J. Membrane Biol. 123, 115–132 (1991)
002226319100113Q

The Journal of
Membrane Biology

Effects of Potassium-Free Media and Ouabain on Epithelial Cell Composition in Toad Urinary Bladder Studied with X-Ray Microanalysis

Joanne M. Bowler, Robert D. Purves,† and Anthony D.C. Macknight
Departments of Physiology and †Pharmacology, University of Otago Medical School, Dunedin, New Zealand

Summary. The technique of X-ray microanalysis was used to study the composition of toad urinary bladder epithelial cells incubated in Na Ringer's and K-free medium, with and without ouabain. Following incubation under short-circuit conditions, portions of tissue were coated with an external albumin standard and plunge-frozen. Cryosections were freeze-dried and analyzed. In Na Ringer's, granular and basal cells, and also the basal portion of the goblet cells, had similar water and ion compositions. In contrast, mitochondria-rich cells contained less Cl and Na. On average, the granular cells and a subpopulation of the basal cells lost K and gained Na after ouabain and in K-free medium alone. However, there was considerable variation from cell to cell in the responses, indicating differences between cells in the availabilities of ion pathways, either as a consequence of differences in the numbers of such pathways or in their control. In contrast, the compositions of both the low Cl, mitochondria-rich cells and a sub-population of the basal cells were little affected by the different incubation conditions. This is consistent with a comparatively low Na permeability of these cells. The results also indicate that (i) much, if not all, of the K in the dominant cell type, the granular cells, is potentially exchangeable with serosal medium Na, and (ii) Na is accumulated from the serosal medium under K-free conditions. They also provide information about the role of the (Na-K)-ATPase in the maintenance of cellular K in K-free medium, being consistent with other evidence that removal of serosal medium K inhibits transepithelial Na transport by decreasing Na entry to the cells from the mucosal medium, rather than solely by inhibiting the basolateral membrane (Na-K)-ATPase.

Key Words toad bladder · ouabain · cell ions · cell volume · X-ray microanalysis

Introduction

An understanding of transepithelial Na transport requires knowledge of the water and ion composition of the transporting cells. Early attempts to define cell composition using standard chemical techniques in a model tight epithelium—the urinary bladder of the toad—were complicated by inclusion of the underlying subepithelial supporting tissues (e.g., Essig & Leaf, 1963; Herrera, 1968). Removal and analysis of the epithelial cell layer alone following incubation of bladders in Ussing chambers overcame this problem, and data for the composition of cells following incubation in Na Ringer's and after a variety of experimental manipulations, including exposure to hormones, amiloride, ouabain and K-free media, were reported (Macknight, DiBona & Leaf, 1980).

Such studies, however, can provide only an average result for all of the cells and give no indication of the effects on the different cell types or of cell to cell variation within any one cell type. Four different cell types can be identified in the toad bladder epithelium (Pak Poy & Bentley, 1960; Peachey & Rasmussen, 1961; Choi, 1963). Three of these—the granular, goblet and mitochondria-rich cells—form a surface layer which is bathed by the urine. The fourth cell type, the basal cell, sits between the basement membrane and the surface cells and does not make contact with the urine (DiBona, Civan & Leaf, 1969). Gap junctions are found only between adjacent basal cells and between basal cells and granular cells (Wade, 1978). The surface cells make up 70% of the cells in the epithelial layer, while the basal cells contribute the remaining 30% (Kraehenbuhl et al., 1979). In bladders from Dominican toads, 80% of the surface cells are granular cells (DiBona, 1978) responsible for transepithelial Na transport (Macknight et al., 1980) and 13% are mitochondria-rich cells (DiBona, 1978), with the remainder being goblet (or mucous) cells. The basal cells have been subdivided into two populations (Kraehenbuhl et al., 1979), the commoner, microfilament-rich cells and undifferentiated cells.

X-ray microanalysis allows examination of the behavior of individual cells in a tissue and overcomes another major disadvantage of chemical analysis, namely the need to determine and correct for extracellular contamination when deriving cell composition. In practice, there are a variety of problems

in applying X-ray microanalysis to epithelia. The ways that they were dealt with are discussed in Materials and Methods.

There are few X-ray microanalysis studies of toad urinary bladder (Rick et al., 1978*b*; Civan, Hall & Gupta, 1980; Rick & DiBona, 1987; Rick, Spancken & Dörge, 1988). This paper presents the results from a large number of measurements in tissues incubated in Na Ringer's under short-circuit conditions to define cellular composition. The effects of incubation in K-free medium and in media containing ouabain, provide information about (i) the nature and the extent of the responses of the different cell types to changes in medium composition; (ii) the exchangeability of the granular cell K, (iii) the source of cell Na during incubation in K-free medium; and (iv) how removal of serosal medium K may inhibit transepithelial Na transport.

Materials and Methods

Incubation Media

Na Ringer's contained (in mM): Na^+, 115; K^+, 3.5; Cl^-, 116.5; Mg^{2+}, 1; Ca^{2+}, 1; SO_4^{2-}, 1; glucose, 10; buffered to pH of 7.8 by HPO_4^{2-}, 2 mM. K-free medium contained (in mM): Na^+, 118.5; Cl^-, 116.5; Mg^{2+}, 1; Ca^{2+}, 1; SO_4^{2-}, 1; glucose, 10; also buffered to pH 7.8 by HPO_4^{2-}. Ouabain (Sigma) was dissolved in the appropriate media to a final concentration of 1 mM. In K-free, Na-free isosmotic medium, *n*-methyl D-glucamine [NMDG] Cl replaced NaCl and KCl and the medium was buffered to pH 7.8 by NMDG phosphate (2 mM).

The osmolalities of all solutions (220–230 mosmol/kg H_2O) were measured with a Wescor vapor pressure osmometer.

Choice and Preparation of External Standard

To quantify the results, the peripheral standard technique was used (Rick, Dörge, & Thurau, 1982). Albumin was preferred to high molecular weight dextrans (Civan et al., 1980), as a considerable loss of mass from dextran samples and much smaller losses from albumin and from cells were observed in preliminary experiments. Bovine albumin (RIA grade—Immuno Chemical Products) was dialyzed for 48–60 hr to remove any diffusible ions present, freeze dried at −70°C and stored at 4°C. Peripheral standards were prepared as a 30% (wt : vol) albumin solution in the appropriate unbuffered Ringer's solution. The water content was determined gravimetrically. Ions were extracted from each dried sample into 10 ml of 3 mM Li in 0.1 M HNO_3 over 48 hr at room temperature. Na and K were measured by flame photometry and Cl by conductometric titration.

Animals and Incubation

Dominican toads (*Bufo marinus*) obtained from National Reagents, Bridgeport, CT, were kept on wood shavings with free access to water and regularly force-fed with ground liver. The toads were double-pithed and the two hemibladders removed.

Hemibladders were pinned, mucosal side upwards, onto a wax-coated Petri dish filled with Na Ringer's solution. Four plastic rings (area approx. 1 cm^2) were slid under each hemibladder, and an O-ring was placed over the top of each ring to hold the tissue in place. Care was taken at every stage to prevent excessive stretching of the tissue and to avoid any contact with the mucosal surface. In most studies each ring was held upright in a beaker of aerated medium and incubated at room temperature (18–20°C). Under these conditions, tissues remain continuously short circuited. Alternatively, rings were inserted in modified Ussing chambers and the short-circuit current (I_{sc}) measured.

Initially, tissues from four toads were incubated in Na Ringer's for 50 to 70 min before being taken for freezing and analysis. In a second set of experiments using bladders from four toads, incubation was begun in Na Ringer's; after 15 min tissues were rinsed with Na Ringer's, and then the medium was replaced by either fresh Na Ringer's, Na Ringer's + ouabain, K-free medium or K-free medium + ouabain. Immediately after the introduction of K-free media, the solution was replaced 3–4 times. It was replaced again after 15 min. No K was detected by flame photometry in the K-free media at the end of the incubations. Different pieces from the same bladder were exposed to each of the media in every experiment. The tissues were incubated under these new conditions for a further 50 to 70 min. Previous studies in toad bladder have shown that this time is sufficient for the complete inhibition of I_{sc} following exposure to ouabain (Macknight, Civan & Leaf, 1975*b*) or to K-free medium (Robinson & Macknight, 1976*a*) and this was confirmed in preliminary experiments.

To determine the source of the cell Na in K-free medium, portions of bladders from two toads were mounted on rings, incubated in Ussing chambers and short circuited. After 15 min, mucosal Na Ringer's was replaced by K-free medium (with or without amiloride) and serosal Na Ringer's by either K-free or K-free, Na-free medium.

After all incubations, rings were removed and very gently touched with Whatman 542 filter paper on both the mucosal and serosal surfaces to remove excess solution. Albumin was applied to the mucosal surface with a Pasteur pipette and the excess removed with filter paper.

To assess the possibility of tissue damage during these procedures, and also to examine whether measurable movements of ions between the medium and cells occurred during freezing or processing, the standard albumin solution contained RbCl (115 mmol/liter) instead of NaCl. Bladders from two toads were incubated in Na Ringer's solution for 30 min. The removal of the excess incubation medium and albumin was handled in one of two ways: (i) the excess Ringer's solution and the albumin were both removed by draining onto filter paper held at the edge of the ring, with the filter paper never touching the mucosal surface within the ring; (ii) the tissue was gently blotted, as above.

Freezing and Cryosectioning

Within 20 sec of removal from the incubation medium, the plastic ring with its tissue was plunged into liquid propane and isopentane [4 : 1] (Jehl et al., 1981). Under liquid nitrogen, a fragment was broken off the frozen tissue sample, wrapped in indium foil and clamped in the chuck of a modified Cryocut I (Burlington Scientific Instruments) fitted to a Sorvall MBT-2 ultramicrotome. Sections (0.3 to 0.5 μm thick) were cut at slow speeds with glass knives (knife temperature −80 to −90°C). Two or more blocks from each piece of frozen tissue were cut and several sections from each block analyzed.

Table 1. The composition of the different cell types

Cell type	*n*	Na	K (mmol/liter)	Cl	P	Dry mass (g/liter)
Granular	63	28 ± 4	108 ± 6	34 ± 2	151 ± 6	250 ± 11
Mitochondria rich	7	9 ± 3	108 ± 14	17 ± 5	139 ± 24	248 ± 20
Goblet	3	23 ± 9	116 ± 29	29 ± 5	155 ± 33	238 ± 18
Basal	20	27 ± 4	163 ± 16	42 ± 5	196 ± 18	307 ± 23

Data expressed per liter of tissue (*see* Materials and Methods) is the mean ±SEM of the number of cells analyzed from sections from four toads.

Table 2. The average composition of the epithelial cells following incubation in Na Ringer's (low Cl cells omitted)

	Na	K	Cl	P
mmol/liter tissue	18 ± 1	156 ± 2	42 ± 1	182 ± 3
mmol/liter cell H_2O	25 ± 1	217 ± 3	57 ± 1	
mmol/kg dry wt	70 ± 2	611 ± 5	164 ± 2	702 ± 8
Water		3.07 ± 0.04 kg/kg dry wt		
Dry mass		261 ± 2 g/liter		

n = 766; bladders were obtained from eight toads. Combined data from both studies is the mean ±SEM.

Sections were sandwiched between a Formvar-coated 3 mm nickel slot grid and a Formvar-coated aluminium specimen holder, transferred to the stage (precooled to below −180°C) of a scanning electron microscope (JEOL JSM-840) and freeze dried at 10^{-4} Pa by warming the stage to room temperature over 10 to 15 min.

Analysis of Sections

Sections were imaged with a transmitted electron detector. Spectra were collected for 100 sec at 15 kV with a Tracor Northern X-ray 30 mm^2 detector connected to a Nucleus AD converter in an Apple *IIe* microcomputer. The probe current (150–250 pA) was measured with a Faraday cup after each spectrum. In the absence of a beam blanking system, a potentially serious source of error in an SEM whose scan generator is phase-locked to the mains frequency is that, for a substantial fraction of the time, the beam is stationary over the regions of the specimen outside the field of view. An auxiliary scan generator free from this defect was developed and used during spectrum acquisition.

Spectra were collected from at least two relatively large areas of albumin above the cells. Data from these areas was pooled to provide an average value. In the epithelial layer, fragments of cells without nuclei and also edges of cells were avoided to reduce the possibility of extracellular contamination. To obtain average cellular values, the area scanned included cytoplasm with a portion of the nucleus. Only one measurement was taken from each cell in any section, except in a relatively few instances where the nucleus and the cytoplasm were measured separately.

Data Analysis and Quantification

Elemental peaks were quantified by filtered least-squares fitting (Apple IIe and Macintosh computers) to a library of mono-elemental peaks (Fuchs & Fuchs, 1981). Library spectra for Na, Mg, Si, P, S, Cl, K and Ca were derived from microcrystals produced by spraying atomized droplets of the appropriate salts onto a Formvar film. Only the smallest of the crystals were analyzed. The relative detector sensitivity to each element was determined by pairwise comparisons in spectra from crystals containing two of the elements. Energy ranges for the library encompassed the *K* lines of Na (0.80–1.32 keV), Mg (1.00–1.52 keV), Al (1.22–1.74 keV), Si (1.48–2.06 keV), P (1.76–2.38 keV), S (2.06–2.72 keV), Cl (2.34–3.10 keV), K (3.08–3.86 keV), Ca (3.42–4.26 keV) and Ni (7.16–8.54 keV) and the *L* lines of Ni (0.54–1.12 keV). White counts were summed over the range 4.6–6.0 keV and corrected for spurious contributions from the Al specimen holder and Ni grid.

Electronic drift of gain and offset in all spectra was corrected numerically (Fuchs & Fuchs, 1981) by reference to a 200,000-count Cu/Al calibration spectrum obtained each day. Absolute concentrations were determined by normalization to the known concentration of Na in the peripheral standard (Rick et al., 1982). The primary outcome of this calculation is a concentration expressed in mmol per liter of tissue. Given a tissue density of 1000 g/liter, this is equivalent to mmol/kg wet wt as it is often expressed. Other representations such as ion content on a dry-weight basis or ion concentration in the cell water were obtained from the measured dry mass of the tissue (as calculated from the white counts in standard and tissue spectra, together with the known density of the standard).

Results

To assess the accuracy of data collection, analysis and quantification, results for Na and K from X-ray microanalysis of sprayed microdroplets of dextran

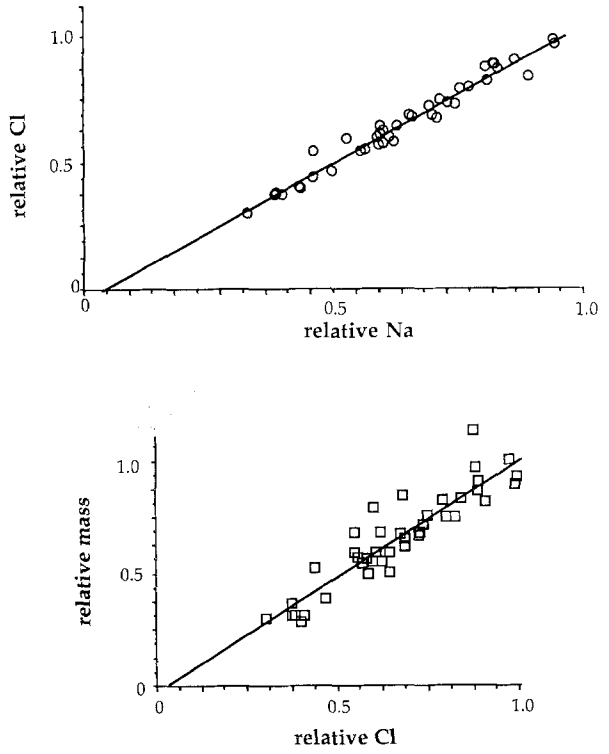


Fig. 1. Relationships between relative ion contents and between relative Cl content and relative mass in albumin droplets. Each point represents a single microdroplet

[10% Dextran containing a range of different concentrations of the nitrate salts of Na and K (0–200 mM) and a fixed LiCl concentration as an internal standard] were compared to those from flame photometry of the parent solutions. There was excellent correlation between the two measurements, with r values of 0.994 for Na and of 0.993 for K. Sprayed microdroplets of albumin, all of the same composition but of different thickness, were also analyzed (Fig. 1). Ideally, all data points should lie on the same line. Deviations from this reflect the inherent variability in the measurements. There is an excellent correlation (r = 0.983) between the relative Cl and Na contents, with a somewhat greater scatter between relative mass and Cl (r value of 0.916 for mass against Cl), illustrating the fact that mass measurements, which rely upon determination of a portion of the continuum, are somewhat less precise than the measurements of elemental peaks.

Assessment of Possible Tissue Damage Prior to Freezing

Figure 2*a* shows a spectrum from the Rb-albumin applied to the surface of the tissue. As illustrated (Fig. 2*b*), there was no subsequent movement of Rb

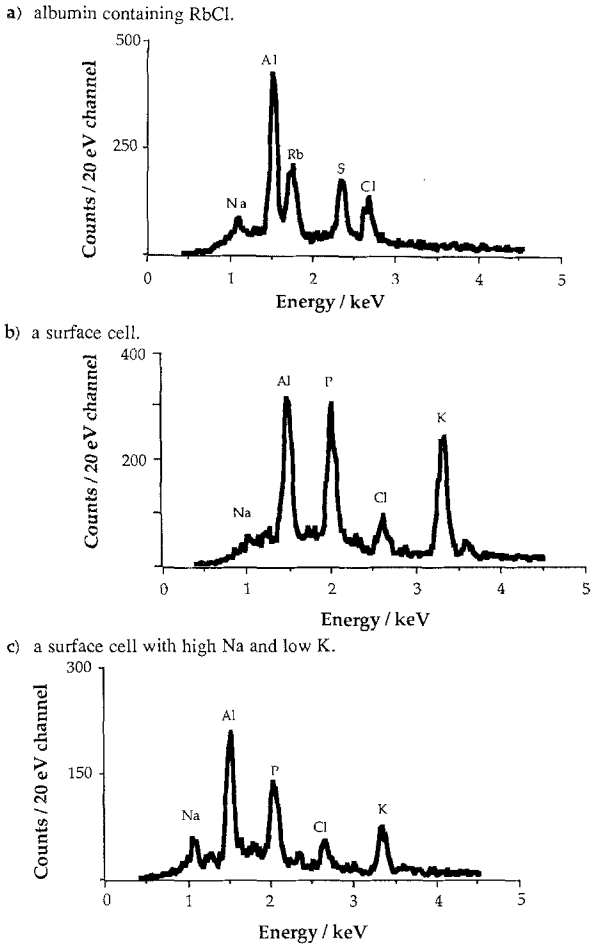


Fig. 2. Spectra from the albumin and from epithelial cells following the application of albumin containing Rb to the mucosal surface

into surface cells following blotting. Furthermore, the few cells identified in the sections from blotted tissue that had a high intracellular Na and low intracellular K (that are discussed later) had not accumulated any Rb (Fig. 2*c*). Thus, there was no evidence that Rb entered the cells to any extent over the 20 sec that they were exposed to the Rb-albumin solution following incubation and prior to freezing.

Appearance of Cryosections

Figure 3 shows a cryosection prepared for X-ray microanalysis after incubation of tissue in Na Ringer's. When the epithelium was cut in transverse section, as in most of Fig. 3, the cell types could often be identified: the granular cells by the dense granules in the apical cytoplasm and by the shape of the cell: the goblet cells by the very prominent mucous granules, and the basal cells by their position be-

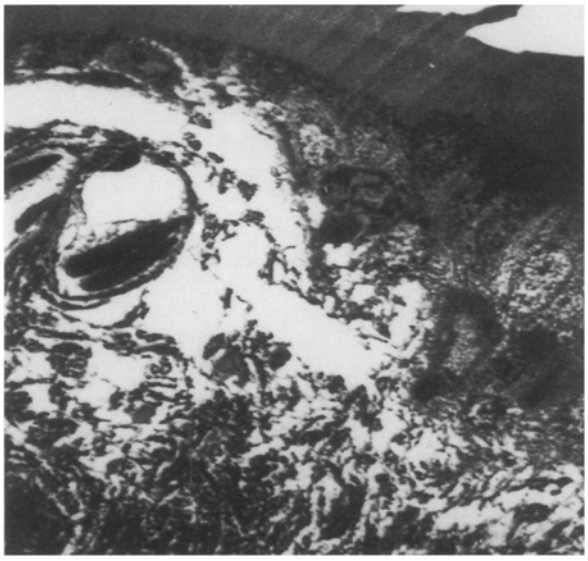


Fig. 3. An electron micrograph of a freeze-dried cryosection from toad bladder epithelium. This illustrates the quality of freezing regularly obtained by plunging tissue into a propane-isopentane mixture. On the surface of the cryosection is a layer of albumin which appears as a homogeneous layer overlying the tissue. An epithelial cell layer can be seen between the albumin and the deeper submucosal tissues

tween the surface cells and the basement membrane. The most difficult cells to distinguish were the mitochondria-rich cells. In some sections they could be identified by their distinctive flask-shape, with the narrow neck sitting near the surface of the bladder, but this required that the cell was sectioned through its apex. Otherwise, they simply appeared as rounded cells beneath the surface. Attempts to identify cells were often defeated, however, by the plane of the section. In sections cut at an oblique angle, as on the right side of Fig. 3, the epithelial layer appears as several cells thick and the normal relationship between surface and basal cells is lost. Also, in bladders stretched at the time of freezing, the epithelium appeared as a single layer of squamous cells whose dimensions were so small that they could not be analyzed because of the close proximity of the basement membrane and submucosa with its high content of Na and Cl.

Cell Composition

Spectra collected under control conditions from the albumin layer and from the different types of epithelial cell are seen in Fig. 4. The relatively high Na and Cl and small K peaks in the albumin contrast with the low Na, high K and lower Cl peaks in the granular and basal cells (Fig. 4*b* and *c*) and in the basal portion of the goblet cells (Fig. 4*d*). Unlike the spectra from the other cell types, that from the mitochondria-rich cell was characterized by a negligible Cl peak (Fig. 4*e*). Occasionally, a surface cell was found with a high Na and low K (Fig. 4*f*).

Figure 5 illustrates the frequency distributions for water and ions for all of the 836 normal epithelial cells analyzed from tissues incubated in Na Ringer's. Table 1 presents data from several transverse sections from one hemibladder in which it was possible to identify the cell types with confidence. The data for the goblet cells was obtained from their basal portion which is free from mucus granules. The major difference between the types was that, on average, the mitochondria-rich cells had lower Cl and Na contents than the others. Low-Cl cells were occasionally seen that could not be positively identified on morphological grounds as mitochondria-rich cells, and, given the difficulties of cell identification, it is also possible that not all mitochondria-rich cells are low in Cl. Altogether, in both groups of control data, 70 out of 836 cells could be classified as 'low-Cl' cells (Cl $<$ 75 mmol/kg dry wt). These averaged water content 2.55 $\pm$ 0.11 kg/kg dry wt, Na 48 $\pm$ 4, K 511 $\pm$ 21, Cl 42 $\pm$ 2, and P 681 $\pm$ 28 (all mmol/kg dry wt).

Table 2 summarizes the average composition of the remaining 766 cells incubated in Na Ringer's. The results are dominated by measurements from granular cells. However, because of the difficulties of recognizing the cell-types, it is likely that the measurements include some basal cells. Goblet cells were excluded from this analysis.

The occasional section had abnormal cells immediately beneath the albumin layer. Usually only a few such cells were seen. Their lateral margins were sometimes indistinct and nuclei not always apparent. Rarely, there was a continuous layer of these cells. When several blocks of tissue were cut from the same hemibladder, such abnormal cells did not appear in every section. They seem, therefore, to occur rather irregularly over the bladder. In the majority of the bladders, no such cells were found in any of the sections. On analysis, such cells had high Na and Cl contents associated with a lowered K content and somewhat reduced P content, as illustrated in the spectrum in Fig. 4*f*. The underlying cells were of normal composition. The average composition of 37 of these unusual cells taken from a number of bladders was water content 3.23 $\pm$ 0.24 kg/kg dry wt, Na 388 $\pm$ 19, K 123 $\pm$ 17, Cl 269 $\pm$ 18, P 472 $\pm$ 25 (all mmol/kg dry wt).

Sites in both the nucleus and the cytoplasm were analyzed in 32 cells (Table 3). Several significant differences in composition between the cytoplasm and the nucleus were noted. The nucleus had a lower

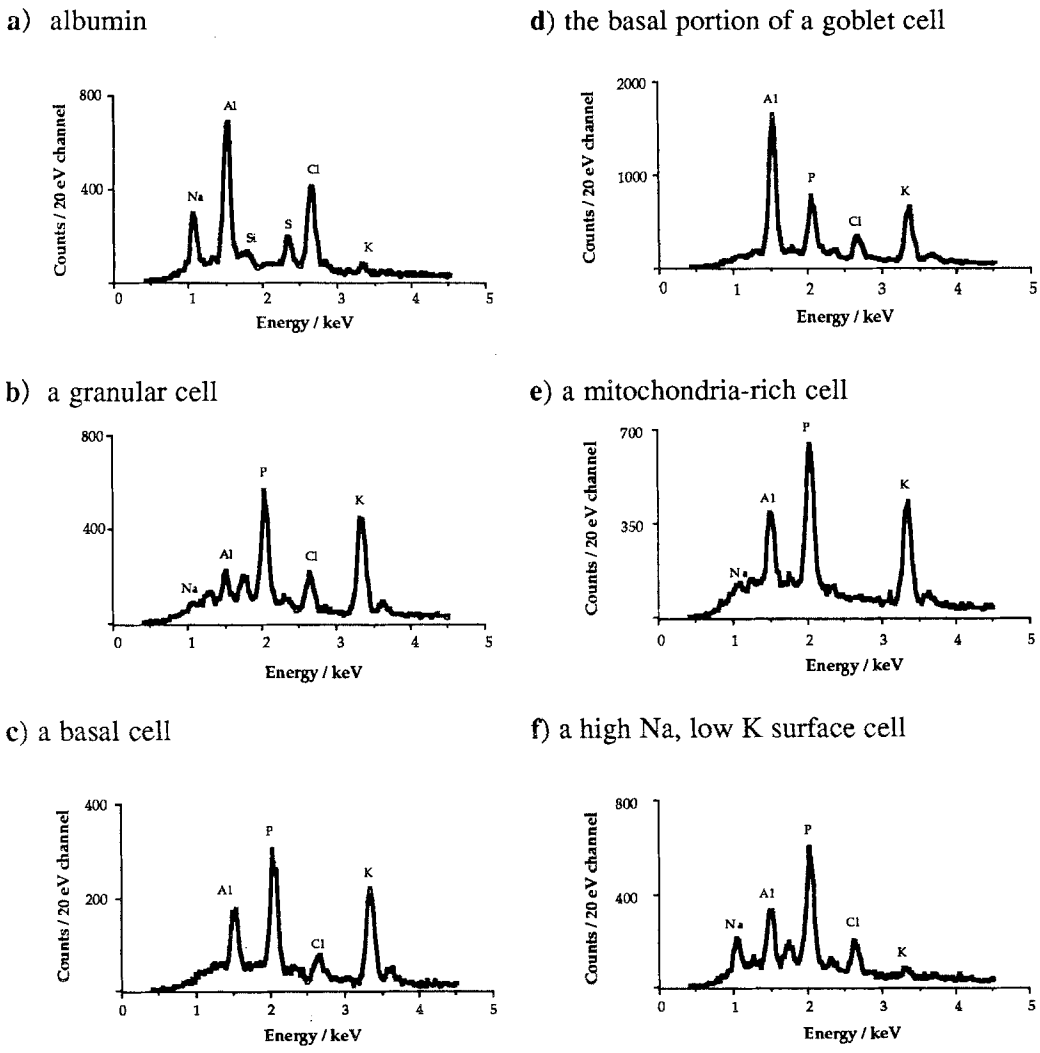


Fig. 4. Typical spectra from the albumin and from the different cell-types

Cl content (mmol/liter wet wt) and a lower dry mass (g/liter). As reported by others (Rick et al., 1978b; Civan et al., 1980), on a dry weight basis, both the K and P contents were significantly higher in the nucleus than in the cytoplasm and, in addition, there was a significantly lower water content in the nucleus. In this study, there were no significant differences in the Na and Cl contents on a dry wt basis.

The only other subcellular structures analyzed were the apical granules in several granular cells and mucus granules in goblet cells. Both contained high levels of S and Ca with low levels of Na, K and P, as reported by others (Rick et al., 1978*b*; Davis et al., 1987).

Effects of K-Free Media and of Ouabain

There were no detectable morphological changes in the granular cells after incubation in K-free media or in media containing ouabain.

K-free Medium + Ouabain

Cells in the epithelium responded differently to the altered medium, as illustrated by representative spectra collected in a single cryosection (Fig. 6). A spectrum from a granular cell (Fig. 6*a*), had a very high Na peak and a low K peak, reflecting the loss

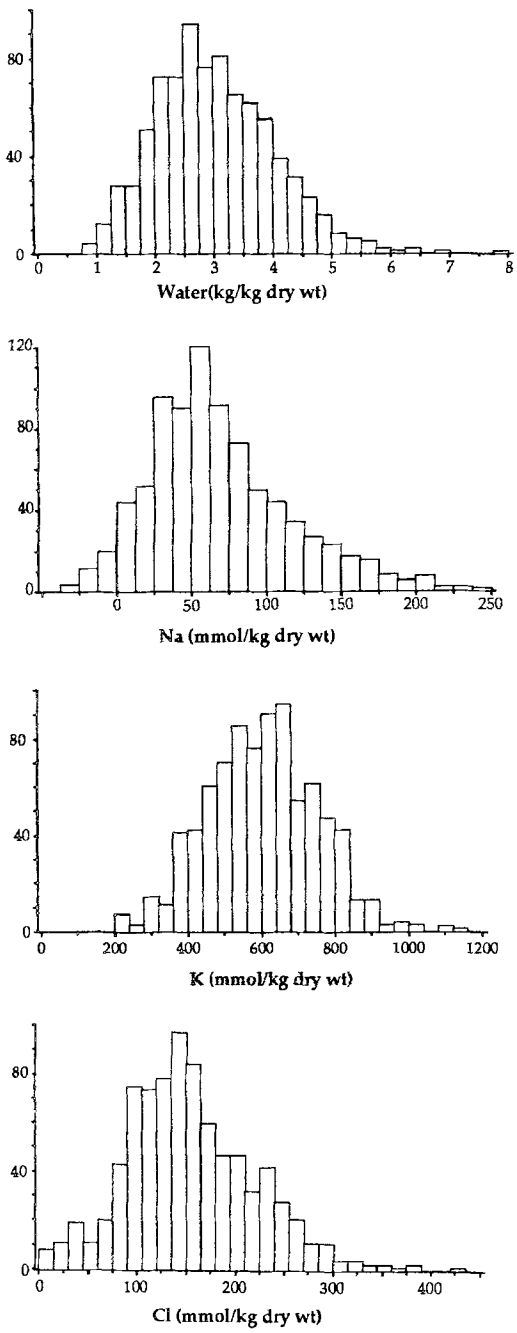


Fig. 5. Frequency distributions for water and ions following incubation in Na Ringer's (n = 836)

of most intracellular K and a large gain in Na. A spectrum from a basal cell (Fig. 6*b*), had a low Na peak with a high K peak and a visible Cl peak, resembling the spectra seen in most of the cell types in Na-Ringer's (Fig. 4*b* and *c*). The spectrum in Fig. 6*c* has a low Na and a high K peak but no apparent Cl peak, an appearance typical of mitochondria-rich cells under control conditions (Fig. 4*e*).

A scatter-plot of cell Na against K (Fig. 7) shows that about 20% of the analyzed cells had a relatively normal cation composition (bold points in the figure). Such cells are referred to subsequently as unaffected cells. The majority of the cells lost K and gained Na and will be referred to as *affected* cells. However, there were appreciable differences in the extent of these changes in the individual granular cells and some of the basal cells. Some cells lost very little K and gained little Na, whereas others lost the bulk of their K which was replaced by Na. These differences in behavior could be quite dramatic, with one cell showing little change in composition situated next to a cell that had lost virtually all of its intracellular K.

Following K-free medium + ouabain (Table 4), the frequency distribution of Na for the affected cells (Fig. 8) has a much greater range than that for the cells in Na Ringer's, with many cells gaining considerable quantities of this ion. The K frequency distribution for this group of cells illustrates that a large number of cells lost most of their K in K-free medium + ouabain. In contrast, the mean water and Cl contents of the affected cells were only a little higher than those in Na Ringer's (Table 4) and there was no greater scatter than that seen under control conditions (illustrated in Fig. 6).

The remaining 20% of the cells (Table 4), consisting predominantly of low Cl cells and a subpopulation of the basal cells, were relatively unaffected. The lower mean Cl content reflects the presence of the low Cl cells in this group.

Incubation in a K-free Medium

Under these conditions, 60% of the cells had exchanged Na for K to a variable extent (Table 5; Fig. 8) with relatively little change in Cl and water. The remainder were relatively unaffected (Table 5). On average, such cells appeared to lose Cl with K, accompanied by water. However, it is difficult to interpret the average behavior of this diverse cell population which, as well as mitochondria-rich cells and a subpopulation of basal cells, must also contain a proportion of the granular cells.

It appears that the Na gained was derived predominantly from the serosal medium. With Na available in the serosal medium, cells lost K (mean K content = 210 ± 15 mmol/kg dry wt, n = 93) and gained Na (Na content = 272 ± 19 mmol/kg dry wt) [Fig. 9]; in a separate set of experiments mucosal amiloride (0.1 mM) did not affect this pattern, although it abolished I_{sc}. But in K-free medium with serosal Na replaced by NMDG (an impermeant cation), mean cell K content (476 ± 10 mmol/kg dry

Table 3. The composition of the nucleus and the cytoplasm in the epithelial cells

A) wet weight

Site	*n*	Na	K	Cl	P	Dry mass
		(mmol/liter)				(g/liter)
Nucleus	32	21 ± 7	154 ± 6	41[a] ± 2	151 ± 9	252[a] ± 13
Cytoplasm	32	22 ± 2	170 ± 10	54 ± 2	172 ± 13	381 ± 21

B) dry weight

Site	*n*	H_2O	Na	K	Cl	P
		(kg/kg dry wt)		(mmol/kg dry wt)		
Nucleus	32	3.11[a] ± 0.18	64 ± 12	605[a] ± 22	167 ± 10	605[a] ± 26
Cytoplasm	32	2.02 ± 0.22	67 ± 10	477 ± 19	166 ± 13	458 ± 21

[a] Significant difference between nucleus and cytoplasm; $P < 0.001$.
Data is the mean ±SEM of the number of cells analyzed from sections from one hemibladder.

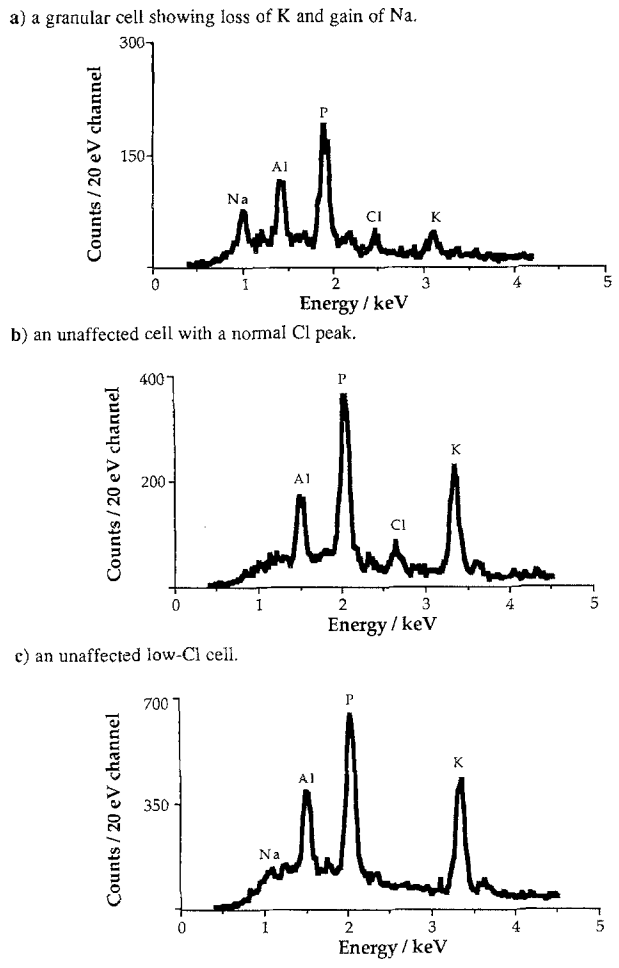


Fig. 6. Spectra from epithelial cells after incubation in K-free medium + ouabain (1 mM)

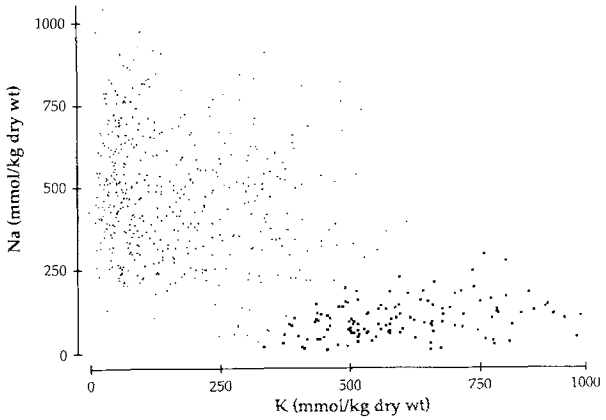


Fig. 7. A scatter-plot of Na against K following incubation in K-free medium + ouabain. A population of relatively unaffected cells is shown by the bold symbols

wt, $n = 67$) remained high and Na (39 ± 3 mmol/kg dry wt) was actually somewhat lower than under control conditions.

Of interest is the fact that in these experiments the combination of a K-free, Na-free serosal medium was associated with an attenuation of the initial increase in I_{sc} that follows exposure to a serosal K-free medium alone (Robinson & Macknight, 1976*a*), together with a persistent residual I_{sc} (Fig. 10). This residual I_{sc} was abolished immediately by mucosal but not serosal amiloride (0.1 mM) and more slowly by serosal ouabain (1 mM). A similar maintenance of I_{sc} with choline substituted for serosal Na in K-free medium has been reported (Essig & Leaf, 1963; Robinson & Macknight, 1976*a*).

Table 4. Effects of K-free medium + ouabain (1 mM) on cell composition

	n	H_2O (kg/kg dry wt)	Na	K (mmol/kg dry wt)	Cl
Control	405	2.89 ± 0.05	76 ± 2	587 ± 6	146 ± 2
Affected	478	3.15 ± 0.06[a]	482 ± 8[a]	163 ± 6[a]	179 ± 8[a]
	Δ	**+0.26**	**+406**	**−424**	**+33**
Unaffected	130	3.03 ± 0.09	95 ± 5	594 ± 1	94 ± 5

Values were significantly different from the control values; [a]$P < 0.001$.
Data is the mean ±SEM of the number of cells analyzed from sections from bladders obtained from four toads. Control data is from all cells (other than the low Cl cells) analyzed from tissues incubated in Na Ringer's in this set of experiments. Affected and unaffected cells were identified in a scatter plot (Fig. 7).

Incubation in Na Ringer's + Ouabain

The majority of the cells responded by gaining Na and losing K to some extent, but again there was marked variability in the changes in Na and K amongst this affected group (Fig. 8). As in K-free medium + ouabain, ~20% of the total cells analyzed, including mitochondria-rich and some basal cells showed relatively little change in composition (Table 6).

Discussion

The results presented here can be summarized as follows:

i) In toad bladders incubated in Na Ringer's, the compositions of granular and basal cells, and of the basal portions of goblet cells, were similar. In contrast, mitochondria-rich cells had lower Cl and Na contents.
ii) Incubation in K-free media and in Na Ringer's + ouabain resulted in variable losses of K and gains of Na by granular cells and a subpopulation of basal cells. In contrast, low Cl, mitochondria-rich cells and a subpopulation of the basal cells were hardly affected over the time course of the exposure.
iii) In K-free, Na-free serosal medium, cells did not lose K or gain Na.

The following issues will be discussed: (i) the validity of the data, (ii) the composition of the different cell types under control conditions, (iii) the responses of the different cell types to changes in medium composition, (iv) differences between results from chemical analysis and X-ray microanalysis, (v) the extent to which cell K is free to exchange with extracellular cations, (vi) the source of cell Na in K-free media, and (vii) how removal of serosal medium K may inhibit transepithelial Na transport.

Validity of Data

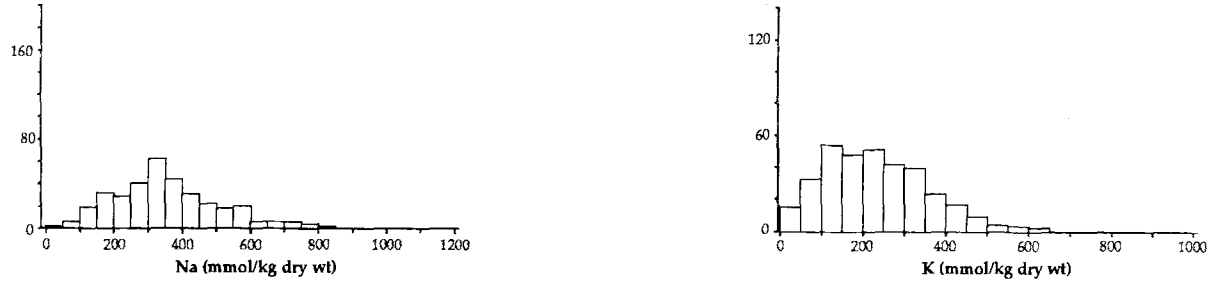
A variety of technical problems can impair the accuracy of measurements made using X-ray microanalysis (Heinrich, 1982). When measurements by flame photometry and X-ray microanalysis of Na and K in dextran droplets were compared, there was an excellent proportionate linear relationship between the data obtained with the two techniques. Also, when albumin droplets of different sizes containing known concentrations of ions and fractional dry mass were analyzed (Fig. 1), there was little spread of the data about the line of equivalence. Over all, the consistency of the data obtained with measurements of the composition of dextran and albumin droplets shows that the amount of variation in measurement due to technical errors was relatively small.

Another possible source of error is ion movements occurring between the extracellular fluid and the cells after incubation. This would be facilitated prior to freezing if apical membranes were damaged during the blotting and application of the albumin. To assess this possibility, cells were exposed to a Rb-albumin solution prior to freezing. There was no evidence that Rb entered the cells over the 20 sec that they were exposed to the solution (Fig. 2). This finding therefore argues against the possibility that acute damage to the apical membrane during the blotting procedure could have resulted in significant alterations in the cell ions. In addition, the exclusion of Rb from the cells also argues against any significant movements of ions between extracellular and cell compartments during subsequent freezing, cryosectioning and freeze drying.

To better understand the contributions of some other sources of error to the scatter in the data (illustrated in Fig. 5), values from tissues incubated in Na Ringer's were subjected to a three-level nested

a) Na Ringer's

b) Na Ringer's + ouabain



c) K-free medium

d) K-free medium + ouabain

Fig. 8. Frequency distributions for Na and K for all normal Cl cells incubated in Na Ringer's and for all affected cells following incubation in K-free media or in Na Ringer's + ouabain. Note that the same scales are used for each Na and K histogram. (Data from these experiments are summarized in Tables 4 to 6). (*a*) Following incubation in Na Ringer's. (*b*) Following incubation in Na Ringer's + ouabain. (*c*) Following incubation in K-free medium. (*d*) Following incubation in K-free medium + ouabain

Table 5. Effects of K-free media on cell composition

	n	H_2O (kg/kg dry wt)	Na	K (mmol/kg dry wt)	Cl
Control	405	2.89 ± 0.05	76 ± 2	587 ± 6	146 ± 2
Affected	323	2.72 ± 0.05[a]	354 ± 8[b]	226 ± 7[b]	128 ± 4[b]
	Δ	**−0.17**	**+278**	**−361**	**−18**
Unaffected	228	2.54 ± 0.06	87 ± 4	510 ± 9	87 ± 3

Values were significantly different from the control values; [a]$P < 0.025$; [b]$P < 0.001$.
Data is the mean ±SEM of the number of cells analyzed from sections from bladders obtained from four toads. Control data is from all cells (other than the low Cl cells) analyzed from tissues incubated in Na Ringer's in this set of experiments. Affected and unaffected cells were identified in a scatter plot (as in Fig. 7), not illustrated here.

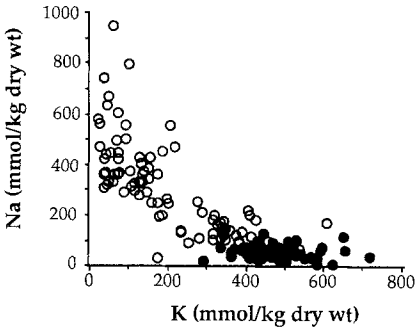


Fig. 9. Scatter plot showing relationships between cell Na and K contents in K-free serosal medium in the presence (○) or absence of serosal medium Na (●). Note that a number of cells in K-free medium alone were relatively unaffected by the conditions

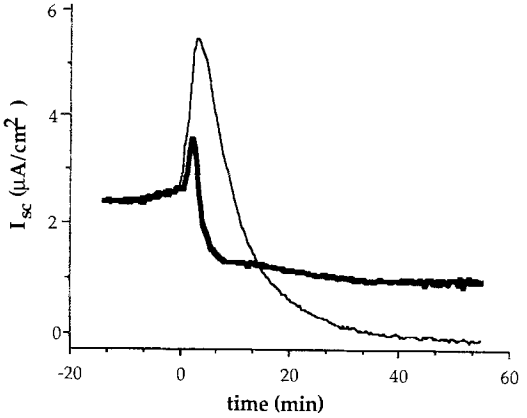


Fig. 10. Effect of K-free (—) or K-free, Na-free serosal medium (—) on the short-circuit current (I_{sc}). Media were changed from Na Ringer's to the experimental media at time 0. Chambers were perfused with media continuously throughout the experiment. Traces are means from four paired experiments on tissue from two toads

analysis of variance with unequal size groups (Sokal & Rohlf, 1981). By far the greatest variation was from cell to cell within an individual section. It did not arise from differences between animals, between blocks or between sections (Table 7). Therefore, such differences, which would include variations in thickness, in albumin composition and in the extent of freezing damage, have not contributed substantially to the differences in composition from cell to cell that are found with X-ray microanalysis in this study.

Part, at least, of the cell to cell variation may reflect the impossibility of analyzing exactly the same representative volume in each cell. For example, any extracellular contamination due to the inclusion of the cell membranes and adherent extracellular ions in the analyzed volume will distort the measurements. Also, the inclusion of different proportions of cytoplasm and nucleus in the analyzed volume will affect the results, since these two compartments differ significantly in their water and elemental compositions (Table 3).

The tissue measurement of Na has its own unique problems as the count rate is relatively low because of the comparative insensitivity of the detector for the low energy X-rays. The problem will be compounded by the low intracellular Na concentration, any self-absorption of the X-rays in the sections, and by the fact that the Na peak occurs in a part of the spectrum where the continuum is high. The variance due to counting errors was estimated as in Fuchs and Fuchs (1981). For Na, the mean variance due to counting error was 26% of the variance from cell to cell, whereas for K the comparable figure was only 2% ($n = 54$).

The least accurate estimate is that of the dry mass of the cells and, therefore, of the cell water content. This reflects several problems, including the possibilities that mass loss from the standard and from the cells is dissimilar and that osmotic shifts of

Table 6. Effects of Na Ringer's + ouabain (1 mM) on cell composition

	n	H_2O (kg/kg dry wt)	Na	K (mmol/kg dry wt)	Cl
Control	405	2.89 ± 0.05	76 ± 2	587 ± 6	146 ± 2
Affected	459	3.29 ± 0.05[a]	327 ± 6[a]	287 ± 5[a]	166 ± 3[a]
	Δ	**+0.40**	**+251**	**−300**	**+20**
Unaffected	120	3.17 ± 0.10	103 ± 6	612 ± 13	127 ± 6

Values were significantly different from the control values; [a]$P < 0.001$.
Data is the mean ± SEM of the number of cells analyzed from sections from bladders obtained from four toads. Control data is from all cells (other than the low Cl cells) analyzed from tissues incubated in Na Ringer's in this set of experiments. Affected and unaffected cells were identified in a scatter plot (as in Fig. 7), not illustrated here.

Table 7. Three-level nested analysis of variance with unequal group sizes

Source	*df*	Sum of squares	Mean square	Variance	%
Na (mmol/liter tissue)					
Animals	3	9131.1	3043.7	32.3	15.8
Blocks	11	3428.6	311.7	6.0	2.9
Sections	29	5537.4	190.9	3.1	1.5
Cells	331	54164.1	163.6	163.6	79.8
Total	374	72261.2			
K (mmol/liter tissue)					
Animals	3	6517.2	2172.4	−122.5	0
Blocks	11	100825.3	9165.9	211.4	10.5
Sections	29	159712.0	5507.3	468.7	23.2
Cells	331	444155.8	1341.9	1341.9	66.4
Total	374	711210.3			
Cl (mmol/liter tissue)					
Animals	3	1006.5	335.5	−1.2	0
Blocks	11	4251.5	386.5	1.2	0.6
Sections	29	11624.9	400.9	24.6	11.8
Cells	331	60324.4	182.2	182.2	87.6
Total	374	77207.3			
Mass (g/liter tissue)					
Animals	3	17580.4	5860.1	24.3	0.4
Blocks	11	59137.7	5376.2	−219.7	0
Sections	29	323796.8	11165.4	726.8	13.3
Cells	331	1557698	4706.0	4706.0	86.2
Total	374	1958213			

(Data were from four animals, 15 blocks, 44 sections, and 375 control cells).

water from the cells to the interstitial spaces may occur during freezing. From the present data, neither of these possibilities can be excluded.

Cell Composition in Na Ringer's

The mean values for water and ions under these conditions are very similar to the X-ray microanalysis data reported by others for tissues incubated in Na Ringer's (Table 8) (Rick et al., 1978*b*; Civan et al., 1980; Rick & DiBona, 1987; Rick et al., 1988). In the earliest toad bladder study, Rick et al. (1978*b*) gave results for each of the four cell types, but reported that the similarities in the shape and in the localization of the mitochondria-rich and goblet cells made morphological identification of these cell populations difficult. Although Civan et al. (1980) noted that the different cell types could be identified, they did not publish detailed values.

The difficulties in recognizing the different cell types in this study are described in Results. Average

Table 8. A comparison of mean values from X-ray microanalysis for toad bladder epithelial cells incubated in sodium Ringer's

A) wet weight

	n	Na	K	Cl	P	Dry mass
		(mmol/liter tissue)				(g/liter)
This study	766	18	156	42	182	261
Rick et al. (1978*b*)	12[a]	13	116	39	129	283
Civan et al. (1980)	30	18	116	28	139	
Rick & DiBona (1987)	59	13	117	34	129	195

B) dry weight

	n	H_2O	Na	K	Cl	P
		(kg/kg) dry wt			(mmol/kg dry wt)	
This study	766	3.07	70	611	164	702
Rick et al. (1978*b*)	12[a]	2.44	42	409	134	455
Civan et al. (1980)	110	3.7	72	543	155	785
Rick & DiBona (1987)	59	4.13	64	598	172	662

C) concentration in cell water

	n	Na	K	Cl
		(mmol/kg water)		
This study	766	25	217	57
Rick et al. (1978*b*)	12[a]	19	162	54
Civan et al. (1980)	30	19	147	42
Rick & DiBona (1987)	59	16	145	42

[a]Figures were obtained by combining the nuclear and cytoplasmic values to obtain average cellular values (assuming the nucleus is 1/7 of the total cell volume).

compositions from a series of sections in which positive cell identification was possible are given in Table 1. Mitochondria-rich cells contained less Cl than the other cell types. Altogether, 70 cells (of 836) were relatively low in Cl. They were also relatively low in Na, in agreement with the recent reports of Rick and DiBona (1987) and Rick et al. (1988). These 70 cells represent ~8% of the total cells analyzed, in good agreement with the estimate that in bladders of Dominican toads mitochondria-rich cells provide some 10% of the total cells in the epithelial layer (Macknight et al., 1980). The lower Na and Cl in these cells supports the contention that the mitochondria-rich cells are not part of a syncytium (Rick et al., 1978*b*).

The average intracellular Cl concentration in the low-Cl cells was 16.5 mmol/liter water. Assuming the same activity coefficient in medium and in cell water, at this concentration Cl would be in electrochemical equilibrium if the membrane potential were −53 mV. Mean granular cell Cl concentrations (Table 8) are significantly above those expected for an equilibrium distribution given membrane potentials of −50 to −70 mV under short-circuit conditions (Donaldson, Leader & Macknight, 1987; Nagel & van Driessche, 1989). This is also true in X-ray microanalytical studies of other tight epithelia [e.g., frog skin (Rick et al., 1978*a*), principal cells of the collecting duct (Sauer et al. 1989)]. The pathways involved in Cl accumulation by the granular and basal cells in the toad bladder remain to be established. Possible candidates include coupled Na-K-2Cl cotransport (or variants thereof) and Cl-HCO_3 exchange (together with Na-H exchange).

Occasionally, in some sections, surface cells had high Na and Cl and low K (Fig. 4*f*). It is possible that such cells are being shed from the bladders to be replaced by the underlying cells which were found to be of normal electrolyte composition. The abnormal cells may, therefore, be regarded as equivalent to the cells found in the outermost layer of the frog skin; in toad urinary bladder they occur sporadically rather than constituting a complete cell layer.

Effects of Different Media on Cell Composition

Unaffected Cells

Mitochondria-rich cells and a population of the basal cells were relatively unaffected by K-free media and by ouabain, in general agreement with Rick et al. (1978*b*). Also, Civan et al. (1980) reported that, even in K-free medium + ouabain, one or more subpopulations of cells (15–20% including the five identified basal cells) retained ≥ 2/3 of the control levels of K.

The smaller effect of ouabain on the mitochondria-rich cells may reflect lower membrane ion permeabilities associated with lower active transport rates, for these cells do not appear to play a major role in transepithelial Na transport (Bobrycki et al., 1981) and, in frog urinary bladder, have fewer ouabain-binding sites on their basolateral membrane (Mills & Ernst, 1975). The differences in the responses of the basal cells to the altered media may reflect differences in transport properties of the two subpopulations of basal cells identified on morphological grounds (Kraehenbuhl et al., 1979).

Whereas in media with ouabain, about 20% of the cell population was relatively unaffected (Fig. 8), in K-free medium alone some 40% of the cells were in this category in a scatter plot. These cells had nevertheless lost some K (Table 5). Much of this loss seems to have been accompanied by Cl, with associated decrease in cell water content, but detailed interpretation of the changes is impossible as this unaffected group must contain some granular cells as well as mitochondria-rich and basal cells.

Affected Cells

Removal of medium K alone resulted, on average, in a net loss of this ion, associated with a somewhat smaller gain in Na and net losses of Cl and water from the ~60% of affected cells (Table 5). Initially, removal of medium K would hyperpolarize the basolateral membrane, leading, in the short-circuited preparation, to an increase in the driving forces for Na entry and Cl exit through conductance pathways. Subsequently, there may be a combination of (i) decreased basolateral membrane potential due to loss of cell K and/or inactivation of basolateral membrane K channels [perhaps as a consequence of the decreased cell volume (Lewis et al., 1985)], (ii) inactivation of apical membrane Na channels (*see* Inhibition of Transepithelial Na Transport by Serosal K-Free Medium below), and (iii) inhibition of the (Na-K)-ATPase. These could account for the decrease in I_{sc} (Fig. 10). Since ouabain significantly enhanced the loss of K (Δ + 63 mmol/kg dry wt, $P < 0.001$) and uptake of Na (Δ + 128 mmol/kg dry wt, $P < 0.001$) in K-free media (Tables 4 and 5), there must be some residual (Na-K)-ATPase activity in the K-free medium alone. This may reflect recycling of K trapped in the lateral intercellular spaces and subepithelial tissues and/or preferential reaccumulation by the pump of K ions as they diffuse from the cell across the basolateral membrane (Civan, 1981).

In the present study 42% of cell K (average including the affected and unaffected cells) was lost into a K-free medium, whereas Civan et al. (1980) reported a loss of only 18%. We bathed both surfaces with K-free media, but there was K available from the mucosal medium in the experiments of Civan et al. (1980). It is possible that there was sufficient K diffusion from the mucosal medium across the tight junctions under their conditions to provide additional K for the pump, thereby minimizing cellular K loss. A similar explanation was advanced by these workers to explain their observed cellular uptake of mucosal Rb. After ouabain, the mean losses of K in the two studies were very similar (56 and 61%).

In both K-free medium and Na Ringer's, ouabain resulted in a small increase, rather than a decrease, in cell water and Cl (Tables 4 and 6) compared to the losses in K-free medium alone, in agreement with data from chemical analysis (Robinson & Macknight, 1976*b*). At 1 mM, the glycoside completely inhibits transepithelial transport in toad bladder even in the presence of 3.5 mM K in the serosal medium (Macknight et al., 1975*b*). Inhibition of (Na-K)-ATPase with membrane depolarization and loss of cell K should result in cell swelling. However, since Cl is accumulated above its equilibrium distribution under control conditions, the rate of such swelling is impossible to predict. For example, based on the data in Table 6, an Na-K-2Cl cotransporter would be expected to result initially in net Cl loss after ouabain, whereas an Na-Cl cotransporter would favor Cl uptake from the start, with cell swelling. Additional factors, including increased basolateral membrane Cl permeability, decreased apical membrane Na permeability and reversal of direction of the Na-H exchanger as cells accumulate Na, may further slow the rate of cell swelling after ouabain.

Consideration only of the mean data neglects the very important finding that the gains of Na and losses of K varied considerably from cell to cell under all three experimental conditions (Fig. 8). Such variation must reflect differences between the cells in the relative availabilities of different transport pathways, either as a consequence of differences in the numbers of such pathways or in their control. For example, since much of the K balances the net negative charge on cell nondiffusible solutes,

appreciable loss of K requires Na to be gained by the cells. Thus cells with relatively few functional pathways for Na entry would be limited in the extent to which they could lose K. It is possible that the variations in granular cell responses reflect differences in the ages of individual cells; for example, as cells age they may lose their capacity for transepithelial transport. So long as Na entry decreased in parallel to any decreased Na pump activity, cell composition would be reasonably preserved under control conditions and it would alter more slowly following exposure to K-free medium or ouabain than would the composition of cells which were transporting at high rates with a relatively large number of functioning Na channels. Unfortunately, little is known about the age-distribution or the rates of turnover of the granular cells.

Results from Chemical Analysis and X-Ray Microanalysis

In tissues bathed on both surfaces with Na Ringer's, means of results from chemical analysis in different studies range between 2.63 to 4.48 kg/kg dry wt for water, and (in mmol/kg dry wt) 142 to 258 for Na, 329 to 513 for K, and 172 to 334 for Cl (Macknight, 1977; Macknight et al., 1971; Macknight, Civan & Leaf, 1975*a*). Therefore, whereas estimates of water and K contents are similar when data from X-ray microanalysis and conventional chemical analysis are compared, the latter technique overestimates cell Na and Cl contents. The reasons for these discrepancies, discussed elsewhere (Macknight, 1990), are not established.

Following exposure to K-free media or to ouabain, cells lost K and gained Na. Although the patterns are similar, the changes observed using chemical analysis were somewhat less than those seen with X-ray microanalysis in the different studies (Table 9). Given that different cell types respond differently to these conditions of incubation, this discrepancy may in part reflect differences in the populations sampled.

Exchangeability of Cell Cations

Based on a number of observations using a variety of techniques, Civan and Shporer (1989) have recently argued that in epithelial cells the great bulk of the intracellular Na and K is readily exchangeable with the cytosolic Na and K, respectively. Average K concentrations (mmol/kg cell water) in the present study exceed those anticipated on the basis of measurements of cell K activity made using ion-selective microelectrodes (DeLong & Civan, 1983) and assuming an activity coefficient of 0.76. This is also true of studies in some other epithelia [e.g., mammalian urinary bladder (Lewis, Wills & Eaton, 1978; Dörge et al., 1988)]. However, given the difficulties experienced in obtaining microelectrode data in toad bladder epithelial cells, as well as the uncertainties in estimates of cell water by X-ray microanalysis, further examination of this point is not justified. In particular, it would be unwarranted to argue that the apparent discrepancy provided evidence for any substantial K compartmentalization within the cytoplasm or immobilization of this ion.

In studies of some intact epithelia, only a fraction of cell K appears to be readily exchangeable with labeled K in the serosal medium [rabbit ileum (Nellans & Schultz, 1976), rabbit colon (Frizzell & Jennings, 1977), frog skin (Cox, 1988)]. For example, in toad bladder a more rapidly-exchanging pool, labeled with a half-time of ~30 min, contained 29% of the cellular K. A second pool, with a half-time of about 9 hr, was never completely labeled over the duration of the studies (Robinson & Macknight, 1976*c*). The present results, however, argue against the interpretation that in toad bladder only about one third of the cell K is able to exchange freely with extracellular cation. In particular, after 50 to 70 min in K-free media, there was a wide variation in response between different cells, with some apparently unaffected by the conditions and others losing considerable quantities of K into K-free medium and virtually all of their K following ouabain (Fig. 8). Much of the K lost was replaced by Na. Thus in the granular cells, at least, most if not all of the K is exchangeable with extracellular cation. Failure to detect this degree of exchangeability using chemical analysis and isotopes may reflect the wide variability in response both within and between the different cell types, with the mean values concealing the different underlying patterns.

The Source of Cell Na in K-free Medium

In Na Ringer's with ouabain, the Na gained by the cells comes predominantly from the mucosal medium. Thus after ouabain, labeled Na of mucosal origin is increased (Macknight et al., 1975*b*) and cells gain more Na and lose more K with Na available from the mucosal medium as well as from the serosal medium as determined both chemically (Macknight et al., 1975*b*) and by X-ray microanalysis (Rick et al., 1978*b*). These results are consistent with those from metabolic studies that indicate that the basolateral membrane is normally relatively impermeable to Na (Canessa, Labarca & Leaf, 1976; Macknight & McLaughlin, 1977).

Table 9. Changes in composition in epithelial cells following incubation in K-free medium ± ouabain and Na Ringer's + ouabain measured using chemical analysis and X-ray microanalysis

	H_2O (kg/kg dry wt)	Na	K (mmol/kg dry wt)	Cl
K-free medium				
Chemical analysis				
Robinson & Macknight (1976*b*)	−0.44	+ 47	−116	−63
X-ray microanalysis				
This study	−0.21	+171	−246	−28
Civan et al. (1980)	−0.40	+150	− 99	−24
K-free medium + ouabain				
Chemical analysis				
Robinson & Macknight (1976*b*)	+0.27	+174	−153	+26
X-ray microanalysis				
This study	+0.23	+325	−329	+23
Civan et al. (1980)	−0.1	+264	−333	−13
Na Ringer's + ouabain				
Chemical analysis				
Macknight et al. (1975*b*)	−0.05	+133	−142	− 4
X-ray microanalysis				
This study	+0.38	+206	−226	+21
Rick et al. (1978*b*)		+296	−219	

In contrast to the findings with ouabain, in isotope-labeling experiments little Na was gained from the mucosal medium when the serosa was bathed by K-free medium (Robinson & Macknight, 1976*b*). This finding is supported here by the striking observation that replacement of serosal medium Na with the impermeant univalent cation NMDG entirely prevented the K loss and associated Na uptake in a K-free medium (Fig. 9). Also, blocking mucosal Na uptake with amiloride did not prevent granular cells losing K and gaining Na. Thus it appears that it is predominantly Na of serosal not mucosal origin that is gained by cells under K-free conditions. This conclusion, however, does not argue against the normal dominance of the apical membrane Na permeability. A gain of some 170 mmol of Na/kg dry wt predominantly across the basolateral membrane by cells incubated in K-free medium for 60 min (Table 5) represents only 10–20% of the Na that would normally flow across the apical membrane in that time (Macknight et al., 1975*b*).

Two additional points arise in considering the effects of K-free, Na-free serosal medium. First, the residual I_{sc} seen under such conditions (Fig. 10; and Essig & Leaf, 1963; Robinson & Macknight, 1976*a*) appears to be carried by Na through a transcellular rather than a paracellular route for mucosal amiloride rapidly, and ouabain more slowly, decreased I_{sc} to zero. Although it is possible that, as in the nystatin study of Garty, Warncke and Lindemann (1987), Na left the cells through amiloride-sensitive basolateral Na channels, serosal amiloride (0.1 mM) did not decrease I_{sc}. Secondly, since Na continued to enter the cells to some extent from the mucosal medium, as evidenced by the residual I_{sc}, the failure of the cells to lose substantial K when the serosal medium was both Na- and K-free, is unlikely to reflect unavailability of Na. Rather it is consistent with an inactivation of basolateral membrane K channels, as described under Na-free serosal conditions by Garty et al. (1987).

Inhibition of Transepithelial Na Transport by Serosal K-free Medium

When serosal K is removed, I_{sc} is first stimulated markedly and then declines with time to zero (Fig. 10; *see also* Robinson & Macknight, 1976*a*). There is evidence that the inhibition of transport is not simply a direct consequence of inhibition of the (Na-K)-ATPase, for *in a K-free medium* (i) I_{sc} is restored by mucosal amphotericin B, which promotes Na entry to the cells (Lichtenstein & Leaf, 1965); and (ii) labeled cell Na of mucosal origin does not increase (Robinson & Macknight, 1976*b*), as would be expected if the primary inhibitory site was the Na-K pump, and as is found following ouabain (Macknight et al., 1975*b*). In the present study, the absence of serosal medium K for 50–60 min, which is invariably associated with virtual cessation of transepithelial

transport, had not completely inhibited the (Na-K)-ATPase, for losses of K into K-free media were greater in the presence of ouabain (Tables 4 and 5).

It appears, therefore, that, as well as any inhibition of the (Na-K)-ATPase, Na entry to the cell from the mucosal medium is also inhibited by incubation in a K-free serosal medium. This conclusion is consistent with the reduced Na flux across this membrane (Essig & Leaf, 1963; Chase & Al-Awqati, 1979). It is possible that this inhibition may result from the raised cell Na and/or decreased K, for transport was not inhibited completely in K-free, Na-free medium under which conditions cell K and Na remained relatively normal. In the absence of serosal medium Na, the reversal of the normal gradients for the Na-H and Na-Ca transporters should result in a decreased cell pH and increased cell Ca. A decrease in cell pH can inhibit apical membrane Na conductance (Harvey & Erhenfeld 1988) and in toad bladder vesicles, the amiloride-sensitive Na flux is inhibited by Ca (Chase & Al-Awqati, 1983; Garty, 1984; Garty & Asher, 1985). However, the fact that I_{sc} was sustained in the K-free, Na-free serosal medium argues against an important inhibitory role of pH and Ca, at least under these specific conditions.

In summary, the technique of X-ray microanalysis has been used to study the composition of toad urinary bladder epithelial cells incubated in Na Ringer's and in K-free medium, with and without ouabain. The granular cells and a subpopulation of the basal cells lost K and gained Na after ouabain and in K-free media, although there was considerable variation in the responses from cell to cell. In contrast, the compositions of the low Cl, mitochondria-rich cells and a subpopulation of the basal cells were little affected by the different incubation conditions.

We are grateful to Drs. A. Dörge, R. Rick and F. Beck for helpful advice and discussions during the establishment of the technique of X-ray microanalysis in our laboratory. Ms. Sylvia Zellhuber-McMillan assisted in the analysis of some of the material and contributed to technical developments and Dr. C.W. McLaughlin assisted in the Ussing chamber experiments. Purchase of the equipment was only possible through grants from the Medical Research Council of New Zealand, the Medical Distribution Committee of the Lottery Board, the University Grants Committee, the Telford Trust, the New Zealand Neurological Foundation and the National Heart Foundation. The work was supported by a Programme Grant from the Medical Research Council of New Zealand.

References

Bobrycki, V.A., Mills, J.W., Macknight, A.D.C., DiBona, D.R. 1981. Structural responses to voltage clamping in the toad urinary bladder: I. The principal role of granular cells in active transport of sodium. *J. Membrane Biol.* **60:**21–33

Canessa, M., Labarca, P., Leaf, A. 1976. Metabolic evidence that serosal sodium does not recycle through the active transepithelial transport pathway of toad bladder. *J. Membrane Biol.* **30:**65–77

Chase, H.S., Jr., Al-Awqati, Q. 1979. Removal of ambient K^+ inhibits net Na^+ transport in toad bladder by reducing Na^+ permeability of the luminal border. *Nature* **281:**494–495

Chase, H.S., Jr., Al-Awqati, Q. 1983. Calcium reduces the sodium permeability of luminal membrane vesicles from toad bladder. *J. Gen. Physiol.* **81:**643–665

Choi, J.K. 1963. The fine structure of the urinary bladder of the toad, *Bufo marinus*. *J. Cell Biol.* **16:** 53–72

Civan, M.M. 1981. Intracellular potassium in toad urinary bladder: The recycling hypothesis. *In:* Epithelial Ion and Water Transport. A.D.C. Macknight and J.P. Leader, editors. pp. 107–116. Raven, New York

Civan, M.M., Hall, T.A., Gupta, B.L. 1980. Microprobe study of toad urinary bladder in the absence of serosal K^+. *J. Membrane Biol.* **55:**187–202

Civan, M.M., Shporer, M. 1989. Physical state of cell sodium. *Curr. Topics Membr. Transp.* **34:**1–19

Cox, T.C. 1988. Potassium dependence of sodium transport in frog skin. *Biochim. Biophys. Acta* **942:**169–178

Davis, W.L., Jones, R.G., Hagler, H.K., Farmer, G.R., Goodman, D.P.G. 1987. Histochemical and elemental localization of calcium in the granular cell subapical granules of the amphibian urinary bladder epithelium. *Anat. Rec.* **218:**229–236

DeLong, J., Civan, M.M. 1983. Microelectrode study of K^+ accumulation by tight epithelia: I. Baseline values of split frog skin and toad urinary bladder. *J. Membrane Biol.* **72:**183–193

DiBona, D.R. 1978. Direct visualization of epithelial morphology in the living amphibian urinary bladder. *J. Membrane Biol.* **Special Issue:**45–70

DiBona, D.R., Civan, M.M., Leaf, A. 1969. The anatomic site of the transepithelial permeability barriers of toad bladder. *J. Cell Biol.* **40:**1–7

Donaldson, P.J., Leader, J.P., Macknight, A.D.C. 1987. Membrane potentials in toad urinary bladder epithelial cells. *Fed Proc.* **46:**1269 (*Abstr.*)

Dörge, A., Wienecke, P., Beck, F., Wörndl, B., Rick, R., Thurau, K. 1988. Na transport compartment in rabbit urinary bladder. *Pfluegers Arch.* **411:**681–687

Essig, A., Leaf, A. 1963. The role of potassium in active transport of sodium by the toad urinary bladder. *J. Gen Physiol.* **46:**505–515

Frizzell, R.A., Jennings, B. 1977. Potassium influx across basolateral membranes of rabbit colon: Relation to sodium absorption. *Fed. Proc.* **36:**360 (*Abstr.*).

Fuchs, H., Fuchs, W. 1981. Foqus: A Fortran program for the quantitative analysis of X-ray spectra from thin biological specimens. *Scan. Electron Microsc.* **2:**377–394

Garty, H. 1984. Amiloride blockable sodium fluxes in toad bladder membrane vesicles. *J. Membrane Biol.* **82:**269–279

Garty, H., Asher, C. 1985. Ca^{2+}-dependent, temperature-sensitive regulation of Na^+ channels in tight epithelia. A study using membrane vesicles. *J. Biol. Chem.* **260:**8330–8335

Garty, H., Warncke, J., Lindemann, B. 1987. An amiloride-sensitive Na^+ conductance in the basolateral membrane of toad urinary bladder. *J. Membrane Biol.* **95:**91–103

Harvey, B., Ehrenfeld, J. 1988. Role of Na^+/H^+ exchange in the control of intracellular pH and cell membrane conductances in frog skin epithelium. *J. Gen Physiol.* **92:**793–810

Heinrich, K.F.J. 1982. The accuracy of quantitation in X-ray

microanalysis, particularly in biological specimens. *Scan. Electron Microsc.* **7**:281–187

Herrera, F.C. 1968. Bio-electric properties and ionic content in toad bladder. *J. Gen. Physiol.* **51**:261*s*–270*s*

Jehl, B., Bauer, R., Dörge, A., Rick, R. 1981. The use of propane/isopentane mixtures for rapid freezing of biological specimens. *J. Microsc.* **123**:307–309

Kraehenbuhl, J.P., Pfeiffer, J., Rossier, M., Rossier, B.C. 1979. Microfilament-rich cells in the toad bladder epithelium. *J. Membrane Biol.* **48**:167–180

Lewis, S.A., Butt, A.G., Bowler, J.M., Leader, J.P., Macknight, A.D.C. 1985. Effects of anions on cellular volume and transepithelial Na^+ transport across toad urinary bladder. *J. Membrane Biol.* **83**:119–137

Lewis, S.A., Wills, N.K., Eaton, D.C. 1978. Basolateral membrane potential of a tight epithelium: Ionic diffusion and electrogenic pumps. *J. Membrane Biol.* **41**:117–148

Lichtenstein, N.S., Leaf, A. 1965. Effect of amphotericin B on the permeability of the toad bladder. *J. Clin. Invest.* **44**:1328–1342

Macknight, A.D.C. 1977. Contribution of mucosal chloride to chloride in toad bladder epithelial cells. *J. Membrane Biol.* **36**: 55–63

Macknight, A.D.C. 1990. Ion and water transport in urinary bladder epithelia. *In:* Handbook of Physiology, Renal Physiology. E.E. Windhager, editor. American Physiological Society, Bethesda (*in press*)

Macknight, A.D.C., Civan, M.M., Leaf, A. 1975*a*. The sodium transport pool in toad urinary bladder epithelial cells. *J. Membrane Biol.* **20**:365–386

Macknight, A.D.C., Civan, M.M., Leaf, A. 1975*b*. Some effects of ouabain on cellular ions and water in epithelial cells of toad urinary bladder. *J. Membrane Biol.* **20**:387–401

Macknight, A.D.C., DiBona, D.R., Leaf, A. 1980. Sodium transport across toad urinary bladder; A model tight epithelium. *Physiol. Rev.* **60**:615–715

Macknight, A.D.C., DiBona, D.R., Leaf, A., Civan, M.M. 1971. Measurement of the composition of epithelial cells from the toad urinary bladder. *J. Membrane Biol.* **6**:108–126.

Macknight, A.D.C., McLaughlin, C.W. 1977. Transepithelial sodium transport and CO_2 production by the toad urinary bladder in the absence of serosal sodium. *J. Physiol.* **269**:767–776

Mills, J.W., Ernst, S.A. 1975. Localization of sodium pump sites in frog urinary bladder. *Biochim. Biophys. Acta* **375**:268–237

Nagel, W., van Driessche, W. 1989. Intracellular potentials of toad urinary bladder. *Pfluegers Arch.* **415**:121–123

Nellans, H.N., Schultz, S.G. 1976. Relations among transepithelial sodium transport, potassium exchange and cell volume in rabbit ileum. *J. Gen. Physiol.* **68**:441–464

Pak Poy, R.F.K., Bentley, P.J. 1960. Fine structure of the epithelial cells of the toad urinary bladder. *Exp. Cell Res.* **20**:235–237

Peachey, L.D., Rasmussen, H. 1961. Structure of the toad's urinary bladder as related to its physiology. *J. Biophys. Biochem. Cytol.* **10**:529–553

Rick, R., DiBona, D.R. 1987. Intracellular solute gradients during osmotic water flow: An electron-microprobe analysis. *J. Membrane Biol.* **96**:85–94

Rick, R., Dörge, A., Arnim, E. von, Thurau, K. 1978*a*. Electron microprobe analysis of frog skin epithelium: Evidence for a syncytial Na transport compartment. *J. Membrane Biol.* **39**:313–331

Rick, R., Dörge, A., Macknight, A.D.C., Leaf, A., Thurau, K. 1978*b*. Electron microprobe analysis of the different epithelial cells of toad urinary bladder: Electrolyte concentrations at different functional states of transepithelial sodium transport. *J. Membrane Biol.* **39**:257–271

Rick, R., Dörge, A., Thurau, K. 1982. Quantitative analysis of electrolytes in frozen dried sections. *J. Microsc.* **125**:239–247

Rick, R., Spancken, G., Dörge, A. 1988. Differential effects of aldosterone and ADH on intracellular electrolytes in the toad urinary bladder epithelium. *J. Membrane Biol.* **101**:275–282

Robinson, B.A., Macknight, A.D.C. 1976*a*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder: I. Effects of different medium potassium concentrations on electrical parameters. *J. Membrane Biol.* **26**:217–238

Robinson, B.A., Macknight, A.D.C. 1976*b*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder: II. Effects of different medium potassium concentrations on epithelial cell composition. *J. Membrane Biol.* **26**:239–268

Robinson, B.A., Macknight, A.D.C. 1976*c*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder: III. Exchangeability of epithelial cellular potassium. *J. Membrane Biol.* **26**:269–286

Sauer, M., Dörge, A., Thurau, K., Beck, F.X. 1989. Effect of ouabain on the electrolyte concentrations in principal and intercalated cells of the isolated perfused cortical collecting duct. *Pfluegers Arch.* **413**:651–655

Sokal, R.R., Rohlf, F.J. 1981. Biometry. (2nd ed.) Freeman, New York

Wade, J.B. 1978. Membrane structural specialization of the toad urinary bladder revealed by the freeze-fracture technique: III. Location, structure and vasopressin dependence of intramembrane particle arrays. *J. Membrane Biol.* **Special Issue**:281–296

Received 18 October 1990; revised 11 March 1991