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DOPA RING-CLEAVAGE IN THE BIOGENESIS OF STIZOLOBIC ACID IN *MUCUNA DEERINGIANA**

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Key Word Index—*Mucuna deeringiana*; Leguminosae; velvet bean; non-protein amino acids; DOPA; stizolobic acid; ring-cleavage.

Abstract—Tyrosine and DOPA are specifically incorporated into stizolobic acid in young leaves of *M. deeringiana*. The biosynthesis of this heterocyclic amino acid must, therefore, involve 4,5-extra-diol ring-cleavage of the aromatic ring of DOPA and subsequent cyclization.

INTRODUCTION

The non-protein amino acid stizolobic acid (**1**) and its isomer, stizolobinic acid (**2**), were originally isolated in minute quantities from seedlings of *Stizolobium hassjoo* [1,2]. On the basis of their structures it was suggested [3] that these α-pyrone derivatives could arise by extra-diol ring-cleavage of DOPA, which occurs in large quantities in the same plant. Such a ring-cleavage pattern is also a key step in betalain biosynthesis from DOPA in the Centrospermae [4,5], suggesting that the same 4,5-extra-diol dioxygenase may be present in these unrelated species. As a prelude to the study of such enzymes, it was considered necessary to examine experimentally the hypothesis that stizolobic acid is biosynthesized specifically from tyrosine and DOPA. Since the genus *Mucuna* is evidently synonymous with *Stizolobium* [6], and also synthesizes large quantities of DOPA [7], *Mucuna deeringiana* plants were chosen as experimental material.

RESULTS AND DISCUSSION

Chromatographic and electrophoretic examination of extracts from *M. deeringiana* plants revealed the presence of an amino acid indistinguishable from authentic stizolobic acid. Typical concentrations in young leaves were 0·3–0·8 μmol/g fr. wt; in mature leaves up to 0·2 μmol and in cotyledons only traces. Comparable concentrations of DOPA were 6–18, 23–56 and 43–85 μmol/g fr. wt respectively. The isomeric stizolobinic acid could also be detected but was present in much lower concentrations. The younger leaves consistently had the highest concentration of stizolobic acid and all radioactive feeding experiments were thus carried out on this material.

Administration of radioactive substrates by petiole uptake or vacuum infiltration proved ineffective as the damaged tissue rapidly blackened from DOPA oxidation. Cuticular administation was found to produce the least damage and best uptake of substrate. The results of feeding radioactive tyrosine and DOPA are shown in Table 1. The specific activities of DOPA, stizolobic acid, and endogenous tyrosine show the relationship that would be expected for a sequence involving ring-3-hydroxylation of tyrosine to form DOPA and subsequent conversion to stizolobic acid. The dilution values are low (3–24) which suggests a close biogenetic relationship but since nothing is known of the size or multiplicity of the intracellular pools this evidence is not conclusive.

Table 1. Incorporation of ^{14}C-tyrosine and ^{14}C-DOPA into DOPA and stizolobic acids in young *M. deeringiana* leaves after 24 hr

Compound fed	Specific activity (dpm/μmol) Tyrosine	DOPA	Stizolobic acid
L-tyrosine[U-^{14}C] 44×10^6 dpm	$4{\cdot}8 \times 10^5$	$1{\cdot}2 \times 10^5$	$2{\cdot}0 \times 10^4$
L-tyrosine[U-^{14}C] 55×10^6 dpm	$1{\cdot}0 \times 10^6$	$1{\cdot}5 \times 10^5$	$6{\cdot}1 \times 10^4$
L-tyrosine[1-^{14}C] 44×10^6 dpm	$8{\cdot}7 \times 10^5$	$1{\cdot}1 \times 10^5$	$6{\cdot}4 \times 10^4$
L-DOPA[U-^{14}C] 17×10^6 dpm	—	$4{\cdot}3 \times 10^5$	$1{\cdot}3 \times 10^5$
L-tyrosine[U-^{14}C 2,3-*side-chain*-^{3}H] T/C = 8·42	T/C = 8·55	—	T/C = 3·97

Doubly-labelled tyrosine [2,3-*sidechain*-^{3}H; U-^{14}C] was tested as a substrate in an attempt to demonstrate specific incorporation of the amino acid into stizolobic acid. The latter, and the reisolated tyrosine, were chromatographically purified until the T/C ratio was constant, which yielded the results shown in Table 1. During the conversion of tyrosine to stizolobic acid, or the purification process, approximately 50% of the tritium label

Table 2. Ninhydrin decarboxylation of amino acids from *M. deeringiana* after labelling from L-tyrosine[1-^{14}C] or L-tyrosine[U-^{14}C]

Isolated amino acid	Precursor	dpm^{14}C in amino acid	dpm^{14}C recovered in CO_2	% of theoretical
L-tyrosine	L-tyrosine[1-^{14}C]	36100	33900	95·3
L-tyrosine	L-tyrosine[1-^{14}C]	17200	16500	95·0
L-stizolobic acid	L-tyrosine[1-^{14}C]	3000	1670	56·2
L-stizolobic acid	L-tyrosine[U-^{14}C]	19300	1200	57·7

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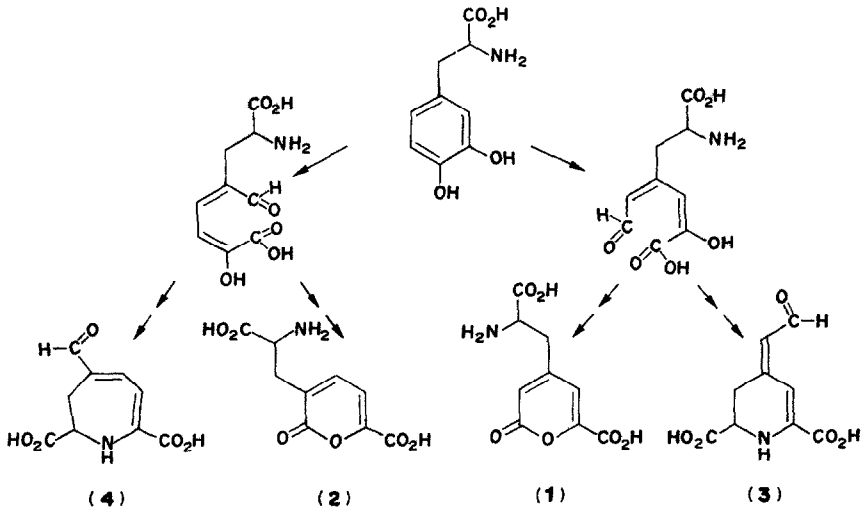

had been lost. Tyrosine-[2,3-*sidechain*-^{3}H] carries half its tritium in each position [8] and the feeding results suggest that one of these positions becomes relatively labile when the side-chain forms part of the stizolobic acid molecule. The latter has been previously suggested to possess an "activated" methylene group in the side-chain [9] which accounts for the atypical colour produced in its reaction with ninhydrin. The tritium attached to such a group is likely to exchange readily with protons in the medium, resulting in complete loss of label from the 3-position of the side-chain.

To obtain more conclusive evidence of specific incorporation, the labelled amino acids obtained after administering L-tyrosine-[1-^{14}C] to young leaves were submitted to ninhydrin decarboxylation (Table 2). This procedure showed that at least 95% of the label in reisolated tyrosine was located in the carboxyl carbon, demonstrating that no substantial degradation and resynthesis had taken place. Decarboxylation of the stizolobic acid sample, however, yielded only 56% of the predicted radioactivity. This result was essentially unaltered by longer heating or use of more carrier. When a sample of uniformly-labelled stizolobic acid was decarboxylated under identical circumstances, however, the same reduced yield of $^{14}CO_2$ was obtained (Table 2). This indicated that the ninhydrin decarboxylation was consistently only 55–60% complete and that the figure of 56% obtained for the carboxyl-labelled samples represented essentially non-randomized labelling of stizolobic acid.

Taken together these results clearly confirm the original hypothesis of stizolobic acid biosynthesis from tyrosine by 4,5-extradiol ring-cleavage of DOPA [3]. It would be very surprising if stizolobinic acid were not likewise a product of 2,3-extra-diol ring-cleavage although no data were obtained for this isomer in the present study.

DOPA metabolism in various genera of higher plants can thus include oxidative fission of the aromatic ring at either extra-diol site (Fig. 1). Catabolism of the ring had earlier been detected in ten species of plants examined in suspension culture [10] but the mode of ring-fission involved is still unknown. Reports have recently appeared of the occurrence in various species of Basidiomycetes of stizolobic acid [11], stizolobinic acid [11], betalamic acid (3) [12] and muscaflavin (4) [13], all probable recyclized products of extradiol ring-fission of DOPA. As yet, no muscaflavin-type structure has been reported from higher plants, nor is there any direct evidence for intra-diol ring-cleavage of DOPA by plants. Since all plants examined do, however, appear to be capable of catabolizing DOPA to some degree [11], more detailed investigations of the enzymology of such reactions should reveal whether this specificity is apparent or real.

EXPERIMENTAL

Plant material. Mucuna deeringiana (cv. Early Jumbo) seed was obtained from the Georgia Seed Development Commission, Athens, Georgia. Seed was surface-sterilized and planted in autoclaved potting soil. Plants were grown at room temp. on a 16 hr light/8 hr dark regime.

Chemicals. L-tyrosine-[U-^{14}C] and L-tyrosine-[2,3-*side-chain*-^{3}H] were obtained from the Radiochemical Centre and L-tyrosine-[1-^{14}C] from New England Nuclear. L-DOPA-[U-^{14}C] was synthesized enzymatically as described previously [14]. L-tyrosine-[2,3-^{3}H; U-^{14}C] was prepared by mixing the appropriate singly-labelled compounds and chromatographically purifying the mixture. All radioactive compounds were counted by liquid scintillation counting.

Administration of radioactive substrates. Washed young leaves (20–30 mm long, *ca* 1 g) were laid out on moist filter paper in glass Petri dishes. The substrate solution containing a small amount of Tween 80 was spread over the leaf surface with a glass rod and the covered dishes left in the light for 24 hr. Leaves were then rinsed free of unabsorbed radioactivity with water and extracted.

Amino acid isolation. Amino acids were isolated from plant material by refluxing exhaustively in 70% EtOH. Extract was conc and extracted with petrol to remove lipid pigments. Aqueous residue was loaded onto a column of Dowex 50W × 8 (200–400 mesh), H^+ form, and eluted with a 0·01–2 M HCl gradient. Amino acids of interest were eluted in the order stizolobic acid, DOPA and tyrosine. Appropriate fractions were pooled and purified by combinations of TLC (Avicel) in (a) 80% phenol, (b) *n*BuOH–HOAc–H_2O (20:5:11) (c) 2% HCO_2H (d) *i*PrOH–NH_4OH–H_2O (6:3:1) and TLE (Avicel) (40V/cm; 40 min.) in (e) pH 1·9 HOAc–HCO_2H and

(f) pH 3·5 ammonium acetate. For stizolobic acid the sequence (a), (e), (b), (f), (c) was used; for DOPA, (b), (e), (c), (f) and for tyrosine, (b), (d), (e), (f), (c). The concentration of each amino acid was determined spectrophotometrically after elution from the Avicel with H_2O.

Amino acid decarboxylation. Purified amino acid samples were decarboxylated using a modified ninhydrin procedure [15]. The CO_2 was trapped in phenylethylamine–MeOH (1:1) which was counted directly by liquid scintillation counting. Since very little stizolobic acid was available, 25 mg L-alanine was added to the radioactive stizolobic acid samples as non-radioactive carrier before decarboxylating them.

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