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DEMONSTRATION OF EXTRACELLULAR SPACE BY FREEZE-DRYING IN THE CEREBELLAR MOLECULAR LAYER

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SUMMARY

In electron micrographs of the molecular layer of the mouse cerebellum frozen within 30 sec of circulatory arrest and subsequently dried at −79 °C an appreciable extracellular space was found between the axons of the granular cells. Tight junctions were regularly observed between pre- and postsynaptic structures and the enveloping glia cells. In micrographs of cerebellum frozen 8 min after decapitation the space between the axons was absent and tight junctions between the nerve fibres were almost exclusively encountered. The extracellular space of asphyxiated and non-asphyxiated tissue in electron micrographs of frozen-dried material is similar to the space in comparable tissues treated by freeze-substitution. These observations suggest that there is an appreciable amount of extracellular material in oxygenated, living tissue which is taken up by cellular elements during asphyxiation.

INTRODUCTION

In a previous communication (Van Harreveld, Crowell & Malhotra, 1965) electron micrographs of the cerebellar molecular layer of the mouse prepared by rapid freezing of the tissue followed by substitution in acetone (containing 2% OsO_4) at −85 °C have been described. In micrographs of the cerebellum frozen within 30 sec of circulatory arrest (decapitation) relatively large areas of extracellular space were seen, dispersed amongst axons of granular cells. When the tissue was frozen 8 min after circulatory arrest, extracellular space was generally absent, and tight junctions were often observed between the plasma membranes of adjacent axons.

During the first few minutes of asphyxiation a marked increase in the impedance of the cerebellar cortex develops (Van Harreveld, 1961). A large part of the measuring current in the cerebellum, as in other tissues, can be expected to be carried by extracellular electrolytes, since the intracellular ions are enveloped by cell membranes which in general impede ion movements (see Cole, 1940; Van Harreveld, 1962; Van Harreveld & Biber, 1962; Van Harreveld & Ochs, 1956). The asphyxial impedance increase has therefore been interpreted as the expression of a movement into cellular elements of extracellular sodium chloride accompanied by water to maintain osmotic equilibrium. This postulate is supported by light-microscope observations of an asphyxial water and chloride transport into fibres of Bergmann and dendrites of Purkinje cells (Van Harreveld, 1961). The findings in electron micrographs of freeze-substituted material mentioned above are in agreement with this concept. It was recognized, however, that the extracellular space in the freeze-substituted cerebellum

may not be a faithful representation of the water distribution in the living tissue, since differences in the concentration of tissue compounds soluble in acetone may cause osmotic adjustments between the extra- and intracellular compartments during substitution. It was therefore considered worth while to preclude the latter possibility by freeze-drying the tissue, and to compare the resulting electron micrographs with those obtained by freeze-substitution.

METHODS

The cerebellum of mice was frozen as described previously (Van Harreveld & Crowell, 1964; Van Harreveld *et al.*, 1965), by bringing the tissue into contact with a highly polished silver surface cooled to about −207 °C and protected from condensation of water, CO_2 and air by dry helium gas. To minimize mechanical disturbance, the exposed cerebellum was left *in situ* and the isolated head of the mouse was lowered on to the metal surface in such a position that the vermis was parallel to the freezing surface. After freezing, the vermis, recognizable by its gyrations, was isolated under liquid nitrogen with the use of a small electric circular saw. To prevent heating, the small block of tissue (about 0·05 cm^3) was wrapped under liquid nitrogen in tissue paper, and quickly transferred to the drying apparatus.

The apparatus used for freeze-drying is schematized in Fig. 1. It consisted of a U-shaped tube about 3 cm wide. The leg *A* in which the water vapour was condensed was kept in a Dewar vessel filled with liquid nitrogen. The leg *B* ended in a ground joint which fitted the vessel *C*. The latter was half-filled with liquid nitrogen before the tissue was transferred to it. The block was positioned in the liquid nitrogen so that the surface of the vermis faced upwards. The vessel *C* and the leg *B* were then placed in a second Dewar vessel filled with solid CO_2 and ethanol. After evaporation of the liquid nitrogen in *C*, the apparatus was evacuated to a pressure of about 10^{-3} mm, using a mercury diffusion pump backed by a mechanical vacuum pump.

The method described above differs from those more commonly employed where the surface on which the water vapour is condensed is brought in proximity to the tissue (see Hanzon & Hermodsson, 1960; Sjöstrand & Baker, 1958). The blocks of tissue used in the present experiments were relatively large. It was therefore considered advantageous to dry the tissue surface suitable for electron microscopy at −79 °C (solid CO_2 temperature), and then speed up the drying of the deeper parts by raising the temperature in controlled steps. The advantage of a short path between tissue and condensation surface is given up in the arrangement used, which, however, allows good control of the temperature of the tissue to be dehydrated in a simple way. The tissue was dried for 7 days at −79 °C. To examine the thickness of the tissue layer dried during this period, some blocks were transferred under liquid nitrogen to isopentane cooled to the temperature of solid CO_2. The dried layer was then rubbed off from the hard, frozen deeper parts with a wad of cotton. Light-microscope preparations from this material showed that the molecular layer and part of the granular layer (at least 200 μ) had been removed. Since only the superficial 10–15 μ of the tissue is usually free of obvious ice crystals (Van Harreveld & Crowell, 1964; Van

Harreveld *et al.*, 1965) and therefore yields acceptable electron micrographs, this period of drying at low temperature seemed ample. The tissue was subsequently dried for 2 days at −25 °C, followed by 2 days at −5 to −10 °C. It was then kept for 1 day in vacuum at room temperature.

After breaking the vacuum the dried tissue was treated for 24 h with osmium tetroxide vapour in vessel *C* closed with the stopper *D*, and then impregnated with propylene oxide. This compound was put in leg *A* of the tube shown in Fig. 1 and

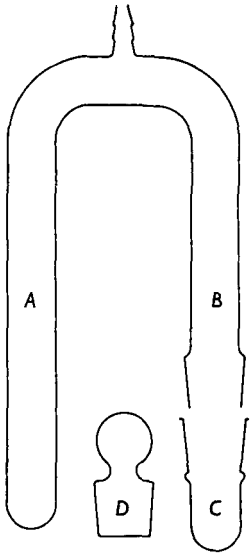

Fig. 1. Apparatus used for drying. See text for explanation.

frozen by immersion in liquid nitrogen. The vessel *C* containing the tissue was sealed to leg *B*. The apparatus was evacuated and after warming to room temperature the propylene oxide was made to flow over into vessel *C*, after which the vacuum was broken.

The tissue was subsequently embedded in Maraglas (Freeman & Spurlock, 1962) and sectioned on an LKB Ultrotome. The sections were studied with a Philips EM 200 electron microscope after staining with lead citrate (Reynolds, 1963).

RESULTS

All the sections have been cut at right angles to the folia so that they are in the plane of the dendritic arborization of the Purkinje cells. The fibres of Bergmann are sectioned longitudinally, and the axons of the granular cells transversely, in this plane. The identification of various cellular elements in the electron micrographs is facilitated by the criteria used by Gray (1961), Palay, McGee-Russell, Gordon & Grillo (1962) and Schultz (1964). Blocks of tissue of sixteen mice were examined.

The quality of preservation in electron micrographs of frozen-dried cerebellar molecular layer is in many respects comparable with that in freeze-substituted material. The plasma membranes are generally continuous and clearly show their tripartite

unit membrane structure in regions where the membranes of two cellular elements do not form tight junctions. The dense line on the cytoplasmic side of the unit membrane is more opaque and wider than the dense line on the outside which makes the unit membrane look asymmetrical (Fig. 7). The whole tripartite structure is about 100 Å wide. The matrix of the mitochondria is extremely dense and their membranous system is therefore not easily discerned. In favourable locations it was noted, however, that no distinct cleft was present between the outer and inner limiting membranes or in the cristae; similar observations have been made in freeze-substituted tissues (Malhotra & Van Harreveld, 1965). The two apposing membranes thus form a five-layered tight junction. A somewhat similar appearance of the mitochondrial membranous system has been described in frozen-dried pancreatic exocrine cells (Sjöstrand & Elfvin, 1964). The synaptic vesicles are also dense in electron micrographs of frozen-dried cerebellum. The ground cytoplasm usually has a frothy appearance, with irregular dispersal of clumps of dense amorphous material.

The electron micrographs shown were produced from cerebellar cortex frozen either within 30 sec after circulatory arrest or from cortex whose exposure and freezing had been delayed for 8 min after decapitation. There were no other differences in the preparative procedures used. Figs. 4, 5 and 8 show superficial parts of the molecular layer of the vermis frozen 8 min after decapitation. The plasma membranes of adjacent nerve fibres in the axon fields form tight junctions, 180–240 Å wide, over most of their surfaces. Hardly any extracellular space is present, except at a few places where three cellular elements abut against each other. At such locations small, often triangular, areas of extracellular space are sometimes seen (arrows in Fig. 4). Tight junctions are also found between plasma membranes of axons and glial processes, dendrites and glia, and between glial elements themselves, resulting in an extreme paucity of extracellular space. Karlsson & Schultz (1965) have recorded a similar abundance of tight junctions in electron micrographs of mammalian central nervous tissue prepared by perfusion with formaldehyde or glutaraldehyde. Judging from the electron density of the ground cytoplasm, it appears that the glial processes are swollen in micrographs of cerebellar cortex frozen 8 min after decapitation (Figs. 4, 5). Also dendrites often look swollen.

Figures 2, 3, 6 and 7 show superficial parts of the molecular layer of the vermis frozen within 30 sec after decapitation. A comparison with electron micrographs of 8-min asphyxiated cerebellum reveals a striking difference in the magnitude of the extracellular space in the axon fields. The nerve fibres in these fields are separated from each other by areas of extracellular space which are sometimes irregular (Fig. 6), but usually the space is rather evenly distributed between the fibres (Figs. 2, 3). Fibres may make contact over small areas where their plasma membranes form short tight junctions (Fig. 7). The plasma membranes of pre- and postsynaptic structures and of the enclosing glia element are usually closely apposed, showing the five-layered tight junction structure (Figs. 2, 3, 6). Consistently triangular spaces were found on both sides of the synaptic membranes between the pre- and postsynaptic structures and the enclosing glia (Fig. 6). The synaptic cleft was about 150 Å wide in frozen-dried tissue and often showed irregularly dispersed electron-dense material in it

(Fig. 6). The glia in the non-asphyxiated preparations does not have the swollen appearance which is characteristic of the micrographs of tissue asphyxiated for 8 min.

The differences in the extracellular space found consistently in the present electron micrographs of the molecular layer frozen 30 sec and 8 min after circulatory arrest are very similar to those observed previously in cerebellar material subjected to freeze-substitution (Van Harreveld *et al.*, 1965). The magnitude of the extracellular space in the axon field of frozen-dried non-asphyxiated tissue is not obviously different from that in freeze-substituted cerebellum.

DISCUSSION

The main difference between freeze-substituted and frozen-dried cerebellum is the frothy appearance of the ground cytoplasm in the frozen-dried material which has also been observed by several other workers (see Durning, 1958; Finck, 1958; Gersh, 1964; Seno & Yoshizawa, 1960) in various similarly treated tissues. But for a greater contrast, the cytoplasmic matrix in the present electron micrographs of the cerebellum resembles that shown by Elfvin (1963) in frozen-dried peripheral nerve. There is in the present micrographs no evidence of the rather sharply outlined ice-crystal artifacts described in frozen-dried material (Hanzon & Hermodsson, 1960; Sjöstrand & Baker, 1958). Furthermore, the methods of freezing and study of the superficial 10 μ of the molecular layer in frozen-dried cerebellum were the same as for freeze-substituted material, which in general did not show any obvious ice crystals (Van Harreveld *et al.*, 1965). Hanzon & Hermodsson (1960) discussed in some detail artifacts which may develop during freeze-drying. They suggested that large surface-tension forces acting during impregnation on the fine structure of the fragile specimen may cause artifacts. These could be responsible for the frothy appearance of the cytoplasm and for occasional breaks in the membrane (Figs. 5, 8).

The asphyxiated and non-asphyxiated cerebella were treated exactly alike but for the difference in the interval between decapitation and freezing. It would for this reason seem likely that the difference in magnitude of extracellular space in the asphyxiated and non-asphyxiated cerebellum is due to a difference in the state of the tissue at the moment of freezing. These observations therefore support the concept, based on physiological findings, that there is present in the normal cerebellar cortex an appreciable amount of extracellular material which is taken up by cellular elements during asphyxiation.

Tight junctions were often found between glia elements and presynaptic structures and between glia and postsynaptic structures in non-asphyxiated freeze-substituted cerebellum. More commonly, however, narrow clefts were present between these tissue elements. In the frozen-dried non-asphyxiated material tight junctions between glia and nervous elements were the usual finding. Although by freeze-drying, in contrast to freeze-substitution, the contact of the tissue with acetone was eliminated, it still had to be impregnated with propylene oxide and Maraglas. During this phase of the tissue preparation osmotic adjustments between extra- and intracellular space would seem possible due to differences in concentration of tissue constituents soluble

in these fluids. Some doubt therefore still remains about the exact representation in the present electron micrographs of the fluid distribution in normal tissue.

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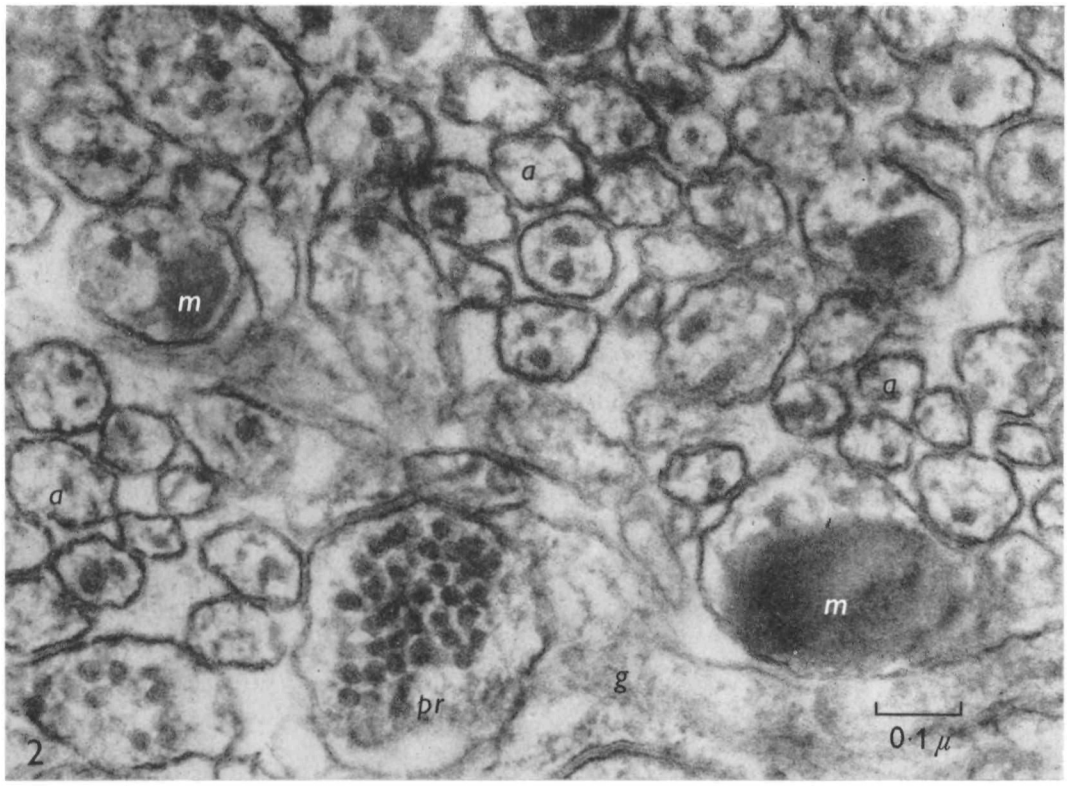

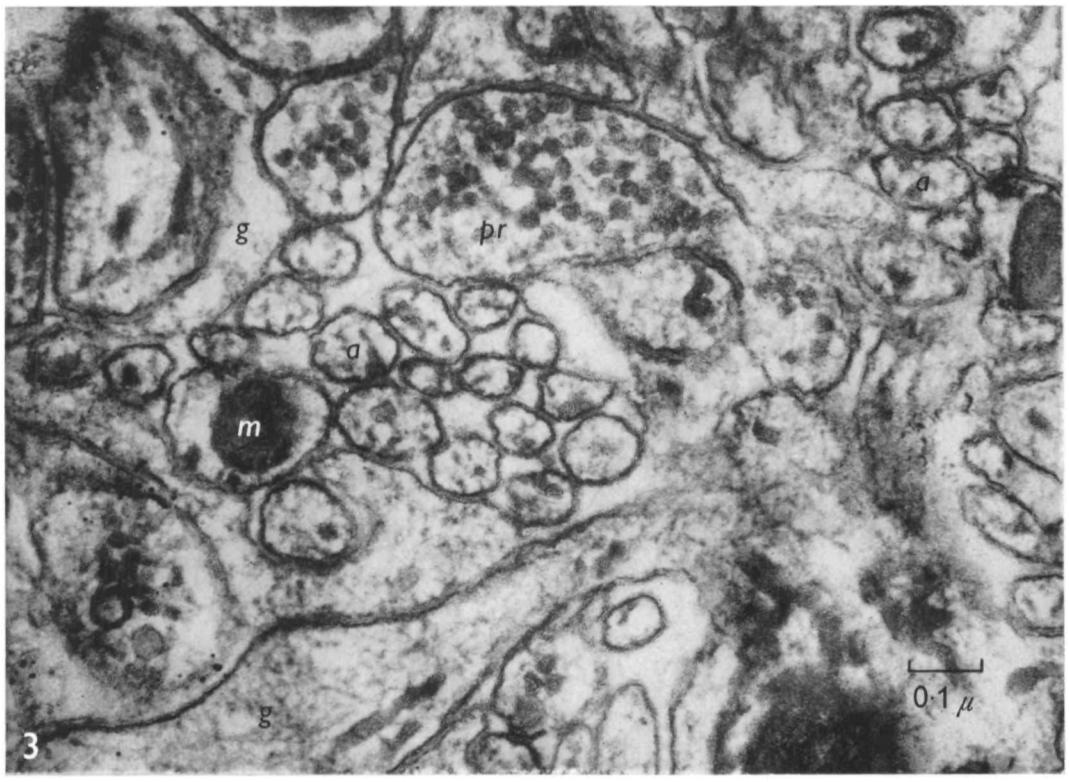

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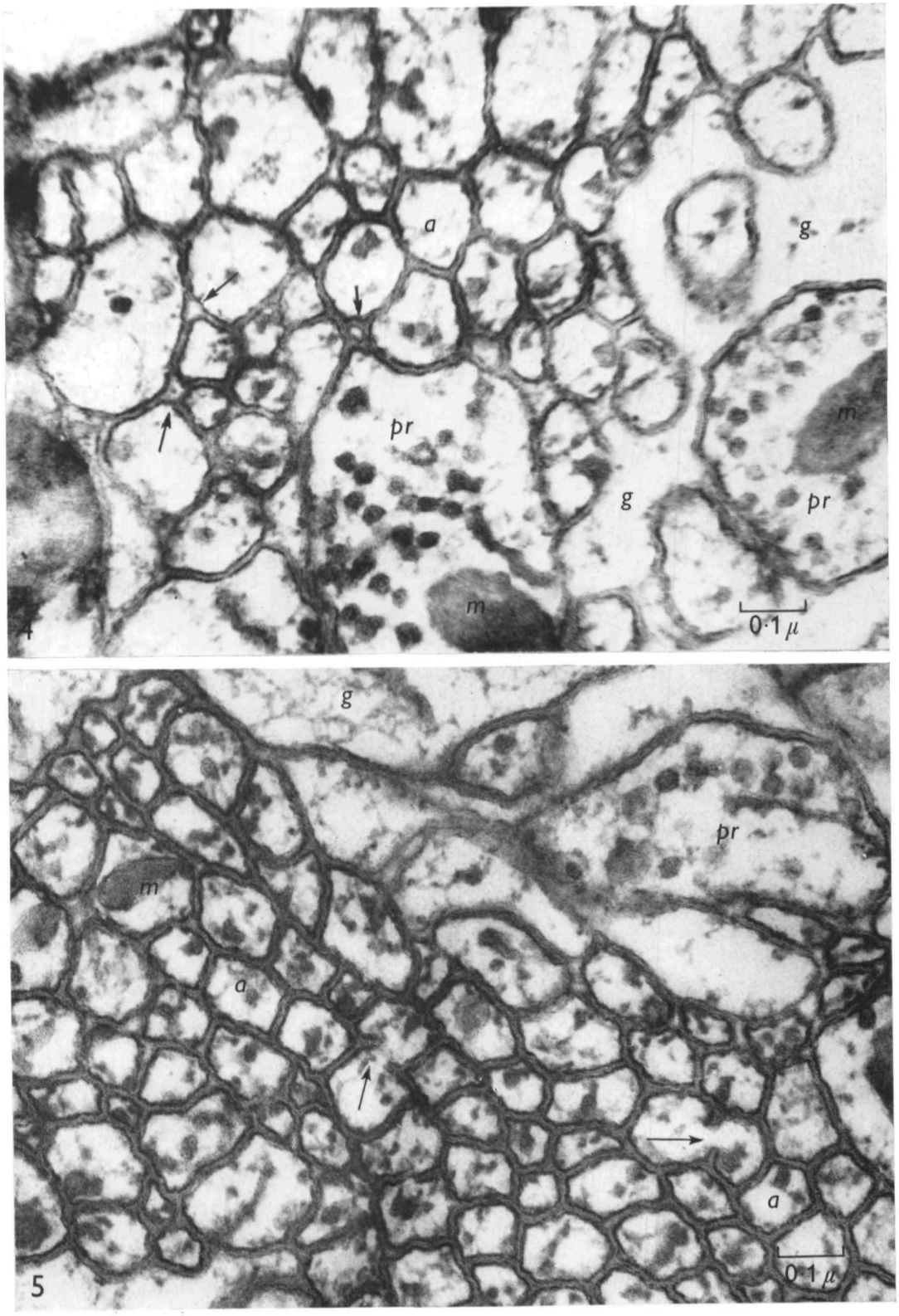
Figs. 2, 3. Micrographs of the molecular layer of the cerebellar cortex frozen within 30 sec of circulatory arrest. Note appreciable areas of extracellular space in the axon fields (*a*). Tight junctions are prevalent between glia and presynaptic endings characterized by synaptic vesicles, and between dendrites and glia. Mitochondria (*m*) are seen as dense bodies of which the structure is not easily discerned. Presynaptic endings (*pr*) and glia (*g*) can be identified.

Figs. 4, 5. Micrographs of the molecular layer of the cerebellar cortex frozen 8 min after circulatory arrest. Note the extreme paucity of extracellular space in the axon fields (*a*). Where three cellular elements meet, small triangular spaces (arrows in Fig. 4) are often seen. Arrows in Fig. 5 show breaks in the membrane. Presynaptic endings (*pr*) and mitochondria (*m*) can be identified. The structures labelled (*g*) are probably glial processes.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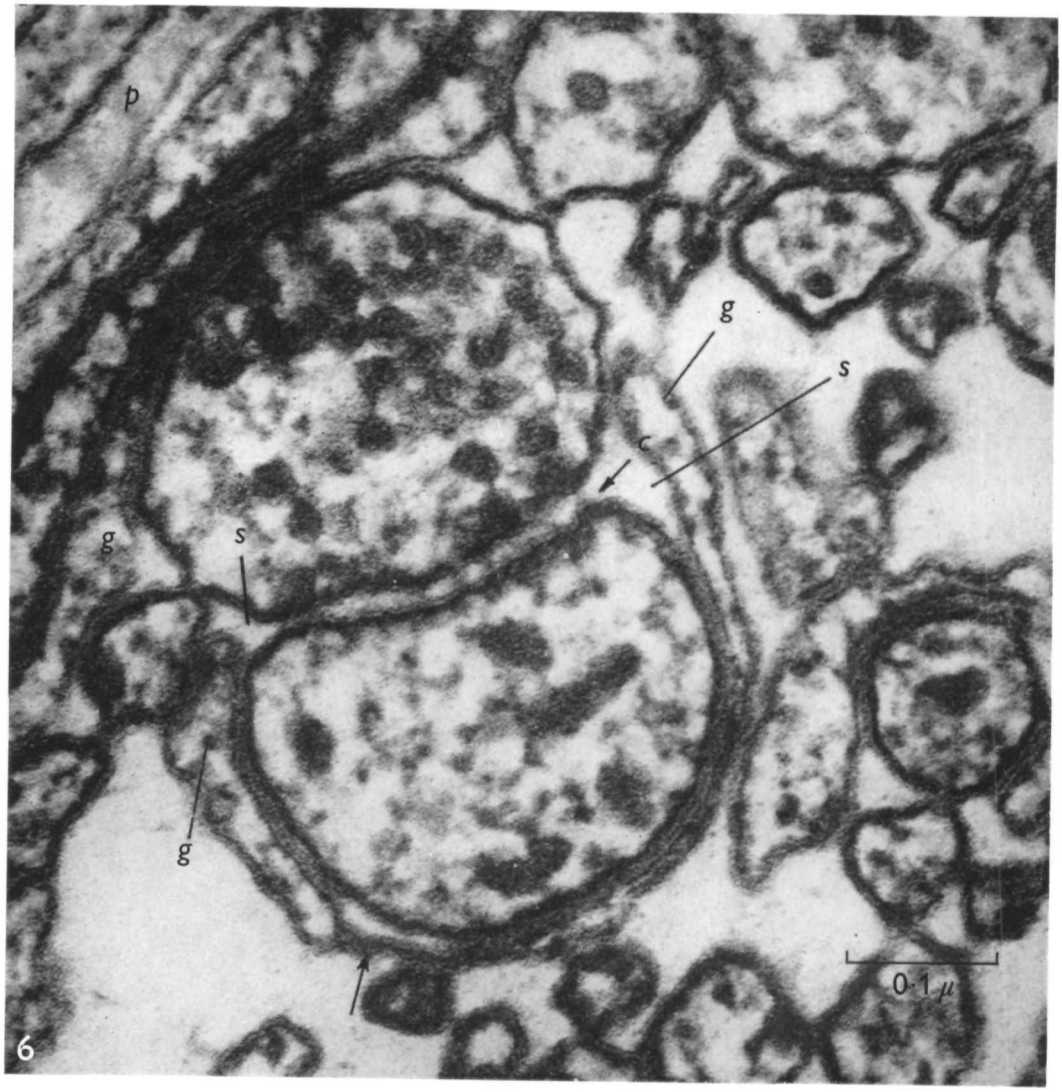

Fig. 6. A synapse lying directly under the pia (*p*). The enclosing glia (*g*) forms tight junctions with the pre- and postsynaptic structures, except at one place (arrow) where a cleft between glia and postsynaptic structure is present. The synaptic cleft (*c*) which contains some material is continuous on both sides with triangular spaces (*s*).

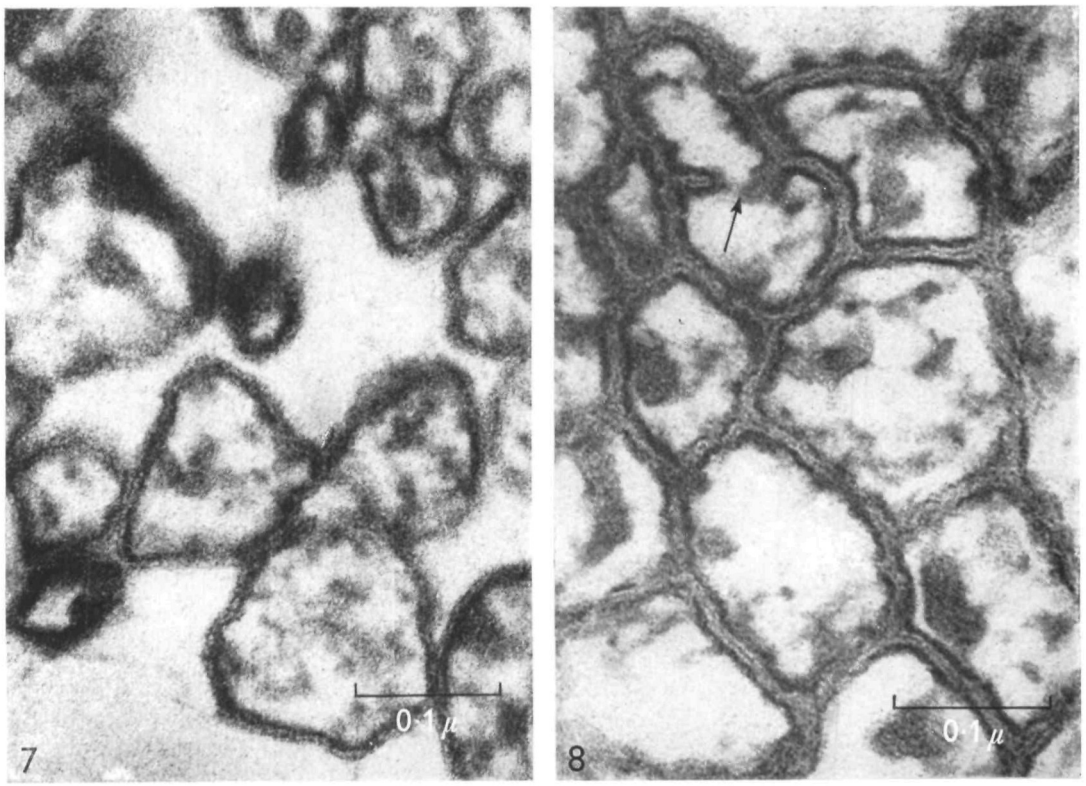

Fig. 7. A magnified view of an axon field from cerebellar cortex frozen within 30 sec after circulatory arrest. Note the abundance of extracellular space. The plasma membrane shows an asymmetric unit membrane structure, except at places where axons touch each other and a tight junction is found over a short distance.

Fig. 8. A magnified view of an axon field from cerebellar cortex frozen 8 min after circulatory arrest. There is hardly any extracellular space present and the axons touch each other to form five-layered tight junctions over large distances. Arrow shows a break in the membrane.