The Biosynthesis of *cis*-4-Hydroxy-L-proline in Sandal (*Santalum album* L.)

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Two isomers of hydroxyproline are present in sandal leaves (*Santalum album* L.), *cis*-4-hydroxy-L-proline in the free state (Radhakrishnan & Giri, 1954) and bound *trans*-4-hydroxy-L-proline, which forms a minor proportion (4%) of total hydroxyproline (Kuttan & Radhakrishnan, 1969). Bound *trans*-4-hydroxy-L-proline in plants (Lamport, 1965) is presumably synthesized by a mechanism similar to that in mammalian systems (Udenfriend, 1966; Kivirikko & Prockop, 1967) by hydroxylation of peptide proline. No information is currently available on the mode of biosynthesis of free *cis*-4-hydroxy-L-proline, although early experiments of Radhakrishnan, Gopalkrishnan & Giri (1961) with unlabelled compounds suggested that proline and ornithine were likely precursors of free *cis*-4-hydroxy-L-proline in sandal. In the present work a number of ^{14}C -labelled compounds have been checked for their ability to act as precursors of *cis*-4-hydroxy-L-proline in sandal.

Experimental. Young apical leaves from a vegetative sandal tree, usually consisting of the tip leaf buds and two adjoining pairs of leaves, were employed; the freshly cut shoots were immersed in solutions of the radioactive precursors (0.2 ml) contained in small glass tubes (0.5 cm $\times$ 2.5 cm). They were then transferred to an evacuating chamber and a vacuum was applied. After the solution had infiltrated (usually 1-2 h) the operation was repeated twice with the addition of 0.1 ml of water to ensure complete infiltration of the radioactive material. Over 95% of the radioactivity supplied was taken up by the leaves in a total period of 4 h. After infiltration the leaves were incubated at room temperature (25-27°C) in a humid chamber for various periods under laboratory conditions of diffused light. At the end of the incubation period the leaves were quantitatively extracted with ethanol (75%, v/v), and then chloroform (3 vol.) was added to the ethanolic extract. After centrifugation, the top aqueous layer containing all the free amino acids was subjected to paper chromatography (streaking) on Whatman no. 3 sheets with phenol-0.2 M-KCl-HCl, pH 1 (50:7, v/v). The position of *cis*-4-hydroxy-L-proline in guide strips was located (Jepson & Smith, 1953), the unsprayed strip corresponding to hydroxyproline was cut out and eluted with water, and the concentrated eluate

was chromatographed with butan-1-ol-acetic acid-water (4:1:1, by vol.). The eluate of the paper strip containing hydroxyproline was fractionated on a Dowex 50 (X 12) column (0.9 cm $\times$ 9 cm) by the procedure of Manning & Meister (1966), but with 0.5 M-HCl for elution for better separation of proline and hydroxyproline. The fractions containing hydroxyproline were pooled and the specific radioactivity was determined. From this value and the amount of hydroxyproline present in the original extract as assayed chemically (Neuman & Logan, 1950) the total radioactivity in each experiment was calculated.

The incorporation of [^{14}C]proline into bound *trans*-4-hydroxy-L-proline present in hydrolysates of the 75%-ethanol-insoluble leaf fraction was studied by the same procedures.

The optical purity of the isolated radioactive imino acids was determined by crystallization in the presence of excess of either of the unlabelled isomers and checking the extent of label in the crystals, and also by paper chromatography of the copper salts (Friedberg, 1954). Both these procedures showed that the hydroxyproline radioactivity was all in *cis*-4-hydroxy-L-proline in the free amino acid fraction and in *trans*-4-hydroxy-L-proline in the bound hydroxyproline fraction.

Results and discussion. The results obtained with a variety of possible precursors (Table 1) show that the degree of incorporation into free *cis*-4-hydroxy-L-proline was about 1.7% with L-proline and 0.6% with ornithine, assuming that only the L-isomer of ornithine was active. Glutamic acid, arginine, pyruvate, acetate and glucose were rather poor precursors.

In a separate series of experiments the time-course of incorporation of [^{14}C]proline and [^{14}C]ornithine into *cis*-4-hydroxy-L-proline was determined at intervals of 4, 8, 12 and 20 h. With L-proline maximum labelling occurred at 8 h after the start of infiltration and this remained constant up to 20 h. The incorporation into *cis*-4-hydroxy-L-proline in this experiment was about 6% of the initial radioactivity of L-proline at the time of maximum labelling and about 2.4% with ornithine (calculated for L-ornithine).

It was considered that proline may initially be converted into bound *trans*-4-hydroxy-L-proline

Table 1. *Incorporation of label from radioactive precursors into cis-4-hydroxy-L-proline*

The experimental details are given in the text. In each case the initial radioactivity used was 2×10^5 c.p.m. The DL-[2- 14 C]ornithine hydrochloride was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. All other labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The total incubation time was 30 h. The weight of fresh leaf tissue in the experiments ranged from 0.32 to 0.51 g. The radioactivity was measured with a Beckman model LS-100 liquid-scintillation counter.

Compound used for infiltration	Radioactivity in <i>cis</i> -4-hydroxy-L-proline		% incorporation of initial radioactivity
	(total c.p.m.)	(c.p.m./g of fresh leaf tissue)	
L-[U- 14 C]Proline	3487	10260	1.74
DL-[2- 14 C]Ornithine hydrochloride	603	1587	0.30
L-[U- 14 C]Glutamic acid	184	438	0.09
L-[U- 14 C]Arginine hydrochloride	195	375	0.09
[3- 14 C]Pyruvate	135	388	0.06
[2- 14 C]Acetate	65	203	0.03
[U- 14 C]Glucose	244	478	0.12

followed by proteolysis to yield the free isomer, which on epimerization would yield *cis*-4-hydroxy-L-proline. To test this, *trans*-4-hydroxy-DL-[2- 14 C]-proline was used in the infiltration experiments. After separation of the two epimers by paper chromatography of the copper salts only a negligible amount of radioactivity was found in *cis*-4-hydroxy-L-proline, suggesting there was no significant epimerization.

Our limited studies on the incorporation of proline into bound *trans*-4-hydroxy-L-proline of sandal leaves showed about 0.6% of the initial proline radioactivity in *trans*-4-hydroxy-L-proline, a result comparable with that obtained by Cleland & Olson (1967).

We have considered the following pathways for the biosynthesis of free *cis*-4-hydroxy-L-proline in sandal on the basis of available information cited in each case, assuming that the enzymes possessed the required stereospecificity.

(1) Pyruvate + glyoxylate $\rightarrow$ γ -hydroxyglutamate $\rightarrow$ 3-hydroxy- Δ^1 -pyrroline-5-carboxylate $\rightarrow$ *cis*-4-hydroxy-L-proline. This route is a reversal of the degradative pathway of *trans*-4-hydroxy-L-proline in mammals (Maitra & Dekker, 1963; Goldstone & Adams, 1962; Adams & Goldstone, 1960) and of its possible synthesis (Efron, Bixby, Hockaday, Smith & Meshorer, 1968). The poor incorporation of pyruvate obtained in the present work does not appear to support this pathway for *cis*-4-hydroxy-L-proline.

(2) Ornithine $\rightarrow$ γ -hydroxyornithine $\rightarrow$ 4-hydroxy- Δ^1 -pyrroline-2-carboxylate $\rightarrow$ *cis*-4-hydroxy-L-proline. This pathway is suggested on the basis of the work of Radhakrishnan & Meister (1957), but the present results show that ornithine

was not superior to proline, and maximum labelling of *cis*-4-hydroxy-L-proline occurred later than that of proline. The observed incorporation, then, may be due to conversion of ornithine into proline.

(3) Proline $\rightarrow$ *cis*-4-hydroxy-L-proline. Proline was the most effective of the various precursors tried and clearly this third pathway is strongly supported. Whether there are intermediates in this conversion or whether it is a direct hydroxylation is not clear from the present experiments. The possible intermediates are Δ^1 -pyrroline-5-carboxylate, 3-hydroxy- Δ^1 -pyrroline-5-carboxylate, and 4-oxo-proline (Smith & Mitoma, 1962). If it is a direct hydroxylation of free L-proline to *cis*-4-hydroxy-L-proline, the enzyme system must exhibit a stereospecificity different from that of the mammalian systems.

In the present work γ -hydroxyglutamate (Fowden, 1965) and γ -hydroxyglutamine (Goldstone & Adams, 1964) are not clearly excluded as intermediates in the plant biosynthesis of *cis*-4-hydroxy-L-proline, and this needs evaluation.

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