

Na⁺ and K⁺ Transport at Basolateral Membranes of Epithelial Cells

II. K⁺ Efflux and Stoichiometry of the Na,K-ATPase

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ABSTRACT Changes of ⁴²K efflux (J_K^{23}) caused by ouabain and/or furosemide were measured in isolated epithelia of frog skin. From the kinetics of ⁴²K influx (J_K^{32}) studied first over 8–9 h, K⁺ appeared to be distributed into readily and poorly exchangeable cellular pools of K⁺. The readily exchangeable pool of K⁺ was increased by amiloride and decreased by ouabain and/or K⁺-free extracellular Ringer solution. ⁴²K efflux studies were carried out with tissues shortcircuited in chambers. Ouabain caused an immediate (<1 min) increase of the ⁴²K efflux to ~174% of control in tissues incubated either in SO₄-Ringer solution or in Cl-Ringer solution containing furosemide. Whereas furosemide had no effect on J_K^{23} in control tissues bathed in Cl-rich or Cl-free solutions, ouabain induced a furosemide-inhibitable and time-dependent increase of a neutral Cl-dependent component of the J_K^{23} . Electroconductive K⁺ transport occurred via a single-filing K⁺ channel with an n' of 2.9. K⁺ efflux before ouabain, normalized to post-ouabain (\pm furosemide) values of short-circuit current, averaged 8–10 μ A/cm². In agreement with the conclusions of the preceding article, the macroscopic stoichiometry of ouabain-inhibitable Na⁺/K⁺ exchange by the pump was variable, ranging between 1.7 and 7.2. With increasing rates of transepithelial Na⁺ transport, pump-mediated K⁺ influx saturated, whereas Na⁺ efflux continued to increase with increases of pump current. In the usual range of transepithelial Na⁺ transport, regulation of Na⁺ transport occurs via changes of pump-mediated Na⁺ efflux, with no obligatory coupling to pump-mediated K⁺ influx.

INTRODUCTION

To examine further the mechanism of K⁺ transport at the basolateral membranes of the epithelial cells of frog skin, studies were undertaken to characterize the

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effects of ouabain and furosemide on the ^{42}K efflux of epithelia bathed with either Cl- or SO_4 -Ringer solution. Despite differences in the assumptions and experimental approach between this and the preceding article (Cox and Helman, 1986a), the macroscopic stoichiometry of pump-mediated Na/K exchange was found to be variable.

MATERIALS AND METHODS

Isolated epithelial sheets of abdominal frog skin were prepared according to the methods of Fisher et al. (1980). Tissues were bathed symmetrically in either a Cl-Ringer solution containing 100 mM NaCl, 2.4 mM KHCO_3 , and 2 mM CaCl_2 , or in an SO_4 -Ringer solution containing 56 mM Na_2SO_4 , 2.4 mM KHCO_3 , and 1.2 mM CaSO_4 . K^+ -free Ringer solution, to be referred to as 0- K^+ Ringer solution, contained 100 mM NaCl, 2.4 mM NaHCO_3 , and 1 mM CaCl_2 .

Tissue Labeling of Cold K^+ with ^{42}K

Single epithelial sheets of tissues were loaded with ^{42}K by incubation in large volumes (500 ml) of Ringer solution containing ^{42}K (0.5–5 $\mu\text{Ci/ml}$). Tissue ^{42}K content was measured (see Cox and Helman, 1986a) at timed intervals for up to 10 h. Tissue dry weight (DW) was measured at the conclusion of each experiment after overnight drying at 100°C. After digestion of the dried tissues in HNO_3 , evaporation of the HNO_3 , and subsequent neutralization to pH ~ 7.0 , K^+ content was measured by flame photometry.

^{42}K Efflux (J_k^{23})

^{42}K efflux studies were carried out with tissues short-circuited in chambers (Cox and Helman, 1983). Tissues were preloaded with ^{42}K for 2–4 h, rinsed briefly (~ 1 min) in isotope-free Ringer solution, and then mounted in the chambers. During control and experimental periods of 10–20 min, the basolateral solution was collected at intervals of 1 min by flushing 3 ml of tracer-free Ringer solution through the basolateral chamber (~ 0.5 ml) directly into counting vials. At the end of an experiment (usually 30 min), the apical solution was collected and counted for ^{42}K activity. The appearance of ^{42}K in the apical solution was $< 1\%$ of the total ^{42}K activity collected in the basolateral solution over the duration of the experiment, which indicates that the apical membranes were essentially impermeable to K^+ .

The short-circuit current, I_{sc} , was measured continuously during control and experimental periods and is given in units of microamperes per square centimeter. For purposes of comparison with the I_{sc} , the fluxes of K^+ (unidirectional and net) are also (when appropriate) expressed in units of microamperes per square centimeter. Where a fraction of K^+ flux is involved in an electroneutral mechanism of transport, expression of its magnitude in units of microamperes per square centimeter should be interpreted to indicate only its magnitude and not a mechanism of transport (see Results).

It has been shown previously and confirmed here again that washout of extracellular spaces is complete within several seconds (Cox and Helman, 1983, 1986a; Stoddard et al., 1985; Stoddard and Helman, 1985). Indeed, within several seconds after treatment of basolateral membranes with drugs that affect isotope fluxes or upon exposure to isotope-free basolateral solution, the extracellular spaces in the latter case were washed free of isotope within the intercellular spaces. This is evidenced in the present studies by step changes of ^{42}K efflux concurrent with treatment of the basolateral membranes with ouabain and/or furosemide. The drugs must, within a few seconds at most, diffuse within the intercellular spaces to the basolateral membranes and change the ^{42}K efflux, and

washout of extracellular ⁴²K must also be completed within at most a few seconds in order to observe "immediate" step changes of ⁴²K efflux (see Results).

Drugs

Both ouabain (Sigma Chemical Co., St. Louis, MO) and furosemide (Hoechst-Roussel Pharmaceuticals, Somerville, NJ) were used at concentrations of 1 mM. Amiloride (Merck, Sharp & Dohme, West Point, PA) was used at 0.1 mM. Solutions containing drugs were prepared immediately before use.

Statistical data are reported as means ± SEM.

RESULTS

Cellular K⁺ Distribution

⁴²K influx occurs primarily via a ouabain-inhibitable Na⁺,K⁺-ATPase (Cox and Helman, 1986a). To determine the rate and extent of isotopic labeling of cellular K⁺, studies identical to those reported in the preceding article were carried out for periods of 8–9 h. Single epithelial sheets were assayed for ⁴²K activity at the time intervals shown in Fig. 1. Although the buildup of tissue ⁴²K activity appeared at first to be essentially monoexponential, with a halftime of ~30–40 min, labeling of cold tissue K⁺ was incomplete after prolonged incubations of up to 10 h. At 8–9 h, only 70.8 ± 4.0 (Cl-Ringer) and 51.4 ± 6.8% (SO₄-Ringer) of cold tissue K⁺ was labeled by ⁴²K (see Table I). At least two exponentials (fast and slow) were required to describe the kinetics of ⁴²K influx (Fig. 1B). The exchangeable fraction of tissue cold K⁺ (fast exponential) averaged 53.3 (Cl-Ringer) and 38.6% (SO₄-Ringer) (Table I). Cold tissue K⁺ averaged 420 (Cl-Ringer) and 310 neq/mg DW (SO₄-Ringer) (Table I). In a larger group of tissues from the same batch of animals, tissue K⁺ averaged 369.4 ± 17.4 (11) (Cl-Ringer) and 283.4 ± 16.3 (16) neq/mg DW (SO₄-Ringer). Accordingly, exchangeable K⁺ was ~193 (Cl-Ringer) and 109 neq/mg DW (SO₄-Ringer). Although intracellular compartmentalization of K⁺ has not been observed by others in studies of frog skin (Curran and Cerejido, 1965; Candia and Zadunaisky, 1972; Ferreira, 1979), there is precedence for the idea of K⁺ compartmentalization derived from studies of toad urinary bladder (Finn and Nellans, 1972; Robinson and Macknight, 1976). Both Curran and Cerejido (1965) and Candia and Zadunaisky (1972) have noted some of the difficulties in assessing the kinetics of ⁴²K influx attributable to the unstirred layers of the corium, and, in this regard, studies with isolated epithelia are preferable. Although Ferreira (1979) has observed two pools for K⁺ in the kinetics of ⁴²K washout (isolated epithelia of *Rana temporaria*), the halftimes of 12.8 (fast pool) and 407 min (slow pool) reported for this preparation are inconsistent with her claim that loading of all tissue K⁺ to specific activity equilibrium was accomplished within 2 h. Given the enormously long halftimes of the poorly exchangeable pool of K⁺, we shall refer to the readily exchangeable K⁺ pool as the "exchangeable pool."

Effects of Amiloride, Ouabain, and K⁺-free Media on Exchangeable K⁺

To further define the magnitude and behavior of the exchangeable K⁺ pool, studies were done to determine the changes of tissue K⁺ caused by high concen-

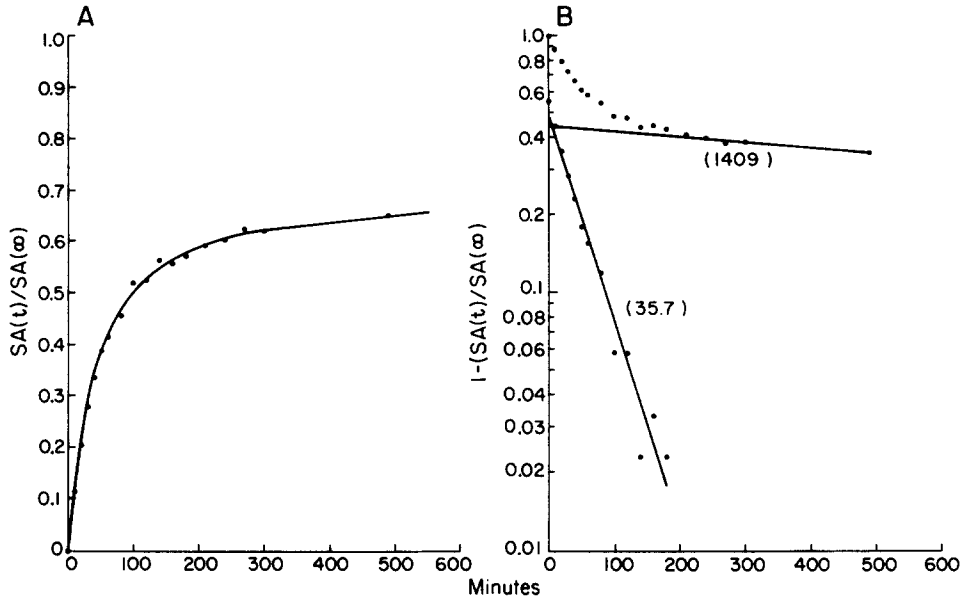


FIGURE 1. Results of typical experiments to measure the kinetics of ^{42}K influx (beaker experiments). (A) ^{42}K influx expressed as a fractional change of specific activity. $\text{SA}(\infty)$ is the specific activity of the extracellular loading solution. $\text{SA}(t)$ is the specific activity of the tissue as a function of loading time (cpm/neq K^+). (B) Data of A replotted in semilogarithmic form. Note the existence of two exponentials with half-times ($t_{1/2}$) of 35.7 and 1,409 min. The K^+ content of the readily exchangeable (fast) pool was $\sim 48\%$ of total tissue K^+ , as estimated from the intercept at the ordinate. The solid line of the fast exponential was drawn according to least-squares linear regression analysis.

trations of amiloride, ouabain, and by K^+ -free solutions, or their combinations. Single epithelial sheets were divided into five pieces. One piece of each tissue served as control, while the others were incubated for 100 min in Cl-Ringer solution containing either 100 μM amiloride, 100 μM amiloride plus 100 μM

TABLE I
Kinetics of ^{42}K Loading of Isolated Epithelia of Frog Skin

	Tissue K <i>neq/mg DW</i>	Fast exponential		Slow exponential		Percent labeling at 8-9 h
		Pool size %	$t_{1/2}$ min	Pool size %	$t_{1/2}$ min	
Cl-Ringer	359	56.7	23.7	44.0	473	78.5
	351	49.2	35.7	44.3	1409	64.9
	550	54.1	29.1	46.6	863	68.9
Mean \pm SEM	420 ± 65	53.3 ± 3.5	29.5 ± 3.5	45.0 ± 0.8	915 ± 271	70.8 ± 4.0
SO_4 -Ringer	350	40.0	41.4	54.8	1663	55.2
	310	50.0	32.1	44.6	2803	60.8
	270	25.8	41.1	73.2	2212	38.2
Mean \pm SEM	310 ± 23	38.6 ± 7.0	38.1 ± 3.0	57.5 ± 8.4	2226 ± 329	51.4 ± 6.8

ouabain, ouabain alone, 0 mM K⁺, or a combination of ouabain and 0 K⁺. The pieces were analyzed by flame photometry for total K⁺.

Summary data are expressed as a percent of the control K⁺ [371.8 ± 14.9 (6) neq/mg DW] in Fig. 2A. In agreement with others (Rick et al., 1978), we observed that amiloride caused a 19.4% mean increase of total K⁺ content and that ouabain in the presence or absence of K⁺ in the bathing solution caused a loss of tissue K⁺. The data were recalculated to reflect the changes of exchangeable K⁺ (see Fig. 2B), assuming that the poorly exchangeable K⁺ remained constant at 46.7% of total K⁺. Amiloride caused exchangeable K⁺ to increase

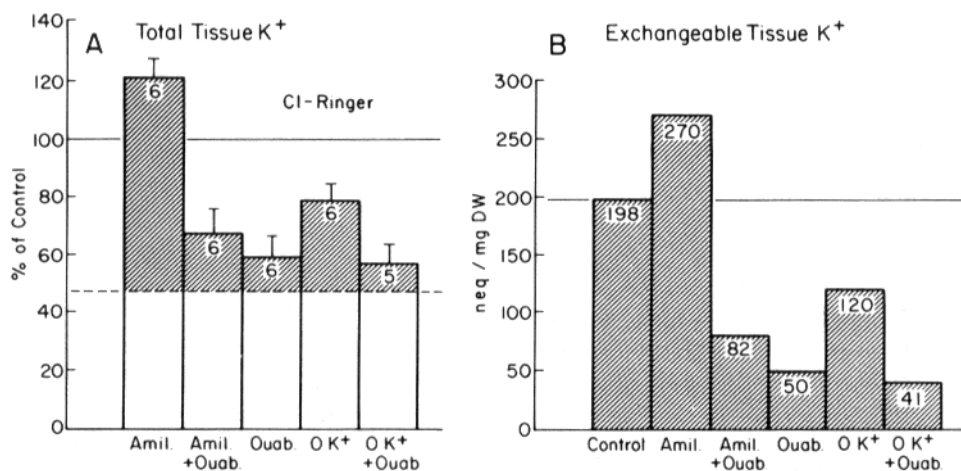


FIGURE 2. Changes of total tissue K⁺ caused by amiloride (100 μ M), ouabain (100 μ M), and/or K⁺-free (0 K⁺) bathing solutions. Tissues were studied in beakers and treated for 100 min. Each of six epithelia were divided into five pieces of ~ 1 cm² each. One served as control and the others served as experimental pieces. (A) Control K⁺ content averaged 371.8 ± 14.9 neq/mg DW. Experimental values are given as a percent of control (mean \pm SEM). The values under the \pm SEM bars are the number of tissues (*n*). The dashed line represents the percent of total tissue K⁺ that exists in the poorly exchangeable pool of K⁺ (see text and Table I). (B) Values of exchangeable tissue K⁺ (neq/mg DW) remaining in tissue after treatment with amiloride, ouabain, and/or 0 K⁺ for 100 min. Control was 198 neq/mg DW. Values were calculated from the difference between the total mean K⁺ content (A) and the mean value of poorly exchangeable K⁺.

from 198 to 270 neq/mg DW (or by 36.4%). Ouabain caused exchangeable K⁺ to fall to 25.3% of control within 100 min after inhibition of the Na,K-ATPase. Although K⁺ removal from the bathing solution caused exchangeable K⁺ to fall to 60.6% of control, it was evident, owing to the ouabain sensitivity of these 0-K⁺-Ringer-bathed tissues (ouabain plus 0 K⁺ = 20.7% of control), that nominal removal of bathing solution K⁺ did not completely inhibit pump activity. This is most likely explained by a nonzero K⁺ concentration in the microenvironment of the cells maintained by the continued loss of K⁺ from the cells.

Neither these data nor those above provide specific information about the nature or location of the poorly exchangeable pool of K⁺. Two intracellular

pools have been identified for Cl^- in other studies of the isolated epithelium of frog skin (Ferreira and Ferreira, 1981; Stoddard et al., 1985; Stoddard, 1984). It would not be difficult to envision compartmentalization and/or binding of K^+ and/or Cl^- within vesicles or other structures that are relatively impermeable to K^+ . K^+ compartmentalization in isolated renal cortical tubules has been reported by Soltoff and Mandel (1984). They suggested, according to the findings of Altschuld et al. (1981), that mitochondria that are relatively K^+ impermeable might contribute to the K^+ compartment poorly accessible to ^{42}K . Given the relatively long half-time for loading of the poorly exchangeable pool of K^+ in the isolated epithelium of the frog skin (Table I), the exchangeable pool behaved for all practical purposes as a single kinetic pool that communicates with the external environment of the cells.

Effect of Ouabain and Furosemide on J_{K}^{23} and I_{sc} (SO_4 -Ringer)

To determine the effects of ouabain on K^+ efflux (J_{K}^{23}), studies were done with tissues short-circuited in chambers. The appearance of ^{42}K in the basolateral solution was measured at intervals of 1 min.

Within 1 min (see Fig. 3), ouabain caused a decrease of I_{sc} to $75.7 \pm 2.0\%$ (14) of control with a concurrent increase of the J_{K}^{23} to $173.1 \pm 11.7\%$ of control (control I_{sc} was $19.7 \pm 3.3 \mu\text{A}/\text{cm}^2$). In separate studies of isolated epithelia bathed with SO_4 -Ringer solution, the basolateral membrane voltage measured with intracellular microelectrodes (V_b) decreased from -78.6 ± 3.2 to -68.9 ± 3.9 (9) mV (Table II) (see also Cox and Helman, 1983, 1986b). After ouabain treatment for 10 min, the tissues were treated further with furosemide. Furosemide caused no acute changes of J_{K}^{23} or I_{sc} (see Figs. 3 and 5). These observations are in accordance with the idea that passive K^+ transport at the basolateral membranes of control tissues is electrodiffusive and that the pumps are electrogenic.

Single-File Diffusion

Since the original findings of Hodgkin and Keynes (1955), it has been observed repeatedly that K^+ channels behave kinetically as single-file-like channels. The K^+ flux ratio is:

$$J_{\text{K}}^{23*}/J_{\text{K}}^{32*} = (I_{\text{K}}^* + FJ_{\text{K}}^{32*})/FJ_{\text{K}}^{32*} = (K_c/K_b)\exp(FV_b/RT)^{n'}, \quad (1)$$

where J_{K}^{32*} and J_{K}^{23*} are the unidirectional passive K^+ fluxes; I_{K}^* is the net K^+ flux via the K^+ channels; K_c and K_b are intracellular and extracellular concentrations of K^+ ; and V_b is the basolateral membrane voltage. We assumed, according to previous measurements, that K_c is ~ 120 mM, that V_b after ouabain is -68.9 mV (Table II, SO_4 -Ringer), and that J_{K}^{32*} of ouabain-poisoned tissues averaged $0.44 \mu\text{A}/\text{cm}^2$ (Cox and Helman, 1986a). Since at the steady state, pump-mediated K^+ influx must equal passive K^+ efflux ($7.4 \mu\text{A}/\text{cm}^2$, as reported in the preceding article, and $8.3 \mu\text{A}/\text{cm}^2$, reported below), I_{K}^* immediately after ouabain was $\sim (7.85 \times 1.74) 13.6 \mu\text{A}/\text{cm}^2$. Solving for n' gave a value of 2.9. This value is in the range measured by others (Hodgkin and Keynes, 1955; Begenisich and De Weer, 1980; Kirk and Dawson, 1983). Assuming that n' is unchanged by ouabain,

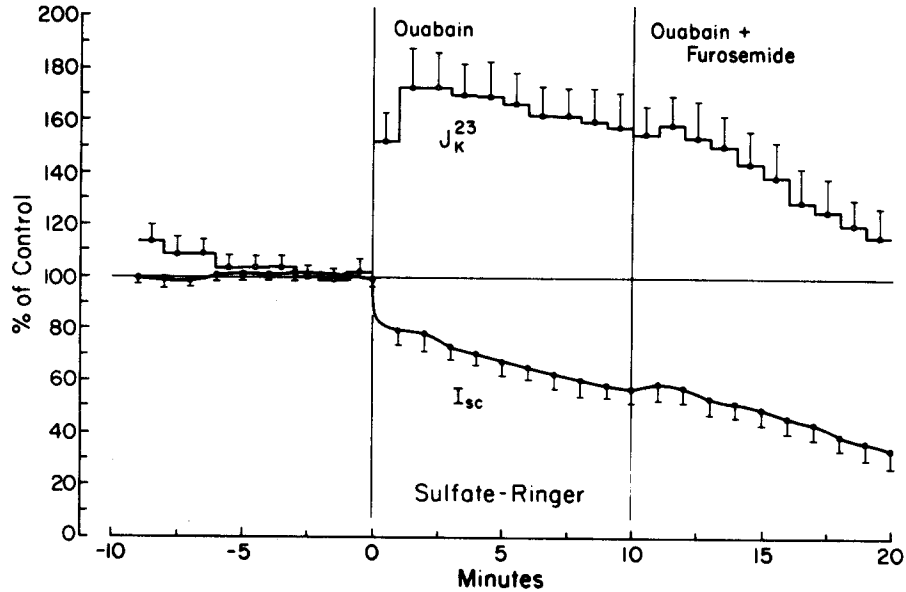


FIGURE 3. Changes of ^{42}K efflux to the basolateral solution (J_K^{23}) and the short-circuit current (I_{sc}) caused by 1 mM ouabain. Tissues were treated additionally with 1 mM furosemide in the presence of ouabain. Epithelia were bathed with SO_4 -Ringer solution. $n = 14$. 100% values were defined immediately before ouabain.

J_K^{32*} before ouabain was calculated to be $0.86 \mu\text{A}/\text{cm}^2$ at the control V_b of -78.6 mV. When compared with the K^+ influx after ouabain of $\sim 0.5 \mu\text{A}/\text{cm}^2$ (Cox and Helman, 1986a), the decrease of J_K^{32*} from control after ouabain was $\sim (0.86 - 0.5) 0.36 \mu\text{A}/\text{cm}^2$ as compared with the increase of J_K^{23*} of $(13.6 - 7.85) 5.8 \mu\text{A}/\text{cm}^2$.

Effect of Ouabain and Furosemide on J_K^{23} (Cl-Ringer)

Studies similar to those above were done with tissues bathed with Cl-Ringer solution. Two experimental protocols were used. First, as shown in Fig. 4A, epithelia were treated first with furosemide for 10 min and then with ouabain in the presence of furosemide. Furosemide caused little or no change of the I_{sc} or

TABLE II
Effect of 1 mM Ouabain on Basolateral Membrane Voltage of Short-Circuited Epithelia of Frog Skin

	V_b	
	SO_4 -Ringer	Cl-Ringer
	mV	mV
Control	-78.6 ± 3.2 (9)	-81.9 ± 4.7 (11)
Ouabain (2-3 min)	-68.9 ± 3.9 (9)	-60.9 ± 7.3 (11)

Values are means \pm SEM (N).

J_K^{23} , although a relatively small transient increase of the I_{sc} was observed over 10 min in several of the tissues. With tissues pretreated with furosemide, ouabain caused changes of J_K^{23} and I_{sc} that were the same as those observed in tissues bathed with SO_4 -Ringer solution. I_{sc} was decreased by ouabain (plus furosemide) within 1 min to $76.5 \pm 2.2\%$ of control, and J_K^{23} was increased to $164.6 \pm 21.1\%$ (6) of control. In a second group of experiments, epithelia were treated first with ouabain and then with furosemide, as shown in Fig. 4B. Although the decreases of I_{sc} caused by ouabain were identical to those reported above ($75.1 \pm 0.7\%$ of control at 1–2 min), the increase of J_K^{23} was smaller at 1–2 min ($137.3 \pm 6.2\%$ [5] of control). J_K^{23} continued to increase during the 10 min of observation. When the tissues were treated thereafter with furosemide, an immediate and unmistak-

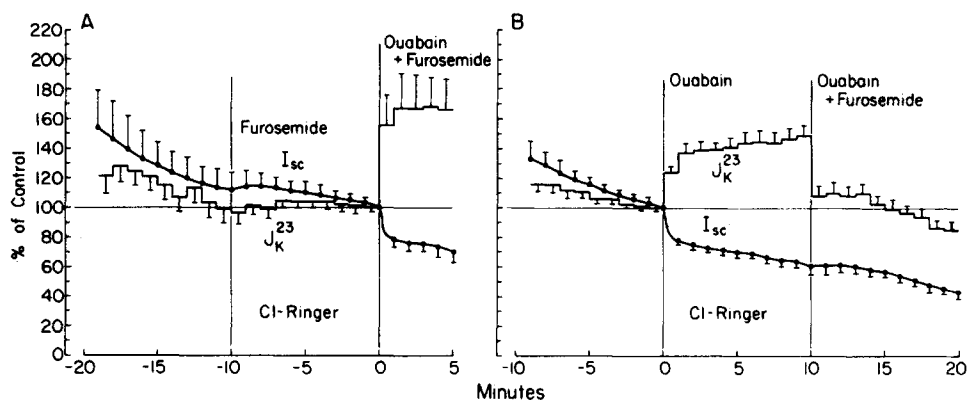


FIGURE 4. Changes of ^{42}K efflux to the basolateral solution (J_K^{23}) and the I_{sc} caused by furosemide and ouabain. Epithelia were bathed with Cl-Ringer solution. (A) Tissues were treated first with furosemide for 10 min and thereafter with ouabain. $n = 6$. 100% values were defined immediately before furosemide. (B) Tissues were treated first with ouabain and then with furosemide. $n = 5$. Notice the inhibition of J_K^{23} by furosemide and absence of change of I_{sc} ; cf. panel A.

able inhibition of the J_K^{23} was observed (148.1 ± 7.2 to $108.9 \pm 7.7\%$ of control) that was not accompanied by a change of I_{sc} . Thus, in the absence of a change of charge transfer across the apical and basolateral membranes of the cells (no change of I_{sc}), the furosemide-inhibitable component of the post-ouabain J_K^{23} was electroneutral. Since there was no acute furosemide sensitivity of the J_K^{23} before ouabain, we inferred (see also Cox and Helman, 1983) that ouabain induced an electroneutral Cl-dependent and furosemide-inhibitable K^+ flux at the basolateral membranes of the cells. If we assume that the 100% value of J_K^{23} corresponded to a K^+ efflux of $\sim 8 \mu A/cm^2$, then the furosemide-inhibitable K^+ efflux, 10 min after ouabain, was in the vicinity of $[8(1.48 - 1.08)] 3.2 \mu A/cm^2$.

Pump-mediated K^+ Flux

Evidence was presented in the preceding article (Cox and Helman, 1986a) to support the idea that K^+ influx saturates, whereas Na^+ efflux continues to increase with increases of pump current. Accordingly, the pump stoichiometry was

variable, ranging from 1.5 at low rates of Na⁺ transport to near 6 at the highest rates of Na transport measured.

To obtain estimates of the pump current and the current carried by K⁺, the data presented above were re-analyzed in the following way. With epithelia short-circuited, net charge transfer must be identical across apical and basolateral membranes of the cells. Therefore, in their control (pre-ouabain) state:

$$I_{sc} = I^P + I_K^* \quad (2)$$

I^P is the net charge transfer ($I_{Na}^P - I_K^P$) via the pump. I_K^* is the net charge carried by K⁺ efflux via K⁺ channels, which at the control steady state must be equal in magnitude to I_K^P .

Since ouabain at high concentrations maximally inhibits the Na,K-ATPase (as evidenced by a 94% inhibition of K⁺ influx [Cox and Helman, 1986a] and an ~80% inhibition of the Na⁺ efflux via the Na,K-ATPase [Cox and Helman, 1983]), we assumed that, after ouabain, $I^P = 0$ and that $I_{sc}^{ouab} = I_K^*$.

$$I_{sc}^{ouab} = I_K^* = FJ_K^{23*} - FJ_K^{32*} \quad (3)$$

Since the K⁺ flux ratio after ouabain was ~32:1, we assumed that FJ_K^{23*} could be equated (within 3%) with the post-ouabain I_{sc} , thereby allowing expression of the ⁴²K efflux in units of current. If, for example, I_{sc} after ouabain was 15 $\mu A/cm^2$, then the ⁴²K efflux at the same time was assigned a value of 15 $\mu A/cm^2$.¹

For studies carried out in Cl-Ringer solution, a similar normalization procedure was used, except that the ⁴²K efflux was normalized to the post-ouabain plus furosemide values of I_{sc} , where we assumed that furosemide inhibited K⁺ flux via the electroneutral mechanism(s) of transport.

FJ_K²³: Experiments with SO₄-Ringer Solution

24 experiments were carried out with epithelia bathed with SO₄-Ringer solution. I_{sc} ranged between 5.6 and 48.6 $\mu A/cm^2$, with a mean of $20.8 \pm 2.8 \mu A/cm^2$ (Table III). Fig. 5 shows the results of a representative experiment (SO₄-Ringer), where the tissue was treated first with ouabain and then with furosemide. Ouabain caused inhibition of I_{sc} and stimulation of J_K^{23} (Fig. 5A). J_K^{23} was normalized to the post-ouabain and ouabain plus furosemide values of I_{sc} , so that on the average there was no mean difference between the post-ouabain values of FJ_K^{23} and I_{sc}^{ouab} (Fig. 5B). As can be seen in Fig. 5A, the ⁴²K efflux "tracked" the I_{sc} fairly well after ~1–2 min of treatment with ouabain. Neglecting any contribution of recycling of Na⁺ via basolateral membranes to the pumps, the minimum current via the pump (I_{min}^P) was calculated as the difference between I_{sc} and FJ_K^{23} ($= I_K^P$) immediately before ouabain. Control FJ_K^{23} (and hence I_K^P) averaged $8.3 \pm 0.9 \mu A/cm^2$, with a range of 3.1–19.0 $\mu A/cm^2$ (Table III). These values are in good agreement with those of I_K^P reported in the preceding article (Cox and Helman, 1986a). We take this as compelling evidence in support of the assumption that the majority if not all of the post-ouabain K⁺ efflux is

¹ The suggestion that I_{sc} after ouabain is the same as FJ_K^{23} was confirmed in experiments where loss of cold K⁺ to a K⁺-free basolateral solution was correlated directly (1.00 ± 0.07) with the simultaneously measured I_{sc} (Cox, 1985).

TABLE III
Effects of Ouabain and Ouabain plus Furosemide on I_{sc} and FJ_K^{23}

	I_{sc}	FJ_K^{23}	I_{min}^p	r_{min}
	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	
SO ₄ -Ringer (N = 24)	20.8 ± 2.8 (5.6–48.6)	8.3 ± 0.9 (3.1–19.0)	12.3 ± 2.0 (1.2–35.6)	2.50 ± 0.19 (1.20–5.00)
Cl-Ringer (N = 19)	21.9 ± 2.2 (10.5–47.0)	10.6 ± 0.9 (6.5–17.7)	11.3 ± 1.6 (3.0–29.7)	2.04 ± 0.13 (1.33–3.89)

Values are means ± SEM (range).

electrodiffusive. It remains impossible, however, to exclude minor amounts of electroneutral K⁺-coupled transport.

I_{min}^p was calculated to average $12.3 \pm 2.0 \mu A/cm^2$, with a range of 1.2 to 35.6 $\mu A/cm^2$ (Table III). From the paired values of I_{sc} and FJ_K^{23} , the apparent minimum stoichiometry of Na/K exchange was found to average 2.50 ± 0.19 , with a range of 1.20–5.00.

FJ_K^{23} and J_{Cl}^{23} : Experiments with Cl-Ringer Solution

Studies similar to those above were carried out with epithelia bathed with Cl-Ringer solution. Normalization of the K⁺ efflux was done assuming that FJ_K^{23} was equal to the post-ouabain plus furosemide values of I_{sc} . Figs. 6 and 7 show the results of representative experiments. Tissues were treated first with ouabain for either 20 (Fig. 6) or 10 min (Fig. 7) and thereafter treated with furosemide. In comparison with SO₄-Ringer-bathed epithelia, the ⁴²K efflux did not “track” I_{sc} after ouabain. Within 1–2 min after ouabain, the values of FJ_K^{23} were the same as those of I_{sc} . Thereafter, FJ_K^{23} continued to increase, diverging from the

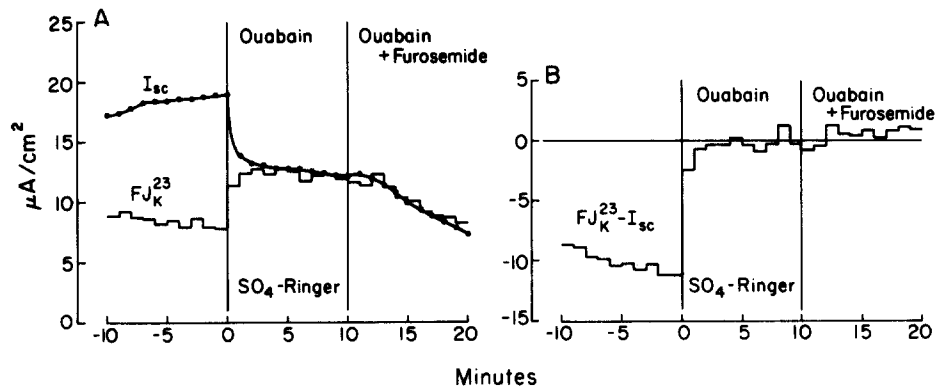


FIGURE 5. Changes of I_{sc} and FJ_K^{23} caused by ouabain and furosemide. Data are from a typical experiment with a tissue bathed with SO₄-Ringer solution. (A) Values of I_{sc} and FJ_K^{23} . See text for method of normalization of J_K^{23} to units of current. (B) Ordinate values are $FJ_K^{23} - I_{sc}$. By definition (see text), I_{sc} after ouabain is equal to FJ_K^{23} . Hence, $(FJ_K^{23} - I_{sc}) = 0$ after ouabain. Before ouabain, $(FJ_K^{23} - I_{sc})$ provides a minimal estimate of the pump current I^p .

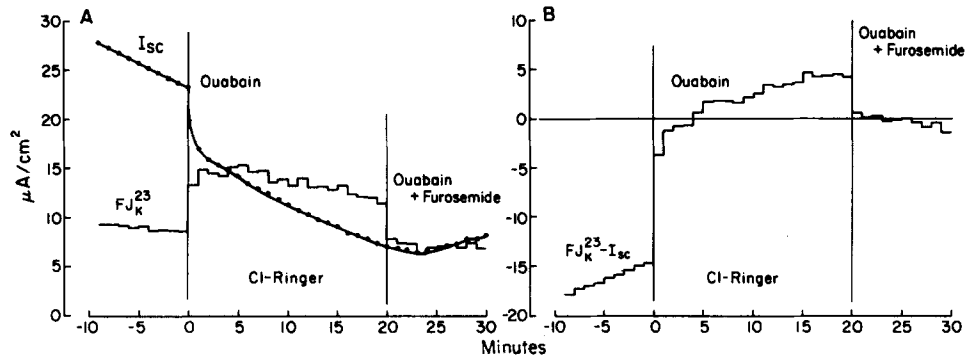


FIGURE 6. Effect of ouabain and furosemide on I_{sc} and FJ_K^{23} (typical experiment, Cl-Ringer solution). See legend to Fig. 5. (A) Values of I_{sc} and FJ_K^{23} . (B) Values of $FJ_K^{23} - I_{sc}$. Notice the furosemide-inhibitable fraction of FJ_K^{23} . Compare with Fig. 5.

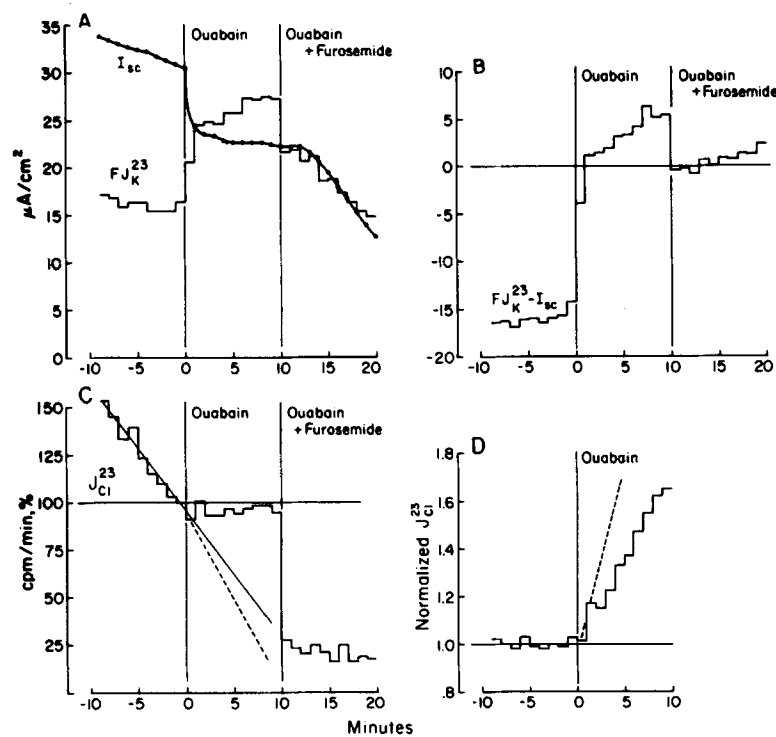


FIGURE 7. Double-label experiments showing changes of ^{42}K (A and B) and ^{36}Cl (C and D) efflux caused by ouabain and furosemide (Cl-Ringer solution). (A) Changes of I_{sc} and FJ_K^{23} . (B) Changes of $FJ_K^{23} - I_{sc}$. (C) The 100% control value was defined immediately before ouabain and all other values were normalized as a percent of control. The decreasing rate of ^{36}Cl efflux (cpm/min) during the control period is attributable to the time rate of change of cellular ^{36}Cl specific activity (see text). (D) Normalized rate of increase of J_{Cl}^{23} caused by ouabain. The dashed line indicates that the rate of increase is underestimated (see text).

continuously decreasing values of I_{sc} . Furosemide caused an immediate inhibition of FJ_K^{23} , with no concurrent change of I_{sc} . Hence, we presumed that the furosemide-inhibitable fraction of the post-ouabain K^+ efflux occurred via an electro-neutral mechanism of transport. Table III summarizes the pre-ouabain control values of I_{sc} , FJ_K^{23} , I_{min}^p , and τ_{min} .

In a final group, experiments were done to measure simultaneously the changes of ^{36}Cl and ^{42}K efflux caused by ouabain. Epithelia were preloaded with ^{42}K as described above and with ^{36}Cl according to methods described by Stoddard (1984) and Stoddard et al. (1985). Fig. 7 shows the results of a representative study. Fig. 7, *A* and *B*, shows the changes of ^{42}K efflux that were described above, where notably ouabain induced a furosemide-inhibitable component of the post-ouabain ^{42}K efflux. The furosemide-inhibitable flux averaged 3.7 ± 0.7 (8) $\mu A/cm^2$ at 10 min and 6.9 ± 1.1 (3) $\mu A/cm^2$ after 20 min of ouabain (Fig. 6). Also notable was the observation that the furosemide-inhibitable ^{42}K efflux did not appear immediately after ouabain (1–2 min) but increased with time after exposure to ouabain ($FJ_K^{23} - I_{sc} = 0.4 \pm 0.5$ [8] $\mu A/cm^2$ between 1 and 2 min after ouabain).

^{36}Cl efflux (J_{Cl}^{23}) was also affected markedly by ouabain. As shown in Fig. 7*C*, the rate of appearance of ^{36}Cl in the basolateral solution decreased continuously during the control period. This was due to the time rate of change of intracellular ^{36}Cl specific activity (Stoddard et al., 1985). After treatment with ouabain, the rate of ^{36}Cl efflux was increased above that expected with reference to the baseline rate of ^{36}Cl efflux established during the control period (solid line of Fig. 7*C*). Although it is impossible to estimate precisely, the dashed line of Fig. 7*C* indicates the deviation of baseline rate of ^{36}Cl efflux owing to the increased rate of change of intracellular ^{36}Cl specific activity. As a rough attempt to normalize for the time-dependent changes of ^{36}Cl specific activity after ouabain, we assumed (as a worst case) a linear extrapolation of the baseline rate of appearance of ^{36}Cl (solid line, Fig. 7*C*). Accordingly, the difference between the observed ^{36}Cl efflux and the linear extrapolated baseline value after ouabain gives an underestimate of the actual percent increase of J_{Cl}^{23} . This is shown also in Fig. 7*D*. J_{Cl}^{23} increased continuously after ouabain, following in parallel the increases of J_K^{23} . This suggests but does not prove, that ouabain induces a co-transport system involving both K^+ and Cl^- . Thus, ouabain not only caused changes of permeability to K^+ but also caused time-dependent changes of permeability to Cl^- . To the extent that J_{Cl}^{23} is considerably larger than J_K^{23} and J_{Na}^{23} (control J_{Cl}^{23} averaged near 21 $\mu A/cm^2$ [Cox and Helman, 1983; Stoddard et al., 1985]), the minimal increase of J_{Cl}^{23} 10 min after ouabain was $\sim(0.7 \times 21)$ 14.7 $\mu A/cm^2$. This far exceeds in magnitude the ouabain-induced increase of FJ_K^{23} ($3.7 \pm 0.7 \mu A/cm^2$ at 10 min), which rules out a simple 1:1 coupling of K^+ and Cl^- . As total tissue Cl^- remains unchanged by ouabain for up to 60–90 min (Rick, R., personal communication), the increases of ^{36}Cl efflux caused by ouabain must reflect changes of Cl^- permeability that could include not only a simple mechanism of Cl^-Cl^- exchange but also cation-coupled co- or countertransport mechanisms. Insufficient information is available at present to resolve this further.

Stoichiometry of the Na,K-ATPase

The conclusion of the preceding article (Cox and Helman, 1986a) was that the stoichiometry of Na/K exchange by the ouabain-inhibitable pump was variable, with r ranging between 1.8 and 7.2. Whereas Na⁺ efflux increased continuously with increases of pump current, K⁺ influx appeared to "saturate," which led to a macroscopic observation of variable coupling ratio.

In the present series of studies, I_K^P of the control state of the tissues was determined by normalization of the electroconductive ⁴²K efflux to the post-ouabain values of short-circuit current. Hence, the values of I_K^P , in units of current, represent net K⁺ charge transfer by the pump. Na⁺ efflux via the pump

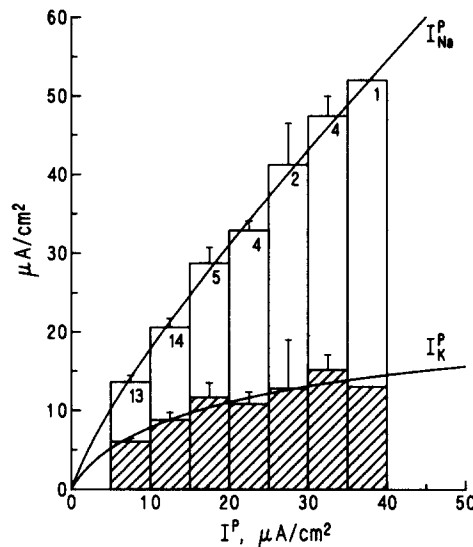


FIGURE 8. Relationships among pump current (I^P), pump-mediated K⁺ influx (I_K^P), and pump-mediated Na⁺ efflux (I_{Na}^P). See text for description. Notice the saturability of K⁺ influx and "linearity" of Na⁺ efflux with increasing I^P at $I_{Na}^P > 10 \mu A/cm^2$. Values below the standard error bars indicate the number of observations.

is the sum of I_{sc} (Na⁺ entry via apical membranes) and net Na⁺ entry via basolateral membranes. To the extent that $I_{sc} \gg$ basolateral membrane Na⁺ entry, I_{sc} measured electrically provides a good approximation of the Na⁺ load of the pumps. For purposes of the following calculation, we have assumed here, as in the preceding article (Cox and Helman, 1986a), that basolateral membrane Na⁺ entry averages $\sim 3.5 \mu A/cm^2$ and is independent of I_{sc} (Stoddard, 1984; Stoddard and Helman, 1985). Accordingly:

$$I_{Na}^P = I_{sc} + 3.5; \quad (4)$$

$$I^P = I_{Na}^P - I_K^P. \quad (5)$$

After calculation of the I_{Na}^P and I^P , the data were grouped into I^P intervals of $5 \mu A$, as shown in Fig. 8, and plotted as an interval histogram. A comparison of

these data with those presented in the preceding article (Cox and Helman, 1986a) leads to the same conclusion—namely, with increases of I^p , K^+ influx appears to saturate, whereas Na^+ efflux continues to increase with increasing Na^+ load to the pump. A least-squares fit of the I_k^p to the empirical relationship,

$$I_k^p = \frac{1}{m + (r^o/I_{Na}^p)}$$

(Eq. 3, Cox and Helman, 1986a) indicated that r^o was 1.56 and m was 0.041. This is shown by the solid line (I_k^p) in Fig. 8. It should be emphasized that the above equation has no known basis in theory, and we have used this empirical relationship only as a means to summarize the findings.

The value of $m = 0.084$ in Cox and Helman (1986a) is about twice the value of 0.041 reported for the present studies. Such a difference in the value of m cannot be taken too seriously in view of the scatter of data points in either group of experiments.

DISCUSSION

Compartmentalization of Cellular K^+

The premise that basolateral membranes of frog skin are highly permeable to K^+ , enunciated originally by Koefoed-Johnsen and Ussing (1958), is not controversial. By virtue of a pump-leak configuration for K^+ transport, pump-mediated K^+ influx is balanced at the steady state by a passive K^+ efflux leading to high intracellular K^+ concentrations in the vicinity of ~120 mM. Such values have been observed in studies of frog skin using electron microprobe analysis (Rick et al., 1978), intracellular ion-selective K^+ electrodes (Garcia-Diaz et al., 1985; Nagel et al., 1981), and measurements of total tissue K^+ and cellular water content (Aceves and Erij, 1971; Fisher et al., 1980; Ferreira, 1979). As none of these methods allows for an assessment of the distribution of K^+ within the cells, kinetic analyses with radioisotopes are useful. There is a precedent for the idea that K^+ is compartmentalized (or bound) within epithelial cells (Finn and Nellans, 1972; Robinson and Macknight, 1976; Soltoff and Mandel, 1984). Studies of the kinetics of ^{42}K loading with the isolated epithelium of frog skin avoid the usual uncertainties imposed by the large unstirred layers of the corium of intact skins as in the studies by Curran and Cereijido (1965) and Candia and Zadunaisky (1972). In the only other study of this kind with isolated epithelia, Ferreira (1979) claimed that all cellular K^+ could be labeled within 2 h. However, this assertion is incompatible with her demonstration that washout of ^{42}K was biexponential, with a halftime of washout of the slow component of 6.8 h (Table I, Fig. 1, Ferreira, 1979). Assuming the latter observation is correct, we would be in agreement that a sizable fraction of intracellular K^+ is either compartmentalized (mitochondria, other intracellular vesicles) or bound.

Assuming that poorly exchangeable K^+ was not responsive to procedures known to affect intracellular K^+ , studies were done to measure the changes of tissue K^+ caused by amiloride, ouabain, and K^+ -free bathing solution. Indeed, in agreement with others, we observed that amiloride caused an increase of cellular

K⁺, whereas ouabain and/or 0 K⁺ caused significant decreases of cellular K⁺. We urge caution in overinterpretation of these data. Not only is it impossible to reduce extracellular [K⁺] to zero during washout of cellular K⁺ into 0-K⁺-Ringer solution, it is also impossible to maintain constancy of the electrochemical potential differences driving K⁺ out of the cells when tissues are treated with ouabain in the presence or absence of K⁺ in the bathing solution. Hence, we made no attempt to determine "rate" coefficients for K⁺ loss after the tissues were exposed to 0 K⁺ and/or ouabain. Nevertheless, these data provided additional evidence in support of the idea that the readily exchangeable pool of K⁺ was sensitive to Na⁺ transport inhibitors. Given that ⁴²K influx proceeds primarily via a ouabain-inhibitable mechanism, we conclude that ⁴²K efflux originates primarily, if not solely, from an intracellular pool of K⁺.

⁴²K Efflux

⁴²K efflux was increased by ouabain concurrent with depolarization of the basolateral membrane voltage. Within 1–2 min, and at the rates of net K⁺ loss measured, there can be little or no change of intracellular K⁺ concentration. Hence, in the absence of significant changes of basolateral membrane electrical resistance (Cox and Helman, 1983), increases of J_K^{23} are probably due to depolarization of V_b . To the extent that the flux ratio for K⁺ in ouabain-poisoned tissues was ~32:1 ($J_K^{23^*}/J_K^{32^*}$), a single-file diffusion factor, n' , of 2.9 was calculated (see Results). Such a value is in the range of 1.5–3.5 reported by others (see, for example, Hodgkin and Keynes, 1955; Begenisich and De Weer, 1980; Kirk and Dawson, 1983) for electrodiffusive K⁺ transport.

With $J_K^{23^*} \gg J_K^{32^*}$, it seemed reasonable to believe that the ⁴²K efflux provided a rather good (to within 3%) estimate of the net K⁺ efflux proceeding via the K⁺ channels. Hence, after ouabain inhibition of the pumps, we assumed that the post-ouabain I_{sc} could be equated with net K⁺ efflux = FJ_K^{23} . Indeed, after ouabain (SO₄-Ringer) or after ouabain plus furosemide (Cl-Ringer), the ⁴²K efflux "tracked" the changes of I_{sc} . Consequently, the FJ_K^{23} values were normalized to the post-ouabain values of I_{sc} . This provided a means of obtaining estimates of the net K⁺ efflux (FJ_K^*) immediately before ouabain, from which the currents I^p and I_K^i were calculated. The FJ_K^{23} reported in Table III (efflux experiments) were not measurably different from the I_K^i (influx experiments) reported in the preceding article (Cox and Helman, 1986a). Thus, we conclude that, despite the differences in methodologies and assumptions, both methods allow for determination of the pump-mediated K⁺ influx.

Ouabain-induced K⁺ and Cl⁻ Efflux

Complicating the analysis for tissues bathed with Cl-Ringer solution is a Cl-dependent, furosemide-inhibitable component of Na⁺ and K⁺ fluxes at the basolateral membranes of the cells. With SO₄-Ringer solution bathing the epithelia, furosemide was without significant effect either before or after treatment of epithelia with ouabain. After ouabain, the ⁴²K efflux tracked I_{sc} , which supports the suggestion that I_{sc} after ouabain was a direct measure of the net K⁺ efflux. However, when epithelia were bathed in Cl-Ringer solution, the FJ_K^{23} efflux after

ouabain did not track the changes of I_{sc} . With increasing time after ouabain, the ^{42}K efflux diverged from I_{sc} , leading to a furosemide-inhibitable fraction of the ^{42}K efflux. As furosemide inhibition of the ^{42}K efflux occurred with neither a consistent change of I_{sc} nor a change of basolateral membrane voltage (Cox and Helman, 1983), it seemed that the furosemide-inhibitable ^{42}K efflux was involved in an electroneutral mechanism of transport.

We also observed that the ^{36}Cl efflux was increased by ouabain. Although parallel changes of ^{42}K and ^{36}Cl efflux are suggestive of a possible coupling mechanism for transport, it remains impossible, as noted in the Results, to exclude other mechanisms of electroneutral transport including simple K/K or Cl/Cl exchangers that are induced by ouabain or, more interestingly, an electroneutral $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ -coupled mechanism (Geck et al., 1980; Kregenow, 1981).

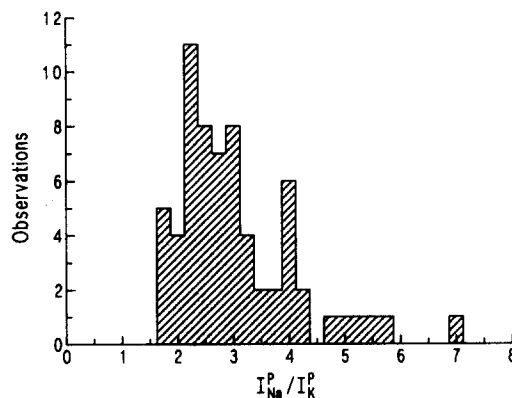


FIGURE 9. Histogram of observed values of stoichiometry of pump-mediated Na/K exchange. Values reported in the preceding article (Cox and Helman, 1986a) were combined with those reported here ($n = 65$).

On the premise that the intracellular Cl^- concentration and content (Rick, R., personal communication) and cellular volume (MacRobbie and Ussing, 1961) remain constant after ouabain, increases of Cl^- efflux after ouabain must be balanced by identical increases of Cl^- influx so that the induced fluxes do not contribute to a net Cl^- transport across the basolateral membranes. Thus, regardless of how the ouabain-induced Na^+ and/or K^+ fluxes are coupled to Cl^- , they would not contribute to a net Na^+ or K^+ flux. In this regard, it has been observed that net Na^+ influx across basolateral membranes is not changed by furosemide, despite inhibition by this drug of the ouabain-induced unidirectional Cl^- fluxes (Stoddard, 1984; Stoddard and Helman, 1985).

Stoichiometry of the Pump

The rates of transepithelial Na^+ transport observed varied spontaneously over their usual large range, providing variable loads of Na^+ to be transported by the basolateral membrane pumps. I_{K}^p appeared to saturate with increasing Na^+ transport, which led to the observation of a variable macroscopic stoichiometry

of the Na/K exchange process. The studies reported here do not give further information on the mechanism(s) of this variability. Nevertheless, the conclusions of the preceding article (Cox and Helman, 1986a) are strengthened, given the differences of assumptions and methods involved in arriving at this conclusion. We note here again the precedent for our findings in the studies of squid axons by Mullins and Brinley (1969; Brinley and Mullins, 1974) and suggest that, in epithelia, regulation of transepithelial Na⁺ transport may occur via a process or state of the pump that does not obligatorily require exchange of Na⁺ for K⁺. Fig. 9 shows a frequency histogram of the macroscopic coupling ratio of all data ($n = 65$) reported in this and the preceding article (Cox and Helman, 1986a). The macroscopic coupling ratio for Na/K ranged between 1.5 and 7.

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