Biochemical Pharmacology, Vol. 18, pp. 1441–1446. Pergamon Press. 1969. Printed in Great Britain

THE ACTIVITY OF TYROSINE HYDROXYLASE AND RELATED ENZYMES OF CATECHOLAMINE BIOSYNTHESIS AND METABOLISM IN DOG KIDNEY— EFFECTS OF DENERVATION

TOSHIHARU NAGATSU,* LYMAN A. RUST and VINCENT DEQUATTRO

Clinical Pharmacology Section, Department of Medicine,
University of Southern California, Los Angeles, Calif., U.S.A.

(*Received* 18 *September* 1968; *accepted* 13 *December* 1968)

Abstract—Renal denervation resulted in the disappearance of norepinephrine and tyrosine hydroxylase activity from both cortex and medulla, while the activities of the other enzymes of norepinephrine biosynthesis and metabolism were unaffected. Of the enzymes which were successfully assayed, only tyrosine hydroxylase was exclusively intraneuronal; the others were mainly extraneuronal. Endogenous inhibitors in the crude homogenates interfered with the assay of dopamine-β-hydroxylase. The relatively high concentration of the regulatory biosynthetic enzyme in the renal medulla suggests that the sympathetic nervous system has an undefined but specific role in this tissue.

IN THE renal cortex, the sympathetic nerves influence renal physiology and electrolyte homeostasis via the arteries and afferent arterioles[1] and the production and release of renin.[2, 3] Histochemical studies of the renal medulla indicate the presence of sympathetic fibers and terminals which are not in association with the blood vessels.[1] This study describes the effect of denervation on the renal distribution of the sympathetic neurotransmitter and the enzymes involved in its biosynthesis and metabolism. The results confirm findings in other tissues[4, 5] that tyrosine hydroxylase is exclusively intraneuronal and, further, the high capacity for neurotransmitter synthesis suggests that the sympathetic nerves have a specific role in the renal medulla.

MATERIALS AND METHODS

L-Tyrosine-^{14}C (uniformly labelled, 352 mc/m-mole), tryptamine-2-^{14}C-bisuccinate (10·3 mc/m-mole), dopamine-β,β-^{3}H (6·2 mc/m-mole) and S-adenosyl-L-methionine (S-methyl-^{14}C; 47 mc/m-mole) were obtained from New England Nuclear Corp. Ring-labelled dopamine-^{3}H was prepared enzymatically from ring-labelled L-DOPA-^{3}H (500 mc/m-mole) obtained from Nuclear of Chicago. L-Tyrosine-^{14}C was purified with alumina.[6] *p*-Bromo-*m*-hydroxybenzoxyamine (Brocresine), iproniazid and harmaline were donated by Lederle, Hoffman-LaRoche and Latabec Laboratories respectively. 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$), L-3,4-dihydroxyphenylalanine (dopa) and epinephrine were obtained from Calbiochem.

Selective surgical denervation of both kidneys was done in 35–45-pound mongrel

* Present address: Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, Chikusa, Nagoya, Japan.

female dogs. The dogs were anesthetized with pentobarbital, the kidneys exposed, and all renal nerves were stripped from the renal blood vessels and ureters. A solution of 5% phenol was applied to the stripped vessels. In sham-operated animals, the kidneys were exposed but not denervated. At 4–5 weeks after operation, the animals were sacrificed and the kidneys, heart and spleen were rapidly excised and immediately frozen on dry ice. Organs were stored at −85° for subsequent enzyme assays. Preliminary study was made of wedge sections (containing mainly cortex) of kidneys. In later studies, kidneys were separated into cortex and medullary portions. The tissues were homogenized for 20–30 sec, twice, in a glass homogenizer in 3 vol. of 0·25 M sucrose. Cooling of the homogenate was maintained during each homogenization. Aliquots of the homogenates were assayed for norepinephrine[7] and protein.[8]

Tyrosine hydroxylase activity was measured by the conversion of tyrosine-^{14}C to dopa-^{14}C.[6] The incubation mixture contained 100 mg tissue, 2 mμmoles L-tyrosine-^{14}C (6×10^5 cpm), 100 mμmoles L-tyrosine, 1·0 μmole $DMPH_4$, 100 μmoles mercaptoethanol, 200 μmoles phosphate buffer (pH 6·4), and 100 mμmoles Brocresine in a final volume of 1·0 ml. The incubation was at 30° in air for 15 min on a metabolic shaker. A nonincubated trichloroacetic acid-precipitated mixture was used as a blank. The formed dopa-^{14}C and carrier dopa were adsorbed on to alumina and eluted and radioassayed. The values were corrected for recovery (mean, 68 per cent). Tissue tyrosine was assayed fluorometrically.[9] In the kidney, tyrosine levels obtained by the fluorescence assay were high, indicating the presence of nitrosonaphthol-reacting phenols other than tyrosine. Therefore, tyrosine was determined in a control and denervated kidney by amino acid analyzer.

Dopa decarboxylase (aromatic L-amino acid decarboxylase) activity was assayed by the conversion of dopa to dopamine, which was subsequently isolated by an IRC-50 (Na^+) column.[10] The incubation mixture contained 50 mg tissue, 2 μmoles iproniazid, 0·5 μmole L-dopa, 150 mμmoles pyridoxal-5-phosphate, and 750 μmoles phosphate buffer, pH 8·0, in a total volume of 2·0 ml. The incubation was for 20 min on a shaker at 37°.

Dopamine-β-hydroxylase activity was assayed by two methods: with dopamine-β,β-^{3}H as substrate, the released tritiated water was assayed;[11–13] with dopamine-^{3}H (ring-labelled) as substrate, the norepinephrine-^{3}H product was isolated by Dowex-50 column and radioassayed.[14] The incubation mixture contained 100 mg tissue and, in micromoles: fumarate, 10; ascorbate, 10; potassium phosphate (pH 6·5), 200; dopamine (including radioactive dopamine), 10; ascorbate, 10; harmaline, 0·2; and enough catalase in a final volume of 1·0 ml. The reaction mixture was incubated for 20 min at 37°.

Monoamine oxidase (MAO) activity was assayed by measuring the conversion of tryptamine-^{14}C to indoleacetic acid.[15] The incubation mixture contained 1·0 mg tissue, 60 mμmoles tryptamine-^{14}C (0·1 μc), and 50 μmoles phosphate buffer, pH 7·4, in a total volume of 300 μl. The incubation was carried out at 37° in air for 20 min on a metabolic shaker. Boiled enzyme was used as a blank.

Catechol-*O*-methyl transferase (COMT) activity was assayed with epinephrine as substrate.[16] An aliquot of the 50,000 *g* supernatant was taken for enzyme assay. The incubation mixture contained 5 μmoles phosphate buffer (pH 7·6), 2·5 μmoles $MgCl_2$, 2·1 mμmoles S-adenosyl-L-methionine (S-methyl-^{14}C; 0·1 μc), 0·25 μmole epinephrine, and 25 μl of the supernatant in a final volume of 50 μl.

RESULTS

Tissue distribution of tyrosine hydroxylase in dog organs. The activity of tyrosine hydroxylase and the concentration of norepinephrine, quantitative indicators of sympathetic nerve supply to an organ, are given for spleen, heart, renal cortex and medulla (Table 1). The ratio of tyrosine hydroxylase activity to the concentrations of norepinephrine was similar in heart, spleen and renal cortex. However, in the renal medulla the ratio was markedly increased. MAO activity did not correlate with either norepinephrine concentration or tyrosine hydroxylase activity.

TABLE 1. NOREPINEPHRINE AND THE ACTIVITIES* OF TYROSINE HYDROXYLASE AND MONOAMINE OXIDASE IN DOG TISSUES

Tissue	Norepinephrine (μg/g)	Tyrosine hydroxylase (mμmoles/g/hr)	Monoamine oxidase (μmoles/g/hr)
Spleen	2·1	16·5	4·1
Left ventricle	1·0	10·1	0·4
Renal cortex	0·5	3·1	5·2
Renal medulla	0·2	4·9	3·7

* L-Tyrosine-^{14}C and tryptamine-^{14}C were substrates for tyrosine hydroxylase and monoamine oxidase respectively. Each value is the mean of three determinations.

Effect of renal denervation on the activity of tyrosine hydroxylase, dopa decarboxylase, dopamine-β-hydroxylase, MAO and COMT in dog kidney. A preliminary study of the effect of renal denervation on the activity of tyrosine hydroxylase and the concentration of norepinephrine was made on wedge sections of dog kidneys. As shown in Table 2, tyrosine hydroxylase activity and norepinephrine concentration decreased to insignificant levels. MAO activity did not change significantly after denervation.

TABLE 2. EFFECT OF RENAL DENERVATION ON NOREPINEPHRINE AND THE ACTIVITIES OF TYROSINE HYDROXYLASE AND MONOAMINE OXIDASE IN THE DOG KIDNEY

	Norepinephrine (μg/g/hr)	Tyrosine hydroxylase (mμmoles/g/hr)	Monoamine oxidase (μmoles/g/hr)
Sham-operated (4)*	0·55 ± 0·07	3·02 ± 0·61	4·69 ± 0·30
Denervated (6)*	0·04 ± 0·03	0·0	5·23 ± 0·45

* Numbers represent organs studied. Data expressed as mean ± S.E.

The relative activities of the enzymes involved in catecholamine biosynthesis and metabolism and the effect of denervation were examined separately in the renal cortex and medulla. The concentration of norepinephrine in cortex expressed in micrograms per gram wet weight was significantly higher than in medulla (0·50 compared to 0·22 μg/g; $P < 0·02$). When expressed per milligram of tissue protein, however, the norepinephrine concentration was not significantly different (Table 3). Further, tyrosine hydroxylase activity per amount of protein was about three times higher in the medulla than in the cortex.

Denervation resulted in almost complete disappearance of tyrosine hydroxylase

TABLE 3. DISTRIBUTION OF RENAL NOREPINEPHRINE AND TYROSINE HYDROXYLASE ACTIVITY PER AMOUNT OF PROTEIN

	Norepinephrine (mμmoles/mg protein)	Tyrosine hydroxylase (mμmoles/mg protein/hr)	Protein (mg/g)
Cortex	22 ± 2	0·022* ± 0·003	139* ± 8
Medulla	18 ± 5	0·067* ± 0·003	73* ± 3

* Values significantly different ($P < 0·001$).

activity (less than 0·1 μμmole/mg protein/hr) from both contex and medulla. The concentration of norepinephrine decreased markedly to less than 0·03 μg/g in the cortex and 0·01 μg/g in the medulla. When dopamine-β-hydroxylase activity was measured by using dopamine-β,β-^{3}H as the substrate, release of tritiated water was observed both in the sham-operated kidney and in the denervated kidney. However, when the formation of norepinephrine from ring-labelled dopamine-^{3}H was measured, no activity was observed in either the sham-operated or denervated kidney. The distribution of the other enzyme activities were as follows (Table 4): dopa decarboxylase was higher in cortex; MAO was distributed equally in cortex and medulla; and COMT activity was higher in medulla. There was no significant effect of denervation on the activities of dopa decarboxylase, MAO or COMT in cortex or medulla (Table 4).

TABLE 4. ACTIVITIES* OF DOPA DECARBOXYLASE, MAO AND COMT IN THE INTACT AND DENERVATED KIDNEY

	Dopa decarboxylase		Monoamine oxidase		Catechol-*O*-methyl transferase	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla
Sham (4)	24·5±2·9†	11·8±2·3†	36·8±6·3	48·9±11·2	1·13±0·14‡	2·22±0·28‡
Denervated (3)	33·9±5·8	10·4±1·5	38·8±3·6	60·9±15·4	1·47±0·07	2·08±0·21

* Values in mμmoles/mg protein/hr.
†, ‡ Sham values are significantly different ($P < 0·02$). There were no significant differences after denervation.

Effect of denervation on nitrosonaphthol-reacting phenols, tyrosine and other amino acids. The nitrosonaphthol fluorescence assay of tyrosine[9] is not specific for tyrosine in kidney. The values found by this method were significantly higher than those obtained by using an amino acid analyzer. The mean values (mμmoles/g ± S.E.) for three sham-operated and three denervated organs were 825 ± 95 and 591 ± 53 respectively. The difference was not significant. The effect of denervation on tyrosine specifically and on other amino acids in dog kidney was examined by using an amino acid analyzer. Although some amino acid values such as valine, methionine and isoleucine decreased slightly after denervation, the changes were minimal. The values (mμmoles/g) for a sham-operated and denervated kidney, respectively, were as follows: tyrosine, 214 and 198; proline, 468 and 377; valine, 598 and 362; methionine, 192 and 120; isoleucine, 293 and 182; phenylalanine, 137 and 163; histidine, 225 and 285; and arginine, 253 and 212.

DISCUSSION

The high levels of tyrosine hydroxylase activity and norepinephrine in both renal cortex and medulla indicate rich sympathetic innervation This is in agreement with recent histochemical evidence.[1] The activity of the rate-limiting enzyme,[17] when expressed per amount of protein, was higher in medulla than in cortex, suggesting increased biosynthetic capacity in medulla. The elevated ratio of tyrosine hydroxylase activity to norepinephrine concentration may indicate deficient storage sites for, or rapid utilization and turnover of, norepinephrine in the renal medulla. An analogous situation exists in the brain where norepinephrine turnover is rapid but the tissue levels are low.[18] Alternatively, there may be preferential formation of dopamine. In any case, the increased tyrosine hydroxylase activity in the medulla compared to that in the more vascular cortex suggests an important nonvascular role for the sympathetic nervous system in the renal medulla.

Denervation resulted in complete disappearance of tyrosine hydroxylase activity from both cortex and medulla, suggesting that the enzyme is exclusively intraneuronal. The reduction of norepinephrine can be accounted for by this loss of tyrosine hydroxylase. This is in agreement with findings in heart[4] and salivary gland.[5] In addition, other studies have indicated that norepinephrine storage capacity is impaired after sympathetic denervation of various organs.[19] The nitrosonaphthol fluorescence assay[9] was not specific for tyrosine in dog kidney, and the decrease of the nitrosonaphthol-reacting phenols after denervation was not significant. Total tyrosine was not affected by denervation, since the amount occurring intraneuronally was relatively small.

The activity of dopa decarboxylase, the second enzyme in norepinephrine biosynthesis, was unchanged by denervation. Although dopa decarboxylase was found to be primarily intraneuronal in certain organs,[20, 21] in the dog kidney the intraneuronal content was small compared to the extraneuronal portion. This is in agreement with findings in denervated dog heart[22] and in rat kidney after immunosympathectomy.[21] The physiological role for this extraneuronal nonspecific aromatic L-amino acid decarboxylase, which occurs in higher concentration in cortex, has not been characterized.

Dopamine-β-hydroxylase is thought to be an intraneuronal enzyme, and its disappearance after denervation would be expected as in the case of tyrosine hydroxylase. However, when the formation of norepinephrine-^{3}H from dopamine-^{3}H (ring-labelled) was measured, no activity of the enzyme could be determined either in the sham-operated kidney or in the denervated kidney, indicating interference of the assay by the presence of endogenous inhibitors. When the release of tritium water from dopamine-β-β-^{3}H was measured, activity was observed both in the sham-operated and denervated kidneys. This suggests that the measurement of tritium water released from dopamine-β,β-^{3}H is not specific for the assay of dopamine-β-hydroxylase in crude kidney homogenate. Similar results were found in brain homogenate, although the release of one molecule of tritium water for the formation of one molecule of norepinephrine has been confirmed in a purified adrenal dopamine-β-hydroxylase system.* Thus, the use of the β-labelled substrate does not appear to be indicated for dopamine-β-hydroxylase assay in crude tissue homogenate.

Denervation did not affect MAO activity, indicating that in kidney as in heart[21] and other organs[23] the intraneuronal concentration of the enzyme is small compared

* C. R. Creveling, personal communication (1968).

to that occurring extraneuronally. There have been conflicting reports of a substantial decrease (42 per cent),[24] and little change, in COMT activity[22] after denervation of dog heart. However, the lack of change in COMT activity in dog kidney after denervation agrees with the widely held concept that it is almost entirely an extraneuronal enzyme.[19] Previous studies in dogs indicated that infused labelled norepinephrine underwent tubular reabsorption or metabolism after glomerular filtration. However, renal denervation or therapy with MAO inhibitors did not affect this reduction of norepinephrine clearance, leading to the observation that COMT may be an important degradative enzyme in the renal tubule.[25] The present findings of increased COMT in the renal medulla emphasize the possible importance of this pathway in the kidney. Renal denervation does not affect the urinary excretion of norepinephrine or its metabolites so that the contribution of the renal sympathetic nerves to total endogenous catecholamine excretion is negligible.[25, *] Further knowledge of renal intraneuronal norepinephrine metabolism may require isotopic studies.

Acknowledgement—The capable technical assistance of Mrs. Shirley Chan and Mr. Dan Meyer is gratefully appreciated.

* L. Rust and V. DeQuattro, submitted for publication.

REFERENCES

1. O. C. McKENNA and E. T. ANGELAKOS, *Circulat. Res.* **22**, 345 (1968).
2. R. M. GORDON, O. KÜCHEL, G. W. LIDDLE and D. P. ISLAND, *J. clin. Invest.* **46**, 599 (1967).
3. R. D. BUNAG, I. H. PAGE and J. W. McCUBBIN, *Circulat. Res.* **19**, 851 (1966).
4. P. E. POOL, J. W. COVELL, M. LEVITT, J. GIBB and E. BRAUNWALD, *Circulat. Res.* **20**, 349 (1967).
5. G. C. SEDVALL and I. J. KOPIN, *Biochem. Pharmac.* **16**, 39 (1967).
6. T. NAGATSU, M. LEVITT and S. UDENFRIEND, *J. biol. Chem.* **239**, 2910 (1964).
7. R. CROUT, *Stand. Meth. clin. Chem.* **3**, 62 (1961).
8. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
9. T. P. WAALKES and S. UDENFRIEND, *J. Lab. clin. Med.* **50**, 733 (1957).
10. W. LOVENBERG, H. WEISSBACH and S. UDENFRIEND, *J. biol. Chem.* **237**, 89 (1962).
11. S. SENOH, C. R. CREVELING, S. UDENFRIEND and B. WITKOP, *J. Am. chem. Soc.* **81**, 6236 (1959).
12. C. R. CREVELING, *Studies on Dopamine β-Oxidase* (Ph.D. thesis). George Washington University Washington, D.C. (1962).
13. M. GOLDSTEIN, N. PROCHOROFF and S. SIRLIN, *Experientia* **21**, 592 (1965).
14. A. BERTLER, A. CARLSSON and E. ROSENGREN, *Acta physiol. scand.* **44**, 273 (1958).
15. R. J. WURTMAN and J. AXELROD, *Biochem. Pharmac.* **12**, 1439 (1963).
16. J. AXELROD and R. TOMCHICK, *J. biol. Chem.* **233**, 702 (1958).
17. M. LEVITT, S. SPECTOR, A. SJOERDSMA and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **148**, 1 (1965).
18. S. UDENFRIEND and P. ZALTZMAN-NIRENBERG, *Science, N.Y.* **142**, 394 (1963).
19. J. AXELROD, *Pharmac. Rev.* **18**, 95 (1966).
20. N. E. ANDEN, T. MAGNUSSON and E. ROSENGREN, *Acta physiol. scand.* **64**, 127 (1965).
21. G. I. KLINGMAN, *J. Pharmac. exp. Ther.* **148**, 14 (1965).
22. L. T. POTTER, T. COOPER, V. L. WILLIAM and D. E. WOLFE, *Circulat. Res.* **16**, 468 (1965).
23. J. H. BURN, F. J. PHILPOT and U. TRENDELENBURG, *Br. J. Pharmac. Chemother.* **9**, 423 (1954).
24. R. CROUT and T. COOPER, *Nature, Lond.* **194**, 387 (1962).
25. H. R. OVERY, R. PFISTER and C. A. CHIDSEY, *J. clin. Invest.* **46**, 482 (1967).