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FREEZE-DRYING AND RELATED PREPARATION TECHNIQUES FOR BIOLOGICAL MICROPROBE ANALYSIS

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Abstract

An X-ray microanalytical and morphological investigation has been carried out on rapidly frozen, freeze-dried or freeze-substituted tissues. A comparison was made between different embedding and polymerisation procedures following freeze-substitution and freeze-drying. The investigation also included an analysis of specimens infiltrated, embedded and polymerised by ultraviolet irradiation at low temperatures with Lowicryl HM20. The morphological preservation of Lowicryl embedded tissue was adequate for the identification of different cell structures like nuclei, mitochondria, lysosomes and different types of endoplasmic reticulum. X-ray microanalytical investigation of low temperature embedded material displayed an elemental composition of cells and organelles similar to that found in freeze-dried cyosections. Compared with freeze-dried cryosections, low temperature embedded material could be sectioned for light microscopy and area of intrest chosen for further thin sectioning. This is of great importance in work with tissues with complicated morphology and heterogenous cell populations.

Key words: freeze-drying, freeze-substitution, low temperature embedding, muscle, liver.

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Introduction

Different specimen preparation methods have recently been used for retention of mobile ions in situ in soft biological tissues. The method of choice (theoretically) is rapid freezing, followed by cryosectioning and analysis in the frozen state in the electron microscope using a cryo-specimen holder, allowing controlled dehydration. However, this method requires special instrumentation such as a cryoultramicrotome, a cryotransfer system and a cryostage. The alternative methods of preparation are rapid freezing followed either by freeze-substitution or freeze-drying followed by embedding in resins. Both methods have been tested in several analytical applications (Chandler 1983, Edelmann 1980, Harvey 1980, Marshall 1980,Ingram et al 1974, Ingram and Ingram 1980, Kaufman 1980, Rebhun 1972). Low temperature embedding media recently developed by Carlemalm et al (1982) are good substitutes to conventional resins for tissue infiltration after freeze-drying or freeze-substitution.

The purpose of the present paper is to describe our experimental conditions when using Lowicryl HM20 embedding medium and to compare it with conventional embedding resins where polymerization is performed at high temperatures. With Lowicryl resin infiltration and polymerization can be performed at -50°C with UV light irradiation. This together with the fact that there is no water production during polymerization of methacrylates would enhance immobilisation and ensure minimal loss of diffusible elements during preparation for X-ray microanalysis (XRMA). The validity of freeze-drying and freeze-substitution have been demonstrated in the present study by using tenotomized rat skeletal muscles known to have high sodium and chlorine and low potassium levels as compared with healthy muscles (Wroblewski and Edström 1984). We have also tested the validity of low temperature preparation methods using liver samples.

Material and methods

Freezing procedure

Liquid propane and a copper forceps cooled in liquid nitrogen were used to assure rapid freezing. Prior to the different water withdrawal procedures the specimens were stored in liquid nitrogen.

Freeze-drying

Freeze-drying was performed in a specially redesigned conventional freeze-dryer (Fig.1) allowing introduction of freeze-dried samples into the resins under vacuum conditions. For this reason tissue samples were transferred from liquid nitrogen (inside the freeze-dryer) onto the surface of a thinly stretched Parafilm covering the top of the cryotubes which contained cooled Lowicryl or frozen Araldite resin. The surface of the Parafilm was perforated by several lines of holes made by small insect needles. Freeze-drying was started at a temperature of about -90°C in the morning. The temperature was kept at about -70°C during 10 h and thereafter slowly raised to -30° for two days. The vacuum was about 0.001 Torr. After freeze-drying the samples were plunged into the embedding medium by breaking the Parafilm surface, all in vacuum and at -30° C. When Lowicryl resin was used, polymerization also was performed at low temperature and in vacuum.

Freeze-substitution

Anhydrous ether was used as substitution fluid. The frozen samples were transferred into a low temperature box in liquid nitrogen and subsequently to cryotubes (Nunc,Denmark) filled with dry ether. Substitution was allowed to proceed at CO_2-ice temperature for 2-3 weeks.After substitution the ether was exchanged for Lowicryl HM20 medium cooled to CO_2 temperature. When epoxy resin was used the temperature in the cold box was slowly raised to room temperature and the substitution fluid exchanged gradually for resin. Molecular sieves were introduced into the cold box to dry the inside atmosphere (mainly CO_2). For work with Lowicryl an oxygen-free atmosphere is of great importance.

Sectioning

Sections (0.2-1.5 µm thick) were cut on a conventional ultramicrotome using a dry knife. Sections were picked up from the knife edge with a hair and transferred to a specially designed carbon specimen holder (Wroblewski and Wroblewski 1982, Wroblewski 1982) with a hole covered by a Formvar film allowing analysis in the scanning-transmission mode. Sections were then pressed down onto the Formvar film. Several sections could be placed on the same specimen holder. After adherence, the sections were coated with a carbon layer and stored dry over molecular sieve until analyzed. Adjacent sections were mounted on the glass slides,stained with toluidine blue and studied simultaneously in the light microscope in order to provide better knowledge of the morphology and preservation of the specimens used for elemental analysis in electron microscope. There was no difference in cutting properties between Lowicryl HM20 and Araldite. We found, however, that tissue sometimes failed to adhere to Lowicryl medium and fell out of the section.

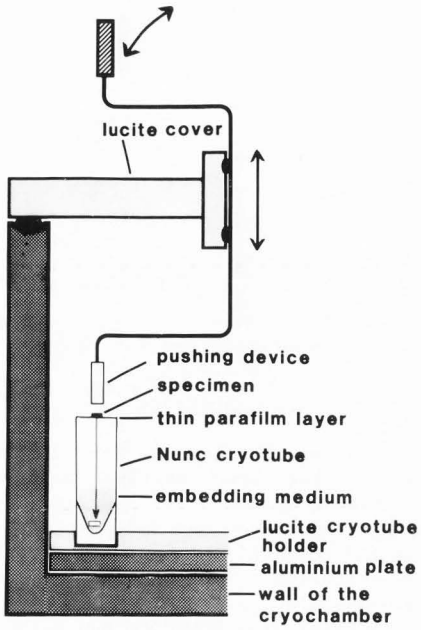

Fig. 1. Schematic drawing of the freeze-drying apparatus. Frozen specimens are transferred from liquid nitrogen to the surface of a Parafilm covering cryotubes filled with resin. After completed freeze-drying the samples were transferred into the resin by means of breaking the Parafilm. After infiltration, samples in Lowicryl were directly polymerized using UV radiation, samples in Araldite were infiltrated at room temperature in vacuum and polymerized in the oven.

Results

Morphological observations

Comparison of morphological preservation of specimens processed in different ways is far from straightforward. This is due to the non-uniform freezing where several factors such as specimen mass and speed of injection into the cryogen may affect the quality of cryofixation. Also injury during dissection might affect final morphology.

Freeze-substitution, freeze-drying, infiltration by resin, polymerization, and cutting are far from standardized, and deviations in procedures may highly affect the appearance of the section. Freeze-dried and freeze-substituted rat skeletal muscle showed relatively uniform morphological preservation (Fig.2). In longitudinal sections I- and A-bands and the Z-lines were easily seen. The nuclei showed electron dense and electron lucid areas. Blood capillaries, strands of collagen and fibroblasts were recognizable between muscle fibres. The nature of other structures was more a question of imagination than of proper visualisation.

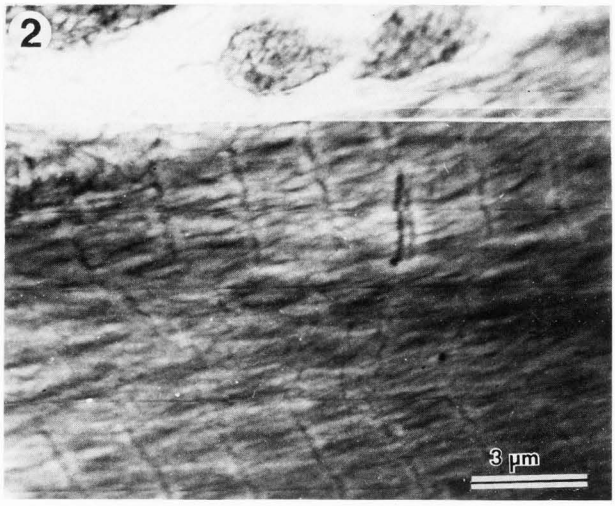

Fig. 2. Scanning transmission electron micrograph of dry cut Lowicryl semithin section of tenotomized rat soleus muscle. Muscles were freeze-substituted in ether, infiltrated and polymerized at low temperatures using ultraviolet light.

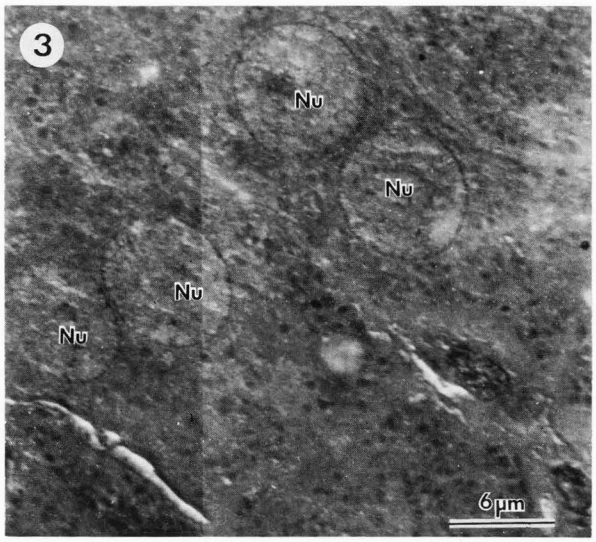

Fig. 3. Low power scanning transmission electron micrograph of freeze-dried, low temperature embedded rat liver. Several hepatocytes with prominent nuclei (Nu) are present. Section thickness 0.3-0.5 µm.

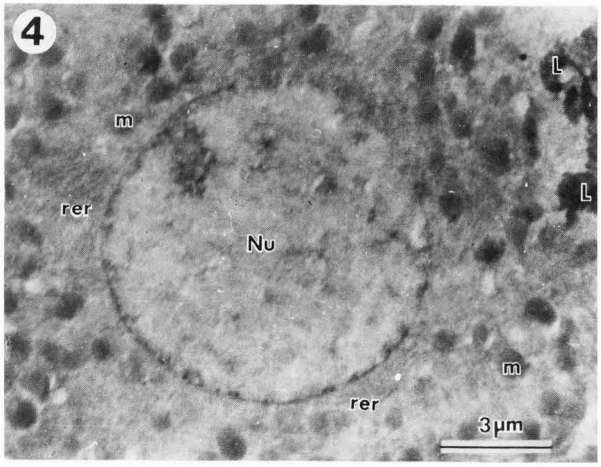

Fig. 4. High power scanning transmission electron micrograph of dry cut chemically unfixed semithin (0.3-0.5 µm) section of freeze-dried and low temperature embedded rat liver. Several parenchymal cells with prominent nuclei (Nu), mitochondria (m), rough endoplasmic reticulum (rer) and lysosomes (L) are present.

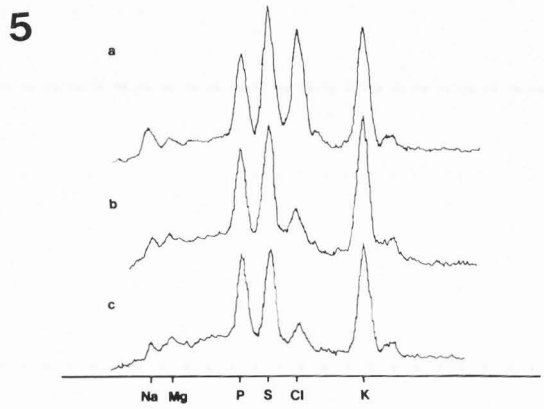

Fig. 5. Elemental spectra of rat muscles processed in different ways. a. Freeze-substituted and embedded in Lowicryl (tenotomized muscle), b. Freeze-dried and embedded in Lowicryl (normal muscle), c. Freeze-dried and embedded in Araldite (normal muscle). Note high sodium and chlorine peaks in tenotomized muscles as compared with normal. Quantitative results are presented in Tables 1 and 2.

Liver cells showed better preservation than skeletal muscle. Numerous mitochondria, lysosomes, lipid droplets, areas of endoplasmic reticulum and nuclei were directly recognizable (Figs. 3 and 4).

Visualisation improved after 10-20 seconds of irradiation by the electron beam. It was evident that mass loss was not equal in Araldite and Lowicryl sections. It is possible that the faster improvement of the image in Lowicryl sections depends not only on a faster mass loss but also on higher contrast of tissues embedded in Lowicryl (Carlemalm et al 1982).

Elemental analysis

Cryosections (6 µm thick) of tenotomized muscle were analysed (Wroblewski and Edström 1983 and 1984). Significantly elevated sodium and chlorine and lowered potassium was found in all tenotomized muscles as compared with contralateral or healthy rat muscle (Table 1, Fig. 5). Similar ion shifts could be recorded in muscles processed by means of freeze-substitution (Table 2), freeze-drying and embedding in vacuum. The only difference was that the concentrations of sodium were smaller in Araldite embedded muscles than in

Table 1

Analysis of cryosectioned muscle

	Tenotomized muscle	Contralateral muscle	Normal muscle
Na	393 (77)	130 (30)	106 (30)
Mg	73 (27)	75 (13)	75 (22)
P	343 (29)	333 (44)	356 (90)
S	396 (67)	483 (50)	432 (95)
Cl	193 (55)	33 (13)	28 (9)
K	356 (44)	433 (99)	496 (63)

Absolute concentrations (mmol/kg dw) of Na, Mg, P, S, Cl and K in rat soleus muscle after tenotomy as compared with non-tenotomized soleus and soleus from unoperated control rats. Study performed on 6 µm thick cryosections cut at -30°C in a conventional cryostat (see Wroblewski and Edström 1983).
Mean and SD of individual mean values of 5 tenotomized and 3 control rats are given. 15-20 analyses per animal were performed.

Table 2

Analysis of freeze-substituted muscle

	Tenotomized muscle	Contralateral muscle
Na	0.28 (0.10)	0.16 (0.05)
Mg	0.14 (0.08)	0.10 (0.04)
P	1.26 (0.22)	1.32 (0.28)
S	1.45 (0.18)	1.76 (0.26)
Cl	0.99 (0.25)	0.62 (0.22)
K	3.04 (0.32)	3.22 (0.36)
n	10	10

Relative intensities (R=P/B) of Na, Mg, P, S, Cl, and K in rat soleus muscle after tenotomy as compared with contralateral non-tenotomized muscles. Study performed on freeze-substituted and Lowicryl infiltrated and polymerized at low temperatures material.
Mean and SD are given. n = number of analyses.

Lowicryl embedded muscles. In normal muscle processed in Lowicryl after freeze-substitution and freeze-drying respectively, the sodium signal (retention of sodium) was higher in Lowicryl embedded material than in material embedded in Araldite (Table 3). In liver specimens (only normal liver was analysed) different elemental spectra were obtained when analysing nuclei, endoplasmic reticulum, mitochondria, or lysosomes (Table 4, Fig. 6). Analytical spectra obtained by scanning several hepatocytes in a Lowicryl section and in 2 µm thick freeze-dried cryosection are shown in Fig. 6. Sodium and magnesium are clearly seen in both the spectra. Those two signals were often difficult to detect in the Araldite section. The chlorine signal was found to be somewhat higher in Lowicryl embedded liver than in freeze-dried cryosections. It is possible that a difference in cell populations within the analysed area partly explains the discrepancy in chlorine concentrations. Analysis of Lowicryl resin free of tissue showed some traces of Si, P and Cl. In hepatocytic lysosome phosphorus, sulphur chlorine, potassium peaks as well as an iron signal were recorded. No sodium or magnesium was detected.

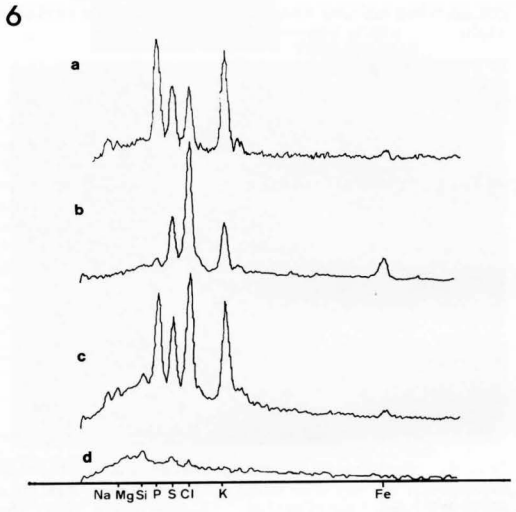

Fig. 6. Elemental spectra from different compartments of rat liver as seen in Figs. 3 and 4. in comparison with freeze-dried cryosections. a. 6 µm thick freeze-dried cryosection, the analysed area includes several liver cells of unknown type. b. spectra from freeze-dried Lowicryl embedded liver lysosome, c. from several parenchymal cells, d. from Lowicryl resin outside the sample. Note high Fe and low P peak in lysosome.

Table 3

Comparison of preparative techniques for muscle

	Freeze-dried		Freeze-substituted	Freeze-dried
	Araldite	Lowicryl	Lowicryl	cryosections
Na	0.08 (0.04)	0.15 (0.08)	0.16 (0.05)	0.20 (0.09)
Mg	0.07 (0.05)	0.09 (0.04)	0.10 (0.04)	0.16 (0.08)
P	0.99 (0.11)	0.91 (0.18)	1.32 (0.28)	1.92 (0.22)
S	1.37 (0.20)	1.26 (0.30)	1.76 (0.26)	2.62 (0.30)
Cl	0.55 (0.09)	0.54 (0.22)	0.62 (0.22)	0.52 (0.11)
K	3.45 (0.61)	3.46 (0.53)	3.22 (0.36)	8.52 (1.01)
n	14	11	10	15

Relative intensities (R=P/B) of Na, Mg, P, S, Cl and K in normal muscle samples after freeze-drying, freeze-substitution followed by different infiltration and polymerization procedures in comparison with freeze-dried cryosections.
Mean and SD are given. n=number of analyses.
1. Muscle samples were freeze-dried and infiltrated with resin under vacuum. Muscles infiltrated in Araldite were polymerized in the oven. Muscles infiltrated with Lowicryl were polymerized in vacuum at -30°C using UV-light.
2. Muscles were freeze-substituted with ether, infiltrated with resin at - 79°C and polymerized at -50°C Muscle samples were taken from the contralateral side of tenotomized rat.
3. 6 µm thick cryosections were cut using a conventional cryostat operating at - 30°C.

Table 4

Comparison of preparative techniques for liver

	Lowicryl sections			Cryosections
	Nuclei	Mitochondria	Lysosomes	
Na	0.19 (0.12)	0.37 (0.18)	0.18 (0.18)	0.63 (0.10)
Mg	0.05 (0.03)	0.12 (0.09)	0.04 (0.01)	0.15 (0.07)
P	1.65 (0.05)	1.23 (0.11)	0.40 (0.17)	2.89 (0.38)
S	0.83 (0.08)	1.32 (0.19)	1.33 (0.16)	1.51 (0.09)
Cl	2.24 (0.06)	2.91 (0.12)	4.03 (0.23)	2.22 (0.25)
K	2.35 (0.05)	3.10 (0.23)	3.33 (0.42)	5.32 (0.22)
Fe			1.66 (0.28)	0.48 (0.23)
n	5	5	5	5

Relative intensities (R=P/B) of Na, Mg, P, S, Cl, K and Fe in different cell compartments in freeze-dried and Lowicryl embedded liver samples in comparison with 2 µm thick freeze-dried cryosections. Mean and SD are given, n=number of observations.

Discussion

Direct embedding of cryofixed, freeze-dried samples has been described earlier in publications concerned with the ultrastructure of different tissues. Freeze-drying fixation was already used in 1943 by Sjöstrand (1943 a,b) and Richards et al. (1943) in studies on the structure of muscle and nerve fibers respectively. Sjöstrand (1943 a,b) when studying structure of biological material in electron microscope had the same criteria for tissue preservation as those which are valid today for preparation for microanalysis. He postulated the use of freeze-drying vacuum fixation without any use of any chemical fixation and staining methods which he considered superfluous as fine biological structures have sufficient contrast factors especially when visualised at high magnification. Sjöstrand's claims about non-chemical fixation and considerations concerning the inherent contrast of biological structures became a reality with the advent of the scanning-transmission electron microscope and the visualisation of biological structures by using Z-contrast. Hanzon and Hermodson (1960) analysed freeze-dried pancreas and described the structural appearance of different artifacts. These authors pointed out that impregnation of freeze-dried tissues with resin is a critical step and used a vacuum chamber allowing insertion of specimens into the resins under vacuum conditions. Elfvin (1963) described the ultrastructure of the plasma membrane and myelin sheath after freeze-drying fixation in comparison with chemical fixation. A different membrane pattern was obtained when using Vestopal and Araldite resin. Monroe et al (1968) described the ultrastructure of cardiac and striated muscles

obtained during specific phases of the contraction cycle by ballistic cryofixation. Muscle fragments were freeze-substituted for 2 weeks in absolute ethanol (-75°C), immersed in a 1:1 mixture of ethanol and Epon at -25° C, embedded and polymerized in Epon. The authors suggested the future use of their method in investigations of mobile ion movements during muscle contraction and relaxation. Wroblewski and Jansson (1975) studied the fine structure of single histochemically classed muscle fibres separated from freeze-dried muscle biopsies. Single fibres were subsequently fixed in glutaraldehyde and osmium and embedded in Spurr's resin. Type I fibres were found to have thicker Z-bands and more mitochondria and lipid droplets than the type II fibres. Edelmann (1978) described a simple technique of freeze-drying of muscle samples together with frozen drops of Spurr's medium. After conducted freeze-drying, the temperature in the chamber was raised followed by liquefaction of the embedding medium and infiltration of the specimens in vacuum. Ingram et al. (1974) used the method of Hanzon and Hermodson (1960) to analyse intra- and extracellular environment of freeze-dried, osmic vapor fixed and epoxy embedded samples. Edelmann (1980) studied the potassium binding sites in striated muscle. The selection of a suitable technique for tissue preparation for analytical electron microscopy often depends on the availability of certain laboratory equipment.Using methods other than the method of choice (cryofixation, cryosectioning, cryotransfer and analysis at low controlled temperatures) a critical evaluation of the preparation procedures should be performed. The animal model of muscle tenotomy characterized by an increased Na and Cl and often lowered K content was used here to establish the usefulness of freeze-drying and freeze-substitution techniques in microanalytical investigations. The significant ion shifts characteristic for tenotomy were found in all preparations. Comparison between elemental composition of 6 µm thick cryosections and plastic embedded sections showed decreased sodium levels especially in Araldite embedded material. Anniko et al (1984) found a lower sodium content in perilymphatic spaces of the inner ear of the adult mice freeze-dried and embedded in Araldite as compared with freeze-dried cryosections. This finding, and our present findings might be partially explained by greater absorption of sodium X-rays in the embedding medium. Since the cochleas were transferred into the embedding media through room atmosphere a minor ion redistribution can be expected to occur. In our experiments, however, freeze-dried muscle samples were transferred into the Araldite medium in vacuum. Redistribution of ions into the embedding media was also checked by analysis of embedding media close to the tissue, but no signs of diffusion could be detected. The higher sodium levels in Lowicryl preparations might be due to a higher degree of ion immobilization and/or quicker mass loss of resin from the sections, decreasing the absorption.

Conclusions

Freeze-drying followed by infiltration and polymerization by UV light at low temperatures and under constant vacuum conditions is an alternative tissue preparation technique for microprobe analysis. It can be achieved by using the nonpolar low-temperature embedding resin (Lowicryl HM20) which allows infiltration and polymerization at temperatures down to - 50° C. Morphological preservation is adequate for identification of structures like mitochondria, lysosomes and different types of endoplasmic reticulum in liver cells. Some physical properties of Lowicryl resins as its mass loss under the electron beam and its high contrast are positive characteristics when analysing 0.2-1.5 µm thick sections. It is likely that the initial preparation step- the cryofixation to a high degree determines the morphological preservation of freeze-dried and embedded tissue.

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Discussion with Reviewers

B.L. Armbruster: What imaging mode (s) was used in the STEM to generate the images shown in Figures 2, 3 and 4 ?
Authors: The images were obtained using bright field STEM.

B.L. Armbruster: Did you try analyses of specimens embedded in the polar resin K4M ?
Authors: No.

L. Edelmann: Have you tested different freeze-substitution and embedding techniques concerning structure preservation of muscle and retention of mobile ions ?
Authors: We have been carrying out freeze substitution using acetone or DMP (dimethoxypropane), but found that retention of ions was best with diethyl ether as substitution medium. The morphological preservation of muscle was about equal as seen in 0.3 - 1 µm thick sections in STEM. Fine structural preservation can, however not be demonstrated in sections of that thickness.

M. Schmitz and R. Meyer: You started the drying procedure at a temperature of -90°C. Where did you measure this temperature?
Authors: As stated in Material and Methods the starting temperature was approximately -90° C. Exact measurement of the starting temperature is difficult. The specimens were brought in LN_2 into the chamber of the freeze-dryer which is also partly filled with LN_2 . After placing the tissue samples on the surface of the Parafilm, the freeze-dryer was closed and evacuated. During this first phase of evacuation the temperature of the condenser increased from LN_2 temperature to the temperature of the dry ice. The temperature measurements were performed on the condenser wall.

M. Schmitz and R. Meyer: Placing your specimen in a high vacuum at relatively long distance from cooled elements and on Parafilm with little thermal conductivity, what do you think is the specimen temperature for freeze drying and the following steps?
Authors: The distance between specimen and condenser (wall of the freeze-dryer) is about 8 mm. The temperature of the specimens is higher, probably several degrees C, than the nominal temperature.

M. Schmitz and R. Meyer: In order to achieve better information about the preservation of the tissue, why didn't you use conventional poststained ultrathin sections for electron microscopy instead of the toluidine stained sections for light microscopy?
Authors: Toluidine stained sections were used as they can give a quick survey of entire specimen, indicating areas of crystal formations. Such sections can be cut dry. Cutting of ultrathin sections, using a knife with a trough filled with water can cause ion redistribution within the entire specimen, if the water comes into contact with the block face.

M. Schmitz and R. Meyer: How do you interpret the data shown in Table 3 that freeze substituted samples reveal a higher P/B ratio compared with freeze-dried samples? How do you explain the relatively small difference in P/B ratio between plastic sections and cryosections ?
Authors: The difference between Lowicryl embedded tissues after freeze-drying and freeze-substitution respectively is rather small and not statistically significant. A much larger

difference is found between freeze-substituted Lowicryl embedded muscles and freeze-dried cryosections. This difference is due to the presence of the Lowicryl medium which increases the total mass of excited volume. As white radiation increases the P/B ratio will be lower in plastic sections. The relatively small difference in P/B ratio between plastic sections and cryosections might be partly explained by a selective mass loss (mainly plastic) in the embedded material.

Reviewer I: When you described how tissue sometimes failed to adhere to Lowicryl medium and fell out of the section, was it well infiltrated at the time?
Authors: Yes in most cases. It is possible that different polymerization conditions in tissue and in the pure resin cause the tissue to fall out of the section. The quality of the sections was better within the tissue than outside. Slower polymerization with more diffuse ultraviolet light might prevent this phenomenon.

Reviewer I: The contrast in your figures is excellent, were the images obtained from unstained tissue? Are samples also fixed with a chemical fixative, or is cryofixation sufficient to stabilize proteins?
Authors: The images were obtained from chemically unfixed, unstained tissue cut dry at room temperature. STEM bright field was used.

Reviewer I: Does Lowicryl spectra contain contaminant peaks from Cl or other elements with atomic number heavier than 16? If there are contaminant peaks, how are they treated?
Authors: In Fig. 6d the spectrum from the Lowicryl resin outside the tissue is presented including some minor peaks- Si, S and Cl. As "contaminant" peaks are very small (less than 1%) in comparison with those obtainable from the tissue (Fig. 6 a-d) no action was taken in the quantitation of the final results.

B.L. Armbruster: In Table 4 were any particular organelles scanned in cryosections or do the values represent total content in the tissue?
Reviewer I: From which part of the hepatocytes were the cryosectioned data obtained in Table 4?
Authors: Data in Table 4 (right column) were obtained by using a selected area raster scanning over several different types of liver cells. Such analytical data are also influenced by the content in the extracellular spaces etc. Of course, use of thin cryosections for comparison with data obtained from freeze-dried and embedded material would be more accurate.

Reviewer I: Do you suggest that Araldite not be used as an embedding medium for electron probe microanalysis because of the apparent loss of Na signal with its use?
Authors: We found better retention of sodium in Lowicryl embedded material than in Araldite. Retention of other elements was about the same. As we found the P/B ratio for most elements to be the same in Lowicryl and Araldite embedded material, and considering higher mass loss in Lowicryl embedding, it is likely that mobile ions diffuse into the Araldite during the embedding and polymerization procedure.

H.K. Hagler: The X-ray spectra presented in Fig.6 suggest extreme loss of plastic during analysis or rediffusion of elements during embedding both of which could produce such high elemental peaks in the presence of the embedding media. Do you have any measurements regarding the mass loss occurring during analysis?
Authors: We have done some preliminary measurements using EELS, which showed rather extensive mass loss during the first seconds of irradiation. The elevated P/B ratio after mass loss indicate selective loss of plastic rather than rediffusion of elements during embedding.

H.K Hagler: Why are you still cutting cryosections at -30°C when it is now widely accepted that these temperatures result in significant redistribution of diffusible elements?
Authors: We have been using semithick and thick cryosections cut at -30°C for a decade in many applications concerning elemental changes in experimental and pathological conditions in several tissues. We found that X-ray microanalysis of such sections can give quick and reliable results concerning the elemental content based on cellular level. Several cells per subject can be analysed and related to histochemical findings studied in an adjacent serial section processed for histochemical staining. No data, however, can be obtained on the subcelluar level (except cell nuclei). No redistribution between the cells has been found. We agree that it will be of interest to have some data (see Table 4) from thin cryosections where analyses can be performed on the organelle level.

Reviewer I: Have you determined whether the increased mass loss with Lowicryl arises from the plastic or from the tissue? It was observed by Ingram and Ingram (Scanning Electron Microsc. 1983; III: 249-254) that more reproducible measurements of tissue water could be made with freeze-dried, plastic embedded tissue if a final drying step at +50° C is observed prior to embedding. If samples are not completely dry before they are embedded, any remaining water will be driven off by the electron beam, unless the sample is cooled during analysis. If the increased mass loss arises from the Lowicryl itself, could that be indication that polymerization with heat drives off volatile substances before the sample is introduced to the electron beam? If that were the case, could the gentle heat , of say, 65°C, be used with Lowicryl samples before they are introduced to the electron beam?
Authors: It is possible to use gentle heat with Lowicryl samples before introducing them into the electron beam. Such a procedure will, however, mainly affect the tissue, and not the Lowicryl medium. If one wants to polymerize Lowicryl by heat, an accelerator has to be added to the Lowicryl formula.