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THE FATE OF HOMOSERINE-^{14}C IN GERMINATING PEAS

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Abstract—Radioactive L-homoserine, fed through the roots, is slowly converted at 25°, to other metabolite by 7- and 14-day-old pea seedlings. The homoserine content was increasing in the younger seedlings and decreasing in the older seedlings, yet the pattern and rate of metabolism appeared very similar except for slightly greater amounts of label appearing in the protein fraction exclusively as threonine in the older seedlings. Over 24 hr, approximately 5% of the label from 1-^{14}C-homoserine appears as CO_2. *O*-Acetyl homoserine, and threonine are substantially labelled in the free amino acid fraction with significant label also appearing in the organic acid fraction. The cystathionine pathway to methionine is probably operative, as methionine, cystathionine and homocysteine of high specific activity were detected, although not consistently.

INTRODUCTION

THE ACCUMULATION of high levels of homoserine in germinating peas has been reported several times.[1–4] Such high levels prompt one to ask whether this amino acid has a special, perhaps unusual metabolic function in this species. Pate *et al.*;[5] Larson and Beevers;[3] and Mitchell and Bidwell[6] have all examined the fate of ^{14}C-homoserine in pea seedlings and their results have thrown some light on the answer to this question. However, a limitation to the work of all three groups was the rather low specific activity of the homoserine that was supplied to the seedlings. The first group worked with seedlings at the 8 leaf stage, by which time the homoserine content is much lower than it is in younger seedlings,[3,4] and possibly the manner in which this amino acid is metabolized may change as development proceeds beyond the germination stage.

In the present work, the fate of homoserine of high specific activity fed to pea seedlings at two different stages of development has been investigated.

RESULTS AND DISCUSSION

Three experiments were conducted. Details about the growth and feeding of the seedlings are summarized in Table 1. In all cases the amount of homoserine taken up was less than 1% of the amount of this amino acid that was found in the seedlings. Thus it is unlikely that the level of feeding caused any abnormal metabolic response.

The seedlings in experiments A and B were both harvested at the same age but the seeds germinated more rapidly in experiment B and consequently it will be noted that the seedlings were considerably larger than those in experiment A. Growth conditions were identical but experiment B was carried out several months later. The explanation for this variation is unknown, but in spite of this, the seedlings in both experiments A and B were harvested at

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[2] J. M. LAWRENCE and D. R. GRANT, *Plant Physiol.* **38**, 561 (1963).
[3] L. A. LARSON and H. BEEVERS, *Plant Physiol.* **40**, 424 (1965).
[4] D. R. GRANT and E. VOELKERT, *Phytochem.* **9**, 985 (1970).
[5] J. S. PATE, J. WALKER and W. WALLACE, *Ann. Bot.* **29**, 475 (1965).
[6] D. J. MITCHELL and R. G. S. BIDWELL, *Can. J. Bot.* **48**, 2037 (1970).

a stage where the homoserine content would be increasing.[3,4] The seedlings in experiment C took up the labelled compound at a stage when homoserine content would have declined considerably from the maximum level.[3,4]

The distribution of the radioactivity among the components analysed, is given in Table 2. An activity balance revealed that 98·7, 99·2 and 104·2% of the administered ^{14}C was recovered in experiments A, B and C respectively.

TABLE 1. SUMMARY OF FEEDING EXPERIMENTS

Experiment	A	B	C
Material fed	1-^{14}C-L-homoserine	U-^{14}C-L-homoserine	1-^{14}C-L-homoserine
Specific activity	14 mCi/m-mol	31·8 mCi/m-mol	14 mCi/m-mol
Amount of activity supplied*	38·9	61·7	37·5
Amount of activity taken up*	13·2	60·6	17·6
Age at start of feeding period	6 days	6 days	12 days
Duration of feeding period	10 hr	10 hr	12 hr
Incubation time†	16 hr	16 hr	36 hr
Age at harvest time	7 days	7 days	14 days
Mean length of root shoot axes‡	44 mm	81 mm	282 mm
Dry weight per 10 seedlings‡			
cotyledons	1·455 g	1·442 g	0·347 g
roots	0·031 g	0·148 g	0·244 g
shoots	0·040 g	0·121 g	0·637 g

* In dpm $\times$ 10^6–10 seedlings were used for each experiment.
† Interval between conclusion of feeding and time of harvesting. During this period the seedling roots were immersed in water.
‡ At harvest time.

There appears to be very little difference in the pattern of label distribution among the fractions in the three experiments if the differences in the length of the feeding and incubation periods are taken into account. The larger seedlings seemed to convert a somewhat higher proportion of the radioactivity into protein, in which threonine was the only detectable radioactive amino acid.

TABLE 2. PER CENT DISTRIBUTION * OF RADIOACTIVITY AMONG ISOLATED FRACTIONS

Fraction	Experiment A	B	C
Respired CO_2	5·08	2·33	11·54
Free amino acids	88·83	90·59	73·10
Organic acids	4·43	2·34	6·69
Soluble carbohydrates	0·30	0·18	0·67
Proteins	0·68	3·68	4·10
Nucleic acids	0·46	0·34	1·84
Lipids	0·06	0·35	0·51
Crude fibre	0·16	0·19	1·55

* Percentage of activity taken up by whole seedlings.

The rate at which homoserine was converted to CO_2 in experiments A and C was approximately twice that observed in experiment B. Since U-^{14}C-homoserine was used in experiment B and 1-^{14}C-homoserine in the other two experiments, the implication is that much of the $^{14}CO_2$ must have arisen from decarboxylation of the number 1 carbon.

TABLE 3. DISTRIBUTION OF ^{14}C ACTIVITY AMONG COTYLEDONS, ROOTS AND SHOOTS

Fraction	Percentage of activity								
	Experiment A			Experiment B			Experiment C		
	Cotyledons	Roots	Shoots	Cotyledons	Roots	Shoots	Cotyledons	Roots	Shoots
Free amino acids	71·0	6·7	22·3	1·3	83·0	15·7	2·8	50·3	46·9
Organic acids	64·6	9·6	31·1	5·2	76·9	17·9	6·0	45·1	48·9
Soluble carbohydrates	74·1	2·7	23·2	13·3	64·6	22·1	4·1	15·6	80·3
Proteins	58·9	5·7	35·4	1·0	24·7	74·3	2·9	27·2	69·8
Nucleic acids	82·1	11·0	6·9	7·1	32·9	60·0	7·8	29·2	63·0
Lipids	68·6	24·4	7·0	2·5	74·3	23·2	3·4	13·2	83·4
Whole seedling	70·7	6·6	22·7	1·5	80·5	18·0	3·3	47·8	48·9

* The activity in all parts of the seedling for each type of fraction was set at 100%.

The distribution of the label among the cotyledons, roots and shoots for each of the fractions isolated is shown in Table 3. It will be noted that in experiment A most of the activity has been translocated away from the roots, with the major portion appearing in the cotyledons and an intermediate amount in the shoots. In experiment B most of the activity has remained in the roots, with very little appearing in the cotyledons. There are two factors which may account for these differences. As already mentioned, the seedlings in experiment A were less developed, and greater participation of the cotyledons in the overall metabolism was to be expected. Second, the humidity of the air circulating through the growth chamber was fairly low in experiment A, thus favouring transpiration and translocation. In experiments B and C, steps were taken to increase the relative humidity of the circulating air so that less water loss to the seedlings by transpiration would be expected. The distribution of label in the 14-day-old seedlings between the roots and shoots was almost equal, with very little activity in the cotyledons. The greater extent of translocation to the shoots in comparison with experiment B probably reflects the longer feeding and incubation period.

The shoots of the seedlings appear to be much more extensively engaged in protein synthesis than the roots. This is in agreement with the observation of Mitchell and Bidwell[6] that light stimulates the conversion of homoserine to alcohol insoluble compounds.

No other free radioactive amino acid except homoserine could be detected in the cotyledons. In the roots and shoots the distribution of ^{14}C among the free amino acids is presented in Table 4. Homoserine accounted for 86% of the activity in the shoots in experiment A and in all other cases the percentage was even higher.

Homoserine has been considered a precursor of threonine in bacteria and fungi as well as in higher plants.[5,7–11] In many species homoserine has been implicated in the formation of methionine, with *O*-acetyl homoserine, cystathionine and homocysteine as intermediates.[7,8,12–14] During the first 2 weeks following germination, there does not appear to

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be anything unusual in the metabolism of homoserine in pea seedlings, insofar as it involves other amino acids. The conversion to threonine as reported by both Goas[11] and Pate *et al.*[5] is confirmed in spite of the fact that Larson and Beevers[3] were unable to detect labelled threonine in their experiments. A comparison of the dilution values in roots or shoots indicates that this conversion is quite direct.

It is also quite probable that pea seedlings synthesize methionine from homoserine via the cystathionine pathway. The results are not conclusive as the labelled intermediates, cystathionine and homocysteine, were each detected only in the shoots in experiment A and labelled methionine was detected only in roots and shoots in experiment C. The failure to detect methionine consistently is probably associated with the ability of pea seedlings to convert methionine to homoserine.[15] As the dilution value for cystathionine is lower than that of *O*-acetyl homoserine the data are not consistent with the latter being a precursor of the former, unless the metabolite pools are compartmented.[16] However, application of the analytical method to authentic cystathionine revealed that the recovery of CO_2 was rather low compared to that of other amino acids. Since the ^{14}C label was on a carboxyl carbon, it is probable that this carbon would be preferentially converted to CO_2 and consequently we suspect that the dilution values reported for this amino acid are too low.

Nigam and Ressler[17] have reported that *Lathyrus sylvestris* converted homoserine to both 2,4-diaminobutyric acid and 2-aminoadipic acid. In peas no detectable activity was found in these two compounds, even though the mass peaks of their silyl derivatives could be distinguished in the gas chromatograms for the 14-day-old seedlings.

TABLE 4. PER CENT DISTRIBUTION OF ^{14}C ACTIVITY AMONG THE FREE AMINO ACIDS

	Experiment A		Experiment B		Experiment C	
	%*	D.V.†	%*	D.V.†	%*	D.V.†
Roots						
Homoserine	96·4	170	97·4	160	94·3	290
Serine			0·1	5800	0·4	1800
Threonine			1·6	330	1·2	560
Aspartic acid	1·6	1050				
Methionine					0·7	480
O-Acetyl homoserine	2·0	240	0·9	1100	3·4	300
Shoots						
Homoserine	86·2	130	96·6	330	90·4	290
Serine	0·5	970	0·2	3900	1·9	2400
Threonine	2·7	270	0·9	640	3·0	560
Homocysteine	0·6	310				
Asparagine	0·5	3900				
O-Acetyl homoserine	4·8	300	2·1	1200	3·4	760
Methionine					1·3	1300
Cystathionine	3·0	150				
Unknown 1	0·9					
Unknown 2	0·7					

* Based on the total activity in the free amino acid fraction of each type of tissue being equal to 100%.
† Dilution value = specific activity of homoserine supplied, divided by specific activity found for each compound after isolation from the seedling tissue.

[15] D. R. GRANT and E. VOELKERT, *Can. J. Biochem.* **49**, 795 (1971).
[16] D. J. MITCHELL and R. G. S. BIDWELL, *Can. J. Bot.* **48**, 2001 (1970).
[17] S. NIGHAM and C. RESSLER, *Biochemistry* **5**, 3426 (1966).

Labelled aspartic acid and asparagine were detected in only one of the experiments, and the dilution values were quite high. It would appear that the well accepted pathway from aspartic acid to homoserine[18] is not readily reversible.

Of the labelled homoserine that was taken up by the seedlings, the portion of the activity that was recovered as homoserine, presumably unchanged, was found to be 85, 88 and 67% in experiments A, B and C respectively. These results confirm that the turn over of labelled homoserine is a rather slow process.[3,5] This is not to say that the metabolism of homoserine is slow relative to that of other amino acids. Because of the high content of naturally occurring homoserine, a small percentage conversion still represents a significant amount of metabolic activity on an absolute scale. During the feeding and incubation periods, homoserine was being converted to other metabolites at average rates of 1·2, 2·4 and 4·0 μmol per seedling per day in experiments A, B and C respectively. Taking into account the differences in the average size of the seedlings and in the distribution of label among the parts, it would appear that for a given amount of tissue, the rate of conversion of homoserine to other metabolites was similar in all three experiments. A comparison can be made with another experiment in which labelled methionine-1-^{14}C was fed to 7-day-old pea seedlings, approximately 60% of the label being converted to other metabolites within 10 hr.[15] In this experiment methionine was converted at an average rate of 1·1 μmol per seedling per day. Even though the turnover rate for methionine was much faster, the absolute rate was similar or perhaps slightly slower than that of homoserine.

The similarity in the overall rate of homoserine metabolism for 7- and 14-day-old seedlings is in agreement with the conclusion reached by Larsen and Beevers[3] that the decrease in homoserine content between the first and second week of growth is a result of a decreased rate of homoserine synthesis rather than an increased rate of utilization.

If one is to ascribe any special role to homoserine in young pea seedlings, it does not appear to be associated with amino acid metabolism. The hypothesis has been put forward that this compound is involved in the mobilization and translocation of C and N reserves, as has been attributed to asparagine in many plant species.[6] The significant extent to which CO_2 and the organic acid fraction became labelled are in agreement with this hypothesis. The possibility still remains that there may be some involvement in the control mechanisms of the plant, perhaps by feedback inhibition of one or more of the seedlings' enzymatic systems.

EXPERIMENTAL

L-Homoserine (U)-^{14}C was purchased from Amersham Searle Corp., with a specific activity of 31·8 mCi/m-mol. Prior to use, the material was purified by paper chromatography using phenol–H_2O (3:1). The purified material was eluted from the paper according to the procedure of Dent.[19]

L-Homoserine-1-^{14}C (specific activity 14 mCi/m-mole) was prepared from L-methionine-1-^{14}C (Calbiochem) according to a modified[20] procedure of Flavin and Slaughter[21] and purified by paper chromatography using BuOH–H_2O–HOAc (12:5:3) and eluted as before. With both compounds, the material was shown to be free from impurities by two dimensional TLC on silica gel G combined with autoradiography.

Growth, feeding and harvesting of seedlings. Pea seeds (*Pisum sativum* L. cv. Alaska) were surface sterilized[4] and germinated in moist cotton wool. After 3 days the seedlings were transplanted to sterile moist quartz sand and subsequently subjected to alternating light–dark cycles. Light was provided by a combination of incandescent and fluorescent lamps at 500 lx. Seedlings to be harvested at 7 days were subjected to cycles of

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12 hr light and 12 hr dark and given H_2O only. Seedlings harvested at 14 days received cycles of 16 hr light and 8 hr dark and were provided with Hoagland's[22] nutrient solution. A constant temperature of 25° was maintained throughout the growth period.

Radioactive substrates were fed by inserting the roots of individual seedlings into small tubes, each containing 200 μl of solution. A small vacuum desiccator was used as a growth chamber and a slow flow of air through the desiccator was maintained by attachment of an aspirator. Respired radioactive CO_2 was collected in a NaOH trap placed between the desiccator and the aspirator. In experiments B and C the air entering the growth chamber was intermittently humidified by bubbling through H_2O. Following the feeding period the seedlings were transferred to similar tubes containing only H_2O for further incubation in the same chamber. The light–dark cycles were not interrupted during the feeding and incubation periods.

At harvest time the seedlings were dissected,[23] measured, and fresh weights determined and immediately frozen in liquid N_2. Dry weights were calculated from fresh weights using moisture content data that was obtained using similar seedlings that were not required for the feeding experiments.

Fractionation and counting of plant material. The fresh frozen material was ground with mortar and pestle with liquid N_2 and subsequently extracted with hot MeOH (4 ml/g fresh wt). This was followed by two more extractions with 80% aqueous MeOH. Further fractionation of the solid and extract was by a combination of the procedures used by Pate *et al.*[5] and by Reisener *et al.*[24] A Nuclear Chicago Unilux-I scintillation system was used for quantitative radioactivity determinations. A modified Bray's solution[25] was employed as most samples were in aqueous solution. Counting efficiency was determined by the channel ratio method.[26]

The free amino acids were further fractionated by gas chromatography of their silyl derivatives with subsequent combustion, flow counting and mass determination as described by Martin.[27] Identification was by comparison to authentic compounds using two temperature programs, and was confirmed by two dimensional TLC and autoradiography. Authentic *O*-acetyl homoserine was prepared from homoserine according to the method of Sheehan *et al.*[28] Further details of all experimental methods may be found elsewhere.[29]

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Key Word Index—Pisum sativum; Leguminoseae; pea; homoserine; threonine; cystathionine; homocysteine.