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OPTIMAL FREEZE-DRYING OF CRYOSECTIONS AND BULK SPECIMENS FOR X-RAY MICROANALYSIS

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Abstract

Electron microscopic investigations of rapidly frozen specimens of striated muscle, either frozen-hydrated or obtained after different dehydration procedures, have shown that the subcellular distribution of the main cellular cation K^+ or its surrogates Rb^+, Cs^+, or Tl^+ does not follow the water distribution but follows certain proteins. Conflicting results obtained by X-ray microanalysis of freeze-dried cryosections are explained by showing that freeze-drying of bulk specimens and cryosections must be carried out for rather long periods at low temperature in order to avoid severe shrinkage and ion redistribution artefacts. Proposals for future freeze-drying studies are derived from the concept that cellular water is organized differently from normal free water and that proteins of living cells are able to selectively adsorb alkali-metal ions.

Key Words: Freeze-drying, freeze-dried cryosections, X-ray microanalysis, low temperature embedding, shrinkage, ion redistribution, potassium binding, cell water, striated muscle

Introduction

The purpose of X-ray microanalysis of biological specimens is the life-like localization of ions in the biological material. During the last 19 years I have used striated muscle as a representative biological system and tried to optimize dehydration procedures (freeze-drying and freeze-substitution) followed by resin embedding without using chemical fixatives in such a way that both structural artefacts and ion redistribution artefacts are minimized. The main findings of these studies are: [1] The best structural preservation is obtained by using long dehydration periods at low temperature (between -80 and -60°C), [2] After such dehydration procedures the subcellular distribution of mobile cellular cations does not follow the (former) water distribution but follows certain proteins (for reviews see Edelmann, 1984a, 1986, 1988, 1991a).

On the one side this latter finding is of great significance for our understanding of cellular organization in general (Ling, 1992a) and for our understanding of phenomena detected after preparative procedures. On the other side results obtained from freeze-dried cryosections are reported in the literature which are in conflict with this finding (see e.g., LeFurgey *et al.*, 1992). It is the purpose of this paper to show that different freeze-drying procedures may lead to different subcellular ion distributions and that meaningful X-ray microanalytical data from freeze-dried preparations are only to be expected after optimized freeze-drying. Suggestions for future freeze-drying studies are given at the end of this paper.

The Biological Problem

With regard to mass, the largest component of living cells is water, the second largest is protein. In number, the most abundant again is water, while the second most abundant is K^+. By and large, the external environment of cells is a salt solution containing water as its largest component and NaCl as its next largest

component. The history of cell physiology shows that the asymmetric distribution of K^+ and Na^+ between cells and their environment as well as cell volume regulation played major roles in developing alternative models of the living cell (see Ling, 1984). According to modern textbooks the living cell is a collection of protein-containing compartments (or a single compartment) filled in first approximation with free water and solutes. Membranes separate the compartments from each other (or from the external environment) and regulate the composition of solutes by means of active and passive transport mechanisms. This largely accepted view is challenged by a minority maintaining that the physical state of the bulk of cellular water is different from that of free water and that most of cellular K^+ is adsorbed (weakly bound) at β- and γ-carboxyl groups of cellular proteins (see e.g., Ling, 1992a,b; Negendank and Edelmann, 1988). According to this view cellular accumulation of K^+ and exclusion of Na^+ are explained by the physical state of water and ions in the cytoplasm of living cells.

Noteworthy is the close connection between the water problem and the K^+ problem in any theory of the cell as can be seen from the following observation: Living frog muscle cells are isotonic with a 0.1 M aqueous NaCl solution; since the total ionic concentration in the cell is about 0.1 M and because K^+ constitutes the bulk of the cations, it follows that cellular K^+ (and anions) **and** cellular water must be free (Hill and Kupalov, 1930; Hill, 1930) **or** if cellular K^+ is bound the cell water must be influenced by macromolecules in such a way that its activity is reduced to that of a 0.1 M NaCl solution.

The Problem of Preparing Biological Material for Analytical Electron Microscopy

The watery biological material in its natural state is not suited for investigation in the vacuum of an electron microscope. It must be stabilized either by conventional chemical fixation, dehydration and embedding procedures or by cryotechniques. Rapid freezing is - according to present knowledge - the best approach to preserve the original composition of a biological specimen at a given time. By means of cryoelectron microscopes it is possible to visualize ultrastructural details in ultrathin frozen-hydrated cryosections prepared from the rapidly frozen specimens (see e.g., McDowall *et al.*, 1983; Michel *et al.*, 1991; Edelmann, 1992). Subcellular localization of elements like K by means of X-ray microanalysis, however, is not (yet) possible with ultrathin frozen-hydrated cryosections because of radiation damage (Zierold and Steinbrecht, 1987). Microanalysis of frozen-hydrated preparations is possible only in thick cryosections or bulk specimens (Hall and Gupta, 1983; Marshall, 1980), the disadvantage of increasing specimen thickness, however, is the loss of spatial resolution. Full use of the high spatial resolution obtainable with ultrathin sections and X-ray microanalysis can be made after freeze-drying of cryosections or after freeze-drying or freeze-substitution of bulk specimens, subsequent embedding, and sectioning. During dehydration and embedding, however, structural artefacts and ion redistribution artefacts cannot be avoided (see e.g., Steinbrecht and Müller, 1987). Only suitable controls may answer the question whether - despite these artefacts - meaningful information can be obtained from these dehydrated specimens. An acceptable control is the original frozen-hydrated preparation. Structural changes can be detected by comparing the ultrastructure seen in ultrathin frozen-hydrated cryosections with that preserved in freeze-dried cryosections or thin sections of dehydrated and embedded material. It is more difficult to evaluate the ion redistribution artefacts introduced by freeze-drying or by dehydration and subsequent embedding.

Choice of a Suitable Biological System

One of the most suited biological materials for the investigation of both the biological problem and the problem of specimen preparation is the striated muscle, in particular the striated muscle of the frog. This is due to the following established facts: [1] Many physiological problems have been studied with this tissue including problems of asymmetric ion distribution, of cell volume regulation, of cellular electrical potentials, and of intracellular cation mobility and activity, [2] The ultrastructure is well known. Proteins are periodically arranged in areas with higher and lower water content as determined by light microscopy (Huxley and Niedergerke, 1958). The water content is about 10% higher in the I band compared to the A band, [3] The putative binding sites for K^+ are β- and γ- carboxyl groups (Ling, 1977a). Myosin contributes about 65% of the β- and γ-carboxyl chains of all the muscle proteins (Ling and Ochsenfeld, 1966) and is found only in the A band, [4] About 80% of the cellular K^+ of isolated living muscle can be replaced **reversibly** with the heavy Cs^+ or Tl^+ ions (Ling and Bohr, 1971; Ling, 1977b).

This means that we can obtain living cells in which the main cellular cation is Cs^+ or Tl^+ instead of K^+. Such cells can be rapidly frozen and we thus achieve a preparation which is particularly well suited for electron microscopic investigation as it contains a large amount of electron-dense particles. Provided these particles are

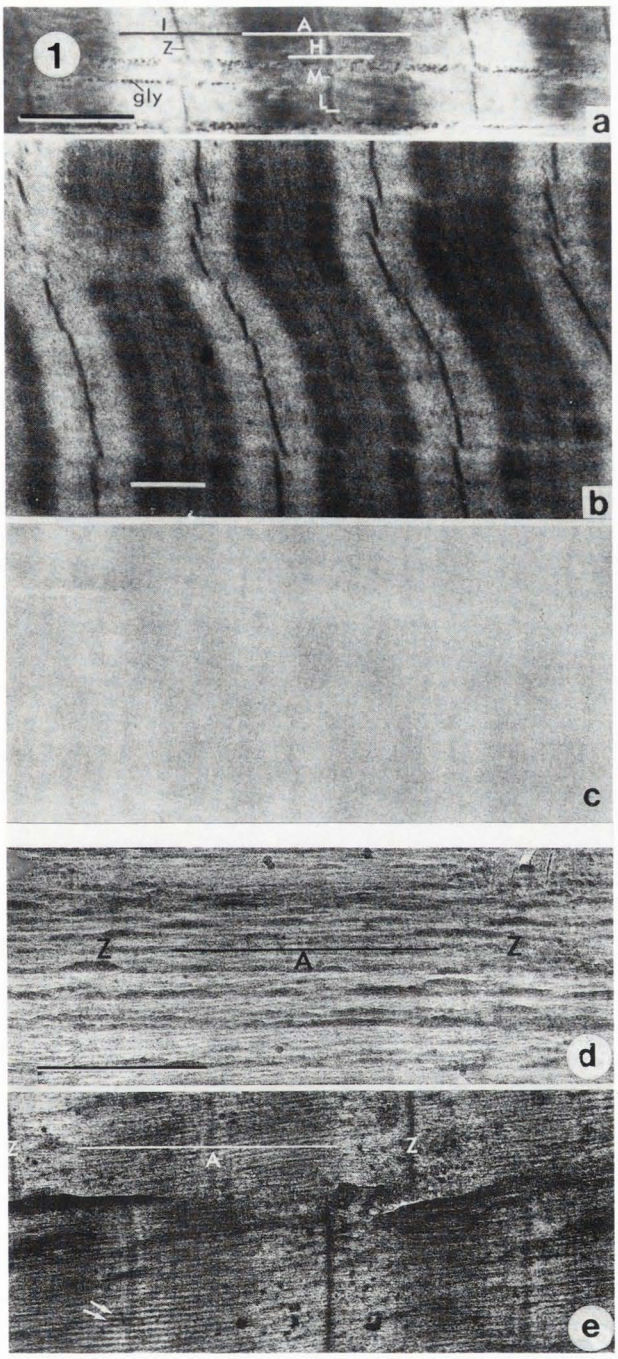
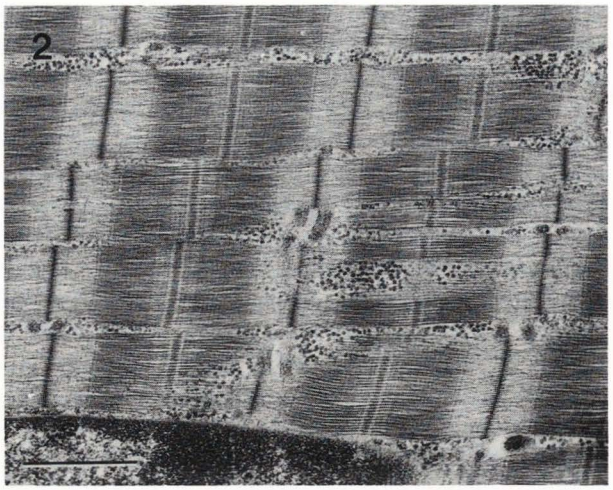


Fig. 1: Subcellular distribution of Cs^+ and Tl^+ in frog sartorius muscle. Living muscles had been loaded with Cs^+ (**a**) and Tl^+ (**b**, **e**) prior to cryofixation. **a-c** Dry-cut sections of freeze-dried and embedded preparations (freeze-drying equipment described by Edelmann, 1978; procedure: freeze-drying for 3 days at -80°C and for additional 6 days at -60°C, breaking the vacuum by dry nitrogen gas, infiltration in Spurr's resin at about -15°C for about 15h, polymerisation at 40-50°C. **a** Cs^+-loaded muscle. **b** Tl^+-loaded muscle. **c** Normal K^+ containing muscle. Abbreviations: **A**, A band; **H**, H zone; **M**, M line; **L**, L zone; **I**, I band; **Z**, Z line; **gly**, glycogen. **d,e** Frozen-hydrated cryosections. **d** Normal K^+ containing muscle. Only very faint ultrastructural details are visible. **e** Tl^+ containing muscle. Dark myosin filaments (arrows) in the A bands (**A**) and dark Z lines (**Z**) indicate preferential Tl^+ accumulation in the living cell. Bars: 1μm. **a** from Edelmann (1977); **b,c** from Edelmann (1984a); **d,e** from Edelmann (1988). Reprinted by permission.

unevenly distributed in the cell we may visualize directly this uneven distribution in frozen-hydrated cryosections by low-dose transmission electron microscopy. The ion distribution can also be detected in freeze-dried cryosections or freeze-dried (freeze-substituted) and embedded preparations.

The Subcellular Distribution of Cellular Cations in Striated Muscle after Different Preparation Procedures

Starting in 1976 several cryotechniques have been used to localize alkali-metal ions and Tl^+ in the striated muscle of the frog by electron microscopic methods. Either muscles with their normal K^+ content or muscles in which about 80% of the cellular K^+ was replaced by Rb^+, Cs^+, or Tl^+ have been investigated. The methods used include analysis of sections of freeze-dried (Edelmann, 1977, 1986) or freeze-substituted (Edelmann, 1988, 1989a,b) and embedded muscle, autoradiography of frozen-hydrated single fibers using ^{86}Rb and ^{134}Cs (Edelmann, 1980a), electron probe X-ray microanalysis of freeze-dried cryosections (Edelmann, 1983) and visualization of Tl^+ in frozen-hydrated cryosections (Edelmann, 1984b), (for reviews see Edelmann, 1984a, 1988). The main findings and conclusions of these studies are the following: In the normal K^+ containing frog skeletal muscle and in muscle loaded with the electron-dense K^+ surrogates Rb^+, Cs^+, or Tl^+ the accumulated ions are preferentially localized within the A bands (see Fig. 1) especially at the two marginal regions and at the Z lines. (This paper does not include a separate Materials and Methods section for the results shown and discussed here; the freeze-drying protocols and used freeze-drying equipments are therefore given in the legends of the shown figures). Of particular importance are the results obtained with frozen-hydrated preparations: [1] Autoradiographs visualizing the Cs^+-distribution in a Cs^+-loaded muscle



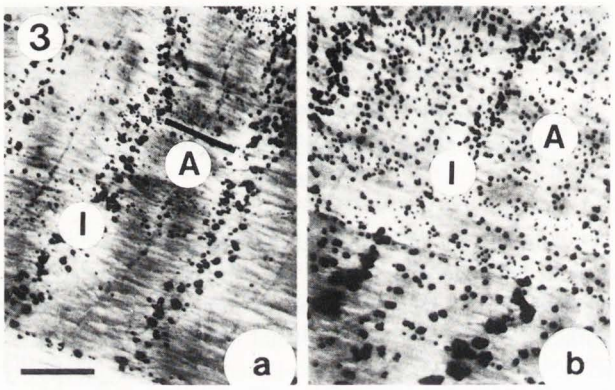


Fig. 2: Chemically unfixed frog sartorius muscle, cryofixed on a LN_2 cooled Cu block, freeze-dried, embedded in Spurr's resin (same procedure as given for Figs. 1a-c); section stained with uranyl acetate and lead citrate. Bar: 1 μm. From Edelmann (1986), reprinted by permission.

Fig. 3: Dry-cut sections of "shortly" freeze-dried and embedded Tl^+-loaded frog sartorius muscle (similar procedure as given for Figs. 1a-c but shorter freeze-drying times: 2d at -80°C, 3d at -60°C). Different distributions of Tl-precipitates in **a** and **b**. Bar: 1 μm. From Edelmann (1984a), reprinted by permission. Note A band (A) shortening (1μm instead of 1.5 μm) and high accumulation of Tl-precipitates in the I bands of **a**.

cell show that the concentration of Cs^+ is low in the I bands and high in the A bands (Edelmann, 1980a), [2] Micrographs of frozen-hydrated cryosections of Tl^+ loaded muscle (Fig. 1e) show that individual filaments (mainly myosin filaments in the A band, but also I band filaments) and Z line proteins are "stained" by Tl^+ (Edelmann, 1988); this implies that most of the cellular Tl^+ ions are bound to proteins and not dissolved in the surrounding water; otherwise a very poor contrasting or even a negative staining of proteins would have to be expected. Note that these results have been obtained with fully hydrated cryosections. The sections have been kept at about -170°C and photographed in a Zeiss EM 10CR at a magnification of 5000 (electron exposure below 1000 e^-/nm^2). No mass loss of the area irradiated during photographing could be detected.

From these electron microscopic studies it is concluded that most of cellular cations are weakly bound (adsorbed) to cellular proteins. A further conclusion is (see above) that cellular water must be differently organized than normal free water or in other words that the cell water activity is determined to a large extent by the interaction of water with certain cellular proteins (Ling, 1992b).

The argument that ion sensitive microelectrodes measure high concentrations of free intracellular K^+ (hence no considerable K^+ binding) has been answered by showing that microelectrodes damage the intracellular organization and are not suited to evaluate the physical state of K^+ in a living cell (Edelmann, 1989c).

The Subcellular Distribution of K^+ in Freeze-dried Cryosections of Striated Muscle

It is now well accepted that freeze-dried cryosections are ideal preparations for the detection of subcellular ion distributions by electron probe X-ray microanalysis. Freeze-dried cryosections are stable in the electron beam, the high electron optical contrast allows good ultrastructural localization of the irradiated area, the X-ray sensitivity in terms of peak/background ratio is high and allows localization of ions with a high spatial resolution (Zierold and Steinbrecht, 1987). We now ask whether an uneven distribution of K^+ in freeze-dried cryosections of the striated muscle has been detected by X-ray microanalysis. To my knowledge, only one report has appeared in print confirming quantitatively that K^+ is preferentially localized in A bands of the striated muscle (von Zglinicki, 1988; see also a qualitative study by Edelmann, 1983). The K^+ content in mmol/g dry weight is higher in the A band than in the I band, it is also higher in the A band if the data are given in mmol/g fresh weight or in mmol/g compartment water. On the other hand, in a recent paper (and on the front cover of the Journal of Microscopy, Vol. 165, Part 2) LeFurgey *et al.* (1992) presented quantitative color images from a rapidly frozen, cryosectioned and freeze-dried frog skeletal muscle fiber

showing distributions of different elements including that of K^+. According to the shown K^+ map, the K^+ content is higher in the I band than in the A band. Although this finding is not discussed explicitly by the authors it is consistent with the view - mentioned in their Discussion - that "K^+ (as well as Na^+ and Cl^+) is assumed to be primarily in solution rather than in bound form". Because of the importance of a correct evaluation of the K^+ distribution in striated muscle a conceivable source of error leading to the mentioned conflicting result is considered in the next section.

Shrinking and Ion Redistribution Artefacts

The procedure finally adopted to obtain good structure preservation (Fig. 2) of freeze-dried embedded muscle (specimen size about $0.1mm^3$, e.g., $0.5x0.5x0.4mm^3$) was: use of a simple freeze drying apparatus based exclusively on cryosorption pumping by zeolite (Edelmann, 1978, 1979, 1986), freeze-drying for 3 days at -80°C and for additional 6 days at -60°C, breaking the vacuum by dry nitrogen gas, infiltration in Spurr medium at about -15°C for about 15h, polymerization at 40-50°C. The ion distributions shown in Figs. 1a, b have been obtained by using the same procedure. Trials to shorten the periods of freeze-drying at low temperature failed: shrinkage and different ion redistribution artefacts could be detected (Edelmann, 1984a, see Fig. 3). Noteworthy is the repeatedly observed finding that a large amount of Tl^+ precipitates in the I band and that the A band is much shorter (about 1 μm) than in the well preserved preparations. (The A band width of living resting muscle is 1.5 μm, hence shrinkage artefacts can easily be detected).

Considering the K^+ distribution in muscle as revealed by LeFurgey *et al.* (1992) we now may ask: Is it conceivable that the freeze-drying used was not suited to avoid shrinkage and ion redistribution artefacts? According to Fig. 13a of the mentioned paper the linear shrinkage of the freeze-dried muscle section is about 40% (i.e., 40% size reduction in one direction or in case of a uniform shrinkage in all 3 dimensions more than 75% volume reduction). This tremendous shrinkage may reflect incomplete freeze-drying below a critical low temperature as discussed in detail by Edelmann (1986); in this case redistribution of water and ions upon warming of the sample (even if it is still in the vacuum of the freeze-drying chamber) cannot be excluded. To be noted are other values of shrinkage of freeze-dried cryosections. During a rather fast drying of a cryosection in the cryoelectron microscope (about 10°C/min), Dubochet *et al.* (1983) observed a linear shrinkage of about 40%. Zierold (1984) found linear shrinkage values of 10-20%. According to our results obtained with freeze-dried embedded muscle one may conclude 1) that shrinkage can be kept small (so far about 5% linear shrinkage) by long freeze-drying at low temperature and 2) that severe shrinkage artefacts may occur together with ion redistribution artefacts. Hence, it appears necessary to produce freeze-dried cryosections with a quality of structure preservation similar to that shown with freeze-dried embedded material.

Optimum Freeze-drying of Cryosections

Trials to minimize shrinkage artefacts of freeze-dried cryosections have been reported earlier (Edelmann, 1986). It could be shown that after freeze-drying of a muscle section for 1h at -100°C followed by 6h at -80°C and slow temperature increase (10°C/h) up to room temperature in a cryoelectron microscope (Zeiss EM 10 CR) the linear shrinkage is about 5%. Since such a procedure is rather impractical a new freeze-drying apparatus was used to further investigate the freeze-drying of cryosections.

The new apparatus is described elsewhere (Sitte *et al.*, 1994). Briefly, it is based on the already mentioned simple freeze-drying apparatus with a built-in cryosorption pump and can be used in the Reichert AFS cryosubstitution apparatus. The freeze-drying chamber may be vented by dry cold nitrogen gas and can be loaded from top in a simple manner. Grids with cryosections are clamped to a temperature controlled specimen support, and a cold trap (-180°C) is positioned about 10 mm above the specimens. The vacuum during freeze-drying is between 10^{-4} and 10^{-5} mbar. Freeze-drying may be carried out at temperatures above -140°C under controlled (e.g. temperature increase) conditions. Consumption of liquid nitrogen during freeze-drying is about 4 l per day.

A freeze-dried cryosection is shown in Fig. 4. The experiments carried out so far show - as expected - that prolonged freeze-drying at low temperature yields cryosections with little shrinkage (see frozen-hydrated cryosection shown in the inset of Fig. 4) and a good structure preservation comparable with that obtained after optimal freeze-drying and embedding. Such cryosections may be warmed up slowly (in the vacuum of the freeze-drying chamber) to room temperature without further shrinkage (Edelmann, 1986).

Future Freeze-drying Studies

The new freeze-drying apparatus allows freeze-drying of cryosections and of bulk specimens which may subsequently be embedded at high or low temperatures

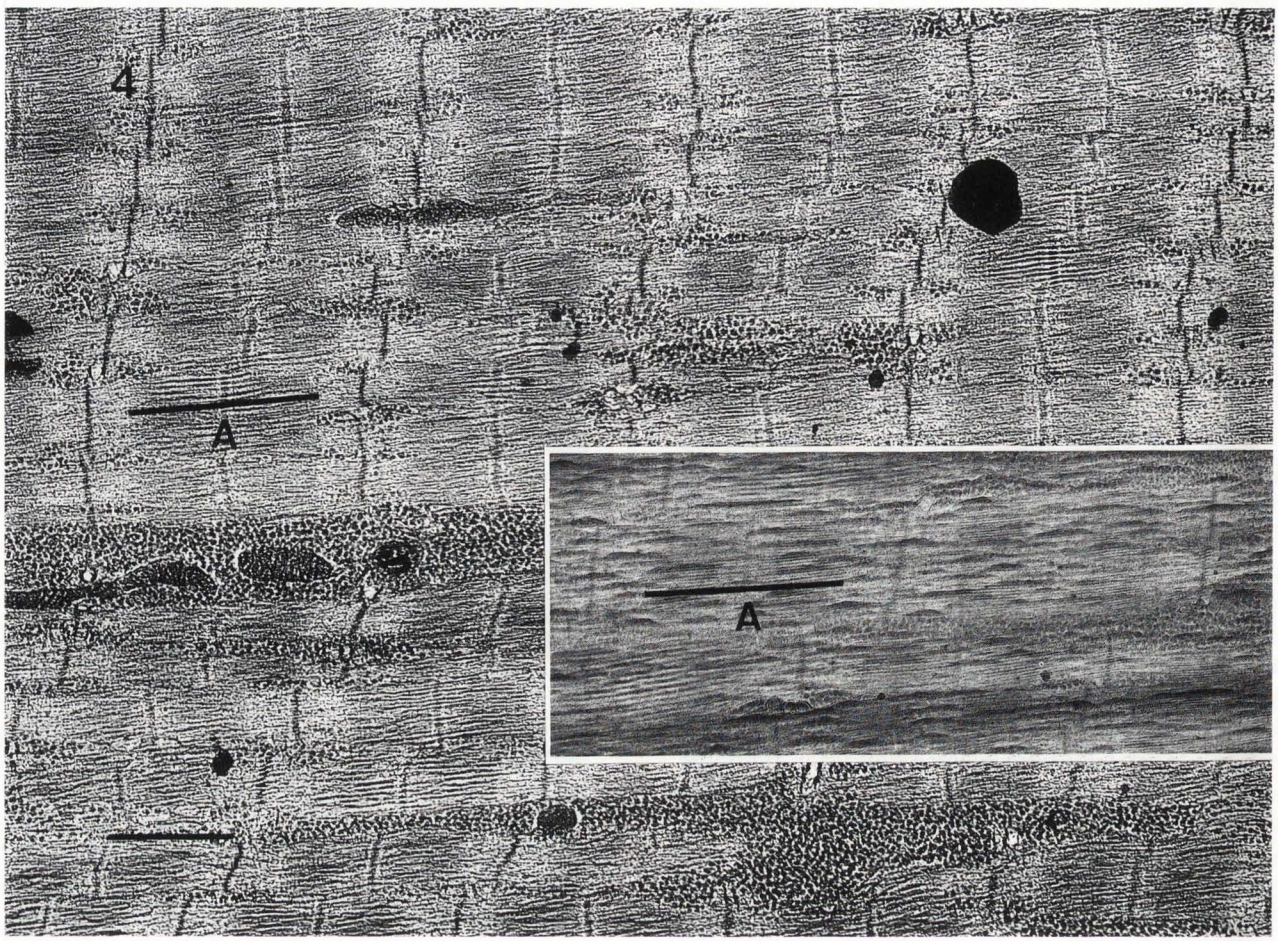
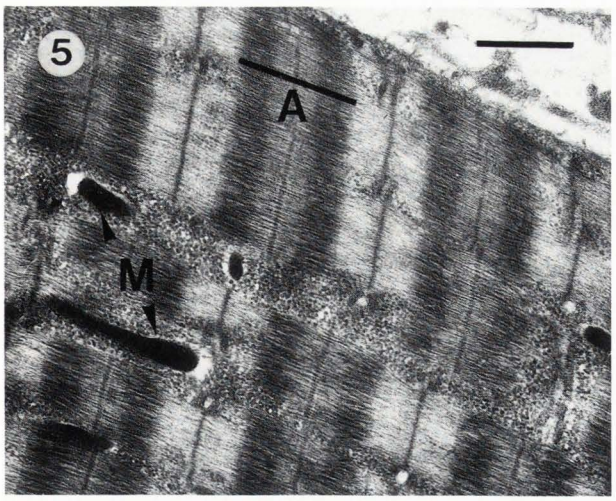


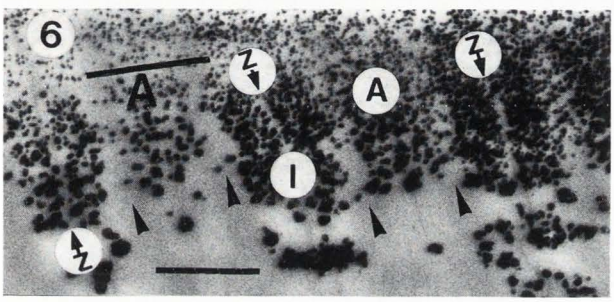
Fig. 4: Freeze-dried cryosection of chemically unfixed frog sartorius muscle. The section has been freeze-dried for 33h at -100°C in the cryosorption freeze-dryer described by Sitte *et al.* (1994), this volume. **A**, A band. **Inset**: Frozen-hydrated cryosection of frog sartorius muscle. Cryosectioning at -165°C; Reichert Ultracut S/FCS, Diatome diamond knife, Simco antistatic device, cutting speed 0.4mm/s, section placed on uncoated 600-mesh copper grid. Both sections have been photographed at a magnification of 4400x in a Zeiss EM 902 with energy-filter, electron exposure below 500 e^-/nm^2. Bar: 1μm. Compare dimensions of A bands (A) and not those of sarcomeres which may be stretched to different degrees in the different preparations.

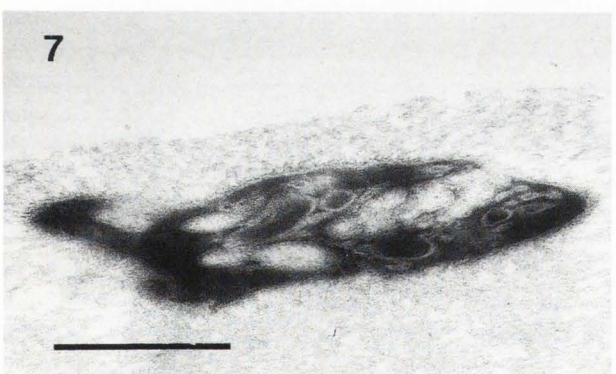
(e.g. Lowicryls) within the apparatus (see Fig. 5). With the possibilities to examine freeze-dried embedded material, and ultrathin cryosections before and after different freeze-drying procedures one may study systematically unsolved problems of freeze-drying a few of which are expressed by the following questions:

Is it possible to freeze-dry partly or completely cryosections of vitrified specimens without changing the ultrastructure seen in the fully hydrated cryosections? Is there a difference in freeze-drying cryosections containing only vitrified ice or only cubic ice (e.g., after warming the specimen above the transition temperature)? Is there a difference in freeze-drying differently frozen vitrified specimens - frozen e.g. on a cold metal block (Sitte *et al.* 1987; Edelmann, 1992) or by high pressure freezing (Moor, 1987; Studer *et al.*, 1989; Michel *et al.*, 1991)? It cannot yet be ruled out that the "slow" cooling during high pressure freezing causes a modification of the cellular organization and that this new organization is then vitrified (Wallén and Hallberg, 1993). Is it possible to obtain a structure preservation similar to that obtainable after freeze-substitution with organic solvents containing no chemical fixatives (see e.g., Edelmann, 1991a)?

In this context a special freeze-drying procedure called "molecular distillation drying" (Linner *et al.*,







1986) should be mentioned. It has been claimed that this technique "can remove amorphous phase tissue water without devitrification or rehydration making possible ultrastructural localization of soluble molecular entities without the problems of alteration, redistribution, and loss". However the proof is missing that it is possible to remove vitrified ice by freeze-drying without changing the ultrastructure seen in frozen-hydrated cryosections. Furthermore, the proof is missing that this procedure yields better structure preservation than for instance prolonged freeze-substitution or freeze-drying carried out between -80°C and -60°C. The artefact of differential shrinkage is clearly not avoided by using this technique

Fig. 5: Freeze-dried frog sartorius muscle, embedded in Lowicryl HM20 (the used freeze-drying and embedding equipment is described by Sitte *et al.*, 1994, this volume). Freeze-drying: 2d -80°C, 1d -60°C, 2d -50°C. Infiltration in HM20 at -30°C for 6h. UV polymerization at -30°C for 1d. Section stained with uranyl acetate and lead citrate. Bar: 1μm. Note the A band width (A) of about 1.2μm, corresponding to a linear shrinkage of about 20%, and the differential shrinkage of the dark mitochondria (M), indicating that longer freeze-drying at low temperature is required to avoid this artefact (see text).

Fig. 6: Tl^+-redistribution artefacts in a dry-cut section of a Tl^+ loaded muscle, freeze-dried and embedded in Lowicryl HM 20 as the muscle shown in Fig. 5. Bar: 1μm. To be noted are many Tl precipitates in I bands (**I**), fewer in A bands (**A**), and large areas in which Tl appears to be absent. This irregular Tl distribution indicates that the freeze-drying procedure used was not suited to stabilize the Tl^+ ions at their original adsorption sites. Note in particular Z lines (Z, arrow) and outer edges of A bands (arrow heads) with relatively few Tl precipitates. Compare with Fig. 1b which shows a preferential accumulation of Tl at these sites.

Fig. 7: Human blood platelet, freeze-dried for 3d at -80°C, 6d at -60°C, embedded in Spurr's medium according to the procedure given for Figs. 1a-c. Section stained with uranyl acetate and lead citrate. Bar: 1μm. (Electron micrograph courtesy of E. Morgenstern). Note the intense staining (for explanation see text). For "normal staining" of a blood platelet after freeze-drying and embedding see Fig. 3 in Edelmann (1979).

(Linner *et al.*, 1986; Fig. 11).

The problem of differential shrinkage should be discussed in more detail. This artefact has often been observed in tissue rich in mitochondria (see e.g., Coulter and Terracio, 1975; Linner *et al.*, 1986; Fig. 5, this paper) which show large halos after freeze-drying. Own experiments have shown that this artefact can almost be avoided by drying at low temperature for much longer periods (3 days at -80°C, 6 days at -60°C **and** 5 days at -50°C see Edelmann, 1986) than proposed for muscle tissue (3 days at -80°C, 6 days at -60°C).

A further problem concerns a conceivable difference between freeze-drying of cryofixed specimens untreated before freezing and specimens chemically fixed before freezing. This question points to the water problem of living cells as mentioned above. If there is a difference between the protein-water interaction of living and that of dead cells (killed e.g., by chemical fixation) or of

protein solutions one may anticipate that the respective interaction will (partly) "survive" a good cryofixation and that different energies are required to remove the water. It should be tested whether the required long freeze-drying at low temperature is reflecting a relatively strong interaction between proteins and water of living cells. This problem may also be tackled by model systems according to the following considerations: Model systems have been found in which the water has reduced solvency for solutes (Ling and Hu, 1988; Ling 1992 a,b; 1993); such models are e.g., aqueous solutions of gelatin, denatured hemoglobin, polyvinylpyrrolidone (PVP), polyethylene oxide (PEO), polyethylene glycol (PEG); these proteins and polymers are called "Extroverts". On the other hand, aqueous solutions of most native proteins do not exhibit reduced solvency (so-called "Introverts"). Most interesting are the findings that the reduction of solvency of the Extroverts is size-dependent (i.e., the larger the solute, the lower the solvency) and that the partial exclusion of solutes from cellular water is similarly size dependent (Ling, 1992a; Ling *et al.*, 1993)).

Finally, I would like to point again to the problem of ion binding which may be tackled further by freeze-drying and X-ray microanalysis. The findings described above strongly support the idea that optimal freeze-drying preserves mobile cellular cations at binding sites available at protein sites of living cells (mobile adsorption). In other words, the protein-ion interaction may be preserved although it must be assumed that it is modified after removal of the water. The binding energy may be enhanced or lowered as seen e.g. after "wrong" freeze-drying (Fig. 3 and Fig. 6; Fig. 6 shows additional interesting redistribution artefacts: Z lines and outer edges of A bands appear with reduced accumulation of Tl. In the well freeze-dried preparations of Figs. 1a and b the electron-dense Cs and Tl are preferentially found at these sites). This signifies that the binding sites captured in a freeze-dried specimen may exert different attractions towards e.g. ionic stains depending on the freeze-drying and embedding procedures used. An example is given in Fig. 7 showing a very intense staining of a blood platelet in a freeze-dried and embedded preparation. This phenomenon has been observed repeatedly in the region of best cryofixation (vitrified area?). A conceivable interpretation is that in this area still more water is retained (lower sublimation rate) due to a relative strong protein-water interaction and that therefore less salt linkages are built up between anionic and cationic sites of proteins in the incompletely dehydrated specimen. Hence a more intense staining at still open sites is to be expected.

Another interesting phenomenon of ion binding has been observed. Well preserved freeze-dried and embedded muscle can be stained by electron-dense alkali-metal ions like Cs^+, a phenomenon which has also been described with freeze-substituted muscle (pure acetone, one week at -80°C, for review see Edelmann, 1991b). The staining pattern is similar to the images of subcellular Cs and Tl distributions shown in Figs. 1a and b. Most interesting is the finding that when using different alkali-metal ions in the staining solution these ions are attracted to different degrees at protein sites (Edelmann, 1980b, 1981). Since this observed selectivity supports the idea that cellular proteins of living cells have the capability of selectively adsorbing K^+over Na^+ (Ling, 1992a, pp 39-67) it would be worthwhile to study with sections of freeze-dried embedded material the ion selectivity and changes of selectivity upon different treatments of the biological material including different procedures of freeze-drying and embedding. Of particular interest would be the behaviour of specimens kept exclusively at low temperatures during freeze-drying and during embedding in Lowicryls (Kellenberger *et al.*, 1986; Kellenberger, 1991).

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Discussion with Reviewers

A. LeFurgey: This paper is basically a critique of a paper by LeFurgey *et al.* published in another journal (LeFurgey *et al.*, 1992). As such it would have seemed more appropriate for the author to have submitted his comments to that journal in order that the readership might have the benefit of continuity.

Author: This paper is basically a discussion on optimal freeze-drying which according to my own experience is essential for meaningful X-ray microanalysis of mobile cellular ions. Two criteria of good freeze-drying of the striated muscle are [1] the quality of structure preservation and [2] a characteristic subcellular distribution of the main cellular cation (K^+ in an untreated muscle). In defending own work I concluded that microanalytical results presented in an important paper by LeFurgey *et al.* (1992) contradict own findings and may possibly be caused by improper freeze-drying of the analyzed freeze-dried cryosections. This explanation should be understood as an invitation to try better freeze-drying and to think about my conflicting results published and discussed in public many times but not considered by LeFurgey *et al.* Since most of my findings are published by Scanning Microscopy, an extended defence of the optimized freeze-drying procedures used appears to be well suited for this journal, in particular in a special issue on biological microanalysis. I am convinced that the included new results obtained by freeze-drying and the new proposals for future experiments will stimulate others to test and improve further this technique.

A. LeFurgey: The author's major argument in support of his contention, that the K distribution in skeletal muscle in the paper of LeFurgey *et al.* is of an artifactual nature, specifically is that it is the result of ion redistribution due to improper freeze-drying. This conclusion is based on previously published hypotheses, repeated at length in the present communication, concerning the distribution of K, rather than on evidence

demonstrating putative preparatory deficiencies. Furthermore, since these hypotheses are mainly based on the distribution of elements with which K has been replaced, the relevance of the distribution of these substitute elements to the K distribution is not immediately apparent.

Author: Considering all my experimental results - not hypotheses - the most likely explanation of the conflicting results is that different subcellular distributions of mobile ions may be obtained after different freeze-drying schedules. (The fact that my experimental results confirmed postulations of hypotheses not well esteemed by the scientific establishment does not justify to ignore these results). It must not be further proved that a grossly shrunken freeze-dried cryosection (compare Fig. 13a of the paper by LeFurgey *et al.* with Fig. 13b) is reflecting a preparatory deficiency. By using a cryofixed Tl^+ loaded muscle and following different freeze-drying schedules one can produce at will well freeze-dried and grossly shrunken freeze-dried preparations exhibiting different subcellular Tl^+ distributions. Since the subcellular Tl^+ distribution is similar in frozen-hydrated and in well freeze-dried preparations I conclude that improper freeze-drying may cause severe ion redistribution artefacts. The relevance of the distribution of substitute elements (Tl, Rb, Cs) to the true K distribution has been discussed in several publications cited in this paper. It has been shown that the subcellular distribution of all these elements is similar in well freeze-dried muscle. Since the mechanism of cellular accumulation and the interaction with cellular proteins is similar for these elements (e.g., reversible replacement) it is expected that a similar but not necessarily identical subcellular redistribution of all these elements may take place during improper freeze-drying. E.g., liberated Tl^+ ions may combine differently than liberated K^+ ions with free or liberated phosphate anions with the result that Tl precipitates are formed but not K precipitates.

A. LeFurgey: The striking potassium distribution in a color panel of quantitative X-ray maps of skeletal muscle fibers was shown in order to illustrate the efficacy and spatial resolution of biological X-ray mapping, which after all was the main purpose of the paper by LeFurgey *et al.* This image set was obtained closest to the stimulus site from a series of samples taken along the entire length of the same point-stimulated skeletal muscle fibre. These new, exciting experiments await further critical evaluation since the elemental distributions are most likely going to be a function of time and distance from the point of stimulation, given an approximate conduction velocity measured on our specimens of about 1.5 to 2.0 m/sec. Before impugning unproved deficiencies of preparatory procedures, the displayed elemental distributions must be evaluated taking into account the precise local volumes i.e., I band versus A band, for any given moment during the time course of the excitation-contraction cycle. While qualitative conclusions can be drawn concerning the relative changes of rather tightly bound elements such as calcium in the JSR (junctional sarcoplasmic reticulum), premature interpretations should not be made from dry weight data of highly diffusible ions (such as K^+ and Na^+), since it is obvious that precise local volume data are essential requirements for quantitative physiological (wet weight) measurements using EPXMA (electron probe X-ray microanalysis). The relevance of, and protocol for, the determination of local water content is well documented in the literature and addressed specifically in the paper of LeFurgey *et al.*

Author: According to the paper by LeFurgey *et al.* the color panel of quantitative X-ray maps was obtained from a single, intact, skeletal muscle fibre; it was not mentioned that it was obtained from a cryosection rather close to the stimulus site of a point stimulated muscle fibre; in addition, the stimulus to freezing interval was not given. I agree completely with "precise local volume data are essential requirements for quantitative physiological (wet weight) measurements using EPXMA". But I suppose that by comparing your shown continuum (Fig. 12) with the concentration image of K (Plate 1) and by taking into account that the freeze-substituted control muscle shown by you (Fig. 13b) does not exhibit signs of local swelling it is evident (and not a premature interpretation) that more K is found in the I band (certainly not less) than in the A band. This finding is in conflict with results discussed in this paper and with results reported by von Zglinicki (1988). One may now consider the possibility that the K distribution is different in a stimulated muscle compared to an unstimulated muscle. Indeed, a subcellular redistribution of K during muscle contraction has been postulated by Ling (1984 p. 574) and discussed in several publications (e.g., Edelmann, 1989a, b). I have carried out experiments with Rb^+, Cs^+ or Tl^+ loaded frog sartorius muscle, frozen a few ms (up to 20 ms) after a single stimulus (unpublished). In sections of freeze-dried embedded muscles I could observe A band shortening (about 10%) but no qualitative difference in the ion distribution when compared with resting muscle. However, later experiments carried out with muscles quick-frozen in tetanus (Edelmann, 1989a) yielded A band shortening of up to 20% and ion redistribution (Cs^+, Tl^+). Whereas the Cs^+ loaded muscle still revealed a slightly preferential Cs^+ accumulation in the

A band, the Tl^+ loaded muscles showed either a preferential accumulation of Tl in the A bands together with Tl precipitates in the I bands or Tl precipitates randomly distributed over the sarcomeres. Taken together, a subcellular redistribution of the main cellular cation during contraction must be considered and is worth to be investigated further by quantitative EPXMA. I doubt however that K^+ will be liberated from the A band a few ms after a single stimulus to such an extent that it is found preferentially in the I band. To be noted is also the similarity between your shown color map of the Ca distribution and the Ca image of an **unstimulated** fibre (Fig. 16a) which is completely different from that of a tetanized fibre (Fig. 16b). The conclusion appears justified that the K map was taken from a part of the muscle not far away from the resting state. I would be delighted if you have already confirmed the postulated redistribution of K during contraction, if not, I propose to repeat your experiments with an optimized freeze-drying procedure.

H.Y. Elder: The quality of your images of freeze-dried muscle done by the "best" drying protocols is superb and you show a clear inverse correlation between the degree of shrinkage and the drying time of the freeze-dry protocol. What do you consider to be the freeze-drying protocol giving the best preservation of structure and fidelity of ion location? Do you think that the long time at -60°C (you specify up to 6 days), or the sequence -80, -60, -50°C is important, or could it be the final slow warm up at 10°C per hour?

Author: It is important to freeze-dry the specimen first at or below -80°C to such an extent that at higher temperatures a transition from cubic to hexagonal ice is prevented which may greatly disturb the original structure; this problem is similar for freeze-drying and freeze-substitution (see e.g., Steinbrecht and Müller, 1987, p. 152). The long freeze-drying time (6 days) at -60°C was empirically found to be "safe" for small muscle bulk specimens which could be infiltrated and polymerized in Spurr medium or Lowicryl K11M maintaining a rather good ultrastructure and a specific subcellular ion distribution (Tl^+, Cs^+, K^+, see Edelmann, 1986). But many more experiments are necessary to determine the shortest freeze-drying protocol yielding similar results. I have finished freeze-drying at -60°C and one may try freeze-drying while warming up slowly the specimen. Freeze-drying is usually carried out that way but the results show in most cases "forbidden" shrinkage artefacts. By freeze-drying thin cryosections it should be possible to determine the critical temperature below which the specimen must be dried sufficiently before it can be warmed up without further shrinkage. However, one problem should be mentioned: bulk specimens and cryosections shrink in all three dimensions during freeze-drying. If a cryosection is tightly fixed in the x-y plane (e.g. on grid bars) one may possibly observe no shrinkage of the freeze-dried cryosection although the shrinkage is very severe in the z-direction. In general, in order to determine the degree of shrinkage of a cryosection it would be necessary to determine the degree of shrinkage in all 3 dimensions (see e.g., Buchanan RA, Leapman RD, O'Connell MF, Reese TS, Andrews SB (1993) Quantitative scanning transmission electron microscopy of ultrathin cryosections: Subcellular organelles in rapidly frozen liver and cerebellar cortex. J Struct Biol **110:** 244-255). Keeping in mind, that the shrinkage in the direction of muscle filaments can be kept small during freeze-drying of bulk specimens (not fixed in space) I tried to find out the conditions necessary to freeze-dry cryosections yielding similar structure preservation as observed in sections of freeze-dried and embedded bulk specimens. Therefore the cryosections have been placed on grids without supporting film and the degree of shrinkage in the direction of filaments was only determined from freeze-dried cryosections which have been fixed only on one side on a grid bar.

Concerning the preservation of ion location I assume that the shrinkage should be kept as small as possible because shrinkage reflects a certain changing interaction between macromolecules and water or a water redistribution which may cause ion redistribution. From the observation that a specimen can be freeze-dried at low temperature to such an extent that it does not further shrink upon warming one may conclude that in this case the whole system including the position of the ions has been greatly stabilized.

H.Y. Elder: Do you think that it will be possible to further improve on freeze-drying protocol to reduce linear shrinkage below even the 5% your best can achieve?

Author: In my opinion, this problem has to be investigated with thin cryosections by freeze-drying them at very low temperature for very long periods. It would be very interesting to know whether shrinkage can be avoided at all and whether the shrinkage is different in differently frozen specimens (e.g., after metal mirror freezing or high pressure freezing, well preserved specimens containing either vitrified or cubic ice).

H.Y. Elder: You consider that there is a difference between the protein/water interaction of living cells and of chemically fixed cells. Is this a function of the

number of reactive protein side chains reduced by fixation additive cross linking reactions? If so, have you observed any differences between different fixatives, e.g. fixation for varying times, or between those producing more cross links (e.g. glutaraldehyde) compared with those making fewer (e.g., formaldehyde), or between additive fixatives (e.g. aldehydes and OsO_4) and denaturing fixatives like the alcohols which remove the hydration shell from the proteins?

Author: At present I cannot answer these questions. I can only repeat and extend the observation that there is a striking similarity between living cells and aqueous solutions of the so called Extroverts (Ling, 1992a,b; 1993; Ling *et al.*, 1993). In both systems the solvency for salts like sodium sulfate, sugars like sucrose or raffinose or amino acids like glycine is **reduced** (indicating that the water is not "normal" but influenced by certain macromolecules) whereas this is not the case in aqueous solutions of the so called Introverts and most likely in chemically fixed biological material (indicating that the water has similar properties as normal free water). One may now study the change of solvency properties of cellular water after treatment of the biological material with different fixatives. In addition, it remains to be determined whether there is a difference (freeze-drying time, shrinkage) when freeze-drying rapidly frozen systems containing differently influenced water.

H.Y. Elder: You mention the greater shrinkage halo round mitochondria than is apparent in the muscle sarcomeres and assume that it denotes freeze-drying shrinkage. Is it not more likely to be an indicator of less than optimal cryofixation; mitochondrial shrinkage changes, following less than optimal cryofixation, are well recognized (e.g. see pp 95-97 in Echlin, P. 1992. *Low Temperature Microscopy and Analysis.* Plenum Press, New York & London, 539pp)?

Author: Halos round mitochondria may be an indicator of less than optimal cryofixation. Whether this is the case may be checked by freeze-substitution. In a well preserved (cryofixed) area mitochondria do not show halos after freeze-substitution in acetone supplemented with OsO_4. But if you freeze-dry the same specimen not long enough at low temperature mitochondria with halos appear in an otherwise well preserved cytoplasm. One may conclude that halos may be the result of two different mechanisms: Either the mitochondria are partly dehydrated during slow cryofixation by ice crystals growing outside the mitochondria or the mitochondria are not sufficiently dehydrated (e.g., by freeze-drying) at low temperature with the result that they shrink upon warming up.

S.B. Andrews: The data presented do not prove a causal link between ion redistribution and freeze-drying time. The micrographs that demonstrate ion redistribution (Figs. 3 and 6) appear to be material which was either thawed before it was dry or rehydrated. In either case, the movement of K or Tl is not surprising. This occurrence is a useful caution for other investigators, but factors other than the time of freeze-drying could have produced this effect. The experiments described here are not sufficient to prove the hypothesis favored by the author. Therefore, some restraint is indicated in interpreting these results. This advice also applies to the author's criticism of the work of LeFurgey *et al.* There are other explanations for why these workers obtained conflicting results besides the artifact identified in the present paper. Does the author really wish to pillory the work of others, especially when this implies a serious error on their part which is not evident form the quality of their dried sections?

H.Y. Elder: Your observations on the correlation of quality of freeze-drying, degree of shrinkage and electrolyte translocation are most interesting. Would you like to speculate further on the mechanism by which a metal ion (or protein-bound metal atom) could be translocated during less than optimal low temperature freeze-drying?

Author: I have shown that Tl^+ loaded muscles which have been freeze-dried for shorter periods than 3 days at -80°C and 6 days at -60°C reveal both unacceptable shrinkage and ion redistribution artefacts after embedding and dry sectioning (Figs. 3 and 6). Most likely these artefacts are csused by events taking place already at temperatures below -50°C. This can be concluded from the fact that the muscle from which Fig. 6 has been obtained (freeze-drying: 2 days -80°C, 1 day -60°C, 2 days -50°C) must have been dried to a higher degree than that shown in Fig. 1b (3 days -80°C, 6 days -60°C) because freeze-drying for 2 days at -50°C corresponds to a freeze-drying time of about 8 days at -60°C (see e.g., Umrath W (1983) Berechnung von Gefrier-trocknungszeiten für die elektronenmikroskopische Präparation [Calculation of freeze-drying times in preparation for electron microscopy] Mikroskopie (Wien) **40**, 9-34). Hence, one may conclude that the visualized ion redistribution occurred in a temperature range between -80°C and -50°C. The possibility that the redistribution may be due to rehydration can be excluded because in both cases (Figs. 3 and 6) freeze-drying and embedding was carried out in an absolutely dry environment. The idea that the specimens thawed before they were dry does not conflict

with my interpretation. On the contrary, it appears logical to conclude that shrinkage values exceeding 5% linear shrinkage are only obtained if a specimen is warmed up (even during continuous freeze-drying) above a critical temperature before it is sufficiently dehydrated. In this case the water molecules which are more or less immobilized at the low temperature become more and more mobile with increasing temperature (whether there is a definite melting point for the remaining water or whether this water is unfrozen remains to be determined). As a result the attractive forces between macromolecules or between intramolecular sites are no 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. This mechanism is similar to that proposed by Kellenberger *et al.* (1986, p11) for freeze-substitution at low temperatures.

It is logical to conclude that alkalimetal ions or other ions which are not tightly bound in living cells may be dislocated during a "sudden" aggregation of macromolecules which occurs together with a redistribution of water. During aggregation (leading to a considerable shrinkage) salt linkages between formerly free positive and negative sites of the macromolecules are formed with the result that these sites are no longer available for the adsorption (very weak binding) of cations like K^+ or certain anions. (Note, that the improper freeze-dried muscle of Fig. 6 shows Z lines and outer edges of A bands with reduced Tl accumulation whereas in well freeze-dried preparations these sites are particularly high in electron-dense Cs or Tl (Figs. 1a, b). The color map of the K distribution published by LeFurgey *et al.* (1992) also shows reduced K at the Z lines and very few K at the outer edges of the A bands). On the other hand, if the water is removed slowly at low temperatures the distance between positive and negative fixed charges does not change suddenly, the attractive forces between mobile (adsorbed) ions and fixed charges are slowly increasing with the result that these ions are stabilized near their original adsorption sites. A consequence of this mechanism is that less cross links (with less energy) are formed between macromolecular sites.

I must emphasize that by mentioning the paper of LeFurgey *et al.* (1992) it is not my intention to pillory the work of others but I want to find out acceptable answers to conflicting results. The results obtained be LeFurgey *et al.* fit very well in my interpretations which may easily be tested. It should be possible to obtain reproducibly freeze-dried cryosections from the same cryofixed specimen which are either shrunken to the extent exhibited in Fig 13a of LeFurgey *et al*'s paper (40% size reduction in one direction) or stabilized at lower shrinkage values. Most interesting would be the answer to the question whether different subcellular distributions of K (and most likely also of other elements like P) can be obtained which are unequivocally related to the degree of shrinkage of the cryosections or to specific freeze-drying protocols respectively.

S.B. Andrews: It is difficult to accept the claim that the cryosections in Figs. 1d and 4 (inset) are fully hydrated, because amplitude contrast appears to be a major component of the contrast mechanism, which is not expected for frozen-hydrated specimens. The high contrast of surface features such as cutting-induced "crevasses" is also not consistent with phase images of good frozen-hydrated cryosections. The questions: How can you be sure that the frozen-hydrated sections (Figs. 1d and 4 (inset)) were fully hydrated when imaged in the electron microscope and not partially freeze-dried? What contrast mechanism(s) produced these images? What electron dose was used to obtain these micrographs?

Author: The frozen-hydrated cryosections (thickness about 100nm) shown in Figs. 1d and e have been recorded rather close to focus in a Zeiss EM 10CR at a magnification of 5 000 (electron exposure below 1 000 e^-/nm^2). The Zeiss MDF system has been used for focusing at a distance from the recorded area in order to minimize electron exposure before photographing. No mass loss of the area irradiated during recording could be detected as judged from second pictures taken at lower magnification (3 000) and reduced electron exposure. The contrast shown in both micrographs is at first approximation an amplitude contrast. The contrast of the ultrastructure of the muscle in Fig. 1d is very poor and the contrast of the compression artefacts is not surprising because of the large differences in mass thickness. To be noted is the fact that it is very difficult to produce very thin "beautiful" frozen-hydrated cryosections from cryofixed living frog sartorius muscles (and it was still more difficult a few years ago) since this muscle is always covered by a few micrometers of extracellular water and connective tissue preventing true vitrification of the muscle by metal mirror freezing (discussed by Edelmann, 1989c, p 45) and as a result preventing optimal cutting. Nevertheless, Fig. 1d is a suitable control for Fig. 1e. The inset of Fig. 4 has been photographed close to focus in a Zeiss EM 902 with zero-loss energy filtering at a magnification of 4 400

(electron exposure below 500 e^-/nm^2). Using the microscope in the zero-loss mode improves the contrast of ultrastructural details of frozen-hydrated cryosections considerably but also intensifies the contrast of artefacts, such as crevasses, folds, and knife marks as has been shown by Michel *et al.* (1991). These authors also demonstrated that crevasses and faint knife marks disappear after a short irradiation time. Own experiments confirmed this finding and I conclude that these artefacts may be used as indicators for a minimal mass loss.

S.B. Andrews: In light of the long freeze-drying times required to minimize, but not entirely eliminate (see Fig. 4), shrinkage according to your techniques, how do you justify the extra time and expense to achieve only limited improvement over the results obtained with faster drying?

Author: The purpose of science is understanding. Despite the many sophisticated techniques now available decades of expensive research have not yet led to a true understanding of the complicated interactions between macromolecules, mobile ions and water in biological systems. As discussed in this paper it appears that a systematic investigation and optimization of the freeze-drying technique may provide answers to open questions concerning the behaviour of the main constituents of living cells. If it turns out that the very best results can only be obtained by long-term freeze-drying at low temperatures the required extra time is certainly not wasted. Furthermore, the waiting time may be used for other work. The used Cryosorption Freeze-Drying system (described by Sitte *et al.*, 1994, this volume) marketed by Leica as Reichert CFD, is well suited for systematic studies of long-term freeze-drying and requires not much extra expense because no mechanical pumps and only 4l of LN_2 per day are required.

S.B. Andrews: Why is X-ray microanalysis a key word?

Author: An important topic of X-ray microanalysis is the localization of mobile elements in biological material. This paper deals with this problem and shows how to control and improve preparative procedures widely used in X-ray microanalysis.