AN ULTRASTRUCTURAL STUDY OF AXONAL CHANGES FOLLOWING CONSTRICTION OF POSTGANGLIONIC BRANCHES OF THE SUPERIOR CERVICAL GANGLION IN THE RAT

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Postganglionic branches were ligated or cut 1 to 2 mm from the superior cervical ganglion in 48 Wistar rats. The axons were examined at intervals from 6 h to 143 days postoperatively. At 6 h the axons were swollen for about 0.6 mm proximal to the ligature, the distended segments containing chiefly vesiculotubular smooth endoplasmic reticulum, 60 to 110 nm dense-cored vesicles and some mitochondria. These organelles were tightly packed close to the ligature and dispersed in amorphous axoplasm further away. Over the next 12 to 30 h many mitochondria became grouped in compact clusters, associated with filaments, near the proximal end of the zone of densely packed organelles, and other organelles appeared in increasing numbers in the same region. These included clumps of small vesicles (some with dense cores), multivesicular bodies of regular form, loops or flattened sacs of membrane, and tubules with an electron-dense content (all of which were probably transported from the cell bodies), and autophagic vacuoles and large cytoplasmic dense bodies, which appeared to form locally within the intra-axonal accumulations. The autophagic vacuoles and dense bodies formed part of a reaction of intra-axonal digestion of the material initially accumulated.

This digestive reaction coincided with the onset of a phase of intracytoplasmic digestion in the cell bodies. The flattened sacs of membrane and the tubules with electron-dense content, and possibly also the multivesicular bodies, appeared to provide material for the intra-axonal formation of autophagic vacuoles and dense bodies. Close to the ligature the tips of the congested axons degenerated and were phagocytosed by Schwann cells. Regenerative axon sprouting (not described in detail in this paper) began within 24 to 38 h and was associated with the appearance of a wispy material in the axons. It was concluded that the relative output of various types of material from the cell bodies may change during the retrograde reaction to axonal injury in these neurons, as part of a coordinated response involving correlation of events in widely separated parts of the neuron.

INTRODUCTION

In neurons whose axons project outside the central nervous system successful axon regeneration can occur. In such neurons the retrograde reaction to axonal injury appears to consist of two main processes: (1) A phase of intracytoplasmic digestion involving increased activity of lysosomes (Bodian & Mellors 1945; Barron & Tuncbay 1964; Holtzman, Novikoff & Villaverde 1967; Matthews & Raisman 1972), which may be related to an observed reduction in the output of transmitter-related material (Hebb & Silver 1965; Boyle & Gillespie 1970; Karlström & Dahlström 1970; Dahlström 1971); and (2) an activation of protein synthetic mechanisms in relation to the regeneration and maturation of the axon (e.g. Brattgård, Edström & Hydén 1958; Watson 1965, 1968; Engh, Schofield, Doty & Robinson 1971). The adrenergic neuron offers the advantage in the study of such processes that material related to the transmitter function (dense-cored vesicles) may be recognized at the ultrastructural level. In an earlier paper (Matthews & Raisman 1972) an account has been given of the morphological changes which occur in the cell bodies of neurons in the superior cervical ganglion of the rat after constriction or section of the axon close to the cell body. The present paper presents a parallel

study of the nerve fibres in the same ganglia as were described in this earlier report. The situation in the neuron responding to axonal injury is complex, in that major cytoplasmic organelles producing material destined for the axon (such as the granular endoplasmic reticulum and the Golgi apparatus) are virtually confined to the cell body, and that, furthermore, the cell body is linked with the axon by multiple flow mechanisms which are as yet imperfectly understood (e.g. Lasek 1970; Dahlström 1971; Karlsson & Sjöstrand 1971*a*). The total response of the neuron will occur partly in the cell body and partly in the axon, and the reactions in these two sites may differ considerably. For these reasons it is important that events in the neuronal cell body be interpreted in the light of concurrent events in the axon, and vice versa.

Numerous ultrastructural, biochemical and histochemical studies have been made of the axonal reaction to local injury. The early consequences of ligation or section of axons involve the accumulation in the proximal axon tips of material apparently carried there by persistent axoplasmic flow. This includes transmitter-related material: in cholinergic axons, choline acetyltransferase (Hebb & Waites 1956) and acetylcholinesterase (Zelená & Lubińska 1962; Lubińska & Niemierko 1971); and in adrenergic axons, noradrenaline (Dahlström 1965; Banks, Mangnall & Mayor 1969). At the ultrastructural level the accumulated material is seen to consist predominantly of vesicles and short segments of tubules, with some mitochondria (e.g. Wettstein & Sotelo 1963; Blümcke, Niedorf & Rode 1966; Zelená, Lubińska & Gutmann 1968; Pellegrino de Iraldi & De Robertis 1968). In the adrenergic axon there are also included dense-cored vesicles of about 60 to 100 nm diameter (Kapeller & Mayor 1967, 1969*a*; Geffen & Ostberg 1969). Following the phase of accumulation, and partially co-extensive with it, there occur a series of ultrastructural changes whose interpretation has been far less clear. These include the formation of autophagic vacuoles and of various dense lamellar bodies and other dense bodies of a type suggesting focal cytoplasmic degradation (e.g. Holtzmann & Novikoff 1965; Blümcke *et al.* 1966; Schlote 1966; Kapeller & Mayor 1967, 1969*a*), the formation of cores of filamentous material (Wettstein & Sotelo 1963; Lampert 1967; Zelená *et al.* 1968), and the appearance of processes containing a characteristic web of fine branching tubular elements of variable diameter (Zelená *et al.* 1968). Some of these features may simply be associated with the disposal of accumulated material, while others may be signs of degeneration, abortive attempts at regeneration, or else may be the ultrastructural correlates of one or other aspect of the actual regenerative process. The present study attempts to examine some of these possibilities by following the time course of the accumulative and degenerative aspects of the axonal reaction, especially in its earlier stages, and relating it to the concurrent events in the cell body. The events relating to the regenerative sprouting will be described in a subsequent article (Matthews & Raisman, in preparation), but specific features of this reaction are referred to in the present article where they become relevant.

Material and methods

Forty-eight young adult male Wistar rats, of about 180 g body weight, were used in this study. Under chloral hydrate anaesthesia the superior cervical ganglion of one side (sometimes both sides) was exposed and the external carotid nerve and/or the internal carotid nerve were either cut (12 ganglia), or were ligated (with 5/0 or 8/0 silk or 10/0 monofilament nylon) and were left undivided (30 ganglia). (These nerves are the major postganglionic branches of the superior cervical ganglion, running along the external and internal carotid arteries respectively.)

The animals were allowed to survive for the following periods: 6, 12 to 13, 24 and 38 h; 2, 2½, 3, 4, 6, 7, 10, 14, 16, 21, 28, 34, 68, 138 and 143 days. At a second operation the ganglion was re-exposed, excised, rapidly divided into three blocks and fixed by immersion for 2 h in chilled 1 % osmium tetroxide, buffered to pH 7.4 with phosphate or with veronal acetate. The unoperated contralateral ganglia were removed and fixed as controls; additional superior cervical ganglia were taken from normal rats.

Sham operations were performed (6 ganglia), in which the ligatures were placed round the nerves but were not tightened, and were removed before closing the wound. These animals were allowed to survive for 4, 7, 14 and 46 days.

After fixation the blocks were washed, dehydrated in graded ethanols and embedded in Araldite. 1 to 2 μm sections were taken, and from these appropriate regions were selected for ultrathin sections. At 6, 12, 13, 24 and 38 h and at 2, 3 and 7 days ultrathin sections extending up to and across the ligature site were examined. Scale drawings were made of these sections as they lay on the grid under the electron microscope and on these drawings the locations of the various changes were charted; this gave distances from the ligature in terms of the fixed and embedded tissue.

Results

The axonal accumulations have been studied both immediately adjacent to the site of ligation and farther away within the ganglion. In the postganglionic nerve trunks at the point of ligation the nerve fibres are probably without exception axonal, but within the ganglion in addition to axons there are many dendrites, which arise from the sympathetic neurons. While it is not possible to make a clear distinction between axons and dendrites in every case, there are certain features by which they may often be differentiated: for example, irregular contour and the presence of ribosomes or granular endoplasmic reticulum suggest dendrites; axons tend to be of regular and rather uniform calibre and to run in bundles. The impression gained from survey of the material suggests strongly that the changes seen in nervous processes within the ganglion after constriction are occurring primarily in the axons of the ganglionic neurons and represent a retrograde axonal reaction.

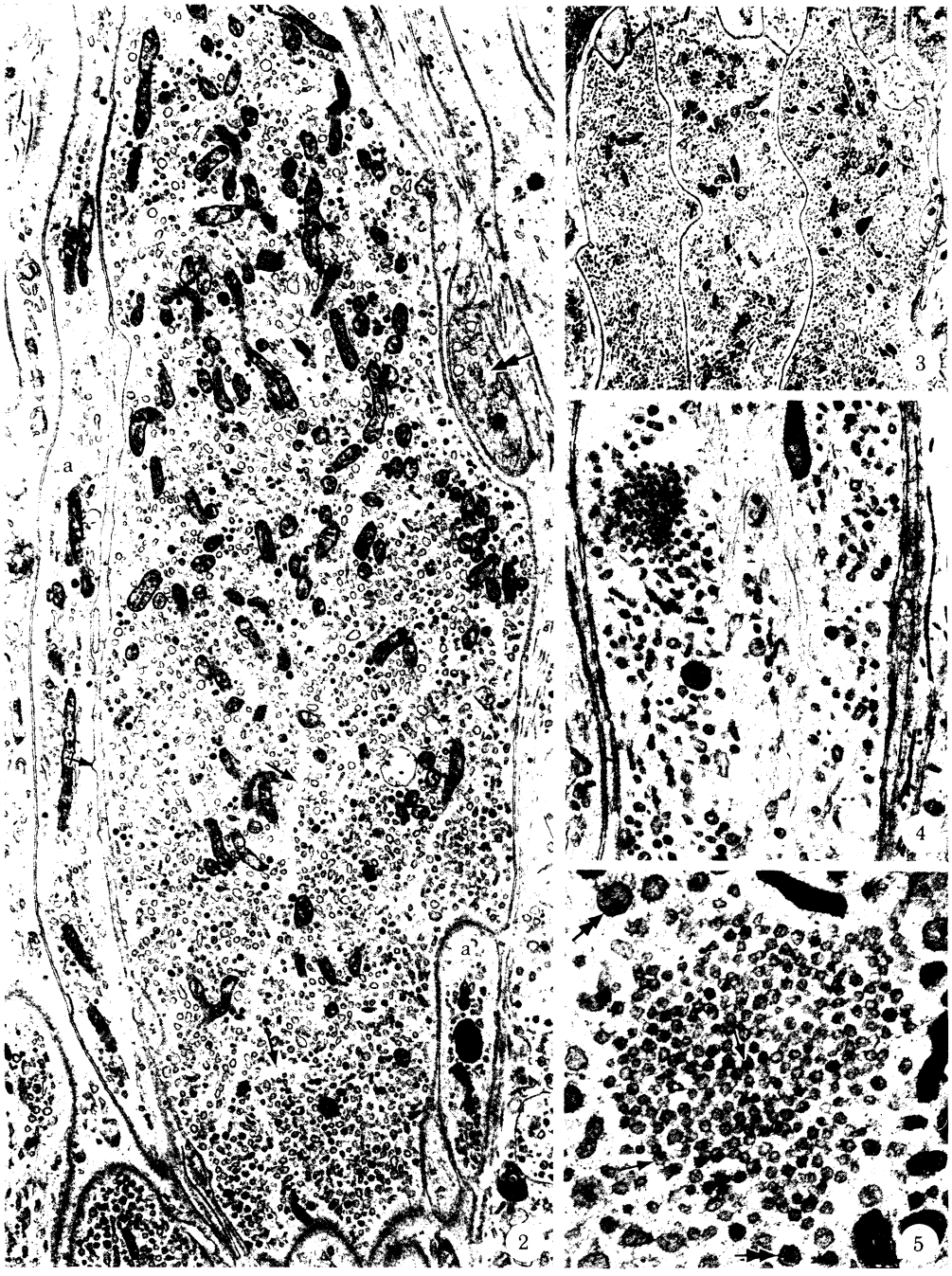
The sham-operated ganglia were not found to differ in any major respects from normal ganglia. For the purpose of comparison of the changes in the nerve fibres with those in the cell bodies described in the previous publication, the experimental material will be divided into the same four groups according to survival times.

Group I: 6 h to 2 days

The earliest effects of ligation are seen in the axons immediately proximal to the site of ligature. Here there are rapid and progressive changes over the first 2 days, particularly during the second day when there develops a phase of intense intra-axonal digestion of accumulated organelles. The changes are therefore described sequentially. Distances from the ligature are given as mm in the fixed and embedded specimen.

At 6 h following ligation, the axons proximal to the ligature are distended over a length of approximately 0.6 mm (figure 1, plate 54). For about half this distance the interstitial connective tissue spaces are virtually obliterated and are marked only by compressed collagen bundles. The sheathing processes of the Schwann cells are greatly attenuated and in many places are no longer present between adjacent axons (e.g. figures 2 and 3, plate 55). Almost all the axons

FIGURE 1. Light micrographic montage of 2 μm section passing through the ligated internal carotid nerve and cranial tip of a superior cervical ganglion, 6 h after ligation. The section was stained with a mixture of methylene blue and Azur II. Scale bar = 0.1 mm. L, ligature material (10/0 monofilament nylon), including parts of the knot. At the level of the ligature and immediately adjacent to it above and below is a compressed zone showing very intense staining. Proximal to this for about 0.4 mm the axons are rather deeply stained. In this region the electron microscope shows them to be greatly distended and packed with organelles. Further proximally (arrows) there is an abrupt change to a region in which the axons are still distended but show unusually light staining. This extends to the level of the closest ganglionic neurons, which form a compact group in the lower left part of the figure. Bundles of axons (double arrows) among the ganglion cells show a normal diameter and staining reaction. (*Facing p.* 482)



FIGURES 2 TO 5. For legends see facing page.

appear swollen, showing diameters of up to approximately 3.5 μm (normal range, 0.1 to 1.8 μm, mean 0.73 μm; Matthews & Raisman, in preparation); and their profiles may be distorted from mutual compression. Farther proximally the interstitial tissue spaces are progressively less narrowed and the axon-sheath relations less disturbed; the swelling of axons is more variable and some axons show diameters of up to 4.5 μm, while others have diameters within the normal range (e.g. figure 2). At approximately 0.6 mm from the ligature, which is about the level of the closest ganglionic neurons (figure 1), the appearances of the axons, interstitial spaces and axon sheaths are within normal limits.

The proximal extent of the intra-axonal accumulations does not increase so rapidly after the first 6 h postoperatively. At 12 to 13 h it is still about 0.6 mm from the ligature, and at 38 h it is not beyond about 0.7 mm in most axons, although more proximal accumulations have begun to appear in some axons. It appears that in most axons the rate of increase in the volume of the accumulated material is not maintained for many hours at its initial level.

Nature of the intra-axonal accumulations

Already at 6 h postoperatively the contents of the distended axons vary according to the distance from the ligature. Within the first 0.35 to 0.4 mm the axons are filled with closely packed organelles (organelle-rich accumulations; darker-staining region in figure 1). Proximal to this is a zone distended with finely granular axoplasm containing sparse organelles of the type seen more distally (dilute accumulations; lighter-staining region in figure 1). The zone of demarcation from the organelle-rich accumulations may be so abrupt that areas of pale granular cytoplasm and masses of closely packed organelles may be seen in the same cross-section of a distended axon (e.g. figure 6, plate 56).

In the zone of closely packed organelles, the most numerous structures are vesicles and short tubular profiles with an interior of slight to moderate electron density, without dense cores and

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DESCRIPTION OF PLATE 55

FIGURES 2 to 5. Early accumulations in axons proximal to the ligature, and subsequent appearance of clumps of small vesicles. In this and all subsequent legends the time given in parentheses is the postoperative survival time. The figure in mm, where given, is the approximate distance from the ligature.

FIGURE 2. An axon greatly distended with vesiculo-tubular material, dense-cored vesicles and mitochondria. Microtubules within the axon lie in disarray (arrows), and are typically flanked by narrow zones of organelle-free cytoplasm. Mitochondria are more numerous towards the upper (proximal) end of the axon. Adjacent axons (e.g. a) have diameters within the normal range. One of these (double arrow, upper right) shows a darkened cytoplasmic matrix and some expanded, empty-looking vesicles: these are interpreted as early degenerative changes. Only a minority of vesicles in the enlarged central axon have the same expanded empty appearance. The satellite sheath of the central axon is widely deficient, so that in places (upper left and lower right margins) this axon lies in contact with adjacent axons, and down much of its left border it and the parallel adjacent axon are separated from an intervening interstitial tissue space by only basement membrane. The latter axon shows an unusual number of shallow coated pits at its surface membrane (e.g. crossed arrow). (12 h, 0.25 mm.) × 12000.

FIGURE 3. Low-power electron micrograph of three axons which are closely apposed, with no intervening satellite cytoplasm, and are much distended. They are filled with accumulations of vesiculo-tubular material, with some mitochondria and early forms of dense bodies. (24 h.) × 8700.

FIGURE 4. Part of an intraganglionic axon showing a central region of filaments, peripheral vesiculo-tubular material and a compact cluster of small vesicles. (24 h.) × 21400.

FIGURE 5. Higher-power electron micrograph of a compact cluster of small vesicles, some of which show dense cores (e.g. arrows). Double arrows, larger dense cored vesicles. This vesicle cluster is from the profile shown in figure 11. (38 h.) × 42000.

with a diameter mainly in the range of 40 to 80 nm (figures 2, 3, 42, 43, plates 55 and 60). The vesicles are often arranged in short rows. The tubules are straight or slightly sinuous and may be varicose, showing a tendency to break up into rows of vesicles. This vesiculo-tubular material closely resembles the type of smooth endoplasmic reticulum which is sparsely distributed along normal axons. Within the accumulated vesiculo-tubular material are scattered numerous dense-cored vesicles, of about 60 to 110 nm diameter, which show an approximately uniform dispersal (figure 2). Rather few mitochondria are present in the distal parts of these accumulations; these are elongated and tend to be alined longitudinally and to be associated with filaments in slender longitudinal bundles of width about equal to the diameter of a mitochondrion. Mitochondria become progressively more numerous further proximally in many axons (e.g. figure 2). By 12 to 13 h the mitochondria are beginning to form compact clusters within the proximal ends of the vesiculo-tubular accumulations. At this level, at 6 h postoperatively, there are also occasional isolated examples of the following organelles: multivesicular bodies (e.g. figure 8, plate 56), cytoplasmic dense bodies (figure 2, lower right, axon a), and clumps of small vesicles (of about 20 to 40 nm diameter) some of which show dense cores (figures 4, 5, plate 55), such as are normally found as clumps within the cell bodies and dendrites of the intraganglionic neurons. Within the dilute accumulations, occasional tubular or flattened sacs of membrane are seen loosely encircling groups of organelles.

Changes in the intra-axonal accumulations over the first 2 days

The principal changes involve (1) the appearance of additional organelles and of new types of material at the proximal ends of the organelle-rich accumulations, and (2) the development there of characteristic groupings of organelles and of a local intra-axonal digestive reaction. These changes do not penetrate far into the organelle-rich accumulations, and in many axons the distal segments of these accumulations show degenerative changes and are apparently phagocytosed by Schwann cells.

(*a*) *Fate of distal segments of organelle-rich accumulations.* Close to the ligature (within 0.1 to 0.2 mm) by 12 to 13 h postoperatively in many of the distended axons the accumulated

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Description of plate 56

Figure 6. A distended axon profile filled partly by an accumulation of organelles similar to that in figure 2 and partly by an extensive, sharply demarcated zone of finely granular or filamentous material. (12 h, 0.3 mm.) × 9300.

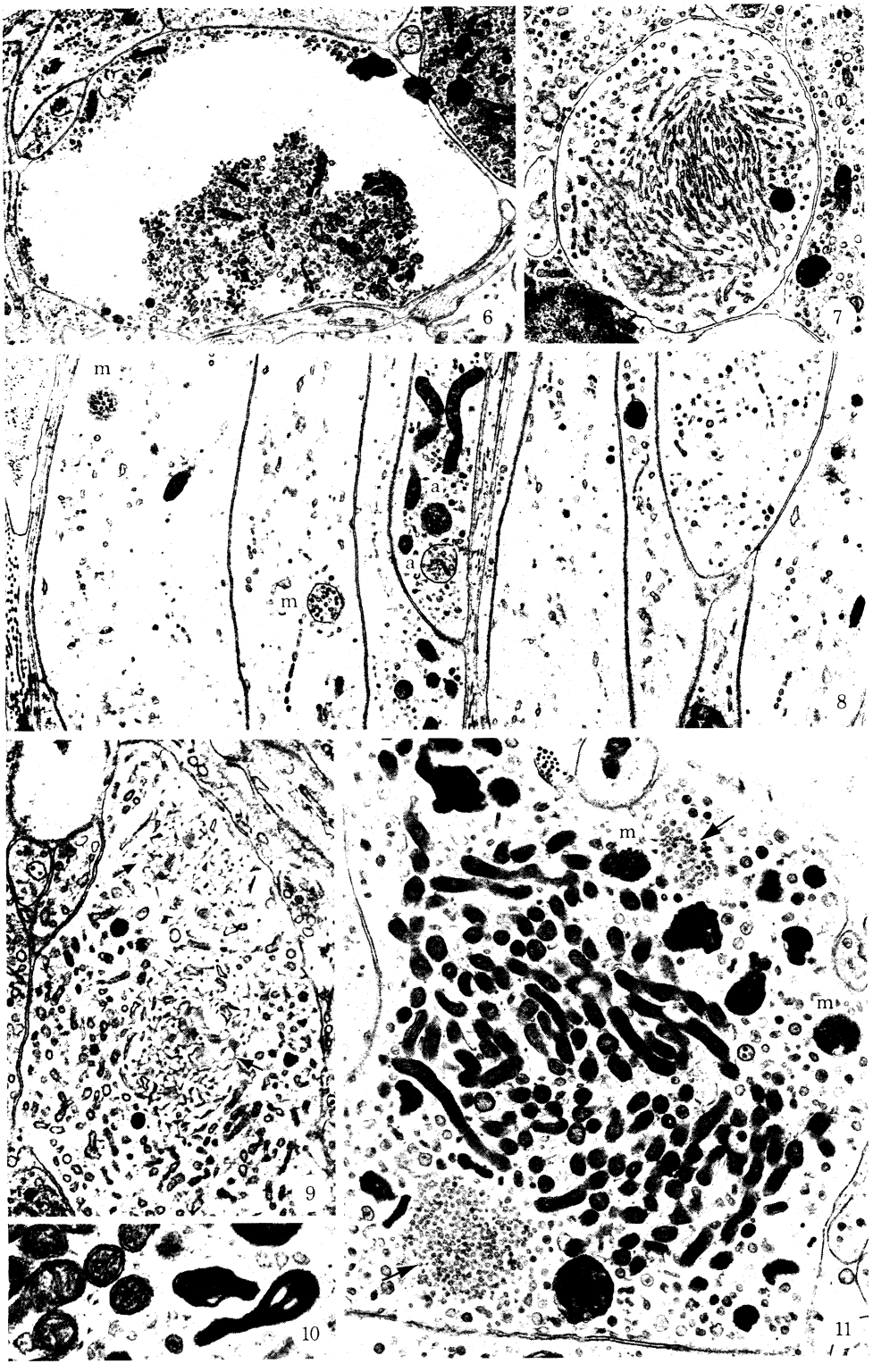
Figure 7. An axonal profile showing a central accumulation of parallel tubules or flattened sacs, somewhat resembling the inner lamellae of the Golgi apparatus. Some vesiculo-tubular material is seen in the same profile at the left, and in adjoining profiles. (12 h, 0.25 to 0.35 mm.) × 15300.

Figure 8. A group of closely packed distended axons (note virtual absence of satellite sheaths) showing relatively dilute accumulations with occasional organelles (chiefly vesiculo-tubular material) widely scattered in a finely granular or filamentous cytoplasmic matrix. m, multivesicular bodies; a, autophagic vacuoles. (38 h, 0.4 to 0.5 mm.) × 12000.

Figure 9. An axon showing an accumulation of vesiculo-tubular material and, in addition, two associated regions of wispy material (arrows), one of which is centrally placed in the profile. (13 h, 0.25 to 0.3 mm.) × 17000.

Figure 10. Part of a large accumulation of mitochondria of normal appearance, showing associated dense lamellated structures which are one type of intra-axonal dense body, and which may be derived from mitochondria. (38 h, 0.3 to 0.4 mm.) × 39000.

Figure 11. A distended axon with a central region of closely packed mitochondria and filaments and a peripheral zone of vesicular material, dense bodies and multivesicular bodies (m). Arrows, clusters of small vesicles, some of which show dense cores. (38 h, 0.4 mm approx.) × 16000.



FIGURES 6 TO 11. For legends see facing page.

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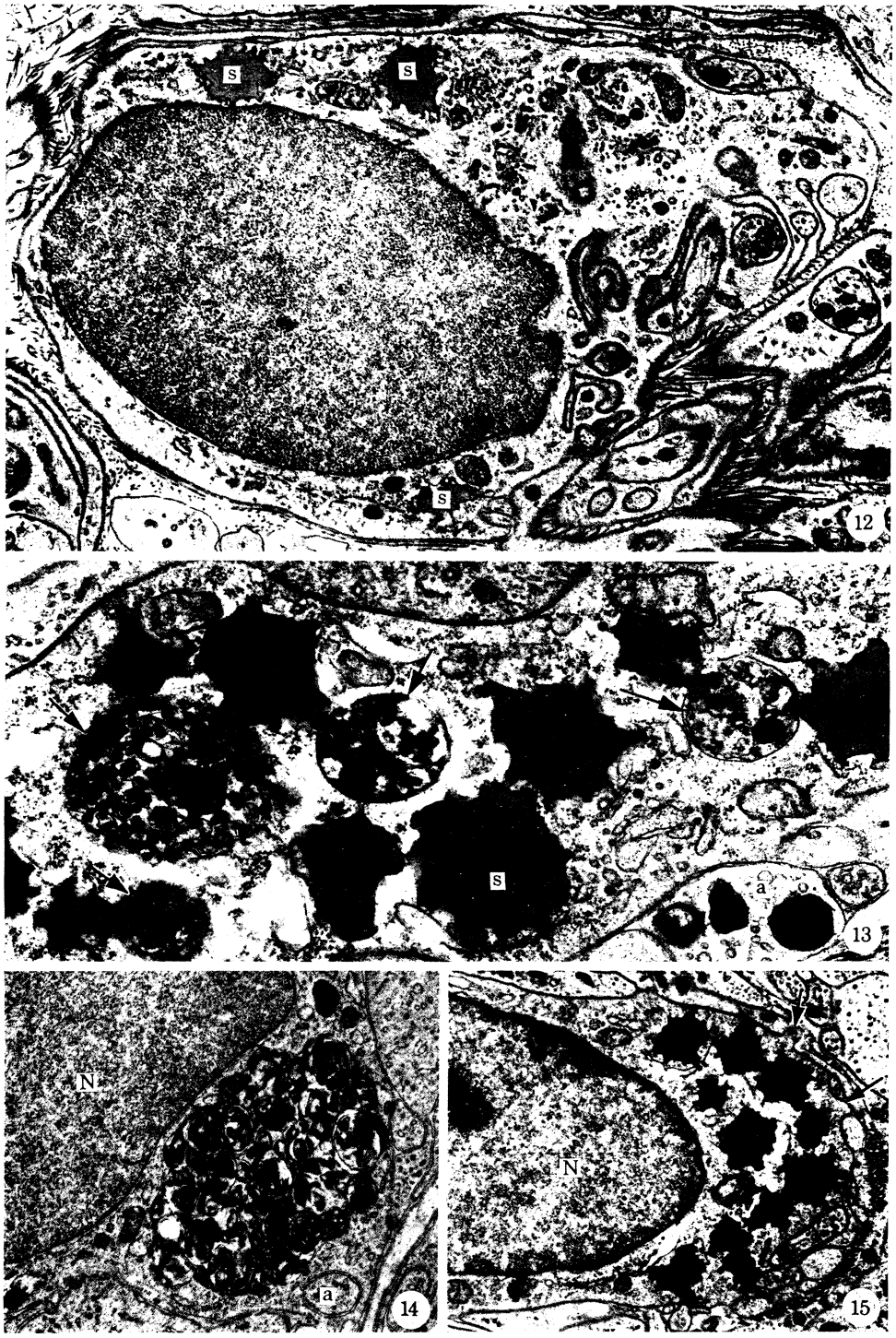

FIGURES 12 to 15. Axonal debris and lipid-like masses in Schwann cells.

FIGURE 12. An intraganglionic Schwann cell with three stellate lipid-like inclusions (s). (12 h, approx. 0.5 mm from ligature.) × 11000.

FIGURE 13. Part of a Schwann cell containing many irregularly stellate lipid-like inclusions (e.g. s) and four rounded masses of darkened and partly degraded axonal cytoplasm showing alteration of accumulated organelles (arrows). These are seen in two cases to be bounded by a single membrane and are presumed to be phagosomes. An adjacent axon (a, lower right), containing some vesicles and dense bodies, has an intact membrane and does not show darkening of its cytoplasmic matrix. (38 h, 0.15 mm approx.) × 21000.

FIGURE 14. Part of a Schwann cell (nucleus, N) containing a large compacted mass of late forms of intra-axonal dense bodies, which has a collapsed, irregular outline and is indistinctly demarcated from the Schwann cytoplasm. This is interpreted as a phagosome of an axonal profile packed with dense bodies. The same Schwann cell is also seen to support an axon of normal appearance (a). (7 days.) × 12300.

FIGURE 15. Part of a Schwann cell (nucleus, N) whose cytoplasm is loaded with stellate, lipid-like inclusions. At its periphery there lie several small, axon-like profiles between which the Schwann cell sends short, blunt processes (arrows), not entirely surrounding them. This is typical of the stage of rounding up of the Schwann cells during the digestion of axonal debris and the beginning of regenerative axon sprouting. (38 h, 0.2 to 0.3 mm.) × 8000.

organelles are swollen and distorted, and some axon membranes are disrupted. These appearances suggest early stages of autolysis. The Schwann cells in this region become more or less rounded up and are apparently no longer providing sheaths for the distended axons, but frequently contain rounded masses of partly degraded and darkened axoplasm surrounded by a single membrane: these are interpreted as phagosomes within the Schwann cells (e.g. figure 13, plate 57). A characteristic inclusion now begins to appear within the cytoplasm of these Schwann cells (an inclusion which in this ganglion is typically associated with evidence that these cells are digesting phagocytosed material) (Matthews, in preparation): this is a stellate or irregular mass of amorphous material of moderate electron density, resembling lipid, which has a slightly denser rim but is not enclosed within the cisternae of the granular endoplasmic reticulum (dense extra-cisternal material; figures 12, 13, 15, plate 58). Its extra-cisternal position and its amorphous, lipid-like character distinguish it from a form of dense intracisternal material which is a later feature of the Schwann cell reaction to the changes in progress (Matthews & Raisman, in preparation). By 38 h postoperatively most of the distended axons within 0.2 mm proximal to the ligature are degenerating and becoming phagocytosed by Schwann cells. The incidence of the lipid-like material within the Schwann cells is highest from about 38 h to 3 days postoperatively, and over this period the phagosomes of axonal debris in these cells darken, their contents ceasing to be recognizable as organelles, and in many cases become transformed into lamellated dense bodies (showing internal stacks or whorls of parallel lamellae). These subsequently evolve into residual dense bodies resembling the lipofuscin bodies of neurons. At 2 to 3 days postoperatively, mitoses of Schwann cells have been found in this zone, and also further proximally.

(*b*) *Proximal segments of organelle-rich accumulations and zone of dilute accumulations.* From 12 to 13 h onward the clustering of *mitochondria* toward the proximal end of the zone of organelle-rich accumulations becomes a characteristic and well-marked feature. By 38 h in many profiles quite massive and closely packed clusters of mitochondria are seen, which typically lie centrally within the profiles, sometimes occupying the whole cross-sectional area (figure 11, plate 56). The mitochondria are elongated and show a small circular cross-sectional profile and a rather dense inter-cristal matrix; they are associated with loosely arranged neurofilaments which tend to lie parallel with their long axes. Within the same zones there appear from 12 to 13 h onward increasing numbers of *multivesicular bodies* and *clumps of small vesicles*; both are frequently seen in association with clusters of mitochondria (figure 11). In addition, *cytoplasmic dense bodies* (of very varied form, e.g. figure 11) and *autophagic vacuoles* (figures 20, 22, 25 to 31, 35, plates 58 and 59) increase greatly in incidence, chiefly from 24 h onward. By 38 h postoperatively a massive reaction of intra-axonal digestion has become established throughout the more proximal regions of the organelle-rich accumulations, chiefly just distal to the mitochondrial clusters. This reaction involves the appearance of large numbers of autophagic vacuoles, masses of cytoplasmic dense bodies and a wide range of forms intermediate between the two, in addition to *flattened sacs of membrane* and *tubules with a dense content*, which may contribute to their formation. Multivesicular bodies may also be involved in the reaction. (See below: the intra-axonal digestive process.)

In some profiles from 12 to 24 h postoperatively, again near the junction of the more dilute and the organelle-rich accumulations, there is a central accumulation of parallel flattened sacs or narrow tubules of smooth endoplasmic reticulum, with a sharply defined membrane and low internal density (figure 7, plate 56). Similar sacs are later found lying singly in profiles

where autophagic vacuoles are forming (see below). Other, occasional, profiles at about the same level from 12 h onward contain small amounts of a wispy material, which takes the form of slender, branching, threadlike tubules, expanding here and there into angular sacs or lakes, often at points of branching (figure 9). This material is more frequently seen from 38 h onward, typically in profiles distended with dilute accumulations, which by now show more variation in types and in concentrations of organelles (e.g. profiles in left-hand half of figure 8). A high incidence of wispy material tends to be associated with evidence of sprouting of new nerve fibres. The first sign of regenerative sprouting has been seen at 24 h postoperatively.

Changes in nerve fibres at more proximal levels, within the ganglion

Few abnormalities are seen in intraganglionic nerve fibres, i.e. more than 1 to 2 mm from the lesion, within the first 24 h. Some fibres are slightly or moderately distended with more or less dilute accumulations, which include vesiculo-tubular material and occasional clumps of small

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Description of plate 58

Figures 16–22. Multivesicular bodies, autophagic vacuoles and intra-axonal dense bodies. Figures 16 to 18 are taken from the region of more dilute intra-axonal accumulations.

Figure 16. Two dilated axonal profiles containing a large number of multivesicular bodies, some lamellated dense bodies and a few dense-cored and other vesicles. The larger axon has a band of microtubules (m). The multivesicular bodies are of the 'regular' form, but some of the vesicles are darkened and flattened. (2 days.) × 21 000.

Figure 17. A multivesicular body of 'regular' form, showing an external halo of fine cytoplasmic filaments, with which additional vesicles are associated. (38 h, 0.6 mm.) × 28 000.

Figure 18. A complex body appearing to consist of a dense body (or darkened autophagic vacuole) and a multivesicular body (which forms a peripheral crescent). The two components are partly separated by a distinct membrane (which is continued as an inner layer of the membrane round the dense body), but share the same limiting membrane. (38 h, 0.55 mm.) × 26 000.

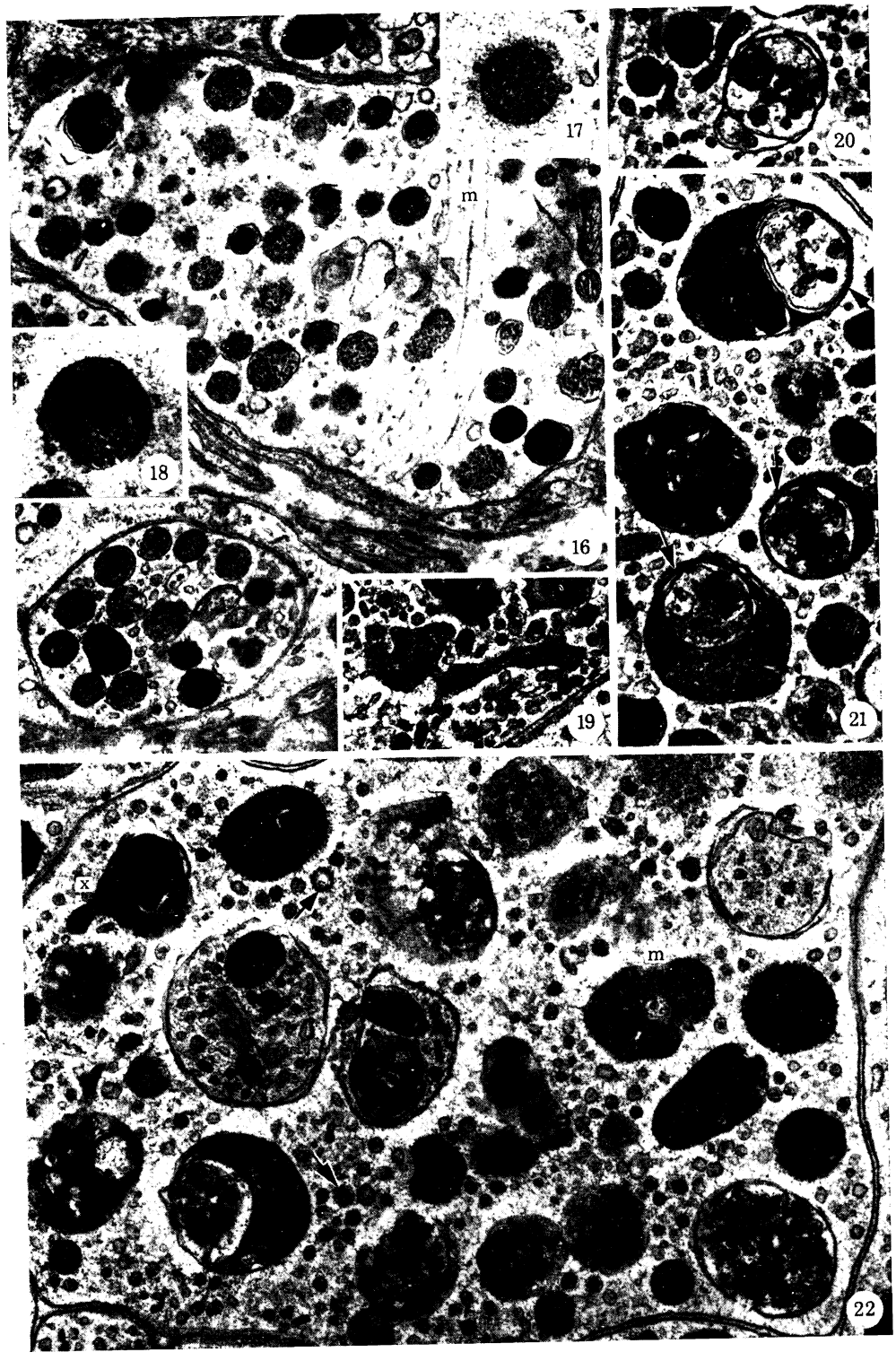
Figures 19 to 22 are from the zone of intra-axonal digestion among the organelle-rich accumulations, within 0.2 to 0.5 mm of the ligature, at 38 h.

Figure 19. A multivesicular body of highly irregular form and condensed appearance, showing a darkened matrix and darkening and irregularity of the many internal vesicles. (38 h.) × 26 300.

Figure 20. A region of axoplasm containing small dense bodies, vesicular material and an autophagic vacuole. The autophagic vacuole has enclosed a multivesicular body and several vesicles, some of them dense-cored. Its membrane is partly double (enclosing two separate loculi below), partly fused to form a single, denser layer. The cytoplasmic matrix of the main vacuole shows patchy dense material, which encroaches on some of the organelles. (38 h.) × 26 000.

Figure 21. Part of a distended axon containing vesicular material, several small dense bodies and a number of large complex dense bodies, three of which (arrows) have incorporated recognizable autophagic vacuoles (with contents at various stages of darkening and degradation). One of these dense bodies (lower left) contains also a multivesicular body, which is indented by the autophagic vacuole. Remnants of organelles (e.g. dense-cored vesicles) and whorled lamellar formations are seen in the dark matrix of the large dense bodies. (38 h.) × 27 300.

Figure 22. A distended axonal profile crowded with dense bodies and with formed and forming autophagic vacuoles (which show various degrees of darkening, especially of the cytoplasmic matrix, e.g. lower right). Two of the autophagic vacuoles (near centre) are enclosing dense bodies (or darkened autophagic vacuoles), and a neighbouring dense body (lower left) has a partly double enclosing membrane and a lighter loculus containing cytoplasmic debris which suggest that it may be derived by transformation and coalescence of autophagic vacuoles. Most of the dense bodies contain recognizable dense-cored vesicles; and many dense-cored and other vesicles, including several coated vesicles (e.g. arrows), are lying free in the cytoplasm. Also present is a large distorted multivesicular body (m). At x (upper left) is seen possible fusion of a small dense body with a larger multilocular dense body. A coated pit is seen at the surface of an adjacent axon (lower left). (38 h.) × 26 000.



FIGURES 16 TO 22. For legends see facing page.

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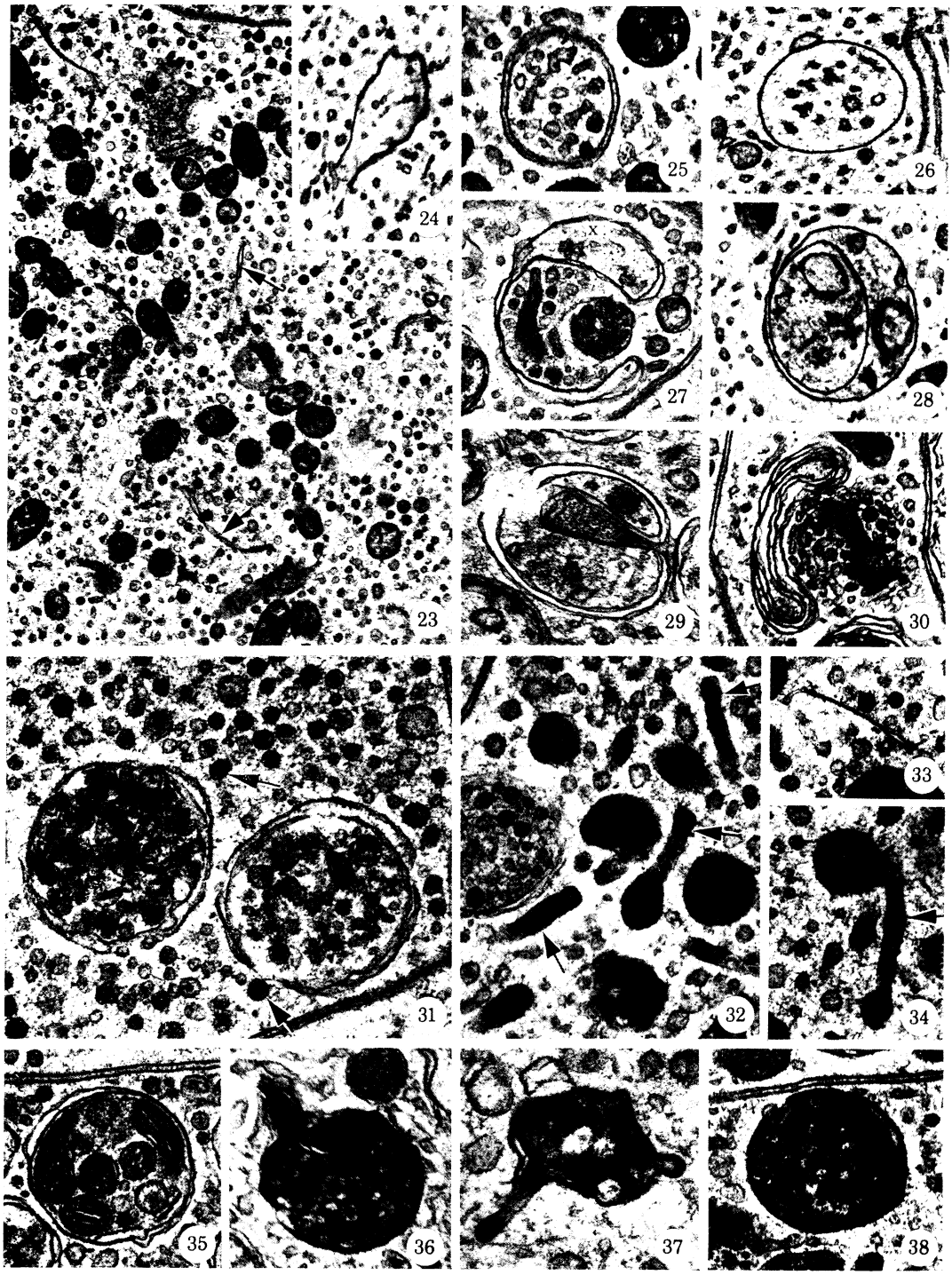


FIGURES 23 TO 38. For legends see facing page.

vesicles (e.g. figure 4) or clusters of mitochondria. By 38 h to 2 days postoperatively there is still little general evidence of distension of intraganglionic nerve fibres. Occasional profiles show an increase of vesiculo-tubular material and others show moderate accumulations of wispy material, with or without distension. In the few profiles which are distended with dilute accumu-

Description of plate 59

Figures 23–38. Formation of autophagic vacuoles. Membranous loops; dense-cored tubules.

Figure 23. Part of a distended axon filled with many dense-cored and other vesicles and containing numerous mitochondria. Among these organelles lie several loops or flattened sacs of membrane (e.g. arrows), which may be precursors of the membranous coverings of autophagic vacuoles. (38 h, region of organelle-rich accumulations.) × 29000.

Figure 24. Probable early stage of autophagic vacuole showing enclosure of an area of cytoplasm within a membranous loop. (6 days, intraganglionic axon.) × 30300.

Figures 25 to 30. Autophagic vacuoles of varying complexity showing various degrees of fusion of membranes with little alteration of contents.

Figure 25. An autophagic vacuole enclosed by paired membranes. It contains apparently unaltered vesicular material. (38 h.) × 27300.

Figure 26. An autophagic vacuole showing reduction of the enclosing membranes to a single dense membrane. Its contents resemble those of the surrounding cytoplasm. (24 h.) × 27300.

Figure 27. Possible stage in formation of a double autophagic vacuole by inclusion of a group of organelles (including dense-cored vesicles and two dense bodies) in a double-layered cup formed of an already apparently closed loop of membranes. More cytoplasmic material is enclosed between the walls of the cup, in its outer loculus (x). The membranes are double in most places, becoming reduced to a single membrane at some points, e.g. along the inner wall. (38 h.) × 33400.

Figure 28. One autophagic vacuole apparently contained within another; possibly a late stage of the process shown in figure 27. The enclosing membranes are almost everywhere reduced to a single layer. The contents of each compartment show slight darkening. (6 days.) × 28000.

Figure 29. An autophagic vacuole with double enclosing membranes, which shows a deep indentation with narrow neck and darkened contents. (2 days.) × 41400.

Figure 30. A compact mass of organelles partly surrounded by redoubled loops of membrane, which may be about to enclose them to form a complex autophagic vacuole or may have retracted from them (cf. figure 27). (38 h.) × 31000.

Figures 31 to 38 are taken from the region of organelle-rich accumulations, during the stage of intra-axonal digestion.

Figure 31. Two autophagic vacuoles lying in an area of axoplasm crowded with accumulated vesicles, many of which are dense-cored (e.g. arrows). The autophagic vacuoles have largely double (unfused) enclosing membranes, but their contents (which include dense-cored and other vesicles) show a patchy darkening, which appears to involve the cytoplasmic matrix more than the organelles. (38 h.) × 49000.

Figures 32 and 34. Tubules (e.g. arrows) with electron-dense content and a marginal halo. Some contain denser rounded masses, which may likewise have a distinct halo (figure 34). Small intra-axonal dense bodies, some with organelle-like inclusions, are also present, and some of the tubules show apparent continuity with these. (38 h.) × 40000.

Figure 33. A loop of partially fused membrane of the type which appears to enclose autophagic vacuoles (cf. figure 23). (38 h.) × 37000.

Figure 35. A number of mitochondria enclosed in an autophagic vacuole. The envelope clearly shows a partial reduction of double enclosing membranes to a single dense membrane. (38 h.) × 40000.

Figure 36. Apparent fusion of a narrow, lamellated dense body with a larger, rounded dense body (which has a partly double enclosing membrane and contains recognizable dense-cored and other vesicles). (3 days.) × 51500.

Figure 37. Dense body containing remnants of vesicles, with possible accretion of further vesicles, and perhaps also of electron-dense tubular material, from the surrounding axoplasm. (13 h.) × 51500.

Figure 38. Rounded dense body showing partly double enclosing membrane, extreme density of matrix and distortion of enclosed organelles, which include dense-cored vesicles. (38 h.) × 41500.

lations, multivesicular bodies may be very numerous (e.g. figure 16); others may contain cytoplasmic dense bodies. Scattered dense bodies containing altered axonal organelles are quite frequently seen in non-distended intraganglionic axons of otherwise normal appearance from this stage onward to about 7 days postoperatively, and this may indicate that some dense bodies are being transported retrogradely along the axons from the region of intra-axonal digestion close to the ligature. Such dense bodies are relatively uncommon in normal axons.

Changes in the intraganglionic neurons (cf. Matthews & Raisman 1972)

Over the first 2 days a chromatolytic reaction becomes established in the cell bodies, and there is also evidence of activation of an intracellular digestive process (involving cytoplasmic dense bodies and autophagic vacuoles). Various organelles which appear in the axonal accumulations begin to decrease in incidence in the cell bodies: these are dense-cored vesicles, clumps of small vesicles and the regular form of multivesicular body.

Changes in the axons distal to the ligature

The changes during the first 24 h resemble those reported by Kapeller & Mayor (1969*b*). At 6 h postoperatively clusters of mitochondria have begun to form in the segments of the axons from about 0.1 to 0.2 mm beyond the ligature, and the axons here are slightly distended. At 12 h postoperatively this region of the axon shows slight to moderate distension with dense accumulations of rather swollen mitochondria, which are associated with compact bundles of filaments and with quite numerous multivesicular bodies (of regular form, see later and cf. Matthews & Raisman 1972). The accumulations also contain scattered cytoplasmic dense bodies and relatively few vesicles, with only occasional dense-cored vesicles. By 3 days postoperatively, only a few degenerating remnants of axons containing mitochondrial accumulations are seen within 0.1 to 0.2 mm beyond the ligature. Beyond this point axonal fragments are rare, the debris having been digested within Schwann cells, and instead are seen many small profiles which are identified as newly formed processes from Schwann cells; they contain ribosomes and are therefore distinguishable from sprouts of axons.

The intra-axonal digestive process

The essential feature of this process appears to be the autophagic digestion of a large proportion of the organelles initially deposited in the proximal axonal stumps, i.e. it involves principally vesiculo-tubular material and dense-cored vesicles. The organelles are digested within autophagic vacuoles and cytoplasmic dense bodies, with a possible involvement of multivesicular bodies. The process is summarized diagrammatically in figure 39.

(1) *Cytoplasmic dense bodies.* Cytoplasmic dense bodies are uncommon in the axons of normal sympathetic neurons but are quite numerous in the cell bodies, where in the normal cell the typical form is of regular, approximately circular profile, 200 nm or more in diameter, with a single limiting membrane, a narrow electron-lucent halo and a rather uniformly granular dense content (Matthews & Raisman 1972). Dense bodies of this type have not been found in the intra-axonal accumulations. From the time of their first appearance the intra-axonal dense bodies show great variation and are markedly irregular in both form (figure 3) and content. Almost without exception they show some form of internal complexity (e.g. figures 10, 11, 20 to 22). Some of the earliest dense bodies are seen in profiles containing clustered mitochondria, and are elongated, showing a lamellated interior (figure 10). Dense bodies do not, however,

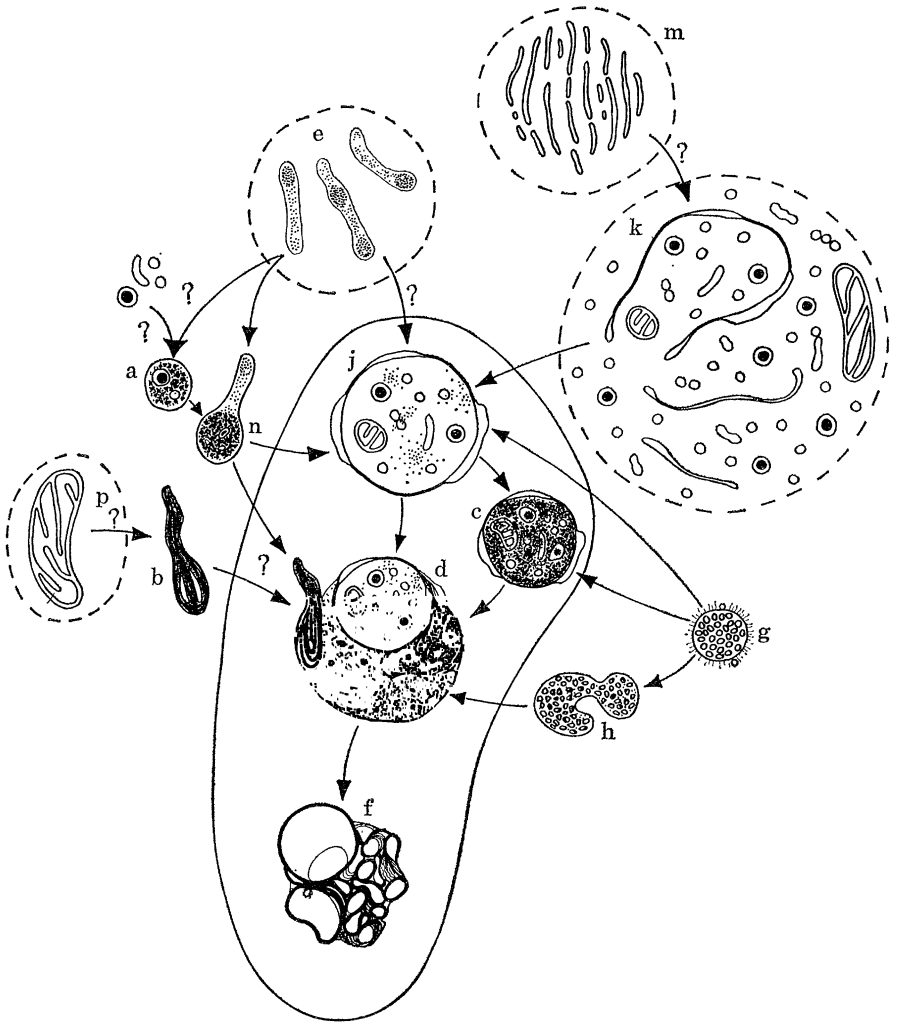


FIGURE 39. Schematic diagram illustrating organelles involved in the intra-axonal digestive reaction, and indicating probable sequences of inter-relationships between them (arrows).

Small intra-axonal dense bodies (a) may be derived from tubular material with a dense content (e), and may enclose and degrade axonal organelles directly. Further dense-cored tubular material may combine with them (n). Types a and n may become incorporated into autophagic vacuoles (j) or into larger, more complex dense bodies (d). Lamellar, elongated dense bodies (b) are found in association with clusters of mitochondria and may behave in a similar way. Autophagic vacuoles (j) appear to form by the enclosing of groups of axonal organelles within loops of double or partly fused membranes (k), which lie scattered in regions where autophagic vacuoles are becoming frequent. These membranes may be derived from groups of parallel flattened sacs or tubules (m), which are found slightly earlier at more proximal levels. The organelles enclosed are chiefly dense-cored vesicles and vesiculo-tubular material, but include also mitochondria. The autophagic vacuole darkens progressively and may give rise to one form of intra-axonal dense body (c). Either before or after this stage is reached it may become included within a large, complex dense body (d). Autophagic vacuoles may acquire digestive enzymes by the incorporation of dense-cored tubules (e), small dense bodies (a, n) and/or multivesicular bodies (g). The axonal multivesicular bodies (g) are initially of a regular form but may darken and show an irregular outline (h). Such large, irregular forms may arise by fusion. Some multivesicular bodies become included within dense bodies (d). Later, dense bodies evolve into multilocular, whorled lamellar forms (f), which may coalesce into massive aggregates.

become frequent in the axonal accumulations until the widespread formation of autophagic vacuoles has begun, between 24 and 38 h postoperatively. By this stage most of the dense bodies contain recognizable vesicles, including dense-cored vesicles (e.g. figures 11, 22, 38). Some of these dense bodies show lamellated portions which resemble the early lamellated dense bodies (figure 36). Forms are seen which suggest that vesicles are being added on at the periphery (figures 37, 38). Autophagic vacuoles are usually present within the same axonal profile. The dense bodies are often composite, showing more than one compartment, or containing a multivesicular body, or containing (or sometimes being contained within) an autophagic vacuole (figures 21, 22). Most of the larger dense bodies seen in the accumulations at this stage appear to be bounded, like autophagic vacuoles, by partly fused double membranes (figures 20, 21). It is therefore likely that some of the intra-axonal dense bodies arise by transformation of autophagic vacuoles, and that dense bodies receive further material for digestion by the incorporation of additional autophagic vacuoles, and perhaps also by the direct uptake of other organelles. There is no indication that the large regular cytoplasmic dense bodies of the cell soma migrate into the axonal accumulations to take part in the digestive reaction there. There is found, however, at the height of the autophagic reaction a type of tubular structure with dense content which might represent a transport form of primary lysosome carrying lytic enzymes (cf. Holtzman 1971). By 38 h postoperatively groups of these tubules are found quite commonly in the proximal parts of the organelle-rich accumulations. The tubules are of about 65 to 90 nm diameter, are bounded by a delicate membrane and show a narrow peripheral clear zone and an electron dense core of finely granular material (figures 32, 34). Their contents may be partly segmented into denser rounded masses, which themselves have a narrow halo and may slightly distend the profile (figure 34). Some of these tubules are seen in continuity with cytoplasmic dense bodies (figures 32, 34).

Within the first 2 days postoperatively most of the intra-axonal dense bodies still contain recognizable organelles. Later their contents become degraded, and darkened whorled forms are seen; a few of these are found already at 38 h (e.g. figure 11).

(2) *Multivesicular bodies.* Multivesicular bodies in neurons, as in other cell types, may be a form of lysosome, since some of their vesicles may show lytic enzyme activity (e.g. Holtzman 1969). The multivesicular bodies which are found in the axonal accumulations with increasing frequency from 12 h postoperatively resemble the 'regular' kind found in the cell bodies (Matthews & Raisman 1972), i.e. they are more or less uniformly and rather closely filled with vesicles of approximately uniform size (figures 8, 16, 17). In the regions of dilute accumulations, multivesicular bodies tend to be circular in profile and to contain spherical, clear-centred vesicles. They may show external haloes of radiating finely filamentous material, with which additional small vesicles are associated (figures 8, 17). In regions of intra-axonal digestion some multivesicular bodies are markedly irregular in outline (e.g. figures 19, 22), their vesicles tend to be flattened and darkened and the inter-vesicular matrix may show increased electron density. Many multivesicular bodies are seen to be incorporated into dense bodies, as described above (figures 18, 21). Not all the multivesicular bodies become involved in the autophagic reaction, however: some are seen later in the shafts and varicosities of profiles which have the characteristics of regenerative axonal sprouts.

(3) *Autophagic vacuoles.* In the zone of intra-axonal digestion at 38 h to 2 days postoperatively, autophagic vacuoles are seen at all stages of formation. Their contents represent all the organelles to be found in this region of the accumulations, in approximate proportion to their frequency

there (e.g. figures 22, 25 to 31, 35). There appears, however, to be a strong tendency to sparing of clusters of mitochondria and clumps of small vesicles.

The autophagic vacuole appears to form by the encircling of an area of cytoplasm by one or more closed loops or flattened sacs of membrane (figure 24) which then link up to enclose it (e.g. figures 22, 25). Within some profiles containing dense vesiculo-tubular accumulations, from 38 h postoperatively, loops or flattened sacs of membrane are seen scattered among the accumulated organelles (figure 23); these are straight or curved and lie singly, but otherwise they resemble the loops or sacs seen massed in the centres of profiles at an earlier stage (figure 7). The loop of membrane may appear fused in places to form a single denser membrane (figure 33). Complete fusion of the paired membranes may occur, leading to the encirclement of organelles by a single membrane, which is thicker and more electron-dense (figures 26, 41, 43, plates 59 and 60). Perhaps because of the frequency with which autophagic vacuoles are forming at this stage, many bizarre forms are seen, which show for example doubling of the loops of membrane to give a second autophagic vacuole enclosed within the first (figures 27 to 29).

Transformation of autophagic vacuoles into dense bodies (figures 20, 22, 31)

The autophagic vacuole next undergoes darkening, which begins patchily in the matrix and progressively involves the contents until the vacuole becomes indistinguishable from a cytoplasmic dense body of the form typical of the intra-axonal accumulations at this stage (e.g. figures 22, 38). The contained organelles are at first still recognizable, in particular the dense-cored vesicles, which retain their haloes and dense cores for some time. The degree of darkening of the autophagic vacuoles appears to be unrelated to the stage of fusion of their limiting membranes (e.g. figures 26, 31) and hence may be independently determined, e.g. by coalescence with primary lysosomes, possibly the tubules with dense content described earlier.

Group II: 2 *to* 7 *days*

During this period and particularly between 4 and 7 days the retrograde reaction in the nerve cell bodies reaches its maximum intensity in terms of morphological alteration, with extreme nuclear displacement, central chromatolysis of Nissl material and conspicuous central aggregation of grossly altered cytoplasmic dense bodies. The cytoplasm of the reacting neurons shows consistent reductions in the incidences of regular multivesicular bodies, dense-cored vesicles and clumps of small vesicles, and an increase in the incidence of autophagic vacuoles (Matthews & Raisman 1972). In addition, there appears a striking sprouting reaction at the surface of the cell body.

Accumulations close to the ligature

Over the same period the removal of the primary accumulations of organelles in the axons immediately proximal to the ligature is almost completed, and the region just proximal to the ligature becomes populated by a dense field of slender newly formed nerve fibres and associated Schwann cells.

At 3 *days* following the constriction the Schwann cells close to the ligature contain late phagosomes. These are highly electron-dense bodies which show stacks of concentric lamellae. These Schwann cells are loaded also with lipid-like material of the type described above, and some of them are dividing. Further proximally (within the first 0.4 mm proximal to the ligature) are seen variably distended and distorted axonal profiles containing some autophagic vacuoles

and many darkening cytoplasmic dense bodies. Most of the dense bodies are now multilocular, and the contained organelles are becoming indistinguishable and beginning to be replaced by multicentric whorls of lamellae. Within these axonal profiles mitochondrial clusters with associated filaments are still frequently seen, but in comparison with earlier stages there are reduced numbers of the other organelles typical of the earlier accumulations. The distended axons now show an increased incidence of coated vesicles and coated pits (from 38 h onward; cf. figure 22). Longitudinal orientation of the organelles and conspicuous numbers of microtubules are seen in many cases, suggesting the re-establishment of forward axoplasmic streaming. A zone of axons distended with dilute accumulations persists proximal to the level of the accumulated dense bodies, up to about 0.8 mm from the ligature. These axons show a high incidence of wispy material and in places many of them now lack Schwann sheaths altogether and lie grouped or singly in the interstitial spaces. Distal to and surrounding the distended axons are seen masses of newly formed fine, sometimes varicose nerve fibres.

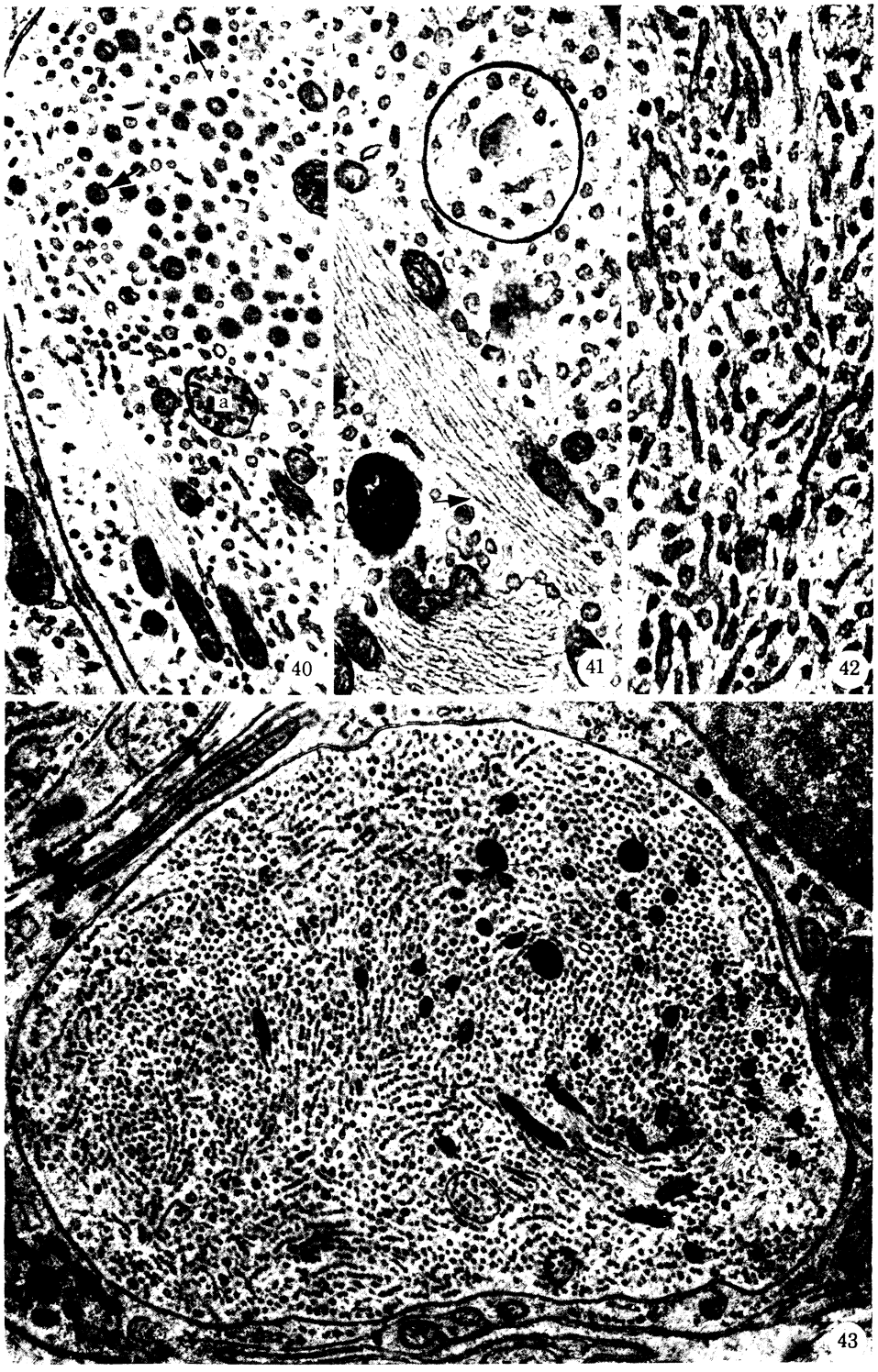
By 7 *days* following the constriction, remnants of the primary accumulations close to the ligature are seen as occasional profiles. These may be quite large, and contain many dark axonal dense bodies of a late form (showing multicentric whorls of lamellae with a tendency toward empty centres, e.g. figures 44 to 47, plates 61 and 62). Sometimes such profiles contain large numbers of mitochondria, of which many appear to be degenerating. The rest of the tissue is crowded here with many fine nerve fibres, which are beginning to become organized into groups associated with Schwann cells.

Accumulations at more proximal levels

Axonal profiles within the ganglion now (during the period 2 to 7 days) frequently show distension with various types of accumulations. These are located mainly within the first 3 mm of the point of ligation, but are also close to their parent cell bodies. Many of the intraganglionic axons develop localized swellings or varicosities linked by narrow interconnecting segments. Some of the distended profiles contain dilute accumulations, as at earlier stages. In addition, organelle-rich accumulations are seen which are of two main types, one consisting essentially of vesiculo-tubular material and the other largely of dense bodies. These reflect the successive stages seen in the earlier accumulations close to the ligature, but here they may occur in adjacent axons or in consecutive varicosities of the same axon, and they differ in other respects from the earlier accumulations.

(1) The profiles containing vesiculo-tubular material are often enormously distended (e.g. figure 43). Their contents are typically arrayed in swirling formations, which suggest local eddying movement rather than axial flow. Relatively fewer dense-cored vesicles are included than in the earlier accumulations, and multivesicular bodies and clusters of small vesicles are absent or rare. Scattered or grouped mitochondria are present, and these are often intimately alined along bundles of filaments (figures 40, 41, 43). Coated vesicles of various sizes may be rather numerous in these profiles, and tend to occur in groups (figure 40). These vesicles are by no means always clear-centred, but may show a darkened interior, dense cores or a variety of other inclusions (figure 40 and cf. Morris, Hudson & Weddell 1972).

(2) The many cytoplasmic dense bodies which are found in the second main type of accumulation tend to be of a late whorled and multilocular form, but are often associated with numerous autophagic vacuoles at various stages of formation and transformation into dense bodies (figures 44 to 47). The range of stages of lytic bodies occurring in such profiles (from early



FIGURES 40 to 43. Vesiculo-tubular material, coated vesicles, association of mitochondria with filaments.

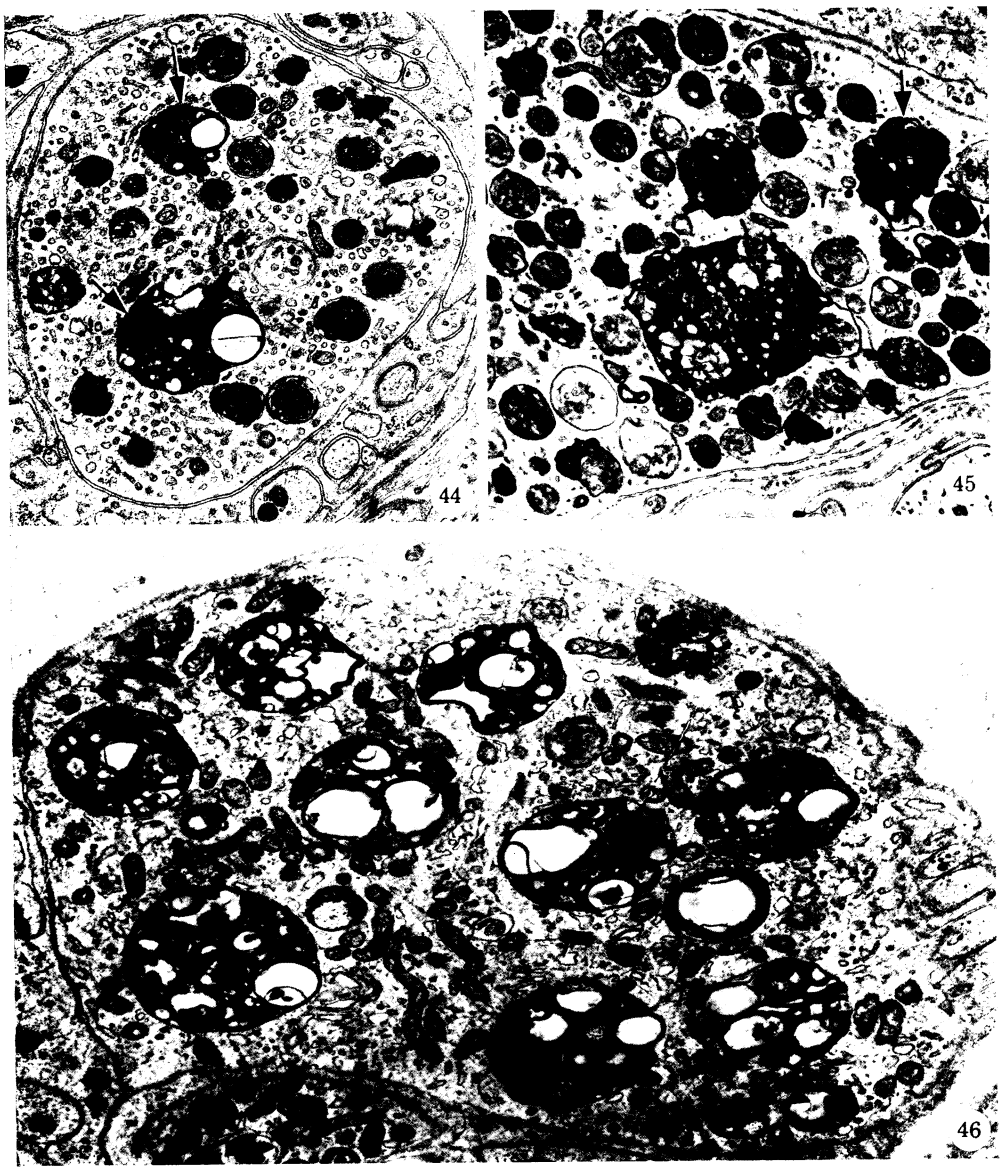
FIGURE 40. Part of a distended axonal profile containing in its upper half a large cluster of coated (alveolate) vesicles (e.g. arrows), which vary considerably in their size and content. The profile also contains vesiculo-tubular material, some of which is alined parallel with a bundle of filaments which runs with associated mitochondria along the left border of the profile. a, autophagic vacuole, which appears to be only partially closed. (6 days, intraganglionic axon.) × 21 000.

FIGURE 41. An area of axoplasm containing an autophagic vacuole, vesiculo-tubular material, a small dense body and compact bundles of filaments with mitochondria (and microtubules, arrow) alined at their margins. (7 days, intraganglionic axon.) × 40 000.

FIGURE 42. Part of a distended axon containing vesiculo-tubular material in mainly tubular form. (6 h, organelle-rich accumulations, 0.1 mm from ligature.) × 39 000.

FIGURE 43. A greatly distended axon containing vesiculo-tubular material in the form chiefly of rows of vesicles, arrayed in swirling patterns, with occasional tubules of similar or slightly smaller diameter. Mitochondria with associated bundles of filaments are also present, chiefly in the lower right quadrant. The mitochondria and filaments, which are mutually alined, are cut in some places longitudinally and in others transversely. (6 days, intraganglionic axon.) × 16 600.

(*Facing p. 492*)

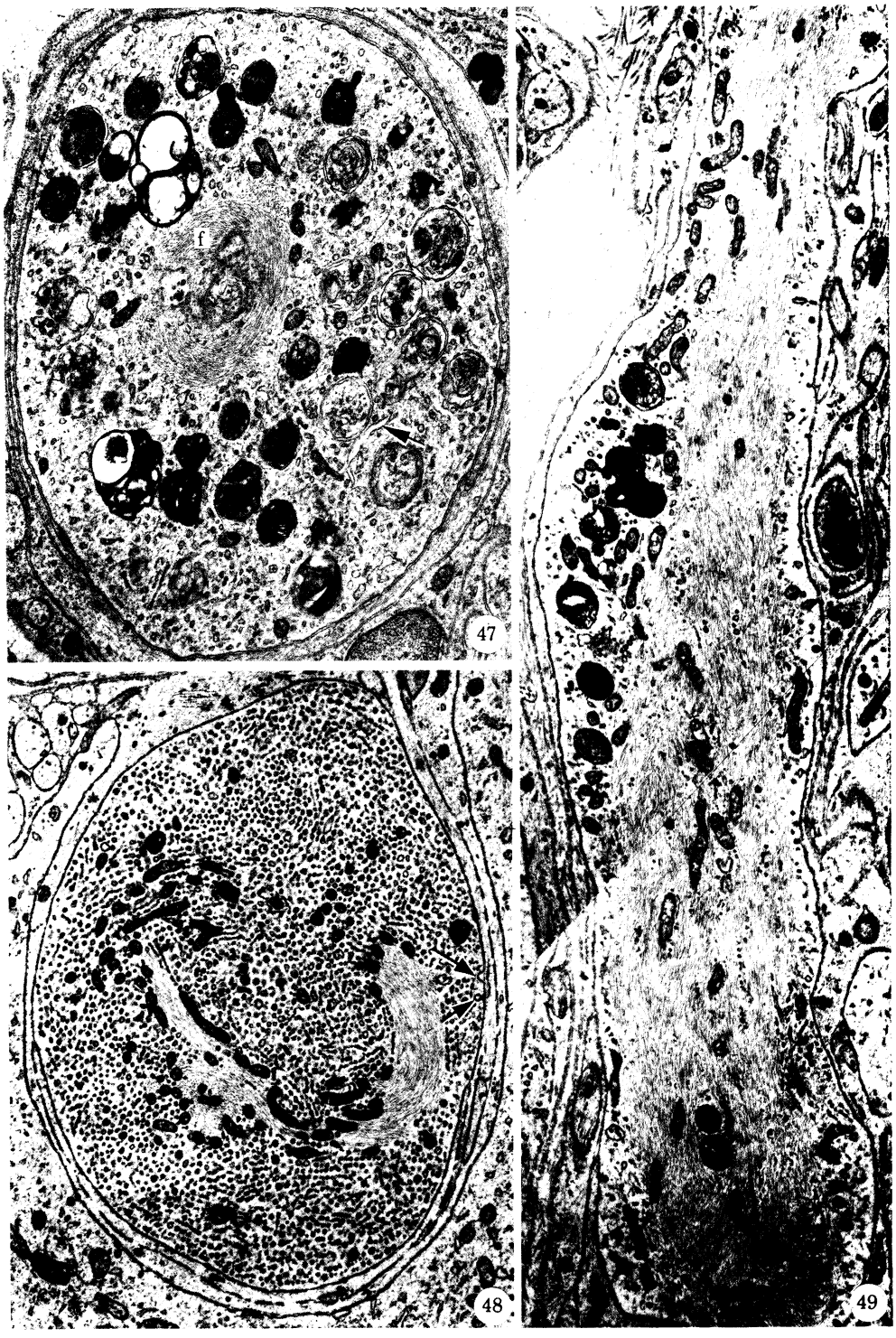


FIGURES 44 to 46. Late forms of dense bodies in intraganglionic axons; agglomerated forms.

FIGURE 44. A distended axon containing vesiculo-tubular material, some mitochondria and a variety of dense bodies and autophagic vacuoles. Arrows, large dense bodies of late agglomerated form showing multicentric highly electron dense whorled lamellar formations with electron lucent centres. (7 days.) × 15600.

FIGURE 45. Part of a much distended axon filled with large numbers of dense bodies and autophagic vacuoles, some of which are coalescing with each other (e.g. arrow) and with a large dense multilocular agglomerate. (6 days.) × 12000.

FIGURE 46. Intraganglionic profile containing a number of large dense agglomerates with multicentric whorled lamellar form, showing electron lucent locules, and several smaller dense bodies of basically similar, but more unitary form. Many mitochondria and some vesicles and sacs of agranular reticulum are also present, the latter in places arranged as in the Golgi formation. This profile is likely to be the base of a large cell process. (7 days.) × 15800.



FIGURES 47 to 49. Cores of filaments in intraganglionic axons.

FIGURE 47. A distended axon profile with a central looped core of parallel cytoplasmic filaments (f). A few mitochondria lie close to the margin of the filament bundle. This may represent an early stage of formation of an organized core. The surrounding area contains vesiculo-tubular material, a loop of membrane (arrow) and a variety of dense bodies, autophagic vacuoles and forms intermediate between the two. The dense bodies include stages of transition to forms showing highly electron dense multicentric whorls with electron lucent locules. (7 days.) × 15000.

FIGURE 48. A distended axon with a central looped core of parallel filaments flanked by alined mitochondria. The rest of the profile is packed with swirling rows of vesicles and tubules, and a few small dense bodies (cf. figure 41). The centre of the profile contains a cluster of coated (alveolate) vesicles (cf. figure 38), and two coated pits possibly representing the formation of coated vesicles are seen at its right border (arrows).

FIGURE 49. A longitudinal section of part of an axon containing a massive core of axially oriented filaments, with which are associated alined mitochondria and rows of vesicles and tubules (vesiculo-tubular material). A narrow peripheral region of the profile is free of filaments but contains mitochondria and vesicles associated with the margin of the bundle; at one point this zone is expanded and contains also some dense bodies and autophagic vacuoles. Coated pits and/or coated vesicles are seen at several points along the surface membrane, especially along the right border of the profile. (6 days.) × 12300.

(*Facing p. 193*)

autophagic vacuoles to late dense bodies) may be much wider than is found in the earlier accumulations close to the ligature at the height of the digestive process, and this suggests that they have formed over a longer period. There is evidence of coalescence of earlier with later forms of lytic bodies (e.g. figure 45); and the complexity of the larger multicentric whorled forms in some profiles suggests that they may have been derived by such a mechanism (figures 44 and 46). This process of coalescence is one way in which the accumulated dense bodies may become condensed over the long term.

Group III: 7 *to* 14 *days*

Over this period the ganglionic neurons are beginning to revert towards a more normal appearance: the nuclear displacement and crenation are becoming less marked, and the cytoplasmic dense bodies are nearing the end of the cycle of change which they undergo.

Accumulations within axonal profiles in the ganglion show predominantly the later forms of dense bodies, and autophagic vacuoles are less numerous, although vesiculo-tubular material may still be present in profiles containing late dense bodies. A highly characteristic complex array of mitochondria and neurofilaments is now seen in many of the distended axonal profiles, where it typically occupies a central position. This array has a central core of densely packed parallel filaments which is usually coiled or looped within the profile (figures 41, 47, 48, plates 60 and 62) but may run longitudinally (figure 49, plate 62). The core of filaments is flanked by elongated mitochondria of small, circular cross-sectional profile, which lie in direct apposition with the filaments and are oriented along the long axis of the filament bundle (figures 48, 49). Microtubules may also be seen alined at the margin of the bundle of filaments (e.g. figure 41). Vesiculo-tubular material, if present, tends to be alined along the filament bundle only where it immediately adjoins the filaments (figure 48). Similar or even larger bundles of filaments with associated mitochondria have been found within cell bodies, both at 12 h close to the lesion and from 7 days onward further away (cf. Matthews & Raisman 1972). These bundles of filaments tend to lie with one edge closely apposed to the nuclear membrane, partly encircling the nucleus, and may be seen to extend from the cell body into the base of a large process. (Such cells at longer survivals are generally shrunken and show a pyknotic nucleus and loss of many organelles.)

Group IV: 14 *to* 143 *days*

At longer survival intervals many ganglionic neurons continue their gradual return to a more normal appearance, and some are becoming atrophic, while others may appear hyperchromatic (Matthews & Raisman 1972).

In the nerve fibres within the ganglion there is a similar return to a more normal appearance. The occurrence of dense bodies and vesiculo-tubular material is progressively more infrequent, and at the longer survivals only an occasional fibre shows distension, typically with a dense agglomeration of late whorled forms of dense bodies.

The fate of the dense bodies in the fibres is not clear, but occasional profiles of Schwann cells have been seen to contain within their cytoplasm condensed, darkened profiles filled with massed dense bodies of an axonal type (e.g. figure 14), which suggests a sequestration and digestion as phagosomes of axonal segments packed with dense bodies. Neither is it clear what happens to axonal profiles distended with other types of accumulations. Histograms of fibre diameters suggest a loss of some of the larger fibres at these late stages (Matthews & Raisman, in preparation),

but this could have occurred by shrinkage of distended segments. It is possible that organelles and accumulated material which have not been degraded are transported away proximo-distally along the axon when the axons are sufficiently regenerated.

Discussion

This study of sympathetic axons after ligation or section close to the cell body has shown that the material which accumulates in the axons proximal to the ligature changes in character with time following the injury. The initial accumulations are succeeded by a wave of material of varied nature which includes precursors of lytic bodies, and this is followed by material characteristic of the stage of regrowth of the axon. The cycle of changes is well-marked, begins early and proceeds rapidly; this is probably due to the close proximity of the lesion to the cell bodies (within 1 to 4 mm).

Nature of initial accumulations

1. *Organelles*

The organelles which accumulate proximal to the ligature in the first 12 h are essentially vesiculo-tubular smooth endoplasmic reticulum, dense-cored vesicles and mitochondria. The proportion of dense-cored vesicles in the initial accumulations is probably not as high as has been reported for constricted hypogastric and splenic nerves (Kapeller & Mayor 1967, 1969*a*; Geffen & Ostberg 1969). All the organelles which are seen can be found, but with a sparser distribution, in normal adrenergic axons (cf. also Elfvin 1958; Kapeller & Mayor 1967). No major changes in the appearance of these organelles are seen between 6 and 12 h postoperatively, apart from early signs of degeneration close to the lesion.

These initial accumulations of organelles therefore probably represent material which is being carried along the axon in the axoplasmic flow at the time of application of the ligature and for some period thereafter. Comparable early accumulations of vesiculo-tubular material have been reported in numerous studies of crushed or ligated axons (see Lubińska 1964; Zelená *et al.* 1968; Pellegrino de Iraldi & De Robertis 1968; Kapeller & Mayor 1969*a*), and various interpretations have been offered as to the origin of this material, e.g. local formation from dilated microtubules (Pellegrino de Iraldi & De Robertis 1968). In the present experiments, microtubules appear to be clearly distinguishable from vesiculo-tubular material, and it is thought probable that this has come virtually unaltered from the cell body.

The speed of accumulation of such a large volume of vesiculo-tubular material and dense-cored vesicles relatively to the volume present in an equivalent length of an uninterrupted axon suggests that these organelles have been transported by the rapid component of the axoplasmic flow (e.g. Dahlström 1965, 1971; McEwen & Grafstein 1968; Karlsson & Sjöstrand 1968; Lasek 1968*a*, *b*, 1970), which has been reported to proceed at rates of 100 to over 400 mm/day in various systems. Studies with radioactive tracers indicate that the rapid component carries material related to the secretory (transmitter) function of the axon terminals (e.g. Schonbach, Schonbach & Cuénod 1971; Hendrickson & Cowan 1971; Karlsson & Sjöstrand 1971*c*); nerve ligation experiments show that in adrenergic nerves this material includes noradrenaline (Dahlström 1965, 1971; Kapeller & Mayor 1967; Banks *et al.* 1969; Boyle & Gillespie 1970) and dopamine β-hydroxylase (Livett, Geffen & Rush 1969), both of which are contained within the dense-cored vesicles of adrenergic nerves (De Potter, Smith & De Schaepdryver 1970). In cholinergic nerves acetylcholinesterase accumulates with a rapid time-course above an obstruc-

tion (Zelená & Lubińska 1962; Lubińska & Niemierko 1971), and acetylcholinesterase has been identified within some of the vesicles and tubules of intra-axonal smooth endoplasmic reticulum (Brzin, Tennyson & Duffy 1966; Schlaepfer & Torack 1966; Kása 1968). In adrenergic axons, however, it is not at present clear what is contained within the smooth endoplasmic reticulum which comprises the vesiculo-tubular material.

Observations on doubly ligated nerves and on isolated nerve segments show that the rapid transport of material related to transmitter function is predominantly centrifugal, almost entirely so in the case of noradrenaline (Dahlström 1965, 1967; Banks *et al.* 1969; Geffen, Hunter & Rush 1969); and this is reflected in the ultrastructure of the early accumulations distal to the ligature (Kapeller & Mayor 1969*b*; present experiments).

2. *Amorphous axoplasm*

The material carried in the rapid axoplasmic flow is largely particulate (McEwen & Grafstein 1968; Kidwai & Ochs 1969; Sjöstrand 1970; Elam & Agranoff 1971); the slow component of the flow consists largely of soluble axoplasm and carries certain soluble enzymes (e.g. dopa decarboxylase, Dahlström & Jonason 1968), but includes also structural protein, e.g. microtubule protein (Grafstein, McEwen & Shelanski 1970; Karlsson & Sjöstrand 1971*b*). Four rates of axoplasmic flow have recently been described by Karlsson & Sjöstrand (1971*a*). In the present experiments the nerve fibres for some distance proximal to the accumulated organelles were from an early stage distended with dilute accumulations of finely granular or amorphous axoplasm containing few organelles. It is possible that this represents soluble axoplasm carried in a slower flow component of the axoplasm and excluded from the tip of the fibre by the accumulation of organelles carried in the most rapid component. It might also be augmented by soluble axoplasm displaced backward from the region closest to the ligature by the forcible crowding of the accumulated organelles, since the forward flow which produced the organelle-rich accumulations had sufficient force in the present experiments to cause disarray of the microtubules (cf. Martinez & Friede 1970), and to distend the axons to four or more times their original diameter, thereby obliterating the interstitial spaces and disrupting the Schwann cell envelopes of the axons. The sharp line of demarcation sometimes observed between accumulated organelles and amorphous axoplasm suggests some cohesive linkage between the rapidly transported organelles and that component of the transport mechanism by which they are moved, i.e. probably the microtubules. The rapid axoplasmic flow is thought to be mediated via microtubules (Schmitt 1969) because, like microtubule structure, it is susceptible to damage by colchicine (Dahlström 1968; Karlsson & Sjöstrand 1969; Kreutzberg 1969; Banks, Mayor, Mitchell & Tomlinson 1971) and by cold (Rodríguez Echandía & Piezzi 1968). Smith (1971) has observed an association of vesicles with side-arms of microtubules in lamprey axons.

Decrease in rate of accumulation of material with time

The length of axon occupied by the densely packed organelles of the initial accumulations proximal to the lesion did not increase rapidly after the first 6 to 12 h following ligation; a comparable finding was reported by Zelená *et al.* (1968). By this stage the process of digestion of the accumulations was not yet far advanced, so it is likely that the rate of accumulation of these organelles had decreased. This implies a reduction in the output of this material from the cell bodies at this stage. In studies involving measurement of the rate of accumulation of transmitter-related material proximal to second ligatures applied at different times after a first, it has been

shown that this rate becomes reduced at some stage during the first few days following the initial ligation, both in cholinergic nerves (Hebb & Silver 1965) and in adrenergic nerves (Boyle & Gillespie 1970; Karlström & Dahlström 1970; Dahlström 1971).

By the time the quantity and/or nature of material sent out from the cell have become altered the cell must also have received and responded to whatever may be the signal for chromatolysis (cf. Cragg 1970). The time of onset of such a response probably depends on the distance of the ligature from the cell body. In the present experiments the ligature was applied close to the cell body, and the signs of a change in the output of material from the cell were early. The onset of the chromatolytic reaction in the cell bodies was similarly early (cf. Härkönen 1964), and was detectable in over 60 % of neurons at 12 h postoperatively (Matthews & Raisman 1972). Chromatolysis was accompanied by a change in the incidence of dense-cored vesicles (of about 80 to 110 nm diameter) in the cell bodies, which had begun to fall by 38 h postoperatively and was consistently below normal from 3 days onwards to at least 14 days postoperatively in the retrogradely reacting neurons (Matthews & Raisman 1972). This *per se* does not necessarily indicate a reduced output of dense-cored vesicles, but suggests such a reduction when taken together with the reduced incidence of dense-cored vesicles in the later accumulations in intra-ganglionic axons.

Change in nature of accumulated material with time

The output of other types of material from the cell bodies may also become altered. Of the organelles which appear secondarily in increasing numbers in the intra-axonal accumulations, the autophagic vacuoles and the cytoplasmic dense bodies appear to be formed locally, but the other organelles (including multivesicular bodies, clumps of small vesicles, flattened sacs of membrane and segments of dense-cored tubules) are probably transported along the axons from the cell bodies. These organelles are not numerous in normal sympathetic postganglionic axons (cf. Elfvin 1958; Kapeller & Mayor 1967; Hökfelt 1969). Kapeller & Mayor (1969*a*) noted clumps of small vesicles in the proximal stumps of constricted sympathetic axons from 8 h onward. It is not clear whether all these organelles form part of an unaltered slower axoplasmic flow, piling up at the point at which flow is impeded by the initial accumulations, or whether the quantity transported increases, or the nature of the organelles changes, in response to axonal injury. The degree of localized accumulation of these organelles suggests at least an increase in their output from the cell relative to the output of material of the initial accumulations. A similar consideration may apply to the wispy intra-axonal material which appears slightly later, at the stage of regenerative axonal sprouting (Matthews & Raisman, in preparation).

Mitochondria appear to behave differently from the other organelles. The mitochondrial clusters which form from an early stage at the proximal ends of the organelle-rich accumulations may be derived both from a secondary accumulation of mitochondria moving centrifugally along the axon and also by retrograde movement of mitochondria out from among the initial accumulations; these clusters are characterized by their homogeneity, i.e. the virtual exclusion of other organelles. It is just beyond the level of the mitochondrial accumulations that the intra-axonal digestive reaction tends to be most intense, and the regenerative sprouting of new nerve fibres begins close to this level. The mitochondrial clusters might be functionally involved in either or both of these processes.

Organelles of the secondary axonal accumulations

1. *Multivesicular bodies and clumps of small (dense-cored) vesicles*

Both the multivesicular bodies and the clumps of small vesicles are apparently transported along the axon from the cell body without undergoing major structural modification. The *multivesicular bodies* which accumulate in the axons are of a 'regular' form, like some of those found in the cell soma of the sympathetic neurons (Matthews & Raisman 1972) and in their dendrites, especially near to postsynaptic sites (e.g. Matthews & Raisman 1969). However, within the cell body and basal dendrites are also found multivesicular bodies which are 'irregular' in form (irregular both in contour and in the size and distribution of their internal vesicles, and often containing a small dark dense body; Matthews & Raisman 1972). Such irregular multivesicular bodies are rare in the axonal accumulations in the present material, although Elfvin (1958) illustrates one in his study of normal splenic axons. He found multivesicular bodies to be relatively common in these axons. Multivesicular bodies in general are regarded as a form of lysosome (e.g. Smith & Farquhar 1966), and the same function has been ascribed to neuronal multivesicular bodies (Holtzman, Novikoff & Villaverde 1967; Holtzman 1969, 1971), since acid phosphatase activity has been demonstrated in some of the internal vesicles. Holtzman (1971) has concluded (from experiments involving ferritin as a marker) that multivesicular bodies in neurons are concerned specifically with the uptake (and degradation/digestion) of exogenous material. In the present experiments they were quite frequently seen to become involved in the formation of autophagic vacuoles and dense bodies in the axonal accumulations, and in such regions they often underwent a darkening of vesicles and matrix and became irregular in contour. It is possible therefore that the regular multivesicular bodies are also preferentially concerned in processes of intracytoplasmic digestion outside the cell body. That they may have yet other functions is suggested by their frequent occurrence at postsynaptic sites in the dendrites, by their presence in the regenerating axonal sprouts (Matthews & Raisman, in preparation) and also by their accumulation, together with many mitochondria and bundles of cytoplasmic filaments, in the proximal ends of the distal stumps just beyond the ligature (present study and Kapeller & Mayor 1969*b*).

The *clumps of small vesicles*, some with dense cores, which are seen in the axonal accumulations are of particular interest. They closely resemble similar clumps which are found in the cell bodies and dendrites of the sympathetic neurons (cf. Taxi 1965; Grillo 1966; Hökfelt 1969; Taxi, Gautron & L'Hermite 1969; Elfvin 1971*a*, *b*; Matthews & Raisman 1972). They are also very similar to the small dense-cored vesicles of the terminal varicosities of sympathetic axons (Richardson 1962, 1964; Wolfe, Potter, Richardson & Axelrod 1962). Occasional clumps of small vesicles have been reported in normal postganglionic axons (Elfvin 1958; Hökfelt 1969), and are found to show dense cores after permanganate fixation (Hökfelt 1969). The demonstration of cores in such vesicles depends on the fixation, and may not be optimal in osmium fixed material (but cf. Pellegrino de Iraldi, Gueudet & Suburo 1971). Within the intra-axonal accumulations, as in the cell body and dendrites and in the more proximal axons, the clumps of small vesicles retain their clumped arrangement, behaving as if coherent, and this suggests that the vesicles are inter-linked. The incidence of such clumps and of regular multivesicular bodies in the cell bodies of the reacting neurons (like that of the larger dense-cored vesicles) had begun to fall by 38 h after axonal injury and was consistently reduced over the period 3 to 14 days postoperatively (Matthews & Raisman 1972). In the intra-axonal accumulations

the clumps of small vesicles are seldom found within autophagic vacuoles or dense bodies; similar small vesicles, some with dense cores, are later found in rows and small groups in the stems and varicosities of the newly formed axonal sprouts (Matthews 1972). The origin of the small dense-cored vesicles of the sympathetic terminal varicosities is uncertain; it has been suggested that they are formed locally in the varicosities in various possible ways, e.g. from the larger dense-cored vesicles which are carried in the rapid axoplasmic flow (for review see Geffen & Livett 1971). The present observations, while not excluding the possibility of local formation of small dense-cored vesicles in the axon terminals, suggest that those which are also present as clumps within the cell body may pass out along the axon in response to an axonal injury, since the incidence of these clumps in the proximal parts of the axons rises at a stage when they are becoming scarce in the cell body. The acquisition of small dense-cored vesicles by the newly formed sprouts would have the effect of reconstituting at an early stage the apparatus of the terminal varicosity (Matthews 1972).

2. *Autophagic vacuoles and intra-axonal dense bodies*

In the present experiments these organelles do not appear to be transported preformed along the axons from the cell bodies, but instead appear to be formed locally in the axon as part of an intra-axonal digestive reaction (see figure 39, p. 489). This reaction is particularly intense during the second postoperative day, and coincides in time with the onset of a process of intracytoplasmic digestion within the cell soma (Matthews & Raisman 1972). Numerous authors have reported dense bodies and/or formations suggesting autophagic vacuoles in cut or ligated axons (e.g. Wettstein & Sotelo 1963; Holtzman & Novikoff 1965; Blümcke *et al.* 1966; Zelená *et al.* 1968; Kapeller & Mayor 1969*a*), including axons of central nervous pathways which do not regenerate (Schlote 1966; Lampert 1967; Barron & Doolin 1968). Such formations also occur in the distal axonal stumps (Schlote 1966), and here small lamellated dense bodies and some multivesicular bodies are regularly found together with the mitochondria which typically accumulate there (Blümcke *et al.* 1966; Holtzman & Novikoff 1965; Kapeller & Mayor 1969*b*). The origin of the various bodies is, however, somewhat uncertain. An interpretation is offered below, based largely on the observations reported here.

Autophagic vacuoles are uncommon both in normal neuronal cell bodies (Holtzman *et al.* 1967; Holtzman 1969; Matthews & Raisman 1972) and in normal axons (cf. Elfvin 1958; Kapeller & Mayor 1967, 1969*a*). Nor are they typical of the initial accumulations, which collect within the first few hours postoperatively. They appear first in the proximal regions of the intra-axonal accumulations of organelles and become very numerous by 38 h postoperatively, when they are seen apparently at all stages of formation, enclosing organelles characteristic of the surrounding accumulations. At about the same time autophagic vacuoles increase in incidence in the cell body (Matthews & Raisman 1972) but here they most often contain organelles which are typical of the cell body rather than the axons, such as granular endoplasmic reticulum and ribosomes. It would therefore appear that it is precursors of the axonal autophagic vacuoles that are carried along the axons from the cell bodies, rather than pre-existing autophagic vacuoles.

Similar considerations apply to the *cytoplasmic dense bodies* which appear in the intra-axonal accumulations. Such dense bodies are infrequent in normal postganglionic axons (e.g. Elfvin 1958; Kapeller & Mayor 1967, 1969*a*). They are very varied in form, having typically a complex internal structure, and do not resemble the 'normal' type of uniformly granular large

cytoplasmic dense bodies of the cell soma. Neither do they resemble the altered somatic dense bodies concurrently seen in the same neurons in the early stages of the reaction to axonal injury. Within the obstructed axons dense bodies begin to appear toward the proximal end of the organelle-rich accumulations, and at first tend to lie among clusters of mitochondria. These dense bodies may be elongated and lamellar in form, and it has been suggested that such forms may represent altered mitochondria (Webster 1962; Lampert 1967; but cf. Holtzman & Novikoff 1965). Others of these early dense bodies are rounded and already contain axonal organelles. Dense bodies later become very numerous in regions of autophagic vacuole formation, and here they show a variety of more complex forms which suggest, *inter alia*, fusion of dense bodies with autophagic vacuoles and/or multivesicular bodies, and transformation of autophagic vacuoles into dense bodies (figure 39, p. 489). The later whorled forms of dense bodies found in the axons from 3 days onward resemble the whorled dense bodies found in the cell soma at such times, and this convergence of form probably represents a common late stage of digestion, indicating a more rapid degradation of protein with the persistence of phospholipids; it is known that these latter may precipitate from solution into whorled lamellar patterns (Swift & Hruban 1964).

Since the autophagic vacuoles and dense bodies appear to form locally within the axons from precursors which are presumably transported from the cell body, two questions arise: first, what is the nature of these precursors, and secondly, in which component of the axoplasmic flow are they carried? The following lines of evidence suggest that the precursor materials do not normally travel in the rapid axoplasmic flow, at any rate in sufficient quantity to account for the intensity of the digestive process.

(1) The formation of dense bodies and autophagic vacuoles does not begin immediately after the axonal ligation, but after an interval of 12 to 24 h, and does not reach its maximal level before the second postoperative day.

(2) The process does not begin until certain other types of material, including multivesicular bodies, have begun to reach the proximal ends of the accumulations in increasing quantity.

(3) The intra-axonal digestive process begins within the proximal end of the organelle-rich accumulations and does not penetrate to their most distant regions. This is what would be expected if a later wave of primary lysosomal material met the accumulated organelles at this point and were impeded from penetrating more distally into the accumulations by a disturbance of axonal transport mechanisms consequent upon the presence of accumulations. The distal regions of the accumulations tend instead to show autolytic changes (darkening of the cytoplasmic matrix, and swelling and deformation of organelles), and are phagocytosed by Schwann cells.

(4) The level of activity of acid phosphatase, a lysosomal enzyme, is low in normal nerves and is initially low adjacent to a crush lesion in nerve, but rises later, at a stage when lysosomal activity is becoming well marked (Holtzman & Novikoff 1965). Since protein formation does not occur to any great extent in axons this suggests that lysosomal enzymes, presumably contained within primary lysosomes (De Duve & Wattiaux 1966; Novikoff 1967; De Duve 1969; Holtzman 1969, 1971), have travelled within the axon as a slower or later component of the axoplasmic flow. The output of such material from the cell bodies may become altered as part of the response to the axonal injury.

Nature of the precursor material of autophagic vacuoles and dense bodies (cf. figure 39)

Limiting membranes for the formation of autophagic vacuoles appear to be provided by the *flattened cisternae (or slender tubules) of smooth endoplasmic reticulum*, somewhat resembling the inner lamellae of the Golgi apparatus, which are first seen grouped in the centres of profiles and later scattered singly among the material of the initial accumulations, in regions where autophagic vacuoles are being formed. These cisternae or tubules are probably a transport form of membrane, and possibly also of lytic enzymes (cf. Sotelo & Palay 1971) for the initiation of autophagic processes in the axon. Membranes of this appearance have been seen enclosing groups of organelles in the early stages of degeneration of preganglionic nerve terminals in the rat superior cervical ganglion (Matthews, in preparation). It is not certain whether these sacs of membrane contain lytic enzymes, i.e. are primary lysosomes (cf. De Duve 1969), but similar membranes in axons may contain acid phosphatase (Sotelo & Palay 1971). Numbers of autophagic vacuoles are however seen in the present material (especially in the later intraganglionic accumulations) which do not show any sign of darkening or degradation of their contents, although their limiting membranes may have reached the stage of complete fusion. This last observation suggests that the enclosing of organelles by limiting membranes could be independent of the acquisition by these autophagic vacuoles of a full complement of digestive enzymes, in neurons as it is in other cells (De Duve & Wattiaux 1966). Other enzymes involved in the autophagic process might be provided by the observed incorporation into autophagic vacuoles of pre-existing dense bodies, of multivesicular bodies and even of mitochondria (which contain monoamine oxidase). In addition, the tubules with dense content which are found in groups in axons at the height of the autophagic reaction may also contribute lytic enzymes. Tubules of this kind have been seen in continuity with dense bodies in the axonal accumulations, and similar tubules are found near the ends of Golgi profiles in the cell bodies at this time (Matthews & Raisman 1972). These might correspond to the acid-phosphatase-containing tubules of the GERL region (Novikoff *et al.* 1971). Lysosomal enzymes have been demonstrated in tubular structures of comparable form in axons (acid phosphatase, Holtzman & Novikoff 1965; aryl sulphatase, Holtzman 1971). Such tubules therefore probably represent an intra-axonal transport form of primary lysosomes (cf. Novikoff 1967; De Duve 1969; Holtzman 1971). Of all the forms seen among the intra-axonal dense bodies in the present study, the dense-cored tubules most closely resemble the type of uniformly granular cytoplasmic dense body which is seen in the cell body; they have a similar internal texture and a halo of comparable width, and they differ most notably in their shape, their small diameter and in the partial segmentation of their contents. In size they are about equal to the smallest of the dense bodies seen in the cell body, and at about this stage (38 h to 2 days) the latter show an increased incidence in some cell profiles in the ganglion, later becoming less frequent than normal.

Difference in the process of intracellular digestion in the axon from that occurring in the cell body

The predominant forms of lysosomes in axon and soma differ considerably and so also, apparently, does the material which is being digested. In the axonal accumulations it is principally organelles which are being digested, via uptake into autophagic vacuoles, which become transformed (or incorporated) into cytoplasmic dense bodies; the formation of these lytic structures apparently depends upon transport forms of primary lysosomal material, as discussed above. In the cell body, although autophagic vacuoles are also seen, the form of

lysosome which is most numerous is the pre-existing regularly granular form of cytoplasmic dense body, characteristic of the cell soma. Both autophagic vacuoles and dense bodies increase in incidence in the soma during the reaction to the axonal lesion. The relatively few autophagic vacuoles take up organelles, principally ribosomes, granular endoplasmic reticulum and mitochondria. The many dense bodies in the soma undergo a series of changes, which involve increasing complexity of internal form, such as the development of stacks or whorls of lamellae, reverting finally to a granular internal structure (Matthews & Raisman 1972). This sequence of changes suggests a process of digestion, but it is not certain what is being digested within these dense bodies.

In the axon the dense bodies form differently, and although they do show a late whorled stage there is not a later conversion within the axon to a uniformly granular form. The ultimate fate of the axonal dense bodies is not certain, but it is possible that some pass retrogradely in the axons toward the cell body, and there is some evidence for eventual phagocytosis by Schwann cells of accumulations of late dense bodies in axons. A remarkable level of intracellular organization is suggested by a response to injury which activates simultaneously appropriate mechanisms for digesting apparently different types of material in different forms at such widely separated sites.

Intra-axonal mitochondria: accumulations and transport

The early localized clustering of mitochondria in association with loosely arranged cytoplasmic filaments, in both proximal and distal axonal stumps, and the later formation in many axons of dense axial bundles or loops of filaments, closely flanked by alined mitochondria, are consistent features of the reaction to axonal injury. Changes of this nature have been found both in peripheral nerves (e.g. Webster 1962; Zelená *et al.* 1968; Kapeller & Mayor 1969*a*, *b*; Morris *et al.* 1972) and in the central nervous system (Lampert 1967; Barron & Doolin 1968). The formation of massive bundles of neurofilaments in damaged axons recalls the neurofilamentous type of orthograde degeneration seen in certain nerve terminals in the central nervous system after axotomy (Colonnier & Guillery 1964; Gray & Guillery 1966; Guillery 1970). Bundles and swirling masses of filaments with peripherally alined mitochondria have also been seen in severely reacting cell bodies in the present experiments (Matthews & Raisman 1972); and clustering of mitochondria in the cell body is typical of the retrograde chromatolytic reaction of ganglionic neurons (e.g. Härkönen 1964; Holtzman *et al.* 1967; Matthews & Raisman 1972).

Masses of alined or tangled filaments are known to develop in neuronal and other cell bodies when the microtubule system for rapid transport of organelles has been disrupted by colchicine or by substances with similar action (e.g. Wisniewski, Shelanski & Terry 1968; Goldman 1971). The nature of the transport mechanism for mitochondria is not yet certain, but it does appear that mitochondria can move in axons independently of microtubules. Thus, colchicine is found not to prevent the transport of mitochondrial enzymes in axons (Kreutzberg 1969; Banks *et al.* 1971), and mitochondria can move bidirectionally in axons (Zelená 1968) at a rate intermediate between the most rapid and the slowest components of the axoplasmic flow (Lasek 1970; Karlsson & Sjöstrand 1971*a*). The alinement of mitochondria along the axes of bundles of parallel filaments, in situations in which the rapid axoplasmic flow is interrupted or obstructed, suggests the possibility of translocation of mitochondria along or together with the bundles of filaments. The mechanism which produces localized clustering of mitochondria might have some importance for the survival of the injured neuron.

In conclusion, the findings reported in this paper, taken together with earlier observations, allow a more precise interpretation of the accumulative and resorptive processes that occur during the response of a peripheral neuron to axonal injury. The main gain is the recognition of a coordination of changes which depends upon an interplay of happenings in the axonal stump and in the cell soma.

Summary

In 48 Wistar rats either or both of the major postganglionic branches of the superior cervical ganglion, i.e. those accompanying the internal and external carotid arteries, were ligated (39 ganglia) or cut (12 ganglia) within 1 to 2 mm of the ganglion. Postoperative changes in the axons both close to the lesion and more proximally, within the ganglion, were studied at intervals ranging from 6 h to 143 days. Control observations were made in unoperated contralateral ganglia and in sham-operated ganglia, and additional observations in ganglia from normal animals.

At 6 h postoperatively the postganglionic axons were swollen with accumulated material for about 0.6 mm proximal to the ligature. In the first 0.3 to 0.4 mm proximal to the ligature they contained close-packed vesiculo-tubular material, 60 to 110 nm dense-cored vesicles and some mitochondria. In the swollen region proximal to this, similar organelles were widely scattered in granular or amorphous axoplasm. Over the next 12 to 30 h increasing numbers of other organelles (secondary accumulations) appeared towards the proximal end of the intra-axonal accumulations. These included increasingly compact groupings of mitochondria, associated with cytoplasmic filaments. Some of the secondarily accumulating organelles, such as clumps of small vesicles (some with dense cores), multivesicular bodies of a regular form, flattened sacs or double lamellae of membrane, and tubules of smooth endoplasmic reticulum with an electron dense content (dense-cored tubules), appeared to be transported from the cell body along the axon. Others, such as autophagic vacuoles and dense bodies, seemed to form locally within the intra-axonal accumulations, and to form part of a reaction of intra-axonal digestion of the accumulated material, involving particularly the organelles of the initial accumulations. The flattened sacs of membrane, dense-cored tubules and possibly the multivesicular bodies appeared to provide material for the formation of autophagic vacuoles and dense bodies. Close to the ligature the distal regions of the congested axons degenerated and were phagocytosed, chiefly over the first 2 to 3 days, by Schwann cells, which become rounded up, accumulated lipid-like intracytoplasmic material and showed mitotic activity. Groups of small (including dense-cored) vesicles and larger dense-cored vesicles became distributed to regenerative axonal sprouts which began to appear from 24 to 38 h postoperatively and were very numerous by the third day. A type of wispy intra-axonal material was also associated with the sprouting reaction. This reaction is not considered in detail in the present paper. The results are discussed in relation to other studies and it is concluded that the relative output of various types of material from the cell bodies may change as a consequence of axonal injury. The phase of intra-axonal digestion is found to coincide in time with a phase of intracytoplasmic digestion within the cell bodies of these retrogradely reacting neurons, and yet to differ from it both in the nature of the material digested and in the predominant type of lysosome involved. Mitochondria may be importantly concerned in the regenerative reorganization. The overall reaction to axonal injury is considered to be a highly coordinated activity, occurring partly in the cell body and partly in the axon, and involving correlation of events in widely separated parts of the neuron.

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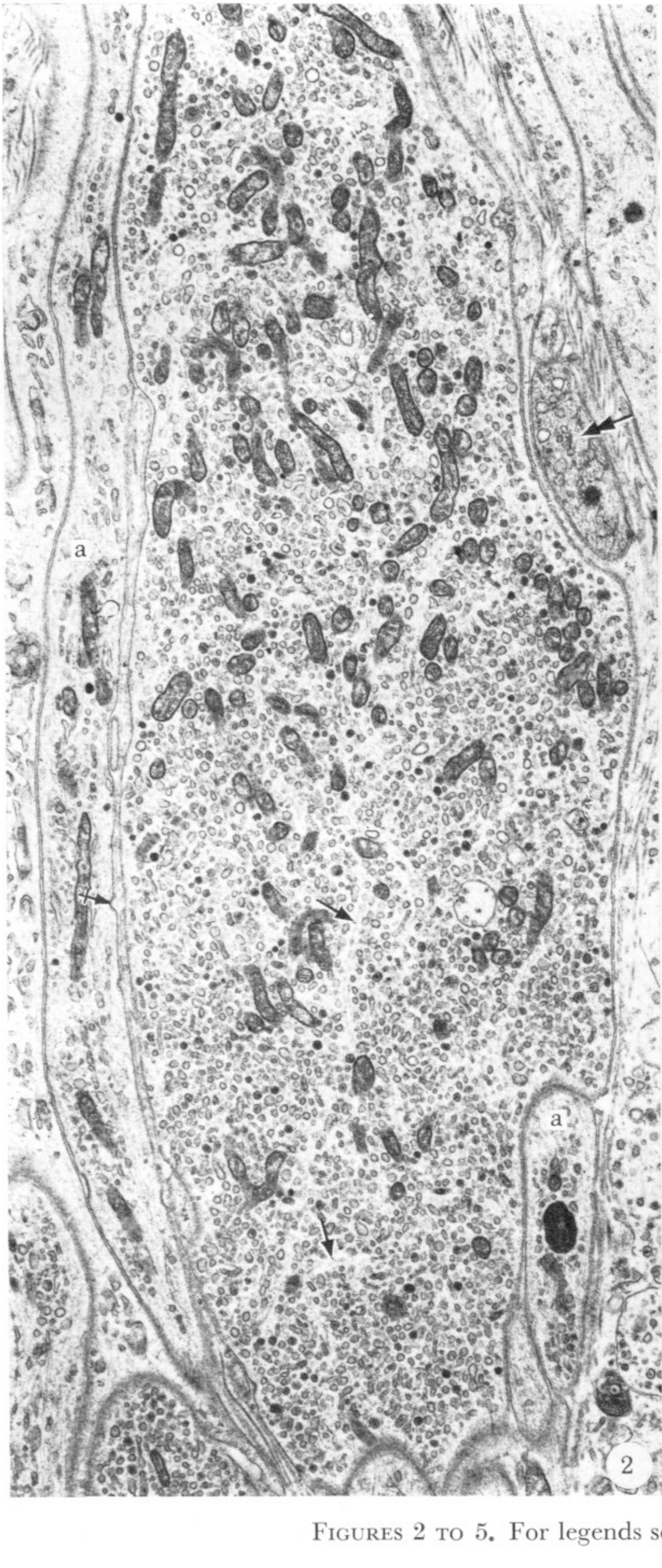
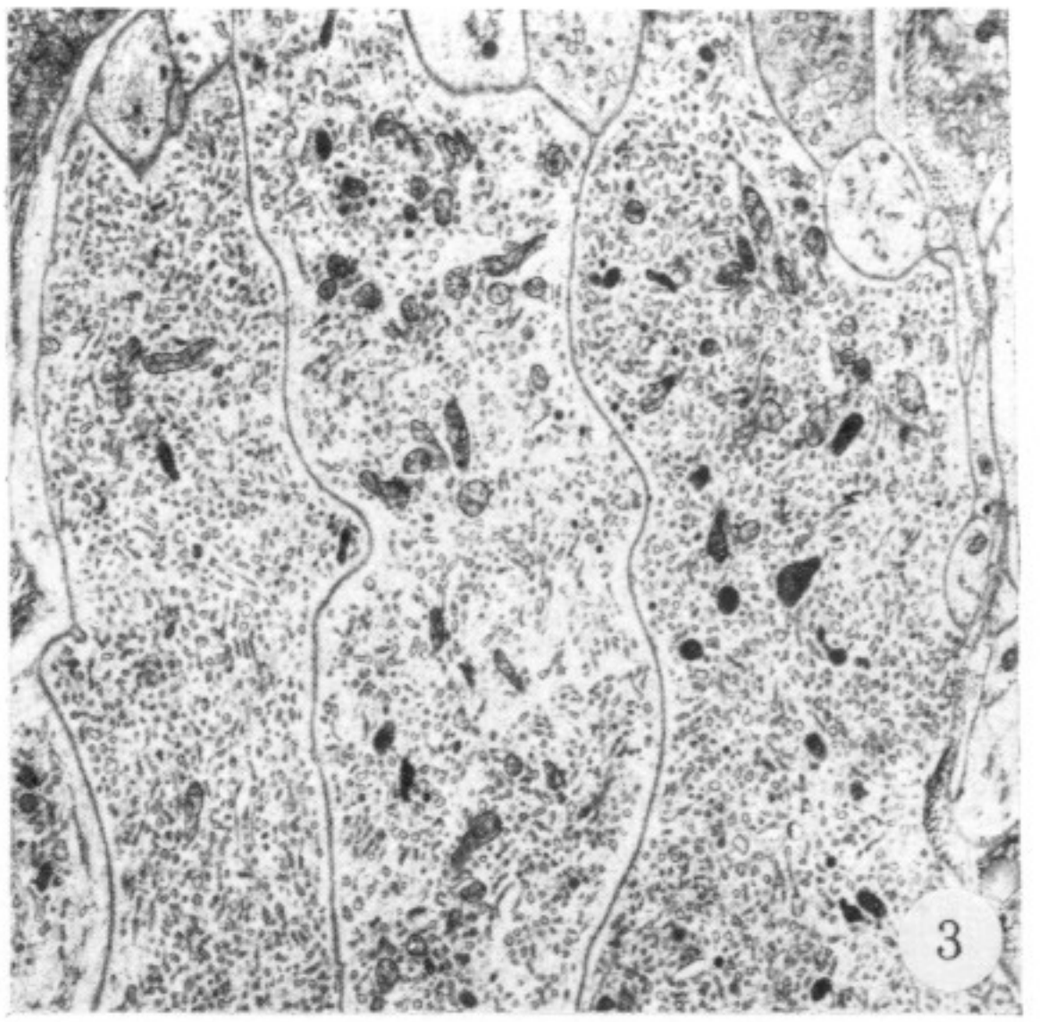
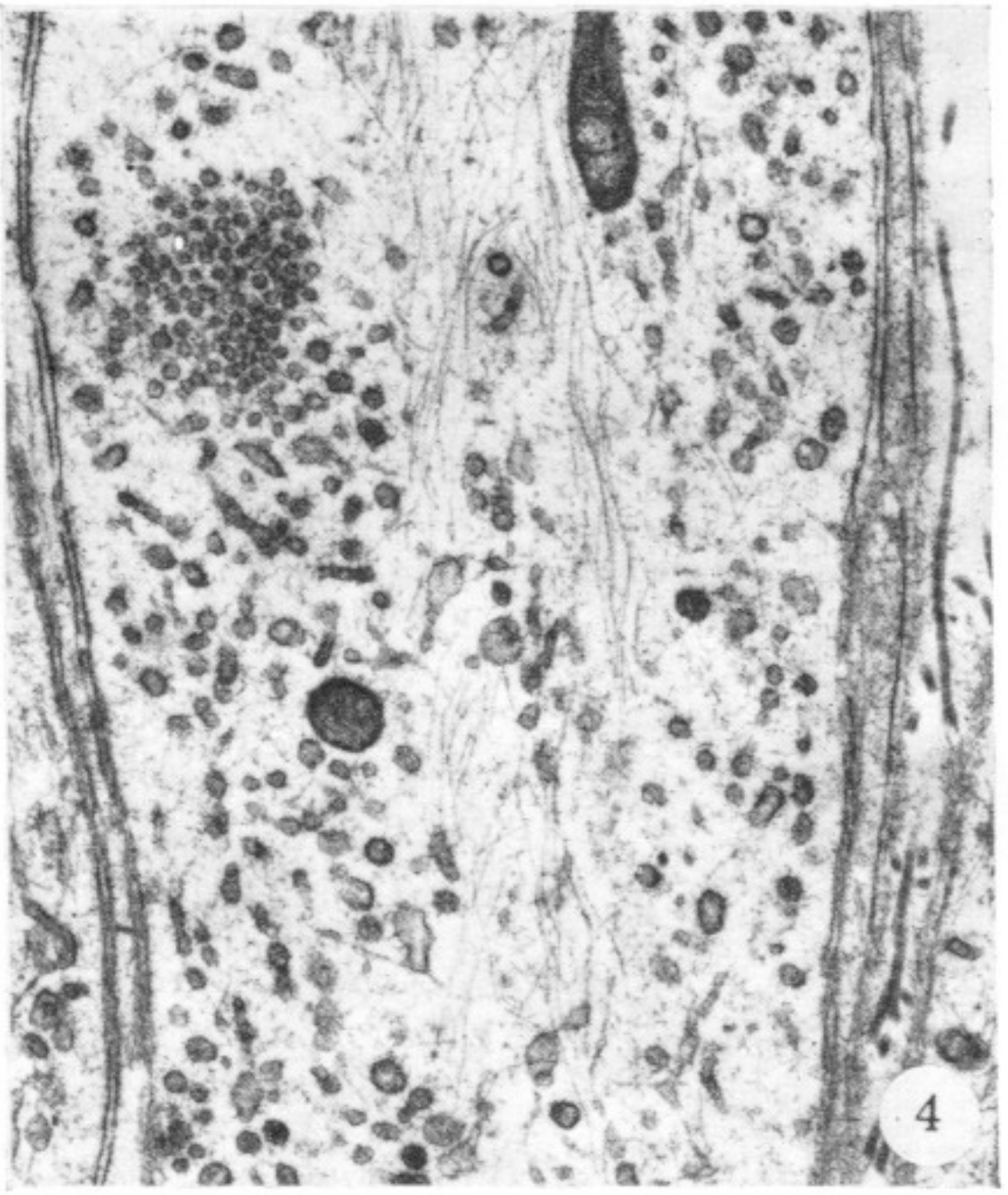
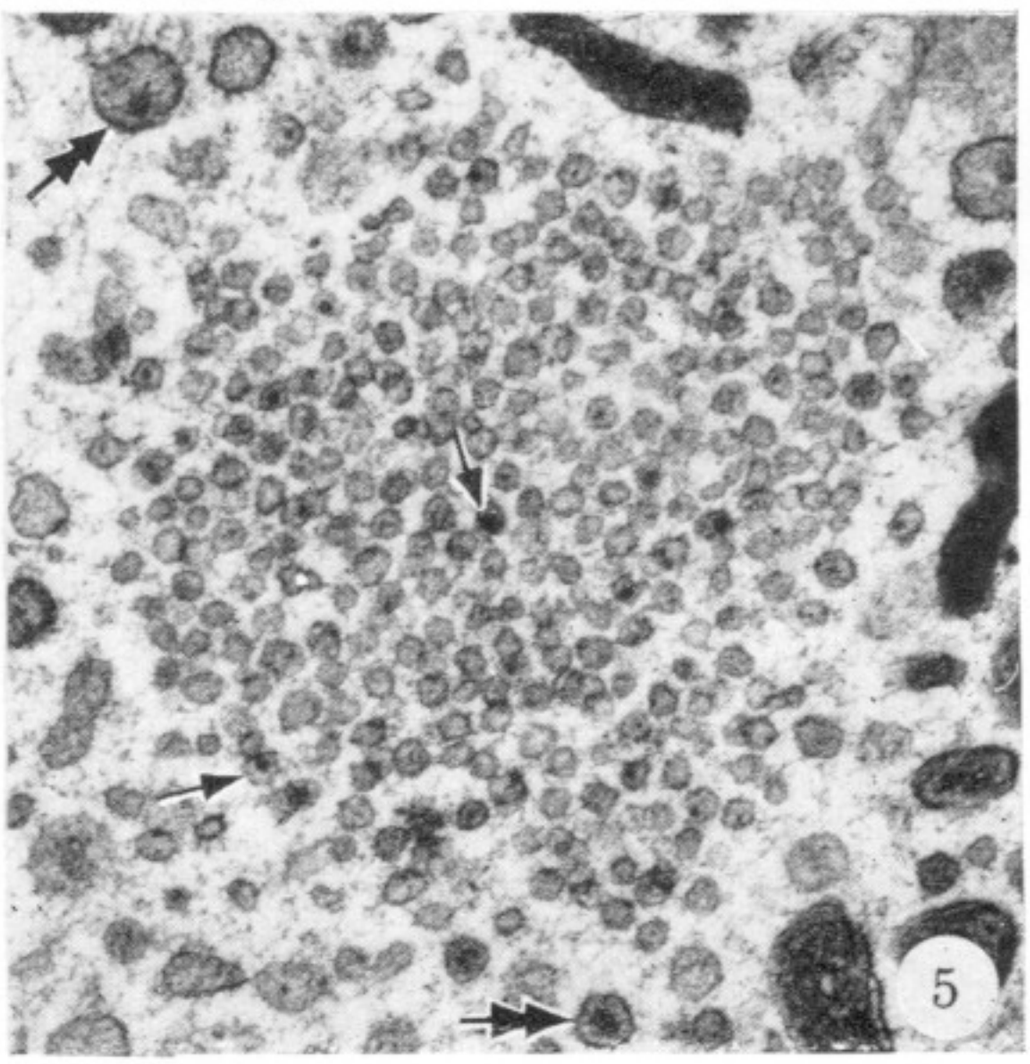
FIGURE 1. Light micrographic montage of 2 μm section passing through the ligated internal carotid nerve and cranial tip of a superior cervical ganglion, 6 h after ligation. The section was stained with a mixture of methylene blue and Azur II. Scale bar = 0.1 mm. L, ligature material (10/0 monofilament nylon), including parts of the knot. At the level of the ligature and immediately adjacent to it above and below is a compressed zone showing very intense staining. Proximal to this for about 0.4 mm the axons are rather deeply stained. In this region the electron microscope shows them to be greatly distended and packed with organelles. Further proximally (arrows) there is an abrupt change to a region in which the axons are still distended but show unusually light staining. This extends to the level of the closest ganglionic neurons, which form a compact group in the lower left part of the figure. Bundles of axons (double arrows) among the ganglion cells show a normal diameter and staining reaction.

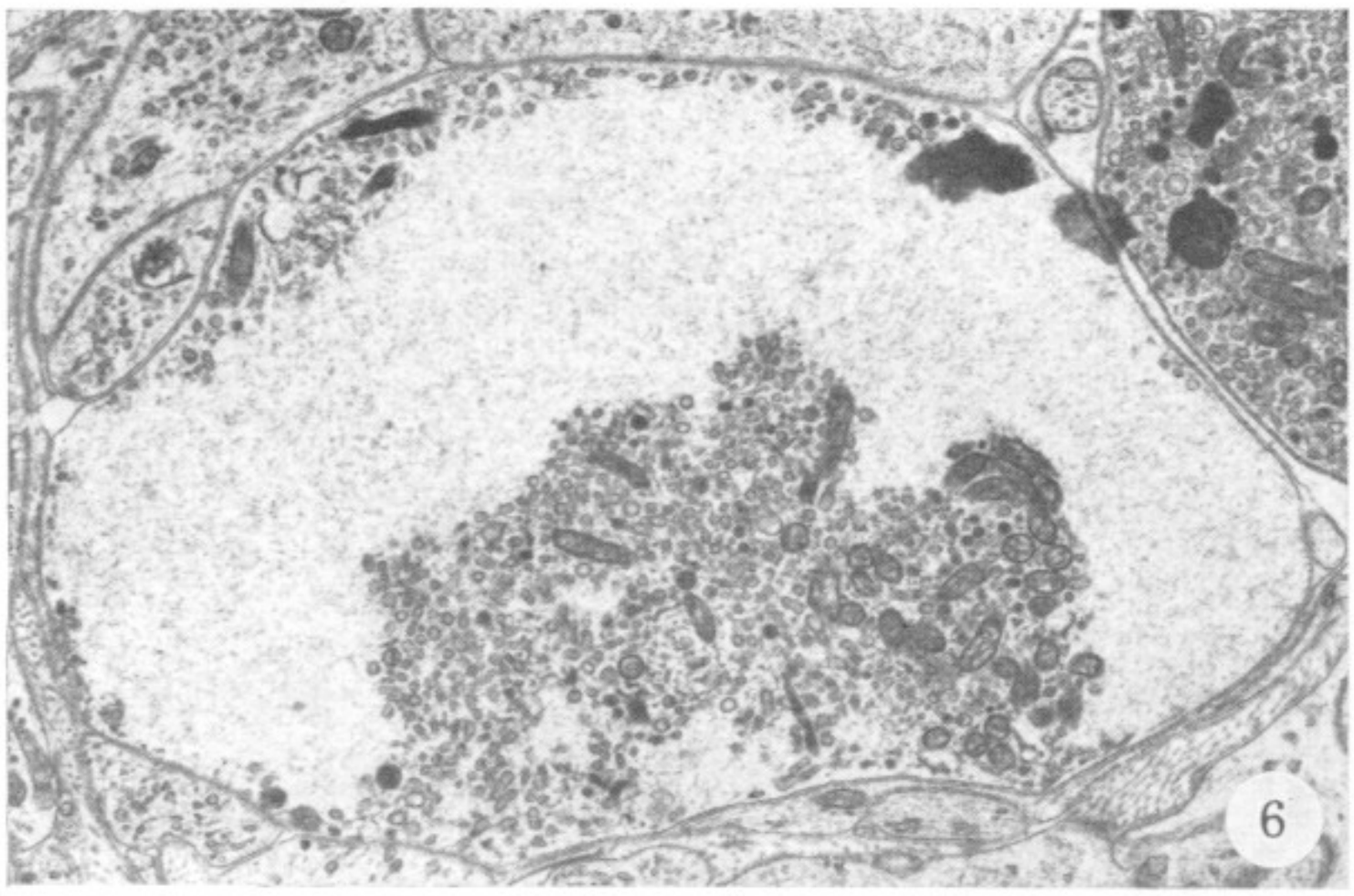


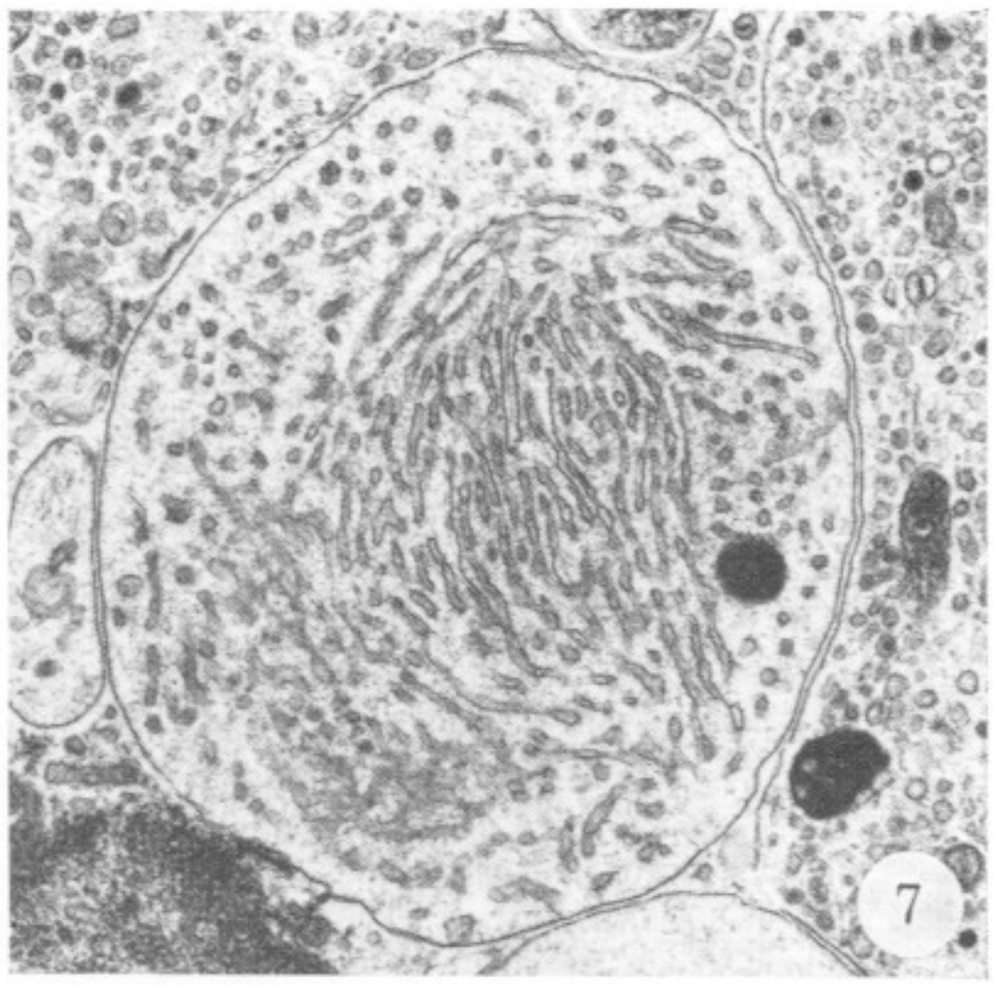


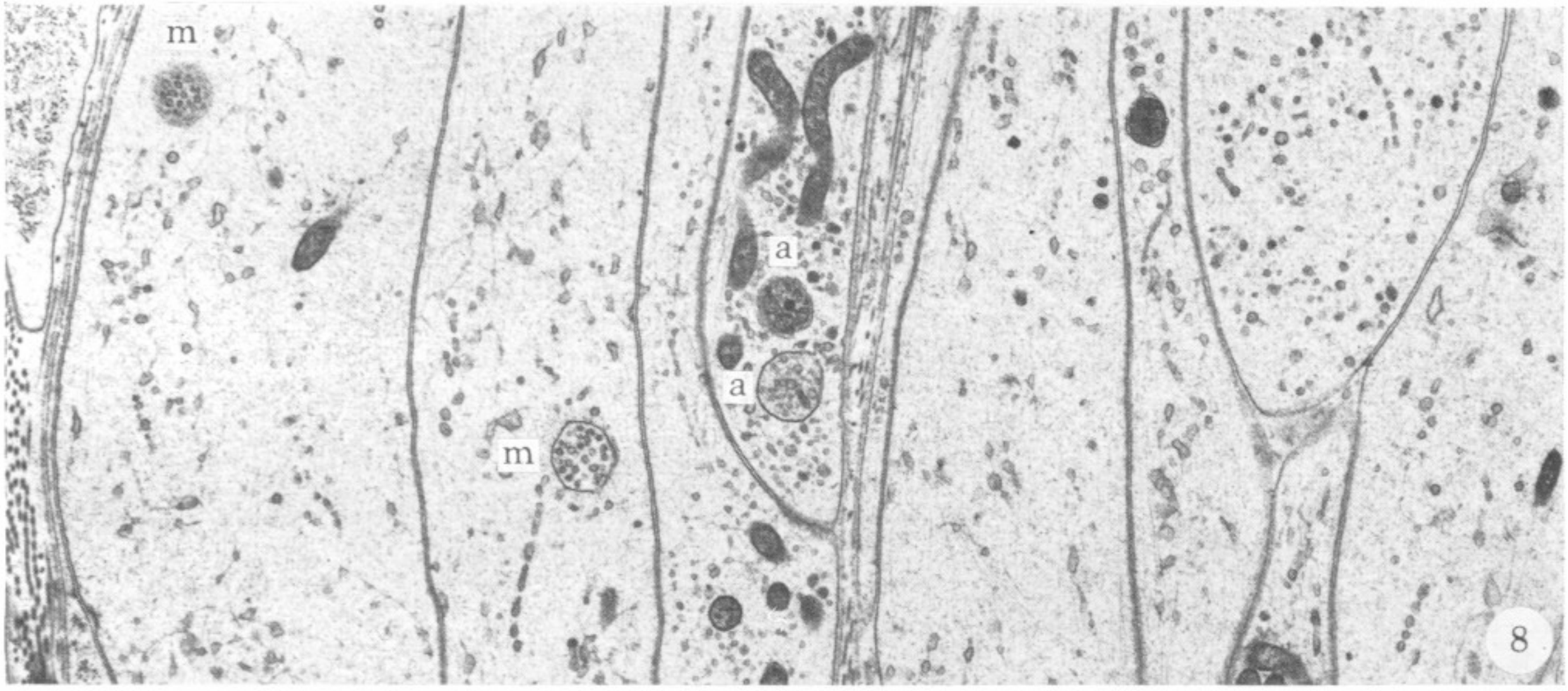


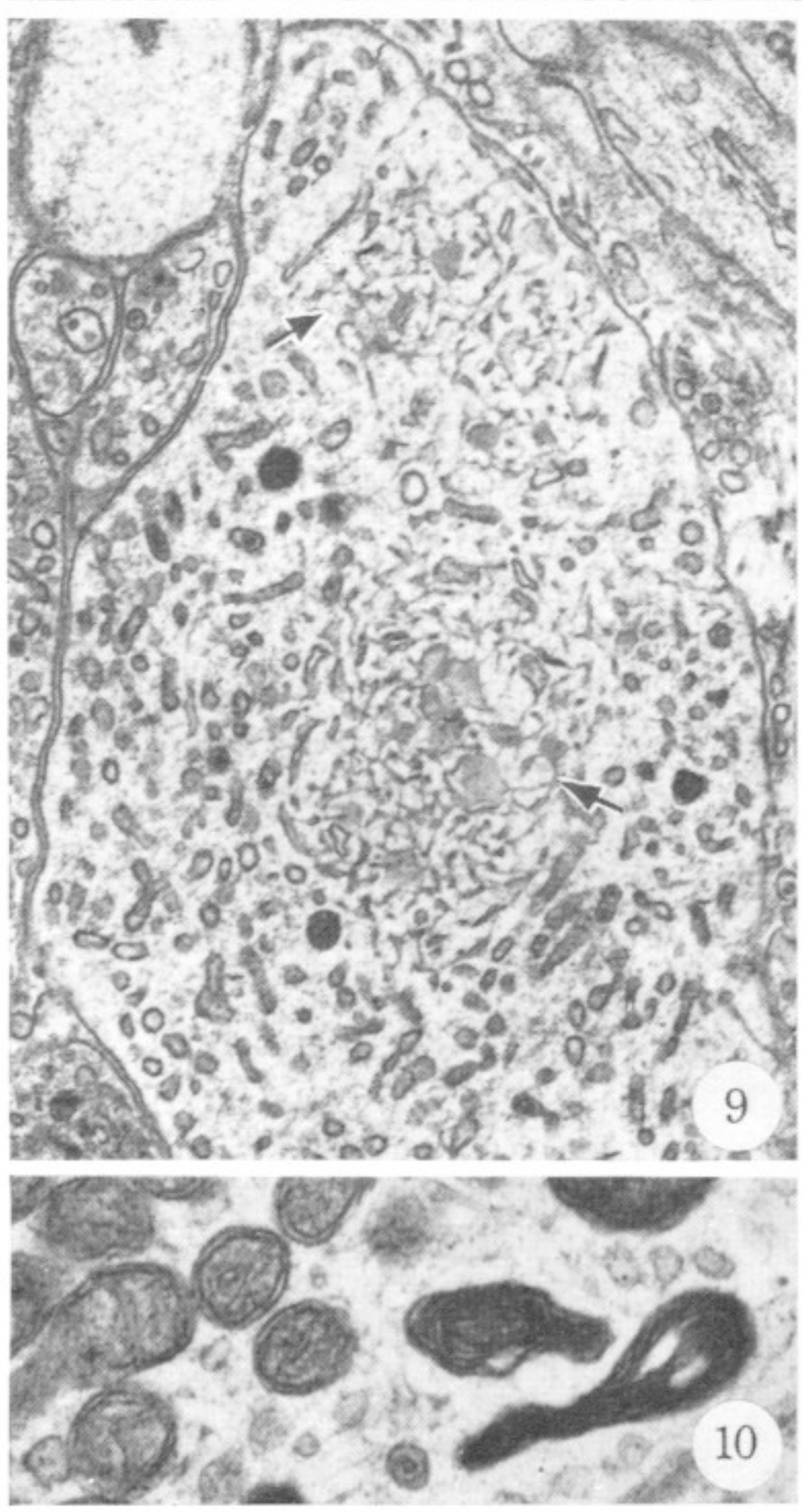


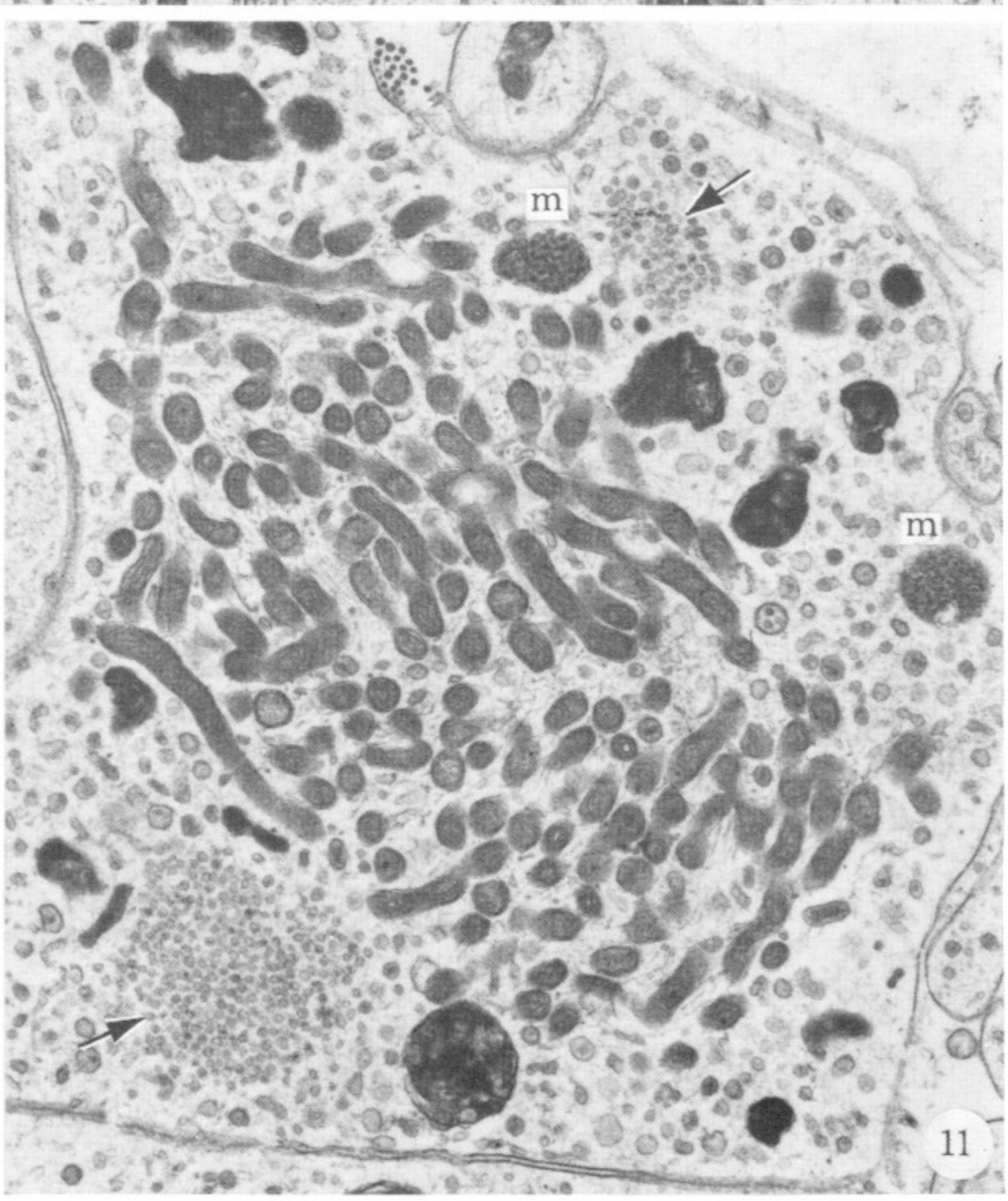
FIGURES 2 TO 5. For legends see facing page.



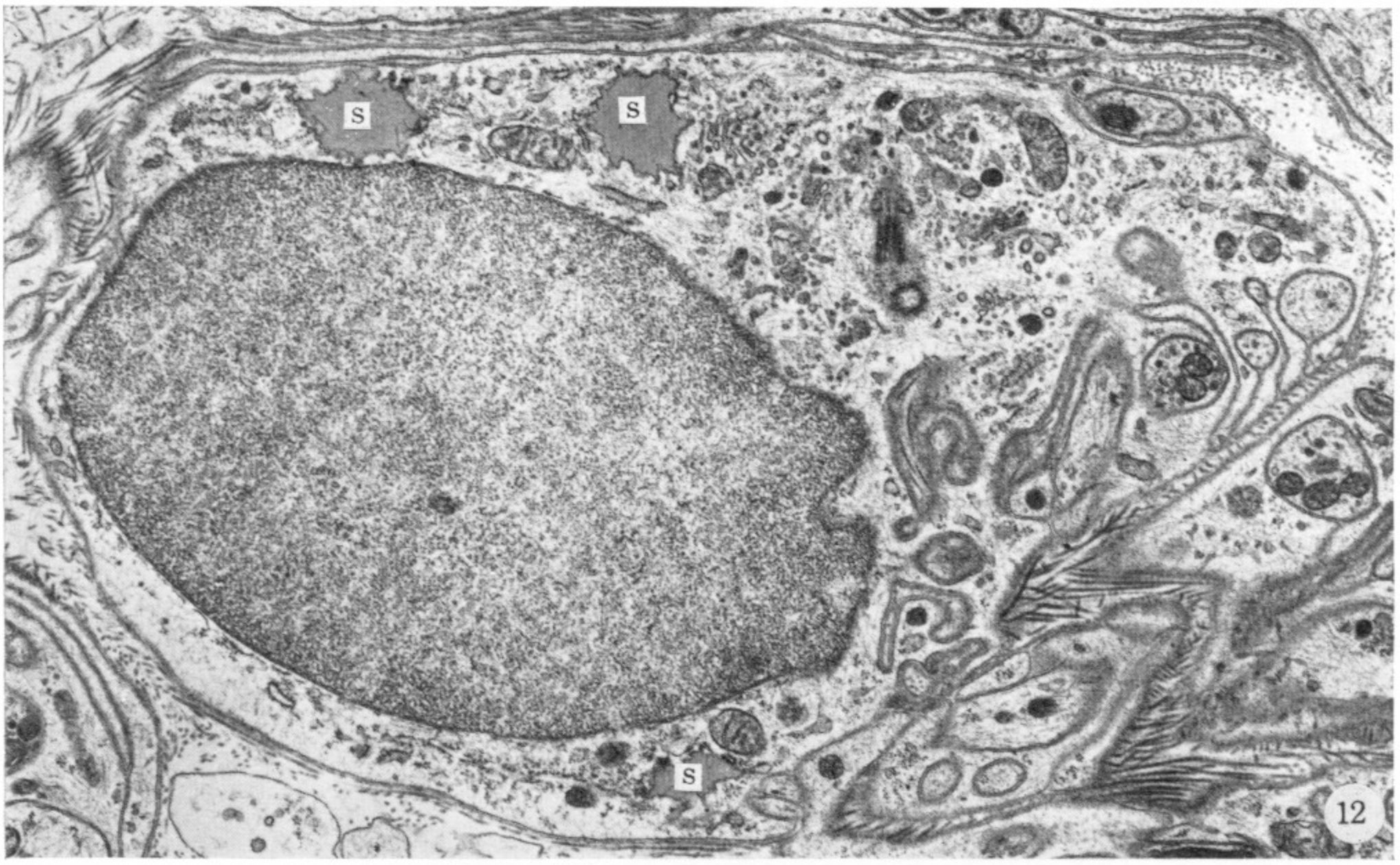


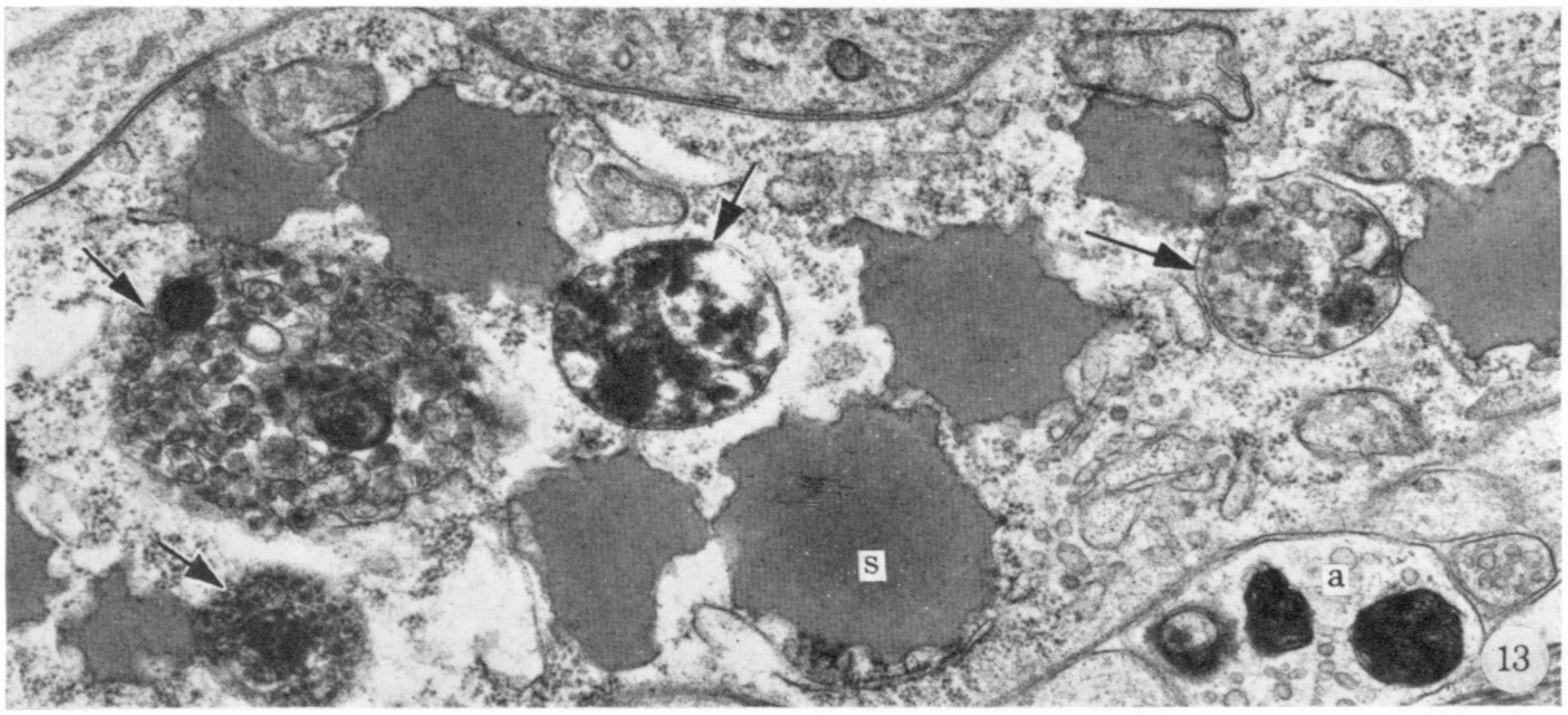


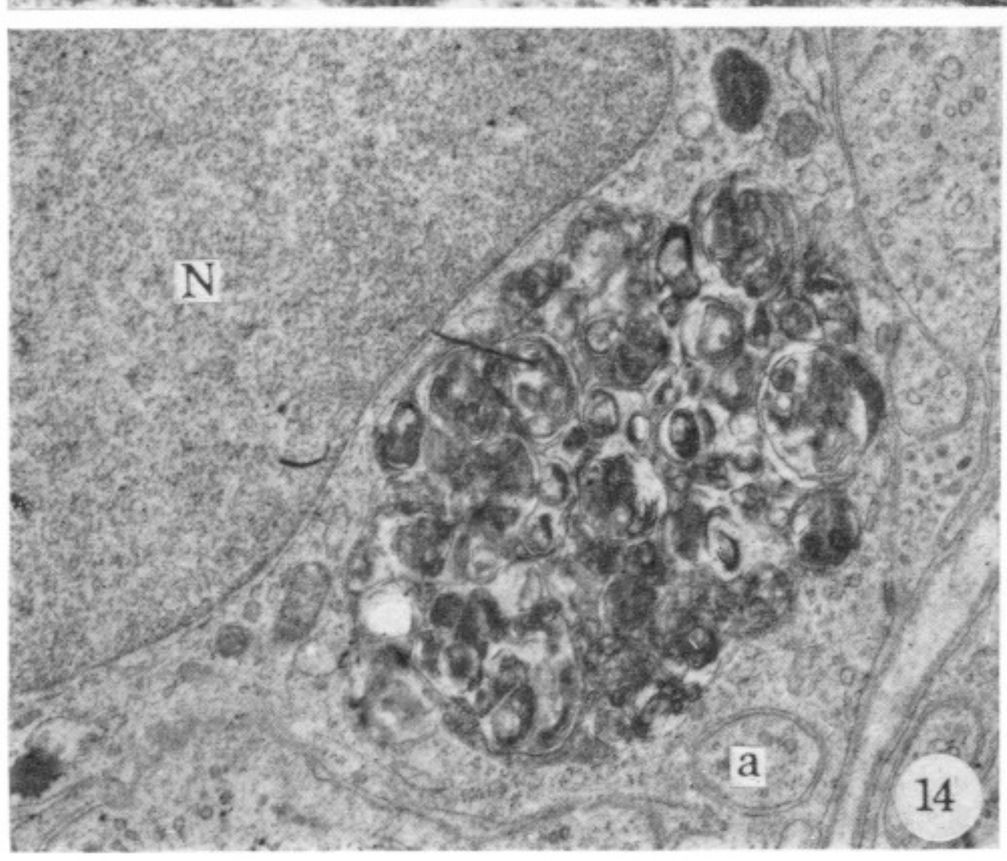


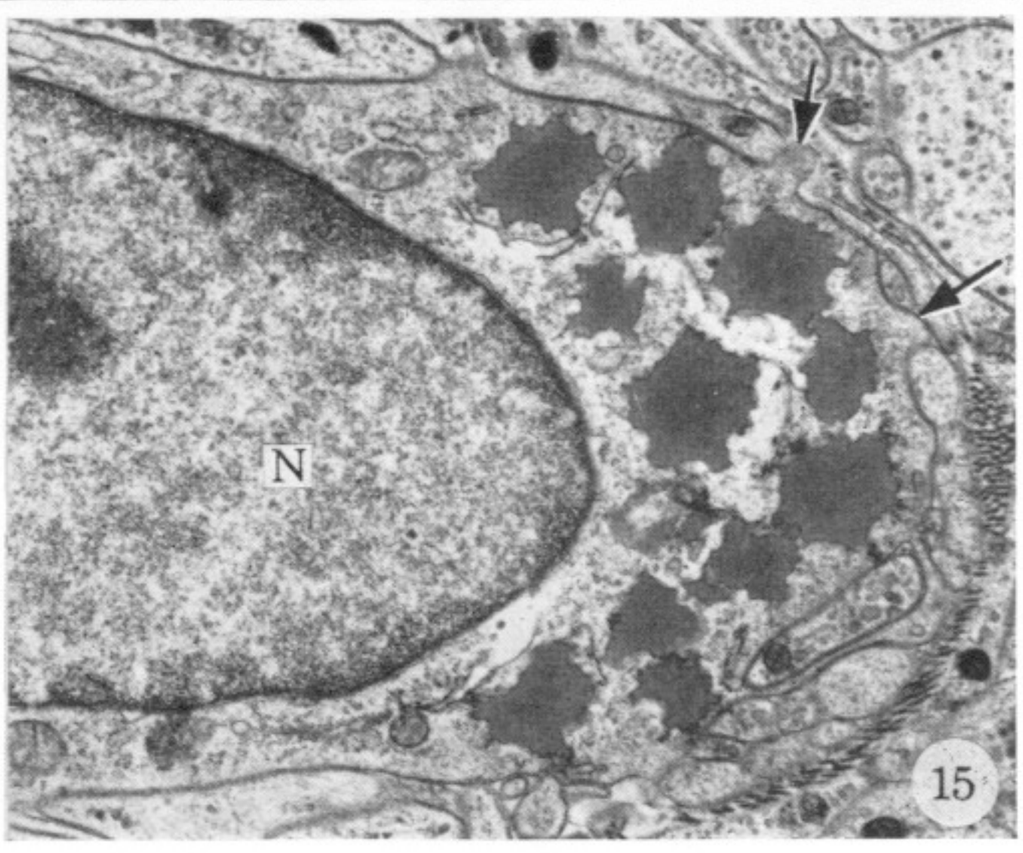


FIGURES 6 TO 11. For legends see facing page.









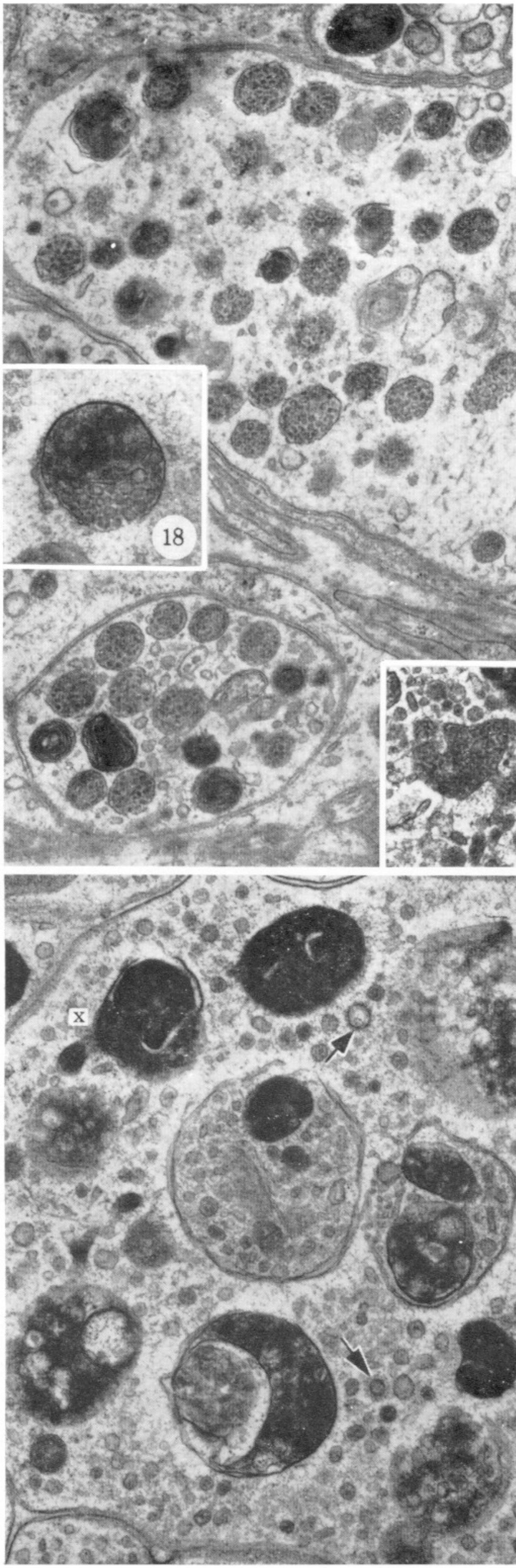
FIGURES 12 to 15. Axonal debris and lipid-like masses in Schwann cells.

FIGURE 12. An intraganglionic Schwann cell with three stellate lipid-like inclusions (s). (12 h, approx. 0.5 mm from ligature.) × 11000.

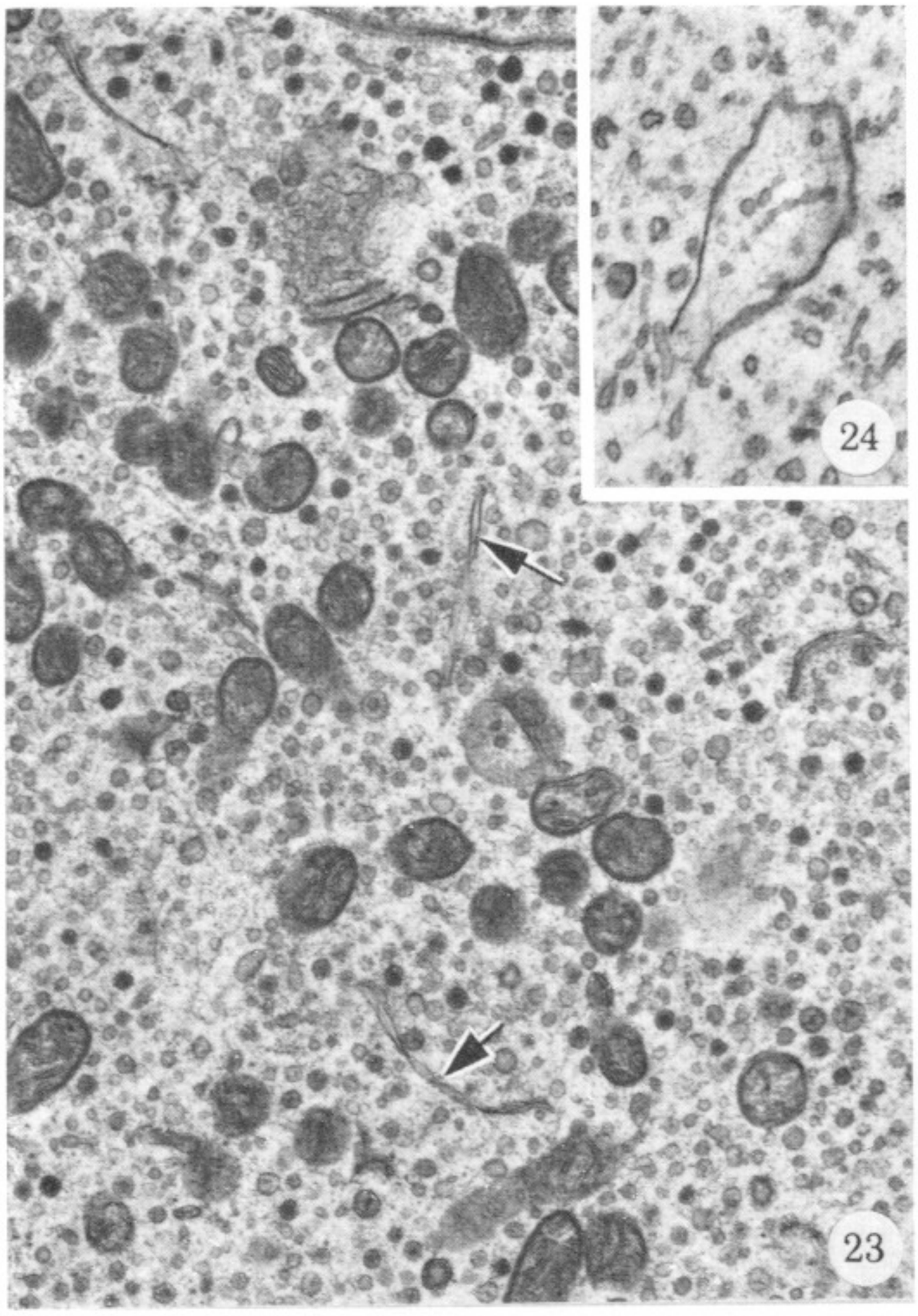
FIGURE 13. Part of a Schwann cell containing many irregularly stellate lipid-like inclusions (e.g. s) and four rounded masses of darkened and partly degraded axonal cytoplasm showing alteration of accumulated organelles (arrows). These are seen in two cases to be bounded by a single membrane and are presumed to be phagosomes. An adjacent axon (a, lower right), containing some vesicles and dense bodies, has an intact membrane and does not show darkening of its cytoplasmic matrix. (38 h, 0.15 mm approx.) × 21000.

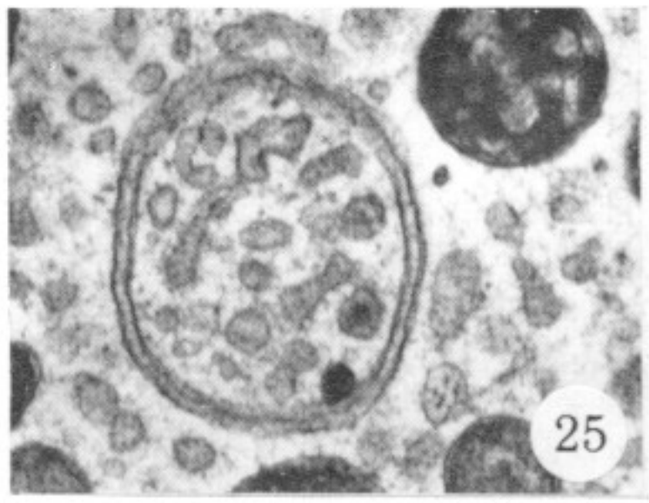
FIGURE 14. Part of a Schwann cell (nucleus, N) containing a large compacted mass of late forms of intra-axonal dense bodies, which has a collapsed, irregular outline and is indistinctly demarcated from the Schwann cytoplasm. This is interpreted as a phagosome of an axonal profile packed with dense bodies. The same Schwann cell is also seen to support an axon of normal appearance (a). (7 days.) × 12300.

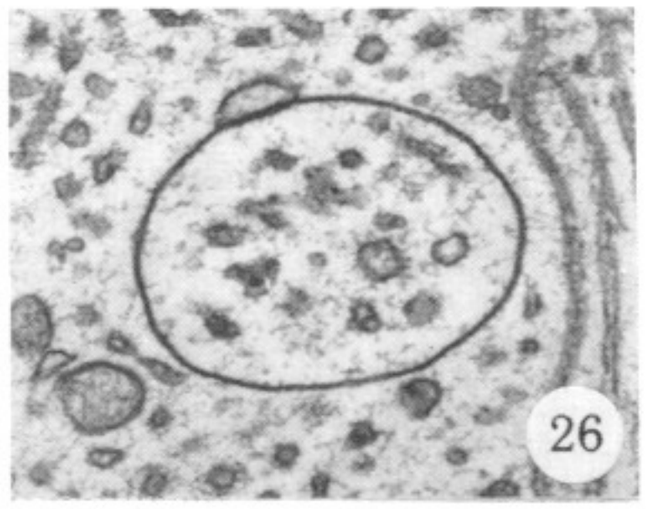
FIGURE 15. Part of a Schwann cell (nucleus, N) whose cytoplasm is loaded with stellate, lipid-like inclusions. At its periphery there lie several small, axon-like profiles between which the Schwann cell sends short, blunt processes (arrows), not entirely surrounding them. This is typical of the stage of rounding up of the Schwann cells during the digestion of axonal debris and the beginning of regenerative axon sprouting. (38 h, 0.2 to 0.3 mm.) × 8000.

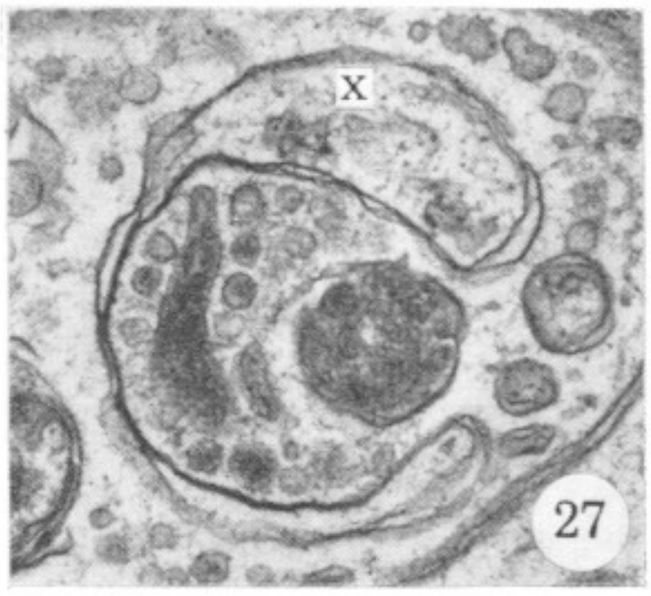


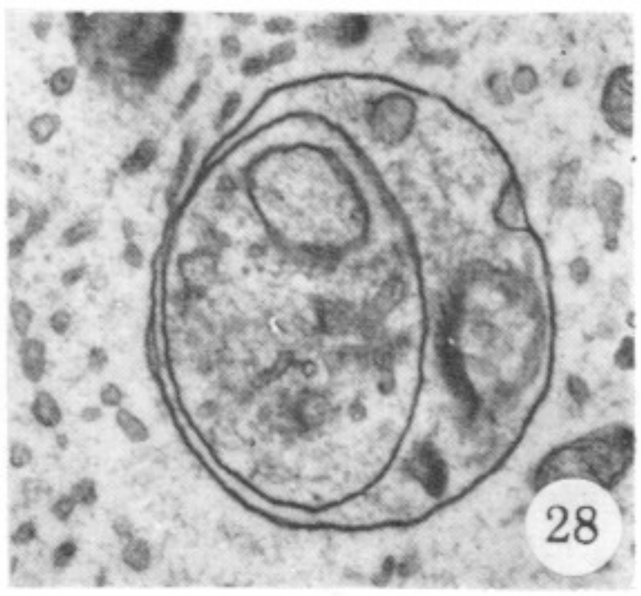
FIGURES 16 TO 22. For legends see facing page.

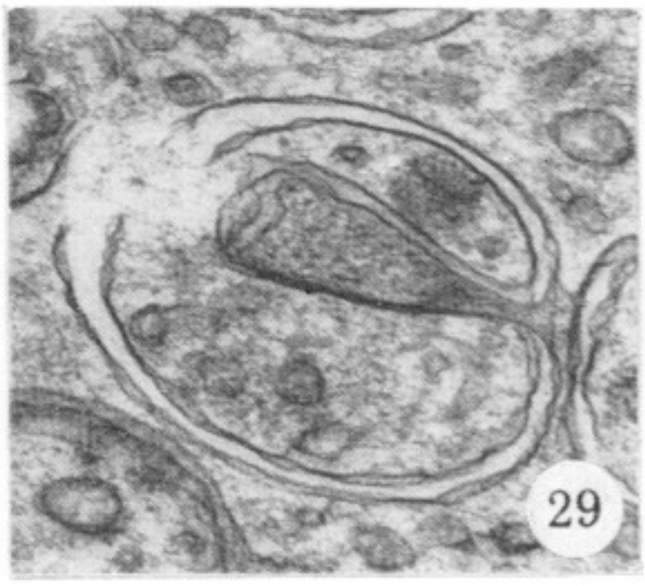


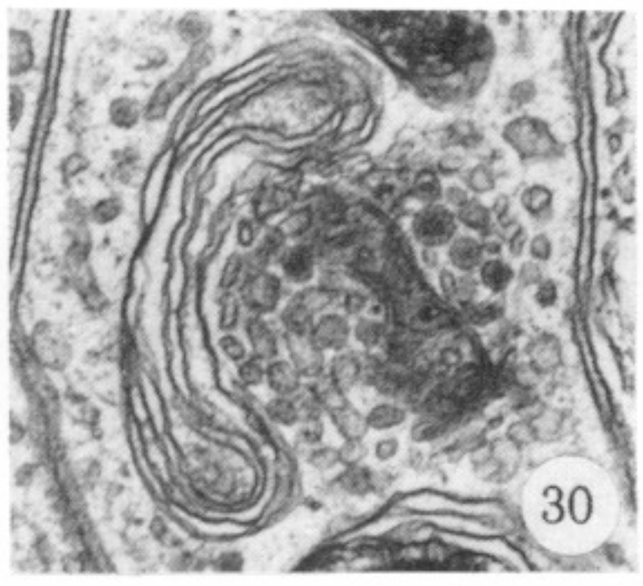


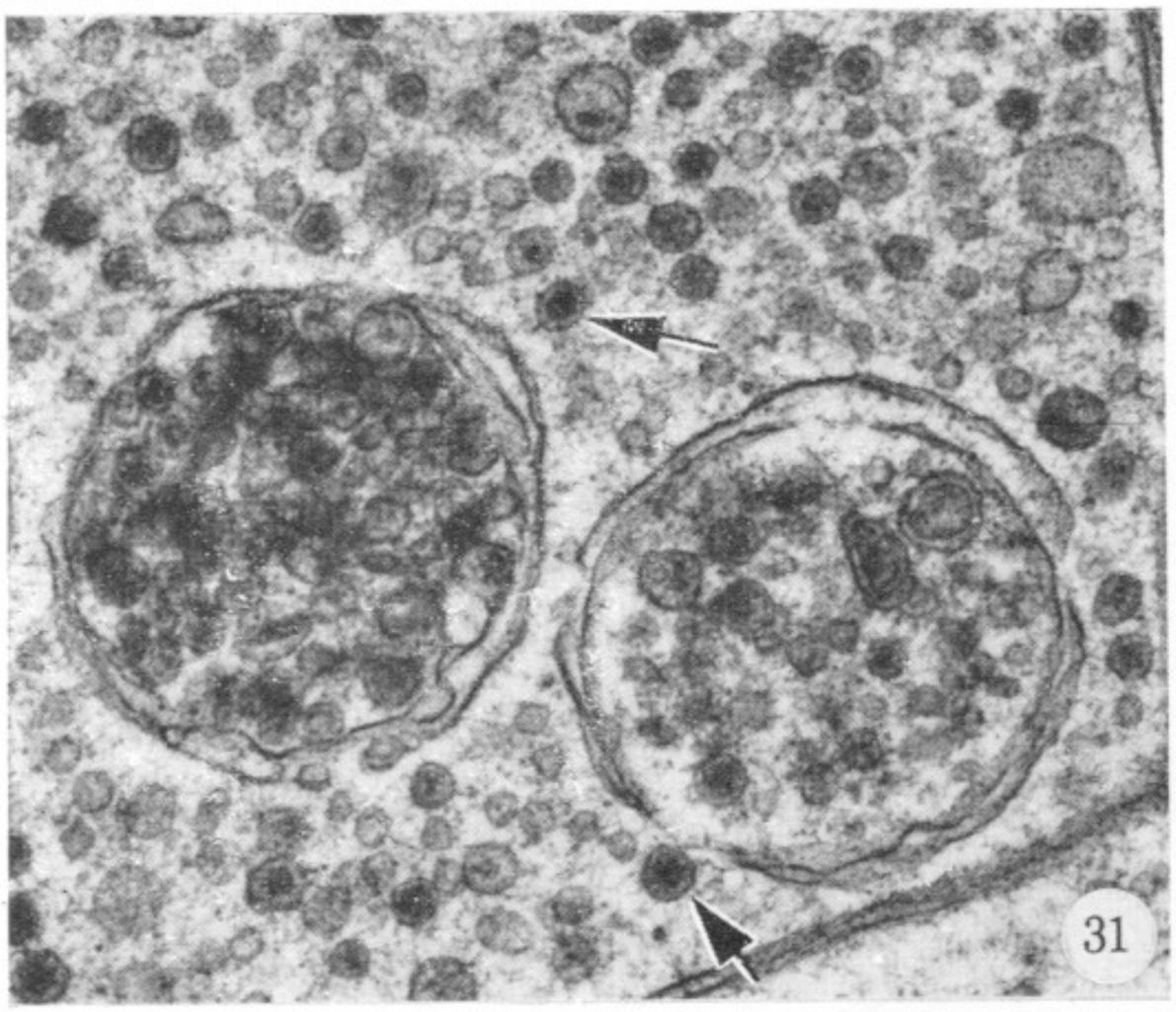


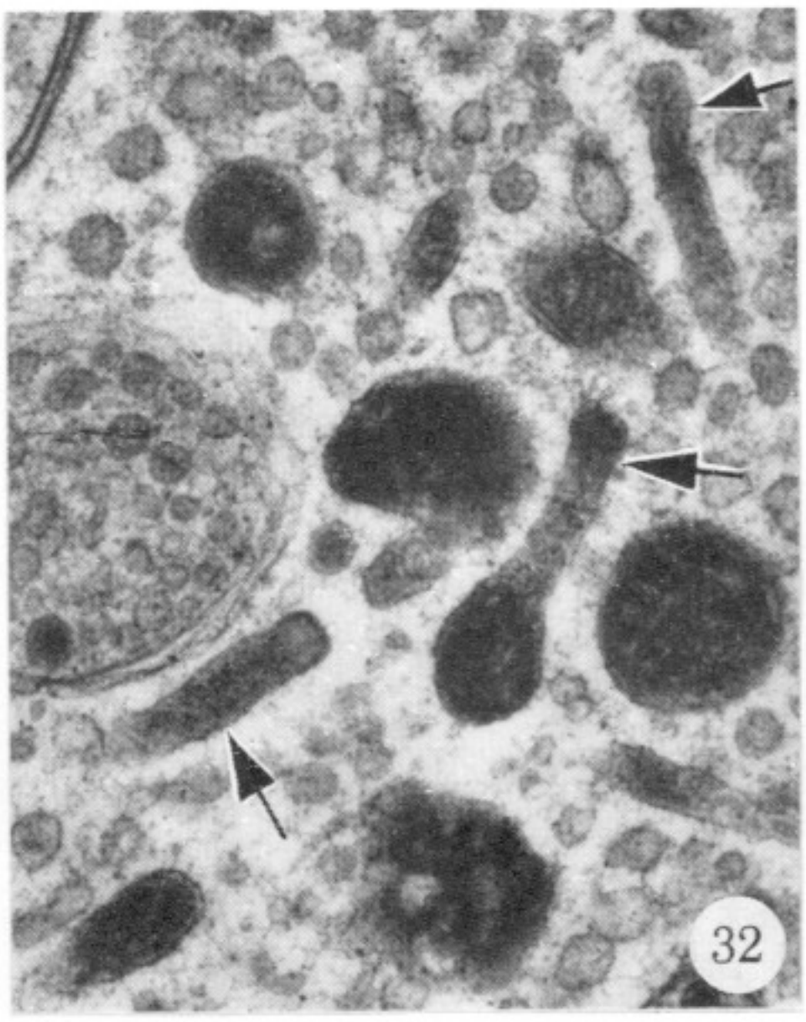


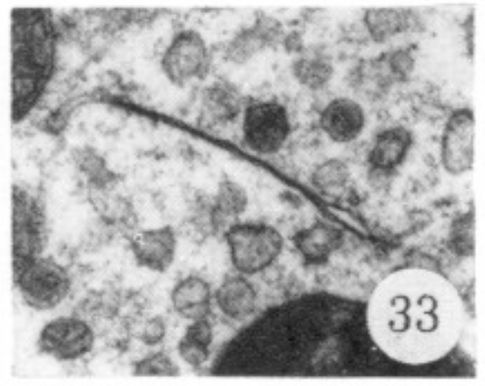


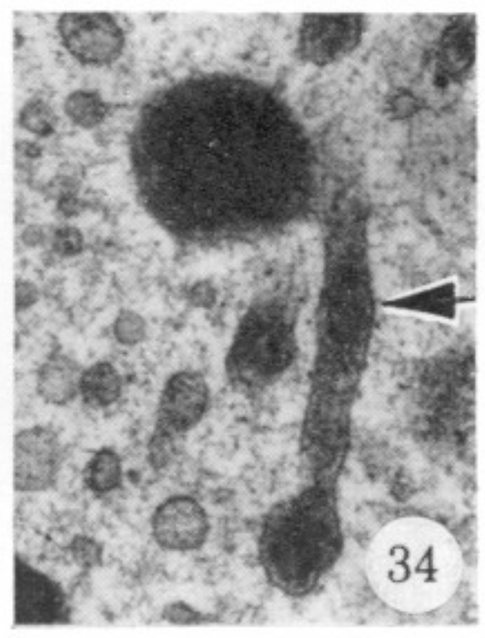


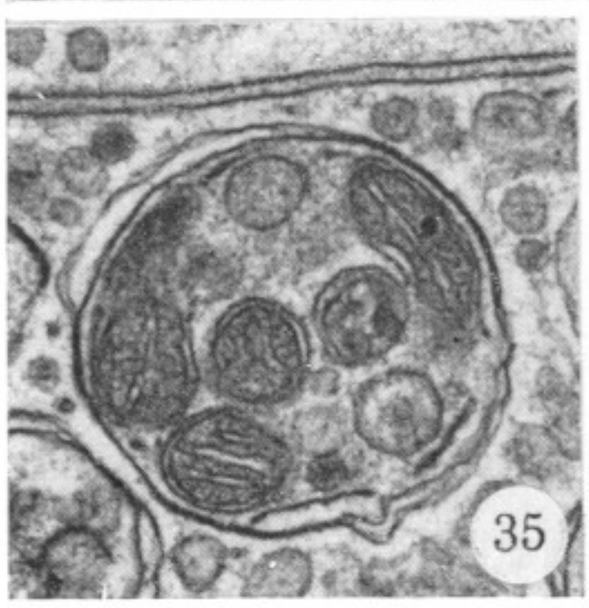


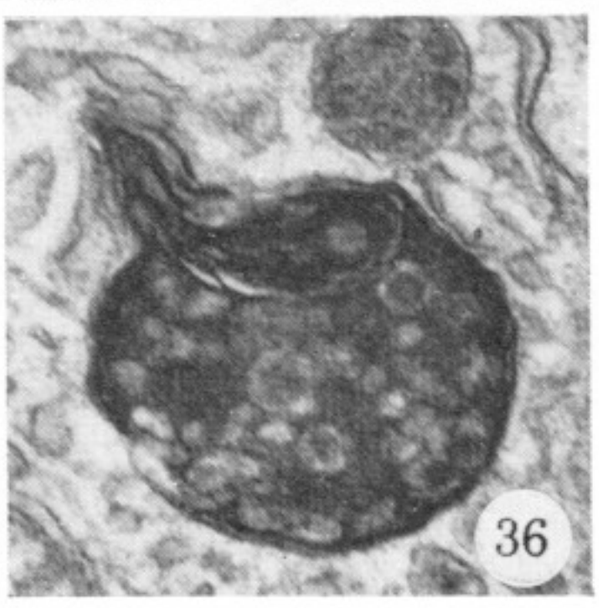


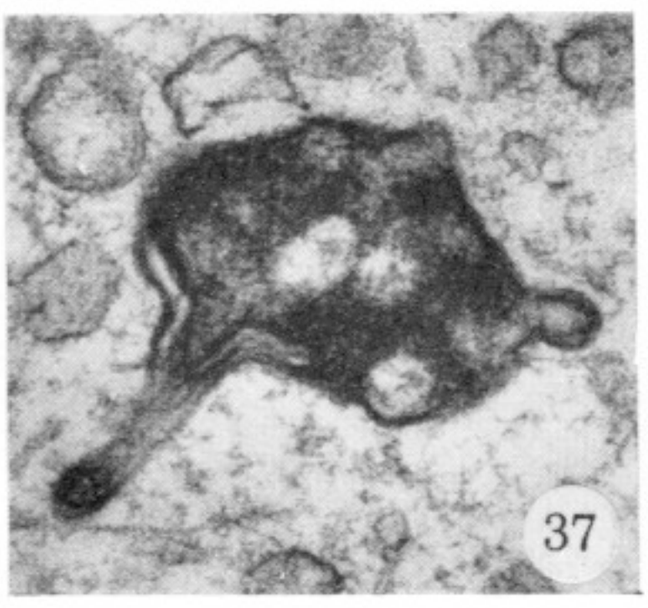


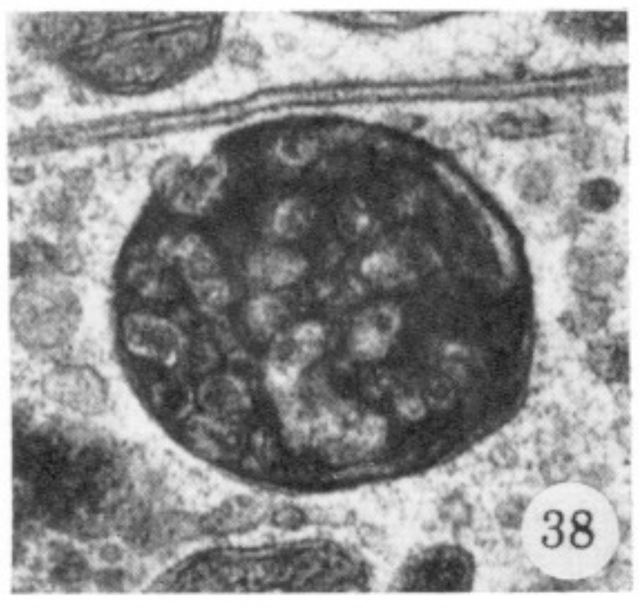




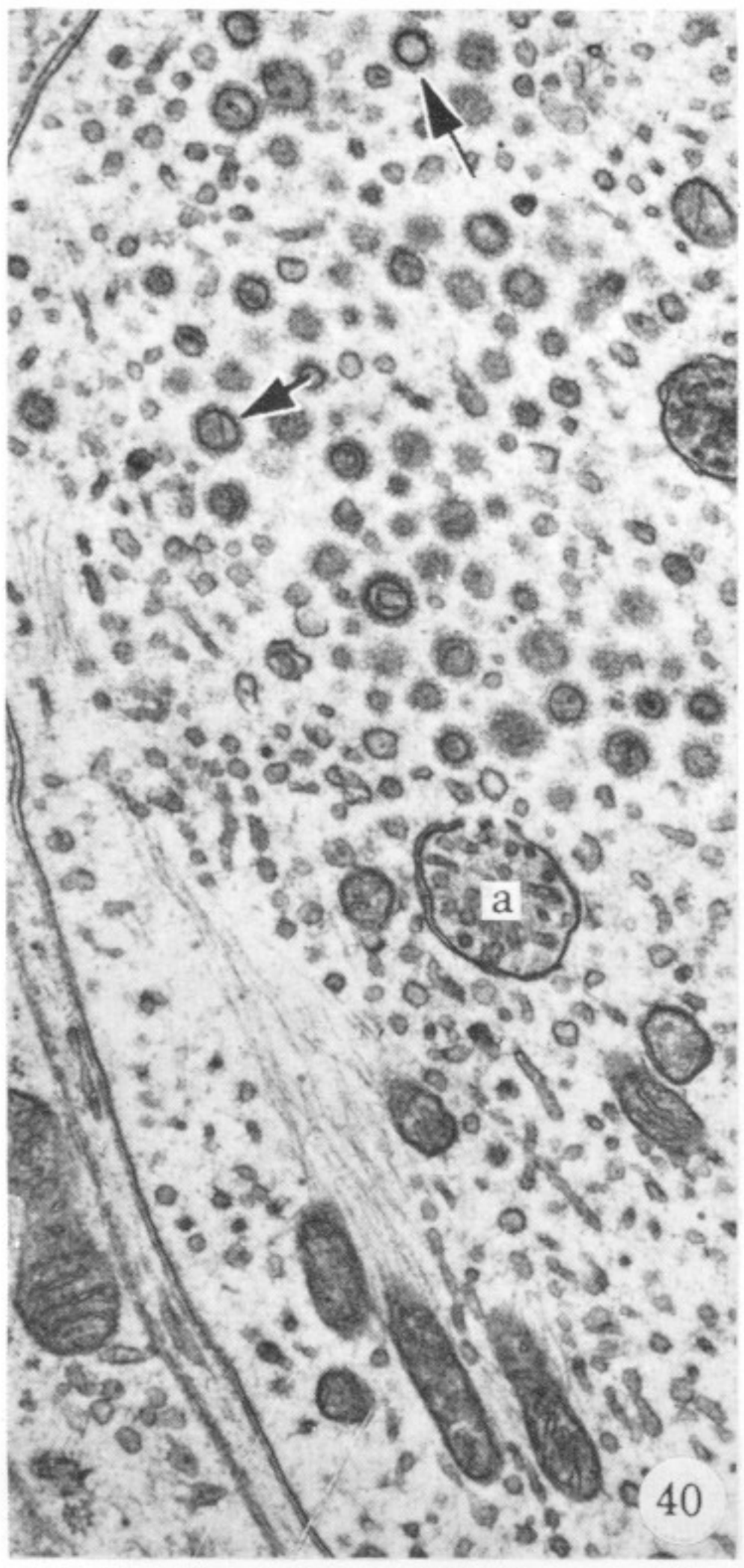


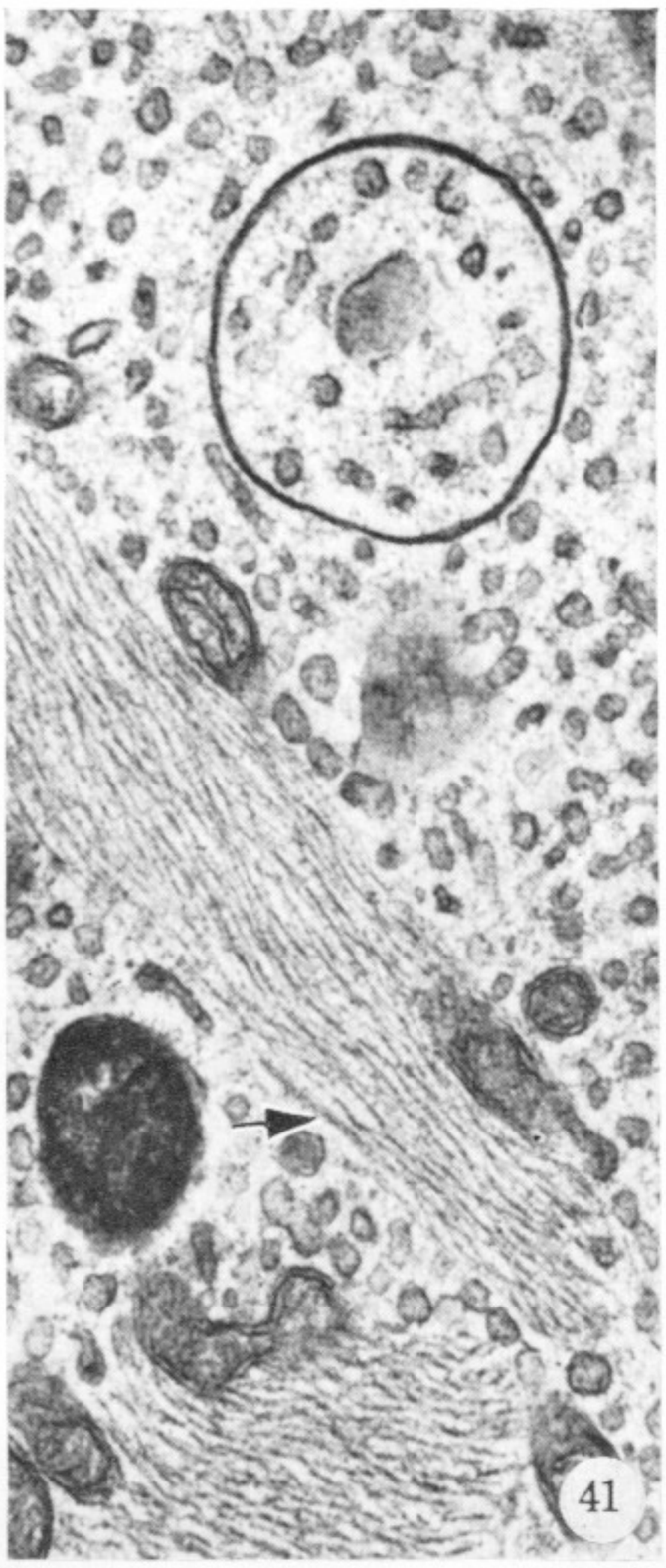


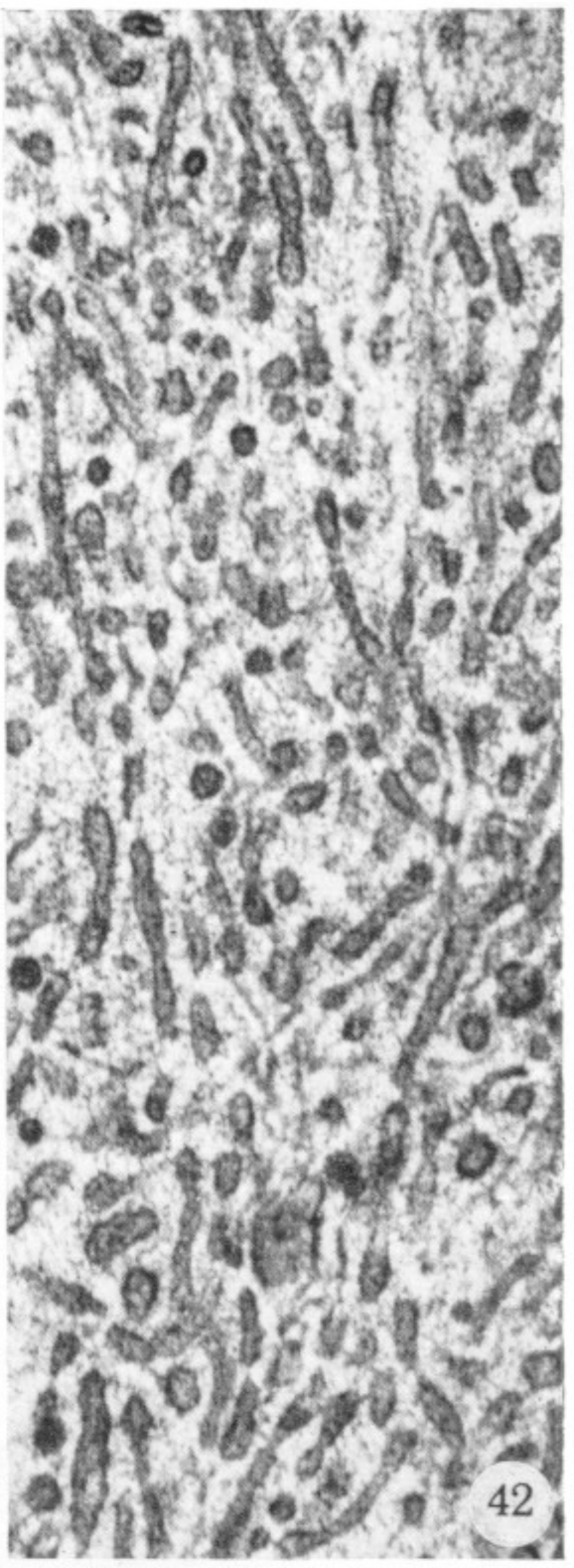


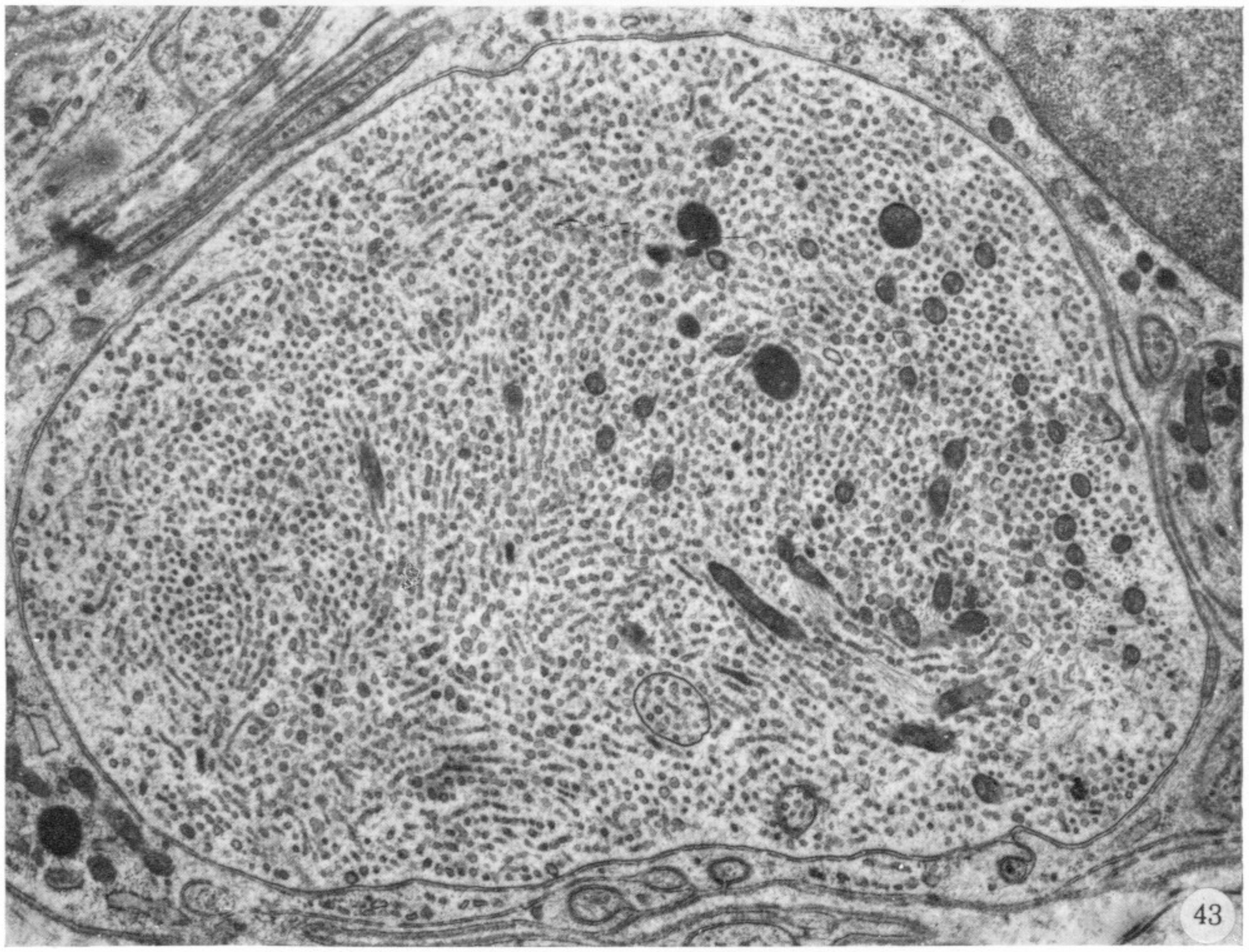


FIGURES 23 TO 38. For legends see facing page.









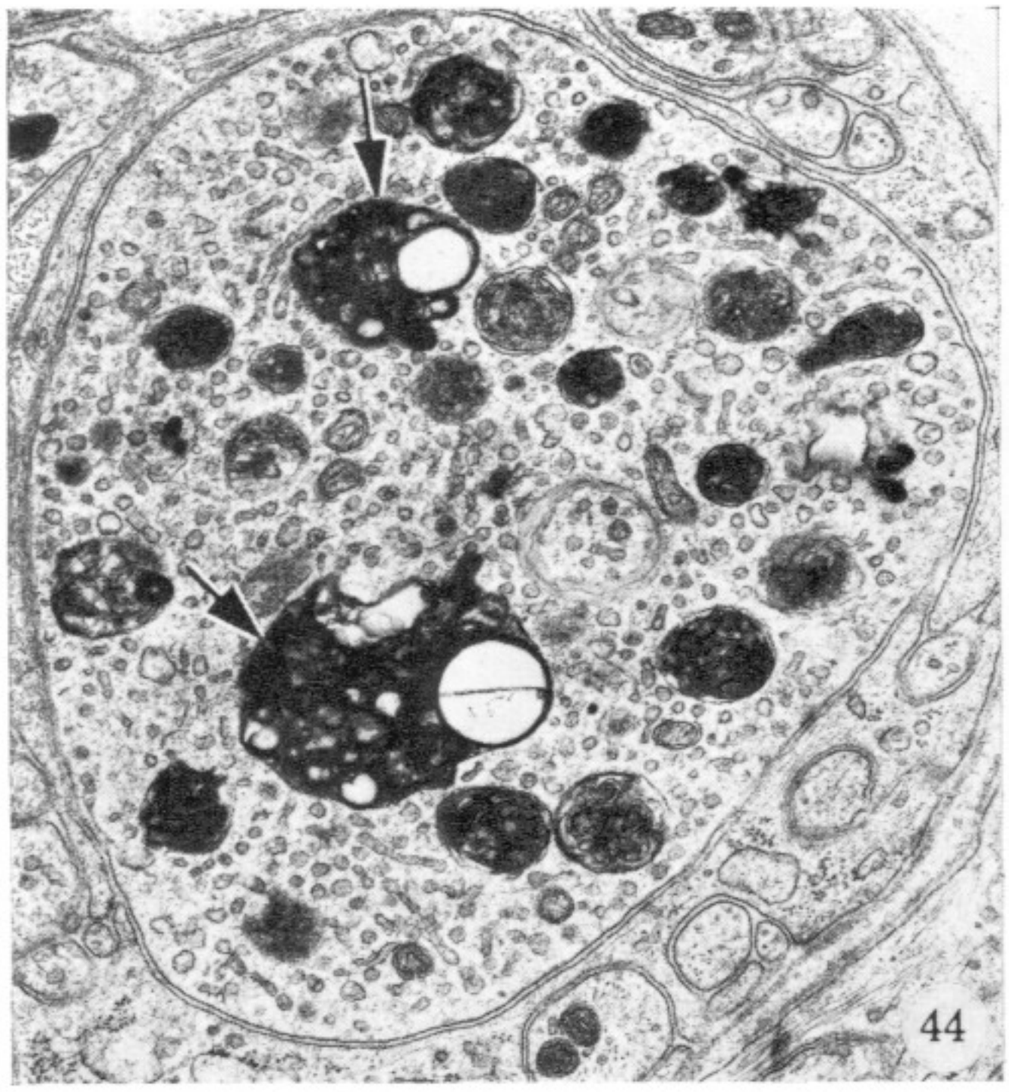
FIGURES 40 to 43. Vesiculo-tubular material, coated vesicles, asscciation of mitochondria with filaments.

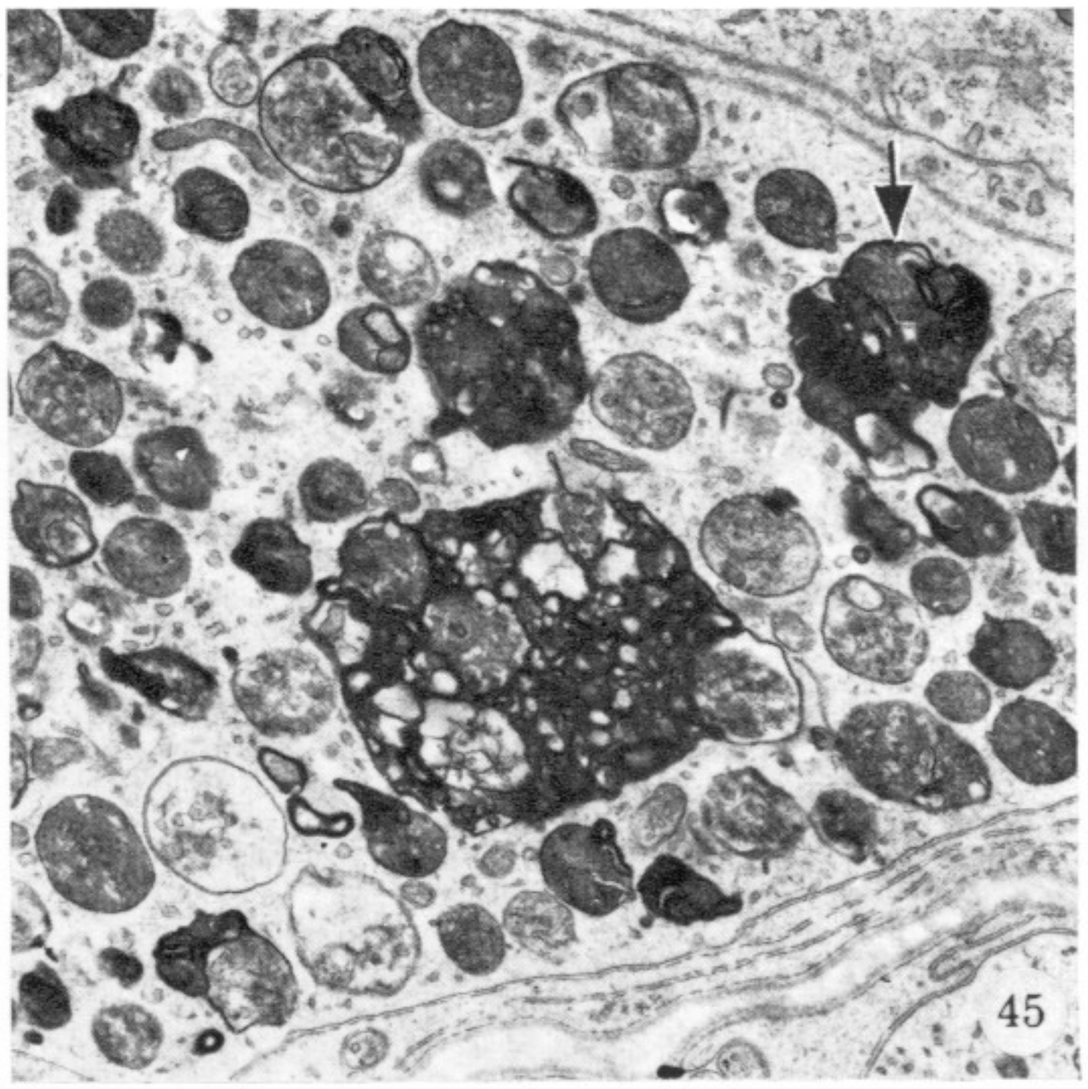
FIGURE 40. Part of a distended axonal profile containing in its upper half a large cluster of coated (alveolate) vesicles (e.g. arrows), which vary considerably in their size and content. The profile also contains vesiculo-tubular material, some of which is alined parallel with a bundle of filaments which runs with associated mitochondria along the left border of the profile. a, autophagic vacuole, which appears to be only partially closed. (6 days, intraganglionic axon.) × 21 000.

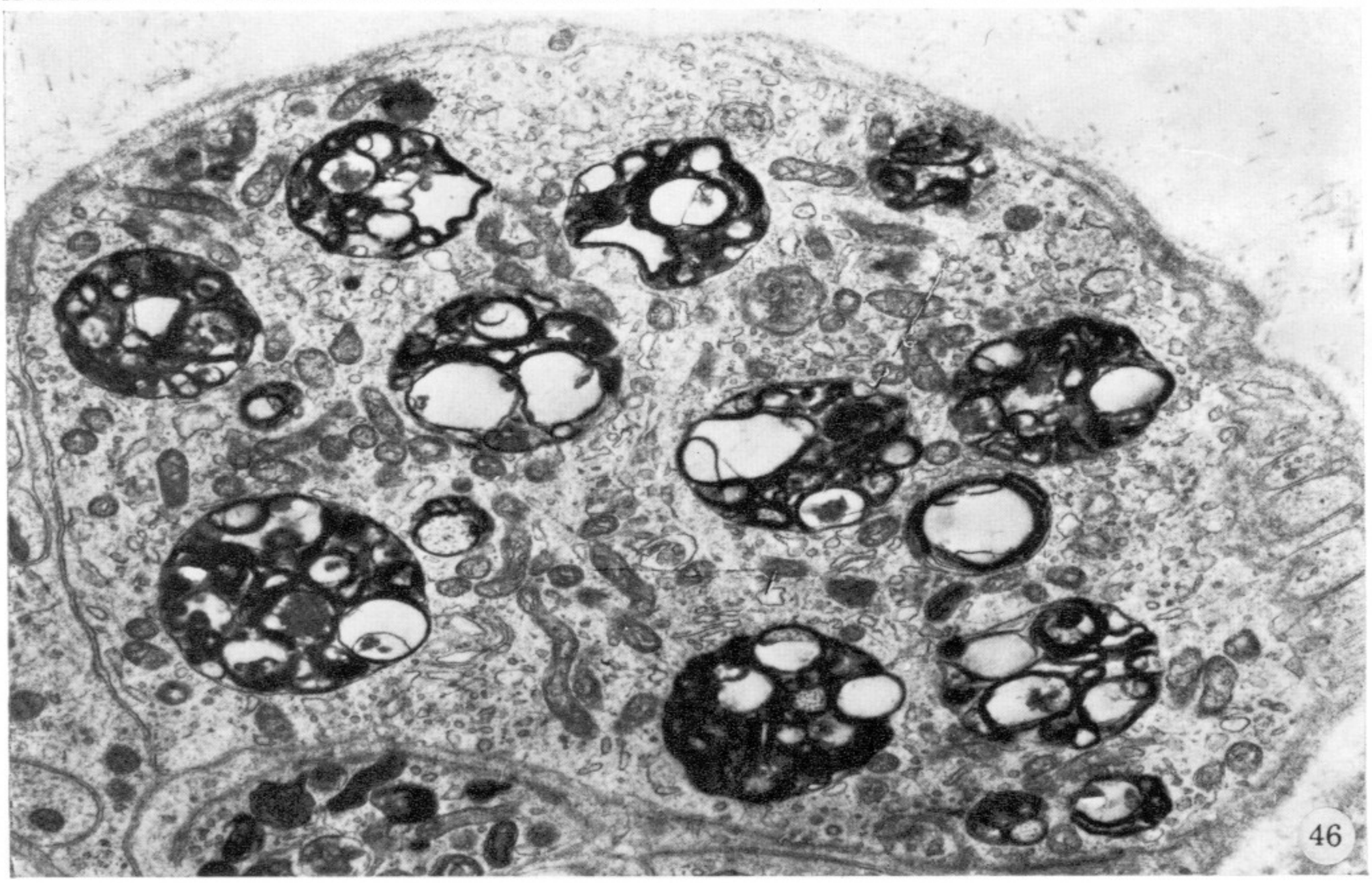
FIGURE 41. An area of axoplasm containing an autophagic vacuole, vesiculo-tubular material, a small dense body and compact bundles of filaments with mitochondria (and microtubules, arrow) alined at their margins. (7 days, intraganglionic axon.) × 40 000.

FIGURE 42. Part of a distended axon containing vesiculo-tubular material in mainly tubular form. (6 h, organelle-rich accumulations, 0.1 mm from ligature.) × 39 000.

FIGURE 43. A greatly distended axon containing vesiculo-tubular material in the form chiefly of rows of vesicles, arrayed in swirling patterns, with occasional tubules of similar or slightly smaller diameter. Mitochondria with associated bundles of filaments are also present, chiefly in the lower right quadrant. The mitochondria and filaments, which are mutually alined, are cut in some places longitudinally and in others transversely. (6 days, intraganglionic axon.) × 16 600.





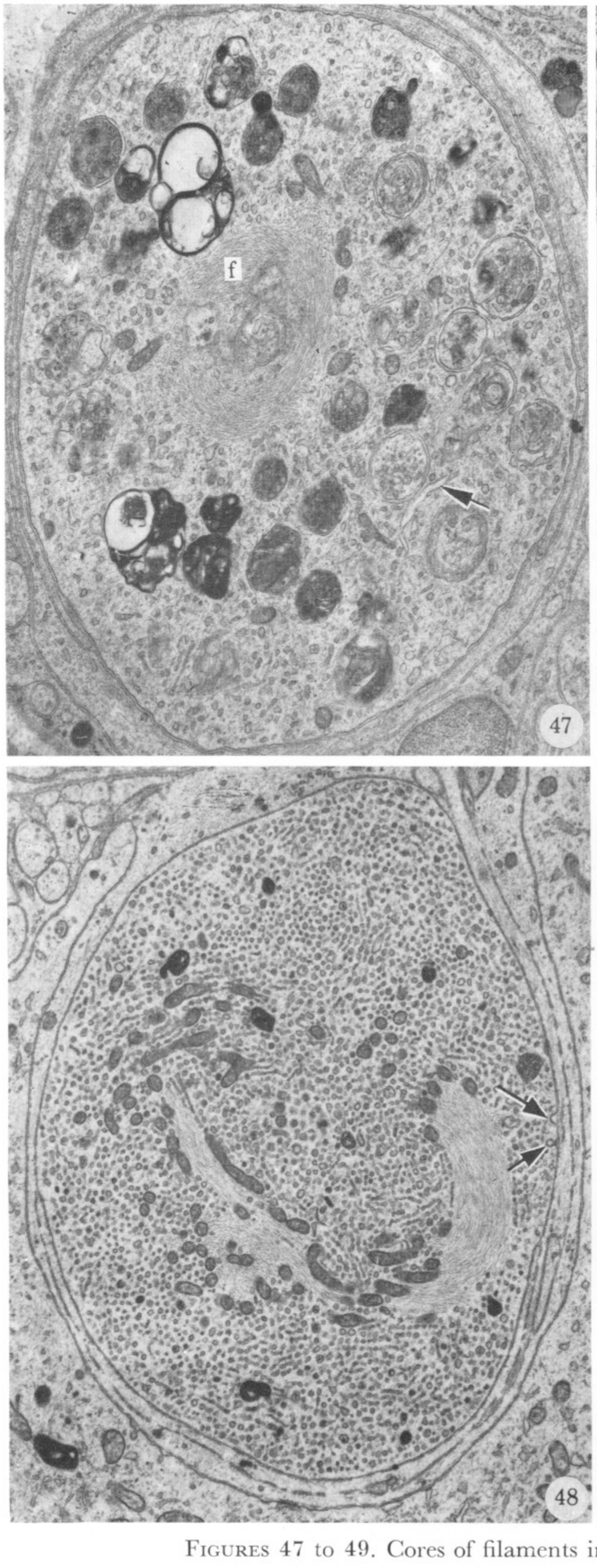


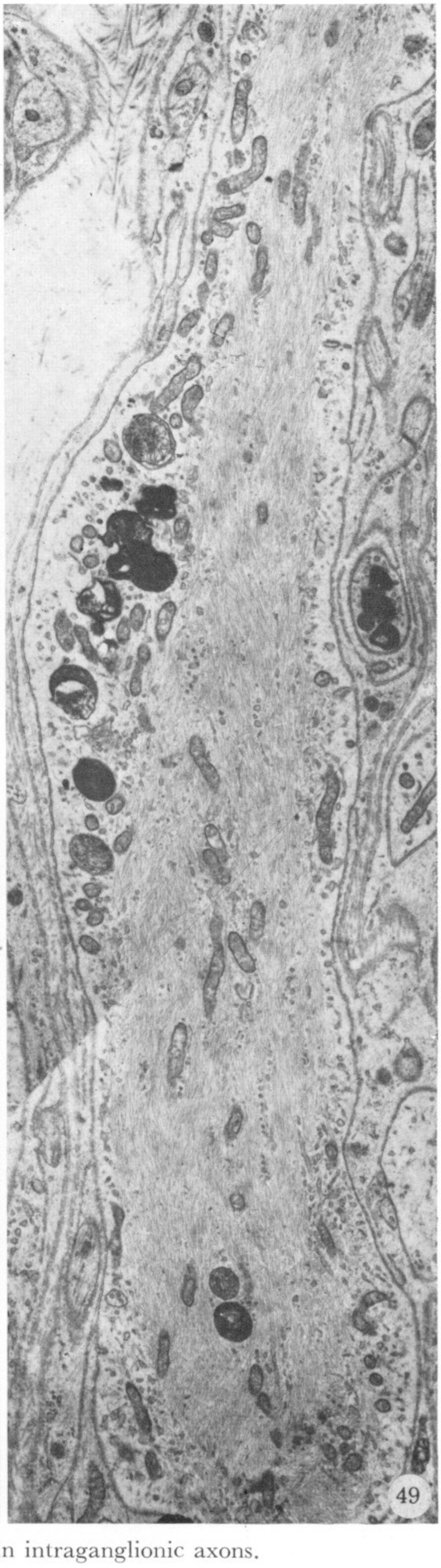
FIGURES 44 to 46. Late forms of dense bodies in intraganglionic axons; agglomerated forms.

FIGURE 44. A distended axon containing vesiculo-tubular material, some mitochondria and a variety of dense bodies and autophagic vacuoles. Arrows, large dense bodies of late agglomerated form showing multicentric highly electron dense whorled lamellar formations with electron lucent centres. (7 days.) × 15600.

FIGURE 45. Part of a much distended axon filled with large numbers of dense bodies and autophagic vacuoles, some of which are coalescing with each other (e.g. arrow) and with a large dense multilocular agglomerate. (6 days.) × 12000.

FIGURE 46. Intraganglionic profile containing a number of large dense agglomerates with multicentric whorled lamellar form, showing electron lucent locules, and several smaller dense bodies of basically similar, but more unitary form. Many mitochondria and some vesicles and sacs of agranular reticulum are also present, the latter in places arranged as in the Golgi formation. This profile is likely to be the base of a large cell process. (7 days.) × 15800.





FIGURES 47 to 49. Cores of filaments in intraganglionic axons.

FIGURE 47. A distended axon profile with a central looped core of parallel cytoplasmic filaments (f). A few mitochondria lie close to the margin of the filament bundle. This may represent an early stage of formation of an organized core. The surrounding area contains vesiculo-tubular material, a loop of membrane (arrow) and a variety of dense bodies, autophagic vacuoles and forms intermediate between the two. The dense bodies include stages of transition to forms showing highly electron dense multicentric whorls with electron lucent locules. (7 days.) $\times 15000$.

FIGURE 48. A distended axon with a central looped core of parallel filaments flanked by alined mitochondria. The rest of the profile is packed with swirling rows of vesicles and tubules, and a few small dense bodies (cf. figure 41). The centre of the profile contains a cluster of coated (alveolate) vesicles (cf. figure 38), and two coated pits possibly representing the formation of coated vesicles are seen at its right border (arrows).

FIGURE 49. A longitudinal section of part of an axon containing a massive core of axially oriented filaments, with which are associated alined mitochondria and rows of vesicles and tubules (vesiculo-tubular material). A narrow peripheral region of the profile is free of filaments but contains mitochondria and vesicles associated with the margin of the bundle; at one point this zone is expanded and contains also some dense bodies and autophagic vacuoles. Coated pits and/or coated vesicles are seen at several points along the surface membrane, especially along the right border of the profile. (6 days.) $\times 12300$.