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Purification and Characterization of Tyrosine Hydroxylase from a Clonal Pheochromocytoma Cell Line

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SUMMARY

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Tyrosine hydroxylase (TH) was purified from pheochromocytoma PC-12 cloned cells by a short and gentle procedure. The enzyme was isolated in pure form and has a molecular weight of approximately 210,000–220,000. SDS electrophoresis yields one single protein band with a molecular weight of approximately 62,000. Antiserum to rat pheochromocytoma TH was obtained in rabbits and immunotitration data show that the antiserum to rat TH reduces the activity of the homologous enzyme more effectively than the activity of the heterologous enzyme. The activity of purified TH is stimulated by a cAMP-dependent protein kinase phosphorylating system (PKP system), and the highest percentage of stimulation is obtained when the enzyme activity is measured at physiological pH's. The stimulation of the purified enzyme by the PKP system results in a reduction of the apparent K_m for the cofactor 6-MePH$_4$ and in an increase of the K_i for dopamine. Incubation of purified TH with the PKP system and [^{32}P]ATP, resulted in incorporation of radioactivity into the 62,000 subunit of the enzyme.

INTRODUCTION

Pheochromocytoma tumors are characterized by the presence of high levels of catecholamine synthesizing enzymes and catecholamines. The availability of a clonal cell line of rat pheochromocytoma (1, 2) provides a useful source for isolation and characterization of catecholamine synthesizing enzymes. Tyrosine hydroxylase (TH)[4] catalyzes the initial step in the biosynthesis of catecholamines, and the enzyme derived from bovine adrenal particles by treatment with digestive enzymes was partially purified (3, 4). However, the native adrenal TH (not treated with digestive enzymes) tends to aggregate and this hampered its extensive purification. Recently we have purified TH from human pheochromocytoma tumors and we have obtained specific antibodies to purified human enzyme (5). The loss of immunocrossreactivity between species could limit the usefulness of heterologous systems for immunochemical and immunohistochemical studies. Since the rat is being frequently utilized as an experimental animal we have purified TH from PC-12 clonal cell lines which were derived from a transplantable rat adrenal medullary pheochromocytoma (6, 7). In this paper we describe the purification and properties of rat pheochromocytoma TH as well as the immunocrossreactivity of rabbit anti-rat TH with TH from various species.

METHODS

Pheochromocytoma PC-12 cells were cultured in 75 cm plastic tissue culture flasks (Falcon Plastics) in medium consisting of 85% RPMI 1640 (Gibco) 10% heat-inactivated horse serum, 5% fetal calf serum, 5% units/ml of penicillin, and 25 μg/ml of streptomycin. Cells were maintained at 37° in an atmosphere of 95% air and 5% CO_2. The medium was changed three times a week. The cells were grown to confluency and harvested by mechanical agitation. Each flask contained approximately 15–20 × 10^6 cells and for each purification procedure 100–150 flasks were used. The purification procedure was carried out at 4°.

For purification of TH the cells were sonicated in potassium phosphate buffer (KP-buffer) (20 mM, pH 6.5) containing 0.25 sucrose and centrifuged for 20 min at

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[4] The abbreviations used are: TH, tyrosine hydroxylate; DA, dopamine.

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45,000 *g*. Sucrose was added to protect the enzyme activity from inactivation following sonication. The enzyme was precipitated from the supernatant fraction by addition of $(NH_4)_2SO_4$ to 80% saturation. After dialysis this fraction was brought to 25% saturation with $(NH_4)_2SO_4$ and the precipitate removed by centrifugation. The supernatant fraction was brought to 35% saturation with $(NH_4)_2SO_4$, the solution centrifuged and the supernatant discarded. The 25–35% pellet was dissolved in a minimum volume of 20 mM KP-buffer, pH 6.5, and this solution (40 mg protein) was applied to a Sepharose 4B column (26 × 1.6 cm). The Sepharose 4B column was preequilibrated with 20 mM KP-buffer, pH 6.5, and the enzyme was eluted with the same buffer at a flow rate of 8 ml/hr. Fractions of 0.75 ml were collected and those with highest specific activity were pooled and applied to linear sucrose density gradient (5–20%) prepared in KP-buffer (20 mM, pH 7.6) containing 10 mM 2-mercaptoethanol. Centrifugation and fractionation were performed as previously described (8). Purified TH is referred to as enzyme preparation obtained following sucrose density gradient step.

Protein was determined by the procedure of Lowry *et al.* (9) or by the microassay of Bradford (10).

Enzyme assay. TH activity was determined by the method of Nagatsu *et al.* (11). The enzyme was incubated if not otherwise stated for 10 min in a mixture containing the following components: 200 mM Tris–acetate buffer, pH 6.0, 37.5 mM 2-mercaptoethanol, 1.0 mM 6-methyltetrahydropteridine (6-MePH$_4$), 1.4 mM ferrous sulfate, and 100 μM L-[3,5-^{3}H]tyrosine (specific activity 1 μCi/50 nmol).

The reaction was stopped by addition of 200 μl 0.1 N NaOH and the reaction mixture was passed through an ion exchange column. The column (0.5 × 1.5 cm in a disposable pipet) consisted of equal volumes of cation exchange resin (Bio-Rad Ag 50W-X8; 200–400 mesh; hydrogen form, lower layer) and of ion exchange resin (Bio-Rad 9; 200–400 mesh; chloride form, upper layer). The reaction mixture was passed through the column and then 0.5 and 1.0 ml of H_2O were passed. Tritiated water was measured in the eluate (11). The enzyme activity is expressed in units; one unit is the amount which catalyzes the formation of 1 μmol of product per hour under standard assay conditions. The apparent K_m values were calculated from the Lineweaver–Burk plot and the K_i values were estimated by the method of Dixon (12). Kinetic data were computed by fitting the data to linear functions by the method of least square analysis.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out by the method of Davis (13) and Ornstein (14). TH activity could be detected on ammonium persulfate catalyzed gels only if the gels were preelectrophoresed or prepared at least one day in advance. Sodium dodecyl sulfate (SDS)–gel electrophoresis was performed in slab gels on the LKB 2117 Multiphor according to the method of Weber and Osborne (15). A protein standard prepared for SDS electrophoresis by Bio-Rad was used for calculating the molecular weights of TH subunits. This preparation contained myosin, β-galastosidase, phosphorylase B, BSA, and ovalbumin.

Isoelectric focusing. The isoelectric point of TH was determined by using LKB Ampholine PAG plates with a pH range of 3.5–9.5, based on the procedure of Vesterberg (16). The pH in the focused gel was measured by placing gel sections (0.5–1.0 mm) in 1 ml of boiled deionized water, homogenizing, and reading the pH 1 hr later on the expanded scale of the pH meter.

Determination of sedimentation coefficients. Linear sucrose density gradients were used for determination of sedimentation coefficients and estimation of molecular weights according to the method of Martin and Ames (17).

Protein standards employed in the sucrose gradients were dissolved in 20 mM KP-buffer, pH 7.6, and mixed with the enzyme preparation. The following protein standards were used: fumarase (from pig heart, Sigma Chemical Co.), γ-globulin (human, Schwarz/Mann), and catalase (bovine, Sigma Chemical Co.).

Immunization of rabbits. About 0.2 mg of purified TH was subjected to disc gel electrophoresis, loading approximately 50 μg of protein on each gel. The protein band was cut out of the gel and homogenized in an equal volume of 0.9% NaCl. After mixing with Freund's adjuvant, the preparation was administered intradermally and intramuscularly to New Zealand white rabbits. Immunization was repeated every 3 weeks until a sufficiently high titer was obtained. Thereafter serum was collected monthly until the titer declined, at which time a booster was administered.

Purification of beef heart protein kinase catalytic subunit. The catalytic subunit of protein kinase was purified according to the procedure of Soderling *et al.* (18). The specific activity of the purified protein kinase was 175 nmol/mg/min at 30° using mixed calf thymus histones (Type IIA, Sigma) as substrates.

Phosphorylation. The effects of phosphorylation on the enzymatic activity and on kinetic parameters were investigated in presence of cAMP-dependent protein kinase (bovine heart, Sigma) while the incorporation of ^{32}P was investigated in presence of purified protein kinase catalytic subunit.

The stimulation of TH activity by phosphorylation was studied in purified enzyme preparations. The enzyme was preincubated for 10 min at 30° in the presence of 40 mM KP-buffer, pH 6.5, 10 mM Mg^{2+}, 0.5 mM ATP, 0.2 mM cAMP, and 40 μg of cAMP-dependent protein kinase.

The tyrosine hydroxylase reaction was subsequently carried out by incubating for 5 min at 37° in a mixture containing 200 mM Tris–acetate buffer at either pH 6.0 or pH 7.2, and all the other components listed for the standard TH assay. The concentration of all the components was the same as in the standard assay but the concentration of 6-MePH$_4$ was only 0.1 mM.

The incorporation of $^{32}PO_4$ into TH was carried out in a mixture containing the following components: purified TH containing 5 μg enzyme protein, 0.5 mM [^{32}P]ATP (with a specific activity of 1 μCi/nmol), 10 mM Mg^{2+}, 2 μg purified protein kinase catalytic subunit. The mixture (200 μl) was incubated for 5 min at 30° and the reaction was terminated by addition of 0.01 M KP-buffer (pH 7.0) containing 1% SDS and 10% 2-mercaptoethanol. After placing the reaction mixture for 2 min in a boiling water bath, an aliquot was applied to a 5% SDS–polyacrylamide slab gel for electrophoresis. The gels were stained and

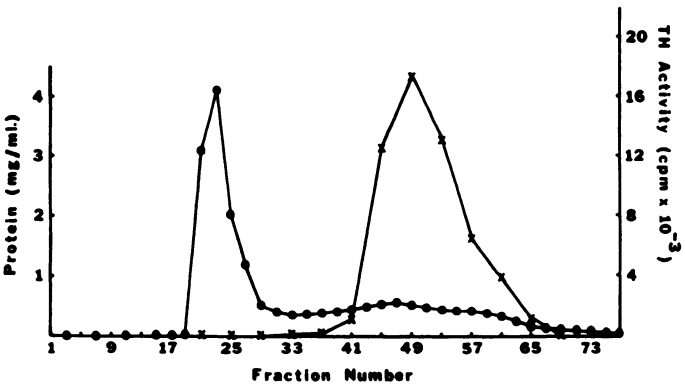


FIG. 1. *Elution profile of TH from Sepharose 4b column*
(—●—) Protein, (—×—) TH activity. The enzyme activity is expressed as cpm/10 μl of eluate per 5 min of incubation time (see METHODS).

TABLE 1
Purification of tyrosine hydroxylase from cultured pheochromocytoma PC-12 cells

Purification step	Total protein	Total[a] activity	Specific activity	Apparent yield
	(mg)	*(units)*	*(units/mg protein)*	*(%)*
$(NH_4)_2SO_4$, 80% ppt	256	71	0.27	—
$(NH_4)_2SO_4$, 25–35% ppt	45	39	0.86	55
Sepharose 4B column	2.1	25	11.4	35
Sucrose density gradient	0.3[b]	6.7	22.4	9

[a] Units are expressed as μmol of product formed for 60 min assay (tyrosine = 10^{-4} M; 6-MePH$_4$ = 10^{-3} M; pH = 6.0).
[b] The protein concentration was low in this fraction and therefore this value is approximate.

cut into sections for measuring radioactivity in a scintillation counter. Purified protein kinase catalytic subunit and purified TH were electrophoresed on the same gel as markers.

RESULTS

Purification of the enzyme. A summary of the purification of TH from rat pheochromocytoma PC-12 cells is shown in Table 1. Following $(NH_4)_2SO_4$ fractionation the enzyme was further purified on a Sepharose 4B column (Fig. 1) and by centrifugation on a linear sucrose density gradient. The overall purification was about 80-fold with an apparent yield of approximately 9%. Initially we concentrated the enzyme in a dialysis tube surrounded by Sephadex G-200 but the concentrated protein lost enzymatic activity within 48 hr. Subsequently, we have omitted the concentration of the enzyme after the elution from the Sepharose 4B column, and stored the diluted enzyme preparation following sucrose density gradient centrifugation at 4°. No appreciable loss of enzyme activity was observed for at least 2 weeks. The total purification procedure could be accomplished in 3 days yielding about 0.3 mg of TH protein.

Electrophoretic and immunoelectrophoretic studies. Polyacrylamide disc gel electrophoresis of enzyme preparation obtained after sucrose gradient centrifugation revealed a single stained protein band. The enzyme activity was associated with the protein stained section of the gel. The immunoelectrophoretic analysis of rat TH antiserum against purified rat pheochromocytoma TH or against partially purified rat pheochromoctyoma TH [after $(NH_4)_2SO_4$ fractionation] showed a single precipitin arc (Fig. 2).

Molecular weight determination. To determine the average sedimentation coefficient of TH, the enzyme as well as various standard proteins were centrifuged on a linear sucrose density gradient. The molecular weight was calculated from the sedimentation coefficient (Fig. 3). The molecular weight of the enzyme was found to be approximately 210,000–220,000.

SDS-Polyacrylamide gel electrophoresis (Fig. 4) shows that the purified enzyme consists of a single protein subunit with a molecular weight approximately equivalent to 62,000.

Isoelectric focusing. Isoelectric focusing of purified TH revealed a single staining protein band that coincided with TH activity. The isoelectric point was determined from three separate experiments and it was found to be 5.3 ± 0.1 at 4°.

Immunocrossreactivity and enzyme inhibition studies with antiserum. On Ouchterlony immunodiffusion a single precipitin line was evident when rat anti-TH was run against partially purified concentrated rat pheochromocytoma TH or human pheochromocytoma TH.

The decrease of enzymatic activity of six THs obtained from different species and tissues by the anti-rat TH is shown in Table 2. A typical dose-dependent relationship between enzyme activity and amount of antiserum was observed. The rat pheochromocytoma TH activity was decreased by approximately 50% with 1.1 μl of antiserum and by approximately 95% with 2.0 μl of antiserum. A larger quantity of antiserum was needed to reach the equivalence point for human, bovine, or mouse enzyme than for rat enzyme. The amount of antiserum required to produce a comparable reduction of rat pheochromocytoma TH compared to rat striatum TH was almost the same. Similarly the amount of antiserum needed to pro-

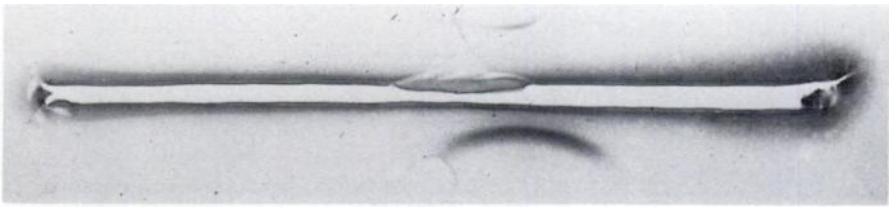


FIG. 2. *Immunoelectrophoretic analysis of purified (upper; 4 μg of protein) and crude (lower; 100 μg of protein) pheochromocytoma TH*
The buffer was barbital–Tris–glycine, pH 8.8 (29). The anode is at left.

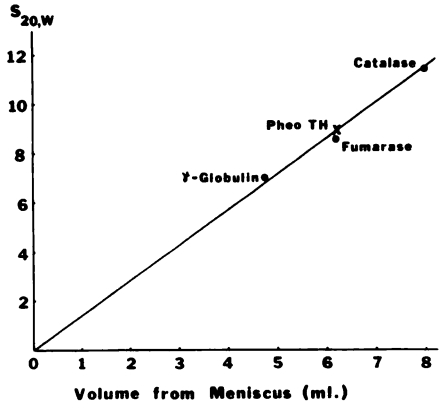


FIG. 3. *Determination of the sedimentation coefficient ($s_{20,w}$) of TH by sucrose density gradient centrifugation (5–20%)*

The results are the means from three experiments ± a SEM of 2–5%. Sucrose gradient was performed in a 13-ml gradient solution of 5–20% sucrose in 20 mM K-P buffer, pH 7.6, containing 10 mM 2-mercaptoethanol. After centrifugation at 40,000 rpm in a Spinco SW 40 rotor for 16 hr at 4°, tubes were punctured from the bottom and fractions of approximately 0.6 ml were collected. The calibrating enzymes and proteins were determined by standard procedures. TH activity was associated with two fractions in the middle of the gradient.

duce a comparable reduction of human pheochromocytoma TH as compared to human striatum TH was also almost the same.

Stimulation by a cAMP-dependent protein kinase phosphorylating system (PKP system). The addition of cAMP-dependent protein kinase, cAMP, Mg^{2+}, and ATP (PKP system) to purified enzyme results in stimulation of TH activity (Table 3). The enzyme activity was not stimulated when cAMP, ATP, or protein kinase was omitted from the incubation mixture.

The percentage stimulation is higher when TH activity is measured at pH 7.2 than at pH 6.0. The phosphorylated enzyme has a broad pH optimum and its enzyme activity is essentially the same at pH 6.0 and at pH 7.2. The activity of the phosphorylated enzyme is higher at pH 7.2 than the activity of the nonphosphorylated enzyme at its pH optimum, pH 6.0.

The addition of dopamine (DA) to the incubation mixture results in an inhibition of the activity of the basal and phosphorylated TH. The percentage stimulation of TH activity by the PKP system is higher in the presence of DA than in the absence of DA. It is also evident from the data in Table 3 that DA inhibits less effectively the activity of the phosphorylated enzyme than the activity of the basal enzyme. In a separate experiment, the DA inhibition constants (K_i) were determined with Dixon plots at pH 6.0 for the basal enzyme and for the phosphorylated enzyme. Phosphorylation increased in the K_i for DA greater than sevenfold. The K_i for DA was found to change from 10.0 ± 1.0 μM for the basal enzyme to 78.0 ± 5.0 μM for the phosphorylated enzyme.

The effect of phosphorylation on the K_m for the pteridine cofactor, 6-$MePH_4$, was also investigated. The results presented in Figs. 5 and 6 show that phosphorylation results in a reduction of the apparent K_m for 6-$MePH_4$. The apparent K_m for 6-$MePH_4$ is higher when the basal enzyme activity is determined at pH 7.2 than at pH 6.0. It is also evident from the data in Figs. 5 and 6 that, upon phosphorylation, the change in the K_m for 6-$MePH_4$ is greater when the enzyme activity is determined at pH 7.2 than at pH 6.0.

Incorporation of ^{32}P to tyrosine hydroxylase. Results

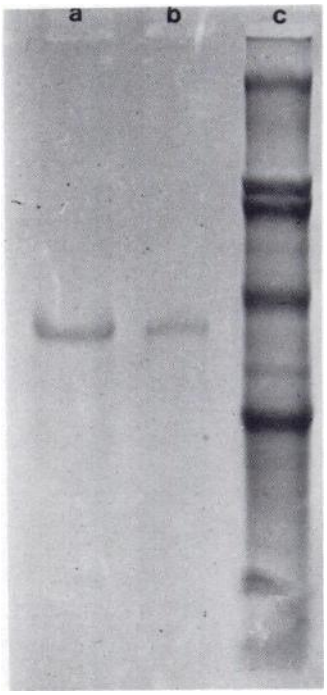


FIG. 4. *SDS gel electrophoretic pattern of purified tyrosine hydroxylase: (a) purified TH (20 μg); (b) purified TH (8 μg); (c) standard proteins.*

TABLE 2

Effect of rat anti-tyrosine hydroxylase on TH activity obtained from different species

Enzyme[a] source	Equivalence point[b]
	(μl ± SEM)
Rat pheochromocytoma	2.20 ± 0.10
Rat striatum	1.99 ± 0.05
Human pheochromocytoma	3.31 ± 0.05
Human striatum	3.06 ± 0.14
Bovine striatum	4.14 ± 0.15
Mouse striatum	4.31 ± 0.08

[a] The tissues were homogenized in KP-buffer (20 mM; pH 7.6) and centrifuged for 20 min at 45,000 *g*. The amount of TH in all tissues was adjusted by dilution with the KP-buffer containing 1% bovine serum albumin so that the activities from all supernatants were similar. The initial activity in each sample (50 μl) was approximately 0.18 unit. The immunochemical titrations were performed by adding varying amounts of immune and preimmune sera (in a final volume of 10 μl) to fixed aliquots of antigen. The reaction was incubated for 1 hr at room temperature, and following centrifugation for 10 min at 10,000 *g*, the supernatant fraction was assayed at pH 6.0 for enzyme activity.

[b] The equivalence point is expressed in μl of antiserum required for a decrease of 100% of enzyme activity, and it was determined by extrapolation from plots of enzyme activity versus volume of antiserum, using the method of least squares. The results are the means from three experiments ± SEM.

presented in Fig. 7 show that ^{32}P is incorporated into the 62,000 mol wt subunit of purified TH. The phosphorylation was dependent on the addition of the catalytic protein kinase subunit. Omission of the catalytic protein kinase subunit from the reaction mixture resulted in a complete loss of the ^{32}P incorporation. The stoichiometry of ^{32}P incorporation was found to vary with the specific activity of TH. Purified enzyme that has lost some activity upon storage incorporated less ^{32}P than the enzymes with a higher specific activity. Under our experimental conditions the highest incorporation achieved with the purified enzyme was approximately 1 mol of ^{32}P/mol TH (assuming the molecular weight of TH to be 240,000).

DISCUSSION

Pheochromocytoma PC-12 cells grown in uncoated tissue culture flasks and maintained at high cell density contain relatively high activity of TH (0.15 unit/mg protein). TH can be purified from these cells by a procedure that requires only four steps. The purified enzyme has a high specific activity and it appears to be homogeneous as judged by the analysis on polyacrylamide disc gel electrophoresis and on SDS–gel electrophoresis. It is noteworthy that on SDS–gel electrophoresis one single band was obtained with a mol wt of approximately 62,000, while it was recently reported that purified brain enzyme yields on SDS–gel electrophoresis three protein bands and purified enzyme from cultured cells of human neuroblastoma yields apparently a single protein band (19). Although the rat brain TH may have different subunits than rat pheochromocytoma TH, it is possible that the short and gentle procedure described in this study minimizes degradation of TH during the purification. Even in this procedure a part of the enzyme activity might be lost during the purification and therefore the calculated yield might be higher than the estimated one (see Table 1).

The purified TH was obtained in sufficient quantities for production of monospecific antibodies in rabbits. Since heterologous immunoreactions are generally weaker than homologous reactions it was of interest to determine the crossreactivity of rat pheochromocytoma anti-TH with TH from different species. The immunotitration data show that anti-rat TH inhibits more effectively the activity of rat pheochromocytoma or brain TH than the activity of TH from other species. Thus, anti-rat pheochromocytoma TH might be more sensitive and specific for immunohistochemical mapping of catecholamine neurons in rats than the previously used anti-bovine or human TH.

The findings that dibutyryl cAMP stimulates TH activity in striatal slices and synaptosomes as manifested by an increase in the biosynthesis of DA from tyrosine (20, 21) have prompted several studies on the stimulation of the enzyme activity (22–25). The stimulation of crude adrenal or brain TH by cAMP is mediated through a protein kinase system (PKP system) and causes a reduction of the apparent K_m value for the cofactor 6-methyltetrahydropteridine and an increase in the K_i for the end product dopamine (22–25). The kinetic changes observed

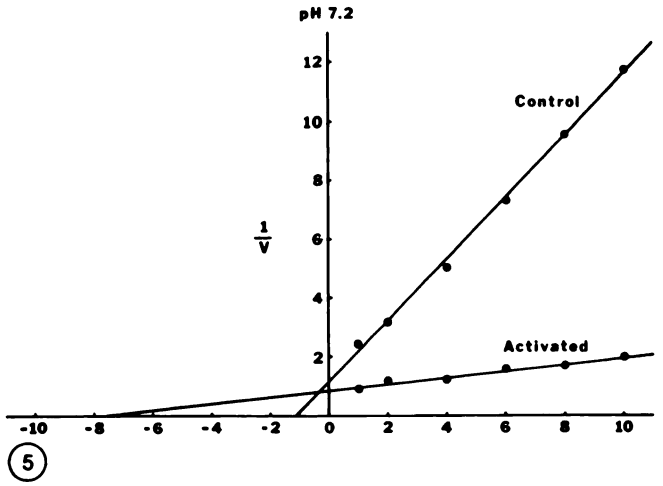
TABLE 3

Effect of pH and of DA on the stimulation of TH by the PKP system

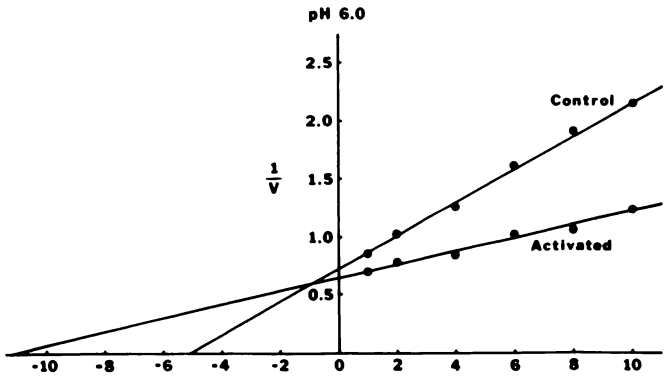
The enzyme activity is expressed as nanomoles of product formed per hour under the standard assay conditions per incubation. The results are the mean ± SEM from three experiments.

Incubation system	TH activity		Percentage stimulation	
	pH 6.0	pH 7.2	pH 6.0	pH 7.2
Control	4.50 ± 0.25	1.00 ± 0.08	—	—
PKP	7.95 ± 0.40	8.28 ± 0.55	75	728
Control + DA[a]	2.56 ± 0.10	0.28 ± 0.02	—	—
PKP + DA	5.75 ± 0.35	4.07 ± 0.25	125	1350

[a] The phosphorylation reaction was carried out as described in the text. DA, where indicated (1.5×10^{-5} M), was added to the incubation mixture together with all other components required for tyrosine hydroxylation.



(5)



(6)

FIGS. 5 and 6. *Lineweaver–Burk plots of control and PKP-activated TH assayed at either pH 7.2 (Fig. 5, top) or pH 6.0 (Fig. 6, bottom)*

The concentration of tyrosine was 100 μM, and the concentration of 6-$MePH_4$ was varied from 0.1 to 1.0 mM. Velocity is reported as nmol product formed per 5 min at 37°. Individual points represent the mean of duplicate samples from three separate experiments. Statistical analysis revealed a significant change in K_m for cofactor at both pH levels, while no significant changes occurred in V_{max} values. The apparent K_m for 6-$MePH_4$ at pH 6.0 was reduced from 0.20 ± 0.01 to 0.09 ± 0.01 mM and at pH 7.2 from 0.92 ± 0.07 to 0.14 ± 0.02 mM.

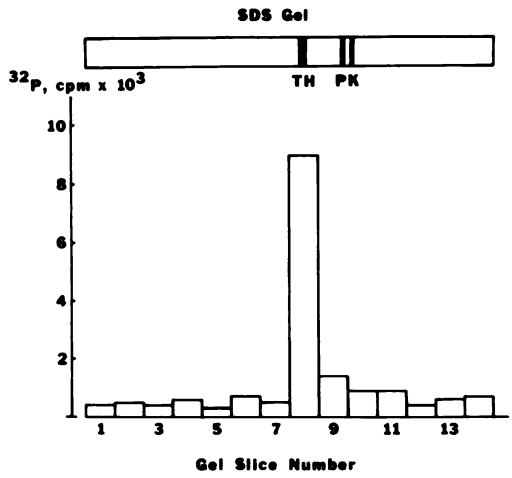


FIG. 7. *$^{32}PO_4$ incorporation into purified TH by the PKP system* ^{32}P was detected only in a single protein band corresponding to the 62,000 mol wt subunit of TH. The ^{32}P was measured in 0.5-cm slices obtained from a 5% SDS–slab gel.

after stimulation of the purified pheochromocytoma enzyme by the PKP system are similar to those observed after stimulation of crude adrenal or brain enzyme preparations. However, recently it was reported that the stimulation of purified striatal TH by the PKP system causes an increase in V_{max} but no change in the K_m for the pterin cofactor. The difference between the kinetic changes observed after stimulation of TH by the PKP system in our study and that of Joh *et al.* (19) could be due to different regulatory properties of the pheochromocytoma TH versus striatal TH. In view of our findings that the reduction in the apparent K_m for the pterin cofactor is more pronounced when the enzyme activity is measured at physiological pH's, it is conceivable that a change in the K_m for pterin would have been observed with the purified striatal enzyme if TH activity had been measured at pH 7.2 and not at pH 5.9 (19).

It is evident from our data that the phosphorylated enzyme has a higher enzymatic activity in physiological pH's than the nonphosphorylated enzyme at its pH optimum, 6.0. These findings and the findings that the K_m for the pterin cofactor of the phosphorylated enzyme is significantly lower than that of the nonphosphorylated enzyme support the idea that phosphorylation of TH may play an important role in the short term regulation of catecholamine biosynthesis.

Initial attempts to phosphorylate TH have failed (24, 26) and it has been postulated that a protein activator and not the enzyme itself is phosphorylated. Subsequently, it has been demonstrated that TH itself is phosphorylated in organ cultures of rat adrenal medulla and superior cervical ganglia (27) as well as in purified striatal enzyme preparations (19, 28). The results of this study show that purified rat pheochromocytoma TH is phosphorylated by the PKP system. The findings that one mole of phosphate was incorporated per mole of TH suggest that only one of the four subunits of TH was phosphorylated or that under our experimental conditions only 25% of the enzyme was phosphorylated. The mechanism of TH phosphorylation is now under further investigation and will be reported in a separate paper.

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