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TYROSINE HYDROXYLASE IN RAT BRAIN—COFACTOR REQUIREMENTS, REGIONAL AND SUBCELLULAR DISTRIBUTION

JOSEPH T. COYLE

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Md. 20014, U.S.A.

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Abstract—A sensitive, radiochemical assay for tyrosine hydroxylase in rat brain is described. The enzyme is stimulated by catalase, dihydropteridine reductase and ferrous ion; maximal activity was found by homogenization in hypotonic buffer containing Triton X–100. Milligram quantities of rat brain tissue could be assayed at saturating concentrations of L-tyrosine. The enzyme is concentrated in the nerve terminal fractions as demonstrated by subcellular distribution studies. Seventy per cent of the activity can be released into the soluble fractions by hypotonic shock.

TYROSINE hydroxylase catalyzes the conversion of tyrosine to 3,4-dihydroxyphenylalanine (dopa).[1] This reaction appears to be the rate-limiting step in the synthesis pathway for catecholamines.[2] Furthermore, evidence has accumulated from studies both *in vitro* and *in vivo* that tyrosine hydroxylase is subject to end product inhibition.[3–5] Thus, the enzyme plays a central role in modulating the biosynthesis of catecholamines.

There have been a number of reports in which assays for brain tyrosine hydroxylase have been used,[6–8] however, there is little information available describing the biochemical characteristics of the enzyme in the brain. Also, most of the assays described lack sensitivity. Based upon the characteristics of highly purified tyrosine hydroxylase as reported by Shiman *et al.*,[9] an extremely sensitive assay for tyrosine hydroxylase in homogenates of rat brain has been developed. Modifications incorporated in the present brain assay that greatly enhance its sensitivity include the solubilization of the enzyme by homogenization with a buffer containing detergent, purification of substrate immediately prior to use, the inclusion of catalase and dihydropteridine reductase in the incubation mixture, and the isolation of the product by chromatography on alumina columns.

MATERIALS AND METHODS

Preparation of tissues. Female Sprague–Dawley rats (150–250 g) obtained from Hormone Assay Lab. (Chicago) were killed by cervical dislocation; their brains were removed and dissected at 5° according to the method of Glowinski and Iversen.[10] Brain tissue was homogenized in 10 vol. (w/v) of ice-cold 0·05 M Tris–HCl buffer (Trizma Base; Sigma Chemical Co.), pH 6·0, containing 0·2% Triton X–100 (v/v; Packard Instrument Co.) with a smooth glass homogenizer with a tightly fitting Teflon pestle (Kontes Glass No. 88600). The homogenates were centrifuged at 10,000 *g* for 10 min and the supernatant fluid was decanted for assay.

Purification of L-[^{3}H]-*tyrosine.* Side chain (2–3) labeled L-[^3H]-tyrosine (specific radioactivity, 13·5 c/m-mole; Amersham/Searle) was purified as follows: 1 mc was diluted to 5 cc with 0·2 N sodium acetate buffer, pH 8·6, and stirred with 400 mg alumina. The alumina suspension was poured over a column of 400 mg of activated alumina and washed with 5 ml water. The total effluent was titrated to pH 3 with 1 N HCl and applied to a Dowex-50 H^+ column (0·5 × 3 cm).[11] The column was washed with 100 ml of water and then 2 ml of 2 N HCl, and the tyrosine was eluted with 20 ml of 2 N HCl. The eluate was evaporated to dryness in a flash evaporator (Laboratory Glass Supply Co.) and the purified L-[^{3}H]-tyrosine was redissolved in 10 ml of absolute ethanol and stored until use at −20°.

Immediately prior to an experiment, a portion of the ethanol solution of L-[^{3}H]-tyrosine was dried under a stream of N_2 and redissolved in 0·2 N sodium acetate buffer, pH 8·6. About 1 mg alumina was added and mixed on a Vortex mixer (Scientific Industries, Inc.). The suspension was centrifuged at 6000 *g* for 10 min and a portion of the supernatant was mixed with an equal volume of a solution of 4 mM L-tyrosine. [^{14}C]-dopa (specific radioactivity, 52 mc/m-mole; Amersham/Searle) was purified by chromatography over alumina and stored until use in 0·2 N HCl at −20°.

Preparation of enzymes. Sheep liver dihydropteridine reductase was partially purified through the second ammonium sulfate step by the method of Kaufman.[12] Five μl of the enzyme solution (54 μg protein) produced maximal stimulation of tyrosine hydroxylase activity isolated from rat brains and adrenal glands. Tyrosine hydroxylase was prepared from frozen rat adrenal glands by homogenization in 5 vol. of isotonic KCl–5 mM Tris–HCl, pH 7·0. The homogenate was centrifuged at 100,000 *g* for 1 hr. The fat-free supernatant was fractionated with ammonium sulfate; the 33–60 per cent pellet was redissolved in a minimal volume of 0·005 M Tris–HCl, pH 7·0, containing 0·1 mM dithiothreitol and dialyzed for 12 hr against 10,000 vol. of the same buffer. The preparation had a specific activity of 280 *p*moles of [^{3}H]-dopa formed/mg protein/min.

Assay procedure. Tyrosine hydroxylase activity in the supernatant fluid of brain homogenates was assayed by a modification of the method of Shiman *et al.*[9] Fifty μl of the supernatant of the brain homogenates were added to 15-ml glass-stoppered centrifuge tubes containing a reaction mixture of 10 μl of 1 M KPO_4 buffer, (pH 5·5), 10 μl of sheep liver dihydropteridine reductase, 1100 units of catalase (Boehringer Chemical Co.) in 10 μl of glass-distilled water, 5 μl of 0·01 M TPNH (Boehringer Chemical Co.) and 5 μl of 6·4 mM 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$; freshly prepared in ice-cold 0·005 M HCl; Aldrich Chemical Co.). Blanks consisting of supernatant fluid heated to 95° for 5 min were run in all experiments as well as internal standards consisting of 15,000 cpm of [^{14}C]-dopa in place of L-[^{3}H]-tyrosine. The reaction was initiated by the addition of 10 μl of 2·0 mM L-[^{3}H]-tyrosine and was incubated for 10–60 min at 37° in room air. The reaction was terminated by the addition of 6 ml of 0·4 N perchloric acid containing 6 μg of carrier L-dopa.

[^{3}H]-dopa was isolated by a modification of the method of McGeer *et al.*[13] The perchlorate-inactivated incubation mixture was centrifuged at 1000 *g* for 10 min; the supernatant was added to a 20-ml beaker containing 30 mg sodium bisulfate, 5 ml of 2% EDTA (w/v), 1·5 ml of 0·35 M KH_2PO_4 and 200 mg alumina. The mixture was stirred and titrated to pH 8·6 with 1 N NaOH. The suspension was then poured over

columns of 200 mg of activated alumina and washed with 20 ml of glass-distilled water. The [^{3}H]-dopa was eluted with 2·5 ml of 0·2 N acetic acid, and the total eluate was counted in 15 ml of Triton phosphor in a Beckman LS–250 scintillation counter with a counting efficiency of 27 per cent. The results were corrected for recovery of [^{14}C]-dopa standard, which was consistently 59–61 per cent.

Chromatography. The product of the tyrosine hydroxylase assay for the rat brain was identified by thin-layer chromatography with the use of cellulose chromatogram sheets (Eastman). Over 95 per cent of the [^{3}H]-product had the same R_f as dopa and a different R_f from that of *O*-methyl-dopa, dopamine, norepinephrine, tyrosine and the deaminated metabolites with solvent systems consisting of: *N*-butanol–methanol–formic acid (20:20:65; v/v), *N*-butanol–glacial acetic acid–water (4:1:1; v/v) and methylethylketone–concentrated formic acid–water (24:1:6; v/v).

Subcellular fractionation. Whole rat brain was homogenized in 0·3 M sucrose and was subjected to subcellular fractionation by the method of Whittaker.[14] The pellets (P_1, P_2, P_3) were homogenized in 0·05 M, pH 6, Tris–HCl buffer containing 0·2% Triton X–100 (v/v) and centrifuged at 10,000 *g* for 10 min. The supernatant fluid was then decanted for assay. S_1 fractions were labeled with L-[^{3}H]-norepinephrine (specific radioactivity, 2·18 c/m-mole; Amersham/Searle) by the method of Coyle and Snyder.[15] In some experiments, the P_2 pellets were hypotonically shocked by resuspension in 0·03 M sucrose and then centrifuged at 100,000 *g* for 60 min to release the soluble enzyme. Linear sucrose gradients (30 ml; 0·3–1·5 M) were prepared according to the method of Bock and Ling.[16] The P_2 fractions were resuspended in 0·3 M sucrose, layered on the gradients and centrifuged for 90 min at 130,000 *g* max at 4° in an SW–27 rotor in a Beckman L2–65B ultracentrifuge; 24 fractions (1·3 ml) were obtained after piercing the bottom of the tube. The monoamine oxidase (MAO; EC 1.4.3.4) activity of the fractions was assayed according to the method of Wurtman and Axelrod.[17] The occluded lactate dehydrogenase (LDH; EC 1.1.1.27) and tyrosine hydroxylase (TH; EC 1.14.3a) in the particulate fractions were solubilized by homogenization with 0·1% Triton X–100 (v/v). The LDH activity of the fractions was determined by the method of Kornberg.[18] The fractions were counted for radioactivity with 10 ml of Bray's phosphor.[19]

Protein. Protein was determined by the method of Lowry *et al.*[20] with bovine serum albumin as standard.

RESULTS

Cofactor requirements for tyrosine hydroxylase in rat brain

$DMPH_4$, the pteridine cofactor, was essential for activity (Table 1). The inclusion of dihydropteridine reductase and TPNH as a regenerating system for pteridine resulted in a 25 per cent stimulation of activity. In contrast, 2-mercaptoethanol (10 or 100 mM), a more commonly used pteridine regenerating system, did not significantly increase the brain enzyme activity even in the presence of ferrous ion. In the absence of catalase, there was an 18-fold increase in the nonenzymatic formation of dopa as well as a 90 per cent inhibition of tyrosine hydroxylase activity. Ferrous ion was not as effective as catalase in stimulating the enzyme and reducing the spontaneous oxidation of tyrosine, although its stimulatory effect appeared to be additive with catalase. The omission of Triton X–100 from the homogenization buffer resulted in the release of 40 per cent less enzyme activity.

TABLE 1. COFACTOR REQUIREMENTS OF TYROSINE HYDROXYLASE IN RAT BRAIN*

Modification	Radioactivity (cpm)		
	Supernatant	Heated supernatant	Difference
Complete system	2954 ± 54	110 ± 5	2844
No $DMPH_4$	174 ± 41	117 ± 10	57
No dihydropteridine reductase	2234 ± 173	109 ± 4	2125
No catalase	2145 ± 122	1795 ± 71	350
No Triton X-100	1865 ± 152	107 ± 6	1758
3-Iodotyrosine (0·2 mM)	105 ± 7	104 ± 7	0
ME (10 mM) instead of PtRase	1774 ± 62	111 ± 4	1663
ME (100 mM) instead of PtRase	2129 ± 63	190 ± 10	1939
Fe^{2+} (3 mM) instead of catalase	2671 ± 120	566 ± 32	2105
Complete system with Fe^{2+} (3 mM)	4135 ± 120	105 ± 11	4030
ME (10 mM) and Fe^{2+} (3 mM) instead of catalase and PtRase	2021 ± 59	478 ± 22	1543
ME (100 mM) and Fe^{2+} (3 mM) instead of catalase and PtRase	2516 ± 121	246 ± 13	2270
ME (100 mM) and Fe^{2+} (6 mM) instead of catalase and PtRase	2492 ± 168	264 ± 27	2228

* Fifty μl of the supernatant fluid from rat brains homogenized in 10 vol. of Tris–Triton buffer was incubated for 30 min in a total volume of 100 μl as described in Methods. An equivalent volume of glass-distilled water was substituted for deleted cofactors. The final concentrations of the substituted cofactors are in parentheses. The values are a mean of five to nine determinations on separate brains with S.E.M. indicated. Abbreviations: $DMPH_4$ is 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; PtRase is dihydropteridine reductase; M.E. is 2-mercaptoethanol.

The specificity of the assay was demonstrable by inhibition of enzyme activity by 3-iodotyrosine to levels observed with heat-inactivated blanks. Chromatographic analysis with three solvent systems indicated that the product was [^{3}H]-dopa and that there was no detectable decarboxylation, *O*-methylation or deamination.

To determine whether the endogenous inhibitors in the brain were overcome by the cofactors, partially purified rat adrenal tyrosine hydroxylase was added to the reaction mixture as an internal standard (Table 2). The exogenous tyrosine hydroxylase retained almost 80 per cent of its activity in the presence of the brain homogenates.

Some kinetic characteristics of tyrosine hydroxylase in rat brain

The activity of tyrosine hydroxylase was proportional to the amount of tissue assayed (Fig. 1). The activity remained linear in respect to the length of incubation for periods up to 60 min (Fig. 2) however, the blank did not increase with time. The K_m for tyrosine of 0·14 mM in the rat brain (Fig. 3) was similar to that reported for tyrosine hydroxylase partially purified from bovine adrenal medulla[9] and bovine caudate nucleus.[23] Thus, 5 mg of brain tissue could be assayed at greater than K_m concentrations of tyrosine with experimental values up to 60 times greater than blank.

Regional distribution of tyrosine hydroxylase in rat brain

The regional distribution of enzyme activity was examined in the adult rat brain (Table 3). The lowest activity was found in the cerebellum, where experimental values

TABLE 2. RECOVERY OF TYROSINE HYDROXYLASE ACTIVITY*

Enzyme preparation	Enzyme activity (pmoles/assay/1 hr)
Whole rat brain homogenate	522 ± 22
Partially purified rat adrenal tyrosine hydroxylase	348 ± 11
Brain homogenate plus partially purified tyrosine hydroxylase	790 ± 18
Recovery of activity of partially purified tyrosine hydroxylase	77 ± 5

* Fifty μl of the supernatant of whole rat brain homogenates, 10 μl of partially purified rat adrenal tyrosine hydroxylase, or both together were assayed in a total volume of 100 μl at 0·2 mM L-[^{3}H]-tyrosine. The results are a mean of eight separate brains with S.E.M. indicated.

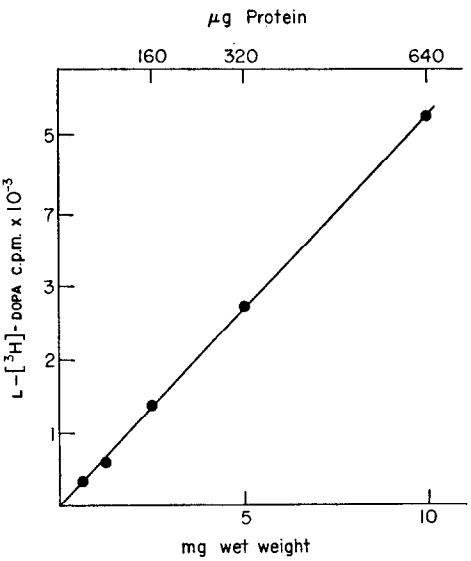

FIG. 1. Enzyme dilution curve for tyrosine hydroxylase activity in brain homogenates. Whole rat brains were homogenized in 5 vol. of Tris–Triton buffer. The supernatant fluids were serially diluted, and 50 μl was assayed under standard conditions with 0·2 mM L-[^{3}H]-tyrosine. The results are plotted in terms of milligrams of protein per assay and the equivalent wet weight of brain. Each point is a mean of four separate brains.

were five times greater than blank after a 30-min incubation. The highest activity was found in the striatum as previously reported by Côté and Fahn[24] and Musacchio *et al.*[6] The levels of enzyme activity in the various regions of the brain correlated with their total content of the catecholamines, dopamine and norepinephrine.[25] It is noteworthy that the striatum (caudate nucleus and putamen) contributes almost 40 per cent to the total activity of whole brain.

Subcellular distribution of tyrosine hydroxylase in rat brain

Over 60 per cent of the enzyme activity sediments in the P_2 fraction and less than one-quarter of the activity remains in the supernatant after centrifugation at 100,000 *g*

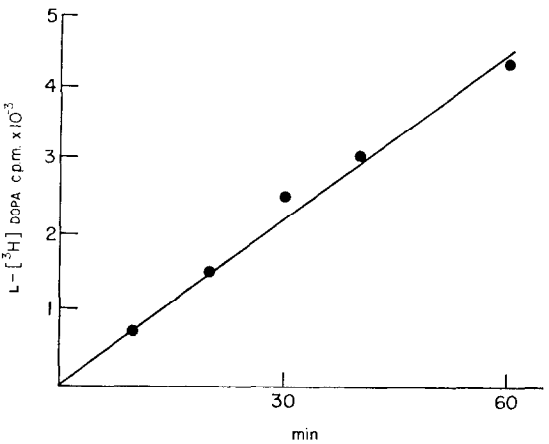

FIG. 2. Relationship between the duration of incubation and the amount of product formed by tyrosine hydroxylase in brain homogenates. Fifty μl of the supernatant fluid of Tris–Triton homogenates of whole rat brain were incubated under standard conditions with 0·2 mM L-[^{3}H]-tyrosine for 10–60 min. Each point is a mean of four separate brains.

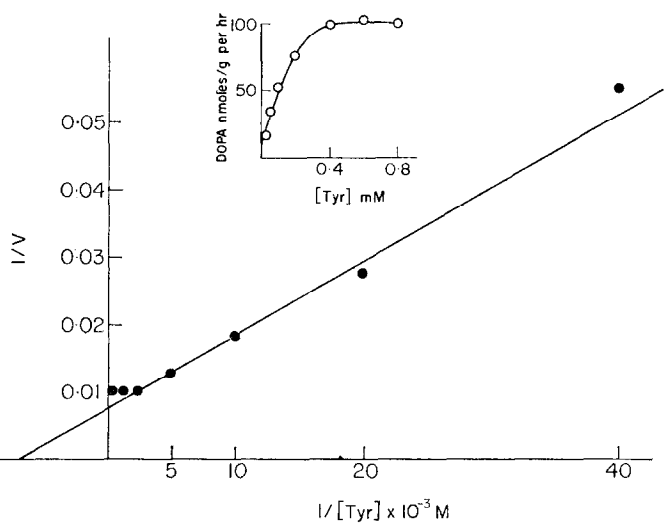

FIG. 3. Lineweaver–Burk plot for tyrosine hydroxylase in brain homogenates. Fifty μl of the supernatant fluid from Tris–Triton homogenates of whole rat brain were incubated under standard conditions for 30 min with L-[^{3}H]-tyrosine varying in concentration from 8×10^{-4} to $2{\cdot}5 \times 10^{-5}$ M. Each point is a mean of eight separate brains. Kinetic constants with S.E.M. were determined by the method of Wilkinson[21] with a program written by Cleland.[22] K_m: $1{\cdot}44 \pm 0{\cdot}10 \times 10^{-4}$ M; V_{max}: 133 ± 4 nmoles/g/hr.

TABLE 3. TYROSINE HYDROXYLASE ACTIVITY IN VARIOUS REGIONS OF ADULT RAT BRAINS*

Region	Enzyme activity† (nmoles/g/hr)	Enzyme activity† (nmoles/mg protein/hr)
Whole brain	77 ± 5	1·22 ± 0·12
Cortex	14 ± 2	0·21 ± 0·02
Cerebellum	7·2 ± 0·5	0·13 ± 0·01
Midbrain-thalamus	27 ± 2	0·58 ± 0·03
Hypothalamus	125 ± 6	2·70 ± 0·15
Medulla-pons	21 ± 1·2	0·47 ± 0·04
Striatum	520 ± 22	9·93 ± 0·40

* Fifty μl of the supernatant of brain tissue homogenized in Tris–Triton was assayed for tyrosine hydroxylase activity under standard conditions at 0·2 mM L-[^{3}H]-tyrosine.

† Results are expressed as nanomoles of dopa formed per gram wet wt. of tissue per hr and per milligram of protein per hr. The values are the mean of at least eleven separate preparations with S.E.M. indicated.

TABLE 4. SUBCELLULAR DISTRIBUTION OF TYROSINE HYDROXYLASE ACTIVITY IN WHOLE RAT BRAIN*

Preparation	Total activity %	Specific activity (nmoles/mg protein/hr)
P_1 (1000 *g*, 10 min)	13·2 ± 0·7	0·271 ± 0·028
P_2 (15,000 *g*, 60 min)	61·5 ± 2·0	0·580 ± 0·046
P_3 (100,000 *g*, 60 min)	2·4 ± 0·2	0·094 ± 0·006
S_3 (100,000 *g*, 60 min)	23·3 ± 0·6	0·351 ± 0·025
P_2 hypotonically shocked pellet (100,000 *g*, 60 min)	17·9 ± 1·8	0·117 ± 0·011
Supernatant (100,000 *g*, 60 min)	82·1 ± 3·6	0·989 ± 0·049

* Subcellular fractions prepared from whole rat brains (Methods) were assayed under standard conditions with 0·2 mM L-[^{3}H]-tyrosine. The results are a mean of ten separate preparations with S.E.M. indicated. The mean recovery of total activity for the brain was 86 per cent.

for 60 min (Table 4). However, over 80 per cent of the activity in the P_2 pellet became soluble after osmotic shock. These findings corroborated the previous results in which it was found that homogenization in hypotonic Tris buffer released only two-thirds of the activity as compared to homogenization with Tris–Triton buffer (Table 1).

To further characterize the subcellular localization of the enzyme, resuspended P_2 pellets were centrifuged on linear sucrose gradients (Fig. 4). The fractions were assayed for: tyrosine hydroxylase; LDH, a cytoplasmic enzyme occluded within synaptosomes; L-[^{3}H]-norepinephrine, a marker for synaptosomes that contain catecholamines; and MAO, an enzyme associated with mitochondria. The particulate tyrosine hydroxylase activity exhibited a distribution similar to that of particulate L-[^{3}H]-norepinephrine and LDH and peaked at a distinctly lower density than did MAO. Thus almost all the tyrosine hydroxylase activity in the P_2 pellet localized in those fractions in the density gradient enriched with synaptosomes.

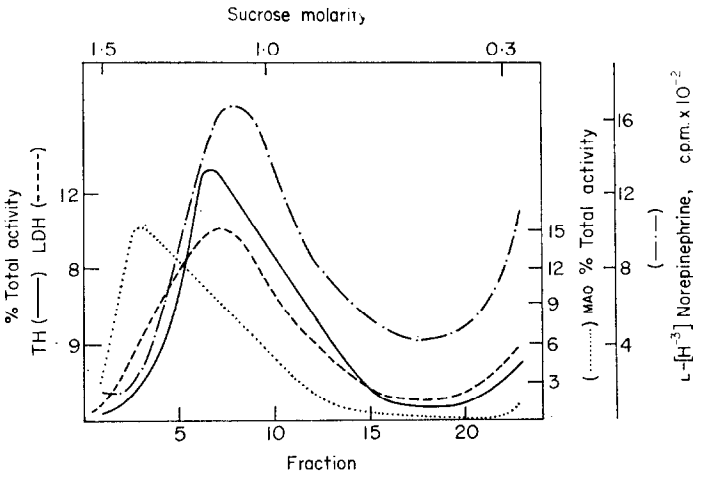

FIG. 4. Sedimentation characteristics of tyrosine hydroxylase, LDH, MAO and L-[^{3}H]-norepinephrine on linear sucrose gradients. P_2 fractions were prepared by the method of Whittaker,[14] resuspended in 0·3 M sucrose and layered on linear sucrose (0·3–1·5 M). After centrifugation, the fractions were assayed for tyrosine hydroxylase, LDH and MAO and counted for tritium. The results are a mean of three experiments.

DISCUSSION

One of the main difficulties encountered in assaying the low levels of tyrosine hydroxylase activity in brain tissue is the effect of endogenous inhibitors.[6] Shiman *et al.*[9] and Kaufman[26] have demonstrated that both partially purified dopamine-β-hydroxylase and tyrosine hydroxylase are stimulated by catalase and that this stimulation is related to the protection of the enzymes from peroxides. Recently, we have found that dopamine-β-hydroxylase activity in brain homogenates is almost completely inhibited in the absence of catalase.[27] This finding suggested that brain homogenates might be particularly prone to the formation of peroxides. The inclusion of catalase in the assay for brain tyrosine hydroxylase resulted in a 10-fold increase in activity. Furthermore, the catalase reduced the nonenzymatic oxidation of tyrosine by almost 20-fold. Unlike the partially purified enzyme,[9] ferrous ion was not as effective as catalase; however, ferrous ion in the presence of catalase resulted in a further stimulation of tyrosine hydroxylase activity.

The formation of dopa by tyrosine hydroxylase is linked to the oxidation of the pteridine; however, pteridine may undergo spontaneous oxidation during incubation.[9] Therefore, a pteridine regenerating system would seem necessary for the maintenance of first-order kinetics. 2-mercaptoethanol, a frequently used regenerating system for pteridines, had no stimulatory effect on the brain enzyme, whereas the TPNH–dihydropteridine reductase system resulted in a 25 per cent increase in activity. The fact that almost 80 per cent of the activity of partially purified rat adrenal tyrosine hydroxylase added to the brain assay was recovered indicates that a major portion of the endogenous inhibitors have been overcome.

The methods used to release the enzyme may have a significant effect on the levels of demonstrable activity. Homogenization of the brain in a hypotonic buffer containing

a detergent released maximal levels of enzyme activity. Homogenization in a hypotonic detergent-free buffer[7] released only 70 per cent of the enzyme activity. With isotonic sucrose homogenates of brain in hypertonic buffer,[13] most of the activity would remain occluded within synaptosomes. Since over 70 per cent of the activity sediments in the P_1 and P_2 fractions, assaying the supernatant of the 15,000 *g* isotonic sucrose homogenate of brain would measure only one-quarter of the brain activity.

The levels of activity found in the present study for brain and various regions are comparable to those reported by Musacchio *et al.*[6] for tyrosine hydroxylase partially purified from the brain and are much higher than those reported for other methods.[7,28] The main advantage of the present method is its high degree of sensitivity, which is due to a combination of several factors. Maximal levels of enzyme activity are liberated by homogenization in buffer containing Triton X–100. Enzyme activity is stimulated by the inclusion of catalase, dihydropteridine reductase and ferrous ion in the incubation mixture. Blank values were reduced to less than 0·02 per cent of the total radioactive substrate in the assay by rapid purification of the L-[^{3}H]-tyrosine immediately prior to incubation and by the reduction of the spontaneous oxidation of tyrosine during incubation with catalase. Although in this study the equivalent of 5 mg of whole brain tissue has been assayed at 0·2 mM L-tyrosine with experimental values up to 60 times greater than blank, it is possible to reduce the amount of tissue assayed to less than milligram quantities. With this method, it has been possible to assay readily tyrosine hydroxylase in regions and in subcellular fractions of fetal rat brains.[29]

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