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Research Article

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Tyrosine Hydroxylase in Human Adrenal and Pheochromocytoma: Localization, Kinetics, and Catecholamine Inhibition

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ABSTRACT The properties of partially purified tyrosine hydroxylase from six pheochromocytomas were compared with partially purified normal human and bovine adrenal medulla enzyme. Substrate and inhibition kinetics, cofactor requirements, and intracellular localization of the enzyme from normal and tumor chromaffin tissue of humans were similar, as was the amount of enzyme activity per gram of tissue. Contrary to previous reports, the sensitivity to catecholamine inhibition of the pheochromocytoma enzyme from the six tumors studied was similar to that of both human and bovine adrenal medulla tyrosine hydroxylase. These results suggest that the excessive synthesis and secretion of catecholamines in some pheochromocytomas is not the result of a reduced sensitivity of tyrosine hydroxylase to catecholamine inhibition.

INTRODUCTION

The formation of dihydroxyphenylalanine (dopa)¹ from tyrosine, catalyzed by tyrosine hydroxylase (TH), is considered to be the rate-limiting step in the biosynthesis of catecholamines (1, 2). In normal sympathetically innervated neural tissues, the biosynthesis is presumably regulated by feedback inhibition of TH by catecholamines in competition with the pteridine cofactor of the enzymic reaction (3).

Most human pheochromocytomas are known to secrete large quantities of catecholamines, as manifested by increased plasma catecholamine levels and urinary excretion of these compounds. It has been suggested that the elevated production of catechols in the pheochromocytoma

may be the consequence of less effective catecholamine feedback regulation of synthesis in the tumor. Roth, Stjärne, Levine, and Giarman (4) observed that alumina-treated low speed supernates prepared from human pheochromocytoma synthesized more catecholamines than did similar preparations from human adrenal medullae of the same patients. The authors reported that catecholamine synthesis in nonfortified homogenates of pheochromocytoma was far less sensitive to inhibition by catechol compounds than similar preparations from normal chromaffin tissue. Nagatsu, Yamamoto, and Nagatsu (5) investigated the properties of TH from human pheochromocytoma, comparing them with similar preparations from bovine adrenal medulla. They observed that the tumor enzyme was stimulated by ferrous ion and 6,7-dimethyltetrahydropterin (DMPH₄), but found that the enzyme was less sensitive to inhibition by norepinephrine and epinephrine. In the presence of low concentrations of catecholamines and high concentrations of DMPH₄, they observed stimulation of enzyme activity. Tyrosine hydroxylase from normal human adrenal medulla was not studied. Both groups concluded that abnormally high synthesis of catechols in pheochromocytoma may be due, in part, to insensitivity of tyrosine hydroxylase to end product feedback inhibition.

When human pheochromocytomas became available in our laboratories, we compared the partially purified TH from the tumor with that of a similarly purified enzyme from normal human adrenal. In preliminary experiments, catechol inhibition of TH was found to be similar in all studies (6). A more thorough investigation of the enzyme was therefore undertaken. The present report describes the results of a study of six pheochromocytomas in our two laboratories. Substrate and inhibition kinetics as well as cofactor requirements are compared in partially purified TH from the tumors as well as from

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¹Abbreviations used in this paper: DBH, dopamine- β -hydroxylase; DMPH₄, 6,7-dimethyltetrahydropterin; dopa, dihydroxyphenylalanine; TH, tyrosine hydroxylase.

TABLE I
Subcellular Localization of Tyrosine Hydroxylase, Dopamine- β -Hydroxylase, and Catecholamines in Human Pheochromocytoma

	Tyrosine hydroxylase activity		Dopamine- β -hydroxylase activity		Catecholamine level*	
	per g tissue‡	per mg protein‡	per g tissue§	per mg protein	per g tissue	per mg protein
	μmoles		μmoles		mg	
Whole homogenate	3.4	0.043	0.047	0.59	2.86	3.5
7,000 g·min supernate	3.4	0.055	0.064	1.35	2.58	4.1
235,000 g·min supernate	4.7	0.199	0.028	1.18	0.62	2.5
235,000 g·min pellet	0.2	0.012	0.083	4.77	1.37	8.0
Chromaffin granule	0.1	0.044	0.214	118.67	0.71	39.2
Chromaffin granule membrane	0.1	—¶	2.665	—¶	none	—¶

* Milligrams catecholamine, 95% norepinephrine.

‡ Activity expressed as micromoles product formed per hour using tyrosine hydroxylase assay 2. Incubations included 100 μM l - ^{14}C -tyrosine, 2 mM DMPH₄, and 1.0 mM ferrous sulfate. All samples dialyzed 4 hr in 100 vol 100 mM imidazole-acetate-glycerol, pH 7.0.

§ Activity expressed as micromoles product formed per hour.

|| Activity expressed as nanomoles product formed per hour.

¶ Protein determination not performed.

Values represent average of duplicate assays done on each fraction from pheochromocytoma No. 1.

normal human and bovine adrenal medullae. The subcellular distribution of the enzyme in the three tissues is also reported since the localization of TH relative to the storage site of catecholamines (chromaffin granule) may be an important factor in feedback regulation of synthesis.

METHODS

Chemicals

The artificial cofactor for the assay of tyrosine hydroxylase, 6,7-dimethyl-5,6,7,8-tetrahydropterin (DMPH₄) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. [$3,5$ - ^3H]- L -Tyrosine (1,000 mCi/mmole) and [1 - ^{14}C]- L -tyrosine (52 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass. [1 - ^{14}C]- L -Tyrosine required no purification; [$3,5$ - ^3H]- L -tyrosine was purified as described by Ikeda, Fahien, and Udenfriend (7). All other chemicals were obtained from standard commercial sources.

Radiochemical assay of tyrosine hydroxylase

Assay 1, isolation and assay of $^3\text{H}_2\text{O}$. Tyrosine hydroxylase activity was determined by a modification of the method of Nagatsu, Levitt, and Udenfriend (8). The assay contained 100 mM Tris-maleate, pH 5.9, 0.5–2.0 mM DMPH₄, 0.5–1.0 mM ferrous sulfate, 50 mM mercaptoethanol, and 200 μM ($3,5$ - ^3H)- L -tyrosine (5.0 $\mu\text{Ci}/\mu\text{mole}$). Incubations were stopped by the addition of glacial acetic acid. The tubes were centrifuged, and a portion of the supernate transferred to a column (0.5 \times 3.0 cm) of Dowex 50-H⁺ (Dow Chemical Co., Midland, Mich.). The tritiated water in the effluent and wash was counted by liquid scintillation spectrometry using 15 ml of Bray's solution (9). The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrophotometer (Packard Instrument Co.,

Inc., Downers Grove, Ill.). Activity, corrected for quenching, is expressed as micromoles $^3\text{H}_2\text{O}$ released per unit time.

Assay 2, formation and assay of $^{14}\text{CO}_2$. This recently developed procedure (10) incorporates the recovery and assay of $^{14}\text{CO}_2$ after quantitative decarboxylation with partially purified hog kidney aromatic L -amino acid decarboxylase of carboxyl-labeled dopa formed from carboxyl-labeled tyrosine. The assay contained 100 mM sodium acetate, pH 6.1, 0.5–2.0 mM DMPH₄, 0.5–1.0 mM ferrous sulfate, 50 mM 2-mercaptoethanol, and 100 μM [1 - ^{14}C]- L -tyrosine (10 $\mu\text{Ci}/\mu\text{mole}$). $^{14}\text{CO}_2$ was collected in 0.2 ml NCS solubilizer (Nuclear-Chicago Corporation, Des Plaines, Ill.) and was counted by liquid scintillation spectrometry using 15 ml toluene containing 0.5 g 1,4-bis(2[4-methyl-5-phenyloxazolyl]) benzene and 4.0 g 2,5-diphenyloxazole per liter. Activity is expressed as micromoles $^{14}\text{CO}_2$ collected per unit time.

Assay of dopamine- β -hydroxylase

Dopamine- β -hydroxylase (DBH) activity was assayed in the presence of 10^{-5} M *para*-chloromercuribenzoate by the procedure of Viveros, Arqueros, Connett, and Kirshner (11). Activity is expressed as micromoles of octopamine formed per unit time.

Assay of catecholamines

Catecholamines were measured by the colorimetric method of von Euler and Hamberg (12) using citrate-phosphate buffer at pH 6.0.

Subcellular tissue fractionation

Human adrenal glands were obtained either postmortem from the pathologist in cases where no adrenal pathology was present or from accident victims who were being maintained as organ donors by the Colorado General Hospital transplantation team. Human pheochromocytomas were ob-

TABLE II
Subcellular Localization of Tyrosine Hydroxylase in Three Chromaffin Tissues

Fraction	Tyrosine hydroxylase activity*†		
	Human adrenal medulla‡	Bovine adrenal medulla‡	Human pheochromocytoma 2
		<i>μmoles</i>	
Whole homogenate	3.57	3.49	2.08
7,000 g·min supernate	3.57	4.43	1.91
235,000 g·min pellet	0.24	2.96	0.80
235,000 g·min supernate	2.38	3.00	4.32

* Micromoles product formed/gram tissue per hour using tyrosine hydroxylase assay 2. Incubations included 100 μM $1\text{-}^{14}\text{C}$ -tyrosine, 2 mM DMPH₄, and 1.0 mM ferrous sulfate.

† All samples dialyzed 4 hr in 100 vol 100 mM imidazole-acetate-glycerol pH 7.0.

‡ Representative of typical distribution pattern for tissue.

Values represent average of duplicate assays done on each fraction.

tained immediately after surgical removal and placed in ice-cold 0.3 M sucrose. Bovine adrenal glands were obtained from the animal at time of slaughter. All tissues were minced and homogenized in 3 vol of 0.3 M sucrose using five strokes with a loose fitting Teflon pestle (0.8 mm clearance). All fractionation procedures were carried out at 0°C.

Homogenates were centrifuged at low speed (7,000 g·min). The sediment contained cell nuclei, unbroken cells, and debris. The cytoplasmic extract was further centrifuged at 235,000 g·min and at 6×10^6 g·min to yield the large granule fraction and microsomal pellet, respectively. Chromaffin granules were isolated by passing 1 ml of the resuspended large granule fraction through 9.0 ml 1.8 M sucrose at 12×10^6 g·min. All samples were assayed after a 4 hr dialysis in 100 mM imidazole-acetate buffer, pH 7.0, containing 10% glycerol. This procedure has been found to remove catecholamines while preserving tyrosine hydroxylase activity.

Partial purification of tyrosine hydroxylase

Soluble tyrosine hydroxylase was used as a starting material for all purifications. The enzyme was either fractionated

TABLE III
Tyrosine Hydroxylase Activities in Pheochromocytoma and Normal Tissues

Tissues	Tyrosine hydroxylase* activity
	<i>μmoles/g tissue per hr</i>
Pheochromocytoma (malignant) 1	3.44
Pheochromocytoma (benign) 2	2.08
Human adrenal medulla	3.57
Rat adrenal medulla	1.57
Bovine adrenal medulla	3.49

* Determined on whole homogenates dialyzed against 100 vol 100 mM imidazole-acetate, pH 7.0–10% glycerol to remove catecholamines using assay 2. Incubation included 100 μM $1\text{-}^{14}\text{C}$ -tyrosine, 2 mM DMPH₄, and 1 mM ferrous sulfate.

with ammonium sulfate (25–37% saturation) and dialyzed 4 hr in 100 mM imidazole-acetate buffer, pH 7.0, containing 10% glycerol, or purified according to Nagatsu et al. (5), (two ammonium sulfate fractionations, 0–40% saturation and then 25–35% saturation, charcoal treatment, and chromatography on a hydroxyapatite column).

RESULTS

Subcellular fractionation of two human pheochromocytomas gave similar results. In the fractionation study shown in Table I, dopamine- β -hydroxylase (DBH) and catecholamines were used as markers for the localization of the chromaffin granules. Tyrosine hydroxylase appeared almost entirely in the soluble fraction while DBH was associated with the chromaffin granules. Table II gives the results of a comparison of TH localization in three tissues: human pheochromocytoma, human adrenal, and bovine adrenal. Both the human

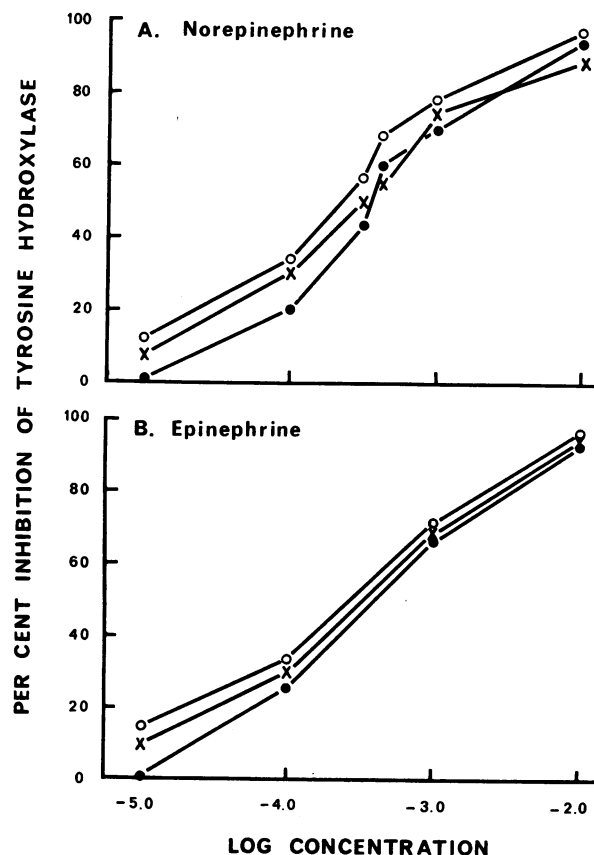


FIGURE 1 Per cent inhibition of normal human adrenal and human pheochromocytoma tyrosine hydroxylase over a wide range of inhibitor concentrations of: (a) norepinephrine and (b) epinephrine. Activity measured using assay 2. Incubation included 100 μM $1\text{-}^{14}\text{C}$ -tyrosine, 2 mM DMPH₄, 1.0 mM ferrous sulfate, and catecholamine as indicated. ●—●, tumor 2; ○—○, tumor 1; ×—×, normal human adrenal.

TABLE IV

Comparison of Cofactor Requirements for Tyrosine Hydroxylase from Three Chromaffin Tissues

Condition	Amount of tyrosine hydroxylase in complete system		
	Bovine adrenal medulla	Human adrenal medulla	Human pheochromocytoma
	%	%	%
Complete system*	100.00	100.00	100.00
-DMPH ₄	0.00	0.00	0.00
-Fe ⁺⁺	0.90	6.40	10.00
1.0 mM α,α' -dipyridyl	0.03	0.04	0.04
1.0 mM α,α' -dipyridyl with 2.0 mM Fe ⁺⁺	96.20	96.50	96.00
+Catalase (2500 U)†	100.00	100.00	100.00
-Fe ⁺⁺ , + catalase (2500 U)	37.50	30.20	24.50
+1.0 mM α,α' -dipyridyl and catalase (5000 U)	0.05	0.02	0.06
+2.0 mM 3-iodotyrosine	0.00	0.00	0.00

* Complete system contained 40 μ moles Na acetate, pH 6.2, 0.020 μ moles l-¹⁴C-tyrosine, 0.40 μ moles DMPH₄, 0.10 μ moles mercaptoethanol, 0.20 μ moles FeSO₄, and water to a final volume of 0.2 ml.

† 1 U decomposes 1 μ mole H₂O₂ per min at pH 7.0 at 25°C while H₂O₂ concentration falls from 10.3 to 9.2 μ moles/ml.

adrenal and pheochromocytoma TH are soluble. Bovine adrenal TH is distributed in all subcellular fractions. This is explicable on the basis of its adsorption to membrane fragments as has been previously reported (13).

Table III shows tyrosine hydroxylase activity in two pheochromocytoma tumors as well as in normal adrenals of human, rat, and cow. The enzyme activities in the tumor tissues are of the same order of magnitude and do not differ significantly from the activities found in normal adrenal medullae of these species.

The properties of TH separated from pheochromocytoma and human adrenal gland were similar to those of the enzyme from bovine adrenal medulla (Table IV). All three enzymes required a pteridine cofactor (DMPH₄). Maximal activity was not possible without 1.0 mM ferrous sulfate; smaller concentrations of iron in the incubation gave proportionately less TH activity. The addition of the iron complexing agent, α,α' -dipyridyl, resulted in the complete loss of activity. Addition of excess iron restored the activity to control levels. Shiman, Akino, and Kaufman (14) have recently suggested that catalase is effective in replacing ferrous iron in tyrosine hydroxylase incubations. In our studies TH activity is between 24.5 and 37.5% of control activity when catalase replaces iron. Excess iron is effective in reversing α,α' -dipyridyl inhibition of the enzyme while catalase is not. 3-Iodotyrosine completely inhibits all three enzyme preparations.

Inhibition of TH from two pheochromocytomas by a wide range of concentrations of catecholamines is shown in Fig. 1. The results of a more thorough study of the inhibition of TH by catecholamines is shown in Table

V. Concentrations of catecholamines identical to those used by Nagatsu et al. (5) were tested for inhibition of the TH from six tumors and normal human adrenal medulla. Contrary to previous reports (4, 5), the enzyme from all tumors exhibited sensitivity to feedback inhibition similar to that found for either human adrenal medulla or bovine adrenal medulla. Inhibition was similar with norepinephrine, epinephrine, or dopamine.

Fig. 2, a and b, illustrates the competitive nature of the catechol inhibition with respect to the pteridine

TABLE V
Effect of Catecholamine on Tyrosine Hydroxylase of Human Adrenal and Chromaffin Cell Tumors

Enzyme source	Amount tyrosine hydroxylase activity at catecholamine concentration (mM)					
	0.00	0.10	0.20	0.33	0.40	0.50
			%			
Human adrenal*	100	75	60	48	—	36
Pheochromocytoma 1*	100	71	—	50	—	42
Pheochromocytoma 2*	100	80	69	49	45	43
Pheochromocytoma 3†	100	71	72	54	—	53
Pheochromocytoma 4‡	100	57	33	35	—	25
Pheochromocytoma 5§	100	—	66	43	—	38
Pheochromocytoma 6§	100	68	47	36	—	29

* Assay 2 carried out with 1.0 mM ferrous sulfate, 2.0 mM DMPH₄, and 28–37% (NH₄)₂SO₄ fraction of 100,000 g supernate as enzyme and epinephrine as inhibitor.

† Assay 1 carried out with 0.5 mM ferrous sulfate, 0.5 mM DMPH₄, and 80% ammonium sulfate fraction of 100,000 g supernate as enzyme and norepinephrine as inhibitor.

‡ Assay 1 carried out with 1.0 mM ferrous sulfate, 2.0 mM DMPH₄, and enzyme purified according to Nagatsu et al. (10) using norepinephrine as inhibitor.

|| As per cent activity in absence of catecholamine.

cofactor (DMPH₄) for both human adrenal and human pheochromocytoma TH. Table VI summarizes the calculated kinetic constants for TH from three sources. The apparent K_m 's for DMPH₄ and tyrosine are similar for the three. The K_i values using epinephrine as inhibitor are approximately the same for the pheochromocytoma and human adrenal medulla enzyme. Norepinephrine gives similar results.

TABLE VI
Kinetic Constants of Tyrosine Hydroxylase from Human Adrenal Medulla and Pheochromocytoma

Constant	Normal human adrenal medulla	Pheochromocytoma No. 1	Pheochromocytoma No. 2
K_m tyrosine, mM	0.079 (1)	0.129 (1)	0.097 (1)
K_m DMPH ₄ , mM	0.240 (2)	0.356 (1)	0.371 (2)
K_i epinephrine, mM	0.037 (4)	0.031 (1)	0.055 (3)

Number in parentheses indicates number of experiments. Activity measured using assay 2. Incubations included 1.0 mM ferrous sulfate.

DISCUSSION

The properties of TH from all three sources examined (bovine adrenal, human adrenal, and human pheochromocytoma) are quite similar. The requirement of DMPH₄ as a cofactor and stimulation of Fe²⁺ have been reported previously for both the bovine enzyme (7, 15) and the pheochromocytoma enzyme (5). Similar dependencies were observed for the TH from the human adrenal (Table IV). All three enzymes were capable of partial activity if catalase replaced iron in the incubation. The fact that only excess iron reverses α, α' -dipyridyl inhibition of TH while catalase is without effect may indicate that a small amount of iron is necessary to activate the enzyme (such as Cu²⁺ activates dopamine- β -hydroxylase) (16). Additional amounts of iron may inhibit the formation of peroxides and catalase could substitute in this role.

The Michaelis constants for DMPH₄ and tyrosine were also quite similar in the enzymes from the two human sources (pheochromocytoma and adrenal medulla). The values agree closely with those published for bovine adrenal TH (7, 15).

The most interesting observation in our series of experiments, however, is that the similarity of properties of the enzyme from normal and tumor tissue extends to sensitivity to catecholamine inhibition of TH. In the cases investigated in our laboratories, biochemical and clinical findings indicated that the tumors were synthesizing and secreting large amounts of catecholamines before surgery. In contrast to previous reports, however, the sensitivity of the enzyme isolated from these (six) tumors did not differ from normal human adrenal TH over a wide range of catechol concentrations (Fig. 1 a and b). No stimulation of TH by catecholamines was found (Table V).

The substrate and inhibition kinetics of tyrosine hydroxylase from pheochromocytoma also approximated those of the enzyme from normal human adrenal (Fig. 2 a and b and Table VI). The reason for the discrepancies between our results and those of Nagatsu et al. (5) and Roth et al. (4) is not clear, but may

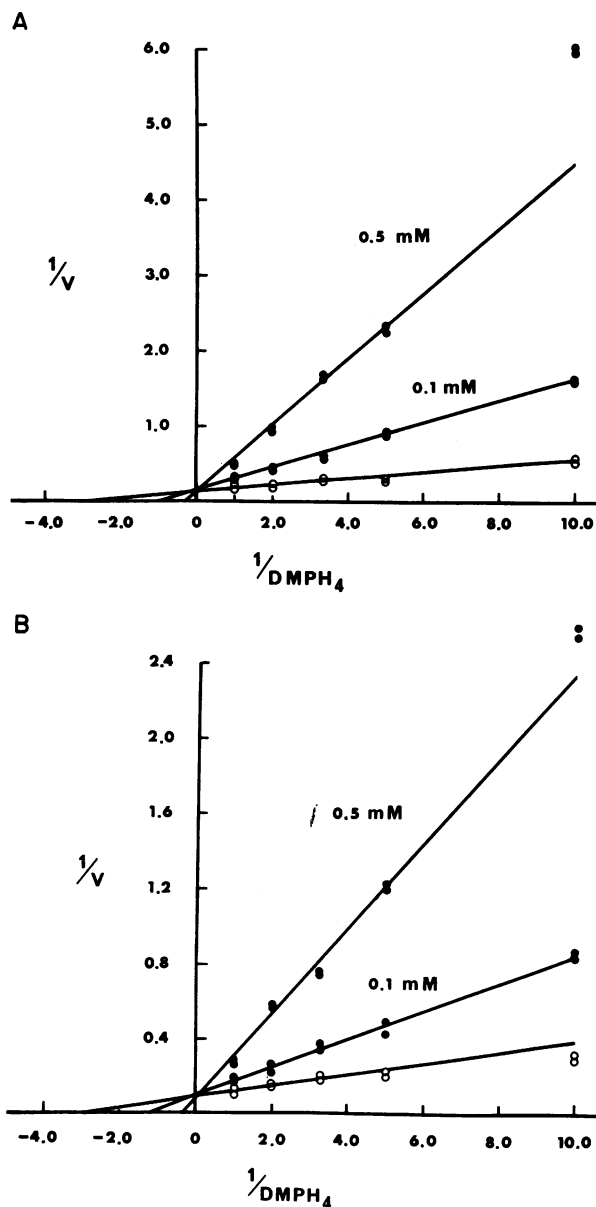


FIGURE 2 Lineweaver-Burk plot of tyrosine hydroxylase activity of (a) human adrenal medulla and (b) human pheochromocytoma as a function of cofactor concentration (millimolar) (open circles). Analysis of inhibition by 0.1 and 0.5 mM epinephrine (closed circles). Activity measured using assay 2.

reflect the existence of distinct types of tyrosine hydroxylases in different chromaffin cell tumors. Alternatively, it is possible that different conditions in the isolation and assay of the different enzymes could account for the results obtained, although, in our studies, several isolation and assay methods were analyzed, including the procedure used by Nagatsu et al. (5).

It thus appears that, in at least some pheochromocytomas (all six that we studied), the mechanism of elevated synthesis and secretion is not via a less sensitive end product feedback inhibition. Roth and co-workers (4) report that in unfortified low speed supernates the level of synthesis per gram of tissue is greater in the tumor than in adrenal preparations from the same patients. Since they did not fortify their system with pterin cofactor, explanations for this could be a higher concentration of enzyme or endogenous cofactor in the tumor tissue or less sensitivity of the tumor enzyme to feedback inhibition. A comparison of the levels of the enzyme in normal adrenal tissue and in two tumors (Table III) indicates that, at least in the tumors we examined, there was no elevation of enzyme levels. Tyrosine hydroxylase activity per gram of tissue is of the same order of magnitude in all the tissues studied. No estimates of pteridine levels have been reported in either normal human chromaffin tissue or pheochromocytoma.

A possible mechanism for the elevated synthesis could be an abnormal distribution of TH relative to the chromaffin granule. Our results (Table I) indicate, however, that TH is similarly located in both the tumor and normal human chromaffin tissue. The localization of the enzyme in the cytosol agrees with that reported for other chromaffin tissues (13).

The finding of catecholamine/ATP ratios in chromaffin granules isolated from tumors which are higher than those found in chromaffin granules from normal adrenal tissue (17, 18) indicates a mechanism of storage different from that characteristic of amines in normal chromaffin granules. The amines in the latter granules are believed to be bound in a stoichiometric relationship to adenine nucleotides. Catecholamines may be taken up more readily and/or bound more tightly by the granules of the tumor tissue. The suggestion that the synthesis rate may be related to an exaggerated uptake and storage mechanism in these granules should be reconsidered since smaller amounts of the amine would be left in the soluble portion of the cell where tyrosine hydroxylase is located and where increased synthesis would occur as a result of reduced levels of the end products capable of inhibiting TH (19, 20).

Lower inhibition by catecholamines of pheochromocytoma tyrosine hydroxylase may be the cause of elevated synthesis in some, but not all, pheochromocytomas.

At present the mechanism of increased synthesis in the pheochromocytomas we have studied remains obscure. It is of course quite possible that the increased levels of circulating catecholamines in patients with pheochromocytomas could be due merely to the increased mass of chromaffin tissue present in these individuals.

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