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Microprobe Study of Toad Urinary Bladder in Absence of Serosal K^+

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Summary. The bulk of the intracellular potassium in mucosal epithelial cells from toad urinary bladder has been previously reported to exchange very slowly with the serosal medium, with a half-time of some 9 hr. This observation, based on chemical analyses of mucosal cell scrapings, has been reexamined with simultaneous diffractive and energy dispersive electron probe X-ray microanalysis. Fifty-three intracellular sites in hydrated sections and 286 sites in dehydrated sections were studied in bladders from eight toads under baseline conditions and after removal of serosal K^+ for 83–133 min, with or without 10^{-2} M ouabain. The baseline data confirm and extend previous examinations of the intracellular ionic composition, and provide the most direct measure of intracellular water thus far available for this tissue. Removal of serosal K^+ reduced the intracellular K^+ content by 20%, increased intracellular Na^+ content threefold, and slightly reduced the intracellular Cl^- and water contents, qualitatively consistent with published chemical analyses. The intracellular Na^+ content of mucosal origin, measured by radioactive tracers and chemical analyses of cell scrapings, has been reported to be unchanged under these conditions. Simultaneous addition of ouabain and removal of external K^+ produced a dramatic fall in intracellular K^+ of more than 80% in a third of the cells and reduced the mean intracellular K^+ content by 60%; 20% of the cells appeared to retain K^+ more effectively than the bulk of the epithelial cell population. We conclude that: (i) the low rate of net exchange of intracellular K^+ with the serosal bulk solution primarily reflects recycling of K^+ across the basolateral membranes, (ii) radioactive tracer and chemical measurements of the intracellular Na^+ pool of mucosal origin substantially underestimate the total intracellular Na^+ content under certain experimental conditions, and (iii) the epithelial cells display a functional heterogeneity of response to the effects of adding ouabain and withdrawing external K^+.

Transepithelial sodium transport is thought to proceed in two stages. Sodium likely enters the transporting cell from the mucosal or outer medium in response to its electrochemical gradient across the apical plasma membrane. Sodium is subsequently extruded across the basolateral membrane by an energy-dependent transfer mechanism or pump. In the classical formulation of Koefoed-Johnsen and Ussing (1958), the sodium pump was also considered responsible for cellular accumulation of potassium from the serosal or inner solution; a fixed stoichiometry was presumed to characterize the relative rates of potassium accumulation and sodium extrusion.

Among other observations, at least two phenomena have been thought not easily accommodated by this simple model. First, Essig and Leaf (1963) noted that, as expected, removal of serosal potassium did inhibit transepithelial sodium transport and did produce a net loss of intracellular potassium and a net gain of intracellular sodium. However, when choline replaced sodium in the serosal medium, removal of serosal potassium was far less effective in producing the changes in transepithelial sodium transport and intracellular electrolyte contents. (Although removal of serosal sodium does produce a concentration gradient favoring net movement of sodium from mucosa to serosa, transepithelial sodium transport is still dependent upon the availability of metabolic energy under these conditions [Essig, 1965].) These early analyses of intracellular electrolyte contents were

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necessarily conducted on samples of whole urinary bladder; 80–90% of the intracellular salt and water contents so measured actually reflect contributions from the subepithelial elements within the tissue (Macknight, Civan & Leaf, 1975). However, chemical analyses of mucosal cell scrapings has confirmed the basic observation that removal of serosal potassium is only half as effective in inducing the net loss of intracellular potassium when choline is substituted for serosal sodium (Robinson & Macknight, 1976*a*). On the other hand, Robinson and Macknight (1976*a*) did not detect any change in the intracellular sodium content of mucosal origin; Rick et al. (1978) have suggested that, at least under the experimental conditions they examined, only the sodium which equilibrates with the mucosal medium represents cellular sodium.

A second particularly puzzling phenomenon was described by Robinson & Macknight (1976*b*). They observed that only a quarter to a third of the intracellular potassium of toad bladder exchanges with the serosal medium with a half-time of a half-hour, while the great bulk of the intracellular potassium was found to exchange with a half-time of some 9 hr. On the basis of these measurements of the rates of exchange of intracellular potassium using ^{42}K, Robinson and Macknight suggested that the epithelial cells contain at least two distinct intracellular pools of potassium. This concept was supported by their observation that either removing serosal potassium (Robinson & Macknight, 1976*a*) or adding ouabain in high concentration (Robinson & Macknight, 1976*b*) reduced the intracellular potassium content by about a third; the net reductions in intracellular potassium produced by these agents were not significantly different from the total baseline potassium content within the rapidly exchanging pool.

One possible interpretation of these two observations might be that most of the intracellular potassium is either compartmentalized and/or immobilized within the transporting cells of toad urinary bladder. However, the potassium activity of the freely exchanging fraction may be of critical importance in regulating sodium entry into the transporting cells.

Two experimental approaches have been applied to examine this hypothesis. Intracellular microelectrodes have been utilized, presuming that the cytosolic potassium constitutes part or all of the freely exchangeable intracellular potasssium pool. Such direct measurements of the intracellular potassium activity have suggested that the cytosolic potassium plays no direct, immediate role in regulating sodium entry into the transporting cells (DeLong & Civan, 1978, 1979); the possibility of indirect, delayed effects has not yet been critically examined. In the present study, we have turned to another approach, electron probe X-ray microanalysis, in order to study the changes in total intracellular electrolyte and water content under baseline conditions and after removing serosal potassium.

Materials and Methods

Animals and Chamber

Female specimens of the toad *Bufo marinus* obtained from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.) were maintained on moist wood chips at 16 °C, and fed small mice at weekly to biweekly intervals. Experiments were conducted at room temperature. Urinary hemibladders were excised from doubly-pithed animals and mounted mucosal surface up over a supporting stainless steel tube. In the first experiment, a small tube, 5.6 mm long and 4.2 mm outer diameter (OD), was capped with an agar pyramid supporting the bladder. In all subsequent experiments, the hemibladder was stretched over a larger tube, 11.5 mm long and 9.5 mm OD. In order to minimize deformation of the tissue during the subsequent quenching process, it was useful to support the serosal surface of the preparation with nylon mesh.

Solutions

Under baseline conditions, the serosal surface of the tissue was bathed with a standard Ringer's solution consisting of (in mM): Na^+, 115.1; K^+, 3.3; Ca^{++}, 0.8; Cl^-, 113.9; HCO_3^-, 2.2; $HPO_4^=$, 1.8; $H_2PO_4^-$, 0.3; the pH was 7.6–7.8 and osmolality 215–225 mosmol. Under experimental conditions, the serosal medium was a K^+-free Ringer's solution, identical with the standard Ringer's solution except for the equimolar replacement of Na^+ for K^+. After mounting the tissue, the initial mucosal medium was always a K^+-free Ringer's solution. Subsequently, the mucosal solution was replaced either by an albumin- or dextran-Ringer's solution, serving both as cryoprotectant and peripheral standard for later analysis.

The albumin-Ringer's solutions contained 127–148 g albumin · (kg wet wt)$^{-1}$. The bovine albumin was obtained as Fraction V powder (Sigma Chemical Co., St. Louis, Mo.), and dialyzed overnight. In the four initial experiments, the solutions contained 7–30 mM Rb^+ in order to help confirm the identity of the medium during microprobe analysis of the frozen hydrated tissue; K^+ crosses the apical plasma membrane only very slowly, and Rb^+ is known to closely mimic K^+ in toad urinary bladder (Robinson & Macknight, 1976*b*). In the four later experiments, no Rb^+ was included. In the absence of Rb^+, the albumin-Ringer's solution contained (in mM): Na^+. 128; Ca^{++}, 0.2; Cl^-, 89.2; HCO_3^-, 2.1; $HPO_4^=$, 1.6; $H_2PO_4^-$, 0.3; the pH and osmolality were similar to standard Ringer's solution. The dextran-Ringer's solution consisted of 194 g dextran (mol wt 255,000, Clinical Grade, Sigma Chemical Co., St. Louis, Mo.) · (kg wet wt)$^{-1}$ dissolved in the K^+-free Ringer's solution.

Bathing the mucosal surface of the preparation with albumin-Ringer's solution did not produce systematic changes in open-circuit electrical potential or resistance. However, random changes were often noted. In this respect, the dextran-Ringer's solution was preferable, consistently producing no change in either electrical parameter.

Electrical Measurements

The transepithelial potential V (serosa positive to mucosa) was measured with matched calomel electrodes in series with agar salt

bridges containing K^+-free Ringer's solution. The voltage was introduced into a preamplifier (Model 602, Keithley Instruments, Cleveland, Ohio), whose output was displayed continuously on a paper chart recorder.

Resistance (R) was measured from the voltage deflections produced by pulses of constant current provided by a constant stimulus isolation unit (Model PSIU 6, Grass Instrument Company, Quincy, Mass.) controlled by a stimulator (Model S 44, Grass Instrument Company). Current was passed across the tissue through a pair of chlorided silver electrodes in series with agar bridges containing K^+-free Ringer's solution. Usually, pulses of 10 μA were applied for 10 sec at intervals of 70 sec. Current was monitored by introducing the differential voltage across a known series resistor into the second channel of the dual pen recorder.

Commonly, chemical (e.g., Macknight et al., 1971) and microprobe (Rick et al., 1978) analyses of toad bladder have been performed on tissues initially maintained in the short-circuited state. The preparations are then usually open-circuited for periods of 30–60 sec or more, prior to the final isolation of the mucosal cells or quenching of the tissue. This period of time is sufficient to potentially permit substantial redistribution of ions between the intra- and extracellular fluids, at least of some tissues (e.g., Coles & Tsacopoulos, 1979). Therefore, it has seemed preferable to maintain the control and experimental tissues under open-circuited conditions throughout the period of observation, monitoring potential and resistance, until the final time of quenching. Short-circuit current (I_s) was calculated as (V/R), in view of the approximately linear current-voltage relationship of toad bladder over the operating range between the open-circuited and short-circuited states (Civan, 1970).

Experimental Procedure

Prior to mounting, the control hemibladder was bathed in aerated standard Ringer's solution and the experimental hemibladder was bathed in K^+-free Ringer's solution either with or without 10^{-2} M ouabain. The serosal surface of the experimental preparation was exposed to K^+-free medium for a total time of 83 to 133 min.

In the first experiment, the tissue and voltage-sensing agar bridges were simultaneously quenched in Freon 13 (monochlorotrifluoromethane, m.p. −181 °C) slush permitting monitoring of the transepithelial potential until the very instant of freezing. However, the necessarily thick layers of mucosal and serosal media complicated the subsequent sectioning and analysis. In the seven other experiments, the agar bridges were removed, the serosal medium drained, and the mucosal medium largely removed by just touching the mucosal surface with filter paper (Whatman No. 40 "Ashless" Filter Paper, W. & R. Balston, Ltd., Maidstone, Kent, England). The tissue and supporting tube were then rapidly thrust into the liquid Freon, using a Teflon tube holder. The draining procedure could be completed within 15 sec; during this brief period, the tissue was probably not greatly perturbed from its steady-state open-circuited condition.

Tissue Preparation

The tissue and tube were left for approximately 60 sec in the Freon 13, shaken free excess Freon, and then stored in liquid nitrogen for future processing.

In the first experiment, it was possible to section the preparation while still mounted on the supporting tube. However, the large mass of underlying agar greatly complicated analysis of the bladder. In all later experiments, the preparation was first separated from the stainless steel tube; at a convenient time, the tissue was gently fractured under liquid nitrogen with a scalpel. Suitable pieces were clamped into a brass chuck lined with a pocket of Indium, as suggested to us by Dr. Roger Rick. The frozen hydrated blocks were then sectioned with a cryomicrotome (SLEE, London, England) at a specimen temperature of −60 to −80 °C. Sections were usually cut 1 μm thick with a steel knife oriented perpendicular to the tissue plane. The sections were then transferred with an eyelash holder to aluminium-coated nylon films mounted on Dural holders. Holders were subsequently transferred to a storage Dewar cooled by liquid nitrogen, using a shroud device previously described (Gupta, Hall & Moreton, 1977; Gupta & Hall, 1979). Tissue hydration was maintained for periods of storage as long as 2–3 weeks in the present study; hydration can be retained for even longer periods, if necessary.

Electron Probe Microanalysis

Microanalysis of the sections was performed with a JEOL JXA-50A Electron Probe Microanalyzer (JEOL Ltd., Tokyo, Japan) equipped with two diffracting spectrometers, a Link Systems energy-dispersive spectrometer with a Kevex detector, a transmission stage cooled by liquid nitrogen to between −160 and −170 °C, and an anticontamination cap cooled by liquid nitrogen to −190 °C. Under the operating conditions used, the temperature of the cold stage was not critical; tissue hydration could be maintained until the stage warmed to approximately −110 °C, at which temperature water is lost *in vaccuo*. RAP (rubidium acid phthalate) and PET (penta-erythritol) crystals were used to monitor Na and Ca, respectively.

The operating voltage was 45 kV. Preliminary scanning transmission electron microscopy was carried out with a current of 0.5 nA. Analyses were conducted with probe currents of 1 to 5 nA, whose stabilities were frequently checked. Counts were usually accumulated over periods of 100 sec "live time" operation by the solid-state detector and 200 sec "real time" by the crystal spectrometers. Although analyses were occasionally conducted with static probes, rasters of appropriate dimensions were usually applied in order to reduce the possibility of mass and elemental loss; areas as large as 1,200 μm^2 were scanned in studying extracellular sites. Determinations associated with significant mass loss were rejected.

Tissue sections were occasionally first analyzed in the hydrated state, and subsequently dehydrated for further analysis. The most straightforward approach was to warm the stage temperature to approximately −110 °C, await dehydration, and then recool the stage. The simpler, quicker approach more commonly used was to withdraw the section holder almost entirely from contact with the cold stage, wait several minutes for dehydration, and then replace the holder on the stage. Because of the considerable difficulty in identifying the cells and subcellular structures in frozen hydrated sections (Fig. 1), most analyses were conducted using dehydrated sections.

Data Reduction

Deconvolution of the complete X-ray energy spectrum (Statham, 1976, 1977) permitted calculation of the characteristic counting rates for Na, P, S, Cl, K, Ca and Rb, as well as the rate for the continuum spectrum, which provided a direct measure of local mass. Frequently, a small amount of Cl was found associated with the substrate film itself, especially in sections which had been dried within the analyzer. This is presumably due to a contamination arising within the column from pump oils, O-rings and plastics, and adsorbed by the cold specimen holder when removed from under the protection of the colder anticontamination cap during dehydration (Gupta et al., 1977; Gupta, 1979). The counting rates for Cl in tissue sections were corrected for his film-associated Cl.

The counting rates for Na and Ca obtained with the crystal spectrometers were corrected for the corresponding background values. In this study, Ca was monitored primarily to help identify goblet cells. The measured characteristic counting rate for Na (W'_{Na}) was corrected for absorption within the specimen with an expression based on Hall's analysis (1971):

$$W_{Na} = W'_{Na} p/(1 - e^{-p}) \tag{1}$$

where

$$p = (6.09)(10^{-2})\, W(3.652 - 1.424 f_D)/(3.67 - 0.39 f_D). \tag{2}$$

W_{Na} is the corrected counting rate for Na, W is the continuum rate in counts $\cdot$ nA^{-1} $\cdot$ sec^{-1}, and f_D is the fractional dry weight of the specimen. Although some rough estimate of f_D is required, the correction is relatively intensitive to this parameter. Correction for absorption of the other characteristic X-rays in a 1-μm section is considered negligible (Gupta et al., 1977) and was omitted from the current analysis.

Conversion of the measured characteristic and continuum counting rates to concentrations was performed by the approach of Hall (1971). The peripheral standard (albumin- or dextran-Ringer's solution) was applied to the mucosal surface for at least 10 min before freezing the tissue.

Conversion factors (*v.* Eq. (3)) were obtained by pooling the counting rates determined for the peripheral standards. In analyzing frozen hydrated sections, it was necessary to limit this averaging procedure to data obtained from the standard contained on the same holder as the specimen under study. The possibilities of random small accumulations of frost or slight degrees of dehydration precluded pooling the unprocessed counting rates measured for hydrated standards from different holders. On the other hand, the instrumental stability of the system used did permit pooling of the results obtained from all of the preparations for each of the dehydrated media used as peripheral standards; this approach was followed in analyzing both the tissue and peripheral standards. Such pooling is possible when the data reduction is based on Eq. (3) (below) since the quantities (W_i/W) are independent of section thickness; W_i is the corrected characteristic counting rate for the element "i" and W is the continuum counting rate.

The peripheral standards were specifically used to calibrate the Na and Cl signals of the mucosal medium. The intracellular Na and Cl counting rates could then be converted directly, while the intracellular P, S, K and Ca signals were converted after first adjusting their counting rates for the separately determined relative sensitivities of the nuclides with respect to Cl.

The final conversion of specimen counting rates to concentrations c_i for each element "i" in mmol $\cdot$ kg^{-1} was performed with Eq. (3) (Hall, 1971):

$$c_i = \frac{(W_i/W)}{(W_i/W)_0} (c_i)_0 \frac{G}{G_0} \tag{3}$$

where the subscript "o" refers to the peripheral standard. G is defined as:

$$G = \sum_i f_i (Z^2/A)_i \tag{4}$$

where f_i is the weight fraction, Z the atomic number, and A the atomic weight of element "i" (Hall, 1971). The value of G could be calculated for each standard from the known composition of the medium. The theoretical values of G for the control and K^+-depleted tissues were based on the microprobe data published for control toad bladder (Rick et al., 1978) and on the induced differences in intracellular composition measured chemically for K^+-depleted tissues (Robinson & Macknight, 1976*a*), respectively. Although (G/G_0) was explicitly included in the calculations, the ratio deviated from unity by no more than 8%, thus affecting the final estimation to only a minor degree.

The most direct way to use Eq. (3) for calculating intracellular elemental concentrations would be to introduce the mean values of (W_i/W) and $(W_i/W)_0$. However, this approach gives inordinate weight to those determinations associated with random underestimates of W. Therefore, the mean and SE were calculated separately for W_i and W and the ratios of the ratios of the mean values were then introduced into Eq. (3) (Palmer, Century & Civan, 1978). Each standard error presented in the current report therefore constitutes a conservative overestimate of the uncertainty associated with each tabulated elemental concentration, since it includes the variance in specimen mass, whereas the variation in local mass actually has no bearing on the calculated elemental concentrations.

We consider that the approach described above constitutes the preferred method of data reduction of the current results. However, it should be emphasized that whether or not the data are pooled does not significantly alter the conclusions, qualitatively or quantitatively.

Results

Morphology

Figure 1 presents electron micrographs of the same section from an experimental hemibladder obtained in hydrated state (upper panel) and after dehydration within the analyzer (lower panel). The low contrast in electron density associated with well-hydrated specimens (upper panel) hampers identification of cellular sites. In practice, it was helpful to first analyze a dehydrated section, and then to identify and analyze hydrated intracellular fields in another holder with a neighboring region of the same block. With this approach, intracellular fields could be satisfactorily studied, although subcellular structures could rarely be localized within the fully hydrated cells of toad bladder.

On the other hand, considerable detail is available from dehydrated unstained frozen sections (Fig. 2). In the majority of the intracellular sites studied, the cell type and intranuclear or cytoplasmic position of the beam could be identified.

X-ray Spectra

Representative X-ray energy spectra from hydrated and dehydrated granular cells from the same tissue section are shown in the upper and lower panels, respectively, of Fig. 3. The continuum X-ray (Brehmsstrahlung) counts are much larger in the upper spectrum, reflecting the considerably greater mass of the hydrated field.

Representative spectra from dehydrated sections are presented in Fig. 4. Qualitative differences can be

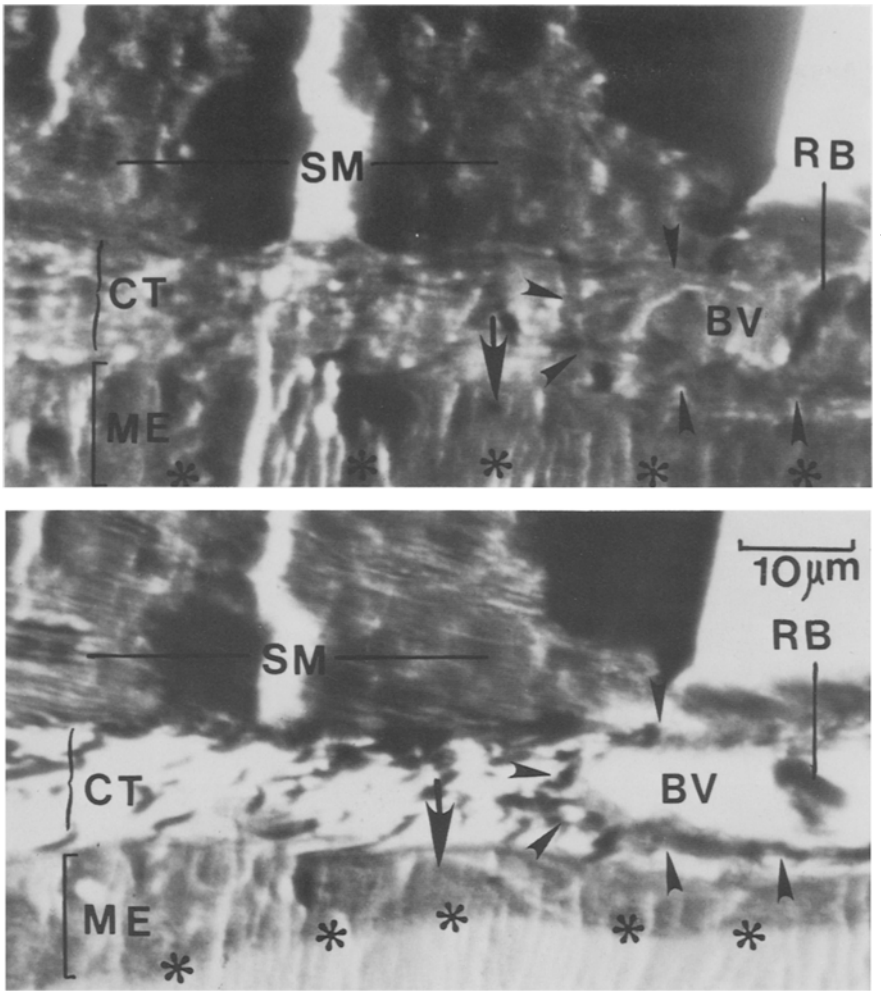

Fig. 1. Micrographs of a toad urinary hemibladder preparation incubated in K^+-free Ringer's solution. Comparison of scanning transmission images from $a \sim 1$-μm thick cryosection in a fully hydrated state (top) and after the same section had been dehydrated within the microanalyzer column (bottom). Major components such as smooth muscle (*SM*), submucosal connective tissue (*CT*), mucosal epithelium (*ME*) and a blood vessel (*BV*) with its wall delineated by arrow heads and containing a red blood cell (*RB*) can be recognized in both images. The boundary between the mucosal surface and dextran containing mucosal Ringer is delineated by asterisks. Note the marked change in contrast and image detail from hydrated to dry state of the section. Aqueous contents filling up the connective tissue spaces and blood vessel in the fully hydrated section have been noticeably lost on dehydration. Corresponding changes in X-ray energy spectra from a granular cell (bold arrow) are shown in Fig. 3.

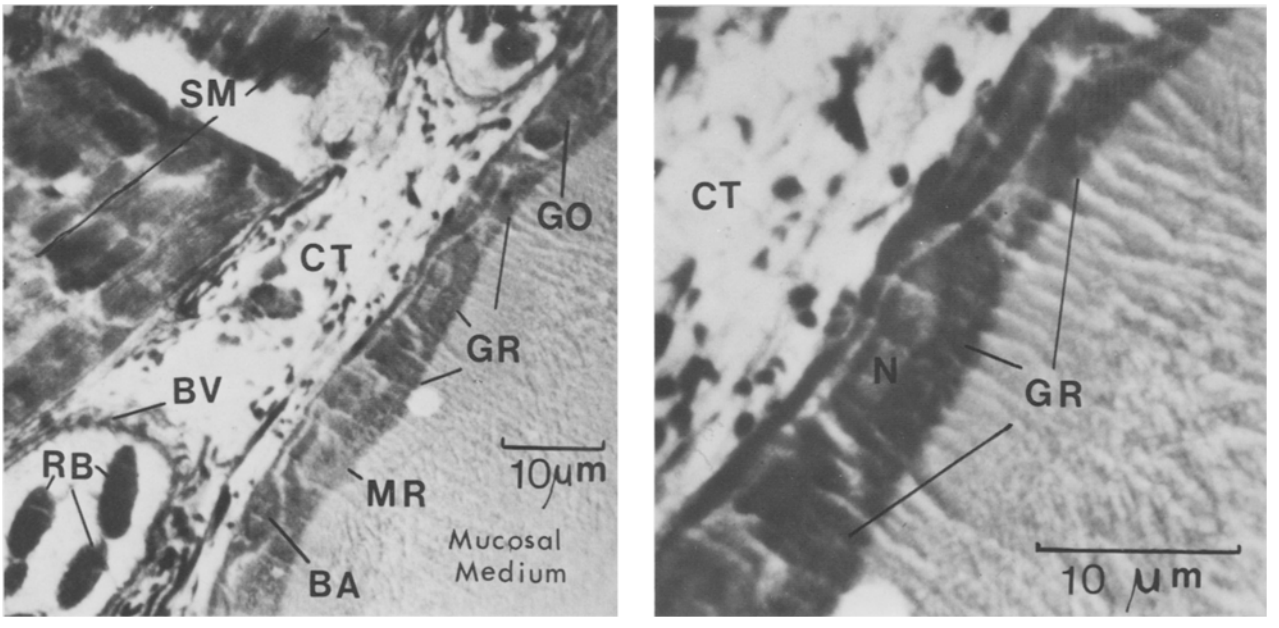

Fig. 2. Scanning transmission images of ~1 μm thick cryosections of toad urinary hemibladder. The micrograph shows smooth muscle (*SM*), submucosal connective tissue (*CT*) with collagen fibers, blood vessels (*BV*) with red blood cells (*RB*), the mucosal epithelium with basal cells (*BA*), a mitochondria rich cell (*MR*), granular cells (*GR*), a mucus-secreting goblet cell (*GO*), and the mucosal medium. A small field from the low magnification image on the left is shown at a higher magnification on the right; a granular cell nucleus with a dense nucleolus is indicated by *N*.

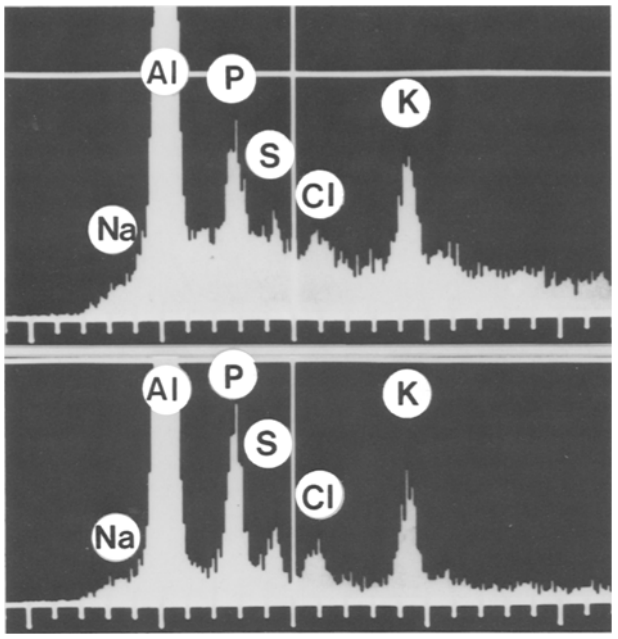

Fig. 3. Comparison of X-ray energy spectra (over the range 1–5 keV) from a granular cell in fully hydrated state (top) and after complete dehydration (bottom) of the section illustrated in Fig. 1. The major change on section dehydration is a great reduction in background continuum X-ray due to loss of water, resulting in highly improved peak-to-background ratios for the characteristic peaks of P, S, Cl and K. In spite of this change, the peak for intracellular Na is hardly discernible from the background. The signal for *Al* is from the Dural section holder and the *Al*-coated substrate film. The toad urinary bladder was bathed in K^+-free serosal and mucosal media. It should be emphasized that (as noted in *Materials & Methods*) the intracellular Na was determined almost exclusively with the crystal spectrometer, rather than from the energy dispersive spectra such as those presented in Figs. 3 and 4. The characteristic counting rate for Na measured with the crystal spectrometer was always appreciably greater than the background counting rate. Under experimental conditions (e.g., Fig. 4*c* and *d*) where the intracellular Na concentrations were high, the Na concentrations estimated with the energy dispersive system simply served as checks of the Na concentrations determined with the wavelength dispersive system.

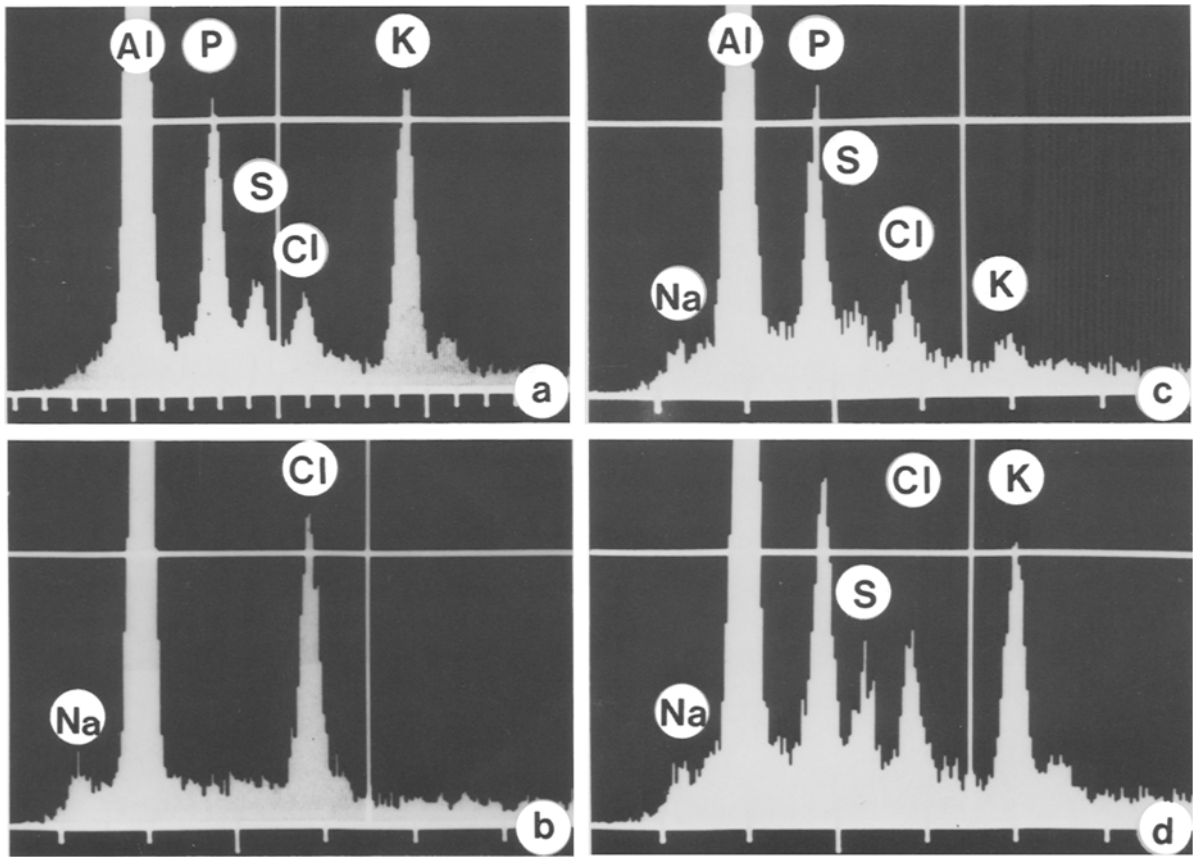

Fig. 4. Representative X-ray energy spectra (1–5 keV) from dehydrated cryosections of toad urinary bladders approximately 1 μm thick: (*a*) from a granular cell nucleus under baseline conditions; (*b*) from mucosal Ringer's solution containing 19.4% (wt/wt) dextran; (*c*) from the nucleus of a granular cell bathed with ouabain in the absence of external K^+ and which has lost more than 90% of its intracellular K^+; and (*d*) from a cell of the same preparation as that of *c*, but which has retained half its intracellular K^+. The *P* signal in *d* is the large peak just to the left of the *S* signal.

Table 1. Initial estimates of intracellular composition of mucosal epithelial cells (other than goblet cells) (Expt. I)

State of Specimens Examined	Na			P			S			Cl			K			Rb		
	ICF	NUC	CYTO	ICF	NUC	CYTO	ICF	NUC	CYTO	ICF	NUC	CYTO	ICF	NUC	CYTO	ICF	NUC	CYTO
Dehydrated	109	101	110	809	949	753	69	78	65	231	251	223	613	765	551	72	72	73
[mmols · (kg dry wt)$^{-1}$]	±25	±34	±28	±132	±196	±143	±10	±14	±11	±33	±51	±33	±98	±168	±100	±12	±18	±13
	(17)	(6)	(11)	(17)	(6)	(11)	(17)	(6)	(11)	(17)	(6)	(11)	(17)	(6)	(11)	(17)	(6)	(11)
Hydrated	9	–	–	98	–	–	20	–	–	26	–	–	46	–	–	42	–	–
[mmols · (kg wet wt)$^{-1}$]	±3			±23			±4			±1			±11			±10		
	(8)			(8)			(8)			(8)			(8)			(8)		

Data are presented in this and succeeding tables as means ± SE, with the number of determinations entered in parentheses. The symbols NUC and CYTO refer to determinations at nuclear sites and cytoplasmic sites, respectively. Entries under ICF refer to all intracellular measurements collectively, including determinations at overlapping nuclear and cytoplasmic sites and at unidentified intracellular positions.

readily appreciated among the spectra from the mucosal medium and intracellular sites under baseline and experimental conditions.

Initial Measurements

Table 1 presents the microprobe results obtained with hydrated and dehydrated sections from the initial experiment. Small areas of tissue had been mounted on agar supports and bathed with a mucosal medium containing 30 mM Rb^+. The mucosal Rb^+ was helpful in rapidly identifying the mucosal medium. However, presumably because of edge damage associated with the unfavorable perimeter-to-area ratio for the tissues studied, significant amounts of mucosal Rb^+ came into contact with the basolateral membranes of the epithelial cells. Since Rb^+ closely mimics K^+ in toad bladder (Robinson & Macknight, 1976*b*), the transporting cells were able to accumulate substantial amounts of intracellular Rb^+, whether or not K^+ was present in the serosal medium. The data of Table 1 obtained with dehydrated sections were taken from the control tissue. The results obtained from the hydrated sections came from the experimental tissue, bathed in a K^+-free serosal medium. The total intracellular K^+ and Rb^+ concentration of the experimental hydrated material is not very different from the K^+ concentrations subsequently measured in other control tissues (Table 3). These microprobe data are consistent with the electrophysiological observations. Because of the availability of Rb^+ to the basolateral surfaces, the removal of serosal K^+ had little effect on the transepithelial potential or short-circuit current (Expt. I, Table 2).

Table 2. Electrical parameters of transepithelial transport

Expt.	Control			Experimental			Experimental conditions
	V (mV)	R (kΩ)	I_s (μA)	V (mV)	R (kΩ)	I_s (μA)	
I	61	1.35	45	45	1.26	36	0 serosal K^+
II	78	0.77	102	–	–	–	–
III	74	2.29	32	27	1.69	16	0 serosal K^+
IV	64	2.26	28	25	2.29	11	0 serosal K^+
V	54	1.09	49	12	1.08	11	0 serosal K^+
VI	48	2.12	22	8	1.58	5	0 serosal K^+
VII	71	1.74	41	−1.3	2.17	−0.6	0 serosal K^+ + ouabain
VIII	66	2.33	28	−4.5	1.72	−2.6	0 serosal K^+ + ouabain

V, R and I_s symbolize the transepithelial potential, resistance, and short-circuit current, respectively. In Expt. I, the mucosal medium contained 30 mm Rb^+.

Table 3. Intracellular composition in hydrated sections

Cell type	Experimental conditions	Mucosal medium	Expts.	*N*	Na	P	S	Cl	K
					[mmols·(kg wet wt)$^{-1}$]				
Epithelial cells (other than goblet cells)	Baseline	Alb-3	V	12	14 ±4	136 ±31	35 ±8	27 ±6	101 ±22
		Dextran	VI	11	21 ±4	137 ±17	34 ±4	33 ±6	122 ±14
		Dextran	VI	7	–	145 ±19	26 ±8	24 ±7	125 ±18
		Mean ±SE			18 ±3	139 ±3	32 ±3	28 ±3	116 ±8
	0 serosal K^+	Alb-3	V	3	43 ±21	203 ±58	38 ±16	26 ±7	118 ±35
	0 serosal K^+ +ouabain	Dextran	VII	7	97 ±17	147 ±18	26 ±4	24 ±4	16 ±9
Goblet cells	Baseline	Alb-3	V	1	26	110	132	53	95
		Dextran	VI	1	21	132	53	24	80
	0 serosal K^+ +oubain	Dextran	VII	3	80 ±28	143 ±49	42 ±20	30 ±8	14 ±7

The compositions of the albumin Ringer's solutions (Alb-1, Alb-2, and Alb-3) and dextran Ringer's solution (Dextran) are described in the *Methods* section. *N* is the number of intracellular determinations.

The initial procedure of tissue mounting proved troublesome in two other respects, as well. Because the underlying support was shaped in the form of a pyramid, it proved difficult to avoid oblique sectioning of the tissue, complicating later cellular identification and analysis. More importantly, the relatively large mass of supporting agar obscured the much smaller fragments of epithelial tissue.

These problems were circumvented by mounting the tissues of Expts. II–VIII (Table 2) as flat sheets over relatively larger tubes. The mucosal medium in Expts. II–IV contained 7 mM Rb^+; cellular uptake of Rb^+ was negligible in these experiments. In Expts. V–VIII, Rb^+ was omitted altogether. As can be appreciated from Table 2, using this second experimental approach, removal of serosal K^+ substantially reduced transepithelial potential and short-circuit current.

Baseline Measurements

Table 3 presents the intracellular ionic compositions of hydrated tissues measured under baseline and experimental conditions. The mean values measured for the intracellular concentrations of P, S, and K under control conditions are 139 ±3, 32 ±3, and 116 ±8 mmol·(kg wet wt)$^{-1}$, respectively, and are almost the same as those estimated by Rick et al. (1978). The current estimates for intracellular sodium and chloride concentrations of 18 ±3 and 28 ±3 mmol·(kg wet wt)$^{-1}$, respectively, are slightly different from theirs. These differences, as well as differences in physiologic conditions, tissue preparation, and data reduction between the two studies, are considered in detail in the *Discussion* section.

An appreciably larger number of analyses were successfully performed on dehydrated tissues (Table 4) because of their better stability under the beam and greater image detail. The values for the means ±SE are entered separately for nuclear (NUC) and cytoplasmic (CYTO) sites. In addition, 15% of the 233 analyses summarized in Table 4 were measured at unidentified intracellular sites; these values are included, together with the nuclear and cytoplasmic data, under a combined heading for the intracellular fluid (ICF). As previously observed for toad bladder, the nuclear phosphorus content in [mmol·(kg dry wt)$^{-1}$] is higher and the nuclear chloride content is lower than the corresponding values in the cytoplasm (Rick et al., 1978). In addition, the nuclear potassium content seems somewhat higher than the cytoplasmic content, consistent with microprobe observations in this and other tissues (Gupta et al., 1976, 1977,

Table 4. Intracellular composition of mucosal epithelial cells (other than goblet cells) measured in dehydrated sections [mmols · (kg dry wt)$^{-1}$]

Experimental contitions	Mucosal medium	Expts.	Na			P			S			Cl			K		
			ICF	NUC	CYTO	ICF	NUC	CYTO	ICF	NUC	CYTO	ICF	NUC	CYTO	ICF	NUC	CYTO
Baseline	Alb-2	II. IV	97 ±19 (33)	66 ±14 (12)	103 ±23 (14)	711 ±130 (33)	796 ±187 (12)	712 ±134 (14)	110 ±21 (33)	85 ±20 (12)	115 ±22 (14)	140 ±27 (33)	97 ±22 (12)	154 ±31 (14)	484 ±87 (33)	499 ±112 (12)	485 ±90 (14)
	Alb-3	V	64 ±10 (32)	66 ±14 (14)	63 ±13 (15)	872 ±139 (32)	1,006 ±190 (14)	734 ±121 (15)	178 ±29 (32)	138 ±29 (14)	206 ±42 (15)	164 ±25 (32)	160 ±34 (14)	160 ±29 (15)	613 ±94 (32)	696 ±149 (14)	515 ±81 (15)
	Dextran	VI, VII	54 ±8 (45)	53 ±10 (22)	55 ±10 (20)	771 ±108 (45)	848 ±120 (22)	696 ±127 (20)	60 ±8 (45)	63 ±9 (22)	56 ±8 (20)	160 ±21 (45)	148 ±21 (22)	167 ±22 (20)	531 ±68 (45)	588 ±79 (22)	472 ±68 (20)
	Mean ±SE		72 ±13	62 ±4	74 ±15	785 ±47	883 ±63	714 ±11	116 ±34	95 ±22	126 ±44	155 ±7	135 ±19	160 ±4	543 ±38	594 ±57	491 ±13
0 serosal K^+	Alb-2	III, IV	211 ±42 (33)	177 ±38 (19)	268 ±62 (11)	875 ±182 (33)	954 ±256 (19)	836 ±186 (11)	119 ±22 (33)	114 ±26 (19)	120 ±23 (11)	120 ±25 (33)	106 ±30 (19)	143 ±34 (11)	456 ±91 (33)	509 ±13 (19)	394 ±86 (11)
	Alb-3	V	220 ±33 (32)	263 ±52 (13)	186 ±35 (12)	874 ±132 (32)	928 ±155 (13)	733 ±147 (12)	183 ±25 (32)	175 ±27 (13)	192 ±31 (12)	101 ±16 (32)	81 ±17 (13)	109 ±20 (12)	412 ±63 (32)	445 ±75 (13)	338 ±66 (12)
	Dextran	VI	235 ±53 (3)	–	235 ±53 (3)	916 ±131 (3)	–	916 ±131 (3)	62 ±8 (3)	–	62 ±8 (3)	171 ±32 (3)	–	171 ±32 (3)	465 ±69 (3)	–	465 ±69 (3)
	Mean ±SE		222 ±7	220 ±32	230 ±24	888 ±14	941 ±150	820 ±53	121 ±35	145 ±19	125 ±38	131 ±21	94 ±17	141 ±18	444 ±16	477 ±38	399 ±37
0 serosal K^+ +ouabain	Dextran	VII, VIII	336 ±50 (55)	369 ±60 (29)	314 ±60 (13)	859 ±128 (55)	808 ±134 (29)	762 ±157 (13)	81 ±12 (55)	79 ±12 (29)	98 ±20 (13)	142 ±24 (55)	140 ±22 (29)	127 ±28 (13)	210 ±42 (55)	159 ±34 (29)	168 ±36 (13)

1978*a*; Gupta, Hall & Naftalin, 1978*b*; Rick et al., 1978; Somlyo, Somlyo & Shuman, 1979; Jones et al., 1979). However, taking into account differences in water content between the nucleus and cytoplasm of the granular cells of toad bladder, the calculated potassium concentrations have been reported to be similar in this tissue (Rick et al., 1978). In the single epithelial preparation where it has been technically feasible to impale the nucleus and cytoplasm of a single cell with microelectrodes, the potassium activity has been found to be the same in nucleoplasm and cytoplasm (Palmer & Civan, 1977).

The intracellular water concentration (c_{water}) can be directly calculated from the microprobe data of Tables 3–5:

$$(c_{water})\,[\text{kg water}\cdot(\text{kg dry wt})^{-1}] = \frac{(c_i)_{Dehydrated}\,[\text{mmol}\cdot(\text{kg dry wt})^{-1}]}{(c_i)_{Hydrated}\,[\text{mmol}\cdot(\text{kg wet wt})^{-1}]} - 1. \quad (5)$$

The calculation can be performed for any intracellular ion "i" studied in both hydrated and dehydrated sections. This approach is similar to that previously applied to other tissues (Gupta et al., 1977, 1978*b*; Gupta & Hall, 1979). Table 6 presents estimates of intracellular water based on analyses of intracellular Na, P, S, Cl, and K, together with the mean ±SE calculated under baseline and experimental conditions. The control value was 3.7±0.4 kg water·(kg dry wt)$^{-1}$ for epithelial cells other than goblet cells, and 1.8±0.2 kg water·(kg dry wt)$^{-1}$ for goblet cells.

Effects of Removing Serosal K^+

The removal of potassium from the serosal medium reduced the intracellular potassium and chloride contents of the mucosal epithelial cells (exclusive of the goblet cells) by some 20% and increased the sodium content by about 200% (Table 4). Three hydrated intracellular sites were also successfully analyzed under this experimental condition; the results suggest an increase in sodium concentration and little change in potassium or chloride concentration, but the uncertainty in the determination is obviously large (Table 3). The water content of these cells was calculated to have decreased by about 10%; the uncertainty of the measurement is of the same order of magnitude as the change itself.

Five intracellular sites within goblet cells and two sites within smooth muscle cells were also studied in dehydrated sections from tissue bathed in K^+-free media (Table 5). Although a larger fraction of the intracellular potassium was lost, these cells still retained approximately half their K^+ contents. A substantial amount of chloride was lost. A large amount of sodium was clearly gained in one smooth muscle,

Table 5. Intracellular composition of goblet and smooth muscle cells in dehydrated sections

Cell type	Experimental conditions	Mucosal medium	Expts.	*N*	Na	P	S	Cl	K
					[mmols·(kg dry wt)$^{-1}$]				
Goblet cell	Baseline	Alb-2	II	3	108 ±21	343 ±88	87 ±27	107 ±20	238 ±61
		Alb-3	V	10	49 ±12	433 ±131	267 ±73	92 ±18	300 ±80
		Mean ±SE			78 ±12	388 ±79	177 ±39	100 ±13	269 ±50
	0 serosal K^+	Alb-3	V	5	76 ±24	328 ±106	227 ±94	67 ±24	159 ±48
	0 serosal K^+ + ouabain	Dextran	VII	7	202 ±55	381 ±96	154 ±70	101 ±28	52 ±19
Smooth muscle	Baseline	Alb-3	V	4	89 ±28	454 ±69	205 ±36	244 ±50	640 ±91
	0 serosal K^+	Alb-2	IV	1	88	617	105	26	376
		Alb-3	V	1	216	371	208	148	298
	0 serosal K^+ + ouabain	Dextran	VII	5	371 ±105	891 ±227	120 ±32	175 ±50	88 ±24

Table 6. Intracellular water content [kg · (kg dry wt)$^{-1}$]

Cell type	Nuclide	Experimental conditions		
		Control	0 serosal K^+	0 serosal K^+ + ouabain
Epithelial cells (other than goblet cells)	Na	3.0±1.0	4.2±2.5	2.5±0.8
	P	4.6±0.4	3.4±1.2	4.8±1.1
	S	2.6±1.1	2.2±1.6	2.1±0.7
	Cl	4.5±0.6	4.0±1.6	4.9±1.4
	K	3.7±0.5	2.8±1.2	[12±8][a]
	Mean±SE	3.7±0.4	3.3±0.4	3.6±0.8
Goblet cells	Na	2.2	–	1.5±1.1
	P	2.2	–	1.6±1.1
	S	0.9	–	2.7±2.4
	Cl	1.6	–	2.4±1.3
	K	2.1	–	2.7±2.3
	Mean±SE	1.8±0.2	–	2.2±0.2

[a] Excluded from calculation of mean.

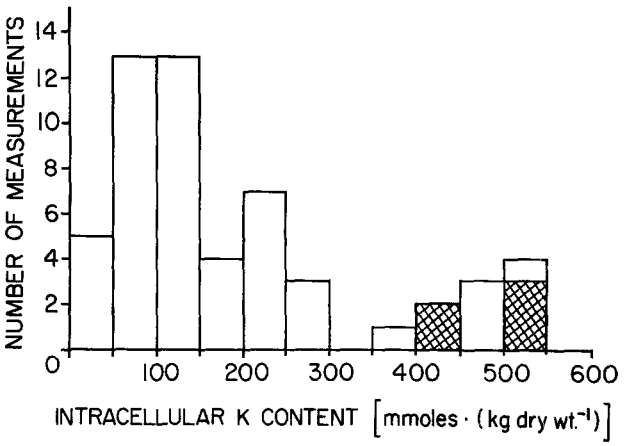

Fig. 5. Frequency distribution of intracellular potassium following concurrent removal of external K^+ and addition of ouabain. A total of 55 measurements were performed at 29 nuclear, 13 cytoplasmic, and 13 unidentified sites within the mucosal epithelium. Data obtained from goblet cells were tabulated separately and not included in this bar graph. Data from basal cells have been included in the bar graph and have also been identified separately by the cross-hatched entries.

with littel change noted in the sodium contents of the other cells within the small sample examined.

Effects of Concurrently Removing Serosal K^+ and Adding Ouabain

The above changes measured by microprobe analysis are qualitatively similar to those observed with chemical analyses of scraped epithelial cells following removal of external potassium (Robinson & Macknight, 1976*a*). However, the basis for the observed surprisingly slow rate of loss of intracellular potassium has been uncertain. In principle, an intrinsic intracellular mechanism (such as subcellular compartmentalization or binding) could be operative. Alternatively, the phenomenon could involve a mechanism extrinsic to the cell, reflecting the structural and functional organization of the tissue as a whole.

In order to further examine these possibilities, tissues were studied after the concurrent removal of serosal potassium and serosal addition of 10^{-2} M ouabain. Under these conditions, active sodium transport was abolished; the transepithelial voltage and short-circuit current were actually reduced to slightly negative values (Expts. VII–VIII, Table 2).

Microprobe analysis of dehydrated sections indicated that the epithelial cells (exclusive of goblet cells) lost an average of about two-thirds of their intracellular potassium contents, gaining corresponding amounts of sodium, with little changes in their chloride contents (Table 4). Similar changes were induced in the goblet and smooth muscle cells examined, except that the mean percentage loss of intracellular potassium (~80%) was greater (Table 5).

It was instructive to plot the frequency distribution of the intracellular potassium contents of the epithelial cells, excluding the goblet cells (Fig. 5). In the presence of ouabain and without serosal K^+, one-third of the cells lost $\geqq$80% of their intracellular potassium, while nearly two-thirds of the cells lost $\geqq$2/3rds of their potassium. The shape of the bar graph (Fig. 5) suggests a bimodal distribution of epithelial cells; 15–20% of the intracellular sites retained $\geqq$2/3rds of their control levels of potassium. It is of interest that this small fraction of potassium-retaining cells included all of the 5 intracellular sites clearly identified to lie within basal cells.

Analysis of hydrated sections also indicated that under these experimental conditions, a very large percentage of the intracellular potassium (~85%) was lost from the epithelial cells in exchange for an accumulation of intracellular sodium, with little change in chloride concentration (Table 3). The intracellular water content was unchanged (Table 6).

Discussion

Baseline Measurements

The present experimental approach has differed in several significant respects from the microprobe study of toad urinary bladder reported by Rick et al. (1978). First, the analysis of both hydrated and dehy-

drated sections gave further assurance that the elemental distributions in the dehydrated specimens were not seriously disturbed by freeze-drying. Second, the normalization of the characteristic X-ray counts against the continuum count obviated the need for any assumptions about uniformity of section thickness or the absence of differential shrinkage of cells and medium. Third, the tissues were both studied *in vitro* and subsequently frozen under open-circuited conditions; thus, the electrical and microprobe data were obtained when the tissue was in the same steady state of transport activity. Usually, as in the previous microprobe study, transport measurements are performed under short-circuited conditions, but the tissues are frozen (or otherwise prepared for analysis) during brief periods under open-circuited conditions. Fourth, both diffracting and energy dispersive spectrometers were utilized in the tissue analysis. Energy dispersive systems are extremely helpful in providing both a measure of specimen mass (the continuum counts) and a measure of the various elemental contents (the characteristic counts), and were used in both studies. However, the analysis for Na is based upon the deconvolution of a very large continuum background in order to measure a much smaller characteristic peak (Figs. 3–4). For this nuclide, crystal spectrometry usually provides greater precision by reducing the background-to-peak ratio by more than an order of magnitude; this disadvantage of energy dispersive spectrometers can be partially overcome by stationing newer highly efficient systems close to the specimen stage, although this point remains controversial (Lechene, 1977).

A fifth major difference between the two microprobe studies of toad bladder lay in the use of the peripheral standards. Rick et al. compared the counting rates obtained at intracellular sites with those in their albumin-Ringer's solution close to the apical plasma membranes of the cell analyzed. In order to ensure a homogeneous distribution of the bulk medium close to the apical membranes, they blotted their tissues with filter paper before exposure to the peripheral standard solution.

In the present study, we wished to avoid possible cell damage and redistribution of ions and water caused by the blotting procedure, itself. Therefore, the tissues were maintained in contact with the albumin- or dextran-Ringer's solution for at least 10 min, in contrast to the 1-min period used by Rick et al. Even so, the macromolecular concentration was lower immediately adjacent to the apical plasma membranes than in the bulk medium $\geqq 2$ μm away.

Another consideration, as well, led us not to adopt the conversation procedure used by Rick et al. Variation in measured characteristic and continuum counting rates within the peripheral standard should reflect both true variation in composition and random sampling errors. It is certainly desirable to minimize these random errors by pooling the separate determinations of the peripheral standard. In addition, under the experimental conditions, true local variation in composition of the mucosal medium caused by unstirred layers and microprecipitates produced during the quenching procedure should have had no significant effect on the ionic composition of the transporting cells. Sodium transport across toad urinary bladder is saturated at mucosal sodium concentrations of 30–40 mM (Frazier, Dempsey & Leaf, 1962); the sodium concentrations used in the current study were severalfold larger. In addition, intracellular K^+ (Robinson & Macknight, 1976*b*) and Cl^- (Macknight, 1977*b*) have been reported to exchange far more slowly across the apical than across the basolateral plasma membranes of the mucosal epithelium.

Despite these experimental differences, the results of the present study obtained under baseline conditions basically confirm and extend the microprobe observations of Rick et al. (1978). The intracellular concentrations of Na^+, K^+ and Cl^- within the transporting cells of toad bladder have been found to be 18 ± 3, 116 ± 8, and 28 ± 3 mol·(kg wet wt)$^{-1}$, respectively (Table 3), while the water content has been calculated to be 3.7 ± 0.4 kg water·(kg dry wt)$^{-1}$ (Table 6). The value for Na^+ concentration is slightly higher than the estimate of some 13 mmol·(kg wet wt)$^{-1}$ reported by Rick et al. (1978). In principle, this small difference could reflect a small degree of overlap of extracellular with intracellular fluid. However, such overlap should also factitiously raise the measured value of intracellular chloride. Rick et al. (1978) reported that their granular cells contained Cl^- concentrations of 41 and 23 mmol·(kg wet wt)$^{-1}$ in the cytoplasm and nucleus, respectively. Since the nuclear volume constitutes only 1/6–1/7th of the total cell volume (D.R. DiBona, *personal communication*) their values should correspond to an average intracellular Cl^- concentration of 38 mmol·(kg wet wt)$^{-1}$ within the granular cells; this value should also constitute an estimate of their mean intracellular chloride concentration for the entire toad bladder insofar as Rick et al. (1978) found similar compositions for the granular, mitochondria rich, and basal cells, and for the basal portions of goblet cells. The present estimate for the baseline intracellular chloride concentration is 28 ± 3 mmol·(kg wet wt)$^{-1}$. a value actually less than theirs. Thus, it seems unlikely that the extracellular fluid contributed significantly to the signals measured at intracellular sites in the present study.

It should be emphasized that in terms of the parameter of greatest physiologic interest, the Na^+ concentration in units of mmol · (kg intracellular water)$^{-1}$, the current estimate is only 20% higher than that calculated by Rick et al. Their reported values for the Na^+ concentration and fractional water content of the granular cell cytoplasm were 13.8 mmol · (kg wet wt)$^{-1}$ and 0.709, respectively, equivalent to a Na^+ concentration of 19 mmol · (kg intracellular water)$^{-1}$. On the basis of the current data, the Na^+ concentration is calculated to be 23 mmol · (kg intracellular water)$^{-1}$. Given the uncertainties of the measurements and the differences in experimental procedure, the two values are in satisfactory agreement.

The intracellular K^+ activity of toad bladder has recently been measured with ion-selective microelectrodes to be 81 ± 2 mM, under conditions minimizing the outward leak of cell potassium around the impalement site (DeLong & Civan, 1979). From this value and the current estimate of intracellular K^+ concentration, the apparent activity coefficient for K^+ within toad bladder is calculated to be 0.55. The intracellular activities of Na^+ and Cl^- have not yet been directly measured with microelectrodes in this tissue.

The baseline water content has been found to be slightly higher than the average value of 3.1 kg water · (kg dry wt)$^{-1}$ obtained by analysis of mucosal cell scrapings, although in any individual experiment, chemical estimates may range from 2.2 to 4.5 kg water · (kg dry wt)$^{-1}$ (Macknight, 1979). It is unlikely that the tissue water content was factitiously raised in the present study. The high measured values of open-circuit potential under baseline conditions (Table 2) indicate that the tissues were not hypoxic. It is also unlikely that water, with/without NaCl, entered the cells during the course of the freezing procedure; such a possibility is incompatible with the findings of elevated Na^+, reduced Cl^-, and identical K^+ concentrations in comparison to the values reported by Rick et al. (1978). Therefore, the present results suggest that the true water content of toad bladder epithelium is at least as high as that estimated from chemical measurements.

Attempts to measure the intracellular Na^+ content and concentration in toad bladder epithelium by chemical analysis of mucosal cell scrapings (Gatzy & Berndt, 1968); Macknight, Leaf & Civan, 1970; Macknight et al., 1971; Lipton & Edelman, 1971: Handler, Preston & Orloff, 1972) have led to puzzling results (Table 7). In the presence of sodium Ringer's solution on both tissue surfaces, the cellular sodium content has been estimated to be very high, approximately 160 mmol · (kg dry wt)$^{-1}$ (Macknight et al., 1975). On the other hand, only 20% of the total chemically measured Na^+ content can be labeled by application of radioactive Na^+ to the mucosal surface. When Na^+ is removed from the serosal medium, the intracellular Na^+ content of mucosal origin (the mucosal pool) measured in mucosal cell scrapings is unchanged; however, the total intracellular Na^+ content is markedly reduced, becoming equal to the intracellular Na^+ content of mucosal origin (Macknight et al., 1975). These data were interpreted to reflect the presence of two chemical pools of intracellular sodium within the mucosal epithelium. The intracellular pool of mucosal origin, most recently estimated to be 40 mmol · (kg dry wt)$^{-1}$ (Macknight, 1979) has been considered to constitute the Na^+ transport pool of physiologic interest. The anatomic site and functional significance of the second and larger pool included within the total chemically determined intracellular Na^+ pool were uncertain.

Table 7. Baseline values of intracellular sodium content in toad urinary bladder estimated by different techniques

Technique	Approximate Na^+ content [mmol · (kg dry wt)$^{-1}$]	Reference
Total chemical content within scraped mucosal cells	160	Macknight, Civan and Leaf, 1975
Intracellular pool of mucosal origin within scraped mucosal cells	40	Macknight, 1979
Electron microprobe analysis	55	Rick et al., 1978; Macknight, 1979
Electron microprobe analysis	72	Present study

Rick et al. (1978) tried to identify the site of the chemically defined intracellular pool of serosal origin by microprobe analysis of the mucosal epithelium in the presence and absence of serosal Na^+. However, they found no measurable difference in the intracellular Na^+ content, amounting to some 55 mmol · (kg dry wt)$^{-1}$ (Macknight, 1979), in the two conditions. Rick et al. (1978) concluded that the chemically measured intracellular sodium of serosal origin was an artifact, reflecting a contribution of extracellular Na^+. Furthermore, they suggested that the intracellular sodium content of mucosal origin measured with radioactive Na^+ was identical with the total intracellular sodium content measured by microprobe analysis, at least under their experimental conditions. The precise nature of the artifact was unclear, but is not likely to have reflected a falsely low estimate of the intracellular water content; the present data indicate that the true value is at least as

large as that estimated chemically. The microprobe results of the current study also confirm that the true intracellular sodium content is considerably smaller than the value estimated from chemical analysis of mucosal scrapings under baseline conditions. However, the results obtained in the absence of serosal K^+ indicate that the intracellular Na^+ content of mucosal origin, measured with radioactive Na^+, is an incomplete measure of the true intracellular content under certain experimental conditions.

Effects of Removing Serosal K^+

Removing potassium from the serosal medium for periods as long as 2 hr reduced the intracellular potassium content by only 20%. This change was accompanied by a substantial increase in intracellular Na^+ of about 150 mmol · (kg dry wt)$^{-1}$, together with reductions in Cl^- and water content of uncertain significance (Tables 4–5). These changes are qualitatively consistent with the changes in ionic and water composition detected by chemical analysis of mucosal scrapings under similar experimental conditions (Robinson & Macknight, 1976*a*), although the current increase in Na^+ is larger and the decrease in Cl^- is smaller than the corresponding changes previously reported. On the other hand, Robinson and Macknight found no significant change whatsoever in the intracellular sodium content of mucosal origin. Clearly, under these experimental conditions, the mucosal Na^+ pool is no longer a valid measure of the true intracellular Na^+ content.

The basis for this dissociation between the mucosal sodium pool and total intracellular sodium content is unclear. However, one possible mechanism bears particular consideration. Several lines of evidence (summarized by Civan, 1977) have suggested that under baseline conditions very little Na^+ enters the transporting epithelial cells of toad bladder across their basolateral membranes. It is possible that the removal of external K^+ leads to an increase in the Na^+ permeability and a recycling of Na^+ across the basolateral membrane; the increased Na^+ permeability could reflect either a change in the bulk membrane or a reversal of direction of the $Na^+ - K^+$ pump.

Effects of Simultaneously Removing Serosal K^+ and Adding Ouabain

The microprobe data obtained in the absence of serosal K^+ confirmed the observation of Robinson and Macknight (1976*a*) that most of the intracellular K^+ of toad bladder epithelium exchanges very slowly with the external bulk media. This phenomenon could have reflected the presence of a small rapidly exchanging intracellular K^+ pool and a separate, much larger slowly exchanging pool, as suggested by Robinson & Macknight (1976*b*). In this event, an intrinsic intracellular mechanism such as subcellular compartmentalization or binding would have been playing a role, although immobilization of intracellular K^+ was highly unlikely (Civan et al., 1976; Shporer & Civan, 1977). Alternatively, the loss of intracellular K^+ might have been slowed by the rapid reabsorption of interstitial K^+ near the basolateral membrane, with recycling of K^+ across this membrane. The results observed following the simultaneous application of ouabain and withdrawal of external K^+ strongly support the second possibility.

The concurrent presence of 10^{-2} M ouabain and absence of serosal K^+ abolished the activity of the $Na^+ - K^+$ exchange pump at the basolateral membrane (Table 2). Thus, K^+ lost from the epithelial cells was free to diffuse into the serosal medium without being subjected to recycling at the basolateral membrane. Under these conditions, microprobe analysis of dehydrated sections indicated an average loss of nearly 2/3rds of the intracellular K^+ from the transporting epithelial cells (Table 4), consistent with microprobe data obtained after the simple addition of ouabain (Rick et al., 1978).

Microprobe analysis of a limited number of epithelial cells in hydrated sections indicated an even larger fall in K^+ concentration of 85% (Table 3). In the absence of a significant change in intracellular water content (Table 6), the percentage losses of intracellular potassium in the hydrated and dehydrated sections should be identical. This observed difference probably reflects the small number of hydrated cells sampled within a functionally heterogeneous population of mucosal epithelial cells. The frequency distribution presented in Fig. 5 strongly suggests that one or more subpopulations of cells retain potassium more avidly than the majority of the epithelial cells under these experimental conditions. A large fraction of these cells appear to be basal cells (Fig. 5). It is unclear whether the increased potassium retentions reflects special membrane properties or the epithelial position of the cells. Specifically, most of the potassium leaking out of the superficial granular cells must pass over the membrane surfaces of the basal cells before entering the serosal bulk medium. A high local external concentration of K^+ could reduce the rate of net passive loss of intracellular K^+ and also interfere with the inhibitory effect of ouabain on the $Na^+ - K^+$ exchange pump (Glynn, 1964); the latter protective effect of external K^+ is , however, likely to be modest in view of the prolonged period of incubation and high serosal concentration of ouabain used in the present study (Glynn, 1957).

Rick et al. (1978) have also reported that the changes in electrolyte composition induced by oua-

bain were somewhat less prominent in the basal cells and much smaller in the mitochondria-rich cells than in the more numerous granular cells. Both their results and the current data may well reflect reduced ouabain binding to the basolateral membranes of the basal cells in comparison with that of the granular cells. Such a phenomenon was observed by Mills and Ernst (1975) in their studies of ^{3}H-oubain binding to frog urinary bladder.

The possibility of the recycling of potassium has long been recognized (e.g., Harris & Burn, 1949; Berridge & Gupta, 1967; Baker, et al., 1969; Lew, Hardy & Ellory, 1973; Phillips, 1977; Ray & Tague, 1978; Gupta et al., 1980), but its possible physiologic significance in transepithelial transport has not been fully and widely appreciated. For example, as noted in the introduction, Essig & Leaf (1963) were the first investigators to report from their analyses of whole urinary hemibladders that removal of serosal K^+ was less effective in altering the intracellular ion composition and in reducing short-circuit current when choline replaced sodium in the serosal medium. This observation was subsequently confirmed by Robinson & Macknight (1976*a*) in their chemical analyses of mucosal cell scrapings, where the very large contributions of the subepithelial elements to the total measured electrolyte and water contents are reduced (Macknight et al., 1975). However, the physical basis for the protective effect of choline has been obscure. In the light of the present data, it seems likely that the phenomenon reflects recycling of potassium at the basolateral membranes of the transporting cells.

In the absence of external potassium, there is a net leakage of K^+ out of the cell. Because of the geometrical constraints of the subepithelial supporting tissue, K^+ cannot instantaneously diffuse into the bulk serosal medium, so that the local concentrations of K^+ near the basolateral membranes are higher than that in the bulk medium. Some of this interstitial K^+ is recumulated by the cells by means of the $Na^+ - K^+$ exchange pump; the rate of net loss of intracellular potassium will be inversely dependent upon the rate of transport activity of the pump. External sodium is known to markedly influence this pump activity (e.g., Garrahan & Glynn, 1967; Baker et al., 1969). The external K^+ concentration required for half-maximal potassium influxes into erythrocytes has been reported to be 1.3–1.5 mM in Na^+ Ringer's solution, and only 0.2 mM in choline Ringer's solution (Garrahan & Glynn, 1967). Thus, at low external concentrations of K^+, the pump functions far more effectively in accumulating potassium in choline Ringer's solution, where the inhibitory effect of external Na^+ is minimized.

The recycling hypothesis presented accommodates a number of otherwise puzzling observations previously published. However, certain points remain unclear. For example, on the average, chemical analysis of scraped mucosal cells suggests that ouabain is only about half as effective in reducing the intracellular potassium content as seems to be the case when estimated by electron microprobe analysis. Second, even after an 8-hr exposure to ^{42}K, Robinson and Macknight (1976*b*) observed that only 40% of the subepithelial tissue potassium had equilibrated with ^{42}K at a time that 60% of the cellular potassium had reached equilibration; the basis for the persistently low specific activity of ^{42}K in the subepithelial tissue is obscure. In the absence of further information, we currently presume that these observations reflect certain unidentified technical limitations associated with chemical analysis of mucosal cell scrapings. The precise basis for these unexplained observations bears further study.

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