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Electron Microprobe Analysis of the Different Epithelial Cells of Toad Urinary Bladder

Electrolyte Concentrations at Different Functional States of Transepithelial Sodium Transport

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Summary. The electrolyte composition of toad urinary bladder epithelial cells has been measured using the technique of electron microprobe analysis. Portions of hemibladders, which had been mounted in chambers and bathed with a variety of media, were layered with albumin solution on their mucosal surfaces and immediately shock-frozen in liquid propane at −180 °C. From the frozen material 1–2 μm thick cryosections were cut and promptly freeze-dried for 12 hr at −80 °C and 10^{-6} Torr. Electron microprobe analysis using a scanning electron microscope, an energy dispersive X-ray detector, and a computer programme, to distinguish between characteristic and uncharacteristic radiations, allowed quantification of cellular ionic concentrations per kg tissue wet wt by comparison of the intensities of the emitted radiations from the cells and from the albumin layer. Granular, mitochondrial-rich, and basal cells, and the basal portions of goblet cells, showed a similar composition, being high in K (about 110 mM/kg wet wt) and low in Na (about 13 mM/kg wet wt). The apical portions of goblet cells were higher in Ca and S and lower in P and K, presumably reflecting the composition of the mucus within them. With Na-Ringer's as the mucosal medium, cells gained Na and lost K, when their serosal surfaces were exposed to ouabain, 10^{-2} M. Replacement of mucosal Na by choline virtually prevented these ouabain-induced changes. Cellular ion contents were unchanged when Na in the serosal medium was replaced by choline. No differences in Na and K concentrations were detected between nuclei and cytoplasm. These results provide independent support for the hypothesis that the cellular Na transport pool in toad bladder epithelial cells derives exclusively from the mucosal medium and that no important recycling of Na occurs from the serosal medium to the cells.

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Amphibian epithelia such as frog skin and toad urinary bladder have been extensively studied in an attempt to understand their role in the transport of solutes and water. In particular, the active transport of Na by these tissues has been a focus of investigations. Since, even across the histologically simple toad bladder, several possible pathways exist by which Na ions can be reabsorbed from urine to body fluids (from mucosal to serosal bathing media), much attention has been directed to the route of active transepithelial transport. Potential pathways are either intercellular spaces with Na penetrating the limiting junctions and never entering cells, or transcellular routes with Na passing through the cells. Since Na from the mucosal medium has been shown to enter the mucosal layer of cells [12] and transepithelial Na transport was found to be coupled to cellular energy metabolism [10], it is most likely that active transport of Na follows a transcellular rather than an intercellular route. However, with several different cell types present in the mucosal layer of the epithelium, it is possible that one or more types may be engaged in transepithelial Na transport. One interpretation of the finding that only a fraction of the cellular Na is exchangeable from the mucosal side [13] might be that only a certain cell type is involved in transepithelial Na transport.

In the present study cellular electrolyte concentrations in the different epithelial cell types of toad urinary bladder under varying conditions of transport were measured using electron microprobe analysis in an attempt to identify the epithelial cell types involved in transepithelial transport of Na and to define the magnitude of the cellular Na transport pool.

Materials and Methods

Female toads of the species *Bufo marinus* (Dominican Republic, National Reagents, Bridgeport, Conn.) were kept in plastic troughs with free access to tap water. Toads were pithed and their urinary bladders dissected and cut into 4–5 pieces. The pieces were then mounted on lucite rings (exposed surface area 3.2 cm^2), which were inserted into Ussing-type chambers. The experiments were performed under short-circuited conditions, using an automatic voltage clamping device. Transepithelial potential difference (PD) was measured every 30 min for 2 min. During the preincubation period the bladders were incubated on both the mucosal and serosal sides in Ringer's solution for some 60 min until the short-circuit current had reached a steady-state value. After preincubation the half chambers were emptied and refilled with new incubation solutions, according to the following protocol:

a) Control: both sides with Ringer's solution

b) Ouabain: both sides with Ringer's solution, serosal medium containing 10^{-2} M ouabain

c) Ouabain, mucosal Na-free: mucosal side with Na-free Ringer's solution, serosal side with Ringer's solution, containing 10^{-2} M ouabain

d) Serosal Na-free: mucosal side with Ringer's solution, serosal side with Na-free Ringer's solution.

The Ringer's solution contained (in mM) 112 NaCl, 3.5 KCl, 1 $CaCl_2$, 1.8 Na_2HPO_4, and 10 glucose. The Na-free Ringer's solution contained (in mM) 120 choline-Cl, 1 $CaCl_2$, 1.8 K_2HPO_4, and 10 glucose. At the end of the experiments the latter solution contained between 0.7 and 0.9 mM Na. All solutions were bubbled with air and had a pH of 7.8. The exchange of solutions was repeated 5 times during the first 30 min of the experimental period. After a further 30–60 min of incubation, the rings were removed from the chambers. The mucosal surface of the bladder was then gently blotted with filter paper and covered with a layer of an appropriate standard albumin solution approximately 20 μm in thickness. A piece 1 cm^2, supported on lens paper, was then cut with a cork borer from the central part of the exposed area of the bladder and immediately plunged into cold propane (−180 °C) for shock-freezing. The time interval that elapsed between removal of the bladder from the chamber and freezing was about 1 min. From the frozen material, 1–2 μm thick cryosections were cut at a temperature of −80 °C from tissue blocks trimmed to (0.2 × 0.2) mm^2. These were promptly freeze-dried for 12 hr at −80 °C and 10^{-6} Torr. The albumin was prepared by dissolving 20 g/100 ml bovine albumin in the respective bathing solution. Since albumin *per se* contains some protein-bound Na, the standard prepared for Na-free Ringer's solution contained some 30 mM Na.

Electron microprobe analysis of the freeze-dried sections was performed in a scanning electron microscope (Cambridge, Stereoscan S4), to which an energy dispersive X-ray detector (EDAX) had been adapted. The acceleration voltage used was 15 kV, and the probe current selected was 0.5 nA. Areas of usually 1–2 μm^2 were scanned for 200 sec and the emitted X-rays were analyzed in the energy range between 0.6 and 4 keV covering the K-lines of the elements from Na to Ca. The discrimination between characteristic and uncharacteristic radiations (Bremsstrahlung) was performed by the use of a computer program [2].

Quantification of electrolyte was achieved by comparing the characteristic radiations of the cells with those of the adherent albumin layer. The cellular dry weight content was estimated by a comparison of the Bremsstrahlung intensities of cell and albumin standard. Details of the preparation of freeze-dried cryosections for microanalysis and the quantification procedure have been described [5, 6].

Results

Identification of the Different Epithelial Cells

Figure 1 shows a scanning transmission electron micrograph of a 1-μm thick freeze-dried cryosection of toad urinary bladder together with a sketch of the epithelium indicating the different cell types. The epithelium is supported by a soft connective tissue (right hand) and is covered on the mucosal surface by the albumin standard layer (left hand). From inspection of the epithelium it is obvious that morphological criteria like the number of endoplasmatic granules or mitochondria, which serve in conventional electron micrographs for differentiation of the various cell types (granular, mitochondria-rich, basal and goblet cells)

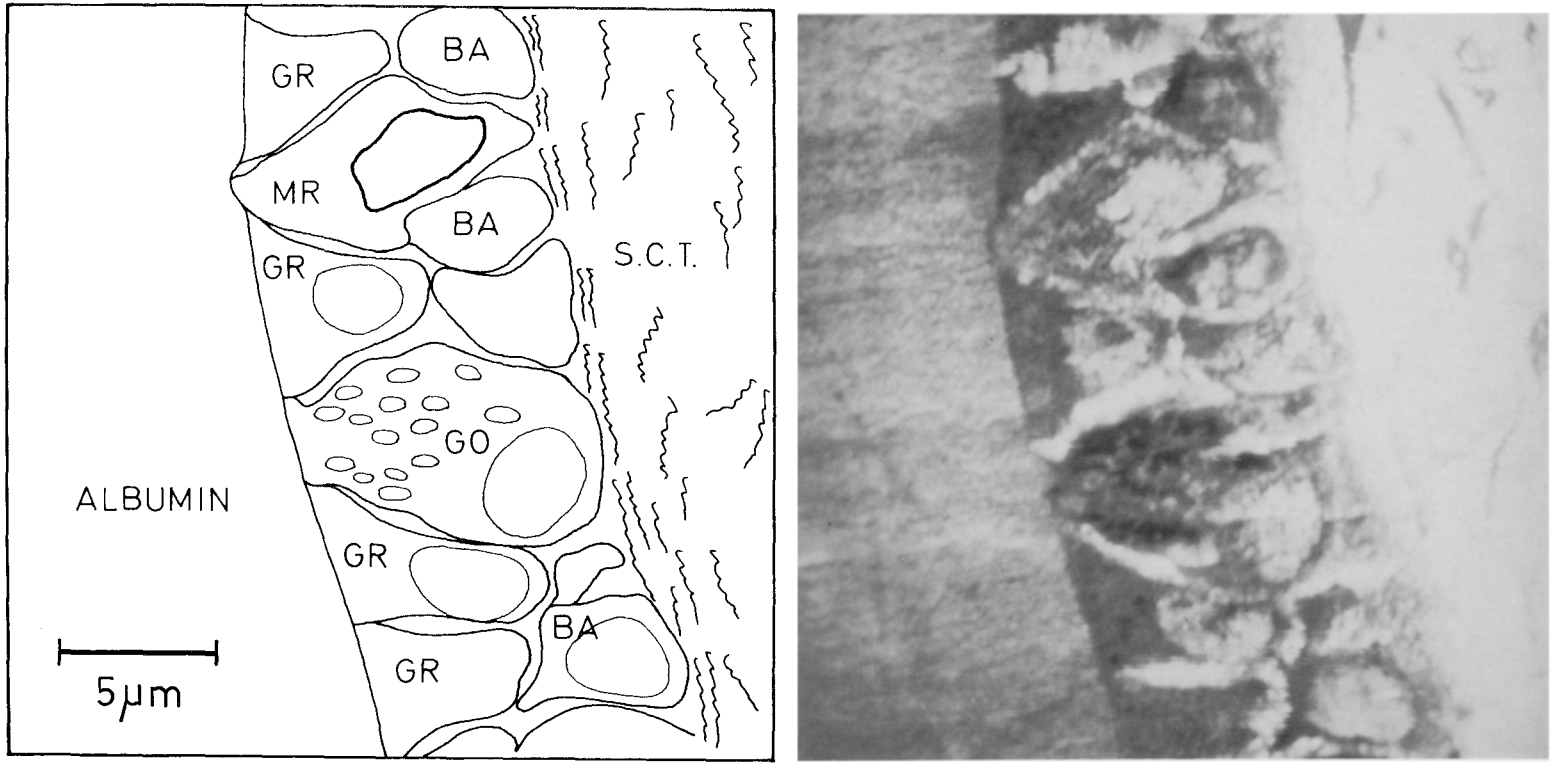

Fig. 1 Scanning transmission electron micrograph of a freeze-dried cryosection of toad urinary bladder epithelium 1 μm thick together with a sketch of the various epithelial cells. *GR* = granular cell; *BA* = basal cell; *MR* = mitochondria-rich cell; *GO* = goblet cell; *S.C.T.* = soft connective tissue. The fine granularity seen in the albumin layer is caused by microcrystals of ice. As one penetrates into the tissue, this granularity increases slightly. Note that the scanning area used during analyses was always much larger than the size of the individual inhomogeneities

are not available in this kind of preparation. However, using mainly their shapes and their localization within the epithelium, the different cell types can be identified. The small basal cells can easily be discriminated, because, in contrast to all the other cells, they show no contact with the mucosal surface. In the superficial cell layer the most frequent cell type (about 70% of the epithelial volume) is characterized by a large apical membrane area. This feature is characteristic of the granular cells. In addition, there are cells which exhibit only a small area of contact with the mucosal solution. These cells, which are most often larger than the granular cells and typically show a pear-like shape, probably represent the mitochondria-rich and goblet cells. Within this population, cells with electron-dense corpuscles (approximately 0.5 – 1 μm thick) on the apical part of the cytoplasm can be distinguished from those with no apparent inclusions, which occasionally show microvilli at the apical surface. It is presumed that the first cell type represents the goblet cells, whereas the second corresponds to the mitochondria-rich cells.

Energy Dispersive X-ray Spectra of the Different Epithelial Cells under Control Conditions

Figure 2 shows six energy dispersive X-ray spectra obtained under control conditions in the albumin standard layer and in the various cell types of the section depicted in Fig. 1. The spectrum of the albumin layer shows a typical extracellular electrolyte pattern: high Na and Cl peaks and a low K peak. Qualitatively, similar spectra with regard to Na, Cl and K were obtained when the electron beam was positioned over intercellular spaces or at the basal margins of the epithelium. In contrast, the cellular spectra show low Na and Cl peaks but high P and K peaks with only minor differences between granular cell, basal cell, mitochondria-rich cell and basal part of the goblet cell. However, the spectrum obtained in the apical part of the cytoplasm of the goblet cell differs from the other cellular spectra in having higher Ca and S peaks and lower P and K peaks.

Na and K Concentration at Different Functional States

Figure 3 shows three energy dispersive X-ray spectra of granular cells under control conditions, after the action of ouabain and after the combined effect of ouabain and mucosal Na-free Ringer's solution. Compared to control, after ouabain the Na peak is increased and the K

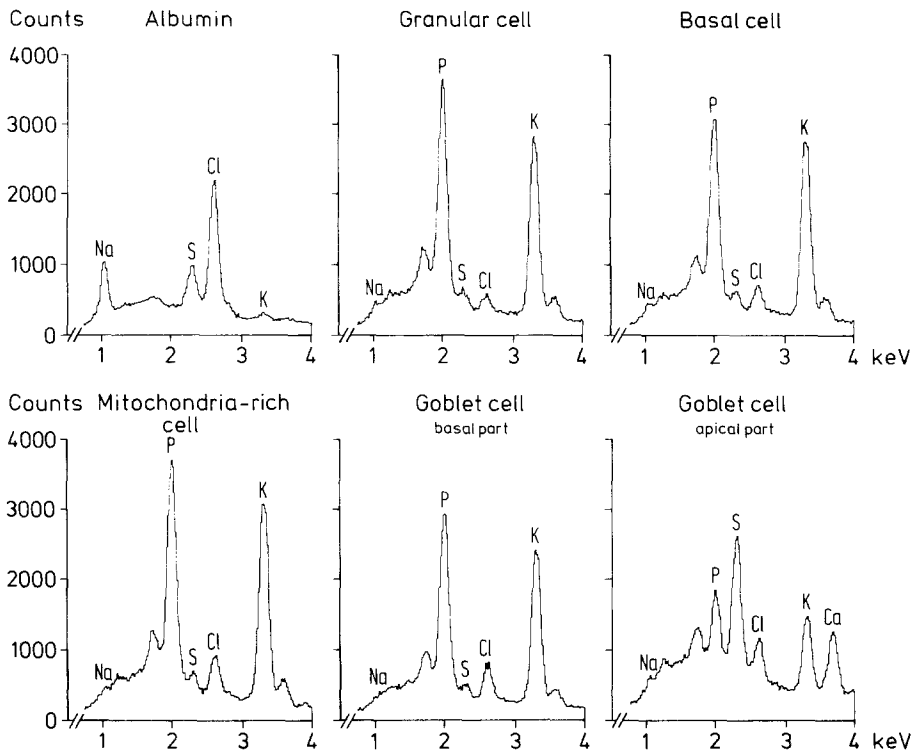

Fig. 2. Energy dispersive X-ray spectra obtained in the albumin layer and the various epithelial cells under control conditions. Measurements were performed in the section depicted in Fig. 1. The major peaks of interest are labelled. In addition, at 1.3 keV there is a small peak corresponding to Mg and at 1.75 keV there is a Si peak originating from slight contamination of the water used for film preparation

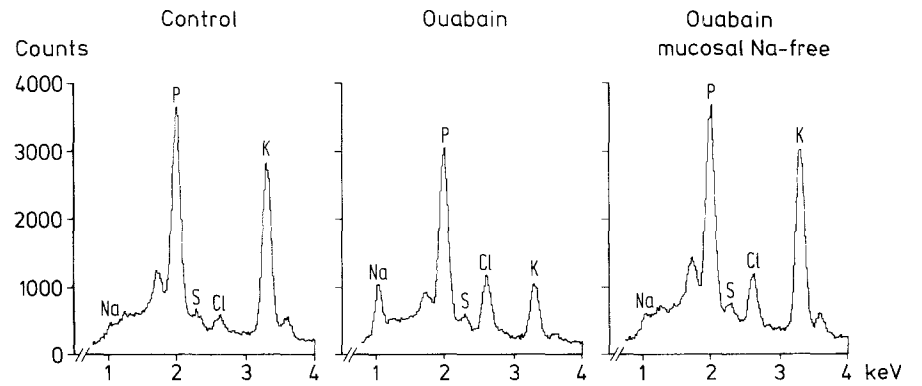

Fig. 3. Energy dispersive X-ray spectra obtained in granular cells under control conditions, after ouabain and after ouabain with mucosal Na-free Ringer's solution

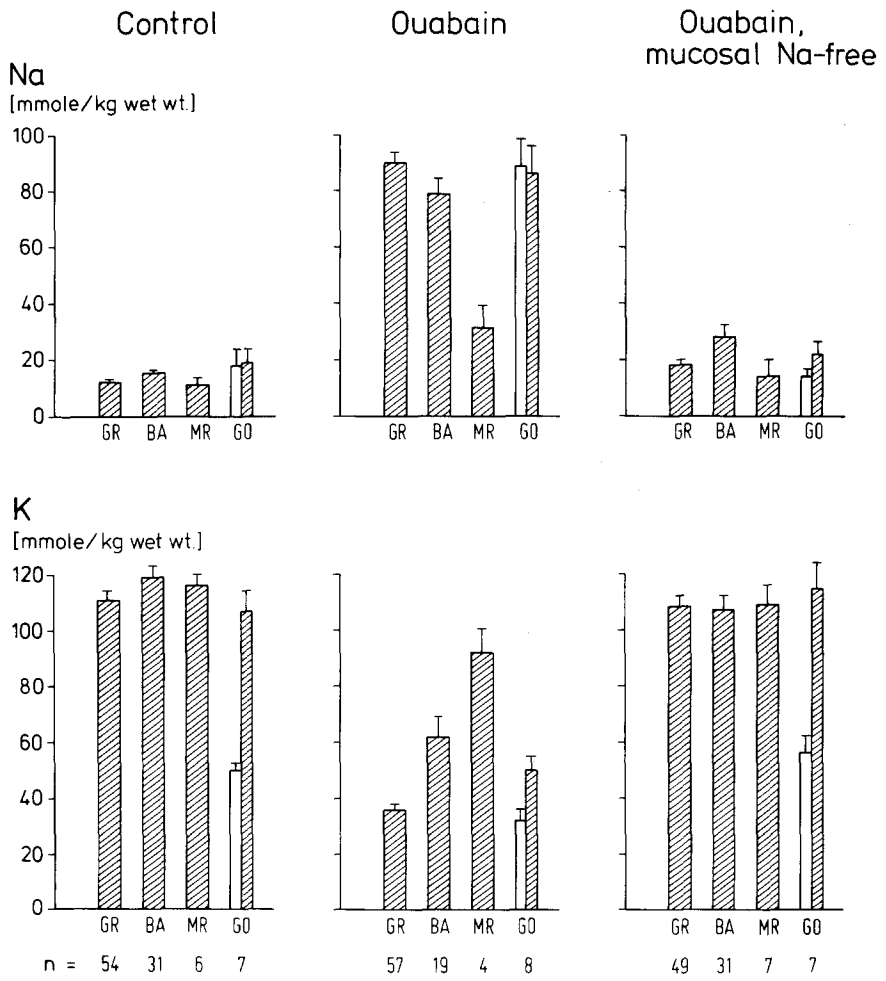

Fig. 4. Cellular Na and K concentration values of the various epithelial cell types under control conditions, after ouabain and after ouabain with mucosal Na-free Ringer's solution. *GR* = granular cells; *BA* = basal cells; *MR* = mitochondria-rich cells; *GO* = goblet cells; unhatched and hatched columns represent measurement in the apical and basal part of goblet cells, respectively. (Mean values, ± SE)

peak is decreased. In the absence of mucosal Na, however, ouabain produced almost no alteration in the Na and K peaks. Figure 4 and Table 1 show the cellular Na and K concentrations of the different cell types under the same three conditions. The data are pooled from 3 animals. For each condition at least 10 sections were analyzed. In the control, the Na concentrations of all four cell types were virtually identical, and, with the exception of the apical part of the goblet cells, this was also true for K. After ouabain the concentration of Na in the

Table 1. Na and K concentrations in toad bladder epithelial cells under control conditions, after ouabain and after ouabain with mucosal Na-free Ringer's solution

Cell type	Control			Ouabain			Ouabain, mucosal Na-free		
	Na	K		Na	K		Na	K	
	mmole/kg wet wt								
Granular	12.2±0.8	110.6±3.2	54	90.1±3.6	35.6±1.9	57	18.1±1.6	108.0±3.8	49
Basal	15.4±1.0	118.0±3.3	31	79.6±5.2	61.3±7.2	19	27.9±3.9	106.6±4.9	31
Mitochondria-rich	11.2±2.5	114.4±3.7	6	31.5±7.6	91.2±7.9	4	14.2±5.6	108.4±6.7	7
Goblet, apical	17.6±5.6	49.6±2.5	7	88.2±9.4	31.8±4.2	8	14.2±2.4	56.0±6.1	7
Goblet, basal	18.9+4.8	106.2+7.1	7	85.7+9.7	49.6+4.7	8	21.7+4.3	113.9+9.0	7

(Mean values ± SE, *n*)

granular cells increased from 12±1 to 90±4 mmole/kg wet wt, whereas the K concentration decreased by about the same extent from 111±3 to 36±2 mmole/kg wet wt. When the mucosal bathing medium was replaced by a nominally Na-free Ringer's solution (Na concentration 0.7 −0.9 mM), this effect was almost abolished. The Na and K concentrations of the granular cells were then found to be 18±2 and 108 ±4 mmole/kg wet wt, respectively.

In the basal cells a similar behavior of the Na and K concentrations was observed under the three experimental conditions. However, the extent of the changes after ouabain were somewhat less pronounced than in the granular cells. In the mitochondria-rich cells after ouabain a much smaller change in cellular Na and K was observed than in the granular cells. In the goblet cells similar Na concentration values were found as in the granular cells. In the basal part of the goblet cells also similar K values were observed, where as in the apical part the K values were considerably lower under all experimental conditions.

After incubation with a Na-free serosal medium with Na Ringer's on the mucosal surface, cellular Na was not detectably different from the low value with Na Ringer's solution bathing both surfaces, and cellular K was unchanged.

Differences between the Elemental Content of Cytoplasm and Nucleus

Table 2 shows the Na, K, P, Cl, Ca and dry weight content of the cytoplasm and nucleus under control conditions. The data comprise

Table 2. Cytoplasmic and nuclear concentrations of Na, K, Ca, P, Cl and dry wt[a]

	Na	K	Ca	P	Cl	dry wt
	mmole/kg wet wt					g/100 g
Cytoplasm	13.8 ± 3.6	115.9 ± 15.3	2.2 ± 1.8	124.5 ± 31.5	40.8 ± 10.1	29.1 ± 4.0
Nucleus	11.2 ± 3.5	118.5 ± 13.7	0.1[b] ± 0.3	153.2[b] ± 17.0	22.9[b] ± 7.3	23.8[b] ± 2.2

[a] Mean values ± SD.
[b] Significantly different from the cytoplasmic value. Paired measurements were made on 12 cells from the same bladder.

measurements from twelve granular cells in which a clear cut morphologic differentiation between cytoplasm and nucleus was possible. For Na and K the small deviations observed between cytoplasm and nucleus were not statistically significant. In contrast, systematic deviations were observed for P, Cl, Ca and dry wt content between cytoplasm and nucleus. The P concentration in the cytoplasm was lower, whereas the Cl, Ca, and dry wt concentrations were higher. Similar differences for the nuclear and cytoplasmic P, Cl, Ca and dry wt values were observed under the other experimental conditions. The Na and K values were again not significantly different. In the goblet cells there was a large difference between nuclear and cytoplasmic Ca concentrations. As in the granular cells, nuclear Ca was zero, but the cytoplasmic Ca concentration ranged from 20 to 60 mmoles/kg wt, seemingly related to the density of the electron-dense corpuscles.

Discussion

Cell Identification

The epithelium of toad urinary bladder is composed of various cell types with possibly different functional relationships to transepithelial Na transport [3, 4, 20]. A method allowing determination of the electrolytes within individual cells should assist in elucidating which cell type contributes to the transport processes. It has previously been shown [5, 6] that electron microprobe analysis of freeze-dried cryosections allows the quantitative determination of electrolytes in individual cells or even in subcellular structures. The preparation of the tissue sections by shock-freezing, cryosectioning and freeze-drying has been chosen to preserve the *in vivo* distributions of readily diffusible electrolytes like Na and K [5, 8]. However, with this preparation technique, the differentiation be-

tween the various cell types is beset with some difficulties. This is especially the case for cells with similar shape and localization within the epithelium, like the mitochondria-rich and goblet cells of toad urinary bladder epithelium. The discrimination between these cells was made in the present study according to the appearance of electron-dense particles in the apical cytoplasm, which showed a markedly high Ca and S concentration. The view, that these corpuscles correspond to mucus vesicles of goblet cells, is supported by the finding of Warner and Coleman [23, 24], that the mucus of small intestine goblet cells of rat and chick has a high Ca content. The high S content may be due to the sulfuric acid mucoproteins of the goblet cell mucus. The fact that the K concentration in the apical part of the goblet cells was always considerably lower than in the basal part or in the nucleus is best explained by a replacement of the apical cytoplasm by mucus vesicles containing a very low K concentration. In addition, the discrimination between mitochondria-rich cells and goblet cells is also based on the observation that in some sections at the apical surface of pear-shaped cells containing no particles, microvilli could be seen. Further support for the view that these cells are the mitochondria-rich cells may be derived from electron microprobe analysis of frog skin sections. In this epithelium the only pearlike cells in the apical layer are mitochondria-rich cells, which have never been found to contain Ca- and S-rich electron dense particles (our *unpublished observations*). However, since the discrimination between both cell types was mainly made according to the Ca and S content, it cannot be excluded that in some cases nonsecreting goblet cells were taken for mitochondria-rich cells.

Cellular Na and K Concentrations

Since no significant differences were found for Na or K concentrations between nuclei and cytoplasm in the epithelial cells, the cells appear to represent a single compartment with respect to the distribution of these ions. It must be stressed, however, that since the microprobe technique cannot discriminate between ions in free solution and ions which might be "bound" to cellular constituents, concentrations as measured by the microprobe need bear no relation to ion activities within the cells. Although no values are available for the ionic composition of toad urinary bladder nuclei, high intranuclear concentrations of Na have been reported for other tissues [9, 21]. These reports, however, are based largely on estimates of ionic content following

isolation in nonaqueous media of nuclei from the cells. The direct electron microprobe analysis of nuclei and cytoplasm does, however, reveal characteristic differences for P, Cl, Ca and dry wt. The higher P concentration in the nuclei reflects the high content of nuclei acids and would account for the lower concentrations of the other free anions, such as Cl.

The higher Ca concentration in cytoplasm as compared with nuclei need not indicate that Ca concentration in the cytosol is higher than in the nuclei. It is more likely a reflection of compartmentation of this ion in mitochondria and/or endoplasmic reticulum.

It is conceivable that irregularities in the thickness of sections could result in differences in ion concentrations between cytoplasm and nucleus. However, since analyses were performed on serial sections, differences due to such irregularities would cancel out. Even within a single section concavities on one surface would be expected to be balanced by convexities on the opposite surface. The absence of a difference between nuclear and cytoplasmic Na and K is consistent with these arguments.

The most striking finding in this study is the very low concentration of Na found in all cell types of the epithelium. On an average of all cells the Na concentration was 13 mmole/kg wet wt. This value is considerably lower than that obtained by chemical analysis of the Na concentration in whole toad bladder tissue [7] and also lower than the values reported for the chemical analysis of scraped epithelial cells [12]. In addition, the cellular K concentrations obtained are higher than those of chemical analysis of whole tissue, though similar to those of scraped epithelial cells [14]. Upon application of ouabain, the cellular Na concentrations rose from the average control value of 13 to 88 mmole/kg wet wt. The cellular K concentrations showed an equivalent but reciprocal change from 114 to 44 mmole/kg wet wt. Changes in the same direction, but to a smaller extent, have been observed after ouabain by using chemical analysis [13].

The low intracellular Na concentrations and the high K concentrations found in the control cannot be ascribed to artifacts during the preparation of the tissue for electron microprobe analysis. If diffusion of Na or K had occured between extra- and intracellular spaces during the preparation, this would lead to increased cellular Na and decreased K values. The discrepancy between the cellular Na and K concentrations obtained by electron microprobe analysis and those obtained by chemical analysis might be explained by an underestimation of the extracel-

lular space by extracellular markers as used in chemical analysis. The calculation of intracellular Na and K concentrations in the chemical analysis would, therefore, be falsified by the inclusion of an additional extracellular compartment containing high Na and low K concentrations. This would, therefore, result in apparently higher cellular Na and lower cellular K concentrations. Overestimation of the intracellular volume might also account for the smaller changes in Na and K concentrations observed by chemical analysis after the action of ouabain. This suspected artifact would be smaller when scraped epithelial cells [14] are analyzed, for no adherent serosal connective tissue is present. However, even in this preparation the artifact involved in the measurement of extracellular volume still appears to be present. In the control, the cellular Na concentrations of scraped epithelial cells were found to be approximately 40 mmole/kg wet wt [14], about 3 times higher than those obtained by electron microprobe analysis. However, about 75% of this Na equilibrated with Na in the serosal medium [12]. The direct measurement with electron microprobe analysis revealed no differences in cellular Na concentration in the presence or absence of serosal Na. There is, therefore, no evidence for such an exchange. This suggests that the cellular Na apparently exchanging with the serosal medium in scraped epithelial cells is located in a noncellular space inaccessible to extracellular markers [12]. Assuming this to be so, only the Na which equilibrates with the mucosal medium represents cellular Na. The close similarity between these values (14 mmole/kg wet wt) in scraped cells [12] and those obtained by electron microprobe analysis is consistent with this view. Since, after removal of Na from the mucosal bathing solution, the gain in cellular Na after ouabain was almost abolished, it can be concluded that Na enters the cells predominately from the mucosal side. Therefore, all the cellular Na fulfills the definition of the Na transport pool [12]. The total amount of cellular Na found in this study agrees well with the Na transport pool in scraped epithelial cells [12] as measured by ^{24}Na exchangeable from the mucosal side.

The present results shed some light on the relative permeabilities of the mucosal and basolateral membranes of the epithelium. Since under short-circuited conditions the electrochemical gradients for movement of Na into the cell can be expected to be the same across both membranes, the almost exclusive exchangeability with the mucosal medium indicates a considerably higher Na permeability of the mucosal membranes compared to the basolateral ones. This conclusion is supported by the finding that removal of serosal Na has only little influence on the rate of CO_2

production by the epithelial cells [1, 15]. If there were considerable basolateral membrane permeability to Na and consequently a large influx of Na from the serosal medium, this would require extrusion of Na of serosal origin with the expenditure of metabolic energy.

Functional Organization of the Epithelium

Since, histologically, the basal cells do not directly contact the mucosal surface [4], the communication of these cells with the mucosal medium can only occur via intercellular junctions to the superficial cells. Therefore, the observation that the Na concentration in the basal cells largely depends on mucosal Na provides evidence for a syncytial organization of the epithelium with respect to this ion. The fact that after ouabain the Na concentration in the basal cells was somewhat lower than in the granular cells may simply reflect a lesser accessibility of these cells from the mucosal medium. Since ouabain was only applied for 60 min it is possible that in the granular cells, and particularly in basal cells, steady-state conditions were not yet achieved. In frog skin epithelial cells, incubation with ouabain for more than 60 min leads to a further increase in Na and decrease in the K concentration as observed with chemical analysis [19], which has been confirmed by electron microprobe analysis (our *unpublished observations*).

The only exception from the syncytial organization of the epithelium may be the mitochondria-rich cells. Although these cells exhibit a direct access to the mucosal medium, a markedly less pronounced cellular exchange of Na and K was observed after ouabain. After ouabain some mitochondria-rich cells with almost unchanged Na and K concentrations were found. These were surrounded by granular cells with high Na and low K values, indicating that the Na exchange between the two cell types is relatively low. The considerably smaller response of the mitochondria-rich cells to ouabain might reflect a lesser content of membrane-bound ouabain-sensitive Na-K-ATPase, as has been described from ouabain-binding studies in frog bladder [16].

The conclusion that the epithelial cells of the toad urinary bladder form a functional syncytium is similar to that previously proposed for the frog skin [18, 22] and for toad bladder [11]. Although seemingly most epithelial cells share in a functional syncytium, this does not clarify whether they are involved to the same extent in transepithelial Na transport. It is even possible that a syncytium is formed only after the action of ouabain. Under the influence of a possible cell swelling, plasma

membranes from adjacent cells may be brought into such a close contact that junctions are formed between them.

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