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## Lowicryl® Letters - No.3

*Trends and Developments in Low Temperature Embedding*

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### A. LOWICRYL HM23 AND K11M: TWO NEW EMBEDDING RESINS FOR VERY LOW TEMPERATURE EMBEDDING.

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The encouraging results achieved with low temperature embedding of freeze-substituted specimens in Lowicryl K4M and HM20. (Bjornsti et al.., 985;Hunziker et al., 1984a and Hunziker and Schenk, 1984b) lead to the obvious question if further advantages could be obtained with still lower temperatures. Maximized cryofixation and minimized chemical fixation should be of advantage for immunocytochemical localization of cell components on sections, since chemical fixation of the biological material modifies the antigens and causes a reduction in the sensitivity of the labeling.

The two new resins HM23, non polar, and K11M, polar, have thus been developed by us. The are based on acrylate and methacrylate esters, as the old Lowicryl HM20 and K4M were, with freezing points that allow applications down to about -60°C and -70°C for K11M and HM23, respectively. The resins have the same very low viscosities at these temperatures as the old ones at -35°C. HM23 and K11M can be used principally in the same way as the old Lowicryl resins for embedding of PLT dehydrated (Progressive Lowering of Temperature, for further details see the instruction in the kit) or freeze-substituted samples. Polymerization is achieved by the same procedure given for HM20 and K4M (UV-polymerization, 360 nm).

The new resins are mixed as follows 1)K11M 19 g monomer + 1.0 g cross-linker +0.1 g initiator (benzoin monomethylether), and II) HM23 18.9 g monomer + 1.1 g cross-linker + 0.1 g initiator (above -50° C benzoin monomethylether and below -50 C Igracure 651). All components are supplied with the kits.

The first test of the resins were done on bacteria with or without bacteriophage infection. This system was chosen since some mutants of phage T4 produce a very large DNA pool in *E. coli* which is difficult to preserve. The low protein content of the DNA pool renders the DNA almost unfixable, since DNA itself does not react sufficiently with aldehydes to be cross-linked enough to prevent a course precipitate to be formed during the preparation procedure. The relatively high resolution required to visualize structural details in the virus related particles in the cytoplasm is an additional factor that makes this specimen, in our opinion, a suitable but demanding test object for a embedding procedure.

The results with the PLT dehydration protocol and the new resins showed that a reduction of the final embedding temperature from -35° C to -60 C gave some improvements, but not sufficient to prevent aggregation of DNA pool. The comparatively high temperatures during the early dehydration steps seem to be the critical part of the PLT dehydration procedure which prevents further improvements by lowering the embedding temperature. One advantage of the new resins is that K11M has a lower viscosity at -35 C than K4M, which can under some circumstances facilitate the infiltration of the samples when processing them according to the "traditional" scheme for K4M.

The only way to circumvent the dehydration steps at the higher temperatures (the first 10 to 20 degrees below 0°C) is offered by rapid freezing and low temperature embedding after freeze-substitution. Lowicryl K11M and HM23 allow such low

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temperatures to be used, -60°C and -70°C, respectively, that it can be questioned whether the reactivity of the aldehydes, which are mixed into the substitution media, is so low that their action as fixative is negligible (Humber, Marti and Muller, 1983)

Therefore, they could perhaps be omitted. Results from freeze-substitution with or without fixative in the substitution media show no significant differences, the DNA pool, of our test system, shows no visible aggregation in both cases. This preliminary result raises hope that the new resins, in conjunction with freeze-substitution, can be used for preparing samples intended for immunocytochemical localization of antigens which are sensitive towards chemical modification by the fixative. Further technical details concerning freeze-substitution in combination with low temperature embedding is treated by Dr. Muller in another contribution in this issue.

The two new resins are made available to those that want to contribute to the improvement of the cryo-embedding technique or have a problem that perhaps can be solved by the new resins.

Main-distributors of electron microscopy accessory supply have already added these new embedding resins to their sales program.

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## B. REINTRODUCTION OF LOW TEMPERATURE EMBEDDING METHODS FOR ELEMENTAL ANALYSIS AT CELL AND ORGANELLE LEVEL.


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### Introduction:

As preparative technique for histological observations freeze-drying and vacuum embedding has been known for nearly a century (Altmann 1890), while drying of tissue prior to observations was used by Leeuwenhoek already in 1720 (Mann 1902).

The Altmann's method involved maintenance of small portions of tissue in vacuum at -20°C, over sulfuric acid in a desiccator. After several days of drying tissues were embedded in vacuum in paraffin wax. The Altmann's method was adopted and further developed by Gersh who in 1932 constructed a forerunner of the modern freeze-drying apparatus.

In electron microscopy fixation by freeze-drying was first used in 1943 (Richards et al. 1943, Sjostrand 1943). Most of the criteria for tissue preservation the authors had at that time are also valid today for preparation of samples for microanalysis and histochemistry on ultrastructural level.

An alternative way of removal of water at low temperature is by the method of freeze-substitution (Simpson 1941). The first step of preparation is the same as that of freeze-drying; very small pieces are rapidly frozen in propane or freons cooled by liquid nitrogen. Dehydration, however, is based on water extraction from the sample in an organic solvent which is performed at low temperature (-60 to -80C). The diffusion of water molecules into the solvent is a relatively slow process which involves several problems. Although freeze-substitution theoretically does not offer full retention of ions it has been extensively used in many analytical investigation because it provides satisfactory morphological preservation of the tissue (Marshall 1980, Harvey 1980).

Fernandez-Moran (1959) used freeze-substitution and low temperature embedding in methacrylate followed by polymerization with UV-light to study ultrastructure of dark and light adopted retinal rods.

We have tested the technique described by Fernandez-Moran using Lowicryl resin in the aspects of elemental and morphological tissue preservation and compared it with the method of freeze-drying and vacuum embedding at low temperatures using Lowicryl HM20 and HM23.

## Materials and Methods

### Freeze-fixation

Biopsies originating from different tissues: liver, skeletal muscle, cochlea and epiphyseal cartilage were used in the present investigation. Directly after dissection tissue samples were frozen by:-plunging the tissue into liquid propane (-189° C) cooled by liquid nitrogen (LN) or -against LN-cooled highly polished copper.

### Freeze-drying and plastic embedding

Frozen samples were transferred into the chamber of the modified freeze-drying apparatus in plastic vials (Nunc cryotubes)filled with LN. Freeze-drying was started at about -90° C(temperature of the freeze-drying chamber) and thereafter continued at -70° C for 10-12 hours. The following 48 hours temperature was slowly raised to -40° C. The vacuum in the freeze-drier was kept constant at 103 Torr. After completed freeze-drying the embedding medium was introduced to the plastic vials with the specimens without breaking the vacuum or changing the temperature in the cryochamber (Wroblewski and Wroblewski 1984). The samples were impregnated with resin overnight under the conditions described above. Lowicryl HM20 (Carlemalm et al. 1982) and HM23, were polymerized by illumination with UV light (360 nm), without changing vacuum or temperature conditions of the freeze-drying apparatus.

### Freeze-substitution

The frozen samples were placed in plastic vials filled with LN into a low-temperature box. Inside the box, the samples were transferred to the cryotubes filled with the substitution fluid, dry ether and molecular sieves (1:1) (Marshall 1980). After freeze-substitution for 2-3 weeks at a temperature of the CO ice (-78°C), ether was removed and stepwise changed for precooled Lowicryl HM20 resin. The resin was then cured at the same temperature for 48 hours by illumination with UV-light.

### Sectioning of freeze-dried and freeze-substituted plastic embedded tissue:

100-200nm thick sections (nominal thickness) were cut with a glass knife using an LKB-Ultrotome (Cryo Nova, LKB, Bromma, Sweden). In order to prevent redistribution and loss of ions no through liquid was used. Sections collected dry from the knife edge with an eyelash were transferred to the formvar-film coated election microscope grids. The sections were pressed to the grids with a polished silver rod. To diminish compression, which occurs during dry cutting of ultrathin sections, the temperature in the cryochamber of the ultrotome was lowered ( in the range of -30° C to -60° C) below the glass transition point temperature of the resins. Plastic sections cut at low temperature were either brought to ambient temperature in a desiccator or transferred to the column of the election microscope by means of the cry-transfer-system.

### Electron microscopy and X-ray microanalysis

Thin and semi-thin plastic sections were analyzed in a Philips 400T electron microscope with a scanning attachment. Energy dispersive X-ray microanalysis was carried out with a Kevex 8000 - analytical system at an accelerating voltage of 120 kV. Both conventional transmission and scanning transmission electron images (bright field) were obtained.

## Results

The method of freeze substitution gave more variable results than the method of freeze drying prior to embedding, which is due to several factors. Freeze substitution involves more steps of preparation than freeze drying. After freezing tissue has to be transferred from LN2 to the cooled substitution fluid which results in a very rapid change of temperature from -196 C 60 -80-90° C. After freeze substitution the samples have to be moved gradually through a series of concentrations of embedding medium and organic solvent solutions which comprises a great risk of loosing or damaging the sample. Some of the organic solvents used routinely as substitution fluids are highly hydroscopic which may lead to a redistribution and loss of ions in the sample. It is also more difficult to maintain oxygen free environment in the low temperature box during long period (2-3 weeks) of freeze substitution. Despite the continual presence of CO ice, the polymerization and cutting properties of the Lowicryl blocks from different experiment varied.

The method of freeze drying and embedding at low temperature and in vacuum proved in our hands more reproducible. Better polymerization of Lowicryl and first of all much shorter time of preparation of the samples was achieved. As the method does not require manipulation (mechanical) of the specimens after placing them into the chamber of the freeze drier the risk of loosing or damaging the specimen is minimized. Maintenance of vacuum and low temperature during most steps of preparation assures an oxygen free atmosphere and therefore homogenous impregnation by and polymerization of the resin.

X-ray microanalytical and morphological results were almost the same in our hands independently of the method of preparation used. However, by freeze drying and embedding in vacuum, morphology of the tissues was somewhat improved. This may be a result of maintaining vacuum during most critical steps of preparation, e.g. impregnation and polymerization.

We have been testing the reability of low temperature embedding techniques in microanalytical investigation by using rat tenotomized muscle. Such muscle was reported to have changes in the cell membrane (Baker and Baldwin 1982) and also characteristic elemental composition as revealed in semithick cryosections (Wroblewski and Edstrom 1983). The muscle is characterized by an increased Na and Cl and frequently lowered K. These significant ions shifts could be detected in low temperature embedded material both after freeze-drying and freeze-substitution.

Techniques of freeze-drying and plastic embedding have been used by Ingram and Ingram (1980) to study the composition of extracellular fluids in skeletal muscle. In our laboratory we have been earlier using the freeze-drying and Araldite embedding technique to study the inner ear tissue. Our investigation was focussed on analysis of fluid content in perilymphatic and endolymphatic spaces. We found sodium content in perilymph to be lower in Araldite embedded material than in semithick cryosections (Anniko et al 1984). This finding and our recent observations (Wroblewski and Wroblewski 1984) might be partially explained by greater absorption of sodium signal in the embedding medium or redistribution of Na during the Araldite impregnation. The redistribution of sodium which occurred in the extracellular fluid-filled spaces was however absent intracellularly. Lowicryl HM20 resin proved to be superior to Araldite, especially when contrast in sections of the tissues embedded in that resin is concerned. This is due to the electron scattering properties of Lowicryl (Carlemalm et al. 1982, Colliex et al. 1984) and selective mass loss of the resin under the electron beam, which results in a contrast enhancement (Wroblewski and Wroblewski 1984).

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**Conclusions:**

1. The freeze-drying and low temperature embedding is alternative preparation method for microprobe analysis. The method is the method of choice when analysis have to be performed on tissues with heterogenous cell populations where the area of interest have to be chosen on light microscopical level.
2. The method allows direct correlation of elemental data with immunohistochemical (such performed on adjacent section).
3. It is likely that initial preparation step-the cryofixation to a high degree- determinates the morphological preservation of low temperature embedded material.
4. Freeze-drying seems to be more convenient and more reliable method of water removal prior to embedding than freeze-substitution.
5. The oxygen free milieu (vacuum) is of great importance for the final polymerization by means of UV-light.
6. For elemental analysis of the content in the extracellular spaces and small fluid filled cavities filled with the salts, the embedding procedure should be performed below -40° C, using chlorine free and low viscosity embedding medium.

## C. PERSPECTIVES OF COMBINING FREEZE SUBSTITUTION AND LOW TEMPERATURE EMBEDDING.

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Generally, the aim of an investigation by electron microscopy is to provide the structural basis for the correlation of structure and function. The structural information is therefore the more valuable, the more completely the structure can be preserved and thus the smaller significant details can be described. Since electron microscopy is the only tool with the power to observe structural details down to molecular dimensions in the context of the complex biological systems, every effort to keep up with the rapid advancement of molecular biology and biochemistry seems to be justified.

By the presently used preparation techniques based on chemical fixation, dehydration and heavy metal staining, we are not able to offer the microscope a specimen for imaging which contains significant structural details below approx. 10 nm (except if on resorts to isolated systems or to periodical structures which in addition help to reduce the effects of the irradiating electron beam). The effects of the conventional procedures (conformational changes, volume changes, extraction of lipids and other small macromolecules, loss of diffusible ions) are continuously demonstrated in the literature (1).

Structural alterations due to the conventional preparation methods can be overcome to a great extent by techniques based on cryofixation. (The term cryofixation refers to the immobilization of the biological sample with high cooling rates, which produce no or only very small ice crystals which do not introduce detectable structural deteriorations.) Cryofixation exerts only physical influence ion the specimen. Freeze fracturing followed by replication allow the cryofixed specimen to be further processed in a purely physical way.

A controlled dehydration procedure is, however, only useful if it can be combined with an embedding resin that leads to a cuttable block in the presence of residual water, both, infiltration and polymerization being performed at the necessary high subzero temperatures. At present, no such embedding resin is available but the frequently used Lowicryls HM20 and K4M represent a close approximation.

The disadvantage of this technique is, however, that it can hardly be used for anything else than the description of specific structural aspects which depend on the fracturing behavior of the sample and its components.

Our aim in combing freeze substitution and low temperature embedding is to find a way to prepare cryofixed specimen for thin sectioning which equals at least the structural preservation of freeze fractured samples. In addition, we want to combine a more significant structural description with the localization of intracellular antigens and diffusible ions.

Water seems to be the most important factor in maintaining the structural and functional integrity. During dehydration and drying, the cells shrink up to 70% of their initial volume (1) and macromolecules may collapse. These temperatures above which different types of macromolecules collapse when exposed to different dehydrating agents (organic solvents, vacuum) were determined by MacKenzie (2). They range form 215K to 263K. These data indicate different interactions between water and different cellular constituents. The presence of cellular water in different states has been demonstrated (3-7). Thus in order to obtain an optimal preservation of the structural and functional integrity, conditions have to be found where enough water is removed from the cells to allow for a successful embedding but where enough water is left to guarantee the preservation of the structural integrity.

Since the removal of the water depends on solvent and temperature (2), freeze substitution may be a useful tool to control the dehydration step. Looking at the solvents most frequently used for freeze substitution experiments, either pure or containing already a certain percentage of water, Humbel (8) could give additional evidence for this assumption.

Successful experiments combining freeze substitution in pure solvents and low temperature embedding are performed at 243 K (8,9). At this temperature, the effects of the substituting solvent are minimized and the infiltration and polymerization of the Lowicryls is routinely possible. If freeze substitution is stopped at much lower temperatures (e.g. at 223 K), not enough water is removed from the biological material. Depending on the nature of the interaction of the water with the cellular structures, locally varying amounts of residual water prevent a sufficient infiltration and polymerization of the Lowicryl HM20 ( K4M is too viscous at 223 K). Upon irradiation in the electron microscope, this results in the rapid formation of small holes usually associated with identical cellular structures. Freeze substitution and low temperature embedding experiments are greatly facilitated using a dedicated instrumentation. The set-up used in our lab is outlined by Humbel et al. (8). Suitable equipment for freeze substitution recently became commercially available by Reichert and for combined freeze substitution and low temperature embedding by Balzers Union.

Since the exposition of biological material to substituting organic solvents such as methanol or acetone at a temperature of 243 K may result in complete dehydration (and therefore in structural alterations of cellular components) as well as in a loss of lipids freeze substitution is often performed in the presence of stabilizing agents. There is evidence for osmium tetroxide reacting already at 203 K with the double bonds of the unsaturated fatty acids (10). Glutaraldehyde in methanol starts to effectively cross-link proteins at 223 K (8).

Thin sections of high pressure frozen (11) muscle tissue, freeze substituted in pure methanol, embedded and polymerized in Lowicryl HM20 at 243 K showed a good structural preservation (9). In addition an M-line protein was labelled by the protein A-gold technique after freeze substitution and low temperature embedding. Growth cartilage showed excellent structural preservation after high pressure freezing followed by freeze substitution in methanol containing 3% glutaraldehyde and low temperature embedding in K4M (12). The demonstration of cryofixed lipid samples by thin sectioning becomes routine by freeze substitution in methanol containing UO2Ac2, GA and OsO4 (13) or in acetone containing OsO4 followed by low temperature embedding and polymerization in HM20 at 243 K (14,15). Osmium tetroxide as a stabilizing agent is well compatible with low temperature embedding if the temperature does not exceed 243 K. Tissue blocks or pellets appear lightly brownish which still allow for UV polymerization.

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