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ELECTRON PROBE X-RAY MICROANALYSIS OF EPITHELIAL CELLS: ASPECTS OF CRYOFIXATION

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Abstract

Content and distribution of diffusible ions in epithelial cells were studied by scanning transmission electron microscopy and energy dispersive electron probe X-ray microanalysis of freeze-dried cryosections from trout kidney, rat liver and Malpighian tubules of *Drosophila* larvae. Cryofixation of small excised kidney and liver samples by rapid immersion into liquid propane resulted in intracellular K/Na-ratios < 1. In contrast, K/Na-ratios > 7 were obtained after *in situ* cryofixation by means of a cryopunching device which allows tissue pieces to be frozen during excision from the intact organ. Isolated hepatocytes cryofixed in a small droplet of culture medium had a K/Na-ratio of 3.7. After culturing the hepatocytes, the K/Na-ratio increased to 24. Effects of extracellular media of different composition on the intracellular element content were studied. Malpighian tubules of *Drosophila* larvae were cryofixed by rapid immersion into liquid propane, and the distribution of K across the cells forming the tubules from the basal to the apical cell membrane was measured. An increasing K gradient was found from the intermediate to the apical cytoplasm. The intracellular K distribution was dependent on ions and transport inhibitors present in the fluid surrounding the Malpighian tubules within the larvae. Content and distribution of ions in epithelial cells sensitively depend on the physiological state immediately before cryofixation. Thus, electron probe X-ray microanalysis of cells and cell functions requires careful selection and control of the cell system to be studied.

Key words: Cryofixation, electrolyte ions, epithelia, hepatocyte, intracellular ions, kidney, liver, Malpighian tubules, X-ray microanalysis.

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Introduction

Epithelial cells are involved in the control of electrolyte ions and water in many organs of animals and man. In order to elucidate the underlying ion transport mechanisms, electron probe X-ray microanalysis (EPXMA) is used for measurements of content and distribution of elements in epithelial cells (see e.g., Gupta *et al.*, 1976; Gupta, 1984; Dörge *et al.*, 1978; Marshall, 1983; LeFurgey *et al.*, 1988; von Zglinicki and Roomans, 1989). However, electrolyte ions are very mobile. The removal of tissue or cell samples from intact epithelia may redistribute the original intracellular element distribution. The diffusion constant of ions such as Na^+, Cl^-, K^+ and Ca^{2+} in aqueous solution at room temperature is about 2×10^{-9} m²/s. From this diffusion constant a diffusion length of 2 μm within 1 ms can be estimated (Zierold, 1991). Therefore, reliable microanalytical experiments on the localization of electrolyte ions in cells and tissues in defined functional states require immobilization by in situ cryofixation techniques as described previously (Hagler *et al.*, 1983, 1989; von Zglinicki *et al.*, 1986; Wendt-Gallitelli and Isenberg, 1989; Zierold and Schäfer, 1988; Zierold, 1989; Zierold, 1993a).

In the following we describe our experiences with EPXMA of three different types of epithelia: Proximal tubules of trout kidney, hepatocytes of rat liver and Malpighian tubules of *Drosophila* larvae. We intend to show that content and distribution of electrolyte ions in epithelial cells sensitively depend on the physiological state immediately before cryofixation.

Materials and Methods

Tissue samples of rat liver and trout kidney were cryofixed by two different methods: Rapid immersion into liquid propane cooled by liquid nitrogen (Fig. 1a) or cryopunching (Fig. 1b).

Immersion into liquid propane

The liver was excised from an anesthetized rat and

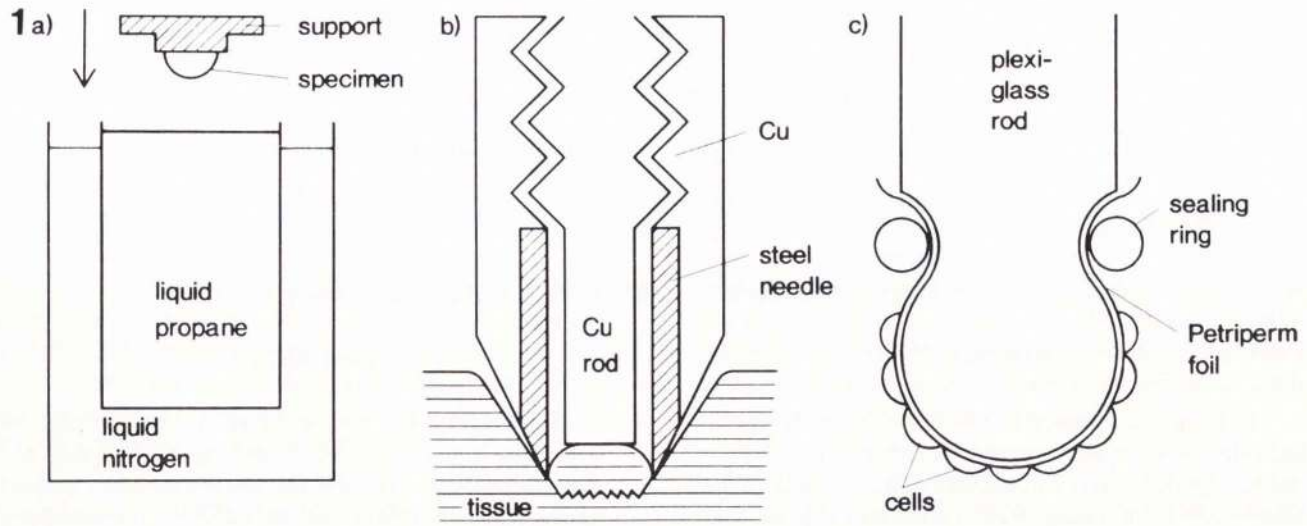


Fig. 1: Schematic drawing of cryofixation techniques used. a) Rapid immersion of a specimen adhering on a gold plated specimen support of 3 mm in size into liquid propane. b) Cryopunching by a precooled steel needle of 2 mm inner diameter surrounded by a Cu cartridge. c) Cultured cells on Petriperm foil stretched over the round end of a plexiglass rod of 2 mm diameter by immersion into liquid propane.

stored in Ringer solution at room temperature. The time interval from the excision of the liver up to cryofixation varied from 10 min up to 1h. A small tissue piece of approximately 1mm in size was excised from the liver lobe and placed onto a gold plated Balzers freeze-etch specimen support held by a modified forceps. The specimen support held by the forceps was plunged into liquid propane by an air pressure driven device with a velocity of 2m/s. The time interval between excision of the tissue sample and cryofixation was up to 10 s. In a similar way small pieces of the kidney of the rainbow trout *Oncorhynchus mykiss* were cryofixed after decapitation of the fish and storing the removed kidney in Ringer solution ventilated by air. For comparison tubules were isolated from the kidney by means of fine forceps under light microscopical control and then cryofixed by plunging into liquid propane as described above.

Cryopunching

Cryopunching was performed automatically by means of a pneumatic device: Disk-shaped cryofixed tissue pieces of 2mm in diameter were obtained by punching a precooled steel needle surrounded by a Cu cartridge with an inner polished Cu rod into the isolated rat liver lobe stored in Ringer solution or into the excised trout kidney as illustrated schematically in Fig. 1b. The delay between excision of liver and kidney from the animal and the moment of cryofixation was the same as described above for cryofixation of tissue pieces. Technical details are described elsewhere (Zierold, 1993b).

Hepatocyte cultures

Hepatocytes were isolated and cultured as described previously (Petzinger *et al.*, 1988). Briefly, the cells were isolated by perfusing rat liver with collagenase in Krebs-Henseleit buffer under bubbling oxygen atmosphere. The cells were washed in Tyrode solution and then suspended in Dulbecco's modified Eagle's medium. Isolated cells were cryofixed by rapid immersion of a small droplet of cell suspension placed on a gold Balzers freeze-etch specimen support into liquid propane as schematically drawn in Fig. 1a. Primary cultures were obtained by plating freshly isolated cells on collagen coated Petriperm dishes (Bachofer GmbH, 72770 Reutlingen, Germany) in culture medium and incubation in CO_2-air (5%/95%) for two days. Within this time the cells formed confluent monolayers on the Petriperm foil. Round areas with a diameter of 10mm were punched out from the cell monolayer grown on Petriperm foil and incubated in experimental solutions as described in the results section. The punched Petriperm foil was stretched over the round-shaped end of a 2mm thick plexiglass rod and cryofixed by rapid immersion into liquid propane as described previously (Zierold, 1989) and schematically drawn in Fig. 1c.

Malpighian tubules

Malpighian tubules of *Drosophila hydei* larvae were excised carefully in Ringer solution under light optical control with a binocular microscope. The tubules were placed on a gold freeze-etch specimen support and cryofixed within 10 s after excision by rapid immersion

Table 1: Element content in proximal tubule cells of trout kidney

	cryofixation of excised tissue samples	cryofixation by cryopunching	cryofixation of isolated tubules
n	15	10	10
d	0.28±0.04	0.30±.05	0.25±0.03
Na	302± 55	62± 22	38±35
Mg	37± 2	36± 13	55±13
P	509± 51	524± 103	589±98
S	185± 31	192± 30	195±23
Cl	212± 31	160± 23	97±17
K	240±134	441± 78	545±59
Ca	5± 5	1± 5	0± 8
K/Na	0.79	7.1	14.3

Data given in mmol/kg dry weight ± standard deviation. n = number of measured cells, d = dry weight fraction.

Table 2: Element content in rat liver cells

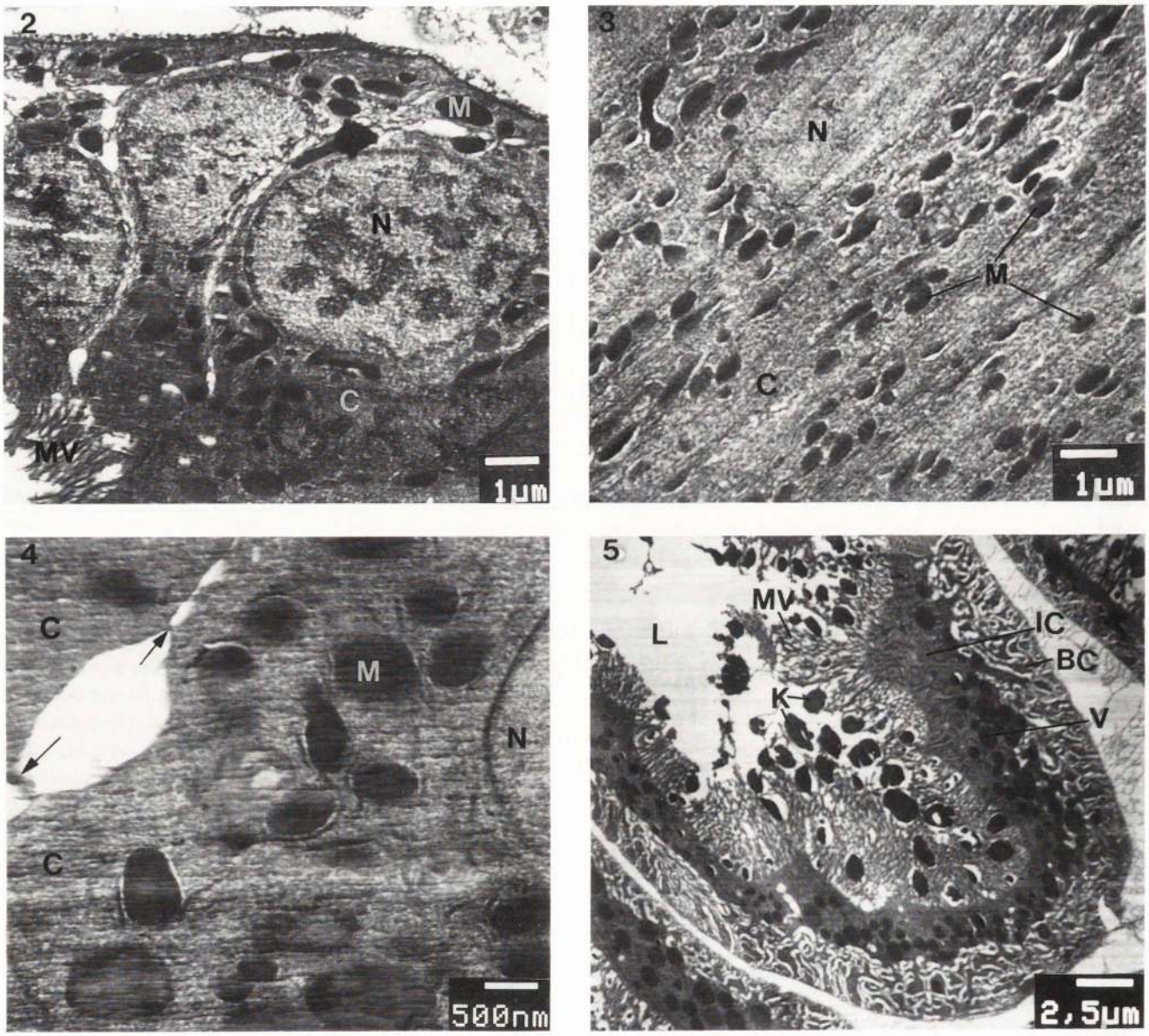
	cryofixation of excised tissue samples	cryofixation by cryopunching	cryofixation of isolated cells	cryofixation of cultured cells	cultured cells in 300 mosm Tyrode sol.	cultured cells 10 min 1 mM ouabain
n	15	20	12	10	15	15
d	0.30±0.05	0.24±0.05	0.29±0.04	0.30±0.05	0.28±0.04	0.25±0.04
Na	311±159	41± 29	58± 23	15± 14	28± 26	502±126
Mg	15± 14	24± 13	20± 5	23± 18	23± 14	33± 24
P	383±103	377± 82	375± 56	346± 68	318± 65	240± 82
S	133± 27	154± 18	149± 12	133± 41	133± 25	114± 21
Cl	275±185	105± 39	98± 26	109± 16	81± 21	345± 89
K	97± 37	298± 59	217± 25	361± 54	260± 46	68± 17
Ca	4± 4	4± 4	1± 2	4± 2	3± 4	21± 17
K/Na	0.31	7.3	3.7	24	9.3	0.14

Data given in mmol/kg dry weight ± standard deviation; n = number of measured cells, d = dry weight portion

into liquid propane according to the method sketched schematically in Fig. 1a. The K transport in Malpighian tubule cells was studied by injection of specific substances into the hemolymph of the larva. The exposure time usually was 10 min. Then the Malpighian tubules were excised from the larval body and cryofixed as described above.

Cryosectioning and analysis

Cryofixed specimens were stored in liquid nitrogen. Cryoultramicrotomy, cryotransfer, freeze-drying, scanning transmission electron microscopy (STEM) and EPXMA were done as described previously (Zierold, 1988). Briefly, approximately 100 nm thick cryosections were cut by means of glass knives at a temperature below 170 K and cryotransferred to the cryo-STEM. After freeze-drying during cryotransfer, cryosections were studied in the cryo-STEM at a temperature of 138 K. Energy dispersive X-ray spectra were measured with an electron beam of 100 kV acceleration voltage and 1.3 nA electron current by use of a SiLi- detector and a multichannel analyzer. Element concentrations related to the dry weight (c_d) were determined according to the peak-to-background method. They were converted into



Figs. 2-5: Morphology of freeze-dried cryosections from epithelial cells. BC = basal cytoplasm, C = cytoplasm, IC = intermediate cytoplasm, K = K-accumulating concretions, L = lumen, M = mitochondria, MV = microvilli, N = nucleus, V = mass-dense vacuoles. **Fig. 2**: Proximal tubule of trout kidney after cryopunching. **Fig. 3**: Rat liver tissue after cryopunching. **Fig. 4**: Cultured rat hepatocytes cryofixed by immersion into liquid propane. Arrows point to contact points of cells growing to a monolayer. **Fig. 5**: Malpighian tubule of a *Drosophila* larva, cryofixed by immersion into liquid propane.

element concentrations related to water (c_w) by means of dry weight fractions (d) determined in intracellular compartments from measurements of the darkfield intensity in STEM:

$$c_w = c_d \cdot d / (1 - d) \qquad (1)$$

Elemental maps were obtained by scanning the electron beam with a resolution of 128x128 pixels across the cryosection area of interest and by collecting X-rays from energy windows related to specific elements or bremsstrahlung background between 4.5 and 6.0 keV (Zierold *et al.*, 1991).

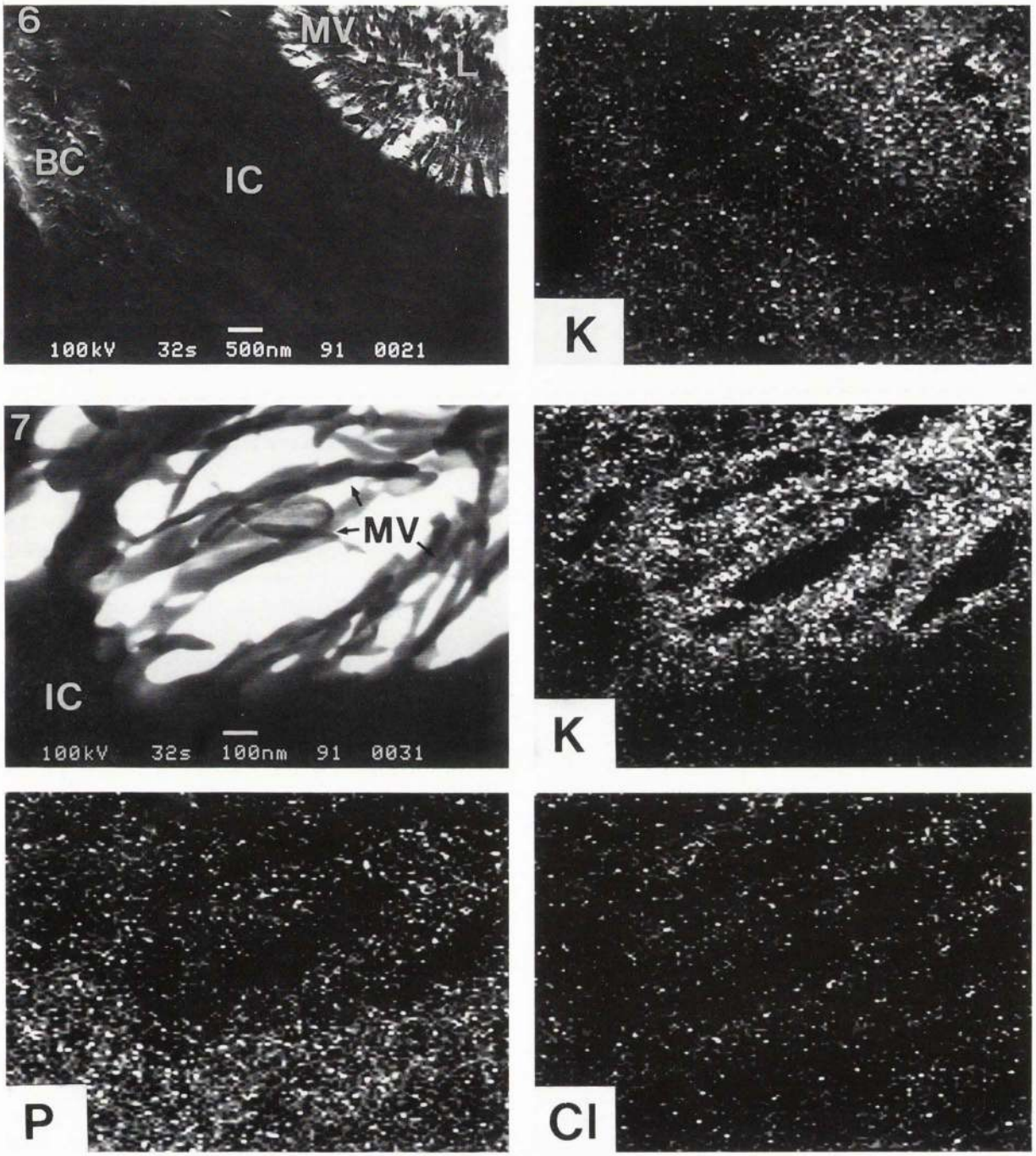


Fig. 6: Elemental mapping of K in *Drosophila* Malpighian tubules. Left: STEM image, right: K distribution. The accumulation of K in the microvilli and the luminal space as compared to the intermediate cytoplasm is evident. BC = basal cytoplasm, IC = intermediate cytoplasm, L = lumen, MV = microvilli.

Fig. 7: Elemental mapping of K, P and Cl in *Drosophila* Malpighian tubules. Upper left: STEM image. K is accumulated in the microvilli. P is found in the intermediate cytoplasm. Low Cl levels are found in the luminal space. IC = intermediate cytoplasm, MV = microvilli.

Results

Fig. 2 shows a cryosection of epithelial cells forming the proximal tubule in trout kidney after freezing the tissue by cryopunching. The basal membrane, cytoplasm, nucleus, mitochondria and apical microvilli can be clearly identified. The cytoplasmic element contents measured by EPXMA after different specimen preparation methods are compiled in Table 1. In comparison to cryopunched tissue or to isolated and then cryofixed tubules a considerably lower K/Na-ratio was found in tissue pieces frozen several seconds after excision from the organ. As can be seen in Table 2, similar differences of the intracellular K- and Na-content were measured in rat liver tissue after cryofixation of excised tissue pieces or cryopunching respectively. A cryosection of cryopunched rat liver tissue is shown in Fig. 3.

In comparison to cells in excised liver tissue specimens, isolated hepatocytes in suspension had low intracellular Na and elevated K content, resulting in a K/Na- ratio of 3.7. The K content in the isolated cells was significantly enhanced after forming a confluent monolayer of cells on Petriperm foil. The cells yielded a K/Na-ratio in the range of 20. Two cultured hepatocytes touching each other can be seen in the cryosection of Fig. 4. Merely the exchange of the culture medium by Tyrode solution of 300 mOsm decreased the intracellular K content and the K/Na-ratio respectively. Cultured cells exposed to 1 mmol/l ouabain for 10 min showed a tremendous decrease of intracellular K and an increase of Na and Cl. In addition a slight increase of cytoplasmic Ca was observed. EPXMA-data from hepatocytes cryofixed in different physiological conditions are compiled in Table 2.

A cryosection of Malpighian tubules of *Drosophila* larvae is shown in Fig. 5. The results presented here are restricted to the content and to gradients of the K distribution within the epithelial cells forming the anterior Malpighian tubules. Despite high statistical variations with standard deviations up to 30% of the mean value, the data in Table 3 provide evidence for a slightly decreasing K gradient from the basal to the intermediate cytoplasm and a steeply increasing K gradient from the intermediate cytoplasm to the cytoplasm of the apical microvilli. The corresponding elemental maps (Figs. 6 and 7) corroborate the heterogeneous K distribution across the cells from the basal to the apical membrane. A similar K distribution was found 10 min after injection of standard Ringer solution into the larva. Injection of K-free Ringer solution resulted in an almost complete depletion of K from the cells. Amiloride, a K/H-transport inhibitor, decreased the water portion in the microvilli resulting in a decrease of the K content related to dry weight and a slight increase of K content related to water whereas the K content of the intermediate and basal cytoplasm was not or only slightly influenced by amiloride. After injection of amiloride together with $BaCl_2$ the intracellular K content increased considerably, in particular in the basal and in the intermediate cytoplasm.

Discussion

Tissue samples

The high intracellular Na and Cl values in comparison to the low K values measured in tissue samples of trout kidney (Table 1) and rat liver (Table 2) cryofixed after excision from the original organ indicate severe cell or membrane damage, presumably due to interruption of blood and oxygen supply by dissection of tissue samples. According to our experience, cryofixation after sample excision is an unsuitable preparation technique for physiological experiments on intracellular diffusible ions. The high K/Na-ratios obtained from cryopunched tissue samples show that in situ cryofixation by freezing during excision considerably improves microanalytical data. However, it remains to be proven, to which extent preparation induced redistribution of elements can be prevented by cryopunching or other *in situ* cryofixation techniques. For example, the cryopunching experiments described above were done with isolated tissues which could be affected also by anaesthesia or the shock of operation. Differences in the intracellular element content, depending on the cryofixation technique either by freezing excised tissue samples or by use of a cryobiopsy needle were reported from experiments with hamster tracheal epithelium by Spencer and Roomans (1989).

Ice crystal segregation patterns in cryosections from cryopunched tissue (Figs. 2 and 3) are similar to those observed after cryofixation by rapid immersion into liquid propane (not shown), indicating that similar cooling rates are achieved by both techniques. Ions in aqueous solution are considered to be displaced by cryofixation and freeze-drying not further than the diameter of one segregation hole. Therefore the accuracy of element localization is determined by the size of the ice crystal segregations appearing as holes after freeze-drying. As ice crystal growth increases with the distance from the tissue surface, the restriction to superficial cell layers is the main limitation of EPXMA of large organs such as liver or kidney.

Isolated and cultured cells

From the preparation technical point of view iso

Table 3: K distribution in the anterior Malpighian tubules, proximal segment of *Drosophila hydei* larvae after injection of different solutions into the larval body.

	control	10 min Ringer solution	10 min K-depleted Ringer solution	10 min 1 mM amiloride	10 min 1 mM amiloride + $BaCl_2$
Microvilli					
c_d	1108±427	1269±480	32±10	640±116	1192±419
d	0.16±0.04	0.16±0.04	0.21±0.02	0.34±0.02	0.25±0.06
c_w	211±81	241±91	9±3	330±60	397±140
n	12	10	4	10	9
Intermediate cytoplasm					
c_d	337±130	409±82	19±3	448±138	581±142
d	0.18±0.04	0.18±0.02	0.27±0.02	0.17±0.04	0.4±0.05
c_w	74±29	90±18	7±1	92±28	387±95
n	18	8	5	5	5
Basal cytoplasm					
c_d	380±132	271±73	4±5	425±108	1923±787
d	0.16±0.04	0.33±0.09	0.20±0.02	0.17±0.03	0.23±0.05
c_w	72±25	134±36	1±1	87±22	574±235
n	12	6	4	6	7

c_d = K content in mmol/kg dry weight, d = dry weight portion, c_w = K content in mmol/kg water, n = number of measurements

lated or cultured cells are specimens of choice for microanalytical experiments for two reasons: [1] Due to their small size they can be cryofixed without or with only minute ice crystal damage (Fig. 4), [2] The cells do not need to be dissected from an intact organ, thus they can be cryofixed within their culture medium or any experimental medium of choice. The measured intracellular K/Na-ratio turned out to be a very sensitive parameter for the integrity of the cell membrane and the vitality of the cell respectively, in agreement with results of previous studies (Zierold and Schäfer, 1988). This aspect is illustrated in particular by the tremendous decrease of intracellular K and the increase of intracellular Na and Cl after exposing the cells to ouabain, a specific inhibitor of the Na/K-ATPase. The observed increase of intracellular Ca is a well known phenomenon accompanying cell damage (Osornio-Vargas *et al.*, 1981; Buja *et al.*, 1985; LeFurgey *et al.*, 1986).

Isolated cells may lose some of the properties they had in the intact tissue. For example, isolated hepatocytes in suspension had consistently less K than cells in cryopunched tissue or cells cultured to a confluent monolayer (Table 2). It is conceivable that epithelial cells such as hepatocytes with functionally different apical and basolateral cell membranes lose polarity by isolation and interruption of intercellular communication. As the elemental composition in cultured hepatocytes and in cells of cryopunched liver tissue was found to be similar, we believe to have sufficient evidence for considering cultured hepatocytes as a good experimental model system for studying electrolyte ion transport in rat liver. However, extrapolation of data from cultured cells to tissue function has to be done with caution.

Isolated tubules

The data obtained from isolated tubules of trout kidney (Table 1) and the results obtained from Malpighian tubules of *Drosophila* larvae (Table 3) elucidate the advantage of this specimen type for EPXMA of epithelial cells in comparison to tissue pieces excised from the intact organ prior to cryofixation. Isolated tubules allow to cut cryosections from intact cells far away from the tubule end damaged by excision whereas cryosections from tissue pieces have to be cut close to the block surface where cells are more or less damaged by excision. Isolated tubules exhibit functionally intact tissue of

small size suitable for physiological experiments and cryofixation as well. This aspect is illustrated by experiments on K transport in Malpighian tubules:

The main function of Malpighian tubules is to transport K from the surrounding hemolymph space into the lumen. Therefore absence of K in the hemolymph space causes a K depletion of the cells. In the luminal space K is found highly concentrated in the fluid phase and accumulated in specific concretions which can be seen in Fig. 5 (Wessing *et al.*, 1992). The data compiled in Table 3 and the elemental maps (Figs. 6 and 7) give evidence for the inhomogeneous K distribution across the cell. Electrolyte gradients across epithelial cells were found by Gupta *et al.* (1976) in *Rhodnius* Malpighian tubules, by Rick and DiBona (1987) in toad urinary bladder and by von Zglinicki and Roomans (1989) in crypt and villus cells of mouse small intestine. We assume that the increased K content in the microvilli in comparison to the neighboured intermediate cytoplasm is due to binding or high affinity of K ions to negative charges of glycosaminoglycans (Wessing and Zierold, 1993). Amiloride was applied to the Malpighian tubules in order to inhibit the apical K/H-transporter (Bertram *et al.*, 1991, Wessing *et al.*, 1993). After application of amiloride alone K ions transferred from the hemolymph space into the cells by the K/Na-ATPase or the K/Na/Cl-cotransporter leave the cells by basally located K channels. Probably these channels are closed by Ba ions. Thus, after application of amiloride with Ba, K ions are inhibited to leave the cells resulting in an increase of the intracellular K content.

Cryofixation

The data obtained from cryofixed tissue samples of trout kidney (Table 1) and of rat liver (the first two columns of Table 2) illustrate the importance of in situ cryofixation techniques for EPXMA of electrolyte ions in epithelial cells. The different cryofixation techniques in use for morphological or microanalytical studies (for review see e.g., Robards and Sleytr, 1985; Bald, 1987; Sitte *et al.*, 1987; Echlin, 1992) should not be evaluated only with respect to technical criteria such as achievable cooling rate, ice crystal size in the specimen or ease of operation. Irrespective of physical and technical properties, the efficiency of a particular cryofixation technique to immobilize a defined functional state of an experimental biological system is of eminent importance for ion localization by EPXMA. For example, high pressure cryofixation is known to provide excellent preservation of cellular ultrastructure with no or minimal ice crystal damage in comparatively large specimens of up to 0.5 mm in size (Moor, 1987). Unfortunately, the time interval necessary to mount an excised tissue sample in the high pressure freezing machine, restricts the efficiency of this method for EPXMA of diffusible ions. However, excellent microanalytical results can be expected from this method applied to cryofixation of sufficiently small organs or epithelial cell compounds such as isolated tubules or salivary glands which can be kept in a defined physiological state for several seconds without the risk of affecting their ion distribution.

The high variation of EPXMA-data of similar types of tissues obtained by different laboratories might be at least partly due to different physiological states the cells had achieved before cryofixation started (LeFurgey *et al.*, 1988; Tobler *et al.*, 1992). It should be kept in mind that aqueous specimens like tissue samples or droplets of cell suspensions exposed to air dry the faster the smaller they are (Hall and Gupta, 1982). Thereby the extracellular osmolarity at the cell surface may increase and affect the intracellular ion distribution. As illustrated by the results compiled in Table 2, the intracellular K/Na-ratio of hepatocytes is very sensitive to changes of the elemental composition of the extracellular medium. Appropriate environmental chambers can help to keep the cells in warm and humid atmosphere until the moment of cryofixation (Bellare *et al.*, 1988; Somlyo *et al.*, 1989; Frederik *et al.*, 1991).

The distribution of electrolyte ions in epithelial cells reacts faster and more sensitively on physiological changes than any other morphologically observable features. Therefore, EPXMA of epithelial cells and cell functions requires careful selection and control of the cell system to be studied until immobilization by cryofixation is achieved.

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Discussion with Reviewers

A.J. Spencer: It is possible to obtain similar K/Na values to those obtained after cryofixation of liver in situ by freezing the tissue immediately after excision (i.e., within a few seconds and without incubation in a bathing medium)? I have obtained values of 57 ± 3 and 529 ± 17 mmol/kg dry weight for Na and K, respectively (a K/Na of 9.2) in rat liver by this method (unpublished observations), A K/Na of 11 was also reported by Beck F-X, Dörge A, Mason J, Rick R, Thurau T (1982) Element concentrations of renal and hepatic cells under potassium depletion. Kidney Int **22**: 250-260. Please comment.

Authors: The consistently low K/Na-ratios which we obtained from tissue samples excised from isolated liver were the reason to develop the cryopunching technique. The comparison of the results obtained from liver tissue (Table 2) indicates that dissection of the liver from the anaesthetized animal and storage of the whole organ in Ringer solution affect the intracellular K/Na-ratio much less than excision of a small sample before cryofixation. Probably the low K/Na-ratio found in samples cryofixed before excision is due to cells close to the sample surface where they were damaged by excision. Perhaps you have succeeded to measure undamaged cells in the bulk of your samples.

H. Elder: The importance of your message, that knowing and maintaining the physiological state of ones cells/tissue at the moment of cryofixation is of paramount importance, is very great and your data demonstrate the point dramatically. However, you have been a bit severe in your choice of tissue protocols for comparison. It is likely that metabolically active organs such as liver and kidney, excised and stored in 'Ringer' for an hour, even if vigorously aerated, will be moribund, if not dead. A fairer comparison for your samples, cryopunched from the organs in situ, might have been samples cut and cryoplunged from organs perfused with oxygenated 'Ringer'. Have you tried such preparations?

Authors: No, we have not yet tried. I agree with you that samples cryopunched from organs in situ or cryobiopsy samples would provide more reliable reference data. However, we consider the result of the cryopunching experiment (Table 2) surprising and important: Cells of isolated liver which was stored up to 1 h in Ringer solution have a remarkably high K/Na-ratio. Obviously the cells still are healthy.

G.M. Roomans: It is remarkable that despite the large changes in K/Na-ratios caused by ouabain treatment of liver cells, the dry weight portion does not change. How do you explain this?

Authors: Liver cells have efficient osmoregulation systems. Probably they are able to restore the original cell volume within 10 min after starting the exposure of the cells to ouabain by changing ion specific membrane conductances.

G.M. Roomans: Is the effect of Tyrode solution statistically significant, and if so, what is the mechanism of this effect?

Authors: The slightly lower K content of cells in Tyrode solution in comparison to those in the culture medium is statistically highly significant as found in several experiments. The error probability is less than 0.001 according to the Student's t-test. We do not know the mechanism of this effect. However, one has to keep in mind that the osmolarity of the used culture medium is 220 mOsm and that of the Tyrode solution 300 mOsm. In addition, the culture medium contains substances missing in the Tyrode solution such as fetal calf serum and hormones.

H. Elder: You state that, in the Malpighian tubules, an increasing K gradient exists from the intermediate

cytoplasm to the cytoplasm of the apical microvilli. Your data do not demonstrate this gradient; it is more like a step at the luminal membrane.Is it not more likely that the undoubtedly high K concentrations imaged in Figs. 6 and 7 are a consequence of potassium migration from the luminal fluid during freeze-drying and settling on the luminal plasma membrane and microvillous membranes? This interpretation would also seem to be borne out by your amiloride experiment (Table 3). Presumably the drug diffuses down the tubules to block the K^+/H^+ pump from the luminal side. If there were a rising cytoplasmic K^+ gradient apically, would you not expect it therefore to be enhanced by amiloride, instead of the observed fall as the previously excreted K^+ then presumably diffuses out of the tubule? In your Ba^{2+} plus amiloride experiment (Table 3), intracellular and particularly basal K^+ rise as would be expected with the basolateral K^+ efflux pathway blocked by the Ba^{2+} but the input pathways by the sodium pump and the secondary active $Na^+/K^+/2Cl^-$ cotransporter are still operative. (Have you tried the effects of ouabain and/or furosemide to block these also?) However, despite the raised intracellular K^+, the 'microvillous' K^+ levels are, if anything, lower than control. This argues that the luminal high K^+levels are freeze-drying extramembranous adsorption artefact.

Authors: X- ray microanalytical spot measurements and K maps argue against your interpretation that the high K content in the microvilli is a freeze-drying artefact. K-ions precipitated from the lumen to the cell surface would result in K accumulations along the border of the microvilli and in low K content in between. This was not observed. K was found to be positively correlated with the distribution of P and dry mass indicating the cytoplasmic location of K. Binding of K to intracellular substances (presumably glycosaminoglycans) in the microvilli is supported by recent studies on the effect of brefeldin. Brefeldin is known to transform the Golgi apparatus, where glycosaminoglycans are formed, into inactive vesicles. After application of brefeldin to the Malpighian tubules the K accumulation was reduced in the microvilli and high in the lumen as in control experiments.

We understand the results of our amiloride experiment by assuming that K transferred into the cell across the basal membrane at least partly flows back into the hemolymph together with water. Thereby the water content in the microvilli decreases causing a slight increase of the K content related to water and a decrease of the K content related to dry weight. Closing of the basally located K channels by Ba leads to a K increase in the whole cell, mainly in the basal cytoplasm.

Application of furosemide caused a decrease of the K and Cl content in the microvilli to approximately 20% of the control values whereas ouabain had no significant effect on the intracellular element distribution.

H. Elder: Do you think that the very large range in dry weight fractions (Table 3) which you found is real? If so, what is your explanation for such variation? If the high apical K^+ is really a freeze-drying artefact rather than a cytoplasmic gradient, then the calculated wet weight concentrations would not be valid anyway.

Authors: Although not completely understood we estimate the dry weight fractions measured in the cells of the Malpighian tubules to be basically real. Of course, there is some uncertainty in the determination of the dry weight fraction by measuring the darkfield intensity in STEM because of inaccuracies in the section thickness. Dry weight fractions determined in more homogeneous systems, e. g. cultured liver cells, vary much less than the data obtained from Malpighian tubules under different functional conditions, indicating the reliability of the method to be reasonable.