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THE INFLUENCE OF DIFFERENT CRYOPREPARATIONS ON THE DISTRIBUTION OF IONS IN BULLFROG MYOCARD CELLS

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Abstract

Bullfrog heart muscle trabecula are shock-frozen in liquid propane cooled by liquid nitrogen and then processed for X-ray microanalysis in two different ways: 1. Freeze-drying followed by vacuum embedding. 2. Cryoultramicrotomy and freeze-drying.

Stained sections of freeze-dried, embedded tissue exhibit detailed ultrastructure, but are useless for X-ray microanalysis. Unstained, dry cut plastic-sections are suitable for X-ray microanalysis, but the ultrastructure appears faint. Higher electron optical contrast and peak-to-background ratio of X-ray spectra are generally obtained in freeze-dried cryosections. Both preparation methods show that the X-ray spectra are influenced by the quality of cryofixation. The phosphorus/potassium ratio in nuclei increases with increasing ice crystal size.

KEY WORDS: Cryofixation, cryosections, freeze-drying, vacuum embedding, quantitative X-ray microanalysis, ice crystal damage, bullfrog heart muscle, cryopreparation.

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Introduction

In the last few years the technique of energy-dispersive X-ray microanalysis (EDS) in the electron microscope has been applied to an increasing number of biological objects and questions (reviewed by Moreton, 1981; Sumner, 1983). In scanning electron microscopy mode (SEM) bulk specimens were analyzed dry and frozen-hydrated (reviewed by Zierold, 1983). In transmission electron microscopy mode (TEM) and in scanning transmission electron microscopy mode (STEM) only sections and other electron transparent objects can be visualized. In preparation of sections various attempts have been made to achieve suitable specimens, where EDS detects elemental distributions resembling the in vivo state. After recognizing the limitations of histochemical precipitation techniques even in combination with EDS (reviewed by Morgan, 1979), cryopreparation techniques were improved by several groups (Somlyo et al., 1977; Wendt-Gallitelli et al., 1980; Hagler et al., 1980).

Presently, there are three different ways to get sections of cryofixed specimens: Freeze-substitution and embedding (reviewed by Harvey, 1982), freeze-drying and vacuum embedding (reviewed by Ingram and Ingram, 1980) and cryoultramicrotomy (reviewed by Roomans et al. 1982; Sitte, 1982). All three methods have one common problem, they depend directly on the quality of cryofixation, i.e., cooling rate and resulting ice crystal size. Additionally, each method has its own restrictions and artifacts. The aim of this paper is to compare two of the three preparation techniques, freeze-drying followed by vacuum embedding as well as cryoultramicrotomy, concerning their quality for EDS of bullfrog heart muscle.

The preparation steps are reviewed according to our knowledge of the literature. The methods applied for this investigation are described separately. Then the results from freeze-dried, embedded samples as well as from cryosections are reported and discussed. Finally both methods are compared.

Cryopreparation

Rapid freezing of fresh biological material is required for many different electron micro-

ted sections. Secondly, the peak/background (P/B) ratio of EDS is remarkably decreased in frozen-hydrated sections compared to freeze-dried sections (Gupta et al., 1977; Ross et al., 1981; Zierold, 1982a, b, 1983). If frozen-hydrated sections are dried within the microscope or in the cryotransfer system, as done in this investigation, contamination artifacts are avoided (Zierold, 1982b, 1983) and the enhanced contrast and increased P/B ratio of EDS enables identification and analysis of structures.

Preparation of cryosections for this investigation

Specimens frozen on Balzer's gold planchettes were sectioned in a Reichert Ultracut^{R} with FC 4 cooling device. Section thickness was about 100 nm. The sections were pressed between two film-coated grids, which were split afterwards, thus leading to two grids with sections. As controls parts of the sectioned tissue blocks were freeze-substituted according to Müller et al. (1980b) and sectioned transversely. Cryosections were transferred to the electron microscope frozen-hydrated by means of a cryotransfer system (Zierold et al., 1981). They were usually freeze-dried in the transfer chamber before analysis. Analyses were carried out in a Siemens Elmiskop ST 100 F^{R}, a scanning transmission electron microscope equipped with a field emission gun and an energy dispersive microanalysis system, detector, USC nuclear semiconductor, and multichannel analyzer, Link Systems, at 100 kV accelerating voltage, as described by Zierold (1982a).

Quantitation of X-ray spectra of freeze-dried cryosections was done by comparison with standards of glycerol-gelatine mixed salt solutions of known concentration, which were prepared in the same way as the tissue specimens. Similar standards are reported by Roomans and Seveus (1977), Hagler et al. (1983), and Zierold et al. (1984). The background values used for this investigation were measured by averaging the continuum irradiation between 4.5 and 5.5 keV.

Results and Discussion

Results and discussion of freeze-dried embedded samples

In this section our results on freeze-dried plastic embedded tissue are demonstrated and the methodological aspects are discussed.

For morphological orientation it is advantageous to use wet cut stained sections. Tissue appearance in these sections differs somewhat from conventionally prepared samples (compare Figs. 1a and c), but all subcellular compartments can be clearly identified. Within the well preserved region (10 - 15/um below the surface of the trabeculae) the tissue is very dense and the membranes appear in negative contrast (Fig. 1c). Compared to these sections unstained wet cut as well as dry cut sections offer a very weak contrast, particularly in well frozen regions cell organelles cannot be visualized, except for nuclei (compare Figs. 1c and d). In EDS only dry cut sections exhibit elemental distributions resembling the in vivo state (compare Figs. 2f with g-k). In myoplasm as well as in nuclei P and K are detectable. The average P/B ratio for K in the myoplasm was 3.2 $\pm$ 0.8 (n=10) (Figs. 2h and k). There are differences in the P/B ratio for P and K between nuclei and surrounding cytoplasm: P/B ratio for P is usually higher in nuclei than in cytoplasm, this holds for the P/B ratio for K, if the nuclei are without any visible ice crystal damage (compare Figs. 2g and j with h and k). In the presence of small ice crystals within the nuclei the P/B ratio for K is about the same as in the surrounding myoplasm (compare Figs. 2j and k). Referring the influence of the ice crystal damage to the phosphorous/potassium (P/K) ratio the P/K ratio in nuclei without ice crystals is found to be 0.85 $\pm$ 0.1 (n=7), whereas in nuclei with recognizable ice crystals the P/K ratio increases to 1.31 $\pm$ 0.1 (n=10).

From morphological point of view freeze-drying causes several artifacts and problems: Freeze-drying induces serious shrinkage up to 30% (Ingram and Ingram, 1975) or 15% according to Boyde and Maconnachie (1981). Additionally, cracks are found in the tissue (Ingram and Ingram, 1980; own observations). Unstained sections, as necessary for EDS, exhibit only a weak contrast. These sections resemble frozen-hydrated sections, in which ice is the embedding medium (Sumner, 1983). The problems of OsO_4-vapour fixation for the enhancement of contrast have already been discussed.

For the application of EDS different processes have to be considered. Ions may be displaced during the drying procedure, in particular in lumina or vacuoles. The possible amount of dislocation by embedding is not known: There has been a discussion in the literature that epoxy resins produce water in one polymerization step (Ingram and Ingram, 1980; Marshall, 1980). But the preservation of K-gradients in neighbouring

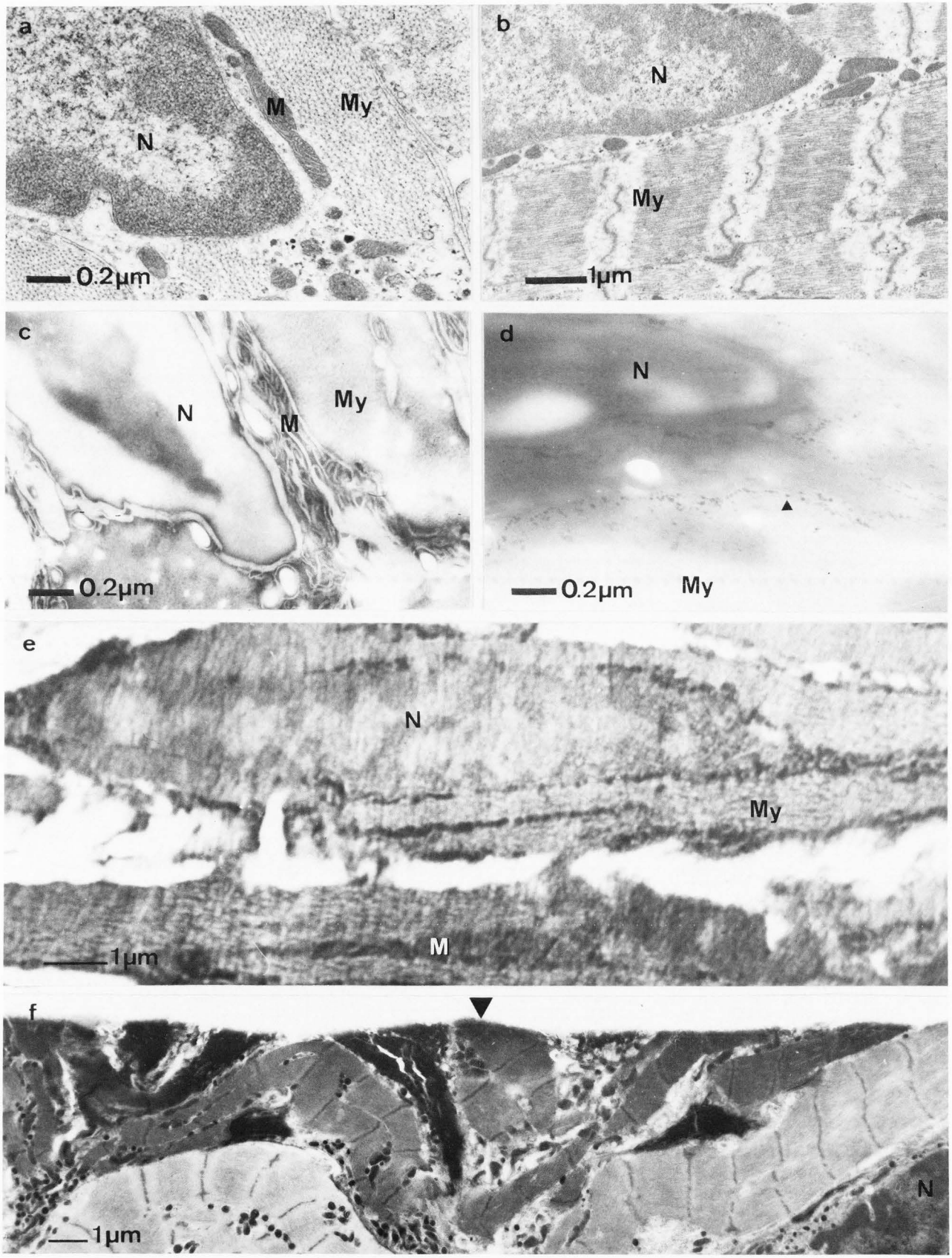
Fig. 1: The influence of different preparation procedures on the appearance of bullfrog atrial cells. N=nucleus; M=mitochondrium; My=myofibril

a, b) Conventionally prepared tissue fixed in 2.5% glutaraldehyde and 1% OsO_4 simultaneously, postfixed in 1% OsO_4 and dehydrated in a graded series of ethanols, sections stained with 0.5% uranyl acetate and 1% lead citrate. Pictures taken in TEM. a) Cross section; b) longitudinal section.

c, d) Freeze-dried, embedded tissue, cross sections, floated on water. c) Section stained in the same way as a and b; d) unstained section, arrowhead points to electron dense Ca-containing granules (see Meyer et al., 1982 a). Pictures taken in TEM.

e) Freeze-dried, longitudinal cryosection. Picture taken in STEM, brightfield divided by darkfield.

f) Freeze-substituted tissue with the plane the cryosections were cut from (arrowhead). Section stained like a, b and c. Picture taken in TEM.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cellular compartments indicates that ion displacement remains small (Figs. 2g-i). K within cells was also detected by Ingram and Ingram (1975), Masters et al. (1979), and Barckhaus et al. (1980).

For quantitation of embedded biological material standards for different elements are available: Na and K (Spurr, 1974), transition elements (Roomans and van Gaal, 1977), halogen elements, Br, Cl, I (Roomans, 1979), and Ca (Hagler et al., 1981). Quantitation of elements in freeze-dried embedded samples is possible (Ingram and Ingram, 1975, 1979). Since the reported values for elemental concentrations are comparable to those detected by other methods, the uncertainties introduced by shrinkage and embedding cannot be evaluated.

Results and discussion of cryosections

In this section our results on cryosectioned samples are demonstrated and the methodological aspects are discussed. From the morphological point of view dried cryosections are clearly inferior to conventionally prepared specimens and to freeze-dried, embedded, stained sections (compare Figs. 1a, b, c, with e), but they exhibit more contrast than unstained freeze-dried embedded ones (compare Fig. 1d and e). Mitochondria, myofibrills, and nuclei can be recognized. This is valid only for regions with very small ice crystals, in regions without any recognizable ice crystals nothing can be visualized within the bullfrog atrial cells. To prove whether the tissue was really frozen very well, the same region was checked in the freeze-substituted parts of the samples (Fig. 1f).

EDS of freeze-dried cryosections resulted in spectra, which exhibit the elements usually found in heart tissue (Figs. 2a-e). Cryosections deliver an excellent P/B ratio, which is 6.9 $\pm$ 2.1 (n=15) in the case of K in myoplasm. The phosphorous/potassium ratio of nuclei increases with increasing ice crystal size comparable to the situation in freeze-dried embedded tissue (compare Figs. 2a with d). Quantitation of X-ray spectra was carried out for P and K in myoplasm and nuclei by comparison with glycerol-gelatine standards. The obtained concentrations are compiled in Table 1 and compared with the results of other investigations carried out on freeze-dried ultrathin cryosections of muscle.

The demonstrated K-concentration in cytoplasm agrees with measurements on mouse myocytes (Dykes et al., 1979), propane-frozen rat heart (Wendt-Gallitelli and Wolburg, 1981), and of skeletal muscle (Somlyo et al., 1977). But the K-concentration of bullfrog atrial cytoplasm is clearly lower than the values in impact frozen rat heart (Wendt-Gallitelli and Wolburg, 1981), in rabbit heart (Hagler et al., 1983), and smooth muscle (Somlyo et al., 1979; 1982). The phosphorus content of bullfrog myoplasm is higher than the concentrations reported by Wendt-Gallitelli and Wolburg (1981) from rat and guinea pig heart, and they are also higher than those obtained from frog skeletal muscle (Somlyo et al., 1977; Gonzales-Serratos et al., 1978) as well as smooth muscle (Somlyo et al., 1979, 1982). However, Hagler et al., (1983) found higher P-concentrations in rabbit heart cytoplasm. Increased K- and P-concentrations in the nuclei compared with the cytoplasm like in bullfrog myocardium were also reported from mouse cardiocytes (Smith et al., 1983) and from smooth muscle (Somlyo et al., 1979, 1982), but not from rat heart (Wendt-Gallitelli and Wolburg, 1981). Thus, the concentrations obtained in this investigation are within the limits reported by other authors. Differences between the measured values of different investigations can depend on species differences and/or differences between types of muscle. But they might also be caused by uncertainties deriving from the method, which are expressed in the high standard deviations too: One effect interfering with the P/B ratio is the size of ice crystals. In regions without any visible ice crystals the P/K ratio in nuclei is usually smaller than in regions with minute ice crystals (comp. Figs. 2a with d and g with j). The P/K ratio in freeze-dried, embedded sections is 1.31 $\pm$ 0.1 for nuclei with recognizable ice crystals, however, 0.85 $\pm$ 0.1 for nuclei without visible ice crystal damage (Figs. 1c, d). This effect is less pronounced in cryosections (comp. Figs. 2a and d), because in cryosections without visible ice crystals cell organelles such as nuclei are not detectable. Thus, the lowest P/K ratios are obtained from nuclei like that in Fig. 1e. Presently, the influence of the ice crystal damage on the P/K ratio of nuclei cannot be explained. However, dependence of P/B ratio of several elements on the ice crystal damage was also found by Wendt-Gallitelli and Wolburg (1981) and by Zierold et al. (1984).

A second effect adding uncertainties to the quantitation of X-ray spectra from freeze-dried cryosections are variations in dry weight portions in different cell compartments, as compared with standards.

Comparison of freeze-dried, embedded samples versus cryosectioned samples

Freeze-drying and vacuum embedding is a method, which can be followed without very expensive equipment, except from the freeze-dryer it is the same as used for conventional electron microscopy.

Preparation of cryosections requires some additional equipment, such as a cryoultramicro-

Fig. 2: X-ray spectra of bullfrog atrial cells. a-e) X-ray spectra of cryosections.
a) Well preserved nucleus;
b) adjacent cytoplasm;
c) outside the section (small extraneous amounts of Cu, Si and Au);
d) nucleus with recognizable ice crystals;
e) adjacent cytoplasm.
f) X-ray spectrum of well preserved nucleus in freeze-dried, embedded tissue, unstained, section floated on water. Only extraneous Cr is detected.
g-k) X-ray spectra of unstained, dry cut sections of freeze-dried, embedded tissue. Extraneous Cr out of scale.
g) Well preserved nucleus;
h) adjacent cytoplasm;
i) outside the section;
j) nucleus with recognizable ice crystals;
k) adjacent cytoplasm.

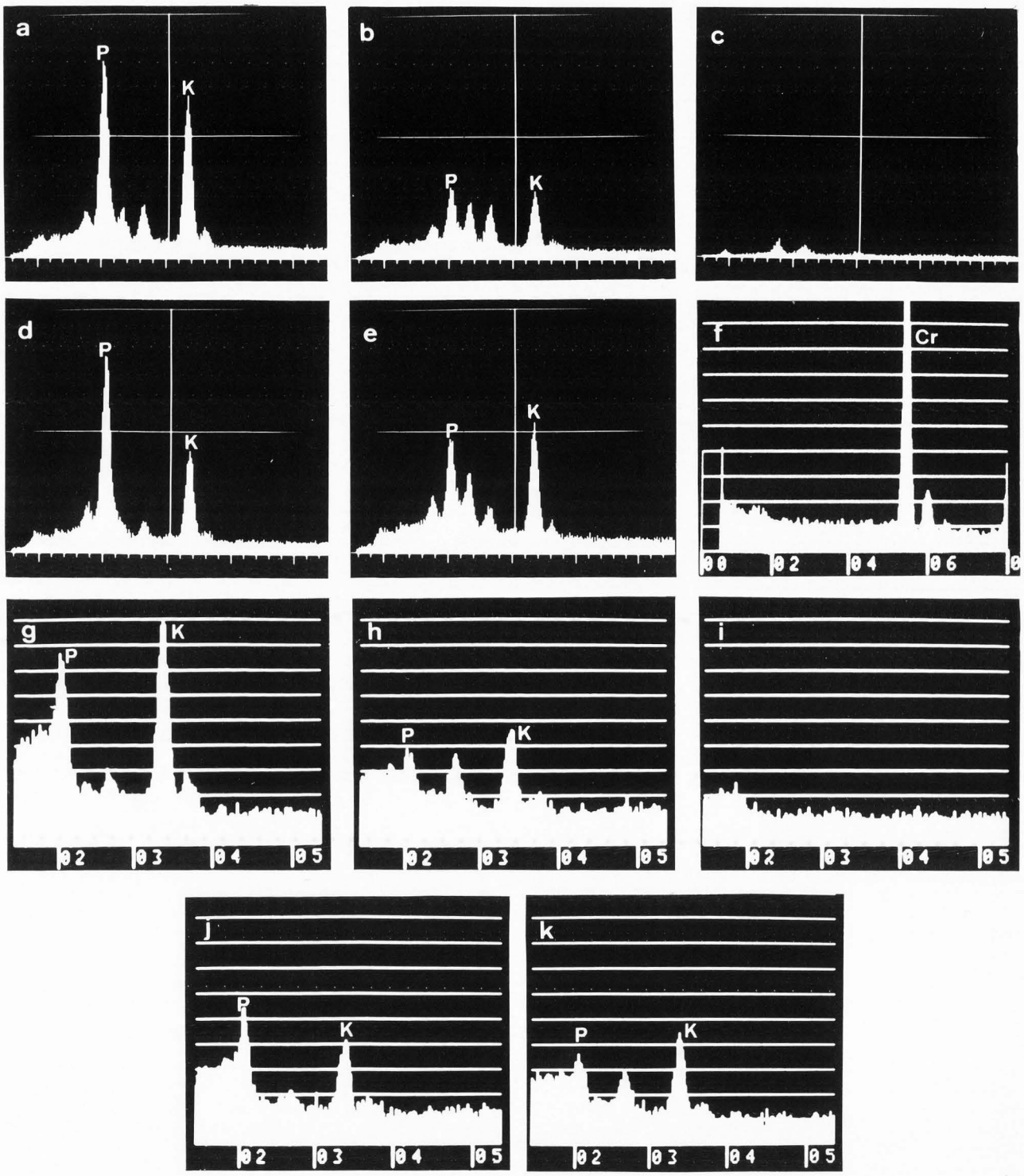
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Table 1: Quantitative X-ray Microanalysis of P and K in Muscle.

Authors	muscle	myoplasm		nuclei	
		P	K	P	K
Dykes et al., 1979	heart, mouse	365±46	335±31	402±58	359±49
Wendt-Gallitelli & Wolburg, 1981	heart, rat, propane	144±47	390±90	108±49	283±80
"	heart, rat, helium	194±49	530±62		
"	heart, guinea pig	76±21	207±48		
Hagler et al., 1983	heart, rabbit	527.5±86.4	594.4±57.1		
Smith et al., 1983	heart, mouse	256±34	263±24	362±46	287±23
Somlyo et al., 1979	skeletal, frog	302±51.3	404±84.5		
Gonzales-Seratos et al., 1978	skeletal, frog	317±12	488±16		
Somlyo et al., 1981	smooth, rabbit	250	592	593	649
This investigation	heart, frog	420±180	390±120	820±330	490±290

mean ± SD; all concentrations in mmol/kg dry weight; n = 15 in this investigation.

tome. But with a modern cryomicrotome the cutting of frozen-hydrated sections is not much more difficult than the dry cutting of embedded sections. Mounting of the sections is approximately the same. Attention has to be paid on the transfer of the sections before drying, and once dry, the sections have to be protected against moisture. But many simple devices for section transfer have been described (Somlyo et al., 1977; Dörge et al., 1978; Wendt-Gallitelli et al., 1980; Hagler et al., 1983). Nevertheless, the use of a cryotransfer system is advisable, because this is the only possibility to exclude uncontrolled artificial damage of the sections.

From morphological point of view freeze-dried, plastic embedded tissue in wet cut, stained sections offers the best resolution. But for EDS these sections are of little use. Dry cut, unstained sections are clearly inferior to freeze-dried cryosections, as long as very small ice crystals are present. In regions without ice crystal damage nothing can be seen in cryosections (Wendt-Gallitelli and Wolburg, 1981, own observations), whereas some structures, such as nuclei and electron dense Ca-deposits (Meyer et al., 1982a) are visible in unstained plastic sections. Therefore, it is not true that cryosections always provide a better morphological resolution than unstained plastic-sections. If dry cut plastic-sections can be prepared as thin as cryosections, the contrast might be improved. For EDS cryosections are clearly superior to plastic sections, because the P/B ratio is about two times higher in cryosections - 6.9 in cryosections versus 3.2 in plastic sections, both calculated from the K-concentration in myoplasm. The interaction of electrons with the embedding medium causes increased "Bremsstrahlung" resulting in a higher background compared to freeze-dried cryosections. With respect to electron optical contrast and X-ray microanalytical P/B ratio unstained plastic sections are similar to frozen-hydrated sections (Gupta and Hall, 1981; Ross et al., 1981; Zierold,1983). This holds for freeze-dried and freeze-substituted specimens (Marshall, 1980). By cutting on anhydrous solutions (see above) thinner sections can be obtained. They offer better P/B ratios with inherent risk of ion dislocation.

The second disadvantage of freeze-dried, plastic embedded samples is the higher risk of elemental dislocation during the preparation procedure. But nevertheless, the preparation procedure does not seem to change natural elemental gradients to a very high extent as K-gradients between different compartments are preserved (see above) and the effects of ice crystal damage on elemental distribution could be detected by both techniques of this investigation.

Quantitation is possible using both techniques and leads to comparable results. As several different effects influence quantitation of elements, it appears obvious from this study that only well frozen specimens with no or minute ice crystal damage can be used for reproducible X-ray micoroanalysis.

Conclusions

Stained sections of freeze-dried, embedded specimens exhibit a more detailed ultrastructure of cells than freeze-dried cryosections. Freeze-dried cryosections generally show more ultra-

structural details than unstained sections of embedded tissue. However, plastic sections pronounce other details than cryosections. For EDS the freeze-dried cryosections are superior to the freeze-dried embedded samples, because they yield higher P/B ratios and have theoretically smaller dislocation artifacts. Reliable EDS requires cryofixation without or at least with minute ice crystals. As both techniques have their own advantages, parallel application is reasonable.

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Discussion with Reviewers

H.K. Hagler: Most of the papers on freeze-drying of tissue address the removal of water prior to plastic embedding but what happens to the lipid in the tissue when it is warmed to room temperature?

Authors: The tissue was infiltrated by the resin at 263 K. Warming up is performed in the presence of resin. The resin possibly dissolves lipid components, as shown by the negative contrast of the membranes in Fig. 2c.

H.K. Hagler: In your work with the cryosections and the coldstage, have you noticed any morphological and/or elemental changes when the tissue is warmed to room temperature in the electron microscope?

Authors: Freeze-dried cryosections stored in the vacuum of the microscope column at room temperature overnight basically exhibit the same morphology as observed immediately after freeze-drying. However, a slight additional shrinkage of structures seems to take place. Differences in the element distribution were not found after such storage, except an increase in the Si-peak, which is always present in varying amounts. Systematic studies on the influence of the storage of freeze-dried cryosections in vacuum were not performed.

H.K. Hagler: What statistical tests were used to draw some of the conclusions?

Authors: No statistical tests were applied to data presented in Table 1.

G.M. Roomans: The fact that P/B ratios in freeze-dried embedded specimens are lower than in freeze-dried cryosections is, of course, due to the presence of resin. Actually, with 80% water in the tissue one might expect a 400% difference between these two specimen types, which is larger than the difference actually demonstrated by you. Could you comment on this?
Comparing Fig. 1d and 1e for contrast is difficult because of the completely different instrumental parameters (CTEM vs bright-darkfield STEM). How do the instrumental parameters in your experience affect contrast?

R. Wroblewski: Why is there a difference in the P/B ratio in embedded material versus freeze-dried?

Authors: In this investigation we used two different microscopes: A Philips EM 300 and a Siemens ST 100 F. The two microscopes provide different specimen contrast. Plastic sections were examined in the EM 300 since we could not obtain enough contrast from these sections in the ST 100 F. Comparing STEM- and TEM-mode in the EM 300, TEM yielded remarkably higher resolution than STEM (comp. Meyer et al., 1982a). The reason for the difference in contrast and resolution obtained by STEM and TEM remains obscure. Since plastic sections did not exhibit enough contrast in the ST 100 F, they had to be analyzed in the EM 300, whereas the study of cryosections requires a cryotransfer system, which was only available in combination with the

ST 100 F.

As these two instruments have different arrangements between detector and specimen, spectra obtained from plastic sections in the EM 300 (Figs. 2f-k) and spectra obtained from cryosections in the ST 100 F (Figs. 2a-e) are influenced by different instrumental parameters. Therefore, the obtained difference in P/B ratio and the theoretically expected difference do not coincide.

R. Wroblewski: Have you considered using semithick cryosections in your investigations?
Authors: Since ultrathin cryosections provided significant amounts of elements combined with a better spatial resolution compared to semithin sections, we did not investigate semithin cryosections.

R. Wroblewski: Background level in unstained, dry cut, embedded sections (Fig. 2g-k) is very high, especially at low energy level. Please comment.
Authors: Spectra obtained from dry cut, plastic sections (Figs. 2g-k) always showed increased background at low energy levels below 2 keV, possibly depending on increased section thickness.

R. Wroblewski: What is your probing area in comparison to crystal size?
Authors: All presented spectra were obtained by spot analyses. Additionally performed analyses in scanning mode did not exhibit differences in elemental distribution. The probing area of the analyses performed in scanning mode was always several times larger than the obtained ice crystal diameter of the specimens.

R. Wroblewski: Have you compared standards (cryocut and freeze-dried versus freeze-dried, embedded)?
Authors: No. A comparison of standards is intended in further work.

R. Wroblewski: You do not comment on the concentrations of other elements such as sodium, chlorine, and sulphur. Why?
Authors: Sodium was never detected in significant amounts. Chlorine and sulphur were not evaluated, since the P/B ratio of these elements and the number of spectra did not allow precise conclusions.

M. Ashraf: Fig. 1e is not in sharp focus.
Authors: Please note that the contrast in Fig. 1e is obtained by the biological material without any heavy metal stain, resulting in weak contrast, particularly in well frozen specimens with small ice crystals.