Scanning Microscopy

Volume 8 | Number 3 Article 14

1994

Low Temperature Embedding of Chemically Unfixed Biological Material After Cryosorption Freeze-Drying

L. Edelmann
Universität des Saarlandes

Scanning Microscopy, Vol. 8, No. 3, 1994 (Pages 551-562) 0891-7035/94$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

LOW TEMPERATURE EMBEDDING OF CHEMICALLY UNFIXED BIOLOGICAL MATERIAL AFTER CRYOSORPTION FREEZE-DRYING

L. Edelmann

Medizinische Biologie, Fachbereich Theoretische Medizin,
Universität des Saarlandes, D-66421 Homburg/Saar, Germany
Telephone number: (49) 6841-166254 / FAX number: (49) 6841-166256

(Received for publication 1994 and in revised form 1994)

Abstract

After freeze-drying of bulk specimens in a newly developed cryosorption freeze-dryer (CFD) a special accessory is used to infiltrate the specimens in Lowicryl HM20 and to polymerize them at low temperature by ultra-violet (UV) irradiation within the CFD chamber in flat embedding moulds. The accessory allows polymerization in a dry, oxygen free environment without the risk of evaporation of volatile components of the resin which may lead to unsatisfactory polymerization. First results demonstrate the quality of structure preservation of biological material not treated with any chemical fixative.

Key Words: Low temperature embedding, Lowicryl HM20, freeze-drying, chemically unfixed biological material, glycogen.

Introduction

Electron microscopic investigations of biological material aim at the understanding of structure and function of living cells. In recent years considerable progress towards this goal was possible mainly due to the improvement of low temperature techniques (see e.g., Steinbrecht and Zierold, 1987). It turned out that the widely used chemical fixatives, which greatly alter the original biological material, can be reduced or even avoided when stabilizing biological specimens by suitable low temperature procedures. Noteworthy are the impressive results obtained after rapid freezing, freeze-substitution (FS) and subsequent embedding in a resin. In particular, FS combined with low temperature embedding (LTE) is now widely used and its impact on biological electron microscopy has been reviewed recently (Hippe-Sanwald, 1993; von Schack and Fakan, 1993; Quintana, 1994). Another well known low temperature technique is freeze-drying (FD) combined with resin embedding (see e.g., Coulter and Terracio, 1977; Edelmann, 1977, 1978, 1979, 1986; Pfaller and Rovan, 1978; Wróblewski and Wróblewski, 1984; Linner *et al.*, 1986; Steinbrecht and Müller, 1987). This procedure has not yet gained a similar general attention as FS probably because of the prevailing opinion that the latter technique is easier. However, FD has major advantages and may be used to produce, in the simplest way, stable preparations of chemically unfixed biological material. This is due to the facts that rapidly frozen specimens can be dehydrated at low temperature by sublimation without the use of organic solvents like alcohols or acetone (as in the FS technique) and that the dried specimens can be infiltrated in a single step with small amounts of a resin and hardened afterwards by polymerization. Keeping in mind that artefactual alterations of the biological material can be reduced by LTE (Kellenberger, 1991), a method has been developed which allows simple resin-infiltration of freeze-dried specimens at low temperature and subsequent ultra-violet (UV) polymerization in a recently described cryosorption freeze-drying apparatus (Sitte *et al.*, 1994). It is the purpose of this paper to show first

results of ultrastructure preservation of different chemically unfixed biological material after cryosorption freeze-drying and LTE in Lowicryl HM20.

Materials and Methods

Cryofixation

Frog sartorius muscle (*Rana pipiens pipiens*) was cryofixed by contact with a falling liquid nitrogen (LN_2) cooled copper block as described by Edelmann (1989). The same cryofixation method was used for freezing of 0.5 mm thick tissue slices (Sitte *et al.*, 1987) from mouse liver. 0.6 μl of platelet rich plasma (Morgenstern *et al.*, 1985) were cryofixed in plastic spacers by using the Reichert MM80 cryofixation unit as described by Sitte *et al.* (1987).

Transfer of cryofixed specimens

Small cryofixed specimens (side length < 0.5 mm) were transferred into the moulds of the LN_2 cooled object table as described by Sitte *et al.* (1994). Briefly, the preparation was carried out inside a styrofoam box partially filled with LN_2 by using a stereo light microscope and fiber optic illumination. By means of a cryomanipulator the loaded object table was transferred into the cryosorption freeze-drying unit (CFD).

Freeze-drying of specimens

After evacuation of the CFD (described by Sitte *et al.*, 1994) by means of a membrane pump (10 mbar) and the built-in cryosorption pump (below 10^{-4} mbar) freeze-drying was started by heating the object table to the desired temperature. Periods of drying temperatures (temperatures of the specimen stage) between -80°C and -40°C are given under **Results**. During the entire FD procedures the pressure inside the CFD was between 10^{-5} and 10^{-4} mbar and a condenser positioned about 20 mm above the specimen stage had a temperature of about -180°C.

Infiltration of specimens with Lowicryl HM20 and UV polymerization

After FD over a period of 10 days, the FD unit was vented by dry cold nitrogen gas and opened. During the following manipulations, the FD chamber was constantly flushed with nitrogen gas. An aluminum tube with connected glass plate was screwed on the object table as set out in Figure 1. The procedure of LTE is described in the figure legend. To be noted were the following steps: In the present case infiltration with Lowicryl HM20 was carried out at a temperature of the object table of -40°C. Lowicryl is not precooled but due to the slow flowing over cold metal the resin is cold when it reaches the specimens. After filling of the moulds with resin (only one filling, no exchange), closing of the Lowicryl-containing specimen stage was closed and the UV lamp was mounted as described in the legend of Figure 1. The UV lamp was not immediately switched on in order to allow infiltration of the dried specimens without UV irradiation. The specimen stage was warmed up to -25°C and after 3 hours, the UV lamp was switched on. After 1 day, the UV irradiated Lowicryl is hardened and the embedded specimens are ready for sectioning. (One may call the mode of UV irradiation "direct", but due to the back-scattering properties of the used Delrin-moulds the Lowicryl is irradiated from all sides and evenly polymerized, see Edelmann, 1989).

Electron microscopy (EM)

Ultrathin sections were obtained by using a Diatome diamond knife. The sections were either stained (method 1) with uranyl acetate and lead citrate for short periods (< 1 minute) or according to the following method (method 2): A 5% lead citrate solution was used in the knife trough. The sections floating on this solution after the cutting process were picked up by grids coated with Formvar films and the adhering fluid is removed with a filter paper. The sections are photographed in a Zeiss EM 902.

Results

Results of different biological material obtained after FD and LTE in Lowicryl HM20 are given in Figures 2-4. The specimens were well infiltrated and polymerized. Sectioning posed no difficulties. As a rule, staining was very intense even after short exposures (< 1 minute) of the sections to the staining solutions.

Figures 2a-2c show micrographs of mouse liver after FD for 2 days at -80°C, 1 day at -70°C, 4 days at -60°C, 2 days at -50°C, and 1 day at -40°C. After infiltration with Lowicryl HM20, UV polymerization was performed for 1 day at -25°C. Figure 2a was obtained after staining of an ultrathin section with uranyl acetate and lead citrate (method 1). The contrast is very intense, mitochondria appear very dark, the nucleus is well preserved and exhibits the usual staining pattern. However, holes are visible in the section, which indicates loss of material during staining according to method 1. Loss of material (although less than shown in Fig. 2a) has also been observed after ultrathin sectioning of the same preparation and floating of the sections on distilled water. By using staining method 2 (exposure to 5% lead citrate only) the sections do not show similar holes as can be seen at low and higher magnification in Figures 2b and 2c. Most interesting is the finding that the sections exhibit large dark areas in which huge amounts of glycogen are preserved and stained. On the other hand, nuclei and mitochondria are not well stained

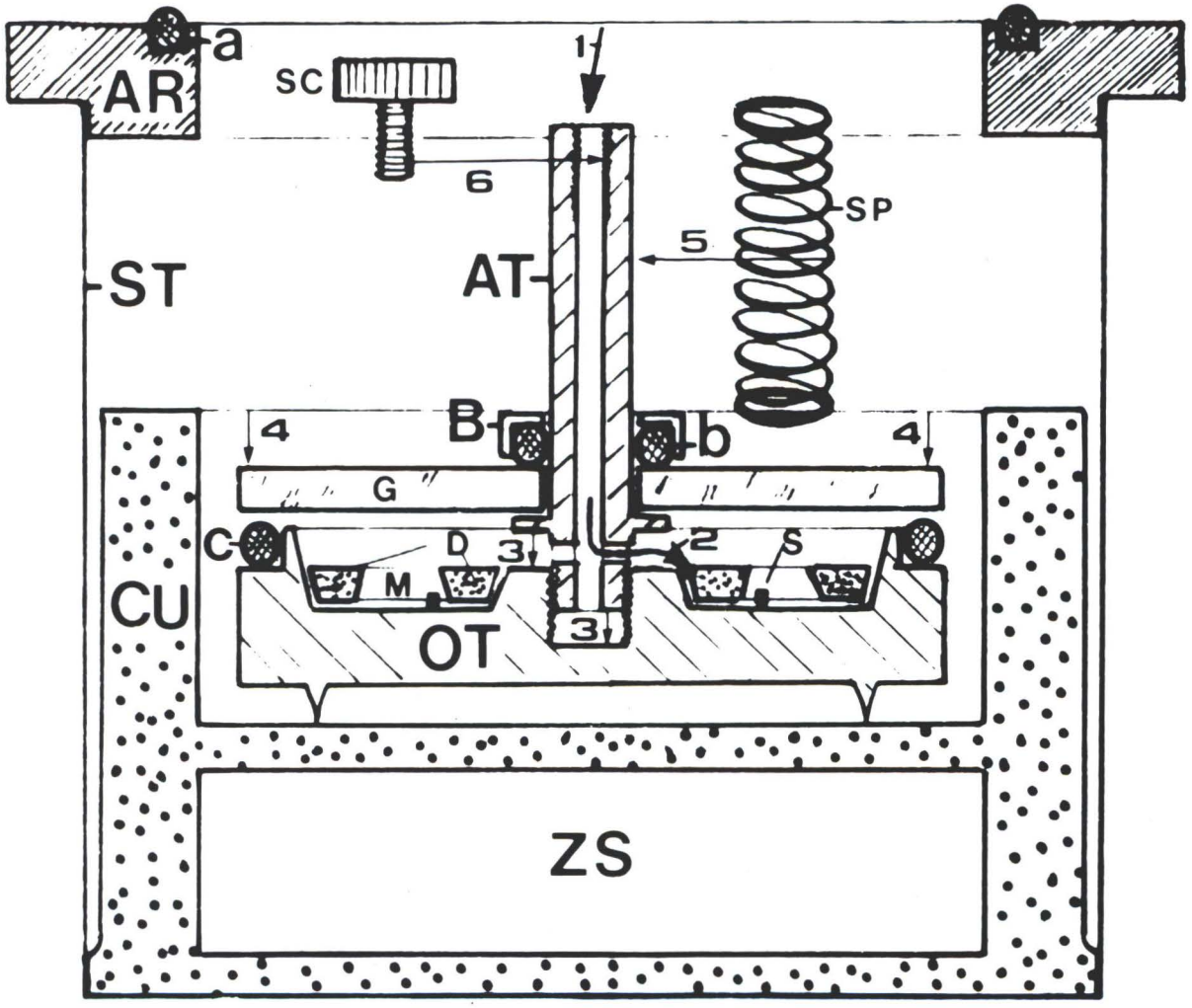
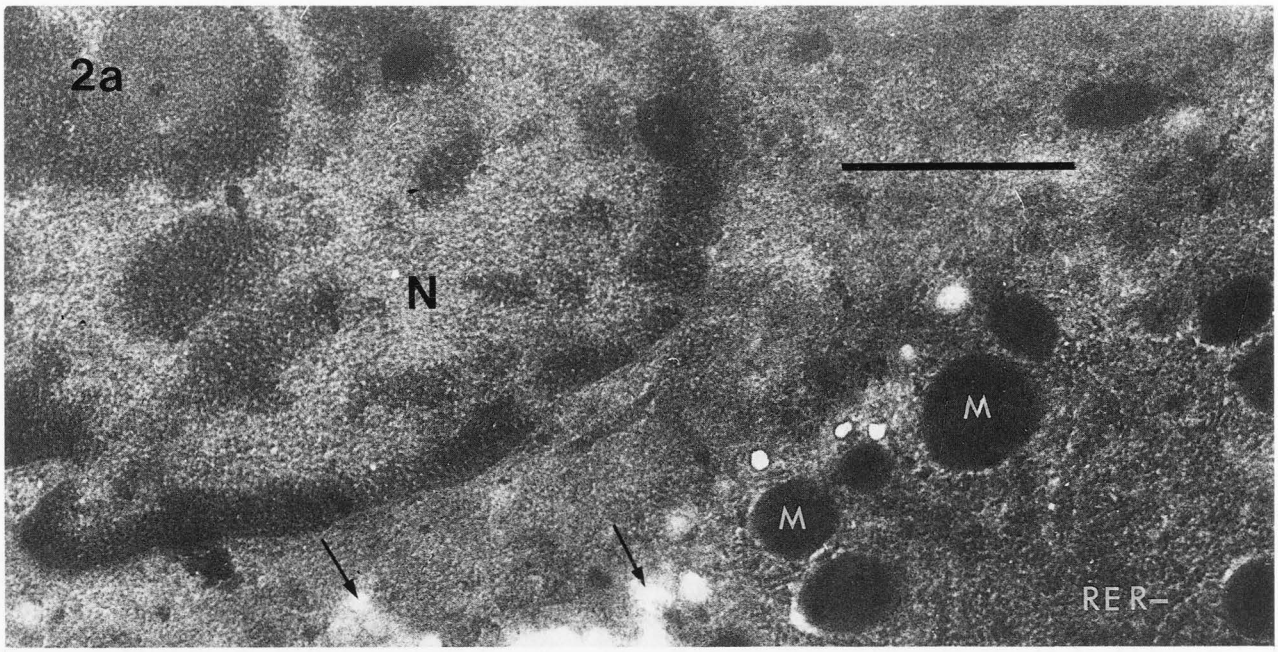


Figure 1. Schematic cross section of object table **OT** with accessory for LTE in the open FD chamber (described in detail by Sitte *et al.*, 1994) which is placed in the neck of a Dewar (not shown). The main parts of the FD chamber are composed as shown: a copper body **CU** (temperature about -185°C during FD) is connected by a stainless steel tube **ST** with an upper aluminum ring **AR** (room temperature). The chamber can be closed by an UV translucent glass plate (not shown) on top of O-ring **a**. **CU** contains a space **ZS** for cryosorption material Zeolite which maintains the required vacuum during FD. Valves between different spaces and electrical connections are not shown.

Procedure of LTE: After FD, the chamber is vented by cold dry nitrogen gas and opened. Aluminum tube **AT** with connected glass plate **G** is screwed into **OT** as shown. By means of a syringe, about 3 ml of Lowicryl is introduced into **AT** (arrow 1). The resin flows (arrow 2) via holes in **AT** slowly into moulds **M** containing dried specimens **S** (the moulds are formed by a plate **D** of DELRIN with holes placed on the **OT** as shown; a similar flat embedding system is used in the Reichert AFS). After filling of the moulds with resin, **AT** is completely screwed into **OT** (arrows 3), spring **SP** is set on mobile bearing **B** of O-ring **b** (arrow 5) and compressed by screw **SC** fixed at arrow 1 on **AT** (arrow 6). By this procedure, the space above the infiltrated specimens is tightly sealed by O-rings **b** and **c** (O-ring **c** has been placed on **OT** before cooling and loading with frozen specimens). After setting a thin walled aluminum tube (not shown) on glass plate **G** (at arrows 4) the FD chamber is closed by fixing a UV translucent glass plate on O-ring **a**. Then, a UV lamp, similar to those used in the Reichert CS-auto and AFS units, is put on this glass plate and switched on. After 1 day of UV polymerization, **OT** is heated to room temperature and removed from the FD chamber. The DELRIN plate bearing the polymerized Lowicryl blocks is removed from the object table. The blocks can be removed from the plate and are ready for sectioning.



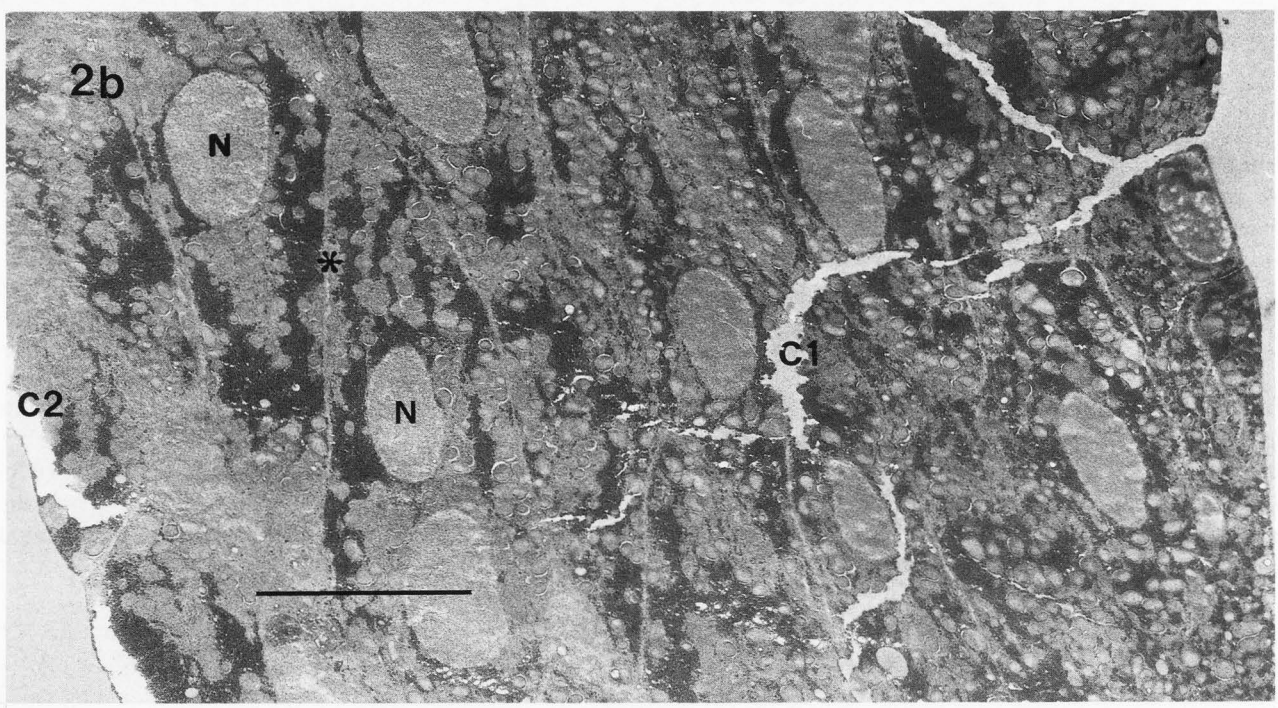
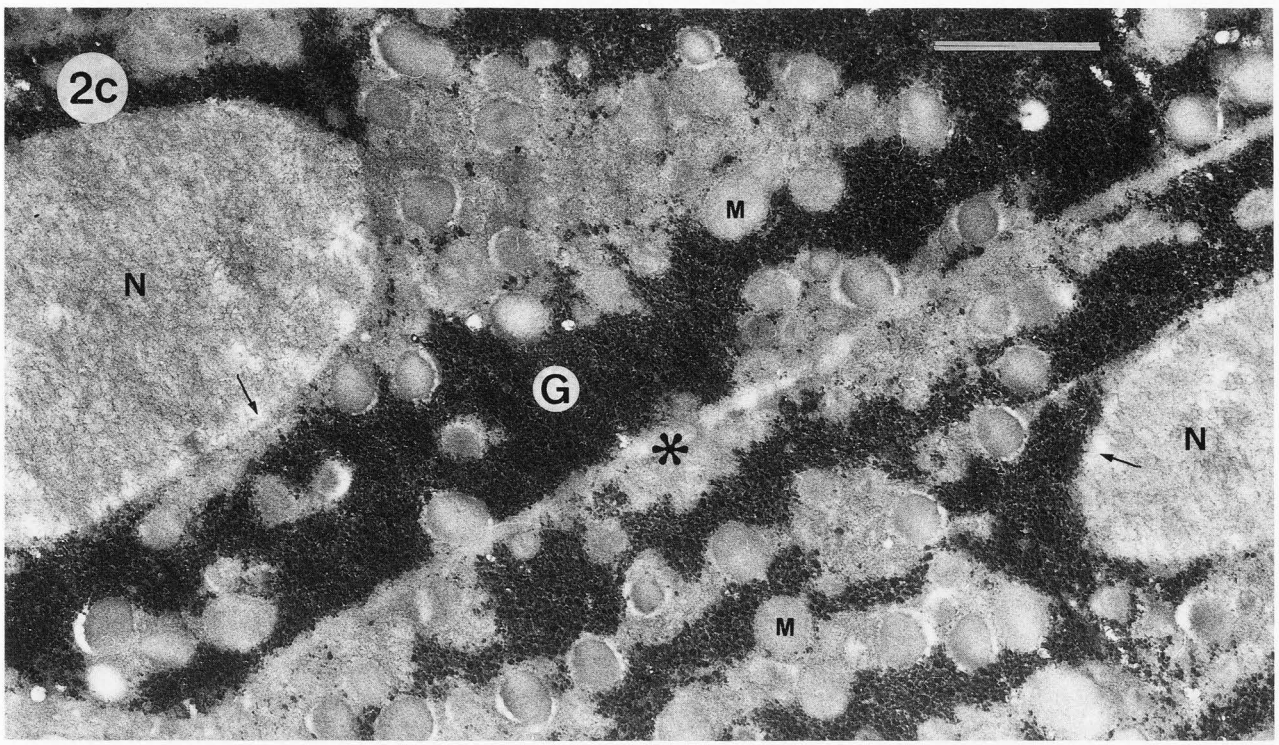


Figure 2 (2a and 2b above, 2c on the facing page). Ultrathin sections of mouse liver after FD and LTE at -25°C (FD: 2 days -80°C, 1 day -70°C, 4 days -60°C, 2 days -50°C, 1 day -40°C). **2a.** After staining with uranyl acetate and lead citrate (method 1); **N**: nucleus, **M**: mitochondria, **RER**: rough endoplasmic reticulum; arrows point to holes in the section which indicate loss of material during wet-cutting and staining according to method 1 (see text). Bar = 1 μm. **2b.** Low magnification after staining with a 5% lead citrate solution (method 2); **N**: nuclei, **C1**: cracks in the freeze-dried tissue before infiltration with resin. **C2**: cracks produced during wet-cutting. Asterisk: center of higher magnification shown in Figure 2c. Bar = 10 μm. **2c (on the facing page).** Higher magnification of a part of Figure 2b (asterisk in both micrographs at the same place); **N**: nuclei, **M**: mitochondria. Note huge amounts of glycogen **G**. Arrows point to areas of heterochromatin (see text). Bar = 2 μm.



and the areas of heterochromatin (outer regions of the nuclei) appear in negative contrast. One has the impression that some nuclear material is extracted after using staining method 2.

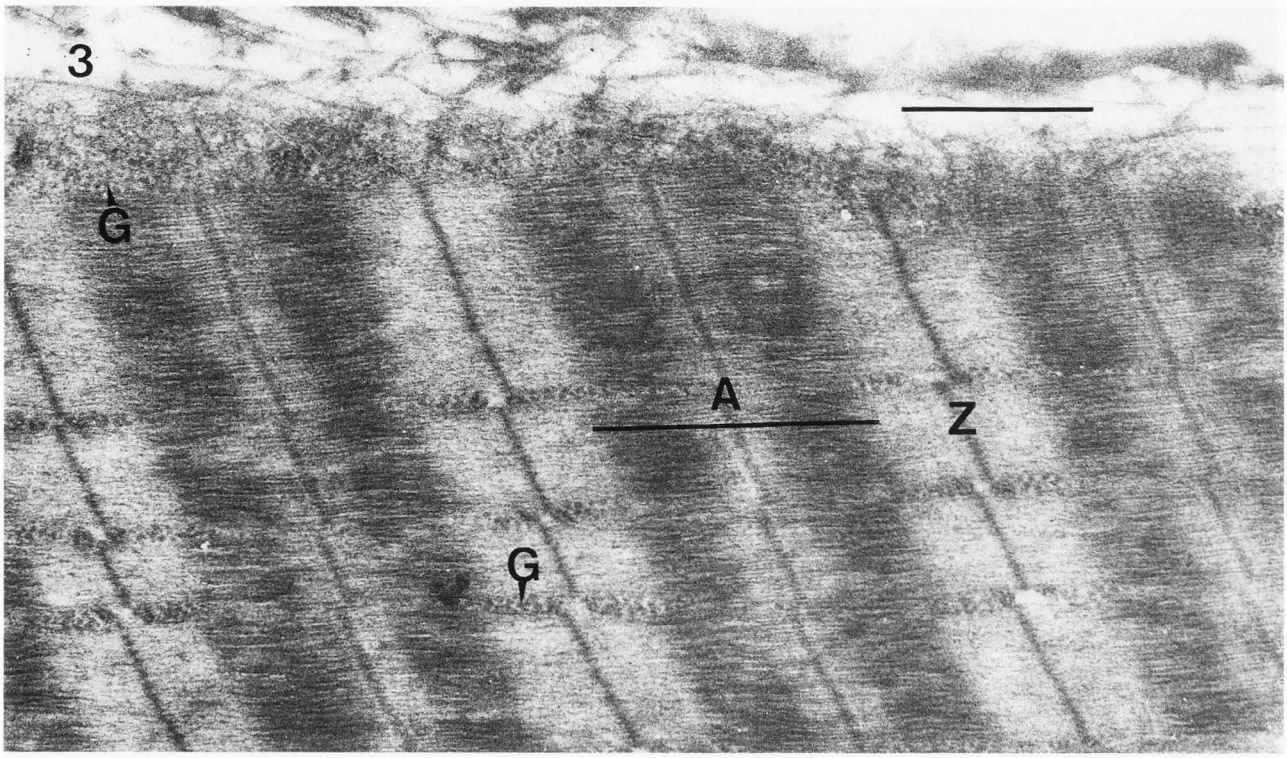
Figure 3 shows a section of a frog sartorius muscle, freeze-dried and embedded in Lowicryl similarly as the liver shown in Figures 2a-2c. The section was stained with uranyl acetate and lead citrate as the section shown in Figure 2a (method 1). Contrary to Figure 2a, glycogen is preserved in this preparation. The ultrastructure of the sarcomeres is preserved in a similar way to that obtained earlier after FD and LTE in K11M (Edelmann, 1986).

An example of a freeze-dried suspension of human blood platelets and leukocytes is given in Figure 4 (staining according to method 1, FD: 2 days -80°C, 1 day -60°C, 2 days -50°C, 1 day -40°C, LTE as in the other preparations).

Discussion

As stated in the **Introduction**, FD combined with resin embedding represents the simplest way to obtain stable chemically unfixed biological material. It is much easier to carry out this procedure than, for instance, FS and LTE. During FS and subsequent embedding of a cryofixed specimen, the sample is first placed into a container with precooled substitution medium. After completing the substitution process, the liquid surrounding the specimen is step-wise replaced with a liquid of increasing concentration of embedding medium. After complete infiltration with resin, the specimen is transferred into a container where polymerization is carried out. The exchange of media and/ or the transfer of specimens during these procedures may pose difficulties, in particular, if small specimens are processed. FD and subsequent embedding of a cryofixed specimen as described here only requires one preparational step, namely the addition of a small amount of resin after FD. During the whole process, no waste of organic liquids or resins is produced, the used resin is hardened and contains the freeze-dried specimen.

By using FD and LTE, it is possible to avoid interactions between proteins or other cellular components and dehydration solvents such as alcohols and acetone (as during FS). As a result of this advantage, together with the minimization of artifacts by resin embedding at low temperature and by omitting any chemical fixative during specimen preparation, it is expected that physicochemical properties of cellular macromolecules (e.g., antigenicity, ion adsorption) can be preserved in a state rather close to their state maintained in living cells. In order to substantiate this optimistic view, future investigations are necessary: The resin embedded chemically unfixed biological material may be used for immunocytochemical work, for X-ray microanalysis (see e.g., elemental spectra obtained from different visualized and identified areas of a dry cut section of frog sartorius


3
G
A
Z
G

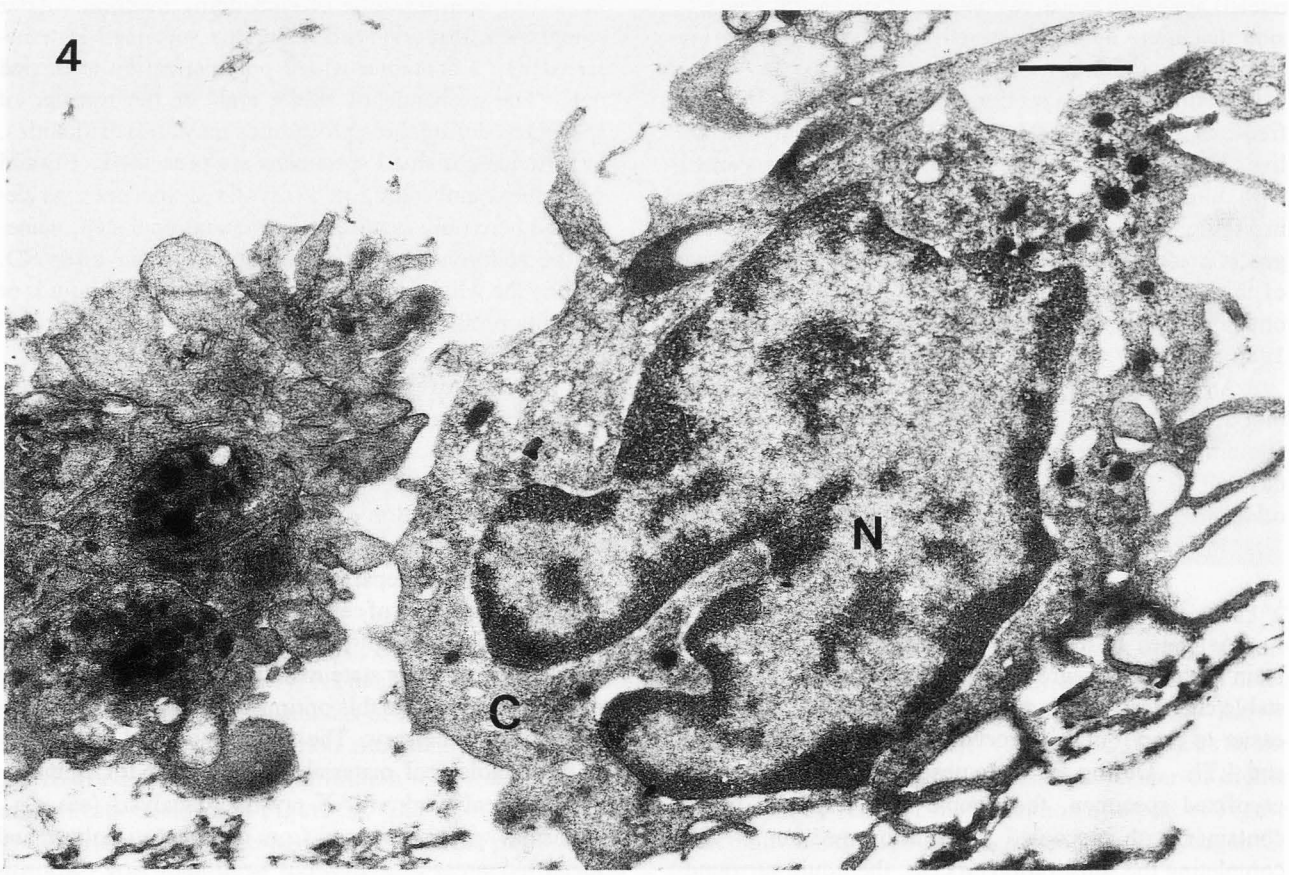

4
N
C

Figure 3 (facing page, top). Ultrathin section of frog sartorius muscle after FD and LTE; same procedure as given in Figure 2. **A**: A band, **Z**: Z line, **G**: glycogen. Note an A band width of about 1.5 μm, which is close to the known A band width of living muscle (see Edelmann, 1994). Bar = 1 μm.

Figure 4 (facing page, bottom). Ultrathin section of a human leucocyte and aggregated blood platelets after FD and LTE (FD: 2 days -80°C, 1 day -60°C, 2 days -50°C, 1 day -40°C). Contact plane of specimen with metal mirror during cryofixation is close to the bottom line of the figure. Note the better structure preservation of the leucocyte (finer granularity of nucleus **N** and cytoplasm **C**) in the lower part. Bar = 0.5 μm.

---

muscle after cryosorption FD and LTE in Lowicryl K11M in Edelmann, 1986, p. 1349), and for investigations concerning the interactions between cellular proteins and different ions like H^+, Na^+, K^+, Ca^{2+} (see e.g., Edelmann, 1991).

In order to obtain good results with the procedure of FD and LTE, the following technical preconditions must be fulfilled:

The specimens to be processed must be very small or very thin in order to minimize the required FD time (see below) and to optimize the quality of UV polymerization. The speed of infiltration with resin as well as the UV intensity "seen" inside the specimen are highly dependent on specimen thickness. Therefore, preparation of small specimens as described under **Materials and Methods** is of prime importance. Best results are obtained with specimens reduced in one side to values < 0.2 mm.

FD should be carried out for prolonged periods between -80°C and -60°C in order to minimize shrinkage artifacts. This problem has been discussed in detail in several papers (Edelmann, 1986, 1994; Sitte *et al.*, 1994). It is important to note that even during continuous FD, a "fast" temperature increase within and above the mentioned temperature interval may lead to severe shrinkage and to redistributions of mobile ions and other components within the biological material (Edelmann, 1994). Furthermore, during shrinkage the space which was originally occupied by water is reduced and infiltration of this space with resin may become impossible after severe FD shrinkage. In this context, the question of whether freeze-dried samples require vacuum infiltration should be discussed (see e.g., Wróblewski and Wróblewski, 1984; Linner *et al.*, 1986). One reason for the necessity of vacuum infiltration could be the increased density of considerably shrunken freeze-dried specimens. According to our own experiments with freeze-dried muscle embedded in Spurr's resin, the structure preservation is much better after infiltration at atmospheric pressure compared to vacuum infiltration. One should keep in mind that gas bubbles may arise in the embedding medium during infiltration *in vacuo* and disrupt labile structures even if the embedding medium has been evacuated before infiltration (Edelmann, 1986). Furthermore, due to the possible evaporation of volatile components of the resin during vacuum infiltration significant changes in resin formulation may result (Mollenhauer, 1993).

As discussed previously (Edelmann, 1989), the container in which UV polymerization of Lowicryl at low temperature is carried out must be tightly sealed; without closing the used flat embedding system a substantial part of the Lowicryl HM20 disappears from the moulds during polymerization and condenses at the cold surfaces of the FD chamber. Furthermore, the polymerization is not complete in the upper part of the polymerized blocks. Such unsatisfactory results are apparently due to a change in resin formulation caused by the evaporation of volatile components of Lowicryl HM20.

To be noted is the observation of artefactual holes in the Lowicryl sections of special biological material (see e.g., Fig. 2a). Such holes are produced during sectioning on distilled water and/or during specific staining procedures; after dry sectioning no holes are observed. Apparently, certain very labile structures have not been perfectly captured in the used resin. It has been shown that even these structures may be stabilized by a suitable different staining method. Whether the observed artifacts of extraction may be reduced only by a suitable treatment of the section or by further prolonged FD or by using Lowicryl K11M instead of HM20 or any other embedding medium remains to be determined.

Conclusions

Freeze-drying combined with low temperature embedding is a simple (most likely the simplest) method to obtain stable preparations of chemically unfixed biological material well suited for sectioning at room temperature and subsequent electron microscopic investigations. It is expected that by further optimization of FD and LTE, artifacts of this stabilization procedure can be further reduced and that still unknown properties of the original biological material can be captured and investigated by using this method.

Acknowledgements

Supported by Zentrale Forschungskommission der Universität des Saarlandes. I would like tho thank Mrs. E. Frank for typing the manuscript.

References

Coulter HD, Terracio L (1977) Preparation of biological tissue for electron microscopy by freeze-drying. Anat Rec **187**: 477-494.

Edelmann L (1977) Potassium adsorption sites in frog sartorius muscle visualized by cesium and thallium under the transmission electron microscope. Physiol Chem Phys **9**: 313-317.

Edelmann L (1978) A simple freeze-drying technique for preparing biological tissue without chemical fixation for electron microscopy. J Microsc **112**: 243-248.

Edelmann L (1979) Freeze-drying of chemically unfixed biological material for electron microscopy. Mikroskopie (Wien) **35**: 31-36.

Edelmann L (1986) Freeze-dried embedded specimens for biological microanalysis. Scanning Electron Microsc **1986**;IV: 1337-1356.

Edelmann L (1989) The contracting muscle: a challenge for freeze-substitution and low temperature embedding. Scanning Microsc **Suppl 3**: 241-252.

Edelmann L (1991) Adsorption staining of freeze-substituted and low temperature embedded frog skeletal muscle with cesium: a new method for the investigation of protein-ion interactions. Scanning Microsc **Suppl 5**: S75-S84.

Edelmann L (1994) Optimal freeze-drying of cryosections and bulk specimens for X-ray microanalysis. Scanning Microsc **Suppl 8**: 67-81.

Hippe-Sanwald S (1993) Impact of freeze-substitution on biological electron microscopy. Microsc Res Tech **24**: 400-422.

Kellenberger E (1991) The potential of cryofixation and freeze-substitution: observations and theoretical considerations. J Microsc **161**: 183-203.

Linner JG, Livesey SA, Harrison DS, Steiner AL (1986) A new technique for removal of amorphous phase tissue water without ice crystal damage: A preparative method for ultrastructural analysis and immunoelectron microscopy. J Histochem Cytochem **34**: 1123-1135.

Mollenhauer HH (1993) Artifacts caused by dehydration and epoxy embedding in transmission electron microscopy. Microsc Res Tech **26**: 496-512.

Morgenstern E, Edelmann L, Reimers HJ, Miyashita C, Haurand M (1985) Fibrinogen distribution on surfaces and in organelles of ADP stimulated human blood platelets. Eur J Cell Biol **38**: 292-300.

Pfaller WP, Rovan E (1978) Preparation of resin embedded unicellular organisms without the use of fixatives and dehydration media. J Microsc **114**: 339-351.

Quintana C (1994) Cryofixation, cryosubstitution, cryoembedding for ultrastructural, immunocytochemical and microanalytical studies. Micron **25**: 63-99.

Sitte H, Edelmann L, Neumann K (1987) Cryofixation without pretreatment at ambient pressure. In: Cryotechniques in Biological Electron Microscopy. Steinbrecht RA, Zierold K (eds.). Springer-Verlag, Berlin. pp 87-114.

Sitte H, Edelmann L, Hässig H, Kleber H, Lang A (1994) A new versatile system for freeze-substitution, freeze-drying and low temperature embedding of biological specimens. Scanning Microsc **Suppl 8**: 47-66.

Steinbrecht RA, Müller M (1987) Freeze-substitution and freeze-drying. In: Cryotechniques in Biological Electron Microscopy. Steinbrecht RA, Zierold K (eds.). Springer-Verlag, Berlin. pp 149-172.

Steinbrecht RA, Zierold K (eds.) (1987) Cryotechniques in Biological Electron Microscopy. Springer Verlag, Berlin.

von Schack ML, Fakan S (1993) The study of the cell nucleus using cryofixation and cryosubstitution. Micron **24**: 507-519.

Wróblewski R, Wróblewski J (1984) Freeze drying and freeze substitution combined with low temperature embedding: Preparation techniques for microprobe analysis of biological soft tissues. Histochemistry **81**: 469-475.

Discussion with Reviewers

J.A. Hobot: The use of FD for structural studies has lagged behind those carried out using freeze-substitution (FS), and it is vital that more work is done to understand FD such that it can be assessed properly alongside FS. Perhaps this equipment, in its simplicity of use, will provide the impetus for such work.

Author: I would like to further emphasize the main point of this comment: Understanding is the purpose of science. So far we do not really understand the interactions between macromolecules, mobile ions and water in biological systems and how these interactions change upon chemical fixation or during cryofixation and subsequent dehydration either by organic solvents or by sublimation at different low temperatures. Optimization of FD may not only lead to an accepted method of structure preservation but also to a better understanding of the molecular mechanisms underlying the specific ion and water distributions in living cells and redistribution of these components during specimen preparation (see Edelmann, 1994).

J.A. Hobot: The author mentions that chemical fixatives greatly alter the original biological material. This statement, although true, is not the whole story as chemical fixation still allows structural studies to be carried out, and it also allows for the preservation of antigenicity within embedded tissue for subsequent immuno-

labelling. Of course, it cannot be used for some applications (e.g., X-ray microanalysis), but the contribution of chemical fixation procedures to the literature is vast, and in many cases valid.

Author: I agree. We know that chemical fixatives drastically change the intracellular ion distributions (K, Na), they change the interactions between different macromolecules and most likely the interactions between certain macromolecules and water (Ling, 1992). It remains the challenging problem to know if, and to understand why, meaningful results are obtained despite the use of chemical fixatives and why we may obtain, in other cases, better or more informative results by omitting chemical fixatives.

J.A. Hobot: The author claims that a major advantage of FD is due to the fact that the frozen specimen can be dehydrated without the use of organic solvents. But, resins are organic solvents too, even in small amounts.

Author: The important point is that the biological material can be dehydrated and stabilized by FD without an organic solvent. The ultrastructure (including subcellular ion distributions) of such stabilized material may be controlled by examination of freeze-dried cryosections (Edelmann, 1994). Different resins, infiltrated at different temperatures may then provide different results of structure preservation and of subcellular ion distribution which can be related to these organic solvents and not to the combined influence of dehydrating solvents (as used during FS) and resins.

J.A. Hobot: The micrographs presented after freeze-drying show some patterns of protein aggregation and also of extraction of cellular components. Could this be due to the efficient removal of water, such that hydration shell water is removed and protein aggregation occurs? Would a possible way to improve structural preservation be by not removing all the water, but by retaining some hydration of the sample by completing the freeze-drying cycle at a lower temperature, and then infiltrating with a more polar acrylic resin, like Lowicryl K11M, rather than with the less polar Lowicryls HM20 or HM23? The more polar acrylic resins will tolerate the presence of water, and experiments in this direction may be revealing. Although the author has worked with these low temperature resins, are there any results or ideas in his experience which point to this path of enquiry being worth pursuing?

A. Allenspach: Are there reasons, other than Lowicryl sectioning, to explain the loss of cytoplasmic matrix?

Author: In my opinion, protein aggregation after FD is dependent on: 1) the quality of cryofixation, 2) the temperature and the speed of water removal, 3) the degree of cross-linking of proteins during dehydration, and 4) the final degree of hydration. Most of these parameters are dependent on the original degree of hydration of the area in question. As discussed earlier, it appears that aggregation of proteins and shrinkage of biological material can be severe if a specimen is warmed up (even during continuous FD) above a critical temperature before it is sufficiently dehydrated (Edelmann, 1994). It has been speculated that: "... in this case the water molecules which are more or less immobilized at the low temperature become more and more mobile with increasing temperature. As a result the attractive forces between macromolecules or between intramolecular sites are not longer balanced by the immobilized intermediate water molecules, the macromolecules aggregate at many areas almost simultaneously and the whole system shrinks. Only if the water is removed slowly at rather low temperature certain few sites are dehydrated first which may combine with sites of opposite polarity thereby stabilizing the system. Further slow dehydration causes step by step more cross links and the tremendous shrinkage can be avoided" (Edelmann, 1994). With these considerations, I do not believe that in the present case, the protein aggregation is due to the efficient (final) removal of water but either due to imperfect cryofixation (see difference of aggregation in the leucocyte: Fig. 4, less in the lower part) or due to 2) or 3) of the above mentioned reasons for aggregation. In any case, the exclusive FD at still lower temperatures and the use of K11M instead of HM20 or HM23 are planned to tackle this problem.

In this context, the quality of structure preservation after FS (e.g., in pure acetone) should be discussed: Although the mechanism of protein aggregation is probably rather similar during FS and FD, there is the observation that (so far) the quality of structure preservation in the well cryofixed area is superior after FS {compare e.g., Fig. 2 of Edelmann (1991) with Fig. 3 of this paper} and that distortions caused by imperfect freezing are more pronounced after FD. The difference could be due to the different polarities of the dehydrating agents (vacuum, organic solvent) and/or the different degree of conformational change of macromolecules caused by the dehydration in a vacuum (FD) or by the increasing concentration of organic solvent around the macromolecules during FS. For instance, it could be that during FS the redistribution of weakly bound ions and other mobile components is facilitated by the presence of an organic solvent with the result that more "unnatural" cross links between proteins are formed than during FD (see Edelmann, 1994). Therefore, it cannot be ruled out that, despite the superior structure preservation after FS, certain physicochemical properties (e.g., ion binding, antigenicity) are better preserved after FD.

Concerning the observed extraction of cellular mate-

rial, there are several possibilities. They are either not really extracted but unstained or aggregated at neighboring sites or they are extracted into the resin during infiltration or extracted during wet cutting or during staining. The extraction shown in Figure 2a could be due to an incomplete dehydration of the areas in question with the result of a localized incomplete polymerization. Similar artifacts can also be observed after FS in pure organic solvents and LTE, in particular if FS is carried out exclusively but not long enough at low temperature. After FS without chemical fixatives, extraction of cellular material is more pronounced when using K11M: thin sections take up water during wet cutting and material which is not sufficiently cross linked is dissolved. I therefore believe that in the case of FD, extraction will not be reduced when using K11M instead of the less polar HM-Lowicryls. But any artefact observed with the different resins may lead to a better understanding of the stabilization procedure. Of interest are of course also comparative studies with resins which have to be heat polymerized.

Reviewer II: The author describes the very, very first results using freeze-drying and embedding in Lowicryl resin HM20 using a commercially available instrument. The publication is far from the level of Edelmann's own paper about the same subject using non-commercial equipment (Edelmann, 1986).
Author: The (Edelmann, 1986) paper as well as two additional cited publications (Edelmann, 1994; Sitte *et al.*, 1994) are important cross-references for the reader of this short contribution which presents new results obtained after FD and LTE in Lowicryl HM20. A critical comparison of the mentioned non-commercial equipment with the CFD shows that the physical parameters (pressure, temperature, time) during FD in the non-commercial freeze-dryer are easily reproduced by using the CFD. Furthermore, the CFD is equipped with important new features including the possibilities of easy infiltration of resin and UV polymerization at low temperature. Hence, not only the experiments carried out with the old freeze-dryer can be repeated but a large variety of new low temperature experiments is possible by using the CFD. It has to be noted that only the result shown in Figure 8 of Edelmann (1986) has been obtained after FD and Lowicryl embedding (K11M). A comparison of that Figure 8 with Figure 3 of this paper shows: The structure preservation of frog sartorius muscle is similar (1) after FD in the non-commercial equipment and subsequent embedding in K11M and (2) after FD and embedding in HM20 by using the CFD. The result of the latter procedure may even be judged as being superior to the former one since glycogen granules are stained in the HM20 preparation; such granules are either absent or unstained in the K11M preparation.

Reviewer II: The middle range of magnification does not reveal, if the described method is good enough to be used in membrane studies (see, Sjöstrand and Kretzer, 1975).
Author: There is no doubt that the results obtained by Sjöstrand and Kretzer can be reproduced by using the CFD. However, it should be noted that the used material had been chemically fixed by glutaraldehyde and partially dehydrated with ethylene glycol before cryofixation and FD. FD of chemically fixed material is outside the scope of this paper. Ultrastructural and physicochemical differences between chemically fixed and untreated biological material are expected and should be investigated by future FD studies.

Reviewer II: The extremely small size of the specimens used (side length below 0.5 mm, for best results < 0.2 mm) makes the usefulness of the method very limited as dissection of such small pieces will restrict the investigation to very uniform tissues. Therefore, in pathological tissues, where morphological alterations are randomly distributed, morphological (or analytical) investigations cannot be performed with adequate accuracy. The dissection procedure of samples of that size will lead to numerous mechanically introduced artifacts.
A. Allenspach: Could you comment on the possibility of structural damage due to procedural manipulation of specimens.
Author: It is well known that dissection of untreated biological tissue may cause severe artifacts including ion and water redistribution artifacts. This problem must be considered when choosing the optimal method of cryofixation of a specific dissected tissue. The results of all follow-up procedures (e.g., cryosectioning, FS, FD) are dependent on the quality of cryofixation which captures the more or less artificial state of tissue after dissection. After cryofixation however the specimen can be reduced in size at liquid nitrogen temperature by means of a scalpel without further introduction of mechanical or ion and water redistribution artifacts. In other words, the dissection procedure of samples of the suggested small size will not lead to artifacts different from those known from other established cryomethods (also see Discussion with Reviewers in Edelmann, 1986). In addition, the specimens need not be extremely small: large but thin tissue slices are well suited for optimal FD and subsequent embedding in Lowicryl or other resins.

Reviewer II: The author claims in the **Discussion** that severe shrinkage is a problem when FD and embedding is performed at high vacuum, citing Linner *et al.* (1986) and Wróblewski and Wróblewski (1984). I could not

find any statement in these publications concerning severe shrinkage.

Author: My claims are different: 1) Inadequate FD may lead to severe shrinkage of biological material; severe shrinkage may be prevented by prolonged FD in a certain low temperature range (either by cryosorption FD or by FD in a vacuum which is maintained by mechanical pumps). 2) Resin infiltration *in vacuo* **may** be required if the freeze-dried specimen is considerably shrunken. I have cited two papers (among many others) describing resin infiltration of freeze-dried material *in vacuo*. The authors of these papers did not consider the problem of shrinkage during FD probably because it is usually very difficult to determine the degree of shrinkage of freeze-dried embedded material. [However, severe shrinkage of mitochondria has been shown by Linner *et al.* (1986) Fig. 11b, see discussion by Edelmann (1994)]. I do not know whether these authors have tried resin infiltration after breaking the vacuum and whether different results have been obtained in that case. I assume however that resin infiltration of considerably shrunken freeze-dried tissue may become a problem when carried out at atmospheric pressure.

M. Malecki: In your work, you have evaluated the procedure at low magnifications. How is the retention of the three-dimensional intracellular architecture within freeze-dried cells at the supramolecular level (e.g., actin network) after exposure to surface tensions of the wave of infiltrating embedment?

Author: This problem has not yet been investigated. It is, however, interesting to note that the structure preservation of a cryosection of frog sartorius muscle, freeze-dried exclusively at low temperature (Edelmann, 1994) is rather similar to the structure preservation seen after FD at low temperature and subsequent embedding in Spurr's resin (Edelmann, 1986) or Lowicryl HM20 (this paper) and ultrathin sectioning. One may, therefore, expect that infiltrating of resin at low temperature (both resins have been infiltrated at low temperature) will not considerably change the intracellular architecture stabilized by FD.

M. Malecki: You consider a possibility of using this procedure as an efficient way for retention of antigenicity: Have you compared efficiency of post-embedding labelling in freeze-dried-embedded versus freeze-substituted (acetone only)-embedded samples?

Author: I have not yet carried out such studies. As discussed above (see answer to J.A. Hobot, 4th question), I assume that the preservation of physicochemical properties of macromolecules is different and probably superior in many cases after FD compared to FS in pure acetone. The observed different staining behavior after FS and after FD supports this idea: The material shown here is intensely stained with uranyl acetate and lead citrate whereas a similar staining of material freeze-substituted in pure acetone and embedded in Lowicryl HM20 usually produces a rather poor contrast.

A. Allenspach: Could you comment on the stabilization of membranous structures by FD?

Author: It is assumed that membranous structures are stabilized after FD as well as after FS in pure acetone and subsequent resin embedding provided these procedures are carried out exclusively at low temperatures. Staining of membranes is rather difficult, however, after these procedures (negative contrast in most cases). Noteworthy is the earlier reported finding that after FD and resin embedding in Spurr medium, the basolateral membrane system of a distal tubule of mouse kidney can be well stained whereas the mitochondria do not show similar well defined membranes (Edelmann, 1986). Future studies may reveal to what extent the appearance of membranes may be modified, e.g., by the use of Os vapor after FD or after FD, embedding, and sectioning or by other specific staining procedures.

A. Allenspach: Could you comment on the preservation, or lack thereof, of extracellular matrix materials: high quality preservation should retain the plasma proteins in the platelet enriched samples, yet little is visible in Figure 4.

Author: The quality of preservation of extracellular matrix materials is dependent on the quality of cryofixation and on the original concentration of matrix material. In the present case, the concentration of extracellular proteins (albumin, immunoglobulin) was very low (about 1%, 99% water). Hence, a stained homogenous network is neither expected after FS in pure organic solvent nor after FD and embedding. A visible extracellular matrix obtained after FD and embedding of platelet rich blood plasma has been published earlier (Edelmann, 1979).

A. Allenspach: You state that samples must be 0.2 mm or less on a side. Does this suggest that freeze-dry techniques with your cryosorption FD unit preserve the ultrastructure of frozen specimens throughout 200 μm? If not, to what depth have specimens exhibited quality ultrastructure?

Author: The quality of structure preservation is dependent on the quality of cryofixation and on the dehydration procedure. Quality ultrastructure is similar after FS in a pure organic solvent and after FD (may be superior after FS, see answer to 4th question of J.A. Hobot) and may be 200 μm in the case of high pressure freezing. The suggestion to use small specimens for optimal

FD was given to avoid particularly long FD times which must be empirically determined for specimens of uncontrolled thickness and to guarantee optimal polymerization by UV irradiation.

Additional References

Ling GN (1992) A Revolution in the Physiology of the Living Cell. RA Krieger Co., Melbourne, FL. Chapter 5: 69-110.

Sjöstrand FS, Kretzer F (1975) A new freeze-drying technique applied to the analysis of the molecular structure of mitochondrial and chloroplast membranes. J Ultrastruct Res **53**: 1-28.