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A Thermodynamic Study of Electroneutral K-Cl Cotransport in pH- and Volume-clamped low K Sheep Erythrocytes with Normal and Low Internal Magnesium

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ABSTRACT Swelling-induced human erythrocyte K-Cl cotransport is membrane potential independent and capable of uphill transport. However, a complete thermodynamic analysis of basal and stimulated K-Cl cotransport, at constant cell volume, is missing. This study was performed in low K sheep red blood cells before and after reducing cellular free Mg into the nanomolar range with the divalent cation ionophore A23187 and a chelator, an intervention known to stimulate K-Cl cotransport. The anion exchange inhibitor 4,4'diisothiocyanato-2,2'disulfonic stilbene was used to clamp intracellular pH and Cl or NO_3 concentrations. Cell volume was maintained constant as external and internal pH differed by more than two units. K-Cl cotransport was calculated from the K effluxes and Rb (as K congener) influxes measured in Cl and NO_3, at constant internal K and external anions, and variable concentrations of extracellular Rb and internal anions, respectively. The external Rb concentration at which net K-Cl cotransport is zero was defined as flux reversal point which changed with internal pH and hence Cl. Plots of the ratio of external Rb concentrations corresponding to the flux reversal points and the internal K concentration versus the ratio of the internal and external Cl concentrations (i.e., the Donnan ratio of the transported ions) yielded slopes near unity for both control and low internal Mg cells. Thus, basal as well as low internal Mg-stimulated net K-Cl cotransport depends on the electrochemical potential gradient of KCl.

KEY WORDS: K-Cl cotransport • sheep erythrocytes • thermodynamics • magnesium • DIDS-pH-clamp

INTRODUCTION

K-Cl cotransport, a secondary active transport pathway, occurs in cells of the erythron such as nucleated red blood cells, reticulocytes, and young red blood cells of a variety of species, in endothelial cells, in certain epithelial cells, and in fish liver cells (reviewed by Lauf et al., 1992). In mature red blood cells, K-Cl cotransport declines with cell aging (Hall and Ellory, 1986; O'Neill, 1989) but less so in low K (LK)[1] red blood cells of ruminants (Lauf et al., 1992) and carnivores (Parker and Dunham, 1989). Because of the apparent persistence of K-Cl cotransport in human red blood cells with certain hemoglobinopathies, such as hemoglobin SS disease, and its potential contribution to abnormal cellular dehydration (Bookchin et al., 1991), interest has

been focussed on regulatory aspects using rabbit and sheep red cells as models (Jennings and Al-Rohil, 1990; Dunham et al., 1993).

In human red blood cells, K-Cl cotransport is considered to operate in an electroneutral fashion: Brugnara et al. (1989) showed Cl-driven uphill K transport at constant membrane potential, Φ_m, and Kaji (1993) varied Φ_m with the Na ionophore hemisodium without affecting K-Cl cotransport. In these two studies, the effect of Φ_m was assessed without deliberate efforts to maintain constant cell volume. For swelling-induced K flux of LK sheep red blood cells in Cl, the experimentally obtained and theoretically predicted net K fluxes disagreed at equilibrium (Delpire and Lauf, 1991a). A similar conclusion was reached in volume-clamped LK sheep red cells with normal and low Mg_i (Lauf et al., 1994). Hence, a thorough thermodynamic evaluation of basal and activated K-Cl cotransport, still lacking for any cell system, was undertaken.

The present study uses the stilbene-derivative DIDS which irreversibly inhibits band 3 protein-mediated anion, i.e., chloride/bicarbonate, exchange (Ship et al., 1977; Gasbjerg et al., 1993), and therefore pH equilibration (Cala, 1980) but only reversibly blocks K-Cl cotransport in LK sheep red cells (Delpire and Lauf, 1992). After pH_i titration with CO_2 or Na_2CO_3 DIDS should permit clamping of the internal Cl concentra-

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[1]*Abbreviations used in this paper:* dcs, dry cell solids; FRP, flux reversal point; LK, low K.

tions, $[Cl]_i$, at different levels. In such cells K effluxes and influxes may be studied at variable $[Cl]_i$ and external K concentrations, $[K]_o$, and with constant internal K and external Cl concentrations, $[K]_i$ and $[Cl]_o$, respectively. Intracellular NO_3 concentrations were varied accordingly. Since there is no kinetic evidence for Cl-dependent K/Rb exchange (Delpire and Lauf, 1991 a, b), the effect on K-Cl cotransport of variable ion products on both sides of the plasma membrane can thus be assessed. By combining this technique with osmotic volume clamping (Zade-Oppen and Lauf, 1990; Lauf et. al., 1994), we unequivocally demonstrate that the electrochemical potentials of both K and Cl are indeed the driving forces for K-Cl cotransport.

MATERIALS AND METHODS

Red Blood Cells

For each experiment, blood was freshly drawn by professional and licensed personnel from the Laboratory of Animal Resources through venipuncture from mixed breed sheep (consistent with laboratory animal guidelines and license #A91). Red cells were homozygous for the LK (LL) genotype as determined by cation and immunologic analysis (Lauf, 1984). Red cells were separated from plasma by centrifugation at 10,000 rpm and washed at that speed twice with 295 mosM NaCl before suspension in further experimental media (see below). Cell suspensions for ion flux determinations were 2.5% (vol/vol).

Chemicals and Solutions

The following solutes of analytic grade were purchased from either Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific (Fair Lawn, NY): NaCl, $NaNO_3$, glucose, ouabain, EDTA, PIPES, BICINE, ($N,N,$bis[2hydroxyethyl]glycine), Tris, sucrose, and $MgCl_2$; A23187 was obtained from Calbiochem Corp., San Diego, CA. Stock solutions were made for A23187 (1.91 mM) in ethanol. DIDS (4,4′-diisocyanato-2,2′disulfonic stilbene), dissolved in buffer solutions, and diamox (Acetoazolamide), dissolved in DMSO, were obtained from Sigma Chemical Co., and ultrapure (Puratronic) RbCl and $RbNO_3$ were obtained from Johnson Matthew Chemicals, Royston, UK. Osmolalities were measured with an Advanced Digimatic Osmometer (model 3DII; Advanced Instruments, Needham Heights, MA), and pH was measured using an Orion Research Ionalyzer 901 (Orion Research Incorporated, Cambridge, MA) and Fisher Microprobe or Orion combination electrodes. Dry weights were determined using a Mettler M5 microbalance on ~150 μl triplicate samples of packed red cells, dried for 48 h in a drying oven at 80°C (Lauf, 1982). The pH values are given for 37°C if not otherwise stated. Treatment and flux solutions are described below.

pH_i-titration with Osmotic Clamping, Mg_i Removal, and DIDS-pH_i Clamping

The bulk of the experiments required a three-step procedure for osmotic clamping, pH_i-titration and Mg_i removal. The osmolalities needed to maintain constant cell volume at various pH_o after pH_i clamping with DIDS were calculated based on the approaches chosen by Zade-Oppen and Lauf (1990) and Lauf et al. (1994) and experimentally confirmed. From a plot of Kg cell water/Kg dry cell solids (dcs) versus variable osmolality (range: 350–220 mosM) for each pH_o (6.5, 7.4/7.5, and 9), the osmolal-

ity was extrapolated yielding close to 1.85 Kg water/kg dsc. This water content was close to that obtained in 250 mosM solutions (see composition below) of pH 9 and only $\sim3\%$ below the original cell volume in plasma. The external anion concentration was fixed at ~100 meq/liter. The salt and water shifts across the cell membrane induced by pH titration were balanced by addition of sucrose at pH 7.4/7.5 and 6.5. Samples were taken within the first 10 min of the flux experiment for determination of cell water, $[Cl]_i$ and pH_i. $[Cl]_i$ was measured with a Buchler chloridometer (Buchler Instruments, Fort Lee, NJ) as published earlier (Lauf, 1982), and pH_i on freeze-thawed red blood cells. $[K]_i$ was measured with a Perkin-Elmer 5000 Atomic Absorption Spectrophotometer (Perkin Elmer Instruments, Norwalk, CT). Ionic concentrations are expressed in meq/liter of original cells (loc) or meq/Kg cell water, and pH in units. Final solution osmolalities are given after appropriate adjustments with sucrose.

To obtain pH_i ~6.5, LK red cells washed in 295 mosM NaCl were resuspended in 330 mosM NaCl and titrated with CO_2 to pH_o 6.5 in a jacketed chamber equilibrated at 0°C. Cells were washed by centrifugation at 7,500 rpm until pH_o was stable (Gunn et al., 1973). Cells were then subjected to Mg_i removal as described below and resuspended in a 10 mM PIPES-buffered solution (4°C) of the same pH as above, containing either 100 mM NaCl (a) or $NaNO_3$ (b) with final osmolalities of 330 mosM in a and 320 mosM in b, respectively. The packed cells were then resuspended in solutions a and b containing 0.1 mM DIDS and 10 μM diamox, a carbonic anhydrase inhibitor, at 37°C, and incubated for 45 min in a shaker bath. Cells were washed twice at 4°C in the same solutions to remove the inhibitors.

For pH_i ~7.4 the titration step was omitted, and washed cells were suspended in 10 mM PIPES-buffered solutions of pH 7.4 or 7.5 at 4°C containing 100 mM NaCl with a final osmolality of 295 mosM. After Mg_i removal (see below), cells were resuspended and incubated for 45 min in fresh PIPES-buffered pH 7.4 or 7.5 media at 37°C with either 100 mM NaCl or $NaNO_3$, with final osmolalities at 295 and 277 mosM, respectively, and containing 0.1 mM DIDS and 10 μM diamox. DIDS and diamox were removed by washing, as described above.

For pH_i ~9, washed LK red cells were loaded into a jacketed chamber, equilibrated at 0°C, and titrated to pH 9 with 100 mM Na_2CO_3 with intermittent washes in 250 mosM NaCl until pH_o was stable. Cells were then suspended in cold 100 mM NaCl buffered to pH 9 with 10 mM BICINE, final osmolality 250 mosM, for Mg_i removal by A23187 and EDTA as described below. Cells were then divided into two aliquots and resuspended in 10 mM BICINE-buffered (pH 9) 100 mM NaCl (a) or $NaNO_3$ (b) with final osmolalities of 250 and 240 mosM, respectively, for DIDS and diamox treatment and subsequent removal of the drugs by washing.

Free Mg_i was lowered into the nanomolar range in pH-titrated and volume-clamped cells with 10 μM A23187 in the presence of 1 mM EDTA (Lauf et al., 1994). To exclude undesired pH equilibration, the ionophore was removed by washing in the presence and absence of BSA. The effectiveness of A23187 removal was functionally tested at each pH value (data not shown).

Measurements of Ion Fluxes, Statistics, and Calculations

K efflux and Rb influx were assessed simultaneously, as described previously (Lauf, 1983; Lauf et al., 1994). Flux solutions contained 0.1 mM ouabain and were buffered with 10 mM PIPES for pH_o 6.5, with 10 mM PIPES/BICINE for pH_o 7.4/7.5 and with 10 mM BICINE for pH_o 9. For cells titrated to pH_i 6.5, ~7.4, and ~9, the osmolalities of the Cl and NO_3 flux media were maintained at 330 and 320 mosM, 295 and 277, and 250 and 240 mosM, respectively, at all pH_o values during the flux experiment. External Rb concentrations, $[Rb]_o$, were 0, 5, 10, 15, 20, 30, 40,

and 60 mM replacing equimolar quantities of $[Na]_o$ keeping the total cation concentration, $[Rb + Na]_o$, constant in both Cl and NO_3 media. For measurement of K loss, samples were taken at about 10, 20, 30, 40, and 50 min, and the supernatants were removed after centrifugation at 10,000 rpm. K loss was measured by atomic absorption spectrophotometry and the pseudo-first-order rate constants (1/h) of K loss were calculated from the supernatant $[K]_o$ at the five time points, corrected for spontaneous hemolysis (<2%), and $[K]_i$ at equilibrium, using a linear regression program essentially as published earlier (Lauf, 1983). Data are presented as K efflux in mmol/(locxh) by multiplying the rate constants of K loss by $[K]_i$. The Cl-dependent K efflux (used interchangingly with K-Cl flux or K-Cl cotransport) is the calculated difference between K efflux measured in Cl and NO_3.

For assessment of ouabain-resistant Rb uptake, 1-ml aliquots of cell suspension were removed at about the same time intervals and placed into test tubes with 8 ml ice-cold 295 mosM Tris-buffered $MgCl_2$, pH 7.4. Cells were separated by centrifugation, washed twice in the same media to remove any Rb_o, and hemolyzed in "hemolyzing solution" (Lauf, 1983; Lauf et al., 1994). Rb uptake was calculated in mmol/(loc) based on (*i*) the hemolysate Rb measured by flame emission spectrophotometry in a Perkin-Elmer 5000 Atomic Absorption Spectrophotometer, (*ii*) the optical density of the hemolyzed cell aliquot, and (*iii*) of 1 ml of the original packed cells measured at 527 nm (Lauf, 1989), respectively. Rb influx in mmol/locxh was calculated from the initial velocity of Rb uptake at 5 time points using linear regression programs available through STATISTIX 4 or the Slide Write Programs. The Cl-dependent Rb influx (also referred to as K-Cl influx or cotransport) is the calculated difference between Rb influx in Cl and NO_3. The kinetic parameters V_{max} and K_m were obtained from Wolf-Hanes or Lineweaver-Burke plots.

Thermodynamics

We derived the conditions for flux equilibrium, i.e., when the net flux is zero, from the standard equation defining the electrochemical potential (μ_j) of the transported ions (see Heinz, 1978). Accordingly:

$$\tilde{\mu}_j = \mu^\circ_j(T) + RT \ln a_j + z_j F \Phi \tag{1}$$

where j = signifies $K_{i,o}$ and $Cl_{i,o}$ for internal and external K and Cl ions, μ°_j = standard chemical potential of ion j, T = absolute temperature, R = gas constant, a_j = activity of ion j ≈ concentration of j, [j], i.e., $[K]_{i,o}$ and $[Cl]_{i,o}$, z_j = charge of ion j; F = Faraday constant, Φ = electrical potential, i.e., difference between the electrical potential inside, Φ_i, or outside, Φ_o, the cell, minus the standard potential.

Thus, for $[K]_i$ and $[Cl]_i$, and for $[K]_o$ and $[Cl]_o$, respectively, Eq. 1 becomes:

$$\tilde{\mu}_{KCli} = \tilde{\mu}_{Ki} + \tilde{\mu}_{Cli} = RT\{\ln([K]_i \cdot [Cl]_i)\} + F\Phi_i(z_K + z_{Cl}),$$
$$\tag{2a}$$

and

$$\tilde{\mu}_{KClo} = \tilde{\mu}_{Ko} + \tilde{\mu}_{Clo} = RT\{\ln([K]_o \cdot [Cl]_o)\} + F\Phi_i(z_K + z_{Cl}).$$
$$\tag{2b}$$

Subtracting Eqs. 2b from 2a yields:

$$\Delta\mu_{KCl} = \Delta\mu_K + \Delta_{Cl} = RT\ln\{([K]_i/[K]_o) \cdot ([Cl]_i/[Cl]_o)\}.$$
$$\tag{3}$$

When $\Delta\mu_{KCl} = 0$, $[K]_o/[K]_i = [Cl]_i/[Cl]_o$, net flux through the K-Cl cotransporter must be zero, i.e., the flux reverses from outward to inward or vice versa (flux reversal point or FRP), at a $[K]_o$ (or $[Rb]_o$ when used as K congener) as per Eq. 4:

$$FRP = [Rb]_o = [K]_o = [K]_i \cdot [Cl]_i/[Cl]_o. \tag{4}$$

The FRPs were calculated from the relationship between net flux rates and the logarithm of the $[Rb]_o/[K]_i$ ratios (Lauf et al., 1994).

RESULTS

Water and Ions in DIDS-treated LK Red Cells after pH Equilibration

Fig. 1 shows the effect of titration with CO_2 to pH_o 6.5 or with $NaCO_3$ to pH_o 9 on cell water and ion concentrations of Cl^-- and NO_3^--equilibrated LK red cells subsequently treated with 0.1 mM DIDS and 10 μM diamox. Cell water and hence volume were maintained near 1.84 Kg/Kg dcs (Fig. 1, *A*, legend), i.e., ~3% below that found at pH 7.4 for normal red cells (see MATERIAL AND METHODS). Like human hemoglobin A (Freedman and Hoffman, 1978), intracellular oxygenated sheep hemoglobin, with its isoelectric point of ~7 (Blunt and Huisman, 1975), provides the fixed and titratable charges. Acidification or alkalinization of the cell suspension caused the expected pH_i shifts in both Cl and NO_3 (Fig. 1, *B*). As expected from the Donnan distribution of permeable cations and anions, Cl and NO_3 either entered or left the red cell compartment through the conductive pathway (Gunn et al., 1989), as measured for Cl in Fig. 1, *C*. Subsequent treatment with DIDS and diamox (to further reduce proton generation by the carbonic anhydrase catalyzed hydration of CO_2) of volume-clamped and pH-titrated (but not pH-clamped) cells did not change this result. Although diamox did not affect the parameters depicted in *A–C*, it was kept throughout the pretreatment step with DIDS.

Water and Ions in DIDS pH_i-clamped Control and Low Mg_i LK Red Cells versus pH_o

Table I displays the cell water content of control and low Mg_i LK red cells with pH_i clamped at different values by DIDS in Cl or NO_3 media. The average mean water contents for each pH_o were close to 1.87 and 1.81 in Cl, and 1.83 and 1.82 in NO_3, in control and low Mg_i cells, respectively, and there was no significant difference between the three pH_o values. Hence, the techniques developed here provided cells with similar volumes at all pH_i /pH_o permutations chosen.

Fig. 2 shows the mean pH_i values of control (*interrupted lines*) and low Mg_i (*solid lines*) LK red cells each at different pH_o (6.5, 7.4/7.5, and 9) imposed on the cells after DIDS treatment. Comparing these data with those in Table I suggests that DIDS effectively clamped pH_i at any pH_o. The fact that low Mg_i cells always displayed
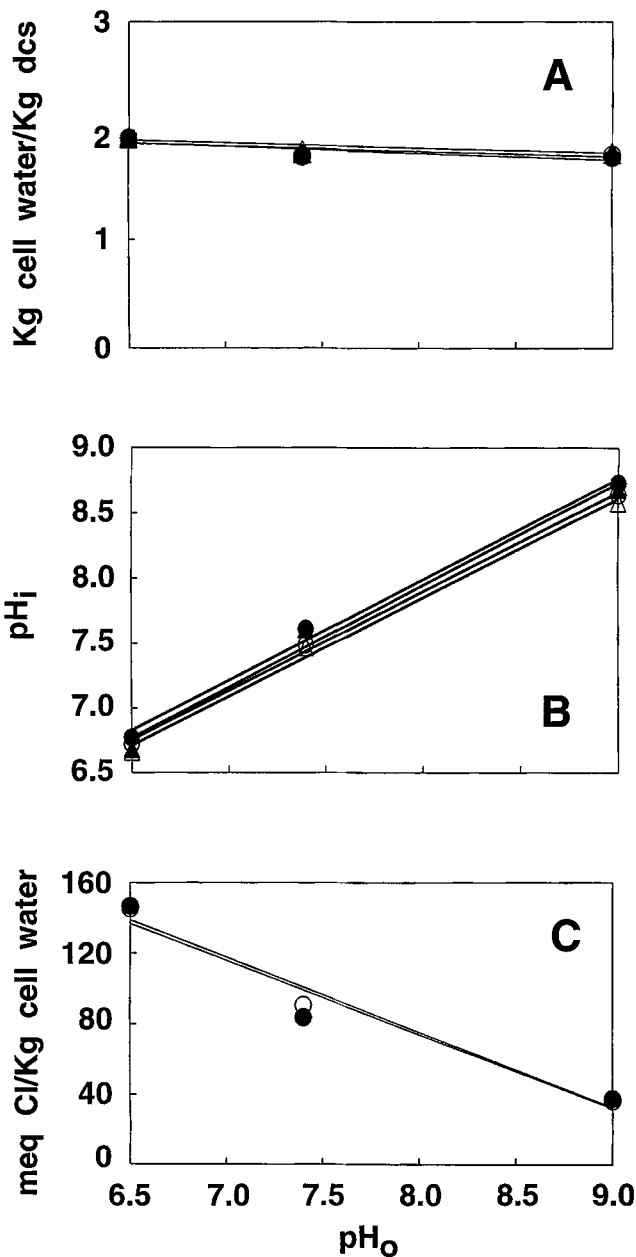
Mean Cell Water* of Control and Low Mg_i LK Red Cells pH_i-clamped by DIDS in Cl or NO_3

	Controls			Low Mg_i	
pH_i	Cl_o	NO_3	pH_i	Cl	NO_3
6.5	1.877 (0.026)	1.781 (0.016)	6.8	1.847 (0.052)	1.862 (0.051)
7.4	1.888 (0.018)	1.865 (0.015)	7.6	1.761 (0.060)	1.780 (0.031)
8.3	1.867 (0.081)	1.840 (0.019)	8.5	1.823 (0.081)	1.812 (0.024)

*Kg/Kg dry cell solids (n = 3, ±SD) for each pH_i at pH_o = 6.5, 7.4/5, and 9.

Figure 1. Comparison of cellular water content (A), intracellular pH, pH_i (B), and chloride concentration, $[Cl]_i$ (C), in LK red cells treated without (controls) or with DIDS and diamox after equilibration at the pH_o values (abscissa). Before treatment with 0.1 mM DIDS and 0.01 mM diamox for 45 min at 37°C, pH_o was titrated to 6.5 with CO_2 and to 9 with Na_2CO_3 in hyperosmotic (350 mosM) and hyposmotic (250 mosM) NaCl or $NaNO_3$, respectively. Symbols of mean values are larger than the SD values. Open and filled symbols for control and DIDS/diamox-treated cells, respectively; circles and triangles for cells in Cl and NO_3, respectively. In A, the mean values (±SD, n = 3) for Kg cell water/Kg dcs were 1.826 (0.033) and 1.858 (0.046) for Cl and NO_3 controls, and 1.814 (0.040) and 1.828 (0.031) for Cl and NO_3-equilibrated DIDS/diamox-treated cells, respectively. In B the equations for the computer-fitted regression lines were pH_i = 0.762 pH_o + 1.798 (r = 0.999), 0.760 pH_o + 1.774 (r = 0.998), 0.774 pH_o + 1.793 (r = 0.997), and 0.785 pH_o + 1.666 (r = 0.993) for Cl and NO_3 controls and Cl and NO_3-equilibrated DIDS-treated cells, respectively. In C

slightly higher pH_i values is not readily explained; it is, however, not important for interpretation of the data shown below. Fig. 3 shows $[Cl]_i$ in cells with pH_i clamped at different pH_o values: $[Cl]_i$ increased from ∼52 to 166, and from 44 to 143 meq/Kg cell water in controls (A) and low Mg_i (B) cells, as pH_i was lowered from 8.3 to 6.5 and from 8.5 to 6.8, respectively (see also Table II). This Cl shift was not affected by the subsequently imposed pH_o as the slopes of all three lines were not significantly different from zero. In contrast to pH_i and $[Cl]_i$, $[K]_i$ remained at 22–25 meq/Kg cell water and did not change with pH_o (Table II).

An inverse change of pH_i and $[Cl]_i$ indicates effective DIDS-clamping which sharply reduced the dissipation of the imposed pH and Cl gradients while pH_o was altered in the subsequent flux experiments.

Ouabain-resistant K and Rb Fluxes in Cl and NO_3 Before and After DIDS Treatment

Although DIDS reversibly inhibits K-Cl cotransport at pH 7.4 (Delpire and Lauf, 1992), potential DIDS effects on K-Cl cotransport at pH values different from pH 7.4 are unknown, particularly at pH 9 where DIDS covalently attaches via the second cyanate group to band 3 protein in human red cells (Jennings and Passow, 1979; Okubo et al., 1994). DIDS also affects passive K fluxes in low ionic strength media suggesting involvement of band 3 protein (Jones and Knauf, 1995), and Maldonado and Cala (1994) labeled a swelling-stimulated K/H exchanger with DIDS in Amphiuma red blood cells.

Fig. 4 shows that in low Mg_i cells, both K effluxes and Rb influxes measured in Cl at 20 mM $[Rb]_o$ were higher the higher the pH of equilibration (A). Most notably, at pH 9 the Rb influxes in Cl controls exceeded the K effluxes, and DIDS stimulated both. Consistent with Delpire and Lauf (1992), no such effects were dis-

the equations were $[Cl]_i$ = −42.4 pH_o + 414.64 (r = 0.986) and −41.8 pH_o + 408.7 (r = 0.969) for Cl controls and DIDS-treated cells, respectively.
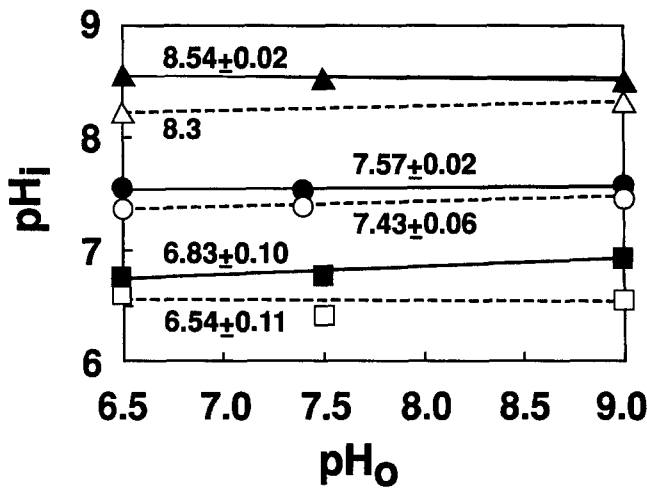
FIGURE 2. Relationship between pH_i and pH_o after pH-clamping with DIDS at pH 6.5, 7.5, and 9. Mean pH_i values ($\pm$SD for $n = 3$) of controls and low Mg_i cells (interrupted and solid lines, respectively) measured for each pH_o.

cerned at lower pH values, particularly at pH 7.4. Fig. 4 B shows that both K and Rb fluxes in NO_3 increased with the pH of equilibration. However, a substantial increase was seen for both fluxes in the presence of DIDS
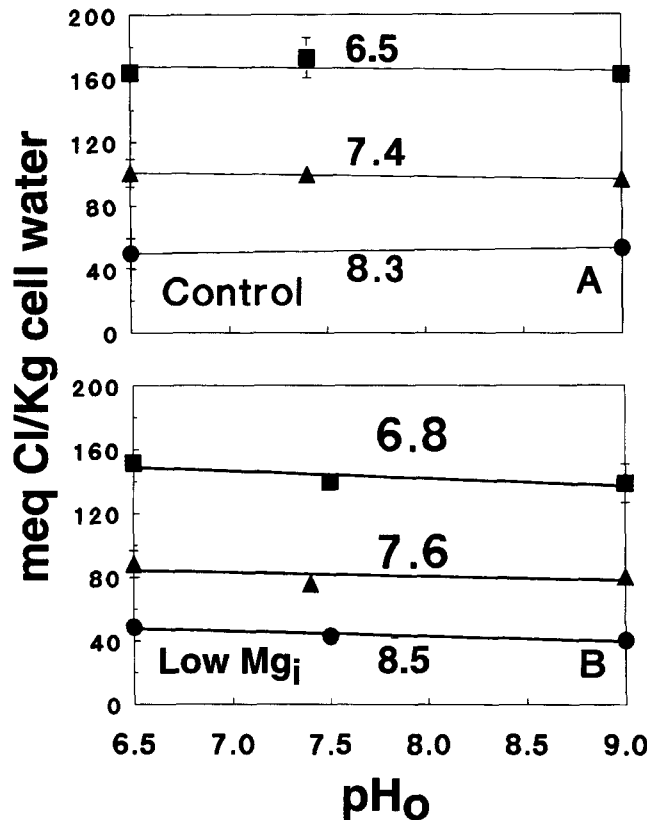

FIGURE 3. Chloride concentrations in control (A) and low Mg_i (B) LK red cells versus pH_o after DIDS clamp at various pH_i values (measured) as indicated by bold numbers. Lines are computer fitted. Mean ion concentrations in meq Cl/Kg cell water ($\pm$SD for $n = 3$ experiments) for the three pH_o values are given in Table II.

TABLE II

Mean Ionic Concentrations* in Control and Low Mg_i LK Red Cells after pH_i Clamping by DIDS

	Controls			Low Mg_i	
pH_i	C	K	pH_i	Cl	K
6.6	166.4 (5.8)	24.4 (1.0)	6.8	143.4 (7.2)	25.1 (0.4)
7.4	98.9 (2.1)	25.3 (0.5)	7.6	81.4 (6.4)	22.8 (0.5)
8.3	52.0 (2)	24.1 (0.7)	8.6	44.2 (4.3)	21.8 (0.3)

*meq/Kg cell water for ($n = 3$, $\pm$SD) for $pH_o = 6.5$, 7.4/5, and 9.

at pH 9 suggesting activation of diffusional K fluxes. C contains the calculated K-Cl cotransport activities (dCl). Although the stimulatory effect of DIDS on Rb influxes in Cl was canceled, the alkaline pH activation remained unabated. The Cl-dependent K effluxes were slightly higher in the presence of DIDS, an effect of probably borderline significance. These data are consistent with alkaline stimulation of K-Cl cotransport, in particular of Rb-Cl influx in the absence of DIDS reported by us earlier for Mg_i-depleted LK red cells (Lauf et al., 1994).

Determination of Flux Reversal Points (FRPs)

The assessment of the FRPs requires measurements of both Cl-dependent Rb influx and K efflux at varying $[Rb]_o$. 18 experiments were carried out to determine the FRPs: 9 in controls and 9 in low Mg_i LK red cells. To illustrate the flux behavior and the assessment of the FRPs, Fig. 5 presents 3 experiments with controls and 6 with low Mg_i cells. Shown are Cl-dependent Rb influx (Fig. 5, A and D) and K efflux (B and E), and net K-Cl cotransport, calculated as the difference between the two former (C and F) as a function of $[Rb]_o$ in volume- and pH_i 6.6-clamped controls (A–C) and in pH_i 6.8- (open symbols) and 8.6-clamped (closed symbols) low Mg_i (D–F) LK red cells, respectively.

Rb-Cl influx. As expected for carrier mediated K-Cl cotransport (Delpire and Lauf, 1991a, b) Cl-dependent Rb influx (Rb-Cl influx) in both control (Fig. 5 A) and low Mg_i (Fig. 5 D) red cells saturated with increasing $[Rb]_o$. Fig. 5, A–C displays the experiments with control cells clamped at pH_i of 6.6 and subsequently exposed to pH_o 6.5, 7.5, and 9. Varying pH_o between 6.5 and 9 had no effect. The average kinetic parameters ($n = 3$, mean $\pm$ SD) calculated from Wolf-Hanes plots from Fig. 5 A, were 0.9 ± 0.23 mmol/(locxh) for the V_{max} values and 35.1 ± 8.8 mM $[Rb]_o$ for the K_m values. Hence pH_i, but not pH_o, determined V_{max}, but not K_m of Rb-Cl influx. These data provide the new information that acid pH activation of K-Cl cotransport in cells with normal Mg_i and unity volume reported by us earlier (Lauf et al., 1994) is due to internal proton effects.
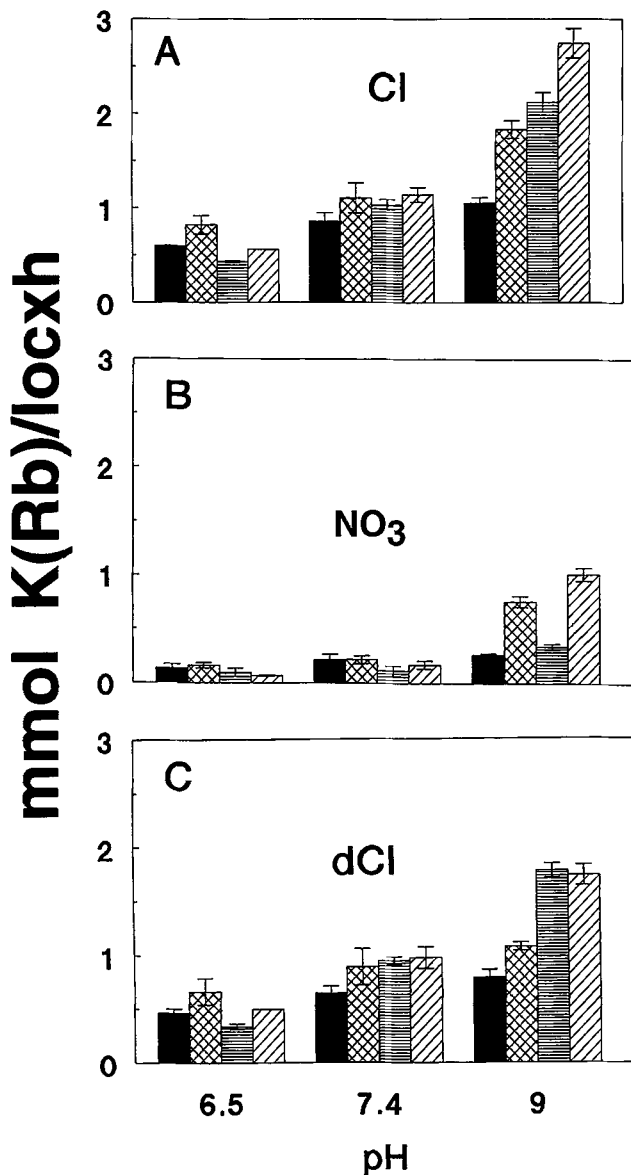
Fig. 5 D shows kinetically indistinguishable saturating

FIGURE 4. Effect of DIDS pretreatment on K and Rb fluxes in osmotically clamped low Mg_i LK red cells, pre-equilibrated at three pH_o values. K and Rb fluxes in Cl (A), NO_3 (B), and K-Cl cotransport (dCl, C). Black and cross-hatched columns denote K efflux in control and DIDS-exposed cells, respectively. Horizontally and diagonally striped columns for Rb influx in controls and DIDS-exposed cells, respectively. $n = 2$ experiments for pH 6.5 and 9 with bars indicating range, and $n = 4$ for pH 7.4, with bars for standard deviations.

Rb-Cl influxes when pH_o was varied from 6.5 to 9 in low Mg_i pH_i 6.8 cells, albeit Rb-Cl influx was more than twice that of controls (A). The average values for V_{max} were 2.46 ± 1.06 mmol/(locxh) and for K_m 31.3 ± 6.6 mM $[Rb]_o$. Compared to the latter as well as to the controls (Fig. 5 A), saturation of Rb-Cl influx at >20 mM$[Rb]_o$ in pH_i 8.6 low Mg_i cells increased by greater than twofold as pH_o was raised from 6.5 to 9 (bold num-
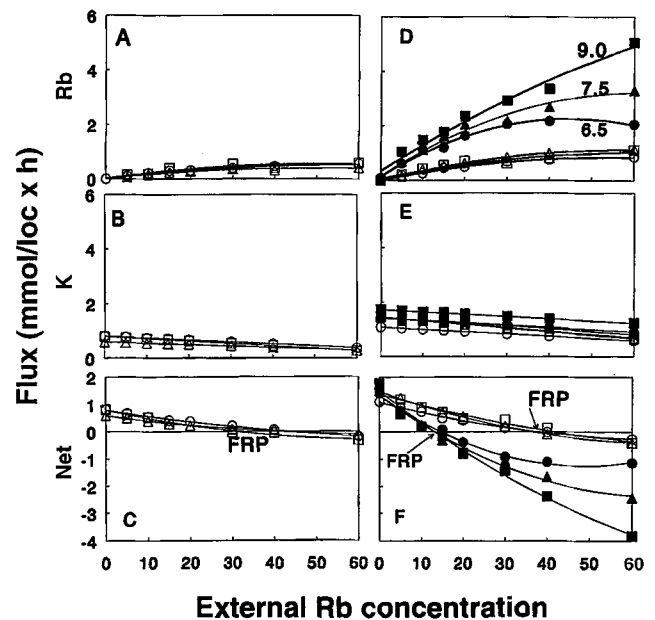

External Rb concentration

FIGURE 5. Determination of flux reversal points (FRP) in control LK red cells with physiologic Mg_i (A–C) and in Mg_i-depleted cells (D–F). Chloride-dependent Rb influxes (A, D) and K effluxes (B, E), calculated from the difference of the Rb influxes and K effluxes in Cl and NO_3, and net K-Cl fluxes (C, F) from the difference between A and D and B and E, respectively, are plotted as function of $[Rb]_o$ in control LK and low Mg_i red cells DIDS-clamped at pH_i 6.8 and 6.6 (open symbols), and at pH_i 8.6 (closed symbols), respectively. Squares, triangles, and circles represent pH_o 9, 7.5, and 6.5, respectively. Lines were computer fitted to a second order polynomial function. FRP indicates the $[Rb]_o$ (in mM) at which net flux is zero.

bers), an effect primarily due to changes in V_{max} (calculated values were 3.6 and 8.5 mmol/(locxh), respectively) but not in K_m (24.1 and 35.9 mM $[Rb]_o$, respectively). At the intermediate pH_o of 7.5, the values for V_{max} were 4.9 mmol/(locxh) and for K_m 31.8 mM$[Rb]_o$. These findings are commensurate with our earlier report that alkaline pH, even in the absence of a pH clamp, increases V_{max} but not K_m of Rb-Cl influx in Mg_i-depleted LK red cells (Lauf et al., 1994) and add the new information that in alkaline pH_i-clamped cells, V_{max} is sensitive to pH_o.

K-Cl efflux. In low Mg_i cells (Fig. 5 E) K-Cl efflux was about twofold larger than in controls (Fig. 5 B) exhibiting a small *trans*-inhibition with increasing $[Rb]_o$. The twofold stimulation in low Mg_i cells over controls is commensurate with the effect of Mg_i removal on Rb-Cl-influx shown above. *Trans*-inhibition has been attributed to the level of $[K]_i$ at the *cis*-side of the transporter as well as to the fact that the slow step of the transporter is the translocation of the loaded form (Delpire and Lauf, 1991a, b). Furthermore, at 25 mM $[Rb]_o$, which is about equivalent to $[K]_i$ (see Table II), lowering Mg_i stimulated K-Cl efflux less than Rb-Cl influx (compare panels E vs. B and D vs. A), in particular at

high pH$_i$ (D and E, filled symbols), and no pH$_o$ effects were evident on K-Cl efflux. The apparent insensitivity of K-Cl efflux to pH$_i$ can be in part explained by the high K$_m$ values of the K-Cl cotransporter reported earlier by us (Delpire and Lauf, 1991a, b), i.e., the pH$_i$ effect should be observed at [K]$_i$ > 25mM as shown in D for Rb influx as function of [Rb]$_o$.

Net K-Cl flux and FRP. The FRPs resulting from the x-axis intercept of the net fluxes, i.e., the differences between the K-Cl efflux (panels B and E) and Rb-Cl influx (panels A and D) are indicated in Fig. 5 C for controls and Fig. 5 F for low Mg$_i$ cells. The FRPs (i.e., the [Rb]$_o$ at which net K-Cl cotransport is zero) were ~40 mM in both low pH$_i$ controls and low Mg$_i$ cells, and ~12 mM in pH$_i$ 8.5 low Mg$_i$ cells. Based on our earlier work on LK sheep red cells in the absence of DIDS, differences between the FRPs signal changes in the driving forces associated with pH titration (Lauf et al., 1994): The shift in the FRPs was commensurate with a greater than threefold decrease of [Cl]$_i$ as pH$_i$ increased from 6.8 to 8.5 (Fig. 3).
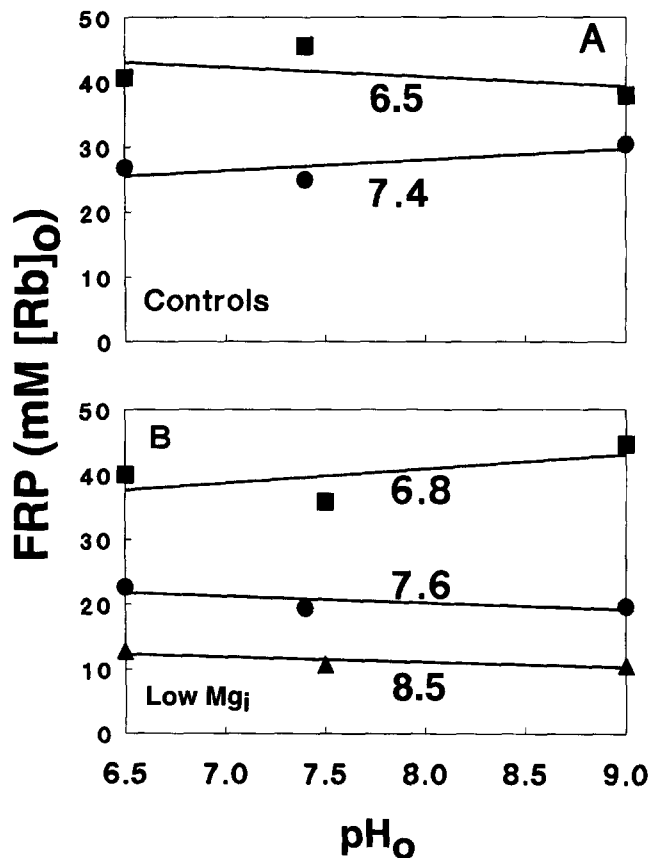
FRPs in Relation to Ion Gradients after DIDS Treatment

The experimentally determined flux reversal points from 15 experiments on control (A) and low Mg$_i$ (B) LK red cells are shown in Fig. 6. In panel A only 6 of the 9 control experiments are displayed since at alkaline pH no significant K-Cl cotransport was measured. The FRPs in controls with pH$_i$ of 7.4 and 6.5 (A), and in low Mg$_i$ cells with pH$_i$ 8.5, 7.6, and 6.8 (B) increased, independently of pH$_o$, from near 30 to above 40 mM [Rb]$_o$, and from near 10 to near 40 mM [Rb]$_o$, respectively. Thus, the FRP values approximately followed the shifts of [Cl]$_i$ in Fig. 3.

Eq. 4 states that the FRPs should be a linear function of the Cl ratio, or more precisely, of [Cl]$_i$ since [Cl]$_o$ was constant, with a slope equal to [K]$_i$. Fig. 7 shows plots of the FRPs from Fig. 6, calculated according to Eq. 4 from the measured [K]$_i$, [Cl]$_i$, and known [Cl]$_o$ as a function of [Cl]$_i$/[Cl]$_o$ for controls (A) and low Mg$_i$ (B) cells. The mean slopes for the data obtained from

FIGURE 6. Flux reversal points for control (A) and low Mg$_i$ LK red cells (B) as a function of pH$_o$ for three different pH$_i$ per condition (numbers). Regression lines were computer fitted. Mean FRPs (mM) [Rb]$_o$ for n = 3 (±SD) were 41.4 (3.9) and 27.4 (2.8) for pH 6.6 and 7.4 controls, and 40.2 (4.5), 20.6 (1.8), and 11.4 (1.2) for pH 6.8, 7.6, and 8.6 low Mg$_i$ cells.
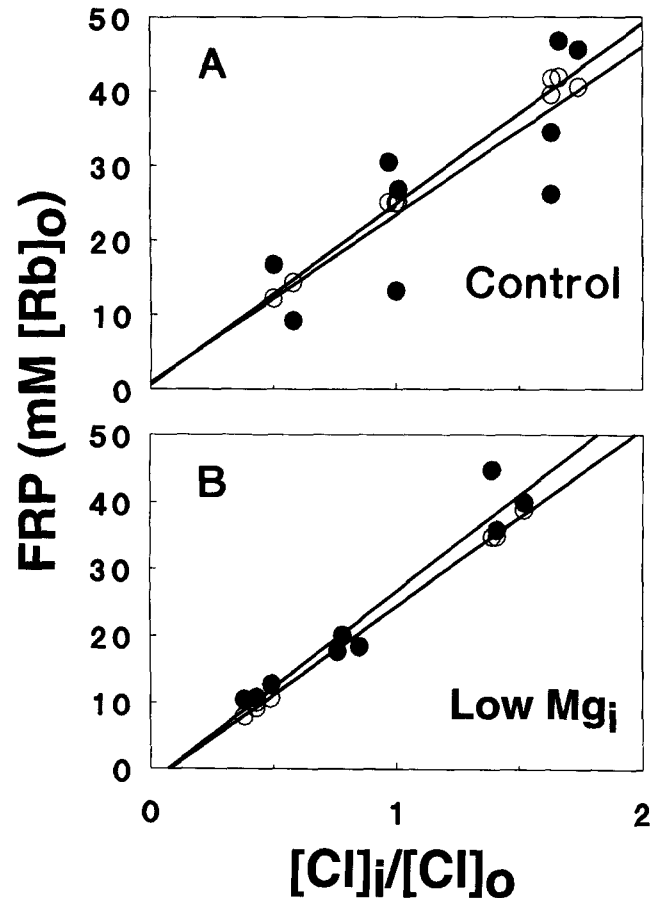

FIGURE 7. FRP as a function of the chloride ratios in controls (A) and low Mg$_i$ (B) LK red cells. The slopes of the regression lines were: (A) 22.6 (r = 0.821, filled circles) and 24.3 (r = 0.995, open circles) mM [K]$_i$, and (B) 28.7 (r = 0.973) and 26.5 (r = 0.997) mM [K]$_i$, for values measured (filled circles) and calculated (open circles) from [K]$_i$ · [Cl]$_i$ /[Cl]$_o$ for controls (A) and low Mg$_i$ (B) cells, respectively.
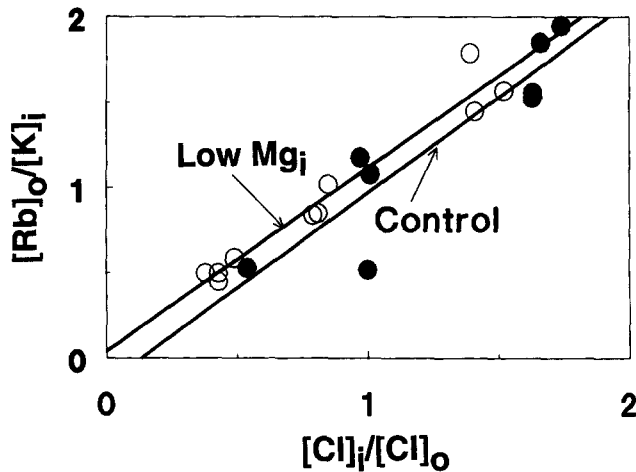
FIGURE 8. Relationship between the ratios of the concentrations of the transported cations $[Rb]_o / [K]_i$, with $[Rb]_o$ determined from the FRP and the measured ratio of $[Cl]_i / [Cl]_o$ in control and low Mg_i LK red cells. The slope for controls (*closed circles*) is 1.12 ($r = 0.912$, $n = 8$ experiments) and for low Mg_i cells 1.08 ($r = 0.976$, $n = 10$ experiments).

the fluxes (*closed symbols*) and those calculated according to Eq. 4 (*open symbols*) were 22.6 and 24.3 mM for controls, and 28.7 and 26.5 mM for low Mg_i cells, respectively, and hence approximated $[K]_i$ (24.6 and 23.3 meq/Kg cell water, see Table II). Thus the product of the chemical gradients was the thermodynamic driving force for both basal and low Mg_i-activated K-Cl cotransport. Consequently, a plot of the Donnan ratio of cations versus anions transported by the carrier should reveal a slope of unity if K-Cl cotransport is electroneutral. In Fig. 8 the slopes of the experimental data relating $[Rb]_o$ (determined from the FRP)/$[K]_i$ to $[Cl]_i / [Cl]_o$ were 1.11 ($r = 0.912$) for controls (*filled symbols*) and 1.08 ($r = 0.976$) for low Mg_i cells (*open symbols*).

DISCUSSION

The principal aim of this study was to fully describe the response of the basal and activated erythrocyte K-Cl cotransporter to its thermodynamic driving forces in volume- and pH-clamped LK sheep red cells. Although uphill transport of K driven by the Cl gradient and independence of K-Cl cotransport from the membrane potential were shown in earlier studies, these were not done at constant (original) cell volume (Brugnara et al., 1989; Kaji, 1993). In our study, DIDS was used to vary simultaneously pH_i and hence $[Cl]_i$ while keeping cell volume and $[K]_i$ constant, and to vary $[K]_o$, using Rb_o as congener, while maintaining $[Cl]_o$ constant (Fig. 3, Table II). Our principal conclusion is that K-Cl cotransport is electroneutral in control and in low Mg_i LK red cells because the experimentally determined FRP (in mM $[Rb]_o$) provided with $[K]_i$ a ratio that

equaled $[Cl]_i / [Cl]_o$, i.e., $[K(Rb)]_o / [K]_i = [Cl]_i / [Cl]_o$. The measured FRPs (i.e., $[Rb]_o$) of the net K-Cl cotransport shifted linearly and, as thermodynamically predicted, with $[Cl]_i / [Cl]_o$ (Figs. 5–7), commensurate with the Donnan ratio of the transported ions (Fig. 8). A transport system with such properties is able to use the chemical potential of one ion (K or Cl) to move the counter ion (Cl or K) against its chemical potential. Furthermore, the thermodynamically derived stoichiometry of the transported K and Cl ions was unity (Fig. 8). Our data do not permit to assess whether unity coupling occurs between one or two K and Cl ions, respectively. In swollen cells Dunham and Ellory (1981) showed sigmoidal kinetics of both K and Cl fluxes in LK sheep red cells with Cl varied on both sides of the membrane. However, our present results (see also the saturation kinetics of Figs. 5) are consistent with earlier kinetic data (Hill coefficient of unity for K, Lauf, 1984). Our findings are also in agreement with our earlier work showing that K-Cl cotransport is independent of Na (Delpire and Lauf, 1991a) and exclude the contribution of proton gradients, particularly at acid pH_i, to the driving force.

Although the data clearly show that K-Cl cotransport is electroneutral, we cannot prove beyond doubt that changes in Φ_m over the pH range studied could have altered unidirectional flux rates. At alkaline pH, DIDS-treated cells are expected to be hyperpolarized which would cause Rb influx > K efflux in NO_3 as shown in Fig. 4 *B* but not electroneutral Rb-Cl flux > K-Cl efflux. If indeed in DIDS-treated cells, the experimentally achieved $[Cl]_i / [Cl]_o$ ratios determine Φ_m, then the permeability (P) to Cl should be much larger than that to K, i.e., $P_{Cl} \gg P_K$. A plot of the FRPs versus the calculated Φ_m ranging from $+12$ to -23 mV between pH 6.5 and 9, respectively, should be linear. However, such an approach may be irrelevant for two reasons. First, DIDS inhibits P_{Cl} in human red cells (Kaplan et al., 1983; Knauf et al., 1983). To test whether DIDS alters P_{Cl} as function of pH we measured P_K in valinomycin-treated control LK cells according to Knauf et al. (1977). The valinomycin-induced changes in P_K were highest at low pH as compared to alkaline pH (data not shown) suggesting that P_{Cl} is affected by DIDS differently at pH 6.5 than at pH 9. Second, at alkaline pH, DIDS augmented both K efflux and Rb influx in NO_3, i.e., the (Cl-independent) P_K, whereas it did not affect K-Cl cotransport (Fig. 4). This indirect experimental evidence suggests that Φ_m indeed affected unidirectional fluxes without having a major effect on K-Cl cotransport, a conclusion agreeing with Brugnara et al. (1989) and Kaji (1993).

The data presented here contrast with two conclusions of our earlier work. First, Zade-Oppen and Lauf (1991) showed that DIDS, present during the flux experiments, stimulates K and Rb fluxes in Cl preferen-

tially at acid pH, whereas no experiments were done in NO₃. In Fig. 4 we again find that DIDS stimulated Rb influx in Cl, however, at alkaline pH, but affected much less K efflux. Later, Delpire and Lauf (1992) showed later that DIDS inhibits K-Cl cotransport reversibly. Besides DIDS, alkaline pH also enhanced Rb influx in NO₃ (Fig. 4 and see also above), i.e., the basal K permeability. This is consistent with recent findings by Ortiz-Carranza et al. (1996b). The present study on Cl-dependent K and Rb fluxes excludes "other" effects of DIDS on K fluxes such as those shown by Jones and Knauf (1985) in human red cells. Delpire and Lauf (1991a) reported a twofold difference between the calculated and measured FRPs, in swollen LK cells and in Cl only, the Cl-dependent component being unknown. Based on the present work this difference may be due to different pH_i and hence [Cl]_i. Lauf et al. (1994) found a similar small difference for net K-Cl cotransport in osmotically "clamped" cells, as opposed to the present study were both osmotic and pH_i were clamped. This discrepancy is more difficult to explain. It is possible that DIDS eliminated K flux components which may have contributed to the small discrepancies in the FRPs of the preceding studies on swollen and volume-clamped cells.

The design of the experiments to demonstrate electroneutrality of K-Cl cotransport provided also new information on the kinetic behavior of the system which needs to be addressed in future work. Whereas the bulk of the kinetic data were consistent with our previous work in volume- but not pH-clamped LK red cells (Lauf et al., 1994), the data in Fig. 5 clearly indicate separate effects of pH_i and pH_o on Rb-Cl influx: in both control and low Mg_i low pH_i cells, internal protons determined Rb-Cl influx, whereas in low Mg_i alkaline pH_i cells both internal and external protons were modulatory. The reason for this discrepancy cannot be explained based on the data presented in this study. We have recently shown elsewhere that alkaline pH per se stimulates K-Cl cotransport, in particular when combined with extraction of Mg by A23187 and EDTA at pH 9 (Ortiz-Carranza et al., 1996a). Consistent with this report, in the present study K-Cl efflux was higher in alkaline pH_i cells (Fig. 5 E, filled symbols) than in controls (Fig. 5 B).

However, Rb-Cl influx in alkaline pH_i cells with low Mg_i was more sensitive to pH_o suggesting complex kinetic effects of protons on K-Cl cotransport. Since by design of the experiments the FRPs respond solely to [Cl]_i and the apparent affinities of the transporter for Rb_o were about equal in 15 experiments (mean values ± SD of 32.6 ± 7.2 and of 37.2 ± 7.1 mM for controls and low Mg_i cells, respectively), the kinetic effects of pH_o on Rb-Cl influx at high [Rb]_o seen in Fig. 5 D are of no consequence regarding the thermodynamic conclusions.

Both basal and low Mg_i-activated K-Cl cotransport behaved thermodynamically identical. Together with the findings of Brugnara et al. (1989) for the NEM-activated K-fluxes in LK sheep red cells and those of Kaji (1993) for the swelling-induced human K-Cl cotransporter, we may conclude that all K-Cl cotransport modes displayed through these activation mechanisms are electroneutral and must obey the Donnan relationship of the transported cations and anions as shown here (Fig. 8). Since DIDS has been recently shown to reduce the Cl permeability of human red blood cells close to that of bilayers (Gasbjerg et al., 1994), yet does not inhibit K-Cl cotransport in human red blood cells (Kaji, 1986), it should be feasible to test this conclusion in red blood cells from other species, including human.

Our demonstration that basal and stimulated K-Cl cotransport modes in LK sheep red cells is electroneutral follows similar approaches to another cotransporter: Geck et al. (1980) established the thermodynamically electroneutral nature of Na-K-2Cl cotransport in Ehrlich tumor cells by demonstrating that the slopes for Cl fluxes were twice those for Na and K fluxes, and Duhm and Göbel (1984) showed that the electrochemical gradient ratios of the transported ions were the determinants also in human red blood cells.

Although the detailed mechanism by which K and Cl are transported across the membrane awaits further clarification, two papers published most recently at the time of completion of the final revision of this study report cloning and functional expression of two isoforms of the K-Cl cotransporter from rabbits, rat and human (Gillen et al, 1996; Payne et al., 1996).

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