J. Membrane Biol. 117, 177–188 (1990)

The Journal of
Membrane Biology

K-Cl Cotransport in LK Sheep Erythrocytes: Kinetics of Stimulation by Cell Swelling

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Summary. The effects of osmotic cell swelling were studied on the kinetics of Cl-dependent K^+ influx, K-Cl cotransport, in erythrocytes from sheep of the low K^+ (LK) phenotype. Swelling ~25% stimulated transport by increasing maximum velocity (J_{max}) ~1.5-fold and by increasing apparent affinity for external K (K_o) nearly twofold. Dithiothreitol (DTT) was shown to be a partial, reversible inhibitor of K-Cl cotransport. It inhibited in cells of normal volume by reducing J_{max} more than twofold; apparent affinity for K_o was increased by DTT, suggesting that DTT stabilizes the transporter-K_o complex. Cell swelling reduced the extent of inhibition by DTT: J_{max} was inhibited by only about one-third in swollen cells, and apparent affinity was only slightly affected. This result suggested that DTT does not act directly on the transporter, but on a hypothetical regulator, an endogenous inhibitor. Swelling relieves inhibition by the regulator, and reduces the effect of DTT. Reducing intracellular Mg^{2+}, Mg_c, stimulated cotransport. Swelling of low-Mg^{2+} cells stimulated transport further, but only by raising apparent affinity for K_o nearly threefold; J_{max} was unaffected. Thus effects of swelling on J_{max} and apparent affinity are separable processes. The inhibitory effects of Mg_c and DTT were shown to be additive, indicating separate modes of action. There appear to be two endogenous inhibitors: the hypothetical regulator, which holds affinity for K_o low; and Mg_c, which affects J_{max}, perhaps by holding some transporters in an inactive form. Swelling stimulates transport by relieving both types of inhibition.

Key Words K-Cl cotransport · sheep erythrocytes · secondary active transport · volume sensor · volume regulation

Introduction

Erythrocytes from sheep of the low $[K]_c$ (LK) phenotype have a Cl-dependent K^+ transport system which is stimulated several-fold by slight (10%) osmotically induced cell swelling (Ellory & Dunham, 1980; Dunham & Ellory, 1981). Because of the Cl dependence, this transport pathway was proposed to be K-Cl cotransport (Ellory & Dunham, 1980; Dunham & Ellory, 1981). Direct evidence for coupling of K^+ and Cl^- fluxes has not been obtained owing to the enormous Cl^- permeability, characteristic of mammalian red cells. We provided early, preliminary evidence for electroneutrality of volume-sensitive, Cl-dependent K^+ transport (Dunham & Ellory, 1981). A recent study provided more direct evidence for K-Cl cotransport in LK sheep red cells; it was demonstrated that a Cl^- gradient could drive K^+ uphill (Brugnara, Van Ha & Tosteson, 1989). Therefore we refer to the Cl-dependent K^+ transport in sheep erythrocytes as K-Cl cotransport.

LK sheep erythrocytes are particularly convenient for the study of K-Cl cotransport. They lack Na-K-Cl cotransport (Dunham & Ellory, 1981) and the Ca-activated K^+ channel (Brown et al., 1978). Furthermore, the Na/K pump rate is low compared to most mammalian erythrocytes (Dunham & Hoffman, 1971). Thus much of K^+ transport in LK cells is by a Cl-dependent, volume-sensitive pathway, the K-Cl cotransporter.

Evidence has also been presented for K-Cl cotransport in red cells of ducks (Kregenow, 1971; Haas & McManus, 1985; Lytle & McManus, 1987), rabbits (Al-Rohil & Jennings, 1989), and pigs (Lauf, Zeidler & Kim, 1984; Kim et al., 1989). K-Cl cotransport is not normally present in human erythrocytes (Duhm, 1987), but there are circumstances under which it can be demonstrated: it can be induced by treatment with N-ethylmaleimide (NEM) (Wiater & Dunham, 1983; Lauf, Adragna & Garay, 1984) and by osmotic swelling (Kaji, 1986). It is demonstrable in resealed human red cell ghosts (Dunham & Logue, 1986; Sachs, 1988; O'Neill, 1989*b*), and in young circulating red cells (Hall & Ellory, 1986; Brugnara & Tosteson, 1987; Canessa et al., 1987). For a recent review, *see* Dunham (1990).

The thermodynamic gradient for K-Cl cotransport is poised to direct a net KCl efflux from cells.

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The physiological functions of K-Cl cotransport are to participate in cell volume regulation by promoting osmotically obliged water effluxes, and in transcellular transport of salt and water in epithelia. Evidence has been presented for a role of K-Cl cotransport in volume regulation in young erythrocytes of sheep (Lauf & Bauer, 1987) and people (Brugnara & Tosteson, 1987; O'Neill, 1989*a*).

Essential to a volume-regulating transport mechanism are a sensor of cell volume, changes in it, and a signal transduction system from the sensor to the regulator. Very little is known of these mechanisms (*see* for reviews Siebens, 1985; Hoffmann & Simonsen, 1989). Volume sensors coupled to transport pathways are undoubtedly ubiquitous, playing central roles in regulation of cell growth, among other functions. There must be different classes of sensors, reporting both increases and decreases in volume. A change in concentration of a critical solute can probably be ruled out as the signal to the volume sensor for K-Cl cotransport in red cells since volume-sensitive K-Cl cotransport has been observed in inside-out vesicles made from LK sheep erythrocytes (Kracke & Dunham, 1989). It is more likely that mechanical changes in the membrane are sensed as cell volume changes, perhaps changes in the relationship between the integral portion of the membrane and the cytoskeleton (Kregenow, 1971; Siebens, 1985; Mills, 1987).

Clues on the nature of the changes in transporters in response to a signal from the volume sensor can be sought by kinetic studies of K^+ influxes in swollen and shrunken cells. There have been a number of measurements of the kinetics of ouabain-insensitive K^+ fluxes in LK sheep red cells (Dunham & Hoffman, 1971; Dunham, 1976*a*; Dunham & Ellory, 1981; Lauf, 1983; Ellory, Hall & Stewart, 1985*a*,*b*; for a review *see* Lauf, 1985*a*). Measurements have been made of total ouabain-insensitive influx, Cl-dependent influx, and swelling-induced influx. Wide ranges of values have been reported for J_{max} (0.6–14 mmol · liter · hr^{-1}) and $K_{1/2}$ (20–100 mM). From a comparison of different reports, it appeared that swelling of cells increases J_{max}, but in no study was a systematic attempt made to compare the kinetic constants of K^+ transport in cells at two volumes and at the full range possible of $[K]_o$'s. In a recent study, measurements were made of the effect of changes in cell volume on kinetic constants of K^+ influx in erythrocytes from human subjects homozygous for hemoglobin C (Brugnara, 1989). Swelling of the cells increased both J_{max} and the apparent affinity for K^+. The studies on kinetics were not carried further. Most of the K^+ influx was presumably Cl dependent, but this was not shown directly. The effects of swelling on kinetics of hemoglobin C cells were similar to ours reported below on sheep cells.

Potentially useful clues about the mechanism of K-Cl cotransport and associated volume sensor have been provided by studies on sulfhydryl groups. Agents which react covalently with sulfhydryl groups (e.g., iodoacetamide, N-ethylmaleimide, diamide) stimulate K-Cl cotransport in sheep red cells (Ellory & Dunham, 1980; Lauf & Theg, 1980; Bauer & Lauf, 1983; Logue et al., 1983; Lauf, 1984, 1987, 1988*b*). Dithiothreitol, which reacts reversibly with sulfhydryl groups, was reported to inhibit K-Cl cotransport in sheep cells (Lauf, 1984), though it was not determined if it was a reversible inhibitor. In kinetic studies, a reversible inhibitor is more useful than covalently binding activators. The studies employing SH-active agents indicate several classes of sulfhydryl groups associated with the K-Cl cotransporter, one of which may not be on the transporter itself (for reviews *see* Lauf, 1985*a*, 1986, 1988*a*).

Another potentially useful probe for the investigation of the relationship between cotransport and the volume sensor is intracellular Mg^{2+}. Reducing $[Mg]_i$ stimulates K-Cl cotransport in red cells of both sheep and humans (Lauf, 1985*b*; Sachs, 1988). The effect of Mg^{2+} is biphasic in that at low concentrations it stimulates cotransport (Sachs, 1988; Kracke & Dunham, 1989). A role for Mg^{2+} in sensing volume changes was suggested by our recent preliminary report that reducing $[Mg]_i$ alters the time course in the increase in cotransport after swelling (Dunham, 1990).

We report here studies on the kinetics of stimulation of K-Cl cotransport in LK cells by osmotic cell swelling, and of the inhibition of cotransport by DTT in normal and swollen cells. We observed that swelling stimulates K^+ influx by increasing both the apparent affinity of the cotransporter for K_o and the maximum velocity. In cells with reduced Mg^+ activity, swelling stimulates cotransport solely by increasing the apparent affinity for K_o. Therefore stimulation by raising affinity to K_o and by increasing maximum velocity are separable phenomena. DTT inhibits much less in swollen cell than in cells of normal volume. Therefore, DTT may act on a regulator, and not directly on the transporter. A preliminary report of some of these results has been published (Dunham, 1990).

Materials and Methods

Cells

Sheep of the Suffolk breed, both LK and HK phenotypes, are maintained on the Vinzant Family Farms, Borodino, New York. Blood was drawn from the jugular vein into heparin as anticoagulant. Eight different LK sheep and one HK sheep were used as donors in these experiments. Within two hr of bleeding, the

erythrocytes were washed free of plasma and white cells by three brief centrifugations and resuspensions. The isotonic medium used for washing contained (in mM): NaCl 145, KCl 5, glucose 5, Tris-HCl 10, pH 7.4. The osmolality was adjusted to 290 mosm/kg (measured using a vapor pressure osmometer, Model 5100C, Wescor, Logan, UT).

Flux Media

The isotonic medium described above was used in a few measurements of fluxes, as indicated. For one set of experiments, a Cl-free form of this medium was used with NO_3^- substituted for all Cl^-. To inhibit the Na/K pump, all cells were incubated 15 min at 37°C in the isotonic medium containing 0.1 mM ouabain; ouabain was also present during measurement of the fluxes.

For the experiments on kinetics of K^+ influx, all incubation media contained (in mM): glucose 5, Tris-HCl 10, pH 7.4. $[K]_o$ was varied from 17 to 125 mM, and $[choline]_o$ was varied reciprocally with $[K]_o$ from 108 to 0 mM. Media were made either with Cl^- salts or with methyl sulfate salts (Cl-free media). Aliquots of these media were adjusted to the desired osmolality, hypotonic (235–245 mosm/kg) or near isotonic (290–310 mosm/kg) by addition of crystalline sucrose.

Cell Volumes

Cells were equilibrated in media of various osmolalities by centrifugation and resuspension three times. Kinetic experiments were carried out on *swollen* cells, i.e., cells equilibrated in hypotonic media and on cells of near *normal* volume, i.e., equilibrated in media near isotonic. Cells in 310 mosm/kg are slightly shrunken (~7%), but will be referred to as normal; fluxes in these cells were not distinguishable from those in cells in 290 mosm/kg (*cf.* Dunham & Ellory, 1981).

In two experiments, relative cell volumes were determined for cells equilibrated in media of 310 or 245 mosm/kg. The volumes were determined from hematocrits and hemoglobin concentrations and were expressed relative to the same measurements made on cells in the physiological medium of 290 mosm/kg, as described before (Dunham & Ellory, 1981). In these two experiments the relative cell volumes were 0.95 ± 0.02 (n = 8) and 1.12 ± 0.01 (n = 8) for shrunken and swollen cells, respectively. The expected relative cell volumes for perfect osmometers are 0.93 and 1.16, respectively.

K^+ Influxes

Unidirectional K^+ influxes were measured using ^{86}Rb by a slight modification of the method described before (Sachs et al., 1974). ^{86}Rb is a good tracer for K^+ in Cl-dependent K^+ transport in sheep erythrocytes (Ellory & Dunham, 1980; Dunham & Ellory, 1981). Ouabain at 0.1 mM was present in all flux media. For cells preincubated with DTT, the same concentration of DTT was present in the flux medium for those cells. ^{86}Rb in the flux media was added in proportion to $[K]_o$, thereby keeping specific activity constant. All fluxes were measured in triplicate. ^{86}Rb influxes were measured for 30 min at 37°C, and stopped by addition of five volumes of an ice-cold solution of $MgCl_2$ (100 mM), Tris-HCl (10 mM), pH 7.4. The cells were washed free of extracellular tracer by centrifugation and resuspension three times in the ice-cold $MgCl_2$ solution. The washed pellets of cells were lysed in distilled water, and samples of the lysates were taken for determination of radioactivity (autogamma counter) and hemoglobin concentration (absorbance at 540 nm). The fluxes, calculated as before (Sachs et al., 1974), are expressed as mmol of K^+ per original liter of packed cells per hour, i.e., at their volume in the medium of 290 mosm/kg.

Reducing Cellular Mg^{2+}

This was accomplished using the method of Flatman (1982). Cells were preincubated in Cl^- medium with the ionophore for divalent cations, A23187, at 10 μm for 20 min, and then washed free of the ionophore by three successive centrifugations and resuspensions. Control experiments were carried out to determine if the effect of this pretreatment, stimulation of Cl-dependent K influx, was due to A23187 and not reduced cellular divalent cation concentration. In the same control experiments attempts were made to attribute changes in flux to changes in cellular Mg^{2+}, as has been shown for ferret red cells (Flatman, 1987). Cells were preincubated with A23187 without Mg^{2+}, and with Mg^{2+} at various concentrations from 0.03 to 1 mM. At Mg^{2+} of 0.4 and lower, transport was stimulated. Similar results were seen by Lauf (1985*b*) with sheep cells. At higher concentrations, up to 1 mM, there was little or no effect. None of the treatments affected Cl-independent K^+ influx more than 10%. Inclusion of EGTA in the preincubation medium had no effect. We conclude that stimulation of Cl-dependent K^+ influx by preincubation with A23187 is due to reduced cellular Mg^{2+}, and that Mg activity is reduced to the micromolar range, consistent with earlier observations (Lauf, 1985*b*; Flatman, 1987).

Analysis of Results

The results from the experiments on the kinetics of K influx were fitted by computer to a Michaelis-Menten equation, $J = J_{max}/[1 + (K_{1/2}/S)]$, where J is the unidirectional K^+ influx, J_{max} is its maximum velocity, $K_{1/2}$ is the apparent Michaelis constant for external K^+ ($[K]_o$ at $J_{max}/2$), and S is the substrate concentration, $[K]_o$. A nonlinear least-squared program was used (Marquardt algorithm), which yielded best-fit curves and estimates of the kinetic constants ± asymptotic standard errors.

Anti-L_1 Antibody

Antiserum was produced by a slight modification of the method described before (Dunham 1976*a*). An HK sheep was immunized with washed, white membranes of LK erythrocytes packed and suspended in Freund's complete adjuvant. Complement in the antiserum was inactivated by heating. The presence of anti-L_1 antibodies was indicated by the inhibition of ouabain-insensitive K^+ influx (Dunham, 1976*a*,*b*).

Materials and Abbreviations

DTT (dithiothreitol) was purchased from Eastman Kodak (Rochester, NY). NEM (N-ethylmaleimide), ouabain, and Tris base [tris(hydroxymethyl)aminomethane] were from Sigma Chemical (St. Louis, MO). Choline Cl was a gift from Syntex Agribusiness (Springfield, MO), and was further purified by recrystallization from hot ethanol. ^{86}Rb was from New England Nuclear (Boston, MA). A23187 was from Calbiochem (La Jolla, CA).

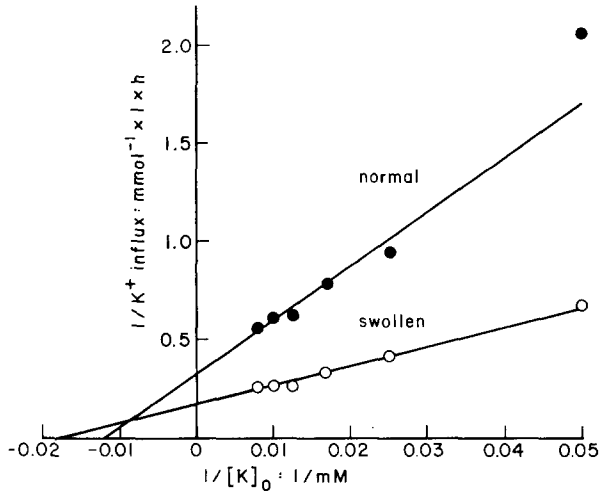


Fig. 1. Double reciprocal plots of ouabain-insensitive Cl-dependent unidirectional K^+ influxes in LK sheep erythrocytes at normal and swollen volumes (osmolalities of the media, 300 and 245 mosm/kg, respectively). Measurements were made in both Cl^- and methyl sulfate media. Results shown are mean values from three separate, identical experiments, on cells from three different sheep, each carried out in triplicate. Six $[K]_o$'s were used (20–120 mM). Fluxes are given in reciprocal mmol · liter^{-1} · hr^{-1}. The lines drawn through the points were obtained from nonlinear least-squares computer fits of the data to an equation for a hyperbola (*see* Materials and Methods). The lines were drawn using means of estimates of the kinetic constants for the three experiments. Nearly the same results were obtained from computer fits to the mean fluxes for the three experiments

$[K]_c$ and $[K]_o$ are cellular and external concentrations of K^+, respectively. K_c and K_o are cellular and external K^+ without reference to concentration. The same notation is used for Mg^{2+}. LK and HK are the low $[K]_c$ and high $[K]_c$ phenotypes of sheep, respectively.

Results

Kinetics of Cl-Dependent K^+ Influx in Normal and Swollen Cells

Figure 1 shows double reciprocal plots of Cl-dependent unidirectional K^+ influxes in cells of normal volume and cells swollen ~25% (osmolalities 290 and 235 mosm/kg, respectively). The results are from three experiments on cells from three different sheep. Cl-dependent influx was obtained from the difference between influxes in Cl^- and Cl-free media (with methyl sulfate the substitute), measured simultaneously. The lines were drawn from computer fits using the means of kinetic constants estimated separately for the data from each experiment. These

Table 1. Kinetic constants for Cl-dependent K^+ influxes in LK sheep erythrocytes of normal and swollen volumes

Volumes	Kinetic constants	
	J_{max}	$K_{1/2}$
Normal	3.1 ± 0.4	84 ± 17
Swollen	6.0 ± 0.9	58 ± 5

J_{max} is in mmol · liter^{-1} · hr^{-1}; $K_{1/2}$ for $[K]_o$ is in mM. The constants are means ± asymptotic SE estimated by computer fit to the mean fluxes shown in Fig. 1.

mean constants are shown in Table 1. Swelling stimulated the maximum influx about twofold and caused a decrease in $K_{1/2}$ of more than 30%. There were several-fold differences in the Cl-dependent K influxes among the different sheep, as we and others have observed before. However, in cells from all three sheep, swelling increased both maximum velocity and apparent affinity for K_o. The statistical significance of the difference between constants will be addressed below when the results of these three experiments and of three other similar experiments are summarized. Insight into how swelling might increase affinity for K_o will come from the results below from experiments on inhibition by DTT. We will also support the view the increase in J_{max} and decrease in $K_{1/2}$ are separable phenomena.

Cl-Independent K^+ Influxes

Figure 2 shows double reciprocal plots of the fluxes in methyl sulfate media, obtained in the three experiments in Fig. 1, and in a fourth experiment carried out in Cl-free media only. Mean fluxes at $[K]_o$ 20–120 mM are shown for normal and swollen cells. Swelling appeared to stimulate slightly at $[K]_o < 80$ mM, but the effect was neither consistent nor significant. An early report noted a lack of an effect of swelling on Cl-independent fluxes in sheep cells (Lauf, 1984). More recently Lauf (1988*c*) showed that K^+ influx was stimulated by swelling in Cl-free media with I^-, NO_3^-, or SCN^- as the substitute anion. It had not been reported earlier that the K^+ influx in Cl-free media is saturable as it appears to be in Fig. 2. It is possible that this effect owes to methyl sulfate serving as a low affinity Cl substitute. Alternatively the transport of K^+ through a ouabain-insensitive, Cl-independent pathway is a saturable function of $[K]_o$, albeit with a low affinity ($K_{1/2} > 100$ mM). The apparent maximum velocity of the Cl-independent influx is substantial (>2 mmol · liter^{-1} · hr^{-1}), but at physiological $[K]_o$, 5 mM, the flux is only about 0.1 mmol · liter^{-1} · hr^{-1}.

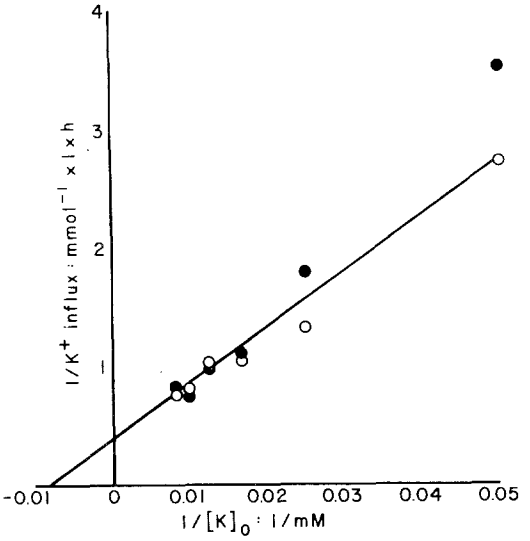


Fig. 2. Double reciprocal plots of ouabain-insensitive Cl-independent unidirectional K^+ influxes in LK sheep erythrocytes with varying $[K]_o$. Cells were of normal (●) or swollen (○) volumes (media of 300 or 245 mosm/kg, respectively). Methyl sulfate was the Cl^- substitute. The line was fitted by a nonlinear least-squares program to the means of the fluxes at the two osmolarities. The kinetic constants generated by the computer fit were: J_{max} = 2.62 ± 0.48 mmol · liter^{-1} · hr^{-1} and $K_{1/2}$ = 119 ± 37 mM (± asymptotic SE). Shown are means from four experiments, the three in Fig. 1 and a fourth one in which only Cl-free media were used.

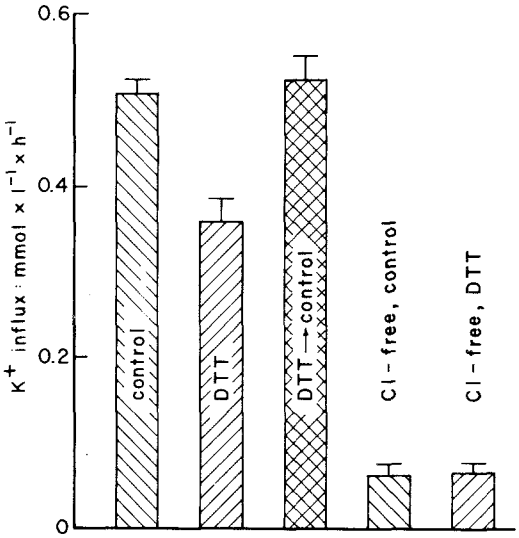


Fig. 3. Reversibility and Cl dependence of the inhibition by dithiothreitol (DTT) of ouabain-insensitive K^+ influx in LK sheep erythrocytes. Cells were washed in an isotonic medium containing (in mM): NaCl 145, KCl 5, glucose 5, Tris-HCl 10, pH 7.4, 290 mosmol/kg. Aliquots of cells were made Cl free by four washes in a medium of similar composition except that all Cl^- was replaced by NO_3^-. Aliquots of Cl^- and NO_3^- cells were incubated for 20 min at 37°C in media containing 0.5 mM DTT. An aliquot of Cl^- cells in DTT was washed free of DTT by two centrifugations and resuspensions in the isotonic Cl^- medium ("DTT→control"). Unidirectional K^+ influxes were measured in triplicate. Shown are means ± SD (n = 3) in mmol · liter^{-1} · hr^{-1}.

Inhibition of K^+ Influx by Dithiothreitol

This reducing agent was employed in the hope that it would help illuminate the effect of cell swelling on kinetics of K^+ influx. Figure 3 shows that incubation of LK sheep erythrocytes with 0.5 mM DTT for 20 min inhibited K^+ influx by about 30%, the same level of inhibition reported earlier for DTT in LK cells (Lauf, 1984). Figure 3 shows two other important features of the inhibition by DTT. First, it is freely reversible; washing the cells by centrifugation after the 20-min incubation with DTT completely abolished inhibition. Second, the inhibition is entirely of the Cl-dependent component of influx; there was no inhibition of K^+ influx by DTT in cells in Cl-free medium (with NO_3^- as the substitute anion). DTT inhibited about one-third of Cl-dependent K influx. The time course of the inhibition by DTT was investigated; maximal effect was reached before 20 min (*results not shown*). Thus in experiments with DTT, its binding was at equilibrium since the preincubation was for 30 min.

A potentially useful feature of the K-Cl cotransporter in LK cells is its association with the L_1 blood group antigen. Alloimmune anti-L_1 antiserum inhibits ouabain-insensitive K^+ transport in LK sheep red cells (Ellory et al., 1972; Dunham, 1976*a*,*b*; Lauf, Stiehl & Joiner, 1977). The effect of the antibody is entirely on the Cl-dependent, volume-sensitive transport system (Dunham & Ellory, 1981). There is a relationship between the critical SH groups and the L_1 antigen because anti-L_1 prevents the stimulation of K transport by NEM (Logue et al., 1983; Lauf, 1984). Therefore, we tested if inhibition by DTT and anti-L_1 could be by a common mechanism. The results in Fig. 4 show that the inhibitory effects of DTT and anti-L_1 antibody, at maximal doses of each, are additive. Therefore, the two agents are apparently not acting by a common mechanism or at the same site. Figure 4 also shows that 0.5 mM DTT caused near maximal inhibition; this concentration of DTT was used in all subsequent experiments. DTT at maximal effect does not inhibit Cl-dependent K^+ influx completely, even though DTT inhibition appears to be specific for this pathway. This same is true of anti-L_1.

Since effects of DTT and anti-L_1 are additive, DTT may not be specific for LK cells. Erythrocytes from sheep of the HK (high $[K]_c$) phenotype have much lower ouabain-insensitive K fluxes than LK cells have (Dunham & Hoffman, 1971). HK cells also lack the L_1 antigen, and their K transport is

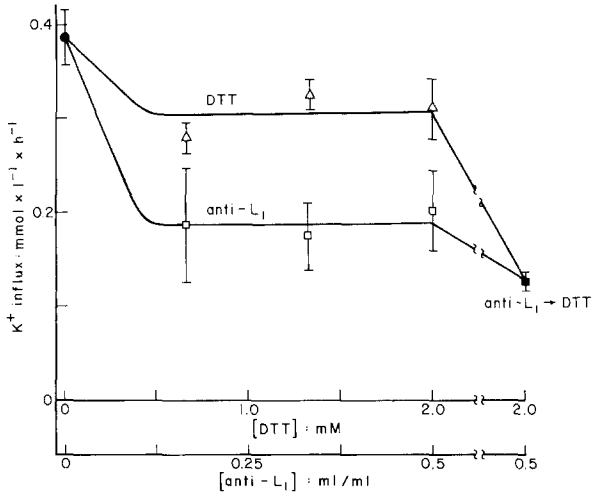


Fig. 4. Inhibitory effects of DTT and anti-L_1 antibody on ouabain-insensitive K^+ influxes in LK sheep erythrocytes. Procedures were the same as in the experiment in Fig. 3. Aliquots of cells were incubated for 20 min at 37°C in anti-L_1 antiserum diluted as indicated with the isotonic medium (ml antiserum/ml medium) and then washed twice. Incubation with media containing DTT was also for 20 min at 37°C. For these cells, DTT was also present during the flux. For cells incubated in both inhibitors, anti-L_1 preceded DTT. Values for fluxes are means ± SD (n = 3). The same results were obtained in another similar experiment

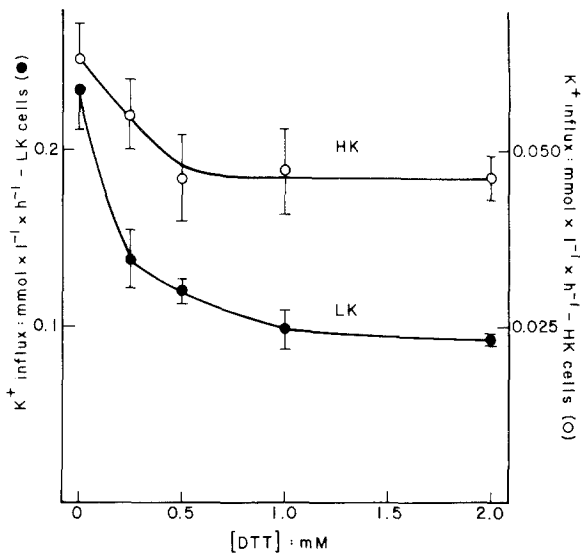


Fig. 5. Inhibitory effect of DTT on ouabain-insensitive unidirectional K^+ influx in HK and LK sheep erythrocytes; concentration-dependence of DTT's effect. Experiments were carried out the same as those in Fig. 3 and Table 2. Shown are means ± SD (n = 3)

unaffected by alloimmune anti-L_1 antiserum. However, ouabain-insensitive K^+ transport in HK sheep cells is stimulated by osmotic cell swelling (Ellory & Dunham, 1980), is partially Cl dependent, and is stimulated by NEM (Fujise & Lauf, 1987). Therefore a fraction of the relatively low ouabain-insensitive K^+ transport in HK cells appears to be through a K-Cl cotransporter. Accordingly DTT was tested on ouabain-insensitive influx in HK cells. As shown in Fig. 5, there was significant inhibition in HK cells. Concentration dependence of inhibition by DTT was compared in HK and LK cells and was found to be similar. The inhibition by DTT in these LK cells was to a greater extent than in the experiments in Figs. 2 and 3, by about 60%. The results in Fig. 5 suggest that DTT may be a general inhibitor of K-Cl cotransport. DTT is probably not an inhibitor of Na-K-Cl cotransport since 1 mM DTT had no effect on unidirectional K^+ influx in human red cells (Wiater & Dunham, 1983), which normally lack K-Cl cotransport (Duhm, 1987). Therefore DTT may be specific for K-Cl cotransport.

We wished to determine if DTT inhibits K-Cl cotransport by interaction with the same sulfhydryl groups at which NEM stimulates cotransport. DTT reduces disulfide bonds and maintains them reduced; NEM alkylates reduced sulfhydryls. Results were obtained which are consistent with the view of common sites of action. Table 2 shows inhibition of K^+ influx by DTT, stimulation of it by NEM, and the failure of DTT to inhibit K^+ influx in NEM-treated cells. This is also consistent with the conclusion of Lauf (1988*b*) that diamide and NEM act at the same locus, and that DTT reverses the effects of diamide.

Table 2. Effects of DTT and NEM on unidirectional K^+ influx in LK sheep erythrocytes

Treatment	K^+ influx (mmol · liter^{-1} · hr^{-1})
Control	0.33 ± 0.04
DTT (0.5 mM)	0.26 ± 0.005
NEM (0.75 mM)	0.93 ± 0.09
NEM → DTT	0.96 ± 0.03

Procedures were the same as for the experiment in Fig. 3. For the NEM treatments, cells were incubated at 0.75 mM NEM in the isotonic Cl^- medium at 10% hematocrit for 15 min, and then washed free of NEM by three successive centrifugations. DTT treatment, following NEM, was carried out as described for Fig. 3. Fluxes are means ± SD (n = 3).

Kinetics of Inhibition by DTT

Figure 6 shows double reciprocal plots of Cl-dependent unidirectional K^+ influxes, ±DTT, in swollen cells (245 mosm/kg media) and in cells near normal volume (310 mosm/kg media). Mean fluxes are

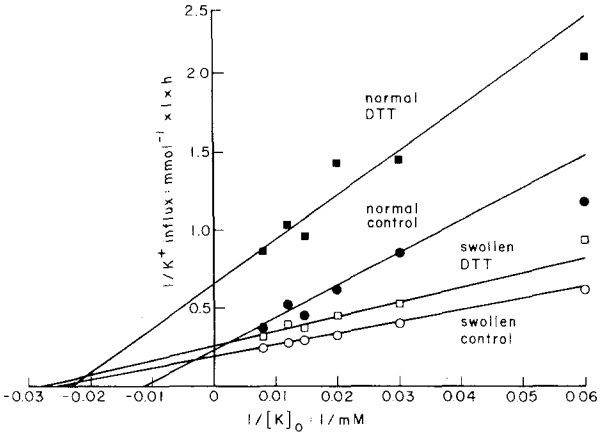


Fig. 6. Double reciprocal plots of Cl-dependent unidirectional K^+ influxes measured at six $[K]_o$'s (17–125 mM) in LK sheep erythrocytes ± DTT, normal and swollen volumes (media of 310 or 245 mosm/kg). Aliquots of cells of both volumes were treated with 0.5 mM DTT (20 min, 37°C) before the flux; DTT was also present during measurement of the fluxes. Results shown are mean values from three experiments on cells from three different sheep. All of these experiments were carried out in Cl medium. Cl-dependent fluxes were calculated by subtracting Cl-independent fluxes calculated for each $[K]_o$ from the kinetic constants for the curve in Fig. 2. Fluxes shown are in reciprocal mmol · liter^{-1} · hr^{-1}. Symbols: ●: normal control; ■: normal DTT; ○: swollen control; □: swollen DTT. The lines drawn through the points are computer fits to the data obtained as described in Materials and Methods. The estimates of kinetic constants generated by computer are shown in Table 3. The lines drawn were obtained using the mean fluxes. Nearly the same results were obtained from computer fits to the individual fluxes, or to the means from the three experiments.

Table 3. Kinetic constants for Cl-dependent unidirectional K^+ influxes in LK sheep erythrocytes of normal and swollen volumes ± DTT

Cells	Kinetic constants			
	Control		DTT-treated	
	J_{max}	$K_{1/2}$	J_{max}	$K_{1/2}$
Normal	4.2 ± 0.7	90 ± 14	1.5 ± 0.1	43 ± 11
Swollen	5.4 ± 0.1	38 ± 2	3.9 ± 0.3	36 ± 8

The constants shown ± asymptotic SE were obtained by computer fit to the mean fluxes from the three experiments in Fig. 6.

Table 4. Kinetic constants of Cl-dependent K^+ influx for cells of normal and swollen volumes from the experiments in Fig. 1 (Table 1) and Fig. 6 (Table 3)

	Kinetic constants				(*n*)
	Normal cells		Swollen cells		
	J_{max}	$K_{1/2}$	J_{max}	$K_{1/2}$	
Table 1	3.1 ± 0.4	84 ± 17	6.0 ± 0.9	58 ± 5	(3)
Table 3	4.2 ± 0.7	90 ± 14	5.4 ± 0.1	38 ± 2	(3)
Mean	3.7 ± 0.8	87 ± 10	5.7 ± 1.0	48 ± 6	(6)

Shown are means ± SE. Units are mM for $K_{1/2}$ and mmol · liter^{-1} · hr^{-1} for J_{max}; *n* is number of experiments. The means for both the $K_{1/2}$'s and J_{max}'s were significantly different between normal and swollen cells ($P = 0.016$ for both constants; randomization test for matched pairs; Siegel, 1956).

shown from three experiments at six $[K]_o$'s (17–125 mM). The cells were from three different sheep (none of these sheep were used in the experiments in Fig. 1). These experiments were carried out in Cl-media. Cl-dependent fluxes were obtained by subtracting Cl-independent fluxes calculated for each $[K]_o$ in Fig. 6 using the kinetic constants from the results in Fig. 2. The justification for this procedure will come from the comparison of the kinetic constants for Cl-dependent fluxes in Fig. 6 with those in Fig. 1, where measurements in Cl- and Cl-free media were made on the same cells at the same time. The mean kinetic constants for the four sets of Cl-dependent fluxes in Fig. 6 are shown in Table 3: shrunken cells ±DTT and swollen cells ±DTT. These constants were calculated in the same way as those in Table 1.

One of the inhibitory effects of DTT in normal cells was to reduce J_{max} more than twofold, as shown in Table 3. There was also a large increase in apparent affinity for K_o caused by DTT; $K_{1/2}$ was reduced in all three experiments, and the mean reduction was ~50%. The inhibition by DTT resembles uncompetitive inhibition, in which the increased apparent affinity is due to preferential binding of the inhibitor to the enzyme-substrate complex and stabilization of the complex. Thus the inhibition of K^+ influx by DTT in cells of normal volume, with the increased apparent affinity for K_o, can be explained in part by stabilization of a transporter-substrate (K_o) complex. However, it is not true uncompetitive inhibition because the lines are not parallel in the double reciprocal plots, ±DTT (Fig. 6).

In swollen cells, DTT had much less inhibitory effect than in normal cells. J_{max} was reduced by only about one-quarter. The $K_{1/2}$, which had been reduced by swelling, was unaffected further by DTT. Thus swelling of the cells mitigates the inhibitory effect of DTT, raising the possibility that DTT does not bind directly to the transporter. Finally the kinetic constants can be compared between DTT-treated cells of normal and swollen volumes. The $K_{1/2}$, reduced by DTT in normal cells, was not reduced further by swelling of DTT-treated cells.

Table 4 summarizes the kinetic constants for control (no DTT, normal Mg^{2+}) cells swollen and

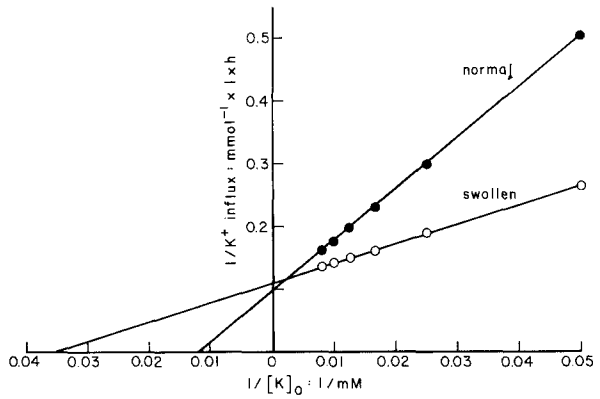


Fig. 7. Double reciprocal plots of Cl-dependent K^+ influxes in low-Mg^{2+} cells. Fluxes were measured at six $[K]_o$'s (20–120 mM) in LK sheep erythrocytes of normal and swollen volumes after pretreatment with A23187 to lower $[Mg]_c$ (*see* Materials and Methods). Osmolalities of the media, both with Cl^- and methyl sulfate, were 295 or 235 mosm/kg. Results are from one experiment carried out in triplicate. The lines through the points were obtained from nonlinear least-squares fits.

near normal volumes from the experiments in Figs. 1 and 6 (Tables 1 and 3). Table 4 is also presented to justify calculating the Cl-dependent fluxes shown in Fig. 6 from Cl-independent fluxes measured in separate experiments. Though there are differences, they are not systematic, and probably are not attributable to the method for obtaining the Cl-dependent fluxes in Fig. 6 and Table 3. The biggest difference between the two sets of results is in the $K_{1/2}$ for swollen cells, but in both cases it is lower than for cells of normal volume. The mean fluxes reinforce the conclusion drawn from the results in Fig. 1 and Table 1: swelling increases the maximum velocity of K-Cl cotransport by about 60%, and also has a substantial effect on the apparent affinity of the transporter for K^+, reducing $K_{1/2}$ nearly 50%.

Kinetics of Cl-Dependent K^+ Influx in Low-Mg^{2+} Cells

Since Mg^{2+} is an inhibitor of K-Cl cotransport in both sheep and human red cells (Lauf, 1985*b*; Sachs, 1988), we investigated the effect of swelling on kinetics of Cl-dependent K^+ influx in cells which had been pretreated with A23187 in order to reduce intracellular Mg^{2+} activity. Figure 7 shows a double reciprocal plot of fluxes from such an experiment. The kinetic constants are in Table 5. Reducing $[Mg]_c$ caused an increase in maximum velocity, compared to control cells, to 10 mmol · liter^{-1} · hr^{-1} from about 6 mmol · liter^{-1} · hr^{-1} in cells of normal volume. Significantly, swelling of low-Mg^{2+} cells did not increase J_{max} of K^+ influx. Swelling-induced stimulation is entirely attributable to an increase of twofold or more in apparent affinity for K_o.

Table 5. Kinetic constants for Cl-dependent K^+ influxes in LK sheep erythrocytes with reduced $[Mg]_c$ and at normal and swollen volumes

Volumes	Kinetic constants	
	J_{max}	$K_{1/2}$
Normal	10.5 ± 2.8	85 ± 44
Swollen	9.0 ± 0.2	28 ± 2

J_{max} is in mmol · liter^{-1} · hr^{-1}; $K_{1/2}$ in mM. The constants ± asymptotic SE were estimated by computer fit to the results from one experiment, shown in Fig. 7.

An experiment was carried out to investigate interactions between DTT, Mg_c, and swelling in modifying K-Cl cotransport. Cells were treated with DTT, with A23187 to lower $[Mg]_c$, and with DTT following A23187. These and control cells were tested in Cl- and methyl sulfate-media at normal and swollen volumes. $[K]_o$ was 10 mM. The Cl-dependent fluxes are shown in Table 6. Reducing $[Mg]_c$ stimulated the flux, as expected. Swelling of both control cells and low-Mg^{2+} cells also stimulated transport. Stimulation of transport by reducing $[Mg]_c$ in cells of normal volume (Table 6) probably is a consequence of an increased J_{max} because swelling of control cells stimulates by increasing both J_{max} and affinity for K_o (Table 4), and swelling of low-Mg^{2+} cells stimulates only by increasing apparent affinity (Table 5). The inhibition by DTT in control cells, both normal and swollen, is greater in Table 6 than in experiments in Figs. 3, 4, and 6 and about the same as in Fig. 5. The variability is probably attributable to variability among cells from different sheep.

The matrix diagram at the bottom of Table 6 may aid in the examination of the results. The results in the matrix are presented as though the experiment had been started with low-Mg^{2+}, swollen cells (0 inhibitory treatments). Aliquots of these cells receive one of the three inhibitory treatments (Mg^{2+}, shrinkage, or DTT), designated 1, 2, or 3, respectively. Each of these three aliquots then receives one of two additional inhibitory treatments. For example shrunken cells receive either Mg^{2+} or DTT, treatments 6 or 7. Treatment 2 followed by 6 yields the same cells as treatments 1 and 4. The three aliquots of cells which have received two inhibitory treatments each receive a third, and one type of cell is obtained, with normal volume and Mg^{2+}, and DTT treated. Procedures entailing removal of an inhibitor

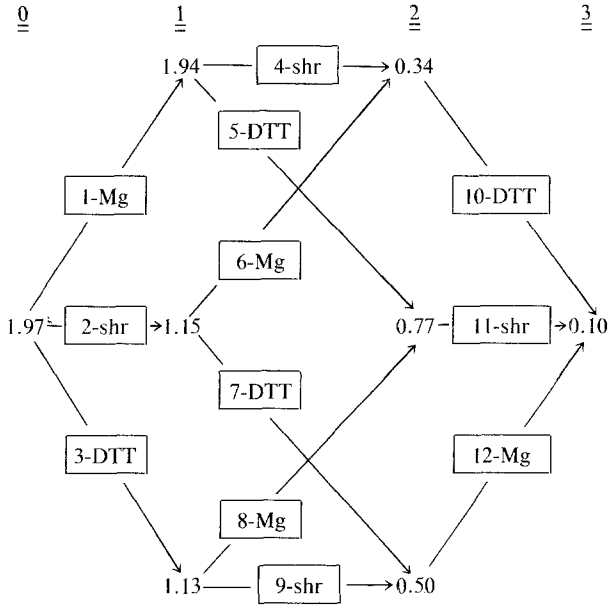
Table 6. Effects of $[Mg]_c$ and DTT on Cl-dependent K^+ influx in LK sheep red cells of normal and swollen volumes

Cell volume	K^+ influx (mmol · liter^{-1} · hr^{-1})			
	Control	Low Mg^{2+}	DTT	Low Mg^{2+} → DTT
Normal	0.34 ± 0.02	1.15 ± 0.02	0.10 ± 0.006	0.50 ± 0.02
Swollen	1.94 ± 0.08	1.97 ± 0.03	0.77 ± 0.02	1.13 ± 0.05

The flux media were similar to those described in Materials and Methods for kinetic experiments; all contained a single $[K]_o$, 10 mM. The osmolalities were 290 (normal) and 235 (swollen). Low-Mg^{2+} cells were prepared as described in Materials and Methods. Treatment with DTT was the same as in Fig. 3 (0.5 mM, 20-min preincubation; also present during the flux). Cl-dependent fluxes were determined as in Fig. 1. Shown are means ± SE of differences. The same results are also displayed in the following matrix (*see* text for explanation).

K^+ influx

Number of inhibitory treatments



Inhibitory treatments: Mg, increased $[Mg]_c$; DTT, 0.5 mM DTT; shr, osmotic cell shrinkage.

are designated by a negative number (e.g., swelling cells of normal $[Mg]_c$ is treatment −4).

Of the 12 treatments in the matrix in Table 6, four had been examined in more detail in Table 3. These were treatments −4 and 10 (swelling and DTT treatment of cells of normal volume and Mg^{2+}), and −11 and 5 (swelling of DTT cells and DTT treatment of swollen cells). Effect of treatment −2 (swelling of low-Mg^{2+} cells) is shown in Table 5.

The result of treatment −1 in Table 6 is surprising: lowering $[Mg]_c$ in swollen cells had no effect on

Table 7. Summary of kinetics constants for K-Cl cotransport in cells of normal and swollen volumes

	Normal		Swollen	
	J_{max}	$K_{1/2}$	J_{max}	$K_{1/2}$
Control	3.7 ± 0.8	87 ± 10	5.7 ± 1.0	48 ± 6
DTT	1.5 ± 0.1	46 ± 10	3.9 ± 0.3	36 ± 8
Low Mg	10.5 ± 2.8	85 ± 44	9.0 ± 0.2	28 ± 2

Control and DTT: means ± SEM, n = 6 and 3, respectively; low Mg: one experiment ± asymptotic SE.

cotransport. Comparison of results in Tables 4 and 5 leads to the prediction that treatment −1 should increase both J_{max} and $K_{1/2}$ about twofold, though the difference in $K_{1/2}$'s is of marginal statistical significance and was obtained in separate experiments. Since the $[K]_o$ in the experiment in Table 6, 10 mM, was well below $K_{1/2}$, a large change in transport in response to treatment −1 need not have been expected.

Of the remaining five treatments in Table 6 not tested in other experiments, four involve combined effects of Mg^{2+} and DTT (treatments 3, 7, 8, and 12). These four treatments all inhibited cotransport to substantial extents, showing that the inhibitory effects of DTT and Mg^{2+} are additive.

Discussion

We have investigated the kinetics of volume-sensitive Cl-dependent K^+ transport, presumably K-Cl cotransport, in LK sheep erythrocytes. We have measured Cl-dependent K^+ influxes in cells near normal volume and in swollen cells as functions of $[K]_o$, and we have looked at the effects on the kinetics of DTT, and of lowering $[Mg]_c$; both DTT and Mg^{2+} are reversible inhibitors of cotransport. Some of the results are summarized in Table 7. Our first observation is that osmotic swelling stimulates K^+ influx by increasing both J_{max} and the apparent affinity for K_o. The second observation is that DTT inhibited K-Cl cotransport in cells of near normal volume by reducing both J_{max} and $K_{1/2}$. Thus DTT appears to inhibit in part by stabilizing the transporter-K_o complex. This resembles uncompetitive inhibition, in which the inhibitor binds preferentially to the complex, thereby raising the apparent affinity for the substrate. The third observation is that DTT had little inhibitory effect on swollen cells. There was no effect on $K_{1/2}$, and J_{max} was reduced less than 30% (in contrast, DTT reduced $K_{1/2}$ more than 50% and

J_{max} more than 60% in cells of normal volume; Table 7). The reduction in the effect of DTT caused by cell swelling is consistent with the view that DTT does not bind directly to the transporter, but rather to an adjacent "regulator." [Lauf (1984) proposed that the site of action of NEM in stimulating K^+ transport is not the transporter, and Table 2 suggests that DTT and NEM act at the same site.] It follows that the putative regulator serves as an endogenous inhibitor, and further, that stimulation of transport by swelling is in part due to relief of the effect of this inhibitor.

The experiment with low-Mg^{2+} cells (Table 5) helps clarify the results on effects of swelling by showing that stimulation by increasing J_{max} and by decreasing $K_{1/2}$ are separable phenomena. Swelling of low-Mg^{2+} cells raises apparent affinity for K_o, but has no effect on J_{max}. The summary of results in Table 7 suggests that lowering $[Mg]_c$ in cells of normal volume stimulates by raising J_{max}, with no effect on $K_{1/2}$. Reducing $[Mg]_c$ may activate latent transport sites. Results in Table 6 show that the inhibitory effects of Mg and DTT are additive. In our scheme, then, there are two endogenous inhibitors of the cotransporter, the hypothetical regulator (DTT's site of action), which controls affinity of functioning transporters, and Mg_c, which reduced J_{max}, perhaps by holding some transporters in an inactive form. Swelling of normal cells reduces both of these modes of inhibition. While there are probably other schemes of interpretation which we cannot rule out, ours fits the observations qualitatively in a straightforward manner.

It might be useful to consider our results in terms of the probable nature of the signal to the volume sensor in response to an increase in cell volume. As stated above the signal is likely to be a mechanical change in the membrane rather than the dilution of a critical cytoplasmic solute (Sachs, 1988; Kracke & Dunham, 1989). The precise nature of the mechanical signal is a complete mystery, except that it is not a pressure, and it does not depend on membrane orientation since inside-out vesicles respond to swelling in the same way as intact cells (Kracke & Dunham, 1989).

Stimulation by swelling may entail reduced inhibition by Mg_c, but not, as argued above, as a consequence of dilution of cytoplasmic Mg^{2+} activity. In addition to the reasons just given, swelling of 25% is unlikely to reduce Mg^{2+} activity enough to stimulate transport significantly (Lauf, 1985*b*). Furthermore intracellular buffering of [Mg] will reduce the extent of the change.

There is a membrane-associated compartment containing ATP used by the Na/K pump (Mercer & Dunham, 1981). We propose that such a compartment also contains the elements of the signal which respond to volume changes, and the sensor of these signals. We propose further that the signal, a response to mechanical rearrangements in the compartment, is a change in concentration of a solute, perhaps Mg, ATP, and/or binding sites for these solutes. These binding sites may include enzymes activated or inhibited by the change in solute concentration in the membrane-associated compartment.

The apparent association of phosphate metabolism to volume sensitivity is complex (Dunham, 1990) and includes some apparent contradictions, e.g., the apparent requirement of phosphorylation by ATP for volume-sensitive K-Cl cotransport (Sachs, 1988; Kracke & Dunham, 1989), and the evidence that activation of a phosphatase accompanies stimulation by swelling (Jennings & Al-Rohil, 1990). We suggested that ATP phosphorylates a regulator and not the transporter (Kracke & Dunham, 1989), and further that the phosphatase proposed by Jennings and Al-Rohil (1990) may be activated by phosphorylation. Conceivably, the regulator we propose here, based on results with DTT, is the phosphatase activated by ATP. This is obviously all highly speculative.

One clear requirement for the system coupling swelling to an increase in cotransport is a high gain; a relatively small volume increase (~10%) stimulates cotransport by several-fold (Dunham & Ellory, 1981). Regulatory systems with high gain often entail a series of enzymes. We have proposed two systems, reduction of inhibition by Mg and by the DTT-binding regulator (how these are activated by swelling, or more specifically by a mechanical change in a membrane compartment, is unclear). The systems we have proposed lead to independent increases in J_{max} and affinity for K_o. These two changes could be in series rather than in parallel, thereby contributing to gain, particularly if the relief of inhibition envisioned leads to activation of enzymes. Obviously these suggestions are also entirely speculative.

Another treatment has been reported to inhibit K-Cl cotransport in LK cells by reducing both J_{max} and $K_{1/2}$. Ellory et al. (1985*a*) reported that reducing the temperature by 10°C during measurement of the flux (from 40 to 30°C) reduced J_{max} of the volume-sensitive component of K^+ influx nearly 10-fold (from 2.9 to 0.3 mmol · liter^{-1} · hr^{-1}), and reduced $K_{1/2}$ for K_o nearly twofold (from 64 to 38 mM). It is unclear why two very different inhibitory treatments, DTT and reduced temperature, have similar effects on kinetics.

We have measured the kinetics of volume-sensitive K^+ influx, and the physiological role of volume-sensitive K-Cl cotransport is to permit net KCl ef-

flux. Thus the question arises of the physiological relevance of our findings. There are a number of observations on K-Cl cotransport in sheep red cells which indicate that it is a reversible system. Indeed the apparent affinities for K^+ are similar, though not identical, for influxes and effluxes (Lauf, 1983, 1985*a*). Therefore our observations on kinetics of unidirectional influxes should apply at least qualitatively to K-Cl cotransport in its physiological mode. It remains to be demonstrated in sheep cells to what extent swelling-induced changes in the properties of the cotransporter operating in the influx mode apply to K-Cl efflux.

This work was supported by grants from the National Institutes of Health, U.S. Public Health Service (DK-28290, DK-33640).

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Received 11 December 1989; revised 8 March 1990