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Photosynthetic Formation of the Aspartate Family of Amino Acids in Isolated Chloroplasts[1]

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ABSTRACT

The metabolism of ^{14}C-labeled aspartic acid, diaminopimelic acid, malic acid and threonine by isolated pea (*Pisum sativum* L.) chloroplasts was examined. Light enhanced the incorporation of [^{14}C]aspartic acid into soluble homoserine, isoleucine, lysine, methionine and threonine and protein-bound aspartic acid plus asparagine, isoleucine, lysine, and threonine. Lysine (2 millimolar) inhibited its own formation as well as that of homoserine, isoleucine and threonine. Threonine (2 millimolar) inhibited its own synthesis and that of homoserine but had only a small effect on isoleucine and lysine formation. Lysine and threonine (2 millimolar each) in combination strongly inhibited their own synthesis as well as that of homoserine. Radioactive [1,7-^{14}C]diaminopimelic acid was readily converted into [^{14}C]threonine in the light and its labeling was reduced by exogenous isoleucine (2 millimolar) or a combination of leucine and valine (2 millimolar each). The strong light stimulation of amino acid formation illustrates the point that photosynthetic energy is used *in situ* for amino acid and protein biosynthesis, not solely for CO_2 fixation.

The important role of plastids in nitrogen metabolism of higher plants is becoming increasingly apparent (15). Wallsgrove *et al.* (26) recently found two important enzymes of nitrogen assimilation, nitrite reductase and glutamate synthase, to be located exclusively in pea chloroplasts.

Aromatic amino acid synthesis has been demonstrated in isolated chloroplasts, and several of the enzymes required are plastid-localized (3). A number of the enzymes required for the synthesis of aspartate derived amino acids are also present in plastids (15), but evidence that chloroplasts are capable of synthesizing the full range of amino acid is limited (14, 22). Here, we report the light-stimulated synthesis of isoleucine, homoserine, lysine, methionine and threonine from ^{14}C-labeled precursors including aspartic acid (Fig. 1) and provide evidence that lysine and threonine function in the regulation of the pathway.

MATERIALS AND METHODS

Growth of Plants. Seeds of *Pisum sativum* L. var. Feltham First were soaked overnight in running tapwater, planted in Vermiculite, and maintained in a controlled environment chamber as previously described (14). Plants 9–11 days old were harvested 30 min into the light cycle.

Isolation, Incubation, and Characterization of Chloroplasts and Mitochondria. A modification of the method of Bottomley *et al.* (5) was used for chloroplast isolation. Leaves and stems were cut with scissors into small pieces and placed in ice-cold filter-sterilized isolation medium consisting of 330 mM sorbitol, 50 mM Tricine (pH 8.4), 2 mM EDTA, 1 mM $MgCl_2$ and 4 mM 2-mercaptoethanol. Tissue was homogenized with a Polytron (setting 5) for 3 s (14). The brei was filtered through two layers of muslin and two layers of Miracloth, placed in glass centrifuge tubes and spun at 2,000*g* for 50 s. The supernatant was decanted and the plastid pellet resuspended in isolation medium with a small piece of 20-μm nylon mesh which had been frayed at the end, rolled into a cylinder and attached to a glass rod. When soaked in the appropriate medium, it allowed gentle resuspension of the pellet without absorbing it. After centrifugation as before, the supernatant was decanted and 5 ml of isolation medium added (except 50 mM EPPS[3] (pH 8.4) replaced Tricine). The "soft pellet" on top was resuspended by briefly swirling and decanted. The remaining "hard pellet" was gently resuspended in either KCl medium (200 mM KCl, 50 mM EPPS (pH 8.3), 6.6 mM $MgCl_2$) or sorbitol medium (300 mM sorbitol, 50 mM EPPS (pH 8.3), 30 mM KCl).

Incubation with labeled precursors (usually for 20 min) was carried out in a shaking water bath at 20 C as described previously (14). In time course experiments aliquots were removed from samples at appropriate intervals and added to an equal volume of 10% (w/v) trichloroacetic acid. Bicarbonate-dependent O_2 evolution was examined with a Rank Bros. (Cambridge) O_2 electrode as described (1), except the following incubation medium was used: 330 mM sorbitol, 50 mM EPPS (pH 8.2), 10 mM $NaHCO_3$, 5 mM $Na_4P_2O_7$, 2 mM ADP, 2 mM EDTA, and 1 mM $MgCl_2$. Evolution of $^{14}CO_2$ from labeled precursors was analyzed as reported by Mazelis *et al.* (19). In this case, samples were incubated in sealed Warburg flasks which contained 0.3 ml of 20% (w/v) trichloroacetic acid in the side arm and the reactions were stopped by tipping the acid into the sample.

Intactness of chloroplast preparations was estimated by ferricyanide-dependent O_2 evolution (16) as well as phase contrast microscopy (24). Chl was determined by the method of Arnon (2).

Marker enzymes were utilized to characterize the chloroplast preparations. Nitrite reductase was employed as a marker for intact chloroplasts and was assayed by the method of Bourne and Miflin (6). The microbody marker, catalase, was assayed by the procedure of Luck (17), and mitochondrial markers, Cyt oxidase

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[3] Abbreviation: EPPS: 4-(2-hydroxyethyl)-1-piperazine propanesulfonic acid.

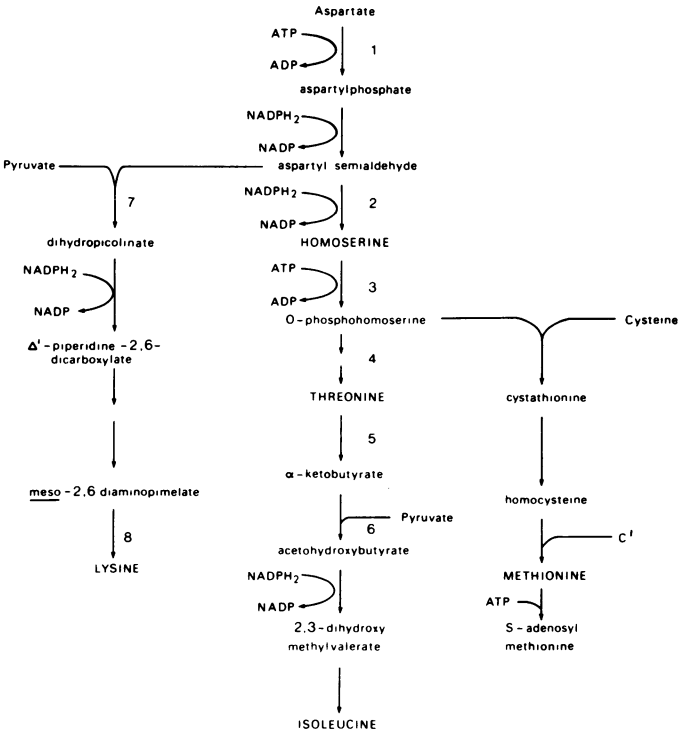

FIG. 1. Biosynthetic pathways for aspartate-derived amino acids. The numbers represent enzymes catalyzing the reaction: 1, aspartate kinase; 2, homoserine dehydrogenase; 3, homoserine kinase; 4, threonine synthase; 5, threonine dehydratase; 6, acetolactate synthase; 7, dihydrodipicolinate synthase; 8, diaminopimelate decarboxylase.

and NAD-dependent glutamate dehydrogenase, were assayed as described by Hackett (12) and Miflin (20), respectively.

Mitochondria were isolated from young pea leaves according to the method of Douce *et al.* (9) except that MOPS buffer was replaced by Hepes.

Analysis of Radioactively Labeled Products. After incubation with labeled precursors, samples were prepared for subsequent analysis by one of two methods. In method A reactions were stopped by addition of an equal volume of ice cold 10% trichloroacetic acid and stored at 4 C for at least 1 h. Precipitated protein was collected by centrifugation and the pellet washed twice with 5% trichloroacetic acid. The supernatants (which contained the free amino acids) were pooled and washed two times with equal volumes of ether. After the pH was adjusted to approximately 2, samples were purified by cation-exchange chromatography and reduced in volume by rotary evaporation (7). In method B samples were frozen in liquid N_2 to stop reactions. Before thawing, methanol-chloroform-water (12:5:3, v/v) was added and the samples extracted as previously described (4). With either method, the insoluble fractions (containing precipitated protein) were hydrolyzed in 6 N HCl plus 7.2 mM 2-mercaptoethanol (7).

Radioactively labeled amino acids from both the soluble and hydrolyzed fractions were analyzed by several separation systems. With all methods amino acids were identified by co-chromatography with authentic compounds. Two-dimensional TLC on cellulose plates (7) was performed twice in the first dimension; initially in methylethyl ketone-acetone-pyridine-water-formic acid (35:35:15:15:2, v/v) for 1.5 h, then in isopropanol-water-formic acid (20:5:1, v/v) for 5.5 h, and in the second dimension (4.5 h) in butanol-acetone-water-diethylamine-triethylamine (10:10:5:1:1, v/v). Radioactivity was determined by a modification of the method of Davies and Miflin (8). Automated amino acid analysis was performed with lithium buffers according to the procedure of Vega and Nunn (25). The column eluate was split, with 10% passing through the analytical system and 90% being collected in test tubes at 18-min intervals. Two-ml aliquots from each tube were added to 8-ml aqueous scintillant (11) and analyzed by scintillation counting at about 80% counting efficiency. The recorder of the amino acid analyzer was equipped with an event marker which allowed comparison of the A_{570} and radioactive peaks. Two-dimensional chromatography/electrophoresis was performed on cellulose thin layer plates. Samples were run electrophoretically in the first dimension in 8% acetic acid and 2% formic acid (pH 2) at 1 kv for 20 min and chromatographically in butanol-acetone-water-diethylamine-triethylamine (10:10:5:1:1, v/v). Samples were also analyzed by one dimensional high voltage electrophoresis on paper (Whatman No. 3MM) in 50 mM veronal buffer (pH 8.4) at 3 kv for 45 min and one dimensional thin-layer electrophoresis in 50 mM K-acetate (pH 5.5) at 1 kv for 15 min.

For protein synthesis experiments, samples were incubated 15 min with [^{14}C]leucine in the light (see above). They were mixed with equal volumes of ice-cold leucine solution (100 mM) and then spotted and dried on Whatman No. 3MM filter papers, which were prepared for scintillation counting by the method of Mans and Novelli (18) and counted at about 70% efficiency in toluene-based scintillant (8 g PPO, 0.5 g POPOP/l toluene).

Chemicals. Radioactively labeled L-[U-^{14}C]aspartic acid (210 mCi/mmol), L-[U-^{14}C]leucine (354 mCi/mmol), [U-^{14}C]malic acid (55 mCi/mmol) and L-[U-^{14}C]threonine (232 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, U.K. and DL-[1,7-^{14}C]diaminopimelic acid (29.6 mCi/mmol) from ICN. Amino acids were obtained from Sigma.

RESULTS AND DISCUSSION

Characteristics of Chloroplast Preparation. By modifying established chloroplast isolation methods (5) (*e.g.* cutting leaves into very small pieces before grinding, reducing homogenization times and generally resuspending plastids) we have been able to obtain routinely chloroplasts of high quality (Table I). Whether determined by ferricyanide-dependent O_2 evolution, phase contrast microscopy or nitrite reductase recovery the preparations always contained more than 85% intact chloroplasts immediately after isolation. The isolated chloroplast preparations evolved O_2 at high rates when incubated with bicarbonate and ADP and incorporated [^{14}C]leucine into trichloroacetic acid-insoluble material at the fastest rates so far recorded (10). Although the chloroplast prepa-

Table I. *Characteristics of Isolated Pea Chloroplast Preparations*

Rates of [^{14}C]leucine incorporation were calculated over the first 15 min incubation.

Characteristics	No. of Experiments	Activity	Activity Obtained in Crude Homogenization
		mg Chl^{-1} h^{-1}	*% of total*
Marker Enzyme			
Cyt oxidase	4	5.3 ± 3.7 μmol	0.7 ± 0.3
NADH-dependent glutamate dehydrogenase	2	0.9 ± 0.2 μmol	1.0 ± 0.3
Catalase	4	8.6 ± 1.0 mmol	0.4 ± 0.2
Nitrite reductase	2	18.6 ± 4.2 μmol	8.4 ± 3.9
Chl	10		7.8 ± 3.3
CO_2-dependent O_2 evolution	26	85 ± 34 μmol	
L-[^{14}C]Leucine incorporation into trichloroacetic acid insoluble material	5	4.1 ± 2.1 nmol	
Percentage intactness [$K_3Fe(CN)_6$ test]	26	(86 ± 10)	

rations were not completely free of mitochondrial and microbody contamination (estimated by assaying Cyt oxidase and NAD-dependent glutamate dehydrogenase for mitochondria and catalase for microbodies) such contamination was relatively low (Table I).

Metabolism of [^{14}C]Aspartic Acid. In both light and dark, approximately 67% of the recovered radioactivity remained in the soluble amino acid fraction, 30% was in soluble, neutral and acidic material (compounds not binding to a Dowex 50 column) and only 0.3% in lipids (chloroform-methanol soluble). While 1.5% was recovered in insoluble material from light-treated samples only 0.2% was in this fraction from dark samples. After acid hydrolysis of insoluble material, approximately 5% remained as humin. Less than 0.1% of the added radioactivity was recovered as $^{14}CO_2$, though this was higher in the dark than the light. Recovery of added radioactivity was 90–95%.

The biosynthesis of amino acids from [^{14}C]aspartic acid by isolated pea chloroplasts is shown in Table II. In the soluble fraction labeling of homoserine, lysine, threonine, isoleucine, and methionine was stimulated by light. Homoserine was the major soluble radioactive product with the ratios of label incorporated being approximately 10:1:1:0.3:0:1 for homoserine, lysine, threonine, isoleucine, and methionine, respectively.

The incorporation of [^{14}C]aspartic acid into isoleucine, lysine, threonine, and, to a lesser extent, homoserine does not proceed linearly for long periods (Fig. 2). We have not attempted to determine the basis for this, although several workers have observed similar kinetics for light-driven protein synthesis in isolated chloroplasts (5, 10, 23). The labeling of homoserine and lysine appeared to be more rapid than that of isoleucine and threonine. The endogenous concentration of homoserine in pea chloroplasts is high (21) which may explain both the accumulation of large amounts of label in this compound as well as the delay in incorporation into threonine and isoleucine.

Using the newly developed chloroplast isolation technique of Mills and Joy (21), we have estimated the endogenous aspartic acid concentration in isolated pea chloroplasts to be about 150 nmol mg Chl^{-1}. If we assume that due to very rapid rates of uptake (13) all the [^{14}C]aspartate is taken up by the chloroplasts and that amino acid biosynthesis reaches a plateau at 10 min we can utilize the data given in Table II to calculate an initial rate of biosynthesis (in nmol mg Chl^{-1}h^{1}) of 92 for homoserine and 10 for lysine. These rates are much lower than those observed for CO_2-dependent O_2 evolution but are equal to or greater than reported rates of amino acid incorporation into protein by isolated chloroplasts (23).

Aspartic acid was also converted into alanine, asparagine and glutamate in reactions which were not light-dependent, suggesting that some mitochondria were present and carrying out the reactions of the tricarboxylic acid cycle.

No additional energy sources or cofactors were required by chloroplast preparations to carry out light-dependent amino acid synthesis. The stimulation by the light is not unexpected since at least six steps in the biosynthesis of the aspartate-derived amino acids require ATP or NADPH (Fig. 1). The subsequent incorporation of the newly formed amino acids into protein also requires ATP and GTP and has been shown for some time to be light dependent (10).

The marked stimulation by light of most of the metabolism observed, and the fact that similar results were obtained under sterile conditions (data not shown) both argue that the amino acid biosynthesis was carried out by the chloroplasts in the preparation. However the preparations also contain mitochondria and peroxisomes (Table I) and also possibly ER-derived membranes. A preparation containing a 66-fold higher level of mitochondria (as judged by Cytoxidase activity) and presumably increased amounts of other potential contaminants was used to test the involvement of non-chloroplast material. There was no increase in labeling of

Table II. *Incorporation of [^{14}C]Aspartic Acid into Aspartate-derived Amino Acids in Isolated Pea Chloroplasts*

One-ml samples containing 500 μg Chl and 10 μCi [^{14}C]aspartic acid were incubated in KCl medium for 20 min. Amino acids were extracted by method B and unless stated separated by TLC which does not resolve leucine from isoleucine.

Amino Acids	Incubation Conditions									
	0 Time		Light			Dark			Fold Stimulation in Light[a]	
	Soluble Fraction	Protein Fraction	Soluble Fraction		Protein Fraction	Soluble Fraction		Protein Fraction	Soluble Fraction	Protein Fraction
	cpm/sample									
	× 10^{-3}	× 10^{-2}	× 10^{-3}		× 10^{-2}	× 10^{-3}		× 10^{-2}		
Aspartic family[b]										
Asp[c]	9,958	98	8,724	9,057	1,912	9,605	9,670	238	0	12
Hse	0	0	589	511		13	11		45–46	
Lys	0	0	64	94	92	27	7	10	2–13	8
Thr	0	0	61	70	82	4	6	7	12–14	13
Ile	0	0	21	13	18	7	7	8	2–3	2
Met	0	0	5	5	0	4	0	0	1–2	
Others										
Ala	103	0	194	168	0	164	172	0	0	
Asn[c]	6		38	36		73	100		0	
Glu	15	0	103		0	166		0	0	

[a] Ranges are given for estimates based on different separation systems.

[b] Amino acids separated by automatic ion-exchange chromatography. Glutamic acid, glutamine, and homoserine are not resolved by this method.

[c] Asparagine in protein is included with aspartic acid.

Table III. *Effect of Mitochondrial Fraction and Incubation Medium on Incorporation of [^{14}C]Aspartic Acid into Aspartic Acid-derived Amino Acids in Isolated Pea Chloroplasts*

One-ml samples containing 406 μg Chl and 2 μCi [^{14}C]aspartic acid were incubated in KCl or sorbitol medium for 20 min. Sixty-six times the amount of mitochondria (as estimated by Cyt oxidase activity) was added to some samples. Amino acids were extracted by method A and samples analyzed by two-dimensional TLC.

Soluble Amino Acids	Incubation Conditions								
	KCl Medium		Fold Stimulation in Light	KCl Medium + Mitochondrial Fraction		Fold Stimulation in Light	Sorbitol Medium		Fold Stimulation in Light
	Light	Dark		Light	Dark		Light	Dark	
	Incorporation			Incorporation			Incorporation		
	cpm × 10^{-2}			*cpm* × 10^{-2}			*cpm* × 10^{-2}		
Asp	18,106	25,207	0	17,157	26,684	0	17,582	26,895	0
Hse	1,286	7	192	204	7	31	1,463	8	188
Lys	120	17	7	50	11	4	136	12	12
Thr	117	7	17	59	4	15	112	4	28
Ile (+Leu)	92	15	6	30	2	13	84	5	18
Met	5	0		8	0		8	0	

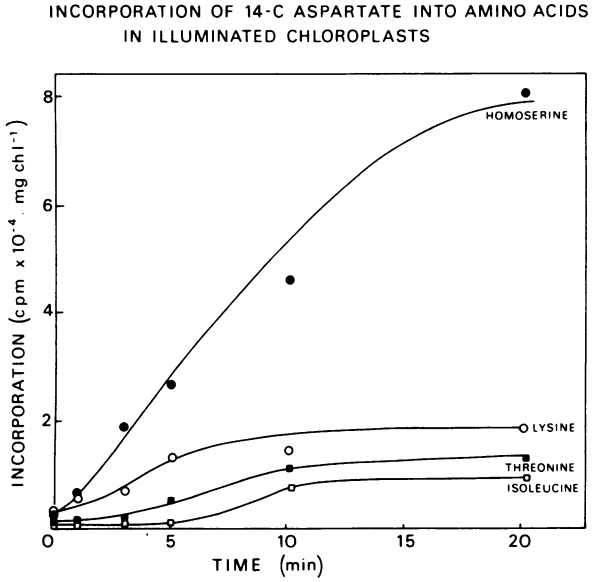

FIG. 2. Time course of light-driven incorporation of radioactivity from [^{14}C]aspartic acid into amino acids by isolated pea chloroplasts. A final volume of 0.7 ml contained 980 μg Chl and 7 μCi [^{14}C]aspartic acid. At indicated intervals, 0.1-ml aliquots were removed and mixed with an equal volume of 10% trichloroacetic acid. Samples were extracted by method A and analyzed by two-dimensional TLC.

amino acids of the aspartic acid family, rather it was generally decreased (Table III).

Table III also shows that incorporation of label into amino acids was the same when the chloroplasts were incubated in either KCl or sorbitol medium. Similarly there is little difference in rates of protein synthesis (5). However, rates of CO_2-dependent O_2 evolution in KCl medium were only about 5% of those in sorbitol medium (data not shown, see also ref. 5).

Our experiments show that the synthesis of amino acids in chloroplasts is subject to feedback regulation (Table IV). Threonine (2 mM) inhibited homoserine synthesis from [^{14}C]aspartic acid as well as its own (both in soluble and protein fractions); consistent with the reported presence of a threonine-sensitive aspartate kinase and a threonine-sensitive homoserine dehydrogenase in pea chloroplasts (27). Threonine did not strongly inhibit lysine formation whereas lysine (2 mM) not only limited its own synthesis but also that of homoserine and threonine suggesting the presence of a lysine-sensitive aspartate kinase in pea leaves and chloroplasts (14). The failure of threonine to inhibit lysine formation is probably because 9- to 11-day-old pea leaves (which were used in this study) appear to contain a predominantly lysine-sensitive aspartate kinase (14). Thus if threonine inhibits aspartate kinase only slightly while greatly affecting homoserine dehydrogenase the amount of 3-aspartylsemialdehyde for lysine synthesis should not be significantly decreased and may even increase (see Fig. 1). This is particularly likely if our estimates of a 9-fold greater flux of carbon down the homoserine branch of the pathway in the absence of added amino acids are correct. Further, some of the carbon label in lysine and isoleucine could have been derived from pyruvic acid (formed by dark metabolism of [^{14}C]aspartic acid) which would be less affected by threonine. Lysine plus threonine (2 mM) reduced their own synthesis as well as that of homoserine to the level of the dark control, consistent with the previous observation (23) that lysine plus threonine markedly lowered light-driven protein synthesis in isolated pea chloroplasts.

Metabolism of Diaminopimelic Acid. Isolated intact chloroplasts readily metabolized DL-[1,7-^{14}C]diaminopimelic acid (Table V). As lysine is produced by decarboxylation of this compound it is possible to follow its production by assaying either $^{14}CO_2$ liberation or labeled lysine formation. As KCl medium is inhibitory to CO_2 fixation (5), little of the evolved $^{14}CO_2$ should be refixed in the light. Agreement between $^{14}CO_2$ liberation and ^{14}C lysine formation was relatively close.

In contrast to the light stimulation of the above reactions pea chloroplasts readily produce lysine from diaminopimelate both in light and dark probably because no high energy compounds are necessary. Incorporation of lysine into chloroplastic protein however was markedly light-dependent. These results not only support other findings presented here (*i.e.* that pea chloroplasts synthesize lysine from aspartic acid; Tables II, III, and IV) but also those of Mazelis *et al.* (19) who found a chloroplast-localized diaminopimelate decarboxylase from *Vicia faba* and Wallsgrove *et al.* (27) who have recently obtained evidence that this enzyme is located exclusively in chloroplasts from pea leaves.

Metabolism of Threonine. Threonine was converted to isoleu-

Table IV. *Effect of Isoleucine, Lysine, and Threonine on Incorporation of [^{14}C]Aspartic Acid into Aspartic Acid-derived Amino Acids by Isolated Pea Chloroplasts*

Aliquots (0.4 ml) containing 100 μg Chl and 2 μCi [^{14}C]aspartic acid were incubated in the light in KCl medium for 20 min. Isoleucine, lysine, and threonine were present at 2 mM each. Amino acids were extracted by method A and analyzed by two-dimensional TLC. The data represent the means of two experiments.

Labeled Compound	Incubation Conditions									
	No Added Amino Acids		+ Lys		+ Thr		+ Lys + Thr		+ Ile	
	Soluble Fraction	Protein Fraction	Soluble Fraction	Protein Fraction	Soluble Fraction	Protein Fraction	Soluble Fraction	Protein Fraction	Soluble Fraction	Protein Fraction
	cpm × 10^{-3}	*cpm × 10^{-2}*	*incorporation as % in absence of amino acids*							
Asp[a]	1674.3	454.5	105	45	105	58	105	59	94	96
Hse	119.8		58		26		3		88	
Lys	10.4	12.2	27	0	89	110	18	0	92	76
Thr	12.0	15.9	45	28	10	0	6	0	84	74
Ile	7.4	1.8	50	0	83	65	69	52	28	0

[a] Label in aspartic acid derived from the protein fraction includes that present in the protein as asparagine.

Table V. *Incorporation of [1,7-^{14}C]Diaminopimelic Acid into Lysine by Isolated Pea Chloroplasts*

Two-ml samples containing 174 μg Chl and 0.2 μCi DL-[1,7-^{14}C]diaminopimelic acid were incubated in KCl medium for 20 min. Radioactivity in lysine was analyzed by one-dimensional thin layer electrophoresis for soluble fractions and two-dimensional TLC for hydrolyzed protein. $^{14}CO_2$ was collected and analyzed as described under "Materials and Methods."

Labeled Compound	Incubation Conditions		Fold Stimulation in Light
	Light	Dark	
	cpm × 10^{-2}		
CO_2	527.8	554.1	0
Lys			
Soluble	617.1	603.9	0
Protein	16.1	1.6	10

Table VI. *Incorporation of [^{14}C]Threonine into Isoleucine by Isolated Pea Chloroplasts*

One-ml samples containing 2 μCi [^{14}C]threonine and 104 and 210 μg Chl in experiments 1 and 2, respectively, were incubated in KCl medium for 20 min. Isoleucine, leucine, and valine were at 2 mM. Samples were extracted by method A and analyzed by two dimensional TLC.

Experiments	Incubation Conditions						
	Light	Dark		Light + Ile		Light + Leu + Val	
	Incorporation	Incorporation	Light Control	Incorporation	Light Control	Incorporation	Light Control
	cpm μg Chl^{-1}		*%*	*cpm μg Chl^{-1}*	*%*	*cpm μg Chl^{-1}*	*%*
1	894	308	34	87	10		
2	833			129	15	361	43

cine by isolated chloroplasts (Table VI) and various lines of evidence suggest that this occurred via the pathway shown in Figure 1. First, label appeared in α-aminobutyrate, the transamination product of α-ketobutyrate. Secondly, isoleucine inhibited its own synthesis consistent with its ability to inhibit threonine dehydratase (15). Thirdly, leucine plus valine also inhibited isoleucine formation as might be expected from their inhibition of isolated acetolactate synthase (15). Isoleucine also inhibits this enzyme (15) but the effect is much smaller than that of leucine plus valine or of isoleucine inhibition of threonine dehydratase. Acetolactate synthase has been shown previously to be located in chloroplasts (20). The failure of threonine to consistently inhibit isoleucine synthesis from ^{14}C-aspartic acid (Table IV) does not necessarily contradict those conclusions drawn from the experiment in Table VI since some label probably enters isoleucine via the formation of [^{14}C]pyruvate.

Metabolism of Malic Acid. Malic acid is readily converted by the chloroplast preparations into amino acids, particularly aspartic acid (Tables VII and VIII). However, since aspartic acid production was not dependent on light and was enhanced by the addition of mitochondria, it is difficult to assess whether the conversion (presumably via oxaloacetate) occurred in the plastids or in the small amounts of contaminating mitochondria. Certainly, plastid involvement is not excluded since increasing the mitochondria 66-fold less than doubled aspartate formation and the plastid preparation formed the aspartic acid-derived amino acids at light-dependent rates comparable to those achieved with [^{14}C]aspartate, irrespective of the level of mitochondrial contamination. Furthermore it is known that: (*a*)much of the total aspartate aminotransferase is associated with the plastids; (*b*) a NADP-malic dehydrogenase which is regulated via the ferredoxin/thioredoxin system is present in spinach chloroplasts (28), and (*c*) plastids accumulate malate from low external concentrations (13). It has been suggested by other workers that chloroplasts cannot synthesize most amino acids from $^{14}CO_2$ but that fixed carbon must be exported to the cytoplasm (or mitochondria) where keto acids are produced (see ref. 15 for a full description of this process); these then reenter the plastid where amino acid synthesis occurs. Our results are consistent with this idea, as malic acid (which could be synthesized in the tricarboxylic acid cycle) appears to be an effective precursor for aspartic acid family amino acids in isolated chloroplasts.

In previous work Mills and Wilson (22) observed a highly labeled compound co-chromatographing with methionine and a lack of label migrating with homoserine. By contrast, we have now found only a small amount of label in compounds co-chromatographing with methionine and a large amount in homoserine. This discrepancy could be explained if the labeled compound Mills and Wilson (22) observed to migrate with methionine was

Table VII. *Incorporation of Radioactivity into Various Fractions after Incubation of [^{14}C]Malic Acid with Isolated Pea Chloroplasts*

One-ml samples containing 406 μg Chl and 2 μCi [^{14}C]malic acid were incubated in KCl medium for 20 min. Sixty-six times the amount of mitochondria (estimated by Cyt oxidase activity) was added to some samples. Samples were extracted by method A (see under "Materials and Methods") and analyzed by two-dimensional TLC. Label in protein was not determined.

Fraction	Incubation Conditions							
	Control				Plus Mitochondria			
	Light		Dark		Light		Dark	
	cpm × 10^{-4}	*% of recovered cpm*	*cpm* × 10^{-4}	*% of recovered cpm*	*cpm* × 10^{-4}	*% of recovered cpm*	*cpm* × 10^{-4}	*% of recovered cpm*
Lipids	14	5	10	3	11	4	10	3
Soluble non-amino acids	200	66	201	68	56	53	111	38
Soluble amino acids	89	29	85	29	123	42	165	56
CO_2	1	0	1	0	5	2	9	3
Totals	304	100	297	100	294	100	296	100

Table VIII. *Incorporation of [^{14}C]Malic Acid into Aspartate-derived Amino Acids by Isolated Pea Chloroplasts and Mitochondria*

One-ml samples containing 406 μg Chl and 2 μCi [^{14}C]malic acid were incubated in KCl medium for 20 min. Sixty-six times the amount of mitochondria (estimated by Cyt oxidase activity) was added to some trials. Samples were extracted by method A and analyzed by two-dimensional TLC.

Soluble Amino Acid	Incubation Conditions						
	Chloroplasts			Chloroplasts plus Mitochondrial Fraction			Mitochondrial Fraction Only
	Light	Dark	Fold Stimulation in Light	Light	Dark	Fold Stimulation in Light	Light
	cpm × 10^{-2}			*cpm* × 10^{-2}			*cpm* × 10^{-2}
Aspartic acid family amino acids							
Asp	7,795	8,432	0	18,197	16,066	0	14,116
Hse	876	51	17	184	41	5	7
Lys	68	8	9	32	14	2	4
Thr	72	9	8	29	7	4	0
Ile (+Leu)	61	10	6	34	18	2	7
Met	2	0		0	1		0
Others							
Ala	31	80	0	172	305	0	393
Glu	31	84	0	177	673	0	654

a derivative of homoserine. Alternatively, the differences could be due to the developmental state of the plants (*e.g.* developmental variation in aspartate kinase activity has recently been found), varietal differences, or alternative methods of sample preparation and analysis.

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