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# Cytochemical studies of protein transport in the nervous system

By E. Holtzman
*Department of Biological Sciences, Columbia University, New York, N.Y.* 10027, *U.S.A.*

[Plates 82 to 90]

## Introduction

The transport of materials within the nervous system has received much attention in recent years. Considerable information has accumulated concerning such subjects as the exchange of substances between the circulation and nervous tissue, the passage down axons of molecules and organelles originating in perikarya and the release and fate of neurotransmitters and neurosecretory agents at synapses and other neuron endings (see, for example, Wolstenholme & Porter 1968; Barondes 1969). Many investigations are underway on modulations of transport during nerve growth and regeneration and in tissue responding to injury or to other experimental alterations.

However, numerous gaps remain. Of central importance to future analysis of intraneuronal transport will be the determination of details of the routes and mechanisms by which various components synthesized in the perikaryon become distributed throughout the cell. It is becoming increasingly clear that different components move along axons at quite different rates (see, for example, McEwen & Grafstein 1968). This heterogeneity may reflect the existence of a variety of intraaxonal transport pathways. Important clues derive from such work as the studies on microtubules reported by D. S. Smith in the present proceedings but much remains to be learned about possible compartmentalization of movement within axons.

Similarly, the organization of perikarya must be further investigated. Neurons possess abundant rough endoplasmic reticulum, many free ribosomes, a well-developed Golgi apparatus and other features consonant with their intensive metabolic activity. Remarkably little is known, however, about most of the molecules synthesized within perikarya and about the division of labour among the organelles of the perikaryal cytoplasm.

The functions of the non-neuronal cells (Schwann cells, glial cells and satellite or capsule cells) that are closely associated with neurons are also poorly understood. Roles in myelin formation and maintenance are well established. But much more information is needed about the extent to which associated non-neuronal cells modify the interactions of neurons with the extracellular medium or perhaps provide molecules important for neuronal functioning.

Well-known machinery exists for the degradation or reabsorption of some of the components released by axons in the course of neurotransmission and related processes. However, release and associated mechanisms probably can account for the fate of only a very small proportion of the molecules and organelles transported into endings (see, for example, Droz 1969). The contributions to turnover at endings of reverse flow (toward the perikaryon; see, for example, Lubinska 1964) and of other mechanisms have yet to be adequately evaluated.

Many such gaps will ultimately be filled through correlation of physiological and biochemical information with morphological findings. The present report deals with investigations of transport and related phenomena in the nervous system based on coordinated use of electron microscopy and enzyme cytochemistry. Cytochemical procedures are available for

demonstration of a variety of enzymes and other components in nervous tissue (cf. Adams 1965; Friede 1966; Novikoff 1967; Holtzman 1969). Particularly useful for our purposes are the methods for demonstration of peroxidase activities and those for detection of lysosomal hydrolases. Horseradish peroxidase is a small protein (mol. mass 40 000) that can be administered via extracellular fluids to organisms or cultured cells and then followed, within tissues and cells, by microscopy of suitably incubated preparations. Lysosomal hydrolases are present in essentially all animal cell types. Study of their transport in neurons provides a means for determining some of the routes by which endogenous proteins are handled by perikarya and axons.

## The accessibility of the neuron surface to exogenous materials

Electrophysiological experiments have indicated that when myelin is not present, inorganic ions and small molecules can rapidly traverse glial and Schwann cell sheaths and reach the surfaces of neurons (Villegas, Caputo & Villegas 1962; Kuffler & Nicholls 1966, Rosenbluth

## Explanation of plates

Tissue preparation was by methods previously described (Holtzman & Novikoff 1965; Holtzman *et al.* 1967; Holtzman & Dominitz 1968; Holtzman & Peterson 1969*a*; Holtzman, Freeman & Kashner 1970). The cultured neurons were similar to those used by Holtzman & Peterson (1969); the irradiation dose was usually 40 000 rad (cf. Masurovsky *et al.* 1967). Initial fixation of tissue was generally with glutaraldehyde (Sabatini, Bensch & Barrnett 1963) except for the lobster material which was fixed with Karnovsky's (1965) glutaraldehyde-formaldehyde mixture. Acid phosphatase was demonstrated with Gomori's (1952) medium, usually with CMP (5-cytidylic acid) as substrate (Novikoff 1963). Aryl sulphatase was demonstrated with a modification of Goldfischer's (1965) procedure (Holtzman & Dominitz 1968) and peroxidase was demonstrated with the medium of Graham & Karnovsky (1966). After postfixation in osmium tetroxide, tissue was embedded in Epon (Luft 1961), sectioned on diamond knives, stained with lead citrate (Reynolds 1963), uranyl acetate (Watson 1958) or with uranyl followed by lead. *En bloc* staining with uranyl acetate (Friend & Farquhar 1967) was often employed. Micrographs were taken with an RCA EMU 3G microscope at initial magnifications of ×2–20 000.

Figures 1 and 2 are from Holtzman *et al.* (1970), figures 11 and 35 are from Holtzman (1969), figure 12 is from Holtzman & Novikoff (1965), and figure 21 is from a micrograph taken by Susan Abrahams of the author's laboratory.

## Description of plate 82

Figures 1 to 3 are from a lobster giant fibre that was dissected from a walking limb, soaked for 20 min in Ringer's solution containing horseradish peroxidase, fixed, frozen and then incubated for peroxidase activity.

Figure 1. Peroxidase reaction product is seen between connective tissue cells (C), in the space (AS) separating the axon (A) from the Schwann cell (S) and in networks of anastomosing tubules (arrows). M indicates a mitochondrion in the axon. (Magn. ×18 000.)

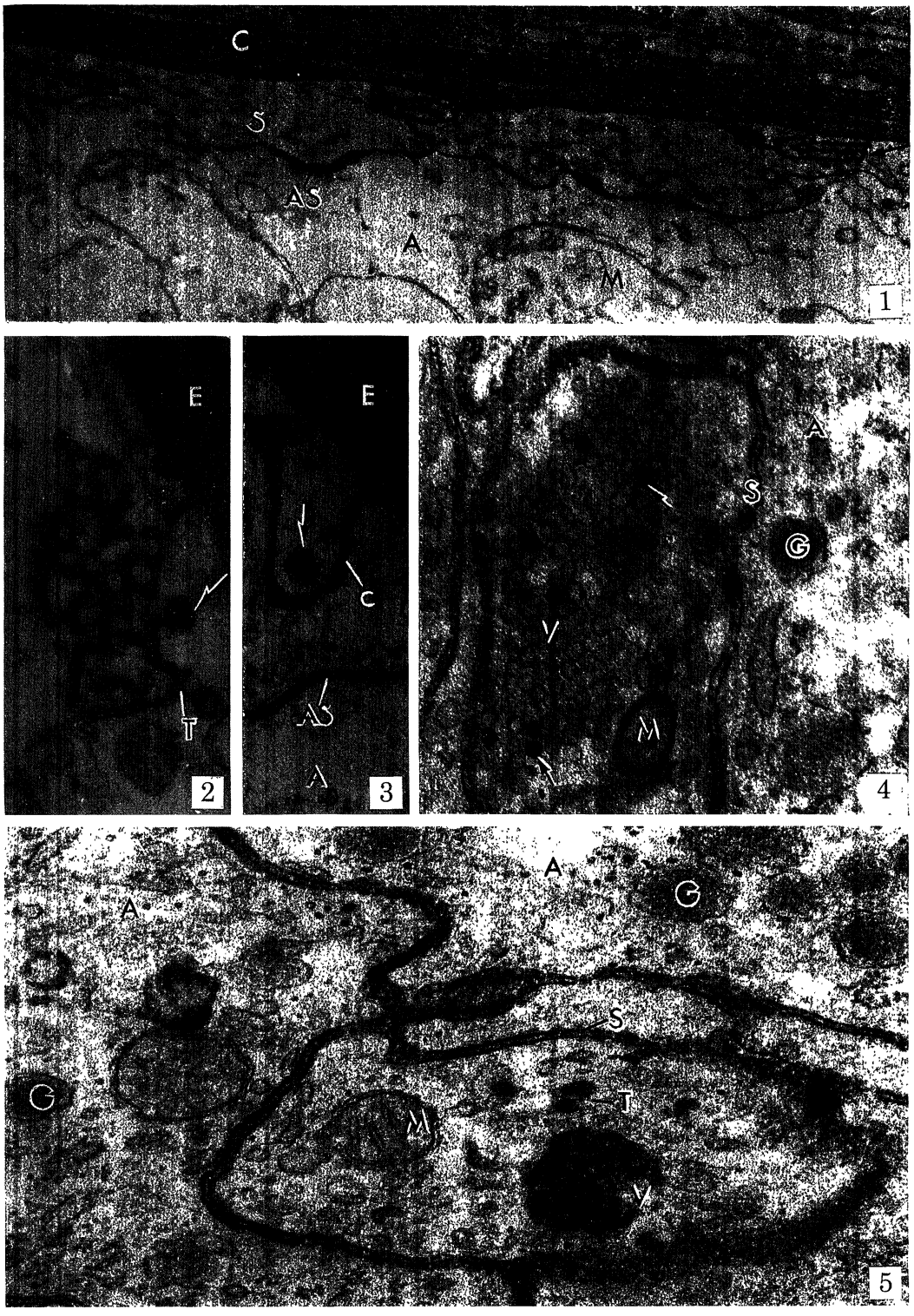
Figure 2. Higher magnification view of a tubule network similar to those shown in figure 1. Some peroxidase reaction product is present in the tubules (T); it is more heavily concentrated in a coated vesicle (arrow) that appears to be budding from the network. The space outside the Schwann cell sheath is seen at E. (Magn. ×48 000.)

Figure 3. Small portion of a Schwann cell showing peroxidase reaction product in a coated vesicle (arrow). E indicates the space outside the Schwann cell sheath, C, a channel in the sheath and AS, the space between axon (A) and Schwann cell. (Magn. ×47 000.)

Figures 4 and 5 are from the adrenal medulla of a rat that received an intravenous injection of peroxidase 35 min before the tissue was fixed. The preparation was then frozen-sectioned and incubated to demonstrate peroxidase activity.

Figure 4. Portion of an axon ending (or *bouton de passage*; see Coupland 1965) near an adrenaline-producing gland cell (A). Peroxidase reaction product is present in several small vesicles (arrows) scattered among the synaptic vesicles (V). Some product also is seen in the space (S) between axon and gland cell. M indicates a mitochrondrion in the axon and G, a secretion granule in the gland cell. (Magn. ×45 000.)

Figure 5. Portion of an axon near an ending adjacent to adrenaline-producing cells (A). Peroxidase reaction product is present in a multivesicular body (V) in the axon. T indicates peroxidase-containing tubules (cf. Birks 1966); other labels are as in figure 4. (Magn. ×52 000.)




FIGURES 1–5. For legends see facing page.

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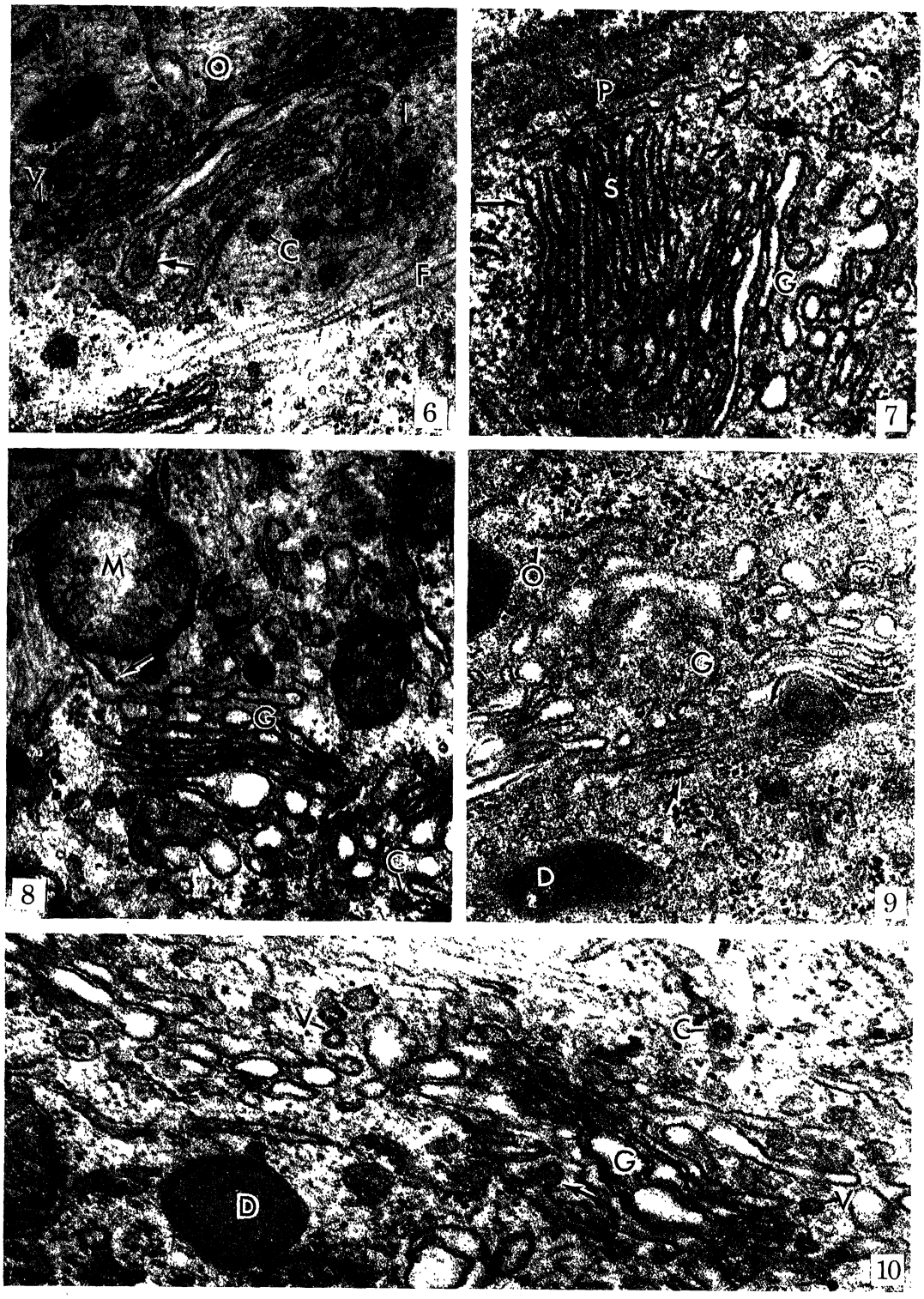


FIGURES 6–10. For legends see facing page.

1968). The sheaths do restrict ion movement to some extent and this can result in detectable alteration of the electrical behaviour of axons (Frankenhauser & Hodgkin 1956; Ritchie & Straub 1957) but, on the whole, extracellular spaces apparently provide ready communication between the neuron surface and the spaces outside the surrounding glial or Schwann cells. Spaces within the sheaths also provide channel systems that may be of physiological importance for control of local ion concentrations near the neuron surface (Kuffler & Nicholls 1966; Rosenbluth 1968).

That larger molecules, up to 10 nm or more in diameter, can also move across the sheaths by passage through extracellular spaces has been shown with a variety of tracers for several classes of vertebrate perikarya and unmyelinated axons (see, for example, Rosenbluth & Wissig 1964; Kaye, Donahue & Pappas 1964; Pappas & Purpura 1966; Brightman 1965, 1968; Waggener, Bunn & Beggs 1965; Becker, Hirano & Zimmerman 1968; Holtzman 1969; Holtzman & Peterson 1969*a*). In invertebrate unmyelinated fibres, the morphology is somewhat different, but the situation is fundamentally similar. Villegas & Villegas (1968) have found that thorium dioxide particles can move to the surfaces of squid axons by way of flat channels that pass across the Schwann cell sheath (see also Baker (1965) for discussion of crab nerves). In lobster giant fibres (Holtzman, Freeman & Kashner 1970) such channels are supplemented by an extensive system of anastomosing tubules that connect the space surrounding the axon with that outside the Schwann cell sheath (figures 1, 2).

For myelinated fibres, the situation is less well understood. Although the axon-Schwann cell space appears not to be readily accessible to peroxidase (Hirano, Becker & Zimmerman 1969), some materials, in addition to inorganic ions, do pass into or across this space from the medium outside the myelin sheath. This has been shown, for example for radioactive amino acids, by autoradiographic studies (Singer 1968); the amino acids are thought to pass through the sheath. Furthermore, a polypeptide of molecular mass 2000 has recently been found to enter the

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## Description of plate 83

Figures 6 to 10 show portions of the Golgi apparatus in perikaraya of cultured mouse dorsal root ganglia.

Figure 6. From a preparation that was not irradiated. The saccules at the outer surface of the Golgi apparatus (O) are often somewhat more dilated and more obviously fenestrated than those at the inner surface (I). Coated vesicles (C; see Holtzman *et al.* 1967; Novikoff 1967) and larger bodies with moderately electron-dense content (arrow) appear to arise from dilatations of sacs or tubules near the inner Golgi surface. Overall, the apparatus is a curved and somewhat twisted network so that it is not always possible to determine which surface or surfaces are shown in a given thin section. However, as in figures 8 and 9, tentative identification can often be made from criteria such as those just outlined (see also Holtzman *et al.* 1967; Novikoff 1967). Small vesicles without coats (V) abound near the Golgi apparatus. Microfilaments are seen at F. (Magn. × 46000.)

Figure 7. Two days after irradiation. Near the Golgi apparatus (G) a stack of smooth-surfaced sacs is present (S). One of the sacs shows ribosomes on the surface facing away from the stack (arrow) suggesting that the sacs arose from endoplasmic reticulum. The plasma membrane is indicated by P. (Magn. × 52000.)

Figure 8. Three days after irradiation. The arrow indicates a cisterna of rough endoplasmic reticulum closely apposed to the outer surface of the Golgi apparatus (G) and showing ribosomes on the cisternal surface facing away from the apparatus. A coated vesicle is seen at C and a mitochondrion is present at M. (Magn. × 50000.)

Figure 9. Two days after irradiation. Cisternae of rough endoplasmic reticulum with ribosomes only on the surfaces facing away from the Golgi apparatus (G) are seen close to the outer (O) and inner (arrow) surfaces of the apparatus. A dense body is indicated by D. (Magn. × 39000.)

Figure 10. From the same ganglion as was used for figure 8. At the arrow a vesicle is seen as if budding from a cisterna of endoplasmic reticulum near the Golgi apparatus (G). D indicates a dense body and C, a coated vesicle. Small Golgi-associated vesicles without coats are present at V. (Magn. × 52000.)

axon-Schwann cell space of mouse myelinated fibres, probably by way of the nodes of Ranvier (Feder, Reese & Brightman 1969).

Channels, such as those shown in figures 1 and 2, provide a quite extensive surface area at which Schwann cells might interact with the extracellular medium. The concept that Schwann cells can control the composition of the fluid immediately surrounding axons, perhaps by active exchange of material (see, for example, Rosenbluth 1968; Villegas 1968) is an attractive one, but direct evidence relating to this possibility is difficult to obtain (see, for example, Kuffler & Nicholls 1966). Some microscopic observations are suggestive, although further work is needed for adequate evaluation. For example, Schwann cells and glial cells are capable of moderately extensive pinocytosis (see, for example, Rosenbluth & Wissig 1964; Kaye *et al.* 1964; Waggener *et al.* 1965; Holtzman & Peterson 1969*a*); in the Schwann cells of lobster giant axons, 'coated' (Roth & Porter 1964) pinocytosis vesicles appear to form from the anastomosing tubules (figures 2 and 3; Holtzman *et al.* 1970). In many of these cases, much of the material taken up has been shown eventually to accumulate in lysosomes (see review in Holtzman 1969). Pinocytosis itself is a somewhat selective process (see review by Stockem & Wohlfarth-Botterman 1969). In addition, its occurrence sometimes reflects or parallels other, more subtle changes in plasma membranes. Thus, Brandt & Freeman (1967) have demonstrated alterations in the electrical properties of the amoeba plasma membrane associated with the induction of pinocytosis. Changes in electrical properties and in water transport characteristics are also observed in toad bladder mucosal epithelial cells under conditions (presence of oxytocin or cyclic AMP) that induce pinocytosis by the mucosal cells (Masur, Holtzman & Eggena 1969; Masur, Holtzman & Walter 1970).

In a wide variety of nervous tissue, one or another cholinesterase is present at surfaces of Schwann cells and other cells associated with neurons (see, for example, reviews by Torack 1965; Novikoff 1967; Tennyson, Brzin & Duffy 1968); such activity is demonstrable, for example, in the tubule networks of lobster Schwann cells (Holtzman *et al.* 1970). Although for many of the locations at which cholinesterase activity is found, the roles of acetylcholine and related compounds are not yet clear, it may be that glial, capsule and Schwann cells can act as 'filters' preventing or limiting passage to and from the neuron surface of neurotransmitters or other

## Description of plate 84

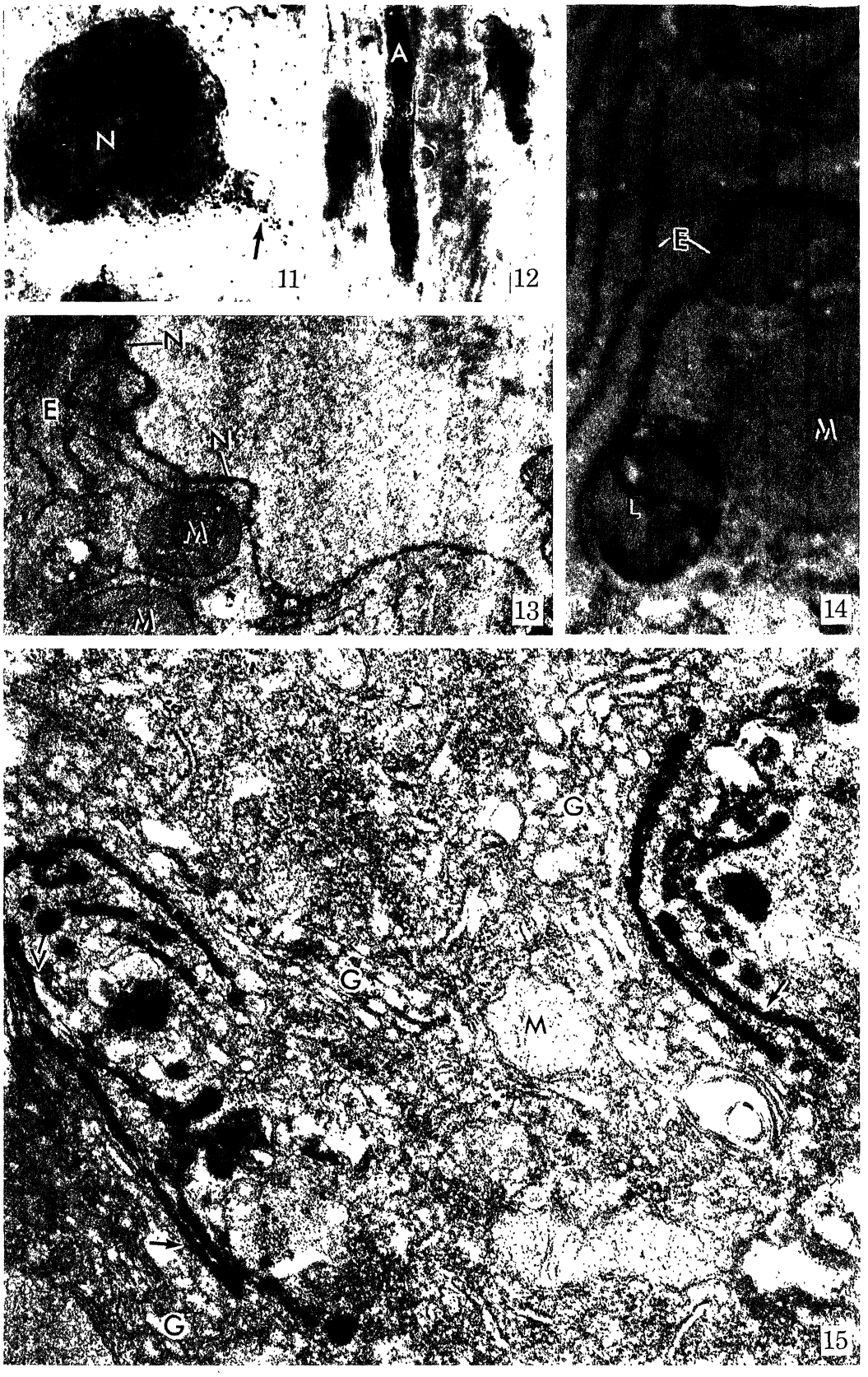
Figure 11. Light micrograph of a neuron from a cultured mouse dorsal root ganglion incubated for aryl sulphatase activity. N indicates the nucleus and the arrow, the initial portion of the axon. Reaction product is seen in many small cytoplasmic bodies; these are lysosomes. (Magn. × 850.)

Figure 12. Light micrograph showing a portion of an acid phosphatase preparation of the distal segment of a rat sciatic nerve 1 day after the nerve had been interrupted. Reaction product within an axon (A) is seen in many small granule-like structures and in an elongate tubule (see Holtzman & Novikoff (1965) for explanation of these appearances). (Magn. × 800.)

Figures 13 to 15 show portions of perikarya from acid phosphatase preparations of cultured mouse dorsal root ganglia 2 days after irradiation. M indicates mitochondria.

Figures 13, 14. Reaction product is present in cisternae of rough endoplasmic reticulum (E) including the nuclear envelope (N). The thin section used for figure 14 shows endoplasmic reticulum from the cytoplasm of the same neuron as was used for figure 13 but unlike figure 13, the section was not stained with uranyl and lead; this was done to permit clear demonstration that the rather sparse reaction product is almost entirely confined within the cisternae (see Kalina & Bubis 1968 for contrasting results). L indicates a lysosome. (Figure 13: magn. × 14000; figure 14: magn. × 65000.)

Figure 15. Reaction product is seen in sacs or tubules (arrows) and in vesicles (V) present near the inner surface of the Golgi apparatus (G). The outer Golgi saccules (G) rarely show reaction product in such preparations although sometimes small amounts are present. (Magn. × 39000.)




FIGURES 11–15. For legends see facing page.

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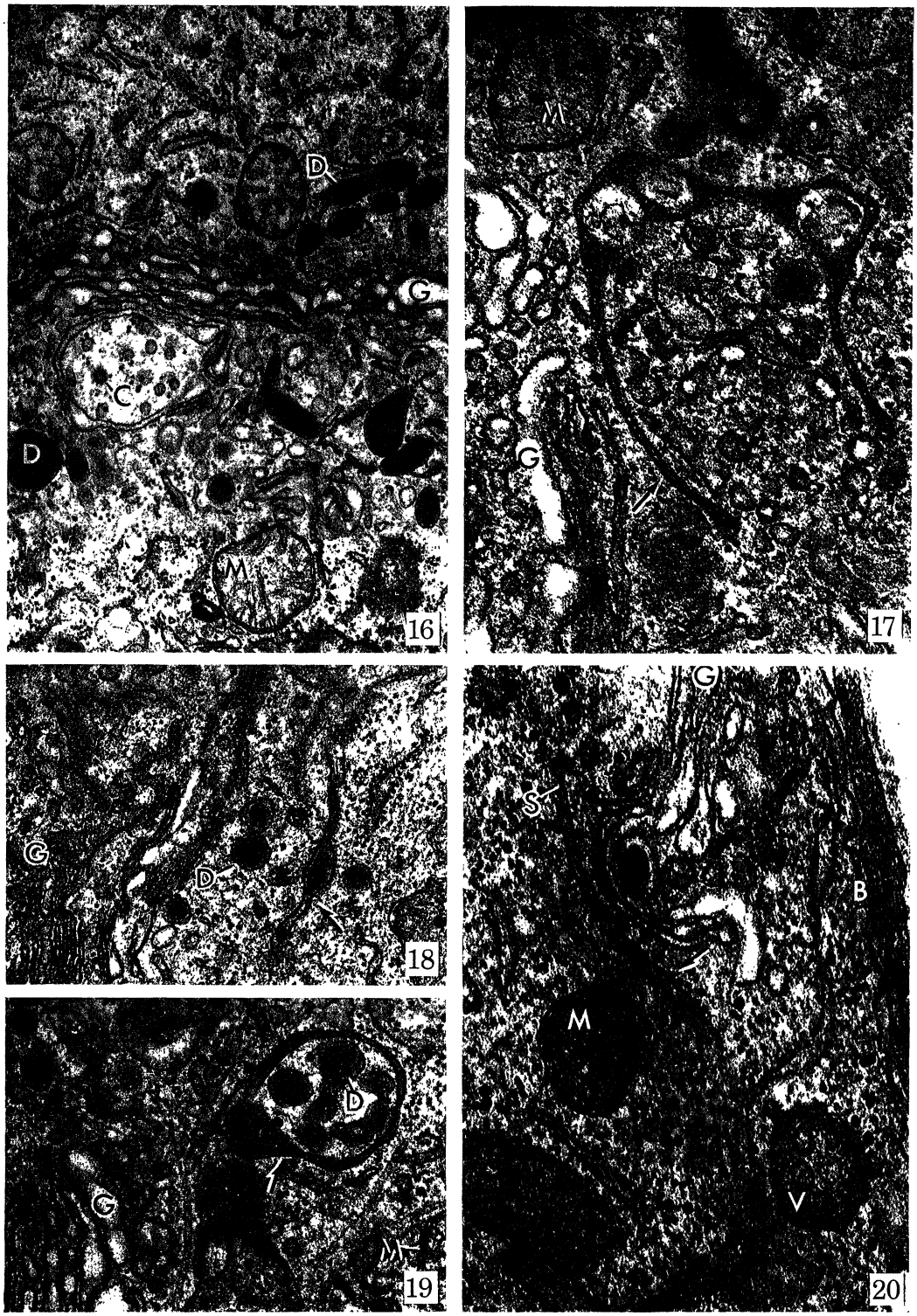


FIGURES 16–20. For legends see facing page.

biologically active molecules. (See also: Trachtenberg & Pollen 1970; Hall, Bownds & Kravitz 1970; Faeder & Salpeter 1970).

The ability of Schwann cells and many other cell types to pinocytose exogenous proteins should be borne in mind in interpreting results such as those from perfusion experiments in which release of components from neurons is evaluated by analysis of the perfusion medium leaving a tissue. Selective uptake and degradation of some of the components by Schwann cells, macrophages, and other cells, such as phagocytes associated with blood vessels might well produce misleading results.

## Uptake of large molecules by neurons

Pinocytosis by living neurons can be observed directly in tissue-cultured material; for example, Pomerat, Hendelman, Raiborn & Massey (1967) have noted the uptake of droplets of extracellular fluid by the endings of growing axons. Electron microscopic studies indicate that a variety of macromolecular tracers can be taken up in pinocytosis vesicles formed at the surfaces of perikarya, unmyelinated axons and axon endings (see, for example, Palay 1963; Rosenbluth & Wissig 1964; Brightman 1965, 1968; Birks 1966; Villegas & Fernandez 1966; Becker *et al.* 1968; Holtzman & Peterson 1969*a*) and morphological observations suggest that the plasma membrane of myelinated axons may also be capable of forming such vesicles (Waxman 1968).

Much of the material taken up by neuronal pinocytosis eventually accumulates in lysosomes (figure 24; see below and Holtzman 1969 for review). As is true of many other tissues (see, for example, Novikoff, Essner & Quintana 1964; DeDuve & Wattiaux 1966; Friend & Farquhar 1967; Holtzman & Dominitz 1968), multivesicular bodies (figures 5, 20 to 24, 34, 39) are prominent among the intraneuronal structures that come to contain exogenous proteins and other tracers; such bodies are a form of lysosome as is demonstrated by their content of acid phosphatase and aryl sulphatase activities (figures 22, 23).

The significance of macromolecule uptake by neurons is yet to be determined. Although there are restrictions, for example on the passage of material into central nervous tissue (see, for example, Becker, Novikoff & Zimmerman 1967; Reese & Karnovsky 1967; Brightman 1968; Rosenbluth 1968) it is possible that neurons normally take up some large molecules that

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## Description of plate 85

Figures 16 to 20 are from a cultured mouse dorsal root ganglion 3 days after irradiation. They show portions of perikarya near the Golgi apparatus (G).

Figure 16. Many dense bodies (D) are seen near the Golgi apparatus. C indicates a coated vesicle and M, a mitochondrion. (Magn. × 36000.)

Figure 17. Present near the inner surface of the Golgi apparatus, is a smooth-surfaced sac (arrow) with moderately electron dense content. M indicates a mitochondrion. (Magn. × 52000.)

Figure 18. The arrow indicates a dilated region of a smooth-surfaced sac with moderately electron-dense content. A small dense body is seen at D. (Magn. × 38000.)

Figure 19. At the arrow a small dense body is seen as if in process of formation by dilatation of a sac. Other dense bodies are indicated by D and the edge of a mitochondrion, by M. (Magn. × 42000.)

Figure 20. A sac (S) with moderately electron-dense content is present near the inner surface of the Golgi apparatus. A few ribosomes seem to be attached to the sac (arrow), although most of its surface lacks ribosomes. By themselves, observations of this sort would be unconvincing, but we do find all the intermediates (e.g. figure 9 at arrow) between rough ER and configurations such as this one or figure 17. M indicates a mitochondrion and V, a multivesicular body. The cell border is seen at B. (Magn. × 58000.)

enter the extracellular medium from the circulation, from Schwann cells and glial cells, from cells upon which the neurons end or even from other neurons. Rosenbluth & Wissig (1964) suggest that pinocytosis may be one mechanism by which a neuron can 'sample' its environment. Along similar lines, pinocytosis might contribute to some of the cellular interactions thought to occur in nervous tissue (see, for example, Hyden (1967), Singer (1968), Rosenbluth (1968); but see also, for example, Kuffler & Nicholls (1966)). However, it should be noted that the direct biological effects of macromolecules taken up by pinocytosis would probably be quite restricted in usual conditions since much of the material taken up rapidly becomes segregated within lysosomes, organelles capable of degrading most types of macromolecules (cf. DeDuve & Wattiaux 1966).

Phenomena similar to pinocytosis have also been implicated in the circulation of membranes in the cell (cf. Bennett 1969). For example, recent work on gland cells (Amsterdam, Ohad & Schramm 1969) supports the proposal that the addition of membrane to the cell surface that takes place during the release of secretion granules is balanced by subsequent formation of small vesicles from the plasma membrane. In this connexion, it is interesting that cells of the adrenal medulla show extensive incorporation of exogenous peroxidase through inclusion in coated vesicles and other membrane-delimited structures (Holtzman & Dominitz 1968). That this might in part represent membrane 'retrieval' similar to that proposed by Amsterdam *et al.* is suggested by observations reported during these proceedings by Dr Grynszpan-Winograd; she has observed vesicles, including many coated vesicles, continuous with and apparently forming from plasma membrane regions where adrenal secretion granule release has just occurred. In addition, Susan Abrahams in our laboratory has recently observed enhanced uptake of peroxidase, suggesting increased rates of pinocytosis, by medulla cells of rats responding to agents, such as insulin, that increase medullary secretion rates; uptake is via tubules and vesicles and much of the tracer accumulates in lysosomes and related bodies which are exceptionally large or numerous within cells that are relatively depleted of secretion granules.

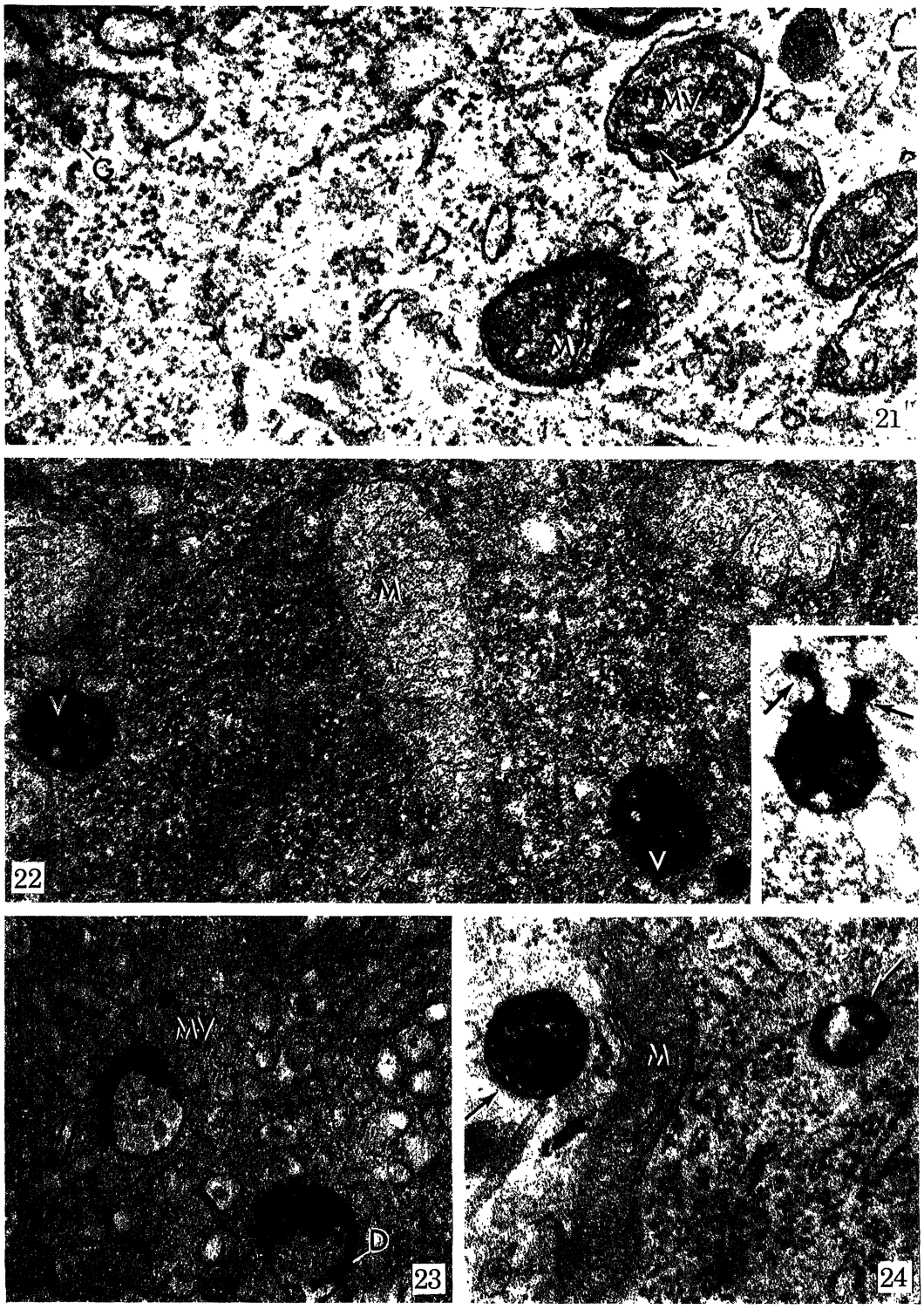
## Description of plate 86

Figure 21. Portion of a perikaryon from a cultured chick sympathetic ganglion. Within a multivesicular body (MV) there is present a small membrane-delimited granule (arrow) similar to granules seen free in the cytoplasm (G). Comparable disposal of secretion granules via multivesticular bodies and other lysosomes of gland cells (Smith & Farquhar 1966; Holtzman & Dominitz 1968) may be a physiologically important control mechanism (cf. Farquhar 1969; Masur & Holtzman 1969). The granule shown in the multivesicular body in this figure is still surrounded by a membrane, whereas some of the gland cell granules have lost their delimiting membranes (see Smith & Farquhar (1966) for the pituitary gland and Holtzman & Dominitz (1968) for the adrenal medulla); this may reflect differences in mode of entry of the granules (see Farquhar's (1969) discussion of fusion vs. more 'conventional' autophagia). Membrane-less granules are also occasionally seen in neuronal multivesicular bodies. M indicates a mitochondrion. (Magn. × 54 000.)

Figure 22. Portion of a perikaryon from an acid phosphatase preparation of a mouse dorsal root ganglion 2 days after irradiation. Reaction product is seen surrounding the vesicles within two multivesicular bodies (V). Part of a mitochondrion is present at M. The insert (lower right) shows another multivesicular body from a similar preparation. Reaction product surrounds the vesicles and also is found in two tubular 'tails' (arrows); such tails are often seen continuous with multivesicular bodies (cf. Holtzman & Dominitz 1968; Holtzman 1969). (Magn. × 52 000.)

Figure 23. Portion of a perikaryon in a sulphatase preparation of the same irradiated ganglion as was used for figure 22. Reaction product is present in a multivesicular body (MV) and in a dense body (D). (Magn. × 52 000.)

Figure 24. Portion of a peroxidase preparation of a perikaryon in the adrenal gland of a rat that had received peroxidase intravenously 35 min before fixation. Reaction product is present in two multivesicular bodies (arrows). M indicates a mitochondrion. (Magn. × 42 000.)




FIGURES 21–24. For legends see facing page.

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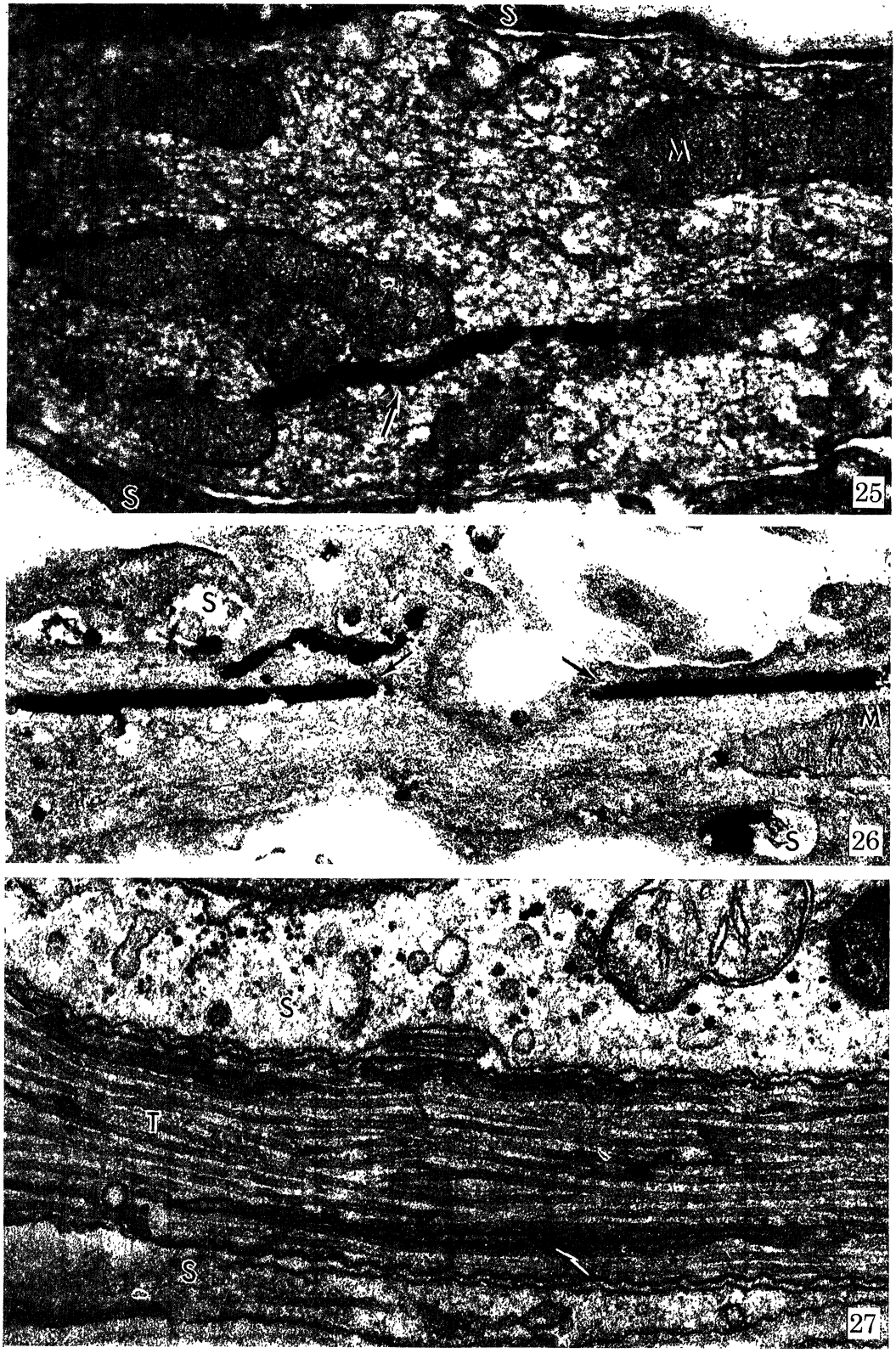


FIGURES 25–27. For legends see facing page.

These observations on gland cells are relevant to studies on axon endings. We along with others (Birks 1966; Villegas & Fernandez 1966; Brightman 1968; Bunt 1969; Zacks & Saito 1969; U. Smith, in these proceedings) have noted apparent uptake of various macromolecular tracers into small tubules and vesicles in several different types of endings (figure 4). Some of the vesicles are of morphology and dimensions similar to the coated vesicles seen elsewhere in neurons. Many, however, are smaller and lack obvious coats; they are similar to the small 'synaptic vesicles' in size and appearance. In individual sections of nerve endings in rat adrenal medulla exposed to peroxidase we have seen as many as 25 small peroxidase-containing vesicles in a single ending. Their frequency and distribution is such that it is quite unlikely that more than a small proportion are still in continuity with the plasma membrane; most appear to lie free in the axoplasm. Further, we have found that in medullas exposed to lanthanum (Revel & Karnovsky 1967) at *all* stages of *fixation*, very few of the nerve-ending tubules or vesicles show the presence of any electron-dense lanthanum deposits; lanthanum readily reaches the axon surface but not the vesicles even in tissue treated to permit simultaneous demonstration of vesicular uptake of peroxidase administered prior to fixation. This argues against the vesicles being artefacts of fixation, a possibility raised by Birks (1966), and also indicates that most peroxidase-containing vesicles were not attached to the cell surface at the time of fixation.

One reasonable explanation for these observations is that vesicles bud from the plasma membrane at axon endings as part of a device that compensates for the fusion of synaptic vesicles with the membrane. Such fusion is generally held to be involved in transmitter release (see also Bunt's 1969 discussion of neurosecretory processes). Techniques are not yet available to determine whether or not the vesicles that come to contain exogenous tracers are delimited by the same bit of membrane that originally bounded a synaptic vesicle. Also, it has proved difficult to demonstrate noteworthy alterations in rates of macromolecule uptake by endings whose rates of transmitter release have been experimentally changed (Birks 1966). Even when hints of such responsiveness are obtained, as in some of our work on peroxidase uptake by electrically stimulated lobster neuromuscular junctions (Holtzman, Freeman & Kashner 1971) the numbers of tracer-containing vesicles thus far observed to form usually fall far short of the numbers expected from the simplest schemes. (For example, it might be expected that one or more reasonably long-lived vesicle forms per ending per impulse during or very soon after repetitive stimulation of crustacean axons; cf. Bittner & Kennedy (1970) and Jones & Kwanbunbumpen (1970).) Conceivably, external tracer can reach only a percentage of the relevant vesicles or, perhaps uptake is a 'slow' process that cannot keep up with transmitter

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## Description of plate 87

Figures 25 to 27 show portions of axons from three different cultured mouse dorsal root ganglia 2 days after irradiation.

Figure 25. From an acid phosphatase preparation. Reaction product in the axon is seen in a tubule or sac (arrow). M indicates a mitochondrion and S, parts of the Schwann cell associated with the axon. (Magn. × 46000.)

Figure 26. From a sulphatase preparation. The arrows indicate reaction product within tubules or sacs in the axoplasm (see also figure 29). The Schwann cells (S) associated with the axon apparently are undergoing degeneration (cf. Masurovsky *et al.* 1967). A mitochondrion in the axon is seen at M. (Magn. × 30000.)

Figure 27. From an unincubated preparation. A tubule or sac of agranular reticulum is seen (arrow) in the axoplasm (see also figure 28); many microtubules (T) also are present. Portions of the associated Schwann cell are indicated by S. (Magn. × 51000.)

release if the stimulus rate is too high or if adequate rest periods are not provided. It should also be borne in mind that if a single thin section of an ending shows 20 or 30 tracer-containing vesicles, as is true in some of our crustacean and mammalian material, the total number of such vesicles in the ending must be in the hundreds or low thousands.

As elsewhere in the neuron, much of the peroxidase incorporated at endings ultimately is found in multivesicular bodies (figure 5). This suggests that many of the peroxidase-containing vesicles eventually become incorporated in lysosomes, presumably by fusing with the membranes surrounding multivesicular bodies as occurs with vesicles containing exogenous material in many other tissues (see Friend & Farquhar (1967) for review). The possibility that some of the vesicles are reused for transmission cannot be ruled out, but at present, it is difficult to confirm experimentally. It is also too early to decide whether or not pinocytosis by axon endings is important for reabsorption of proteins such as chromogranins or of other components released to the extracellular space during transmission and related activities.

## Some features of the organization of perikarya

The Golgi apparatus has long been known to be very well developed in perikarya of most neurons (Golgi 1898; Novikoff & Goldfischer 1961; Shanthaveerappa & Bourne 1965) but surprisingly little is known of its functioning in the neuronal economy. By autoradiography, Droz (1969) has demonstrated that as in many other cell types, proteins synthesized on the rough endoplasmic reticulum are transported to the Golgi apparatus and also that the apparatus plays a central role in the synthesis of some carbohydrate-containing macromolecules. In adrenergic and neurosecretory neurons, membrane-delimited granules similar to those found at the axon endings appear to form within the Golgi apparatus (see, for example, Grillo & Palay 1962; Osinchak 1964; Scharrer 1965); the possibility has been raised by several contributors to the present proceedings that some of the vesicles of cholinergic endings may have a similar origin. For the most part, however, the interrelations of the neuronal Golgi apparatus

## Description of plate 88

Figures 28 to 33 are from mouse dorsal root ganglion cultures.

Figure 28. From an axon 1 day after irradiation. The arrow indicates a smooth-surfaced membrane delimited tubule or sac within the axoplasm. A microtubule is present at T, microfilaments are shown at F and parts of the associated Schwann cell are seen at S. (Magn. × 47000.)

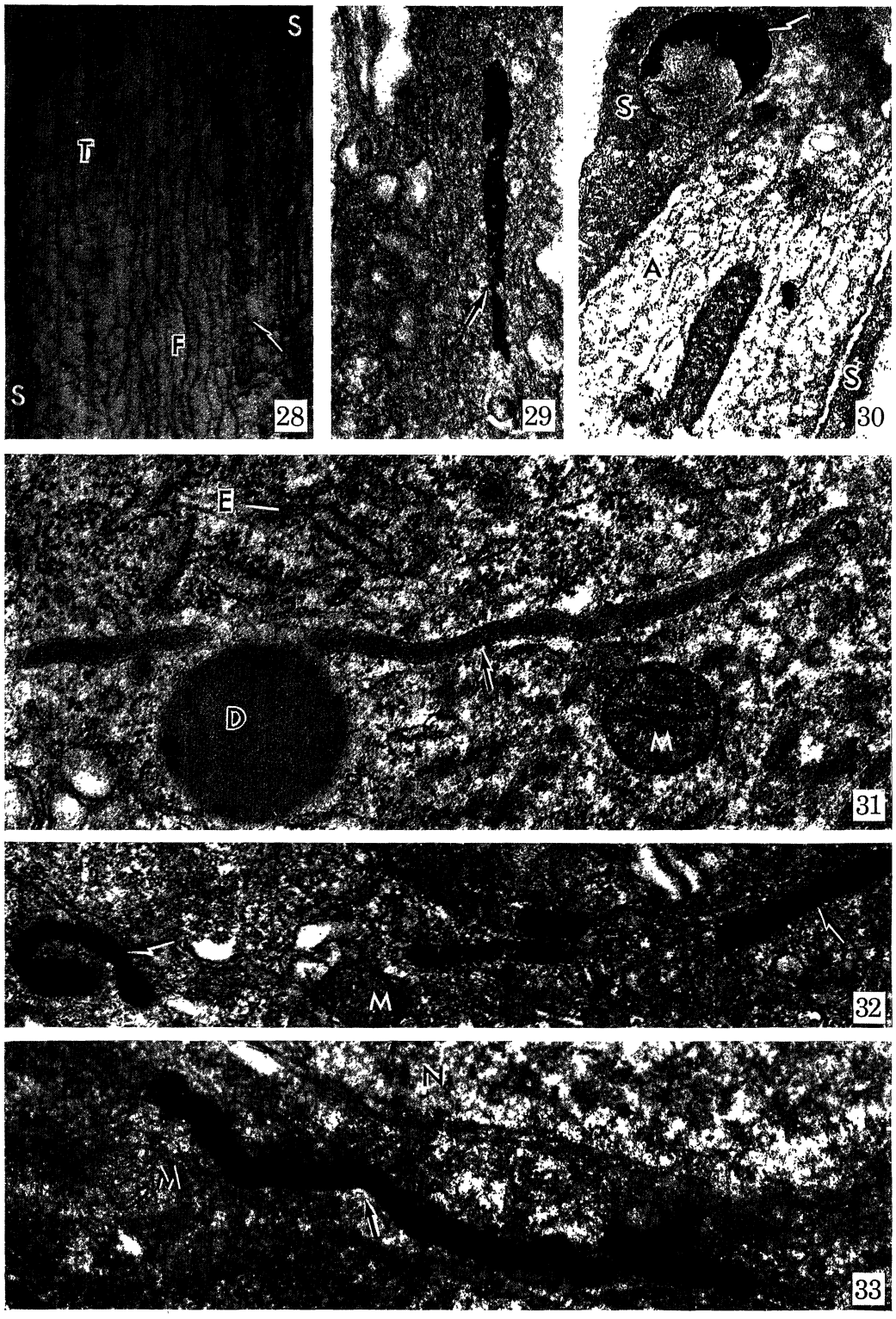
Figure 29. Portion of a sulphatase preparation showing an axon 2 days after irradiation. Reaction product is seen (arrow) in a smooth surfaced tubule or sac in the axoplasm. (Magn. × 49000.)

Figure 30. From the same preparation as was used for figure 29. Reaction product is present in a dense body (arrow) in the cytoplasm of a Schwann cell (S). M indicates a mitochondrion in the axon (A) with which this Schwann cell is associated. (Magn. × 38000.)

Figure 31. Portion of a perikaryon 6 days after irradiation at 20000 rad. The arrow indicates a smooth-surfaced tubule or sac with moderately electron-dense content. It may eventually be of interest that the width of this structure is similar to the width of nearby cisternae of rough endoplasmic reticulum (E). Some such tubules or sacs appear to fragment into small dense bodies. A large dense body is present at D and a mitochondrion at M. (Magn. × 51000.)

Figure 32. Portion of a sulphatase preparation showing part of a perikaryon 2 days after irradiation. Reaction product is seen in structures of the type shown in figure 31 (arrows). A mitochondrion is seen at M. (Magn. × 42000.)

Figure 33. Portion of an acid phosphatase preparation showing part of a perikaryon 2 days after irradiation. Reaction product is seen in a structure of the type shown in figure 31 (arrow). M indicates a mitochondrion and N, the edge of the nucleus. (Magn. × 39000.)




FIGURES 28–33. For legends see facing page.

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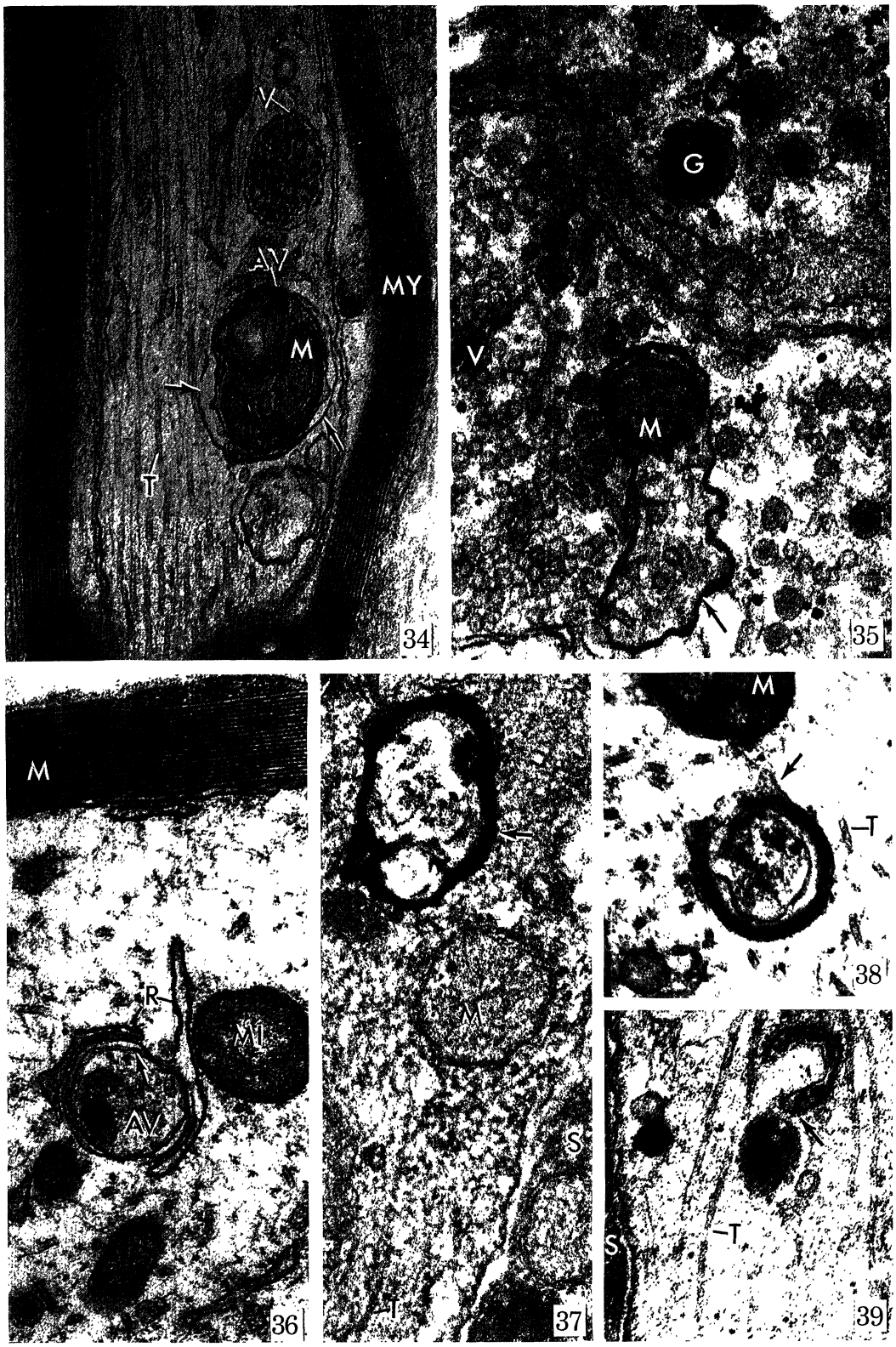


FIGURES 34–39. For legends see facing page.

with other organelles and the identity, significance and fate of the molecules processed by the apparatus are poorly understood.

Lysosomes are also quite abundant in perikarya (figure 11; see reviews by DeDuve & Wattiaux 1966; Novikoff 1967; Holtzman 1969; Koenig 1969). Through electron microscopy coupled with cytochemical demonstration of acid hydrolase activity, several distinct types of neuronal structures can be identified as lysosomes. The identification is tentative since until recently only one of the lysosomal hydrolases, acid phosphatase, was reliably demonstrable in the electron microscope; new techniques for enzymes such as aryl sulphatase (Goldfischer 1965) have now permitted demonstration of other acid hydrolases in some of the acid phosphatase-containing structures both within neurons and in other cells of the nervous system (figures 11, 22, 23, 25 to 33; cf. Holtzman 1969). Among the lysosomes normally present in neurons are: autophagic vacuoles (figure 35) in which the cell sequesters and degrades a portion of its own cytoplasm; multivesicular bodies, primarily involved in handling exogenous materials (cf. the preceding section of this report) although some play roles resembling autophagia (figure 21; see Holtzman & Albala 1966; Smith & Farquhar 1966; Holtzman, Novikoff & Villaverde 1967; Holtzman & Dominitz 1968; Farquhar 1969); small Golgi-associated vesicles (figure 15) that probably are 'primary lysosomes' transporting hydrolases to other membrane-delimited bodies with which they fuse (some of the vesicles are 'coated' (Holtzman *et al.* 1967)); dense bodies of various kinds (figures 9, 10, 16, 19, 23 and 31).

Both in neurons and in other cells, many types of lysosomes appear to form near the Golgi apparatus (see, for example, Novikoff *et al.* 1964; Novikoff & Shin 1964; DeDuve & Wattiaux 1966; Holtzman *et al.* 1967; Holtzman 1969; Friend & Farquhar 1967; Bainton & Farquhar 1966, 1968, 1970). Based on his study of neurons, Novikoff (1967) has advanced the proposal that G.e.r.l., a special region of Golgi-associated endoplasmic reticulum, is responsible for transport of hydrolases from ribosomes bound to the endoplasmic reticulum to the Golgi region and for

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## Description of plate 89

**Figure 34.** Portion of an axon from a mouse dorsal root ganglion culture 2 days after irradiation. AV indicates an autophagic vacuole containing a mitochondrion (M). The membrane system delimiting the vacuole appears to involve two membranes (arrows) as might be expected if it arose from a sac. A multivesicular body is seen at V, a microtubule at T and myelin at MY. (Magn. × 48 000.)

**Figure 35.** An axon ending (or *bouton de passage*; cf. Coupland 1965) from a rat adrenal medulla. The arrow indicates an autophagic vacuole containing a mitochondrion (M). Synaptic vesicles are seen at V and a granule in a nearby adrenaline-producing cell is present at G. (Magn. × 49 000.)

**Figure 36.** Portion of an axon from a mouse dorsal root ganglion culture. The structure at AV probably is a lysosome of the autophagic vacuole type; a region of axoplasm apparently is enclosed within a system that consists of two membranes (arrow; cf. figure 34). Agranular reticulum (R) is closely associated with the surface of the structure. MI indicates a mitochondrion and M, myelin. (Magn. × 45 000.)

**Figure 37.** Portion of an axon, not far from the perikaryon, in an acid phosphatase preparation of a mouse dorsal root ganglion culture 2 days after irradiation. At the arrow, a sac containing reaction product is seen as if surrounding a region of axoplasm. M indicates a mitochondrion, T, a microtubule and S, part of the Schwann cell associated with the axon. (Magn. × 52 000.)

**Figure 38.** From an axon in a mouse dorsal root ganglion culture. The structure at the arrow can be interpreted as a smooth-surfaced sac in process of forming a multilayered membrane system that surrounds a small region of the axoplasm. M indicates the edge of a mitochondrion and T, a microtubule. (Magn. × 54 000.)

**Figure 39.** Portion of an axon from a mouse dorsal root ganglion culture. 'Cup-like' structures of the type shown at the arrow appear to give rise to multivesicular bodies (Holtzman *et al.* 1967; Holtzman & Dominitz 1968; Holtzman 1969). S indicates part of the Schwann cell associated with the axon and T, a microtubule. (Magn. × 47 000.)

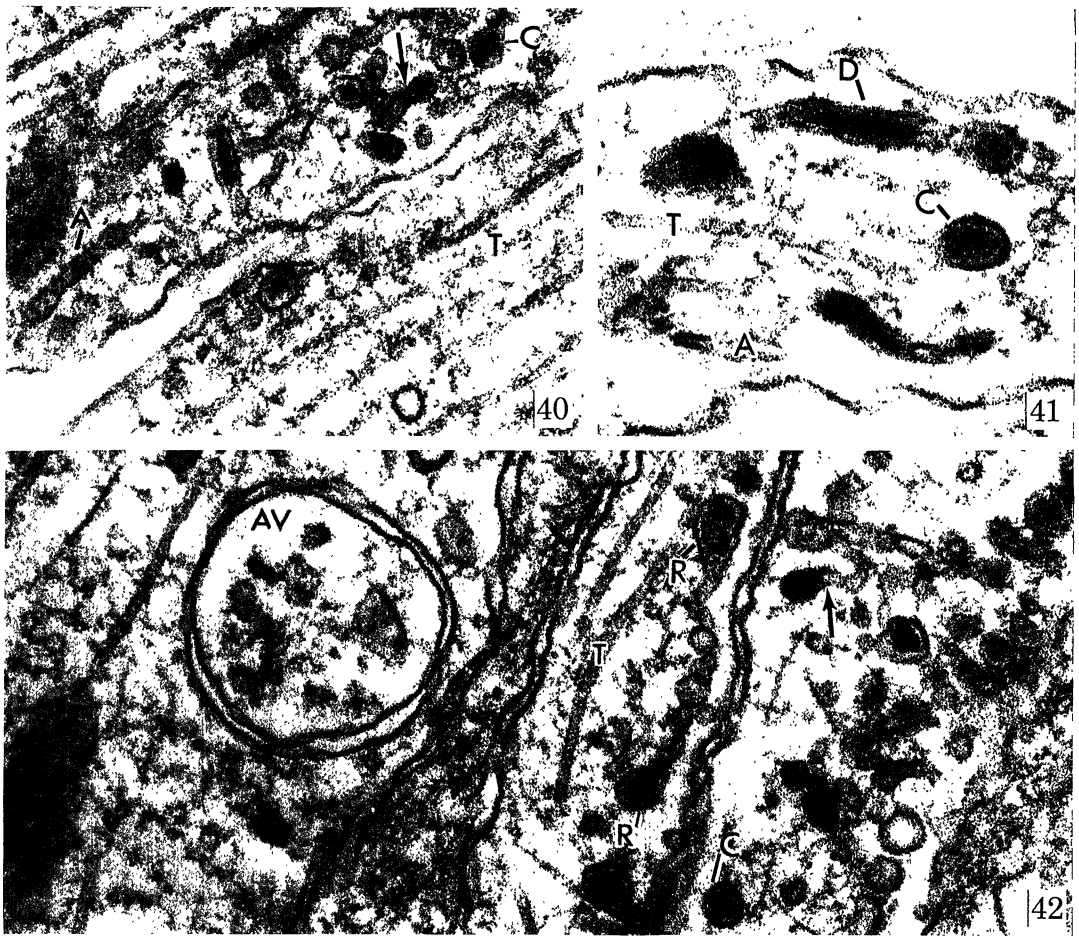
packaging of the hydrolases into several types of lysosomes. Subsequently, similar configurations of interrelated elements of the Golgi apparatus and endoplasmic reticulum have been identified in several cell types (including secretory cells of the adrenal medulla (Holtzman & Dominitz 1968)) and tentatively implicated in transport of a number of components other than acid hydrolases (see, for example, Novikoff & Biempica 1966; Holtzman *et al.* 1967; Novikoff, Albala & Biempica 1968).

The interrelations of Golgi apparatus and endoplasmic reticulum and the formation of lysosomes can be studied to advantage under abnormal conditions in which many lysosomes are formed and patterns of intracellular transport presumably are altered. This is true, for example, in the response to irradiation. Thus, in irradiated cultured KB cells, Lane & Novikoff (1965) have demonstrated extensive lysosome formation accompanied by the accumulation of acid phosphatase activity in the endoplasmic reticulum. Several investigators have reported dramatic changes involving lysosomes in irradiated neurons (see, for example, Kagan, Brownson & Sutter 1962; Andres 1963; Pick 1965; Forssman, Tinquely, Posternak & Rouiller 1966; Masurovsky, Bunge & Bunge 1967; Holtzman & Peterson 1969*b*).

Irradiation of cultured mouse dorsal root ganglia results in the accumulation of lysosomes in the centre of the perikaryon (Holtzman 1969; Holtzman & Peterson 1969*b*) and the formation of large numbers of dense bodies (figures 9, 10, 16, 31) and autophagic vacuoles (cf. Masurovsky *et al.* 1967; Holtzman 1969; Holtzman & Peterson 1969*b*). Acid phosphatase is demonstrable in the rough endoplasmic reticulum of some of the injured perikarya (figures 13, 14); such activity is found only very rarely in normal neurons (Novikoff 1965). Acid phosphatase is also found in membrane systems associated with the Golgi apparatus (figure 15).

The Golgi apparatus of a 'normal' cultured mouse neuron is shown in figure 6. As is true of many cell types, the apparatus shows an obvious morphological polarity that probably reflects functional differences between inner and outer surfaces. Endoplasmic reticulum in configurations suggesting involvement in transport of material to the Golgi region is found occasionally in normal mouse neurons (Holtzman 1969). Similar appearances are encountered more frequently after irradiation. Figure 10 shows a vesicle apparently in process of budding from the rough endoplasmic reticulum near the Golgi apparatus; such vesicles have been implicated in transport of protein in several cell types (Faure-Fremiet, Favard & Carasso 1962; Friend 1965), notably the exocrine cells of the mammalian pancreas (Jamieson & Palade 1965, 1967, 1968). In figure 8 a cisterna of rough endoplasmic reticulum is seen with ribosomes only on the surface facing away from the Golgi apparatus; this type of relationship may indicate transformation of cisternae of endoplasmic reticulum into Golgi saccules by close apposition with the outer surface of the Golgi apparatus and loss of ribosomes (Essner & Novikoff 1962; Novikoff & Biempica 1966; Novikoff, Roheim & Quintana 1966; Flickenger 1969*a*, *b*). Sometimes unusal configurations, such as the one shown in figure 7, are encountered. Most frequent in irradiated preparations, however, is close association of endoplasmic reticulum with the inner Golgi surface (figure 9); in this region it often is difficult, and may not be meaningful to distinguish sharply between Golgi saccules and cisternae of smooth endoplasmic reticulum presumably derived from rough endoplasmic reticulum by loss of ribosomes (cf. Novikoff 1967; Holtzman & Dominitz 1968; Holtzman 1969).

The dense bodies that accumulate after irradiation arise from dilated regions of smooth-membrane delimited tubules or sacs in which a moderately electron dense content is present. These structures are sometimes interspersed among cisternae of endoplasmic reticulum (figures




FIGURES 40–42. Portions of axons from chick sympathetic ganglia grown in culture (from work by S. Teichberg in in our laboratory). The cells shown in figures 41 and 42 were exposed to 5-hydroxydopamine; the one in figure 40 was not. T indicates microtubules, A, agranular reticulum and C, dense-cored vesicles. AV designates an autophagic vacuole.

The configurations shown at the arrows, suggest that dense-cored vesicles may arise by dilatation of regions of agranular reticulum in which a dense content (D in figure 41) accumulates. Similar configurations are seen near the Golgi apparatus and have also been noted in other sympathetic nervous systems preparations (for example, in the axons studied by Pellegrino de Iraldi & de Robertis (1968), although the interpretation by these investigators differs somewhat from ours). Structures such as the ones shown at R in figure 42 may eventually prove to be of interest for analysis of the formation of dense cores; we have the impression from study of the effects of reserpine and hydroxydopa compounds that electron dense material, resembling that in the cores, may be deposited in association with structures with the appearance of membrane-delimited vesicles that accumulate within the agranular reticulum. The source of such 'vesicles' might be the reticulum membrane but analysis of the phenomenon is complicated by several factors, including the possible presence of virus-like particles in some of the cultured neurons. (Figure 40: magn. ×55000; figure 41: magn. ×75000; figure 42: magn. ×60000.)

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31 to 33) and some show no obvious relationship to the Golgi apparatus. The sources of the many dense bodies that do arise in the Golgi region (figures 17 to 19) include membrane systems located near the inner surface of the Golgi apparatus (figure 17) and showing acid phosphatase activity (figure 15). These are the systems Novikoff (1967) designates G.e.r.l. While the sacs or tubules are usually smooth-surfaced (i.e. they lack ribosomes), configurations such as the one shown in figure 20 suggest that they may derive from Golgi-associated endoplasmic reticulum.

It is, of course, necessary to be cautious in drawing conclusions about directions of transport and processes of transformation from static electron micrographs. With this limitation, the observations just cited suggest that transport between the endoplasmic reticulum and the Golgi region in neurons may involve several different routes and mechanisms (see also Holtzman & Dominitz (1968), for similar observations on adrenal medulla cells). It will be of interest to determine whether different components are transported in different ways and how the cell controls the appropriate admixing, segregation and packaging of materials processed by the Golgi apparatus and associated systems (cf., for example, Bainton & Farquhar 1966, 1968, 1970).

### Axonal lysosomes, agranular reticulum and dense-cored vesicles

Lysosomes are relatively infrequent in normal axons, although a few are regularly found both along the axon and in endings. We have already discussed multivesicular bodies that sequester exogenous material. Autophagic vacuoles also are encountered (figure 35) as well as dense bodies of several types (cf. Holtzman & Novikoff 1965; Holtzman 1969). Presumably, these lysosomes contribute to normal turnover of axonal constituents. Structures that appear to be autophagic vacuoles and multivesicular bodies (figure 39) in process of formation are also encountered in axons suggesting that at least some of the lysosomes are formed locally rather than transported from the perikaryon.

After injury, large numbers of lysosomes are found in focal accumulations within axons (figure 34; Webster 1962; Holtzman & Novikoff 1965; Masurovsky *et al.* 1967; Holtzman 1969). Acid phosphatase and aryl sulphatase activities are demonstrable in many of these bodies. Focal accumulation of acid phosphatase activity is seen shortly after injury even in the distal segment of interrupted axons (figure 12; Gould & Holt 1961; Holtzman & Novikoff 1965). Apparently this represents accumulation or unmasking of enzyme previously present in the axon since in the absence of continuity with the perikaryon there is no obvious source of newly synthesized protein (see also Lubinska's (1964) discussion of cholinesterase accumulation under similar circumstances).

Since lysosomal hydrolases can digest essentially all components of the axon, enzyme transport must normally depend on some system that restricts contact between the hydrolases and their potential substrates. Normally, little hydrolase activity is demonstrable in axons other than that found in the lysosomes. However, in interrupted axons, acid phosphatase is detectable in smooth surfaced sacs or tubules thought to represent a form of agranular endoplasmic reticulum (Holtzman & Novikoff 1965). The situation is similar in irradiated neurons in which both acid phosphatase and aryl sulphatase activities are demonstrable in the axonal agranular reticulum during the first few days after injury (figures 25 to 29). At least some of the lysosomes in injured axons appear to arise from the agranular reticulum by processes involving the enclosing of regions of axoplasm within reticulum-derived membrane systems (figures 34, 36 to

38; see also Holtzman & Novikoff 1965; Blumcke & Niedorf 1965; Holtzman 1969). (Morphological studies suggest that autophagic vacuoles in perikarya also arise by the enclosing of cytoplasmic regions within membrane systems derived from pre-existing tubules or sacs (Holtzman *et al.* 1967; Holtzman 1969) and in cytochemical preparations of irradiated neurons, acid phosphatase-containing sacs are sometimes seen as if surrounding areas of cytoplasm. Rough endoplasmic reticulum is often very closely associated with the membranes that delimit newly forming autophagic vacuoles in injured perikarya (see also Masurovsky *et al.* 1967; Terry, Wisniewski & Johnson 1970) suggesting origin of the membranes from the reticulum (cf. Novikoff & Shin 1964; Beaulaton 1967), although this may not be the only mode of formation (see DeDuve & Wattiaux 1966; Frank & Christensen 1968; Ericsson 1969).)

These findings indicate that the agranular reticulum of axons represents a route for intra-axonal transport of one class of enzymes, the lysosomal hydrolases. There are reports that acetylcholinesterase may also be carried by the agranular reticulum (see, for example, Tennyson *et al.* 1968). Presumably transport within this compartment could occur at rates different from transport in other axonal compartments which might help explain the heterogeneity of rates observed for movement of different components within axons. Recent observations by Saul Teichberg in our laboratory suggest that among the components carried by the reticulum are some that become packaged into dense-cored vesicles, 50 to 80 nm in diameter, and resembling vesicles often found at synapses (figures 40–42).

## Summary and conclusions

The findings reviewed in the present report bear on several aspects of uptake, transport and intracellular digestion by neurons and associated cells. Through use of microscopically demonstrable tracers, it has been shown that relatively large molecules can readily cross Schwann and glial cell sheaths and thus reach the neuronal surface. Subsequently, such molecules can gain access to the interior of neurons by inclusion in membrane-delimited vesicles and tubules formed by the plasma membranes surrounding perikarya, axons and endings. Such vesicle formation also may be involved in the circulation of membranes between the cell surface and the cytoplasm.

Exogenous macromolecules that enter neurons tend to be sequestered in lysosomes; some membranes that participate in their transport become incorporated by lysosome membranes. Endogenous molecules and organelles may be digested in autophagic vacuoles. Although quantitative information is not available, it is likely that lysosomes play important roles in normal turnover of a variety of neuronal constituents in all parts of the cytoplasm.

Intracellular transport of lysosomal hydrolases depends on the endoplasmic reticulum and Golgi apparatus. In perikarya, several routes exist by which these enzymes and other proteins might pass from the rough endoplasmic reticulum to the Golgi region. Among these, the best present candidates for hydrolase transport pathways are the cisternae of endoplasmic reticulum that are associated with the inner surface of the Golgi apparatus. In axons, the agranular reticulum appears to fulfil a similar function. Some types of dense-cored vesicles also appear to form both near the Golgi apparatus and from axonal agranular reticulum.

These conclusions probably apply not only to the particular 'marker' molecules studied, but also to other components for which reliable techniques of microscopic demonstration are not yet available. They also illustrate the fact that work on nervous tissue responding to abnormal

conditions can contribute useful insight into normal mechanisms (cf., for example, the studies on lysosomes reviewed above (see also Holtzman 1969), Olssons's (1968) findings on pathological change in vascular permeability and Whetsall & Bunge's (1969) investigation of alterations in the Golgi apparatus of ouabain-treated neurons).

Finally, it should be borne in mind that in investigating intracellular localization, microscopists and biochemists tend, for technical reasons, to concentrate on macromolecules and on relatively large and well-defined structures, usually those that are delimited by membranes. Many of the most interesting phenomena in nervous tissue depend on inorganic ions or on small molecules and some may involve important enzyme systems that are not associated with readily recognizable organelles. Roles of structures such as the microfilaments that abound in neurons are very incompletely understood. A number of these components have been studied electrophysiologically or by biochemistry and autoradiography and a few promising cytochemical methods are in process of development. Integration of such studies with morphological work already has added many significant details to the analysis of neuronal organization (for example, Pappas & Bennett's (1966) observations on electrotonic junctions) and is a key area for future research.

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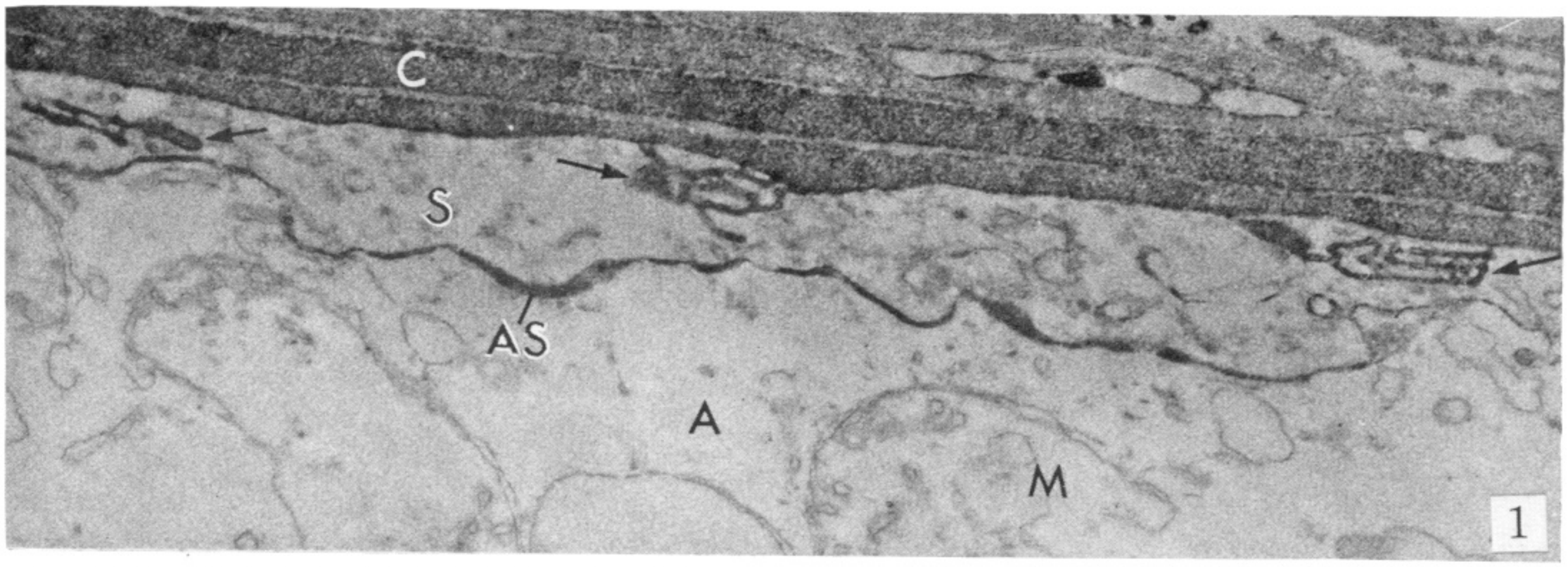
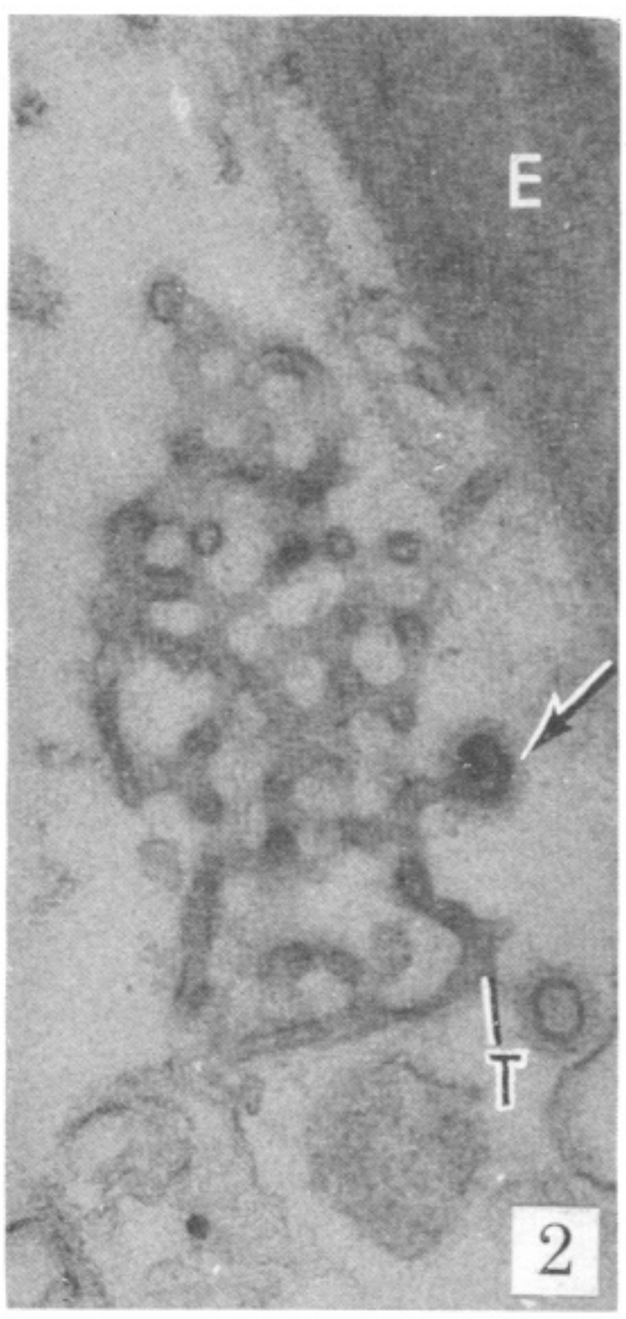
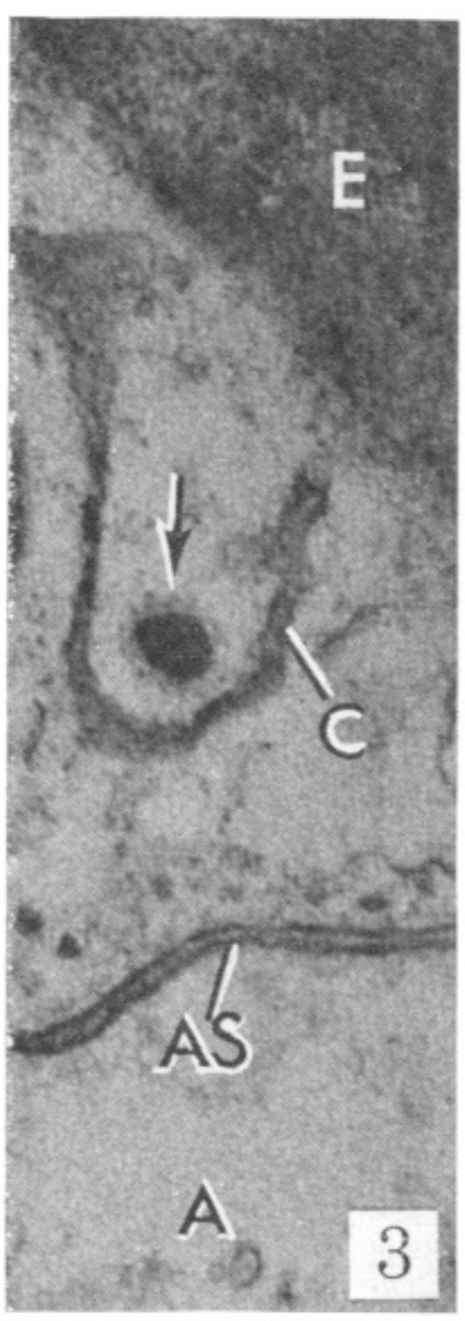
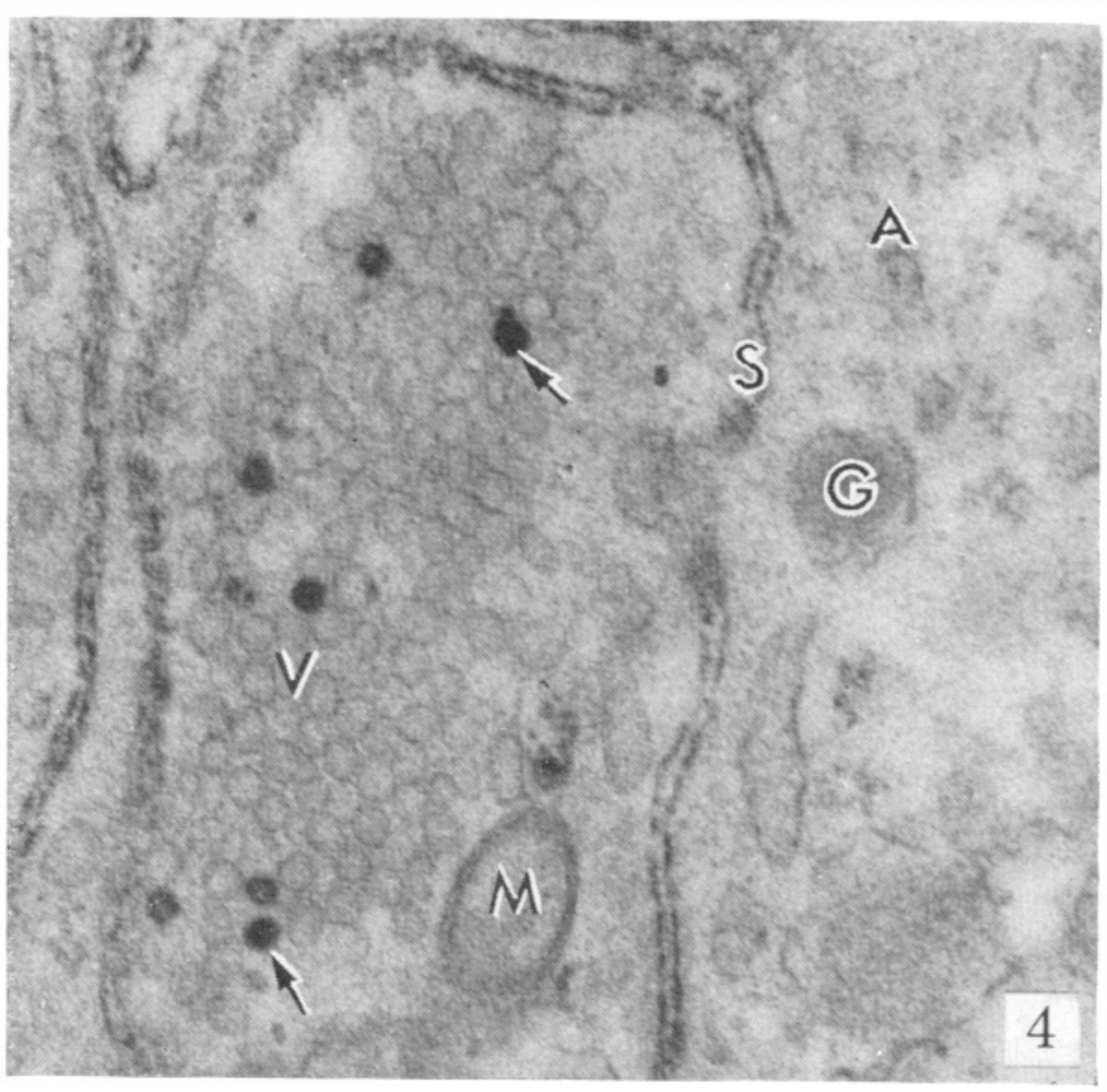
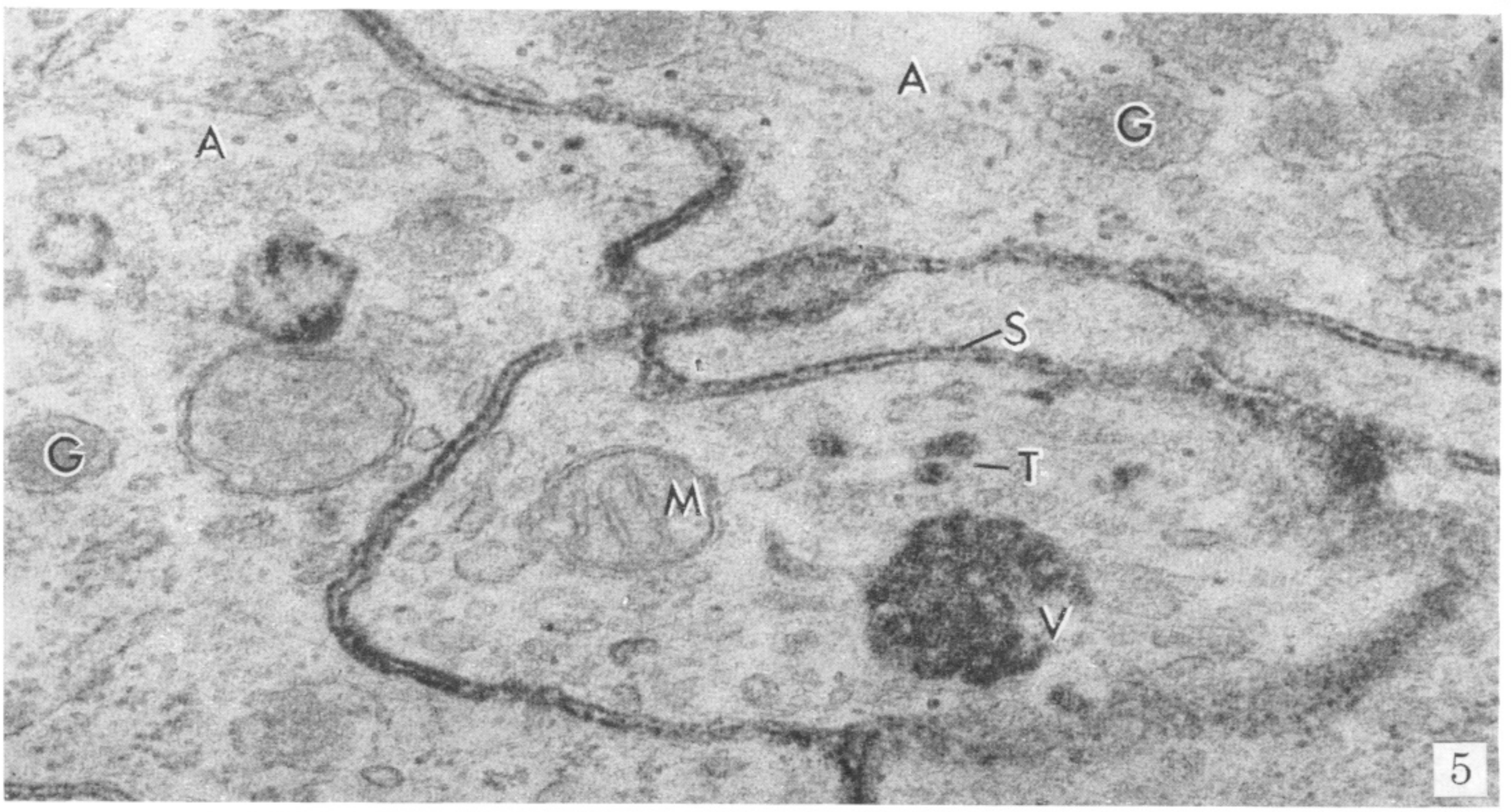


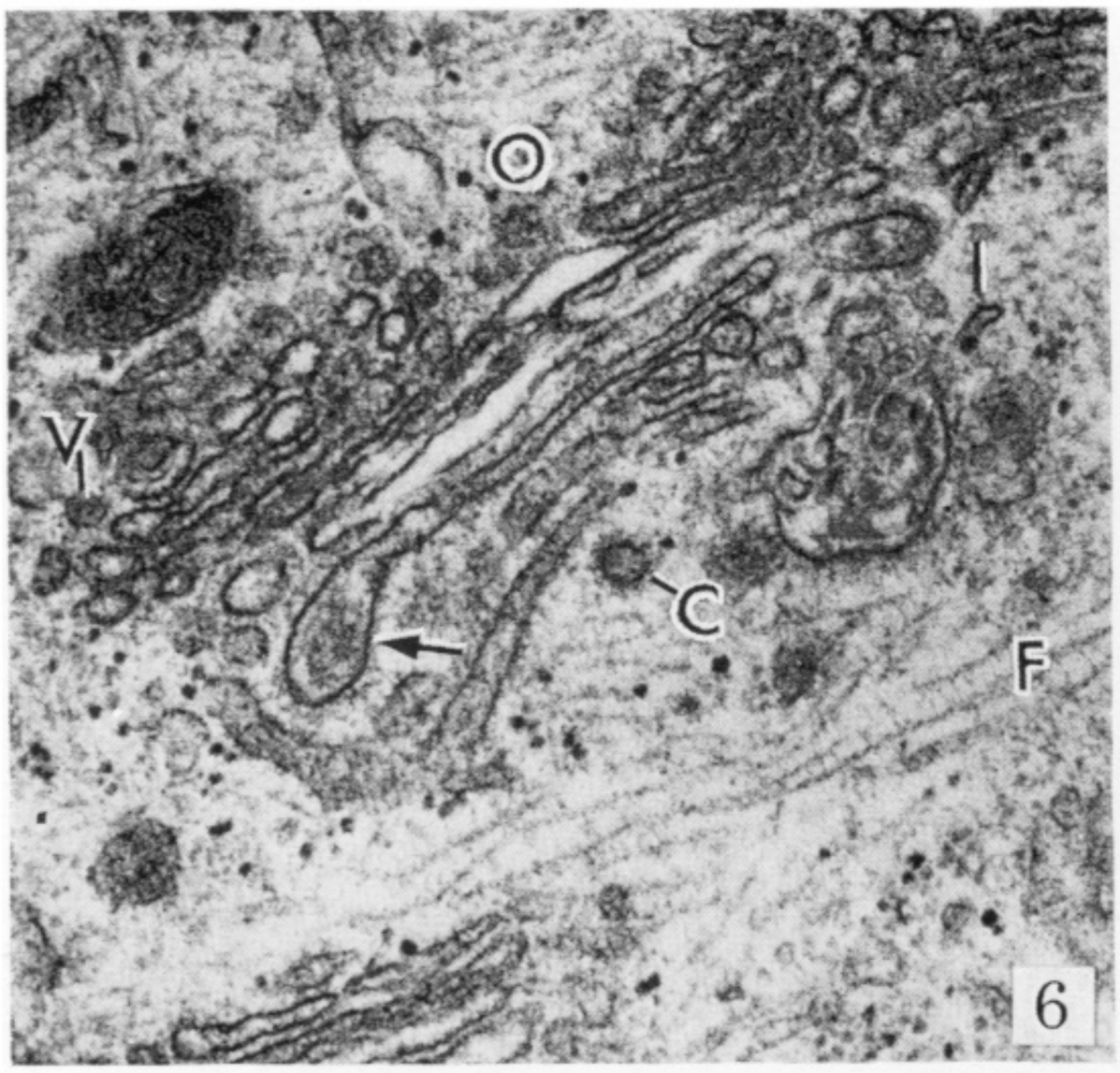



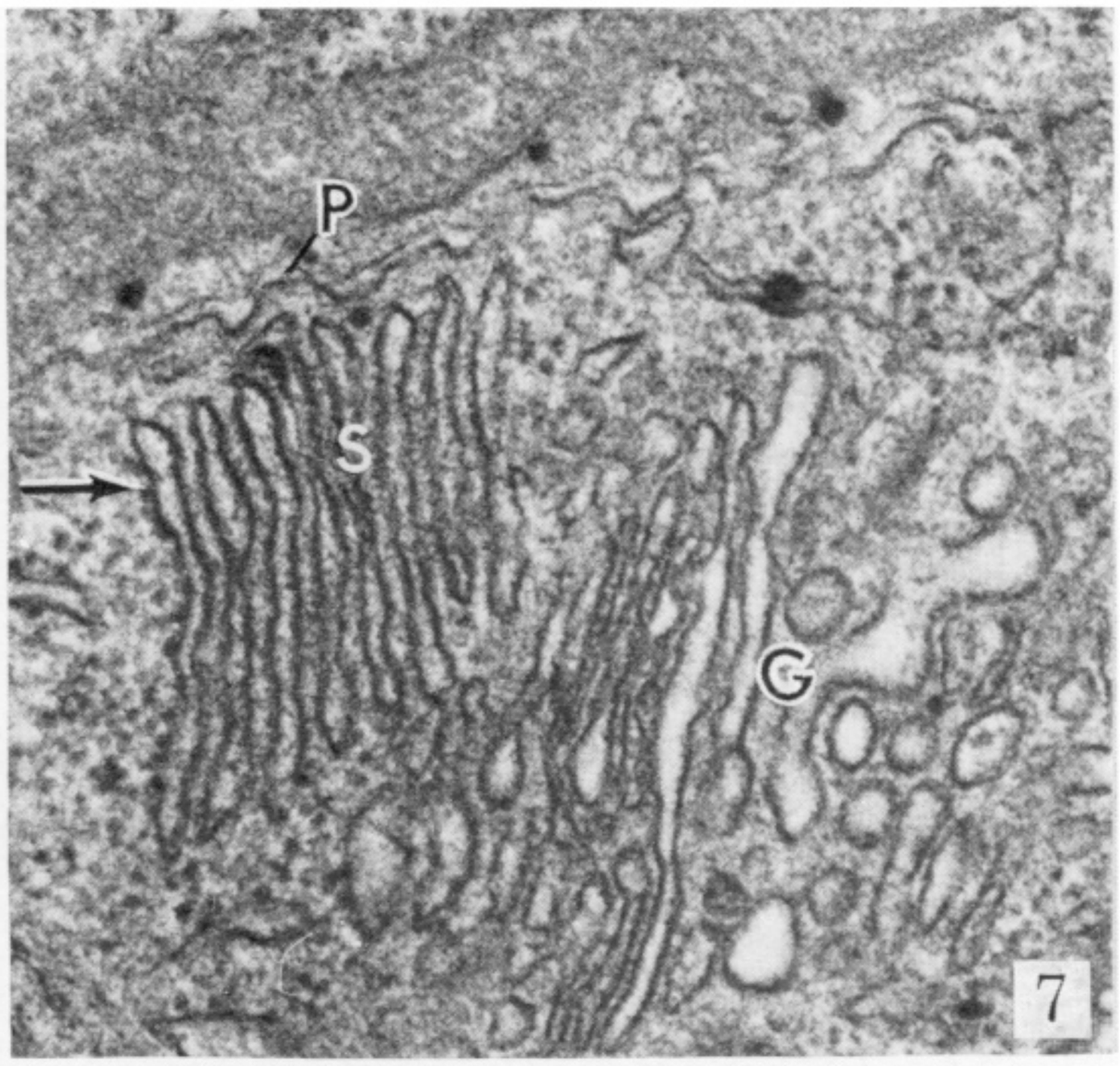



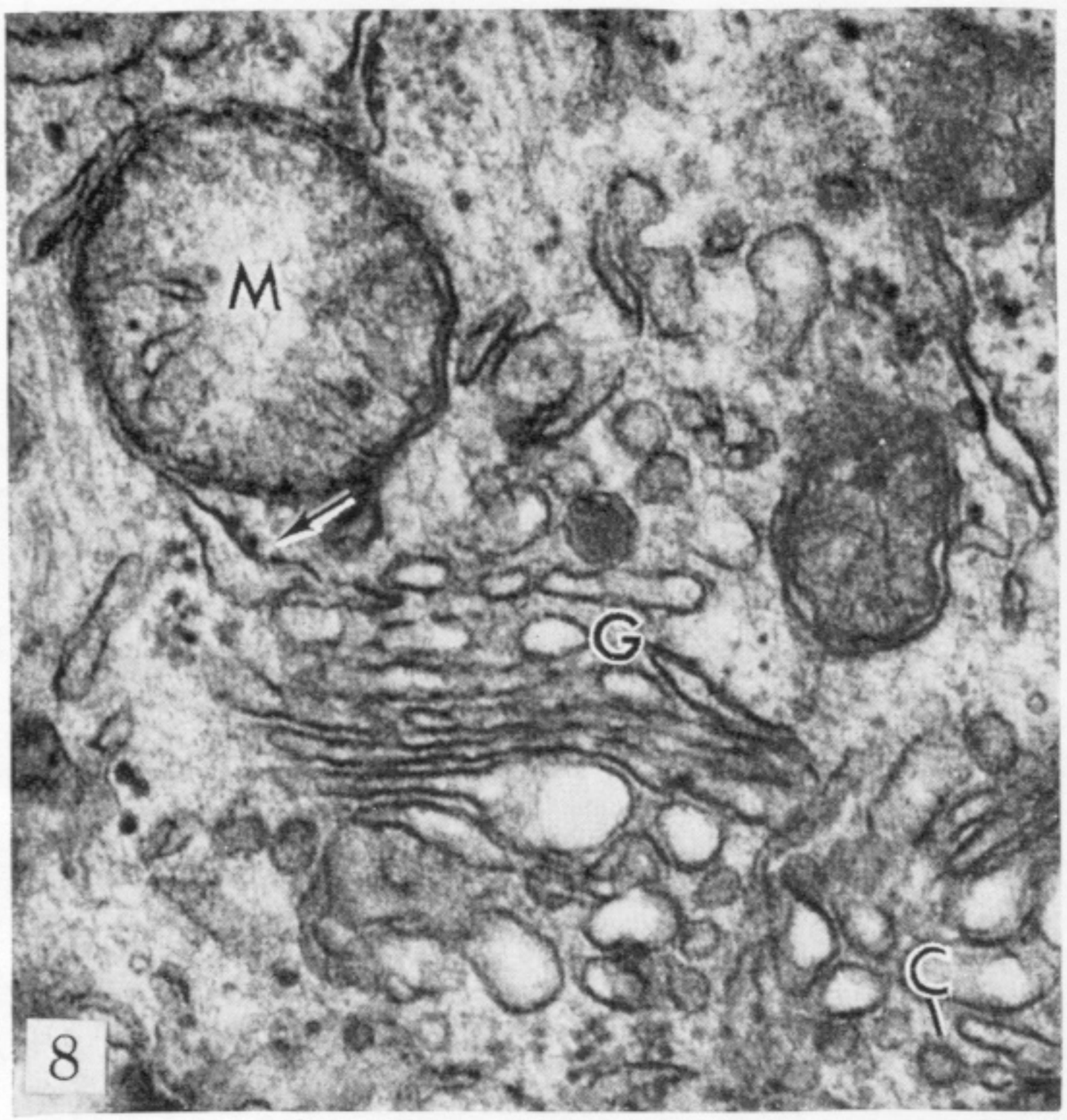



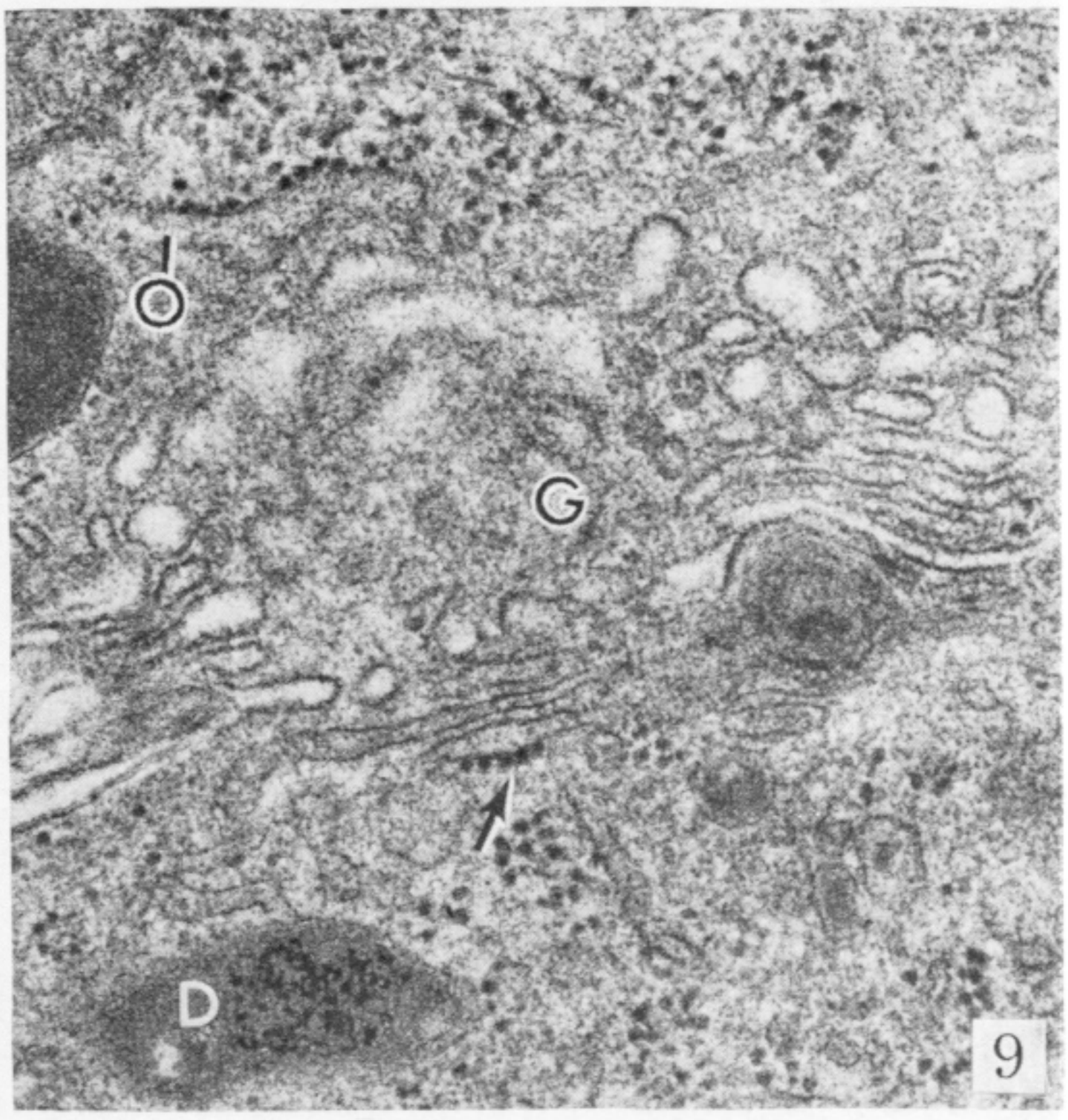



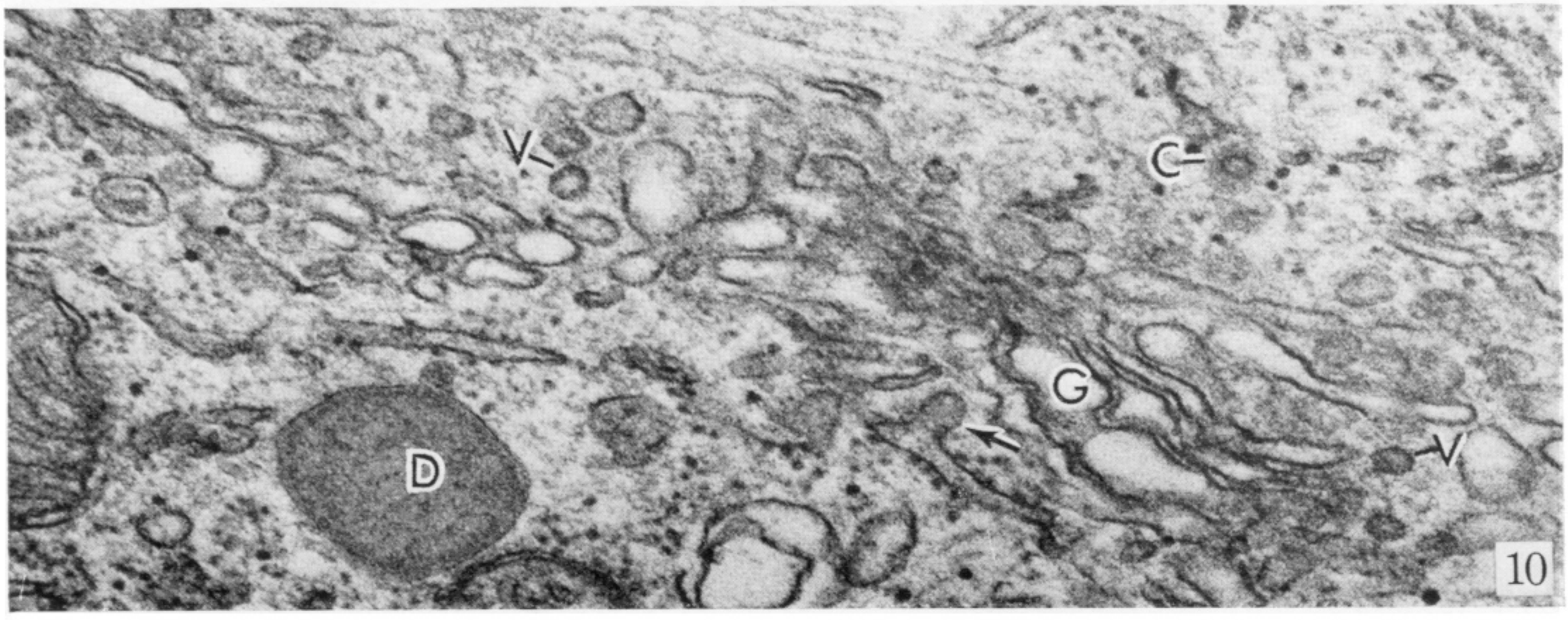

FIGURES 1–5. For legends see facing page.



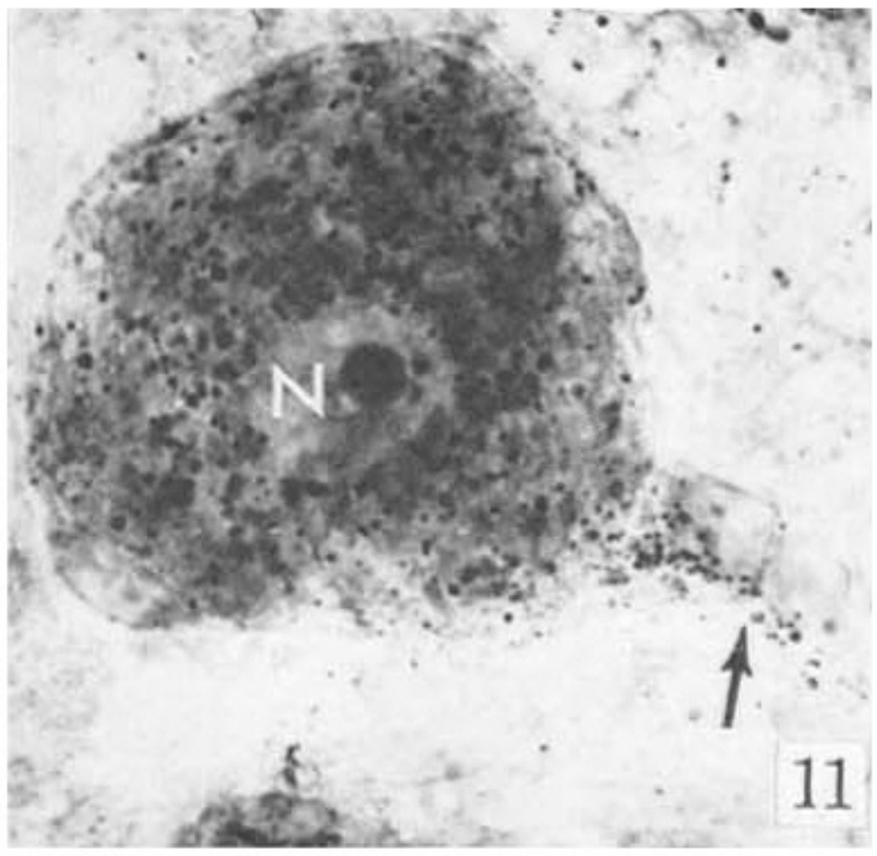



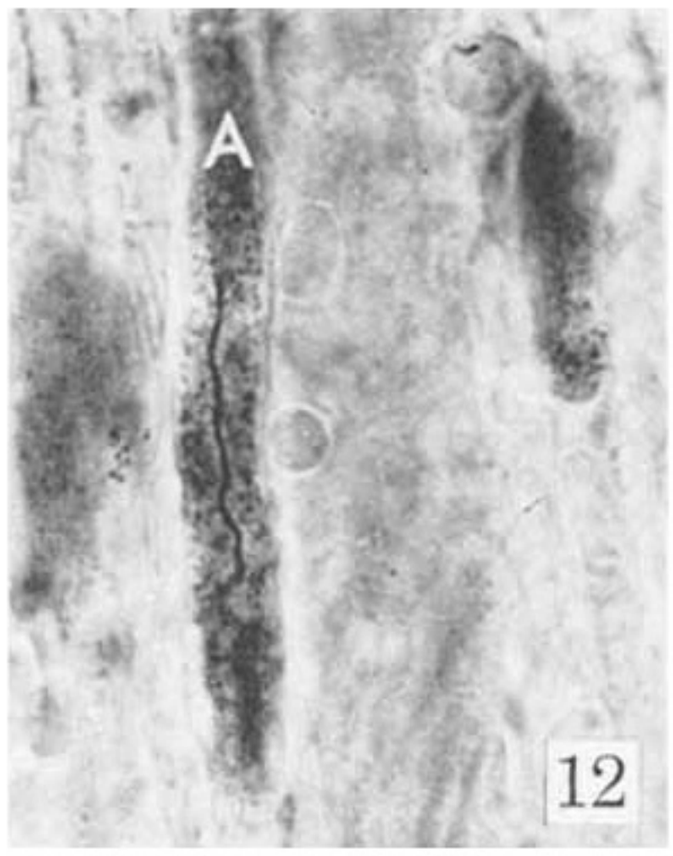



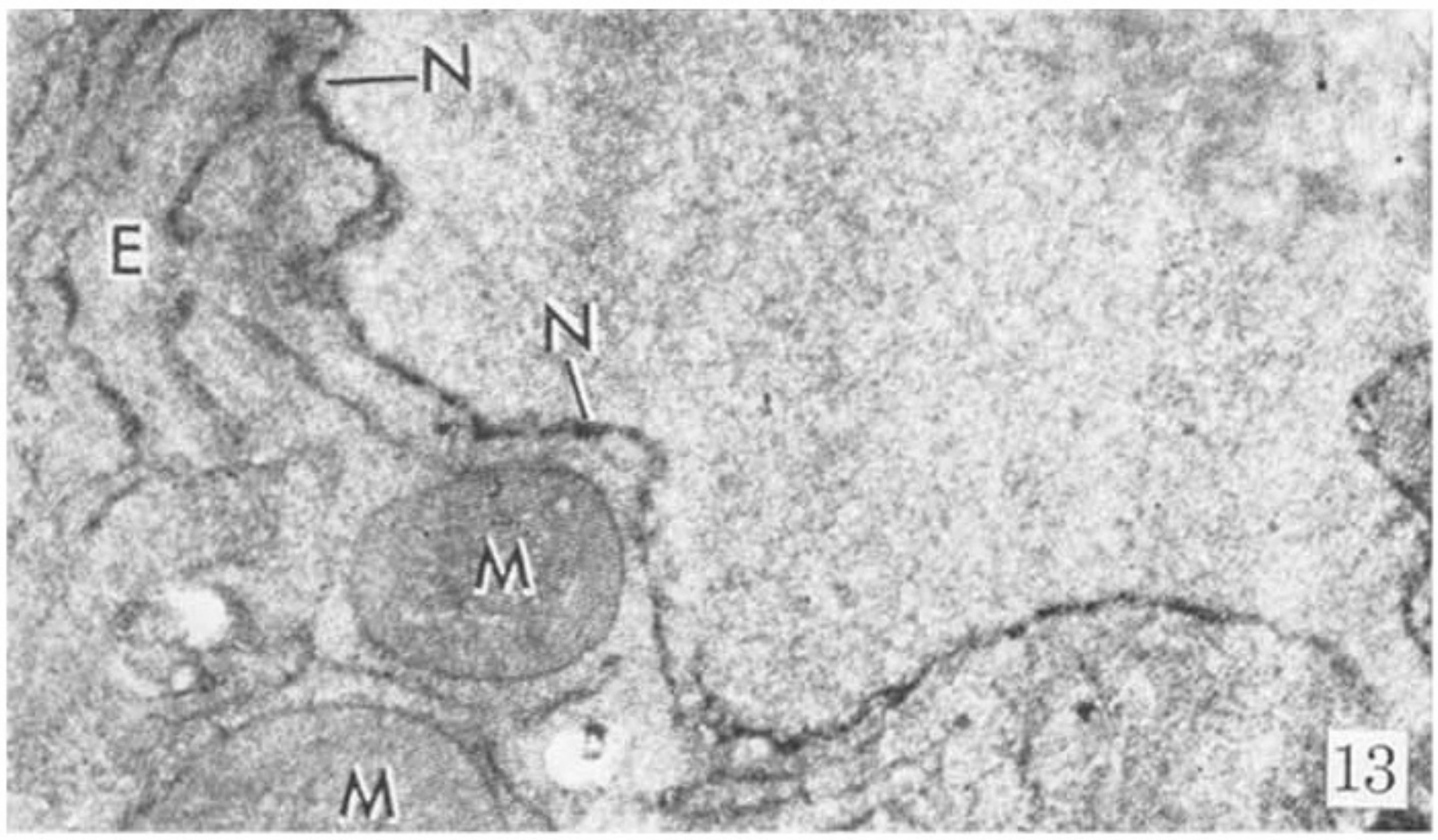



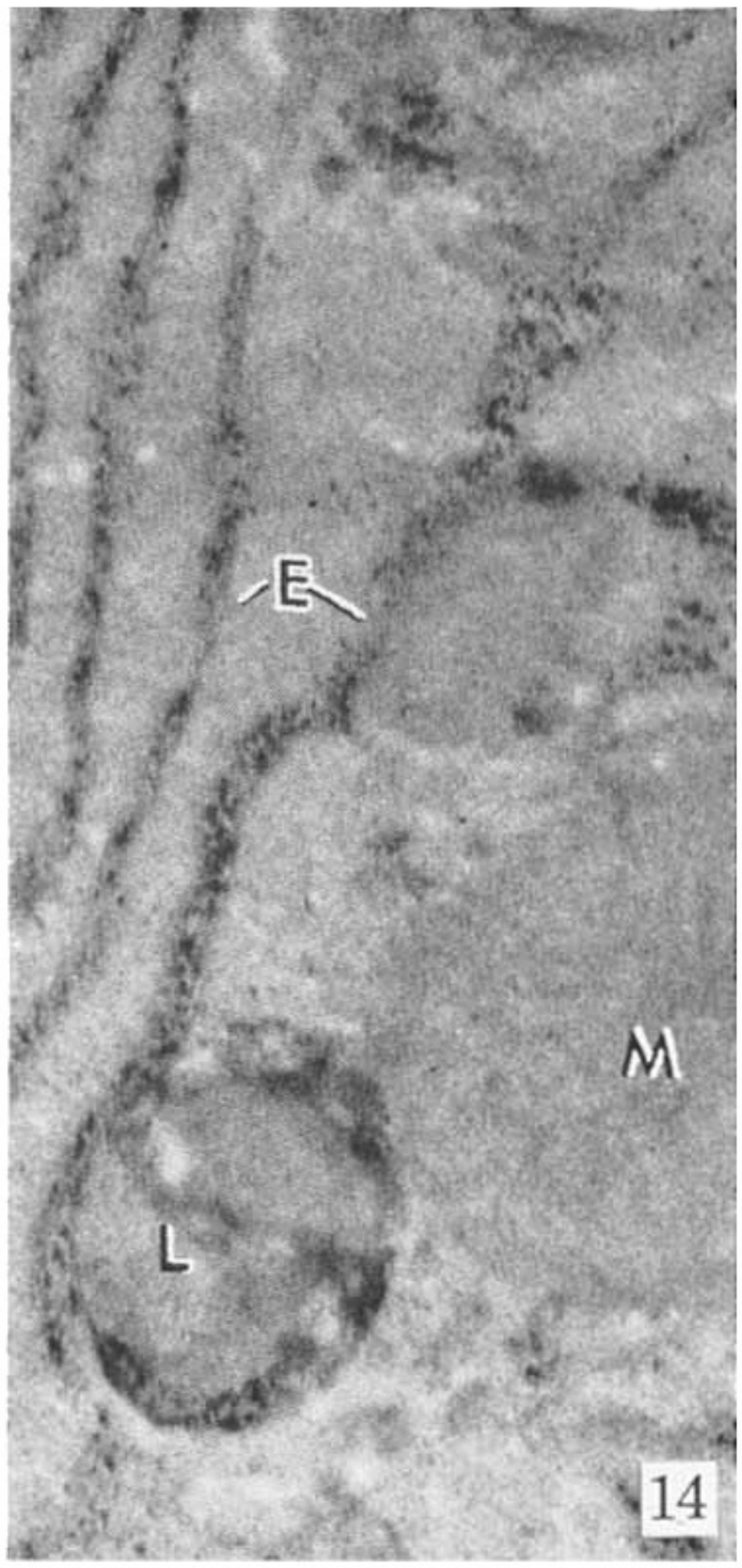



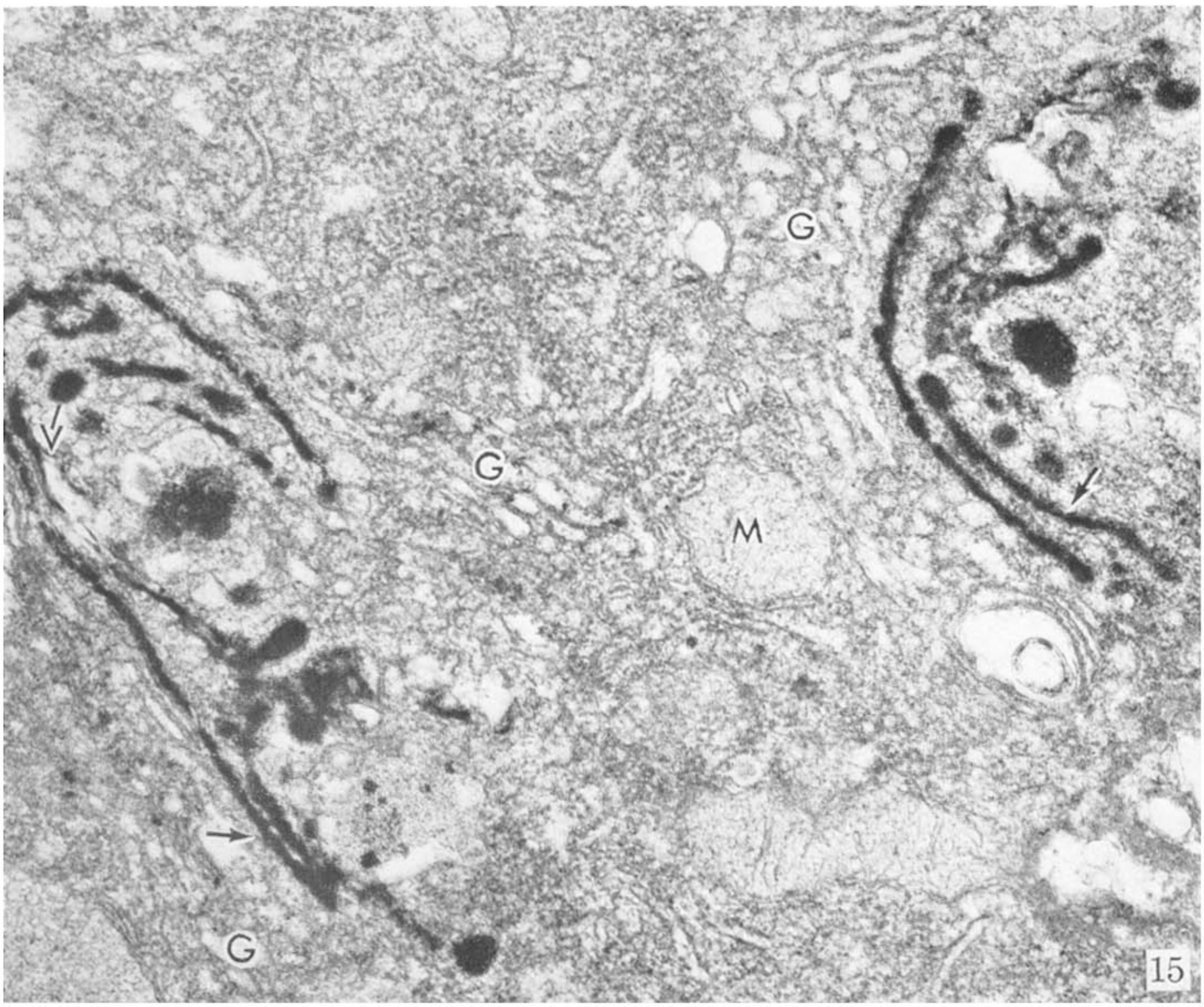

FIGURES 6–10. For legends see facing page.



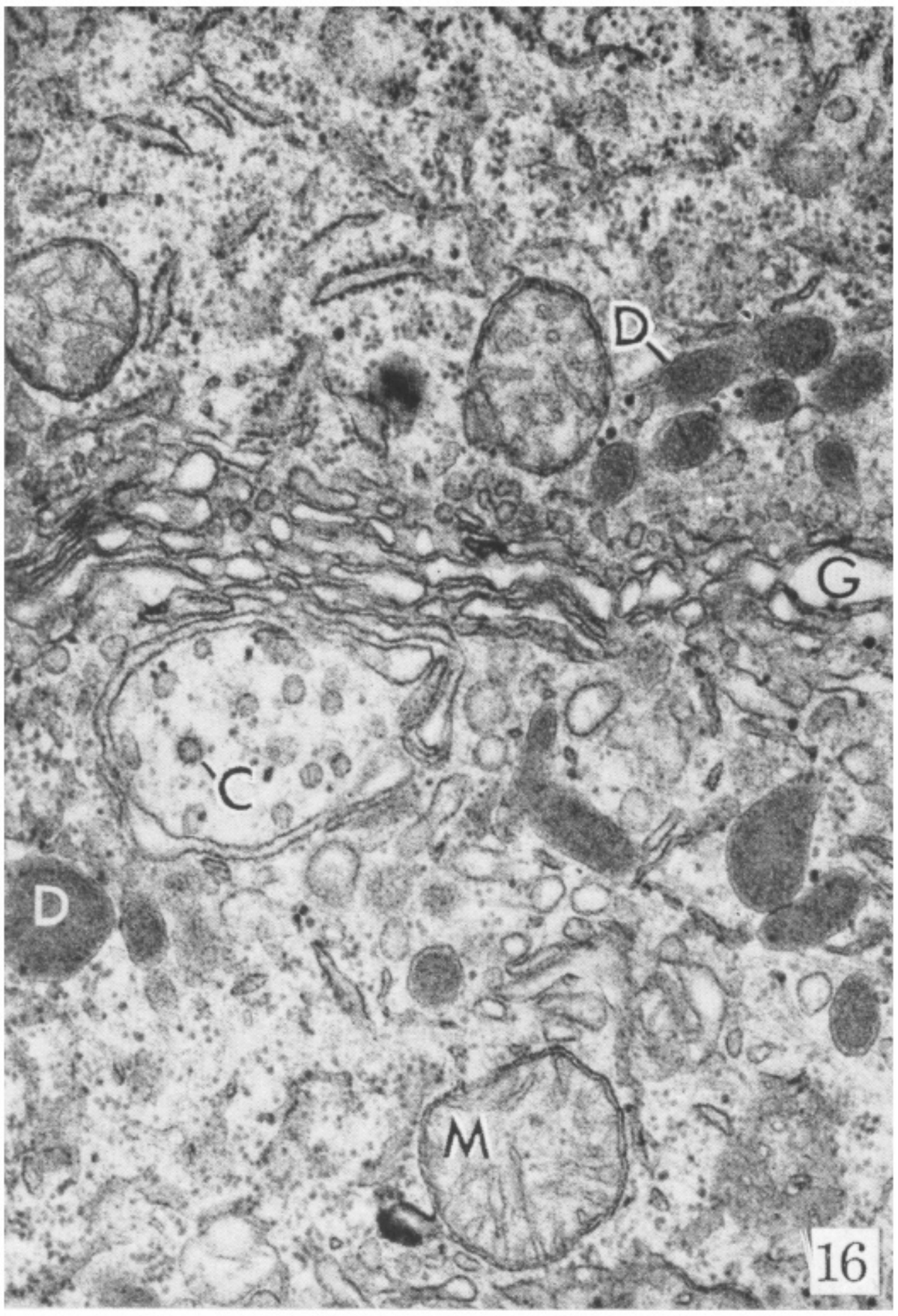



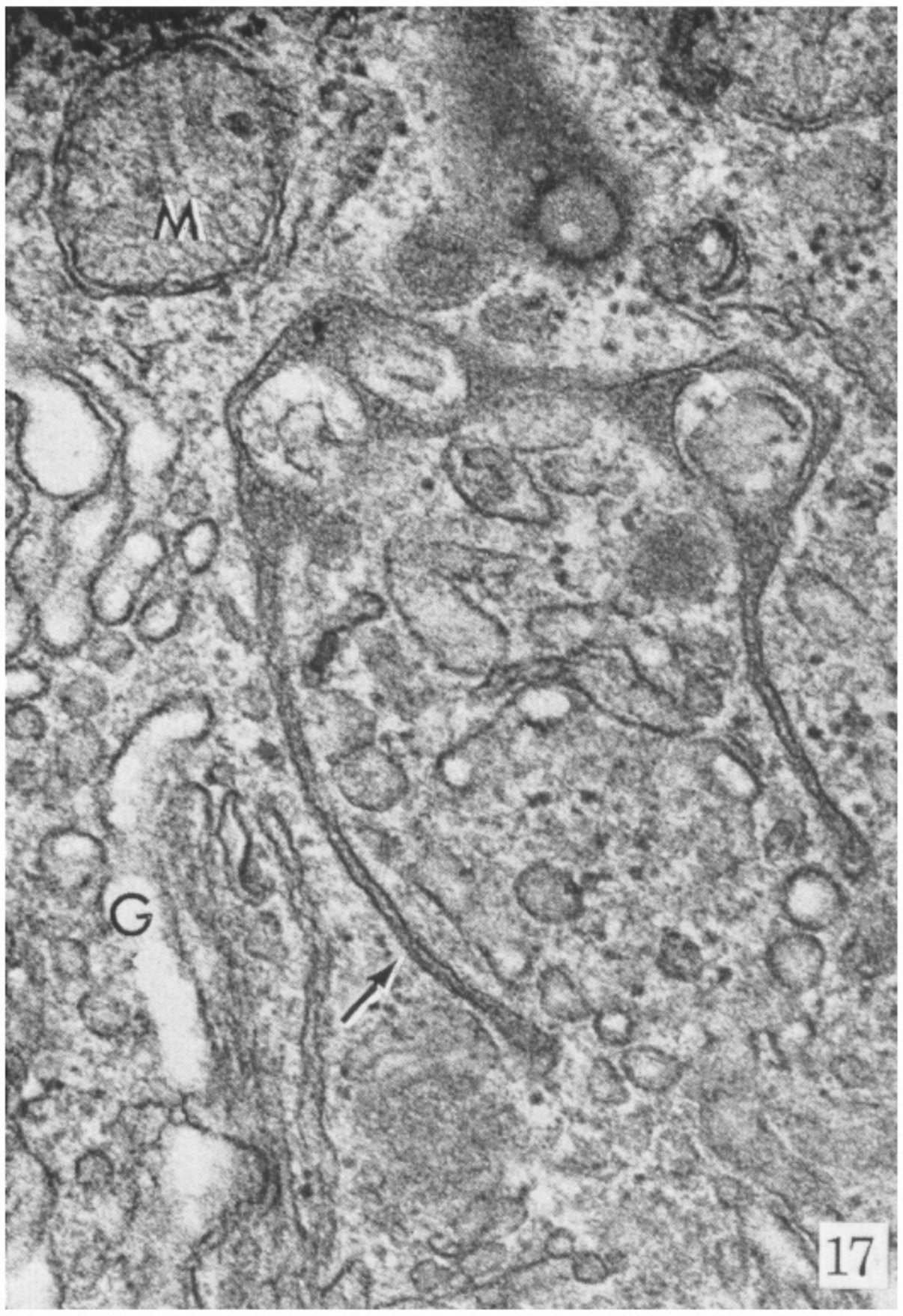



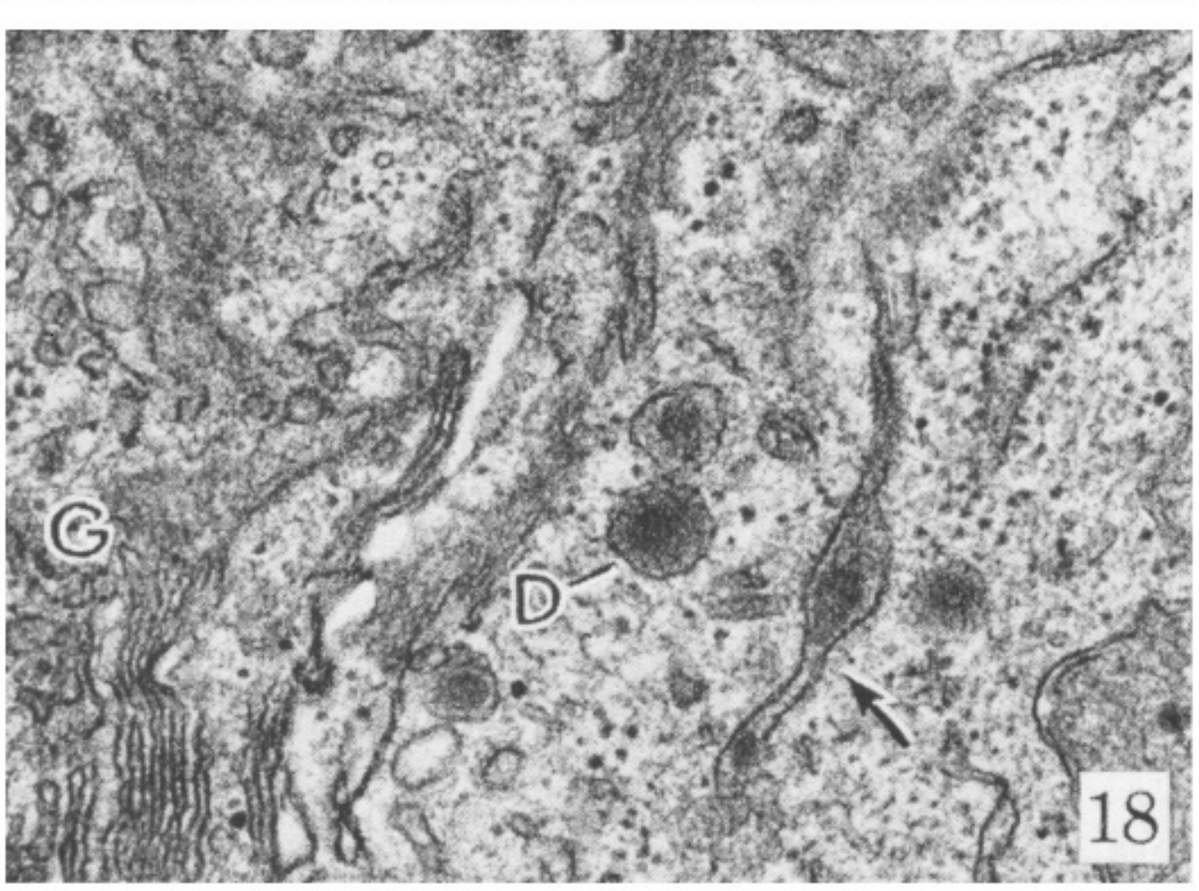



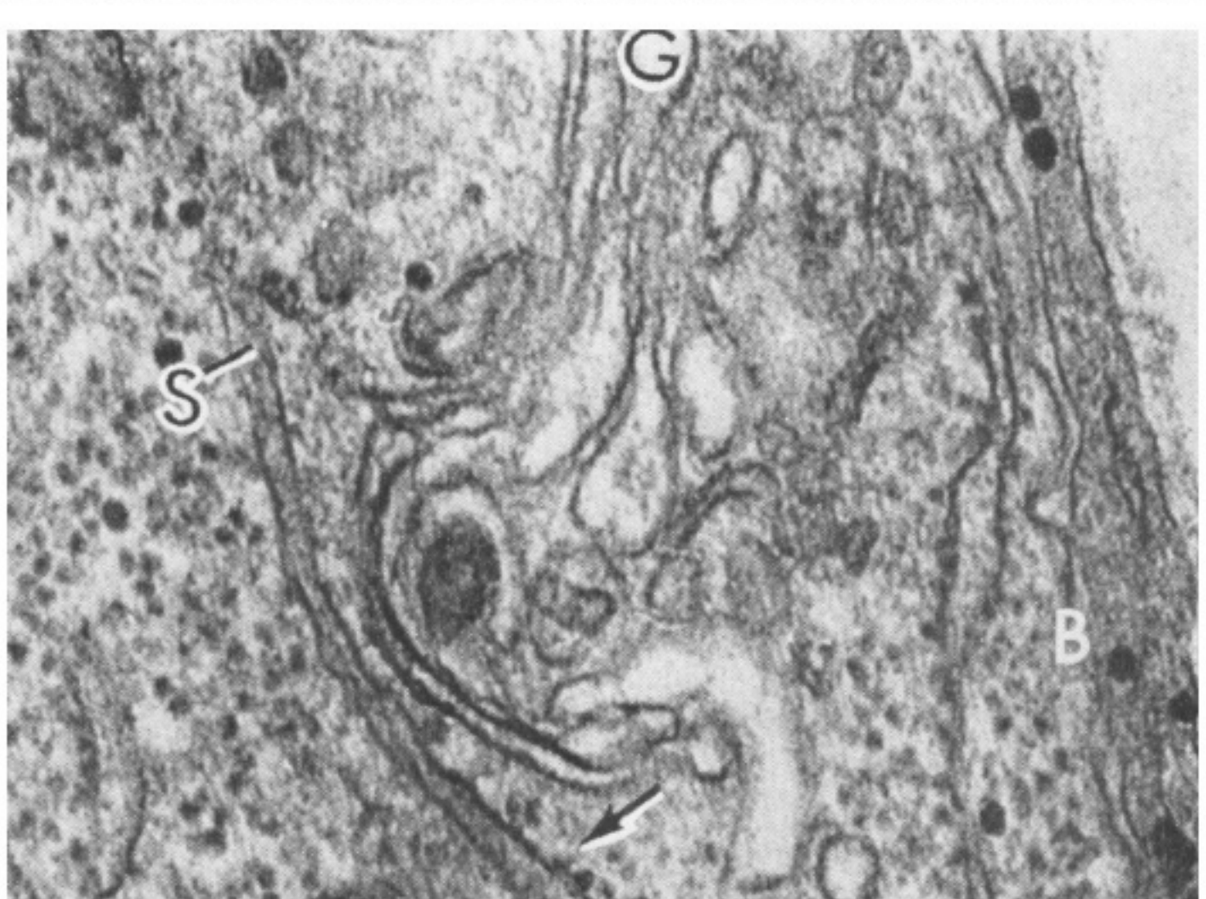



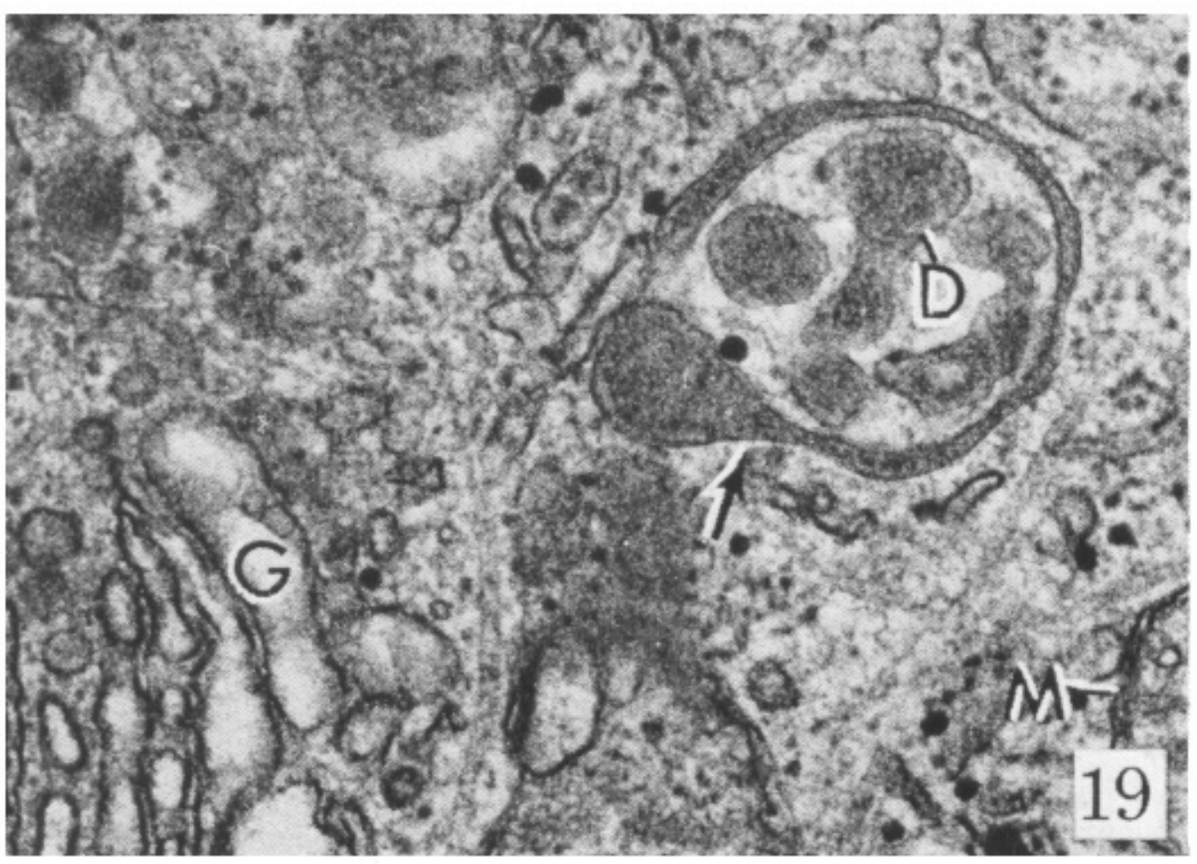

FIGURES 11–15. For legends see facing page.

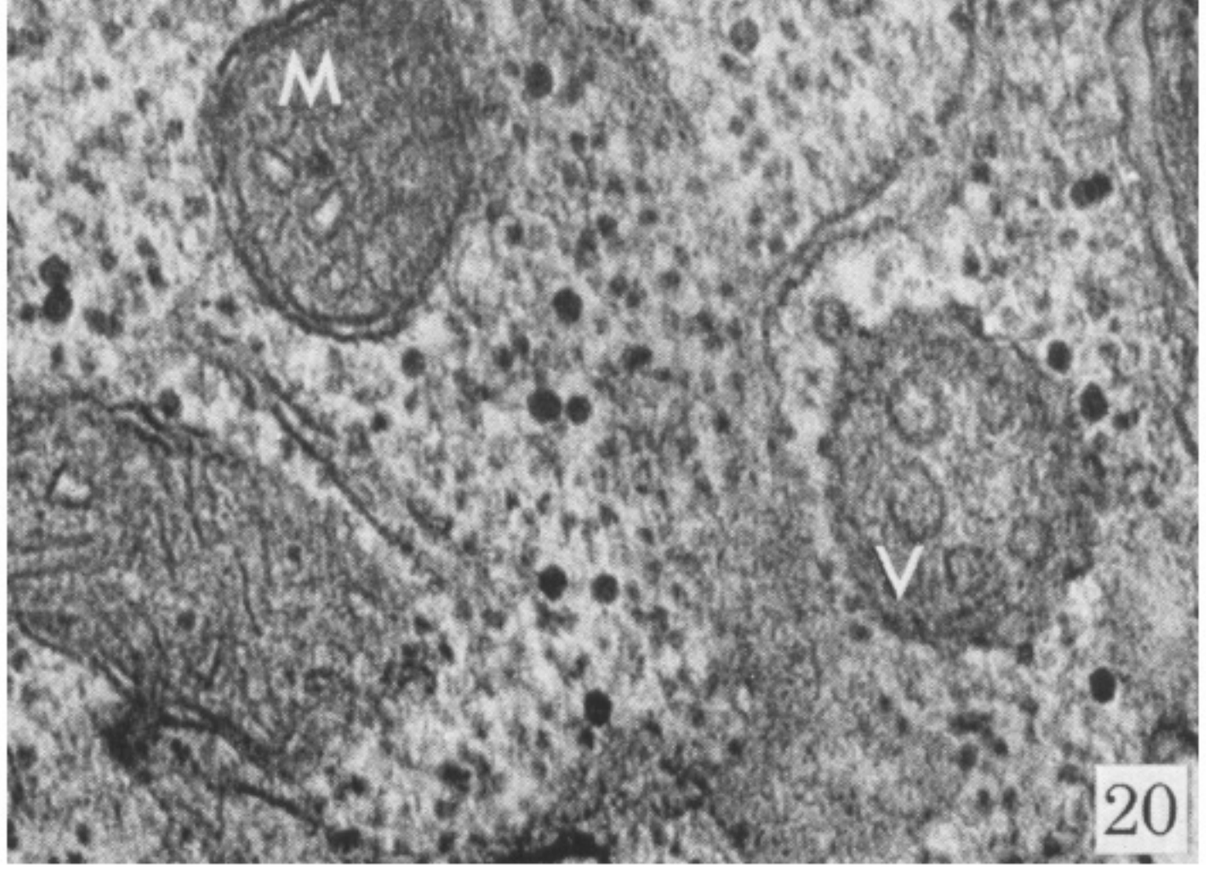


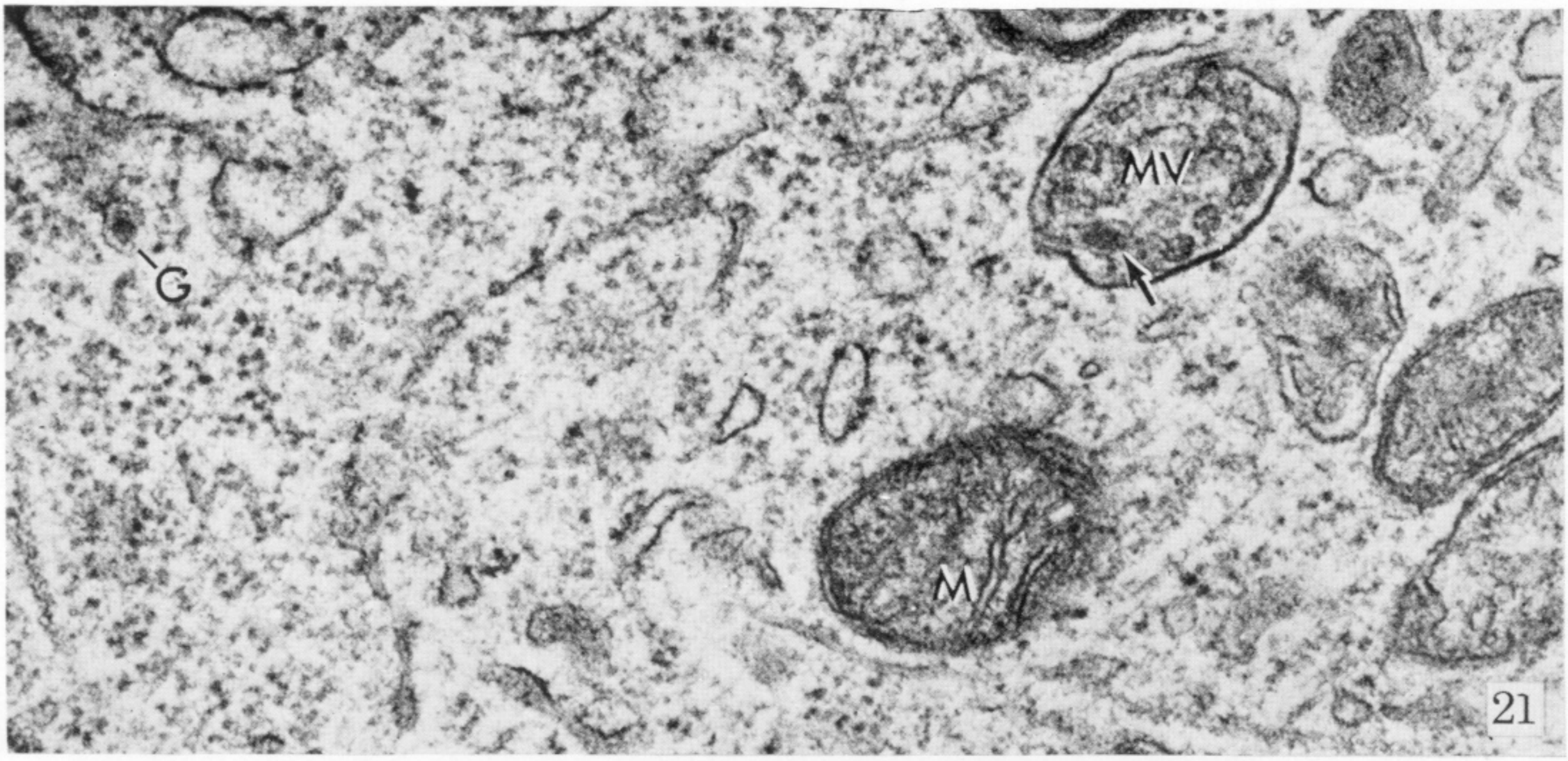



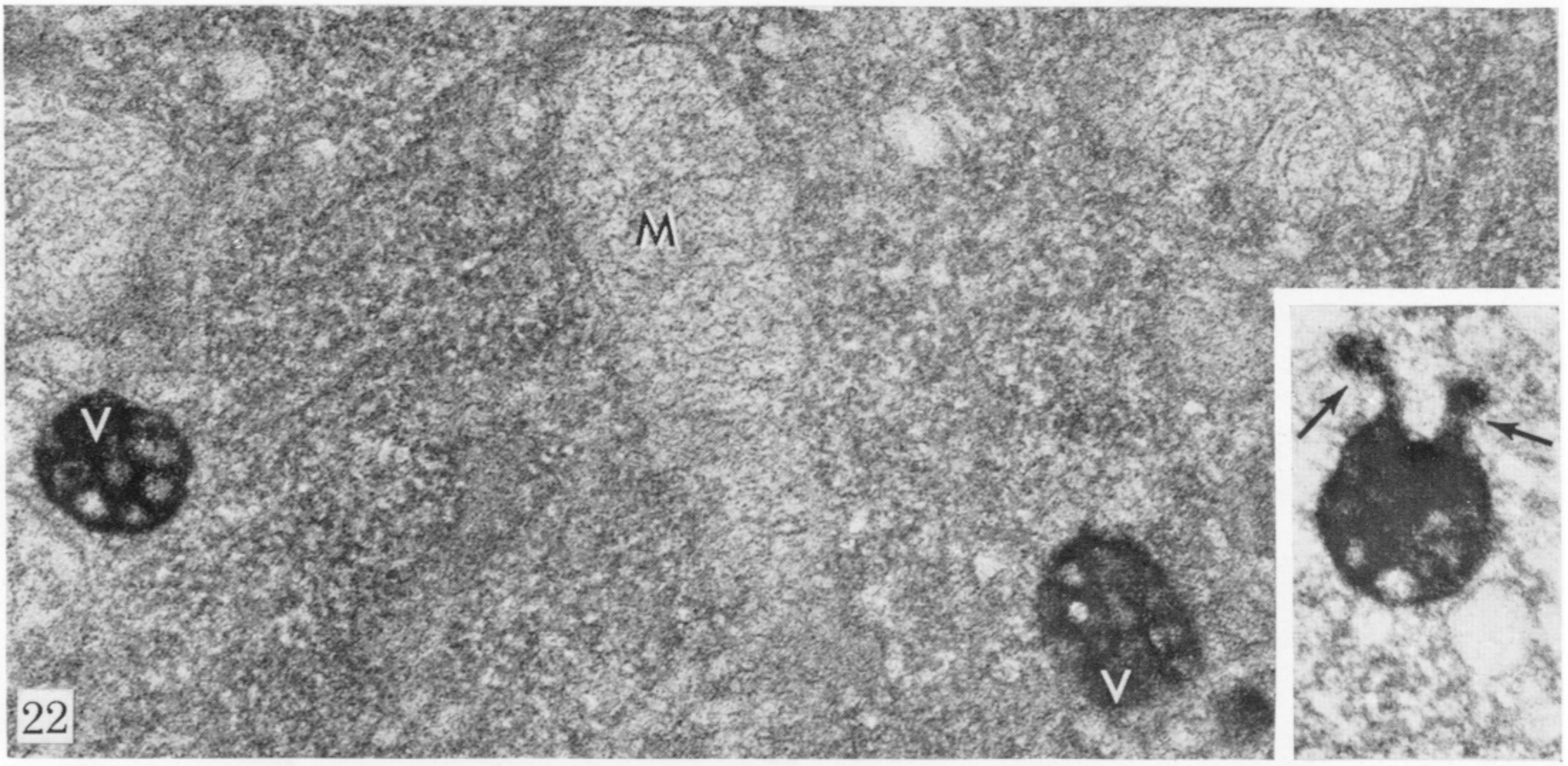



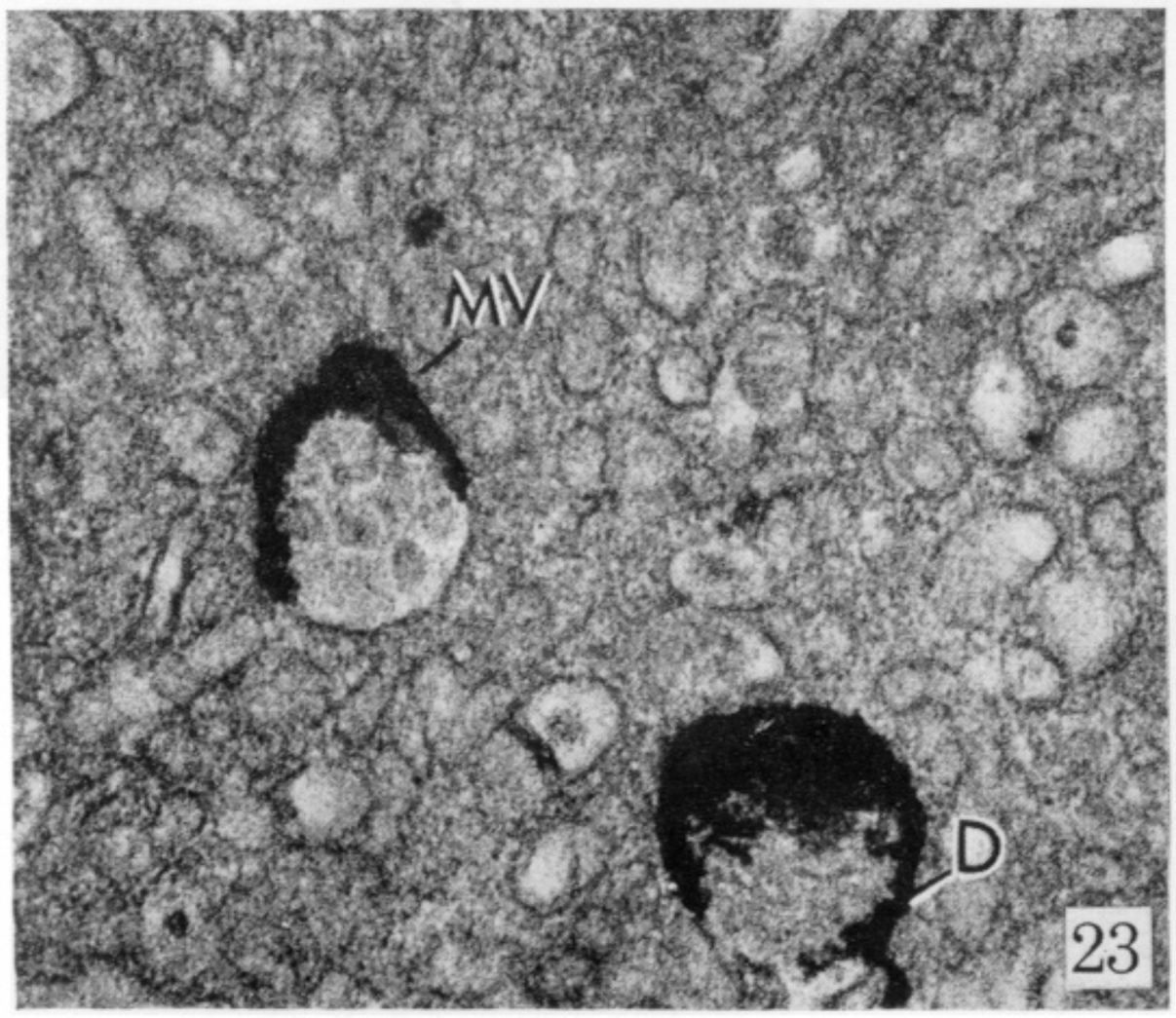



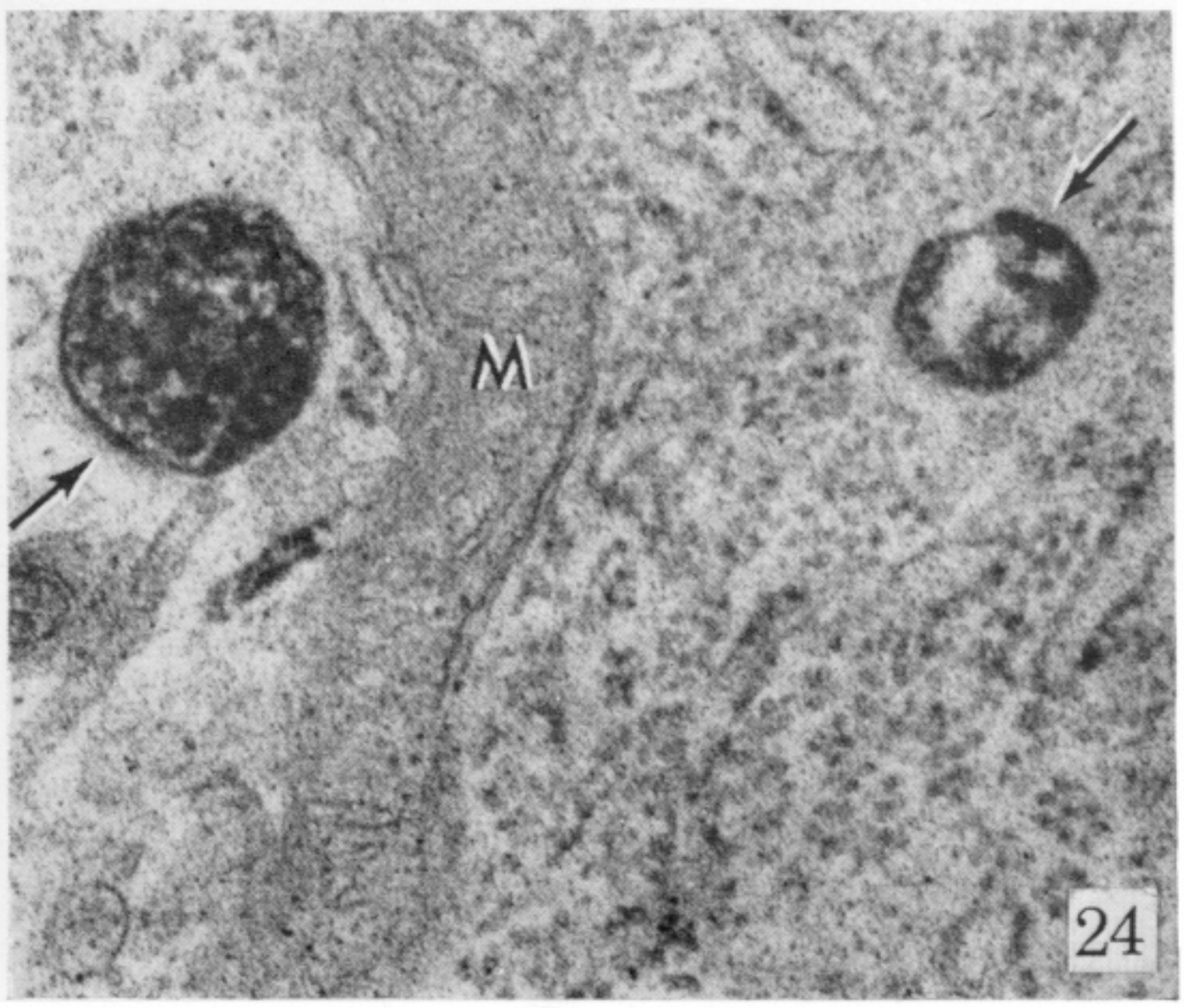




FIGURES 16–20. For legends see facing page.

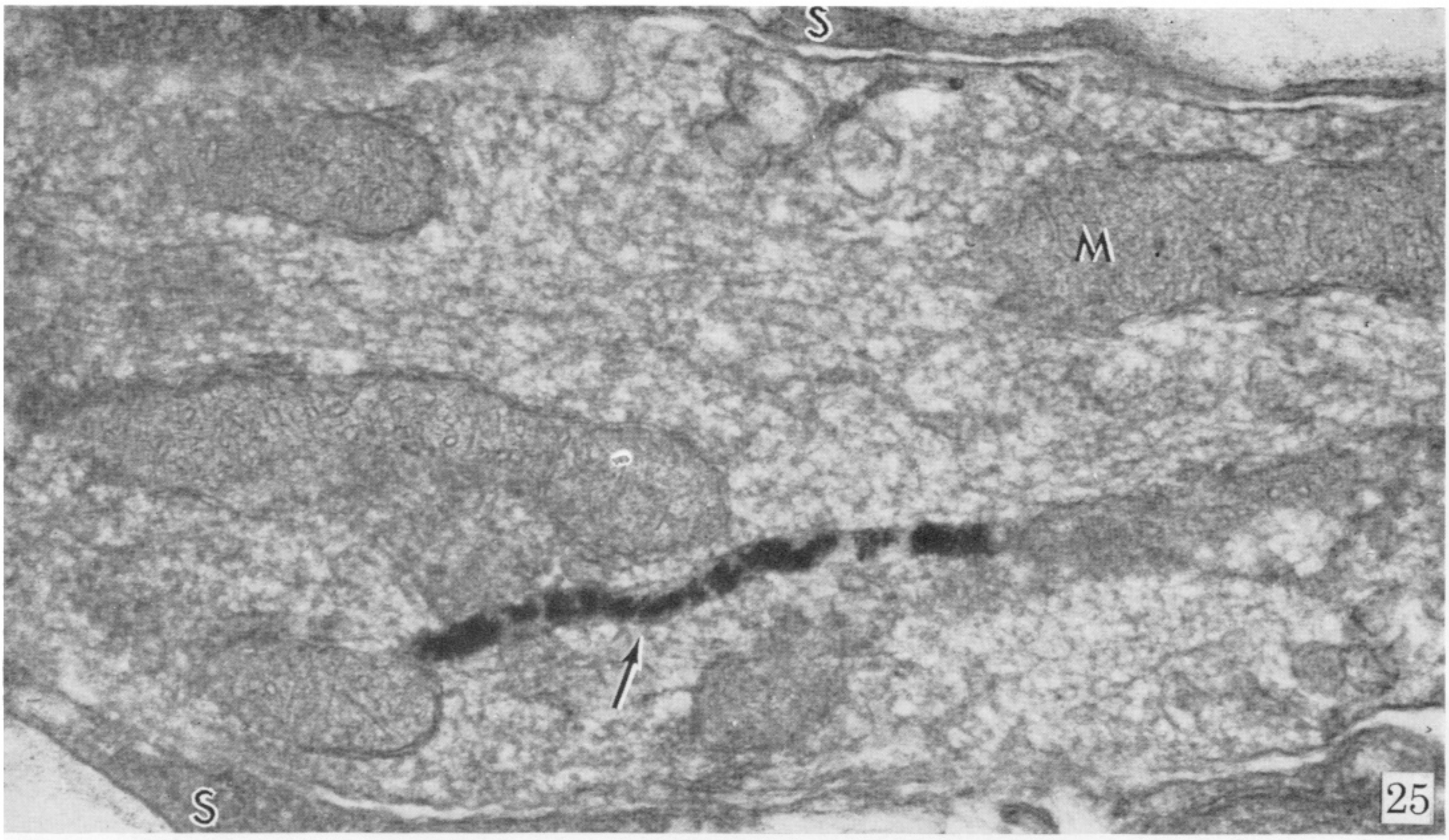


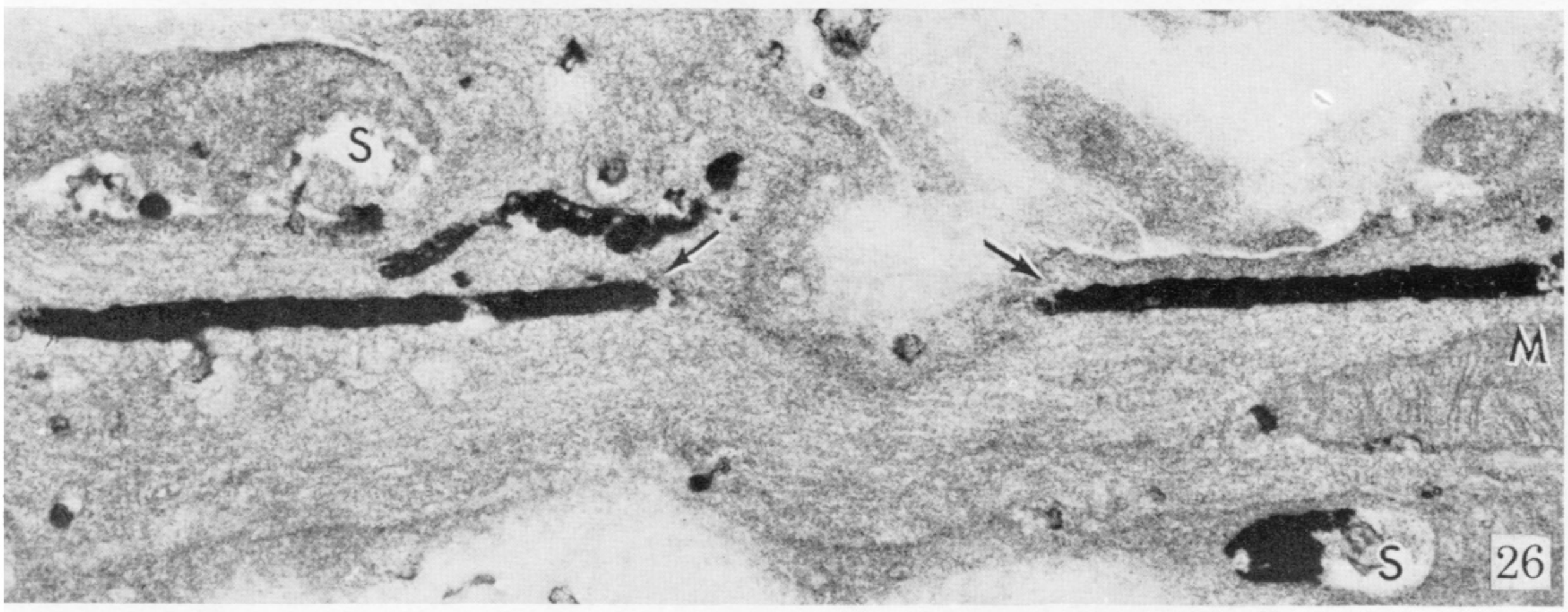



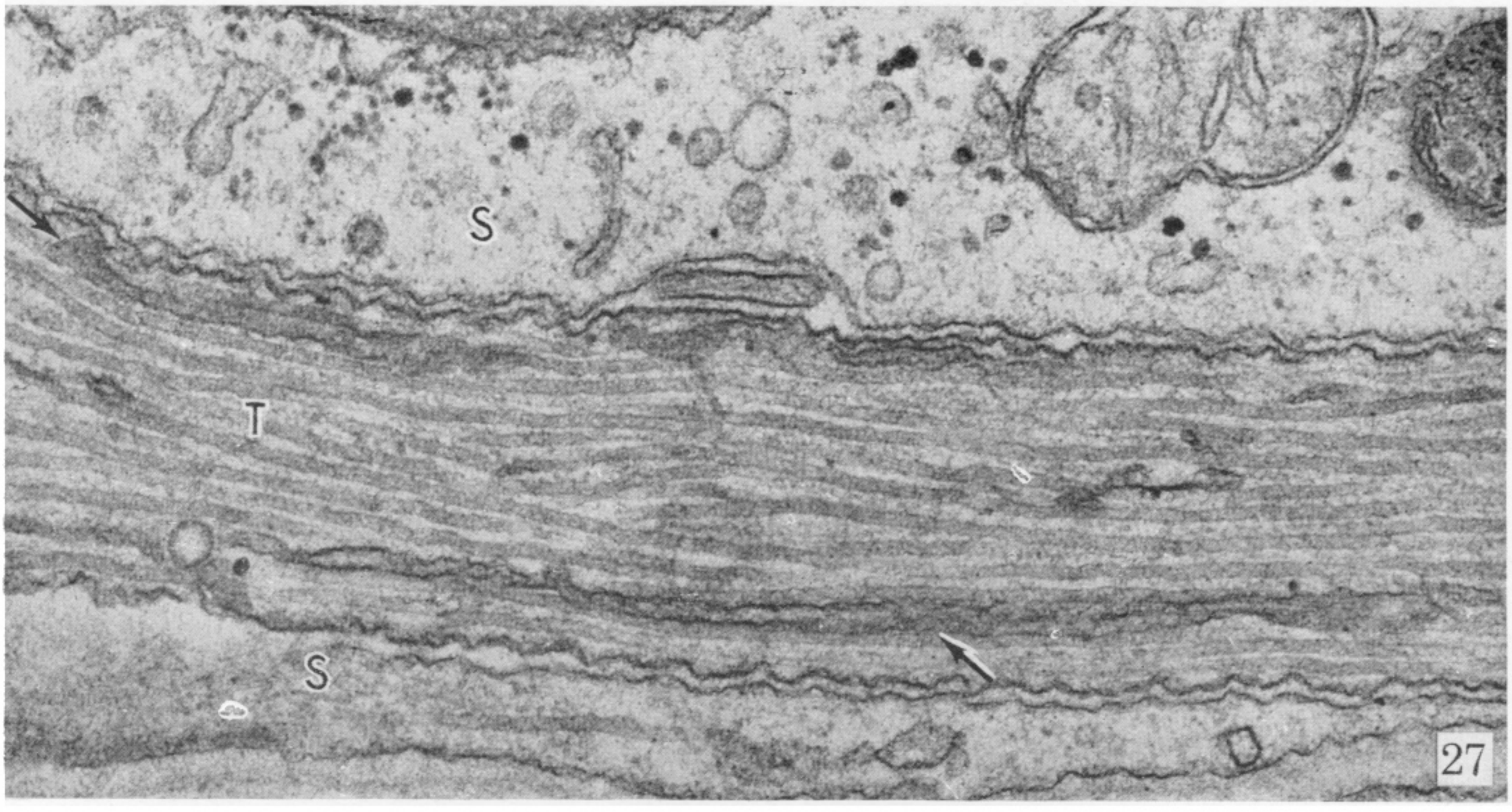



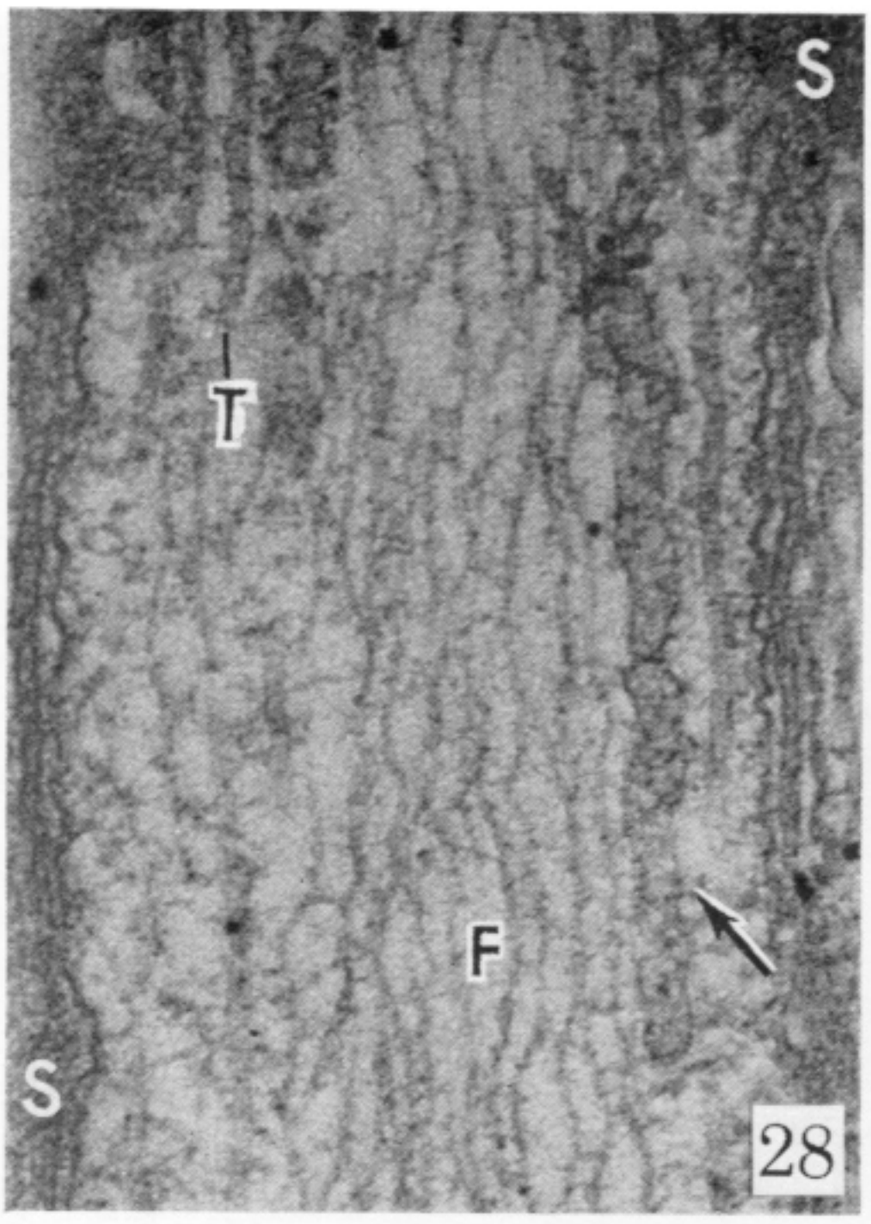

FIGURES 21–24. For legends see facing page.

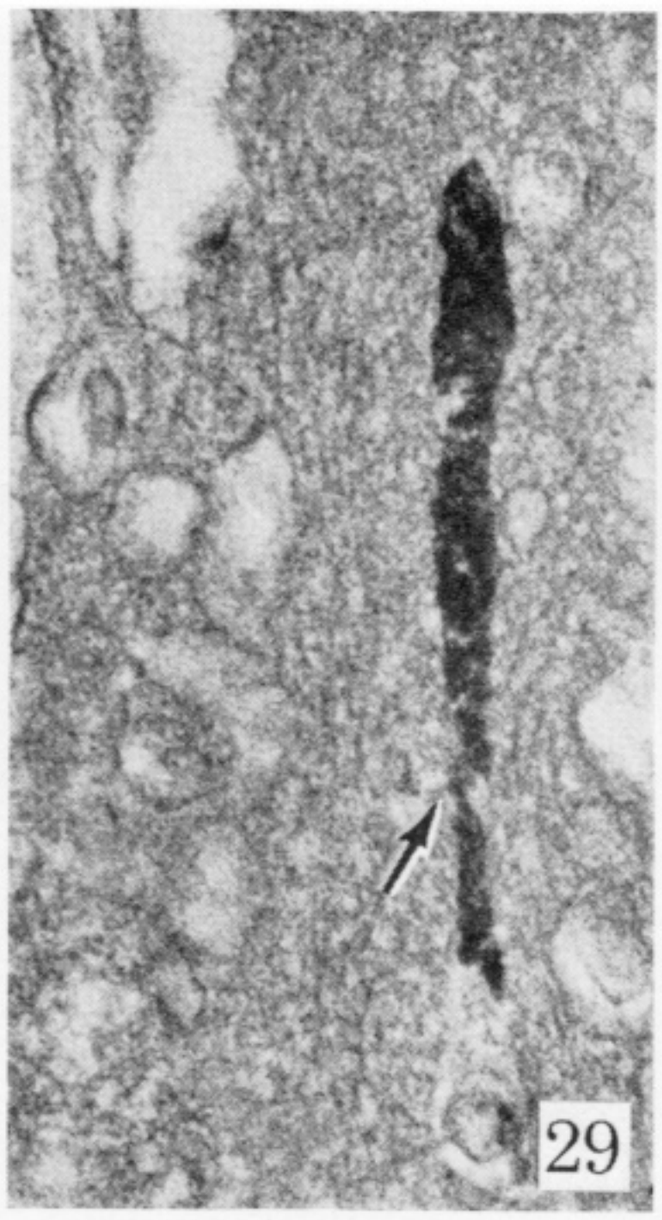


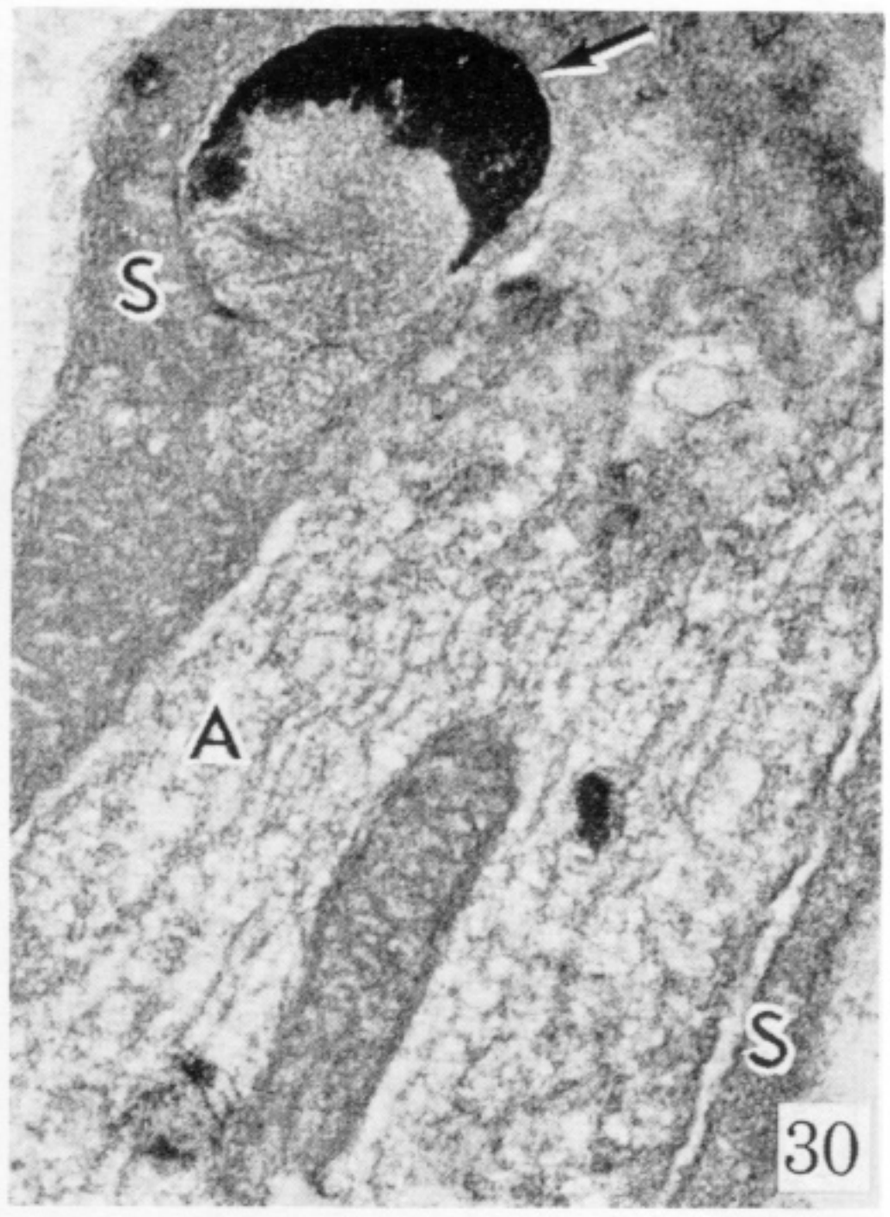



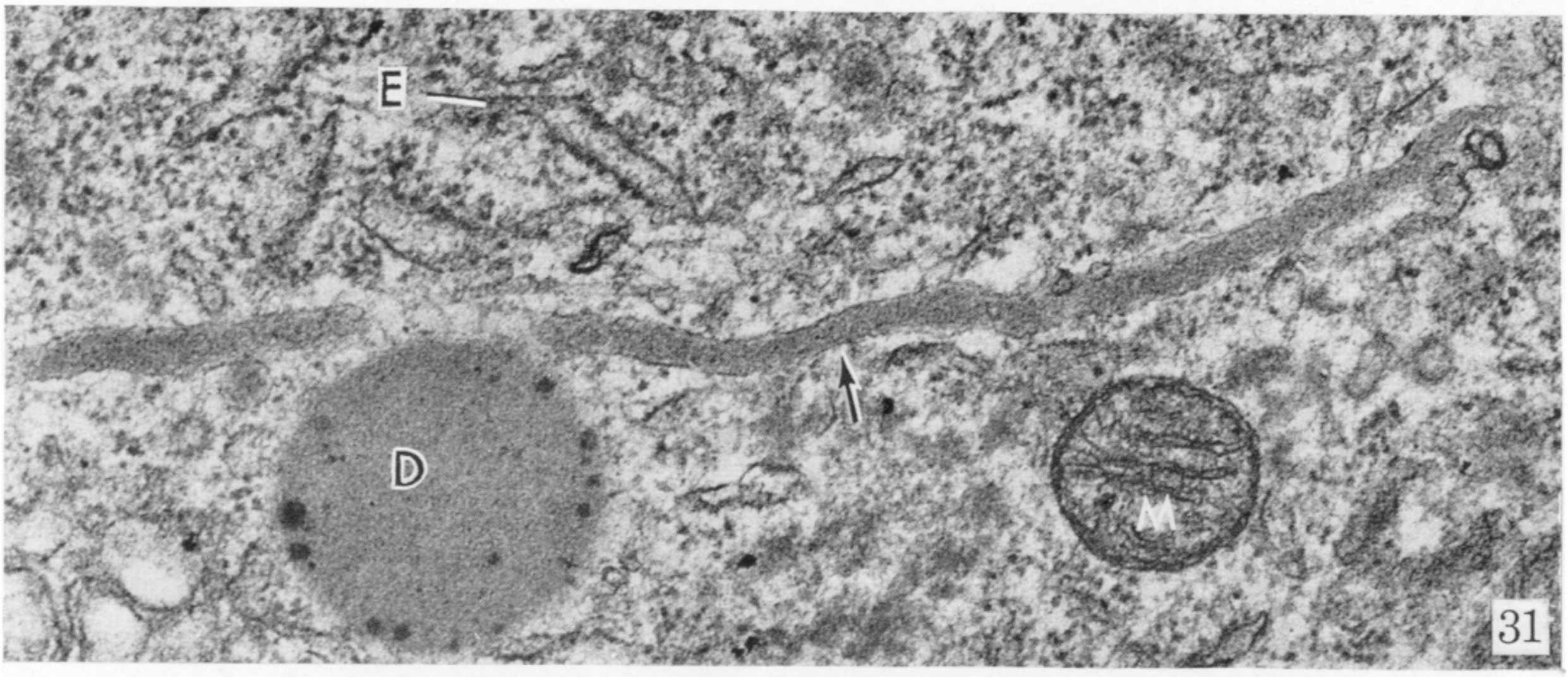



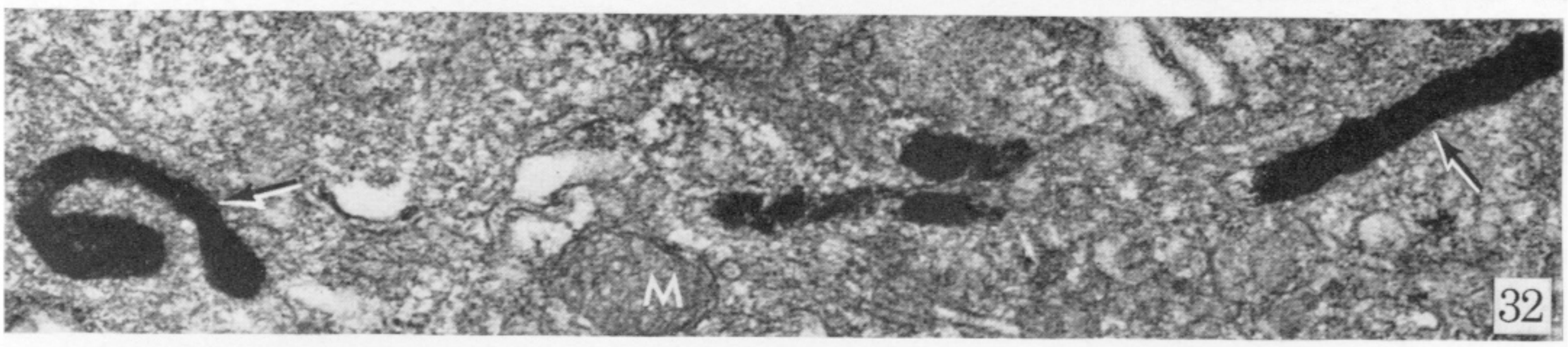

FIGURES 25–27. For legends see facing page.

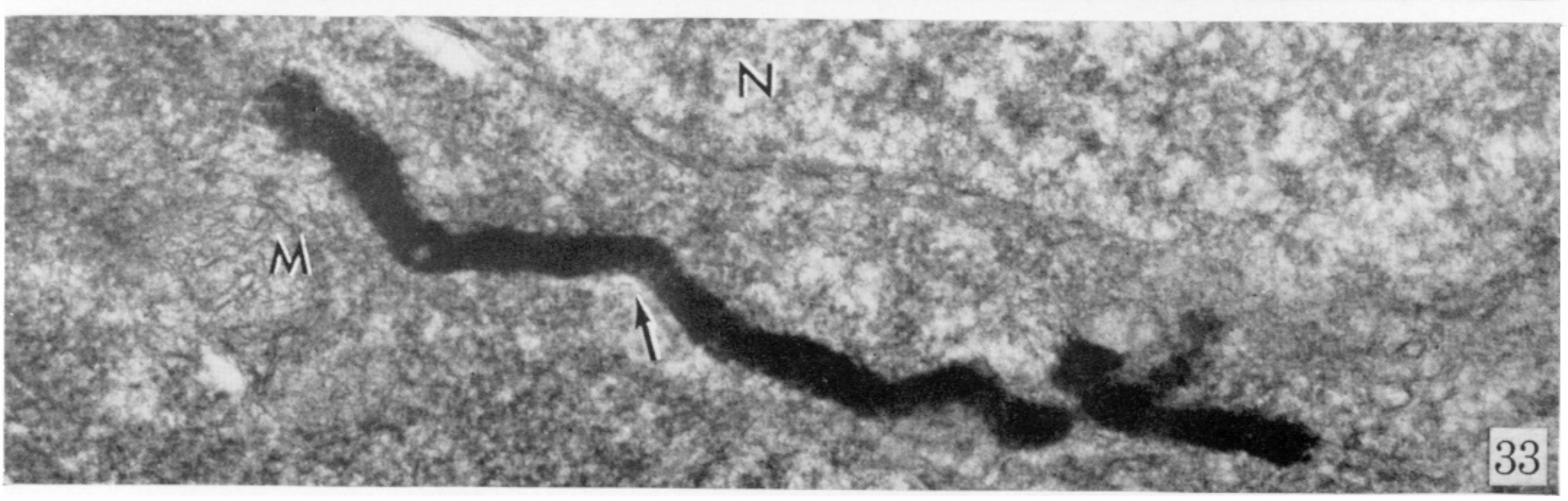


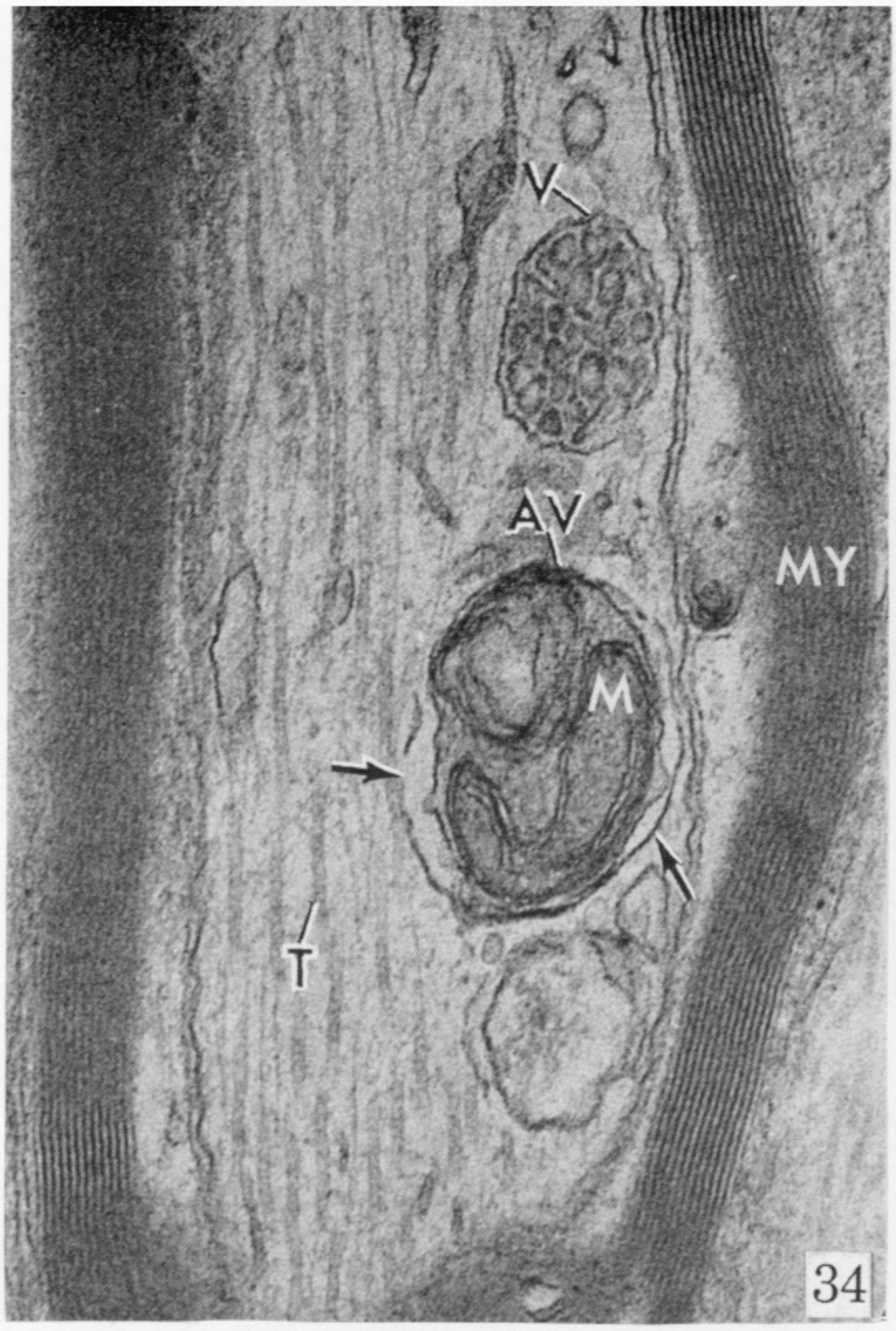



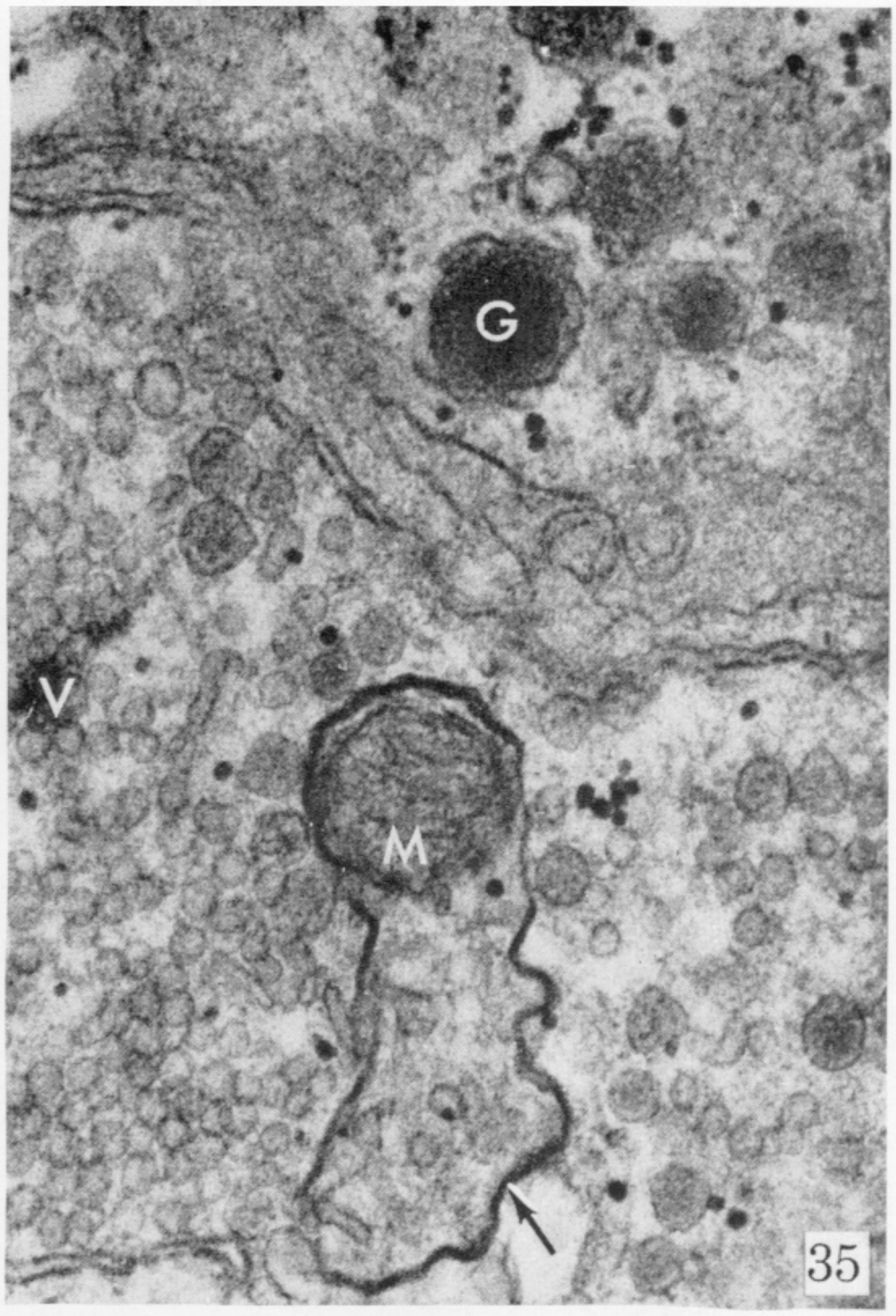



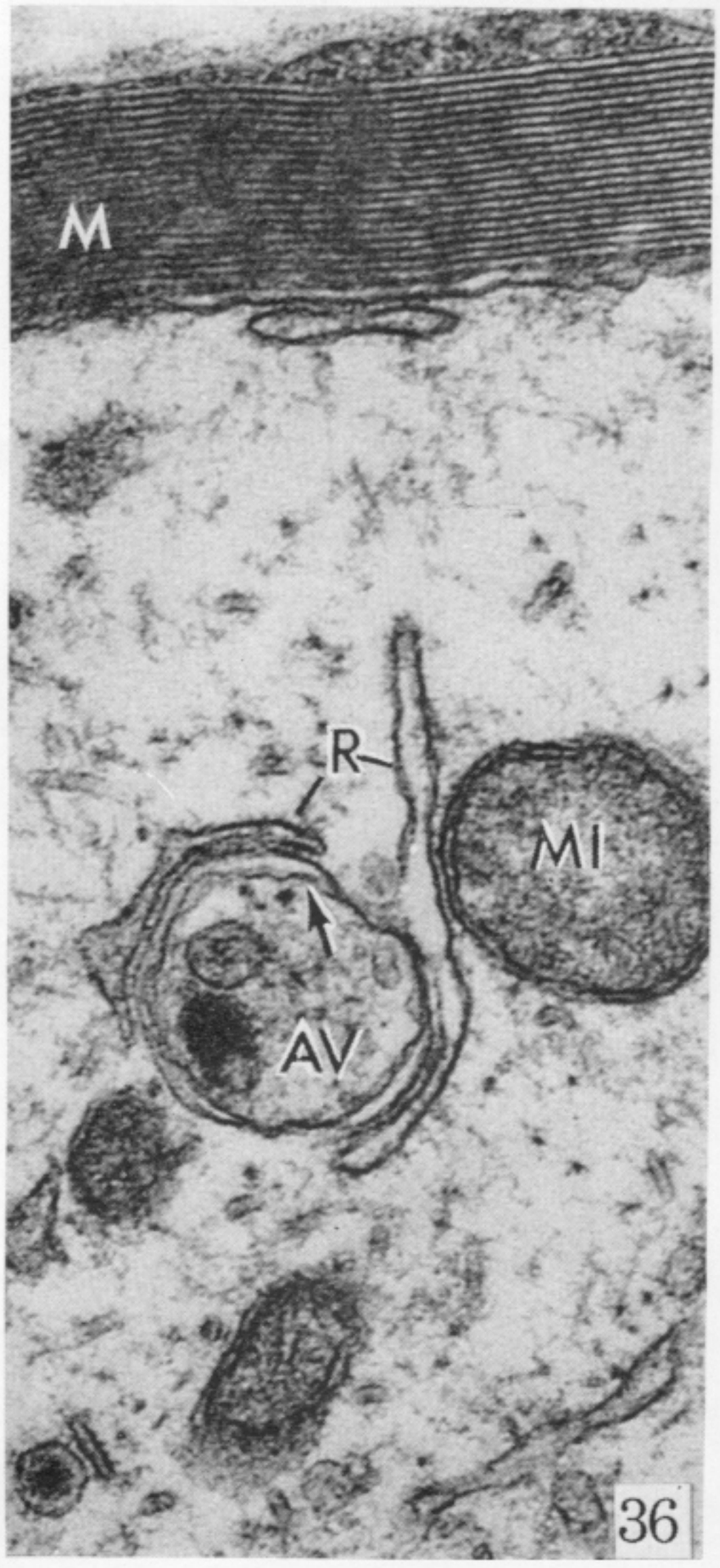



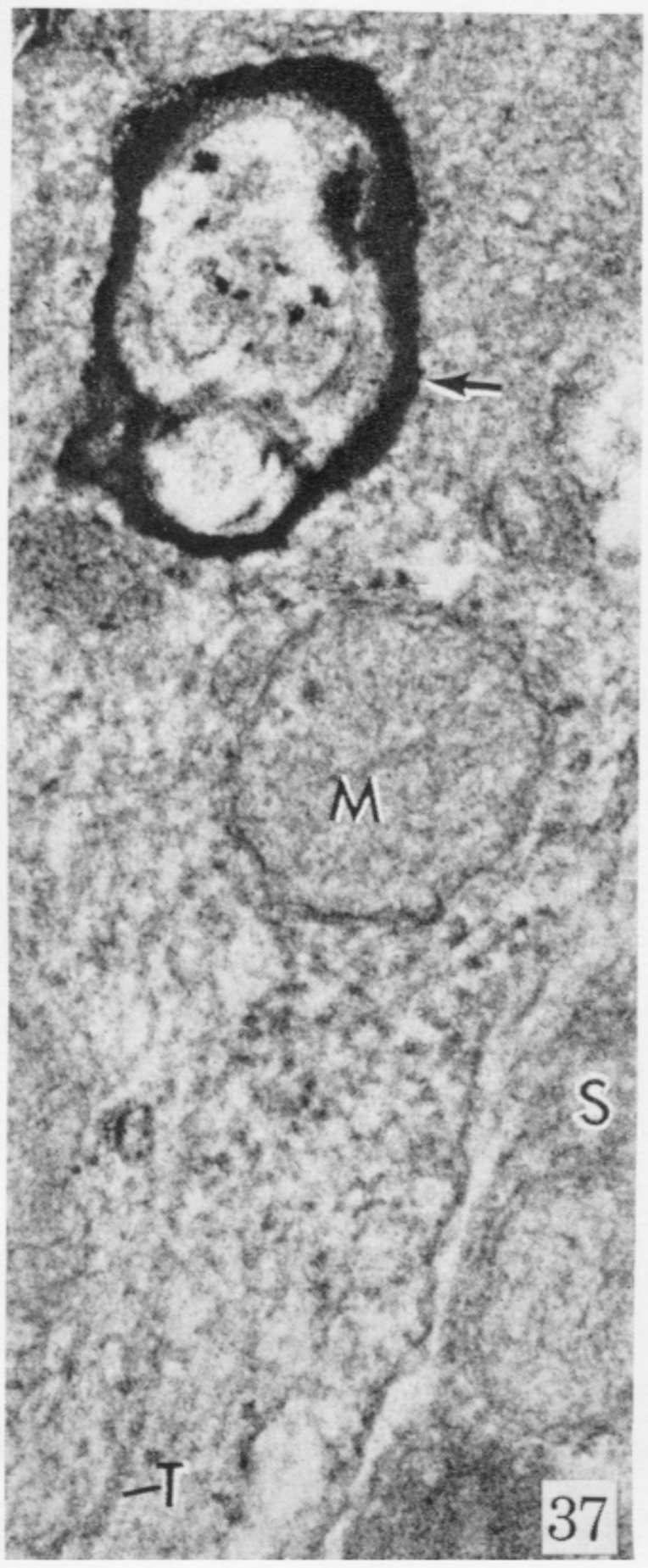



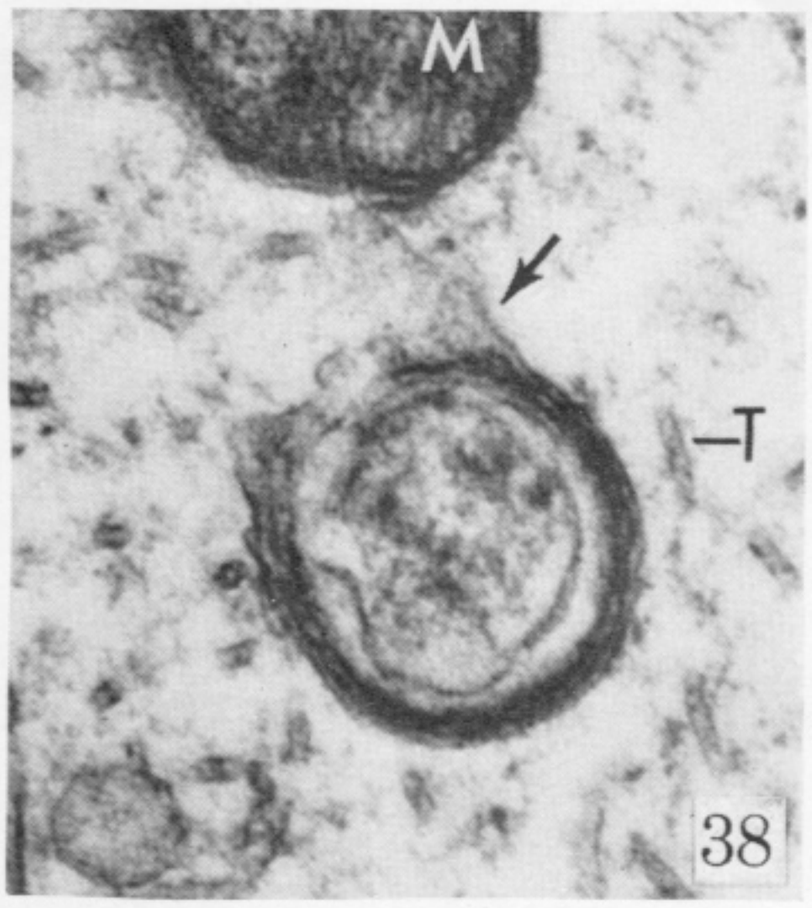

FIGURES 28–33. For legends see facing page.

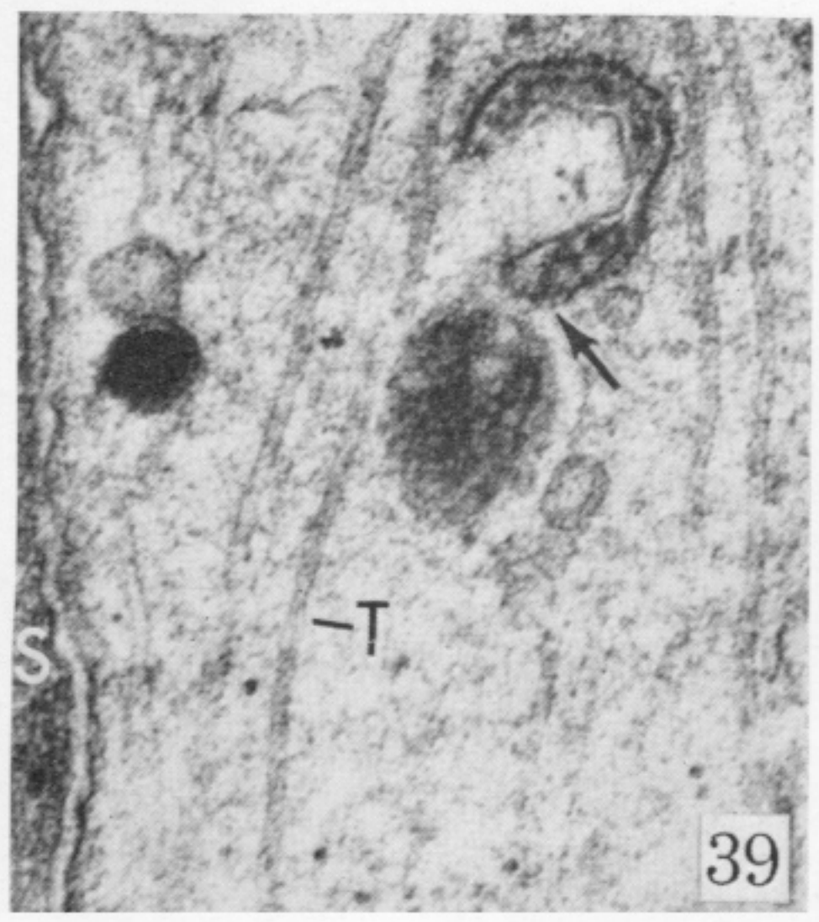


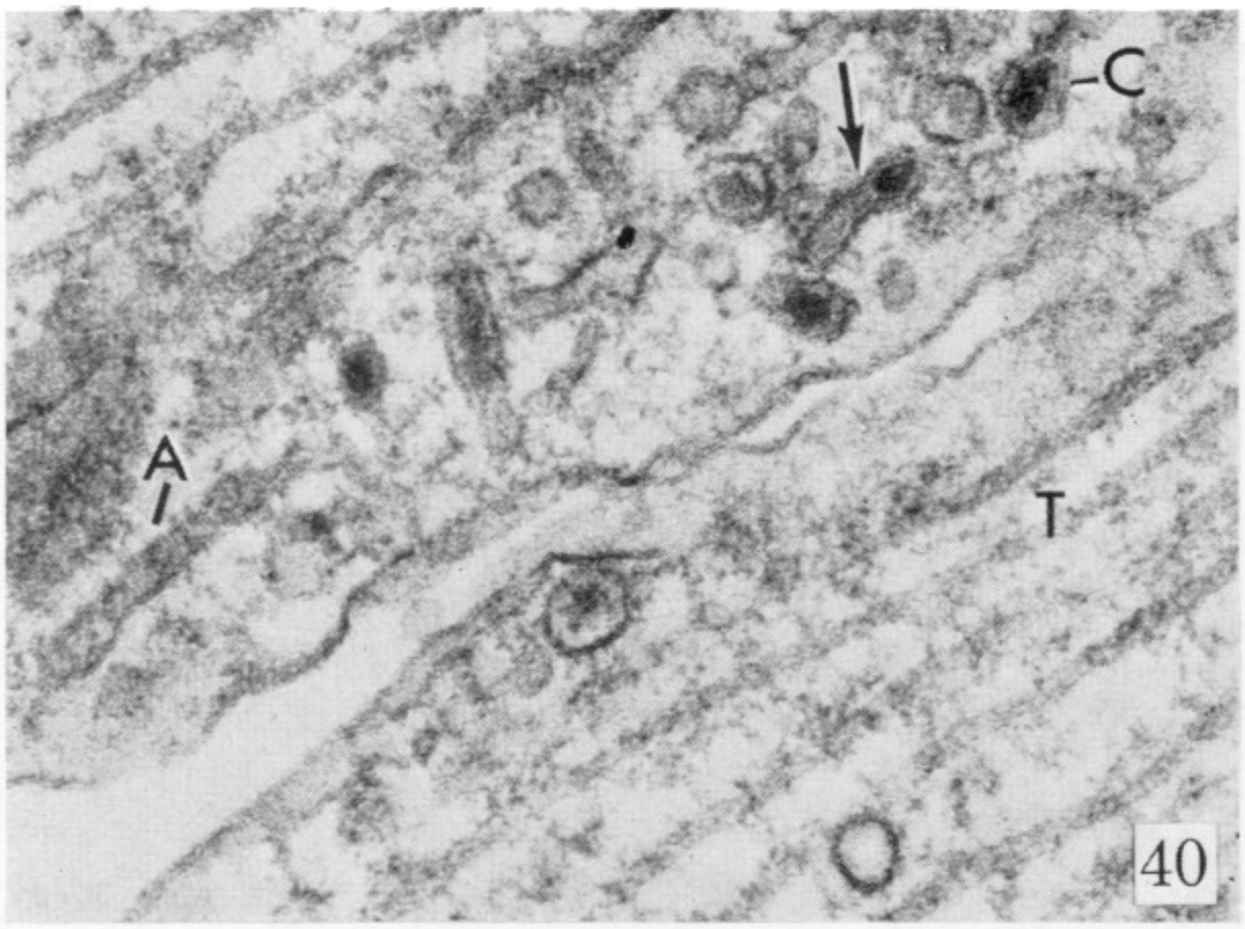



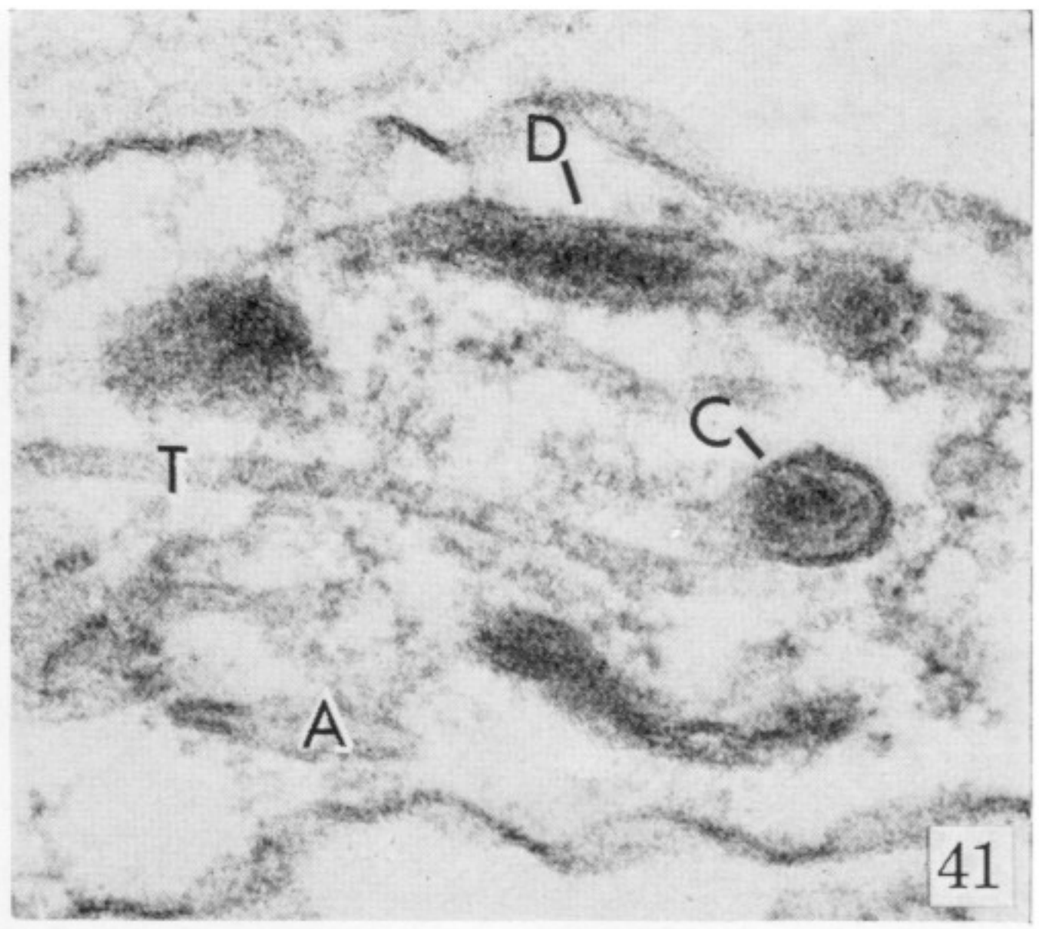



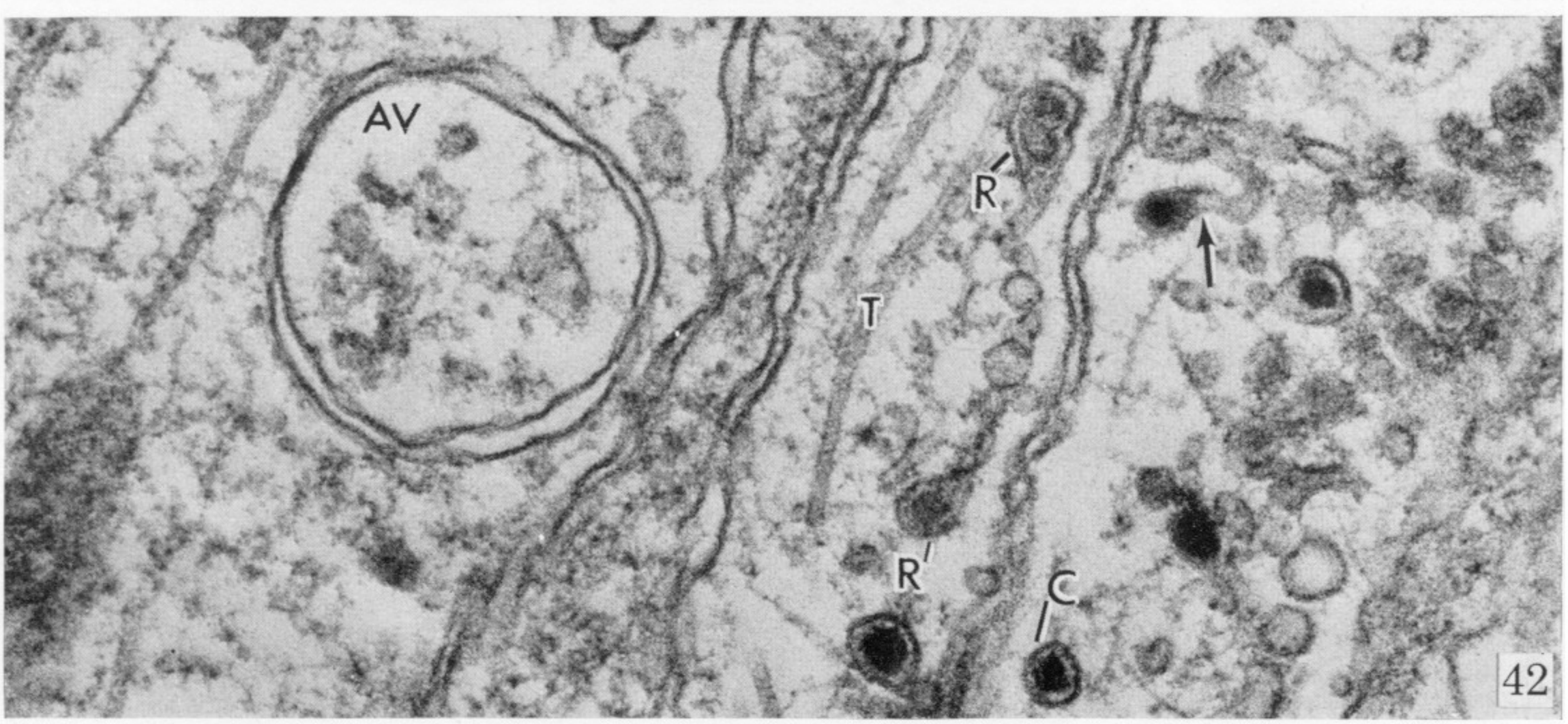




FIGURES 34–39. For legends see facing page.




FIGURES 40–42. Portions of axons from chick sympathetic ganglia grown in culture (from work by S. Teichberg in in our laboratory). The cells shown in figures 41 and 42 were exposed to 5-hydroxydopamine; the one in figure 40 was not. T indicates microtubules, A, agranular reticulum and C, dense-cored vesicles. AV designates an autophagic vacuole.

The configurations shown at the arrows, suggest that dense-cored vesicles may arise by dilatation of regions of agranular reticulum in which a dense content (D in figure 41) accumulates. Similar configurations are seen near the Golgi apparatus and have also been noted in other sympathetic nervous systems preparations (for example, in the axons studied by Pellegrino de Iraldi & de Robertis (1968), although the interpretation by these investigators differs somewhat from ours). Structures such as the ones shown at R in figure 42 may eventually prove to be of interest for analysis of the formation of dense cores; we have the impression from study of the effects of reserpine and hydroxydopa compounds that electron dense material, resembling that in the cores, may be deposited in association with structures with the appearance of membrane-delimited vesicles that accumulate within the agranular reticulum. The source of such 'vesicles' might be the reticulum membrane but analysis of the phenomenon is complicated by several factors, including the possible presence of virus-like particles in some of the cultured neurons. (Figure 40: magn. $\times 55000$; figure 41: magn. $\times 75000$; figure 42: magn. $\times 60000$.)