CONVERSION OF L-TYROSINE TO 3,4-DIHYDROXYPHENYLALANINE BY CELL-FREE PREPARATIONS OF BRAIN AND SYMPATHETICALLY INNERVATED TISSUES

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From isotopic studies it is known that the first step in the biosynthesis of noradrenaline involves the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). Although the overall conversion of tyrosine to noradrenaline can be demonstrated in various intact organs (1,2,3) and in tissue slices (4,5,6), nothing is known about the cellular catalyst responsible for the first step in the biosynthesis. It has now been possible to obtain mitochondrial fractions from brain and adrenal medulla which can hydroxylate L-tyrosine to DOPA. Some properties of this system are presented in the present report.

tyrosine ⟶ DOPA ⟶ DOPAmine ⟶ noradrenaline

MATERIALS AND METHODS

L-Tyrosine-U-C^{14} (300 μc/μmole), D-tyrosine-1-C^{14} (23.9 μc/μmole), DL-m-tyrosine-H^3 (26.5 μc/μmole), tyramine-1-C^{14} (1.83 μc/μmole), and DL-DOPA-H^3 (223 μc/μmole) were obtained from New England Nuclear Corp. N'-(Methyl,3-hydroxybenzyl) hydrazine (pargyline) was kindly donated by Dr. A. Sjoerdsma. N'-Methyl-N'-3-hydroxyphenylhydrazine (NSD-1034) was kindly donated by Dr. E. Costa. α-Propyl-3,4-dihydroxyphenylacetamide was kindly donated by Dr. A. Carlsson. DL-α-Methyl-tyrosine was obtained from Merck Sharp & Dohme Research Laboratories, m-tyrosine from H. M. Chemical Co.,

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D-tyrosine from Mann Research Laboratories, Inc. Alumina was obtained from M. Woelm. Eschwege and treated according to the method of Crout (7).

Unless otherwise specified incubations were carried out in air in a total volume of 4 ml containing 0.05 to 0.1 µg of tyrosine-C^{14} (100,000 to 200,000 c.p.m.), potassium phosphate buffer pH 6.4 (400 µmole), 4 µmoles of the decarboxylase inhibitor NSD-1034, and 4 µmoles of the monoamine oxidase inhibitor, pargyline. The incubation was stopped by the addition of 3 ml of 20% trichloroacetic acid. Twenty µg of nonradioactive L-DOPA (and in some instances DOPAmine) were added as carrier and the beaker was rinsed with 5 ml of water. Catechols were adsorbed onto alumina according to the method of Crout (7). The alumina columns were then washed twice with 15 ml volumes of water and then eluted with 6 ml of 0.3 N acetic acid. A 2 ml aliquot of the acetic acid eluate containing DOPA-C^{14} was transferred to a counting vial. Ten ml of Bray's solution (8) was added and radioactivity measured in a scintillation spectrometer. When 20 µg of DOPA or DOPAmine were carried through this procedure they were recovered to the extent of about 85%.

Tyrosine-C^{14} from commercial sources contained appreciable amounts of material which assayed as catechols by this procedure. However, by treating the labelled tyrosine solutions twice with alumina as described above and then passing the solutions over IRC-50 (Na^+ form) columns it was possible to remove almost all catechol-like interferences; 500,000 c.p.m. of purified tyrosine yielded less than 50 c.p.m. through the procedure.

RESULTS

In a preliminary survey homogenates or 15,000 x G particle fractions of a number of tissues of the guinea pig were tested for their ability to convert L-tyrosine to DOPA. Tissues heated at 80° for 10 minutes were used as controls in each case. As can be seen in Table I adrenal medulla and brain stem were most active. Homogenates and particles derived from brain stems of rats, rabbits, cows and hogs exhibited activities compara-

ble to those of the guinea pig preparations. When homogenates of brain stem were prepared in 0.25 M sucrose and subjected to differential centrifugation essentially all the activity was found to be associated with tnose particles sedimenting at 15,000 x G (Table II).

TABLE I

Conversion of L-tyrosine to DOPA by guinea pig tissues. The data presented in this table are typical of data obtained in many similar experiments. The controls were heated at 80° for 10 min before adding substrates. Particles represent the sediment obtained by centrifugation at 15,000 x G and prepared as described in the text.

Tissue	DOPA formed (mμmoles/g/hr)
Brain stem particles	7.53
Brain stem particles (heated)	0.03
Whole adrenal homogenate	13.60
Whole adrenal (heated)	0.12
Heart particles	0.05
Heart particles (heated)	0.00
Spleen particles	0.49
Spleen particles (heated)	0.17
Liver particles	0.29
Liver particles (heated)	0.05
Kidney particles	0.07
Kidney particles (heated)	0.02

TABLE II

Intracellular localization of hog brain tyrosine hydroxylase activity

Cell fraction	DOPA formed (c.p.m./g/hr)
Whole homogenate	3540
15,000 x G particles	4580
15,000 x G supernatant	760

That the product of the reaction was a catechol was shown by its adsorption onto alumina at pH 8.5 and subsequent elution with acetic acid. When acetic acid eluates (of samples incubated in the presence of the decarboxylase inhibitor NSD-1034) were evaporated to dryness under nitrogen and subjected to chromatography on paper only one radioactive spot appeared having the same R_f as authentic DOPA (Fig. 1). When incubations were carried out in the absence of the decarboxylase inhibitor similar amounts of catechol were formed but most of the material was now DOPAmine (Fig. 1). Enzymatically formed DOPA and synthetic DOPA were also found to be indistinguishable in their migrations on paper electrophoresis, 22 cm on Whatman No. 3 MM paper at 5 K volts for 1.5 hour using 4% formic acid as medium, and on column chromatography using IRC-50 (Na^+ form) and Dowex 50 (H^+ form) columns.

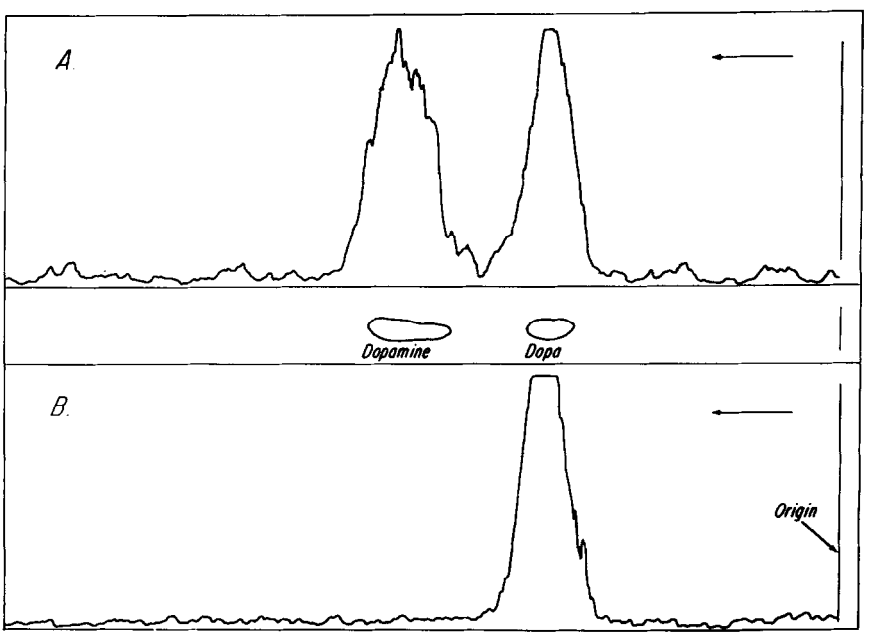

Fig. 1. Chromatographic properties of enzymatically formed catechols. A. In the absence of a decarboxylase inhibitor, total catechols formed in experiment 9619 c.p.m. B. In the presence of a decarboxylase inhibitor, total catechols formed in experiment 12,942 c.p.m. The control spots were visualized by spraying with 0.25% potassium ferricyanide and then exposing to ammonia vapors.

The fact that heated preparations lost their activity is only indirect evidence of an enzymatic catalysis, particularly in view of previous reports of nonenzymatic catechol production from phenols (9,6). However, the marked specificity of the reaction is definite evidence of enzyme catalysis. Thus, in a given experiment with 10^{-5} M tyrosine having 100,000 c.p.m. L-tyrosine yielded 7,500 c.p.m. By contrast comparable amounts of D-tyrosine gave absolutely no labelling in DOPA and did not inhibit the conversion of L-tyrosine. No conversion was observed even when D-tyrosine levels were raised to 10^{-3} M. In addition, tyramine and the meta isomer of tyrosine, 3-hydroxyphenylalanine, were absolutely inactive. The particles did not metabolize L-DOPA to any appreciable extent in the presence of the decarboxylase inhibitor.

With brain stem particles which had been washed once with isotonic KCl containing 10^{-4} M mercaptoethanol activity was maximal at pH 6.4. At this pH DOPA formation was linear for at least 30 minutes and then fell off. Oxygen was found to be an absolute requirement but high oxygen tension inhibited DOPA formation. Other factors such as TPNH, DPNH, ATP, and FAD produced little effect. Brain stem particles from ascorbic acid deficient guinea pigs (animals showing definite symptoms of scurvy) had activity comparable to normal controls. In the particulate preparation the only suggestion of a cofactor was the inhibition observed with α-α-dipyridyl (10^{-3} M) which may be indicative of a Fe^{++} involvement. Other agents such as dinitrophenol, azide, p-chloromercuribenzoate, thiourea, diethyldithiocarbamate and versene (at 10^{-4} to 10^{-3} M) did not inhibit appreciably.

Of specific interest was the inhibition observed with some tyrosine analogues. p-Fluoro-D,L-phenylalanine and α-methyl-D,L-tyrosine at 10^{-4} M inhibited to the extent of about 60% and 90% respectively using 2×10^{-5} M substrate. α-Propyl-3,4-dihydroxyphenylacetamide was also an effective inhibitor producing 80% inhibition at 10^{-4} M and 40% inhibition

at 10^{-5} M. Other catechol compounds, including DOPA, were also effective as inhibitors of this reaction.

DISCUSSION

This is certainly not the first demonstration of an enzymatic mechanism for conversion of tyrosine to DOPA. Such a conversion is known to occur during melanin formation and to be catalyzed by the enzyme tyrosinase. However, the enzyme in brain and sympathetic tissue is not tyrosinase because (a) it does not metabolize DOPA, (b) it is not affected by inhibitors of tyrosinase such as thiourea and diethyldithiocarbamate, (c) tyrosine oxidation is inhibited rather than stimulated by DOPA, and (d) D-tyrosine is not a substrate as in the case of tyrosinase. The pH optimum, tissue localization and failure to observe pigment formation are also evidence that the enzyme reported here is not tyrosinase but is in effect a tyrosine hydroxylase.

There have been many attempts in this and other laboratories to demonstrate the initial enzyme step in noradrenaline biosynthesis. The failure to detect it before this was, we believe, due to technical problems in purifying the substrate and in using sufficiently small amounts of tyrosine and tissue to minimize nonenzymatic hydroxylation processes which are artifacts of in vitro experimentation.

Following studies with inhibitors of the other two steps in noradrenaline synthesis we have suggested that the first step, tyrosine hydroxylation, must be the rate-limiting step (10). The Km of the brain tyrosine hydroxylase has been found to be between 10^{-6} and 10^{-5} M. By contrast the Km value for DOPA decarboxylation by aromatic L-amino acid decarboxylase is greater than 10^{-4} M; for purified dopamine-β oxidase it is about 10^{-3} M. The significance of these relative Km values with respect to noradrenaline production in intact tissues is being investigated using the isolated perfused guinea pig heart.*

* Levitt, M., Spector, S., Sjoerdsma, A., and Udenfriend, S., to be published.

The finding of this enzyme in brain and sympathetically innervated tissues is conclusive evidence that noradrenaline is synthesized locally and is not transported to the various organs by the blood (3,11). The presence of such high concentrations of tyrosine hydroxylase in brain is an excellent corroboration of the *in vivo* findings regarding rates of noradrenaline synthesis by McGeer *et al.* (11) and by Udenfriend and Zaltzman-Nirenberg (12).

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