

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

I. Stoichiometry of the Na,K-ATPase

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ABSTRACT The stoichiometry of pump-mediated Na/K exchange was studied in isolated epithelial sheets of frog skin. ⁴²K influx across basolateral membranes was measured with tissues in a steady state and incubated in either beakers or in chambers. The short-circuit current provided estimates of Na⁺ influx at the apical membranes of the cells. ⁴²K influx of tissues bathed in Cl⁻ or SO₄⁻-Ringer solution averaged ~8 μA/cm². Ouabain inhibited 94% of the ⁴²K influx. Furosemide was without effect on pre-ouabain-treated tissues but inhibited a ouabain-induced and Cl⁻-dependent component of ⁴²K influx. After taking into account the contribution of the Na⁺ load to the pump by way of basolateral membrane recycling of Na⁺, the stoichiometry was found to increase from ~2 to 6 as the pump-mediated Na⁺ transport rate increased from 10 to 70 μA/cm². Extrapolation of the data to low rates of Na⁺ transport (<10 μA/cm²) indicated that the stoichiometry would be in the vicinity of 3:2. As pump-mediated K⁺ influx saturates with increasing rates of Na⁺ transport, Na⁺ efflux cannot be obligatorily coupled to K⁺ influx at all rates of transepithelial Na⁺ transport. These results are similar to those of Mullins and Brinley (1969. *Journal of General Physiology*. 53:504-740) in studies of the squid axon.

INTRODUCTION

In this and the following articles (Cox and Helman, 1986*a, b*), we address the question of the apparent stoichiometry of the exchange of Na⁺ for K⁺ by the ouabain-inhibitable Na,K-ATPase of the epithelial cells of frog skin. As this question is of particular importance in assessing the manner by which epithelial tissues absorb Na⁺ and regulate their transepithelial Na⁺ transport, and as we had previously observed (Cox and Helman, 1983*b*) that ouabain "induces" a Cl⁻-dependent and furosemide-inhibitable Na⁺ flux at the basolateral membranes of the cells of frog skin, we undertook studies to define the changes of the basolateral membrane K⁺ fluxes caused by ouabain and/or furosemide. In part, our purpose

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was to determine to what extent changes by ouabain of the so-called "ouabain-insensitive" K^+ flux would bias assessment of the stoichiometry of the Na/K exchange process by the pump.¹ Our studies were made possible by the availability of large sheets of isolated epithelia of frog skin, where unstirred layers of the corium at the basolateral surface were removed and where, for all practical purposes, measurements of the kinetics of isotopic fluxes were not compromised by the remaining unstirred layer of the intercellular spaces.

MATERIALS AND METHODS

Preparation of Tissues

Isolated epithelial sheets of the abdominal region of frog skin have been used in a wide variety of electrophysiological and isotopic studies of this Na^+ -transporting epithelium (Aceves and Erlj, 1971; Biber et al., 1972; Aceves, 1977a; Ferreira, 1979; Nielsen, 1979; Fisher et al., 1980). In all ways known to us, isolated epithelia prepared in the absence of applied hydrostatic pressure behave in a manner identical to their parent intact tissues studied in vitro, retaining both drug and hormonal sensitivities at both the apical and basolateral surfaces of the cells. Isolated epithelia were prepared by the method of Fisher et al. (1980). The corium was separated from the epithelium without the use of applied hydrostatic pressure after preincubation of the basolateral surface of intact tissues in Ringer solution containing collagenase (~0.4 mg/ml CLS II, Worthington Biochemical Corp., Freehold, NJ) for 2 h, leaving the isolated sheets of tissues glued (Zipbond, Tescom Corporation, Minneapolis, MN) to Lucite rings. The area of tissue obtained was limited only by the size of the frogs. In the present studies, 10 cm² of isolated epithelium was prepared from each frog. The tissues were studied with two experimental protocols, to be referred to as "beaker studies" and "chamber studies." Northern *Rana pipiens* (Nasco Biologicals, Oshkosh, WI) were used in all studies, and the experiments were done at room temperature.

For studies done in beakers, a single epithelium provided paired control and experimental pieces of tissue (~2–3 cm² each). The incubation medium was either a Cl-Ringer solution containing 100 mM NaCl, 2.4 mM KHCO₃, and 2 mM CaCl₂ or an SO₄-Ringer solution containing 56 mM Na₂SO₄, 2.4 mM KHCO₃, and 1.2 mM CaSO₄. Solutions were equilibrated with room air, and the pH was ~8.1. ⁴²K was included in the Ringer solution at ~0.1–0.5 μCi/ml and was prepared at the Nuclear Research Facility at the University of Illinois, Urbana, IL, as ⁴²K₂CO₃ with subsequent conversion to either ⁴²KCl or ⁴²K₂SO₄. ⁴²K activity was measured in the usual ways with correction for isotopic decay. From the amounts of ⁴²K accumulated by the tissue at timed intervals (see Results) and the specific activity of the loading solution (counts per minute per nanoequivalent) the K^+ influx (J_k^{42}) was calculated and expressed in units of either nanomoles per square centimeter per second or microamperes per square centimeter (FJ_k^{42}). Ouabain (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 10⁻⁵ M and was freshly prepared immediately before use. Furosemide (Hoechst Roussel Pharmaceuticals, Somerville, NJ) was used at a concentration of 10⁻³ M.

¹ It is often assumed that "ouabain-insensitive fluxes" provide a measure of the leak fluxes in parallel to the ouabain-inhibitable Na,K-ATPase. By virtue of ouabain related or "induced" changes of the leak fluxes, the terminology "ouabain-insensitive fluxes" should probably be abandoned when changes of electrochemical potential differences and/or changes of mechanisms of the leak fluxes occur consequent to ouabain inhibition of the Na,K-ATPase.

Epithelia were also studied in chamber experiments. They were continuously short-circuited using methods described in detail previously (Cox and Helman, 1983*b*). To measure K^+ influx, SO_4 -Ringer solution containing ^{42}K was flushed into the basolateral chamber for timed intervals of 5–6 min (see Results). At the termination of the experiments, tissues were dried at $100^\circ C$ overnight to give the milligrams dry weight per square centimeter of tissue.

Summary data are reported as means \pm SEM (*N*).

RESULTS

Beaker Experiments

K^+ transport between intra- and extracellular fluids occurs primarily, if not solely, via the basolateral membranes of the epithelial cells of frog skin (see below and Cox and Helman, 1986*a*). This idea follows the original postulate of Koefoed-Johnsen and Ussing (1958), who suggested that pump-mediated K^+ influx is balanced at the steady state by an outwardly directed passive K^+ efflux. Ouabain has been used to inhibit Na,K-ATPase activity, thereby allowing partitioning of unidirectional tracer fluxes into so-called ouabain-sensitive (pump) and ouabain-insensitive (leak) components. Implicit in this notion, however, is the assumption that the ouabain-insensitive leak flux remains constant, being independent of direct or indirect consequences of pump inhibition that may occur via changes of electrochemical potential difference and/or K^+ permeability (see below).

We set out to measure the ^{42}K unidirectional influx, J_K^{32} . Following previous 3-compartment notation, compartment 3 is defined as the basolateral solution, and compartment 2 is defined as the intracellular compartment of the epithelial cell.² Studies were done first in beakers that permitted several pieces of the same epithelium to be used as either control or experimental pieces.

Isolated epithelia were incubated in Ringer solution during all phases of the studies to ensure that the tissues were in a steady state of ion transport at the time of measurement of the J_K^{32} . A single epithelium was divided into two to three pieces of ~ 2 – 3 cm^2 each. The actual area of each piece was calculated after determination of the tissue dry weight at the conclusion of the experiments. In a separate group, six tissues averaged 1.4 ± 0.2 mg dry weight/ cm^2 . Since the cells contain an enormous quantity of K^+ (see Cox and Helman, 1986*a*), the initial rates of ^{42}K influx were expected to be essentially linear owing to isotopic dilution of the ^{42}K entering the cellular pool of K^+ .

The method used to measure J_K^{32} was modified from the procedure of Curran

² Despite the "apparent" morphological complexity of the tissue, we are unaware of any data, electrophysiological or other, inconsistent with the notion that, physiologically, the epithelium behaves as a relatively simple three-compartment model. Na,K-ATPase activity has been found only at the basolateral membrane of the cells (DiBona and Mills, 1979), and the stratified cell layers are of one cell type, with the exception of a relatively minor ($\sim 1\%$) contribution of mitochondria-rich cells. Although the intracellular compartment may be further subcompartmentalized, the issue of localization of basolateral membrane influx and efflux is readily resolved by the demonstrated sensitivity of the ion fluxes to drugs known to act on mechanisms resident in the basolateral plasma membranes of the cells.

and Cereijido (1965). At zero time, the tissues were transferred to a beaker containing ^{42}K -Ringer solution. At intervals of 10 min, the tissues were removed from the loading solution, rinsed with isotope-free Ringer solution for 20 s, and transferred to a gamma counting tube containing 1 ml of isotope-free Ringer solution. ^{42}K activity was measured for 1 min, and thereafter the tissue was returned to the ^{42}K loading solution for an additional 10 min of ^{42}K loading. After removal of the tissue from the gamma counting tube, the blank tube (without tissue) was recounted for remaining ^{42}K activity originating from either incomplete washout of the extracellular space and/or loss of ^{42}K from the cells. As the remaining counts per minute in the blank tubes averaged only $2.6 \pm 0.4\%$ (13) of the total counts (tissue and blank), it was certain that for all practical arguments, extracellular ^{42}K washout was virtually complete within 20 s and that loss of cellular ^{42}K was negligible.

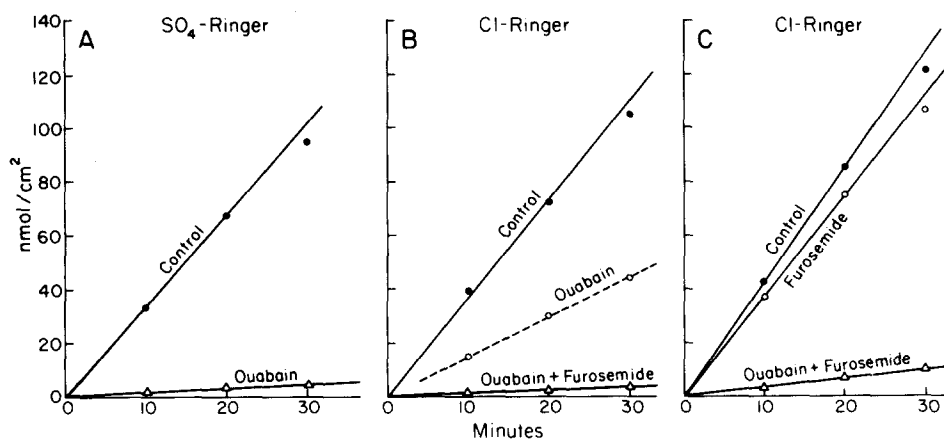


FIGURE 1. Influx of ^{42}K by isolated epithelia incubated in beakers containing Cl-Ringer or SO_4 -Ringer. Experimental pieces of tissue were exposed at zero time to Ringer solution containing ^{42}K and either ouabain (1 mM) or ouabain (1 mM) plus furosemide (1 mM). Slopes of lines yield values of J_{K}^{32} .

Fig. 1 shows the results of typical experiments. The J_{K}^{32} was measured from the slopes of the time-amount relationships. It should be noted that the slopes passed through the origin and were linear for at least 20 min. At 30 min, especially for control tissues, there was a small systematic deviation from linearity as expected owing to increasing specific activity of cellular ^{42}K .

In order to permit direct comparison of the magnitudes of J_{K}^{32} and the short-circuit current (see below), we have summarized the control values in Table I in units of microamperes per square centimeter (FJ_{K}^{32}). The average values of FJ_{K}^{32} in Cl-Ringer or SO_4 -Ringer solution were similar (8.9 ± 1.5 and $7.4 \pm 0.7 \mu\text{A}/\text{cm}^2$, respectively). However, the control FJ_{K}^{32} varied widely among epithelia, and this variability, as will be shown below, was correlated with spontaneous difference in the rate of transepithelial Na^+ transport. Ouabain at 1 mM causes a maximal and irreversible inhibition of the Na,K -ATPase within seconds in this preparation (Cox and Helman, 1983b). As can be observed in Fig. 1A (see also

data summary in Table I), ouabain inhibited 94% of the FJ_K^{32} when epithelia were bathed in the Cl-free SO_4 -Ringer solution. Therefore, ^{42}K influx occurred primarily via a ouabain-inhibitable mechanism, i.e., via the Na,K-ATPase.

In tissues incubated in Cl-Ringer solution, ouabain caused inhibition of FJ_K^{32} (Fig. 1B), but only to a mean of $30.0 \pm 2.0\%$ of control (Table I). In view of previous findings of a ouabain-induced Na^+ efflux that was Cl^- dependent and furosemide inhibitable (Cox and Helman, 1983b),³ we tested for the combined action of ouabain and furosemide on FJ_K^{32} . Together, these drugs inhibited FJ_K^{32} to a mean value of $6.2 \pm 1.0\%$ of control (Table I, Fig. 1, B and C) that was not different from the value observed with Cl^- -free Ringer-bathed tissues treated with ouabain alone. Furosemide alone had no consistent effect on FJ_K^{32} (Fig. 1C and Table I). Furosemide sensitivity of the FJ_K^{32} appeared after tissues were

TABLE I
Effect of Ouabain and/or Furosemide on FJ_K^{32} of Frog Skin (Beaker Experiments)

	K ⁺ influx	
	Cl-Ringer	SO ₄ -Ringer
Control ($\mu A/cm^2$)	8.9 \pm 1.5 (11) (3.3–17.1)	7.4 \pm 0.7 (14) (2.8–11.8)
	Experimental/control	
Furosemide (1 mM)	0.931 \pm 0.102 (7) (0.683–1.482)	—
Ouabain (1 mM)	0.301 \pm 0.022 (6) (0.253–0.331)	0.061 \pm 0.016 (6) (0.011–0.108)
Ouabain + furosemide	0.062 \pm 0.010 (8) (0.012–0.124)	—

Values are means \pm SEM (N) (range).

poisoned with ouabain. We presume, therefore, that the “ouabain-insensitive” K^+ influx of Cl-Ringer-bathed tissues is changed by “ouabain-induced” K^+ influx. It is also noteworthy that furosemide, either before or after ouabain, caused no important change of electrophysiology of the cells (Cox and Helman, 1983b). Hence, it can be inferred that ouabain-induced K^+ influx occurs via an electro-neutral mechanism of K^+ transport. Regardless of the precise mechanism of these ouabain-induced fluxes, they lead to sizable errors in the assessment of the pump-leak mechanisms of cation transport at the basolateral membrane of the cells. Inasmuch as the ouabain-inhibitable K^+ influx accounts for ~94% of the total influx in SO_4 -Ringer and since ouabain plus furosemide-inhibitable K^+ influx accounts for ~94% of the total K^+ influx in Cl-Ringer, it is reasonable to conclude that K^+ influx of control tissues is mediated primarily via the Na,K-ATPase.

³ Incubation of tissues in the hypo-osmotic SO_4 -Ringer solution may lead to changes of cell volume, intracellular pH, and other processes that contribute to the furosemide sensitivity and Cl^- dependence of the ouabain-induced cation fluxes. We have not yet, regardless of methods available to us, detected consistent significant differences of transport physiology in epithelia equilibrated in either Ringer solution despite differences of the osmolality of the solution or replacement of Cl^- by SO_4^- .

When isolated epithelia are challenged with high concentrations of ouabain, basolateral membrane voltage, V_b , depolarizes within seconds (Cox and Helman, 1983*b*, 1986*a, b*). Accordingly, ouabain-insensitive values of ^{42}K influx must be corrected for this change of voltage, so that a partition can be made between pre-ouabain pump-mediated K^+ influx and the leak influx via the K^+ channels. As will be reported in the following paper (Cox and Helman, 1986*a*), the K^+ channels exhibit the phenomenon of single filing (Hodgkin and Keynes, 1955), with an n' of ~ 2.9 . Eq. 1 takes into account the idea that the unidirectional fluxes are not independent of each other, as may occur in a channel where ions proceed in a single file. Thus, defining I_k^p as the influx via the pump and FJ_k^{32*} as the unidirectional K^+ influx via the K^+ channels:

$$\frac{I_k^p + FJ_k^{32*}}{FJ_k^{32*}} = \left[\frac{K_c}{K_b} \exp(FV_b/RT) \right]^{2.9} \quad (1)$$

Since I_k^p averaged $8.3 \mu\text{A}/\text{cm}^2$ (see Cox and Helman, 1986*a*) and was similar in value to the ouabain-inhibitable K^+ influx (Table I), the FJ_k^{32*} of the control period (pre-ouabain) could be calculated and compared with the FJ_k^{32*} after ouabain. Taking K_c to be 120 mM (Rick et al., 1978; Fisher et al., 1980) and V_b to be -78.6 mV (see Table II, Cox and Helman, 1986*a*), we calculated that the control K^+ influx via the K^+ channel (FJ_k^{32*}) was $0.86 \mu\text{A}/\text{cm}^2$. After ouabain and/or furosemide, the FJ_k^{32*} was $0.06 \times 7.4 = 0.44 \mu\text{A}/\text{cm}^2$ (SO_4 -Ringer) and $0.06 \times 8.9 = 0.54 \mu\text{A}/\text{cm}^2$ (Cl-Ringer). The difference between the values of FJ_k^{32*} in control and ouabain-treated tissues is probably due to depolarization of V_b caused by ouabain inhibition of the Na,K-ATPase. Assuming that the control K^+ influx via the leak is $0.86 \mu\text{A}/\text{cm}^2$, K^+ influx via the pump is $\sim 8.3/(0.86 + 8.3)$, or 90.6% of the control FJ_k^{32} . Thus, to a good approximation, changes of V_b by ouabain would not seriously affect determination of the pump-mediated K^+ influx and hence the I_k^p . For practical purposes, in the calculations below, we have assumed that $I_k^p = 0.906 FJ_k^{32}$.

Chamber Experiments

Studies similar to those above were done with tissues mounted in chambers and bathed with SO_4 -Ringer solution. Na^+ entry into the cells via apical membranes was measured directly as the short-circuit current (Ussing and Zerahn, 1951). ^{42}K influx across apical membranes did not occur since ^{42}K was added only to the basolateral solution.

After stabilization of the I_{sc} for 10–30 min, ^{42}K -Ringer solution was flushed into the basolateral chamber for timed intervals of 5–6 min. To end the influx period, the chamber was again flushed with tracer-free solution containing 1 mM ouabain to inhibit pump-mediated ^{42}K influx. Immediately thereafter (1–2 min), the tissue was removed from the chamber and 0.38 cm^2 of tissue was punched out and counted for ^{42}K activity. Both I_{sc} and FJ_k^{32} are expressed in units of microamperes per square centimeter.

The results of 21 experiments are shown in Fig. 2. The short-circuit current ranged between 4.8 and $61.3 \mu\text{A}/\text{cm}^2$. FJ_k^{32} ranged between 4.3 and $11.8 \mu\text{A}/$

cm^2 , with a mean of $7.91 \pm 0.5 \mu\text{A}/\text{cm}^2$. These values are not significantly different from those reported in Table I for tissues studied in beakers. The FJ_K^{32} appeared to "saturate" with increasing I_{sc} reaching maximal values of 10–12 $\mu\text{A}/\text{cm}^2$. If the stoichiometry of the Na/K exchange process were fixed, then the relationship between FJ_K^{32} and I_{sc} would be linear. This was not observed. Given the enormous differences between the I_{sc} and FJ_K^{32} , especially at the higher I_{sc} , and the improbability of measurement errors of the FJ_K^{32} of tens of microamperes per square centimeters, it seemed most unlikely that the stoichiometry of the Na/K exchange process of the pump was constant. It should be recalled (a) that FJ_K^{32} overestimates I_K^p by $\sim 9\%$ (see above), and (b) that to this point we have assumed that apical membrane Na^+ entry provides the only source of Na^+ load

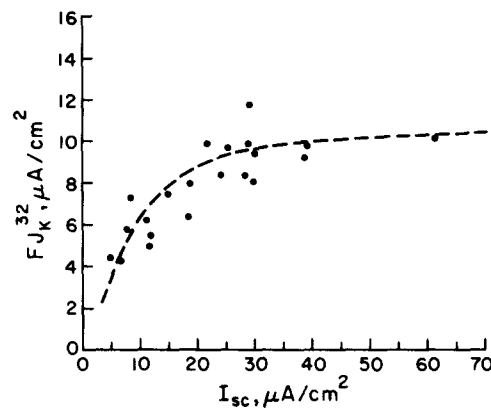


FIGURE 2. Relationship between I_{sc} and FJ_K^{32} . The dashed line was drawn by eye.

to the pump, with no contribution from Na^+ entry via basolateral membranes. Accordingly, estimates of the stoichiometry $I_{Na}^p/I_K^p \equiv r$ from the ratio I_{sc}/FJ_K^{32} provide a minimal estimate of r , where I_{Na}^p is defined as the pump-mediated Na^+ efflux. I_{sc}/FJ_K^{32} averaged 2.7 ± 0.3 (21), with a range of 1.1–6.0.

In studies carried out under the same conditions used here, Stoddard and Helman (1985) found an electroneutral Na^+ influx ($\sim 3.5 \mu\text{A}/\text{cm}^2$) via basolateral membranes. This Na^+ entry into the cells was essentially constant and independent of the I_{sc} . Although at higher I_{sc} , a basolateral membrane Na^+ entry of $3.5 \mu\text{A}/\text{cm}^2$ would contribute a relatively small amount to the I_{Na}^p , its importance would increase as I_{sc} fell toward zero. To obtain values of I_{Na}^p that more closely approximated the total Na^+ load of the pumps, we assumed that $I_{Na}^p = I_{sc} + 3.5$ at all values of I_{sc} .

Values of I_{Na}^p thus calculated are plotted in Fig. 3A against the values of I_K^p . The dashed line indicates the expected behavior if the pumps exchanged Na^+ for K^+ at a fixed ratio of 3:2. In every experiment, the I_{Na}^p/I_K^p was $>3:2$, deviating markedly from 3:2 with increasing I_{Na}^p .

Fig. 3B gives the values of coupling ratio (r) as a function of I_{Na}^p . Despite the scatter of data points, there was an apparent linear relationship of the form:

$$r = m \cdot I_{Na}^p + r^0. \quad (2)$$

The slope (m) of the relationship was 0.084, and the intercept (r^0) was 1.41, with a 95% confidence interval of 0.94–1.88 as calculated according to Kleinbaum and Kupper (1978). For I_{Na}^p between 10 and 65 $\mu\text{A}/\text{cm}^2$, r increased from ~ 2 to ~ 6 . If this trend were extrapolated to lower Na^+ currents ($<10 \mu\text{A}/\text{cm}^2$), stoichiometries at or near 3:2 would be observed. We conclude from this that the pump stoichiometry varies directly with Na^+ transport rate with values of $\sim 3:2$ at low rates of transepithelial Na^+ transport.

Eq. 2 can be transformed to:

$$I_K^p = \frac{1}{m + (r^0/I_{Na}^p)} \quad (3)$$

This saturating relationship between I_K^p and I_{Na}^p is shown by the solid line in Fig. 3A.

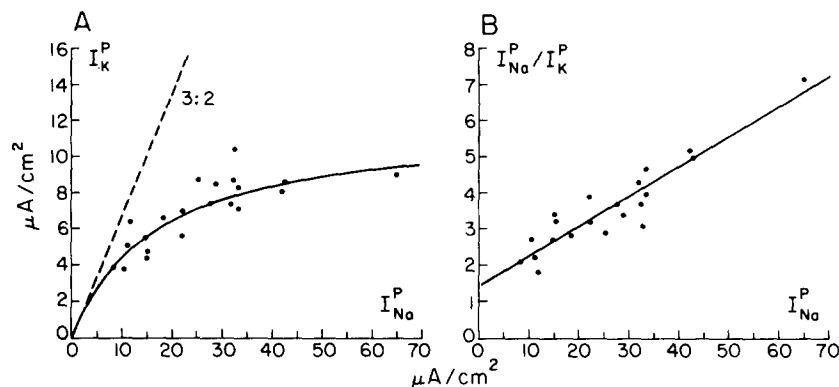


FIGURE 3. Relationship between I_{Na}^p and I_K^p (A) and between I_{Na}^p and coupling ratio $r = I_{Na}^p/I_K^p$ (B). The data of B were fitted by least-squares linear regression analysis to Eq. 2 of the text, and the solid line of A was drawn according to Eq. 3. As $I_{Na}^p \rightarrow \infty$, $I_K^p = 1/m = 11.9 \mu\text{A}/\text{cm}^2$.

To show the relative contributions of current carried by Na^+ and K^+ through the pump ($I^p \equiv I_{Na}^p - I_K^p$), we graphed the I_{Na}^p and I_K^p as a function of I^p as shown in Fig. 4, according to Eq. 3. With increasing I^p , pump-mediated K^+ influx approached saturation, whereas Na^+ influx increased almost linearly. Remarkably, these relationships are virtually the same as those reported by Mullins and Brinley (1969; Brinley and Mullins, 1974), who studied squid axon and who first reported that the stoichiometry of the Na^+/K^+ exchange process varies with Na^+ transport rate.

Ba⁺⁺ Method (According to Nielsen) for Stoichiometry

Nielsen (1979) has suggested a method for determination of the stoichiometry of the Na^+/K^+ pump. His method rests on the assumptions that (a) Ba^{++} at high concentrations inhibits completely electrodiffusive K^+ transport, and (b) Ba^{++} has no effect on the pump. In the case of a 3:2 Na/K exchange stoichiometry, Nielsen suggested that abolition of K^+ efflux by Ba^{++} would cause a 67% inhibition

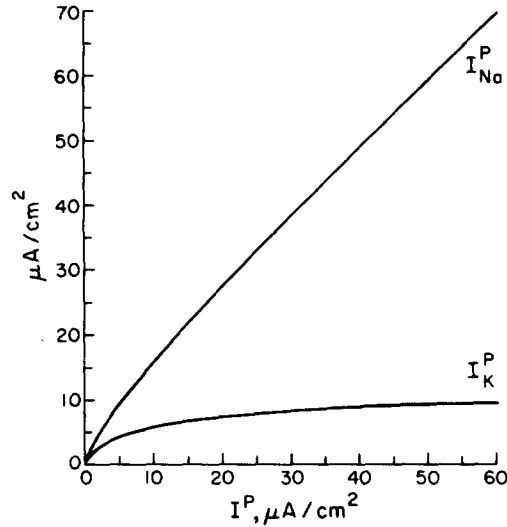


FIGURE 4. Contribution of Na^+ and K^+ currents to the net current of the pump. $I_P = I_{\text{Na}}^P - I_{\text{K}}^P$. The I_P vs. I_{Na}^P and I_P vs. I_{K}^P relationships were drawn according to Eq. 3. Note that as I_{K}^P approaches "saturation," I_{Na}^P continued to increase almost linearly. Between I^P of 10 and 60 $\mu\text{A}/\text{cm}^2$, I_{K}^P increased by 3.7 $\mu\text{A}/\text{cm}^2$, whereas I_{Na}^P increased by 60.0 $\mu\text{A}/\text{cm}^2$.

of the I_{sc} , provided that the I^P is independent of the basolateral membrane voltage, i.e., that the pump behaves as a constant current source. If this suggestion is correct, it must follow also that after inhibition of the pump by ouabain, Ba^{++} should cause 100% inhibition of the I_{sc} , as K^+ is presumed to be the only charge-carrying species at the basolateral membranes of the cells.

These ideas were tested with isolated epithelia short-circuited in chambers and bathed with Cl-Ringer solution. As the I_{sc} and its changes can be measured with high resolution and precision, we would have expected to observe highly reproducible percent changes of I_{sc} if r were constant among epithelia.

Fig. 5 shows the changes of I_{sc} caused by 5 mM Ba^{++} in a typical experiment.

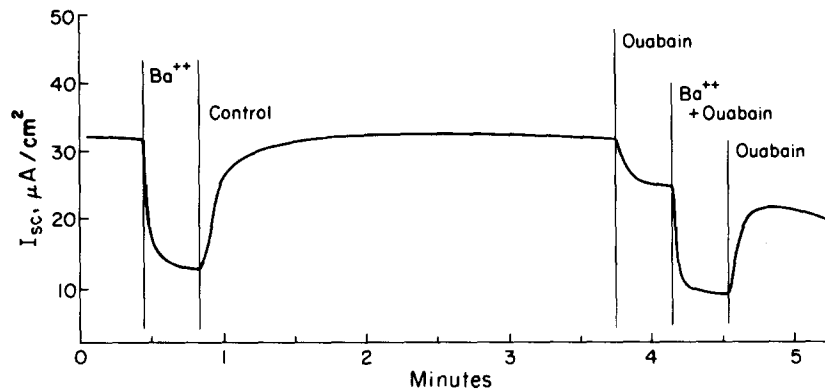


FIGURE 5. Changes of I_{sc} caused by 5 mM Ba^{++} in the basolateral solution before and after 0.1 mM ouabain.

Within 30 s, the I_{sc} was inhibited, and the inhibition was reversible upon removal of Ba^{++} from the basolateral solution. Thereafter, the tissue was exposed to 0.1 mM ouabain for 1 min and treated again with 5 mM Ba^{++} in the presence of ouabain. The results of 23 experiments are summarized in Table II.

The control I_{sc} averaged $18.5 \mu A/cm^2$; 5 mM Ba^{++} inhibited the I_{sc} by 51–69%. Taken at face value, this would correspond to pump stoichiometries in the range of ~2:1–3:2.

Ouabain alone inhibited the I_{sc} by ~25%. In the presence of ouabain, 5 mM Ba^{++} caused virtually the same mean percent inhibition of the I_{sc} as observed in control tissues, i.e., 47–79%. This is in marked contrast to the predicted drop of I_{sc} to zero (see above). As it is impossible to explain away I_{sc} values of the magnitude observed after Ba^{++} plus ouabain, we conclude that this method does not provide reliable estimates of stoichiometry. Although Ba^{++} appears to inhibit K^+ efflux, the efflux of ^{42}K is inhibited by only 48% by 5 mM Ba^{++} (Cox and Helman, 1983a), which demonstrates that a fundamental assumption of this method is not valid. We therefore conclude that estimates of stoichiometry derived from Ba^{++} inhibition of the I_{sc} are fortuitous in giving values in the range of 3:2.

TABLE II
Ba⁺⁺ Inhibition of I_{sc} in Control and Ouabain-poisoned Epithelia

	Control	Ouabain	5 mM Ba^{++}	Percent inhibition by Ba^{++}
	$\mu A/cm^2$	$\mu A/cm^2$	$\mu A/cm^2$	
$N = 23$	18.5 ± 1.4 (7.9–32.2)	—	7.2 ± 0.6 (3.2–12.6)	61.0 ± 1.0 (51.0–68.8)
$N = 23$	—	13.8 ± 1.1 (6.3–24.7)	5.1 ± 0.5 (2.2–10.8)	63.3 ± 2.1 (47.3–79.3)

Values are means \pm SEM (range). Tissues were bathed in Cl-Ringer solution.

DISCUSSION

Compelling evidence exists both for and against the hypothesis that the stoichiometry of the ubiquitous Na,K-ATPase is fixed, with a ratio of 3:2. Indeed, since the original observations of a pump stoichiometry of 3:2 in studies of human red blood cell by Post and Jolly (1957; Post et al., 1967), the results of numerous studies in epithelial and nonepithelial cells have left the answer to the question of fixed vs. variable stoichiometry unresolved. A challenge to the 3:2 hypothesis was presented by Mullins and Brinley (1969; Brinley and Mullins, 1974) from studies of squid axons, and to our knowledge, their suggestion of variable stoichiometry has not been refuted. Over the past 20 years or so, numerous reports have appeared quoting stoichiometries that range between ~1 and 6, and in our review of the literature, we could find no compelling reasons to dismiss all observations at variance with the 3:2 fixed stoichiometry hypothesis.

Major reviews since 1972 (Thomas, 1972; Sjodin, 1982; Beaugé, 1984; Gadsby, 1984; Glynn, 1984) have cited observations of variable stoichiometry

from many tissues where various methods have been used to determine the stoichiometry. Several attempts have been made to measure the stoichiometry of the Na,K-ATPase in frog skin (Curran and Cereijido, 1965; Biber et al., 1972; Candia and Zadunaisky, 1972; Aceves, 1977a; Nielsen, 1979; Nagel, 1980). Although uncertainties owing to the unstirred layers of the corium and owing to ouabain-induced cation fluxes may in part compromise the interpretation of some of these data, these investigators, with the exception of Nielsen, have presented data inconsistent with the idea that the stoichiometry of Na/K exchange is 3:2. As we found that 5 mM Ba⁺⁺ inhibits K⁺ efflux by only 48% (Cox and Helman, 1983a), and since the percent inhibition of the I_{sc} by Ba⁺⁺ is the same in control tissues and after ouabain inhibition of the pumps, we must conclude that the stoichiometry estimated by this method is unreliable. We therefore turned to isotopic methods, where the stoichiometry could be estimated at the steady state of ion transport, thereby avoiding the possibility that drugs and/or other interventions may in and of themselves alter the relationships between Na⁺ and K⁺ transport via the pump.

Na⁺ Transport by the Pump

In addition to the usual housekeeping functions of the Na,K-ATPase, epithelial tissues, especially those of renal distal tubular origin, are noted for their ability to absorb Na⁺ at highly variable rates commensurate with their known regulatory role in maintaining constancy of extracellular Na⁺ concentration. Hormones and drugs are known to modulate the rate of transepithelial Na⁺ transport, providing at the steady state highly variable loads of Na⁺ to be transported by the pump. In model tissues like frog skin and toad urinary bladder, these variable rates of Na⁺ transport are reflected in wide-ranging rates of transepithelial Na⁺ transport, measured most often by the short-circuit current method of Ussing and Zerahn (1951). This method has proven advantageous because rates of Na⁺ transport measured electrically provide quantitative accuracy of the Na⁺ entry into the cells and subsequently the Na⁺ load of the pump at the basolateral membrane of the cells. Thus, in the context of the present studies, measurement of Na⁺ transport is readily accomplished with relatively little room for argument as to its magnitude. It can be stated that maximum errors of 5% are certainly a liberal overestimate of the maximum uncertainty in the determination of the rate of apical membrane Na⁺ entry into the cells.

A more serious source of error in the determination of the pump-mediated Na⁺ efflux arises from the possibility of passive basolateral Na⁺ influx, with subsequent recycling via the pump (see review by Macknight et al., 1980). Under the circumstances of the present studies, Stoddard and Helman (1985) observed a net electroneutral Na⁺ entry of ~3.5 $\mu\text{A}/\text{cm}^2$. At the usual rates of transepithelial Na⁺ transport (20–30 $\mu\text{A}/\text{cm}^2$), basolateral membrane net Na⁺ influx amounts to ~10–20% of the Na⁺ load to the pump. Since this flux is independent of the I_{sc} , its importance, in practical terms, increases as the spontaneous rate of transepithelial Na⁺ transport falls. Hence, we have taken this into account when attempting to deal with the issue of stoichiometries (see below).

K⁺ Transport by the Pump

The direct measurement of basolateral K⁺ transport has been complicated by unstirred layers of connective tissue in epithelial tissues, and this problem has for all practical purposes been circumvented by the use of isolated epithelia of frog skin. At the rate of K⁺ transport by this epithelium, our calculations indicated that ⁴²K influx was at the very most underestimated by 2.5%. Errors of this kind would not alter in any significant way the data or their interpretation. The amount of ⁴²K taken up across the basolateral surface of the tissue was a linear function of time (for at least 20 min in our studies), which confirms the observations of Biber et al. (1972), who measured the ⁴²K influx for periods of up to 4 min. Neither the J_K^{32} nor the I_{sc} was different for epithelia bathed with Cl-Ringer or the Cl-free SO₄-Ringer solution, which rules out any important Cl-dependent co-transport of K⁺ under control conditions. Since the average FJ_K^{32} is ~8 μA/cm² and the average I_{sc} is near 22 μA/cm², the stoichiometry of the Na,K-ATPase is >3:2. If the pump stoichiometry were in fact 3:2, and the I_{sc} were taken as a minimum estimate of the I_{Na}^p , then FJ_K^{32} would have to be, at a minimum, ~14 μA/cm² (⁴²K influx via K⁺ channels assumed to be zero). Since we have measured K⁺ efflux by an electrical method (Cox and Helman, 1986a) and found it to be the same as FJ_K^{32} , we conclude that I_{sc}/FJ_K^{32} is >3:2. When corrected for basolateral membrane Na⁺ recycling and FJ_K^{32*} via the K⁺ channels, the stoichiometry ($r = I_{Na}^p/I_K^p$) was, in every tissue, >3:2.

Ouabain-insensitive FJ_K^{32}*

Implicit in the above assessment of the data and the ultimate determination of the r is knowledge of the actual magnitude of the I_K^p . As measured, the ⁴²K influx FJ_K^{32} is partitioned between the pump (I_K^p) and the K⁺ channel FJ_K^{32*} . FJ_K^{32*} is most often estimated from the ouabain-insensitive FJ_K^{32} . As we were aware of the existence of ouabain-induced Na⁺ fluxes in frog skin and indeed in other tissues, notably squid axons and red cells (Mullins and Brinley, 1969; Lew and Beaugé, 1979), the possibility of ouabain-induced basolateral K⁺ fluxes was studied. In the absence of Cl⁻ from the bathing solution, ouabain caused a marked inhibition of FJ_K^{32} to 6% of control, which is consistent with the notion that ⁴²K influx occurred primarily via the Na,K-ATPase. Before inhibition of the Na,K-ATPase with ouabain, furosemide caused no significant change of the K⁺ influx. After ouabain, however, the "ouabain-insensitive" ⁴²K influx (30% of the control FJ_K^{32}) was sensitive to furosemide. Indeed, when tissues were treated in combination with ouabain and furosemide, the ouabain-insensitive ⁴²K influx was 6% of control and identical to that observed with tissues bathed with Cl-free Ringer solution. As acute sensitivity to furosemide (seconds) of the Na⁺ and K⁺ effluxes is manifest when Cl⁻ but not SO₄²⁻ was present in the bathing solution, and as no concurrent changes have been observed electrically in measurements of the I_{sc} and intracellular voltage, as was observed before in studies of Na⁺ efflux (Cox and Helman, 1983b), we conclude that ouabain induces a neutral Cl-dependent and furosemide-inhibitable process of K⁺ transport at the basolateral membrane of the cells. This idea will be explored further in the following article (Cox and

Helman, 1986a). The mechanism of this effect of ouabain is unknown from our experiments, but it is interesting to note the observations of Fossel and Solomon (1981, 1983). They suggested the existence of an intimate association between the Na,K-ATPase and the anion transport band 3 protein of human red blood cells. Thus, it may be that ouabain binding to the Na,K-ATPase alters the nature of anion transport processes linked to cation transport. In the context of the present studies, the existence of a Cl⁻-dependent induced component of ⁴²K influx would lead to a serious underestimation of I_K . Fortunately, this can be avoided by carrying out studies in SO₄-Ringer solution or by inclusion of furosemide in studies carried out in Cl-Ringer solution. No systematic study of other anion dependence has been attempted.

A second source of error in the determination of the ⁴²K influx via K⁺ channels arises from the change of basolateral membrane voltage caused by ouabain. Within seconds, ouabain at $\geq 10^{-4}$ M maximally depolarizes V_b and hence decreases electroconductive ⁴²K influx. Evidence presented in the following article (Cox and Helman, 1986a) is consistent with a single-file-like process for K⁺ transport with an n' value near 3. When we calculated the control ⁴²K influx via the K⁺ channels (FJ_K^{32*}), its value of 0.86 $\mu\text{A}/\text{cm}^2$ was greater than that observed from the ouabain-insensitive ⁴²K influx ($\sim 0.5 \mu\text{A}/\text{cm}^2$). This is to be expected owing to the change of V_b caused by ouabain. To the extent that the change of FJ_K^{32*} after ouabain is relatively small as compared with the total ⁴²K influx (FJ_K^{32}), the pump-mediated fraction of FJ_K^{32} was in the vicinity of 91%. Thus, the stoichiometry calculated from the ratio I_{Na}^p/I_K^p , assuming that $I_K^p = 0.91 FJ_K^{32}$, should provide reasonably accurate estimates of r .

Stoichiometry of the Pump

When r was plotted against I_{Na}^p , a linear relationship was observed (see Fig. 3B). Transformation of Eq. 2 indicated a saturable-like relationship between I_K^p and I_{Na}^p ; i.e., as shown in Fig. 3A, the K⁺ influx appeared to increase toward a maximum as pump-mediated Na⁺ efflux increased. Hence, there appeared to be no fixed macroscopic coupling between Na⁺ and K⁺ transport, and, as defined, r is variable.

Our experiments were not designed to ascertain the mechanism of variable stoichiometry of the pump. However, we observed that as the demand for Na⁺ extrusion by the pump was decreased toward the minimum "housekeeping" transport rate of the Na,K-ATPase, the stoichiometry fell toward 3:2. Probably, if tissues with low rates of transepithelial Na⁺ transport ($I_{sc} < 10 \mu\text{A}/\text{cm}^2$) had been selected and studied as a group, a mean pump stoichiometry near 3:2 would have been measured. However, I_{sc} in frog skin is normally $>10 \mu\text{A}/\text{cm}^2$, and in the range of 10–70 $\mu\text{A}/\text{cm}^2$ the stoichiometry increased linearly with pump-mediated Na⁺ extrusion. As K⁺ influx appeared to saturate with increasing Na⁺ transport, the pumps must be capable of increasing Na⁺ efflux uncoupled to K⁺ influx. If in fact K⁺ influx saturates completely at the higher rates of Na⁺ transport, then clearly the pumps must be capable of uncoupled Na⁺ efflux (at least to K⁺). As the magnitudes of the fluxes could be equated to electrical

currents (see Cox and Helman, 1986a), the “uncoupled” fraction of Na^+ efflux might represent a mode of the pump (Na^+ alone, uncoupled Na^+ efflux) that operates when the demand for Na^+ extrusion is increased.

There are numerous ways in which a pump capable of several modes of transport (see reviews by Lew and Beaugé, 1979; Glynn, 1984) can lead to macroscopic observations of variable stoichiometry. At one extreme, it is possible to envision a single enzyme molecule capable of exchanging 3 Na^+ for 2 K^+ or transporting 3 Na^+ for nothing (Na^+ -alone, Na^+ -for-nothing mode), thereby retaining the notion that the enzyme contains three sites for activation of the Na^+ efflux.⁴ Depending on the statistical distribution of the states of the molecule

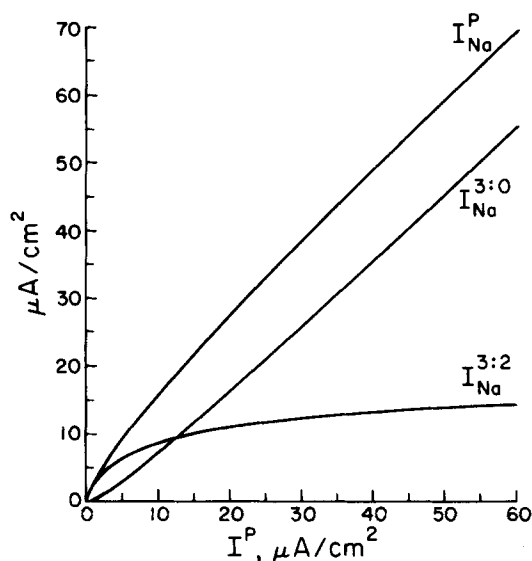


FIGURE 6. Hypothetical partitioning of Na^+ efflux via a pump with fixed 3:2 stoichiometry ($I_{\text{Na}}^{3:2}$) and a pump operating in an Na^+ -for-nothing mode ($I_{\text{Na}}^{3:0}$). Note that at low rates of Na^+ transport, Na^+ efflux is primarily via a 3:2 exchanger, whereas at higher I^P , increases of I^P occur primarily via the Na^+ -for-nothing mode of the pump.

at the time of enzyme turnover, the macroscopic stoichiometry would vary between 3:2 and ∞ . At the other extreme, it is possible to envision enzyme molecules inhibitable by ouabain that, by virtue of their lipid environment and/or other factors of membrane or environmental origin, influence the state of the enzyme so that a fraction of the pool of enzyme is either in a 3:2 state of Na/K exchange or in a state of Na^+ -for-nothing transport. This, too, would lead to observations of variable macroscopic stoichiometry $>3:2$. Regardless of these or other speculations of molecular mechanism, it will ultimately be necessary to

⁴ Calculations according to Mountcastle (1980) indicate that sufficient energy in conversion of ATP to ADP (Nunnally et al., 1983) is available for the pumps to operate in a 3 Na^+ -for-nothing mode of transport.

resolve further how the Na^+ load to the pump is related to K^+ transport. We have, as shown in Fig. 6, attempted to obtain as a first approximation an estimate of the amount of K^+ -dependent Na^+ efflux and K^+ -independent Na^+ efflux. We made the assumption that Na^+ efflux coupled to K^+ influx occurs via a mode of the pump that is fixed at 3:2 at all transport rates. Accordingly, the line shown for $I_{\text{Na}}^{3:2}$ was calculated with the data of Fig. 4, where $I_{\text{Na}}^{3:2} = 1.5 I_{\text{K}}^{\text{p}}$. The difference between total Na^+ efflux I_{Na}^{p} and $I_{\text{Na}}^{3:2}$ provided the data shown by the line $I_{\text{Na}}^{3:0}$. If such reasoning is tenable, then at physiological rates of transepithelial Na^+ transport, the 3:2 exchange mode of the pump would contribute to the "house-keeping" functions of the pump but little to the overall regulation of transepithelial Na^+ transport. Indeed, according to this view, given the near-linear relationship between uncoupled Na^+ efflux and I_{p} , it may be that cells alter their ability to regulate Na^+ extrusion by using a K^+ -independent, Na^+ -for-nothing mode of Na^+ transport. This suggestion remains to be proven. Nevertheless, it remains clear that with variable stoichiometry, there is no obligatory coupling, in the physiological sense, between Na^+ extrusion and K^+ transport by cells of this epithelium.

We wish to acknowledge the technical assistance of Mrs. Shashi Gupta, who performed a portion of these studies.

This work was supported by AM 16663 from the National Institutes of Health. Dr. Cox was sponsored by National Institutes of Health postdoctoral fellowship GM 07357.

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

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