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HOMOAGMATINE FROM *LATHYRUS SATIVUS* SEEDLINGS

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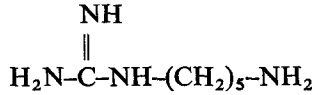
Key Word Index—*Lathyrus sativus*; Leguminosae; chick pea; isolation and characterization; homoagmatine; homoarginine; germination; changes in concentration.

Abstract—A new guanidino amine has been isolated from *Lathyrus sativus* seedlings and characterized as homoagmatine on the basis of various physico-chemical criteria including IR spectrum and comparison with that chemically synthesized. Homoagmatine is accumulated in the embryos axis while its precursor, homoarginine, is lost from the cotyledons. However, there was a progressive increase in homoarginine content of the embryo axis during development. Since the amine content of the whole seedlings corresponded to nearly 20–25% of net decrease in homoarginine levels, it is concluded that the catabolism of homoarginine through homoagmatine represents a major pathway of metabolism of the amino acid.

INTRODUCTION

IN 1963, we have reported on the isolation and characterization of L-homoarginine from the seeds of *Lathyrus sativus*.[1] Several other *Lathyrus* species have been shown to contain this unusual amino acid.[2,3] In *L. tingitanus*, this guanidino amino acid served as the precursor of lathyrine, through a series of conversions involving γ-hydroxyhomoarginine and its lactone,[4,5] as postulated earlier.[1] Since neither lathyrine nor any of the hydroxy derivatives of homoarginine are detectable in *L. sativus*, alternative routes of metabolism of this amino acid have to be envisaged. In studying the catabolism of homoarginine in *L. sativus*, Cheema *et al.*[6] concluded that the degradation of homoarginine through the urea cycle could represent at best only a minor pathway.

In the course of investigations on the biosynthesis and metabolism of polyamines in *L. sativus* during embryonic development and seedling growth, we found substantial amounts of a Sakaguchi- and ninhydrin positive amine which did not correspond to any of the hitherto recognized guanidino amines. This paper deals with the isolation of this substance from etiolated *L. sativus* seedlings and its characterization as homoagmatine (I) by various physico-chemical criteria and by comparison with chemically synthesized homoagmatine. To the best of our knowledge, this is the first report on the natural occurrence of this guanidino amine.



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RESULTS AND DISCUSSION

Approximately 5 g of *L. sativus* seedlings was extracted with 3 vol. (w/v) of 0·4 M perchloric acid. After removal of cell debris by centrifugation and perchlorate as K-salt, the amines were extracted into butanol from the aqueous phase as described in the Experimental. The butanol layer when concentrated and subjected to circular paper chromatography followed by spray with ninhydrin and Sakaguchi reagents revealed the presence of at least 7 ninhydrin positive compounds. Only two of these (with R_f 0·69 and 0·79) were guanidino compounds. The guanidino amine with R_f of 0·69 co-chromatographed with agmatine, while the other behaved differently when compared to any of the known guanidino amines.

Chromatography of the butanol extract on Dowex-50 (H^+) with 0·5–4 M HCl gradient did not clearly separate the guanidino amines from the other amino compounds. In order to further characterize the new amine, a large scale isolation and purification was carried out on seedlings as described in the Experimental. From the perchlorate-free extract applied to a Dowex-50 column, amino acids and water soluble non-cationic impurities were removed by washing with 4N NH_4OH, while most of the other amines were eluted from the resin with 1 M piperidine[7] as revealed by PC. Subsequent washing of the column with distilled water served to remove most of the piperidine from the resin column. However, traces of piperidine remaining with the resin at this stage interfered with the column chromatographic resolution of the guanidino amines by direct elution with an HCl gradient. Elution from the column with 6 N HCl and extraction into butanol at alkaline pH obviated this difficulty and yielded an amine fraction which when rechromatographed on another Dowex-50 column with an HCl gradient resulted in clear-cut separation of the new guanidino amine from agmatine and traces of other contaminants. The amine so obtained crystallized as the hydrochloride and a yield of 141·5 mg corresponding to *ca.* 85% recovery was realized.

TABLE 1. R_f OF AGMATINE, HOMOAGMATINE AND THE ISOLATED GUANIDINO AMINE ON PCs

Solvent system (v/v)	Agmatine	R_f Isolated guanidino amine	Synthetic homoagmatine
n-BuOH–HOAc–pyridine–H_2O (4:1:1:2)	0 39	0·43	0 43
PhOH–0 067 M HCl–KCl pH 2 (4:1)*	0·69	0·79	0 79
PrOH-conc. HCl–H_2O (8:1:1)	0·59	0·65	0 65
n-BuOH–MeCOEt–NH_4OH–H_2O (5:3:1:1)	0·17	0·21	0·21
PrOH–triethylamine–H_2O (85:3:15)	0·17	0 25	0·25

* Circular chromatography. In all other cases, ascending technique was used.

The purity of the isolated compound was ascertained by PC in a variety of solvent systems. R_fs obtained for the new amine together with those of agmatine and synthetic homoagmatine are listed in Table 1. In paper electropherograms, the guanidino amine moved as a single band well separated from the amino acids and other amines but not from synthetic homoagmatine. Since the amine hydrochloride (m.p. 135–137°) was highly hygroscopic, it was converted into diflavianate and dipicrate derivatives. Elemental analysis for the N in these derivatives gave the following: diflavianate (m.p. 170–172°) (Found: N,

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14·00. Calc. for $C_6H_{16}N_42$. $C_{10}H_6N_2O_8S$; N–14·50%); dipicrate (m.p. 197–200°) (Found: N, 23·65. Calc. for $C_6H_{16}N_42.C_6H_3N_3O_7$: N, 23·26%). Further proof of its identity as homoagmatine was obtained by comparison of the properties of the *L. sativus* amine and its derivatives with those of the synthetic compound prepared as outlined in the Experimental. Finally, IR spectra in a KBr disc of the isolated and synthetic homoagmatine were identical and superimposable. The spectrum showed characteristic absorptions as follows: stretching vibrations (guanidium: 3275–3407 cm^{-1}, NH_3^+: 3130 cm^{-1}, C–N 1170 cm^{-1}); bending vibrations (NH_3^+: 1490, 1610 cm^{-1}, guanidium: 1650, 1680 cm^{-1}).

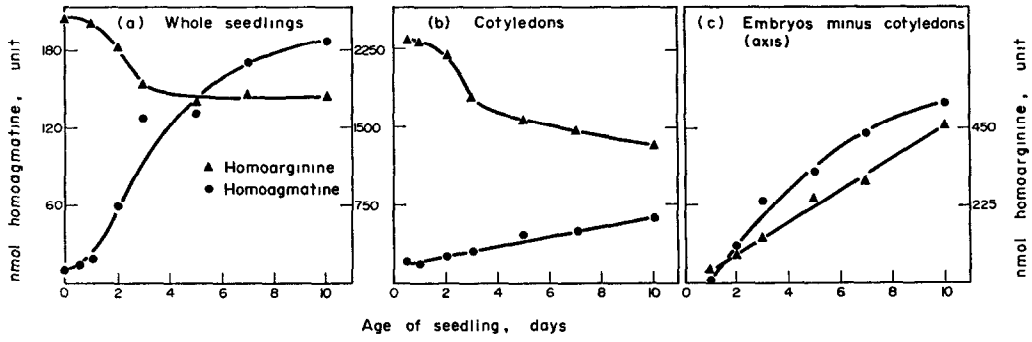

FIG. 1. QUANTITATIVE CHANGES IN HOMOARGININE AND HOMOAGMATINE CONCENTRATIONS OF *Lathyrus sativus* SEEDLINGS WITH GROWTH.
Unit represents a whole seedling in (a), a pair of cotyledons in (b), and an embryo axis in (c). Data represent mean value of 6 independent determinations and s.e. was less than 5% of the values indicated.

Having established unequivocally its chemical structure, it was of interest to quantitate the changes in the contents of this amine in the whole seedlings, the cotyledons and the embryo axis at different developmental stages, and to correlate these changes with alterations in the levels of homoarginine from which the amine presumably arises by decarboxylation. The data obtained are presented in Fig. 1. The disappearance of homoarginine in the seedlings was rapid in the initial stages and the amino acid level remained nearly constant at later stages of seedling growth. Concomitantly, homoagmatine levels increased steadily throughout, accounting for nearly 20% of total homoarginine disappeared during 10 days of growth. However, the relative concentrations of homoarginine and homoagmatine differ markedly in cotyledons and embryo axis (Fig. 1b and c). The major portion of homoarginine metabolized during the growth period could be accounted for in terms of its disappearance from cotyledons. The amine content of this tissue rose very slowly with the age of the seedlings, the increase corresponding to less than 18% of total homoagmatine produced and to less than 5% of total homoarginine metabolized. In contrast, the embryo axis accumulated the amino acid progressively, presumably due to translocation from the cotyledons. Some *de novo* synthesis of homoarginine in the growing embryo axis cannot, however, be ruled out. Homoagmatine concentrations increased sharply with growth reaching a level of *ca.* 140 nmol per axis at 10 days representing *ca.* 80% of the total amine elaborated. On the basis of the amino acid content of the embryo this accounts for nearly 30% conversion to amine. The embryo thus appears to be the major site of decarboxy lation of the amino acid while translocation from the cotyledons cannot be ruled out entirely at this stage.

It must be emphasized that these calculations do not take into consideration the turnover of homoagmatine and conversion into other metabolites, nor the *de novo* synthesis of the homoarginine and may, therefore, be an under estimate of the actual magnitude of homoarginine → homoagmatine conversion. In higher plants, elegant studies of Smith and his associates[8] have shown that agmatine, the decarboxylation product of arginine, is the major precursor of putrescine and hence of polyamines. In preliminary experiments, we have encountered in growing *L. sativus* seedlings parallel accumulation in substantial amounts of an aliphatic amine with properties similar to cadaverine. From the extent of homoagmatine accumulation in growing seedlings the catabolism of homoarginine through decarboxylation appears to represent a major pathway of the amino acid degradation. By analogy with the agmatine pathway of polyamine biogenesis, homoagmatine might similarly give rise to analogous vital metabolites. Enhanced elaboration of di- and poly-amines in rapidly growing systems like plant[9,10] and chick embryos[11] has been well documented.

EXPERIMENTAL

Materials. Lathyrus sativus seeds were obtained from the Plant Breeding section of Indian Agricultural Research Institute, New Delhi. L-Homoarginine was purchased from Nutritional Biochemicals, cadaverine dihydrochloride from Calbiochem and agmatine sulphate from Sigma Chemical Co. Dowex-50 resins were of analytical grade (Biorad). *O*-Methyl isouronium sulphate was kindly donated by Prof. L. K. Ramachandran of Osmania University, Hyderabad, A P.

Germination and growth conditions. The seeds of *L. sativus* were surface sterilized by immersion for 3 min in 0 5 M NaClO and washed thoroughly with dist. H_2O. After H_2O imbibition for 12 hr, the seeds were germinated and allowed to grow in the dark on moistened Whatman No. 1 filter paper discs placed on a wet sterile cotton layer in sterile Petri dishes in an incubator at 25–28°. The seedlings thus grown were found to be free from detectable microbial contamination.

Preparation of amine fraction from the seedlings. 5 g of whole seedlings were finely ground with acid-washed glass-powder in a chilled pestle and mortar and extracted with 3 vol. of cold 0·4 M $HClO_4$. After removing cell debris by centrifugation at 5000 *g* at 4°, the supernatant was adjusted to pH 8 with 40% KOH. Precipitated $KClO_4$ was removed by centrifugation and from the clear extract, adjusted to pH 13 with KOH and saturated with alkaline salt mixture (Na_2SO_4–Na_3PO_4 $12H_2O$, 7:1, w/w), the amines were extracted into 3 vol. of *n*-BuOH[12]. The BuOH layer, clarified by centrifugation, was evaporated to dryness in a flash evaporator at 60°. The residue was dissolved in a minimal vol. of H_2O.

Large scale isolation of homoagmatine 1850 g of 3-day-old seedlings (corresponding to 750 g dry seeds) were macerated in 3 vol. of cold 0·4 M $HClO_4$ for 5 min in a precooled Waring blender at maximum speed. After filtration through cheese cloth, the extract was clarified by centrifugation at 8000 *g* for 15 min in a Sorvall (RC 2-B) centrifuge at 4°. The supernatant was adjusted to pH 9 with 40% KOH and the precipitated $KClO_4$ removed by centrifugation. The extract was concentrated to 1 l. in a flash evaporator and sediment, if any, removed by centrifugation at 25 000 *g* for 30 min It was then loaded onto a column (1·4 × 35 cm) of Dowex 50 × 4 (200–400 mesh) H^+ form and was washed sequentially with 4 M NH_4OH (1 l.) H_2O (2 l.), 1 M piperidine (750 ml) and H_2O (2 l). The resin was freed of residual piperidine by further washing with 0·25 M HCl till effluent had a pH *ca.* 3. Final elution with 6 M HCl (1 l) secured complete recovery of guanidino amines from the column. This fraction was evaporated to dryness *in vacuo* at 60° and the residue dissolved in 30 ml H_2O. After adjusting the pH to 13 with KOH and saturating with KCl, the amines were extracted into 3 vol. of BuOH. The BuOH extract was taken to dryness at 60° under vacuum and the residue dissolved in 10 ml H_2O at pH 4. It was then applied to a second Dowex-50 × 4 H^+ column (1·8 × 62 cm) and the column washed with H_2O till pH of the effluent was neutral. The guanidino amines were fractionated on the column by two consecutive linear gradients Alternate fractions were analyzed with FDNB[13] reagent for amino groups and with Sakaguchi reagent as modified by Smith[14] for guanidino function. Of the four distinct well resolved peaks detected by FDNB reagent, the first two, eluted in 1·5 to 3·5 M HCl, were not

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Sakaguchi-positive. The last two, eluted in 3·5 to 4·5 M HCl, contained both the amino group and a guanidino moiety. When the fractions under these two peaks were concentrated and analysed by PC, the first guanidino compound corresponded to agmatine, while the second was the new guanidino amine detected in the amine fraction described earlier. The fractions corresponding to this region were concentrated to 8 ml, decolourized by boiling with activated charcoal and the amine precipitated as hydrochloride with Me_2CO. Excess Me_2CO was removed, the amine hydrochloride was purified by repeated precipitation from H_2O solution with Me_2CO and dried in a desiccator. The final yield was 141·5 mg.

Preparation of picrate derivative. 10 mg of the guanidino amine in 10 ml H_2O was heated with an excess of saturated picric acid in H_2O. Excess of picric acid was removed by repeated Et_2O extraction. When the aq. layer was concentrated to a small vol. and cooled, the material crystallized in yellow needles. Recrystallization in hot H_2O yielded 27 mg.

Preparation of flavianate derivative. 12 mg of the amine hydrochloride in 1 ml H_2O was treated with an equal vol. of 15% flavianic acid in H_2O. The mixture was heated at 100° for 30 min and allowed to attain room temp. slowly. Flavianate derivative which deposited as bright yellow plates was washed with 0·5% flavianic acid and then with EtOH. It was recrystallized by the procedure of Vickery[15] and dried at 105° for 2 hr. Yield: 65 mg.

Micro analysis of picrate and flavianate derivatives for N. *N*-Analysis was performed by Micro Analytical Laboratory of Department of Organic Chemistry of this Institute.

Quantitative determination of homoarginine in the acid-soluble fraction. The acid-soluble fraction was prepared as detailed earlier. Perchlorate-free extract adjusted to pH 5 was loaded onto a Dowex-50 × 8, H^+ column (1 × 5 cm), washed with 40 ml H_2O and the amino acids were eluted with 50 ml of 4 M NH_4OH. This fraction was evaporated to dryness *in vacuo* at 55°. The residue was dissolved in 6 ml 0·2 M sodium citrate buffer pH 2·4 and clarified by centrifugation at 5000 *g* for 10 min. An aliquot of this was used for homoarginine estimation with an automatic amino acid analyser (Evans Electroselenium Ltd., U.K. model 193). The analysis was carried out at 50° on a 0·9 × 15 cm column at a buffer flow rate of 90 ml/hr using 0·35 M sodium citrate buffer pH 5·28 ± 0·02. Homoarginine was eluted after and well-resolved from arginine between 85 and 115 ml of eluting buffer. The amino acid concentration in the samples was calculated according to the standard procedure (Spackman *et al.*).[16]

Quantitative estimation of homoagmatine. The preparation of the crude amine-fraction has already been described. The aqueous extract containing the amine was passed through a small column (1 × 5 cm) of Dowex-50 × 8 (20–50 mesh) and washed successively with 50 ml 4 M NH_4OH, 500 ml of H_2O and 60 ml 0 4 M HCl. The amine was eluted with 30 ml 6 M HCl. The HCl eluate was taken to dryness *in vacuo* at 60° and dissolved in a known vol. of H_2O. An aliquot of this was subjected to circular PC on a buffered Whatman No. 1 filter paper in PhOH–0·067 M HCl–KCl buffer pH 2 (4:1, v/v). After an Et_2O wash, to remove traces of PhOH, the dried chromatograms were sprayed with 0·3% ninhydrin in Me_2CO and heated at 70° for 90 min and the coloured bands corresponding to homoagmatine were cut and eluted with HOAc–EtOH–H_2O (4:3:1) containing cadmium acetate (2 mg/ml) and absorbance at 510 nm was determined with a Beckman DU-2 spectrophotometer. The amine contents were quantitated by using a standard curve obtained by subjecting standard amounts of homoagmatine to similar procedure.

Paper electrophoresis. Strips (3 × 50 cm) of Whatman No. 1 filter paper were used and the buffer employed was 0·065 M sulphosalicylic acid pH 3·5. Homoagmatine moved to a distance of 19·7 cm when a potential of 800 V was applied for 90 min.

IR spectrum. This was taken in KBr pellet using a Carl Zeiss UR-10 IR Spectrophotometer. Amine hydrochloride–KBr ratio was 1:300 (w/w).

Chemical synthesis. This was achieved by guanidation of one of the amino groups of cadaverine with *O*-methyl isourea.[17] 2 mmol cadaverine was treated with 2·1 mmol of *O*-methyl isouronium sulphate and 1·08 ml 2 M NaOH in a total vol. of 3 ml at 2–5°. The mixture was allowed to stand at 20–25° for 5 days. After adjusting the pH to around 7, it was applied to a Dowex-50 × 4 (200–400 mesh) H^+ column (0·8 × 30·0 cm), washed with 200 ml H_2O and eluted with a linear gradient of HCl (0·5–4 M) 600 ml. Unreacted cadaverine was eluted between 149 and 198 ml, homoagmatine (Sakaguchi and ninhydrin +ve) between 215 and 347 ml and diguanidocadaverine (Sakaguchi +ve but ninhydrin −ve) between 406 and 581 ml of the eluting gradient. Homoagmatine fractions were pooled and concentrated. The amine hydrochloride was purified and recrystallized as under large scale isolation of the amine from *L. sativus* seedlings. A yield of 150 mg of the purified amine was realized.

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