FREEZE-FRACTURING OF NERVE GROWTH CONES AND YOUNG FIBERS

A Study of Developing Plasma Membrane

KARL H. PFENNINGER and RICHARD P. BUNGE

From the Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri 63110. Dr. Pfenninger's present address is Section of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510.

ABSTRACT

Neural and non-neural cellular processes have been studied in organotypic cultures of spinal cord and olfactory bulb by means of the freeze-fracturing technique. Identification of specific cellular elements in replicas has been achieved by comparison with thin-sectioned material in which differences in shape and contents are evident. Freeze-fracturing reveals that neural growth cones may be distinguished from glial pseudopodia by the low number of intramembranous particles within their plasma membrane; the counts of particles within the growth cone membrane average $85/\mu m^2$ (for the inner leaflet) as opposed to hundreds per square micrometer in glial pseudopodia. Whereas the intramembranous particle number in glial pseudopodia is only slightly lower than in their perikaryal plasmalemma, the number of particles in outgrowing axons increases about eightfold from the periphery towards the perikaryon. Furthermore, with prolonged time of growth in culture, the particle density in the young nerve fibers increases by about the same factor. The same phenomenon, i.e. a low intramembranous particle level at earlier stages and an increase in numbers as the nerve fiber matures, is observed in fetal nerve tissue in vivo. These findings suggest that the plasmalemma of the outgrowing nerve, and especially of the growth cone, is immature and that maturation is accompanied by the insertion of intramembranous particles. Furthermore, these data indicate that the chemistry of the growth cone membrane is distinct from that of the neuron soma which may be significant for the mechanisms of guidance and recognition in the growing nerve tip.

The growth cone of the elongating nerve fiber is a highly specialized region remarkable for its complex functional capacity. Displaying a vigorous intrinsic motility and rapid advancement, this portion of the growing nerve cell is able to invade a variety of tissues, expressing the requisite properties both to follow the proper route of growth, and to recognize and relate to a specific target cell. It has long been known that the motile activities and form of the growing nerve fiber and its growth cone in tissue culture resemble their in vivo counterparts (Harrison, 1910; reviewed by Bunge, 1973). More recently, it has been shown that the functional capacities expressed in vitro include the

formation of synapses (reviewed by Shimada and Fischman, 1973; Crain, 1966; Bunge et al., 1974), and in some cases these are known to form with the expected selectivity (Olson and Bunge, 1973). The outgrowth of axons from cultured nerve tissue thus provides a suitable system for detailed study of growing nerve fibers.

It seemed reasonable to undertake a thorough examination of the surface membrane of the growth cone in a search for morphological structures which might provide the basis for the capacities mentioned above, in particular the mechanisms of guidance and recognition. In the present study, we have employed a newly developed method of freeze-fracturing through monolayer regions of cultured tissues[1] which has allowed an investigation of the intramembranous structure (especially the particle density) of outgrowing axons and supporting cells from cultured spinal cord (SC) and olfactory bulb (OB). The content of intramembranous particles is considered particularly relevant to this investigation because of the high particle concentration demonstrated by membranes of known functional complexity in many differentiated cells. Some of the data presented below have been published earlier in abstract form (Pfenninger, 1972; Pfenninger and Bunge, 1973).

MATERIALS AND METHODS

Tissue Culture Techniques

Explants of SC (cross sections 0.5–1 mm thick) and OB (cut in half), both stripped of meninges, were obtained from Holtzman albino rat fetuses at 14.5–15.5, and 16–17 days of gestation, respectively. SC explants, for longer cultivation (up to 40–50 days), were similarly prepared, but with dorsal root ganglia and meninges attached. Isolated superior cervical ganglion neurons were prepared from albino rats just before, or immediately after, birth according to the methods described elsewhere (Bray, 1970). The explants were prepared in Leibovitz's medium containing 200 U/ml of penicillin G. They were transferred into collagen-coated shallow Aclar wells contained in Falcon no. 1006 Petri dishes in which the plastic bottom had been replaced by a glass window (Bunge and Wood, 1973). In those cultures which were later used for freeze cleaving, two explants were placed at the edge on opposite sides of 3-mm honeycomb gold grids embedded in collagen before polymerization. Thus, part of the outgrowth would later be supported by the

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[1] Pfenninger, K. H., and E. R. Rinderer. 1974. Freeze-fracturing of nerve tissue cultures. A method for the cleavage of cell monolayers. Manuscript in preparation.

collagen-coated grids.[1] Similarly, the isolated ganglion cells were seeded on collagen-coated gold grids for later support. The culture medium consisted of 65% Eagle's minimum essential medium, 25% human placental serum, 10% chick embryo extract, supplemented to 6.40 mg/ml glucose without antibiotics. The dishes were kept at 35°C in a moist atmosphere containing 5% carbon dioxide. The duration of growth ranged from 5 to 50 days for spinal cord and from 5 to 11 days for olfactory bulb cultures. The superior cervical ganglion neurons were cultivated for 24–36 h only.

For freeze-fracturing, we selected cultures in which nerve fibers were dispersed and displayed numerous active growth cones, preferentially some distance ahead of the majority of non-neuronal cells which had migrated out of the explant. Cultures which showed meningeal contamination, i.e., growth of fibroblasts, were discarded. The first fixative, consisting of 4% formaldehyde (freshly made from paraformaldehyde) and 0.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.3) with 0.002% anhydrous $CaCl_2$ (Vaughn and Peters, 1967), or 2.5% glutaraldehyde in Earle's balanced salt solution, was slowly infused (2–3 drops/min) from the rim of the Aclar dish into the tissue culture medium (Bunge, 1973). During the procedure, the culture was maintained at 35°C. After 15 min, the initial fixative was gradually replaced by 4% glutaraldehyde in 0.15 M phosphate buffer with 0.002% $CaCl_2$ added and kept at room temperature for 45 min. In our hands this two-step fixation procedure gave better results than simpler protocols. The fixative was then replaced by 0.2 M cacodylate or phosphate buffer at pH 7.3. After 30–120 min of washing at 4°C, 25% cold glycerol in 0.1 M cacodylate or phosphate buffer (pH 7.3) was slowly added to, and finally substituted for, the previous buffer. About 30 min later, the explants were carefully cut away from the fiber outgrowth which was still supported by the grids. The gold grids were then cautiously removed from the culture dish and placed between special carriers and, finally, the assembled sandwich was quickly frozen in Freon 22 at liquid nitrogen temperature. With the aid of a special tool, the sandwiches were fractured in a Balzers BA 360 apparatus at −110°C in a vacuum better than 8 × 10^{-7} torr. This technique, with special reference to the problems of cell monolayer cleavage, is described in detail elsewhere.[1] Replication was usually performed after 30–90 s of "etching" but, in some cases, immediately followed the fracturing of the specimens. Although true etching is not possible in glycerinated specimens it seemed that the procedure described above made the appearance of intramembranous particles somewhat clearer. However, systematic differences between "etched" and unetched specimens could not be established. The platinum-carbon replicas were produced with regular carbon rod evaporators, or (more often and with much greater accuracy and reproducibility) electron beam guns were used in connection with a quartz crystal

film thickness monitor to obtain about 20 Å platinum and 250 Å carbon. The replicas were floated off in distilled water, cleaned overnight in a commercial bleach (Clorox), rinsed in several changes of distilled water, and finally picked up on Formvar-coated grids.

The tissue for *thin sectioning* was grown as described above but without the gold grid support. After the same aldehyde fixation and washing procedures as for freeze fracturing, these cultures were postfixed with 2% OsO_4 in 0.1 M cacodylate buffer, pH 7.3, for 60 min at 4°C. After subsequent uranyl acetate block staining (Karnovsky, 1967), the specimens underwent ethanolic dehydration and were embedded in Epon-Araldite (Mollenhauer, 1964). All these steps were carried out while the tissue was still attached to the collagen layer in the Aclar dish. After polymerization of the resin, the Aclar plastic well was easily removed. Selected areas of the specimens were then cut out of the flat Epon-Araldite disks and mounted on a block of the same resin for thin sectioning at a 90° angle or in parallel to the bottom of the culture. These sections were then stained with lead citrate (Reynolds, 1963). For counting of intramembranous particles, flat and well-preserved areas were randomly selected. To insure such sampling, all growth cones encountered (identified on the basis of their shape) were used for measurements. Regardless of their content of intramembranous particles, nerve fiber membranes were photographed (at × 30,000–50,000 magnification) for quantitative analysis in different areas of several fiber tracts. The areas in which particles were counted measured at

least 0.7 μm^2 in each membrane with the exception of very slender nerve fibers where the regions were sometimes as small as 0.2 μm^2. In Table I, the total sizes of the samples are indicated in each case. Only those particles which protruded clearly from the membrane face with a sharply outlined shadow were counted; these particles had a diameter in the range of 70–120 Å.

RESULTS

Histology of Outgrowth from Spinal Cord and Olfactory Bulb Explants

After a lag period of about 1 day following transfer into the culture dish, small nerve fiber sprouts appear along the edge of the explants. Within the next few days, the nerve fibers grow rapidly to form long bundles (Figs. 1 and 3). Considerable variability exists in the degree of fasciculation. In SC cultures, hundreds of fine nerve fibers tend to aggregate into thick bundles, whereas the outgrowth of OB explants is more often dispersed, containing only thin strands of nerve fibers. At their tips, the individual fibers spread out to form growth cones. Accordingly, the bundles also broaden at their ends but they are often about the same size or even smaller than the single growth cones of isolated superior cervical

TABLE I

Particle Densities in the Plasmalemma of Developing Neurons and Supporting Cells

Cell part	Tissue	Days in culture	Particles/μm^2 (mean ± SEM*)			
			Inner leaflet		External leaflet	
Cultured neurons						
Nerve growth cone	OB	11	93.8 ± 9.9	(4/3.0)	32.6 ± 9.0	(4/8.4)
	SC	5, 13, 50	78.3 ± 9.6	(6/16.0)	23	(2/13.1)
Nerve fibers	SC	5	53.3 ± 3.3	(10/7.7)		
		7	70.0 ± 6.8	(10/7.1)		
		13	445 ± 66	(10/3.0)		
	SC + DRG	40	527 ± 60	(10/2.0)		
Perikaryon	SCG	1	660 ± 80	(6/4.1)		
Presynaptic membrane	SC + DRG	40	735	(2/0.4)		
Nerve fibers, not cultured	Embryonic	15 d gest.	112 ± 13	(4/1.6)		
	SC	17 d gest.	743	(2/0.4)		
Supporting cells in culture	OB	11	488 ± 106	(4/4.2)	48.8 ± 11.6	(4/11.9)

OB, olfactory bulb; SC, spinal cord; SC + DRG, spinal cord with dorsal root ganglia attached; d gest., days of gestation.
* SEM, standard error of the mean. The figures in parentheses indicate the sample size (number of different cells and total area measured in square micrometers, respectively).

ganglion (SCG) cells grown in culture (Bunge, 1973) and their gross morphology is quite comparable. Under phase-contrast optics (Figs. 1 and 2), the filopodia extending from the growth cones can often be recognized, and active movement and advancement of the cones can be seen. The strongly refractile large vacuoles described by others (Pomerat et al., 1967) in cultured nerve tissue were not observed in the nerve growth cones of our preparations.

A varying number of non-neuronal cells migrate from the explants together with the nerve fibers. Three main types can be distinguished and are similar in OB and SC cultures: (a) The vast majority of these supporting elements are small, polygonal cells with numerous processes identified as neuroglia (Figs. 1 and 3). They are mostly clustered near the proximal parts of the nerve fiber bundles. As can be seen in Fig. 3, their processes may contact the nerve fibers and sometimes bear growth cone-like terminal enlargements with fine processes. These glial elements are henceforth referred to as pseudopodia. Occasionally, cell bodies of the size of the polygonal cells are contained within the contour of the nerve fascicles (Figs. 1 and 2). In this case, no cell processes are found to leave these bundles. (b) Much less frequently encountered are large, flattened epithelioid cells in the periphery of the fiber outgrowth (Figs. 1 and 2). They are so thinly spread that their borders are often difficult to detect, but their large clear nuclei with several nucleoli stand out conspicuously. These cells are likely also to occur in more proximal parts of the outgrowth but cannot easily be identified there because of the mass of overlying cellular elements. The epithelioid cells appear to form a kind of carpet to which the nerve tips preferentially attach (cf. Grainger and James, 1970). Nerve growth cones, however, are also found in cell-free areas, directly on the collagen substrate. (c) The third type of cell is roughly round and is seen scattered mainly over the more proximal parts of the fiber outgrowth. It exhibits numerous lamellopodia, and increases in number with the age of the culture. These are phagocytic cells common in this and other types of explant cultures.

These observations on living tissues provide the basis for the understanding of the freeze-fractured replicas. The survey picture in Fig. 4 shows clusters of polygonal supporting cell bodies related to fascicles composed of countless slender nerve fibers. Several of these fibers are exposed over many tens of micrometers. Sometimes, groups of nerve fibers are covered over a substantial length by an apparently tightly adherent membrane, probably belonging to a glial process. At higher magnification (Figs. 5 and 6), the nerve fibers are seen to vary considerably in diameter, not only between the individual fibers but also along their axes; many have a somewhat beaded appearance (Fig. 5). The average fiber diameter is about 0.5 μm, but sizes of 0.2-1.1 μm are also found. Whereas the nerve fibers are tubular (with roughly circular profiles) (Fig. 7) and exhibit few branching points, other fascicle components branch frequently, are quite variable in shape, and appear to envelop some of the nerve fibers. According to their contents, these elements can be clearly identified as glial (see below). Cross sections of the embedded fiber outgrowth (Fig. 7) yield a different but quite compatible perspective of the topography of the cultures. Fig. 7 shows small portions of two epithelioid cells lying on the collagen layer of the culture dish bottom. On top of the more superficial epithelioid cell, the profile of one of the small polygonal glial cells can be seen almost to its full extent. Part of a nerve fascicle composed of over 100 fibers overlies this glial element.

Identification of the Nerve Growth Cone

In thin sections, nerve growth cones are identified either on the basis of their typical shape or on the basis of their contents. The contents of the shafts of nerve fibers can be seen in Figs. 6 and 7. The nerve fiber contains within its clear cytoplasm many microtubules, some neurofilaments, mitochondria, a few cisterns of smooth endoplasmic reticulum, some 1,200-Å dense-cored vesicles and, occasionally, clusters of clear vesicles with diameters averaging about 1,500 Å. Nerve fibers in older cultures also contain increasing numbers of small clear vesicles, about 500 Å in diameter, which are probably synaptic vesicles. In the growth cone (Figs. 8 and 9), the family of organelles is similar to that of the fibers but most striking are large amounts of smooth membrane in cisterns of various sizes and shapes, numerous dense-cored vesicles, clusters of the clear 1,500-Å vesicles, groups of mitochondria (not seen in the plane of section of Figs. 8 and 9), and an extensive network of microfilaments both within the filopodia and underlying the plasmalemma of the cone (Yamada et al., 1971; Bunge, 1973). Occasionally, bundles of
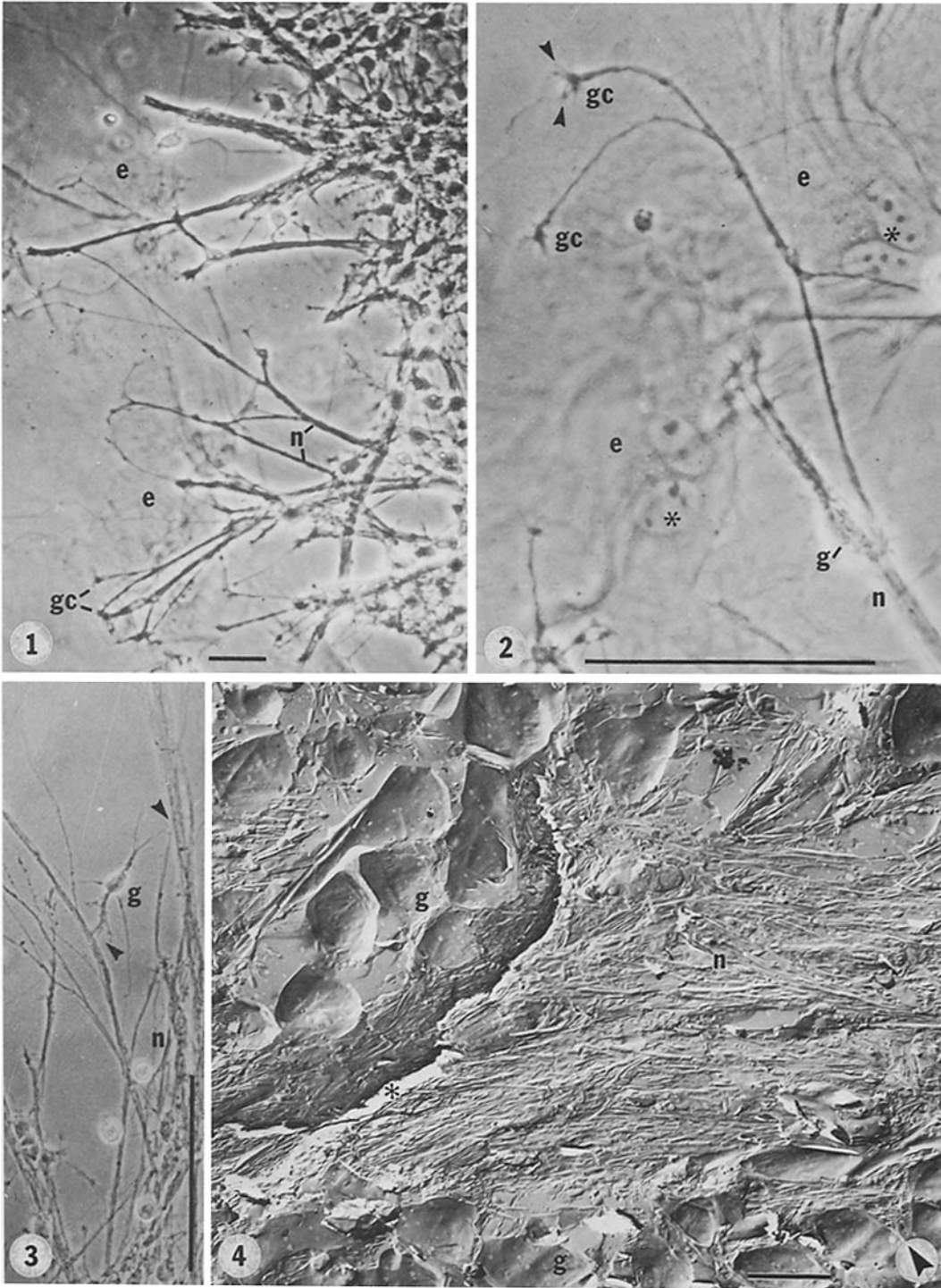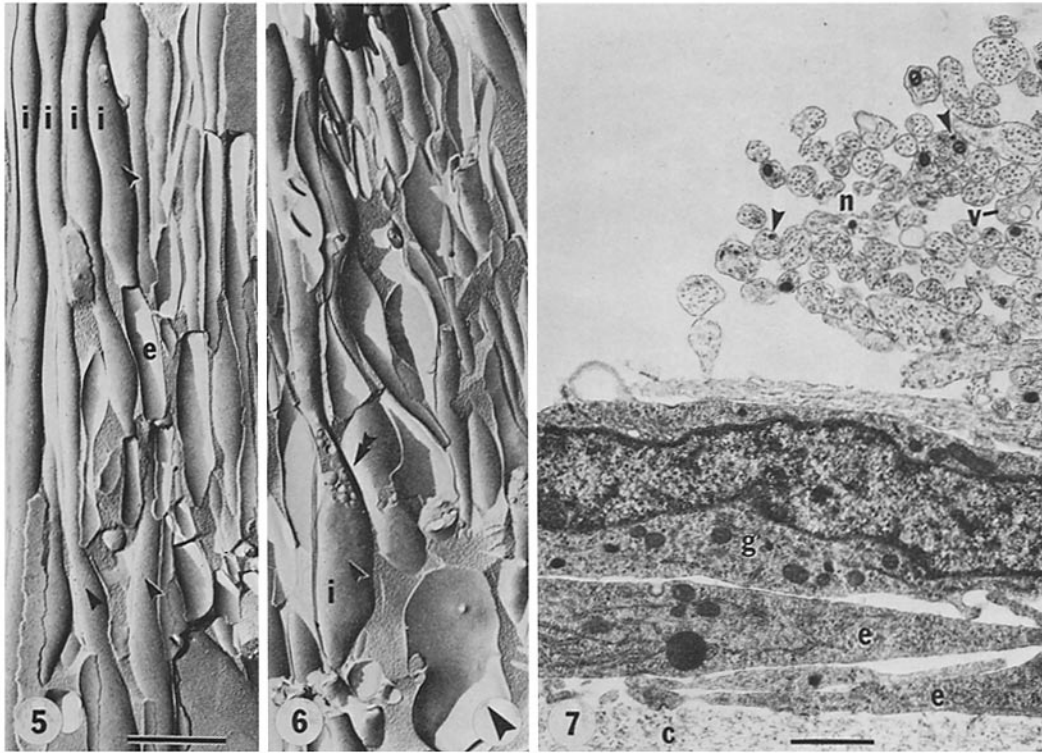
FIGURES 5 and 6   Closer view of nerve fiber bundles from SC cultures grown for 13 days. In these pictures, the fracture has most frequently exposed the inner plasmalemmal leaflets (*i*); the external leaflet (*e*) can be seen only occasionally. The fibers appear slightly beaded and the thicker portions often contain clusters of 1,500-Å vesicles (double arrowhead). Note the low density of intramembranous particles (arrowheads). Arrowhead on white disk, shadowing direction. × 13,100; calibration 1 μm.

FIGURE 7   Cross section through the outgrowth of a 5-day old SC culture. A glial cell (*g*) overlies two flat processes of putative epithelioid cells (*e*). On this cellular carpet, a bundle of nerve fibers (*n*) can be seen. Their cross sections are characterized by numerous microtubules, some mitochondria, some large dense-cored vesicles (arrowheads), and an occasional aggregate of clear 1,500-Å vesicles (*v*). *c*, collagen substrate. × 10,600; calibration, 1 μm.

FIGURES 1-3   Phase-contrast micrographs of the outgrowth of nerve tissue cultures. The low magnification of Fig. 1 illustrates the bundles of nerve fibers (*n*) radiating from the explant which lies on the right-hand side but is not seen in the picture. At their ends, most nerve fibers show an enlargement containing several growth cones (*gc*). Many polygonal cells, probably of glial nature, are associated with the proximal parts of the fiber bundles whereas very flat cells called epithelioid cells (*e*) have migrated farther from the explant where they form a preferential substrate for the growth cones. The typical shape of the nerve growth cone groups (*gc*) with numerous filopodia (arrowheads) can be better seen in Fig. 2. There, nerve fiber bundles (*n*) lie on epithelioid cells (*e*) whose nuclei are marked by asterisks. The thicker bundle has a glial cell (*g*) incorporated into its shaft. As seen in Fig. 3 (arrowheads), the processes or pseudopodia of glial cells (*g*) often reach out for neighboring nerve fiber bundles (*n*). Figs. 1 and 2, SC tissue grown for 5 days in vitro, × 82 and × 430, respectively; calibrations, 0.1 mm. Fig. 3, OB tissue cultured for 4 days, × 290; calibration, 0.1 mm.

FIGURE 4   Survey picture of the freeze-cleave replica of a proximal region in a SC culture that was grown for 5 days. This area contains an abundance of nerve fibers (*n*) and numerous glial cell bodies (*g*) and corresponds to the image obtained by phase-contrast microscopy from the more proximal outgrowth areas shown in Fig. 1. The asterisk indicates a rupture in the replica. Arrowhead on white disk, shadowing direction. × 1,180; calibration, 10 μm.
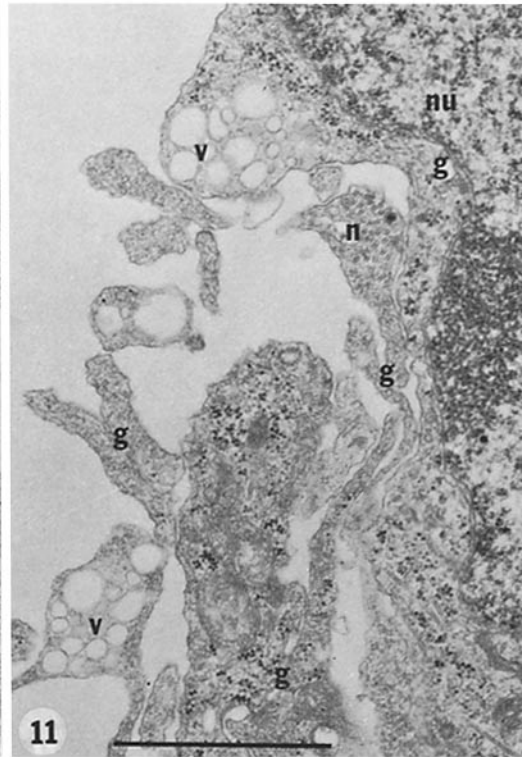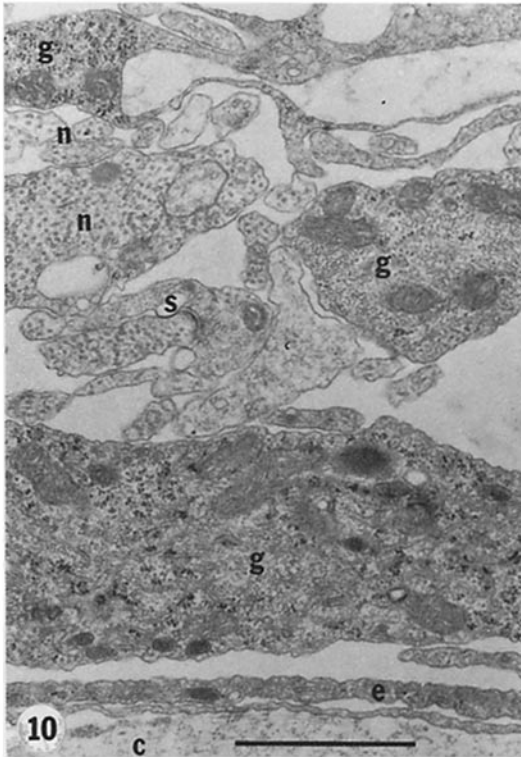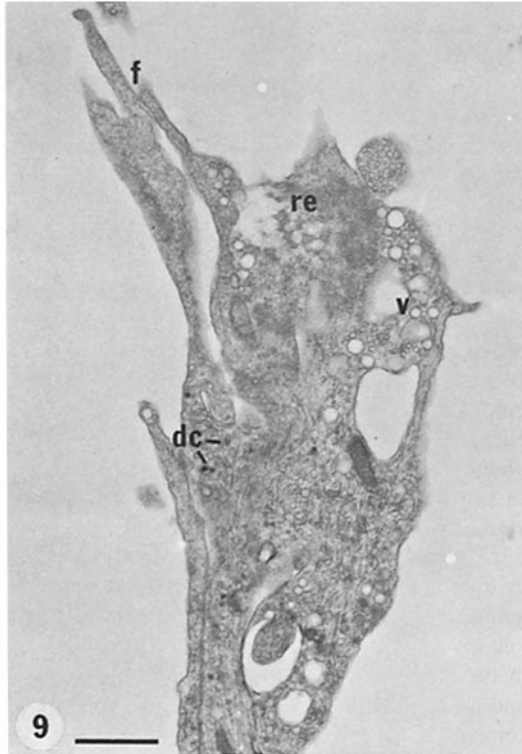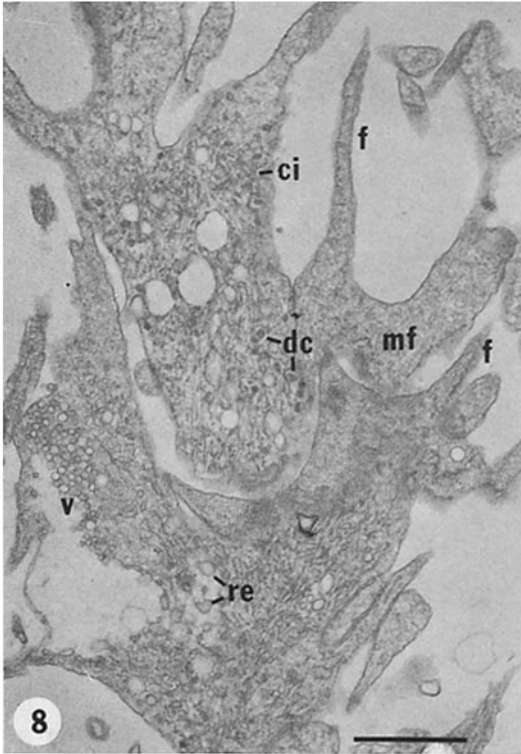
larger 80–100-Å filaments are observed. In addition, multivesicular bodies, dense bodies, and other lysosomal structures are frequently encountered. Ribosomes, however, do not occur in these peripheral fibers or nerve growth cones at the time intervals studied. As with the nerve growth cone, the pseudopodia of glial and epithelioid cells are the enlarged tips of processes but their stems are often quite short (Figs. 3, 15). These pseudopodia occur within the fascicles, free in the periphery of the outgrowth, or between neighboring epithelioid and glial cells. Although they are sometimes difficult to distinguish from the nerve growth cone because of their similarity in shape, the glial and epithelioid pseudopodia contain a strikingly different set of organelles (Figs. 10, 11). Ribosomes are abundant in the darker cytoplasmic matrix, as well as in the pseudopodia; 1,200-Å dense-cored vesicles are very rare if not absent; and large bundles of filaments are usually found throughout the cytoplasm and not primarily beneath the plasmalemma. Furthermore, smooth membrane cisterns and microtubules occur only in small amounts. As in the nerve growth cone, however, dense bodies and mitochondria, as well as 1,500-Å vesicles are common.

Because in thin cell processes the cleavage plane runs primarily in the plasmalemma and normally does not expose the cytoplasmic contents, the only criteria for the recognition of nerve growth cones vs. glial pseudopodia in replicas are their shape and origin. The characteristics employed to identify the nerve growth cone in this study are: (a) a slender nerve fiber stem extending over many micrometers, often coming out of a nerve fascicle, (b) a polygonal enlargement at the tip whose (c) edge is frequently ruffling and from which (d)

numerous small processes, the so-called filopodia, arise. Such growth cones are shown in Figs. 12, 13, and 15. The freeze-cleaved cones are revealed over greater areas than with thin sectioning since the fracture follows the plasmalemma whereas sections, although in parallel to the culture dish bottom, rarely expose more than small lengths of the intertwined processes (Figs. 8, 9). The membrane of the nerve growth cone in Fig. 16 bulges outward to form a protrusion which is surrounded by a circle of small pits. In a growth cone seen from an intracellular vantage point (i.e., in its external leaflet), Fig. 14 shows two depressions surrounded by a circle of small protuberances which represent a complementary view of the mounds described above.[2]

As can be seen in Fig. 13, ruffling of the nerve fiber surfaces occasionally occurs also at some distance from the nerve growth cone. This may represent the formation of a new branch with an additional growth cone. Fig. 13 B shows that, in this area, part of the nerve fiber is thinly spread out over the collagen surface and that the spiky processes tend to follow the reconstituted collagen fibrils.

Freeze-fractured pseudopodia which originate from a putative epithelioid cell are shown in Fig. 15. The differences between these processes and the nerve growth cone are quite evident. The bases

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[2] These specialized plasmalemmal regions (termed growth zones; Pfenninger and Bunge, 1973) will be described in detail in a forthcoming paper in which it will be shown that they are related to clusters of the 1,500-Å vesicles described above. The membranes of these vesicles are distinguished by their lack of intramembranous particles.

FIGURES 8 and 9   Thin sections of nerve growth cones from a 5-day old SC culture. The sectioning plane is parallel to the culture dish bottom. Parts of at least three overlying growth cones can be seen in Fig. 8 whereas the main part of one cone with a few accompanying fibers is illustrated in Fig. 9. Note the typical shape of the growth cone and its characteristic organelles (see text). ci, membranous cisterns; dc, large dense-cored vesicles; f, filopodia; mf, microfilaments; re, reticular system of membranes which seems related to aggregations of clear vesicles (v) averaging 1,500 Å. × 15,200 (Fig. 8) and × 10,900 (Fig. 9); calibrations, 1 μm.

FIGURES 10 and 11   Cross sections of glial (g) elements in the proximal outgrowth of a 5-day old SC culture. Note the branching pattern of the glial processes and their high density of ribosomes and filaments which clearly distinguish them from the neuronal elements (n). The glial cell in Fig. 11 bears a characteristic moundlike protrusion filled with clear vesicles (v). Such structures can also be seen on slender processes (lower left-hand corner). c, collagen substrate; e, epithelioid cell; nu, nucleus; s, synapse, probably not fully developed. × 23,800 (Fig. 10) and × 29,000 (Fig. 11); calibrations 1 μm.
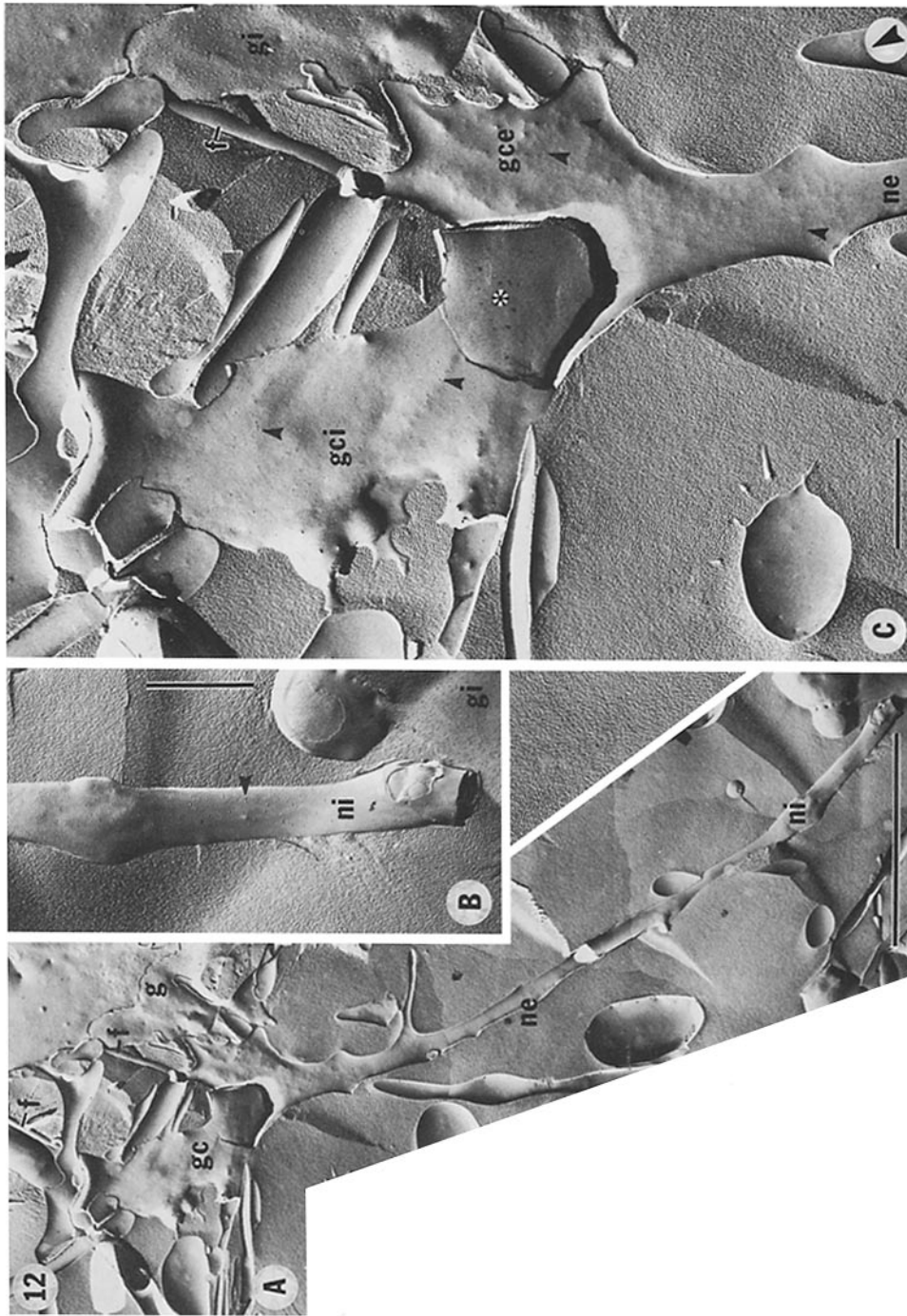
of the numerous epithelioid filopodia are broader than the stem of the neighboring nerve growth cone and are directly continuous with the perikaryon. Apart from their membrane properties which are described below, the glial and epithelioid filopodia are morphologically very similar to their neuronal counterparts and also seem to reach out to touch the surfaces of other cellular elements. The mounds surrounded by a circle of pits which are observed in the nerve growth cone are also quite often found in supporting cells (Fig. 17). They are not preferentially located on pseudopodia or along the edge of the cells, however, and stand out in greater contrast than the neuronal mounds due to their special membrane properties (see below).

Membrane Properties of Neuronal and Glial Pseudopodia

The following descriptions are based on the widely accepted view that the freeze-fracturing process delaminates cell membranes by cleaving between the two layers of the lipid core, thus exposing intramembranous faces for replication (Branton, 1966; Pinto da Silva and Branton, 1970; Tillack and Marchesi, 1970; Wehrli et al., 1970). This concept has been shown to apply also for nervous tissue (Pfenninger et al., 1972). Since the membranes in fractured cultures bear all the basic characteristics of fractured blocks of native nerve tissue it is reasonable to assume that the same fracturing rules apply for the cultured tissue. The two exposed membrane faces are referred to as outer or external leaflet (as seen from an intracellular vantage point, also called elsewhere the B face) and cytoplasmic or inner leaflet (as seen from a vantage point outside the cell; also called elsewhere the A face).

SUPPORTING CELLS: A fairly uniform distribution of intramembranous particles over the entire cell is typical of the non-neural elements. From cell to cell, the particle density varies considerably, probably because they belong to different types (glial or epithelioid) which are frequently difficult to distinguish in replicas and/or because they are in different functional stages. Whereas the particle number in the perikaryal plasmalemma averages around $448/\mu m^2$ in the inner leaflet, with figures of up to $700/\mu m^2$ and as low as $200/\mu m^2$, the average counts in the external leaflet are $49/\mu m^2$, i.e., only about one-ninth of those in the inner leaflet. Pseudopodia often contain fewer membrane particles, with the density being around $270/\mu m^2$ for the inner plasmalemmal leaflet. However, the mound areas described above contrast with their surroundings by the almost complete absence of intramembranous particles (Fig. 17). The size of particles in the plasma membrane of the supporting cells is not uniform; their diameter varies between 70 and 120 Å.

NEURONAL PROCESSES: Observations on the neurons are strikingly different from those on supporting cells. When compared with supporting elements in the same region of the outgrowth, the low particle density of the growth cone plasmalemma is evident (Figs. 12-16). In the inner leaflet, the average density and standard error of the mean are $78.3 \pm 9.6/\mu m^2$ for cones from SC grown for 5, 13, or 50 days in vitro, and $93.8 \pm 9.9/\mu m^2$ for OB cones grown for 11 days in vitro (see also Table I). The two figures seem different although the significance at the 0.1 level is marginal; this result will need further confirmation. As can be seen particularly well in Fig. 13 A and B, particle counts are even lower in the outer leaflets

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FIGURE 12 Freeze-cleave pictures of a nerve growth cone from an OB culture grown for 11 days. The survey picture (Fig. 12 A) which is composed of several electron micrographs shows a slender nerve fiber whose inner (ni) and external (ne) plasmalemmal leaflets are alternately exposed. This fiber bears a terminal enlargement, the growth cone (gc), from which filopodia (f) extend. g, glial cell. × 5,900; calibration 5 μm. Fig. 12 B shows the inner plasmalemmal leaflet of the nerve fiber (ni) at higher magnification at a distance of about 17 μm proximal from the growth cone in Fig. 12 A. Note the paucity of intramembranous particles (arrowhead). gi, inner plasmalemmal leaflet of glial cell. × 15,000; calibration, 1 μm. In Fig. 12 C, the morphology of the freeze-fractured growth cone can be seen in detail. Particularly striking is the low density of intramembranous particles (arrowheads) in the inner plasmalemmal leaflet (gci; compare with the neighboring glial inner leaflet, gi). The external leaflet (gce) contains even fewer particles. For further description see text. The asterisk marks a partially dislocated piece of growth cone cytoplasm and adjacent inner leaflet. × 15,800; calibration, 1 μm. Arrowhead on white disk, approximate shadowing direction for all figures.
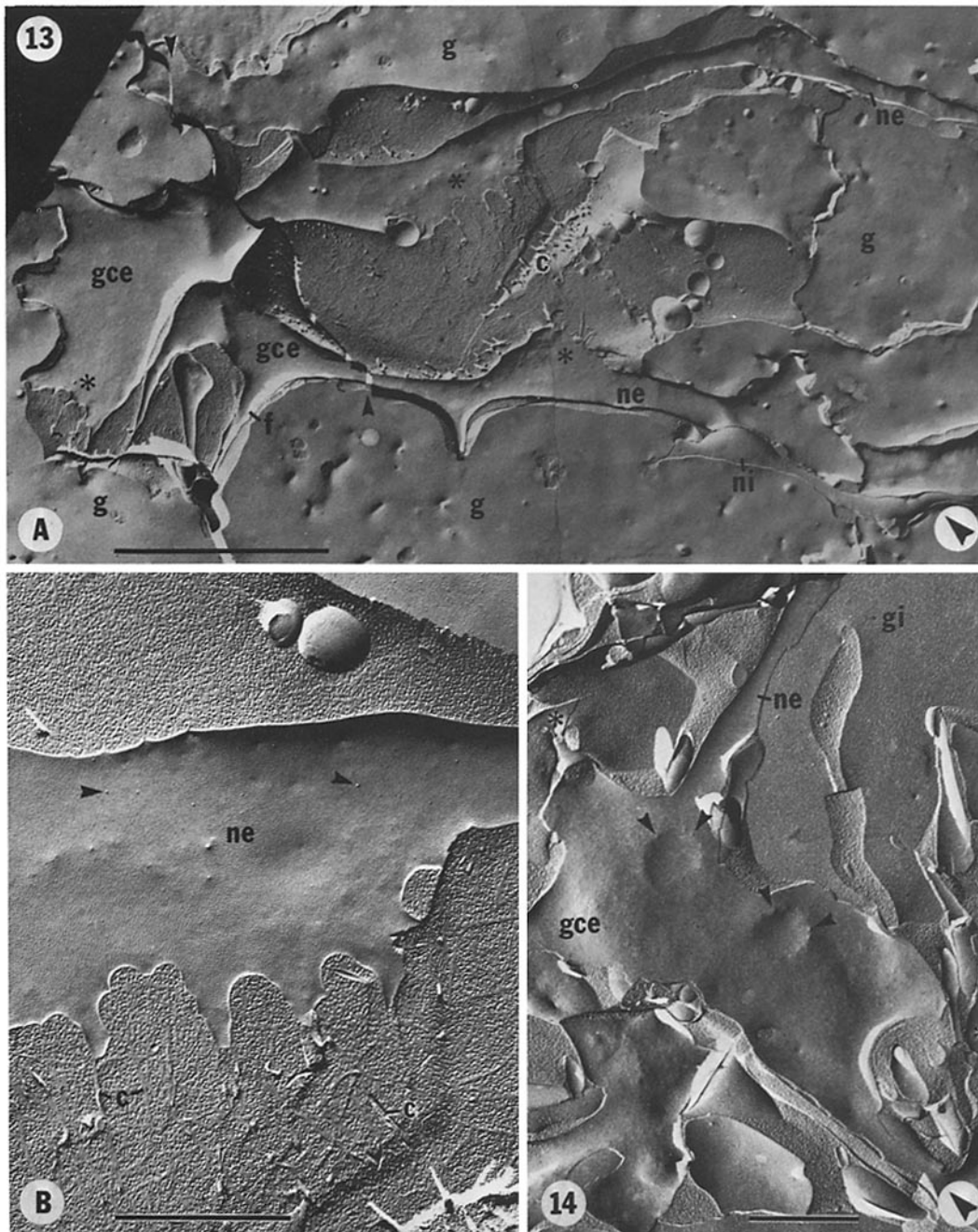
FIGURE 13 A survey picture (composed of two electron micrographs) showing three nerve fibers (*ni*, inner leaflet; *ne* external leaflet) running between the broad processes of unidentified supporting cells (*g*). The arrowheads indicate a small crack in the replica. The collagen fibrils (*c*) of the culture substrate can be seen in the center. The fibers whose external leaflets are exposed terminate in growth cones (*gce*, external leaflets) one of which is broadly spread out and exhibits a ruffling area (asterisk). The other is only partially visible but shows slender filopodia (*f*). Both nerve fibers have spreading or ruffling areas (asterisks) somewhat more proximal from the cones. The upper ruffling area is shown at higher magnification in Fig. 13 B. The ruffles tend to follow collagen fibers (*c*) of the substrate. Note the almost complete absence of intramembranous particles (arrowheads), particularly near the ruffles. The membrane matrix, however, exhibits a fine pattern of small bumps. OB tissue, cultured for 11 days. × 6,100 (Fig. 13 A), × 25,200 (Fig. 13 B); calibrations, 5 μm (Fig. 13 A), 1 μm (Fig. 13 B). Arrowhead on white disk, shadowing direction.

FIGURE 14 External leaflet of a nerve fiber (*ne*) terminating in a growth cone (*gce*) with several processes (asterisks). Note the two depressions surrounded by small protuberances (arrowheads). *gi*, supporting cell, inner plasmalemmal leaflet. SC and dorsal root ganglia cultured for 50 days. × 15,700; calibration, 1 μm.

of the growth cone membrane; the average densities are $23/\mu m^2$ (SC) and $32.6 \pm 9.0/\mu m^2$ (OB). In ruffling, spreading areas of the tip or a few tens of micrometers more proximal, particles may be entirely absent in the outer leaflet (Fig. 13 B) and very scarce in the inner leaflet (Fig. 12 B). In much older cultures, axonal enlargements sometimes contain synaptic vesicles and the inner axolemmal leaflet of one such area exhibited about 290 particles/μm^2. The inner leaflets of two clearly identified presynaptic membranes (Pfenninger et al., 1972) which were found in a 40-day old culture of SC and adjacent dorsal root ganglia contained 545 and 924 particles/μm^2.

In three cases, the particle density could be measured at a known distance from the growth cone. 17 μm from the tip, the inner leaflet of the nerve fiber shown in Fig. 12 A and B contained only 46 particles/μm^2, i.e., even less than the attached growth cone (65/μm^2). A similar decrease was not evident in the outer leaflet of the upper fiber shown in Fig. 13 A. However, as we proceed to more *proximal fiber bundles*, the particle density in the inner leaflets of randomly selected cultured SC nerve fibers is only $53.3 \pm 3.3/\mu m^2$ and $70.0 \pm 6.8/\mu m^2$ in 5- and 7-day old cultures, respectively (Table I). The counts in the 5- but not in the 7-day old fibers are significantly lower ($P = 0.05$ in the t test) than the ones in the growth cones. With increasing age of the cultures, however, the particle counts in the axolemma greatly increase. At day 13, the density is $445 \pm 66/\mu m^2$ and at day 40, in a culture with SC and dorsal root ganglia, counts of $527 \pm 60/\mu m^2$ were noted.

Freeze-fracturing of native fetal SC reveals the same membrane phenomena (Figs. 18–20). On the 15th day of gestation, most fibers contain very few intramembranous particles (approximately 110/μm^2 for the inner leaflet). 2 days later, such fibers are still frequently encountered but, in many cases, the particle density has increased to about 700/μm^2 and more. It appears that particle-rich and particle-poor fibers generally belong to different tracts with more or less uniform particle counts.

Since it is presently not possible to fracture and identify those neurons within the cultured SC or OB that give rise to the outgrowing nerve fibers it was necessary to use neurons of the peripheral nervous system from the SCG to obtain the particle counts in the perikaryal plasmalemma. After cultivation in vitro for 1 day, when these cells

have already developed processes of considerable lengths (Bray, 1973; Bunge, 1973), the average particle density in the inner plasmalemmal leaflet of the soma is $660 \pm 80/\mu m^2$. Our observations indicate that the density of intramembranous particles in the processes of these cells is low, similar to that of the young axons described above. As in the supporting cells, the moundlike areas in the neuronal elements are almost particle-free and stand out clearly when arising from the particle-rich perikaryon. On the growth cone and the young nerve fibers, however, they are more difficult to identify because, in both cases, the membrane faces reveal so few particles (Fig. 14).

As in the supporting elements, the intramembranous particles in neuronal processes range in size from 70 to 120 Å. Our observations do not indicate the existence of distinct particle classes with constant diameter or a systematic change in size with aging of the nerve fibers. But in the perikaryal plasmalemma of the isolated SCG neurons, the particles were found to be somewhat larger, varying in size between 90 and 130 Å. On growth cones (Figs. 12 and 13) as well as on neuronal and non-neuronal mounds (Figs. 16 and 17), i.e., in all particle-poor areas, the matrix of both membrane faces is often not as smooth as is usually observed in freeze-cleave preparations; slightly elevated small bumps of an estimated diameter of 60–80 Å are quite regularly observed. These structures are not protruding from the membrane faces, as the particles appear to be, but are molded into both the inner and the outer membrane leaflets at a high density, giving these faces a cobblestone pattern (Figs. 13 B and 17).

DISCUSSION

Comparison of Axonal and Glial Pseudopodia

The similar shapes of axonal and supporting cell pseudopodia, and especially of the filopodia at their ends, suggest similar functions. The exploring motion of the filopodia seen in living cultures and their variably transient attachment to materials along their way suggest that the filopodia are probing devices of both the neuronal and the glial pseudopodia. There are, however, important morphological distinctions between the two types of cell processes. In addition to variations in the amount of endoplasmic reticulum membrane and
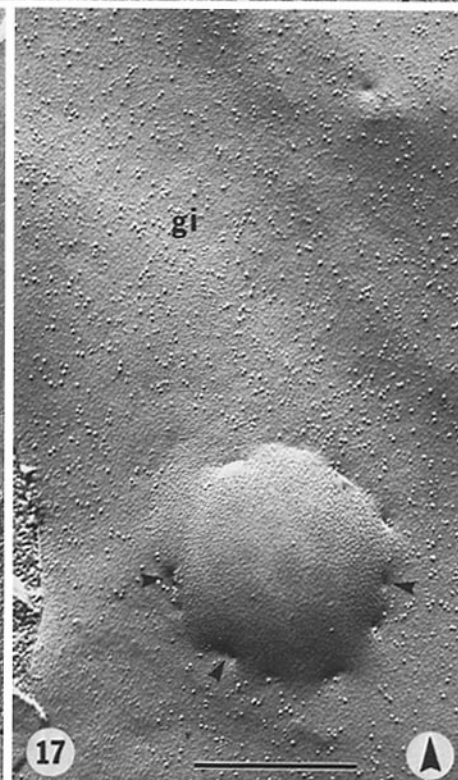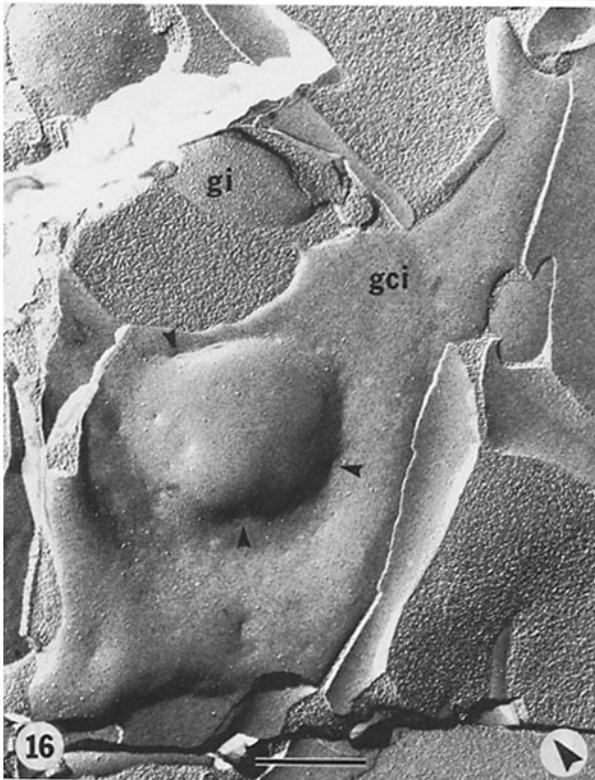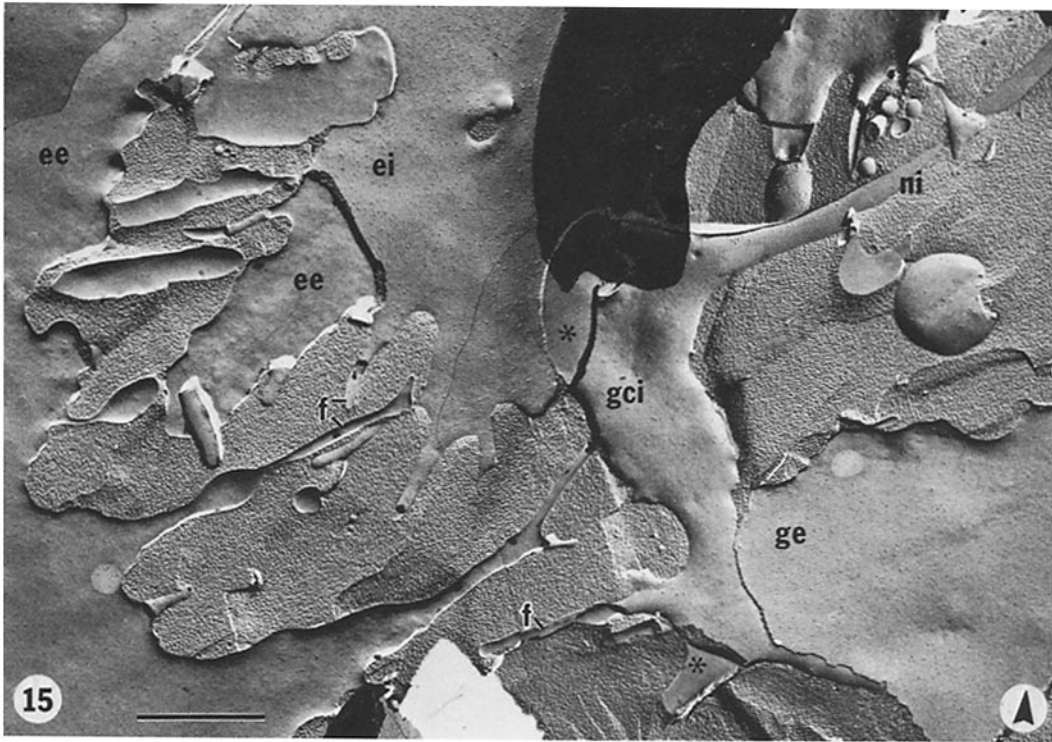
lysosomal structures, the most distinguishing properties are the content of (a) ribosomes, (b) large dense-cored vesicles, and (c) intramembranous particles. The large, 1,200-Å dense-cored vesicles which distinctly mark nerve cell processes occur in the nerve growth cones in much larger numbers than in the adult nerve terminals (for review see e.g., Pfenninger, 1973). This is true not only for adrenergic (Bunge, 1973) but for a variety of (very probably nonadrenergic) nerve fibers (cf. Yamada et al., 1971). The fact that glial pseudopodia contain several times as many intraplasmalemmal particles as the nerve growth cones indicates that functional properties of the two cell processes must differ considerably in spite of their similarity in shape and movement.

The Nature of the Growth Cone Plasmalemma

The major findings of this study are (a) that the membranes of growing nerve fibers contain few intramembranous particles, (b) that the density of these particles is exceptionally low in the growth cone and increases as one approaches the perikaryon, and (c) that there is a gradual increase in particle number throughout the nerve fiber as a function of time. Whereas the meaning of intramembranous particles is not understood, it has generally been observed that complex membranes with a high protein content, such as those of certain intercellular junctions (Goodenough and Revel, 1970; McNutt and Weinstein, 1970), chloroplasts (Goodenough and Staehelin, 1971), and mitochondria (Wrigglesworth et al., 1970), host many particles and that less complex, protein-poor membranes such those in the myelin sheath (Bran-

ton, 1967) are relatively particle-free. In some types of cells, the intramembranous particles seem to mark the areas where certain glycoproteins penetrate the cell membrane (Pinto da Silva et al., 1971; Tillack et al., 1972). Also, the synaptic junction which develops after the nerve tip has contacted its target cell contains large amounts of glycoprotein (Brunngraber et al., 1967) and numerous intramembranous particles (Pfenninger et al., 1972). Thus, the complex roles and fate of the growth cone led us to expect a plethora of particles within its membrane, particularly, because a considerable amount of carbohydrate has been demonstrated cytochemically on its surface[3] (James and Tresman, 1972). The apparent paradox in these considerations may be resolved if the carbohydrate components of the growth cone plasmalemma are contributed largely by glycolipids, or by a type of glycoprotein that is not visible as intramembranous structure.

As we seek an explanation for the particle distribution in the growing neuron, an important factor is its unique development, i.e., the formation of the axon (or dendrites) which represents a well-defined site of substantial and long-lasting vectorial growth involving membrane expansion. The study of the position of particulate surface labels (at the light microscopical level) and of distances between perikaryon, branch points, and tips of growing nerve fibers (Bray, 1970, 1973), as well as the observation that severed axons continue

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[3] The growth cone exhibits extensive binding (sensitive to α-methyl-mannoside) of concanavalin A (Pfenninger and M. Bunge, 1973, unpublished observations), a lectin which associates with α-D-glucose or α-D-mannose (Lis and Sharon, 1973).

FIGURE 15 Outgrowth periphery with a nerve growth cone (gci, inner leaflet) and pseudopodia of two putative epithelioid cells (ee, external; ei, inner leaflets) in an OB culture grown for 11 days. Pseudopodia of supporting cells also bear filopodia (f) but are continuous with the perikaryon through a broad base and contain numerous plasmalemmal intramembranous particles. The nerve growth cone, by contrast, is attached to a slender fiber (ni, inner leaflet) and is poor in plasmalemmal particles. The asterisks mark small areas of exposed external leaflet of the growth cone. ge, external leaflet of the plasmalemma of an unidentified supporting cell. Arrowhead on white disk, shadowing direction. Composite of three electron micrographs. × 17,200; calibration, 1 μm.

FIGURES 16 and 17 Moundlike protrusions of the plasmalemmae (inner leaflets) of a nerve growth cone (gci) and a glial cell (gi). These mounds are characterized by the almost complete absence of intramembranous particles, particularly striking in Fig. 17. Furthermore, they are surrounded by a circle of pits (arrowheads). Arrowheads on white disks, shadowing directions. Fig. 16, SC and dorsal root ganglia grown for 50 days in culture; × 29,500. Fig. 17, OB grown for 11 days in vitro; × 42,800. Calibrations, 0.5 μm.
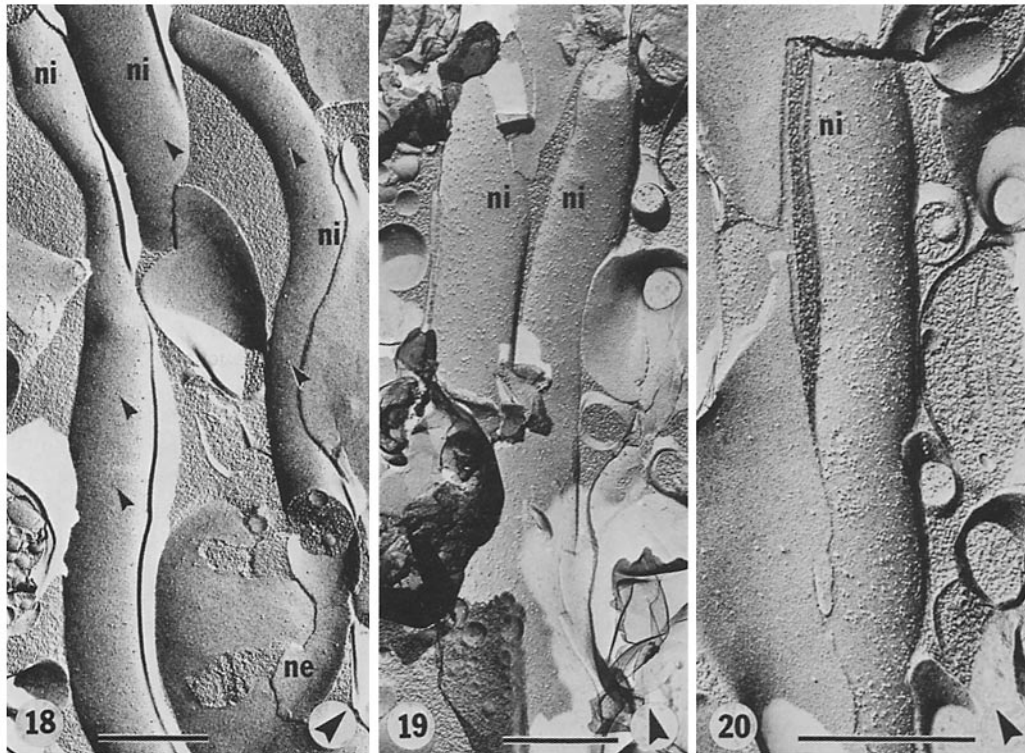
FIGURES 18–20   Nerve fibers (*ni*, inner plasmalemmal leaflets) in rat embryonic SC at 15 (Fig. 18) and 17 days of gestation (Figs. 19, 20). Note the pronounced paucity of intramembranous particles (arrowheads) at the earlier stage (Fig. 18) whereas, in the 17-day old embryo, the plasmalemmae of certain groups of nerve fibers have reached considerable levels of particle density. *ne*, external leaflet of nerve fiber plasmalemma. Arrowheads on white disks, shadowing directions. × 29,300 (Fig. 18), × 31,000 (Fig. 19), and × 47,400 (Fig. 20). Calibrations, 0.5 μm.

to grow for several hours (Hughes, 1953), suggest that membrane addition during nerve elongation occurs primarily at the growth cone, i.e., at a peripheral, clearly defined site. Localized membrane growth was also suggested for the assembly of the retinal rod outer segment membranes (Hall et al., 1969). If the membrane inserted into the plasmalemma of the growth cone is particle-free, such a mechanism of localized, peripheral membrane addition would readily explain our data. Indeed, one possible source of new plasmalemmal components is the particle-free, 1,500-Å vesicles found along the nerve fiber and in the growth cone (Pfenninger and Bunge, 1973). This possibility is now being investigated further. The alternative hypothesis, i.e., that membrane expansion occurs by random and widespread incorporation of membrane units (for review see e.g., Singer and Rothfield, 1973) cannot be ruled out at present but is not as easily compatible with our data.

Regardless of the mechanism of membrane addition, the morphological changes in the plasmalemma during the development of the nerve fiber indicate that the neuron produces an incomplete (particle-poor) "membrane matrix" initially (cf. the "Urmembrane" of Singer and Rothfield, 1973) and that only subsequently does the membrane mature by gradual insertion of special functional sites represented by intramembranous particles. This view of *stepwise membrane formation* has also been suggested by changes in membrane structure and composition during the dvelopment of synchronized hamster cells (Scott et al., 1971) and of chloroplasts (for review see Singer and Rothfield, 1973). As to the origin and sojourn of the intramembranous particles (see e.g., Singer and Nicolson, 1972; Bretscher, 1973), they are, because of their presumed proteinaceous nature, most likely synthesized in the ribosome-containing perikaryon. The particles would then have

to travel the length of the axon either in the cytoplasm (before reaching a peripheral site of insertion) or within the axolemma (by translational movement if they are added to the membrane at the perikaryon). Particle-loaded membranous structures which could represent special particle carriers have not been observed in our material.

The data presented above and the concept of stepwise membrane formation imply the presence of a gradient of particle density and, thus, of membrane maturation within the plasmalemma of the developing neuron. This gradient may be useful in explaining differing functional properties of specific parts of the neuronal surface. Certainly, the axonal growth cone membrane is morphologically, and therefore also biochemically, different from that in the other parts of the neuron. Because of the unique functional capacities of the growth cone, this is not a surprising conclusion. Dendrite growth bears many resemblances to axonal growth (Skoff and Hamburger, 1974), and thus the same principles may apply and a gradient of membrane maturation on the dendrite may explain the acceptance of different synaptic inputs in specific dendritic zones.

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