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Freeze-substitution and the preservation of diffusible ions

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SUMMARY

Freeze-substitution of biological material in pure acetone followed by low-temperature embedding in the Lowicryls K11M and HM23 yields stable preparations well suited for sectioning and subsequent morphological and microanalytical studies. Transmission electron microscopy of dry-cut sections shows that diffusible cellular thallium ions (Tl^+) of Tl^+-loaded muscle are localized at similar protein sites in freeze-substituted as in frozen-hydrated preparations. A comparison of X-ray microanalytical data obtained from freeze-dried cryosections and sections of freeze-substituted normal (potassium-containing) muscle shows that K^+ ion retention in the freeze-substituted sample is highly dependent on the freeze-substitution procedure used; so far, in the best case, about 67% of the cellular K^+ is retained after freeze-substitution in pure acetone and low-temperature embedding. It is concluded that the retention of diffusible cellular ions is dependent on their interactions with cellular macromolecules during the preparative steps and that ion retention may be increased by further optimizing freeze-substitution and low-temperature embedding.

INTRODUCTION

Ultra-rapid freezing (cryofixation) of biological material yields preparations in which all components (water, macromolecules and diffusible substances) are immobilized in a life-like state. After cryotransfer of thin cryofixed specimens, or of frozen-hydrated cryosections, into a cryo-electron microscope the ultrastructure of these specimens may be visualized (Dubochet *et al.*, 1987), but in most cases it is not yet possible to identify and localize diffusible macromolecules and ions with a high spatial resolution within these frozen-hydrated preparations. In order to carry out such investigations, different techniques are used to dehydrate well-cryofixed biological material and to produce stable specimens which can be analysed routinely in an electron microscope. Stable preparations are freeze-dried cryosections (stable only under certain conditions), or sections obtained from resin-embedded, freeze-dried or freeze-substituted material. In the latter case the frozen specimen is dehydrated at low temperature by means of an organic solvent; the solvent is subsequently replaced by a resin which can be polymerized at low or high temperatures (see, e.g., Steinbrecht & Müller, 1987;

Edelmann, 1989a). It is currently assumed that freeze-dried cryosections are best suited for X-ray microanalysis of diffusible ions at an ultrastructural level and that X-ray microanalysis of these specimens should be carried out to obtain the most reliable data.

On the other hand, many reports exist which prove that diffusible ions can be retained in biological preparations after freeze-drying and resin embedding (for reviews see Ingram & Ingram, 1980; Edelmann, 1986; Steinbrecht & Müller, 1987) or even after freeze-substitution and embedding (for reviews and further references see van Zyl *et al.*, 1976; Marshall, 1980; Harvey, 1980, 1982; Wróblewski & Wróblewski, 1984; Steinbrecht & Müller, 1987; Zierold & Steinbrecht, 1987). Many scientists working with plant material have claimed that when using diethyl ether or acetone as substitution liquids, mobile ions can be preserved within cells or subcellular compartments (Harvey, 1980). Despite these claims there are strong reservations to the belief that meaningful microanalysis can be carried out with freeze-substituted and resin-embedded material. The reasons for these reservations arise from some rather 'unsuccessful' attempts to preserve diffusible ions by freeze-substitution (Roos & Barnard, 1986; Zierold & Schäfer, 1988); and furthermore, there is as yet no fully quantitative comparison of data obtained from properly freeze-substituted embedded material and freeze-dried cryosections. Subcellular redistribution cannot be ruled out even if most of the cellular ions are retained within the freeze-substituted specimen.

The purpose of this paper is to offer new strategies to investigate the problem of the extent to which diffusible ions may be captured in freeze-substituted and resin-embedded material.

THEORETICAL CONSIDERATIONS

It is widely assumed that the validity of preparation procedures for microanalytical work can be judged by the degree of concentration differences of mobile cellular ions measured between neighbouring membrane-enclosed compartments of the dehydrated specimen. However, this view need not be correct. Depending on the degree of dehydration and on the properties of substitution and embedding media in the case of freeze-substitution, the affinity of fixed charges of proteins in a specific compartment towards certain ions of opposite sign may increase the extent to which preferred ions migrate through membranes and accumulate at these proteins to a higher degree than in a living cell. Even if it is assumed that membranes maintain their integrity at low temperature—which does not mean ion-impermeability—we do not know the conditions necessary to impede migrations of ions through membranes either by diffusion or by an adsorption–desorption mechanism. Therefore we cannot trust a priori the results obtained, from any dehydrated specimen (including a freeze-dried cryosection). In other words, since the interactions between fixed charges and mobile ions in living cells are certainly not the same as those found in dehydrated specimens, it is necessary to know where mobile ions are localized in the living cells or in a well cryofixed frozen-hydrated preparation before any conclusions can be drawn from microanalytical results obtained from dehydrated preparations (Gupta & Hall, 1979). Unfortunately, no X-ray microanalytical techniques are yet available for the quantitative detection of diffusible ions with a high spatial resolution in ultrathin frozen-hydrated cryosections (Zierold, 1986).

Nevertheless, according to the following considerations, it is possible to localize precisely certain mobile cellular ions in such frozen hydrated preparations. Many physiologists have studied the accumulation of rubidium (Rb^+), caesium (Cs^+), and thallium ions (Tl^+) in living cells with the aim of gaining better understanding of the molecular mechanism of potassium ion (K^+) accumulation, as well as the molecular mechanisms responsible for electrophysiological phenomena (for review see

Edelmann, 1984a). These studies have unequivocally shown that Rb^+, Cs^+, and Tl^+ all accumulate in living cells according to the same mechanisms as K^+, which is the main cellular cation of most living cells. In particular, experiments with living isolated frog muscle revealed that about 80% of the cellular K^+ can be replaced reversibly by the heavy, electron-dense Tl^+ ions (Ling, 1977). This means that living cells can be obtained in which the main cellular cation is Tl^+ instead of K^+. Similarly to the behaviour of K^+ ions in normal cells, the mobile intracellular Tl^+ ions are in constant exchange with extracellular Tl^+ ions and the dying cell loses the accumulated Tl^+ ions during chemical fixation. However, the living Tl^+-loaded cell can be cryofixed and we thus obtain a preparation which is particularly well suited to electron microscopic investigations as it contains a large amount of electron-dense particles. Provided these particles are unevenly distributed in the cell, it should be possible to visualize directly this uneven distribution in frozen-hydrated cryosections by transmission electron microscopy.

Experiments of this kind have been carried out (Edelmann, 1984b, 1988) and the results are shown in Fig. 1. When comparing frozen-hydrated cryosections of Tl^+-loaded muscle (Fig. 1a) with those of normal K^+-containing muscle (Fig. 1b), it was found that the electron-dense Tl^+ ions are mainly localized in the A-bands and Z-lines; more precisely, the Tl^+ ions accumulate at individual protein filaments of the muscle preparation.

This result has far-reaching significance because it shows—in contrast to the conclusions derived from physiological and electro-physiological experiments—that the

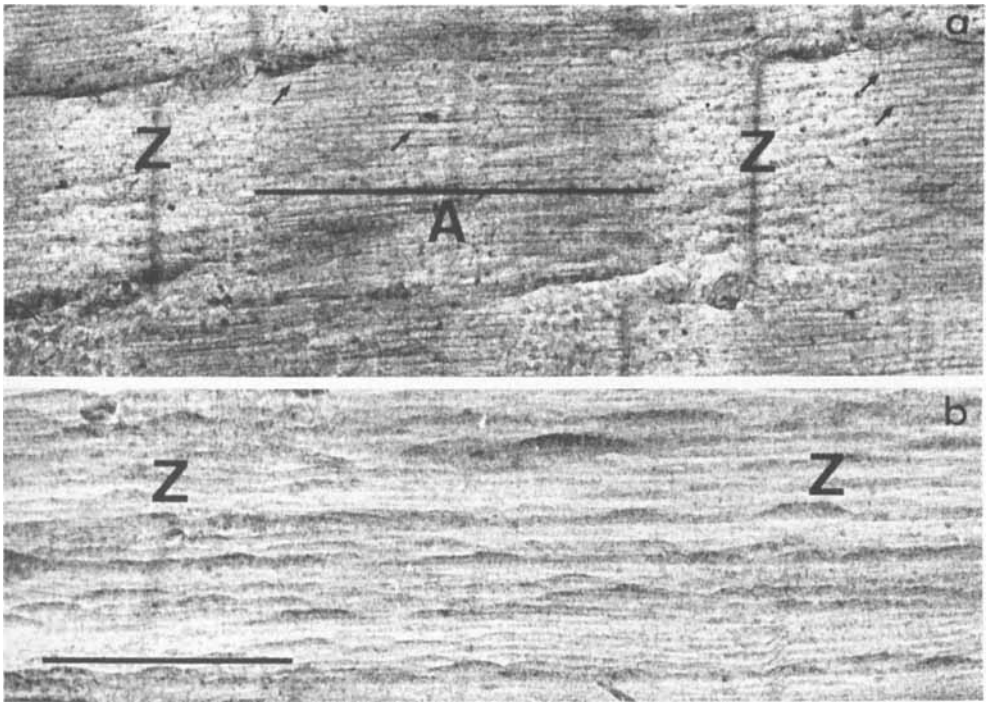


Fig. 1. Frozen-hydrated cryosections (about 0·1 μm thick) of frog sartorius muscle. The sections were kept at about 100 K and photographed in a Zeiss EM10CR at a magnification of 5000 (electron exposure below 1000 e^-/nm^2). (a) Tl^+-loaded muscle (see text). Dark myosin filaments (arrows) in the A bands (A) and dark Z lines (Z) indicate sites of preferential Tl^+ accumulation in the living cell. (b) Normal K^+-containing muscle. Only very faint ultrastructural detail can be seen. Scale bar = 1 μm.

main cellular cation is not homogeneously distributed in the cellular water (see Edelmann, 1988, 1989b); rather, the ions are in close contact with proteins probably adsorbed to β- and γ-carboxyl groups (comparable to the well-known mobile adsorption of K^+ and other ions at glass surfaces) as postulated in the association–induction hypothesis (Ling, 1984; see also Edelmann 1989c).

If we now want to develop a freeze substitution and embedding technique suitable to retain the mobile ions at the same subcellular sites as visualized in the frozen-hydrated preparation the prime question is: which conditions are necessary to maintain the ability of ion adsorption of cellular proteins and to minimize changes of ultrastructure, including extraction of macromolecules? Considering the findings on the extraction of macromolecules (Weibull *et al.*, 1984; Weibull & Christiansson, 1986), and that the configurational changes of proteins caused by organic solvents are reduced when the specimen is kept at low temperatures during dehydration and embedding (Kellenberger *et al.*, 1986), it may be expected that cellular ions are eventually preserved at their original protein sites if freeze-substitution and embedding are carried out exclusively at low temperatures. By using the Lowicryls K11M and HM23 there are no difficulties in carrying out these steps below $-60^{\circ}C$ (see, e.g., Edelmann, 1989a).

Zierold & Schäfer (1988) consider that organic solvents with a low dielectric constant (DC) are best suited for ion retention because the electrostatic forces between fixed charges of the tissue and ions of opposite sign are increasing during substitution in proportion to the ratio between the DC of water (80·3) and the DC of the solvent (e.g. DC of diethyl ether: 4·3). Bearing in mind our finding that mobile ions may be already in close contact with fixed charges in the living hydrated cell, this consideration need not be correct (see Debye, 1929). Furthermore, the finding of van Zyl *et al.* (1976) that many more ions are retained when using acetone (DC: 21·4) instead of ethanol (DC: 25·1) shows that ion retention is probably not mainly dependent on the DC of the used substitution fluid. (Note that the dielectric constants are given at room temperature; they are much higher at the low temperatures used in freeze-substitution.) Since it is not yet possible to deduce theoretically the organic solvent optimally suited for ion retention during freeze-substitution, we propose to investigate further diethyl ether and acetone, which have been reported to give the highest ion retention in biological material (Harvey, 1980). Since freeze-substitution with diethyl ether is extremely slow at low temperature (Humbel & Müller, 1986), and because preservation even of very labile biological structures can be obtained by using pure acetone (Hobot *et al.*, 1987), this latter solvent may be the best choice for obtaining good structure preservation within a reasonable length of time.

The experiments described in the next section were aimed at investigating the following problems: is it possible (i) to maintain the ultrastructure of muscle tissue by using pure acetone as substitution liquid?, (ii) to retain Tl^+ in Tl^+-loaded muscle at the same sites as visualized in the hydrated preparation?, and (iii) to retain K^+ in normal K^+-containing muscle after freeze-substitution and low-temperature embedding?

MATERIALS AND METHODS

Frog sartorius and semitendinosus muscles were isolated from Northern American leopard frogs (*Rana pipiens pipiens*, Schreber) and used either when containing a normal amount of K^+, or after the major part of cellular K^+ (about 80%) was replaced by Tl^+ (Ling, 1977). Resting muscles were cryofixed by rapid contact with a liquid nitrogen (LN_2)-cooled copper block as described elsewhere (Edelmann, 1989a). Small pieces of frozen tissue were transferred into the Reichert-Jung CS-auto

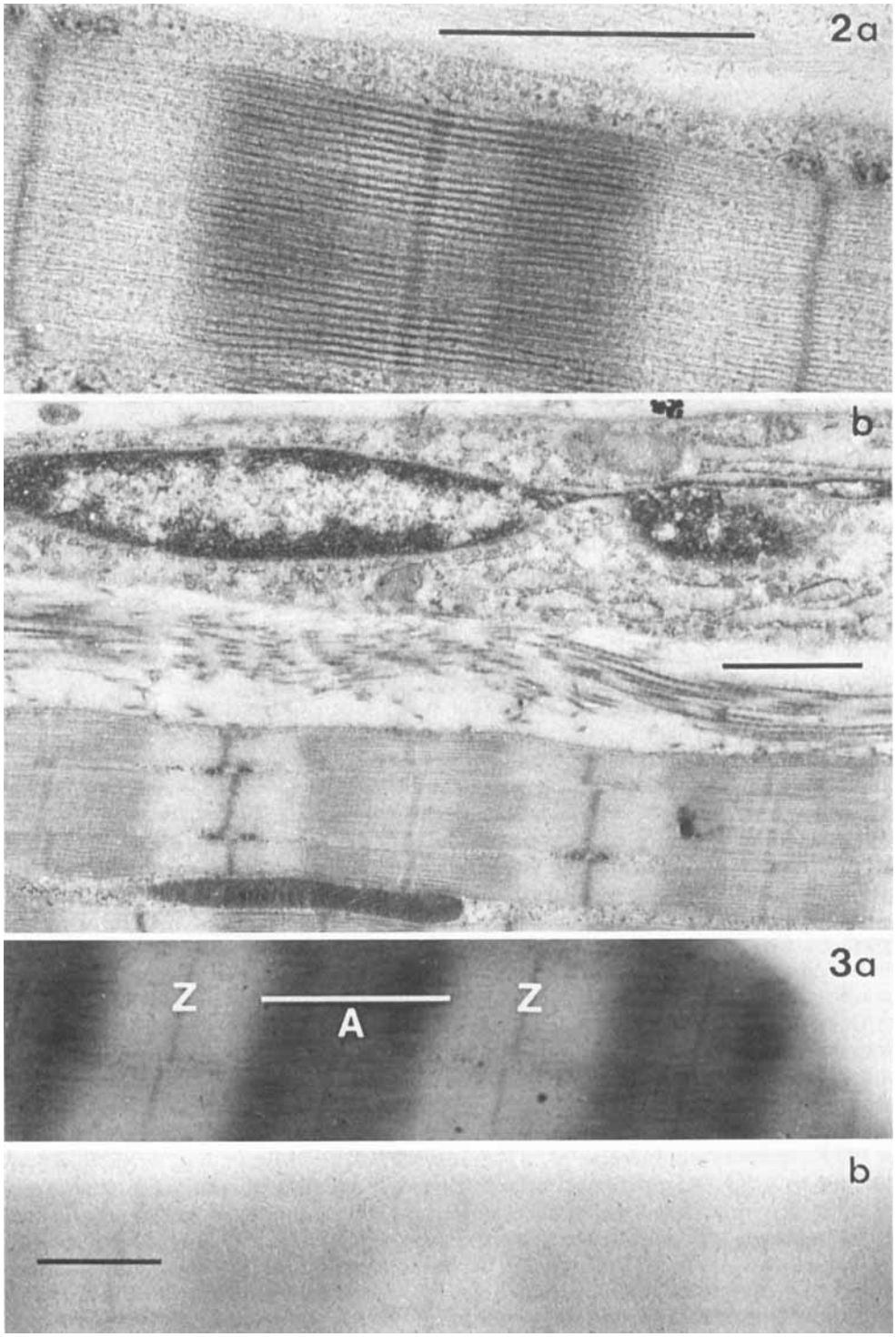
cryosubstitution unit. Freeze-substitution was carried out in pure acetone for up to 7 days at 193 K (for different procedures see below and legends of Figs. 2–4). Afterwards the muscles were infiltrated with Lowicryl (K11M or HM23), transferred into fresh embedding medium in flat embedding moulds and polymerized by UV irradiation at 213 K for 24 h. Samples embedded in HM23 were further irradiated by UV light for 2 days at 223 K. All these steps were carried out inside the CS-auto (Edelmann, 1989a). The polymerized specimens were warmed to room temperature (without further UV irradition) and removed from the moulds. Diatome diamond knives were used to obtain ultrathin and 0·2–0·3 μm dry-cut sections (dry-cutting is necessary to avoid ion loss which occurs during wet-cutting). Cutting of dry sections was facilitated by using a discharge device (Ziegener + Frick, Beilstein) as suggested by H. Gnägi, Diatome (personal communication). X-ray microanalysis of dry-cut sections was carried out in a Siemens Elmiskop ST100F as described by Zierold (1988). Cryosections of cryofixed muscle were cut in a Reichert FC4-Ultracut cryo-ultramicrotome. Cryotransfer of the sections into the ST100F, freeze-drying (30 min) and X-ray microanalysis were carried out as described by Zierold (1988). The X-ray microanalytical work was carried out in collaboration with K. Zierold, Dortmund.

RESULTS

Figure 2 demonstrates the quality of structure preservation of resting skeletal frog muscle after freeze-substitution for 7 days in pure acetone followed by low-temperature embedding either in K11M or in HM23.

Figure 3a reveals the distribution pattern of electron-dense Tl^+ ions in a well-preserved area of a muscle preparation (see figure legend). It is evident that cellular Tl^+ ions are preferentially retained at the same subcellular places as in frozen-hydrated preparations (Fig. 1a), namely at A bands and Z lines. It is noteworthy that no leaching of the ions (i.e. less contrast) can be observed at the edge of the open-ended muscle cell (broken after cryofixation) where the muscle was directly exposed to substitution and embedding fluids. (The similarity between Figs. 1a and 3a is not yet complete. This is due to the fact that the dry-cut section of the freeze-substituted muscle is rather thick. Such sections can easily be obtained and it is rather difficult to produce much thinner sections of Lowicryl K11M at room temperature. If needed, the section thickness may eventually by reduced by cutting at low temperature.)

The X-ray microanalytical results obtained from a freeze-dried cryosection and from sections of the same muscle after freeze-substitution and low-temperature embedding are given in Fig. 4. Although an exact quantitative comparison between the amounts of potassium found in the analysed preparations cannot yet be given, the ratios of potassium (K) and sulphur (S) peak areas found in the different preparations may be concerned. Assuming that S is retained completely in both the freeze-substituted specimen and in the freeze-dried cryosection, and that K^+ is retained completely in the freeze-dried cryosection, a retention of about 67% of the cellular K^+ is obtained under the conditions described in Fig. 4(d) (freeze-substitution for 2 days at 193 K, temperature increase of 20 K/h up to 293 K, infiltration with HM23 for 16 h, cooling to 213 K and polymerization). It was interesting to note that less K was retained after other low-temperature procedures. We found up to 50% retention of K in preparations which had been substituted for 7 days at 193 K and subsequently embedded in K11M or HM23 at 213 K. Much less K-retention was found (Fig. 4e) if a freeze-substituted muscle had been left in acetone for 16 h at 293 K before infiltration with HM23 and polymerization at low temperature (compare figure legends of Fig. 4d and e).


2a
b
3a
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A
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DISCUSSION

A well-preserved ultrastructure is a prerequisite for meaningful microanalysis of biological material at a subcellular level; otherwise a redistribution of ions cannot be excluded. Inadequate freeze-drying or freeze-substitution results in diffusible ions being trapped between redistributing, condensing macromolecules, with the result that although the ions remain within the biological specimen they are no longer found at or near the same place they occupied in the living cell. So far, the best preservation of ultrastructure in a stable dehydrated preparation—without using a chemical fixative—can be obtained with freeze-substitution (see Fig. 2 and discussion by Edelmann, 1989a). The problem of how to achieve a complete retention of mobile ions within this stable preparation is still unsolved. Although it was demonstrated that diffusible Tl^+ ions are distributed similarly within a freeze-substituted and low-temperature embedded specimen and in a frozen-hydrated cryosection, the X-ray microanalytical data show that considerable amounts of K^+ ions are lost during the freeze-substitution and low-temperature embedding procedures used so far. Whether there is a quantitative difference between Tl^+ and K^+ retention remains to be determined. From the above-mentioned Tl^+–K^+ replacement studies (Ling, 1977) it was concluded that Tl^+ is adsorbed at cellular proteins with approximately twice the adsorption energy of K^+. Therefore, it is expected that it is easier to retain Tl^+ than K^+ in a freeze-substituted resin-embedded preparation. The observed K^+ retention is nevertheless very promising. The finding that most of the K^+ ions are lost from the freeze-substituted preparation upon exposure to acetone at room temperature for 16 h, whereas far less ions are lost if the sample is infiltrated with Lowicryl immediately after warming to room temperature, suggests that a further improvement in ion retention is possible if the temperature at which acetone is replaced by Lowicryl is reduced. At this lower temperature, however, the degree of dehydration should be similar to that occurring after exposure of the specimen to acetone at 193 K for 2 days and higher temperatures, as described in Fig. 4(d). It is assumed that freeze-substitution at 193 K for 7 days is not long enough to obtain such a degree of dehydration and that therefore more K^+ ions are lost than with the procedure described in Fig. 4(d). It may be argued that the procedure described in Fig. 4(d) implies that low-temperature embedding is not necessary to obtain the results shown in Fig. 4(d). This may be true, but a higher degree of ion retention would be expected if freeze-substitution, embedding and polymerization were carried out exclusively at low temperatures; systematic studies are necessary to test this assumption.

From these considerations, and the fact that the results have been obtained from small pieces of open-ended muscle cells (broken after cryofixation), it is concluded that the observed degree of K^+ retention is independent of membrane properties, but is dependent on the interactions of the ions with fixed charges of cytoplasmic macromolecules in the presence of the remaining water molecules and organic solvents (substitution fluid and embedding medium) to which the preparation is exposed. In order to find an optimized freeze-substitution and embedding procedure for the

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Fig. 2. Ultrathin sections of frog sartorius muscle after freeze-substitution in pure acetone (7 days, 193 K) and subsequent low-temperature embedding at 213 K in the Lowicryls K11M (a) or HM23 (b); uranyl acetate and lead citrate staining. Scale bars = 1 μm.

Fig. 3. Transmission electron micrographs of 0·3 μm-thick sections of frog muscles after freeze-substitution in pure acetone (7 days, 193 K) and low-temperature embedding in Lowicryl K11M (213 K). (a) Tl^+-loaded semitendinosus muscle (see text) which shows preferential accumulation of electron-dense Tl^+ ions at A bands (A) and Z lines (Z). (b) Control: normal K^+-containing muscle showing rather poor contrast. Scale bar = 1 μm.


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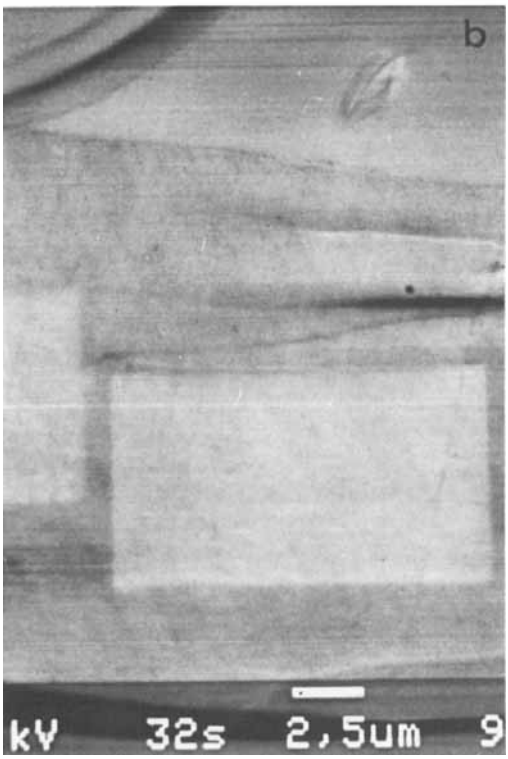
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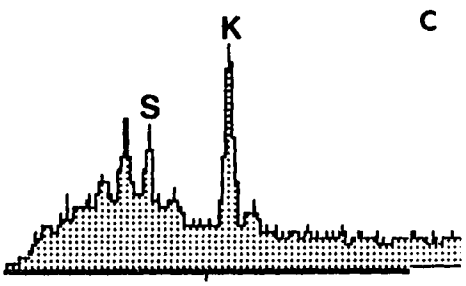
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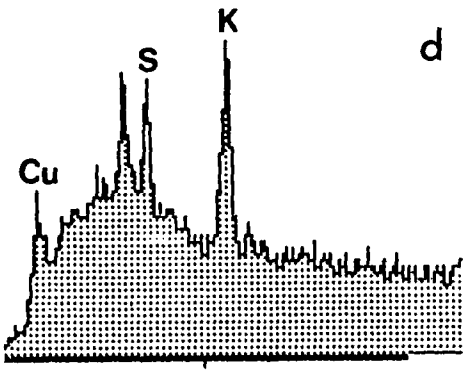
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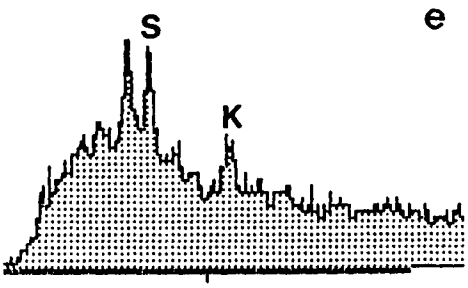
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retention of ions it would therefore be worthwhile to investigate binding of ions at subcellular structures of freeze-substituted material under different conditions. The following experiments may produce improvements in freeze-substitution and low-temperature embedding.

(1) Ion retention may be determined by analysing sections of preparations dehydrated by different substitution fluids at varying temperatures (see Morgan, 1978). The technique would be as follows: after dehydration the specimens would be cooled below the temperature at which the substitution fluid solidifies and then cryosectioned. The solid substitution medium is then removed by sublimation in a vacuum chamber, and the sections are analysed like freeze-dried cryosections. Such investigations should yield the conditions necessary to optimize ion retention during dehydration. If ion retention is dependent on ion association at cellular proteins, free and adsorbed ions can be determined by this method. In addition, these experiments should reveal whether ion loss or redistribution is dependent upon structural preservation.

(2) After understanding the events which occurred during dehydration with substitution fluids, it is possible to investigate the ion loss or redistribution caused by embedding at different temperatures in different resins and subsequent polymerization at different temperatures.

(3) Since we assume that ions can be retained during freeze-substitution and embedding if the ability of proteins to adsorb ions is maintained during the preparative steps, we suggest carrying out staining experiments, as described earlier (Edelmann, 1984a). In these, sections are exposed to solutions containing electron-dense ions (e.g. Cs^+) which are supposed to be adsorbed to proteins at the same sites as for example K^+. First experiments of this kind have already been undertaken by Edelmann (1989b) and a typical result is shown in Fig. 5. An intense staining of muscle proteins is observed with Cs^+. The alkali-metal ions accumulate at the same protein sites as the Tl^+ ions in living cells as visualized in frozen-hydrated cryosections (Fig. 1). It is expected that the degree of staining (which can be quantified by X-ray microanalysis) may be related to the degree of the retention of mobile cellular ions during free-substitution and embedding. It should be noted that chemically fixed and resin-embedded muscle cannot be stained with Cs^+, indicating that the proteins have lost their ability to adsorb monovalent ions (Edelmann, 1984a).

COMPARISON OF FREEZE SUBSTITUTION WITH OTHER TECHNIQUES

Compared to other preparation techniques for analytical electron microscopy freeze-substitution has the advantage that reproducible results can be obtained in every laboratory because the specimens are always immersed in a liquid whose temperature can be controlled precisely. The method is easy and yields durable specimens which can be handled by well-known sectioning and staining techniques. The method preserves fine structure without using chemical fixatives, better, than for instance, the preservation after freeze-drying and embedding (Edelmann, 1989a).

Fig. 4. Typical X-ray spectra obtained from sections of frog sartorius muscle. (a) Freeze-dried cryosection. (b) Section (about 0·2 μm thick) of freeze-substituted and low-temperature embedded (HM23) muscle. The spectra shown in (c)–(f) were obtained from rather large areas (e.g. $7 \times 12\,\mu m^2$), see irradiated area in (b) of the sections. (c) Spectrum of a freeze-dried cryosection. (d) Spectrum of a muscle after freeze-substitution and low-temperature embedding (2 days pure acetone at 193 K, temperature increase 20 K/h up to 293 K, infiltration in HM23 at 293 K for 16 h, cooling to 213 K and polymerization). (e) Spectrum of a muscle after freeze-substitution and low-temperature embedding (2 days pure acetone at 193 K, temperature increase 20 K/h up to 293 K, 16 h 193 K, cooling to 213 K, infiltration in HM23 and polymerization. (f) Spectrum of a section of pure HM23.

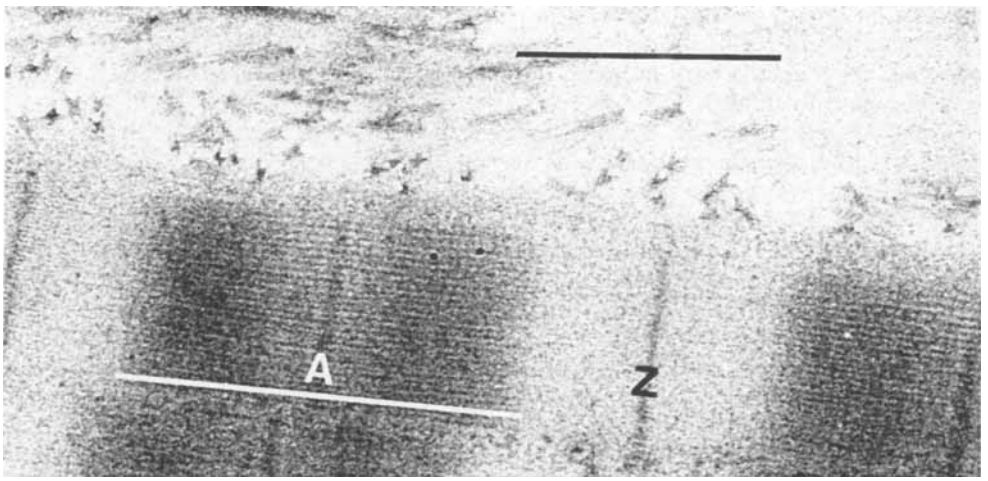

Fig. 5. Section (0·1 μm thick) of frog sartorius muscle after freeze-substitution in pure acetone (7 days, 193 K) and low-temperature embedding (K11M, 213 K); the sections were exposed to a solution containing 100 mM LiCl and 10 mM CsCl as described by Edelmann (1984a). Mainly myosin filaments of the A band (A) and proteins of the Z line (Z) bind the electron-dense Cs^+ ions. Scale bar = 1 μm.

Although it has been claimed that freeze-drying at very low temperatures should yield better structural preservation than freeze-substitution, because the specimen can be dehydrated at lower temperatures (Linner *et al.*, 1986), the experimental proof is missing. Comparative studies with vitrified specimens (Dubochet *et al.*, 1987) are necessary to clarify this issue.

If we consider the retention of mobile cellular ions in biological specimens and their quantitative evaluation by microanalytical methods, it is currently assumed that freeze-dried cryosections offer the best preservation of mobile ions at a subcellular level. Even this method has its problems, as can be seen from the following example. Over the last 14 years (Edelmann, 1977) it has been shown that K^+ or electron-dense surrogates are preferentially localized in the A bands of the striated muscle in freeze-dried and embedded samples, in frozen-hydrated material, and in freeze-substituted and embedded preparations (for reviews see Edelmann, 1984a, 1989a). This finding could be confirmed only once by quantitative X-ray microanalysis (von Zglinicki, 1988), despite the fact that muscle tissue has been analysed worldwide by many scientists. This example points to the problem of subcellular ion redistribution which may occur during improper freeze-drying of cryosections, or of bulk specimens which are subsequently embedded in a resin (see Edelmann, 1986). In other words, redistribution of mobile cellular ions at a subcellular level is conceivable during all dehydration procedures currently used for analytical electron microscopy; furthermore, ion loss or redistribution is similarly conceivable during embedding of freeze-dried or of freeze-substituted biological material. This implies that more control experiments with frozen-hydrated preparations are needed. However, absolute quantification of ion concentrations in thin, frozen-hydrated cryosections by means of X-ray microanalysis is not possible due to the fact that the high electron doses which are required damage the analysed area. A possible solution to this problem is the use of biological material which has been loaded with electron-dense mobile ions. Thin, frozen-hydrated cryosections of such material can be photographed (low dose) without damaging the specimen. The distribution of electron-dense particles visualized in the micrograph can be evaluated by microdensitometry. The problem of ion redistribution during

preparative procedures can then be investigated by analysing freeze-dried cryosections or sections of freeze-dried and embedded or freeze-substituted and embedded preparations for the same biological material. Taken together, experiments are feasible which allow the control of all preparative steps and which may lead to generally accepted cryomethods suitable for the localization of mobile cellular ions of biological material at an ultrastructural level.

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Note added in proof. After finishing the manuscript a paper dealing with the preservation of diffusible ions in resin-embedded, freeze-dried and freeze-substituted specimens appeared in print (Condron, R.J. & Marshall, A.T. (1990) A comparison of three low temperature techniques of specimen preparation for X-ray microanalysis. *Scanning Microsc.* **4,** 439–447.) A quantitative X-ray microanalytical comparison of intracellular ion concentrations in two tissues (chicken kidney and duck nasal gland), prepared as bulk frozen-hydrated, embedded freeze-dried and freeze-substituted (20% acrolein in diethyl ether) samples, shows that there are similar losses of K^+ and P (30–40%) in freeze-dried and freeze-substituted samples in both types of tissue.