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PRESERVATION OF EXTRACELLULAR SPACE DURING FIXATION OF THE BRAIN FOR ELECTRON MICROSCOPY

ABSTRACT. Adult mammalian brain contains about 20% extracellular space, but fixatives cause the cellular processes to ingest the extracellular fluid, and the space is not preserved in electron micrographs prepared by any of the conventional methods. This distortion can be prevented by replacing part of the sodium chloride in the extracellular fluid by an impermeant solute such as sucrose. To do this, the blood-brain barrier can be opened by vascular perfusion at 300 mmHg pressure, or the barrier can be bypassed by immersion of thin slices of fresh brain in the impermeant solution. In either case, addition of aldehyde fixatives and conventional processing then leads to the preservation of extracellular space in electron micrographs. Both procedures are as easy to use for routine fixation as conventional methods. In well fixed tissue the cellular processes are different in size, shape and electron density from the inflated profiles seen after the ingestion of extracellular fluid that accompanies conventional fixation. Moreover, extracellular space is found to separate widely some cellular elements, while leaving others contiguous.

Introduction

VAN HARREVLD (1972) has reviewed three independent lines of evidence that indicate that about 20% of the volume of adult mammalian brain consists of extracellular space. When the subarachnoid space is perfused with artificial cerebrospinal fluid containing a radioactive marker substance that remains extracellular, the concentration of marker in the brain rises to a plateau. This plateau concentration is 17–20% of the concentration in the perfusing fluid when sucrose or inulin are used in rabbit, cat, dog or monkey brain (Levin *et al.*, 1970). Independent confirmation is provided by the fact that the electrical resistance of brain tissue is four or five times greater than that of cerebrospinal fluid. The electrical measuring current is largely confined to the extracellular fluid by the high resistance of cell membranes, and calculations reviewed by

Van Harreveld (1972) indicate that a volume of 15–20% extracellular fluid is needed to match the resistance observed. Extracellular space has been observed by electron microscopy in brain tissue after extremely rapid freezing, substitution of ice by acetone or ethanol containing osmium tetroxide, and plastic embedding (Van Harreveld and Steiner, 1970). Only the outer 15 µm of tissue is frozen fast enough to avoid damage by ice crystals, however, and the method has not come into use as the standard procedure for electron microscopy of brain tissue.

After conventional procedures with liquid fixatives an extracellular space of less than 5% is present in brain tissue (Karlsson and Schultz, 1965; Gonzalez-Aguilar, 1969). The reasons for this have been analysed by Van Harreveld and his colleagues who combined the rapid freezing method with components of the perfusion procedure. Most of the extracellular space is lost if the tissue is made anoxic before freezing, but 1–3·5 min of anoxia is needed to bring this about (Van Harreveld and Ochs, 1956). However, perfusion of brain with aldehyde fixative before

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freezing causes an almost immediate loss of extracellular space (Van Harreveld and Khattab, 1968) due to the movement of sodium and chloride ions from the extracellular fluid into the neuronal and glial cell processes. This ionic movement is accompanied by water which expands the cellular processes to occupy the extracellular space. A possible mechanism by which anoxia or fixatives could produce this cellular swelling has been investigated by Bito and Myers (1972).

It should be possible to prevent this process occurring during fixation by replacing the sodium chloride in the extracellular fluid by a substance that is unable to enter cells. This replacement would have to occur before anoxia or fixatives could start the swelling process. Unfortunately, a molecule unable to permeate the brain cells would also be unable to cross the blood-brain barrier. The barrier can be bypassed by immersion of slices of brain tissue, or opened by perfusion at a high pressure (300 mmHg) (Rapoport, 1976). Both methods have been used here to replace the extracellular fluid with an isotonic solution of sucrose. Addition of an aldehyde fixative then leads to the preservation of a large extracellular space. However, the standard aldehyde fixative containing 4% formaldehyde and 1% glutaraldehyde has an osmolarity of 1.4 M compared with the osmolarity of cerebrospinal fluid of only 0.3 M. Although these aldehydes will rapidly cross the cell membranes, their initial presence in the extracellular fluid will cause some water to leave the cells. After the aldehydes have entered the cells they will start to fix the proteins, and the returning water may not restore the cellular processes to their original shapes. This may not matter with conventional fixation because the entry of water from the extracellular space distorts most profiles of cellular processes to polygonal shapes. But if the extracellular space is preserved, the shape of the cell profiles is sensitive to shrinkage, and the sudden addition of 1.4 M aldehydes to the isotonic perfusate can cause concave distortions.

For the present work, a perfusion apparatus has been set up so that the concentration of aldehydes in the perfusate can be made to rise slowly and continuously from zero to the desired value. Similarly, the change prior

to embedding in plastic from an aqueous solution to absolute alcohol has been made continuously to avoid distortions. The final methods are quick, convenient and reliable to use, and give a new insight into the sizes, shapes and electron densities of cellular processes, and reveal an uneven distribution of extracellular space.

Materials and Methods

Mice and rats were rapidly anaesthetized in a strong chloroform vapour.

Perfusion

This must start within 1 min of opening the chest, and the pressure to be contained is 300 mmHg. The cannula was a No. 14 hypodermic needle squared off at the end and provided with a ground neck (C in Fig. 1). This was inserted in a cut in the left

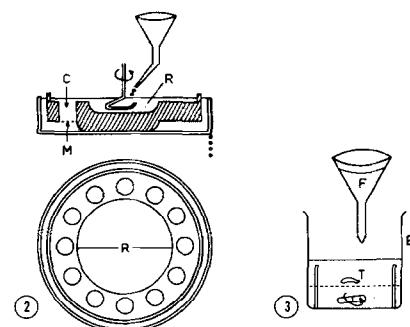
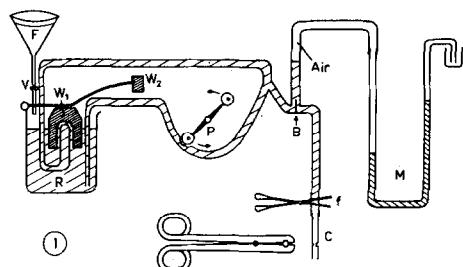


Fig. 1. A pumped circuit for perfusion at 100 or 300 mmHg with a continuous rise in concentration of fixative. See text.

Fig. 2. A stirred reservoir and chambers for dehydrating tissues in a steadily rising concentration of alcohol.

Fig. 3. A stirred beaker for immersing tissue to be fixed in a steadily increasing concentration of aldehydes.

cardiac ventricle and passed up into the ascending aorta. A circular clip made by drilling a pair of artery forceps (Fig. 1) and covering the jaws with thin plastic tubing was applied to the aorta to grip the ground neck of the cannula. The right auricle was opened, and the descending aorta clipped if the spinal cord was not to be studied. Perfusion started about 40 sec after opening the chest.

A roller pump (P in Fig. 1) operating on 3 mm internal diameter tubing with two rollers at 200 rev/min was used to pump solution from a reservoir R of 400 ml capacity back to a pressure release valve W_1 in the same reservoir. The aperture of this valve was 8 mm and the fitted weight W_1 of 77 g allowed a pressure of 100 mmHg to develop in the flowing perfusate. A further weight W_2 of 84 g was adjustable on a hinged arm so that the pressure could be raised to 300 mmHg. The flow in the pumped circuit was 600 ml/min, and part of this was bled off to the cannula (C) and also connected to a mercury manometer (M) in the manner shown. This ensured that air bubbles did not enter the output, and the baffle (B) at the T junction together with the air in the connection to the manometer smoothed out the pulsatile pressure from the pump.

With the cannula clamped in place, the clip f was removed, the pump was switched on and brought up to 200 rev/min over a period of 5 sec to avoid a rapid pressure transient. When 100 ml of perfusate had been passed and 100 ml was left in the reservoir R, a second solution containing fixative was poured into the funnel F and the flow rate adjusted at V so that the level of fluid in the reservoir stayed approximately constant. The fluid escaping from the release valve W_1 provided rapid stirring of the reservoir, and the concentration of fixative in the perfusate increased smoothly. When 100 ml of fixative had been passed, the weight W_2 was slowly lifted off, and a second 100 ml of fixative perfused at 100 mmHg pressure.

Slices of brain tissue about 1 mm thick were immersed in the same fixative for 48 hr. Plates of tissue less than 0.5 mm thick were then cut and immersed in 1 ml of fixative in a vial that was cooled to 4°C. Then 0.2 ml of a 6% solution of osmium tetroxide in water (0.24 M) was slowly added to give a final osmium concentration

of 1% in the fixative solution. When formaldehyde was used in the perfusate, the tissue was washed in the perfusate solution without formaldehyde before addition of osmium tetroxide. After fixation for 16–24 hr, the tissue was washed in 0.1 M sodium acetate buffer at pH 4.5, stained in 2% uranyl acetate in water for 2 hr, and dehydrated in the chambers of the apparatus shown in Fig. 2. These chambers (C) were filled with water, and absolute alcohol was slowly run into the central stirred reservoir (R in Fig. 2). Diluted alcohol passed through the mesh bottoms (M) of the chambers (C) containing the tissue, overflowed the outer rim and ran to waste. The total volume of fluid filling the apparatus was 75 ml and 150 ml of absolute alcohol was passed in 15 min, raising the concentration of alcohol in the reservoir to 86% according to calculation. The tissue was then put back into vials of absolute alcohol, and embedded in Araldite in the usual manner.

Immersion

The skull of a rat or mouse that was deeply anaesthetized with chloroform was quickly and widely opened, and a slice of brain tissue less than 1 mm thick cut with a new razor blade. The tissue (T) was immersed in 50 ml of sucrose solution in a magnetically stirred beaker (B in Fig. 3). After 90–120 sec, 50 ml of a second solution containing aldehyde fixatives was run into the stirred beaker from the funnel over a period of 2–3 min. The tissue was then treated as in the paragraph above.

Solutions

For both perfusion and immersion fixation, the first solution was 0.135 M sucrose with 0.135 Osm sodium phosphate buffer at pH 7.3 and 0.002 M calcium chloride. The second solution was the same with the addition of 1% glutaraldehyde for perfusion or 2% glutaraldehyde for immersion fixation. Formaldehyde or acrolein can be added with the glutaraldehyde. The two solutions were made by dissolving 18.5 g sucrose and 4.1 g $\text{Na}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ in 400 ml water, adding 0.5 ml of a 5% solution of calcium chloride, and bringing the pH to 7.3 by addition of a saturated solution of sodium hydroxide.

Estimation of extracellular space

A grid of 100 red points 2 cm apart on a sheet of transparent film was placed over each photomicrograph, and the number of points lying over extracellular space was counted for ten placements of the grid.

Results

After perfusion or immersion of tissue in isotonic sucrose the addition of aldehyde fixatives and conventional processing led to the preservation of a large extracellular space in electron micrographs. The space was much more than 20% of brain volume, and cellular processes, especially axon terminals, were severely shrunken and condensed. The conventional fixative that was used contained 4% formaldehyde and 1% glutaraldehyde, and had an osmolarity of 1.4 M due to the aldehydes. It seemed possible that the shrinkage of cellular processes could be due to this excessive osmolarity, but when 1% glutaraldehyde, having an osmolarity of 0.1 M, was slowly added with the isotonic sucrose shrinkage still occurred. It was then realized that an aldehyde fixative would increase the permeability of cell membranes so that small molecules could leave the cells, but evidently sucrose could not enter them. It was thus desirable to replace part of the sucrose with an isotonic solution of a substance that could enter the cells in the presence of fixative.

An isotonic solution containing 0.14 M sucrose together with 0.14 osmolar sodium phosphate buffer was then used for perfusion and immersion, and 1% glutaraldehyde in the same mixture used for fixation. This procedure gave an extracellular space of about 20%, and the cellular processes were not shrunken or condensed as before. However, the lamellae of myelin were not ad-

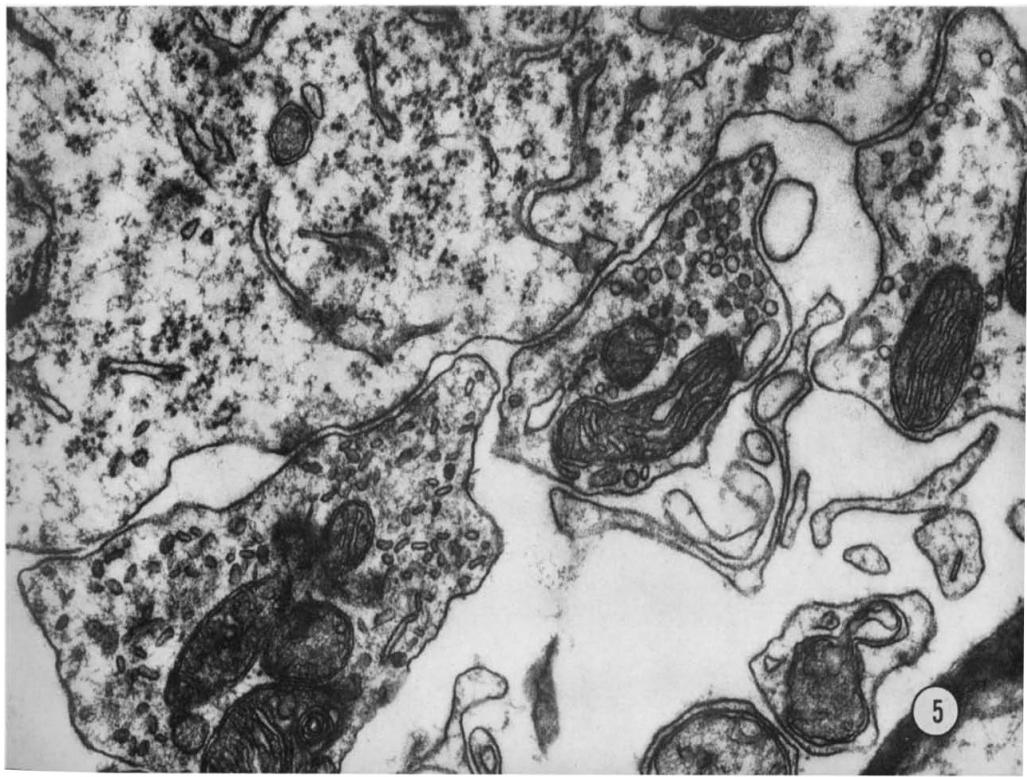
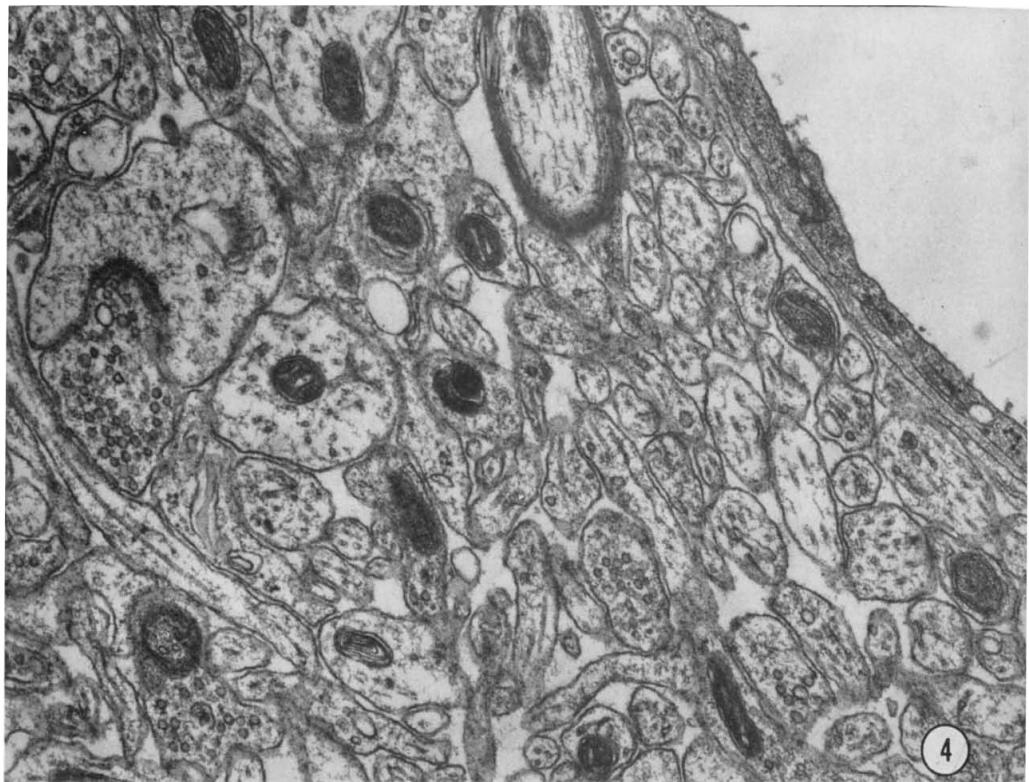
herent, and the conditions of fixation were varied in 194 experiments seeking improvement. Sucrose was replaced with glucose, HEPES or calcium chloride in isotonic solution with preservation of extracellular space, but without advantage. Sodium phosphate was replaced with sodium cacodylate, sulphate, acetate or dichromate, or with urea or glycerol in isotonic solutions, but again without advantage. The proportion of impermeant solute to permeant solute has been varied, and the size of the extracellular space decreased as expected with more of the permeant solute, but the fixation of myelin was unaffected. The alkalinity of the solutions was varied by one unit of pH on either side of pH 7.3 without any marked effect upon ultrastructural preservation. The addition of calcium chloride to the point of precipitation with the phosphate buffer seemed to improve the adhesion of myelin lamellae. It was noticed that 1% glutaraldehyde by itself made the tissue more yellow than when formaldehyde was present as well, and it seemed possible that the two aldehyde groups in the glutaraldehyde molecule might cross link the lamellae of myelin more strongly in the absence of formaldehyde. However, concentrations of 1–5% glutaraldehyde in the presence or absence of formaldehyde or acrolein gave similar preservation of myelin. The addition of 1% formaldehyde to 1% glutaraldehyde possibly improved the definition of cell membranes, but the differences seemed small.

Perfusion pressure

The rate of rise of perfusion pressure to 300 mmHg was found to be important, for a sudden rise led to shrinkage and condensation of the ultrastructure of cellular processes. This effect is avoided if the pumping rate is raised from 0 to 200 rev/min over a period

Fig. 4. Rat cerebral cortex, parietal area, near a blood capillary fixed after opening the blood-brain barrier by perfusion at 300 mmHg pressure. $\times 30,000$.

Fig. 5. Rat spinal cord, ventral horn, showing three synapses on a cell body fixed by perfusion at 300 mmHg pressure. $\times 42,000$.



of about 5 sec after the cannula is in place and the clip f in Fig. 1 removed. The ultra-structure of cellular processes adjacent to the wall of a blood capillary in rat cerebral cortex is shown in Fig. 4. The high pressure of perfusion does not seem to have distorted the tissue, an extracellular space of about 15% is present, and myelin is not seriously disarranged. The tight junctions between the endothelial cells in the capillaries were not seen to be opened after perfusion at 300 mmHg pressure. However, perfusion at 100 mmHg pressure with the same solutions did not preserve extracellular space. After opening the blood-brain barrier by perfusion at 300 mmHg, all blocks of tissue examined in 18 perfused rats had preserved extracellular space, and the blocks included cerebral and cerebellar cortex, thalamus and spinal cord. Synapses were not always invested closely with glial processes, and this is illustrated in Fig. 5 by three synapses on a cell body in the ventral horn of the spinal cord. The isotonic solutions used were found to differentiate round and flattened vesicles clearly.

Fixation by immersion

The difficulties of opening the blood-brain barrier can be avoided and the barrier bypassed by immersing slices of fresh brain in isotonic sucrose solution to replace the extracellular fluid. The tissue (T in Fig. 3) was cut at less than 1 mm thickness with a new razor blade and placed on a submerged horizontal mesh in the magnetically stirred beaker B which contained 0.3 M sucrose. The loss of ions from the cut slice of brain immersed in sucrose was followed by measuring the electrical conductance of the stirred solution as a function of time. The conductance was found to approach exponentially to an

asymptote with a time constant of 75 sec. After 90 sec of immersion, when the fixative began to run in, the tissue had lost 66% of the readily lost ions. After 4 min of immersion, when the full fixative concentration would have been reached, the loss of ions was close to the asymptotic level. A solution of sodium chloride (0.9%) was added in 10 μ l aliquots to calibrate the conductance. The asymptotic loss of ions was found to be equivalent to 45 μ M NaCl/g brain tissue.

The solutions found optimal for perfusion fixation were also satisfactory for immersion fixation, and were used to fix 32 slices of mouse, rat, monkey and human brain as set out under Methods.

Extracellular space was preserved throughout the thickness of the tissue, and the quality of fixation, particularly of myelin, appeared to be at least as good as after perfusion at 300 mmHg pressure. Superficial parts of the brain such as cerebral (Fig. 8) and cerebellar cortex (Fig. 9) and deep structures such as thalamus (Figs. 6, 7) were equally well fixed. The time of immersion in sucrose before the addition of aldehydes was varied from 1 to 3 min without apparent effect, but omission of the sucrose treatment by placing the tissue directly into the final mixture of sucrose, phosphate and aldehyde did not preserve extracellular space.

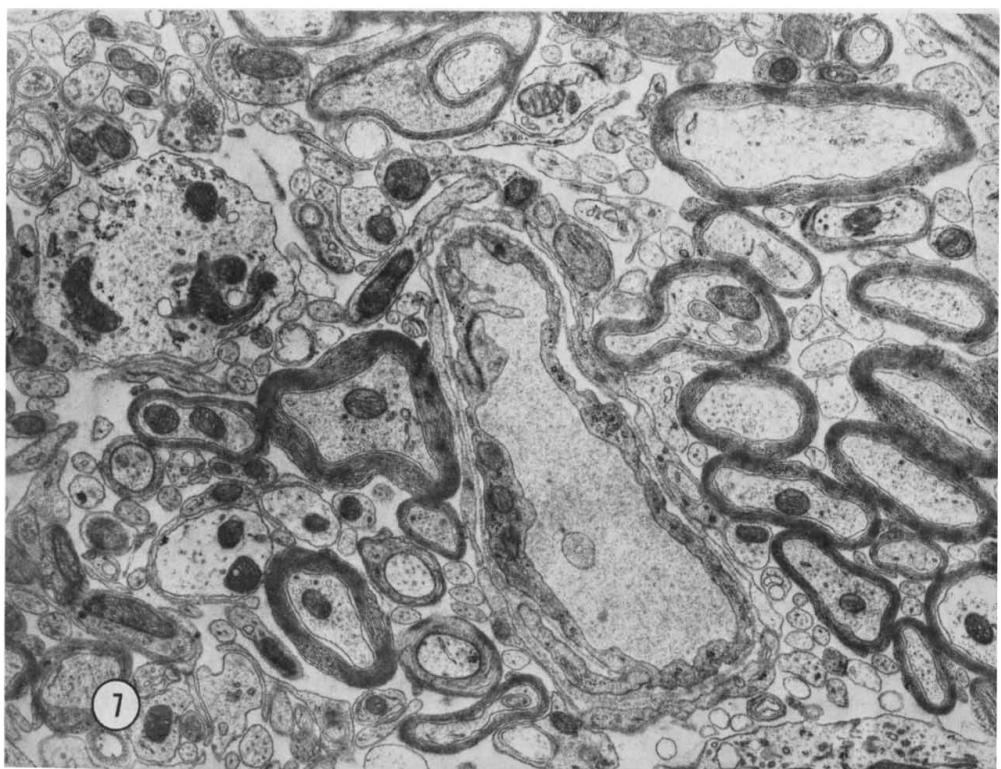
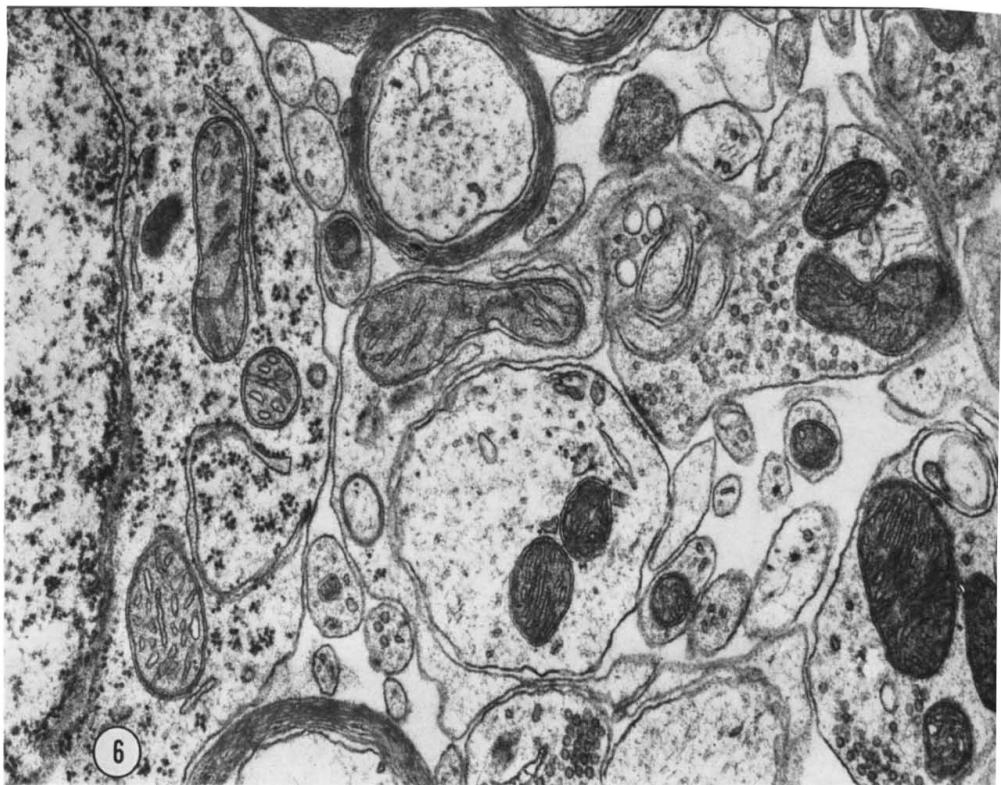
Discussion

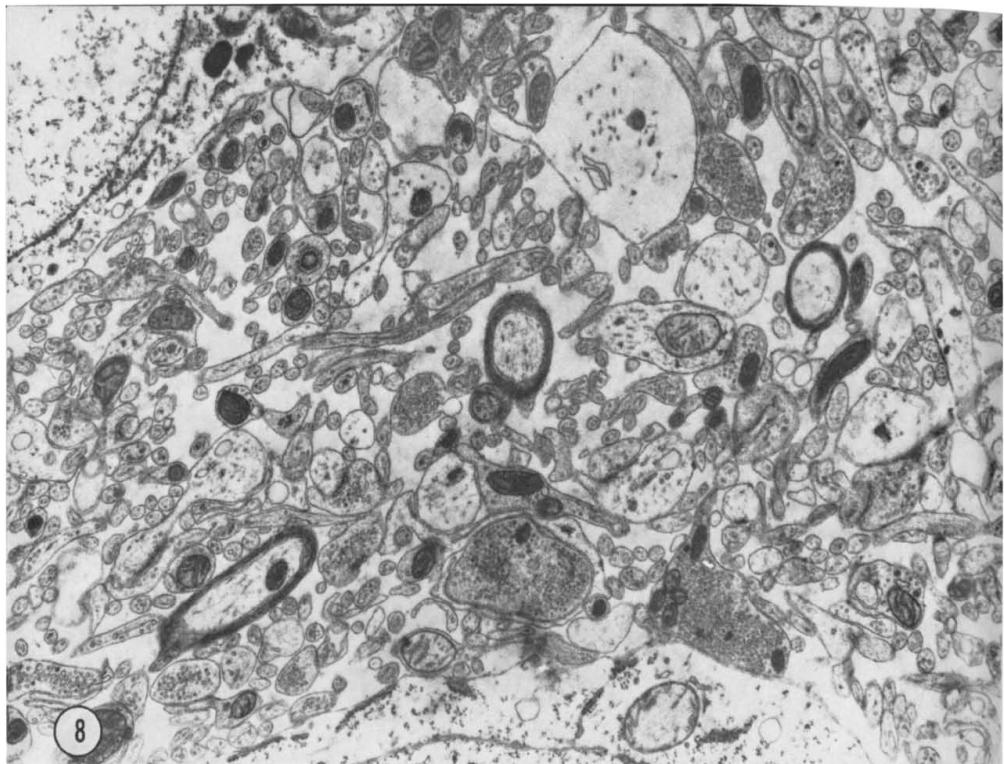
The fixation procedures described above are substantially different from the conventional methods, and the resulting electron micrographs differ in several ways from the usual result. Although 194 experiments were done to test the effects of the many variables involved, it is unlikely that the procedures adopted are incapable of improvement.

Figs. 6, 7. Mouse thalamus (lateral geniculate body) fixed by immersion. $\times 30,000$ (Fig. 6); $\times 13,000$ (Fig. 7).

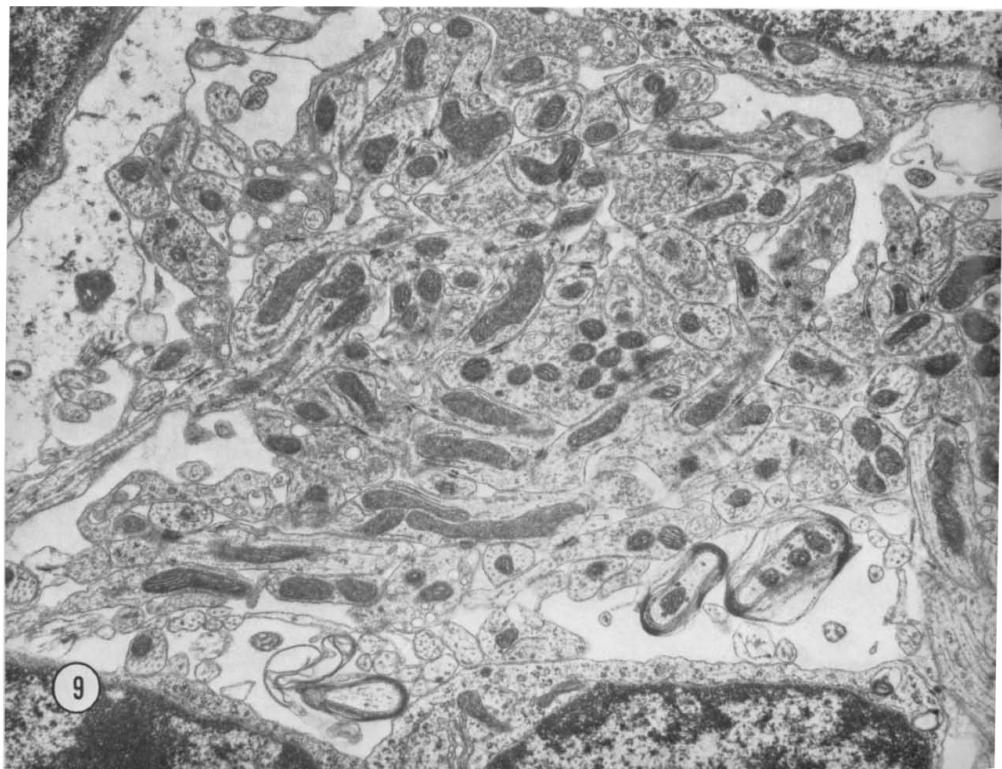
Fig. 8. Mouse cerebral cortex, parietal area fixed by immersion. $\times 13,000$.

Fig. 9. Rat cerebellar cortex, granular layer, showing a synaptic glomerulus and granule cells fixed by immersion. $\times 13,000$.





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However, the methods are reliable and no more trouble to use than conventional methods. They yield an acceptable quality of fixation though some disturbance of myelin lamellae is produced. The fact that an extracellular space of 20% of brain volume is missing from conventional electron micrographs is not acknowledged by electron microscopists, even in a standard atlas such as that by Peters *et al.* (1970). It is now easy to avoid the distortions produced by this loss of extracellular space, and I hope that the achievement of Van Harreveld in analysing the problem will become recognized.

Study of nervous tissue with preserved extracellular space can be expected to advance knowledge in three areas that are of interest to physiology. First, the space is unevenly distributed and there may be significance in which cellular processes are contiguous and which are separated by space. In Fig. 9 the synaptic glomerulus contains little space but is widely separated from the surrounding cell bodies. This is true also of the synaptic glomeruli in the thalamus, and should be helpful in indicating which processes are not associated with a glomerulus. When two different kinds of structure are seen to be contiguous, this is no longer due merely to loss of space. The appearance of the space makes it easier to understand how dendrites can retract and subsequently regrow when a motor axon loses and then regains synaptic contact with muscle fibres (Sumner and Watson, 1971).

Secondly, the extracellular clefts available for diffusion of substances to and from blood capillaries are clearly extensive in cross-sectional area (Figs. 4, 7). The argument of Kuffler and Nicholls (1966, p. 27) that clefts of 15 nM would only retard the diffusion of a molecule as large as sucrose by 18% compared with free solution is misleading because the total cross-sectional area of the 15 nM clefts around a capillary in tissue that has been fixed conventionally is small compared to the true extracellular space available for diffusion. Many synapses in the cerebral and cerebellar cortex, spinal cord and thalamus are not closely invested by glial cell processes. Instead a large extracellular space is continuous with the synaptic cleft (Fig. 5) and the diffusion of transmitters would not be retarded by a small cross-sectional area of 15 nM clefts. The widths of the gaps at synaptic appositions and at small-gap junctions appear to be unaffected by the method of fixation.

Thirdly, different kinds of neurons and glial cells have been distinguished by size and electron density. Thus in cerebral cortex, pyramidal cells and their axons and dendrites are larger and paler than those of stellate interneurons. These differences have been recognized after ingestion of extracellular fluid during conventional fixation. They are still present in tissue with preserved extracellular spaces, and it will be interesting to determine whether further distinctions between cell processes of different origin can be recognized.

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