A LIGHT AND ELECTRON MICROSCOPE STUDY OF LONG-TERM ORGANIZED CULTURES OF RAT DORSAL ROOT GANGLIA

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ABSTRACT

Dorsal root ganglia from fetal rats were explanted on collagen-coated coverslips and carried in Maximow double-coverslip assemblies for periods up to 3 months. These cultured ganglia were studied in the living state, in stained whole mounts, and in sections after OsO_4 fixation and Epon embedment. From the central cluster of nerve cell bodies, neurites emerge to form a rich network of fascicles which often reach the edge of the carrying coverslip. The neurons resemble their in vivo counterparts in nuclear and cytoplasmic content and organization; e.g., they appear as "light" or "dark" cells, depending on the amount of cytoplasmic neurofilaments. Satellite cells form a complete investment around the neuronal soma and are themselves everywhere covered by basement membrane. The neuron-satellite cell boundary is complicated by spinelike processes arising from the neuronal soma. Neuron size, myelinated fiber diameter, and internode length in the cultures do not reach the larger of the values known for ganglion and peripheral nerve in situ (30). Unmyelinated and myelinated nerve fibers and associated Schwann cells and endoneurial and perineurial components are organized into typical fascicles. The relationship of the Schwann cell and its single myelinated fiber or numerous unmyelinated fibers and the properties of myelin, such as lamellar spacing, mesaxons, Schmidt-Lanterman clefts, nodes of Ranvier, and protuberances, mimic the in vivo pattern. It is concluded that cultivation of fetal rat dorsal root ganglia by this technique fosters maturation and long-term maintenance of all the elements that comprise this cellular community in vivo (except vascular components) and, furthermore, allows these various components to relate faithfully to one another to produce an organotypic model of sensory ganglion tissue.

INTRODUCTION

A series of technical advances (reviewed in 1) has made possible the maturation and long-term maintenance of many parts of the nervous system in culture: sympathetic ganglia (2-4), spinal (5) and cranial ganglia (6, 7), spinal cord (8), cerebellum (9-11), brain stem (12), and cerebrum (13), among others. Of these various types of nervous tissue preparations, the sensory ganglion provides one of the most useful cytological models. The explant becomes thin enough for direct high-power light microscopic observation of neurons in the living state. Highly organized outgrowth areas offer a similar opportunity for direct observation of unmyelinated and myelinated neurites and their supporting elements. Such cultures have proven useful for (*a*) direct observation of cytoplasmic

movements presumably involved in transport within the neuron and axon (14); (*b*) studies of the relation of the Schwann cell to the myelin sheath during myelinogenesis (15, 16); and (*c*) observations on the pattern of myelin or neuron response to various insults (reviewed in 1 plus recent references 6, 17, 18).

In order to establish the suitability of this in vitro system for experimental electron microscopic studies, it was necessary to extend the investigation of cytological fidelity to the ultrastructural level. Preliminary observations had indicated that cultured dorsal root ganglia were highly promising for such studies (19, 20). Rat rather than chick ganglion cultures were chosen for electron microscopic study because formation of myelin sheaths and organization of nerve fibers into fascicles are more extensive (see below). There are now available a number of papers describing the fine structure of adult rat spinal ganglion in situ (reviewed in 21 and 22), and it is the purpose of this communication to demonstrate fine structural similarities between these normal adult ganglia and their long-term cultured counterparts. This is the first of a group of ultrastructural studies of cultured rat dorsal root ganglia. The reports immediately following (23, 24) present a detailed description of the effects of in vitro X-irradiation on the mature cultures.

MATERIALS AND METHODS

Cervical or lumbar (occasionally thoracic) dorsal root ganglia were removed from fetal rats after 16½–19 days in utero. Ganglia taken from the older fetuses were preferred for electron microscopic study because they retain, even in long-term cultures, a number of layers of neurons in the explant area. One or two ganglia were placed on a No. 1 round (22 mm in diameter) collagen-coated (25) coverslip and maintained in a Maximow double-coverslip assembly. The cultures were fed at explantation and twice a week thereafter with one drop of a complex feeding mixture, after washing in balanced salt solution (BSS). This feed consisted of one part human placental serum, one part 9-day chick embryo extract, one part bovine serum ultrafiltrate, and one part BSS. The mixture was supplemented with glucose to give a final concentration of 600 mg % and with penicillin to give a concentration of about 200 units per ml. Light microscopic observations were made directly on the living culture (up to 600 × magnification) or on the whole mount after fixation and staining for myelin (Sudan black B), Nissl substance (toluidine blue), nerve fibers (Holmes' silver), or connective tissue elements (Foot-Bielschowsky).

For electron microscopy, representative ganglia were selected at intervals after 4 wk in vitro. (Neuronal maturation and widespread myelination require 6–8 wk in culture.) They were rinsed free of feeding medium with BSS, fixed in Veronal acetate–buffered OsO_4, dehydrated in ethanol, embedded in Epon, sectioned for light or electron microscopy, and examined in a Zeiss light microscope or an RCA EMU 3G electron microscope. The details of handling this type of culture for electron microscopy are given in reference 26.[1] 22 of the cultures so prepared were studied electron microscopically (after 24, 32, 33, 37, 39, 42, 56, 72, 77, or 79 days in vitro). No indication of infection by bacteria or higher microorganisms was encountered in the electron micrographs.

The cultures were thus analyzed: (*a*) in the living state in the Maximow assembly, (*b*) in the whole mount after fixation and staining on the original coverslip, (*c*) in toluidine blue–stained (28) 1 or 2 μ-sections of plastic-embedded cultures ("semithin sections"), and/or (*d*) in lead citrate–stained (29) thin sections viewed in the electron microscope. In *c* and *d*, realignment of the tissue blocks as described in reference 26 allowed examination of the culture in desired planes of section.

OBSERVATIONS AND COMMENTS

In explanting the dorsal root ganglion, the central and peripheral branches of the dorsal root are cut near the edge of the ganglion. In culture the neurons remain in a central, more or less compact mass while the nerve fibers grow out for a distance of 5-10 mm, thus reaching areas near the edge of the coverslip. These neurites may sprout from the cut ends of the original root or they may radiate from all parts of the explant. The outgrowing

[1] The method involves embedding the culture without its removal from the coverslip. The consistently good fixation of this cultured tissue may be due, at least in part, to the lack of mechanical handling, an idea likewise entertained by Webster and Collins (81) for in situ ganglion. The problem of removing the carrying coverslip from the culture after embedding directly on glass was solved by Robbins and Gonatas (27) by initially coating the glass surface with a heavy coat of carbon. Their technique works well in the present culture system, but the carbon coat must be limited to the central region of the carrying coverslip to give the collagen coat an adequate foothold on the coverslip periphery. Our present experience indicates that cultures develop normally on coverslips so prepared.

nerve processes may traverse a zone near the explant in considerable disarray and then become organized into bundles more peripherally (Fig. 1). Each bundle contains both myelinated and unmyelinated fibers and their accompanying Schwann cells. The neuronal mass is encapsulated by connective tissue (Fig. 2), and fiber bundles are associated with perineurial sheaths (Fig. 10). Each of these areas will be described separately with correlated light and electron microscopic observations under the headings: neuronal somas, satellite cells, and nerve fiber bundles.

Neuronal Somas

In the living culture (Fig. 3), neuronal somas are seen in a scattered monolayer or, more often, in a number of layers. The neurons do not migrate. The somas are round, elliptical, or angular in shape and measure 11.5–50 μ in diameter (30), as compared to < 18–75 μ for adult rat lumbar ganglia neurons in vivo (21). The vesicular nucleus occupies about one-half the neuron diameter in the smaller neurons, about one-third in the larger. It is near the cell center but rarely appears exactly central; it is almost always eccentric in the smallest neurons. The rounded nuclear profile rarely shows indentations. Most cultures contain a few binucleate neurons which are the largest in size. The nucleolus, one to three per nucleus, is clearly resolved against the lighter nucleoplasm and occupies about one-fourth the diameter of the nucleus. The cytoplasm displays a fine granularity which is more concentrated in the perinuclear area. The quality of this granularity is considered to be an index of the general health of the neuron—when it becomes more or less extensive or coarser or becomes differently distributed, the health of the neuron is in question. A culture which contains 3–5% neurons with altered cytoplasmic granularity is not considered abnormal.

In whole mount preparations stained with Sudan black, the normal fine cytoplasmic granularity is particularly well seen. The moderately dense granules are generally dispersed throughout the cytoplasm, but in some large neurons they are more concentrated around the nucleus. Occasionally, one sees, scattered through the cytoplasm, more dense 1–2 μ droplets thought to be neutral fat. Clear axon hillock regions are seldom found and the path of the emerging axon is difficult to follow in these preparations. In formalin-fixed whole mounts stained for Nissl substance, neurons show discrete Nissl bodies scattered throughout the cytoplasm or, in some of the larger neurons, concentrated more peripherally.

The general organization and placement of neurons is best seen *in semi-thin sections* (Figs. 2, 4). In cross-section, the neurons can be seen grouped or scattered in a number of layers among fibers and Schwann cells, all of these structures encased within a sheath of flattened connective tissue. The smaller neurons tend to be grouped together more closely and, possibly as a consequence, tend to be more angular in shape than the larger neurons (as in reference 21). The neurons are not flattened in the plane of the coverslip. Cytoplasmic organization varies as illustrated in Fig. 4. Neuron 1 (n_1) is small and contains fairly dense and homogeneous cytoplasm. Neuron 2 (n_2), on the other hand, is larger with lighter and less homogeneous cytoplasm in which one can discern small light patches, particularly in the perinuclear region. These light spots correspond to the areas containing neurofilaments as seen in the electron microscope. In neuron 3, the differing granularity of the perinuclear and peripheral zones is more marked. Neuron 4, a binucleate cell, demonstrates that a lighter axon hillock area may sometimes be found in these preparations and that the exiting axon is straight, as is often the case. Coiling of the axon into a glomerulus has not been observed in any of the light microscopic preparations thus far. Fig. 4 also illustrates the frequent position of the nucleolus flattened against the nuclear membrane, as Andres (21) noted in situ. Bourne and Tewari (31) have ascribed functional significance to this configuration in dorsal root ganglion neurons in vivo.

In the electron microscope, the spheroidal or elliptical profile of the *nucleus* may occasionally display irregular shallow undulations or indentations (Fig. 5); only rarely are deep indentations or folds visible. The nucleus is usually situated in a paracentral rather than central position as Andres (21) found in rat spinal ganglia in situ. Chromatin is finely dispersed with only occasional foci of clumping, generally not near the nuclear envelope. Often one or two nucleoli are visible. The nucleolus is made up of the usual components, i.e., dense granules and less well-defined units of smaller diameter. The latter are disposed as dense aggregates or, less frequently, as light patches, and are presumed to be the filaments observed by others. Nucleolar components are organized into a tightly woven nu-

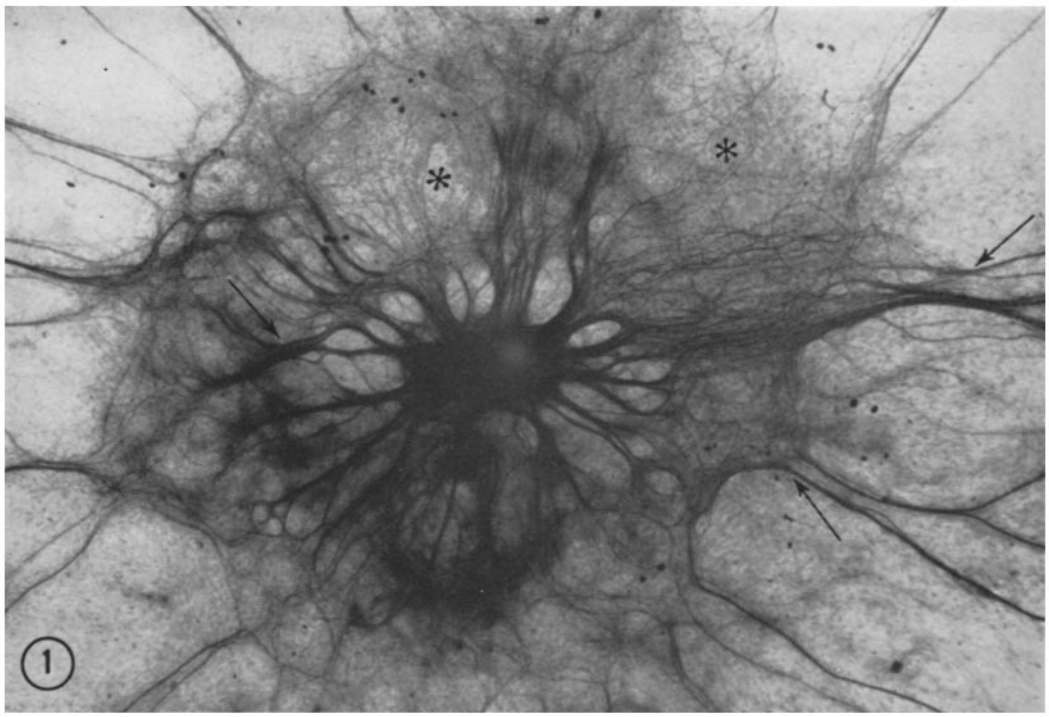


FIGURE 1 This is a survey photomicrograph of a dorsal root ganglion culture. Extending from the central dense area, which contains the neuronal cell bodies, are nerve fibers gathered into fascicles (arrows) of varying thickness. Some fibers pass through a zone (best shown at *) where they lose their fascicular arrangement and occur singly. About one-half the length of the peripherally directed fascicles is shown; these fascicles terminate near the edge of the carrying coverslip. Adventitial cell nuclei create the background pattern. Whole mount fixed with OsO_4 and stained with Sudan black. × 20.

cleolonema. Protuberances from nucleoli and nucleolar satellites noted in vivo (21) were not observed. Often skirting the nucleolus, and in other areas of the nucleus as well, are loosely clustered, irregularly shaped, highly dense particles ~400 A in diameter. Comparable particles have been noted in rat spinal ganglia in vivo (32). The nuclear envelope is studded with pores. When the envelope has been sectioned tangentially (Fig. 7), the pores are seen to be 680–1050 or 300–500 A in outside or inside diameter, respectively, and may display a central density. Dimensions reported by others studying mammalian spinal ganglion neurons in vivo are: 850 A (outside diameter, 33), 280–360 A (inside diameter, 34) and 1000–1100 A or 400–500 A (outside or inside diameter, 21). The inner membrane of the envelope appears thicker, denser and smoother than the ruffled outer membrane, as Palay and Palade (34) and Andres (21) pointed out. The outer membrane may be dotted with ribosomes or may display vesicle-like or cisternal outpocketings.

In thin sections, *mitochondria* appear round, ellipsoidal or rodlike, with cristae mitochondriales oriented in different directions but usually in a plane perpendicular to their length. The mitochondrial matrix is moderately dense and contains particles of much greater density (Fig. 7). These mitochondria are typical for neurons (34). Occasionally, irregularly contoured mitochondria display a matrix of diminished density with cristae mitochondriales situated mainly peripherally. They may occur near normal-appearing mitochondria. Similar mitochondria were found by Cervós-Navarro (22) in rat spinal ganglia in vivo though he noted that these two forms of mitochondria did not occur in the same neuron.

A *Golgi area* appears as a typical straight or curved complex of closely packed cisternae and vacuoles surrounded by sprays of small vesicles

(Fig. 7). In a thin section, a number of such complexes are scattered around the nucleus (Fig. 6); their placement in a complex reticular network around the nucleus as shown by the light microscopist (35) can be easily envisioned. In rare cases, a cisterna appears partially "coated." Coated vesicles are frequently encountered in the vicinity of these Golgi complexes (or near the plasmalemma). Dense-cored vesicles may be present in Golgi areas and, in rare instances, appear coated.

Also associated with the Golgi complex are the *multivesicular bodies* first described in neurons by Palay and Palade (34). At one extreme, the multivesicular body may be large, irregularly shaped, and seemingly empty but for a vesicle or two. At the other, it appears as a smaller sphere filled with dark vesicles or discs and patches of dense substance arranged in a dense matrix (comparable to "dark" and "light" multivesicular bodies, ref. 36). All types may display vesicle-like outpocketings and a plaque (rarely two) of fuzzy or radially striated dense substance on the outside of a flattened portion of the limiting membrane (Fig. 7). Such configurations have been described primarily in central nervous tissue (reviewed in 26) but also in toad spinal ganglion (36). Multivesicular bodies appear most frequently in association with the Golgi complex or in the vicinity of the plasmalemma. (Those near the somal surface more often seem to be of the irregularly shaped, empty-appearing type.)

Dense bodies (lysosomes?), seen by many electron microscopists studying neurons, are scattered, usually sparsely, throughout the perinuclear cytoplasm. Some of the smaller ones contain barely discernible vesicles, reminiscent of the multivesicular bodies. Others of varying shape are uniformly dense or may exhibit aggregates of highly dense particles and/or stacked linear densities (Fig. 7). They lack the peripherally located vacuoles of contrasting density which characterize the lipofuscin found by other investigators (21, 22, 37, 38) in mammalian spinal ganglion neurons; this is undoubtedly due to the young age of the cultured tissue. *Lipid droplets*, encountered occasionally in the perinuclear or peripheral cytoplasm, have a dense, wavy profile and an empty-appearing interior. *Centrioles* and *cilia* are present very rarely.

Nissl substance, or granular endoplasmic reticulum (34), occurs throughout small somas or principally in the periphery of large perikarya. In small cell bodies, the Nissl substance appears uniformly distributed (Fig. 5) whereas in the larger somas it may appear as small discrete bodies, as large aggregates (*ns*, Fig. 6), or more rarely as a thick, dense, nearly uninterrupted ring as Andres (21) noted in rat dorsal root ganglia in situ. The discrete Nissl bodies are noted in those somas containing prominent neurofilaments. Nissl substance often extends to the plasmalemma. The cisternae and vacuoles of endoplasmic reticulum are usually only partially covered by ribosomes; most ribosomes, in the form of rosettes, lie free in the intervening cytoplasmic matrix. The cisternae usually display little apparent orientation when viewed in a plane either perpendicular or parallel to the coverslip on which they are grown, but they occasionally may be situated in the parallel arrays illustrated for rat dorsal root ganglia in vivo (22, 34, 80). When the cisternal membrane has been grazed during sectioning (Fig. 7) the ribosomes are in typical single or double rows which may be straight,

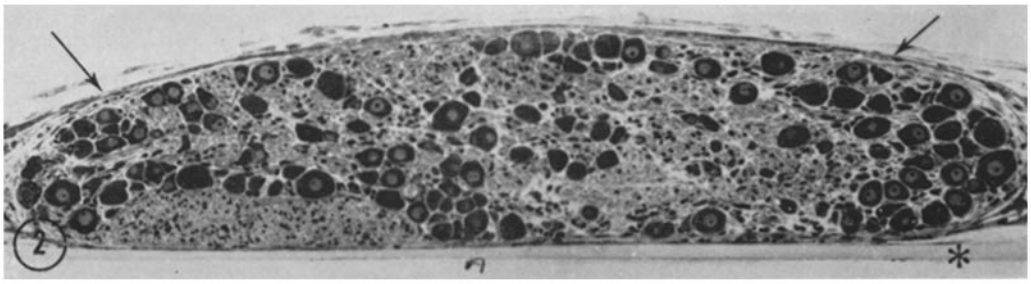


FIGURE 2 A particularly thick explant region is illustrated in this figure. This stained semi-thin section was cut in a plane perpendicular to the glass coverslip. The explant region, composed of neurons and numerous fiber bundles, is surrounded by a sheath of highly flattened cells (arrows). The bottom of the culture is at the lower edge of the figure. Beneath the culture the collagen film coating the coverslip appears as a clear zone (*). × 360.

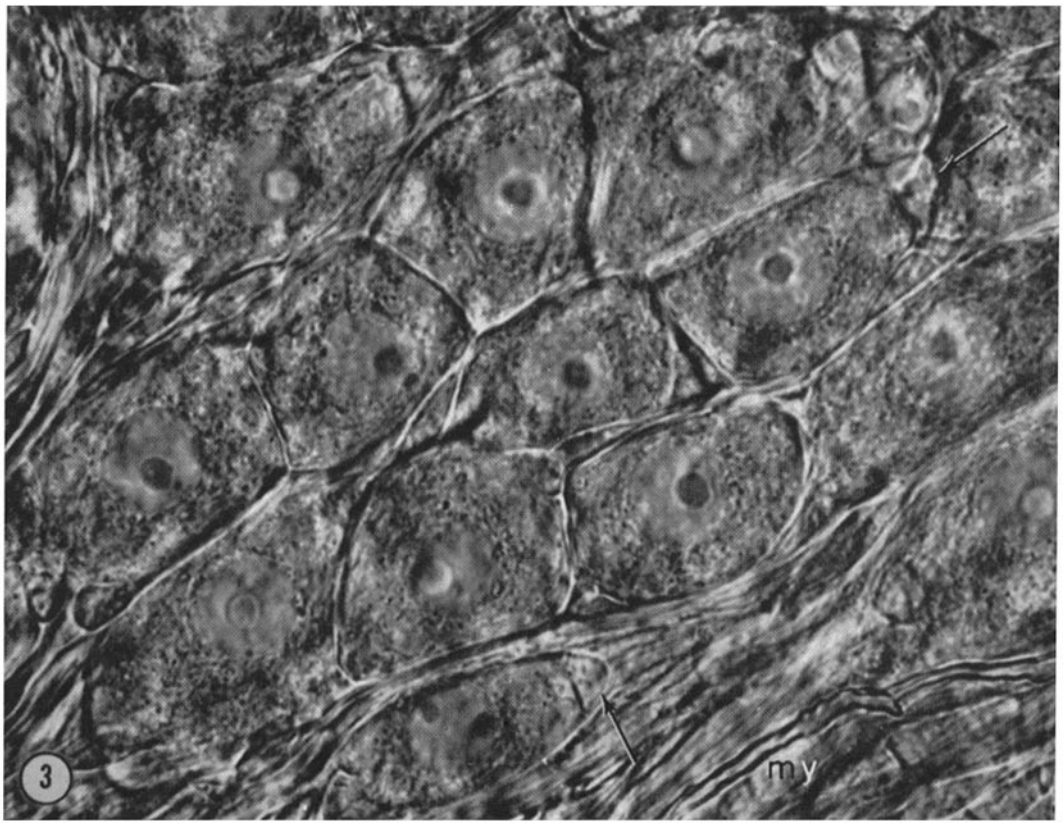


FIGURE 3 This photomicrograph shows the appearance of living neurons. Nucleoli are prominent in the light nucleoplasm; the cytoplasmic granularity illustrated here is typical for healthy neurons. Most of the cells shown have assumed an angular outline. Related satellite cells (arrows) and a myelin sheath (*my*) may be seen. Bright-field microscopy with 40 × fluorite oil lens with 1 mm working distance. × 1,050.

curved, e- or c-shaped, or looped, or form a circle as in situ (34).

Subsurface cisternae are present as small vacuoles, as larger vacuoles or cisternae partially covered by ribosomes (Fig. 7), or as ribosome-lacking, highly flattened cisternae which follow closely the ruffled contour of the plasmalemma. Very rarely a cisterna is seen to veer away from the subsurface position into the cytoplasm where it widens and displays more ribosomes, thus becoming indistinguishable from granular endoplasmic reticulum. The relationship between subsurface cisternae and granular reticulum is far less conspicuous than it is in spinal cord cultures (26; or cord in situ, 39, 45). Also, the cultured ganglion subsurface cisternae are less broad and less highly flattened than those in cultured spinal cord, in agreement with conclusions drawn from a study of in situ rat tissue (39).

Typical *neurofilaments* are present, predominantly in the wide perinuclear zone with the mitochondria, Golgi apparatus, and dense bodies (Fig. 7). Their diameter generally falls into the 70–100 A size range indicated by others (21, 34). The smallest neurons contain only a few which are hardly noticeable among the other cytoplasmic constituents. The largest somas display far more neurofilaments which are often arrayed in loose bundles. Moreover, these aggregates create prominent "roads" in the cytoplasm ("Plasmastrassen," Andres, 21), predominantly in the perinuclear area, but also spreading peripherally where they may seemingly divide the Nissl substance into discrete bodies (Fig. 7) (as in reference 34, also).

Fibrillar areas or linear streaks lacking the normal fine cytoplasmic granularity have been observed in living neurons by a number of investigators; Pomerat et al. (14) have clearly demonstrated the rapid movement of dense particles in these regions by means of time-lapse cinematography. Accompanying the neurofilaments are *tubules* similar to the microtubules found in many mammalian cell types, including neuronal somas (40–42, among others). Their outside diameter in our samples averages 230 A. All neurons contain at least a few such tubules. They are often seen lying near and parallel to the plasma membrane.

In the preceding description, *differences between large and small neurons* have been mentioned. The smallest neurons tend to be grouped together, are more angular in shape, contain Nissl substance dispersed throughout the perikaryon, and are nearly devoid of neurofilaments. The largest neurons, on the other hand, tend to occur singly, are more rounded, display a perinuclear zone in which mitochondria, Golgi complexes, and dense bodies are concentrated, and contain Nissl substance which is more concentrated in the periphery of the perikaryon. Their most distinctive feature is the dilution of these cytoplasmic components by low density areas containing neurofilaments. These are, therefore, the cells which other electron microscopists have labeled "light" (33, 37, 41) or "Type A" (21, 43) as opposed to the small "dark" or "Type B" neurons. Andres (21) has further subdivided each type of neuron on the basis of nuclear properties, perikaryal size, predominance of "roads," axonal diameter and spiralling (glomerulus), and axonal ensheathment (myelin). The small number of exiting neurites viewed and the wealth of transitional forms found militate against such elaborate classification in our preparations.

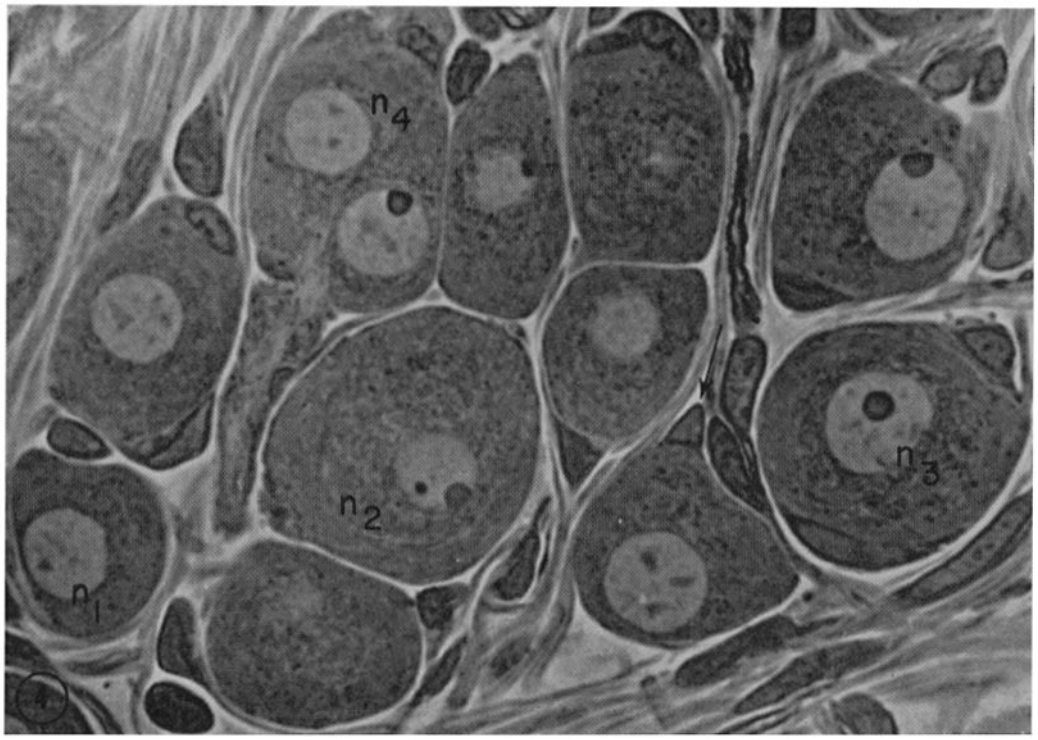


FIGURE 4 Variously sized and differently organized neurons (see text) are shown in this photomicrograph of a stained semi-thin section. As usual, the larger neurons are more rounded than the smaller neurons. A rarely seen axon hillock region is present in the binucleated neuron (n_4). The largest neurons (e.g., n_2) display lighter areas ("roads") in the cytoplasm. A "dunce cap" satellite cell is designated by an arrow. × 1,100.

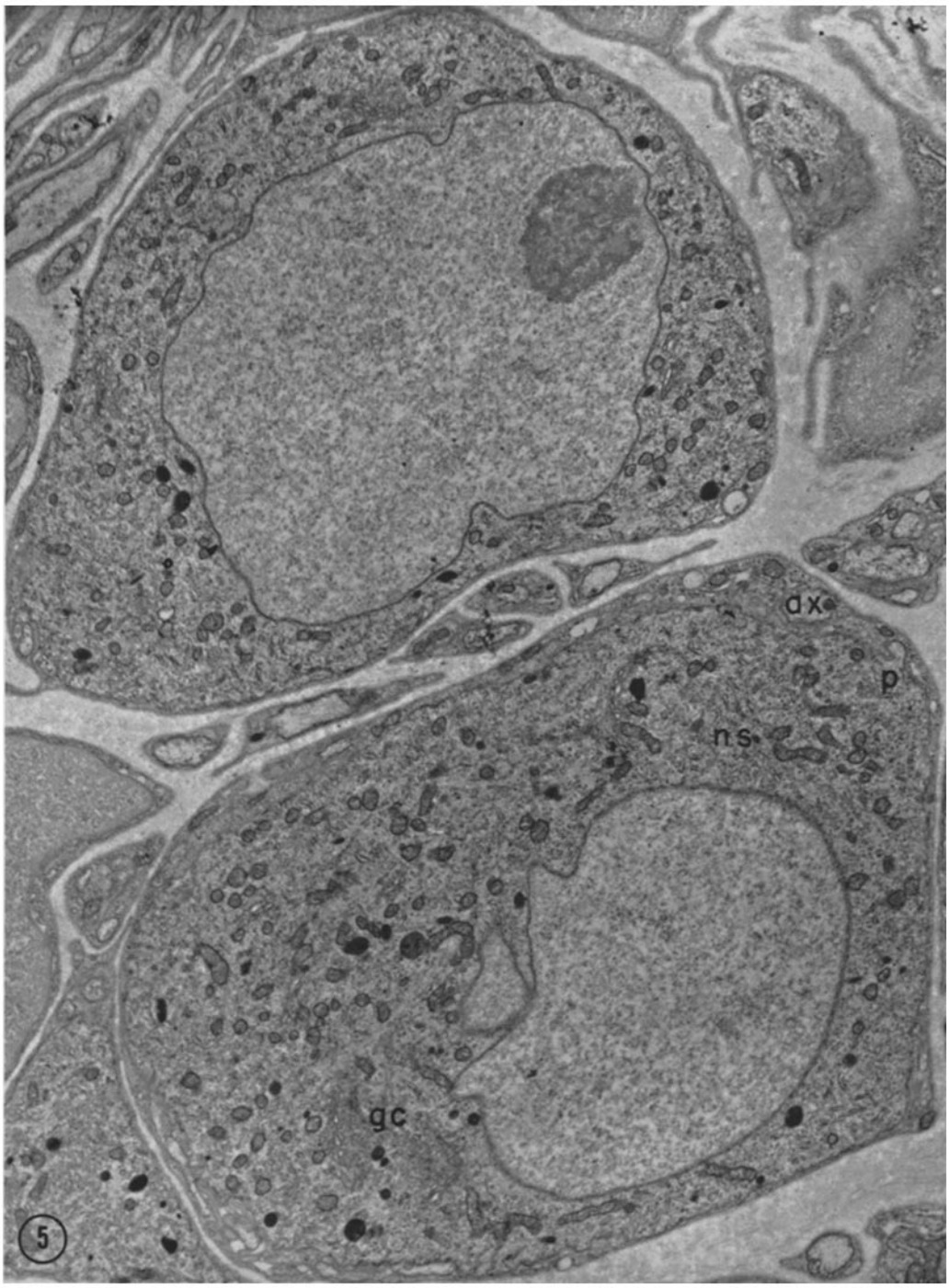


FIGURE 5 This electron micrograph portrays the small neurons in this culture system. Their organelles are quite evenly distributed throughout the cytoplasm, and neurofilaments are rare. A spinelike process emerges from the lower neuron at *p*. The neurons are completely covered by satellite cells. Invaginated into one of the satellite cells is an axon (*ax*); at higher magnification, a mesaxon can be followed from this axon to the satellite cell outer surface. *gc* = Golgi complex; *ns* = Nissl substance. × 8,500.

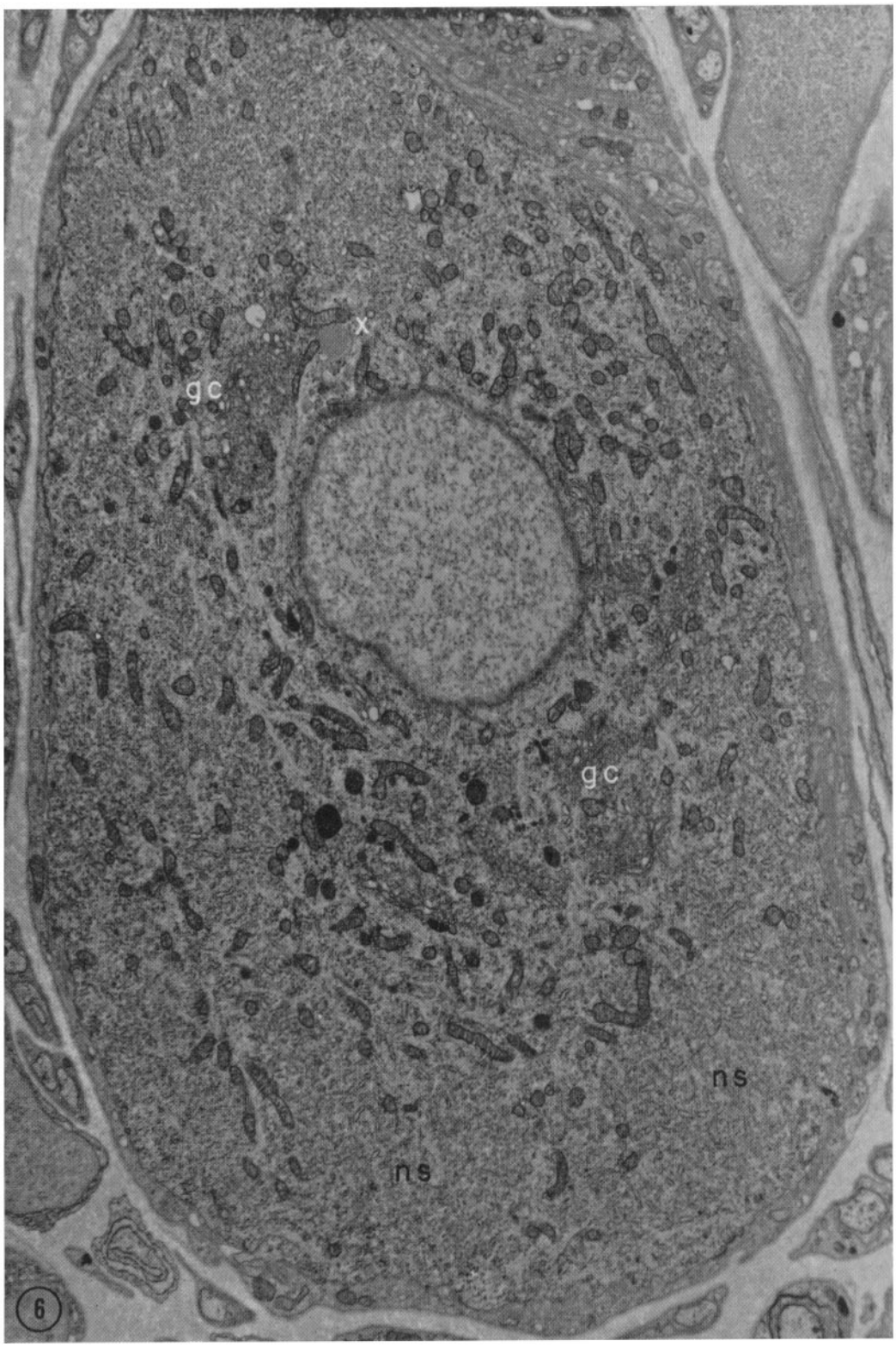


FIGURE 6 Features of the large cultured neurons are shown in this electron micrograph. Mitochondria, Golgi zones (*gc*), dense bodies, and slender bundles of neurofilaments occupy a wide perinuclear zone. Nissl substance (*ns*) is concentrated more peripherally. Satellite cell cytoplasm everywhere ensheathes the neuron surface. The aggregated granular material of unknown nature at *x* is frequently seen in cultured neurons. × 8,500.

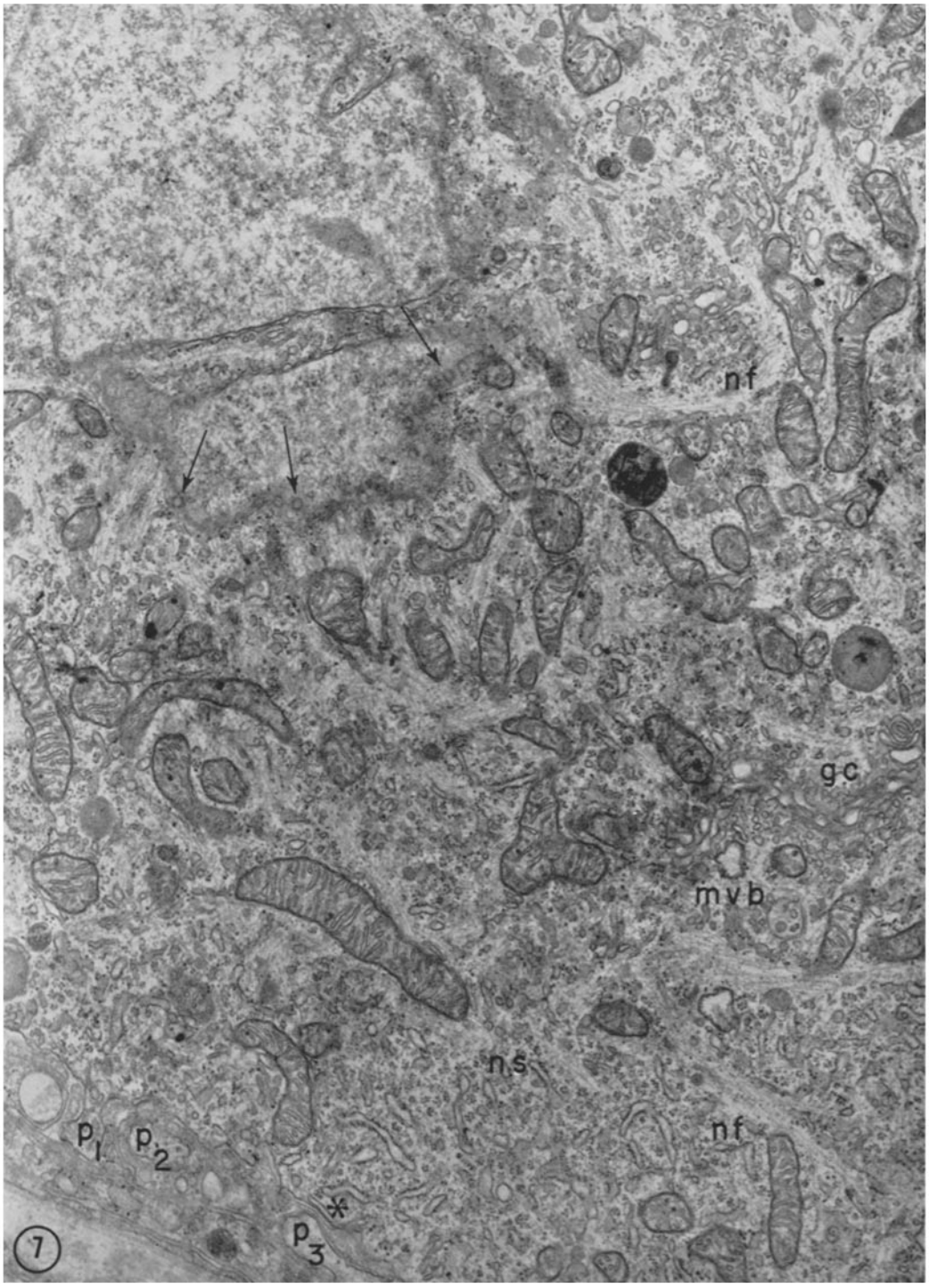


FIGURE 7 This electron micrograph illustrates at higher magnification the type of cytoplasm characteristic of larger neurons. Mitochondria, Golgi complex (*gc*), Nissl substance (*ns*), dense bodies, neurofilaments (*nf*), a multivesicular body (*mvb*), and nuclear pores (at arrows) are included. A subsurface cisterna is indicated by an asterisk. Projections (p_1–p_3) from the neuronal soma, only one of which arises at this level (p_1), are commonly found embedded in satellite cell cytoplasm. × 20,000.

It appears to us that the larger the neuron, the more prominent are the bundles of filaments and resulting cytoplasmic partitioning. Andres reviews the literature suggesting a correlation between the neuronal types and different sensory modalities. The consistent variation in cytoplasmic organization found by electron microscopists should help to resolve the long-standing question (35) concerning the role of faulty preservation in creating different types of dorsal root ganglion neurons.

Single particles presumed to be *glycogen* are occasionally found throughout the cytoplasm or grouped in sizeable aggregates most often pocketed just beneath the plasma membrane. These spheroidal or irregularly shaped particles range from ~190 to 350 A in diameter and stain intensely with the lead solution employed, but not with aqueous uranyl acetate. In these properties, they correspond to particles identified as beta-glycogen in other tissues, as summarized by Revel (44). A few times, we have observed these particles to be enmeshed in a large array of oriented membranous cisternae. This configuration resembles the "glycogen body" found in ethionine-treated liver by Steiner et al. (63). Some of the involved cisternae were continuous with cisternae of granular endoplasmic reticulum. Regarding mammalian dorsal root ganglia, glycogen has thus far been described as occurring normally only in embryonic stages (41, 46).

The neuronal plasmalemma consistently appears more ruffled (Fig. 8) than the apposed plasma membrane of the satellite cell, as indicated previously (21, 22). *The neuron-satellite cell boundary* is greatly complicated by a plethora of processes from both the neuron and the satellite cell, as many electron microscopists have documented (21, 37, 48–52). Slender evaginations of indeterminate length arise from the perikaryon (large or small) and invaginate attenuated or perinuclear portions of satellite cells (Fig. 5) or extend along the gap between soma and satellite cell (p_3, Fig. 7). These "spines" do not completely penetrate the satellite cell sheath and, therefore, do not appose the basement membrane on the outer surface of the satellite cell. Cross-sections of similar appearing processes in which their connection to the soma is not present are surrounded by a circle rather than a mesaxon of satellite plasmalemma (p_2, Fig. 7). Similar to neuronal processes demonstrated in electron mi-

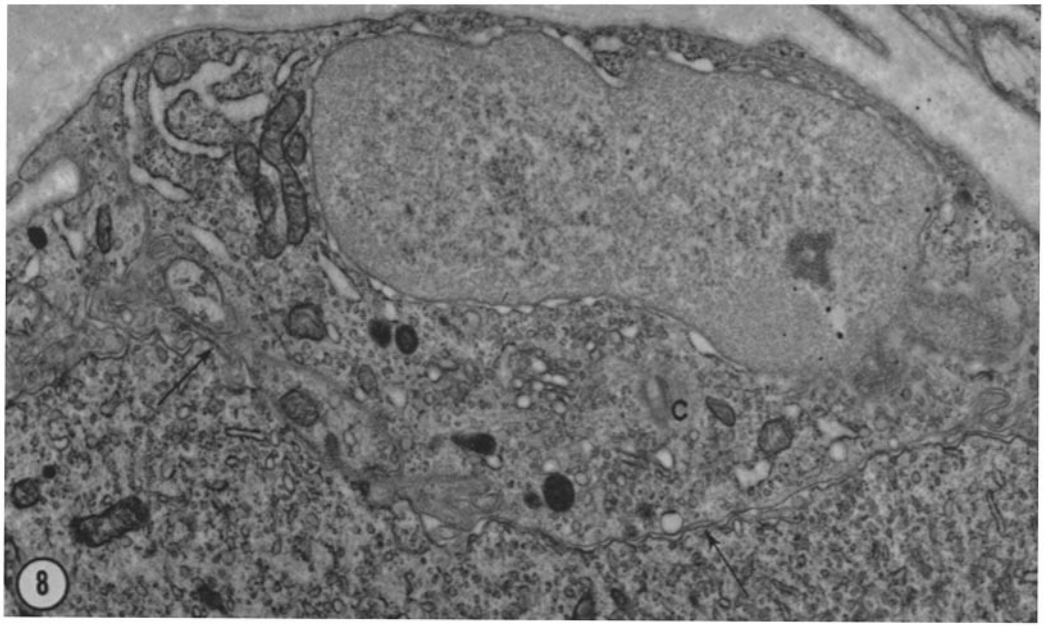


FIGURE 8 This neuronal satellite cell contains the usual cytoplasmic organelles, including cisternae of granular endoplasmic reticulum. A centriole (*c*) appears at this level. The chromatin pattern is characteristic for satellite cells and Schwann cells in the cultures. The closely apposed satellite cell and neuron plasma membranes are indicated by arrows. × 16,500.

croscopic studies of in vivo adult mammalian spinal ganglia (37, 48–50, 52, 53), these may represent the pericellular and intracapsular network of fine unmyelinated fibers (subcapsular dendrites?) observed in the light microscope (reviewed in 49, 54). Additional neurite-like processes harbored within satellite cell cytoplasm differ from those just described in two ways. They are larger in diameter than the slender somal spines (but not so large as the main axon) and they are surrounded by an externally directed mesaxon (of satellite cell membrane), indicative of an origin outside the neuronal perikaryon. Are these branches of the exiting axon (recurrent collaterals?) or axons from other neurons? Rosenbluth and Palay (50) found "axonal twigs" surrounded by a mesaxon of capsule cell membrane in rat dorsal root ganglia in vivo. In a study of dissociated chick spinal ganglion cells maintainted in vitro, Nakai (55) demonstrated the formation of collateral processes which sometimes turned back to the region of the perikaryon. Whatever these processes may be, neither we nor anyone else have found electron microscopic evidence that they establish typical synaptic connections with the soma.

In general, the *main axon* does not exit from a well demarcated axon hillock region. This may be, in part, a reflection of the somewhat reduced size of neurons in the cultures, because Andres (21) rarely encountered hillock areas in the smaller spinal ganglion neurons. The exiting axon may contain Nissl substance in its most proximal portion, occasionally in the form of well oriented, long cisternae of endoplasmic reticulum less densely covered with ribosomes and more shallow in depth than in the neighboring perikaryon. Such configurations have been found in proximal dendrites of spinal cord motor neurons by Bodian (45). In thin sections, evidence for a glomerulus is generally lacking; in only a few cases, two or three or more portions of the axon may be seen in one plane. This finding is in agreement with conclusions drawn from in vivo studies. Pannese (52) points out that the glomerulus is rather uncommon in rat dorsal root ganglia and that those present display relatively few coils. The glomerulus appears with increasing maturity of the tissue. Andres (21) reports that only the larger dorsal root ganglion neurons display a well developed glomerulus.

Satellite Cells

In the living culture, satellite cells are consistently associated with neuronal perikarya. The smallest

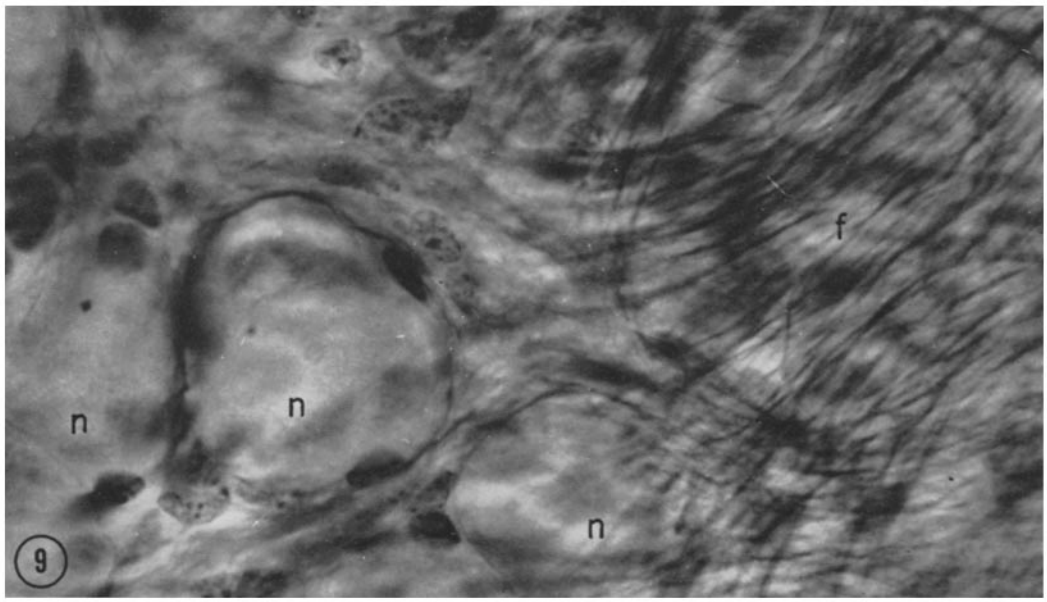


FIGURE 9 This photomicrograph illustrates the presence of reticulin following Foot-Bielschowsky silver impregnation. The reticulin is oriented as a capsule around the neuron (*n*)–satellite cell complex and outlines the nerve fibers in an emerging fascicle (*f*). × 1,050.

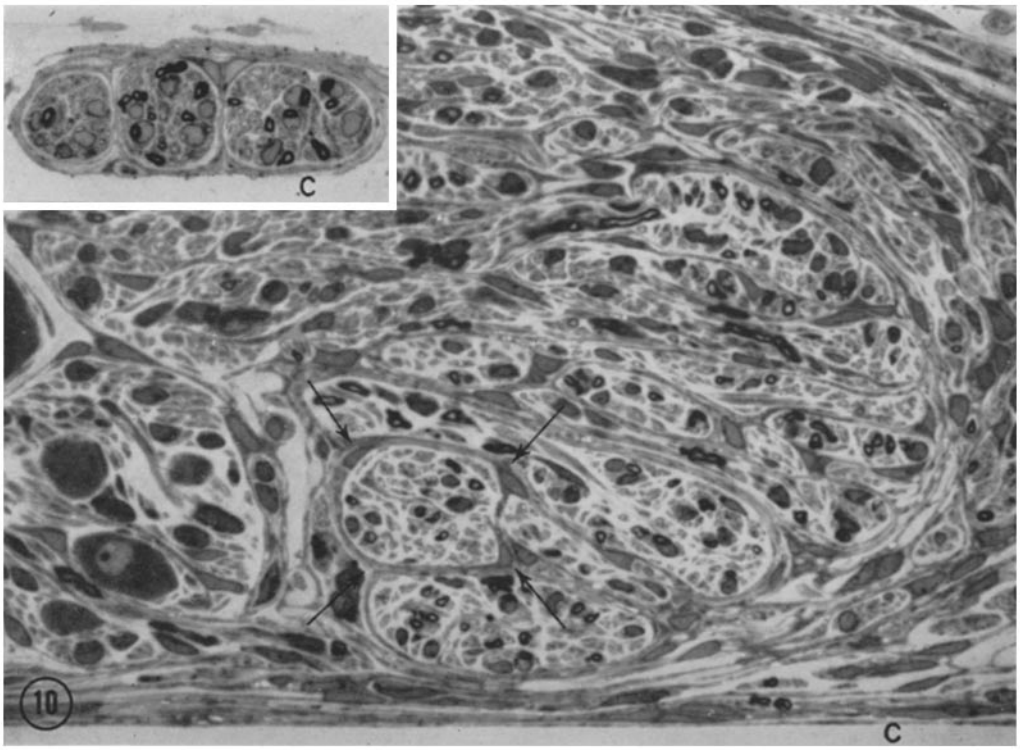


FIGURE 10 Well organized nerve fiber bundles are shown in these stained semi-thin sections of the explant region and, in the inset, of the outgrowth (~0.5 cm from the explant). Flattened perineurial cells (arrows) typically enclose a number of unmyelinated and myelinated fibers associated with Schwann cells. The collagen substrate is at *c*. Figure, × 590; inset, × 1,150.

somas are invested by two or three cells, and as many as ten to 12 may be found on the surface of the largest neuronal cell bodies.

In semi-thin sections, no more than two satellite cell nuclei are found at one level (Fig. 4). Some nuclei are elliptical and sit on a flattened portion of the neuronal soma in such a way that the resulting topography conforms to the curvature of the soma. Other nuclei are nearly triangular and perch atop the neuron like "dunce caps" (Fig. 4). In addition, there are some nuclei which appear perineuronal, but are actually separated from the soma by basement membrane and collagen (as revealed by electron microscopy). Thus, they are related to the satellite cell-neuron complex, much as pericytes are situated on blood vessels. Nuclei are nearer the outer membrane of the satellite cell than the inner one. Nucleoli are visible. The nucleoplasm is more dense than that in the neuron. This density is comparable to that in Schwann cell nuclei and is definitely greater than in the nucleoplasm of the fibroblasts. This is substantiated in electron micrographs—the arrangement of components in satellite and Schwann cell nuclei appears identical and contrasts with the more dispersed chromatin pattern in fibroblasts.

In the electron microscope, the satellite cell sheath is seen to cover the neuron completely (Figs. 5, 6). Substances outside the satellite cell must reach the neuron either by traversing the satellite cell or by moving through the small gap between neighboring satellite cells, as earlier electron microscope studies implied (21, 37, 49, 51, 52). There is recent physiological evidence for substantial movement of material through such narrow intercellular gaps in nervous tissue (reviewed in 56). The satellite cell layer is thin over much of the neuronal soma. More than one layer may be present. The space between satellite cell and soma is fairly constant except where neuronal "spines" arise or groups of entangled, slender satellite processes indent the neuron (as in vivo, 50); here the space is uneven

(as in 21) and increased. Foci of intertwined satellite cell processes are more often associated with the larger neurons. Whereas satellite cell processes may cause shallow indentations in the neuronal profile, they do not invaginate the neuron in the way that neuronal "spines" tunnel into satellite cell cytoplasm.

Chromatin is more concentrated in the satellite cell nucleus than in the neuronal nucleus (as mentioned in 37, 51, 53) and, in the best preserved nuclei, clearly consists of two components. Near the nuclear envelope there is mainly fine-textured material whereas, more centrally, some nuclear components are larger and more dense, a pattern similar to that illustrated by Hay and Revel (57) in interphase nuclei of regenerating salamander limbs.

The most striking feature of the perinuclear cytoplasm is the common occurrence of oriented cisternae of granular endoplasmic reticulum. Such parallel arrays in situ have been observed by Pannese (52), but not by others (21, 37, 49, 51, 53) The cisternae, sometimes branched, may be densely covered by ribosomes and contain filamentous material. In some areas, cytoplasmic density is increased by the close packing of cisternae of granular endoplasmic reticulum and mitochondria. Golgi complex consists of the usual vacuoles, cisternae, and vesicles. The lysosome-like bodies may be spotted with a more dense material. Multivesicular bodies, coated vesicles, centrioles, and an occasional filament are present. Microtubules are clearly visible and, particularly in the attenuated portions, follow the cell contour. Vesicles are numerous, as earlier investigators found (21, 52, 58), but may be nearly twice as large (600–1300 A) as their in vivo counterparts; they are more often lined up near the membrane facing the neuronal soma rather than the outer membrane, in agreement with observations made in vivo (21, 52). Some of these appear coated. Many vacuoles of varying size are seen. Some of the larger vacuoles and the often widened nuclear envelope and cisternae of granular endoplasmic reticulum probably result from the preparative procedure.

The outer plasma membrane is smoother than the membrane facing the neuronal soma and is everywhere covered by a basement membrane. This basement membrane closely follows the cell contour, including the slender processes (or flat sheets?) of cytoplasm emanating from the satellite cells, but typically does not extend into the gap between apposed satellite cells. The number and complexity of evaginations from the outer surface of the satellite cell is greater in older cultures. The pericyte-like satellite cell referred to earlier is separated from the perineuronal satellite cell by the two apposing basement membranes and intervening collagen fibrils. It is not related to any nerve fibers. The difficulty one has in distinguishing these from Schwann cells points to their morphological similarity. Wyburn (49) calls them Schwann cells.

Nerve Fiber Bundles

In the living culture, bundles of nerve fibers are seen to radiate out from the explant (Fig. 1). Schwann cell nuclei are spindle-shaped, whether they be related to myelinated or unmyelinated fibers (as in Fig. 1, reference 24), and on myelinated fibers they are usually located midway between nodes and are fitted into a curvature of the sheath, partially constricting the enclosed fiber. In these mature cultures, myelin sheaths are rarely smooth; rippling and bleblike projections occur frequently along the internode and may be further exaggerated into long, finger-like projections near the node (17, 59; as in Fig. 1, reference 24). The length of the internode averages $\sim$128 μ, varying from 54 to 246 μ. These internodes are substantially shorter than their in vivo counterparts, as has been pointed out in reference 30. Occasionally, miniature internodes ($\sim$10 μ long) are observed, much as greatly shortened ("intercalated") internodes are seen rarely in normal adult peripheral nerve (60) and more often in regenerating nerve (61). Details of the frequently observed Schmidt-Lanterman clefts, which form and may undergo alterations during in vitro existence, appear elsewhere (1, 17, 62). Since the clefts are found in living cultures, they cannot be considered to result from preparative procedures (1). In the polarizing microscope internodes are birefringent (1; as in Fig. 8, reference 24). Areas between fascicles contain characteristically flattened fibroblasts. During the first few days of culture, capillaries migrate from the explant in both solid and tubular forms; in time, they lose their distinctive organization and may no longer be recognizable.

In the whole mount stained with Sudan black, the entire internode is sudanophilic and the myelin features just described are confirmed. By comparing cultures before and after OsO_4 fixation and

Sudan black staining, it was observed that this type of preparation faithfully preserves myelin irregularities and causes little shrinkage; i.e., internodal length is decreased by less than 2%. Branching of the myelinated neurites is observed, always at a node of Ranvier. With the Holmes' silver method, nerve fibers are seen to terminate as a simple spray of branches with bouton swellings among fibroblasts in the outgrowth. Functional aspects of these terminals are not known but it may be noted that similar appearing endings on muscle ribbons in other preparations participate in reflex arc activity (8; E. R. Peterson and S. M. Crain, unpublished observations). With the Foot-Bielschowsky silver stain, thin black fibrous sheaths are present in fascicle areas and in capsules around neurons, indicating the widespread occurrence of reticulin (Fig. 9).

In semi-thin sections, a fascicle (designated by arrows in Fig. 10) is demarcated by a complete or partial sheath of perineurium and usually contains, regardless of the number of fibers, a few myelinated and many more unmyelinated fibers plus associated Schwann cells. Following thorough acquaintance with the living cultures, it was surprising to find such high proportions of unmyelinated fibers in heavily myelinated cultures both in these preparations and in electron micrographs. The perineurial cells are greatly flattened and display nuclei which are lighter than those in nearby Schwann cells. The seemingly clear areas evident in Fig. 10 will be shown below to contain endoneurial components.

In the electron microscope, a typical fascicle is seen to consist of a perineurial sheath partially or completely enclosing Schwann cells and their mye-

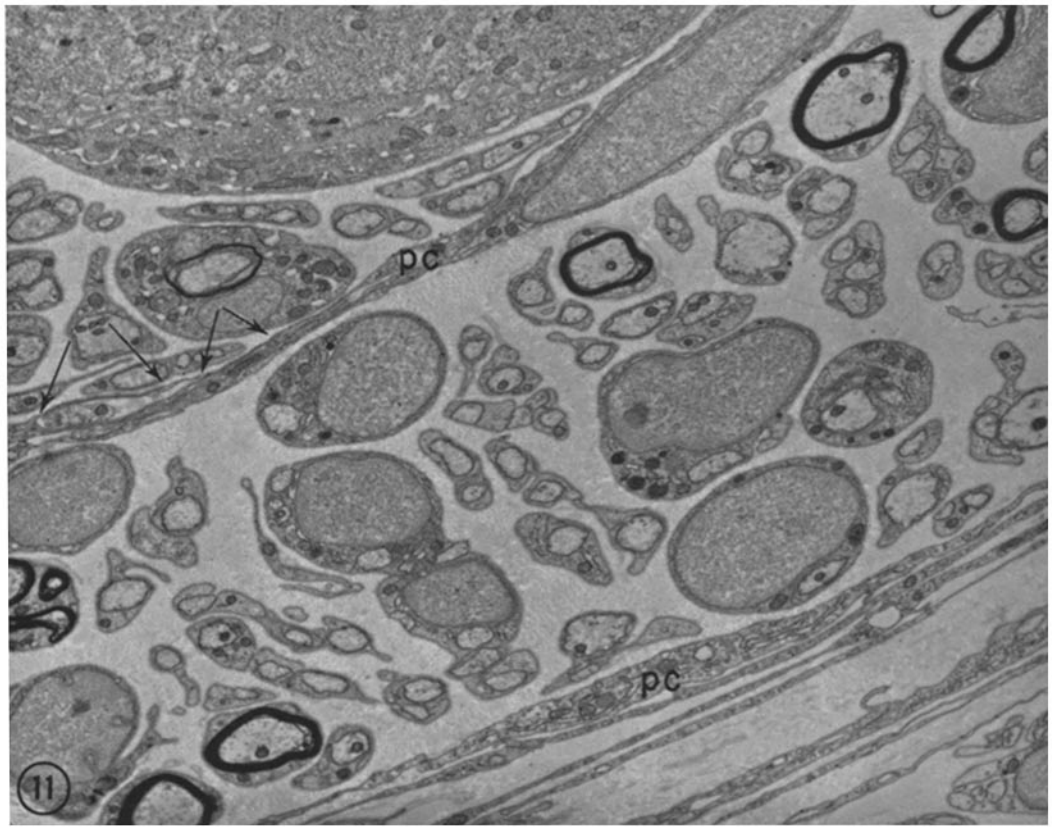


FIGURE 11 The components of a nerve fiber bundle are depicted in this electron micrograph. Enclosed by the flattened perineurial cells (*pc*) are Schwann cells and their related unmyelinated and myelinated fibers. Note that the myelinated fibers occur singly in a Schwann cell. The two pairs of arrows indicate the span of regions of close contact between perineurial cells (see text). × 7,000.

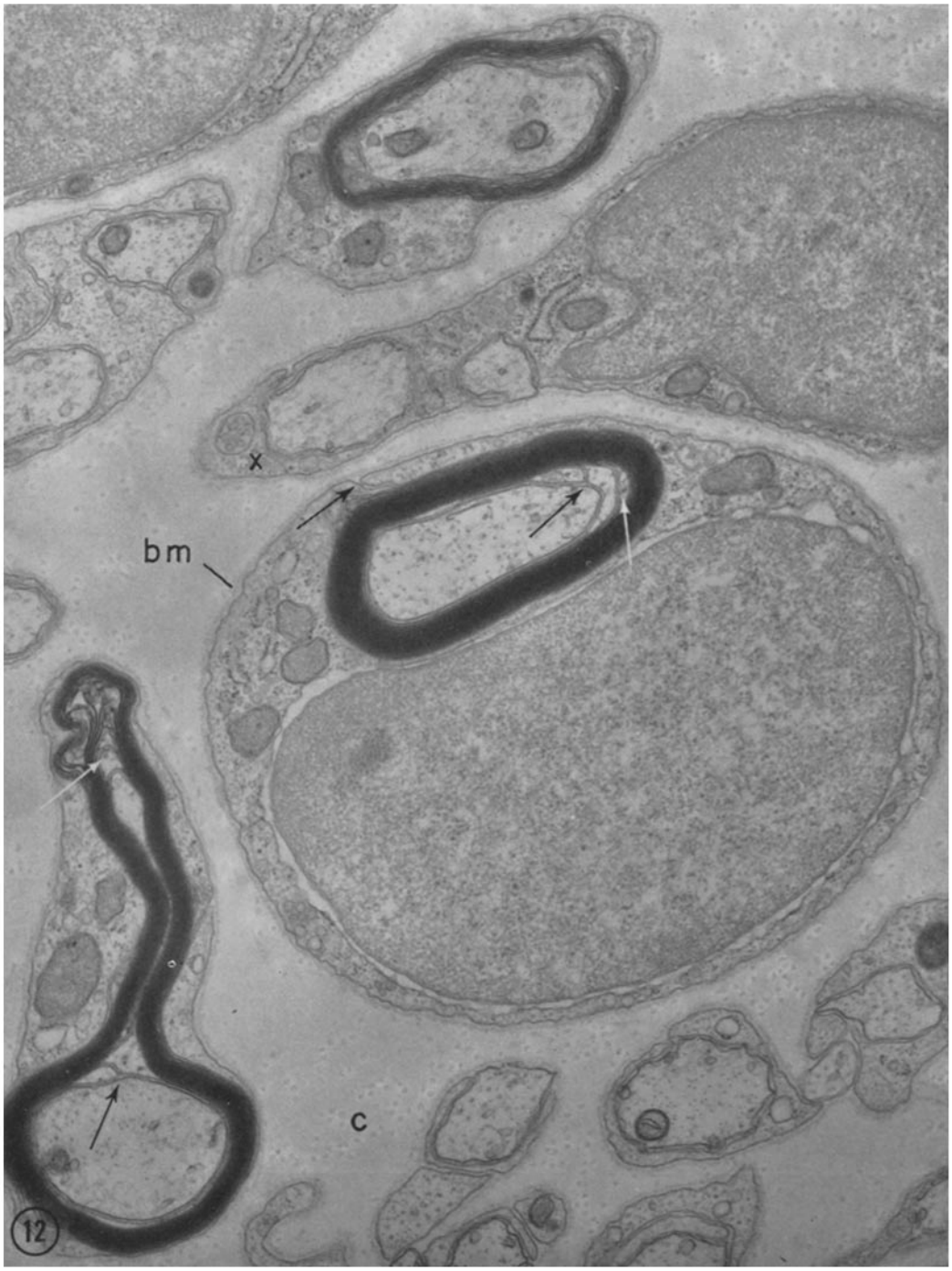


FIGURE 12 The Schwann cell–nerve fiber relationship, basement membrane (*bm*), and collagen fibrils (*c*) are illustrated in this electron micrograph. Inner or outer mesaxons appear at the black arrows. White arrows point to microtubules in Schwann cell cytoplasm. A Schwann cell multivesicular body with a plaque of fuzzy substance on its exterior is seen at *x*. × 25,000.

linated and unmyelinated fibers, basement membrane, and collagen fibrils (Figs. 11, 12). This architecture is present in the explant and also in the outgrowth zone which is organized entirely in vitro. The *perineurium* is composed of concentric layers (one or usually four or five or sometimes more) of interdigitating flattened cells. The usual intercellular gap between these layers is in some places widened locally (Fig. 11) or for some distance (and may contain collagen), or is diminished, thereby allowing closer cell contact. These regions of close contact, commonly seen in vivo (64, 65), may be of considerable length (referred to in Fig. 11) and average ~180 A from one cytoplasmic surface to the other. In perineurial cell cytoplasm, long cisternae of granular endoplasmic reticulum and small vesicles are prominent and particles presumed to be glycogen are often seen scattered

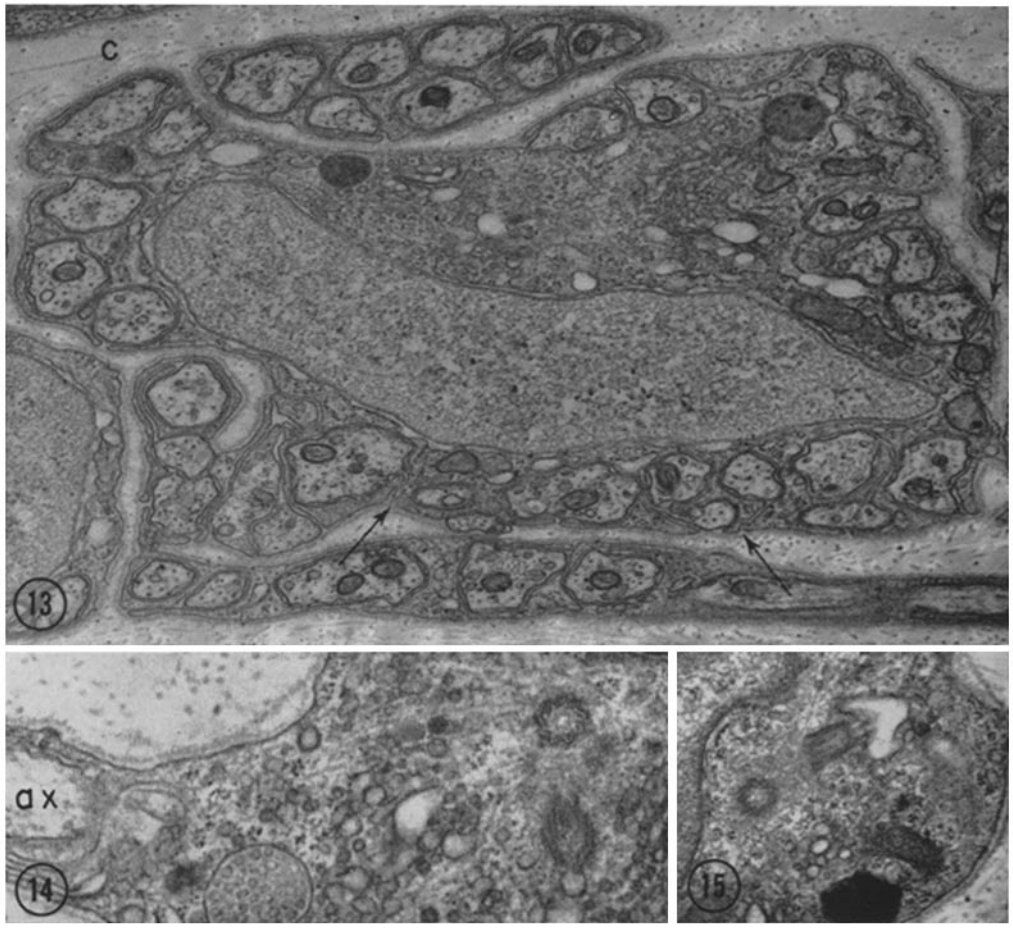


FIGURE 13 This figure demonstrates the number of unmyelinated axons that may be related to one Schwann cell. Most of the axons are completely invested, resulting in a typical mesaxon configuration (arrows). The basement membrane covering of the Schwann cell and collagen fibrils (*c*) are also illustrated. × 21,000.

FIGURES 14 and 15 Centrioles and associated cilia are shown in these portions of Schwann cell cytoplasm. (An identifying axon (*ax*) is present in one area.) In Fig. 14, the centriole is best seen; in it are the typical peripheral triplets of tubule-like elements. In Fig. 15, the cilium and surrounding ciliary vesicle are better shown. It is common to see centrioles and cilia in this relationship in cultured Schwann cells. Fig. 14, × 28,500; Fig. 15, × 23,000.

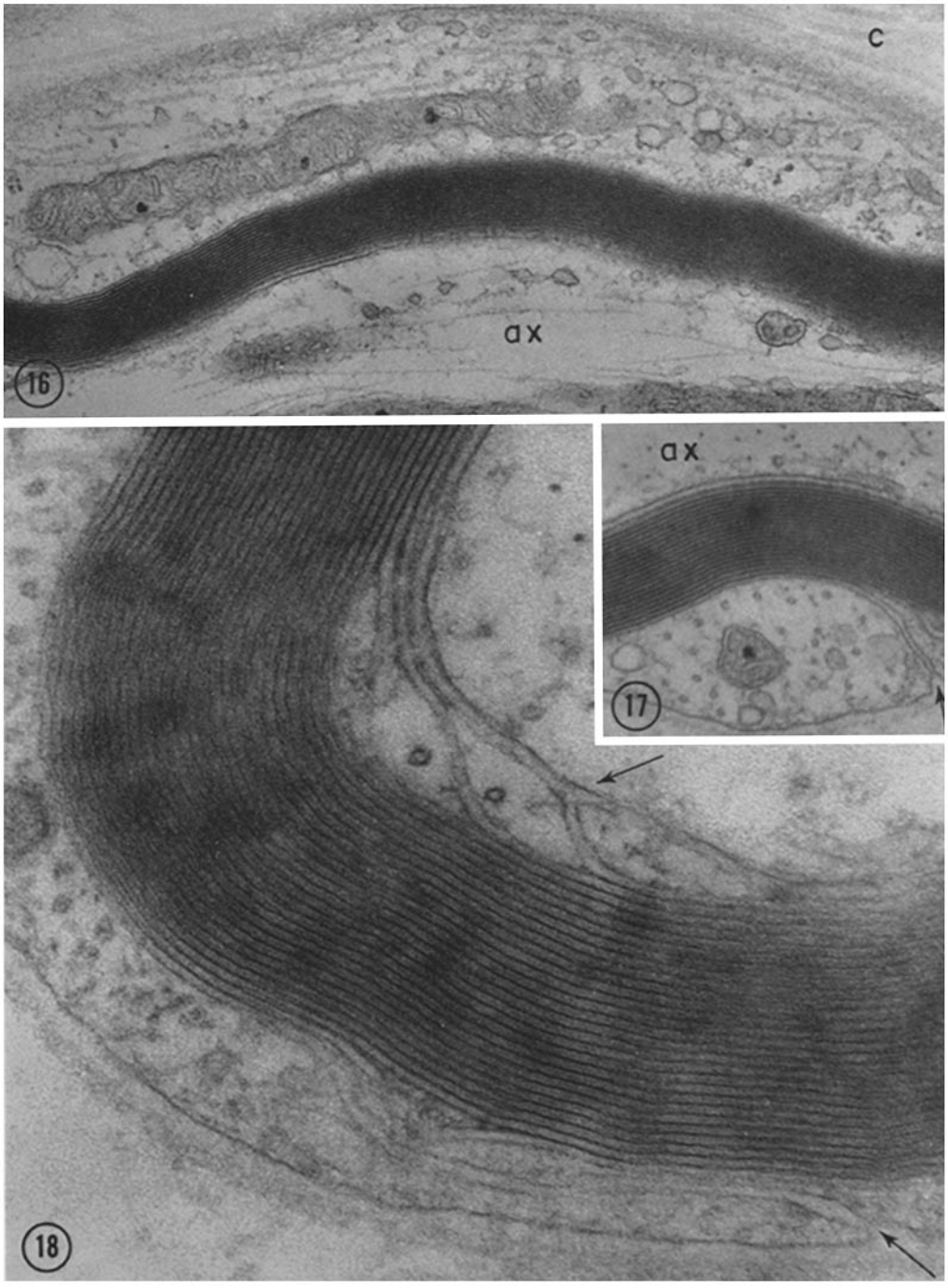
throughout or appear in aggregates. A perineurial sheath is covered internally and externally by basement membranes, but many of the intermediate layers of this sheath are not, as Waggener et al. (66) have observed in rat sciatic nerve. The fine structure of the perineurium in culture closely resembles that described for mammalian peripheral nerve in situ by Thomas (64) and Gamble (65), among others. Within the perineurium, an infrequent fibroblast or histiocyte may be found. The fibroblast is here identified by its lack of basement membrane, lighter cytoplasm compared to the Schwann cell, prominent granular endoplasmic reticulum which is often oriented in parallel arrays and filled with filamentous material, large vacuoles bounded by agranular membrane, and bundles of filaments in the cytoplasm. These cells are triangular or crescentic with elongated processes. This description of fibroblasts fits that presented by others (64, 65) for peripheral nerve and dorsal root in situ. The histiocyte is distinguished from the other cell types by its ruffled, outline, which often gives rise to slender processes, its lack of basement membrane, predominance of vesicles and vacuoles, paucity of granular endoplasmic reticulum, and an occasional heterogeneous appearing inclusion body or multivesicular body that is larger and more irregularly shaped than elsewhere in the culture.

The *Schwann cell* nucleus is composed of two differently appearing chromatin components (Figs. 12, 13) as in the satellite cell nucleus. Evenly spaced particles are arrayed on the inner membrane of the nuclear envelope. The gap between the inner and outer membranes varies because the outer membrane is irregularly wavy and may form infrequent outpocketings containing dense material. Ribosomes are regularly arrayed on certain areas of the outer membrane, particularly those areas facing the cytocentrum. The cytoplasm contains granular endoplasmic reticulum in the form of scattered long, sometimes branched, widened cisternae which contain filamentous material and are regularly and densely covered with ribosomes. An occasional cisterna may be greatly distended by accumulations of contained substance. Multivesicular bodies resemble those in neurons, including the plaque of dense substance adhering to the outer surface. Vesicles of varying size are numerous. An occasional filament is seen. Filaments have been noted in the Schwann cells of cat splenic nerve (67). Microtubules averaging 230 A are present throughout Schwann cell cytoplasm. They are particularly noticeable in the cytoplasmic collar inside and outside the myelin sheath where they lie parallel to the fiber length (Figs. 16–18, 20). In the paranodal cytoplasmic spiral and in cleft cytoplasm, however, they curve around the axis cylinder. Thus, when the fiber is sectioned longitudinally, cross- or obliquely sectioned tubules are seen in the paranodal (Fig. 20) or cleft (Fig. 19) cytoplasm whereas, when the fiber is sectioned transversely, tubules appear in longitudinal section following the curve of terminating cytoplasm. The tubules are also found in Schwann cells investing unmyelinated fibers. Sandborn et al. (42) have reported the presence of microtubules in Schwann cells related to rat sympathetic nerve fibers. Centrioles and cilia are encountered occasionally (Figs. 14, 15) but only in Schwann cells related to unmyelinated fibers. This is in contrast to a claim made by Barton (68) that centrioles are present only in myelin-related Schwann cells but is in agreement with a conclusion reached by Grillo and Palay (69). The cilium is usually seen to arise from one of a pair of centrioles and contains a large central vesicle, a distinctive feature of Schwann cells (69). Other morphological differ-

FIGURE 16 This electron micrograph illustrates a myelinated fiber (*ax*) and investing Schwann cell in longitudinal section. Lengths of Schwann cell microtubules are seen running parallel to the fiber (and may be compared to the axoplasmic filaments). Collagen fibrils (*c*) have also been sectioned longitudinally. × 38,000.

FIGURE 17 Microtubules appear as circles when the Schwann cell and related fiber are transversely sectioned. Cross-sectioned neurofilaments are present in the axon (*ax*). An outer mesaxon is also shown (arrow). × 45,000.

FIGURE 18 This electron micrograph illustrates the similarity of myelin formed in vitro to that observed in vivo (see text). Mesaxons are indicated by arrows. Microtubules are present in the Schwann cell cytoplasm internal and external to the sheath. × 110,000.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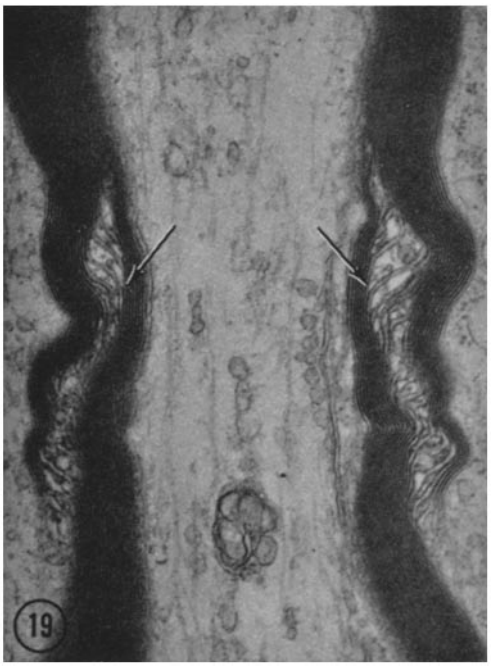


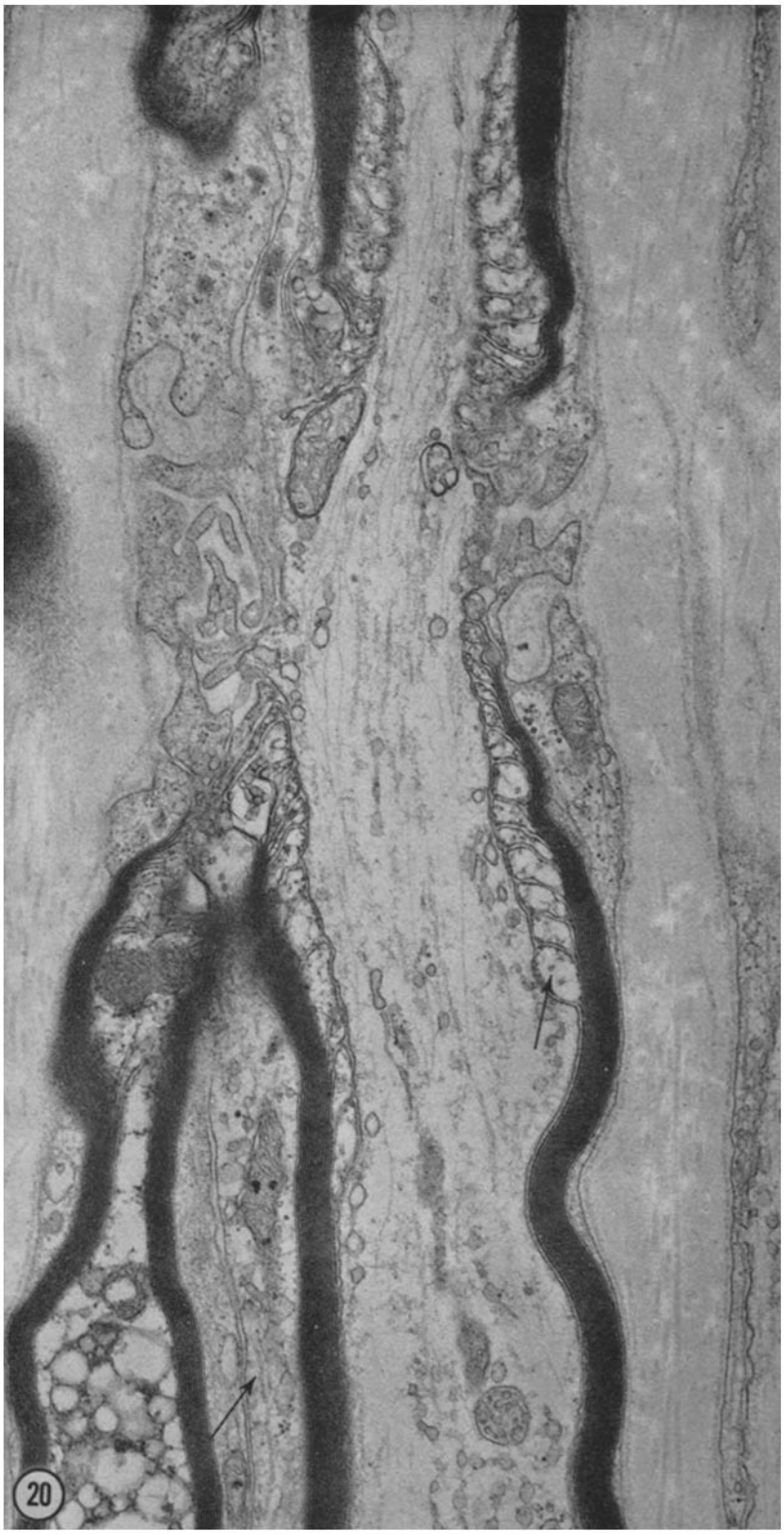
FIGURE 19 A Schmidt-Lanterman cleft appears in this electron micrograph. At the arrows, the components of the major dense line separate and enclose Schwann cell cytoplasm. Within this cytoplasm of the longitudinally sectioned sheath, transversely sectioned microtubules are present (near arrows). The position of these circular profiles suggests that they belong to the same microtubule. × 31,500.

ences between the cytoplasm of Schwann cells with unmyelinated fibers and that of Schwann cells with myelinated fibers were not apparent.

Schwann cells are typically coated with *basement membrane* (Figs. 12–18). Usually there is but one layer and it closely follows the cell contour, also covering the nerve fibers where they are not completely encircled by Schwann cell cytoplasm. Occasionally, the basement membrane veers away from the Schwann cell in folds, thus appearing as an irregular, oversized envelope (Fig. 12). Gamble (65) mentions the very occasional presence of bleb-like projections of basement membrane in adult rat dorsal root. The proportion of folded projections observed in the cultures is probably more than that found normally after maturation has occurred. Highly folded basement membranes are common in degenerating dorsal root (70–72) in which they are thought to result from loss in volume of contained structures; i.e., loss of axons. Occasionally, multiple layers of basement membrane, many of them incomplete, surround a Schwann cell. Within these layers an occasional collagen fibril may be detected (Fig. 12). Gamble (65) found multiple basement membrane layers and bundles of collagen among these layers in adult rat sural nerve and, to a lesser degree, in rat dorsal root. Collagen fibrils were found frequently among multiple laminae in regenerating rat dorsal roots by Nathaniel and Pease (72) who used this and additional evidence to suggest that the Schwann cell can both form and polymerize tropocollagen. Light microscopic indication of reticulin formation by Schwann cells in vitro has been offered by Peterson and Murray who found that the pattern of newly formed reticulin fibers conforms to Schwann cell orientation (5), depends upon the nutritional status of the Schwann cell (97), and does not exhibit a spatial relationship to adventitious fibroblasts or other cells.

Nerve fibers typically contain neurofilaments, various tubular components, mitochondria, and vesicles or vacuoles of varying size (Fig. 20). Mitochondrial cristae most often lie in a plane perpendicular to the length of the mitochondrion. Vacuoles are frequently situated close to the axolemma. Particles presumed to be glycogen are observed infrequently. According to random measurements obtained from light and electron micrographs, fiber diameter measures from 0.2 to 2.6 μ for the axon itself, or up to 4 μ if the myelin sheath is included. Diameters of fiber plus myelin may reach 13 μ in dorsal roots of 6-month-old rats (77). The

FIGURE 20 This electron micrograph shows one of the nodes found regularly along myelinated fibers in the cultured ganglia. Typically, the myelin lamellae terminate in loops, the innermost loop occurring farthest from the node. Microtubules are present in the Schwann cell cytoplasm outside the sheath and inside a loop (arrows). Near the node is a long slender projection of myelin which is seen to be continuous with the lower myelin sheath at another level. Schwann cell processes with their overlying basement membrane interdigitate at the node. × 27 500.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parameters of myelinated nerve fiber diameter as well as internode length and neuronal soma size (described briefly in reference 30) will be considered in more detail in a future publication. The smaller fibers are invested with cytoplasm of Schwann cells which form the well known mesaxons (Fig. 13) (73, 74). The larger neurites occur singly in a Schwann cell and are usually myelinated as in situ (74, 75). Myelinated fibers always occur singly in a Schwann cell (Figs. 11, 12). An occasional configuration resembling a second myelinated fiber is seen, upon closer inspection, to lack an axon and to be, therefore, a myelin evagination. The occurrence of multiple unmyelinated or single myelinated fibers within a Schwann cell has been reported already for cultured dorsal root ganglia (19). The critical size for myelination in the culture is ~ 1 μ, i.e., in electron micrographs few fibers below 1 μ in diameter are myelinated whereas most fibers greater than 1 μ display myelin sheaths (30). Gasser (73) found that cat dorsal root unmyelinated fibers in electron micrographs generally measured 0.15–0.5 μ, but trailed off to 1.0 μ. In a study of sensory nerves in mouse cornea, Whitear (76) found that fibers measured 0.15 to just over 2 μ in electron micrographs; in some graphs of these data, it appeared that myelination occurred as the fibers approached 1 μ in diameter. Peters and Muir (75) state that, in developing peripheral nerve, axons undergoing myelination measure 2–4 μ whereas unmyelinated grouped axons are 1 μ or less in diameter. From a light microscope study of mammalian dorsal and ventral roots, Duncan (77) surmised that the critical size for myelination was around 1 μ. In rare cases in the cultured ganglion, a fiber may contain an accumulation of pleomorphic dense bodies displaying areas resembling myelin figures, interpreted as a sign of degeneration (23, 78; see also 79). A rare fiber (deteriorating?, reference 23) contains mitochondria which have rounded up from their usual long slender shape, with granular substance rather than cristae mitochondriales occupying the central region. Although the dorsal root ganglion culture is a fairly stable population, from time to time neurons may degenerate for no apparent reason.

Myelin sheaths display the typical lamellated structure with a repeating period of $\sim$120-150 A (Fig. 18), a range equaling that for in vivo peripheral nerve (47, 80, 81) after OsO_4 fixation and epoxy resin embedding. The cultured myelin sheaths vary little in thickness. The inner and outer mesaxons are shown in Figs. 12, 17, and 18. Occasionally, an axon is surrounded by many turns of a spiraled mesaxon which probably would have been compacted during further maturation. A few turns of Schwann cell cytoplasm around an axon are noted frequently; some myelination is undoubtedly continuing at a month or more in vitro. Schmidt-Lanterman clefts are visible in both transverse and longitudinal (Fig. 19) sections. As described earlier (82), a cleft is a series of splits in myelin lamellae at the major dense line with portions of Schwann cell cytoplasm enclosed, and it may be regular, as pictured in the figure, or a more complex and irregular structure. Nodes of Ranvier are covered by interdigitating Schwann cell processes within a continuous basement membrane, and the paranodal myelin terminates in loops, the loop nearest the node arising from the outermost myelin unit (Fig. 20) (as in situ 83–85). In addition to tubules, many dense particles are found within the terminating loops and, as Elfvin (85) points out, the particles are often clustered on the membrane adjacent to the axolemma and at times resemble ribosomes. Evenly spaced particles dot the gap between some terminating Schwann loops and axolemma in a fashion similar to that shown so clearly in hedgehog nerve by Bargmann and Lindner (86). In a few cases, we have noted a stack of desmosome-like densities (including the locally widened intercellular gaps) between neighboring regions of Schwann cell cytoplasm in node and cleft areas; these configurations resemble those shown among paranodal loops in rat vestibular ganglion (87) and rat sciatic nerve (88). Especially at the node and occasionally elsewhere along the sheath, long outpocketings of myelin lie parallel to the sheath length (Fig. 20). We consider these loops to be continuous with the sheath (though they may appear not to be, because of the plane of thin sectioning) and to be devoid of axoplasm. In cross-section, local bulges and loops are commonly seen and some sheaths appear larger than their contained axons, resulting in comma- or banjo-like (Fig. 12) configurations. These irregularities are not preparative artifacts because they are observed in living cultures; nor are variations produced solely as a result of in vitro existence for myelin loops and folds, including long outpocketings near nodes, exist in mammalian peripheral nerve in situ (80).

Endoneurial *collagen* measures 200–450 A in diameter as compared to measurements of 250–

550 A (65) or 300–500 A (21) in rat dorsal root in vivo. The distribution of this collagen ranges from an uneven sparse to a more uniform, fairly dense accumulation always associated with a mottled background suggesting the presence of additional matrix substance. Generally, these fibrils are neither gathered into bundles nor as closely compacted as they appear to be in peripheral nerve in vivo (64). Furthermore, they are not condensed into a continuous sheath around the nerve fiber, and the collagen present is not organized into two layers, the inner and outer endoneurial sheaths (Figs. 12, 20). These findings agree with Gamble's observations of rat dorsal root in situ (65) showing that, in contrast to more distal peripheral nerve, the amount of collagen is smaller and is not organized into two endoneurial sheaths. The collagen associated with cultured fibers shows some characteristics of the inner endoneurial sheath (or the sheath of Plenk and Laidlaw, reference 89); i.e., though most fibrils run parallel to nerve fiber length, there is some variation in their orientation, the fibrils are not densely packed, and the presence of intervening matrix is indicated. As Thomas (89) summarizes, it is the inner endoneurial sheath that is the argyrophilic or reticulin layer, the reticulin staining probably depending on the associated carbohydrate in the matrix. This, then, would explain why reticulin rather than collagen staining is heavy in the cultures (Fig. 9). The fibrils per se cannot be identified as collagen or reticulin components electron microscopically (90). Some of the collagen fibrils found in the explant have been carried into the culture at explantation but many and particularly those in the outgrowth form in vitro.

FURTHER COMMENTS

Fascicle Formation

The gathering of lengthening nerve fibers into histiotypic fascicles, even in distal regions of the outgrowth, is one of the most striking aspects of the cultured rat dorsal root ganglion (Fig. 10). This organization of the outgrowing fibers involves both the convergence of fibers into bundles (fascicle formation) and the exchange of nerve fibers between bundles (plexus formation). The final result is a series of interconnected fascicles separated by large fields of connective tissue devoid of nerve fibers (Fig. 1). These fascicles contain both myelinated and unmyelinated fibers, Schwann cells, connective tissue elements, and associated perineurial constituents. Organization of the perineurium is attained even though the fibers grow out on a flat surface; this is in contrast to the view held by Nakai (cultured chick ganglia, 91), who claims that a three-dimensional network is necessary for development of perineurial elements in vitro.

The problem of what may influence the course of outgrowing nerve fibers has been extensively studied in recent decades (93). The fascicle formation that occurs in the ganglion cultures grown on a two-dimensional collagen substrate may appear at first glance difficult to explain on the basis of concepts such as contact guidance and selective fasciculation. The relationship between connective tissue and nerve fibers may be a critical factor in the cultures described in the present study. When well encapsulated ganglia are explanted, nerve fibers emerge as stout fascicles, primarily at the central and peripheral roots but also at other points where the capsule has weakened and can be penetrated. When the capsular investment is minimal (due to rupture or deliberate removal or because the ganglion is taken at a very early fetal age), nerve fibers emerge without becoming associated either as an even radial outgrowth or as a loose tangle of unoriented elements. The nonaggregated fibers do, however, often become grouped into bundles in more distal regions of the outgrowth (see Fig. 1).

Whereas the presence of capsular tissue would help to explain the formation of primary fascicles near the explant, the factors operating in the more distal regions to induce fascicle formation are less clear. In addition to the partially ruptured capsule, vestiges of meninges are commonly explanted with the ganglia (unless deliberately removed by painstaking dissection). Both of these connective tissue elements migrate and proliferate rapidly over the collagen surface and soon outstrip the early burst of neuritic outgrowth. This modifies the surface of the collagen in areas extending far from the original explant by introducing inhomogeneities which may mark out linear paths for the outgrowing nerve fiber. Thus the neurites may be guided by contact with this connective tissue terrain. Weiss (98), under the general term of "contact guidance," has repeatedly stressed the importance of the orientation of the components of the substrate or surrounding matrix in the determination of the course taken by nerve fibers in vitro.

Other factors influencing the formation of nerve fascicles have been proposed. Based primarily on in vivo experiments (92, 93), it has been suggested that pioneering fibers, upon reaching their peripheral termination, become endowed with a selective adhesiveness which draws subsequent outgrowing fibers into the same pathway. Weiss (93) concludes that this "selective fasciculation" occurs only if the pioneering fiber finds adequate peripheral termination. The extensive fascicle formation described in the present paper would appear to contradict this contention unless the simple endings found in our ganglion cultures can be considered adequate peripheral termination. The physiology of sensory nerve endings in culture is not known. Although Lumsden (94) clearly illustrated the presence of nerve endings in cultures of chick dorsal root ganglia, he preferred to consider that fascicle formation is controlled by forces within the neuron rather than by influences dependent upon neuritic termination. Another factor of possible significance in fascicle formation is the formation of filopodia (from the growth cone or nearby lateral regions of the fiber) which seek out and sometimes adhere to similar projections from other fibers, subsequently pulling the nerve fibers together as they retract (95, 96). Much remains to be learned of the influences on nerve fiber course and organization; the special contribution of the present study is the demonstration of the complex three-dimensional organization which may occur in cultured nerve tissue, an organization involving the histiotypic interrelationship of connective tissue, Schwann cell, and nerve fiber elements.

Rat vs. Chick Cultures

In vitro development of rat dorsal root ganglia differs in a number of ways from that of the chick dorsal root ganglia described earlier (5). The study of chick ganglion cultures was done with a feeding medium containing ~100 mg % glucose; higher levels of glucose do not appear to enhance development. This amount of glucose will foster neuronal maturation in rat ganglion cultures, but myelin formation is greatly enhanced if the glucose level is raised to 600 mg %. The collagen substrate so useful for long-term culture of rat ganglia is not sufficient for the maintenance of chick ganglia beyond the 4th wk in vitro. If the collagen is supplemented with a plasma clot during the 3rd wk, the otherwise imminent acute degeneration is prevented. Mature neurons in cultured chick ganglia are larger than those in rat cultures, with the proportion of cytoplasmic to nuclear mass being greater. During Nissl development, an intermediate pattern of large peripheral masses and small central particles is observed in chick but not in rat cultured neurons. The Nissl bodies of matured rat neurons are smaller than those in matured chick neurons. An abundance of neurofibrils is readily demonstrated, in chick neurons, with Bodian's and Holmes' silver stains; a fine neurofibrillar network is seen in rat neurons only after Holmes' stain. Rat ganglion neurons, in general, display more associated satellite cells than the usual two to four satellite cells investing chick neurons. In the chick culture, well developed reticulin sheaths are demonstrable by the 2nd wk in vitro (prior to myelination), whereas in rat cultures more delicate reticulin sheaths usually appear after myelination has begun. The period of myelination is similar in both culture systems, but myelin formation is far more extensive in the rat tissue; nerve fibers from the rat neurons are usually continuously enveloped by myelin segments far into the outgrowth, whereas chick fibers more often display shorter groupings of internodes, or even isolated segments (99). Rat myelin is readily stained by the standard Luxol fast blue technique; chick myelin is stained by this method only after chromation. Finally, fascicle formation is more prominent in rat ganglion cultures than in chick ganglion cultures. It is not known whether this is influenced by one obvious difference in the two types of ganglia, i.e., the presence on rat ganglia of meningeal remnants which are absent from chick ganglia (because of their location outside the vertebral column). Additional details and illustrations of points of difference between rat and chick cultures appear in reference 16.

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