AN ELECTRON MICROSCOPE STUDY OF CULTURED RAT SPINAL CORD

RICHARD P. BUNGE, MARY BARTLETT BUNGE, and EDITH R. PETERSON

From the Departments of Anatomy and Surgery, Columbia University College of Physicians and Surgeons, New York

ABSTRACT

Explants prepared from 17- to 18-day fetal rat spinal cord were allowed to mature in culture; such preparations have been shown to differentiate and myelinate *in vitro* (61) and to be capable of complex bioelectric activity (14–16). At 23, 35, or 76 days, the cultures were fixed (without removal from the coverslip) in buffered OsO_4, embedded in Epon, sectioned, and stained for light and electron microscopy. These mature explants generally are composed of several strata of neurons with an overlying zone of neuropil. The remarkable cytological similarity between *in vivo* and *in vitro* nervous tissues is established by the following observations. Cells and processes in the central culture mass are generally closely packed together with little intervening space. Neurons exhibit well developed Nissl bodies, elaborate Golgi regions, and subsurface cisternae. Axosomatic and axodendritic synapses, including synaptic junctions between axons and dendritic spines, are present. Typical synaptic vesicles and increased membrane densities are seen at the terminals. Variations in synaptic fine structure (Type 1 and Type 2 synapses of Gray) are visible. Some characteristics of the cultured spinal cord resemble infrequently observed specializations of *in vivo* central nervous tissue. Neuronal somas may display minute synapse-bearing projections. Occasionally, synaptic vesicles are grouped in a crystal-like array. A variety of glial cells, many apparently at intermediate stages of differentiation, are found throughout the otherwise mature explant. There is ultrastructural evidence of extensive glycogen deposits in some glial processes and scattered glycogen particles in neuronal terminals. This is the first description of the ultrastructure of cultured spinal cord. Where possible, correlation is made between the ultrastructural data and the known physiological properties of these cultures.

INTRODUCTION

The biologist undertakes the formidable task of establishing and maintaining organized nervous tissue cultures in the hope of having in hand as exact a model of *in vivo* material as possible. The more exact the model is, the more meaningful are the results derived from the experimental system that tissue culture so uniquely provides. To the accumulated evidence of the structural and functional fidelity of cultured nervous tissue, this paper adds electron microscopic data on cultured mammalian spinal cord which demonstrate that a remarkable ultrastructural similarity exists between *in vivo* and *in vitro* systems. It is one of a group of integrated morphological and physiological studies. The initial study (61) describes the methods of obtaining organized spinal cord cultures, outlines the pattern of development, and illustrates the organization of the mature cultures.

A second study (15, 16) demonstrates that cultures so prepared are capable of complex bioelectric responses to electric stimuli and suggests that the complex excitatory phenomena obtained in electrophysiologic recordings could take place only if the cultures contained multiple chains of synaptically linked neurons. The ultrastructural aspects of the mature cultures presented in this third paper of the series help clarify some of the questions raised by light microscopic examinations and demonstrate that the synaptic junctions predicted by the physiological studies do in fact exist.

The accumulation of evidence that nervous tissue could differentiate and mature in culture was accelerated in 1956 by the demonstration of systematic neuronal maturation and myelin formation in chick spinal ganglia maintained for long periods *in vitro* (62). First Hild (38), using roller tubes, then Bornstein and Murray (5), utilizing Maximow assemblies, were able to obtain myelination of fragments of central nervous system (cerebellum) grown *in vitro*. Deitch and coworkers (18, 19) used cultured avian dorsal root ganglia to demonstrate that Nissl bodies are present in living neurons. A recent report (8) emphasized the ultrastructural similarities between *in vivo* and *in vitro* rat dorsal root ganglia.

Despite the attainment of such cytological sophistication in culture, the earliest electrophysiological studies of cultured cerebellum by Hild and Tasaki (39) suggested that it was unlikely that synapses were present in cultured nervous tissue. Electron microscope studies of central nervous system (CNS) cultures by Duncan and Hild (23) and Lumsden and Piper (42) do not mention finding synaptic configurations. The ultrastructural study by Ross, Bornstein, and Lehrer (73) of cerebellar cultures, while demonstrating that CNS cellular elements were faithfully reproduced *in vitro* and illustrating sequential stages of myelin formation, did not report finding synapses in these cultures.

Now evidence has become available from a variety of sources that cultured CNS does contain synaptic links between cellular elements. A number of years ago, Périer and de Harven (58) obtained electron micrographs of definitive synaptic areas in cultured cerebellum. Recently Crain and Peterson (15), in a preliminary report prior to the present studies, presented electrophysiological data that could best be explained by the occurrence of synapses in spinal cord cultures. Wolf (82) has reported light microscopic evidence from silver-stained preparations of cultured cerebellum that synaptic end bulbs occur in relation to nerve cells of the Purkinje type. Light microscopic evidence of synapses in cultured spinal cord is submitted in a companion paper to the present study (61). Bornstein and Pappas (4, 56) and Crain (13) have obtained electron microscopic and physiological evidence of synapses in cultured mouse cerebrum.[1] Geiger's (25) claim to have identified synapses in cultures of adult mammalian cortex does not seem justified, for the unconvincing light microscopic evidence is not strengthened by the presentation of electron microscopic or electrophysiological data. There is, in addition, a serious problem of positively identifying cells as neurons in Geiger's cultures (61).

In the present paper it is possible to demonstrate that virtually all general ultrastructural characteristics of mammalian CNS tissue can be found in cultures of rat spinal cord. These characteristic configurations, now well established by many electron microscope studies, include: (*a*) the elaborate Nissl bodies of neuronal cytoplasm, (*b*) the close packing of cell processes with the consequent paucity of intercellular space, (*c*) a variety of synaptic associations, (*d*) the occurrence of subsurface cisternae, (*e*) an extensive system of tubules and filaments in neuronal cytoplasm, (*f*) the occurrence of a variety of glial cell types, and (*g*) the characteristic pattern of the CNS myelin sheath. In addition, the present paper illustrates certain features of cultured CNS that are less commonly observed *in vivo*. These include (*a*) highly ordered arrays of synaptic vesicles, (*b*) synapse-bearing "spines" on neuronal somas, (*c*) non-membrane-bounded granular cytoplasmic aggregates, and (*d*) the extensive occurrence of glycogen.

The authors expect that the establishment of the ultrastructural fidelity of spinal cord *in vitro* will be the prelude to a series of experimental modifications of cultured central nervous system tissue under conditions which allow a unique control of the immediate tissue environment as well as the opportunity for continuous direct observation.

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[1] Very recently, Callas has reported electron microscopic evidence of synapses in cultured rat brain stem and cerebellum (*Anat. Rec.*, 1964, **148**, 267).

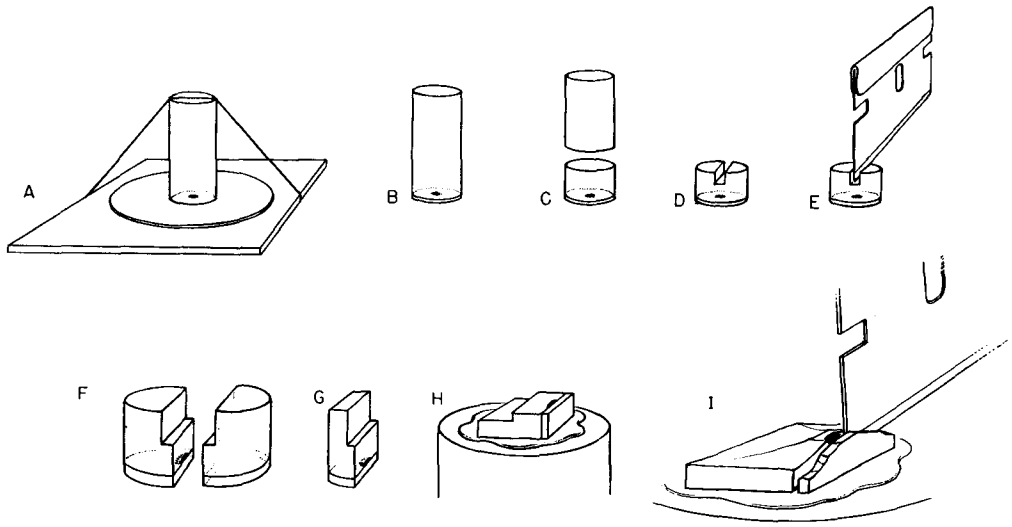


FIGURE 1 This diagram illustrates the steps in embedding and orienting the explant and in the subsequent removal of the coverglass. The explant remains on its carrying coverslip during fixation, dehydration, and infiltration. For embedding, a small piece of cardboard is placed beneath the coverslip to provide rigidity. A gelatin cylinder is centered over the culture and a rubber band placed around the cylinder and cardboard base (*A*); the cylinder is then filled with Epon. After curing, that part of the coverslip projecting beyond the cylinder base is trimmed away along with the cardboard (*B*). The bottom fourth of the cylinder is separated by a saw cut (*C*). The part removed, still covered on the bottom surface by the coverslip, is partially sawed through over the area of the explant (*D*). This break is completed by placing a razor blade into the incomplete saw cut and striking sharply (*E*). The resultant break will pass through or near the culture and through the underlying glass coverslip (*F*). The rounded surface is trimmed (*G*) so that the fragment may be mounted (as in *H*) on the flat end of a blank capsule. This fragment can now be trimmed to a standard rectangular block face and the culture cut in cross-section. During the course of the trimming, efforts are made to separate the coverslip from the plastic as illustrated in *I*. Usually, large flakes of the coverslip come free "uncovering" an adequate area of the adjacent explant for sectioning. Sometimes the coverslip will break into fine pieces and these must be scraped from the plastic surface. Occasionally it may be necessary to trim away the deepest zone of the culture adjacent to the coverslip to insure complete removal of the glass.

MATERIALS AND METHODS

Cervical or lumbar spinal cord was removed from rat fetuses after 17 to 18 days' gestation and explanted as half cross-sections on collagen-coated coverslips in Maximow double-coverslip assemblies. The details of media composition and renewal are given in a companion paper (61). Serial observations allowed the selection of 14 representative, mature explants for electron microscope study. At 23, 35, or 76 days *in vitro*, the coverslips carrying these cultures were placed in a Columbia dish and rinsed 3 times with Simms' balanced salt solution to remove the protein-containing culture medium. Immediately after the final rinse, the dish was filled with Veronal-acetate-buffered (pH 7.4 to 7.5) (45) 2 per cent OsO_4 containing 0.05 per cent $CaCl_2$ at room temperature. The temperature was lowered to 4°C and fixation continued for a total of 45 to 60 minutes.

The coverslips remained in the Columbia dish during ethanol dehydration and the initial phases of Epon infiltration (modified Luft's method, 41). After an overnight soaking in the Epon 812 mixture at room temperature, the coverslip was removed from the dish and placed culture side up on a square piece of stiff cardboard. The rounded bottom of an 00 gelatin capsule was cut off to convert the capsule to a cylinder (see Fig. 1, *a*); this cylinder was centered over the explant. A No. 8 rubber band was placed around the cardboard base and gelatin cylinder to insure a tight seal. The cylinder was filled with Epon mix and the entire assembly was cured 1 day at 35°C, 1 day at 45°C, and 7 days at 60°C. The realignment procedure, designed to obtain the desired tissue orientation and to allow the eventual removal of the glass coverslip, is illustrated in Fig. 1. Rare and erratic success has attended our efforts to

remove the coverslip from the block by cooling on dry ice (40) or in a dry ice-acetone mixture.

Sectioning was done on a Porter-Blum (Model 1) microtome with a glass or DuPont diamond knife. One to 2 μ sections were stained with Richardson's toluidine blue solution (67) and photographed in Zeiss phase contrast. Sections picked up on carbon-reinforced collodion films were stained with lead citrate according to Reynolds (66). These preparations were examined and photographed in an RCA EMU 3G microscope at magnifications ranging from 2000 to 31,000.

OBSERVATIONS

Light Microscopy

A detailed description of the unsectioned culture—living or fixed and stained—is given in the light microscopic study (61). The observations on whole mount preparations are most precise at the explant border or in the outgrowth zone, for the thickness of the explant itself makes difficult any exacting examination of the constituent cellular elements (other than large neurons). Therefore, stained semithin sections of the explant were prepared and are described below. Our present observations are confined to the explant area.

Fig. 2 diagrams a cross-section of the entire thickness of the explant. The dome-shaped profile at the top is the surface exposed to the feeding medium; the flat edge at the bottom lies against the coverslip. Sections cut in this plane generally show three zones: an overlying zone of neuropil relatively free of cell bodies (Fig. 3), a middle zone containing the majority of cells (Figs. 3 and 4), and a deep third zone of necrosis where nutrition is apparently inadequate. In the cultures chosen for electron microscopy, the thickness of the necrotic zone generally was as diagrammed but it in fact varies from culture to culture.

The free surface of the culture may show no developed sheath, although cells (which electron microscopy shows to be glial) often align themselves intermittently along the surface (Fig. 3). The meninges, often explanted with the cord fragment, affect the pattern of the outgrowth but they do not spread over the surface of the explant unless deposited there (61). The surface cells are swollen and separated by considerable space. In contrast, the underlying neuropil shows little extracellular space. Cells in the neuropil zone are mostly glial; scattered neurons sometimes are visible.

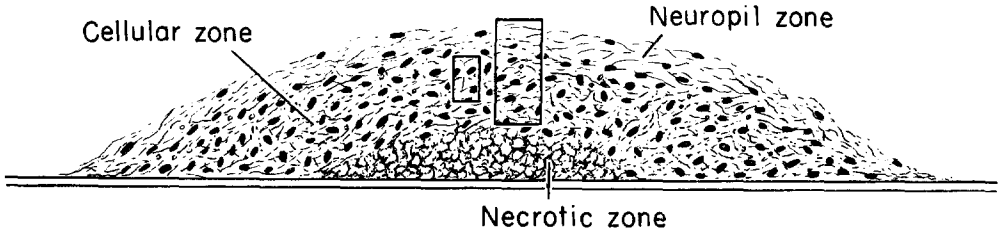
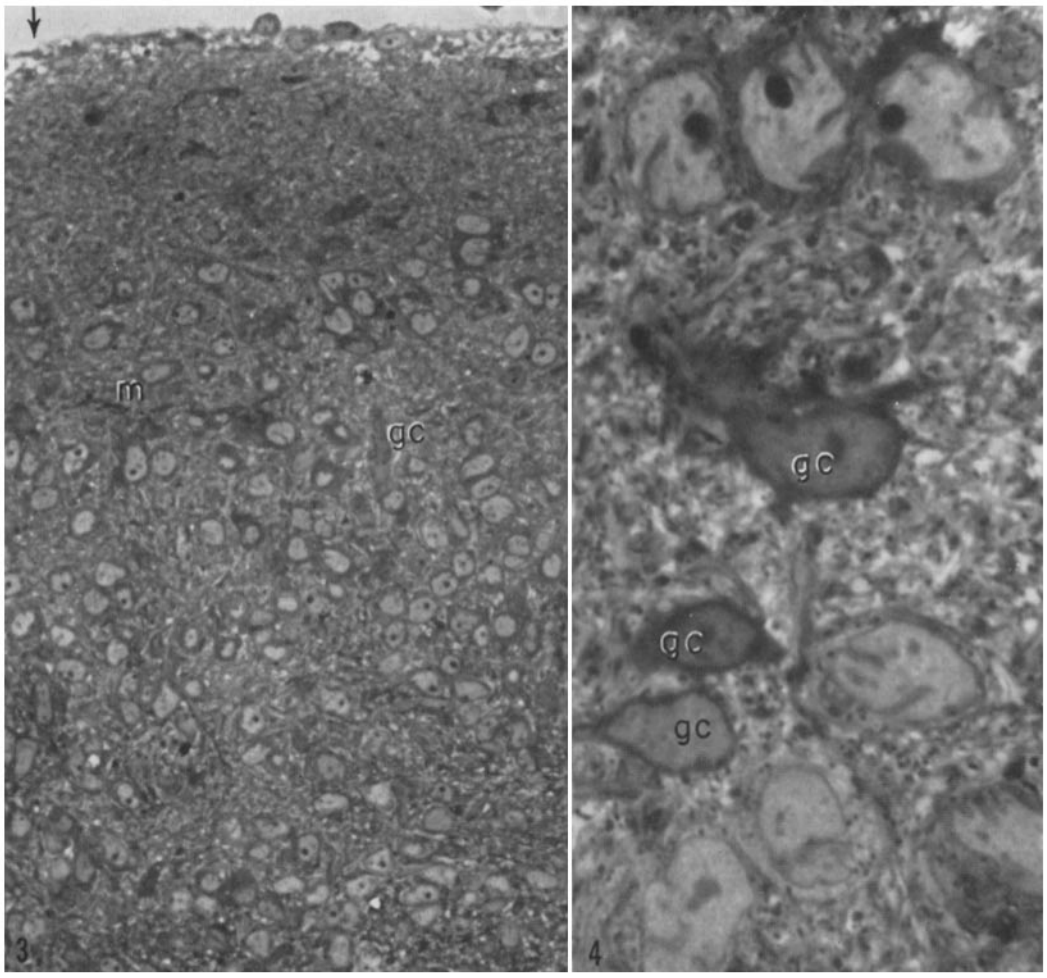


FIGURE 2 This is a diagrammatic representation of a cross-section of the mature spinal cord explant. The most superficial tissue is largely neuropil; most neurons are found in a deeper broad cellular zone. Many of the large explants have a zone of necrosis occupying the deepest area adjacent to the coverglass. In cultures used for the present study, the relative size of the three zones is approximately as illustrated. Figs. 3 and 4 are representative of the histology in the areas enclosed by the large and small rectangles.

A broad spectrum of glia is seen. One end of the spectrum is represented by a large cell with extraordinarily dense nucleus, cytoplasm, and processes (Fig. 4). The cytoplasm contains numerous large dense droplets. This cell is undoubtedly the intermediate type of glia described in more detail below. A small cell containing a clearly defined dense nucleus and poorly defined cytoplasm, probably an oligodendrocyte, represents the opposite end of the glial spectrum.

In the deeper cellular zone, glial cells are also found but the prominent cell is the neuron. The neuronal nucleus is large, frequently remarkably folded, and pale with a prominent nucleolus (Fig. 4). It is surrounded by pale granule-filled cytoplasm extending into processes of greatly varying dimensions. Some comet-shaped neurons appear to extend themselves as a single large process.



Figs. 3 and 4 are photomicrographs of 1 μ sections of epon-embedded tissue stained with toluidine blue. The remaining figures are electron micrographs of OsO_4-fixed, Epon-embedded, and lead citrate–stained tissue.

FIGURE 3 This figure is representative of the area of a culture enclosed by the larger rectangle in Fig. 2. The surface of the culture (arrow) is formed by a few cell perikarya and many flattened processes. There is no meningeal or ependymal sheath over this culture. The suggestion of extracellular space immediately below the surface is confirmed by electron microscopy. Underneath this area is the neuropil zone where the cell processes are closely packed. The deeper portion of the explant contains most of the perikarya of neurons and glia. Neuronal nuclei are light and display prominent nucleoli. Nuclei of glial cells (*gc*) appear more dense and sometimes more elongated than their neuronal counterparts. A small amount of myelin is present at *m*. 23 days *in vitro* (d.i.v.). × 770.

FIGURE 4 These cells are found in an area represented by the smaller rectangle in Fig. 2. The light nuclei, some of which contain prominent nucleoli, identify the neurons. Indentations and folds of these nuclei are apparent. The denser nuclei belong to glial cells (*gc*). One of the glial cells contains a considerable amount of equally dense cytoplasm extending into many processes. The electron microscopic appearance of such a cell may be seen in Fig. 26. Between the cells is neuropil. 76 d.i.v. × 4000.

Some regions of the neuronal zone may be occupied by islands of relatively cell-free neuropil.

As the necrotic zone is approached, cellular processes appear more and more dilated. In the necrotic area, large spaces and debris-containing processes give the zone an alveolar appearance. The electron microscope study was confined to areas well above this region.

Electron Microscopy

In the central portion of the explant, cell somas and processes usually are closely packed together with little intervening extracellular space (Figs. 5, 17, 19), as in central nervous tissue *in vivo*. Superficial and lateral areas of the explant, on the other hand, contain varying amounts of intercellular space (Fig. 21).

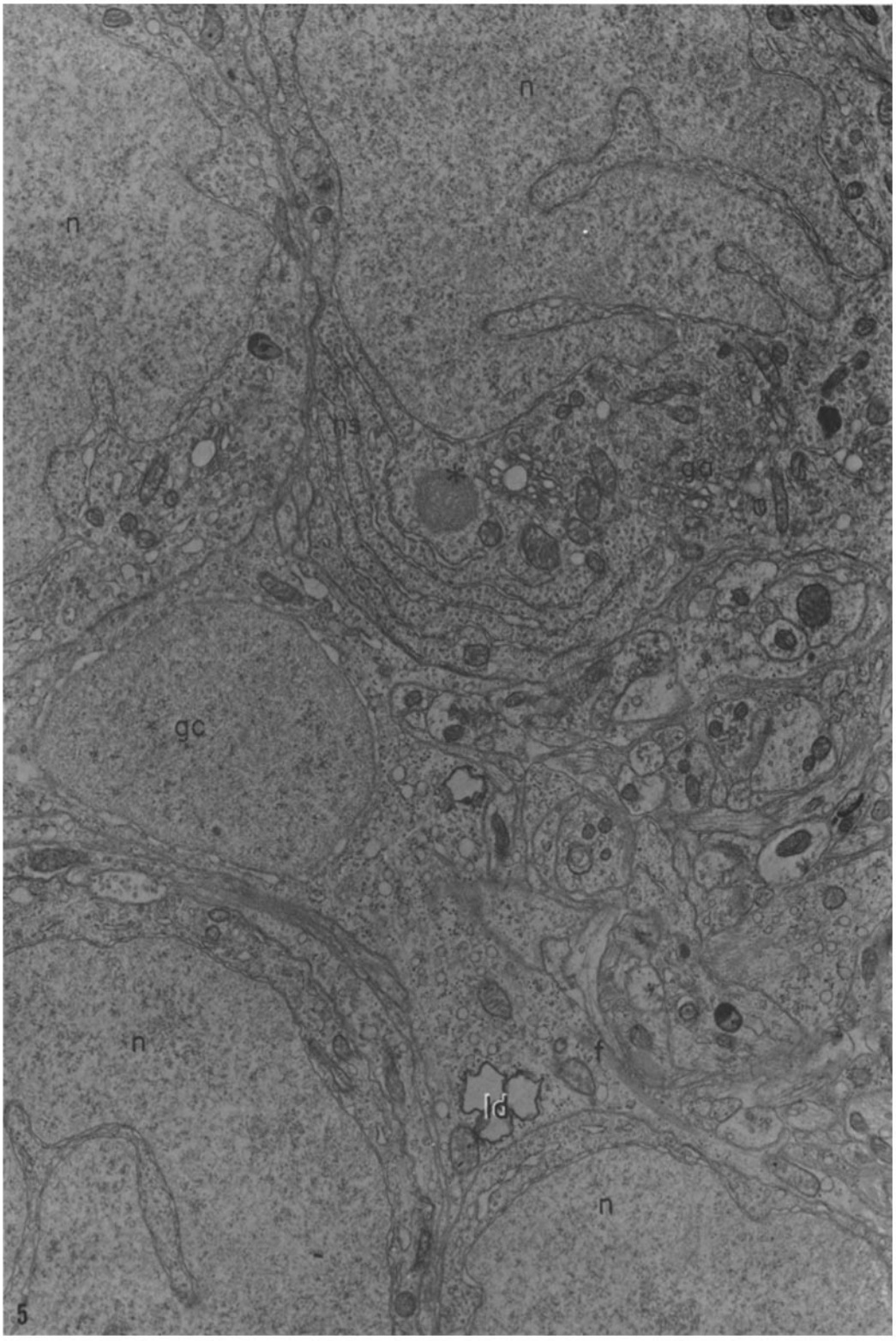
Neuronal Somas

Most neurons are identified by their size and shape, characteristic Nissl substance, and nuclear morphology. Although neurons are often found in groups of two or three, intervening layers of flattened glial processes generally prevent their direct contact (Fig. 5). Transversely sectioned, the perikaryon is approximately ovoid but irregularly indented by the many neighboring processes and cells. A few neurons appear comma-shaped with the nucleus occupying the thicker portion of the comma and Nissl substance sometimes filling the narrowing part (Fig. 19). In thin sections two processes may be visible in continuity with the cell body; usually only one is captured at one level. Occasionally, minute "spines" complicate the perikaryal profile. The largest neuronal soma found measured 30 μ which may represent only part of the dimension; the smallest neurons, undoubtedly not identifiable as such with the light microscope, measured 8 to 10 μ. These smallest somas exhibit only a narrow rim of cytoplasm. The variations in shape and size probably reflect the differing types of neurons included in the original explant rather than the effect of *in vitro* life.

Throughout the large *nuclei* the chromatin is quite uniformly dispersed with usually no clumping visible near the envelope (Fig. 5). Typical nucleoli are prominent, occasionally two being visible in a thin section of a nucleus. Nuclei in the small neurons display some indentations, are usually centrally placed, and follow the perikaryal outline. The larger neurons have more highly indented and folded nuclei which are eccentrically located; the folds and indentations are confined mainly to that portion of the nucleus facing the main aggregate of cytoplasmic organelles (Fig. 5). The folds may be so extensive that the nucleus appears lobed. The folds leave trapped "within" the nucleus islands of cytoplasmic constituents (mainly clustered ribosomes, sometimes vesicles and vacuoles, occasionally a mitochondrion or dense body, rarely a cisterna of the granular endoplasmic reticulum). In these features—eccentric nuclei with folds oriented toward the "central zone"—they resemble the secretory neurons described by Palay (50). Ross, Bornstein, and Lehrer (73) found dramatically indented neuronal nuclei in their cultures of rat cerebellum. This remarkable indentation is not inevitable in cultured neurons, however, for cultured rat dorsal root neurons contain large rounded nuclei without substantial irregularities (9). Nuclear pores are visible as perforations in the transversely sectioned envelope as Palay and Palade (53) first saw them, or as circular structures measuring $\sim$ 900 A, outside diameter, when the envelope has been sliced tangentially. As commonly observed, the outer membrane of the envelope is more ruffled than the inner one. Ribosomes are associated with the outer membrane.

The cytoplasm is stocked with the usual organelles. Neuronal *mitochondria* resemble those first described (53) in that they are round or ovoid or slender rods. The latter may be straight, curved or bent, sometimes exhibiting branches. Within the moderately dense mitochondrial matrix the cristae are easily resolved, traversing the

Figure 5 This general view shows portions of four neurons (*n*), a glial cell (*gc*), and neuropil. In one of these neurons can be seen organized Nissl substance (*ns*), a cytoplasmic aggregate of unknown nature (*), Golgi apparatus (*ga*), and a number of mitochondria. The nucleus displays folds opposite this collection of organelles. We consider the glial cell to be of the astrocytic type. It contains a bundle of filaments (*f*), scattered dense particles interpreted as glycogen, and lipid droplets (*ld*). In the neuropil are a number of neurites and glial processes variously oriented and in close apposition as *in vivo*. 35 d.i.v. × 12,000.


n
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diameter or length of the mitochondrion or sometimes differing in direction within the same mitochondrion. Particles are not visible as they are in glial mitochondria.

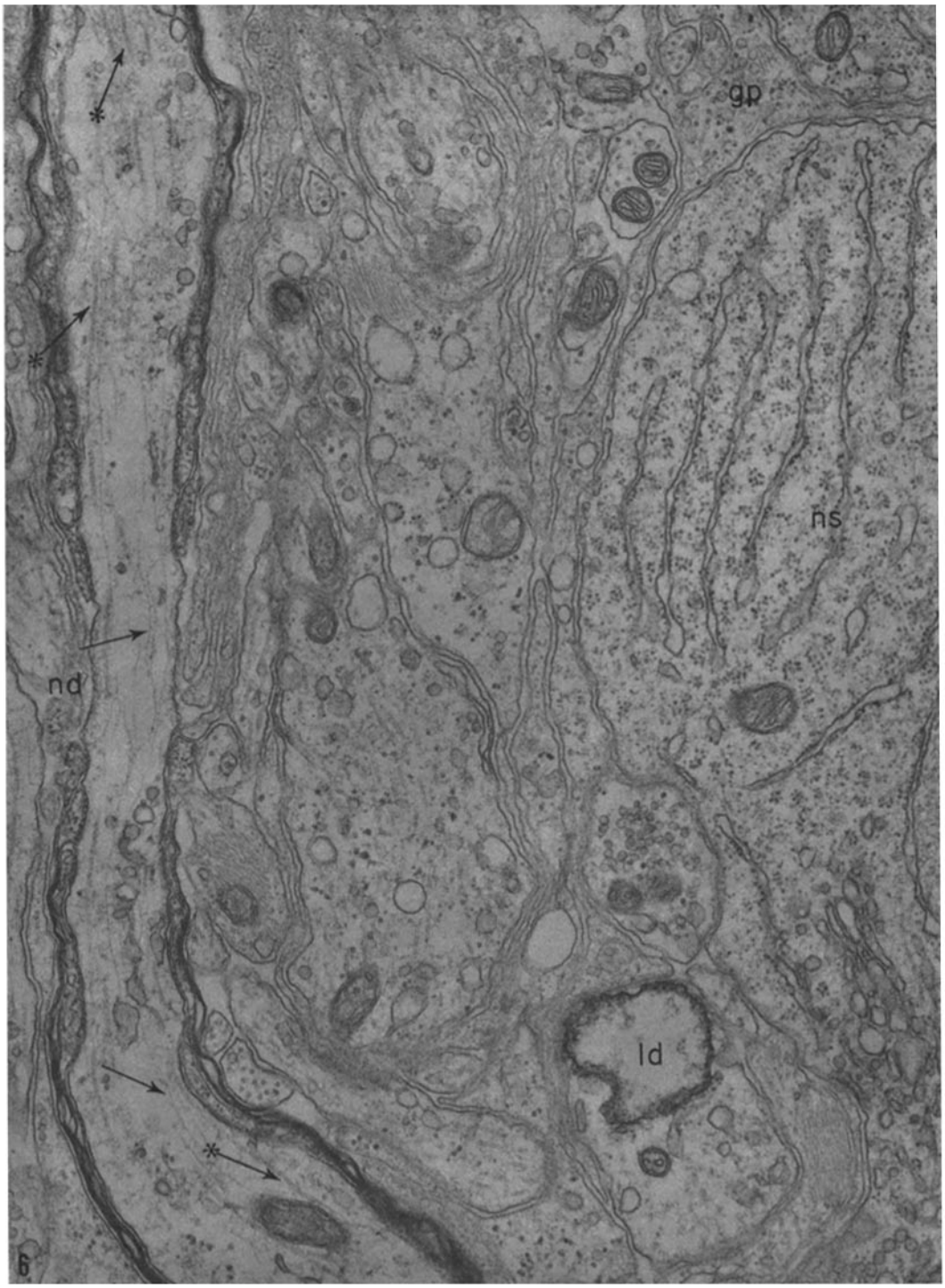
The *Golgi complex* typically consists of elongated and vacuolar profiles surrounded by a profusion of smaller vesicles in rows or clusters (Figs. 5, 8, 9, and 11). Some vesicles appear to be merging with or budding from the vacuoles or cisternae; they always appear more dense than the latter, possibly reflecting their shallowness rather than contained material. The complex is prominent, sometimes reaching a quarter of the way around the perikaryon at one level.

Occasionally associated with the Golgi complex are round or irregularly oval *granule-containing vesicles* (Fig. 7). There is a constant hiatus between the granule and enveloping membrane of the vesicle. More rarely, a granule of similar size and density can be detected within a Golgi cisterna where it is presumably forming (Fig. 7), or a granulated vesicle is seen in contiguity with a cisterna (Fig. 8). Such granule-containing vesicles may be either precursors of the larger dense inclusions scattered throughout the perikaryon or, more likely, related to granulated vesicles of similar size and appearance located in the neurites, particularly in their terminating portions (see below).

Often associated with the Golgi apparatus are the frequently occurring *multivesicular bodies* (first observed by Palay and Palade, 53). At one extreme, they are spherical with a matrix as dense as the evenly dispersed vesicles within (Fig. 9). At the other, these bodies present an irregularly round profile as large or slightly larger than the largest Golgi vacuoles, seemingly empty but for one or a few dense vesicles (Fig. 10). The observation that the latter type is surrounded by vesicles and often displays vesicle-like evaginations or invaginations at the outer membrane suggests vesicle traffic in or out. Though the vesicles surrounding the multivesicular body are spherical, the profiles of the enclosed vesicles may appear as thick-rimmed discs or circlets with circumferential thickenings (Fig. 18). Scattered membranous fragments and poorly defined dense patches are occasionally detected among the enclosed vesicles. The membrane delimiting the multivesicular body was found broken only very rarely. Occasionally on a flattened portion of the surface of these bodies there is a dense plaque (which cannot be attributed to obliquity of section) (Fig. 10) like those Pappas and Purpura (57) found on dendritic multivesicular bodies in cat cortex and Palay (52) observed in rat Purkinje cells. Pappas and Purpura likened this thickening to those observed on the postsynaptic membrane of dendrites. Speculation of a specific relationship to synaptic morphology is complicated by our finding similar thickenings on glial multivesicular bodies in the cultures (see below).

Additional inclusions are round or oval dense bodies frequently larger than mitochondria. These bodies vary in density and may contain particles, stacked linear densities or, more rarely, barely visible small vesicles. Remnants of cristae mitochondriales or other cytoplasmic organelles are not seen. Occasionally the single limiting membrane appears slightly larger than the dense mass inside. Presumably the lysosomes are among this population. Also present are single vacuoles and vesicles, lipid droplets exhibiting a dense wavy outline (Fig. 5), and vesicles coated with a radially oriented filamentous material (Fig. 11) often found near the Golgi complex or plasmalemma (and probably similar to those Roth and Porter have shown to be concerned with protein uptake at the cell surface, reference 74). An inclusion apparently peculiar to these cultured neurons is shown in Fig. 5. It is a large round aggregate of granular or filamentous material less densely stained and of smaller diameter than ribosomes. Not bounded by membrane, it is often found in association with a Nissl body. A few centrioles and

FIGURE 6 This area contains a myelinated axon in which neurofilaments (arrows) and tubules (starred arrows) may be directly compared. Vesicles of assorted shapes and sizes, some of them presumably tubule dilatations, are also present. The myelin terminates to form a node (*nd*) 0.85 μ long. The outermost myelin unit terminates nearest the node as usual. The neuron at the right contains organized Nissl substance (*ns*). A glial process (*gp*) bifurcates to ensheath this neuron. One of the processes of the neuropil contains a lipid droplet (*ld*). 35 d.i.v. × 30,000.


gp
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cilia (Fig. 11) are seen; with the electron microscope, these organelles have been found in some mature CNS neurons *in vivo* (17, 44, 50, 51, 54, 55).

Nissl substance, composed of ribonucleoprotein particles in association with the endoplasmic reticulum as first discovered by Palay and Palade (53), is prominent (Figs. 5, 6, 18, and 19). The membrane comprising the slender rippling cisternae is only intermittently studded with ribosomes. When the cisternal membrane is tangentially sectioned, the associated ribosomes may appear as single or double rows which are straight, curved, e-shaped or looped, or form a circle. Actually the bulk of the ribosomes is not attached to membrane but is scattered between the cisternae of a Nissl body (Figs. 6 and 18) or throughout the remainder of the cytoplasm (noticeably filling the cytoplasmic invaginations into nuclei, for example). These free ribosomes usually appear in clusters.

Within the frequent dilatations that characterize these cisternae, filamentous material is often visible. A cisterna may occasionally be seen to branch. Infrequently a cisterna is seen to be receiving or dispatching small vesicles like those in Golgi areas. Some of the long cisternae of the granular endoplasmic reticulum may be intimately aligned along the mitochondrial surface, even to the point of partly encircling some mito-

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Figs. 7 through 13 are portions of neurons.

FIGURE 7 At the border of this Golgi complex is a vesicle, ~950 A in diameter, containing a dense particle which measures ~600 A (arrow). A granule of similar density and size appears within a cisterna of the Golgi complex (starred arrow). 76 d.i.v. × 37,000.

FIGURE 8 Associated with this Golgi complex is a granulated vesicle which appears to be confluent with one of the cisternae (arrow). The enclosed dense granule measures ~650 A in diameter, comparable with those pictured in Fig. 7. Just outside this area were four granulated vesicles nestled among the components of this Golgi apparatus. 35 d.i.v. × 37,000.

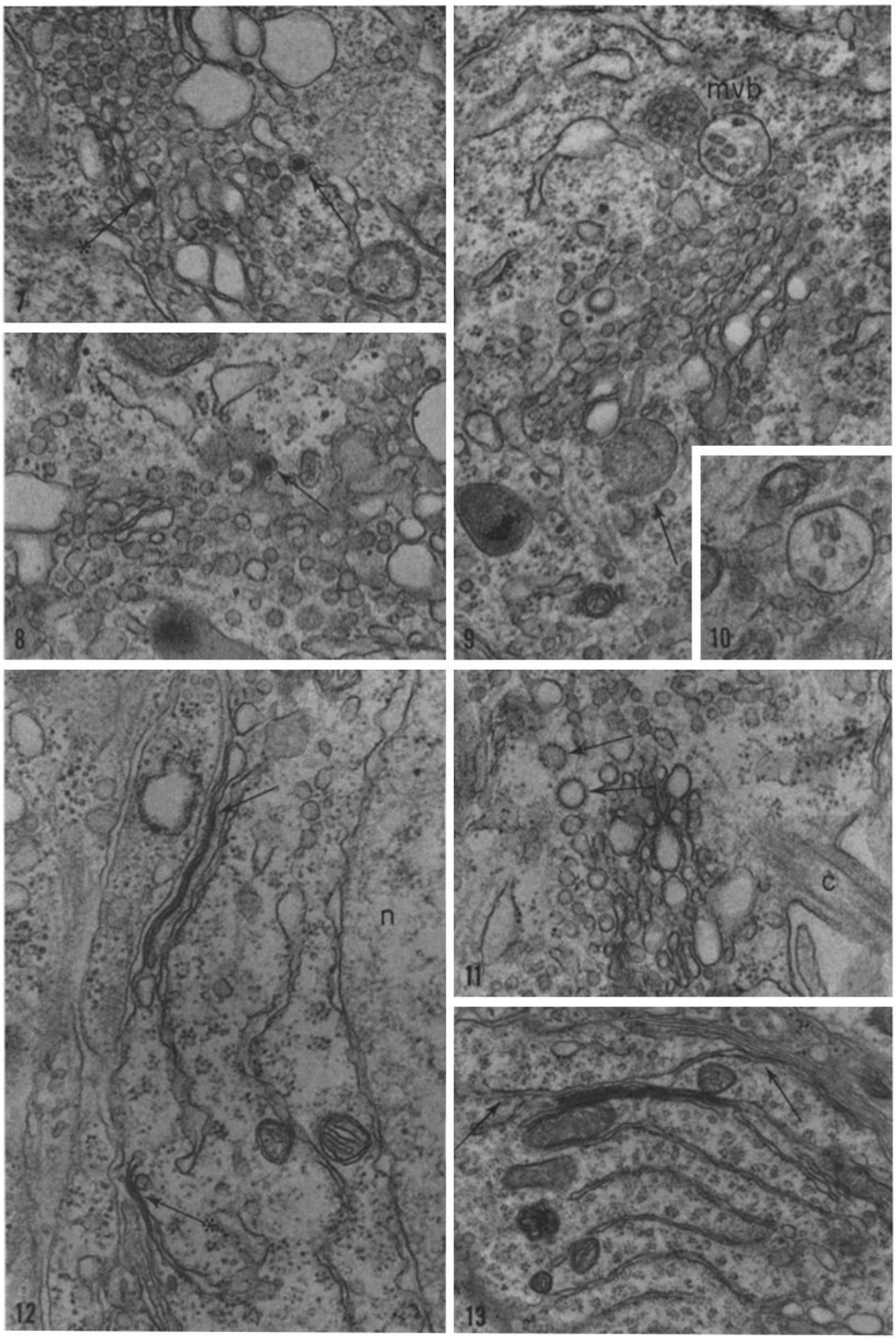
FIGURE 9 Multivesicular bodies (*mvb*) are often found in groups of two or three near Golgi areas and vary in density as shown here. The matrix of the multivesicular body on the left is of such a density that the enclosed vesicles are barely discernible. The occurrence of multivesicular bodies like these, as well as additional transitional types, suggests that they may be one source of dense bodies like the one below (arrow). 23 d.i.v. × 37,000.

FIGURE 10 This multivesicular body exhibits dense material on the surface of a flattened portion of its limiting membrane. In micrographs such as this, there is a suggestion that this substance is arranged as a coil or as radial filaments along the surface. 35 d.i.v. × 41,000.

FIGURE 11 Interrupting the contour of this neuron is a cilium (*c*). It has been sectioned obliquely, and so determination of its inner structure is precluded. Also in this figure is a Golgi complex and two vesicles coated with radial filaments (arrows). 35 d.i.v. × 37,000.

FIGURE 12 Subsurface cisternae are illustrated here. The upper one (at arrow) is 1 μ long and is greatly flattened, measuring only ~190 A from outer surface to outer surface of apposed membrane. Parallel to it is a cisterna which is dotted with ribosomes on its lateral portions and cytoplasmic surface. The starred arrow points to a cisterna which is devoid of ribosomes and flattened where it is in the subsurface position. (The neuronal nucleus is designated by *n*.) 35 d.i.v. × 37,000.

FIGURE 13 In this area, portions of neighboring partly granular cisternae are closely apposed and highly flattened, thus forming a myelin-like fragment. An additional cisterna (arrows), similarly dotted with particles on only some portions of its surface, appears to be related to this configuration and also to be subjacent to the plasmalemma. 23 d.i.v. × 24,000.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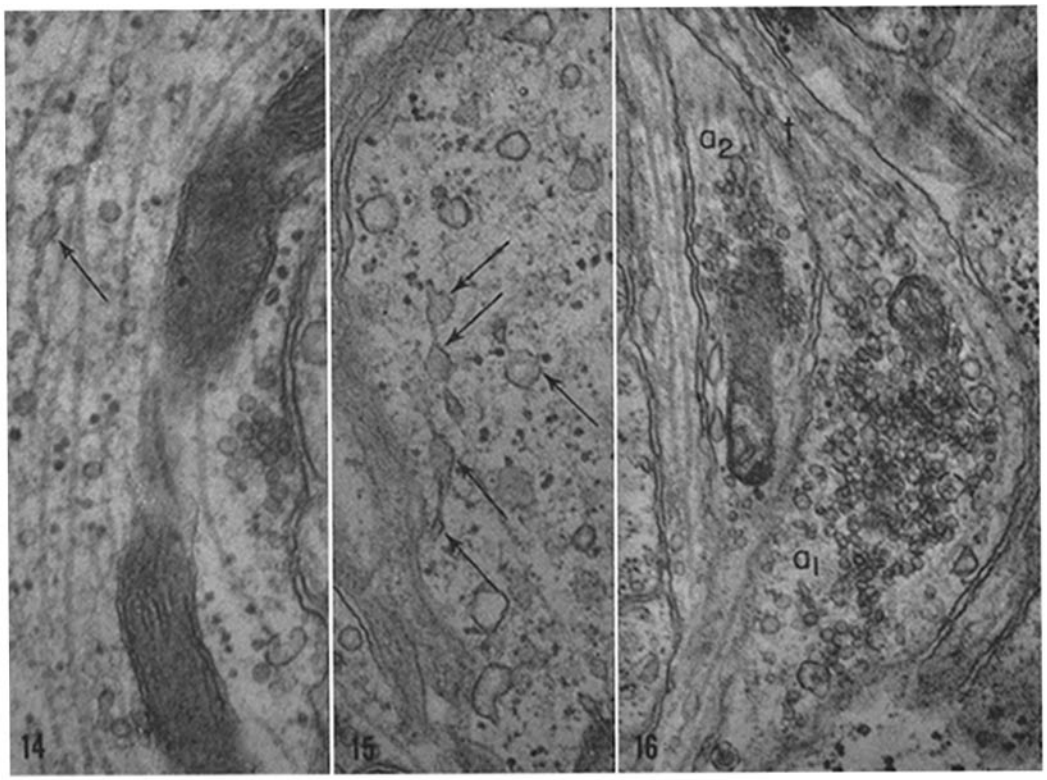


FIGURE 14 This micrograph shows the tubules found in neurites. They measure ~220 A and are often remarkably straight. Some tubules display dilatations such as the one included here (arrow). 76 d.i.v. × 50,500.

FIGURE 15 In this segment of a dendrite are examples of dilatations in the tubular system (arrows). The dense particles are probably glycogen. 35 d.i.v. × 37,000.

FIGURE 16 This micrograph contains a process positively identified as an axon (a_1) because of the synaptic vesicles clustered within it. In addition to these vesicles, tubules (t) are present particularly in its narrowed portion. Tubules and synaptic vesicles are seen together, not quite so well, in a neighboring axon, a_2. 35 d.i.v. × 36,000.

chondria. At one extreme, a neuron contains a few scattered cisternae which roughly follow the curvature of the nucleus. At the other, many long cisternae lie in parallel array (Fig. 19). Nissl bodies are generally found at the perikaryon periphery. Sometimes the inner cisternae of a Nissl body form nearly complete rings, the outer components roughly following this configuration. Near the Nissl substance, a few whorls of more closely packed membrane which contain some scattered apparently unassociated ribosomes are visible. Similar configurations have been seen *in vivo* ("onion-like corpuscles," reference 53).

The variation in amount and organization of Nissl substance again undoubtedly reflects the differing types of neurons present. Such variation occurs *in vivo* (53). The most well developed Nissl substance we have found probably identifies the motor neuron; Palay and Palade (53) found that of all the neurons they studied electron microscopically the motor neurons contained the most well organized Nissl bodies. On the other hand, neurons in which cisternae are not aggregated are commonly found *in vivo* (37, 53, 57, 76). Though the eccentric indented nuclei of some cultured neurons may suggest a chromatolytic condition,

the well organized Nissl substance, a more reliable indicator of the neuronal state, attests to their maturity.

Related to the granular endoplasmic reticulum are the *subsurface cisternae* found frequently in these neurons. It is the presence of these structures that allows us to identify with certainty some of the smaller neurons; in nervous tissue the cisternae have been found only in nerve cells (70). Subsurface cisternae measure 1½ μ or less in breadth and may be flattened such that the apposing portions of the cisternal membrane and enclosed lumen altogether measure only 180 A (Fig. 12). A ribosome-dotted cisterna may approach the plasmalemma, follow it closely for a distance (here devoid of ribosomes and its lumen narrowed) then leave the vicinity of the membrane and, again displaying ribosomes, wander medially perhaps into the depths of a Nissl body. Or, such a profile may lie just below a subsurface cisterna that is almost completely flattened with the apposing membranes nearly touching and lacking in ribosomes (Fig. 12). In this case, ribosomes are found only on the cytoplasmic side of the innermost cisterna. Either of these configurations may be teamed with additional granule-studded cisternae lying roughly parallel below. A singly occurring cisterna whose entire extent is parallel to the plasmalemma displays fewer ribosomes on its cytoplasmic side as the degree of flattening increases; ribosomes or other organelles are never found between the cisterna and the plasmalemma. Varying configurations may be found within one perikaryon.

These configurations resemble closely the ones that Rosenbluth (70) discovered in cord, cortex,

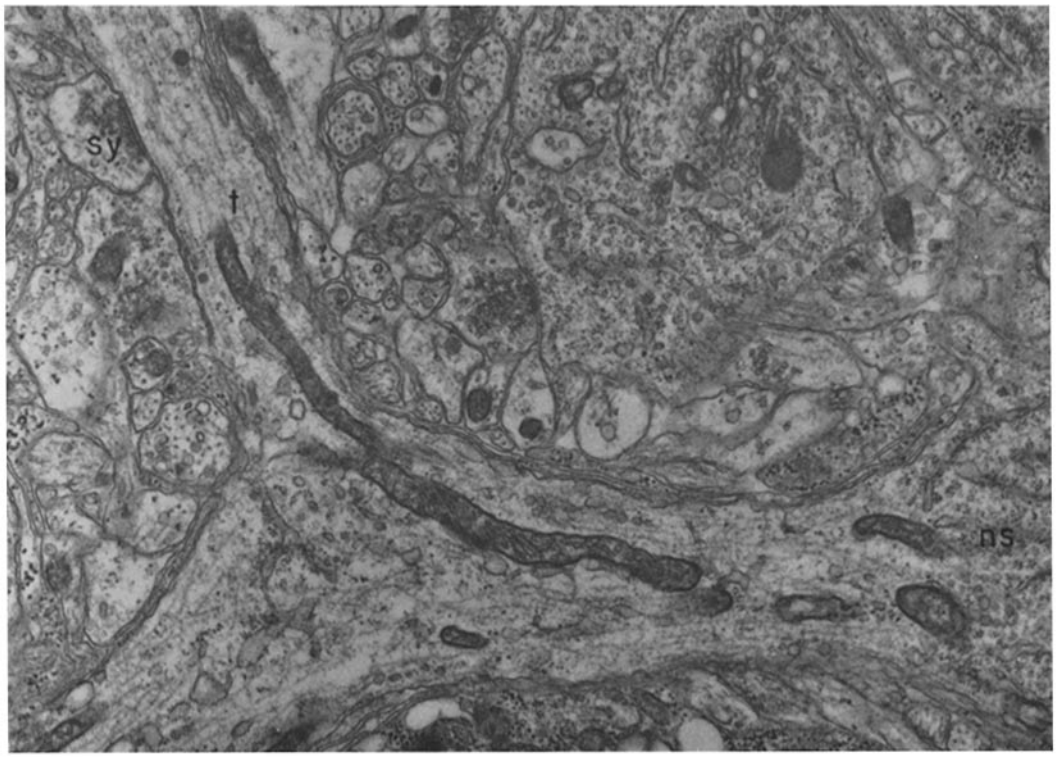


FIGURE 17 This branching dendrite contains considerable Nissl substance (*ns*) in its stoutest portion and mostly tubules (*t*) within the narrowest segment included here. On the latter is a terminating axon. This axodendritic synapse (*sy*) is characterized by an accumulation of synaptic vesicles and thickened apposed membranes of increased density. Surrounding portions of the dendrite are highly flattened glial processes containing some dense particles presumed to be glycogen. Note the uniformity of the extracellular space. 23 d.i.v. × 17,000.

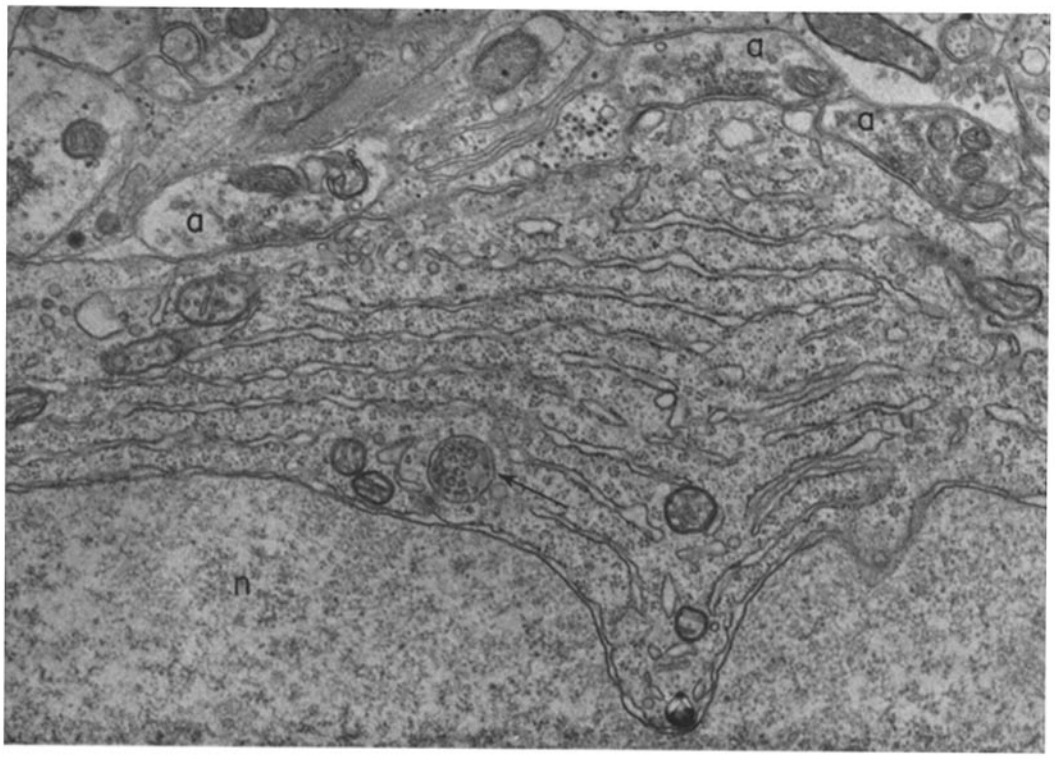
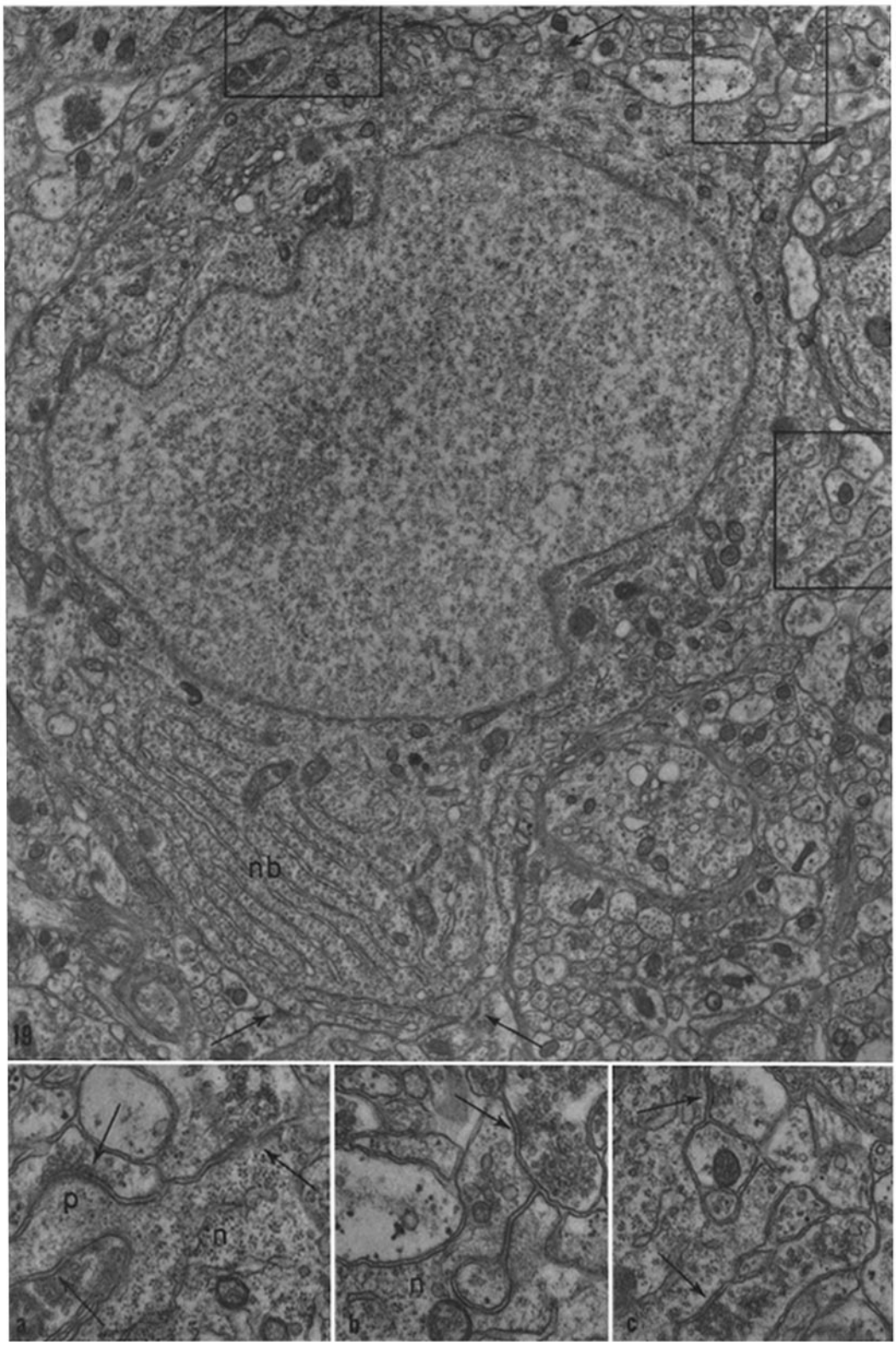


FIGURE 18 Three axons (*a*) are terminating on this portion of a neuron. The accumulation of synaptic vesicles is the most prominent feature of these typical axosomatic synapses. Inside the neuron characteristically are ribosomes, free and associated with oriented cisternae of the endoplasmic reticulum, mitochondria, vesicles and, at the arrow, a multivesicular body. Part of the nucleus (*n*) is present. 35 d.i.v. × 22,000.

and ganglia. Furthermore, the characteristics that he found to typify spinal cord subsurface cisternae *in vivo* may be observed here; *i.e.*, the cisternae tend to be broad and thus more frequently highly flattened and devoid of ribosomes on the cytoplasmic side and are often accompanied by a stack of cisternae below. The subsurface cisternae appear not to be in relation with mitochondria, as suggested elsewhere (37, 70), or with synaptic areas on the plasmalemma, as observations by others may (30, 57) or may not (37, 70) indicate. In rare instances, we have noted a cisterna of the granular endoplasmic reticulum to traverse the perikaryon, at one end apposing the nuclear

FIGURE 19 This neuron contains a Nissl body (*nb*) as well as the usual organelles. On its surface are nine synapses at this level. Some synaptic junctions are designated by arrows, while the remainder (enclosed within rectangles) are enlarged below. Within insert *a* are three synapses (arrows). Two of them are found on a cytoplasmic projection (*p*) of the neuronal soma (*n*). Insert *b* illustrates another synaptic junction (arrow) between axon and an evagination from the cell body (*n*). Material can be seen within the synaptic cleft. Within insert *c*, two axons are terminating on the soma (*n*) at the arrows. All these synapses, whether on the soma or on the "spines", are Type 2 according to Gray's classification (26). Note in the large figure that extracellular space is minimal as in central nervous tissue *in vivo*. 23 d.i.v. × 11,500; Inserts *a* and *b*, × 26,500; Insert *c*, × 20,500.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envelope and at the other, forming a subsurface cisterna.

Rosenbluth (70) detected stacks of flattened cisternae deep in the neuronal cytoplasm which resemble those associated with subsurface cisternae. There is a comparable situation in the cultured neurons. We have found, away from the surface, a few parallel arrays of flattened cisternae displaying ribosomes only on their widened lateral portions. In one grouping (Fig. 13), the flattened cisternae apposed one another so closely that they appeared to form a myelin-like fragment with major dense lines; one of the cisternae involved could be followed to the plasmalemma where it assumed a subsurface position.

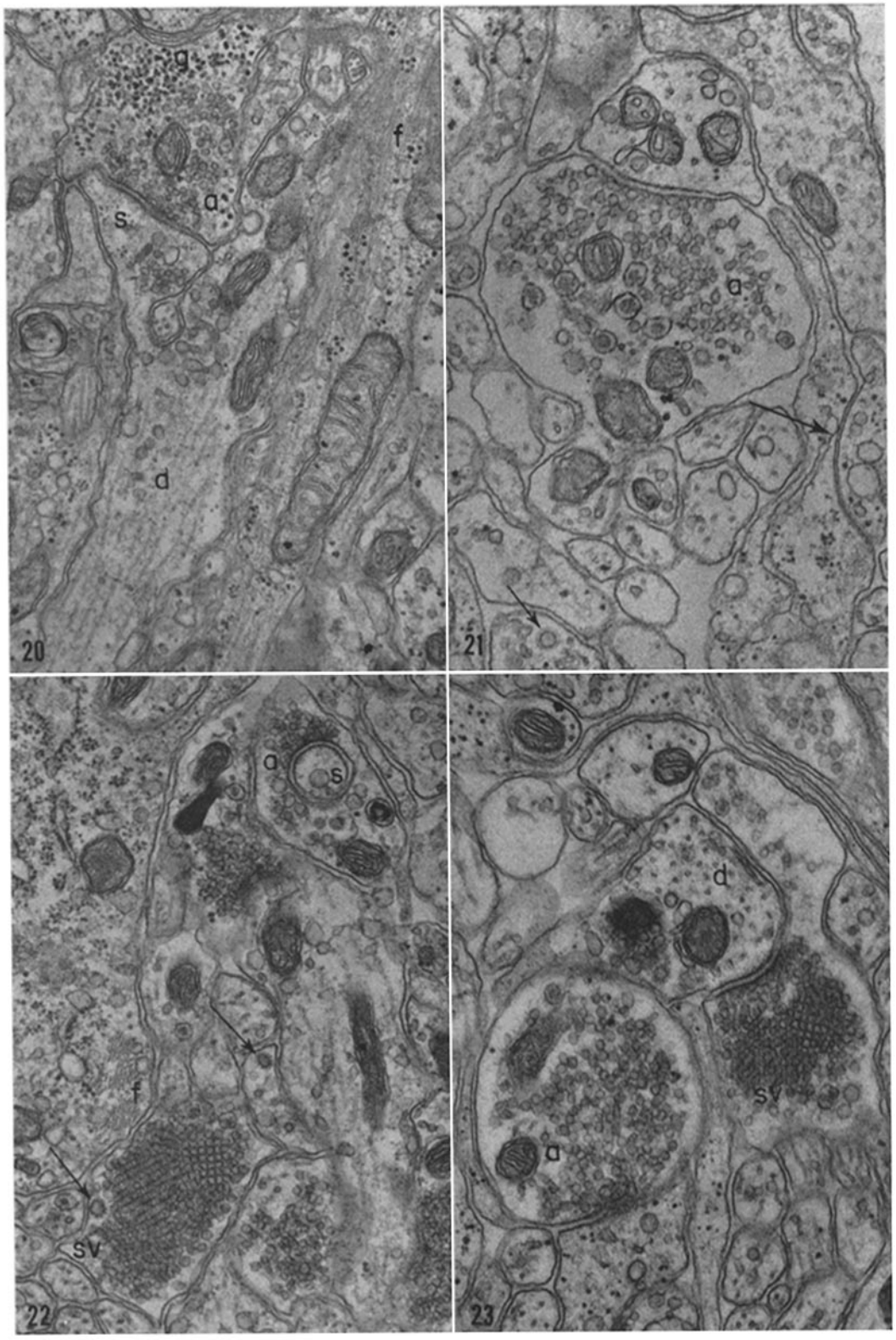
Few, if any, *neurofilaments* are visible in the perikaryon; they are found predominantly in the axons. This finding correlates with the experience that success in silver staining these perikarya is limited (61), for silver impregnation apparently depends, at least in part, on the presence of neurofilaments (33, 35, 78). Long slender *tubules*, however, are conspicuous in the perikarya. Absent from areas of organized Nissl substance, they appear either dispersed throughout the central zone or as loose bundles often swerving around the nucleus toward a dendritic trunk. At times the tubules seem to merge with the nuclear envelope but this impression may result from tangential sectioning of an envelope with adjacent tubules. The tubules are particularly prominent in Golgi regions. These structures measure 200 to 240 A, averaging 220 A, in outside diameter. They are, therefore, comparable in diameter with those previously described in central nervous tissue (3, 26, 46, 57, 69, 79, 80, 83) and in a variety of other tissues (reviewed by Slautterback, reference 77). In our preparations, the membrane (?) delineating the tubules consistently appears thinner and less dense than either plasmalemmal or mitochondrial membrane; this is true for both longitudinally and transversely sectioned tubules. It is of interest that tubules are not visible after $KMnO_4$ fixation (26, 68), further suggesting that they may not consist of the usual lipoprotein complex comprising typical unit membrane. Palay (46, 47) noted first that tubules, or canaliculi in his words, exhibited occasional enlargements, these swellings possibly representing the smooth endoplasmic reticulum. We also detect occasional dilatations in some of the tubules (to a limited extent here but to a greater degree in dendrites where tubules are most concentrated). In longitudinal section, the tubules showing dilatations differ from the majority in that

FIGURE 20 This dendrite (*d*) displays a spine (*s*) on which an axon is terminating. The spine contains only particles and vesicles of varying sizes. The axon with which it synapses (*a*) contains synaptic vesicles and particles interpreted as glycogen (g). Beside the dendrite is a glial process identified by its filaments (*f*). This process also contains particles, presumed to be glycogen, and mitochondria which display dense particles. Glial and neuronal mitochondria may be compared directly here; characteristically those occupying the neurite are smaller (as noted *in vivo*, reference 54) and lack particles. 35 d.i.v. × 27,000.

FIGURE 21 This synapsing axon (*a*) contains both typical synaptic vesicles and larger granule-containing vesicles. The synaptic vesicles average ~500 A here; the larger vesicles measure ~1250 A, the contained granules measuring ~650 A in diameter. The large arrow points to an area where glial cell membranes come closer together and are more parallel than usual. The small arrow points to a "coated" vesicle. Intercellular space is not so uniform here. 35 d.i.v. × 35,000.

FIGURE 22 Synaptic vesicles (*sv*) may occasionally be clustered together in an ordered array as illustrated here. One axon (*a*) is terminating on a dendritic spine (*s*) which has invaginated into it. A few granulated vesicles and "coated" vesicles (arrows) are scattered throughout this area. Glial filaments (*f*) may also be seen. 76 d.i.v. × 31,500.

FIGURE 23 An ordered array of synaptic vesicles (*sv*) may be seen at the level of a synapse in this illustration. (The array may be directly compared to the usual appearance of synaptic vesicles in an adjacent axon, *a*). Material can be seen in the synaptic cleft. Within the synapsing dendrite (*d*), and other neurites as well, a number of tubules have been sectioned transversely. 76 d.i.v. × 35,000.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their limiting "membrane" appears slightly more dense and their course is tortuous rather than straight (Figs. 14 to 16). This raises the possibility, then, that there exists in neuronal cytoplasm two separate systems of tubular structures, functioning perhaps in one case as a transport system and in the other as support.

Neuronal Processes and Synapses

Proximal dendritic trunks resemble perikaryal cytoplasm in their complement of organelles except that cisternae of the granular endoplasmic reticulum are less frequent and usually unoriented, subsurface cisternae are less numerous and occur only singly, and tubules are more concentrated and become aligned along the length of the dendrite. The mitochondria are oriented parallel to the length of the process as usual; the density of the mitochondrial matrix is similar to that observed perinuclearly. In thinner dendrites the tubules are the most prominent organelles (Figs. 14, 17, and 20). Some of the tubules display conspicuous dilatations. Scattered among them are an occasional neurofilament, clusters of ribosomes, mitochondria, vacuoles, vesicles (sometimes in chains and infrequently containing a dense granule), and multivesicular bodies. The multivesicular body found in the processes is more often of the type that is filled with dense vesicles evenly distributed in a dense matrix. A branching dendrite we observed appears in Fig. 17. As a dendrite continues to narrow it contains fewer and fewer organelles and can be distinguished from axons of similar size only with difficulty or not at all, as Palay (47) has noted. Spines may be found on the dendrites (Fig. 20). They contain only vesicles that vary in size and particles that vary in clarity, the most punctate resembling ribosomes. Neither tubules nor spine apparatuses are present. Gray (27) has reported that tubules do not enter the dendritic spines in rat visual cortex and, with Guillery (34), has noted that the spine apparatus occurs only very rarely in rat or cat cord.

Axons contain mitochondria, assorted vesicles, in rare cases multivesicular bodies and single subsurface cisternae, scattered particles presumed to be glycogen (see below), neurofilaments, and tubules. Some processes in which tubules are found may be definitely identified as axons because of the presence of myelin (Fig. 6) or contiguity with an enlarged terminal containing the characteristic synaptic vesicles (Fig. 16). In the latter case, tubules are particularly prominent in narrowed portions near the bouton; if these segments were cut transversely they would fit the usual picture of a fine dendrite. Many authors studying *in vivo* central nervous tissue (3, 26, 37, 46, 47, 69, 79, 80, 83) have reported finding tubules in axons though usually not so frequently as in dendrites. The tubules, therefore, generally cannot be used to distinguish between small dendrites and axons, as many of these authors have also pointed out. The axons lack Nissl substance in contrast with the larger dendrites; this is characteristic (47, 64). The axons appear not to be so straight as the dendrites, for only the latter are seen as long segments in a thin section. Wolfe (83) reached a similar conclusion in his study of rat area postrema. Some axons are ensheathed with myelin which displays the usual laminated structure. Typical sheaths exhibit the inner mesaxons, outer loops and nodes (Fig. 6) that we find in cat spinal cord *in vivo* (11). That the myelin sheaths formed *in vitro* resemble their counterparts *in vivo* has been demonstrated earlier in cultured cerebellum by Périer and de Harven (59), and by Ross, Bornstein, and Lehrer (73).

Synaptic junctions are visible throughout the explant. Axons are found terminating on neuronal somas (Figs. 18 and 19), dendritic trunks, dendrites (Fig. 17), and dendritic spines (Figs. 20 and 22). As many as nine axons were found synapsing on a single soma at one level (Fig. 19); more than one axon may be seen terminating on a single dendrite, or one axon may terminate on a number of dendrites. Synapses on very fine dendritic terminals, as reported by Pappas and Purpura (57) in cat neocortex, were not found in the cultures. The synapse-bearing spines are foot-like processes (Fig. 20) or invaginations into the presynaptic process (Fig. 22). Gray has shown that spines carry synaptic junctions (27) and, furthermore, that both these types of spines are synapsing elements in mammalian spinal cord *in vivo* (30). Note in Fig. 19 that synapses also are located on slender evaginations which arise from the neuronal perikaryon much as spines emerge from dendrites. The perikaryal "spines" contain only vesicles and particles as in the case of dendritic spines but they may be longer and more slender. One could argue that these are dendritic spines, the parent dendrite being out of the section, but this seems unlikely because spines were not seen issuing from dendrites

so close to the cell body. We know of no published reports of such perikaryal evaginations other than mention in an abstract by Wolfe (83) that a "synaptic peg" containing only vaguely granular cytoplasm may emerge from a neuron perikaryon in the area postrema of the rat.

The synapses we find meet the specifications that are by now well known (21, 22, 46, 48); on the presynaptic side are gathered typical vesicles measuring 300 to 650 A (average = 450) occurring singly or in aggregates, some of them clustered on the synaptic membrane, and the pre- and postsynaptic membranes are more dense and sometimes appear thickened. The larger axon terminals contain the expected cluster of mitochondria but the more numerous smaller ones do not. An aggregation of mitochondria in the terminating axon is not always seen *in vivo* (20, 21, 26, 43, 85). Typically, neurofilaments and tubules are absent from the presynaptic terminals. In the cultured cord, extracellular material is usually visible in the synaptic cleft.

Some of the axodendritic synapses manifest additional specializations. The synaptic cleft is enlarged and filled with particularly dense extracellular substance, and the occurrence of additional dense material just inside the postsynaptic membrane makes this membrane appear further thickened (Fig. 24); these features correspond to those described by Gray (26) for certain axodendritic synapses. These more complex junctions he labeled Type 1 to distinguish them from the more commonly occurring synapses described above (which he called Type 2). All synapses on dendritic spines and some of the synapses on dendrite trunks were Type 1; all axosomatic synapses were Type 2. Our results agree in part. Type 1 junctions are found only between axons and some dendrites and their spines, as Gray observed, but not all dendritic spines display Type 1 synapses. It is becoming apparent that Type I synapses are more prominent in some areas of the central nervous system than in others (3, 21, 26, 35, 36, 46, 48, 57, 79, 80). Perhaps some but certainly not all of this variation from area to area derives from the use of phosphotungstic acid as a stain, because we (Fig. 24) as well as others (3, 20, 37, 57, 80) have been able to demonstrate Type 1 synapses without it. Gray (26) reported that Type 1 synaptic thickenings cover a greater proportion of the apposed surfaces of the synapsing processes. This difference was not seen in the cultured cord. All the axosomatic synapses, whether involving the soma or the perikaryal "spine," are Type 2, as the *in vivo* work has demonstrated.

Occasionally we find in the larger axon terminals dense particles which are presumed to be glycogen (Fig. 20); the case that these are glycogen will be argued subsequently. Yamamoto (86) has recently identified similar particles as glycogen in presynaptic terminals of bullfrog sympathetic ganglia; Robertson *et al.* (69) consider glycogen to be present in some boutons terminaux in goldfish brain. Also found sometimes among the synaptic vesicles are larger granule-containing vesicles (Fig. 21). They measure 750 to 1250 A (average = 1000); the granules inside are 350 to 750 A (average = 550) in diameter. The loose-fitting membrane frequently varies in density or appears fragmented. Hardly ever are these vesicles concentrated in an ending by themselves; they usually accompany synaptic vesicles. We do not believe that the mixed population observed here could result from different planes of section through granular vesicles (67) (see Fig. 21). Some granulated vesicles are thought to contain catecholamines (67); Wolfe *et al.* (84) have demonstrated by electron microscopic autoradiography that tritiated norepinephrine is associated with granular vesicles of adrenergic nerve endings. Multivesicular bodies may be found near the synapse (more frequently on the dendritic side) but this hardly seems to be their preferential location because they are present throughout the dendrites and axons. For central nervous tissue *in vivo*, other authors (3, 79, 80) have published a similar statement, while Pappas and Purpura (57) believe that multivesicular bodies are preferentially situated near synapses. Lastly, the synaptic vesicles have been observed in a highly ordered array not heretofore reported. In a few axon terminals, a large number of vesicles are grouped together such that they resemble a crystalline array (Figs. 22 and 23). These configurations are not found in axons terminating on perikarya. More arrays were found in the oldest cultures. It seems possible that these arrays could arise from an "excess" of vesicles; in the oldest culture there were more vesicles in a terminal, and even those not in ordered arrays sometimes appeared to be more tightly clustered than usual. The synaptic vesicles could conceivably be accumulating in presynaptic terminals that are inactive or only infrequently

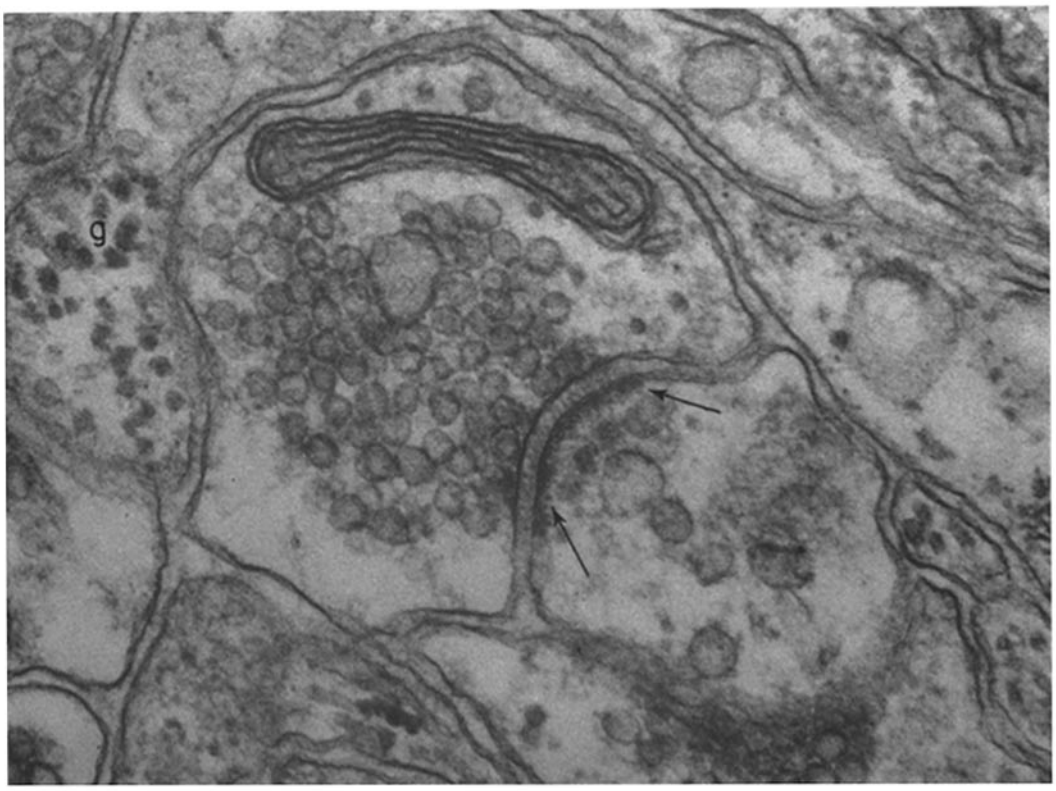


FIGURE 24 Some axodendritic synapses show specializations that are known to occur *in vivo*. This junction displays a widened cleft in which some substance is prominent, and the postsynaptic membrane appears greatly thickened due to the presence of additional dense material (arrows). These features identify this synapse as Type 1 according to Gray (26); they are demonstrated here without phosphotungstic acid staining. Particles thought to be glycogen (*g*), measuring ~350 A here, are present in an adjacent process. 76 d.i.v. × 99,000.

stimulated. Similar crystal-like arrays of vesicles have been found recently *in vivo*; Brightman (6) has observed them in endings distributed throughout the subependymal neuropil of rat brain and regards the arrays as an example of storage of protein structures.

Do we find any evidence of presynaptic inhibitory mechanisms? The synaptic configuration that could provide the morphological basis for this inhibition would involve an axon synapsing with a second axon which in turn would terminate on a dendrite (24). Gray (31, 32) claims to have found such complexes in cat spinal cord. No similar sequence of endings has been found in the present study. The probability of observing the entire sequence is, of course, much less than finding just the axoaxonic junction. But the difficulty here is to prove morphologically that the postsynaptic process is indeed an axon. Thus, some of the synapses thought to be axodendritic may actually be axoaxonic. Perhaps any questionable junctions should instead be labeled axoneuritic. Considering the problems of identification, to the best of our knowledge we have not seen any neurites synapsing with glial processes. (Likewise, we have not noted any neurites attached to glial processes by desmosome-like structures, such as Schultz *et al.*, reference 76, and Robertson *et al.*, reference 69, have considered, perhaps prematurely, because of the identification difficulties.)

GLIA

Glial cell bodies are found everywhere in the explant, the profusion of their processes contribut-

ing substantially to the neuropil. Some glia are identified as *oligodendrocytes*. These are small cells exhibiting a smooth contour interrupted only infrequently by short stout processes. The spherical nucleus, rarely indented, contains uniformly dispersed chromatin except for some clumping near the nuclear envelope; typical nucleoli may be seen therein. The cytoplasm contains mitochondria larger than those in the neurons. Most ribosomes are in clusters free in the cytoplasm; the remaining are associated with an occasional cisterna of the endoplasmic reticulum. Sometimes a few such granular cisternae lie in parallel array. The oligodendrocyte, like the neuron, contains tubules. We have observed these tubules in oligodendrocytes of kitten cord (7), and Palay *et al.* (54) have found them in oligodendrocytes of adult rat CNS. Fine filaments and particles presumed to be glycogen, characteristic of the other glia in these cultures, are not seen.

Some typical *fibrous astrocytes* are found. They contain bundles of typical glial filaments measuring 70 to 100 A in diameter and similar to those described by many investigators of central nervous tissue (10, 11, 28, 43, 49, 54 among others). It is the presence of these filaments that allows us to identify as glial many of the processes in the tangle of neuropil. Scattered among the filaments is a wealth of fine particles presumed to be glycogen. Without the lead staining, these cells would present the more typical "watery" appearance described so often. Typical organelles are present also, among them a few elements of the granular endoplasmic reticulum here taking the form of vacuoles, an occasional lipid droplet, and a few tubules. This cell must dispatch numerous proc-

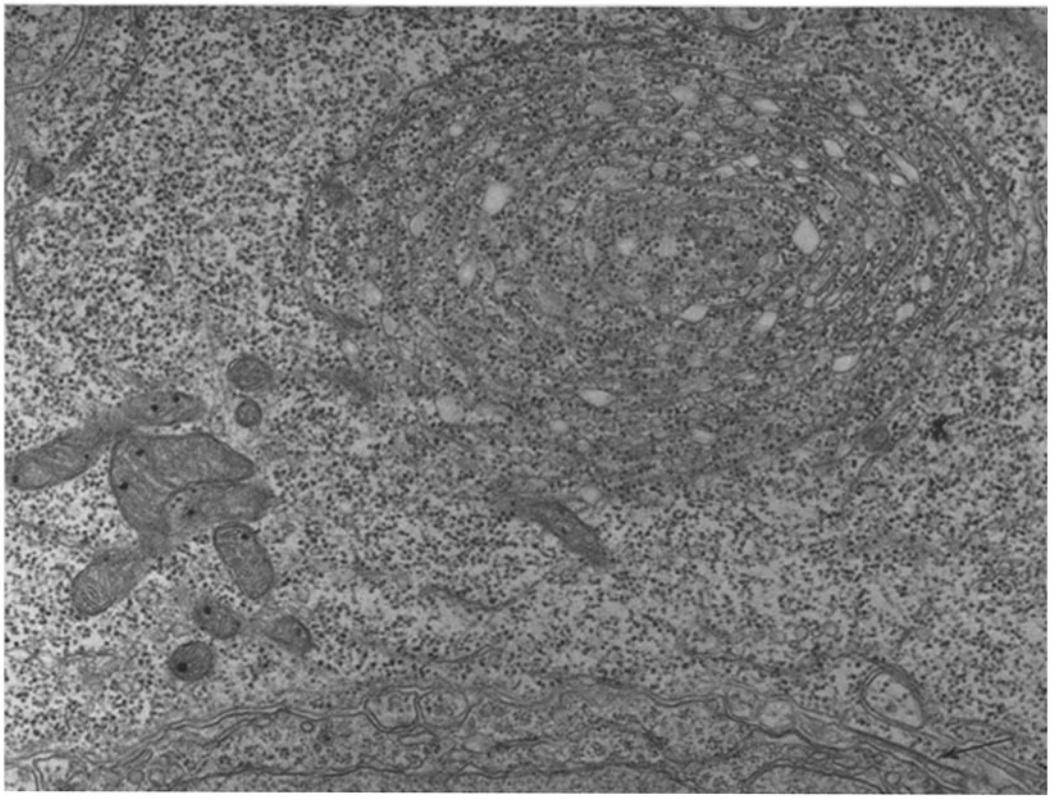


FIGURE 25 This portion of a glial cell is filled with particles that we consider to be glycogen. (These particles can be seen to be larger than the ribosomes associated with the endoplasmic reticulum in the cell below.) Cisternae of agranular membrane are arranged in a whorl or occur singly throughout the area. The mitochondria contain dense particles, as is typical of glia in these cultures. The arrow points to an area where this glial cell closely apposes an adjacent glial process. 76 d.i.v. × 25,500.

esses, for a number of them are always visible at one level. These two glial types resemble those found *in situ* and conform to the classification we (10) have described for *in vivo* cord white matter. Palay and colleagues (54), employing initial perfusion of the CNS with OsO_4 for improved preservation, arrived at a similar conclusion.

The most prominent glial cell in the cultures is one that displays features of both the oligodendrocyte and fibrous astrocyte (Fig. 26). This *intermediate cell* is larger than either of the other types and is stellate-shaped even in thin sections, some of the processes becoming greatly flattened. The nucleus is elongated, showing small indentations or occasionally appearing in two parts due to indentation by a bundle of filaments (Fig. 26). Its outline is not usually highly indented or folded as is that of the neuronal nucleus. This difference was noted also in cultures of cerebellum (73). The chromatin is not quite so uniformly dispersed as that in neurons; some clumping is visible near the nuclear envelope. A nucleolus may be present. When a section grazes the envelope, nuclear pores are visible (Fig. 26). We should note that the nuclear envelope in this cell type often appears artifactually enlarged, as do some vacuoles in the cytoplasm. This cell type is more difficult to preserve than the neuron.

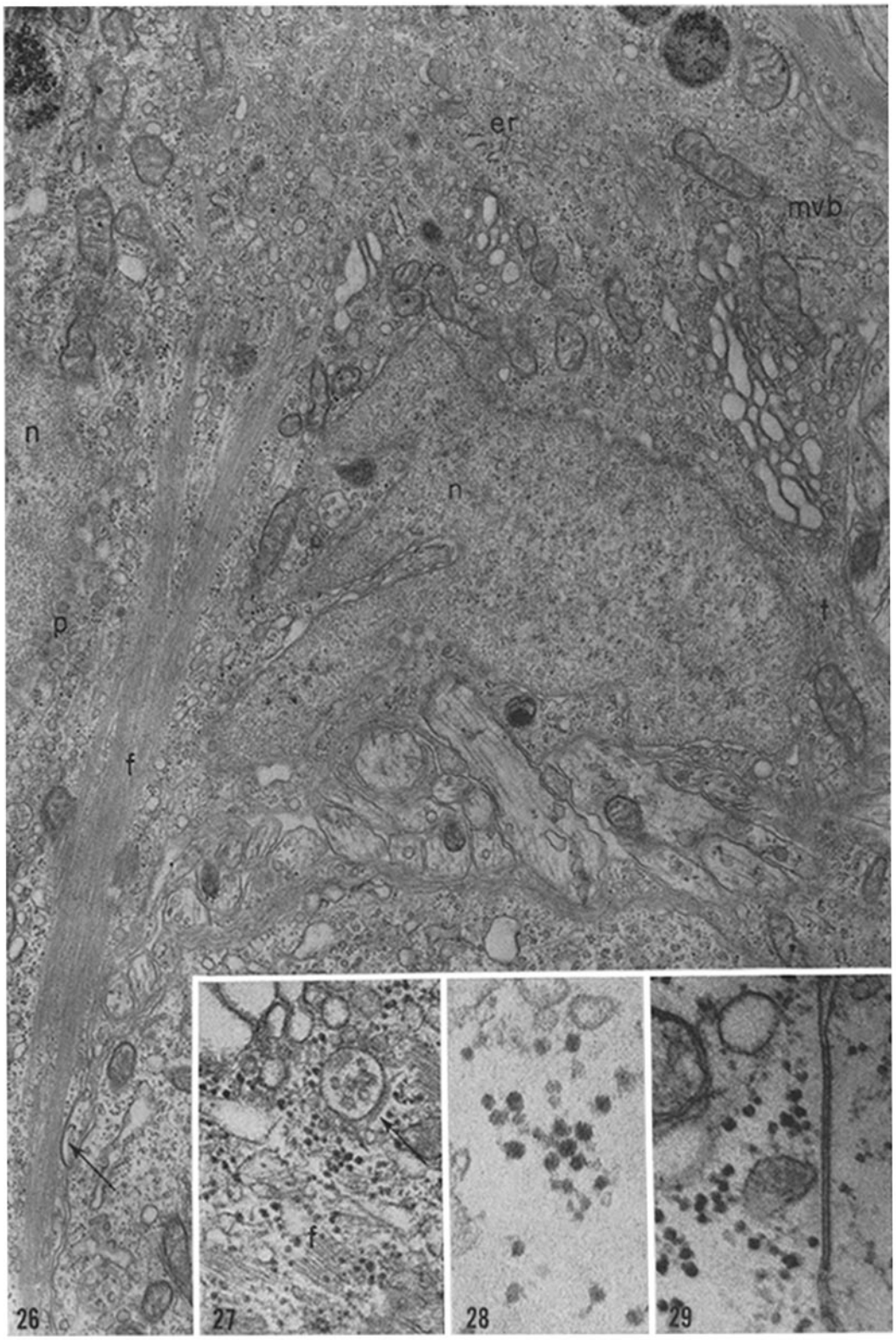
The perinuclear cytoplasmic organelles are closely packed, the ribosomes, glycogen particles, and filaments in particular contributing to the density. The mitochondria are larger than those in neurons and, unlike neuronal mitochondria, display dense particles (Fig. 20). Most of the ribosomes occur in clusters free in the cytoplasm while a few are attached to sparsely occurring short cisternae or small vacuoles of the endoplasmic reticulum. A few of these cisternae may be parallel to one another. When cisternae are cut tangentially the usual loops, e's, and strings of ribosomes are seen. In rare cases a ribosome-dotted cisterna appears engorged with flocculent material. Multivesicular bodies are similar to those described above, including, in some instances, a plaque of fuzzy material on a flattened portion of the surface of the limiting membrane (Fig. 27). Golgi complex, vesicles (including some coated ones), and vacuoles are present. Dense bodies, larger than mitochondria, are round or irregularly shaped and may be speckled with denser particles or

FIGURE 26 This glial cell can be labeled neither oligodendrocyte (because bundles of filaments are present) nor fibrous astrocyte (because of the unusual density of the cytoplasm). Bundles of filaments (*f*), many of them obliquely sectioned and thus not readily apparent, free ribosomes, and particles thought to be glycogen all contribute to this density. Some ribosomes are associated with the endoplasmic reticulum (*er*). One of the multivesicular bodies (*mvb*) is slightly flattened, and there is a suggestion of a plaque of material on its surface. Tubules (*t*) are present. Large dense bodies, as seen at the top of this micrograph, are frequent in this cell type and are visible in the light microscope (Fig. 4). The nucleus (*n*) appears in two parts at this level and displays pores (*p*) measuring ~1000 A. Between this cell and a neighboring glial process is a "junction" where their plasmalemmas are unusually close and parallel (arrow). 35 d.i.v. × 19,500.

FIGURE 27 Multivesicular body in a glial cell. The arrow points to an area on the surface displaying a moderately dense plaque of material. These plaques are also found on neuronal multivesicular bodies. Inside, vesicles and an unbounded dense patch are present. Glial filaments (*f*) and particles thought to be glycogen are present in the surrounding cytoplasm. 35 d.i.v. × 41,000.

FIGURE 28 This figure shows a number of particles interpreted as glycogen. Here they measure ~250 A. Each particle appears to be composed of a number of smaller units. Whether this reflects their true structure or is artifactual, as has been suggested, is not known. 35 d.i.v. × 117,000.

FIGURE 29 This micrograph illustrates the type of close apposition and parallelism that glial cell plasmalemmas may exhibit in these cord cultures. Such areas are found only between fibril- and glycogen-containing glia. The distance from the cytoplasmic surface of one membrane to the cytoplasmic surface of the other is ~200 A. 35 d.i.v. × 60,000.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contain stacks of linear inclusions. In general these dense bodies are more heterogeneous in appearance than comparable structures in the neurons. Rarely it appeared that a mitochondrion had been included. An occasional lipid droplet, a few cilia, and some tubules are seen. Filaments, similar to those in the fibrous astrocytes, usually occur in slender bundles which become more prominent with time. Short segments of unusual membrane proximity and parallelism are found between processes that usually contain both filaments and glycogen particles (Figs. 26 and 29). (The processes may, therefore, belong to definitive astrocytes as well as to this intermediate cell type.) Similar junctions have been seen between fibril-containing glial processes *in vivo* (29, 60, 69). Particles interpreted as glycogen are scattered among the organelles of this intermediate cell type and, near the cell periphery, may accumulate in large deposits.

This brings us to a consideration of the nature of the particles referred to so far as glycogen. These particles stain intensely with the lead solution employed. There is some variation in density among them. Comparable particles are buried within a faintly mottled area in unstained sections or become only faintly visible with uranyl acetate stain. The particles average about twice the size of ribosomes, 350 A (range = 200 to 450), and are spheroid. They are neither membrane-bounded nor attached to membrane although they may lie near or among cisternae of agranular membrane. In size, shape, and staining properties, these particles correspond to those positively identified as glycogen in other tissues by Revel *et al.* (65). Yamamoto (86), studying sympathetic ganglia in which comparison is possible between clumps of such particles in the electron microscope and comparable areas in preparations stained for light microscopy, identified similar particles as glycogen. In addition to this finding of glycogen in bullfrog sympathetic ganglia, Rosenbluth has observed similar particles in neurons in gastropod visceral ganglion (72) and toad spinal ganglion (71). We have found identical particles in cultured rat spinal ganglia (9).

Large processes, more probably peripheral portions of perikarya from which the nucleus and dense perinuclear cytoplasm are barely excluded, are found that contain mainly glycogen. Some of the processes are portions of this intermediate cell type; others may derive from the fibrous astrocytes which also contain glycogen particles. In such a glycogen deposit, only the particles may be present or they may be accompanied by a few meandering cisternae of agranular membrane or by a large whorl of cisternae (Fig. 25). Here, as elsewhere in the culture, the glycogen particle may be an aggregate of smaller units (Fig. 28). Most of the large glycogen deposits are located near the top of the culture.

The *surface of the culture* is composed of glial cells; the cultures selected for electron microscopy were explanted without their meningeal ensheathment. The most outstanding characteristic is that the glial cells in contact with the nutrient or just below always appear markedly swollen. Their surfaces display processes in various contortions and there are numerous vesicles suggestive of pinocytotic activity. The mitochondria and sparse components of the granular endoplasmic reticulum appear swollen, too. Scattered glycogen particles, typical filaments, occasional tubules, vesicles, and vacuoles are usually seen. The organelles may be widely dispersed. The condition of these cells is thought to result from their position at the surface rather than from defects in preservation, because these cells always appear as described, no matter what the state of preservation below, and there is essentially no membrane breakage. Cells at the surface of rat dorsal root ganglion cultures are similarly affected (9). Extracellular space is also increased in this area.

DISCUSSION

Throughout this description we have stressed the cytological similarities between cultured spinal cord and *in vivo* central nervous system tissue. It must be pointed out that it is not yet clear how much of this remarkable state of differentiation is established in culture. It does seem clear that the following events occur during the course of *in vitro* existence. (*a*) Because the neuron at the time of explantation is known to be small with few processes and to lack Nissl bodies, it seems certain that the many processes and well organized Nissl substance of the cultured neuron indicate differentiation in culture. (*b*) Because myelin sheaths cannot be seen by light microscopy at the time of explantation but can be subsequently seen to appear, to increase in number, and to become thicker, it seems certain that myelin forms *in vitro* (61). (*c*) Because the recurrent culture pattern of a neuropil zone overlying the neurons occurs

despite the fact that explants must fall initially into every conceivable orientation, it seems certain that the complex entanglement of dendritic, axonic, and glial processes in the superficial zone organizes in culture. Lastly, (*d*) because synapses are found frequently in this neuropil zone, it seems very likely that some form during *in vitro* life.

Further evidence for this final point—that synapses are formed *in vitro*—comes from preliminary investigation of the early development of spinal cord explants.[2] When explants are prepared, as they were in the present study, from 17- to 18-day rat fetuses and fixed and embedded rather than cultured, subsequent electron microscopic examination reveals that the tissue is in a very immature state. Neurons and glia are scarcely distinguishable. Ultrastructural configurations that characterize synapses in mature cultures are extremely rare. When these preparations are compared with mature cultures in which synapses are found in nearly every field, there seems little doubt that synapses form *in vitro*. How might this observed paucity of synapses at explantation be reconciled with the data of Windle and Baxter (81) indicating that spinal reflexes are present beginning at 15½ days in rat fetuses? Either these reflexes function with only a small fraction of the adult number of synapses, or transmission occurs at membrane contacts which are not yet recognizable as typical synapses. The answer to this question will depend on an analysis of what types of membrane contact actually comprise transmitting links between cells. It may be possible to obtain this information from a coordinated electrophysiological and cytological study systematically following younger explants of spinal cord which, free of synaptic transmission at the time of explantation, later exhibit such transmission; such a study is presently under way.

Synapses found in the mature spinal cord cultures resemble those present in central nervous tissue *in vivo*. They display the characteristic accumulation of synaptic vesicles, increased density and thickenings of apposing membranes and, in the larger terminals, mitochondria (21, 43, 46, 48, 85). Furthermore, certain additional features found by Gray (26) are seen. In some axodendritic synapses the cleft is widened and contains dense material, and in the postsynaptic process there is additional substance associated with the membrane (Type 1 synapse). More specifically, as in cord *in vivo* (30), the cord cultures contain synapses on dendritic spines which are stubby processes or invaginations into the presynaptic terminal. It should be pointed out that there are some synaptic structures—by no means found generally but nevertheless present in cord *in vivo*—that are not seen in the cultures. Rings of neurofilaments found in a small percentage of mammalian cord boutons by Gray and Guillery (33) are lacking. More complex vesicles observed by Gray (30) to be dispersed among the usual synaptic vesicles were not seen. Ordered densities just inside the presynaptic membrane or dense particles below the postsynaptic membrane found by Gray (30, 32) may not have been visible in the cultured cord because phosphotungstic acid staining was not employed. This point should be investigated.

The final organization of the cord cultures that the electron microscope reveals, *i.e.*, a variety of synaptic types occurring among nerve processes in a dense neuropil zone and among neurons in deeper regions, could provide the basis for some of the complex bioelectric patterns characteristic of these cultures. Evoked and spontaneously occurring barrages of spikes and subsequent slow waves suggest the presence of complex synaptic networks (16). The electron microscope shows that the morphological basis for a complex network is present. The current state of knowledge concerned with the general problem of correlating synaptic morphology and distribution with physiological function allows us to speculate only tentatively regarding a possible cytological basis for the 5–15/second diphasic oscillatory after-discharges evoked in some of the spinal cord cultures (14). Such phenomena are common in neuronally isolated CNS tissue *in situ* and have been explained in a number of ways (63). It has been suggested that the diphasic after-discharges in the cultures may depend on the establishment of inhibitory "phasing" mechanisms (13, 14).

Recently data have become available regarding the possible location of synapses concerned with postsynaptic inhibition. Andersen *et al.* have presented evidence that inhibitory synapses occur on the somas of hippocampal pyramidal and cerebellar Purkinje cells (1, 2). They mention the

[2] Additional bioelectric and electron microscope data now available further support the contention that synapses form *in vitro* (Crain, S. M., Bunge, R. P., Bunge, M. B., and Peterson, E. R., *J. Cell Biol.*, 1964, **23**, 114A).

possibility that the location of inhibitory synapses on a restricted portion of the neuron, the cell soma, may be a general feature of the mammalian CNS. (It is interesting in this light that De Lorenzo (20) found synapses on neuron somas in cortex to lack the acetylcholinesterase staining reaction that certain neighboring axodendritic junctions exhibited.) The ultrastructure of the synapses that Andersen *et al.* implicate in hippocampal inhibition is typical of axosomatic synapses (Type 2, Gray, reference 26) (36). Ultrastructurally similar axosomatic synapses occur frequently on cultured spinal cord neurons and may provide at least part of the basis for the postulated inhibitory mechanism. A consideration of the morphological aspects of presynaptic inhibition has been included in the observations. Both the physiological suggestion of inhibitory mechanisms and the possible anatomical basis for these mechanisms require further investigation.

While the cytological fidelity of the cultures has been emphasized, much remains to be learned of the histological organization. Two observed departures from normal CNS histology may be mentioned. When meninges are explanted with the CNS fragment, they usually remain confined to those regions where they were initially deposited and show no proclivity to ensheath the explant or to infiltrate it. The explants are free of blood vessels. Because there are no vessels, the explant must be nourished from the surface of the culture, the only area in contact with the nutrient. If the explant is too thick the deepest central region becomes and remains necrotic. The maximum thickness of explant that appears by light microscopic examination to be well maintained is 200 μ in depth. Because of the nutritional problem forced upon cultured CNS the presence of glycogen-rich glial processes, especially near the culture surface, is not surprising. Rosenbluth (72) has studied the gastropod visceral ganglion which is part of the CNS and, being avascular, is apparently nourished exclusively from a surrounding vascular sinus. It is interesting that in this ganglion he has found the glia to contain large amounts of glycogen. The spinal cord cultures, encountering a similar nutrition problem, appear to have developed a similar system of conveying or storing carbohydrates.

The glial cells we have observed in the cord cultures present a spectrum of types suggesting stages of differentiation. Most glial cells are of an intermediate type. They can be labeled neither oligodendrocyte, because of the filaments, nor astrocyte, because of the density (particularly ribosomal) of the cytoplasm. This glial type has been found in a number of areas and conditions. (*a*) We (10, 11) have found that the glial cells reacting to reform myelin in demyelinated adult cat spinal cord *in vivo* are of this type. (*b*) We (7) have observed this cell type to be a frequent resident cell in developing kitten spinal cord. (*c*) Schultz and Pease (75) have commented on its frequency in areas of rat cortex reacting to stab wound injury and in normally developing rat corpus callosum. Finally, (*d*) Ross *et al.* (73) found most glial cells in their rat cerebellar cultures to be dense with organelles including filaments in varying amounts. The presence of both the intermediate and mature glial types in the spinal cord cultures suggests that maturation of the glia is progressing slowly. The increase in bundles of filaments in older cultures specifically implicates fibrous astrocyte formation. How much of the progressive glial development observed represents normal glial maturation and how much represents reaction to the trauma of explantation we do not yet know.

Thus it appears that these spinal cord cultures should offer great potential in the study of factors influencing glial filament formation and the directly related problem of CNS scar formation. Because fibrillar glial cells appear to be forming slowly in the cultures, this system offers a unique opportunity to attempt to inhibit or alter the formation of the scarring cells of many neuropathological processes—the fibrous astrocytes.

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