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PARTIAL SEPARATION AND PROPERTIES OF TYROSINE HYDROXYLASE FROM THE HUMAN PHEOCHROMOCYTOMA: EFFECT OF NOREPINEPHRINE

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SUMMARY

Tyrosine hydroxylase was partially purified from the human pheochromocytoma. Properties of the pheochromocytoma enzyme were similar to those of the bovine adrenal enzyme. The enzyme required tetrahydropteridine as a co-factor and was markedly activated by Fe^{2+}. Tyrosine hydroxylase isolated from the human pheochromocytoma was found to be less sensitive to the inhibition by norepinephrine than the enzyme from the bovine adrenal medulla, either in the presence or absence of Fe^{2+}. It is suggested that the uncontrolled excessive production of norepinephrine in the pheochromocytoma could be partly due to altered sensitivity of tyrosine hydroxylase to norepinephrine inhibition.

INTRODUCTION

Tyrosine hydroxylase[1], which catalyzes the conversion of tyrosine to DOPA, was found to be in the first and rate-limiting step in the biosynthesis of norepinephrine[2]. It was reported that in normal sympathetically innervated tissues such as the brain, heart, spleen and adrenal medulla, the biosynthesis of norepinephrine is regulated by the feed-back inhibition of tyrosine hydroxylase by norepinephrine[3–5].

Pheochromocytoma tissue is characterized by a high rate of synthesis and secretion of catecholamines, but it was recently reported that the high rate of release of catecholamines in the pheochromocytoma cannot be the result of defective storage[6]. Since the feed-back regulation at the tyrosine hydroxylase stage was known to be a main regulatory mechanism in the biosynthesis of norepinephrine in normal tissues, it was of interest to investigate whether or not tyrosine hydroxylase in the pheochromocytoma is susceptible to the end-product inhibition by norepinephrine. ROTH *et al.*[7] have reported that tyrosine hydroxylase from the pheochromocytoma was far less sensitive to the inhibition by catechol compounds than was the similar enzyme preparation from either normal human adrenal gland or bovine splenic nerve tissue.

Abbreviation: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

They used as an enzyme preparation the high-speed supernatant from the pheochromocytoma homogenate, which was treated with alumina in order to remove the soluble extra-granular catecholamines.

This communication describes the partial separation and properties of tyrosine hydroxylase from the pheochromocytoma and the altered sensitivity of the pheochromocytoma enzyme to the inhibition by catecholamines. Tyrosine hydroxylase of the bovine adrenal medulla was also isolated by the same procedure and used as a control enzyme from a normal tissue. A preliminary report was presented at the IVth International Congress on Pteridines[8].

MATERIALS AND METHODS

The pheochromocytoma tissues were kindly supplied by Dr. Torikai (Tohoku University, Sendai, Japan). Frozen tissues stored in a deep-freezer were used. Beef adrenals were obtained fresh, packed in ice from the slaughterhouse, and the medullary portions were dissected. They were stored frozen at −20°. Florisil was purchased from Floridin Co., and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) from Calbiochem.

Tyrosine hydroxylase activity was measured by a spectrofluorometric method[9], with a modification. The incubation mixture contained (in μmoles): acetate buffer (pH 6.0), 200; L-tyrosine, 0.1; mercaptoethanol, 100; $DMPH_4$, 1; an appropriate amount of the enzyme preparation and water to 1.0 ml. In some experiments, 2 μmoles of Fe^{2+} (freshly prepared $FeSO_4$ solution) were included in the incubation mixture. The incubation was carried out at 30° for 15 min in a metabolic shaker. For the blank incubation, D-tyrosine was used as substrate instead of L-tyrosine. Ten nmoles of DOPA were added to another blank incubation as internal standard. Reaction was stopped by the addition of 50 μl of glacial acetic acid and the incubation mixture was centrifuged in order to remove protein. The precipitate was washed with 1 ml of 2% acetic acid and centrifuged. The combined supernatant was passed through two columns fitted together sequentially; the top column contained Florisil (100/200 mesh, 0.6 cm × 4.0 cm), which had previously been washed with 2% acetic acid until the effluent was pH 4.0 (ref. 10), and the bottom column contained Amberlite CG-120 (Na^+) (Type I, 0.6 cm × 4.0 cm), which had previously been washed with 5 M NaOH and water. The effluent from the two columns was discarded. Both columns were washed successively with 5 ml of 2% acetic acid and the washings were discarded. The Amberlite CG-120 column was then separated and washed with 10 ml of water. DOPA was eluted with 10 ml of 0.1 M sodium acetate buffer (pH 6.5). To 2.0 ml of the eluate was added 0.1 ml of 0.25% $K_3Fe(CN)_6$. After 3 min, 1.0 ml of a mixture of 2% ascorbic acid–20% NaOH (1:9, v/v) was added and the resultant fluorescence was measured at 480 nm, with the excitation light at 356 nm, using an Aminco–Bowman spectrophotofluorometer. The DOPA formed enzymatically was calculated from the value of the internal standard by means of the following equation:

$$\frac{F\,(\mathrm{L}) - F\,(\mathrm{D})}{F\,(\mathrm{D} + \mathrm{IS}) - F\,(\mathrm{D})} \times 10 \text{ nmoles}$$

where $F(\mathrm{L})$ = reading of L-tyrosine incubation, $F(\mathrm{D})$ = reading of D-tyrosine incu-

bation and $F(D + IS)$ = reading of D-tyrosine *plus* DOPA (internal standard, 10 nmoles) incubation.

Catecholamines were assayed according to the method of A. F. HOGANS (a sensitive method for the simultaneous estimation of norepinephrine and dopamine in tissue, personal communication), with a slight modification. An aliquot of the homogenate of the pheochromocytoma was deproteinized with 5% trichloroacetic acid. 10-μl aliquots of the supernatant (equivalent to 2 mg of the tissue) were pipetted into four tubes. For the assay of dopamine and norepinephrine, 2 ml of phosphate buffer (0.1 M, pH 6.5) and 1.0 ml of 4% EDTA were added to two tubes (experimental and blank). For the assay of epinephrine, 2 ml of phosphate buffer, adjusted to pH 3.5 with glacial acetic acid, and 1.0 ml of 4% EDTA were added to the other two tubes (experimental and blank). To each tube, 0.2 ml of iodine solution (4.8 g KI and 0.25 g I_2 in 100 ml water) was added. Exactly 2 min later 0.5 ml of alkaline sulfite (5 ml of 12.6% anhydrous Na_2SO_3 made up to 25 ml with 5 M NaOH) was added. For the blanks, the additions of iodine and alkaline sulfite were reversed. Exactly 2 min later 0.6 ml of 5 M acetic acid was added. The solutions were heated in a boiling water bath for 5 min, then cooled rapidly by immersion in cold water. The intensities of fluorescence in the resulting solutions were determined in an Aminco-Bowman spectrophotofluorometer at the following wave lengths (excitation (nm)/fluorescence (nm)): dopamine and norepinephrine tubes, 310/365 and 385/480; epinephrine tubes, 410/500. Samples of 0.1 μg of dopamine, norepinephrine and epinephrine in 10 μl of 0.01 M HCl in two tubes (experimental and blank) were carried through the entire reaction.

The concentrations of dopamine (D), norepinephrine (N) and epinephrine (E) in the tissue sample are estimated differentially by solving the following simultaneous equations.

$$\frac{F(D365)}{0.1}\,D = F(S365),\ \frac{F(N480)}{0.1}\,N + \frac{F(E480)}{0.1}\,E = F(S480),\ \frac{F(N500)}{0.1}\,N +$$

$$\frac{F(E500)}{0.1}\,E = F(S500)$$

where: (blanks were subtracted from all values), $F(D365)$ = reading of 0.1 μg dopamine at 365 nm (sample oxidized at pH 6.5); $F(N480)$ = reading of 0.1 μg norepinephrine at 480 nm (sample oxidized at pH 6.5); $F(E480)$ = reading of 0.1 μg epinephrine at 480 nm (sample oxidized at pH 6.5); $F(N500)$ = reading of 0.1 μg norepinephrine at 500 nm (sample oxidized at pH 3.5). This final value was small. $F(E500)$ = reading of 0.1 μg epinephrine at 500 nm (sample oxidized at pH 3.5); $F(S365)$ = reading of sample at 365 nm (sample oxidized at pH 6.5); $F(S480)$ = reading of sample at 480 nm (sample oxidized at pH 6.5); $F(S500)$ = reading of sample at 500 nm (sample oxidized at pH 3.5).

DOPA interfered in the assay of dopamine. The value of dopamine obtained here is the sum of dopamine and dopa. However, the amount of DOPA in pheochromocytoma tissue is generally very low[11].

Polyacrylamide-gel electrophoresis was carried out according to the method of DAVIS[12].

RESULTS

Partial separation of tyrosine hydroxylase from the human pheochromocytoma and the bovine adrenal medulla

All steps were carried out at 0–4°. All buffers were prepared by dilution of 1 M potassium phosphate, pH 7.5, to the desired concentration. Dialysis was performed overnight against at least a 40-fold excess of the same buffer, which was changed twice. Precipitates were collected by centrifugation for 20 min at 15 000 × *g*.

Fractionation with $(NH_4)_2SO_4$. Tissues were homogenized twice with 2 vol. of 0.1 M buffer in an Ultra Turrax homogenizer for 1 min. The homogenate was centrifuged at 100 000 × *g* for 60 min and the supernatant was carefully removed. To each 100 ml of the supernatant, 67 ml of saturated $(NH_4)_2SO_4$ solution (adjusted to pH 7.5 with NH_4OH) were added dropwise with constant mixing (40% satn.). The suspension was stirred for 20 min and centrifuged. The precipitate was dissolved in 5 mM buffer (100 ml per 100 g starting material) and dialyzed against 5 mM buffer. To each 100 ml of the dialyzed solution, 33 ml of saturated $(NH_4)_2SO_4$ solution was added (25% satn.) and after 20 min of mixing the solution was centrifuged. To the supernatant, 19 ml of saturated $(NH_4)_2SO_4$ solution was added (35% satn.) and after a further 20 min of mixing, the solution was again centrifuged and the supernatant discarded. The precipitate was dissolved in 5 mM buffer (75 ml per 100 g of the starting material). The colour of the enzyme solution from the pheochromocytoma at this stage was brown.

Charcoal treatment[13]. To each 20 ml of the solution was added 0.5 g of charcoal. The suspension was stirred for 30 min and the charcoal was removed by centrifugation. The solution was dialyzed against 5 mM buffer and then centrifuged whereupon the supernatant containing the enzyme activity became nearly colorless.

Hydroxyapatite column. Hydroxyapatite (1 g for 100 mg protein) and powdered cellulose (1:2, w/w) were mixed well, equilibrated with 5 mM buffer and packed in a column (1.2 cm diameter). After application of the enzyme solution onto the column, gradient elution was performed with 50 ml increments of phosphate buffer of increasing concentration (5, 25, 50, 70, 100 and 400 mM). Peaks of tyrosine hydroxylase activity from the bovine adrenal medulla appeared at 5 mM and 50 mM. Specific activity in the 50-mM fraction was increased about 4-fold. Most of the enzyme

TABLE I

PURIFICATION OF TYROSINE HYDROXYLASE FROM THE PHEOCHROMOCYTOMA

Purification step	*Protein (mg)*	*Total activity (nmoles/min)*	*Specific activity (nmoles/min per mg protein)*
Homogenate*	1160	121	0.10
High-speed supernatant	857	117	0.14
$(NH_4)_2SO_4$ fractionation	64.6	360	5.6
Charcoal treatment	43.9	255	5.8
Hydroxyapatite column	4.4	88	20.0

* Pheochromocytoma tissue, Case 1, 9.3 g.

activity in the pheochromocytoma was eluted with 50 mM buffer. The method of isolation of the enzyme from a pheochromocytoma is summarized in Table I. Since there was a gain in units of enzyme activity during the procedure, especially at the $(NH_4)_2SO_4$ step, the actual degree of purification is unknown. The enzyme preparation after hydroxyapatite chromatography became unstable and the activity was lost gradually, even at $-20°$. Polyacrylamide-gel disc electrophoresis[12] of the pheochromocytoma enzyme showed three stained protein bands.

TABLE II

TYROSINE HYDROXYLASE ACTIVITIES AND CATECHOLAMINE CONCENTRATIONS IN THE PHEOCHROMOCYTOMAS

Pheochromocytoma	*Catecholamines (mg/g)*		*Tyrosine hydroxylase* (nmoles/min per mg protein)*
Case 1	Dopamine	0.067	5.8
	Norepinephrine	6.02	
	Epinephrine	0.922	
Case 2	Dopamine	0.073	0.63
	Norepinephrine	0.485	
	Epinephrine	0.770	

* Enzyme preparation after charcoal treatment.

Catecholamine concentrations and tyrosine hydroxylase activities in the pheochromocytomas

As shown in Table II, the pheochromocytoma which had the higher catecholamine concentrations showed the higher tyrosine hydroxylase activity.

Properties of tyrosine hydroxylase separated from the pheochromocytoma

Properties of tyrosine hydroxylase separated from the pheochromocytoma were found to be similar to those of the enzyme from the bovine adrenal medulla[1]. The pheochromocytoma enzyme required tetrahydropteridine as co-factor; in the absence of tetrahydropteridine, no enzyme activity was observed. The addition of mercaptoethanol which protects the pteridine co-factor from aerobic oxidation, was necessary in order to attain the maximal activity.

A characteristic property of tyrosine hydroxylase isolated from the pheochromocytoma was a marked (2–5-fold) activation by Fe^{2+} (Table III). Bovine adrenal enzyme also showed a slight activation on the addition of Fe^{2+}, but the degree of stimulation by Fe^{2+} was considerably different with each batch of the adrenal enzymes, as shown in Table III.

The effect of norepinephrine on tyrosine hydroxylase separated from the pheochromocytoma was investigated. Since the enzyme activity became unstable after hydroxyapatite column chromatography, most of the following experiments were carried out on the preparation after charcoal treatment. As shown in Fig. 1, the enzyme from the bovine adrenal medulla was inhibited significantly by norepinephrine at concentrations between 0.1 and 1 mM. This result agreed with those of

TABLE III

EFFECT OF Fe^{2+} ON TYROSINE HYDROXYLASE FROM THE HUMAN PHEOCHROMOCYTOMA OR THE BOVINE ADRENAL MEDULLA

Fe^{2+} (mM)	*Percent of control activity*					
	Adrenal enzyme				*Pheochromocytoma enzyme*	
	No. 1	*No. 2*	*No. 3*	*No. 4*	*No. 1*	*No. 2*
0 (control)	100	100	100	100	100	100
2	84	109	113	143	495	240

previous reports[1,14]. In contrast, tyrosine hydroxylase from the pheochromocytoma was less sensitive to the inhibition by norepinephrine. Tyrosine hydroxylase from the pheochromocytoma was activated by norepinephrine at low concentrations around 0.1 mM and inhibited at high concentrations to a lesser extent than was the adrenal enzyme (Fig. 1).

Kinetic studies on the effect of norepinephrine on tyrosine hydroxylase from the bovine adrenal medulla and from the pheochromocytoma were carried out. As shown in Fig. 2, norepinephrine inhibited adrenal tyrosine hydroxylase in competition with $DMPH_4$. This agreed with the results of a previous report[14]. The K_m value of $DMPH_4$ was calculated to be 1 mM and the K_i value of norepinephrine 0.1 mM. Tyrosine hydroxylase from the pheochromocytoma was also inhibited by norepinephrine in competition with $DMPH_4$, but to a lesser extent. Substrate inhibition was observed at concentrations of $DMPH_4$ higher than 0.5 mM (Fig. 3). The apparent K_m value of $DMPH_4$ was 0.4 mM and the K_i value of norepinephrine 0.3 mM. The substrate inhibition was removed by the presence of low concentrations of norepine-

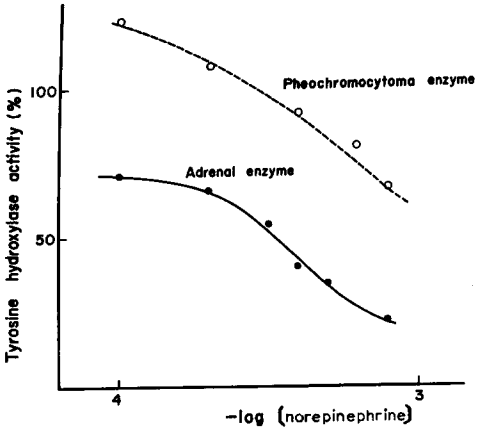
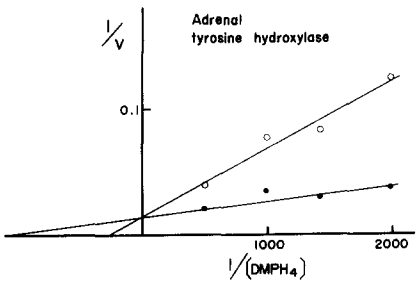


Fig. 1. Effect of norepinephrine on tyrosine hydroxylases from the bovine adrenal medulla and from the human pheochromocytoma in the absence of Fe^{2+}. Standard incubation mixture including 1 mM of $DMPH_4$ was used. The enzyme used was from the charcoal treatment stage. The assay was carried out as described in MATERIALS AND METHODS.



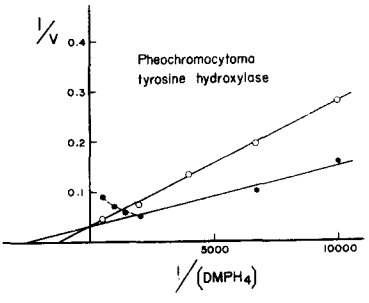


Fig. 2. Lineweaver–Burk plot of $DMPH_4$ concentration against tyrosine hydroxylase activity of the bovine adrenal medulla with and without norepinephrine, 0.3 mM. The enzyme used was from the charcoal streatment step. The assay was carried out as described in MATERIALS AND METHODS. The velocities are expressed as nmoles of DOPA formed per 15 min. The substrate concentration is expressed in moles. ●—●, enzyme alone; ○—○, enzyme with 0.3 mM norepinephrine.

Fig. 3. Lineweaver–Burk plot of $DMPH_4$ concentration against tyrosine hydroxylase activity of the pheochromocytoma with and without norepinephrine, 0.3 mM. The enzyme used was from the charcoal treatment step. The assay was carried out as described in MATERIALS AND METHODS. The velocities are expressed as nmoles of DOPA formed per 15 min. The substrate concentration is expressed in moles. ●—●, enzyme alone; ○—○, enzyme with 0.3 mM norepinephrine.

phrine. Therefore, activation by norepinephrine was observed at higher concentrations of $DMPH_4$.

Since tyrosine hydroxylase of the pheochromocytoma was markedly activated by Fe^{2+} (Table III), the effect of norepinephrine on the enzyme was re-examined in the presence of Fe^{2+} (2 mM). When high concentrations of catecholamines were added in the presence of Fe^{2+}, a purple color appeared, due to the formation of complexes between added Fe^{2+} and the catechols. However, as shown in Table IV, at concentrations of between 0.1 mM and 0.5 mM, the pheochromocytoma enzyme was also found to be less sensitive to the inhibition of norepinephrine just as in the absence of Fe^{2+}. Therefore, the possibility that the lower inhibition of the pheochromocytoma enzyme by norepinephrine is due to a deficiency in endogenous Fe^{2+} bound to the

TABLE IV

EFFECT OF NOREPINEPHRINE ON TYROSINE HYDROXYLASE OF THE PHEOCHROMOCYTOMA IN THE PRESENCE OF Fe^{2+}

Catecholamine	*Concn. (mM)*	*Percent of control activity**	
		Adrenal enzyme	*Pheochromocytoma enzyme*
Control	–	100	100
Norepinephrine	0.1	78	139
	0.2	54	140
	0.3	62	125
	0.5	25	42
Epinephrine	0.2	81	124

* Fe^{2+}, 2 mM; $DMPH_4$, 1 mM.

enzyme protein is not likely. The pheochromocytoma enzyme was also insensitive to the inhibition by epinephrine (Table IV).

DISCUSSION

Tyrosine hydroxylase was partially separated from the human pheochromocytoma. Since the total number of units of enzyme activity increased during the purification procedure, especially at the $(NH_4)_2SO_4$ fractionation step, the real degree of purification is unknown. However, this enzyme must still be crude, as judged by the increase in total units and specific activities (Table I), as well as by the results of polyacrylamide-gel electrophoresis.

It appears that the pheochromocytoma containing high concentrations of catecholamines also possessed a high tyrosine hydroxylase activity (Table II).

Tyrosine hydroxylase in the pheochromocytoma could be purified by procedures similar to those used for the purification of the same enzyme from bovine adrenal medulla. The properties of the pheochromocytoma enzyme were similar to those of the adrenal enzymes. The pheochromocytoma enzyme also required tetrahydropteridine as a co-factor.

One difference noted between tyrosine hydroxylase preparations from the adrenal medulla and those from the pheochromocytoma was the degree of stimulation by Fe^{2+}. The pheochromocytoma enzyme was markedly activated by Fe^{2+}. As shown in Table III, a 1.0–1.5-fold activation was observed in tyrosine hydroxylase preparations from the bovine adrenal medulla, whereas a 2.4–5-fold activation occurred in the pheochromocytoma enzymes.

The pheochromocytoma enzyme was found to be less sensitive to inhibition by norepinephrine (Fig. 1). At a concentration of 0.1 mM of norepinephrine, the pheochromocytoma enzyme showed activation rather than inhibition. The K_m value of $DMPH_4$ for the pheochromocytoma enzyme was lower than that for the adrenal enzyme, whereas the K_i value of norepinephrine for the pheochromocytoma enzyme was higher than that for the adrenal enzyme. The pheochromocytoma enzyme, therefore, appears to have a high affinity for $DMPH_4$ and a low affinity for norepinephrine. The pheochromocytoma enzyme was inhibited by $DMPH_4$ at concentrations higher than 0.5 mM, whereas the bovine adrenal enzyme was inhibited at concentrations higher than 2 mM. Norepinephrine at low concentrations (0.1 mM) reversed the inhibition of the pheochromocytoma enzyme caused by a high concentration of pteridine, and stimulated the activity. The possibility that this insensitivity to norepinephrine is due to a deficiency in endogenous Fe^{2+} bound to the enzyme is not likely, since the insensitivity was observed equally as well in the presence of 2 mM of Fe^{2+} (Table IV).

This difference in the sensitivity to norepinephrine inhibition may be caused by the conformational alteration of the enzyme protein. Preliminary results have indicated that the molecular weights of both tyrosine hydroxylases of the adrenal medulla and the pheochromocytoma are similar, when measured by the gel-filtration method.

Since the pheochromocytoma enzyme was still crude, the lower inhibition by norepinephrine may be due to other components in the enzyme preparation and may not be a characteristic of the pheochromocytoma enzyme. In the case of the bovine

adrenal enzyme, the degree of inhibition by norepinephrine did not change significantly with the enzyme preparations of different purities. However, a higher purification of the pheochromocytoma enzyme is necessary before a definite conclusion may be drawn. Another possibility for the differences between the bovine adrenal and the pheochromocytoma enzymes is that a species difference might be involved. ROTH *et al.*[7] reported that the human adrenal enzyme is as sensitive to norepinephrine inhibition as is the bovine splenic nerve enzyme. However, in order to exclude the possibility of species differences, the human adrenal enzyme would be a better control for the pheochromocytoma enzyme.

If the lower inhibition by norepinephrine of the pheochromocytoma tyrosine hydroxylase is a characteristic of the enzyme *in vivo*, the uncontrolled excessive production of norepinephrine in pheochromocytoma tissue could be accounted for, at least partly, by the lack of feed-back inhibition at the tyrosine hydroxylase stage.

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