LOCALIZATION OF TRITIATED NOREPINEPHRINE IN VASCULAR SYMPATHETIC AXONS OF THE RAT INTESTINE AND MESENTERY BY ELECTRON MICROSCOPE RADIOAUTOGRAPHY

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ABSTRACT

The distribution of infused tritiated norepinephrine (NE-^{3}H) in small mesenteric arteries and intestinal arterioles in rats was investigated with electron microscopic radioautography. Silver grains, indicating the presence of the tritium label on the sections, were found lying mainly over axon bundles, but some were present over collagen and smooth muscle cells. Axons with the highest concentrations of silver grains had been sectioned at points where they were naked of Schwann cell sheath, were dilated into varicosities, and contained small granular vesicles. This finding was taken as confirmatory circumstantial evidence that the small granular vesicles were the sites of uptake and storage of NE. The short interval between the start of infusion and the fixation of the tissue appeared to rule out any process other than a direct uptake of NE by the peripheral axons. If axonal sites of uptake of NE-^{3}H correspond to sites of release of NE, then the evidence suggests that such sites of release are widespread over the terminal part of the axon and are not confined to those parts of the axon which are in close contact with smooth muscle cells. Since the fixation and embedding procedures will remove NE which is not strongly bound to tissues, the localization of NE-^{3}H in the radioautographs does not necessarily correspond to the distribution of all the NE present in vivo.

INTRODUCTION

Electron microscopic radioautography with tritiated norepinephrine (NE-^{3}H) has provided evidence for the uptake of norepinephrine (NE) in adrenergic nerves in tissues such as the pineal body (Wolfe et al., 1962), atrial myocardium (Wolfe and Potter, 1963), hypothalamus (Aghajanian and Bloom, 1966, 1967), vas deferens and intestinal submucosa (Taxi and Droz, 1966), and brain (Lenn, 1967).

We have previously shown that in rats small mesenteric arteries and intestinal submucosal arterioles are convenient tissues for the investigation of the innervation of vascular smooth muscle (Devine and Simpson, 1967). In the present study we have investigated the uptake of NE-^{3}H into axons lying around these vessels. A preliminary report has already been published (Devine, 1967).

METHODS

250 μc (30 μg) of DL-7-norepinephrine-^{3}H (specific activity 1.4 c/mmole; Radiochemical Centre, Amersham, England) were infused via the femoral vein into each of two anesthetized rats. The blood

pressure of the rats was monitored and the infusion rate was adjusted so that the blood pressure did not rise above 180 mm Hg. The infusion time was 20 min. After 5 min after the cessation of infusion, the blood pressure returned to normal, and tissues were removed for electron microscopy and NE assay. Portions of the jejunum and mesentery were fixed in 1% osmium tetroxide in Veronal-acetate buffer (pH 7.3) for 5 hr. Tissues were dehydrated in alcohol and embedded in Epon 812.

Radioautography

Thin sections (gold interference colors) were cut with a Porter-Blum ultramicrotome and placed on Formvar-coated, carbon-stabilized copper grids. The sections were stained with uranyl nitrate followed by aqueous lead citrate (Saito and Matsunaga, 1966) and then carboned (Bachmann and Salpeter, 1965). The grids were attached at their edges to a microscope slide with double-sided adhesive tape, and Ilford L4 nuclear research emulsion was placed over the grids with a wire loop (Caro and van Tubergen, 1962; Maunsbach, 1966). The grids were stored in the dark with a dessicant at 4°C for periods ranging from 5 to 14 wk. All the results in the present paper are based on exposure times of 10 wk, with the exception of Fig. 6. The grids were developed in freshly filtered Kodak D19B solution for 5 min at 15°C, fixed in an acid fixer for 5 min, washed briefly in distilled water, and then dried. The sections were viewed in a Hitachi HU11A electron microscope. Structural details in the tissue were best seen after removal of the gelatin with 0.1% sodium hydroxide and restaining of the sections with saturated uranyl acetate solution.

Norepinephrine Assay and Radioactivity Estimations

The mesenteries were removed and homogenized, and the NE content was assayed fluorimetrically (Laverty and Sharman, 1965). The radioactivity of NE extracted from the mesentery was measured in a scintillation counter.

Statistical Evaluation of Results

Photomicrographs were taken of areas where axons lay near small mesenteric arteries and intestinal arterioles (the axons were all within 8 μ of the smooth muscle cells at the closest point). In all micrographs smooth muscle cells, adventitia, and axon bundles were present in varying proportions. Counts were made of the number of silver grains which lay over or within 0.2 μ of (*a*) axon bundles, (*b*) smooth muscle, or (*c*) which lay over adventitia (elastic tissue, fibroblasts, collagen, and adjacent extracellular space). The area of tissue was obtained by placing a suitably dimensioned, squared, transparent grid over the micrographs. The concentration of silver grains over the various tissues in each micrograph was expressed as grains per 100 μ^2. The means and standard deviations (SD) thus obtained (from 29 micrographs from intestinal arterioles and from 26 micrographs from small mesenteric arteries) are shown in Fig. 1. The significance of the difference between means was tested with Students *t* test. Schwann cell was inevitably included in measuring the area of some axon bundles, as it was often difficult to distinguish between axons and Schwann cells at the magnifications used (10,000–19,500). A total of 735 silver grains were counted, of which 438 were lying over nerve axons.

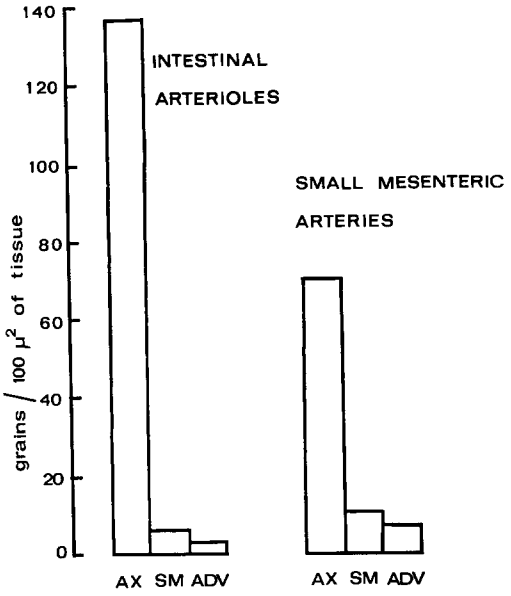


FIGURE 1 Distribution of silver grains over axon bundles (*AX*), smooth muscle cells (*SM*), and adventitia (*ADV*) in electron microscope radioautographs of intestinal arterioles and small mesenteric arteries (expressed as grains per 100 μ^2 of tissue) after 10 wk exposure.

RESULTS

Radioautography

The characteristic silver grains were seen over nerves, smooth muscle cells, and adventitia (Figs. 2–10), and also occasionally over areas of embedding medium at the edge of the section. This latter "background count" amounted to about 0.5 grains per 100 μ^2 for the intestinal arterioles and about 2.0 grains per 100 μ^2 for the small

mesenteric arteries. The number of silver grains (Fig. 1) over the bundles of axons was far higher than the numbers of such grains lying over areas of smooth muscle or lying over the adventitia both in intestinal arterioles (Figs. 4–7) and in small mesenteric arteries (Figs. 2, 3, and 8). The grain count per 100 μ^2 over the axons in the intestinal arterioles (136.1 ± 25.1 SD) is significantly higher ($P < .001$) than the grain count per 100 μ^2 over the smooth muscle cells (6.5 ± 6.6 SD). Similarly, the grain count per 100 μ^2 over the axons in the small mesenteric arteries (70.5 ± 36.5 SD) is significantly higher ($P < .001$) than the grain count per 100 μ^2 over the smooth muscle cells (10.8 ± 10.2 SD). The large standard deviations reflect large variations in grain count; in general, axons which were devoid of Schwann cells had many overlying silver grains (see Fig. 5), whereas axons which were almost completely enveloped by Schwann cell had few overlying silver grains (see Fig. 4).

Most axons with overlying silver grains contained small granular vesicles (Figs. 2, 3, 5–8). Usually the silver grains lay over varicosities of the axons as can be seen in two nearly consecutive sections (Figs. 2, 3) and over areas devoid of Schwann cell covering (compare Figs. 4, 5, and 7). However, direct statistical evidence that silver grains were more numerous over varicosities (Figs. 2, 3) than over narrowed portions of axons was difficult to obtain because of the comparatively few examples of longitudinal sections of axons seen.

The distribution of silver grains over the smooth muscle cells did not show any definite pattern. However, occasionally high concentrations were found in some areas of smooth muscle cells (Fig. 9), sometimes near the cell surface (Figs. 4, 8–10).

The distribution of silver grains over collagen fibrils and fibroblasts in the adventitia did not appear to show any pattern.

The large difference in concentrations of silver grains over axons from intestinal arterioles (136.1 ± 25.1 SD; see Fig. 1) compared with axons from small mesenteric arteries (70.5 ± 36.5 SD) was significant ($P < .001$). In contrast, there was no significant difference ($0.05 < P < 0.1$) between small mesenteric arteries and intestinal arterioles with respect to concentrations of silver grains over smooth muscle cells.

Norepinephrine Estimation

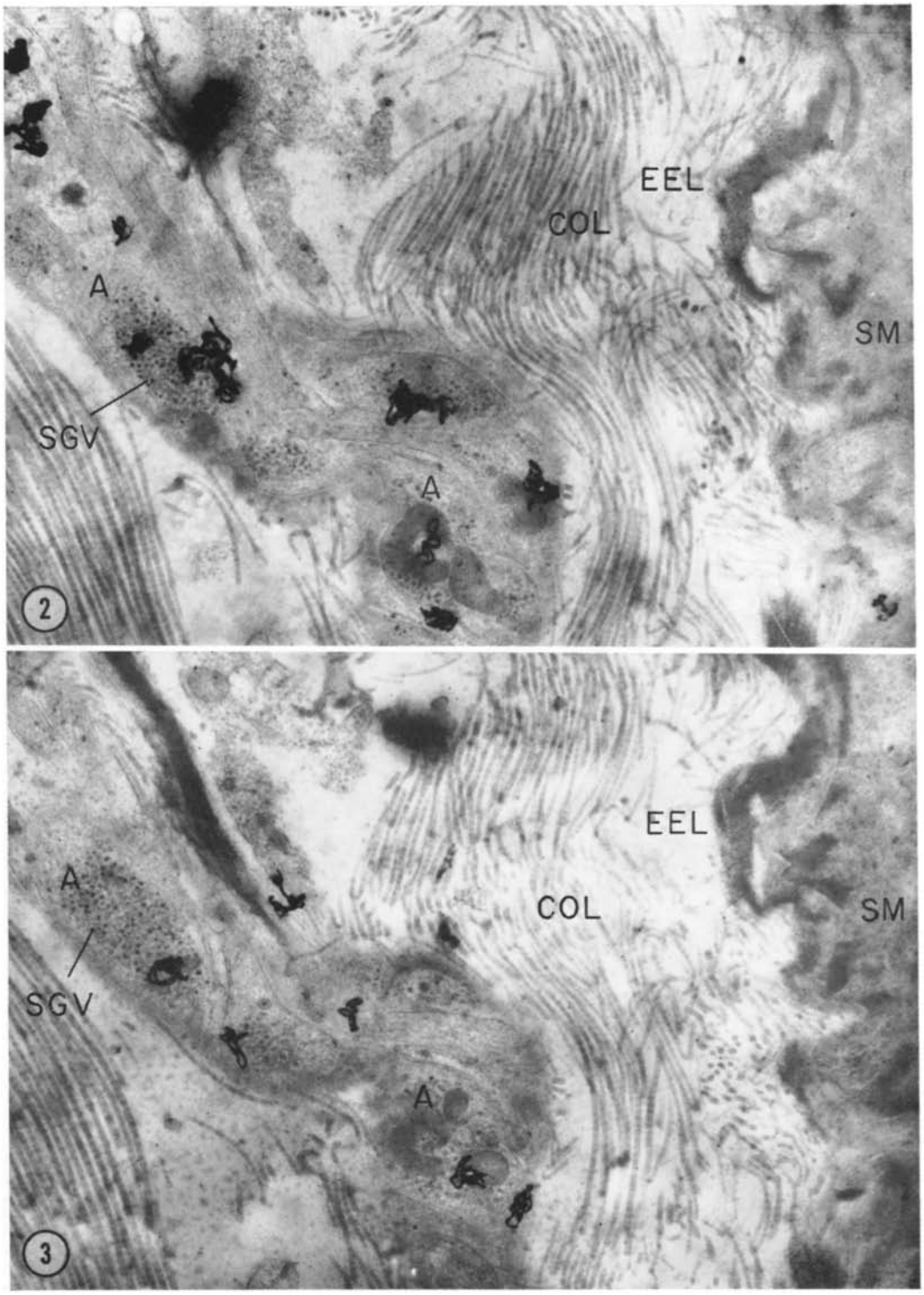
The concentration of NE in the mesentery, measured by fluorimetric assay, was 0.12 μg/g of wet tissue uncorrected for recovery of NE which was 80%. By measurement of the radioactivity, it was calculated that about 25% of the NE in the mesentery was derived from the infused NE-^{3}H.

DISCUSSION

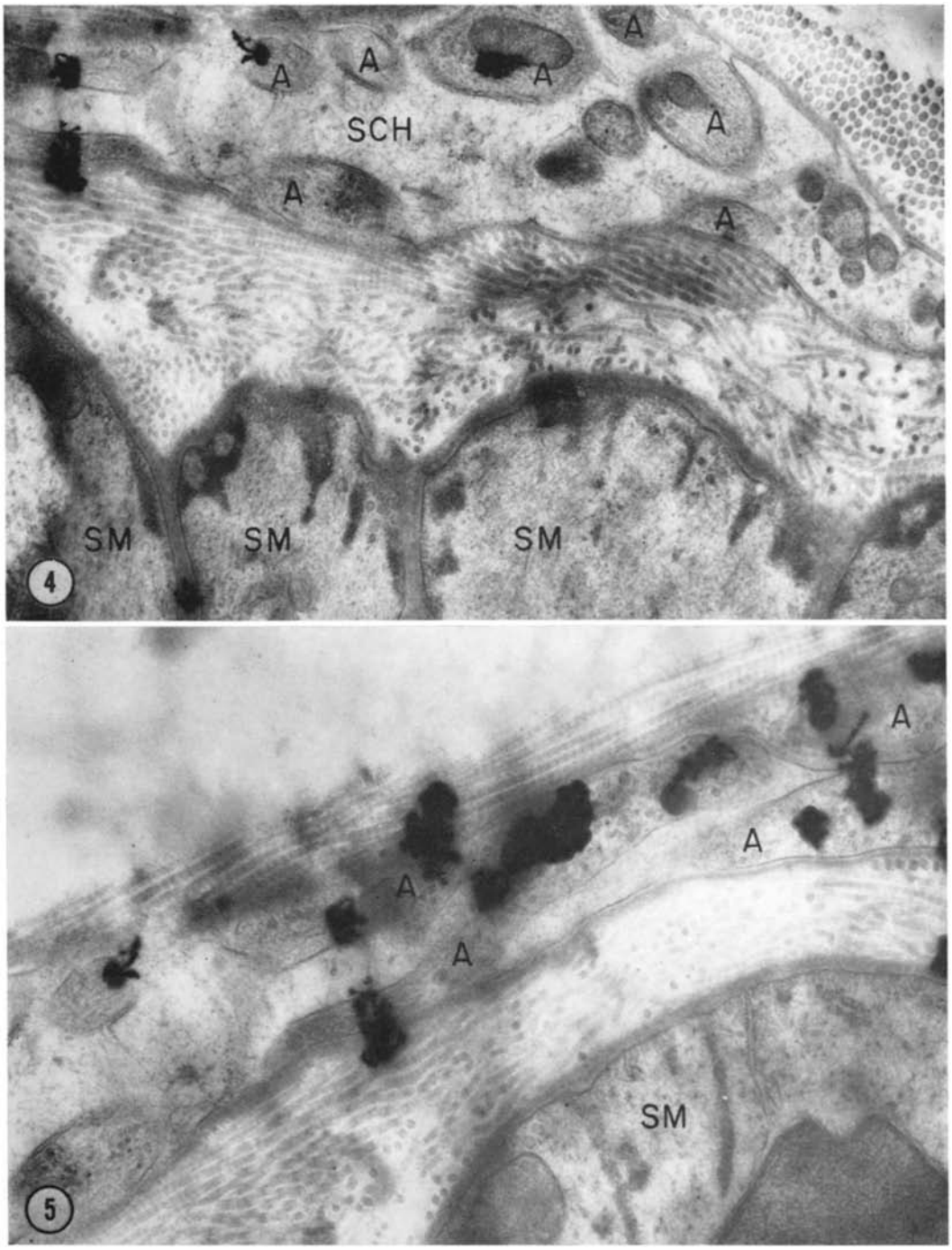
The present study has demonstrated that NE-^{3}H is taken up in vivo by the axons of the autonomic ground plexus around small blood vessels. Similar uptake has been demonstrated in the pineal body (Wolfe et al., 1962), hypothalamus (Aghajanian and Bloom, 1966, 1967), vas deferens and intestinal submucosa (Taxi and Droz, 1966), and brain (Lenn, 1967). A mechanism of peripheral uptake of NE into sympathetic axons (Rosell et al., 1963; Gillespie and Kirpekar, 1966 *a*) and nerve granules (von Euler and Lishajko, 1963; Stjärne, 1964) is, of course, a vital part of present day concepts of sympathetic function.

The present results, therefore, were predictable. They are, nevertheless, of interest in themselves, and they raise a number of points. For instance, the maximum time of exposure of the animals to the NE-^{3}H was 25 min, a time presumably too short to permit other than a direct uptake of the NE-^{3}H by the distal parts of the peripheral axons. Uptake of the NE-^{3}H by the nerve cell body and its subsequent passage down the axon would take several hours, according to the calculations of Dahlström and Häggendal (1966) who estimated that axonal flow proceeds at only a few millimeters per hour.

While uptake of NE-^{3}H into the adventitia of vessels has been demonstrated by light microscopic radioautography (Gillespie and Kirpekar, 1966 *b*) and uptake of NE into the autonomic ground plexus after reserpine treatment has been demonstrated by fluorescence microscope studies (Malmfors, 1965), the present results show that the axons themselves are, in fact, the sites of uptake. The proportion of silver grains found over tissue other than axons was small, though all types of tissue present (smooth muscle, endothelium, fibroblasts, and collagen) had more silver grains than did areas of the embedding medium. Presumably these tissue components were able to bind the NE-^{3}H and thus prevent its removal by the fixation procedures. Aghajanian and Bloom (1967) have shown that only about



FIGURES 2 and 3 Two near consecutive sections of a longitudinally sectioned bundle of axons (*A*), almost devoid of Schwann cell, with silver grains overlying the small granular vesicles (*SGV*) in the varicosities. The axons are lying beside the smooth muscle cells (*SM*) of a small mesenteric artery at a region where the external elastic lamina (*EEL*) is deficient. *COL*, collagen. 10 wk exposure. × 19,500.



FIGURES 4 and 5 Parts of the same obliquely sectioned axon bundle lying close to several smooth muscle cells (*SM*) of an intestinal arteriole. Those axons (Fig. 4) which are almost completely embedded within the Schwann cell (*SCH*) do not have as many overlying silver grains as the more naked axons shown in Fig. 5. There is one silver grain lying between two smooth muscle cells, and another lies on the surface of a smooth muscle cell. 10 wk exposure. *A*, bundle of axons. Fig. 4, × 21,000; Fig. 5, × 26,000.

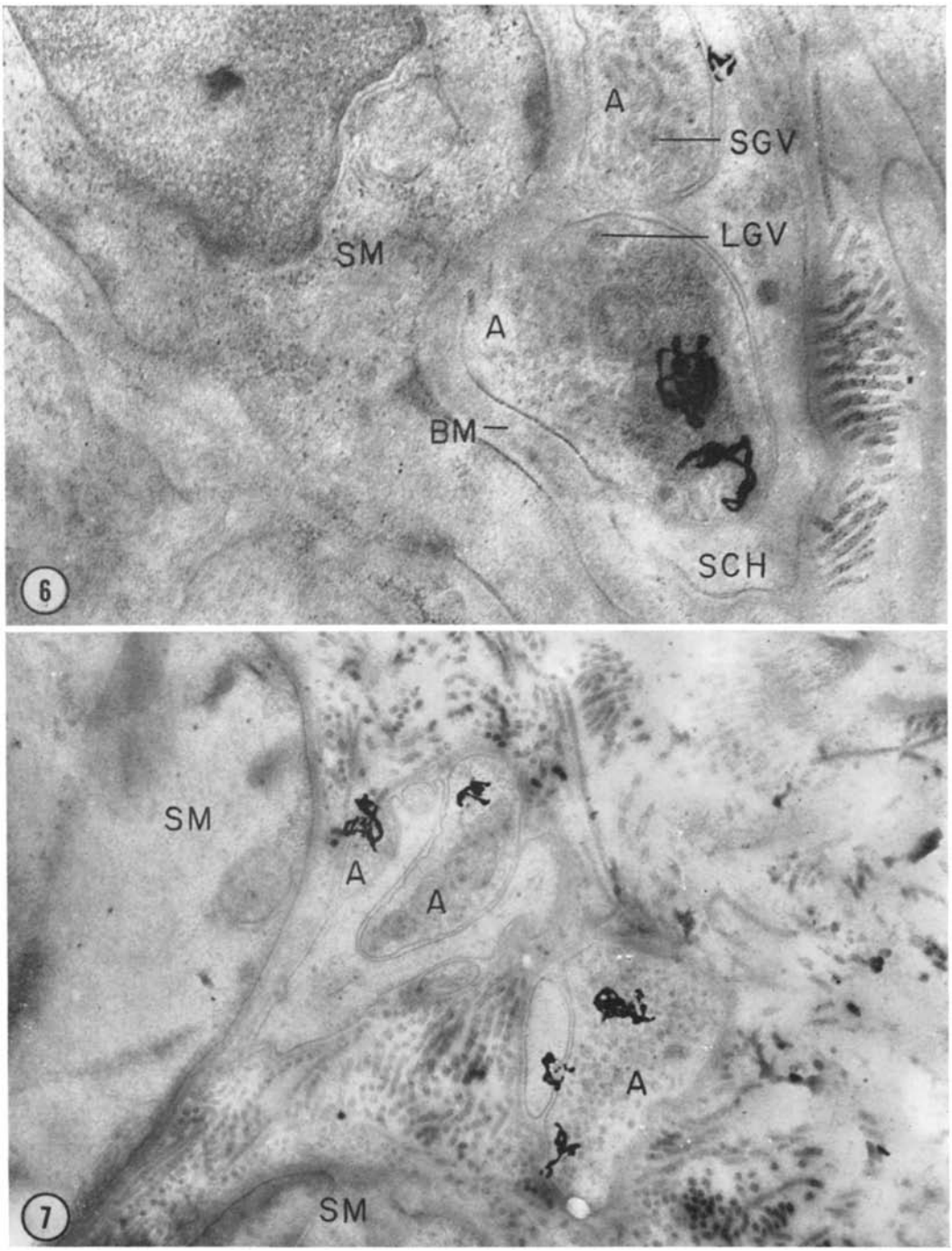


FIGURE 6 Two axons (*A*), partially devoid of Schwann cell sheath (*SCH*), lying close (1500 A) to a smooth muscle cell (*SM*) of an intestinal arteriole and separated from it only by basement membrane (*BM*). Silver grains are present over one of the axons and over the Schwann cell within 0.2 μ of the other axon. Both axons contain small granular vesicles (*SGV*), and one axon contains large granular vesicles (*LGV*). 6 wk exposure. × 38,000.

FIGURE 7 An axon bundle lying between two smooth muscle cells (*SM*) of an intestinal arteriole. Silver grains lie exclusively over the axons (*A*). 10 wk exposure. × 20,800.

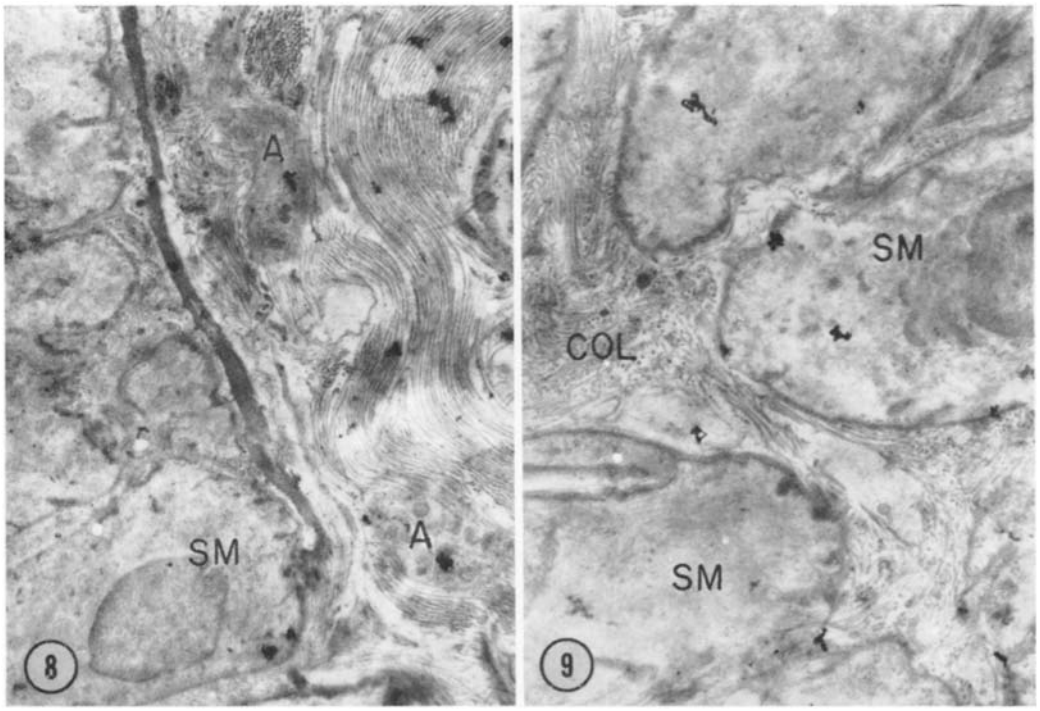


FIGURE 8 Low power survey picture of axon bundles (*A*) lying beside smooth muscle cells of a small mesenteric artery. Silver grains are lying over axons, a smooth muscle cell, external elastic lamina and collagen. 10 wk exposure. *SM*, smooth muscle cells. × 7,000.

FIGURE 9 An unusually large number of silver grains lie over smooth muscle cells of a small mesenteric artery. Some silver grains appear to be associated with the muscle membrane. 10 wk exposure. *COL*, collagen. × 10,000.

35% of the NE-^{3}H is retained in the final tissue after fixation. Clearly, then, the final radioautographic picture may be considerably modified by the degree of binding of NE by different tissues and is not necessarily a reliable indicator of the distribution of all the NE during life.

Little difference was found in the number of silver grains lying over smooth muscle cells of small mesenteric arteries, on the one hand, and of intestinal arterioles on the other. However, the uptake of NE into the axons appeared to be quantitatively different in the two types of blood vessel (Fig. 1), more silver grains lying over the axons in intestinal arterioles than over axons in small mesenteric arteries. This finding may merely reflect differences in effects of fixation and histological processing on the axons of the two types of vessel, although they were always processed simultaneously, or it may reflect a real difference. The distances between nerve and muscle previously observed in rat arterioles were smaller than in rat small mesenteric arteries (Devine and Simpson, 1967), and this difference could be associated with slight differences in the release and uptake of NE by the axons.

The question arises whether sites of NE uptake are necessarily also sites of NE release. It seems reasonable to suppose that a naked axon close to a smooth muscle cell (see Fig. 6) will be a site of both uptake and release of NE. However, uptake of NE can evidently occur in axons quite distant from vascular smooth muscle (see Figs. 4 and 5) or separated from smooth muscle by the external elastic lamina (Figs. 2 and 3). This observation suggests either that uptake is a more widespread phenomenon than release or that release of NE around blood vessels occurs in a rather diffuse manner.

In general, in the present study, the axons were well filled with small granular vesicles. The

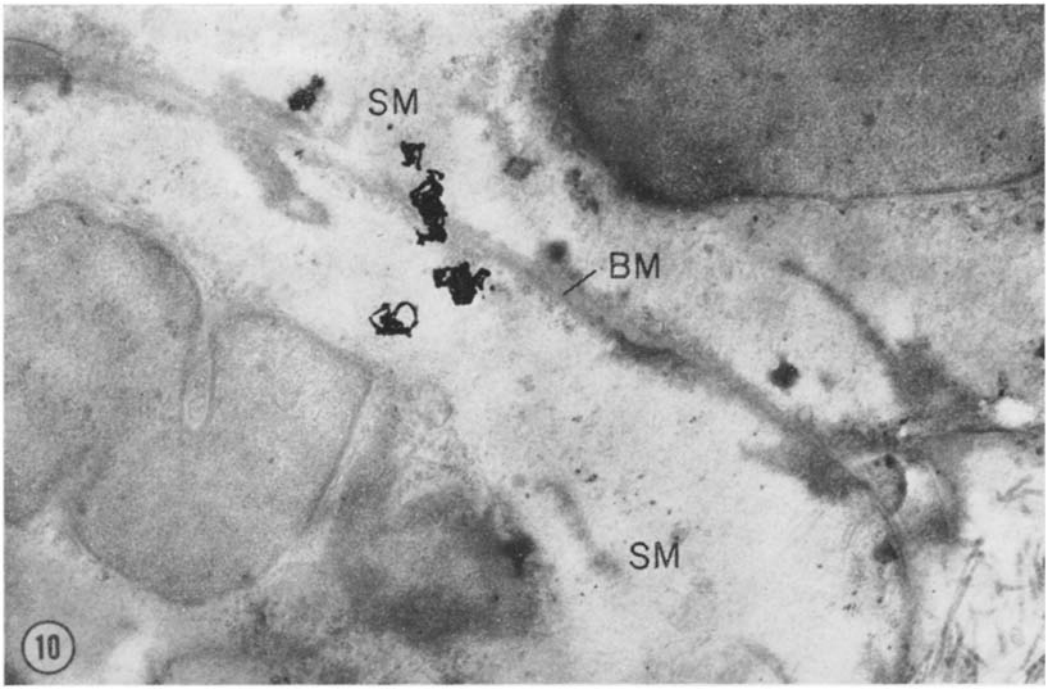


FIGURE 10 Silver grains overlying the basement membrane (*BM*) and adjacent portions of two smooth muscle cells (*SM*) of an intestinal arteriole. 10 wk exposure. × 19,200.

radioautographic resolution is, of course, insufficient to localize the tritium label to a given vesicle; with Ilford L4 nuclear research emulsion and 100-mμ sections, the resolution has been estimated to be of the order of 0.1 (Caro, 1962) or 0.185 μ (Bachman and Salpeter, 1965). However, the association of silver grains with granule-containing axons is sufficiently close to be taken as confirmation of an NE uptake and storage function for the small granular vesicles.

The large granular vesicles can also be seen in axons in the present material, but they are intermingled with the small granular vesicles; no evidence of their function has, therefore, emerged from the present work.

Rhodin (1967) has described invaginations into vascular smooth muscle cells of structures which he believes to be nerve endings. We have seen similar structures in the past (Devine and Simpson, 1968) and have believed them to be invaginations of one smooth muscle cell into another. In the present investigations we have not found any evidence to suggest that nerve endings capable of taking up NE exist as invaginations into smooth muscle cells.

The morphological evidence for specialized NE receptor areas in vascular smooth muscle is not yet definite (Devine and Simpson, 1967), though areas suggestive of such specialization can be found particularly in sheep vascular smooth muscle (Simpson and Devine, 1966). It is of interest that we have not found any consistently high concentrations of silver grains over smooth muscle at points of neuromuscular contact. Occasionally, however, several silver grains were grouped at the surface of a smooth muscle cell (Fig. 10), and it is tempting to think that a binding site for NE may have been present. Gillespie and Hamilton (1966) and Avakian and Gillespie (1968), using higher concentrations of NE in vitro than we have used for our in vivo studies, found that the NE fluorescence was not confined to localized regions of the smooth muscle membrane but was present throughout the whole length of the smooth muscle cell. However, the fact that all parts of the muscle cell can take up NE when it is present in high concentrations does

not necessarily mean that specialized receptor areas do not exist.

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