Biochemical Pharmacology, Vol. 17, pp. 1470–1473. Pergamon Press. 1968. Printed in Great Britain

Subcellular distribution of adrenal tyrosine hydroxylase*†

(*Received* 23 *October* 1967; *accepted* 22 *December* 1967)

THE HYDROXYLATION of tyrosine to dopa is considered to be the rate-limiting step in the biosynthesis of catecholamines.[1] There are indications that this step is regulated *in vivo* by sympathetic nerve activity: decentralization produces a decrease in the amount of norepinephrine synthesized from tyrosine but not from dopa or dopamine,[2] while increased sympathetic activity increases catecholamine synthesis from tyrosine, but not from other precursors.[3] The mechanism of this regulation is not known, but it is possible that end product inhibition may play a role, since tyrosine hydroxylase (TH) is known to be inhibited by catecholamines.[4] Catecholamines are considered to be mostly localized in granular structures in the adrenal gland[5] and in the sympathetic nerve endings.[6] The study of the subcellular localization of TH may therefore be of importance in understanding the mechanism of its regulation.

Several preliminary experiments in this laboratory showed that the tyrosine hydroxylase activity in the rat adrenal gland is found in the high speed supernatant fraction. However, before accepting that tyrosine hydroxylase is not a particulate enzyme, at least two possibilities should be carefully examined:

(1) The TH activity of the high speed supernatant may be an artifact of homogenization due to particle destruction and enzyme leakage. If this were the case, a gentle homogenization should leave some enzyme in the particulate fraction. The particle-bound enzyme might be undetectable, however, if either the substrate or cofactors could not penetrate the particles, or if the enzyme were completely inhibited by the high catecholamine content of the particles.

(2) TH may be located in light particles that do not precipitate when spun for 1 hr at 100,000 *g*. Experiments were therefore designed to investigate these possibilities.

MATERIALS AND METHODS

In order to keep particle destruction and possible enzyme leakage to a minimum, rat adrenal glands were mainly used in this study because they can be easily homogenized with a Teflon pestle homogeniser driven by hand. However, some experiments were performed with beef adrenal glands. Sprague-Dawley rats weighing 180–200 g were killed by a blow on the head. The adrenal glands were rapidly removed and chilled in ice-cold isotonic sucrose or KCl. The adrenal glands in groups of 2 or 4 were homogenized in 7–10 ml of 0·15 M KCl or in 0·32 M sucrose buffered at pH 7·0 with potassium phosphate or Tris-HCl at a final concentration of 0·005 M. All the procedures were carried out at 4° to 5° unless otherwise stated. The adrenal gland homogenate was in all cases centrifuged at 1000 *g* for 15 min to separate unbroken cells, cell debris and nuclei. The 1000 *g* supernatant fractions was then subjected to an average centrifugal force of 100,000 *g* for 1 hr. In some cases the 100,000 *g* supernatant was recentrifuged at 150,000 *g* (average force) for 3 hr. Tyrosine hydroxylase activity was precipitated from the high speed supernatants by the addition of equal volumes of a saturated solution of $(NH_4)_2SO_4$ buffered at pH 7·0 with potassium phosphate at a final concentration of 0·02 M. The precipitate was collected by centrifugation at 30,000 *g* for 20 min and it was dissolved in 0·6 or 1·0 ml of 0·005 M potassium phosphate buffer at pH 7·0. The particles precipitating with high speed centrifugation were subjected to different procedures in attempts to detect tyrosine hydroxylase activity. The particulate fractions were resuspended in hypotonic solutions with a small glass homogenizer in order to favor catecholamine losses and particle destruction. Prolonged dialysis and sonication were also used, and their respective effects are described in the tables.

Some experiments using beef adrenal gland were performed. Fresh beef adrenal glands were dissected free of cortical tissue and were homogenized at low speed in a glass homogenizer. The methods used were otherwise similar to those used in the rat adrenal experiments.

* Supported by National Institutes of Health Grant HE 10333.

† A summary of these results has been presented at the 1967 Fall Meeting of A.S.P.E.T. held at Washington, D.C. (*Pharmacologist*, **9**, 210, (1967).

Tyrosine hydroxylase activity was determined by the method of Nagatsu *et al.*[7] Samples were incubated for 10 or 15 min at 25° and the reaction was stopped by the addition of 0·1 ml of 30% trichloroacetic acid.

RESULTS AND DISCUSSION

It can be seen in Table 1 that practically no TH activity precipitated at 100,000 *g* for 1 hr. No enzyme activity was detected even after the particles obtained with 100,000 *g* were dialyzed against

TABLE 1. TYROSINE HYDROXYLASE ACTIVITY IN RAT ADRENAL GLAND SUBCELLULAR FRACTIONS*

Homogenate No.	Sediment at 100,000 *g* nondialyzed		100,000 *g* Supernatant
1 KCl		0·15	6·37
2 KCl		0·09	5·92
3 Suc		0·15	4·43
4 Suc		0·12	2·98
	Sediment at 100,000 *g* 1 hr (dialyzed)	Sediment at 150,000 *g* 3 hr	Supernatant from both previous centrifugations
5 KCl	0·11	0·12	4·56
6 KCl	0·28	0·44	5·39

* Groups of 2 adrenal glands were homogenized in isotonic KCl (KCl) or sucrose (suc). The 1000 *g* supernatant was centrifuged at 100,000 *g* for 1 hr and separated into a supernatant and a particulate fraction. The supernatants of homogenates 5 and 6 were subjected to an additional centrifugation at 150,000 *g* (average force) for 3 hr. Tyrosine hydroxylase activity is expressed as millimicromoles of H^3OH formed in 15 min by two glands.

hypotonic (0·005 M) Tris-HCl buffer, pH 7·0, for 18 hr in order to remove the particle-bound epinephrine. When the 100,000 *g* supernatant fraction was subjected to an additional centrifugation at higher speed (150,000 *g*) for longer periods, no significant TH activity was precipitated (Table 1).

The findings that no TH activity can be solubilized from the 100,000 *g* sediment even after several procedures, and that a gentle homogenization of the whole gland liberates all the detectable enzyme activity are indicative that TH is a soluble enzyme and not particle-bound.

In other experiments attempts were made to liberate TH activity by sonication of a particulate fraction. As shown in Table 2, no activity was detected.

Experiments using fresh beef adrenal glands consistently agreed with the results obtained with the rat. Almost 90 per cent of the TH activity was found in the supernatant fraction, even when high centrifugal forces were used (Table 3).

The small percentage of TH activity present in the high speed sediment of beef adrenals can be accounted for by aggregation and precipitation of proteins. Tyrosine hydroxylase preparations that previously remained in the supernatant fraction, when subjected to high speed centrifugation, will yield precipitates with enzymatic activity when they are recentrifuged after standing for some time. This phenomenon is exaggerated by such procedures as filtering through Sephadex G-200 in a dilute buffer (0·005 M) followed by freezing and thawing. The sedimentable enzyme maintains a considerable

fraction of its original activity.* This phenomenon may explain, at least in part, why this enzyme has been described to be particulate[8] as well as some of the difficulties encountered during its purification.

The results presented in this paper strongly suggest that TH is a cytoplasmic enzyme. Our observations are supported by similar findings in the cat adrenal gland.† Furthermore, it has been reported that in tissue extracts of bovine splenic nerve, TH is also localized in the high speed supernatant.[9]

TABLE 2. EFFECT OF SONICATION ON THE RAT ADRENAL 100,000 *g* SEDIMENT*

	mμMoles H^3OH in 15 min
Supernatant	12·15
Sediment	0·00
Sonicated sediment recentrifuged	
Supernatant	0·06
Sediment	0·70

* Two groups of 4 adrenal glands were homogenized in isotonic sucrose buffered at pH 7. The 100,000 *g* sediment was resuspended in 0·005 M potassium phosphate buffer at pH 7 in a glass homogenizer and sonicated for a total of 45 sec (excluding interruptions to minimize a rise in temperature). The sonicated resuspended sidement was then centrifuged at 100,000 *g* for 1 hr. The results are the average of 2 experiments and are expressed as millimicromoles of H^3OH formed in 15 min at 25° by 4 adrenal glands.

TABLE 3. TYROSINE HYDROXYLASE ACTIVITY* IN BEEF ADRENAL GLAND SUBCELLULAR FRACTIONS

Experiment No.	Centrifugal force	Sediment (mμmoles)	Supernatant (mμmoles)	(%)
1†	150,000 *g*	71	665	90·3
		73	637	89·7
2†	100,000 *g*	56	349	86·2
		45	284	86·3

* Tyrosine hydroxylase activity is expressed as millimicromoles of H^3OH formed in 10 min at 25° per g of gland.

† Experiment 1. Beef adrenal medulla was homogenized in 3 vol. of isotonic KCl and 1 ml of the 1000 *g* supernatant obtained was layered upon a solution of isotonic sucrose and centrifuged in the Spinco SW39 head at 39,000 rpm for 1 hr. The sediment was resuspended in 1 ml of 0·005 M Tris-HCl buffer, pH 7. The supernatants and resuspended pellets were dialyzed overnight against a large excess of the same buffer. Tyrosine hydroxylase activity was precipitated from the supernatant fractions as described in Methods.

† Experiment 2. Adrenal medulla was homogenized in 10 vol. of isotonic KCl. The 1000 *g* supernatant was subjected to 100,000 *g* for 1 hr. Resuspension of sediment, dialysis and tyrosine hydroxylase assay as in experiment 1.

The regulation of catecholamine synthesis would be expected to occur at the cytoplasmic level. If TH activity *in vivo* is regulated by a feedback mechanism,[10] the free catecholamines should be the regulatory pool. The conclusion is in agreement with the view that the concentration of free catecholamine is one of the regulating factors in biosynthesis.[11] However, the localization of TH outside the granules

* J. M. Musacchio, unpublished observation.

† N. Kirshner, personal communication.

is not in accord with Udenfriend's hypothesis[12] that a particle contains all of the enzymes necessary for catecholamine synthesis.

Department of Pharmacology,
New York University School of Medicine,
New York, N.Y., U.S.A.

JOSE M. MUSACCHIO

REFERENCES

1. M. LEVITT, S. SPECTOR, A. SJOERDSMA and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **148**, 1 (1965).
2. J. M. MUSACCHIO and V. K. WEISS, *Pharmacologist* **7**, 156 (1965).
3. R. GORDON, J. V. O. REID, A. SJOERDSMA and S. UDENFRIEND, *Molec. Pharmac.* **2**, 606 (1966).
4. S. UDENFRIEND, P. ZALTZMAN-NIRENBERG and T. NAGATSU, *Biochem. Pharmac.* **14**, 837 (1965).
5. H. BLASCHKO and A. D. WELCH, *Arch. exp. Path. Pharmak.* **219**, 17 (1953).
6. U. S. VON EULER and N. A. HILLARP, *Nature, Lond.* **177**, 44 (1956).
7. T. NAGATSU, M. LEVITT and S. UDENFRIEND, *Analyt. Biochem.* **9**, 122 (1964).
9. L. STJÄRNE and F. LISHAJKO, *Biochem. Pharmac.* **16**, 1719 (1967).
10. E. COSTA and N. H. NEFF, *2nd Symposium of the Parkinson's Disease Information and Research Center* (29–30 November, 1965), pp. 141–155. Raven Press, New York (1966).
11. A. ALOUSI and N WEINER, *Proc. natn. Acad. Sci. U.S.A.* **56**, 1491 (1966).
12. S. UDENFRIEND, *Harvey Lect.* **ser. 60**, 57 (1964).

Biochemical Pharmacology, Vol. 17, pp. 1473–1476 Pergamon Press. 1968. Printed in Great Britain

The influence of aerobic and anaerobic incubations on the uptake of serotonin by blood platelets*

(*Received* 12 *May* 1967; *accepted* 9 *February* 1968)

THE ABILITY of blood platelets to concentrate serotonin against large gradients is well established,[1] and it appears that this is due to an energy-requiring transport mechanism.[2, 3] Various lines of evidence lead to this conclusion: for example, uptake is inhibited by low temperature,[4, 5] by certain metabolic inhibitors,[6] and by drugs such as reserpine.[5] On the other hand, there are contradictory data in the literature with respect to the effect of anaerobiosis on the uptake of serotonin. Weissbach and Redfield[2] showed, for example, that a nitrogen atmosphere clearly suppressed the uptake of serotonin by platelets. Stacy[7] and Hughes and Brodie[8] claimed that the uptake of serotonin was equal in aerobic and anaerobic conditions.

* Supported in part by grants from the New Haven Heart Association and the United States Public Health Service (HE 09057).