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THE BASIS FOR SILVER STAINING OF SYNAPSES OF THE MAMMALIAN SPINAL CORD: A LIGHT AND ELECTRON MICROSCOPE STUDY

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Neurofibrillae have been known to anatomists for many years (Schultze, 1862, 1871; Fromann, 1864). They can be stained by silver methods and often occur in axons, dendrites and nerve cell bodies. Also in some regions they mark the terminal parts of axons, where they form characteristic rings, clubs and reticulated structures, the classical 'boutons terminaux' (Cajal, 1911; Bartelmez & Hoerr, 1933; Bodian, 1937). Neurofilaments, fine osmiophilic threads 60–100 Å in diameter, have been described in electron micrographs by Schmitt & Green (1950), Fernández-Morán (1954), de Robertis & Bennet (1955) and Palay & Palade (1955). These authors have suggested that the neurofilaments form the basis of the neurofibrillae of light microscopists.

Boycott, Gray & Guillery (1960, 1961) have recently described neurofilamentous rings in the terminal bags in certain parts of the lizard brain, where neurofibrillar rings are found in great numbers. The present investigation was undertaken to determine whether similar neurofilamentous structures occur in the mammalian spinal cord, where the classical boutons are also plentiful. In addition, blocks stained with silver for terminal boutons by the classical methods of light microscopists have been examined by electron microscopy to determine whether silver is deposited on the neurofilaments and whether any other synaptic structures take up silver when such methods are used.

The presence of neurofilamentous rings in boutons was readily confirmed. The second question proved more difficult to answer, for it was impossible to obtain suitably fixed material for electron microscopy showing both neurofilaments and silver deposits in the same preparation. However, by a process of elimination other organelles could be excluded, leaving little doubt that, in the preparations used, the neurofilaments are in fact responsible for the silver deposits.

METHODS

Several difficulties were encountered. Obtaining good fixation of spinal cord for electron microscopy was found to be a major problem. Perfusion techniques and thin slicing of frozen cord before immersion in fixative both proved unsatisfactory. The method (*b*) described below was finally adopted, but it entails the risk of mechanical damage. Another difficulty was to obtain silver-stained material sufficiently well fixed for electron microscopy. The ideal method, silver staining of osmium-fixed material, proved impossible. Method (*c*) (below) proved the most effective, although it will be seen that the electron micrographs of this material are much inferior to those obtained by conventional methods of osmium fixation.

Light microscopy. (*a*) Cats and rats were perfused with formol saline and the cords were stored in formol saline for 10 days. Blocks about 5–7 mm across were then impregnated by Bielschowsky's pyridine block method (see Romeis, 1948). A part of each block was then cut as frozen sections and the remainder of those blocks that showed successful impregnation were cut in two. One part was embedded in paraffin for light microscopy, and the other used for electron microscopy (see *c*). Other blocks were prepared by the block silver method of Cajal (see Wyckoff & Young, 1956) for light microscopy only.

Electron microscopy. (*b*) Slices of rat and cat cord were placed in 1 % osmium tetroxide in saline, buffered at pH 7·4 with veronal acetate. The slices were immediately chopped into very small pieces (preferably less than 0·3 mm thick in any direction) with a razor blade. They were transferred to fresh fixative and maintained for 3 hr with continuous agitation at approximately 4° C. Ethanol was used for dehydration, followed by staining for 3 hr in 1 % phosphotungstic acid (PTA) in absolute alcohol. The pieces were finally embedded in Araldite for sectioning (see Gray, 1959).

(*c*) The Bielschowsky material (see *a*) was cut into 0·2 mm slices to ensure good penetration of the stain and subsequently of the embedding medium. They were prepared for electron microscopy by ethanol dehydration and PTA staining and were embedded in Araldite (see *b*).

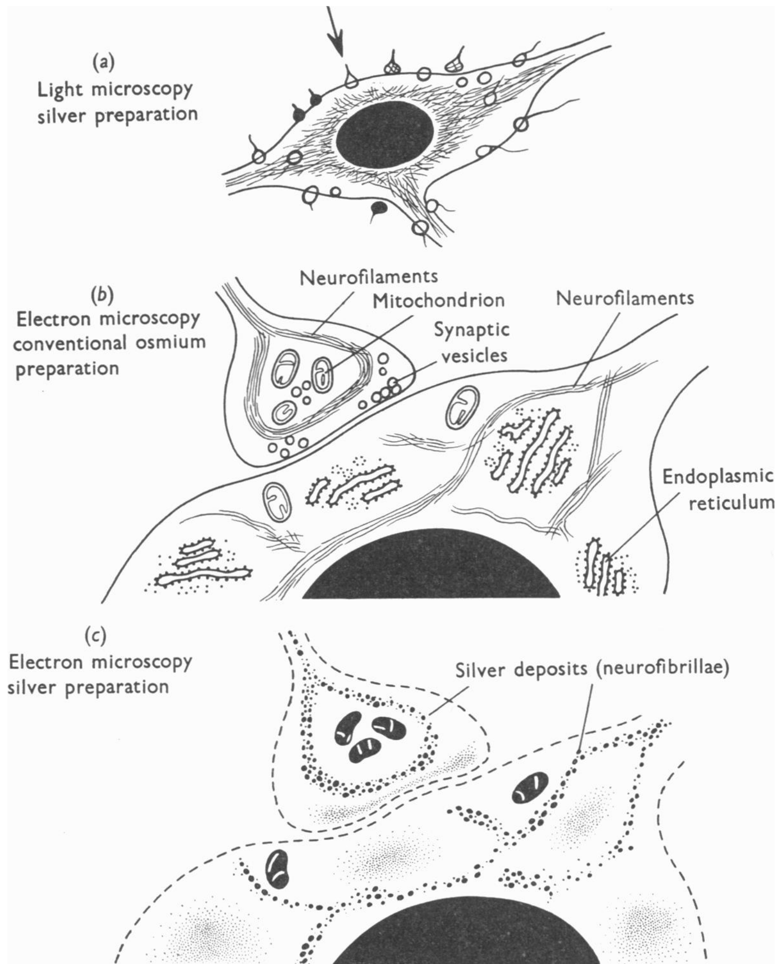
RESULTS

Electron microscopy of osmium-fixed material

The electron micrographs of the cat's spinal cord showed a number of terminal bags in which neurofilaments could be clearly observed (Text-fig. 1; Pl. 1; Pl. 2, fig. 1; Pl. 3, fig. 2). The terminal bags could be identified by their characteristic synaptic vesicles, about 500 Å in diameter, mitochondria and localized thickenings of the synaptic membranes. As far as can be determined by present techniques the neurofilaments of the terminal bag are structurally identical with the filaments that can be observed in myelinated axons (Pl. 1, nf_1), neuronal perikarya (see Palay & Palade, 1955) and some dendrites. Twenty to forty filaments are usually seen forming a bundle near the periphery of the synaptic bag. The cytoplasm containing the individual filaments is characteristically pale and free from synaptic vesicles. The bundles usually appear as profiles that can best be regarded as sections of rings or loops of filaments cut in various planes. A group of mitochondria occurs characteristically within the ring. Thus Pl. 1 shows most of the ring included in the section, whereas Pl. 3, fig. 2,

shows a ring cut radially and represented as two groups of dots, each dot being a sectioned filament.

Structurally these neurofilamentous rings are similar to the rings described in the lizard brain (Boycott *et al.* 1961) and it is reasonable to



Text-fig. 1. Diagrams of spinal cord synapses. In (*a*) ring-shaped, club-shaped (solid) and reticulated boutons are seen in silver preparations by light microscopy. The arrow indicates a ring bouton selected for illustration in (*b*) and (*c*) by electron microscopy.

suppose that in both situations the neurofilaments (or the pale cytoplasm in which they lie) take up silver stains to give the characteristic picture of ring-shaped terminal boutons (Pl. 5, fig. 1). Occasionally the filaments form an irregular network within the terminal bag (Pl. 2, fig. 1). It is

probable that such structures would appear as the reticulated or club-shaped endings of light microscopists (Text-fig. 1; Pl. 5, fig. 1).

The rat material that has been examined showed fewer neurofilaments in the terminal bags. Occasional endings with filaments could be found (Pl. 2, fig. 2). The pale cytoplasm (*pc*) around the filaments is clearly shown, but the number of filaments is markedly lower than that shown in the cat material. These observations by electron microscopy support the findings by light microscopy that silver preparations of rat cord generally show fewer rings than do similar preparations of cat cord (Phalen & Davenport, 1937).

Electron microscopy of silver-stained material

Silver preparations of spinal cord seen with the light microscope show the well known annular, club or reticulated boutons (Text-fig. 1). Plate 4, fig, 2, and Pl. 5, fig. 1, are taken from a block Cajal preparation, and show these structures clearly. The Bielschowsky material generally showed the boutons less intensely impregnated, but the morphology of the boutons was the same. In both types of silver preparation only some of the boutons appear continuous with the neurofibrillae of the preterminal axons. The cell bodies contain pale nuclei and these are surrounded by coarse or fine neuro-fibrillar aggregates (Pl. 5, figs. 1 and 2), which sometimes extend into the basal region of the dendrites.

Examination of ultra-thin sections of this silver-stained material by electron microscopy presents a quite different picture because (*a*) of greatly increased magnification and (*b*) many structures that are invisible by light microscopy can be observed because of their electron dense properties. Plate 4, fig. 1, is a low power electron micrograph of silver-stained material. Membrane structures are seen to be poorly preserved, but comparison with micrographs of the conventional osmium-fixed material (see above) makes the general organization readily understandable. In Pl. 4, fig. 1, the neuronal fibre plexus is seen (above) with a part of the perikaryon of a large ventral horn neurone with a row of boutons (*b*) at its surface (below). The silver deposits appear as dark strands or irregular masses that at higher magnifications (Pl. 3, fig. 1; Pl. 5, fig. 3) are seen to consist of discrete electron-dense particles, from 20–150 Å in diameter. In the fibre plexus (Pl. 4, fig. 1, and Pl. 3, figs. 1 and 3, at higher magnification) the silver can be clearly seen lying within the axoplasm and not in the myelin sheath (see Peters, 1955).

There are only two commonly occurring organelles in osmium preparations of myelinated axons of the spinal cord. They are the neurofilaments and the mitochondria (Pl. 1, nf_1 and m_1). In silver preparations the mitochondria appear as vague dense bodies, but they can nevertheless be

identified by their cristae. They show no impregnation at all (Pl. 3, figs. 1, 3, and Pl. 4, fig. 1). It thus appears probable that the silver is deposited upon the only other common known differentiated part of the axon, the neurofilaments (compare Meyer, 1957).

Observations on the nerve cell body confirm this. Fine or coarse strands of silver particles can be seen ramifying through the cytoplasm (Pl. 4, fig. 1, and Pl. 5, fig. 3). In electron micrographs of osmium-fixed material the neurofilaments show a similar distribution in the cell body (Palay & Palade, 1955). Again, the mitochondria of these regions appear entirely non-argyrophilic.

Finally, in Pl. 4, fig. 1, a row of boutons (*b*) is shown in contact with a nerve cell body. Two boutons are shown at higher magnification in Pl. 5, fig. 3. It is clear that neither the bouton cytoplasm, nor its surface membrane or mitochondria are argyrophilic. The synaptic vesicles, since they are membrane structures, are poorly preserved in the silvered material. A vague non-argyrophilic mass (*sv*?) can be seen near the synaptic membrane (in Pl. 5, fig. 3, for example). This mass might represent synaptic vesicles, but since rather similar masses appear in perikaryal cytoplasm (Pl. 4, fig. 1) it is more likely to be protein material precipitated by the formalin fixation. However, axosomatic boutons of mammalian cord invariably contain aggregates of synaptic vesicles, as is shown by osmium-fixed material (compare Pl. 3, fig. 2), while many boutons in the electron micrographs of silver preparations contain no silver particles in sufficient aggregations to be within the limits of resolution of light microscopy. So there can be little doubt that the synaptic vesicles are not responsible for the argyrophilia within a bouton.

The only remaining differentiated parts of the bouton that are known are the neurofilaments and their surrounding of pale cytoplasm. One or two strands of silver can be seen in boutons in Pl. 4, fig. 1 (b_1 and b_2). These could be oblique sections of rings. A more or less complete ring formed by silver deposits is shown in a bouton in Pl. 5, fig. 4. These, without doubt, correspond in position to the loops of neurofilaments. The fine silver particles clearly form linear aggregates (see Pl. 5, figs. 3 and 4), probably along the reaction product formed when the neurofilament (or the immediately adjacent cytoplasm) is fixed with formalin.

DISCUSSION

Neurofilaments have been demonstrated by electron microscopy in the terminal presynaptic bags of axons of the spinal cord of the cat and rat. These filaments are often arranged to form rings that match the classical terminal boutons of light microscopy in shape and in approximate size. The

structures that can be seen in the terminal bag of spinal cord axons include surface membrane, mitochondria, synaptic vesicles and neurofilaments. The first three do not take up silver selectively in material impregnated for boutons nor do they have the right shape to form a possible basis for the neurofibrillar terminal boutons. It is thus highly probable that the silver is deposited in the neurofilamentous region of the synapse, probably on the fixation products of the filaments themselves.

Since terminal bags vary in the amount of neurofilamentous material that they contain it is relevant to ask whether this variation may have any functional significance. Several possibilities for further investigation arise. The number of neurofilaments may be related to size of terminal bag, number of mitochondria, or discharge activity of the axon. Thus in the lizard brain it was found that changes in the neurofilaments of boutons could be produced by altering environmental temperature, and these changes were accompanied by changes in the mitochondria in the same region (Boycott & Guillery, 1959; Boycott *et al.* 1960, 1961).

A great number of terminal bags show no neurofilaments in electron micrographs, fewer even in the rat than in the cat. While it is not possible without serial sections to state that they are absent in a given terminal, it is clear that many synaptic terminals have no neurofilaments. The variable effectiveness of the silver stains of light microscopy in different parts of the central nervous system and in different species is thus related to the presence or absence of neurofilaments in the synaptic terminal rather than to an inefficiency of the silver technique. On the other hand, mitochondria are almost invariably present in spinal cord synaptic terminals and this agrees with the observation that more synapses can be displayed by light microscopy with mitochondrial methods than can ever be seen with classical silver methods (Armstrong, Richardson & Young, 1956). It emphasizes that silver methods can never give more than a partial picture of synaptic organization.

SUMMARY

1. Bundles of neurofilaments, often orientated in the form of a ring, can be observed by electron microscopy in a small proportion of the pre-synaptic bags of axon terminals of the spinal cord.

2. These neurofilaments form the basis of the argyrophilic material that constitutes the classical neurofibrillar rings and clubs of light microscopy. The possibility that other neuronal structures, the cytoplasm and surface membranes, myelin sheaths, mitochondria and synaptic vesicles, are argyrophilic can be excluded.

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EXPLANATION OF PLATES

In all plates letters indicate structures thus: *b*, bouton; *m*, mitochondrion; *my*, myelin sheath; *nb*, neurofibrillae; *nf*, neurofilaments; *pc*, pale cytoplasm containing neurofilaments; *post*, post-synaptic cytoplasm; *si*, silver deposits; *sv*, synaptic vesicles.

PLATE 1

Fig. 1. Electron micrograph. Cat cord. A presynaptic bag containing a ring of neurofilaments surrounding a group of mitochondria; synaptic vesicles are also present. (Below) myelinated axon.

PLATE 2

Fig. 1. Electron micrograph. Cat cord. Presynaptic bag containing an irregular arrangement of neurofilaments.

Fig. 2. Electron micrograph. Rat cord. Presynaptic bag containing a few neurofilaments situated in a clear zone of the cytoplasm.

PLATE 3

Fig. 1. Electron micrograph. Cat cord. Silver deposits within a myelinated axon.

Fig. 2. Electron micrograph. Cat cord. Dots within the circles represent a radial section of a ring of neurofilaments.

Fig. 3. Electron micrograph. Cat cord. Silver deposits within a myelinated axon.

PLATE 4

Fig. 1. Electron micrograph. Cat cord. Silver-stained preparation. Fibre plexus (above) and large ventral horn cell (below) with a row of boutons.

Fig. 2. Low-power light micrograph. Cat cord. Cajal silver preparation of large neurone with boutons.

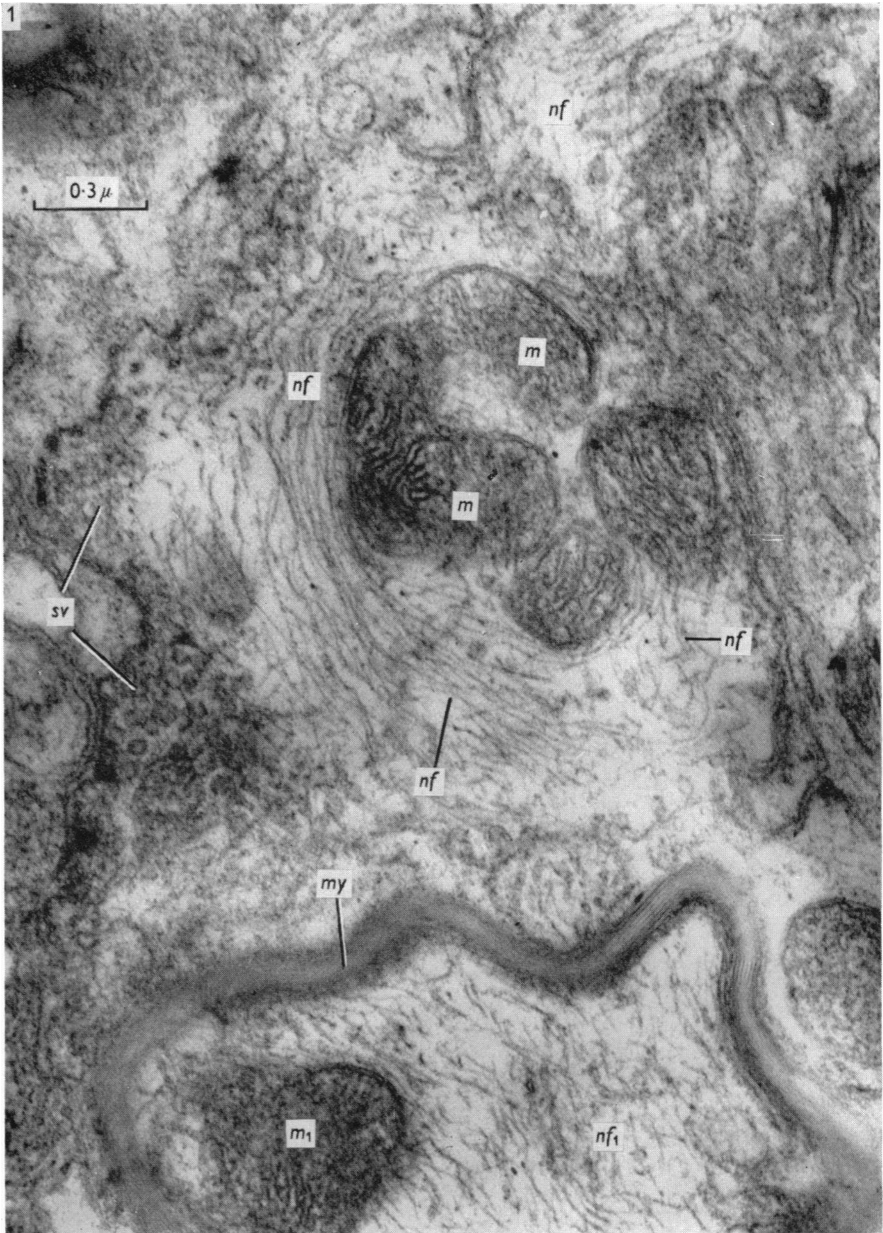
PLATE 5

Fig. 1. Light micrograph. Cat cord. Cajal silver preparation of large neurone with boutons and fibre plexus.

Fig. 2. Light micrograph. Cat cord. Neurone with neurofibrillae extending into basal regions of dendrites.

Fig. 3. Electron micrograph. Cat cord. Axo-somatic boutons in a silvered preparation.

Fig. 4. Electron micrograph. Cat cord. Axo-somatic bouton showing ring of silver particles.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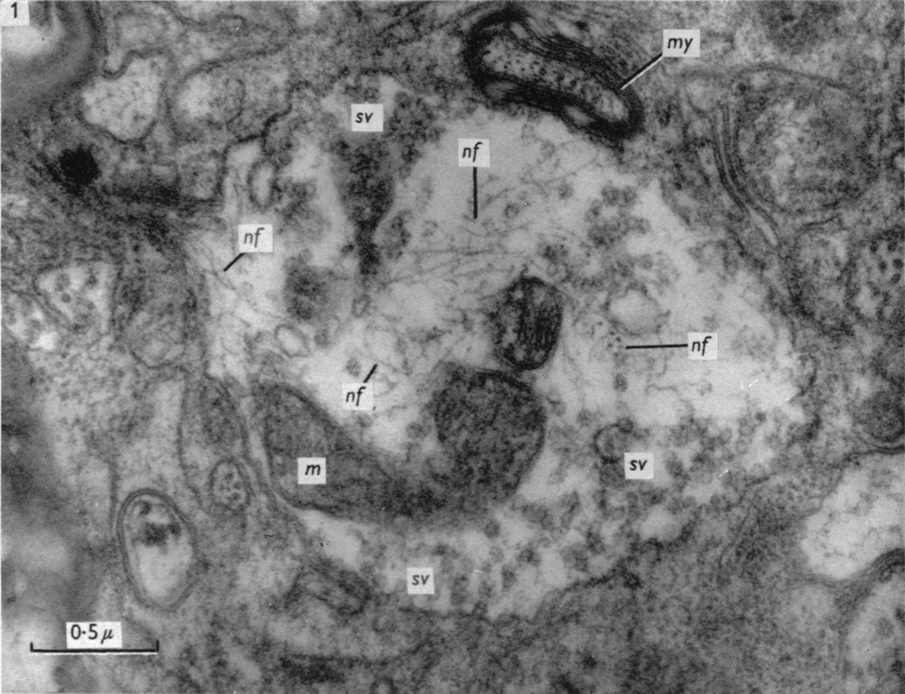

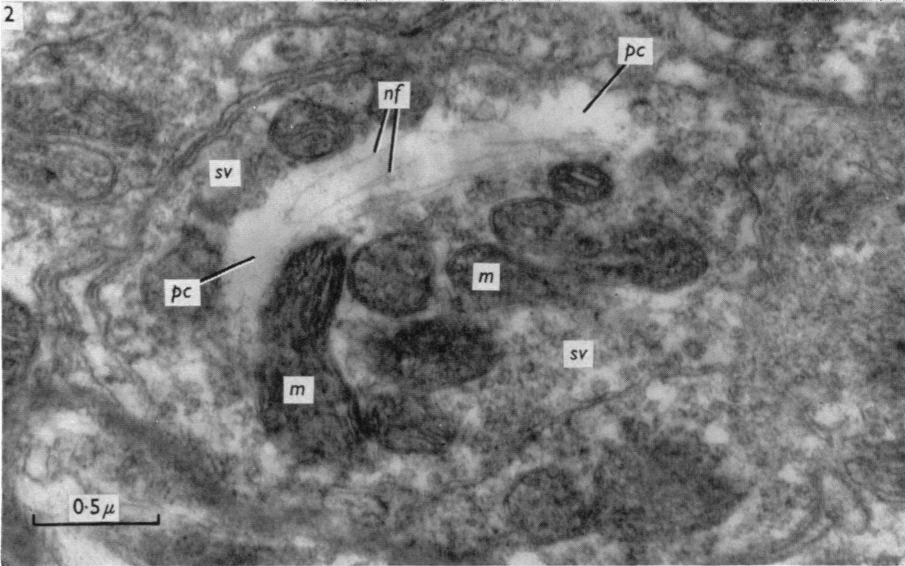

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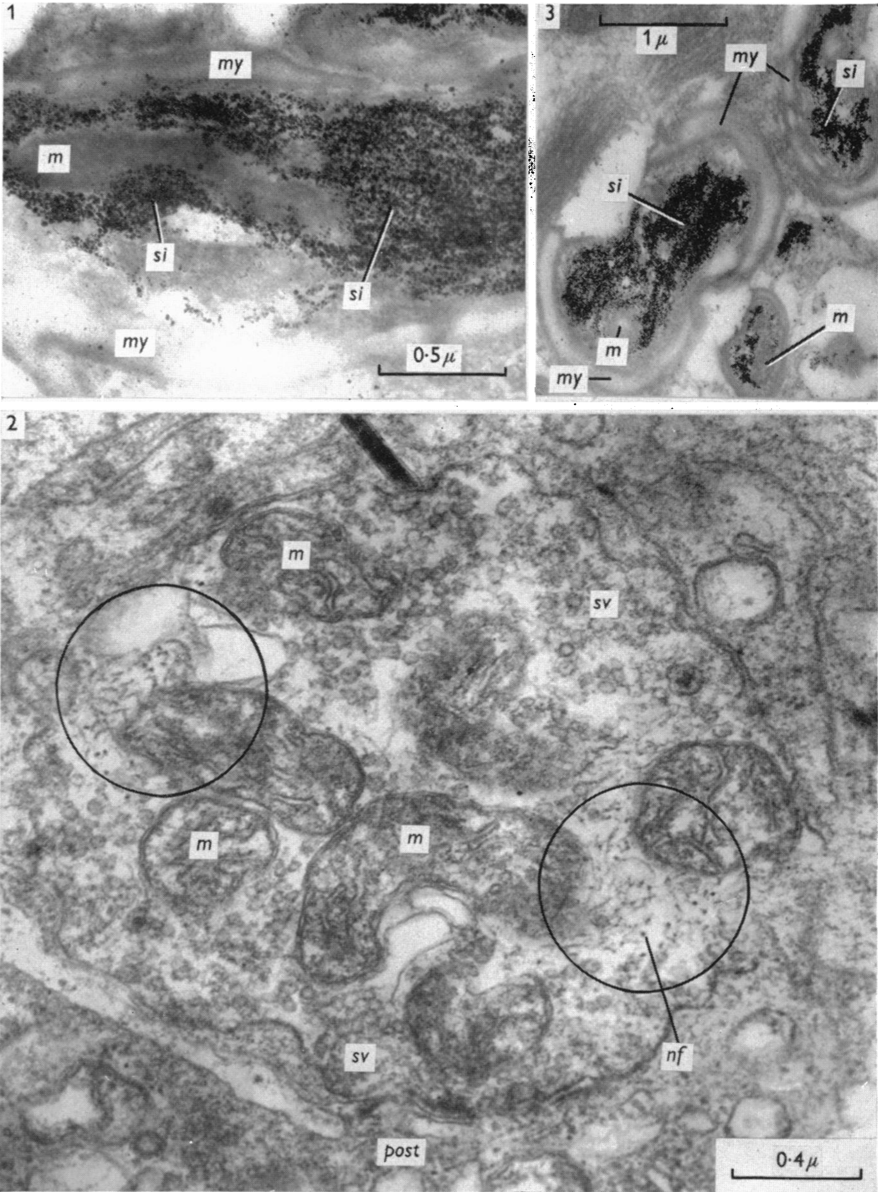

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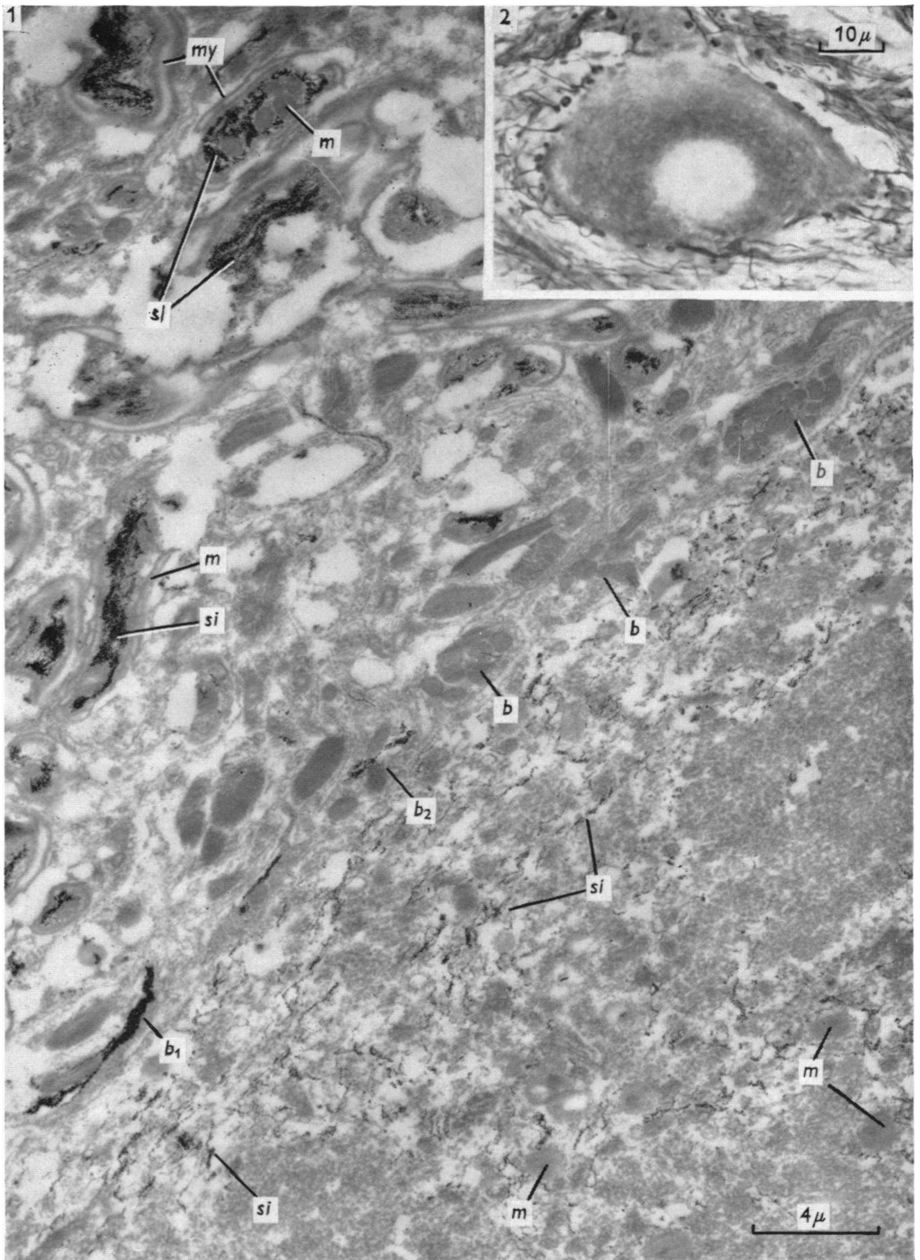

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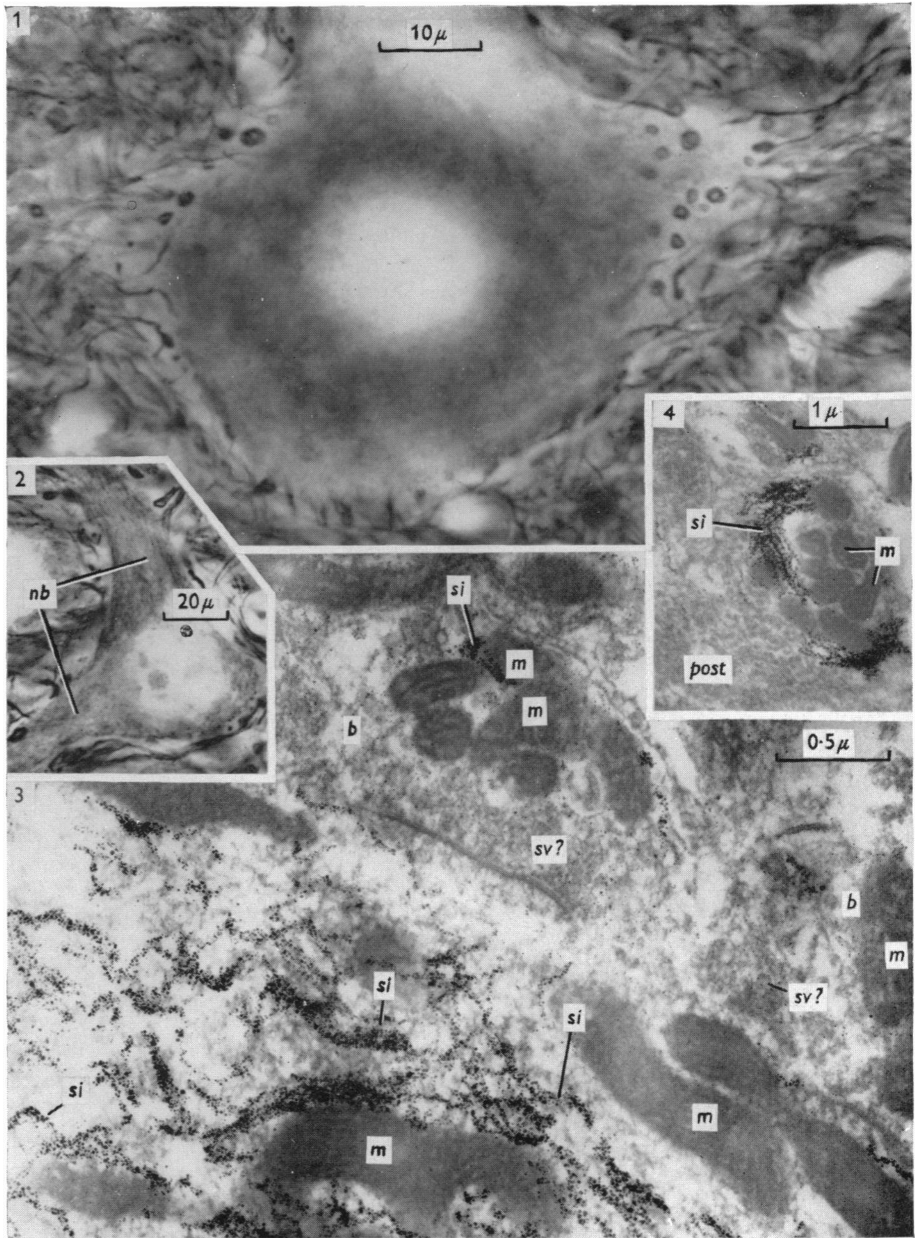

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