Biochimica et Biophysica Acta, 445 (1976) 567—578

BBA 67932

STUDIES ON PHENYLALANINE AND TYROSINE HYDROXYLATION BY RAT BRAIN TYROSINE HYDROXYLASE

IRA KATZ, TOM LLOYD and SEYMOUR KAUFMAN

Laboratory of Neurochemistry, National Institute of Mental Health, Building 36, Room 3D-30, Bethesda, Md. 20013 (U.S.A.)

(Received March 9th, 1976)

Summary

Tyrosine hydroxylase (EC 1.14.16.2), presumably the rate-limiting enzyme in the biosynthesis of catecholamines, is known to catalyze the hydroxylation of both phenylalanine and tyrosine. Using both an isolated enzyme preparation and a synaptosomal preparation, where some architectural integrity of the tissue has been preserved, we have attempted to evaluate the manner in which these two substrates are hydroxylated by rat brain tyrosine hydroxylase. In the presence of tetrahydrobiopterin the isolated enzyme catalyzes the hydroxylation of phenylalanine to 3,4-dihydroxyphenylalanine with the release of free tyrosine as an obligatory intermediate. In contrast, the rat brain striatal synaptosomal preparation in the presence of endogenous cofactor converts phenylalanine to 3,4-dihydroxyphenylalanine without the release of free tyrosine.

Introduction

In 1965, Ikeda et al. [1] reported that tyrosine hydroxylase (EC 1.14.16.2) could catalyze the hydroxylation of L-phenylalanine. These workers found that when either crude brain or adrenal tyrosine hydroxylase preparations were tested in the presence of the synthetic cofactor, $DMPH_4$, the rate of L-phenylalanine hydroxylation was 4—6% that of L-tyrosine hydroxylation. Ikeda et al. [2] also showed that this hydroxylation of phenylalanine was not catalyzed by a specific liver type of phenylalanine hydroxylase, but was mediated by a tyrosine hydroxylase which could convert L-phenylalanine to DOPA. They

Abbreviations: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; DOPA, 3,4-dihydroxyphenylalanine, Hepes; N-2-hydroxyethylpiperazine-N-2-ethanesulfonate; NSD-1034, N-methyl-N-3-hydroxyphenyl hydrazine; 6-MPH_4, 2-amino-4-hydroxy-6-methyltetrahydropteridine; TLC, thin layer chromatography.

suggested that the reaction proceeded in two steps with free tyrosine as the intermediate. Subsequently, Shiman et al. [3] confirmed and extended the above observations with highly purified bovine adrenal tyrosine hydroxylase. These workers discovered that, in the presence of a natural cofactor, tetrahydrobiopterin, the rate of L-phenylalanine hydroxylation was equal to or exceeded the rate of tyrosine hydroxylation. Thus, the extent of phenylalanine hydroxylation appears to depend on the structure of the pterin cofactor. Recently a number of groups have found phenylalanine hydroxylating activity in synaptosomes [4] and in unsupplemented homogenates [5,6,7] from areas of the rat's brain that are known to be rich in tyrosine hydroxylase activity.

In this paper, we report a comparison of the tyrosine hydroxylase-catalyzed hydroxylation of phenylalanine as observed with the isolated enzyme in the presence of tetrahydrobiopterin, and with a synaptosome-enriched fraction from the corpus striatum, a tissue preparation high in tyrosine hydroxylase activity. As previously shown [4], synaptosome fractions catalyze the hydroxylation of phenylalanine to DOPA at a rate comparable to that for the hydroxylation of tryrosine to DOPA. We show that although, as expected, phenylamine hydroxylations catalyzed by the isolated enzyme and by synaptosomes have many features in common, there are significant differences between the two systems. These differences are discussed in terms of the manner in which properties of the cellular environment can influence the catalytic properties of intracellular enzyme systems.

Experimental procedures

Materials

The aromatic L-amino acid decarboxylase inhibitor NSD-1034 (*N*-methyl-*N*-3-hydroxyphenylhydrazine) was obtained through the generosity of J.G.B. Howes of Smith and Nephew Research, Ltd., Harlow, Essex, England. L-[U-^{14}C]phenylalanine (specific activity 384 Ci/mol), L-[U-^{14}C]tyrosine (specific activity 368 Ci/mol), and L-[3,5-^{3}H]tyrosine (specific activity 1.0 Ci/mmol) were obtained from New England Nuclear, $DMPH_4$ and 3-iodo-L-tyrosine were purchased from the Aldrich Chemical Co. Tetrahydrobiopterin and 6-MPH_4 were synthesized in this laboratory by Dr. Sheldon Milstien. Crystalline catalase in suspension was purchased from the Boehringer Mannheim Corp.

Methods

A crude synaptosomal fraction (P_2) was prepared from male Sprague-Dawley rats (250 g) as follows. After killing by decapitation, the brains were removed, and the corpus striatum was dissected and immediately placed in 0.3 M sucrose at 0°C. Approximately 80—100 mg of tissue was obtained per rat. The P_2 fraction containing mitochondria, myelin, and synaptosomes, was prepared by the method of Gray and Whittaker [8]. It was washed once in 0.3 M sucrose, resedimented by centrifugation at 20 000 × *g* for 20 min and then resuspended in 0.3 M sucrose, usually to a total volume of 100 μl suspension for each brain used in the preparation. Analysis of the material prepared in this manner gave 1.0—1.2 mg of protein per brain.

In each experiment, 50 μl of the P_2 suspension were added to 200 μl of a

buffer containing 150 mM NaCl, 2 mM KCl, 1.25 mM potassium phosphate pH 6.8, 3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 25 mM Tris/Hepes buffer pH 6.8, and 25 mM glucose. The DOPA decarboxylase inhibitor, NSD-1034, was added in saturating amounts ($1-5 \cdot 10^{-4}$ M). Incubations were performed with constant shaking under air at 37° C. Reactions were terminated by addition of 250 µl of 10% trichloroacetic acid; the precipitated protein was removed by centrifugation. Samples were analyzed for product formation after separation of tyrosine, DOPA and phenylalanine by one or more of three analytical procedures which are described below.

The partially purified rat brain tyrosine hydroxylase was prepared immediately before use, although it was found to retain its activity for several weeks after rapid freezing. The rat brains used for preparation of this enzyme were removed, and placed in ice-cold 0.3 M sucrose; the striata were dissected and homogenized in 10 volumes 0.002 M potassium phosphate, pH 7.0 in an all glass Duall homogenizer. The homogenate was centrifuged at 45 000 × *g* for 30 min and the clear extract was brought to 55% saturation with respect to ammonium sulfate by the addition of 350 mg powdered ammonium sulfate per ml of extract. After equilibration, the precipitate was collected by centrifugation at 15 000 × *g* for 15 min and then dissolved in a minimum volume of 0.02 M potassium phosphate, pH 6.8, containing 8% sucrose (protein concentration varied from 11.0 to 14.0 mg/ml).

The assay of the isolated enzyme was carried out in a total volume of either 100 or 250 µl, depending upon method of product analysis, and contained the following components: 100 mM potassium phosphate pH 6.2, 20 mM β-mercaptoethanol (or 1.0 mM NADPH and sheep liver dihydropteridine reductase in excess), 8000 units catalase per ml, 0.2 mM NSD-1034, phenylalanine or tyrosine as described in the text and figure legends, $DMPH_4$ or tetrahydrobiopterin as described in the text and figure legends, 1.0—1.5 mg isolated rat brain tyrosine hydroxylase and water to a final volume of either 100 or 250 µl. Incubations were carried out in a metabolic shaker at 37° C for 10 min and terminated by addition of sufficient 100% trichloroacetic acid to make the final mixture 5% with respect to trichloroacetic acid.

DOPA formation was measured by (A) the tritium-release assay [9], (B) column chromatography on aluminum oxide [10], or by thin layer chromatography. DOPA and tyrosine formation from phenylalanine were measured by chromatography of the supernatant fractions from reaction mixtures on Eastman precoated cellulose (No. 6064) sheets utilizing the solvent system: *n*-propanol/H_2O/NH_4OH (8 : 1 : 1, by vol.). This solvent system routinely yielded R_f values of 0.66, 0.39, and 0.13 for phenylalanine, tyrosine and DOPA respectively. In a second TLC system, we used MN 300 cellulose coated glass plates (Uniplate, from Analtech, Inc.) with the solvent system: *n*-butanol/acetone/NH_4OH/H_2O (4 : 4 : 2 : 1, by vol.). This TLC system routinely yielded R_f values of 0.75, 0.50 and origin-0.30 for phenylalanine, tyrosine and DOPA, respectively. After chromatography, the separated amino acids were visualized by spraying with ninhydrin, the three appropriate areas from each chromatogram were scraped off, and radioactivity was determined by liquid scintillation spectrometry. In some experiments, tyrosine and DOPA were determined after chromatography of reaction mixtures on an amino acid

analyzer column [11]. Protein was determined by the method of Lowry et al. [12] with the use of bovine serum albumin as the standard.

Results

Hydroxylation of phenylalanine and tyrosine by isolated rat brain tyrosine hydroxylase

In the presence of $DMPH_4$, rat brain tyrosine hydroxylase (isolated as described under Methods) was found to catalyze the hydroxylation of tyrosine at a much faster rate than that of phenylalanine (Fig. 1). As previously reported by Ikeda et al. enzyme activity with phenylalanine as the substrate is about 5% of that with tyrosine. When tetrahydrobiopterin was used as cofactor, however, rat brain tyrosine hydroxylase utilized phenylalanine nearly as well as tyrosine (Fig. 2). The K_m values for tetrahydrobiopterin (0.20 mM for the conversion of phenylalanine to tyrosine and 0.25 mM for the conversion of tyrosine to DOPA) are about onefifth the K_m value for $DMPH_4$ for conversion of tyrosine to DOPA (1.25 mM). In this respect, the brain enzyme is qualitatively similar to bovine adrenal tyrosine hydroxylase which also exhibits a lower K_m towards tetrahydrobiopterin (0.1 mM), than towards $DMPH_4$ (0.3 mM) [13].

A further study of the substrate specificity of the isolated brain enzyme is illustrated in Fig. 3 where enzymatic activity, measured as product formed in the presence of tetrahydrobiopterin as cofactor, is plotted as a function of either phenylalanine or tyrosine concentration. The severe substrate inhibition observed when tyrosine is the substrate is similar to that previously reported for bovine adrenal tyrosine hydroxylase [3]. When phenylalanine was used as substrate, there was no inhibition by excess phenylalanine of tyrosine forma-

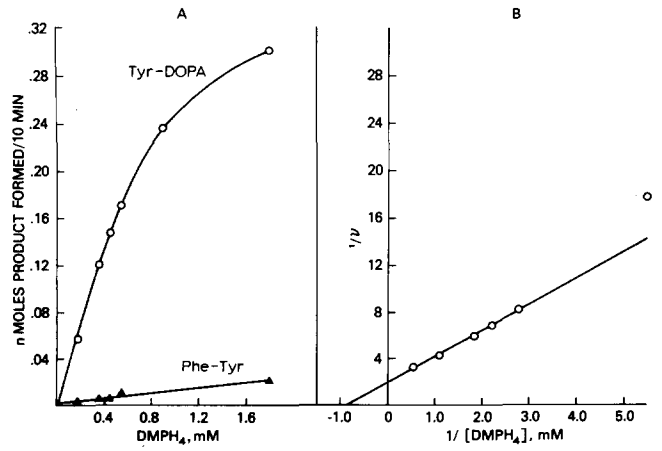

Fig. 1. The effect of $DMPH_4$ on the hydroxylation of phenylalanine and tyrosine by isolated rat brain tyrosine hydroxylase. (A) linear graphical representation and (B) data plotted by the method of Lineweaver-Burk. Each incubation contained in addition to the components described in the Methods: 1.20 mg rat brain tyrosine hydroxylase preparation, either 1.0 μCi [3,5-^{3}H]tyrosine or 0.9 μCi [^{14}C]phenylalanine, the enzymatic cofactor regenerating system, $2 \cdot 10^{-5}$ M of the appropriate amino acid, and the concentrations of $DMPH_4$ described in the figure. Incubation was at 37°C for 10 min. DOPA formation from [^{3}H]tyrosine was determined by the tritium release assay and [^{14}C]tyrosine formation from [^{14}C]-phenylalanine was isolated by thin layer chromatography.

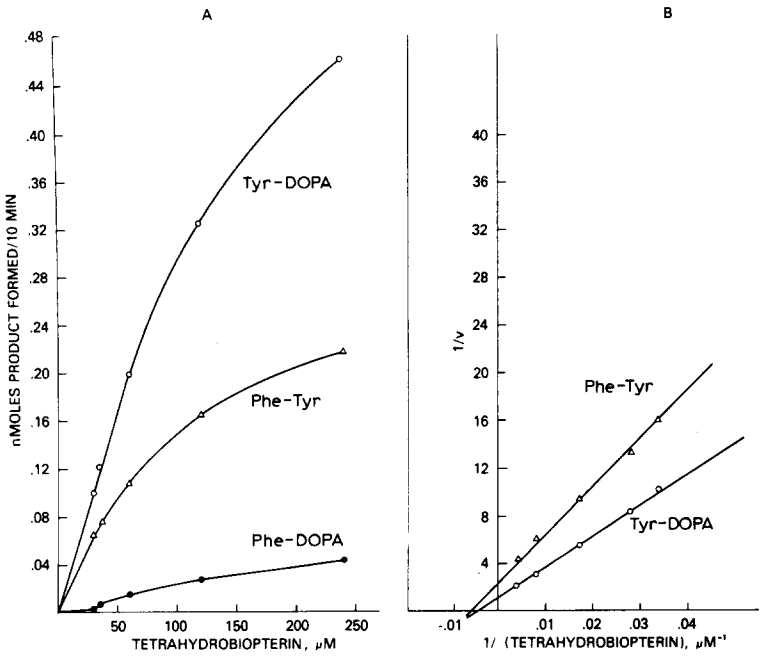

Fig. 2. The effect of tetrahydrobiopterin on the hydroxylation of phenylalanine and tyrosine by isolated rat brain tyrosine hydroxylase. The conditions employed in this experiment and the display of data are identical to those described for Fig. 1, the only difference being the use of tetrahydrobiopterin as the co-factor in this experiment.

tion. Inhibition of DOPA formation, however, was observed. The inhibition of DOPA formation as a function of phenylalanine concentration is not due to the accumulation of tyrosine to an inhibitory concentration, i.e., 30 μM. It is most likely due to competition between phenylalanine and newly formed tyrosine for the active site of the enzyme and thus suggests that free tyrosine is an intermediate in the conversion of phenylalanine to DOPA.

The time course of product formation from both precursors was investigated in a series of experiments similar to those described in Fig. 3. Initially, the conversions of tyrosine to DOPA, and of phenylalanine to tyrosine were linear with the time of incubation, whereas that of phenylalanine to DOPA was parabolic, that is, linear with the square of the time of incubation. This last finding is consistent with the idea that tyrosine is a free intermediate in the conversion of phenylalanine to DOPA. Apparently, the rate of DOPA formation, at any instant of time, depends upon the concentration of tyrosine present in the reaction medium. Since the tyrosine concentration, and, therefore, the rate of DOPA formation, increases with time, the amount of DOPA formed increases as the square of the time of incubation.

We further investigated the mechanism of conversion of phenylalanine to DOPA with a series of double-labelling experiments. With the use of a mixture of [^{3}H]phenylalanine and [^{14}C]tyrosine as substrates for isolated tyrosine hydroxylase, the ratio of $^3H/^{14}C$ in tyrosine compared to that in DOPA at the end of the incubation may be used to determine the reaction pathway. Thus, if

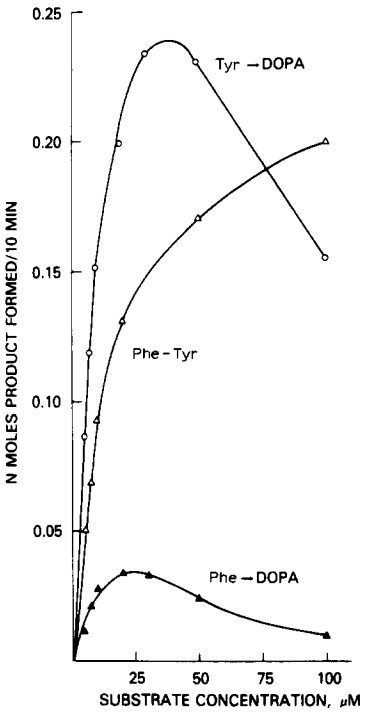

Fig. 3. Isolated rat brain tyrosine hydroxylase activity as a function of either phenylalanine or tyrosine in the presence of tetrahydrobiopterin. Each incubation contained, in addition to the components described in Methods, 1.20 mg rat brain tyrosine hydroxylase, the enzymatic cofactor regenerating system, either 1.0 μCi [3,5-^{3}H]tyrosine or 0.9 μCi [^{14}C]phenylalanine, 0.115 mM tetrahydrobiopterin, and sufficient non-radioactive phenylalanine or tyrosine to produce the concentrations described. Incubation was at 37°C for 10 min. DOPA formation from [^{3}H]tyrosine was determined by the tritium release assay and [^{14}C]tyrosine formation from [^{14}C]phenylalanine was isolated by thin layer chromatography.

DOPA were formed from phenylalanine without the formation of free tyrosine as an intermediate, the ratio of ^{3}H/^{14}C in DOPA would be much higher than the ratio of ^{3}H/^{14}C in tyrosine at the end of the reaction. Alternatively, if all of the DOPA which is formed from phenylalanine proceeds through free tyrosine as an intermediate, the ratio of ^{3}H/^{14}C in DOPA would be equal to the time-averaged value of ^{3}H/^{14}C in tyrosine (time averaging of the values is necessary since [^{14}C]tyrosine decreases, whereas [^{3}H]tyrosine increases, during the incubation). With these considerations in mind, it can be predicted that a ratio [^{3}H/^{14}C DOPA]/[^{3}H/^{14}C tyrosine] greater than 0.50, must mean that some DOPA is formed from phenylalanine by a pathway that does not involve free tyrosine as an intermediate. On the other hand, if free tyrosine is an obligatory intermediate (and if the rate of product formation is linear with time) this ratio should be 0.50. The data presented in Table I show that the [^{3}H/^{14}C DOPA]/[^{3}H/^{14}C tyrosine] ratio is slightly less than 0.50 and indicate, therefore, that with isolated rat brain tyrosine hydroxylase free tyrosine is an obligatory intermediate in the conversion of phenylalanine to DOPA.

TABLE I

SIMULTANEOUS MEASUREMENT OF HYDROXYLATION OF [^{14}C]TYROSINE AND [^{3}H]PHENYLALANINE BY ISOLATED RAT BRAIN TYROSINE HYDROXYLASE

The rat brain tyrosine hydroxylase used in this study was prepared as described in Methods except that only striata were used as the tissue source. Each incubation contained the following components in a total volume of 100 μl: 10 μmol potassium phosphate, pH 6.1, 5 μmol β-mercaptoethanol, 400 units catalase, 25 nmol tetrahydrobiopterin, 2 nmol L-[^{3}H]phenylalanine containing $3.68 \cdot 10^{6}$ cpm, 2 nmol L-[^{14}C]-tyrosine containing $2.6 \cdot 10^{5}$ cpm, 100 nmol NSD-1034 and 300 μg tyrosine hydroxylase preparation. Incubation was 37°C for 15 min terminated by the addition of 5 μl 100% trichloroacetic acid and tyrosine and DOPA analyzed as described in the Methods. Blank incubations were made by using either boiled enzyme or adding 50 nmol 3-iodo-L-tyrosine per incubation. Typical blank values were as follows (in nanomoles): tyrosine: ^{3}H, 0.01; DOPA: ^{3}H, 0.02; ^{14}C, 0.005. Qualitatively similar data was obtained at two other enzyme levels.

Compound	Product formed (nmol)		Ratios	
	^{3}H	^{14}C	^{3}H/^{14}C	[^{3}H]DOPA/[^{14}C]DOPA / [^{3}H]tyrosine/[^{14}C]tyrosine
Tyrosine	0.124	0.670	0.185	0.43
DOPA	0.025	0.315	0.080	

Hydroxylation of phenylalanine and tyrosine by rat striatal P_2 fractions

As discussed above, we studied the phenylalanine and tyrosine hydroxylating activities of a more highly organized tissue preparation to be able to compare its properties with those of the isolated enzyme. For this purpose, we used a rat striatal P_2 fraction, a preparation known to be rich in dopaminergic synaptosomes. Tissue from a rat striatal P_2 fraction suspended in a physiological salt solution (as described in Methods) exhibits tyrosine hydroxylase activity as previously described. In the presence of saturating concentrations of the DOPA decarboxylase inhibitor, NSD-1034, the rate of formation of labelled DOPA from labelled tyrosine is linear with time of incubation and tissue concentration and is inhibited by phenylalanine and by low concentrations of 3-iodo-L-tyrosine.

The tyrosine hydroxylase activity in these experiments was measured without added cofactor, suggesting that the enzyme was functioning with endogenous cofactor retained within the synaptosomes. To confirm this possibility, disruption of synaptosomal preparations was examined. In the experiment described in Table II, synaptosome preparations were incubated in the presence and absence of the detergent, Triton X-100. In the presence of the detergent tyrosine hydroxylation is abolished, presumably due to lysis of the synaptosome and subsequent dilution of the previously sequestered cofactor. This interpretation is supported by the further demonstration that when exogenous cofactor is added back to the detergent-treated synaptosomes, tyrosine hydroxylation is restored. Separate experiments indicated that the detergent under these conditions, was without effect on the isolated enzyme.

After having established the general characteristics of tyrosine hydroxylation catalyzed by rat striatal P_2 preparations, the activity of such preparations in catalyzing the hydroxylation of phenylalanine was studied. The conversion of phenylalanine to tyrosine and DOPA as a function of time of incubation and

TABLE II

EFFECT OF TRITON X-100 ON TYROSINE HYDROXYLASE ACTIVITY FROM RAT STRIATAL P_2 FRACTION

Rat striatal P_2 fraction derived from one hemisphere and suspended in 100 μl 0.3 M sucrose was added to 400 μl of the physiological buffer described in the Methods which also contained the following: 50 mM β-mercaptoethanol, 10^{-4} M NSD-1034, 20 μM L-[^{14}C]tyrosine (220 000 cpm) and 2000 units catalase. Samples were incubated for 30 min at 37° C, the reactions terminated by the addition of trichloroacetic acid and the samples were analyzed for [^{14}C]DOPA formation by chromatography on alumina.

Experiment	Additions	nmoles DOPA/30 min
1	None	0.33
2	Triton X-100 (1% v/v)	0
3	Triton X-100 (1% v/v) plus 700 μM 6-MPH$_4$	0.76

tissue concentration is shown in Fig. 4. It is of interest to compare the time course of DOPA formation from phenylalanine observed in synaptosomes with that seen with the isolated enzyme. With synaptosomes, the conversion is roughly linear with time, whereas with the isolated enzyme, as discussed above, the rate is linear with time squared. These results indicate that with synaptosomes it is not necessary to build up a high concentration of tyrosine derived from phenylalanine before significant amounts of DOPA can be formed. As can be seen in Table III, hydroxylation of phenylalanine to tyrosine and DOPA, like that of tyrosine to DOPA, is inhibited by low levels of 3-iodotyrosine, a specific tyrosine hydroxylase inhibitor, a finding that supports the conclusion that the hydroxylation of phenylalanine, like that of tyrosine, is catalyzed by tyrosine hydroxylase within the synaptosomes.

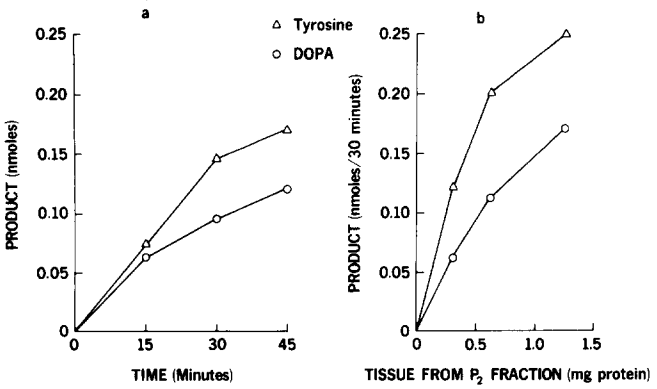

Fig. 4. Effect of time and tissue concentration on phenylalanine hydroxylation by rat striatal P_2 fraction. (A) 50 μl of a rat striatal P_2 fraction in 0.30 M sucrose was added to 200 μl of the physiological buffer described in the Methods and incubated for the intervals indicated above. (B) varying amounts of P_2 tissue in 50 μl of a 0.30 M sucrose suspension were added to 200 μl of the physiological buffer described in the Methods. The blanks for (A) contained no tissue. Incubation was at 37° C for 30 min. The incubation mixtures for both sets of experiments also contained 20 μM L-[^{3}H]phenylalanine (containing 220 000 cpm) and $4 \cdot 10^{-4}$ M NSD-1034. The reactions were terminated by the addition of trichloroacetic acid and product formation was analyzed by TLC. It should be noted that there is some difference of tyrosine hydroxylase specific activity in the data for (A) and (B). Some variation of activity was observed between synaptosomal preparations from day to day, but data obtained from each preparation was routinely reproducible.

TABLE III

3-IODO-L-TYROSINE INHIBITION OF PHENYLALANINE HYDROXYLATION BY RAT STRIATAL P_2 FRACTION

Rat striatal P_2 fraction from one hemisphere was suspended in 50 μl 0.3 M sucrose and added to 200 μl of the physiological buffer described in the Methods which also contained 10^{-4} M NSD-1034 and 20 μM [^{14}C]phenylalanine (containing 220 000 cpm). Incubation was at 37°C for 30 min, terminated by the addition of trichloroacetic acid and product formation was analyzed by TLC.

3-Iodo-L-tyrosine concentration (M)	nmol product/30 min		% inhibition	
	Tyrosine	DOPA	Tyrosine	DOPA
0	0.150	0.100	—	—
$2 \cdot 10^{-7}$	0.110	0.060	27	40
$5 \cdot 10^{-7}$	0.100	0.048	33	52
$4 \cdot 10^{-5}$	0	0	100	100

We previously demonstrated (Table II) that synaptosomal tyrosine hydroxylase activity is dependent upon the presence of a sequestered endogenous cofactor. Since this system shows activity toward phenylalanine as well as tyrosine, the endogenous cofactor must be one capable of stimulating both activities; i.e. it must function like tetrahydrobiopterin rather than like $DMPH_4$ [3]. This conclusion is strengthened by the effects observed upon supplementing P_2 suspensions with $DMPH_4$ (Table IV). $DMPH_4$ inhibits the hydroxylation of phenylalanine, whereas it stimulates that of tyrosine. The inhibition of the phenylalanine hydroxylation is probably due to competition between the endogenous and exogenous cofactors leading to net displacement of the endogenous pterin by one incapable of promoting tyrosine formation from phenylalanine. The stimulation of tyrosine hydroxylation represents either enhancement of the activity of tyrosine hydroxylase, unsaturated with respect to endogenous cofactor in intact synaptosomes, or activation of any enzyme that might be present in synaptosome ghosts devoid of cofactor.

Having shown that the P_2 preparation can convert phenylalanine to DOPA,

TABLE IV

EFFECT OF $DMPH_4$ ON TYROSINE HYDROXYLASE ACTIVITY OF RAT STRIATAL P_2 FRACTION

Rat striatal P_2 fraction from one hemisphere was suspended in 50 μl 0.3 M sucrose and added to 200 μl of the physiological buffer described in the Methods. The final reaction mixture also contained 10^{-4} M NSD-1034, 20 mM β-mercaptoethanol, 0.50 mM $FeSO_4$, and either [^{3}H]phenylalanine (220 000 cpm) or [^{14}C]tyrosine (220 000 cpm). Incubation was for 20 min at 37°C, terminated by the addition of trichloroacetic acid and product formation was analyzed by TLC.

Substrate	$DMPH_4$ (800 μM final conc.)	Product formed (nmol/20 min)	
		Tyrosine	DOPA
20 μM phenylalanine	—	0.095	0.054
20 μM phenylalanine	+	0.022	0.021
20 μM tyrosine	—	—	0.220
20 μM tyrosine	+	—	0.353

TABLE V

SIMULTANEOUS MEASUREMENT OF HYDROXYLATION OF [^{14}C]TYROSINE AND [^{3}H]PHENYLALANINE BY RAT STRIATAL P_2 FRACTION

P_2 fraction derived from rat striatum of one hemisphere and suspended in 50 μl 0.30 M sucrose was added to 200 μl of the physiological buffer described in the Methods which also contained the following: $4 \cdot 10^{-4}$ M NSD-1034, 20 μM [^{3}H]phenylalanine ($2 \cdot 10^{6}$ cpm), and 20 μM [^{14}C]tyrosine, ($2 \cdot 10^{5}$ cpm). Blank tubes contained, in addition, 10^{-4} M 3-iodo-L-tyrosine. Tubes were incubated at 37°C for 30 min, the incubations terminated by the addition of trichloroacetic acid, and carrier amino acids and the products analyzed by both the TLC and amino-acid analyzer techniques as described in the Methods. Qualitatively similar data were obtained in experiments carried out at 16 μM phenylalanine and 16 μM tyrosine. Typical blank values were as follows: (in nmol) [^{3}H]tyrosine: 0.05; [^{3}H]DOPA: 0.01; [^{14}C]DOPA: 0.055.

Product formed	nmol		Ratios	
	^{3}H	^{14}C	^{3}H/^{14}C	[^{3}H]DOPA/[^{14}C]DOPA / [^{3}H]Tyrosine/[^{14}C]tyrosine
Tyrosine	0.073	4.50	0.016	4.17
DOPA	0.030	0.45	0.0677	

we investigated the mechanism of this conversion with a double labelling experiment similar to the one described previously for the isolated enzyme. The results are shown in Table V. After incubation of a striatal P_2 suspension with [^{3}H]phenylalanine and [^{14}C]tyrosine, the ratio ^{3}H/^{14}C found in DOPA is much greater than the time-averaged ratio found in tyrosine. As discussed before, this result indicates that [^{3}H]tyrosine present in solution is not the major precursor of [^{3}H]DOPA. Thus, with synaptosomes, in contrast to the isolated enzyme, free tyrosine, i.e. tyrosine that rapidly equilibrates with tyrosine in the medium is not an obligatory intermediate in the conversion of phenylalanine to DOPA.

Discussion

We have studied tyrosine and phenylalanine hydroxylation in two preparations obtained from the rat corpus striatum: an ammonium sulfate precipitate of a hypotonic extract supplemented as appropriate for assay of tyrosine hydroxylase, and an unsupplemented P_2 fraction prepared by the method of Gray and Whittaker [8]. The enzyme in the ammonium sulfate precipitate behaved in most respects, including substrate and cofactor specificity, in a manner similar to that of the highly purified tyrosine hydroxylase from the bovine adrenal medulla [3]. The P_2 fraction, a synaptosome-enriched preparation, was similar in many respects, but strikingly different in others. The significance of these differences depends on whether the hydroxylating enzyme that is within the synaptosome is tyrosine hydroxylase.

There are several lines of evidence suggesting that both the tyrosine and phenylalanine hydroxylating activity of striatal synaptosomes is, in fact, due to tyrosine hydroxylase that is compartmentalized within the synaptosomes together with an endogenous tetrahydrobiopterin-like cofactor and a cofactor regenerating system:

(1) Both tyrosine and phenylalanine hydroxylation are inhibited by low con-

centrations of 3-iodotyrosine, a specific tyrosine hydroxylase inhibitor.

(2) Hydroxylation of either tyrosine or phenylalanine can be inhibited by the other amino acid substrate, as expected if the reactions are catalyzed by the same enzyme.

(3) Hydroxylation of tyrosine and phenylalanine is inhibited when synaptosomes are disrupted, as expected if catalytic activity required a diffusable component such as a cofactor. Tyrosine hydroxylation by synaptosomes is stimulated, whereas phenylalanine hydroxylation is inhibited by exogenous dimethyltetrahydropterin, as expected for reactions catalyzed by tyrosine hydroxylase.

As already shown, there are several differences between synaptosomes and the isolated enzyme in the properties of phenylalanine hydroxylating activity. On the assumption that hydroxylations of both phenylalanine and tyrosine in synaptosomes are catalyzed by tyrosine hydroxylase, there are two possible explanations for these differences. First, it is conceivable that the basic mechanism of the hydroxylation reaction is different in the synaptosome than it is with the isolated enzyme. For example, the observed differences could be explained if tyrosine hydroxylase within the synaptosome could catalyze a direct conversion of phenylalanine to DOPA with two hydroxylations occurring in a single encounter of the enzyme with the substrate. Although a change in fundamental catalytic mechanism of the enzyme could account for the observed differences, there is little precedent for such a change, and this possibility must be regarded as remote. A more likely explanation for these kinetic differences is that they reflect differences in the enzyme's environment. Thus, the characteristics of the hydroxylation reaction that are seen with the P_2 fraction are the expected ones if the tyrosine that is formed from phenylalanine within the synaptosome did not rapidly distribute through the assay medium, and did not rapidly equilibrate with any external tyrosine present. Clearly, lack of equilibration of newly formed tyrosine with the tyrosine in the medium implies that unidirectional transport away from the enzyme of tyrosine derived from phenylalanine proceeds at a rate comparable to that of tyrosine formation. Since methods for preparing homogenous preparations of dopaminergic synaptosomes are not currently available, this possibility cannot be directly tested. Comparison of the phenylalanine hydroxylating activity of the synaptosome and the isolated enzyme, however, does lead to some conclusions regarding the relative rates of transport and reaction in the synaptosome. The effectiveness of nerve termini in catalyzing the conversion of phenylalanine to DOPA apparently depends upon the properties of the transport systems of the membrane, as well as upon the properties of the enzyme itself. If transport across the membrane were very rapid relative to the hydroxylation reaction, these environment effects could not be operative and we would, therefore, expect the synaptosome to behave more like the isolated enzyme; that is, the synaptosome would be less effective in catalyzing the conversion of phenylalanine to DOPA. Given the physical similarity of the substrates involved, and the fact that the specificity of amino acid transport systems is dependent upon the physical properties of the side chains, the above considerations, quite likely apply not only to the conversion of phenylalanine to tyrosine but also to the intrasynaptosomal conversion of tyrosine to dopamine.

Finally, these considerations raise the possibility that transport may be the

rate-limiting step in synaptosomal tyrosine hydroxylase activity. Although, as discussed above, the relevant transport experiments cannot be performed, there is some indirect evidence on this point. The fact that 3-iodotyrosine, a potent tyrosine hydroxylase inhibitor that is competitive with the amino acid substrate, efficiently inhibits the synaptosomal reaction at low (submicromolar) concentrations, suggests that the hydroxylation reaction is not seriously limited by transport [14]. Rather, the rates of transport and the rate of the hydroxylation reaction must be fairly closely balanced.

References

1 Ikeda, M., Levitt, M. and Udenfriend, S. (1965) Biochem. Biophys. Res. Commun. 18, 482—488
2 Ikeda, M., Levitt, M. and Udenfriend, S. (1967) Arch. Biochem. and Biophys. 120, 420—427
3 Shiman, R., Akino, M. and Kaufman, S. (1971) J. Biol. Chem. 246, 1330—1340
4 Karobath, M. and Baldessarini, R.J. (1972) Nat. New Biol. 236, 206—208
5 Bagchi, S.P. and Zarycki, E.P. (1972) Biochem. Pharmacol. 21, 584—589
6 Bagchi, S.P. and Zarycki, E.P. (1970) Life Sci. 9, 111—119
7 Bagchi, S.P. and Zarycki, E.P. (1973) Biochem. Pharmacol. 22, 1353—1368
8 Gray, F.G. and Whittaker, V.P. (1962) J. Anat. 96, 79—88
9 Nagatsu, T., Levitt, M. and Udenfriend, S. (1964) Anal. Biochem. 9, 122—126
10 Weiner, N., Draskoczy, P.R. and Burack, W.R. (1962) J. Pharmacol. Exptl. Ther. 137, 47—55
11 Tong, J.H., D'Iorio, A. and Benoiton, L. (1971) Biochem. Biophys. Res. Commun. 44, 229—236
12 Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Shiman, R. and Kaufman, S. (1970) in: Methods in Enzymology (Tabor, H. and Tabor, C.W., eds.), 17A, pp. 609—615, Academic Press, N.Y.
14 Engasser, J.-M. and Horvath, C. (1974) Biochemistry 13, 3845—3849