Phytochemistry 1971, Vol. 10. pp. 1523 to 1528. Pergamon Press. Printed in England.

IMINO ACID BIOSYNTHESIS IN *DELONIX REGIA*

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(Received 30 September 1970)

Abstract-The biosynthesis of L-azetidine-2-carboxylic acid and *trans*-3-hydroxy-L-proline has been studied in *Delonix regia* seedlings by labelled precursor feeding techniques. α,γ-Diaminobutyric acid was incorporated into azetidine-2-carboxylic acid more efficiently than homoserine, methionine or aspartic acid. More radioactivity from proline was found in *trans*-3-hydroxyproline after 2 day's than after 4 day's metabolism, indicating a continuous turnover of the hydroxyimino acid in seedlings.

INTRODUCTION

L-AZETIDINE-2-carboxylic acid (A2C) was isolated from *Convallaria majalis* (Lily-of-the-valley) and *Polygonatum officinale* independently by two groups[1–3] in 1955. The imino acid remained as a product characteristic of the Liliaceae until its recent isolation from the legume, *Delonix regia*.[4] A2C is absent from the dried seed of *Delonix,* but it is produced rapidly during the early stages of seedling growth. Seeds and seedlings of *Delonix* also contain high concentrations of another unusual imino acid, *trans*-3-hydroxy-L-proline (3HP), present in an unbound form.[4]

Several studies concerned with A2C biosynthesis have been reported. Early investigations in which putative precursors of A2C were supplied to excised root, rhizome or leaf material of C. *majalis* or *P. officinale*, failed to give metabolically significant labelling of A2C.[5–6] More recently, emphasis has been placed on methionine as a biogenetic precursor of A2C. Leete[7] has reported that 1·67% of the activity supplied as [1-^{14}C] methionine to the base of shoots of intact plants of C. *majalis* was incorporated into A2C during seven days, whilst Su and Levenberg[8] have worked with excised, young leaves of C. *majalis* and shown that various specifically-labelled methionines were incorporated into A2C in 0·25–0·88% yield during 3 days. Leete[7] reiterated an idea proposed originally by Schlenk and Dainko[9] that δ-adenosylmethionine may play an important role as an activated intermediate conducive to ring closure during A2C synthesis from methionine. However, Su and Levenberg[8] interpreted their findings more cautiously, realizing that the available evidence did not eliminate the possibility that methionine was converted to a sulphur-free metabolically-related derivative prior to the utilization of its carbon atoms for A2C synthesis. Data now presented show that the carbon atoms of two such related compounds, homoserine and a,y-diaminobutyric acid, are incorporated into A2C by *Delonix* seedlings more effectively than is carbon from methionine.

[1] L. FOWDEN, ***Nature* 176, 347 (1955).**
[2] A. I. VIRTANEN and P. LINKO, ***Acta Chem. Scand. 9,551 (1955).***
[3] L. FOWDEN, ***Biochem. J. 64,323 (1956).***
[4] M-L. SUNG and L. FOWDEN, ***Phytochem.* 7, 2061 *(1968).***
[5] L. FOWDEN and M. BRYANT, ***Biochem. J. 71, 210* (1959).**
[6] P. LINKO, ***Acta Chem. Scand.* 12,** 101 (1958).
[7] E. J. LEETE, ***J. Am. Chem. Soc.* 86,** 3162 *(1964).*
[8] E. F. W. SU and B. LEVENBERG, ***Acta Chem. Scand. 21,493 (1967).***
[9] F. SCHLENK and J. L. DAINKO, U.S. ***Atomic Energy Commission Reports of the Argonne National Lab.*** ANL-6200, 94 (1960).

The synthesis by plants of 3HP has not been studied previously, although detailed biosynthetic investigations have been reported for the isomeric *trans*- and *cis*-4-hydroxy-L-prolines. Tram-4-hydroxyproline probably occurs only in protein-bound form in plants; its formation apparently involves a direct oxygenative attack at the C-4 atom of proline residues already bound in peptide combination," *i.e.* a process similar to that yielding trans-4-hydroxyproline in collagen. Cis-4-hydroxyproline, which represents an important constituent of the non-protein nitrogen pool of *Santalum album*, also appears to be formed directly from proline.[11] Experiments now reported have established a similar facile conversion of proline into 3HP in growing *Delonix* seedlings.

RESULTS AND DISCUSSION

Studies with Azetidine-2-carboxylic Acid (A2C)

During the early growth of *Delonix* seedlings, A2C synthesis unmistakeably occurs, for the imino acid cannot be detected by routine chromatographic procedures in extracts of dry seeds, whereas it constitutes an important component of the soluble nitrogen fraction obtained from seedlings having radicles 5 cm long. Developing seedlings of *Delonix* therefore should form a more suitable system for the study of A2C biosynthesis than the liliaceous plant organs used in previous investigations, for there is only limited information regarding the main location and period of A2C synthesis in *Convallaria* and *Polygonatum* plants.

For the biosynthetic experiments, *Delonix*, seeds, whose testas had been chipped, were germinated at 30" in moistened vermiculite. Seedlings having radicles just emerging through the testa were selected, and supplied with solutions containing the various ^{14}C-labelled amino acids. When uptake was complete (20-30 hr), the seedlings were again transferred to vermiculite, so that a total period of 70-80 hr was allowed for metabolism of the labelled compounds. During this metabolic period at 30°, the radicles attained a length of 6-10 cm. Any unabsorbed ^{14}C-labelled substance was washed completely from the surface of the seedlings prior to extraction with 75 %, (v/v) ethanol : the extract was processed to yield an amino acid fraction, whose individual components were separated on two-dimensional paper chromatograms. Radioactive compounds were located by radioautography and their activities determined by surface scanning using a Geiger-Miiller system.

Five ^{14}C-labelled amino acids (DL-[1-^{14}C] aspartic acid, DL-[4-^{14}C] aspartic acid, DL-[4-^{14}C]-α,γ-diaminobutyric acid, DL-[4-^{14}C] homoserine, and L-[1-^{14}C]-methionine) were supplied to the *Delonix* seedlings: each seedling received 5 μc radioactivity. The distribution of radioactivity between the components of the soluble amino acid pool of the various seedling extracts is shown in Table 1: the data express the radioactivity present in each amino acid as percentages of that initially supplied to each group of seedlings.

The experiments indicate that in these legume seedlings, both homoserine and diaminobutyric acid were utilized better than methionine for A2C formation, although the former two compounds were DL-racemates in contrast with methionine which was the L-isomer. The two types of specifically-labelled aspartic acid were incorporated into A2C about as efficiently as methionine, when their racemic nature is taken into consideration.

All the ^{14}C-precursors effectively labelled y-methyleneglutamine which, like A2C, is absent from dry *Delonix* seed, but present in high concentration in young seedlings.[4]

[10] E. R. STOUT and G. J. FRITZ, *Plant Physiol. 41, 197 (1966).*
[11] A. N. RADHAKRISHNAN, personal communication.

TABLE 1. THE DISTRIBUTION OF ^{14}C FROM VARIOUS LABELLED PRECURSORS* AMONG THE COMPOUNDS OF THE FREE AMINO ACID POOL OF SEEDLINGS OF *Delonix regia*. DATA ARE EXPRESSED AS PERCENTAGES OF THE RADIOACTIVITY ENTERING EACH AMINO ACID DURING A METABOLIC PERIOD OF 70-80 hr AT 30°

Labelled amino acid formed	% ^{14}C Incorporation into soluble amino acids from:				
	[1-^{14}C]-DL-Aspartate	[4-^{14}C]-DL-Aspartate	[4-^{14}C]-DL-Homoserine	[4-^{14}C]-DL-α,γ-Diaminobutyrate	[1-^{14}C]-L-Methionine
Aspartic acid	0.28	0·52	0.10	0.052	0·004
Homoserine	—	—	0.34	1·66	—
α,γ-Diaminobutyric acid	—	—	0·034	3·04	—
Methionine			—	—	0·20
A2C	0042	0060	0·28	0.60	0.11
γ-Methyleneglutamine	0.30	0.38	0.46	0.12	0·26
γ-Methyleneglutamic acid	0008	0.01	0·036	0002	0.010
Asparagine	0·89	1.21	1·61	0·40	0·24
Glutamic acid	0·01	0.016	0.026	0.012	0.012
Thrwnine	0004	0004	0.80	—	0.016
N^{γ}-Acetyl-α,γ-diaminobutyric acid				3.54	

* Details of quantities and specific activities of ^{14}C-labelled compounds supplied are given in text.

Asparagine concentrations also increase strikingly during the early phase of germination, and substantial labelling of this amide occurred, especially after supplying ^{14}C-aspartates, or ^{14}C-homoserine, which can yield aspartate by C-terminal oxidation. As expected, threonine was also a notable labelled product following ^{14}C-homoserine metabolism.

In general, only a very small percentage of the radioactivity supplied remained in the original amino acid. These observations probably reflect the facile use of the ^{14}C-precursors for protein synthesis, a process that would form the dominant metabolic pathway for amino acid utilization in the developing radicle and smaller plumule. The fact that diaminobutyric acid is metabolically more remotely concerned with protein synthesis is reflected in the higher residual activity found in this free amino acid. Considerable conversion of diaminobutyric acid into its γ-acetyl derivative was obtained, and may be compared with the similar conversion demonstrated in another legume, *Onobrychis viciifolia*, by Brown and Fowden.[12] The fate of the D-isomers is not known. Perhaps they were converted into the L-forms by racemases. Alternatively, they may have conjugated with malonyl residues (compare Rosa and Neish)[13] and, as N-malonyl derivatives, they would have been discarded as anions during the separation of the amino acid fraction from seedlings using cation-exchange resins.

When the present results with *Delonix* seedlings are considered in relation to the earlier studies of A2C biosynthesis in liliaceous plants, it is apparent that utilization of methionine via an activated *S*-adenosyl derivative, as suggested by Leete,[7] cannot represent the main synthetic pathway in the legume. The more likely situation is that an intact C_4 chain from methionine, homoserine or diaminobutyric acid, or from compounds such as aspartic acid that can be converted readily into one of these compounds, can be incorporated into A2C, probably via some other type of activated intermediate as yet unidentified. The efficiency of incorporation of each precursor amino acid into A2C then would depend in part upon

[12] D. H. BROWN and L. FOWDEN, *Phytochem.* 5,887 (1966).
[13] N. ROSA and A. C. NEISH, *Can. J. Biochem. 46,797* (1968).

the relative rates at which compounds were converted into such a common intermediate. These relative rates may vary considerably between the different species producing A2C, and thereby explain the differing conclusions reached for the legume and for *Convallaria* regarding the principal precursor of A2C.

Studies with 3-Hydroxyproline (3HP)

3HP forms the major component of the soluble-nitrogen pool extracted from dry seed of *Delonix:* nevertheless some additional 3HP is synthesized during the early stages of seed growth for dry seeds contain about 720 μg, seeds with radicles just emerging, 950 μg, and seedlings with radicles about 4 cm long, 1160 μg 3HP/g dry wt.

Seeds with emergent radicles were supplied [U-^{14}C]-L-proline (2·5 μc per seedling): the seeds were grown further at 30" to give total metabolic periods of 64 and 116 hr (radicles about 3 and 7 cm length, respectively) before extraction and analysis of ^{14}C distribution among the free and protein-bound amino acids. Details of the ^{14}C activity present in the different products of proline metabolism are reported in Table 2 as percentages of the initial ^{14}C supplied to each seedling. Clearly, 3HP is the principal labelled product arising from ^{14}C-proline; activity found in the 3-hydroxy derivative exceeded that remaining in free proline at both sampling times. When seedlings were extracted 64 hr after commencing the supply of ^{14}C-proline, activity in 3HP was almost three times greater than that in the imino acid isolated from seedlings after 116 hr metabolism. It is then clear that considerable degradation of 3HP molecules also occurs in these early stages of seed growth.

Asparagine again represented another principal labelled product, its activity increasing with longer periods of metabolism. Other characterized amino acids of the soluble-

TABLE 2. THE DISTRIBTUION OF ^{14}C AMONG SOME FREE AND PROTEIN-BOUND AMINO ACIDS IN *Delonix regia* SEEDLINGS FED [U-^{14}C] PROLINE.* DATA ARE EXPRESSED AS % OF THE ^{14}C SUPPLIED ACCUMULATING IN EACH COMPOUND

Labelled amino acid formed	% ^{14}C Incorporated after metabolism of [U-^{14}C] proline for: 64 hr	116hr
Free		
Proline	1.58	0.76
3HP	5.46	1·92
Aspartic acid	0.03	0.03
Glutamic acid	0.04	0.04
y-Methyleneglutamic acid	0.04	0.03
y-Methyleneglutamine	0.14	0·15
Serine	0.02	0·02
Asparagine	0·56	1·84
α,γ-Diaminobutyric acid	0.22	0·04
A	0.94	0·74
B	0.28	0·26
C	0.46	1·66
Protein-bound		
Proline	8.8	—
Glutamic acid	0·64	—
4-Hydroxyproline	0·48	—

* See text for amount and specific activity of [U-^{14}C] proline supplied.

nitrogen pool contained only low levels of ^{14}C, but radioautographs showed the presence of three other labelled compounds (A, B and C) in the cationic fraction; their identities were not further investigated, but they did not correspond with ninhydrin-positive areas on chromatograms.

A soluble protein fraction (see Experimental) obtained from seedlings metabolizing ^{14}C-proline for 64 hr was hydrolysed and the constituent amino acids separated on a two-dimensional chromatogram. Radioautography, followed by ^{14}C-assay, showed ^{14}C was present only in proline, glutamic acid and 4-hydroxyproline. ^{14}C present in these three compounds was 8·8, 0·64 and 0·48%, respectively, of the activity supplied initially as [U-^{14}C]-proline. 3HP was not detected in the mixture of amino acids derived after protein hydrolysis.

The formation of 3HP in young *Delonix* seedlings would appear to result from a direct utilization of ^{14}C-proline, for its arises under circumstances where all other components of the soluble-nitrogen fraction possess little ^{14}C-activity. Extensive metabolism of proline resulting in an indirect labelling of 3HP would seem improbable, for under such circumstances the ^{14}C-label of proline would be unlikely to accumulate so specifically in 3HP. Experiments involving $^{18}O_2$ are now required to establish whether its formation from proline involves a direct oxygenative attack at the C-3 ring atom.

EXPERIMENTAL

Radioactive Compounds

L-[U-^{14}C] proline (15·0 μc/μmole) was supplied by the Radiochemical Centre, Amersham, L-[1-^{14}C]-methionine (10·9 μc/μmole) and DL-[4-^{14}C]-α,γ-diaminobutyric acid (10·0 μc/μmole) by Service Molecules Marquees, CEA, Gif-sur-Yvette, France, and DL-[1-^{14}C] aspartic acid (10.6 μc/μmole), DL-[4-^{14}C] aspartic acid (11·7 μc/μmole) and DL-[4-^{14}C] homoserine (11.4 μc/μmole) by Schwarz Bioresearch, Inc., New York.

Supply of "T-Compounds to Seedlings

After chipping the impervious testas, seeds of *Delonix* were planted in moist vermiculite at 30". Seedlings were selected for ^{14}C-labelling experiments as soon as the radicles began to extend i.e. when they were 5-10 mm in length. The tips of the radicles were immersed in solutions of the ^{14}C-amino acids: volumes were restricted such that uptake was complete in not more than 18 hr. Then the seedlings were replanted in vermiculite and grown further in the dark at 30" to complete the metabolic periods indicated in Tables 1 and 2. Finally, the seedlings were harvested, any adhering testas being discarded, and washed carefully to remove unabsorbed ^{14}C-compounds. They were macerated finely in 75 % (v/v) ethanol and extraction was continued with constant shaking for 24 hr. After centrifuging, the solid residue was re-extracted, and the two aqueous-ethanolic fractions combined.

Chromatography and Radioactive Assay

Cationic fractions were prepared from the aqueous-ethanolic extracts using small Zeokarb 225 columns following the procedure of Dunnill and Fowden.[14] Aliquots of these fractions, equivalent to 0·4 g fr. wt. seedlings, were applied to paper chromatograms (Whatman 3MM paper), which were developed in 75% (w/w) phenol, in the presence of NH_3 vapour, followed by *n*-BuOH–HOAc–H_2O (90:10:29, by vol.) as second solvent. Radioautographs of the two-dimensional chromatograms were prepared, and ^{14}C-activities of individual labelled compounds were determined using a Geiger end window counting tube attached to an Isotopes Development Ltd. 1700 scaler.

Separation of Free and Bound Amino Acid Fractions

Seedlings that had received ^{14}C-proline were ground finely in 0·1 M phosphate buffer, pH 8.1, containing 0·1% Teepol 530 as a wetting agent; the macerate was shaken for 24 hr and then centrifuged to recover a supernatant and residue fraction. The solid residue was re-extracted with additional buffer, and the supernatants combined. Soluble protein present in the extract was precipitated and coagulated by adjustment to pH 4·3 with acetic acid, followed by warming to 60° for 10 min. It was recovered by centrifuging, and then washed successively with 5 % (w/v) trichloroacetic acid, 80 % (v/v) ethanol, 50 % (v/v) acetone, acetone and

[14] P M. DUNNILL and L. FOWDEN, *Phytochem. 4,933 (1965).*

ether to yield a fine dry powder. The aqueous supernatant obtained at this centrifuging contained the free amino acid from the seedlings; they were separated from other soluble compounds using small Zeokarb 225 columns.[14] Protein was hydrolysed to the constituent amino acids using 6N-HCl at 100" for 18 hr.

Acknowledgement-Miss M.-L. Sung held a Commonwealth postgraduate studentship during the course of this work. *Delonix* seed was kindly sent to us by Professor B. L. Thrower (Hong Kong).