A STUDY OF EXTRACELLULAR SPACE IN CENTRAL NERVOUS TISSUE BY FREEZE-SUBSTITUTION

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ABSTRACT

It was attempted to preserve the water distribution in central nervous tissue by rapid freezing followed by substitution fixation at low temperature. The vermis of the cerebellum of white mice was frozen by bringing it into contact with a polished silver mirror maintained at a temperature of about −207°C. The tissue was subjected to substitution fixation in acetone containing 2 per cent OsO_4 at −85°C for 2 days, and then prepared for electron microscopy by embedding in Maraglas, sectioning, and staining with lead citrate or uranyl acetate and lead. Cerebellum frozen within 30 seconds of circulatory arrest was compared with cerebellum frozen after 8 minutes' asphyxiation. From impedance measurements under these conditions, it could be expected that in the former tissue the electrolyte and water distribution is similar to that in the normal, oxygenated cerebellum, whereas in the asphyxiated tissue a transport of water and electrolytes into the intracellular compartment has taken place. Electron micrographs of tissue frozen shortly after circulatory arrest revealed the presence of an appreciable extracellular space between the axons of granular layer cells. Between glia, dendrites, and presynaptic endings the usual narrow clefts and even tight junctions were found. Also the synaptic cleft was of the usual width (250 to 300 A). In asphyxiated tissue, the extracellular space between the axons is either completely obliterated (tight junctions) or reduced to narrow clefts between apposing cell surfaces.

An apparent paucity of extracellular space in the central nervous system has been demonstrated with the electron microscope by many authors. Horstmann and Meves (15) determined the extracellular space from the mean distances between the cell membranes (about 200 A) and the mean diameters of the cellular elements. A value not exceeding 5 per cent was in this way computed for the extracellular volume in the shark's optic tectum. An extracellular space of 3 to 5 per cent of the tissue volume has been estimated for mammalian central nervous tissue (17). Recently, a tight apposition of cell membranes in central nervous tissue was described in material fixed by perfusion with glutaraldehyde or formaldehyde, reducing the extracellular spaces even more (16).

These findings apparently conflict with the relatively high concentration of the traditionally extracellular ions, sodium and chloride, in the central nervous system. On the assumption that all the sodium and chloride is extracellular and that the extracellular material has the same ionic composition as cerebrospinal fluid, Manery (19) and Manery and Hastings (20) estimated a chloride space of 25 to 35 per cent and a sodium space of 38 per cent of the tissue volume. Woodbury *et al.* (45) found after 8 hours' equilibration with Cl^{36} a chloride space of 25 per cent. Aprison *et al.* (2) reported estimates of extracellular water

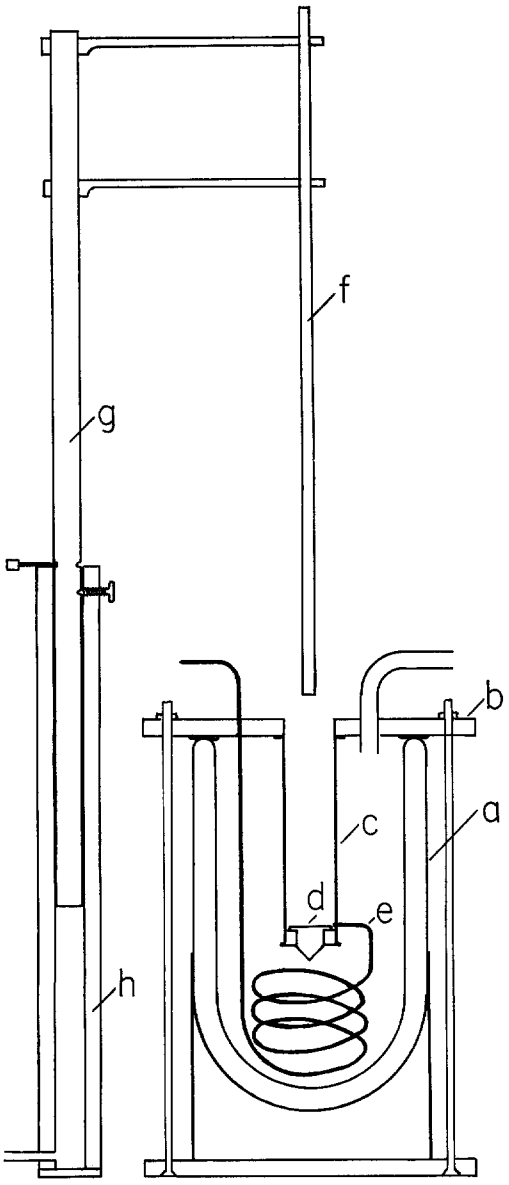


FIGURE 1 The apparatus for rapid freezing consisted of a Dewar vessel (*a*) closed with a bakelite plate (*b*). A brass tube (*c*) was fastened to the bakelite under a hole in the plate. The bottom of the tube was formed by a silver plug (*d*) the upper surface of which had been polished mirror smooth. The pressure in the Dewar vessel which was filled with liquid nitrogen could be reduced by evacuating N_2 through a tube passing through the bakelite plate. Helium, after flowing through a spiral (*e*) immersed in the liquid nitrogen, was released over the surface of the silver plug. A glass plate placed over the hole in the bakelite retarded the escape of helium from tube *c*. The tissue was attached to a wooden stick (*f*) which was fastened to a piston (*g*) moving in a barrel (*h*). By regulating the outflow of air from the barrel, the speed of descent of the tissue could be controlled.

varying from 36 per cent for the cerebral cortex to 27 per cent for the medulla. Since some of the sodium and chloride is present in the cellular elements, for instance in the neurons (7), the sodium and chloride spaces will be larger than the actual extracellular space. Methods for determining extracellular space based on the comparison in plasma and tissue of the concentrations of compounds which do not readily pass cell membranes are less suitable for the central nervous system due to the presence of the blood-brain barrier. From such efforts, estimates between 4 per cent and 22 per cent have resulted, depending on the compounds used and the measures taken to circumvent the barrier (1, 6, 25, 34, 44).

The extracellular space can also be estimated from the electrical impedance of a tissue. The currents used for impedance measurements are carried mainly by extracellular electrolytes, since the high resistance of the limiting cell membranes impedes movements of intracellular ions. It has been possible to compute accurately the volume percentage of the particles in cell suspensions, which can be considered as models for tissues, from the specific resistances of the suspension and of the substrate (5, 12). A mean specific impedance of 208 to 256 Ω cm has been determined for the cerebral cortex (11, 24, 40). From this low specific impedance, which is not more than 4 to 5 times that of a tyrode solution, an extracellular space in excess of 25 per cent was estimated (43).

Although there are uncertainties in the indirect estimates of extracellular space in central nervous tissue, the values obtained are so high that they stand in striking contrast with the electron microscope observations. These discrepancies can be reconciled by assuming that the sodium and chloride present in the tissue is contained in cellular elements, especially in the glia (17, 32, 37). Furthermore, to account for the low specific impedance of the tissue, the assumption has to be made that the membranes of glia cells are ion permeable and do not markedly impede current flow (17, 37, 42, 43). Alternately, it has been suggested that an appreciable space exists in the living nervous tissue, but that the preparation necessary for electron microscopy results in a transport of extracellular material into the intracellular

compartment (37). There is evidence that the water and electrolyte distribution in central nervous tissue is labile and that transport of extracellular material into the intracellular compartment occurs on relatively slight provocation. For instance, asphyxiation of central nervous tissue results within minutes in a marked increase of its impedance, suggesting that electrolytes have disappeared from the extracellular space (36–38, 41). The latter conclusion is supported by the observation with the light microscope of an asphyxial transport of chloride and water into dendrites (35–38, 42, 43) and into certain glial elements of the cerebellum (fibers of Bergmann, 36). Comparable impedance changes and a similar transport of chloride were observed during spreading depression (37, 42). It was postulated that the transport of electrolytes into the intracellular compartment is caused by an increase in permeability of the cell membranes for sodium and perhaps for other inorganic ions like chloride and potassium. If it is also assumed that the membrane remains impermeable for large intracellular organic ions, then Donnan forces can be expected to cause a transport of extracellular sodium chloride into the intracellular compartment. Water will have to accompany the electrolyte transport to maintain osmotic equilibrium.

If short periods of asphyxiation or the functional changes occurring during spreading depression can produce appreciable shifts of electrolytes and water into the intracellular compartment, it is not inconceivable that the strong stimulation and chemical alterations caused by the application of fixatives result in a similar material transfer. In the present investigation, it was attempted to preserve the water distribution as present in the living tissue by the use of a freeze-substitution technique.

METHODS

The method of freeze-substitution used in the present study has been described in detail elsewhere (39). The tissue was frozen rapidly by bringing it into contact with a heavy silver plug, polished mirror smooth and cooled to the temperature of liquid nitrogen under reduced pressure (about −207°C). The silver surface was protected against the condensation of water and air by a stream of cold dry helium gas flowing over it. The tissue to be frozen was lowered onto the polished silver surface by a guiding system which made it possible to let it descend through the cold helium at a controlled velocity (30 to 40 cm/sec). The apparatus is schematized in Fig. 1.

The cerebellar vermis of white mice anesthetized with urethane (0.25 to 0.3 cc of a 20 per cent solution) was used exclusively in this investigation. This tissue was chosen because spreading depression, which causes similar, although less pronounced, electrolyte and water movements as asphyxiation (37, 42), is not readily elicited in the cerebellum by slight mechanical stimuli as is the case in the cerebral cortex (9, 27, 36). In order to minimize the mechanical disturbance of the tissue, the cerebellum was not removed from the animal, but the whole head was placed on the carrier of the guiding system. A suitably shaped brass strip was introduced into the mouth and kept in place by closing the jaws tightly on the strip with a ligature circling the head and passing under the zygomatic arcs. The strip was fastened to the carrier at such an angle that the vermis became parallel with the surface of the silver plug. This part of the procedure was carried out while respiration and circulation were intact. The body was then severed from the head with a scissors' cut immediately caudal to the cerebellum. Two cuts were made through the lateral regions of the occipital bone, and the part of the skull covering the cerebellum was lifted off. Blood and fluid on the cerebellum were removed by gently touching the surface with tissue paper (Kleenex) moistened with Ringer's solution. The carrier was then lowered on the freezing surface. The tube, which carried the silver plug at its lower end (Fig. 1, *c*), was filled with liquid nitrogen. The head was kept in there for 2 to 3 minutes and then transferred to a Dewar vessel filled with liquid nitrogen. The cerebellum was flattened on contact with the silver surface, but the cerebellar vermis and hemispheres could be recognized after fixation by the characteristic gyration.

The tissue was then subjected to substitution fixation at −85°C in a 2 per cent osmium tetroxide solution in acetone for 2 days. In some experiments it was shown that after that time most of the molecular layer can be rubbed with a wad of cotton from the cerebellum kept at low temperature (−80°C). This indicates that a layer of 50 to 100 μ thick becomes substituted in 2 days. The head was warmed in three steps (−25°C, 4°C, and room temperature) while still in the substitution medium. The vermis was isolated and cut into blocks, each consisting of a single folium. These were kept for 2 to 3 hours in repeatedly changed acetone, then in propylene oxide, and finally embedded in Maraglas (10). All sections were cut on an LKB Ultrotome at right angles with the folia. Sections about 1 μ thick were stained with methylene blue and Azure II (28) for examination by light microscopy. Thin sections for electron microscopy were stained with lead citrate (26) or with uranyl acetate (13) followed by the lead stain of

Millonig (21). Electron micrographs were taken with a Philips EM-200.

The study of the 1-μ-thick sections made it possible to discard unsuitable blocks. The flattening of the tissue on contact with the silver surface can be expected to cause distortion of the tissue structure. By accepting only blocks of which the thick sections showed the normal position and direction of recognizable tissue elements in the molecular layer, areas were selected which had first contacted the silver and thus were frozen before they could be disturbed mechanically. Only a narrow layer of tissue, often about 10 μ thick, yields electron micrographs which do not exhibit ice crystals. Blocks from which sections examined with the light microscope showed ice crystals in the molecular layer could be discarded, since experience had shown them to be unsuitable for electron microscopy. Also, blocks in which the pia was unusually thick, covered with a large blood vessel, or was separated from the cortex by an appreciable space were discarded, since such surface structures reduce the ice crystal–free region of the cerebellar tissue which can be used for electron microscopy.

RESULTS

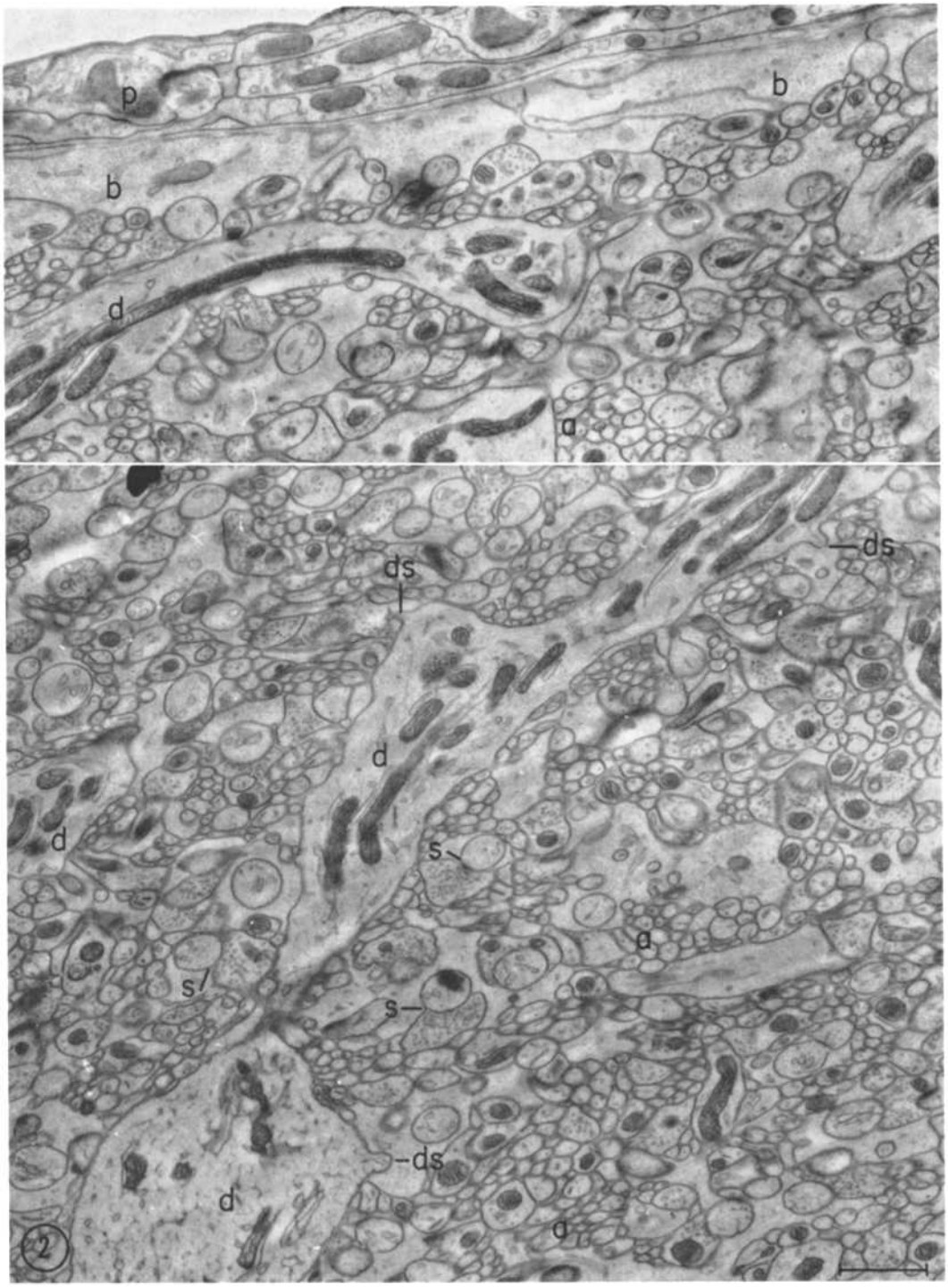
In poorly frozen preparations, ghosts of ice crystals are recognizable as light areas, distinctly and sharply separated from each other by dark lines, which represent the protoplasm concentrated by the formation of crystals consisting of pure H_2O. In well frozen preparations the surface layer is free from such crystals, but in deeper layers ice crystals are always present especially in the nucleus and cytoplasm of cells, and in large dendrites. Fig. 9 demonstrates ice crystals in a cell body; Fig. 2 shows the crystals in a dendrite. Fig. 9 also shows the damage that results from the formation of ice crystals: the cellular inclusions generally get disorganized and sometimes even lose their clear identity. Also, the nuclear membrane in this figure is severely distorted. Still deeper, the ice crystals become larger and more uniformly distributed throughout the tissue. In such regions, the organization of the tissue and of the individual cellular elements is unsuitable for electron microscopy.

In electron micrographs without recognizable ghosts of ice crystals, the criteria now in general use for evaluation of satisfactory fixation (Palade, 22; Palay *et al.*, 23) are easily met with. Such micrographs show sharply delimited, continuous cell membranes, clearly identifiable membranous cellular inclusions, (*e.g.*, endoplasmic reticulum, mitochondria, Golgi apparatus, synaptic vesicles), and uniformly fine ground cytoplasm (see Figs. 5, 6, 8, 10, 15, 16). The tissue which meets the above requirements and is uniformly satisfactorily preserved is always found in the superficial part of the molecular layer immediately beneath the pia. An estimate of the depth of this layer from the

Abbreviations

a, Axon
as, Astrocyte
b, Fiber of Bergmann
c, Collagen fibers
d, Dendrite
ds, Dendritic spine
er, Endoplasmic reticulum
g, Glia
ga, Golgi apparatus
m, Mitochondrion
n, Nucleus
p, Pia
pb, Process of Bergmann fiber
ps, Presynaptic ending
s, Synapse

FIGURE 2 Micrograph of a large field of the cerebellar molecular layer frozen 8 minutes after decapitation. The dendrite (*d*) in the upper part of the figure is a continuation of the one seen in the lower part, as indicated by the mitochondria in the overlap. The dendrite is characterized by the presence of spines (*ds*). The pia (*p*) of the cerebellum is shown in the upper part of the figure. Immediately underneath the pia are end-feet of Bergmann fibers (*b*). The profiles of axons (*a*) are present in groups throughout the micrograph (axon fields). Numerous synapses (*s*) are recognizable by the vesicles in the presynaptic terminals. Many relatively large structures which show few inclusions are scattered amongst the axon fields; these are difficult to identify. They may be parts of glia or of dendrites. Ghosts of ice crystals are recognizable as light areas surrounded by relatively electron-opaque material in the lower part of the dendrite. Note the absence of appreciable extracellular space. (Lead citrate; the calibration line indicates 1 μ).



electron micrographs gives a figure of approximately 10 μ. The limitations of the method restrict this investigation to a study of the very surface of the molecular layer of the vermis.

Identification of Cellular Elements in Electron Micrographs

The identification of cellular elements in electron micrographs of central nervous tissue made by the following authors has been adopted in the present paper. Gray (14) has made a careful study of the fine structure of the cerebellum, with appropriate control by light microscopy. Palay and his colleagues (23) have published excellent electron micrographs of the cerebellum and have pointed out criteria for the identification of some of the cellular elements, such as oligodendrocytes and fibrous astrocytes. More recently, Schultz (31) has also provided some data for identification of the macroglial elements.

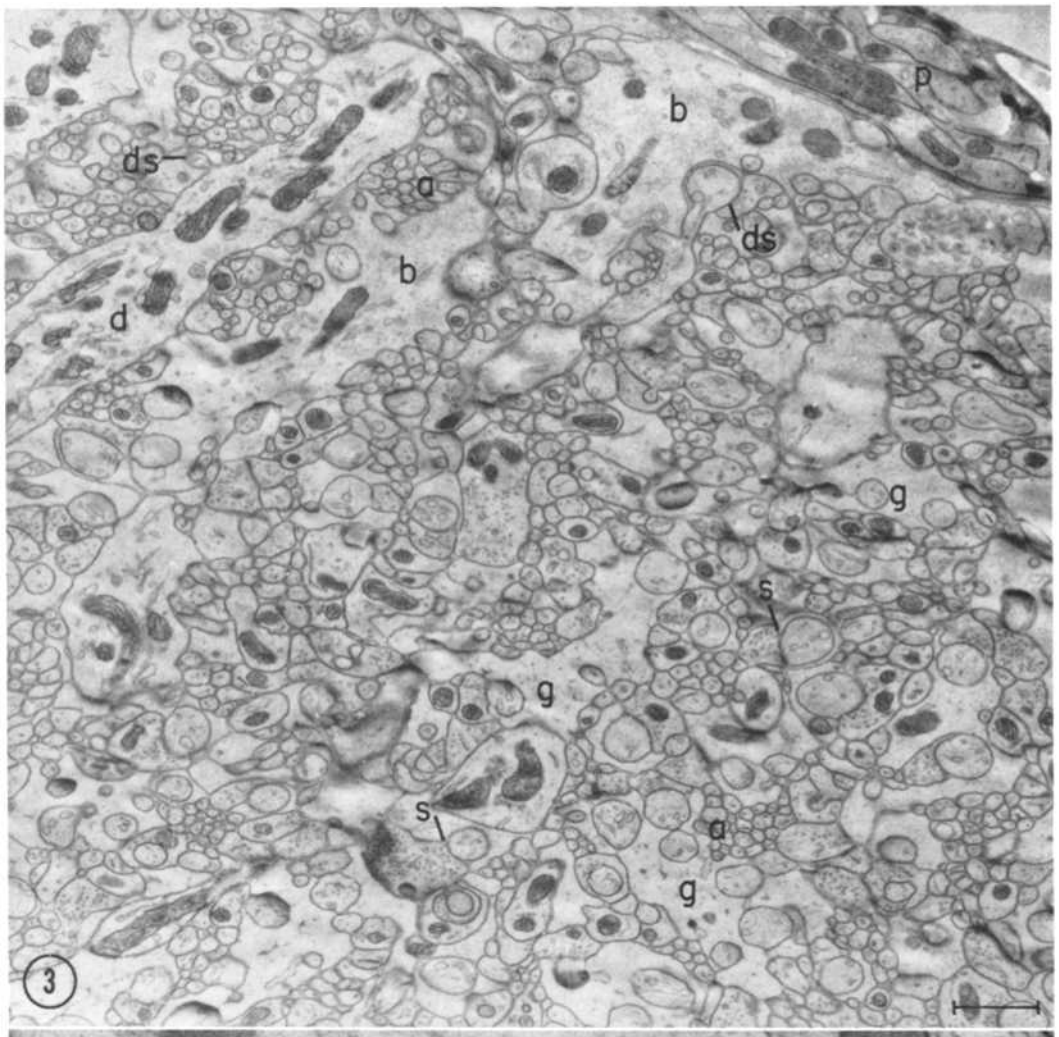
All electron micrographs published in the present paper have been produced from sections cut at right angles to the folia so that they are in the plane of the dendritic arborization of the Purkinje cells. This plane cuts the fibers of Bergmann longitudinally. These fibers run straight upward from cell bodies situated in the layer of Purkinje cells and form end-feet on the surface of the molecular layer (33). The axon branches of the granular cells are cut at right angles. The dendrites of the Purkinje cells can be identified in the electron micrographs by the presence of characteristic "thorns" or "spines" on their surface, which serve as postsynaptic processes for axodendritic synapses (14). Examples of such "spines" are shown in Figs. 2 to 4. Often the section passes only through the spines, and the relationship with the main dendritic branch is not obvious. They can then be identified only when associated with presynaptic endings characterized by synaptic vesicles (Figs. 2, 3, 5, 10 to 14). A dendrite can be distinguished from a fiber of Bergmann by the fact that the latter forms an end-foot, which runs immediately underneath and parallel to the pia (Figs. 3, 6, 7, 13). Dendrites stop short of the pia. The distinction between the two may become uncertain if the fiber of Bergmann cannot be followed right up to the pia. To facilitate identification, electron micrographs have been selected which show the formation of end-feet by fibers of Bergmann. If the cytoplasm of a Bergmann fiber is compared with that of a dendritic branch lying close by, it is noticed that the mitochondria are more numerous in the dendrite (Figs. 3, 6, and 13). Moreover, there appear to be more elements of the endoplasmic reticulum dispersed in the cytoplasm of the dendrites than in fibers of Bergmann.

The fibers of Bergmann give out branches along their length which invade the surrounding tissue. These branches do not look like the well defined and club-like spines of the dendrites, but are more irregular (Fig. 7). The electron micrographs show numerous glial elements scattered throughout the tissue. These show few cellular inclusions and may, at least in part, be sections of the branches of Bergmann fibers (Figs. 2 to 6, 11, 14, 15).

The end-foot of the fiber of Bergmann shown in Figs. 7 and 8 exhibits a special arrangement of the elements of the endoplasmic reticulum enclosed within a membrane-delimited, rounded structure. It does not seem to have been described before in the available literature on the molecular layer of the cerebellum and has been very infrequently seen during the present study. In ultrathin sections, the elements of the endoplasmic reticulum, constituting this organelle, appear either as sausage-like

FIGURE 3 Micrograph of an area of the asphyxiated molecular layer similar to that in Fig. 2. A fiber of Bergmann (*b*) is seen forming an end-foot under the pia (*p*). Another fiber of Bergmann is seen between the dendrite (*d*) on the left and the Bergmann fiber forming the end-foot. The micrograph exhibits the superficial 16 μ of the molecular layer. Ghosts of ice crystals begin to appear in the deepest regions only. (Lead citrate; calibration line indicates 1 μ).

FIGURE 4 A detail of Fig. 3 (right upper quadrant) is shown at higher magnification. A structure, probably a dendritic spine (*ds*), is present which seems to form a synapse. Narrow clefts are present between adjacent cellular elements. Where three axon or other cell processes meet, triangular spaces (arrows) are found. An irregular glial structure (*g*) is surrounded by axon fields. (Lead citrate; calibration line indicates 1 μ).



cisternae or circular-to-oval profiles, packed together in no well defined order. The membrane that forms the limiting boundary of this organelle and the membranes of the endoplasmic reticulum enclosed in it have the usual unit membrane structure (Fig. 8), and are of about equal thickness (~ 75 A). This organelle has a superficial resemblance to the "growth spiral" described by Robertson (30), consisting of a single spirally wrapped cisterna of the endoplasmic reticulum in the cytoplasm of Schwann cells in developing mouse sciatic nerve.

In addition to the fibers of Bergmann, dendrites of the Purkinje cells, and pre- and postsynaptic structures, numerous profiles of unmyelinated axons of the granular cells appear in the electron micrographs of the molecular layer of the cerebellum. These are seen as small (about 0.2 μ in diameter) rounded, or polygonal structures which generally appear in groups, axon fields (Figs. 2, 3, 5, 10 to 13, 16).

The identification of various cell bodies in electron micrographs of the cerebellar molecular layer is often difficult, because many sections pass through only small regions of cytoplasm, which may poorly represent the entire cell body. None of the cell bodies illustrated in Figs. 9, 10, and 12 seems to be that of a neuron. A vertebrate perikaryon is generally characterized by the presence of aggregates of elements of the endoplasmic reticulum in association with clusters of ribosomes that constitute Nissl bodies; and these micrographs do not exhibit such an organization. The cell body shown in Fig. 9 appears to be an astrocyte rather than oligodendrocyte because the cytoplasm is not densely packed with ribosomes, which is a characteristic of the oligodendrocytes (23, 31). Moreover, oligodendrocytes have a narrow rim of cytoplasm and dense nuclear chromatin (31). The cell body shown in Fig. 10 is difficult to identify; the section has not passed through the nucleus, but the appearance and size of the cytoplasm suggest that it may be an astrocyte. The small fraction of cytoplasm seen in Fig. 12 may also be that of an astrocyte.

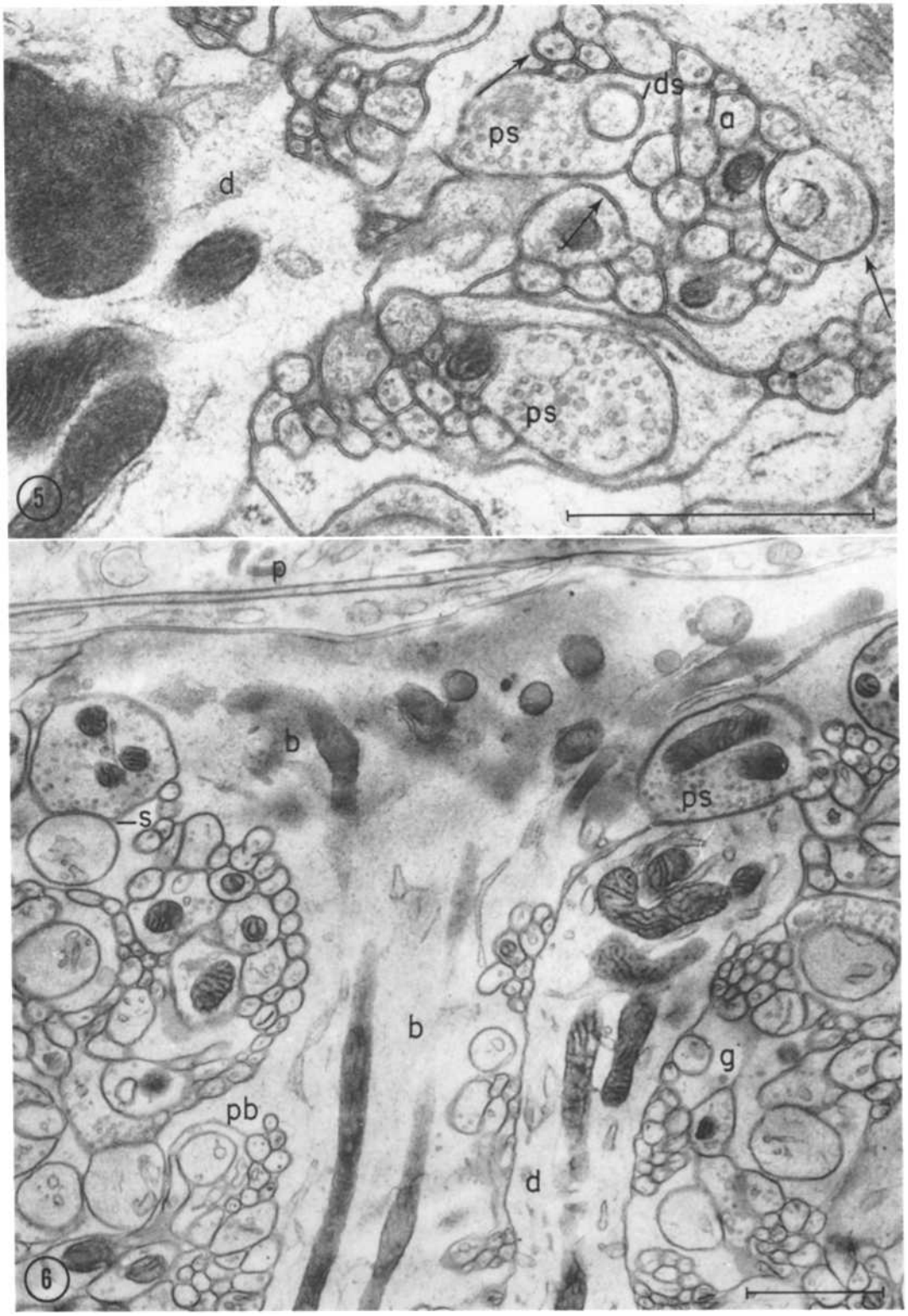
A survey of the electron micrographs of the cerebellar molecular layer produced by the freeze-substitution technique shows that the quality of preservation of fine structural details of cellular elements is comparable to that seen by routine fixation with OsO_4. However, the mitochondria frequently show an unusual feature inasmuch as the usual, about 100 A, gap between the outer and inner limiting membranes and within the cristae is not seen. The mitochondrial membranes are represented by three dense lines separated by two pale lines of about equal width. The total thickness is about 120 A; and they resemble the tight junctions of epithelial cells (8) and also the external compound membrane in Schwann cells described by Robertson (30). This appearance of the mitochondria has been seen not only in the cerebellum, but also in the exocrine cells of the pancreas and in liver cells. This will be discussed in more detail in a future paper (Malhotra and Van Harreveld, *J. Ultrastruct. Research*, 1965, in press).

Differences between Asphyxiated and Non-Asphyxiated Tissue in Electron Micrographs

In part of the experiments, the cerebellum was frozen between 25 and 30 seconds after decapi-

FIGURE 5 This micrograph shows the paucity of extracellular space in the molecular layer frozen 8 minutes after decapitation. Note the presence of tight junctions (arrows) between adjacent cellular elements. The large structure on the left is probably a dendrite (*d*). An invagination of a dendritic spine (*ds*) into a presynaptic structure (*ps*) is seen. (Lead citrate; calibration line indicates 1 μ).

FIGURE 6 In this figure of molecular layer asphyxiated for 8 minutes, a fiber of Bergmann (*b*), and a dendrite (*d*) can be compared. The fiber of Bergmann forms an end-foot under the pia (*p*) and shows a process (*pb*) on the left. The dendrite forms a synapse directly with a presynaptic structure (*ps*). Mitochondria and elements of the endoplasmic reticulum are more numerous in the dendrite than in the Bergmann fiber. The identity of the homogeneous, rounded, membrane-delimited bodies in the end-foot of the fiber of Bergmann is not obvious; these may be lipid inclusions. (Lead citrate; calibration line indicates 1 μ).



tation. It is known that, during such a period of oxygen deprivation due to circulatory arrest, the impedance of the tissue does not increase markedly (36). According to the concept discussed in the Introduction, this would indicate that no appreciable shifts of electrolytes and water have taken place during this period. The water distribution in the tissue at the moment of freezing would, therefore, represent that of the living cerebellum. In other experiments, the tissue was frozen 8 minutes after circulatory arrest. Such a period of asphyxiation causes a major (40 to 70 per cent) increase in tissue impedance, which would indicate that an appreciable part of the extracellular electrolytes accompanied by water has moved into the intracellular compartment. It was, therefore, of interest to compare the extracellular space of cerebellum asphyxiated for 8 minutes with that of cerebellar tissue deprived of oxygen for less than 30 seconds in electron micrographs obtained by the freeze-substitution technique.

Figs. 2 to 10 are electron micrographs of the superficial parts of the molecular layer of the vermis of mice which had been decapitated about 8 minutes before freezing. The general appearance of the various cellular structures is essentially similar to that seen in comparable electron micrographs of cerebellar tissue prepared by *ex situ* or by perfusion fixation with OsO_4 (Gray, 14; Palay *et al.*, 23; Smith, 33). The cells, dendrites, axons, presynaptic structures, and processes of glial cells are all closely packed and clearly defined, giving the impression of uniformly satisfactory fixation. A study of suitably stained sections at high magnification showed in many preparations tight junctions (zonula occludens) between adjacent cellular elements. These junctions shown in Fig. 5 exhibit a five-layered structure typically seen in epithelial cells (8). The total thickness of the membranes at the junctions is about 150 A. Similar tight junctions have been described recently by Karlsson and Schultz (16) in mammalian central nervous tissue fixed by perfusion with glutaraldehyde or formaldehyde and subsequent treatment with OsO_4. In micrographs of other blocks (Fig. 4) the total thickness of the apposed membranes is slightly larger (up to about 250 A), and no middle dense line formed by fusion of the outer lamellae of the apposing membranes is seen; an actual narrow cleft may be present in these instances. Such preparations show small but definite, often triangular areas of extracellular space in which three cellular elements meet (Fig. 4). The synaptic gap was found to be of the order of 250 A; a similar value has been reported by Gray (14) in *ex situ* OsO_4-fixed cerebellar tissue.

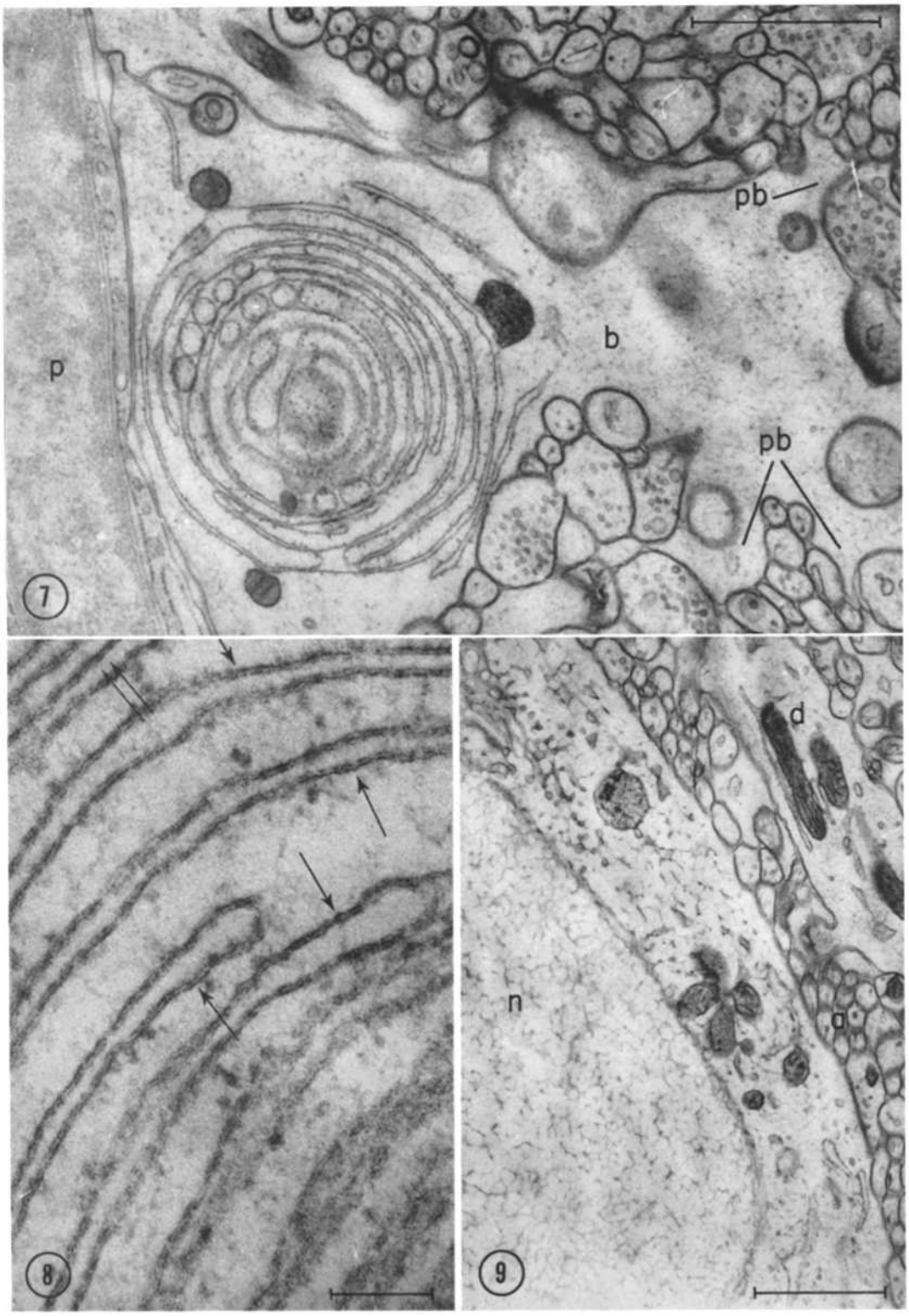
Figs. 11 to 17 are electron micrographs of cerebellar molecular layer frozen within 30 seconds of decapitation. There are consistent differences as compared with the micrographs of 8-minute asphyxiated cerebellum. The most striking difference is the presence of appreciable extracellular space between the axons, which were never tightly packed as in the asphyxiated preparations (Figs. 11 to 17). These spaces are usually uniformly dispersed amongst the axons. However, in a few instances, rather large extracellular lakes were present in the axon fields (Fig. 12). Many axons touch each other over only small areas. With higher magnification, such areas of contact showed in a few instances the tight junction structure (Fig. 17); but in most preparations no middle dense line was apparent (Fig. 16).

The clefts between the other cellular elements of the molecular layer are similar to those found in preparations fixed with OsO_4 either *ex situ* or by

FIGURE 7 This figure shows an unusual formation of the endoplasmic reticulum enclosed by a membrane in the end-foot of a fiber of Bergmann (*b*) described in detail in the text. Processes of the Bergmann fibers (*pb*) are shown. The preparation is of 8-minutes asphyxiated cerebellum. (Lead citrate; calibration line indicates 1 μ).

FIGURE 8 The typical unit membrane appearance in the endoplasmic reticulum (arrows) and in the limiting membrane (double arrow) of the structure shown in Fig. 7. (Lead citrate; calibration line indicates 0.1 μ).

FIGURE 9 Nucleus (*n*) and cytoplasm of a cell identified as an astrocyte in asphyxiated cerebellum. This cell was situated some distance from the surface and shows ghosts of ice crystals and deformed cytoplasmic inclusions. (Lead citrate; calibration line indicates 1 μ].



perfusion. In most electron micrographs, the total distance between the inner lamellae of the apposing cell membranes of glia and pre- or postsynaptic structures is of the order of 200 to 250 A (Figs. 15 and 16). However, in a few preparations tight junctions were also observed between these structures (Fig. 17). In these instances, the distance between the inner lamellae was smaller (150 A). The synaptic cleft in non-asphyxiated tissue was of the order of 250 to 300 A (Fig. 11), which is similar to the value in asphyxiated material.

Blocks of 16 mouse cerebellums frozen within 30 seconds after decapitation were examined. The resulting electron micrographs consistently showed an appreciable extracellular space in the axon fields. With a modification of the Chalkley method (18), it was attempted to make a quantitative estimate of this space. In 6 suitable electron micrographs the extracellular space varied between 18.1 and 25.5 per cent of the tissue volume, with a mean of 23.6 per cent. Similar determinations on micrographs of 8-minute asphyxiated preparations yielded a value of less than 6 per cent.

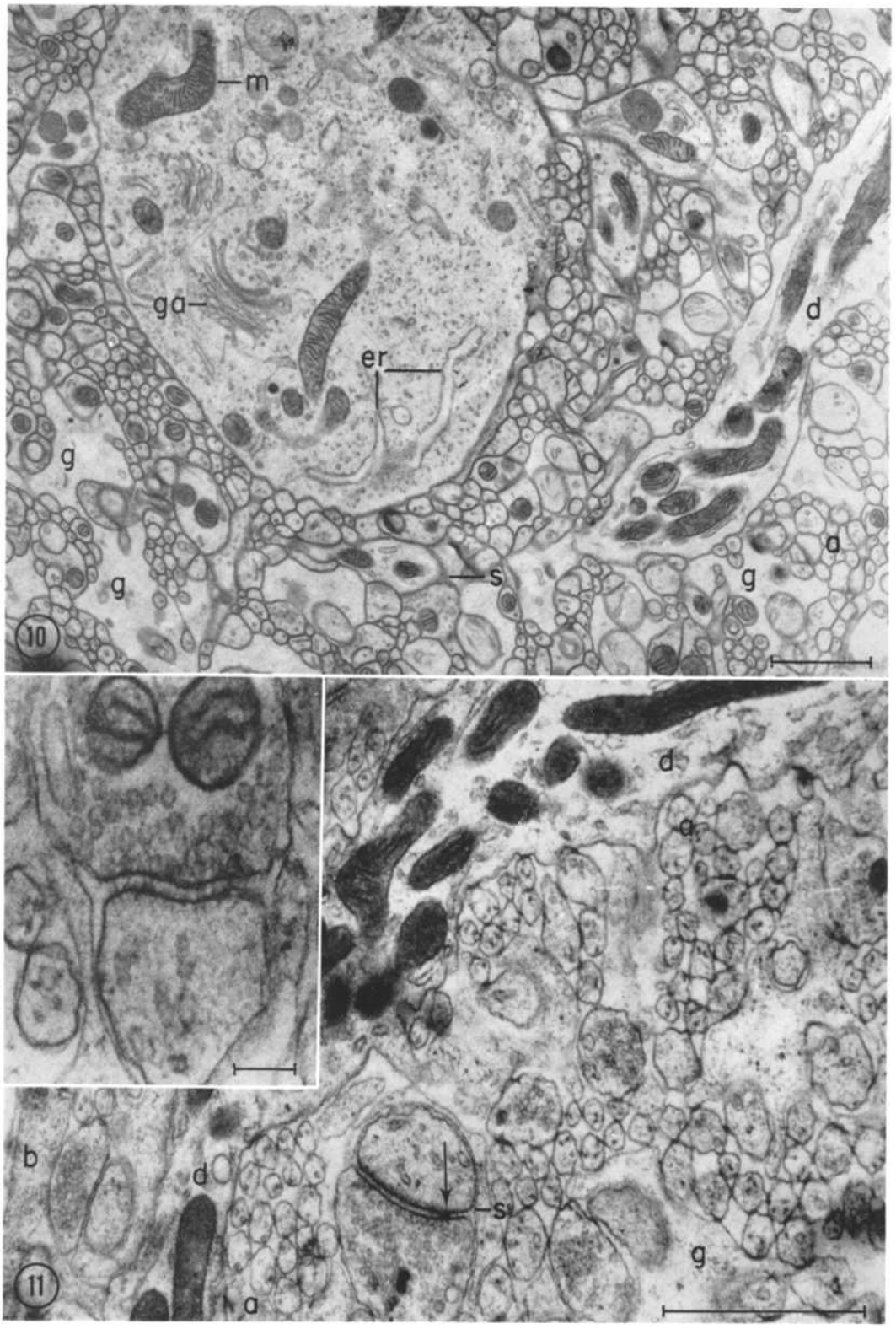
With the light microscope, a transport of chloride into the fibers of Bergmann and dendrites of Purkinje cells was observed and a swelling of the Bergmann fibers was demonstrated (36). In the 1-μ-thick sections of Maraglas-embedded material of asphyxiated cerebellum, swollen Bergmann fibers were visible as lightly stained structures running from the surface of the cerebellum into the molecular layer. Also, the dendrites of Purkinje cells appeared swollen in this material. In similar sections of non-asphyxiated cerebellum, the molecular layer was more or less homogeneous, and Bergmann fibers and dendrites could be identified only with some difficulty since they did not stand out as lighter-colored structures. These differences are not as obvious in the electron micrographs of asphyxiated and non-asphyxiated material, but in Figs. 6 and 7 the fibers of Bergmann look definitely swollen.

DISCUSSION

Methods of fixation, which give excellent preservation of the fine structure of central nervous tissue, have been described by Palay *et al.* (23) and by Bodian and Taylor (4). The quality of fixation is judged by the preservation of fine cytological and anatomical details and is generally described as satisfactory, if the criteria adopted by Palade (22) and by Palay *et al.* (23) are met with. In the absence of any direct evidence on the fine structure of the living tissue, one is forced to make use of indirect criteria for the evaluation of electron micrographs. From the exhaustive discussion and tenable arguments put forth by Palay *et al.* (23), one is reasonably well convinced that modern electron microscopy reveals as life-like a representation of the central nervous system as is possible with current technical procedures. One cannot be sure, however, that the micrographs obtained in this way faithfully represent each and every feature of the living tissue. As mentioned above, there are discrepancies between estimates of the amount of extracellular space based on biochemi-

FIGURE 10 This micrograph shows the cytoplasm of a cell identified as an astrocyte in the molecular layer of an 8-minute asphyxiated preparation. This cell was located close to the surface and shows well preserved cytoplasmic inclusions: *e.g.*, endoplasmic reticulum (*er*), mitochondria (*m*), and Golgi apparatus (*ga*). The uniformly dispersed minute granules in the cytoplasm are presumably ribosomes. Surrounding the cell are the usual nerve and glia elements of the molecular layer. (Uranyl acetate and lead; calibration line indicates 1 μ).

FIGURE 11 Micrograph of a part of the molecular layer of a cerebellum frozen within 30 seconds after decapitation. The end-foot of a Bergmann fiber (*b*) is present on the left. A dendrite (*d*) extends through the field. Axons (*a*), synapses (*s*), and glia (*g*) can be easily distinguished. Note that the axons are not closely packed as in the asphyxiated preparations, but that the axon fields exhibit abundant extracellular space. The axons contact each other only over small areas. The synaptic cleft in the large synapse (*s*) is obliterated at one point (arrow). A similar appearance of the synaptic clefts is also seen in Fig. 12. The inset of Fig. 11 shows at high magnification the usual structure of a synapse in the molecular layer of a preparation frozen within 30 seconds of decapitation (Uranyl acetate and lead; calibration line indicates 0.1 μ).



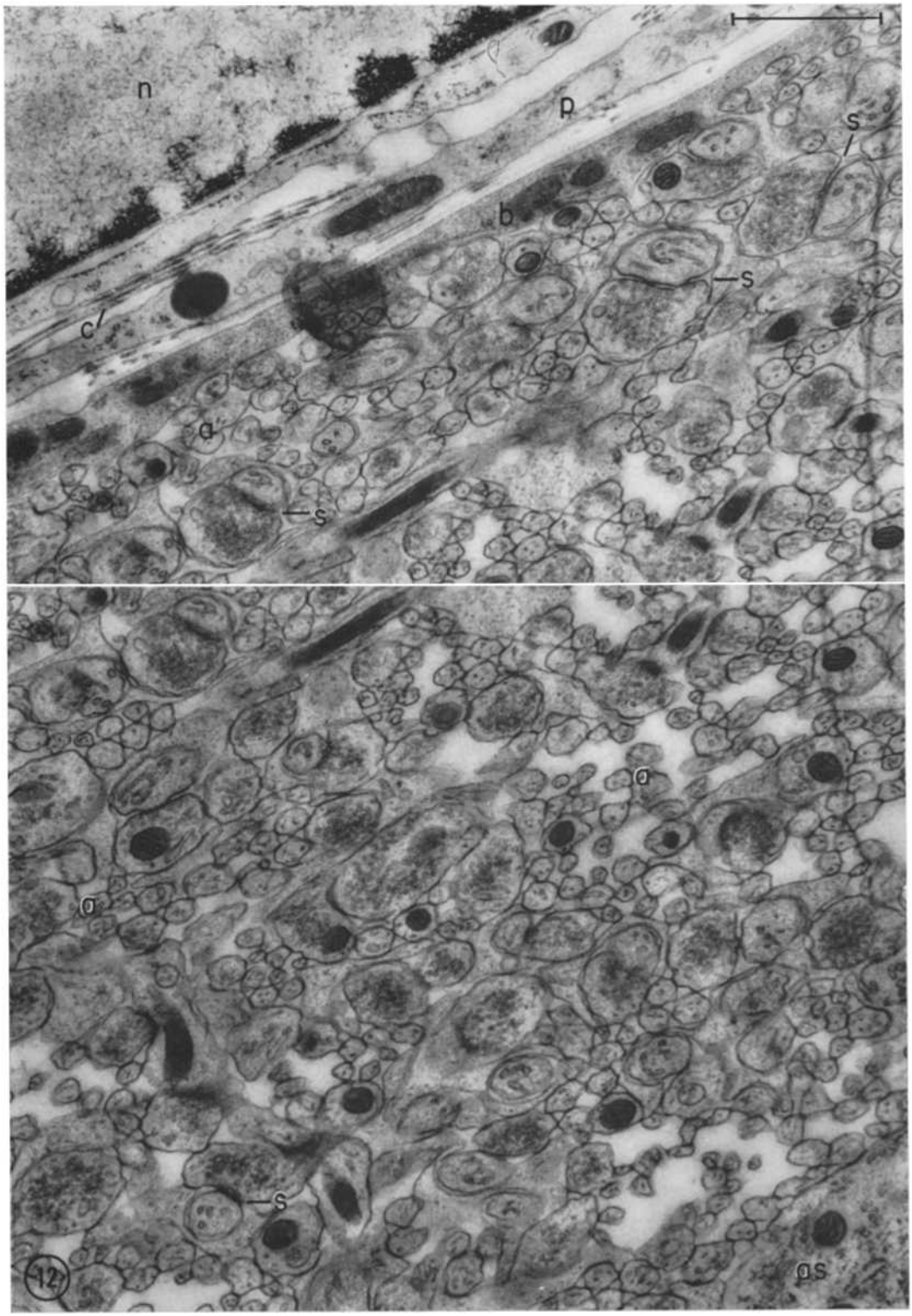
cal and physiological findings and the results of electron microscopy. A search for special methods designed to give information on this specific point seems, therefore, desirable.

The electron micrographs produced by the method of rapid freezing and substitution fixation used in the present investigation meet the criteria of satisfactory fixation. The cell membranes are sharply represented and are generally continuous. The fine structure of cellular organelles seems to be preserved as well as with any other method of fixation. Similar observations have been made on other tissues such as liver (39), and more recently on exocrine cells of pancreas (Malhotra and Van Harreveld, *J. Ultrastruct. Research*, 1965, in press) and on dorsal spinal roots (Malhotra and Van Harreveld, *Anat. Rec.*, 1965, in press). Exactly the same method of freeze-substitution was applied to cerebellum frozen within 30 seconds of decapitation and to tissue asphyxiated for 8 minutes. It would seem, therefore, that the differences in extracellular space observed in these tissues are due to the state of the tissue at the time of freezing. If the micrographs are taken at face value, they indicate that in cerebellar tissue shortly deprived of its oxygen supply, there is an appreciable amount of extracellular material which disappears during a prolonged period of asphyxiation.

The above conclusion implies that the method of freeze-substitution used in the present investigation preserves the water distribution in the tissue more faithfully than the usual chemical fixation. The following considerations seem to be pertinent in this respect. Instantaneous freezing will obviously preserve the water distribution. When the tissue is frozen slowly, however, the formation of crystals of pure ice will concentrate the solutes. The increased osmoconcentration may attract water from regions in which no ice is formed. If ice forms uniformly and at the same rate throughout the tissue, no water transfer can be expected. In the present experiments, the rate of freezing was probably high and uniform in the surface layers of the tissue, since no evidence of ice crystal formation was found. In the deeper parts, small ice crystals are often discernible especially in cells and dendrites, indicating that freezing in these layers may not have been uniform. Although the conditions for the above-mentioned water transport were thus more favorable in the deeper regions, no significant differences between the amounts of extracellular space in surface and deep layers are noted in the micrographs shown in Figs. 2 and 12. This may be taken as an indication that water movement due to unequal freezing has no important effect in the tissue layers considered in the present investigation.

The membranes in regions which do not exhibit obvious ghosts of ice crystals are well preserved. This may indicate that the membranes do not sustain severe mechanical damage during the process of freezing. The action of acetone as a fixative seems to be lost at low temperature (Baker, 3). Also, osmium tetroxide has no apparent effect on the tissue at a temperature of −85°C since the color of the tissue remains unchanged after 1 to 2 days in the substitution fluid. No chemical fixation thus seems to occur during substitution. The tissue was kept for about an hour at −25°C before it was warmed to 4°C, and finally to room temperature. Fixation by OsO_4 occurs at −25°C, as shown by the blackening of the tissue which, in this way, is chemically fixed before it is brought to room temperature. Since the membranes are apparently not seriously affected by freezing and during substitution fixation, it would seem possible that the mechanism for the electrolyte and water transport, postulated to cause the paucity of extracellular space in routinely

FIGURE 12 A large field of the cerebellar molecular layer frozen within 30 seconds of decapitation, comparable to Fig. 2, which exhibits a similar field of asphyxiated tissue. There is some overlap of the upper and lower part of the figure. The left upper corner of the figure shows a nucleus (*n*) probably of a fibrocyte which may contain minute ice crystals. Collagen fibers (*c*) are visible in the pia. Most of the other structures underneath the end-feet of Bergmann fibers (*b*) are either profiles of axons (*a*) or synapses (*s*). No obvious ice crystals are found even in the deepest region of the nervous tissue shown here. The fraction of the cytoplasm visible in the lower right corner is probably of an astrocyte (*as*). Note the presence of abundant extracellular space amongst the axons (Uranyl acetate and lead; calibration line indicates 1 μ).

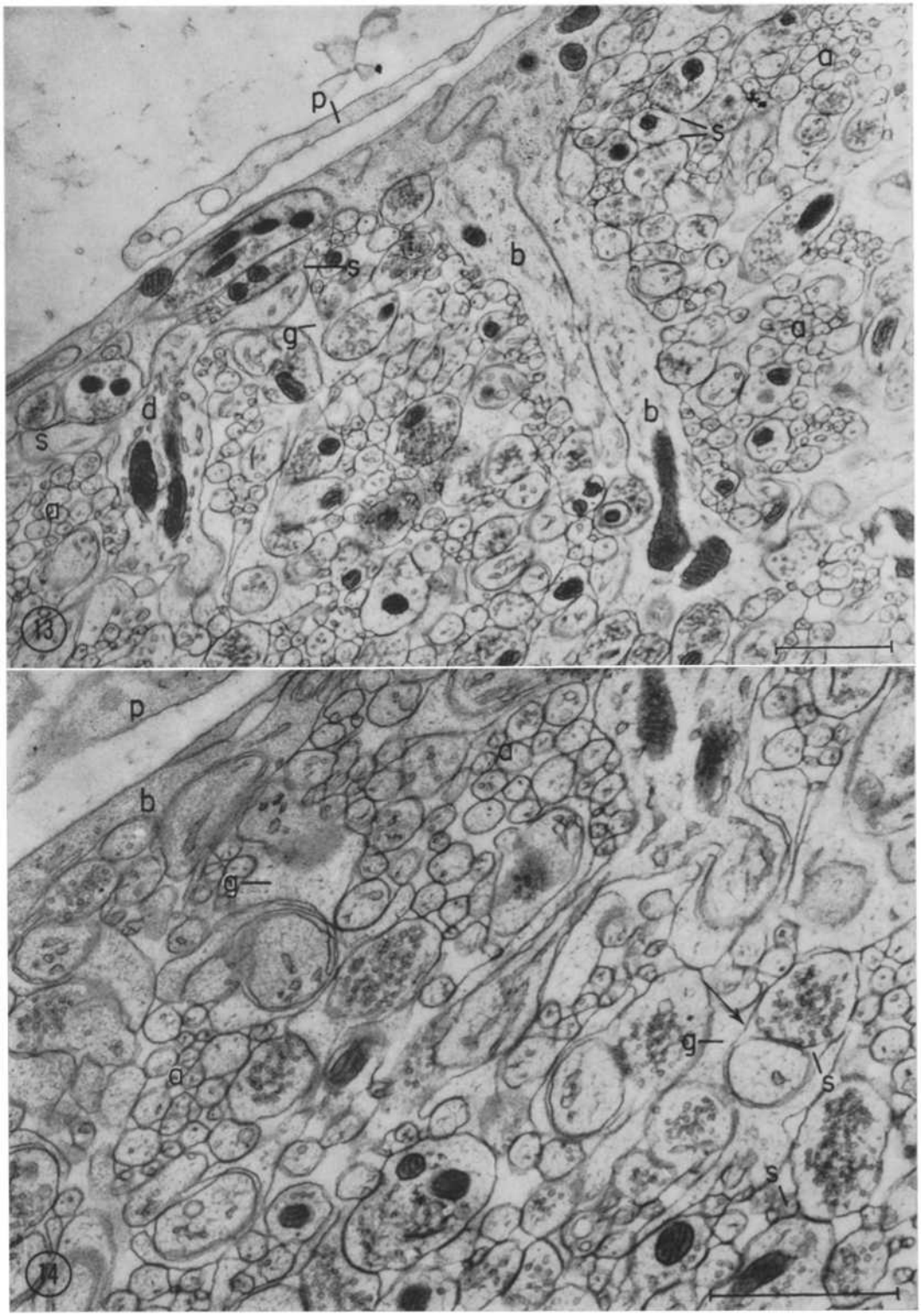


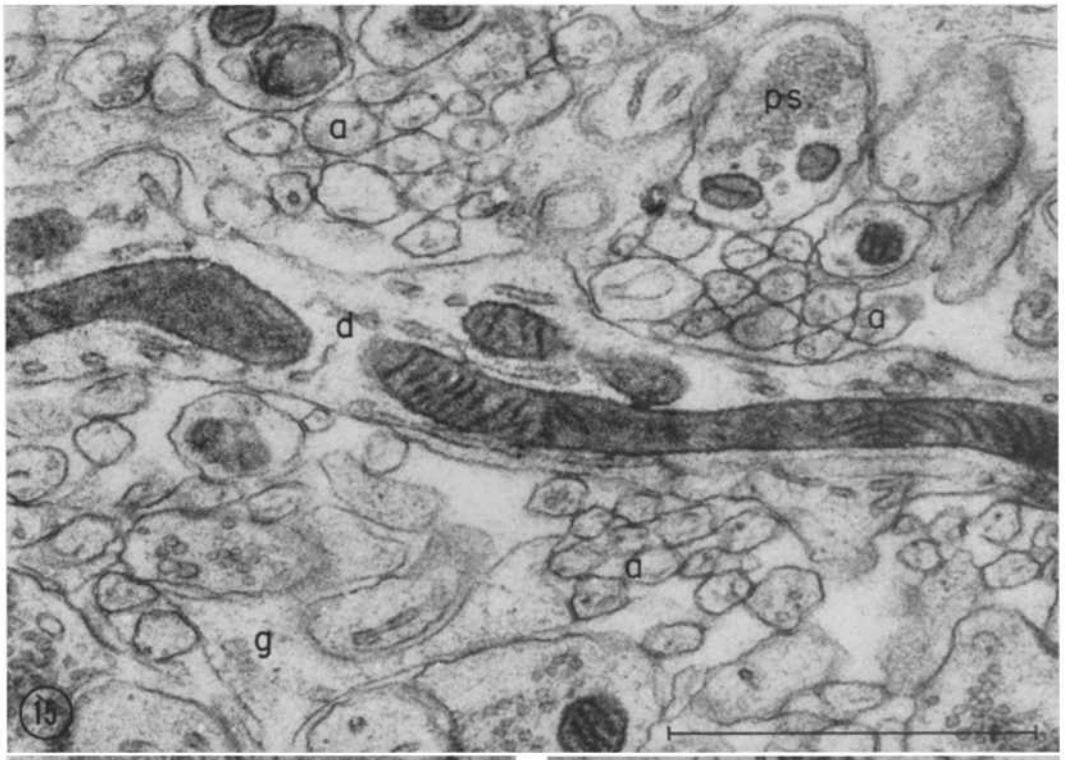
fixed preparations, is potentially still operative after the substitution medium has replaced the ice in the tissue. The conditions under which substitution occurs may prevent such events, however. The amount of water in acetone in equilibrium with ice diminishes as the temperature is lowered. The water content of acetone under these conditions was determined from the nuclear magnetic resonance of the water protons and of the naturally occurring C^{13} in acetone. In Fig. 18 *A*, the water content of acetone is plotted against the temperature. At the temperature of substitution (−85°C) the water content is about 2 per cent. If a frozen preparation is placed in 100 per cent acetone at this temperature, the solvent will proceed to dissolve ice from the tissue. The water released locally diffuses into the surrounding acetone, and in this way the plane of substitution slowly penetrates deeper and deeper into the tissue. At no time, however, will the water concentration rise above 2 per cent, and the ice in the tissue is in this way replaced directly by 98 per cent acetone. The solubility of the tissue constituents in this solvent is likely to be quite different from that in water. For instance, Fig. 18 *B* shows the solubility of sodium chloride in acetone in equilibrium with ice at various temperatures. The solubility of NaCl at −85°C is very low (0.0018 per cent). The mechanism postulated to account for the transport of extracellular electrolytes and water into the intracellular compartment during asphyxiation and spreading depression (see Introduction) and perhaps also during routine fixation with OsO_4 would, therefore, be greatly retarded and perhaps prevented by the relative insolubility of NaCl in the substituting acetone at −85°C. Other tissue components may be soluble in acetone, and concentration differences across cell membranes may cause osmotic adjustments between the extra- and intracellular compartments. There is, therefore, no guarantee that the distribution of the acetone in the tissue is a faithful representation of the original water distribution. The mean extracellular volume determined in the electron micrographs may, for this reason, not accurately represent the magnitude of the extracellular space.

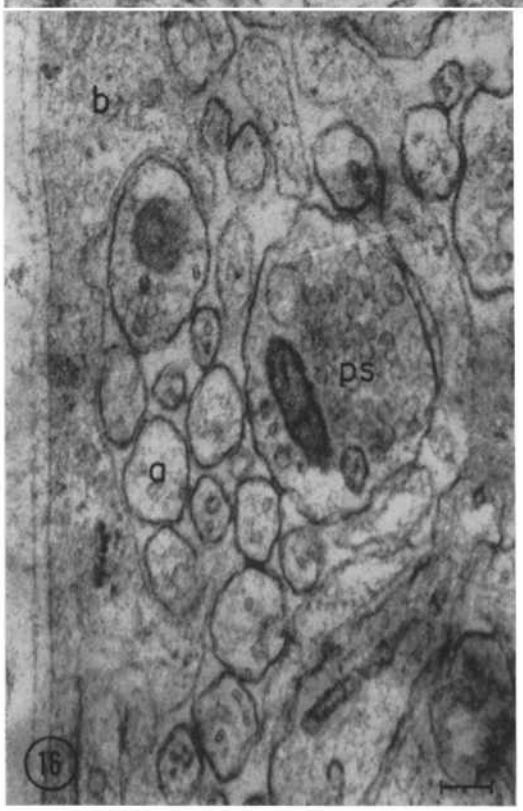
It follows from the above discussion that the faithful preservation of the water distribution in the living tissue by the method of freeze-substitution may not readily be achieved. The consistent difference in the extent of extracellular space in cerebellum frozen within 30 seconds and after 8 minutes of asphyxiation must, however, as mentioned above, be ascribed to differences in the tissue at the moment of freezing. Although perhaps other explanations are possible, the most simple and direct one would be that these findings are the expression of differences in water distribution which are preserved by the method of freeze-substitution. Such an assumption would make it unnecessary to assume an unusually high sodium chloride content of glia elements, since much of this compound would find a place in the extracellular space. It would, furthermore, give a ready explanation of the low impedance of central nervous tissue. Finally, the differences in extracellular space found in tissue deprived of oxygen for 30 seconds and for 8 minutes are in good agreement with the asphyxial impedance changes and chloride and water transport reported previously.

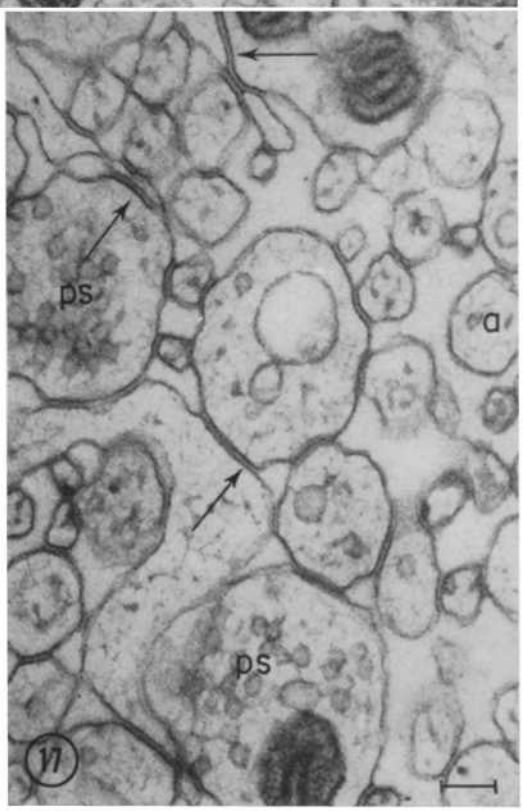
In electron micrographs of non-asphyxiated cerebellar tissue subjected to substitution fixation at low temperature the spaces between glial, dendritic, and presynaptic structures are similar to those found in tissue fixed with OsO_4 either *ex situ* or by perfusion. Also, the width of the synaptic gap is similar. There is, however, considerable space between the fibers in the axon fields in contrast to the findings in routinely fixed tissue. In general, this space is rather uniformly distributed between the axons. Whether larger spaces as shown in Fig. 12 are real cannot be decided, especially since processes such as em-

FIGURES 13 and 14 Micrographs of superficial molecular layer of the cerebellum frozen within 30 seconds after decapitation. In Fig. 13, two Bergmann fibers (*b*) are shown forming end-feet under the pia (*p*); a dendrite (*d*) with two synaptic contacts (*s*) is exhibited on the left. A comparison with electron micrographs from tissue frozen 8 minutes after decapitation will show that there is considerably more extracellular space in the axon fields. In Fig. 14, a triangular extracellular space (arrow) is present on both sides of the synapse (*s*), between the pre- and postsynaptic structures and the surrounding glia (*g*) (Lead citrate; calibration lines indicate 1 μ).









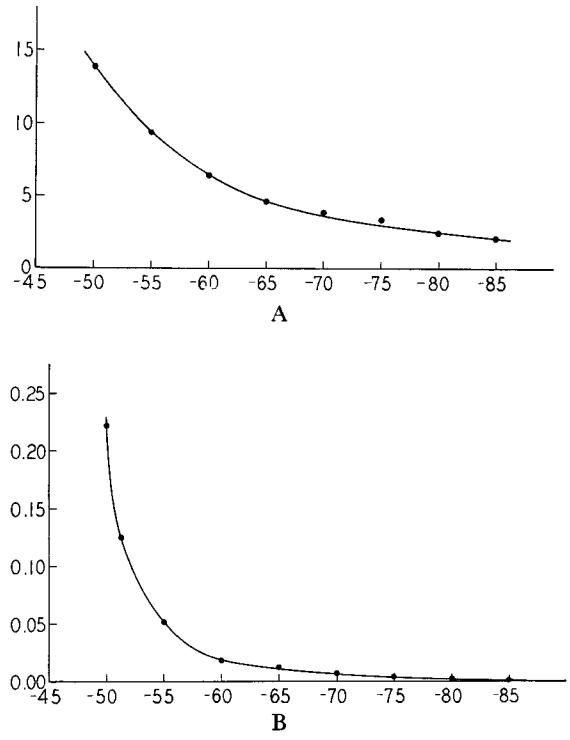


FIGURE 18 *A*, The water content (volume per cent) of acetone in equilibrium with ice is plotted on the ordinate; the temperature on the abscissa. *B*, The solubility of NaCl (weight per 100 cc) in acetone in equilibrium with ice is plotted on the ordinate; the temperature on the abscissa.

bedding may well have displaced such apparently loosely connected cellular elements as the axons seem to be. In asphyxiated tissue, the spaces between the tissue elements, including those in the axon fields are narrow, often resulting in tight junctions, as has been observed after fixation by perfusion with glutaraldehyde and formaldehyde (16).

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(*References start on p. 136*)

FIGURES 15 to 17 Molecular layer frozen within 30 seconds of decapitation shown at high magnification. Fig. 15 shows a dendrite (*d*) running from left to right containing two long mitochondria. There is ample extracellular space present in the axon fields (*a*). Fig. 16 shows an axon field immediately underneath the end-foot of a Bergmann fiber (*b*). The preparation shown in Fig. 17 perhaps contains minute ice crystals. The membranes between the cellular elements show tight junctions (arrows) at many places. (Fig. 15, lead citrate; calibration line indicates 1 μ. Fig. 16, uranyl acetate and lead; calibration line indicates 0.1 μ. Fig. 17, lead citrate; calibration line indicates 0.1 μ).

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