FIXATION OF NEURAL TISSUES FOR ELECTRON MICROSCOPY BY PERFUSION WITH SOLUTIONS OF OSMIUM TETROXIDE

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ABSTRACT

This paper describes in detail a method for obtaining nearly uniform fixation of the nervous system by vascular perfusion with solutions of osmium tetroxide. Criteria are given for evaluating the degree of success achieved in the preservation of all the cellular components of the nervous system. The method permits analysis of the structural relations between cells at the electron microscopic level to an extent that has not been possible heretofore.

One of the greatest impediments to the electron microscopy of the nervous system has been the inadequacy of the methods currently available for processing neural tissues. This technical deficiency is less evident in studies of the peripheral nervous system than in those of the central nervous system, where the exquisite susceptibility of the tissue to trauma and anoxia leads to severe, recognizable artifacts unless great care is taken to avoid them. Among the currently more successful methods are four varieties of immersion fixation. In the first, the tissue is simply excised from the living anesthetized animal and quickly diced into small fragments in a pool of fixative. In the second, adjuvants are added to the fixative in order to increase its penetrating power (23). In the third, the tissue is fixed *in situ* by injecting the fixative into the ventricles (29) or into the subarachnoid space (11), or by flooding the exposed peripheral nerve with fixative (39), and after a suitable interval the tissue is excised and diced as before. In the fourth method, the tissue is pretreated *in situ* with a topical application of hyaluronidase before immersion in the fixative (30).

All of these methods have serious disadvantages for the student of the nervous system. They limit investigation to those regions that can be reached by a fixative acting upon free surfaces, *i.e.*, to the immediately subpial or subependymal regions of the central nervous system or to the smallest nerves and the outer fibers of the large nerves. Because their results are unpredictable, they force the investigator to select for study areas, even single cells and nerve fibers, on the basis of chance agreement with criteria for good preservation. Thus they make comparisons between different specimens difficult or impossible. They also result in gross distortions of the central nervous system so that identification of cytoarchitectonic features, such as the nuclei of the brain stem or the layers of the cerebral cortex, is hazardous at best. The consequence of these drawbacks has been to confine electron microscopic work on the nervous system almost entirely to certain general cytological problems,

for example, the fine structure of neurons, glia, nerve fibers, and synapses. Even in this relatively restricted area of investigation, the resolution of comparatively simple problems, such as the identification and characterization of the neuroglia, has been seriously encumbered by unsatisfactory methods of tissue preservation. Perhaps the most serious handicap has been attached to the field of experimental analysis because of the difficulties in distinguishing between preparative artifacts and the effects of experiment when an adequate picture of the undisturbed nervous system has not been at hand.

Satisfactory preservation of the nervous system requires rapid and uniform penetration of the fixing agent into all parts without prior injury to the tissue by anoxia or manipulation. The only way to accomplish this aim and to obviate the drawbacks of immersion fixation is perfusion of the fixing agent through the vascular channels. This paper presents in detail a method for obtaining successful fixation of the central nervous system by means of vascular perfusion with solutions of osmium tetroxide.

MATERIALS AND METHODS

The procedure for perfusion of the brain of the rat will be described first because it is the simplest and is the prototype for the other procedures to follow.

After a fast of 24 to 48 hours, apparently healthy, young adult rats of the Osborne-Mendel strain (100 to 250 gm) were anesthetized by an intraperitoneal injection of a 3.5 per cent solution of chloral hydrate (35 mg per 100 gm of body weight) (10). The animals became insensitive to painful stimuli within 3 to 5 minutes without passing through an excitatory stage. The animals were then shaved and pinned to a dissecting board with the ventral side up. The trachea was exposed through a midline incision in the neck and a no. 18 needle was inserted into the trachea through a hole made by removing one or two cartilaginous tracheal rings below the level of the thyroid gland. The needle was attached by means of a 1 inch length of rubber tubing to a glass Y tube. One limb of the Y led through a rubber tube to a tank of 95 per cent O_2/5 per cent CO_2 and the other limb was left free. The animal was able to breathe normally through the open limb of the Y tube, as the short connecting tube increased the dead space only slightly. Artificial respiration could be administered under minimal pressure (flow of 2 liters per minute), just enough to inflate the lungs gently, by fluttering the operator's forefinger over the tip of the free limb of the Y tube.

The midventral incision was then continued down over the abdomen and the skin was reflected on either side. A nick was made in the chest wall at the left costophrenic angle. The lungs immediately collapsed and artificial respiration was begun. This was carefully regulated to simulate the depth and rate of normal respiration. The color of the nose, ears, and paws became pink. After artificial respiration had become established the anterior rib cage was isolated by cutting along the midaxillary line on either side and along the costal attachment of the diaphragm with blunt-tipped dressing scissors. Injuring the lungs was avoided by deflating them momentarily before placing the blades of the scissors in position for each cut. The rib cage was then elevated, hinging at the first costosternal joint, and a hemostat was placed across it in order to clamp the internal mammary arteries. The rib cage was then removed by cutting along the hemostat with scissors. Care had to be exercised during this maneuver to avoid obstructing the trachea.

This procedure exposed the heart and the great vessels. The pericardium was then slit with iridectomy scissors, and the thymus, which covers the arch of the aorta, was carefully removed. A fine curved forceps was inserted under the arch of the aorta and a length of no. 2 suture thread was drawn through. The thread was looped about the root of the aorta as a loose knot in preparation for tying the perfusion cannula in place. At this point usually a few moments were allowed for recovery of respiration and cardiac action. The apex of the heart was grasped with a fine forceps and 1 ml of 1.0 per cent sodium nitrite was injected slowly over a period of a minute into the left ventricular cavity from a syringe and no. 27 needle that had been previously wetted with heparin. Another minute was allowed for recovery of normal cardiac action. Next a clamp was placed upon the descending aorta at about the middle of its length. The tip of the heart was then amputated and artificial respiration was discontinued. A glass cannula, which was fashioned with a bulbous tip 2 mm in outside diameter and which formed the end of the perfusion apparatus (Fig. 1), was quickly inserted into the left ventricle, through the aortic valve, and tied into place by means of the previously placed ligature.

The perfusion was begun with 6 to 7 ml of a balanced salt solution and immediately followed with 100 to 150 ml of 1 per cent osmium tetroxide in buffered acetate-veronal solution at pH 7.35–7.4 (25, 26). Both the saline solution and the initial portion of the osmium tetroxide solution were warmed by means of heating tapes wrapped around the rubber tubing of the perfusion apparatus. The temperature of the heating tapes was adjusted so that the rubber tubing became warm, but not hot, to the touch. In one test, the temperature of the perfusion fluid delivered from the tip of the cannula under these conditions was 31 °C. The bulk of the fixing solu-

tion, held in a reservoir, was chilled to ice temperature by means of a mixture of solid CO_2 ("dry ice") and ice cubes. Perfusion was carried out under a head of pressure equivalent to 5 feet of water. After the initial flow had washed the blood out of the vascular tree and osmium tetroxide was detectable in the return gushing out of the open right ventricle, the heating tapes were disconnected and the flow was reduced to a slow trickle so that 20 to 40 minutes were required to pass a total of 150 ml through. The return was collected in a pan beneath the animal board. The entire procedure from the opening of the chest to the beginning of the perfusion took only 2 to 3 minutes. Less than 60 seconds elapsed from the cessation of artificial respiration to the first sign of fixation (blackening of the nose and ears).

The perfusion apparatus (Fig. 1) consisted of a glass reservoir shaped like a Dewar flask[1] from which a rubber tube led to the glass cannula. The inner chamber of this flask was open to the air and was filled with a mixture of ice cubes and chips of solid CO_2. The outer chamber was closed except for a narrow funnel inlet and an air outlet at the top, and an outlet at the bottom to which the rubber tube was attached. The fixing solution was poured into the outer chamber through the funnel. The rubber[2] outlet tube was interrupted about midway by a Y tube, the side arm of which led to a second reservoir containing the saline solution. This arrangement had the advantage of permitting only a small volume of saline to be used and facilitated the filling of the tubing without entrapping air bubbles. In filling the apparatus the fixing solution was poured into the main reservoir while the tubing was clamped close to its origin (Fig. 1, *A*) and again just below the Y tube (*D*). Removal of the first clamp (*A*) allowed the fixative to flow down the tube and up into the side arm of the Y. Any entrapped bubbles were removed by compressing the rubber tubing intermittently or by allowing the solution to flow back and forth several times. Then a clamp (*B*) was placed on the tubing just above the Y and the second reservoir was filled with saline. The clamp (*D*) below the Y was then removed and the tubing from the second reservoir to the glass cannula was washed through with the saline solution until no more vapors of osmium tetroxide could be detected in the outflow. The tubing above the side arm was clamped (*C*) and a clamp (*E*) was placed on the rubber tubing above the cannula. When the perfusion was started the clamps above the cannula and below the first reservoir were removed.

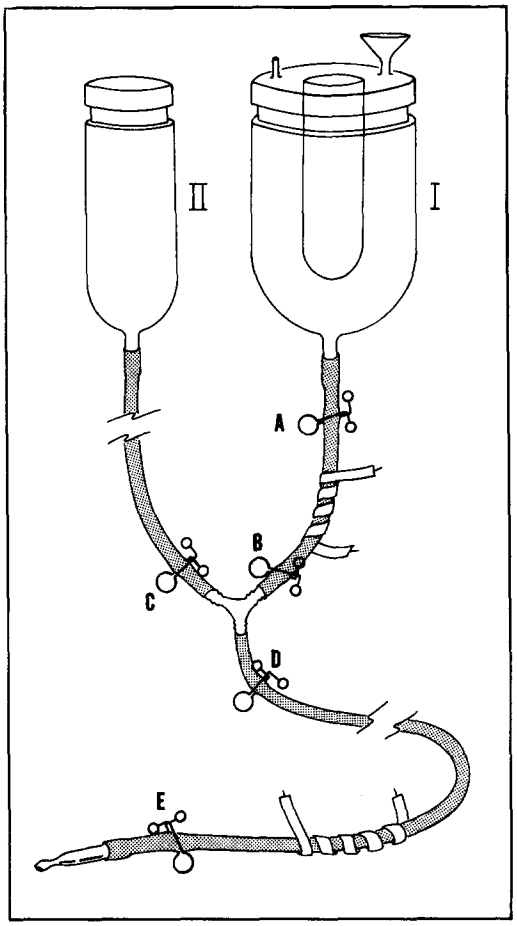


FIGURE 1

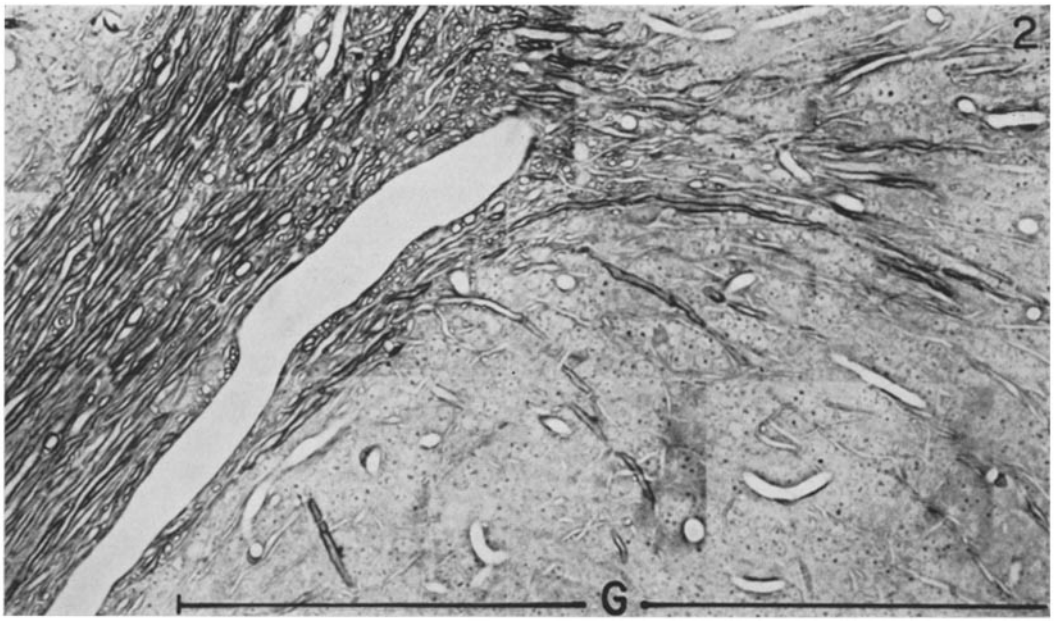
Diagram of the perfusion apparatus. Flask I is a double-walled, Dewar-type reservoir the inner chamber of which is filled with the ice and solid carbon dioxide used to cool the fixing solution that occupies the outer chamber. Flask II is a simple, graduated cylindrical vessel containing the saline solution. Heating tapes are wrapped around the rubber tubing above the Y tube and above the outlet cannula. The letters *A*, *B*, *C*, *D*, and *E* represent the positions of clamps used during the filling of the apparatus. During the perfusion only clamp *C* is left in position. Further description in text.

The composition of the balanced salt solution was as follows:

mMoles/liter		gm/liter
2.5	$CaCl_2$ (anhydrous)	0.28
1.1	$MgCl_2 \cdot 6H_2O$	0.22
4.0	KCl	0.30
116.0	NaCl	6.80
26.0	$NaHCO_3$	2.20
1.2	$NaH_2PO_4 \cdot H_2O$	0.16

[1] The modified Dewar flask was suggested by Professor Ernst Scharrer, Department of Anatomy, Albert Einstein College of Medicine, New York.

[2] The best quality surgical "amber" rubber tubing was used. Although it blackened immediately upon exposure to osmium tetroxide, it remained flexible for many months of use.



FIGURES 2 AND 3

Phase contrast micrograph of an unstained section of cerebellum (rat). This picture, representing only a strip 1 mm long from a much larger tissue block, was made by fitting together 25 separate photomicrographs of adjacent microscopic fields taken at a higher magnification. The figures show the empty and slightly distended vessels which are the mark of perfused tissue. On the left (Fig. 2), the white matter at the core of the folium sends a spray of myelinated nerve fibers across the granule cell layer (*G*), while on the right (Fig. 3), the Purkinje cells (*P*) in a vertical row spread their dendrites fanwise through the molecular layer (*M*). The figures illustrate the compactness and coherence of specimens resulting from perfusion fixation. Notice the absence of splits and empty spaces between the white matter and the granule cell layer and around the Purkinje cells. Absence of dark and light cells is also noteworthy. × 350.

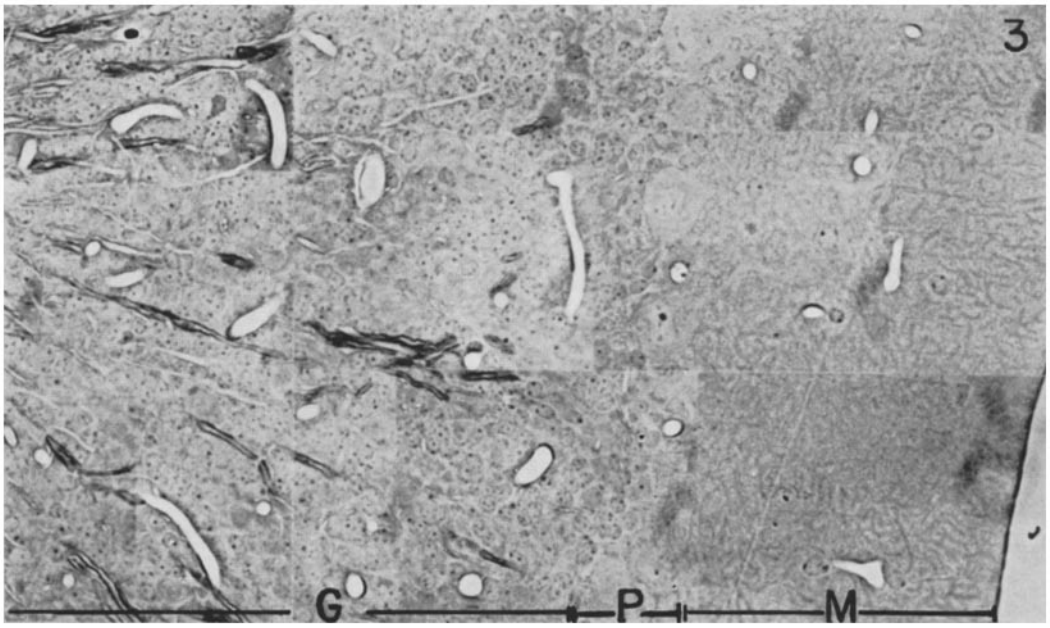
The pH of this solution was adjusted to 7.2–7.3 by bubbling a small amount of carbon dioxide through it immediately before use.

The fixative was a 1 per cent solution of osmium tetroxide, in acetate-veronal buffer at pH 7.35–7.4 (25), to which had been added 5.4 mg of calcium chloride (anhydrous) per ml of final solution.

This procedure provided fixation of the entire brain, cranial nerves, cervical spinal cord, and cervical dorsal root ganglia. The rest of the spinal cord was usually not completely preserved, although large parts of the gray matter in the thoracic and lumbar cord were adequately fixed.

Preservation of the entire spinal cord, lower medulla oblongata, and almost all spinal ganglia was obtained by either of two variations on the method described above. In the first, inserting the cannula into the heart was preceded by placing hemostats upon the iliac arteries, the inferior and superior mesenteric arteries, and the two common carotid arteries, in that order. In the second variation, the simpler and more effective one, the glass cannula was replaced by a no. 18 needle which was inserted into the abdominal aorta at the point of its bifurcation, and a clamp was placed upon the descending limb of the aortic arch in the thorax. The perfusion fluid was allowed to flow in the retrograde direction and egress was obtained by opening the right ventricle and atrium immediately after onset of the flow. Clamping the mesenteric arteries was unnecessary. With this method incomplete perfusion of the intestines, pancreas, and liver was obtained.

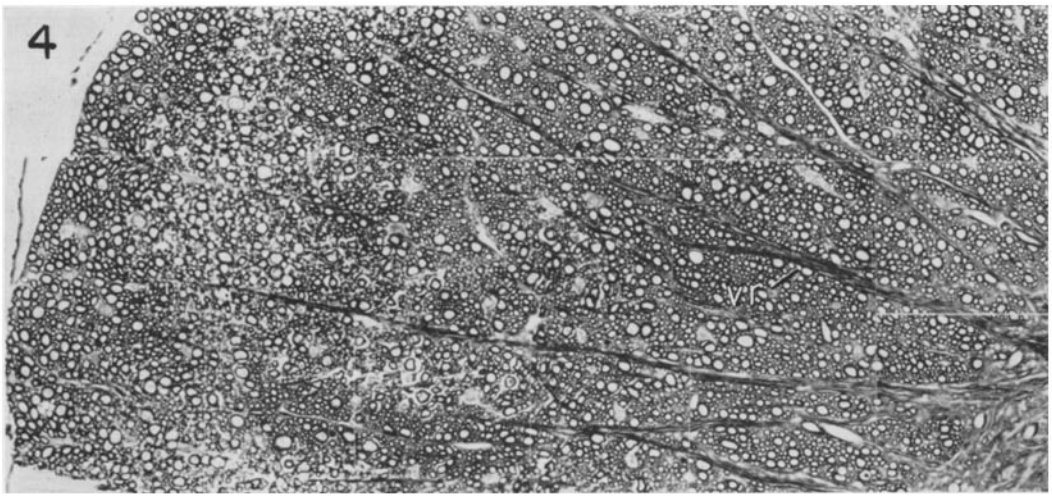
Preservation of the brain of the goldfish was secured by essentially the same procedure. The only modifications introduced were those required by the vascular anatomy of this animal and the difference in tonicity of the blood. The fish (20 cm long) was anesthetized by allowing it to swim in a 1:4000 solution of tricaine methanesulfonate (MS, 222, Sandoz) until it became quiescent. It was then removed to a dissecting board to which it was pinned with the ventral side upward. Water containing 1:8000 tri-



caine was allowed to flow through its mouth and over its gills by means of a tube leading from a reservoir. The flow was controlled by a Hoffman clamp. Parts of the opercula were removed to increase visibility. The heart was exposed by removing the pectoral girdle and slitting the pericardium. A ligature was placed around the root of the conus arteriosus but not tied. Then 0.5 ml of 0.7 per cent sodium nitrite was injected into the ventricle slowly from a syringe previously wetted with heparin. In quick succession, the tip of the ventricle was amputated, the glass cannula leading from the perfusion apparatus was inserted through the ventricle into the conus arteriosus, the ligature was tied, and the perfusion fluid allowed to flow. Egress was permitted by incising the sinus venosus. The blood was washed out by approximately 5 ml of the mammalian balanced salt solution diluted to 70 per cent of its concentration by distilled water. Within a minute the gills became pale as the blood was washed out and then brown to black as the osmium tetroxide replaced the salt solution. Perfusion by 100 ml of 1 per cent OsO_4 (pH 7.35–7.4) was allowed to continue for about 30 minutes before beginning the dissection. The entire brain, eyes, and olfactory bulbs were preserved by this method, but the spinal cord was never successfully fixed.

This procedure could not be used for small goldfish about 7 or 8 cm long. For these animals the perfusion apparatus consisted of a 25 ml glass syringe nested in a tray filled with ice and attached to a polyethylene tube to which was fitted a no. 29 needle. A glass bubble trap was inserted into the tubing so that the combined volume of trap and tubing was no more than 5 ml. The tubing and trap were filled with bubble-free balanced salt solution and kept in a dish of warm water. The syringe was filled with 1 per cent osmium tetroxide in acetate-veronal buffer (pH 7.35–7.4). The fish was prepared as before, the sinus venosus was incised, and the needle tip was inserted into the ventricle and conus arteriosus. With one hand the needle was held in place by means of a fine curved forceps and with the other hand the plunger of the syringe was depressed to deliver the perfusion fluid at a steady rate of 1 ml per minute. Mechanical devices for propelling the fluid were found to be too cumbersome and inflexible to be valuable.

In all cases, the brain or spinal cord and ganglia were exposed as rapidly as possible after the conclusion of the perfusion and thick, completely transverse sections were made by cutting with fresh razor blades. The surfaces were examined under a dissecting microscope at 9 × magnification. After the procedure had been mastered, the specimens were discarded if the entire section was not blackened (see Results). Spotty blackening along the major vascular channels was regarded as a mark of failure. The large pieces were temporarily stored in weighing bottles capped by ground glass stoppers and containing a chilled osmium tetroxide solution similar to the perfusion fluid. The pieces were then examined at leisure. Care was taken so that during dissection and blocking the tissue was never allowed to dry. Areas of interest were selected, diced into 1 to 4 mm^2 fragments, and transferred to fresh 1 per cent buffered osmium tetroxide and stored



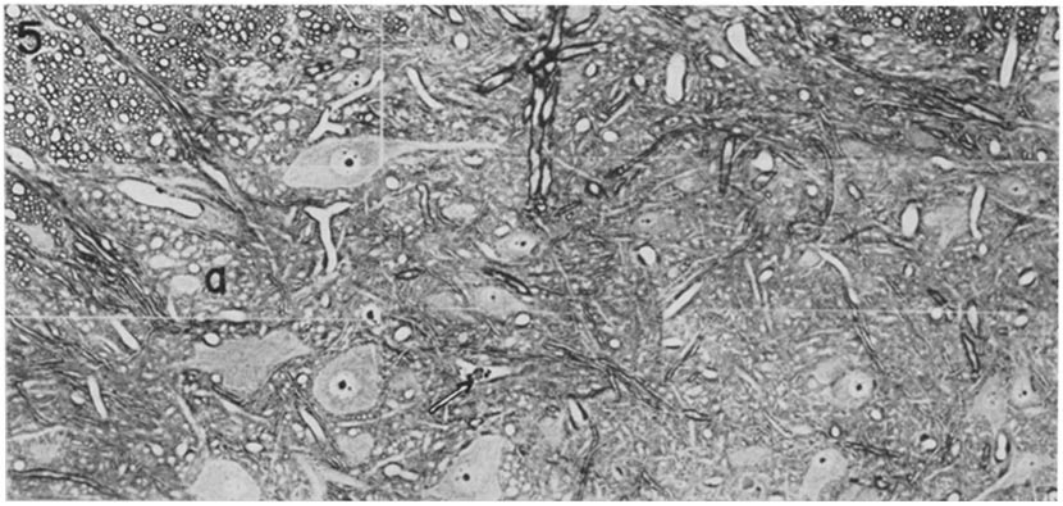
FIGURES 4 AND 5

Phase contrast micrograph of an unstained section of the sacral spinal cord (rat). The picture represents a strip, nearly 1.5 mm long, of a larger tissue block consisting of a transverse section of the whole spinal cord. It was made by fitting together 37 separate pictures of adjacent microscopic fields taken at a higher magnification. The picture includes the ventral funiculus (Fig. 4) and the lateral part of the ventral gray column (Fig. 5). The intermingled gray and white matter on the right (Fig. 5) and the deeper two-thirds of the ventral funiculus (Fig. 4) display the compactness and coherence of satisfactory fixation, but an irregular zone on the left (Fig. 4) consists almost exclusively of swollen and distorted myelinated fibers. Beyond this zone there is a superficial shell of nerve fibers in a fairly good state of preservation. The large motoneurons in the ventral horn display their characteristic multipolar shape, Nissl bodies, and vesicular nuclei with prominent nucleoli. They are immersed in the neuropil without any intervening clear spaces. At higher magnification it may be seen that the thin light zone around some motoneurons is filled with boutons terminaux. The clusters of rounded light spots (Fig. 5, *a*) at the edge of the gray matter are transverse sections of the unmyelinated initial segments of the axons leaving the ventral horn motoneurons. These axons acquire myelin sheaths at the edge of the gray matter and course out through the ventral funiculus as long dark streaks (Fig. 4, *vr*) to become the ventral rootlets at the surface of the cord. Red cells remaining in a few vessels (arrow) indicate that the perfusion was incomplete and provide the reason for the incomplete fixation of this specimen. × 200.

in a refrigerator at about 4°C. Total fixation time, including the duration of the perfusion and dissection, was 2 to 4 hours. At the end of this period the tissue fragments were rinsed 3 times in acetate-veronal buffer of the same pH and concentration as that used in the fixing solution and were dehydrated in an ascending series of methanol concentrations: 25 per cent, 35 per cent, and 50 per cent, at 10 minutes each, and 70 per cent, 80 per cent, 95 per cent, and 100 per cent, at 15 to 20 minutes each. In the initial experiments the tissues were embedded in a 15:85 mixture of prepolymerized methyl and butyl methacrylates (9). In later experiments the tissues were embedded in Epon 812 (Shell) according to Luft (22) and use of the methacrylates was discontinued.

Sections were cut with glass knives on Porter-Blum microtomes. Thick (1 to 2 μ) sections mounted in glycerol were examined in Leitz or Reichert phase contrast microscopes. Thin sections (silver or gold interference colors) were picked up on carbon-coated copper grids, stained with saturated aqueous lead hydroxide or uranyl acetate (38), and examined in the RCA 3D or 3E electron microscope.

Of the numerous variations performed in arriving at the procedures described above, the only one of sufficient importance to be mentioned here was the introduction of calcium ions into the saline and fixing solutions. In the early attempts calcium was omitted. In one experiment (1 rat) the fixing solution consisted of 1 per cent osmium tetroxide, buffered with acetate-veronal at pH 7.48, to which had been added 0.13 mg calcium chloride and 43 mg sucrose per ml of solution. In another experiment (involving 3 rats) only 0.13 mg $CaCl_2$ per ml was added to the fixing solu-



tion and sucrose was omitted. The results of perfusion with these three solutions were compared with that of perfusion with the standard solution containing a large excess of calcium, *i.e.* 5.4 mg per ml.

RESULTS

Gross Examination and Optical Microscopy

Successful perfusion with buffered osmium tetroxide resulted in a brain, spinal cord, or other organs which appeared completely black to the unaided eye. Upon dissection, the tissue was firm, almost brittle, having the consistency of a hard-boiled egg. A new razor blade sliced through it cleanly and smoothly without compression, protrusion, or collapse of the cut surface or distortion of the form of the organ.

In the slices, the internal architectural features of the brain or spinal cord could be identified by examination with a dissecting microscope at 9 × magnification. With the source of illumination adjusted to the appropriate angle and brilliance, the gray matter of the cerebral cortex, the neuropil, and the nuclear regions of the brain stem appeared a dull, dark gray, and the white matter, both in the great tracts and commissures and in the slender bundles that interlace everywhere, all appeared a glistening black. The major nuclei and tracts of the brain stem and diencephalon were easily recognizable in the slices of a successfully perfused brain, just as in a section carefully stained by some modification of the Weigert method. Consequently, selected regions of especial interest could be identified and then removed and cut into small cubes or rectangular blocks without compression or distortion and attendant loss of orientation. The peripheral and cranial nerves and their ganglia appeared black throughout and so rigid that lengths of the nerves could be dehydrated and embedded without any other support to keep them straight and extended.

When the perfusion was unsuccessful, the brain or spinal cord appeared spottily blackened with white intervening regions. The organs were soft and easily compressed. In most of these cases the external surfaces appeared completely black, but examination of the slices with the dissecting microscope revealed that only a thin shell of tissue 1 mm or less in thickness had been fixed. Internally the blackened areas were distributed according to the pattern of the larger vessels. Apparently the capillaries had first been washed free of blood but did not subsequently remain patent. After our initial experiments such specimens were discarded, because electron microscopy of thin sections from these areas showed that only the tissues immediately surrounding the larger vessels were adequately preserved.

Even in the most successful perfusions, a few small white spots, usually not visible to the unaided eye, but detectable upon 9 × magnification, persisted in the slices. The most common site of focal failure was the center of the corpus callosum. Other sites were unpredictable and usually asymmetrical.

Phase Contrast Microscopy

The quality of preservation in individual blocks of tissue was evaluated by phase contrast

microscopy of thick sections (1 to 2 μ) taken from the embedded blocks. Two samples of this quality are shown in Figs. 2 to 3 and 4 to 5. Actually these illustrations represent only narrow strips from the sections, which measured about 2 mm square. Figs. 2 and 3, showing approximately half the thickness of a folium of the cerebellum, illustrate one of the principal criteria for evaluation—integrity of the tissue throughout its thickness. The blood vessels appear slightly distended and empty. The white matter at the core of the folium is compact, and the individual myelinated fibers that traverse the granule cell layer of the cortex display no swellings, distortions, or surrounding haloes. The three layers of the cortex merge one into the other and into the white matter without any breaks. The Purkinje cells rest directly upon the granule cell layer and extend their dendrites into the molecular layer without any clefts or empty spaces. Throughout the section "dark" and "light" cells (13, 35) are nowhere to be seen.

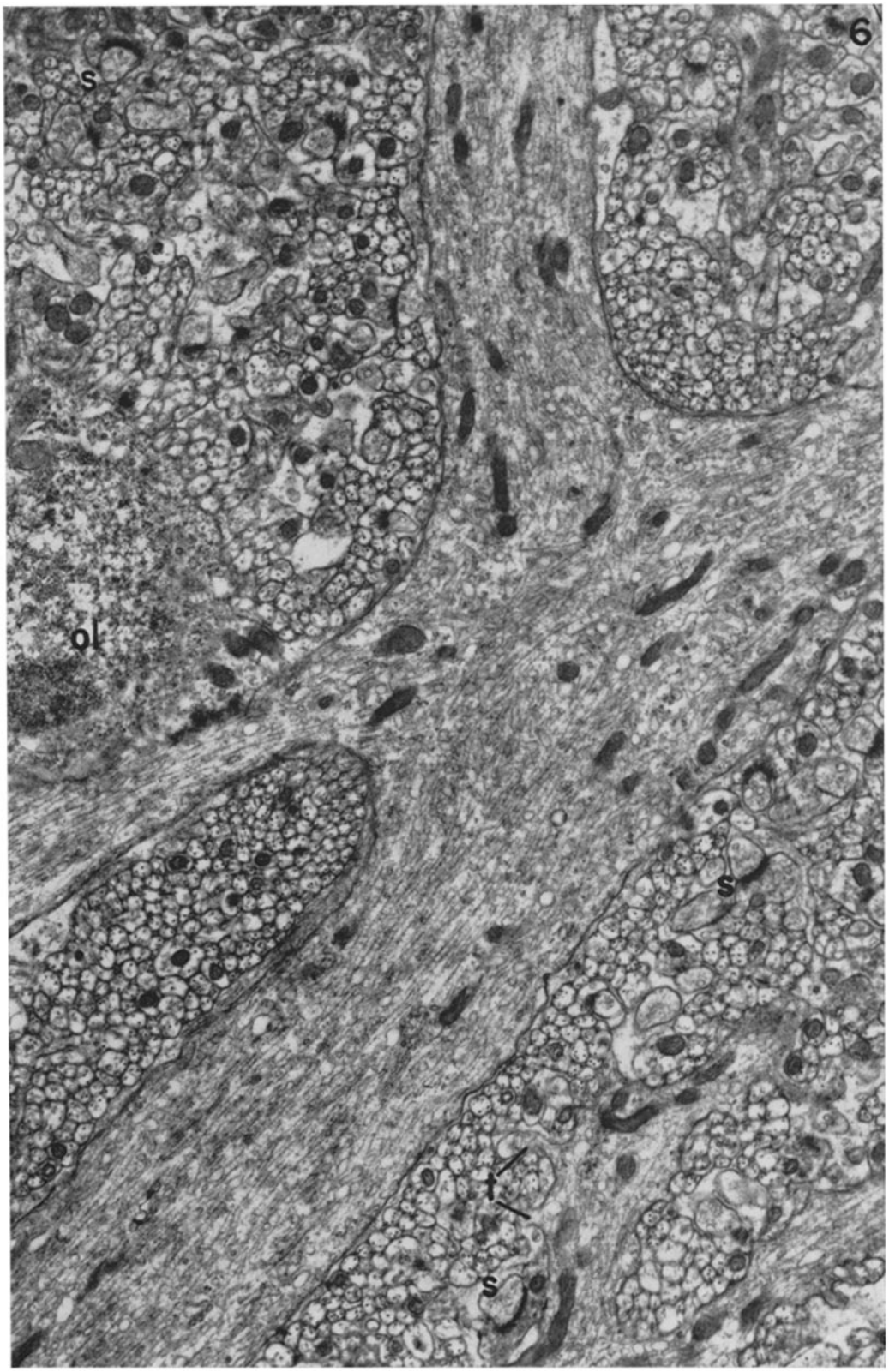
At higher magnifications, details within the cells become visible. The nuclear outline is sharp, thin, and smooth; nucleoli and small chromatin masses are discernible within the homogeneous nuclear sap. In the cytoplasm, the mitochondria appear as small, dense dots or slender filaments. Nissl bodies appear as dark masses with soft outlines. Vacuoles and empty spaces are generally absent, but small, dense droplets, or lumps of pigment can be seen in the larger neurons. Neuroglial cells are most easily distinguished in the white matter, where they lie completely surrounded by myelinated nerve fibers among which they extend their processes. Nuclei suspended in clear spaces are lacking. In a word, at the level of phase contrast microscopy, the organization of the nervous tissue appears intact after successful perfusion.

A less successful but nevertheless quite useful specimen is illustrated in Figs. 4 and 5, which show a strip of gray and white matter from the ventral horn and ventral funiculus of the sacral spinal cord. In these phase contrast photomicrographs, the gray matter and a broad zone of the surrounding white matter appear to be intact, as does a thin superficial shell of the funiculus. In between, however, there is an irregular region in which most of the myelinated fibers are shrunken or exploded and the neuroglia lies in empty or coarsely precipitated beds.

These pictures show not only how phase contrast microscopy can be used to evaluate the quality of the preservation, but also how extensive the areas of adequate fixation may be expected to be. Since perfusion of the fixing solution maintains the integrity of the tissue, orientation and selection of interesting areas for thin sectioning may be undertaken after study of thick sections like those illustrated. Unfortunately, thin sectioning of large block faces, especially of Epon-embedded specimens containing large amounts of myelin, is still an unsolved technical problem. Consequently, in this study the embedded blocks were trimmed so that faces smaller than 1 mm square were presented for thin sectioning. For analysis of the architecture of larger regions, composites from several blocks were necessary. It was possible, however, to obtain thin sections from blocks containing the full

FIGURE 6

Electron micrograph of the molecular layer of the cerebellum (rat). The primary dendrite of a Purkinje cell spreads diagonally across the figure and divides into secondary branches. It is filled with long, fine canaliculi, dark, slender mitochondria, small vesicles, and tubules. Its surface is smooth and is covered by thin, light axons belonging to the climbing fibers, which in this figure do not happen to show their synaptic relations to the dendrite. The section just grazes the surface of an oligodendrogliocyte (*ol*) at the left. At the lower right, tertiary branchlets of a Purkinje cell dendrite send out small processes or thorns (*t*), which contact terminal expansions of the granule cell axons. The plane of the section favors transverse sections of these thorn-granule cell axon synapses, some of which are indicated by the letter *s*. Nearly all of the small polygonal or circular profiles, in which the Purkinje cell dendrite is immersed, are transverse sections of granule cell axons which are in the ratio of 200,000 or 300,000 to 1 Purkinje cell (17). The number of synaptic junctions per unit area of field is considerably higher in the more superficial regions of the molecular layer than in the deep zone shown here. × 11,000.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thickness of a cerebellar folium such as is illustrated in Figs. 2 and 3.

Electron Microscopy

Figs. 6 to 11 are electron micrographs of representative fields from the cerebellum and spinal cord of the rat, and Fig. 12 is from the brain of the goldfish. The magnifications are necessarily low in order to show the extent of uniform preservation. The details of each picture are described and identified in the legends. The important characteristic to be noted in these figures, as in the phase contrast micrographs, is the large area of intact tissue, in which clefts, shrinkage or retraction spaces, explosions, vacuoles, disrupted limiting membranes, and dehiscences all are absent. Each cellular component fits precisely against its neighbors according to the architectonics of the region, axons against dendrites, neuron against neuron, capillary endothelium against basement membrane and that against neuroglia, myelinated fiber against myelinated fiber, and, throughout the whole tissue, the amazingly complex feltwork of the neuropil forming a close contexture that fills every interstice between the cells and their major processes. No deep extracellular spaces or lakes can be seen except around the larger penetrating vessels (the Virchow-Robin spaces).

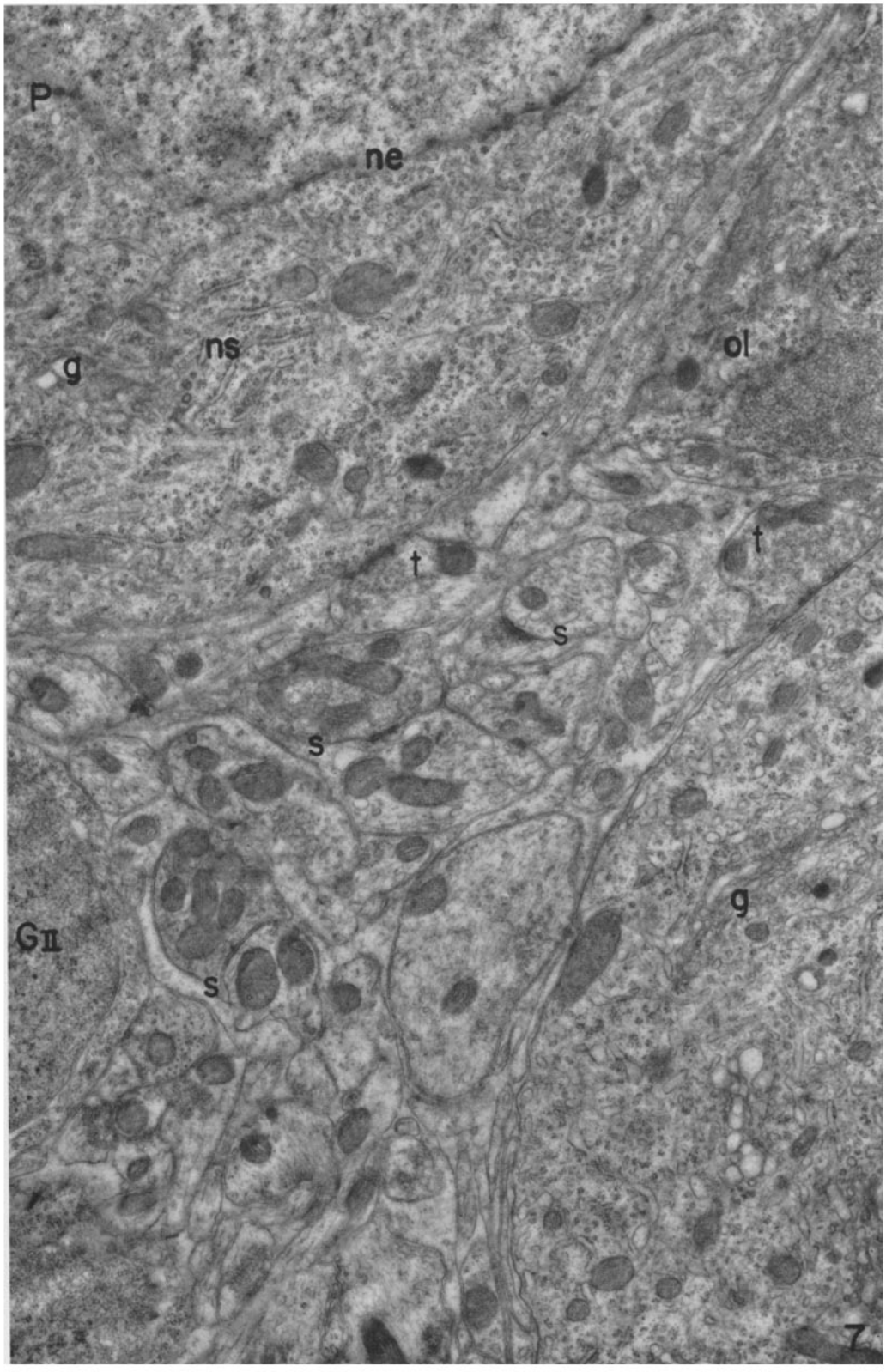
In addition, the internal structures of each cell display all the signs of adequate preservation. The nuclei are finely and compactly granular; the cytoplasm is densely packed with organelles, filaments, and ribonucleoprotein particles. The mitochondria and the endoplasmic reticulum exhibit little or no evidence of swelling or disruption. Filaments and canaliculi are aligned according to the longitudinal axes of the processes which contain them. Within all cell types, large, clear areas occupied by seas of embedding plastic are wanting. All of these characteristics attest to the nearly uniform excellence of the preservation obtained by successful perfusion with osmium tetroxide.

Neuroglia

Two types of neuroglial cells are readily distinguishable in electron micrographs of perfused tissue from the central nervous system. The first is a relatively small cell with a rounded nucleus and a narrow rim of cytoplasm, which is crowded with ribonucleoprotein particles (Fig. 13). The endoplasmic reticulum is usually disposed in the form of one or two broad cisternae concentric with the nuclear envelope. Processes are not usually encountered, but when they are included in the section they often display fine, long canaliculi similar to those characteristically found in the dendrites of neurons (Fig. 6). The nuclear envelope and the endoplasmic reticulum exhibit a much greater tendency to swell or distend than do those of any other cell in our experience. The boundary or plasmalemma of this cell is very difficult to follow, as it seems

FIGURE 7

Electron micrograph of the cerebellar cortex (rat), showing the boundary between the Purkinje cell layer and the granule cell layer. Parts of two Purkinje cells (*P*), one Golgi type II cell (*G II*), and one oligodendrogliocyte (*ol*) are included within the field. The cytoplasm of the Purkinje cells is crowded with the characteristically dispersed Nissl substance (*ns*), an elaborate Golgi complex (*g*), and small mitochondria. The nuclear envelope (*ne*) of the cell at the top of the figure has been sectioned obliquely and displays a number of distinct pores. Each Purkinje cell is encompassed by profiles of the basket fibers, some of which terminate in expansions (*t*) containing mitochondria and clustered vesicles. Where these terminals abut against the surface of the Purkinje cell their surface membrane is thickened and dense. The Golgi type II cell has a large nucleus and only a thin rim of cytoplasm. In this figure none of its processes is shown. An insufficient extent of the oligodendrogliocyte is included in this figure to permit identification. It was, however, recognizable in the composite or mosaic covering a large area of neighboring fields, of which this figure is only one. The chink intervening between these four cells is packed with axons of the basket cells and dendrites belonging to other cells, possibly the Golgi II cell and granule cells. Many of these axons and dendrites are in synaptic relation (*s*). × 16,000.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always to lie tangential to the plane of section over most of the perimeter of the cell. The mitochondria are sparse, usually elongated and with numerous ill defined, transversely oriented cristae. This cell is here identified as the oligodendrogliocyte. It is commonly encountered both in gray and in white matter, often in rows between myelinated fibers and as a satellite to the large neurons.

The second neuroglial cell (Fig. 14) is usually larger than the first and has either a rounded or an irregularly elongated nucleus. Its cytoplasm is much more voluminous and is occupied by prominent bundles of very fine, long filaments, sometimes oriented in swirls about the nucleus and nearly always extending into the several processes. The endoplasmic reticulum consists of dispersed small vesicles and short, bent tubules. The ribonucleoprotein particles, although present, are not conspicuous. Consequently, the cytoplasm appears lighter than that of the first cell. The mitochondria are few but larger than those in neurons. The limiting membrane of this cell is more readily followed than that of the first cell. It extends over numerous processes or arms, which insinuate themselves between myelinated nerve fibers or along the basement membranes of capillaries. Consequently the cell usually has a stellate shape, even in thin sections. This cell is here identified as the fibrous astrocyte.

Effects of Calcium Ions

When calcium ions were omitted from the perfusing solution, the limiting membranes of many, but not all, cells displayed irregularly spaced interruptions. The surface membranes of certain cells (for example, neurons and Schwann cells in the spinal ganglia, smooth muscle cells in the intestine, pancreatic acinar cells) are especially susceptible to this kind of damage. The cells in the central nervous system are less obviously affected. The damage is illustrated in Fig. 15, which shows adjacent surfaces of three pancreatic acinar cells. The surface membranes are discontinuous, as if fragments of the plasmalemma had disappeared, with little disturbance in the arrangement of the subsurface structures and with no interruption of the overlying basement membrane.

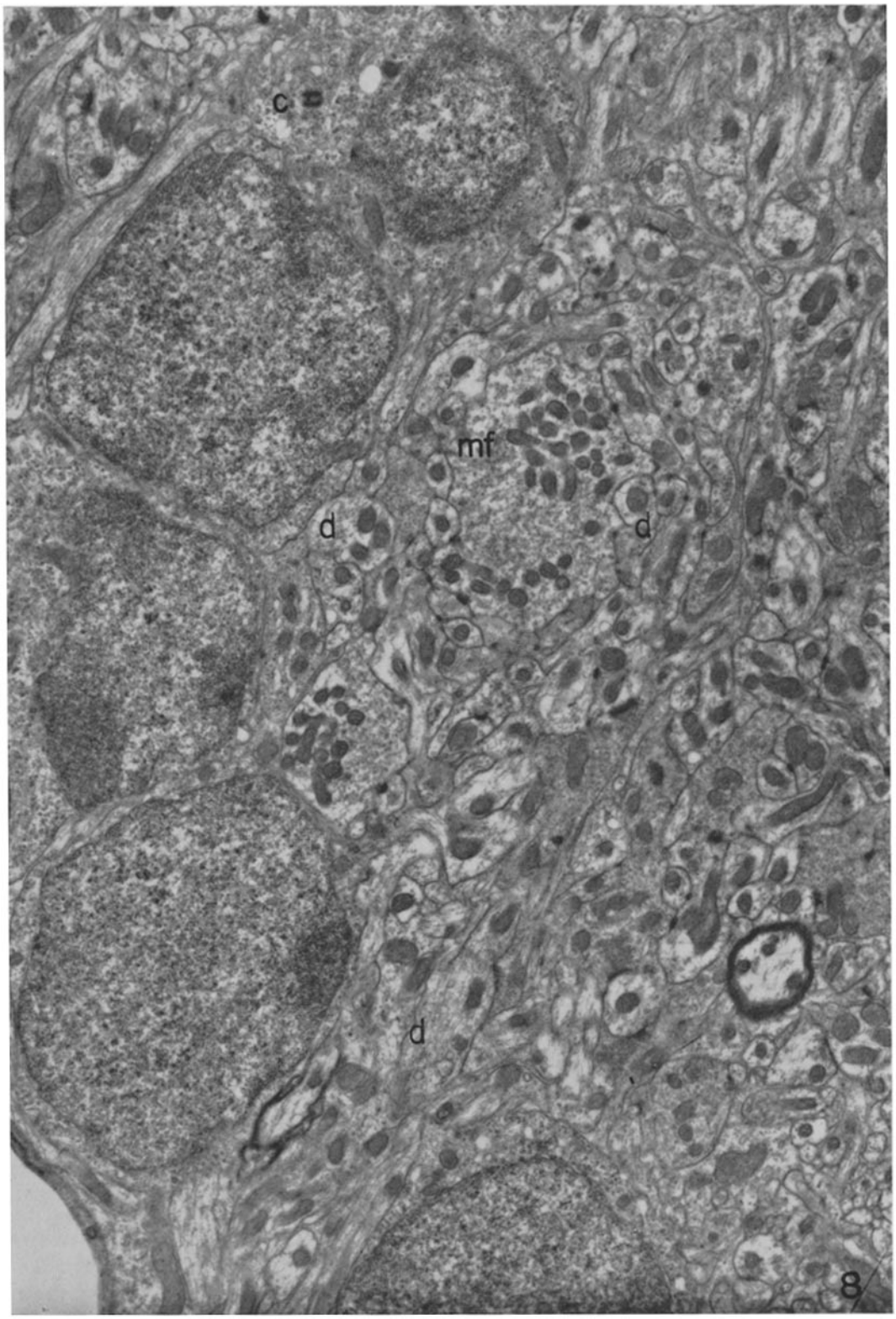
When 0.13 mg $CaCl_2$ was added to each milliliter of the fixative these discontinuities were not prevented. When, however, the excessive amount of 5.4 mg (approximately 0.5 per cent *w/v*) was added per milliliter of fixative, the membranes of all cells in all the tissues examined remained intact and continuous (Fig. 16). The internal cytoplasmic membranes (nuclear envelope, the endoplasmic reticulum, and mitochondria) of the cells studied were not apparently susceptible to damage associated with calcium deficiency in the fixative.

COMMENTARY ON THE METHOD

The procedure described in this paper was developed by the method of trial and error using numerous animals over a 3 year period. It requires skill and speed in surgical dissection and attention to the

FIGURE 8

Electron micrograph of the granule cell layer of the cerebellar cortex (rat). The field includes an arc formed by five granule cells and filled with a tangle of interlocking synapsing nerve fibers, a glomerulus or protoplasmic islet. A capillary passes across the left lower corner of the figure. The granule cells are notable for their small size, their scanty cytoplasm populated by ribonucleoprotein granules, and the small number of processes. A centriole (*c*) may be seen in the cytoplasm of the uppermost granule cell. The glomerulus consists of a central, branching axonal mass, the ramifying tip of a mossy fiber (*mf*), and a peripheral complex of branching dendrites (*d*) originating from the granule cells. The terminal structure of the mossy fiber is packed with mitochondria and small vesicles. The smaller subsidiary masses of similar content represent sections through the branches of the mossy fiber. The lighter, small, polygonal profiles of the granule cell dendrites (*d*) frequently display the typical dark patches where their surfaces contact the axons. The single myelinated fiber in the field is probably the myelinated preterminal portion of the same or another mossy fiber. The elongated processes are profiles of dendrites entering the glomerulus. The coherence of the tissue is noteworthy. × 10,000.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minute details in the sequence of operations. Investigators who are unfamiliar by previous experience with the general technique of perfusion would do well to practice with the more commonly used fixatives, such as 10 per cent formalin and Zenker-formal. As these solutions are incomparably easier to work with than osmium tetroxide, success with them does not require artificial respiration, warming, or great speed. Of course they also do not yield results that are worth examining in the electron microscope. Once one has learned the appropriate skill, the method described here should give excellent results at each attempt. The only danger in its practice is that, because each successful perfusion yields large quantities of material, the investigator may forget the details of the technique before he has any need to repeat it.

The most common reasons for failure of the perfusion are errors in carrying out the method; for example, perforating the aorta by clumsy dissection, failure to avoid air bubbles entrapped in the tubing, forgetting the injection of sodium nitrite, or allowing the animal to die before onset of the perfusion, either from an overdose of anesthetic or from crude dissection. Another cause of failure is the use of excessive amounts of saline solutions to wash out the blood.

A further reason for disappointing results is exposure of the central nervous system too soon after the onset of perfusion. Cammermeyer (12, 13) has shown that dark and light cells and other postmortem artifacts can still be produced by handling of the central nervous system before the perfused fixative has had time to harden the tissues. Our experience with osmium tetroxide solutions confirms Cammermeyer's results with other fixatives. Although osmium tetroxide acts very rapidly, a perfusion which lasts 40 minutes yields more homogeneous fixation than one lasting 15 to 20 minutes. Prolonging the perfusion beyond this time, however, results in such brittle tissue that dissection is difficult.

It cannot be too strongly emphasized that adequate precautions must be taken by the investigator to avoid exposure of his eyes and respiratory tract to the vapors of osmium tetroxide during the perfusion procedure and subsequent handling of the specimens. The entire procedure can be carried out in an operating chemical hood. Unless the hood is specially constructed, however, both the surgical procedures and the dissection are likely to be awkward. In our experience, a well ventilated room provided sufficient protection if a stream of air was directed over the operative field away from the experimenters. An additional important precaution is to wear goggles to protect the eyes during the perfusion and the dissection. The perfusion procedure itself was less dangerous in this respect than the dissection, because, once started, the perfusion could be left to flow unattended, whereas dissection often involved 2 or more hours of continuous exposure.

The following paragraphs of miscellaneous comments are offered in order to explain the reasoning behind some of the apparently arbitrary details in the technique.

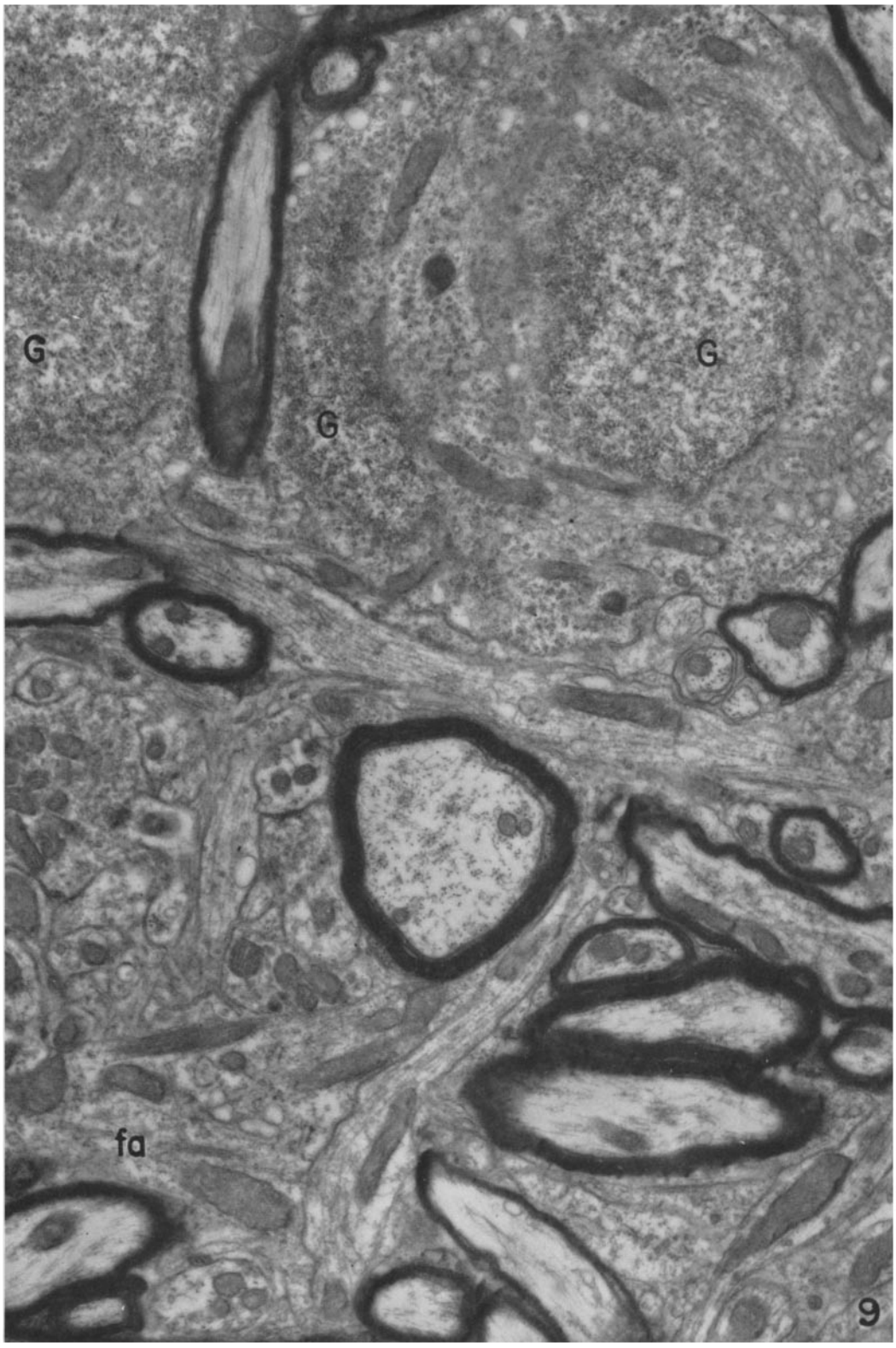
It is important to emphasize that the method of perfusion requires healthy animals. Attempts to perfuse sickly animals, or animals dying from circulatory collapse or excessive anesthesia, invariably failed.

Because the lungs immediately collapse upon opening of the thoracic cavity and because the cells of the central nervous system are extremely susceptible to anoxia, artificial respiration is a necessity in this procedure. Depending upon the skill and care of the surgeon, the period between opening of the chest and starting the perfusion may be anywhere from 2 to 15 minutes. In early attempts to arrive at an effective procedure, ordinary air from the common laboratory tap was used for inflating the lungs. This method was soon abandoned because it was difficult to control the air pressure and because the perfusion consistently failed. Pure oxygen was used a few times but it, too, was followed by failures, as should have been expected from the fact that pure oxygen induces constriction of cerebral vessels (19). Finally, mixtures of 3 or 5 per cent CO_2 and 97 or 95 per cent oxygen were chosen because CO_2 not only causes dilatation of the cerebral vessels (19) but also contributes to the buffering system of the blood and tissues. In administering the gas it is important to avoid overinflating the lungs, as they are easily damaged.

Sodium nitrite is the only vasodilating drug that was tested, because it gave satisfactory results on the first trial. Use of a vasodilator is essential for the suc-

FIGURE 9

Electron micrograph of the deep zone of the granular layer in the cerebellar cortex (rat). Three granule cells (*G*) lie at the top of the figure, and the edge of a glomerulus appears at the left lower border. The myelinated fibers are probably to be identified, according to their size, as the axons of Purkinje cells on their way to the central white matter of the folium (see Fig. 2 also). A process of a fibrous astrocyte (*fa*) enters the picture at the left margin and partly encircles the large myelinated fiber in the center. The continuity of white and gray matter in this heterogeneous field is evident. × 13,000.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cess of vascular perfusion with osmium tetroxide solutions. On occasions when we have intentionally omitted or have forgotten the nitrite, the perfusion has always failed.

Warming of the initial perfusion fluids is also an important step in the procedure. Preliminary and unsuccessful trials were made with precooled solutions and with solutions at room temperature. The explanation for these failures is not immediately obvious. Possibly chilled osmium tetroxide induces arteriolar constriction which prevents passage of the fixative into the capillary bed. In contrast, warm solutions may dilate the arterioles, and once they have become immobilized in an open position, further perfusion with a chilled solution becomes possible. Moreover, it should be remembered that warming the solutions 10–15°C above room temperature significantly increases the diffusion rate of the solutes.

In 1944 Baker (1) recommended the use of calcium ions in fixatives for light microscopy. In 1956 Pappas (31) noted that 0.01 per cent $CaCl_2$ in the fixative improved the preservation of *Amoeba proteus* for electron microscopy. Porter and Pappas (33) reported that 0.001 per cent $CaCl_2$ was necessary for successful fixation of chick embryo skin (previously disclosed in Palade's review of fixing procedures (26)). Off and on since 1953 the senior author has added calcium chloride to solutions of osmium tetroxide without noticing any significant difference in the preservation of membranes at the electron microscopic level. Following Baker's strong recommendation of calcium ions in 1958 (2), we added 0.5 per cent $CaCl_2$ to our perfusion fluid in the hope that it would improve the preservation of myelin sheaths in the central nervous system. It had no effect upon the myelin, but it did improve the preservation of cell surface membranes. After the introduction of epoxy embedding media, the advantages of fixatives containing calcium ions were even more evident than they had been when methacrylate was used. Convincing evidence was obtained by the experiments reported above (Figs. 15 and 16).

In a study of decalcification of the liver by *in vivo* perfusion with ethylenediaminetetraacetic acid, Leeson and Kalant (21) noticed defects in the surface membranes of separated cells similar to those reported here. Because the cytoplasmic structures under these defects were intact, they concluded that the breaks were produced during handling of the tissues after fixation. This reasonable inference suggests that calcium ions in some way may stabilize the plasmalemma against damage in the dehydrating and embedding media. The mechanism of action of calcium ions is not clear, however, and why a great excess of calcium ions should be needed seems equally mysterious. It has been suggested that calcium ions play a role in controlling the permeability of the surface membrane (36). Possibly, they may improve the coherence of the membrane by cross-linking phospholipids. The experiments of Leeson and Kalant (21) demonstrated neatly that calcium ions are important in maintaining the cohesiveness of cells in the liver. It would be interesting to know whether this finding can be extended to the cells of the central nervous system.

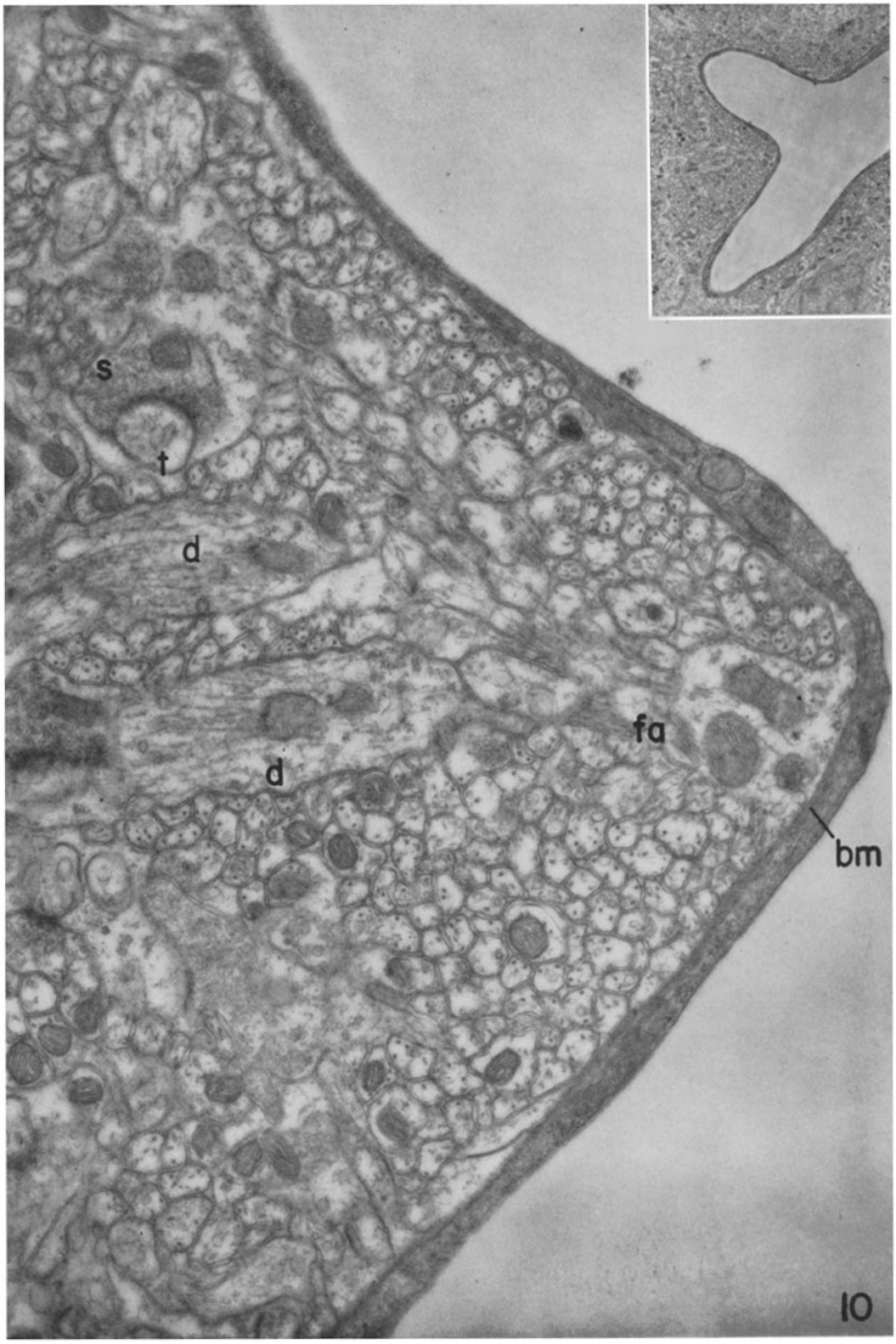
After perfusion fixation, the principal differences between central nervous tissue embedded in methacrylate as compared with Epon concerned the preservation of myelin and the delineation of surface membranes. The myelin sheath in methacrylate-embedded tissue invariably displayed explosions, distortions, and focal disarray of the lamellae. These disturbances were almost completely eliminated merely by embedding the tissue in Epon; those which were not could be attributed to faulty sectioning. It is well known that in epoxy-embedded tissues, surface membranes appear sharper and cleaner than they do in methacrylate-embedded tissues.

DISCUSSION

In cytology fixation aims at a satisfactory preservation of the cell as a whole; it does not aim at the best possible preservation of a given cell component with disregard to the fate of the others.—Palade (26).

FIGURE 10

Electron micrograph of a branching capillary and surrounding neuropil in the molecular layer of the cerebellar cortex (rat). The inset shows the general topography. The large picture shows the thin endothelial lining of the capillary lying against a dark basement membrane (*bm*). A blunt process of a fibrous astrocyte (*fa*) approaches the bifurcation of the capillary and spreads out as a thin layer over the basement membrane. Several profiles of tertiary branches of Purkinje cell dendrites (*d*) may be seen. The transverse section of a dendritic thorn (*t*) capped by a synaptic terminal (*s*) of a granule cell axon appears at the upper left. The numerous, closely packed, polygonal profiles belong to other granule cell axons (parallel fibers). × 21,000; inset, × 2000.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Although numerous neuroanatomists have extolled the advantages of perfusion fixation for the study of the nervous system by optical microscopy (*e.g.*, 6–8, 12, 13, 20, 35), electron microscopists have not displayed much interest in it. Whether this neglect is due to the relatively high cost of the most favored fixing agent, fear of the vapors of osmium tetroxide, ignorance of the method, or unsuccessful trials is not known. Only a few investigators have reported their use of the method. In early work on the electron microscopy of normal tissues, Dalton (14–16) found that the slow penetration of solutions containing osmium tetroxide required perfusion in order to obtain satisfactory fixation. He stated (16) that perfusion of the liver through the hepatic portal vein was easy and that satisfactory perfusion of the intestine, kidney, and testis could be obtained after preparation of the animal with atropine or sodium nitrite (14). Perfusion of the central nervous system was, however, unsuccessful in his hands. Bargmann and Knoop used the method for fixation of the lung in amphibia and mammals (3, 4) and of the neurohypophysis in kittens and dogs (5). Nylen and Scott (24) obtained fairly satisfactory fixation of growing teeth by means of perfusion, and Horstmann and Meves (18) mention that they used the method for fixing the brain of one kitten but do not provide any illustrations of their results. In addition to these scanty reports, however, it is known that several experienced investigators have attempted vascular perfusion with osmium tetroxide solutions, and that, being unable to obtain satisfactory results, they have abandoned it as a preparative technique. Pease (32) declares boldly that vascular perfusion with osmium tetroxide "is doomed to failure in all except very special cases."

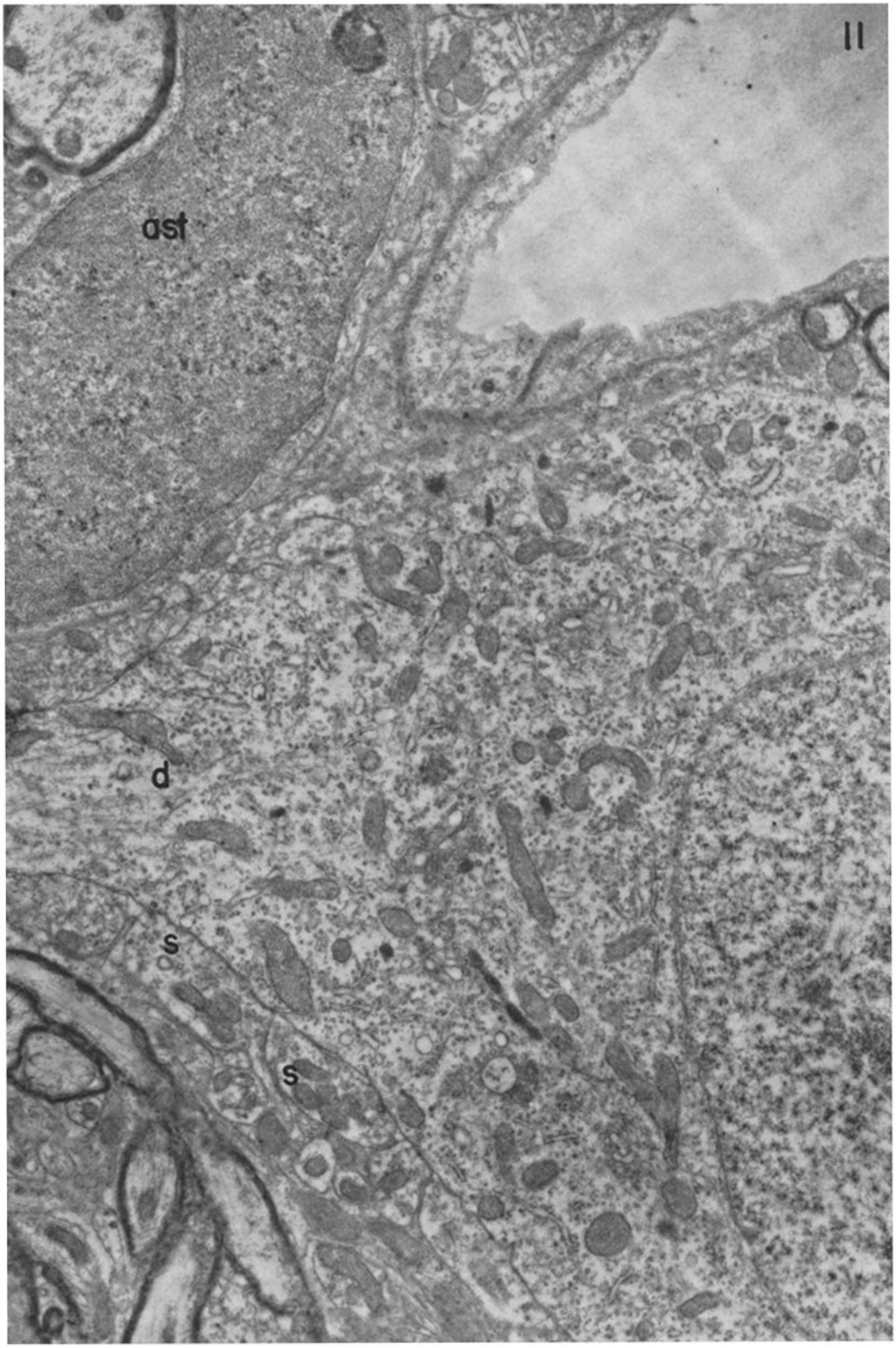
The method presented in this paper is described in detail so that even one previously inexperienced with perfusion should be able to obtain satisfactory preservation of the central nervous system after he has achieved the necessary skill. It should not be assumed, however, that merely going through the motions of the technique will assure adequate fixation. Examination of the tissue with the dissecting microscope, the phase contrast microscope, and finally the electron microscope is necessary to evaluate the success of each attempt. As Bodian (7) has pointed out, failure to obtain good fixation is not failure of the method but failure of its execution.

In an early discussion of fixation for electron microscopy, Palade (26) set forth a number of useful criteria for judging the adequacy of cellular preservation at the electron microscope level. In general terms these were (*a*) correspondence with the appearance of the living tissue as far as can be ascertained by optical microscopy, and (*b*) agreement with the images constructed from evidence provided by such indirect means as polarization microscopy, x-ray diffraction, cell physiology, and cytochemistry. As Palade epitomized the fundamental prerequisite, "Fixation should be true to nature." In more specific terms he listed a host of negative qualities the absence of which, it was proposed, denotes satisfactory fixation—precipitates in the cytoplasm and nucleoplasm, large discontinuities in the cell membrane, empty spaces, clumping and retraction, swelling of mitochondria, distension of the endoplasmic reticulum, disarray of orderly structures, thickening of membranes, and vacuolization of lipid inclusions. It is extremely difficult to set up specific standards for judging the adequacy of fixation except in these negative terms. More positively, however, they may all be subsumed under such indefinite words as continuity, coherence, and delicacy.

A very serious difficulty arises when we seek to apply these criteria to the central nervous system. We have no means whatever for knowing

FIGURE 11

Electron micrograph of the sacral spinal cord (rat) showing part of a large motoneuron, an astrocyte (*ast*), and a capillary. The neuron contains the usual complement of organelles–mitochondria, Nissl substance, Golgi complex, multivesicular bodies, etc. A dendrite (*d*) passes out of the cell body at the left. Boutons (*s*) containing mitochondria and synaptic vesicles are aligned upon the surface of the neuron and its dendrite, especially along their lower margin. Myelinated fibers appear in the left upper and lower corners. Notice the compactness of the tissue. × 11,000.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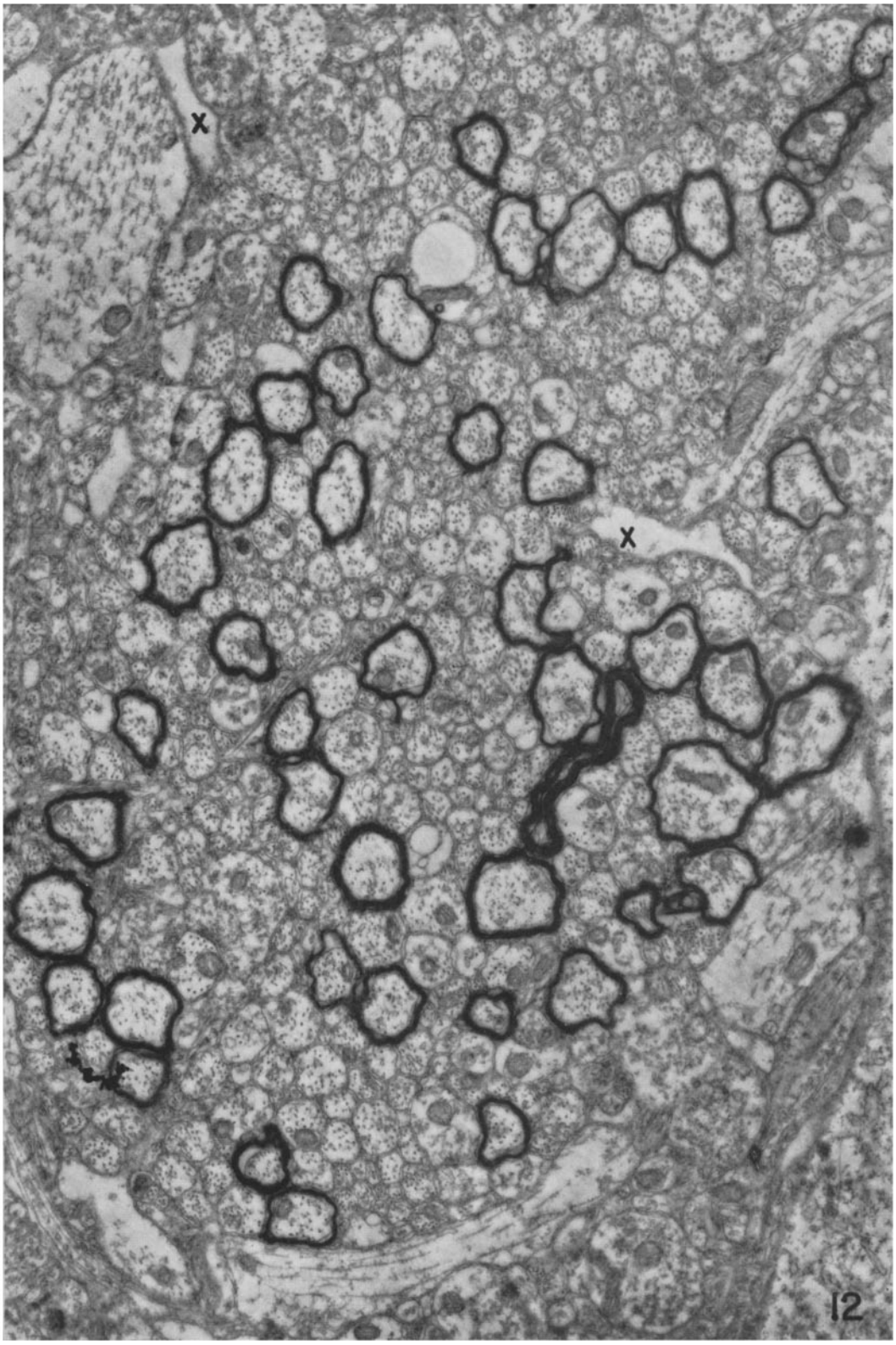
what the intact central nervous system actually looks like in the living state, for it has never been examined. We are forced, therefore, to rely entirely upon the indirect methods mentioned above for a picture of the living tissue. Unfortunately, even these methods are not easily adapted to the living brain or spinal cord. Most of the evidence believed to apply to the central nervous system has been gained from studies of the peripheral nervous system and even, by extension, from studies of other tissues and does not necessarily apply to the organization of the central nervous system. Under these circumstances there is no very strong reason to expect that the interpretation of data obtained from indirect methods (*e.g.*, the determination of the volume of the extracellular space) is necessarily any more reliable than that obtained from examining electron micrographs. At the same time, however, the law of parsimony obliges us to believe that the principles of biological action are identical throughout the organism, and by the same token we are obliged to expect that the principles of fine structure that apply, for instance, to living cells in tissue cultures also apply to living cells in the central nervous system. Therefore, we must assume that the criteria for adequate fixation that are derived from a study of living cells by both direct and indirect methods are equally applicable to cells which we cannot study in the living. With respect to the central nervous system, this means that, as Bodian (8) has phrased it, "In the absence of observations on living tissues, the use of refined cytological methods should form the basis of cytological analyses of structures which, like the synapse, cannot be satisfactorily observed in the living state." Such a "refined cytological method" is fixation by vascular perfusion.

Nevertheless, it should be pointed out that when individual neurons in material fixed by perfusion are compared with the best preserved cells in material fixed by immersion, only insignificant differences can be discerned. This result can hardly cause surprise in view of the fact that the criteria for satisfactory preservation are the same in both cases. Consequently, present ideas concerning the intracellular organization of the neuron (that is, the structure of the perikaryon and the internal morphology of dendrites, axons, and terminals) cannot be expected to undergo much revision as a result of perfusion fixation. Where adequate preservation has not previously been attained, as in the neuroglia and the neuropil, new conceptions can be expected to develop on the basis of the more satisfactory picture now accessible for study.

There is, however, an important difference between the aims of cytological fixation and the requirements fundamental to morphological study of the central nervous system. The currently accepted standards for adequate fixation relate almost entirely to intracellular structures. So long as the investigator is concerned with such objects as mitochondria, nuclear pores, the endoplasmic reticulum, secretory granules, and the surface membrane, he can afford to ignore those cells in his material that do not fulfill the criteria for good fixation and can limit his attention to those that do. But the moment he becomes concerned with how cells are put together to constitute a tissue or an organ, he must become interested in achieving good fixation of every cell in his material, and of the intercellular structures as well. In other words, we may paraphrase Palade's principle quoted at the head of this section in the following manner: In cytology fixation aims at a satisfactory preservation of the tissue or organ as a whole; it does not aim at the best possible preservation of a given cell type with disregard to the fate of the others. As regards the central nervous system, this dictum implies that we can expect to learn little or nothing about its organization at the level of fine structure from methods of fixation, like those in current use, which preserve only a small pro-

FIGURE 12

Electron micrograph of a tract containing both myelinated and unmyelinated nerve fibers in the hypothalamus of the goldfish. The tissue shows several empty spaces (*x*) bounded by cell membranes which indicate incomplete preservation. Other micrographs of cells from the nervous system of the goldfish may be found in references 27, 28, and 34. × 14,000.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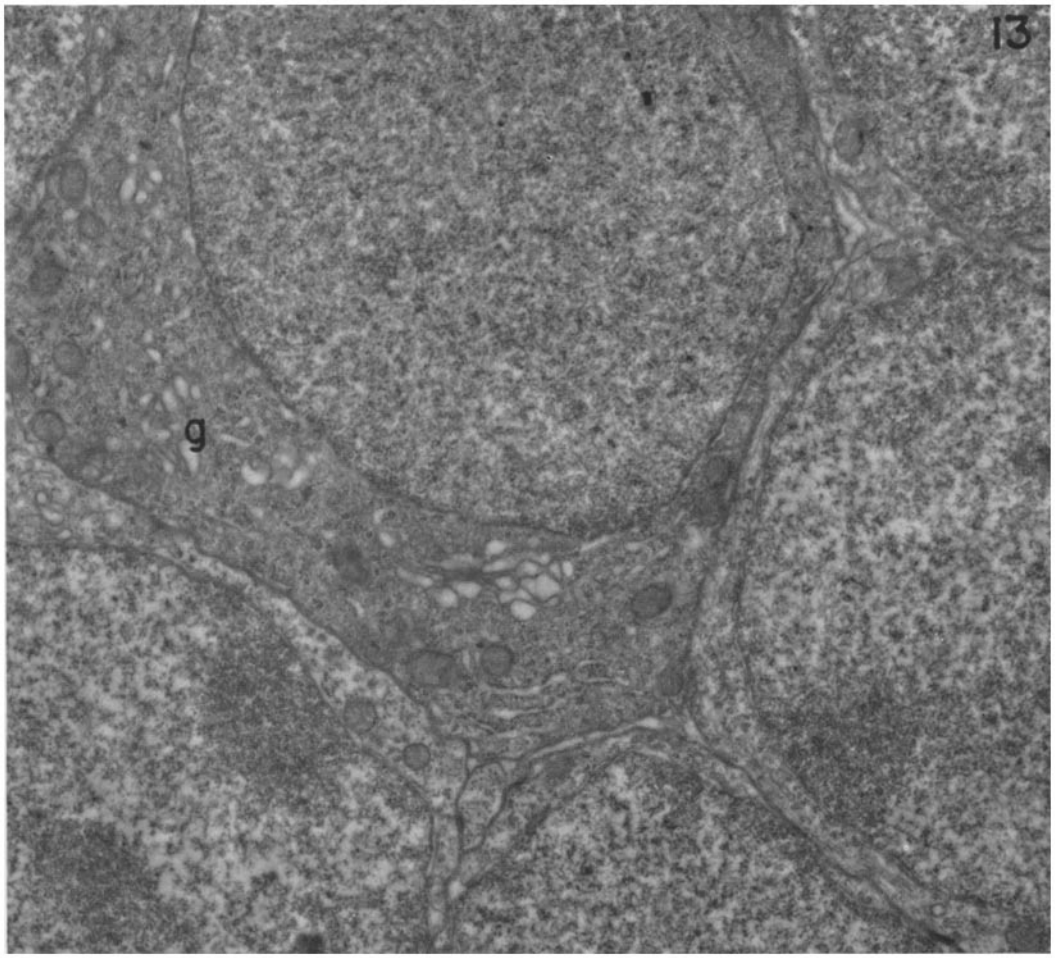

FIGURE 13

Electron micrograph of an oligodendrogliocyte in the granule cell layer of the cerebellar cortex (rat). This cell is characterized by a rounded nucleus and a cytoplasm packed with ribonucleoprotein particles. Although the cytoplasm is usually restricted to a thin shell about the nucleus, this figure was selected to show the region of the Golgi complex (*g*) where the cytoplasm is somewhat more voluminous. The glial mitochondria are slightly larger than those in the surrounding granule cells. The cytoplasm of the granule cells, in contrast to that of the oligodendrogliocyte, is pale and contains fewer ribonucleoprotein particles. Their nuclei also appear to be more loosely organized. Notice that the oligodendrogliocyte is in immediate contact with each of the five granule cells surrounding it in this field. × 14,000.

portion of its cells and produce either swollen ghosts or shriveled and precipitated carcasses of the great majority.

Successful fixation of the central nervous system by vascular perfusion with osmium tetroxide results in specimens that are homogeneously compact and uniform. Empty spaces, torn cell membranes, swollen myelin sheaths, distended endoplasmic reticulum, swollen mitochondria, and coarsely precipitated nuclei—all are absent. "Watery" cells, dark cells, and light cells are all equally missing. Empty pericapillary spaces are non-existent. The cells, which almost exclusively comprise the central nervous system, fill every part of the specimen. Each cell together with its processes fits into the surrounding tissue

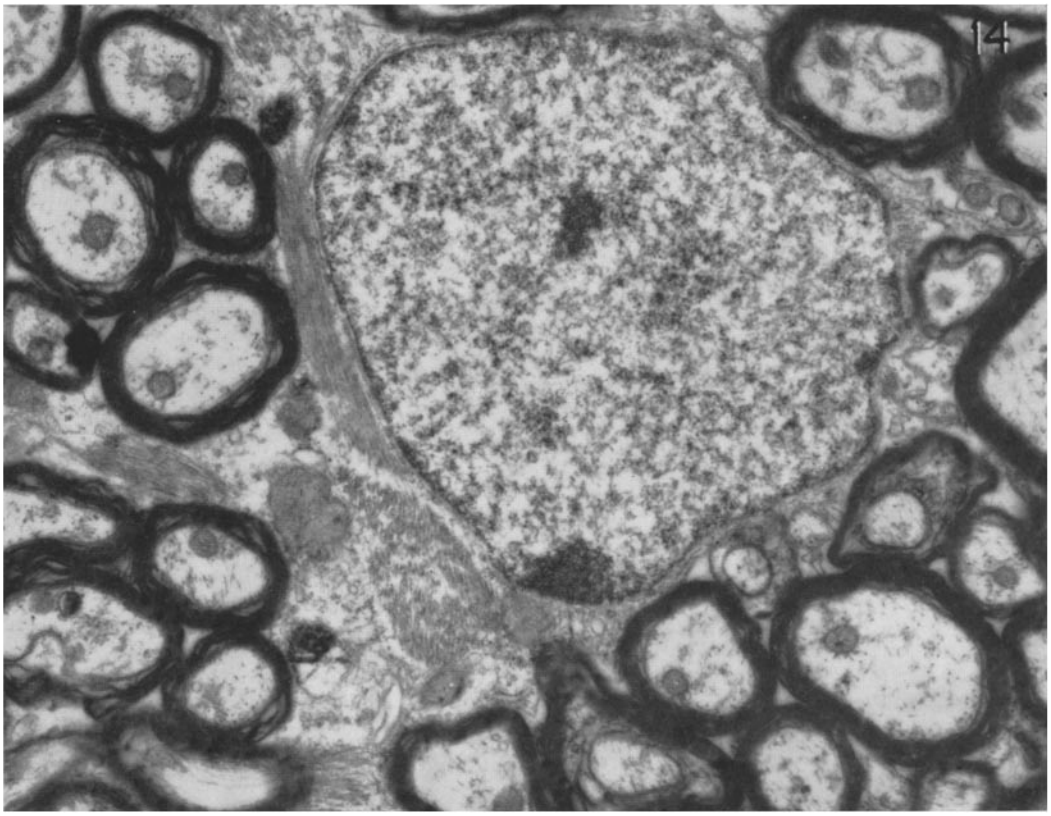

FIGURE 14

Electron micrograph of a fibrous astrocyte in the white matter of the cerebellum (rat). The nucleus is slightly irregular (probably a transverse section) and the cytoplasm contains few ribonucleoprotein particles and little endoplasmic reticulum. Sheaves of fine filaments are prominent. The cell displays numerous processes extending in all directions from the perinuclear region. × 14,000.

with a precision that forcefully reminds the observer that he is dealing with a neuroepithelium. Actually, the superiority of perfusion fixation over immersion fixation is obvious to anyone who has applied it successfully and hardly needs an elaborate defense. The difference between the devastated tissue resulting from immersion fixation and the coherent, intact tissue obtained by perfusion fixation is at once evident on even superficial examination.

The significance of adequate fixation for the furtherance of investigation into the organization of the nervous system cannot be overemphasized. For the first time it is possible to obtain most of the brain and spinal cord of mammals and lower vertebrates intact so that the organization of these intricate structures can be analyzed electron microscopically in a way comparable to the penetrating work of numerous investigators at the light microscope level. This does not mean that the electron microscope should be used for tract-tracing and the construction of wiring diagrams. The traditional methods of light microscopy are much more efficient for this purpose.

The merit of electron microscopy appears at the point where these traditional methods reach their limit. Light microscopy cannot define the topographical relations between cells—either neurons or neuroglia—either because the boundaries of cells cannot be distinguished or because the methods do not reveal all components in place. Light microscopy has enjoyed an enormous advantage in having specific methods for certain

types of neurons, certain types of neuroglia, for axons, cell bodies, or dendrites. By these methods it is possible to restrict one's attention to the specific object stained and to remain unconfused by the greatest part of the nervous tissue, which remains unstained. Even the magnificently useful method of Golgi derives its usefulness as much from what it does not demonstrate as from what it does. It allows the investigator to select for study one out of seventy or more neurons (37) and to remain undistracted by the fact that he is studying only about 1 per cent of the neural components in a given region. In electron microscopy this advantage is replaced by the apparent disadvantage of demonstrating all the cellular elements at once and in place. In the past decade this disadvantage has seemed to restrict the usefulness of electron microscopy for exploring the complex structure of the central nervous system and it has been used only to a limited extent even in the peripheral nervous system. It has now become evident that this disadvantage is spurious. It can be turned to real advantage by adequate fixation procedures, which reveal the neural tissue in its full detail with every cell intact, in its normal place, and in proximity with its associates. It is now possible to analyze these relations, which are the essence of the morphology of the nervous system and the substrate of its organized function.

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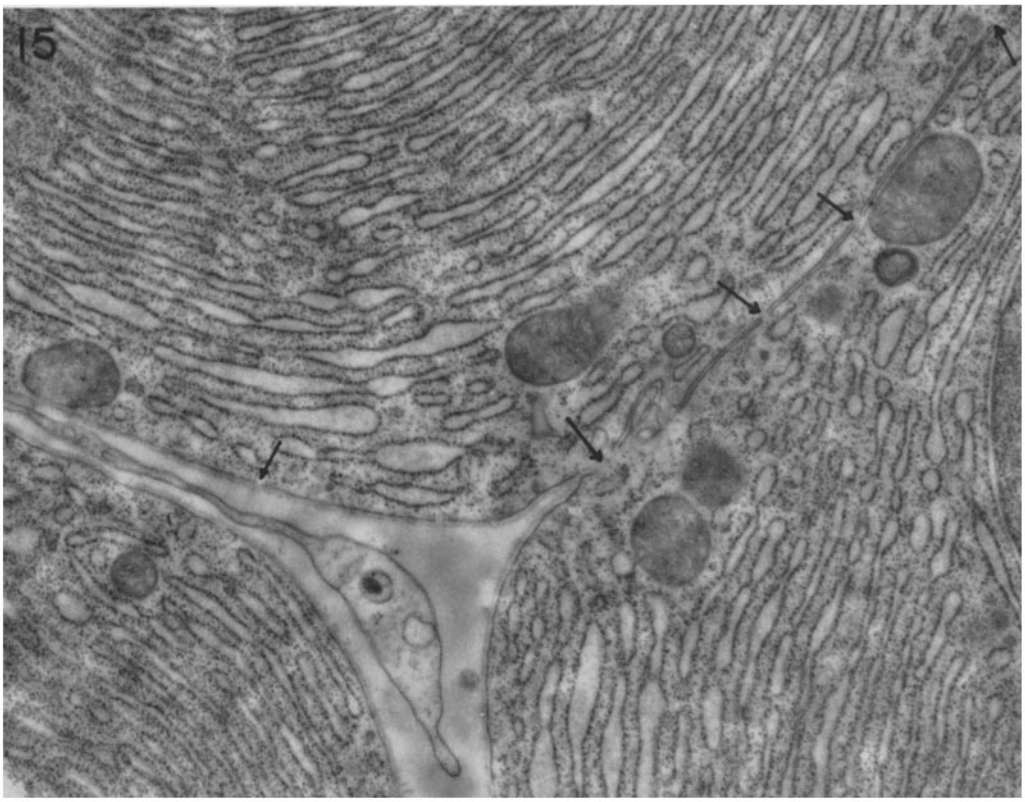
FIGURE 15

Electron micrograph of three acinar cells from the pancreas of a rat perfused with a solution of osmium tetroxide containing 0.13 mg $CaCl_2$ per ml. Breaks in the plasmalemmas of these cells are indicated by arrows. Note that the basement membrane is not interrupted. × 19,000.

FIGURE 16

Electron micrograph of two acinar cells from the pancreas of a rat perfused with a solution of osmium tetroxide containing 5.4 mg $CaCl_2$ per ml. The plasmalemmas of these cells are intact. × 27,000.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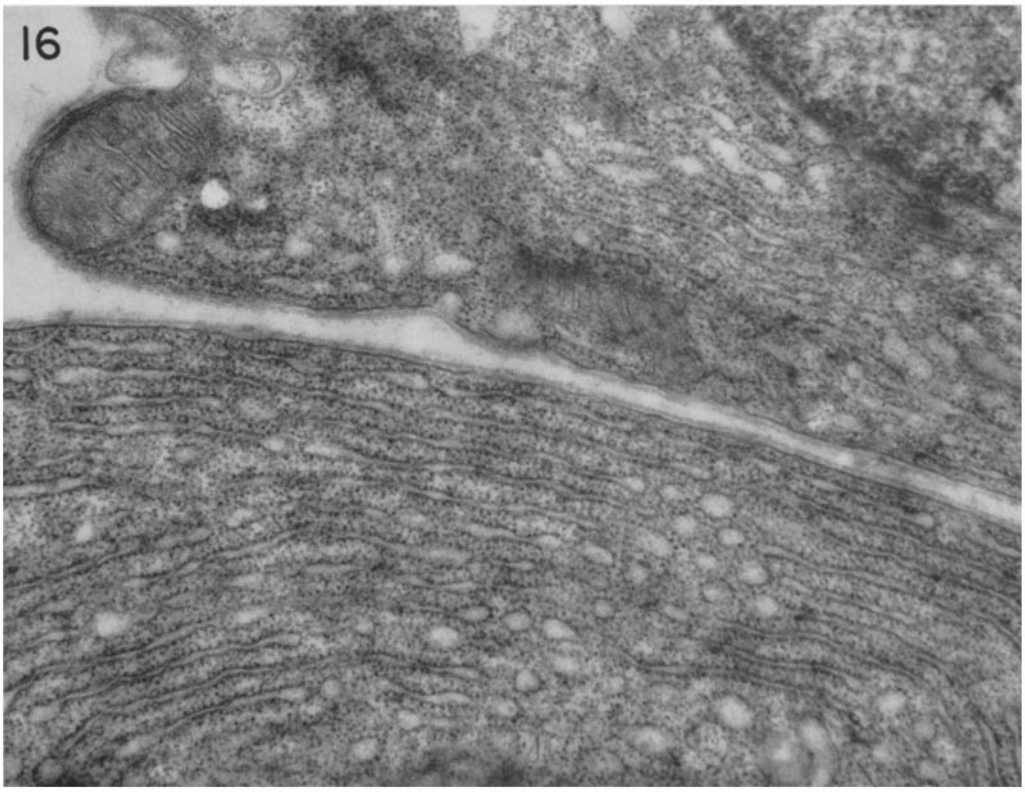
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