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The vesiculated axons in relation to arteriolar smooth muscle in the pancreas. A fine structural and quantitative study

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INTRODUCTION

Exact neurovascular relationships upon the walls of small arteries and arterioles are difficult to determine with the light microscope, and it is significant that no definitive nerve endings (boutons or other specializations) have been recognized upon arteriolar smooth muscle by this means. The first account of an electron microscopic study of presumptive vasomotor nerves is that of Lever & Esterhuizen (1961). Working on pancreatic arterioles they describe vesiculated axons lying in the adventitial coat which in places are not covered by Schwann cell processes and there separated from smooth muscle only by basement membrane and an interval which varied in width from 1000 to 4000 Å. More recently, Apenzeller (1964) has described a similar neurovascular interval of some 600–800 Å upon arterioles in the external ear of the rat. A closer neurovascular approximation than 600 Å has not so far been reported and there has been some hesitation in describing as nerve endings axons which remain at this or greater distance from their targets even though many of them are denuded of Schwann covering opposite the muscle and contain concentrations of microvesicles and mitochondria. It has already been suggested that quanta of transmitter substance are conveyed at nerve terminals within microvesicles both in cholinergic (De Robertis & Bennett, 1955) and in adrenergic nerves (Von Euler & Hillarp, 1956; Lever & Esterhuizen, 1961; De Robertis & De Iraldi, 1961).

Rosenblueth (1950) has mooted that diffusion of transmitter substance might occur over a considerable distance from adrenergic nerves to certain types of muscle and in their paper on the turtle atrium Fawcett & Selby (1958) have published micrographs depicting intervals of about 1500 Å between pre- and post-synaptic components at presumptive nerve terminals: they do not specify whether the terminals should be regarded as adrenergic or cholinergic.

In this paper we have shown that there is a significantly larger number of microvesicle contacts on the exposed than on the Schwann-covered aspect of axons at presumptive arteriolar nerve terminals (Lever & Esterhuizen, 1961; Lever & Graham, 1964). Moreover, we have observed membrane continuity between microvesicles and axolemma in some instances, an appearance which suggests strongly the probability of humoral release into the neuromuscular interval.

MATERIALS AND METHODS

For this investigation eight young adult guinea-pigs were selected at random from a pool of similar animals aged 6–8 months. Material for electron microscopy was obtained at laporotomy under ether anaesthesia, small specimens from the tail of the pancreas being fixed for 2 hr. in veronal acetate-buffered 1 % osmic acid solution and subsequently ethanol-dehydrated and embedded in methacrylate for fine sectioning. Sections exhibiting gold-silver interference colours by reflected light were stained on the grid by potassium plumbite (Lever, 1960) and photographed at a screen magnification of $\times 10^4$ in a Siemens electron microscope.

The appearance of the nerves in relation to arteriolar walls was studied and quantitative estimations (§§ 1 and 2 below) were made on certain axons in the electron photographs taken. Micrographs were printed in each instance to a final specimen magnification of 3×10^4. Axons were considered suitable for measurement only if: (*a*) part or all (see §§ 1 and 2 below) of their surface was denuded of Schwann cell covering, (*b*) this denuded surface was separated from arteriolar muscle by an interval not exceeding 4000 Å (usually 1000 Å) containing no more definitive structure than basement membrane, (*c*) their axolemmal membranes were well defined and therefore their length and the area enclosed by them were measurable.

(1) *Estimation of microvesicle population in axons selected by criteria a, b, c (above).* Cross-sectional areas of these axons were individually measured with a planimeter and a count of all microvesicles within every area was made. Microvesicles were each defined as being between 300 and 1000 Å in diameter (the vast majority measured between 300 and 500 Å), clearly bounded by a single membrane but containing no internal membranes. In all, fifty-nine axons from a total of eight animals were measured and for each axon a population figure of vesicles per unit area of axoplasm was obtained. In the present sense the area of axoplasm included everything contained by the axolemma and no deductions were made for space occupied by neuro-filaments or mitochondria. Some of the axons measured were completely naked of Schwann covering (see (*a*) above).

(2) *Estimation of microvesicle contacts on exposed and Schwann cell-covered aspects of the same axon.* Axons were selected by criteria *a*, *b*, *c* (above) but those completely denuded of Schwann covering were not measured since our objective was a comparison of vesicle contacts on exposed/covered aspects of the same axon. By means of a map measurer (opisometer) the lengths of (*a*) Schwann cell-covered, and (*b*) exposed surfaces of each selected axon were estimated. The number of microvesicles in direct contact or in communication with the axolemmal membrane on both its covered and exposed aspects was counted for every measured axon. Results were expressed as vesicles/unit length of surface covered or exposed. In all a total of forty-one axons was examined from seven animals. All statistical calculations were checked on a S.T.C. 'Zebra' computer.

RESULTS

Neurovascular alignment

There is generally no difficulty in locating arterioles and their accompanying nerves in any specimen of pancreas by means of the phase-contrast microscope before fine sections are cut. The nerves lie in the perivascular tissue spaces together with collagen and fibroblasts. At a fine structural level the well-known Schwann cell relationship to unmyelinated axons can be observed in these nerves—that is to say a number of axons are seen to be enwrapped by processes of individual Schwann cells (Pl. 1, fig. 1), each axon being suspended by mesaxonal invaginations of Schwann plasma membrane (Hess, 1956).

Larger order nerves may be composed of a number of Schwann-axon bundles each containing as many as three dozen axons per Schwann cell cross-section. These nerves are located at 2–3μ or more from arteriolar wall: in general the larger the nerve the farther is it from the wall.

One or more perineural sheaths consisting of flat epithelial-like cells were found around the larger nerve bundles. Between nerve bundle and sheath and between individual layers of the sheath, collagen may be found as Shanthaveerappa, Hope & Bourne (1963) have described for other peripheral nerves. Similar type sheath cells may separate Schwann-axon bundles from arteriolar walls (Pl. 1, fig. 1) and it sometimes appears that the arterioles themselves may be invested by incomplete sheaths of these cells lying as near as 100 mμ or as far as 1 μ from their walls (Pl. 1, fig. 1 and Pl. 3, fig. 3). Progressing nearer the vessel wall individual Schwann-axon bundles penetrate within this perivascular sheath and thereafter lie in the arteriolar adventitia: axons in these bundles may number between 1 and 24 (usually about 6). The intervals between muscle cells on their adventitial aspect are often much wider than the intermuscular (1000 Å) spaces in the media and not uncommonly Schwann-axon bundles or individual naked axons, although remaining in the adventitia, are recessed into these intervals in relation to more than one muscle cell (Pl. 4, fig. 4). *No nervous penetration of the media has been observed and the ultimate neurovascular relationships occur across the medio-adventitial border.*

Axon-muscle relationships

In the present series the closest observed approach of neurites to muscle is 830 Å (Pl. 4, fig. 4) but greater distances are frequently encountered between smooth muscle plasma membrane and axons naked of Schwann covering (Pl. 1, fig. 1, Pl. 2, fig. 2, Pl. 3, fig. 3 and Pl. 6, fig. 6). In Pl. 1, fig. 1, uncovered axons are seen to separate from smooth muscle by intervals of a micron or more containing sheath cell processes as well as basement membrane. The closest approach of nerve to muscle is only possible once the Schwann-axon bundles have penetrated beyond this perivascular sheath (Pl. 4, fig. 4, and Pl. 5, fig. 5). In this most intimate relationship nerve and muscle are separated by an interval as narrow as 830 Å or as wide as 4000 Å. Basement membrane lines both nervous and muscular sides of the interval but may also be present between these linings and it often appears continuous from side to side across the neuromuscular space when this is as narrow as 1000 Å ($\pm$50 Å) (Pl. 4, fig. 4).

In the single sections it is of course not possible to estimate what proportion of the surface of a terminal axon is denuded of Schwann covering and at the same time is taking part in the kind of close neuromuscular relationship just described. Photographs such as Pl. 2, fig. 2, suggest that such naked areas may be extensive. Indeed it is never difficult, given the correct field, to locate denuded axons in intimate relationship to arteriolar smooth muscle and not infrequently individual axons in this situation can be seen to lack Schwann covering around their whole circumference.

Because of the probability that an axon may be intimately and significantly related to arteriolar muscle over a large part of its (axonal) surface it was thought advisable to refer to 'nerve terminal areas' rather than use the term 'nerve endings' in the present context. It is clear that individual axons may possess nerve terminal areas in relation to more than one muscle cell (Pl. 4, fig. 4) and it is also apparent (Pl. 2, fig. 2) that individual muscle cells may be closely related to terminal areas on more than one axon.

Characteristic features in terminal and near terminal axons

In all but the largest nerves—i.e. those lying at several microns distance from arteriolar walls—the following inclusions were found, in greater or lesser concentration, to be characteristic of terminal or near terminal axons (Pl. 3, fig. 3, Pl. 4, fig. 4, Pl. 5, fig. 5 and Pl. 6, fig. 6): (1) Clusters of two or more mitochondria. (2) Microvesicles ranging in size from 300 to 1000 Å overall diameter: there are two size categories of these, a larger-sized vesicle—10 % of the total number—measuring 600–1000 Å and a more numerous but smaller-sized vesicle of 300–500 Å (Pl. 3, fig. 3 and Pl. 6, fig. 6). All microvesicles are individually bounded by single membranes but have no internal membranes (see earlier definition in Materials and Methods).

Most of the larger vesicles show complete fillings of a finely granular material of greater electron density than the surrounding axoplasm. On the other hand, the smaller vesicles (measuring 300–500 Å) are more variable in content. Some contain dense osmiophilic centres surrounded by halos of less dense material or halos of no discernible density which extend out to the enclosing membrane (Pl. 6, fig. 6). Others are less distinctive, possessing no definitive core or centre but having an amorphous material content of an electron density equal to or very slightly more than that of the surrounding axoplasm. In some axon cross-sections (Pl. 2, fig. 2) all of the smaller range vesicles may be of this less distinctive type, while in other axons (Pl. 6, fig. 6) they may be a mixture of vesicles: some with obvious dense centres and others, of the less distinctive type, with an overall content of only moderate electron density. Only very rarely do the microvesicles in any one axon all contain granular centres.

Attempts to enumerate granulated vesicles of the 300–500 Å range proved unrewarding since although those with obvious cores could be recognized easily there were innumerable vesicle sections with a granule content so inconspicuous as to be virtually unidentifiable. Apropos such an appraisal it is worth noting that granulated vesicles would appear agranular, or only fractionally granular, in sections passing to one side of, rather than through, their central cores. We have therefore been unable to make accurate estimates of the ratio granulated/non-granulated vesicles. It was however possible to record the overall population of microvesicles in terminal axons as well as their disposition within the axon.

Microvesicle population in terminal axons. It is worth emphasizing here that the figures given in Table 1 refer to total vesicle counts regardless of size range.

From Table 1 it is clear that there is wide variation in the microvesicle population of terminal axons not only from one animal to another but also between comparable axons within the same animal.

Disposition of microvesicles in terminal axons. Table 2 presents evidence that in terminal axons (as defined in Materials and Methods) there are significantly more microvesicles in contact or direct communication with the plasma membrane on the uncovered aspect, facing towards the arteriolar smooth muscle, than on the aspect

Table 1. *Counts of the vesicles per unit area of axoplasm in terminal axons*

Animals	No. of samples $= n$	No. of vesicles per unit area $\bar{x}$	No. of vesicles per unit area S.D.
1	12	6·34	3·14
2	6	4·43	0·92
3	12	7·54	4·70
4	12	4·88	2·08
5	7	6·30	2·40
6	3	4·43	2·47
7	4	9·00	2·77
8	3	6·70	1·49
Totals	59	6·19	3·29

Table 2. *Comparisons of vesicle contacts with covered and exposed surfaces of terminal axons*

Animal	No. of readings $= n$	Means of vesicle contacts per unit length axolemma (*a*) Covered	Means of vesicle contacts per unit length axolemma (*b*) Exposed	Differences (*a*)-(*b*) Mean	Differences (*a*)-(*b*) S.D.	Differences (*a*)-(*b*) T	P
1	7	1·63 (S.D. *0·93*)	2·09 (S.D. *1·35*)	1·11	1·16	2·35	$0{\cdot}05 < P < 0{\cdot}10$
2	5	1·41 (S.D. *0·55*)	3·20 (S.D. *1·37*)	1·79	1·23	2·75	$0{\cdot}05 < P < 0{\cdot}10$
3	7	2·07 (S.D. *1·26*)	4·33 (S.D. *2·87*)	2·89	2·46	2·87	$0{\cdot}02 < P < 0{\cdot}05$
4	9	1·36 (S.D. *0·65*)	1·94 (S.D. *1·25*)	1·03	0·81	3·63	$0{\cdot}002 < P < 0{\cdot}01$
5	6	1·48 (S.D. *0·53*)	2·68 (S.D. *1·37*)	1·43	1·15	2·78	$0{\cdot}02 < P < 0{\cdot}05$
6	4	1·00 (S.D. *0·19*)	1·98 (S.D. *0·93*)	0·98	0·96	1·75	$0{\cdot}10 < P < 0{\cdot}20$
7	3	1·90 (S.D. *0·99*)	3·40 (S.D. *0·43*)	1·50	0·94	2·25	$0{\cdot}10 < P < 0{\cdot}20$
Totals	41	1·56 (S.D. *0·87*)	2·75 (S.D. *1·85*)	1·54	1·55	6·30	$P < 0{\cdot}001$

which has a Schwann cell covering. (See earlier sections for description of Schwann-axon and neuromuscular relationships.) This result is unequivocal when comparisons are made for the group as a whole (i.e. $P < 0{\cdot}001$ for forty-one axons from seven animals), although it can be seen that for some individual animals (1, 2, 6 and 7) there is a 10% probability that the differences in the means could be accounted for by chance: the high individual values for P in these animals might be statistically explained by the corresponding low values for n (Table 2).

A 'streaming' or crowding of microvesicles towards the uncovered surface is often seen as a characteristic feature of terminal axons (Pl. 3, fig. 3, Pl. 4, fig. 4 and Pl. 5, fig. 5). Membrane communications (Pl. 2, fig. 2*a*) between axolemma and vesicles of both large (600–1000 Å) and small (300–500 Å) size ranges are sufficiently often found at these situations to be described as not uncommon, but sometimes they are difficult to identify, being often obscured in electron micrographs within areas of increased osmiophilia extending across the axolemma (Pl. 4, fig. 4 and Pl. 5, fig. 5). These areas of increased density are not easy to define or to describe: they include a strip of axoplasm perhaps 300–400 Å wide immediately adjacent to the plasma membrane, the plasma membrane itself and its overlying basement membrane. They may be as much as 3000 Å or less than 800 Å in length but often they are not well enough defined to be readily measured.

DISCUSSION

In a paper on the disposition of vesiculated nerve processes in smooth muscle, Thaemert (1964) comments that intimate neuromuscular relationships on arteriolar walls 'seem to be very rare' and he further adds that because the interval between axon and muscle at these related areas is as much as 770 Å they may not constitute functional neuromuscular junctions. As previously mentioned Appenzeller (1964) has described the nearest approach of nerve processes to muscle on arteriolar walls as 600 Å and, from the material examined in the present investigation, an interval of 820–1000 Å is reported. In spite of the divergence of these figures, which might be accounted for by differences of embedding procedure and optical variations in electron microscopes between one laboratory and another, they provide evidence from three independent sources that the closest relationship of nerve to muscle on arteriolar walls is across a space about three times wider than the typical intra-synaptic space found at myoneural junctions in skeletal muscle (Birks, Huxley & Katz, 1960) and at other cholinergic nerve endings.

One of the most noteworthy findings in the present investigation has been that individual arteriolar nerves may have an intimate and significant relationship to muscle over relatively extensive terminal areas. At these areas axons are denuded of Schwann covering and separated from muscle by basement membrane within an interval varying from 830 to 4000 Å. It is also clear (Pl. 4, fig. 4) that more than one muscle cell may be so served by a single axon, a finding which was made earlier by Richardson (1962) in describing neuromuscular relationships in the vas deferens. Furthermore, several axons may serve individual muscle cells (Pl. 2, fig. 2). It is inappropriate therefore to refer to nerve 'endings' on arteriolar walls; instead those parts of the axon bearing the intimate relationships with smooth muscle described in this paper should be referred to as 'nerve terminal areas'. That these nerve terminal areas are synaptic in nature cannot of course be proved by this investigation, but the finding of more microvesicles in surface contact or *communication* (Pl. 2, fig. 2*a*) at these areas than elsewhere upon the axolemmal surface supports this concept provided that microvesicles are regarded as purveyors of or in some way connected with the transmitter substance.

Although arteriolar nerves in the splanchnic region are most likely sympathetic in nature on morphological evidence (Mitchell, 1953), proof of their identity has not been provided in this investigation and it would therefore be unwise to do more than speculate upon the nature of the material contained within the microvesicles at their terminals. Von Euler & Hillarp (1956) and Von Euler & Heller (1963) have shown that in suspensions from homogenates of adrenergic (splenic) nerves, noradrenaline is present in a granule-bound form. They further suggest that each of these granules is covered by a membrane since rapid release of noradrenaline into free solution occurs when granule suspensions are treated with detergents. The present demonstration of microvesicles and their contained material within terminal axons would appear to be directly complementary to Von Euler's findings more especially as we have also shown a significant accumulation of microvesicles upon the free surface of these axons most nearly related to smooth muscle.

SUMMARY

1. The fine structure of presumptive post-ganglionic sympathetic nerves in close relation to smooth muscle cells of arterioles in the pancreas of the guinea-pig is described.

2. No nervous penetration of the media has been observed and the ultimate neuromuscular relationships occur across the medio-adventitial border.

3. Variable lengths (areas) of axon, denuded of Schwann cell covering, are intimately related to smooth muscle across an interval measuring 830–4000 Å and containing basement membrane.

4. Regions of most intimate neuromuscular relationship are referred to as 'nerve terminal areas' rather than 'nerve endings'.

5. One axon may be so related to more than one smooth muscle cell and individual muscle cells can be so related to more than one axon.

6. The axoplasm of terminal axons is characterized by gatherings of mitochondria and microvesicles. There are two sizes of vesicle: large (10% of the number) 600–1000 Å d: small 300–500 Å d. Both contain variable amounts of electron dense material. In some of the smaller vesicles this may appear as a dense central granule surrounded by a halo of less dense material.

7. In terminal axons streaming of the vesicles towards the uncovered surface of the neurite can be seen; contact and apparent continuity with the axolemmal membrane is also revealed.

8. A count of the number of vesicles per unit area of 'terminal' axoplasm was made in fifty-nine axons from eight animals. There is a wide variation in this population within each animal and between animals.

9. There is a greater number of vesicles in contact or continuity per unit length of axolemmal membrane on the naked than on the Schwann cell-covered side of terminal axons. This difference is statistically significant.

10. It is postulated that the 'nerve terminal areas' are sites of humoral release.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Nerve bundles in relation to sheath cells (sh) near an arteriolar wall. Note: arteriolar muscle (M); Schwann cytoplasm (S); axons (A); collagen (C). The axon A1 contains a concentration of microvesicles (mv) and a gathering of mitochondria (mt) and is denuded of Schwann covering over most of its surface: the denuded area is covered only by basement membrane (b) but is separated by an interval of some $0{\cdot}6\,\mu$ from muscle surface (↔). The denuded axon A2 is separated from muscle by sheath cells and a distance of $2{\cdot}3\,\mu$. ×30,000.

PLATE 2

Fig. 2. Schwann-axon bundle arching around an arteriolar muscle cell (M). The bundle is separated from the muscle surface by an interval of variable width ($0{\cdot}2$–$0{\cdot}5\,\mu$, i.e. 2000–5000 Å) containing basement membrane (b). The long axon A1 has no Schwann covering over that part of its surface (...) facing directly towards the muscle but in the upper part of the micrograph it is seen to be more deeply incorporated in the Schwann-axon bundle. Note separate concentrations of microvesicles (V1, V2 and V3) within the same axon. Note also the diffuse area of increased electron density across the exposed surface of the axon A2. The single muscle cell (M) is related (across the neuromuscular interval) to the exposed surface of more than one axon (A1, A2, A3 and A4). ×35,000.

Fig. 2*a*. Communication between a microvesicle and the axolemmal membrane is encircled at ○. The axon surface is denuded of Schwann cytoplasm and separated from muscle cell (M) surface by an interval of 2800 Å containing basement membrane (b). ×35,000.

Plate 3

Fig. 3. Arteriolar muscle (M), sheath cell processes (SC) and an axon (A) denuded of Schwann cell covering over that aspect of its surface facing towards the vessel wall. On its other aspect the axon is covered by Schwann cell membranes (S). Within the axoplasm note mitochondria (mt), large-sized microvesicles (V1) and small-sized microvesicles (V2). In general the large vesicles are filled with a moderately dense granular material while the small vesicles may have a content of similar or slightly greater density than the surrounding axoplasm or a dense central core within a peripheral less dense area. Note crowding or streaming of microvesicles towards (↑) the exposed axolemma and the deficiency (⌶) in the cell sheath between axon and muscle. × 60,000.

Plate 4

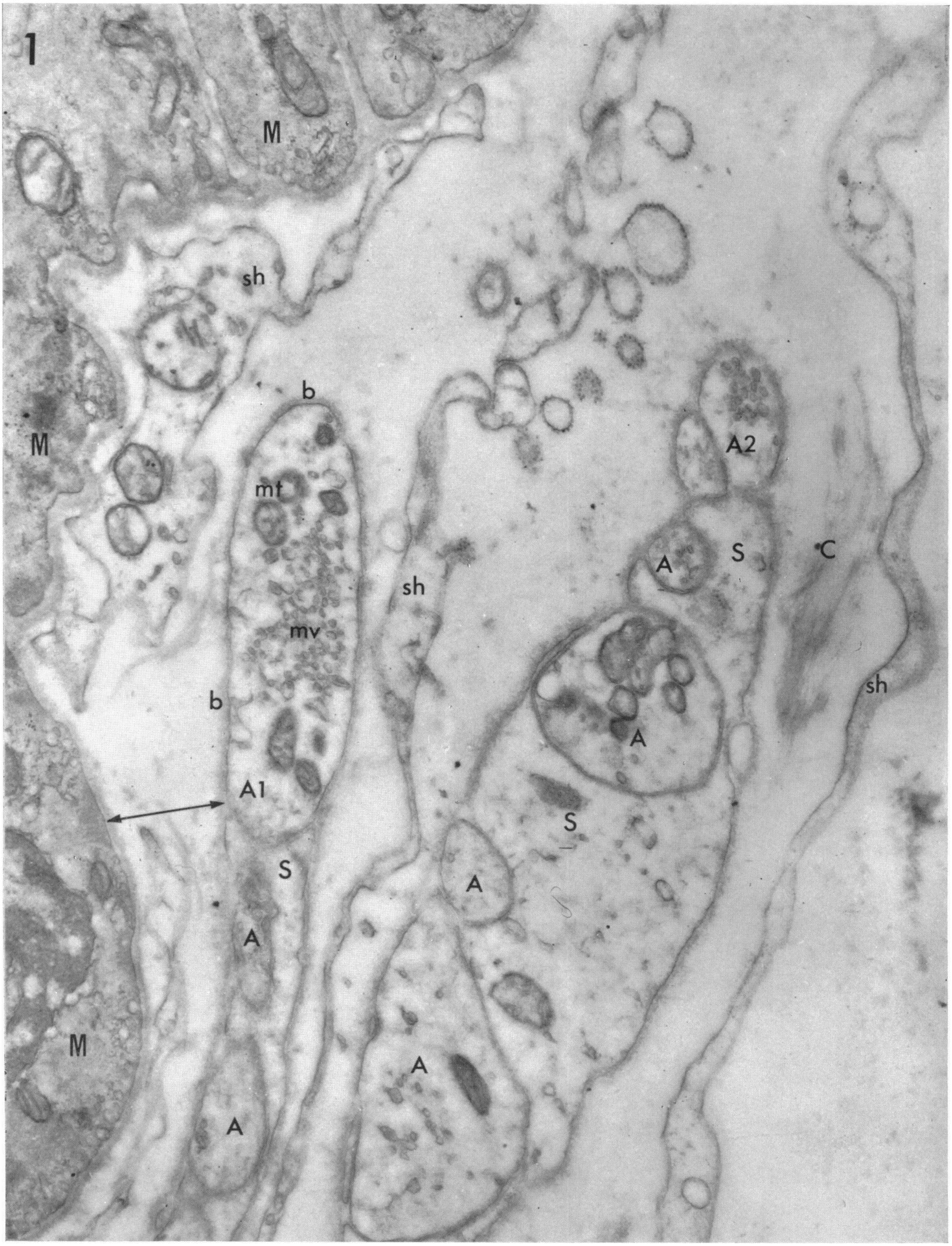
Fig. 4. A terminal axon (A) intimately related to two muscle cells (M1 and M2). At its narrowest the neuromuscular interval (↑) measures 830 Å and is bridged by basement membrane. In axons such as A there are significantly more microvesicles in contact or communication (see fig. 2*a*) with the axolemma on exposed than on Schwann cell-covered surfaces (S). Axolemmal-microvesicle contacts are particularly obvious in this micrograph over that part of the axon surface which is most closely apposed to the muscle. × 60,000.

Plate 5

Fig. 5. Terminal axons A1, A2, A3 and A4 in the adventitia of an arteriole are naked over a large part of their surfaces except for a covering of basement membrane (b). On these exposed axon surfaces, referred to in the text as 'nerve terminal areas', microvesicle contacts are numerous. Actual continuity of microvesicle with axolemma (see fig. 2*a*) is often obscured by a diffuse osmiophilia on both sides of the axolemma as at the areas indicated (↑). In this micrograph the narrowest neuromuscular interval is 2800 Å. Note sheath cell process (sc); also several large-sized microvesicles in A4 and A1 (see description in fig. 3). × 60,000.

Plate 6

Fig. 6. A long expanded axon lying in the adventitia of an arteriole. The surface of the axon towards the arteriolar muscle (M) is for the most part covered by Schwann cell membranes (S) but a nerve terminal area covered only by basement membrane can also be seen (↑). At this site several microvesicles with dense central cores are in close proximity to the axolemma which is indistinctly visualized because of an area of increased osmiophilia (see also ↑ fig. 5). This axon can properly be referred to as terminal and contains concentrations of the smaller-sized microvesicles many of which show a dense central granule surrounded by a halo of less dense material all contained within (individual) enclosing membranes (see also fig. 3). Neurofilaments (n) may also be observed. × 70,000.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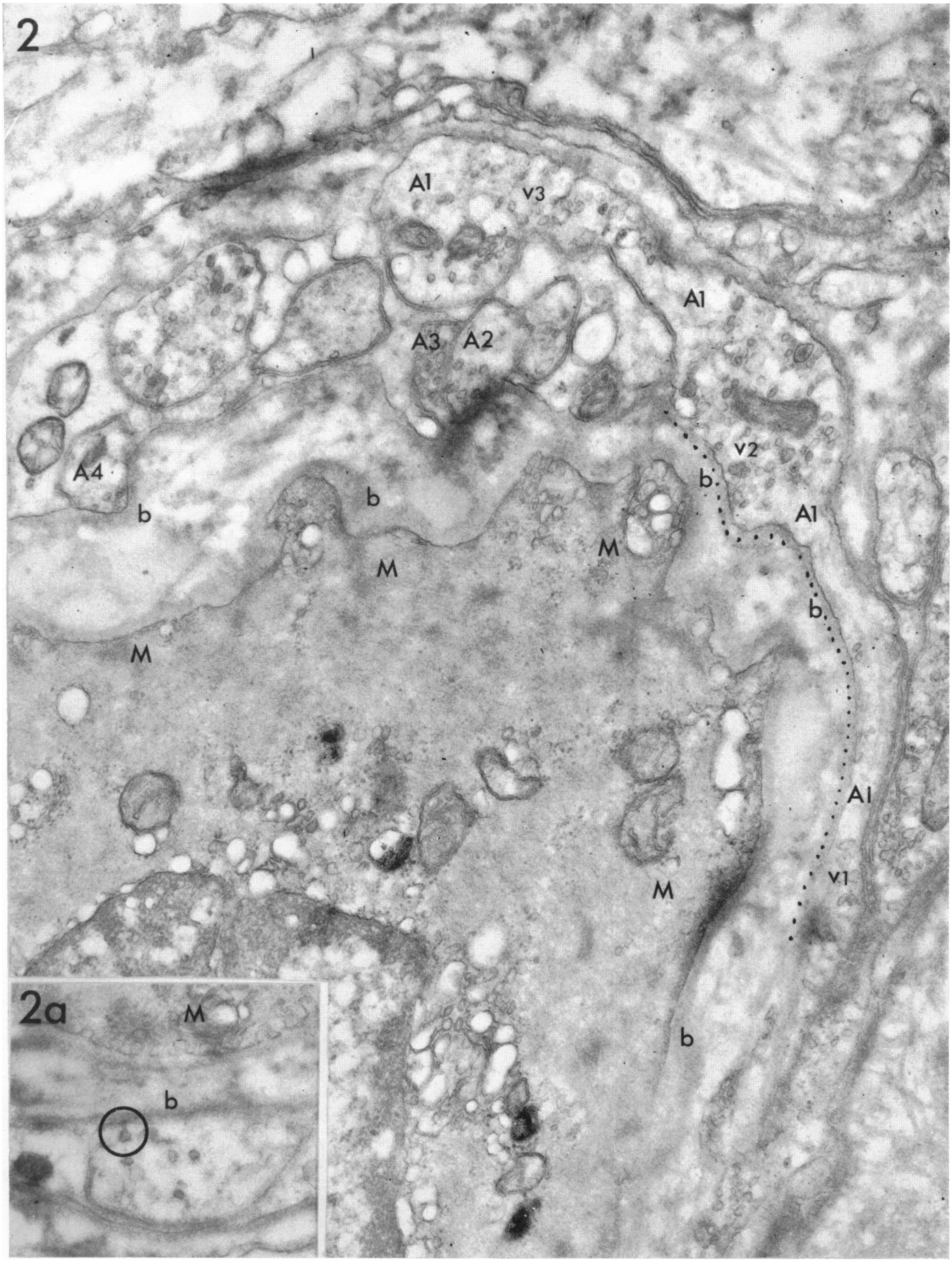
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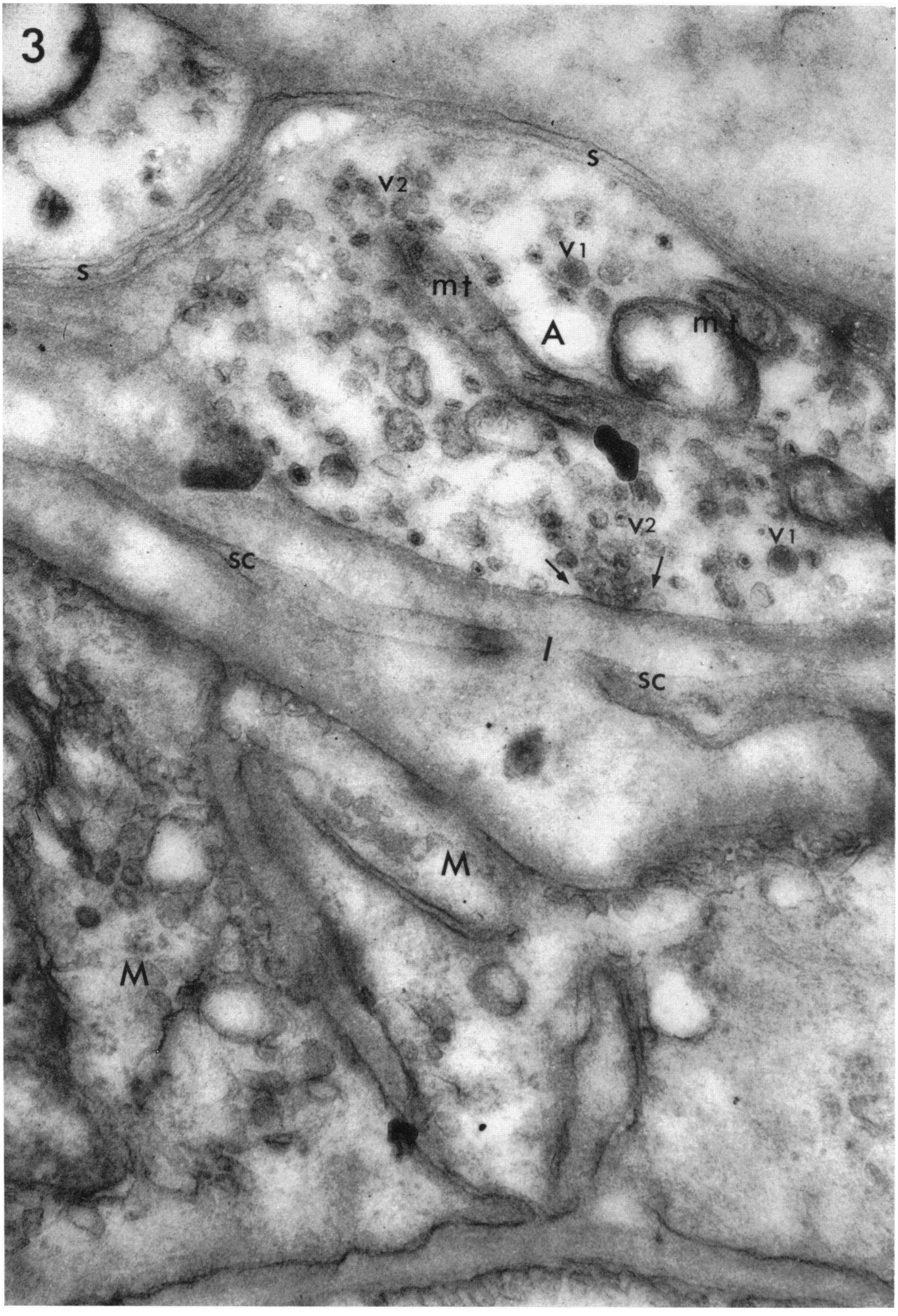
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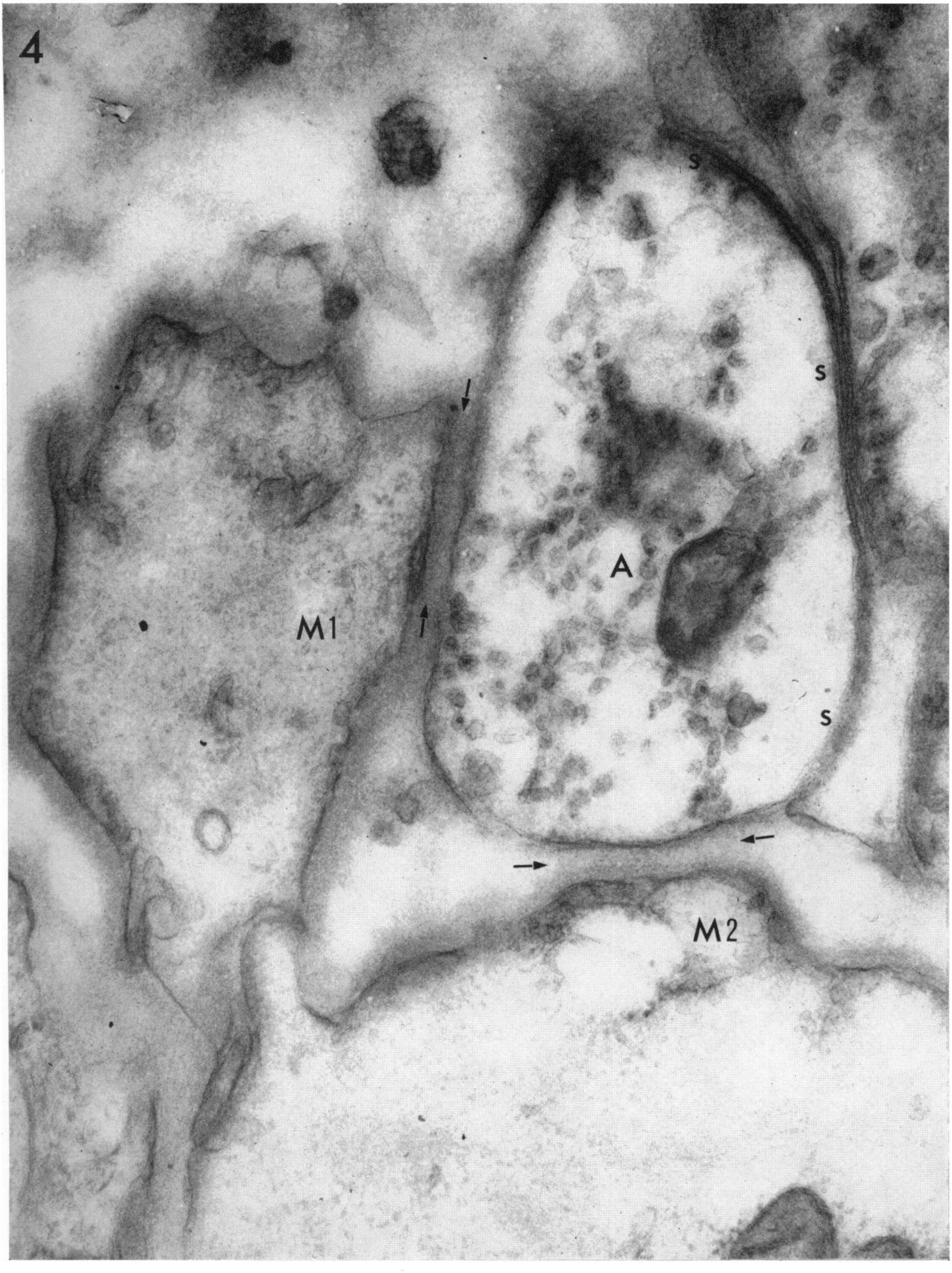
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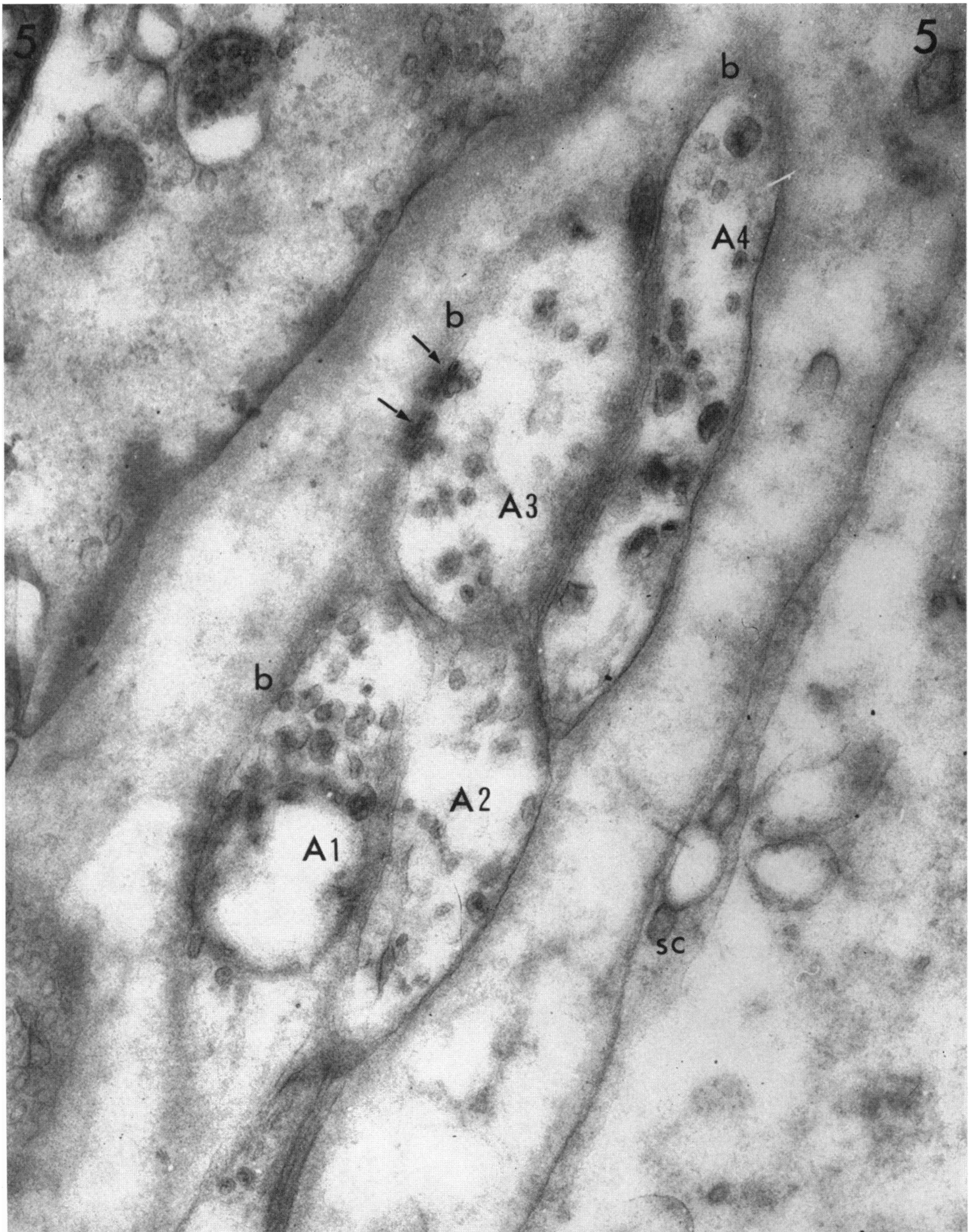
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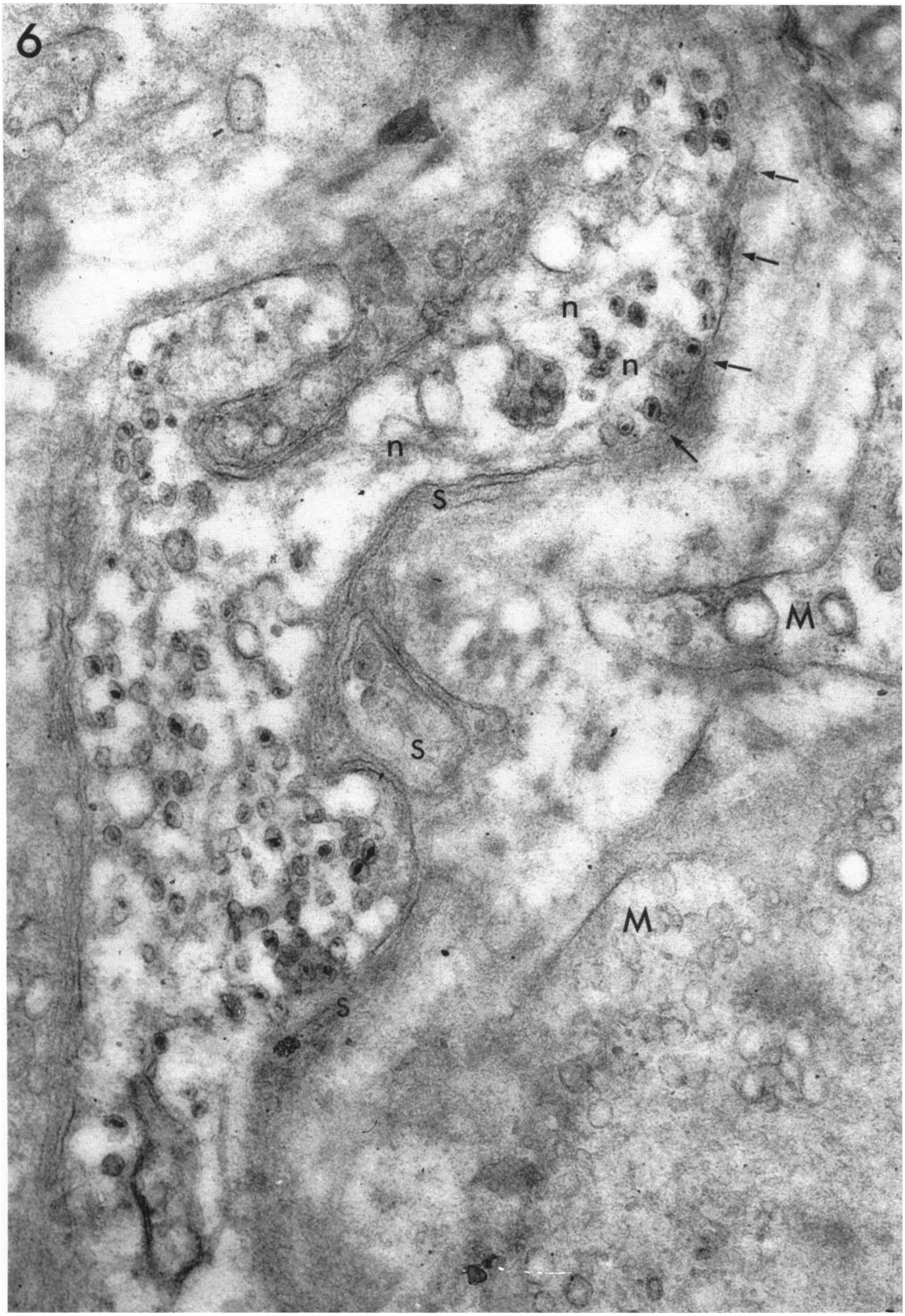
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