

Furosemide-sensitive Na and K Fluxes in Human Red Cells

Net Uphill Na Extrusion and Equilibrium Properties

CARLO BRUGNARA, MITZY CANESSA, DANIELE CUSI, and DANIEL C. TOSTESON

From the Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115, and Università degli Studi di Milano, Istituto di Clinica Medica I, Scuola di Specializzazione in Nefrologia Medica, Milano, Italy

ABSTRACT This paper reports experiments designed to find the concentrations of internal and external Na and K at which inward and outward furosemide-sensitive (FS) Na and K fluxes are equal, so that there is no net FS movement of Na and K. The red cell cation content was modified by using the ionophore nystatin, varying cell Na (Na_i) from 0 to 34 mM (K substitution, high-K cells) and cell K (K_i) from 0 to 30 mM (Na substitution, high-Na cells). All incubation media contained NaCl ($\text{Na}_o = 130$ or 120 mM), and KCl ($\text{K}_o = 0$ – 30 mM). In high-K cells, incubated in the absence of K_o , there was net extrusion of Na through the FS pathway. The net FS Na extrusion increased when Na_i was increased. Low concentrations of K_o (0–6 mM) slightly stimulated, whereas higher concentrations of K_o inhibited, FS Na efflux. Increasing K_o stimulated the FS Na influx ($\text{K}_{0.5} = 4$ mM). Under conditions similar to those that occur in vivo ($\text{Na}_i = 10$, $\text{K}_i = 130$, $\text{Na}_o = 130$, $\text{K}_o = 4$ mM, $\text{Cl}_i/\text{Cl}_o = 0.7$), net extrusion of Na occurs through the FS pathway (180 – 250 $\mu\text{mol/liter cell}\cdot\text{h}$). The concentration of K_o at which the FS Na influx and efflux and the FS K influx and efflux become equal increased when Na_i increased in high-K cells and when K_i was increased in high-Na cells. The net FS Na and K fluxes both approached zero at similar internal and external Na and K concentrations. In high-K cells, under conditions when net Na and K fluxes were near zero, the ratio of FS Na to FS K unidirectional flux was found to be 2:3. In high-K cells, the empirical expression $(\text{Na}_i/\text{Na}_o)^2(\text{K}_i/\text{K}_o)^3$ remained at constant value (apparent equilibrium constant, $K_{eq}^{app} \pm \text{SEM} = 22 \pm 2$) for each set of internal and external cation concentrations at which there was no net Na flux. These results indicate that in the physiological region of concentrations of internal and external Na, K, and Cl, the stoichiometry of the FS Na and K fluxes is 2 Na:3 K. In high-Na cells under conditions when net FS Na and K fluxes were near

Address reprint requests to Dr. Carlo Brugnara, Department of Physiology, Harvard Medical School, 25 Shattuck St., Boston, MA 02115.

zero, the ratio of FS Na to FS K unidirectional fluxes was 3:2 (1). In high-Na cells, the empirical expression $(\text{Na}_i/\text{Na}_o)^3(\text{K}_i/\text{K}_o)^{2(1)}$ remained at a constant value (apparent equilibrium constant, $K_{eq}^{app} \pm \text{SEM} = 0.85 \pm 0.13$) for each set of internal and external cation concentrations at which net FS Na and K fluxes were zero. Evidently, the stoichiometry of FS Na to FS K unidirectional fluxes varies with the concentrations of these cations.

INTRODUCTION

Several observations on the behavior of the Na and K fluxes in the presence of ouabain indicate that more than one transport system regulates the translocation of these cations across the human red cell membrane. Hoffman and Kregenow (1966) suggested the presence of a second pump in human red cells, which could perform Na extrusion in the presence of ouabain. Several studies have since demonstrated that several different transport systems couple the movements of Na, K, and Li through ion translocating pathways in human red cells. Wiley and Cooper (1974) proposed that *cis*-stimulated Na and K influxes were driven by a furosemide-sensitive (FS) Na/K cotransport system. Studies carried out by Garay et al. (1981) characterized outward Na/K cotransport in the presence of high MgCl_2 concentrations (75 mM). The Cl dependence of the FS Na and K movements was studied by Chipperfield (1980, 1981) and Dunham et al. (1980). The kinetics and stoichiometry of an Li/Na exchange system were characterized by Haas et al. (1975), Duhm et al. (1976), Pandey et al. (1978), and Sarkadi et al. (1978). Canessa et al. (1982) described several properties of the Li/Na exchange pathway that distinguish it from Na/K cotransport. Funder et al. (1978) showed that the facilitated monovalent anion exchange system can transport Na and Li, but not K, in the form of NaCO_3^- or LiCO_3^- ion pairs.

In recent years, the following cell types have been shown to carry out coupled FS Na and K transport, which requires the presence of Cl: intestinal epithelia (Mush et al., 1982), glial cells (Johnson et al., 1982), squid axon (Russel, 1983), kidney proximal tubule (McRoberts et al., 1982), and shark rectal glands (Han-nafin et al., 1983).

The Na/K/Cl cotransport system in avian red cells has been extensively studied. Kregenow (1971) and Riddick et al. (1971) showed the effect of hypertonicity, norepinephrine, and cyclic AMP on cation transport in duck red cells. Schmidt and McManus (1977*a, b*) and Haas et al. (1982) investigated the properties of this system in response to cell shrinkage and norepinephrine stimulation.

The experiments described in this paper were designed to provide information about the apparent equilibrium positions and stoichiometry of the FS Na and K fluxes in human red cells.

In this paper, we describe the dependence on internal and external Na and K concentrations of the equilibrium position for outward and inward FS Na and K movements. The data reveal that net FS Na and K movement is in the outward direction when external and internal Na and K concentrations are in the physiological range. At the equilibrium position, when inward and outward FS Na and K fluxes are equal, the apparent stoichiometry is 2 Na:3 K. In human red cells with high-Na, low-K content, at the equilibrium position of the FS Na and K fluxes, the stoichiometry of FS fluxes can be 3 Na:2 K or 3 Na:1 K.

Preliminary results of this work have been reported (Canessa et al., 1983, 1984; Brugnara et al., 1985).

MATERIALS AND METHODS

All the experiments were performed according to the methods section of the following paper (Canessa et al., 1986).

RESULTS

FS Na and K Fluxes in High-K Cells

Effect of external K on FS Na efflux. The FS Na efflux can be inhibited by external Na or external K. Fig. 1 shows the effect of external K on the FS Na

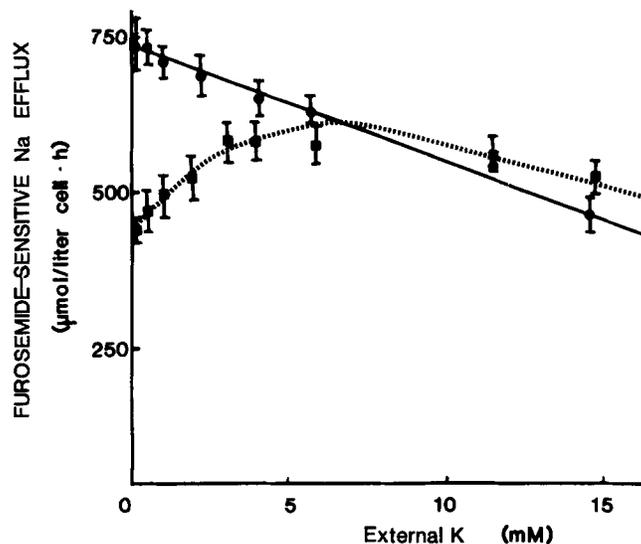


FIGURE 1. The effect of external K on the FS Na efflux (\pm SE) into Na (■) and choline (●) media. Cell Na = 16.5 mmol/liter cells; cell K = 80.5 mmol/liter cells. The Na media contained (mM): 130 NaCl, 0–20 KCl, and 20–0 choline Cl. The choline media contained 150–130 choline Cl and 0–20 KCl. All media contained (mM): 1 MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the efflux media was 2%. The incubation times were 5 and 65 min. ²²Na was used as a tracer. (Subject D.C. Similar results were obtained in the other two subjects.)

efflux from fresh cells in the presence and absence of external Na. It can be seen that in the absence of external K, external Na (130 mM) produced a marked inhibition of the FS Na efflux. On the other hand, external K promoted an inhibition of the FS Na efflux into Na-free media, as shown by Brugnara et al. (1983). However, when both ions were present in the medium, external K promoted a small but significant stimulation of FS Na efflux between 0 and 8 mM. Thus, when external K was increased in a medium containing Na, the inhibition of the FS Na efflux produced by external Na alone seemed to be completely released (Fig. 1).

Fig. 2 shows the effect of K_o on the FS Na efflux from cells loaded by the nystatin procedure to contain 4, 8, and 22 mmol Na/liter cells. It can be seen that the stimulation of FS Na efflux by external K was a function of cell Na. At cell Na = 4 mmol/liter cell, there was little stimulation by external K of the Na efflux. At cell Na = 22 mmol/liter cell, external K greatly stimulated the FS Na efflux. These results confirm those reported by Chipperfield (1981) in outdated erythrocytes. The $K_{0.5}$ for external K to stimulate FS Na efflux was 2.5 ± 0.4 mM (\pm SD; $n = 6$) and was independent of the cell Na content. The concentration of external K required to release maximally the inhibition produced by external Na increased from 5 to 10 mM when internal Na was raised from 4 to 22 mmol/liter cell.

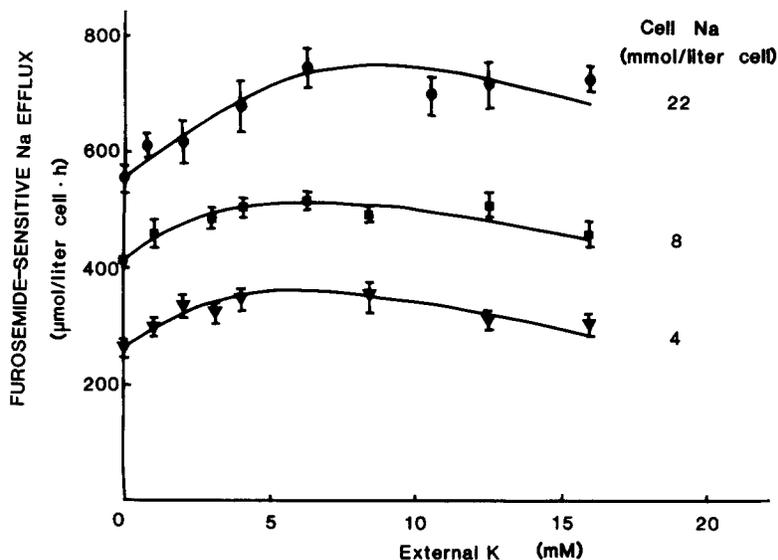


FIGURE 2. The effect of external K on FS Na influx (\pm SE) into Na media at different cell Na. Cell Na was varied with the nystatin loading procedure at the expense of K to 4, 8, and 22 mmol/liter cells. The external media contained (mM): 130 NaCl, 0–20 KCl and 20–0 choline chloride, 1 MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the efflux media was 2%. The incubation times were 5 and 65 min. ²²Na was used as a tracer. (Subject C.B. Similar results were obtained in the other two subjects.)

External K stimulates FS Na influx. Fig. 3 shows the effect of external K on the FS Na influx when internal Na was varied from 0.5 to 22 mmol/liter cell. External K stimulated the ouabain-resistant (OR) and FS Na influx. The $K_{0.5}$ for the stimulation by external K of the FS Na influx was 4.5 mM (as reported by Wiley and Cooper, 1974), and in fresh cells the V_{max} was ~ 500 μ mol/liter cells · h. It can also be seen in Fig. 3 that the increase of cell Na in the physiological range markedly stimulated FS Na influx. Table I shows the kinetic parameters of the OR Na influx in the three subjects. Two components of the Na influx

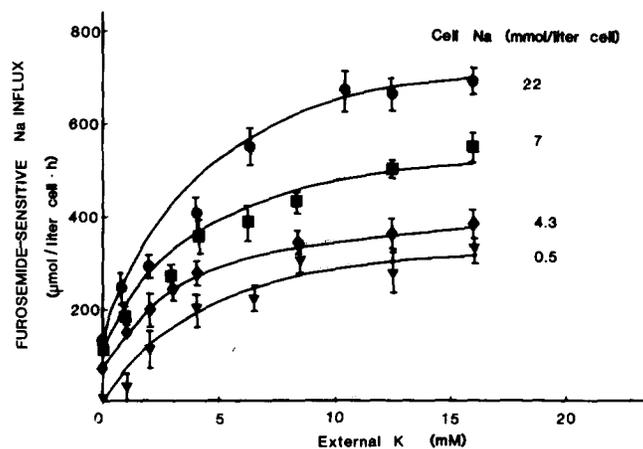


FIGURE 3. Effect of external K on the FS Na influx (\pm SE) at different cell Na. Cell Na was varied with the nystatin loading procedure at the expense of K to 0.5, 4.3, 7, and 22 mmol/liter cells. The external media contained (mM): 130 NaCl, 0–20 KCl and 20–0 choline chloride, 1 MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the influx media was 5%. The incubation times were 5 and 65 min. ²²Na was used as a tracer. (Subject C.B. Similar results were obtained in the other two subjects.)

TABLE I
Kinetic Parameters of FS Na Influx into Human Red Cells

Subject	Cell Na <i>mmol/liter cell</i>	Na influx; K _o stimulated			
		FS		OR	
		K _{0.5} for K _o <i>mM</i>	V _{max} <i>µmol/liter cell · h</i>	K _{0.5} for K _o <i>mM</i>	V _{max} <i>µmol/liter cell · h</i>
C.B.	0.5	4.9 \pm 1.3	450 \pm 50	3.4 \pm 0.4	475 \pm 30
	4.3	3.6 \pm 0.3	385 \pm 25	3.1 \pm 0.4	360 \pm 25
	7.0	3.5 \pm 0.2	490 \pm 45	4.8 \pm 0.8	520 \pm 60
	22.0	3.8 \pm 0.2	710 \pm 35	8.3 \pm 2.2	810 \pm 100
A.K.	4.0	4.8 \pm 1.1	500 \pm 60	5.1 \pm 0.4	540 \pm 25
	11.5	5.0 \pm 1.3	680 \pm 105	3.0 \pm 0.5	745 \pm 40
	22.4	3.5 \pm 0.3	775 \pm 25	3.8 \pm 0.5	975 \pm 80
D.C.	11.5	5.7 \pm 0.6	620 \pm 20	4.1 \pm 0.5	560 \pm 30
	Mean \pm SD	4.4 \pm 0.8		4.5 \pm 1.6	

The external media contained (mM): 130 NaCl, 0–20 KCl and 20–0 choline chloride, 1 MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, 0.1 ouabain, with and without 1 furosemide. The hematocrit of the influx media was 5%. The incubation times were 5 and 65 min. ²²Na was used as a tracer. The K_o-stimulated component of the OR Na influx was calculated as the difference between OR Na influx in the presence and absence of different concentrations of external K. The K_{0.5} and V_{max} (\pm SEM) were calculated from the Eadie plot, after subtracting the value of the Na influx in the absence of external K. To enable comparison between V_{max} of FS and OR Na influx, the value of FS Na influx in the absence of external K should be added to the FS component.

were studied: (a) the FS Na influx and (b) the K_o - (or *cis*-) stimulated Na influx (estimated by subtracting from the OR Na influx at any external K concentration the value of the OR Na influx in the absence of external K). The latter provides an estimate of the Na/K cotransport that is independent of the inhibitory action of furosemide. The V_{max} values of both the FS and the *cis*-stimulated (K_o -stimulated) Na influx were increased when cell Na was increased above 4 mmol/liter cell.

For a given concentration of external K, the value of the *cis*-stimulated component of the OR Na influx (Na/K cotransport) was slightly lower than the FS component. The reason for this is that there was a small FS component of the Na influx even in the absence of external K.

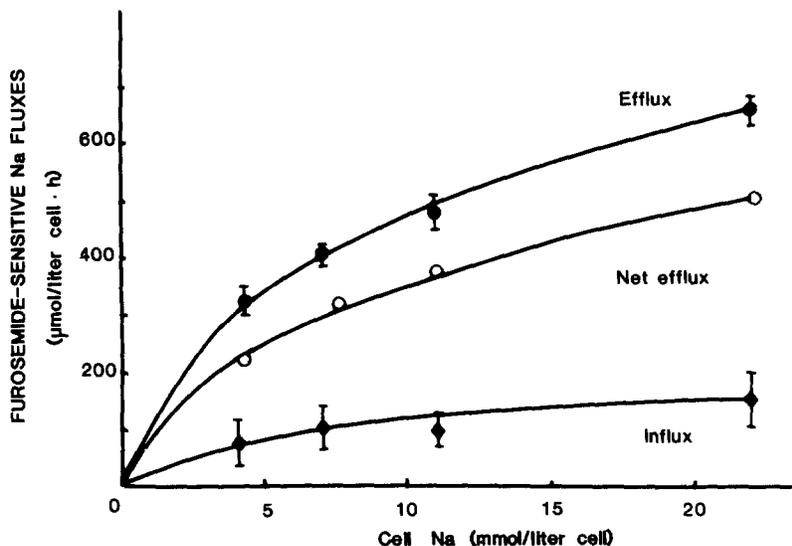


FIGURE 4. Effect of internal Na on the FS Na efflux and influx (\pm SE) in the absence of external K. The experimental conditions were identical to those in Figs. 2 and 3. (Subject D.C. Similar results were obtained in the other two subjects.)

Net uphill FS Na extrusion in the absence and presence of external K. Fig. 4 shows that when the cells were incubated in media containing only Na (130 mM), the FS Na efflux was always larger than the influx, despite the large inward electrochemical Na gradient. This net FS Na efflux increased with increasing cell Na concentration.

Figs. 5–7 show simultaneous measurements of FS Na efflux and influx as a function of external K in cells containing 4.7, 10, and 22 mmol Na/liter cells. It can be seen that for all three cellular Na contents and up to 5–6 mM K_o , the FS Na efflux was larger than the influx. There was net Na efflux at 4 mM K_o from cells with an Na content similar to that of fresh cells (Fig. 6). Similarly, at low cell Na (Fig. 5), there was still FS net Na extrusion when K_o was 4 mM. At cell Na higher than the physiological steady state, the FS net Na extrusion at 4 mM K_o increased to 300 μ mol/liter cell · h (Fig. 7). Fig. 8 shows the effect of external

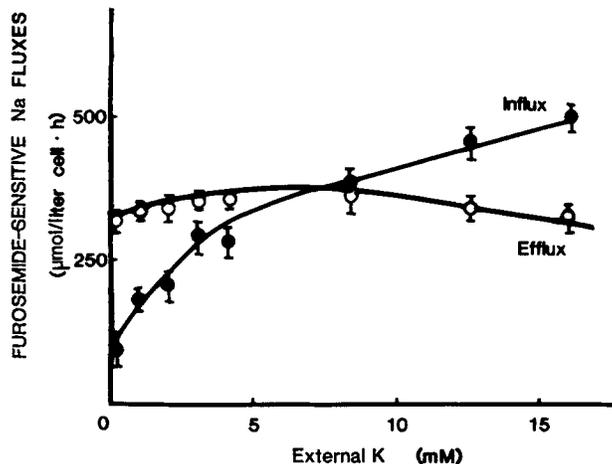


FIGURE 5. Simultaneous measurement of FS Na efflux and influx (\pm SE) in cells containing 4.7 (Na) and 90 (K) mmol/liter cells. The experimental conditions were identical to those in Figs. 2 and 3. (Subject C.B. Similar results were obtained in the other two subjects.)

K on the net FS Na extrusion at three different cell Na levels. The net uphill outward FS movement of Na was progressively inhibited by the increase of external K.

Effect of Cl removal (nitrate substitution) on the net FS Na extrusion. Table II shows measurements of FS Na efflux and influx in cells that contained 11 mmol

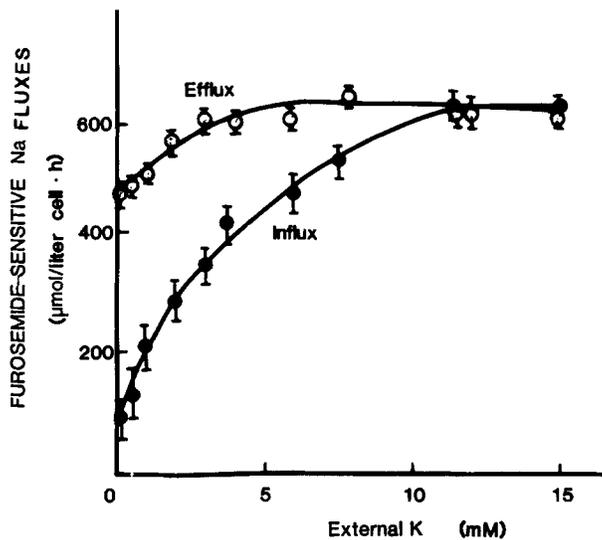


FIGURE 6. Simultaneous measurement of FS Na efflux and influx (\pm SE) in cells containing 10 (Na) and 85 (K) mmol/liter cells. The experimental conditions were identical to those in Figs. 2 and 3. (Subject A.K. Similar results were obtained in the other two subjects.)

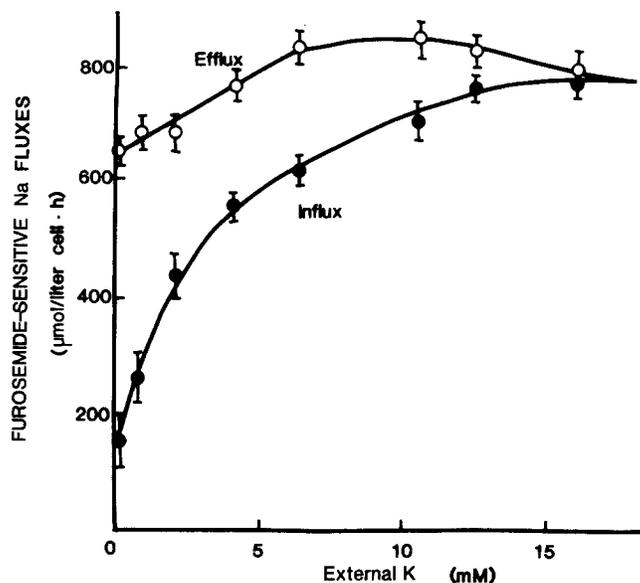


FIGURE 7. Simultaneous measurement of FS Na efflux and influx (\pm SE) in cells containing Na 22 (Na) and 73 (K) mmol/liter cells. The experimental conditions were identical to those in Figs. 2 and 3. (Subject A.K. Similar results were obtained in the other two subjects.)

Na/liter cell and were incubated in medium containing 130 mM NaCl and 4 mM KCl. Under these conditions, there was a net FS Na extrusion of 100 μ mol/liter cell·h. When Cl was replaced by nitrate, there was a marked inhibition (80%) of the FS Na efflux and a stimulation of the FS Na influx. Therefore, Cl replacement by nitrate produced a net FS Na influx of 600 μ mol/liter cell·h.

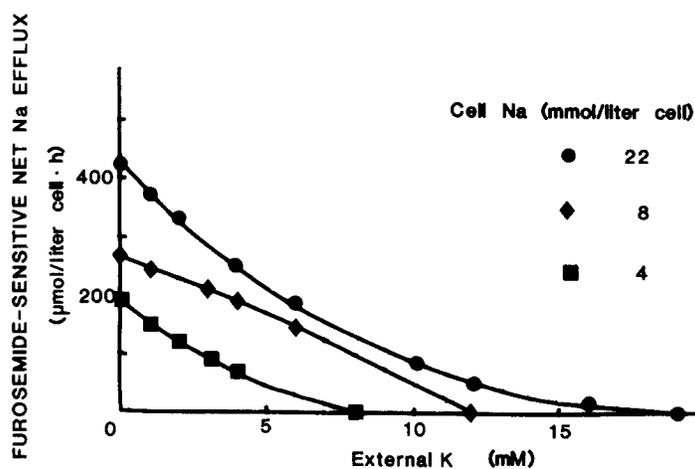


FIGURE 8. Effect of external K on the FS net Na extrusion at three different intracellular Na concentrations (4, 8, and 22 mmol/liter cells). (Subject C.B. Similar results were obtained in the other two subjects.)

TABLE II
Cl Dependence of the Net FS Na Extrusion

	Na efflux			Na influx			Net movement	
	OR	FR	FS	OR	FR	FS	FS	FR
	$\mu\text{mol/liter cell}\cdot\text{h}$			$\mu\text{mol/liter cell}\cdot\text{h}$			$\mu\text{mol/liter cell}\cdot\text{h}$	
Cl	780±24	290±15	490±28	1245±42	860±33	385±53	-105	+570
Nitrate	460±15	355±17	105±23	2135±47	1550±47	585±66	+480	+1195

The cellular Na content was 11 mmol/liter cell. The flux media contained (mM): 130 Na, 4 K, 16 glucamine, and 1 Mg (Cl or nitrate). All the media contained (mM): 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit was 2% for the efflux and 5% for the influx (subject D.C.). Similar results were obtained in another subject. (Values ± SE of the fluxes.)

We also observed that increasing external K (from 0 to 20 mM) did not stimulate Na efflux and influx in nitrate medium. It can also be seen in Table II that the FR Na fluxes were nitrate-stimulated or chloride-inhibited. Similar results were obtained using bumetanide (0.01 mM) instead of furosemide. The bumetanide-sensitive Na efflux was reduced by nitrate to 5% of the value in Cl (from 500 to 20 $\mu\text{mol/liter cell}\cdot\text{h}$) and was not affected when external K was increased from 0 to 20 mM. However, there was still a sizable bumetanide-sensitive component of the Na influx (200 $\mu\text{mol/liter cell}\cdot\text{h}$) from nitrate media. Therefore, both FS and bumetanide-sensitive net Na extrusions were abolished by nitrate replacement. The marked increase of the Na influx in nitrate media (K_o independent)

TABLE III
Equilibrium of the FS Na Fluxes

$n\text{Na}_o + m\text{K}_o \rightleftharpoons n\text{Na}_i + m\text{K}_i$							
$K_{eq} = \left(\frac{\text{Na}_i}{\text{Na}_o}\right)^n \left(\frac{\text{K}_i}{\text{K}_o}\right)^m$							
Subject	Na_i	Na_o	K_i	K_o	$\frac{n}{1} \frac{m}{\text{Na}:1 \text{ K}}$	$\frac{n}{3} \frac{m}{\text{Na}:2 \text{ K}}$	$\frac{n}{2} \frac{m}{\text{Na}:3 \text{ K}}$
	mM	mM	mM	mM	K_{eq}	K_{eq}	K_{eq}
C.B.	6.6	130	134	7.5	0.91	0.04	14.7
	11.7	130	144	12	1.08	0.11	14.0
	34.0	130	113	19	1.55	0.63	14.4
Coefficient of variation (%)					23	100	2
A.K.	6.6	130	139	7	1.0	0.05	20.2
	18.5	130	131	12	1.55	0.34	26.3
	35.4	130	113	16	1.92	1.01	26.1
Coefficient of variation (%)					62	86	12
D.C.	9.1	130	120	7	1.20	0.10	24.7
	15.7	130	126	10	1.52	0.28	29.2
	23.0	130	110	12	1.62	0.47	24.1
Coefficient of variation (%)					12	53	9
Mean ± SD					1.37±0.33	0.34±0.31	21.5±5.7
Mean coefficient of variation					32±21	80±20	8±4

was similar to that described by Funder and Wieth (1967). We also found (data not shown) that DIDS (150 μM) totally inhibited the increase of the Na influx produced by nitrate.

Equilibrium for the FS Na fluxes in high-K cells. Table III shows the internal and external Na and K concentrations at which the FS Na fluxes were at

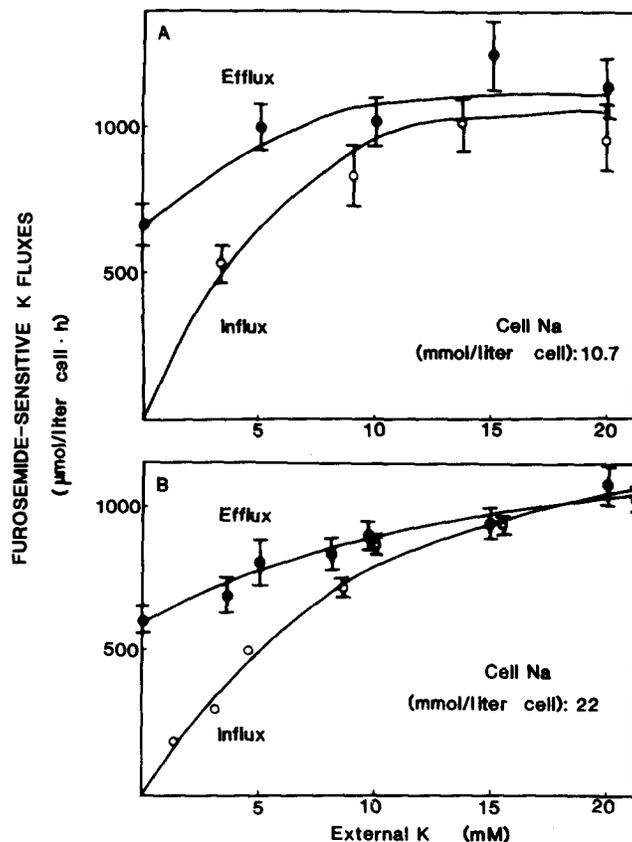


FIGURE 9. Simultaneous measurement of FS K efflux and influx (\pm SE) in cells containing (A) 10.5 (Na) and (B) 22 (Na) mmol/liter cells. Experimental conditions were identical to those in Figs. 2 and 3. ^{42}K was used as a tracer. The incubation times for the influx were 5 and 25 min. (A, subject A.K.; B, subject C.B. Each experiment was repeated in the other subject with similar results.)

equilibrium. Assuming that the transport of Na and K is due to an interaction of these two ions on both sides of the membrane, the cotransport reaction can be written



where n and m are the stoichiometric coefficients for Na and K, respectively. Since the Cl concentration inside and outside the cells was the same in all these experiments, Cl is not included in this description of the transport process.

The empirical function $(\text{Na}_i/\text{Na}_o)^n (\text{K}_i/\text{K}_o)^m$ can be used to estimate an apparent equilibrium constant ($K_{\text{eq}}^{\text{app}}$) for this reaction (Tosteson, 1981). The $K_{\text{eq}}^{\text{app}}$ of this transport reaction was calculated assuming different stoichiometric ratios for the reactants (Table III). We compared the values for $K_{\text{eq}}^{\text{app}}$ calculated from the different sets of internal and external K and Na concentrations at which net FS Na transport was zero. From the results summarized in Table III, it is evident that the calculated values for $K_{\text{eq}}^{\text{app}}$ are constant for every subject when the ratio of Na to K transport is assumed to be 2:3. The coefficient of variation for $K_{\text{eq}}^{\text{app}}$ calculated from the different sets of internal and external cation concentrations

TABLE IV
Stoichiometry of the FS Na and K Fluxes at Equilibrium

	OR	FR	FS	FS Na/K fluxes ratio
<i>μmol/liter cell·h</i>				
(A) $\text{Na}_o = 130, \text{K}_o = 7.5, \text{Na}_i = 6.6, \text{K}_i = 134 \text{ mM}$				
Na efflux	570±20	220±10	350±22	0.60
Na influx	1,010±24	670±7	340±25	0.55
K efflux	1,300±25	720±61	580±66	0.60
K influx	660±10	40±3	620±10	0.55
(B) $\text{Na}_o = 130, \text{K}_o = 12, \text{Na}_i = 12, \text{K}_i = 144 \text{ mM}$				
Na efflux	850±11	340±15	510±19	0.74
Na influx	1,200±5	700±41	500±41	0.78
K efflux	1,670±85	980±5	690±85	0.74
K influx	760±15	120±9	640±17	0.78
(C) $\text{Na}_o = 130, \text{K}_o = 19, \text{Na}_i = 34, \text{K}_i = 113 \text{ mM}$				
Na efflux	1,530±6	800±48	730±48	0.67
Na influx	1,550±26	845±35	705±44	0.69
K efflux	1,950±43	860±78	1,090±89	0.67
K influx	1,220±43	200±10	1,020±44	0.69
			Mean ± SD	0.67±0.08

The intracellular Na content was varied with the nystatin loading procedure. The external media contained (mM): 130 NaCl, 0–20 KCl and 20–0 choline Cl, 1 MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the media was 5% (influx) and 2% (efflux). The incubation times were 5 and 65 min (K influx, 5 and 25 min). ²³Na and ⁴²K were used as tracers. Subject C.B. Similar results were obtained in another subject (A.K.).

for every subject was less for this model than for models that assumed a 3 Na:2 K ratio or a strict coupling of 1 Na:1 K.

Equilibrium positions for the FS K fluxes. The determination of equilibrium position for FS K fluxes involved some experimental difficulties. The ⁴²K influx was not linear during the experimental time period used for Na flux measurement, and was underestimated by 40% using a protocol with 1 h incubation. Hence, shorter times (25 min) were used to measure the ⁴²K influx from Na-containing media.

Fig. 9 shows simultaneous measurements of FS K efflux and influx in cells containing 10.7 (A) and 22 (B) mmol Na/liter cell. External K stimulated FS K efflux and influx, and the FS K fluxes approached equilibrium at internal and

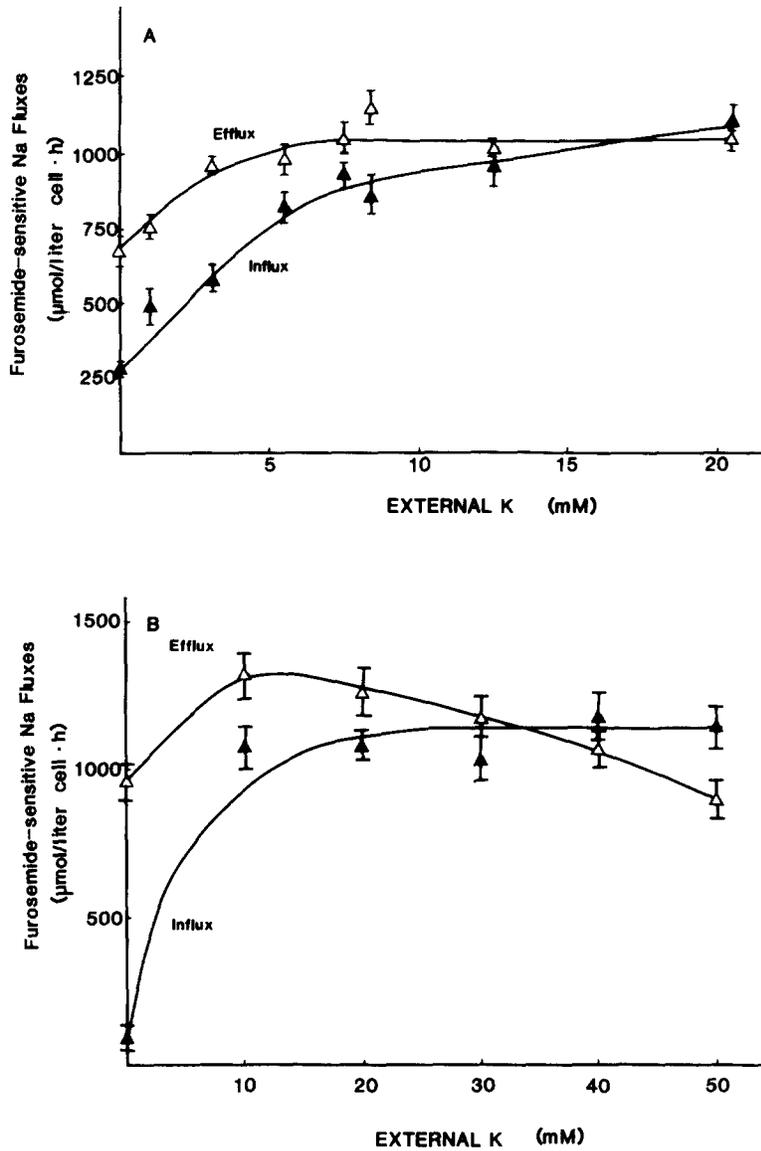


FIGURE 10. Simultaneous measurement of FS Na efflux and influx (\pm SE), as a function of external K, in (A) cells containing 80 (Na) and 12 (K) mmol/liter cell (subject C.B.) and (B) cells containing 76 (Na) and 26.6 (K) mmol/liter cell (subject A.K.). The intracellular content was varied with the nystatin loading procedure. The cells were incubated in media containing (mM): 120 NaCl (100 for B), 0–30 KCl and 30–0 choline Cl, 1 MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the flux media was 2% for the efflux and 5% for the influx. ²²Na was used as a tracer. The experiment in A was repeated in one other subject and the experiment in B was repeated in the other two subjects.

external Na and K concentrations that were not significantly different from those required to bring net FS Na flux to zero. As in the case of the FS Na fluxes, when the cellular Na content was increased, higher concentrations of external K were needed to bring the FS K fluxes to equilibrium.

Stoichiometry of FS Na and K fluxes at the equilibrium position. Table IV shows the values of the four unidirectional Na and K fluxes in cells with low, normal, and high Na content. The measured stoichiometric ratio of FS Na and K fluxes averaged 2:3 at the equilibrium position of FS Na fluxes. The experimentally measured stoichiometry fits with the 2 Na:3 K model estimated from the dependence of the equilibrium position of FS Na transport on internal and external Na and K concentrations (Table III).

TABLE V
Equilibrium of the FS Na Fluxes at High Cellular Na

$$n\text{Na}_o + m\text{K}_o \rightleftharpoons n\text{Na}_i + m\text{K}_i$$

$$K_{\text{eq}} = \left(\frac{\text{Na}_i}{\text{Na}_o} \right)^n \left(\frac{\text{K}_i}{\text{K}_o} \right)^m$$

Sub- ject	Cation composition				Equilibrium constant at Na:K ratio				
	Na _i	Na _o	K _i	K _o	$\frac{n}{m}$ 3:1	$\frac{n}{m}$ 3:2	$\frac{n}{m}$ 2:1	$\frac{n}{m}$ 1:1	$\frac{n}{m}$ 2:3
	mM	mM	mM	mM					
C.B.	133	120	1	3	0.45	0.15	0.41	0.37	0.045
	114	120	17	15	0.97	1.10	1.02	1.08	1.31
	104	120	34	30	0.74	0.84	0.85	0.98	1.09
D.C.	119	120	14	15	0.91	0.85	0.92	0.92	0.80
	106	120	31	40*	0.53	0.41	0.60	0.68	0.36
A.K.	109	100	38	35	1.41	1.53	1.29	1.18	1.52
	Mean ± SD				0.85±0.31	0.81±0.45	0.85±0.28	0.87±0.27	0.85±0.52
	Coefficient of variation (%)				37	55	33	31	61

* The point was extrapolated from the efflux slope.

FS Na and K Fluxes in High-Na Cells

FS Na fluxes in high-Na, low-K cells. We further tested the hypothesis that the equilibrium position for inward and outward FS Na fluxes allows an estimate of the stoichiometry of the transport reaction. Red cells were made to contain Na as the main cation and cell K was varied from 0 to 22 mmol/liter cell. These cells were incubated in media containing 120 mM NaCl and 0–30 mM KCl for the measurement of FS Na and K fluxes. Under these experimental conditions, Na and K concentration gradients across the cell membrane are small compared with the case in which high-K cells are incubated in high-Na, low-K media.

When the cellular K content was 12 and 22 mmol/liter cell (Fig. 10), there was net FS Na extrusion in the absence of external K. External K stimulated both FS Na efflux and influx, but net FS Na extrusion was reduced by external

K, because the FS Na influx was stimulated to a greater extent than FS Na efflux. The FS Na fluxes approached equilibrium at 15 mM K_o in cells containing 12 mmol K/liter cell and at 30 mM K_o in cells containing 22 mmol K/liter cell. Thus, more external K was required to bring the FS Na fluxes to equilibrium when internal K was increased.

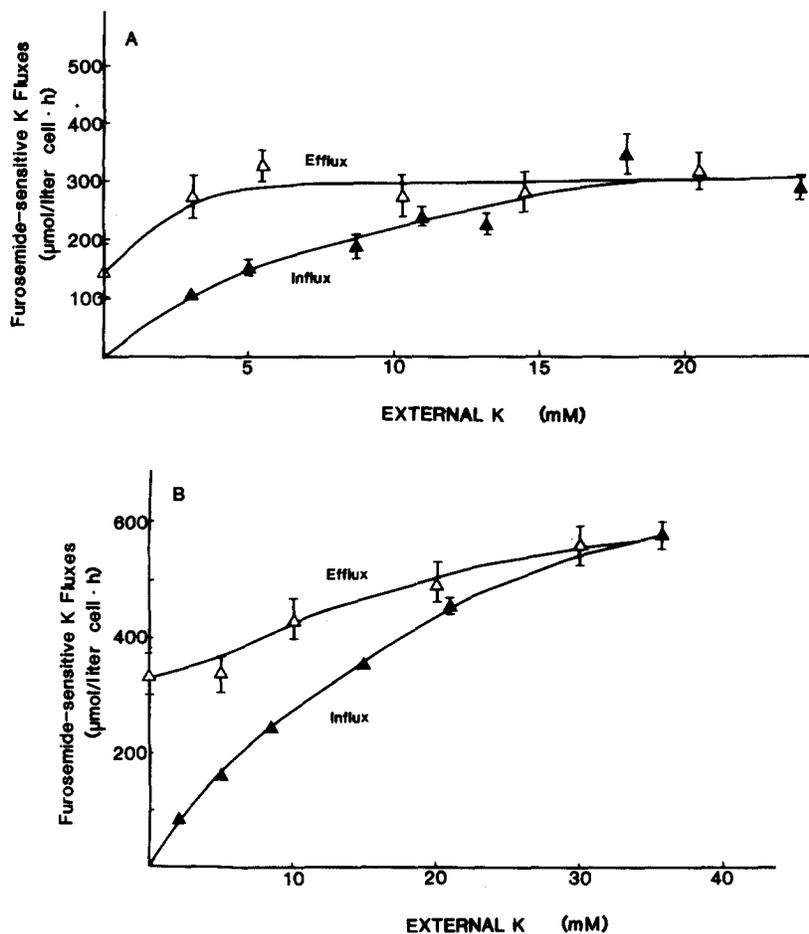


FIGURE 11. Simultaneous measurement of FS K efflux and influx (\pm SE), as a function of external K in (A) cells containing 84 (Na) and 10 (K) mmol/liter cell (subject C.B.) and (B) cells containing 73 (Na) and 22 (K) mmol/liter cell (subject D.C.). The intracellular content was varied with the nystatin loading procedure. The cells were incubated in media containing (mM): 120 NaCl, 0–30 KCl and 30–0 choline Cl, 1 MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, 10 glucose, and 0.1 ouabain, with and without 1 furosemide. The hematocrit of the flux media was 2% for the efflux and 5% for the influx. ⁴²K was used as a tracer. Each experiment was repeated in the other subject with similar results.

Several stoichiometric ratios of FS Na and K fluxes were tested to calculate the apparent equilibrium constant for a cotransport reaction (Eq. 1). Some of these models are shown in Table V. It can be seen that the coefficient of variation was lower (31%) for 1 Na:1 K, 2 Na:1 K (33%), and 3 Na:1 K (37%), and was higher for 2 Na:3 K (61%) and for 3 Na:2 K (55%).

FS K fluxes in high-Na, low-K cells. Fig. 11 shows the behavior of FS K fluxes in high-Na, low-K cells (10 and 21 mmol K/liter cell). Increasing external K stimulated both FS K efflux and influx. Internal K not only stimulated FS K efflux, but also *trans*-stimulated the FS K influx. The V_{max} of the FS K influx increased from 450 to 970 $\mu\text{mol/liter cell}\cdot\text{h}$ when the internal K was raised from 10 to 21 mmol/liter cell. This *trans* effect was similar to the stimulation by internal Na of the FS Na influx observed in high-K, low-Na cells.

TABLE VI
Stoichiometry of FS Na and K Fluxes at the Equilibrium Position in High-Na Cells

Subject	K_o	Na_o	K_o	FS fluxes					
				Na efflux	K efflux	Na/K ratio	Na influx	K influx	Na/K ratio
				$\mu\text{mol/liter cell}\cdot\text{h}$			$\mu\text{mol/liter cell}\cdot\text{h}$		
C.B.	12	80	15	1,020 \pm 32	300 \pm 40	3.4	1,000 \pm 50	290 \pm 30	3.4
	24	76	30	1,000 \pm 85	350 \pm 80	2.9	930 \pm 65	390 \pm 20	2.4
D.C.	10	84	15	700 \pm 40	350 \pm 50	2.0	710 \pm 90	370 \pm 25	1.9
	22	73	40	700*	550 \pm 40	1.3	700 \pm 40	580 \pm 30	1.2
				Mean \pm SD		2.4 \pm 0.8			2.2 \pm 0.8

The values reported for the fluxes are those at the equilibrium point between efflux and influx.

* This point was extrapolated from the efflux slope.

The external K concentration at which the FS K fluxes reached equilibrium was a function of the internal K concentration (Fig. 11). The Na and K concentrations at which the FS K fluxes reached equilibrium were similar to those required for the FS Na fluxes (compare Figs. 10 and 11).

Stoichiometry of the FS Na/K fluxes in high-Na, low-K cells. Table VI shows the measured stoichiometry of the FS Na and K fluxes at their equilibrium positions. It can be seen that the experimentally measured stoichiometry in high-Na cells varied between 3 Na:2 K and 3 Na:1 K. These results indicate that the stoichiometry of the FS fluxes is variable with the internal Na and K contents.

DISCUSSION

Net Uphill Na Extrusion Through the FS Pathway

The small size of the FS Na fluxes in human red cells made it difficult to demonstrate net FS Na loss under physiological ionic conditions ($Na_i = 10$ nM; $K_i = 140$ nM; $Na_o = 140$ nM; $K_o = 4$ mM; $Cl_i/Cl_o = 0.7$). The simultaneous

measurement of FS ^{22}Na efflux and influx has provided experimental evidence that in human red cells incubated in media not containing bicarbonate, there is net uphill FS extrusion of Na when the K and Na concentrations (and the Cl distribution ratio) are in the physiological range.

In the absence of external K, there was maximal net uphill FS extrusion of Na (Fig. 8). External K produced a slight stimulation of the FS Na efflux and a large stimulation of the FS Na influx. The rate of net FS Na extrusion in the presence of 4 mM external K markedly increased with the increase of cell Na from 4 to 34 mM (Figs. 5–8).

The present results confirm an early finding of Hoffman and Kregenow (1966), which suggested the presence of a second pump in human red cells that can perform net Na extrusion in the presence of ouabain. Sachs (1971) also showed a ouabain-insensitive FS net Na efflux when cells were incubated for 18 h in a medium containing only a slightly higher Na concentration than was present in the cells. Beaugé (1975) showed that OR net Na gain decreased when cell Na was increased. Measurements of net fluxes over 16 h performed by Wiley and Cooper (1974) showed that in the presence of ouabain, the net Na gain was increased by 5 mM K in the medium. Garay et al. (1981) showed that cells loaded with 100 mmol/liter cell of Na perform an OR-FS Na extrusion when incubated into 140 mM NaCl medium. Canessa et al. (1981) showed that at $K_o = 0$, in cells loaded with equal amounts of Na and K, there was an FS outward uphill movement of Na driven by the outward K gradient. Duhm and Göbel (1984) also showed that in fresh human red cells incubated for 24 h in a plasma-like medium, furosemide accelerated Na gain and retarded K loss.

The operation of this second Na pump appears to be coupled to the dissipation of the outward K gradient, as expected for a cotransport system. An increase of external K reduced or abolished the FS net Na extrusion (Fig. 8).

The net FS net Na extrusion was also Cl dependent (Table II). The replacement of Cl with nitrate greatly reduced OR-FS Na efflux into Na plus K medium, as reported by Chipperfield (1981). In contrast to the effect on the Na efflux, nitrate greatly increased the OR-FS Na influx. This result is in agreement with the findings of Funder and Wieth (1967). The latter authors showed a 36% stimulation of OR Na influx by nitrate, compared with Cl in media containing 22 mM bicarbonate. In bicarbonate-free media, we found a 72% stimulation by nitrate, compared with Cl, and this stimulation was completely inhibited by DIDS. Evidently, more work is needed to explain the effect of nitrate on the Na permeability.

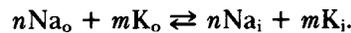
The operation of this second Na pump is coupled to metabolism. Hoffman and Kregenow (1966) reported that 8 h of metabolic depletion did not block the second pump, but it did inhibit the OS Na extrusion. Furthermore, Dagher et al. (1985) showed that when red cells were starved for >12 h and the ATP content was reduced below 100 $\mu\text{mol/liter}$ cell, the FS net Na extrusion was inhibited.

Equilibrium Properties and Stoichiometry of FS Fluxes in High-K Cells

The external K concentration required to equilibrate FS Na efflux with influx was a function of the internal Na concentration. At low internal Na, the fluxes

were at equilibrium at 7 mM K_o . At physiological cell Na, the fluxes equilibrated at 12 mM K_o . At 22 mmol/liter cell of Na_i , 18 mM K_o was needed to bring the transport of Na to equilibrium.

From an analysis of the equilibrium position of the FS Na fluxes, it is possible to distinguish between several different models for the FS transport reaction, with each model having different stoichiometric coefficients (Tosteson, 1981). We have calculated the apparent equilibrium constant, K_{eq}^{app} , for several stoichiometries of a cotransport reaction of the form:



An important assumption underlying this approach is that the reaction describes all net FS Na and K movement. 1:1 exchange of Na and/or K that is furosemide sensitive does not invalidate the approach. The results of some of these calculations are shown in Table III. These results show that the 2 Na:3 K model yields the lowest variance of K_{eq}^{app} calculated from three different sets of internal and external concentrations, in the red cells of each of three different subjects. The experimentally measured ratio of Na to K unidirectional FS fluxes at equilibrium also agrees with the 2 Na:3 K model in high-K cells (Table IV).

The "apparent" equilibrium constant for a transport reaction with this stoichiometric ratio can be written

$$K_{eq}^{app} = (Na_i/Na_o)^2 \times (K_i/K_o)^3.$$

In the three subjects studied, the K_{eq}^{app} varied between 14 and 29, which is within the limits of error for the experimental protocol of this study (Table III). This estimate of K_{eq}^{app} differs from the K_{eq}^{app} for a 1:1 stoichiometric ratio expected for a tightly coupled Na/K cotransport. Thus, these results suggest that other types of ion movement with different coupling ratios may operate in parallel with the Na/K cotransport. In the following paper (Canessa et al., 1986), we provide experimental evidence for the operation of a K_i/K_o exchange pathway, in parallel with the Na/K cotransport, under similar ionic conditions.

In this formulation of the transport reaction, we did not include Cl, because the Cl concentration was kept constant in all of the experiments used to calculate K_{eq}^{app} . Furthermore, because of the rapid rate of facilitated Cl exchange in human red cells, and the fact that such exchange is furosemide sensitive, it is difficult to measure the Cl fluxes that are part of the Na/K cotransport system. Therefore, it is not possible to compare the stoichiometry derived from calculations of K_{eq}^{app} with the stoichiometry estimated from flux measurements. It is feasible and will be of interest to measure the effect of varying the ratio of internal to external Cl concentration on the values of K_{eq}^{app} for the Na/K cotransport reaction as formulated here.

Stoichiometry and Equilibrium Properties of FS Fluxes in High-Na Cells

Another set of experiments was performed to study the behavior of the FS Na and K fluxes in the absence of the large inward Na and outward K gradients normally present in high-K, low-Na cells. The equilibrium position for inward and outward FS fluxes and the stoichiometry of FS Na and K fluxes were studied

in cells that contained high Na (90–70 mmol/liter cell) and low K (0–22 mmol/liter cell) and were incubated in media with 120 mM NaCl and 0–30 mM KCl.

In the absence of external K, net FS Na extrusion into 120 mM NaCl medium occurred in the range of cell K content tested (0–22 mmol/liter cell). External K stimulated FS Na influx more than FS Na efflux (Fig. 10), so that FS Na fluxes reached equilibrium. The external K concentration at which the FS Na efflux was equal to the FS Na influx was a function of the cell K content (Fig. 10 and Table V). Different stoichiometric models were tested for the FS Na/K cotransport reaction, as was done above in high-K, low-Na cells. Unfortunately, the larger error of the fluxes in high-Na cells did not allow an accurate determination of the stoichiometry, as was possible in high-K cells (see Table V).

Measurements of FS K fluxes under similar conditions showed that both FS Na and K fluxes reached equilibrium at similar sets of internal and external Na and K concentrations (Fig. 11). The stoichiometry estimated from measurements of the fluxes was in agreement with a 3 Na:1 K or 3 Na:2 K model (Table VI).

These results suggest that the stoichiometric ratio of the FS Na and K fluxes at equilibrium varies with the concentrations of Na and K in cells and medium. We show in the following paper (Canessa et al., 1986) that there are different modes of FS Na and K transport (inward and outward cotransport of Na and K, Na_i/Na_o and K_i/K_o exchange, and uncoupled K and Na efflux). According to the internal and external Na and K concentrations, each of these modes contributes to a different extent to the total FS Na or K movement. Therefore, the different stoichiometries of FS Na and K transport observed under different experimental conditions can be interpreted as resulting from the different ratios of some of these modes.

Several other studies have provided measurements of FS or bumetanide-sensitive Na and K fluxes under a variety of internal and external Na and K ratios, most of them not at equilibrium. Table VII summarizes various studies reporting the stoichiometry of FS Na and K fluxes. Different methods were used to obtain the data reported in Table VII. Simultaneous measurements of unidirectional Na, K, and Cl influxes were performed in squid axon (Russel, 1983). The degree of cooperativity for binding affinities of unidirectional Na, K, and Cl influxes was determined in MDCK cells (McRoberts et al., 1982). The unidirectional Na, K, and Cl influxes were measured in Ehrlich tumor cells (Geck et al., 1980). The net Na, K, and Cl fluxes were measured in duck red cells (Haas et al., 1982). Dunham et al. (1980) and Ellory et al. (1982) measured the stoichiometry of inward Na/K cotransport in human red cells under different experimental conditions (using media with 100 NaCl and 0–50 KCl for the K influx and with 100 KCl and 0–50 NaCl for the Na influx). Our results provide evidence that the stoichiometry of the FS Na and K fluxes in human red cells is not a fixed 1:1 stoichiometry, but is determined by the Na and K concentrations on both sides of the red cell membrane.

Physiological Functions of Na/K/Cl Cotransport

Inward Na/K cotransport has been reported to be present in many cells, such as the MDCK line (McRoberts et al., 1982), Henle's loop (Greger and Schlatter, 1981), flounder intestine (Mush et al., 1982), glial cells (Johnson et al., 1982),

Ehrlich ascites tumor cells (Geck et al., 1980; Geck and Heinz, 1980), squid axon (Russel, 1983), shark rectal glands (Hannafin et al., 1983), vascular smooth muscle (Kreye et al., 1981), and endothelial cells (Brock et al., 1984).

There are several possible physiological roles for the coupled movement of Na, K, and Cl. Such a system can use the electrochemical potential gradient of one of the three partners to pump one or both of the other partners. Thus, the system can function as an Na, K, or Cl pump, depending on the relative concentration of the ions on the two sides of the membrane. Some of the systems in which Na/K/Cl cotransport performs as an Na pump under physiological conditions include human red cells (present paper) and kidney slices (Whittembury and Grantham, 1976). Examples of cells in which the cotransport acts as a Cl pump include the shark rectal gland (Silva et al., 1977; Hannafin et al. 1983) and the thick ascending limb of Henle's loop of rabbit kidney (Greger and

TABLE VII
Stoichiometry of the Na, K, and Cl Fluxes Through the Na/K/Cl Cotransport

Reference	Cells	Stoichiometry		
		Na	K	Cl
Dunham et al. (1980)	Human red cells	1	5	—
Geck et al. (1980)	Ehrlich cells	1	1	2
Garay et al. (1981)	Human red cells	1	1	
Greger and Schlatter (1981)	Rabbit kidney	1	1	2
Haas et al. (1982)	Duck red cells	1	1	2
McRoberts et al. (1982)	MDCK cell line	1	1	2
Mush et al. (1982)	Flounder intestine	1	1	1.5
Russel (1983)	Squid axon	2	1	3
Brock et al. (1984)	Vascular endothelium	1	3	—
Canessa et al. (1983), Brugnara et al. (1986)	Human red cells	2	3	—
Brugnara et al. (1986)	Human red cells	3	2 (1)	—

Schlatter, 1981; Koenig et al., 1983). Because the Na/K/Cl cotransport produces net solute movement, it also can play a role in volume regulation (Kregenow, 1971; Schmidt and McManus, 1977a) and in the movement of fluid across epithelia.

In summary, our results indicate that in human red cells, there is FS net extrusion of Na under physiological conditions. The FS net Na extrusion is regulated by internal Na and external K concentrations. The stoichiometry of the FS fluxes in the physiological range of operation is 3 K:2 Na.

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