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PREPARATION OF CULTURED AND ISOLATED CELLS FOR X-RAY MICROANALYSIS

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Abstract

Various electron microscopical preparation techniques are compared with regard to the preservation of the intracellular element distribution as determined by X-ray microanalysis in scanning and scanning transmission electron microscopy. By use of chemical agents for fixation and dehydration ions are redistributed and washed out. This is also true for freeze-substitution. Whole cells are prepared by cryofixation followed by freeze-drying. Interference of intracellular measurements by extracellular elements can be avoided by appropriate washing the cells before cryofixation. The washing medium has to be carefully selected in order to avoid distortions of the original intracellular element content. These problems are circumvented by the preparation of freeze-dried cryosections from cryofixed cells. This is demonstrated by data of the intracellular elemental composition in cultured cells (fibroblasts, Staphylococcus aureus bacteria) and in cells isolated from rat tissue (kidney papillary collecting duct and liver). Cryofixation of a single cell in a defined functional state is illustrated by results obtained from streaming Amoeba proteus cells, cryofixed under light microscopical control.

The main conclusion is that X-ray microanalysis of cells in functional states requires cryofixation and cryopreparation techniques which have to be adapted to the particular cell biological problem to be investigated.

Key Words: cell, cryofixation, cryopreparation, cryosection, electron probe microanalysis, intracellular ions, X-ray microanalysis.

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Introduction

Measurements of the element content in biological cells and in intracellular compartments are useful in many studies of the physiology of cells and tissues (Moreton, 1981; Hall, 1986; Zierold and Steinbrecht, 1987; LeFurgey et al., 1988). For example, the activity of ion transport systems in membranes can be determined by measuring the ion concentration in the membrane-bounded compartment by X-ray microanalysis as depending on various functional parameters such as the extracellular concentration of ions, presence of inhibitors, or drugs. Other examples are the storage and depletion of specific elements in granules or in other intracellular structures, vesicular transport, and cytotic processes. The use of electron probe microanalytical methods in such investigations is justified only, if the distribution of elements in cells or intracellular compartments is the same in the electron microscopical specimen as in the cells in the functional state of interest. In other words: Preparation must not change biology. This condition is very hard to accomplish and in its strict sense it is even self-contradictory. This very crucial problem in the application of electron probe microanalysis in biology is discussed in the literature by several authors. For review see, for example, Morgan et al., 1978; Morgan, 1980; Chandler, 1985. It is reduced, however, by the limited spatial resolution as depending on probe size and specimen type. The analytical spatial resolution in a whole mount specimen studied in a scanning electron microscope (SEM) is in the range of 2 µm, depending on the acceleration voltage used. 100 nm thick freeze-dried cryosections in a 100 kV scanning transmission electron microscope (STEM) can be studied with a lateral analytical resolution of 30 nm. Thus, any preparation artefacts, including the displacement of ions, have to be limited to volumes with a diameter smaller than the achieved analytical resolution.

In the following paper X-ray microanalytical methods and preparation techniques are described and compared with respect to their capability to preserve the element distribution in cells in defined physiological states. The effect of chemical agents as used in chemical fixation and

freeze substitution is shown by X-ray spectra obtained from cultured fibroblast cells in SEM. X-ray microanalysis of cryosections in STEM provides the most reliable and detailed information on the element distribution in cells. This is demonstrated by experiments with fibroblast cells and Staphylococcus aureus bacteria. The problems involved in the preparation of tissue cells and their isolation are shown by experiments with rat kidney and rat liver cells. The importance of cryofixation of single cells under light microscopical control is illustrated by microanalysis of whole cells and cryosections of Amoeba proteus. Finally, the reported results are discussed with respect to electron microscopic preparation methods, viability criteria for cells, and the problems involved with the microanalysis of cells in defined functional states.

X-ray microanalytical methods

Whole cells were analyzed by an EDAX energy dispersive X-ray microanalyser in the ETEC Autoscan scanning electron microscope as described by Zierold (1981). The accelerating voltage of the primary electron beam was 12.5 kV, the probe current was about 0.5 nA. Microanalysis of freeze-dried cryosections was carried out by means of an energy dispersive X-ray microanalysis system (USC nuclear semiconductor detector combined with a Link multichannel analyzer) in a Siemens Elmiskop ST 100 F scanning transmission electron microscope equipped with a cold stage. During analysis, the cryosections were kept at a temperature of 138 K. X-rays were generated in the specimen by a 100 kV electron beam of 4 nA. Dry weight element concentrations were evaluated from the X-ray spectra by means of the Link Quantem FLS program based on the peak-to-background continuum method (Hall and Gupta, 1982). For calibration standard specimens were prepared from mixtures consisting of 20 % dextran and 80 % electrolyte solution of known concentration. The dry weight portions in the sections were estimated by measuring the relative darkfield intensity in STEM. Details of quantitative evaluation are described by Zierold (1986a).

Fixation and dehydration methods

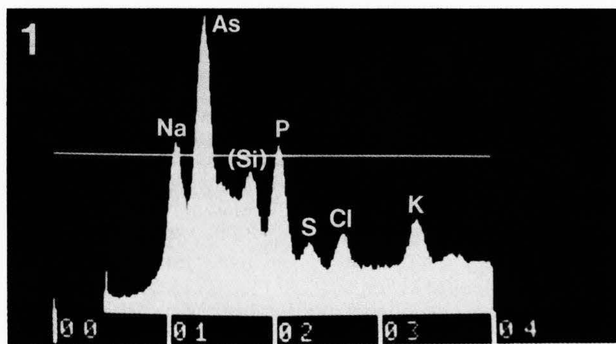
As water is inconsistent with the vacuum necessary for electron microscopy, cells have to be studied either in the frozen-hydrated or in the dehydrated state. Frozen-hydrated specimens, however, cannot be used for X-ray microanalysis because of weak electron optical contrast, high radiation damage in thin-sections, and electrical charging in thick specimens (Zierold, 1983). Concerning X-ray microanalysis of frozen-hydrated specimens the reader is referred to Gupta and Hall (1981), Marshall (1980a, 1987), Zierold (1983).

Various dehydration methods were tested by the use of L929 fibroblast cells grown on plastic coverslips. After dehydration, the specimens were coated by carbon. For electron microscopy of cells macromolecular structures are usually stabilized by chemical fixation with aldehydes and then dehydrated by an ascending alcohol series. In this case we used ethanol. Fig. 1 shows a typical X-ray spectrum of a cultured fibroblast cell after fixation with 3 % glutaraldehyde in cacodylate buffer for 10 min and air drying. Most of the potassium is lost. The arsenic peak is caused by the cacodylate buffer. Glutaraldehyde fixation followed by ethanol dehydration reduces the potassium content even more and adds calcium to the cell, probably an artefact by the drying agent of the ethanol (Fig. 2). The X-ray spectra in Figs. 1 and 2 illustrate, that chemical fixation and dehydration methods are not useful to localize diffusible elements in cells.

The alternative is cryofixation of the cells, which means solidification of the cells by rapid freezing to a temperature below 170 K. Cryofixation methods have been reviewed extensively by Plattner and Bachmann (1982), Robards and Sleytr (1985), Sitte et al. (1987). We froze the cells grown on Thermanox plastic chips by propane jet freezing or by plunging into liquid propane (Zierold, 1981). The following dehydration was made either by freeze-substitution or freeze-drying (Zierold and Schäfer, 1983).

Freeze-substitution means the exchange of ice against an organic solvent at low temperature. We used the freeze-substitution program as recommended by Müller et al. (1980a): The substitution medium consisted of methanol containing 0.5 % uranyl acetate, 1 % OsO_4 and 3 % glutaraldehyde. The frozen cells were exposed to this medium for 8 h at 178 K, 8 h at 213 K, and 8 h at 243 K. Then, the specimens were warmed up to room temperature and air-dried. In order to find out the effect of freeze-substitution alone on the intracellular element content embedding in plastic was omitted. The dry specimens were coated by carbon and analyzed in SEM. Fig. 3 shows the result: Sulfur and chlorine are almost completely lost from the cells. The high uranium peak due to the used uranyl acetate covers up

Figs. 1-8: X-ray spectra obtained from cultured fibroblast cells in SEM after different preparation methods. The horizontal scale indicates X-ray energy (keV), the full width of the vertical scale is 2500 counts. Brackets mark extraneous X-ray peaks which are not generated in the cells.
Fig. 1: Chemical fixation by 3% glutaraldehyde, air drying.
Fig. 2: Chemical fixation by 3% glutaraldehyde followed by dehydration in an ascending series of ethanol, air drying.
Fig. 3: Cryofixation, freeze-substitution by methanol with 1% OsO_4, 3% glutaraldehyde, 0.5% uranyl acetate, as described in the text.
Fig. 4: Cryofixation, freeze-substitution by methanol.
Fig. 5: Cryofixation, freeze-substitution by the same medium as described in Fig. 3, without uranyl acetate.
Fig. 6: Cryofixation, freeze-substitution by the same medium as described in Fig. 3, without OsO_4.
Fig. 7: Cryofixation of the cell within the culture medium, freeze-drying.
Fig. 8: Cryofixation after washing the cells with 300 mMol/l ammonium acetate, freeze-drying.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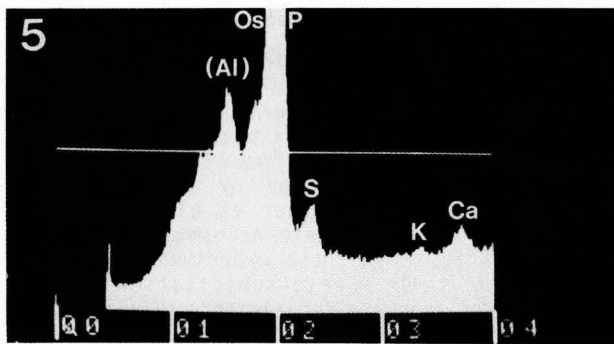
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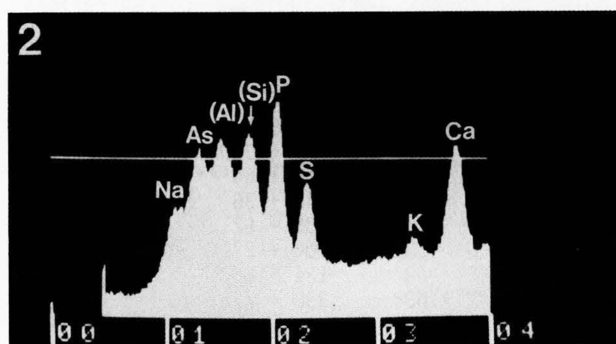
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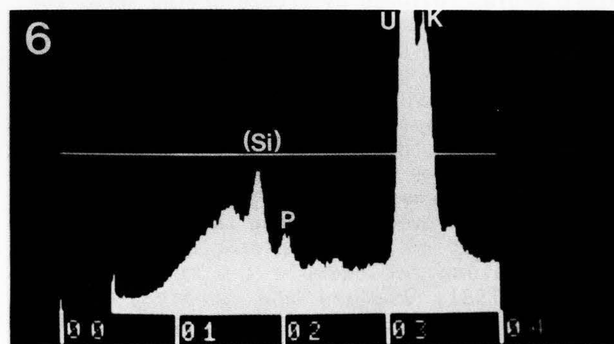
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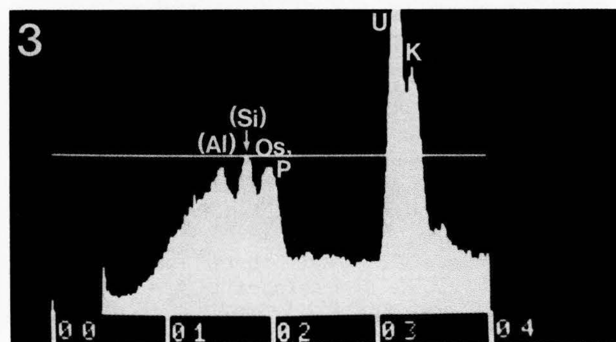
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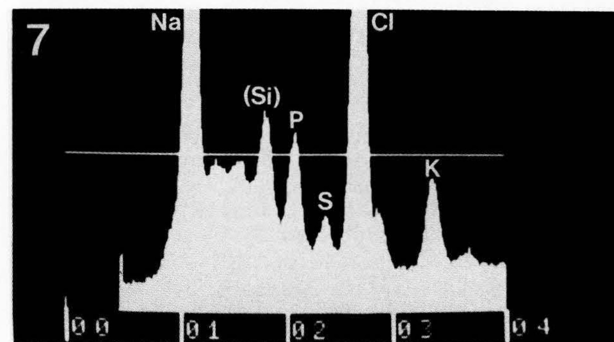
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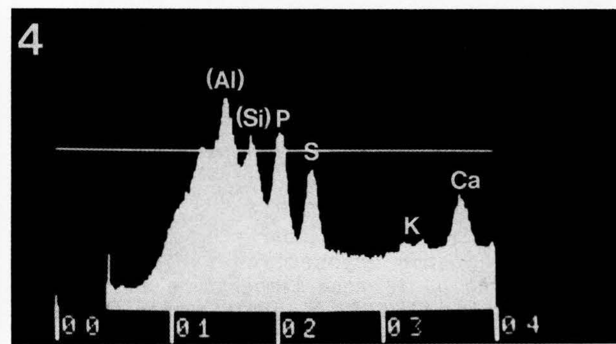
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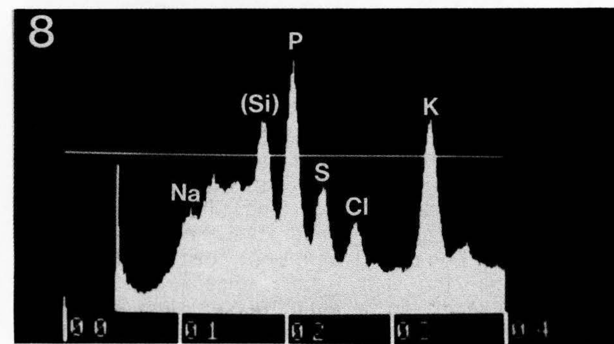
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potassium. Therefore, the cells were freeze-substituted according to the same temperature schedule by pure methanol. The resulting X-ray spectrum (Fig. 4) proves that methanol dissolves almost all potassium and chlorine. The additional calcium is an artefact similar to that in Fig. 2. The X-ray spectra in Figs. 5 and 6 were obtained from cells freeze-substituted by the original medium recommended by Müller et al. (1980a), without either uranyl acetate or osmium tetroxide, respectively. From these experiments it can be concluded that freeze-substitution with methanol is not adequate for element localization in cells. Similar observations were made by using acetone instead of methanol. The problem of element localization after freeze-substitution will be treated below in the discussion.

Cryofixation of the cells within their culture medium followed by freeze-drying seems to be the preparation method of choice for X-ray microanalytical element localization. Unfortunately, X-ray spectra from whole cells prepared in this way contain peaks of intracellular and extracellular elements, as the extracellular elements, mainly sodium and chlorine, precipitate on the cell surface after freeze-drying (Fig. 7). In some cases the interfering extracellular medium can be removed by washing the cells with a volatile buffer of the same osmolarity as the culture medium, for example ammonium acetate (Zierold, 1981; Zierold and Schäfer, 1983; Zierold et al., 1979). This is demonstrated by fibroblast cells in culture (Figs. 8 and 9). However, this washing step may disturb the ion composition in cells, as will be discussed below. Because of these uncertainties, alterations of the element distribution in cells caused by physiological experiments or by handling before cryofixation were studied on cryosections.

Cultured cells in suspension

One advantage of cryosections for X-ray microanalysis in comparison to whole cells is that the extracellular medium does not interfere with the measurement of the intracellular element content. This is demonstrated by L929 fibroblast cells in suspension. Small droplets were frozen between two copper foils, as described in detail previously (Zierold, 1986b). From the frozen droplets 100 nm thick cryosections were cut by a dry glass knife in a Reichert FC4-Ultracut cryoultramicrotome at a temperature below 170 K. The sections were freeze-dried and cryotransferred to the Siemens Elmiskop ST 100F STEM. For details on analysis conditions and data evaluation the reader is referred to Zierold (1986 a,b). Fig. 10 shows cryosectioned fibroblast cells. Major compartments such as the nucleus, cytoplasm, and mitochondria can be clearly identified. Data on the element distribution in these compartments were reported previously (Zierold, 1986a). In Table 1 data on the cytoplasmic element composition and the dry weight portion obtained from three different preparations of this cell line are compiled. The data coincide with each other, except for sodium in one case and, to a much lesser degree, for potassium in another case. These deviations might be caused by handling differences immediately before cryofixation, such as temperature changes or alterations in the extracellular osmolarity after centrifugation. In general, however, the coincidence of the data demonstrates the reliability of the preparation technique.

Table 1

Element content in the cytoplasm of fibroblast cells (L929) in suspension as found in three different preparations (I, II, III) in mMol/kg dry weight ± standard deviation. n = number of cells, d = dry weight portion. * denotes a significant difference to the K value in experiment III with the error probability of 0.05. *** denotes a significant difference to the Na value in experiment I with the error probability of 0.001. Horizontal bars indicate concentrations below the detection limit.

	I	II	III
n	10	8	10
d	0.17 ± 0.03	0.16 ± 0.02	0.18 ± 0.03
Na	61 ± 11	115 ± 64***	81 ± 25
Mg	68 ± 21	86 ± 36	61 ± 22
P	360 ± 83	385 ± 62	348 ± 47
S	176 ± 28	200 ± 27	199 ± 29
Cl	124 ± 60	131 ± 56	113 ± 16
K	372 ± 83*	338 ± 75	292 ± 37
Ca	-	-	-

This statement is corroborated by X-ray microanalysis of cryosections from *Staphylococcus aureus* bacteria. Before cryofixation, these cells were kept either in isotonic culture medium or in hypertonic medium containing 10 % NaCl. Cryosectioned bacterial cells appear as homogeneous electron dense discs without intracellular structure (Fig. 11). The intracellular element content coincides in both cell preparations,

Fig. 9: Cultured fibroblast cells, washed with ammonium acetate, cryofixed, and freeze-dried in SEM. bar = 10 µm.

Fig. 10: Cryosection of fibroblast cells in suspension in STEM. C = cytoplasm, M = mitochondria, N = nucleus. bar = 2.5 µm.

Fig. 11: Cryosection of *Staphylococcus aureus* cells in suspension, STEM. bar = 500 nm.

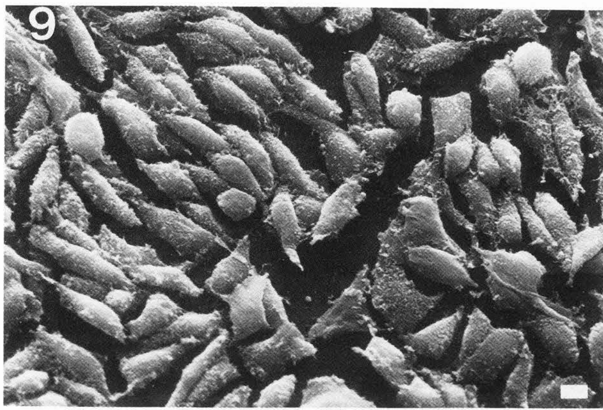
Fig. 12: Cryosection of papillary collecting duct tubules, isolated from rat kidney, STEM. C = cytoplasm, M = mitochondria, N = nucleus. bar = 2.5 µm.

Fig. 13: Cryosection of rat liver tissue, STEM. C = cytoplasm, ER = endoplasmic reticulum, M = mitochondria, N = nucleus. bar = 2.5 µm.

Fig. 14: Cryosection of an isolated rat liver cell in suspension, STEM. C = cytoplasm, M = mitochondria, N = nucleus. bar = 2.5 µm.

Fig. 15: Cryosection of rat liver cells cultured on gas permeable foil, STEM. C = cytoplasm, M = mitochondria, N = nucleus. bar = 1 µm.

Fig. 16: The same cryosection as in Fig. 15, after warming up to room temperature and 30 min storage in air, STEM. C, M, and N denote the same structures as in Fig. 15. bar = 1 µm.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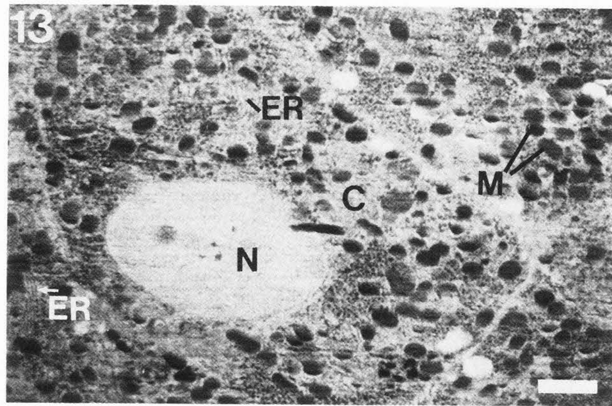
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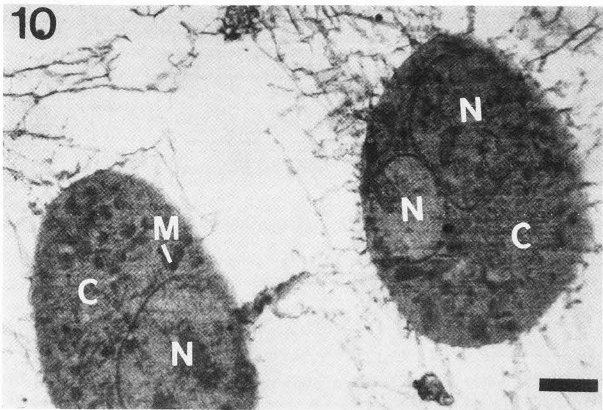
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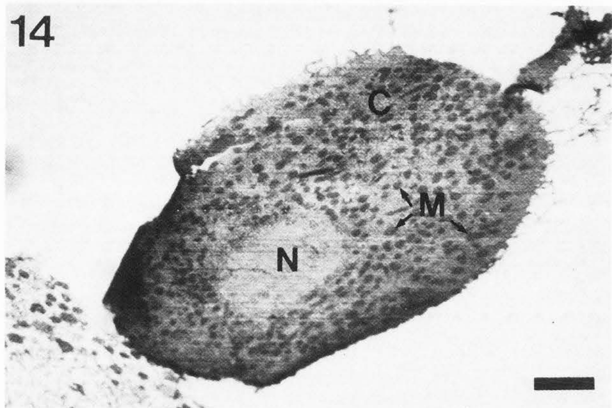
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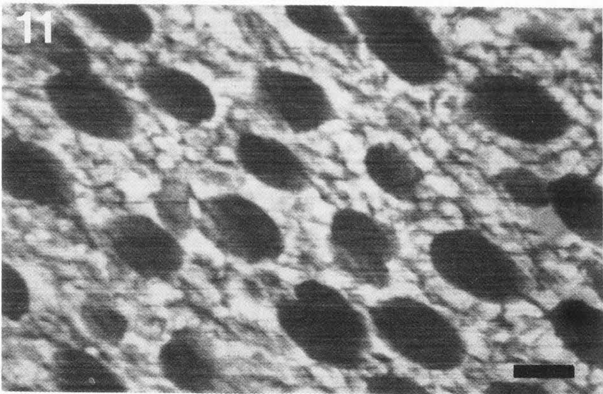
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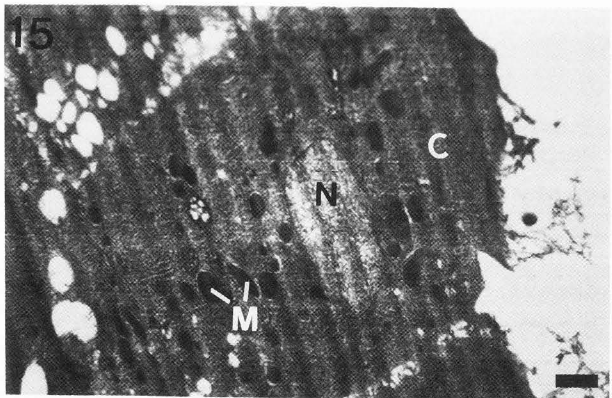
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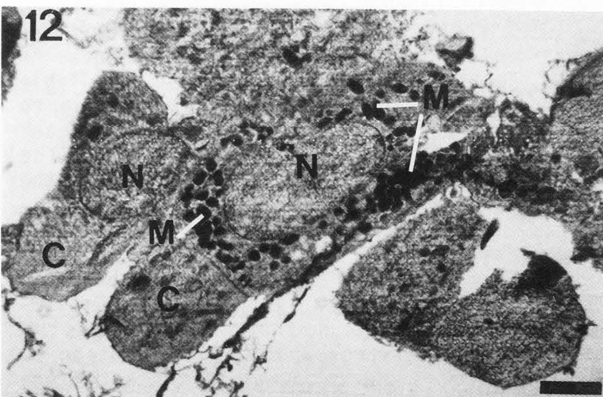
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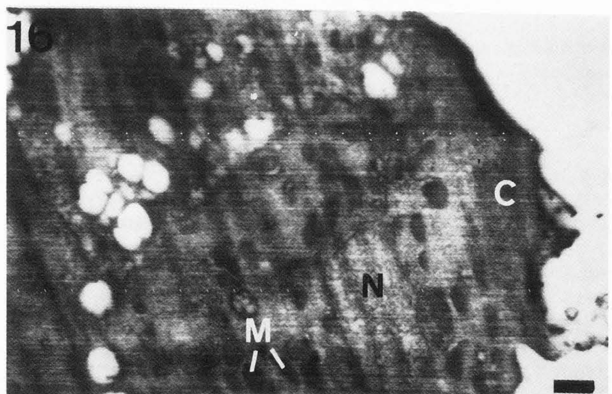
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except for sodium and chlorine, because the cells obviously do not tolerate such high extracellular salt concentration (Table 2). Furthermore, the high standard deviation for sodium, chlorine, and potassium found in the cell preparation exposed to the NaCl-rich medium indicates that not all cells react in the same way. Cells with much sodium and chlorine contain a low amount of potassium and vice versa. Such detailed information on intercellular variations can be achieved only by measuring the element content in single cells. A more detailed analysis of the data compiled in Table 2 will be given in the discussion part below (Table 8).

Table 2

Element content in Staphylococcus aureus cells in isotonic and NaCl-rich medium in mMol/kg dry weight ± standard deviation. n = number of cells, d = dry weight portion. *** denotes a significant difference with the error probability of 0.001. Horizontal bars indicate concentrations below the detection limit.

	Isotonic medium	Isotonic medium + 10 % NaCl
n	20	20
d	0.15 ± 0.03	0.15 ± 0.03
Na	326 ± 74	651 ± 317***
Mg	94 ± 19	100 ± 28
P	383 ± 52	413 ± 39
S	101 ± 12	109 ± 36
Cl	284 ± 61	952 ± 360***
K	381 ± 76	397 ± 212
Ca	-	-

Isolated tissue cells

The preparation of tissue cells for electron probe microanalysis turns out to be difficult, as samples generally have to be dissected from the intact tissue. Such an operation often disturbs the ion distribution in the sample and makes further analysis useless. This is illustrated by data obtained from cryosections of the papilla of rat kidney, compiled in Table 3. The high mean values and standard deviations for sodium and chlorine indicate severe cell damage. An alternative idea to overcome this problem is to isolate the cells by enzymatic digestion and to let them recover in an appropriate nutrition medium. Such a procedure for the preparation of isolated tubule fragments containing papillary collecting duct cells was described by Stokes et al. (1987). Fig. 12 shows a cryosection of such tubule fragments, the cytoplasmic element content is listed in Table 3. By comparison with the tissue values it can be seen that the concentrations of phosphorus and sulfur are about the same in the tissue and in the isolated tubules, whereas the potassium-to-sodium ratio is remarkably higher in the isolated cells. Although further cell damage by the isolation procedure cannot be completely excluded, such tubule fragments have proved to be reliable models to study ion transport systems in papillary collecting duct cells.

Table 3

Element content in the cytoplasm of papillary collecting duct cells of rat kidney, as prepared by dissection of tissue and after isolation of tubule fragments, in mMol/kg dry weight ± standard deviation. n = number of cells, $\bar{d}$ = dry weight portion. *** denotes a significant difference with the error probability of 0.001. Horizontal bars indicate concentrations below the detection limit.

	tissue	isolated tubules
n	12	12
d	0.15 ± 0.04	0.20 ± 0.05
Na	230 ± 278	66 ± 31
Mg	-	-
P	441 ± 86	490 ± 64
S	250 ± 124	215 ± 24
Cl	250 ± 313	171 ± 51
K	154 ± 68	344 ± 105***
Ca	-	-

In a similar way rat liver cells were prepared for X-ray microanalysis (Petzinger and Frimmer, 1988; Petzinger et al., in press): The cells were isolated by digestion with collagenase, washed and suspended in Tyrode buffer. In addition, Petzinger and coworkers were able to culture the cells on gas-permeable support films. Liver cells as appearing in tissue, in cell suspension and grown in culture are shown in Figs. 13-15. Fig. 16 is an image of the same cryosection as in Fig. 15 after storage at the laboratory atmosphere for 30 min. The comparison of the same structures in Figs. 15 and 16 illustrates the importance of cryotransfer or at least of a closed transfer system for the identification of morphological details in cryosections. The element content measured in the cytoplasm of rat liver cells after three different preparations (tissue samples, isolated and cultured cells) are compiled in Table 4. The injury of the tissue samples, which were obtained from the dissected organ in Ringer solution is obvious, as can be seen by the low K/Na ratio. In isolated cells this ratio appears remarkably recovered. Culturing on the gas-permeable support again increases the cytoplasmic potassium content. The differences in the element content as depending on the cell preparation before cryofixation are not uniform in all intracellular compartments. As can be seen by comparison of Table 4 with Table 5, the variation in the element content due to cell isolation and culturing is less significant in mitochondria than in the cytoplasm. The calcium accumulation in the mitochondria of the cells in the tissue samples is a strong indication for cell damage. Obviously, the cells recover after isolation and culturing.

Unlike in cultured fibroblasts and bacteria we observed remarkable variations in the cytoplasmic phosphorus, sulfur, and potassium content up to 50 % from one cell preparation to the other. It is unknown whether these differences were caused by different physiological and metabolic states of the livers the cells were

isolated from or whether these results were caused by small variations in the handling before cryofixation. Despite these variations in the absolute values of element concentrations, the qualitative reaction on the application of inhibitors on ion transport systems such as ouabain remains the same: Decrease of potassium, increase of sodium and chlorine in the cells (Petzinger et al., in press). We conclude from this experience that every microanalytical determination of the intracellular element content after a physiological experiment has to be compared with measurements of control cells taken from the same cell preparation.

Table 4

Element content in the cytoplasm of rat liver cells as found in tissue samples, isolated cells in suspension, and in cell cultures grown on gas permeable foil in mMol/kg dry weight ± standard deviation. n = number of cells, d = dry weight portion. *, **, and *** denote significant differences to the values in the left hand column with the error probability of 0.05, 0.01, and 0.001, respectively. Horizontal bars indicate concentrations below the detection limit.

	tissue	isolated cells	cultured cells
n	15	20	12
d	0.26 ± 0.05	0.32 ± 0.04	0.30 ± 0.04
Na	300 ± 162	–***	–
Mg	26 ± 25	26 ± 17	41 ± 14*
P	387 ± 158	265 ± 76**	235 ± 54
S	201 ± 60	211 ± 17	185 ± 24**
Cl	403 ± 194	93 ± 26***	62 ± 9***
K	226 ± 77	137 ± 26***	421 ± 48***
Ca	–	–	–

Table 5

Element content in the mitochondria of rat liver cells as found in tissue samples, isolated cells in suspension, and in cell cultures grown on gas permeable foil in mMol/kg dry weight ± standard deviation. n = number of cells, d = dry weight portion. *, **, and *** denote significant differences to the values in the left hand column with the error probability of 0.05, 0.01, and 0.001, respectively. Horizontal bars indicate concentrations below the detection limit.

	tissue	isolated cells	cultured cells
n	10	10	9
d	0.60 ± 0.09	0.45 ± 0.06	0.50 ± 0.06
Na	66 ± 28	–***	–
Mg	33 ± 22	32 ± 19	22 ± 17
P	199 ± 74	156 ± 46	108 ± 51*
S	250 ± 79	263 ± 30	213 ± 47*
Cl	67 ± 12	29 ± 8***	15 ± 12**
K	148 ± 65	101 ± 32	128 ± 59
Ca	11 ± 8	–***	–

Cryofixation under light microscopical control

The proper preservation of cells for microanalysis may require the cryofixation under light microscopical control. This is illustrated by experiments on the calcium distribution in streaming *Amoeba proteus* cells. These unicellular animals move in their culture medium by cytoplasmic streaming which is correlated with the formation and retraction of protrusions of the cell called pseudopodia. Disturbed or damaged cells stop the cytoplasmic streaming, round up, and retract pseudopodes. This may be caused by toxic substances in their environment or even by mechanical shock caused by the transfer of the specimen support containing the water droplet with the single ameba to the freezing device. Such a disturbed cell is shown in Fig. 17. In order to catch the definite state of cytoplasmic streaming observed by the binocular microscope, the propane jet freezing device according to Müller et al. (1980b) was modified to a propane shower, as illustrated by Figs. 18 and 19: The propane container cooled by liquid nitrogen is connected with a horizontally positioned pipe with a small hole at the bottom of the left end. Single amebae were prepared in water droplets of about 1 mm diameter on specimen holders within a plastic dish positioned below the binocular microscope. As soon as the cytoplasm streams in one clearly recognizable direction, the movable propane dewar with the horizontal pipe is shifted towards the left until it meets the chock. Simultaneously, light guides switch on the nitrogen gas valve, and nitrogen gas pushes the cooled propane through the pipe and the hole at the left end down to the specimen. Thus, the cells are cryofixed within about 0.1 s after light microscopical observation. Details of this device are described also by Zierold and Schäfer (1987). Fig. 20 shows the same ameba in the light microscopical image immediately before freezing and after cryofixation by the described method and freeze-drying in SEM. X-ray spectra obtained from whole cells exhibit remarkable differences in the calcium distribution in various parts of the cells: In some regions no or only low amounts of calcium were found, similar to the spectrum of the pseudopode in Fig. 21, in other regions the calcium concentration is remarkably enhanced as in the hind end spectrum in Fig. 22. In whole cells structures containing calcium cannot be localized more precisely. In cryofractured and freeze-dried cells electron dense vesicles can be revealed in SEM which may indicate the presence of calcium, however, the large X-ray excitation volume of 1-5 μm in these specimens makes the unambiguous calcium localization difficult (Fig. 23). By X-ray microanalysis of cryosections the existence of two obviously different electron dense vesicles was proved (Fig. 24): One type of vesicles contains very much phosphorus, calcium, and magnesium (V_1), the other type contains approximately no calcium and only small amounts of magnesium, phosphorus, and sulfur (V_2). The results are compiled in Table 6. The biological function of these vesicles and their distribution within the cells is not yet completely understood. The informa-

tion, however, which was obtained by X-ray microanalysis of whole cells in SEM and of cryosections in STEM demonstrates the importance of freezing single cells in a defined state of locomotion under light microscopical control.

Table 6

Element content in the cytoplasm, electron dense granules, and Ca-, Mg-rich granules of amoeba proteus cells, in mMol/kg dry weight ± standard deviation n = number of measurements. Horizontal bars indicate concentrations below the detection limit.

	cytoplasm	Ca-,Mg-rich granules	electron dense granules
n	10	12	8
Na	63 ± 79	13 ± 10	25 ± 17
Mg	105 ± 47	782 ± 513	32 ± 19
P	364 ± 103	3384 ± 974	76 ± 37
S	358 ± 100	-	223 ± 46
Cl	208 ± 112	-	59 ± 15
K	308 ± 93	29 ± 19	62 ± 22
Ca	20 ± 25	235 ± 156	-

Discussion

Concerning fixation of cells for electron probe microanalysis there is general agreement that cryofixation is the most appropriate technique to preserve the distribution of intracellular components as close as possible to the native state. Although not free from artefacts, for example deformations by ice crystal growth and the formation of segregation zones, it is still the only method to localize diffusible substances in an electron microscopical specimen (Zierold and Steinbrecht, 1987). Chemical fixation methods keep their importance only in studies of tightly bound elements and in some histochemical procedures where X-ray microanalysis may be useful to identify precipitates and unsoluble reaction products (Sumner, 1984). As cells mainly consist of water, from a dogmatic point of view they should be studied in the frozen-hydrated state. There are some attempts to do that, as described by Gupta and Hall (1981), Marshall (1980a, 1987), Zierold (1983,1986b), but the high mass loss in ultrathin cryosections, electrical charging in thick specimens, and the very low electron optical contrast reduce the capability of this method to few objects of biological interest. Microanalysis in the submicrometer range is almost impossible. The only way out is dehydration of the cells with the risk of displacement of diffusible substances. In particular the ion composition in water-rich compartments such as vacuoles or extracellular spaces will undergo redistribution resulting in random precipitates and aggregates. The experience with X-ray microanalysis of intracellular compartments of at least 10 % dry mass portion has not provided any indication for segregation or precipitation of elements within homogeneous compartments. From this observation we conclude that ions are not displaced further than the diameter of the segregation zones left after dehydration in biological specimens.

Fortunately, most elements of interest such as Na, Mg, P, S, Cl, K, and Ca are not volatile and bind with their counterions or with electrically charged molecules in the specimen. This is a prerequisite for the analysis in the vacuum of the electron microscope. Theoretically, S and Cl could be lost in the vacuum of the electron microscope as these elements may form gaseous molecules such as SO_2 and Cl_2. Also because of this risk, a low specimen temperature is recommended for electron probe microanalysis.

First X-ray microanalytical measurements were carried out on air-dried cells (see e.g. Kirk et al., 1974; Lechène et al. 1977 or the list in the review paper by Wróblewski and Roomans, 1984). The method works well in rigid cells with bound elements such as bacterial spores (Scherrer and Gerhardt, 1977) or algae such as diatomes or protozoa with a mineralized architecture (Pedersen et al., 1981; Noll and Zierold, 1981, 1984). After fixation with aldehydes and dehydration with alcohol at least bound metals and some cations can be detected in bacteria (Sigee et al., 1985). In more complex cells air-drying may cause deformations by shrinkage or bursting thus affecting the intracellular ion composition. Therefore, less crude

Fig. 17: Amoeba proteus cell after cryofixation and freeze-drying in SEM. The cell appears disturbed by mechanical shock due to transfer to the freezing device. C = cell, pp = pseudopodia. bar = 1 µm.

Fig. 18: Propane jet modified to the propane shower device. ch = chock, LN_2 = liquid nitrogen device, M = binocular light microscope, P = horizontal pipe of the propane jet.

Fig. 19: Propane shower device as in Fig. 18. ch = chock, M = binocular light microscope, p = horizontal pipe of the propane jet, s = place of the specimen in a plastic petridish. The arrow indicates the direction the propane jet is moved for cryofixation.

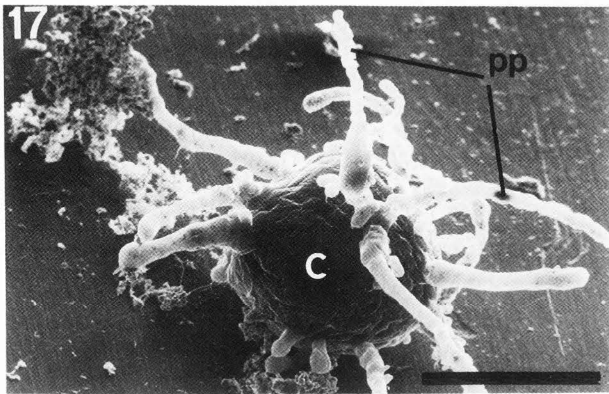
Fig. 20: The left side shows a streaming ameba in light microscopy. The arrow indicates the direction of cytoplasmic streaming. The right side shows the same ameba after cryofixation and freeze-drying in SEM. a and b mark areas where the X-ray spectra in Figs. 21 and 22 are obtained from. pp = pseudopodia. bar = 1 µm.

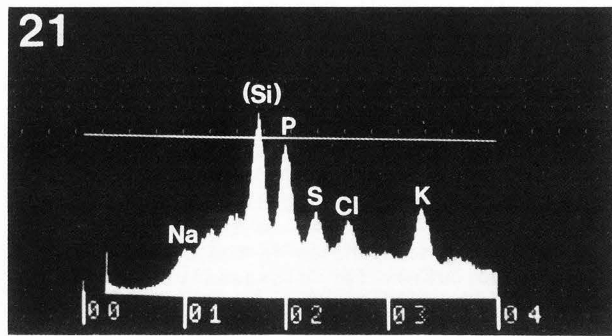
Fig. 21: X-ray spectrum obtained from area "a" in Fig. 20. Horizontal scale indicates X-ray energy (keV). Full width of vertical scale = 2500 counts. Brackets mark extraneous peaks.

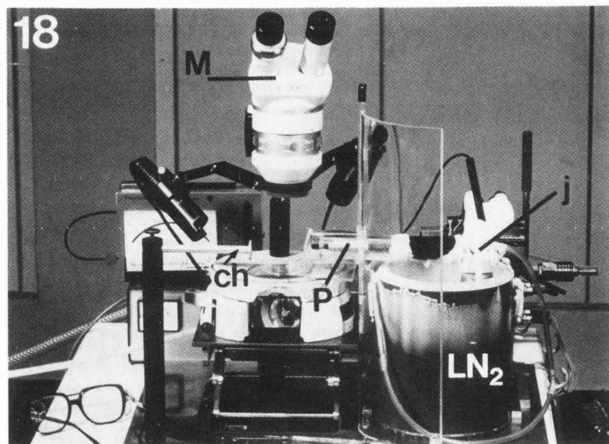
Fig. 22: X-ray spectrum obtained from area "b" in Fig. 20. Horizontal scale indicates X-ray energy (keV). Full width of vertical scale = 2500 counts. Brackets mark extraneous peaks.

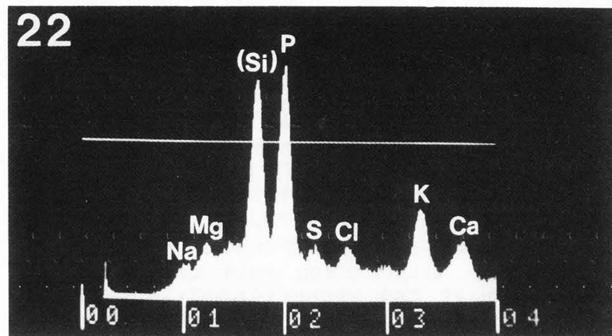
Fig. 23: Cryofractured ameba in SEM. The inset shows a central part of the cell in higher magnification. C = cytoplasm, pp = pseudopodium, V = electron dense vesicles. bar = 10 µm.

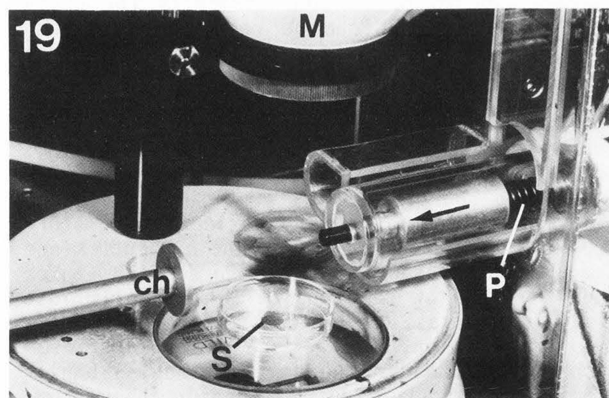
Fig. 24: Cryosection of an amoeba proteus cell. V_1 = vesicles containing much Ca and Mg. V_2 = electron dense vesicles without Ca. bar = 500 nm.

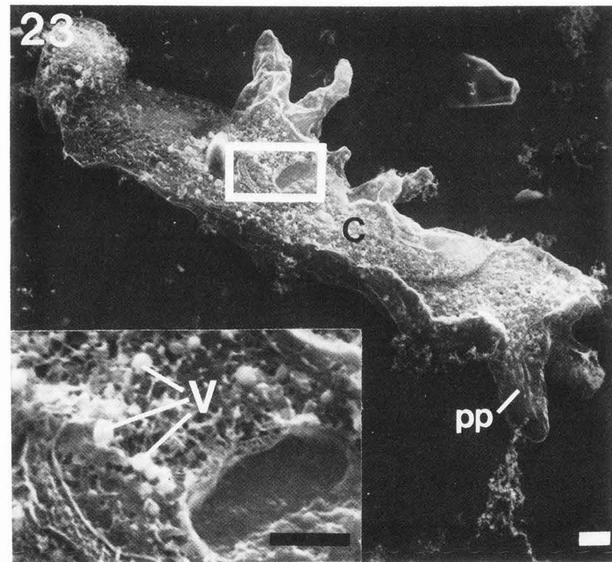

17
pp
C

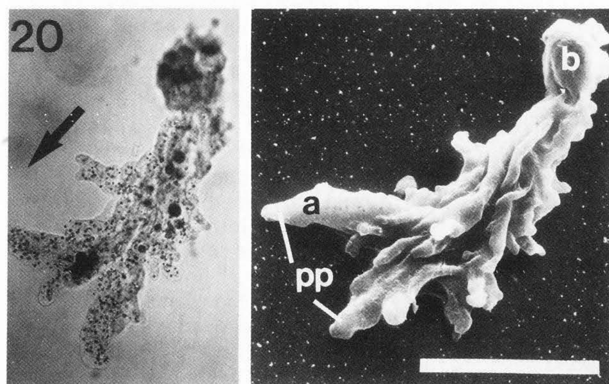

21
(Si)
P
S
Cl
K
Na

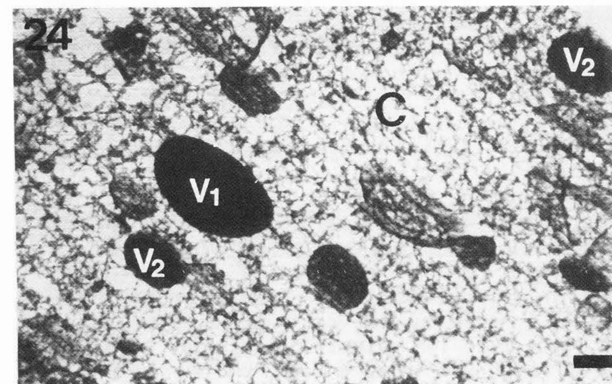

18
M
j
ch
P
LN2


22
(Si)
P
Mg
Na
S
Cl
K
Ca


19
M
ch
P
S


23
C
V
pp


20
b
a
pp


24
C
V2
V1
V2

and more sophisticated dehydration methods were developed.

The use of freeze-substitution as dehydration method for X-ray microanalysis is discussed very controversially in the literature (see e.g. Marshall, 1980b; Harvey, 1982; Zierold et al., 1978; Zierold and Schäfer, 1983; Wróblewski and Wróblewski, 1986; Steinbrecht and Müller, 1987; Zierold and Steinbrecht, 1987, and the X-ray spectra shown in Figs. 3-6 in this paper). The reason for the discrepancies in the evaluation of freeze-substitution for microanalysis may be caused by differences in the aim of the intended investigation and in the use of biological objects, substitution media, and protocols: It makes a great difference whether attention is focused only on the qualitative proof for the existence of one element of interest in a particular cell or whether alterations of the whole ionic composition are studied on a quantitative scale. Ion preservation after freeze-substitution may depend on the cell type. For example plant cells and animal tissue cells differ remarkably in the rigidity of the cell wall and in their water content, parameters which presumably determine the substitution rate considerably.

Looking at the reports of "successful" use of freeze-substitution, it is remarkable that most authors have tried diethylether or (in few cases) acetone, whereas ion retention after substitution with ethanol or methanol is observed rarely. The reason for these findings probably is the degree of polarity of the substitution medium which is characterized by its dielectric constant. The dielectric constants of some media important in dehydration are compiled in Table 7.

Table 7

Dielectric constants of various dehydration media

Medium	Dielectric constant
water	80.3
vacuum	1.0
methanol	33.5
ethanol	25.1
acetone	14.8
diethylether	4.3

Freeze-substitution in a polar medium (high dielectric constant) keeps the ions in solution and washes them off, whereas the exchange of water by an apolar medium increases the forces between the ions and surface charges of neighboured molecules. As a consequence ions are bound to each other and to surface charges of macromolecules. For example the mere removal of water, respectively the exchange of water by vacuum increases the electrical forces by the factor of 80. The relatively apolar liquid, diethylether unfortunately needs a very long time to penetrate membranes at low temperature. Therefore, it seems unfeasible from a practical point of view (Humbel and Müller, 1986; Steinbrecht and Müller, 1987). Considering Table 7 vacuum with the dielectric constant 1 is completely apolar and, therefore, it should be the best dehydration medium with respect to ion retention in cells. This idea is realized by freeze-drying. We conclude from our hitherto experience that freeze-substitution is unsuitable for measurements of the ionic composition of cells, whereas after freeze-drying the element content in cells as well as ion concentration gradients across intra- and extracellular compartments appear well preserved.

Intracellular ion distribution may be compromised by embedding freeze-dried specimens in plastic for ultramicrotomy. The data reported by Roos and Barnard (1985) and by Wróblewski and Wróblewski (1986) indicate such effects. However, there are not enough comparative studies available to evaluate the effect of plastic embedding on the intracellular ion distribution. Such effects may critically depend on details of the dehydration and embedding protocol (Edelmann, 1986). Furthermore, the peak-to-background ratios of the X-ray spectra from plastic sections are found to be less than half of the value obtained from cryosections, as caused by the high X-ray continuum portion due to the embedding plastic material (Meyer et al., 1985; Wróblewski and Wróblewski, 1986).

Microanalysis of freeze-dried whole cells can be disturbed by extracellular elements which precipitate on the cell surface (Fig. 7). We have tried to avoid this interference by washing the cells by an isotonic medium such as ammonium acetate consisting of volatile substances which vanish by freeze-drying. The method works well in the case of cultured skeletal muscle cells (Zierold et al., 1979), glioma cells (Zierold, 1981), or fibroblasts (see Fig. 8). Abraham et al. (1985) have found that fibroblasts may be washed also by distilled water without loss of intracellular elements. This washing step, however, has to be checked carefully for every cell type before it can be recommended. James-Kracke et al. (1980) have measured a 33 % loss of potassium from cultured vascular smooth muscle cells after washing with ammonium acetate. Wróblewski et al. (1983) have found that cultured chondrocytes, washed by sucrose before freezing, provided the most satisfactory intracellular ion composition whereas washing with distilled water or ammonium acetate caused considerable distortions in the intracellular element content. Kinne et al. (1985) observed that cultured TALH (thick ascending limb of the loop of Henle) cells lost nearly all potassium by contact with ammonium acetate solution. In this case the potassium could be retained in the cells by adding barium to the washing medium. By this action the barium-sensitive potassium channel was closed. These examples illustrate that various cells may be very sensitive for alterations of their extracellular environment immediately before freezing. These problems are avoided by freezing cells within their culture medium or within any other extracellular medium of interest and by preparing cryosections from these frozen specimens.

Although single and cultured cells because of their small size are the object of choice for cryofixation and cryosectioning, there are relatively few applications, however, with increasing tendency. Roomans and Sevéus (1976) studied the uptake of rubidium and cesium in yeast cells by X-ray microanalysis of cryosections. Masters et al. (1979) prepared cryosections of macrophages for X-ray microanalysis.

X-ray distribution maps for various elements obtained from cryosections of Bacillus cereus and Bacillus coagulans cells were published by Stewart et al. (1980, 1981). Chang et al. (1986) studied the element content in cryosections of E.coli bacteria as depending on the growth phase. James-Kracke et al. (1980) determined the element distribution in the cytoplasm, nucleus, and mitochondria of cultured vascular smooth muscle cells by X-ray microanalysis of cryosections. Warley et al. (1983a) prepared cryosections of HeLa cells in culture for X-ray microanalysis. They found that the intracellular sodium, chlorine, and phosphorus concentration varied with the cell cycle (Warley et al., 1983b). Buja et al (1985) studied the progressive alteration of the element distribution accompanied by ultrastructural changes in cultured myocytes with impaired energy metabolism. Warley (1986, 1987) has studied the intracellular element distribution in thymocytes as depending on the composition of the extracellular medium and on recovery conditions after cell isolation from tissue. Zierold et al. (1984) analyzed cryosections of fibroblasts grown on gold freeze-etch supports. Potassium and chlorine were determined by X-ray microanalysis in cryosectioned skeletal muscle fibers and compared with measurements by means of ion-sensitive electrodes (Acker et al., 1985). Some observations on isolated and cultured cells indicate that the potassium concentration in cells grown on a solid support is higher than in isolated cells in suspension. This can be seen by comparison of data from rat liver cells in Table 4 and by data obtained from fibroblasts (Zierold et al., 1984; Zierold, 1986a).

One reason to measure the element content in cells isolated from tissues is to study the properties of membrane transport systems without interference by the heterogeneous composition and function of most tissues (Kinne, 1983). This method was applied for example to prepare kidney epithelial cells and renal tubules for X-ray microanalytical measurements (Larsson et al., 1986; Le Furgey et al., 1986; Stokes et al., 1987). As the isolation procedure may damage the cells, criteria are required to determine the viability of isolated cells. The leakage of cells due to membrane damage was tested by the uptake of an extracellular dye such as trypan blue as can be observed in light microscopy. Baur et al. (1975) found that only severely damaged cells can be recognized by this method. A similar method for X-ray microanalysis was reported by Walker et al. (1984): These authors tested the integrity of the plasma membrane of macrophages by the celluar uptake of Erythrosin B, a substance containing iodine. Then, the viability of the cells was determined by measuring the potassium/iodine ratio. According to Baur et al. (1975) neither oxygen consumption nor the ATP/ADP ratio are sensitive criteria for the viability of isolated liver cells whereas ^{14}C-uridine incorporation and the stimulation of cellular respiration by 1 mM succinate turned out to be sensitive tests. The latter is based on the observation that damaged liver cell membranes are impermeable for succinate. Therefore, it cannot be used for cells containing a succinate transport system as for example proximal tubule cells in kidney (Kinne, 1983). Calcium accumulation in mitochondria and to a lesser extent in the cytoplasm, as found in crudely dissected liver tissue samples (Table 5) was often reported as indication for cell damage (Osornio-Vargas et al., 1981; Buja et al., 1985; LeFurgey et al., 1986).

The membrane potential and the intracellular ion concentrations (low sodium, high potassium) were found to be the most sensitive criteria for the viability of various mammalian cells (Baur et al., 1975; Kinne, 1983; Wróblewski and Edstrom, 1984; Abraham et al., 1985; Acker et al., 1985; LeFurgey et al., 1986). Table 8 shows the distribution of the potassium-to-sodium ratio (K/Na) in cells calculated from the dry weight element concentration data compiled in Tables 1, 2, and 3. In fibroblast cells the K/Na ratio is remarkably higher than 1. The cells obviously are intact. In isotonic medium most S.aureus bacteria have a K/Na ratio above 1. In hypertonic medium (isotonic medium + 10 % NaCl) this ratio drops below 1 for the majority of the cells. As there are some cells with unchanged ionic composition in hypertonic medium, the standard deviation of Na, K, and Cl in Table 2 is enlarged. In kidney papillary collecting duct cells dissected from the tissue the distribution of the K/Na ratio indicates a few intact and many injured cells. This evaluation agrees with the high standard deviation of the corresponding data in Table 3. After enzymatic isolation and recovery in an adequate nutrition medium most of the cells achieve their high K/Na ratio. The data analysis in Table 8 illustrates the capacity of X-ray

Table 8

Potassium-to-sodium ratio (K/Na) as determined from the X-ray microanalytical data of various cell preparations. n = number of cells.

K/Na ratio:	0-1	1-2	2-3	3-4	4-5	>5
Fibroblasts (Tab. 1), n=28	0	2	5	5	6	10
S.aureus bacteria in isotonic medium (Tab. 2), n=20	6	11	3	0	0	0
S.aureus bacteria in isotonic medium + 10% NaCl (Tab. 2), n=20	12	4	4	0	0	0
Kidney papillary collecting duct cells dissected from the tissue (Tab. 3), n=12	5	2	1	1	0	3
Isolated kidney papillary collecting duct cells (Tab. 3), n=12	0	1	2	0	1	8

microanalysis of single cells to determine the degree of cell injury and the homogeneity of the studied cell population.

The complexity of viability criteria makes clear the importance of cryofixation of cells in defined conditions in order to study cellular functions as related to ion movements. The freezing of streaming amebae under light microscopical control as illustrated by Figs. 17-24 is only one example. Somlyo et al. (1977, 1985) and Wendt-Gallitelli and Wolburg (1984) have measured calcium shifts in single muscle fibers which were cryofixed in defined states of electrical excitation. These are a few successful examples for X-ray microanalytical element localization in cells frozen in defined functional states. This is much more difficult to do in cells in tissues and complex organs as indicated by the data in Tables 3, 4, 5, and 8. For example, Hagler and Buja (1984) and von Zglinicki et al. (1986) have developed special devices to freeze tissue samples in situ. In general, however, the adequate adaption of cryofixation techniques to cells in defined functional states remains a challenge for the future.

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Discussion with Reviewers

J. Wróblewski: In Table 7 dielectric constants of various dehydration media are given. It is evident that apart from vacuum the best dehydration medium is diethylether and worst methanol. Why did you use methanol? Have you tried to use also diethylether and if so how did it affect the elemental composition compared to freeze-drying alone?

Authors: We have done some preliminary X-ray microanalytical measurements of muscle tissue bulk specimens freeze-substituted by diethylether and acetone (Zierold et al., 1978). As the observed ion loss was higher than after freeze-drying we have not further investigated this alternative.

J. Wróblewski: The freeze-substitution according to Müller et al. (1980a) is suitable for morphological investigations only. Could you comment why you chose to apply it for elemental studies and not for example the method proposed for biological X-ray microanalysis by Marshall (1980)?

Authors: A priori there is no reason why a preparation technique which preserves the ultrastructure should not maintain also the ion distribution in cells. It was disappointing for us to see that most freeze-substitution agents giving excellent ultrastructural preservation, for example methanol or acetone, wash out more or less the diffusible ions. This does not imply that freeze-substitution cannot be used for specific microanalytical purposes. It might still be a useful technique for qualitative tests whether a specific (bound) element is present in a cell or not. However, the use of polar media for dehydration always causes solution of charged ions resulting in more or less redistribution and washing out. This property makes most freeze-substitution media unreliable for quantitative studies of the ion composition of cells and cell organelles. We have no experience with the acroleindiethylether method described by Marshall (1980b). Unfortunately diethylether needs very long substitution times to exchange the water.

A. Warley: I find the results for fixation and freeze substitution interesting, though I am not clear about the Ca artefact. Do the authors think

that this element is added from one of the solutions used? That the peak is intensified by dehydration or that the element is migrating and collecting giving high concentrations in one particular area?
Authors: We do not know where the Ca (Figs. 2, 4,5) comes from. Probably it is taken up from the chemical agents used. A precipitation of intracellular Ca would not cause thus high peaks measured in whole cells.

J. Wróblewski: Fibroblast are to my knowledge best cultured as monolayers. I, therefore, assume that you had to remove the cells from their support enzymatically before cryofixation. If so, the "physiological state" of the cells is changed quite dramatically. Could you comment on the effects of the enzyme on the metabolic state of the cells in relation to ionic composition?
Authors: As compared to previous results (Zierold et al. 1984) the concentrations of phosphorus and potassium measured in cells in suspension (Table 1) are lower, whereas sulfur remains constant. We do not know whether the differences are caused by the enzyme used for cell isolation (trypsin) or by the different environmental conditions the cells meet with.

A. Warley: Although the K/Na ratio in rat liver cells (Table 4) is improved in the isolated cells compared to the tissue cells, it would appear that K as well as Na has been lost from these cells. Does the concentration of K improve with time in culture?
Authors: We have measured cells cultured for 1 or 2 days only. In these preparations we have not found significant differences. Microanalytical studies on alterations of the intracellular element content with culture time are intended with other epithelial cells.

Th. von Zglinicki: The Staphylococcus aureus cells in the hypertonic medium contain relatively low amounts of NaCl in comparison with the medium. Their membranes seem to be well in order as compared to, e.g., liver tissue cells prepared under severe trauma (Table 4, first column) which appear swollen as shown by their low dry mass portion. However, their K/Na ratio is higher than that in Staphylococcus cells in hypertonic medium. In this respect I would not recommend the use of the K/Na ratio as a general viability test. It is well known that cells can recover from the high Na-low K situation (cryopreserved cells, for instance) and, on the other hand, cells may die before membrane permeability for the monovalents increases.
Authors: Low viability according to the proposed definition of low K/Na ratio does not mean that the cell may not recover. The K/Na ratio is just used as a measure for the actual physiological state of a cell.

A. Warley: Do the authors consider that the success of preparing tissue samples for X-ray microanalysis may also depend on the tissue studied and on the conditions immediately before cryofixation? I can understand that the dissection of kidney papilla which lie deep in the tissue would lead to damage and high levels of Na and Cl. What was the preparative procedure used for liver tissue which led to high levels of Na and Cl in these cells? I ask this because several authors have published microanalysis results for liver tissue in which such high concentrations were not encountered (e.g. Cameron et al., Phys. Chem. Physics & Med. NMR 16, 1984, 521).
G. Roomans: Cryofixation of kidney in situ is known to be difficult because the kidney is very sensitive to anoxia. The poor results on liver in situ are somewhat surprising, since better results have been published in the literature with comparable freezing techniques. The high sodium and cloride is not consistent with the nice ultrastructure in the cryosection. The membrane damage apparent from the elemental concentration should give rise to cell swelling and therefore poor cryosections. Please comment!
Authors: The data on rat liver tissue samples shown in Table 4 were obtained from a dissected liver in Ringer's solution. In situ cryofixation was not used. The experiment was done only to emphasize this crucial point of biological X-ray microanalysis: The measurement of reliable data requires the maintenance of a defined functional state of the cell or the tissue to be studied. This can be achieved easier with isolated or cultured cells than with an intact organ. We are aware of the fact that by appropriate cryofixation (in situ cryofixation as described for example by Hagler and Buja, 1984, or by von Zglinicki et al., 1986) considerably improved data, representing better the physiological state of the tissue are obtained.

Concerning the relationship between morphology and elemental composition we have found that the correlation is rather weak: Cells with large ice crystal segregation zones indicating high water content and/or vacuoles in most cases have a low K/Na ratio. Cells with well preserved ultrastructure may have high or low K/Na ratio independent of their morphological appearance. We have not found that the quality of cryosections depends on membrane damage. However, cells with large ice crystal damage are more difficult to cut than well frozen cells.

Reviewer V: With reference to Fig. 12: Do collecting tubule fragments consist of individual cells or are several cells maintained intact, with in situ apical-basolateral orientation? Can apical microvilli be discerned in cryosections?
Authors: The isolated fragments consist of clusters of 10-30 still adhering cells, as observed by parallel SEM studies. In some cases also microvilli could be discerned in cryosections of these cell clusters.

Reviewer V: With reference to Fig. 15: Have the authors been able to obtain cryosections parallel to the growth surface from cells grown on Thermanox or on gas permeable membranes? What is plane of sectioning of cells in Fig. 15?
Authors: Fibroblast and muscle cells grown on Thermanox coverslips were cut almost parallel, slightly oblique to the support surface after gluing a chip of the plastic support on the cryoultramicrotome holder by liquid heptane

(Steinbrecht RA, Zierold K (1984). A cryoembedding method for cutting ultrathin cryosections from small frozen specimens. J. Microsc. 136, 69-75). However, we have not succeeded to get cryosections from very flat growing cells.

The liver cells shown in Fig. 15 were grown on gas permeable foil. This foil was stretched over the half sphere shaped end of a 2 mm thick plexiglass rod. This device was frozen and then mounted into the cryoultramicrotome arm. Thus sections were cut from cells parallel to the growth surface.

Reviewer V: With reference to Figs. 18,19: What is rate of freezing with propane shower device?

Authors: The freezing rate depends on the pressure used to push the liquid propane onto the specimen and on the specimen size and support. The higher the propane pressure, the higher was the cooling rate. However, too high propane pressure may shoot the specimen away from the support. A propane pressure of 300 kPa turned out to be the optimum in the described device. Using this propane pressure and 0.1 mm thick thermocouples placed on glass support below a water droplet of 1-2 mm in size we have measured a cooling rate of 4400 K/s $\pm$ 40% standard deviation for the temperature decrease between 273 K and 173 K. The high standard deviation is mainly caused by differences in the size of the water droplet placed upon the thermocouple. Without the water droplet and using 400 kPa propane pressure 9900 K/s $\pm$ 17% were measured by the same thermocouple on glass support.

Th. von Zglinicki: It would be interesting to compare the elemental concentrations of granules in *Amoeba proteus* with those of mitochondria, which can also store high amounts of Ca and P under pathological conditions.

Authors: The Ca amounts we have found in mitochondria of damaged cells is much lower than observed in the granules of amebae.

G. Roomans: What is the lowest concentration of sodium and magnesium that can be detected in your system?

Authors: The detection limit for sodium in a single 100 s spectrum is approximately 30 mMol/kg dry weight, and for magnesium 20 mMol/kg dry weight.