FINE STRUCTURE OF NERVE FIBERS AND GROWTH CONES OF ISOLATED SYMPATHETIC NEURONS IN CULTURE

MARY BARTLETT BUNGE

From The Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115 and (present address) The Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

The leading tips of elongating nerve fibers are enlarged into "growth cones" which are seen in tissue culture to continually undergo changes in conformation and to foster numerous transitory slender extensions (filopodia) and/or a veillike ruffling sheet. After explantation of 1-day-old rat superior cervical ganglia (as pieces or as individual neurons), nerve fibers and tips were photographed during growth and through the initial stages of aldehyde fixation and then relocated after embedding in plastic. Electron microscopy of serially sectioned tips revealed the following. The moving parts of the cone, the peripheral flange and filopodia, contained a distinctive apparently filamentous feltwork from which all organelles except membranous structures were excluded; microtubules were notably absent from these areas. The cone interior contained varied forms of agranular endoplasmic reticulum, vacuoles, vesicles, coated vesicles, mitochondria, microtubules, and occasional neurofilaments and polysomes. Dense-cored vesicles and lysosomal structures were also present and appeared to be formed locally, at least in part from reticulum. The possible roles of the various forms of agranular membranous components are discussed and it is suggested that structures involved in both the assembly and degradation of membrane are present in the cone. The content of these growing tips resembles that in sensory neuron growth cones studied by others.

INTRODUCTION

Harrison, in his seminal paper of 1910 (15), stressed the importance of protoplasmic movement in the formation of nerve fibers. He wrote, "... it is [the] laying down of the primary nerve paths by means of a form of protoplasmic movement ... that constitutes the specifically intricate problem in the development of the nervous system. ... In order to discover the factors which influence the formation of the nerve paths, we must, therefore ... take into consideration this property of protoplasmic movement ..." (p. 832, reference 15). Harrison observed that the motility exhibited by the forming fibers was largely limited to the nerve fiber tips which were enlarged, highly active, and provided with very fine processes or pseudopodia. He recognized these terminal enlargements to be the growth cones ("cônes d'accroissement") that Ramón y Cajal had previously discovered in histological preparations of developing and regenerating nervous tissue.

The behavior of growth cones when in contact with cellular or noncellular substrates is difficult to investigate in the intact animal. One of

the few successful observations in vivo was that of Speidel (35) on growing nerve fibers in the transparent tail fin of tadpoles. Speidel found that the appearance and the rate of growth of the fibers were similar to those observed in Harrison's in vitro preparations. As tissue culture techniques have improved they have offered increasingly favorable opportunities for the study of the fiber growth. Whereas the movement of growth cones in culture may be fast enough to be seen by direct microscope observation, the behavior of the growing tip is best analyzed by the use of time-lapse photography. Nowhere is the movement of growth cones better illustrated than in one of the time-lapse films of cultured nervous tissue prepared by Pomerat and his collaborators (32).

Whereas time-lapse studies give information about the form and rate of fiber extension, basic questions concerning the underlying mechanisms for motility and growth have only recently begun to be studied. First, what structures in the growing tip are responsible for its movement? By combining electron microscopy with colchicine or cytochalasin treatment of cultured nerve fibers, Yamada et al. (41, 42) concluded that a microfilamentous network found in the cone periphery (and in the fine processes arising from it) is involved in fiber elongation by way of maintaining the shape and participating in the movement of the tip. Second, at what point along the lengthening fiber is new surface added? Data bearing on this question, obtained by marking the growing tip with microscopically visible particles, was reported by Bray (7). The evidence pointed to the growth cone as the site of surface addition. The present study, undertaken in collaboration with Dr. Dennis Bray, reports fine structural data bearing on both of these questions.

Typical growth cones also develop in neurons which are grown singly in culture, as reviewed in reference 8. This system was chosen for much of the present electron microscope study to ensure identification of all areas of the nerve fiber. Conflicting descriptions of the fine structure of growth cones from tissue *in situ*, considered in detail in the Discussion, may be due at least in part to identification difficulties. Electron microscopy of the tips of cultured chick sensory nerve fibers was reported recently by Yamada et al., as cited above (42). The findings in the present study of rat sympathetic motor neurons were generally similar, but have the added weight that the immediate past behavior of particular tips was known through previous time-lapse or still photography. Special care was taken in a few cases to photograph neurons shortly before fixation (to establish that the fiber was growing at that time) and through the initial stages of aldehyde fixation (to assess the fidelity of preservation) and then to relocate them in the plastic for serial thin sectioning and examination in the electron microscope. The outline of the cell in the final electron micrographs could be correlated with the pictures taken before fixation, and in the samples chosen there was no visible evidence of morphological change occurring with this procedure. In this paper emphasis is placed on the growth cones but other areas of the cultured sympathetic neuron are described as well. A preliminary report of some of the electron microscope findings on growth cones has appeared (9). Light microscope observations on the isolated sympathetic neurons appear in a companion paper by Bray (8).

MATERIALS AND METHODS

Details of culturing the explanted neurons have been published previously (7) and appear in abbreviated form in the accompanying paper (8). The source of nerve cells was the superior cervical ganglion from day-old rat pups. Neurons were explanted either as single cells after mechanical dissociation or in pieces of intact ganglion by Dennis Bray. The material was placed on glass cover slips which had been coated first with carbon (to allow the eventual separation of the polymerized embedding plastic from the cover slip, reference 33) and secondarily with rat-tail collagen (6). (As explained in reference 7, the cover slip was secured with paraffin to the bottom of a plastic culture dish, covering a hole 11 mm in diameter; in this way a shallow well was formed.) The culture medium included nerve growth factor. Temperature and evaporation of the culture medium during observation in a Nikon inverted microscope were carefully controlled (8). In order to minimize agitation of the samples, methylcellulose was added to increase the viscosity of the culture medium (7), and for all the changes of solutions described below a glass ring was placed around the well to confine fluid changes to that smaller area rather than the entire culture dish. The cultures were usually fixed for electron microscopy after 17–22 h in vitro.

The cultures were fixed at room temperature for a total time of from 0.5 h to 2 h in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde (TAAB Laboratories, Emmer Green, Reading, England) in

0.1 M or 0.15 M phosphate buffer containing 0.002% $CaCl_2$ (39). Without removing the culture medium, the fixative was introduced slowly (10–20 ml/h, by means of fine plastic tubing) onto the floor of the culture dish (at least 0.5 cm from the well) rather than into the well to prevent blebbing of the growth cone which otherwise was occasionally observed. Cell movement stopped abruptly within 5 min of the beginning of the perfusion and both the shape and the size of the growth cone being observed were maintained if all precautions had been taken. After 30 min the culture was removed from the microscope.

The cultures were further fixed at room temperature in 2% osmium tetroxide in phosphate buffer (three periods of 15–30 min each), washed in sodium maleate buffer of pH 5.2 (four periods of 5 min each), and stained for 15–45 min in 1% uranyl acetate in this buffer (24). They were again rinsed in maleate buffer (two periods of 5 min each) and dehydrated in a graded series of ethanol solutions. Propylene oxide could not be used because it dissolved the plastic culture dish. An Epon or Araldite-Epon mixture (27) was poured directly into the dish, allowed to stand overnight at room temperature, and then cured for 24 h at 50°C and for 24 h at 60°C. Gentle pressure applied with a razor blade tip freed the coverslip, leaving the tissue near the surface of the plastic.

Finally, the cell under investigation was relocated in the plastic under phase contrast microscopy by reference to light photomicrographs, and a circle ~1 mm in diameter was inscribed around it by rotating a modified microscope objective carrying an eccentrically mounted diamond point. The specimen was serially sectioned with a diamond knife on a Sorvall Porter-Blum MT-2 ultramicrotome parallel to the surface of the cover slip, beginning at the collagen surface. The sections were picked up on 150-mesh or 200-mesh (EFFA general, Ernest F. Fullam, Inc., Schenectady, N. Y.) grids which were covered with carbon-stabilized collodion or Formvar films. After staining with lead citrate (40), the sections were examined in a Philips 200 or 300 electron microscope. Serial sections of 12 growth cones were examined in the electron microscope.

RESULTS

When first explanted the isolated neuron appeared as a rounded, highly refractile cell in which an eccentric nucleus and nucleolus were prominent. Such a cell began to send out processes some 12 h after explantation (8). The straight thin processes displayed active terminal enlargements (growth cones) which moved as much as 40 μm/h and began to branch. Slender lateral extensions appeared occasionally along the fiber.[1] The growth cones were variable, sometimes appearing thin and spread out (Fig. 1), at other times appearing spindle- or triangular-shaped with numerous slender processes (filopodia) (Figs. 14 and 16). Sketches of representative configurations of the cultured sympathetic neurons appear in Fig. 2 of the accompanying paper (8).

At the fine structural level, the morphology of the isolated cells studied closely resembled that of developing neurons. The cell body contained many polysomes and some granular endoplasmic reticulum. In their extensions, microtubules and neurofilaments were present in longitudinal alignment and occasional ribosomal clusters were also seen. In addition, dense-cored vesicles were always seen throughout the nerve cell. These constituted a useful distinguishing feature because they were absent from cells considered to be nonneuronal in the cultured ganglion pieces. Slender extensions of nonneuronal cells appeared very different for they contained mainly ribosomal clusters along with some agranular membranous elements, mitochondria, lipid droplets and, in some areas, a thick stratum of longitudinally oriented filaments subjacent to the plasma membrane, a feature not seen in neuronal extensions.

Neuron A

Neuron A will be described in detail because it was in some respects the most successful sample; this isolated bipolar cell was observed and photographed during growth and was therefore known to be growing at the time of fixation, and all areas of one of its fibers were studied in serial thin sections. When first observed, the dark triangular area (*e*, Fig. 1) was the tip. Subsequently a branch appeared and developed into a new spread-out area (*a*, Fig. 1) while the original tip area began to retract. Representative areas of this fiber are shown here (Figs. 2–8). The shape of each area as seen in the electron microscope corresponded very well with that observed in the light microscope (*vide infra*). The fiber was single and had no sheath of any kind.

In the spread-out area, *a* (Fig. 2), and in the

[1] Since developing axons could not be distinguished from dendrites in these short-term cultures, the term nerve *fiber* was used in this paper to refer to all slender processes put out by the nerve cell.

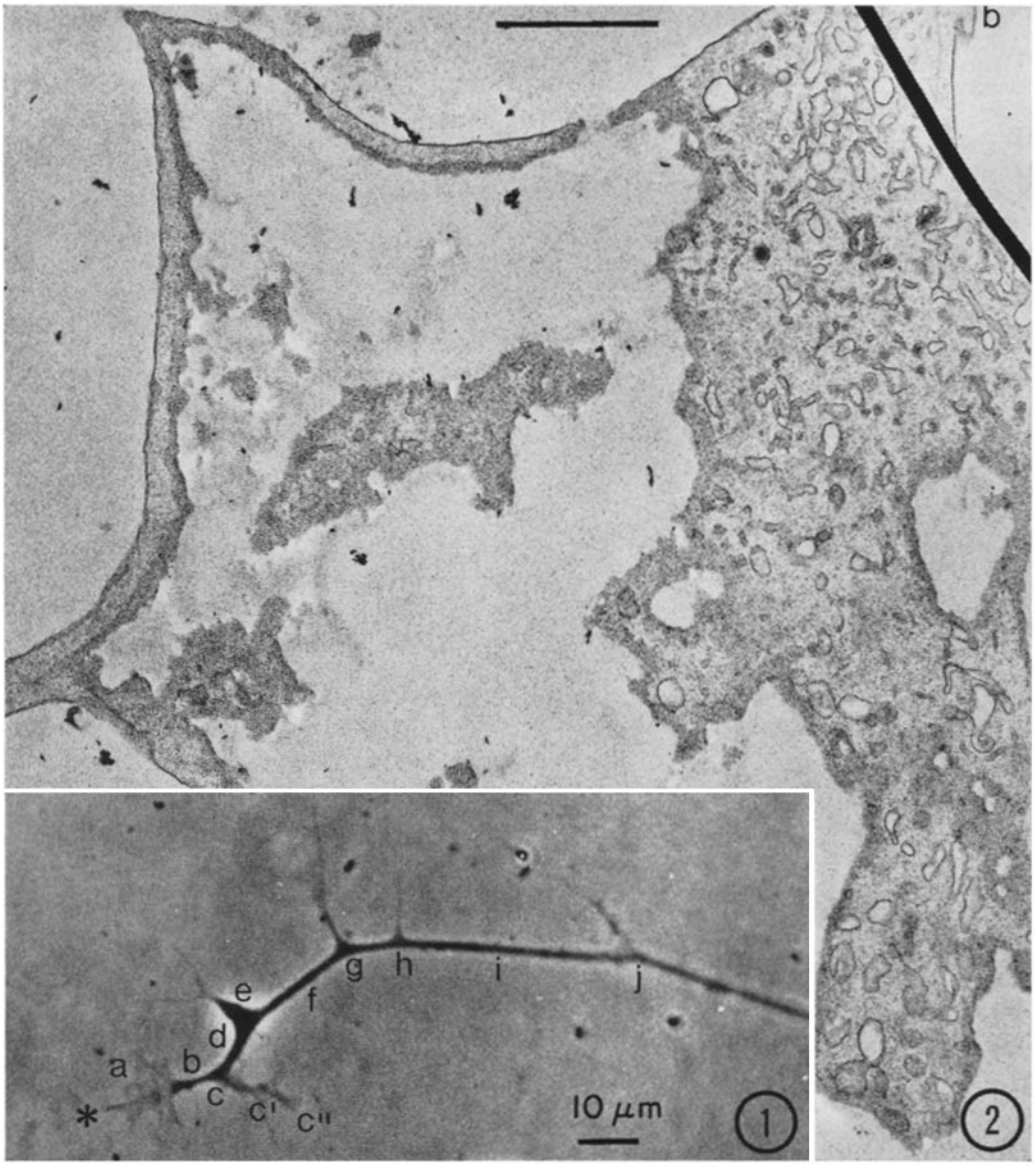
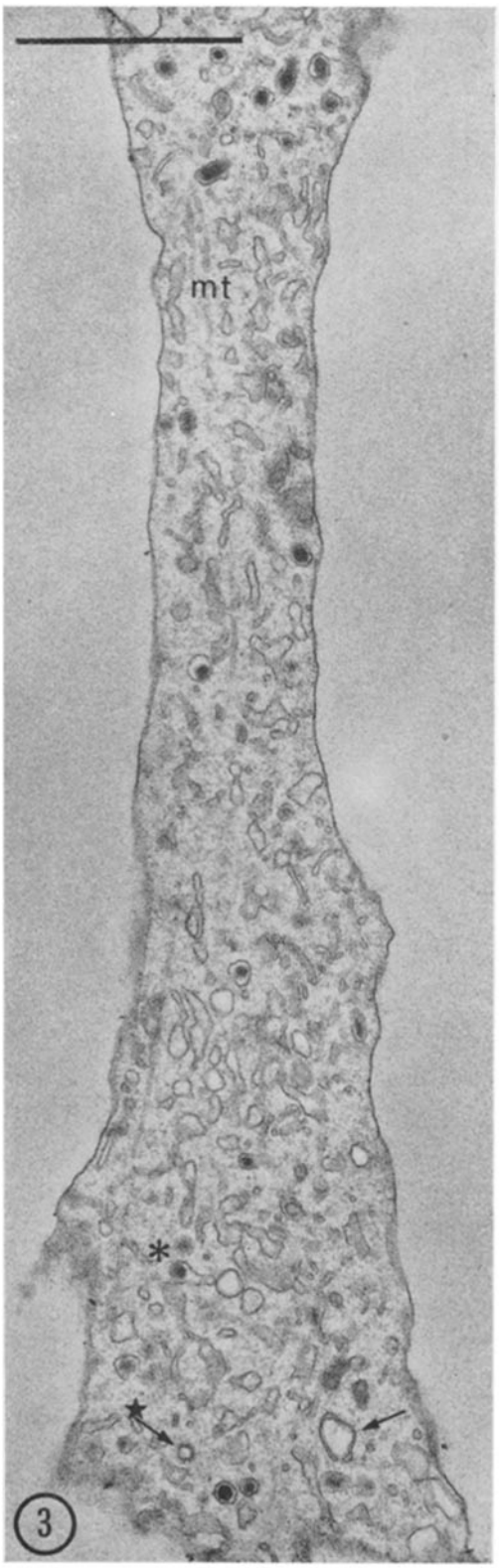


FIGURE 1 One of two extensions of isolated neuron A, observed and fixed during growth. The leading spike (*) of the enlarged termination is ~350 μm from the cell body. The spread out area, *a*, had recently begun to flourish whereas the previous tip area, *e*, was in the process of retracting. Bar, 10 μm. × 835.

FIGURE 2 Electron micrograph of neuron A, part of growth cone *a* of Fig. 1. The leading spike of the cone (shown at *, Fig. 1) is out of the figure, below the bottom of this page; area *b* starts at the upper right of the figure. The main components are membrane bound vacuoles, vesicles, and cisternae and also a moderately dense matrix. The leading spike consisted of this matrix plus a stream of vacuoles. Only the thicker areas, a peripheral lip and the central core, are present in this section. Bar, 1 μm. × 21,000.

leading spike (*), agranular membranous elements were suspended in a moderately dense matrix. The matrix alone filled the area subjacent to the plasmalemma around most of the tip. In area *b* (Fig. 3), agranular membrane, some of it in the form of branching cisternal elements, dense-cored granules or rods (the density sometimes being located within the reticulum sacs),



and coated vesicles were present. Microtubules were rare; a few are shown in the figure. Neurofilaments were not visible. All three levels of this portion appeared similar.

Parts of area *c* (Fig. 4) were seen in four sections. This spread-out region resembled that in *a* in that in the most distal portion (*c''*) and in the filopodia, only vacuoles and the dense matrix were seen; midway (*c'*) these were found along with long, meandering cisternae of agranular reticulum, and in the area more proximal to the fiber (*c*) these components were joined by vesicles and branching cisternae of endoplasmic reticulum, dense-cored structures, and coated vesicles. The dense matrix material alluded to here and in *a* (as well as in other tips; *vide infra*) probably corresponds to the microfilamentous network described by Yamada et al. (42). Again, the larger neurofilaments were absent and microtubules were rare as in *a*.

In the *d* portion of the nerve fiber, however, some neurofilaments were seen, more microtubules were present, and mitochondria were found (Fig. 5). Cisternae of smooth reticulum (some of which branched and reached a subsurface position) and dense-cored structures were still prominent. Note the vesicles and vacuoles characteristically filling a protuberance of the fiber. Vacuole clusters were observed occasionally along the fiber (in protuberances, as here) or near the origin of a lateral extension (at *g*, *h*, and *j* which is shown in Fig. 8).

The dark triangular area, *e*, was in the process of retracting at the time of fixation. It was therefore of interest to find a large myelin figure with altered cytoplasmic contents, suggestive of autolytic breakdown (Fig. 6). This presumed autophagic vacuole was present in the seven thin sections of *e* which were examined. Other configurations suggesting autophagic vacuole formation, double membrane bounded bodies and cup-shaped bodies, were noted at this and other levels. Neurofilaments and microtubules were present in

FIGURE 3 Neuron A, area *b* of Fig. 1. Membrane bound structures predominate. Among these are a number of dense-cored vesicles, a nearly closed cup-shaped body (arrow), and a coated vesicle (starred arrow). One of the dense cores appears within the reticulum at*. Microtubules (*mt*) are rare. Bar, 1 μm. × 30,000.

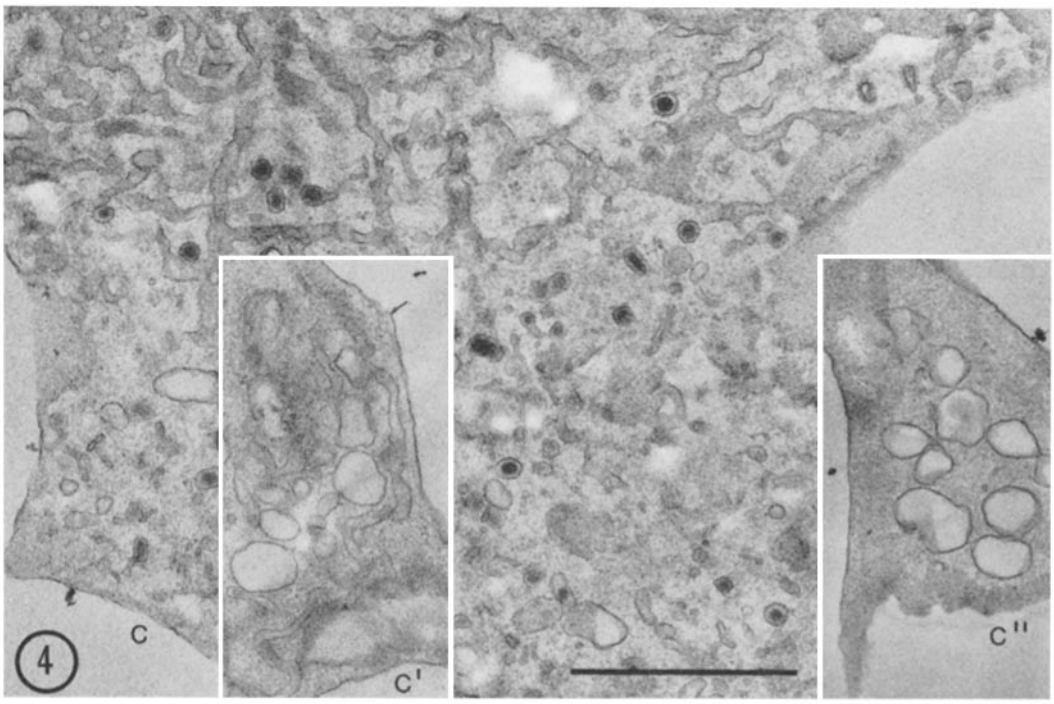


FIGURE 4 Neuron A, areas *c*, *c'*, *c''* of Fig. 1. Spread out area *c* emerges from the fiber (which runs horizontally at the top of the figure) where numerous components of endoplasmic reticulum are seen. Area *c'*, located about halfway between the fiber and the tip, contains long meandering tubules of agranular membrane, vacuoles, coated vesicles (not shown), and matrix material. Area *c''*, at the distal tip, harbors a cluster of vacuoles. Bar, 1 μm. Fig. 4c, × 30,000. Fig. 4c', 27,000. Fig. 4 c'', 30,000.

e, largely confined to that area continuous with the nerve fiber (*d* and *f*); in all seven levels examined no microtubules were observed to veer in the direction of the tip of *e*. Clusters of particles (ribosomes?) were seen in *e*, outside as well as inside the myelin figure, but were clearer at levels other than this one. The two filopodia at the tip of *e* (refer to Fig. 1) contained only the dense matrix material.

The thick area of *g* was observed at six levels in the electron microscope. In this area were various smooth membranous elements (including branching cisternae, curving cisternae of narrower caliber, vesicles, and vacuoles), groups of particles presumed by their arrangement to be polysomes, and dense-cored structures. Microtubules were present in that portion continuous with the fiber on either side of the projection (Fig. 7) but unlike the retracting enlargement at *e*, an occasional microtubule veered into the lateral extension and coursed along its length. Another area, *j*, from which an extension arises, is illustrated in Fig. 8.

Other Neurons

The fine structural data presented below are based upon the study of additional neurons, some of which branched and therefore had more than one growth cone. Many of the areas of interest for each neuron were photographed at more than one level in the electron microscope.

CELL SOMAS: Within the cytoplasm free polysomes were abundant (Fig. 9). Some usually unoriented cisternae of granular endoplasmic reticulum were present, in some cases curving to a subsurface position beneath the plasmalemma. Other organelles residing in the soma included long cisternae of agranular reticulum, mitochondria, Golgi apparatus, multivesicular bodies, dense bodies, and dense-cored vesicles. Dense bodies made up primarily of myelin figures were seen inside, protruding from, or just outside the soma, suggesting that they were being shed by the neuron. Filaments were rare. Some microtubules were present, coursing in all directions in contrast

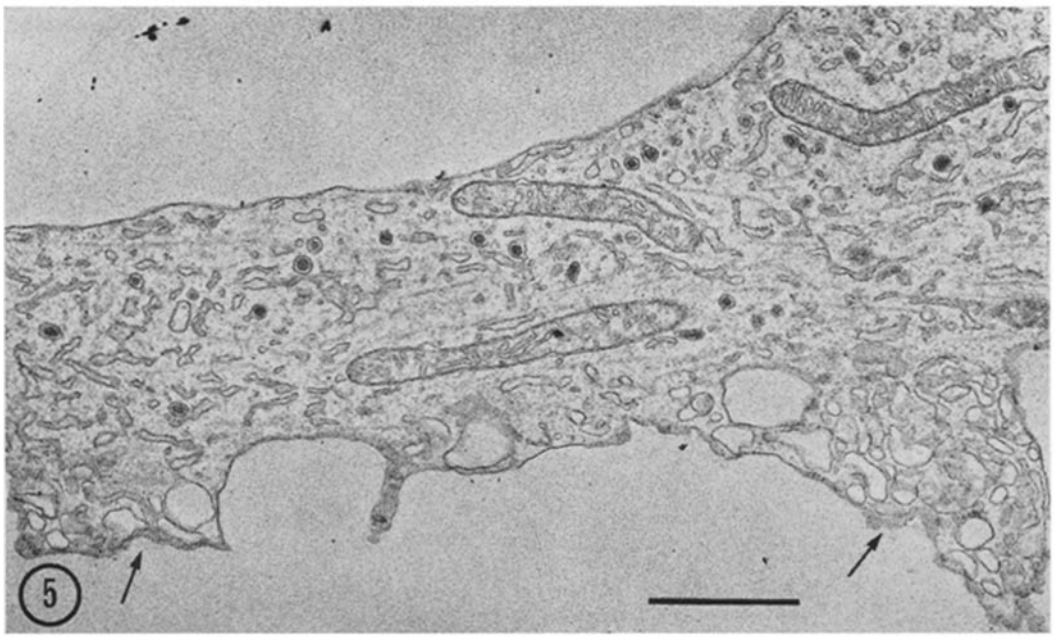


FIGURE 5 Neuron A, area *d* of Fig. 1. In addition to the organelles seen previously, mitochondria are now visible in this stouter portion of the fiber. An occasional microtubule and neurofilament are present. Within two protuberances (arrows) are clusters of vesicles and vacuoles. Bar, 1 μm. × 20,000.

to their characteristic parallel orientation in the nerve fiber.

FIBERS: Axons could not be distinguished from dendrites by light microscopy (8); at the electron microscope level, the specializations of the axon initial segment, the plasmalemmal undercoating and the clustered microtubules (30), were not found.

The nerve fiber as it emerged from the soma (Fig. 10) and elsewhere (Figs. 11–13) contained smooth endoplasmic reticulum (often branched and occasionally containing dense material), long mitochondria lying parallel to the fiber length, polysomes, microtubules, occasional neurofilaments, coated vesicles, dense-cored structures, multivesicular bodies, dense bodies, autophagic vacuoles, and distinctive narrowed and curved cisternae of agranular membrane (cuplike bodies?; see below). In surveying a length of fiber, it appeared that the polysomes were more prominent in the vicinity of or clustered around or on the mitochondria (Fig. 11) and that smooth reticulum was often in close apposition to a mitochondrion (Fig. 15). Subsurface cisternae of smooth membrane were often seen. Granular endoplasmic reticulum was never observed. Although transverse sections of fibers were not available for counting, it did appear in longitudinal sections that microtubules were more numerous in areas closer to the cell body.

Coated vesicles (~900 Å in diameter) were seen to be continuous with the fiber plasmalemma (Fig. 13), and if the surface membrane was dotted with precipitate so also were the local interiorly located coated vesicles. Thus, it was surmised that at least some of the coated vesicles arise from the fiber surface.

Rarely a group of dense-cored vesicles, dense bodies, the distinctive curved agranular cisternae, and also multivesicular bodies and autophagic vacuoles (both of which often displayed curving "tails") were arrayed in a line as if confined to the same channel in the fiber (Fig. 12), a phenomenon also noted by Yamada et al. (42) although different organelles were involved. Occasionally, a large, empty-appearing vacuole was found in a protuberance of the fiber (Fig. 13), suggestive of a pinocytotic droplet moving along the periphery of the process; its position is comparable to that of a pinocytotic droplet shown in a light micrograph by Pomerat et al. (Fig. 12 b, reference 32).

Small foci of cytoplasm contained within two membranes were often seen and will be discussed in the section on "growth cone". In addition,

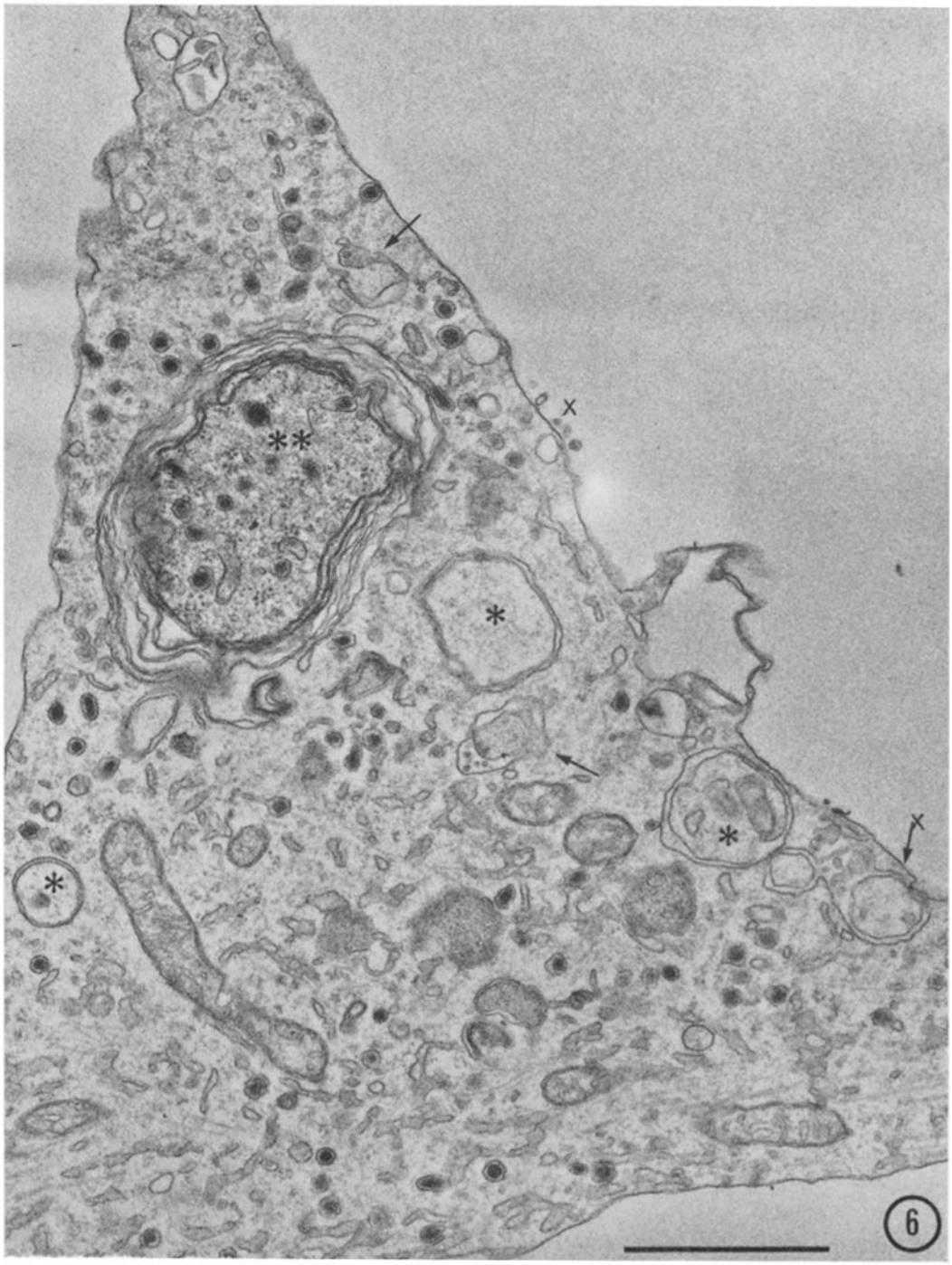


FIGURE 6 This electron micrograph illustrates the contents of area *e* (of Fig. 1) from neuron A. Of note here is the large body (**) containing dense-cored vesicles and particles. This structure and others (*) are considered to be various stages in the formation of autophagic vacuoles as are the cup-shaped bodies (at arrows). If the presence of the material (here at *x* and in other areas) near the outside of the plasmalemma can serve as an indicator, then the formation of autophagic vacuoles via cup-shaped bodies from the surface membrane (at *x*→) might be suggested. Bar, 1 μm. × 30,000.

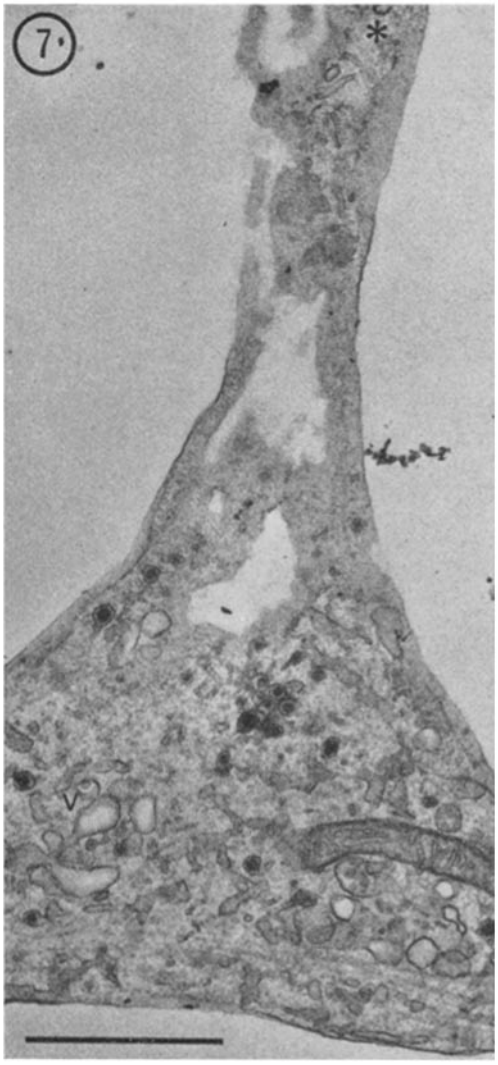


FIGURE 7 Neuron A, area *g* of Fig. 1. A lateral extension containing matrix material and membranous components (*). Dense-cored vesicles are often clustered as here. Vacuoles, similar to those at *v*, were seen at other levels to be grouped near the plasmalemma at the junction between the fiber and its extension. Vacuoles were also noted along the length of the extension. Bar, 1 μm. × 26,000.

there may be present a finer endoplasmic reticulum system than that mentioned and illustrated above; in the finer type, membrane-bounded dilatations alternate with channels that may be as thin as or thinner than neighboring microtubules. Dense core material was not observed in this type of reticulum which was less noticeable in fiber areas containing higher amounts of the larger caliber reticulum. As can be seen in Figs. 13 and 15 the fibers of the solitary neurons studied occur singly and are unsheathed.

BRANCH POINTS: All of the organelles seen in the fiber may be found in this area; branch points appeared not to have any morphological specializations or regional discontinuities in the cytoplasm. One point that should be made, however, concerns the orientation of the microtubules. The microtubules were seen to splay out from the parent fiber into the daughter ones, mainly following the curve of the newly formed branches. Microtubules also had formed beneath the straight surface of the membrane connecting the two daughter branches (neuron C, Fig. 15; see Fig. 14 for orientation). The presence of oriented microtubules along the curve of the daughter processes was particularly striking in neuron D (Fig. 22; for exact position, see * in Fig. 16).

GROWTH CONES: In the electron microscope, the organelles were usually abundant in the thicker portion of the enlarged termination, diminishing in concentration near the periphery of the tip and in the filopodia (Figs. 17, 18). Growth cones of different cells varied mainly in the concentration of organelles; growth cones from the same cell were very similar in content. For example, in neuron D the size and the configuration of the three tips (plus processes emerging from the soma) varied at the time of fixation but the cytoplasmic content was strikingly similar (compare *e*, Fig. 18 with *f*, Fig. 21).

Within the thin irregular peripheral flange of the tip and in the filopodia, membranous structures could be found scattered throughout the matrix material. This matrix substance, undoubtedly comparable to the microfilamentous network described by Yamada et al. (41, 42), usually appeared as particles of varying size but linear or filamentous structures as thin as approximately 30 Å in diameter could also be discerned. Polygonal forms could be detected within this meshwork as Yamada et al. noted; in addition, a particle was often seen to be enclosed within them (Fig. 19). Suspended in the matrix were streams of agranular reticulum or small foci of membranous tubules and vesicles. On the basis of size difference, these elements were presumed to be different from the large empty-appearing membrane-bounded (pinocytotic?) vacuoles observed occasionally within the matrix material and elsewhere (compare Figs. 7, *, and 18 with Figs. 5, 8, and 13).

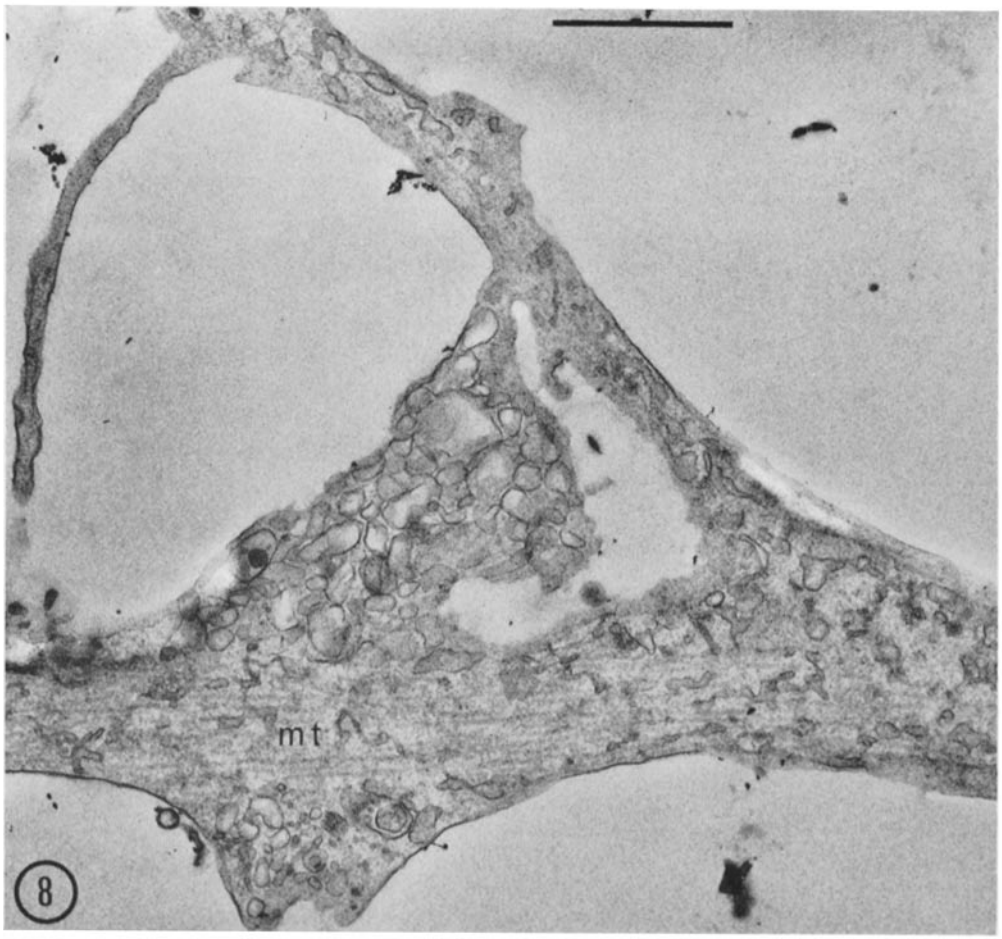
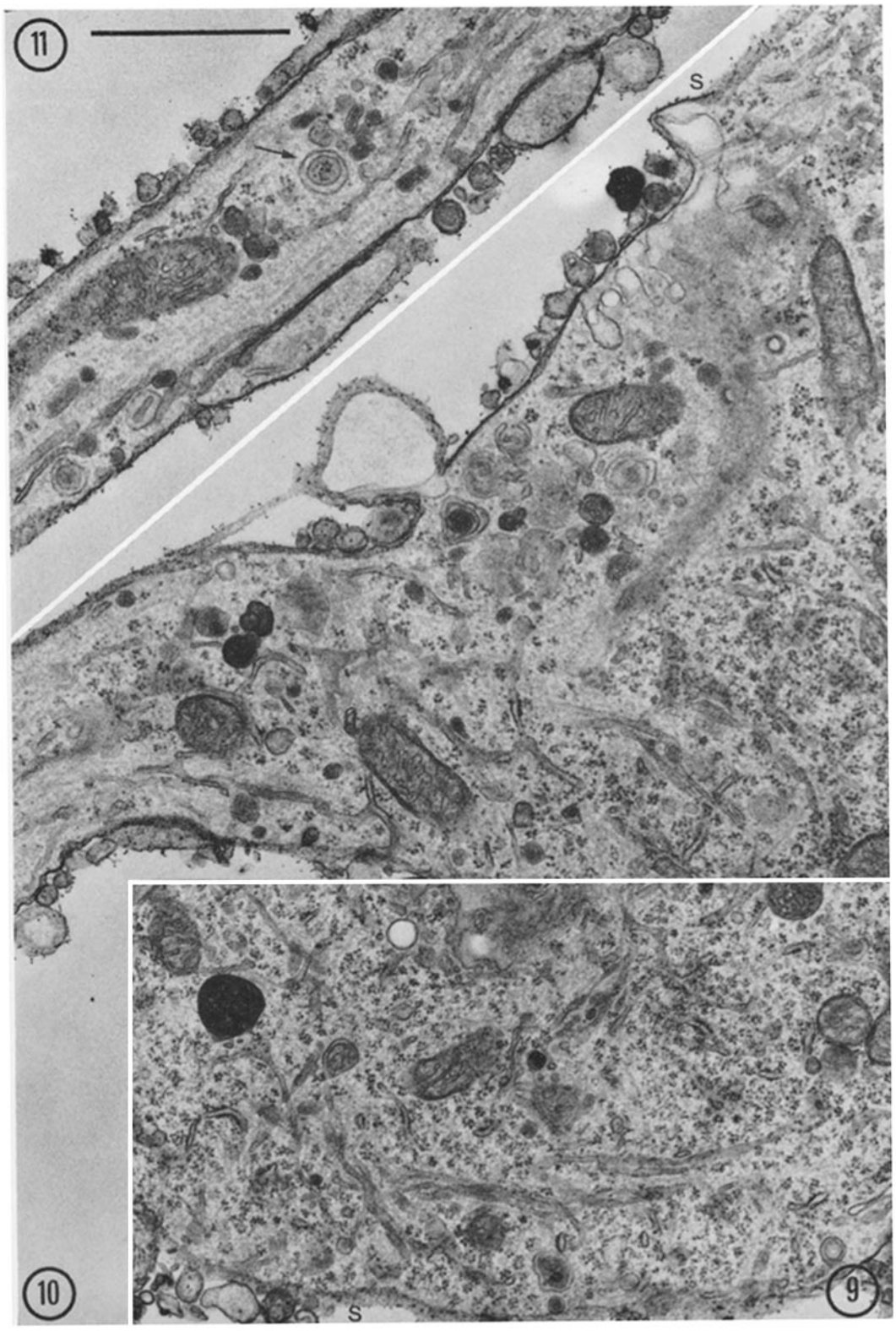


FIGURE 8 Neuron A, area *j* of Fig. 1. At the site where a thin extension arises from the fiber (which runs horizontally here) there is a mass of vacuoles. Microtubules (*mt*) are numerous in this more proximal portion of the fiber. Note that here as in all other areas of neuron A illustrated, the fiber has no sheath. Bar, 1 μm. × 24,000.

The smooth endoplasmic reticulum in the thicker portion of the growth cone varied in amount from cell to cell and assumed a variety of forms. The reticulum sacs (or vesicles) varied in length, girth, and branching. Dense rods or spheres appeared within the reticulum and in some cases appeared to be budding from it. Distinctive curved and narrowed structures were

FIGURES 9, 10, and 11 Areas of isolated neuron C. In Figs. 9 and 10, portions of the soma are shown. The somal surface (*s*) is not covered by other cells but only by some debris. The cell body contains a wealth of free polysomes, some granular endoplasmic reticulum, long elements of agranular endoplasmic reticulum, mitochondria, and dense bodies. The exiting fiber, illustrated in Fig. 10, contains polysomes, branching agranular reticulum, and mitochondria. Farther along, before its bifurcation (see Fig. 14), the fiber also contains dense-cored vesicles and microtubules (which appear faint in this preparation). A portion of cytoplasm enclosed within two membranes is indicated by the arrow. Bar, 1 μm. Fig. 9, × 27,000. Fig. 10, × 28,000. Fig. 11, × 31,000.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seen to arise from reticulum elements (Figs. 18, 20); within their lumina could be found small vesicles or dense material which caused a local bulge. Some cytoplasmic organelles, particularly dense-cored vesicles (Fig. 18), could be seen in the cytoplasmic bay of these C-shaped structures or within single- or double-walled bodies (autophagic vacuoles). All these images suggest that from reticulum cisternae cuplike bodies form and segregate areas of cytoplasm with closure of the rim of the cup (see Fig. 21). In some cases the two apposing membranes of the cisterna eventually fuse, resulting in a single-walled autophagic vacuole. Throughout the nerve cell small areas of cytoplasm enclosed within two membranes were seen. Many of these are interpreted as being stages in autophagic vacuole formation, before fusion of the membranes of the bounding cisterna.

Occasionally, vesicle-containing sacs were seen to be attached to dense bodies (Fig. 18 and as in Fig. 22). The dense bodies often contained large myelin figures as well as some small vesicles. Coated vesicles were present, both internally and contiguous with the surface membrane. Dense-

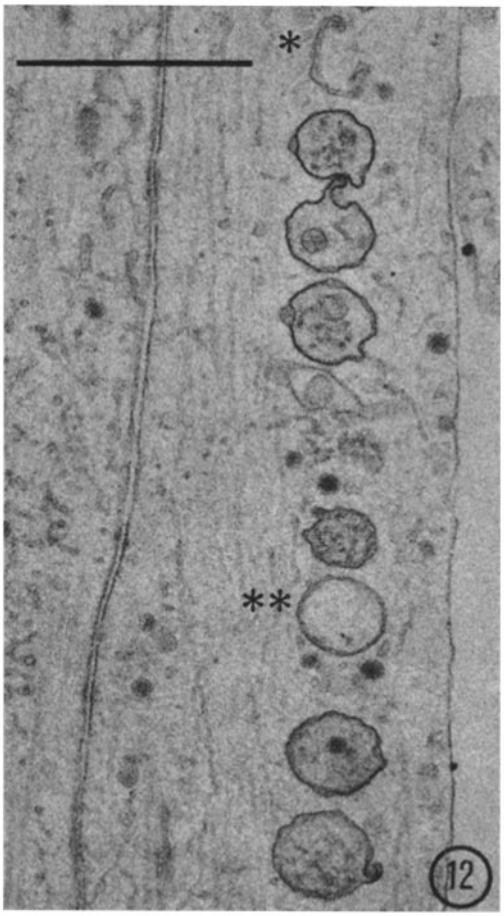


FIGURE 12 In one of these two fibers there is a row of organelles considered to be autophagic vacuoles. Early stages in their formation are represented by the C-shaped body (*) and the round body bounded by a double membrane (**). The limiting membranes of the others have become largely fused. Portion of fiber from neuron B in an explanted ganglion piece. Bar, 1 μm. × 31,000.

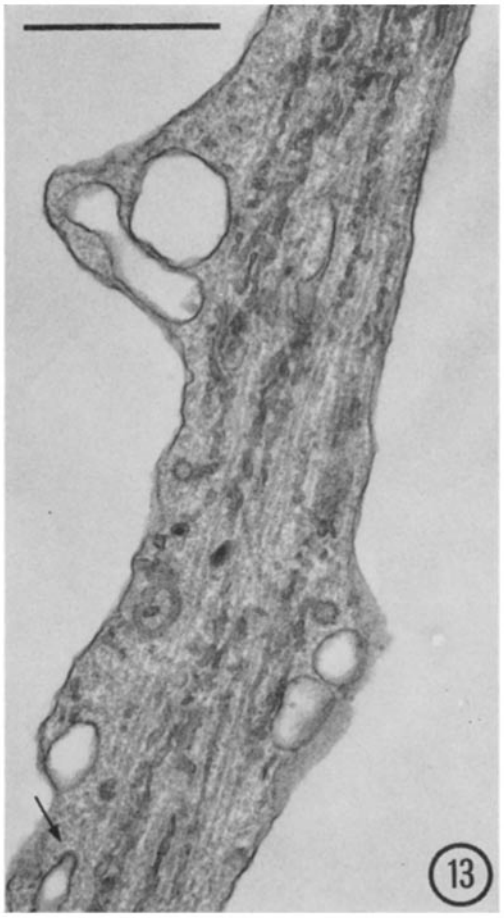


FIGURE 13. The vacuoles causing bulges in this nerve fiber are undoubtedly pinocytotic elements which have been observed in living fibers (see text). Numerous microtubules and agranular reticulum elements are also visible. A coated vesicle is continuous with the surface membrane (arrow). From area *d* (see Fig. 16); isolated neuron D. Bar, 1 μm. × 26,000.

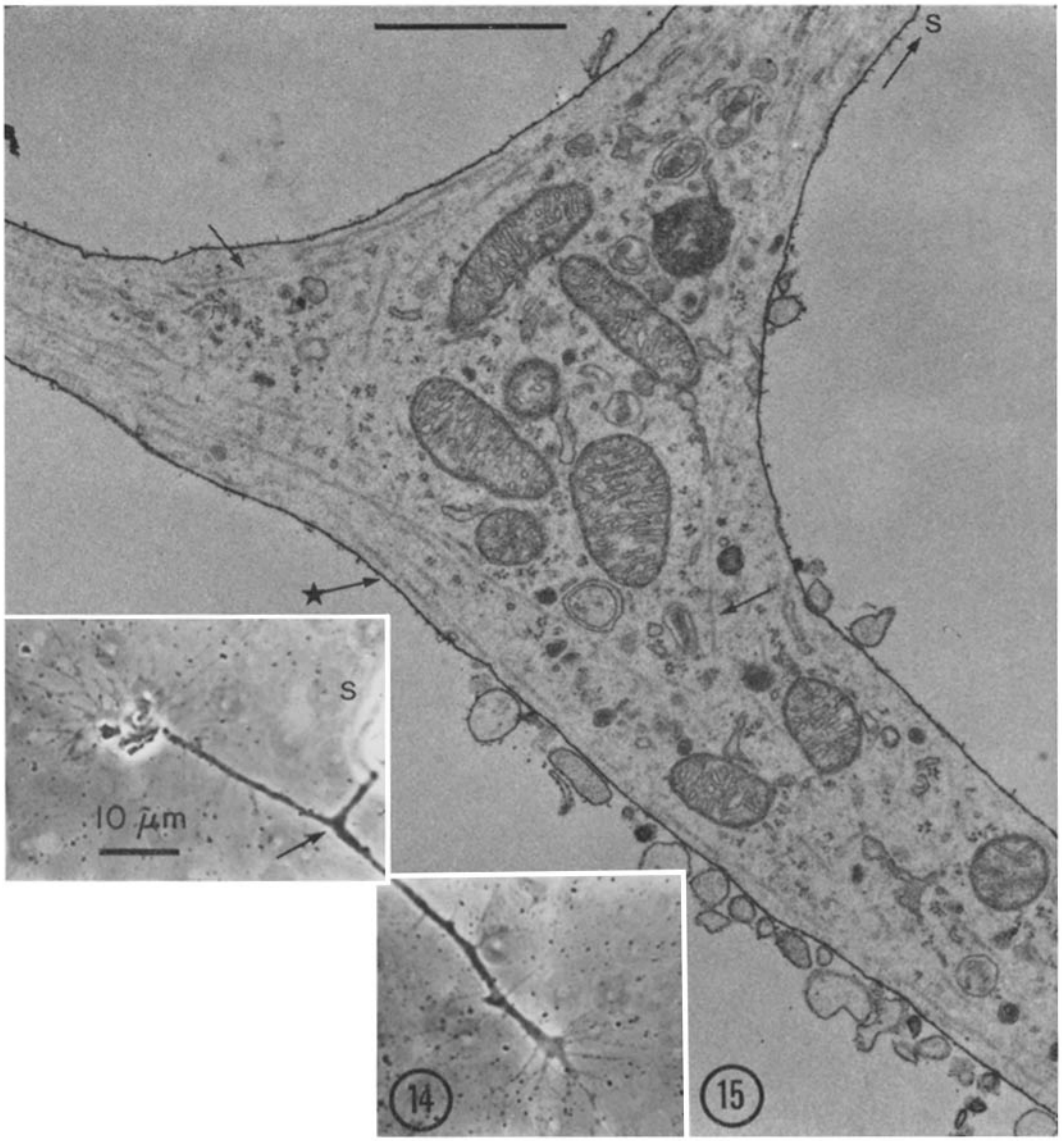


FIGURE 14 Light micrograph of isolated neuron C. Portions of the neuronal soma (*s*) and exiting fiber are illustrated in Figs. 9–11. The point at which the fiber branches (arrow) is shown in Fig. 15. Both tips were characterized by numerous long slender filopodia which in the electron microscope were seen to contain mainly matrix material. Bar, 10 μm. × 1,060.

FIGURE 15 Bifurcation of fiber of neuron C. Microtubules splay out at the branching point (arrows) and also have formed at right angles to the parent fiber (see starred arrow). Polysomes are present. Agranular reticulum components lie near the mitochondria. *s*, cell soma. Bar, 1 μm. × 25,000.

cored structures, mainly granules but also rods, were prominent. They exhibited a high degree of variation in size and density; dense-cored vesicles ranged from 650 to 1600 Å in diameter. Polysomes were scattered sparsely throughout the thicker portion of the cone. Mitochondria were concentrated in the thickest part of the tip (Fig. 18). Some microtubules (220–300 Å in diameter) extended from the fiber into the tip: those near the surface of the proximal cone followed the smooth

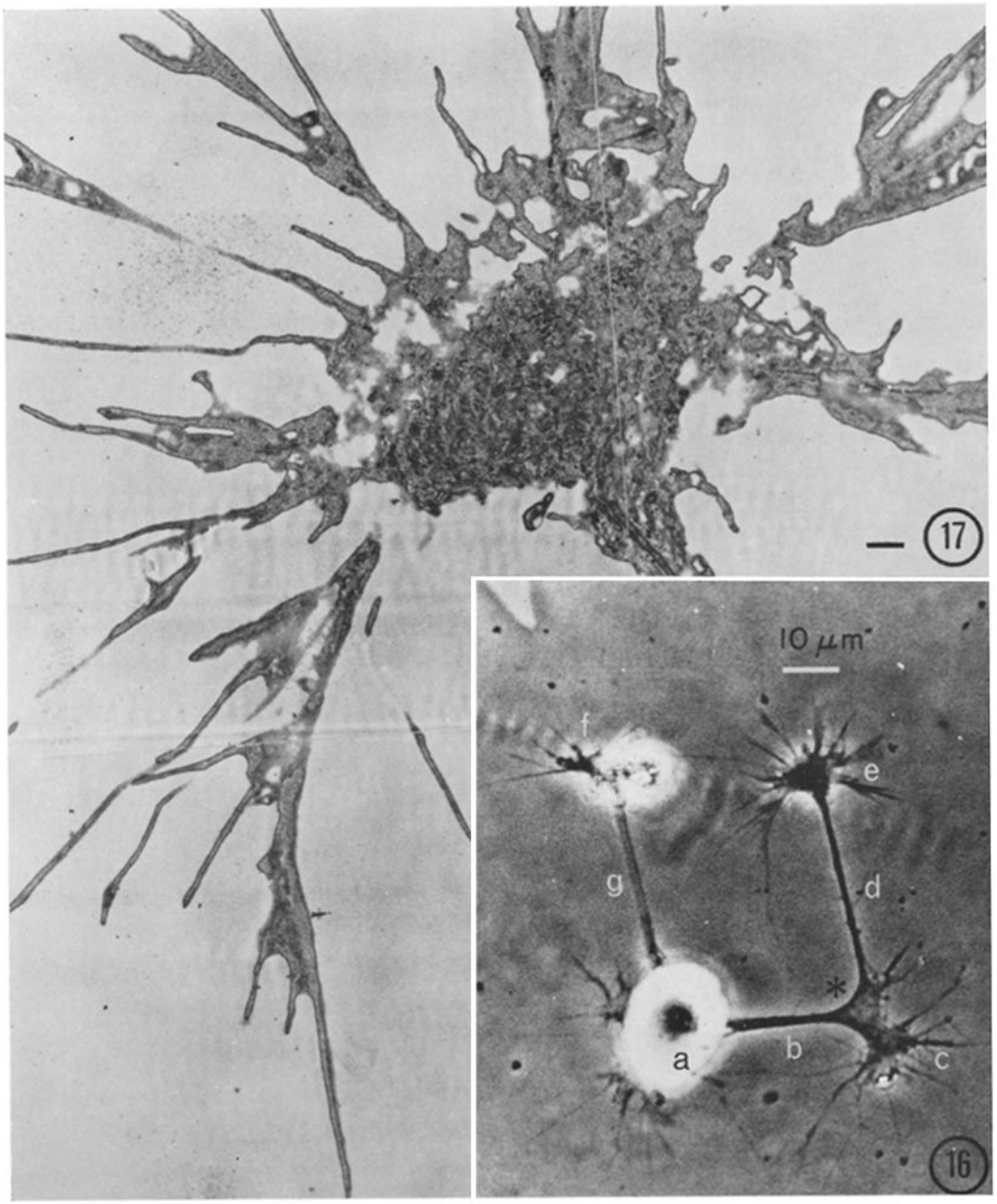


FIGURE 16 Light micrograph of isolated neuron D. The highly refractile portion, *a*, is the cell body which gives rise to two fibers, *g* and *b*. There are three growth cones, *f*, *c*, and *e*. The additional processes emerging from *a* have fine structural characteristics of growth cones. Electron micrographs of some of these lettered areas follow; the area near the asterisk appears in Fig. 22. Bar, 10 μm. × 790.

FIGURE 17 Low magnification electron micrograph of growth cone *e* of Fig. 16 from neuron D. Nearly all the filopodia visible in the light micrograph (Fig. 16) are present in this thin section. The thick area of the tip is filled with organelles (see Fig. 18) whereas the filopodia consist mainly of the matrix material. Bar, 1 μm. × 5,400.

outward curvature whereas those in the tip interior coursed in every direction (as in Fig. 22). At the point where the more distal tip contour became irregular and contained the characteristic matrix feltwork, microtubules vanished. They consistently were absent from the peripheral cytoplasm or filopodia (Figs. 18, 19). The few neurofilaments present in general followed the distribution of the microtubules. The neurofilaments measured 60–110 Å in diameter in contrast to the finer matrix elements discussed above.

A few examples of continuity between cytoplasmic membrane and this surface membrane were found (Fig. 23). The most convincing examples were noted in areas where a membranous sheet appeared folded, suggesting that the area was ruffling at the time of fixation. The immediately adjacent cytoplasmic membranes were in a distinctive array in these areas (Fig. 23), and also in a number of areas where the cytoplasmic surface of the cell membrane (which rested on collagen) had been grazed in thin sectioning. These areas of continuity as well as the distinctive arrays of membranes were clearly within nerve fibers (which contained dense-cored vesicles) but their exact relationship to growth cones was not always clear.

DISCUSSION

Growth Cone Content

The growth cones of rat sympathetic motor neurons are similar in many respects to those of chick sensory ganglion cells grown in vitro as studied by Yamada and his colleagues (42). In both studies the moving parts of the tip, the peripheral flange and filopodia, were filled with a distinctive fine meshwork from which all other cytoplasmic organelles except some membranous structures were excluded. Also, in both investigations occasional microtubules were found in the thicker central portion of the tip but they did not invade the meshwork area. Yamada and coworkers (41, 42) concluded from their investigations that axon elongation depends upon both the presence of microtubules which serve as a skeletal support for the established fiber, preventing its collapse, and the meshwork which maintains the structural integrity and participates in the movement of the growth cone. (In our study numerous oriented microtubules were situated in the peripheral cytoplasm of branching points, suggesting that they contribute to the stability of these areas as well.) In both studies, other organelles were located in the thicker or more central region of the cone. These included smooth endoplasmic reticulum sacs and vesicles, dense-cored and coated vesicles, mitochondria, and neurofilaments up to 110 Å in diameter.

The community of organelles in the growth cones of cultured sympathetic neurons also resembles that in the growing tip of the rabbit dorsal root neuroblast *in situ* studied by Tennyson (38). In the tip enlargement, she found channels of agranular reticulum, scattered microtubules and neurofilaments, numerous mitochondria and dense bodies, and occasional polysomes. In addition, she reported that the peripheral flange of cytoplasm and the filopodia were filled only with a "finely filamentous matrix" and occasional small vesicles. Widely dilated agranular cisternae, some of which appeared fused with the surface membrane, were interpreted as the pinocytotic droplets seen by Pomerat et al. (32) in living growth cones.

The variety of organelles in the tips discussed thus far makes it clear that these tips differ from the enlargements identified as growth cones by Bodian (5) and del Cerro and Snider (11). These investigators found bulbs or protuberances about 0.5 μm in diameter (5) containing only large clear vesicles (1,100 Å in diameter, reference 11) and some coated vesicles suspended in cytoplasm of very low density. Kawana and collaborators (25) identified as growth cones structures which were similar to these in that they were small bulges containing segregated masses of membranous elements (termed tubules and sacs of smooth reticulum rather than vesicles) in areas of low density. But, in addition, filopodia with a filamentous matrix were seen to arise from the bulges, and other organelles (though in separate regions) sometimes were found in the bulges. These profiles were encountered in random sections of fetal monkey spinal cord (5) or developing rat or cat cerebellar cortex (11, 25); there were no light microscope counterparts showing the expected enlargements of the tip in the range of up to 5 μm wide and 10 μm long (reviewed in reference 38). Grainger and James (12) found similar aggregates of vesicles (400–2,000 Å) not only near the ending but also along the length of cultured chick spinal cord fibers.

In this study clusters of vacuoles and vesicles were also found along the fiber (in excrescences;

Fig. 5) or near the origin of a lateral extension (Fig. 8). In neither case did they constitute a tip and only very rarely were such configurations seen in the tip area. Have these areas resulted from inadequate preservation? It would seem that the method of fixation of single neurons, if not the fixative, was close to ideal and, also, that in the other areas of the nerve cell the preservation was adequate. Del Cerro and Snider (11) made the point that the vesicle aggregates were present after either aldehyde or osmium tetroxide fixation. However, are certain areas of agranular reticulum, for instance, more labile and therefore more difficult to preserve? These clusters of vesicles do appear to be "specialized" areas because other types of organelles are excluded and the cytoplasm is very low in density. Other questions remain, such as whether growth cones differ depending upon their location in the nervous system or their axonal or dendritic nature.

It should be emphasized that vesicle or vacuole clusters are not unique to neurons. Tennyson (38) noted that supporting cells occasionally exhibited vesicle aggregates. Guillery et al. (14) observed areas of closely packed vesicles (1,000–2,000 Å) in both nerve fibers and glia of cultured mouse spinal cord as did Grainger and James (12) who studied cultured chick spinal cord. Clusters of vesicles in a similar size range also were observed in cultured muscle cells by James and Tresman (23).

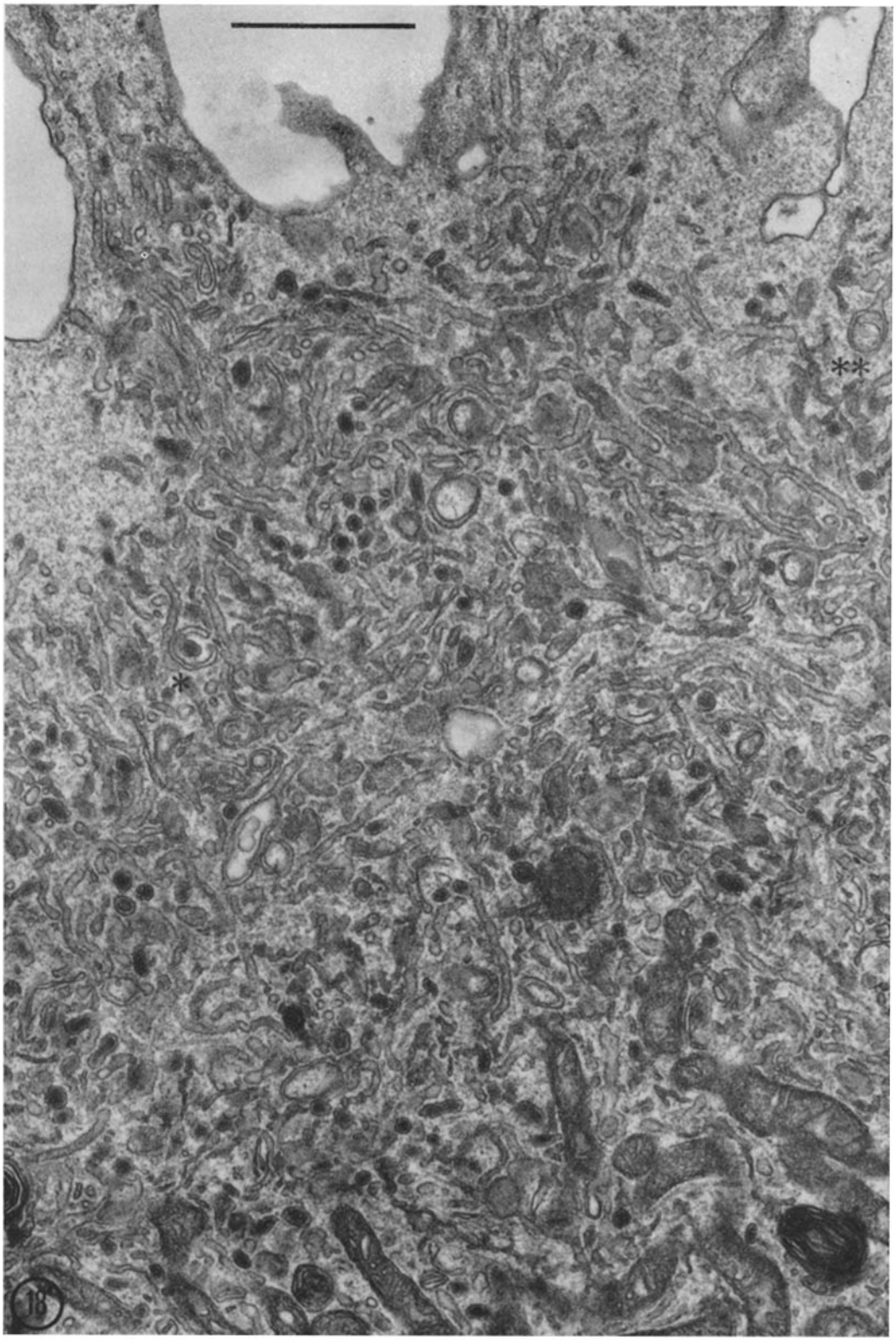
One possibility is that these are regions of (macro) pinocytosis. It is known that the extracellular fluid taken up at the ends of growing axons is contained in large droplets which subsequently move centripetally along the fiber (reviewed in reference 32). The vesicle aggregates always occur near the surface or occupy a bulge from the surface. In cultured human sarcoma cells, for example, Gropp (13) has described the light microscope appearance of pinocytosis in undulating regions as the appearance of an irregular light gray lake beneath the cell surface which subsequently becomes a more refractile white droplet before starting to migrate interiorly. Would the interior of the initial lake seen in the light microscope appear as a collection of large vesicles or small vacuoles in the electron microscope? A few examples from a variety of tissues shown in Rose's "Atlas of Vertebrate Cells in Tissue Culture" (34), if they have been correctly identified as pinocytotic areas, would indicate that this is possible. Presumably, the vesicles would then coalesce to form one or a few much larger vacuoles which start to move centripetally.

It must be conceded, however, that the vesicle aggregates may move in the opposite direction, that is, that they are destined for the cell surface rather than the cell interior as just discussed. In an electron microscope study of phytohaemagglutinin-stimulated lymphocytes (4) strikingly similar "vesiculated blebs" were shown to occur on long cytoplasmic extensions (or "uropods") of the lymphocytes. These vesicles of 850 Å diameter did not contain ferritin which had been added to the culture medium whereas ferritin was found within nearby lysosomal bodies. Biberfeld (4) conjectured that the vesicles were shed from the lymphocyte. Clearly, tracer work is needed to determine the direction in which the vesicle aggregates are moving in neuronal extensions.

Activities of Agranular Reticulum

SURFACE MEMBRANE FORMATION: Agranular endoplasmic reticulum was always present in growth cones of cultured sympathetic neurons, in some cases in very high amounts (see Fig. 18). What functions might this organelle subserve? An obvious possibility is that it contributes to the formation of new growth cone membrane, as Yamada et al. (42) and we (9) have suggested. It seems likely that nerve processes in culture

FIGURE 18 Electron micrograph of part of growth cone *e* (neuron D), at a level different from that in Fig. 17. (Its fiber *d* is out of the figure, at lower right.) Agranular membranous elements predominate. A number of C-shaped structures are present; a dense-cored vesicle is found within one (*), and another is continuous with reticulum (**). A dense body containing both membranous whorls and vesicles is at the lower right. Mitochondria are clustered in the thickest portion of the tip. Near the plasmalemma is the matrix material; it is this material that also fills filopodia along with membranous components (see top left). Bar, 1 μm. × 29,000.



grow by assembly at their distal ends, since fibers will continue to lengthen for several hours after amputation from the cell body (22) and features such as branch points are left behind by advancing tips (8). More directly, the behavior of glass or carmine particles on the surface indicates addition of membrane at the tips (7). The presence of abundant membrane within the tips, as one of only two visible cell constituents at the leading edge, is consistent with the idea that the tip is the site of addition of new cell surface.

Some examples of continuity between cytoplasmic membrane and the plasma membrane were seen. The few observed in the growth cones selected for study were not particularly convincing. By far the most striking examples were present in folded membranous sheets (undulating membranes?) found randomly in outgrowth from a ganglion piece, arising from processes identified as neural by the presence of dense-cored vesicles (Fig. 23). These continuities appear different from the vesicle aggregates which were discussed above in relation to (macro)pinocytosis; the membrane configurations in continuity with the surface are neither rounded nor coated as would be expected of (micro)pinocytotic vesicles (reviewed in reference 17), a point also made by Spooner et al. (36). In these areas the immediately adjacent cytoplasmic membranes were found to be oriented in a very distinctive pattern, as illustrated in Fig. 23. It was only in areas adjacent to the cytoplasmic side of the surface membrane that this pattern was observed.

The folds in the cell surface suggested that ruffling was present at the time of fixation (Fig. 23). It is of interest to note that Yamada et al. (42) did not mention such continuities in their paper on growth cones but did present pictures of continuities very similar to those shown in Fig. 23 for the leading or ruffling edge of glia (36). Is the addition of components to the surface membrane accelerated in a ruffling area to such a degree as to be visible in electron micrographs? Abercrombie and co-workers (1) claim that the veillike leading and ruffling sheet (lamellipodium) of a moving fibroblast in culture is the region of rapid assembly of new surface. And yet, when these regions are examined in the electron microscope the thin lamellipodium is filled only with a "faintly fibrillar" material (similar to the meshwork seen in the neuronal filopodia) and at the base of it there may be a string or small cluster of vesicles along with larger vacuoles, none of which are in continuity with the plasmalemma (2). One would expect to find similar thin sheets in growth cones because, at least at times, portions of the cone exhibit ruffling. Is it possible that the process of addition of components to surface membrane is completed during the first minute(s) of aldehyde fixation as Heuser and Reese (reference 16, *vide infra*) proposed for synaptic vesicle exocytosis? This could help explain the infrequency of observed continuities between cytoplasmic and surface membrane.

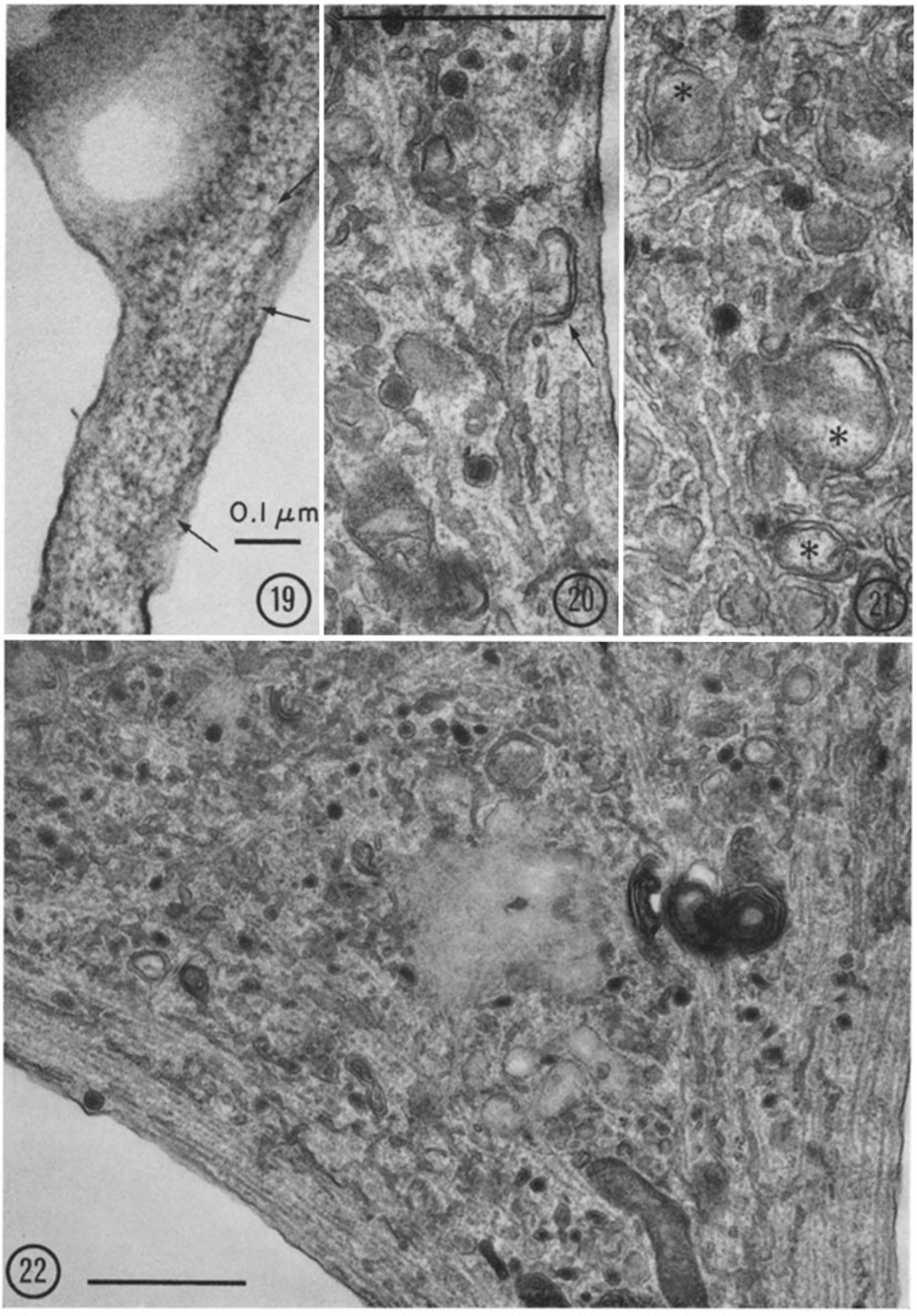
Contribution of cytoplasmic membranes to the surface membrane is certainly not without precedent. Secretory granules when leaving the cell lose their Golgi complex-derived encircling membrane to the surface membrane. A great deal of discussion now surrounds the fate of the synaptic vesicle membrane: does it, too, at least in some cases, fuse with the presynaptic surface mem-

FIGURE 19 Portion of a filopodium arising from area *a* of Fig. 16, neuron D. Within certain areas of the matrix material, polygonal structures may be discerned (arrows). Bar, 0.1 μm. × 103,000.

FIGURE 20 From the proximal portion of area *e*, neuron D. A C-shaped structure (arrow) in continuity with endoplasmic reticulum. This is considered to be an initial stage in the formation of autophagic vacuoles. Bar, 1 μm. × 40,000.

FIGURE 21 From area *f* of Fig. 16, neuron D. Three areas of cytoplasm (*) have been sequestered within endoplasmic reticulum. These configurations are interpreted as slightly later stages in autophagic vacuole formation than that shown in Fig. 20. × 43,000.

FIGURE 22 Neuron D. The area shown here is near the asterisk in Fig. 16; fiber *b* is beneath the figure and fiber *d* is out of the figure at the left. Numerous parallel microtubules correspond to the curvature of the surface. More interiorly they, along with a few neurofilaments, course in various directions. Bar, 1 μm. × 25,000.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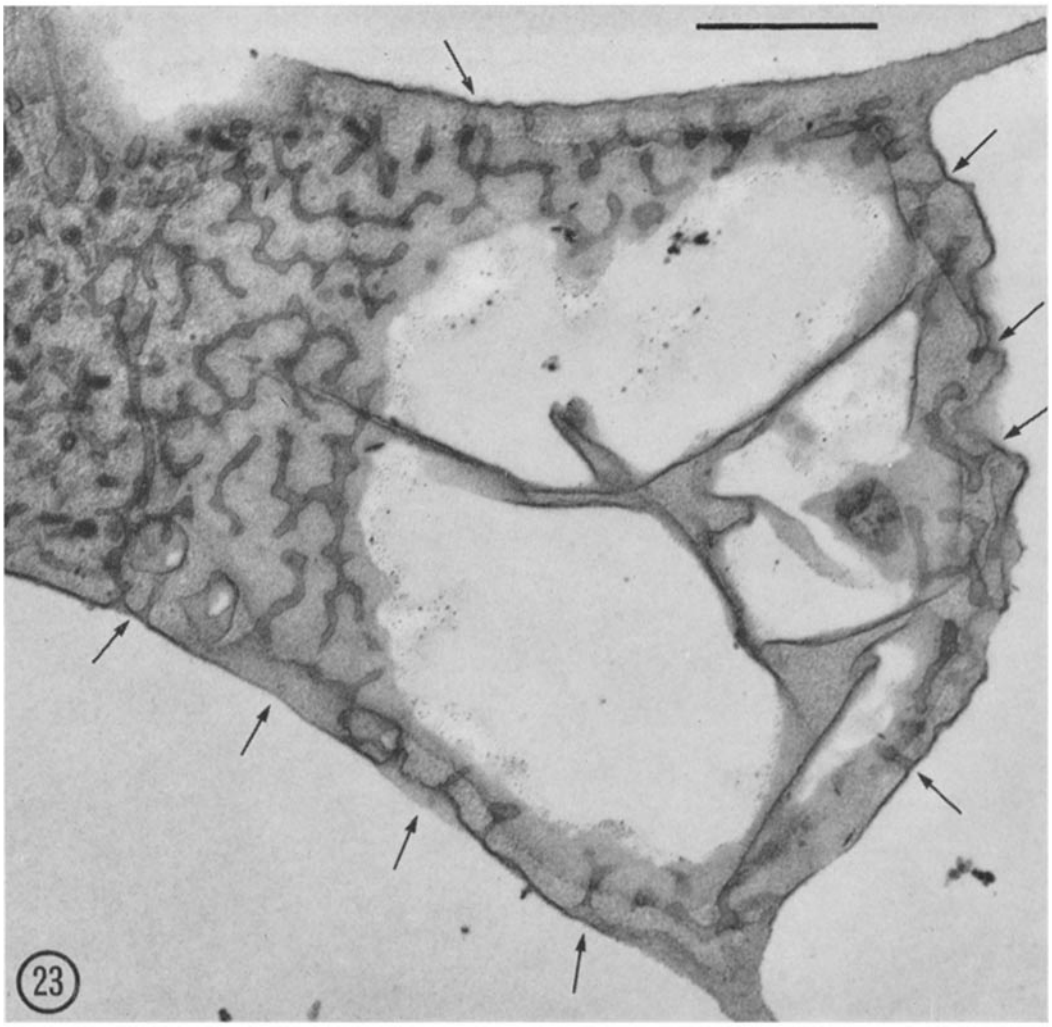

FIGURE 23 Spread-out area of a nerve fiber in the outgrowth from a ganglion piece. Several areas of continuity between cytoplasmic membrane and the surface membrane are indicated by arrows. Membranous folds and matrix material are visible near the center of the figure. Bar, 1 μm. × 25,000.

brane? Recent studies by Clark et al. (10) and Heuser and Reese (16) presented evidence that the membrane of synaptic vesicles fuses with the presynaptic membrane at frog neuromuscular junctions. Furthermore, Heuser and Reese showed that peroxidase was taken up by coated vesicles formed at the lateral surfaces of the axonal endings and subsequently appeared in agranular cisternae which they considered to be a source of new synaptic vesicles. Thus, cisternal membrane was implicated in recycling of surface membrane. It would seem, then, that some contribution of the conspicuous endoplasmic reticulum of the growing tip to surface membrane is a likely possibility but the mechanism by which one contributes to the other remains unknown.

DENSE-CORED VESICLE FORMATION: Dense material appeared within endoplasmic reticulum sacs and also budding from it along the fiber and in the tip regions of the cultured superior cervical ganglion neurons. From these observations it was concluded that dense-cored vesicles formed from reticulum in peripheral regions of the neuron rather than, or in addition to, being formed in

the Golgi region and thence transported to the tip. This is in complete accord with the conclusion reached by Teichberg et al. (37) who studied cultured embryonic chick sympathetic neurons. They reported that dense-cored vesicles were formed by budding from agranular reticulum in the nerve fiber as well as from Golgi-related membranous systems in the perikaryon.

The dense-cored vesicles observed in the present study varied greatly in internal density and diameter (650–1,600 Å) and presumably represent a heterogeneous group functionally. Dense-cored vesicles averaging 400 Å in diameter are considered to carry the biogenic amine transmitters; larger dense-cored vesicles (averaging 900 Å), ubiquitous in nervous tissue, may in certain regions be related to transmitter storage or metabolism but they differ from the smaller granulated vesicles in a number of staining reactions (reviewed in reference 31). Granules attaining a diameter of 1,200 Å or more have been regarded as neurosecretory (28). It should be noted that dense-cored vesicles were found in the growth cones of cultured *dorsal root ganglion* neurons by Yamada and co-workers (42). Recently, Pappas et al. (29) have raised the question of the role of dense-cored vesicles in cholinergic synaptic regions; they observed numerous dense-cored vesicles in nerve endings at motor end plates which had formed in rodent spinal cord-skeletal muscle long-term cultures. We are thus reminded of the question posed by Lentz (26) regarding the possibility of certain dense-cored vesicles serving as carriers of "trophic substances" during regeneration. In his study of nerves in regenerating forelimbs of adult newts, Lentz found vesicles, 1,000–1,100 Å in diameter and with interior density, which he considered to be different from the smaller 800 Å diameter dense-cored vesicles found normally; and ascribed to these larger elements the possible role of conveying trophic material.

DEGRADATIVE PROCESSES: In the cultured sympathetic nerve fibers and tips there are numerous structures considered to be members of the lysosomal family. These are C-shaped structures, often continuous with agranular reticulum cisternae (Fig. 20), single- or double-walled autophagic vacuoles, and dense bodies. Some of the autophagic vacuoles possess "tails" as shown in Fig. 12. All of these images suggest that, from the reticulum, cup- or goblet-like bodies form and eventually segregate an area of the cytoplasm with closure of the rim of the cup (Fig. 21). The tail (or stem of the goblet) would thus be a remnant of the sac from which the new body had been formed (see Fig. 18). This digestive vacuole may or may not have a single boundary, depending upon whether or not the cisternal membranes had fused. The cytoplasmic foci bounded by two membranes, described in the "Results" section of this paper, are probably in many cases developing autophagic vacuoles before fusion of the membranes of the parent cisternae. Subsequently, the autophagic vacuoles would take on the appearance of dense bodies with their contained vesicles and myelin figures (Fig. 18). Vesicles were sometimes noted within the newly forming cuplike bodies.

These various stages in lysosome formation, then, develop locally as well as in the perikaryon, the necessary enzymes presumably having been transported down the fiber via the reticulum (20). Holtzman and Peterson (21), studying mouse dorsal root ganglion in culture and rat adrenal medulla, found exogenous horseradish peroxidase within cuplike bodies (as well as coated vesicles and multivesicular bodies) and tentatively identified the cuplike structures as precursors of multivesicular bodies, a type of lysosomal body. Furthermore, similar findings obtained for perikarya and fibers, indicating to them uptake and lysis of proteinaceous material in many areas of the neuron.

In the extensions and particularly in the tips of the cultured sympathetic neurons there was an occasional image suggestive of cup-shaped body formation via a long neck (or tubule) from the *surface* membrane, as Holtzman and Dominitz (19) have proposed in the case of another cell type. If a cuplike structure were formed from invaginated surface membrane, a cross section of the cup would show cytoplasm encircled by a double membrane, a structure often seen near the plasmalemma (Fig. 6). If new fiber surface is assembled only at the developing tip a superfluity of membrane would develop in retracting areas such as area *e* in neuron A. Is the excess membrane removed by being taken into the cytoplasm as cup-shaped bodies which later acquire lysosomal enzymes and become autophagic vacuoles? It is even possible that the membrane components ending up in a myelin-figure type dense body are eventually sloughed off, for a few

examples of this type of body were observed protruding from the neuronal surface.

There are numerous instances, such as secretory activity, in which cell surface must increase and yet the cell does not continue to increase in size. The work of Heuser and Reese has been mentioned above: the addition of synaptic vesicle membrane to presynaptic membrane is considered to be balanced by coated vesicle formation elsewhere in the ending (16). In this case membrane is recirculated. However, there is evidence that in cells secreting their product by means of exocytosis, the resulting increase in surface membrane is balanced by increased formation of small pinocytotic vesicles from the surface membrane (reviewed in references 18, 3). The membrane taken in by this route would then enter the lysosomal system and be at least partially degraded.

In sum, then, we would expect to find mechanisms for both membrane assembly and breakdown (or recycling) at the nerve fiber tip. The present fine structural study suggests that there are candidates for both of these activities. The prominence of smooth membranous structures in various forms raises the question as to whether some of these contribute to the surface membrane of the growing tip. In thin membranous sheets (with folds, therefore ruffling?) continuities between cytoplasmic and surface membrane were seen. In these as well as other spread-out areas, the cytoplasmic membranes assumed a highly distinctive array near the plasmalemma. Various stages of presumed lysosomal bodies (cup-shaped bodies, autophagic vacuoles, and dense bodies) were observed in the growth cone. It is concluded that the formation of lytic bodies occurs in the fiber tip and suggested that they play a role in surface membrane breakdown.

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