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The Influence of High Pressure Freezing on Mammalian Nerve Tissue

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Summary. Vitrification of biological specimens in liquid nitrogen can be achieved under high pressure (2,100 bars). This procedure obviates the use of aldehyde fixation and cryoprotection (glycerol). The present work demonstrates its applicability to the freeze-etching of mammalian brain tissue. Freeze-fracture replicas from rat cerebellar cortex and subfornical organ prepared by this method are compared to conventionally processed material using aldehyde fixation, glycerination and freezing with Freon. The formation of large ice crystals is prevented in tissue blocks up to 0.5 mm thick; deep etching is markedly enhanced. Cytoplasmic microstructures such as mitochondrial cristae, microtubules and microfilaments, are readily observable against a finely granulated cytosol matrix. An additional advantage is the combined application with freeze-substitution.

Key words: High pressure (2,100 bars) freezing – Freeze-etching – Nerve tissue – Rat.

Cryofixation is one of the most critical steps in freeze-etching cells and tissues (Moor 1973). The aim of cryofixation is to pass between the freezing and recrystallization points within a time limit that prevents ice crystals from becoming larger than about 100 to 200 Å. The solidification of a specimen in such a microcrystalline state is called vitrification in physical terms, the problem of cryofixation consists in removing heat from the specimen more rapidly than it can be produced by crystallization. Because heat can only be extracted from a specimen through its surface by contact with a cooler medium, heat transfer at the surface, heat capacity and conductance of the specimen play a prominent role. The limiting factor is the low heat conductance of water, which in deeper regions of the specimen slows down the freezing rate drastically, in comparison with that of the surface, and

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often beyond a cooling rate that is necessary for vitrification. Although using the most suitable contact media and lowest possible temperature, only very superficial layers of a specimen (1 to 10 μm in thickness) can virtually be vitrified. If vitrification of thicker layers or whole tissue blocks is required, the physical properties of the object must be altered. One possibility is offered by the application of cryoprotectants (Moor 1964) which reduce the freezing point and crystal growth, but elevate the recrystallization temperature and hence reduce the freezing rate which is otherwise necessary for vitrification. In order to prevent deleterious cytological effects and improve membrane permeability of cryoprotectants, generally a "mild" aldehyde fixation precedes the glycerol treatment. Theoretical considerations and practical experience suggest that such treatments prior to freezing are by no means without ill effects (Moor 1973; McIntyre et al. 1974; Sandri et al. 1977).

Therefore, an alternative method was sought and a technique developed based on the application of high hydrostatic pressure for the following reason: In principle, the pressure in the range of 2,100 bars has the same effect as a cryoprotectant in a concentration of about 20% (Riehle 1968; Moor 1973). Hence, with both techniques samples of a thickness up to about 0.5 mm can be vitrified. On the one hand, high-pressure excludes the influence of any chemicals, on the other hand, it is known to be lethal. However, we have been able to show that by reducing the time of pressurizing below 0.1 sec., the deleterious effects of the physical interactions can be greatly reduced or even prevented (Moor and Höchli 1970).

In the initial approach of resolving the technical problems of high pressure freezing, we were able to demonstrate the predicted improvements in cryofixation of thicker specimens, but the apparatus was not applicable for routine work with tissue blocks (Riehle 1968). Therefore, we developed a new pressure-freezer based on the original concept of Riehle and by profiting from the contributions of Hoechli (Riehle and Höchli 1973). Appropriatively 10 years were required until all the technical problems were solved (Moor 1977). Since 1978 we have been able to apply pressure freezing routinely to nerves and other tissues and to collect valuable experience, some of which shall be reported in the present paper.

Materials and Methods

1. Preparation of the Tissue. Adult albino rats anesthetized with sodium pentobarbital were used for this study. Cerebellar cortex and subfornical organ were rapidly exposed and within 1 min dissected from the brain. Small tissue blocks (0.5 mm in diameter) were immersed in a droplet of cacodylate buffer with 6.8% saccharose and subsequently pressure-frozen.

For conventional cryofixation, the deeply anesthetized animals were perfused with buffered (0.05 M phosphate, pH 7.4 paraformaldehyde (4%). Small tissue blocks were then dissected and immersed in buffered (0.1 M phosphate, pH 7.4) glutaraldehyde (5%) for 2 to 4 h at room temperature; after washing overnight in 0.2 M phosphate buffer with 6.8% sucrose (pH 7.4) at 4° C, the specimens were immersed in Ringer solution containing 25% glycerol for at least 30 min and then subjected to freezing in Freon.

2. Freezing Techniques. For pressure-freezing, a tissue block is placed in the cavity of a conventional golden Balzers spezimen supporting platelet (⌀ 3 mm). A second platelet, with a dome-shaped cavity, is placed on top of the first (Fig. 2) in the conventional manner for preparation of double-replicas.

However, in this case, the second platelet is covered with a thin layer of egg-lecithin in order to enhance fracturing along its surface and hence to provide a frozen specimen that may be "cut" with the freezing-ultramicrotome (Moor et al. 1961). The assembled specimen-sandwich (Fig. 2) is then inserted into a holder that fits into the pressure chamber of the pressure-freeze apparatus (see below). After pressurizing and freezing, which lasts about 0.5 s, the sandwich is withdrawn from the apparatus and immediately immersed in liquid nitrogen where the second platelet is removed and the specimen, adhering to the first platelet, can be stored until use.

For conventional freezing a tissue block is placed on a BALZERS specimen support (as mentioned above), cooled by dipping into liquid Freon 22 at –150° C and then stored under liquid nitrogen (Moor and Mühlethaler 1963).

3. Apparatus for Pressure-Freezing. A description of all the technical details contributing to a highly reproducible and rapid processing cannot be dealt with in the present report. Here, emphasis will be concentrated on some aspects of the biological application, while the technology will be published elsewhere. Therefore, only a short description of the apparatus is provided in an attempt to explain the operation of the apparatus and to give an idea of the lines along which technical problems were solved.

In principle, liquid nitrogen is used as cryogen and as pressurizing medium at the same time. The solution of the problem is represented diagrammatically in Fig. 1: the hydraulic pump (*HP*) forces oil into the pressure accumulator (*PA*) compressing a gas balloon (*GB*), preloaded with 250 bars, to about 350 bars. The pressure valve (*PV*) releases the pressurizing oil through the low pressure line (*LL*) into the low pressure cylinder (*LC*) and forces the pressure piston (*PP*) downward. The high-pressure cylinder (*HC*) is mounted in a dewar flask (*DV*) filled with liquid nitrogen (LN_2). In this cylinder, the lower part of the piston, the diameter of which is reduced from 80 to 26 mm, compresses liquid nitrogen and forces it through the high-pressure line (*HL*) into the pressure chamber (*PC*). There, two jets of pressurized liquid nitrogen are injected from opposite sides onto the surface of the specimen-sandwich. As shown in cross section (*CS*), the nitrogen escapes from the chamber through two narrow apertures (*NE*, ⌀ 0.3 mm). Thereby, a volume of about 100 ml liquid nitrogen is forced through the chamber in less than 0.5 s, while a pressure of more than 2,100 bars is maintained. After one application the piston is moved upward by the action of pressurized air, which enters at the bottom of the low pressure cylinder. The rising piston allows liquid nitrogen to enter the high pressure cylinder through a valve at the bottom of the pressure system (indicated between *H* and *C*). The oil in the low pressure cylinder is returned through a separate line (not indicated) to the hydraulic pump.

The liquid nitrogen jet on the specimen was monitored by measuring simultaneously temperature fall and pressure rise in the high pressure chamber. For this purpose, a thermoelectric couple (Philips chromel-alumel thermocoax ⌀ 0.34 mm) mounted in a sandwich, a Kistler high pressure quartz transducer mounted in the side wall of the pressure chamber and a Gould digital storage oscilloscope for simultaneous recording was used. Temperature fall and pressure rise (T_1 and p in Fig. 4) occur simultaneously and reach –100° C and 2,100 bars, respectively, after about 30 msec. This indicates that under these conditions freezing may start at low pressure conditions. In fact, the samples showed extensive ice crystal formation. Therefore, it was necessary to delay the cooling until a pressure of about 2,100 bars is reached. This was obtained by adding a warm pressurizing medium preceding the liquid nitrogen. For this purpose, an "injector" was inserted (Fig. 3) immediately below the pressure chamber into the high pressure line: Through valve V_1 a small volume of isopropyl alcohol (*IPA*) can be injected into the container (*C*). Injector and pressure chamber are thermocontrolled. Valve V_2 prevents the contact between the warm alcohol and the LN_2-containing part of the high pressure line. Such contact would block the line with solid alcohol. At the beginning of a pressure application, the liquid nitrogen opens valve V_2, and alcohol (about 2 ml) escapes through the pressure chamber. During this time a pressure of about 2,100 bars develops, while the specimen is kept warm (T_2 and p in Fig. 4). The delay between cooling and pressure rise is just sufficient for high-pressure freezing and yet too short for causing damage due to pressurizing and/or to the contact between unstabilized specimen and alcohol.

4. Freeze-Etching. Etching of the frozen material is performed in a more or less conventional manner: In a BALZERS BA 510 M apparatus (Moor 1971) equipped with a Meissner trap and electron beam heated evaporators, the specimens are kept under a vacuum lower than 10^{-7} Torr. Etching is performed at –110° C for 1 min for pressure-frozen and 5 min for Freon-frozen specimens. Replication is obtained by platinum-carbon shadow casting (25 Å, 45° angle) and carbon evaporation (250 Å, 90° angle). After

withdrawal of the specimens from the apparatus and thawing of the tissue, the replicas are cleaned by subsequent immersion in 100% methanol, water, chromic acid (35% anhydrid), water, bleach and double distilled water. The replicas are then mounted on 75/300 mesh formvar-coated grids.

5. Microscopy and Photography. The replicas were viewed in a Siemens Elmiskop 102, photographed on Kodak Electron Image Film 4463 and reproduced as positives (white shadows). The nomenclature of Branton et al. (1975) concerning freeze-fracture membrane was adopted.

Results

1. General Remarks. The new methodology guarantees high reproducibility of pressure-freezing, i.e., pressurizing and cooling including the delay between the two processes.

In addition, rapid reloading of the apparatus after each application allows the handling of more than 30 samples within one hour, and 70 to 80% of the sandwiches can be successfully cleaved and fractured with the freezing ultramicrotome.

Nerve tissue is extremely delicate, and this property makes the microdissection of unfixed material notoriously difficult. For this reason, we have chosen two distinct brain regions that are readily accessible and rather compact, as a basis for a comparative study: cerebellar cortex and subfornical organ. The latter is particularly suitable in view of its pinhead size and ease with which it can be peeled off from the fornix. It was found that specimens can be vitrified by pressure-freezing at a thickness of up to 0.5 mm.

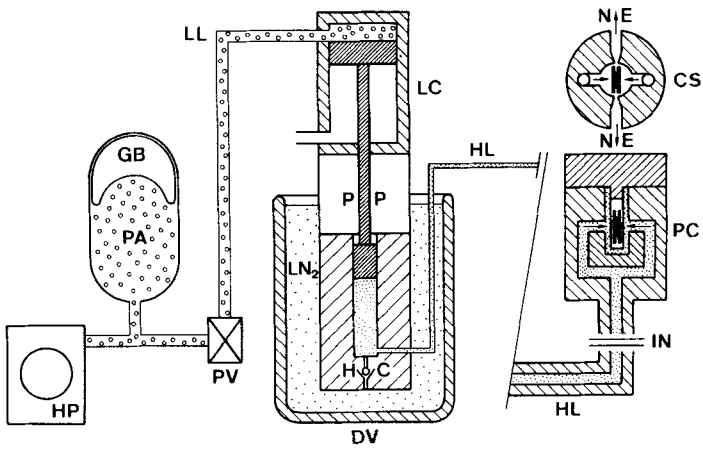


Fig. 1. Diagram of the high pressure freezing apparatus. The operation is explained in the text. Abbreviations: *DV* Dewar flask, *GB* gas balloon, *HC* high pressure cylinder, *HL* high pressure line, *HP* hydraulic pump, *IN* position of the injection chamber (see Fig. 3), *LC* low pressure cylinder, *LL* low pressure line, *PA* pressure accumulator, *PC* freezing chamber containing a specimen sandwich, *PP* pressure piston, *PV* pressure valve. *Small circles* oil; *dots* liquid nitrogen (LN_2). Cross section (*CS*) of the high pressure chamber is shown at the upper right. *NE* narrow apertures for gas escape

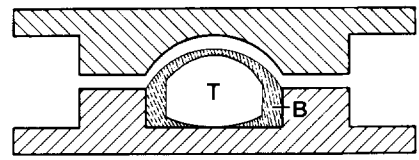


Fig. 2. Profile of specimen-sandwich. *B* buffer solution (droplet), *T* tissue block. The diagram shows the course of cleavage, when the upper platelet is removed after freezing

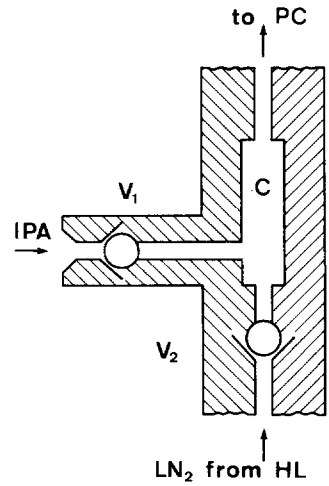


Fig. 3. Injection chamber (*C*) for uptake of isopropyl alcohol (*IPA*). This chamber is inserted into the high pressure line at its entry into the high pressure chamber (*PC*) (see Fig. 1). V_1 and V_2: pressure valves regulating the injection of isopropyl alcohol, which precedes liquid nitrogen in order to delay the cooling (see Fig. 4)

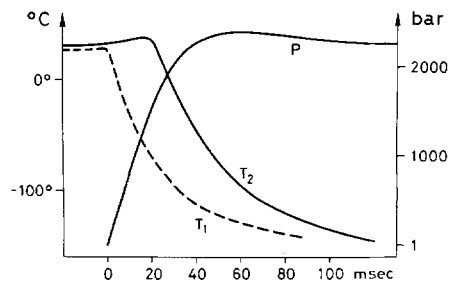


Fig. 4. Temperature (T_1 and T_2) and pressure (p) curves during high pressure freezing. T_1 represents the fall in temperature due to liquid nitrogen injection; it occurs simultaneous with a rise in pressure. T_2 represents the delayed fall to temperature due to the preceding injection of isopropyl alcohol

2. Neuronal Cytoplasm. Samples taken from the cerebellar cortex including Purkinje cell perikarya and dendrites, subjected to conventional cryofixation and 5 min etching, are compared with pressure-frozen samples etched for 1 min. The difference is illustrated in Figs. 5 and 6. Pressure-frozen perikaryal cytoplasm exhibits much deeper etching than a conventionally treated area in spite of a reduced etching time. The enhanced etching allows demonstration of structural details such as microtubules and microfilaments (Fig. 5b), which are often obscured in the glycerol-containing background of specimens processed with conventional techniques (Fig. 5a). An additional reason for the obvious difference between these pictures may be the lack of chemical treatment in the case of pressure-frozen material. Fibrillar and microtubular structures are known to be quite sensitive to both fixatives and high pressure. The results show that the described technique

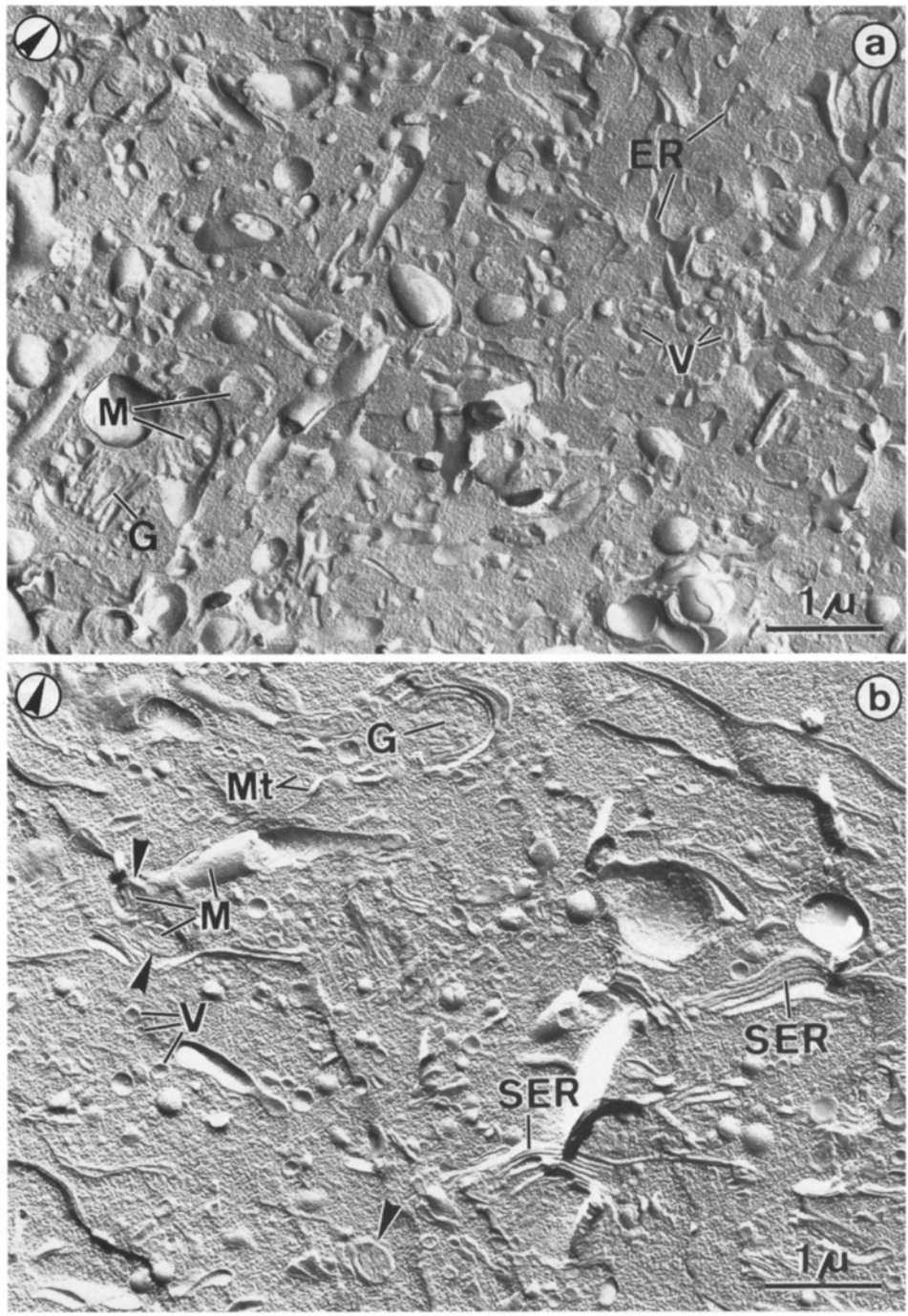


Fig. 5a and b. Purkinje cell cytoplasm, rat cerebellar cortex, containing Golgi complexes (*G*), endoplasmic reticulum (*ER*), microtubuli (*MT*), vesicles (*V*), mitochondria (*M*). Primary magnification × 8000. **a** Aldehyde fixation, glycerol; frozen with Freon: etching time 5 min. Golgi complexes and SER are dilated and distorted; mitochondrial cristae and microtubuli hardly visible. **b** High pressure freezing of tissue pretreated with cacodylate buffer, etching time 1 min. Golgi complexes and ER appear undilated and regularly arranged; mitochondrial cristae and microtubuli are visible (*arrowheads*). Enhancement of etchability of the cytosol as compared with conventional method in (**a**). The *encircled arrows* in this and all the subsequent figures represent the direction of shadow casting

provides a pressurizing period that is short enough to preserve microtubular structures.

3. Endomembrane Systems. The profiles of endoplasmic reticulum and Golgi cisternae exhibit a swollen aspect in fixed and glycerinated cells, which cannot be observed after pressure-freezing (Figs. 5, 6). Such swelling is still more pronounced when unfixed cells are glycerinated (Moor 1971). This would indicate that aldehyde fixation is capable of reducing, but not of preventing the swelling (or shrinkage) of membrane-bound structures.

4. Mitochondria and Nuclear Envelope. Great differences may be encountered in the state of swelling concerning mitochondria and the perinuclear compartment. A comparison of the membrane structure of both "double membrane systems" shows a certain similarity in the response to the applied freezing technique. After fixation and glycerination, the fracture plane follows evenly along the outer or inner membrane, exhibiting a smooth matrix and only few particles. After pressure-freezing of untreated material, the fracture plane more often jumps from the inner to the outer membrane and vice versa. Plaques of material can be observed that seem to be left on the fracture face of the inner nuclear (nucleoplasmic) membrane (*PF*). Membrane particles are more numerous and exhibit a tendency to form linear and reticular arrays (Fig. 7b). The *EF* of the outer mitochondrial membrane, while completely smooth after fixation and glycerination, shows reticular clefts after pressure-freezing (Figs. 6a, 9b). To a lesser extent, similar observations can also be made with respect to other membranes. The fracture plane alternates between the two halves of a membrane more frequently during the cleavage of a chemically untreated specimen, thereby protraying more "inhomogeneities" of a membrane than after the conventional procedure.

5. Cytoplasmic Membranes. An exception to the above mentioned rule is seen in certain myelin structures: Tight junctions in the myelinated axon exhibit less irregularities of the fracture plane after pressure-freezing, and the rows of particles are more clearly visible and show a more linear alignment. On the other hand, the complementary imprints of the particles in the matrix of the opposite half of the membrane are rather faint and often not visible (Fig. 8b, d). A similar observation has been made with respect to gap junctions: The imprints of the gap particles in the opposite membrane leaflet are less pronounced or absent after pressure-freezing (Fig. 10). The myelin stacks seem to be more regular and compact in high pressure-frozen specimens, while after conventional treatment myelin often appears swollen.

Discussion

The comparison of conventionally and pressure-frozen nerve tissue portrayed by freeze-fracture etching shows that the structural features of the specimen are preserved in a quite similar state. One could speculate that now, on the basis of a

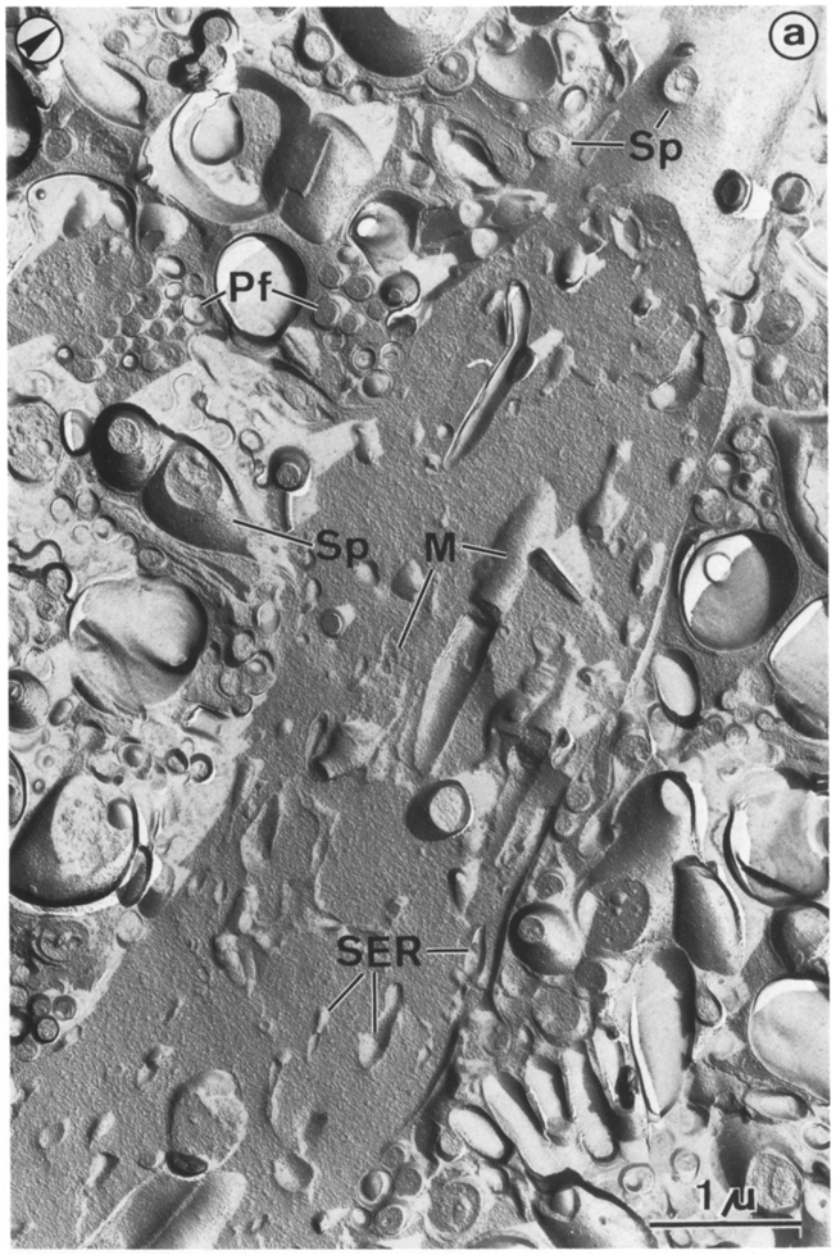
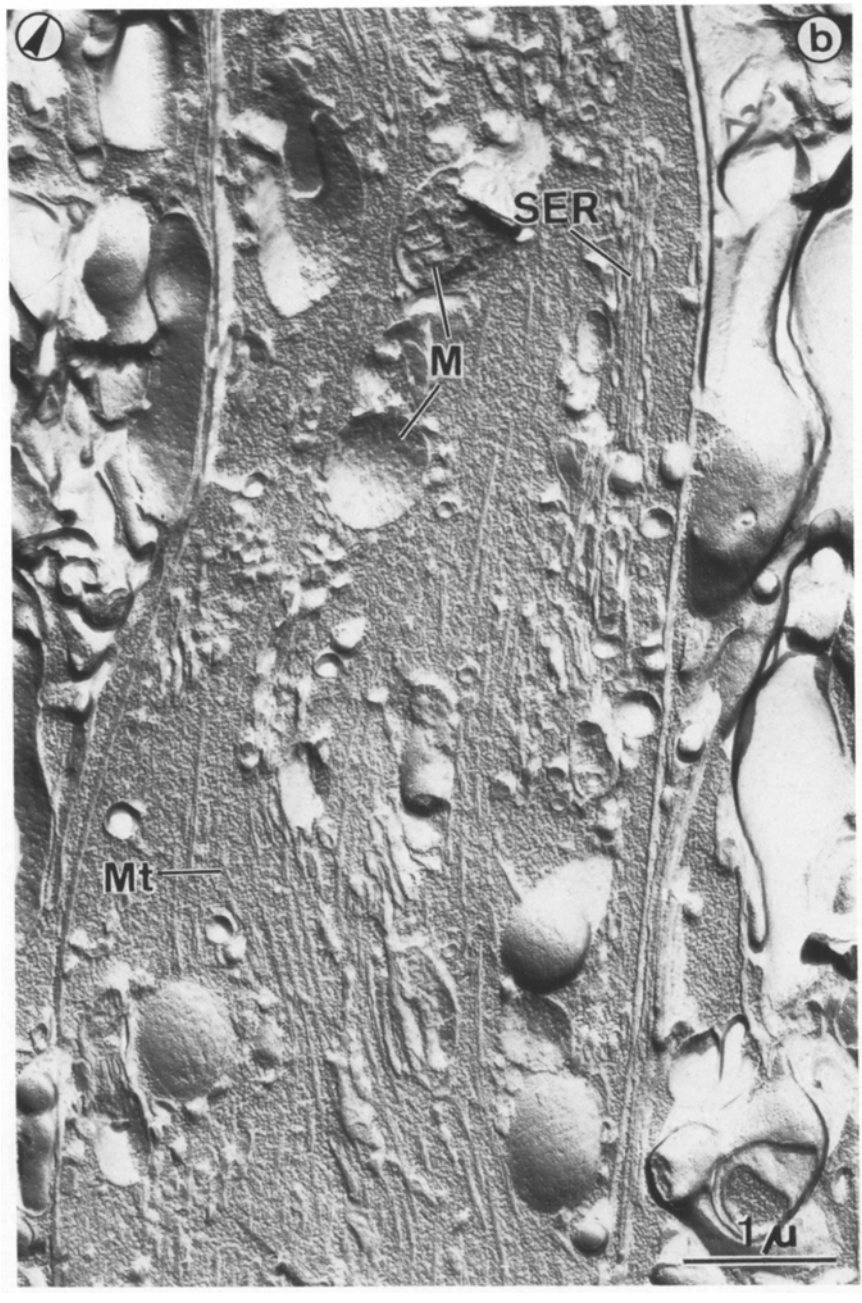


Fig. 6a and b. Dendrite of Purkinje cell, rat cerebellar cortex. Primary magnification ×20,000. **a** Conventional method. Etching time 5 min. Cytoplasm with low etchability contains mitochondria and tubular *SER*. *SP* dendritic spines, *Pf* parallel fibres. **b** High-pressure freezing. Etching time 1 min. Cytoplasm with increased etchability shows microtubuli (*Mt*), mitochondria (*M*) and smooth endoplasmic reticulum (*SER*)


b
SER
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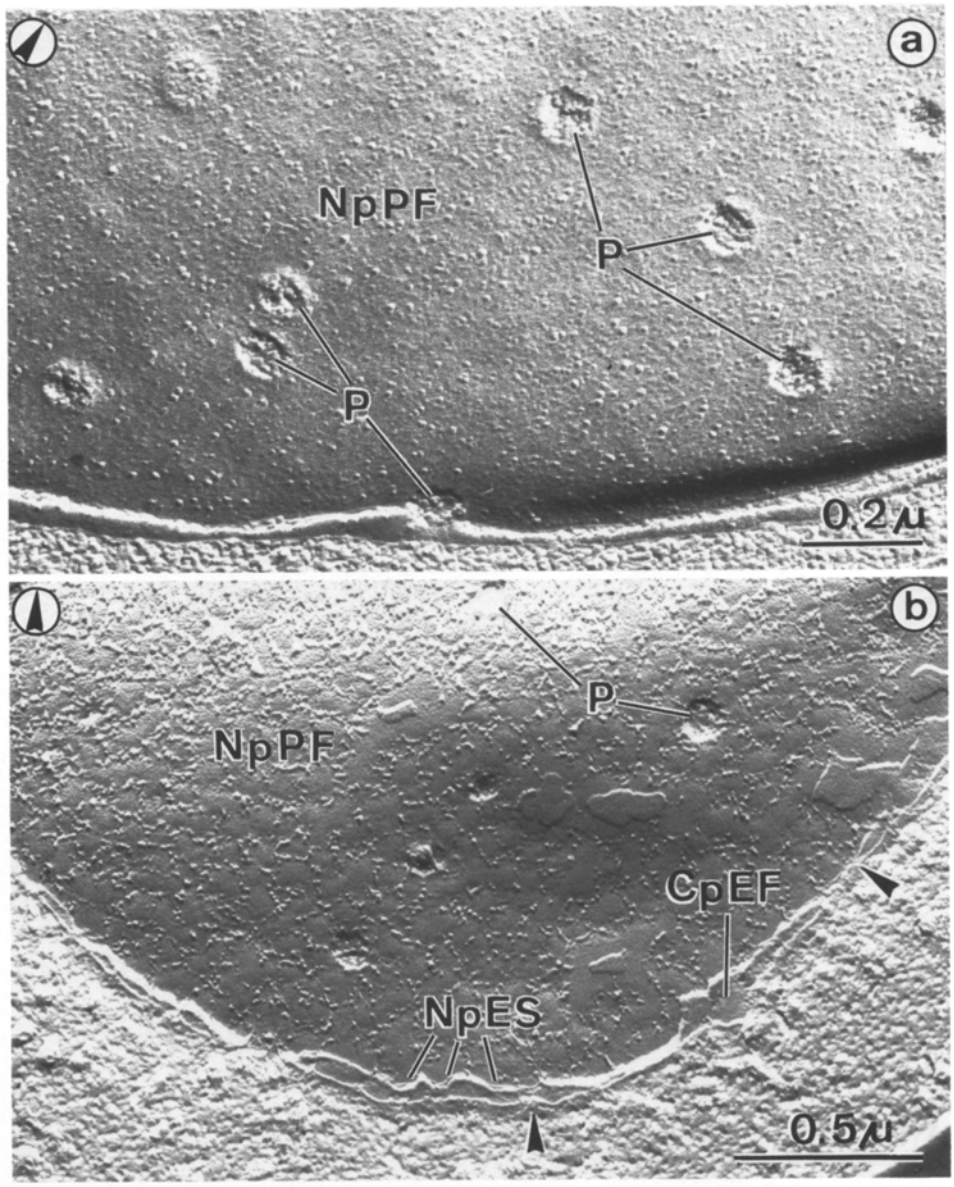


Fig. 7 a and b. Nuclear envelope of glial cell showing nuclear pores (*P*); rat cerebellar cortex. Primary magnification ×20,000. *Np* nucleoplasmic (inner) membrane; *Cp* cytoplasmic (outer) membrane. **a** Conventional method. Etching time 5 min. **b** High pressure freezing. Etching time 1 min. Note the distinct border of nuclear pores and the relatively smooth membrane face in (**a**), and the low profiles and irregular contours of nuclear pores in (**b**). The nucleoplasmic PF in (**b**) appears to contain more particles of various sizes, which are arranged in short chains forming an irregular network. Small polygonal membrane fragments (*arrowheads*) may represent the outer nucleoplasmic surface of the nuclear envelope (*NpES*). In contrast to (**a**), the etching exposes here not only inner membrane faces of the inner (*NpPF*) and outer (*CpEF*) nuclear envelope, but also external surfaces as well. This is best seen at the circumferential fracture line (*arrowheads*)

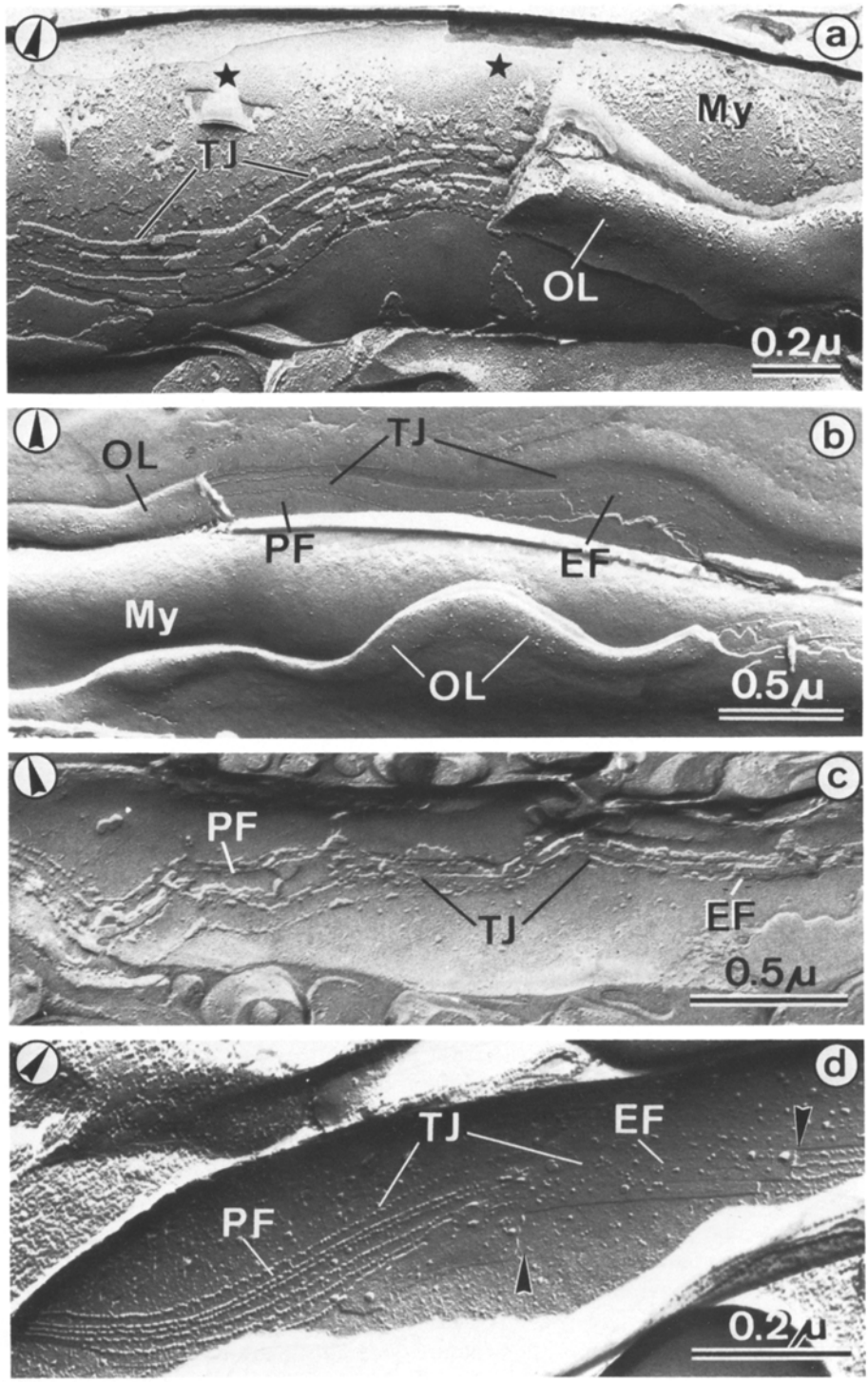
preparation technique that works without any chemical treatment and stabilizes the living object nearly instantaneously, the most reliable recording of cellular ultrastructure is realized and, because the results of this technique are comparable with conventional cryofixation and fixation for thin section purposes, can be used as a standard in evaluating the reliability of data derived from other methods. However, this conclusion may be slightly exaggerated and misleading, for every method has its own field of application and its special value. Pressure-freezing contributes to the preservation and presentation of cellular ultrastructure not so much in terms of quality, but rather in certain areas by supplying additional information. These new aspects are briefly discussed below.

Most probably the osmotic conditions varying between the various cellular compartments are less altered by pressure-freezing than by the conventional method. Therefore, differences in the shape of organelles as well as variations in extracellular spaces can be observed. However, the opposite view cannot be excluded, because it is not known how rapidly perfusion fixation with aldehydes break down the diffusion barriers, on the one hand, and what kind of alterations are introduced by dissecting and compressing an unstabilized, living piece of tissue, on the other hand.

The smooth and homogeneous aspect of membrane fracture faces after conventional cryofixation seems to be a certificate for structural integrity and hence for artifact-free replication. Such pictures confirm the assumption that fracturing separates the two leaflets of a membrane very regularly and that the cleavage plane is only interrupted at the site of specialized intramembranous structures. Replicas of pressure-frozen specimens indicate that membrane structures may be more complex than previously assumed, since new details can be visualized that still require explanation. For example, membrane fragments are observed that most probably represent areas where the cleavage plane follows the membrane surface rather that the center, thereby exposing additional features of the mosaic structure of membranes.

The appearance and distribution of intramembranous particles (the sense of the term not being fully self-explanatory), may differ greatly between specimens prepared in alternative ways. The question arises whether linear and reticular arrays or a more random distribution of the particles reflects the natural state of membranes. Two things may have happened: On the one hand, aldehyde fixation could be responsible for stabilizing proteins and keeping them in the original position, while in unfixed specimens such structures are mobile and could be subject to drifting and clustering during the period of tissue preparation. Phase separation of segregation phenomena, as encountered in relatively slowly frozen membranes, could be induced by pressurizing, however, the time left for such alterations is extremely short (about 30 ms). On the other hand, the action of buffers and/or fixatives and/or cryotectants could weaken interactions between membrane components, i.e., enhance their mobility and thereby provoking a uniform distribution of originally aligned or clustered elements.

An additional phenomenon, which awaits elucidation, is the lower degree of complementarity in certain pressure-frozen specimens. Since the first paper on improved replication of membrane structures with ultrahigh vacuum freeze-etching at –196° C (Gross et al. 1978), the assumption has been generally adopted that the


a
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structures exposed in membrane fracture faces (the authors used unfixed and unglycerinated yeast cells) are susceptible to alterations when prepared at –100 or –110° C, and that the structures of the ER-face, which in most cases is a lipid replica of the PF-face, are most sensitive. Heat input by disrupting structures during the cleavage process most probably elevates the temperature in the zone of fracturing, thus, plastic deformation and/or local melting of membrane components should be taken into account. Such phenomena can be reduced or eliminated when cleaving occurs far below –100° C. In our own specimens, we compared samples etched at –110° C and found differences in conservation of EF-face structures in tight- and gap junctions. They are obviously prepared in a critical temperature range, and therefore already small differences in rigidity and/or melting temperature may be responsible for sufficient or insufficient structural stability. Therefore, we may assume that chemical fixation could stabilize membrane structure, e.g., by "hardening" the lipids. As a consequence, unfixed specimens would be more sensitive and should be freeze-fractured at much lower temperatures, if structural complementarity of fracture faces is required.

The application of high hydrostatic pressure can be easily criticized, due to its lethal nature. Much less is known about its mode of operation: Main factors may be conformational alteration and inactivation of essential proteins, the decay of microtubules and microfilaments causing irreparable disorder and the rupture of membranes. In an initial attempt to obtain information on possible damage caused by pressure-freezing, we investigated survival rates of *Euglena* cells pressurized for different periods of time (Moor and Hoechli 1970). These experiments showed that the briefer the period of exposure to pressure, the greater is the pressure that can be tolerated. About 90 % of the *Euglena* cells survived when subjected to 2,100 bars for not more than 0.1 s. In our apparatus, this time is reduced by a factor of 3. This indicates that we actually portray cells that have been frozen in the living state. The limited experience with pressure-frozen specimen indicates that the portrayed cells are free of pressure artifacts: membranes and microtubules are well preserved as shown in the illustrations.

Improved etchability of the cytoplasm in pressure-frozen material is explained by the absence of highly concentrated dissolved cryoprotectants. Therefore, cytoplasmic structures, such as microtubules, microfilaments, "side arms" and

Fig. 8a–d. Myelinated axon in cerebellar cortex of the rat. Outer loop (*OL*) with typical belt of tight junctions (*TJ*). Primary magnification × 10,000 in (**a, b, c**) and × 20,000 in (**d**). **a** Conventional method. Etching time 5 min. Nerve fiber, external view. The fractured outer loop exposes multistranded tight junctions. The fracture plane is irregular and exposes severely fragmented membrane leaflets. The myelin (*My*) membrane faces contain bulging areas which lack particles (*asterisks*). **b** High pressure freezing. Etching time 1 min. Two parallel nerve fibers, external view. The myelin sheet (*My*) and the membrane of the outer loop is smooth. The fracture plane is clearly defined in the region of the tight junctions with distinct steps between external and cytoplasmic faces (*EF, PF*). The tight junctional "grooves" in the EF are not readily visible. **c** Conventional method. Etching time 5 min. Outer loop membrane (*EF, PF*) with tight junctions showing the same irregularities as in Fig. 8a. **d** High pressure freezing. Etching time 1 min. Same situation as in Fig. 8c. Tight junctional strands and fracture planes are clearly defined. The "grooves" in the EF face representing the complementary impressions of the tight junctional strands of particles in the PF face are hardly visible (*arrowheads*)

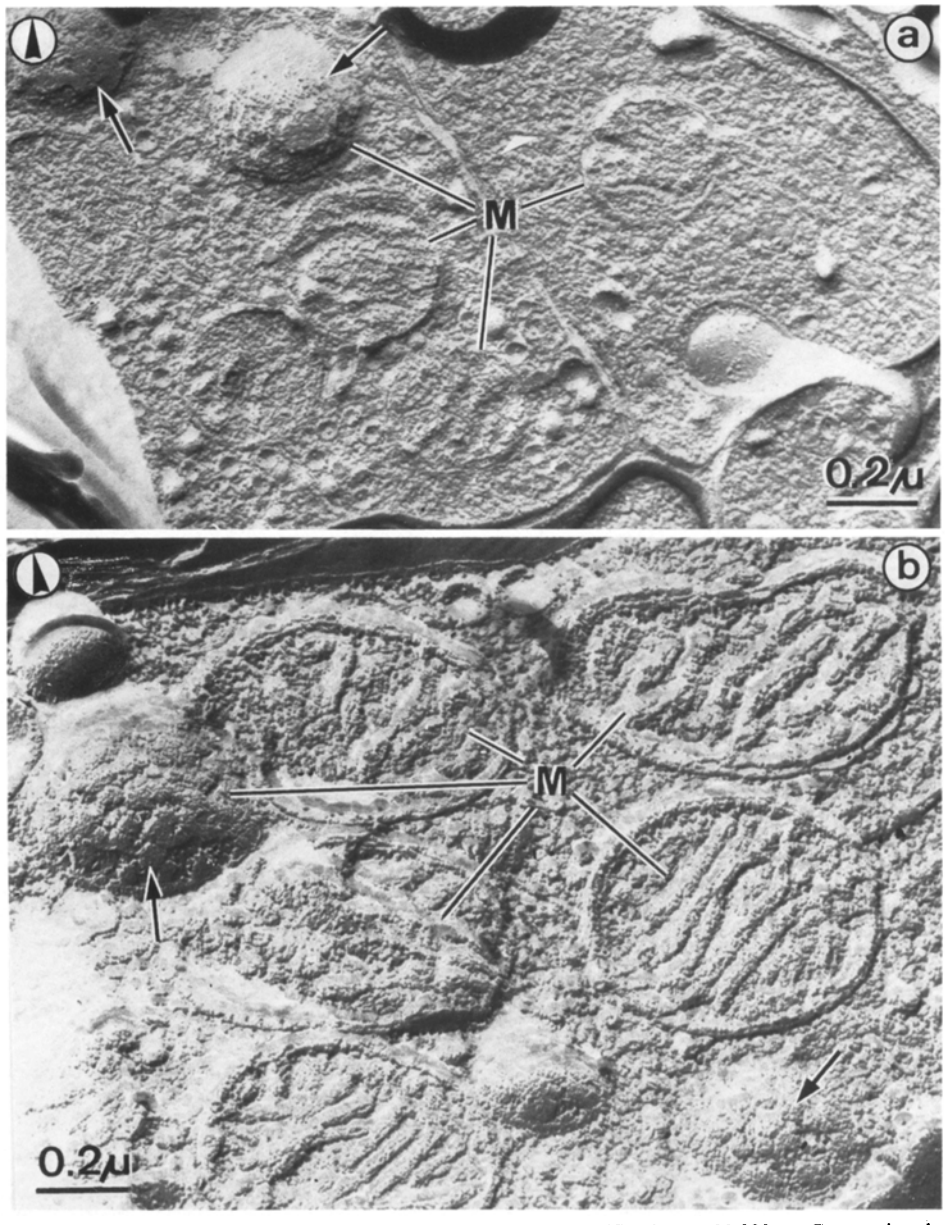

Fig. 9a and b. Mitochondria (*M*) in nerve axon. Primary magnification ×20,000. **a** Conventional freezing: Note the low etchability and hence the blurred appearance of membrane structure in cross-fracture. The face view of the wall exhibits a rather smooth structure plane (*arrows*). **b** High-pressure freezing; 1 min etching. Note the distinct appearance of the double membrane of wall and cristae and the face-view of the wall exhibiting many discontinuities in the course of the fracture plane (*arrows*)

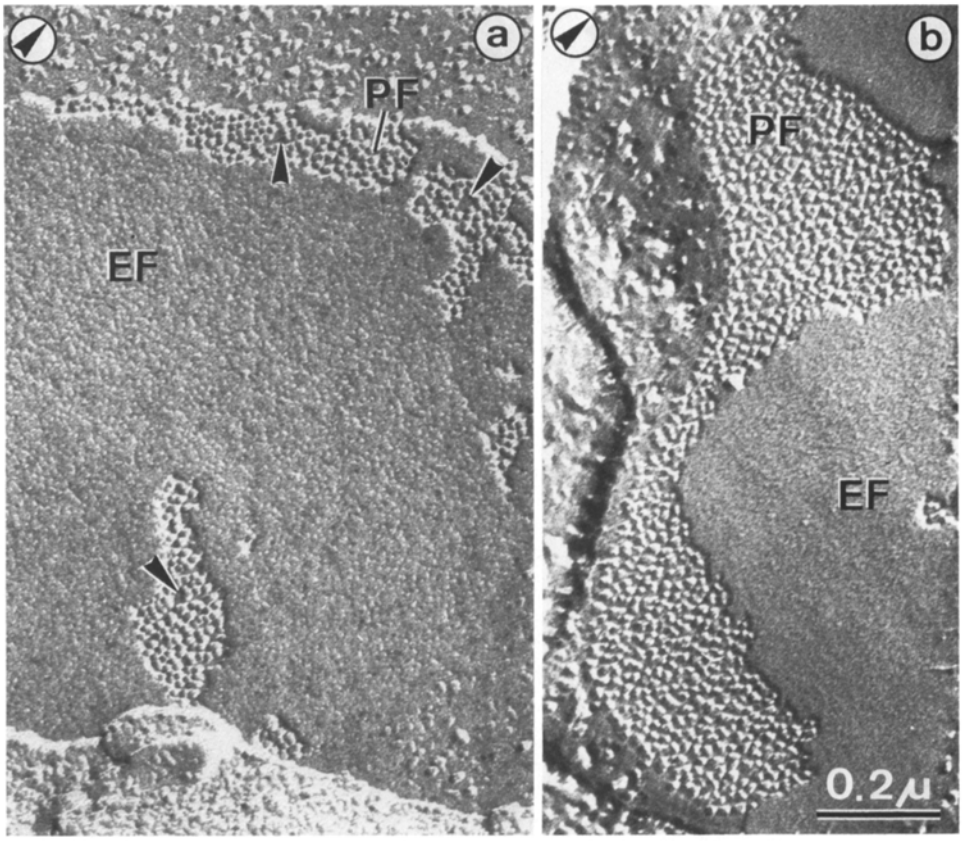

Fig. 10a and b. Gap junctions between glial cells, rat cerebellar cortex. Primary magnification. × 20,000. **a** Conventional method. Etching time 5 min. **b** High pressure freezing. Etching time 1 min. Note that the PF particles in (**b**) are more regularly arranged; there are no "missing particles" (*arrowheads*). On the other hand, the complementary EF pits seen in (**a**) are missing in (**b**)

anchor proteins may be particularly well exposed for portraying with heavy metal coating.

The application of pressure-freezing is not restricted to freeze-etching; it offers promising results in combination with freeze-substitution, conventional embedding and thin sectioning. Properly frozen and at very low temperature, substituted and chemically fixed specimens may offer enhanced preservation of fragile structures and soluble components. Such features are prerequisites for cytochemical reactions and the application of energy dispersive analysis of X-rays.

References

Branton D, Bullivant S, Gilula NB, Karnovsky MJ, Moor H, Mühlethaler K, Northcote DH, Packer L, Satir B, Satir P, Speth V, Staehelin LA, Steere RL, Weinstein RS (1975) Freeze-etching nomenclature. Science Wash 190:54–56

Gross H, Bas E, Moor H (1978) Freeze-fracturing in ultra high vacuum at −196° C. J Cell Biol 76:712–728

McIntyre JA, Gilula NB, Karnovsky MJ (1974) Cryoprotectant-induced redistribution of intramembranous particles in mouse lymphocytes. J Cell Biol 60:192–203

Moor H (1964) Die Gefrierfixation lebender Zellen und ihre Anwendung in der Elektronenmikroskopie. Z Zellforsch 62:546–580

Moor H (1971) Recent progress in the freeze-etching technique. Phil Trans R Soc Lond B 261:121–131

Moor H (1973) Cryotechnology for the structural analysis of biological material. In: Benedetti EL, Favard P (eds) Freeze-etching techniques and applications. Société Française de Microscopie Electronique, Paris, p 11–19

Moor H (1977) Limitations and prospects of freeze-fixation and freeze-etching. In: Thirty-Fifth Annual EMSA-Meeting, 1977, Proc Electron Microscopical Society of America, p 334–337

Moor H, Mühlethaler K (1963) Fine structure in frozen-etched yeast cells. J Cell Biol 17:609–628

Moor H, Hoechli M (1970) The influence of high-pressure freezing on living cells. In: Favard P (ed) Proc 7th Intern Congr Electron Microsc Grenoble, Soc Franc Microsc Electron, Paris, Vol 1, p 449–450

Riehle U (1968) Ueber die Vitrifizierung verdünnter wässriger Lösungen. Diss. Nr. 4271, ETH Zürich, Juris Verlag, Zürich

Riehle U, Hoechli M (1973) The theory and technique of high pressure freezing. In: Benedetti EL, Favard P (eds) Freeze-etching technique and applications. Société Française de Microscopie Electronique, Paris, p 31–61

Sandri C, Van Buren JM, Akert K (1977) Membrane morphology of the vertebrate nervous system. A study with freeze-etch technique. In: Progress in Brain Research, Elsevier, Amsterdam, Vol 46, p 1–384

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