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Synthesis and Interconversion of Amino Acids in Developing Cotyledons of Pea (*Pisum sativum* L.)

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ABSTRACT

Freshly isolated cotyledons from 10-day developing pea (*Pisum sativum*) seeds were fed radiolabeled precursors for 5 hours, and the specific radioactivity of the free and total protein amino acids was determined using a dansylation procedure. When the seven most abundant amino acids in phloem exudate of pea fruits (asparagine, serine, glutamine, homoserine, alanine, aspartate, glycine) were fed singly, their carbon was distributed widely among the aliphatic amino acids, proline and tryptophan; sporadic labeling of tyrosine and histidine also occurred. Feeding of glucose led to relatively greater labeling of aromatic amino acids including phenylalanine. The data support the involvement of known plant pathways in these interconversions. Labeling patterns were consistent with participation of the cyanoalanine pathway in the conversion of serine to homoserine, and with the synthesis of histidine from adenosine. All of the labeled amino acids were incorporated into protein.

Synthesis of reserves by the developing seed requires a continuous supply of organic solutes from the parent plant, which arrive predominantly in the phloem. In legumes the carbon chains of these solutes have undergone a complex process of recycling between leaf, root, stem, and pericarp, with incorporation of amino nitrogen in nodules and further processing at each subsequent site (11, 18) including the cross-transfer from xylem to phloem (19). Studying the composition of fruit phloem sap of *Pisum sativum*, Lewis and Pate (12) found about seven times as much carbon to be in the form of sugars as in amino acids. Of the latter, on a molar basis nearly 90% was present as asparagine, serine, glutamine, homoserine, alanine, aspartate, and glycine; many amino acids required for protein synthesis occurred in only trace amounts or were not detected. On the other hand, when $^{14}CO_2$ was fed to a leaf, the specific radioactivity of the amino acids in protein of seeds at that node was relatively uniform (12). This suggested that the seeds themselves must actively synthesize those amino acids present in low concentration or not detected in the phloem sap. Totipotency in amino acid synthesis is also indicated by the apparent ability of developing lipin seeds (1) and cultured pea cotyledons (16) to use asparagine as sole N source.

The above evidence is, however, indirect. The possibility remains that the attached seed could be incapable of synthesis of certain amino acids, yet by efficient incorporation of the trace amounts entering in the phloem sap, avoid limitation of protein synthesis. The aim of the present study was to assess the capacity of the isolated developing pea cotyledon for synthesis of amino acids from phloem sap precursors and for their incorporation into protein. Of particular interest were the sulfur amino acids, whose low content limits the nutritional value of legume seed protein for humans and other monogastric animals.

MATERIALS AND METHODS

Labeled Precursors. L-[U-^{14}C]asparagine (151 mCi/mmol), L-[U-^{14}C]serine (159 mCi/mmol), L-[U-^{14}C]glutamine (48 mCi/mmol), L-[U-^{14}C]homoserine (21 mCi/mmol), L-[U-^{14}C]alanine (173 mCi/mmol), L-[U-^{14}C]aspartic acid (224 mCi/mmol), [U-^{14}C]glycine (114 mCi/mmol), D-[U-^{14}C]glucose (281 mCi/mmol), L-[U-^{14}C]phenylalanine (522 mCi/mmol), and [2-^{3}H]adenosine (22 Ci/mmol) were obtained from Amersham.

Plant Material and Precursor Feeding. Plants of *P. sativum* L. cv. Greenfeast were raised in a growth cabinet as described by Millerd and Spencer (15), and pods were collected 10 days after anthesis. Using sterile gloves and scalpel, cotyledon pairs (wt: 180-240 mg) were dissected from the seeds carefully to avoid bruising or cutting injury, except for the small cut necessary for removal of the embryonic axis. Each cotyledon was laid, adaxial (flat) surface downward, on a 20-μl drop of radioactive precursor resting on a piece of Parafilm (freshly exposed surface) in a Petri dish, whose lid was lined with moist filter paper. The dish was placed near a south-facing window under natural illumination (*i.e.* out of direct sunlight) for 5 hr at 20 to 22 C, then the cotyledons were swirled briefly in a large volume of distilled H_2O, blotted dry, and dropped into liquid N_2.

Determination of Trichloroacetic Acid-soluble and -insoluble Radioactivity. Each pair of cotyledons was ground in a mortar with 2 ml of 5% trichloroacetic acid containing 0.2% casein hydrolysate, transferred quantitatively to a 10-ml cylinder, heated 15 min at 90 C to discharge amino acids from tRNA, allowed to cool, and the suspension made up to volume. For radioactivity in insoluble material, a 2-ml aliquot was filtered through an Oxoid membrane and the residue was washed five times with 5 ml of 5% trichloroacetic acid-0.2% casein hydrolysate, once with 0.5 ml of 80% ethanol, and once with 0.5 ml of ether. The membrane with residue was then heated at 50 C for 1 hr with 1 ml of Soluene (Beckman) in a scintillation vial with occasional shaking. After cooling, 9 ml of Triton-toluene-based scintillation fluid was added and the radioactivity counted. For soluble radioactivity, an aliquot of suspension was centrifuged at 10,000*g*, and a 1-ml aliquot of supernatant scintillation was counted as above.

Determination of Amount and Specific Radioactivity of Amino Acids. The rationale and development of this procedure are given elsewhere (13). Each pair of frozen, labeled cotyledons was homogenized, while thawing, with 1 ml of phenol-acetic acid-water (1:1:1, w/v/v). After centrifugation the residue was re-extracted with 1 ml PAW[1] and discarded. Total protein was precipitated from the combined supernatant by stirring in 5

[1] Abbreviation: PAW: phenol-acetic acid-water.

volumes of acetone and leaving overnight in the cold. The protein was collected by centrifugation and washed by resuspension in 80% ethanol (2 ml), heating briefly at 90 C. After centrifugation the ethanolic and acetone-PAW supernatants were combined to give the free amino acid fraction, which was purified by successive extraction with light petroleum (b.p. 60–80 C, 3 volumes), diethyl ether (2 volumes) plus water (0.25 vol), and lastly diethyl ether (2 volumes).

The protein pellet was reduced and carboxymethylated by a slightly modified Crestfield *et al.* (3) procedure and dialyzed against 50 mM $NaHCO_3$ before being hydrolyzed *in vacuo* for 24 hr at 110 C with 6 N HCl containing a little mercaptoethanol and phenol. Following removal of HCl by rotary evaporation the hydrolysate was freed from NH_3 by dissolving in 0.2 M $NaHCO_3$, taking to dryness again, and redissolving in water. The free amino acid fraction was reduced and carboxymethylated by making it 0.2 M in $NaHCO_3$, adjusting to pH 8.5 with NaOH, and treating with 2 mM dithiothreitol under N_2 for 30 min followed by 10 mM iodoacetic acid under N_2 for 60 min. Excess iodoacetic acid was destroyed with 20 mM mercaptoethanol.

An aliquot from each amino acid fraction, equivalent to 10 μg α-amino-N, was dansylated for 5 hr at 37 C in a reaction mixture containing 0.1 M $NaHCO_3$, 5 mM dansyl chloride, and 50% acetone. The reaction products were evaporated to dryness, dissolved in 0.1 N HCl, and passed through a 1-ml column of Porapak Q resin. After washing the column with 0.1 N acetic acid, the dansyl amino acids were eluted with 80% acetone. The eluate was taken to dryness and the residue dissolved in ethanol for TLC.

The dansyl amino acids were separated on plastic-backed polyamide layers (15 × 15 cm) (Schleicher and Schuell F1700) in the following solvent systems. For protein amino acids the first dimension solvent was 1.5% formic acid and the second was benzene-acetic acid (9:1; twice) followed by butyl acetate-methanol-acetic acid (30:20:1). For free amino acids, two chromatograms (A and B) were run. Chromatogram A, for the dansyl derivatives of Trp, Lys, Phe, Tyr, Leu, Ile, Val, Pro, Gly, Met(O), αAbu, γAbu, βAla, and Ala, was developed in the same system as above with omission of the last solvent. Chromatogram B, for the derivatives of CMC, Asp, Glu, Ser, Thr, Hse, Gln, Asn, Arg, and His, was developed in 0.5% formic acid (first dimension) and chloroform-methyl ethyl ketone-acetic acid (8:1:1) (second dimension). Representative separations are shown in reference 13. After location of the spots under long wave UV light they, together with appropriate blank spots, were cut out and placed in scintillation vials, paying scrupulous attention to cleanliness, and eluted overnight in the dark with 95% ethanol-1% triethylamine. The fluorescence of a small aliquot of the eluate was measured, using a Turner model 111 fluorometer with No. 7-60 primary filter, No. 2A plus No. 65A secondary filters, and the microcuvettes supplied with the instrument. Following addition of phosphor, the radioactivity of the remaining eluate was measured using a Packard TriCarb scintillation counter (see below). The fluorescence value, after correction for fluorescence of the solvent and of the blank spot, was converted to amount of dansyl amino acid present in the eluate, using a value previously determined for the pure compound. This enabled calculation of the specific radioactivity.

In order to obtain figures for the total amino acid pools it was necessary to correct the amount of eluted dansyl derivative for losses during extraction, derivatization, and elution. Extraction losses, as judged by recovery of Leu and Asn, were insignificant. A combined figure for dansylation efficiency and elution recovery was obtained for each amino acid by adding it to pea cotyledon extract (so as to increase its α-amino-N concentration by not more than 10%) before dansylation, chromatography and elution. This figure ranged from 41% for His (measured as α-dansyl-His) to 102% for Ala.

Statistical Analysis of Radioactivity Data. For assessment of the significance of low levels of radioactivity the following procedure was used. Ten "background" dials, containing elution solvent and scintillation fluid only, were interspersed among the sample vials from each experiment and all vials were counted to 1,000 counts for two cycles. The eluates of the blank spots (see above) were included in each run; they invariably showed only background levels of radioactivity. Then if $\bar{d}$ is the difference of the mean radioactivity of a given experimental vial from the mean of all background vials, its estimated standard deviation $\hat{\sigma}_{\bar{d}}$ is given by

$$\hat{\sigma}\frac{2}{d} = \frac{11}{20}(BMS) \quad (1)$$

where *BMS* is the "between vials" variance of the background vials. The quotient $\bar{d}/\hat{\sigma}_{\bar{d}}$ gives the t value with 9 degrees of freedom, which is compared with that in the t-table for a significance level of 0.1%. This was taken as the criterion for significant labeling of an amino acid.

RESULTS AND DISCUSSION

Values for the total pools of amino acids in a typical pair of cotyledons 10 days after anthesis are shown in Table I. In view of its acid lability, the figures for tryptophan are only approximate. Conspicuous are the large pools of free alanine, arginine, asparagine, glutamine, homoserine, and threonine, and the very small pools of cysteine, methionine, and tryptophan both free and in protein. Since in the 10-day seed synthesis of storage globulins has only just begun (15), it is likely that the protein amino acid pool mainly reflects the average composition of the metabolic and structural proteins of the cotyledon. The composition of the free amino acid pool differs markedly from that of phloem sap (12) in its very high level of arginine, possibly a temporary storage compound, and low levels of serine, glycine, and aspartate, all of which are likely to be in heavy demand for synthesis of other amino acids. Alternatively, the relative levels of these

Table I. Total Pools of Amino Acids in a Pair of 10-Day Pea Cotyledons

Amino Acid	Protein	Free
	μmol/g fresh wt.	
αAbu*		0.69
γAbu		2.0
Ala	10.0	33.1
βAla		0.87
Arg	9.2	33.8
Asp		2.3
Asn	12.1	16.6
Cys	1.4	0.75
Glu		11.2
Gln	12.3	26.6
Gly	13.1	1.54
His	3.4	1.09
Hse		6.4
Ile	9.0	2.4
Leu	17.7	1.38
Lys	9.4	1.8
Met	1.7	0.80
Phe	9.4	0.67
Pro	2.0	0.49
Ser	11.8	1.9
Thr	6.6	16.0
Trp	0.48	0.36
Tyr	7.0	0.37
Val	13.0	5.9

* Abbreviations as in (10).

and other amino acids may be the result of specific control mechanisms.

Exposure of the adaxial surface of the cotyledon to labeled amino acids resulted in rapid distribution of radioisotope through the tissue. This was shown by feeding cotyledons for the usual period (5 hr) with [U-^{14}C]glycine or [U-^{14}C]aspartate, then washing them and cutting each cotyledon, parallel to the adaxial surface, into five to six freehand sections. When these were dispersed in warm Soluene and scintillation-counted it was found that the section farthest from the adaxial surface (this was also the smallest section) contained 23% (glycine) or 12% (aspartate) as much radioactivity as the closest one (mean of two experiments).

The time course of appearance of ^{14}C in the trichloroacetic acid-soluble and -insoluble fractions of cotyledons fed with [U-^{14}C]aspartate is shown in Figure 1. While the soluble (free amino acid) fraction accumulated label at a constant rate until it reached a plateau at about 5 hr, the insoluble (protein) fraction showed an initial lag followed by a relatively constant rate of incorporation between 3 and 8 hr. Since one aim of this study was to learn whether the seed can incorporate each of its synthesized amino acids into protein, 5 hr was selected as the precursor-feeding period in subsequent experiments.

Although developing pea cotyledons from the growth cabinet are essentially free of bacteria (16), it was necessary to confirm that under the present incubation conditions bacterial contamination was insignificant. Following incubation on droplets of [^{14}C]asparagine or [^{14}C]glycine, cotyledons (each with its droplet) were shaken in sterile H_2O which was plated out onto Oxoid nutrient agar and incubated at 25 C. After 2 days no colonies had developed; after 5 days there was a maximum of nine colonies/cotyledon.

In the first series of feeding experiments the seven major phloem sap amino acids (12), uniformly ^{14}C-labeled, were each fed to freshly isolated cotyledons for 5 hr. The resulting specific radioactivities of the amino acids in the protein and nonprotein fractions are shown in Table II. In order to gain more information about the synthesis of aromatic amino acids and histidine, further experiments were performed with uniformly labeled [^{14}C]glucose and [^{14}C]phenylalanine, and specifically labeled [^{3}H]adenosine as precursors; the results are given in Table III. Not included in these tables is the significant labeling found in several as yet unidentified compounds, which is the subject of continuing investigation.

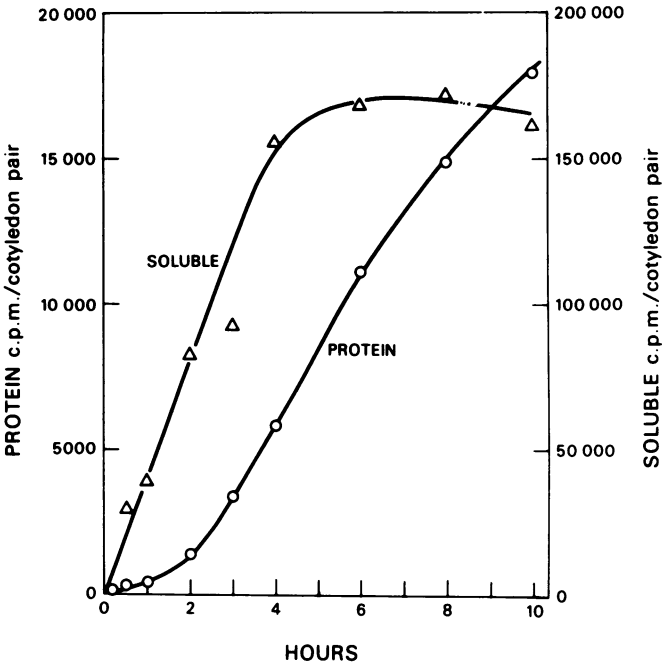

FIG. 1. Time course of appearance of label in protein and trichloroacetic acid-soluble fractions of 10-day pea cotyledons following the feeding of [U-^{14}C]aspartate. A set of cotyledon pairs with weights matched to 250 ± 5 mg was used.

Table II. Specific activity of pea cotyledon amino acids after feeding as ^{14}C-labeled precursor each of the 7 major phloem sap amino acids

One μCi of ^{14}C presented to each cotyledon. Amino acid abbreviations as in (10).

Precursor	Protein Amino Acids	Free Amino Acids
	dpm/nmole	
L-Asparagine-^{14}C(U)	Asx(565), Glx(20), Lys(15)	Asn(373), Asp(306), Met(160), Trp(150), Glu(81), Pro(40), βAla(39), γAbu(36), Ile(16), Hse(15), Lys(11), Val(4.4), Ala(3.7), Arg(2.3)
L-Serine-^{14}C(U)	Ser(427), Cys(244), Gly(125), Met(14), Ala(4.5), Leu(3.5), Val(3.2), Glx(1.5)	Ser(590), Gly(210), Cys(100), Met(79), βAla(66), γAbu(16), Val(7.6), Asn(6.5), Thr(2.4), Ala(1.2)
L-Glutamine-^{14}C(U)	Glx(71), Pro(24), Arg(18), Asx(17)	Gln(315), Asp(216), Met(206), γAbu(156), Glu(120), Pro(109), Arg(8.5), Ala(4.8)
L-Homoserine-^{14}C(U)	Thr(63), Met(43), Ile(28), Gly(12), Ser(9), Leu(1.5)	Thr(210), Trp(155), Hse(125), Met(110) Asp(80), αAbu(60), Gly(53), Ile(16), Leu(14), Ser(13), γAbu(6.0), Val(1.8), Ala(0.5)
L-Alanine-^{14}C(U)	Ala(54), Leu(29), Val(18), Glx(16), Lys(14), Asx(8.7), Pro(8.5), Ile(4.8), Arg(4.3)	Ala(100), Tyr(99), Glu(95), γAbu(89), Leu(72), Asp(50), Lys(13), Gly(9.9), Val(5.7), Arg(3.4) Gln(2.9)
L-Aspartate-^{14}C(U)	Asx(25), Leu(9.3), Glx(7.7) *	Asp(1580), Glu(240), Trp(160) Met(78), αAbu(71), βAla(69), γAbu(43), His(28), Ala(21), Thr(5.0), Arg(2.1)
Glycine-^{14}C(U)	Ser(260), Gly(250), Cys(170), Met(57)	Gly(920), Ser(780), Met(270), His(230), Cys(110), Asp(103), Leu(57), Trp(39), Glu(11), Asn(6.3), Gln(3.6), Thr(2.6), Ala(1.2), Arg(1.2)

* No Trp recovered from hydrolyzate

Table III. Specific activity of pea cotyledon amino acids after feeding ^{14}C-Glucose, ^{14}C-Phenylalanine, or ^{3}H-Adenosine

Precursor	Protein Amino Acids	Free Amino Acids
	dpm/nmole	
D-Glucose-^{14}C(U), 2 μCi presented	Tyr(70), Cys(55), Phe(43), Val(27), Ala(17), Asx(9.1), Leu(8.8), Ser(5.4), Glx(4.8)	Asp(154), Cys(130), Phe(130), His(92), Tyr(77), Glu(59), βAla(40), γAbu(37), Leu(31), Ala(15), Val(7.0), Arg(2.7), Gln(1.7)
L-Phenylalanine-^{14}C(U), 1 μCi presented	Phe(1400), Trp(61), Tyr(16), Ser(4.5), Val(4.4), Lys(3.2), Gly(3.1)	Phe(12600), Tyr(203), Thr(1.5)
Adenosine-2-^{3}H, 20 μCi presented	His(4100), Arg(106), Phe(54), Cys(47), Ser(15)	His(1800), Met(1500), Lys(670), Cys(460)

In general, the drop in specific radioactivity (dilution of label) between two amino acids should be least when they are separated by fewest metabolic steps. Disparate pool sizes, however, can cause a substantial difference in specific radioactivity between two neighboring compounds in a pathway. On the other hand, if two amino acids exhibit comparable specific radioactivity and are adjacent members of a well established plant pathway, then the simplest hypothesis is that this pathway is operating in the cotyledon. Even here the possibility exists, at least theoretically, that the observed result reflects some effect of compartmentation on labeling kinetics. Bearing these problems of interpretation in mind, only the salient aspects of the data will be considered in what follows.

Each of the seven phloem sap amino acids was extensively metabolized to other amino acids in both its own and other biogenetic families. The labeling patterns are for the most part consistent with accepted pathways in plants (2, 6, 7). Those pathways which appear to be operative in the pea cotyledon are shown in Figure 2. Photosynthetic reactions are not included, since photosynthesis by the seed of the field pea has been found (4) to make a negligible contribution to its carbon economy.

Cysteine and methionine were labeled to relatively high specific radioactivity (Table II), suggesting that there is no marked limitation to synthesis by supply of reduced sulfur. The labeling of methionine is as expected if 4-carbon precursors yield the C_4 chain and the methyl group is provided by glycine or serine via the C_1 pool. Data of this type do not permit conclusions about the mechanism of homocysteine synthesis (5); in any case this amino acid and cystathionine could not be detected reliably with the present methodology.

Radiocarbon from homoserine was recovered in amino acids agreeing substantially with those labeled in pea shoot protein (20) (Table II). The heavy labeling of O-acetylhomoserine found by Pate *et al.* (20) and other workers (8) could not be confirmed because O→N acyl transfer at alkaline pH (9) would prevent dansylation of this compound. The low specific radioactivity of amino acids in the glutamate and pyruvate families supports the concept (17) that interconversions within the aspartate family occur in a compartment removed from the tricarboxylic acid cycle. Further support comes from the glucose-feeding experiment (Table III), in which radioactivity was not detected in any member of the aspartate family except aspartate itself. For the labeling of serine and glycine by homoserine no explanation based on known plant pathways can be offered at present.

The amides, particularly asparagine, were extensively converted by the cotyledons to other amino acids (Table II), as already recorded for pea shoots (20) and lupin seeds (1) fed through the transpiration stream of whole shoots. Asparagine itself was not labeled from 4-carbon precursors but was labeled from serine and glycine. This is consistent with a synthesis of asparagine via β-cyanoalanine (2), which would also account for

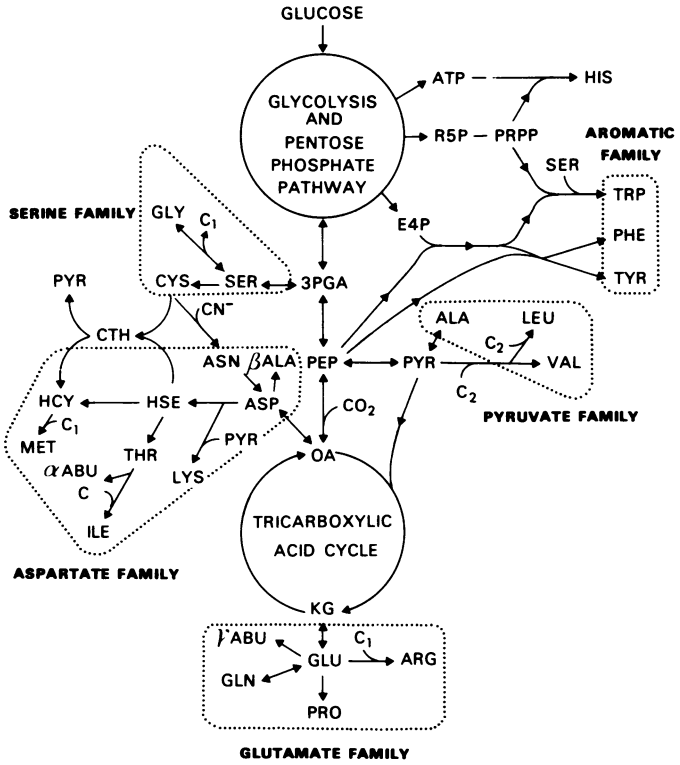

FIG. 2. Biosynthetic pathways for amino acid carbon chains in the pea cotyledon, that are consistent with the present data. Abbreviations: CTH: cystathionine; E4P: erythrose 4-phosphate; OA: oxaloacetate; KG: α-ketoglutarate; PEP: phosphoenolpyruvate; 3PGA: 3-phosphoglycerate; PRPP: 5-phosphoribosyl 1-pyrophosphate. Amino acid abbreviations as in (10). C_1 and C_2 represent one- and two-carbon fragments.

the observed labeling of aspartate family amino acids by serine and glycine. Unfortunately, lack of positive identification of the dansyl derivative has so far prevented the detection of labeling in β-cyanoalanine.

The aromatic amino acids, with the exception of tryptophan, were poorly labeled (tyrosine) or not labeled at all (phenylalanine) from the seven phloem sap amino acids (Table II). The feeding of glucose, which is the form of sucrose is the major carbon source for the developing pea seed (12), resulted in the labeling of these compounds, as well as many aliphatic amino acids, to fairly high specific radioactivity (Table III). When phenylalanine was fed, extensive degradation was indicated by the large decrease in specific radioactivity between recovered phenylalanine and the other labeled products. Tryptophan was

labeled from 4-carbon precursors and glycine but not by serine, alanine, or glucose. This is an unexpected result, for which an explanation is not yet apparent. The evidence also does not allow a conclusion as to whether the cotyledon can carry out *de novo* synthesis of the benzene or indole ring systems.

Histidine, whose synthesis is not well understood in plants (2), was labeled only from aspartate and glycine among the phloem sap amino acids (Table II). In microorganisms (14) glycine contributes carbon atoms to positions (4 and 5) of the purine ring of ATP which do not appear in histidine. Nevertheless, the preferential labeling of histidine by [2-^{3}H]adenosine (Table III) indicates that the early steps of histidine synthesis are the same in pea cotyledons as in microorganisms. Simultaneous catabolism of the purine ring to urea, which contains the C-2 atom (14) is suggested by the appearance of tritium in arginine.

The present study has shown that the isolated pea cotyledon has the capacity to make its own amino acids for protein synthesis, using precursors that are supplied in large amounts in the phloem and pathways that appear to conform largely to accepted synthetic routes in plants. The regulation of these pathways, and the question whether the flux through them is sufficient to account for the high rate of protein synthesis in the developing cotyledon, are interesting topics for future research.

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