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Extracellular space in the cerebral cortex of the mouse

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INTRODUCTION

An appreciable extracellular space has been demonstrated in electron micrographs of the molecular layer of the cerebellar cortex of mice prepared by freeze-substitution, a method of fixation designed to preserve the water distribution in the tissue more faithfully than can be achieved by immersion or perfusion fixation (Van Harreveld, Crowell & Malhotra, 1965). The tissue for these preparations had been frozen within 30 s of circulatory arrest. In micrographs of cerebellum which had been asphyxiated for 8 min before freezing the extracellular material had almost completely disappeared. This finding is in agreement with the assumption that a loss of extracellular material occurs during oxygen deprivation, which was first postulated from a marked asphyxial impedance increase of the cortex (Van Harreveld & Ochs, 1956). Since the measuring current in a tissue is mainly carried by extracellular ions a marked increase in impedance would indicate a loss of electrolytes from the extracellular compartment. It was suggested that these electrolytes accompanied by water to maintain osmotic equilibrium are taken up by cellular elements. This concept was supported by the demonstration of an asphyxial transport of water and chloride into dendrites of Purkinje cells and into (glial) fibres of Bergmann (Van Harreveld, 1961). The cerebellar cortex was chosen for the investigation with the electron microscope because spreading depression which causes a similar impedance increase (Leão & Ferreira, 1953; Freygang & Landau, 1955; Van Harreveld & Ochs, 1957) and changes in chloride and water distribution (Van Harreveld, 1958; Van Harreveld & Schadé, 1959) as asphyxiation, is not readily elicited in the cerebellum (Fifková *et al.* 1961; Rhoton, Goldring & O'Leary, 1960; Van Harreveld, 1961).

In the present investigation the freeze-substituted cerebral cortex of mice was examined with the electron microscope. Since asphyxiation causes a marked impedance increase in the cerebral cortex (Leão & Ferreira, 1953; Van Harreveld & Ochs, 1956) and a transport of water (Van Harreveld, 1957) and chloride (Van Harreveld & Schadé, 1959) into cellular elements (apical dendrites) similar to that observed in the cerebellar tissue, micrographs of cerebral cortices frozen shortly after circulatory arrest and of asphyxiated tissue were compared. The cerebral cortex is sensitive to mechanical disturbances and may respond to such stimuli with spreading depression. Ways were therefore sought to counteract the effects of the procedures necessary to expose the cortex which tend to result in mechanical stimulation.

METHODS

Cortical impedance was measured at 1000 cyc./s using silver-silver chloride electrodes placed a few millimetres apart on the cortical surface with the bridge set-up described previously (Van Harreveld & Ochs, 1956). Slow potential changes, led off between a silver-silver chloride electrode placed on the cortical surface and a subcutaneous indifferent electrode, were recorded with a Grass polygraph.

The method of freezing on a silver block cooled with liquid nitrogen at reduced pressure to a temperature below −200 °C was described previously (Van Harreveld & Crowell, 1964; Van Harreveld *et al.* 1965). The head of a mouse was fixed on a wooden stick which could be clipped to the carrier system used to bring the tissue in contact with the cold metal surface. A brass strip fastened to the stick was introduced into the mouth of the anesthetized (urethane) and tracheotomized mouse. A ligature, circling the head and passing under the zygomatic arch, closed the jaws tightly on the metal strip which was positioned in such a way that the exposed cortex of one hemisphere contacted the cold metal when the preparation was lowered on the freezing surface. The cortex was exposed by removing a large part of the calvarium on one side by cutting the bone with a high-speed dental drill. To prevent injury to the brain it was attempted not to cut entirely through the skull, but to weaken the bone to such extent that a bone plate could easily be lifted off. The dura remained intact, and had to be removed with fine jeweller's forceps under a binocular microscope. All these preparations were carried out while the respiration and circulation were intact. When the moment of freezing had arrived the head was cut off, and the wooden stick was clipped on the carrier which was then lowered on the freezing surface. The unavoidable period of circulatory arrest between decapitation and freezing was between 20 and 30 s.

The frozen tissue was then subjected to substitution fixation at −85 °C in a 2% osmium tetroxide solution in acetone. After 2 d the head was warmed to room temperature in 3 steps (−25 °C, 4 °C and room temperature). Blocks of cortical tissue were cut and kept for 2–3 h in repeatedly changed acetone, followed by propylene oxide. They were finally embedded in Maraglass (Freeman & Spurlock, 1962). Sections about 1 μ thick, cut on an LKB Ultrotome, were stained with methylene blue and Azure II (Richardson, Jarett & Finke, 1960) for light microscope study. Thin sections for electron microscopy were stained with lead citrate (Reynolds, 1963). Tissue blocks of about 40 mice were used in the investigation.

RESULTS

Asphyxial impedance changes and asphyxial potentials. The impedance of the cerebral cortex of the mouse measured at 1000 cyc/s shows a characteristic increase after circulatory arrest. Figure 1 shows these changes plotted as the reciprocal of the impedance (conductivity). During the first 2 min the loss in conductivity is slight, and may be caused, at least in part, by a loss of blood of relatively high conductivity from the cortex. Then there is a precipitous drop in which the cortex loses almost one-third of its conductivity in about 1 min, followed by a more gradual decline. These asphyxial changes are very similar to those observed in other mammals (Van Harreveld &

Ochs, 1956; Van Harreveld, 1957). The latency of the rapid drop is shorter (1–2 min) than in rabbits (2–3 min). The large drop in conductivity would indicate that, in the mouse as in other mammals, an asphyxial transport of extracellular material into the cellular compartment of the cortex occurs 1 to 2 min after start of oxygen deprivation (Van Harreveld, 1957; Van Harreveld & Schadé, 1959).

A negative shift of the cortical surface with respect to an indifferent electrode was recorded after circulatory arrest in the mouse (Fig. 2), which resembles the asphyxial potentials described in the rabbit (Leão, 1947, 1951). The latency varied between 1 and 2 min; the potential seemed to coincide with the rapid impedance increase (Van Harreveld, 1957).

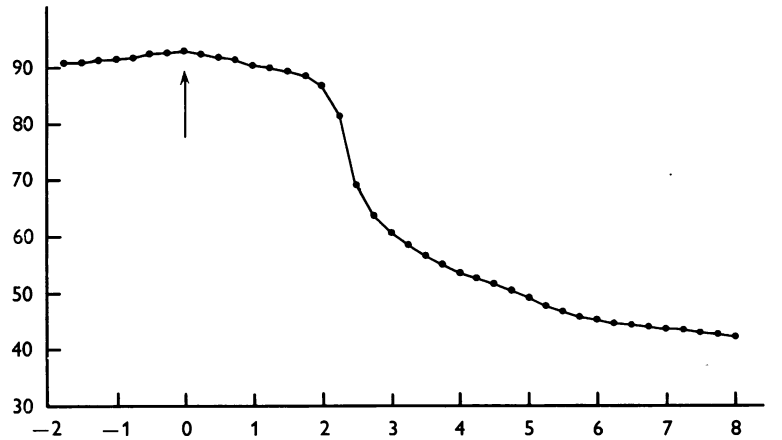


Fig. 1. Conductivity changes of the mouse cerebral cortex during asphyxiation. At the arrow the aorta is severed. On the abscissa the conductivity is plotted (arbitrary unit); on the ordinate time min.

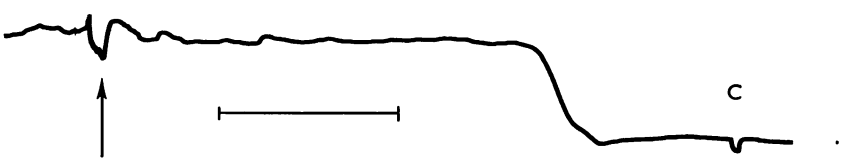
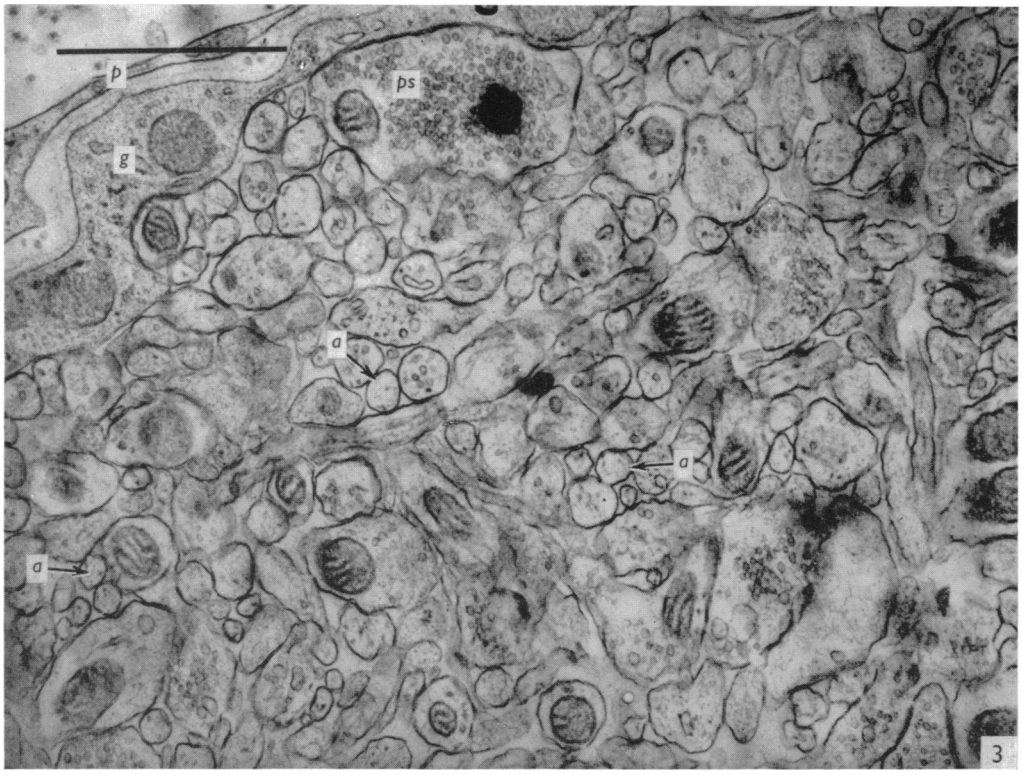


Fig. 2. Asphyxial potential, led off from the cortical surface with respect to an indifferent electrode. The aorta is severed at the arrow. The horizontal calibration line indicates 1 min, at C a 2 mV calibration.

In eight mice prepared for slow potential recording no potential changes indicating the presence of spreading depression were noted for 10 min after exposure of the cortex. After that time a spreading depression could be elicited by stimulating the cortex with a cotton pledget moistened with 20 % KCl solution. These observations indicate that the cerebral cortex of the mouse is not prone to produce spontaneous spreading depressions, but can do so on stimulation.

Electron micrography of oxygenated and asphyxiated cortex. The method of freeze-substitution has the limitation that only the surface of the tissue layer is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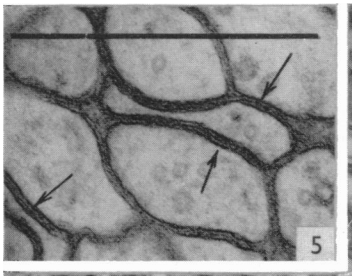
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frozen so rapidly that no visible ice crystals are formed (Van Harreveld & Crowell, 1964; Van Harreveld *et al.* 1965). Even in a small animal such as the mouse an appreciable part of this tissue layer consists of the pia-glial membrane. Therefore not more than the superficial 10 μ to 15 μ of the nervous tissue proper yields electron micrographs in which the tissue structure is not distorted by ice crystals. All the micrographs discussed in the present study depict for this reason the very surface of the cortical molecular layer. The preservation of the tissue elements in this layer is generally satisfactory. The membranes are intact and continuous, cellular organelles such as mitochondria, endoplasmic reticulum and synaptic vesicles are well preserved (Van Harreveld *et al.* 1965). The fine structure of the superficial cortical neuropil has been described by Pappas & Purpura (1961).

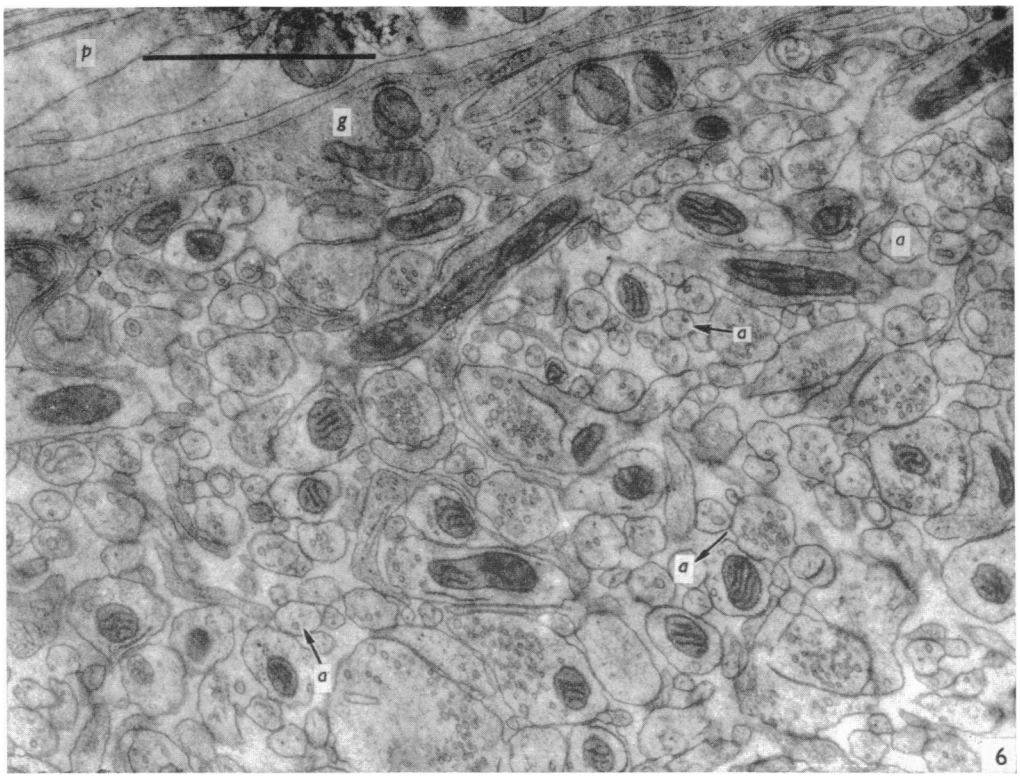
Figure 4 shows a micrograph of cortex frozen 6 min after decapitation. The calvarium and dura over the hemisphere were removed just before freezing. The micrographs of such tissues are characterized by an extreme paucity of extracellular material. Small triangular extracellular spaces are sometimes found where three cellular elements meet, but in most places the cell membranes are closely apposed, forming five-layered tight junctions consisting of two layers of electron transparent material sandwiched between three electron dense layers (Fig. 5). The identification of cellular elements in this region of the cortex is difficult. In addition to the pia and pia-glial membrane, presynaptic structures can be identified by the synaptic vesicles. The smallest profiles may be those of non-myelinated axons. The larger structures which cannot be identified as presynaptic endings may be dendrites, dendritic spine or glia.

The micrographs of asphyxiated tissue were compared with those of preparations decapitated immediately after exposure of the cortex and frozen within 30 s of circulatory arrest. No asphyxial impedance increase can be expected to have developed during this short period of asphyxiation. Figure 3 shows a micrograph obtained from this material. The same cellular elements are observed as found in Fig. 4. However, there is present in this micrograph an appreciable extracellular space. This space is found especially between the small profiles (non-myelinated axons), a distribution of extracellular material which was also observed in the cerebellum (Van Harreveld *et al.* 1965) and in the white matter (Malhotra & Van Harreveld. 1966). Not all cortices showed this abundance of extracellular material. In some micrographs there was considerably less extracellular space than in Fig. 3 but a paucity of extracellular material as seen in asphyxiated cortices (Fig. 4) was never observed.

The various structures present in micrographs of asphyxiated cortex (Fig. 4) show marked differences in electron density. The small profiles and presynaptic endings are in general rather dense, whereas other elements are more transparent. This may be due to a relative paucity of intracellular inclusions in these structures in addition to a greater transparency of the matrix. In the oxygenated cortex (Fig. 3) the electron

Fig. 3. Oxygenated cerebral cortex frozen within 30 s after exposure. *a*, non-myelinated axons; *d*, dendrite; *g*, glia; *p*, pia; *ps*, presynaptic ending. The calibration line in all figures indicates 1 μm.

Figs. 4, 5. Cerebral cortex asphyxiated for 6 min *in situ*, frozen shortly after exposure. Arrows in Fig. 5 indicate 5-layered tight junctions.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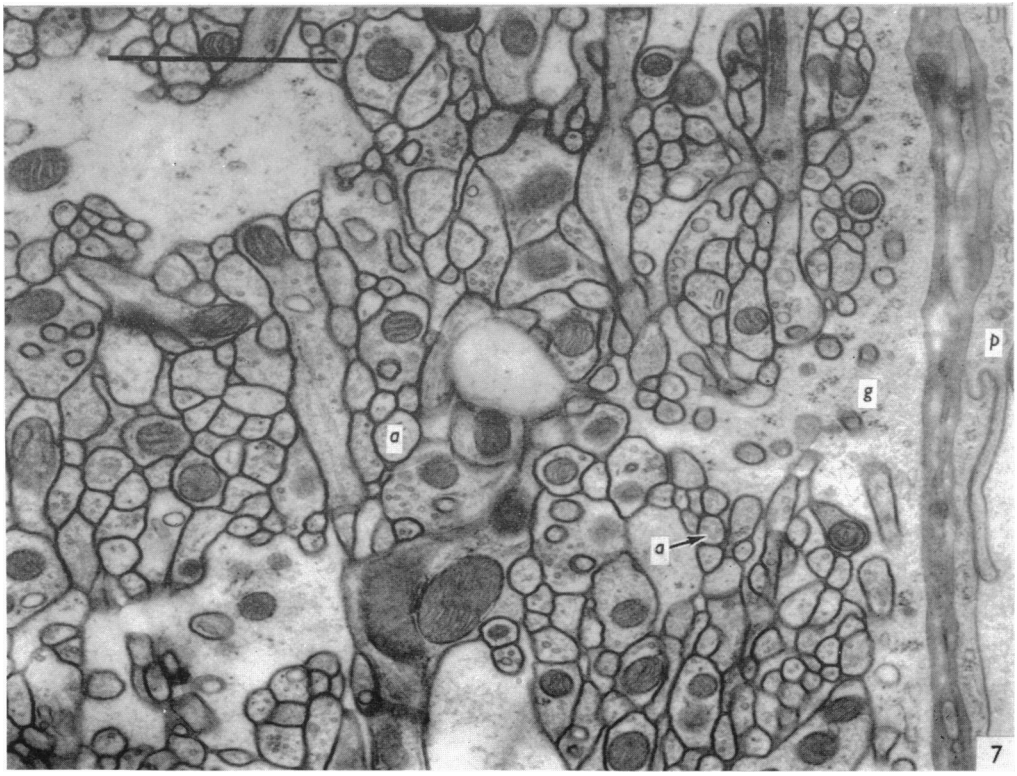
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density is more uniform. This would suggest that the transparent elements in asphyxiated cortex (which often also seem larger) are swollen. This and the loss of extracellular material in asphyxiated cortex are in good agreement with the asphyxial transport of extracellular material into certain cellular elements (dendrites) observed with the light microscope (Van Harreveld, 1957; Van Harreveld & Schadé, 1959).

The exposure of the cortex cannot in general be accomplished without stimulation and damage of the tissue. The cut in the bone had to be as deep as possible to facilitate the removal of the bone plate. This may result in small injuries to the cortical tissue. Furthermore, the removal of the plate causes consistently mechanical disturbances of the cortex. Removal of the dura is always accompanied by transient deformations of the cortex. Finally the dura can rarely be removed without minor injuries to the underlying tissue. Even when the cortex appears to be in an optimal condition it thus has been exposed to rather severe mechanical stimulation. Such stimulation may cause spreading depression, but even if the threshold for this phenomenon is not reached, changes in the electrolyte and water distribution of the tissue might well be produced. The varying degree of mechanical stimulation may therefore be the cause of the observed differences in abundance of extracellular material in cortices frozen immediately after their exposure.

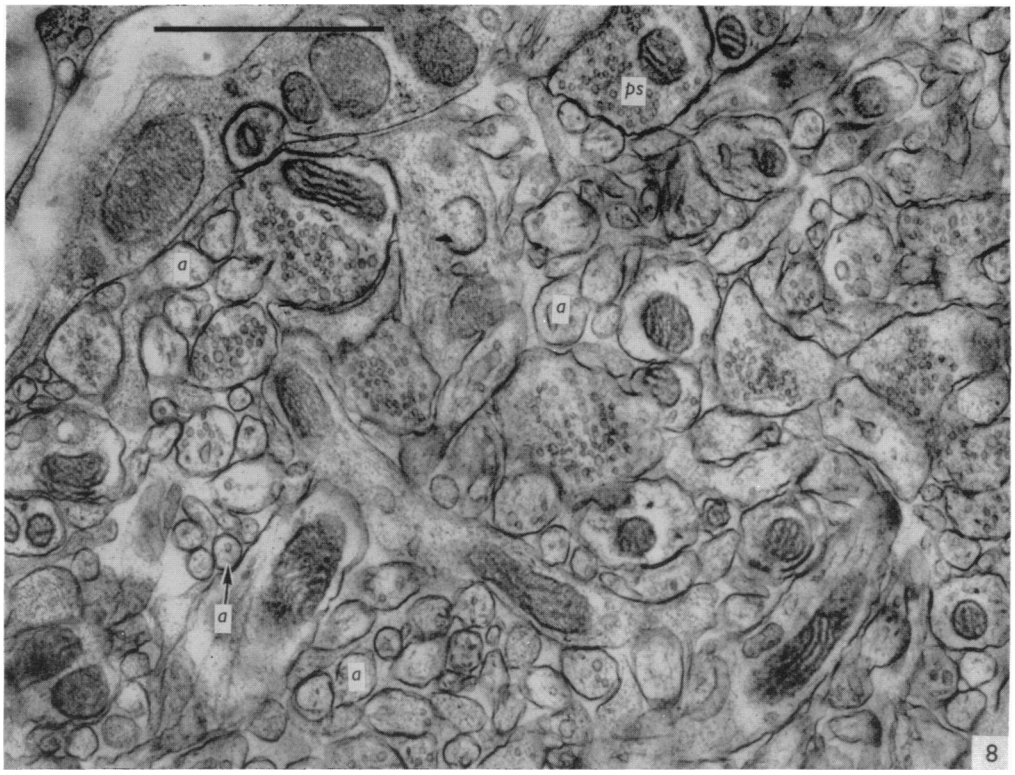
Since the effects of mechanical stimulation of central nervous tissue are reversible, when not too severe, it might be possible to restore a more normal water distribution in the tissue by freezing after a period of recovery. To investigate this possibility, a small cover adapted to the shape of the skull and lined on the inside with filter paper wetted with Ringer's solution was placed on the skull immediately after exposure of the cortex. The cover which formed a moist chamber prevented evaporation from the cortex. After a period of 5 min the chamber was removed and the cortex was frozen. Electron micrographs from this material invariably showed an appreciable extracellular space as shown in Fig. 6. Again the extracellular material is present mainly between the small profiles which may represent non-myelinated axons. Between other cellular elements (for example glia and presynaptic structures) narrow slits are present.

Electron micrographs of cerebral cortex treated in the way described above, but asphyxiated by arresting the circulation while the cortex was kept in the moist chamber for 5 min showed an almost complete absence of extracellular material (Fig. 7). The electron density of the various structures in these micrographs is quite unequal. Again the small profiles and presynaptic endings are in general dense as contrasted with large apparently swollen structures of greater transparency. The micrographs of oxygenated cortex (Fig. 6) show a much more uniform electron density.

In other experiments Ringer's solution at body temperature was made to flow over the cortex for 5–6 min before freezing; again providing for a period during which the cortex can recover from mechanical stimulation. Both normal and Ringer's solution with about half the K concentration (as in the cerebrospinal fluid) were used.

Fig. 6 Oxygenated cortex frozen after having been kept for 5 min after exposure in a moist chamber.

Fig. 7. Cerebral cortex asphyxiated for 5 min while in the moist chamber.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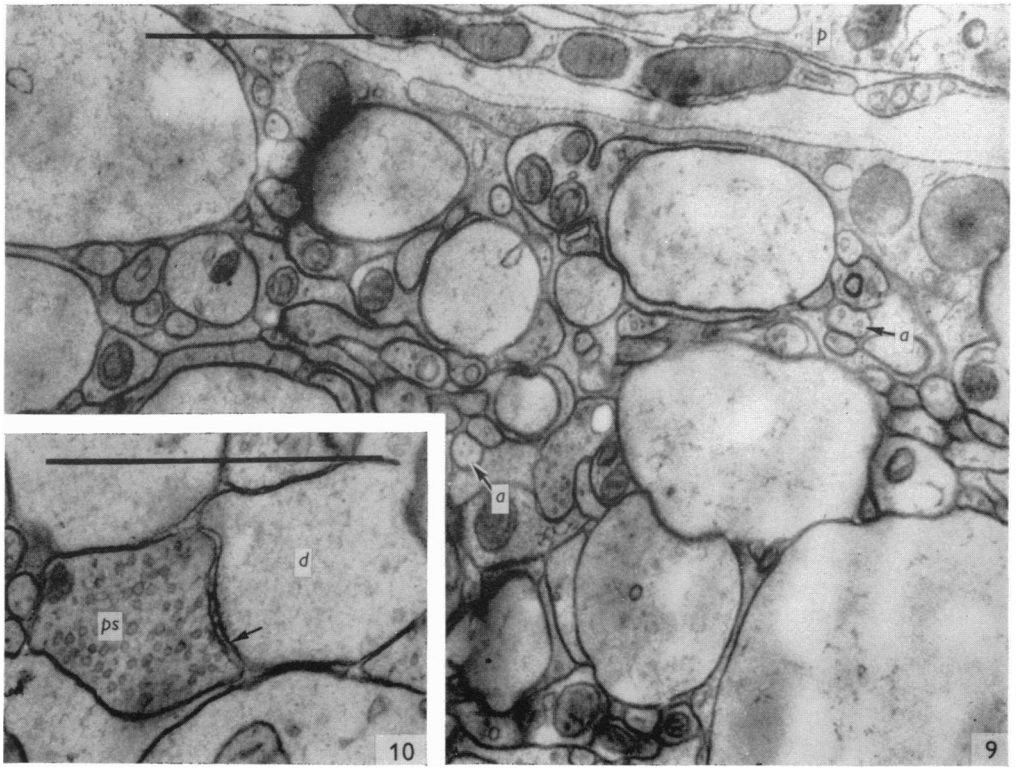
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The micrographs of this material resembled those obtained after recovery of the cortex in the moist chamber. As shown in Fig. 8 an appreciable extracellular space is present especially between the small profiles. The impression was gained that extracellular material is more abundant in micrographs from material treated with low K than with normal Ringer's solution.

To investigate the effect of circulatory arrest the head was separated from the body after exposure of the cortex and starting the flow of Ringer's solution; 6 min later the cortex was frozen. The micrographs of such tissues had the typical appearance shown in Fig. 9. There was again practically no extracellular space but certain elements were enormously swollen. Much more so than in the micrographs of cortices asphyxiated *in situ* or kept in the moist chamber for 5 min. The small profiles (non-myelinated axons) and presynaptic endings did not participate in this swelling. Some of the swollen structures seemed to form a synapse with a preterminal ending (Fig. 10) identifying them as dendrites or dendritic spines. This may suggest that other swollen structures are also dendrites even if they cannot be identified as such. The glial membrane on the cortical surface is not obviously swollen in micrographs of tissue asphyxiated *in situ* (Fig. 4), in the moist chamber (Fig. 7) or while bathed with Ringer's solution (Fig. 9), which may perhaps be taken as an indication that swelling of the cerebral cortical glia is not marked during oxygen deprivation.

The swelling of cellular elements in tissue bathed with Ringer's solution during asphyxiation is so great that the material taken up by these tissue elements cannot be derived entirely from the extracellular material. Part of this fluid may therefore have come from the Ringer's solution flowing over the cortex during the asphyxial period. The swollen elements are large enough to be seen with the light microscope in sections 1 μm thick. They were found mainly in the surface of the molecular layer, supporting the concept that much of the material in these swollen structures was derived from the bathing fluid.

DISCUSSION

An appreciable extracellular space was generally observed in electron micrographs of oxygenated cerebral cortex prepared by freeze-substitution. The extent and distribution of this space between non-myelinated axons was similar to that found in cerebellar cortex (Van Harreveld *et al.* 1965) and in white matter (Malhotra & Van Harreveld, 1966) prepared in the same way. Asphyxiation eliminated this space so completely that the membranes between the cellular elements exhibited tight junctions. Furthermore, evidence for swollen cellular elements was found. Whereas electron micrographs of asphyxiated material invariably were characterized by an extreme paucity of extracellular space, those of oxygenated tissue frozen immediately after exposure of the cortex varied considerably in the amount of extracellular material. It was suggested that these differences can be accounted for by varying degrees of mechanical stimulation during exposure of the cortex. Indeed, providing for a period

Fig. 8. Oxygenated cortex frozen after having been bathed for 6 min with Ringer's solution (1/2 normal K concentration) after exposure.

Figs. 9, 10. Cerebral cortex asphyxiated for 6 min while being bathed with Ringer's solution.

Fig. 10 shows a synaptic contact (arrow) between a presynaptic ending and a swollen dendritic structure.

during which the tissue can recover from this unavoidable stimulation resulted in electron micrographs in which the magnitude of the extracellular space showed much greater consistency. The differences in the micrographs of oxygenated and asphyxiated cerebral cortex are in good agreement with the marked impedance increase of the cortex which develops with a latency of 1–2 min after circulatory arrest and the transport of water (Van Harreveld, 1957) and chloride (Van Harreveld & Schadé, 1959) into apical dendrites observed with the light microscope.

As remarked previously (Van Harreveld *et al.* 1965) the extracellular space observed in non-asphyxiated cortex prepared by freeze-substitution may not represent exactly the water distribution in the living tissue, since differences in concentration of tissue components soluble in acetone used for substitution and in propylene oxide and Maraglas for embedding may cause osmotic adjustments between the intra- and extracellular compartment. The contact with acetone was eliminated by preparing central nervous tissue by freeze-drying (Van Harreveld & Malhotra, 1966). In electron micrographs of non-asphyxiated cerebellar cortex the magnitude of the extracellular space between the non-myelinated axons was not different in this material from that in micrographs of freeze-substituted tissue, enhancing the likelihood that the extracellular space found actually represents that of the normal, oxygenated cortex.

Since the surface of the molecular layer is rich in non-myelinated axons between which the extracellular material is present, but poor in large tissue elements such as perikarya, large dendrites and glia cell bodies, the average extracellular space in the entire cortex may be considerably smaller than would appear from the micrographs of the surface layer only (Horstmann & Meves, 1959). Bourke, Greenberg & Tower, (1965) determined the sucrose- and inulin spaces of the cerebral cortex in several species and observed an increase of these spaces with the brain weight. In guinea-pigs the spaces were 19·4 %, in rabbits 22·5 %, in cats 28·0 % and in monkeys 32·8 % of the tissue volume. Assuming that this relationship can be extrapolated to a small animal such as the mouse a cortical sucrose- or inulin (extracellular) space of 8·5 % was computed. If the prevalence of extracellular material between non-myelinated axons holds true for the entire cortex, then the species differences in extracellular space observed by Bourke *et al.* (1965) could be due to a relationship between the abundance of these structures in the cortex and the brain size.

SUMMARY

The surface layer of the cerebral cortex of the mouse was investigated by rapid freezing of the tissue followed by substitution fixation at low temperature. Electron micrographs of this material showed in general an appreciable extracellular space when the cortex had been frozen shortly after circulatory arrest. The extracellular material was present especially between non-myelinated axons. A similar location of extracellular material has been observed in the cerebellar cortex and in central white matter.

The extracellular material had practically disappeared in cortices asphyxiated for 5–6 min before freezing. Micrographs of this tissue showed tight junctions between cellular elements, and certain tissue elements appeared to be swollen. The effects of

asphyxiation on the cerebral cortex parallels that on cerebellar cortex, and is consistent with an asphyxial impedance increase and a transport of water and chloride into cellular elements observed with the light microscope in both cerebral and cerebellar cortex.

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