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FREEZE SUBSTITUTION AND LOW TEMPERATURE EMBEDDING

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Abstract

The problems of conventional EM-preparation techniques based on chemical fixation may be overcome to a considerable extent by freeze substitution techniques. Although at present substitution cannot be performed at sufficiently low temperatures to prevent the recrystallization of vitrified aqueous specimens, thin sectioned biological samples show an improved information density. If freeze-substitution is combined with conventional embedding above 273 K (Epon/Araldite, Spurr's resin) the substituting organic solvent must contain stabilizing agents such as osmiumtetroxide, glutaraldehyde or uranylions. In combination with low temperature embedding procedures (Lowicryl) completely unfixed samples are obtained, which are suitable for immunolabelling and electron spectroscopic experiments. Water in its different dynamic states is considered to be the most important factor in maintaining the structural and functional integrity. Thus, the main advantage of freeze substitution is a better control over the removal of the cellular water, necessary for subsequent plastic embedding.

Key Words: Rapid freezing, Freeze-substitution, Low temperature embedding, Dehydration, Electron spectroscopic imaging.

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Introduction

Freeze substitution (= dissolution of the frozen water by an organic solvent at low temperatures) is a technique originally developed in light microscopy. It was introduced to electron microscopy approximately 30 years ago. For a review see reference (10, 13). Freeze substitution is frequently used to prepare cryofixed biological samples for X-ray microanalysis (33). An improved structural preservation has also been demonstrated (18, 19, 30, 36). If freeze substitution and embedding can be performed under controlled conditions, not only the preservation of the structural but also of the functional integrity (e.g. enzyme activities, antigenicity) may be achieved. Here we report experiments directed towards this goal.

In order to obtain more detailed, reliable structural information (= improved resolution), an improved preservation of the biological structures is needed first. During chemical fixation, immobilization and stabilization of the structures occur relatively slowly and there remains always an uncertainty whether or to what extent a structural detail is a function of the applied preparation procedure (22). Membranes change their osmotic behaviour unpredictably, thus causing dimensional changes of organelles and other structural features. The fixatives may denature or depolymerize macromolecules (8). Prior to embedding in hydrophobic plastics, the fixed samples have to be dehydrated. There is some evidence (6, 32) that water in its different dynamic states is the most important factor in maintaining the structural and functional integrity. Upon dehydration the cells shrink up to 30 % of their initial volume (3) and macromolecules may collapse. The temperatures at which different types of macromolecules collapse when exposed to different dehydrating agents (organic solvents, vacuum) were determined by MacKenzie (24) who used a freeze substituting and a freeze drying light microscope. These temperatures range from 215 K to 263 K.

In contrast to conventional chemical fixation, freeze substitution may allow to control the immobilization, stabilization and dehydration steps separately.

Immobilization by cryofixation

The most limiting prerequisites for successful freeze substitution experiments are cryofixation techniques. These must produce very high freezing rates which lead to the solidification of the aqueous biological material in the vitreous state or at least in a microcrystalline state, where the ice crystals are so small, that they do not introduce any detectable structural alterations. Several techniques are available for the rapid freezing of thin aqueous layers of suspensions up to approximately 20 µm (spray-freezing (2), propane-jet freezing (27, 28, 20), metal mirror freezing (37, 15, 11), plunge freezing (7, 12). We obtain most reproducible results by freezing suspensions sandwiched between two low mass copper platelets in a two sided propane jet (20). The specimen sandwich is prepared by dipping a 12 µm thick gold grid into the supsension. The grid is then placed between the copper platelets, where it serves as a spacer. The very carefully manufactured copper platelets together with the spacer guarantee for a constant specimen geometry and thus for a high reproducibility. The frozen specimen sandwiches are used for freeze fracture as well as for freeze substitution experiments. It is assumed, that similar results are obtained by freezing such sandwiches using an optimized plunging system. Heat can only be extracted from the specimen through the surface by conduction. Because of the low thermal conductivity of the water only a thin superficial layer of tissues is frozen rapidly enough to prevent the formation of detectable ice crystals. Thicker samples are only sufficiently well frozen, if the physical properties of the water are changed. This is achieved by the application of cryoprotectants which are frequently used in combination with aldehyde prefixation, or by rapid freezing under a pressure of 2100 bar. By high pressure freezing (35, 26, 29), the freezing point is depressed to 251 K and the crystal growth and nucleation rates are drastically reduced. Under this condition, the freezing rate obtained in the center of a 600 µm thick sample is still sufficient to prevent the formation of detectable ice crystals.

Equipment for freeze substitution and low temperature embedding

In order to facilitate freeze substitution and low temperature embedding experiments, we built a special cryostat. The cryostat consists of a cylindrical brass container, the temperature of which is controlled by a 3 point regulator via a Pt 100 resistor. Cooling and heating are achieved by liquid nitrogen and heat cartridges respectively (Fig. 1A). A presetable control unit is used that changes the temperature automatically. It can be programmed for 4 different temperatures (between 77 K and 273 K), kept constant for different times (1 min - 100 days) (Fig. 1D). We routinely freeze substitute in Eppendorf tubes (1.5 ml). Whereas the handling of tissues is very simple, suspensions have to be pelleted, when the media are changed during freeze substitution and embedding. After freeze substitution even very small objects like liposomes (14, 31) are spun down in a Beckman microfuge B. In order to maintain the necessary high subzero temperatures, we placed the microfuge in a normal household freezer and surrounded it with a copper-cylinder cooled by liquid nitrogen (Fig. 1B). With the help of a two point regulator, temperatures down to 183 K are maintained, the centrifuge working properly at this low temperature. After centrifugation for 5 min, the temperature of the samples increased by about 10-25 K. Low temperature embedding with Lowicryl and UV polymerization are performed in the same Eppendorf tubes. The tubes are suspended in a rack which stands in a reflecting stainless steel container that fits into the cryostat (Fig. 1C).

The stainless steel container is filled with ethanol to guarantee an optimal cooling of the samples. In order to minimize the effect of oxygen, by which a component of the Lowicryl necessary for polymerization is inactivated, the Eppendorf tubes are completely filled and their covers closed. Indirect UV-radiation is achieved by placing a cover on top of the samples. This set-up guarantees that the polymerization starts at the tip of the tubes, where the tissue piece or the small pellet is located. Low temperature polymerization is performed at 243 K overnight with 2 UV lamps (Sylvania F8T4/350BL) situated 10 cm above the sample tubes and completed by additional UV irradiation at room temperature (Fig. 1A).

Based on the instrumentation outlined above, an apparatus suitable for freeze-substitution and low temperature embedding was made commercially available by Balzers Union, Balzers, Fürstentum Liechtenstein.

Dehydration by freeze substitution

It is generally assumed that in biological samples the danger of ice crystal growth and recrystallization is minimal at temperatures below 188 K. Since vitrified water is only stable below 143 K an ideal freeze substituent would of course dissolve ice sufficiently well at temperatures below 143 K (35). But so far we have not been able to perform freeze substitution experiments below 183 K in reasonable time.

In order to learn more about the substitution process we examined the substitution at 183 K, 213 K and at 243 K with diethylether (13, 21), acetone (37, 23, 10, 34, 36) and methanol (41, 30). As a test specimen, we used 1 µl of tritiated water, soaked into a 3 mm disc of filter paper. The soaked filter paper was frozen in liquid nitrogen and immersed into 1 ml of the substituent (16). At different time intervals the released radioactivity was assessed by scintillation counting. Fig. 2 shows, that at 183 K dry methanol and acetone dissolved the ice

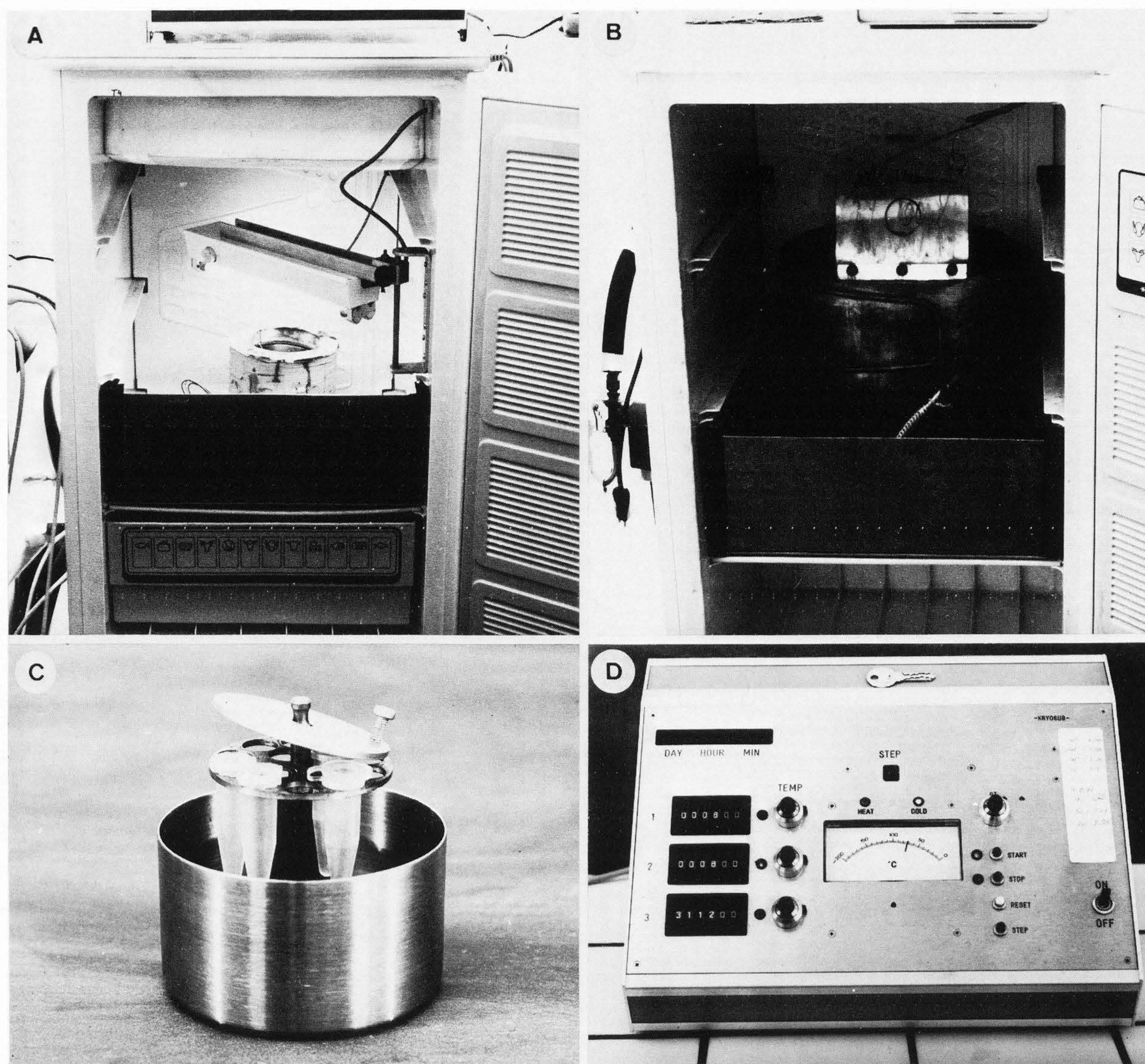


Fig. 1: Illustrates the equipment used. A) The cryostat placed in a freezer with the ultra-violet (UV)-lamps for low temperature polymerization. B) A Beckman Microfuge B, surrounded with a LN_2 cooled copper cylinder for centrifugation down to 183 K. C) The reflecting stainless steel container, used for low temperature polymerization. D) Temperature control unit. For explanations see text.

specimen within a very short time whereas diethylether did not dissolve any detectable amount within 8 h. At 213 K diethylether dissolved only a little bit of ice, whereas acetone and methanol substituted very fast. At 243 K ether is also capable of dissolving all the ice within 8 h.

Since we are interested in finding ways to control the residual water content, i.e., how much water has to be left so that the cells maintain their structural and functional integrity, and how much water has to be removed to allow a successful plastic embedding, we studied the substitution capacity of the solvents containing already a certain amount of water. At 183 K the substitution capacity of acetone (Fig. 3) is already prolonged 4 times in the presence of 1 % of water whereas methanol (Fig. 4) substitutes rapidly with a water content of up to 10 % at 183 K. Thus the combination of these solvents with different water contents may allow for a controlled dehydration.

Stabilization during freeze substitution

If freeze substitution is performed with a substituent containing fixatives, the stabilization of biological structures takes place at the

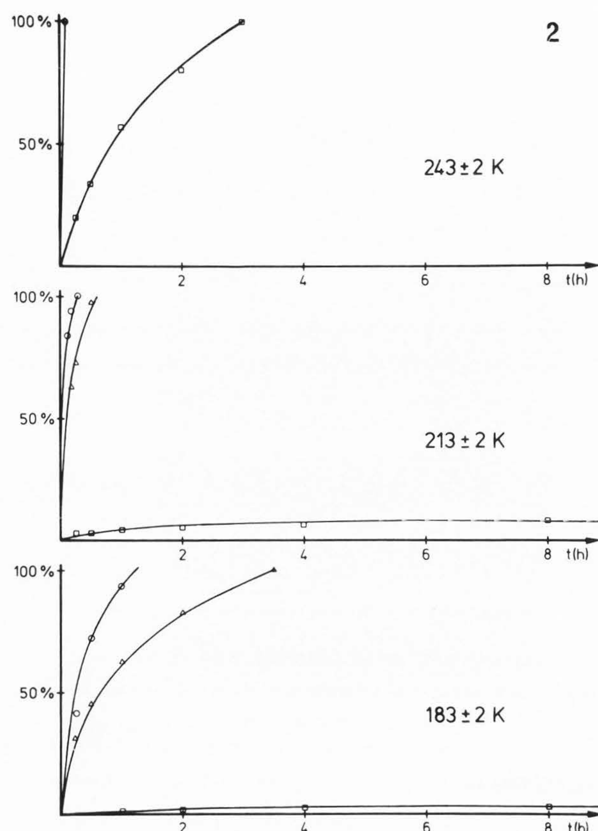


Fig. 2: Substitution speed of different solvents at 183 K, 213 K and 243 K. o methanol, ▵ acetone, ▫ diethylether. Ordinate: relative amount of ^3H_2O dissolved. Abscissa: time of substitution.

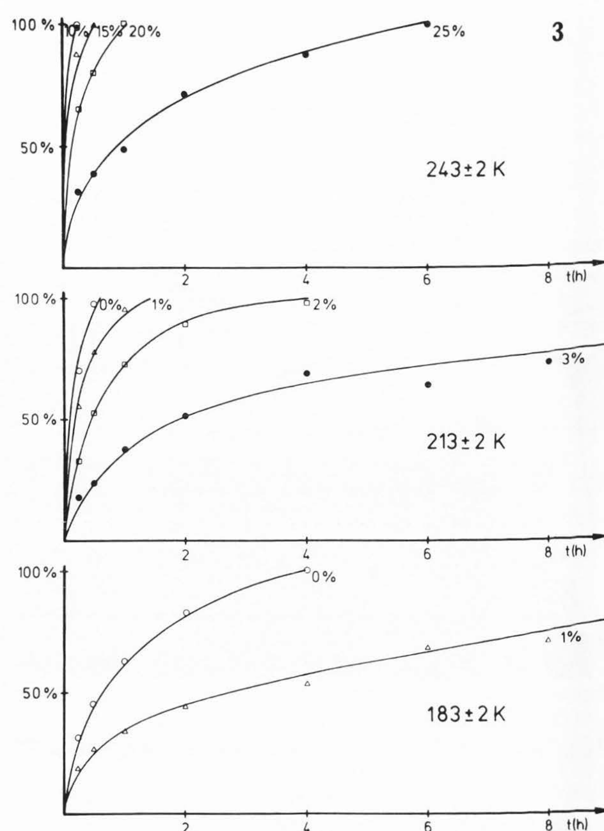


Fig. 3: Substitution by acetone with different water contents. Ordinate: relative amount of ^3H_2O dissolved. Abscissa: time of substitution.

ice-solvent interphase. Fixatives may be necessary to reduce solvent effects such as the loss of lipids and other low molecular constituents and conformational changes of the proteins. Excellent structural preservation is obtained by OsO_4 in acetone or methanol (18, 19, 36). Aldehydes like acrolein (41, 25) or glutaraldehyde (30) have been used either alone or in combination with OsO_4 and/or uranylacetate. The presence of fixatives is necessary, if freeze substitution is combined with conventional embedding. Whereas an excellent structural preservation is obtained, the functional integrity may be affected. Nevertheless, Inoué (18) still found preserved antigenic sites after freeze substitution at 193 K in acetone containing 2-5 % of OsO_4, followed by embedding in Epon/Araldite. There is experimental evidence that uranylacetate reacts at the lowest temperature. The reaction of OsO_4 with the double bonds of unsaturated fatty acids has been reported to occur at 203 K (40).

In order to get some estimate of the action of glutaraldehyde, an aqueous 2 % BSA solution was freeze-substituted in methanol containing 3 % glutaraldehyde (16) (Fig. 5). Ten µl of the BSA solution was injected into the substitution medium kept at 183 K in the cryostat. After 8 h the medium was warmed up to the examination temperature. At intervals of 15-60 min (depending on the temperature) the reaction of the glutaraldehyde was stopped by removing the substitution medium and subsequent washing in pure methanol at the temperature of the experiment. The crosslinked protein was pelleted in a Beckman microfuge B, which can be operated at temperatures down to 183 K. The specimen was then warmed up to room temperature and washed with bidistilled water to ensure that all soluble protein was removed. The crosslinked protein was dissolved in 1 N sodium hydroxide and determined according to Lowry (23).

The choice of fixative may depend on the substituent, on the embedding procedure and whether a structural and/or functional preservation is to be achieved. If freeze substitution is used to prepare specimens for X-ray microanalysis of diffusible ions, an apolar solvent (e.g., diethylether) without any fixatives is preferred (13).

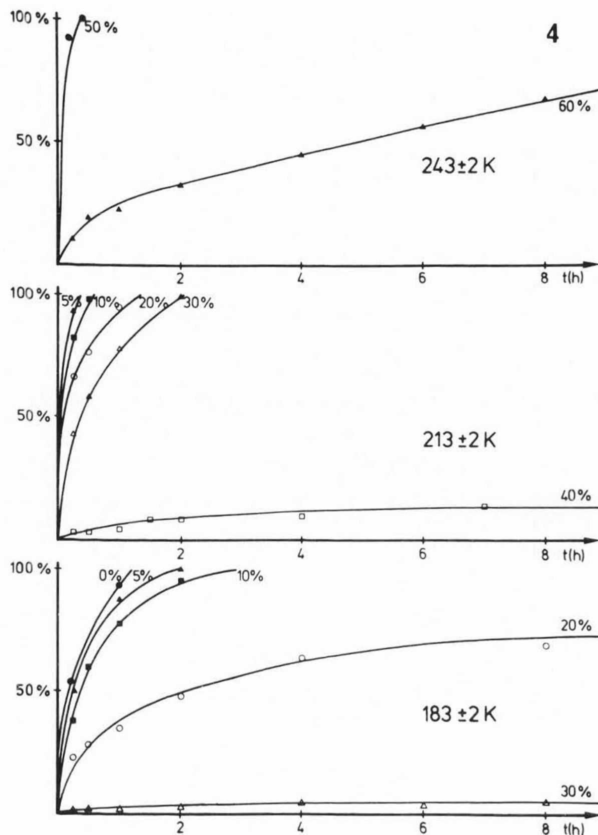
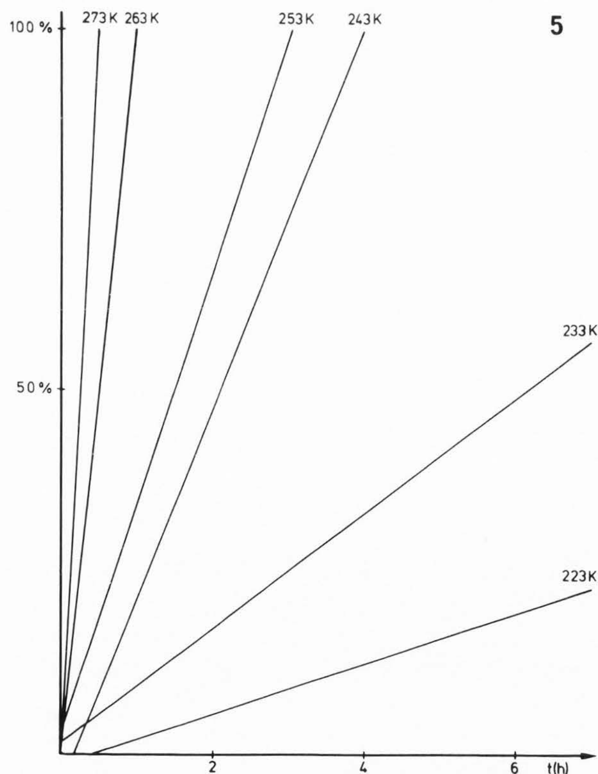


Fig. 4: Substitution by methanol with different water contents. Ordinate: relative amount of $^{3}H_2O$ dissolved. Abscissa: time of substitution.



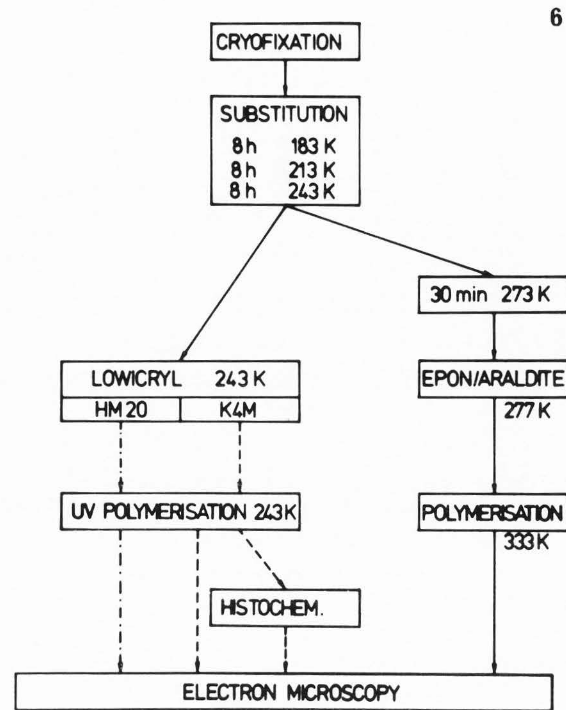


Fig. 6: Schematic representation of the embedding procedures.

Embedding of freeze-substituted samples

The substitution and embedding procedures which we use are schematically depicted in Fig. 6 (30). For routine applications, a complex substitution medium is used. It consists of methanol containing 1 % OsO_4, 0.5 % UO_2Ac_2, 3 % GA and 3 % water (30). The cryofixed specimen is immersed in 1.5 ml of the substitution medium kept at 183 K. The substitution is usually accomplished after 24 h, the specimen being kept at 183 K, 213 K and 243 K for 8 h at each temperature. Prior to embedding in Epon/Araldite, an additional step of 30 min at 273 K is added. The substitution medium is then replaced by anhydrous acetone. Embedding is done in the conventional way at 277 K, followed by polymerization at 333 K. With this routine protocol an improved structural preservation could be demonstrated with many biological samples (14, 9, 17). The routine processing of lipid vesicles prepared of different kinds of lipids was possible with this technique followed by conventional or low temperature embedding (14, 31, 38) and periodic protein structures of bacillus spore coats (9) were much better preserved than by conventional procedures.

If freeze substitution is used in combination with low temperature embedding in Lowicryl K4M or HM20 (4) the complex medium described above can be used as well. If the temperature does not

Fig. 5: Crosslinking properties of 3 % glutaraldehyde in methanol at different temperatures. Ordinate: relative amount of crosslinked protein. Abscissa: time of fixation.

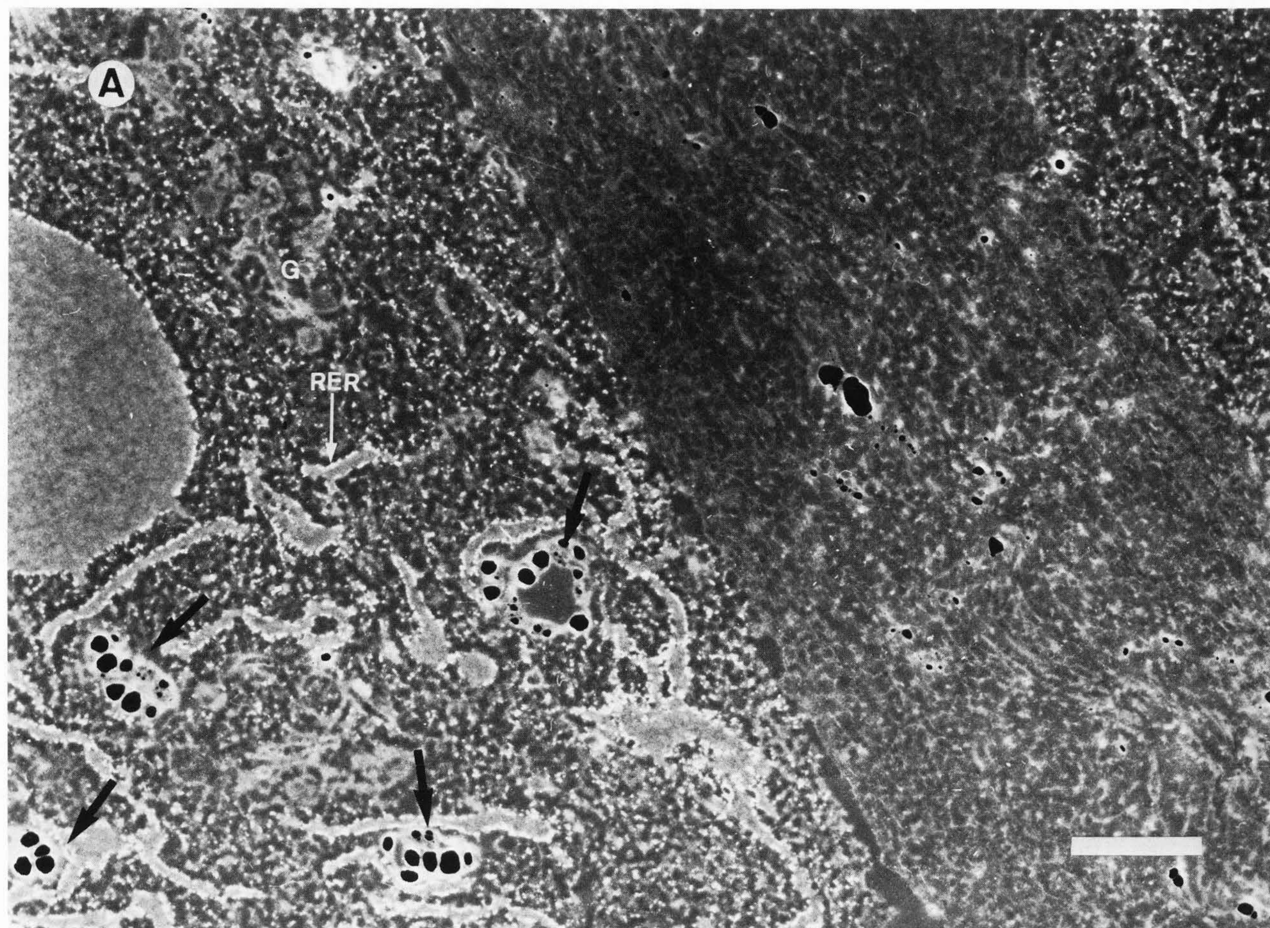


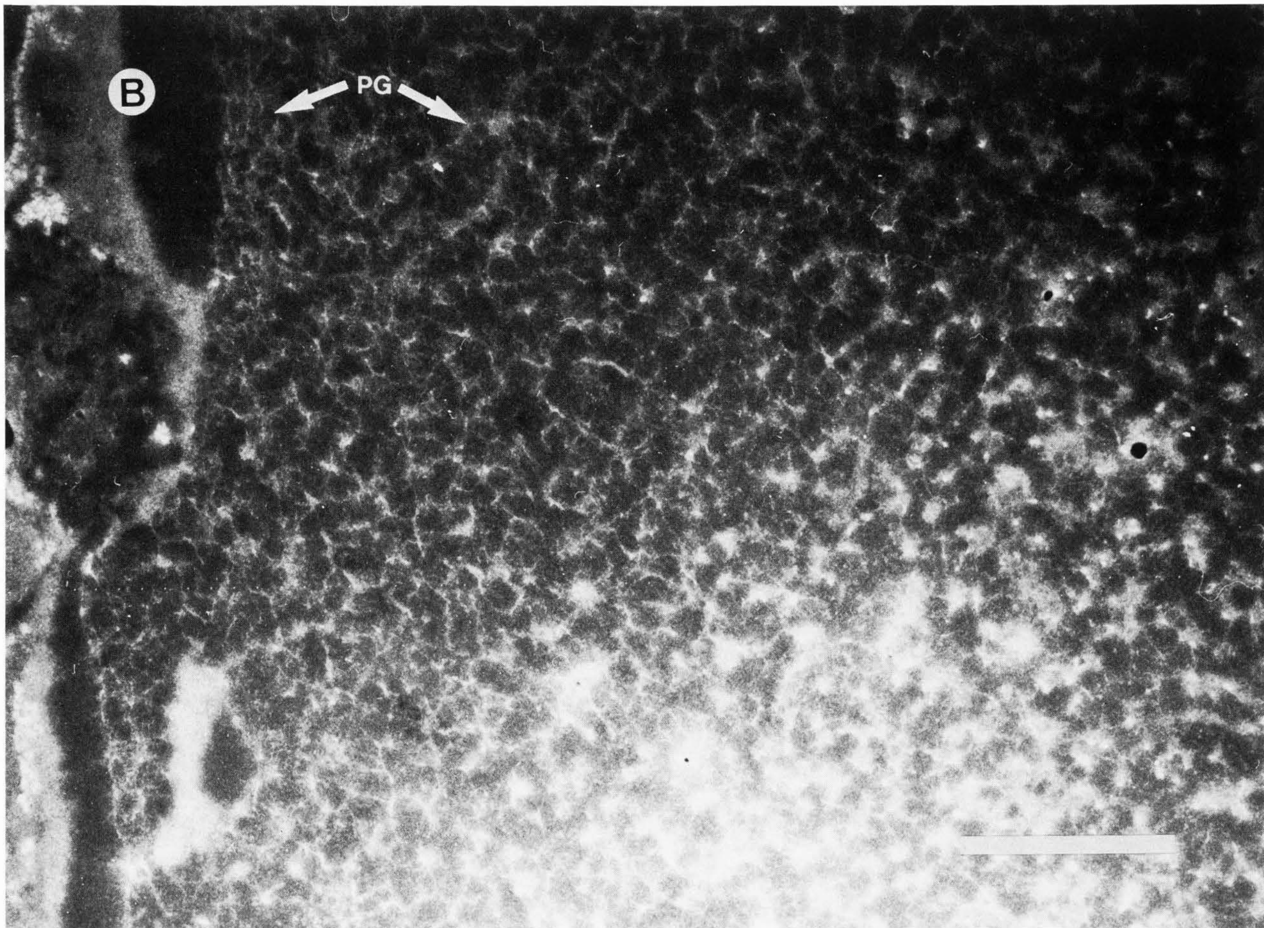
Fig. 7: Shows a portion of high pressure frozen, freeze-substituted and Lowicryl HM20 embedded rat growth cartilage. The very thin section is not fixed or stained at all. Micrographs are taken at an energy loss $\Delta E = 200 \pm 10$ eV. In Fig. 7A, the arrows point to imperfectly embedded structures within the chondrocyte. Note the appearance of the rough endoplasmic reticulum (RER) and the Golgi complex (G). Bar = 1 µm. Fig. 7B (see facing page) shows the matrix between the chondrocytes at a higher magnification (bar = 0.5 µm). Note the excellent contrast of the proteoglycans (PG).

exceed 243 K tissue blocks or pellets appear light brownish, which still allows for UV polymerization at 243 K.

Positive results are also obtained, however, by using the pure solvent or the solvent containing only one of the stabilizing agents (16, 17). For low temperature embedding, attention has to be paid that the sample never gets too warm, until polymerization is completed. In practical work 243 K is a compromise. If the temperature is kept too low, not all the water (which is supposed to be necessary for the maintenance of the structural integrity) is removed, therefore embedding into the apolar, hydrophobic HM20 becomes incomplete. Below 243 K, the polar K4M, which can be polymerized in the presence of a few percent of water, becomes too viscous for successful embedding.

Different solvents (and embedding media) may extract different molecular constituents and exert different effects on the structure of proteins and other macromolecules. The collapse temperatures of different cell constituents (24) seem to depend on the polarity of the solvent. Because of the higher polarity the collapse temperature for methanol is lower than that for acetone. The collapse temperature of the polar K4M, which is very useful for immunocytochemical studies, is probably between the one of acetone and methanol. In practical experiments the structure of biological material freeze substituted in the absence of any fixative seems to be more strongly influenced by the polar K4M than by the apolar HM20. The hydrophilic K4M is probably too viscous to be used at the low temperatures necessary to prevent collapse phenomena and the extraction of lipids (39).

The demonstration of the ultrastructure of rapidly frozen unfixed material freeze-substituted in pure solvents and low temperature embedded is very difficult. Post-staining of the thin sections with uranylions or leadcitrate shows usually very little effect. Nice results have been obtained by ratio contrast in a STEM (5).



Similar results are obtained by using a TEM equipped with an energy filter (1). Fig. 7 shows a portion of chemically untreated, high pressure frozen rat growth cartilage, freeze-substituted in pure methanol, embedded and polymerized in Lowicryl HM20 at 223 K. The very thin sections (invisible on the surface of the knife trough liquid) were not post-stained at all. The micrographs were taken with inelastically scattered electrons ($\Delta E = 200 \pm 10$ eV) using a Zeiss EM 902. Cellular structures can be clearly identified (Fig. 7A). Note the good contrast of the proteoglycan molecules (PG) of the matrix (Fig. 7B). Such thin, unsupported sections are not very stable in the electron beam and small holes are formed rapidly. Since these holes (arrows) seem to be related to identical cellular structures (mitochondria), we assume that they represent areas of incomplete penetration and polymerization of the Lowicryl which might be due to the presence of residual water. In these structures, the interaction between the structures and the associated water may be strong enough to prevent the complete substitution by methanol at 223 K.

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