Na\textsuperscript{+} and K\textsuperscript{+} Transport at Basolateral Membranes of Epithelial Cells

\textit{II. K\textsuperscript{+} Efflux and Stoichiometry of the Na,K-ATPase}

THOMAS C. COX and SANDY I. HELMAN

From the Department of Physiology and Biophysics, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

\textbf{ABSTRACT} Changes of K\textsuperscript{+} efflux (\(J_{\text{K}}\)) caused by ouabain and/or furosemide were measured in isolated epithelia of frog skin. From the kinetics of K\textsuperscript{+} influx (\(J_{\text{K}}\)) studied first over 8-9 h, K\textsuperscript{+} appeared to be distributed into readily and poorly exchangeable cellular pools of K\textsuperscript{+}. The readily exchangeable pool of K\textsuperscript{+} was increased by amiloride and decreased by ouabain and/or K\textsuperscript{+}-free extracellular Ringer solution. 42K efflux studies were carried out with tissues short-circuited in chambers. Ouabain caused an immediate (<1 min) increase of the 42K efflux to \(\sim 174\%\) of control in tissues incubated either in SO\textsubscript{4}-Ringer solution or in Cl-Ringer solution containing furosemide. Whereas furosemide had no effect on \(J_{\text{K}}\) 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 \(J_{\text{K}}\). Electroconductive K\textsuperscript{+} transport occurred via a single-filing K\textsuperscript{+} channel with an \(n'\) of 2.9. K\textsuperscript{+} efflux before ouabain, normalized to post-ouabain (\(\pm\) furosemide) values of short-circuit current, averaged 8-10 \(\mu\text{A/cm}^2\). In agreement with the conclusions of the preceding article, the macroscopic stoichiometry of ouabain-inhibitable Na\textsuperscript{+}/K\textsuperscript{+} exchange by the pump was variable, ranging between 1.7 and 7.2. With increasing rates of transepithelial Na\textsuperscript{+} transport, pump-mediated K\textsuperscript{+} influx saturated, whereas Na\textsuperscript{+} efflux continued to increase with increases of pump current. In the usual range of transepithelial Na\textsuperscript{+} transport, regulation of Na\textsuperscript{+} transport occurs via changes of pump-mediated Na\textsuperscript{+} efflux, with no obligatory coupling to pump-mediated K\textsuperscript{+} influx.

\textbf{INTRODUCTION}

To examine further the mechanism of K\textsuperscript{+} transport at the basolateral membranes of the epithelial cells of frog skin, studies were undertaken to characterize the...
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$_2$SO$_4$, 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 μCi/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$^{2+}$)**

$^{42}$K efflux studies were carried out with tissues short-circuited in chambers (Cox and Helman, 1985). 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 \(^{42}\text{K}\) must also be completed within at most a few seconds in order to observe "immediate" step changes of \(^{42}\text{K}\) 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**

\(^{42}\text{K}\) influx occurs primarily via a ouabain-inhibitable \(\text{Na}^+,\text{K}^+\)-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 \(^{42}\text{K}\) activity at the time intervals shown in Fig. 1. Although the buildup of tissue \(^{42}\text{K}\) activity appeared at first to be essentially monoexponential, with a halftime of \(\sim 30–40\) 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% (SO\(_4\)-Ringer) of cold tissue \(K^+\) was labeled by \(^{42}\text{K}\) (see Table I). At least two exponentials (fast and slow) were required to describe the kinetics of \(^{42}\text{K}\) influx (Fig. 1B). The exchangeable fraction of tissue cold \(K^+\) (fast exponential) averaged 53.3 (Cl-Ringer) and 38.6% (SO\(_4\)-Ringer) (Table I). Cold tissue \(K^+\) averaged 420 (Cl-Ringer) and 310 neq/mg DW (SO\(_4\)-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 (SO\(_4\)-Ringer). Accordingly, exchangeable \(K^+\) was \(\sim 193\) (Cl-Ringer) and 109 neq/mg DW (SO\(_4\)-Ringer). Although intracellular compartmentalization of \(K^+\) has not been observed by others in studies of frog skin (Curran and Cereijido, 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 Cereijido (1965) and Candia and Zadunaisky (1972) have noted some of the difficulties in assessing the kinetics of \(^{42}\text{K}\) 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 \(^{42}\text{K}\) 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. SA(0) is the specific activity of the extracellular loading solution. 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 ~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.

Concentrations 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 by $K^+$-free solutions, or their combinations.

| TABLE I |
|--------|
| Kinetics of $^{42}$K Loading of Isolated Epithelia of Frog Skin |
| Tissue K Pool size $t_{1/2}$ % min % 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 46.6 865 68.9 |
| Mean ± SEM 420±65 55.3±2.5 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 ± SEM 310±25 58.6±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 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 ⁴²K. Given the relatively long halftime 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 Jₖ and Iₜ (SO₄-Ringer)**

To determine the effects of ouabain on K⁺ efflux (Jₖ), studies were done with tissues short-circuited in chambers. The appearance of ⁴²K in the basolateral solution was measured at intervals of 1 min. Within 1 min (see Fig. 3), ouabain caused a decrease of Iₜ to 75.7 ± 2.0% (14) of control with a concurrent increase of the Jₖ to 173.1 ± 11.7% of control (control Iₜ was 19.7 ± 3.3 μA/cm²). In separate studies of isolated epithelia bathed with SO₄-Ringer solution, the basolateral membrane voltage measured with intracellular microelectrodes (Vₜ) 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 Jₖ or Iₜ (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:

\[
\frac{J_{k}^{32}}{J_{k}^{22}} = \frac{(I_{K} + FJ_{k}^{32})}{FJ_{k}^{22}} = \left(\frac{K_{c}}{K_{b}}\right)\exp\left(FV_{b}/RT\right)^{n'},
\]

where \(J_{k}^{32}\) and \(J_{k}^{22}\) 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, SO₄-Ringer), and that \(J_{k}^{22}\) of ouabain-poisoned tissues averaged 0.44 μA/cm² (Cox and Helman, 1986a). Since at the steady state, pump-mediated K⁺ influx must equal passive K⁺ efflux (7.4 μA/cm², as reported in the preceding article, and 8.3 μA/cm², reported below), \(I_{K}\) immediately after ouabain was ~(7.85 × 1.74) 13.6 μA/cm². 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 $^{42}\text{K efflux to the basolateral solution (} J_{K}^{2+} \text{)}$ 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.

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

**Effect of Ouabain and Furosemide on $J_{K}^{2+}$ (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** |

|       | $V_b$ (mV) |
|-------|------------|
|       | SO$_4$-Ringer | Cl-Ringer  |
| 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 ± 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 ± 2.2% of control, and $J_{K}^{23}$ was increased to 164.6 ± 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 ± 0.7% of control at 1–2 min), the increase of $J_{K}^{23}$ was smaller at 1–2 min (137.3 ± 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-
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:

\[ I_{sc} = I^p + I^K. \]  

(2)

\(I^p\) is the net charge transfer (\(I^K_{oa} - I^K\)) 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 \(I^K_{oa}\).

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 \(I^K_{oa} = I^K_{sc}\).

\[ I^K_{sc} = I^K = FfK^* - FfK^{52^*}. \]  

(3)

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

For studies carried out in Cl-Ringers solution, a similar normalization procedure was used, except that the \(^{42}\)K 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.

**FfK\(^{52}\): Experiments with SO\(_4\)-Ringer Solution**

24 experiments were carried out with epithelia bathed with SO\(_4\)-Ringer solution. \(I_{sc}\) ranged between 5.6 and 48.6 \(\mu\)A/cm\(^2\), with a mean of 20.8 ± 2.8 \(\mu\)A/cm\(^2\) (Table III). Fig. 5 shows the results of a representative experiment (SO\(_4\)-Ringer), where the tissue was treated first with ouabain and then with furosemide. Ouabain caused inhibition of \(I_{sc}\) and stimulation of \(FfK\) (Fig. 5A). \(FfK\) 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 \(FfK\) and \(I^K_{sc}\) (Fig. 5B). As can be seen in Fig. 5A, the \(^{42}\)K efflux “tracked” the \(I_{sc}\) fairly well after \(-1\) to 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 (\(I^p_{min}\)) was calculated as the difference between \(I_{sc}\) and \(FfK\) (= \(I^K\)) immediately before ouabain. Control \(FfK\) (and hence \(I^K\)) averaged 8.3 ± 0.9 \(\mu\)A/cm\(^2\), with a range of 5.1–19.0 \(\mu\)A/cm\(^2\) (Table III). These values are in good agreement with those of \(I^K\) 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 \(FfK\) 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).
Values are means ± SEM (range).

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

I_p,min was calculated to average 12.3 ± 2.0 μA/cm², with a range of 1.2 to 35.6 μA/cm² (Table III). From the paired values of I_c and FJK, the apparent minimum stoichiometry of Na/K exchange was found to average 2.50 ± 0.19, with a range of 1.20–5.00.

**TABLE III**

|                | I_c   | FJK⁺ | I_p,min | r_p,min |
|----------------|-------|------|---------|---------|
| SO₄-Ringer     | 20.8±2.8 | 8.3±0.9 | 12.3±2.0 | 2.50±0.19 |
| (N = 24)       | (5.6–48.6) | (3.1–19.0) | (1.2–35.6) | (1.20–5.00) |
| Cl-Ringer      | 21.9±2.2  | 10.6±0.9 | 11.3±1.6 | 2.04±0.13  |
| (N = 19)       | (10.5–47.0) | (6.5–17.7) | (3.0–29.7) | (1.33–3.89) |

**FIGURE 5.** Changes of I_c and FJK⁺ caused by ouabain and furosemide. Data are from a typical experiment with a tissue bathed with SO₄-Ringer solution. (A) Values of I_c and FJK⁺. See text for method of normalization of FJK⁺ to units of current. (B) Ordinate values are FJK⁺ - I_c. By definition (see text), I_c after ouabain is equal to FJK⁺. Hence, (FJK⁺ - I_c) = 0 after ouabain. Before ouabain, (FJK⁺ - I_c) provides a minimal estimate of the pump current I_p.
Figure 6. Effect of ouabain and furosemide on $I_K$ and $F^{23}_{JK}$ (typical experiment, Cl-Ringer solution). See legend to Fig. 5. (A) Values of $I_K$ and $F^{23}_{JK}$. (B) Values of $F^{23}_{JK} - I_K$. Notice the furosemide-inhibitable fraction of $F^{23}_{JK}$. 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 $I_K$ and $F^{23}_{JK}$. (B) Changes of $F^{23}_{JK} - I_K$. (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 $F^{23}_{JK}$ caused by ouabain. The dashed line indicates that the rate of increase is underestimated (see text).
continuously decreasing values of \( I_c \). Furosemide caused an immediate inhibition of \( \bar{F}J_{Kr}^a \), with no concurrent change of \( I_c \). 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 \( I_c \), \( E \bar{F}J_{Kr}^a \), \( I_{min} \), and \( r_{min} \).

In a final group, experiments were done to measure simultaneously the changes of \( ^{36}\text{Cl} \) and \( ^{42}\text{K} \) efflux caused by ouabain. Epithelia were preloaded with \( ^{42}\text{K} \) as described above and with \( ^{36}\text{Cl} \) 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 \( ^{42}\text{K} \) efflux that were described above, where notably ouabain induced a furosemide-inhibitable component of the post-ouabain \( ^{42}\text{K} \) efflux. The furosemide-inhibitable flux averaged \( 3.7 \pm 0.7 \) (8) \( \mu A/cm^2 \) at 10 min and \( 6.9 \pm 1.1 \) (3) \( \mu A/cm^2 \) after 20 min of ouabain (Fig. 6). Also notable was the observation that the furosemide-inhibitable \( ^{42}\text{K} \) efflux did not appear immediately after ouabain (1–2 min) but increased with time after exposure to ouabain (\( E \bar{F}J_{Kr}^a - I_c = 0.4 \pm 0.5 \) [8] \( \mu A/cm^2 \) between 1 and 2 min after ouabain).

\( ^{36}\text{Cl} \) efflux \( (J_{Cl}^{36}) \) was also affected markedly by ouabain. As shown in Fig. 7C, the rate of appearance of \( ^{36}\text{Cl} \) in the basolateral solution decreased continuously during the control period. This was due to the time rate of change of intracellular \( ^{36}\text{Cl} \) specific activity (Stoddard et al., 1985). After treatment with ouabain, the rate of \( ^{36}\text{Cl} \) efflux was increased above that expected with reference to the baseline rate of \( ^{36}\text{Cl} \) efflux established during the control period (solid line of Fig. 7C). Although it is impossible to estimate precisely, the dashed line of Fig. 7C indicates the deviation of baseline rate of \( ^{36}\text{Cl} \) efflux owing to the increased rate of change of intracellular \( ^{36}\text{Cl} \) specific activity. As a rough attempt to normalize for the time-dependent changes of \( ^{36}\text{Cl} \) specific activity after ouabain, we assumed (as a worst case) a linear extrapolation of the baseline rate of appearance of \( ^{36}\text{Cl} \) (solid line, Fig. 7C). Accordingly, the difference between the observed \( ^{36}\text{Cl} \) efflux and the linear extrapolated baseline value after ouabain gives an underestimate of the actual percent increase of \( J_{Cl}^{36} \). This is shown also in Fig. 7D. \( J_{Cl}^{36} \) increased continuously after ouabain, following in parallel the increases of \( \bar{E}J_{Kr}^a \). This suggests but does not prove, that ouabain induces a cotransport system involving both \( K^+ \) and \( \text{Cl}^- \). Thus, ouabain not only caused changes of permeability to \( K^+ \) but also caused time-dependent changes of permeability to \( \text{Cl}^- \). To the extent that \( J_{Cl}^{36} \) is considerably larger than \( \bar{E}J_{Kr}^a \) and \( J_{Kr}^{36} \) (control \( \bar{E}J_{Kr}^a \) averaged near 21 \( \mu A/cm^2 \) [Cox and Helman, 1983; Stoddard et al., 1985]), the minimal increase of \( J_{Cl}^{36} \) 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 \( E \bar{F}J_{Kr}^a \) (3.7 \( \pm 0.7 \) \( \mu A/cm^2 \) at 10 min), which rules out a simple 1:1 coupling of \( K^+ \) and \( \text{Cl}^- \). As total tissue \( \text{Cl}^- \) remains unchanged by ouabain for up to 60–90 min (Rick, R., personal communication), the increases of \( ^{36}\text{Cl} \) efflux caused by ouabain must reflect changes of \( \text{Cl}^- \) permeability that could include not only a simple mechanism of \( \text{Cl}^- \)-\( \text{Cl}^- \) 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 $^{42}$K 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](image_url)

**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 $I_{K}^p > 10$ $\mu$A/cm$^2$. Values below the standard error bars indicate the number of observations.

is the sum of $I_{c}$ (Na$^+$ entry via apical membranes) and net Na$^+$ entry via basolateral membranes. To the extent that $I_{c} \gg$ basolateral membrane Na$^+$ entry, $I_{c}$ 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 $I_{c}$ (Stoddard, 1984; Stoddard and Helman, 1985). Accordingly:

$$I_{Na}^p = I_{c} + 3.5;$$

$$I^p = I_{Na}^p - I_{K}^p.$$  

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,

\[
I_K^p = \frac{1}{m + (r^o/I_{Ko})}
\]

(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 \( \sim 120 \) 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 Erlij, 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 1, 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 fK⁺ efflux are probably due to depolarization of Vb. To the extent that the flux ratio for K⁺ in ouabain-poisoned tissues was ~32:1 (fK⁺ efflux/fK⁺ influx), 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 fK⁺ influx > fK⁺ efflux, 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 Iₑ could be equated with net K⁺ efflux = FjK⁺ efflux. Indeed, after ouabain (SO₄-Ringer) or after ouabain plus furosemide (Cl-Ringer), the ⁴²K efflux "tracked" the changes of Iₑ. Consequently, the FjK⁺ efflux values were normalized to the post-ouabain values of Iₑ. This provided a means of obtaining estimates of the net K⁺ efflux (FjK⁺ efflux) immediately before ouabain, from which the currents Iₑ and I_K were calculated. The FjK⁺ efflux reported in Table III (efflux experiments) were not measurably different from the I_K values (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 Iₑ, which supports the suggestion that Iₑ after ouabain was a direct measure of the net K⁺ efflux. However, when epithelia were bathed in Cl-Ringer solution, the FjK⁺ 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 Na*/K*/2Cl$^-$-coupled mechanism (Geck et al., 1980; Kregenow, 1981).

![figure 9](image)

**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. $I^p$ 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.

We thank Robert Sorensen for help in carrying out a portion of these studies. Amiloride was a gift from Merck, Sharp & Dohme Research Laboratories, West Point, PA. This work was supported by grant AM 16663 from the National Institutes of Health. Dr. Cox was sponsored by NIH postdoctoral fellowship GM 07357.

Original version received 18 March 1985 and accepted version received 9 December 1985.

REFERENCES

Aceves, J., and D. Erlj. 1971. Sodium transport across the isolated epithelium of the frog skin. *Journal of Physiology.* 212:195–210.

Altschuld, R., C. Hohl, A. Ansel, and G. P. Brierly. 1981. Compartmentalization of K⁺ in isolated adult rat heart cells. *Archives of Biochemistry and Biophysics.* 209:173–184.

Begenisich, T., and P. De Weer. 1980. Potassium flux ratio in voltage-clamped squid giant axon. *Journal of General Physiology.* 76:83–98.

Brinley, F. J., Jr., and L. J. Mullins. 1974. Effects of membrane potential on sodium and potassium fluxes in squid axons. *Annals of the New York Academy of Sciences.* 242:406–433.

Candia, O. A., and J. A. Zadunaisky. 1972. Potassium flux and sodium transport in the isolated frog skin. *Biochimica et Biophysica Acta.* 255:517–529.

Cox, T. C. 1985. Correlation of net K⁺ efflux and $I_C$ of ouabain-treated skin. *Federation Proceedings.* 44:644. (Abstr.)

Cox, T. C., and S. I. Helman. 1983. Effects of ouabain and furosemide on basolateral membrane Na⁺ efflux of frog skin. *American Journal of Physiology.* 245:F312–F321.

Cox, T. C., and S. I. Helman. 1986a. Na⁺ and K⁺ transport at basolateral membranes of epithelial cells. I. Stoichiometry of the Na,K-ATPase. *Journal of General Physiology.* 87:467–483.

Cox, T. C., and S. I. Helman. 1986b. Na⁺ and K⁺ transport at the basolateral membranes of epithelial cells. III. Voltage independence of basolateral membrane Na⁺ efflux. *Journal of General Physiology.* 87:503–509.

Curran, P. F., and M. Cereijido. 1965. K fluxes in frog skin. *Journal of General Physiology.* 48:1011–1053.

Ferreira, K. T. G. 1979. The relationship of K⁺ efflux at the inner surface of the isolated frog skin epithelium to the short-circuit current. *Biochimica et Biophysica Acta.* 555:13–25.

Ferreira, K. T. G., and H. G. Ferreira. 1981. The regulation of volume and ion composition in frog skin. *Biochimica et Biophysica Acta.* 646:193–202.

Finn, A. L., and H. Nellans. 1972. The kinetics and distribution of potassium in the toad bladder. *Journal of Membrane Biology.* 8:189–203.
Fisher, R. S., D. Eriij, and S. I. Helman. 1980. Intracellular voltage of isolated epithelial of frog skin. Apical and basolateral cell punctures. Journal of General Physiology. 76:447-453.

Garcia-Diaz, J. F., L. M. Baxendale, G. Klemperer, and A. Essig. 1985. Cell K activity in frog skin in the presence and absence of cell current. Journal of Membrane Biology. 30:143-158.

Geck, P., C. Pietrzyk, B. C. Burckhardt, B. Pfeiffer, and E. Heinz. 1980. Electrically silent cotransport of Na⁺, K⁺, and Cl⁻ in Ehrlich cells. Biochimica et Biophysica Acta. 600:432-447.

Hodgkin, A. L., and R. D. Keynes. 1955. The potassium permeability of a giant nerve fibre. Journal of Physiology. 128:61-88.

Kirk, K. L., and D. C. Dawson. 1983. Basolateral potassium channel in turtle colon. Evidence for single-file ion flow. Journal of General Physiology. 82:297-313.

Koefoed-Johnsen, V., and H. H. Ussing. 1958. The nature of the frog skin potential. Acta Physiologica Scandinavica. 42:298-308.

Kregenow, F. M. 1981. Osmoregulatory salt transporting mechanisms: control of cell volume in anisotonic media. Annual Review of Physiology. 43:493-505.

MacRobbie, E. A. C., and H. H. Ussing. 1961. Osmotic behaviour of the epithelial cells of frog skin. Acta Physiologica Scandinavica. 53:348-365.

Mullins, L. J., and F. J. Brinley, Jr. 1969. Potassium fluxes in dialyzed squid axons. Journal of General Physiology. 53:504-570.

Nagel, W., J. F. Garcia-Diaz, and W. McD. Armstrong. 1981. Intracellular ionic activities in frog skin. Journal of Membrane Biology. 61:127-134.

Rick, R., A. Dörge, E. von Arnim, and K. Thurau. 1978. Electron microprobe analysis of frog skin epithelium: evidence for a syncitial sodium transport compartment. Journal of Membrane Biology. 39:313-331.

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

Soltoff, S. P., and L. J. Mandel. 1984. Active ion transport in the renal proximal tubule. I. Transport and metabolic studies. Journal of General Physiology. 84:601-622.

Stoddard, J. S. 1984. Influence of CO₂ on electrophysiology and ionic permeability of the basolateral membrane of frog skin. Ph.D. Dissertation. University of Illinois, Urbana, IL. 152 pp.

Stoddard, J. S., and S. I. Helman. 1985. Dependence of intracellular Na⁺ concentration on apical and basolateral membrane Na⁺ influx in frog skin. American Journal of Physiology. 249:F662-F671.

Stoddard, J. S., E. Jakobsson, and S. I. Helman. 1985. Basolateral membrane chloride transport in isolated epithelia of frog skin (R. pipiens). American Journal of Physiology. 249:C318-C329.