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ASSAY FOR TYROSINE HYDROXYLASE IN HYPOTHALAMIC HOMOGENATES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A highly sensitive and reliable assay for tyrosine hydroxylase (TH) activity in hypothalamic homogenates of male rats using high-performance liquid chromatography with electrochemical detection is described. Modification of sample preparation and chromatographic conditions led to a complete separation of L-3,4-dihydroxyphenylalanine (L-DOPA) and α-methyldopa from all interfering catecholamines and their metabolites. This assay is highly sensitive; 2 pmol of L-DOPA formed enzymatically could be measured. We were able to determine TH activity in tissue pieces weighing less than 1 mg. TH activity was not changed after storage for three months at $-80\,^{\circ}C$. In hypothalamic homogenates the Michaelis constant (K_M) for L-tyrosine was 80.5 ± 6.5 μmol/l and the maximal velocity (V_{max}) was 132.5 ± 10.5 pmol/mg of protein per min for L-DOPA formed enzymatically.

INTRODUCTION

Tyrosine hydroxylase (TH, EC 1.14.16.2) is a monooxygenase that catalyses the formation of L-3,4-dihydroxyphenylalanine (DOPA) from L-tyrosine in peripheral and central catecholaminergic neurons and in chromaffin cells of adrenal medulla [1]. The formation of DOPA is the rate-limiting step in the catecholamine synthesis [1,2]. The enzyme is a mixed-function oxidase, requiring molecular oxygen [3] and a reduced pterin [1,4] as co-substrates. Ferrous ion appears to be required for optimal activity [1,5].

In most studies of TH, radiochemical methods using L-[U-^{14}C]tyrosine [1], L-[3,5-^{3}H]tyrosine [6,7] or L-[1-^{14}C]tyrosine [8] as a substrate have been utilized. These tests have generally been considered the most accurate. Fluorometric methods [9,10] have been reported, but the sensitivity of the fluorescence assay seems to be insufficient for tissues with low activity. The low sensitivity of the fluorescence assay can be enhanced by combination with high-performance liq-

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uid chromatography (HPLC) and fluorescence detection of DOPA [11]. Though the fluorometric method was the first non-isotopic assay for TH widely applicable to any crude tissues, progress in neurosciences required an extremely sensitive method permitting the assay of TH in less than milligram amounts of brain punches. For this purpose HPLC with electrochemical detection (ED) to measure enzymically formed DOPA was introduced. HPLC–ED has enabled us to determine femtomole levels of endogenous biogenic amines (for review see refs. 12–15) and their main metabolites [16–18]. This HPLC–ED method was applied to the assay of TH [19–23] and is of interest for routine use.

In this paper a reliable HPLC–ED assay for TH activity in hypothalamic homogenates of the rat brain is described. Modifications concerning sample preparation, TH assay and HPLC conditions are reported, and the kinetic properties of TH in hypothalamic homogenates are described. This method enables us to determine TH activity in hypothalamic homogenates of rat brain, not only under physiological conditions but also in pathological situations were TH activity might be reduced [24].

EXPERIMENTAL

Reagents

Sucrose, L-tyrosine, D-tyrosine, 3-iodo-L-tyrosine, 6-methyl-5,6,7,8-tetrahydrobiopterin (6-MPH$_4$), ferrous ammonium sulphate, L-3,4-dihydroxyphenylalanine (DOPA), 2-methyl-(3,4-dihydroxyphenyl)-L-alanine (α-methyldopa), 3,4-dihydroxyphenylglycol (DHPG), norepinephrine hydrochloride (NE), L-epinephrine (E), DL-3,4-dihydroxymandelic acid (DOMA), 3-methoxy-4-hydroxyphenylglycol (MOPEG), DL-normetanephrine hydrochloride (NMN), 3,4-dihydroxyphenylethylamine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine creatine sulphate complex (5-HT), 3-methoxy-4-hydroxyphenylacetic acid (HVA), DL-4-hydroxy-3-methoxymandelic acid (VMA), 3-methoxytyramine (3-MT), 5-hydroxyindole-3-acetic acid dicycloammonium salt (5-HIAA) and 2-mercaptoethanol were obtained from Sigma (St. Louis, MO, U.S.A.); perchloric acid was from Boehringer (Mannheim, F.R.G.); aluminium oxide was from Waters Assoc. (Milford, MA, U.S.A.); sodium octyl sulphonate was from Fluka (Buchs, Switzerland) and Bio-Rex 70 (200–400 mesh, sodium form) was from Bio-Rad Labs. (Richmond, CA, U.S.A.). Glass-distilled methanol and all other reagents (analytical-reagent grade) were supplied by E. Merck (Darmstadt, F.R.G.). The water was purified by a Milli-Q Reagent water system from Millipore (Bedford, MA, U.S.A.). 6-MPH$_4$ was used as cofactor; the 10 m*M* solution was prepared once a week in 1.0 *M* 2-mercaptoethanol and stored at $-20\,^{\circ}C$, protected from light.

Sample preparation

Male Wistar rats (200 g) were obtained from Süddeutsche Versuchstierfarm (Tuttlingen, F.R.G.). After animals had adapted to the new environmental conditions, they were quickly removed from their cages and sacrificed by decapitation. Brains were removed and immediately frozen on dry ice. Medio-basal hypothalami (MBH) were punched out of the frozen brains with a needle (4 mm

I.D.). The ventral limit of the punch was the chiasma opticum, the dorsal limit were the mamillary bodies. The lateral limits were the lateral sulci of the hypothalamus. The heights of the tissue cylinders were 2 mm. Each tissue piece contained the ventromedial hypothalamic (hy) nucleus (nu), the dorsomedial hy nu, the para- and periventricular hy nu, parts of the arcuate hy nu and small parts of the lateral hypothalamus. The mean weight of the tissue pieces was 30.3 ± 5.0 mg. Each of the tissue pieces was then placed in 550 μl of 0.32 *M* sucrose and homogenized with an ultrasonic cell disrupter (Model B 15, Branson, Danberg, CT, U.S.A.). These homogenates were stored at $-80°C$ until assay. Aliquots were taken from the homogenates for the analysis of protein according to the method of Lowry et al. [25].

Assay of tyrosine hydroxylase

All TH assays were performed according to the procedures of Nagatsu et al. [20] and Kato et al. [21] with slight modifications. The standard incubation mixture consisted of the following components in a volume of 100 μl (final concentrations in parentheses): 30 μl of 0.5 *M* potassium phosphate buffer pH 6.0, 10 μl of 10 m*M* 6-MPH_4 (1 m*M*) in 1 *M* 2-mercaptoethanol (100 m*M*), 10 μl of 10 m*M* ferrous ammonium sulphate (1 m*M*), 20 μl of 1 m*M* L-tyrosine (200 μM or various concentrations for kinetic studies) and 30 μl of 0.32 *M* sucrose (100 m*M*) containing enzyme. For blank incubation, D-tyrosine was used as substrate (100 μM) instead of L-tyrosine, and 100 pmol of DOPA were added to another blank incubation as an internal standard for DOPA. The tyrosine hydroxylase inhibitor 3-iodo-L-tyrosine (100 μM) was added to both blanks in order to prevent DOPA formation from D-tyrosine, which contains some L-tyrosine. Incubation was performed at 37°C for 20 min and the reaction was stopped with 0.33 *M* perchloric acid (4°C) containing 100 pmol of α-methyldopa as an internal standard and 20 μl of 0.2 *M* EDTA. After 10 min 100 μl of 1 *M* potassium carbonate and 1 ml of 1 *M* Tris–HCl were added to adjust the pH to 8.0–8.5, and the mixture was then centrifuged at 1600 *g* for 10 min at 4°C. The clear supernatant was passed through two columns (Baker disposable filtration column, dual 20-μm frits, 1 ml) (J.T. Baker, Phillipsburg, NY, U.S.A.) fitted together sequentially. The upper column contained 1 ml of a Bio-Rex 70 suspension (5 g of Bio-Rex 70 were suspended in 20 ml of water), and the bottom column contained 100 mg of aluminium oxide. The effluent through both columns was discarded. Both columns were washed with 2 ml of 0.02 *M* Tris and 3 ml of water. DOPA and α-methyldopa were passed through the Bio-Rex 70 column and absorbed on the second aluminium oxide column, which was separated and washed with 1 ml of water twice and with 50 μl of 0.5 *M* hydrochloric acid once. DOPA and α-methyldopa were eluted with 250 μl of 0.5 *M* hydrochloric acid. The eluates were stored at $-80°C$ or directly analysed by HPLC (25 μl of the eluate).

HPLC analysis

The HPLC system consisted of a Model 590 programmable solvent-delivery pump and an automatic injection system (WISP Model 710 B) obtained from Waters Assoc. The 5-μm C_{18} column (250 mm × 4 mm I.D., LiChrosorb RP-18,

EC 250-4) protected by a guard column (containing Perisorb RP-18, 30–40 μm) was from E. Merck. The E656/641 electrochemical detector from Methrom (Herisau, Switzerland) was used with a glassy carbon electrode. The detector potential was normally maintained at +0.65 V versus an Ag/AgCl reference electrode. The mobile phase was a mixture of 0.1 *M* potassium phosphate buffer (pH 3.6), 0.5 m*M* sodium octyl sulphonate, 0.1 m*M* EDTA and 5% methanol (v/v). The flow-rate was 0.8 ml/min. Under these conditions the retention times were: DOPA, 7.3 min; α-methyldopa, 15.9 min.

Standards and calculations

The stock solutions of the standards of DOPA and α-methyldopa were prepared in 0.1 *M* potassium phosphate buffer (pH 3.6) at a concentration of 10 m*M* and stored at −80°C. On the day of the experiment they were diluted with mobile phase to a final concentration of 100 pmol per TH assay.

The amount of DOPA formed enzymatically by TH was calculated by the equation:

$$\frac{R(\text{L}) - R(\text{D})}{R(\text{D+S}) - R(\text{D})} \times 100 \text{ pmol}$$

where R is the ratio of peak heights (peak height of DOPA/peak height of α-methyldopa), $R(\text{L})$ being that from the L-tyrosine incubation, $R(\text{D})$ from the D-tyrosine incubation and $R(\text{D+S})$ that of D-tyrosine plus DOPA (internal standard, 100 pmol).

RESULTS AND DISCUSSION

Chromatographic conditions

DOPA and α-methyldopa have to be separated completely from all other possibly interfering catecholamines and their metabolites. Therefore the chromatographic pattern concerning the double-column procedure and the HPLC system of some known catecholamines (DA, DOMA, DOPAC, DOPA, NE, E, α-methyldopa, MOPEG, VMA, 5-HIAA, HVA, NMN, 5-HT and 3-MT) was investigated. Given amounts of the tested catecholamines (concentration was between 2.5 and 12.5 pmol per 25 μl) are added to the standard incubation mixture (see Experimental) containing 1–2 mg of hypothalamic homogenates. When 1 ml of the Bio-Rex 70 (200–400 mesh) suspension was used in the upper column, 65% of DA, 70% of NE, 31.5% of E and 35% of NMN were retained. The other catecholamines were eluted to the second column, which contained 100 mg of aluminium oxide. Here 79% of α-methyldopa, 82% of DOPAC, 113.6% of 5-HIAA, 83% of MOPEG, 22% of NMN and 84% of DOPA were retained. Parts of the catecholamines could not be recovered; they might have been irreversibly fixed on one of the columns or eluted without retention. Several compounds could not be detected by HPLC under the conditions chosen for the standard assay. Whilst DOMA and VMA were in the front peak, HVA, 5-HIAA, 5-HT and 3-MT had long retention times leading to peaks that could hardly be evaluated.

With this double-column procedure we were able to separate the main part of NE from DOPA; the remaining small part of NE, which is reversibly fixed on the

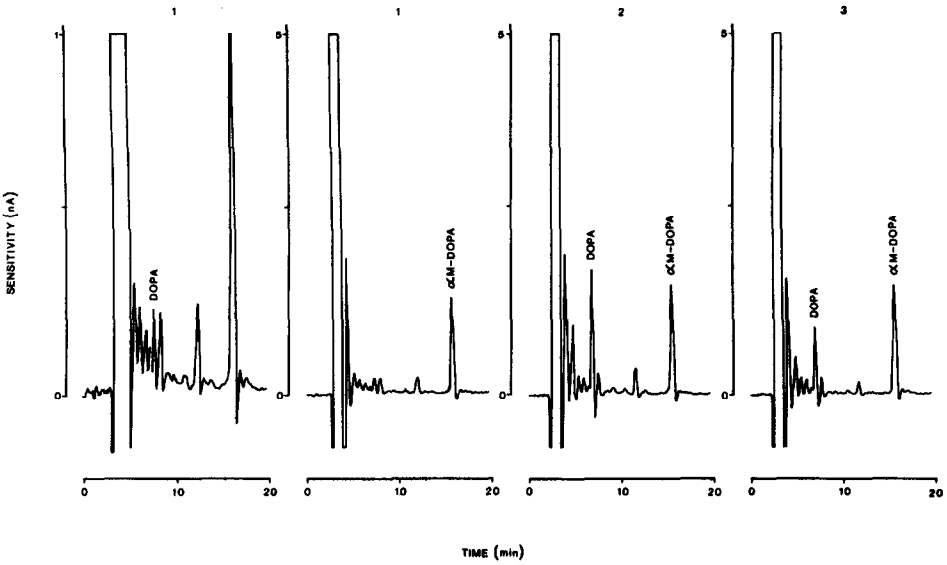

Fig. 1. HPLC elution pattern of TH incubation mixtures with hypothalamic homogenates as enzyme. (1) Blank incubation with D-tyrosine; (2) 100 pmol of DOPA were added as an internal standard to a blank incubation with D-tyrosine; (3) experimental incubation with L-tyrosine. After incubation, 100 pmol of α-methyldopa (αM-DOPA) were added to each sample. DOPA in D-tyrosine incubation (1) had to be measured with higher sensitivity.

aluminium oxide column, does not interfere with DOPA determination (Fig. 1). Secondly we see that α-methyldopa is a suitable, accurate candidate as internal standard, because it has the same recovery rate as DOPA (ca. 80%) and does not interfere with any other catecholaminergic substance under the chromatographic conditions described in Experimental.

Using the mobile phase of Nagatsu et al. [20] or Kato et al. [21] we were not able to detect DOPA and α-methyldopa. Therefore the chelate former EDTA and the ion-pairing reagent sodium octyl sulphonate were added to phosphate buffer (pH 3.6) containing 5% (v/v) methanol. Under these conditions a chromatographic pattern of the TH reaction in hypothalamic homogenates as shown in Fig. 1 is obtained. The charged functional groups of the amines interact with the ion-pairing reagent in the mobile phase and on the stationary phase, increasing the retention times of the amines [12]. With methanol as an organic modifier, the retention time of DOPA was shortened to such a degree that the DOPA peak was close to the front peak but did not interfere with it. The different columns used in the experiments of Nagatsu et al. [20] and Kato et al. [21] (Yanapak ODS) and in our experiments (LiChrosorb RP-18) could be one of the reasons leading to different mobile phases.

Sensitivity of the detector

The electrochemical detector is very sensitive to catechol compounds as DOPA and α-methyldopa. The relation between the injected amount of standards and the detector response was linear for DOPA and α-methyldopa in the tested range

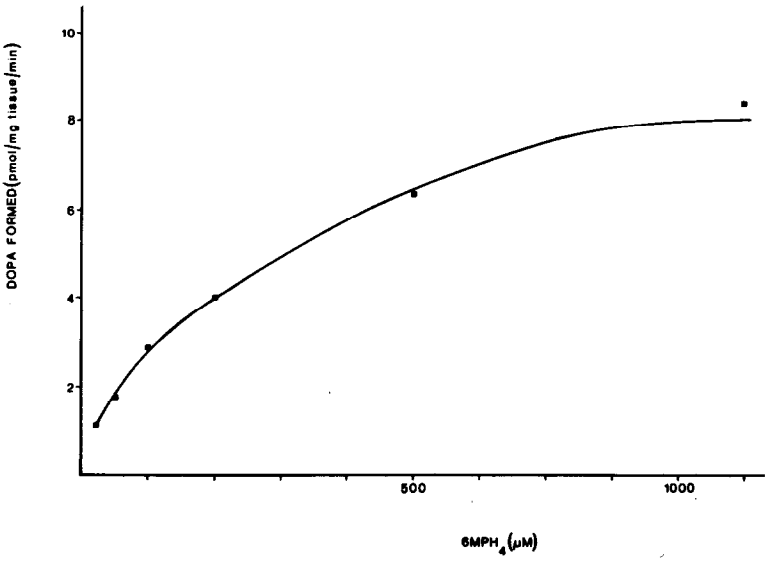

Fig. 2. Rate of DOPA formation using a homogenate of hypothalamus as enzyme at 37 °C. A standard incubation system was used, as described in Experimental, containing different amounts of 6-MPH_4.

(0.5–100 pmol). The sensitivity of the measurement varied mainly according to the condition of the detector. The noise of the detector cell was the limiting factor, and this was primarily dependent on the condition of the reference electrode. The detection limits at a signal-to-noise ratio of 3, expressed in terms of pmol of the components injected, were 0.09 ± 0.01 (DOPA) and 0.1 ± 0.01 (α-methyldopa) ($n=8$).

Assay of tyrosine hydroxylase

In order to evaluate optimal conditions for TH assay, its dependence on co-substrate concentration, tissue concentration and incubation time was tested. The results are shown in Figs. 2–4. It can be seen that 1 m*M* 6-MPH_4, which is available in the standard assay, is the optimal co-substrate concentration. 2-Mercaptoethanol was used as antioxidant, because ascorbic acid, the antioxidant used by Kato et al. [21], leads to a larger front peak that interferes with the peak of DOPA. Fig. 3 shows that we were able to measure TH activity in very small tissue pieces (below 1 mg). The sensitivity could even be improved by the newer generation of electrochemical detectors. Our hypothalamic tissue pieces normally weighed between 1 and 2 mg. This range gives the best precision. Under these conditions the within-assay variability was ca. 6% ($n=8$) and the inter-assay variability was 11% ($n=10$). The reaction rate was linear up to 4 mg of tissue. Fig. 4 shows a linear reaction rate up to an incubation time of 30 min. To test the stability of TH in crude homogenates, these homogenates were stored for different times at -80 °C. Fig. 5 indicates TH activity as a function of storage time. TH activity remained stable over a period of three months. Perhaps even longer times of storage are possible.

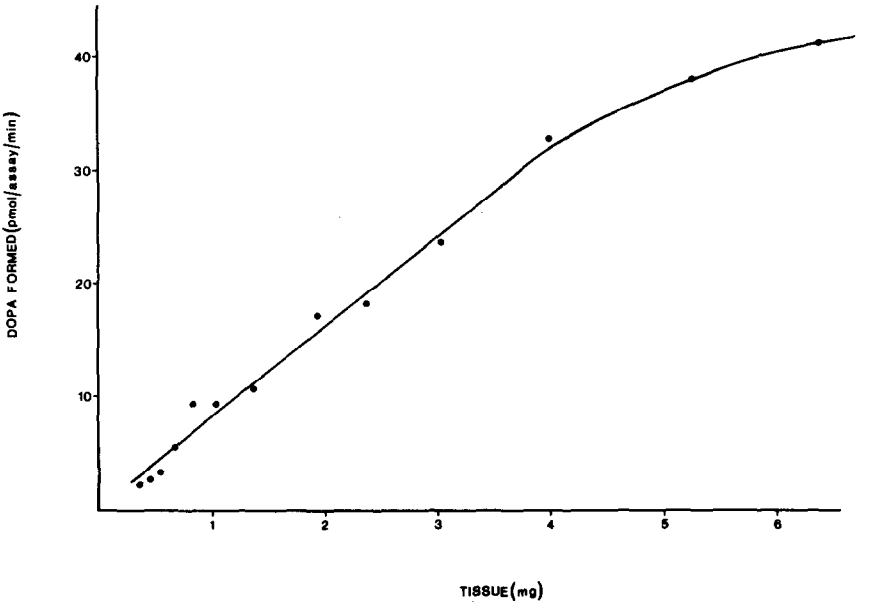

Fig. 3. TH activity in homogenates of the medio-basal hypothalamus of the rat as a function of enzyme concentration. A standard incubation system was used, as described in Experimental.

Michaelis constant (K_M) and maximal velocity (V_{max}) in hypothalamic homogenates

Using this highly sensitive assay method, we were able to study the kinetics of

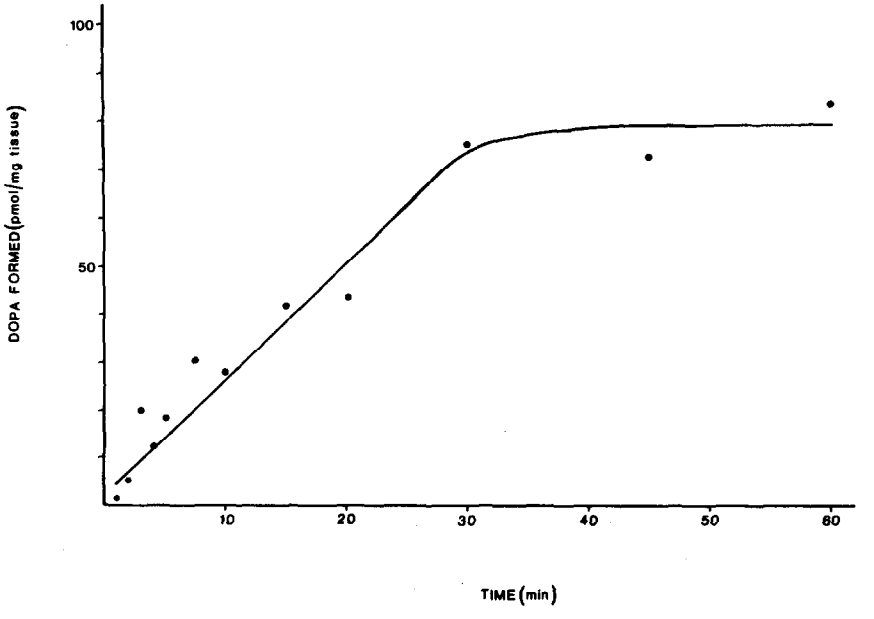

Fig. 4. TH activity in homogenates of rat brain containing ca. 4 mg of tissue as a function of incubation time. The other parameters of enzyme assay were used as described in Experimental.

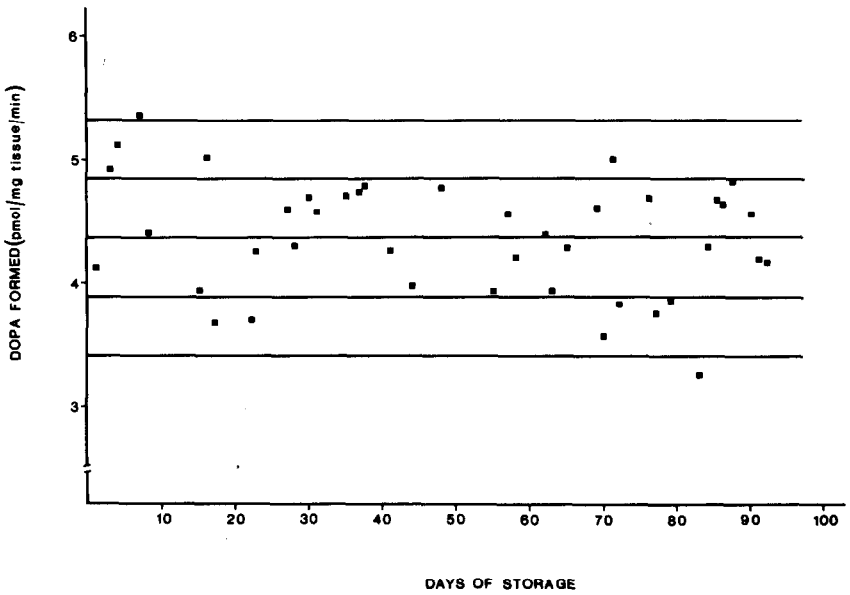

Fig. 5. TH activity in homogenates of rat brain containing ca. 4 mg of tissue as a function of storage time. Homogenates were stored at −80°C until assay.

TH in the MBH not only in control animals, but also in starved and semistarved rats, where a reduced TH activity is expected [24]. In order to determine V_{max} and K_M for tyrosine, we measured TH activity in hypothalamic homogenates of every rat with different L-tyrosine concentrations (30, 50, 75, 100 and 200 μM). Fig. 6 gives an example of the effect of L-tyrosine on DOPA formation in the MBH of the male rat. The evaluation of K_M and M_{max} resulted from Lineweaver–Burk plots (Fig. 7). The K_M value (mean ± S.D.) ($n=8$) for L-tyrosine in the presence of 1 mM 6-MPH$_4$ was 80.5 ± 18.4 μmol/l. The V_{max} value (mean ± S.D.) ($n=8$), using the same data, was 132.5 ± 29.7 pmol of DOPA formed per mg protein per min. This value is ca. three times higher than that of Hirata et al. [23]. The hypothalamic areas used for TH assay by these authors were different from those used in our experiments. This may be the cause of the different values for K_M and M_{max}.

CONCLUSION

This study offers a simple, reliable, rapid and stable method for measuring TH activity in homogenates of the MBH of the rat. The assay is highly sensitive; the limit of sensitivity was ca. 2 pmol DOPA formed enzymatically; this limit is lower than that achieved by Nagatsu et al. [20]. The sensitivity is only determined by the blank value, which derives either from DOPA formed by the non-enzymatic reaction or from DOPA contained in a crude enzyme preparation [1,20].

This method can be used to study the kinetics of TH, not only in homogenates of MBH under different physiological and pathological conditions, but also in

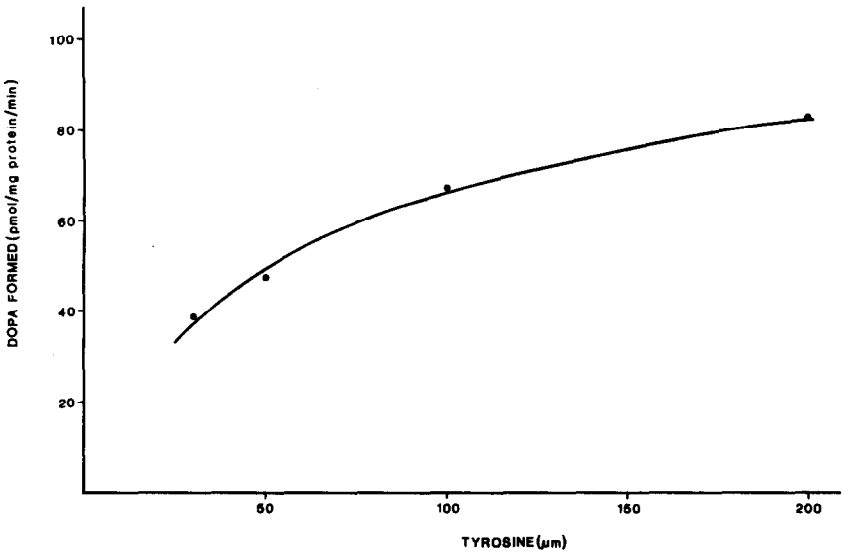

Fig. 6. Rate of DOPA formation using hypothalamic homogenates as enzyme. A standard incubation system was used, as described in Experimental, containing different amounts of L-tyrosine (30, 50, 75, 100 and 200 μM).

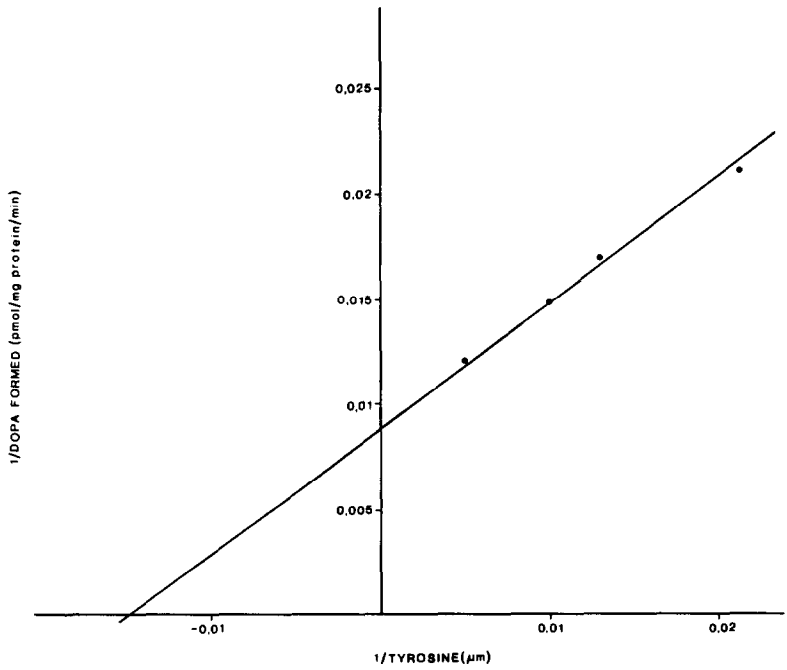

Fig. 7. Lineweaver-Burke diagram for TH activity (expressed as pmol of DOPA formed per min and per mg protein). TH activity was measured at different tyrosine concentrations (50, 75, 100 and 200 μM).

brain areas with very low TH activity (cortex, hippocampus) [21,23] or in different brain nuclei (e.g. different hypothalamic nuclei) containing only small amounts of tissue. It should be mentioned that the sensitivity of the HPLC determination of DOPA may be increased even further by using the new generation of electrochemical detectors with very high sensitivity.

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