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# CRYOPREPARATION OF TISSUE FOR CLINICAL APPLICATIONS OF X-RAY MICROANALYSIS

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## Abstract

A number of diseases is associated with changes in ion and/or water distribution at the tissue or cell level, and X-ray microanalysis can be used to investigate the relationship between the disease process and the changes in elemental distribution. For analysis of diffusible elements by X-ray microanalysis, the tissue has to be prepared by cryotechniques. To carry out studies of this kind in a clinical environment poses a number of problems. Some of these problems occur already before the tissue is frozen, others are caused by the practical and ethical limitations that are imposed on the freezing method itself when human tissue is to be used. The use of cryostat sections for analysis at the cellular level, and of *in vitro* systems and cell cultures in which sampling and cryopreparation can be separated in time and place can be useful alternatives.

**Key Words**: X-ray microanalysis, human pathology, ions, freezing, cryosectioning, cell cultures, *in vitro* systems

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## Introduction

X-ray microanalysis has been extensively used in the study of human diseases (Shelburne *et al.*, 1989). With regard to the preparation methods used, one can distinguish four groups of specimens: (1) specimens needing little or no preparation, other than a conductive (carbon) coating, e.g., hair, nails, gall stones and kidney stones; (2) specimens that are digested prior to analysis since only the particulate content, not the tissue itself is important; (3) specimens prepared by conventional aldehyde fixation, possibly also osmium fixation, dehydration, embedding and wet sectioning, for analysis of elements that are very tightly bound to tissue structures and where only qualitative analysis is of importance; (4) specimens prepared by cryotechniques: rapid freezing followed by sectioning, or possibly by freeze-substitution or embedding of freeze-dried tissue; bulk specimens can be analyzed in the frozen-hydrated state or freeze-dried. Even though some problems remain, quantitative microanalysis of biological specimens is virtually routine, which means that pathologically significant changes in concentrations of intrinsic elements (such as Na, Cl, K and Ca) can be demonstrated (Roomans 1980, 1981, 1990).

The most frequent application of X-ray microanalysis in pathology is the analysis of particulates in e.g., lung, either in digested specimens or *in situ* (Churg, 1989; Roggli, 1991; Abraham *et al.*, 1991). Only relatively few applications concern the localization of diffusible elements. A number of diseases is caused by defects in the regulation of ion or water transport mechanisms. The epithelial cells form the main barrier between the outside environment and the rest of the body and one of their main functions is to regulate the transport of ions and water. A recent survey (LeFurgey *et al.*, 1988) shows that many epithelia have been the subject of physiological studies by X-ray microanalysis, but as yet only two epithelial diseases, cystic fibrosis and diarrhoea, have been the subject of microanalytical

studies. Our own research has centered on transepithelial ion transport, since the congenital hereditary disease cystic fibrosis (CF) is caused by a defective regulation of chloride (and water) secretion by epithelial cells. In patients with this disease, the chloride channel in the apical membrane of the epithelial cells cannot be opened by cAMP. In the airways and the pancreas, this results in the production of viscous (water-deficient) secretion blocking the smaller airways or the pancreatic ducts, respectively. A study on hamster tracheal epithelium (Spencer and Roomans, 1989) showed that $\beta$-adrenergic stimulation resulted in a significant decrease of the cellular chloride content, indicating a net chloride efflux from the cells. This decrease in chloride content was also observed after stimulation with cAMP in normal cultured human respiratory epithelial cells, but not in respiratory epithelial cells from patients with cystic fibrosis, which confirms that chloride efflux in CF cells cannot be activated by cAMP (Sagström *et al.*, 1990b). A defective chloride transport in the sweat gland duct was demonstrated in a microprobe study by Wilson *et al.* (1988). It is unclear whether the defect in chloride transport in CF epithelial cells can also be found in other cells, such as fibroblasts. Our recent data (von Euler and Roomans, 1991) show, however, that cAMP-stimulated chloride efflux from CF fibroblasts is not significantly different from normal. Also changes in other elements than chloride have been observed in epithelial cells of CF patients such as labial gland cells (Izutsu *et al.*, 1985) and bronchial goblet cells (Roomans *et al.*, 1986). It has been attempted to produce an animal model for cystic fibrosis where the sequence of pathological tissue changes, starting from the defective transepithelial chloride transport to the full-blown clinical symptoms could be studied. This was done by inhibiting the transepithelial chloride transport by long-term treatment with diuretics (Scarlett *et al.*, 1988; Sagström *et al.*, 1990a, Mörk *et al.*, 1991). Although it could be shown that the intracellular chloride concentration decreased, which reduces or abolishes the gradient driving chloride efflux through the apical membrane, and physiological experiments confirmed that fluid transport was partially inhibited, the changes in tissue structure were relatively minor, even if treatment was continued for a period of up to 13 months, and significant CF-like symptoms could not be demonstrated.

The normal intestinal epithelium was studied by Gupta *et al.* (1978) and more recently by von Zglinicki and Roomans (1989a) and Sjöqvist and Beeuwkes (1989). The diarrhoea in, e.g., cholera is caused by a continued activation of chloride channels, because the breakdown of cAMP is inhibited by the bacterial toxin. Hence X-ray microanalysis was used to investigate the activation of chloride efflux in the different cells types constituting the epithelium. Vaso-intestinal peptide (VIP) is a known stimulator of fluid secretion in the intestine and VIP stimulation significantly decreases the chloride and potassium content of the crypt cells (von Zglinicki and Roomans, 1990). Also stimulation with isoproterenol caused a decrease of the cellular chloride concentration in crypt cells (von Zglinicki & Roomans, 1989b); this decrease could be inhibited by the adenylate cyclase inhibitor alloxan and is therefore likely to be mediated by cAMP. Absorptive diarrhoea, on the other hand, is assumed to be caused by inhibition of water influx in the villus tip cells. However, Spencer *et al.* (1990) showed that diarrhoea induced by rotavirus-infection is a very complicated process affecting both villus tip and villus base cells.

Other diseases cause secondary alterations in ion distribution at the cellular or subcellular level. Increase of cellular calcium levels and of the Na/K ratio after cardiac ischemia is well-known (Trump *et al.*, 1979; Singh *et al.*, 1983) even though the exact mechanism behind these changes has not yet been elucidated. The presence of foreign bodies in tissue is known to give rise to calcifications (Khan and Wilkinson, 1990) which can be an important clinical problem in the use of biomaterials (Pathak *et al.*, 1991).

In still another group of cases, the relationship between changes in ion distribution and disease process is not clear. Several lines of evidence indicate a relationship between the intracellular ionic environment and cellular proliferation. Cone (1971) proposed that stimulation of quiescent cells to divide was coupled to an influx of $Na^+$ ions into the cells. An abnormally high $Na^+$ concentration in cancer cells would be part of the defective regulation of proliferation in these cells. Energy-dispersive X-ray microanalysis has provided an excellent method to test this hypothesis. Cameron *et al.* (1980) compared a variety of tumor cells with their non-tumor counterparts and found large and significant increases of both Na and Cl concentrations in the tumor cells, but not of other elements. However, when a group of rapidly dividing normal (non-tumor) cells was compared to slow-growing counterparts, a smaller difference in Na and Cl was noted, but in addition, the rapidly dividing cells had higher concentrations of Mg, P and K. Significantly elevated concentrations of Mg, P and K were also found in epidermal cells in affected areas in patients with psoriasis (non-proliferating areas from the same patients were used as controls) (Grundin *et al.*, 1985). The increased level of P may due to an increased concentration of nucleic acids, that are preferably stabilized by $K^+$ and $Mg^{2+}$ ions. It seems that an increased intracellular $Na^+$ concentration can be related to mitogenesis, but the much higher $Na^+$ concentration in cancer cells suggests an even stronger link between Na

and oncogenesis (Cameron and Smith, 1989). Other systems showing elevated intracellular Na concentrations are neoplastic mouse mammary tissue (Smith *et al.*, 1981), colon cells of rats treated with the carcinogen 1,2-dimethylhydrazine (DMH) (Cameron and Smith, 1989), Morris hepatoma, as well as in regenerating liver cells, and liver cells stimulated to proliferate by treatment with tri-iodothyronine (Pieri *et al.* 1983, 1984). Increased intracellular Na/K ratios were established in a number of human tumors *in situ*: invasive urogenital cancers (Zs.-Nagy *et al.*, 1981), thyroid tumors (Zs.-Nagy *et al.*, 1983) and laryngeal tumors (Zs. Nagy *et al.*, 1987), as well as in tumors of the oral mucosa (Wroblewski *et al.*, 1983a) and human prostate neoplasms (Tvedt *et al.* 1987). Interestingly, amiloride, an inhibitor of the $Na^+$-$H^+$ antiport mechanism, not only specifically decreased the intracellular Na concentration in a number of tumors *in vivo* (a mammary adenocarcinoma and a hepatoma) but also inhibited the growth of these tumors in a dose-dependent fashion (Sparks *et al.*, 1983). Cameron and Hunter (1983) found that amiloride decreased intracellular Na in rapidly proliferating intestinal crypt cells, and Szolgai-Daniel *et al.* (1991) recently published similar effects of amiloride on a glioma cell line and on cultured colon carcinoma cells. Recently, our group found that chronic amiloride treatment results in a markedly decreased intracellular Na/K ratio in intestinal epithelial of mice treated *in utero* with amiloride, while the effect on adult animals is very small (Fig. 1) (Mörk *et al.*, 1991; von Euler *et al.*, 1992).

This brief and non-exhaustive survey shows that X-ray microanalysis can be applied to elucidate aspects of a disease process. While highly interesting work can and should be done on experimental animals, some problems may only be solved by analysis of human tissue. Localization of ions at the cellular or subcellular level is known to require the use of cryopreparative techniques, and since the use of such techniques in a clinical setting has only been carried out very infrequently, special methods have to be developed.

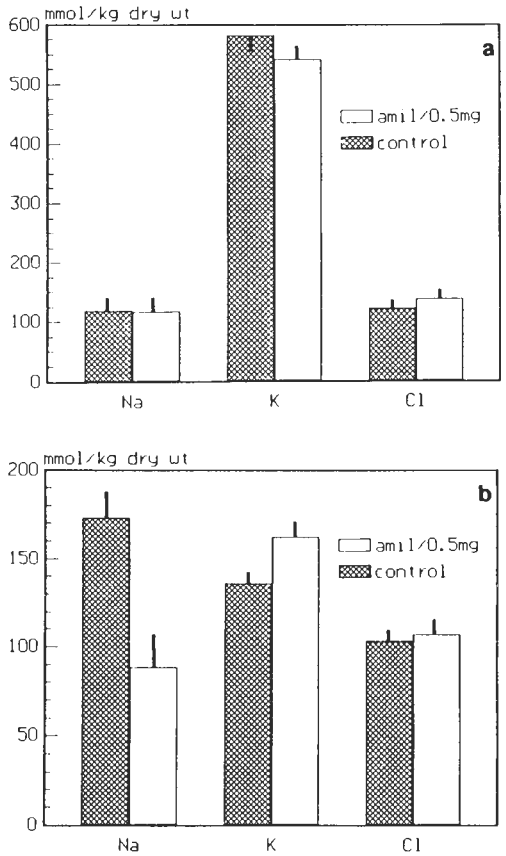



**Figure 1.** Effects of chronic treatment with amiloride on elemental content of mouse enterocytes; (a) adult animals, treated for 2 months with a daily dose of 0.5 mg/kg body weight, (b) newborn mice (day 2), of which the mothers had been treated during the entire pregnancy with the same dose of amiloride. X-ray microanalysis was carried out on 16 $\mu$m thick cryosections in a scanning electron microscope at 20 kV. In adult animals amiloride has no significant effect on any of the elements, but in newborn mice treated *in utero* a significant decrease of the Na concentration was noted. (Vertical bars indicate standard error, n=8).

## Cryopreparation of Tissue for X-ray Microanalysis

The extensive experience with freezing of animal material has resulted in consensus on the following points:

1. Freezing should be carried out as fast as possible to avoid the formation of ice crystals that may displace ions and destroy tissue structure.

2. Damage to the tissue prior to freezing should be avoided.

The occurrence and extent of ice crystal formation during freezing of biological tissue has been extensively described (Frederik and Busing, 1981; Elder *et al.*, 1982; Robards and Sleytr, 1985; Steinbrecht, 1985; Echlin, 1991). In freeze-dried tissue sections, ice crystal formation during freezing is evident from "holes" ("ice crystal imprints" or "ice crystal damage") in the tissue; it is assumed that these imprints represent the ice crystals present when the tissue was in the frozen hydrated state. It is now generally assumed that the ice crystals formed in the cells during freezing are large, branched structures, and that only one or a few ice crystals per cell are formed. The imprints observed in the freeze dried tissue would actually represent cross sections through the branches of the large ice crystals. In the outer layer of properly frozen tissue no visible ice

crystal imprints are present; imprint size increases in general with increasing distance from the specimen surface. However, there are additional factors that may affect ice crystal imprint size, such as local water content, extent of hydration water, and the degree of protein aggregation (Cameron *et al.*, 1988). Since biological tissue is a poor conductor of heat, the use of small samples is a prerequisite and even then only a small, outer layer of the sample can be frozen without visible ice crystal "damage".

In practice, "plunging" (plunging the tissue in a liquid coolant), "spraying" (spraying the specimen with a liquid coolant), and "slamming" (slamming the specimen against a cooled metal block) are used. Propane and ethane are more suitable liquid coolants than the freons, and much better than liquid nitrogen (at its boiling point), in which an thermally insulating layer surrounding the specimen develops (Bald, 1984). Except for very thin specimens, "slamming" is probably more suitable than the use of liquid coolants (Bald, 1985). The tissue is frozen against liquid nitrogen or liquid helium-cooled metal; silver is only at very low temperatures and even then only marginally better than copper (Bald, 1985). In some cases use of high-pressure freezing, which gives excellent results for morphological work (Müller and Moor, 1983) may give good results also for X-ray microanalysis (Zierold *et al.*, 1991), provided the preparation of the biological specimen prior to freezing by this method does not interfere with the cellular ion distribution.

Even in work with experimental animals which can be done under well-controlled conditions, damage to the tissue prior to freezing should be avoided. Dissecting the sample may introduce ion shifts because of anoxia (von Zglinicki *et al.*, 1986) even within time periods of a minute. Also, with small samples, loss of water by evaporation may occur. Therefore, methods for *in-situ* freezing, such as cooled clamps (Ingram and Ingram, 1983; Hagler and Buja, 1983; Warley, 1989) and cryobioptic needles (von Zglinicki *et al.*, 1986) have been developed.

If analysis at the subcellular level is required, freezing is followed by cryoultramicrotomy at very low temperature (Roomans *et al.*, 1982). The mechanism by which sections are obtained at temperatures below -100°C has been discussed extensively. The early view that cryosectioning should be performed at as low a temperature as possible was challenged by Saubermann *et al.* (1977) who advocated the use of much higher temperatures on the grounds that the process of thin sectioning at low temperature would transfer so much energy to the section that partial melting would be possible. The view of Saubermann *et al.* (1977) sprung mainly from theoretical considerations based on the "metal-machining" theory. On the other hand, Karp *et al.* (1982) showed in a practical experiment that the increase in temperature during cryosectioning around -100°C could not be more than a few degrees. Most groups hence continue to section below around -100°C whenever possible, to stay well under the recrystallization point of water in biological tissue, which most believe is in the range of -70 to -90°C. Recently, however, Kirk *et al.* (1991) showed that both "fracturing" and "cutting" could occur during preparation of thin cryosections, and that the latter process might be associated with superficial melting of the sections. The frozen sections can be transferred to the electron microscope at low temperature, and be freeze-dried in the vacuum of the microscope. Although the risk for rehydration of the cryosections and the redistribution of ions that could be a consequence of rehydration was recognized fairy early on (Roomans and Sevéus, 1976; Sevéus *et al.*, 1978; Frederik, 1982) it was difficult to prevent this artefact, as a study of micrographs of cryosections from the early days of biological X-ray microanalysis shows. According to Hagler and Buja (1986) "external freeze-drying" in a separate instrument carries a great risk of rehydration and these authors therefore strongly recommended freeze-drying in the electron microscope, after transfer at low temperature. However, von Zglinicki and Uhrik (1988) showed that with careful handling of externally freeze-dried sections artifacts could be avoided. Freeze-dried sections transferred to room air should always be slightly warmer than the surrounding air to avoid condensation of moisture on the sections and they should be carbon-coated immediately (von Zglinicki and Zierold, 1989). Thin frozen-hydrated sections cannot be analyzed because of extensive mass loss (Zierold, 1988) but analysis of frozen-hydrated sections of 1-2 $\mu$m thick is well possible (Hall, 1986; Gupta, 1989).

The main alternatives for the preparation of thin cryosections are freeze-substitution and sectioning of freeze-dried (low-temperature) embedded tissue (Wroblewski *et al.*, 1988). Comparative studies have given conflicting results. According to Roos and Barnard (1986) freeze-substitution resulted in extensive losses of diffusible elements, but their results may have been due to an unfortunate choice of substitution fluid. More careful studies by Condron and Marshall (1990) and Edelmann (1991) show losses not larger than 30% for diffusible elements.

Analysis at the cellular level can be carried out on frozen-hydrated bulk tissue (Marshall, 1988), freeze-fractured freeze-dried bulk samples (Zs.-Nagy, 1989) or freeze-dried cryostat sections (Wroblewski *et al.*, 1988; McMillan and Roomans, 1990). Such specimens are relatively easy to prepare and analyze and can also be

prepared from tissue that has not been frozen under optimal conditions. The usefulness of these specimens in the field of experimental and clinical pathology will be discussed below.

Although methods for cryopreparation of tissue *in principle* have been firmly established, the application of these established principles to analysis of human tissue in a clinical setting meets with problems.

## Problems in the Pre-Freezing Stage

The specific problems associated with obtaining human tissue for microanalytical studies can be divided into problems occurring before freezing the tissue and problems occurring during the freezing.

If tissue is to be obtained after the death of the patient, it may not be possible to control the time between death and sampling because of legal or practical reasons. Delays of several hours, up to a day, may easily occur. In such cases, post-mortem changes in the ion distribution have to be taken into account (Kuypers and Roomans, 1980; Roomans and Wroblewski, 1985): the cellular concentrations of Na, Cl and Ca will increase, those of K and Mg will decrease. No systematic studies on human tissue have as yet been carried out to investigate whether such material is still useful for X-ray microanalysis. In a pilot study of animal material it could be shown, that changes in cellular ion content induced before death (mimicking a pathological process) could be distinguished even after a post-mortem period of 24 h (Roomans and Wroblewski, 1985).

If tissue is to be obtained by biopsy, this may have to be performed under local or general anesthesia out of consideration for the patient; effects of anesthetics on ion distribution have not been investigated by X-ray microanalysis, but from what is known about the mechanism of action of several anesthetics it is evident that they may indeed affect ion and water distribution. This does not necessarily preclude the use of biopsies obtained during anesthesia: in a study on hamster tracheal epithelium we could show that a physiological process such as chloride efflux induced by $\beta$-adrenergic stimulation persisted during anesthesia (Spencer and Roomans, 1989). On the other hand, when tissue is taken without anesthesia the excitement or tension (adrenalin) might be a complicating factor.

Patients of whom the biopsy is taken may be undergoing drug treatment for the disease investigated or for unrelated diseases or conditions (e.g., contraceptives). Such treatment has to be recorded in the experimental protocol and the possible implications on ion and water distribution considered.

While it may be possible to motivate a patient to undergo a biopsy for diagnostic or research purposes, it may be difficult or unethical to obtain control material when the biopsy procedure is particularly painful or carries some clinical risk. The problem is exacerbated if special requirements such as age- or sex-matching are important.

## Problems in the Freezing Stage

As described above, a certain consensus has been obtained on freezing methods for biological X-ray microanalysis. However, when freezing human tissue it may not be possible to use *in-situ* freezing techniques. This could be because the tissue is unaccessible for an *in situ* method: it would be unethical to use open biopsy procedures, e.g., in the case of a liver biopsy. *In situ* freezing techniques also cause damage to the surrounding tissue which may be unacceptable. When freezing is to be carried out in the operating theater considerations of safety and sterility apply that may make it impossible to use particular freezing methods. The use of inflammable liquids such as propane or ethane may be prohibited, and the use of large equipment may be unpractical. Finally, a well known problem in ultrastructural pathology is that in many disease processes changes may be unevenly distributed in the tissue. In morphological studies this makes it generally necessary to start with a light microscopical investigation. Similarly, for X-ray microanalysis the use of very small samples may give erroneous results and the initial investigation of relatively large samples may be necessary.

## Alternative Methods

If the best methods for freezing and preparation can not be used, can one still use X-ray microanalysis for localization of diffusible ions in human tissue? Several ways around the problems encountered can be proposed. One possibility is to accept a sub-optimal freezing method and to adapt the preparative method to a poorly frozen specimen. Ice crystal formation in poorly frozen specimens has two consequences: (1) analysis at high resolution is not possible since the smallest volume that can analyzed should be significantly larger than the average ice-crystal size; (2) cutting of thin frozen sections from poorly frozen tissue is impossible. As alternatives to thin cryosections, thick and semi-thick cryostat sections, or freeze-dried or frozen-hydrated cryofractured bulk specimens can be used. These specimens only permit low resolution analysis (several $\mu$m) and can easily be prepared from poorly frozen tissue. Also sections of freeze-substituted or freeze-dried embedded tissue can be used. This type of specimen can also be obtained from non-optimally frozen tissue (Roomans *et al.*, 1986; Wroblewski and Wróblewski,

1986), and the resolution of analysis can be adapted to the ice crystal size. The second possibility is to separate the removal of the tissue in time (and space) from the freezing of the sample. This can be done by incubation of the tissue *in vitro* or by using primary tissue cultures or cell cultures.

**Analysis at the Cellular Level**

Preparation and analysis of thick (Wróblewski *et al.*, 1978, 1987; McMillan and Roomans, 1990) and semi-thick (Wroblewski *et al.*, 1983b) sections specifically for clinical work allows the analysis of rather large pieces of tissues if necessary. In this method, the tissue can be frozen in liquid propane or with an "*in situ*" method, but even the use of liquid nitrogen gives acceptable, though especially with regard to morphology, inferior results. The tissue is sectioned at -20° to -30°C (preferably at the lower temperature) in a conventional cryostat. For analysis at 20 kV in a scanning microscope or in a transmission microscope with a scanning attachment, 16 $\mu$m thick sections are needed to ensure that the analytical volume is contained within the cell. If a lower accelerating voltage is used, sections can be thinner. Sections are pressed in the cryostat with a brass weight onto a specimen support, in our case a Formvar film-covered graphite plate, and freeze-dried (McMillan and Roomans, 1990). Successfully cryosectioned and freeze-dried sections have a white color; accidental melting or rehydration of the section gives rise to a grey color; these sections have very poor morphology and often display low elemental concentrations (Fig 2a,b). The sections should be coated with carbon and can then, in our experience, be stored for at least several weeks in a desiccator without changes in the analytical results. Quantitative analysis of thick cryostat sections gives results that are well comparable with those obtained on thin cryosections of the same tissue, as shown by Forslind *et al.* (1985) for skin.

For relatively small cells it is better to prepare semi-thick (2-6 $\mu$m) cryosections. Instead of a massive graphite plate, a graphite plate with a hole covered by a Formvar-film is used (Wroblewski *et al.*, 1983b, 1988). The sections are mounted over the hole, and freeze-dried as described above for thick sections. Analysis is carried out in the scanning transmission mode at high accelerating voltage. Semi-thick sections have the advantage of a somewhat higher resolution and, because of the higher accelerating voltage that can be used, a better peak-to-background ratio.

The analysis of (semi-)thick cryosections has been used in various fields of pathology, e.g., muscle pathology (Wróblewski and Edström, 1989), dermatology (Forslind, 1989), inner ear pathology (Anniko and Wróblewski, 1989), and neuropathology (Wróblewski, 1989). Similarly, the use of cryofractured, freeze dried bulk specimens (Zs.-Nagy *et al.*, 1977) in cancer research has been reviewed by Zs.-Nagy (1989).

***In vitro* Systems**

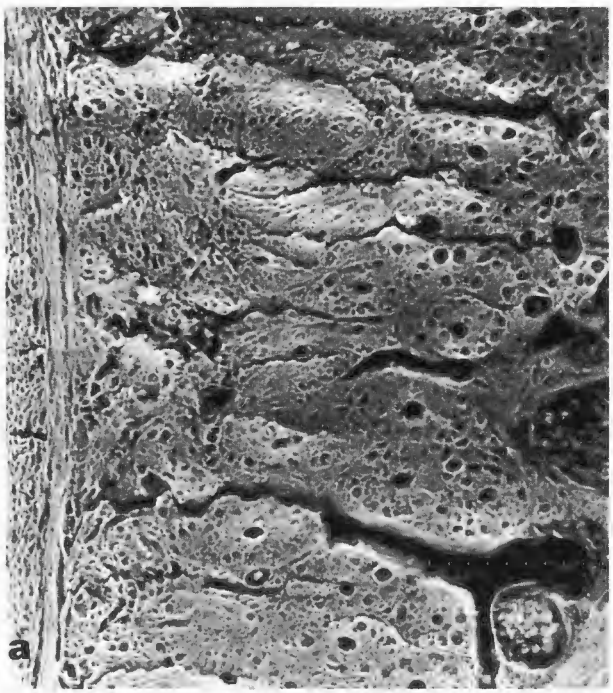
It may be possible that the use of *in vitro* systems, where the tissue can recover from the trauma of the biopsy can alleviate some of the problems described above. Use of an *in vitro* system uncouples the biopsy procedure and the freezing procedure both in time and in place and would allow the use of the most sophisticated freezing techniques in the laboratory without any of the restrictions imposed in a clinical environment. *In vitro* systems are well developed for a variety of tissues, e.g., muscle, where they allow X-ray microanalytical data to be correlated with measurements of other properties of the tissue (Ingram *et al.*, 1989; Wendt-Galiitelli and Isenberg, 1989). In addition, *in vitro* systems allow the investigator to carry out experiments (e.g., to expose the tissue to hormones, neurotransmittors, or toxins) in a way that would not be possible in *in situ* experiments.

Isolated cells that are used without subsequent culturing should be considered as a special sub-group of the *in vitro* systems. Warley (1986, 1989) showed that many factors in the preparation procedure, such as centrifugation speed and suspending medium were of great significance for the elemental composition and the viability of the cells.

A very intriguing question is whether the elemental composition and the subcellular ion distribution in such *in vitro* preparations are identical with the *in situ* situation. Our experiments with epithelial cells (von Euler *et al.*, 1983; Müller *et al.*, 1985; Roomans *et al.*, 1989) indicate that although the elemental composition of incubated tissue is not the same as that of tissue *in situ*, physiological processes such as ion shifts occurring during e.g., secretory stimulation, can be measured (Fig. 3). However, apart from the work of Warley (1986) on thymocytes the problem has not been very systematically studied and very little attention has been given to possible improvements of the incubation procedure.

**Cell Cultures**

Both primary and permanent cell cultures can be used to study ion distribution. Again, the biopsy procedure and the freezing procedure are separated in place and time which creates the possibility for both to be carried out in an optimal way. Also in cultured cells X-ray microanalysis can be combined with other methods of investigation (Hagler *et al.*, 1989). Methods for the preparation of cell cultures and single cell systems for X-ray microanalysis have been reviewed by Wroblewski and Roomans (1984) and Warley (1987): in particular in the latter paper, problems that may occur in some of the preparative steps are carefully considered.



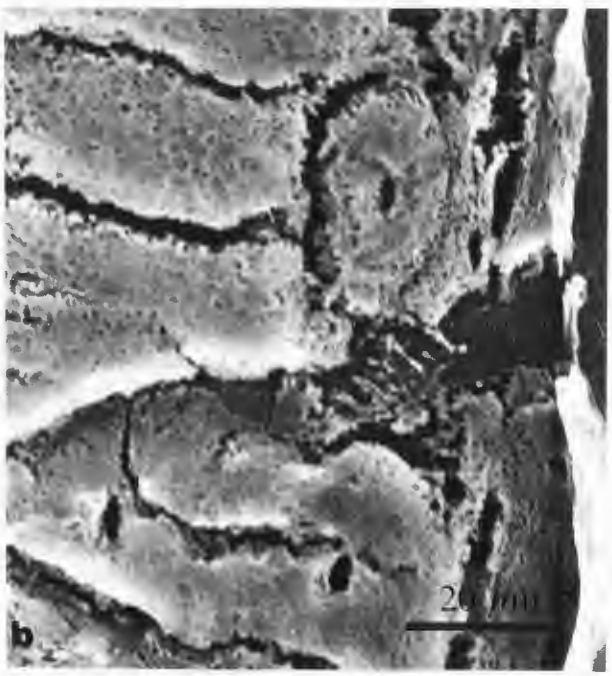




**Figure 2.** Thick cryosections of mouse intestine as viewed in the scanning electron microscope. (a) Correctly freeze-dried section in which tissue structure can be easily seen, (b) (partially) melted section (McMillan and Roomans, 1990).

Analysis at the subcellular level may be carried out on cryosections of cultured cells. Although freezing of

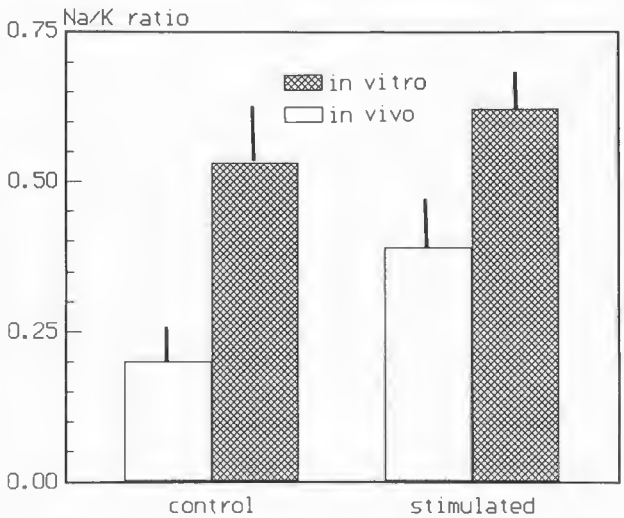



**Figure 3.** Effect of maximal cholinergic stimulation on the Na/K ratio in rat submandibular gland acinar cells. The effect of cholinergic stimulation *in vitro* is qualitatively the same as that *in vivo* but quantitatively much less pronounced. Figure based on data presented in Roomans *et al.* (1989). (Vertical bars indicate standard error, n=8).

cell monolayers gives good results, the subsequent handling of the specimens for cryosectioning requires some dexterity. Special methods to freeze cell monolayers so that ultrathin cryosections can be prepared have been described by Zierold *et al.* (1984) and Tvedt *et al.* (1988). In the method of Tvedt *et al.* (1988) the cell layer is covered by a layer of a polyvinylpyrrollidone (PVP) solution containing ions, which supports the cells and makes the monolayer easier to handle; in addition the PVP layer can be used as a standard for quantitative analysis.

If only analysis at the cellular level is required, freeze-dried whole mounts of cultured cells can be used. This requires that the culture medium is completely removed before freezing and replaced by a medium that does not contain any elements interfering with the analysis. Of course, the rinsing procedure should not affect the ionic composition of the cells. Among rinsing fluids commonly used are distilled water (which some cells actually withstand during a short period), and isotonic ammonium acetate (a volatile buffer) or isotonic solutions of macromolecules (sucrose, mannitol). Rinsing with sucrose or mannitol can be considered safe from a physiological point of view, but these substances may leave remnants after freeze-drying that may be difficult to remove and that may interfere with the analysis. Therefore, an attempt should always be made to use water or a volatile buffer as rinsing fluid. Ideally, the results obtained with different rinsing fluids should

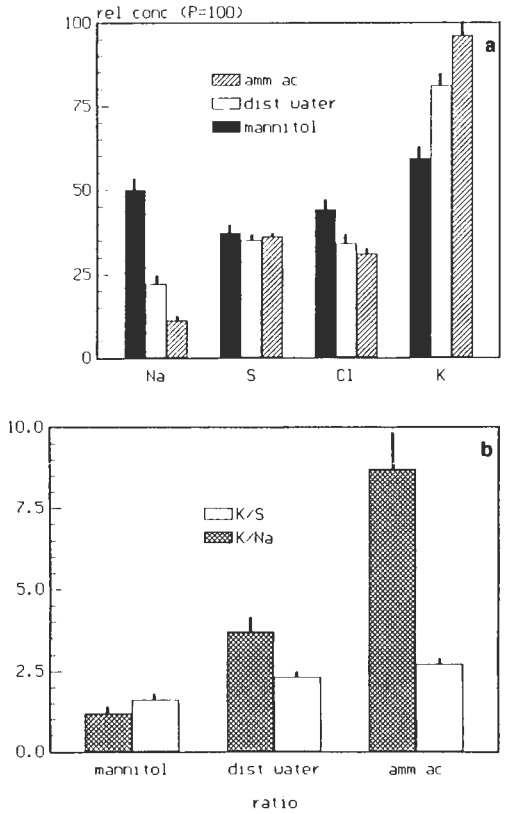



**Figure 4.** Effect of the rinsing procedure (rinsing with 0.3 M mannitol, 0.15 M ammonium acetate, or distilled water, respectively) on the elemental composition of human fibroblasts. The cells were cultured and analyzed on Nucleopore filters, hence the data were expressed as relative concentrations; (a) data expressed relative to phosphorus, (b) K/Na ratio and K/S ratio for the different treatments. It can be seen that mannitol rinsing gives a low K/Na ratio, indicating that the culture medium has not been totally removed; the best rinsing is obtained with ammonium acetate. Ammonium acetate also gives the higher K/S ratio indicating minimal leakage of diffusible ions. The data in (a) show that the sulfur content is not affected by the rinsing procedure. (Vertical bars indicate standard error, n=10).

be compared with those obtained on thin cryosections. If this cannot be done, the K/Na ratio and the K/S ratio can be used to compare different rinsing techniques. The rationale for selecting these ratios is that if the rinsing does not remove the extracellular medium (that mainly contains Na and Cl) effectively, a low K/Na ratio will be found. Since the S content of the cell is mainly due to the cell proteins that will not be affected by the rinsing procedure, a low K/S ratio is indicative of leakage of diffusible ions (Fig. 4). In addition to these criteria, observation of cell morphology under the electron microscope may give useful information. While these criteria can be used to select the best of a number of rinsing methods, they provide no absolute guarantee that no changes have been caused even by the best method.

Cells may be cultured on a solid substrate or on a thin film. If cells are grown and analyzed on a solid substrate, the beam will generally completely penetrate the cells and excite the underlying substrate, which makes fully quantitative analysis impossible. Semi-quantitative analysis can be carried out using elemental ratios (e.g., normalization against phosphorus or sulfur, Na/K ratios), which sometimes in an indirect way (e.g., assuming that the electrolyte content is constant) can be converted into absolute values. This technique has been used by Lechene and coworkers in a number of studies (reviewed by Lechene, 1989). If cells can be made to grow on a thin substrate, e.g., titanium grids covered with a Formvar film, this makes fully quantitative analysis possible. In addition, since analysis can be carried out in the (scanning) transmission mode at high accelerating voltage (100 kV), the sensitivity of the analysis is improved (von Euler and Roomans, 1991).

While cultured cells may not have exactly the same composition as their *in situ* counterparts, and, in particular with primary cultures, aging has to be considered very carefully, physiological processes at the cell level can be investigated as well in cell cultures as in tissue *in situ*; in fact, use of cell cultures gives the investigator a much larger freedom in choosing experimental conditions.

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## Discussion with Reviewers

**B. Forslind**: Propane and ethane are efficient lipid solvents. Could you comment on the effects on cell membranes that can be expected before, during, and after the freezing process? In this context a comparison with the effects of local anesthetics would be interesting.
**Author**: While it is true that propane and ethane are efficient lipid solvents at room temperature, the solubility of lipids in propane and ethane will decrease markedly at the temperatures used for freezing, close to -190°C. Evidently, samples should not be exposed to propane or ethane at room temperature prior to freezing. Hence, there should be no reason to suspect an effect of these coolants before or during freezing. During sectioning (or freeze-drying or freeze-substitution) temperatures are higher and some loss of membrane lipids might theoretically be possible, although as far as I am aware no solid data exist on this point. However, the water in the cell remains frozen and the ions insolubilized; in a frozen cell the structural integrity of the cell membrane is of no consequence for ion distribution.

The situation is of course entirely different for local anesthetics that affect membrane lipids, because the tissue is exposed to the anesthetic at room temperature and in a situation where the cell membrane still is regulating ion transport and hence has to be structurally intact.

**B. Forslind**: A consequence of your discussion of X-ray microanalysis at subcellular resolution is that we may assume that the diffusion effects during preparation seen at analysis of bulk specimens are comparatively restricted. This means that subcellular changes in elemental content cancel out due to the large volumes analyzed in bulk specimens. For this approximation to hold what linear relations should exist between the electron beam cross section and the cell cross section in the area of analysis?
**Author**: The spatial resolution of analysis in bulk specimens is not so much determined by the diameter of the beam but by the scattering of the electrons within the

specimen, which is a function of the accelerating voltage and specimen density. Already in early studies of bulk specimen analysis (see e.g., Zs. Nagy *et al.*, 1977, Wróblewski *et al.*, 1978) beam penetration was determined experimentally. For graphs describing the (non-linear) relationship between accelerating voltage and spatial resolution, see e.g., Roomans (1981, 1990).

**L. Edelmann**: You discussed the problem of replacing the extracellular medium of cultured cells with a medium which does not contain elements interfering with the analysis. Did someone consider the possibility of removing the extracellular fluid by centrifuging (see Ling and Walton, 1975)?
**Author**: I doubt that centrifugation would remove all of the extracellular fluid; small remnants can be important if the sample is small in relation to the extracellular fluid, e.g., a monolayer of cells . In addition, the work of Warley (1987) has shown that centrifugation itself may cause changes in elemental content. However, if no suitable rinsing method can be found for a particular system, it might be worthwhile to investigate this avenue.

**T. von Zglinicki**: In general, non-optimally frozen tissue has to be used due to the rather large sample size for analysis at cellular resolution. Slow freezing disrupts cellular membranes and will lead to redistribution of ions between intra- and extracellular spaces at least in a rim along the plasma membrane with a width in the order of the diameters of the ice-crystal branches. Do you have indications whether this redistribution zone is much larger than that, maybe even comparable to typical cell sizes, or not?
**Author**: Even for analysis at cellular resolution small samples can be used, and our data indicate that slow freezing can introduce noticeable redistribution even at the cellular level (McMillan and Roomans, 1990). However, such redistribution at the cellular level introduced by slow freezing may still be much smaller than changes introduced by pathological processes (Roomans and Wroblewski, 1985). I have no doubt that slow freezing causes changes in ion distribution at the subcellular level.

**T. von Zglinicki**: To study clinically relevant changes in ion distribution you suggest several methods which are known to induce such changes themselves. In my eyes this is a dangerous procedure, because the underlying assumption, namely that the preparation produces the same artefacts in the control group and in the group(s) under study, might be completely wrong. If this situation cannot be avoided, different preparation schedules should always be used and the results compared before interpreting the data. Would you agree on that?
**Author**: There is no general answer to this question. The tendency to increasingly use *in vitro* systems and cell cultures instead of *in situ* systems is not specific for microanalytical investigations but is spread throughout the spectrum of biomedical disciplines. Use of *in vitro* systems and cell cultures allows a greater range of physiological experiments to be carried out, minimizes the use of animals, and minimizes the need for experimental animals and humans to be exposed to potentially harmful or unpleasant experiments. It has also become quite clear that only relatively few problems can be attacked with the "golden standard" of specimens, i.e., thin cryosections of *in situ* frozen tissue. Of course, a number of questions have to be answered: Is the *in vitro* system physiologically and morphologically relevant to the biological problem investigated? What are the differences between the *in vitro* system and the *in situ* system? Does the specimen preparation procedure chosen allow relevant results to be obtained? I agree with you that, indeed, whenever feasible, parallel approaches have to be compared to check for any systematic errors.

**L. Edelmann**: Could you provide a conclusion stating the most appropriate techniques, suggestions for optimum equipment and trends of future investigations?
**Author**: Such a general conclusion would be difficult to give in a way acceptable to all. As stated in the answer to the previous question, I believe that increased use of *in vitro* systems and cell cultures will extend the possible applications of X-ray microanalysis to biomedical problems. Although there is a general agreement on the "golden standard" of specimen preparation a dogmatic adherence to thin cryosections as exclusive preparation method would severely limit the number of problems to which microanalysis can be applied, as becomes clear from the literature. Methods and equipment will have to be selected in accordance with the problems; I do not believe that a single method or piece of equipment can solve all problems to which X-ray microanalysis can be applied.

## Additional Reference

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