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THE DISTRIBUTION OF LABELED NOREPINEPHRINE WITHIN SYMPATHETIC NERVE TERMINALS STUDIED WITH ELECTRON MICROSCOPE RADIOAUTOGRAPHY

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ABSTRACT

The distribution of radioactivity in association with sympathetic nerve terminals and intraneuronal organelles 30 min after the administration of tritiated norepinephrine (NE-^{3}H) was studied by electron microscope radioautography with recently developed quantitative methods of analysis reported in the accompanying paper (Salpeter et al., 1969). Nerves from the pineal body and the adrenal capsule were examined. It was found that nerve terminals containing vesicles were heavily labeled. (These terminals were not necessarily in contact with some innervated structure.) There was no selective labeling of either the intraneuronal mitochondria or the relatively small population of large (~1000 A) dense core granules. Small vesicles (~500 A), some of which have a dense internal granule, could not be analysed separately because they are closely packed and occupy ~60% of the volume in terminals. Because of the extensive distribution of these small granular and agranular vesicles in the radioactive terminals, they remain the most likely site for norepinephrine binding. Yet although the vesicles were uniformly distributed within the nerve terminals, it appears that the radioactivity was not. There appeared to be a somewhat higher concentration of radioactivity at the periphery of the nerve terminals than in the center. The usefulness of the method of analysis used in this study for determining the location of bound H^{3}NE pools in the nerve is discussed.

INTRODUCTION

Circulating norepinephrine-^{3}H (NE^{3}H) is taken up by peripheral tissue, and there is considerable evidence that this exogenous norepinephrine is bound by sympathetic nerve endings (see review by Iverson, 1967, chapters 7 and 9). In homogenates of tissue with an extensive adrenergic innervation, the highest proportion of bound NE^{3}H occurs in microsomal subfractions consisting of numerous small granules (Euler, 1966; Potter and Axelrod, 1963 *a*; Taylor et al., 1966; Austin et al., 1967). These isolated granules which have the property of binding catecholamines have yielded considerable information regarding the kinetics of norepinephrine (NE) binding (Euler et al., 1963; Potter and Axelrod, 1963 *b*; Euler, 1966; Stjärne, 1966; Potter, 1966; Euler and Lishajko, 1967).

When viewed with the electron microscope, terminal axons of peripheral adrenergic nerves appear to contain numerous vesicles, many of them with an electron-opaque core (Richardson, 1962; 1966 *a*). On the basis of extensive histochemical and pharmacological studies it has been proposed that endogenous norepinephrine normally occurs within these small granular vesicles

(Pellegrino de Iraldi and DeRobertis, 1963; Richardson, 1966 *b*; Bloom and Barrnett, 1966; Bondareff and Gordon, 1966), and that these vesicles are sites for the binding of exogenous NE^3H (Wolfe et al., 1962; Lever et al., 1968; Devine and Simpson, 1968).

The uptake and binding properties of isolated nerve particles are not, however, sufficient to explain all the phenomena of norepinephrine uptake and release by intact nerves, especially in connection with the location of norepinephrine pools (reviewed by Iverson, 1967, chapter 7). An unequal distribution of accumulated exogenous norepinephrine within nerve terminals in relation to the plasma membrane has been suggested to account for observed differences in the availability of this accumulated norepinephrine for subsequent release (Crout et al., 1962; Crout, 1964).

Electron microscope radioautography provides a tool for visualizing the distribution of NE^3H in undisrupted tissues in vivo. It has been used to confirm that the NE^3H which is retained in peripheral tissue is associated with sympathetic nerves (Wolfe et al., 1962; Wolfe and Potter, 1963). In the present study electron microscope radioautography was used to examine in detail the distribution of accumulated NE^3H within the nerve by using the method of analysis described in the companion paper (Salpeter et al., 1969).

MATERIALS AND METHODS

Two young albino mice, 5–12 wk old, were injected through the caudal vein with 10 μc of DL-NE^3H in 0.01 ml saline per gram body weight. The DL-noradrenaline-7-T hydrochloride was obtained from the Nuclear Chicago Corporation (Des Plaines, Ill.) at 2.6 c/mmole. 30 min after injection, the mice were sacrificed by cervical dislocation, and the adrenal and pineal organs were fixed in 5% cacodylate-buffered glutaraldehyde (pH 7.2) for 1 hr in the cold. After several washes in cacodylate-sucrose buffer, pieces of each organ (1–3 mm^3) were soaked in 1% OsO_4, pH 7.2 (Caulfield, 1957), for 1 hr, then rapidly dehydrated in graded concentrations of alcohol, and, after final dehydration in acetone, embedded in Araldite.

To determine the loss of radioactivity from fresh tissue during tissue processing for radioautography, a separate group of mice was given intravenous NE^3H, and 30 min later known weights of pineal and heart tissue were digested in Hyamine (Packard Instrument Company, Inc., LaGrange, Ill.) or NCS (Nuclear Chicago) and counted in dioxane cocktail in a scintillation counter. Similarly, known weights of tissue from the same organs were fixed in glutaraldehyde, washed and dehydrated as described above, and solubilized in Hyamine or NCS for scintillation counting. All the fixative and dehydration solutions for each tissue sample were pooled, reduced in volume to 0.5 ml by evaporation under a stream of nitrogen at 45°C, and counted in dioxane cocktail. Appropriate control samples containing known amounts of NE^3H were treated in the same way and counted at the same time.

There is considerable evidence that reserpine prevents the binding of norepinephrine to axonal granules but does not inhibit the initial uptake of NE by the axon into a nonbound fraction (Kopin et al., 1962; Iversen et al., 1965). This nonbound pool of NE is, however, quickly destroyed by monoamine oxidase inside the axon. One group of animals was given reserpine (10 mg/kg body wt) followed 2¾, 6¼, 8¼, 12¼, or 23¾ hr later by a single injection of norepinephrine-^3H (0.6 mg/kg body wt). Iproniazid (50 mg/kg body wt) was given 15 min before the NE^3H to inhibit monoamine oxidase and to favor the retention of NE^3H taken up by the nerve. 15 min after the NE^3H, both the adrenal and pineal organs were fixed, embedded, and prepared for radioautography.

Preparation of Radioautograms

For light microscope radioautography, thick sections (0.5–1 μ thick) were cut from each block of tissue, transferred onto glass slides, and covered with a layer of Ilford L4 emulsion (purple to blue interference color, i.e. 1500–1700 A thick). For a correlation between interference color and emulsion thickness, see Salpeter and Bachmann, 1964, 1965. These preparations were exposed for 3–39 days and were developed in Microdol X (Eastman Kodak Co., Rochester, N.Y.) or D19 (Kodak).

For electron microscope radioautography, the method of Salpeter and Bachmann was used (1964, 1965). Thin sections (pale gold interference color, ~1000 A thick) were transferred to collodion-coated slides, stained with uranyl acetate, vacuum coated with ~50 A of carbon, and coated with a monolayer of Kodak NTE emulsion (silver to pale gold interference color, ~700 A thick). Radioautograms were exposed for 39 or 66 days in an atmosphere of helium and developed in Dektol for 2 min at 23°C. Processed radioautographic specimens were photographed in an RCA EMU3C or Philips 300 electron microscope.

Analysis of Electron Microscope Radioautograms

Electron micrographs were enlarged to a final magnification of 30,000. The micrographs were ana-

lyzed by determining grain densities (grains per unit area). Wherever possible, these were tabulated as density distribution around relevant structures. The structures of interest were whole nerve terminals, intraneuronal mitochondria, large dense core granules, and small vesicles. Grain densities were obtained as proposed in the companion paper (Section IV *b*, Salpeter et al., 1969). We first obtained the distribution of developed grains with distance from the suspected source. A similar distribution was then obtained for random points (placebo points) which were punched on the prints in the form of a grid. When the number of grains per unit distance was divided by the number of points in the same unit distance, an accurate correction for area was made, and a true density distribution was obtained. (The location of a developed grain was determined by its center. A thin plastic mask which had concentric circles of increasing radii marked around a common perforated center was placed over each grain. The grain was fitted symmetrically inside the smallest circle which would encompass it, and the center was punched with a needle through the print.) All measurements were tabulated in units of a distance, HD, as discussed in the companion paper (Salpeter et al., 1969). HD is the distance from a line source in a radioautographic specimen within which half the developed grains fall. HD is thus an experimental measure of resolution. It was shown (Salpeter et al., 1969) that if grain density distributions are tabulated in distance units of HD from any source, these distributions have a universal shape which is independent of the resolution. Experimental grain density distributions tabulated in this way can be compared with theoretical distributions expected from radioactive sources differing in shape. The shape of the experimental source can then be described more accurately. HD for the specimen in this study (i.e. pale gold section, monolayer Kodak NTE, Dektol developed) is approximately 1000 A.

RESULTS

Fine Structure

The mouse adrenal capsule is approximately 6–10 μ thick and contains numerous fibroblasts and collagen fibers. In electron micrographs of the capsule, unmyelinated axons, singly or in groups, partially enveloped by Schwann cell cytoplasm, were seen between bundles of collagen fibers and frequently near to arterioles. Myelinated axons were only infrequently observed in the capsule. It is known that the innervation of the adrenal, including the capsule, is principally adrenergic (Marley and Prout, 1968; Coupland, 1965).

The diameters of the unmyelinated axons ranged from 4,000 to 20,000 A, most being between 4,000 and 10,000 A. The enlarged axonal profiles may correspond to the varicosities described on the light microscope level (Hillarp, 1959). Small mitochondria, either scattered or in groups, were a common feature of most axons. Some axons contained numerous small vesicles (average diameter 470 A, range 150–800 A) with a very prominent dense core (see Fig. 1). Large dense core granules (average diameter 1,000 A, range 500–1,500 A) were also seen in most axons. The large granules are similar in size to the Type I particles, and the smaller vesicles resemble Types II and III particles observed in autonomic nerves (Grillo and Palay, 1963). All those unmyelinated axons which contained small vesicles or large granules of the types described above, whether the axons occurred singly or in groups, will be called nerve terminals. Neurotubules and filaments were only occasionally seen in the axons which primarily contained vesicles. Axons containing primarily neurotubules and filaments but few mitochondria and no vesicles were not included in the category of nerve terminals.

In the pineal body, unmyelinated axons, either alone or in groups and partially enveloped in sheath cell cytoplasm, were frequently seen near small blood vessels, but many axons were also seen between pinealocytes and apparently unassociated with blood vessels. The axons resembled those in the adrenal capsule, both with respect to their size and to the presence of granular and agranular vesicles. The predominantly adrenergic innervation of the pineal organ has been demonstrated (Ariëns-Kappers, 1960). A more detailed account of the fine structure of the tissues used in this study will be published elsewhere.

Retention of Radioactivity

To determine how much norepinephrine was retained in the tissues during histological processing, the radioactivity in unfixed and fixed tissues and in fixing and dehydrating fluids was monitored by scintillation counting. About 20% of the activity which was present in the unfixed tissue was removed during fixation and dehydration. We are therefore studying the distribution of a large fraction of the total norepinephrine taken up by the tissue.

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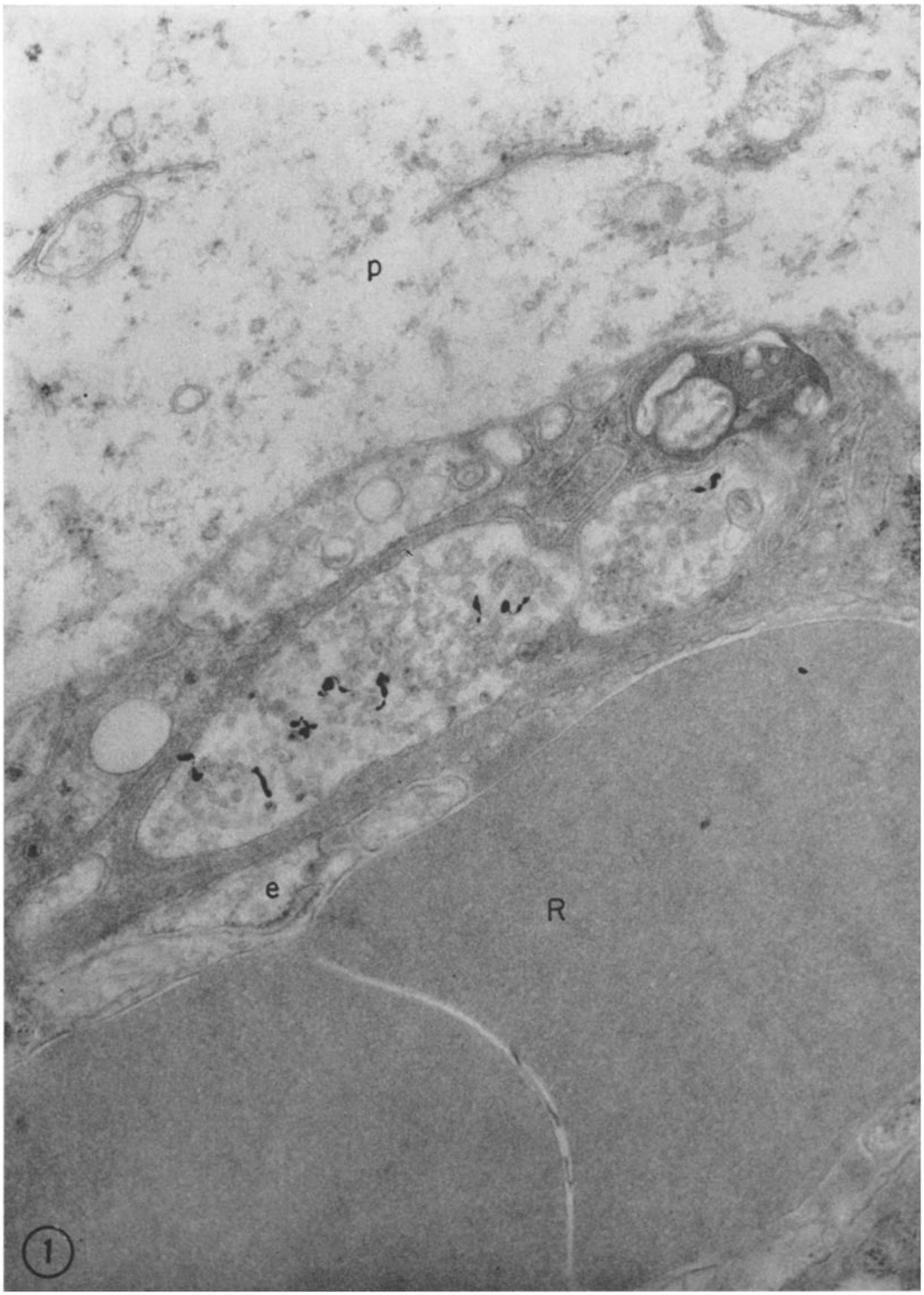

FIGURE 1 Electron microscope radioautogram of mouse pineal organ after intravenous norepinephrine-^{3}H. Developed grains occur over two nerve terminals which are close to, but not touching, capillary endothelium and a pinealocyte. The nerve terminals contain numerous small vesicles (~500 A diameter). *t*, nerve terminal; *e*, endothelium; *p*, pinealocyte; *R*, erythrocyte. × 61,000.

Radioautography

Light Microscope Level

In light microscope radioautograms from animals which were given intravenous tritiated norepinephrine alone, there were small heavily labeled areas with circular or elongated profiles in the pineal organ and the adrenal capsule. (The adrenal medulla and cortex were not included in this study.) The heavily labeled regions were usually arranged in groups around the blood vessels. A small number of similarly labeled areas occurred individually, unassociated with blood vessels. In the pineal organ these were in close contact with pinealocytes. Pinealocytes were not significantly labeled even after exposure periods which caused the heavily labeled areas to appear as dense masses of developed silver grains. Erythrocytes were moderately labeled in both organs.

RESERPINIZED CONTROLS: There was no apparent incorporation of label into the adrenal capsule or pineal organ of mice if the animals were given the reserpine 2¾, 6¼, or 8¼ hr before NE^{3}H. When the time between reserpine and NE^{3}H injections was increased to 12¼ hr without any other change in the times and doses of each compound, there was a weak to moderate incorporation of radioactivity into discrete areas of the kind observed in the adrenal capsule and pineal organ of nonreserpinized animals. When the time between reserpine and NE^{3}H was extended to 23¾ hr, the radioautographs were almost identical with those from animals which had not received reserpine or iproniazid. This result indicates that the radioactivity seen in the tissue is associated with bound norepinephrine rather than with the uptake of some nonspecific breakdown product. Since there is good evidence that reserpine does not prevent the uptake of exogenous norepinephrine into sympathetic nerves but merely prevents the binding of this norepinephrine into a monoamine oxidase protected pool (Kopin et al., 1962; Iversen et al., 1965; reviewed by Iversen, 1967, ch. 8), and since we obtained no radioautograms after short periods of reserpine treatment followed by iproniazid, we must assume that we retain only the bound label in tissue processed for electron microscope radioautography.

Electron Microscope Level

Electron microscope radioautograms were prepared of adrenal and pineal organs from those animals which received tritiated norepinephrine alone (no parallel studies of reserpinized animals were performed). Developed grains seen in these radioautograms were concentrated over and around nonmyelinated nerve terminals presumed to be adrenergic (Figs. 1–3). Similarly, as observed in light microscope radioautograms, developed grains sometimes occurred over erythrocytes in higher concentration than over other nonneuronal structures.

GRAIN DENSITY RELATIVE TO NERVE TERMINALS: The first structure to be analyzed was the nerve terminal as a whole. When scanning many electron microscope radioautograms, we saw numerous axonal areas with a heavy concentration of developed grains. These were invariably nerve terminals as defined previously, i.e. nervous material packed with the characteristic vesicles. About two out of three such terminals were labeled. The nonterminal axons were rarely labeled and were not analyzed in this study.

When analyzing isolated terminals, the distance from all grains and placebo points was measured to the nearest location on the terminal plasma membrane (a plus sign indicated that the grain or point was outside the terminal, and a minus sign indicated that it was inside). Grains and points which measured up to 4000 A (4 HD units) outside and inside the nerve terminals were included. Any grains or points within 4 HD units of an isolated nerve terminal and which were also closer than 4 HD units to the next nearest terminal were excluded from the tabulation. This was done in order to eliminate areas which would be expected to have a grain density considerably elevated due to cross-scatter from two adjacent terminals. By treating the placebo points in the same way as the grains, there was an accurate area correction for the rejected region. A density distribution was then plotted and compared with the theoretical distributions expected from isolated single sources, as given in the companion paper (Salpeter et al., 1969).

For purposes of grain analysis, a bundle of touching or almost touching terminals was treated as a single unit. The grain density distribution around such bundles was obtained as follows. All measurements were made to the axonal plasma membrane which was at the periphery of the bundle. Only those intraneuronal grains and points which came within 4000 A (4 HD) of this outer plasma membrane were included. This in effect

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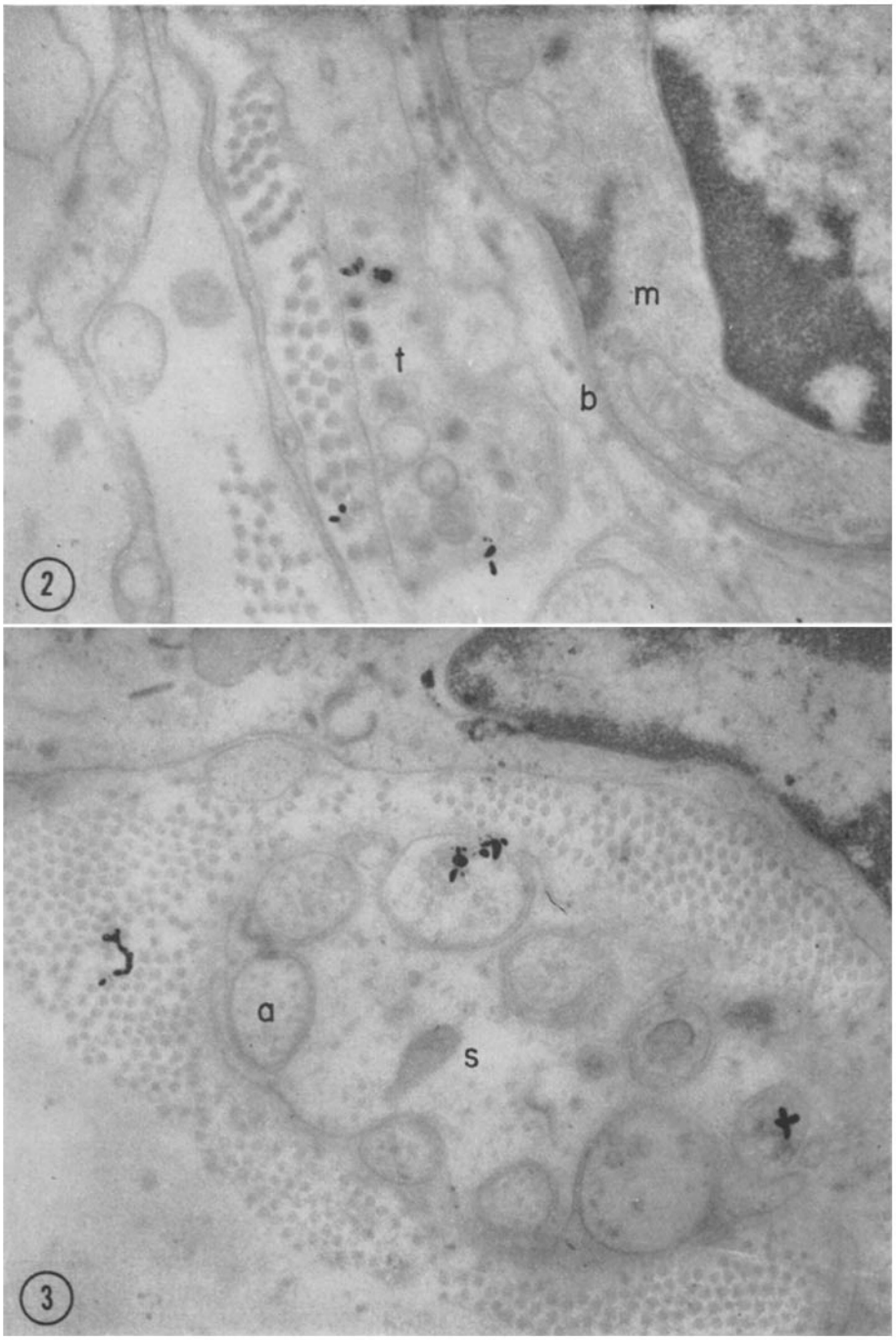

FIGURE 2 Electron microscope radioautogram of mouse adrenal capsule after intravenous norepinephrine-^{3}H. Developed grains are associated with a single terminal axon (*t*) which is close to a smooth muscle cell (*m*) but separated from it by a basement membrane (*b*). × 32,000.

FIGURE 3 Electron microscope radioautogram of mouse adrenal capsule after intravenous norepinephrine-^{3}H. A bundle of axons (*a*) can be seen; all of them are associated with one Schwann cell (*s*) which is separated from a muscle cell by collagen fibrils. One grain is scattered outside the bundle. The other grains are over axons containing small vesicles. × 32,000.

included only terminals at the periphery of any bundle.

Since expected grain density depends on the size of the source (see Salpeter et al., 1969), a typical value for the radius (r) of the nerve terminal had to be obtained. This can be done using the formula

$$r = \frac{x}{1 - 1/\sqrt{2}} = 3.42x$$

where x is the distance from the limiting membrane into the nerve terminal within which half the total placebo points fall. For this purpose all points within nerve bundles and individual terminals were counted to obtain the value for total placebo points. x is thus the width of an annulus inside the limiting membrane with an area equal to half the total area of the terminal. Using this formula, the typical radius was found to be 6000 A (or 6 HD units). The experimental density distribution is plotted as a histogram (Fig. 4). The superimposed (dashed) curve is the expected theoretical distribution for a uniformly labeled circular source of radius 6 HD obtained from the theoretical distributions in the companion paper (Salpeter et al., 1969, Fig. 10 *a*). It can be seen that the experimental grain distribution *outside* the nerve fits the theoretical distribution very well. This indicates that the tissue around the nerve terminal is not radioactive, and that the few grains seen over this tissue merely reflect scatter from the radioactive terminal. The experimental grain distribution inside the terminal does not completely fit the theoretical distribution expected if the radioactivity were uniformly distributed throughout the terminal. If a structure is uniformly labeled the grain density is highest at its center and decreases progressively towards the periphery. The difference between center and periphery could be as much as a factor of two if one has a large circle the size of a nerve terminal (Figs. 10 and 16, Salpeter et al., 1969). This is because the center is completely surrounded by radioactivity, and the grain density at the center reflects the cross-scatter from its radioactive surroundings. At the periphery, however, the radioactivity is only on one side. In the experimental histogram, the density rises only slightly towards the center, suggesting that the radioactivity is not uniformly distributed but is somewhat higher at the periphery.

One can, by combining the theoretical curves (Salpeter et al., 1969, Figs. 10 *a* and 9 *a*) for a solid disc and a hollow circle in different ratios, obtain a curve which fits the experimental data best. Such a composite curve is drawn over the histogram in Fig. 4 (solid curve). The fractional representation from the solid curve (q) was 0.7 and from the hollow circle ($1 - q$) was 0.3. In Section IV *c* and Fig. 18 of the companion paper (Salpeter et al., 1969) there is a discussion of how such a composite curve and the values for q and $1 - q$ can give information regarding the relative contribution to the experimental density curve of a uniformly distributed radioactive component and one which is selectively concentrated at the periphery. This analysis tells us that the present experimental distribution is compatible with one expected if 86% of the radioactivity was uniformly distributed throughout the nerve terminal and the remaining 14% all concentrated only at the periphery (on or within 1 HD of the plasma membrane).

GRAIN DISTRIBUTION RELATIVE TO TWO INTRANEURONAL ORGANELLES: Two organelles are sufficiently dispersed within the nerve terminal to permit grain density distributions to be plotted around them. These are the mitochondria and the large (approximately 1000 A) Type I dense core granules. The analyses were similar to those employed for the whole terminal.

1. *Mitochondria.* The mitochondria can be approximated to circular sources, 1 HD unit in radius. For their analysis, only grains or points within the terminal were included in the tabulation. The distribution of grain density relative to the mitochondria is given in Fig. 5. The distance of each grain and point over the mitochondria and up to 4 HD outside them was measured to the limiting membrane of the nearest mitochondrion. No grains or points were included, even if they were within 4 HD units of a mitochondrion if they were also closer than 3 HD units to a neighboring mitochondrion. The reason for doing this again stems from the need to exclude areas of potentially elevated grain density due to cross-scatter from two adjacent mitochondria if the mitochondria are indeed radioactive. The exclusion of points as well as grains corrects for this excluded zone. The solid curve drawn over the histogram gives the expected grain densities if the mitochondria had been the only labeled structure in the vicinity. There is no relation between the experimental histogram and the theoretical distribution. There is not even a slight peak of density over the mito-

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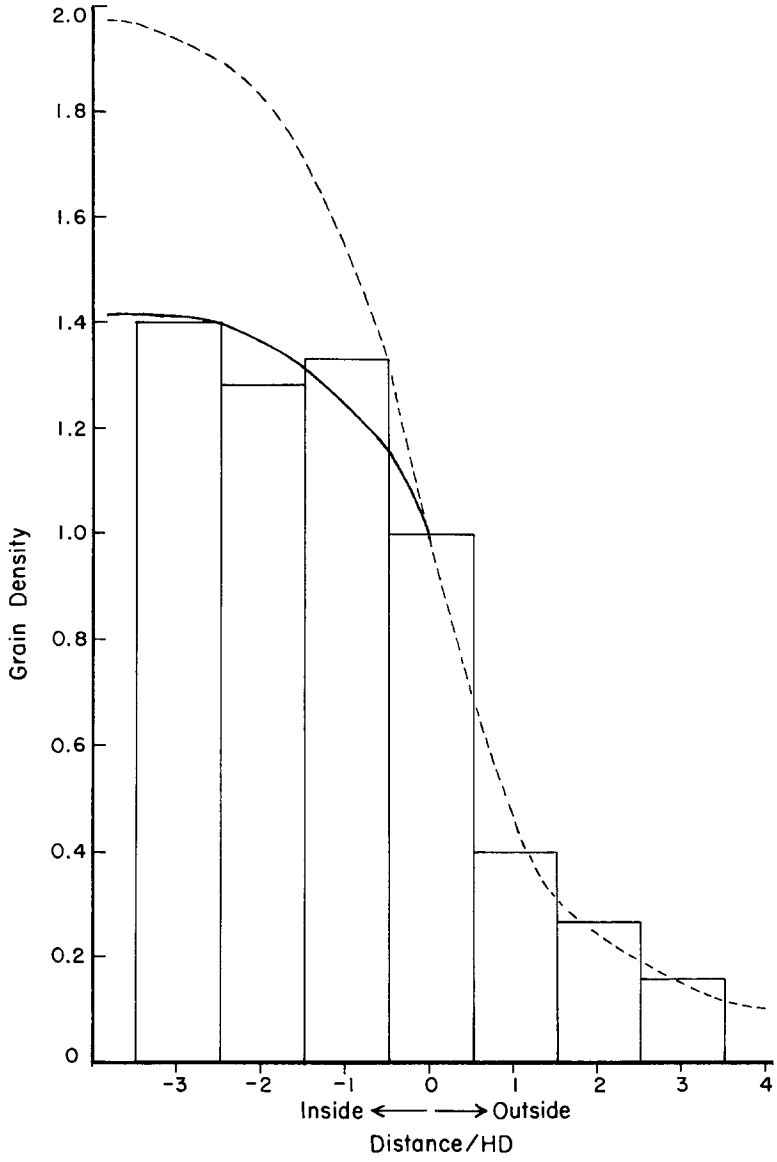

FIGURE 4 A histogram of the experimental distribution of developed grain density over and around terminal axons in the mouse adrenal capsule and pineal. The superimposed dashed curve corresponds to the distribution expected for a uniformly labeled circular disc source equal in diameter to a typical nerve terminal. The solid curve, which fits the experimental histogram, was generated by combining the expected distribution for a uniformly labeled disc and one labeled only at the periphery (see text).

chondria. It appears clear, therefore, that the mitochondria are not selective sites for norepinephrine binding—a finding consistent with other reports in the literature. Because of the small size of mitochondria relative to the radioautographic resolution, cross-scatter from their radioactive surroundings could easily give a grain density over the mitochondria as high as that recorded here. The mitochondria may in fact be completely devoid of any radioactivity.

2. *Large Dense Core Granules.* The grain density around the large dense core granules was deter-

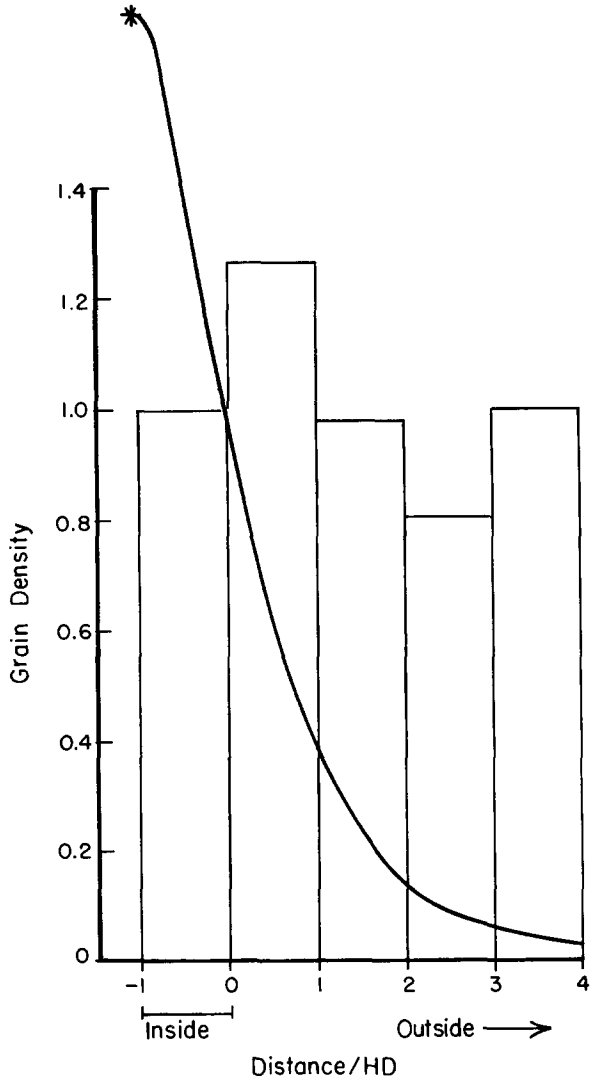

FIGURE 5 A histogram of the developed grain density distribution relative to intraneuronal mitochondria. The solid curve gives expected grain densities had the mitochondria been the only labeled structure. There is no relation between histogram and theoretical distribution, indicating that mitochondria are not selective sites for norepinephrine-^{3}H binding.

mined similarly to that for the mitochondria (the radii of these circular sources are 0.5 HD). The density distribution is given in the form of a histogram in Fig. 6. Again, it does not conform with the distribution expected if these granules were either the only sources or even the sites of some selective concentration of radioactivity. Compare the experimental histogram with the theoretical distribution drawn as a solid curve over it in Fig. 6. The theoretical curve gives the expected distribution if the granules were labeled (from Fig. 10, Salpeter et al., 1969). By the same argument as pertains to the mitochondria, one cannot decide between the two alternatives, i.e. whether these granules are completely devoid of radioactivity or whether they are merely no more radioactive than other constituents of nerve axoplasm.

SMALL VESICLES: The only remaining intraneuronal organelles are the small vesicles (approximately 500 A) with or without the dense core (Type II vesicles). By determining the proportion of the total placebo points per terminal which occurred over small vesicles, it was found that they occupy more than 60% of the area of these terminals, and that they were uniformly distributed throughout the terminals. Because of the close packing of these vesicles, it is impossible to obtain a histogram of grain distribution around

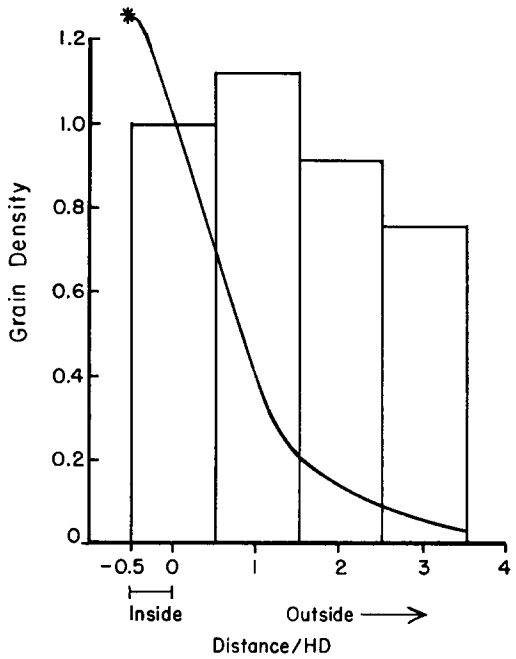

FIGURE 6 A histogram of the developed grain density distribution relative to large dense core granules (~1000 A). The solid curve corresponds to the expected distribution if the granules were selectively labeled. There is no correspondence between experimental and theoretical distributions, indicating that these granules were not selective sites for norepinephrine-^{3}H binding.

them. Since they occupy such a large proportion of the space within which the tritiated norepinephrine is bound, it is reasonable to assume that the binding of norepinephrine is indeed associated with the vesicles as others have suggested. We cannot resolve, however, whether this binding takes place inside or outside the granules, or whether the existence of a dense core in an electron micrograph provides any information regarding the presence or absence of catecholamines-^{3}H.

OVER-ALL DENSITY DISTRIBUTION: Dahlström et al. (1966) estimate that the amount of endogenous norepinephrine in vas deferens and iris of albino rat equals about 5×10^{-3} pg norepinephrine per varicosity with an average volume of 2–8 μ^3 (thus $\sim 10^{-3}$ pg/μ^3). It is difficult to relate the varicosities described by the light microscope histochemist with the vesicle-packed dilations in the electron microscope, since the varicosities may in fact consist of several closely packed terminals in one bundle. A comparison per μ^3 of tissue was thus considered to be more accurate. From Bachmann and Salpeter (1967) we know that, under the specimen conditions employed in our study, one developed grain results on the average from 23 ± 30% radioactive decays in the tissue. The average grain density of the nerve terminals was 0.215 grains per placebo point in radioautograms developed after 66 days. (There are 1.17 placebo points/μ^2.) Since the specific activity of the injected norepinephrine was 2.6 c/mmole, and the thickness of the specimen was ~1000 A, and the exposure time was 66 days, we calculate that this number of developed grains represents an uptake of 2.5×10^{-3} pg (±30%) of injected norepinephrine per μ^3 of terminal. The values were about the same for the adrenal capsule and pineal organ and were ~2.5 times the endogenous store in the rat tissue reported by Dahlström et al. (1966).

There are obvious risks and potential errors in a comparison involving different organs and different species, yet it is noteworthy that these results are in line with those reported in the literature on uptake of circulating norepinephrine-^{3}H by nerve terminals in relation to the endogenous stores (Iversen, 1963; Crout, 1964).

DISCUSSION

Using a new method for analysing electron microscope radioautograms, we have determined that the grain distribution with respect to vesicle-filled nerve terminals is consistent with an hypothesis that norepinephrine-^{3}H is bound inside such terminals, and that the grains seen outside them are merely the result of scatter. The radioactive terminals were not necessarily in contact with innervated structures, such as perivascular smooth muscle cells or pinealocytes, but were often separated from them by basement membrane and frequently seen in isolation within connective tissue. In many instances the radioactive terminals were even part of large nerve bundles, enveloped by Schwann cells. If one assumes an association between the sites of heavy binding of ^{3}HNE and the stores of transmitter, one must conclude that in the adrenal capsule and pineal body transmitter release may occur at considerable distance from innervated structures. Such was suggested by Rosenblueth (1950) and Devine and Simpson (1968).

Within the nerve terminals the radioactivity was not associated with either mitochondria or the large (~1000 A) sparsely distributed granules containing a dense core. Smaller (~500 A) vesicles which constitute the mass of the volume of the

terminals remain the most likely sites for norepinephrine binding. They were present in large numbers in all the radioactive structures examined. This is consistent with the conclusions reached by others (Bloom and Barrnett, 1966; Wolfe et al., 1962; Richardson, 1966 *b*).

One of the most interesting aspects of this study is the suggestion that the norepinephrine-^{3}H, which was taken up by the sympathetic nerve terminals, was not uniformly distributed internally. There appears to be a slightly higher concentration at the periphery than in the center. Yet the small vesicles, the most likely candidates for the sites of binding, were shown to be uniformly distributed. If this is so, one must conclude that the vesicles at the periphery were able to bind more of the radioactive norepinephrine than did those vesicles in the interior.

Experiments on turnover time, metabolism, and release of bound norepinephrine-^{3}H have led to the conclusion that the norepinephrine enters at least two pools in the sympathetic nerve terminals (Potter and Axelrod, 1963 *a* and *b*; Iversen, 1963; Crout, 1964; Kopin, 1966). Circulating norepinephrine-^{3}H is bound first in a small pool, with a relatively rapid turnover time (half-life of a few hours), which is readily available to release by tyramine and is metabolized by 0-methylation. This small pool, which is released as transmitter, contains a larger fraction of the total bound radioactive norepinephrine shortly after its administration than does the rest of the nerve. With time, however, exchange does occur, and the specific activity of the tyramine-resistant pool rises. This tyramine-resistant pool is the larger pool, has a half-life of ~1 day, is accessible to release by reserpine, and is destroyed mainly by deamination (Potter and Axelrod, 1963 *a* and *b*). It has been suggested that different modes of binding of the catecholamine to nerve granules may account for the pools and their different accessibility for chemical releasers (Musacchio et al., 1965). On the other hand, Crout (1964) postulates that the difference resides primarily in the location of the bound norepinephrine within the nerve terminal. According to Crout, the "bound" norepinephrine-^{3}H released as a transmitter (i.e. the small tyramine accessible pool) is bound mainly to granules lying near the axonal membrane, and the norepinephrine in deeper granules is the tyramine-resistant pool. If this were so, one would expect that during the early postinjection periods there would be a higher concentration of radioactivity at the periphery of the terminal. The method for analyzing electron microscope radioautograms employed here provides a tool for testing hypotheses regarding the intraneuronal distribution of radioactivity. The data obtained in this study appear to be consistent with Crout's hypothesis. Further experiments giving the relative distribution of norepinephrine-^{3}H between periphery and center of the nerve, with time after injection or after chemical release, thus could prove definitive for testing the predictions of the Crout hypothesis.

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