Biochemical Pharmacology, Vol. 31, No. 9, pp. 1729–1734, 1982
Printed in Great Britain.

0006-2952/82/091729-06 $03.00/0

STUDIES ON THE REGULATORY MECHANISM OF THE TYROSINE HYDROXYLASE SYSTEM IN ADRENAL SLICES BY USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

AKIFUMI TOGARI, TAKESHI KATO and TOSHIHARU NAGATSU*

Laboratory of Cell Physiology, Department of Life Chemistry, Graduate School at Nagatsuta, Tokyo Institute of Technology, Yokohama 227, Japan

(*Received* 9 *February* 1981; *accepted* 28 *October* 1981)

Abstract—A new method was developed to study the tyrosine hydroxylase (TH) system in rat adrenal slices by high-performance liquid chromatography (HPLC) with electrochemical detection (ED). TH activity was measured by determining DOPA, formed in adrenal slices containing all of the components of the TH system in the presence of an inhibitor of aromatic L-amino acid decarboxylase (NSD-1055), using a new and highly sensitive HPLC–ED method. The properties of the TH system in the slices were also examined. High K^+ (52 mM) stimulated the formation of DOPA in the slices; this stimulation was not observed in Ca^{2+}-free medium. Furthermore, the addition of ethyleneglycol bis (β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) to a Ca^{2+}-free medium not only abolished the stimulation by high K^+ but also significantly inhibited the conversion of L-tyrosine to DOPA. This suggests that intracellular Ca^{2+} may regulate TH activity in the adrenal medulla. The enzyme in homogenates of normal adrenal slices had two different K_m values for the tetrahydropterin cofactor. Alteration of enzyme kinetics using homogenates of adrenal slices that had been treated in various ways suggests that changes in the proportions of the two forms of TH produced by intracellular Ca^{2+} may regulate the activity of this enzyme in the adrenal medulla.

Tyrosine hydroxylase (TH,† EC 1.14.16.2) is a monooxygenase that catalyzes the formation of DOPA from L-tyrosine in peripheral and central catecholaminergic neurons and chromaffin cells of the adrenal medulla [1]. Regulation of TH activity has been studied extensively in dopaminergic and adrenergic tissues, and multiple mechanisms of TH regulation in these tissues have been proposed [2].

In most procedures for measuring TH activity, a tissue extract is made and saturating amounts of a reduced pterin cofactor with a chemical or enzymatic tetrahydropterin-regenerating system and tyrosine are added to the reaction mixture, so that the rate of formation of DOPA equals the V_{max} and is proportional to the amount of TH present. However, to study the physiological regulation of the TH system, it would be necessary to determine the rate of conversion of tyrosine to DOPA in a relatively intact tissue preparation such as tissue slices, in which all of the components of the enzyme system may be present at physiological levels. Using such an assay system of TH, the effects of various pharmacological and physiological interventions on the hydroxylating system could be assessed. For this purpose we have applied the recently developed high-performance liquid chromatography (HPLC) with electrochemical detection (ED) to measure enzymatically formed DOPA in tissue slices. HPLC–ED has enabled us to determine femtomole levels of endogenous biogenic monoamines [3–8]. This HPLC–ED method was applied to the assay of TH [9] and offers attractive features for routine use. By using this HPLC–ED assay for TH [9] we have estimated the DOPA formed in adrenal slices and studied the *in situ* regulatory mechanism of TH.

Stimulus–secretion coupling in chromaffin cells and in adrenergic neurons is accompanied by an acute increase in the rate of catecholamine synthesis [10–14]. It has been demonstrated that Ca^{2+} is a requirement for secretion to occur from chromaffin cells [15, 16]. Furthermore, it has been proposed that the release of Ca^{2+} from some intracellular store could activate the TH in rat pheochromocytoma cells [17]. These results suggest that intracellular Ca^{2+} may be one of the factors that regulate the TH activity.

In the present paper, we describe the development of a simple HPLC–ED method for measuring the activity of TH in adrenal slices. Some of the properties of the TH assay system in adrenal slices and the role of intracellular Ca^{2+} in TH regulation are also examined.

MATERIALS AND METHODS

Materials. Male Wistar rats weighing 220–280 g were used for all experiments. Rats were housed five per cage, at 24°, with *ad lib.* food and water and a 12 hr light–dark cycle.

* Author to whom correspondence should be addressed.

† Abbreviations: TH, tyrosine hydroxylase; HPLC, high-performance liquid chromatography; ED, electrochemical detection; and $6MPH_4$, 6-methyl-5,6,7,8-tetrahydropterin.

L-Tyrosine, D-tyrosine, 2-mercaptoethanol and alumina oxide were obtained from the Wako Pure Chemical Co. (Osaka, Japan); 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$) from the Calbiochem-Behring Corp. (Los Angeles, CA, U.S.A.); DL-α-methyl-DOPA, DL-α-methyl-*p*-tyrosine and EGTA [ethyleneglycol bis (β-aminoethylether)-*N*,*N*'-tetraacetic acid] from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); and catalase from the Boehringer Mannheim Corp. (Mannheim, West Germany) NSD-1055 (brocresine; 4-bromo-3-hydroxybenzyloxyamine hydrochloride) was a gift from the Sankyo Co., (Tokyo, Japan). All other chemicals used were of analytical grade.

Krebs-bicarbonate solution was composed of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.1 mM $MgSO_4$, 1.4 mM KH_2PO_4 and 8 mM glucose. Incubation solution was continuously bubbled with 95% O_2–5% CO_2. The pH of the medium was 7.2 following equilibration with 95% O_2–5% CO_2. In some experiments, Krebs-bicarbonate medium in which $CaCl_2$ was omitted or replaced by 1 mM EGTA was used.

Measurement of DOPA formation in adrenal slices. Rats were decapitated and the adrenal glands were quickly removed and placed in cold Krebs-bicarbonate solution. The whole adrenals were blotted carefully, weighed, and sliced with a razor blade (thickness: 0.5 mm). The slices were then placed in 20 ml of Krebs-bicarbonate solution maintained at 37° and bubbled with 95% O_2–5% CO_2. After 30 min of equilibration, 1 ml of the incubation medium, containing 1 mM NSD-1055, an inhibitor of aromatic L-amino acid decarboxylase, and 0.1 mM L-tyrosine, was renewed, and the slices were further incubated at 37°. After incubation, the slices and the medium were homogenized with ethanol for the estimation of DOPA as described below.

Kinetic studies of tyrosine hydroxylase. The adrenal slices, incubated at 37° for 30 min in various conditions were separated from the medium and rinsed prior to homogenization. They were homogenized with 9 vol. of 0.25 M sucrose, and the homogenates were quickly used for kinetic studies of TH. The final concentration of catecholamines in the incubation mixture for the TH assay *in vitro* was about 10^{-6} M. At this concentration of catecholamine, the kinetic values for TH were not affected. Enzyme activity was assayed according to a modification of the method of Nagatsu *et al.* [9]. The standard incubation mixture consisted of the following components in a total volume of 500 μl (final concentrations in parentheses) 50 μl of 1 M potassium phosphate buffer, pH 6.34 (100 mM), 100 μl of 1 mM L-tyrosine in 0.01 M HCl (200 μM or at various concentrations for kinetic studies), 50 μl of 10 mM 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$, 1 mM or at various concentrations for kinetics) in 1 M 2-mercaptoethanol (100 mM), 20 μl of 2 mg/ml catalase (40 μg/500 μl), 20 μl of 0.25 M sucrose (10 mM) containing enzyme, and water. For the blank incubation, D-tyrosine was used as a substrate instead of L-tyrosine, and 3-iodotyrosine (0.2 mM) was added to avoid the enzymatic formation of DOPA from the L-tyrosine that was present in the D-tyrosine in small amounts as a contaminant. DOPA (1 nmole or 500 pmoles) was added to another blank incubation as an internal standard. Incubation was at 30° for 10 min, and DOPA that formed was assayed by HPLC–ED as described below. With this assay method, the reaction proceeded linearly with time for 15 min at 30° and the reaction rate was linear up to 2 mg of tissue.

Estimation of DOPA. For the estimation of DOPA in both slices and homogenates, the reaction was stopped in an ice bath with 4.5 ml of ethanol solution containing 1 nmole or 500 pmoles of α-methyl-DOPA as an internal standard and 100 μl of 0.2 M EDTA; the mixture was homogenized in a glass Potter homogenizer and centrifuged at 3000 rpm for 30 min at 4°. To the supernatant fraction was added 500 μl of 2.5 M Tris–HCl buffer (pH 8.6) to adjust the pH to 8.0–8.5 and, then, 500 mg of acid-washed alumina. The mixture was shaken for 2.5 min to absorb DOPA. The supernatant fraction was discarded, and the alumina was twice washed with 5 ml of distilled water. After washing the alumina, 1 ml of 0.5 M HCl was added and DOPA was eluted for 1 min with shaking. The recovery of DOPA from alumina was 60%, the same as that of α-methyl-DOPA. A 50-μl aliquot of the eluate was injected into the high-performance liquid chromatograph (Yanaco L-2000) with an Yanaco VMD-100 electrochemical detector and a column (25 cm × 0.4 cm i.d.) packed with Yanapak ODS-T (particle size 10 μm) (Yanagimoto Manufacturing Co., Fushimi-ku, Kyoto, Japan). The mobile phase was 0.1 M phosphate and methanol (100:6.5, v/v) with a flow rate of 0.7 ml/min. The detector potential was set at 0.8 V above the Ag/AgCl electrode. Under these conditions the retention times were: solvent front, 2.2 min; DOPA, 7.2 min; and α-methyl-DOPA, 17.8 min.

RESULTS

To estimate TH activity in adrenal slices by measuring the conversion of L-tyrosine to DOPA, slices were incubated with or without exogenous L-tyrosine and with NSD-1055, an inhibitor of aromatic L-amino acid decarboxylase, in a Krebs-bicarbonate solution after equilibration for 30 min in the absence of exogenous tyrosine and NSD-1055. Figure 1 illustrates the effects of increasing concentrations of NSD-1055 on the rate of DOPA formation in adrenal slices. The formation of DOPA increased almost linearly between the concentrations of 10^{-5} and 10^{-7} M NSD-1055 and reached a plateau at about 0.5 mM. In the absence of NSD-1055, 4.6 pmoles of DOPA per 30 min was formed in the adrenal slices under these conditions. The effects of increasing concentrations of L-tyrosine on the rates of DOPA formation are shown in Fig. 2. The rate of hydroxylation of tyrosine in adrenal slices was linear with respect to the concentration of exogenous L-tyrosine; 160 pmoles of DOPA was formed from endogenous tyrosine. Usually, 1 mM NSD-1055 and 100 μM exogenous L-tyrosine were used in the slice experiments. The effect of α-methyl-*p*-tyrosine, an inhibitor of TH, on the formation of DOPA from L-tyrosine is shown in Fig. 3. α-Methyl-*p*-tyrosine (0.25 to 1 mM) depressed the formation of DOPA dose-

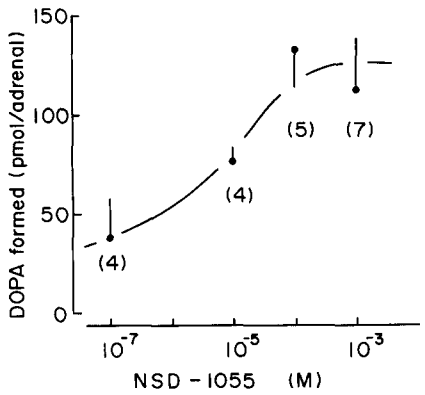

Fig. 1. DOPA formation in rat adrenal slices in the presence of NSD-1055. After equilibration at 37° for 30 min in a Krebs-bicarbonate solution, adrenal slices were incubated for 30 min at 37° in the presence of the indicated concentration of NSD-1055 and 100 μM L-tyrosine. DOPA formed is expressed as picomoles per one adrenal. The numbers in parentheses indicate the numbers of individual experiments. Data are plotted as the means, and vertical bars illustrate the S.E.M.

dependently, and a complete inhibition was observed at 1 mM α-methyl-*p*-tyrosine.

Figure 4 shows that DOPA formation was linear for 60 min. The adrenal slices were equilibrated for 30 min with Krebs-bicarbonate solution containing the normal low concentration of K^+, 4.7 mM.

When the slices were depolarized for 30 min in a Krebs-bicarbonate solution containing 52 mM K^+ and then transferred to the normal, low $[K^+]$, Krebs-bicarbonate solution containing L-tyrosine and NSD-1055, there was a significant increase in DOPA formation. As can be seen in Fig. 4, when slices were incubated for 15 min with L-tyrosine and NSD-1055 following 30 min of K^+-depolarization, there was a 76.9% increase in the formation of DOPA compared to non-depolarized control slices.

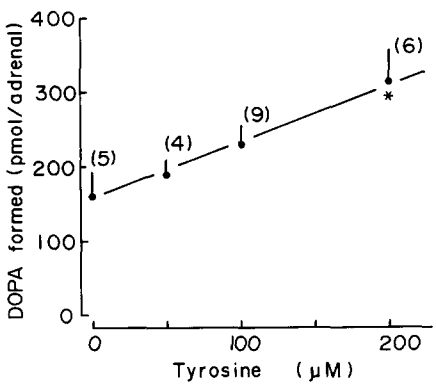

Fig. 2. Effect of L-tyrosine on DOPA formation in rat adrenal slices. After equilibration at 37° for 30 min in a Krebs-bicarbonate solution, adrenal slices were incubated for 60 min at 37° in the presence of the indicated concentration of L-tyrosine and 1 mM NSD-1055. DOPA formed is expressed as picomoles per one adrenal. The numbers in parentheses indicate the numbers of individual experiments. Data are plotted as the means, and vertical bars illustrate the S.E.M. Key: (*) statistical difference between the activity without exogenous tyrosine and that with exogenous tyrosine ($P < 0.05$), calculated according to Student's *t*-test.

After prior incubation with 52 mM K^+, adrenaline and noradrenaline contents were 40.7 ± 3.6 and 14.3 ± 4.5 nmoles/adrenal, respectively, and we did not observe significant differences in catecholamine contents between 0 and 15 min. When the high K^+ medium was maintained throughout the 60 min of incubation with tyrosine and NSD-1055, TH activity was stimulated about 4-fold. As shown in Fig. 4, high K^+ stimulated significantly the TH activity only during the first 15-min period. It is, therefore, speculated that the TH activated by prior incubation with high K^+ remained an active form only for 15 min and that, thereafter, may have been converted to a less active form.

The ability of K^+-depolarization to increase the rate of formation of DOPA from L-tyrosine was examined in a Ca^{2+}-free medium. The results in Table 1 show that, when Ca^{2+} was omitted from the control incubation medium, stimulation by high K^+ was not observed, although a slight, but not statistically significant, activation of TH in the Ca^{2+}-free medium was observed. In many studies, when Ca^{2+} was introduced into medium which had been Ca^{2+}-free, a brisk uptake of Ca^{2+} appeared to take place, associated with exocytotic release of neurotransmitter. This slight activation may have been due to brisk uptake of Ca^{2+}. Furthermore, the addition of a Ca^{2+} chelator, EGTA, to a Ca^{2+}-free incubation medium not only abolished the stimulation by high K^+ but also significantly inhibited the conversion of L-tyrosine to DOPA.

To assess whether the effect of the EGTA was a reversible phenomenon or not, the adrenal slice was washed with normal Krebs-bicarbonate solution for 30 min after the incubation in high-EGTA, Ca^{2+}-free medium. After washing, 92.1% of the TH activity was recovered. This result indicated that, in the high-EGTA, Ca^{2+}-free medium, TH was neither irreversibly inactivated nor denatured.

The kinetic parameters of TH were determined using homogenates of adrenal slices equilibrated for 30 min in normal medium, high K^+ medium, or high-EGTA, Ca^{2+}-free medium. The adrenal enzyme, following the equilibration in the normal Krebs-bicarbonate solution, did not obey normal Michaelis–Menten kinetics toward $6MPH_4$. As shown in Fig. 5A, two different K_m values were obtained depending on whether the concentrations of $6MPH_4$ were lower or higher than 100 μM. At concentrations lower than 100 μM, a relatively low K_m value of about 100 μM was observed, whereas at concentrations higher than 100 μM the K_m value was about 350 μM. In the adrenal enzyme of slices equilibrated with high-EGTA, Ca^{2+}-free Krebs-bicarbonate solution for 30 min, such deviation from Michaelis–Menten kinetics was more pronounced. In contrast, the enzyme activated in high K^+ gave a single low K_m value of about 100 μM. The apparent K_m values, for tyrosine, of adrenal enzyme in homogenates of slices incubated under the three different conditions were similar and were about 20 μM, respectively, when the TH assay was performed in the presence of 1 mM $6MPH_4$. As shown in Fig. 5B, the Lineweaver–Burk plots for tyrosine gave straight lines, suggesting that the enzyme obeyed Michaelis–Menten kinetics.

Table 1. Effect of Ca^{2+} removal on K^+-induced activation of DOPA formation in rat adrenal slices*

Incubation condition	DOPA formed (pmole/adrenal)		
	4.7mM K^+	52mM K^+	(%)
Normal	57.2 ± 7.8(7)	101.2 ± 4.7(4)	176.9†
Ca^{2+} free	68.7 ± 16.6(5)	91.9 ± 28.1(5)	133.8
Ca^{2+} free + EGTA	10.9 ± 0.6(3)	11.9 ± 0.8(3)	109.2

* After equilibration in a normal K^+, or high K^+, Krebs-bicarbonate solution under the indicated conditions for 30 min at 37°, adrenal slices were incubated for 15 min at 37° in a Krebs-bicarbonate solution containing 1 mM NSD-1055 and 100 μM tyrosine. DOPA formed was expressed as picomoles per one adrenal. Values in parentheses indicate the numbers of individual experiments. Results are means ± S.E.M. In the right-hand column, the DOPA formed in the presence of 52 mM K^+ is expressed as a percentage of that from samples incubated in the presence of 4.7 mM K^+.

† Statistical difference between the response of normal medium and that of high K^+ medium ($P < 0.05$).

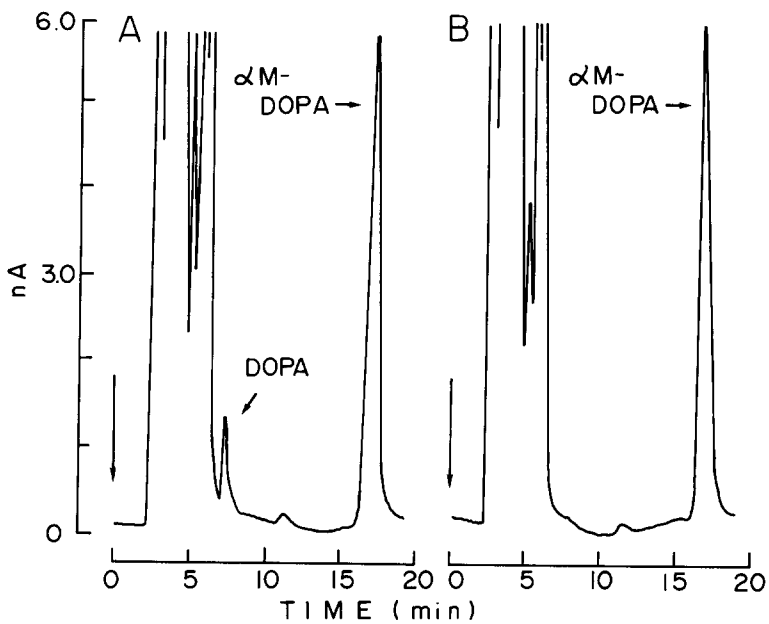

Fig. 3. Typical HPLC–ED pattern of endogenous DOPA formed from L-tyrosine in rat adrenal slices. (A) After equilibration at 37° for 30 min in a Krebs-bicarbonate solution, adrenal slices were incubated with 100 μM L-tyrosine and 1 mM NSD-1055 for 30 min at 37°. (B) After equilibration with 1 mM α-methyl-*p*-tyrosine in a Krebs-bicarbonate solution for 30 min at 37°, adrenal slices were incubated with 1 mM α-methyl-*p*-tyrosine, 100 μM L-tyrosine and 1 mM NSD-1055 at 37° for 30 min. The downward arrow indicates the time when samples were injected. Solvent front contained the peaks of noradrenaline and adrenaline. One nanomole of α-methyl-DOPA (α-M-DOPA) was added to each sample after the incubation, as an internal standard. The experimental conditions are described in Materials and Methods.

DISCUSSION

We developed a new method of TH assay in rat adrenal slices using HPLC–ED. All of the components of the TH system, i.e. the tetrahydropterin cofactor (probably tetrahydrobiopterin), dihydropteridine reductase, tyrosine, and the hydroxylase itself, are present under conditions which should be representative of the actual physiological state of the animal. By employing an inhibitor of aromatic L-amino acid decarboxylase, we were able to measure TH activity by determining the DOPA formed from tyrosine in rat adrenal slices. As shown in Fig. 1, the optimal concentration of NSD-1055 to estimate the hydroxylase system in adrenal slices was 1 mM. An HPLC–ED method for the TH assay [8] has many advantages. For example, the method is highly sensitive, simple and rapid, and economical since radioactively labeled substrate and a liquid scintillation spectrometer are not needed. We applied the HPLC–ED method to the determination of endogenous DOPA formed from tyrosine in rat adrenal slices. As shown in Fig. 3, the DOPA peak detected by HPLC–ED was completely abolished in the incubation with 1 mM α-methyl-*p*-tyrosine, a potent TH inhibitor. This indicates that DOPA formation from tyrosine in adrenal slices depends upon TH activity.

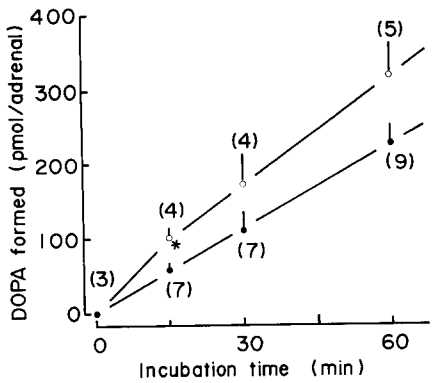

Fig. 4. Time course of DOPA formation in rat adrenal slices in normal or high K^+ medium. After equilibration with normal K^+ (●) or high K^+ (○) Krebs-bicarbonate solution at 37° for 30 min, adrenal slices were incubated for various periods of time in a Krebs-bicarbonate solution containing 1 mM NSD-1055 and 100 μM L-tyrosine at 37°. DOPA formed was expressed as picomoles per one adrenal. The numbers in parentheses indicate the numbers of individual experiments. Data are plotted as the means, and vertical bars illustrate the S.E.M. Key: (*) statistical difference between control and treated groups ($P < 0.05$), calculated according to Student's *t*-test.

The experimental system developed in this study to measure TH activity in adrenal slices consisted of two stages. Adrenal slices were first equilibrated with test or control medium and then transferred to fresh medium containing NSD-1055 and L-tyrosine for the TH assay incubation. In this way, the activity of TH in adrenal slices, manipulated under various circumstances, always could be measured under the same conditions.

Incubation of adrenal slices in medium containing high K^+ resulted in stimulation of DOPA formation. The action of high K^+ was dependent upon the presence of extracellular Ca^{2+}. In contrast, the addition of EGTA to a Ca^{2+}-free medium significantly inhibited the conversion of L-tyrosine to DOPA. The striking effect of Ca^{2+}-free medium plus EGTA versus Ca^{2+}-free medium is considered to be due to inhibition of TH. However, alternative possibilities are also probable. First, prior incubation with EGTA may inhibit the uptake of tyrosine, and the formation of DOPA could then be reduced because of reduced substrate availability. However, since the tissue concentration of tyrosine was as high as about 100 μM, the reduced uptake, if any, may not have affected the DOPA formation so markedly. Second, prior incubation with EGTA may inhibit the uptake of NSD-1055, and therefore DOPA would be converted to catecholamine which would not be detected in the assay employed. However, EGTA was only included in the prior incubation mixture and not during the incubation with NSD-1055. Thus, the uptake of NSD-1055 may not have been affected. Third, EGTA may produce a persistent effect on the membrane, allowing newly formed DOPA to leak out of slices into the medium. However, since our assay method measured DOPA in both the slices and the medium, such a change would not have affected our assay of enzymatically formed DOPA. It is concluded, therefore, that the effect observed may have been directly on the enzyme.

The experiments indicate that decrease of intracellular Ca^{2+} concentration reduced the TH activity in adrenal slices. It is well known that an increase in the frequency of nerve cell impulses cause an increase in the frequency of depolarization of the neuronal terminals, with a consequent increase in the influx of Ca^{2+}. It seems likely, therefore, that the effects of high K^+ on DOPA formation may have

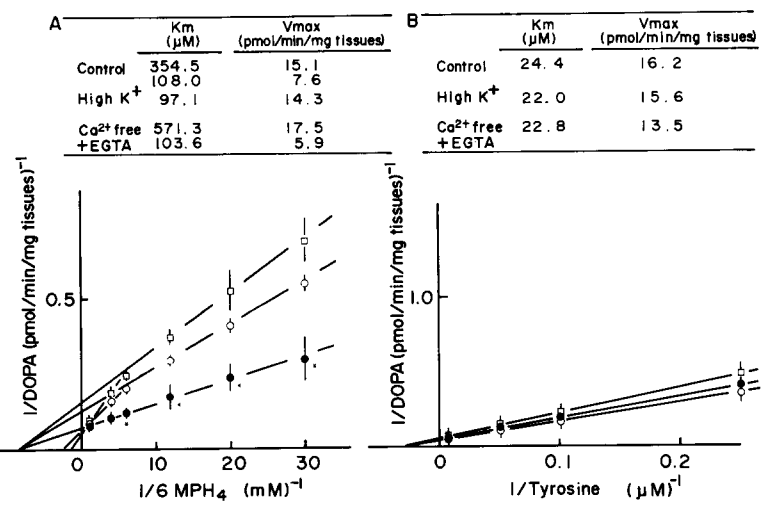

Fig. 5. Lineweaver–Burk plots illustrating the effect of 6-methyl-tetrahydropterin (A) or tyrosine (B) on the rate of DOPA formation by TH in homogenates of rat adrenal gland slices. TH activity was measured in homogenates of slices that had been equilibrated with normal (○), high K^+ (●) or high-EGTA, Ca^{2+}-free (□) medium for 30 min. The values shown are means from three experiments ± S.E.M. (A) Concentration of tyrosine, 2×10^{-4} M. (B) Concentration of 6-methyl-5,6,7,8-tetrahydropterin, 1×10^{-3} M. The K_m values and maximum velocities were determined from Lineweaver–Burk plots. Key: (*) statistical difference between control and treated groups ($P < 0.05$), calculated according to Student's *t*-test.

been mediated by uptake of Ca^{2+} into the cells. Morgenroth *et al.* [18] reported that addition of Ca^{2+} to a crude TH preparation of noradrenergic regions of the brain produced a marked activation of TH. In pheochromocytoma cells, the activation of TH by lasalocid, a Ca^{2+} ionophore, is not dependent upon the presence of extracellular Ca^{2+} [17]. Our results, together with the above-mentioned reports, suggest that intracellular Ca^{2+} may regulate TH activity in the adrenal gland.

In the present studies, the kinetic parameters of TH in adrenal slices homogenates were found to be considerably modified when adrenal slices were incubated prior to enzyme assay *in vitro*. The homogenates prepared from normal adrenal slices had two different K_m values for the tetrahydropterin cofactor, depending upon its concentrations. This result suggests that the enzyme may exist in two forms, a less active form with a relatively low affinity for pterin cofactor and a more active form in which the affinity for pterin cofactor is enhanced. In contrast, homogenates of adrenal slices depolarized by high K^+ had a single, low K_m value for pterin cofactor with classical Michaelis–Menten kinetics. This activation of TH was also associated with an increase in the V_{max} of the enzyme for its pterin cofactor. Very similar kinetic alterations of TH of the rat adrenal medulla after the application of acute stress have been reported by Masserano and Weiner [19]. They indicated that the kinetic change of adrenal TH is essentially similar to the effect of acute stress on the cyclic AMP-dependent protein kinase system. In recent years, there have been a number of studies which have shown that direct enzyme phosphorylation [20] causes activation of TH associated with kinetic changes. Joh *et al.* [21] have reported that activation by phosphorylation increases the V_{max} of TH without changing the K_m of the enzyme for pterin cofactor. On the other hand, it was reported that the K_m for pterin cofactor is decreased by phosphorylation, with no change in V_{max} [22, 23].

In summary, the observations reported here indicate that incubation of adrenal slices in a high K^+ medium brings about an increase in the activity, as well as changes in the kinetic properties, of TH. This activation in the enzyme did not occur in Ca^{2+}-free medium, and it seemed to be dependent on Ca^{2+} uptake in chromaffin cells. In addition, the inactivation of the enzyme observed in high-EGTA, Ca^{2+}-free medium suggests that TH activity may be affected by changes of intracellular Ca^{2+} concentration. On the basis of this evidence, we speculate that TH activity may be regulated through a Ca^{2+}-dependent event which is influenced by changes in intracellular Ca^{2+} concentration. Further experiments on the physiological significance of the two forms of TH with different K_m values for the pterin cofactor should be helpful in elucidating the details of the mechanism involved in TH activation.

REFERENCES

1. T. Nagatsu, M. Levitt and S. Udenfriend, *J. biol. Chem.* **239**, 2910 (1964).
2. T. Nagatsu, in *Advances in Experimental Medicine: A Centenary Tribute to Claude Bernard* (Eds. H. Parvez and S. Parvez), p. 527. Elsevier/North-Holland Biomedical Press, New York, (1980).
3. C. J. Refshauge, P. T. Kissinger, R. Dreinign, L. Blank, R. Freemand and R. N. Adams, *Life Sci.* **14**, 311 (1974).
4. R. M. Riggin, A. L. Shmidt and P. T. Kissinger, *J. pharm. Sci.* **64**, 680 (1975).
5. P. T. Kissinger, R. M. Riggin and R. L. Alcorn, *Biochem. Med.* **13**, 299 (1975).
6. L. J. Felice and P. T. Kissinger, *Analyt. Chem.* **48**, 794 (1976).
7. H. Hashimoto and Y. Maruyama, *J. Chromat.* **152**, 387 (1978).
8. Y. Maruyama and M. Kusaka, *Life Sci.* **23**, 1603 (1978).
9. T. Nagatsu, K. Oka and T. Kato, *J. Chromat.* **163**, 247 (1979).
10. W. C. Holland and H. J. Schumann, *Br. J. Pharmac. Chemother.* **11**, 449 (1956).
11. S. Bygdeman and U. S. von Euler, *Acta physiol. scand.* **44**, 375 (1958).
12. A. Alousi and N. Weiner, *Proc. natn. Acad. Sci. U.S.A.* **56**, 1941 (1966).
13. R. H. Roth, L. Stjarne and U. S. von Euler, *Life Sci.* **5**, 1071 (1966).
14. M. C. Boadle-Biber, J. Hughes and R. H. Roth, *Br. J. Pharmac.* **40**, 702 (1970).
15. W. W. Douglas and R. P. Rubin, *J. Physiol.* **159**, 40 (1961).
16. W. W. Douglas, *Br. J. Pharmac.* **34**, 451 (1968).
17. M. Chalfie, L. Settipani and R. L. Perlman, *Biochem. Pharmac.* **27**, 673 (1978).
18. V. H. Morgenroth III, M. C. Boadle-Biber and R. H. Roth, *Molec. Pharmac.* **12**, 427 (1975).
19. J. M. Masserano and N. Weiner, *Molec. Pharmac.* **16**, 513 (1979).
20. T. Yamauchi and H. Fujisawa, *J. biol. Chem.* **254**, 503 (1979).
21. T. H. Joh, D. H. Park and D. J. Reis, *Proc. natn. Acad. Sci. U.S.A.* **75**, 4744 (1978).
22. A. M. Edelman, J. D. Raese, M. A. Lazar and J. D. Barchas, *Commun. Psychopharmac.* **2**, 461 (1978).
23. M. M. Ames, P. Lerner and W. Lovenberg, *J. biol. Chem.* **253**, 27 (1978).