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AMINE BIOSYNTHESIS IN *LATHYRUS SATIVUS* SEEDLINGS

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Key Word Index—*Lathyrus sativus*; Leguminosae; chick pea; homoarginine; arginine; homoagmatine; agmatine; cadaverine; spermidine; spermine; infiltration; competition; biosynthesis.

Abstract—The biosynthesis of certain amines in *Lathyrus sativus* seedlings was studied in isolated shoots and cotyledons. In shoots, arginine was about 14 times more efficient than ornithine for the synthesis of agmatine, putrescine, spermidine and spermine. Isotope dilution experiments, and the changes in specific activities of the 4 amines with time when ^{14}C-arginine served as the precursor, indicated that putrescine and the polyamines were formed mainly from arginine, via agmatine. Similar experiments showed that cadaverine was formed at least in part from homoarginine, though lysine was *ca* 4 times more effective as a precursor. The pattern of changes in specific activity of homoagmatine and cadaverine with time when ^{14}C-homoarginine served as the precursor support the conclusion that homoarginine and arginine follow analogous metabolic routes in the biosynthesis of putrescine and cadaverine respectively.

INTRODUCTION

BIOGENESIS of the polyamines, spermidine and spermine has been extensively investigated in microbial and animal systems.[1] In rat liver[2,3] and ventral prostrate,[3] ornithine decarboxylation gives rise to putrescine which in turn condenses with the propylamino moiety of decarboxylated *S*-adenosylmethionine to form spermidine and spermine. In *Escherichia coli*, agmatine formed from arginine is converted in one step to putrescine and urea by agmatine ureohydrolase.[4] However, detailed investigations on poly and diamine biosynthesis are comparatively few with plant systems. Studies with potassium deficient barley leaves have shown that arginine is decarboxylated to form agmatine, which undergoes a two step enzymatic conversion to putrescine, with *N*-carbamyl putrescine as the intermediate.[5]

Lathyrus sativus seeds contain high concentrations of L-homoarginine.[6] In a recent study, we isolated and characterized homoagmatine from the *L. sativus* seedlings and demonstrated that this amine is a major metabolite of homoarginine during seedling development.[7] We have now found that cadaverine also accumulates during seedling growth, together with substantial quantities of agmatine, putrescine and spermidine, the postulated intermediates of polyamine synthesis in higher plants.[8] As two sets of homologous metabolites, viz agmatine–homoagmatine and putrescine–cadaverine are elaborated by the

[1] TABOR, H. and TABOR, C. W. (1964) *Pharmacol. Rev.* **16**, 245.
[2] PEGG, A. E. and WILLIAMS-ASHMAN, H. G. (1969) *Biochem. J.* **115**, 241.
[3] PEGG, A. E. and WILLIAMS-ASHMAN, H. G. (1968) *Biochem. J.* **108**, 533.
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[6] RAO, S. L. N., RAMACHANDRAN, L. K. and ADIGA, P. R. (1963) *Biochemistry* **2**, 298.
[7] RAMAKRISHNA, S. and ADIGA, P. R. (1973) *Phytochemistry* **12**, 2691.
[8] RAMAKRISHNA, S. and ADIGA, P. R. To be published.

seedling, it was considered that arginine and homoarginine may undergo similar metabolic conversions. To test this hypothesis we have studied the conversion of labelled guanidino amino acids into the respective amine metabolites in seedlings.

RESULTS AND DISCUSSION

The possibility that in *L. sativus*, the two guanidino amino acids undergo similar metabolic conversions to give rise to the respective guanidino-, di- and possibly polyamines (at least in the case of arginine), was explored in short term infiltration experiments using ^{14}C-labelled amino acids. The cut shoots of 4-day-old etiolated seedlings were fed with ^{14}C-ornithine and ^{14}C-arginine and allowed to metabolize the amino acids for a further period of 6 hr. The data presented in Table 1 show that both arginine and ornithine served as efficient precursors of agmatine, putrescine, spermidine and spermine. The sum total of the label incorporated into these four amines at 6 hr was 7·5% with ornithine, and 16·4% with arginine, on the basis of ^{14}C-amino acids supplied initially. These estimates were made after correcting for the loss of $^{14}CO_2$ during metabolism. The determination of the pool sizes of amino acids in the shoot extracts gave the following values: arginine, 586 nmol/g fr. wt, ornithine 91 nmol/g fr. wt. Using these values, the calculated amount of arginine metabolized to agmatine, putrescine, spermidine and spermine per g fr. wt of shoots was 96·0 nmol. This was in comparison to 6·8 nmol obtained with ornithine as precursor. Thus arginine appears to be at least 14× more efficient as a precursor than ornithine for amine synthesis. However, the label incorporated from ^{14}C-ornithine into agmatine represented nearly 40% of the total activity in the 4 amines. Thus it appears that ornithine served as a precursor of putrescine and hence of the polyamines mainly by conversion to arginine, probably in the urea cycle. However, the possibility of ornithine giving rise to putrescine by alternative, albeit minor routes in *L. sativus* cannot be ruled out at present. Ornithine decarboxylase has been demonstrated in other plant systems.[9] Moreover, ornithine could give rise to putrescine via citrulline and *N*-carbamyl putrescine as demonstrated in sesame leaves.[10] Further, the present data (Table 1) showing a higher proportion of the label in putrescine relative to agmatine with ^{14}C-ornithine as the precursor, compared with ^{14}C-arginine, is suggestive of a route of putrescine formation via citrulline in *L. sativus*. However, drastic reduction in the efficiency of conversion of ^{14}C-ornithine to all the four amines in the presence of exogenous unlabelled arginine (Table 1) indicates that if such alternative pathways of putrescine biosynthesis exist, they are relatively unimportant.

The conversion of ^{14}C-arginine to agmatine, putrescine, spermidine and spermine indicate that quantitatively, the more remote the amine from arginine in the biosynthetic sequence, the lower was the efficiency of incorporation of the label, a pattern expected from the operation of the proposed biosynthetic pathway, which is also supported by the isotope dilution experiments (Table 1). With arginine as precursor, unlabelled ornithine (5 μmol) reduced (by *ca* 20%) incorporation of label into agmatine and hence proportionally to subsequent products of the sequence. It is probable that enhanced arginine formation resulting from exogenously supplied ornithine and hence dilution of pool size of ^{14}C-arginine is responsible for this finding. Alternatively, ornithine might interfere with the arginine → agmatine step by inhibiting arginine decarboxylase.[8] By comparison, agmatine, *N*-carbamylputrescine and putrescine were more effective in reducing incorpor-

[9] HASSE, K., RATYCH, O. T. and SALNIKOW, J. (1967) *Z. Phys. Chem.* **348**, 843.
[10] CROCOMO, O. J., BASSO, L. C. and BRASIL, O. G. (1970) *Phytochemistry* **9**, 1487.

TABLE 1. INCORPORATION OF RADIOACTIVITY FROM ^{14}C-ORNITHINE AND ^{14}C-ARGININE INTO THE AMINES BY *Lathyrus sativus* SHOOTS AND ITS INHIBITION IN THE PRESENCE OF UNLABELLED INTERMEDIATES

Radioactive precursor	Unlabelled intermediate used (5 μmol)	(cpm/g fr. wt) Agmatine	Putrescine	Spermidine	Spermine	Total % conversion of ^{14}C
L-Ornithine-[1-^{14}C]	None	11400 (3·04)	13900 (3·70)	2500 (0·50)	1100 (0·25)	7·49
	Arginine	2400 (0·59)	3900 (0·96)	1200 (0·29)	570 (0·14)	1·98
	None	29200 (7·61)	16100 (5·20)	9700 (3·16)	1200 (0·40)	16·37
	Ornithine	24100 (6·23)	11200 (3·60)	5600 (1·82)	690 (0·22)	11·87
	Agamatine	20300 (5·10)	9700 (3·02)	2200 (0·72)	390 (0·13)	8·97
	N-carbamyl putrescine	21900 (5·90)	9500 (3·21)	1900 (0·66)	530 (0·18)	9·95
	Putrescine	28100 (7·43)	7900 (2·61)	1800 (0·60)	410 (0·14)	9·78

4×10^5 cpm Of ^{14}C-amino acids were used for each experiment. In the competition experiments 5 μmol of the presumed intermediate was mixed with the radioactive amino acid and infiltrated as described in the text. The weight of the shoots was 0·80–0·92 g and the values represented are averages of triplicate experiments. The amines were separated from the amino acids by ion-exchange chromatography and the radioactivity in the various amines separated by PC was determined. The numbers in parentheses correspond to % of conversion of labelled precursor amino acid to respective amines.

ation of label from arginine into subsequent metabolites, in agreement with their being true intermediates in the sequence of reactions leading to the polyamines.[5]

Similar infiltration experiments were also carried out with ^{14}C-homoarginine and ^{14}C-lysine in order to determine whether cadaverine biosynthesis follows an analogous metabolic sequence (Table 2). With ^{14}C-lysine as precursor, cadaverine became radioactive to a substantial degree after 6 hr; accounting for nearly 5·2% of the total labelled amino acid fed to the shoots. But, unlike the labelling of agmatine observed with ^{14}C-ornithine (Table 1), homoagmatine was devoid of radioactivity under these conditions indicating that cadaverine arose by direct decarboxylation of lysine and not through prior conversion to homoarginine and then to homoagmatine. This is not unexpected in view of the demonstration of lysine decarboxylase in other legumes.[11] With ^{14}C-homoarginine, both homoagmatine and cadaverine were radioactive, as expected, accounting for the utilization 1·78% of the labelled amino acid supplied. The pool sizes of lysine and homoarginine in the shoots were respectively 634 and 1580 nmol/g fr. wt. The amount of lysine metabolized for amine formation therefore amounted to 33 nmol/g fr. wt, while the amount of homoarginine was 30 nmol/g fr. wt. However, ^{14}C in cadaverine accounted for only 7·8 nmol of

[11] AMBE, L. and SOHONIE, K. (1959) *J. Sci. Ind. Res.* **18c**, 135.

TABLE 2. INCORPORATION OF RADIOACTIVITY FROM ^{14}C-LYSINE AND ^{14}C-HOMOARGININE INTO THE AMINES BY *Lathyrus sativus* SHOOTS AND ITS INHIBITION IN THE PRESENCE OF UNLABELLED INTERMEDIATES

Radioactive precursor	Unlabelled intermediate used (5 μmol)	cpm/g fr. wt in Homoagmatine	Cadaverine	Total % conversion of ^{14}C
L-Lysine-[U-^{14}C]	None	Nil	17500 (5·22)	5·22
	Homoarginine	Nil	15500 (4·85)	4·85
	None	2600 (1·31)	900 (0·47)	1·78
	Lysine	1000 (0·53)	600 (0·32)	0·85
	Homoagmatine	1300 (0·69)	700 (0·36)	1·05
	N-Carbamyl cadaverine	1400 (0·72)	500 (0·29)	1·01

* L-Homoarginine-[1 to 6-^{14}C] is synthesized from L-lysine-[U-^{14}C] as given in the text. For details see the legend for Table 1. 2×10^5 cpm of ^{14}C-homoarginine and 4×10^5 cpm of ^{14}C-lysine were taken.

homoarginine metabolized. Thus, while there is little doubt that a part of the cadaverine accumulating in *L. sativus* arises from homoarginine through homoagmatine, the pathway of direct decarboxylation of lysine to give rise to the diamine seems to be nearly 3× more efficient in this respect. This apparently lower efficiency of homoarginine utilization for diamine formation might be due to a requirement for a three step metabolic sequence (by analogy with the pathway from arginine to putrescine[5]) as against the single enzymatic step involved in the conversion of lysine to cadaverine. However, from the point of view of the physiology of the seedlings, the homoarginine pathway of cadaverine biogenesis might be significant in view of relative abundance of homoarginine in the tissues, and as other anabolic reactions, such as protein synthesis, requiring continued supply of free lysine may not compete for homoarginine.

Strong support for the hypothesis that the intermediary steps involved in the conversion of homoarginine to cadaverine are analogous to those proposed earlier for the arginine to putrescine sequence was obtained from competition experiments with unlabelled homoagmatine and *N*-carbamylcadaverine (Table 2). Both of these expected intermediates severely restricted ^{14}C-homoarginine utilization for cadaverine formation to degrees comparable to those observed earlier with ^{14}C-arginine conversion for putrescine production (Table 2). The only apparent difference between the two systems was the inhibition of ^{14}C-guanidino amino acid utilization for cadaverine and putrescine synthesis by unlabelled lysine and ornithine, respectively. Lysine was apparently more potent than ornithine in this respect and unlike ornithine, was not converted to the corresponding guanidino compound. The question of the biosynthetic route for homoarginine itself in *L. sativus*, obviously unconnected with the urea cycle enzymes, remains speculative at present.

With a view to substantiate further the co-existence of two parallel sets of reaction sequences leading to the biosynthesis of various amines from arginine and homoarginine, the ^{14}C-guanidino amino acids were infiltrated into shoots and slices of isolated cotyledons of *L. sativus* for 4 hr and the specific activities of the individual amines metabolized were determined at intervals thereafter. The changing patterns of specific activities of agmatine, putrescine, spermidine and spermine observed on ^{14}C-arginine feeding are shown in Fig. 1. With shoots, the specific activity of agmatine at 1 hr after uptake exceeded those of the other amines. But, as is evident from the shape of the curve, it was clearly falling rapidly at this time, apparently having reached its maximum earlier during the period of isotope administration itself (Fig. 1a). By contrast, the specific activity of its immediate product, putrescine, reached its peak at 4 hr and then declined, while those of spermidine and spermine did so after a further lapse of 2 hr. Similar profiles of specific activity curves obtained with cotyledons (Fig. 1b) lends further support to the existence of expected precursor–product relationships among these amines. The only difference appears to lie in the comparatively slower rates of amine biogenesis in the cotyledons, which may be related to the differential permeability of the tissues for the amino acid.

When similar experiments were conducted with ^{14}C-homoarginine the pattern that emerged was again clearly indicative of the operation of analogous metabolic sequence of reactions. With shoots (Fig. 2a) the specific activity of homoagmatine attained a peak around 4 hr, then slightly declined, remaining constant thereafter. During this interval, that of cadaverine rose slowly to a plateau around 6 hr. With cotyledons (Fig. 2b), homoagmatine production appears to be faster, since the guanidino amine presumably attained maximum specific activity during the infiltration period itself and at 2 hr following administration of the amino acid, it was clearly declining. The rate of cadaverine for-

mation progressively increased during this period, levelling off around 6 hr. The relationship between homoagmatine and cadaverine is therefore clearly that of a precursor and product in this plant tissue.

Thus, on the basis of the isotope conversion data as well as the pattern of specific activity changes observed among the proposed intermediates, the major biogenetic pathway of putrescine, and hence of polyamines, appears to follow a course identical to that shown in potassium deficient barley leaves.[5] The demonstration that homoarginine, like arginine undergoes similar metabolic conversion to vital products like the diamine, cadaverine, suggests that the guanidino amino acid is not merely a nitrogen-rich storage constituent in the seed. It now appears to have an important function in the developmental biology of the seedlings. The relationship between di and polyamines with nucleic acid and protein synthesis concerned in growth[12] establishes the importance of these pathways.

[12] BACHRACH. U. (1970) *Ann. Rev. Microbiol.* **24**, 109.

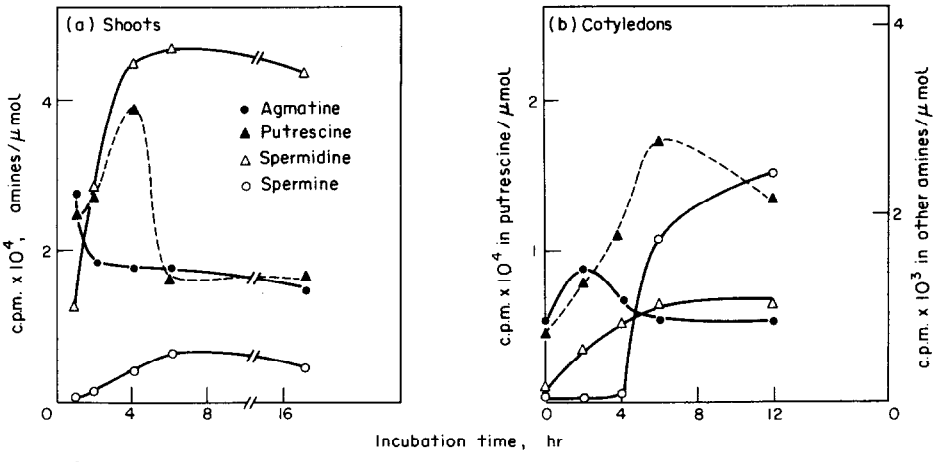

FIG. 1. CHANGES IN THE SP. ACT. OF AGMATINE, PUTRESCINE, SPERMIDINE AND SPERMINE WITH TIME WITH ^{14}C-ARGININE AS PRECURSOR IN *Lathyrus sativus* TISSUES.
Data are the means of closely agreeing triplicate values.

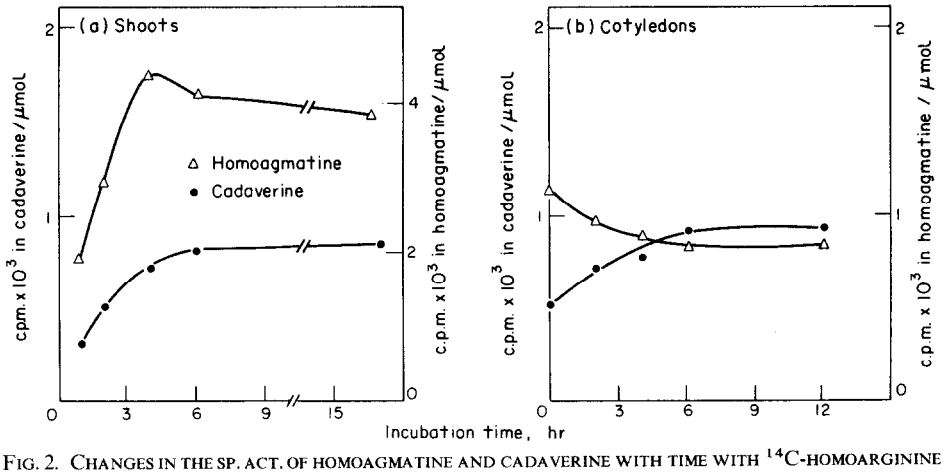

FIG. 2. CHANGES IN THE SP. ACT. OF HOMOAGMATINE AND CADAVERINE WITH TIME WITH ^{14}C-HOMOARGININE AS PRECURSOR IN *Lathyrus sativus* TISSUES.
Data are the means of closely agreeing triplicate values.

EXPERIMENTAL

Materials. Lathyrus sativus seeds were obtained from the Plant Breeding Section of the Indian Agricultural Research Institute (New Delhi, India). Unlabelled amino acids, putrescine dihydrochloride, agmatine sulphate and spermidine trihydrochloride were purchased from Sigma Chemical Co., (St. Louis, M.O., U.S.A.). L-Homoarginine was a product of Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Cadaverine (2 HCl) and spermine (4 HCl) were purchased from Calbiochem (Los Angeles, Calif., U.S.A.). *N*-Carbamylputrescine and *N*-carbamylcadaverine were synthesized according to Smith and Garraway.[13] Homoagmatine was prepared by guanidation of cadaverine with *O*-methyl isourea.[7] L-Arginine-[U-^{14}C] (sp. act. 66·0 mCi/mmol), L-lysine-[U-^{14}C] (sp. act. 99·0 mCi/mmol) were purchased from Bhabha Atomic Research Centre (Trombay, Bombay, India) L-Ornithine-[U-^{14}C] (sp. act. 200 mCi/mmol) was a product of New England Nuclear Corp., Boston, Mass., U.S.A. L-Homoarginine-[1 to 6-^{14}C] (sp. act. 1·9 mCi/mmol) was a gift. L-Homoarginine-[1 to 6-^{14}C] was synthesized from L-lysine-[U-^{14}C] (sp. act. 99·0 mCi/mmol) by guanidation as detailed below.

Preparation of ^{14}C-homoarginine from ^{14}C-lysine. To 100 μCi of L-lysine-[U-^{14}C] in 1 ml H_2O, 100 μmol of lysine was added as carrier. About 100 μmol of $CuSO_4$ was added to form the Cu complex. 150 μmol of *O*-methyl isouronium sulphate was added followed by addition of 0·5 ml 2 N NaOH. After standing for 5 days at room temp. the reaction mixture was acidified with HCl and the Cu removed with H_2S. Excess H_2S was expelled by aeration. The pH was adjusted to 4 and the soln applied to a Dowex 50 × 2 (200–400 mesh, H^+ form) 1·2 × 30 cm column and eluted with an HCl linear gradient (0–3·4 M). The effluent was collected in 3·5 ml fractions. Labelled homoarginine was eluted well resolved (fractions 55–70) from lysine (fractions 22–36). Fractions corresponding to ^{14}C-homoarginine was concentrated *in vacuo* at 60° and purified further. The purity was established by PC using n-BuOH–HOAc–C_5H_5N–H_2O (4:1:1:2), and PhOH–0·067 M HCl–KCl pH < 2 (4:1).

Infiltration experiments. 4-day-old *L. sativus* seedlings (germinated and grown as described earlier[7]) were used for all the infiltration studies. The seedlings were washed with H_2O and the cotyledons excised. The epicotyl (shoot) was cut from the embryo axis at the joint of shoot and root. The epicotyls (800–920 mg fr. wt) or slices of cotyledons, 0·5 mm thick (1·7–2·0 g fr. wt) were immediately dipped in the test soln containing the precursor amino acid in 0·25 ml of H_2O in a glass tube (1 × 5 cm) and infiltrated under vacuum.[14] After 3 hr most of the soln was infiltrated and 0·1 ml H_2O was added. The material was then incubated with H_2O at room temp. 25° in a moist chamber for specified periods of time. In the isotope dilution experiments radioactive material was mixed with 5 μmol of the unlabelled compound and infiltration was carried out as described above.

Preparation of acid soluble fraction. The tissue after incubation was homogenized in 3 vol of 0·4 M $HClO_4$. After centrifugation, the extract was freed from perchlorate by precipitation with KOH.[15]

Quantitative estimation of amino acids. The amino acid pool was obtained from the acid soluble fraction by the procedure described by Ramakrishna and Adiga,[7] and the quantities of lysine, arginine and homoarginine were determined with an automatic amino acid analyser, on a short column (0·9 × 15 cm) using 0·35 M Na citrate buffer pH 5·28 ± 0·02 at 50°. For the estimation of ornithine, the acidic and neutral amino acids were removed.[16] The ornithine-containing fraction was then analyzed[17] for the amino acid on the short column of the amino acid analyzer using 0·38 M citrate buffer of pH 4·26 ± 0·02 at 30°. The amino acid conc. were calculated by the procedure of Spackman *et al.*[18]

Quantitative determination of amines. The amine fraction was prepared from an acid-soluble extract[15] and the amines were separated by circular PC on a buffered Whatman No. 1 paper using PhOH–0·067 M HCl–KCl buffer pH < 2 (4:1) as solvent and the amine concn. determined as described earlier.[15]

Measurement of radioactivity. After PC, the radioactivity in the zones corresponding to the amines was measured with a Liquid Scintillation Counter in 0·5% PPO in toluene with an efficiency of 80% for ^{14}C on paper. Quenching corrections were made.

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