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SUBCELLULAR DISTRIBUTION OF NOREPINEPHRINE IN THE NORMAL AND SURGICALLY DENERVATED CAT HEART

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Abstract—An analysis of the subcellular distribution of both endogenous and administered norepinephrine in homogenates of cat heart has been carried out. Total surgical denervation of the heart resulted in both a reduction of the total amount of administered norepinephrine retained in tissue and a specific interference with microsomal binding. The altered subcellular distribution in denervated hearts indicated that microsomal binding of norepinephrine is dependent on the presence of neural structures. The microsomal structures binding norepinephrine could be purified considerably by centrifugation through a sucrose density gradient, but in these purified fractions morphological evidence has been presented to indicate that even these fractions are contaminated by non-neural elements.

SYMPATHETIC nerves have been demonstrated to be responsible for the uptake and binding of exogenous norepinephrine[1] and it has been shown by autoradiographic techniques that the norepinephrine retained in tissues with a sympathetic innervation is present in neural structures.[2, 3] Furthermore, electron microscopic studies of fixed tissue sections have suggested that vesicles with central dense cores are responsible for the storage of norepinephrine within the neuron.[4, 5] Analysis of the distribution of norepinephrine in tissue homogenates has indicated that a predominant portion of this amine is associated with a microsomal fraction.[6–8] However, it has not been possible to demonstrate that the isolated norepinephrine-binding microsomes are morphologically similar to the vesicular structures observed *in situ*.[9] The purpose of the present study was to define the subcellular distribution of norepinephrine in the cat heart and to examine the morphology of the specific particulate fraction with which this amine is bound. Total surgical denervation of the heart was also studied to determine the effect of this procedure on the uptake and distribution of exogenous radio-labeled norepinephrine and on the morphology of this specific particulate fraction in these hearts.

METHODS

These studies were performed on 24 control mongrel cats weighing from 1·75 to 3·24 kg and 6 denervated cats of similar weight. Surgical denervation of the heart was

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accomplished by mediastinal ablation of extrinsic cardiac nerves as previously described,[10] and these animals were studied 1–4 weeks after denervation.

d,l-Norepinephrine-7-^{3}H was administered intravenously to the animals either 5 or 30 min prior to sacrifice. The control animals received between 0·045 and 7·0 μg of the amine/kg as the free base; the denervated animals all received 7·0 μg/kg. The specific activity of *d,l*-norepinephrine-7-^{3}H was determined by radioactive and fluorometric assay of the purified catecholamine which was used in these studies after adsorption onto and elution from aluminum oxide (5·95 and 9·52 mc/μmole). In control animals (receiving 0·7 and 7·0 μg/kg) this material was diluted with a calculated amount of unlabeled *d,l*-norepinephrine to achieve lower specific activities. The animals were anesthetized with pentobarbital (Nembutal) (25 mg/kg) i.p. and the heart removed immediately. The left ventricle was rapidly dissected, trimmed free of fat, washed with ice-cold saline, and weighed. The material was then passed through a muscle press and homogenized in 5 volumes of 0·25 M sucrose containing 5×10^{-3} M phosphate buffer, pH 7·4, and 10^{-3} M $MgCl_2$. Homogenization was carried out at 1000 rev/min in a loosely fitting all-glass Duall tissue grinder and was limited to 25 strokes. All operations were conducted on ice in a room refrigerated to 4°.

The homogenate was subjected to two differential centrifugation procedures. For a complete subcellular fractionation, four particulate and one supernatant fractions were obtained initially: (a) 1000 *g*–10-min fraction containing nuclei, unbroken cells, and cellular debris; (b) 10,000 *g*–10-min fraction containing the heavier mitochondria; (c) 25,000 *g*–10-min fraction containing smaller and fragmented mitochondria together with some heavier microsomes; (d) 100,000 *g*–60-min microsomal fraction and its supernatant. For all subsequent analyses of the subcellular distribution in the broken cell fraction a shorter procedure was employed in order to avoid changes in structure and loss of the amine from the particulate material with time. The coarse particulate and mitochondrial material was removed by centrifugation in a Spinco AH 40 rotor for 5 min at a setting of 40,000 rev/min to yield the supernatant fraction (S_2) containing only microsomal and soluble material. The distribution of norepinephrine in this fraction was then determined by further centrifugation at 100,000 *g* to estimate the content of microsomal and soluble material. Analysis of these subcellular fractions was carried out after deproteinization and extraction with perchloric acid. After adsorption onto and elution from aluminum oxide[11] the eluates were analyzed for radioactivity[12] in a liquid scintillation spectrometer and for norepinephrine by fluorometric analysis after oxidation to the trihydroxindole.[13, 14] In the experiments in which the retention of radioactive norepinephrine was studied, the exogenous amine was expressed as millimicrograms per gram tissue weight, a value which was determined from the specific activity of the administered norepinephrine.

The norepinephrine binding to particulate material in the S_2 fraction was also analyzed by centrifugation of this material through a continuous sucrose density gradient prepared by a procedure similar to that described by Michaelson *et al.*[9] Sucrose, 0·1 M, was introduced dropwise into 2·6 ml of 1·4 M sucrose in a constant-volume mixing chamber. The resulting solution was developed up to 4·5 ml in a 5·5-ml centrifuge tube. This gave a gradient which varied between 1·4 M and 0·32 M sucrose, and the difference in existing concentrations between the mixing chamber and diluent was an exponential function of the volume added. One ml of the S_2 fraction

was then carefully layered on the surface and centrifuged at 125,000 *g* for 1 hr in a Spinco SW-39 swinging-bucket head. Fractions were obtained by drop collection and, after addition of counting solution, were analyzed for total radioactivity in a liquid scintillation spectrometer.

The region selected for electron microscopic examination was diluted with 5×10^{-3}M phosphate buffer to a sucrose concentration of 0·25 M and then centrifuged at 125,000 *g* for 60 min in the swinging-bucket head. When small regions were removed, the cellulose tube was prepared previously by placing a small concave agar base in the bottom of the tube. The 5% agar solution was made up in 0·25 M sucrose to eliminate disruption of the agar base during centrifugation. With this technique, minute quantities of material could be identified and fixed with 1% osmium tetroxide buffered with phosphate, pH 7·0. The ends of the tubes were pared off with a razor blade and the darkened microsomal pellet was removed with a needle and transferred to a fresh fixative for 1 hr. The material was cut into blocks, dehydrated in methanol. and embedded in Araldite. Individual sections were double-stained with uranyl acetate and lead citrate and examined with an RCA EMU 3E electron microscope.

Protein was determined colorimetrically with the Folin phenol reagent.[15] For the RNA determination, pooled fractions from two to three identical gradients were washed two times with cold 2% perchloric acid and the RNA solubilized by 10% perchloric acid at 70° for 10 min. The RNA was assayed by u.v. absorption of this extract,[16] modified by using microtechniques to detect optical absorption in 0·25 ml with a Zeiss spectrophotometer.

RESULTS

The homogenates of left ventricles of four control cats contained 1·24 ± 0·15 (S.E.M.) μg norepinephrine/g. Upon fractionation of this homogenate by differential centrifugation (Fig. 1) it was found that most of the norepinephrine, 68·0 ± 2·0 per cent, was recovered in the lowest-speed sediment. In the higher-speed fractions, representing ruptured cell material, the norepinephrine was recovered principally in

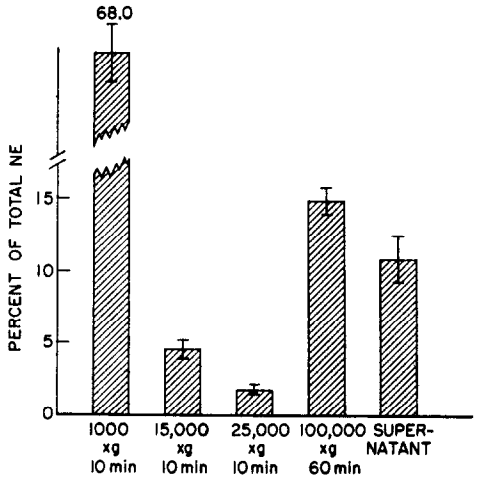

FIG. 1. Subcellular distribution of endogenous norepinephrine in the left ventricle of the normal cat heart. The mean values for 4 cats together with their standard errors are shown in the figure.

the 100,000 *g* and the soluble fractions, which contained 14·7 ± 0·8 per cent and 10·5 ± 2·1 per cent respectively. Smaller amounts, 4·6 ± 0·5 per cent and 1·8 ± 0·2 per cent were recovered in the 10,000 *g* and 25,000 *g* fractions. After elimination of the lower-speed sediments by the rapid method of preparation (see Methods), examinations of the ruptured cell fraction, S_2, in seven control cats disclosed that the microsomal and soluble fractions of this preparation contained nearly equal amounts of norepinephrine, averaging 50·4 ± 2·5 per cent and 49·6 ± 2·5 per cent respectively. In the denervated hearts, endogenous norepinephrine was essentially undetectable, averaging only 0·02 ± 0·005 μg/g, and no attempt was made to analyze for its distribution in these homogenates.

The uptake and distribution of radioactive norepinephrine in the left ventricle of control cats was compared to that of denervated cats (Table 1), The homogenate

TABLE 1. RETENTION AND SUBCELLULAR DISTRIBUTION OF RADIOACTIVE NOREPINEPHRINE IN CONTROL AND DENERVATED CAT HEARTS

Animal	Time* (min)	^{3}H-NE Retention		
		Homogenate	Microsomal (mμg/g)	Soluble
Control (4)	30	42·8 ±2·3	6·49 ±0·55	6·24 ±0·66
Control (1)	5	90·9	12·4	11·8
Denervated (4)	30	0·36 ±0·08	0·005 ±0·002	0·210 ±0·005
Denervated (1)	5	2·1	0·071	1·0

* Time after intravenous injection of μg *d,l*-norepinephrine-7-^{3}H/kg.

contained 42·8 ± 2·3 mμg radioactive norepinephrine/g in the control hearts, whereas this value was markedly reduced in the denervated animals to 0·36 ± 0·08 mμg/g ($P < 0·01$). The subcellular distribution in control hearts showed that 6·49 ± 0·55 and 6·24 ± 0·76 mμg/g were present in the microsomal and soluble fractions, paralleling the endogenous norepinephrine; in the denervated hearts significantly reduced values were found, 0·005 ± 0·002 and 0·210 ± 0·050 mμg/g ($P < 0·01$, for both comparisons). The relative distribution between the microsomal and soluble fractions was significantly altered in the denervated hearts with about 40 times as much present in the soluble fraction as in the microsomal fraction, compared to the control hearts, in which nearly equal quantities were present in the two fractions. The plasma concentration of radioactive norepinephrine in the denervated animal measured in one experiment was 0·73 mμg/ml 30 min after its administration compared to 0·50 mμg/g in the heart at the same time. A comparison of the uptake and subcellular distribution 5 min after the administration of radioactive norepinephrine was also made and, although this involved only one comparison, the previously mentioned differences between control and denervated hearts were also apparent at this time (Table 1).

In order to investigate whether the subcellular distribution of exogenous norepinephrine remained constant as diminished quantities are retained in the tissue, smaller amounts of the amine were administered to control animals, and the retention and

distribution in the heart were measured (Fig. 2). By extending the amount administered over an almost 200-fold range in the normal animal (0·045 to 7·0 μg/kg), a comparison could be made of the subcellular distribution in the normal heart at a dose giving comparable retention to that found in the denervated heart. A linear relationship (calculated logarithmic slope of unity) is obtained between binding to the microsomal

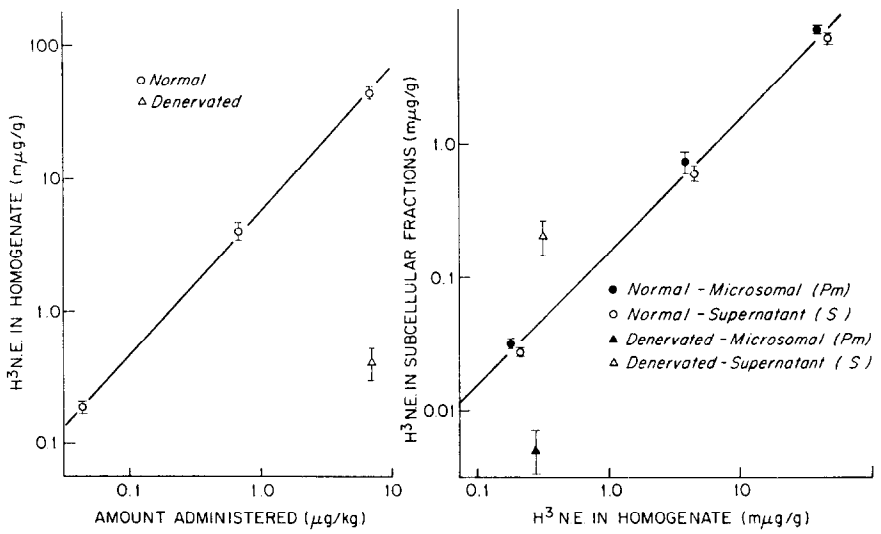

FIG. 2. Relationship of the administered dose of radioactive norepinephrine (^{3}H-NE) to retention and subcellular distribution of the material in control and denervated cat hearts. Left panel: retention of ^{3}H-NE in the total homogenate 30 min after administration of 0·045, 0·7, and 7·0 μg ^{3}H-NE/kg; control hearts (○) and denervated hearts (△). Right panel: subcellular distribution of ^{3}H-NE at the different levels of concentration in the homogenate which represent total retention; control microsomal (●) and soluble (○), and denervated microsomal (▲) and soluble (△). The means and standard errors of the mean are representative of 4 animals at each point.

fractions and total retention of norepinephrine in the homogenate. A similar relationship also exists for the supernatant fraction. In comparison, the content of norepinephrine in the microsomal and supernatant fractions of the denervated heart deviates from the content in these fractions of the control hearts by nearly a cycle on the logarithmic scale. Thus, the content of exogenous norepinephrine found in the microsomal fraction is 10 per cent that of the normal hearts at a similar level of total uptake, and the supernatant fraction is nearly ten times greater than this fraction in the normal hearts.

Density gradient centrifugation was employed in order to characterize further the norepinephrine-binding microsomal fraction. The characteristic pattern of total radioactivity for control animals (Fig. 3) could be obtained by using either the S_2 fraction or the supernatant fraction obtained after centrifugation at 25,000 *g* for 10 min (Fig. 1). Also, changes in the amount of norepinephrine administered (0·045–7 μg/kg) and variable times of removal of the heart after injection (5–30 min) did not effect a change in this pattern. A major peak was uniformly present at a density

corresponding to 0·50 M sucrose with a smaller, secondary peak apparent at a sucrose molarity of 1·0 to 1·1. With the denervated hearts, none of the radioactivity in the homogenate was detected in the sucrose gradient either 5 or 30 min after radioactive norepinephrine administration, and all the radioactivity remained in the soluble fraction (Fig. 3).

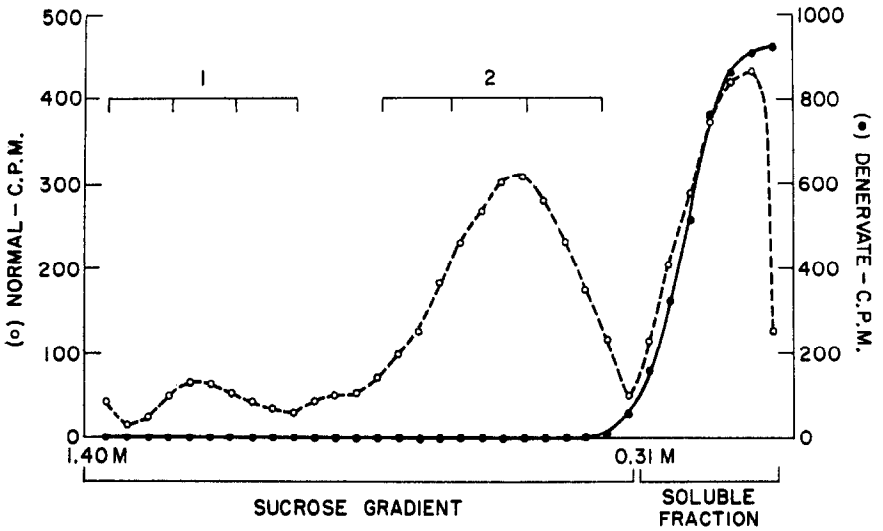

FIG. 3. The distribution of total radioactivity in a continuous sucrose density gradient for the homogenate of a normal and surgically denervated cat heart when 1·0 ml of the S_2 fraction was layered over the gradient. The brackets designate the three portions of regions 1 and 2 that were collected individually for electron microscopic examination. Ten times the dose in terms of total radioactivity was administered to the denervated animal in order to obtain the curves shown here.

The morphology of the norepinephrine-binding particulate material within the gradient was examined by removing the areas which contained a localization of radioactivity (regions 1 and 2 in Fig. 3). Examination of region 2, the region of peak activity, showed a variety of vesicular material (Fig. 4, a and b). No morphological difference, however, could be detected in these respective fractions when the normal and denervated hearts were compared, nor was there any variation in the quantity of material observed. Region 1 for both groups of hearts also contained microvesicular structures of a nature similar to that demonstrated in Fig. 4, but these appeared to be aggregated and matted with a more dense amorphous material, which may account for the different sedimentation properties of these vesicles.

Further separation of the vesicular population within the major peak of radioactivity of the gradient was attempted by the use of the agar base tubes which permitted collection and handling of smaller portions of the gradient. With this modification, minute quantities of particulate material could be examined, owing to its darkening on the agar surface upon fixation. It is apparent that some separation of the particles, on the basis of their ultrastructure, is achieved in the three portions of region 2 with the majority of larger vesicular material being in the lower portion (Fig. 4, c, d, and e).

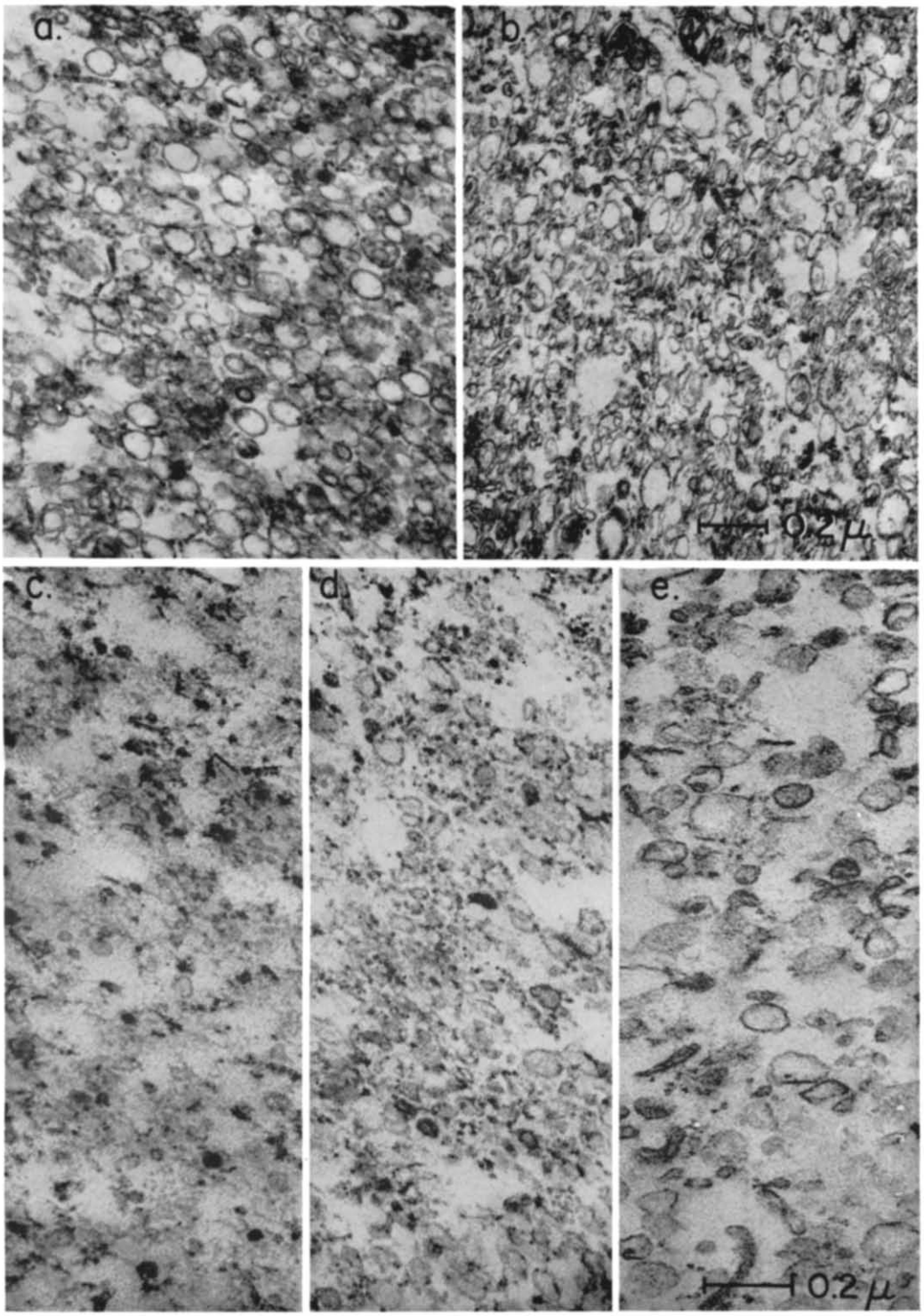

FIG. 4. Electron micrographs. a: Region 2, the region of peak activity in the normal cat heart. b: Corresponding region in the denervated cat heart. c, d, e: The normal cat heart of the three portions of region 2 shown in Fig. 3. The fractions from left to right extend from lower to higher sucrose molarity.

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However, no difference between these respective subfractions in the normal and denervated heart could be noted. No concentration of the dense-cored vesicles which are characteristic of adrenergic nerve terminals *in situ* was found in any of the subfractions, and the predominant number of vesicles observed appeared to be of larger dimensions than the agranular types observed within nerve terminals.[4]

By using this density gradient centrifugation technique, a significant purification of microsomal-bound norepinephrine in terms of protein and RNA was effected, as

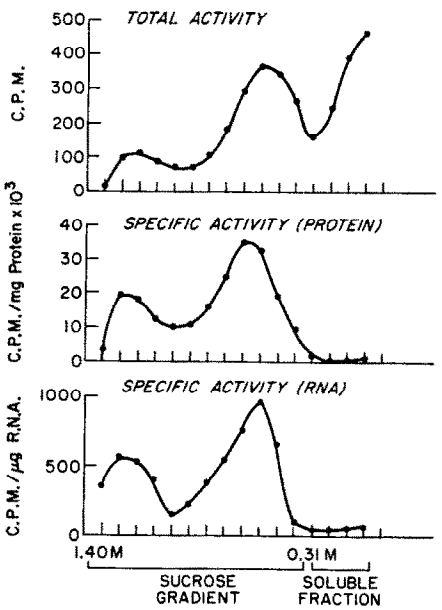

FIG. 5. Total radioactivity (top panel) and specific activity when related to protein (middle panel) and RNA (bottom panel) of the S_2 fraction from normal cat heart in the density gradient shown in Fig. 3. Individual 20-drop fractions were collected and analyzed for the above variables.

evidenced by the peak regions within the total microsomal fraction (Fig. 5). In addition, the specific activity of microsomal-bound norepinephrine was found to be markedly greater than the specific activity of the soluble fraction when related to either protein or RNA. There are still two separate peaks within the gradient even when radioactivity is expressed as specific activity. However, the results of the study of the ultrastructure of the secondary peak indicated that this material was similar to that in the primary peak, but aggregated to form a more dense fraction.

DISCUSSION

The microsomal fraction of heart homogenates has been found to contain a large portion of the norepinephrine recovered in the subcellular fractions of this tissue,[6-8] and to contain amine with a greatly enhanced specific activity when related to protein concentration.[8, 17] In the present investigation a careful analysis of the distribution of endogenous norepinephrine in such homogenates has confirmed these findings and indicated that, although there is a small but significant quantity of norepinephrine in

the mitochondrial and heavy microsomal fractions, a large part of the particle-bound amine is in a small, light microsomal fraction (Fig. 1). The significance of soluble norepinephrine is unclear, since some of the fraction may represent particle-bound amine which has been solubilized during the homogenization procedure.

Although postganglionic sympathetic denervation previously has been shown to reduce the retention of exogenous norepinephrine in tissues,[1, 18] the effect of denervation on microsomal binding of the amine had not been quantified. The present observations indicate that denervation results in a pronounced reduction of not only the total retention of norepinephrine in the heart but also the relative content retained in the microsomal fraction. Thus, the material retained in the denervated heart is virtually all in the soluble fraction of the homogenate. In control animals an almost constant percentage of exogenous norepinephrine was recovered in the microsomal fraction regardless of the quantity administered, and thus the amount bound in the tissue (Fig. 2, left and right); similar observations have been reported in studies using the rabbit heart.[17] It is clear, therefore, that the absence of norepinephrine in the microsomal fraction of the denervated hearts is not a result of the minimal amounts of amine retained in the tissue and subthreshold amounts available for binding. From these observations it may be inferred that microsomal binding is the consequence of the interaction of the administered amine with a specific neural structure and not simply an entrapment or adsorption of a certain percentage of the amine on non-neural cardiac microsomes.

Analysis of the sucrose gradient patterns provided a definition of the density characteristics of the microsomal structures binding norepinephrine. Most of the particulate material with which the amine is associated equilibrates with 0·5 M sucrose, thus having density characteristics similar to the binding material purified from other mammalian species.[9, 17] The secondary peak equilibrating with higher concentration of sucrose appears to be artifactual, from examination of this fraction under the electron microscope, resulting from the entrapment of vesicular structures in dense amorphous material.

The morphological identification of norepinephrine-binding structures proved to be disappointing in spite of the fact that a considerable degree of chemical purity of these structures was achieved (Fig. 5). Distinct ultrastructural differences were seen within a localized region of the gradient, yet a concentration of graular or agranular vesicular material of 30–60 mμ diameter was not apparent within any portion. These are the dimensions of the vesicular material found within nerve endings of tissues which have a sympathetic innervation.[4] When osmium tetroxide is used as a tissue fixative, approximately 30 per cent of the vesicular material in the adrenergic nerve endings stain with a dense core[4, 5] and presumably reflect the presence of norepinephrine. It is not clear why these dense-cored granular vesicles have not been identified in the purified microsomal fractions. Although it must be considered that such vesicles may be more dense and consequently sediment with the mitochondrial fraction, this does not seem likely, in view of the much higher content of norepinephrine in the microsomal, compared to the mitochondrial, fraction. In similar experiments with rat heart homogenates,[19] the method of fixation was varied particularly in order to determine if there was an optimal pH for preservation of granular vesicles. Osmium tetroxide buffered with phosphate in the range pH 5·5–7·5, or with bicarbonate in more alkaline solutions, gave essentially the same preservation of the vesicular material

in the pellets, and it was clear that the pH of the fixative had no influence whatever in preserving a sizable population of granular vesicles. Similar experiments with glutaraldehyde fixation followed by osmium tetroxide still failed to reveal the granular vesicles. It is unlikely, therefore, that loss of these characteristic structures in cat heart homogenates can be attributed to imperfect fixation.

It is possible that the homogenization and tissue fractionation techniques caused a partial loss of norepinephrine and/or binding substances within the vesicle with concomitant loss of the dense-cored property, or such vesicles may have been so diluted in non-neural microsomes that their presence could not be appreciated morphologically. The lack of morphological difference between the relatively highly purified microsomal fractions obtained from the control and denervated hearts lends support to the latter hypothesis. Upon homogenization, the formation of vesicular structures has been observed from cell membranes[20] and other "membranous" cell components,[21] and this factor may dilute out the existing vesicles of the nerve ending. The lack of ability to demonstrate the presence of dense-cored vesicles within these microsomal fractions and the failure to demonstrate the absence of a specific type of vesicle in the denervated hearts may arise from the same reason; that is, the extensive contamination of even these purified norepinephrine-binding microsomes with large quantities of endoplasmic reticulum and cell membranes of the cardiac muscle cells. The possibility must also be considered that the adrenergic nerves have not degenerated in the denervated hearts and that, in spite of the functional deterioration which has occurred, as judged by the absence of norepinephrine, neural vesicular structures persist in the isolated subcellar fractions from these hearts.

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