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BIOSYNTHESIS OF 3,4-DIHYDROXYPHENYLALANINE IN *VICIA FABA*

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Key Work Index—*Vicia faba*; Leguminosae; broad bean; biosynthesis; aromatic amino acids; 3,4-dihydroxyphenylalanine; DOPA; phenylalanine; tyrosine.

Abstract—Radioactive shikimic acid and L-tyrosine were shown to be efficient precursors of 3,4-dihydroxyphenylalanine (DOPA) in *Vicia faba*. [1-^{14}C]Acetate and L[U-^{14}C]phenylalanine were not incorporated into tyrosine or DOPA. Thus the synthesis of DOPA occurs via the shikimic acid pathway and tyrosine or a very closely related metabolite. Phenolase was present in etiolated plants in much larger quantities after a brief light exposure whereas DOPA concentration was relatively constant during all stages of plant growth. Partially purified phenolase did not catalyze the conversion of tyrosine to DOPA and does not appear to have a role in DOPA synthesis.

INTRODUCTION

L-3,4-DIHYDROXYPHENYLALANINE (DOPA), a non-protein amino acid, has been detected in only a limited number of plants.[1,2] It is present in relatively high concentrations in the seeds of a few legumes,[2] the latex of *Euphorbia lathyrus*[3] and the leaves of *Vicia faba*.[4]

The synthesis of DOPA in plants has not been studied in detail but is generally believed to result from the hydroxylation of tyrosine catalyzed by a phenolase (polyphenol oxidase) possessing monophenolase (tyrosinase) activity.[5,6] Kenten[7] and Robb *et al.*[8] have reported that the phenolase of *Vicia faba* has monophenolase activity; that is, it will catalyze the *o*-hydroxylation of monophenols. Furthermore, Liss[3] has reported that ^{14}C from L[1-^{14}C]-tyrosine was incorporated into DOPA in *Euphorbia lathyrus*. However, it is anomalous that DOPA has been detected in only a limited number of plants and in vastly varying concentrations whereas phenolases are widely distributed.[9]

The available evidence points to the conversion of tyrosine to DOPA by a phenolase. However, alternate routes for DOPA synthesis have not been ruled out. In addition the existence of a specific tyrosine hydroxylase similar to that which occurs in animal tissues[10] should be considered.

The present work was undertaken to learn more about the biosynthesis of DOPA in *Vicia faba*.

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RESULTS

Plant material was analyzed for DOPA at various stages of growth in order to ascertain the most suitable time to study its synthesis. Table 1 shows that DOPA is rapidly synthesized upon germination, both in green plants and in etiolated seedlings, and that its synthesis continues during plant growth. The concentration in either etiolated or green tissue remains relatively constant during growth when calculated on a fresh weight basis. The dry seed contains a small amount of DOPA which disappears upon germination as shown by analysis of the residual cotyledons on the plants.

TABLE 1. L-3,4-DIHYDROXYPHENYLALANINE CONTENT OF *Vicia faba*

	Age of plants*	Wt of plant	DOPA in plant	Concn of DOPA
A	Plants grown in greenhouse			
	0 (seed)	(1·6) g	(1·1) μmol	(0·7) μmol/g
	2	5·0	87·5	17·5
	4	9·5	179	18·8
	7	10·0	265	26·5
	14	18·5	347	18·8
B	Etiolated plants			
	1	0·18	3·1	17·4
	4	0·90	15·2	17·1
	7	2·27	28·3	12·5

* From time of germination.

The results of feeding several ^{14}C-labeled precursors to intact green plants and excised hypocotyls of etiolated seedlings are shown in Tables 2 and 3. [G-^{14}C]Shikimic acid was an efficient precursor of both phenylalanine and tyrosine as expected since these precursor-product relationships have been reasonably well established in higher plants.[11,12] Shikimic acid also was incorporated into DOPA whereas sodium [1-^{14}C]acetate was not incorporated into any of these compounds. These results demonstrate that *de novo* synthesis occurs from compounds related to the shikimic acid pathway rather than by way of the

TABLE 2. INCORPORATION OF ^{14}C LABELED COMPOUNDS INTO PHENYLALANINE, TYROSINE AND DIHYDROXYPHENYLALANINE BY INTACT, GREEN PLANTS

Compound fed*	DOPA Incorporation	DOPA Sp. act. (× 10²)	Phenylalanine Incorporation	Phenylalanine Sp. act. (× 10²)	Tyrosine Incorporation	Tyrosine Sp. act. (× 10²)
Sodium [1-^{14}C]acetate	n.d.†	—	n.d.	—	n.d.	—
[G-^{14}C]Shikimic acid	1·3%	0·05 (μCi/μmol)	3·1%	6·1 (μCi/μmol)	6·7%	7·8 (μCi/μmol)
L[U-^{14}C]Phenylalanine	n.d.	—	7·0	20·4	n.d.	—
L[U-^{14}C]Tyrosine	10·1	0·16	n.d.	—	4·8	23·7

* 10 μCi of each compound fed to 1 plant (wt 10 ± 0·50 g).
† Not detected in quantities significantly above background.

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polyacetate[13] or other pathway utilizing acetate directly for synthesis of aromatic compounds.

The conversion of tyrosine to DOPA in green seedlings and etiolated hypocotyls was about 10 and 26% respectively for the conditions used. The absence of ^{14}C in phenylalanine was expected. It is interesting to note that ^{14}C was not detected in tyrosine or (DOPA) when L[U-^{14}C]phenylalanine was administered. This result confirms studies by McCalla and Neish[14] and demonstrates that hydroxylation of phenylalanine to form tyrosine does not occur or at best is of minor significance in the broad bean. Furthermore, the lack of conversion of phenylalanine to DOPA shows that tyrosine or a closely related metabolite is obligatory for its synthesis.

TABLE 3. INCORPORATION OF ^{14}C LABELED COMPOUNDS IN TO PHENYLALANINE, TYROSINE AND DIHYDROXYPHENYLALANINE BY EXCISED HYPOCOTYLS OF ETIOLATED SEEDLINGS

Compound fed*	DOPA		Phenylalanine		Tyrosine	
	Incorporation (%)	Sp. act. ($\times 10^2$)	Incorporation (%)	Sp. act. ($\times 10^2$)	Incorporation (%)	Sp. act. ($\times 10^2$)
Sodium [1-^{14}C]acetate	n.d.†	—	n.d.	—	n.d.	—
[G-^{14}C]Shikimic acid	4·9	1·1 (μCi/μmol)	1·0	1·20 (μCi/μmol)	4·4	4·6 (μCi/μmol)
L[U-^{14}C]Phenylalanine	n.d.	—	50·0	77·5	n.d.	—
L[U-^{14}C]Tyrosine	26·5	6·0	n.d.	—	43·0	39·9

* 5 μCi fed to 3 hypocotyls (wt = 1·6 ± 0·1 g).
† Not detected in quantities significantly above background.

The phenolase activity in a homogenate of etiolated plants before and after exposure to light is shown in Table 4. Phenolase activity was present at only very low levels in homogenates made from plants maintained in the dark. If the plants were brought into the light 12 hr before harvest (Expts. 1 and 2) or if given light for 1·5 or 6 hr (Expt. 3) and then returned to darkness, there occurred increases of 20–30-fold in the activity of the enzyme in the plant homogenates. Although the phenolase was barely detectable in the dark grown plants described in Table 4, it should be stressed that DOPA had been synthesized and was present at the approximate concentrations described in Table 1.

Table 5 lists some of the properties of the phenolase partially purified from plant tissue that has been exposed to light. These properties show that this enzyme is similar to the ones isolated from photosynthesizing plants,[7,8] with the important exception that the hydroxylation of tyrosine could not be demonstrated with the former enzyme.

DISCUSSION

The results obtained when radioactive shikimic acid and tyrosine were fed to green or etiolated *Vicia faba* tissues provide direct evidence that these compounds are involved in the synthesis of DOPA. Since [1-^{14}C]acetate was not a precursor of DOPA, tyrosine, or phenylalanine, the shikimic acid pathway would appear to be the major source of carbon

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TABLE 4. EFFECT OF LIGHT ON THE AMOUNT OF PHENOLASE IN EXTRACTS OF ETIOLATED PLANTS

Experiment	Growth conditions	Phenolase activity × 10^2 (units*/g tissue)
1	No light	0·05
1	12 hr light prior to harvest	1·22
2	No light	0·08
2	12 hr light prior to harvest	0·43
3	No light	0·03
3	1·5 hr light 20 hr prior to harvest	0·92
3	6 hr of light 20 hr prior to harvest	1·00

* 1 unit of enzyme is that amount catalyzing formation of 1 μmol dopachrome per min.

TABLE 5. PROPERTIES OF THE PARTIALLY PURIFIED PHENOLASE OBTAINED AFTER EXPOSURE TO LIGHT ETIOLATED *Vicia faba* SEEDLINGS

MW (estimated with Sephadex G200)	45 000	Inhibitors of DOPA oxidation (>95%)	10^{-4} M KCN, 10^{-4} M sodium diethyldithiocarbamate 5 × 10^{-4} M phenylhydrazine
pH optimum	5·2		
Substrates oxidized	DOPA, catechol, chlorogenic acid	Activator	Sodium dodecylsulfate (required for detectable activity)
Substrates not oxidized	L-Tyrosine, *p*-cresol, hydroquinone, guaiacol		

for these amino acids. Since DOPA was not synthesized from phenylalanine, it may be inferred that aromatic compounds derived from phenylalanine (e.g. cinnamic acid, *p*-coumaric acid and caffeic acid) also are not directly involved in DOPA synthesis.

The extensive incorporation (26·5%) of tyrosine into DOPA by etiolated hypocotyls of *Vicia faba* would indicate its direct conversion in such tissues, possibly catalyzed by a tyrosine hydroxylase. The *o*-hydroxylation of tyrosine by a specific tyrosine hydroxylase[15] from beef adrenal medulla has been extensively studied. However, our efforts to demonstrate such an enzyme either in etiolated or green seedlings of *Vicia faba* have not been successful.[16] Although the ability of phenolases from a variety of sources[9] including *Vicia faba*[7,8] to catalyze *o*-hydroxylation is established, the etiolated hypocotyls contain little of this activity. Moreover, when the amount of that activity is increased by exposure of the tissue to light, the enzyme is still unable to hydroxylate tyrosine. Therefore, the phenolase activity that is present in the etiolated hypocotyls of *Vicia faba* does not appear to be of physiological significance in the biosynthesis of DOPA in that tissue.

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The lack of conversion of phenylalanine to tyrosine in the present study in still another species (*Vicia faba*) again raises the question of the major route for the synthesis of tyrosine in higher plants. Both Towers and Subba Rao[17] and Leete *et al.*[18] have pointed out that the conversion of phenylalanine to tyrosine is a severely limited process in most plants that have been investigated. To the species which these authors list may be added others.[19,20] On the other hand, Neish *et al.*[14,21,22] observed the ready conversion of shikimic acid to both tyrosine and phenylalanine in a number of plants, thereby providing some of the earliest evidence that these amino acids are formed in higher plants, as in microorganisms, by independent parallel pathways from prephenic acid (for review see Ref. 12). Although Nair and Vining[23] have described a phenylalanine hydroxylase in spinach extracts, Fritz and Aman[24] were unable to obtain evidence for the hydroxylation of phenylalanine *in vivo* in experiments specifically designed for that purpose. Attempts in this laboratory[25] to confirm the occurrence of a phenylalanine hydroxylase in enzyme preparations from spinach as well as other plants have not been successful. Similar attempts by Kindl[26] to demonstrate the enzyme in *Astilbe chinensis* were negative although in this instance the apparent absence of the hydroxylase was used to explain the low levels of *p*-hydroxy phenolic acids that were observed.

EXPERIMENTAL

Plant material. Vicia faba seedlings (long pod fava beans, Atlee Burpee Co., Riverside, CA.) were grown in a greenhouse in flats containing Vermiculite. Etiolated plants were obtained by germinating semisterile seeds in the dark on sterile, moist germination paper or in trays of sterile Vermiculite.

Radioactive compounds. Sodium [1-^{14}C]acetate (2 mCi/mmol), [G-^{14}C]shikimic acid (1·86 mCi/mmol), and L[U-^{14}C]phenylalanine (369 mCi/mmol) were purchased from New England Nuclear Corporation. L[U-^{14}C]tyrosine (475 mCi/mmol) was obtained from Nuclear Chicago Corporation. All radioactive compounds were chromatographically pure and were administered to plants without further dilution with non-radioactive carrier.

Administration of isotopic compounds. 9-day-old seedlings were removed from the greenhouse and placed in flasks after removal of their cotyledons. 10 μCi of each isotopic compound was dissolved in 1 ml H_2O and placed in the flask. The solutions were taken up in *ca* 1 hr. To insure complete uptake, 1 ml H_2O was added and allowed to be absorbed by the plant. This process was repeated and then sufficient nutrient solution[27] added to cover the roots. The plants were placed under 'Gro-lux' fluorescent lamps in a laboratory hood and harvested after 24 hr. Assays of residual nutrient solutions showed that less than 1% of the administered ^{14}C remained in each flask. 3-Day-old plants were removed from trays and the hypocotyls excised with a razor blade under H_2O. The stem of the hypocotyl was placed in a small test tube and 5 μCi of isotopic compounds administered in 0·2 ml H_2O in each case. Sufficient nutrient solution was added to keep the base of the stems covered. The plant tissue was harvested 8 hr after feeding. In each case no more than 2% of the ^{14}C remained in the nutrient solution.

Extraction of plant material for amino acid analysis. Plants were extracted in a semi-micro Waring blender with a solvent consisting of 1 part 95% EtOH and 1 part 0·02 N HCl containing 1% ascorbic acid.[28] The homogenate was warmed on the steam bath for 30 min, filtered through Mira cloth, and centrifuged at 20 000 *g* for 10 min. The supernatant solution was stored at 4° until analysis.

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Isolation of tyrosine, phenylalanine, and dihydroxyphenylalanine and the assay for radioactivity. A model K-8000 VG amino acid analyzer (Phoenix Precision Instrument Co., Philadelphia) was used to isolate and determine concentrations of the amino acids. The column was 60 × 0·9 cm and contained a cation exchange resin of styrene polymer with 8% divinylbenzene cross linking. Column temp. was 60°. Elution was performed with sodium citrate, 0·35 M with respect to Na^+ and at pH 5·015. Assays for ^{14}C were achieved with a flow cell coupled to the amino acid analyzer (2 ml anthracene packed flow cell and a Unilux Mark II Nuclear Chicago scintillation counter). The scintillation counter was calibrated with standard amino acids and all counts were corrected to dpm. The DOPA content of etiolated plants was determined by chromatographing the extract on thin layer plates of silica gel in BuOH–HOAc–H_2O (4:1:2). The area of the chromatogram containing DOPA was removed and extracted with 0·02 N HCl. DOPA was estimated by the colorimetric procedure of Shiman *et al.*[18] and the results obtained were in agreement with analysis made on the amino acid analyzer.

Extraction and partial purification of phenolase. Etiolated plants were ground with a mortar and pestle at 4° in a grinding medium of 0·05M Tris-HCl buffer, pH 7·8. The grinding medium contained 10% by wt of Dowex-1 ion exchange resin[29] and was 5 mM with respect to dithiothreitol. The homogenate was squeezed through 2 layers of Mira cloth and centrifuged at 500 *g* for 10 min. The supernatant was immediately transferred to a Sephadex G25 column and eluted with 0·05 M sodium phosphate buffer pH 7·0 in order to remove low MW substances, especially DOPA, from the protein fraction. Routine assays for phenolase in plant tissue were made using this crude homogenate. Additional purification procedures on the crude homogenate were undertaken to characterize the enzyme. The precipitate from a 45–75% ammonium sulfate saturation was further purified on Sephadex G200 and DEAE-cellulose columns.[30] There was only one phenolase peak in each case and the resulting purification was about 20 fold with a yield of *ca.* 30% of the original activity.

Assay of enzyme activity. Phenolase was assayed routinely by measuring dopachrome formation from DOPA[31] at pH 5·2. Other diphenol substrates were assayed using the ascorbic acid method.[32] Monophenolase activity was assayed with *p*-cresol[33] and L-tyrosine.[31] Also tyrosine was incubated in the presence of ascorbic acid or mercaptoethanol and DOPA synthesis assayed by the colorimetric procedure of Shiman *et al.*[15] Sodium dodecyl sulfate was added to all assay mixtures in order that any latent phenolase activity[7] would be detected.

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