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# Enhancement of In Vivo Tyrosine Hydroxylation in the Rat Adrenal Gland Under Hypoxic Conditions

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**Abstract:** We examined the effects of hypoxia (8% $O_2$) on in vivo tyrosine hydroxylation, a rate-limiting step for catecholamine synthesis, in the rat adrenal gland. The hydroxylation rate was determined by measuring the rate of accumulation of 3,4-dihydroxyphenylalanine (DOPA) after decarboxylase inhibition. One hour after hypoxic exposure, DOPA accumulation decreased to 60% of control values, but within 2 h it doubled. At 2 h, the apparent $K_m$ values for tyrosine and for biopterin cofactor of tyrosine hydroxylase (TH) in the soluble fraction were unchanged, whereas the $V_{max}$ value increased by 30%. The content of total or reduced biopterin was unchanged, but the content of tyrosine increased by 80%. Tyrosine administration had little effect on DOPA accumulation under room air conditions but enhanced DOPA accumulation under hypoxia. After denervation of the adrenal gland, the hypoxia-induced increase in DOPA accumulation and in the $V_{max}$ value was abolished, whereas the hypoxia-induced increase in tyrosine content was persistent. These results suggest that in vivo tyrosine hydroxylation is enhanced under hypoxia, although availability of oxygen is reduced. The enhancement is the result of both an increase in tyrosine content coupled with increased sensitivity of TH to changes in tyrosine tissue content and of an increase in dependence of TH on tyrosine levels. The increase in the sensitivity of TH and in the $V_{max}$ value is neurally induced, whereas the increase in tyrosine content is regulated by a different mechanism. **Key Words:** Tyrosine hydroxylase—Adrenal gland—Hypoxia—Tyrosine—Biopterin—3,4-Dihydroxyphenylalanine accumulation. **Hayashi Y. et al.** Enhancement of in vivo tyrosine hydroxylation in the rat adrenal gland under hypoxic conditions. *J. Neurochem.* **54,** 1115–1121 (1990).

It is generally accepted that in response to various stimuli that increase the secretion of catecholamines (CA) from the adrenal medulla into the plasma, the biosynthesis of CA in the adrenal gland increases to meet the increased demand for CA (Holland and Schümann, 1956; Bygdeman et al., 1960; Hökfelt and Bygdeman, 1961; Gordon et al., 1966; Dairman and Udenfriend, 1970; Kvetnansky et al., 1971*b*). This increase in the biosynthesis of CA is believed to occur as a result of an increase in the activity of tyrosine hydroxylase (L-tyrosine, tetrahydropteridine:oxygen oxidoreductase, EC 1.14.16.2; TH), a rate-limiting enzyme for the biosynthesis of CA, which catalyzes the formation of 3,4-dihydroxyphenylalanine (DOPA) from L-tyrosine and oxygen (Nagatsu et al., 1964). Among various possible 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 following increase in afferent nerve activity lead to a rapid activation of TH, which is often expressed as an increased affinity of TH for its pteridine cofactor (short-term regulation) (Masserano and Weiner, 1979, 1981; Masserano et al., 1981; Fluharty et al., 1985). In contrast, prolonged increases in CA release lead to a gradual elevation in the maximal velocity of the TH-catalyzed reaction, owing to the apparent formation of additional enzyme molecules (long-term regulation) (Mueller et al., 1969; Thoenen et al., 1969; Kvetnansky et al., 1971*a*; Hoeldtke et al., 1974; Chuang and Costa, 1974; Tank et al., 1985).

Several lines of evidence suggest that systemic hypoxia is one of the stimuli that increase the secretion of CA from the adrenal gland into the plasma (Steinsland

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*Abbreviations used:* $BH_4$, 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin; CA, catecholamine(s); DHBA, 3,4-dihydroxybenzylamine; DOPA, 3,4-dihydroxyphenylalanine; NSD 1015, *m*-hydroxybenzylhydrazine; TH, tyrosine hydroxylase.

et al., 1970; Myles and Ducker, 1971; Bloom et al., 1977; Lee et al., 1987). However, because hypoxia is a condition in which the tissue level of oxygen, a substrate for TH, is decreased, we wondered if CA biosynthesis could be increased under hypoxic conditions. Therefore, in the present investigation, we examined the effects of hypoxia on in vivo tyrosine hydroxylation in the adrenal gland, using a nonisotopic method for determination of in vivo tyrosine hydroxylation, which we recently developed (Hayashi et al., 1988).

## MATERIALS AND METHODS

### Reagents

Commercial sources of reagents were as follows: L-[1-$^{14}$C]tyrosine (56 mCi/mmol), from Amersham, Arlington Heights, IL, U.S.A.; *m*-hydroxybenzylhydrazine (NSD 1015) dihydrochloride and 3,4-dihydroxybenzylamine (DHBA) hydrobromide, from Aldrich, Milwaukee, WI, U.S.A.; DOPA, catalase, 3-iodo-L-tyrosine, and L-tyrosine ethylester, from Sigma, St. Louis, MO, U.S.A.; acid-washed alumina, L-tyrosine, and pyridoxal phosphate, from Wako Pure Chemical Industries, Osaka, Japan; Bio-Rex 70 (200–400 mesh, sodium form) and Dowex 50W-X8 (200–400 mesh, $H^+$ form), from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Sephadex G-25, from Pharmacia Fine Chemicals, Uppsala, Sweden; 6*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin ($BH_4$) and L-*erythro*-biopterin were generous gifts from Suntory Biomedical Institute, Osaka, Japan.

### Hypoxic exposure

Because exposure to hypoxia of 8% $O_2$ in $N_2$ induced an increase in CA turnover in the rat adrenal gland as previously reported by Lee et al. (1987), we used this concentration of oxygen in the present investigation. Male Wistar rats, weighing 200–250 g, were exposed to room air or to hypoxia (8% $O_2$) for various time periods in a chamber as described previously by Miwa et al. (1986). Either room air or a gas mixture of 8% $O_2$ in $N_2$ was continuously passed through the chamber at a flow rate of 4 L/min. The gas mixture in the chamber was circulated through a $CO_2$ scrubber containing soda lime to prevent accumulation of carbon dioxide. The concentrations of oxygen and carbon dioxide in the chamber were continuously monitored. The $O_2$ concentration under control and hypoxic conditions was 21.0 ± 0.1% and 8.0 ± 0.1%, respectively, throughout the experiments. The $CO_2$ concentration was 0.04 ± 0.01% under both control and hypoxic conditions. Both hypoxic and control rats were kept at a constant room temperature of 25 ± 0.5°C and were deprived of food and water during the experimental periods to avoid effects of nutritional changes.

### Estimation of in vivo tyrosine hydroxylation

In vivo tyrosine hydroxylation in the adrenal gland and the brain was estimated from measurements of the rate of accumulation of DOPA following decarboxylase inhibition. Because DOPA accumulated in the adrenal gland and the brain linearly up to 30 min after an intraperitoneal injection of NSD 1015 (100 mg/kg) as described previously by Hayashi et al. (1988), we estimated the rate of in vivo tyrosine hydroxylation by measuring the amount of DOPA accumulated during the 20 min immediately following the injection of NSD 1015. After exposure to room air or hypoxia for various time periods, rats were intraperitoneally injected with 100 mg/kg of NSD 1015 and the same atmospheric conditions were maintained for an additional 20 min. At the indicated time, the rats were decapitated and the brain and a pair of adrenal glands were rapidly removed.

DOPA accumulated in the adrenal gland was determined according to the method that we recently described (Hayashi et al., 1988). In brief, a pair of adrenal glands was homogenized in 1 ml of 0.1 *M* perchloric acid containing 1 m*M* EDTA and 1 mg/ml sodium bisulfite and the homogenate was centrifuged at 17,500 *g* for 15 min at 4°C. The pH of the supernatant (0.9 ml) 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 3,000 *g* for 10 min at 4°C. The resultant supernatant (750 μl) was applied onto columns (8 mm × 38 mm) of Bio-Rex 70. The effluent and the next 1.5-ml fraction of ice-cold water were collected for determination of DOPA. Following addition of 200 pmol of DHBA as an internal standard and 25 mg of acid-washed alumina to the eluate from each column, 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 distilled water. After washing, DOPA and DHBA were eluted with 300 μl of 0.5 *M* HCl containing 1 m*M* EDTA and 1 mg/ml of sodium bisulfite.

For determination of DOPA accumulation in the brain, the whole brain was homogenized in 10 ml of 0.1 *M* perchloric acid containing 1 m*M* EDTA and 1 mg/ml of sodium bisulfite and the homogenate was centrifuged at 17,500 *g* for 15 min. DOPA in the supernatant was extracted using the alumina batch method as described previously by Miwa et al. (1986).

An aliquot of the alumina eluate was analyzed using HPLC with electrochemical detection. For determination of DOPA in the adrenal gland, the chromatographic mobile phase consisted of 0.1 *M* potassium phosphate buffer (pH 3.20) containing 2% methanol, 400 mg/L sodium heptane sulfonate, and 100 μ*M* EDTA. The chromatographic mobile phase for determination of DOPA in the brain contained 6% methanol in place of 2% methanol. The mobile phase was pumped at 0.7 ml/min. The potential of the electrochemical detector was set at +0.65 V against the Ag/AgCl reference electrode. The working electrode was of glassy carbon and the auxiliary electrode was of stainless steel.

### Assay of tyrosine

The concentrations of tyrosine in the adrenal gland and the brain were determined by the method of Holman and Snape (1983) using HPLC with electrochemical detection. The adrenal gland and the brain were homogenized in 1 ml and 10 ml, respectively, of 0.1 *M* perchloric acid containing 1 m*M* EDTA and 1 mg/ml of sodium bisulfite and the homogenates were centrifuged at 17,500 *g* for 15 min at 4°C. The supernatant was directly analyzed for tyrosine using HPLC with electrochemical detection. The chromatographic mobile phase consisted of 0.1 *M* potassium phosphate buffer (pH 2.90) containing 6% methanol, 200 mg/L sodium heptane sulfonate, and 100 μ*M* EDTA, and was pumped at a flow rate of 0.7 ml/min. The potential of the electrochemical detector was set at +0.8 V against the Ag/AgCl reference electrode.

### Assay of biopterin

Biopterin was assayed using HPLC with fluorescence detection according to the method of Fukushima and Nixon (1980) as modified by Miwa et al. (1985). A pair of adrenal

glands was homogenized in 4.5 ml of 0.1 *M* phosphoric acid. For the assay of total (reduced + oxidized) biopterin, the homogenate (2 ml) was mixed with 0.22 ml of 2 *M* trichloroacetic acid and 0.18 ml of the iodine solution (1% $I_2$/2% KI in distilled water); for the assay of alkali-stable (oxidized) biopterin, the homogenate (2 ml) was mixed with 0.23 ml of 2 *M* NaOH and 0.27 ml of the iodine solution. These mixtures were incubated at 25°C for 1 h. After incubation, 0.25 ml of 2 *M* trichloroacetic acid was added only to the mixture for alkali-stable biopterin. Excess iodine was reduced by adding a 1% ascorbic acid solution. After centrifugation at 27,000 *g* for 15 min, the resulting supernatant was applied to a column (0.7 cm × 1 cm) of Dowex 50W-X8 ($H^+$ form, 200–400 mesh). The column was washed with 6 ml of cold distilled water and pteridines were eluted with 3 ml of 1 *M* $NH_4OH$. The eluate was neutralized with 0.5 ml of glacial acetic acid and analyzed by HPLC with fluorescence detection. The reduced biopterin was determined by subtracting oxidized biopterin from total biopterin. The chromatographic mobile phase was 0.1 *M* sodium acetate buffer (pH 3.7) and was delivered at a flow rate of 0.7 ml/min. A fluorescence spectrometer was used with excitation at 350 nm and emission at 450 nm.

### HPLC system

The HPLC system consisted of a Hitachi LC 655 pump, a Hitachi 655A-40 automated sample injector, a Bioanalytical System LC 4B electrochemical detector, a Hitachi F1000 fluorescence spectrometer, and a $5C_{18}$ reverse-phase column (4.6 × 200 mm).

### Assay of TH in vitro

Because it has been suggested that decapitation itself activates TH in the adrenal gland (Masserano and Weiner, 1979), a pair of adrenal glands was removed under anesthesia with sodium pentobarbital (60 mg/kg, i.p.). A pair of adrenal glands was homogenized in 1 ml of 50 m*M* Tris-acetate buffer (pH 7.4) and the homogenate was centrifuged at 39,000 *g* for 15 min at 4°C. The resulting supernatant then was passed through a Sephadex G-25 column (12 cm × 0.9 cm) to remove CA that otherwise might inhibit TH activity. The column was washed with 2 ml of homogenization buffer and then TH protein was eluted with 3 ml of the same buffer. The TH activity of the soluble fraction of the adrenal glands was determined by means of the coupled decarboxylase assay of Waymire et al. (1971) as modified by Watanabe et al. (1981). Unless otherwise specified, the standard assay mixture (200 μl) contained: 100 m*M* HEPES buffer (pH 7.1), 30 μ*M* [1-$^{14}$C]tyrosine (sp act, 5.6–11.2 Ci/mol), 1 m*M* $BH_4$, 0.2 mg/ml catalase, 100 m*M* mercaptoethanol, and enzyme preparations. The final pH of the reaction mixture was 6.8 at 30°C. Following a 10-min incubation at 30°C, 800 μl of a mixture containing the following ingredients was added to terminate L-DOPA formation and to promote its quantitative decarboxylation at pH 7.8: 3-iodotyrosine (final concentration, 2 m*M*); pyridoxal 5-phosphate (0.1 m*M*); potassium phosphate buffer, pH 8.2 (100 m*M*); EDTA (1 m*M*); and excess hog kidney aromatic L-amino acid decarboxylase, purified according to Christenson et al. (1970). Following a second 20-min incubation at 30°C, the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid and the mixture was incubated further for 60 min at 30°C. Radioactive $^{14}CO_2$ was trapped by connecting assay tubes via thick-walled rubber tubing to counting vials containing GF/B filter soaked in 0.3 ml of β-phenylethylamine solution (20% vol/vol in methanol). Radioactivity was determined by liquid scintillation counting after addition of LSC Complete.

Protein concentrations were determined by the method of Lowry et al. (1951).

### Denervation of the adrenal gland

Rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and were immobilized in the prone position. The longitudinal skin incision, which extended downward about 3 cm from just below the lowest rib, was made 0.5 cm lateral to the midline. Following blunt dissection of muscle layers, the left adrenal gland was exposed and the left greater splanchnic nerve was excised carefully under a dissecting microscope so as not to damage small arteries and veins.

### Statistical analysis

All results were expressed as means ± SEM. The data were subjected to a two-way analysis of variance, and, when significant *F* values were encountered, the Newman–Keuls multiple-range test was used to test for significant differences between treatment means (Steel and Torric, 1960). A probability level of $p < 0.05$ was considered significant.

## RESULTS

Figure 1 shows the time course of the effects of hypoxia on DOPA accumulation in the adrenal gland and the brain following administration of NSD 1015 (100 mg/kg). DOPA accumulation in the adrenal gland decreased to about 60% of the control value 1 h after exposure to hypoxia, but within 2 h, it had increased to about 200% of the control value. The increase was maintained up to 6 h after exposure to hypoxia (Fig. 1A). As previously reported (Davis and Carlsson, 1973; Miwa et al., 1986), DOPA accumulation in the brain decreased to about 60% of the control value 1 h after exposure to hypoxia and remained low up to 6 h (Fig. 1B).

To determine whether the increase in DOPA accumulation in the adrenal gland, as an index of in vivo

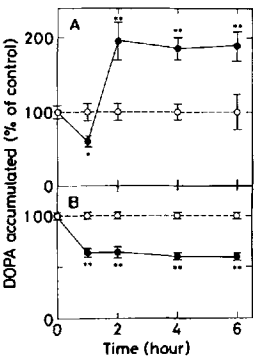


**FIG. 1.** Time course of effects of hypoxia (8% $O_2$ in $N_2$) on DOPA accumulation in the adrenal gland (**A**) and the brain (**B**) following decarboxylase inhibition. Rats were exposed to either room air (○ – – ○) or hypoxia (● —— ●) for various periods and were intraperitoneally injected with NSD 1015 (100 mg/kg), an inhibitor of aromatic L-amino acid decarboxylase, 20 min before they were killed. At the indicated times, the rats were decapitated and the DOPA accumulated in the adrenal gland and the brain was measured as described under Materials and Methods. Each value is expressed as a percentage of the control value obtained under room air and is the mean ± SEM of eight determinations. Control values for the adrenal gland (pmol/g of tissue/20 min): 0 h, 920 ± 98; 1 h, 1,040 ± 134; 2 h, 850 ± 74; 4 h, 778 ± 83; 6 h, 700 ± 91. Control values for the brain (pmol/g of tissue/20 min): 0 h, 398 ± 23; 1 h, 441 ± 11; 2 h, 442 ± 31; 4 h, 367 ± 28; 6 h, 369 ± 20. $^*p < 0.05$; $^{**}p < 0.01$; significantly different from the control values obtained under room air.

**TABLE 1.** *Effects of 2-h exposure to hypoxia on $K_m$ and $V_{max}$ of TH in the soluble fraction of the rat adrenal gland*

| | Room air | Hypoxia |
|---|---|---|
| $K_m$ for $BH_4$ ($\mu M$) | 86.2 ± 8.7 | 82.4 ± 6.6 |
| $K_m$ for tyrosine ($\mu M$) | 19.6 ± 1.9 | 18.9 ± 4.6 |
| $V_{max}$ (nmol/mg of protein/h) | 49.4 ± 3.2 | 66.5 ± 6.9[a] |

After 2-h exposure to room air or to hypoxia (8% $O_2$ in $N_2$), rats were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). A pair of adrenal glands was removed, homogenized, and centrifuged. The resulting supernatant was passed through a Sephadex G-25 column (12 cm × 0.9 cm) and the 3-ml fraction following the void volume (2 ml) was collected. TH activity was determined as described under Materials and Methods. For determination of kinetic parameters ($K_m$ and $V_{max}$) of TH, the concentration of tyrosine in the reaction mixture was varied from 1.5 $\mu M$ to 30 $\mu M$ with that of $BH_4$ fixed at 1 m$M$, or the concentration of $BH_4$ was varied from 20 $\mu M$ to 1 m$M$ with that of tyrosine fixed at 30 $\mu M$. Apparent kinetic parameters ($K_m$ and $V_{max}$) were determined from least-squares linear regression analysis of the linear double-reciprocal plots. The values are means ± SEM of five determinations obtained from five different pairs of adrenal glands.

[a] $p < 0.05$; significantly different from control values.

tyrosine hydroxylation, is a result of a change in the kinetic parameters of TH, we examined the Michaelis constant ($K_m$) and the maximal velocity ($V_{max}$) of TH using the soluble fraction prepared from the adrenal glands of the rats exposed to either room air or hypoxia for 2 h (Table 1). The $K_m$ value for tyrosine or pteridine cofactor was unaffected by the 2-h exposure to hypoxia but the $V_{max}$ value increased by about 30%. Because the 100% increase in DOPA accumulation cannot be explained quantitatively by just a 30% increase in the $V_{max}$ value, we then attempted to determine the content of a substrate (tyrosine) and a cofactor ($BH_4$) for TH in the adrenal gland under both control and experimental conditions, considering the possibility that in vivo tyrosine hydroxylation is enhanced by increased availability of either the substrate, the cofactor, or both.

Table 2 shows the effects of 2-h exposure to hypoxia on the content of total and reduced biopterins in the adrenal gland. In the adrenal gland, reduced biopterin ($BH_4$) represented about 97% of total biopterin. Two hours after the exposure to hypoxia, the content of total and reduced biopterins in the adrenal gland was unchanged. Similarly, the content of total or reduced biopterins was also unchanged in the brain (data not shown).

**TABLE 2.** *Effects of 2-h exposure to hypoxia on the content of total and reduced biopterins in the rat adrenal gland*

| | Biopterin content (nmol/g of tissue) | |
|---|---|---|
| | Room air | Hypoxia |
| Total biopterin | 9.03 ± 1.13 | 9.45 ± 1.37 |
| Reduced biopterin | 8.78 ± 1.10 | 9.12 ± 1.36 |

After 2-h exposure to room air or hypoxia (8% $O_2$ in $N_2$), rats were decapitated and a pair of adrenal glands was removed. The content of total and reduced biopterins in the adrenal gland was determined as described under Materials and Methods. Values are means ± SEM of six determinations.

Table 3 shows the effects of hypoxia on the content of tyrosine in the adrenal gland and the brain. The content of tyrosine was not significantly affected in either the adrenal gland or the brain after 1-h exposure to hypoxia, but increased by about 80% and 150%, respectively, after 2-h exposure. These results strongly suggest that an increase in in vivo tyrosine hydroxylation in the adrenal gland is the result of an increase in tissue levels of tyrosine. However, because it is considered that TH is almost fully saturated with tyrosine (Carlsson and Lindqvist, 1978), it is questionable whether the increased availability of tyrosine could enhance the in vivo tyrosine hydroxylation in the adrenal gland. Therefore, we then examined the effects of tyrosine administration on in vivo tyrosine hydroxylation in the adrenal gland under both control and experimental conditions.

Figure 2 shows the effects of tyrosine administration on DOPA accumulation and tyrosine content in the adrenal gland and the brain under room air and hypoxic conditions. Under room air conditions, tyrosine content in the adrenal gland (Fig. 2A) and the brain (Fig. 2B) increased remarkably following administration of tyrosine ethylester (100 mg/kg as free amino acid, i.p.), whereas no significant increase in DOPA accumulation was observed in either tissue (Fig. 2C and D). In contrast, after 2-h exposure to hypoxia, both DOPA accumulation (Fig. 2C) and tyrosine content (Fig. 2A) in the adrenal gland considerably increased following administration of tyrosine ethylester. Exogenous tyrosine, however, had little effect on DOPA accumulation in the brain (Fig. 2D), although endogenous tyrosine content increased considerably (Fig. 2B).

Next, to examine the mechanism by which in vivo tyrosine hydroxylation and tyrosine content in the adrenal gland are increased by hypoxia, we determined the effects of adrenal denervation on the hypoxia-in-

**TABLE 3.** *Effects of hypoxia on tyrosine content in the adrenal gland and the brain of rats*

| | Tyrosine content (nmol/g of tissue) | |
|---|---|---|
| | Room air | Hypoxia |
| Adrenal gland | | |
| 1-h exposure | 82.9 ± 5.0 | 73.9 ± 4.2 |
| 2-h exposure | 89.0 ± 3.2 | 161.8 ± 14.7[a] |
| Brain | | |
| 1-h exposure | 73.2 ± 4.9 | 70.8 ± 3.9 |
| 2-h exposure | 82.4 ± 3.0 | 199.8 ± 23.5[a] |

After exposure to room air or to hypoxia (8% $O_2$ in $N_2$) for 1 h or 2 h, rats were decapitated and the concentrations of tyrosine in the adrenal gland and the brain were determined as described under Materials and Methods. Values are means ± SEM of six determinations.

[a] $p < 0.01$; significantly different from values of the control group, which was exposed to room air.

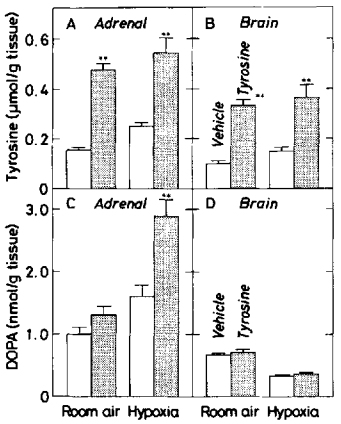


**FIG. 2.** Effects of tyrosine administration on tyrosine content (**upper panels**) and in vivo tyrosine hydroxylation (**lower panels**) in the adrenal gland (**left panels**) and the brain (**right panels**) under room air or hypoxia. After exposure to either room air or hypoxia for 1 h and 10 min, rats were intraperitoneally injected with saline (vehicle, open columns) or tyrosine ethylester (100 mg/kg as free acid form, stippled columns) solution. Thirty minutes after the injection, the animals were intraperitoneally injected with 100 mg/kg of NSD 1015 and were decapitated 20 min thereafter. The content of tyrosine and DOPA in the adrenal gland and the brain was determined as described under Materials and Methods. Each value is mean ± SEM of six determinations. **Group receiving tyrosine differs significantly from group receiving vehicle only at $p < 0.01$.

duced increase in in vivo tyrosine hydroxylation and tyrosine content (Fig. 3). For this purpose, rats, the left adrenal gland of which had been surgically denervated 5–7 days before, were exposed to either room air or hypoxia for 2 h. After exposure to hypoxia, tyrosine content increased in both the intact and the denervated adrenal glands (Fig. 3A). DOPA accumulation increased in the intact adrenal gland, but was unaffected in the denervated gland (Fig. 3B).

Table 4 shows the effects of adrenal denervation on hypoxia-induced changes in kinetic parameters of TH. After 2-h exposure to hypoxia, the $V_{max}$ value of TH-catalyzed reaction increased without a change in the $K_m$ value on the intact side as described above (Table 1), but the increase in the $V_{max}$ value was abolished by denervation.

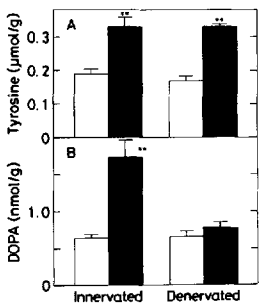


**FIG. 3.** Effects of 2-h hypoxia on tyrosine content (**A**) and in vivo tyrosine hydroxylation (**B**) in the innervated and denervated adrenal glands. The rats, whose left adrenal glands had been surgically denervated 5–7 days before, were exposed to either room air (open columns) or hypoxia (filled columns) for 2 h. They were intraperitoneally injected with NSD 1015 (100 mg/kg) 20 min before they were killed. DOPA accumulation following administration of NSD 1015 and the concentrations of tyrosine in the adrenal glands were determined as described under Materials and Methods. **$p < 0.01$; significantly different from control values obtained under room air.

## DISCUSSION

DOPA accumulation following decarboxylase inhibition is considered to be a good index of in vivo tyrosine hydroxylation (Sharman, 1981). In the present investigation, in vivo tyrosine hydroxylation in the brain was inhibited under hypoxic conditions (Fig. 1B). These results are in good agreement with previous reports (Davis and Carlsson, 1973; Miwa et al., 1986), and are considered to be a result of the decreased availability of oxygen, one of the substrates for TH. In contrast, in vivo tyrosine hydroxylation in the adrenal gland was unexpectedly enhanced following an initial decrease under hypoxic conditions (Fig. 1A), although tissue levels of oxygen must still be considered to be quite low.

In the present study, the content of endogenous tyrosine was found to be increased in the adrenal gland and the brain (Table 3). Under control conditions, tyrosine administration had little effect on in vivo tyrosine hydroxylation in either the adrenal gland or the brain (Fig. 2C and D), although tissue levels of tyrosine increased by more than 100% following administration of exogenous tyrosine (Fig. 2A and B). These results are in good agreement with previous reports (Carlsson and Lindqvist, 1978) and suggest that TH in both the adrenal gland and the brain is almost fully saturated with its substrate tyrosine under control conditions. However, under hypoxic conditions, tyrosine administration produced a further increase in in vivo tyrosine hydroxylation in the adrenal gland (Fig. 2C), but not in the brain (Fig. 2D), suggesting that under hypoxic conditions, the TH in the adrenal gland becomes sensitive to increased tissue levels of tyrosine. These results are in line with previous reports that under certain experimental conditions that activate CA-containing neurons, administration of exogenous tyrosine could enhance CA biosynthesis in the brain (Gibson and Wurtman, 1978; Sved et al., 1979*a,b*; Melamed et al., 1980; Sved and Fernstrom, 1981; Reinstein et al., 1984), retina (Gibson et al., 1983); and peripheral sympathoadrenal system (Alonso et al., 1980; Conlay et al., 1981). According to these reports, neither the tissue level of tyrosine nor CA biosynthesis was increased in the absence of exogenous tyrosine. These results taken together suggest that in vivo tyrosine hydroxylation in the adrenal gland is enhanced under hypoxic conditions, mainly via the following mechanisms: (1) TH becomes sensitive to an increase in tissue levels of tyrosine, and (2) tissue levels of tyrosine are increased.

The kinetic study of TH in the soluble fraction of the adrenal gland showed that under hypoxic conditions, TH in the adrenal gland was activated: the $V_{max}$ value of TH-catalyzed reaction increased without a change in the $K_m$ value (Table 1). Independently of the increased sensitivity of TH to the increased tissue levels of tyrosine, this increase in the $V_{max}$ value could also contribute to the enhancement of in vivo tyrosine hy-

**TABLE 4.** *Effects of adrenal denervation on hypoxia-induced changes in kinetic constants of TH in the adrenal gland*

| | Intact side | | Denervated side | |
|---|---|---|---|---|
| | Room air | Hypoxia | Room air | Hypoxia |
| $K_m$ for $BH_4$ ($\mu M$) | 80.6 ± 7.6 | 82.3 ± 7.1 | 76.5 ± 7.8 | 79.1 ± 7.1 |
| $K_m$ for tyrosine ($\mu M$) | 18.6 ± 2.5 | 19.9 ± 3.6 | 20.7 ± 3.1 | 21.4 ± 4.2 |
| $V_{max}$ (nmol/mg of protein/h) | 45.2 ± 4.2 | 72.4 ± 8.4[a] | 40.8 ± 5.6 | 41.2 ± 6.7 |

The rats, whose left adrenal glands had been surgically denervated 7 days before, were exposed to room air or to hypoxia for 2 h. Each adrenal gland was homogenized separately and the kinetic parameters of TH in the soluble fraction was determined as described in the footnote to Table 1.

[a] $p < 0.05$; significantly different from control values obtained under room air on the intact side or the denervated side.

droxylation. Thus, the present investigation showed that the enhancement of in vivo tyrosine hydroxylation by hypoxia was the result of both the increased tissue levels of tyrosine coupled with the increased sensitivity of TH to tyrosine levels and of the increase in the $V_{max}$ value of the TH-catalyzed reaction.

After denervation of the adrenal gland, the hypoxia-induced enhancement of in vivo tyrosine hydroxylation in the adrenal gland was abolished, whereas the hypoxia-induced increase in tissue levels of tyrosine was persistent (Fig. 3), suggesting that the TH of the denervated adrenal gland is insensitive to an increase in tissue levels of tyrosine. After denervation of the adrenal gland, the increase in the $V_{max}$ value of TH-catalyzed reaction was also abolished (Table 4). The activity of the greater splanchnic nerve which innervates the adrenal gland is considered to be increased under hypoxic conditions, because both the concentration of adrenaline in the plasma and the turnover rate of adrenaline in the adrenal gland are increased under these conditions (see introductory section). Thus, these results suggest that both the increased sensitivity of TH to an increase in tissue levels of tyrosine and the activation of TH (an increase in $V_{max}$ value) are induced by the increased activity of the greater splanchnic nerve, whereas tissue levels of tyrosine are regulated by a different mechanism. The mechanism by which tissue levels of tyrosine are regulated is, at present, unknown but is now under investigation in our laboratory.

As to the mechanism by which TH becomes sensitive to the changes in tyrosine levels when the adrenal chromaffin cells and TH are activated, it is unlikely that the modified kinetic constants of the activated enzyme account for this effect. No change in substrate affinity occurs, although the $V_{max}$ value increased. Hence, the sensitivity of the enzymatic reaction to changes in local substrate level should be the same in the activated and inactivated state of TH. A more likely explanation may be that when the adrenal chromaffin cells and TH are activated, and CA biosynthesis is increased, the local pool of tyrosine is depleted. If so, the tyrosine concentration would come closer to the $K_m$ value of the enzyme, thereby increasing the enzyme's sensitivity to variations in tyrosine levels. This notion has actually been suggested before, and data have been published indicating that when brain dopamine neurons are activated, the local tyrosine pool may be depleted (Westerink and Wirix, 1983).

It is reported that this type of change in kinetic parameters of TH is observed, either when TH is phosphorylated by $Ca^{2+}$/calmodulin-dependent protein kinase (El Mestikawy et al., 1983) or when additional enzyme molecules are formed (Mueller et al., 1969; Chuang and Costa, 1974; Hoeldtke et al., 1974). However, it is at present unknown which activation system is operating under hypoxic conditions. The molecular mechanism by which the maximal velocity of TH-catalyzed reaction is increased under hypoxic conditions is now under investigation in our laboratory.

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