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PASSIVE POTASSIUM TRANSPORT IN LOW POTASSIUM SHEEP RED CELLS: DEPENDENCE UPON CELL VOLUME AND CHLORIDE

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SUMMARY

The major pathway of passive K influx (ouabain-insensitive) was characterized in low-K type (LK) red cells of sheep.

1. Passive K transport in these cells was highly sensitive to variations in cell volume; it increased threefold or more in cells swollen osmotically by 10%, and decreased up to twofold in cells shrunken 5–10%. Active K influx was insensitive to changes in cell volume. Three different methods for varying cell volume osmotically all gave similar results.

2. The volume-sensitive pathway was specific for K in that Na influx did not vary with changes in cell volume.

3. The volume-sensitive K influx was a saturable function of external K concentration. It was slightly inhibited by Na, whereas K influx in shrunken cells was unaffected by Na.

4. Passive K influx was dependent on the major anion in the medium in that replacement of Cl with any of six other anions resulted in a reduction of K influx by 50–80% (replacement of Cl by Br caused an increase in K influx). The activation of K influx by Cl followed sigmoid kinetics.

5. Passive K influx is inhibited by anti-L antibody. The antibody affected only that portion of influx which was Cl-dependent and volume-sensitive. Of the subfractions of the antibody, it is anti-L_1 which inhibits passive K transport.

6. Pretreatment of cells with iodoacetamide reduced the sensitivity of K influx to cell volume in that the influx was reduced in swollen IAA-treated cells and increased in shrunken IAA-cells.

7. Intracellular Ca has no role in altering passive K transport in LK sheep cells.

Therefore, the major pathway of passive K transport in LK sheep red cells is sensitive to changes in cell volume, specific for K, dependent on Cl, and inhibited by anti-L_1 antibody. The minor pathway, observed in shrunken cells, has none of these properties.

INTRODUCTION

The Na/K pump represents the major pathway for K transport in most mammalian cells, but evidence has been accumulating recently for other specific and significant mechanisms of K transport. For example, a characteristic of ouabain-insensitive K transport in certain cells is a dependence on chloride. Such an effect has been demonstrated for cardiac cells (Carmeliet & Verdonck, 1977), Ehrlich ascites cells

(Geck, Heinz, Pietrzyk & Pfeiffer, 1978; Geck, Pietrzyk, Burckhardt, Pfeiffer & Heinz, 1980; Bakker-Grunwald, Andrew & Neville, 1980), avian red cells (Schmidt & McManus, 1977*b*; Kregenow & Caryk, 1979), and recently human red cells (Dunham Stewart & Ellory, 1980; Chipperfield, 1980; see also Funder & Wieth, 1967, and Cotterrell & Whittam, 1971). Another parameter affecting K permeability in some cases is cell volume. In 1937 Davson showed in red cells of rabbit, horse, and ox an increased K permeability when the cells were swollen osmotically, in contrast with human or pig red cells. Similar volume effects have also been reported for red cells of dog and cat (Davson, 1940; Parker, 1977) and duck (Kregenow, 1974; Schmidt & McManus, 1977*a*, *b*), and also certain other cell types (e.g. see Roti Roti & Rothstein, 1973; Hendil & Hoffman, 1974; Doljanski, Ben-Sasson, Reich & Grover, 1974). In the present work we demonstrate the presence in LK sheep red cells of a relatively large ouabain-insensitive K flux which is both sensitive to changes in cell volume and dependent on chloride.

Red cells of sheep are interesting in that the species shows a genetically controlled dimorphism of 'K type', presenting two phenotypes with normal high (HK) or low (LK) intracellular K concentrations. A blood group antigen designated L is specifically associated with the LK phenotype, and it has been demonstrated that sensitization of LK cells with the anti-L antibody increases K influx through the Na/K pump (Ellory & Tucker, 1969) and decreases the ouabain-insensitive K influx component (Ellory, Sachs, Dunham & Hoffman, 1972; Dunham, 1976*a*; Lauf, Parmalee, Snyder & Tosteson, 1977). We have looked at the relationship between the volume sensitive (and Cl sensitive) passive K influx and the effect of anti-L on this pathway of K transport.

An important question regarding K transport outside the Na pump is its relationship to transport of other ions and its possible energy dependence. For human red cells the fact that K activates passive Na transport and Na activates passive K transport has been offered as evidence for Na/K cotransport (Sachs, 1971; Beauge & Adragna, 1971; Wiley & Cooper, 1974). The apparent Na/K cotransport system shares certain features with the Cl-activated K transport system (Dunham *et al.* 1980) and with the ouabain-insensitive 'pump II' of Hoffman & Kregenow (1966). It has been tentatively proposed that these systems may be cotransport mechanisms capable in some circumstances of performing transport against a gradient (Sachs, 1971; Sachs, Knauf & Dunham, 1975). Metabolic depletion in human (Sachs, 1971; Beaugé & Adragna, 1971) and dog (Romualdez, Sha'afi, Lange & Solomon, 1972) red cells alters passive K and Na movements, although no direct involvement of ATP in these effects has been demonstrated. Nevertheless it is clearly relevant to investigate the effects of inhibitors of metabolism on the volume-dependent K-transport system in LK sheep red cells.

A preliminary report of some of the observations presented here has been published (Ellory & Dunham, 1980).

METHODS

Blood was drawn by venipuncture from adult Dorset or Welsh Mountain sheep (with heparin as anticoagulant). The sheep were LK (determined by flame photometry of haemolysates of washed red cells); unless otherwise specified, the sheep were heterozygous (LM) at the locus controlling the 'K type' (determined by tests for lysis using the appropriate immune reagents; Tucker, 1965). The

cells were washed by centrifugation and resuspension in isotonic saline (NaCl 150 mM, glucose 5 mM, Tris-HCl 10 mM, pH 7·5).

Fluxes. Unidirectional influxes of K and Na were measured as described previously (Dunham & Ellory, 1980). Either ^{42}K or ^{86}Rb was used as a tracer for K influx (^{86}Rb unless otherwise specified). As will be shown the two isotopes gave similar results. ^{22}Na or ^{24}Na was used as a tracer for Na influx. Passive K influx (ouabain-insensitive) was taken as that measured with ouabain at 0·05 mM. Active K influx (oubain-inhibitable) was the difference between total influx and passive influx. There was no inhibition of Na influx by ouabain. Fluxes are given as either μmol or mmol per litre of packed cells per hour. The number of determinations, n, refers to parallel estimations in experiments using the same batch of cells. Errors are expressed as S.E. of means.

Cell volumes: alteration and measurement. Cell volumes were varied by one of three methods. In the first two, isotonic saline was diluted 10–15% with distilled water. The osmolarity of aliquots of this medium was increased by adding crystalline NaCl or sucrose up to 50 or 100 mM, respectively. Aliquots of cells were equilibrated with one of these media by repeated centrifugation and resuspension (4 ×) at room temperature. These two methods are referred to as the 'sucrose method' or the 'NaCl method.'

In some experiments cell volumes were varied by increasing reversibly membrane permeability to cations using the ionophore nystatin as described previously (Dunham & Blostein, 1976). Briefly the cells were exposed to nystatin solutions containing KCl (0–50 mM), NaCl (135–85 mM) ([KCl] + [NaCl] = 135 mM); Tris-HCl (10 mM) and nystatin (50 μg/ml). (Stock solutions of nystatin were made the day before use at 5 mg/ml. in methanol.) The solutions also contained sucrose between 40 and 80 mM. After incubation with nystatin, cells were washed free of the ionophore by centrifugation as described before (Dunham & Blostein, 1976). All aliquots of cells were then suspended in isotonic saline. As determined by the method given below, the volume of cells exposed to nystatin with about 60 mM-sucrose was near the volume of fresh cells. Cells exposed to nystatin with 80 mM-sucrose were shrunken when suspended in the saline with 150 mM-NaCl and cells exposed to nystatin with 40 mM-sucrose ended up swollen. This method of altering cell volume is referred to as the 'nystatin method'.

Cell volumes were measured on suspensions of cells with high haematocrit (30–50%; this to increase the accuracy of the measurement) by relating the haematocrit of the suspension and the optical density of cyanomethaemoglobin (in Drabkin's solution) in a dilution of a lysate of the suspension (measured at 540 nm). The o.d./ml. packed cells was calculated and related to the o.d./ml. of fresh (untreated) cells which was determined routinely and was always within 1% of 250. The volume of treated cells is expressed as r.c.v. (relative cell volume), i.e. the ratio of their volume to that of fresh cells: the ratio of o.d./ml., fresh cells to treated cells. The maximum range of cell volumes employed was 0·85–1·25. All fluxes were corrected so as to express them per litre of fresh cells (r.c.v. = 1·00), irrespective of the measured cell volumes during the experiments.

Replacement of chloride. Cells were washed 3 times in isotonic media with Cl replaced by another monovalent permeant anion at 150 mM. The solutions contained HEPES (neutralized with NaOH), rather than Tris-HCl, as a buffer. The cell suspensions were washed 3 times, equilibrated 30 min at 37 °C, washed once, equilibrated another 30 min, and washed 2 more times.

Antiserum. Alloimmune sheep anti-L antiserum was raised and prepared as described before (Tucker & Ellory, 1970; Dunham, 1976*b*). Cells were incubated with antiserum at 5% haematocrit at 37 °C for 30 min, and then washed twice by centrifugation before altering volumes and measuring fluxes.

For one experiment anti-L serum was separated into its two specificities, anti-L_1 and anti-L_p (Ellory & Tucker, 1970; Lauf, Parmalee, Snyder & Tosteson, 1971; Dunham, 1976*b*). The separation was carried out by absorptions with trypsinized LK sheep cells, which bind anti-L_1 but not anti-L_p. The absorbed serum contained anti-L_p; adsorbed anti-L_1 was then eluted from the trypsinized cells using an isotonic medium at pH 3·3 containing glycine-HCl. This method will be described in detail elsewhere (Smalley, Tucker, Dunham & Ellory, 1981).

Chemicals. Radioisotopes (^{22}Na, ^{24}Na, ^{42}K, ^{86}Rb) were obtained as chlorides in aqueous solution from International Chemical Corporation (Irvin, CA, U.S.A.) or from Amersham Radiochemical Centre (Amersham, Bucks., U.K.). Choline Cl was obtained either from Syntex Agri Business, Inc. (Springfield, MO, U.S.A.) or from Sigma Chemical Co. (London, U.K.). Choline Cl from Sigma was purified by recrystallization from a hot ethanol solution; that from Syntex was not purified. Ouabain was obtained from Sigma. HEPES and methyl sulphate salts were obtained from British Drug Houses (Poole, Dorset, U.K.).

Symbols and abbreviations. $[X]_c$ and $[X]_o$ indicate the intracelluar and external concentrations, respectively, of the solute X; X_c and X_o are used to indicate intracellular and external X without reference to concentration. HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Tris, tris-hydroxylmethylaminomethane; $MeSO_4$; methylsulphate; Ac, acetate; Iseth, isethionate; IAA, iodoacetamide; r.c.v., relative cell volume; V_{max}, maximum velocity of influx; $K_{\frac{1}{2}}$, substrate concentration at half-maximal flux (apparent Michaelis constant).

RESULTS

Passive K influx and cell volume. The relationship between passive K influx in LK sheep red cells and the volume of the cells is shown in Fig. 1. The volumes in this experiment were varied using the nystatin method. There was approximately an eightfold increase in passive K influx in cells with their volumes increased 10% and

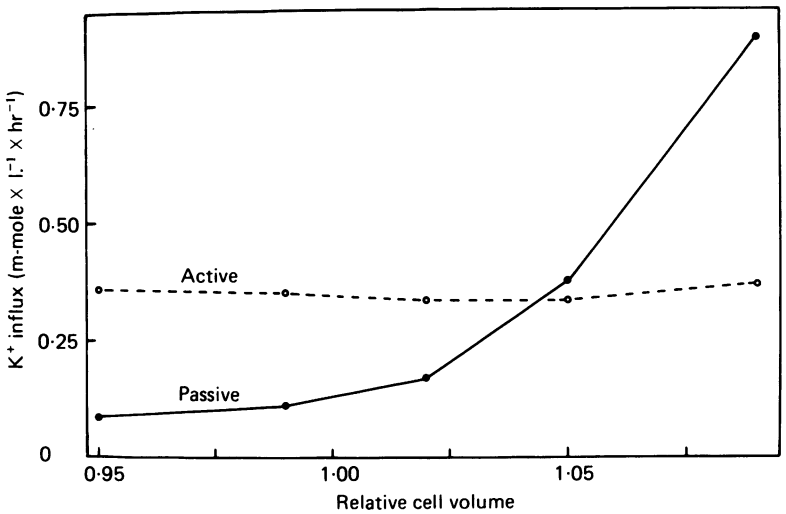

Fig. 1. The effect of varying cell volume on active (ouabain-inhibitable) and passive (ouabain-insensitive) K influx in LK sheep red cells. Cell volumes were varied using the nystatin method. $[K]_o$ = 5 mM (n = 2).

about a twofold decrease in cells shrunken 5%. Active K influx was nearly constant over this range of volumes, with perhaps a slight increase in shrunken cells as compared with swollen ones.

It is possible that the apparent sensitivity to volume of passive K influx is a consequence of some aspect of the method used to vary volume, and not the change in volume itself. We therefore measured passive K influx in cells with volumes varied by two other techniques, the sucrose and NaCl methods. Fig. 2 shows that the passive K influx varies with volume when it was changed by these two other methods as well. The effect on K transport is not likely to be a consequence of changes in electrochemical driving force since the three methods of varying volume can be expected to cause different patterns of changes in transmembrane electrical potential difference. Shrinkage by the sucrose method will cause depolarization ($[Cl]_i$ is increasing while $[Cl]_o$ is constant) and shrinkage by the NaCl method will cause a slight hyperpolarization (Freedman & Hoffman, 1979). Varying cell volume by the nystatin

method should cause little change in membrane potential since the aliquots of cells with different volumes all have the same salt concentrations; the volumes differ because the amounts of salt per cell vary. The concentrations of impermeant anions will vary, but their contribution to voltage will be slight.

The volume-dependent changes in K influx are completely reversible in both cells shrunken before swelling and swollen before shrinking (Ellory & Dunham, 1980).

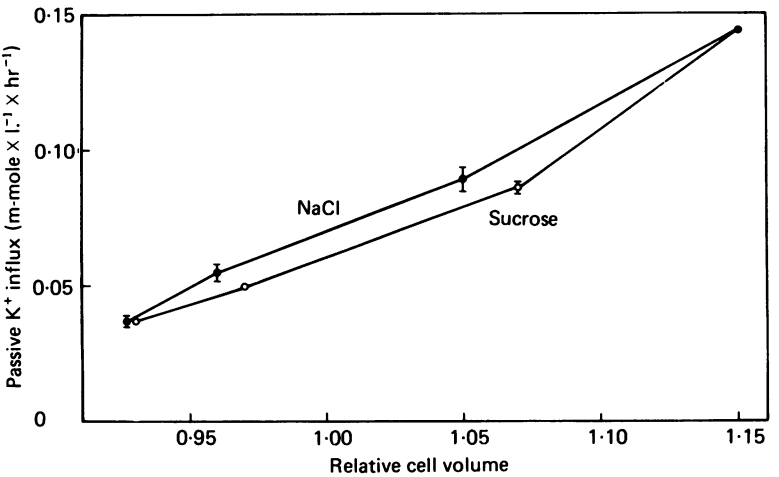

Fig. 2. Passive K influx in LK sheep red cells with their volumes varied by either the NaCl or the sucrose method. $[K]_o$ = 5 mM (n = 3).

Specificity of volume-dependent transport. Fig. 3 shows passive influxes of K and Na in cells of various volumes (r.c.v. 0·94–1·17). K influx varied threefold while Na influx varied 5%, showing that the volume-sensitive cation transport is selective for K. In this experiment active K influx is nearly invariant with changes in volume, with perhaps a slight increase in shrunken cells. The independence of Na influx of cell volume is further evidence that the change in K influx with changing volume is due to a specific effect rather than a consequence of changes in driving force due to a change in membrane potential. It is pertinent to note here that K efflux from sheep cells varied with cell volume in the same way as K influx (Ellory & Dunham, 1980), confirming that the volume-dependence is unrelated to changes in membrane potential (in which case efflux would have decreased if influx increased). Finally, the concomitant changes in influx and efflux also show that the volume sensitivity is not due to the changes in $[K]_c$ associated with changes in volume.

The shapes of the curves relating volume and K influx differ between experiments. As volume is increased, the slope of the curve may increase (Figs. 1 and 8), remain constant (Figs. 2 and 9); more complex curves were also obtained (Fig. 3). The significance of this varibility is not known, but none of the conclusions drawn depended on the shapes of these curves.

Kinetics of volume-dependent passive K transport. Passive K transport in LK sheep cells had been shown earlier to saturate as $[K]_o$ was raised (Dunham & Hoffman, 1971; Dunham, 1976*a*). (The much lower passive K influx in HK sheep cells was a linear

function of $[K]_o$.) It was also shown that replacing Na_o with choline resulted in a slight increase in passive K influx (Dunham, 1976*a*), indicating that the passive flux may normally be inhibited by Na. Fig. 4 shows the kinetics of passive K influx with varying $[K]_o$. The experiment was carried out with cells at two volumes, and with each of these types of cells in Na-media or choline-(Na-free) media. The lines in Fig. 4 were calculated using the Micaelis–Menten equation from the constants, V_{max} and

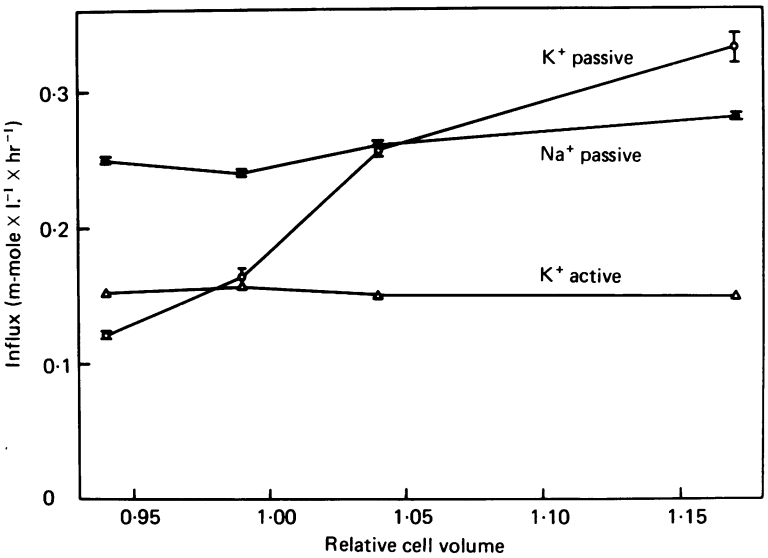

Fig. 3. Influxes of K and Na into LK sheep red cells with volumes varied by the sucrose method. Both passive and active K influxes are shown. Ouabain had no effect on Na influx. $[K]_o$ = 5 mM (n = 3). Similar results were obtained in three other experiments, including two in which cell volume was varied by the nystatin method.

$K_{\frac{1}{2}}$, obtained from double reciprocal plots of the data and given in the legend of Fig. 4. The results confirm the saturation kinetics of the passive K influx. For swollen cells in Na the $K_{\frac{1}{2}}$, 47 mM, was considerably higher than that previously reported (7·4 mM; Dunham 1976*a*), but in that earlier paper it was stated that the $K_{\frac{1}{2}}$ was often much higher than 7·4 mM. The results in Fig. 4 also confirm the increase in passive K influx in Na-free media (Dunham, 1976*a*).

Fig. 4 also shows passive K influx in shrunken cells, in both Na- and choline-media. In these cells there was no inhibition of K influx by Na. (Though the resolution was not great with these low fluxes, if they differed, the ones in Na were slightly higher than in choline.) The nature of the inhibition of K influx caused by shrinkage is complex (both $K_{\frac{1}{2}}$ and V_{max} changed) but the effect on V_{max} was the more striking (about a fourfold decrease). Since Na has no effect in shrunken cells, it follows that the volume-sensitive and Na-sensitive components of K influx are the same.

To show that ^{86}Rb, the tracer used in the experiment in Fig. 4 and most others in this study, is valid for measuring K influx, the kinetics of passive K influx were measured using ^{42}K as a tracer in swollen and shrunken cells (Na media). The results are shown in Fig. 5. The kinetic constants, given in the legend, are similar to those obtained using ^{86}Rb in swollen and shrunken cells in Na media (see legend, Fig. 4)

except for the V_{max} in swollen cells. This difference is certainly due in part to the difference in cell volumes (r.c.v. 1·18 and 1·22), though this may not be a sufficient explanation.

Replacement of Cl. Preliminary results presented earlier showed that equilibrating LK sheep cells in media with any of several permeant anions results in a marked reduction in passive K influx (Ellory & Dunham, 1980). $MeSO_4$, SO_4, and acetate all had this effect, and a low concentration of Cl (10 mM) doubled the passive K influx in cells in Cl-free $MeSO_4$ medium. It was concluded that the effect of Cl-free medium was due to the absence of Cl, and not to specific inhibitory effects of the substituted anions.

Recently Lauf & Theg (1980) presented results on the effects of replacing Cl on passive K efflux which are in conflict with ours. They found no effect of replacing

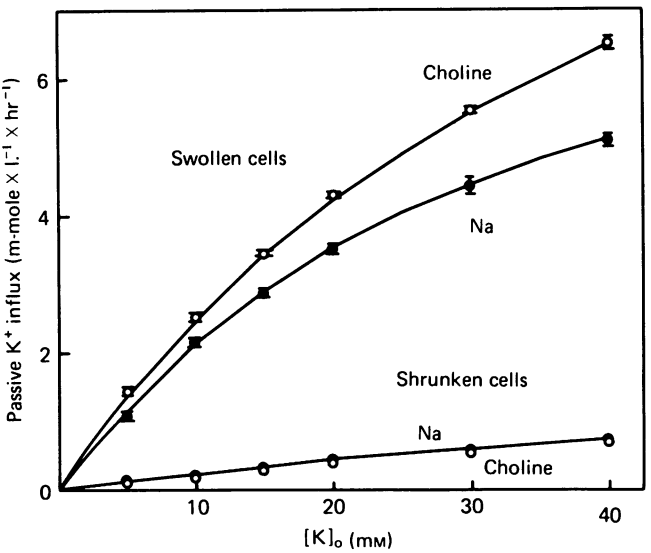

Fig. 4. The kinetics of passive K influx in LK sheep red cells (measured using ^{86}Rb). Fluxes were measured in cells at two volumes, varied by the sucrose method. For cells in Na, the Na concentration in the medium was constant (110 mM). K concentration was varied with total cation concentration kept constant (150 mM) with appropriate concentrations of choline. For fluxes measured in Na-free media, K and choline were varied reciprocally. The lines through the points were calculated as described in the text using the kinetic constants given below. The errors are not represented in the Figure when they were smaller than the points ($n = 3$).

Kinetic constants

	Swollen cells (r.c.v. = 1·22)		Shrunken cells (r.c.v. = 0·97)
	Choline	Na	Choline and Na
V_{max}	14	9·4	2·2
$K_{\frac{1}{2}}$	47	34	89

V_{max} is in m-mole/l. × hr; $K_{\frac{1}{2}}$ is in mM; r.c.v. is relative cell volume. The results were indistinguishable for shrunken cells in Na and choline, and one line was calculated from one set of constants for both conditions.

Cl with Br, a slight increase with SO_4, and only small (and statistically insignificant) decreases with NO_3 and PO_4. In light of this apparent controversy, we sought to confirm and extend our earlier preliminary observation, that K flux is reduced by replacement of Cl. Fig. 6 shows the results of an experiment in which seven monovalent, permeant anions were tested as Cl substitutes. There was a large increase in passive K influx with Br, which was surprising since passive K fluxes in human

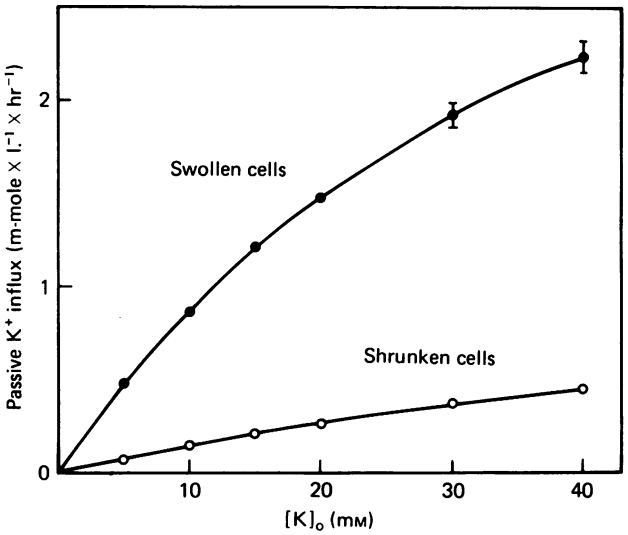

Fig. 5. Kinetics of passive K influx in LK sheep cells measured using ^{42}K. The experiment was carried out in the same way as in Fig. 4, except no fluxes were measured in Na-free media. The lines were calculated as described in the text using the kinetic constants given below.

	Kinetic constants	
	Swollen cells (r.c.v. = 1·18)	Shrunken cells (r.c.v. = 0·91)
V_{max}	4·6	1·7
$K_{\frac{1}{2}}$	42	100

The symbols and their units are the same as in the legend for Fig. 4.

red cells were the same or slightly reduced in Br medium as compared with Cl medium (Funder & Wieth, 1967; Dunham *et al.* 1980). There is no apparent explanation for this curious difference between sheep and human red cells. With all of the other anions, K influx was inhibited, a finding consistent with our preliminary results (Ellory & Dunham, 1980), but at odds with the results of Lauf & Theg. The greatest reduction in Fig. 6 was with acetate. This was a consistent finding, though the reduction in the flux was not always as large as in this experiment.

For reasons not entirely clear, the cells sometimes swelled in the Cl-free media (this was observed in all media in the experiment in Fig. 6; relative cell volumes are given in the Figure), and this increase in volume will cause an increased K influx. However, only with Br was there an increase in K influx, and the small increase in volume in cells in Br medium (3%) accounts for only a small part of the increase in K influx.

Fig. 7 shows the kinetics of the activation of passive K influx by $[Cl]_o$ ([Cl]) was varied by replacement with $MeSO_4$). At each [Cl], aliquots of cells were obtained with three cell volumes (by addition of sucrose); the r.c.vs were 1·00, 0·94, and 0·83.

In an attempt to determine the type of curve best fitting the data, each set of values was fitted to three models. The first was the Hill equation:

$$v = \frac{V_{max}}{(K_H/1 + [Cl]_o^h)} + B, \quad (1)$$

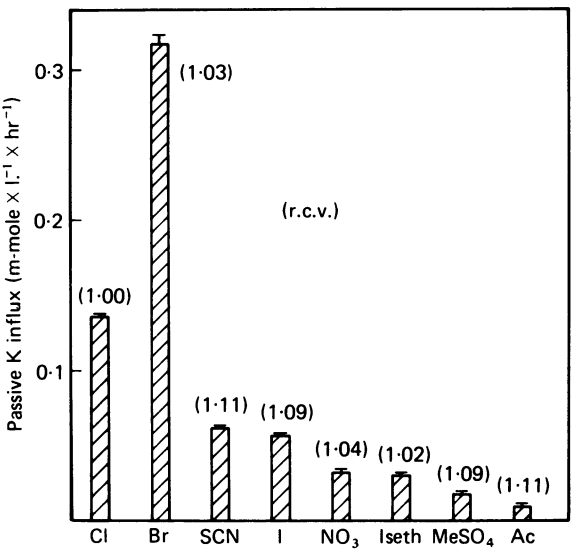

Fig. 6. Passive K influxes in LK sheep red cells equilibrated in media with various permeant monovalent anions substituted for chloride, all at 150 mM. The media were buffered with HEPES/Tris (10 mM) rather then Tris·HCl. The cells were equilibrated with the various Cl-free media as described in Methods. Cell volumes were then measured for each aliquot of cells, and are shown in the figure as relative cell volumes. Similar results were obtained in two other experiments. $[K]_o = 5$ mM ($n = 3$).

where v is K influx, and V_{max}, K_H, B, and h are parameters to be estimated (see Table 1 for their units.) K_H comprises the interaction factors between binding sites on each transport pathway and also the intrinsic association constants; B is the intercept at $[Cl]_o = 0$; h is the Hill coefficient, related to the number of interacting substrate binding sites per pathway, and also to the interaction between the sites (Segel, 1975; Rodland & Dunham, 1980). To determine if the data are fit better by a hyperbola than a sigmoid curve, they were fitted to eqn. (1) with $h = 1$. They were also fitted to a linear model:

$$v = [Cl]_o \cdot \alpha + \beta, \quad (2)$$

where α and β are constants (slope and intercept, respectively). The parameters of these models were estimated by successive iteration using a non-linear least-square computer program. The lines in Fig. 7 were calculated from parameters estimated from the data using eqn. (1). Table 1 shows these estimated parameters and also those estimated using the hyperbolic model [$h = 1$ in eqn. (1)], and the linear model [eqn. (2)]. Table 1 also shows the residual sum of squares for the fit of each of the three

sets of data to the three models. This term is the sum of the squares of the differences between the experimentally obtained values and those obtained using the estimated parameters.

The residual sums of squares can be used to compare the contributions each model makes to explaining the data. At the bottom of Table 1 is the summary of an analysis of the variance in using the sigmoid model and the hyperbolic model, and an F test

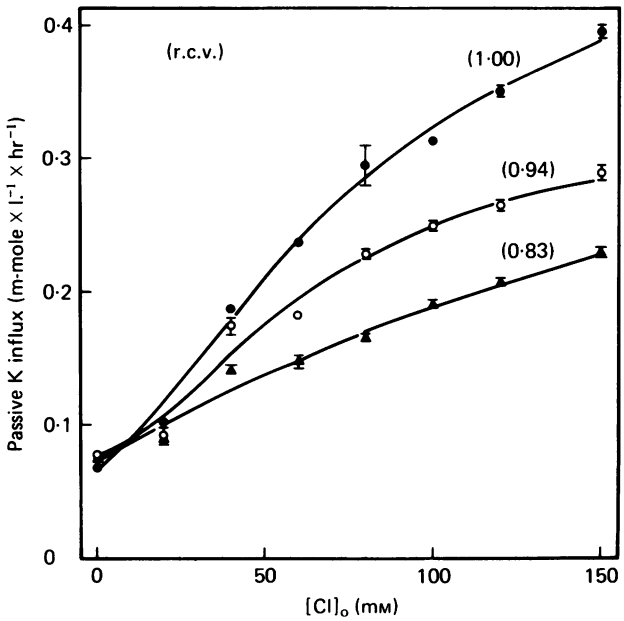

Fig. 7. The kinetics of the activation by Cl of passive K influx in LK sheep red cells. $[Cl]_o$ was varied by replacement with $MeSO_4$ ($[Cl]_o + [MeSO_4]_o = 150$ mM). At each $[Cl]_o$, media were made with varying osmolarities by addition of sucrose such that a set of aliquots of cells with the full range of $[Cl]_o$ was obtained at each of three different cell volumes. Cells were equilibrated in each of the media as described in Methods before the measurements of r.c.v. $[K]_o = 7{\cdot}5$ mM ($n = 6$). Similar results were obtained, at r.c.v. 0·98–1·02, in three other experiments with $MeSO_4$ as the substitute anion, in two with NO_3, and in one with Ac. The curves were calculated from eqn. (1) as described in the text. The estimates of the parameters in eqn. (1) are given in Table 1.

of the significance of the reduction in the residual variation by the sigmoid model (i.e. the consequence of adding h to the hyperbolic equation) in the data for r.c.v. = 1·00. The addition of h reduced the variation significantly ($P = 0{\cdot}10$), so the data for r.c.v. = 1·00 are better fit by a sigmoid than a hyperbolic model. The residual sum of squares for r.c.v. = 0·94 was less for the sigmoid model than the hyperbolic, but the reduction was not significant, and probably can be accounted for by the lower number of degrees of freedom. For r.c.v. = 1·00 and 0·94 the residual sums of squares were much greater for the linear model than for either the sigmoid or hyperbolic models; the differences in residual sums of squares probably cannot be accounted for in terms of the differences in numbers of degrees of freedom. However, the difference between the models precludes a rigorous test of the significance of the reduction in variation. For r.c.v. = 0·83, no clear argument can be made; the best model is not

sigmoid (estimated $h = 0{\cdot}99$), and the hyperbolic model was not much better than the linear one.

To determine the specificity of the effect of Cl-substitution on cation transport, cells were equilibrated in a Cl-free medium with $MeSO_4$ as the principal anion. The cells were then either swollen or shrunken (sucrose method) and placed in media with equimolar concentrations of Na and K (68 mM). (The cells had been pre-equilibrated with ouabain to avoid inhibition of binding by the high $[K]_o$.) Unidirectional K and Na influxes were measured in parallel, identical aliquots. As Table 2 shows, replacing Cl with $MeSO_4$ reduced K influx eightfold in swollen cells (and more than twofold in shrunken cells), but resulted in no decrease (actually a slight increase) in Na influx in either shrunken or swollen cells. Therefore, the effect of Cl substitution on K influx is specific for K. Furthermore the specificity of the volume-dependence for K shown in Fig. 3 is confirmed in Table 2 under somewhat different conditions than in Fig. 3,

TABLE 1. Kinetics of activation by Cl of passive K influx: parameters estimated as described in the text for three models using the data shown in Fig. 7. The models are: (*a*) a sigmoid model [eqn. (1), the Hill equation); (*b*) a hyperbolic model (obtained by setting the exponent, h, in eqn. (1) equal to 1·0); and (*c*) a linear model (eqn. (2)). The definitions of the symbols for the parameters are given in the text. Their units are: V_{max}, B, β: μmole × l.$^{-1}$ × hr^{-1}; K_H: m-mole/l. RSS is residual sum of squares; d.f. is number of degrees of freedom. At the bottom of the table are the results of an analysis of the variance associated with the estimated parameters for the data for r.c.v. = 1·00 and the sigmoid and hyperbolic models. From the residual sums of squares, F was calculated, and a probability was determined for the improvement of the explanation of the data by the addition of the parameter h.

Estimated parameters	Relative cell volume 1·00	0·94	0·83
(*a*) Sigmoid model (eqn. (1); 4 d.f.)			
V_{max}	453	271	562
K_H	820	949	380
h	1·52	1·62	0·99
B	64·7	73·3	72·8
RSS	450	786	393
(*b*) Hyperbolic model (h = 1·0 in eqn. (1); 5 d.f.)			
V_{max}	870	498	570
K_H	232	181	397
B	56·0	67·3	73·1
RSS	942	1132	392
(*c*) Linear model (eqn. (2); 6 d.f.)			
α	2·26	1·49	1·05
β	82·1	88·6	80·6
RSS	3174	2604	591

Analysis of variance, r.c.v. = 1·00

Sources of variation (*s*)	d.f.	RSS	F
Total s minus s due to: V_{max}, K_H, B, h	5	450	
Total s minus s due to: V_{max}, K_H, B,	4	942	
s due to h	1	492	4·37 (1,4) (P = 0·10)

namely equal concentrations of Na and K. Table 2 also shows that there is no effect of changing cell volume on K influx in the absence of Cl. Therefore the volume-sensitive and Cl-dependent K transport systems are the same.

Effect of anti-L on volume-dependent K influx. It was shown earlier that anti-L antibody inhibits passive K transport in LK sheep cells (Dunham, 1976*a*; Lauf, Stiehl

TABLE 2. Specificity of volume- and Cl-dependent passive cation influxes in LK sheep red cells. Influxes of K and Na were measured simultaneously in cells equilibrated in media with either Cl or $MeSO_4$ as the principal anion (150 mM). $[K]_o$ and $[Na]_o$ were equal (68 mM). Aliquots of cells were either swollen or shrunken by the sucrose method. Cells were pre-equilibrated with ouabain (0·05 mM, (0·05 mM, 5 min, 37 °C) ($n = 3$). Similar results were obtained in three other experiments.

Anion	Relative cell volume	Passive influx (m-mole/l. × hr) K	Na
Cl	1·18	5·62 ± 0·07	2·21 ± 0·02
Cl	0·95	1·73 ± 0·02	2·03 ± 0·02
$MeSO_4$	1·12	0·67 ± 0·04	2·60 ± 0·03
$MeSO_4$	0·90	0·63 ± 0·04	2·31 ± 0·03

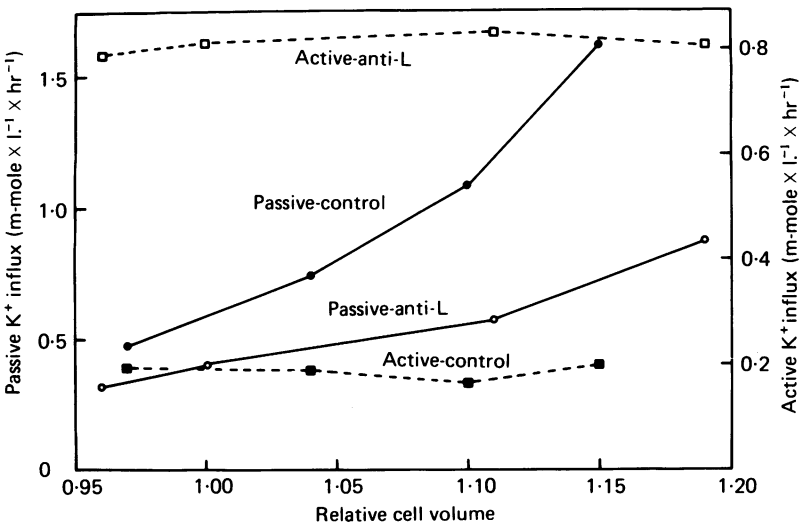

Fig. 8. The effect of preincubation with anti-L antibody on active and passive K influx in LK sheep red cells with their volumes varied by the sucrose method. Similar results were obtained in one other experiment. $[K]_o = 7·5$ mM ($n = 2$).

& Joiner, 1977). The results of the experiment in Fig. 8 show that this inhibition is relatively less in shrunken cells than in swollen cells. Shown are active K influxes (dashed lines) in cells at the various volumes, both untreated (control) and pretreated with anti-L and the passive influxes (solid lines) for the two types of cells. In swollen cells (r.c.v. ≈ 1·15) anti-L inhibited passive K transport by nearly 1 m-mole × hr^{-1} × $l.^{-1}$ (more than twofold), whereas in shrunken cells (r.c.v. ≈ 0·96) inhibition was only 0·2 m-mole × $l.^{-1}$ × hr^{-1} (30%). In cells shrunken further (r.c.v. ≈ 0·92), anti-L had no effect on passive K influx (results not shown). These

results are consistent with identity of the volume-dependent and anti-L-inhibitable passive K influx pathways. We showed earlier that the effect of anti-L on passive transport is specific for K in that there is no effect on ouabain-insensitive Na efflux while active Na efflux was stimulated several fold (Dunham, 1976*a*).

The experiment in Table 3 addresses the question, are the Cl-dependent and anti-L-sensitive pathways of passive K influx the same? Anti-L reduced the passive

TABLE 3. The effects of the replacement of Cl and of anti-L antibody on active and passive K influxes in LK sheep red cells. Cells were first incubated with anti-L serum. Then aliquots of both anti-L treated cells and control cells were equilibrated in media with either Cl or $MeSO_4$ as the principal anion (150 mM). Cell volumes in all aliquots were about the same, as shown. $[K]_o$ = 5 mM (n = 3)

Treatment	Relative cell volume	K influx (μmole × l.$^{-1}$ × hr^{-1}) Active	Passive
Cl-control	1·02	98± 3	24±1
Cl-anti-L	1·04	531± 4	12±2
$MeSO_4$-control	1·08	72± 4	13±3
$MeSO_4$-anti-L	1·05	540±13	12±1

TABLE 4. The effects of the purified anti-L antibodies, anti-L_p and anti-L_1 on passive and active K influxes in LK sheep cells at two cell volumes. Anti-L_p and anti-L_1 were prepared as described in Methods. The fluxes were also measured in cells treated with unfractionated serum. The cells were from a homozygous (LL) sheep. $[K]_o$ = 7·5 mM (n = 3)

Treatment of cells	Influx	K influx (μmole × l.$^{-1}$ × hr^{-1}) relative cell volume 0·96	1·08
Control	Passive	87± 3	143± 3
Anti-L_p	Passive	100±19	131± 3
Anti-L_1	Passive	58± 4	83± 4
Anti-L (unfrac.)	Passive	3± 1	56± 4
Control	Active	98± 2	93± 2
Anti-L_1	Active	105± 4	107±11
Anti-L_p	Active	362±32	414±21
Anti-L (unfrac.)	Active	421±42	486±56

flux in Cl-medium, but had no effect on the already reduced flux in cells in $MeSO_4$-medium. Though the magnitudes of the effects of anti-L and replacement of Cl were not as great as they were in other experiments presented above, we conclude that the Cl-dependent and anti-L-sensitive passive K transport systems are the same.

In Table 3 there was a slight reduction in the active K influx after replacement of Cl, but this was not a consistent finding. The active flux was stimulated more than fivefold by anti-L, and was the same in anti-L-treated cells in Cl- and $MeSO_4$-medium.

It has also been shown that anti-L serum contains antibodies of two specificities. Anti-L_p stimulates the active flux (Ellory & Tucker, 1970; Lauf *et al.* 1971) and anti-L_1 inhibits passive K transport (Dunham, 1976*b*). Several methods are available for separating these antibodies (Smalley *et al.* 1981). The data in Table 4 confirm that, in LL cells (from a homozygous sheep), anti-L_1, affects only passive transport and

anti-L_p affects only active transport. This was true at two cell volumes. As expected there was no volume sensitivity of the active flux, but there was of the passive flux. Furthermore, as shown in Fig. 8, the sensitivity of the passive flux to anti-L_1 was greater with the larger cell volume.

Effect of iodoacetamide. Fig. 9 shows the active and passive K influxes in cells which had been preincubated for 90 min in a medium containing iodoacetamide (IAA). After this 'metabolic depletion', the volumes of the cells were varied and the fluxes

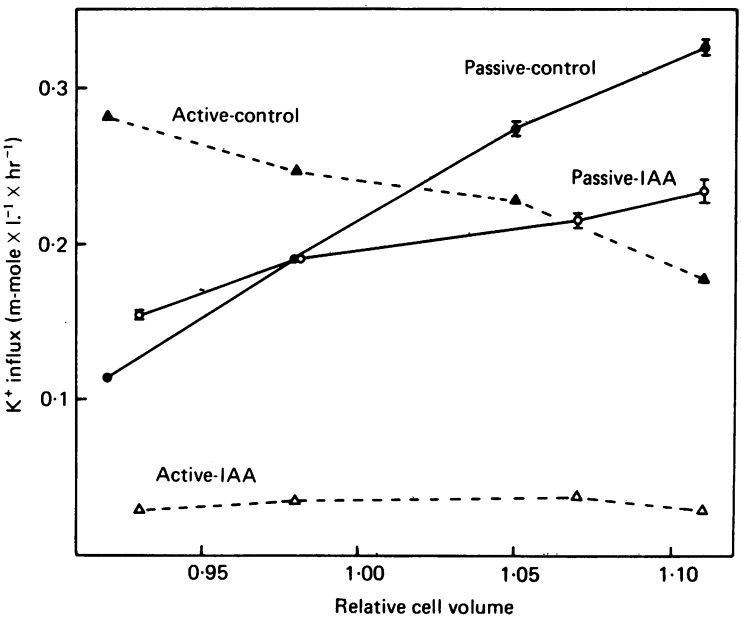

Fig. 9. Effect of iodoacetamide (IAA) on passive and active K influxes in LK sheep red cells with volumes varied by the sucrose method. Before setting the volumes, an aliquot of cells was incubated for 90 min at 37 °C with iodoacetamide (6 mM) and glucose (5 mM). The fluxes for these cells are indicated IAA. Control cells were incubated for the same time with glucose only. $[K]_o = 5$ mM ($n = 3$). Similar results were obtained in four other experiments, including one in which the volumes were varied by the nystatin method, and the fluxes were measured using ^{42}K.

measured. The active flux was reduced at least 80 % in the depleted cells (dashed lines, Fig. 9). (In this experiment the active flux was increased in control cells by decreasing cell volume, perhaps due to an increase in the concentration of some intracellular constituent; as results presented elsewhere in this paper show, this was not a consistent finding.) Incubation with IAA may or may not affect cell volumes; in any case the volumes were measured after the incubation with the inhibitor.

The effect of IAA on the passive K flux was to decrease it in swollen cells and increase it in shrunken cells. With IAA there is a cell volume (0·97 r.c.v. in this experiment) at which the treatment had no effect on passive K influx. This finding is reproducible (the same result was obtained in five separate experiments, and there were none in which it was not). However, the relative cell volume at which IAA-

treatment has no effect on K transport varied somewhat from experiment to experiment (0·97–1·04). Therefore, IAA seems to reduce or increase transport by reducing sensitivity to volume changes. A similar finding appears in our preliminary report (Ellory & Dunham, 1980). We drew the conclusion that the action of IAA was by depletion of a cellular metabolite, though it is difficult to rule out the possibility of an action through modification of membrane proteins associated with the transport system.

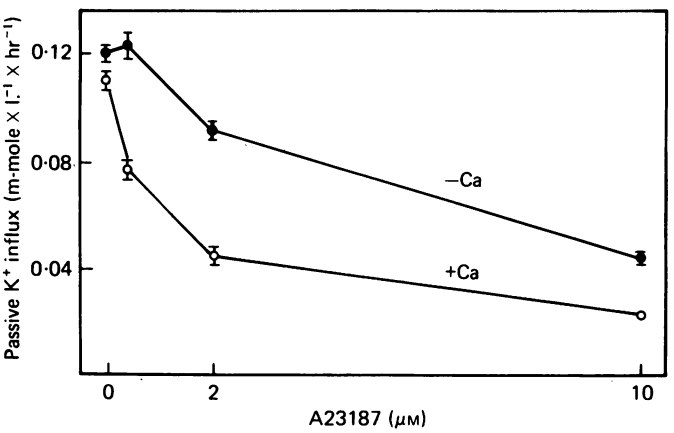

Fig. 10. The effect of A23187 (ionophore for divalent cations) and A23187 + Ca (1·0 mM) on passive K influx in LK sheep red cells. The relative cell volume was 1·0. The cells were preequilibrated in the various concentrations of A23187 or A23187 + Ca for 15 min before measurement of the fluxes. $[K]_o$ = 5 mM (n = 3).

Effect of Ca on passive K transport. A possible explanation for the increase in passive K transport in swollen cells might lie with an effect of Ca similar to the Gardos effect (Ca-activated K permeability increase) in human and other types of red cells (Gardos, 1958; Lew & Beaugé, 1979). Like the volume-sensitive K transport, the Gardos effect is specific for K. However, this seems an unlikely explanation since active transport is unchanged (or only slightly inhibited) by swelling, and Ca is known to be a potent inhibitor of active Na/K transport (Hoffman, 1962). Furthermore, it has been reported that there is no Gardos effect in red cells of adult sheep (Brown, Ellory, Young & Lew, 1978).

We sought to confirm the absence of Ca-induced K transport in sheep cells, and to make certain that Ca plays no other kind of role in the volume-sensitive K transport. One way to promote the Gardos effect is to allow Ca entry into cells using the ionophore A23187. Fig. 10 shows the effects on passive K influx of preequilibration with various concentrations of A23187. The experiment was carried out both without added Ca and with Ca at 1·0 mM. Ca added without ionophore had no effect. The effect of ionophore without added Ca, a decrease in K transport, is surprising and difficult to explain. It was shown previously that A23187 + EGTA (1 mM) has the same effect as ionophore alone (Ellory & Dunham, 1980). Therefore, the effect of ionophore is not likely to be due to entry of the low concentration Ca normally present. It was also shown the the effect of A23187 is not a consequence of redistribution of Mg

(J. C. Ellory and P. B. Dunham, unpublished results). Furthermore ionomycin, an ionophore for divalent cations chemically unrelated to A23187 (Liu, Slusarch, Astle, Trejo, Brown & Meyers, 1978), had the same effect as A23187 (Ellory & Dunham, 1980).

Nevertheless, we can confirm that there is no Gardos effect in LK sheep red cells, and that the specific increase of passive K influx in swollen sheep cells is not due to Ca entry. Since Ca causes a decrease in passive K influx, there is the possibility that shrinking the cells allows Ca entry. This is also unlikely since A23187 + Ca results in inhibition of active K influx in both swollen and shrunken cells (Ellory & Dunham, 1980), and the pump in osmotically shrunken cells was generally unchanged and occasionally increased.

DISCUSSION

This report characterizes the major pathway of passive K transport in LK sheep red cells. This pathway is sensitive to changes in cell volume and is dependent on chloride. It is specific for K (Na is excluded and is inhibitory), shows saturation kinetics, and is inhibited by the anti-L_1 antibody. A central question in studying this system is whether each of these characteristics is shared by a common pathway. Passive K transport, often defined as ouabain-insensitive ^{42}K or ^{86}Rb influx, represents several distinct components in human red cells, dominated by the Cl-dependent pathway (Dunham *et al.* 1980), but including others such as the true electrodiffusional leak, the Ca-activated (Gardos) channel, and transport through the anion channel as the KCO_3 ion (Funder & Wieth, 1980). For LK sheep cells, the magnitude of the changes in ouabain-insensitive K influx (> twofold) which can be achieved by three methods (reducing cell volume, replacing Cl, adding anti-L) means a single transport system for K influx in cells at normal volume in the presence of Cl.

There are several points of additional evidence in support of single predominant pathway for passive K transport LK sheep cells. First, removing Cl has no effect in anti-L-treated cells (Table 2). Secondly, anti-L has little or no effect in shrunken cells (Fig. 8). Third, reducing cell volume has no effect in the absence of Cl (Table 2). Na inhibits passive K influx in swollen cells but not in shrunken ones, suggesting that Na affects only the dominant pathway in cells of normal volume. It follows that, in shrunken cells, passive K transport which is inhibited neither by Na, nor by anti-L, nor by replacement of Cl, is by a single pathway, perhaps electrodiffusion.

The volume sensitivity of passive cation transport has been studied in some depth in avian red cells (Kregenow, 1974, 1977; Schmidt & McManus, 1977*a*, *b*; McManus & Schmidt, 1978) where it is related to the ability of the cells to restore their volume after hypo- or hyperosmotic challenges. It is not known if sheep red cells are capable of regulating their volume (there is one report of regulation of cell volume in human red cells (Poznansky & Solomon, 1972*b*)). In avian red blood cells the volume-sensitive cation-transport system is Na/K cotransport (McManus & Schmidt, 1978; Gardner, Aurbach, Spiegel & Brown, 1976). Cl also plays a role, a reflection either of its dominance in determining the electrical membrane potential (Schmidt & McManus, 1977*b*) or of K/Cl transport (Kregenow & Caryk, 1979). The activation by Cl of K

transport in sheep red cells may reflect cotransport, though this would be difficult to demonstrate owing to the enormous Cl fluxes, compared to K fluxes. There is no K/Na cotransport in sheep red cells (Dunham, 1976*a*); in human red cells the apparent K/Na cotransport is identical with the Cl-activated K and Na transport (Dunham & Sellers, 1980). Geck *et al.* (1980) have recently provided evidence for an ouabain-insensitive transport pathway in Ehrlich ascites cells which carries all three ions: Na, K, and Cl. The transport is apparently electroneutral since the stoichiometry is 1 Na : 1 K : 2 Cl.

The kinetics of activation of K influx by Cl in LK sheep cells were best fitted by a sigmoid curve, at least in cells near normal volume (Fig. 7). The simplest interpretation of these results is that activation of passive K transport by Cl requires binding of Cl ions at more than one site per K 'channel.' (In considering the kinetics of the activation of K transport by Cl, it must be considered that [Cl] was varied both inside and outside the cells. If Cl binding at sites at both membrane surfaces modifies K transport, this will also contribute to the complexity of the kinetics, particularly if the affinities differ.)

The cotransport system proposed for Ehlrlich ascites cells (Geck *et al.* 1980) would be expected to have sigmoid kinetics for its activation by Cl. A similar system may exist in human red cells, where Cl activates the K transport (and therefore maybe Na/K cotransport) with sigmoid kinetics (Dunham *et al.* 1980). The situation in sheep cells is less certain since Na/K cotransport is lacking. One might suppose K/Cl cotransport with a stoichiometry of 2 K : 2 Cl, but the kinetics of activation by K are clearly not sigmoid (Fig. 4).

Poznansky & Solomon (1972*a*, *b*) found volume-dependent changes in K and Na transport in human red cells, though volume-insensitive K transport has been reported by others (Davson, 1937; Ellory & Dunham, 1980). Poznansky & Solomon observed no selectivity: the magnitudes of the changes were about the same for Na and K. Swelling the cells caused increases in the influxes and decreases in the effluxes, as though changes in transport were due to changes in driving force and not permeability. In a recent preliminary report (Adragna, Canessa, Bize, Garay & Tosteson, 1980) volume-sensitive passive effluxes of Na and K were suggested. Interestingly, the stoichiometry of Na/K cotransport was said to be affected.

The results of this study pose three related questions: How does anti-L_1 inhibit passive transport? What is the 'sensor' mediating the response of K transport to chages in cell volume? How does IAA reduce sensitivity to changes in cell volume? It is likely, but by no means certain, that the L_1 antigen and the passive K transport 'channel' are not identical molecular entities. By analogy with L_p antigen and the Na/K pump, L_1 antigen and the K channel interact in a manner which is modified by the binding of the antibody to the antigen.

The mode of action of IAA in reducing the sensitivity to volume is obviously not clear. There may be either a direct action on sulphydryl groups of the membrane, or an indirect effect through inhibition of glycolysis and the depletion of an intracellular metabolite (Parker, 1977).

The 'sensor' of the changes in volume is also an enigma. There are two general kinds of possibilities: (i) the concentration of a cytoplasmic constituent, or (ii) the conformation of a membrane element. It is difficult to imagine what such a solute

might be (we have shown that it is not Ca). It may be easier (though not much) to imagine volume-sensitivity through membrane-associated elements whose conformation changes with shrinking and swelling of the cells.

In relating these three questions to one another, we can speculate that IAA reduces sensitivity to volume by interacting with the sensor, a membrane entity separate from the K channel but associated with it. The L_1 antigen and the volume 'sensor' are unlikely to be identical, but if both interact with the K channel, they may interact with each other; the relevant experiments have not been done.

The kinetics of K influx modifed by the various treatments provide some further information about the passive K transport pathway. For example it was shown earlier that anti-L reduced the V_{max} of the flux but not the $K_{\frac{1}{2}}$ (Dunham, 1976*a*), suggesting that the antibody reduces the number of channels available to K without modifying the affinity for K of the functioning channels remaining. Regarding the action of Na on passive K influx, both V_{max} and $K_{\frac{1}{2}}$ were higher in choline (Na-free) media, so the inhibition by Na is not simple competition. Changes in V_{max} and $K_{\frac{1}{2}}$ in the same direction suggest uncompetitive inhibition, but in double reciprocal plots of the data for swollen cells from Fig. 4, the lines were not parallel as they would be for 'simple' uncompetitive inhibition. Nevertheless, since Na lowers both V_{max} and $K_{\frac{1}{2}}$, one can conclude tentatively that Na does not bind directly at the K loading sites, but does bind with a preference to channels with K already associated with them, rather than to the free form of the channel (which would be competitive inhibition), or with no preference between K-associated and free forms (noncompetitive inhibition). From the kinetics, it also appears that reducing cell volume, as with anti-L treatment, inhibits the flux by reducing the number of available channels since V_{max} was reduced several fold in shrunken cells.

Some caution must be exercised in interpreting the kinetics in the swollen cells, for the curves, and the constants used to fit them, are for the sum of fluxes through more than one type of pathway. Nevertheless, most of passive K influx in swollen cells is by one mechanism and the conclusions drawn seem reasonably secure.

These various points support the conclusion of a major component of K influx in sheep red cells which is sensitive to volume, dependent on chloride, inhibited by anti-L, and slightly inhibited by Na. The minor component (the major component in shrunken cells) has none of these characteristics.

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