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Dissociation of Cellular K^+ Accumulation from Net Na^+ Transport by Toad Urinary Bladder

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Summary. A number of published data suggest a variable stoichiometry between the rates of cellular potassium uptake and net sodium transport (J_{Na}) across the urinary bladder of the toad. This problem was examined by simultaneously studying the intracellular chemical activity of potassium (a_K) with open-tip K^+-selective microelectrodes and micropipets, and monitoring J_{Na} by measuring the short-circuit current (SCC). When bathed in the short-circuited state with solutions containing an a_K of 2.7 mM, the mean $\pm$ SEM values for intracellular a_K were 43 ± 0.6 mM.

Ouabain, at a concentration of 10^{-2} M, reduced intracellular a_K by 56–67% and SCC by 96–100%. At 5×10^{-4} M, ouabain reversibly reduced intracellular a_K by 40–55%, and SCC by 63–68%; the inhibition of SCC was only partly reversible during the period of observation.

Removal of external potassium reduced intracellular a_K by 69–80% and SCC by 51–76%. Restoration of external potassium entirely returned intracellular a_K to its control value, but only partially reversed the inhibition of SCC during the period of study. Furthermore, recovery of a_K began 19–43 min before that of SCC; recovery of a_K was 90–97% complete before any increase in SCC could be measured. Although other interpretations are possible, the simplest interpretation of the data is that the processes responsible for potassium accumulation and transepithelial sodium transport are not identical. We propose the existence of a separate transfer mechanism at the basolateral cell membrane, responsible for accumulating intracellular potassium, and not directly coupled to active sodium transport.

Intracellular potassium is thought to be an important regulator of the transepithelial transport of potassium (Giebisch, 1975) and sodium (Essig & Leaf, 1963). The chemical activity or subcellular distribution of intracellular potassium may also play a role in modulating a wide range of cellular functions, including: cell volume regulation (Kregenow,

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1971*a*, 1971*b*, 1974; Macknight & Leaf, 1977), protein production (Lubin, 1963, 1967; Conway, 1964; Näslund & Hultin, 1971), chromosomal activity (Lezzi & Gilbert, 1970), blast transformation (Quastel & Kaplan, 1970; Negendank & Collier, 1976; Daniele & Holian, 1976), and fertilization (Tupper, 1973). Despite the importance of intracellular potassium, its kinetics of uptake, the nature of the mechanisms responsible for its accumulation, and its subcellular distribution are not yet fully defined.

The working models used by most investigators over the past 20 years in studying sodium transport across epithelia are modifications of the formulation by Koefoed-Johnsen and Ussing (1958). In their view, the sodium-potassium exchange process in the basolateral membranes of the transporting cells provided the basis both for active transepithelial sodium movement and for intracellular potassium accumulation. If this concept were correct, we might expect to observe a stoichiometric ratio between the rate of potassium uptake from the serosal or inner medium into the cell, and the rate of sodium extrusion in the opposite direction. Studies of this point have been inconclusive. Although some of the published data are supportive (Biber, Aceves & Mandel, 1972; Finn & Nellans, 1972), many experimental results suggest that no fixed coupling exists between potassium uptake and sodium extrusion (Essig & Leaf, 1963; Curran & Cereijido, 1965; Essig, 1965; Biber *et al.*, 1972; Candia & Zadunaisky, 1972; Giebisch, Sullivan & Whittembury, 1973; Nellans & Schultz, 1976; Robinson & Macknight, 1976*c*; Kimura *et al.*, 1977; Kimura & Fujimoto, 1977; Valenzo & Hoshiko, 1977).

The inability of investigators to find a characteristic stoichiometry for the sodium-potassium pump of epithelia may reflect a highly variable linkage between potassium uptake and sodium extrusion. A limited slippage in the stoichiometry has been reported for squid axon; Mullins and Brinley have found that between one and three sodium ions may be extruded in exchange for one potassium ion (Mullins & Brinley, 1969; Brinley & Mullins, 1974). Under certain nonphysiologic conditions, (Glynn & Hoffman, 1971; Lew, Hardy & Ellory, 1973; Simons, 1974), the stoichiometry of the pump can be made to depart considerably more widely from the Na^+/K^+ ratio of 3:2 observed under more physiologic conditions (Sen & Post, 1964; Whittam & Ager, 1965; Garrahan & Glynn, 1967). Whether or not the stoichiometry of a single exchange mechanism can change substantially in epithelia under less extreme circumstances is unknown.

An alternative interpretation would be that very little, if any, of the cellular potassium is coupled to sodium extrusion. For example, essentially uncoupled sodium extrusion would result in net charge transfer, establishing an electrical gradient for secondary potassium accumulation (Ussing, 1949). This possibility can be examined directly by comparing the differences in electrical potential and electrochemical potential for potassium across the basolateral plasma membranes of transporting cells. Khuri and his collaborators have, in fact, reported results compatible with this concept in *Necturus* proximal tubule (Khuri *et al.*, 1972*b*), and in rat proximal (Khuri, Agulian & Bogharian, 1974) and distal tubule (Khuri, Agulian and Kalloghlian, 1972*a*). On the other hand, potassium accumulation has been thought to exceed an electrochemical equilibrium distribution in frog skin (Janáček, Morel & Bourguet, 1968), frog intestine (Lee & Armstrong, 1972), *Chironomus* salivary glands (Palmer & Civan, 1975), and urinary bladders of the toad *Bufo bufo japonicus* (Kimura *et al.*, 1977), and frog (Kimura & Fujimoto, 1977). Because of technical problems (Civan, 1978) associated with most of these studies, and the general difficulty in making the measurements, this question is still considered unresolved (Macknight, 1977).

In the current study, we have used intracellular microelectrodes to further examine the relationship between sodium extrusion and potassium accumulation by the urinary bladder of the toad *Bufo marinus*. The urinary bladder of toads obtained from the Dominican Republic is particularly favorable because the short-circuit current can be entirely accounted for by the rate of net sodium transport across the tissue (Leaf, Anderson & Page, 1958), and because extensive information is already available concerning the total intracellular ionic contents under a variety of experimental conditions, and concerning the rates of exchange between intra- and extracellular potassium (Macknight *et al.*, 1971; Handler, Preston & Orloff, 1972; Macknight, Civan & Leaf, 1975*b*; Robinson & Macknight, 1976*a–c*). The transport properties of urinary bladders are known to differ greatly from species to species, and even among different subspecies (Davies, Martin & Sharp, 1968). The results of the present study are in agreement with the steady-state observations reported by Kimura *et al.* (1977) and Kimura and Fujimoto (1977) for other amphibian urinary bladder preparations. However, the primary concern of this work has been to define the time courses of changes induced experimentally in the intracellular electrochemical potential for potassium and in the short-circuit current. The findings have been presented in preliminary form elsewhere (DeLong & Civan, 1978).

Materials and Methods

Animals and Chamber

Female specimens of the toad *Bufo marinus* were obtained from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.) and maintained on moist sphagnum moss at room temperature. Urinary hemibladders were excised from doubly-pithed animals, and mounted mucosal surface up between the two halves of a Lucite chamber similar to that previously described in detail (Civan & Frazier, 1968). The preparations were supported mechanically by a Millipore filter and underlying fenestrated stainless steel disc. The serosal medium was aerated in a reservoir of 0.5–2 liters, and either recirculated or discarded after a single passage through the chamber. The mucosal medium was unstirred and replaced periodically.

Pressure on the bladder's serosal surface was reduced by approximately 10 torr, by placing the serosal system under vacuum and adjusting the rate of aeration through a glass tube in series with a porous glass barrier. This pressure gradient was helpful in immobilizing the tissue for intracellular recording.

Although not critical to the present studies, edge damage was reduced by modifying the earlier chamber. The tissue was clamped between the two Lucite surfaces, as before, but the underlying surface was fitted with a silicone rubber gasket; the enclosed tissue area was 6.2 cm^2. The Lucite borders constituting the outer annulus of this total area were treated with silicone grease (High Vacuum Grease, Dow Corning Corp., Midland, Mich.), a technique used effectively to reduce electrical shunting through damaged tissue (Higgins *et al.*, 1975; Lewis & Diamond, 1976; Civan & DiBona, 1978). The central area of tissue actually exposed to the mucosal and serosal media was only 1.9 cm^2.

Solutions

Under baseline conditions, the tissues were bathed with a standard Ringer's solution consisting of (mM): Na^+ 115.1; K^+, 3.3; Ca^{2+}, 0.8; Cl^-, 113.9; HCO_3^-, 2.2; HPO_4^{2-}, 1.8; $H_2PO_4^-$, 0.3; the pH was 7.6–7.8 and osmolality 215–225 mosmol. In some experiments, a K^+-free Ringer's solution was used, identical with the standard Ringer's solution except for the equimolar replacement of Na^+ for K^+.

Micropipets and Microelectrodes

Micropipets were drawn from Pyrex capillary glass tubing, 0.8 mm OD, 0.6 mm ID (Glass Company of America, Bargaintown, N.J.) using a horizontal micropipet puller (Model M-1, Industrial Science Associates, Inc., Ridgewood, N.Y.). The glass tubing contained a 100-μm glass capillary fused to its inner surface, facilitating filling of the micropipet. A drop of 3-M KCl solution was placed in the barrel; the tip then filled by capillary action. The remainder of the micropipet was finally backfilled by injecting 3 M KCl solution through a syringe equipped with a Millipore filter. The micropipets usually had resistances of 10–30 MΩ and were rejected if the resistance was less than 8 MΩ or if the tip potential exceeded 3 mV.

Liquid-resin K^+-selective microelectrodes (Walker, 1971) were prepared from similar open-tip micropipets, as previously described (Palmer & Civan, 1975, 1977). First, the inner glass surface was coated with dimethyldichlorosilane vapor; best results were obtained

for the micropipets currently used by exposing the glass for 90 sec to vapor from a 50% solution of silane in xylene. After baking the micropipets overnight at approximately 100 °C, a small volume of liquid potassium ion exchanger (#477317, Corning Glass, Corning, N.Y.) was introduced into the barrel; the tip filled spontaneously over a length of 1–2 mm. Finally, the microelectrodes were backfilled with 0.5 M KCl.

The microelectrode response (E_K) could be characterized by the empirical relationship:

$$E_K = E_O + S \log (a_K + k a_{NA}) + \psi \quad (1)$$

where E_O, S and k are constants, a_K and a_{Na} are the chemical activities for potassium and sodium, respectively, and ψ is the electrical potential. The electrodes were calibrated in solutions consisting of: 100 mM KCl, 50 mM KCl, and the Ringer's solutions. This procedure permitted calculation of S and of k. The K^+-selective microelectrodes used fulfilled four criteria: (i) $S \geqq 58$ mV, (ii) $k \leqq 0.04$, (iii) E_K stable in each solution during the period of observation (usually $\geqq 60$ sec), and (iv) electrode resistance $\geqq 10^{10}\ \Omega$. The calibrations were conducted in solutions containing Cl^- as the principal anion. However, E_K does not seem to depend on the anion composition of the solution studied; E_K is unaffected by replacing Cl^- with $H_2PO_4^-$, at constant a_K (Palmer & Civan, 1977).

Since these electrodes are characterized by very low values of k (0.01–0.04), no correction for intracellular sodium was applied to the data. Intracellular values of E_K were considered to reflect only intracellular potassium. Previous control studies have demonstrated that these liquid-resin microelectrodes provide the same estimates of a_K within *Aplysia* neurons (Kunze & Brown, 1971) and frog striated muscle (Palmer, Century & Civan, 1978) as do K^+-selective glass microelectrodes. Furthermore, E_K measured with liquid-resin K^+-microelectrodes agrees closely with the reversal potential measured for the light-induced outward potassium current of *Aplysia* giant neurons (Brown & Brown, 1973). Thus, the electrodes used provide a reliable measure of the intracellular electrochemical activity for potassium.

Electronics and Manipulation

The micropipets and microelectrodes were mounted on modified Huxley micromanipulators (Huxley, 1961) in Lucite housings containing 3-M and 0.5-M KCl solutions, respectively, in contact with silver-silver chloride electrodes. The voltage output of the micropipet or microelectrode was connected to a preamplifier (Model 602, Keithley Instruments, Cleveland, Ohio) with an input impedance of at least $10^{14}\ \Omega$ and an offset current of less than 5×10^{-15} A. The preamplifier output was displayed continuously on a storage oscilloscope.

The transepithelial electrical potential was also measured with a pair of chlorided silver electrodes connected to the chamber by 3-M NaCl agar bridges. The output from the mucosal solution was electrically grounded. The serosal output voltage was introduced into a similar preamplifier, whose output was displayed on a second trace of the oscilloscope..

Electrical current sufficient to reduce the transepithelial potential to zero was provided by a calibrated constant current stimulus isolation unit (Model PSIU 6, Grass Instrument Company, Quincy, Mass.) controlled by a stimulator (Model S44, Grass Instrument Company). Current was passed across the tissue through a set of chlorided silver electrodes in series with 3-M NaCl agar bridges. Superimposed pulses of constant current were also applied to the tissue with a similar stimulus isolation unit and pulse generator. The pulses were usually adjusted to hyperpolarize the tissue by 10–20 mV for periods of 10 sec.

Experimental Procedure

The position of the exploring microelectrode or micropipet tip was determined electrically. Since a substantial difference in E_K was observed between the intra- and extracellular fluids, the position of the microelectrode tip could be determined from the voltage output of the electrode. However, in the short-circuited state, the intracellular electrical potential has been reported to be relatively small (Frazier, 1962; Civan & Frazier, 1968; Reuss & Finn, 1974). It was therefore useful to monitor the relative resistance between the micropipet or microelectrode tip and reference mucosal solution and between the serosal and mucosal media.

After intracellular recording, usually for periods of $\geqq 30$ sec, the microelectrode was withdrawn. If the calibration of the K^+-microelectrode changed during the course of the experiment, all measurements obtained with that electrode were rejected. The resistance and tip potential of each micropipet were also measured before and after each impalement; if the tip potential changed by more than 3 mV, the results of that penetration were discarded.

In the first half of the experiments performed, the intracellular values of a_K and electrical potential were measured during alternate periods with K^+-selective microelectrodes and open-tip micropipets, respectively, both during baseline and experimental conditions. However, it soon became clear that under short-circuit conditions, the intracellular electrical potential was small and did not change significantly during the course of the experimental conditions employed (-4 ± 0.06 mV, measured in 138 penetrations). Therefore, in the second half of the experiments, the intracellular electrical potential was assumed unchanged, and only the intracellular electrochemical activity for K^+ was measured. This approach permitted much more frequent monitoring of the intracellular a_K, allowing for a more precise comparison of the time courses of intracellular potassium activity and short-circuit current during the experimental perturbations.

The results of each impalement were photographed on Polaroid film with an oscilloscope camera for further data analysis. Unless otherwise stated, values reported in the text are the means $\pm$ SEM.

Results

Figures 1*a–b* and 2*a–b* reflect the results obtained in 5–10% of the penetrations performed. In Fig. 1*a*, the transepithelial potential (upper trace) responded to each of two pulses of constant current. No response was observed for E_K (lower trace) when the microelectrode was in the mucosal solution, because of the negligible resistance interposed between the microelectrode tip and reference electrode. Upon impaling the cell, E_K abruptly increased by about 50 mV and subsequently responded to the second pulse of transepithelial current. Except for the transient response to the current pulse, E_K remained entirely stable for approximately 22 sec, until the microelectrode was withdrawn into the reference medium. Such stability of the intracellular trace can be obtained both under baseline conditions (Figs. 1*a*, 2*a*) and during experimental periods (Figs. 1*b*, 2*b*).

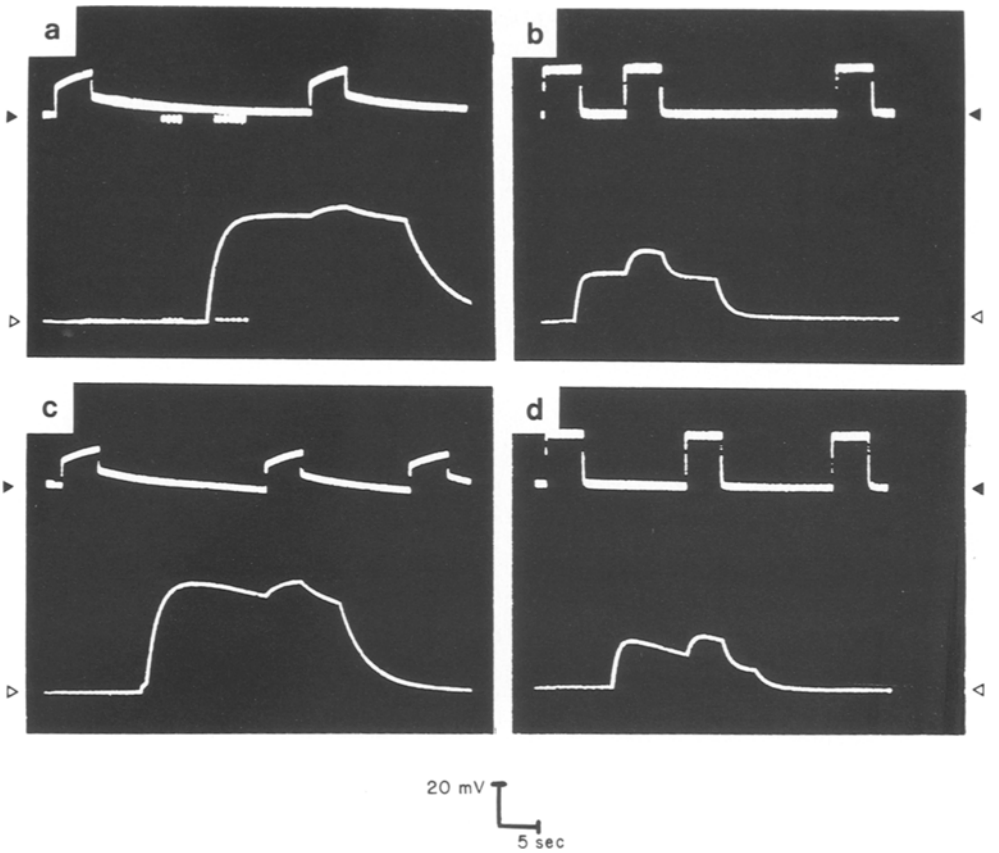

Fig. 1. Effect of ouabain on intracellular electrochemical activity of potassium. The upper trace presents the transepithelial potential (ψ_S-ψ_M) as a function of time. The lower trace is a record of the difference in electrical potential (E_K) between the exploring K^+-selective microelectrode and the reference mucosal solution. The closed and open triangles identify the null positions for the upper and lower traces, respectively. With the microelectrode tip in the mucosal medium, 10-μA pulses of constant current displaced the transepithelial potential from the short-circuited state, without producing any deflection in E_K. Upon impaling a cell, E_K rapidly rose to a new value. In 5–10% of the impalements, E_K displayed an extended plateau, remaining stable for periods of up to 30 sec before withdrawal of the tip (*a–b*); in most of the penetrations, E_K displayed only a brief plateau, beginning to decline only a few sec after the impalement (*c–d*). With the microelectrode tip in the cell, both (ψ_s-ψ_m) and E_K responded to pulses of constant current. Records *a* and *c* were taken during the initial control period. *b* and *d* were taken 36 and 43 min, respectively, after introducing 10^{-2} M ouabain into the serosal medium. The peak values of E_K from the impalements with brief plateaus (*c* and *d*) are close to the corresponding stable values of E_K from impalements having extended plateaus (*a* and *b*). The interrupted lines of *a* indicate the null positions for the upper and lower traces after withdrawal of the microelectrode. The calculated value of the intracellular chemical activity for potassium, and the measured values of short-circuit current for the four frames were: (*a*) 47 mM, 60 μA; (*b*) 13 mM, 12 μA; (*c*) 48 mM, 70 μA; and (*d*) 13 mM, 7 μA

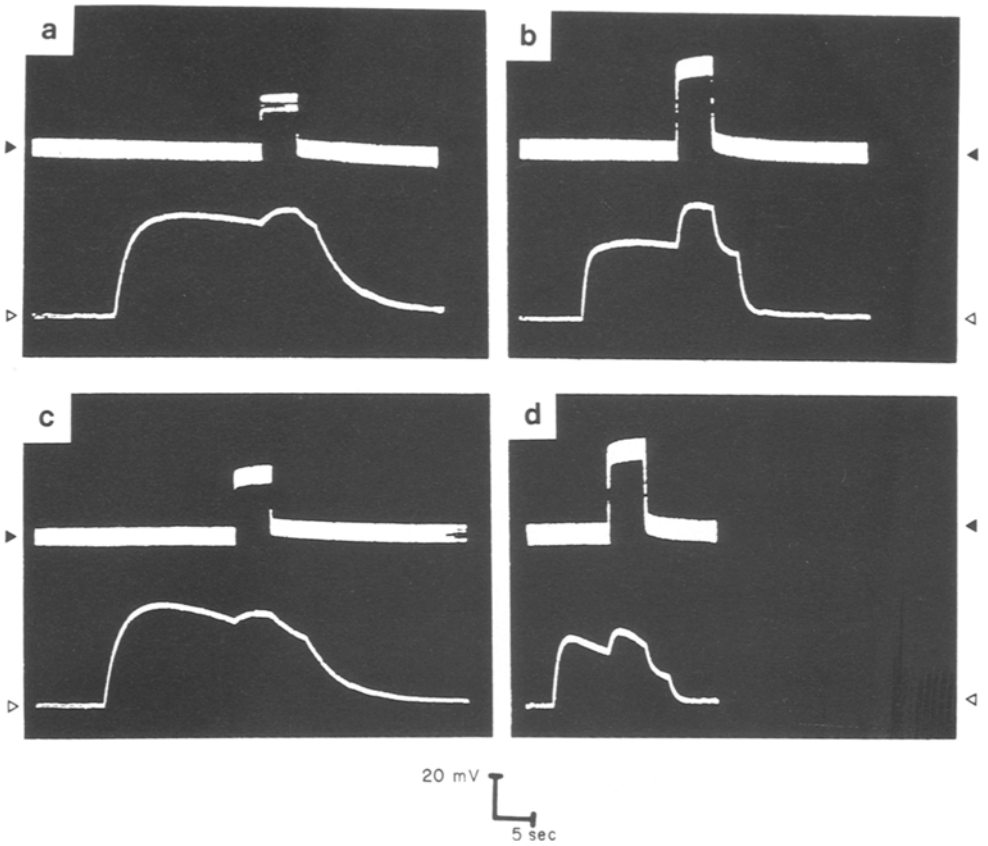

Fig. 2. Effect of removing external potassium on intracellular electrochemical activity of potassium. Records *a* and *c* were obtained during the initial control period. Subsequently, the tissue was incubated for 82 min in nominally potassium-free media. *b* and *d* were taken 10 and 7 min, respectively, after restoring potassium to the mucosal and serosal solutions. As in Fig. 1, the peak values of E_K are similar for impalements taken under similar conditions (*a* and *c*, *b* and *d*), whether an extended plateau (*a–b*) or a brief plateau (*c–d*) is noted for E_K. Constant current pulses of 5 μA were applied in *a* and *c*, and of 3 μA in *b* and *d*. The values of intracellular chemical activity of potassium and of short-circuit current for the four panels were: (*a*) 38 mM, 25 μA; (*b*) 24 mM, 7 μA; (*c*) 38 mM, 22 μA; and (*d*) 21 mM, 7 μA

Figures 1 *c–d* and 2 *c–d* are representative of the time courses of E_K noted during the majority of intracellular impalements. Upon advance of the microelectrode tip into the cell, E_K rises to a substantially higher value and, after a variable period of a few seconds duration, begins to decline. Following penetration of cells with open-tip micropipets, no substantial change in the intracellular electrical potential was noted

throughout the period of declining E_K. Thus, in the period of declining E_K some potassium escapes from the cell, reducing the chemical activity of intracellular K^+. On the other hand, the peak intracellular values of E_K from such penetrations are indistinguishable from the constant values obtained from impalements exhibiting stable time courses.

Table 1 presents comparative data obtained from 12 tissues under control and experimental conditions. Comparison is made between each average value of E_K, estimated from one or more impalements displaying a stable time course, with that estimated from impalements of the same tissue at about the same time, but characterized by a brief voltage plateau. The mean difference ±SEM for the entire series of measurements is

Table 1. Comparison of intracellular potassium activities (in mM) measured during impalements characterized by extended or brief periods of stability

Experimental conditions	Exp. No.	Extended plateau	Brief plateau	Difference
Control	1	44 (21)	43 (14)	−1
	2	43 (2)	42 (3)	−1
	3	48 (1)	48 (8)	0
	4	46 (1)	40 (12)	−6
	5	44 (1)	45 (10)	1
	6	41 (4)	41 (11)	0
	9	47 (3)	45 (10)	−2
	10	44 (1)	42 (15)	−2
	11	46 (3)	44 (14)	−2
	12	38 (1)	41 (12)	3
Ouabain	2	13 (4)	15 (8)	2
	4	22 (1)	18 (14)	−4
	10	20 (1)	18 (17)	−2
	11	27 (1)	25 (20)	−2
Restoration of external K^+	8	42 (1)	45 (18)	3
	9	48 (1)	44 (22)	−4
	Mean			−1.1
	±SEM			±0.63

Examples of the two types of intracellular records are presented in Figs. 1 and 2. Each tabulated value in columns 3 and 4 is the mean of the number of measurements given in parentheses. In each row, the two sets of records were obtained from the same tissue under the same conditions at approximately the same time. Application of the Kolmogorov-Smirnov test as previously described (Siegel & Civan, 1976) indicates that the distributuon of the differences between the paired sets of measurements is not significantly different (at the 0.20 probability level) from a *t*-distribution. Therefore, the Student *t*-test can be applied; the mean difference in activity of −1.1 mM is thereby found insignificantly different from zero, at the 0.10 probability level.

1.1 ± 0.6 mV, a value insignificantly different from zero. Because of this result, the difference in E_K between the intracellular fluid and mucosal medium was consistently measured as the peak deflection after impalement.

Using this approach and Eq. (1), the average intracellular value of a_K was found to be 43 ± 0.6 mM for tissues bathed with the standard Ringer's solution containing potassium at an activity of 2.7 mM. Thus, the apparent equilibrium potential for K^+ would be -71 mV within the cell with respect to the external medium; this value is 18 times larger than the measured value of -4 ± 0.06 mV. Thus, in the present experiments, the difference in electrochemical potential $[(\Delta\mu_K)_{cs}/F]$ across the basolateral membrane was $71-4=67$ mV.

In three preliminary experiments (Table 2), ouabain was applied to the serosal surface of the toad bladder at a concentration of 10^{-2} M. After 60 min, the short-circuit current had fallen to $1 \pm 0.1\%$, and the average intracellular value of a_K had fallen to $38 \pm 3.2\%$ of their initial control values. The intracellular electrical potential had, however, not changed under short-circuited conditions. Ouabain's effect on short-circuit current preceded its effect on intracellular potassium activity.

In order to examine the time course of the effects of adding and

Table 2. Magnitudes of effects on intracellular potassium activity (a_K) and short-circuit current (SCC) associated with adding ouabain or removing external K^+

Exp.	$(a_K)_i$	$(a_K)_e$	$\frac{(a_K)_e}{(a_K)_i}$	$(SCC)_i$	$(SSC)_e$	$\frac{(SCC)_e}{(SSC)_i}$	$(a_K)_f$	$\frac{(a_K)_f}{(a_K)_i}$	$(SCC)_f$	$\frac{(SCC)_f}{(SCC)_i}$
	mM	mM		μA	μA		mM		μA	
Ouabain (10^{-2} M)	42 ± 0.8	14 ± 0.6	0.333	56	2	0.036	--	--	--	--
	48 ± 1.2	17 ± 0.7	0.354	17	0	0	--	--	--	--
	41 ± 1.0	18 ± 0.9	0.439	34	0	0	--	--	--	--
Ouabain (5×10^{-4} M)	42 ± 0.5	19 ± 0.3	0.452	34	11	0.324	41 ± 1.4	0.976	16	0.470
	44 ± 0.5	25 ± 0.6	0.568	33	12	0.364	42 ± 0.4	0.955	21	0.636
	43 ± 0.8	26 ± 0.6	0.605	19	7	0.368	41 ± 0.8	0.953	13	0.684
Removal of external K^+	45 ± 1.0	9 ± 0.3	0.200	32	10	0.313	--	--	--	--
	42 ± 0.6	11 ± 0.4	0.263	30	8	0.267	--	--	--	--
	41 ± 0.8	10 ± 0.3	0.244	25	6	0.240	--	--	--	--
	42 ± 0.5	13 ± 0.3	0.310	43	21	0.488	45 ± 0.6	1.071	35	0.814
	45 ± 0.6	12 ± 0.6	0.267	52	18	0.346	45 ± 0.8	1.000	33	0.635
	40 ± 0.7	11 ± 0.6	0.275	19	7	0.368	42 ± 1.1	1.050	10	0.526

The subscripts i, e, and f refer to measurements obtained during the initial control, experimental, and final control periods, respectively.

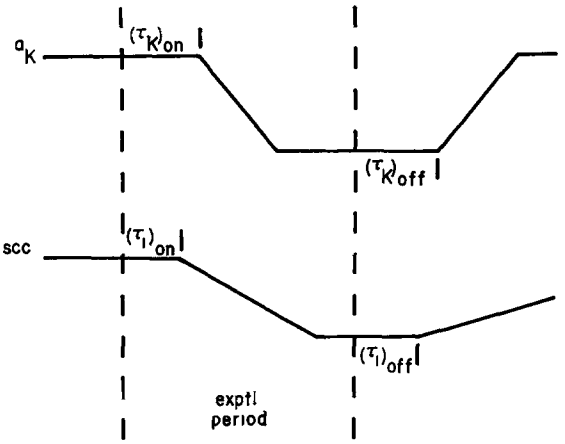

Fig. 3. Definition of time parameters, τ

removing ouabain, another series of three hemibladders was studied at a lower dose (5×10^{-4} M) (Figs. 1 and 4, Table 2). After 41–66 min, the average short-circuit current and intracellular a_K had fallen to $35 \pm 1.4\%$ and $54 \pm 4.6\%$ of their control values, respectively. Upon removal of ouabain, the short-circuit current recovered only partially, rising to $60 \pm 6.5\%$ of the initial control value. However, potassium accumulation was almost entirely reversible; intracellular a_K was restored to $96 \pm 0.7\%$ of the pretreatment value.

In each of the three experiments, ouabain reduced the short-circuit current 9–14 min before changes were noted in the intracellular value of a_K (Table 3, Fig. 4). Recovery from the effects of ouabain was slow, and it was difficult to determine the precise times of onset of recovery (Fig. 4). In two of the three experiments, the short-circuit current began to rise 21 and 53 min before recovery of intracellular a_K began; in the third experiment, recovery of short-circuit current appeared to lag behind that of intracellular a_K (Table 3).

Removal of potassium from the serosal and mucosal media also reduced short-circuit current and intracellular potassium activity (Figs. 2 and 5, Table 2) but the time courses of the onset and recovery of the effects differed from those associated with ouabain. No change was noted in the intracellular electrical potential, measured under short-circuited conditions. After bathing six hemibladders with K^+-free Ringer's solution for 36–68 min, the short-circuit current and intracellular potassium activity had fallen to $35 \pm 3.6\%$ and $26 \pm 1.5\%$, respectively, of their control

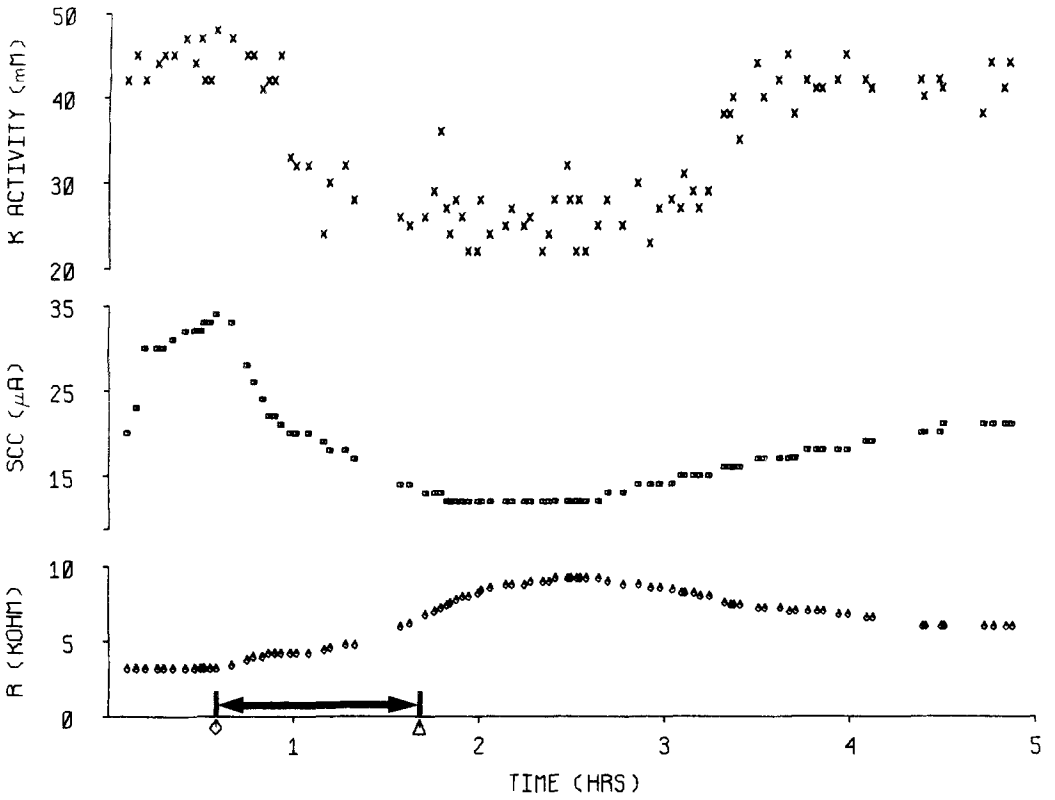

Fig. 4. Time courses of effects associated with ouabain on intracellular chemical activity of potassium, short-circuit current (SCC), and transepithelial electrical resistance (R). The tissue was bathed with serosal Ringer's solution containing 5×10^{-4} M ouabain during the time period indicated by the solid arrow and bracketed by the rhomboid and triangular symbols

Table 3. Time courses (in min) of effects on intracellular potassium activity and short-circuit current associated with adding ouabain or removing external K^+

Exp.	$(\tau_K)_{on}$	$(\tau_I)_{on}$	$(\tau_I)_{on} - (\tau_K)_{on}$	$(\tau_K)_{off}$	$(\tau_I)_{off}$	$(\tau_I)_{off} - (\tau_K)_{off}$	Experimental period
Ouabain	20	3	−17	97	44	−53	65
(5×10^{-4} M)	24	10	−14	82	61	−21	66
	22	13	− 9	34	44	10	41
Removal	--	--	--	6	39	33	91
of K^+	10	18	8	1	20	19	83
	7	16	9	5	48	43	85

As illustrated in Fig. 3, $(\tau_K)_{on}$ and $(\tau_I)_{on}$ refer to the time delays between the beginning of the experimental period and the first reduction in a_K and short-circuit current, respectively. A negative value for $(\tau_I)_{on}$- $(\tau_K)_{on}$ indicates that the change in a_K followed that in short-circuit current. The analogous parameters $(\tau_K)_{off}$ and $(\tau_I)_{off}$ refer to the time delays following the return to baseline conditions.

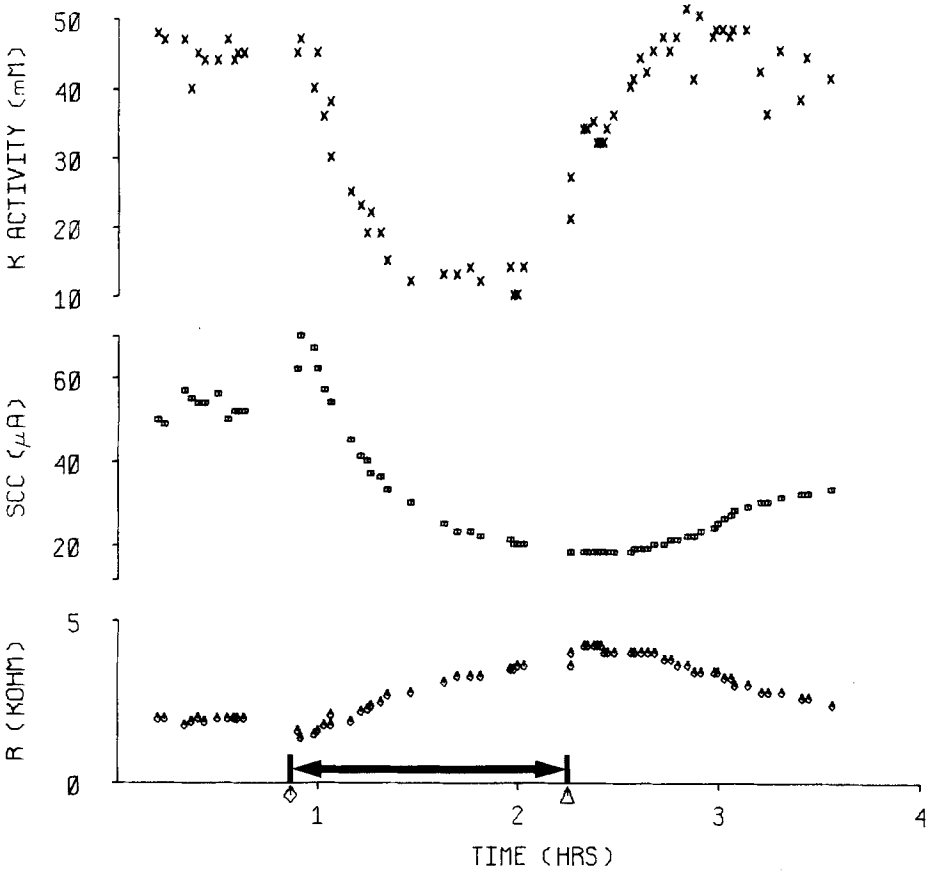

Fig. 5. Time courses of effects associated with removing external potassium on intracellular chemical activity of potassium, short-circuit current (SCC) and transepithelial electrical resistance (R). The tissue was bathed with potassium-free mucosal and serosal media during the time period indicated by the solid arrow and bracketed by the rhomboid and triangle

values (Table 2). After restoring potassium to the media, the short-circuit current returned to $66 \pm 8.4\%$ of the initial control value, while a_K was entirely reversed to $104 \pm 2.1\%$ of its preexperimental value (Table 2). In contrast to the experience with ouabain, removal and subsequent restoration of potassium from the bathing media elicited changes, first in intracellular a_K and only later in short-circuit current. After removing potassium from the external solutions, intracellular potassium activity began to fall 8–9 min before any reduction was noted in the short-circuit current. After returning potassium to the bathing media, intracellular a_K began its recovery 19–43 min before that of the short-circuit current (Table 3).

The time courses of intracellular a_K and short-circuit current differed

in another respect, as well. An initial stimulation of short-circuit current was noted, as previously reported (Essig, 1965; Finn, Handler & Orloff, 1967; Robinson & Macknight, 1976*a*,*b*), without any transient rise in intracellular potassium activity. In order to examine whether a transient change in the intracellular electrical potential might have been masking changes in intracellular a_K, two experiments were performed in which only the intracellular electrical potential and short-circuit current were monitored throughout the course of removing and restoring external potassium. No significant deviation from the average intracellular potential of -4 mV was observed at any point in the experiment.

It will be noted in Figs. 4 and 5 that the transepithelial resistance was inversely related to the short-circuit current, but could not be correlated with the intracellular potassium activity during and after the experimental period. This inverse correlation was consistently noted and has been observed in high resistance epithelia both under baseline conditions (Higgins *et al.*, 1975; Erlij, 1976; Lewis & Diamond, 1976) and following administration of aldosterone (Civan & Hoffman, 1971; Gruber, Knauf & Frömter, 1973; Spooner & Edelman, 1975) or vasopressin (Yonath & Civan, 1971).

The fraction of the total transepithelial resistance noted across the apical permeability barrier varied from about 0.2 to 0.6. Although the fractional apical resistance appeared higher in some impalements following either addition of ouabain (Fig. 1) or removal of external K^+ (Fig. 2), this observation was not consistent. If anything, the fractional apical resistance appeared to decrease after the experimental periods, in comparison to the baseline values obtained some hours earlier.

Discussion

The current results permit estimation of the intracellular potassium activity for purposes of comparison with previous estimates of intracellular potassium concentration (c_K); these data agree with those from previous electrometric studies of amphibian urinary bladders (Kimura & Fujimoto, 1977; Kimura *et al.*, 1977). However, the major observation of the present study was the dissociation between potassium accumulation and short-circuit current, established by comparing their time courses during and following two experimental conditions.

The measurements of intracellular a_K were subject to at least three potential sources of error: junction potentials, leakage of cell potassium during impalements, and interdiffusion potentials.

A systematic error can be introduced into determinations of membrane potential by changes in junction potential at the micropipette tip between extracellular and intracellular fluids. Recent evidence has suggested that such junction potentials can be estimated by application of the Henderson equation (Palmer & Civan, 1977). Using the values of cytoplasmic c_K, c_{Cl} and c_{Na} estimated from chemical and tracer analysis of whole mucosal cell scrapings (Macknight, Civan & Leaf, 1975*a*) and the current electrometric estimate of c_K, the change in junction potential can be calculated by this approach to be no more than 1.0 to 2.2 mV (micropipette positive with respect to cell).

In general, data obtained with intracellular microelectrodes provide a lower bound to the true chemical gradient for potassium across the plasma membrane. Some potassium must leak out of the cell around the tip of the microelectrode because of damage inevitably associated with the impalement; such leakage was, in fact, observed after the first several seconds in the majority of the penetrations. However, the error associated with loss of cell potassium is likely to be small. In 5–10% of the impalements, the intracellular value obtained with the K^+-selective microelectrode was entirely stable throughout the period of observation of some 20–30 sec, and was equal to the peak value noted with the other penetrations.

Perhaps the most significant possible error in the present electrometric measurements was an interdiffusion potential around the pipette tip inevitably arising from some outward leakage of intracellular potassium and anions and entry of sodium and chloride. The consistent value of -4 ± 0.06 mV obtained for the intracellular potential during many penetrations under short-circuited conditions suggests that the membrane potential was truly small; otherwise, some variation in potential might have been expected, depending on the degree of membrane potential. The apical membrane potential presumably reflects the ionic concentration gradients, relative ionic permeabilities, and the energy dissipations associated with movement of charges across the plasma membrane; thus, the absence of measurable changes in membrane potential during the two experimental conditions may have reflected the interplay of several mutually antagonistic effects.

Alternatively, the constancy of the membrane potential and the relatively low and variable fractional apical resistance may have reflected appreciable shunting around the micropipette tip. This possibility is supported by the report of appreciably higher values of the fractional apical resistance in *Necturus* urinary baldders impaled from the serosal,

rather than the mucosal, surface (Higgins, Gebler & Frömter, 1977); the same absolute leak damage was thought to provide a larger fraction of the total conductance measured during mucosal impalements, because of the much larger apical than basolateral membrane resistance. On the other hand, other investigators have reported identical electrical potential profiles across toad urinary bladder (Kimura *et al.*, 1977) and frog urinary bladder (Kimura & Fujimoto, 1977) measured during mucosal and serosal impalements. Thus, the precise role of interdiffusion potentials in the present study is uncertain. The measurements suggest that the apical membrane potential is small, but its exact value is not yet rigorously established.

It should be emphasized that the geometries of the micropipettes and microelectrodes were identical and that the same impalement procedure was used in measuring the intracellular values of ψ and E_K. Thus, an interdiffusion potential should affect both measurements equally. Calculations of the intracellular potassium activity, dependent upon $(E_K\text{-}\psi)$, are therefore unaffected by the uncertainty in the absolute membrane potential. This reasoning is supported by comparing the present results with those obtained previously (Kimura *et al.*, 1977). From the relative electrical resistances of the micropipettes used in two studies, and taking account of the different filling solutions, we may calculate that the tips of the current micropipettes and microelectrodes were more than 2.4 times smaller than those used previously. On this basis, membrane damage is likely to have been of greater significance in the earlier study. Nevertheless, the results are very similar, despite the fact that Kimura *et al.* impaled cells from a different species of toad under open-circuited, rather than short-circuited conditions. The present estimate of intracellular a_K is 43 ± 0.6 mM, close to the earlier estimate of 41 ± 0.5 mM.

Assuming the activity coefficient of intracellular potassium to be 0.76, the value characterizing solutions of NaCl at the ionic strength of Ringer's solution, the measured value for a_K is equivalent to a c_K of 57 mM. Correction for the maximum calculated contribution of the junction potential would raise the estimate for c_K to 62 mM. These values represent some 40–50% of the total intracellular c_K measured chemically; similar observations have been reported in electrophysiologic studies of rat proximal tubule (Khuri *et al.*, 1974) and frog urinary bladder (Kimura & Fujimoto, 1977). This fraction is in rough agreement with the fraction of rapidly exchanging potassium described in the same preparation. Approximately $^1/_4$ to $^1/_3$ of the total intracellular potassium content exchanges with a half-time of 30 min, while the remainder exchanges with

a half-time of about 9 hr (Robinson & Macknight, 1976*c*). The site of the presumed subcellular compartmentalization remains to be determined.

The current results support, but do not prove, the concept that the transporting cells of this tissue accumulate potassium from the serosal medium against an electrochemical gradient $[(\Delta\bar{\mu}_K)_{cs}/F]$ of 67 mV; only a negligible fraction of the intracelluar potassium has been shown to enter from the mucosal medium (Robinson & Macknight, 1976*c*). Similar gradients have been obtained for the urinary bladder of *Bufo bufo japonicus* (57 mV; Kimura *et al.*, 1977) and for frog urinary bladder (67 mV; Kimura & Fujimoto, 1977) under open-circuited conditions.

Effect of Ouabain

Addition of serosal ouabain was found to decrease both short-circuit current and intracellular potassium activity. This observation is in agreement with previous studies of intracellular potassium pools of toad urinary bladder, based on chemical analyses of isolated mucosal cells (Macknight *et al.*, 1975*b*), electron probe microanalysis of whole tissue (Rick *et al.*, 1978) and intracellular electrometric measurements (Kimura *et al.*, 1977). This observation does not agree with the results of kinetic washout studies of radioactive potassium from whole tissue, where no change was noted in either of the two identified intracellular potassium pools (Finn & Nellans, 1972). Since the transporting cells contribute only about 19% of the total intracellular potassium content of whole tissue (Macknight *et al.*, 1975*a*), and since the potassium transport pools seem to constitute only a fraction of this value, the kinetics of washout may not provide a sufficiently precise measure of the potassium transport pools to detect changes associated with ouabain.

The magnitude of the fall in intracellular potassium activity induced by ouabain agrees closely with the change noted in intracellular potassium concentration measured in bladders from the same subspecies under similar experimental conditions. After incubating hemibladders for 60 min in the short-circuited state, Macknight *et al.* (1975*b*) found that the intracellular potassium content fell by 102 ± 14 mmoles/kg dry wt in transporting cells maintaining a water content of 2.9 kg/kg dry wt. Assuming an intracellular activity coefficient of 0.76, the observed changes in chemical content would have been equivalent to a fall in potassium activity of 27 mM. Under these conditions, the average fall

in intracellular a_K calculated from the present electrometric measurements (Table 2) is 27 ± 2 mM.

Kimura *et al.* (1977) have reported comparable results despite the difference in experimental tissue and conditions previously noted. After bathing their tissue with 5×10^{-4} ouabain for 210 min, they found that intracellular potassium activity fell by 30–32 mM.

The time courses of the effects of adding and removing ouabain are not very different from predictions based on current concepts of epithelial sodium and potassium transport. If a single mechanism were responsible for both potassium accumulation and net transepithelial sodium transport, ouabain should inhibit both transport functions *pari passu.* However, the time required for potassium to leak passively out of the cell would introduce a time delay before a change in a_K could be measured; the magnitude of the delay both in adding and removing ouabain would be inversely dependent upon the initial size of the intracellular potassium pool.

In only one respect were the results obtained with ouabain unexpected. Removal of ouabain permitted the cells to reaccumulate potassium to within 90% of their intracellular activities, while the short-circuit current recovered very little. If a single pump indeed subserves both transport functions, we must invoke some change in the state of the tissue between the initial control and later recovery periods. For example, the passive potassium permeability of the basolateral membrane may have decreased, the stoichiometric ratio of sodium extruded to potassium accumulated may have significantly fallen, or the rate of backflux of sodium from serosa-to-mucosa may have increased.

Effect of Removing External Potassium

Removal of external potassium produced an effect on intracellular potassium activity roughly similar to that on intracellular potassium concentration described previously. Robinson and Macknight (1976*b*) found that incubation for 60 min in a potassium-free serosal medium reduced the average intracellular potassium concentration by 19 ± 3 mM. Again assuming an intracellular activity coefficient of 0.76, the fall in average intracellular c_K would correspond to a fall in a_K of 14 mM. From the results presented in Table 2, the average change in intracellular a_K measured in the present study was 32 ± 1 mM, twice the predicted value, but close to the fall of 28 mM reported for frog urinary bladder (Kimura & Fujimoto, 1977). Although addition of serosal ouabain or

removal of serosal potassium appeared to reduce the total potassium content by about the same amount (Robinson & Macknight, 1976*c*), incubation for 36–85 min in potassium-free media in the present study consistently reduced the intracellular potassium activity to a greater extent (by 69–80%) than did incubation for comparable periods of time (31–154 min) in 10^{-2} M ouabain (by 56–67%) (Table 2). The half-time for the fall in intracellular a_K following removal of external potassium may have been slightly shorter (10–30 min) (Fig. 5) than that characterizing changes in c_K (30 min).

The time courses of the effects produced by restoring external potassium were entirely unexpected. Return of external potassium produced a rapid and complete restoration of intracellular potassium activity to its pretreatment control value. This process was 90–97% complete before any recovery whatsoever could be observed in short-circuit current (Fig. 5), demonstrating that potassium accumulation and short-circuit current can be entirely dissociated in toad bladder. However, this datum might not necessarily reflect a dissociation of potassium accumulation from net transepithelial sodium transport. Specifically, (i) the short-circuit current might have been an insufficiently sensitive measure of net sodium transport, (ii) baseline net sodium transport may have been independent of the serosal cations, and (iii) the short-circuit current may not have reflected basolateral Na^+-K^+ exchange.

A rough calculation may be performed in order to examine whether a measurable increase in short-circuit current would necessarily be associated with the amount of potassium reaccumulated by a Na^+-K^+ exchange pump inoperative without external potassium. The dry wt of the transporting cells scraped from the mucosal surface of hemibladders currently used (Macknight *et al.*, 1975*a*) is about 4 mg. In the present experiments, approximately 31% of the total tissue area was studied. Thus, the dry wt of the transporting cells studied was about 1.2 mg. In Fig. 5, restoration of potassium to the bathing media increased the intracellular potassium activity from 12 to 45 mM, over the 20-min period preceding the rise in short-circuit current. The transporting cells contain approximately 2.7 kg water/kg dry wt in the absence of serosal potassium, and some 3.1 kg water/kg dry wt in its presence (Robsinson & Macknight, 1976*b*). Making the simplifying assumptions of uniformity of potassium distribution and equality of the intra and extracellular activity coefficients for potassium, restoration of external potassium was likely associated with the net uptake of 0.18 μmoles by the section of tissue studied. If this potassium accumulation were coupled in a 1:1 exchange with

cellular sodium during the course of transepithelial sodium transport, the short-circuit current should have nearly doubled, undergoing an average increase of 15 μA over the 20-min period. Thus, the expected increase would have been an order of magnitude greater than the uncertainty in measuring the short-circuit current.

The possibility exists that the baseline short-circuit current in the nominal absence of external potassium reflected Na^+-Na^+ exchange through the sodium pump, where the number of sodium ions extruded exceeded that accumulated; for example, the pump may have ejected 3 sodium ions in exchange for taking up 2 sodium ions. It is conceivable that the pump operated at precisely the same rate after restoration of external potassium, but at a more physiologic stoichiometry, let us say at 3 Na^+/2 K^+. Although conceivable, it is hardly likely that the rate of net sodium transport would be entirely unaffected by the identity of the exchanging ion. Certainly, the rate constant for the ouabain-sensitive sodium efflux from human erythrocytes is $2^1/_2$–3 times greater in the presence than in the absence of external potassium (Glynn & Hoffman, 1971). Furthermore, if the restoration of potassium could abruptly restore the normal stoichiometry of the pump, permitting reaccumulation of potassium, it is unclear why the short-circuit current would not also be stimulated at the same time.

The results do not necessarily preclude the possibility that the Na^+-K^+ exchange pump was extruding some sodium from the cell while accumulating potassium from the serosal medium across the basolateral membrane. Some Na^+-K^+ exchange could be restored across the basolateral membrane in the absence of net sodium entry across the apical membrane without increasing the short-circuit current. However, appreciably less sodium could be extruded than potassium accumulated by this mechanism. Removal of serosal potassium causes the transporting cells of toad urinary bladder to lose 116 ± 8 mmoles potassium/kg dry wt and to gain 47 ± 11 mmoles sodium/kg dry wt (Robinson & Macknight, 1976*b*). Therefore, the pump could extrude no more than about one ion of sodium in exchange for three ions of potassium, in the absence of apical sodium entry into the cell. This estimate probably represents a distinct overestimate for the Na^+-K^+ exchange since some of the potassium accumulated can diffuse back into the serosal medium across the basolateral membrane, while very little recycling of sodium occurs across this membrane (Sharp & Leaf, 1966; Coplon & Maffly, 1972; Canessa, Labarca & Leaf, 1976; Hong & Essig, 1976).

Thus, on the basis of the above considerations, the data of Fig. 5

appear to establish a true dissociation between potassium accumulation and net transepithelial sodium transport. This observation supports the concept that removal of external potassium reduces net sodium transport not simply by reducing the potassium activity of the solution bathing the external surface of the sodium pump (Essig & Leaf, 1963; Robinson & Macknight, 1976*b*). Although the local external potassium activity at the membrane pump site may have been less than that of the bulk serosal medium because of unstirred water layers, nearly complete restoration of intracellular potassium activity occurred without stimulating any measurable net transepithelial sodium transport.

The delayed time course of recovery for short-circuit current, in comparison to that for intracellular a_K, strongly suggests that some intracellular event is playing a crucial role in restoring transepithelial sodium transport. It seems unlikely that the cytoplasmic potassium activity has a direct effect on stimulating net transport, because of the prolonged lag time of 19–43 min between the onset of increase in intracellular a_K and that in short-circuit current (Table 3, Fig. 5). The precise sequence of intracellular events occurring during this period remains to be defined. Shrinkage, *per se*, of cell volume (Ussing, 1965) is unlikely to be playing a major role; when the serosal potassium-free medium is hypotonic, cell volume increases, but the transporting cells still lose intracellular potassium and the short-circuit current is markedly reduced (Robinson & Macknight, 1976*b*).

Although agreement is incomplete (Finn & Nellans, 1972; Biber *et al.*, 1972), some investigators have noted little change in the cellular uptake of potassium under a number of conditions either stimulating or inhibiting transepithelial sodium transport (Essig & Leaf, 1963; Curran & Cereijido, 1965; Essig, 1965; Biber *et al.*, 1972; Candia & Zadunaisky, 1972; Giebisch *et al.*, 1973; Macknight *et al.*, 1975*a*,*b*; Nellans & Schultz, 1976; Robinson & Macknight, 1976*c*; Kimura *et al.*, 1977; Kimura & Fujimoto, 1977; Valenzo & Hoshiko, 1977). The data obtained following restoration of external potassium in the current study probably constitute the most striking example thus far reported of dissociation between potassium accumulation and transepithelial sodium transport.

Working Hypothesis

The results of the present study may be interpreted most simply in terms of separate transfer mechanisms subserving homocellular potassium and transepithelial sodium transport. We suggest that the sodium

pump can be inhibited by ouabain and is largely uncoupled to potassium accumulation. Such characteristics have been noted in a heterogeneous reconstitution system for vesicles incorporating Na, K-activated adenosinetriphosphatase from canine renal medulla (Goldin & Tong, 1974), but have not been observed for similar vesicles formed in a more purified system (Goldin, 1977).

In addition, we propose the existence of a separate transfer mechanism at the basolateral cell membrane, which is responsible for the accumulation of intracellular potassium. It seems likely, albeit not yet rigorously established, that such accumulation proceeds against an electrochemical gradient for potassium. If indeed present, the potassium pump can be at least partially inhibited by ouabain.

It should be emphasized that this interpretation is not unique and is presented simply as a working hypothesis for further study. The results of the present study could also be interpreted within the more traditional framework of a single Na^+-K^+ exchange pump, responsible for both sodium extrusion and potassium accumulation. However, such a mechanism would necessarily be capable of altering the stoichiometry of exchange dramatically between the extremes of nearly pure cellular extrusion of sodium and nearly uncoupled accumulation of potassium.

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