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SIMPLE ASSAY PROCEDURE FOR TYROSINE HYDROXYLASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY EMPLOYING COULOMETRIC DETECTION WITH MINIMAL SAMPLE PREPARATION

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SUMMARY

A simple assay procedure for tyrosine hydroxylase activity in crude tissue samples was devised that requires minimal sample preparation and use of high-performance liquid chromatography with coulometric electrochemical detection. After incubation of enzyme samples, such as human brain homogenates or rat pheochromocytoma PC12h cells, with L-tyrosine and a tetrahydropterin cofactor, in the presence or absence of *p*-bromobenzyloxyamine, an inhibitor of aromatic L-amino acid decarboxylase, the reaction was terminated by addition of an equal volume of 0.1 *M* perchloric acid. For quantitation of L-DOPA produced, the sample was centrifuged, filtered and directly applied to the chromatographic apparatus connected to a coulometric electrochemical detector. This method makes redundant a time-consuming step in the previous methods, purification and concentration of L-DOPA or dopamine using alumina. The reaction conditions for the assay of tyrosine hydroxylase activity in brain homogenates and PC12h cells were re-examined by this method. Both tyrosine hydroxylase samples required a naturally occurring cofactor, (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin [(6*R*)BH_4], catalase and NSD-1055 for the full activity, and tyrosine hydroxylase in human brain homogenates required Fe^{2+} ions for its full activity. (6*R*)BH_4 proved to be a more effective cofactor than a synthetic cofactor, (6*RS*)-methyl-5,6,7,8-tetrahydropterin, which is commonly used for this assay.

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INTRODUCTION

Tyrosine hydroxylase [TH, tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] is one of the most important enzymes that regulate the level of catecholamines in brain under physiological and pathological conditions [1–3]. For the assay of TH activity, both radioisotopic methods [1,4] and high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [5,6] are commonly used. Similar methods have been applied to brain synaptosomes for the detection of dopamine [7] or to brain slices for the detection of L-DOPA [8]. However, such methods generally require a time-consuming step to purify L-DOPA or dopamine by alumina adsorption. In some tissues, especially some brain regions, TH activity is extremely low, so that dopamine or L-DOPA has to be concentrated before analysis by HPLC.

This report describes a very sensitive HPLC-ED method, in which L-DOPA formed during incubation is quantitatively determined without pretreatment with alumina. With this method, we re-examined the reaction conditions for assay of TH activity in human brain homogenates and in rat clonal pheochromocytoma PC12h cells.

EXPERIMENTAL

Chemicals and reagents

L-DOPA, L- and D-tyrosine, (6*RS*)-methyl-5,6,7,8-tetrahydropterin dihydrochloride (6MPH$_4$), Triton X-100, and *n*-octyl or *n*-heptyl β-D-glucopyranoside were purchased from Sigma (St. Louis, MO, U.S.A.), 3-iodo-L-tyrosine and sodium octanesulphonate were from Aldrich (Milwaukee, WI, U.S.A.), and catalase prepared from bovine liver was from Boehringer (Mannheim, F.R.G.). CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate, and CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate were purchased from Bio-Rad (Richmond, CA, U.S.A.), *p*-Bromobenzyloxyamine (NSD-1055) was from Nakarai (Kyoto, Japan). Perchloric acid was of the grade for amino acid analysis, methanol was of HPLC grade, and other chemicals were of analytical grade. (6*R*)-L-*erythro*-5,6,7,8-Tetrahydrobiopterin hydrochloride [(6*R*)BH$_4$] was synthesized according to the method of Matsuura et al. [9], and was kindly donated by Dr. S. Matsuura, Department of Chemistry, College of General Education, Nagoya University (Nagoya, Japan).

HPLC assay of L-DOPA

For the quantitation of L-DOPA, a Shimadzu HPLC apparatus, LC-3A (Shimadzu, Kyoto, Japan), connected with a Coulochem electrochemical detector, 5100A (ESA, Bedford, MA, U.S.A.) was used. The conditions of the electrodes were as follows: the voltage of a conditioning cell, Model 5021, was set at +250 mV and those of the first and the second electrode of an analytical cell, Model 5011, were set +50 mV and −300 mV, respectively. The output of the second electrode was monitored. A Cosmosil 5C$_{18}$ reversed-phase column (250 mm×4.6 mm I.D.) was connected to a Cosmosil precolumn, 10C$_{18}$ (50 mm×4.6 mm I.D.)

(Nakarai, Kyoto, Japan). The mobile phase was an aqueous solution of 90 m*M* sodium acetate, 35 m*M* citric acid, 130 μM disodium EDTA and 230 μM sodium *n*-octanesulphonate, containing 10.5% methanol. The sample was eluted at a flow-rate of 0.8 ml/min and at room temperature. The quantitation of the L-DOPA produced was carried out by comparison of the peak area with that of standard.

Enzyme samples

Brains were obtained at autopsy within 8 h of death from control patients without neurological diseases. Grey and white matter were isolated from the frontal lobes and homogenized with 10 v/w of 10 m*M* potassium phosphate buffer (pH 7.4) and centrifuged with a Centricut centrifuge tube (Type 20, Biofield, Tokyo, Japan), which excludes molecules of molecular mass less than 20 000. The supernatant (usually 1 ml) was diluted with 5 ml of 10 m*M* potassium phosphate buffer (pH 7.4), re-centrifuged in a Centricut tube at 1000 *g* for 60 min, and the washing procedure repeated once more. Samples were stored at −80 °C until use.

A clonal rat pheochromocytoma cell line, PC12h [10], was kindly supplied by H. Kuzuya, Fujita-Gakuen Health University (Toyoake, Aichi, Japan) and cultured as described previously [11]. The cells were gathered by centrifugation at 800 *g* for 10 min, washed with phosphate-buffered saline, suspended in 10 m*M* potassium phosphate buffer (pH 7.4) and sonicated for 30 s in a Branson sonicator at power level 40.

Assay for TH activity

The TH samples, brain homogenates (500–700 μg protein) or PC12h cells (50 μg protein), were incubated with 200 μM L-tyrosine in a 100-μl total volume of the reaction mixture in 100 m*M* sodium acetate–acetic acid buffer (pH 6.0), which contained 1 m*M* (6*R*)BH_4 or 6MPH_4, 10 μg of catalase and 1 m*M* NSD-1055, an inhibitor or aromatic L-amino acid decarboxylase (AADC). (6*R*)BH_4 or 6MPH_4 solution was made to be 10 m*M* in 1 *M* mercaptoethanol. In the case of brain homogenates, 2 m*M* ferrous ammonium sulphate was added to the reaction mixture. The incubation mixture, except for tyrosine and the pteridin cofactor, was preincubated at 37 °C for 5 min, and the reaction was started by adding tyrosine and the pterin cofactor. After incubation at 37 °C for 10 min, the reaction was terminated by addition of 100 μl of 0.1 *M* perchloric acid, containing 0.4 m*M* sodium metabisulphite and 0.1 m*M* disodium EDTA. After vortexing. the sample was allowed to stand in an ice-bath for 10 min, then centrifuged at 1000 *g* for 10 min, and the supernatant was filtered through a Millex-GS filter (Millipore, Bedford, MA, U.S.A.). As blank, a similar reaction mixture containing D-tyrosine instead of the L-isomer and 100 μM 3-iodo-L-tyrosine was used. The sample was subjected to HPLC and the amount of L-DOPA was measured as described above. TH activity in PC12h cells was also measured by our previous method [5].

Protein assay

Protein concentrations were measured according to Bradford [12], with bovine γ-globulin as standard, after the sample was dissolved in 0.1 *M* sodium hydroxide.

RESULTS AND DISCUSSION

As shown in Fig. 1, the voltammograms of L-DOPA and L-tyrosine were quite different; L-tyrosine requires a much greater applied potential for oxidation. At lower potentials, L-DOPA can be measured without interference from a large amount of L-tyrosine present in the reaction mixture. The sensitivity of this HPLC-ED method was sufficient to measure 25 fmol L-DOPA, when the limit of sensitivity was defined by a signal-to-noise ratio of 5. Using these HPLC conditions, the TH samples were analysed after incubation with L-tyrosine and as shown in Fig. 2, the peak corresponding to L-DOPA was clearly separated from other peaks. These data also showed that the blank with D-tyrosine instead of the L-isomer and 3-iodo-L-tyrosine, a potent inhibitor of TH, was necessary for the quantitation of L-DOPA produced (Fig. 2, III). A natural cofactor, $(6R)BH_4$, increased TH activity in PC12h cell homogenates markedly (Fig. 2, II and IV).

The optimal reaction conditions for the assay of TH activity in PC12h cells were re-examined. As shown in Figs. 3 and 4 (line I), the amount of L-DOPA produced was dependent on the amount of PC12h cell protein and the reaction time. The linearity of the reaction was confirmed from 5 μg up to 200 μg of protein of PC12h cells, and up to 10 min incubation time. The endogenous L-DOPA concentration in PC12h cells was found to be 0.39 ± 0.04 pmol/mg of protein, and is less than 10% of the amount of L-DOPA produced by TH in the assay from L-tyrosine under standard reaction conditions. Kinetic data on the effects of the concentrations of $(6R)BH_4$ and of L-tyrosine are shown in Fig. 5 and in Table I. To validate this method, TH activity in PC12h cell homogenates was measured by our previous method using activated alumina [5]. The values of TH activity obtained by the two methods were virtually the same: 3.39 ± 0.02 nmol/min per mg protein and 3.68 ± 0.30 nmol/min per mg protein by the previous method and by the newly devised method, respectively.

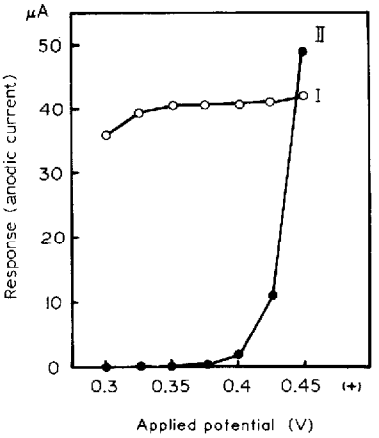

Fig. 1. Voltammograms for L-DOPA and L-tyrosine. Each point is the mean of the triplicate measurements at each potential. The injected amounts of L-DOPA and L-tyrosine were 50 pmol and 5 nmol, respectively. The HPLC conditions are described in the text. Curves: I = L-DOPA; II = L-tyrosine.

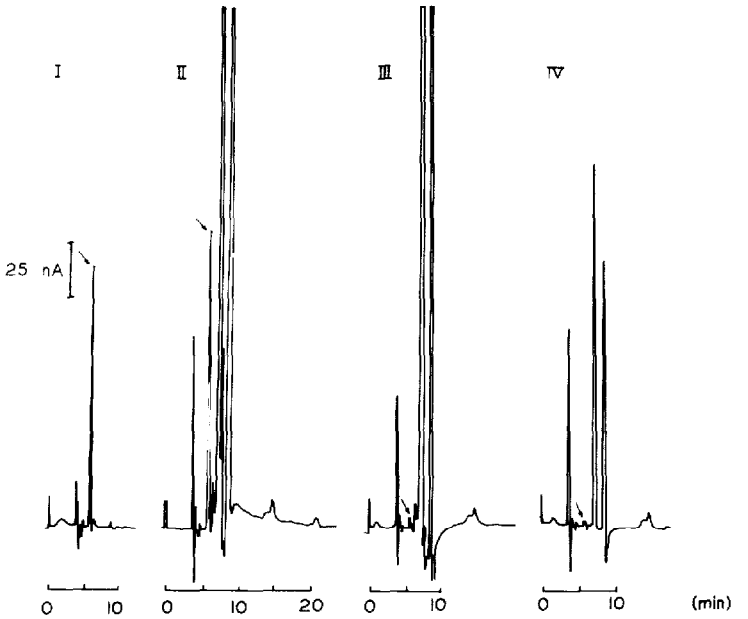

Fig. 2. Chromatograms of standard L-DOPA and the reaction mixture of PC12h cells with L-tyrosine. PC12h cells (50 μg of protein) were sonicated and treated with L-tyrosine as described in the text. (I) 25 pmol of L-DOPA; (II) the complete reaction mixture; (III) D-tyrosine was used instead of L-isomer and 100 μM 3-iodo-L-tyrosine was added; (IV) the reaction mixture without (6*R*)BH_4.

The maximal velocity (V_{max}) of TH activity in PC12h cells was much higher in the presence of (6*R*)BH_4 than in the presence of a synthetic cofactor, 6MPH_4, as summarized in Table I. Fig. 5 shows also that TH had two different kinetic patterns toward (6*R*)BH_4: with a higher Michaelis constant (K_m) and V_{max} value and with a lower K_m and V_{max} value. In the presence of (6*R*)BH_4, a substrate inhibition, which was first reported by Fisher and Kaufman [13], was observed with higher concentrations of L-tyrosine. These kinetic properties are almost the same as reported using TH purified from bovine adrenal medulla [14]. The effects of the reaction conditions on TH activity are summarized in Table II. NSD-1055, an inhibitor of AADC, did not affect the formation of L-DOPA at pH 6.0, where AADC is not active: 99.6% of the control. On the other hand, at pH 7.0, where the pH is near to the optimal for the activity of AADC (pH 7.2) [15], formation of L-DOPA was not detectable in the absence of NSD-1055.

To apply this method to the assay of TH activity in brain tissue, homogenates of grey and white matter of human brain cortex were incubated with L-tyrosine in the presence of (6*R*)BH_4, catalase, NSD-1055 and Fe^{2+}, and L-DOPA produced was measured. As shown in Fig. 6, TH activity could be detected in the homogenates of grey matter, but the activity in white matter homogenates was negligibly small: 4.68 ± 0.17 and 0.38 ± 0.02 pmol/min per mg protein for grey and white matter homogenates, respectively. As summarized in Table II, the washing procedure was required to get the full activity of TH in brain homogenates. The effects of cofactors, such as (6*R*)BH_4, catalase, NSD-1055, and Fe^{2+} on TH ac-

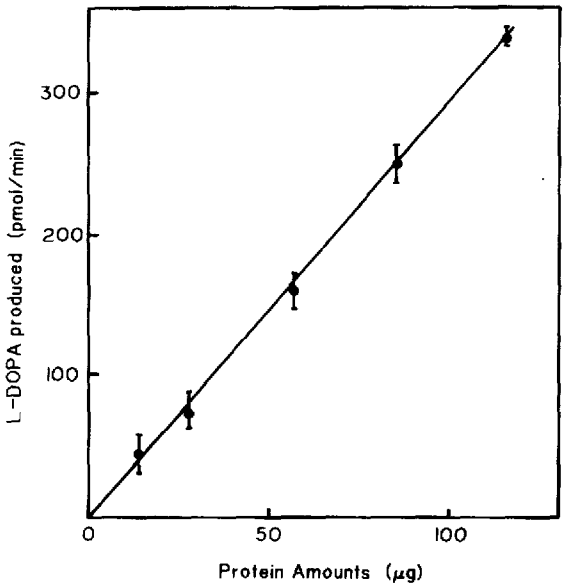

Fig. 3. Effects of protein amounts of PC12h cells on L-DOPA formation. L-Tyrosine (0.2 m*M*) was incubated with various amounts of PC12h cell protein in the presence of 1 m*M* $6MPH_4$, 100 μg of catalase and 1 m*M* NSD-1055. After incubation at 37°C for 10 min, the sample was treated as described in the text and applied to the HPLC apparatus. The HPLC conditions are described in the text. Each plot represents the mean and S.D. of duplicate measurements of two experiments.

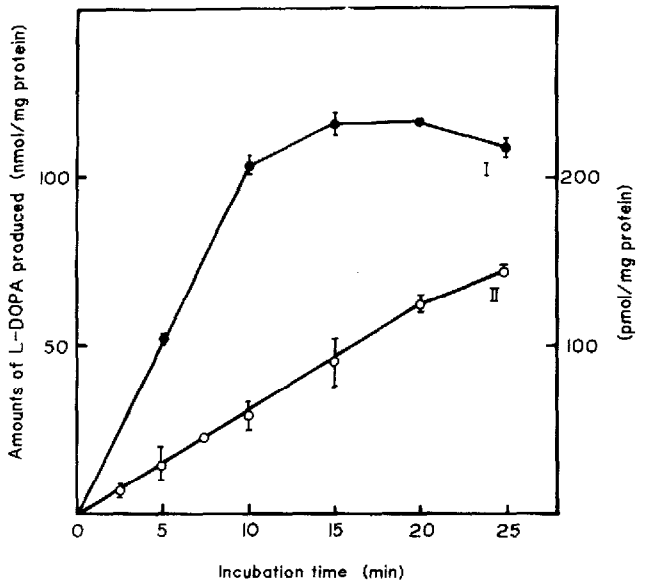

Fig. 4. Effects of reaction time on L-DOPA formation. PC12h cells (50 μg of protein) and brain homogenates (500 μg of protein) were incubated at 37°C with 0.2 m*M* L-tyrosine, in the presence of 1 m*M* (6*R*)BH_4, 100 μg of catalase and 1 m*M* NSD-1055. For brain homogenates, 2 m*M* Fe^{2+} was added. The amounts of L-DOPA produced were measured as described in the text. Curves: I = PC12h cells; II = brain homogenates. The unit of activity on the left is for I, and that on the right for II. Each plot represents the mean and S.D. of triplicate measurements of two experiments.

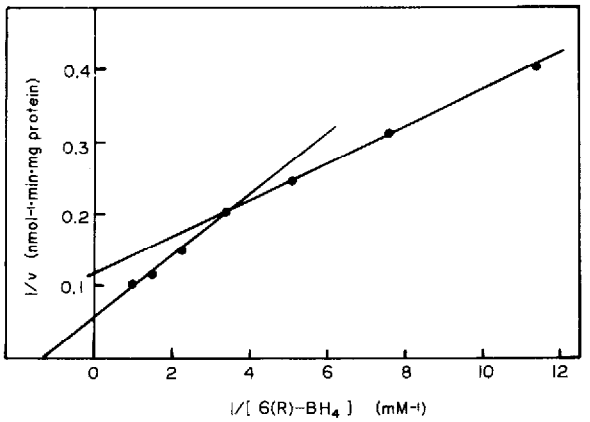

Fig. 5. Effect of the concentration of $(6R)BH_4$ on L-DOPA formation. PC12h cells (50 μg of protein) were incubated with 0.2 m*M* L-tyrosine at 37°C for 10 min with various concentrations of $(6R)BH_4$ in the presence of 100 μg of catalase and 1 m*M* NSD-1055. The amounts of L-DOPA produced were measured as described in the text. The reciprocal of the concentration of $(6R)BH_4$ was plotted against that of the reaction velocity, which was expressed as nmol of L-DOPA produced per min per mg PC12h cell protein, according to Lineweaver and Burk. Each plot represents the mean of the triplicate measurements of two experiments.

TABLE I

KINETIC PROPERTIES OF TH IN PC12h

Values are the mean and S.D. of triplicate measurements of two experiments.

	K_m (μM)	V_{max} (nmol/min per mg protein)
For L-tyrosine*		
Using $(6R)BH_4$	45.9 ± 8.9	19.3 ± 2.8
Using $6MPH_4$	68.5 ± 18.0	6.67 ± 2.50
For $(6R)BH_4$**		
	K_{m1}: 461 ± 67	V_{max1}: 14.3 ± 0.9
	K_{m2}: 204 ± 13	V_{max2}: 8.25 ± 0.35

*The concentrations of both $(6R)BH_4$ and $6MPH_4$ were 1 m*M*.
**The concentration of L-tyrosine was 0.2 m*M*.

tivity are also summarized in Table II. TH in brain homogenates required essentially Fe^{2+} ion for its full activity, and the relation of the Fe^{2+} concentration and the activation of TH activity is shown in Fig. 7, curve I. The K_m and the V_{max} values were 0.75 ± 0.10 m*M* and 19.8 ± 1.2 pmol/min per mg protein, respectively. On the other hand, TH activity in PC12h cells was not increased by Fe^{2+} ion, as shown in Fig. 7, curve II. TH activity in brain homogenates was also increased by addition of $(6R)BH_4$, as shown in Table II. The K_m and the V_{max} value of $(6R)BH_4$ were 78.9 ± 11.2 μM and 15.6 ± 1.9 pmol/min per mg protein, respectively, in the presence of 200 μM L-tyrosine and 2 m*M* Fe^{2+}. TH activity increased linearly with the reaction time up to 20 min under standard reaction conditions (Fig. 4, line II). TH activity in grey matter homogenate is 12.5 ± 0.42

TABLE II

EFFECTS OF REACTION CONDITIONS ON TH ACTIVITY IN PC12h CELLS AND HUMAN BRAIN HOMOGENATES

TH activity in PC12h cells (70 μg of protein) or homogenates of human brain cortex (550 μg of protein for washed sample, or 640 μg of protein for non-washed sample) was measured as described under Experimental. The value represents the mean of triplicate measurements, obtained by subtracting the blank values.

Reaction conditions	TH activity			
	PC12h cells (nmol/min per mg protein)		Brain homogenates, pH 6.0 (pmol/min per mg protein)	
	pH 6.0	pH 7.0	Washed	Non-washed
Complete system	5.24	1.54	12.52	4.68
Minus L-tyrosine	0.27	0.47	0.24	0.78
Minus (6*R*)BH_4	0.14	0.0	0.70	0.0
Minus catalase	5.22	1.66	3.06	2.61
Minus NSD-1055	5.22	0.0	11.70	4.57
Minus Fe^{2+}	2.53	1.41	2.50	0.0
Blank	0.14	0.09	1.11	1.96

pmol/min per mg protein in the complete reaction system containing Fe^{2+} (mean and S.D. of triplicate measurements of two experiments). The presence of detergents, such as Triton X-100, Tween-20, *n*-octyl or *n*-heptyl β-D-glucoside, CHAPS, CHAPSO, or cholic acids, in the reaction mixture did not affect TH activity in brain homogenates.

Hitherto, two HPLC-ED methods have been successfully applied to measure TH activity in synaptosomes or tissue slices, by quantitation of dopamine [7] or L-DOPA [8]. The former method by Messripour and Clark [7] is based on an assumption that the activity of AADC is greater than that of TH, and that L-DOPA produced from L-tyrosine by TH can be completely converted into dopamine. The second method, reported by us [8] to measure TH activity in tissue slices, is based on the quantitation of L-DOPA by inhibition of AADC with NSD-1055. It was applied to measure the extremely low TH activity in slices from various regions of rat brain [8] and proved to be effective for following the changes of TH activity in situ after addition of neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [16]. The method reported here is based on a similar idea, and the adaptation of a highly sensitive and selective ED apparatus enabled us to minimize the preparation step of DOPA formed.

This paper also shows that TH activity was much higher after the addition of a natural cofactor, (6*R*)BH_4 instead of a synthetic cofactor, $6MPH_4$, indicating that for an exact estimation of TH activity, the natural type of cofactor is preferable. Oxygen is known to be another substrate of TH activity [14], but in the experiments reported here the oxygen concentration was not controlled.

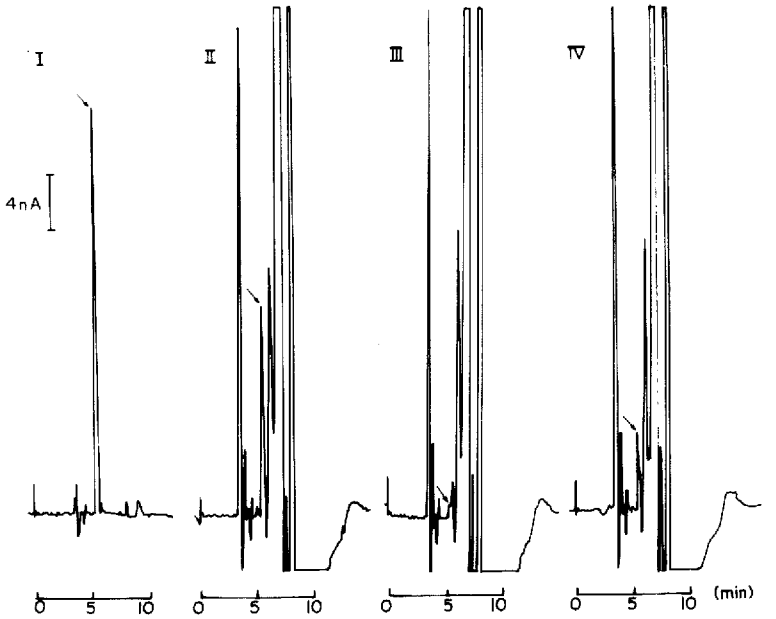

Fig. 6. Chromatograms of the reaction mixture of grey matter homogenates with L- or D-tyrosine. Grey (350 μg of protein) and white matter homogenates (450 μg of protein) were incubated at pH 6.0 with 0.2 m*M* L- or D-tyrosine at 37°C for 10 min. The samples were prepared as described in the text. The filtered sample (1 μl) was applied to the HPLC apparatus, and the HPLC conditions were as described in the text. (I) Standard L-DOPA (1 pmol); (II) reaction product of grey matter homogenates in the complete reaction mixture; (III) grey matter homogenates after incubation in the reaction mixture minus L-tyrosine; (IV) reaction product of white matter homogenates.

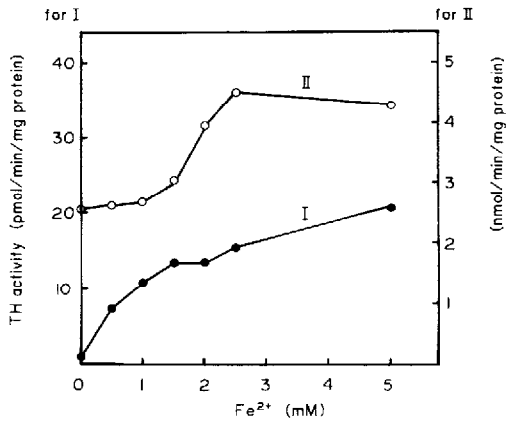

Fig. 7. Effect of Fe^{2+} concentration on TH activity in brain homogenates and PC12h cells. Human brain homogenates (550 μg of protein) or PC12h cells (50 μg of protein) were incubated with L-tyrosine at 37°C for 10 min, in the presence of 1 m*M* (6*R*)BH_4, 100 μg of catalase, 1 m*M* NSD-1055 and various concentrations of Fe^{2+} ion. As the blank, the homogenates were incubated in the presence of 200 m*M* D-tyrosine and 100 μ*M* 3-iodo-L-tyrosine. Curves: I = human brain grey matter homogenates; II = PC12h cells.

ACKNOWLEDGEMENT

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