Biological X-ray microanalysis of ions and water: artefacts and future strategies

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The purpose of electron microscopic investigations of biological material is to understand how living cells are constructed and how they function. We know that if we introduce a stable biological specimen into an electron microscope we can obtain information on the ultrastructure of the biological material and by collecting X-rays generated by the interaction of the electron beam with a given area of the specimen also obtain information on the kind and amount of elements present in the irradiated area (electron probe X-ray microanalysis, EPXMA). However, biological material in its natural environment is not stable. In number of particles, the largest component of living cells is water, the second most abundant is K+ and both components are highly mobile in living cells. Before a biological specimen can be examined in the vacuum of the electron microscope it must be stabilized somehow and we may assume that any stabilization procedure which is able to capture the original water distribution (or that space which was originally occupied by water) as well as the original K+ distribution may also be suited to immobilize all other cellular components at or near their original position.

The main preparation procedures currently used for EPXMA [21] start with specimen sampling followed by freezing. The specimen may then be investigated in the frozen-hydrated state (bulk specimen or frozen-hydrated cryosection) or after freeze-drying (e.g., freeze-dried cryosection) or after the combination of dehydration (freeze-drying or freeze-substitution) and embedding in resin (bulk specimen or dry-cut section). All these procedures introduce artefacts. Thus the development of EPXMA in biological research was and will be highly dependent on the evaluation and minimization of preparative artefacts.

It is not the purpose of this contribution to review in detail the different preparation and analytical detection methods and the many applications (for a review of the history of EPXMA and references see Hall [11]; a review on the reliability of intracellular water and ion distributions as measured by X-ray microanalysis was given by von Zglinicki [17]). Instead, several open questions mainly concerning possible artefacts are discussed; these problems came up during the efforts to localize K+ or electron-dense K+ surrogates such as Cs+ and Tl+ in the striated muscle [3-10]. Furthermore, suggestions for future strategies in biological EPXMA are given.

The studies with striated muscles were carried out with freeze-dried or freeze-substituted and embedded preparations and with freeze-dried and frozen-hydrated cryosections. The different preparations showed correspondingly that K+ and/or the K+ surrogates are mainly localized in the A bands and at Z lines as postulated by Ling [15]. Redistribution artefacts could also be visualized (the use of electron-dense ions allowed the direct visualization of subcellular ion distributions and redistributions in differently prepared thin sections by transmission electron microscopy). It became evident that the following possible artefacts are challenging problems for specimen preparation: redistribution of ions and water before freezing [7,8,18], during freezing [1], during freeze-drying [3-6] and during freeze-substitution [8], redistribution of ions during embedding [3,8], during exposure of sections to atmosphere and during electron irradiation of sections [3,4].

The main weakness of current EPXMA is probably the fact that the precise subcellular water distribution and the quantification of ion concentrations in small subcellular areas is not possible by using thin frozen-hydrated cryosections because the required high electron doses damage the analysed area (for EPXMA of frozen-hydrated preparations see, e.g., [13,14]). Nevertheless, in order to improve the dehydration methods used for EPXMA more control experiments with frozen-hydrated preparations are necessary.

Frozen-hydrated cryosections of even thickness showing ultrastructural details can now be prepared by using new cryo-ultramicrotomes and antistatic devices (Fig.1). Such sections can be freeze-dried under controlled conditions and photographed in different states of dehydration. When freeze-drying is carried out for a sufficient length of time at a low temperature [5] a linear (and uniform!) shrinkage of cryosections of about 5% can be obtained (important for the correct evaluation of ion and water distributions).

Frozen-hydrated cryosections of biological material which has been loaded with mobile electron-dense ions before freezing can be photographed (low dose) without damaging the specimen. The distribution of electron-dense particles visualized in the micrographs can be evaluated by microdensitometry. The problem of ion redistribution during preparative procedures can then be investigated by analysing freeze-dried cryosections or sections of freeze-dried or freeze-substituted and embedded preparations of the same biological material.

In the past most of EPXMA has been carried out with freeze-dried cryosections and the following arguments speak in favour of this method: freeze-dried cryosections are stable in the electron beam and exhibit a high electron optical contrast; lower ion concentrations can be detected than in sections of embedded material and the local dry weight content (and hence the former local water content) can directly be determined [20]. In addition to these advantages an embedding technique which allows a complete retention of mobile ions like K+ in the specimen has not yet been published (Condron and Marshall [2] and Edelmann [9] report losses of K+ of 30-40% in freeze-dried and/or freeze-substituted and embedded material). Nevertheless, further improvement of embedding techniques is recommended for the following reasons:

1) With a new method proposed by Hall [12] the local dry weight content and (former) water content of embedded biological material can be evaluated by EPXMA (the method requires a tag element in the embedding medium [14]).
2) A main goal of electron microscopic investigation of biological material is to evaluate the relations between cellular ultrastructure, properties of cellular macromolecules, and cellular ion distributions. Hence, it appears worthwhile to develop a preparation technique yielding stable preparations which can be used at will for morphological, immunocytochemical, and microanalytical studies.
3) Reproducible freeze-drying of cryosections still poses severe difficulties (e.g., non-uniform shrinkage can be seen in many published micrographs). Reproducible dehydration during freeze-drying or freeze-substitution of bulk specimens appears to be much easier. Once an optimized dehydration and embedding procedure is established, stable and permanently available preparations can be obtained in every laboratory.
4) Several microanalytical techniques require stable sections of even thickness (e.g. EELS, LAMMA, microdensitometry); for these techniques sections of plastic-embedded material are better suited than freeze-dried cryosections.
5) The interaction of "physiological" ions like H+, Na+, K+, Ca++ with cellular proteins can be studied with plastic-embedded material but not with freeze-dried cryosections (adsorption staining experiments, [10]).
6) Free and bound cellular ions may possibly be determined with plastic-embedded material but not with freeze-dried cryosections [8].

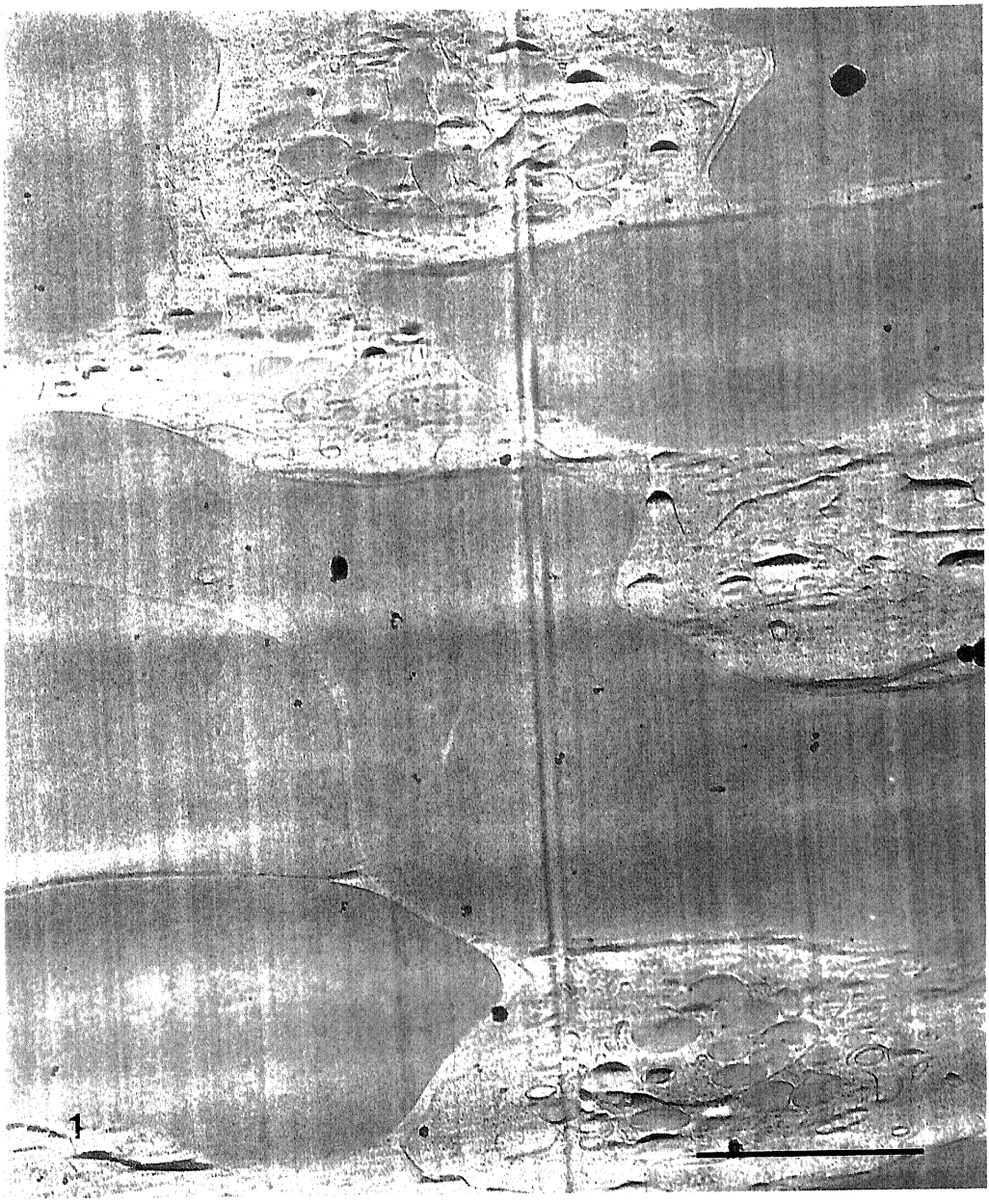

Fig.1. Frozen-hydrated cryosection of a suspension of chemically unfixed human red blood cells and blood platelets (centrifuged for 25 min at 1000g). The suspension is cryofixed as described by Sitte et al. [17] at 77K using a Reichert MM80E. The frozen specimen is glued onto a specimen carrier with liquid soap (as suggested by K. Neumann). Cryosectioning at 108K: Reichert Ultracut-S/FCS, Diatome diamond knife, Simco antistatic device, cutting speed 0.4mm/s. The section is photographed at a magnification of 7000x in a Zeiss EM902 with energy-filter. Bar=1μm.

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