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RELIABILITY OF INTRACELLULAR WATER AND ION DISTRIBUTIONS AS MEASURED BY X-RAY MICROANALYSIS - A REVIEW

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Abstract

X-ray microanalysis can be an important tool to reveal the spatial relationships between polyelectrolytes, ions, and water as they occur within cells and tissues *in vivo*. To reach this goal, at least two of these three closely interrelated variables should be measured independently. Moreover, the absence of systematic errors should be proven.

The present review discusses the probability of artificial ion and water shifts between intracellular compartments due to the growth of dendritic ice crystals much larger than the cross-sectioned remnants commonly seen in frozen-dried sections. Considering the possible mechanism of ice crystal growth it is concluded that ions and water are not translocated over large distances.

Moreover, problems associated with the preparation of a sample for water content estimations are discussed here. The importance of an appropriate pre-freezing treatment is highlighted, as is the importance of fast freezing. The risk of artificial water shifts between compartments with different freezing properties is discussed and the absence of clefts between compartments or haloes around them as seen in frozen-dried sections is taken as an appropriate criterion.

Constancy of section thickness and retention of full hydration of cryosections are necessary prerequisites for many of the techniques and conditions to fulfill these requirements are given.

Key Words: X-ray microanalysis, cryofixation, water content, ions, ion binding, ice crystals

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Introduction

All important biomacromolecules are polyelectrolytes. That means, that their conformation is determined by the local concentrations of ions they interact with. Ions, therefore, are important basic regulators of life processes, and the local concentrations of inorganic ions, water, and polyelectrolytes are intimately interrelated.

Cryomicroscopy combined with X-ray microanalysis is one of the few techniques allowing measurements of ion and water concentrations with subcellular resolution. Results of many X-ray microanalytical investigations speak in favor of a co-compartmentalisation of ions and polyelectrolytes in cells. Whether this might be a preparation artefact shall be considered first.

By far, most of the X-ray microanalytical measurements have been performed on frozen-dried specimens, yielding relative ion concentrations on a dry mass basis. That is because radiolysis of water under the electron beam sets narrow limits to the examination of biological specimens in the hydrated state. True ultrastructural analytical resolution in hydrated specimens has as yet only been achieved in exceptional cases (Gupta 1989). The restriction to frozen-dried specimens often meant neglecting the measurement of the local water content as the third important component apart from ions and dry mass. This neglect might become a serious problem because the local water content changes both spatially, i.e., between compartments, and temporally, depending on the physiological state of the cell in question. In a study of the aging process in heart muscle and liver cells of the rat, for instance, it was found that ion concentrations per dry mass did not change significantly in different intracellular compartments with aging (von Zglinicki and Bimmler 1987a). However, dehydration of mitochondria with advancing age and under correspondent experimental conditions was found, and this is probably an important mechanism in the deterioration of the energetic apparatus during aging (von Zglinicki 1988a, von Zglinicki *et al.* 1991). In other words, dry mass can by no means be regarded as a stable reference value for ion concentration measurements.

Methods to measure local water contents in the cryomicroscope have been reviewed recently, highlighting the different assumptions and requirements of the techniques (von Zglinicki 1990). The present review will focus onto preparative requirements for the unbiased measurement of water distribution by those techniques.

The Intracellular Distribution of Ions and Water - Artefact or Not?

How ions, water and biopolymers in cells interact is a matter of considerable, and often controversial, debate (see, for instance, Negendank 1989). This is not astonishing because of the far-reaching implications of possible answers for any physico-chemical theory of the basic processes of life (Ling 1988).

A promising way to add hard facts to that discussion is the examination of, and the comparison between, intracellular distributions of polyelectrolytes, water, and ions. This is the task of cryo-electron microscopy and X-ray microanalysis, and there are a number of approaches. Intracellular ion and dry mass gradients have been demonstrated more than a decade ago (Gupta *et al.* 1978). Muscle, of course, is a favorite object for distribution studies, and increasing evidence has been collected for a co-compartmentalisation of K and muscle proteins along the myofibril (for review, see Edelmann 1989). This pattern has been verified not only for K, but also for the anion Cl in heart muscle (von Zglinicki 1988b). Essentially the same type of results was obtained in studies of ion and water concentrations in nuclei. In 1985, evidence gained so far allowed the development of a hypothesis about a substantial amount of ion binding in nuclei (Cameron 1985). Measuring ion concentrations in condensed and decondensed regions, we established a positive correlation between dry mass density and local ion content in rat liver nuclei (von Zglinicki and Bimmler 1987b). K- and Cl-distributions measured in isolated nuclei (von Zglinicki *et al.* 1989) were found to be in accord with a cooperative binding similar to counterion condensation as suggested by Manning (1978) for pure nucleic acids.

A compilation of results obtained by X-ray microanalysis, mainly performed on frozen-dried sections, indicates a substantial co-compartmentalisation of anorganic ions with intracellular, and also extracellular (Gupta 1989), polyelectrolytes. This is in accord with a number of results from different techniques (see, for instance, Kellermayer *et al.* 1986, Cameron *et al.* 1988, Negendank 1989) but is seemingly opposed to the view of ions being free in solution as held in physiological textbooks. The question arises, whether this apparent binding of ions as seen in the cryomicroscope might be an artefact of the technique.

To evaluate this we shall assume that ions are freely dissolved in the cellular water *in vivo* and check possible artefacts that could reverse the assumed distribution. Artefacts may arise during different preparation steps and especially during the prefreezing treatment (von Zglinicki 1989, and below). The important step in the current context, however, is the freezing of the tissue, because a phase separation occurs as soon as ice crystals are formed. Ice crystals grow from pure water, excluding all ions and polyelectrolytes, forcing the co-compartmentalisation of ions and polyelectrolytic dry mass in the eutectic segregation zones around the crystals. On a microscopic scale, artificial binding of ions to polyelectrolytes can be avoided only if freezing is fast enough not to produce ice crystals at all, i.e. if the water in the sample becomes vitrified.

Due to poor heat conductivity, tissue samples are too large to be vitrified without pretreatment. Both the treatment of tissue with cryoprotectants (Barnard 1987) and the necessary treatment of a sample before high--pressure freezing (Moor 1987) might induce artificial ion shifts and both treatments are, in general, not acceptable for X-ray microanalytical studies of intracellular ion and water distributions. Tissue subjected to X-ray microanalytical examination will, therefore, normally contain ice crystals. That means, that even the demonstration of co-compartmentalisation of ions and polyelectrolytic mass in frozen-hydrated sections by Edelmann (1988) as opposed to most other studies performed on frozen-dried sections does not prove ion binding *in vivo* as long as vitrification has not been established.

The question to ask is whether the artificial ion shifts necessarily induced during ice crystal formation are with certainty smaller than the analytical resolution or not. Ice crystal remnants as seen in, e.g., frozen-dried thin sections will easily be smaller than about 100 nm in diameter. Ions displaced by such a distance would still remain within one and the same compartment and the differences in concentrations measured between different compartments must then be due to some sort of binding *in vivo*. However, it has been shown by electron diffraction in frozen-hydrated sections that only cubic ice crystals, generally with diameters well below 100 nm, are not interconnected. In most cases, either due to slow freezing or to recrystallisation, hexagonal ice will be produced. Although ice crystal traces as seen in thin sections might still be in the order of 100 nm, they might be all interconnected as branches within one or very few large dendritic ice crystals per cell (Chang *et al.* 1983, Dubochet and McDowall 1984). Therefore, ions free in solution could theoretically become displaced over rather large distances during dendritic growth of hexagonal ice crystals (Fig. 1).

The corresponding macroscopic pattern would be

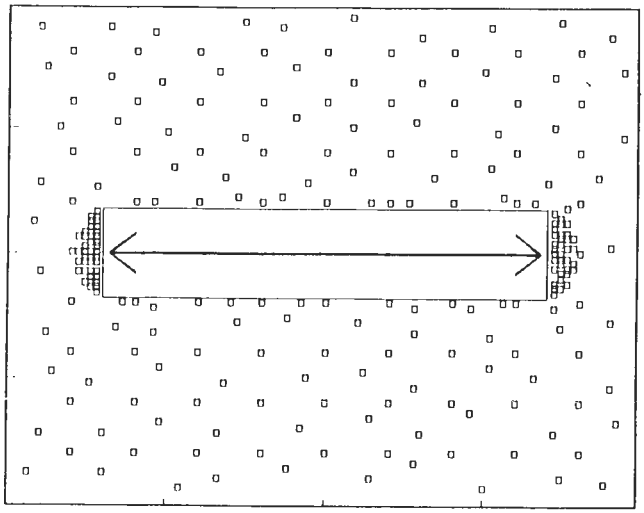

Fig. 1: Model of a dendritic ice crystal growing "out of itself", sweeping ions away over rather large distances in the main direction of growth. Symbols: ▬▬ growth direction, ———— ice, □ ions.

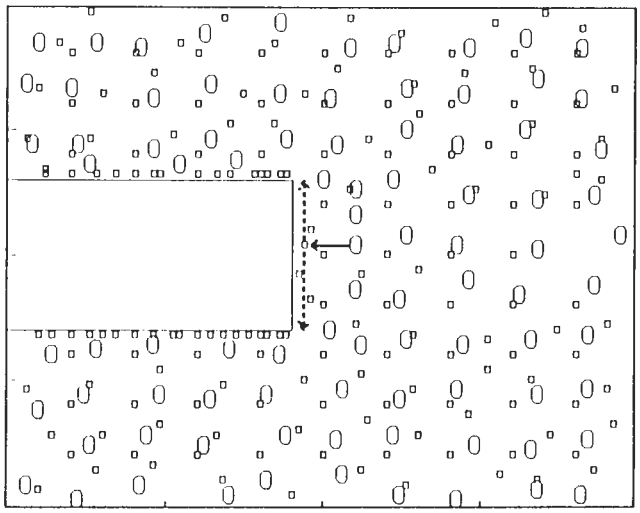

Fig. 2: Model of a dendritic ice crystal growing by diffusion of water molecules out of the solution onto its surface. Ions are squeezed away from the principal surface of growth by the water molecules diffusing onto it and are deposited close to the non-growing surface. Symbols: - - - - ion diffusion, ▬▬ water diffusion, ———— ice, O water, □ ions.

that of a glacier leaving a terminal moraine of ions, maybe deposited onto preexisting "mountains" of polyelectrolytes at its borders. But ice crystal growth out of a solution is not comparable to the movement of a glacier. Water molecules have to move to the surface of a crystal from the outside to make it grow. Within a cellular system, it has been experimentally shown that the diffusion of water molecules is small enough not to cause any significant displacement of water on an organelle scale, even between compartments with different water content and, probably, different rates of subcooling. This holds for freezing rates easily obtainable in the outer zones of tissue by conventional fast freezing techniques (von Zglinicki *et al.* 1987). Ions between the diffusing water molecules and the present surface of the crystal cannot be swept away in the main direction of growth. Rather, they are squeezed aside between the already existing crystal surface and the new molecules attaching to it (Fig. 2).

This model is, of course, oversimplified. Whether the average distance between water molecules within the liquid phase is larger than that in the crystal will depend on the amount of solute present. Anyway, reorientation of water molecules occurs at the surface of the ice crystal and has the same effect of ion displacement. If this results in any accumulation of ions in front of the principal surface of growth, water activity becomes reduced there and water molecules have to diffuse onto the crystal from the outside.

Actually, the eutectic boundaries around crystals grown out of a binary solution are never thicker at the tip of a dendritic crystal as compared to its sides (compare, for instance, Fig. 8 of Dubochet *et al.* 1982). This consideration holds even in binary solutions of water and ions, e.g., in extracellular spaces lacking appreciable amounts of dry mass. The practical problem with the analysis of apoplastic spaces (Echlin 1989) is due to the fact that ice crystals can grow to rather large sizes in every direction, displacing ions easily over distances comparable to the sizes of the compartment in question. Under those circumstances, even analysis of frozen-hydrated specimens can become meaningless. The most important action of macromolecules in intracellular compartments is, therefore, the suppression of the freezing point, leading to considerably smaller ice crystals. Moreover, they may act as additional ion traps (Fig. 3).

Summarizing, displacement of ions over distances larger than the ice crystal damage as seen in sections can most probably be safely neglected. In other words, cryopreparation techniques combined with microanalysis in the present state cannot reveal directly the molecular mechanisms of the interactions of polyelectrolytes, water, and ions. Nevertheless, they enable the unbiased estimation of the distribution of water and ions between intracellular compartments with dimensions that are large as compared to the size of the ice crystal remnants. If this is taken for granted, the results cited above demonstrate a substantial co-compartmentalization of

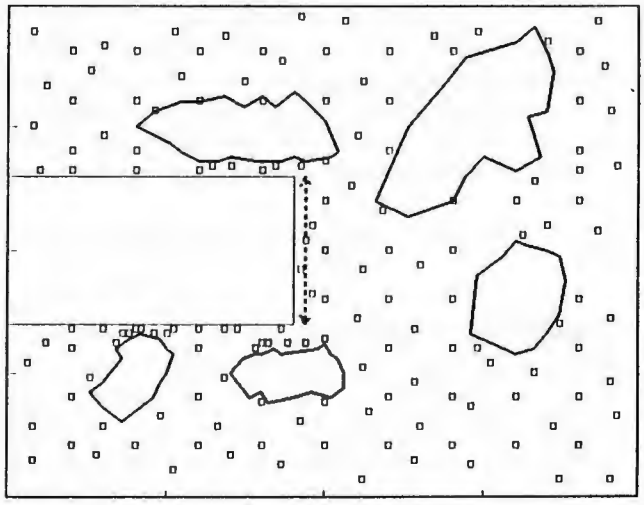

Fig. 3: Model of an ice crystal growing in a solution containing polyelectrolytes. Ions displaced by the growing ice crystal are partly precipitated on the charged surface of the next polyelectrolyte. Water molecules are omitted here for clarity of the figure.

Symbols: - - - - ion diffusion, ▬▬ polyelectrolytes, ——— ice, ▫ ions.

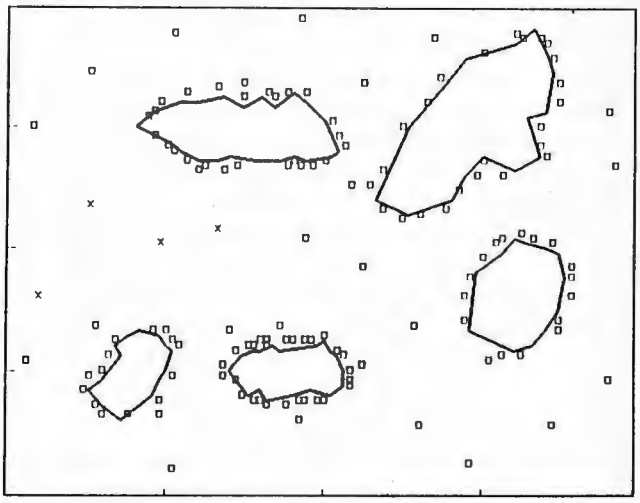

Fig. 4: Model of the probable *in-vivo* distribution of ions in a solution containing polyelectrolytes. Most of the ions are in close association to the charged surfaces of the polyelectrolytes. Only very few ions (marked by asterisks) are displaced during ice crystal growth.

Symbols: ▬▬ polyelectrolytes, x ions (displaced), ▫ ions.

ions with fixed charges *in vivo*. Therefore, the model given in Fig. 4 displaying most of the ions in close association to polyelectrolytes might resemble the real situation best. Possible binding mechanisms can be deduced from the studies performed on isolated nuclei in varying salt concentrations (von Zglinicki *et al.* 1989). It should be borne in mind that a substantial co-compartmentalisation of ions with polyelectrolytes is in fact the result to expect from the physico-chemical point of view (Manning 1978).

The Measurement of Intracellular Water Distribution

Local water concentrations can be estimated by either: 1. determination of the oxygen concentration in frozen-hydrated (bulk) samples using a windowless X-ray detector (Marshall 1980); 2. determination of the mass density of sections by any of the electron beam--specimen interactions resulting in a signal linearly related to mass thickness, either in the frozen-hydrated and frozen-dried state or in frozen-dried sections alone; or 3. determination of element concentrations by fully quantitative X-ray microanalysis in thicker sections or bulk specimens both in the frozen hydrated and dried state (Gupta and Hall 1981).

These different possibilities have been recently reviewed with emphasis laid on the assumptions and limitations of the techniques used for measurement and quantitation (von Zglinicki 1990). Special attention was paid to the problems of mass loss occurring mainly in hydrated specimens and to shrinkage during freeze-drying. However, it was presupposed that the frozen specimen resembles closely the *in-vivo* distribution of ions and water at the onset of the measurement. Here, the problems of preparation will, therefore, be dealt with. The commonly used preparation steps are pre-freezing treatment, freezing itself, and, in many cases, sectioning. These shall be considered in turn.

Prefreezing treatment

Prefreezing treatment starts already before killing the experimental animal, and so does the possibility of inducing artificial ion and water shifts in the cells later under study. Although it is known that seasonal and diurnal alterations of ion and water content at least in a few organs occur, information concerning ion and water shifts in tissues under those influences, under stress, or under the action of anesthetics is sparse. However, morphological changes are known to occur under those influences (David 1969) and extreme caution is advisable if ion and water concentrations in the *in-vivo* state are to be measured.

Killing the animals before sampling leads to hypoxia and, most often, ischemia in its organs. Some tissues, like skeletal muscle, are not very sensitive to short-time ischemia. On the other hand, significant ion shifts have been measured after about 20 sec of preparative ischemia in heart muscle and liver cells (von Zglinicki

et al. 1986). The brain is known to become edematous during short periods of ischemia, which would render any measurement of cellular water content meaningless (David 1969). Last, but not least, the sampling of a tissue specimen might cause trauma to the cells under study with its well-known consequence of a more or less complete equilibrium between intra- and extracellular ion concentrations.

A number of corresponding sampling techniques has been developed including the Cryogun manufactured by Chang *et al.* (1980), freon "popsicles" as used by Somlyo *et al.* (1985) in the study of liver specimens (see also Bond and Jaraki 1989), the Cryoneedle (von Zglinicki *et al.* 1986), and chilled copper pliers (Hagler *et al.* 1983), developed later into the Cryosnapper (Hagler *et al.* 1989). The basic idea of all these systems is to shorten the time lag between killing the animal and cryofixation and, especially, to start sampling and freezing of a tissue specimen simultaneously. Everyone who aims at measuring *in-vivo* ion and water distributions is strongly advised to follow these lines of thought closely.

The situation becomes more relaxed if tissues or cells *in vitro* are to be considered. Cells or tissues are allowed to recover from the isolation process and to reach a physiologically well-defined state, much closer to an ideal sample for X-ray microanalysis than a living animal. Actually, from the point of view of a microanalyst, the establishment of a *in-vitro* model is a solution to the pretreatment problem mentioned above just contrary to the simultaneous cryofixation approach. Instead of aiming at true *in-vivo* information, one obtains data from a rather well-characterized model system. Instead of aiming at simultaneous sampling and freezing, one clearly separates both processes in time, obtaining a defined state in between. Freezing, therefore, can be optimized better than when it is compromised by simultaneous sampling (Nassar *et al.* 1986, Wendt-Gallitelli and Isenberg 1989).

The remaining problem stems from the fact that the tissue *in vitro* is surrounded by a buffer which has to be removed as much as possible in order not to retard the freezing rate in the tissue. On the other hand, minute amounts of water will dry rather fast (Hall and Gupta 1982, Nassar *et al.* 1986) constituting a hyperosmotic environment to the specimen. To reach a satisfactory compromise requires careful measurements of the water loss and skillful withdrawal of water (Nassar *et al.* 1986). A humidity chamber decreases the risk of pre-freezing dehydration (Gupta and Hall 1981).

In a number of cases, cryoprotectants are used to improve the quality of freezing. Low-molecular weight cryoprotectants are in most cases unsuitable for X-ray microanalytical studies due to their high osmotic pressure, which requires prefixation of living cells (Robards and Sleytr 1985, p. 73). However, high-molecular weight cryoprotectants like dextran are commonly used, most often as external standards for ion and water concentrations (Rick *et al.* 1982, Gupta and Hall 1979, Gupta 1989). Those standards are, of course, isosmotic. However, they increase the viscosity of the water dramatically. As speculated by Barnard (1987), this increase in viscosity might actually be the reason for the freezing point depression exerted by those substances. Transport is grossly retarded in tissues surrounded by high-molecular weight cryoprotectants (Barnard *et al.* 1984). Although this effect is reversible, it cannot be stated in general terms whether an external dextran-salt solution, for example, might cause intracellular water and ion shifts or not (Saubermann *et al.* 1986a,b). If external standards/cryoprotectants are used, results should be compared with those obtained from untreated samples, if possible.

High pressure freezing (Moor 1987) is another possibility to obtain large well-frozen, often even vitrified samples. Recently the yield of adequately frozen samples has been considerably increased by replacing the extracellular water with 1-hexadecane, a chemically inert, water-insoluble mineral oil (Studer *et al.* 1989). The problem of the technique is again pretreatment. Rather long times (in the order of minutes) seem to be required to mount a tissue sample in the specimen chamber for freezing. The tissues are subjected to severe anoxia during that period. Microanalytical investigations testing the consequences of that type of preparative anoxia have not been published yet.

Freezing

As evident from the foregoing paragraph, freezing techniques for use in ion and water distribution studies have to be compatible with the lowest level of pre-freezing artefacts possible. Those techniques have been extensively reviewed (Plattner and Bachmann 1982, Robards and Sleytr 1985, Steinbrecht and Zierold 1987). The best that can be obtained from them is an about 20μm thick superficial layer of well-frozen tissue (Echlin 1989), "well-frozen" meaning that ice crystal remnants in this layer are small compared to the size of the commonly analyzed ultrastructural compartments, i.e., that their diameters are in the order of less than 100 nm. Although microanalysts learned to live with that situation, it has serious drawbacks. The risk of ion displacement due to ice crystal formation was already discussed above. Moreover, preparation of samples becomes extremely complicated or even impossible when tissues become more complicated in their spatial organization or when they contain larger fractions of water as, for instance, most plant tissues. It would be highly desirable, therefore, to improve high-pressure freezing by

bringing it more close to an in-vitro cryofixation technique. From such a development, an important impact onto microanalytical techniques could be expected.

Ion displacement as a freezing artefact was discussed at length in the first part of this review. Ice crystal growth might also displace water. The obvious displacement is due to the eutectic growth of crystals, and for this the same considerations as discussed for the ions should hold. In other words, water displacement within ice crystals should be not significantly more than the diameter of the dendritic ice crystals.

However, water shifts might occur even between compartments during freezing: Freezing point depression evidently is different in different compartments. Under comparable freezing conditions, ice crystal size as seen in sections varies in parallel with the local water content (Cameron *et al.* 1988). Therefore, water is expected to freeze first in compartments with a lower dry mass. These compartments become hypertonic, and adjacent compartments with still unfrozen water will be dehydrated by osmotic forces. Evidently the degree of dehydration depends on the freezing rate. It has been confirmed that mitochondria are not significantly dehydrated under freezing conditions leading to ice crystal remnants in the surrounding medium smaller than about 100nm (von Zglinicki *et al.* 1987). However, under slightly worse freezing conditions, haloes around mitochondria are frequently observed in frozen dried sections which are interpreted as indicators of an osmotically driven efflux of water (von Zglinicki 1988a). Those haloes might also be the result of differential shrinkage during freeze--drying. Whatever the exact mechanism, the absence of haloes around or of clefts between compartments can be taken as a sign of good freezing and is a necessary prerequisite for the measurement of water distributions.

Sectioning

Two aspects of cryosectioning have to be considered if the measurement of water distributions at the subcellular level is aimed at: section thickness has to be constant if mass thickness is used as a measure of water content, and full retention of hydration has to be ensured if frozen hydrated sections serve as "standards" for the estimation of water content.

Although there is good evidence against through section melting during cutting (Frederik and Busing 1981, Karp *et al.* 1982, Chang *et al.* 1983), sectioning is still poorly understood from the physical point of view (Zierold 1988). Sections are compressed by 30-60% in the cutting direction and crevasses and chatter occur, changing the thickness of sections (Chang *et al.* 1983). Even more important than these rather regular changes, which can easily be seen in the electron microscope, are stepwise changes of section thickness at the borderline between compartments. These steps occur if cutting actually proceeds as fracturing. An impressive example is given by Ornberg (1989, Fig. 2b) and it is shown that the probability of fracturing increases with increasing ice crystal damage.

From measurements of the relative mass thickness of the same mitochondria in adjacent serial cryosections with a nominal thickness of about 100nm (von Zglinicki *et al.* 1987) a variation of section thickness between mitochondria and medium of about 10% can be calculated. This value is in good accordance with data obtained from catalase crystal cryosections (Chang *et al.* 1983) and sets a lower limit to the variation of water distribution data obtained from mass thickness measurements, but should not significantly bias mean values.

The rate of dehydration of sections in the cryomicrotome depends heavily on the design of the cryochamber. Chang *et al.* (1983) reported a dehydration rate of about 1nm/sec at 183 K in a Reichert FC4 cryoultramicrotome, about one order of magnitude less than the rate in vacuum, while Zingsheim (1984) measured a decrease of sublimation rate by four to five orders of magnitude in a Burlington Cryocut I, leading to negligible sublimation rates even at rather high temperatures. It can be expected that cryomicrotomes like the FC4 which are effectively protected against the entrance of atmospheric air into the cryochamber should have rather high sublimation rates, while those often older ones which allow easy entrance of moist air might facilitate the deposition of frost instead of dehydration even at rather high temperatures.

Assuming the data given by Chang *et al.* (1983) to be an upper limit of dehydration rates in a cryoultramicrotome, cutting should be done at chamber temperatures below 160K in order to avoid significant water loss from 100nm sections. This is surely possible with ultrathin sections, however, the same data would require cutting at about 170K for 1μm sections, assuming that it takes about 20 - 30min to collect enough sections for one grid. In most cases, thick sections have to be cut at considerably higher temperatures. It is very advisable, therefore, to check the dehydration rate in the cryotome in question experimentally, if thick sections are going to be used in the frozen hydrated state.

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Discussion with Reviewers

L. Edelmann: I propose to mention at least the resin embedding techniques and their advantages and disadvantages, in particular, the possibility of controlling freezing and structure preservation best by freeze-substitution.

Author: Although properly used resin embedding techniques can preserve treatment-dependent differences between ion distributions in different samples, they are generally not able to preserve ion and water distributions as they are in biological specimens *in vivo* and are outside the scope of this review, therefore.

L. Edelmann: You wrote that the demonstration of co-compartmentalisation of ions and polyelectrolytic dry mass in frozen-hydrated sections (Edelmann 1988) does not prove ion binding *in vivo* as long as vitrification has not been established. This sentence at this place may be interpreted in different ways and may lead to wrong statements. In fact, electron diffraction of the well frozen parts of the cited frozen-hydrated muscle sections usually yielded very faint rings (indicating cubic ice) in a predominantly diffuse diffraction pattern. Hence, vitrification has not been established and one may conclude that the observed results are of no value. I do not agree with this conclusion for the following reasons: Despite the fact that vitrification has not been established, segregation artefacts are not visible in the mentioned frozen-hydrated cryosections. Hence, according to your own reasoning (later in the text) the observed preferential accumulation of electrondense ions at Z lines and in the A bands cannot be due to ion redistributions caused by the freezing process. Even the regular intense staining of the myosin filaments with Tl^+ is most likely not caused by ice crystal growth in the area

between neighboring myosin filaments because in this area usually very fine ultrastructural details (cross-bridges, actin filaments) are visible indicating that segregation artefacts - if present - must be smaller than about 10 nm. Taken together the observed co-compartmentalisation of ions and proteins must be due to some kind of weak ion binding or ion association or adsorption.

Author: Your conclusions are completely right. However, the point I wanted to make is that this conclusions are based on the assumption that significant artefacts did not occur during preparation, which in your case is practically only freezing. As we have learned, this assumption is not a priori self-evident, even if no major structural damage is seen in the frozen samples. What I tried to do in this paper is to summarize evidence for this assumption being possibly true.

L. Edelmann: I propose to add at least some sentences on possible artefacts caused by freeze-drying of the cryosections and by electron irradiation during X-ray microanalysis. Besides of possible shrinkage artefacts, ion redistribution artefacts have been observed during improper freeze-drying of bulk specimens which were subsequently embedded in a resin and after irradiation of a dry-cut section of resin embedded muscle after intense electron irradiation (Edelmann 1984). Similar events may occur within cryosections during freeze-drying and electron irradiation. Furthermore, Na redistribution has been observed in freeze-dried cryosections of muscle (see Edelmann 1988a). In my opinion it is important to further investigate these artefacts and to propose suitable control experiments.

Author: The topic of radiation damage in general was outside the scope of this paper and I should simply refer to two recent reviews by Echlin (1991) and Lamvik (1991). However, you are quite right, there is an important interrelationship between incomplete freeze-drying and radiation damage: Radiolysis of water produces free radicals with high yield, and it has been repeatedly shown that mixtures of organic materials and water are more radiation sensitive than dry organic mass and even pure water alone (Dubochet *et al.* 1982, Talmon *et al.* 1986), even if the contain only spurious amounts of water as, e.g., after incomplete freeze-drying (Zierold 1988). The practical problem, especially if dealing with freeze-drying of sections in the electron microscope, is that the actual temperature of the specimen is not well controlled. The choice is between the risk of recrystallization and that of improper freeze-drying, then.

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