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Regulatory Structure of the Biosynthetic Pathway for the Aspartate Family of Amino Acids in *Lemna paucicostata* Hegelm. 6746, with Special Reference to the Role of Aspartokinase

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ABSTRACT

Comprehensive studies were made with *Lemna paucicostata* Hegelm. 6746 of the effects of combinations of lysine, methionine, and threonine on growth rates, soluble amino acid contents, aspartokinase activities, and fluxes of 4-carbon moieties from aspartate through the aspartokinase step into the amino acids of the aspartate family. These studies show that flux *in vitro* through the aspartokinase step is insensitive to inhibition by lysine or threonine, and confirm previous *in vitro* data in establishing that aspartokinase *in vivo* is present in two orders of magnitude excess of its requirements. No evidence of channeling of the products of the lysine- and threonine-sensitive aspartokinases was obtained, either form of the enzyme alone being more than adequate for the combined *in vivo* flux through the aspartokinase step. The marked insensitivity of flux through the aspartokinase step to inhibition by lysine or threonine strongly suggests that inhibition of aspartokinase by these amino acids is not normally a major factor in regulation of entry of 4-carbon units into the aspartate family of amino acids. Direct measurement of fluxes of 4-carbon units demonstrated that: (a) Lysine strongly feedback regulates its own synthesis, probably at the step catalyzed by dihydrodipicolinate synthase. (b) Threonine alone does not regulate its own synthesis *in vivo*, thereby confirming previous studies of the metabolism of [^{14}C]threonine and [^{14}C]homoserine in *Lemna*. This finding excludes not only aspartokinases as an important regulatory determinant of threonine synthesis, but also two other enzymes (homoserine dehydrogenase and threonine synthase) suggested to fulfill this role. Complete inhibition of threonine synthesis was observed only in the combined presence of accumulated threonine and lysine. The physiological significance of this single example of apparent regulation of flux at the aspartokinase step, albeit under unusually stringent conditions of aspartokinase inhibition, remains to be determined. (c) Isoleucine strongly inhibits its own synthesis, probably at threonine dehydratase, without causing compensatory reduction in threonine synthesis. A fundamentally changed scheme for regulation of synthesis of the aspartate family of amino acids is presented that has important implications for improvement of the nutritional contents of these amino acids in plants.

Recent *in vitro* studies of aspartokinase activity (EC 2.7.2.4) in *Lemna paucicostata* (22) demonstrated that the maximum capacity (V_{max}) to catalyze this reaction exceeds the rate at which it is required to function *in vivo*. Estimations from such studies of the rates of aspartokinase *in vivo* led us to question the widely held beliefs (35, 40–42) that this enzymic step is the overall 'rate-limiting'[2] one for entry of 4-carbon units into the aspartate family of amino acids, and that feedback inhibition of aspartokinases by lysine and threonine is a major factor in regulating flux into the aspartate family of amino acids. The current work extends our understanding of the regulatory role of aspartokinase by means of comprehensive studies of the effects of aspartate-derived amino acids on the growth, amino acid composition, aspartokinase activities, and *in vivo* fluxes into the aspartate family of amino acids. These data now provide definitive evidence that the step catalyzed by aspartokinase is not normally an important site for regulation of entry of 4-carbon units into the aspartate family of amino acids. The work also confirms a previous report (21) that threonine alone does not regulate its own synthesis and further demonstrates that synthesis of this amino acid is strongly inhibited only in the combined presence of threonine and lysine. These findings lead to fundamental changes in our understanding of the regulatory patterns of biosynthesis of the aspartate family of amino acids and have important implications for improving the nutritional contents of lysine, threonine, and methionine in crop plants.

MATERIALS AND METHODS

General Methods

Methods for ion exchange chromatography have been described (21). The following solvents were used for paper chromatography: solvent A, 2-propanol:88% HCOOH:H_2O (7:1:2, v/v); solvent B, 1-butanol:propionic acid:water (250:124:175, v/v); solvent C, 95% ethanol:7.25 mM NH_4OH (90:10, v/v). Paper chromatograms were run descending, and unless stated otherwise, were allowed to develop overnight to allow the solvent to almost reach the end of the paper. Polyamide plates for TLC were obtained from Pierce. The source of other chemicals has been described (21). Methods

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[2] As generally recommended (30, 39), the term 'rate-limiting' ('pacemaker,' 'bottle neck,' etc.) is avoided, and is used here only for the sake of accurate reporting of other workers' conclusions.

for determination of soluble (45) and total (17) protein have been described.

Growth of Plants

Plants (*Lemna paucicostata* Hegelm. 6746) were grown in standard medium (17) with 20 μM sulfate. Except where noted, plants growing with amino acid supplements were pregrown with the supplement under study for no less than four doublings to achieve a steady state. The number of fronds and the volume of medium were adjusted so that no more than 20% depletion of supplement from the medium occurred. Where not determined experimentally, amino acid uptakes were estimated on the basis of parameters reported by Datko and Mudd (16).

Assay of Aspartokinase

Enzyme extracts for determination of aspartokinase activities were prepared as described (22) from 80 to 270 fronds, and assayed by the standard assay in the absence of NH_2OH (22). Aspartokinase activity was measured in both pellet and combined supernatant fractions and, for plants grown under every culture condition examined, such activity was recovered predominantly (78–95%) in the combined supernatant fraction. For determinations of the relative proportions of aspartokinase inhibited by lysine and threonine, activities were measured in the presence of NH_2OH (22). Thus, lysine-sensitive aspartokinase was determined from the decrease in activity resulting from the addition of 5 mM lysine. The relatively small proportion of threonine-sensitive activity was calculated from the decrease in aspartokinase activity measured in the presence of 5 mM lysine plus 5 mM threonine compared to that determined in the presence of 5 mM lysine alone. For example, for plants growing with 66 μM lysine, aspartokinase activity assayed in the presence of 5 mM lysine was 20% of that of the uninhibited activity, yielding a value of 80% lysine-sensitive activity. Aspartokinase activity assayed in the presence of lysine plus threonine was 8% of that determined with lysine alone. Therefore, threonine-sensitive activity = 92% × 20% = 18% of the total activity.

Assay of Tissue Contents of Soluble Amino Acids

Plants (140–300 fronds) were harvested, washed four times with standard medium to ensure removal of any residual amino acid from the medium, and homogenized in ice-cold methanol. The homogenate was fractionated into a methanol-water-soluble fraction and a chloroform-methanol-insoluble pellet as described (37). The pellet was washed sequentially with methanol and twice with 10% TCA. After removal of TCA by ether extraction, the washes were combined with the methanol-water-soluble fraction, and the combined fraction was subjected to automated amino acid analysis by conventional ion exchange chromatography (ninhydrin determination) on an LKB 4150 α-amino acid analyzer (LKB Biochrom Ltd., Cambridge, England).

The small content of soluble lysine in control plants was determined as follows: Control plants (3440 fronds, 842 colonies) were harvested, washed once with standard medium, and homogenized in 10% TCA containing 1.35×10^5 dpm (0.175 nmol) of authentic L-[U-^{14}C]lysine (Amersham) to serve as marker, and to permit correction for losses of lysine. The homogenate was separated into TCA-soluble and -insoluble fractions by centrifugation. The TCA-insoluble fraction was washed with 10% TCA, and the wash and TCA-soluble fraction combined. This solution was extracted with ether, then chromatographed on Dowex 50-NH_4^+ to yield a basic amino acid fraction. ^{14}C was determined in an aliquot of this fraction to measure recovery of lysine (89%). The remainder of the fraction was evaporated to dryness to remove NH_4OH, dissolved in water, and divided into two aliquots. One aliquot (A) was assayed directly on the amino acid analyzer (with ninhydrin). The other aliquot, after further addition of L-[U-^{14}C]lysine (1.35×10^5 dpm, 0.175 nmol), was incubated with 1.8 μg of L-lysine decarboxylase (Sigma Chemical Company, type VIII purified from *Bacterium cadaveris*) in a final volume of 40 μL of 50 mM Na acetate (pH 5.8). Incubation was for 20 min at 25°C. The reaction was terminated by addition of 1 μL of 0.1 M *p*-chloromercuriphenylsulfonic acid, and a 10 μL sample was chromatographed with solvent C for 18 h. The distribution of ^{14}C on the chromatogram showed more than 95% conversion of lysine (peak at 6 cm from origin) to cadaverine (peak at 23–25 cm from origin), demonstrating essentially complete decarboxylation of lysine. Automated amino acid analysis of the reaction mixture remaining provided a measure of the amount of ninhydrin-positive material resistant to lysine decarboxylase. This determination showed that 77% of the total value for lysine given by direct assay of aliqout A was sensitive to lysine decarboxylase. The amount of soluble lysine (nmol/frond) was calculated from the expression: nmol/frond in aliquot A × 77%/89%. Corrections were made for the relatively small amounts of lysine added as [^{14}C] lysine.

Analysis of ^{14}C Compounds

Plants were harvested and fractionated as described into TCA-soluble and -insoluble fractions (21).

CO_2

CO_2 was analyzed as described elsewhere (21).

TCA-Soluble Fraction

To the TCA-soluble fraction were added known amounts of L-[3-^{3}H]threonine, L-[4,5-^{3}H]isoleucine, L-[^{3}H$_3$C]methionine, and L-[4,5-^{3}H]lysine. L-[2,3-^{3}H]Aspartate was also added in those experiments in which soluble [^{14}C]aspartate was determined. After removal of TCA by ether extraction, the solution was adjusted to pH 6 to 7 (bromcresol purple), and fractionated at 4°C on a column of Dowex-NH_4^+. The fraction not retained on the column contained neutral and acidic amino acids together with nonamino acids. The fraction eluted from the column contained basic amino acids and the sulfonium compounds, AdoMet[3] and *S*-methylmethionine.

[3] Abbreviations: AdoMet, *S*-adenosylmethionine; *S*-methylmethionine, L-methionine methylsulfonium salt; MR, multiplication rate as defined by Hillman (26) = 7224/doubling time in h.

The basic fraction was evaporated to dryness and the residue boiled for 30 min with 0.1 M ammonium acetate (pH 5.0). This procedure converts AdoMet to homoserine and 5′-methylthioadenosine, leaving *S*-methylmethionine and lysine unchanged (20). After adjustment of pH to between 6 and 7, the solution was chromatographed at room temperature on Dowex 50-NH_4^+. Material not retained by the column was chromatographed with solvent B containing 10 mM 2-mercaptoethanol in order to resolve homoserine and 5′-methylthioadenosine from each other and from any ^{14}C contaminants. An upper estimate of ^{14}C in the 4-carbon moiety of AdoMet was given by the relative proportion of ^{14}C on the chromatogram corresponding to the R_F for homoserine (0.3). Material eluted from the column with 3 N NH_4OH was subjected to chromatography with solvent B to resolve *S*-methylmethionine and lysine. The area on the chromatogram indicated by marker [^3H]lysine was eluted, further purified by chromatography with solvent B for 45 h, and an upper estimate of the amount of [^{14}C]lysine calculated from the ratio of $^{14}C/^3H$ in the discrete peak obtained. In some experiments, the *S*-methylmethionine eluate obtained after chromatography with solvent B was incubated at 100°C for 1 h in 0.05 M K borate (pH 8.3), to convert *S*-methylmethionine to homoserine (and dimethylsulfide) (13). After addition of marker [^3H]homoserine, the incubation mixture was subjected to chromatography on Dowex 50-H^+, and the fraction (NH_4OH eluate) containing homoserine chromatographed with solvent A. The amount of ^{14}C originally present in the 4-carbon moiety of *S*-methylmethionine was calculated from the amount of ^{14}C comigrating with marker [^3H]homoserine.

The fraction (described above) containing neutral and acidic amino acids and nonamino acids was applied to a column of Dowex 50-H^+. Radioactivity not retained by the column was a measure of ^{14}C in nonamino acids. Radioactivity retained by the column was eluted with NH_4OH, and fractionated by chromatography on Dowex 1 into neutral and acidic amino acids (21). In certain experiments, the latter fraction was chromatographed with solvent A, and [^{14}C]aspartate determined from the ratio of $^{14}C/^3H$ in the peak of radioactivity corresponding to [^3H]aspartate. The neutral amino acid fraction was subjected to chromatography with solvent B and ^{14}C in methionine, threonine, and an upper limit of that in homoserine, determined as described (21). [^{14}C]Isoleucine was further purified as the dansyl derivative, primarily to remove any traces of [^{14}C]leucine. Derivatization and TLC of dansyl derivatives were performed according to Macnicol (33), except that the dried products were extracted with ethyl acetate (43) (instead of chromatographed on Porapak Q [33]) prior to chromatography in benzene:acetic acid (9:1, v/v). Dansyl isoleucine, detected on the TLC plate by fluorescence and as tritiated marker, was eluted with 95% ethanol:1% triethylamine for determination of $^{14}C/^3H$.

TCA-Insoluble Fraction

The TCA-insoluble fraction was hydrolyzed in mercaptoethanesulfonic acid (20). L-[2,3-^3H]Aspartate and the other authentic tritiated compounds listed under "TCA-Soluble Fraction" were added to the hydrolyzate. The solution was deacidified by chromatography on either Rexyn 203 (20) or Dowex 50-H^+ and basic, neutral, and acidic amino acid fractions isolated essentially as described for soluble amino acids. The basic fraction was chromatographed on paper with solvent B for 30 h. Material moving with marker [^3H]lysine was eluted and chromatographed with solvent C for 50 h. Values for [^{14}C]lysine were calculated from the ratio of $^{14}C/^3H$ in the peaks of radioactivity obtained after chromatography with the latter solvent. Values for ^{14}C in the neutral amino acids were determined as described for soluble amino acids. The acidic amino acid fraction was chromatographed with solvent A to yield two peaks of ^{14}C, one comigrating with [^3H]aspartate, the other with the same mobility as authentic glutamate. [^{14}C]Aspartate was determined from the ratio of $^{14}C/^3H$ in the aspartate peak; [^{14}C]glutamate was determined from the amount of ^{14}C in the glutamate peak relative to that in the aspartate peak.

Calculation of Results

The amount of radioactivity in a given compound was usually expressed as a percent of the total radioactivity taken up by plants, *i.e.* the sum of radioactivity in TCA-soluble and -insoluble fractions and CO_2. For most determinations, known amounts of authentic tritiated amino acids were added to the TCA-insoluble and -soluble fractions and amounts of ^{14}C in each amino acid calculated from the ratio of $^{14}C/^3H$ in the purified amino acid. For the sulfonium compounds, where authentic tritiated compounds were not added, nonspecific losses during paper chromatography were corrected for by determination of the proportion of radioactivity accompanying a particular compound during a given procedure, calculated on the basis of the total radioactivity recovered during that procedure. The amount of radioactivity relative to the original total was calculated as the product of the fractional contributions for that compound during successive analytical steps. Results obtained by this method were in good agreement with those obtained by correction of any losses based on recovery of authentic tritiated compounds. Values were reported as upper limits when the radiopurity of these compounds was not established.

RESULTS

Uptake of Amino Acid Supplements, and Effects on Soluble Amino Acid Pools

To attain optimal conditions for detecting any effects of amino acid supplements on growth, on levels of aspartokinase, and on feedback regulation, it was desired to provide concentrations of supplemental amino acids that allowed for uptake of each amino acid in amounts that: (a) were comparable to the amounts normally synthesized by the plants, as indicated by the contents of the appropriate protein amino acids, and (b) resulted in an appreciable increase in the tissue soluble pool size of the amino acid in question. The concentrations of medium supplements necessary to provide uptake of these amounts were initially estimated by use of the measured parameters of the transport systems for neutral and basic amino acids by *Lemna*, taking into account any mutual inhibition of transport (16). These concentrations were then

Table I. *Uptakes of Amino Acids Relative to Estimated Amounts Normally Synthesized*

Cultures were allowed to grow for 1.3 to 2.7 doublings in the presence of radioactive amino acid. Amino acid uptakes were determined from combined radioactivity in TCA-soluble and -insoluble fractions (16). Depletion of amino acids from the medium was no more than 2% for lysine and 16% for the other amino acids. Values in parentheses are the standard errors of two determinations; all other values are single determinations. Uptake, in nmol/frond·doubling, was calculated as described (21) and expressed as a multiple of the appropriate protein amino acid(s) content. Thus, uptake (nmol/frond·doubling) of lysine and methionine were expressed relative to amounts (nmol/frond) of protein lysine and methionine, respectively. Uptake of threonine was expressed relative to the sum of protein threonine and its product, isoleucine. Protein amino acids of experimental cultures were estimated by multiplying the protein amino acid content of control cultures by the ratio of total protein content of the experimental culture/total protein content of control culture. The following values of protein amino acids of control cultures (nmol/frond) were used: protein methionine, 1.0 (23); protein threonine 4.5 (21); protein isoleucine, 3.4 (21). A value of 4.5 nmol/frond protein lysine in control cultures was calculated by multiplying the value for protein threonine (4.5 nmol/frond) by the mean value of 1.0 (SE, 0.06) for the ratio of protein [^{14}C]lysine/protein [^{14}C]threonine in control cultures (Table IV). The following respective ratios of total protein content in experimental/control cultures (SE; number of determinations) were determined for plants growing under the following conditions (listed from top to bottom of table): 1.3 (0.19; 2), 0.51 (0.05; 3), 0.48 (0.05; 4), 0.87 (0.05; 2), 1.2 (0.22; 2), 0.54 (0.04; 2), 0.55 (0.09; 3), 0.67 (0.08; 2), 0.67 (0.03; 3), 1.0 (0.2; 2), 0.70 (0.08; 3).

Amino Acid Supplement (μM)	Amino Acid Uptake		
	Lysine	Threonine	Methionine
		-fold[a]	
Lysine (66)	1.8		
Threonine (3.5)		1.0	
Threonine (8.0)		3.5 (0.25)	
Methionine (1.7)			2.8
Lysine (75), methionine (2.1)	1.2		3.1
Threonine (5.0), methionine (0.8)		1.0	1.5
Threonine (9.1), methionine (0.8)		1.7 (0.19)	1.3 (0.15)
Threonine (10), methionine (3.4)		1.0	3.3 (0.33)
Threonine (18), methionine (3.4)		1.7	3.2
Lysine (85), threonine (12), methionine (4.0)	1.4	1.0	2.9
Lysine (85), threonine (18), methionine (3.4)	3.3	2.3	3.4

[a] Amino acid uptake expressed as a multiple of the appropriate protein amino acid content.

adjusted on the basis of the results of preliminary experiments. Table I summarizes the concentrations of supplemental amino acids which were finally used and the measured uptake of each amino acid at these concentrations relative to the estimated amount of that amino acid normally synthesized. These results show that each amino acid was taken up in amounts at least equal to the content of the appropriate protein amino acid(s).

Table II presents comparable data for the tissue contents of soluble amino acids. With the exception of plants adapted to growth with lysine plus threonine,[4] addition of each supplemental amino acid to the medium resulted in a 40-fold, or more, expansion of that amino acid in the soluble pool of the plant. Asparagine and glutamine were the only other amino acids to show appreciable increases in pool size due to amino acid supplementation.

Aspartokinase Activities of Amino Acid Supplemented Plants

Table III demonstrates that plants growing under steady state conditions showed no major changes in either the total aspartokinase activities or the relative proportions sensitive to inhibition by lysine or threonine. Plants exposed to the acute toxic effects of 85 μM lysine plus 8.0 μM threonine under nonsteady state conditions showed a progressive reduction of aspartokinase activities with increasing time of exposure, without significant change in the relative amounts of lysine- and threonine-sensitive activities. The significance of the results of Table III will be discussed later. Their immediate

[4] Increases in soluble lysine and threonine in plants adapted to growth on these combined amino acids were much less than those observed for other conditions of supplementation with one or both of these amino acids. Thus, plants adapted to growth in 36 μM lysine plus 3 μM threonine contained a pool of soluble threonine not appreciably higher than that of control plants. In these plants endogenous synthesis of threonine was reduced by approximately 46% (Table V) or 0.46 × 7.9 = 3.6 nmol/frond · doubling. A reduction of this magnitude could make a significant contribution to the differences between the amounts of soluble threonine accumulating in adapted plants, and in plants grown with threonine alone. In the latter plants, threonine synthesis is not regulated. A similar argument cannot be invoked to explain the reduced accumulation of lysine in adapted plants, since lysine synthesis is essentially completely inhibited under all culture conditions studied with lysine in the medium. The reduced accumulation of soluble lysine in plants growing with lysine plus threonine may reflect the relatively low concentrations of lysine in this medium, and corresponding decreased uptake of lysine.

Table II. *Soluble Amino Acid Contents of Plants*

Cultures were grown under steady state conditions essentially as described in "Materials and Methods" with the following exceptions: Plants were adapted to steady state growth (MR = 170) with 36 μM lysine plus 3 μM threonine by prior culture of the inoculum in this medium (47). Plants were adapted to steady state growth on 11 μM lysine plus 5.2 μM threonine by pregrowth of a small inoculum of control plants for 3 d with 11 μM lysine alone. Plants adapted in this way were transferred to medium containing 11 μM lysine plus 5.2 μM threonine and allowed to grow for a further 3 d. Finally, an inoculum of 30 fronds of the latter culture was transferred to 2.4 L of identical medium, and plants were harvested after 2.7 doublings; the MR was 135. Except for methionine (footnote c) and lysine (footnote d) in control cultures, amino acids were determined with an automated amino acid analyzer at a lower limit of detection of approximately 0.05 nmol/frond. None of the contents of leucine, tyrosine, or phenylalanine exceeded this lower limit; these amino acids have been omitted from the table. Based on a mean frond volume of 0.509 μL (15), tissue concentrations (mM) may be estimated by multiplying reported values by 1/0.509 = 1.96. Although not determined directly, the threonine content of plants supplemented with 3.5 μM threonine was estimated to lie within the range of 1.8 nmol/frond (determined for plants supplemented with 2.7 μM threonine (21)) and 6.3 nmol/frond (reported here for plants supplemented with 8 μM threonine).

Soluble Tissue Amino Acid	Amino Acid Supplement in Growth Medium (μM)								
	None	Lys (66)	Thr (8.0)	Met (1.7)	Lys (75) Met (2.1)	Thr (9.1) Met (0.8)	Lys (36) Thr (3.0)	Lys (11) Thr (5.2)	Lys (85) Thr (18) Met (3.4)
	nmol/frond (-fold)[a]								
Aspartate	0.42	0.55	0.41	0.43	0.53	0.32	0.60	0.64	0.56
Threonine	0.098	0.13	6.3 (64)	0.17	0.21	≤4.4 (≤45)[b]	0.18	0.63 (6)	3.8 (39)
Serine	0.29	0.23	0.31	0.55	0.59	0.36	0.17	0.35	0.55
Asparagine	0.36	4.3 (12)	2.7 (7)	0.56	2.2 (6)	1.6 (4)	1.2 (3)	1.9 (5)	3.3 (9)
Glutamate	0.61	0.74	0.49	0.49	0.65	0.42	0.61	0.67	0.65
Glutamine	0.88	2.8 (3.2)	10 (11)	1.1	2.0	5.0 (8)	1.7	2.6	2.7 (3)
Glycine	<0.05	<0.05	<0.05	0.07	<0.05	<0.05	0.37 (≥7)	<0.05	0.33 (≥7)
Alanine	0.22	0.43	0.22	0.24	0.39	0.14	<0.05	0.39	<0.05
Valine	<0.05	<0.05	<0.05	0.09	<0.05	<0.05	<0.05	<0.05	0.12
Half-cystine	<0.05	0.13	<0.05	<0.05	0.19	<0.05	<0.05	<0.05	<0.05
Methionine	0.0075[c]	<0.05	<0.05	1.1 (147)	0.74 (99)	[b]	<0.05	<0.05	0.55 (73)
Isoleucine	<0.05	<0.05	0.18	<0.05	0.10	0.11	<0.05	<0.05	0.17 (≥3)
Lysine	0.015[d]	2.6 (173)	<0.05	<0.05	1.6 (107)	<0.05	0.64 (43)	0.22 (15)	2.6 (173)

[a] Values in parentheses are ratios of values for experimental plants/corresponding value for control plants. Ratios less than three are not reported, since they are of doubtful significance. [b] Although methionine was routinely recovered in the unoxidized form, in this sample it was present exclusively as the sulfoxide. The reason for this unusual result is not clear. It may have resulted from the presence of peroxide impurities in the ether used during the extraction procedure. Since methionine sulfoxide and threonine are not completely resolved, the value of threonine is reported as an upper estimate. [c] Determined in separate studies with $^{35}SO_4^{2-}$ (15). [d] Determined enzymically as described in "Materials and Methods."

importance is to demonstrate that derepression of aspartokinase is not an important factor to be considered in interpretation of the studies to be described below on the effects of supplements on growth rates and fluxes.

Effects of Aspartate-Derived Amino Acids on Growth

It is well documented, both with *L. paucicostata* (14) and other plants (8, 35), that supplementation with lysine plus threonine results in potent growth inhibition. Growth inhibition is caused by methionine deprivation (8, 14, 35) believed to result from the combined inhibition of the lysine- and threonine-sensitive forms of aspartokinase (35). The current work systematically examines the effects of all seven combinations of lysine, threonine, and methionine for analogous effects on growth (Fig. 1). Except for plants growing with lysine plus threonine, growth rates were determined under steady state conditions. The results of Figure 1 confirm the severe growth inhibition by lysine plus threonine and its reversal by methionine. None of the other combinations of lysine, threonine, and methionine caused appreciable growth inhibition. Specifically, these experiments show that: (a) Supplementation with threonine did not result in a growth requirement for lysine or methionine, and additional supplementation of threonine with methionine did not result in a growth requirement for lysine. While partial inhibition of growth was observed at a higher concentration of threonine (8.0 μM), the amount of threonine taken up far exceeded (three-fold) the combined amount of protein threonine plus isoleucine (Table I), and resulted in a tissue concentration of soluble threonine (Table II) at least an order of magnitude greater than that required for complete inhibition of threonine-sensitive aspartokinase (22). The partial inhibition of growth at this high concentration of threonine may well be due to effects on reactions other than that catalyzed by aspartokinase. (b) Supplementation with lysine did not result in a growth requirement for threonine or methionine, and additional supplementation of lysine with methionine did not result in a growth requirement for threonine. (c) Supplementation with methionine did not result in a growth requirement for threonine or lysine.

The possibility was considered that in these steady state experiments any inhibitory effects of supplements may have been masked by 'adaptation' of plants to inhibitory conditions during growth of inocula. Any inhibitory effects of supplements would not then be fully expressed when these plants

Table III. *Aspartokinase Activities of Plant Extracts*

Plants supplemented with 85 μM lysine plus 8.0 μM threonine were grown in two separate cultures. One was inoculated with control plants (91 fronds, 20 colonies) and harvested after 49 h (92 fronds, 52 colonies); the second (inoculum 67 fronds, 18 colonies) was harvested after 100 h (82 fronds, 47 colonies). These culture showed the progressive decrease in growth rates and other abnormalities characteristic of plants toxified with lysine plus threonine (14). All other plants were cultured under steady state conditions. Plants grown with 11 μM lysine plus 5.2 μM threonine had been adapted as described in the legend to Table II. Aspartokinase activities were determined with the standard assay in the absence of NH_2OH. Except as noted in footnote c, lysine- and threonine-sensitivities were determined in the presence of NH_2OH. Aspartokinase activities for plants grown with lysine plus threonine are single determinations. All other values are means of determinations made with two cultures (SE in parentheses).

Amino Acid Supplement	Aspartokinase Activity		Aspartokinase Activity Sensitive to:	
	nmol/min/mg protein	nmol/min/frond	Lysine	Threonine
	% of mean control[a]		% [b]	
None (control)	100	100	82	16
Lys (66)	78 (7)	108 (29)	80	18
Thr (8.0)	61 (4)	41 (0.4)	84	14
Met (1.7)	81 (6)	77 (20)	83	16
Lys (75) + Met (2.1)	90 (20)	119 (36)	83	16
Thr (9.1) + Met (0.8)	58 (13)	31 (7)	86	12
Lys (85) + Thr (8.0)				
49 h	62	58	95[c]	ND[d]
100 h	31	12	81	ND
Lys (36) + Thr (3)	94	146	86	11
Lys (11) + Thr (5.2)	75	97	ND	ND
Lys (85) + Thr (18) + Met (3.4)	94 (1)	122 (21)	86	12

[a] Activities of aspartokinase are expressed as a percentage of the respective mean values of 38 nmol/min/mg protein and 70 pmol/min/frond for control plants (22). [b] Lysine- and threonine-sensitive activities are expressed as percentages of uninhibited activities. [c] Determined with the standard assay (absence of NH_2OH), corresponding to a mean value of 93% in control plants (22). [d] Not determined.

were in turn used as inocula for determination of growth rates. Such adaptation of *L. paucicostata* has been reported to sublethal concentrations of lysine plus threonine (47). To examine whether adaptation may have masked any inhibitory effects of amino acid supplements, 'initial' growth rates and frond/colony ratios were determined with cultures initiated with control (naive) plants (Fig. 2). No lag in growth rate characteristic of that seen in plants undergoing adaptation was detected. Initial growth rates of supplemented cultures relative to that of control plants were in good agreement with the results of the steady state growth rate experiments illustrated in Figure 1. Figure 2 further reports frond/colony ratios. Morphological abnormality is indicated when this ratio falls outside the range of approximately 3 to 5. Plants supplemented with lysine plus threonine showed the abnormally low frond/colony ratios characteristic of plants subjected to lethal combinations of these amino acids (47). Lysine was the only other supplement that resulted in abnormal frond/colony ratios. The latter increased progressively with time in plants grown in the presence of this amino acid.

Taken together, these results not only confirm the well-established inhibitory effect on growth of lysine plus threonine (and its reversal by methionine), but, more importantly, systematically demonstrate that only this combination, among all of those possible for lysine, threonine, and methionine, appreciably affected the growth rate.

Fluxes of 4-Carbon Units from Aspartate into the Aspartate Family and Its Component Amino Acids

To provide quantitative assessments of the physiological roles and regulatory significance of the two forms of aspartokinase, cultures of *Lemna* were grown under steady state conditions in the presence of the amino acid supplements and tracer [U-^{14}C]aspartate. Net fluxes of 4-carbon units into each member of the aspartate family of amino acids could then be determined from the relative accumulation of ^{14}C in these amino acids. Table IV summarizes the labeling patterns of the amino acids of primary interest—lysine, threonine, isoleucine, and methionine. Except for plants cultured in the combined presence of lysine and threonine (experiments 13 and 14), these amino acids comprised at least 7% of the recovered radioactivity. For any particular culture, the labeling pattern for the aspartate family amino acids provides a measure of the relative net *in vivo* flux into the respective amino acid.[5] To compensate for differences between various cultures in growth rates and in the specific activities of tissue [^{14}C] aspartate, ^{14}C was normalized to protein [^{14}C]aspartate. The

[5] For cultures containing threonine in the supplement, the specific activity of [^{14}C]threonine is diluted by an undetermined amount by threonine taken up from the medium, so that ^{14}C in isoleucine no longer provides a valid measure of relative flux into this compound.

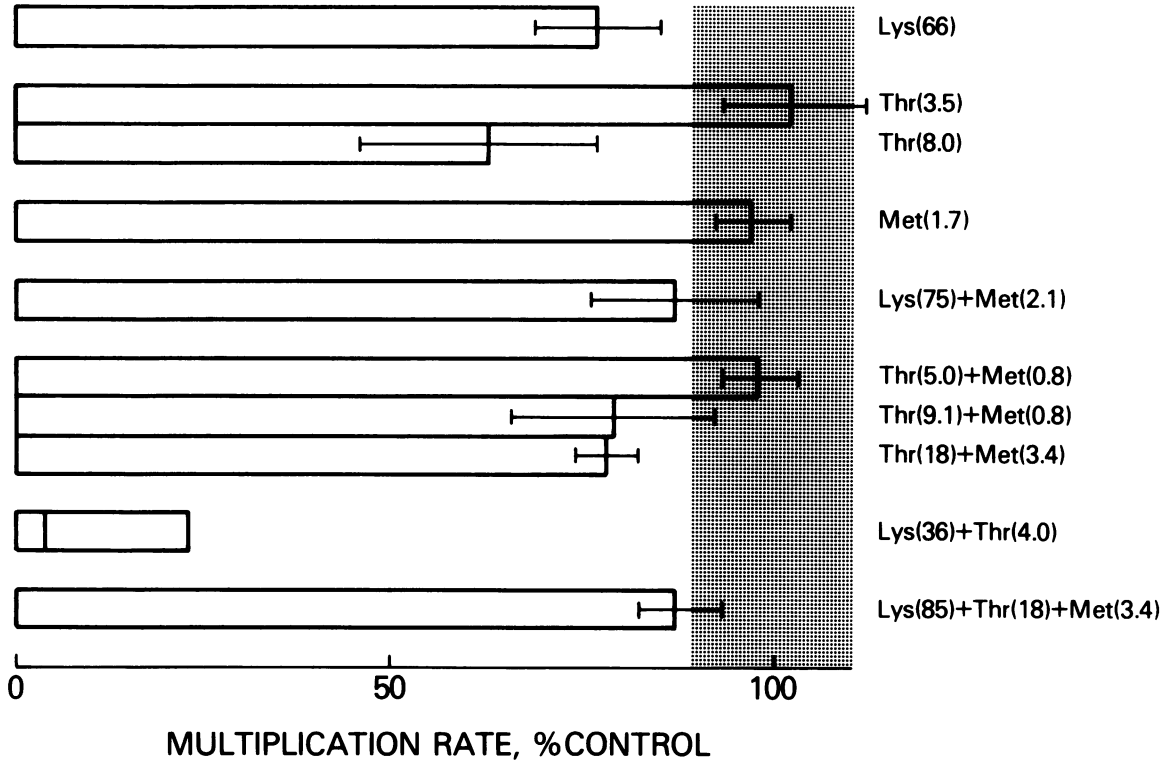

Figure 1. Effects of aspartate-derived amino acids on growth rates. Multiplication rates are expressed as percentages of the mean of 190 determined with control plants (14). The shaded area indicates the range of twice the SD for control cultures. With the exception of those grown with lysine plus threonine, plants were grown under steady state conditions as described in "Materials and Methods," with multiplication rates reported as the mean (±SD, indicated by bars) determined with at least three cultures. Values for 36 μM lysine plus 4.0 μM threonine show the range (3–23% of control) calculated for 12 cultures and measured over intervals of 6 or 7 d after inoculation with control plants (14). Values in parentheses show μM concentrations of supplements.

results so obtained permitted comparison of fluxes between cultures growing under a variety of conditions. Table V summarizes these results for the following key reactions in biosynthesis of the aspartate family of amino acids: the aspartokinase step (AK), the branches leading to synthesis of lysine (L) and O-phosphohomoserine (HSD), to threonine/isoleucine (TI) and methionine (M), and the conversion of threonine to isoleucine (TD). Fluxes in Table V are expressed as a percentage of the total flux through the aspartokinase step in control plants. To better illustrate the effects of supplement on any particular step, fluxes are also expressed in parentheses as percentages of that in control plants through the step under consideration.

DISCUSSION

Regulatory Properties of Aspartokinase

In common with many plants, *Lemna paucicostata* contains two forms of aspartokinase. The dominant form, comprising over 80% of total aspartokinase activity, is inhibited by lysine; inhibition by lysine is increased by AdoMet, which by itself has no effect. Most of the remaining activity is inhibited by threonine (22). Previous *in vitro* studies (22) suggested that the potential capacity of both the lysine- and threonine-sensitive forms of aspartokinase in *L. paucicostata* was each in excess of the requirement *in vivo* for combined synthesis of the aspartate family of amino acids. Definitive proof of this suggestion is provided by the growth experiments and studies of fluxes reported in the present paper.

Before discussing these lines of evidence, however, it should be noted that no evidence of derepression of either lysine- or threonine-sensitive aspartokinase was obtained under any of the steady state growth conditions examined (Table III). The results with plants grown with 36 μM lysine plus 3 μM threonine show that an increased level of aspartokinase is not a factor in adaptation of plants to growth under these conditions. Plants exposed to toxic concentrations of lysine plus threonine under nonsteady state conditions showed a progressive reduction of aspartokinase activity with increasing time of exposure, but no significant change in relative amounts of lysine- and threonine-sensitive activities. The significance of changes under the latter conditions is difficult to assess, since these plants were highly toxified and had virtually ceased growth at the time samples were taken for aspartokinase assays. It is noteworthy that even under these conditions, ones that would be expected to be optimal for derepression, no such effect was observed. Similar conclusions were drawn by Rognes *et al.* in studies of excised barley embryos grown with combinations of lysine, threonine, isoleucine, and methionine (42).

Evidence that lysine-sensitive aspartokinase acting alone can support the combined flux *in vivo* into the aspartate family of amino acids is provided by studies of plants growing with threonine, or threonine plus methionine. The concentra-

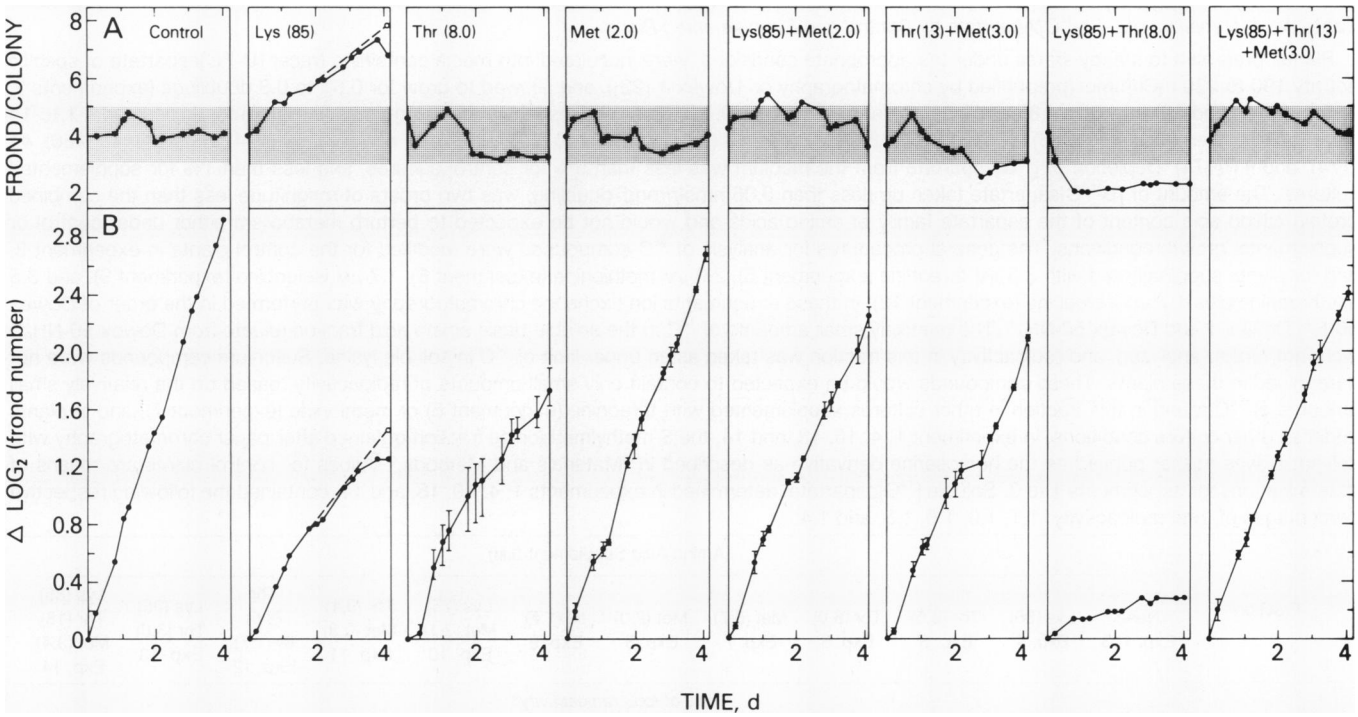

Figure 2. Growth of control (naive) plants in the presence of aspartate-derived amino acids. All cultures were initiated with control plants. Separate experiments with control plants incubated over a 4 h period confirmed that individual amino acids were taken up at rates which would exceed the rates of accumulation of the appropriate protein amino acid(s) of control cultures. Thus, uptake of lysine (nmol/frond·doubling) ranged from 240 to 270% that of the protein lysine content (nmol/frond) of control cultures. Corresponding values for threonine and methionine uptake were 140 to 142%, and 275 to 340%, respectively. Growth curves of control plants and those supplemented with 85 μM lysine alone or combined with 8.0 μM threonine were each based on a single culture. Growth curves reported with 8.0 μM threonine are the means of triplicate cultures. Those for all other cultures are the means of duplicate cultures. Ranges are indicated by vertical bars. The shaded area indicates the range for frond/colony ratios previously observed for 227 control cultures (14). Plants growing with 85 μM lysine were harvested at the end of the experiment for determination of frond and colony numbers. These determinations provided more reliable measurements of growth and frond/colony ratios (shown by open circles and broken lines), since the high frond/colony ratios of these plants precluded accurate measurement of frond number while the plants were floating intact on the growth media. This was especially true during the latter stages of growth.

tions of soluble threonine in these plants (Table II) were far in excess of the concentration of approximately 1 mM determined by *in vitro* studies (22) to result in complete inhibition of threonine-sensitive aspartokinase. Therefore, only the lysine-sensitive form of aspartokinase, present at levels comparable to that in control plants (Table III), would be active in these plants. That this activity is adequate for the combined flux through the aspartokinase step into the amino acids of the aspartate family is shown by the following: First, supplementation with threonine (or threonine plus methionine) does not result in a growth requirement for lysine or methionine (Figs. 1 and 2). Second, supplementation with threonine alone, or combined with isoleucine or methionine does not appreciably reduce flux through the aspartokinase step (Table V).

Analogous experiments with supplements containing lysine show that threonine-sensitive aspartokinase alone is also adequate for combined flux *in vivo* into this aspartate family of amino acids. In plants growing with lysine, lysine plus methionine, or adapted to growth with lysine plus threonine, the tissue concentrations of lysine (Table II) would be expected to cause essentially complete inhibition of lysine-sensitive aspartokinase (22). Flux through the aspartokinase step in these plants would therefore be entirely dependent upon activity of the threonine-sensitive form of the enzyme. That this activity alone is adequate for synthesis at least of threonine, isoleucine, and methionine is evidenced by growth rates with lysine and lysine plus methionine being comparable to that of control plants (Figs. 1 and 2). Conclusive proof was provided by determination of fluxes through the aspartokinase step for plants grown with lysine in the supplement, shown in Table V.

Table VI provides further support for the conclusion that even the threonine-sensitive form provides an excess of aspartokinase activity. The calculations detailed in this table demonstrate that the 65 to 69% of control flux attained by plants growing in 66 μM lysine was achieved with only 13% of the uninhibited activity of threonine-sensitive aspartokinase, acting alone. Therefore, in theory, control flux could be sustained with only 13%/0.65 = 20% of the uninhibited threonine-sensitive activity. Comparable values for two other lysine-containing cultures were 16 to 26% (Table VI). Thus, even the minor (threonine-sensitive) form is present in approximately 5-fold excess of the total *in vivo* requirement of the aspartokinase step. This form comprises only 7% of the total aspartokinase activity (22). It follows that 1 to 2% of the

Table IV. *Metabolism of L-[U-^{14}C]Aspartate by Control and Supplemented Plants*

Plants, pregrown to steady states under the appropriate conditions, were inoculated into media containing tracer [U-^{14}C]aspartate of specific activity 190 to 230 mCi/mmol (prepurified by chromatography on Dowex 1 [22]), and allowed to grow for 0.61 to 0.8 doublings (experiments 2, 3, 5 to 9, 11, and 12) or 4.6 to 4.8 doublings (experiments 1, 4, 10, 13, and 14). Respective doubling times (in hours) for experiments 1 to 14 were (MR in parenthesis): 35 (208), 40 (181), 32 (226), 50 (144), 45 (162), 79 (91), 46 (157), 33 (218), 43 (167), 50 (144), 58 (124), 43 (166), 41 (174), and 41 (174). Depletion of [^{14}C]aspartate from the medium was less than 9% for control cultures, and less than 1% for supplemented cultures. The amount of [U-^{14}C]aspartate taken up (less than 0.06 nmol/frond·doubling) was two orders of magnitude less than the combined protein amino acid content of the aspartate family of amino acids and would not be expected to perturb metabolism either under control or supplemental growth conditions. The general procedures for analysis of ^{14}C compounds were modified for the control plants in experiment 3, and for plants supplemented with 3.5 μM threonine (experiment 5), 2.0 μM methionine (experiment 8), 1.7 μM isoleucine (experiment 9), and 3.5 μM threonine plus 1.7 μM isoleucine (experiment 12). In these experiments ion exchange chromatography was performed in the order of Dowex 50-H$^+$, Dowex 1 and Dowex 50-NH$_4^+$. The relatively small amounts of ^{14}C in the soluble basic amino acid fraction (eluate from Dowex 50-NH$_4^+$) were not further analyzed, and radioactivity in this fraction was taken as an upper limit of ^{14}C in soluble lysine. Sulfonium compounds were not determined in these plants. These compounds would be expected to contain only small amounts of radioactivity, based on the relatively small amounts of ^{14}C found in this fraction in other cultures supplemented with threonine (experiment 6) or methionine (experiment 7), and in plants under all other culture conditions. In experiment 1, 4, 10, 13, and 14, the *S*-methylmethionine fraction obtained after paper chromatography with solvent B was further purified as the homoserine derivative as described in "Materials and Methods." Values for control plants are means of determinations for experiments 1 to 3. Soluble [^{14}C]aspartate, determined in experiments 1, 4, 10, 13, and 14, contained the following respective percentages of total radioactivity: 1.1, 1.0, 1.0, 1.5, and 1.4.

Fraction	Amino Acid Supplement (μM)											
	None Exp. 1–3	Lys (66) Exp. 4	Thr (3.5) Exp. 5	Thr (8.0) Exp. 6	Met (0.7) Exp. 7	Met (2.0) Exp. 8	Ile (1.7) Exp. 9	Lys (75) Met (2.1) Exp. 10	Thr (9.1) Met (0.8) Exp. 11	Thr (3.5) Ile (1.7) Exp. 12	Lys (36) Thr (3.0) Exp. 13	Lys (85) Thr (18) Met (3.4) Exp. 14
	% of total radioactivity[a]											
TCA-insoluble	47	45	32	25	48	47	44	48	28	33	46	46
Aspartate	10	10	8.3	4.7	8.8	11	9.8	8.7	5.9	8.8	9.5	11
Lysine	4.8	≤0.06	4.0	1.9	4.3	5.5	5.0	≤0.05	2.8	4.2	<0.06	≤0.03
Threonine	4.8	4.5	1.6	0.34	4.0	5.7	3.8	4.3	0.38	2.0	2.3	0.06
Isoleucine	4.0	4.2	1.7	0.36	2.9	4.7	1.3	4.5	0.42	1.5	2.2	0.33
Methionine	1.4	1.5	1.4	0.48	0.18	0.21	1.8	0.18	0.11	1.1	1.5	≤0.02
TCA-soluble	17	12	29	32	17	21	28	13	25	39	15	18
Lysine	≤0.02	≤0.02	≤0.25[b]	≤0.05	≤0.02	≤0.17[b]	≤0.20[b]	≤0.02	0.08	≤0.91[b]	≤0.01	≤0.02
Threonine	0.44	0.15	4.2	4.5	0.34	0.62	4.5	0.18	3.3	7.6	0.09	0.03
Isoleucine	≤0.02	≤0.05	≤0.16	0.12	0.03	≤0.15	≤0.13	≤0.08	0.08	≤0.20	≤0.10	≤0.05
Methionine	≤0.02	≤0.07	≤0.07	0.05	0.09	0.4	≤0.03	0.20	0.11	≤0.13	≤0.06	≤0.04
AdoMet[c]	≤0.002	≤0.01	ND[d]	0.06	0.04	ND	ND	0.04	≤0.02	ND	≤0.01	≤0.01
S-MeMet[c,e]	≤0.007	0.03	ND	≤0.13	≤0.07	ND	ND	0.03	≤0.02	ND	≤0.01	≤0.02
Homoserine	≤0.01	≤0.03	≤0.013	≤0.15	≤0.04	≤0.05	≤0.07	≤0.04	≤0.08	≤0.19	≤0.04	≤0.02
CO_2	35	43	39	43	35	32	28	39	47	28	39	36

[a] Each value expressed as a percentage of total radioactivity in TCA-soluble and -insoluble fractions and CO_2. Values of 0.1% or greater were rounded off to two significant figures. Smaller values were rounded off to one significant figure. [b] Upper limit based on total ^{14}C in the basic amino acid fraction (see legend). [c] Values are for the 4-carbon moiety. [d] Not determined. [e] *S*-Methylmethionine.

total uninhibited activity of aspartokinase should suffice for the combined flux through the aspartokinase step. This estimate agrees well with our earlier *in vitro* studies (22) which indicated that the maximum capacity of the uninhibited enzyme is in 42-fold excess of its *in vivo* requirement.

Regulatory Features of the Biosynthetic Pathway for the Aspartate Family of Amino Acids

The findings reported in this, and in the companion (22) paper permit us to put forth a revised tentative outline of the main regulatory features of the biosynthetic pathways leading to each of the aspartate-derived amino acids. This outline is summarized in Figure 3, and discussed in more detail in the following sections.

Lack of Channeling of the Products of the Two Aspartokinases

The results reported in Table V are clearly inconsistent with a hypothetical model in which the products of threonine-sensitive aspartokinase are channeled into the threonine/isoleucine branch, while the products of lysine-sensitive aspartokinase are channeled into the lysine and methionine branches (21). This model would predict that inhibition of threonine-sensitive aspartokinase would specifically inhibit flux into the threonine/isoleucine branch, while inhibition of lysine-sensitive aspartokinase would specifically inhibit flux into the lysine and methionine branches. Contrary to these predictions, severe inhibition of threonine-sensitive aspartokinase in plants supplemented with threonine, threonine plus isoleucine, or threonine plus methionine caused little or no

Table V. *Net Flux Through Aspartokinase and Key Reactions in Biosynthesis of Aspartate-Derived Amino Acids*

Each of the steps through which flux was calculated is illustrated in Figure 3. Flux through threonine dehydratase was calculated from radioactivity in isoleucine. Flux through this step was not determined for plants containing threonine in the supplement (see text footnote 5). Flux through the methionine branch was calculated from radioactivity in methionine and its products, *S*-methylmethionine and AdoMet. Flux through the threonine/isoleucine branch was calculated from the combined ^{14}C in threonine and isoleucine. Flux through the lysine branch was calculated from ^{14}C in lysine. Flux through homoserine dehydrogenase is equal to the combined flux into the methionine and threonine/isoleucine branches. Flux through aspartokinase equals the combined flux into the lysine, methionine, and threonine/isoleucine branches. Details of calculation of fluxes are as follows: (a) The values listed in Table IV were compiled as indicated above, and divided by the corresponding value for protein [^{14}C] aspartate in the same plant culture. The ratios so obtained for the aspartokinase step for the three control cultures yielded a mean value of 1.545. (b) Ratios obtained in (a) for each branch or step and each culture condition were expressed as a percentage of the control value of 1.545. (c) Values in parentheses were obtained by expressing values of (b) as percentage of the value for the same step or branch for control plants. Values arbitrarily set at 100% are in brackets. The lower value for each range is based only on those compounds considered to be radiopure, while the upper limit also includes maximum values estimated for compounds not radiopure. Values were rounded off to the nearest whole number. Experiment numbers refer to those in Table IV.

Step or Branch	Amino Acid Supplement (μM)											
	None Exp. 1–3	Lys (66) Exp. 4	Thr (3.5) Exp. 5	Thr (8.0) Exp. 6	Met (0.7) Exp. 7	Met (2.0) Exp. 8	Ile (1.7) Exp. 9	Lys (75) Met (2.1) Exp. 10	Thr (9.1) Met (0.8) Exp. 11	Thr (3.5) Ile (1.7) Exp. 12	Lys (36) Thr (3.0) Exp. 13	Lys (85) Thr (18) Met (3.4) Exp. 14
	% control flux through AK (% control flux through step)											
Aspartokinase (AK)	[100]	65–69	101–104	108–110	87–88	101–103	109–111	70–71	80–81	120–130	42–44	3–4
	([100])	(65–69)	(101–104)	(108–110)	(87–88)	(101–103)	(109–111)	(70–71)	(80–81)	(120–130)	(42–44)	(3–4)
Lysine branch (L)	31	≤0.5	31–33	26–27	32	32–33	33–34	≤0.5	31	31–38	≤0.5	≤0.3
	([100])	(≤2)	(103–106)	(84–87)	(103)	(103–106)	(106–110)	(≤2)	(103)	(103–123)	(≤2)	(≤1)
Homoserine dehydrogenase (HSD)	69	65–68	70–71	82–83	55–56	68	76–77	70–71	49–50	89–92	42–43	3
	([100])	(94–98)	(101–103)	(119–120)	(80–81)	(99)	(110–112)	(101–103)	(71–72)	(129–133)	(61–62)	(4)
Threonine/isoleucine branch (TI)	60	55–57	58–59	74	53	65–66	63–64	67	46	82–83	31–32	2–3
	([100])	(95–98)	(100–102)	(127)	(92)	(112–113)	(108–110)	(115)	(78)	(140–143)	(53–55)	(3–5)
Methionine branch (M)	9	10–11	11–12	8–10	2–3	4	12–13	3	2–3	8–9	10–11	≤0.5
	([100])	(111–122)	(122–133)	(89–111)	(22–33)	(44)	(133–144)	(33)	(22–33)	(89–100)	(111–122)	(≤6)
Threonine dehydratase (TD)	26	27	ND[a]	ND	22	27–28	9–10	33–34	ND	ND	ND	ND
	([100])	(104)			(85)	(104–108)	(35–38)	(127–131)				

[a] Not determined.

reduction in flux of 4-carbon units from aspartate into the threonine/isoleucine branch (Table V). Further, inhibition of lysine-sensitive aspartokinase in lysine-supplemented plants had no effect on methionine synthesis, as judged by growth rates (Figs. 1 and 2) or fluxes of carbon from [^{14}C]aspartate (Table V). These findings corroborate conclusions previously drawn on the basis of labeling patterns of [^{14}C]homoserine (21).

Inhibition of Aspartokinase Is Not a Major Factor in Regulation of Flux into the Aspartate Family of Amino Acids

The belief that feedback inhibition of aspartokinase by lysine and/or threonine is an important means of regulation of synthesis of the aspartate family of amino acids under physiological conditions has been widely expressed (35, 40–42). This belief is thrown into question by the present observations that aspartokinase activity is present in large excess of the amount needed physiologically, and that flux through aspartokinase is insensitive to inhibition by lysine or threonine. These findings indicate that *in vivo* lysine and threonine do not normally limit aspartokinase activity severely enough to regulate flux through the aspartokinase step and that aspartokinase may not play the major role generally ascribed to it in regulating entry of 4-carbon units into the aspartate family of amino acids.

A 'flux control coefficient' (30) is a property of an enzyme 'embedded in [an] intact system' (30), and is defined as the fractional change in flux relative to a small fractional change in enzyme. This property has been used to quantitate the extent to which particular steps in a pathway contribute to the overall regulation of flux. High values approaching unity for these terms indicate that flux responds almost proportionally to changes in enzyme activity, and define steps that play major roles in regulation. By contrast, enzymes exhibiting low values exercise relatively little control over flux. The low values of the apparent flux control coefficient of aspartokinase estimated in Table VI indicate that this enzyme exercises little control over the flux through the aspartokinase step.

In the absence of significant control by lysine and threonine, what are the factors that normally limit the large potential excess capacity of aspartokinase *in vivo*? In theory, net flux

Table VI. *Excess Capacity of the Minor (Threonine-Sensitive) Form of Aspartokinase Alone in Supporting in vivo Flux Through the Aspartokinase Step*

Supplement (μM)	Flux Through Aspartokinase	Aspartokinase Activity		Aspartokinase Activity Required for Control Flux		Apparent Flux Control Coefficient[a]
		Threonine-Sensitive	Total	Threonine-Sensitive	Total	
	% control[b]	% uninhibited[c]		% uninhibited[d]		
Lys (66)	65–69	13	0.9	20	1	<0.3
Lys (75) + Met (2.1)	70–71	11	0.8	16	1	<0.3
Lys (36) + Thr (3.0)	42–44	11	0.8	26	2	<0.6

[a] Calculated by dividing the decrease in flux [*e.g.* 35% with Lys (66) plants] by the corresponding decrease (99.1%) in total aspartokinase activity. Kacser and Burns (28, 29) have pointed out that experimental determination of flux control coefficients should be based on the effects of fluxes of small changes in enyzme activity, and that flux control coefficients ultimately approach unity as enzyme activity approaches zero. Consequently, the values of the apparent flux control coefficient determined here for greater than 99% inhibition of aspartokinase provide gross upper limits of the true values. [b] Values from Table V. [c] Tissue concentrations of lysine (Table II) would be expected to cause essentially complete inhibition of lysine-sensitive aspartokinase (22). Estimates of activities of threonine-sensitive aspartokinase remaining in supplemented cultures, expressed as a percentage of the corresponding uninhibited value, were calculated from the tissue concentrations of threonine (Table II) and the effect of threonine concentration on threonine-sensitive aspartokinase (22). Remaining total aspartokinase activities, expressed as a percentage of total uninhibited aspartokinase activity, were calculated by multiplying the threonine-sensitive value by 7%, the best estimate of the proportion of total aspartokinase present in the threonine-sensitive form (22). As discussed by Giovanelli *et al.* (22), the latter value is considered a better approximation than those in Table III determined in the presence of NH_2OH. [d] Calculated by multiplying value in columns 2 or 3 by 100/smaller value in column 1.

through the aspartokinase step could be limited by concentrations of aspartate, ATP or Mg^{2+}, back-reaction of aspartyl phosphate to aspartate by reversal of the aspartokinase reaction (3) or by phosphatase action, unfavorable pH, etc. Several considerations lead us to favor the possibility that aspartate concentration is an important limiting factor. First, the concentration of 0.83 mM aspartate determined in control plants (Table II) and the K_m for aspartate of 10 mM (22) indicate that aspartokinase may operate *in vivo* at only 8% of V_{max}. Second, if one considers flux through the aspartokinase step to be limited normally by aspartate concentration, a plausible explanation is provided of the observed insensitivity of flux through the aspartokinase step to inhibition of aspartokinase activity: Inhibition of aspartokinase *in vivo* by supplemental lysine or threonine should result in an accumulation of aspartate. If aspartokinase normally operates far below its K_m, the increased steady state concentration of aspartate would then compensate for the inhibition of enzyme activity and maintain a normal flux through the aspartokinase step.

The end result of feedback inhibition of aspartokinase would then be to regulate the concentration of aspartate, leaving flux catalyzed by the enzyme essentially unaffected. The results of Table II are consistent with the same elevated pool of aspartate being a precursor also for asparagine synthesis, since appreciable accumulation of asparagine was observed in plants accumulating inhibitory concentrations of lysine and/or threonine, but not of methionine.[6] A weakness of this line of reasoning is brought out by the data in Table II: The tissue concentrations of total soluble aspartate in plants grown with lysine and/or threonine were not significantly different from that in control plants. This apparent difficulty may be due to compartmentation of aspartic acid, *i.e.* the tissue concentrations of total soluble aspartate reported in Table II may not be a sensitive measure of changes of this amino acid in chloroplasts at the site of aspartokinase activity. Indeed, Mitchell and Bidwell (36) have reported that the pathways for synthesis of asparagine and homoserine (a precursor of methionine and threonine) in pea roots are compartmented from externally supplied aspartate.

Possible sites at which regulation of aspartate concentration may occur would include synthesis of aspartate and/or its transport to the site of aspartokinase in the chloroplast, and competition by other pathways for this amino acid. An analysis of the regulatory characteristics of branched pathways (11, 12) shows that competition by other pathways for aspartate could in theory control flux into the aspartate family without necessarily affecting total flux into aspartate. A possible example of this type of control is suggested by the marked accumulation of soluble asparagine in all plants grown in media containing lysine, a finding consistent with diversion of at least some of the aspartate spared from lysine synthesis into asparagine synthesis.[7] Synthesis of asparagine, as well as other pathways involving competition for aspartate, are included as potential regulatory sites in the comprehensive

[6] Glutamine also accumulates in plants grown with lysine (and threonine). Asparagine and glutamine are known to accumulate in plants subjected to a variety of conditions of environmental stress (44). At least some of the accumulation of asparagine in lysine-supplemented plants may result from stress incurred under these growth conditions.

[7] Smaller increases in asparagine pools were also observed in plants supplemented with threonine, which caused no significant reduction in flow through the aspartokinase step. As discussed in footnote 6, interpretation of changes in pool sizes of asparagine is probably complicated by the additional effects of environmental stress on these pools.

outline of the regulatory patterns of biosynthesis of the aspartate family of amino acids shown in Figure 3.

Regulation of Lysine Biosynthesis

Lysine strongly feedback regulates its own synthesis to 2% or less of the rate in control plants, without affecting flux into the other branches of the aspartate pathway (Table V). Bright *et al.* (6) have observed similar specific feedback regulation by lysine of its own synthesis in germinating wheat embryos, and less stringent effects have been reported in carrot cell suspension cultures (18). (For reasons previously documented [21], earlier reports, other than those cited here, of lysine controlling its own synthesis fall short of proving this point.) It is likely that the step catalyzed by dihydrodipicolinate synthase (EC 2.7.2.4) is the major site for regulation by lysine of the rate of its own synthesis. This enzyme catalyzes the first committing step in the lysine branch, and is strongly feedback inhibited by lysine (32, 35).

Reduction of flux into lysine is accompanied by a corresponding reduction in net flux through the aspartokinase step (Table V). This reduction is unlikely to be caused by inhibition of the lysine-sensitive form of aspartokinase, since the threonine-sensitive form alone is more than adequate for flux into the combined amino acids of the aspartate pathway. To account for the observed reduction, we propose that the known free reversibility of the reactions catalyzed by aspartate-semialdehyde dehydrogenase (EC 1.2.1.11) (4) and aspartokinase (3) permits any aspartate-semialdehyde or aspartyl phosphate which might tend to accumulate due to the block at dihydrodipicolinate synthase to be reconverted to aspartate.

Regulation of Threonine Biosynthesis

Measurements of fluxes of 4-carbon units from aspartate (Table V) confirm previous findings (21) based on metabolism of [^{14}C]homoserine and [^{14}C]threonine that threonine and/or isoleucine causes little, if any, change in net flux through any of the steps on the pathway to threonine. This finding excludes not only aspartokinase as a major regulatory site for entry of 4-carbon units into the threonine/isoleucine branch under normal growth conditions, but also other steps on the pathway to threonine. Exclusion of the step catalyzed by threonine synthase (EC 4.2.99.2) confirms previous *in vitro* studies of this enzyme (24). Exclusion of homoserine dehydrogenase (EC 1.1.1.3) as a major regulatory determinant *in vivo* indicates either a low flux control coefficient for inhibition of this step, or that the inhibition of this enzyme by threonine observed *in vitro* (8) does not occur *in vivo*. Support for the latter interpretation is provided by the finding that stringent inhibition of homoserine dehydrogenase by threonine was not observed when the assay for this enzyme was performed with concentrations of substrates (aspartic semialdehyde and NADPH) and pH more closely approximating those existing *in vivo* (9).

Stringent regulation of threonine synthesis is observed only when flux through the aspartokinase step becomes limited by severe inhibition of both threonine- and lysine-sensitive aspartokinases. Such regulation at the aspartokinase step is probably a laboratory artifact of little physiological significance. The concentrations of lysine and threonine required to limit flux through the aspartokinase step are at least an order of magnitude greater than those found under normal growth conditions (Table II). Further, such regulation at this step carries with it the penalty of methionine deprivation.

Regulation of Methionine Biosynthesis

Subject to the inaccuracies of measurement of the relatively small amounts of ^{14}C entering the methionine branch, the results of Table V are consistent with our previous demonstration (20) that methionine supplementation reduces the flux of 4-carbon units into this amino acid. Methionine supplementation had no detectable effect on fluxes through the other branches, and none of the other aspartate-derived amino acids were effective in reducing flux into methionine. Cystathionine synthesis, the step catalyzed by cystathionine γ-synthase[8], has previously been shown to be a major site for regulation of the methionine branch (20, 45). It was further established by titration of cystathionine γ-synthase with the active-site-directed inhibitor, propargylglycine (46), that a decrease in the activity of this enzyme is not by itself sufficient to regulate flux through this step, and that additional effects must be involved. One such effect is thought to be the allosteric stimulation of threonine synthase by AdoMet (24, 34). As increased methionine production leads to the accumulation of AdoMet, this stimulation of threonine synthase would tend to divert O-phosphohomoserine normally used for cystathionine synthesis into the threonine branch. It has further been proposed (8, 19) that any resulting increased concentration of threonine would in turn reduce flux through aspartokinase in two ways: (a) by direct inhibition of threonine-sensitive aspartokinase, and (b), by inhibition of homoserine dehydrogenase, thereby causing an overproduction of lysine and inhibition (with AdoMet) of lysine-sensitive aspartokinase. Our findings confirm and extend evidence previously presented (21) that these postulated effects due to threonine accumulation do not play an important role: The concentration of soluble threonine in methionine-supplemented plants was increased by less than twofold (Table II) above that of control plants, an increase negligible compared to those caused by supplemental threonine. Even the latter did not cause a significant reduction in flux through the aspartokinase and homoserine dehydrogenase steps. Finally, no increase in soluble lysine was detected in methionine-supplemented plants that was comparable to that required for reduction of flux through lysine-sensitive aspartokinase.

It is further noted that the soluble threonine concentration would have been expected to increase by a maximum of ninefold if all 4-carbon units normally used for methionine synthesis had been diverted to threonine.[9] Since this did not

[8] A valid EC number has not been assigned to plant cystathionine γ-synthase. EC 4.2.99.2 specifies *O*-succinylhomoserine as substrate and is not an appropriate designation for the plant enzyme which uses only *O*-phosphohomoserine as its physiological substrate.

[9] *De novo* synthesis of methionine is reduced from 1.0 nmol/frond in control plants to approximately 0.21 nmol/frond in methionine-supplemented plants (20). A maximum increase in concentration of $1 - 0.21 = 0.79$ nmol/frond would therefore be expected if all 4-carbon units normally used for methionine synthesis were diverted to soluble threonine. This is an increase of $(0.79 + 0.098)/0.098$, or ninefold above that of control cultures.

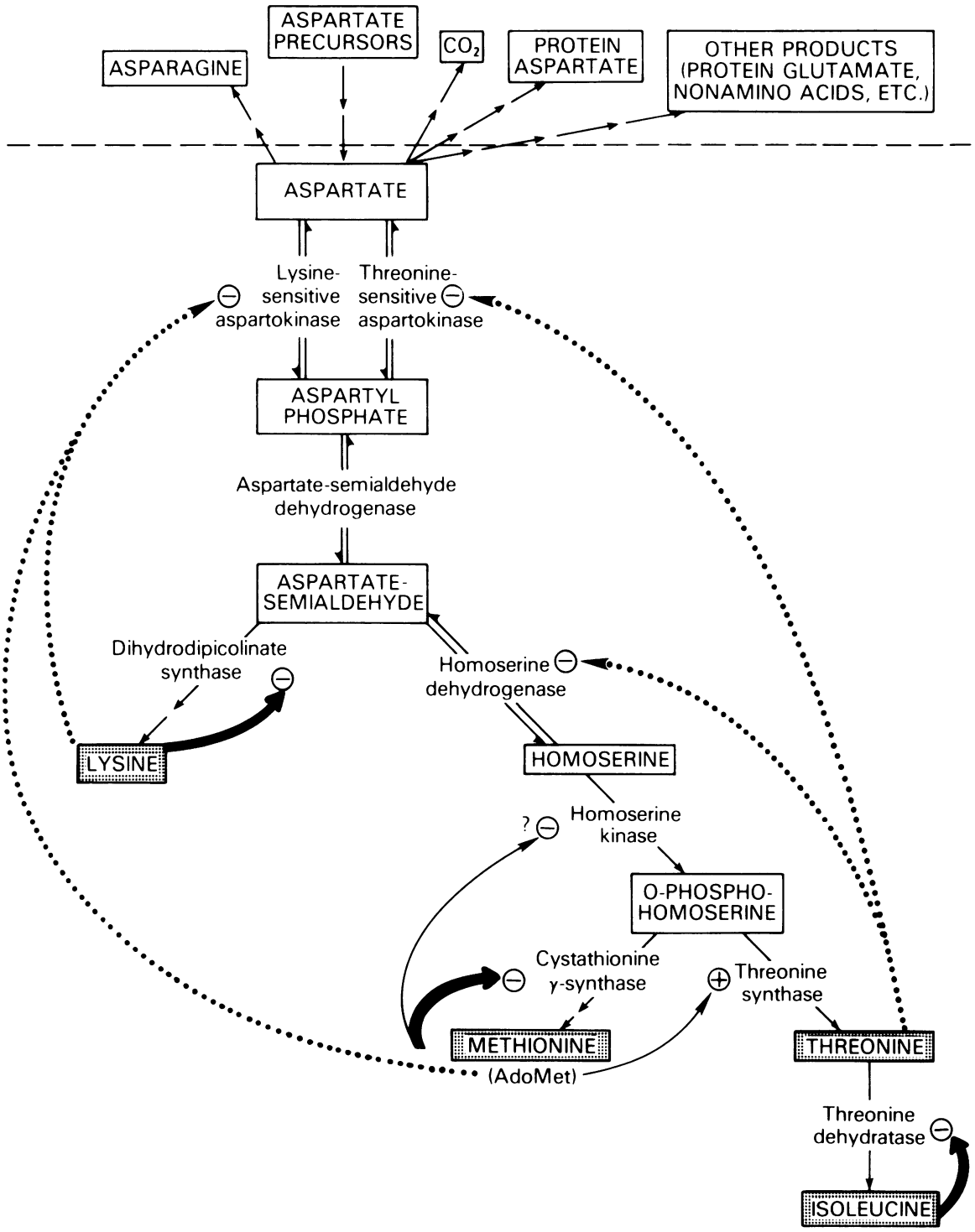

Figure 3. Proposed regulatory structure of the biosynthetic pathway for the aspartate family of amino acids in *L. paucicostata*. Reactions below the broken line known to be reversible *in vitro* are shown by double-barbed arrows; essentially irreversible reactions, by a single barb. Regulatory interactions are indicated with a minus or plus sign, designating inhibition or stimulation, respectively, of the target site. Bold arrows indicate probable sites at which major feedback regulation of flux occurs—the steps catalyzed by dihydrodipicolinate synthase for lysine synthesis, threonine dehydratase for isoleucine synthesis, and cystathionine γ-synthase for methionine synthesis. As discussed in the test, feedback regulation of cystathionine γ-synthase alone is not sufficient to control flux through this step, and additional effects are implicated in such control. One such effect is the stimulation of threonine synthase by AdoMet, although the precise relationship of this effect to regulation of methionine synthesis remains to be clarified. An additional effect may occur at the step catalyzed by homoserine kinase. Threonine synthesis is not appreciably feedback regulated. Dotted arrows indicate regulatory effects demonstrated *in vitro*, which the present work indicates are not normally important for regulation of flux. Flux through aspartokinase is limited by the activity of that enzyme only during severe inhibition of both lysine- and threonine-sensitive aspartokinases in the combined presence of both of these amino acids. Inclusion of pathway(s) for synthesis of

occur, it follows that in the presence of supplemental methionine there is a decrease in the net synthesis of O-phosphohomoserine, suggesting that under these circumstances there may be a partial inhibition of homoserine kinase activity. Support for this speculation has been provided by the studies of Muhitch and Wilson (38) that indicate the presence in pea leaves of separate AdoMet-sensitive and -insensitive homoserine kinase isozymes. Based on their studies of the enzyme from radish leaves, Baum *et al.* (1) have proposed an alternative scheme for regulation of homoserine kinase involving allosteric inhibition of a single species by AdoMet and isoleucine.

Regulation of Isoleucine Biosynthesis

The present studies show that supplements containing isoleucine appreciably reduced flux into isoleucine, without affecting flux into the threonine/isoleucine branch. This pattern of regulation is consistent with our previous observation that isoleucine-supplemented plants exhibit a greatly reduced rate of conversion of threonine to isoleucine, accompanied by an increased pool size of soluble threonine (21). Together with the reports that threonine dehydratase (EC 4.2.1.16) is strongly inhibited *in vitro* by isoleucine (8, 31), our combined studies indicate that feedback inhibition of threonine dehydratase is probably a major means of regulation of isoleucine synthesis *in vivo*, and that such regulation is not accompanied by compensatory reduction in threonine synthesis.

Implications for Improving the Nutritional Contents of Lysine, Threonine, and Methionine in Crop Plants

To the extent that *Lemna* is representative of other higher plants (22), our current understanding provides valuable guidance for attempts to enhance the nutritional value of cereals by increasing their contents of lysine and threonine, and of legumes by increasing their contents of methionine. A commonly used strategy in such attempts has been to select for plant variants with diminished sensitivity of aspartokinase to feedback inhibition by lysine and/or threonine (7). In the light of our present knowledge, this approach appears to present at least two areas of major difficulties:

First, even if flux through the aspartokinase step were increased by such a maneuver, the resulting increase in synthesis of the total amount of amino acids of the aspartate family might be unfavorably distributed from a nutritional point of view. Methionine (20), lysine (this paper), and isoleucine (21, this paper) each strongly feedback regulates its own respective synthesis, while threonine does not (21, this paper). Given this situation, one might expect that increased flux through aspartokinase would result in increased synthesis of threonine, but not of other aspartate-derived amino acids. Indeed, a number of plant variants selected for resistance to growth with lysine plus threonine, and containing aspartokinase activity resistant to inhibition by lysine, do show increases in soluble threonine which are large compared to any increases in the other soluble aspartate family amino acids (5, 10, 25).

Second, a loss of sensitivity of either of the aspartokinases to feedback inhibition by its respective amino acid is neither a necessary nor a sufficient condition to ensure that a sustained increased flux through aspartokinase is actually achieved. Indeed, the observed low flux control coefficient of aspartokinase indicates that an increase in the activity of either of these enzymes will not be reflected by a corresponding fractional increase in flux, assuming other factors remain the same. In agreement, when both protein and soluble aspartate-derived amino acids were determined in a plant mutant containing aspartokinase resistant to inhibition (by lysine), only small increases in total contents of these amino acids were observed—6% for threonine and methionine, and none for lysine and isoleucine (5). If the suggestion, discussed above, as to the limiting role of aspartate is correct, an increase in total aspartokinase activity will soon be offset by a decrease in aspartate concentration, with flux restored to the initial rate. Conversely, an increase in the supply of aspartate might serve to increase the concentration of this amino acid, and so to increase the flux through the aspartokinases without need for changes in their sensitivities to feedback inhibition. This line of discussion raises with some urgency questions as to what factors govern the supply of aspartate at its site of utilization by aspartokinases in chloroplasts. Further information is needed as to whether aspartate synthesis and/or transport into chloroplasts is subject to feedback regulation, the effectors of any such putative regulation, and the effect of an increased supply of aspartate on other pathways competing for this compound.

A tactic which would be expected to avoid at least the first of these major difficulties would be to provide plants with a

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aspartate and of additional pathways competing for metabolism of aspartate (shown above broken line) provides a comprehensive framework for study of regulatory patterns, in contrast to previous analyses exemplified by the 'truncated' pathway (29, 39) shown below the broken line. Among the additional pathways competing for aspartate with synthesis of the aspartate-derived amino acids are synthesis of protein aspartate and of CO_2 (Table IV). Protein glutamate and nonamino acids also were heavily labeled with ^{14}C from aspartate. For example, protein glutamate accumulated from 73% (experiment 14) to 96% (experiment 6) of the amount of ^{14}C in protein aspartate. Nonamino acids contained 7.1% of the total radioactivity in control plants; in supplemented plants, from 2.7% (experiment 4) to 9.1% (experiment 5). No attempt was made to identify the latter compounds. Incorporation of ^{14}C from aspartate into glutamate probably proceeds by transamination of aspartate to oxaloacetate, conversion of the latter to α-ketoglutarate *via* Krebs cycle reactions, and transamination of α-ketoglutarate to glutamate. Labeling of Krebs cycle intermediates could account also for the appearance of ^{14}C in CO_2 and nonamino acids. Note that the relative amounts of ^{14}C in CO_2, protein glutamate, or nonamino acids are not necessarily measures of the corresponding relative net fluxes of aspartate carbon into these compounds as compared to the fluxes into aspartate-derived amino acids. Reversibility of all reactions between aspartate and the given compound, and the fact that the specific radioactivities of the precursors of certain compounds are not necessarily identical to that of the soluble aspartate precursor for synthesis of amino acids of the aspartate family may each cause ambiguity in relating ^{14}C accumulations to net relative fluxes.

'sink' which would remove the soluble amino acids which are the effectors of known regulatory sites in the biosynthetic pathway. For example, increased synthesis of lysine could possibly be facilitated by conversion of soluble lysine to a polypeptide form through introduction of DNA coding for polylysine synthesis. Accumulation of lysine in the peptide form would avoid the feedback inhibition that would accompany an increased concentration of soluble lysine. Similarly, increased synthesis of methionine might be facilitated by insertion of DNA coding for polymethionine synthesis. The DNA (or DNAs) specifying the polypeptide (or polypeptides) could, of course, be adjusted to attain the nutritionally desirable balance of stored lysine and methionine. Conversion of threonine to a polypeptide derivative may not be necessary, since this amino acid does not feedback regulate its own synthesis.

Although the possibility is more speculative, it may be that the tactic in question will have a favorable effect also in the second area of difficulty. If the rate of supply of aspartate is subject to feedback regulation by one or more of the soluble aspartate-derived amino acids, removal of such effectors into a polypeptide sink may help to increase the rate of delivery of aspartate to chloroplasts, thereby tending to increase its concentration and the flux through the aspartokinases.

Plants infected with crown gall (*Agrobacterium tumefaciens*) appear to provide a natural model for this proposed strategy. During infection with certain strains of *A. tumefaciens*, large amounts of lysine are synthesized and converted to the derivative *N*-α-propionyl-L-lysine (lysopine). The latter accumulates in the transformed cells and is used as a source of C, N and energy for the bacterium (2). Of further interest in connection with this strategy, the feasibility of expressing synthetic DNA fragments coding for polypeptides rich in lysine, methionine, threonine and other essential amino acids, and designed for their digestibility by animal proteases, has been demonstrated in *Escherichia coli* (27). For the proposed strategy to be successful, expression of peptides enriched in these essential amino acids must not disrupt the normal metabolism or physiology of the plant. Recent studies by Wallace *et al.* (48) offer at least some encouragement in this regard. Maize zeins normally totally lack lysine and tryptophan. Wallace and coworkers mutagenized zein DNA in such a manner that it would code for substitution or insertion of as many as two lysines and two tryptophans. During expression of the mutagenized DNA's in frog oocytes, no effects were noted on translation, signal peptide cleavage, stability of the modified zeins, or ability of these proteins to assemble into structures resembling maize protein bodies.

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