ULTRASTRUCTURE AND FUNCTION OF GROWTH CONES AND AXONS OF CULTURED NERVE CELLS

KENNETH M. YAMADA, BRIAN S. SPOONER,
and NORMAN K. WESSELLS

From the Department of Biological Sciences, Stanford University, Stanford, California 94305

ABSTRACT

Dorsal root ganglion nerve cells undergoing axon elongation in vitro have been analyzed ultrastructurally. The growth cone at the axonal tip contains smooth endoplasmic reticulum, vesicles, neurofilaments, occasional microtubules, and a network of 50-A in diameter microfilaments. The filamentous network fills the periphery of the growth cone and is the only structure found in microspikes. Elements of the network are oriented parallel to the axis of microspikes, but exhibit little orientation in the growth cone. Cytochalasin B causes rounding up of growth cones, retraction of microspikes, and cessation of axon elongation. The latter biological effect correlates with an ultrastructural alteration in the filamentous network of growth cones and microspikes. No other organelle appears to be affected by the drug. Removal of cytochalasin allows reinitiation of growth cone–microspike activity, and elongation begins anew. Such recovery will occur in the presence of the protein synthesis inhibitor cycloheximide, and in the absence of exogenous nerve growth factor. The neurofilaments and microtubules of axons are regularly spaced. Fine filaments indistinguishable from those in the growth cone interconnect neurofilaments, vesicles, microtubules, and plasma membrane. This filamentous network could provide the structural basis for the initiation of lateral microspikes and perhaps of collateral axons, besides playing a role in axonal transport.

INTRODUCTION

Electron microscope descriptions of structures thought to be growth cones[1] have been reported for the developing nerve cells of monkey spinal cord (Bodian, 1966), rat cerebellum (del Cerro and Snider, 1968), and rabbit dorsal root ganglia (Tennyson, 1970). Due to the complex tissue architecture of even embryonic nervous system in vivo, unequivocal identification and study have been difficult. Growth cones in vivo have been identified by their bulbous shapes ("varicosities"),

and were found to contain large quantities of smooth endoplasmic reticulum and vesicles. Although Bodian (1966) and del Cerro and Snider (1968) found no other organelles in growth cones of 44–47 day fetal monkey spinal cord and 1–12 day postnatal rat cerebellum, Tennyson (1970) reported finding microtubules, neurofilaments, mitochondria, and other organelles in the growth cones of 11–12-day fetal rabbit dorsal root neuroblasts; in addition, long, thin processes containing "a finely filamentous matrix material" extended outward from such growth cones. These processes were thought to correspond to the filopodia (microspikes and flat membranous extensions)

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[1] For purposes of clarity, "growth cone" refers to the conical enlargment at the tip of elongating axons, from which microspikes or membranous structures may extend.

which extend from growth cones of nerve cells in culture (Harrison, 1910; Nakai and Kawasaki, 1959; Pomerat et al., 1967).

Chick embryo dorsal root ganglia can be dissociated and the cells grown in vitro (Nakai, 1956; Scott et al., 1969; Yamada et al., 1970). Such cells develop axons, tipped with growth cones and microspikes, and elongate on a plastic substratum free from contacts with any cells. The growth cones of these cells can be identified unequivocally by both phase-contrast and electron microscopy, undistorted by the numerous tissue contacts which occur in vivo. Yamada et al. (1970) have reported in preliminary form that such growth cones and microspikes contain a filamentous network, the integrity of which is essential to axon elongation. That conclusion was based on experiments involving cytochalasin B, a drug that disrupts contractile microfilament systems involved in cytokinesis (Schroeder, 1969) and epithelial morphogenesis (Spooner and Wessells, 1970; Wrenn and Wessells, 1970). In this paper, a more complete description of normal, cytochalasin-, and colchicine-treated nerve cells is provided, and the contribution of various organelles to axon elongation is discussed.

MATERIALS AND METHODS

Cultures

Lumbosacral dorsal root ganglia from 8-day White Leghorn chick embryos were dissociated with 0.25% trypsin as described previously (Yamada et al., 1970). The culture medium consisted of modified medium F-12 supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.; Spooner, 1970) and nerve growth factor, present as a 1:300,000 homogenate (15–20 strokes of a loose-fitting Dounce homogenizer) of submandibular glands from a highly inbred line of BALB/c adult male mice (Cohen, 1960). 2 ml of cell suspension containing approximately 2×10^5 cells was added to each 35-mm in diameter plastic Petri-style culture dish, (Falcon Plastic Division of B-D Laboratories, Inc., Los Angeles, Calif.), and incubated at 37°C in a humidified 5% CO_2 incubator for 18–25 hr. Whole dorsal root ganglion explants were cultured as described previously (Yamada et al., 1970).

Drugs

Cytochalasin stock solution contained 1 mg of cytochalasin B (Carter, 1967) per ml of dimethyl-sulfoxide (Matheson, Coleman, and Bell, Cincinnati, Ohio). In order to add this drug to a culture, approximately 1.5 ml of culture medium was removed and mixed with a sample of the drug stock solution; the mixture was then gently pipetted back into the culture dish, resulting in a final cytochalasin concentration of 1, 7, or 10 μg/ml of medium. Control cultures were left untouched, or a sample of medium was removed and replaced, or a sample was removed, mixed with dimethylsulfoxide (1% final concentration) and returned to the dish. For "recovery" experiments, cultures were incubated in 1, 7, or 10 μg/ml of cytochalasin for 18 hr; the cultures were washed five times with normal cytochalasin-free medium and reincubated.

In experiments using cycloheximide (Actidione, Upjohn Co., Kalamazoo, Mich.) to inhibit protein synthesis, the drug was present in all rinses and in culture medium at a concentration of 20 μg/ml $(7 \times 10^{-5} \text{ M})$. Colchicine (Calbiochem, Los Angeles, Calif.) was used at 1 μg/ml $(2.5 \times 10^{-6} \text{ M})$ for 18 hr before fixation for electron microscopy. Both colchicine and cycloheximide stock solutions were made up in Hanks' balanced salt solution.

Electron Microscopy

To fix a culture, approximately 1.5 ml of medium was removed from the dish, and 2–3 ml of warm (37°C) 2.5% glutaraldehyde with paraformaldehyde (Sorenson's 0.07 M phosphate buffer, pH 7.4; 30 min) was added gently, following previously described methods (Wrenn and Wessells, 1969). The cultures were postfixed in osmium tetroxide (1% in Veronal-acetate, pH 7.4, 1 hr, 4°C), and embedded in Epon directly in the dish. After polymerization at 60°C for 3 days, the resulting Epon discs were pried away from the bottom of the culture dish. Cell areas were selected with an inverted microscope, and blocks were cut and mounted on Epon blanks with sealing wax (Spooner, 1970). The first 1–2 μ of the block were thin-sectioned parallel to the plane of the original culture-dish substratum (i.e., also parallel to the plane of axon elongation) using a Sorvall MT-2 ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn.). The sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined with a Hitachi HU-11E electron microscope.

Axonal cross-sections were obtained from whole dorsal root ganglion explants, cultured and prepared for electron microscopy as described previously (Yamada et al., 1970). The "nearest neighbor" distance between microtubules or neurofilaments was the center-to-center distance between each such structure and its nearest neighbor.

Radioautography

Cell cultures were labelled with leucine-^{3}H (New England Nuclear Corp., Boston, Mass.; 58.2 Ci/mmole; 1 μCi/ml of medium; 2 or 4 hr, 37°C). The culture medium was then aspirated gently, and the cultures were fixed in formalin-glacial acetic acid–70% ethanol (1:1:18) for 30 min. The fixed cells were rinsed five times in 70% ethanol over an 18 hr period to remove unincorporated label, then rinsed twice in distilled water. Liquid emulsion (Ilford K5, Ilford Ltd., Ilford, Essex, England) was was melted and diluted 1:1 with distilled water, pipetted onto the fixed cells, and swirled several times. Excess emulsion was poured off, and the dishes were drained and allowed to dry overnight at room temperature. They were then stored 4–8 days at 5°C in a light-tight box containing a desiccant. The emulsion was developed for exactly 2 min in D19 developer (Kodak), rinsed in water, and fixed. Silver grains were counted over nerve cell bodies (round, very phase refractile, often with eccentric nucleus and two prominent nucleoli, clearly free of glial cells, and possessing an axon).

RESULTS

The tips of elongating axons consist of expanded regions, the growth cones, from which varying numbers of long, thin microspike processes extend outward (Fig. 1, a–c). The continual movement of these microspikes can be observed by phase-contrast microscopy. A typical microspike might extend from the leading edge of a growth cone over a period of 30–60 sec, attaining a length of 10–20 μ, swing erratically from side to side for 1–5 min covering an arc of 10° in 1–4 sec, and retract in 30–60 sec. In addition to microspikes, flat, thin membranous structures also occasionally expand and retract from the advancing growth cone. Such observations are in general agreement with cinematographic descriptions of growth cone activity in vitro by Hughes (1953), Nakai and Kawasaki (1959), and Pomerat et al. (1967).

Ultrastructurally, the growth cone is characterized by large amounts of smooth endoplasmic reticulum, several classes of vesicles, neurofilaments, occasional microtubules (neurotubules), and by a peripheral filamentous network which extends into the microspikes (Figs. 2–7). The smooth endoplasmic reticulum in the growth cone is located centrally, and often forms interconnecting channels containing an electron-lucent material (Figs. 2, 3, 5 a). Many of the nearby vesicles are bounded by single membranes and resemble portions of smooth endoplasmic reticulum. Other

vesicles or granules contain an electron-opaque material, and are similar to dense-core granules described by Nathaniel and Nathaniel (1966) and others in synaptic endings. Finally, typical "coated" vesicles (Roth and Porter, 1964), very similar to pinocytotic vesicles pinching off from the plasma membrane (Figs. 3, 4; see also Pomerat et al., 1967), are found both at the surface and within the growth cone (Fig. 3). The smooth endoplasmic reticulum and the three types of vesicles are concentrated toward the center of the growth cone, while a filamentous network constitutes the periphery.

At the core of each growth cone, surrounded by the vesicles and smooth endoplasmic reticulum, is often a cluster of neurofilaments (Figs. 2, 3), a continuation of the neurofilaments that extend down the center of the axon. Such neurofilaments average 100 A in diameter and have frequent "sidearms" (Wuerker and Palay, 1969). The sidearms are 40–70 A wide, up to 150 A long, and are oriented at varying angles to the neurofilament axis (most often at right angles). The sidearms are often spaced periodically along the neurofilament axis at intervals of 200–250 A, although distances between adjacent sidearms may vary from 200 A to 450 A or more.

Most axonal microtubules terminate near the point where the axon expands into the growth cone. Some microtubules, however, protrude into the growth cone, passing through or just peripheral to the central mass of smooth endoplasmic reticulum (Fig. 3). In no instance have microtubules been seen to approach close to the plasma membrane of the growth cone or to its microspikes.

Instead, the only organelle found consistently in the region beneath the growth cone plasma membrane and within microspikes is a network of filaments 40–60 A in diameter. These "microfilaments" are interconnected and form a complex meshwork of polygonal structures (Figs. 2–7). All other organelles are excluded from the peripheral growth cone and microspikes, except for the occasional presence of (a) dense or coated vesicles, and of (b) smooth-walled vesicles that may have originated from the Golgi apparatus or, more directly, from the smooth endoplasmic reticulum in the growth cone (Figs. 2, 3). In a few cases, the latter vesicles are even seen within the core of microspikes, a location consistent with the hypothesis that they may be involved in formation or degradation of microspike plasma membrane.
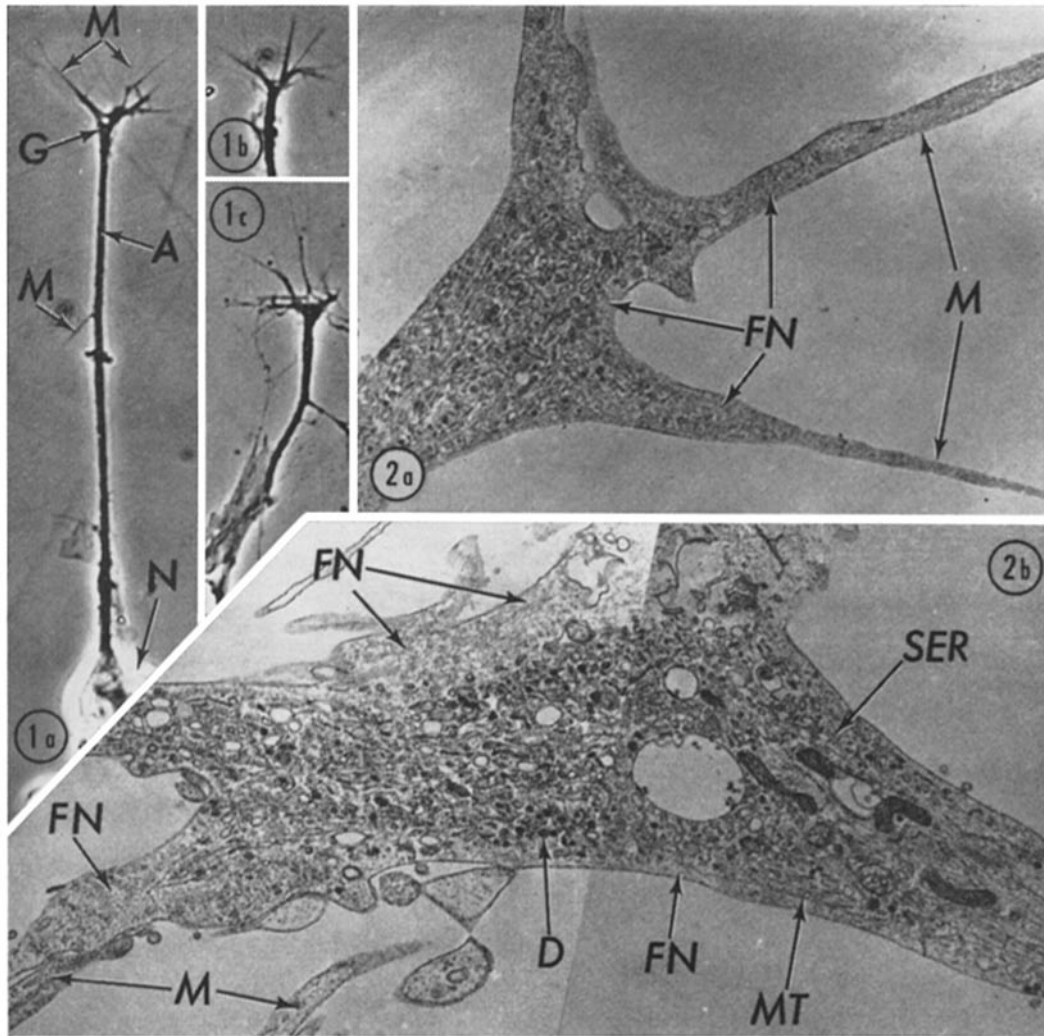
FIGURE 1 Phase-contrast micrographs of dorsal root ganglion cell growth cones and axons. Growth cone, *G*; microspikes, *M*; axon, *A*; nerve cell body, *N*. × 580.

FIGURES 2 *a* and 2 *b* Survey views of axonal growth cones. Microspikes (*M*) and the peripheral portions of growth cones are filled with the filamentous network (*FN*). Smooth endoplasmic reticulum, *SER*; microtubules, *MT*; dense granules, *D*. Fig. 2 *a*, × 12,000; Fig. 2 *b*, × 12,800.

The pattern of the polygonal network in the growth cone itself shows few consistent asymmetries of shape (Figs. 3–5). Thus, in places of a typical thin section where the network is in a plane which permits observation of the angular relationships between filamentous elements of the network, the "polygons" composing the network are generally symmetrical. Furthermore, few regions of oriented sets of polygons are discerned. A common irregularity in pattern, and one that is seen in most growth cones, consists of localized, dense, closely spaced portions of the meshwork (Figs. 4–6).

In contrast, the polygonal meshwork within microspikes and, occasionally, in the area of growth cone near their bases shows consistent asymmetries in pattern (Figs. 3, 6, 7). Such orientation of the meshwork, parenthetically, is not consistent with the idea that the whole network is a randomly arranged fixation artifact. In virtually every microspike examined in detail (52/56), re-
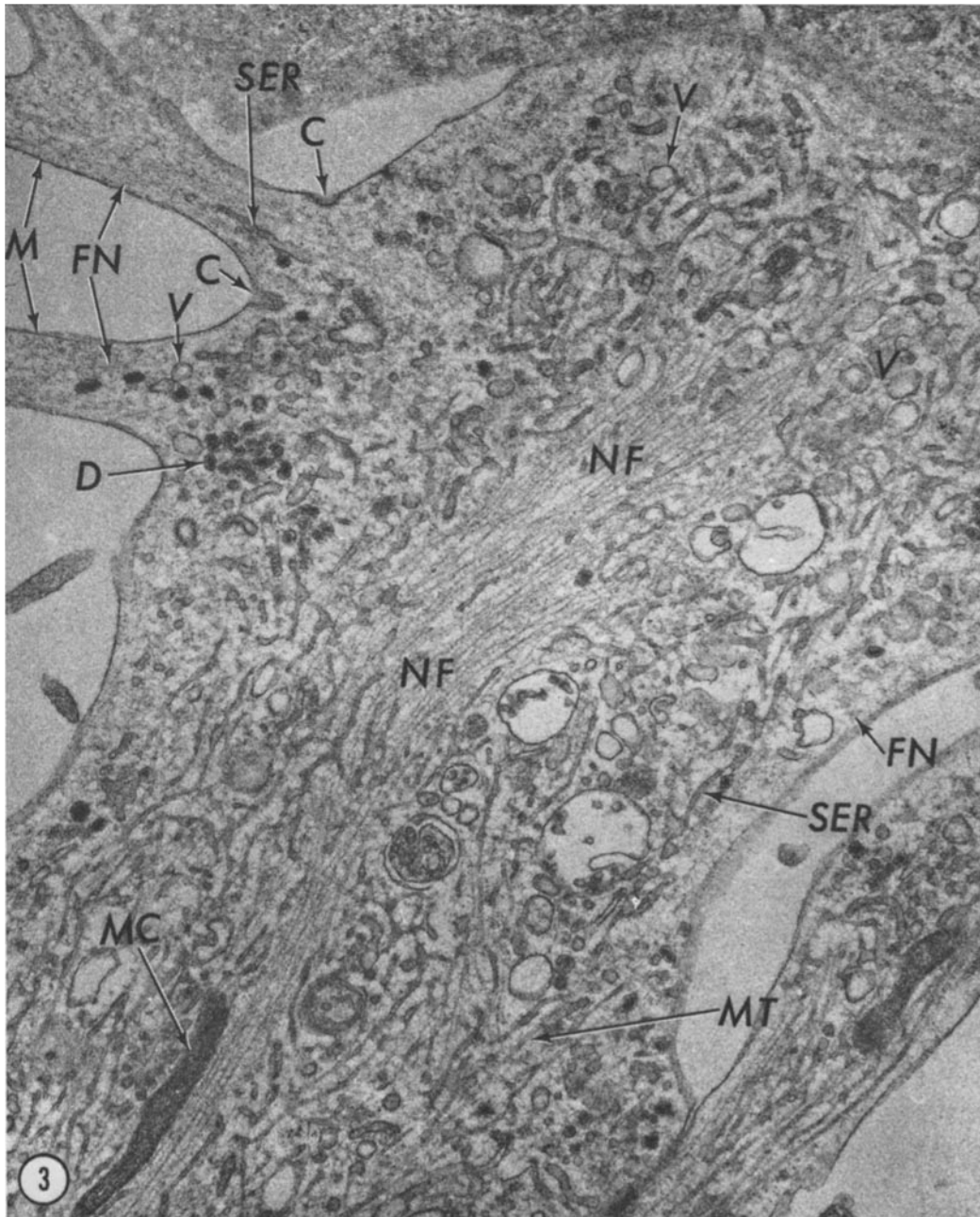
FIGURE 3   A dorsal root ganglion cell growth cone. Note that the filamentous network (*FN*) is the predominant organelle within the microspikes (*M*), that smooth endoplasmic reticulum (*SER*) extends into the base of one microspike, and that coated vesicles (*C*) arise near its base. A bundle of neurofilaments (*NF*) occupies the core of the growth cone, and few microtubules (*MT*) extend into this region of the nerve cell. Near the top of this photograph, the growth cone abuts against a glial cell. Smooth-walled vesicles, *V*; dense-core granules, *D*; mitochondrion, *MC*. × 13,500.

gions of polygons in the network are elongated so as to possess a long axis parallel to the axis of the microspike. The lateral microfilament "sides" of such polygons are closer to each other than are those of the growth cone polygons. Such orientation, when viewed in situations where a series of similarly elongated polygons stretches down the microspike, gives the impression, on cursory examination, of long "filaments" running down the microspike. This, in fact, is probably not the case, since careful examination invariably reveals that the "filaments" are discontinuous, and that the observer's eye is actually jumping from one polygon side to the next as the "filaments" are traced along the microspike.

As indicated above, the polygonal meshwork is also occasionally oriented or more densely packed in the growth cone regions near the base of microspikes. These areas can be best described after discussing the relationship of the filamentous network to the plasma membrane. Along the edge of the growth cone and the microspikes, individual filaments of the network approach the plasma membrane and appear to insert on its inner aspect (Figs. 4–6). In favorable sections a series of such points of insertion are located some 200–300 A apart along the membrane. Particularly at the base of a microspike, where the radius of curvature of the plasma membrane is small, the insertion points may appear to be more closely spaced. The adjacent portion of the polygonal network is consequently deformed, with the result that polygons appear narrower and more tightly packed than elsewhere in the growth cone.

Besides extending outward from the leading edge of active growth cones, microspikes also protrude laterally from axons. Nakai (1956), Pomerat et al. (1967), and others have reported that microspikes can form, wave about, and disappear at any point along an elongating axon of a nerve cell in vitro. Ultrastructurally, such lateral microspikes are identical with those protruding from growth cones: they contain a microfilamentous network in which asymmetric polygons are oriented with their long axis parallel to the long axis of the microspike (Figs. 8–10). Small vesicles resembling those seen in the vicinity of the smooth endoplasmic reticulum of the growth cone may also be found in the center of such microspikes (similar vesicles are also seen within the axon per se; see below). The base of these lateral microspikes is usually a broad mound, the predominant internal organelle of which is the filamentous network.

In the axon beneath the microspike mound, a microtubule or several microtubules may bend laterally toward the base of the microspike. The filament network has been observed to insert upon such microtubules, and they may function in some sense as an anchoring point for the lateral structure. These microtubules are almost certainly part of the normal axonal neurotubular systme, since they can be traced for varying distances proximal and distal from the base of the microspike, just as can be done with microtubules at any point along the axon. In rare cases, a single microtubule has been observed to extend out into a long lateral microspike (Fig. 10). Such microspikes contain the usual filamentous network, and may have a bulbous ending containing small vesicles similar to those thought to originate from smooth endoplasmic reticulum. If one or a few microtubules are sufficient to act as stabilizing skeletal elements, then these rarely observed cases may represent an early phase of collateral axon formation. Thus, initially a lateral microspike may form, and later may become stabilized by acquisition of microtubules; elongation of the collateral could then occur if the expanded bulbous portion began to function as a separate growth cone system, complete with vesicles, filamentous network, and its own subsidiary microspikes.

Axon Ultrastructure

The axons of dorsal root ganglion nerve cells in vitro are long, straight cylinders containing numerous 240–280-A in diameter neurotubules and 90–110-A in diameter neurofilaments oriented parallel to the axonal axis (Fig. 11). In cross-sections of axons (obtained from whole ganglionic explants), the 100-A neurofilaments are usually in a bundle, often more to the center of the axon, while the neurotubules are often more peripheral (Fig. 15; see also Fig. 11). The neurofilaments and neurotubules are regularly spaced: the nearest-neighbor distance for neurofilaments is approximately 420 A, and for neurotubules is 750 A (Fig. 16). Long, thin mitochondria, sacs of smooth endoplasmic reticulum, and vesicles are oriented along the axis of axons (Fig. 11). Occasional ribosomal clusters, but no rough endoplasmic reticulum, are found in axons.

Sidearms or filamentous side connections are
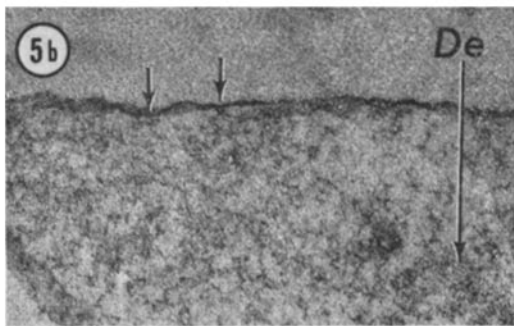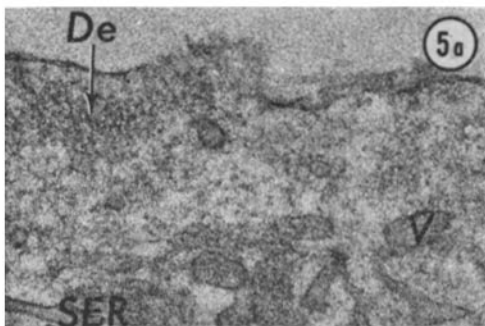
observed on axonal neurofilaments, microtubules, and vesicles (Figs. 9, 11–15). Sidearms (Wuerker and Palay, 1969) are spaced at intervals of about 200–250 A along neurofilaments, and individually are about 40–70 A in width and of variable length, depending on the distance between adjacent neurofilaments. Sidearms, or perhaps connecting links with the filamentous network of the axon, also occur along microtubules at intervals of roughly 200 A. Individual filaments of the mesh-work are often seen to approach the microtubule surface at right angles. In places, long, branching portions of the network arise from a single point on the microtubule surface (Fig. 12). Whether such cases represent the exception or the rule is difficult to decide, since the plane of section through the cylindrical axon would be critical in visualizing the structure of the proposed three-dimensional filamentous network. Filamentous sidearms are often seen to connect the surface of vesicles or pieces of smooth endoplasmic reticulum with the microtubules and neurofilaments of the axon (Figs. 9, 11–13, 15). Finally, a narrow zone of filamentous network lies between microtubules and the plasma membrane of the axon (Figs. 9, 11, 12); this network is indistinguishable from the polygonal network of the growth cone.

Cytochalasin Effects on Nerve Cells

Cytochalasin application results in "wilting" and shortening of microspikes, which subsequently retract into the axonal tip. Initial wilting occurs in 1–3 min and growth cones are often completely "rounded up" within 10 min. Proximally directed "peristaltic" waves along the axon may lead to eventual retraction of *some* of the axons. Of greater interest are the axonal tips that round up but do not retract: such nerves cease elongating and remain of constant length until cytochalasin is removed from the culture medium (Yamada et al., 1970; Fig. 22).

When viewed with the electron microscope, retracting microspikes are twisted, short, and broad in comparison to controls. The filamentous network within these microspikes is no longer oriented, but instead appears denser and disoriented (Yamada et al., 1970). In the growth cones which have rounded up and completely retracted all microspikes, the peripheral filamentous network likewise appears unusually densely packed (Figs. 17 *a*, 17 *b*, 18, 21). These dense regions are hard to distinguish from the small, localized dense areas seen on occasion in normal untreated growth cones (see above, Fig. 5).

Lateral microspikes respond similarly to cytochalasin—they appear curved, short, and broad, and their filamentous network loses its orientation. Eventually all that remains of a lateral microspike is a mound of densely packed filamentous material without orientation. In places, both within the rounded axonal tip and along the sides of axons, areas of polygons with normal spacing can be found. Nevertheless, the predominant condition suggests that cytochalasin causes a collapse or contraction of the polygonal network. This structural alteration correlates with the cessation of growth cone and microspike function, and with the halt of axonal elongation.

Electron microscope observations indicate that cytochalasin is not cytotoxic (see also Carter, 1967). The microtubular system (Figs. 19, 20), the neurofilament sytem (Figs. 19, 20), the smooth endoplasmic reticulum (Figs. 17 *a*, 19, 21), and other organelles of the axon and cell body seem structurally unaeffcted by the drug for as long as 16 hr of continuous treatment with 10 µg/ml of cytochalasin. Sidearms and cross-connections are still seen on the microtubules, neurofilaments, and vesicles within the axon.

Furthermore, incorporation of labeled amino acids into hot acid-insoluble material in organ cultures of whole dorsal root ganglia was previously reported (Yamada et al., 1970) to con-

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Figure 4   The filamentous network at the front of a growth cone. Note the polygons of various sizes (arrows), the dense regions (*De*) of the network, and the points where elements of the network approach the inner surface of the plasma membrane (broad arrows). X 115,000.

Figures 5 *a* and 5 *b*   Views of the filamentous network of growth cones showing densities (*De*), and proximity of the network to the plasma membrane. Minute round (spherical?) bodies (arrows) sometimes occur at sites where the network appears to insert on plasma membrane *SER*, smooth endoplasmic reticulum; *V*, smooth-walled vesicle. Fig. 5 *a*, X 67,000; Fig 5 *b*, X 54,000.
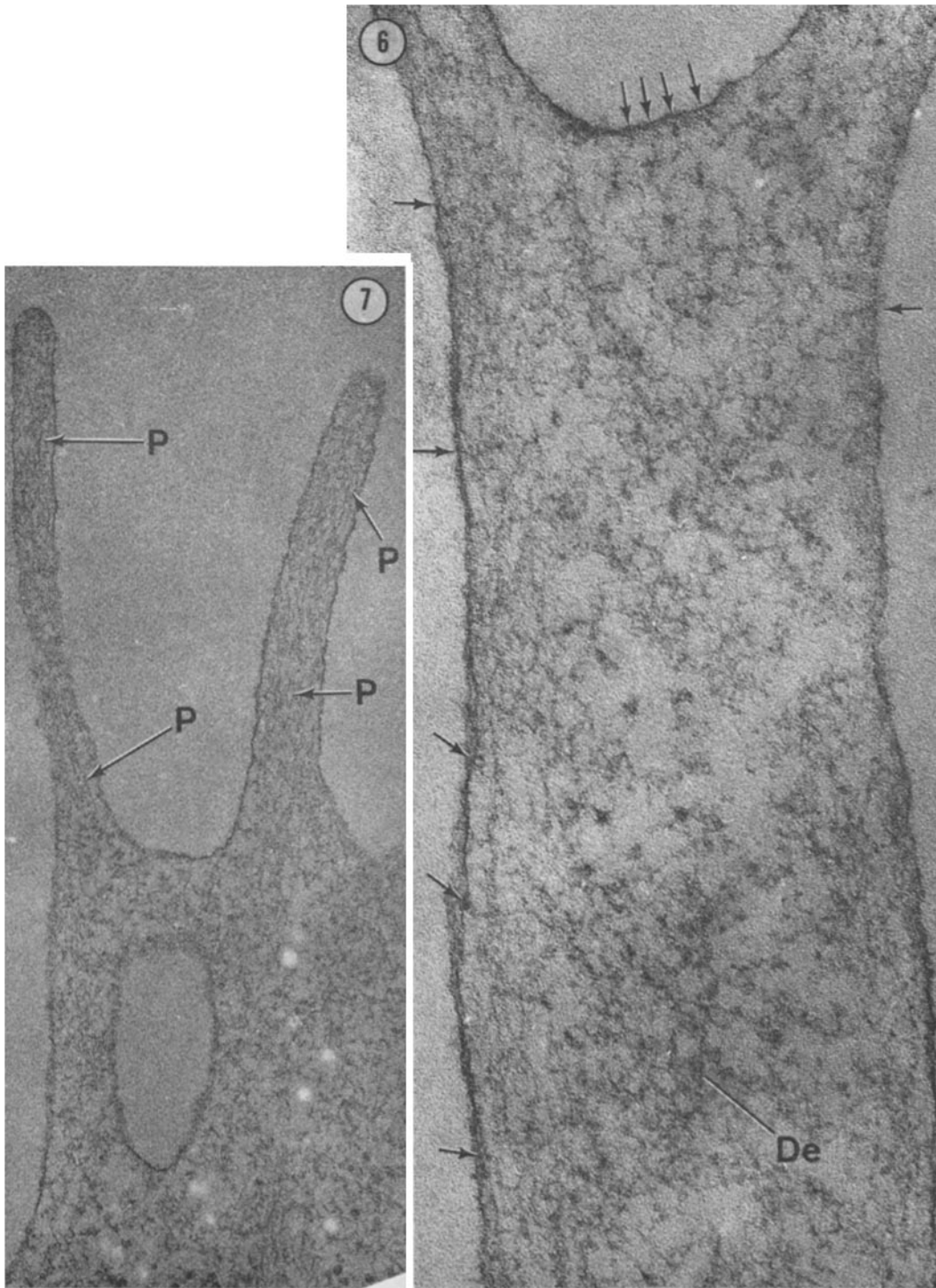
FIGURE 6 The base of a broad microspike showing the filamentous network. Note the asymmetric pattern of polygons on the left and at the lower right. A density (*De*) is present, and the network approaches the plasma membrane at many sites (arrows). Note the absence of microtubules and other organelles from this broad cellular extension. × 129,000.

FIGURE 7 The asymmetric pattern of polygons (*P*) is seen in these microspikes, whereas a more symmetrical pattern is found at their bases. × 55,000.
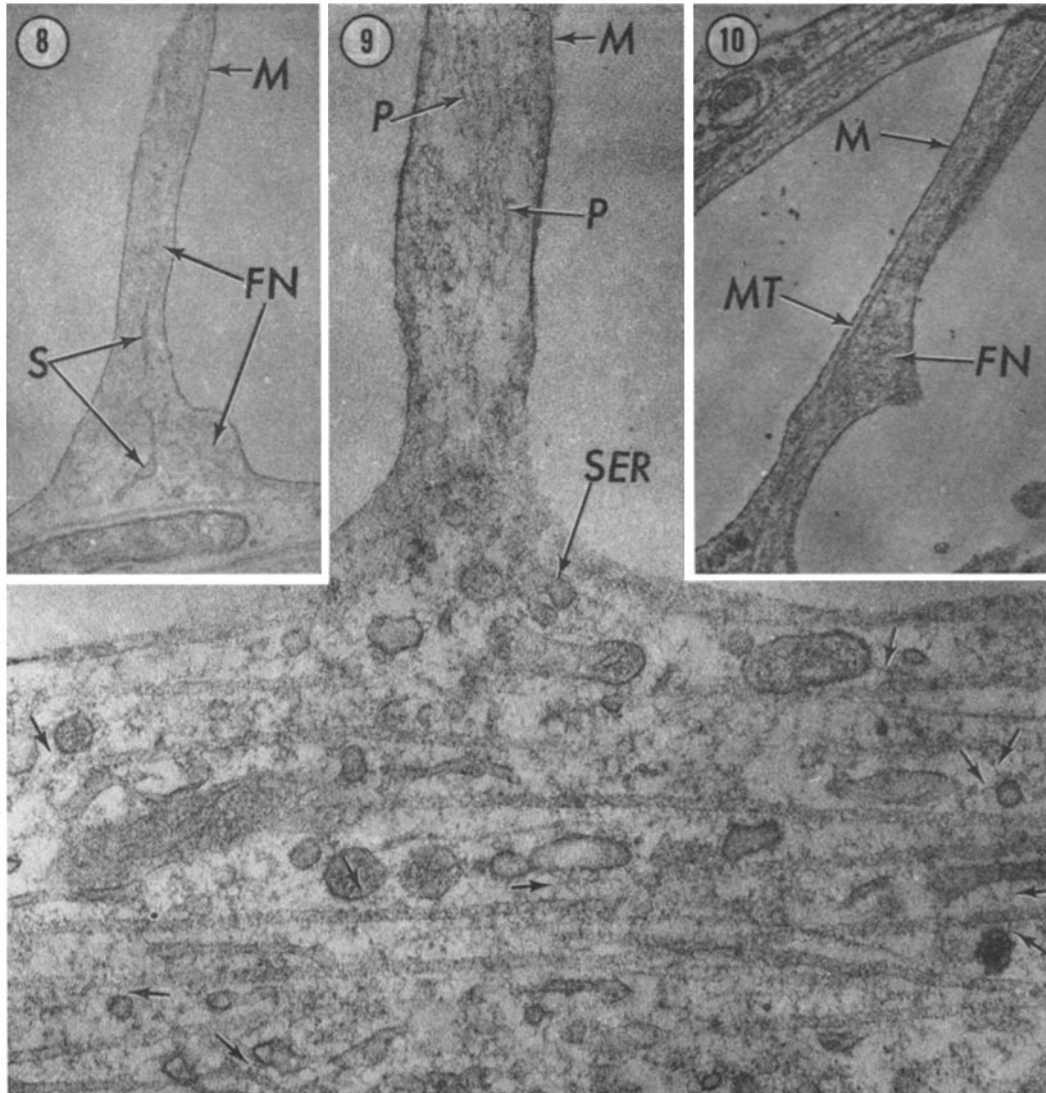
FIGURE 8 A lateral microspike (*M*) located on the side of an axon. The expanded basal mound and the spike itself are filled with filamentous network (*FN*), and a small extension of the smooth endoplasmic reticulum (*S*). × 35,000.

FIGURE 9 A lateral microspike (*M*) showing asymmetric polygons (*P*) of the filamentous network and a small accumulation of smooth endoplasmic reticulum (*SER*) near the base. Filamentous connections (arrows) between the vesicles and longitudinal elements of the axon, and between neurofilaments and microtubules, are present. × 69,000.

FIGURE 10 A thick lateral microspike (*M*) containing a microtubule (*MT*). Other lateral microspikes may have expanded ends filled with vesicles. These states may represent early stages of collateral axon development. *FN*, filamentous network. × 18,500.
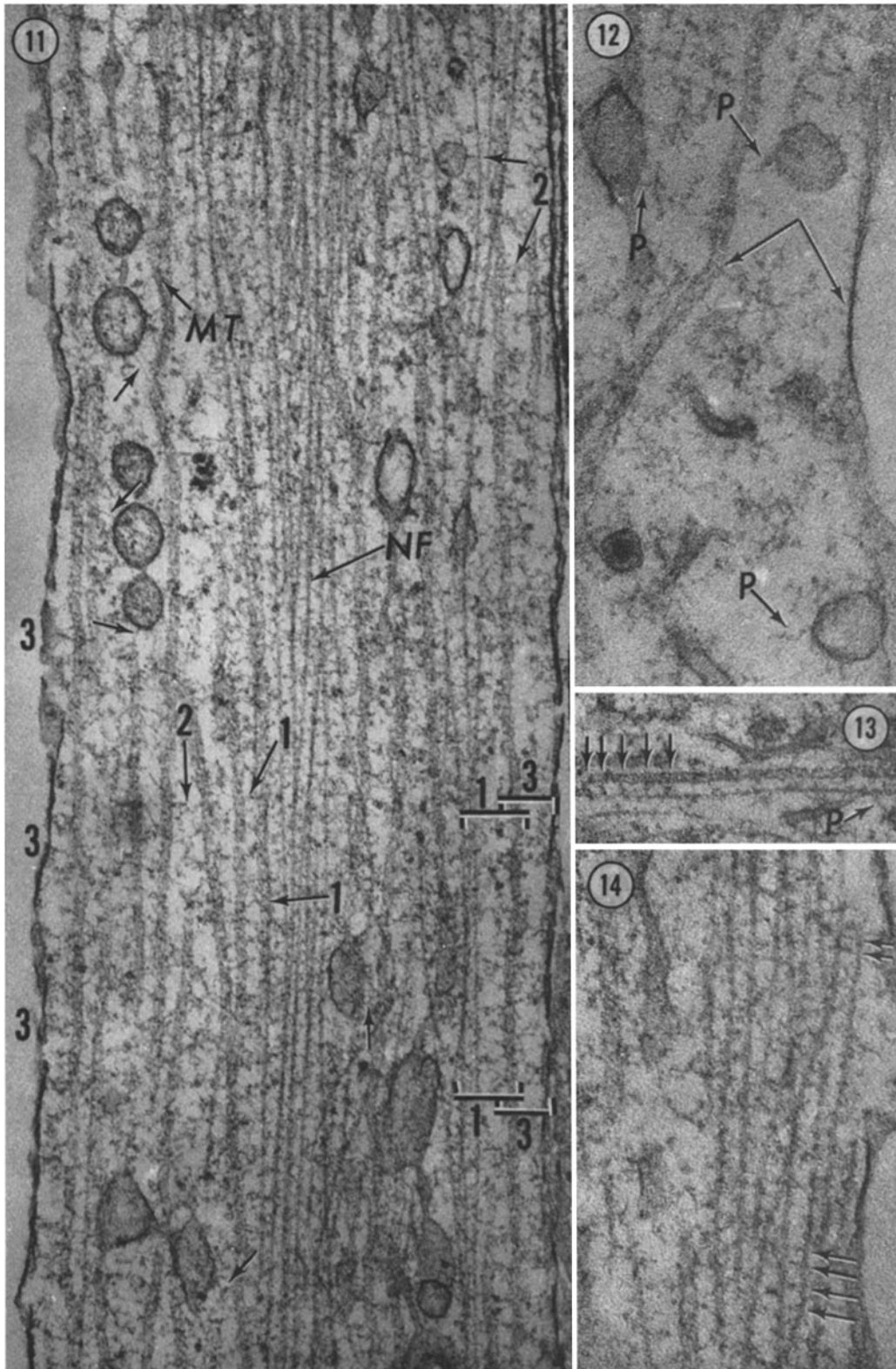
tinue, although at a decreased rate (60–80% of controls). However, radioautographic analysis of leucine-^{3}H incorporation into isolated single nerve cells in cell culture reveals no significant decrease in the number of grains per cytochalasin-treated cell *versus* control cells (Table I). That these counts are indeed representative of the rate of protein synthesis is suggested by the elimination of 96% of the grains in nerve cell populations cultured in the presence of the protein synthesis inhibitor cycloheximide (Table II).

Recovery from Cytochalasin

2–3 hr after removal of 7 μg/ml of cytochalasin B from cultures (1–2 hr after removal of 1 μg/ml and 2–4 hr after removal of 10 μg/ml), growth cones reinitiate activity (Fig. 22). Axons elongate at 15–40 μ/hr, similar to the 20–40 μ/hr measured for untreated axons. Early in the recovery period, many elongating axons exhibit complex, bizarre growth cones consisting of convoluted or multiple undulating membranes (Fig. 22, *d*). Such complex growth cones may exhibit very few microspikes, suggesting that microspikes per se are not necessary for growth cone function and elongation, although the many flat, narrow processes protruding from the undulating membranes may be their deranged analogues. Microspikes reappear on the growth cones by 3–5 hr, although usually reduced in number when compared with controls.

If the recovery from cytochalasin is allowed to proceed in the presence of 7×10^{-5} M cycloheximide, growth cone activity begins and axon elongation proceeds normally, in some cases for over 24 hr (Fig. 22). Such recovery is analogous to the recovery of undulating membrane activity

and locomotion in glial cells treated with cytochalasin and allowed to recover in cycloheximide (Spooner et al., 1971). Axons elongating in the presence of cycloheximide become extremely thin and spindly, suggesting that elongation continued even though contribution of material to fill the axon was reduced. Radioautography indicates that cycloheximide reduces nerve cell incorporation of leucine-^{3}H during a pulse from 0 to 4 hr after removal of cytochalasin by 96% (Table II).

Finally, if recovery from cytochalasin is allowed to proceed in the absence of nerve growth factor in the rinsing and incubation media, normal recovery of growth cone–microspike activity takes place, and elongation continues for over 24 hr (Fig. 22). Thus exogenous nerve growth factor is not necessary for normal recovery from cytochalasin and axonal elongation for at least a day.

Ultrastructure of Colchicine-Treated Cells

Colchicine was utilized in a previous study (Yamada et al., 1970) to analyze the role of microtubules in axon elongation. Nerve cells fixed after 18 hr in colchicine (2.5×10^{-6} M) contain virtually no microtubules (Fig. 23). Instead, the cell body, as well as infrequent cylindrical structures interpreted to be residual stumps of incompletely retracted axons, contains wide swaths of tangled 100-A filaments, similar to those reported in colchicine-treated nerve cells and axons in vivo (Bunge and Bunge, 1968; Wisniewski et al., 1968).

DISCUSSION

The examination of axon endings on neurons cultured as single cells on a flat substratum has elimi-

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FIGURE 11 A portion of a dorsal root ganglion cell axon. Note that the neurofilaments (*NF*) are at least as "straight" as the supposed skeletal microtubules (*MT*). Sidearms and links with the filamentous meshwork can be seen in many places, so that links between neurofilaments and microtubules (*1*), between microtubules (*2*), and between microtubules and plasma membrane (*3*) are frequent. In addition, links between the vesicles and sacs of smooth endoplasmic reticulum and the longitudinal structures are evident (arrows). $\times$ 51,000.

FIGURE 12 A very thin section of an axon, showing a portion of the filamentous network (arrows) running from the plasma membrane to a microtubule. Note the filamentous projections (*P*) from vesicles. $\times$ 120,000.

FIGURE 13 A favorable view of possible linkage (arrows) between a neurofilament and a microtubule. Note the filamentous projection (*P*) extending from the vesicle to the neurofilament. $\times$ 100,000.

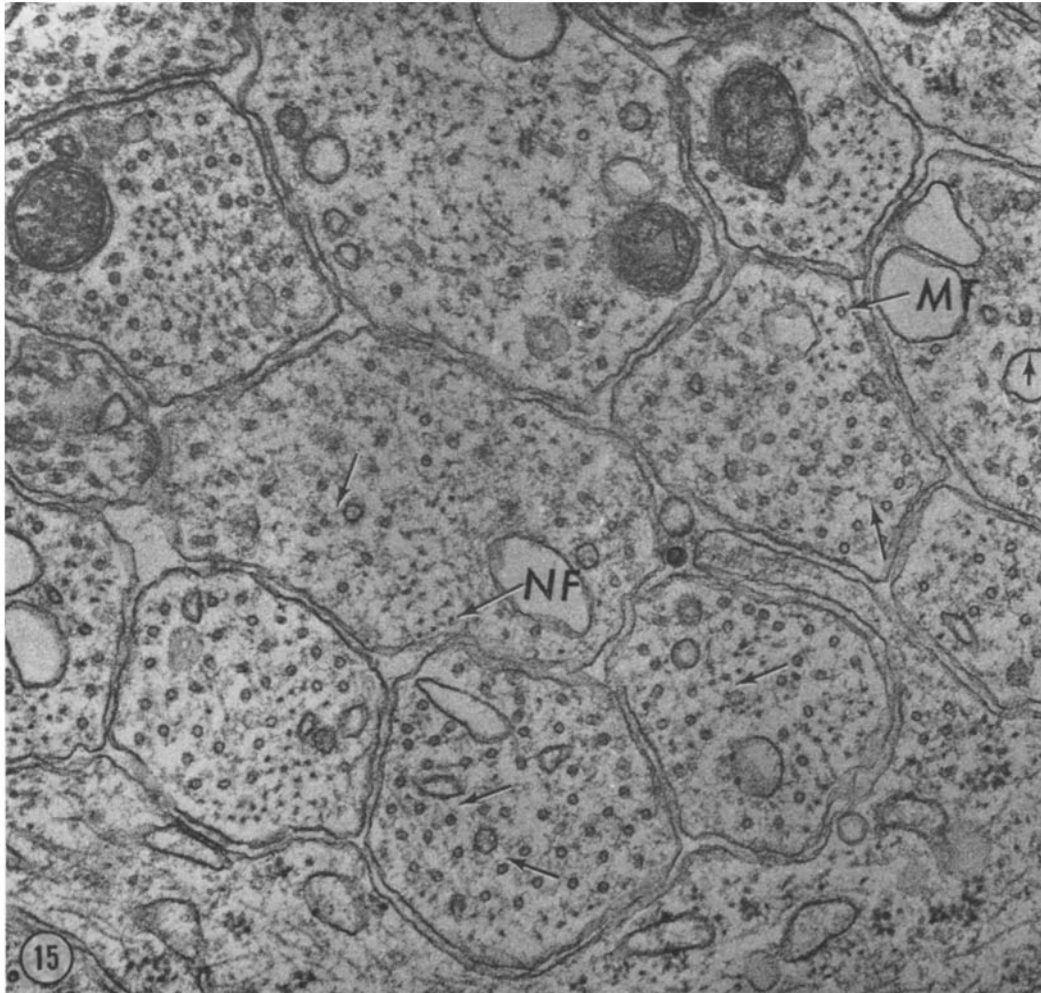FIGURE 14 Sidearms (arrows) of neurofilaments. $\times$ 125,000.

Figure 15 A cross-section of axons near the edge of an intact dorsal root ganglion grown in organ culture in the presence of nerve growth factor. Neurofilaments (*NF*) tend to be organized into bundles, and are regularly spaced. Microtubules (*MT*) are also spaced regularly, and show evidence of being interconnected with the filamentous network (arrows); in addition, the various vesicles are often seen to be connected with that network. × 52,000.

nated the uncertainties of growth cone identification inherent in in vivo studies, thus facilitating ultrastructural analysis. In light of the fine structure observed in such growth cones, it is of interest to attempt to explain the following phenomena: (*a*) microspikes continually arise from the growth cone, wave about over the substratum, and retract (Harrison, 1910; Pomerat et al., 1967); (*b*) the leading edge of the growth cone functions as an undulating membrane locomotory organelle; and (*c*) as the apparent site of axon elongation (Bray,

1970), the growth cone may be involved in deposition of new plasma membrane for the elongating axonal cylinder. Similarly, the fine structure of the axon may be related to: (*a*) axonal transport, (*b*) the structural integrity of the axon, and (*c*) the production of lateral microspikes and collateral axons. In discussing these processes, organelles of particular interest include the Golgi apparatus, smooth endoplasmic reticulum, vesicles, microtubules, neurofilaments, and the filamentous meshwork of growth cone and axon.

Golgi Apparatus, Smooth Endoplasmic Reticulum, and Vesicles

Embryonic nerve cells undergoing elongation in vitro, either in whole dorsal root ganglion explants, or as isolated single cells upon a substratum, contain an extensive Golgi complex in the cell body, from which lines of vesicles extend into the axon hillock (Fig. 24). At any point along the axon itself, similar vesicles, as well as elongated membranous sacs, are scattered among the microtubules or in the region between the microtubules and the central neurofilament bundle (Fig. 11). The arrangement of vesicles, sacs, and mitochondria in lines along the axis of the axon suggests the presence of "corridors" along which such structures are transported (see Martinez and Friede, 1970). In growth cones, a large accumulation of vesicles and membranous sacs occurs; the latter structures are indistinguishable from smooth endoplasmic reticulum of the cell body. In view of Bray's observations (1970), suggesting that new surface materials of an elongating axon are added in the region of the growth cone, it seems reasonable to assumed that vesicles and membranous sacs originate from the Golgi complex of the cell body and are transported down the axon to the growth cone, where they accumulate and serve as a source of new axonal membrane.
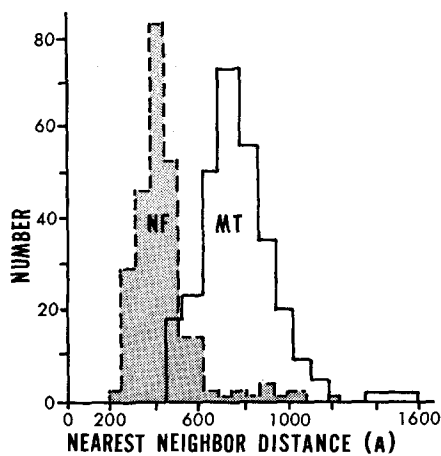

FIGURE 16 The distribution of nearest-neighbor distances for neurofilaments (NF) and for microtubules (MT). Note the different distributions of these axonal structures; the mean of nearest-neighbor distances of neurofilaments is 420 A ± 130 A (SD), and of microtubules is 750 A ± 170 A.

Microtubules (Neurotubules)

The numerous microtubules in the axon are regularly spaced, encircling a central bundle of neurofilaments. The microtubular system may be thought of as a skeletal system that is necessary but not sufficient for axonal elongation (Yamada et al., 1970). Disruption of microtubules with colchicine resulted in axonal retraction, only secondarily affecting growth cone activity. Thus, microspikes and growth cones continued functioning in colchicine until colchicine-induced axonal instability and retraction pulled the axon tips free of the substratum. Continued function of the growth cone as an undulating-membrane locomotory organelle is directly analogous to the continued undulating membrane activity and locomotion of cultured glial cells and fibroblasts treated with colchicine (Spooner et al., 1971). It seems reasonable to conclude that the microtubular systems of axons provide skeletal support for the extremely long, thin, straight, and unsupported (by other cells) axons of cultured embryonic nerve cells. A similar role for microtubules has been proposed, from indirect evidence, for axons in vivo (Porter, 1966; Peters and Vaughn, 1967; Lyser, 1968).

The microtubular system may also play a role in axonal transport (e.g., of vesicles). The observation that fast transport in mature axons in vivo is sensitive to colchicine (Kreutzberg, 1969; Dahlström, 1969) is consistent with this hypothesis. In addition, the observation that fine filaments connect vesicles with microtubules and neurofilaments in elongating axons suggests the possibility that such filaments are involved in propelling vesicles along an axon (see also Schmitt, 1969). Similar short "sidearm" and longer filamentous connections between microtubules and vesicles are seen in lamprey central nervous cells (Smith et al., 1970), and strong arguments are presented that such connecting links and microtubules are involved in transport of vesicles. In fact, contractility of such fine filaments could account for the bidirectional movement of particles along elongating axons in vitro at rates of 5 μ/min or faster (Hughes, 1953; Nakai, 1956; Pomerat et al. 1967).

Neurofilaments

Perhaps the greatest enigma of the axon and growth cone is the function of the neurofilaments (see Smith et al., 1970 for literature and discussion). It has been suggested that 100-A neurofilaments

are products of microtubule dissociation (Peters and Vaughn, 1967), or even artifacts of fixation (Metuzals, 1969). However, Schmitt (1968, 1969) considers neurofilaments to be unique organelles, possibly involved in axonal transport. Neurofilaments are exceedingly straight and well defined in axons grown on a flat substratum in vitro (Figs. 11, 19). Sidearms and longer thin filaments are regularly spaced along neurofilaments, possibly connecting them together to form bundles, or even connecting them with microtubules and vesicles. The presence of neurofilaments in the core of the growth cone may be consistent with the idea that neurofilaments are involved in transport of materials along the axon into the growth cone.

Growth Cone Filamentous Network

The filamentous network of growth cones and microspikes can be thought of as the organelle responsible for locomotory behavior. The growth cone itself appears to function as an undulating membrane (Hughes, 1953; Abercrombie, 1961), analogous to the undulating membrane of glial cells (Spooner et al., 1971) and fibroblasts.[2] In all such cases, a microfilamentous meshwork inserts upon the plasma membrane. Cytochalasin B causes rounding up of growth cones, microspike retraction, and cessation of axon elongation, while its application to glial cells or to fibroblasts results in an inward collapse of undulating membrane and cell margin, and a halt in cell movement (Carter, 1967; Spooner et al., 1971).[2] Ultrastructurally, the meshwork of growth cones, of glial cells, and of fibroblasts is altered after cytochalasin treatment. All of these systems recover normal locomotory function when cytochalasin is removed, even if protein synthesis is inhibited. Furthermore, the locomotory systems are initially unaffected by col-

chicine (although axon retraction secondarily disrupts growth cones). Ultrastructurally, the growth cone–microspike filamentous network cannot be examined following colchicine treatment because of axon retraction; however, the network in the functional undulating membrane of glial cells remains intact even after 18 hr of colchicine treatment. This evidence indicates that the growth cone functions as an undulating membrane and that the filamentous meshwork plays a key role in that function. Since cytochalasin affects 50-A filament systems thought to be contractile (Wessells et al., 1971), the filamentous network of growth cones, and of other undulating membranes, may also be contractile organelles (see also Spooner et al., 1971). Contractility of the network could account for formation and movement of microspikes as follows. Contraction of a circular zone of the meshwork around a point on the surface of the growth cone could result in formation of a cylindrical microspike (accompanied by the appropriate membrane adjustments which must occur whether or not this hypothesis is correct). Thus, contractility pulling the lateral sides of polygons toward one another could generate the elongated polygons observed in microspikes; furthermore, since the polygonal meshwork inserts on the plasma membrane, such a contraction would cause narrowing of the presumptive microspike into a cylindrical structure.

Contraction or relaxation of the meshwork at one side of the base of a newly formed microspike could then result in lateral movement of the whole microspike; thus, the microspike would move from side to side as a stiff rod, bending only at the base, just as is observed (Nakai and Kawasaki, 1959; Taylor, 1966).

Axonal Filamentous Network

The presence of a filamentous meshwork along the sides of the axon relates on one hand to the ability of all such regions to form lateral micro-

[2] Taylor, E., and N. K. Wessells. 1971. Microfilaments and heart fibroblast locomotion. Manuscript in preparation.

FIGURES 17 a and 17 b. Two cytochalasin-treated growth cones that have rounded up and retracted their microspikes. The filamentous network (FN) appears unusually dense throughout the growth cone cytoplasm. Smooth endoplasmic reticulum, SER; dense core granules, D. × 54,000.

FIGURE 18 Another view of the network in a cytochalasin-treated growth cone; as in the other cases, the filamentous network (FN) appears more densely packed, but not completely dispersed by the drug. × 62,000.
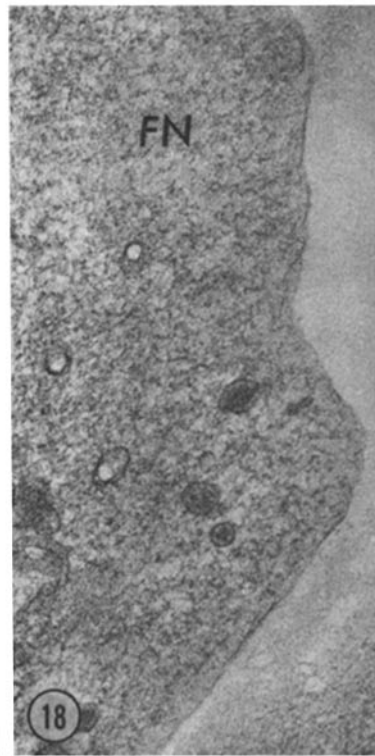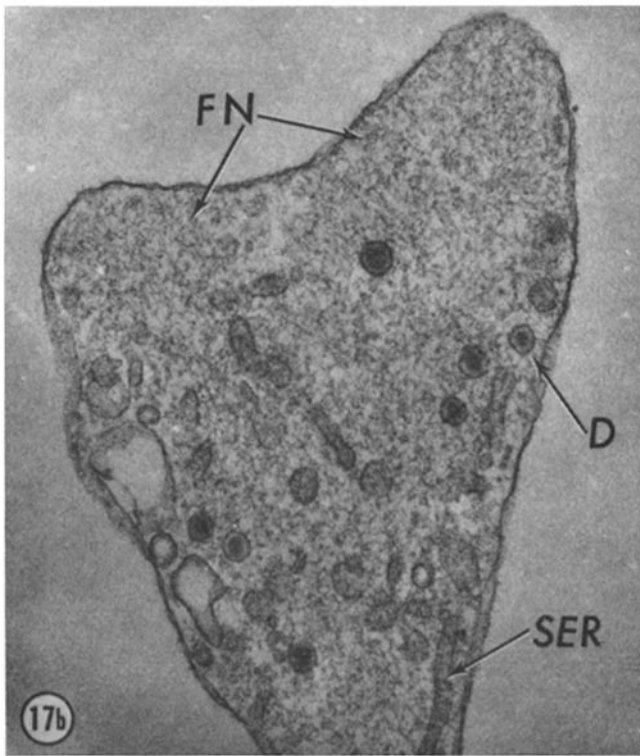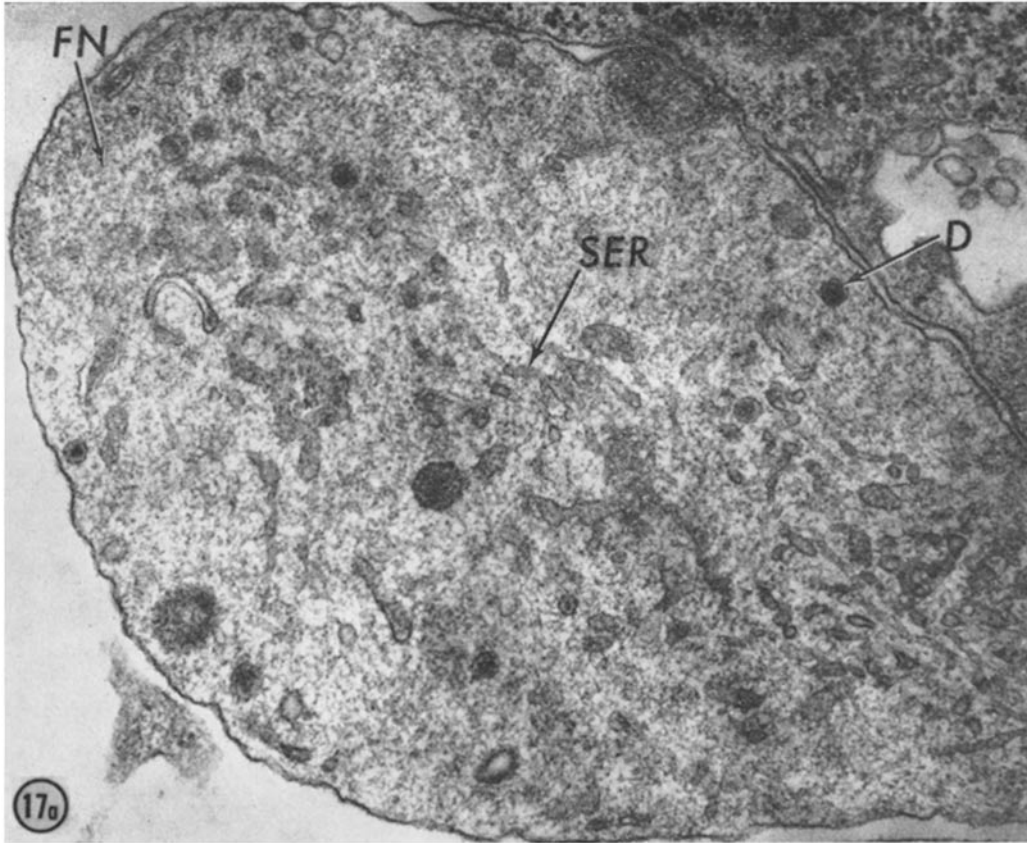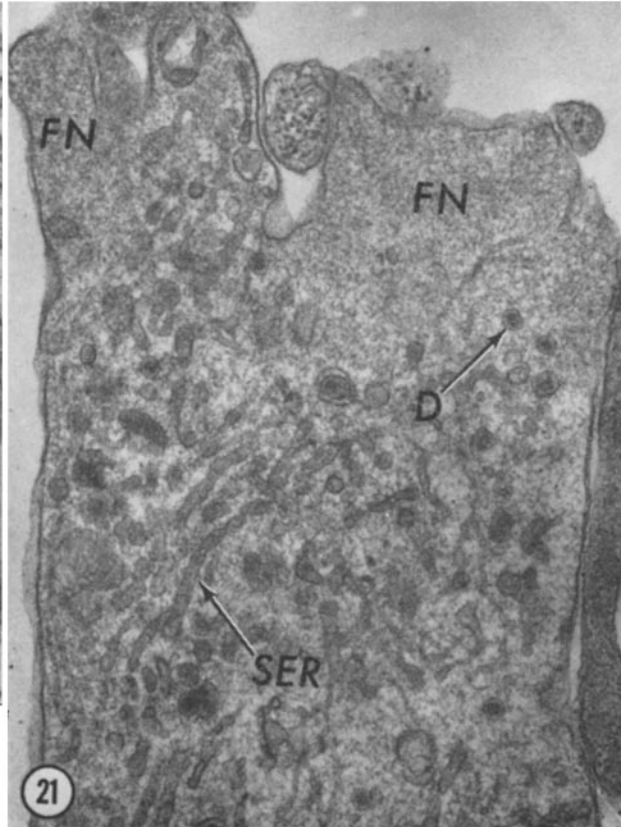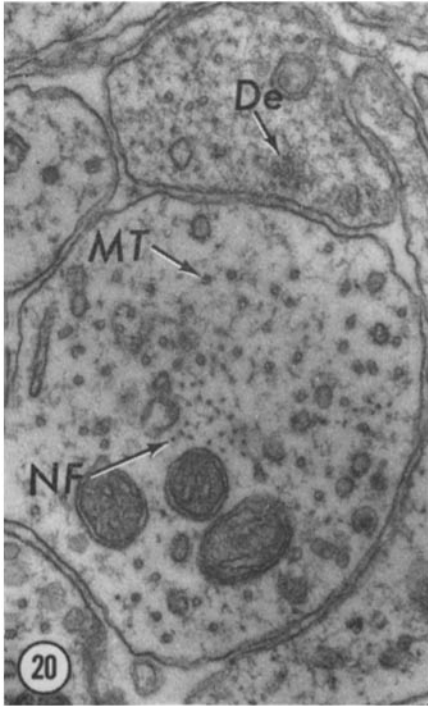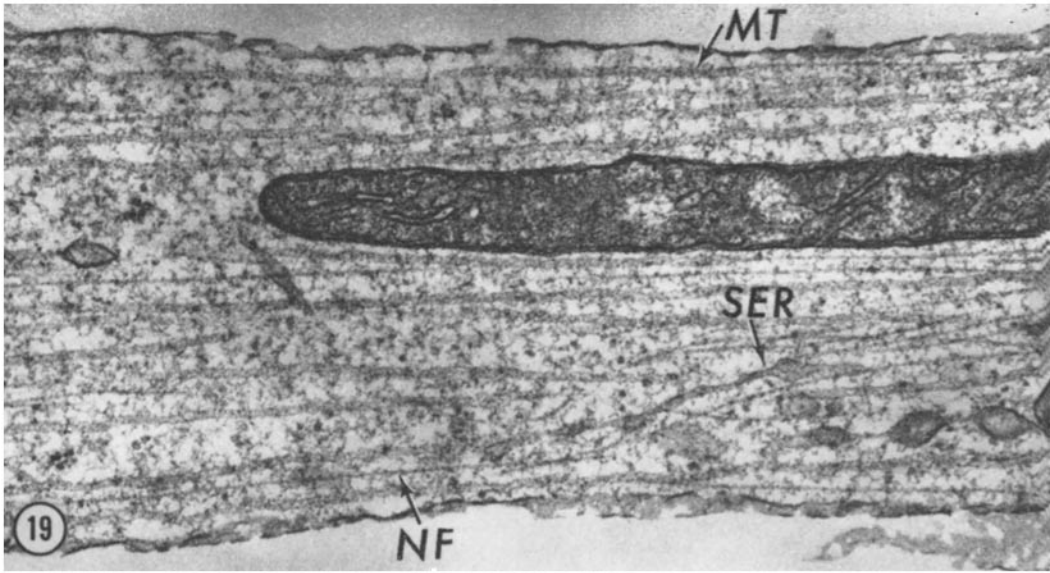
TABLE I		
Leucine-^{3}H Incorporation into Control and Cytochalasin-Treated Nerve Cells		
	Control	Cytochalasin
Grains per cell	41.6 ± 11.1	43.5 ± 13.6

Dorsal root ganglion cells were cultured 18 hr, then half of the cultures received 7 μg/ml of cytochalasin; controls were left untouched. After incubation for another 18 hr, the cells were labeled for 2 hr in leucine-^{3}H in the respective media. Silver grains were counted over isolated nerve cells, and expressed as mean ± 1 SD; number was 200 in each case.

TABLE II		
Leucine-^{3}H Incorporation into Control and Cycloheximide-Treated Nerve Cells during Recovery from Cytochalasin Treatment		
	Control	Cycloheximide
Grains per cell	41.6 ± 10.3	1.7 ± 1.3

Dorsal root ganglion cells were cultured 18 hr, then all cultures recived 7 μg/ml of cytochalasin for 18 more hr. After drug removal, the cells were incubated 4 hr in leucine-^{3}H in normal or cycloheximide-containing medium. Mean ± 1 SD; number was 100 for control, 200 for cycloheximide.

spikes, and on the other to the hypothesized linkage of the plasma membrane to the skeletal elements of the axon by a contractile or a structural web. The proposed capability of the filamentous network lining the axon and growth cone to participate in formation of microspikes, and ultimately to generate axon collaterals with new growth cones, is analogous to the capability of the filamentous network at the periphery of glial cells to participate in formation of new undulating membranes, changing the direction of cellular locomotion (Spooner et al., 1971).

Sidearms or thin filamentous side connections similar to those found on axonal neurofilaments, microtubules, and vesicles have been previously described on rat anterior horn cell axon neurofilaments (Wuerker and Palay, 1969), on rabbit dorsal root neuroblast axon microtubules (Tennyson, 1970), between *Haemanthus* and HeLa cell mitotic spindle microtubules and vesicles (Hepler et al., 1970), and surrounding coated vesicles

(Roth and Porter, 1964; Kanaseki and Kadota, 1969). Wuerker and Palay (1969) have suggested the possible existence of a web of threadlike "filamentous material" in anterior horn cell axons, as have Metuzals and Izzard (1969) in squid giant axons, as well as Yamada et al. (1970) in elongating axons of cultured embryonic sensory ganglion cells. A ruthenium red-staining material (suggesting acid mucopolysaccharides) has been demonstrated around axonal microtubules, neurofilaments, and membranes (Tani and Ametani, 1970), as well as under plasma membrane at sites of microfilament insertion in cleaving marine eggs (Szollosi, 1970). We propose that axonal microtubles, neurofilaments, vesicles, and plasma membrane are interconnected by a complex three-dimensional filamentous network or lattice which may insert on these organelles either via their sidearms or into a material so far defined only as staining with ruthenium red. If this axonal filamentous network is contractile, it might be involved in axonal transport.

FIGURE 19 A longitudinal section through an axon cultured in the presence of cytochalasin. Microtubules (*MT*), neurofilaments (*NF*), and vesicular components of the axon appear as in control axons. Sidearms or filamentous connecting links between the various organelles can be seen to resemble those in control axons. *SER*, smooth endoplasmic reticulum. × 46,000.

FIGURE 20 A cross-section near the periphery of a cultured dorsal root ganglion that had been treated with cytochalasin for 16 hr. Cross-sections of microtubules (*MT*), neurofilaments (*NF*), and vesicular structures are seen. Note the region (*De*) that resembles the dense filamentous network found in growth cones after cytochalasin treatment. × 47,000.

FIGURE 21 A growth cone of a cytochalasin-treated cell showing extensive smooth endoplasmic reticulum (*SER*), dense-core granules (*D*), and other vesicles. The dense filamentous material (*FN*) characteristic of treated cells is also seen. × 54,000.
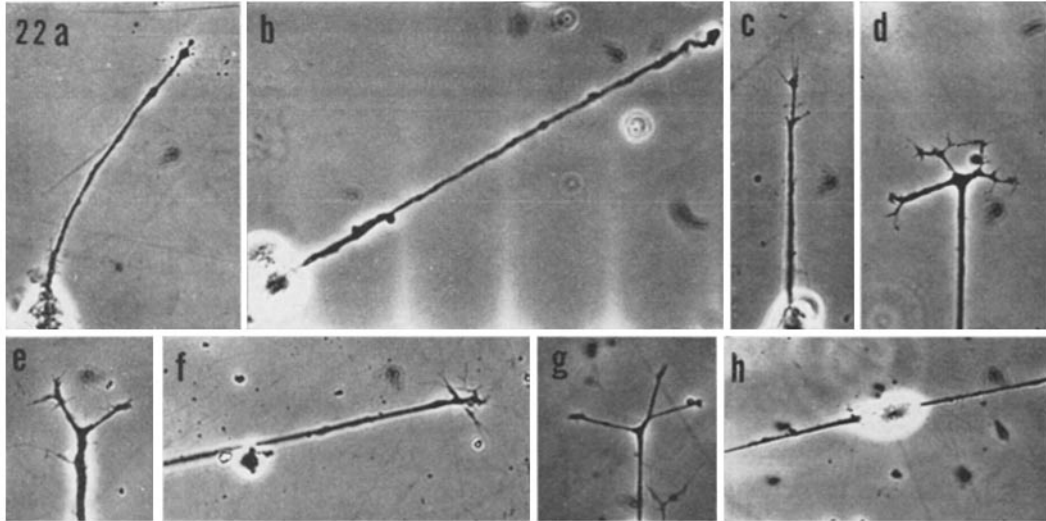
FIGURE 22, a–h (a, b) Dorsal root ganglion nerve cells cultured 18 hr, then treated for 18 hr with 7 μg/ml of cytochalasin B. Note that the axons remain, but that their bulbous ends are rounded and without microspikes. (c, d, e) Recovery from cytochalasin, 3 hr after drug removal. Cells were cultured for 18 hr, treated with 7 μg/ml of cytochalsin for 18 hr, then washed free of the drug. Note the reappearance of functional growth cones and microspikes; the axonal ending in (d) is especially complex.

(f) Elongating axon 25 hr after recovery from cytochalasin with nerve growth factor absent from the medium. The growth cone and microspike appear normal.

(g, h) Growth cones and axons of cells cultured in cycloheximide for 25 hr after removal of cytochalasin. Note the thin, spindly appearance of the axons. Fig. 22, × 450.

A Model for Axon Elongation

The following speculative summary seeks to present a unified scheme for axon elongation (see Fig. 25).

Membranous vesicles and sacs arise in the Golgi apparatus, are transported along the axon, and accumulate in the growth cone. They then fuse with the plasma membrane, serving as the source of new membrane for axonal elongation.

The axon itself is supported by a skeleton of microtubules, surrounding a bundle of neurofilaments. The microtubules and neurofilaments are regularly spaced by interconnecting sidearms or filamentous meshwork. Transport of vesicles or other materials may result from interactions with the meshwork or the sidearms.

Finally, the leading edge of the growth cone functions as an undulating membrane to produce locomotion. Microspikes may function in movement per se (Weiss, 1941), or as tactile (Nakai and Kawasaki, 1959), or chemical (Sperry, 1963) probes of the environment as the growth cone advances. In its triple role as locomotory organelle, as a site of deposition of new surface material for the elongating axon, and as a source of microspikes, the growth cone becomes the key to axon elongation.

FIGURES 23 a, 23 b, and 23 c Nerve cells treated with colchicine for 18 hr. Fig. 23 a, densely packed 100-A filaments (F), with intermixed vesicles and mitochondria. × 7500. Fig. 23 b, interwoven swath of 100-A filaments (F) adjacent to the nucleus. Note how the bands of 100-A filaments exclude other organelles. × 7500. Fig. 23 c, part of a circular cross-section of cytoplasm similar to that in Fig. 23 a. × 45,000.

FIGURE 24 A string of vesicles (arrows) extending from the Golgi apparatus into the axonal hillock. Such vesicles may be the source of the smooth-walled vesicles found all along the axon. × 19,000.
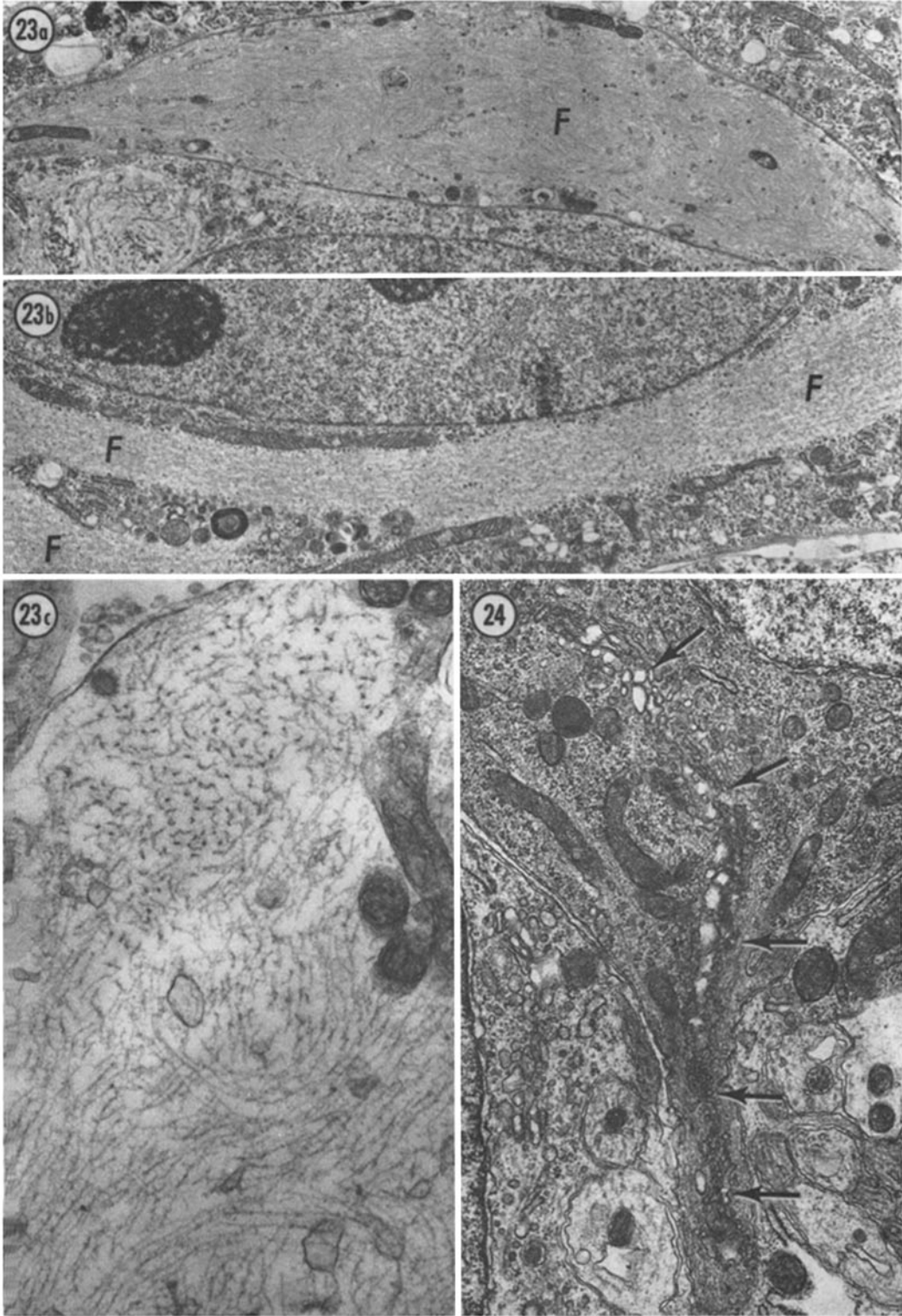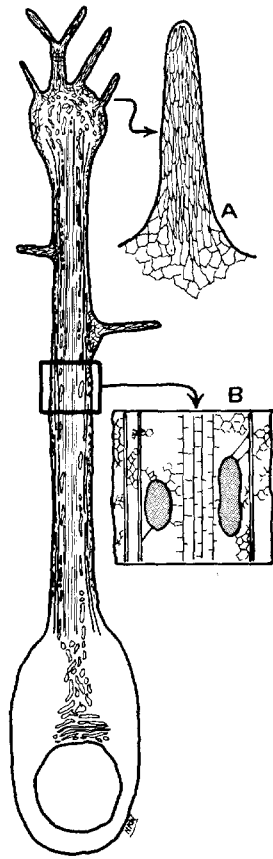
FIGURE 25 Our conception of an elongating axon of a spinal ganglion cell based on data from this paper. Neurofilaments extend up the center of the axon and microtubules are seen to each side, nearer the plasma membrane. Vesicles and sacs of the smooth endoplasmic reticulum are depicted arising in the Golgi region and extending up the axon into the growth cone. In *inset* A the filamentous network is shown, and the elongated polygonal structures characteristic of microspikes are indicated. In B two vesicles (gray) are shown linked (a) to the microtubule system by long, straight connecting links or by means of the filamentous network, and (b) to the neurofilament system via the network or by shorter sidearms. Connections between plasma membrane and microtubules, sets of microtubules, and microtubules and neurofilaments are also shown. Note that, throughout these sketches, the relative size of different structures is *not* shown in the same scale.

REFERENCES

ABERCROMBIE, M. 1961. The bases of the locomotory behaviour of fibroblasts. *Exp. Cell Res. Suppl.* 8:188.

BODIAN, D. 1966. Development of fine structure of spinal cord in monkey fetuses. I. The motoneuron neuropil at the time of onset of reflex activity. *Johns Hopkins Med. J.* 119:129.

BRAY, D. 1970. Surface movements during the growth of a single explanted neurons. *Proc. Nat. Acad. Sci. U.S.A.* 65:905.

BUNGE, R., and M. BUNGE. 1968. Electron microscopic observations on colchicine induced changes in neuronal cytoplasm. *Anat. Rec.* 160:323.

CARTER, S. B. 1967. Effects of cytochalasins on mammalian cells. *Nature (London).* 213:261.

COHEN, S. 1960. Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. *Proc. Nat. Acad. Sci. U.S.A.* 46:302.

DAHLSTRÖM, A. 1969. Synthesis, transport, and life-span of amine storage granules in sympathetic adrenergic neurons. *Symp. Int. Soc. Cell Biol.* 8:153.

DEL CERRO, M. P., and R. S. SNIDER. 1968. Studies on the developing cerebellum. Ultrastructure of the growth cones. *J. Comp. Neurol.* 133:341.

HARRISON, R. G. 1910. The outgrowth of the nerve fiber as a mode of protoplasmic movement. *J. Exp. Zool.* 9:787.

HEPLER, P. K., J. R. MCINTOSH, and S. CLELAND. 1970. Intermicrotubule bridges in mitotic spindle apparatus. *J. Cell Biol.* 45:438.

HUGHES, A. 1953. The growth of embryonic neurites. A study on cultures of chick neural tissues. *J. Anat.* 87:150.

KANASEKI, T., and K. KADOTA. 1969. The "vesicle in a basket." A morphological study of the coated vesicle isolated from the nerve endings of the guinea pig brain, with special reference to the mechanism of membrane movements. *J. Cell Biol.* 42:202.

KREUTZBERG, G. W. 1969. Neuronal dynamics and axonal flow. IV. Blockage of intra-axonal enzyme transport by colchicine. *Proc. Nat. Acad. Sci. U.S.A.* 62:722.

LYSER, K. M. 1968. Early differentiation of motor neuroblasts in the chick embryo as studied by electron microscopy. II. Microtubules and neurofilaments. *Develop. Biol.* 17:117.

MARTINEZ, A. J., and R. L. FRIEDE. 1970. Accumulation of axoplasmic organelles in swollen nerve fibers. *Brain Res.* **19**:183.

METUZALS, J. 1969. Configuration of a filamentous network in the axoplasm of the squid (*Loligo pealii* L.) giant nerve fiber. *J. Cell Biol.* **43**:480.

METUZALS, J., and C. S. IZZARD. 1969. Spatial patterns of threadlike elements in the axoplasm of the giant nerve fiber of the squid (*Loligo pealii* L.) as disclosed by differential interference microscopy and by electron microscopy. *J. Cell Biol.* **43**:456.

NAKAI, J. 1956. Dissociated dorsal root ganglia in tissue culture. *Amer. J. Anat.* **99**:81.

NAKAI, J., and Y. KAWASAKI. 1959. Studies on the mechanism determining the course of nerve fibers in tissue culture. I. The reaction of the growth cone to various obstructions. *Z. Zellforsch. Mikrosk. Anat.* **51**:108.

NATHANIEL, E. J. H., and D. R. NATHANIEL. 1966. The ultrastructural features of the synapses in the posterior horn of the spinal cord in the rat. *J. Ultrastruct. Res.* **14**:540.

PETERS, A., and J. E. VAUGHN. 1967. Microtubules and filaments in the axons and astrocytes of early postnatal rat optic nerves. *J. Cell Biol.* **32**:113.

POMERAT, C. M., W. J. HENDELMAN, C. W. RAIBORN, JR., and J. F. MASSEY. 1967. Dynamic activities of nervous tissue *in vitro*. *In* The Neuron. H. Hyden, editor. American Elsevier Publishing Co., Inc., New York. 119.

PORTER, K. R. 1966. Cytoplasmic microtubules and their functions. *Principles Biomol. Organ. Ciba Found. Symp.* 308.

ROTH, T. F., and K. R. PORTER. 1964. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. *J. Cell Biol.* **20**:313.

SCHMITT, F. O. 1968. The molecular biology of neuronal fibrous proteins. *Neurosci. Res. Program Bull.* **6**:119.

SCHMITT, F. O. 1969. Fibrous proteins and neuronal dynamics. *Symp. Int. Soc. Cell Biol.* **8**:95.

SCHROEDER, T. E. 1969. The role of "contractile ring" filaments in dividing *Arbacia* egg. *Biol. Bull.* **137**:413.

SCOTT, B. S., V. E. ENGLEBERT, and K. C. FISHER. 1969. Morphological and electrophysiological characteristics of dissociated chick embryonic spinal ganglion cells in culture. *Exp. Neurol.* **23**:230.

SMITH, D. S., U. JÄRLFORS, and R. BERANEK. 1970. The organization of synaptic axoplasm in the lamprey (*Petromyzon marinus*) central nervous system. *J. Cell Biol.* **46**:199.

SPERRY, R. W. 1963. Chemoaffinity in the orderly growth of nerve fiber patterns of connections. *Proc. Nat. Acad. Sci. U.S.A.* **50**:703.

SPOONER, B. S. 1970. The expression of differentiation by chick embryo thyroid in cell culture. I. Functional and fine structural stability in mass and clonal culture. *J. Cell. Physiol.* **75**:33.

SPOONER, B. S., and N. K. WESSELLS. 1970. Effects of cytochalasin B upon microfilaments involved in morphogenesis of salivary epithelium. *Proc. Nat. Acad. Sci. U.S.A.* **66**:360.

SPOONER, B. S., K. M. YAMADA, and N. K. WESSELLS. 1971. Microfilaments and cell locomotion. *J. Cell Biol.* **49**:000.

SZOLLOSI, D. 1970. Cortical cytoplasmic filaments of cleaving eggs: A structural element corresponding to the contractile ring. *J. Cell Biol.* **44**:192.

TANI, E., and T. AMETANI. 1970. Substructure of microtubules in brain nerve cells as revealed by ruthenium red. *J. Cell Biol.* **46**:159.

TAYLOR, A. C. 1966. Microtubules in the microspikes and cortical cytoplasm of isolated cells. *J. Cell Biol.* **28**:155.

TENNYSON, V. M. 1970. The fine structure of the axon and growth cone of the dorsal root neuroblast of the rabbit embryo. *J. Cell Biol.* **44**:62.

VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.

WEISS, P. 1941. Nerve patterns: The mechanics of nerve growth. *Symp. Soc. Study Develop. Growth* **5**(*Suppl.*):163.

WESSELLS, N. K., B. S. SPOONER, J. F. ASH, M. O. BRADLEY, M. LUDUENA, E. TAYLOR, J. T. WRENN, and K. M. YAMADA. 1971. Microfilaments in cellular and developmental processes. *Science* (*Washington*). **171**:135.

WISNIEWSKI, H., M. L. SHELANSKI, and R. D. TERRY. 1968. Effects of mitotic spindle inhibitors on neurotubules and neurofilaments in anterior horn cells. *J. Cell Biol.* **38**:224.

WRENN, J. T., and N. K. WESSELLS. 1969. An ultrastructural study of lens invagination in the mouse. *J. Exp. Zool.* **171**:359.

WRENN, J. T., and N. K. WESSELLS. 1970. Cytochalasin B: Effects upon microfilaments involved in morphogenesis of estrogen-induced glands of oviduct. *Proc. Nat. Acad. Sci. U.S.A.* **66**:904.

WUERKER, R. B., and S. L. PALAY. 1969. Neurofilaments and microtubules in anterior horn cells of the rat. *Tissue and Cell.* **1**:387.

YAMADA, K. M., B. S. SPOONER, and N. K. WESSELLS. 1970. Axon growth: Roles of microfilaments and microtubules. *Proc. Nat. Acad. Sci. U.S.A.* **66**:1206.