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Measurement and Stoichiometry of Bumetanide-Sensitive (2Na : 1K : 3Cl) Cotransport in Ferret Red Cells

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Summary. The bumetanide-sensitive uptake of Na^+, $K^+(Rb^+)$ and Cl^- has been measured at 21°C in ferret red cells treated with (SITS + DIDS) to minimize anion flux via capnophorin (Band 3). During the time course of the influx experiments tracer uptake was a first-order rate process. At normal levels of external Na^+ (150 mM) the bumetanide-sensitive uptake of K^+ was dependent on Cl^- and represented almost all of the K^+ uptake, the residual flux demonstrating linear concentration dependence. The uptake of Na^+ and Cl^- was only partially inhibited by bumetanide indicating that pathways other than (Na + K + Cl) cotransport participate in these fluxes. The diuretic-sensitive uptake of Na^+ or Cl^- was, however, abolished by the removal of K^+ or the complementary ion indicating that bumetanide-sensitive fluxes of Na^+, K^+ and Cl^- are closely coupled. At very low levels of $[Na]_o$ (< 5 mM) K^+ influx demonstrated complex kinetics, and there was evidence of the unmasking of a bumetanide-sensitive Na^+-independent K^+ transport pathway. The stoichiometry of bumetanide-sensitive tracer uptake was 2Na : 1K : 3Cl both in cells suspended in a low and a high K^+-containing medium. The bumetanide-sensitive flux was markedly reduced by ATP depletion. We conclude that a bumetanide-sensitive cotransport of (2Na : 1K : 3Cl) occurs as an electroneutral complex across the ferret red cell membrane.

Key Words sodium-potassium chloride cotransport · red blood cells · bumetanide · stoichiometry · ferret

Introduction

It is well established that carrier-mediated transport of Na^+ and K^+ in red cells continues in the presence of the Na^+/K^+ pump inhibitor, ouabain (e.g. Hoffman & Kregenow, 1966). A number of transport systems have been shown to participate in these ouabain-insensitive fluxes, and their characterization has relied, to a large extent, on the use of selective inhibitors. Perhaps the best known of these agents are the high-ceiling "loop" diuretics, including furosemide, and the more potent bumetanide (Wiley & Cooper, 1974; Ellory & Stewart, 1982). These compounds have been found to inhibit a substantial fraction of ouabain-insensitive Na^+ and K^+ transport in a wide variety of cell types (for review, *see* Ellory et al., 1982). Another important feature of the diuretic-sensitive pathway is its dependence on Cl^-, as replacement of this anion by, for example NO_3^-, reduces ouabain-insensitive Na^+ and K^+ cotransport to the same level as that seen in the presence of furosemide (Chipperfield, 1980; Dunham et al., 1980). The role of Cl^- is, however, unclear. A central question is whether Cl ions move across the membrane with Na^+ and K^+ or whether the anion is acting merely at an activator site.

Evidence obtained from a variety of sources suggests that Cl^- is indeed transported with the cations as a diuretic-sensitive electroneutral complex (Geck et al., 1980; Haas et al., 1982). Direct evidence of diuretic-sensitive Cl^- transport has not yet been obtained in mammalian red cells due to the presence of the Band 3 anion exchange system, capnophorin. This protein mediates massive Cl^--HCO_3^- exchange (>70 mol/liter cells · min at 37°C). (Lambert & Lowe, 1980) and this totally obscures any Cl^- flux which might be associated with the cotransport system. Despite the availability of the highly potent stilbene-derivative anion transport inhibitors, SITS and DIDS (Knauf & Rothstein, 1971), following treatment with these compounds the residual Cl^- flux (about 50 mmol/liter cells · hr) is still too large compared to cotransport (0.5 mmol/liter cells · hr, at 37°C) in human red cells.

Recently, however, red cells from the ferret (*Mustela putoris furo L.*) which in common with dog red cells (Parker, 1977) lack the Na^+/K^+ pump, have been shown to possess an enormous capacity for Na^+ and K^+ transport through a bumetanide-sensitive pathway (Flatman, 1983). Indeed, the diuretic-sensitive transport of cations in these cells is more than an order of magnitude greater than in human red cells. Thus the possibility arose that ferret red cells treated with SITS and DIDS may demonstrate a detectable bumetanide-sensitive Cl^- flux

associated with the cotransport of Na^+ and K^+. At normal body temperatures (39°C in the ferret) the Cl^- flux remaining after (SITS + DIDS) treatment was found to be still too large, and so we took advantage of the higher temperature coefficient of capnophorin compared to (Na + K) cotransport (Brahm, 1977; Stewart et al., 1980), by working at 21°C. Under these conditions we detected a significant bumetanide-sensitive Cl^- flux coupled to bumetanide-sensitive Na^+ and K^+ transport.

The experiments described in the present study show that the stoichiometry of unidirectional radiotracer influxes is 2Na : 1K : 3Cl, and therefore the flux is electroneutral. A preliminary account of some of this work has already been presented (Ellory & Hall, 1984).

Materials and Methods

Blood

Blood was collected into heparinized syringes from adult male and female, fitch and albino ferrets (500 to 1100 g) anesthetized by halothane. Red cells were washed three times by centrifugation and aspiration in ice-cold medium of the following composition (mM): NaCl 150; KCl 10; HEPES or MOPS 15; glucose 10; (pH 7.4 at 21°C), and the buffy coat removed. Experiments were performed only on erythrocytes collected the same day, the cells being stored at 5°C until the start of the flux studies. The incubation temperature used throughout this study was 21° ± 0.5°C with the hematocrit in the range 8 to 12%. The exact hematocrit was determined using the cyanmethemoglobin method as described by Flatman and Andrews (1983).

(SITS + DIDS) Treatment

Fresh red cells at about 10% hematocrit were washed in the above incubation medium containing 0.05 mM SITS or DIDS three times each by centrifugation and aspiration. The cells were then incubated for 30 min at 21°C in SITS-containing medium and centrifuged. The supernatant was aspirated, and then the cells were treated in the DIDS medium in the same manner. Finally, the cells were washed three times alternately in SITS and DIDS media before the erythrocytes were ready for the tracer flux studies.

This procedure was found not to alter the Na^+, K^+ or Cl^- contents of fresh ferret red cells. Ion content was determined as described (*see below*) before and after SITS and DIDS treatment. In three separate animals the Na^+, K^+ and Cl^- content of fresh ferret red cells was 143 ± 6.4, 6.2 ± 0.4 and 125 ± 4.3 mM/liter cell water, respectively. In the same donors' cells following (SITS + DIDS) treatment these values were unchanged (Na^+, 146 ± 8.2; K^+, 6.0 ± 0.5; and Cl^-, 128 ± 6.3 mM/liter cell water).

Tracer Uptake

Radioisotope influx in most experiments was studied using ^{22}Na, ^{86}Rb as a tracer for K^+, and ^{36}Cl. As the flux times for these experiments were short, the radioactivity of the flux medium was high at about 20 μCi/ml for the cations, and at about 10 μCi/ml for ^{36}Cl. In one experiment, ^{42}K was introduced in addition to ^{86}Rb and bumetanide-sensitive uptake compared. As no difference was noted between the fluxes calculated using the two isotopes, the latter would appear to be a satisfactory tracer for K^+ in these experiments. For most flux studies, cells treated with SITS and DIDS as described above were suspended at about 10% hematocrit in the following standard flux medium (mM): NaCl 150; KCl 10; HEPES or MOPS 15; glucose 10; EGTA 0.05; SITS 0.05; DIDS 0.05 (pH 7.4 at 21°C). Uptake was studied in the presence and absence of a maximally inhibiting dose of bumetanide (0.1 mM) (*see* Fig. 2), the difference between these fluxes representing the bumetanide-sensitive influx.

In a series of experiments (Figs. 3 and 4) the dependence of Na^+ and K^+ uptake on external Na^+ and K^+, respectively, was investigated. For Na^+ uptake studies a medium of the following composition was used (mM): NMDG-Cl (50 to 100); KCl 50; NaCl (0 to 50); glucose 10; HEPES 15; EGTA 0.05; SITS 0.05; DIDS 0.05 (pH 7.4 at 21°C). For K^+ influx experiments NaCl was maintained at 50 mM. When K^+ uptake was studied with varying $[Na]_o$ (Fig. 5), the values of $[K]_o$ are given in the figure legend. When $[Na]_o$ or $[K]_o$ were altered, NMDG was used to maintain isotonicity, with appropriate correction for changes in pH. For some other studies (Table 2) bumetanide-sensitive uptake was determined in a high K^+-containing medium of the following composition (mM): $NaCH_3SO_4$ 75; KCl 75; HEPES 15; glucose 10; EGTA 0.05; SITS 0.05; DIDS 0.05 (pH 7.4 at 21°C with Tris base).

When the influence of Cl^- removal on bumetanide-sensitive cation fluxes was investigated (Table 1) $MeSO_4^-$ was used as the replacement anion. The incubation media used in these studies were made using the following constituents as appropriate (mM): Na (Cl or $MeSO_4$) 130; NMDG Cl or KCl 25; HEPES 15; glucose 10; EGTA 0.05; SITS 0.05; DIDS 0.05 (pH 7.4 with Tris base or HCl at 21°C; addition of the latter raised the $[Cl]_o$ by about 5 mM). When anion replacement was required the method of Dunham et al. (1980) was employed. $MeSO_4^-$ has been shown to be a suitable chloride replacement ion for studies on cotransport in human red cells (Dunham et al., 1980).

The cell suspension was equilibrated at the experimental temperature (21°C) for 10 min after which the flux was started by the addition of a small volume of the appropriate isotope and the suspension mixed thoroughly. As soon as possible after this (usually after 20 sec) triplicate samples (0.2 ml) of cell suspension were decanted into Eppendorf microcentrifuge tubes containing 0.4 ml of the oil di-*n*-butylphthalate, and 0.9 ml of ice-cold incubation medium. The tubes were centrifuged immediately (10,000 × *g*, 20 sec) thus separating the cells from the aqueous phase, and timing for the flux was begun. The isotope activity in the cells was determined by aspirating off the supernatant and most of the oil, and processing the cell pellet as previously described (Hall & Willis, 1984). After protein precipitation with TCA, an aliquot of the supernatant was added to 3 ml of scintillation fluid (Picofluor 30, Packard) and radioactivity measured in a Packard β scintillation spectrometer.

The specific activity of the flux medium used during each experiment was determined (a) by measuring the radioactivity of samples of supernatant from the actual cell suspensions, and (b) the measured concentrations of $[Na]_o$, $[K]_o$ and $[Cl]_o$ immediately after the flux was completed.

An attempt was also made to measure bumetanide-sensitive fluxes using the more conventional technique of washing cells at the end of the flux by aspiration and centrifugation (*see* Young & Ellory, 1982) with ice-cold SITS medium. In two experiments the

bumetanide-sensitive fluxes of Na^+ and K^+ were in good agreement with those determined by the oil separation method described above.

ATP Depletion

Cellular ATP levels were reduced by incubating fresh-washed ferret red cells at 39°C (5% hematocrit) for 22 hr in a medium of the following composition (mM): NaCl 160; KCl 10; MOPS 15 (pH 7.4). ATP levels were determined using the luciferin-luciferase method of Brown (1982).

Measurement of $[Na]_i$, $[K]_i$ and $[Cl]_i$

Intracellular and extracellular concentrations of Na^+ and K^+ were measured by flame photometry (Evans Electroselenium Ltd.). Intracellular cations were determined by washing cells quickly in ice-cold medium of the following composition (mM): $MgCl_2$ 106; Tris 10; (pH 7.4 at 5°C). The cells were then lysed in distilled water and the Na^+ and K^+ concentrations estimated. Extracellular concentrations of cations were measured in samples of supernatant following centrifugation of the cell suspension (10,000 × g, 20 sec). $[Cl]_i$ was measured by determining the chloride distribution ratio (r) using ^{36}Cl (*see* Flatman & Lew, 1980). The external chloride concentration was measured by chloridometry (CMT 10 chloride titrator, Radiometer, Copenhagen). Cell water (f_w) (in units of liters of water per liter of cells) was estimated by drying samples of red cells to constant weight at 105°C, and correcting for the trapped extracellular space. $[Cl]_i$ is given by

$$[Cl]_i = f_w \frac{[Cl]_o}{r}.$$

Reagents and Isotopes

Analytical grade reagents were obtained from BDH Ltd., Poole, Dorset, and solutions prepared with glass-distilled water. EGTA, HEPES, MOPS, DIDS, Tris, NMDG and di-*n*-butylpthalate were purchased from Sigma Ltd., and SITS was the disodium salt from BDH Ltd. Sodium methylsulfate was obtained from ICN Pharmaceuticals Inc., Plainview, N.Y. Bumetanide was a gift from Leo Laboratories, Princes Risborough, Bucks., and was dissolved in distilled water with a little Tris base to give a final pH of about 7.0.

^{22}Na, ^{86}Rb and ^{36}Cl were obtained from Amersham International. Both $H^{36}Cl$ and $Na^{36}Cl$ were used, the acid being adjusted to approximately pH 7.0 with Tris base. ^{42}K was made by the Universities Research Reactor (Warrington, England) as $^{42}K_2CO_3$ which was neutralized with excess HCl, evaporated to dryness and dissolved in a small volume of distilled water to give approximately 2 mCi/ml.

Abbreviations

SITS: 4-acetamido-4′-iso-thiocyanatostilbene-2,2′-disulfonic acid disodium salt, DIDS: 4,4′-diisothiocyanato-2,2′-stilbene disulfonate, HEPES: N-2-hydroxyethylpiperazine N′-2-ethane-sulfonic acid, MOPS: 4-morpholinepropanesulfonic acid, Tris: tris-(hydroxymethyl) aminomethane, EGTA: ethyleneglycol-*bis*-(β-aminoethylether)-N,N,N′,N′,-tetraacetic acid, TCA: trichloroacetic acid, NMDG: *n*-methyl D-glucamine, $MeSO_4^-$: methylsulfate.

$[X]_i$ and $[X]_o$ represent intracellular and extracellular concentrations, respectively, of the solute X; X_i and X_o indicate intracellular and extracellular X without reference to concentration. k represents the rate constants, $K_{1/2}$ the inhibitor concentration giving apparent half-maximal inhibition, K_m is the substrate concentration at apparent half-maximal flux (apparent Michaelis constant), V_{max} the apparent maximal velocity of the flux and α the proportionality constant (in units of mmol/liter cells · hr/mM) relating the slope of the nonsaturable component of cation influx to the external cation concentration.

Presentation of Data

Results are given as means (± SEM) of triplicate determinations on the blood of one animal unless a value n is given. N/S indicates not significantly different ($P > 0.01$), and the errors are not represented in the figures for those points where they are smaller than the symbols. Curves for the kinetic uptake data (except where otherwise stated) were fitted using a two-component model (*see* Dunham et al., 1980) representing saturable and nonsaturable components. The apparent kinetic constants K_m and V_{max} were computed as described in the Results.

Results

Time Course of Isotope Uptake in Ferret Red Cells

It was important initially to confirm that tracer uptake was a linear function with time during the course of the influx experiments. This was tested by observing influx of ^{22}Na, ^{86}Rb and ^{36}Cl in (SITS + DIDS)-treated ferret red cells with and without bumetanide. The results (Fig. 1) demonstrated that uptake of all three isotopes may be described by a first-order rate equation.

Further analysis of the data enables an estimation of the stoichiometry of bumetanide-sensitive uptake to be made. By multiplying the influx rate constant by the extracellular ion concentration, uptake in units of mmol/liter cells · hr is obtained (Hall & Willis, 1984). Thus for the data presented in Fig. 1, the bumetanide-sensitive uptake of Na^+, K^+ or Cl^- is 7.34, 3.94 and 11.75 mmol/liter cells · hr, respectively, yielding an apparent stoichiometry (taking $K^+ = 1.0$) of 1.9:1.0:3.0 (Na/K/Cl) (*see also* Table 2). As these data show that the uptake of the three ions demonstrated first-order kinetics over the time span investigated, (Fig. 1), in subsequent experiments a single time point could be taken, and this was usually after 10 min.

Bumetanide-sensitive tracer uptake always represented a significant fraction of total influx for that tracer. Quantitative differences in Cl^- transport be-

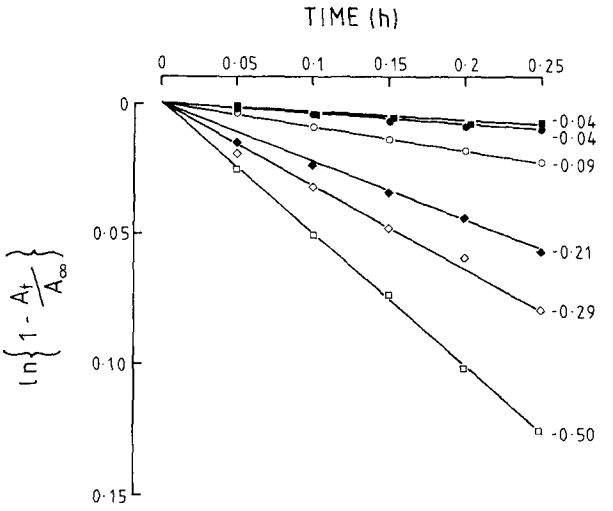

Fig. 1. Time course of tracer uptake in ferret red cells. Influx of Na^+, K^+ and Cl^- was studied in (SITS + DIDS)-treated cells suspended in the standard incubation medium in the presence (●, ■ and ♦, respectively), and absence (○, □ and ◇, respectively) of bumetanide (0.1 mM). Also shown are the rate constants for influx calculated using a first-order rate equation of the form $-kt = \ln(1 - A_t/A_\infty)$, where k = the rate constant of uptake (i.e., the slope of the linear regression), t = the time in hours after the first sample (*see* Materials and Methods), A_t = the intracellular radioisotope activity at time t, A_∞ the intracellular radioisotope activity at equilibrium and was calculated from the radioactivity in the incubation medium, the hematocrit, and the intracellular and extracellular concentrations of the appropriate ion (*see* Flatman, 1983)

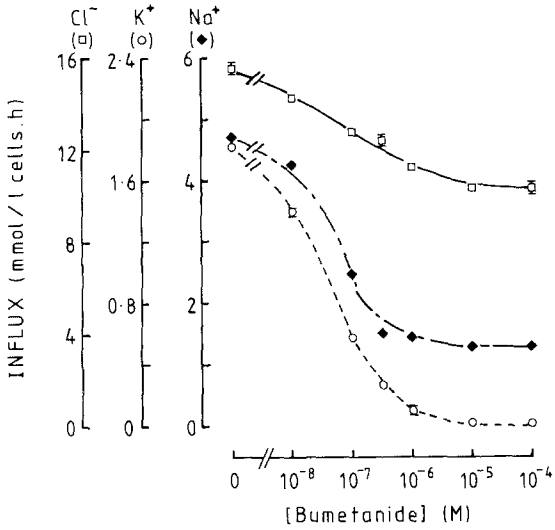

Fig. 2. Inhibition of Na^+, K^+ and Cl^- uptake by bumetanide. Influx of Na^+ (♦), K^+ (○) and Cl^- (□) was determined in fresh (SITS + DIDS)-treated ferret red cells suspended in the standard incubation medium as described (*see* Materials and Methods) at various concentrations of bumetanide. Note the different ordinates for the three ions. In this experiment the bumetanide-sensitive fluxes (at 0.1 mM) of Na^+, K^+ and Cl^- were 3.3, 1.8 and 5.3 mmol/liter cells · hr, respectively

tween donors was noted, and this was probably due to the differing extents to which the anion transport protein (Band 3) was inhibited by the (SITS + DIDS) treatment.

Properties of Bumetanide-Sensitive Tracer Uptake in Ferret Red Cells

The above results suggested that the influx of Na^+, K^+ and Cl^- shared a common (bumetanide-sensitive) carrier. It was important however, to study the kinetic properties of Na^+ and K^+ translocation through this pathway in an attempt to obtain further evidence of the relationship between the movement of these ions and their diuretic sensitivity.

Dose-Response Curves for Bumetanide Inhibition

If the three ions (Na^+, K^+ and Cl^-) shared a common transport system then one would expect the $K_{1/2}$ for bumetanide inhibition of uptake to be very similar. The results shown in Fig. 2 confirmed this expectation. Values for the $K_{1/2}$ of K^+, Na^+ and Cl^- influx were estimated to be 4, 3 and 2 × 10^{-7} M, respectively. For all three ions, inhibition was maximal at 10^{-5} M, and in another identical experiment similar values for the $K_{1/2}$ were obtained with no further inhibition of influx up to a bumetanide concentration of 5 × 10^{-4} M. In all further experiments, bumetanide at a concentration of 0.1 mM was used.

It can also be seen (Fig. 2) that at maximal bumetanide concentrations there was still a significant uptake of Na^+ and Cl^-, in contrast to K^+ influx which was almost totally abolished (*see below*).

Kinetic Aspects of Bumetanide-Sensitive Na^+ and K^+ Uptake

K^+ uptake vs. $[K]_o$. Figure 3 shows the effect of raising $[K]_o$ on K^+ influx in the presence and absence of bumetanide. Apparent kinetic constants were calculated by plotting $[S]/v$ against $[S]$, where $[S]$ is the substrate concentration ($[K]_o$ in these experiments), and v, uptake in units of mmol/liter cells · hr. The slope of the resulting graph is the reciprocal of the V_{max}, and the x-intercept, the negative value of the K_m (Cornish-Bowden, 1976).

Values for the K_m and V_{max} for bumetanide-sensitive K^+ uptake calculated from the data presented in Fig. 3 were 10.2 mM and 2.7 mmol/liter cells · hr, respectively. In the presence of bumetanide there

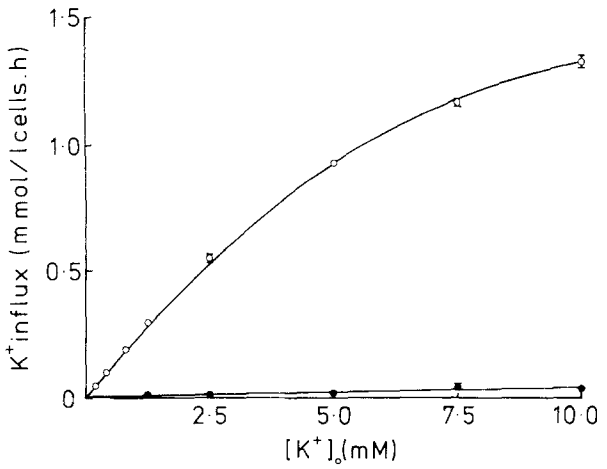

Fig. 3. Effect of raising $[K^+]_o$ on K^+ uptake in (SITS + DIDS)-treated ferret red cells. K^+ influx was studied in the presence (●) and absence (○) of bumetanide (0.1 mM). For these experiments K^+ was replaced by NMDG with $[Na]_o$ maintained at 50 mM. (For exact composition of incubation media *see* Materials and Methods.)

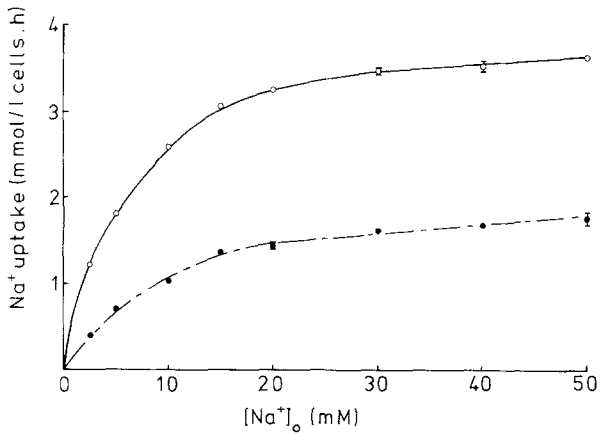

Fig. 4. Activation of Na^+ uptake by $[Na]_o$ in (SITS + DIDS)-treated ferret red cells. Uptake was monitored in the presence (●) and absence (○) of bumetanide (0.1 mM). Na^+ was replaced by NMDG and $[K]_o$ maintained at 50 mM. (For exact composition *see* Materials and Methods.)

was no evidence of a saturable K^+ flux, the linear function demonstrating a slope of 0.004 mmol/liter cells · hr/mM. These data therefore suggest that K^+ uptake under these experimental conditions occurs through two pathways, (1) a bumetanide-sensitive mechanism, and (2) a "passive" residual flow demonstrating linear concentration dependence.

Na^+ Uptake vs. $[Na]_o$. The dependence of Na^+ uptake on external Na^+ in the presence and absence of bumetanide is shown in Fig. 4. In contrast to K^+ fluxes (Fig. 3) a saturable flux remained in the presence of bumetanide. The K_m and V_{max} for the bumetanide-sensitive flux in this experiment were calculated to be 2.7 mM and 1.9 mmol/liter cells · hr. Treating the residual Na^+ flux (i.e., that remaining after bumetanide treatment) as the sum of saturable and linear components, the V_{max} was similar to the bumetanide-sensitive flux (2.1 mmol/liter cells · hr); however the $K_{1/2}$ was significantly higher (10 mM). The slope of the linear component was 0.006 mmol/liter cells · hr/mM and this is similar to that observed for "passive" K^+ uptake (Fig. 3).

K^+ Uptake vs. $[Na]_o$. If the stoichiometry of bumetanide-sensitive cation uptake is 2Na : 1K, then the activation of diuretic-sensitive K^+ uptake by Na^+ should be sigmoidal at low values of $[Na]_o$. Unfortunately, this is a difficult experiment to perform as ferret red cells possess a high $[Na]_i$ (*see* Materials and Methods) and therefore the loss of cell Na^+ during the incubation period may lead to a significant alteration in the $[Na]_o$ from the set value. Nevertheless, an example of the results from one of these experiments is presented (Fig. 5*a*) with the measured values of $[Na]_o$ in the supernatants given as the abscissa.

The final $[Na]_o$ of the "Na^+-free" incubation medium immediately after the flux period was 250 μM with a corresponding bumetanide-sensitive flux of 0.55 ± 0.008 mmol/liter cells · hr. Raising $[Na]_o$ increased the diuretic-sensitive flux in a saturable manner. In the presence of bumetanide, K^+ uptake was reduced, and this flux was independent of $[Na]_o$ remaining constant at about 0.65 mmol/liter cells · hr.

In red cells from another donor, higher values of $[Na]_o$ (Fig. 5*b*) almost completely saturated the bumetanide-sensitive pathway. The K_m and V_{max} were 21.5 mM and 2.24 mmol/liter cells · hr, respectively, and the resulting curve (*see* Materials and Methods) well described the experimental data. The addition of bumetanide abolished the saturable flux, and the residual K^+ uptake remaining unaffected by raising $[Na]_o$ at about 0.1 mmol/liter cells · hr.

As described in the Discussion, the contribution of this bumetanide-sensitive K^+ pathway with increasing levels of $[Na]_o$ is unknown and hence interpretation of these experiments is difficult. Although a meaningful kinetic analysis of the data obtained in low $[Na]_o$ media cannot be performed the gross form of the curve (Fig. 5*a*) in the absence of bumetanide does, however, suggest sigmoidicity, although the complexity of the experiment excludes a firm conclusion on this point. This is in contrast to the kinetic properties of the bumetanide-sensitive pathway present in media of higher Na^+ concentration where the data are satisfactorily fitted by a single saturable component which obeys Michaelis-Menten kinetics (Fig. 5*b*).

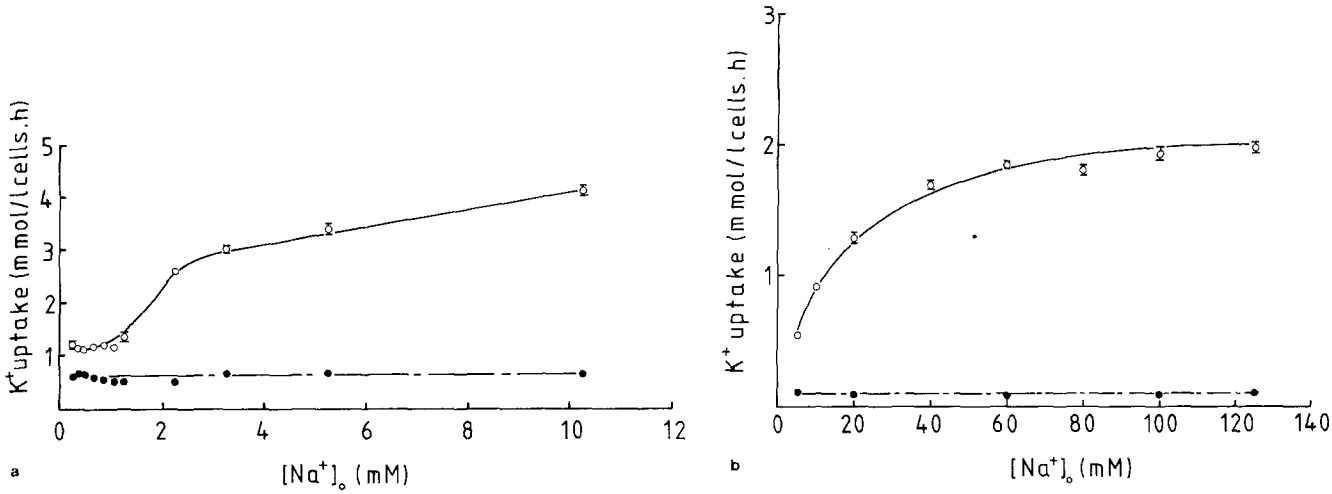

Fig. 5. (*a*) Effect of low $[Na]_o$ on bumetanide-sensitive K^+ uptake in (SITS + DIDS)-treated ferret red cells. Uptake was measured with (●) and without (○) bumetanide (0.1 mM), Na^+ was replaced by NMDG and $[K]_o$ was maintained at 50 mM. The exact level of $[Na]_o$ was determined by flame photometry (*see* Materials and Methods). The curve was drawn by eye, and in another identical experiment on blood from another animal similar results were obtained. (*b*) Effect of higher concentrations of external Na^+ on K^+ uptake in ferret red cells. The experimental conditions are the same as for Fig. 5*a*, except that $[K]_o$ was 25 mM and these data were obtained from the blood of a different donor

Table 1. Effect of anion replacement on bumetanide-sensitive Na^+ and K^+ transport in ferret red cells[a]

		Uptake (mmol/liter cells · hr)			
		Anion	Control	+ Bumetanide	Difference
Na^+ uptake:					
(i)	$[K^+]_o$ = 0 mM	Cl^-	1.055 ± 0.042	1.095 ± 0.058	N/S
		$MeSO_4^-$	1.200 ± 0.051	1.125 ± 0.031	N/S
(ii)	$[K^+]_o$ = 25 mM	Cl^-	3.406 ± 0.081	1.304 ± 0.019	2.1
		$MeSO_4^-$	1.360 ± 0.170	1.286 ± 0.074	N/S
K^+ uptake:					
	$[K^+]_o$ = 25 mM	Cl^-	1.277 ± 0.014	0.042 ± 0.002	1.2
		$MeSO_4^-$	0.077 ± 0.003	0.081 ± 0.004	N/S

[a] Cl^- replacement by methylsulfate ($MeSO_4^-$) was achieved as described in Materials and Methods, and radiotracer uptake in the appropriate flux medium (*see* Materials and Methods) determined at 21°C in the presence and absence of bumetanide (0.1 mM). K^+ was replaced by NMDG, (n = 3) and $[Na]_o$ was 130 mM.

Effect of Na^+, K^+ or Cl^- Substitution on Bumetanide-Sensitive Fluxes. The previous sections suggested that bumetanide-sensitive transport of Na^+, K^+ and Cl^- occurred through a common carrier although other pathways also mediate transport of these ions. The diuretic-sensitive flux should rely on the presence of all three ions and one would expect therefore that the removal of one of these ions should abolish the bumetanide-sensitive flux of the others.

First of all, the influence of Na^+ or K^+ replacement on bumetanide-sensitive Cl^- uptake was studied. In one experiment, Cl^- influx with $[Na]_o$ and $[Cl]_o$ = 150 mM, and $[K]_o$ = 10 mM was 55.02 ± 1.69 mmol/liter cells · hr, falling to 49.11 ± 0.93 with the addition of bumetanide. The control flux in a nominally Na^+-free medium ($[Na]_o$ about 250 μM; NMDG replacement) was reduced to 48.01 ± 1.32, and there was no further significant decrease in uptake with the addition of bumetanide (49.57 ± 0.96). The next series of experiments investigated the effect of K^+ removal on Cl^- influx. Cl^- uptake with $[Na]_o$, $[K]_o$ and $[Cl]_o$ at the same concentrations described above but in cells from another donor was 40.04 ± 0.66 mmol/liter cells · hr. The addition of bumetanide reduced this flux to 29.91 ± 0.55. In the

absence of K^+ (NMDG replacement) Cl^- uptake was 28.10 ± 0.14 and in the presence of bumetanide the flux was unaltered (28.55 ± 0.35).

These data therefore suggest that bumetanide-sensitive Cl^- uptake is dependent on both $[Na]_o$ and $[K]_o$.

The replacement of Cl^- by the substitute anion, $MeSO_4^-$, should have the same effect on Na^+ and K^+ fluxes in ferret red cells as bumetanide addition. The results on anion replacement are shown in Table 1. In the absence of external K^+ there was no bumetanide-sensitive Na^+ uptake whether Cl^- or methylsulfate was the dominant anion. When the $[K]_o$ was raised to 25 mM a bumetanide-sensitive Na^+ uptake was revealed. Replacement of Cl^- by methylsulfate abolished the bumetanide-sensitive component and therefore both K^+ and Cl^- have to be present in order to demonstrate a bumetanide-sensitive Na^+ flux.

K^+ uptake in ferret red cells suspended in a Cl^- medium was significantly reduced by bumetanide addition (Table 1). The absence of a diuretic-sensitive K^+ flux when Cl^- was replaced by methylsulfate indicates that this transport system is dependent on Cl^-. Notice, however, that bumetanide-insensitive Na^+ and K^+ uptake in a methylsulfate medium was marginally higher than in a Cl^- medium. Despite this, in the presence of methylsulfate, bumetanide addition did not further reduce Na^+ or K^+ uptake.

The Stoichiometry of Bumetanide-Sensitive Transport

Table 2 shows pooled data from nine different donors where the bumetanide-sensitive uptake of Na^+, K^+ and Cl^- was determined under two experimental conditions in a low and a high K^+-containing medium (*see* Materials and Methods for the composition). It can be seen that the stoichiometry of the bumetanide-sensitive isotope flux was the same (2Na : 1K : 3Cl) in both K^+ media.

In a further series of experiments, the influence of metabolic substrate depletion on the stoichiometry of bumetanide-sensitive Na^+ and K^+ fluxes was measured. Depletion was achieved as described (*see* Materials and Methods) and ferret red cell ATP levels were monitored. In one experiment the cellular ATP level in fresh cells was 0.41 mM, falling to 0.02 mM after incubation in the depleting medium. In fresh cells the bumetanide-sensitive uptake of Na^+ and K^+ was 2.30 ± 0.06, and 1.22 ± 0.02, respectively, and these values fell to 0.07 ± 0.01 and 0.03 ± 0.00, respectively, following depletion. Thus the bumetanide-sensitive flux is markedly inhibited by metabolic depletion; however the coupling ratio, within the accuracy of the measurements, remains at about 2Na : 1K under both conditions.

Table 2. Stoichiometry of bumetanide-sensitive Na^+, K^+ and Cl^- uptake in ferret red cells[a]

		Influx (mmol/liter cells · hr)		
	(*n*)	Na^+	K^+	Cl^-
Low $[K^+]_o$	6	4.46 ± 0.43	2.20 ± 0.22	7.20 ± 0.83
Stoichiometry (K^+ = 1)		2.05 ± 0.13	1.00	3.38 ± 0.44
High $[K^+]_o$	3	3.38 ± 0.25	2.16 ± 0.16	6.33 ± 0.46
Stoichiometry (K^+ = 1)		1.80 ± 0.21	1.00	2.94 ± 0.04

[a] Influx was studied as described (*see* Materials and Methods) in (SITS + DIDS)-treated cells at 21°C, in a low (10 mM) K^+-containing standard incubation medium, and a high (75 mM) K^+-containing medium (*see* Materials and Methods for exact composition). When present, bumetanide was used at 0.1 mM. The number of different donors is represented by *n*.

Discussion

The present results demonstrate that bumetanide-sensitive radiotracer fluxes of Na^+, K^+ and Cl^- may be measured in (SITS + DIDS)-treated ferret red cells at 21°C. Of particular interest is the association of the diuretic-sensitive Cl^- flux with those of Na^+ and K^+, and this deserves full discussion.

In ferret red cells under the experimental conditions described here, Na^+ and K^+ uptake occur via several pathways. At high values of $[Na]_o$, K^+ uptake is almost exclusively (>90%, *see* Fig. 3) mediated by the bumetanide-sensitive mechanism which is also dependent on the presence of Cl^- in the incubation medium (Table 1). In the presence of bumetanide, K^+ uptake demonstrated linear concentration dependence suggesting influx was mediated by the residual electrodiffusional "leak" (*see* Hall & Willis, 1984). These findings make the possibility that at 21°C K^+ uptake occurs through any other carrier-mediated transport with a reasonable affinity, for example, the Na^+/K^+ pump, unlikely.

Na^+ uptake is more complex, and is mediated by several systems. At normal $[K]_o$ (10 mM), approximately half of Na^+ influx is coupled to bumetanide-sensitive (K^+ + Cl^-) transport (Fig. 4, Table 1). The Na^+ flux remaining after bumetanide treatment represents at least two components. The flux is clearly insensitive to bumetanide (Figs. 2 and 4)

and persists after Cl^- substitution (Table 1) by methylsulfate. The K_m for the bumetanide-sensitive fraction is low (0.8 mM) compared to the diuretic-insensitive flux (3.5 mM) although the V_{max} for both is similar (about 4 mmol/liter cells · hr). Although the precise nature of the bumetanide-insensitive flux is unclear, at least part of this flux will be "passive" (i.e., showing linear concentration dependence, Fig. 4). The contribution of this pathway to the total bumetanide-insensitive flux may be estimated, assuming the nonsaturable uptake has a slope of 0.006 mmol/liter cells · hr/mM (Fig. 4), to be 0.30 mmol/liter cells · hr at a sodium concentration of 50 mM.

Experiments designed to investigate the kinetic properties of Na^+-activated K^+ uptake (Fig. 5) suggest that under certain conditions other cation transport pathways may be unmasked. Thus at very low values of $[Na]_o$ (about 250 μM) a bumetanide-sensitive K^+ uptake is observed. This diuretic-sensitive K^+ influx in essentially Na^+-free conditions has previously been reported in human and sheep red cells (Wiley & Cooper, 1974; Dunham & Benjamin, 1984). Increasing $[Na]_o$ progressively decreases the magnitude of this flux in sheep erythrocytes, so that at "normal" values of $[Na]_o$ (150 mM) diuretic-sensitive K^+ uptake via this pathway is negligible (Dunham & Ellory, 1981). Clearly the presence of at least two bumetanide-sensitive K^+ uptake systems, one inhibited and the other stimulated by raising $[Na]_o$, make a definitive analysis of kinetic data such as that presented in Fig. 5*a*, an extremely complex task.

In the present study we have not attempted to investigate the kinetics of bumetanide-sensitive Cl^- fluxes. This is because in contrast to Na^+ and K^+ transport, bumetanide-insensitive Cl^- fluxes are very large and therefore the bumetanide-sensitive component represents only a small fraction of total Cl^- uptake (*see* Fig. 1 and Table 2). Furthermore, as the Cl^- flux is so high, it would be difficult, if not impossible, to control the internal and external Cl^- concentrations independently during the course of the experiment. It would be vital to have this control in order to interpret data obtained from this type of experiment.

In the context of a kinetic analysis of the cotransport system, it should be noted that this is a complex system to analyze in terms of apparent affinities. This is because there will be internal and external binding sites for the three ions, which may interact with each other. Further, each species may itself have multiple binding sites. It is therefore difficult to interpret ion substitution experiments unequivocally in terms of apparent kinetic constants. Nevertheless, simple Michaelis-Menten kinetics are obeyed by the Na^+ and K^+ influx experiments (Figs. 3 and 4). Therefore, pragmatically, the best measure of the stoichiometry would be the simultaneous study of bumetanide-sensitive Na^+, K^+ and Cl^- fluxes under the same experimental conditions.

Flatman (1983) observed a higher bumetanide-sensitive flux of Na^+ than K^+, and proposed a stoichiometry of between 1 and 3 Na^+ per K^+. A more accurate measurement of the coupling ratio has been achieved in the present study by using a lowered experimental temperature which markedly reduced the fluxes from the enormously high equilibration rates obtained at 39°C (Flatman, 1983). It is conceivable that the ratio may change with temperature although there is evidence to show that the stoichiometry of cotransport in other cell types remains constant under a variety of experimental conditions (Geck et al., 1980; Haas et al., 1982). Reducing temperature has, however, been found to alter the kinetic properties of some carrier-mediated processes (Ellory & Willis, 1982; Ellory et al., 1983; Hall & Willis, 1984).

The stoichiometry of bumetanide-sensitive cotransport in ferret red cells contrasts with that of the catecholamine-stimulated, furosemide-sensitive system observed in duck red cells (Haas et al., 1982) and the diuretic-inhibitable flux in Ehrlich cells (Geck et al., 1980) where net fluxes yield the same apparent stoichiometry of 1Na:1K:2Cl. A similar stoichiometry to that seen in the present investigation may, however, exist in the squid giant axon (Russell, 1983). In human red cells a ratio of 1Na:2K has been found for bumetanide-sensitive isotope fluxes (Ellory et al., 1982), although for net efflux from red cells loaded with cations using the PCMBS technique a ratio of 1Na:1K has been reported (Garay et al., 1981).

It is important to emphasize that the measurement of stoichiometry using unidirectional tracer fluxes may also include exchange pathways. Ferret red cells may have additional Na^+ or K^+ transport systems inhibitable by bumetanide, which could influence the apparent coupling ratio. It seems probable that these erythrocytes with their high $[Na]_i$ and low $[K]_i$ may demonstrate some of the transport pathways identified in red cells from other carnivores (*see* Parker, 1977). The role of these alternative pathways is difficult to assess, although as the present experiments were performed in the presence of EGTA it seems unlikely that transport systems activated by Ca^{2+} are involved.

Whether or not ferret red cells represent a good model system for studying diuretic-sensitive transport must remain an open question. A comparison with various properties of the well-characterized bumetanide-sensitive pathway in human red cells shows some similarities, but also some important differences. The transport capacity of the bumetanide-sensitive Na^+ or K^+ flux is more than an order of magnitude greater in ferret red cells than in

human cells (Ellory et al., 1982; Flatman, 1983). On the other hand values for the $K_{1/2}$ for the bumetanide inhibition of K^+ uptake are in agreement (ferret, 4×10^{-7} M, Fig. 2; human, 2×10^{-7} M, Ellory & Stewart, 1982), suggesting that we may still be dealing with the same basic system. There are, however, considerable differences between the kinetic properties of the systems. At 21°C in human red cells, values for the K_m and V_{max} of bumetanide-sensitive K^+ uptake are about 3 mM and 0.6 mmol/liter cells · hr, respectively (Ellory et al., 1983) compared with 10 and 2.7, respectively, in ferret red cells (Fig. 3). For diuretic-sensitive Na^+ uptake, values for the K_m and V_{max} in human red cells were 6 mM and 0.08 mmol/liter cells · hr, respectively, in marked contrast to the same apparent constants in ferret red cells (2.7 and 1.9, respectively).

The diuretic-sensitive pathway in ferret red cells may be altered in terms of stoichiometry and kinetics, in view of ferret red cell cation composition which is similar to that of other carnivorous species, but atypical among mammalian species (Parker, 1977; Flatman & Andrews, 1983). It is tempting to link the high capacity of this cotransport system in ferret red cells with the unusual intracellular composition, and the lack of the conventional Na^+/K^+ pump. At the present time, however, the physiological role of this cotransporter in these cells still remains an unanswered question.

It seems likely, however, that the bumetanide-sensitive cotransport system in both ferret and human red cells, and perhaps also in other cell types, represents the same basic pathway. As the present paper demonstrates bumetanide-sensitive Cl^- transport in ferret red cells is associated with the simultaneous movement of Na^+ and K^+, it seems reasonable to suggest that where bumetanide-sensitive (Na + K) transport has been identified, Cl^- movement may also occur through the same transporter.

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