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THE SUBCELLULAR LOCALIZATION OF TRITIUM AFTER INCUBATION OF HOMOGENATES OF RAT BRAIN WITH ^{3}H-NOREPINEPHRINE*

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Abstract—Density gradient centrifugation of rat brain homogenates, after their incubation at 37° with ^{3}H-norepinephrine (NE) revealed an uneven distribution of tritium among the five discrete fractions obtained. The bulk (76 per cent) of the tritium was found in the upper two fractions (A and B): the fraction lying between 0·29 and 0·88 M sucrose (B) contained 46 per cent of the total tritium in the gradient. Pretreatment of the animal with reserpine, or incubation with ^{3}H-NE at 4°, decreased the percentage of tritium in fraction B to approximately 36 per cent. The percentage distribution of tritium was uninfluenced by a fifty-fold increase in the quantity of ^{3}H-NE added to the incubation medium. In contrast, the absolute amount of tritium in B bore a linear relationship to the concentration of added amine. The simultaneous addition of both nonisotopic NE and ^{3}H-NE to the incubation medium decreased the tritium content of fraction B; the decrease was roughly proportional to the amount of nonisotopic NE present. Since over 80 per cent of the tritium in fractions A and B could be accounted for by unchanged norepinephrine, it is suggested that in this study the distribution of tritium in brain homogenates may accurately reflect that of endogenous catecholamine in intact neural structures.

PREVIOUS studies[1–3] have indicated that the endogenous norepinephrine and 5-hydroxytryptamine of mammalian brain is stored predominantly within subcellular structures. The uptake of NE by homogenates of rat brain after incubation in media containing NE has recently been demonstrated[4] and suggests that exogenous catecholamine may also be bound to similar cell organelles. To investigate this possibility in more detail, rat-brain homogenates, incubated with tritium-labeled NE, have been subjected to density gradient centrifugation and the distribution of tritium in specific subcellular fractions determined.

METHODS

Subcellular fractions were prepared at 4° from freshly excised whole brains of decapitated male rats (200 g, Sprague–Dawley). Two brains were pooled and homogenized (in a glass homogenizer with a Teflon pestle) for 65 sec in 9 volumes 0·29 M sucrose containing 0·1% edathamil (Versene). Nuclei and cell debris were removed by centrifugation at 900 × *g* for 10 min. A 25-ml aliquot of the cloudy supernatant fraction (I) was decanted and incubated with ^{3}H-NE for 30 min at either 37° or 4°,

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according to the requirements of the particular experiment. In one experiment 1·0 μg ^{3}H-NE* was added to the supernatant fraction (I), which was then immediately treated as described below (i.e. at zero time of incubation).

Incubation mixtures were centrifuged at 105,000 × *g* for 30 min and the sediment resuspended in 10 ml 0·29 M sucrose prior to its being layered over a sucrose density gradient. The supernatant (II) fluid from this centrifugation was used for the determination of total *nonparticulate* tritium. The sucrose density gradient was composed of upper and lower layers of 10 ml 0·88 M and 10 ml of 1·45 M sucrose respectively (Fig. 1). The resuspended sediment was layered over this gradient and centrifuged

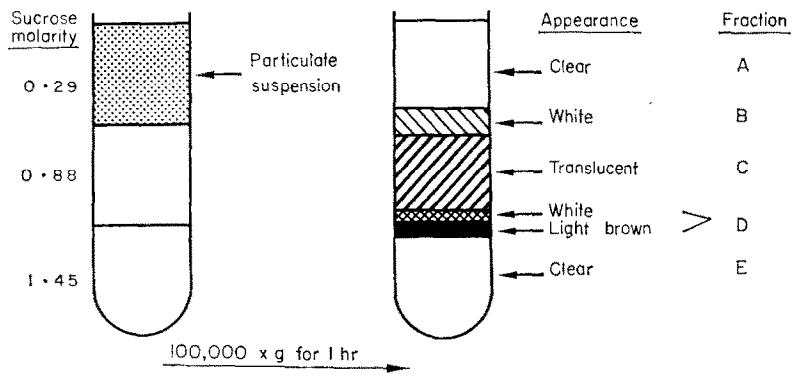

FIG. 1. Appearance of sucrose density gradient before and after centrifugation of rat brain homogenates.

for 1 hr at 100,000 × *g*. Immediately after ultracentrifugation the subcellular fractions were separated by means of a Spinco tube cutter, and the total tritium of each fraction was determined as described below.

The purity of the ^{3}H-NE used was confirmed by comparing the R_f of the sample with that of authentic DL-norepinephrine hydrochloride (California Biochemical Corp.) in a solvent system[5] consisting of butanol: acetic acid: water (4:1:1). All amounts of ^{3}H-NE added to the incubation medium refer to the base. Each fraction from the density gradient was removed and added to 4 ml perchloric acid (0·4 M). After separation of the protein precipitates by centrifugation at 30,000 × *g* for 10 min, the supernatant fluid was retained and the sediment re-extracted with an additional 2 ml perchloric acid (0·4 M). After a second centrifugation the supernatant material was combined with that from the first. The volume of the combined supernatant was measured and a 0·75 ml aliquot added to a counting vial containing 5 ml absolute ethanol and 10 ml phosphor (8 g 2,5-diphenyloxazole, 0·1 g *p*-bis-2-(5-phenyloxazolyl)benzene, 2 l. toluene and 1 l. absolute ethanol).

Sufficient perchloric acid (4·0 M) was added to the supernatant (II) obtained after incubation with ^{3}H-NE to render the solution 0·4 M with respect to the acid; after centrifugation and re-extraction of the precipitate with 2 ml 0·4 M perchloric acid, total tritium was determined as described above for the density gradient fractions. Determination of radioactivity was carried out with a Packard liquid scintillation

* ^{3}H-NE used was DL-norepinephrine-7-^{3}H-hydrochloride, specific activity 15·7 μc/μg, obtained from the New England Nuclear Corp.

spectrometer. Results, expressed as counts per minute, (cpm) are uncorrected for quenching, which was uniformly less than 10 per cent (tritiated water internal standards).

Since NE may be estimated specifically by means of an aluminum oxide extraction,[6] the method described below was used in two experiments to determine the percentage of the total tritium that was associated with unchanged norepinephrine in each fraction of the density gradient. After the addition of 0·1 volume of 1 % (w/v) disodium ethylenediamine tetraacetate, the perchloric acid extracts of all density fractions were brought to pH 8·4 (glass electrode, constant stirring) by means of 5·0 N potassium carbonate (to pH 6) and 0·5 N potassium carbonate. The precipitate that formed was allowed to settle and the clear supernatant fluid poured over 1 g aluminum oxide (Woelm alumina, neutral grade*) in a glass tube. The mixture was shaken vigorously for 10 min, after which the alumina was allowed to settle, and the supernatant fluid was aspirated and discarded. The alumina was washed by shaking for 5 min with 10 ml glass distilled water, followed by aspiration of the water. Removal of the ^{3}H-NE from the aluminia was achieved by shaking for 10 min with three successive 3-ml portions of hydrochloric acid (0·2 M). After each period of shaking the alumina was allowed to settle and the clear supernatant removed by aspiration. The three portions of hydrochloric acid were combined and the tritium of a 0·75 ml aliquot determined as described above. The recovery of ^{3}H-NE, added to each fraction before extraction with perchloric acid, averaged 70 per cent (range, 65 to 82 per cent, in eight separate determinations).

RESULTS

Subcellular distribution of tritium after incubation of homogenates with ^{3}H-norepinephrine

Subfractionation of rat brain homogenates by the method described produced five distinct fractions which were closely reproducible in each series of experiments. The layers of the density gradient have been designated by the letters A, B, C, D, and E as shown in Fig. 1. In the gradient used no distinct pellet was formed on the bottom of the tube, probably because of the high density of the lowest sucrose layer.

Analysis of subcellular fractions prepared from homogenates of whole rat brain which were incubated with 1·0 μg ^{3}H-NE for 30 min at 37°, revealed that a major portion of the total recovered tritium was found in fractions B (46 per cent) and A (30 per cent) (Fig. 1 and Table 1). The percentage uptake by fraction B remained relatively constant despite a fifty-fold increase in the concentration of ^{3}H-NE added to the incubation medium (Table 1). In contrast, the absolute amount of tritium retained by this fraction was directly related to the concentration of amine in the incubation media (Table 1).

The overall uptake of tritium by subcellular fractions appears to be time dependent Thus, the distribution of tritium among fractions obtained at zero time of incubation (see Methods), from media containing 1·0 μg ^{3}H-NE was quite different from that seen after 30 min of incubation with the same amount of ^{3}H-NE (Table 2).

* Before use the alumina was acid washed and dried in a manner similar to that recently described by Anton and Sayre.[7]

TABLE 1. DISTRIBUTION OF TRITIUM AMONG DENSITY GRADIENT FRACTIONS OF RAT BRAIN HOMOGENATES AFTER INCUBATION WITH ^{3}H-NOREPINEPHRINE

Results are expressed as per cent of total tritium in density gradient tube with corrresponding absolute value (cpm $\times$ 10^4) within parentheses.

NE added to incubation medium		Temperature of incubation	Fraction					Tritium in density gradient (as % of total tritium* recovered)
^{3}H-labeled (μg)	Unlabeled (μg)	(°C)	A	B	C	D	E	
0·1	—	37	30·4 (0·38)	41·6 (0·53)	13·7 (0·7)	11·9 (0·2)	2·4 (0·03)	—
1·0	—	37	29·7 (3·8)†	45·7 (5·8)	9·4 (1·1)	14·4 (1·8)	0·7 (0·08)	14·3
5·0	—	37	31·6 (16·1)	41·2 (21·0)	11·2 (5·7)	15·7 (8·0)	0·4 (0·20)	13·6
1·0	4·0	37	27·8 (2·8)	49·0 (4·9)	8·3 (0·8)	14·2 (1·4)	0·4 (0·04)	11·8
1·0	9·0	37	37·0 (3·6)	42·3 (4·1)	11·8 (1·2)	9·5 (0·9)	0·5 (0·05)	11·5
1·0	—	4	39·8 (4·2)	37·2 (3·9)	10·6 (1·1)	7·8 (0·8)	4·6 (0·5)	13·2
1·0 (reserpine‡)	—	37	35·3 (4·0)§	36·3 (4·1)	12·4 (1·4)	15·6 (1·8)	0·4 (0·04)	13·4

* Total tritium recovered = total tritium of density gradient and tritium of supernatant after centrifugation (105,000 $\times$ g for 30 min) of the incubated homogenate.

† Average of 4 experiments.

‡ Animals given reserpine, 5 mg/kg, 18 hr before use.

§ Average of 2 experiments.

When incubation of homogenates with 1·0 μg ^{3}H-NE was carried out at 4°, there was a decreased retention of tritium by fraction B, whereas that found in fraction A was increased (Table 1).

The percentage of the total tritium* recovered in subcellular fractions was remarkably constant in all experiments. The mean recovery was 13·0 per cent (range, 11·5 to 14·3 per cent) of the total tritium (Table 1).

TABLE 2. EFFECT OF DURATION OF INCUBATION ON DISTRIBUTION OF TRITIUM AMONG SUBCELLULAR FRACTIONS OF RAT BRAIN HOMOGENATES*

Subcellular fraction	Incubation period	
	0 min	30 min
	% of total tritium in density gradient	
A	83	30
B	9	46
C	6	9
D	2	14
E	0	1

* Homogenates incubated with 1·0 μg ^{3}H-NE.

Table 3 lists, for each fraction of the density gradient, the percentage of the total tritium measured that was associated specifically with norepinephrine. It can be seen that, in all fractions, the bulk of the tritium estimated could be accounted for by unchanged norepinephrine.

TABLE 3. PERCENTAGE OF TOTAL TRITIUM OF DENSITY GRADIENT FRACTIONS WHICH WAS ASSOCIATED WITH NOREPINEPHRINE*

Fraction	Per cent†
A	85·2
B	91·3
C	79·4
D	100·7
E	87·9

* 1·0 μg ^{3}H-norepinephrine added to incubation mixture.

†The values given are the means of two separate determinations and have been corrected for an average recovery of 70% (see Methods).

Effect of nonisotopic norepinephrine on the uptake of tritium by fraction B

In these experiments brain homogenates were incubated at 37° for 30 min with either 1·0 μg ^{3}H-NE and 4·0 μg NE or 1·0 μg ^{3}H-NE and 9·0 μg NE. The addition of nonisotopic NE decreased the tritium uptake by fraction B, and the magnitude of this reduction was apparently dependent on the amount of nontritiated NE added to the incubation medium. Table 1 shows that the presence in the incubation medium

* The percentage of total tritium refers to the following ratio: (^{3}H total of density gradient)/(^{3}H total in supernatant after 105,000 × *g* centrifugation + ^{3}H total of density gradient) × 100/1.

of 4·0 μg or 9·0 μg of the unlabeled amine along with 1·0 μg ^{3}H-NE decreased the amount of tritium in fraction B by 0·9 $\times$ 10^4 cpm (16 per cent) or by 1·7 $\times$ 10^4 cpm (29 per cent), respectively, when compared with the control values.

Effect of pretreatment with reserpine on the uptake of tritium by subcellular fractions

Homogenates prepared from the brains of rats which had received reserpine (5 mg/kg i.p.) 18 hr prior to the experimental period were incubated with 1·0 μg ^{3}H-NE and subcellular fractions prepared as before. Reserpine (Table 1) abolished the gradient of tritium normally observed between fractions A and B. Thus in homogenates of brain from reserpine-treated rats, the amounts of tritium in fractions A and B were 4·0 $\times$ 10^4 cpm (35 per cent of total uptake in the gradient) and 4·2 $\times$ 10^4 cpm (36 per cent) respectively; in control experiments the corresponding values were 3·8 $\times$ 10^4 cpm (30 per cent) and 5·8 $\times$ 10^4 cpm (46 per cent). In reserpine-treated preparations the absolute amount of tritium in fraction B was decreased by 30 per cent as compared with control values.

DISCUSSION

The uptake of tritium by subcellular fractions prepared from rat brain homogenates indicates that the binding capacity of the different fractions is not uniform and is maximal in the fractions we have designated A and B. Fraction A contained 30 per cent and fraction B 46 per cent of the total tritium taken up by the homogenate. This distribution remained constant despite a 50-fold increment (0·1 to 5·0 μg ^{3}H-NE) in the quantity of ^{3}H-NE added to the incubation medium. The remainder of the tritium found in the density gradient (approximately 25 per cent) appears to be equally distributed between fractions C and D with no obvious discrimination. It is of interest that either pretreatment of the animals with reserpine or incubation of brain homogenates at 4° alters the selective binding capacity of fraction B and thus eliminates the gradient of tritium normally observed between fractions A and B.

The tritium present in fraction B probably represents material truly particle bound since this fraction when prepared from similar homogenates at zero time of incubation (i.e. effectively nonincubated) contained only 9 per cent of the total tritium compared to 46 per cent after 30 min of incubation at 37° (Table 2). In nonincubated samples, 83 per cent of the tritium was found in fraction A and, in this case, was probably associated with NE or its metabolites either in a free form or bound to soluble protein.

Michaelson and Whittaker[3] have shown that the density gradient fraction of rat brain homogenates, which localized between 0·32 M and 0·9 M sucrose consisted of a bulky white layer containing only small quantities of acetylcholine and 5-hydroxytryptamine. This fraction was rich in myelin and microsomal structures and appears to correspond closely in sedimentation characteristics to fraction B in our work and in which we have observed maximal subcellular retention of tritium. Unfortunately, detailed electron microscopic examination of fraction B was not possible at the time of this investigation, but such a study is contemplated for the future. Localization of the endogenous catecholamine of dog hypothalamus in a similar density gradient fraction (at approximately 0·8 M sucrose) has been demonstrated by Chrusciel.[8] It therefore appears that after the incubation of rat brain homogenates with ^{3}H-NE, most of the tritium associated with particulate material is recovered

in the fraction that also contains the bulk of the endogenous catecholamine. Since a significant portion of the retained tritium which was measured in these studies could be accounted for by unchanged NE (Table 3), it appears that the distribution of exogenous NE in rat brain homogenates may accurately reflect that of the endogenous amine in intact neural structures.

REFERENCES

1. A. Bertler, N. A. Hillarp and E. Rosengren, *Acta physiol. Scand.* **50**, 113 (1960).
2. H. Weil-Malherbe and A. D. Bone, *Nature (Lond.)*. **180**, 1050 (1957).
3. I. A. Michaelson and V. P. Whittaker, *Biochem. Pharmacol.* **11**, 505 (1962).
4. B. L. Mirkin, N. J. Giarman and D. X. Freedman, *Biochem. Pharmacol.* **12**, 214 (1963).
5. L. G. Whitby, J. Axelrod and H. Weil-Malherbe, *J. Pharmacol. exp. Ther.* **132**, 193 (1961).
6. I. J. Kopin, J. Axelrod and E. K. Gordon, *J. biol. Chem.* **236**, 2109 (1961).
7. A. H. Anton and D. F. Sayre, *J. Pharmacol. exp. Ther.* **138**, 360 (1962).
8. T. L. Chrusciel, *Adrenergic Mechanisms*, J. R. Vane, G. E. W. Wolstenholme and M. O'Connor, Eds., p. 539. Churchill, London (1960).