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# UPTAKE OF NOREPINEPHRINE BY ISOLATED PINEAL BODIES

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**Abstract**—Isolated pineal bodies of the cat, incubated in a Krebs-Ringer bicarbonate medium containing 5 mμg/ml of DL-norepinephrine-7$^3$H, took up radioactivity to a concentration approximately 25 times that in the medium. The corresponding concentration ratio in pineal slices of calf was about 4. The uptake was inhibited by reserpine and ouabain and apparently required a carrier mechanism. Of the total isotope in the slices of calf pineal, about 30 per cent represents unchanged norepinephrine and the remainder acidic products of oxidation deamination by monoamine oxidase.

BECAUSE of the blood-brain barrier, certain drugs including sulfaguanidine and N-acetyl-4-aminoantipyrine,[1, 2] as well as a number of inorganic ions,[3–5] pass extremely slowly from the general circulation into the brain. There are certain special areas in the central nervous system, however, that appear to lie outside the blood-brain barrier. These regions include the pineal body, the area postrema, the pituitary, the intercolumnar tubercle, and the supraoptic crest.[6] Although circulating catecholamines penetrate most parts of the brain with difficulty,[7] the first four of these special regions recently have been shown to take up isotopically labeled epinephrine from plasma.[8–10] The mechanism by which catecholamines concentrate in these tissues must be more complicated than a simple equilibration of the intra- and extracellular amine achieved by diffusion through the cell membrane, since considerably higher levels of epinephrine are found in the pituitary than in the plasma during its infusion.[8–10] Preliminary experiments with the pineal body and the area postrema[9] have indicated that these organs also concentrate epinephrine. Both of these tissues have been reported to contain relatively high concentrations of catecholamines,[11, 12] and it seemed possible that some mechanism for the inward transport of amines might be a factor in maintaining these levels.

Although the blood-brain barrier makes it impracticable to demonstrate uptake of intravenously injected catecholamines, isolated brain slices incubated with catecholamines *in vitro* will concentrate catecholamines.[13] Uptake is inhibited by reserpine, chlorpromazine, and a number of other drugs.[14, 15] Since the concentrating mechanism becomes saturated at levels not greatly in excess of those at which the catecholamines

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act pharmacologically,[16] the inhibitory effect of reserpine is more easily demonstrated at concentrations of catecholamine below about 100 mμg/ml.

The present investigation of the uptake of tritiated norepinephrine (NE) by the isolated pineal bodies of cats and calves shows that these structures have the ability to concentrate NE.

## EXPERIMENTAL

DL-NE-7-$^3$H bitartrate, specific activity 5 mc/mg, was obtained from the New England Nuclear Corporation. The amine was stored as a stock solution in 0·01 N HCl containing a trace of bisulfite. Immediately before use it was diluted in Krebs-Ringer bicarbonate buffer containing glucose, fumarate, pyruvate, and glutamate as energy sources.[17] Pineal bodies, dissected from cats that had been killed by cervical dislocation, were carefully freed of adhering tissue and bisected by a single cut with a razor blade. Each gland was incubated separately in 2 ml of the Krebs-Ringer buffer for 60 min at 37 °C in an atmosphere of $O_2$ and $CO_2$ (95:5). At the end of the incubation period the tissue was washed as rapidly as possible in two changes of ice-cold 0·9% NaCl, blotted twice on filter paper and weighed on an analytical balance. The pineal bodies ranged in weight from 1·6 to 3·0 mg, the average value from 11 cats being 1·8 mg per gland. Each gland was homogenized in 5 ml of methanol in a Potter homogenizer. After centrifugation of the homogenate, 4 ml of the clear supernatant solution was added to 10 ml of a toluene solution containing 0·4 per cent of 2,5-diphenyloxazole (PPO) and 0·01 per cent of *β-bis*(2-(5-phenyloxazolyl)) benzene (POPOP) for counting in a liquid scintillation spectrometer. Calf pineals were removed immediately after the death of the animal and transported in ice to the laboratory. Approximately 1 to 1·5 hr elapsed between removal of the tissue and the incubations. The tissues were cut longitudinally into slices weighing about 50 mg, and each slice was incubated in 2 ml of medium as noted above.

For determination of the radioactivity in the medium, aliquots of 0·1 ml were added to 5 ml of methanol, and 4 ml of the methanolic solution was added to 10 ml of the phosphor solution. Under the conditions of these experiments, background was approximately 100 counts per min; 0·1 ml of a medium containing 5 mμg of NE-7-$^3$H per ml gave approximately 400 cpm.

The radioactive components in tissue that had been incubated with DL-NE-7-$^3$H were identified by chromatography in the presence of carrier quantities of known catecholamines. For this purpose, pooled calf pineal slices amounting to approximately 1 g of tissue were homogenized in 13 ml of 0·4 M percholoric acid at 0°. A mixture containing approximately 1 mg each of NE, epinephrine, and normetanephrine, and 0·5 mg each of 3,4-dihydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid was added to the homogenate. After centrifugation to remove the precipitated protein, the cold supernatant solution was titrated to about pH 4·5 (bromphenol blue indicator) by addition of chilled 0·5 M $K_2CO_3$. The precipitated potassium perchlorate was removed by centrifugation, and the clear supernatant solution was lyopholized. The residue was taken up in 1·5 ml of the chromatographic solvent and placed on a column containing 12 g of powdered Whatman no. 1 filter paper through which the same solvent had been allowed to pass for several hr. Two-ml fractions were collected, and the positions of the carrier amines were determined by measuring the optical densities at 280 mμ. An aliquot of each eluate was evaporated to dryness under a stream of air,

taken into 0·5 ml of methanol, and added to 10 ml of the toluene solution of PPO and POPOP for counting.

The chromatographic solvent was the upper layer which separated when 5 volumes of *n*-butanol, 1 volume of 85% formic acid and 4 volumes of water were mixed. For best results it was found desirable to let the solvent mixture stand for several days before use.

The absorbent for these columns was prepared by homogenizing sheets of Whatman no. 1 filter paper in deionized water with a Waring Blendor. The powder was washed with hot HCl, water, butanol, acetone, and water according to the procedure of Dengler.[18]

## RESULTS

The pineal bodies from 11 cats were incubated for 60 min in a medium containing 5 mμg/ml of DL-NE-7-$^3$H, and the level of radioactivity in the tissue was measured. Under the conditions of these experiments the observed counts per pineal body ranged from approximately 40 to 480 cpm over a background of approximately 100 cpm. Each sample was counted until a total of 2000 cpm had accumulated. The results

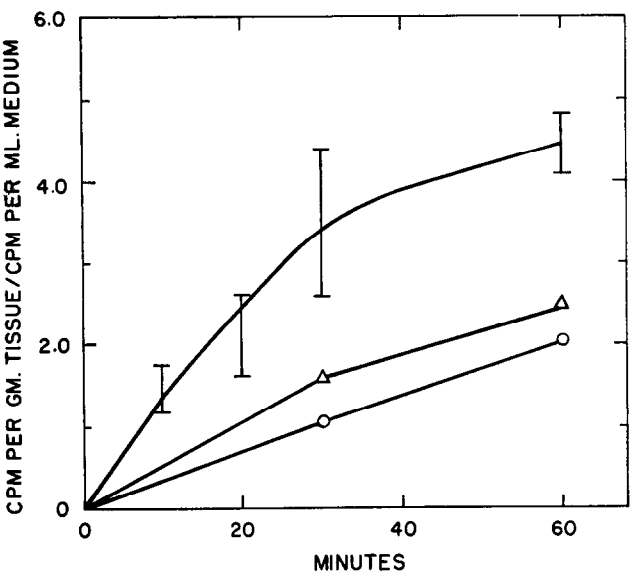


FIG. 1. Ratio of concentration of radioactivity in slices of calf pineal to concentration in Krebs-Ringer bicarbonate medium containing 5 mμg DL-NE-7-$^3$H per ml. Vertical bars represent standard deviation from mean value for 6 slices; △ = average of 4 slices incubated in presence of $10^{-5}$ M ouabain; O = average of 4 slices incubated in presence of $10^{-6}$ M reserpine.

were expressed as a ratio, R, of concentration of the isotope in tissue to that in the medium. Ratios ranging from 5·0 to 59·8 were observed. The mean value of all determinations of R was 25·8 ± a standard deviation of 16·1. These ratios were considerably greater than the value of approximately 4 which is commonly observed when slices of cerebral cortex or heart are incubated under comparable conditions.[13]

Of three pineals from cats that had received 3 mg of reserpine per kg intraperitoneally 24 hr before removal of the organ, one showed no measurable uptake of NE, and the other gave ratios of 4·1 and 4·7, indicating that uptake of catecholamine by the pineal body is inhibited by this drug.

Since the low weight of the cat pineal body (about 2 mg) made it difficult to obtain adequate amounts of tissue, further investigation of the uptake of NE was carried out in the slices of calf pineal. When these slices were incubated in the presence of 5 mμg of NE-7-$^3$H under the usual conditions, the concentration of radioactive isotope in the tissues rose to several times that in the medium (Fig. 1). The uptake of labeled NE was markedly inhibited by the presence of either $10^{-6}$ M reserpine of $10^{-5}$ M ouabain in the medium.

Slices of calf pineal did not concentrate NE as effectively as did the cat gland. This may have been due to the unavoidable delay in transporting the pineal slices to the laboratory.

TABLE 1. EFFECT OF CONCENTRATION OF DL-NE-7-$^3$H ON UPTAKE IN SLICES OF CALF PINEAL*

| NE in medium | | NE in slices at 20 min | | Ratio |
|---|---|---|---|---|
| (mμg/ml) | (cpm/μl) | (mμg/g) | (cpm/μg) | |
| 5 | 3·7 | 17·8 | 13·3 | 3·6 |
| 25 | 18·5 | 109 | 80·5 | 4·35 |
| 100 | 74·0 | 340 | 251 | 3·4 |
| 200 | 148·0 | 360 | 266 | 1·8 |

* Slices weighed from 20 to 50 mg. Each value represents the average from 4 incubations.

Table 1 illustrates the effect of changing concentrations of NE in the medium on uptake of radioactivity by pineal slices that were incubated for 20 min.

In all of the experiments thus far described, the results have been expressed as radioactivity in the slices without regard to what the chemical identity of the labeled molecules might be. To determine how much of the radioactivity taken into pineal slices remained as unchanged NE, a group of pooled slices weighing a total of 0·64 g was incubated for 60 min in a medium containing 10 mμg/ml of labeled NE. At the end of that time the slices were washed rapidly in cold 0·9% NaCl and homogenized in 13 ml of cold 0·4 N perchloric acid. The perchloric acid extract was prepared for chromatography on powdered filter paper as described earlier. Seventy-nine per cent of the radioactivity emerging from the column could be accounted for as NE, and most of the remaining 21 per cent appeared in the fraction containing the acidic products resulting from the action of monoamine oxidase (Fig. 2). Since the fraction of ultraviolet-absorbing substances emerging in tubes 6 to 12 is distorted by the presence of bromphenol blue, the radioactivity appearing in this area can not be definitely identified as catechol acids. The small radioactive fraction occurring approximately in tubes 42 to 48 is unknown. The specific activities (expressed as cpm per ml/optical density at 280 mμ) of the material in tubes 33 through 37 were 1830, 2090, 2030, 1960,

and 2090.The similarity of these figures suggests that the material in these tubes was identical with the carrier NE.

## DISCUSSION

It has been reported that reserpine prevents the active transport of 5-hydroxytryptamine (5HT) and probably of catecholamines into platelets.[19] It also inhibits uptake of NE in isolated slices from several parts of brain.[13, 16] Inhibition of NE uptake *in vivo* has also been reported.[20]

Data from the experiments *in vitro* quoted above indicate that there are two components to the flux of isotopic NE into tissue slices. One of these appears to be simple diffusion. The second component, which is responsible for the ability of these tissues to concentrate the amine, has a number of properties of active transport. These include the ability to transport the amine against a concentration gradient, dependence on a supply of metabolic energy, and responses to changing temperature and NE concentration that are characteristic of enzymatic reactions. It is this second component that is inhibited by reserpine.[16]

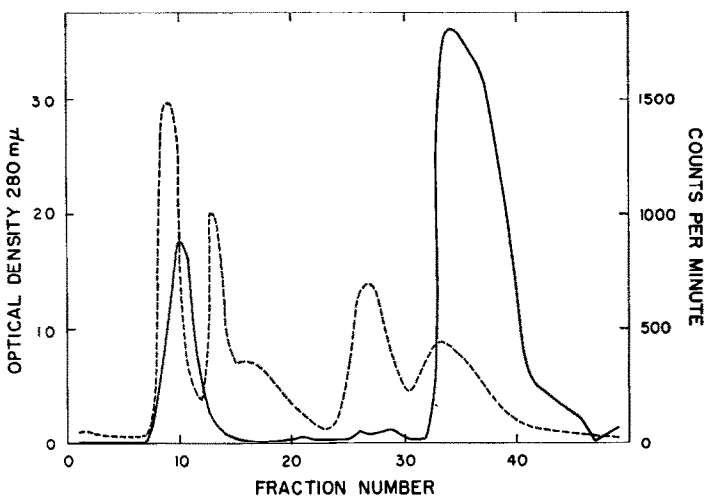


FIG. 2. Chromatography on powdered filter paper of the radioactive substances in slices of calf pineal incubated 60 min with 10 mμg/ml of DL-NE-7-$^3$H. ———— = total radioactivity; — — — — = optical density at 280 mμ of carrier substances added to the homogenate. The peaks represent in order from left to right: (1) a mixture of 3-methoxy-4-hydroxymandelic acid, 3,4-dihydroxymandelic acid, and traces of bromphenol blue used in titration of the $HClO_4$; (2) normetanephrine; (3) epinephrine; and (4) norepinephrine.

Ouabain appears to inhibit the active transport of a number of substrates. It blocks the active uptake of cations in several tissues,[21–23] of iodide in thyroid,[24] and of 5HT in platelets.[25]

The inhibitory action of ouabain and reserpine on the uptake of NE by pineal slices suggested that the amine might enter by active transport. On the other hand it is

equally possible that the isotopic NE could simply exchange with a pool of NE that is adsorbed on some intracellular structure.

Ideally this question could be resolved by observing the effect of varying concentrations of extracellular NE on the initial rate of uptake. In experiments carried out with isotopes, the initial rate of entry of radioactivity is a measure of the unidirectional inward flux. If this flux were simply passive diffusion through a porous membrane, the initial rate of uptake should be a linear function of the concentration of isotopic amine in the medium. If, on the other hand, association of the NE with a carrier molecule is a prerequisite for passage through the membrane, the rate of entry of the isotope will be proportional to the concentration of carrier-NE complex in the membrane. In the latter instance, this rate may be expected to increase with increasing amine concentrations up to the point at which the carrier becomes saturated with NE. The relationship between the rate of entry and amine concentration will thus resemble the well known Michaelis-Menten expression for the effect of substrate concentration on the rate of an enzymatic reaction.

These considerations have been discussed at length in a recent review on membrane carriers by Wilbrandt and Rosenberg[26] and in a paper by Hughes and Brodie.[19]

Since the uptake of isotope in pineal slices is approximately a linear function of time for the first 20 min of incubation (Fig. 1), the amount of isotope incorporated into the tissue at this time was taken as a measure of the initial rate of uptake in the experiments reported in Table 1. Precise measurements of uptake are difficult, since there is always an uncertainty of approximately $\pm 15$ per cent in the values for the concentration of radioactivity in tissues. The data of Table 1, therefore, represent somewhat imprecisely the effect of concentration on the rate of uptake.

Nevertheless it is clear from this table that the rate of uptake of NE in these tissues is not a linear function of concentration. Were this so, the ratio, R, of the concentration of radioactivity in the tissue to the concentration in the medium should remain constant. The fact that this ratio is not constant but diminishes with increasing NE concentration can be explained by assuming that the flux of NE into the pineal, like that into slices from brain proper, has two components. That part of the flux involving interaction with a carrier would reach a maximal rate once the carrier had been saturated. The data of Table 1 suggest that saturation must occur somewhere in the vicinity of 50 to 100 m$\mu$g/ml. Above this concentration, the amount of NE entering the tissue in a given time by this mechanism could not further increase. The influx by passive diffusion, however, would not be subject to this limitation and would increase indefinitely with increasing concentration. At very high concentrations the fixed amount entering in 20 min by the carrier mechanism would become an ever smaller part of the total uptake, and the ratio, R, would diminish.

It appears from the results reported here that circulating catecholamines may enter the pineal body not only because this structure lies outside the blood-brain barrier, but also because it has an active mechanism for taking up amine. Whether this uptake occurs by active transport is not yet certain, but it is clear that the amine must enter by way of a carrier mechanism and that the uptake is markedly inhibited by drugs that are known to inhibit active transport. In these respects the isolated pineal body resembles slices of brain and of sympathetically innervated tissue. Like these other

tissues, the pineal is known to maintain stores of catecholamine,[11] and it is possible that the uptake mechanism plays a role in maintaining these stores.

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