USE OF THE FROZEN THIN SECTION IN ELEMENT ANALYSIS

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X-ray microanalysis (XMA) combined with the use of the electron microscope and energy-dispersive X-ray spectrometer has become a useful method for the element analysis of biological materials. In particular, the use of a scanning transmission electron microscope (STEM) permits the analysis of the element composition of minute structures in thin sections. XMA is applicable to a wide field of biology and medicine because of its relative efficiency of detection and accuracy of element identification (Russ, 1972). However, application is still limited mainly because of the difficulties involved in specimen preparation and the sensitivity of the instrument.

In order to determine the composition and distribution of the elements in the natural state, the biological specimens must be prepared in such a way that all substances are held in situ without their translocation or loss during the preparation, while at the same time the fine structures must be well preserved and identifiable. Once the tissue or cell is subjected to chemical treatment such as fixation, dehydration and embedding during specimen preparation, not only are diffusable substances translocated or lost, but also unnatural chemicals used are added to the specimen. Thus, frozen thin sections (cryosections) of unfixed fresh materials which are not exposed to any chemical solution during the whole procedures can be expected to be the most effective and even ideal specimens for XMA. The usefulness of cryosections for XMA has been emphasized by several investigators (Russ, 1972, Gehring et al., 1972, Appleton, 1974, Panessa and Russ, 1975, Sjöström and Thornell, 1975, Yamada et al., 1975a, b, Sandborg and Russ, 1976, Mizuhira, 1976). The purpose of this paper is to report upon the use of cryosections of various animal tissues and cells in XMA.

MATERIALS AND METHODS

Mouse sternohyoid and sternothyroid muscles, mouse cardiac muscles, newt and frog leg muscles and frog eyes were used in this study. The tissues were freshly dissected out and cut into small pieces. These pieces were then mounted on copper sample holders and quickly frozen either by immersing the samples into freon 22 cooled by liquid nitrogen or directly into liquid nitrogen or by making contact with copper blocks cooled by liquid nitrogen, basically according to the procedures described by Christensen (1971). Cryosections were first dry-cut at setting of 100–200 nm in thickness with a diamond knife on a Sorvall MT-2 ultramicrotome fitted with a cryokit (FTS/LTC-2) and then mounted onto collodion-filmed and carbon-coated copper grids and either air-dried or freeze-dried in vacuo (Yonehara...
et al., 1975).

A Hitachi H-500 analytical electron microscope equipped with a Kevex 5100 energy-dispersive X-ray spectrometer was employed for the XMA. Thin sections were first viewed under the transmission electron microscope (TEM) and then examined under the STEM as images. The following conditions were used for the stationary spot analyses: accelerating voltages: 25, 50 and 75 kV, spot size: 5–50 nm, electron beam currents: $5 \times 10^{-11}$ to $5 \times 10^{-10}$ amperes (absorbed current on the copper), analyzing times: 100–200 sec.

RESULTS AND DISCUSSION

Effects of Chemical Fixation

Spectra from the analysis of cryosections varied depending on how the tissues had been pretreated (Yamada et al., 1975a). The cryosections were cut from mouse cardiac and skeletal muscles which were either unfixed or fixed with 3% glutaraldehyde. When spot analysis was made on the myofibrils (A-bands), the difference in spectra between fixed and unfixed samples was striking. First of all, in the unfixed samples the myofibrils showed a prominent peak of potassium (K) whereas no peak of K was discerned in the fixed samples (Fig. 1) (Yamada et al., 1975b). Similar spectra were obtained by Sjöström and Thornell (1975) on unfixed, frozen and dry-cut muscle fibers. Elements of glutaraldehyde, namely carbon (C),

![Fig. 1. Energy-dispersive X-ray spectrum of spot analysis on the myofibril (A-band) from a freeze-dried cryosection of a fresh frog sartorius muscle. Prominent peaks are present at P, S, Cl and K. Two peaks of Cu (K$_{\alpha,\beta}$) on the right are from the copper grid. Spot size: 30 nm, 100 sec, 75 kV.](image-url)
hydrogen (H) and oxygen (O), were not detected by the instrument due to their low X-ray energy. When a buffer was used for fixation, its elements appeared prominently; sodium (Na) and P in sodium phosphate buffer, and Cl in s-Collidine buffer. Those elements were easily removed by washing the cryosections with water before drying. In this respect, the cryosection from tissues fixed and subsequently immersed in known concentrations of electrolytes may serve as the experimental models for quantitative XMA of intracellular diffusible substances.

Similar results were also obtained from the analysis of whole-mounted air-dried samples of mouse erythrocytes (Yamada et al., 1975a). Significant peaks of Na, P, S, Cl, K and iron (Fe) were produced from the fresh, untreated erythrocytes (Fig. 2a). The cryosections from the fresh blood showed almost the same spectra as those from the fresh whole-mount, although each peak was much reduced (see Fig. 5b). The erythrocytes previously washed with physiological saline (0.9% NaCl) exhibited prominent peaks of Na and Cl together with peaks of well preserved K, P, S and Fe (Fig. 2b). The increased counts of Na and Cl probably reflect the crystallized NaCl on the surface of the erythrocytes. It would also seem that the physiological saline may well retain K within the cell. Once erythrocytes were fixed with 3% glutaraldehyde in water, the peak of K could be hardly detected and P and Cl were also substantially reduced. Fixation with buffered glutaraldehyde simply added the prominent peaks of the elements composing the buffer (Fig. 2c).

From the analysis, it is evident that diffusible substances such as Na, P, Cl and K leak out during chemical fixation. On the other hand, certain elements tightly bound to proteins or structures are apparently retained in chemically fixed samples. However, it is still possible that some kinds and amounts of elements are not only translocated but also removed during fixation, even though they are expected to be bound.

Structural Preservation and Visualization

Air-dried cryosection: When the cryosections from unfixed tissues were dried by blowing dry air on them, the fine structures seemed to be fairly well preserved.
However, the contrast was too low to identify all the ultrastructural details under the TEM. The use of a scanning transmission system did not improve the visibility of the fine structures even though the contrast could be enhanced. Because of poor structural identification it was often hard to analyze the precise organelles in such cryosection. Interestingly enough, the contrast was higher in the cryosections of aldehyde-fixed tissues and was increased even more after washing them with water. This was due to the removal of diffusible and soluble substances.

When the cryosections were air-dried, electron-dense granular precipitates were sometimes found in certain limited positions inside the cells. For example, the locations of the precipitate in skeletal muscle fibers were interpreted to be the mitochondria and sarcoplasmic reticulum based on their distribution pattern (Figs. 3a and b). Similar precipitates were noted in other types of cells in nitrogen-gas dried cryosections by Christensen (1972) and in air-dried tissue spread preparations by Takaya (1975). The spot analysis on the precipitate revealed very prominent peaks of Ca and P (Fig. 4). In freeze-dried cryosections, however, no precipitate was found in the mitochondria and sarcoplasmic reticulum. The mitochondria were seen as ill-defined oval bodies of moderate electron density and a prominent peak of Ca was not produced on these organelles. This discrepancy can be explained by the assumption that in XMA Ca is much more effectively detected in the crystalized form than in the evenly dispersed form even if the same amount of Ca is contained within an electron-excited volume of the section. It is interesting to conjecture about what happens to the cryosection during air-drying. The section must melt before drying and some electrolytes may be crystalized out as drying proceeds. If so, crystalization may depend on the concentration of electrolytes and the speed of water evaporation. Indeed, the precipitates tended to be formed

![Fig. 3. Air-dried cryosections of a mouse fresh sternothyroid muscle. Fig. 3a: transverse section of 2 muscle fibers (M₁, M₂). ×14,000. Fig. 3b: longitudinal section. ×27,000. Note numerous dense precipitates at locations corresponding to the mitochondria and sarcoplasmic reticulum.](image-url)
in larger quantities when the section was allowed to dry more slowly.

Freeze-dried cryosection: Even if it is an instantaneous event, melting may cause some translocation of diffusable substances. To avoid such a possibility, freeze-drying is a theoretically ideal method for specimen preparation. We developed a freeze-drying device which can be used in an ordinary vacuum evaporator (Yonehara et al., 1976). A similar device (vacuum transfer stage) was recently described by Hodson and Williams (1976). Cryosections mounted on grids were placed in the device precooled either at liquid nitrogen temperature or at cryokit chamber temperature and were then freeze-dried in an evaporator. Freeze-dried cryosections prepared in this way turned out to be more or less porous but with an relatively increased contrast, as compared to the air-dried cryosections (Figs. 5a and b). The structural details in freeze-dried cryosections were often disturbed by porosity (also Gehring et al., 1972, Hodson and Williams, 1976). There are 3 possible ways to explain this; (1) the porosity resulted from ice crystal formation during the freezing of the tissue, (2) recrystalization of ice in the cryokit chamber, and (3) mechanical destruction during drying in vacuo as discussed by Appleton (1974). The speed of freezing is apparently crucial. Some authors claimed that only the superficial layer of the tissue is better preserved ultrastructurally, since slow freezing causes serious ice crystal damage (Hodson and Williams, 1976). If this is the case, our preparation may possibly be from a deeper layer. Drying the cryosections may also cause some translocation of diffusable electrolytes. Hence,
one possibility is analysis of the cryosection while still in a frozen state. Porosity should be reduced before the freeze-dried cryosections are used in XMA. Appleton (1974) reported that slow freeze-drying at atmospheric pressure within the cryostat gave the best results. It is also important to store the sections in a dry atmosphere, since exposure of dried sections to humid air may cause redistribution of certain electrolytes (Appleton, 1974). In any case, analysis can be performed on the

![Fig. 5. Freeze-dried cryosection of the frog iris and a XMA spectrum from the same section. Fig. 5a: A capillary is filled with nucleated erythrocytes (R). Several pigment cells are seen around the capillary. ×4,000. Fig. 5b: The spectrum shows a significant peak of Fe (Kα, see marking bars) in addition to peaks at P, Cl, S and K. Compare the spectrum to that from the fresh whole-mount mouse erythrocyte (Fig. 2a). Spot size: 30 nm, 100 sec, 75 kV.](image)

![Fig. 6. Air-dried cryosection of a melanocyte of frog iris (Fig. 6a, ×8,000) and a XMA spectrum from melanin granules in the same section (Fig. 6b). Note the prominent peaks at Ca and Mg in addition to the peaks at Na, P, S, Cl and K. Spot size: 20 nm, 100 sec, 75 kV.](image)
organelles which are recognizable and only slightly disturbed by the porosity. It is interesting that the analysis of various cells and organelles such as erythrocytes, nuclei and pigment granules showed almost the same spectra in both the freeze-dried and air-dried sections.

In the analysis of frog ocular pigment granules, significant peaks were detected on melanin granules in the iris and choroid at Na, Mg, Cl, K and Ca (Figs. 6a and b), whereas the fuscin granules in the pigment epithelial cell produced peaks of Na, S, Cl, K, Ca and Zn (Yamada and Ishikawa, 1976) (Figs. 7a–d). Such prominent peaks were not discerned on the pigment granules of the fixed and embedded sections.
Specimen Damage and Contamination

There is no precise information on how much the biological specimens, especially cryosections, are damaged by the electron beam during analysis. Some elements may be evaporated more easily than others. To test this possibility, thin sections of an Epon block in which iodine (I) had been dissolved were analyzed. As a 100 sec analysis was successively repeated, the peak of Cl was progressively reduced whereas the peak of I was well reproduced. It also sometimes happened that on the cryosections of erythrocytes the peak of Fe was apparent in the first 10–20 sec and became obscure afterward. Such phenomena may be explained by either beam damage or contamination. The latter absorbs the characteristic X-ray or generates background X-ray to mask the peak (Panessa and Russ, 1975).

A significant peak of Si was occasionally shown on the cryosections. One should be cautious as to whether or not the peak of Si arises from its natural existence in the tissues. In some cases, such peaks may be produced by contamination during the specimen preparation and/or analysis.

Choice for Analyzing Conditions

The spectra from the analysis varied depending upon the analyzing conditions used. To get a better spectrum, the proper conditions for particular elements should be chosen. When the relative amount of elements is estimated, one should employ the same conditions throughout the whole analysis: accelerating voltage, beam current, spot size and analyzing time. The basic data is now being accumulated for the choice of analyzing conditions (Panessa and Russ, 1975, Fowler and Goyer, 1975, Futaesaku et al., 1975, Mizuhira, 1976). It is not clear how far one can apply the data from experimental models to the cryosections of unfixed tissues and cells.

CONCLUSION

The frozen thin sections of various unfixed fresh tissues were used in XMA. The analysis demonstrated that in the frozen sections highly diffusible elements were well retained in loco and detected. The usefulness and limitations of XMA using the frozen sections were discussed, emphasizing that, before the frozen sections would be generally used for the XMA, improvements in technique for specimen preparation and more of basic knowledge for the choice of analytical conditions are needed.

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REFERENCES


