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ELECTRON MICROPROBE ANALYSIS OF ELECTROLYTES IN WHOLE CULTURED EPITHELIAL CELLS

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Abstract

Microprobe analysis was used to determine electrolyte contents in whole epithelial sheets of A6 cells and to investigate the most critical points of this method. Analysis of dextran standard sections of different thickness revealed that low accelerating voltages of about 10 kV are best suited for whole freeze-dried cells on thick supports, since 5 μm thick sections are not penetrated by 10 kV electrons. Washing of A6 cells for 10 sec with distilled water led to cell swelling of about 40%, but the molar concentration ratios and the concentrations per dry weight (dw) were not altered. Washing for 60 sec with distilled water caused a further increase in cell volume (120%) and loss of cellular K and Cl (90 mmol/kg dw). Washing with isotonic NH_4-acetate led to a loss of cell Cl already after 10 sec.

To characterize the Na transport compartment, A6 cells cultured on permeable supports were washed for 5 sec with distilled water, freeze-dried, and analyzed. Inhibition of transepithelial Na transport by ouabain increased Na/P from 0.15 ± 0.07 to 0.75 ± 0.03 and Cl/P from 0.21 ± 0.001 to 0.38 ± 0.003 while K/P decreased from 0.83 ± 0.08 to 0.32 ± 0.03. The changes in cell Na and K contents can be explained by K/Na exchange; the increase in Cl content indicates some cell swelling. Since the ouabain-induced changes could be prevented by apical amiloride, the apical membrane provides the most important pathway for Na entry in A6 cells.

Key Words: Epithelial A6 cells, accelerating voltage, cell washing, electron microprobe analysis, permeable support, cellular electrolyte concentrations, amiloride, ouabain, transepithelial sodium transport.

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Introduction

Two principal methods have been used for preparing tissue for electron microprobe analysis of cell element concentrations: [1] measurements on tissue sections which yield element concentrations per cell volume or dry weight and [2] analysis of whole freeze-dried cells which usually provides concentration ratios.

Over the last 15 years, we have used the electron microprobe to analyze tissue sections coated with an albumin standard layer to determine cell electrolyte concentrations in a variety of epithelial structures (Rick *et al.*, 1978a, 1978b; Buchinger *et al.*, 1989). The results obtained under control conditions revealed remarkable uniformity among all epithelia analyzed so far: low Na concentration, high K concentration and the Cl concentration somewhere between. Nevertheless, knowledge of the absolute concentrations has still considerable advantage in many cases. Analyzing whole cells, on the other hand, avoids the complex and time-consuming step of cryosectioning. Furthermore, elemental ratios and their alterations determined with this technique provide meaningful results, by which many processes involved in transepithelial transport can be characterized (Lechene, 1989; Rome *et al.*, 1989). To obtain from concentration ratios similar concentration values as those referred to cellular dry weight or protein mass, the relative concentrations of P and S can be taken as reference for the other elements (Lechene, 1983; von Euler *et al.*, 1993). The concentrations of P and S have been regarded as a measure of the cellular mass content, since these elements are to a large extent tightly bound to matrix structures within the cell.

Applying the "ratio method" requires selection of a suitable accelerating voltage for analysis and of an ideal washing solution to remove the incubation medium. Whereas the first requirement means that the X-rays of interest are accurately assessed the second point implies that the washing solution neither influences the elemental composition of the cell nor contains elements which

interfere with the analysis.

The present paper is concerned with the determination of the cellular element composition of whole A6 epithelia cultured on thick permeable supports. The rationale of the investigation was to combine transepithelial electrical measurements with those of electron microprobe analysis. A6 cells, a continuous cell line originating from the distal renal tubulus of *Xenopus laevis*, have become an important model for studying single components of transepithelial Na transport (Perkins and Handler, 1981; Sariban-Sohraby *et al.*, 1984; Fidelman and Watlington, 1987; Paccolat *et al.*, 1987; Wills and Millinoff, 1990). To characterize further the Na transport compartment, the elemental ratios were determined after blocking transepithelial Na transport by the diuretic amiloride and by the glycoside ouabain. To establish the best conditions for preparation and analysis, measurements were also performed on sections of A6 cells and of dextran standards. These investigations revealed that reliable concentration ratios can be obtained from A6 cells washed for a short time with distilled water and analyzed at relatively low accelerating voltages.

Materials and Methods

Cell culture

Renal A6 cells (*Xenopus laevis*) obtained from American Type Culture Collection (ATCC, Bethesda, USA) were grown to confluence in culture flasks (Greiner, Frickenhausen, FRG, 658170, area 75 cm^2). The cells were detached from the bottom of the culture flasks using a Trypsin EDTA solution (Gibco, Eggenstein, FRG, 043530) for subculture. After adding fresh culture medium to inactivate the enzyme, cells were centrifugated in a cryocentrifuge (K2S, Hettich, Tuttlingen, FRG) at 120 g at 4°C for one minute. The supernatant was discarded and the cells were resuspended in fresh culture medium. The isolated A6 cells were seeded either again in culture flasks at a density of approximately 0.5 x 10^5 cells/cm^2 or on collagen-coated Millicell plastic filters (Millipore, Eschborn, FRG, Millicell PICM 01250 or PIHA 01250, area 0.8 cm^2) at a density of 10^6 cells/cm^2. For collagen coating, 10 mg rat tail collagen Type I (Sigma, Heidelberg, FRG, C7661) was dissolved in 4 ml of 0.2% acetic acid. Cells were fed three times a week with a medium containing 35% Leibovitz F15 medium (Gibco 041-01415M), 35% HAM F12 medium (Gibco 041-01765M) 19% water, and 9% fetal calf serum (Gibco 011-062990H), supplemented with 300 mg/l L-glutamine, 12.5 ml/l penicillin-streptomycin solution (Sigma P0781), and $NaHCO_3$ (Gibco 043-0530H) to a final concentration of 675 mg/l. Osmolality and pH of the incubation medium were 247 mosm/l and 7.4, respectively. The cells grew in a humified atmosphere in an incubator (B5060 EK ICO_2, Heraeus, Hanau, FRG) gassed with 2% CO_2 at 27°C. For the experiments cells at passages 73-74 were used.

Preparation

Dextran standards. A standard solution consisting of dextran and electrolytes was prepared by dissolving 1 g dextran in 4 ml of a solution containing equal concentrations (100 mmol/l) of NaCl and KH_2PO_4. Droplets of this solution were placed onto small aluminium cups and shock-frozen in a propane/isopentane mixture at liquid N_2 temperature (Jehl *et al.*, 1981). Sections with nominal thicknesses of 1, 5 and 10 μm were cut in a modified ultracryomicrotome (Reichert OmU3, Vienna, Austria) with a steel knife at -90°C. The sections were sandwiched between two formvar films and freeze-dried at -80°C and 10^{-6} mbar.

Cells. The essential procedures for preparing A6 cells for electron microprobe analysis are illustrated in Fig. 1. Beginning at day 10 after seeding, the epithelial layers were checked daily for confluence both by light microscopy and by measuring transepithelial resistance in a special Ussing-type chamber under sterile conditions. If the transepithelial resistance exceeded 2 $k\Omega \bullet cm^2$, the epithelia were processed as shown in Fig. 1. In some experiments, short circuit current (SCC) was determined before processing (see below). To improve tissue washing, the plastic wall of the filter cup was sawn off. The washing media were isotonic solutions of NaCl, NH_4-acetate or sucrose or distilled water, all at 4°C. Epithelia were washed for periods of 5, 10 or 60 sec in 200 ml washing solution stirred by a magnetic stirrer.

Whole-cell or sectioned samples were prepared from epithelia processed as above. To obtain whole-cell preparations, the epithelia were frozen at liquid N_2 temperature in a propane/isopentane mixture, freeze-dried at -80°C and covered with a thin carbon layer in a coating device (Med 010, Balzers Union, Liechtenstein). To prepare sectioned samples, the epithelia were dipped twice into an albumin standard solution before freezing. The albumin standard solution was prepared by dissolving 1 g albumin in 4 ml of the washing medium. Sections about 1 μm thick were cut from the frozen specimen, sandwiched between two formvar films, and freeze-dried.

Chamber experiments

The Millipore filter cups with the confluent A6

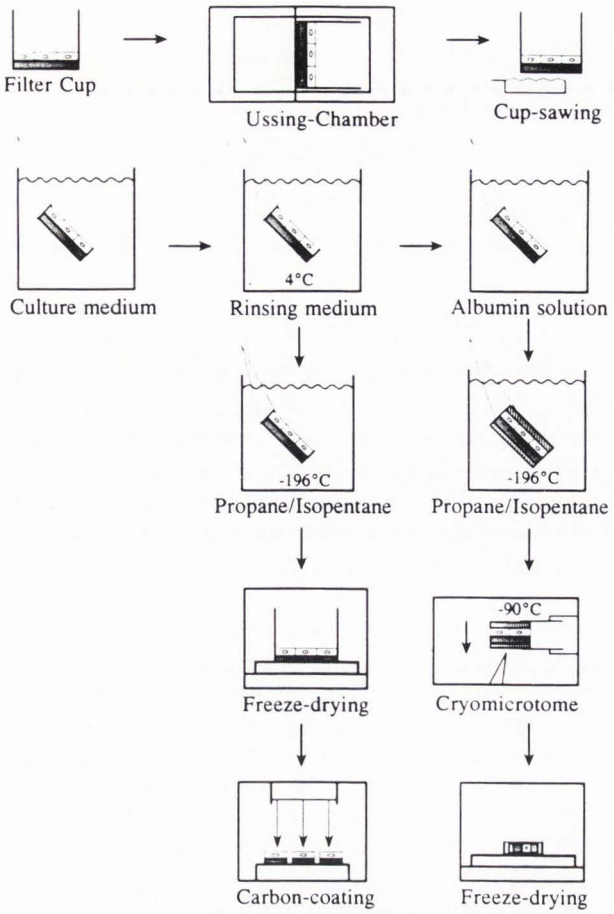

Fig. 1: Preparation of whole freeze-dried A6 epithelia and freeze-dried cyrosections of A6 cells.

monolayers were inserted into Ussing type chambers and perfused at 27°C. Short circuit current (SCC) was measured with an automatic voltage clamping device (Frankenberger, Germering, Germany). After stabilization of the SCC, ouabain (10^{-4}M) was applied for 20 - 30 min to the basal side either alone or after apical application of amiloride (10^{-5}M) for about 10 min. At the end of the experiments the filter cups were removed from the chambers and whole cell samples were prepared as described in Fig. 1. The incubation solution contained (in mM): NaCl 70, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 1, KH_2PO_4 1, $NaHCO_3$ 18 and Hepes 5. The solution was equilibrated with 95% O_2, 5% CO_2 and had a pH of 7.4 and an osmolality of 170 mosm/l.

Electron microprobe analysis

Analysis of the samples was performed in a scanning electron microscope (S150, Cambridge Instruments, Cambridge, UK) with an energy dispersive X-ray detector system (LINK Systems, High Wycombe, UK). Sections of the dextran standards and the cell

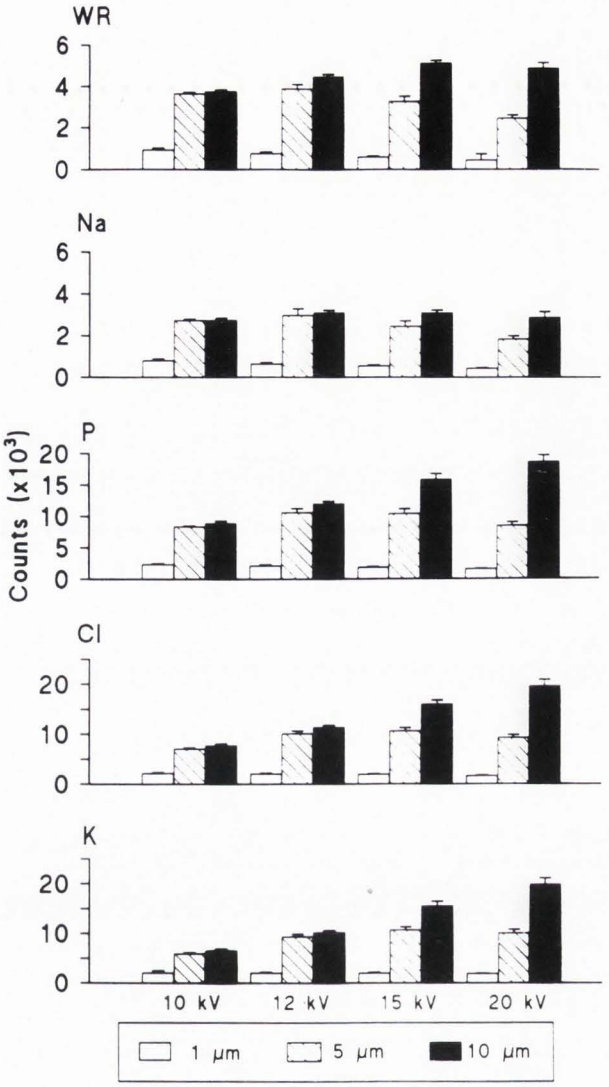

Fig. 2: Intensities of white radiation (WR), Na, Cl, P and K of dextran standard sections of different thickness at different accelerating voltages.

sections were viewed in the scanning transmission mode, whereas the carbon-coated whole cells were visualized in the secondary electron mode. The dextran standard sections were analyzed at acceleration voltages between 10 and 20 kV, the epithelial sections at 20 kV and the whole cells at 12 kV. The probe current was kept constant at either 0.1 nA (dextran standard sections) or 0.2 nA. In all cases areas of 0.5 - 1 μm^2 were scanned for 100 sec for analysis. Discrimination between the element-characteristic X-rays and the background radiation was performed by a computer program (Bauer and Rick, 1978). Element-characteristic X-ray intensities obtained from whole cell preparations were transformed into molar concentration ratios by factors derived from measurements of the dextran standard

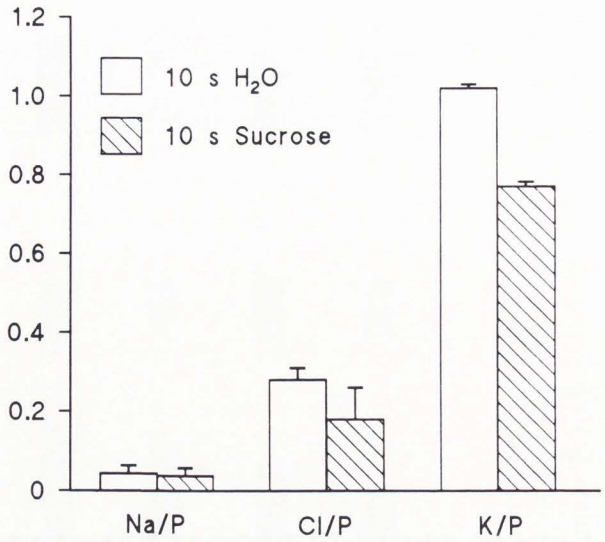

Fig. 3: Na/P, Cl/P and K/P ratios of whole cell preparations of A6 cells (epithelia) after washing for 10 sec with either distilled water or isotonic sucrose.

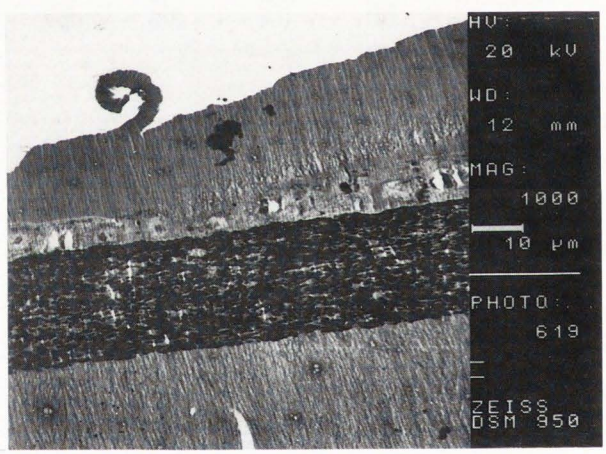

Fig. 4: Scanning transmission electron micrograph of a freeze-dried cryosection of A6 cells.

sections. Cell element concentrations (in mmol/kg ww) and dry weight contents (g/100 g ww) were obtained from measurements of the epithelial sections by direct comparison of the X-ray spectra obtained from the cells with those of the albumin standard layer adherent to the apical cell surface. More detailed descriptions of the quantification procedure may be found elsewhere (Dörge *et al.*, 1978; Rick *et al.*, 1982).

Statistics

Intracellular concentrations and SCC are given as means ± SD. In figures means ± 2 SEM are shown. Student's t-test was used to assess the significance of differences in means. A p value of less than 0.05 was regarded as statistically significant.

Results and Discussion

Accelerating voltage

As demonstrated by Euler and coworkers (von Euler *et al.*, 1993; von Euler and Roomans, 1992) cells attached to thin films and analyzed at relatively high accelerating voltages can be regarded as thin in the context of electron microprobe analysis. This offers the main advantage that quantification procedures developed for thin sections can be used. However, if the cells are placed on solid or thick permeable supports low energy electrons, which do not, or only partially, penetrate the cells might be used for analysis. This would ensure that contribution of X-rays generated in the support is, at most, very small.

To test which accelerating voltage is best suited for epithelial sheets on a thick permeable support, energy dispersive X-ray spectra were recorded from freeze-dried dextran standard sections of different thickness at accelerating voltages between 10 and 20 kV. These sections were sandwiched between thin formvar films. Figure 2 shows the X-ray intensity of the white radiation (WR) and Na, P, Cl and K. The WR and the characteristic intensities of all elements in the 1-μm thick section decrease with increasing accelerating voltage. As expected, for thicker sections the intensities were much higher at each accelerating voltage. Depending on the section thickness and the energy of X-rays, maximal X-ray intensities for 5 and 10 μm thick sections were observed at different accelerating voltages. WR and element-characteristic intensities at 20 kV thus reflect, more or less, the thickness of the section. It should be noted that for 5 and 10 μm thick sections WR and the element-characteristic intensities are the same at 10 kV. This finding suggests that 10 kV electrons can no longer pass through a section thicker than 5 μm. On this assumption, and taking into account that a 20 g% dextran solution with the chosen electrolyte concentrations resembles the composition of biological cells, it can be anticipated that with an accelerating voltage of 10 kV and cells more than 5 μm thick, the X-rays are exclusively generated within the cell.

Table 1 shows the intensity ratios for Na/P, Cl/P and K/P of the dextran standard sections. At first glance these ratios look very similar. However, whereas the Na/P ratio is independent of the section thickness at 10 kV it is inversely related to section thickness at higher accelerating voltages. Since the thickness-dependent reduction in the Na/P ratio is more

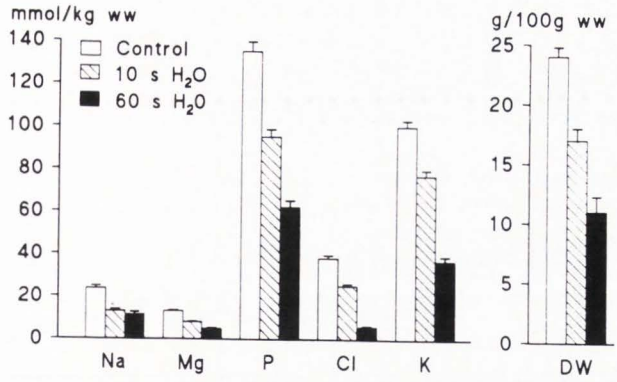

Fig. 5: Cellular element concentrations (mmol/kg wet weight) and dry weight contents (DW) as obtained from freeze-dried cryosections of A6 cells under control conditions and after washing the cultured epithelia for 10 or 60 sec with distilled water.

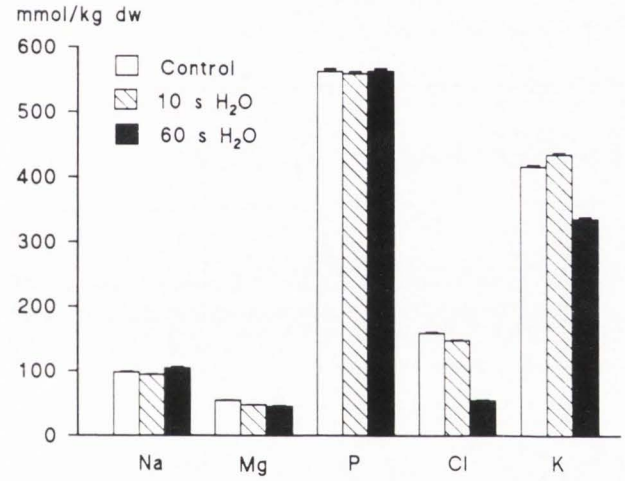

Fig. 6: Cellular element concentrations (mmol/kg dry weight) as obtained from freeze-dried cryosections of A6 cells under control conditions and after washing the cultured epithelia for 10 or 60 sec with distilled water.

pronounced at higher accelerating voltages, at which deeper regions of the thicker sections are excited, the observation is best explained by larger absorption of the low-energy X-rays emitted by Na within thicker sections. Furthermore, the K/P and Cl/P ratios obtained from thicker sections at 10 kV, are significantly smaller than the corresponding ratios from 1-μm thick sections. This difference becomes smaller with increasing accelerating voltage and almost disappears at 15 and 20 kV. The differences in the K/P and Cl/P ratios between the thick and thin specimens at low accelerating voltages are, of course, explained by lower excitation of K and Cl compared with P in the thicker sections. Since we assume that at low accelerating voltages the electrons

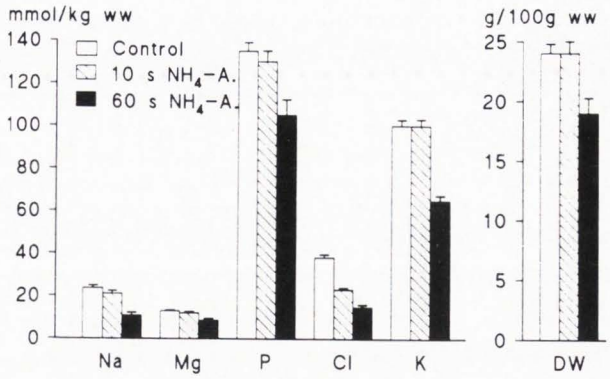

Fig. 7: Cellular element concentrations (mmol/kg wet weight) and dry weight contents (DW) as obtained from freeze-dried cryosections of A6 cells under control conditions and after washing the cultured epithelia for 10 and 60 sec with isotonic NH_4-acetate.

loose almost all their energy in the thick sections, such behaviour must be expected. This result is in accordance with data of Abraham and coworkers (1985), although they found no thickness dependency of K/P ratios according to the thickness in fibroblasts on solid graphite discs. It must be noted that these measurements were obtained at 11 kV, at which the influence of thickness on the K/P ratio should be smaller than at 10 kV, and that local inhomogeneities of K and P concentrations in fibroblasts might be somewhat larger than in standards.

Summarizing the results obtained from the dextran standard sections, the following conclusions can be drawn with respect to biological cells: a) Analyzing relatively large cells at high accelerating voltage leads to an absorption of X-rays emitted by Na. b) The ratio of the elemental characteristic intensities is somewhat dependent on the height or diameter of the cells at low accelerating voltage. c) If cells higher than 5 μm are analyzed at 10 kV, the support does not contribute to the X-ray spectra. The latter point offers the possibility that the WR can be taken as reference for cellular elements; and furthermore that elements within the support do not lead to erroneous results. Thus, low accelerating voltages should be given preference for analysis of freeze-dried cells plated on a thick permeable support.

Cell washing

Cell washing, the most critical point in the analysis of whole cells, is necessary to remove remnants of the incubation medium from the cell surface. Commonly used rinsing solutions are isotonic solutions of sucrose, NaCl, and NH_4-acetate or distilled water. The main criteria usually applied to select a satisfactory rinsing

Table 1. X-ray intensity ratios of dextran standard sections of different thicknesses (T) at different accelerating voltages.

T (μm)	Na/P	Cl/P	K/P
10 kV			
1	0.35±0.07	0.96±0.03	0.91±0.05
5	0.36±0.04	0.88±0.03*	0.75±0.08*
10	0.35±0.03	0.89±0.03*	0.71±0.06*
12 kV			
1	0.33±0.03	1.04±0.08	1.02±0.06
5	0.29±0.01	0.97±0.03	0.91±0.06
10	0.28±0.03	0.96±0.02	0.85±0.03*
15 kV			
1	0.30±0.03	1.08±0.05	1.08±0.08
5	0.26±0.01	1.06±0.03	1.06±0.03
10	0.23±0.03*	1.00±0.08	0.92±0.09
20 kV			
1	0.29±0.02	1.12±0.06	1.18±0.06
5	0.23±0.01*	1.13±0.02	1.18±0.07
10	0.17±0.02*	1.15±0.03	1.21±0.03

Mean values ± SD, (n = 15); * significantly different compared to the data of 1 μm thick sections ($p < 0.05$).

medium are high ratios of K/Na, K/P, and K/S (Abraham *et al.*, 1985; Roomans 1991). These criteria are based on the facts that P and S are mainly bound to the organic matrix and are thus unaffected by the washing procedure and that compared with cell K concentration the Na concentration is low under control conditions. Although these criteria might be useful to select the "best" washing medium, there is no guarantee that even the "best" procedure leaves the cell element composition undisturbed. One possibility for revealing any such perturbation is to determine the absolute cell concentrations in freeze-dried cryosections.

To find an optimal washing procedure for A6 cells grown on permeable supports, measurements were performed on whole freeze-dried cells and on freeze-dried cryosections. Washing the epithelia with isotonic NaCl or sucrose left substantial amounts of the washing medium on the cells. In the case of NaCl the remnants were so substantial that only Na and Cl peaks emerged from the spectra. In the case of sucrose typical cell spectra were recorded only where the sugar layer was thinnest or detached. Figure 3 illustrates such measurements in comparison to data obtained after washing with distilled water. The concentration ratios of

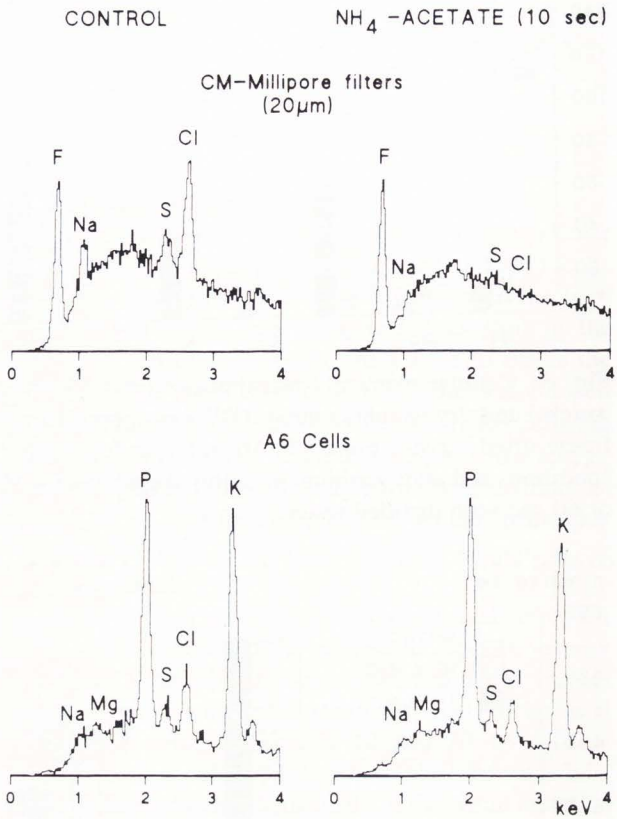

Fig. 8: X-ray spectra of CM-Millipore-filters and A6 cells from freeze-dried cryosections. Measurements were taken from an unwashed preparation and after washing the epithelial sheet for 10 sec isotonic NH_4-acetate.

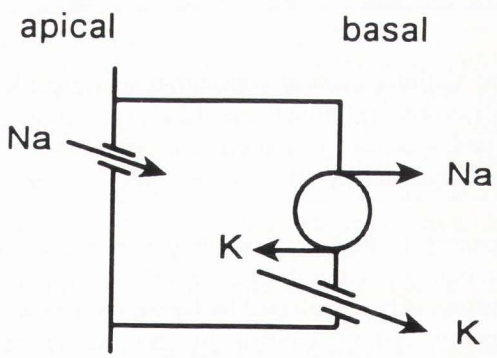

Fig. 9: Model of transepithelial Na transport in tight epithelia.

Cl/P and K/P were significantly smaller after washing with isotonic sucrose. This might indicate that sucrose was still present on the epithelial surface. Based on these results, only distilled water and isotonic NH_4-acetate were further used to investigate the effect of the washing procedure on freeze-dried cryosections.

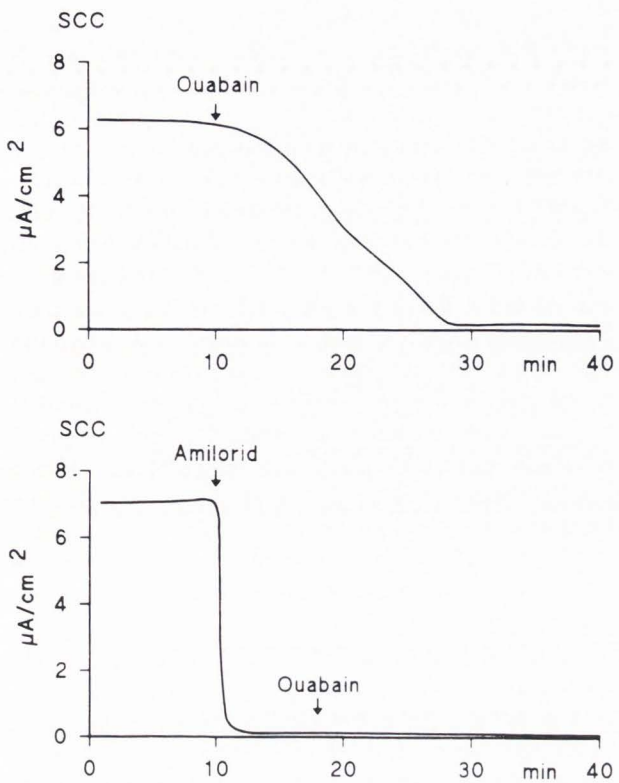

Fig. 10: Short circuit-current (SCC) of A6 epithelia under control conditions, after basal ouabain (10^{-4}M) and after the successive application of apical amiloride (10^{-5}M) and basal ouabain (10^{-4}M).

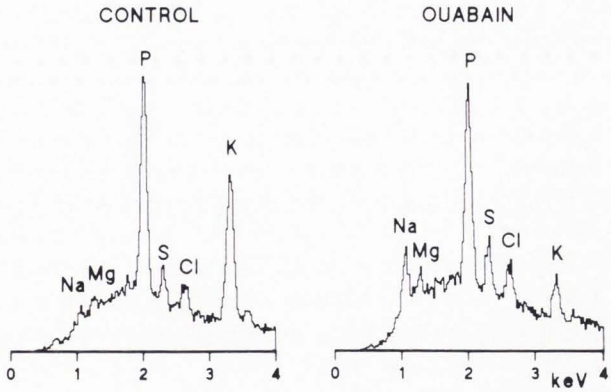

Fig. 11: X-ray spectra of whole freeze-dried A6 cells under control conditions and after basal application of ouabain (10^{-4}M). The preparations were washed for 5 sec with distilled water.

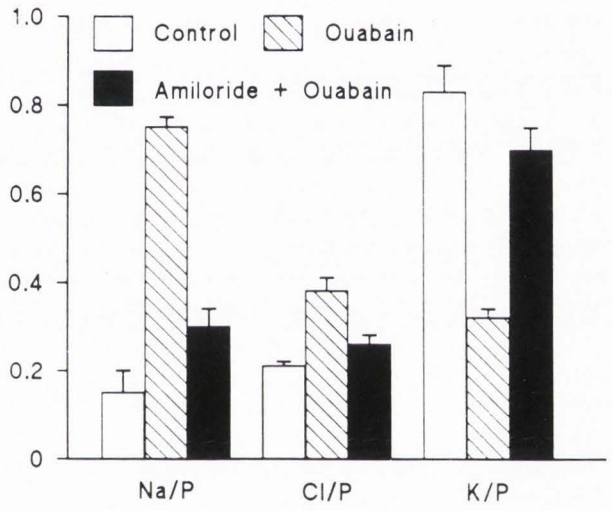

Fig. 12: Elemental contents (Na/P, Cl/P and K/P ratios) of whole, freeze-dried A6 cells under control conditions, after basal ouabain (10^{-4}M) and after the successive application of apical amiloride (10^{-5}M) and basal ouabain (10^{-4}M).

Figure 4 shows a scanning transmission electron micrograph of such a freeze-dried cryosection of an A6 epithelium grown on a 20-μm thick Millipore filter under control conditions. X-ray spectra were recorded from the cells, the adherent albumin standard layer and from the filter. Figure 5 shows the cell element concentrations in mmol/kg ww and the dry weight content of A6 cells under control conditions and after washing for 10 and 60 sec with distilled water. With distilled water washing, both element concentrations and dry weight content decreased with increasing washing time. The decrease in the dry weight content indicates cell swelling due to water influx. The cells were swollen by about 40% after 10 sec and by about 120% after 60 sec.

To test whether the decrease in the cell element concentrations are solely caused by water uptake, the element concentrations were expressed in mmol/kg dry weight as shown in Fig. 6. The dry weight concentrations of Na, Mg and P were not affected by the washing procedure. This suggests that even washing for 60 sec does not lead to loss of these elements from the cells. This also seems to hold for Cl and K after washing for 10 sec. However, after distilled water washing for 60 sec, the Cl and K concentrations were significantly reduced (by about 90 mmol/kg dry weight), indicating a substantial loss of these elements. Such a loss might be explained by high cell membrane permeability for both K and Cl under such conditions. As demonstrated by cellular potential measurements (Granitzer *et al.*, 1992, 1993), A6 cells exhibit a high K, but under control conditions negligible Cl conductance. Cell swelling caused by hypoosmotic media leads to a drastic increase in the basolateral Cl

conductivity. It is thus possible that opening of Cl conductive channels due to cell swelling accounts for Cl loss after distilled water washing.

The essential conclusion from these results is that brief washing of A6 cells with distilled water does not lead to substantial loss of cell elements. Concentration ratios obtained from whole, freeze-dried cells should thus not be affected by this washing procedure. This seems not to be the case if A6 cells are washed with isotonic NH_4-acetate (Fig. 7). Although the dry weight content and the concentrations of Na, Mg, P and K were hardly affected by a 10 sec wash with NH_4-acetate, the Cl concentration was significantly decreased. After a 60 sec wash the dry weight content and the concentrations of all elements were significantly reduced indicating cellular swelling, possibly due to cell uptake of acetate and water (Macknight *et al.*, 1988). At the moment no reasonable explanation can be offered for the loss of only Cl after the 10 sec wash with NH_4-acetate. Further experiments on tissue sections, washed weither in isotonic sucrose or NH_4-acetate, may reveal either Cl loss is a specific effect of NH_4-acetate.

Another problem which might be of importance in analyzing small, whole, freeze-dried cells is the element composition of the support. The question arises as to whether the electrolytes of the incubation medium are completely washed out of the filter. Figure 8 shows X-ray spectra obtained from relatively thin (20-μm thick) Millipore CM-filters before and after washing the preparation for 10 sec in isotonic NH_4-acetate. For comparison, spectra of adjacent epithelial cells are also given. Without washing, the spectrum from the filter exhibits much higher Na and Cl peaks than that from the cell. After washing the Na and Cl peaks in the filter spectrum were reduced to almost zero. On average, the Na and Cl concentrations decreased to 2.9 $\pm$ 1.6 and 3.4 $\pm$ 1.1 after a 10 sec wash and to 0.1 $\pm$ 0.1 and 3.9 $\pm$ 2.2 mmol/kg ww after a 60 sec wash, respectively. The spectra of the filters also disclose the presence of F originating from the support material. This might be of practical significance in analyzing whole freeze-dried cells since the appearance of such a F peak would indicate that the underlying support was excited. Similar results as with NH_4-acetate were obtained with distilled water. In this regard it should be mentioned that the elution of incubation electrolytes from a much thicker permeable support (Millipore-filter, 100 μm) was much less successful. For example, compared with control (unwashed filters) the Na concentration decreased from 105.9 $\pm$ 25.6 to 33.3 $\pm$ 2.2 after 10 sec and to 17.0 $\pm$ 4.8 mmol/kg ww after 60 sec washing with NH_4-acetate.

Transepithelial Na-transport

The continuous A6 cell line has become an important model for studying transepithelial Na transport. Figure 9 shows the Na transport model derived from investigation of Na-transporting, tight epithelia. As in other tight epithelia, Na enters A6 cells passively via apical Na channels and is then actively extruded across the basal membrane by the Na/K-pump. The passive entrance step can be blocked by the diuretic amiloride (Perkins and Handler, 1981) and the active exit step by the glycoside ouabain. To characterize further the Na transport compartment, we determined the cell electrolyte contents on whole freeze-dried sheets of A6 cells before and after blocking transepithelial Na transport with ouabain or amiloride. The epithelia grown on 20-μm thick CM-filters were washed for 5 sec with distilled water and analyzed at 12 kV accelerating voltage.

Figure 10 shows a typical short circuit current (SCC) measurement performed in Ussing-type chambers under control conditions and after blocking the transepithelial Na transport by ouabain and amiloride. After application of ouabain to the basal side the SCC, as a measure of transepithelial Na transport decreased within 20 min to almost zero. Apical application of amiloride decreased the SCC within 1 min to almost zero; additional application of ouabain had no further effect. On average, the SCC decreased after ouabain from 8.8 $\pm$ 3.0 to 1.7 $\pm$ 1.7 $\mu A/cm^2$ and from 8.2 $\pm$ 3.6 to 0.44 $\pm$ 0.75 $\mu A/cm^2$ after amiloride.

Typical X-ray spectra obtained from whole cell preparations under control conditions and after ouabain are given in Fig. 11. Compared with control spectrum the spectrum after ouabain shows higher Na and Cl peaks and a lower K peak. Figure 12 shows the contents of Na, Cl and K referred to P under control conditions, after ouabain and after sequential application of amiloride and ouabain. After ouabain the Na content increased from 0.15 to 0.75 and the K content decreased by almost the same amount from 0.83 to 0.32, indicating exchange of intracellular K for extracelluar Na. The concomitant increase in the Cl content after ouabain might be due to some degree of cell swelling caused by an influx of NaCl and water. The ouabain-induced electrolyte changes were almost completely prevented when amiloride was applied prior to ouabain.

From these results it can be concluded that Na enters the cell mainly by the amiloride-sensitive Na channels in the apical membrane. This also implies that the Na permeability of the basolateral membrane of A6 cells is relatively low. These patterns, which are different from those of Na-transporting mammalian epithelia such as rabbit urinary bladder (Dörge *et al.*,

1988) or colon (A. Dörge, unpublished results), which exhibit high basolateral Na permeability, are in good agreement with data obtained from skin and bladder of frog and toad (Rick *et al.*, 1978a, 1978b; Rick *et al.*, 1988).

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Discussion with Reviewers

T. von Zglinicki: Can your cell preparation be regarded as having a flat surface? If not, how do you cope with surface orientation-dependent absorption of low energy X-rays?
Authors: Pictures in the literature and our own sections imply some differences in cell height from one place to another in cultured A6 epithelia. However, the differences between neighbouring cells are rather small. Furthermore, taking into account that the specimen is tilted to the detector by 15° and that the detector is located about 1 cm above the specimen, site-dependent differences in the absorption of low energy X-rays should be unimportant.

T. von Zglinicki: What is the driving mechanism for the Cl influx and the resultant cell swelling under ouabain? Could the Cl increase be indicative for a deviation from equimolarity of Na/K exchange?
Authors: It is conceivable that A6 cells are depolarized after ouabain with consequent passive Cl uptake via Cl channels. Since this Cl has to be accompanied by a cation (possibly Na) the overall exchange of cellular K with extracellular Na is not equimolar.

R. Wróblewski: In Figs. 5 and 7 you present very interesting wet weight measurements. Why do you then present your experimental data (Fig. 12) only as ratios? Knowledge of the final absolute concentrations presented in mmol/kg wet weight is of great interest especially after the swelling of the cells described by you.
Authors: Figs. 5 and 7 show data from freeze-dried cryosections covered with an albumin standard solution and quantification in terms of element concentrations (mmol/kg ww) is straightforward. In contrast, Fig. 12 presents results from whole freeze-dried cells where only molar concentration ratios can directly be derived from the X-ray data.

R. Wróblewski: Epithelial cells are highly polarized cells with a basal nucleus and apical cytoplasm. Therefore, analytical data are arising from the apical cytoplasm and the nucleus. However, depending of the height of the cells and location of the nucleus, the content of the analyzed cell will vary. Therefore, in Fig. 12 one should use not only the ratio to P but also that to S. Why is sulfur omitted in most of your results?
Authors: Measurements on cryosections have demonstrated certain differences in Na, K, Cl and P concentrations between nucleus and cytoplasm of epithelial cells. The differences, however, are not very large (approximately 10%) so that varying contributions of nucleus and cytoplasm to the volume excited during analysis should not notably influence the results. S was not taken as reference element since the measurements were not performed on a cold stage so that most of the cell S disappears during analysis.

G.M. Roomans: Do you, e.g., from TEM data, know the actual thickness of A6 cells?
Authors: According to scanning transmission micrographs of freeze-dried cryosections the mean thickness of A6 cells was 6 μm.

G.M. Roomans: Have you carried out rinsing experiments shorter than 10 seconds (we routinely rinse only 2-4 seconds)?
Authors: A6 epithelia prepared for whole cell analysis were usually washed for 5 sec with distilled water. The data obtained from these preparations (Figs. 11 and 12) were not significantly different from those of epithelia washed for 10 sec.

G.M. Roomans: Do you propose exchange of Cl for acetate to explain your results?
Authors: An exchange of cellular Cl with extracellular acetate could of course explain the Cl loss after washing the cells for 10 sec with isotonic NH_4-acetate. Direct evidence, however, for such a mechanism is not yet available.