Biochemical Pharmacology, 1964, Vol. 13, pp. 1–12. Pergamon Press Ltd., Printed in Great Britain.

THE RETENTION OF EXOGENOUS NOREPINEPHRINE BY RABBIT TISSUES*

C. N. GILLIS

Laboratory of Psychopharmacology, Departments of Pharmacology and Psychiatry, Yale University School of Medicine, New Haven, Conn., U.S.A.

(*Received* 5 *July* 1963; *accepted* 13 *August* 1963)

Abstract—The retention of ^{3}H-norepinephrine by rabbit heart and by spleen was linearly related to the dose administered, over the range 0·75 to 10·0 μg per kg. The percentage distribution of the labeled amine among the nuclear, mitochondrial, and microsomal fractions of sucrose homogenates of heart, prepared by differential centrifugation, remained constant, regardless of the dose of amine administered. In the microsomal fraction, which contained a maximum of 6 per cent of the amine in the total homogenate, the concentration of ^{3}H-norepinephrine, in terms of protein (i.e. the relative specific activity), was up to fourteen times that found in the total homogenate. The ability of microsomal elements to concentrate exogenous amine reached a saturation point when approximately 3·0 μg per kg of ^{3}H-norepinephrine was administered; the simultaneous administration of nonradioactive and tritium-labeled norepinephrine greatly depressed the relative specific activity of the amine in this fraction. Microsomal fractions, prepared by centrifugation of heart homogenates over a discrete sucrose density gradient, retained up to 28 per cent of the total ^{3}H-norepinephrine in the homogenates; the relative specific activity of the amine in this fraction, however, was always lower than that of the corresponding fraction isolated by differential centrifugation. It is suggested that concentration of exogenous norepinephrine by microsomal elements can account for almost all of the labeled amine retained by heart. Further, it appears that the technique of differential centrifugation yields a sample of these elements that is less contaminated by other structures with the same sedimentation characteristics (but which do not contribute to the retention of exogenous norepinephrine) than are those obtained by sucrose density gradient centrifugation. If microsomal structures are responsible for the retention of ^{3}H-norepinephrine, and this property is saturated at 3·0 μg/kg, then the linear relationship between dose administered and *total* heart retention may be explained by postulating the existence of discrete locations of these structures, which vary in the ease with which exogenous amine can reach them.

THE ABILITY of sympathetically innervated organs to retain systemically administered norepinephrine (NE) is well documented.[1–4] In these studies the administration of NE, in doses much larger than those causing maximal cardiovascular effects (usually in the mg/kg range), resulted in a net accumulation of the amine by the organs under observation. The extent of such uptake when the blood levels of amine are much lower, however, is not clear. After the administration to cats of intravenous infusions of NE, which were considered sufficient in magnitude to produce blood levels similar

* This work was supported by Grant H-7249 from the United States Public Health Service and was presented in part at the April 1963 meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J.

to those achieved by maximal suprarenal stimulation, von Euler[5] could detect no significant elevation of the catecholamine contents of heart, spleen, liver, or kidney. A successful demonstration of uptake under these experimental conditions, however, may have required a greater sensitivity in the analytical methods used for the determination of NE than was available; in addition, when small doses of amine are given, there may be relatively little actual uptake (i.e. increased total organ content), but significant exchange with endogenous amine might occur that would not be reflected by an increased amount of NE in the organs. The availability of tritiated norepinephrine (^{3}H-NE) of high specific activity allowed Whitby *et al.*[6] to demonstrate that the heart and spleen of cats retained relatively large amounts of the labeled amine at a time when its concentration in the plasma was low. These authors used 25 μg of ^{3}H-NE base/kg body weight. Such a dose would be expected to produce a blood level of amine, at least initially, that would be considered much higher than normal. The present investigation was therefore carried out to determine the degree of retention of NE after the administration of a range of low doses of the amine. The demonstration of significant retention of amine by tissues, when the blood levels of amine were not well above the physiological maximum, is believed to be important in considering the quantitative contribution of such retention to the endogenous NE within the tissues.

Cardiac tissue shares with brain,[7] adrenal medulla,[8, 9] and splenic nerve[10] the localization of its catecholamine largely within subcellular organelles.[11, 12] Potter and Axelrod[13] have reported that 30 min after the intravenous administration of ^{3}H-NE to animals the isotopic compound, as well as endogenous NE, is located almost exclusively within the microsomal fraction of rat heart homogenates. A second purpose of this work was to determine whether the subcellular distribution of ^{3}H-NE in the rabbit heart was measurably influenced by varying the amount of amine administered *in vivo*.

METHODS

Albino rabbits of either sex received an intravenous injection of 10 to 15 mg pentobarbital/kg body weight; this was followed by the appropriate dose of ^{3}H-NE.* Thirty minutes later the heart and spleen were rapidly removed, washed in several changes of chilled, 0·9% solution of sodium chloride, blotted, and weighed. Subsequent homogenization was carried out with either 0·29 M sucrose or 0·4 M perchloric acid, as described below. All doses of ^{3}H-NE mentioned in this paper refer to the amount of ^{3}H-NE base administered per kilogram body weight.

Subcellular fractionation of heart homogenates. Preliminary homogenization of the heart was carried out in 6 volumes of chilled, 0·29 M sucrose, in a Virtis '45' homogenizer for 30 sec. The resulting suspension was homogenized further for 1 min by means of a glass homogenizing tube with a ground-glass pestle. This two-stage homogenization could be completed in 3–4 min and was used in preference to tissue disruption carried out entirely in a ground-glass homogenizer, since the latter procedure often took as long as 10 min for adequate tissue breakdown. A 2-ml aliquot of the

* Norepinephrine-7-^{3}H(*d,l*), as the hydrochloride, was supplied by the New England Nuclear Corp. The purity of the sample used was confirmed by paper chromatography, as described by Whitby *et al.*[6]

homogenate was removed and extracted with perchloric acid, as described below. The remainder of the homogenate was subfractionated by means of a differential centrifugation technique, the details of which are given in Table 1. In one series of experiments, subcellular fractionation was achieved by centrifugation of the initial homogenate over a discrete sucrose gradient consisting of upper and lower layers,

TABLE 1. DIFFERENTIAL CENTRIFUGATION TECHNIQUE USED FOR THE SUBCELLULAR FRACTIONATION OF HEART HOMOGENATES

Fraction	Conditions of sedimentation
Nuclear*	900 *g* (15 min)
Mitochondrial	21,000 *g* (15 min)
Microsomal	105,000 *g* (30 min)
(Supernatant)†	

* The nuclear fraction, after initial sedimentation at 900 *g*, was dispersed in 5 ml of 0·29 M sucrose and recentrifuged for an additional period of 15 min under the same conditions. The cloudy supernatant portions from both centrifugations were combined for subsequent treatment.

† The supernatant fraction was the clear fluid remaining above the microsomal pellet.

each of 10 ml, of 0·8 M and 1·2 M sucrose respectively. A 10-ml aliquot of the initial homogenate was carefully layered over this gradient and centrifuged at 100,000 *g* for 1 hr. The appearance of the density gradient, before and after centrifugation, is shown

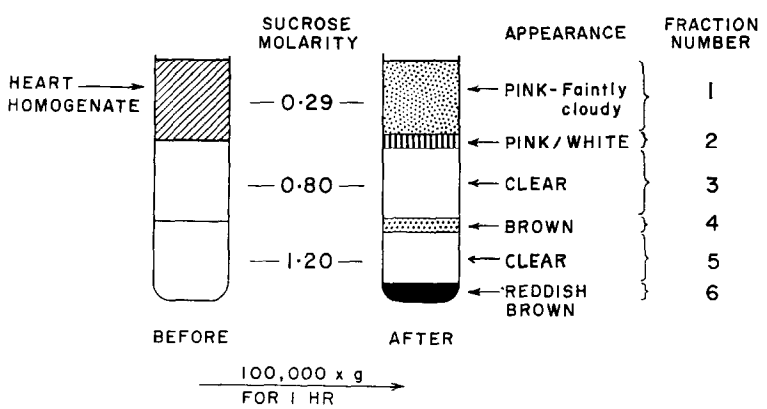

FIG. 1. The appearance of sucrose density gradient, before and after centrifugation of heart homogenates.

in Fig. 1. Immediately after ultracentrifugation, the fractions were separated by means of a Spinco tube cutter, and the amount of ^{3}H-NE in each fraction was determined as described below. In this series of experiments the remainder of the initial homogenate was subfractionated by the differential centrifugation technique given in Table 1.

Determination of ^{3}H-NE *in subcellular fractions of heart homogenates.* 4 ml of perchloric acid (0·4 M) was added to each subfraction of the homogenate, whether prepared by differential or by density gradient centrifugation. One-tenth volume of perchloric acid (4·0 M) was added to supernatant fractions. After separation of the resulting 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 of perchloric acid (0·4 M). After a second centrifugation the supernatant fluid was combined with that from the first. After the addition of 0·1 volume of 1% (w/v) sodium EDTA, the perchloric acid extracts of all fractions were adjusted to pH 8·4 with solutions of potassium carbonate (initially with 5·0 N and subsequently with 0·5 N). The precipitate that formed was allowed to settle and the clear supernatant fluid poured over 1 g of aluminium oxide* 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 separated 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 alumina 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 fluid removed by aspiration. The three portions of hydrochloric acid were combined and the tritium of a 0·1-ml aliquot was determined in a liquid scintillation counter, with 10 ml of a dioxane-phosphor.[15] Corrections for quenching (approximately 15 per cent) were made by adding internal standards of tritiated water to each sample. Recovery of ^{3}H-NE, added to each fraction before extraction with perchloric acid, averaged 70 per cent (range 65–82 per cent) in eight separate determinations. No corrections for incomplete recovery have been made in the results presented.

Determination of total ^{3}H-NE *in heart and spleen.* Both heart and spleen were homogenized for 1 min in 20 ml perchloric acid (0·4 M), in a Virtis model '45' homogenizer. The tissue homogenates were centrifuged at 30,000 *g* for 10 min, after which the supernatant was retained and the precipitate re-extracted with an additional 4 ml of perchloric acid (0·4 M). The supernatant fraction from a second centrifugation (30,000 *g* for 10 min) was combined with that from the first and the ^{3}H-NE of the extract estimated as described above for subcellular fractions.

Determination of protein. The protein of subcellular fractions, after perchloric acid extraction, was measured by a biuret technique using serum albumin as a standard.

Electron microscopy†. Microsomal fractions of heart, isolated by differential centrifugation, were fixed in buffered osmium tetroxide,[16] embedded in Epon and sectioned on an ultramicrotome (LKB) at 300–500 Å thickness. The microsomal fraction isolated on a sucrose density gradient was similarly treated after being firmly packed by dispersion in 0·29 M sucrose, followed by centrifugation at 105,000 *g* for 30 min. Electron micrographs were examined in an RCA (EMU-3F) electron microscope.

Where the significance of data was evaluated statistically, student's 't' test was the method used.

* Woelm alumina, neutral grade was employed. Before use the alumina was acid washed and dried in a manner similar to that recently described by Anton and Sayre.[14]

† I am indebted to Dr. J. G. Wood of the Department of Anatomy, Yale University School of Medicine, for preparing and examining the electron micrographs.

RESULTS

Retention of ^{3}H-NE *by heart and spleen*

The retention of ^{3}H-NE by total rabbit heart and spleen, 30 min after the intravenous administration of 0·75 to 10 μg of the amine, is illustrated in Fig. 2. It can be seen that, within the experimental error, the retention of labeled amine was linearly related to the dose administered. Fig. 2 also demonstrates that the heart retains more ^{3}H-NE than does spleen.

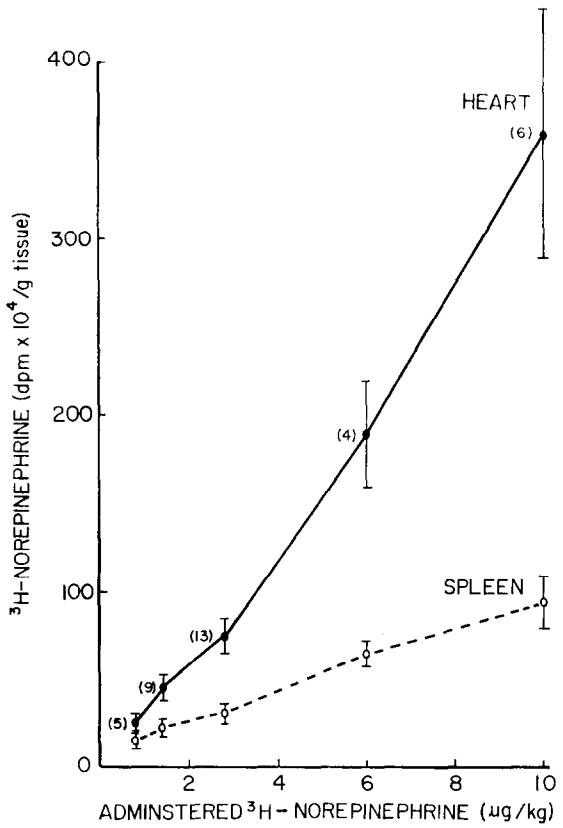

FIG. 2. The retention of ^{3}H-norepinephrine by heart and spleen, 30 min after the intravenous administration of different doses of the amine. The figures in parentheses refer to the number of animals used for the determination of amine retention in both tissues at each dose level used. In this and in all subsequent figures the vertical lines describe the standard error of the mean values.

Concentration of ^{3}H-NE *in subcellular fractions of heart*

The results of experiments in which subfractionation of heart homogenates was carried out 30 min after the intravenous administration of 0·75 to 10 μg of ^{3}H-NE are presented in Fig. 3. The labeled amine in each fraction was expressed in terms of protein (referred to subsequently as the relative specific activity of ^{3}H-NE). Fig. 3 demonstrates that the relative specific activity of the ^{3}H-NE in the microsomal fraction was from three to ten times that of the total homogenate; in one experiment a 14-fold concentration was evident. The relative specific activity of ^{3}H-NE in the nuclear and

mitochondrial fractions and in the total homogenates was not significantly different at any of the dosages used. Over the dose range 0·75 to 2·8 μg of ^{3}H-NE, the relative specific activity of the amine in the microsomal fraction increased linearly; with 6·0 μg the relationship departed from linearity and, in fact, the relative specific activity values after either 6 μg or 10 μg of the amine did not differ significantly. Since the

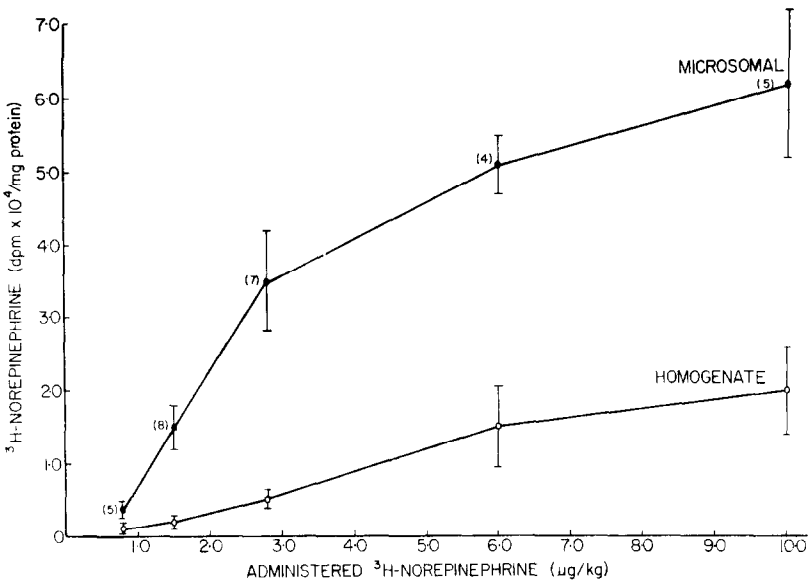

FIG. 3. The concentration of ^{3}H-norepinephrine (in terms of protein) in the microsomal fraction and in the total homogenate of heart. The values for amine retained by the nuclear and mitochondrial fractions have been omitted for the sake of clarity; however, they were almost identical with those illustrated for the total homogenate. Figures within parentheses refer to the number of animals receiving each dose of ^{3}H-norepinephrine.

ability of microsomal elements to concentrate ^{3}H-NE therefore appears to be limited and to have reached a saturation point after the administration of approximately 3 μg of the amine, it was expected that the simultaneous administration of unlabeled NE and ^{3}H-NE should decrease the relative specific activity of the tritiated amine in this fraction. Accordingly three experiments were carried out in which doses of 20 μg of nonradioactive NE and 2·8 μg of ^{3}H-NE/kg were mixed in the same syringe and administered to the animals simultaneously. In these experiments the values for the relative specific activity of ^{3}H-NE in the microsomal fraction were 0·56, 1·19 and 1·04 (disintegrations per min $\times$ 10^4/mg protein). In the seven control experiments (i.e. those in which only 2·8 μg ^{3}H-NE/kg was given), the corresponding mean value was 3·52 $\pm$ 0·71 (standard error of the mean).

Four experiments were carried out in which the microsomal pellet was washed by gentle rehomogenization in 0·29 M sucrose and again sedimented by centrifugation at 105,000 *g* for 30 min. These experiments demonstrated that an average of 66·5 per cent (52 per cent to 77 per cent) of the labeled amine was retained after such treatment.

The subcellular localization of ^{3}H-NE *in heart*

It can be seen (Fig. 4) that over 80 per cent of the amine in the homogenate was distributed approximately equally between the nuclear and the supernatant fractions. The proportions of ^{3}H-NE in the nuclear, mitochondrial, and supernatant fractions after 0·75 μg of the amine were not significantly different from those in the same fractions after any of the doses used; however, the percentage of the amine found in the microsomal fraction after 2·8 μg was significantly greater ($P < 0{\cdot}02$) than after the other doses used.

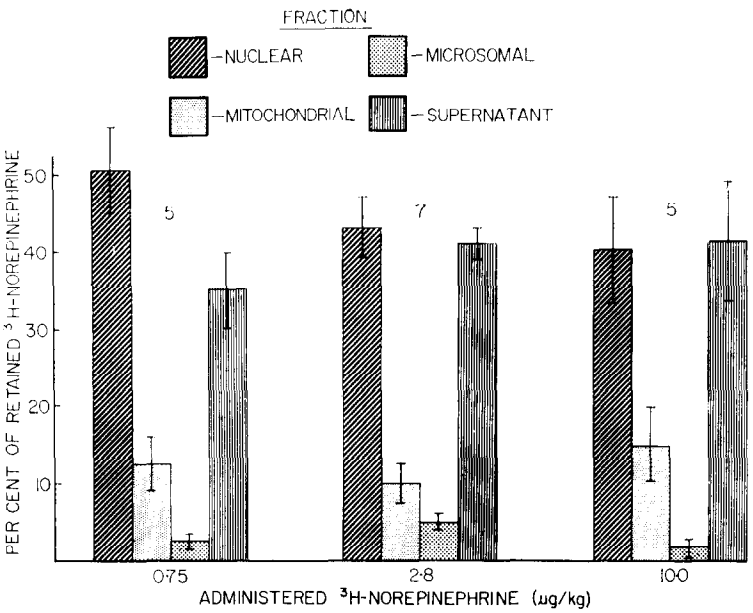

FIG. 4. Histogram indicating the percentage distribution of ^{3}H-norepinephrine among the subcellular fractions of heart after the administration of 0·75 μg, 2·8 μg, and 6·0 μg, respectively, of the amine per kg. The percentage distribution in all but the microsomal fraction, after 1·5 μg or 6·0 μg of ^{3}H-norepinephrine per kg was not significantly different from those shown in this figure. The figure above each set of bars indicates the number of animals used at each dose level.

To determine whether the relatively large amount (40 per cent, Fig. 4) of amine found in the nuclear fraction could be ccounted for by microsomal elements which had not been liberated by homogenization, the following experiments were carried out. After its initial preparation by centrifugation at 900 *g*, the nuclear fraction was rehomogenized and layered over 20 ml of 0·8 M sucrose. The appearance of the gradient, after centrifugation at 100,000 *g* for 1 hr, is illustrated in Fig. 5. The ^{3}H-NE of each fraction was determined as described earlier (see Methods); the results of these experiments are given in Table 2. While it is clear (Table 2) that a considerable portion (25–63 per cent) of the amine in the nuclear fraction was retained despite the rehomogenization of this fraction and its passage through hypertonic sucrose, a variable amount (12–33 per cent) was liberated by such treatment (fraction B, Table 2).

The small (1·8–6·1 per cent) proportion of ^{3}H-NE found in the microsomal fraction was surprising, in view of the report[13] describing the localization of the ^{3}H-NE

almost exclusively in the microsomal fraction of rat heart. In the work of Potter and Axelrod,[13] subfractionation was achieved by centrifugation of heart homogenates over a continuous sucrose density gradient. Even allowing for the different techniques and species used in the present work and that of Potter and Axelrod, the very large quantitative discrepancy merited further investigation. Accordingly, experiments were

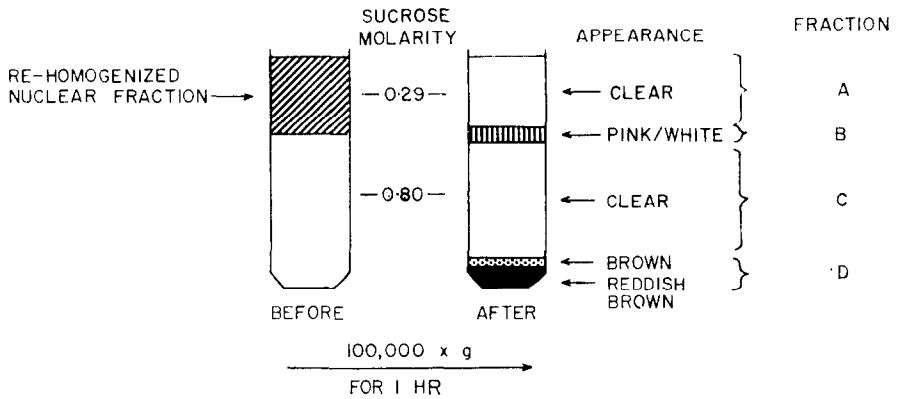

FIG. 5. Appearance of tube before and after centrifugation of rehomogenized nuclear fraction of heart.

performed in which the technique of discrete sucrose density gradient centrifugation (as described in Methods) was used to prepare subcellular fractions. The discontinuous sucrose gradient was used deliberately in order to achieve a more complete separatation of the various layers of subcellular elements than is possible with a continuous sucrose

TABLE 2. THE DISTRIBUTION OF ^{3}H-NOREPINEPHRINE AFTER REHOMOGENIZATION OF NUCLEAR FRACTIONS OF HEART HOMOGENATES

After rehomogenization, the nuclear fraction was centrifuged over 0·8 M sucrose.

^{3}H-NE, μg/kg	Fraction*			
	A	B	C	D
1·5	20·9	14·2	13·6	51·3
1·5	36·2	32·5	6·2	25·1
6·0	15·5	12·2	9·1	63·3
6·0	18·0	23·8	15·6	42·6

* Per cent of ^{3}H-norepinephrine in the original nuclear fraction; see Fig. 5.

gradient. The percentage of the ^{3}H-NE of the total homogenate that was found in the microsomal fraction (i.e., that sedimenting at the junction between 0·29 M and 0·8 M sucrose) and its relative specific activity, as well as the data for the corresponding fraction* obtained by differential centrifugation, are given in Table 3. The percentage

* Fractions obtained by differential and density gradient centrifugation techniques are described as 'corresponding' only in the very limited sense of indicating that they contained the same general category of subcellular particle (see Fig. 6).

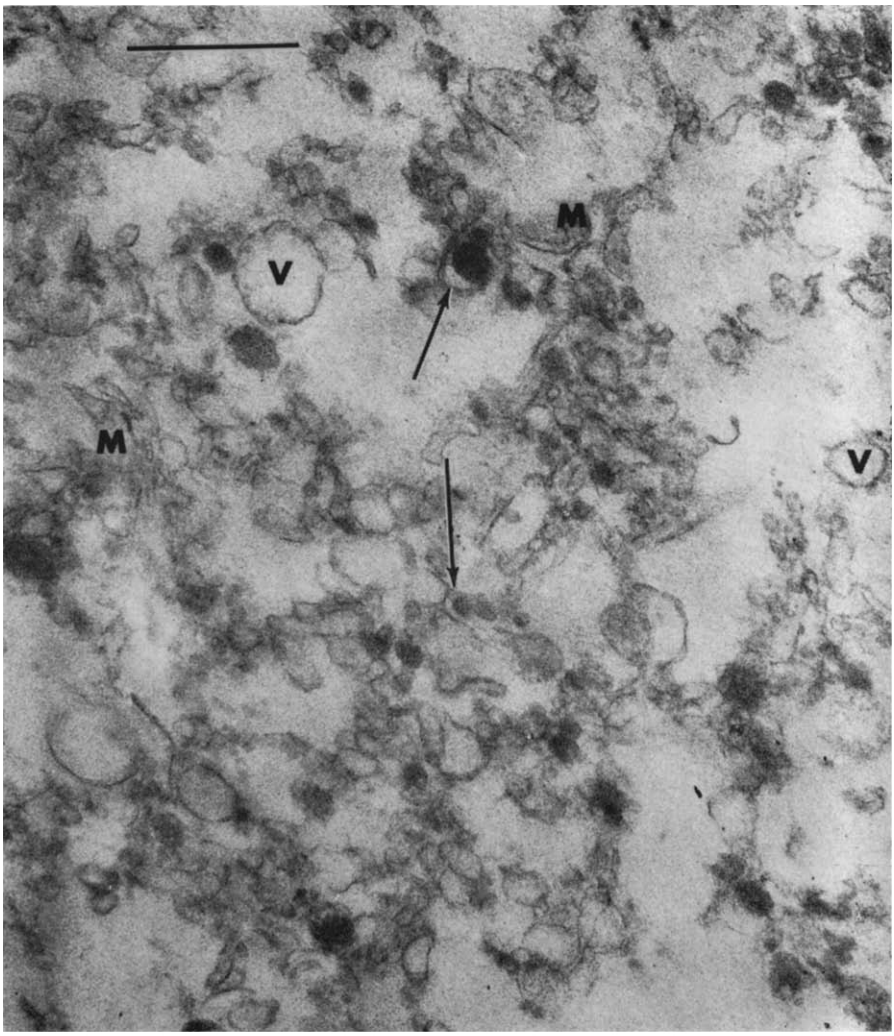

FIG. 6. Electron micrograph of the microsomal fraction of heart, prepared by the differential centrifugation technique described in Table 1. The bar in the upper left corner represents 0·5 μ. Sarcoplasmic reticular vesicles (V) and membranes (M) are visible, together with densely stained structures, some of which appear to be surrounded by a membrane (arrow).

facing page 8

of ^{3}H-NE found in the microsomal fraction obtained by density gradient centrifugation was consistently higher than that found in the microsomal fraction isolated by differential centrifugation. However, the relative specific activity of the labeled amine in the corresponding fraction, prepared by differential centrifugation, was higher than that obtained by the density gradient procedure (Table 3). This difference was particularly marked when 1·5 μg of ^{3}H-NE was used, the relative specific activity of the amine in the differential and density gradient microsomal fractions differing in this case by a factor of approximately 7. Comparison of the relative specific activity of ^{3}H-NE in the nuclear and mitochondrial fractions, prepared by both techniques from the same homogenate, revealed no consistent or statistically significant differences.

TABLE 3. PERCENTAGE OF TOTAL ^{3}H-NOREPINEPHRINE OF HEART HOMOGENATES RECOVERED IN MICROSOMAL FRACTIONS PREPARED BY DIFFERENTIAL AND BY DENSITY GRADIENT CENTRIFUGATION*

	Microsomal fraction			
	Prepared by differential centrifugation		Prepared by density gradient centrifugation	
^{3}H-NE, μg/kg	(dpm × 10^4/mg protein)	Total ^{3}H-NE, in homog. %	(dpm × 10^4/mg protein)	Total ^{3}H-NE, in homog. %
1·5	3·29	5·1	0·39	15·5
1·5	3·46	0·5	0·56	9·1
6·0	4·50	2·8	1·69	12·2
6·0	6·98	0·6	5·08	17·8
10·0	6·54	0·2	5·86	20·1
10·0	7·21	4·1	6·31	27·9

* Also listed are the corresponding values for the concentration of ^{3}H-NE/mg of protein in those fractions.

When a heart homogenate prepared from an untreated rabbit was mixed with the ^{3}H-NE and layered over an identical sucrose gradient and was then centrifuged at 100,000 *g* for 1 hr, over 85 per cent of the amine remained in the supernatant material; thus the possibility of nonspecific adsorption of tritiated amine by subcellular organelles can be discounted.

The microsomal fraction of heart, prepared by the density gradient centrifugation technique used in this study, would be expected to contain labeled amine contributed by the small amount of soluble cytoplasm that necessarily would be removed in the separation of the fraction with a Spinco tube cutter. It was important, therefore, to determine what portion of the ^{3}H-NE found in this fraction was truly particle bound. In two experiments the microsomal fraction, prepared by density gradient centrifugation, was isolated by means of the tube cutter and centrifuged at 105,000 *g* for 1 hr, after which the ^{3}H-NE of the supernatant fraction and the pellet was determined by perchloric acid extraction. It was found that 59 per cent and 71 per cent respectively, of the ^{3}H-NE was retained by the sedimented microsomal material and must therefore have been bound originally to a particulate element of the homogenate.

Electron microscopic examination of heart microsomal fractions

Figure 6 is an electron micrograph of a heart microsomal fraction, isolated by differential centrifugation. The electron micrographs of heart microsomal fractions

were similar whether the fractions were isolated by differential or by density gradient centrifugation.

Vesicles, sarcoplasmic reticular membranes, and densely stained structures can be seen and, in some cases, appear to be surrounded by a membrane.

DISCUSSION

The data presented in Fig. 2 indicate that both the heart and spleen are capable of retaining significant quantities of exogenous NE, even when the administered dose of amine is low. The decreased retention of amine by spleen over the entire dose range used reveals a species difference, since Whitby *et al.*[6] found that the amounts of ^{3}H-NE in cat heart and spleen were similar either 2 min or 2 hr after the intravenous administration of the amine. Significant retention of the amine by the heart and spleen of rabbit after the intravenous administration of 0·75 μg of ^{3}H-NE demonstrates that, regardless of the nature of the process responsible for such retention, it is able to function when the blood levels of NE are low. If the ^{3}H-NE in these tissues represents net accumulation, then it is clear that such a process could operate to remove very low levels of NE circulating in the blood.

Figure 3 shows that when doses above 2·8 μg of ^{3}H-NE were used, there was a definite trend toward saturation of the ability of the microsomal constituents to retain the exogenous NE; in fact, there were no significant differences among the relative specific activity figures for ^{3}H-NE in the microsomal fraction after the administration of 2·8 μg, 6 μg, or 10 μg, respectively, of the tritiated amine. The demonstration of the decreased retention of ^{3}H-NE , when both tritiated and nonradioactive NE were administered simultaneously, offers some support for the concept of a definite limit in the capacity of the microsomal elements to concentrate exogenous NE, Dengler *et al.*[17] have reported saturation of a concentrating mechanism for NE; this was demonstrated by the use of cat heart slices *in vitro*.

It must also be considered whether the saturation observed could be accounted for by some change in the cardiovascular response to the doses of amine administered, which appeared to produce such saturation. It is generally agreed[18] that NE causes an increase in coronary flow of the heart *in situ*. There is no evidence for a qualitative difference in the coronary arterial response to intravenous NE that is dependent upon the dose of amine administered. It must be concluded, therefore, that the change in the dose-concentration pattern of the microsomal fraction is the result of a cellular or subcellular phenomenon and was not caused by a qualitatively different hemodynamic change after the administration of amine doses larger than approximately 3 μg ^{3}H-NE/kg.

Part, if not all, of the amine in the nuclear fraction can be explained by microsomal elements which remained as contaminants within this fraction; the structure of cardiac muscle is compatible with such an explanation. This suggestion is supported by the fact that rehomogenization of the nuclear fraction apparently liberated subcellular elements, with the sedimentation characteristic of microsomal particles; these contained from 12 per cent to 33 per cent of the amine in the original nuclear fraction. Stronger evidence in support of this suggestion is provided by the demonstration that the subcellular distribution of amine did not change significantly, regardless of the dose of ^{3}H-NE administered (Fig. 4). If the ^{3}H-NE is associated with microsomal elements, then its percentage distribution would be a function only of the number

of such elements in each particulate fraction; this, in turn, would depend largely on the degree of homogenization. The variation in the subcellular distribution found, therefore, with different doses of ^{3}H-NE would reflect only the varying degree of tissue disruption achieved during the homogenization of each heart. On the other hand, if the ^{3}H-NE was retained selectively by subcellular elements *other* than microsomal particles, one would expect to see, after the administration of increasing doses of ^{3}H-NE, a change in the percentage of amine that would be largely restricted to the fraction containing these nonmicrosomal elements.

If it is accepted that almost all of the ^{3}H-NE is associated with the microsomal fraction, then it is necessary to explain the linear relationship between the dose of amine administered and retention by the whole heart, despite saturation of the capacity of such microsomal elements to bind the amine. This could be explained by assuming that there exist, within the heart, pools of subcellular particles containing structures that will ultimately sediment along with microsomes, and that differ in the ease with which ^{3}H-NE is able to reach them. With low doses of amine it is possible that a readily accessible pool of storage sites is utilized. Higher doses (perhaps above 3 μg/kg in the rabbit), by virtue of their producing considerably greater initial blood levels of ^{3}H-NE, may favor penetration of the amine to functionally similar, but less accessible, storage locations. The possibility of morphologically distinct locations of 'storage vesicles' for NE in the adrenergic nerves of the rat heart has recently been proposed.[19]

Density gradient centrifugation of heart homogenates gave a microsomal fraction containing, in this study, up to 28 per cent of the total ^{3}H-NE retained. Although the percentage of amine found in this fraction was consistently greater than in the microsomal fraction prepared by differential centrifugation, its relative specific activity was lower (Table 2). This was particularly evident when a dose of 1·5 μg ^{3}H-NE/kg was used. It is probable that subcellular elements unrelated to the retention of ^{3}H-NE would contaminate the microsomal fraction obtained by density gradient to a greater degree than in that resulting from differential centrifugation. Such contaminants would be expected to contribute to the protein but not to the values for ^{3}H-NE and, therefore, would lower the calculated relative specific activity of the amine. As the absolute amount of retained amine increased after the administration of either 6 μg or 10 μg of ^{3}H-NE, the relative specific activity of the amine in microsomal fractions from density gradient centrifugation would increase, since the protein determination would not be expected to show a concomitant increase. It follows from these considerations that the technique of differential centrifugation yields a less contaminated sample of the structures present within the microsomal fraction which, it is suggested, are responsible for concentrating exogenous NE.

In interpreting the results of this study it is essential to consider whether the labeled ^{3}H-NE in the heart and spleen represented true uptake, implying net accumulation, or was simply the result of exchange with the endogenous pools of amine. Stromblad and Nickerson[4] also have referred to this problem. The only definitive proof of uptake would be a measured net increase in the amine content of the tissues. However, it is unlikely that even the most sensitive biological or fluorimetric assays available could measure accurately the very small increases in total amine content that would be expected after the administration of the relatively small doses of ^{3}H-NE used in this study. Since a primary aim of this work was to use lower doses of amine, the use of

sufficient quantities of NE to demonstrate net accumulation was precluded. Kopin *et al.*[20] were able to show definite uptake of ^{3}H-NE by the perfused heart of rats treated previously with reserpine. When the endogenous NE concentrations are normal, however, as in the present study, it cannot be stated with certainty that the ^{3}H-NE retained does in fact represent net uptake.

Acknowledgement—I am grateful to Miss J. Kinch and Miss C. Garzon for their careful technical assistance during this project.

REFERENCES

1. W. RAAB, *Exp. Med. Surg.* **1**, 188 (1943).
2. M. NICKERSON, J. BERGHOUT and R. N. HAMMERSTROM, *Amer. J. Physiol.* **160**, 479 (1950).
3. W. RAAB and W. GIGEE, *Circulat. Res.* **3**, 553 (1955).
4. B. C. R. STROMBLAD and M. NICKERSON, *J. Pharmacol. exp. Ther.* **134**, 154 (1960).
5. U. S. VON EULER, *Circulat. Res.* **4**, 647 (1956).
6. L. G. WHITBY, J. AXELROD and H. WEIL-MALHERBE, *J. Pharmacol. exp. Ther.* **132**, 193 (1961).
7. A. BERTLER, N. A. HILLARP and E. ROSENGREN, *Acta physiol. scand.* **50**, 113 (1960).
8. H. BLASCHKO and A. D. WELCH, Naunyn-Schmiedeberg's *Arch. exp. Path. Pharmak.* **219**, 17 (1953).
9. N. A. HILLARP and B. NILSON, *Acta physiol. scand.* **31**, Suppl. 113 (1954).
10. U. S. VON EULER, *Acta physiol. scand.* **43**, 155 (1958).
11. E. OSTLUND, G. BLOOM, J. ADAMS-RAY, M. RITZEN, M. SIEGMAN, H. NORDENSTAM, F. LISHAJKO and U. S. VON EULER, *Nature (Lond.)* **188**, 324 (1960).
12. A. WEGMANN and K. KAKO, *Nature (Lond.)* **192**, 978 (1961).
13. L. T. POTTER and J. AXELROD, *Nature (Lond.)* **194**, 581 (1962).
14. A. H. ANTON and D. F. SAYRE, *J. Pharmacol. exp. Ther.* **138**, 360 (1962).
15. T. BRAY, *Analyt. Biochem.* **1**, 279 (1960).
16. J. B. CAULFIELD, *J. biophys. biochem. Cytol.* **3**, 827 (1957).
17. H. J. DENGLER, H. E. SPIEGEL and E. O. TITUS, *Science* **133**, 1072 (1961).
18. H. D. GREEN and J. H. KEPCHAR, *Physiol. Rev.* **39**, 617 (1959).
19. L. T. POTTER and J. AXELROD, *J. Pharmacol. exp. Ther.* **140**, 199 (1963).
20. I. J. KOPIN, G. HERTTING and E. K. GORDON, *J. Pharmacol. exp. Ther.* **138**, 34 (1962).