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A Nonisotopic Method for Determination of the *in Vivo* Activities of Tyrosine Hydroxylase in the Rat Adrenal Gland

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A rapid and reliable method for determination of *in vivo* activities of tyrosine hydroxylase in the rat adrenal gland is presented. This method involves determining the rate of accumulation of 3,4-dihydroxyphenylalanine (Dopa) in the adrenal gland after decarboxylase inhibition by NSD 1015, using HPLC with electrochemical detection after purification of the acid-deproteinized tissue extract with Bio-Rex 70 columns followed by alumina batch method. Purification of the sample with alumina adsorption alone, a method usually used for purification of catecholamines and Dopa, was ineffective: epinephrine and norepinephrine, which are present in high concentrations, interfered with an accurate determination of Dopa, and dopamine, which is retained strongly on the reverse-phase column, interfered with a rapid analysis. Purification with Sephadex G-10 columns followed by alumina adsorption was also ineffective. After purification with columns of weak cation-exchange resins such as Bio-Rex 70 or Amberlite CG-50 followed by alumina adsorption, most of the epinephrine and norepinephrine was removed and dopamine was eliminated. Thus a rapid and accurate determination of Dopa could be made. Of the two cation exchangers, Bio-Rex 70 was more effective. Accumulation of Dopa in the adrenal gland was linear up to 30 min after administration of NSD 1015 and a plateau was reached with doses over 10 mg/kg. Using this method, we investigated the effects of immobilization stress, reserpine, and hypoxia on *in vivo* activities of tyrosine hydroxylase in the adrenal gland.

KEY WORDS: adrenal gland; tyrosine hydroxylase; Dopa accumulation; immobilization; reserpine; hypoxia.

In response to various stimuli, the adrenal gland increases the secretion of catecholamines (CA),[2] epinephrine (E), and norepinephrine (NE) (1–7). To meet the altered conditions of demand for CA, the biosynthesis of CA in the adrenal gland is reported to increase (1,8–12). This increase in the biosynthesis of CA is believed to occur as a result of an increase in the activities of tyrosine hydroxylase (TH) (L-tyrosine, tetrahydropteridine:oxygen oxidoreductase, EC 1.14.16.2), a rate-limiting enzyme for the biosynthesis of CA, which catalyzes the formation of 3,4-L-dihydroxyphenylalanine (Dopa) from L-tyrosine (13). Among the mechanisms by which CA synthesis and secretion are coupled, two types of changes in TH seem to be particularly important. Short-term increases in CA release lead to a rapid activation of TH, which often expresses itself as an increased affinity of TH for its pteridine cofactor (14–17). In contrast, prolonged increases in CA release lead to a gradual elevation in the maximal velocity of the TH-

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[2] Abbreviations used: CA, catecholamines; E, epinephrine; NE, norepinephrine; TH, tyrosine hydroxylase; Dopa, 3,4-dihydroxyphenylalanine; AADC, aromatic L-amino acid decarboxylase; HPLC-ECD, high-performance liquid chromatography with electrochemical detection; NSD 1015, *m*-hydroxybenzylhydrazine; DHBA, 3,4-dihydroxybenzylamine; DA, dopamine.

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catalyzed reaction, owing to the apparent formation of additional enzyme molecules (18–23). However, it has not been confirmed whether the *in vivo* activities of TH in the adrenal gland are increased. Furthermore, it is totally unknown whether TH activities of the adrenal gland *in vivo* are regulated by tissue levels of substrates or the pteridine cofactor. This stems from the lacking of a reliable method for determination of the TH activities *in vivo*. A method for determination of *in vivo* TH activities in the brain, as first described by Carlsson *et al.* (24), involves determining the rate of accumulation of Dopa after administration of an inhibitor of aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28). At present, the Dopa accumulated is usually determined using high-performance liquid chromatography with electrochemical detection (HPLC-ECD), after purification of the acid-deproteinized tissue extract with activated alumina (25–28). However, such methods are not directly applicable for determination of *in vivo* TH activities in the adrenal gland, since total activities of TH in the gland are considerably low compared with those of the brain (13), and NE and E, which are present in very high concentrations in the adrenal gland, interfere with an accurate measurement of Dopa (26). Therefore, it is desirable to eliminate NE and E before determination of Dopa with HPLC-ECD.

In the present study, we attempted to purify the sample with columns of weak cation-exchange resins such as Bio-Rex 70 and Amberlite CG-50, or Sephadex G-10, which are reported to be effective for purification of CA and their metabolites (29–31), before purification with alumina adsorption and we found that Bio-Rex 70 is the most suitable for that purpose. We then examined the effects of immobilization stress and reserpine treatment, which are reported to increase TH activities as determined *in vitro* (18,19,32), and of exposure to hypoxia, which is expected to decrease the formation of Dopa by decreasing the concentration of oxygen, a substrate for TH, as observed in the brain (28,33), on TH activities of the adrenal gland *in vivo*.

MATERIALS AND METHODS

Reagents. Commercial sources of reagents were as follows: *m*-hydroxybenzylhydrazine (NSD 1015) dihydrochloride and 3,4-dihydroxybenzylamine (DHBA) hydrobromide from Aldrich (Milwaukee, WI); E bitartrate and reserpine from Sigma (St. Louis, MO); Dopa, dopamine (DA) hydrochloride, NE bitartrate, and acid-washed alumina from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); Bio-Rex 70 (200–400 mesh, sodium form) from Bio-Rad Laboratories (Richmond, CA); Amberlite CG-50 (type I) from Rohm and Haas Co. (Philadelphia, PA); and Sephadex G-10 from Pharmacia Fine Chemicals Co. (Uppsula, Sweden).

Standard stock solutions of CA and amino acids were prepared in 0.1 M HCl containing 1 mg/ml sodium bisulfite and 1 mM EDTA and were stored at −80°C. NSD 1015 was dissolved in physiological saline solution and reserpine in 20% ascorbic acid solution.

Bio-Rex 70 was prepared as described by Riggin and Kissinger (29). The resin was washed successively with 3 M HCl, 3 M NaOH, 3 M CH_3COOH, 1 M NH_4COOH (pH 6.5), and finally with 0.1 M NH_4COOH (pH 6.5). The resin was suspended and stored in 0.1 M NH_4COOH (pH 6.5). Amberlite CG-50 was prepared according to Karasawa *et al.* (34). The resin was washed by cycling through the acid and sodium form with 2 M HCl and 2 M NaOH and finally with water. The resin was equilibrated with 0.1 M NaH_2PO_4–K_2HPO_4 buffer (pH 6.1) and stored in the same buffer. Sephadex G-10 was washed with 10 mM HCOOH containing 100 μM EDTA and was then suspended in the same solution as described by Westerink and Mulder (31).

Preparation of tissue extracts. Male Wister rats, weighing 200–250 g, were intraperitoneally injected with 1 ml of saline or NSD

1015 solution and were decapitated at various times thereafter. The adrenal glands were removed and homogenized in 1 ml of 0.1 M perchloric acid containing 1 mg/ml sodium bisulfite and 1 mM EDTA. The homogenate was centrifuged at 17,300*g* for 15 min at 4°C and the resultant supernatant was used for the following experiments.

Purification and analysis of Dopa. For purification of Dopa with columns of Bio-Rex 70 or Amberlite CG-50, the pH of the supernatant (900 μl) was adjusted to 6–7 by adding 70 μl of 1 M K_2CO_3. After cooling in an ice bath for 20 min, the precipitated $KClO_4$ was removed by centrifugation at 3000*g* for 10 min at 4°C. The resultant supernatant (750 μl) was applied onto columns of Bio-Rex 70 or Amberlite CG-50 (8 × 38 mm). The effluent and the first 1.5-ml fraction of ice-cooled water were collected. For purification with columns of Sephadex G-10, 750 μl of the acid-deproteinized tissue extract was directly applied onto Sephadex G-10 columns (7 × 60 mm). After application of the sample, the columns were washed with 3.5 ml of 10 mM HCOOH containing 100 μM EDTA, and then Dopa was eluted from the column with the next 3 ml of the same solution.

After addition of 200 pmol of DHBA as an internal standard and 25 mg of acid-washed alumina to the eluate from these columns, the pH of the solution was adjusted to 8.3–8.4 with 1 M Tris–HCl buffer (pH 8.6). The mixture was vigorously shaken for 20 min. The supernatant was discarded and the alumina was washed three times with cold redistilled water. After washing, Dopa and DHBA were eluted with 300 μl of 0.5 M HCl containing 1 mg/ml sodium bisulfite and 1 mM EDTA. An aliquot of the eluate was injected onto HPLC-ECD. The chromatographic mobile phase was 0.1 M potassium phosphate buffer (pH 3.20) containing 2% methanol, 400 mg/liter sodium heptanesulfonate, and 100 μM EDTA, which was pumped at 0.7 ml/min. The potential of the electrochemical detector was set at +0.60 V against the Ag/AgCl reference electrode. The working electrode was of glassy carbon and the auxiliary electrode was of stainless steel. The HPLC system consisted of a Hitachi (Tokyo, Japan) LC 655 pump, a Hitachi 655A-40 automated sample injector, a Bioanalytical System LC 4B electrochemical detector, and a $5C_{18}$ reverse-phase column (4.6 × 150 mm).

Immobilization stress, reserpine treatment, and exposure to hypoxia. Rats were subjected to immobilization stress (2.5- and 24-h duration), reserpine treatment (5 mg/kg/day, ip, on 3 successive days), and a 1-h exposure to hypoxia (8% O_2 in N_2) as described (2,18,28).

Identification of Dopa by its conversion to DA by purified AADC. Hog AADC, which was purified to homogeneity according to the method of Christenson *et al.* (35), was used for the identification of Dopa. After purification of the acid-deproteinized tissue extract with Bio-Rex 70 columns followed by alumina adsorption, the pH of the eluate from acid-washed alumina was adjusted to 7.8–7.9

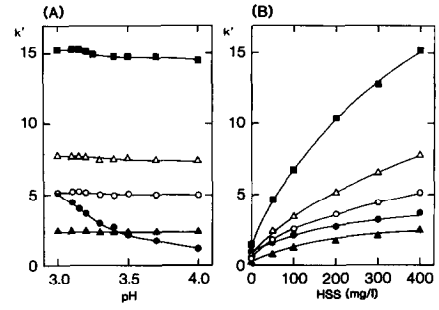

FIG. 1. Effects of the pH and the concentration of sodium heptanesulfonate (HSS) of the mobile phase on the retention time of Dopa, NE, E, DHBA, and DA with the concentration of methanol fixed at 2%. (A) The pH of the mobile phase was varied between 3.0 and 4.0 with the concentration of HSS fixed at 400 mg/liter. (B) The concentration of HSS was varied with the pH of the mobile phase fixed at 3.20. The retention time was expressed in terms of the capacity factor (*k'*). Mobile phase: 0.1 M potassium phosphate buffer containing 2% methanol, 100 μM EDTA, and varying concentrations of HSS. Flow rate: 0.7 ml/min; applied potential: +0.6 V; column: $5C_{18}$ reverse-phase column (4.6 × 150 mm). Dopa, ●; NE, ▲; E, ○; DHBA, △; DA, ■.

with 2 M Tris–HCl buffer (pH 8.6). An aliquot of the solution was incubated with an excess of purified AADC in 50 mM potassium phosphate buffer (pH 7.9) containing 1 mM pyridoxal phosphate at 30°C for 10 min. The reaction was stopped by addition of 1 M perchloric acid. After centrifugation, an aliquot of the supernatant was analyzed for Dopa and DA using HPLC-ECD.

Statistical analysis. All results are expressed as means ± SEM Student's t test for unpaired comparison was used for statistical analyses, and a probability level of $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Figure 1 shows the effects of the pH and the concentration of sodium heptanesulfonate, an ion-pairing agent, of the mobile phase on the retention time of NE, Dopa, E, and DA, with the concentration of methanol fixed at 2%. For the analysis of Dopa, the pH of the mobile phase was critical: the retention time of NE, E, and DA, expressed in terms of the capacity factor (k'), was little changed with an increase in the pH of the mobile phase, whereas that of Dopa was decreased. The optimal pH for separation of Dopa from CA was about 3.20 (Fig. 1A). On the other hand, as the concentration of sodium heptanesulfonate was increased with the pH fixed at 3.20, the retention time of Dopa as well as CA was increased (Fig. 1B), the data showing that to achieve an adequate separation of Dopa from CA, the concentration of sodium heptanesulfonate in the mobile phase should be as high as possible. In the present experiments, we analyzed samples using 0.1 M potassium phosphate buffer (pH 3.20) containing 2% methanol and 400 mg/liters sodium heptanesulfonate.

We compared various methods for purification of the acid-deproteinized tissue extract prepared from rats injected with NSD 1015 (100 mg/kg) before determination of Dopa using HPLC-ECD. As reported (26), after purification of the sample with alumina adsorption alone, a method usually used for purification of CA and Dopa (25–28), large peaks of E and NE interfered with an accurate determination of Dopa using HPLC-ECD. Moreover, DA, which was retained strongly on the reverse-phase column, dis-

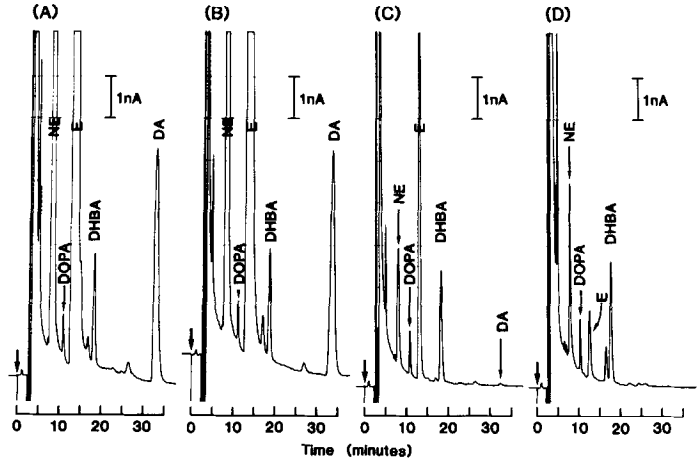

FIG. 2. Chromatograms of the adrenal gland after purification with alumina adsorption alone (A) or after purification with columns of Sephadex G-10 (B), Amberlite CG-50 (C), or Bio-Rex 70 (D) followed by alumina adsorption. Rats were intraperitoneally injected with NSD 1015 (100 mg/kg) and decapitated 20 min thereafter. Dopa which had accumulated in the adrenal gland was determined using HPLC-ECD after purification with four methods.

FIG. 3. Chromatograms of the adrenal gland after incubation in the absence (A) or presence (B) of purified aromatic L-amino acid decarboxylase. Acid-deproteinized tissue extract, prepared from rats injected with NSD 1015 (100 mg/kg), was processed with columns of Bio-Rex 70 followed by activated alumina. After adjustment of the pH of the eluate from activated alumina, the solution was incubated at 30°C for 10 min in the absence or presence of purified aromatic L-amino acid decarboxylase and the reaction was stopped by addition of 1 M perchloric acid. After centrifugation, an aliquot of the supernatant was injected onto HPLC-ECD.

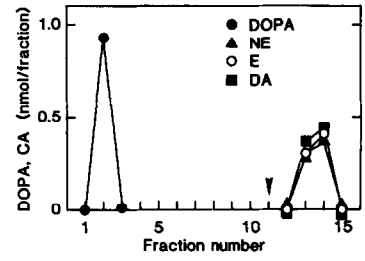

FIG. 4. Elution profile of Dopa, NE, E, and DA from Bio-Rex 70 columns. Dopa, NE, E, and DA (1 nmol in 1 ml) were applied onto Bio-Rex 70 columns (8 × 38 mm). After application of the sample, elution was performed with 10 ml of water followed by 0.5 M HCl. Fractions of 1 ml were collected. An arrowhead indicates a change of eluant from water to 0.5 M HCl.

turbed a rapid analysis of the sample (Fig. 2A). After purification with Sephadex G-10 columns, which are reported to be effective for purification of CA and Dopa (31), followed by alumina adsorption, essentially similar results were obtained (Fig. 2B). In contrast, after purification of the sample with columns of weak cation-exchange resins such as Bio-Rex 70 or Amberlite CG-50 followed by alumina adsorption, the peaks of NE and E became much smaller and the peak of Dopa was clearly separated from these interfering substances (Figs. 2C and 2D). Furthermore, DA was almost completely eliminated. These data show that after purification of the sample with columns of weak cation-exchange resins followed by alumina adsorption, Dopa accumulated in the adrenal gland after administration of an inhibitor of AADC can be determined accurately and rapidly. Of the two cation-exchange resins, Bio-Rex 70 seems more suitable for the assay, since the resin removed NE and E more effectively than did Amberlite CG-50.

We then attempted to identify the electrochemically active material that accumulated after administration of NSD 1015 and exhibited a retention time identical to that of authentic Dopa, as Dopa by its conversion to DA after incubation with purified AADC (Fig. 3). Acid-deproteinized tissue extract,

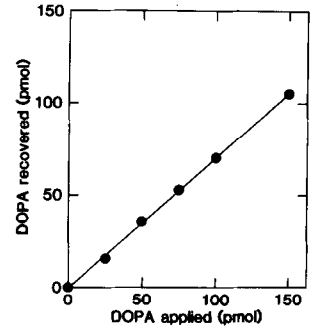

FIG. 5. A standard curve obtained with an authentic Dopa carried through purification procedures with columns of Bio-Rex 70 followed by alumina adsorption. Various amounts of Dopa were applied onto Bio-Rex 70 columns and Dopa was eluted with 1.5 ml of water which was combined with the column effluent. Dopa in the combined solution was extracted using alumina batch method as described under Materials and Methods.

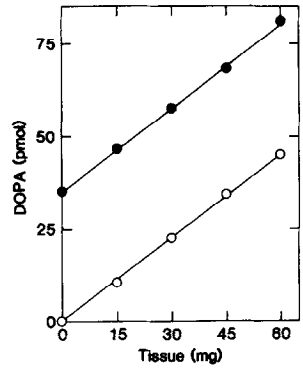

FIG. 6. Tissue dependency and recovery of authentic Dopa in the presence of the acid-deproteinized tissue extract. Various amounts of the pooled acid-deproteinized tissue extract prepared from rats injected with NSD 1015 (100 mg/kg), with (●) or without (○) addition of 50 pmol of authentic Dopa, were extracted as described in the legend to Fig. 5.

prepared from rats injected with NSD 1015 (100 mg/kg), was processed with columns of Bio-Rex 70 followed by alumina adsorption. When the alumina eluate was incubated with purified AADC after pH adjustment, the peak corresponding to Dopa almost completely disappeared with a concomitant appearance of the peak corresponding to DA. Thus, the electrochemically active material that exhibited a retention time identical to that of authentic Dopa was considered to be Dopa.

Figure 4 shows an elution profile of Dopa, NE, E, and DA from Bio-Rex 70 columns. Dopa was eluted in the effluent and during washings with water, whereas NE, E, and DA were eluted after a change of eluant from water to 0.5 M HCl. Thus, we collected the effluent and the first 1.5-ml fraction of water for analysis of Dopa.

A standard curve obtained with an authentic Dopa carried through purification procedures with columns of Bio-Rex 70 followed by alumina adsorption was linear up to 150 pmol of Dopa and the recovery of Dopa through the two steps was 70 ± 2% (Fig. 5). The amount of endogenous Dopa increased linearly with increasing amounts of tissue extract prepared from rats injected with NSD 1015 (100 mg/kg). The recovery of 50 pmol of authentic Dopa was unaffected in the presence of various amounts of the tissue extract (Fig. 6).

Figure 7 shows the time course of accumulation of Dopa in the adrenal gland after an intraperitoneal injection of NSD 1015 (100 mg/kg). Dopa accumulated linearly at least up to 30 min, a finding comparable to that observed for the brain (24).

Figure 8 shows Dopa accumulation after administration of various doses of NSD 1015. Dopa accumulation in the adrenal gland reached a plateau after a NSD 1015 dose of 10 mg/kg, a minimum dose tested. The dose–response relationships were also examined in the heart and brain. Dopa accumulation in the heart also reached a plateau after a dose of 10 mg/kg. In contrast, Dopa accumulation in the brain reached a plateau after a dose of 50 mg/kg, which was in good agreement with the results of Carlsson *et al.* (24).

To demonstrate the validity of the present method, we examined the effects of immobilization stress (2.5- and 24-h duration), re-

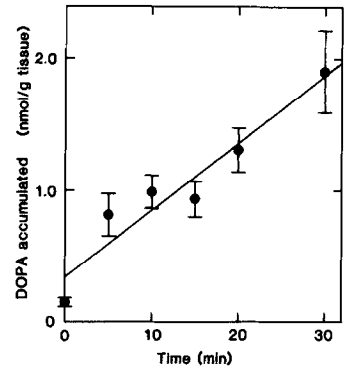

FIG. 7. Time course of accumulation of Dopa in the adrenal gland after administration of NSD 1015. Rats were intraperitoneally injected with NSD 1015 (100 mg/kg) and decapitated at various times thereafter. Dopa which had accumulated in the adrenal gland was determined using HPLC-ECD after purification with columns of Bio-Rex 70 followed by alumina adsorption. Each point represents the mean ± SE of four determinations.

serpine treatment, and 1-h exposure to hypoxia on the *in vivo* TH activities in the adrenal gland (Table 1). Immobilization stresses of 2.5- and 24-h duration and reserpine treatment (5 mg/kg/day, ip, 3 days), which are reported to increase TH activities as assayed *in vitro* (18,19,32), increased Dopa accumulation after administration of NSD 1015 by about 50, 230, and 140%, respectively. In contrast, exposure to hypoxia for 1 h, which is expected to decrease the *in vivo* TH activities by decreasing the concentration of oxygen, a substrate for TH, as observed in the brain (28,33), decreased Dopa accumulation by about 40%. These results show that the present method is valid for determination of the *in vivo* activities of TH in the adrenal glands.

Using the present method, we are investigating the regulatory mechanisms of TH activities in the adrenal gland under various conditions, which might involve changes not only in kinetic parameters of TH but also in tissue levels of substrates or pteridine cofactor.

TABLE 1

EFFECTS OF IMMOBILIZATION STRESS (2.5- AND 24-h DURATION), RESERPINE TREATMENT, AND 1-h EXPOSURE TO HYPOXIA ON DOPA ACCUMULATION IN THE ADRENAL GLAND AFTER AN INTRAPERITONEAL INJECTION OF NSD 1015

	Dopa accumulated (pmol/g tissue/20 min)	
Treatment	Control	Treated
Immobilization		
2.5 h	1294 ± 171	1980 ± 156**
24 h	1202 ± 107	3992 ± 455**
Reserpine	873 ± 210	2064 ± 250**
Hypoxia	1120 ± 145	680 ± 88*

Note. Values are means ± SE of six determinations.
* $P < 0.05$; ** $P < 0.01$; significantly different from control values.

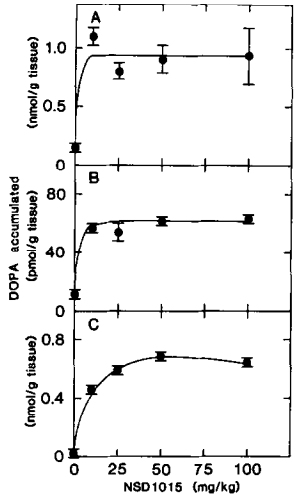

FIG. 8. Effects of various doses of NSD 1015 on Dopa accumulation in the adrenal gland (A), heart (B), and brain (C). Rats were intraperitoneally injected with various doses of NSD 1015 and decapitated 20 min thereafter. Dopa which had accumulated in the adrenal gland was determined after purification of the sample with columns of Bio-Rex 70 followed by alumina adsorption. Dopa in the heart and brain was determined after purification with alumina adsorption alone. Each point represents the mean ±SE of four determinations.

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