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Effect of reserpine on the activity of adrenal enzymes involved in the synthesis of adrenaline

B. BHAGAT, W. J. BURKE AND J. W. DAVIS

Departments of Physiology and Biochemistry, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, Missouri 63104, USA

Summary

1. After administration of reserpine to rats, the tyrosine hydroxylase (TH) and phenylethanolamine-N-methyl transferase (PNMT) activity in their adrenal glands was found to be increased under *in vitro* conditions.

2. The increase in TH activity occurred at 12–18 h after reserpine whereas the PNMT activity increased at 30 hours. Unlike the TH, the increase in PNMT activity did not appear to be neuronally mediated since ganglion blockade by chlorisondamine failed to antagonize the reserpine-induced increase in PNMT activity. The increase in PNMT activity may be a response to increased utilization of catecholamines.

3. Hypophysectomy resulted in a diminution of the activities of both enzymes ; the activity of TH, but not of PNMT, could be partially restored by reserpine. ACTH restored the activities of both enzymes almost to normal.

4. The differential effect of reserpine suggests that the activities of these two enzymes are controlled by different mechanisms.

Introduction

Procedures which cause an increase in the activity of sympathetic nerves, such as direct nerve stimulation (Alousi & Weiner, 1966 ; Bhagat, 1967 ; Sedvall & Kopin, 1967a ; Sedvall & Kopin, 1967b) or a reflex increase in sympathetic discharge (Bhagat & Friedman, 1969 ; Dairman, Gordon, Spector, Sjoerdsma & Udenfriend, 1968 ; Dairman & Udenfriend, 1970 ; Gordon, Spector, Sjoerdsma & Udenfriend, 1966) cause an almost immediate increase in the rate of synthesis of catecholamines due, perhaps, to increased activity of tyrosine hydroxylase (TH). Because the increase in enzymatic activity occurs without an increase in the amount of enzyme (Dairman *et al.*, 1968 ; Sedvall & Kopin, 1967b), this phenomenon may be a consequence of the release of tyrosine hydroxylase from end-product inhibition.

Recently, Axelrod and co-workers (Mueller, Thoenen & Axelrod, 1969a, b ; Thoenen, Mueller & Axelrod, 1969a, b) have shown that an increase in TH activity occurred in adrenal glands and in nerve tissues after (1) destruction of postganglionic nerve endings by 6-hydroxydopamine, or (2) chronic administration of reserpine or phenoxybenzamine. TH activity, however, no longer increased in the adrenal gland if the splanchnic nerve was sectioned before treatment with the drugs. Thus, the elevations in the TH activity appeared to be due to a prolonged increase in sympathoadrenal activity.

Phenylethanolamine-N-methyl transferase (PNMT), another enzyme involved in the biosynthesis of catecholamines converts noradrenaline to adrenaline in the adrenal medulla (Axelrod, 1962). It is therefore of interest to determine (1) if administration of reserpine can also produce an elevation in PNMT activity of the adrenal gland, and (2) if the activities of TH and PNMT are regulated by the same mechanisms.

Methods

Hypophysectomized and sham-operated female Sprague-Dawley rats were obtained from Hormone Assay Inc., Chicago, Ill. All animals were maintained postoperatively on normal laboratory diet fortified with oranges.

Tyrosine hydroxylase activity

Animals were killed by a blow on the head; the adrenal glands were rapidly removed and homogenized in 2 ml of ice-cold 0·25 M sucrose. One millilitre of each homogenate was acidified with 9·0 ml of 0·4 N perchloric acid for the assay of catecholamines. Portions (0·14 ml) of the homogenate were assayed for TH activity by a modification (Mueller *et al.*, 1969a) of the method of Levitt, Gibb, Daly, Lipton & Udenfriend, 1967. The incubation medium for each assay contained: 57·85 nmol of potassium phosphate buffer, pH 6·0; 138·4 nmol of 2-amino-6, 7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine (pteridine) (Aldrich Chem. Co., Milwaukee, Wisc.); 2·857 nmol of 3,5-^{3}H-tyrosine (3·34 μCi) (New England Nuclear Corp., Boston, Mass.); and 0·17 ml of 0·25 M sucrose. The final volume was 50 μl before the addition of homogenate.

After incubation of the samples in a metabolic shaker for 20 min at 37° C, the reaction was stopped by the addition of 1·12 ml of 5% trichloroacetic acid. The mixture was centrifuged and the supernatant was passed through a column (0·6 × 5 cm) of Dowex 50W–X4 (H^+). The tritiated water formed by the hydroxylation of the tyrosine was eluted from the column with 1 ml of distilled water and collected in scintillation vials. Blanks were treated according to the procedure outlined above except that 0·14 ml of 0·25 M sucrose was substituted for the homogenate. Instagel (10 ml) (Packard Instrument Co.) was added to the samples for counting and the radioactivity determined in a Nuclear Chicago Scintillation Counter (model 720). All samples were counted at an efficiency of at least 10%. Before use, the 3,5 ^{3}H-tyrosine was purified by the method outlined by Mueller *et al.* (1969a) and the specific activity adjusted to 1·17 mCi/μmol with L-tyrosine; the sample was stored in 0·01 N HCl at 5° C.

Phenylethanolamine-N-methyl transferase (PNMT) activity

Excised adrenal glands from one animal were homogenized in 1·8 ml of a solution of 0·15 M KCl containing 2 mM mercaptoethanol. The supernatant liquid obtained after centrifugation of the homogenate at 100,000 *g* for 20 min was dialysed overnight against 3·5 mM potassium phosphate buffer pH 7·7 containing 2 mM mercaptoethanol.

PNMT activity in a 50 μl aliquot of dialysed supernatant fraction was assayed by a modification of the method of Wurtman & Axelrod (1965). The medium for each assay contained 37·5 μg DL-normetadrenaline HCl, 1 μmol of S-adenosyl meth-

ionine (methyl-^{14}C) (58 μCi/μmol), 10 μmol of phosphate buffer, pH 7·9, and 0·25 μmol of KCN in a total volume of 255 μl. After incubation for 30 min at 37° C the reaction was stopped by the addition of 0·5 ml of 0·5 M sodium borate buffer, pH 10. Addition of KCN provided more reproducible activities that were as much as 30% (average 28%) greater than samples not containing KCN; the exact role of this agent, however, is yet to be evaluated. The ^{14}C-metadrenaline formed was extracted into 6 ml of toluene: isoamyl alcohol (3:2, v/v). The ^{14}C counts in 4 ml portions of the extract were determined by use of a Packard Tri Carb Liquid Scintillation Spectrometer after addition of 1 ml of absolute ethanol and 10 ml of Bray's solution (Bray, 1960). Corrections for ^{14}C S-adenosyl-methionine and endogenously formed radioactive adrenaline were determined in experiments identical to those described above except that normetadrenaline was omitted. The efficiency of the counting procedure was approximately 80%.

PNMT activity in adrenal medullary tissue

The specific activity of PNMT was determined in the adrenal medullae. Cortical tissue was carefully dissected under a microscope and discarded. The medullae were homogenized and assayed as described above. The protein (Lowry, Rosebrough, Farr & Randall, 1951) and DNA (Burton, 1956) content of the tissue was assayed so that the specific activity of PNMT from this source could be determined.

Catecholamine content of tissues

Adrenal catecholamines were adsorbed on to a column of aluminum oxide at pH 8·6 from which they were eluted with 0·05 N perchloric acid (Anton & Sayre, 1962). The endogenous adrenaline was converted to its fluorescent trihydroxyindole derivative by oxidation with potassium ferricyanide at pH 2 (Euler & Lishajko, 1961). The catecholamine concentration is expressed as adrenaline per pair of adrenal glands. No correction for the recovery (68–85%) was made in the final calculation.

The following drugs were used: adrenal corticotrophic hormone (cortocotrophin, National Drug Co.), chlorisondamine-HCl (Ciba Pharm. Co.) and reserpine (Serpasil, Ciba). Details of dosage time schedules and routes of administration are given in the appropriate section of the **Results**. Tests for statistical significance were made using Student's *t* test (Snedecor, 1956).

Results

Effect of reserpine on adrenal enzymes

After an initial, intraperitoneal injection of reserpine (2·5 mg/kg), rats received 1 mg/kg at 24 h intervals; control animals received similar volumes (approximately 0·2 ml) of 0·15 M sodium chloride. The animals were killed at various times thereafter and their adrenal glands analysed for catecholamine content and activities of TH and PNMT. While reserpine caused a significant increase in the TH-activity 12–18 h after reserpine administration, the activity of PNMT was not measurably elevated at that time (Table 1). PNMT activity began, however, to increase 30 h after reserpine and continued to rise for the ensuing 114 h (Fig. 1). Adrenal catecholamine concentrations began to decrease 18–24 h after the initial injection of reserpine and by 144 h they were down to 38·5% of the control values.

TABLE 1. *Effect of reserpine and the pituitary gland on PNMT and TH activities in rat adrenal gland*

Expt.	Treatment	Body wt. (g)	Adrenal wt. (mg)	PNMT activity* per pair adrenal glands	TH activity† per pair adrenal glands	Catecholamines (μg/pair adrenal glands)
I.	Saline	200±6·2	40·6±1·6	14·9±0·6	27·1±2·8	21·3±1·7
	Reserpine (I)	191±6·4	46·5±4·2	15·2±0·6	36·1±2·5‡	13·3±2·2‡
II.	Sham-operated+saline	197±5·2	39·0±2·4	10·4±0·3	26·0±3·1	23·0±1·9
	Hypox+saline	171±5·4‡	21·7±2·6‡	4·1±0·2‡	16·7±2·1‡	15·1±1·7‡
	Hypox+reserpine (II)	178±6·7‡	22·4±2·0‡	4·0±0·1‡	28·6±2·7	12·1±2·6‡
III.	Sham-operated+saline	200±5·0	39·9±3·1	—	28·4±3·0	31·1±2·1
	Hypox+saline	178±6·0‡	19·4±1·2‡	—	17·8±0·6‡	21·3±2·1‡
	Hypox+ACTH	186±5·0‡	36·4±1·0	—	26·9±2·3	22·5±1·5‡

Reserpine (I): 2·5 mg/kg i.p. 12–18 h before measuring TH and PNMT activity and catecholamine content.
Reserpine (II): 1 mg/kg i.p. every 12 h starting 24 h (TH activity and catecholamines) or 72 h (PNMT activity) before death.
Hypox: hypophysectomy 14 days before the experiment.
ACTH: 4 IU/day for 4 days, starting 10 days after hypox.
* Nanomoles ^{14}C-metadrenaline formed per hour. † Nanomoles $^{3}H_2O$ formed per hour. ‡ Significantly different ($P<0·05$) when compared with respective control in each vertical column.

In some experiments in which the animals were killed 72 h after starting the reserpine treatment, one adrenal gland was assayed for PNMT activity while only the medullary tissue of the opposite adrenal gland of each animal was assayed for specific enzymatic activity based both on its protein and DNA content. The results, summarized in Table 2, indicate that reserpine caused a significant increase in the

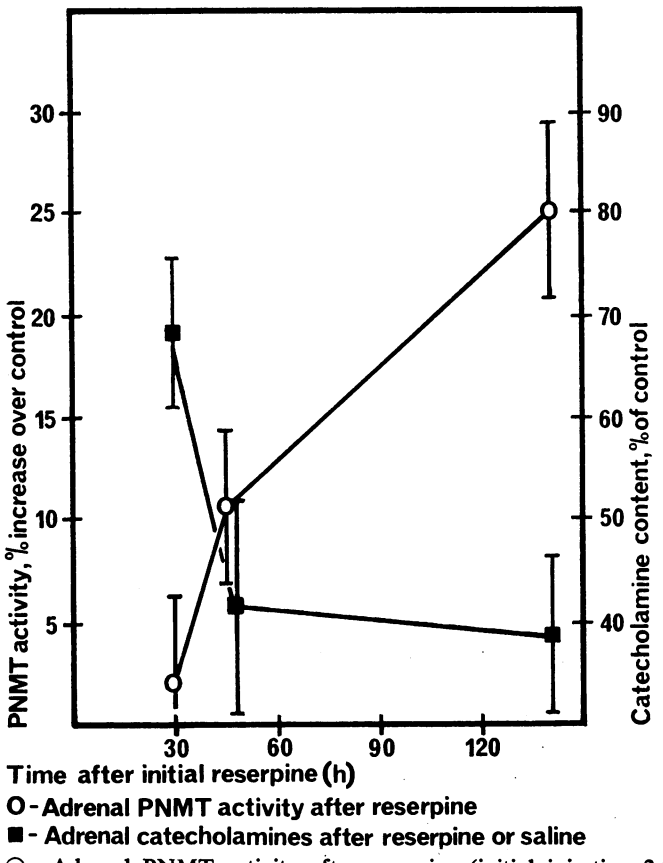

FIG. 1. ○——○: Adrenal PNMT activity after reserpine (initial injection 2·5 mg/kg, i.p.; 1 mg/kg every 24 h thereafter) expressed as percent of mean activity in control rats (0·2 ml 0·15 M NaCl every 24 h). ■——■: Adrenal content of catecholamines (expressed as percent of control values) at different times after the initial injection of reserpine (experimental) or saline (control). Means ± S.E. of five or six rats.

TABLE 2. *Effect of reserpine on PNMT activity in the whole adrenal and adrenal medullae of rats*

	PNMT activity*		
	Control Mean ± S.E.M.	Reserpine treated Mean ± S.E.M.	*P* values†
Activity/whole adrenal gland	4·63 ± 0·08	5·83 ± 0·24	<0·01
Medullary tissue of opposite glands:			
Activity/mg protein	98·81 ± 9·97	85·10 ± 3·30	N.S.
Activity/mg DNA	545·7 ± 94·4	667·3 ± 89·1	N.S.

Reserpine: After an initial injection of reserpine (2·5 mg/kg, i.p.) the animals received 1 mg/kg at 24 h and 48 hours. Controls: 0·2 ml 0·9% NaCl. All animals were killed 72 h after the first injection.
† Difference between the reserpine treated and control animals. Each value is the mean ± S.E. of the mean from six animals. * Nanomoles ^{14}C-metadrenaline formed per hour.

total PNMT activity of the whole adrenal gland. In adrenal medullary tissue, however, the enzymatic activity per mg protein or per cell (activity per mg DNA) did not change. The greater variance in the latter values prevents evaluation of whether PNMT activity increases more rapidly than tissue growth.

Effect of chlorisondamine on the changes of PNMT activity in the adrenal gland induced by reserpine

Table 3 shows the results of experiments on rats which received an initial injection of reserpine (2·5 mg/kg body wt.) and 1 mg/kg 24 h later. Control rats received similar volumes (approximately 0·2 ml) of 0·15 M sodium chloride. Twelve hours after the initial injections, some animals were given chlorisondamine HCl (5 mg/kg, i.m.). To maintain the ganglionic blockade, injections of chlorisondamine were repeated every 12 hours. Other animals received, instead of chlorisondamine, approximately 0·2 ml 0·15 M NaCl. Forty-eight hours after the beginning of the experiment, all animals were killed and the activities of TH and PNMT in the adrenal glands were measured. Chlorisondamine alone did not significantly affect the activity of either enzyme. It antagonized the reserpine-induced increase in tyrosine hydroxylase activity but not the increase in PNMT activity.

Effect of reserpine on adrenal enzyme activities in hypophysectomized rats

Rats were hypophysectomized 14 days before the experiment. Only 1 mg reserpine/kg (i.p.) was administered twice daily as hypophysectomized animals are very sensitive to reserpine. The control animals received approximately 0·2 ml of 0·15 M NaCl. The rats were killed 24 h and 72 h after the beginning of the reserpine treatment and the whole adrenal glands were assayed for enzyme activity. The results show that reserpine increased the lowered activity of adrenal TH in hypophysectomized rats but the PNMT activity remained low (Table 1). If hypophysectomized rats were treated with ACTH (4 units per day for 4–6 days) the adrenal weight, and the TH and PNMT activities rose to normal values ; the catecholamines were further reduced (Tables 1 and 3).

In other experiments, reserpine (1 mg/kg i.p.) was administered daily for 4 days beginning 2 days after ACTH treatment; control animals received approximately 0·2 ml of 0·15 M NaCl instead of reserpine. The combined treatment with reserpine

TABLE 3. *Effect of ganglion blockade on the reserpine-induced elevation of PNMT and TH activity*

Treatment	Adrenal wt. (mg)	PNMT activity* Per pair of adrenal glands	TH activity† Per pair of adrenal glands
Chlorisondamine‡	45·3±0·8	8·38±0·40	25·3±1·8
Reserpine	48·0±2·2	10·80±0·84§	39·1±1·9¶
Reserpine+chlorisondamine	48·0±2·0	11·21±0·74§	26·7±2·8

Chlorisondamine: 5 mg/kg i.m. every 12 h beginning 12 h after the initial injection of reserpine or 0·9% NaCl. Reserpine: 2·5 mg/kg body weight initially; 1 mg/kg 24 h later. All animals (6 for each treatment) killed 48 h after the initial reserpine injection. * Nanomoles of ^{14}C-metadrenaline formed per hour. † Nanomoles of $^{3}H_2O$ formed per hour. ‡ In a separate experiment, chlorisondamine injections provided results that were not significantly different from control animals injected with 0·9% saline on the same schedule. § Significantly higher ($P<0·05$) than chlorisondamine treated. ¶ Significantly higher ($P<0·01$) than other means in column.

and ACTH promoted a slight increase ($P>0.05$) in PNMT activity over that elicited by ACTH alone (Table 4).

Discussion

Two mechanisms appear to be concerned in the regulation of TH activity, which is the rate limiting enzyme in the biosynthesis of catecholamines (Kvetnaňsky, Weise & Kopin, 1970). One mechanism is end-product inhibition of the enzymatic activity; the other is enzyme induction (Dairman & Udenfriend, 1970; Mueller *et al.*, 1969a, b; Thoenen *et al.*, 1969a, b).

The induced biosynthesis of tyrosine hydroxylase is a response evoked only by prolonged nerve stimulation and thus is a long-term adaptation to increased activity of sympathetic nerves. 'Chemical sympathectomy' with 6 hydroxydopamine, or repeated administration of high doses of phenoxybenzamine or reserpine, produce a neuronally mediated increase in TH activity as measured in homogenates of adrenal tissue (Mueller *et al.*, 1969a, b; Thoenen *et al.*, 1969a, b).

In the studies reported in this paper, treatment with reserpine not only caused elevation of adrenal TH activity but also a significant increase in adrenal PNMT-activity. The temporal aspects of the changes were, however, different for the two enzymes. TH activity increased 12–18 h after reserpine, while that of PNMT was only elevated 30 h after administration of reserpine.

Reserpine does not cause induction of adrenal TH if the splanchnic nerves innervating the adrenal gland are severed before treatment with the drug (Thoenen *et al.*, 1969a). Likewise, interruption of sympatho-adrenal activity at the ganglionic level by chlorisondamine, a long acting ganglionic blocking agent, prevents reserpine induction of tyrosine hydroxylase. Unexpectedly, however, chlorisondamine did not antagonize the increase in PNMT activity caused by reserpine. Consistent with our results are the observations of Kvetnaňsky *et al.* (1970) who found that repeated immobilization of animals led to increased activities of both enzymes in the adrenal gland. Denervation of the adrenal gland prevented the increase in activity of tyrosine hydroxylase but not that of PNMT. Together these results suggest that the control of PNMT and TH activities are exerted via different mechanisms.

Thus, while nerve impulses may be of primary importance in increasing the activity of TH they are not necessary for elevation of PNMT activity. The heigh-

TABLE 4. *Effect of combination of ACTH and reserpine on adrenal PNMT activity in hypophysectomized rats*

Treatment	PNMT activity* per pair adrenals	Catecholamine (μg/pair of adrenals)
Controls	5·1±0·3	18·3±1·8
ACTH	9·0±0·4†	21·7±2·0
ACTH+reserpine	10·4±0·7	4·5±0·4‡

Rats were hypophysectomized 28 days before the experiment. ACTH (4 IU/day i.m.) was given beginning 22 days after hypophysectomy. Reserpine (1 mg/kg body weight) was given daily beginning 2 days after initial ACTH injection. Controls: 0·2 ml 0·9% NaCl. All animals were killed 6 days after the beginning of the ACTH treatment and the adrenal PNMT activity and catecholamines were determined. Each value represents the mean ± S.E.M. of six experiments. Total catecholamines are expressed as adrenaline. * Nanomoles ^{14}C-metadrenaline formed per hour. † $P<0.01$, ACTH vs. saline alone. ‡ $P<0.01$, ACTH vs. reserpine+ACTH.

tened PNMT activities upon treatment with reserpine may therefore be a drug effect on the enzyme and/or a compensatory response to the enhanced utilization of catecholamines. Reserpine itself did not show activation of PNMT activity if added to the tissue homogenates; rather it inhibited slightly the conversion of normetadrenaline to metadrenaline (unpublished observation). Since diminished tissue concentrations of catecholamines in the face of more rapid biosynthesis indicate enhanced utilization of these neurohormones, it is possible that the increase in PNMT activity is an adaptation to the stimulated utilization of catecholamines.

While reserpine stimulated the activities of both enzymes in intact animals, in hypophysectomized rats only the activity of TH was raised significantly.

Since both the TH and PNMT activities in the adrenal glands of hypophysectomized rats were considerably lower than in those of sham-operated animals, and since ACTH therapy of hypophysectomized rats restored the activity of both enzymes almost to normal, it is apparent that ACTH is involved, either directly or indirectly, in the normal maintenance of these adrenal medullary enzymes.

Although the administration of ACTH after hypophysectomy restored the activities of TH and PNMT, it did not raise the activities of these enzymes in unoperated animals. Elevation of TH activity above control values requires a reflex stimulation of the sympathetic nervous system for a prolonged period of time (Mueller *et al.*, 1969a) whereas the increase in PNMT activity above control values does not seem to be neuronally mediated.

The large responses in activity of PNMT which can be elicited by glucocorticoids or ACTH (Wurtman & Axelrod, 1966), suggests that this is the more important mechanism for providing optimum biosynthesis of adrenaline. Stimulation in activity of PNMT may also occur as a result of increased utilization of adrenaline. This second control may be useful to the organism in responding to stimuli not evoking release of steroids from the adrenal cortex.

The differential effect of reserpine on PNMT and tyrosine hydroxylase points out a difference in the control of the activities of each enzyme. If increased activity were related to increased biosynthesis of the enzymes, then the results observed after reserpine would indicate a control of the biosynthesis of the two enzymes by separate genomes.

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