphate. Regulation of lysine sensitive aspartate kinase activity by calcium and calmodulin has also been proposed in spinach [41], in which calmodulin was one of the two subunits of the enzyme [47]. However, two other reports utilising carrot [48] and maize [49] aspartate kinase isoenzymes, were unable to support such a regulatory role.

The distribution of the aspartate kinase isoenzymes may vary, dependent upon plant tissue and stage of growth. Lea *et al.* [33] and Bryan [11] suggested that the lysine-sensitive aspartate kinase isoenzyme was predominant in rapidly growing cells such as cell cultures. In general, this isoenzyme has been shown to represent approximately 80% of total aspartate kinase activity in all plants tested so far, with the exception of *Coix lacryma-jobi* [50], soybean cotyledons and callus culture [34] and carrot root [51, 52], in which the

Table 1. Aspartate kinase isolated from higher plants

Plant*	Feedback Inhibition†
Arabidopsis thaliana	Lys, Thr [36, 84, 85]
Barley	Lys, Thr, LT, LM, LS, LSAM [24–26, 35]
Carrot	Lys, Thr [28–31]
Cucumber	Lys, Thr, LT [37]
Lemna paucicostata	Lys, Thr, LSAM [38]
Maize	Lys, Thr, LT, LTM, LSAM, SAM [17–23]
Mustard	Lys, Thr [37]
Oat	Lys, Thr [39]
Pea	Lys, Thr [32, 33]
Radish	Lys, Thr [37]
Rice	Lys, Thr, LT [37, 39, 40]
Rye	Lys, Thr, LT [39]
Sorghum	Lys, Thr, LT [39]
Soybean	Lys, Thr [34]
Spinach	Lys, Thr [41]
Sunflower	Lys, Thr [37]
Tobacco	Lys, LSAM [42, 43]
Wheat	Lys, Thr, LT [39, 44, 45]
Vinca rosea	Lys, Thr [46]

* Feedback inhibition may vary according to the tissue of the plant tested. All different tissues tested for the plants listed were considered together.

† Lys = lysine, Thr = threonine, SAM = *S*-adenosylmethionine, LSAM = lysine plus *S*-adenosylmethionine concerted inhibition, LT = lysine plus threonine concerted inhibition, LM = lysine plus methionine concerted inhibition, LTM = lysine plus threonine plus methionine concerted inhibition.

threonine-sensitive aspartate kinase isoenzyme accounts for approximately 60–70% of total aspartate kinase activity.

The lysine-sensitive aspartate kinase has been purified to near homogeneity from carrot [31] and maize [19, 21] tissue culture cells. Anion exchange and gel filtration chromatography proved to be the best protein separation media for the purification of aspartate kinase. In barley leaves, three peaks of aspartate kinase activity could be eluted from a DEAE-cellulose column and were characterised as independent isoenzymes and labelled AKI (threonine-sensitive), AKII and AKIII (both lysine-sensitive or lysine plus *S*-adenosylmethionine-sensitive) in elution order [25]. In maize, two lysine-sensitive aspartate kinase isoenzymes were eluted from anion exchange chromatography columns [19, 21], while the threonine-sensitive isoenzyme overlapped with the first peak of lysine-sensitive aspartate kinase isoenzyme in elution order [21].

Establishment of the precise M_r of the aspartate kinase isoenzymes has caused some problems. Lysine-sensitive aspartate kinase of carrot exhibited a M_r ranging from 100 000 to 258 000 according to the method used [31]. A M_r of 258 000 for the holenzyme agreed with a M_r of 254 000 composed of 49 000 and 60 000 subunits for the maize isoenzyme determined by Dotson *et al.* [19], who also reported a M_r of 113 000 when analysis was carried out on non-denaturing gels, suggesting that the M_r of 113 000 was probably due to dissociation of a tetramer that retained aspartate kinase activity in the dissociated state. Azevedo *et al.* [21] reported a M_r of 180 000 for the threonine-sensitive aspartate kinase and a M_r of approximately 140 000 for the lysine-sensitive aspartate kinase isoenzyme of maize, determined by gel filtration chromatography and by non-denaturing gel electrophoresis.

Homoserine dehydrogenase (EC 1.1.1.3) catalyses the conversion of aspartate semialdehyde to homoserine in the presence of the coenzymes NADH or NADPH [9] and is located mainly in the chloroplast [53, 54]. Homoserine dehydrogenase has been well characterised in prokaryotes such as *E. coli* [55], in which it is feedback inhibited by threonine. It has also been extensively studied in crude or partially extracts of higher plants [32, 54, 56–58].

Two isoenzymes of homoserine dehydrogenase, one sensitive and the other resistant to threonine inhibition, have been observed in plants and purified to homogeneity from maize suspension cultures and seedlings [59, 60] and the enzyme kinetics studied in detail [60–62]. The isoenzymes differ in size, with the threonine resistant isoenzyme exhibiting a M_r of 70 000 and the sensitive isoenzyme a M_r of 190 000 [59]. The allosteric threonine sensitive homoserine dehydrogenase isoenzyme can be easily altered and desensitized [63], and is almost certainly involved in amino acid biosynthesis, while the physiological function of the cytoplasmic threonine resistant isoenzyme remains unclear [11].

In *E. coli* [63, 65], three aspartate kinase isoenzymes have been identified and two of these isoenzymes also exhibited homoserine dehydrogenase activity. The bifunctional protein AKI-HSDHI was inhibited by threonine whereas AKII-HSDHII was repressed by methionine. The third isoenzyme, AKIII which contained only aspartate kinase activity, was inhibited by lysine [64]. In plants, Wilson *et al.* [66] reported the existence of a bifunctional protein containing aspartate kinase and homoserine dehydrogenase activities in carrot, but the feedback pattern was not determined. Similarities were noted between the amino acid sequence of the *E. coli* AKI-HSDHI and the carrot enzyme. Azevedo *et al.* [22] reported biochemical evidence supporting the co-purification of threonine-sensitive aspartate kinase and homoserine dehydrogenase from maize. All these results strongly supported the early results obtained in pea [32], which presented the first evidence in plants, for the existence of a bifunctional protein containing threonine sensitive aspartate kinase and homoserine dehydrogenase activities similar to that identified in *E. coli* [64].

A very large number of questions has now been raised in relation to the regulation of the pathway. For instance, is the bifunctional protein a common feature in plants? Are there other bifunctional iso-

enzymes involving the lysine sensitive aspartate kinase? What are the implications for the plant mutants, which overproduce threonine and lysine? Is the threonine isoenzyme of aspartate kinase functionally more important for the control of the pathway?

The most up to date view of the role of aspartate kinase and homoserine dehydrogenase in plants has been provided by the molecular analysis recently reported by several groups. Weisemann and Matthews [67] cloned cDNAs encoding bifunctional aspartate kinase and homoserine dehydrogenase activities from carrot. An amplified fragment of the gene from carrot cDNA allowed the identification of two overlapping clones from the cDNA library. Using the carrot cDNA as a probe, cDNA clones were isolated from soybean cDNA libraries [68] and the comparison of the cDNA sequences of carrot and soybean showed a homology higher than 85%, and a higher homology to the *E. coli thrA* gene encoding AKI-HSDHI than to the *metL* gene encoding AKII-HSDHII. A small family of at least three genes has now been shown to be present in the soybean [69].

The molecular cloning of a plant aspartate kinase-homoserine dehydrogenase genomic sequence was first reported for *Arabidopsis thaliana* [70]. The carrot aspartate kinase-homoserine dehydrogenase gene was used to isolate the analogous gene from a genomic DNA library of *Arabidopsis thaliana*. The results for the nucleic acid sequence revealed the presence of two upstream regulatory elements, a chloroplast transit peptide and a sequence homologous to that found in γ-glutamyl kinase [71].

The *Arabidopsis gene* (EMBL/GenBank/DDBJ-Accession no: X71363 and X71364) was named as the plant *thrA* homologue gene, coding for the threonine sensitive aspartate kinase-homoserine dehydrogenase enzyme, and was shown to be present as a single copy [70]. However, Aas [72] has recently reported the isolation of an aspartate kinase-homoserine dehydrogenase cDNA from *Arabidopsis thaliana* by functional complementation of a yeast mutant. The sequence of the nucleotides and amino acids differed considerably from that previously reported by Ghislain *et al.* [70], suggesting that there are at least two separate genes coding for the enzyme in *Arabidopsis thaliana*.

Another recent report [73] provided the characterization of the maize genes that encode aspartate kinase-homoserine dehydrogenase. The full length cDNAs, pAKHSDH1 and pAKHSDH2, and the partial cDNA, pAKHSDH3, exhibited high homology to the aspartate kinase and homoserine dehydrogenase monofunctional enzymes of prokaryotes and *Saccharomyces cerevisiae*, and to the aspartate kinase-homoserine dehydrogenase bifunctional proteins of prokaryotes, yeast and carrot and *Arabidopsis thaliana*. Feedback properties showed that the aspartate kinase and homoserine dehydrogenase were both inhibited by threonine [73]. Both AKI-HSDHI and AKII-HSDHII (amino acid sequences deduced from pAKHSDHI and pAKHSDHII) contained putative transit peptides, which would confirm the chloroplast localisation of the two enzymes [74]. The genetic data for the maize enzymes showed that at least five genes encode aspartate kinase; three for the bifunctional aspartate kinase-homoserine dehydrogenase and two for the monofunctional aspartate kinase [73]. pAKHSDH1 and pAKHSDH2 were mapped to chromosomes 4L and 2S, respectively [73], while *ask*1 and *ask*2 for monofunctional aspartate kinase isoenzymes were mapped on chromosomes 7S [75] and 2L [73], respectively. A very exciting recent finding has been the first report of the isolation of a cDNA clone encoding a monofunctional aspartate kinase from an *Arabidopsis thaliana* library, using a homologous PCR fragment as a hybridising probe. The absence of a homoserine dehydrogenase gene encoding at the carboxyl terminus of the peptide suggests that the cDNA encodes the lysine-sensitive enzyme. Southern blot analysis indicated that there are two forms of the gene present in *Arabidopsis thaliana* [76].

The analysis of all these results showed that a new review of the pathway is necessary. They showed striking similarity between the regulation of the pathway in plants and microorganisms in several aspects, such as feedback regulation by amino acid end products, the presence of different sets of isoenzymes, the existence of bifunctional proteins and the isolation of mutants overproducing amino acids.

The selection of mutants has been shown to be an important tool in improving the nutritional quality of some plants [39, 77] and has helped considerably in increasing our understanding of the regulation of the pathway. Several mutants have been isolated in plants by the use of tissue culture technique. Some of these mutants have shown altered regulatory patterns of enzymes that are less sensitive or completely insensitive to feedback inhibition by amino acids end product or their analogues. For instance, barley mutants selected for lysine plus threonine resistance such as R3004, R2501 and R3202, showed different patterns of inhibition [25; for review see 27]. In the mutant R3004, AKIII was less sensitive to lysine inhibition while AKII was insensitive to lysine inhibition in R2502 and R3202 [25, 26, 78]. The mutations led to overproduction of threonine and to a lesser extent, lysine. Genetic analysis showed that the two genes designated *ltr*1 and *ltr*2 are structural loci for AKII and AKIII [25].

In maize, two dominant mutants, LT19 and LT20 were selected [79] and exhibited overproduction of threonine and serine in maize kernels [79, 80]. Two genes, *ask*1 and *ask*2 for the mutants LT29 and LT20, respectively, were mutated and encoded aspartate kinase isoenzymes altered in their sensitivity to lysine [81]. Further analysis of double mutants LT19/opaque-2 showed that the *opaque*-2 gene may regulate the *ask*1 gene based on an intensification effect on amino acid and protein synthesis in the maize endosperm [75]. Furthermore, the *ask*1 gene is linked to the *opaque*-2 gene [75] and recent evidence for a possible regulation

of aspartate kinase by *opaque*-2 has been reported [23].

In *Nicotiana sylvestris*, a mutant plant (RLT 70) was isolated that was resistant to lysine plus threonine. The leaves and seeds exhibited a 45- and 70-fold increase in soluble threonine, respectively. Aspartate kinase isolated from the plant was completely insensitive to lysine and the trait was shown to be inherited via a single dominant gene [82]. The use of the tobacco mutant (RLT 70) following crossing with a lysine insensitive dihydrodipicolinate synthase mutant [83] will be discussed in a later section.

In *Arabidopsis thaliana*, two mutants resistant to lysine plus threonine (RLT 40 and RLT 4), were also shown to accumulate threonine 4- and 8-fold, respectively. Only a partial loss of lysine sensitivity was detected in the aspartate kinase isolated from both mutants [36, 84]. Tzchori *et al.* [85] have suggested that the regulation of threonine synthesis in *Arabidopsis thaliana* is much more relaxed than in other plant species. This could be explained by the presence of a feedback insensitive form of aspartate kinase [84].

Homoserine kinase

Homoserine kinase (EC 2.7.1.39) catalyses a reaction common to threonine, isoleucine and methionine synthesis, in which homoserine is converted in the presence of ATP to yield *O*-phosphohomoserine and ADP. Homoserine kinase together with aspartate kinase, homoserine dehydrogenase and threonine synthase, are located in the chloroplast [74, 86]. In plants, homoserine kinase has been isolated and partially purified in rather crude preparations from pea leaves [86], pea seedlings [87], radish leaves [88] and barley leaves [89, 90]. The first purification of a plant homoserine kinase to homogeneity was carried out with wheat germ [91].

The properties of homoserine kinase from microorganisms have been well documented [92, 93], but there have been few reports from higher plants, as homoserine is not considered to be a branch point metabolite. These small number of reports, compared to other regulatory enzymes of the pathway, have exhibited very inconsistent results. Muhitch and Wilson [86] identified both thylakoid-associated and soluble homoserine kinase activities in pea chloroplast, but were unable to conclude that they were distinct isoenzymes. However, other workers have not been available to find experimental evidence for the existence of homoserine kinase isoenzymes [87, 89–91].

The regulatory properties observed for homoserine kinase among the plant species, have also been shown to differ considerably. Aarnes [89] was unable to identify feedback inhibition of homoserine kinase extracted from barley seedlings, by the amino acids threonine, methionine or isoleucine. Homoserine kinase purified from wheat germ was not significantly influenced by any of the aspartate-derived amino acids, at least at physiologically relevant concentrations, nor by *S*-adenosylmethionine, which is known to be a regulatory metabolite of the pathway. Considering the results reported in the literature, Riesmeier *et al.* [91] have suggested that in monocotyledonous plants, homoserine kinase is not a regulatory enzyme of the aspartic acid pathway and that isoenzymes are probably not present. In contrast, homoserine kinase from pea seedlings was subject to inhibition by isoleucine, valine, ornithine and *S*-adenosylmethionine, while threonine, methionine and lysine alone, or in combination, had little effect [87]. Homoserine metabolism in pea and some related species is unusual in that large soluble pools are present. Pea is also the higher plant known to synthesise *O*-acetylhomoserine [94]. In pea, homoserine probably acts as a nitrogen storage and transport compound as well as being involved in amino acid biosynthesis. Homoserine kinase isolated from pea has a low affinity for homoserine [87], which could account for the large pool sizes. A mutant of pea lacking ferredoxin dependent glutamate synthase, contained low levels of homoserine and was unable to convert ^{15}N-glutamine to homoserine when exposed to air [95]. Isoleucine, threonine and *S*-adenosylmethionine were effective inhibitors of radish leaf threonine kinase activity, while *S*-adenosylmethionine and isoleucine showed a marked synergistic inhibition [88]. Homoserine kinase has also been assayed in extracts of methionine-overproducing soybean callus lines. The enzyme specific activity was depressed by 45–73% in the mutant lines compared to the activity observed for the wild type [96].

Large differences have also been noted for the native M_r of plant homoserine kinases. Employing Sephadex G200 gel filtration chromatography, Thoen *et al.* [87] separated two peaks of homoserine kinase acitivity corresponding to 240 000 and 120 000 from pea seedlings whilst homoserine kinase from barley seedlings had a M_r estimated of about 150 000 [89]. In both cases, the M_r for the enzyme were much higher than that for bacteria [92, 93]. The enzyme purified to homogeneity from wheat germ exhibited a native M_r estimated to be 75 000 which existed as a homodimer with a subunit M_r of 36 000 [91].

At the present time, there has been no report of the isolation of a gene encoding homoserine kinase from higher plants. Genes coding for the enzyme have been isolated from bacteria [92, 98] and yeast [99, 100], and considerable similarity between the sequences noted.

Threonine synthase

Threonine is synthesised from aspartate in five steps. The last step involves the irreversible conversion of *O*-phosphohomoserine into threonine which is catalysed by the enzyme threonine synthase (EC 4.2.99.2). Threonine synthase has been partially purified from sugar beet leaves [101], radish leaves [102], pea seedlings [101], barley seedlings [90, 103] and *Lemna paucicostata* [104, 105]. The results obtained in these plants species for threonine synthase are very

similar and showed that the enzyme appears to be relatively stable during purification procedures [106]. Threonine synthase has also been shown to be located in the chloroplast [74]. Isoenzymes of threonine synthase have not been detected and a M_r of approximately 190000 has been determined for a peak of pea seedlings threonine synthase activity eluted from Sephadex G200 [102]. The enzyme is not feedback inhibited by threonine, methionine or isoleucine [103–105], but conditions of methionine excess reduce the enzyme activity in barley and *Lemna* [103, 105]. Giovanelli *et al.* [105] have also shown that inorganic phosphate inhibited *Lemna* threonine synthase activity and that AMP is a potent and structurally specific inhibitor of threonine synthase.

Probably, the most important characteristic of plant threonine synthase is that the enzyme is markedly stimulated by *S*-adenosylmethionine [101, 102, 104] in a reversible and co-operative manner, consistent with a possible role in regulating methionine biosynthesis in plants [104]. The availability of *S*-adenosylmethionine could determine the relative amount of *O*-phosphohomoserine that is converted to cystathionine and methionine or threonine.

Greenberg *et al.* [96] tested the activity of threonine synthase in methionine-overproducing soybean callus lines and observed that the enzyme specific activity was depressed by 26–43% compared to the wild type. The inhibition patterns by cysteine varied among the mutant lines with two cell lines showing significant decrease in the inhibition by cysteine. The study of these mutants indicated that a depressed threonine synthase activity may increase the availability of *O*-phosphohomoserine for the competing methionine biosynthesis branch of the pathway [86]. The gene encoding threonine synthase has been isolated from a number of bacteria [107] and yeast [108]. More recently the *Arabidopsis thaliana* gene has been expressed in *E. coli*. The N-terminal section of the recombinant enzyme was shown to be involved in the activation by *S*-adenosylmethionine [109].

LYSINE SYNTHESIS

Aspartate semialdehyde dehydrogenase

Aspartate semialdehyde dehydrogenase (EC 1.2.1.11) catalyses the NADPH dependent reduction of aspartyl phosphate to aspartate semialdehyde. The enzyme has been poorly characterised in higher plants and was initially detected in pea seedlings [9]. Gengenbach *et al.* [18] measured enzyme activity in maize shoot, root, kernel, callus and suspension culture extracts. Aspartate semialdehyde dehydrogenase isolated from maize suspension culture cells was not feedback inhibited by 10 mM lysine, threonine or isoleucine, although some sensitivity to methionine was detected [18]. The enzyme in *E. coli* has a native M_r of 77500 and subunit M_rs of 38000–40000 determined by SDS-PAGE or gene cloning [110]. The kinetic mechanism of the enzyme reaction has been studied in detail in *E. coli* and has been shown to require an essential cysteine residue [111]. The *asd* gene encoding the enzyme has been isolated from a number of bacteria and shown to be part of the *dap* operon in *Bacillus subtilis* [112], but not in *Streptomyces* [113].

Dihydrodipicolinate synthase

The enzyme dihydrodipicolinate synthase (EC 4.2.1.52) is the first enzyme of the lysine biosynthesis branch of the aspartic acid metabolic pathway and its fundamental role in regulating lysine biosynthesis has been demonstrated [11, 114–120]. Dihydrodipicolinate synthase catalyses the condensation of pyruvate and aspartate semialdehyde into dihydrodipicolinate. The enzyme has been isolated, purified and characterised from a variety of plants such as maize [114–116], wheat [117], spinach [118], pea [119] and tobacco [120]. In contrast to the presence of isoenzymes of aspartate kinase and homoserine dehydrogenase, only one form of dihydrodipicolinate synthase sensitive to lysine feedback inhibition has been observed in plants. Results obtained with a highly purified dihydrodipicolinate synthase from wheat suspension cultures, indicated that lysine is a competitive inhibitor with respect to aspartate semialdehyde and a none competitive inhibitor with respect to pyruvate [117]. The M_r determined for plant dihydrodipicolinate synthase varied a little among the plant species studied. A M_r of 115000 was estimated for the spinach enzyme [118], 123000 for wheat dihydrodipicolinate synthase with subunits of 32000–25000 [117], 130000 for maize with four subunits of 38000 [115], 164000 for tobacco with four subunits of 38500 [120] and 127000 for pea with three subunits of 43000 [119]. The binding site of lysine in the *E. coli* enzyme and the reaction mechanism have been studied in detail by X-ray crystallography and NMR-spectroscopy [121].

The gene encoding dihydrodipicolinate synthase has also been characterised in plants. A putative full-length cDNA clone (EMBL/Data Bank Accession no: X52850) encoding sequences for both the catalytic function and regulatory properties of maize dihydrodipicolinate synthase has been isolated by direct genetic selection in an *E. coli dapA* auxotroph [116]. The M_r of 35854, predicted from the deduced amino acid sequence, was very close to the M_r of 38000 determined by SDS-PAGE for the purified dihydrodipicolinate synthase from maize. In wheat, sequence analysis of two cDNA clones has shown minor variation in the amino acid sequence of the mature polypeptide subunit with 21 amino acid differences out of 326, which indicated the presence of at least two functional dihydrodipicolinate synthase genes [122]. cDNA clones encoding the enzyme have also been isolated from poplar, *Arabidopsis thaliana* [123] and soybean [124].

Comparisons of maize and wheat dihydro-

dipicolinate synthase transit sequences showed no homology except in the region immediately adjacent to the start of the mature protein. However, when the sequence of the mature maize enzyme [116] was compared with the two wheat cDNAs [122], the lengths of the mature polypeptides were the same (326 amino acids) and they showed 86–66% homology at the amino acid level, indicating that the functional mature dihydrodipicolinate synthase polypeptide sequence is highly conserved in these two cereal species [116].

Lysine and its analogue, *S*-aminoethylcysteine (AEC), have been largely used to select resistant plants *in vitro*, but the mutants obtained showed very little or no alteration in lysine levels and in many cases dihydrodipicolinate synthase enzymology was not reported or was, still, sensitive to lysine inhibition.

Barley mutants resistant to AEC, designated R906 and R4407, were selected by their ability to grow in the presence of 0.25 mM AEC [125]. Genetic analysis was carried out and showed that the mutations were determined by two allelic genes *aec1a* and *aec1b*. Biochemical analysis revealed that the resistance to AEC in these mutants was due to reduced uptake of AEC in the roots [125].

AEC resistant mutants of *Arabidopsis*, [36, 126], wheat [127] and maize [128] provided results very similar to the ones obtained for the barley mutants [125]. All these mutants were of little use for the study of the aspartic acid metabolic pathway. Other resistant mutants showing higher levels of lysine have been obtained in rice [129] and in pearl millet [130], however, enzymology tests in both cases were not carried out. Jacobsen [131] also selected AEC resistant cell lines of potato exhibiting an increase on the level of soluble amino acids, but not related to a specific accumulation of lysine, threonine and methionine.

One mutant of *Nicotiana sylvestris* regenerated from protoplast culture was resistant to AEC (RAEC-1) and exhibited 28-fold increase in soluble lysine in the leaf. In the initial heterozygous mutant, 50% of the dihydrodipicolinate synthase was insensitive to feedback inhibition by lysine. By subsequent crossing, a homozygous mutant was obtained in which the dihydrodipicolinate synthase was totally insensitive to inhibition by 10 mM lysine [132].

Frankard *et al.* [83] crossed the homozygous AEC resistant mutant of tobacco RAEC-1 that overproduced lysine with the homozygous mutant RLT 70 [82], that was resistant to lysine plus threonine and overproduced threonine. The sensitivities to lysine of aspartate kinase and dihydrodipicolinate synthase isolated from the homozygous parents and the heterozygous progeny are shown in Table 2. The level of soluble lysine in the leaves of the heterozygous double mutant (RAEC-1 × RLT 70) was 20-fold higher than the wild type and was 30% of the total soluble amino acid pool.

When compared to the lysine overproducing parent (RAEC-1), the double mutant contained higher levels of soluble lysine in the leaves (1785 nmol g^{-1} as compared to 1380 nmol g^{-1}). In contrast, the concentration of soluble threonine in the double mutant was greatly reduced when compared to the parent RLT 70 (51 nmol g^{-1} as compared to 18 779 nmol g^{-1}) and was even lower than the wild type (335 nmol g^{-1}). The data confirmed that dihydrodipicolinate synthase normally exerts a strong control over the aspartate pathway and can cause a drain of aspartate semialdehyde to lysine, when deregulated. Unfortunately, the double mutant plants exhibited an abnormal phenotype, characterised by a reduction in leaf blade area and an absence of stem elongation and flower formation [83]. The gene encoding the lysine-insensitive form of *Nicotiana sylvestris* dihydrodipicolinate synthase [132] has now been cloned. Amino acid sequence comparisons indicated that there had been a substitution of leucine for asparagine, which correspond to a dinucleotide mutation [133]. Mutant forms of maize dihydrodipicolinate synthase have been obtained by mutating the enzyme expressed in *E. coli*. Single amino acid substitution at one of the amino acid residues 157, 162, or 166, caused the formation of a lysine-insensitive enzyme [134]. As discussed previously the higher plant dihydrodipicolinate synthase is normally very sensitive to feedback inhibition by lysine. However the *E. coli* enzyme is less sensitive to lysine and the *dapA* gene of *E. coli* [135] has been used to transform plants. Shaul and Galili [136] used two types of construct utilising the 35S promoter, including one that contained the pea *rbcS-3A* chloroplast transit peptide. An increase in the soluble lysine concentration was detected in tobacco plants that contained *E. coli* dihydrodipicolinate synthase in the chloroplast. No increase in lysine was detected in plants when the enzyme was expressed in the cytoplasm, confirming the chloroplast localisation of the pathway [74]. No increases in soluble methionine or threonine were detected in the transformed plants. In a similar series

Table 2. Comparison of the inhibition of aspartate kinase (AK) and dihydrodipicolinate synthase (DHDPS) activities in mutant and wild type tobacco. Data expressed as percentage inhibition of total activity, lysine added at 10 mM. Data from Frankard *et al.* [83]

Genotype	AK	DHDPS
Wild type	80	100
RAEC-1 × RLT 70:		
double heterozygote	40	50
RAEC-1:		
heterozygote	80	50
homozygote	80	0
RLT 70:		
heterozygote	40	100
homozygote	0	100

of experiments, Glassman [137] used the *dapA* gene with 35S promoter and a chemically synthesised chloroplast transit peptide. AEC-resistant transformed plants were shown to contain an elevated concentration of soluble lysine, with a maximum increase of almost 100-fold as compared to the wild type plants. Unfortunately, the plants obtained by both groups of workers, that contained elevated levels of lysine, were observed to have unusual growth characteristics. Young leaves, in particular, contained low levels of chlorophyll, that often appeared in a mozaic pattern [136, 137].

In a second series of experiments Shaul and Galili [138] utilised a mutant *lysC* gene from *E. coli* that encoded aspartate kinase that was insensitive to feedback inhibition by lysine [139]. Plants expressing the *lysC* gene attached to a chloroplast transit peptide exhibited an increase in soluble threonine ranging from 2- to 9-fold. However, significant increases in soluble threonine were also detected when the *lysC* gene was expressed in the cytoplasm. When progenies of the transformed plants were grown in the glasshouse, greater effects were noted. In E-26 plants, homozygous for the chloroplast located *E. coli* aspartate kinase enzyme, the concentrations of soluble threonine, isoleucine and lysine in leaves were increased 83-, 5.5- and 20-fold, respectively, when compared to the leaves of control plants.

When transgenic tobacco plants were constructed that contained both the *dapA* and *lysC* genes of *E. coli* [140], the concentration of soluble lysine was greatly increased when compared to the plants containing the *dapA* gene alone. This increase in lysine was accompanied by a reduction in soluble threonine, when compared to the plants containing the *lysC* gene alone. This data confirms that originally obtained with the double mutant of tobacco [83], that a lysine insensitive dihydrodipicolinate can successfully compete with homoserine dehydrogenase and direct aspartate semialdehyde for lysine synthesis.

When the *lysC* gene was fused to a chloroplast transit peptide and the seed specific promoter of the bean phaseolin gene [141], transformed plants exhibited a 14- to 17-fold increase in the soluble threonine concentration of the seed [142]. Although no increase in protein threonine was detected, small increases in soluble and protein bound methionine were observed in the seeds.

When the *E. coli dapA* gene was expressed in tobacco using the seed-specific promoter of the bean phaseolin gene, there was no evidence of increased soluble lysine in the mature seed [143]. Similar results were obtained if the *E. coli lysC* gene was also present. Karchi *et al.* [143] were however able to demonstrate that at earlier stages of seed development, there was evidence of enhanced soluble lysine in the seed, although this was only in the order of a two-fold increase. The authors concluded that the absence of elevated soluble lysine in the tobacco seed was due to an increased rate of catabolism. This possibility will be discussed in a later section. However, more recently Falco *et al.* [144] have achieved a considerable degree of success in the production of transgenic canola and soybean seeds with increased lysine. The authors followed a similar approach to that proposed by Karchi *et al.* [143], but used the *dapA* gene from *Corynebacterium glutamicum* ATCC 13032 which encoded a lysine insensitive dihydrodipicolinate synthase [145]. Expression of the *C. glutamicum* dihydrodipicolinate synthase, lead to a greater than 100-fold increase in the concentrations of soluble lysine in the canola seeds. The soluble lysine increase was great enough to cause a doubling in the total lysine content of the seed (6 → 12% total amino acids). Expression of a mutated *E. coli lysC* gene along with the *C. glutamicum dapA* gene did not cause any additional increase in the soluble lysine content.

Soybean seeds expressing the *C. glutamicum dapA* gene together with the *E. coli lysC* gene exhibited a greater increase in soluble lysine than those expressing the *dapA* gene alone, in contrast to the results in canola. In the best transformed line analysed, the total lysine content increased over five-fold, from 5.9 to 34% of the total amino acids. At the end of their paper, Falco *et al.* [144] carried out an interesting calculation. They argued that the current cost of adding crystalline lysine as a supplement in animal feed is currently $1.20 per lb. Transgenic lines that have double the seed lysine content contain about 3 lbs of additional lysine per 100 lbs of soybean meal. Thus soybean seeds with double the normal lysine content would be worth an additional $3.60 per 100 lbs over normal commodity seeds, which could represent a 30–35% increase in value.

Increases in soluble lysine following the expression of *E. coli* dihydrodipicolinate synthase in transgenic tobacco [146] and barley [147] have also been demonstrated. Surprisingly transgenic lines of barley transformed with the *E. coli* lysine-insensitive form of aspartate kinase did not contain increased concentrations of threonine [147]. This result conflicts with the previous demonstration that barley mutants expressing a lysine-insensitive form of aspartate kinase, accumulated high concentrations of threonine [25–27, 77, 78]. *Arabidopsis thaliana* transformed with *E. coli* dihydrodipicolinate synthase overproduced lysine, but there was little correlation between the concentration of soluble lysine and the measurable dihydrodipicolinate synthase activity [85]. Whilst in the high lysine containing plants an abnormal phenotype was observed in young leaves, at later stages of development, the plants appeared normal. The expression of *E. coli* aspartate kinase, only resulted in a very small increase in soluble threonine [85]. The results suggest that *Arabidopsis thaliana* has an unusual pattern of regulation of the aspartate pathway and that there may be more variation between species, than was first though possible. Maize cell suspension cultures transformed with an altered form of maize dihydrodipicolinate synthase less sensitive to

lysine inhibition, have also been shown to contain high concentrations of soluble lysine [148].

Dihydrodipicolinate reductase and diaminopimelate decarboxylase

Two other enzymes, dihydrodipicolinate reductase (EC 1.3.1.26) and diaminopimelate (EC 4.1.1.20), involved in lysine synthesis branch of the pathway have also been studied in plants. Dihydrodipicolinate reductase catalyses the pyridine nucleotide-linked reduction of dihydrodipicolinic acid to tetradipicolinic acid. This enzyme had been partially purified from maize kernels [149] and was inhibited by compounds that contained structures similar to dihydrodipicolinic acid. The most potent inhibitor was dipicolinic acid, whereas oxidised pyridine nucleotides inhibited the activity slightly. A M_r of 84000 was determined by gel filtration chromatography on Sephacryl S200 [149]. The 3-dimensional structure [150] and kinetics [151] of the enzyme in *E. coli* have been studied in detail.

The last step in lysine biosynthesis involves the pyridoxal phosphate-dependent decarboxylation of *meso*-diaminopimelic acid to lysine, a reaction that is catalysed by the enzyme diaminopimelate decarboxylase. This enzyme, which is solely localised in the chloroplast [152], has been studied in *Lemna perpusilla* [153], *Vicia faba* [152], wheat [154] and maize [155]. The plant enzymes show similarities in their properties but differences when compared to the bacterial enzyme. The K_m of the plant enzyme for diaminopimelate is much lower than that of bacteria, varying from 0.14 to 0.3 mM in plants [152, 153, 155] and 1.7 to 2.8 mM in bacteria [156, 157]. A M_r of 75000 to 85000 has been estimated for the plant enzyme [154, 155] while in *E. coli* it is 200000 [156]. The bacterial enzyme requires pyridoxal phosphate as cofactor [158] which is not the case for the plant enzyme [152, 153].

LYSINE CATABOLISM

Most of the data on lysine catabolism in higher plants, comes from studies on the incorporation and metabolism of the radiolabelled amino acid by plant tissues. Feeding with [^{14}C]-lysine leads to incorporation of radiolabel into α-amino adipic acid and glutamic acid in wheat [159, 160] and saccharopine and diaminopimelic acid in maize and barley [161–163]. In developing endosperm of maize and barley, radiolabel from lysine was found primarily in glutamic acid and proline [162, 163]. These findings indicate that lysine is mostly catabolised in plants via saccharopine. The first enzymatic evidence for the operation of the saccharopine pathway in plants was obtained by the demonstration of the activity of lysine-ketoglutarate reductase (LKR; EC 1.5.1.8) in developing maize endosperm [164]. It is known that the first two steps involved in the lysine catabolism in plants are similar to that found in mammals and are catalysed by the bifunctional enzyme, lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) [165, 166]. In contrast, in yeast and fungi, the LKR and SDH (EC 1.5.1.9) enzymes catalyse the final steps of lysine biosynthesis and are present as two separate polypeptides [167–169].

Recent evidence suggests that parallel regulatory mechanisms shared by common compounds and/or factors exist in the lysine biosynthetic route in yeast and the lysine catabolic pathway in plants and mammals. In yeast, lysine inhibits the expression of *lys*1 and *lys*9 genes [170, 171], while α-aminoadipic-α-semialdehyde (AASA) induces the expression of these and other related genes [172]. On the other hand, in plant and mammals lysine has been shown to induce its own degradation. Treatment with exogenous lysine, dramatically increased the activity of LKR in developing tobacco seeds and rat liver mitochondria [143, 173, 174].

The LKR/SDH enzyme

The native mammalian LKR/SDH is a homotetramer of 460000, composed of four subunits of 115000 [165], while the plant enzyme is a homodimer of 260000 constituted by two subunits of 125000 [166]. The LKR and SDH from yeast and fungi are monomers of 49000 and 73000 that are encoded by two separate genes, *lys*1 and *lys*9, respectively [170, 171]. It is interesting that the sum of the sizes of yeast LKR and SDH correspond to the sizes of the bifunctional polypeptides of mammals and plants.

The bovine and maize LKR and SDH activities reside, as adjacent domains, in a bifunctional polypeptide [165, 166]. The existence of two covalently linked domains in a polypeptide, catalysing sequential steps of a reaction, has many physiological advantages, e.g. the production of the equimolar amounts of the translation products [175]. Substrate-channelling, allows the product of the first reaction step to be channelled faster to the second catalytic domain, rather than be released into the medium [175].

The LKR domain of LKR/SDH from maize is activated by Ca^{2+} in the μM range (P. Arruda, unpublished results). Active LKR and SDH domains can be obtained by limited proteolysis, suggesting that the domains containing each enzyme activity are independently folded [165, 166]. This apparently independent folding, may explain why Ca^{2+} activates the LKR domain but does not affect the SDH domain of the maize enzyme. The physiological role of the activation of the maize LKR by Ca^{2+} is unknown. Moreover, the effect of Ca^{2+} on the mammalian enzyme is different to that observed for the maize enzyme. The LKR/SDH from rat liver is induced by glucagon treatment [176]. Although glucagon is known to increase the intramitochondrial Ca^{2+} concentration, Ca^{2+} does not affect the activity of LKR

from bovine and rat liver [173; Arruda, P., unpublished results].

Regulation of the plant LKR/SDH

The pattern of LKR activity in developing maize endosperm is co-ordinated with the rate of zein (the most abundant storage proteins of the endosperm) accumulation and total nitrogen input [177, 178]. Furthermore, the opaque-2 mutation that reduces the accumulation of specific zein polypeptides [179–181] also affects the catabolism of lysine. [^{14}C]-Lysine is degraded to a lesser extent in the opaque-2 mutant than in normal endosperm [162]. The amount of enzyme activity was two- to three-fold lower in the mutant than in normal endosperm [178]. In addition, the pattern of LKR reduction was correlated with the pattern of reduction in zein synthesis in the opaque-2 endosperm. Since the *opaque*-2 gene encodes a tbZIP protein that regulates the transcription of several zein genes [182, 183], it is possible that the LKR gene could also be under the control of this transcriptional factor.

When the *dapA* gene encoding *E. coli* dihydrodipicolinate synthase was expressed in tobacco seeds, there was evidence of only a small transient accumulation of lysine during seed development (see previous section [143]). This transient increase in lysine was correlated with an increase in LKR activity in the seeds of the transgenic plants [143]. LKR activity could also be increased in the wild type plants by the injection of lysine into the developing pods. In the leaves of tobacco, where lysine can accumulate to very high concentrations [136–138], LKR activity could not be detected in the control or transgenic plants [143]. In the experiments of Falco *et al.* [144], high concentration of lysine were obtained in the seeds of transgenic canola and soybean, expressing the *C. glutamicum* gene (see previous section). Although LKR activity was not measured, even in these plants, evidence of lysine catabolism was obtained. Transgenic canola accumulated α-aminoadipic acid, whilst soybean accumulated saccharopine. The differences in the catabolism of lysine between the two species is not clear.

The data presented above clearly indicate that an increase in the soluble lysine pool of plant seeds, can increase the activity of the enzymes involved in the catabolytic pathway. It is possible that this lysine-dependent LKR-SDH induction is mediated by Ca^{2+} ions. In maize, the lysine-dependent induction of LKR-SDH can be explained by the relative high concentrations of free lysine translocated from other tissues to the developing endosperm [184]. Since zeins, the most abundant proteins accumulating in the endosperm, are devoid of lysine residues, the demand for lysine during endosperm development would be very low. Considering that synthesis and translocation would provide more lysine than is needed, the excess of lysine could serve for LKR-SDH induction. In the opaque-2 endosperm the lower LKR-SDH activity may be, at least in part, responsible for the high lysine content of the endosperm.

METHIONINE SYNTHESIS

Methionine biosynthesis, as can be seen from Fig. 1, involves the transfer of sulphur from the C_3 skeleton of cysteine to the C_4 skeleton of homoserine. In this section this process termed trans-sulphuration, will be considered in conjunction with the biosynthesis of cysteine from serine and sulphide, methylation of homocysteine, and the biosynthesis and metabolism of *S*-adenosylmethionine (AdoMet). Synthesis of sulphide by the ATP and ferredoxin dependent reduction of sulphate is beyond the scope of this review. However, this process (known as assimilatory sulphate reduction) has been covered recently in several detailed reviews [185–189].

The biosynthesis of cysteine

O-Acetylserine(thiol)-lyase (*O*-acetylserine sulphhydrylase, EC 4.2.99.8) catalyses the biosynthesis of cysteine from *O*-acetylserine and sulphide. The physiological *O*-ester substrate *O*-acetylserine, is synthesised by serine acetyltransferase (EC 2.3.1.30).

Serine acetyltransferase has been partially purified from a variety of plant species [190–192] but has only been completely separated from *O*-acetylserine(thiol)-lyase in highly purified extracts of spinach leaves [193]. Isoenzymes have been detected [194, 195] and, in *Pisum sativum*, localised within the mitochondria (76% of total enzyme activity), cytosol and plastids [196]. cDNA clones of serine acetyltransferase, isolated by functional complementation, encode polypeptides with estimated M_rs in the range 32 000 to 34 000 [196–201]. The recombinant protein, isolated from a *Citrullus vulgaris* cDNA library, was inhibited by low concentrations (I_{50} 2.9 μM) of cysteine suggesting a possible regulatory role of *O*-acetylserine in cysteine biosynthesis [198]. However, this may not be a general phenomena, as other studies have shown that serine acetyltransferase was only inhibited at unphysiological concentrations of cysteine [192, 194].

O-Acetylserine(thiol)-lyase has been partially purified from a variety of plant tissues [see for example: 202–204] and to apparent homogeneity from spinach leaves [205, 206]. In spinach chloroplasts, the enzyme has a M_r of 68 000 and is composed of subunits of M_r 35 000, each containing one pyridoxal phosphate cofactor [205–207]. Enzyme activity was optimal between pH 7.5 and 8.5 and product inhibition was not physiologically significant [205]. Studies of *O*-acetylserine(thiol)-lyase isoenzymes, performed in spinach leaves [208], cauliflower (*Brassica oleracea*) inflorescence [209] and *Datura innoxia* [210], indicated three isoenzymes located respectively in the plastidic (major isoenzyme), cytosolic and mitochondrial compartments.

Further evidence in support of multiple *O*-ace-

tylserine(thiol)-lyase isoenzymes has been provided following the isolation and sequencing of cDNA clones encoding the chloroplastic [206, 211–216], cytosolic [211, 214, 217–219] and mitochondrial [220] isoenzymes. Analysis of *O*-acetylserine(thiol)-lyase transit peptides, fused to bacterial β-glucuronidase, demonstrated these cDNAs have strict organelle and suborganelle specificity [221]. Amino acid sequences deduced from the cDNA clones, have M_r estimated to range from 34 000 to 39 500. The sequences show high homology with each other and with *O*-acetylserine(thiol)-lyase counterparts from bacteria. Further phylogenetic analysis has shown that the enzyme evolved from a common ancestor into five families—three in higher plants and two in bacteria [219, 220].

At present it is not possible to determine what physiological roles the compartment specific isoenzymes play in metabolism. It has been suggested that the plant cell may be unable to transport cysteine between cellular compartments and therefore requires three isoenzymes for protein synthesis [208]. Alternatively, the different isoenzymes could be involved in discrete physiological roles such as protein catabolism [221] or glutathione accumulation [204, 210].

In vitro, the affinity of *O*-acetylserine(thiol)-lyase for the substrate *O*-acetylserine is relatively low with an apparent K_m of 1.3 mM [205]. It has been inferred that strong association of serine acetyltransferase with *O*-acetylserine(thiol)-lyase during purification was due to the formation of a supramolecular complex [191, 205]. Such a complex, containing both enzyme activities, has been detected following gel filtration [191, 193, 198]. Substrate channelling by the complex could, therefore, compensate for the low affinity of *O*-acetylserine(thiol)-lyase for *O*-acetylserine [198, 205]. However, as the contribution of substrate channelling (that is non-equilibrium of a substrate with the bulk phase) to metabolic flux is disputed [222], further structural and kinetic studies of the complex are required to confirm this suggestion.

Changes in the level of *O*-acetylserine(thiol)-lyase in response to sulphur supply have not been observed in *Lemna minor* [233], tobacco cells (*Nicotiana tabacum*) [224], or cultured tomato roots [225]. However, in response to sulphur starvation, the specific activity of the enzyme was observed to increase in maize [226] and tobacco cells [227]. In addition, *O*-acetylserine(thiol)-lyase mRNA levels increase during sulphur starvation [211, 217, 221] and *Capsicum annuum* fruit development [206]. From these conflicting results it is not possible to determine the influence of sulphur supply on *O*-acetylserine(thiol)-lyase specific activity or isoenzyme distribution.

Further work suggests that cysteine biosynthesis is regulated, not by *O*-acetylserine(thiol)-lyase, but at the level of sulphate assimilation and *O*-acetylserine supply [for example: 225, 228, 229]. However, in conflict with these results, plants fed excess sulphur (as sulphate, sulphite or sulphide) accumulated high levels of cysteine without additional *O*-acetylserine [230]. Unfortunately in many experimental systems it is not possible to determine whether this was part of the normal physiological response or a method of detoxifying excess sulphur [321]. In conclusion, general regulatory schemes for cysteine biosynthesis, totally consistent with experimental observations are as yet impossible to construct [see also: 187, 230].

The enzymes unique to methionine biosynthesis

Cystathionine γ-synthase (EC 4.2.99.9), the first enzyme unique to methionine biosynthesis, catalyses the synthesis of cystathionine from *O*-phosphohomoserine and cysteine (Fig. 1). Plants from the most of the major phylogenetic divisions were only observed to utilise *O*-phophohomoserine as the physiological 2-aminobutyryl donor [232, 233]. This contrast with prokaryotes (such as *E. coli* or *Salmonella typhimurium*) that use *O*-succinylhomoserine, or in the case of fungi and yeasts, *O*-acetylhomoserine [234, 235].

Cystathionine γ-synthase has been partially purified from the leaves of barley [236] and wheat [237] and to apparent homogeneity from spinach chloroplasts [238]. Cystathionine γ-synthase has not been observed to exist as isoenzymes and was shown to be located in the chloroplasts of barley leaves and spinach leaves [74]. Homologous cystathionine γ-synthase had a native M_r of 215 000 and appeared to be a tetramer composed of two molecular species of 50 000 and 53 000 [238]. The enzyme required PLP for activity and had a pH optimum between 7.4 and 7.5 [237, 238]. A genomic and cDNA clone for cystathionine γ-synthase has been isolated from *Arabidopsis thaliana*, encoding a protein with an estimated M_r of 60 000 [239]. The presence of an amino terminus, resembling a plastid transport peptide, would appear to confirm localization of this enzyme to the plastid [238, 74].

While cystathionine γ-synthase was not susceptible to feedback inhibition by products of the aspartate pathway [236, 237], it was susceptible to orthophosphate product inhibition [105, 238]. It has been proposed that *in vivo* the orthophosphate to *O*-phophohomoserine ratio could modulate cystathionine γ-synthase activity [105]. The level of extractable cystathionine γ-synthase activity was reduced following growth in the presence of exogenous methionine [103, 240–242]. For example, in *Lemna paucicostata* the specific activity of cystathionine γ-synthase was reduced to 15% of the control plants following growth in a medium containing 2 μM methionine [242]. Furthermore, when the flow of carbon into methionine was reduced (e.g. by the use of inhibitors) the specific activity of cystathionine γ-synthase compared to control plants was observed to increase [103, 242]. These observations are consistent with repression and derepression of the enzyme.

Cystathionine β-lyase (EC 4.4.1.8), the second enzyme unique to methionine biosynthesis, cleaves cystathionine to yield homocysteine, pyruvate and

ammonia (Fig. 1). The enzyme has been partially purified [243, 244] and purified to apparent homogeneity from spinach leaves [245, 246] and *E. colonum* cell suspension cultures [247].

In spinach chloroplasts and *E. colonum*, cystathionine β-lyase was homotetramer with M_r of 160 000–170 000 [245, 247]. In agreement with these figures, an *Arabidopsis thaliana* cDNA clone, containing a putative transit peptide has a mature M_r of 43 000 [248]. However, in a preparation from spinach leaves the enzyme, also a tetramer, had a M_r of 210 000 [246]. Cystathionine β-lyase required PLP for activity, had a pH optimum between 8.3 and 9.0 and was not susceptible to feedback inhibition by products of the aspartate pathway [245–247]. Cytosolic and plastidic isoenzymes were identified by subcellular fractionation of barley leaves [74] and ion-exchange chromatography of spinach leaf extracts [245]. It is possible that isoenzymes are required because, as has been suggested for cysteine [208], homocysteine can not be moved across the plastid membrane. However, cystathionine β-lyase isoenzymes were not observed in maize [249], *E. colonum* plants [247] and *A. thaliana* [248]. It would thus seem probable that, during localization studies, contaminating enzyme activity from non-specific C-S lyases was interpreted as cystathionine β-lyase activity and that homocysteine can be moved between cellular compartments.

The cysteine lyases (C-S lyases) are a diverse group of enzymes that can cleave cystine to thiosulphate, pyruvate and ammonia [250]. Most of these enzymes have been found to be essentially inactive toward cystathionine [251–254]. Cystathionine β-lyase from spinach and *E. colonum* was inactive towards cystine [245, 247]. It would therefore appear that cystathionine β-lyase and the C-S lyases perform exclusive metabolic roles. Furthermore, an enzyme capable of cleaving cystine was not observed in extracts of *E. colonum* cell suspension cultures or plants [247]. This indicates that cystine catabolism may not be ubiquitous in plant metabolism.

Together the reactions of cystathionine β-lyase and cystathionine γ-synthase effect the transfer of the thiol moiety from the C_3 skeleton of cysteine to the C_4 skeleton of homocysteine in a process termed transsulphuration. Formation of homocysteine by direct sulph-hydration of *O*-phosphohomoserine has been proposed based on the studies in crude extracts of spinach [255, 256]. In addition, cystathionine γ-synthase had the capacity *in vitro* to form homocysteine by direct sulph-hydration [237, 238]. However, labelling studies in *Lemna paucicostata* showed the flux of [^{35}S] sulphate into cysteine, cystathionine and homocysteine (i.e. trans-sulphuration as opposed to direct sulph-hydration) accounted for more than 90% of homocysteine biosynthesis [257]. Similar results had been observed in the phylogenetically distinct algae *Chlorella sorokiniana* [258] leading to the conclusion that 'trans-sulphuration accounts for essentially all homocysteine biosynthesis in the plant kingdom' [259]. This has been confirmed following isolation of a cystathionine β-lyase deficient mutant of *Nicotiana plumbaginifolia* that required homocysteine or methionine for growth [260].

The final reaction of methionine biosynthesis, the methylation of homocysteine, is catalysed by methionine synthase (EC 2.1.1.14; Fig. 1). The methyl group is derived from ^{5}N-methyltetrahydrofolate in a cobalamin independent reaction [261–263]. It would appear that the triglutamate form of ^{5}N-methyltetrahydrofolate, not the monoglutamate form, is the methyl donor for *de novo* methionine biosynthesis [263–265].

Methionine synthase has been detected in pea leaves [264] and carrot [266] and partially purified from pea seeds [262]. A cDNA clone has been isolated from *Catharanthus roseus* encoding a polypeptide with an estimated M_r of 85 000 [265]. Methionine synthase has been located in the cytosol by subcellular fractionation of barley leaves [74] and immunoblotting [265].

S-*adenosylmethionine* (*AdoMet*) *biosynthesis*

AdoMet biosynthesis and metabolism will also be considered with methionine biosynthesis due to its regulatory influence on the aspartate pathway and its importance in methionine cycling.

Biosynthesis of AdoMet from methionine and ATP is catalysed by *S*-adenosylmethionine synthetase (EC 2.5.1.6; Fig. 1). This enzyme has been partially purified from pea seedlings [267] and purified to homogeneity from germinating wheat embryos [268] and *Glycine max* [269]. Genomic and cDNA *S*-adenosylmethionine synthesis clones, isolated from a wide variety of species [see for example 270–273] belong to a small multigene family composed of at least three independent genes.

In *G. max S*-adenosylmethionine synthetase was a dimer of M_r 110 000 [269] whereas in pea epicotyls [274] and wheat aleurone [275] the enzyme was dimer of M_r 174 000 and 181 000, respectively. Product inhibition by AdoMet was observed in *G. max* [269]. However, in pea seedlings this was only evident at unphysiological concentrations [267].

Following induction by gibberellic acid, two additional isoenzymes of *S*-adenosylmethionine synthetase could be separated by ion-exchange chromatography [274, 275]. These were homo and heterodimers formed by association of the products of one constitutive and one induced gene. Up-regulation was also observed following salt stress [271] and tissue wounding [276]. *Arabidopsis thaliana S*-adenosylmethionine synthetase, expressed primarily in the vascular tissue, appears to be required to provide high levels of AdoMet for lignification [277].

Regulation of methionine and AdoMet biosynthesis—an overview

As discussed above, cystathionine γ-synthase would appear to be regulated by repression and derepression.

However, the effect of cystathionine γ-synthase activity on the rate of methionine biosynthesis was determined *in vivo* using the irreversible active-site directed inhibitor propargylglycine [278]. Reduction in cystathionine γ-synthase activity by approximately 88% only resulted in a 18% decrease in methionine biosynthesis *in vivo* regulation of methionine biosynthesis [242], it was concluded that, in addition to the regulation of cystathionine γ-synthase activity other regulatory factors must operate [240].

AdoMet metabolism

The methionine component of AdoMet functions as the major methyl donor in plants [263], and as the precursor for polyamine [279] and ethylene [280–282] biosynthesis. Each of these processes will be considered briefly with reference to sulphur cycling.

The nucleoside adenosylhomocysteine (AdoHcy) is formed as a by-product following methylation by AdoMet. This is hydrolysed to homocysteine and adenosine in a reaction catalysed by adenosylhomocysteinase (EC 3.3.1.1). Hydrolysis is the only known pathway of AdoHcy metabolism [283]. The enzyme has been partially purified from spinach leaves [284] and purified to homogeneity from lupin (*Lupinus luteus*) [285]. In lupin the enzyme was a homodimer of M_r 110 000 and showed optimum activity between pH 8.5 and 9.0.

Homocysteine is re-methylated to methionine by methionine synthase as described previously. Therefore, it is apparent that AdoMet methylation does not result in a net loss of sulphur moiety of methionine. This was confirmed by following the fate of [^{35}S, U-^{14}C] methionine in *Lemna paucicostata* (Fig. 2) [241]. It can be seen in Fig. 2, that the flux of cystathionine into homocysteine is equal to the flux of methionine into protein, indicating protein synthesis to be the only major sink for methionine. It should also be noted that, while the formation of AdoHcy does not result in a net loss of methionine, the flux through this compound is approximately four times greater than the flux into protein.

In addition to methylation, AdoMet is used as the precursor for polyamine [279] and in some plant tissues ethylene [280–282] biosynthesis. In each case the C_4 skeleton of methionine is utilised for biosynthesis while the 5′-methylthioadenosine by-product (containing the methyl and sulphur moiety of methionine) is recycled to methionine (Fig. 2). Subsequently, formate and pyrophosphate are released from 5′-methylthioribose to form 2-keto-4-methylthiobutyrate which is converted to methionine by an aminotransferase [286]. Therefore, as a consequence of 5′-methylthioadenosine recycling, biosynthesis of the polyamines or ethylene does not result in a net loss of methionine [286]. This is also apparent from the fluxes in *Lemna paucicostata* [241].

ISOLEUCINE SYNTHESIS

Threonine deaminase

The first committed step of isoleucine synthesis is the dehydration and deamination of threonine to yield 2-oxobutyrate and ammonia, catalysed by threonine deaminase (also known as threonine dehydratase, EC 4.2.1.16). In microorganisms, two forms of the enzymes are present, one form is inhibited by threonine and is considered to be 'biosynthetic'. A second form is not subject to feedback inhibition and has been termed the 'biodegradative' form [287]. A model

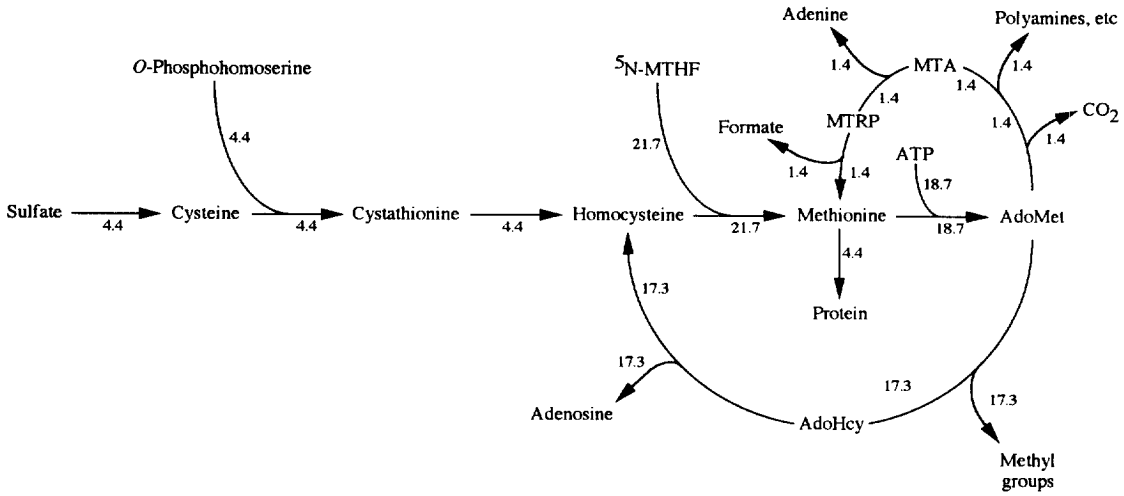

Fig. 2. Estimated fluxes through the major known pathways of methionine metabolism in *Lemna paucicostata*. Fluxes expressed as nmol per colony doubling. Abbreviated intermediates: ^{5}N-MTHF, ^{5}N-methyltetrahydrofolate; ATP, adenosine 5′ triphosphate; MTA, 5′-methylthioadenosine; MTRP, methylthioribose-1-phosphate; AdoMet, *S*-adenosylmethionine; AdoHcy, adenosylhomocysteine. Adapted from Giovanelli *et al.* [241].

for the homotropic cooperativity of the *E. coli* biosynthetic enzyme has recently been proposed [288]. The presence of the biosynthetic form of threonine deaminase in higher plants has been known for some time. The enzyme is subject to feedback inhibition by isoleucine [289–291], and is localised in the chloroplast [289]. In maize, two forms could be separated by ion exchange chromatography, but the properties of the enzymes were very similar, including the sensitivity to threonine [292]. A potential herbicidal compound 2-(1-cyclohexene-3(*R*)-yl-5-glycine has been shown to inhibit threonine deaminase competitively. The compound prevented the growth of maize cells and *Arabidopsis thaliana*, a process that could be reversed by 2-oxobutyrate and other intermediates in the pathways of isoleucine synthesis [293].

Mutants of tobacco have been isolated that require isoleucine for growth and have been shown to lack threonine deaminase [294, 295]. Complementation of the *Nicotiana plumbaginifolia* mutant with the *ilv*1 gene of *Saccharomyces cerevisiae* has confirmed the role of threonine deaminase in isoleucine synthesis [296]. A line of *Rosa* cells has been isolated that was resistant to the isoleucine analogue, *O*-methylthreonine, although the threonine deaminase was shown to insensitive to isoleucine, further studies were not carried out [297]. More recently a mutant (GM 11b) of the intact plant *Arabidopsis thaliana* has been isolated that was resistant to *O*-methylthreonine [298]. The resistance was controlled by a single nuclear dominant gene *omr*1, which was mapped to chromosome 3. The threonine deaminase in the mutant plant was shown to be 53-fold less sensitive to isoleucine than the wild type enzyme. The soluble amino acid content of the mutant, exhibited a 20-fold increase in the concentration of isoleucine. It was concluded that this high concentration of isoleucine, prevented incorporation of *O*-methylthreonine into protein, and hence conferred resistance of the mutant.

Samach *et al.* [299] isolated at 55000 polypeptide P3, that had been shown to be one of the most abundant proteins in tomato flowers, polyclonal antisera was raised and the gene isolated. Comparison of the amino acid sequence with threonine deaminase isolated from yeast and bacteria indicated a greater than 40% overall similarity and a near identity at the important catalytic regions. The first 80 amino acids at the amino terminal appeared to contain a transit peptide for transport into the chloroplast. Northern blot analysis indicated that threonine deaminase mRNA was present in at least 50-fold higher levels in sepals and 500-fold higher in the remainder of the flower, than in leaves, roots and seeds. The threonine deaminase protein was shown to accumulate in the parenchymal cells of petals, stamens and sepals. Samach *et al.* [299] argued that it was unlikely that the threonine deaminase protein was involved in isoleucine biosynthesis, but that it played an 'hitherto unknown structural role'. In a study on the response of leaves to mechanical wounding, abscisic acid, methyl jasmonate and wounding were shown to induce the synthesis of threonine deaminase mRNA in potato [300]. Later studies showed that aspirin, a mammalian cyclooxygenase-mediated reaction inhibitor, prevented the accumulation of threonine deaminase mRNA following wounding [301]. The deduced amino acid sequence of a cDNA clone encoding the enzyme isolated from chickpea, exhibited at 61% homology with the tomato sequence. The highest transcript levels were again detected in the flowers [302].

Following the unusual results concerning the regulation of threonine deaminase at the level of transcription, Szamosi *et al.* [303] carried out a detailed examination of the enzyme activity in tomato plants. High levels of activity were detected in sepals, which were six-fold higher than in young leaves. This difference did not however account for the 50- to 500-fold higher mRNA levels detected by Samach *et al.* [299] and would indicate that the majority of the protein is enzymically inactive in flowers. In older senescing leaves, Szamozi *et al.* [303], demonstrated that there was a sudden increase in threonine deaminase activity. The new enzyme activity was virtually insensitive to inhibition by isoleucine, had a higher pH optimum and a lower molecular mass. The authors argued that in senescing leaves, a biodegradative form of the enzyme was required which could metabolise threonine and serine derived from protein breakdown and liberate ammonia for glutamine synthesis and transport.

Acetohydroxyacid synthase

Acetohydroxyacid synthase (AHAS; also known as acetolactate synthase, EC 4.1.3.18), catalyses two parallel reactions, condensation of 2-oxobutyrate and pyruvate to yield acetohydroxybutyrate and condensation of two molecules of pyruvate to form acetolactate. AHAS requires divalent metal cations, thiamine pyrophosphate and FAD for full activity and enzyme stability [304] The enzyme has been purified from maize and shown to have a monomeric form of M_r 55000 [305, 306], which may aggregate into a number of oligomeric forms up to a maximum M_r of 440000. In *Arabidopsis thaliana*, the enzyme exists as a dimer with a subunit M_r of 65000 [307], in barley there are two 'L' and 'S' forms, with native M_rs of 440000 and 200000 and a subunit of 58000 [308]. In *Brassica napus* a dimer was again detected, but two subunits of 65000 and 66000 were identified by Western blot analysis [309]. In wheat, three subunits of 58000, 57000 and 15000 were detected, with a native M_r of 128000 [310].

Plant AHAS is inhibited individually by each of the three branched chain amino acids or co-operatively by leucine plus valine [311, 312]. The inhibition by the end product amino acids in maize requires the enzyme to be in at least the dimer form [306]. Two forms of the enzyme have been identified in ethiolated barley shoots and *Brassica napus* cotyledons, following ion

exchange chromatography, small differences in the sensitivity of the enzymes to inhibition by leucine, valine and isoleucine were detected [308, 309]. Kinetic studies have indicated that the inhibition by the three branched chain amino acids in mixed competitive with respect to pyruvate [313–315]. In *Arabidopsis thaliana*, the enzyme is subject to 60% inhibition of 1 mM valine plus leucine. However, when the gene encoding the enzyme was expressed in *E. coli*, the enzyme was no longer subject to inhibition. The authors proposed that there may be a second subunit of the enzyme protein that may be involved in the binding of the branched chain amino acids [307].

The branched chain amino acids have also been shown to inhibit the growth of plants. Valine inhibited the growth of barley embryos, which was relieved by isoleucine, but increased the leucine. Leucine inhibition however required the presence of both valine and isoleucine for reversal [316]. Similar results were obtained with *Spirodela polyrhiza*. The authors argued that the combination of leucine and valine inhibited AHAS activity and prevented the synthesis of isoleucine. Isoleucine also inhibited growth, and the soluble pool of valine increased, it was proposed that this inhibition was not due to action on AHAS activity, but the prevention of the conversion of valine to leucine [317]. This suggestion has been confirmed by recent data on leucine biosynthesis [318].

Seven valine resistant tobacco protoplast mutants were regenerated into whole plants and four were found to have defects in amino acids uptake [319]. When AHAS was isolated from the three additional mutants, the regulatory characteristics of the enzyme were shown to be altered and the activity was less sensitive to inhibition by leucine and valine [320]. Wu *et al.* [321] isolated a mutant of *Arabidopsis thaliana* that was resistant to growth inhibition by valine, but not isoleucine and leucine. AHAS isolated from the mutant was less sensitive to all three branched chain amino acids. This data suggests that the growth inhibitory action of isoleucine and leucine is not due to a direct action on AHAS activity. The AHAS gene of valine resistant tobacco has been cloned and sequenced and the mutation shown to be due to a change of serine and leucine at position 214 [322].

Utilising the yeast gene as a heterologous probe, Mazur *et al.* [323] isolated genomic clones encoding the AHAS gene in *Arabidopsis thaliana* and *Nicotiana tabacum*. The deduced amino acids sequences of the two proteins were aligned with that of yeast and *E. coli*; 84% of the amino acid and 73% of the nucleotides were conserved the two plant genes, both of which lacked introns. Although putative chloroplast transit sequences were identified, there was little homology between the two plant peptides.

Two genes encoding AHAS have been detected in tobacco and designated *SurA* and *SurB* [324]. In all the organs and development stages tested, the relative expression of the two AHAS genes was co-ordinately modulated. The transcripts of *SurB* was always present in higher levels than that of *SurA*, but a constant ratio of gene expression was always maintained, indicating a common regulatory mechanism. *In situ* hybridisation studies indicated an ubiquitous expression of AHAS mRNA throughout most tissues, with the greatest accumulation in metabolically active or rapidly dividing cell types of root, stem and flowers. Such expression levels confirm the requirement for the biosynthesis of branched chain amino acids in rapidly growing areas of the plant. The AHAS gene family of the allotetraploid of cotton *Gossypium hirsutum* [325] has been cloned and characterized. Four of the six genes are organized as tandem pairs, in which the genes are separated by only 2–3 kb. Among the four genes in the two tandem pairs, at least two are functional. These genes exhibited either low level constitutive low level expression or high specific expression in reproductive tissues [326].

Brassica napus is an allotetraploid, believed to have originated from a cross between *B. campestris* the A genome donor and *B. oleracea* the C genome donor. Five AHAS genes have been identified in *B. napus* of which four been have cloned and sequenced [327]. *AHAS*1 and *AHAS*2 were constitutively expressed in a wide range of somatic and reproductive tissues. The strong sequence homologies of both coding and non-coding regions of *AHAS*1 and *AHAS*3 indicated a common origin and function [327, 328]. *AHAS*2 was shown to be structurally distinct from the other genes and was only expressed in mature ovules and extra embryonic tissue of immature seeds. *AHAS*4 was not expressed in any tissue studied and probably represents an pseudogene [328].

Three major classes of compounds have been shown to be potent inhibitors of AHAS, sulphonylureas, imidazolinones and triazolopyrimidines. The chemistry of these compounds has been described in detail [6, 7, 329]. The compounds all have strong herbicidal activity at very low application rates (2–75 g hectare^{-1}). The kinetics of enzyme inhibition is beyond the scope of this review, but have been studied in depth [310, 313, 330–332]. K_i values for the sulphonylurea herbicides are in the region of 5–20 nM, whilst the K_i for the imidazolinone herbicides are in the range of 2–60 μM. Despite the differences in inhibitor constants listed above, both classes of herbicides have similar application rates. The discrepancy between the *in vivo* and *in vitro* effectiveness, may be explained by the greater tendency of the herbicides to induce irreversible loss of enzyme activity [333, 334].

The application of AHAS inhibitors induces the accumulation of the substitute 2-oxobutyrate and the transamination production 2-aminobutyrate and it was proposed that this accumulation caused the death of micro-organisms and plants [335]. However, Shaner and Singh [336] were able to show in maize, using the herbicide imazaquin, that there was no correlation between the pool sizes of 2-oxobutyrate and 2-aminobutyrate and the growth inhibitory effects. The authors argued that the herbicidal action was due

to a starvation of branched chain amino acids, in particular valine and leucine.

Höfgen *et al.* [337] transformed potato with a chimaeric antisense gene under the control of the CAMV 35S promotion. The AHAS activity in one transformant was reduced to 14.4% of normal activity and the plants exhibited typical symptoms of herbicide treatments. Although low levels of 2-aminobutyrate were detected in the antisense transformed plants, there was no accumulation of 2-oxobutyrate. The soluble acid pools were increased in the antisense transformed plants, but were not as high as wild type plants treated with the herbicide Scepter. Similar increases in soluble amino acids due to protein breakdown have been reported previously with the herbicide Chlorsulphuron [338]. An interesting observation was that the growth inhibitory effects detected in the antisense transformed plants could not be reversed by the addition of isoleucine, leucine and valine, although some reversal was obtained with casamino acids. The authors concluded that an imbalance in the soluble amino acids in particular those of lysine and threonine may be responsible for the growth inhibition.

There has been considerable interest in obtaining plants that are resistant to the toxic effects of the AHAS specific herbicides. Mutant lines of tobacco [240], *Arabidopsis thaliana* [342], soybean [336], canola [342], *Gossypium hirsutum* [343], *Datura innoxia* [344] and maize [345, 346] have been isolated that show varying degrees of resistance to the herbicides. A resistant biotype of *Sonchus oleraceus* has developed in Australia following field selection with chlorsulphuron for 8 consecutive years [347].

The transformation of the *Arabidopsis thaliana* gene (*csr*-1) coding for the mutant AHAS [340] has been shown to confer resistance in tobacco to the sulphonylurea herbicides [348, 349, 350]. Tourneur *et al.* [351] have used the P70 promoter and the *csr*-1 mutant gene to produce transgenic tobacco that have a 1500-fold increase in resistance to chlorosulphuron. The activity of AHAS was increased 12-fold and the plants were also resistant to the external supply of valine. However, there was no increase in the soluble level of valine, leucine or isoleucine indicating that the enzyme is not the rate limiting step in the pathway. When the mutant gene of *Brassica napus*, *AHAS3R*, that coded for resistance to chlorosulphuron was introduced into tobacco, the gene was expressed under the control of the CAMV 35S promoter and leader sequences. However, evidence was presented to suggest that the total level of AHAS activity in the leaves was regulated by a post-translational mechanism [352]. This result is in conflict with that described previously [351].

Using a three-dimensional model of the AHAS-inhibitor complex, Ott *et al.* [330], rationally designed substitution mutations in the amino acid sequence of the enzyme protein. Following biochemical analysis of the mutant AHAS enzymes expressed in *E. coli*, the expression of the selected mutant AHAS genes in tobacco was shown to confer herbicide resistance [330].

The alteration in the amino acid sequence of the sulphonylurea resistant enzyme has been shown to be a single point mutation affecting proline 197 in *Arabidopsis thaliana* [348] and 196 in tobacco [353]. Imidazolinone resistance has been shown to be due to a change in serine at position 653 to aspartate in *Arabidopsis thaliana* [354] and alanine at position 56 to threonine in maize [355]. Bernasconi *et al.* isolated the AHAS gene from naturally occurring imiadazolinone tolerant lines of cocklebur, AHAS isolated from the Missouri line was highly insensitive to all the herbicide classes. Sequencing indicated that the mutation was due to a change at position 552 of tryptophan to leucine. The normal AHAS cDNA from cocklebur, fused with glutathione *S*-transferase was functionally expressed in *E. coli*. All the possible point mutations affecting tryptophan at position 552 were investigated by site directed mutagenesis. Only the tryptophan to leucine mutation yielded an active enzyme which was resistant to a wide range of AHAS directed herbicides. The authors concluded that the study validated the use of laboratory models to predict mutations that might occur in natural populations. A second naturally occurring resistant mutant of cocklebur has now been isolated that has a change of alanine to valine at position 183 [357].

Acetohydroxy acid isomeroreductase

Acetohydroxy acid isomeroreductase (AHRI, EC 1.1.1.86; also known as ketol acid reductoisomerase, KARI) catalyses an unusual two step reaction in the synthesis of isoleucine. 2-Aceto-2-hydroxybutyrate is converted to 3-hydroxy-3-methyl-2-oxopentanoate in an alkyl migration, which is followed by an NADPH dependent reduction to yield 2,3-dihydroxy-methylvalerate. In a similar reaction in the synthesis of leucine and valine, the enzyme also catalyses the conversion of 2-acetolactate to 2,3-dihydroxy-isovalerate [358–361]. The enzyme has been purified to homogeneity from spinach chloroplasts and shown to be a tetramer with a subunit M_r of 59 000. As three distinct peaks of AHRI activity could be eluted from ion exchange columns, it was postulated that the native enzyme was a combination of non-identical subunits with similar M_r [359]. In later studies when the gene for the spinach enzyme was over-expressed in *E. coli*, it was concluded that AHRI is a dimer of identical subunit [362]. The enzyme has also been purified from etiolated shoots of barley and was shown to be a homodimer with a subunit M_r of 59 000 [363].

Following the identification of a range of potent herbicidal inhibitors of the previous enzyme (AHAS) in the pathway of isoleucine synthesis, the kinetic properties of AHRI have been studied in detail. Both the spinach and bacterial enzymes obey an ordered mechanism in which NADPH binds first followed by

the substrate (2-acetolactate or 2-aceto-2-hydroxybutyrate). Inhibition studies using the inactive substrate analogue 2-hydroxy-2-methyl-3-oxopentanoate, however, indicated that there was some flexibility in the plant enzyme active site and that the substrate and NADPH could bind randomly [362]. Although the K_m for 2-aceto-2-hydroxybutyrate for the spinach chloroplast enzyme was shown to be the same as 2-acetolactate (10 μM), the V_{max} for the former was six-fold higher. AHRI purified from barley was found to have a K_m for 2-acetolactate of 38 μM and for 2-aceto-2-hydroxybutyrate of 4.3 μM, with corresponding V_{max} values of 0.16 μmol min^{-1} mg^{-1} and 1.8 μmol min^{-1} mg^{-1} [363]. Thus, the data from spinach and barley, both indicated a partitioning between the rates of synthesis of the branched chain amino acids [362, 363].

AHRI from plants has been shown to be subject to potent inhibition by Hoe 704 (2-methylphosphinoyl-2-hydroxyacetic acid) and IpOHA (*N*-hydroxy-*N*-isopropyloxanate) [364, 365], although both compounds only act as weak herbicides when compared to the AHAS inhibitors [366]. Using 2-acetolactate and 2-aceto-2-hydroxybutyrate as substrates the K_i values of Hoe 704 for the barley enzyme were 0.4 and 0.19 μM, respectively. Attempts to obtain K_i values for IpOHA were unsuccessful due to the slow binding of the inhibitor. However, an I_{50} value of IpOHA for the barley enzyme suggested that it was a more potent inhibitor than Hoe 704 [363]. In a similar series of experiments with spinach AHRI, IpOHA and Hoe 704 were shown to act as competitive tight binding inhibitors. However, the time needed to achieve 90% inhibition was extremely long (in the order of hours) [367].

A full length cDNA clone encoding the chloroplast AHRI has been isolated from a spinach library. The derived amino acid sequence indicated that the precursor protein contained 595 amino acids including a transit peptide of 72 amino acids. The deduced amino acid sequence exhibited a 23% identity with the deduced sequences of AHRI isolated from *E. coli* and *Saccharomyces cerevisiae* [368]. Analysis of a full length cDNA clone encoding AHRI from *Arabidopsis thaliana*, indicated that the spinach and *Arabidopsis thaliana* sequences were conserved in the mature protein regions, but were divergent in the transit peptide and processing sites [369]. A full length genomic clone of the *Arabidopsis thaliana* AHRI gene was shown to contain nine introns. Southern blot analysis of both the *Arabidopsis thaliana* and spinach genes indicated that they existed as single copies [370].

Alignment of the prokaryotic and plant sequences of AHRI indicated the presence of 5 conserved regions designated domains I, II, III, IV and V. Domain I was proposed to be the NADPH binding site. Mg^{2+}, which has a very high affinity for the enzyme (K_m, 6 μM), was shown to bind to both domains III and IV. Dumas *et al.* [371] proposed that domain III plays a role in the reductive half reaction, whereas domain IV is involved in the isomerisation half reaction.

Dihydroxy acid dehydratase

Dihydroxy acid dehydratase (2,3-dihydroxy acid hydrolase, EC 4.2.1.9) catalyses the fourth step in isoleucine synthesis, which involves the dehydration and tautomerisation of 2,3-dihydroxymethylvalerate to yield 2-oxo-ethylvalerate. The enzyme has been purified to homogeneity from spinach leaves, where the enzyme exists as a dimer with a subunit M_r of 63 000. Spinach dihydroxy acid dehydratase contains a [2Fe-2S] cluster which is a novel finding for enzymes of the hydrolyase class [372]. The enzyme was also independently purified from spinach and shown to have a subunit M_r of 62 000. A range of novel compounds were designed and synthesised as potential inhibitors; 4-fluoro-2,3-dihydroxyisovaleric acid was shown to be the most potent and also to have herbicidal activity [373]. A mutant of *Datura innoxia* requiring isoleucine and valine for growth has been shown to lack dihydroxy acid dehydratase [374].

Aminotransferase

The final step in the synthesis of isoleucine is the transamination of 2-oxo-3-methylvalerate, although the oxo acid may also be involved in the synthesis of medium chain fatty acids [375]. Two forms of branched chain amino acid aminotranferases (EC 2.6.1.42) have been detected in barley [376] and soybean [377]. In barley, the two forms have the same native M_r (95 000), whereas in soybean, M_rs of 69 000 and 93 000 were determined. In barley, using glutamate as the amino donor, the relative of oxo acid utilisation were 2-oxoisocapraote > 2-oxo-3-methylvalerate > 2-oxoisovalerate [378].

EPILOGUE

In the light of the large number of experiments carried out with transgenic plants in the last five years, it is now interesting to reassess the five conclusions listed in the Introduction that were originally proposed by Giovanelli *et al.* [13].

1. In the majority of the plants studied, the insertion of an unregulated aspartate kinase gene has led to an increased flux through the pathway, suggesting that the enzyme does exert some control. The one clear exception is the recent study on *Arabidopsis thaliana* [85], the properties of which may be more closely related to *Lemna paucicostata*.
2. All studies have confirmed that the key regulatory step in the pathway is at dihydrodipicolinate synthase.
3. The majority of transgenic plants have shown that after aspartate kinase, homoserine dehydrogenase and threonine synthase are not involved in the regulation of threonine synthesis.

4. No transgenic plants have yet been constructed that have a significant increase in soluble methionine, suggesting that there is an important regulatory step after phosphohomoserine. As yet molecular studies have not confirmed that the gene encoding cystathionine-γ-synthase is subject to regulation by methionine.
5. Studies on the pathway from threonine to isoleucine have made tremendous progress, almost totally due to the commercial importance of the herbicides that target the enzyme acetohydroxyacid synthase. However the key regulatory steps is still thought to be that controlled by the threonine deaminase enzyme.

We hope that in the next five years, transgenic crop plants will be available to farmers that contain elevated levels of all the aspartate derived amino acids, lysine, threonine, isoleucine and methionine in the seed and will be therefore of tremendous nutritional value.

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