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Whole brain tyrosine hydroxylase activity during the development of deoxycorticosterone acetate-1% sodium chloride-induced hypertension in rats

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Water was chosen as the optimal medium for the extraction of tyrosine hydroxylase (TH) from rat brain. Determination of TH activity in crude homogenates failed to exhibit a linear relationship between enzyme concentration and measured activity, however, when a supernatant was used, a linear relationship existed. At a time when TH activity is maximally increased in the locus coeruleus after reserpine treatment (Reis et al 1975; Zigmond 1979) (2·5 mg kg^{-1} day^{-1} for 3 days, kill 24 h after last dose) we could detect no alterations in whole brain TH, however if treatment continued for 4 days and animals were killed 72 h after the last dose it was possible to detect increases in TH activity in various brain regions. These results suggest that local changes in brain TH activity are not revealed in measurements made on whole brain. The early rise in blood pressure, following the administration of deoxycorticosterone acetate (doca) and 1% NaCl to male Wistar rats, was accompanied by bradycardia. Whole brain TH activity was determined in these hypertensive animals 6–21 days after the commencement of treatment and the results failed to confirm the reported elevation of TH activity (Rylett et al 1976). The results are discussed with reference to the TH assay employed.

Alterations in catecholaminergic nerve activity are associated with parallel changes in transmitter synthesis in vivo (Olivario & Stjarne 1965; Gordon et al 1966a, b; Sedvall & Kopin 1967) and in vitro (Alousi & Weiner 1966; Roth et al 1967; Weiner & Rabadjija 1968; Cloutier & Weiner 1973). Increased transmitter production involves either activation of the enzyme catalysing the rate-limiting step, tyrosine hydroxylase (TH, E.C. 1.14.16.2) during periods of acute stimulation (Roth et al 1975; Bustos et al 1978; Weiner et al 1978) or increased synthesis of TH during prolonged periods of stimulation (Thoenen et al 1969; Thoenen 1970; Joh et al 1973). Thus tyrosine hydroxylase may reflect catecholaminergic nerve activity, furthermore, Bacopoulos & Bhatnagar (1977) have shown in varios brain regions a relationship between TH activity and noradrenaline concentration or turnover.

In rats made hypertensive by the administration of deoxycorticosterone acetate (doca) and 1% NaCl, an overactive sympathetic nervos system has been reported (DeChamplain et al 1969; DeChamplain & Van Ameringen 1972); there is also strong evidence to suggest that central catecholamines play an important role in regulating sympathetic drive (Chalmers 1975). From results obtained following the measurement of catecholamine turnover (Nakamura et al 1971; Van Ameringen et al 1977), catecholamine levels (Zamir et al 1979) and TH activity (Nagoaka & Lovenberg 1977) in brain areas of doca/1% NaCl treated rats it appears that a decrease or inability to increase neuronal activity in certain central catecholaminergic neurons may be responsible for the increased sympathetic tone and blood pressure. It would therefore be expected, and has been shown by Nagoaka & Lovenberg (1977), that the activity of TH measured in brain areas of doca/1% NaCl hypertensive animals will be decreased or at least unchanged. However, results from earlier work in this laboratory (Rylett et al 1975) showed that in hypertensive rats which had been treated for 2 weeks with doca and 1% NaCl there was an increase in whole brain TH activity and this parameter apparently correlated with systolic blood pressure.

This paper describes the results obtained when an attempt to evaluate the findings of Rylett et al (1976) failed to confirm the original observation and therefore reconciles an apparent contradiction in the literature.

MATERIALS AND METHODS

Male rats were randomly divided into groups of 5–7 and received one of the following treatments.

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(a) *Reserpine.* Reserpine (Sigma) 2·5 mg kg^{-1} daily, was administered subcutaneously (s.c.) for 3 days to 7 Sprague Dawley rats (100–150 g) or for 4 days to 6 Sprague Dawley rats (200–250 g), these animals were killed 24 or 72 h after the last dose, respectively. An equal number of control animals received 0·2 ml vehicle s.c. containing 0·5 mg citric acid, 4 μl benzyl alcohol, 20 μl Tween 80 (Sigma) and 176 μl distilled water.

(b) *Doca in oil.* Wistar rats (n = 20, 200–250 g) were injected with doca (Organon 5 mg ml^{-1}) in oil s.c. every 3 days at a dose of 12.5 mg kg^{-1}. 22 control rats received an equivalent volume of arachis oil. Rats were killed 8, 11, 14 and 21 days after the commencement of treatment.

(c) *Doca implant.* Wistar rats (n = 27, 125–150 g, were anaesthetized with ether and a 25 mg pellet of doca (Organon) was implanted s.c., at the back of the neck. 25 control rats were subjected to a sham operation. These animals were killed 6, 10, 14, 17 and 20 days after the commencement of treatment.

Food and drink were freely available, but, for animals receiving doca, water was replaced by 1% NaCl in tap water. Blood pressure and heart rate were measured indirectly by the tail-cuff method; rats were familarized with this technique before the start of each experiment.

Animals were killed between 0900–1200 h by a hard blow on the thoracic spine. The brain and adrenals were rapidly removed, washed with ice-cold distilled water and placed in ice, any adhering fat or connective tissue being removed. For the measurement of TH activity in brain regions, the brain was dissected by the method of Glowinski & Iversen (1966). The adrenals were homogenized in 2 ml ice-cold 10 mM sodium acetate buffer pH 6·1 containing 0·25 M sucrose, the whole brain in 15 ml ice-cold distilled water. Hypothalamus, cerebellum, 'locus coeruleus' (a 1–1·5 mm thick slice of tissue, removed from the pons-medulla oblongata following a coronal cut at the level of the cerebellar peduncles, comprising mainly of the locus coeruleus and the trigeminal motor nucleus), pons-medulla oblongata minus the 'locus coeruleus', and the rest of brian were homogenized in 1·5, 2·3, 1·5 (two tissue samples pooled), 2·0 and 10·0 ml ice-cold distilled water respectively. The crude homogenates were centrifuged at 27 000 *g* for 15 min and the supernatant was stored at −70 °C or assayed immediately. Deep-frozen samples were thawed quickly at 37 °C 10 min before assay.

A modification of the method of Waymire et al (1971) was employed for measuring TH activity. The coupling enzyme, L-aromatic amino acid decarboxylase (EC 4.1.26 L, dopa decarboxylase) was partially purified from pig renal cortex as described by Waymire et al (1971), chromatographed on a column of Sephadex G25 (Pharmacia) which was equilibrated and developed with 10 mM sodium phosphate buffer pH 7·00, and stored at −70 °C, in 1·5 ml batches, in 20% glycerol.

The assay mixture contained (in a total volume of 500 μl) μmol: sodium acetate buffer pH 6·1 100; ferous sulphate 0·5; 2-amino-4-hydroxy-6,7-dimethyl 5,6,7,8-tetrahydropteridine hydrochloride ($DMPH_4$ Sigma) 100; sodium phosphate buffer pH 7·4 10; 2-mercaptoethanol 20; [1-^{14}C]L-tyrosine 0·05 (2 μCi μmol^{-1}); pyridoxal phosphate 5 nmol; dopa decarboxylase extract (excess) 100 μl and adrenal or brain supernatant 100 μl. The $DMPH_4$ was dissolved in 0·143 M 2-mercaptoethanol containing 0·1 M sodium phosphate buffer—this mixture and the ferrous sulphate solution were freshly prepared.

The tissue extract and the solution containing the rest of the assay ingredients, with the exception of tyrosine, were incubated separately for 5 min at 37 °C. The tyrosine was added to the mixture immediately before the assay was initiated by injecting the latter through a rubber septum in a side arm and, after 20 min incubation with vigorous shaking, the reaction was terminated by injection of 500 μl 10% (w/v) trichloracetic acid. The $^{14}CO_2$ liberated during the reaction was trapped in NCS tissue solubilizer (Searle/Amersham) on a paper wick. Total absorption of $^{14}CO_2$ was ensured by a further 2 h incubation with shaking at 37 °C. The wick was then removed from the well and both were placed in 10 ml 0·6% 2-[4′-t-butylphenyl-S-(4′-biphenyl)1,3,4-oxadiazide] (butyl PBD, Hopkins and Williams Ltd) in tolulene and counted using a Packard 3320 Tri-carb refrigerated liquid scintillation spectrometer with external standard channels ratio quench correction (efficiency 65–80%).

All assays were performed in triplicate, control incubations were carried out in which tissue extract was replaced by water. A pooled whole brain supernatant (stored at −70 °C) was included in each set of assays to ensure reproducibility and agreement between sets of determinations. Protein concentration was determined in tripilcate using the method of Gornall et al (1949). One unit (U) of enzyme activity represents the amount of enzyme catalysing the formation of 1 μmol product in 1 min.

Statistical significance testing was by using the Student's *t*-test.

Table 1. A comparison of different homogenization media used for extracting TH from whole rat brain.

Homogenization Medium	†Homogenate protein mg ml^{-1}	†Homogenate TH μU mg protein^{-1}	*Supernatant protein mg ml^{-1}	*Supernatant TH μU/ mg prot	‡Resuspended Pellet protein mg ml^{-1}	‡Resuspended Pellet TH μU mg protein^{-1}	Overall Recovery of TH Homogenate = 100%
Water	46·35	4·42	14·3	14·5 (57%)	44·9	1·55	83
Tris 0·05 (pH 6·0)	47·5	3·41	13·5	9·58 (50%)	52·3	2·24	99
Tris 0·05 M + 0·2% Triton X-100 (pH 6·0)	57·9	3·35	20·2	10·9 (71%)	50·2	0·75	84
Potassium phosphate 0·1 M (pH 7·0)	47·9	3·94	13·6	7·73 (35%)	46·6	1·61	61
Potassium phosphate + 0·2% Triton X-100 (pH 7·0)	54·9	4·06	22·2	11·2 (70%)	48·6	0·86	82

† whole brains were homogenized in 5 vols of medium.
* crude homogenates were centrifuged at 27000 $g \times 15$ min.
‡ the pellet was resuspended in 2·5 ml of the appropriate buffer.
Values in parentheses indicate the proportion of total TH activity remaining in the supernatant.

RESULTS

Table 1 shows the effect of the homogenization medium on TH activity in whole brain homogenate, 27000 $g \times 15$ min supernatant and resuspended pellet. The highest activity in supernatant was observed when the procedure was carried out in media containing Triton X-100 but the increase was at the expense of increased protein extraction generally as shown in Table 1. The crude homogenate prepared in water, or in 0·01 M potassium phosphate buffer pH 7·0 containing 0·13 M potassium chloride, did not show a linear relationship between homogenate volume and enzyme activity per mg protein. Linearity was observed in both cases when the supernatant was used. Homogenization in Tris buffer showed the highest overall recovery but this was associated with low levels of TH activity in the homogenate and supernatant. On the whole, water was considered the most advantageous medium giving good recovery and the highest specific activity in the supernatant.

The supernatant from whole brain homogenate prepared in this way showed linearity with enzyme activity up to 45 μU and with time up to 25 min. The supernatant from adrenals homogenized in 0·01 M sodium acetate pH 6·1 containing 0·32 M sucrose also showed linearity with enzyme activity and time to 70 μU and 25 min respectively. Blanks, without tissue supernatant, were usually about 7% of the maximum observed activity.

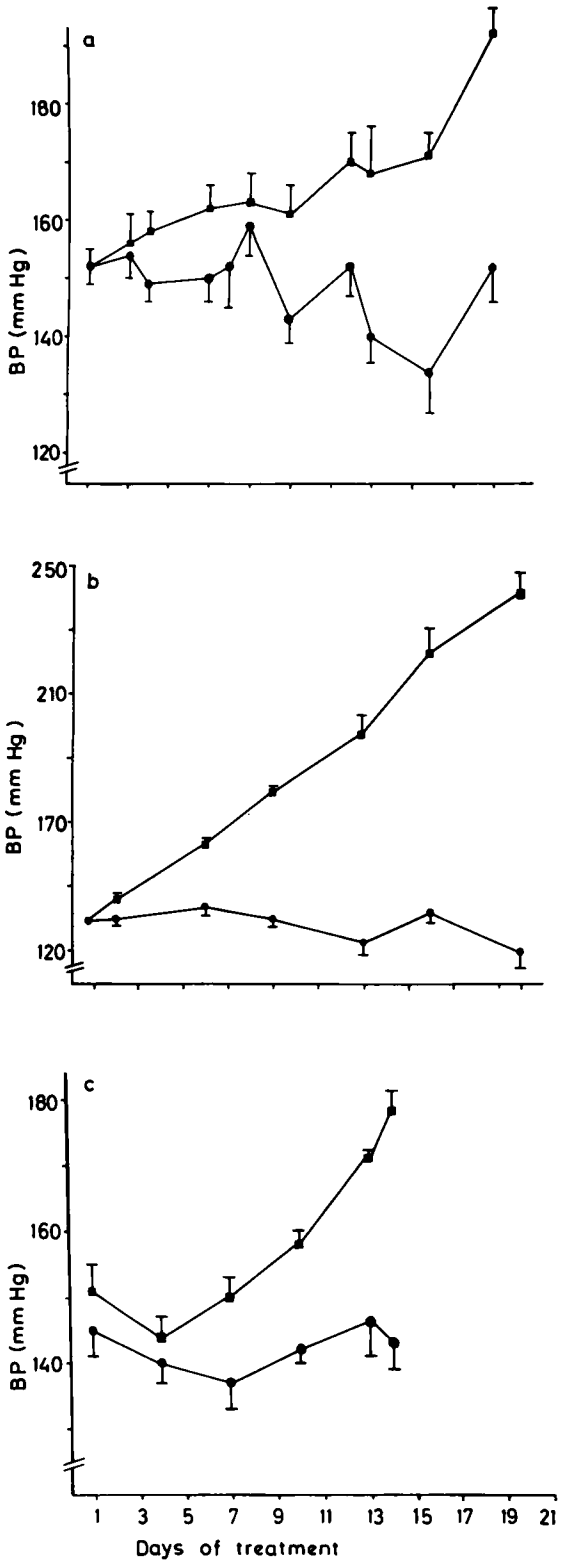
Table 2. The effect of reserpine treatment on tissue supernatant TH activity (μU mg protein^{-1} ± standard error). (a) animals received reserpine 2·5 mg kg^{-1} daily for 3 days and were killed 24 h after the last dose. (b) animals received reserpine 2·5 mg kg^{-1} daily for 4 days and were killed 72 h after the last dose. Control animals received vehicle only. Number of animals in parentheses.

Tissue	Control TH	Reserpine TH	Reserpine % of Control
(a) Adrenals	48·7 ± 4·8 (7)	121·0 ± 12·0 (7)	248
Whole brain	28·2 ± 1·2 (7)	26·7 ± 1·4 (7)	94
(b) Adrenals	28·1 ± 3·0 (6)	155 ± 34·0 (6)	553
'Locus coeruleus'	10·3 ± 1·6 (3)	35·8 ± 3·8 (3)	348
Cerebellum	2·0 ± 0·1 (6)	4·1 ± 0·4 (6)	205
Hypothalamus	64·3 ± 3·1 (6)	75·2 ± 6·1 (6)	117
'Pons-medulla oblong'	8·1 ± 2·0 (6)	9·3 ± 0·5 (6)	115
Rest of brain	25·9 ± 1·7 (6)	25·7 ± 1·5 (6)	99

Table 3. Whole brain TH activity (μU mg protein^{-1}) in rats treated with doca and saline. ± standard error. Number of animals in parentheses.

Treatment	Days of Treatment				
	8	11	14		21
(a) Control	20·0 ± 0·8 (6)	22·2 ± 1·0 (6)	22·5 ± 0·8 (5)		18·8 ± 2·4 (5)
(b) Doca/saline	19·3 ± 1·5 (6)	22·6 ± 1·5 (5)	21·7 ± 0·8 (5)		21·9 ± 1·8 (5)
	6	10	14	17	20
(c) Control	22·7 ± 1·0 (5)	26·6 ± 1·8 (5)	24·7 ± 0·7 (5)	24·6 ± 0·8 (5)	23·1 ± 0·5 (5)
(d) Doca/saline	25·9 ± 1·6 (5)	25·1 ± 1·1 (5)	26·4 ± 1·1 (5)	22·7 ± 1·0 (6)	22·7 ± 0·8 (6)

(a) Arachis oil 2·5 mg kg^{-1} (s.c.) once every 3 days. (b) Doca 12·5 mg kg^{-1}, 5 mg ml^{-1} in oil (s.c.) once every 3 days and 1% NaCl to drink. (c) sham operation only. (d) 25 mg Doca pellet implant s.c. and 1% NaCl to drink.



Under these conditions, the tyrosine hydroxylase activity of adrenal glands from rats treated with reserpine for 3 days and killed 24 h after the last dose showed a significant increase (150% $P < 0.001$), whereas the enzyme was not increased in whole brain (Table 2). Alternatively, when reserpine treatment was continued for 4 days and the animals were killed 72 h after the last dose, the TH activity in the brain had increased by 250, 110, 20 and 15% in the 'locus coeruleus', cerebellum, hypothalamus and 'pons-medulla oblongata' respectively (Table 2). Enzyme activity in the adrenals of these animals had also risen by 450% (Table 2).

Rats receiving doca in oil and 1% NaCl to drink showed a slow continuous rise in systolic blood pressure over 21 days (Fig. 1a). This elevation was significant by day 7 and at day 21 the b.p. had risen to 192 mm Hg (control 152 mm Hg). Table 3 shows that whole brain tyrosine hydroxylase activity on days 8, 11, 14 and 21 of treatment was not significantly changed.

In similar animals, which received doca as a subcutaneous implant, the b.p. rise was also continuous but much more rapid (Fig. 1b) being significantly elevated by day 6 and reaching 240 mm Hg by day 20 (control 130 mm Hg). There was no significant difference in whole brain TH activity at any time during the development of hypertension (Table 3).

Both groups of animals receiving doca and 1·0% NaCl showed a reduced heart rate. This was most pronounced in rats treated with the doca implant and greatest at blood pressures below 200 mm Hg (Fig. 2).

DISCUSSION

When the procedure reported earlier using the crude homogenate (Rylett et al 1976) was repeated and failed to show an increase in whole brain TH activity, all the steps involved were fully reinvestigated. It has been shown that homogenzation of the brain in distilled water gives the best material for assay and,

FIG. 1. The systolic blood pressure of rats (mm Hg) (mean ± standard error) during the administration in (a) doca in oil (12·5 mg kg^{-1}, 5 mg ml^{-1}) s.c. once every 3 days and 1% NaCl to drink ■—■; controls received arachis oil only (2·5 mg kg^{-1}), s.c. once every 3 days ●—●. (b) Doca pellet implant (25 mg) s.c. and 1% NaCl to drink ■—■; controls received a sham operation only ●—●. (c) Doca in oil (12·5 mg kg^{-1}, 5 mg ml^{-1}) s.c. once every 3 days and 1% NaCl to drink ■—■; controls received arachis oil only (2·5 mg kg^{-1}), s.c. once every 3 days ●—● results from Rylett et al (1976).

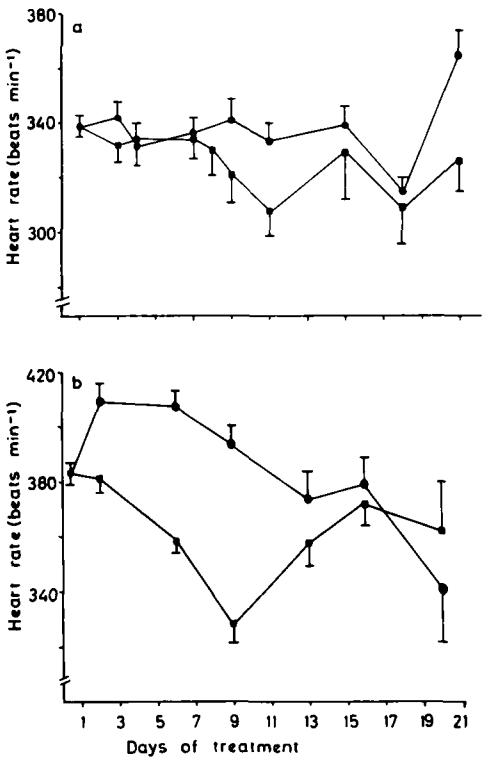


FIG. 2. The heart rate of rats (beats min^{-1}) (mean ± standard error) during the administration in (a) doca in oil (12·5 mg kg^{-1}, 5 mg ml^{-1}) s.c. once every 3 days and 1% NaCl to drink ■—■; controls received arachis oil only (2·5 mg kg^{-1}), s.c. once every 3 days ●—●. (b) Doca pellet implant (25 mg) s.c. and 1% NaCl to drink ■—■; controls received a sham operation only ●—●.

unless the homogenate is centrifuged there is no linear relationship between the amount of enzyme added and enzyme activity per mg protein. A possible explanation for this is binding of dopa to cellular fragments in crude homogenate or an inadequacy in the protein assay when insoluble material is present. The values reported here for rat whole brain TH activity agree closely with those reported by other workers (Cicero et al 1972; Coyle 1972) and are 20 fold greater than those quoted by Rylett et al (1976). These factors, when taken into consideration, must cast some doubt on the TH activity values quoted by Rylett et al (1976).

Reserpine treatment elevates adrenal TH activity (Mueller et al 1969; Joh et al 1973; Pfeffer et al 1975) by increasing the quantity of enzyme protein as a result of drug-induced sympathoadrenal overactivity (Iggo & Vogt 1960). After a 3 day course of reserpine, an increase in adrenal TH activity was observed. However, at a time when TH is maximally increased in the locus coeruleus and is beginning to rise in other brain areas (hypothalamus, cerebellum and substantia nigra) (Reis et al 1975; Zigmond 1979), we could detect no change in whole brain TH activity (Table 2). After rats were treated with reserpine for 4 days and killed 3 days later, the TH activity was elevated in the 'locus coeruleus', cerebellum, hypothalamus and 'pons-medulla oblongata'. These results agree with those of Reis et al (1975) and Zigmond (1979) and show the assay is capable of detecting a rise in brain TH activity. We conclude that whole brain TH activity does not reflect localized, yet pertinent, alterations in enzyme activity and therefore the state of catecholaminergic neurons.

There are clear differences in the hypertensive response corresponding to various treatments as shown in Fig. 1. There is also a difference in control b.p. values for animals receiving doca in oil or as an implant, but this may be related to the age of the animals. The linear rise in systolic pressure is much faster for the smaller animals receiving doca implant. In spite of the clear differences between control and test groups in both doca 1·0% NaCl treatments there is no difference in whole brain TH activity. It is most difficult however, to make a comparison between these changes and the earlier observations (Fig. 1c) where the b.p. exhibited a rapid increase after 7 days of treatment.

The conditions we used in which doca was injected were similar to those used by Rylett et al (1976). It is possible that altered animal handling or the difference in the onset of hypertension may be responsible for the absence of an increase in whole brain TH activity. The most likely explanation however, would be that the reported rise in whole brain TH activity was artifactual. Using an improved tyrosine hydroxylase assay and material which showed a linear relationship between measured activity and amount of enzyme, we have shown that local changes in brain TH are not revealed by measurements on whole brain and that the b.p. rise following doca 1·0% NaCl treatment is not accompanied by a parallel change in the whole brain TH activity.

In the literature there is growing evidence that altered handling of noradrenaline is seen in various brain regions of the doca/saline-treated rat (Nakamura et al 1971; Yamori et al 1973; Van Ameringen et al 1977; Zamir et al 1979). It is suggested that central noradrenergic neurons may play a passive role in the development or

maintenance of the hypertension, in that they allow blood pressure to rise because of an inability to increase activity in neuronal circuits opposing b.p. rises. None of the previously mentioned studies have shown that the changes seen are causing, or result from, the blood pressure increase. It is possible that early changes (within the first week of doca/saline treatment) actively involved in raising b.p. may have been overlooked in studies on established hypertension. Clearly a more detailed biochemical approach is required to reveal any relationship existing between changes in central noradrenergic neuron activity and b.p. during the development and maintenance of experimental hypertension and to locate any 'neurogenic trigger mechanism'.

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