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DOPA DECARBOXYLASE IN *CYTISUS SCOPARIUS*

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Abstract—*Cytisus scoparius* (Scots broom) contains a DOPA decarboxylase which shows little stereospecificity between D- and L-DOPA, and is virtually inactive against tyrosine and phenylalanine. This enzyme requires oxygen for activity and is inhibited strongly by –SH containing reagents and diethyldithiocarbamate. All of the aerial parts of the plant tested showed some decarboxylase activity, with the largest amount of enzyme located in young branches.

INTRODUCTION

THE COMPOUND 3-hydroxytyramine* (3-HT, β-3,4-dihydroxyphenyl dopamine ethylamine) occurs in small amounts in a number of angiosperms[1] and in considerably higher concentrations in the green alga, *Monostroma fuscum*[2] (Postels and Ruprecht) Wittrock. Schmalfuss and Heider[3] were the first to identify 3-HT in plants, in the broom, *Cytisus* (= *Sarothamnus*) *scoparius* (L) Link. Jaminet[4] observed seasonal variation in the quantities of 3-HT and related compounds in this plant and found that their concentrations varied in different tissues. He reported that 3-HT reached its highest concentration in green fruits in the spring and in branches collected late in the autumn. Tyramine and 3-hydroxy-*N*-methyl tyramine (epinine) reached their maximum levels in the spring and were found mainly in the flowers.

The occurrence of DOPA DC (3,4-dihydroxyphenylalanine carboxy-lyase, E.C. 4.1.1.26) in plants might be inferred from the numerous reports of 3-HT in plants. Apparently the only reference to DOPA DC in plants is our previous report on this enzyme in the marine alga *Monostroma fuscum*.[5] Because of the rather unusual characteristics of the *Monostroma* enzyme, we wished to compare it with one from a higher plant.

RESULTS AND DISCUSSION

Effect of pH and buffer ions on DOPA DC

Young, leafy branches of *Cytisus* were homogenized in water in a Waring blendor for 1 min and portions of this homogenate were added to potassium phosphate buffers and subjected to our standard DC assay. Optimal activity of the DOPA DC is at pH 8, but the peak for activity as a function of pH is broad, and activities at pH 7·0 and 9·0 are about 90% of the pH 8·0 level. Activity falls off sharply below pH 6·5. A small amount of non-enzymic

* Abbreviations used include: 3-hydroxytyramine, 3-HT; 3,4-dihydroxyphenylalanine, DOPA; decarboxylase, DC; D- and L-amino acid oxidase, DAAO and LAAO, respectively; sodium diethyldithiocarbamate, DIECA; *p*-chloromercuribenzoic acid, *p*-CMB.

[1] S. UDENFRIEND, W. LOVENBERG and A. SJOERDSMA, *Arch. Biochem. Biophys.* **85**, 487 (1959).
[2] R. D. TOCHER and J. S. CRAIGIE, *Can. J. Bot.* **44**, 605 (1966).
[3] H. SCHMALFUSS and A. HEIDER, *Biochem. Z.* **236**, 226 (1931).
[4] FR. JAMINET, *J. Pharm. Belgique* **8**, 23 (1953).
[5] R. D. TOCHER and CAROL A. TOCHER, Abstr. XI *Int. Botan. Congr.* p. 219 (1969).

decarboxylation of DOPA occurs at the highest pH values and corrections were made by using boiled enzyme as a control. Most subsequent experiments were performed at pH 7·0. *Cytisus* DOPA DC varied little in its activity in potassium phosphate, Tris, *N*-Tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), *N,N*-bis(2-hydroxyethyl)-glycine (bicine) or glycylglycine when these buffers were compared at 0·05 M, pH 7·5. By contrast, the DOPA DC of the alga *Monostroma*[5] is several times more active in phosphate buffer than in organic buffers.

Substrate Specificity

In view of the repeated reports of tyramine and 3-HT in *Cytisus*,[3,4,6,7] Correale and Cortese[7] suggested two pathways by which 3-HT might be produced in *Cytisus*, as follows:

1. Tyrosine $\xrightarrow{-CO_2}$ Tyramine $\xrightarrow{+OH}$ 3-HT

2. Tyrosine $\xrightarrow{+OH}$ DOPA $\xrightarrow{-CO_2}$ 3-HT

Carboxyl-^{14}C-labeled tyrosine and DOPA provide a method to distinguish between these two sequences. If DOPA is an intermediate between tyrosine and 3-HT, unlabeled DOPA should dilute the radioactivity from carboxyl-^{14}C-tyrosine. *Cytisus* contains phenolase with a weak cresolase (*o*-hydroxylase) activity towards monophenols which expresses itself by a small amount of oxygen uptake in tyrosine-containing reaction flasks. Thus, it is not possible to distinguish between the two sequences on the basis of oxygen consumption since it is common to both.

Carboxyl-^{14}C-labeled phenylalanine, tyrosine or DOPA were incubated with *Cytisus* enzyme to measure the extent of their decarboxylation. Other flasks contained labeled tyrosine together with unlabeled DOPA. The results are shown in Table 1.

TABLE 1. SUBSTRATE SPECIFICITY OF *Cytisus* DECARBOXYLASE*

Substrate	Amount in reaction flasks, μmol	Specific activity dpm μmol^{-1} × 10^{-3}	$^{14}CO_2$ released dpm
L-Tyrosine	4	25	470
L-Phenylalanine	8	3	156
L-DOPA (D,L-label)†	8	5	7540
L-Tyrosine (^{14}C)	4	25	243
+ L-DOPA (^{12}C)	8		

* Warburg reaction flasks contained 4·0 ml of fluid including 280 μmol of potassium phosphate at pH 7·0, 1·0 ml of supernatant solution (10 000 *g*) of homogenized young branch tips, and substrate as indicated.

† The tracer compound was commercial carboxyl-^{14}C-D,L-DOPA, the carrier pure L-DOPA.

The addition of unlabeled DOPA to labeled tyrosine did reduce the amount of radioactive CO_2 by half, and this apparent dilution effect is consistent with reaction sequence 2,

[6] O. WOLFES, *E. Merck's Jahresbericht* **50**, 111 (1936).
[7] P. CORREALE and I. CORTESE, *Naturwiss.* **40**, 57 (1953).

although possible inhibition of tyrosine decarboxylation by DOPA cannot be ruled out. Even stronger evidence for the second sequence is the large extent by which DOPA decarboxylation exceeds that of tyrosine or phenylalanine. The amount of $^{14}CO_2$ produced from tyrosine was low, suggesting that the conversion of tyrosine to DOPA is probably a rate-limiting step in the biosynthesis of 3-HT.

Stereospecificity

Until recently, carboxyl-^{14}C-DOPA was available commercially only as the D,L-mixture. In most experiments we assumed that the large dilution of the ^{14}C-D,L-DOPA by unlabeled L-DOPA would negate any effect of the labeled D-DOPA. Nevertheless, when the labeled D,L-mixture was diluted with unlabeled D-DOPA, decarboxylation was about 10% greater than when the L-form was used. The enhanced D-DOPA decarboxylation was consistent in many experiments, whether the enzyme was prepared from different parts of the plant, or was stored at 4° for several days, or was purified by different methods. Two batches of labeled D,L-DOPA, diluted to the usual specific activity of our assays were treated with either DAAO or LAAO. Unfortunately, the pH optimum of hog kidney DAAO is about 8·3, at which pH all of the DOPA was oxidized to black, melanin-like material. Below pH 8, where DOPA is more stable against oxidation, the DAAO was virtually inactive. Crude LAAO from snake venom has optimal activity at pH 7·3, where only slight oxidation of DOPA occurred. When the LAAO reaction was complete, the mixture was acidified and the deaminated acids were extracted into ether. The specific activity of the unchanged D-DOPA was estimated by liquid scintillation counting and measuring the UV absorbance. Decarboxylase activity towards purified ^{14}C-D-DOPA was compared with D-DOPA labeled with the mixed D,L-isomers, and the results are shown in Table 2 as Experiment 1. In terms of the

TABLE 2. COMPARISON OF THE DECARBOXYLATION OF D- AND L-DOPA

Substrate and specific activity	$^{14}CO_2$ released dpm × 10^{-3}
Experiment 1*	
D-DOPA (3·1 μmol)	1·97
+ ^{14}C-D-DOPA purified with LAAO (29 500 dpm)	
D-DOPA (3·1 μmol)	1·80
+D,L-^{14}C-DOPA (29 200 dpm)	
Experiment 2†	
D-DOPA (10 μmol)	12·5
+D,L-^{14}C-DOPA (50 600 dpm)	
L-DOPA (10 μmol)	11·2
+D,L-^{14}C-DOPA (50 600 dpm)	
L-DOPA (10 μmol)	11·3
+L-^{14}C-DOPA (50 600 dpm)	

* Incubates (2·6 ml) contained 160 μmol of potassium phosphate at pH 7·0 and 2·0 mg of lyophilized, purified enzyme from ripe seed pods.

† Incubates contained the same fluid volume and buffer. The enzyme was a homogenate of young leafy branch tips filtered through cheesecloth. An amount corresponding to about 200 mg of fresh tissue was used in each assay.

D-isomer, the mixed ^{14}C-D,L-DOPA had almost exactly one-half the specific activity of the purified ^{14}C-D-DOPA. If the enzyme were specific for D-DOPA, the amount of radioactivity released should presumably have been only half that released when purified D-DOPA was used. However, since the activity of the $^{14}CO_2$ was almost the same whether the D- or D,L-DOPA was used in substrate, both D and L forms must be decarboxylated at about the same rates. The unwanted L-isomer in Experiment 1 represented only 0·1 nmol, diluted 3×10^4 times with D-DOPA.

Pure carboxyl-^{14}C-L-DOPA is now available from Amersham/Searle Corporation. Experiment 2 (Table 2) presents the results obtained when *Cytisus* DOPA DC acted on the mixed isomers and on the pure L-form, and shows the slightly greater decarboxylation which was consistently found when the D-isomer was used as the carrier. In experiment 2 there is essentially no difference in the decarboxylation of the two L-form reactions, although the first has only half the specific activity with respect to the L-isomer, again indicating that the enzyme decarboxylates the D- and L-DOPA at similar rates. This property of the *Cytisus* enzyme contrasts with the DOPA DCs found in various mammalian tissues. The latter are considered to be strictly specific for the L-isomer.[8]

Influence of Oxygen

In an early experiment we tried to reduce the phenolase catalyzed oxidation of DOPA by gassing the Warburg reaction flasks with nitrogen. It was immediately clear that oxygen is required for the decarboxylation reaction. The dependence of the reaction on the presence of oxygen is illustrated for two experiments in Table 3. This dependence on oxygen was

TABLE 3. THE EFFECT OF OXYGEN ON *Cytisus* DOPA DC

Gas phase	$^{14}CO_2$ released by seed pod enzyme* dpm	$^{14}CO_2$ released by branch tip enzyme†
100% Nitrogen	204	68
2% Oxygen–98% nitrogen	1270	2350
Air	2280	12800
100% Oxygen	2420	14300
Air—boiled enzyme	120	70

* Incubates (2·6 ml) contained 2·0 mg of enzyme solubilized from ripe seed pods, 10 μmol L-DOPA + 48 000 dpm ^{14}C-D,L-DOPA and 160 μmol of potassium phosphate at pH 7·0. Reactions were gassed for 10 min before and terminated 60 min after enzyme and substrate were mixed.

† Incubates (2·6 ml) contained 1·6 ml of filtered homogenate of young branch tips equivalent to approximately 200 mg of tissue, 10 μmol of L-DOPA + 36 200 dpm ^{14}C-D,L-DOPA and 160 μmol of potassium phosphate at pH 7·0. Reactions were gassed for 10 min before and terminated 30 min after enzyme and substrate were mixed.

unexpected, since little attempt has been made to exclude oxygen in studies with mammalian DOPA DC. Our experience[5] with the plant DOPA DCs has been that the amount of oxygen uptake observed during the reaction was usually proportional to the amount of DOPA which was decarboxylated. We cannot rule out the possibility that phenolase is implicated

[8] P. HOLTZ, *Naturwiss.* **27**, 724 (1939).

in the decarboxylation. Mason and others[9] have observed that the carboxyl group of dopachrome, an oxidation product of DOPA, is labilized in neutral solution by anions and Zn^{2+}. Loss of the carboxyl group produces 5,6-dihydroxyindole.

Enzyme distribution in Cytisus *tissues*

Plant tissues were collected in late May and again in July from the same shrub. The May collection was divided into flowers, green fruits, leaves and young branches which had been stripped of leaves. The July collection was separated into dry fruits, leaves and young branches. The tissues were weighed and homogenized in pH 7·0, 0·1 M potassium phosphate buffer, filtered and assayed immediately. Enzyme activities in the different plant parts are shown in Table 4. Young branches had the highest DOPA DC activity. The dry fruits were

TABLE 4. DISTRIBUTION OF L-DOPA DC IN *Cytisus**

Tissue	$^{14}CO_2$ released dpm × 10^{-3}†
Flowers	2·7
Green fruits	3·7
Dry fruits	22
Leaves	48
Young branches, leaves removed	73

* Reactions contained 2·6 ml of fluid with homogenate equivalent to approximately 200 mg of tissue, 10 μmol of L-DOPA + 37 200 dpm D,L-^{14}C-DOPA, and 160 μmol of potassium phosphate at pH 7·0. Reaction time was 30 min.

† To allow comparison of tissues collected at different times, DPM activity is given as the $^{14}CO_2$ released by the enzyme contained in 1·0 g of fresh tissue. Leaves and branches in the two collections gave similar results.

the most difficult to homogenize completely because they were leathery, and failure to grind them completely may have yielded lower results. Jaminet's report[4] that the highest 3-HT concentration in *Cytisus* occurred in green fruits seems at variance with our finding of low DOPA DC activity in this tissue. We extracted green fruits with hot 80% ethanol and obtained the UV spectrum. The λ_{max} of the green fruit extract, 270 nm, did not correspond to the following reference compounds dissolved in 80% ethanol: L-tyrosine, 275 nm; tyramine. HCl, 276 nm; L-DOPA, 281 nm; or 3-HT.HCl, 280 nm. In our sample, DOPA or 3-HT were not significant contributors to UV absorbance.

Inhibitors of DOPA DC

Various crude tissue homogenates and partially purified enzyme preparations were tested for inhibition by several metal-complexing chemicals, thiol reagents and substrate analogs with the results shown in Table 5. DIECA is somewhat specific as a complexing agent for copper, and several copper containing enzymes are sensitive to it. There was considerable inhibition by the thiol reagents mercaptoethanol, dithiothreitol and cysteine. Inhibition by these latter three compounds suggests that disulfide bonds are required for the functioning of the enzyme. The lack of inhibition by *p*CMB gives some evidence that free –SH groups

[9] H. S. MASON, *Adv. in Enzymol.* **16**, 105; esp. pp. 114–117 (1955).

TABLE 5. INFLUENCE OF INHIBITORS

Compound	Final concentration (M)* $\times 10^{-4}$	(%) Inhibition
DIECA	0·25	33
	1	100
EDTA	10	10
α,α′-Dipyridyl	12	7
2,9-Dimethyl-1,10-phenanthroline	5	0
NaN_3	12	5
$CuCl_2$	10	70
KCN	12†	56
Thiourea	12	23
Phenylthiourea	12	97
Mercaptoethanol	1·5	60
	10	100
Dithiothreitol	38	99
Cysteine	38	80
*p*CMB	1	0
α-Methyl-D,L-DOPA	120	85
L-Mimosine	3·8	0
	38	13

* In each experiment, the compound was incubated with the enzyme for 10 min before the reaction was started by adding 10 μmol of L-DOPA + D,L-^{14}C-DOPA. During this incubation, the inhibitor concentration was 1·5–2·6 times the final concentration given.

† The actual CN^- concentration may have been reduced by absorption of HCN gas in the alkali trap of the reaction vessel.

are not required for activity. The metal-complexing agents tested gave contradictory results. Little or no inhibition by EDTA, dimethylphenanthroline, azide or dipyridyl conflicts with the inhibition by CN^-, DIECA and phenylthiourea. Firm conclusions cannot be drawn as to a possible role of metals in the functioning of this enzyme. The inhibition by α-methyl-DOPA is probably competitive with DOPA because increasing the DOPA concentration partially overcame the inhibition. L-Mimosine is the pyridone analog of DOPA, and it inhibited only slightly. L-Mimosine was a potent inhibitor of the *Monostroma* DOPA DC.[5]

Pyridoxal Phosphate

Pyridoxal phosphate is an essential prosthetic group of mammalian DOPA DC[10] and exogenous pyridoxal phosphate stimulates DOPA DC independently of that present as tightly bound prosthetic group. In *Cytisus* we found little stimulation (0–10%) of DOPA DC by exogenous pyridoxal phosphate even after the enzyme had been thoroughly dialyzed against water or dilute buffer. Our negative results do not eliminate the possibility of enzyme-bound pyridoxal phosphate in the *Cytisus* enzyme.

Purification Attempts

Enzyme purification, when used in the sense of increasing activity relative to protein concentration, was particularly difficult because most methods of estimating protein concentration were not suitable for use with tissue extracts of this plant. *Cytisus* tissues contain

[10] J. AWAPARA, R. SANDMAN and C. HANLY, *Arch. Biochem. Biophys.* **98**, 520 (1962).

copious amounts of soluble nitrogenous compounds and also compounds with a high UV absorbance near 270 nm which are presumably aromatic. Extracts turned grey-brown when mixed with alkaline biuret reagent. In terms of dry weight of lyophilized enzyme relative to the dry weight of the tissue from which it was derived, an approximately 20-fold 'purification' with low yield was obtained. Ripe fruits were chopped and homogenized for 5 min in a blender with 8 vol. of cold, 0·1 M, pH 7·0 potassium phosphate buffer per unit of tissue weight. The brei was filtered through cheesecloth and centrifuged at 10 000 *g* for 10 min. The resulting supernatant fraction contained about half of the total homogenate activity. Unfortunately, activity in the supernatant fraction rapidly disappeared even when stored under nitrogen and this fraction was therefore discarded. The precipitate was suspended in 2% sodium lauryl sulfate and stored in the cold for 46 hr. The suspension was then centrifuged for 10 min at 10 000 *g* and the precipitate discarded. About two-thirds of the activity was solubilized and 70% of the enzyme activity precipitated when the solution was brought to 25% saturation with $(NH_4)_2SO_4$. After centrifuging, the enzyme was dissolved in water, dialyzed overnight against water and lyophilized. In this form it was stable in the freezer for 6–12 months. Reliable protein determinations (biuret) were only obtained after the salt precipitation step. Insoluble polyvinylpyrrolidone, although frequently useful for binding phenolic materials in protein extracts,[11] was of little use in our purification attempts. Recently, we have had good success with Lam and Shaw's method[12] of grinding plant tissue with Dowex 1 × 8 anion exchange resin which had been previously equilibrated with the buffer used in grinding. By this method the unstable supernatant enzyme retained activity for several days when stored cold under N_2.

EXPERIMENTAL

Decarboxylase assay. Reactions were carried out in triplicate in a Warburg apparatus. The total volume of reaction mixture was usually 2·6 ml including 100–160 μmol potassium phosphate buffer at pH 7·0. D,L-DOPA-carboxyl-^{14}C was diluted with unlabeled DOPA to a specific activity of 3000–10 000 dpm/μmol. The substrate, usually 10 μmol, was added to the reaction mixture after 10 min of temperature equilibration. The reactions were terminated usually after 30 min by tipping in 0·3 ml of 3 N H_2SO_4 to release CO_2 from solution. Release of this gas was complete within 30 min and it was trapped on a folded square of filter paper moistened with 0·1 ml of 10% NaOH in the center well of the Warburg flasks. The filter papers were transferred to vials and counted by liquid scintillation using 10 ml of toluene–EtOH (2/1) counting fluid containing 4·0 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis(2-(5-phenyloxazolyl))benzene (POPOP) per liter. Counting efficiency was 70% (± 5%) as estimated by channels ratio. The quantitative recovery of $^{14}CO_2$ and the counting efficiency were verified by using Warburg flasks containing known amounts of $NaH^{14}CO_3$ manipulated in the same manner as the experimental assays.

Plant tissue. Cytisus scoparius samples were collected at various sites in Portland and along the Oregon Coast. Tissue was either used within 3 hr of collection or stored in plastic bags at 4° for use within 3 days.

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[11] W. D. Loomis and J. Battaile, *Phytochem.* **5**, 423 (1966).
[12] T. H. Lam and M. Shaw, *Biochem. Biophys. Res. Commun.* **39**, 965 (1970).

Key Word Index—Cytisus scoparius; Leguminosae; DOPA decarboxylase; amines; dopamine.