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# Thiol-Dependent Passive $K^+Cl^-$ Transport in Sheep Red Blood Cells: VI. Functional Heterogeneity and Immunologic Identity with Volume-Stimulated $K^+(Rb^+)$ Fluxes

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**Summary.** Ouabain-resistant (OR), volume- or N-ethylmaleimide (NEM)-stimulated $K^+(Rb^+)Cl^-$ fluxes were measured in low-$K^+$ sheep red cells and found to be functionally separate but immunologically similar. In anisosmotic solutions both $K^+$ effluxes and $Rb^+$ influxes of NEM-treated and control cells were additive. In contrast to the NEM-stimulated $K^+Cl^-$ flux, metabolic depletion did not reduce $K^+Cl^-$ flux of normal or swollen cells. The anion preference of OR $K^+$ efflux in NEM-treated cells was $Br^- > Cl^- > HCO_3^- = F^- \gg I^- = NO_3^- = CNS^-$, and hence consistent with a reported $Br^- > Cl^- > NO_3^-$ sequence of the volume-dependent $K^+Cl^-$ transport. Alloimmune anti-$L_l$ antibodies known to decrease passive $K^+$ fluxes in low $K^+$ cells reduced by 51% both volume- and NEM-stimulated, furosemide-sensitive $Rb^+Cl^-$ fluxes suggesting their immunologic identity, a conclusion also supported by anti-$L_l$ absorption studies. Since pretreatment with anti-$L_l$ prevented the activation of $Rb^+$ influx by NEM, and the impermeant glutathionmaleimide-I did not stimulate $Rb^+Cl^-$ influx, the NEM reactive SH groups must be located apart from the $L_l$ antigen either within the membrane or on its cytoplasmic face. A model is proposed consisting of a $K^+Cl^-$ transport path(s) regulated by a protein with two functional subunits or domains: a chemically ($C_S$) and a volume ($V_S$)-stimulated domain, both interfacing with the $L_l$ surface antigen. Attachment of alloanti-$L_l$ from the outside reduces $K^+Cl^-$ transport stimulated through $C_S$ by NEM or $V_S$ by cell swelling.

**Key Words** sheep red cells · ouabain-insensitive $K^+$ fluxes · chloride dependence · cell volume · sulfhydryl groups · N-ethylmaleimide · membrane L-antigen

## Introduction

In LK sheep red cells about 30 to 40% of the $K^+$ transport is $Cl^-$-dependent ($K^+Cl^-$ transport) and can be stimulated several-fold by cell swelling [9, 11] or by treatment with the sulfhydryl (SH) reagent NEM [17, 24]. The presence of OR $K^+Cl^-$ transport in mature LK red cells and in reticulocytes of both LK and high $K^+$ (HK) sheep [18] but not in HK sheep and goat red cells [24] suggests a role in the maturational development of the LK erythrocytes. Recently, NEM has been shown to activate $K^+Cl^-$ transport also in human red cells [22, 30] and in pig erythrocytes [26]. In sheep, similarities between the NEM- and the volume-stimulated $K^+Cl^-$ transport systems such as low affinity for external $K^+(Rb^+)$ [9, 17] and furosemide [19] have led to the tacit assumption that it is the small basal OR $K^+Cl^-$ flux which is activated by the two different stimuli [17, 27]. However, recent data that the NEM-stimulated but not the basal, presumably volume-sensitive $K^+Cl^-$ transport in LK cells is metabolically dependent [20] requires a more thorough evaluation of the identities of both the NEM- and the volume-responsive $K^+Cl^-$ fluxes in LK sheep red cells and their relation to the basal $K^+Cl^-$ flux.

It was found that the NEM-stimulated $K^+Cl^-$ flux in LK sheep red cells was volume independent while the basal and the hyposmotically activated fluxes are refractory to metabolic manipulation. Thus the NEM-stimulated fluxes appeared to be operationally different from the basal and volume-activated fluxes. However, the anion dependence of the NEM-stimulated $K^+Cl^-$ flux agreed with that reported for volume-sensitive fluxes [27]. Both NEM- and volume-stimulated $K^+Cl^-$ fluxes were immunologically identical because, by binding to a site different from that for NEM, anti-$L_l$-antibodies, known to decrease passive $K^+$ fluxes in LK cells [7, 8, 23], reduced $K^+$ fluxes in normal, swollen and NEM-treated LK cells to a similar degree. These data are consistent with the presence of an oligomeric $K^+Cl^-$ transport system with interfacing functional subunits or domains stimulated by NEM or cell swelling and inhibited by the action of the $L_l$-antibody. Preliminary reports of this work have appeared elsewhere [16, 21, 25].

## Materials and Methods

*Erythrocytes* were derived from *Dorset LK and HK sheep* as described previously [17]. The cellular $K^+$ concentration $[K^+]_c$

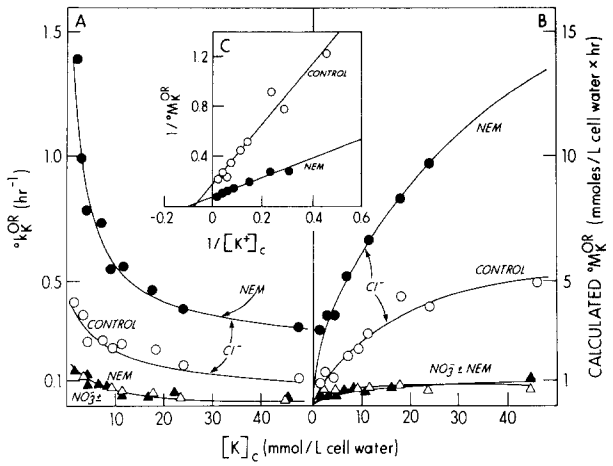



**Fig. 1.** Rate constants ($^o k_K^{OR}$, Panel *A*) of ouabain-resistant (OR) $K^+$ effluxes of LK cells as a function of cell $K^+$ concentration ($[K^+]_c$, mmol/liter cell $H_2O$) under zero-trans conditions. In panel *B*, $^oM_{Rb}^{OR}$ has been calculated from $^ok_K^{OR} \cdot [K^+]_c$. Insert *C*: Inverse plot of $^oM_K^{OR}$ versus $[K^+]_c$. Open and filled symbols for controls and NEM-treated cells, respectively. Circles and triangles for $Cl^-$ and $NO_3^-$ media, respectively

(in mmol/liter cells) of the LK and HK red cells used were: 16.8 and 11.0 for LK (LL) 15 and 53, and 95.0 for HK (MM) 20, respectively. As indicated, for each of the experiments under Results the erythrocytes were usually first washed in isosmotic solutions (295 ± 5 mOsM) with $NaNO_3$ (145 mM) as the principal salt buffered with either 10 mM Tris (Tris hydroxymethylaminomethane)/$HNO_3$, Tris/HCl, Tris/MOPS (morpholinopropane sulfonic acid) or $H_2PO_4/HPO_4$ adjusted to pH 7.4 with the appropriate acid to correct for temperature changes in the Tris-buffered solutions. A stock red cell suspension of about 80% hematocrit was kept on ice prior to further processing. All media contained $10^{-4}$ M ouabain.

*Treatment with N-ethylmaleimide* (NEM) was carried out in one of the above buffers as described before [17] with the modification that in most experiments the treatment pH was 7.4 instead of 7.1 and that the unreacted excess NEM was removed by two washes of the cells in their respective buffers. This step ensured also the removal of cellular $K^+$ that leaked out during pretreatment. For stimulation of the volume-dependent $K^+Cl^-$ transport, NEM-treated or control cells were exposed to phosphate-buffered (10 mM, pH 7.4) solutions of different osmolalities adjusted by varying the molarities of $NaNO_3$ and NaCl. The osmolalities were determined with an Advanced Digimatic Osmometer Model 3DII (Advanced Instruments, Needham Heights, Mass.) The variability between the experiments is given in the Figure legends.

In order to change the cellular cation composition of both LK and HK cells, the *nystatin procedure* [5] was used as described earlier [13]. Prior to the nystatin treatment, cells were washed in ouabain-free, isosmotic NaCl media buffered with Tris/HCl to pH 7.4 at 0°C. Washed cells were suspended at a hematocrit of 2% in ice-cold (0°C) isosmotic solutions containing 30 μg nystatin/ml (Squibb Pharmaceuticals) with $[K^+]_o$, and $[Na^+]_o$, the concentrations of $K^+$ and $Na^+$ varied inversely with respect to each other from 0 to 150 mM. After 30 min the cells were pelleted and washed seven times with large volumes of isosmotic reconstitution media described before [13] at temperatures between 30 and 37°C to remove nystatin. Finally, cells were equilibrated and washed in buffered isosmotic $NaNO_3$ solutions prior to treatment with NEM as described above.

*Metabolic depletion* of LK sheep red cells prior to treatment with NEM was carried out by starvation for 16 hr at 37°C in glucose-free phosphate-buffered media essentially as described before [20]. Controls were kept in glucose-containing media. All solutions had antibiotics added. Cellular metabolism was recovered with glucose and inosine in phosphate-buffered solutions, and cellular ATP levels were measured enzymatically in the neutralized perchloric acid extracts of control, depleted and repleted cell suspensions as described before [20].

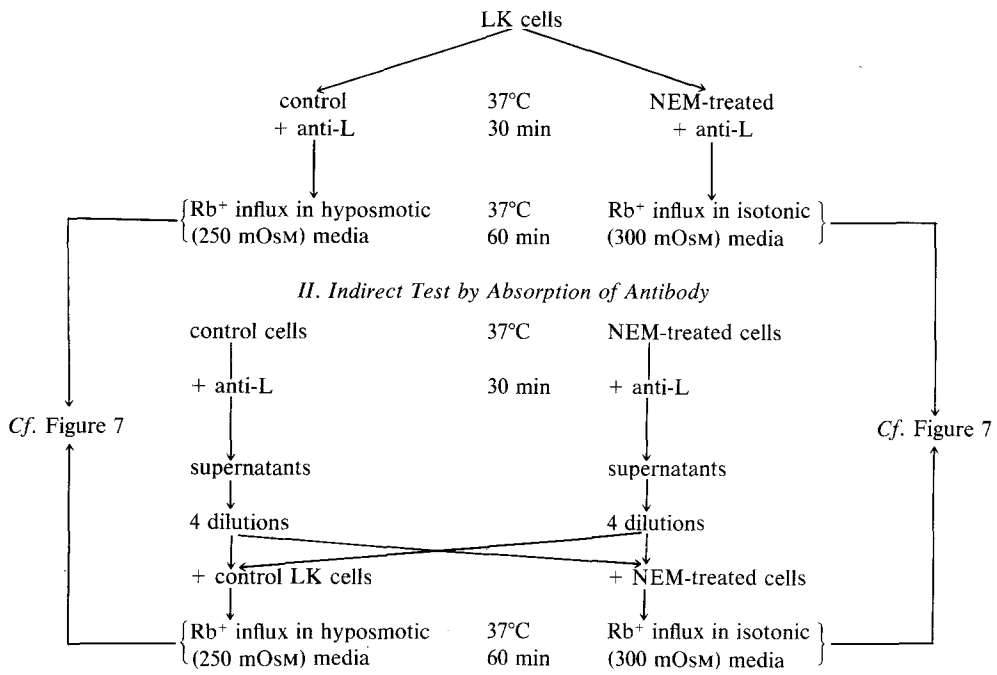
In the *anion replacement experiments*, it was assumed that the anions replacing $Cl^-$ would almost instantly equilibrate via band 3 protein, lately named kapnophorin [31], once the cells were placed into the respective experimental solution. Replacement of $Cl^-$ by $Br^-$, $F^-$, $I^-$, $NO_3^-$ and $CNS^-$ was based on simple equimolar substitution of NaCl with the $Na^+$ salts of these anions. In the case of $HCO_3^-$ a more elaborate procedure was necessary in order to maintain the pH between 7 and 8. Based on the Henderson-Hasselbach equation, $[pH = pK_a(6,1) + \log [HCO_3^-]/[CO_2]]$, the $CO_2$ concentration was varied by titrating, with a pH-stat system (Radiometer, Copenhagen), 10% $CO_2$ to the temperature-equilibrated $NaHCO_3$ solutions. For each $[HCO_3^-]_o$ the pH-stat was set at the $pH_o$ value calculated from the Henderson-Hasselbach equation. In a separate experiment, the $pH_o$ was kept constant at 7.9 for two different $[HCO_3^-]_o$ using 2.5% $CO_2$ for 37.5 mM $[HCO_3^-]_o$ and 5% $CO_2$ for 75 mM $[HCO_3^-]_o$, respectively. An aliquot of packed NEM-treated LK cells suspended in isosmotic $NaNO_3$ was added to the incubation solution to give a final suspension with a 4% hematocrit and thereby the efflux of $K^+$ was started.

In the *immunological studies*, a total of six different preparations of alloimmune anti-L sera obtained from Dr. B.A. Rasmusen (Animal Genetics Laboratory, College of Agriculture, University of Illinois, Urbana, Ill.) and stored over years below −20°C were used following complement inactivation as described earlier [23]. These antisera contain two anti-L antibodies, anti-$L_p$ reacting with the $Na^+K^+$ pump and anti-$L_f$ reducing OR $K^+$ fluxes in LK cells [7, 8, 15, 23]. As the present studies are concerned only with OR $K^+$ fluxes and the effect of anti-$L_f$, only the terms $L_f$-antibody and $L_f$-antigen will be used [15]. Prior to the experiments involving antibodies, all sera were dialyzed exhaustively against the media used in the individual test tubes for $Rb^+$ influx. For testing the effect of anti-$L_f$ 0.2 ml of a 50% cell suspension was mixed with 0.8 ml Tris/MOPS buffered (isosmotic or hyposmotic) NaCl or with 0.8 ml of a dilution of dialyzed anti-L serum. After 30-min incubation at 37°C, flux media containing ultrapure RbCl (Johnson Matthey Chemicals, Hertfordshire, England) and furosemide (a gift from Hoechst-Roussel Pharmaceuticals, Inc., Sommerville, N.J.) were added to yield final concentrations of 10 and 1 mM, respectively, and a suspension hematocrit of 4 to 5%.

Absorption of anti-$L_f$ by control and NEM-treated LK cells was done by incubating equal volumes of packed red cells with undiluted (i.e. at the dilution as obtained), dialyzed anti-L serum for 30 min at 37°C. After a sharp centrifugation at 15,000 rpm (Sorval R2B centrifuge) the cells were discarded and the supernatant containing the residual anti-$L_f$ antibody activity was diluted for subsequent testing on freshly NEM-treated or hyposmotically swollen LK red cells, as described for unabsorbed anti-$L_f$ serum above. A flow scheme of the steps involving absorption and testing of anti-$L_f$ is represented in Table 1.

**Table 1.**

*I. Direct Test for Presence of Antigens*




In the *transport studies,* cellular and extracellular cations, mainly $Na^+$,$K^+$ and $Rb^+$ were determined with a Perkin-Elmer atomic absorption photometer type 460 as described in previous publications [17, 23, 24]. The methods for determination of ${}^{o}k_K^{OR}$ ($hr^{-1}$), the $K^+$ efflux rate constants, in the presence of ouabain from 5 to 6 time point samples during about 60-min flux incubation time, as well as for OR $Rb^+$ influx, ${}^{i}M_{Rb}^{OR}$ (mmol/liter cells · hr), were published in detail elsewhere [17]. With some exceptions most experiments were done with transconcentrations of 10 mM $[Rb^+]_o$ in order to assay simultaneously for $K^+$ efflux and $Rb^+$ influx in $Na^+$ media. The $Cl^-$-specific and the furosemide-sensitive (FS) $K^+$ flux components were derived from the difference between $K^+(Rb^+)$ flux in $Cl^-$ and $NO_3^-$, and $Cl^-$ with and without $10^{-3}$ M furosemide, respectively. It has been shown previously that $10^{-3}$ M furosemide in the presence of 10 mM $[Rb^+]_o$ fully inhibited ouabain-insensitive $K^+$ fluxes in $Cl^-$ media, and the degree of inhibition was similar to that achieved by $NO_3^-$ replacement [19]. Most of the materials such as salts, buffers, sulfhydryl chemicals like dithiothreitol (DTT) and N-ethylmaleimide (NEM) were identical to those reported in preceding communications [17, 19]. The impermeant maleimide [1] *glutathione-maleimide-I* was a generous gift from Dr. R.E. Abbott, Department of Biochemistry, Columbia University, College of Physicians and Surgeons, New York.

## Results

### Dependence of $K^+$ Efflux on Cellular $K^+$

Since NEM stimulated primarily $V_{max}$ of $Cl^-$-dependent $K^+$ or $Rb^+$ influx without affecting the low external affinity for the two cations [17], it remained to be seen whether the same holds true for activation of $K^+$ efflux by cellular $K^+$ under zero-trans conditions. Figure 1*A* shows a plot of the ${}^{o}k_K^{OR}$ values as a function of $[K^+]_c$ (expressed in mmol/liter cell $H_2O$) for control and NEM-treated LK cells in $Cl^-$ and $NO_3^-$ media, respectively. All measured points fell on curved lines, most pronounced in NEM-treated cells suspended in $Cl^-$, indicating saturating flux kinetics as verified in Fig. 1*B*, which plots ${}^{o}M_K^{OR}$, the OR $K^+$ efflux, as the product of $[K^+]_c \cdot {}^{o}k_K^{OR}$ versus $[K^+]_c$. From the Lineweaver-Burk plot in the insert (Fig. 1*C*), it can be seen that NEM activated $V_{max}$ but only slightly affected the apparent affinity of cellular $K^+$ (≈10 mM) which was close to the external affinity for $K^+$ or $Rb^+$ ions reported earlier [17]. In contrast, in HK red cells with $[K^+]_c$ lowered to the levels of normal LK cells $Cl^-$-dependent $K^+$ flux was absent since there was no statistically significant difference between $K^+$ efflux measured in $Cl^-$ or $NO_3^-$, and NEM did not affect either flux.

### $K^+(Rb^+)$ Fluxes in Anisosmotic Media

From the above results, it was assumed that NEM activated the basal $K^+Cl^-$ flux that is also stimulated by cell swelling [9, 11]. However, a functional distinction of NEM- and volume-activated $K^+(Rb^+)$

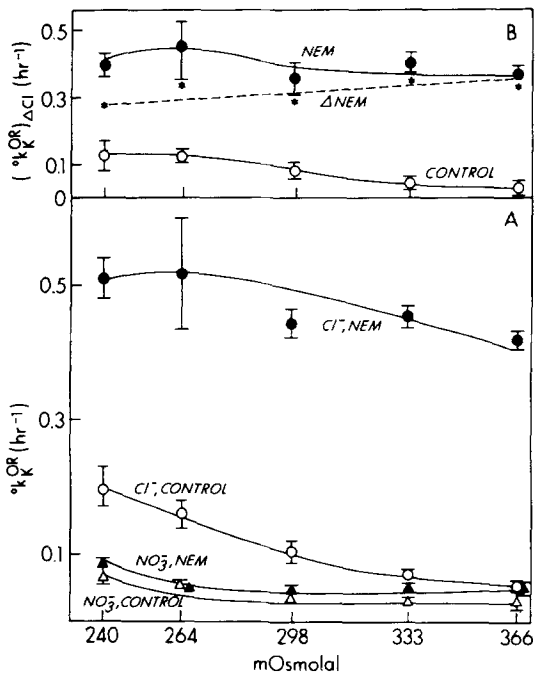



**Fig. 2.** Rate constants, $^{o}k_K^{OR}$, of ouabain-resistant $K^+$ effluxes in control (open symbols) and NEM-treated (closed symbols) LK cells into $Cl^-$ (circles) or $NO_3^-$ (triangles) $Na^+$ media of different osmolalities with 10 mM $[Rb^+]_o$. Bars indicate SEM values for four independent experiments on the same animal. Each osmolality varied by less than 1% SEM ($n = 8$). The slope of the dotted line was calculated from regression analysis of the ΔNEM rate constants and was not different from zero. The ΔNEM rate constant is the difference between the $Cl^-$ specific $^{o}k_K^{OR}$ in control and NEM-treated cells (Panel *B*)

fluxes became apparent, because the $^{o}k_K^{OR}$ values of both LK controls and NEM-treated cells measured at five different osmolarities and in $Cl^-$ or $NO_3^-$ media increased in hyposmotic and decreased in hyperosmotic media, respectively (Fig. 2*A*). There was also a small and statistically significant increment of $^{o}k_K^{OR}$ in hyposmotic $NO_3^-$ in both controls and NEM-treated LK cells. Treatment with NEM shifted the curve connecting the $^{o}k_K^{OR}$ values in $Cl^-$ medium parallel upwards indicating the additive nature of the NEM effect. Hence, the activity of the NEM-specific component was about 0.3 $hr^{-1}$ at all osmolalities tested (Fig. 2*B*). Figure 3 *A*,*B* provides the same information calculated from $Rb^+$ influx ($^{i}M_{Rb}^{OR}$) experiments. Note again the slight increment of $^{i}M_{Rb}^{OR}$ in $NO_3^-$ for both controls and NEM-treated cells. Figure 3*B* leaves no doubt that the NEM-stimulated $Rb^+Cl^-$ flux was additive to the volume-stimulated $Rb^+Cl^-$ flux throughout the range of osmolalities tested.

## Metabolic Manipulations

Previously it was shown that the NEM-stimulated $Cl^-$-dependent $K^+(Rb^+)$ fluxes were abolished and regenerated upon metabolic depletion by 16-hr starvation or by 2 deoxy-D-glucose feeding, and subsequent repletion in media containing glucose and inosine, respectively [20]. However, the basal $Cl^-$-dependent $K^+(Rb^+)$ fluxes of control cells were little affected by these maneuvers. Therefore, it was of interest to test whether prior metabolic depletion would reduce $Cl^-$-dependent $K^+(Rb^+)$ fluxes in control cells exposed to hyposmotic shock.

Table 2 shows the $^{o}M_{Rb}^{OR}$ and $^{o}k_K^{OR}$ values determined in isosmotic (297 mOsm) or hyposmotic (246 mOsM) $Cl^-$ or $NO_3^-$ media, as well as $[K^+]_c$ for each condition. Note that there was not very much effect of starvation on $^{o}k_K^{OR}$ values in $NO_3^-$ or in $Cl^-$-suspended cells. Figure 4*A*,*B* presents the $Cl^-$-dependent $Rb^+$ influx and $K^+$ efflux ($^{o}k_K^{OR}$) data computed from Table 2 together with the ATP levels measured at the start of the flux experiments. For comparison, the $Cl^-$-dependent $Rb^+(K^+)$ flux values of NEM-treated LK cells and their respective ATP concentrations reported earlier [20] were included in the diagram (bars designated IN). In contrast to the NEM-stimulated, the hyposmotically augmented as well as the basal $Rb^+(K^+)$ fluxes did not respond to metabolic depletion to the levels indicated by the ATP data. Quite to the contrary, the $Cl^-$-dependent $Rb^+$ fluxes of starved cells in hyposmotic media (bars designated H) were higher than those of isosmotic controls (bars designated I), and of about equal magnitude in repleted cells. A similar result was obtained for $Cl^-$-dependent $K^+$ efflux (Fig. 4*B*). Hence, as compared to the NEM-activated component, the basal and the volume-stimulated $K^+(Rb^+)$ fluxes were much more refractory to metabolic manipulation.

## $Cl^-$ Replacement by Lyotropic Anions

It was previously reported that anions of the lyotropic series activated volume-sensitive $K^+$ fluxes with the decreasing order: $Br^- > Cl^- > NO_3^-$ [27]. Figure 5 shows the fractional activation of NEM-stimulated $K^+$ efflux under zero-trans conditions by several anions of the lyotropic series, replacing $Cl^-$ over the full range between 0 and 150 mM. With respect to their effects on NEM-activated $K^+$ efflux, three groups of anions can be distinguished. 1. $Br^-$ stimulated $K^+$ efflux as it gradually replaced $Cl^-$. 2. $F^-$ and $HCO_3^-$ inhibited $K^+Cl^-$ flux in hyperbolic fashion, becoming more effective when at least 30% of the total $Cl^-$ was replaced. 3. $I^-$, $NO_3^-$ and $CNS^-$ were the most potent inhibitors of $K^+$ efflux. The data permit the conclusion that anions of groups 1 and 3 had higher affinities for the transport system than those of group 2. By aligning the point of 50% activation or inhibition, the following anion series resulted:

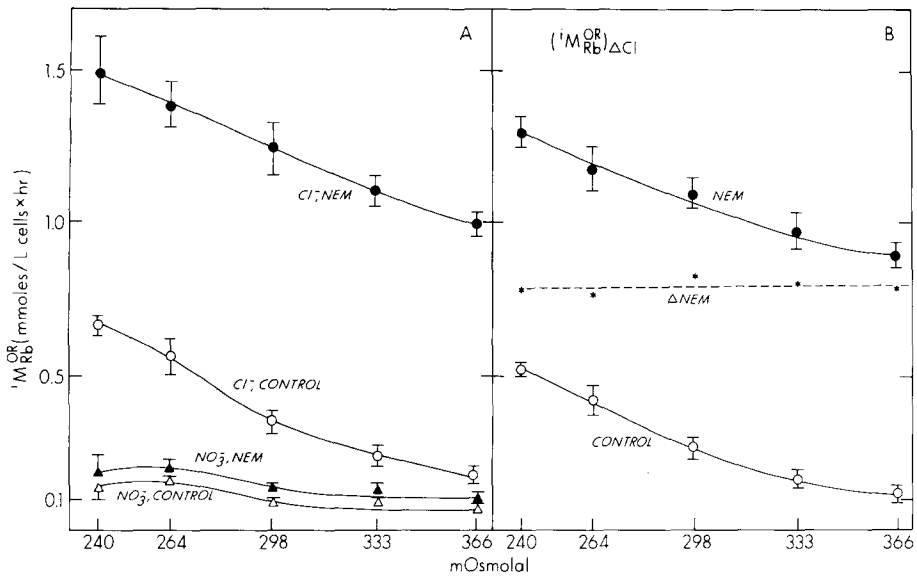



**Fig. 3.** Ouabain-resistant influx of $Rb^+$, $^iM^{OR}_{Rb}$, in control (open symbols) and NEM-treated (closed symbols) LK cells in $Cl^-$ (circles) or $NO_3^-$ (triangles). $Na^+$ media of different osmolalities with 10 mM $[Rb^+]_o$. Bars indicate SEM values for four independent experiments on the same animal as in Fig. 2. The slope of the dotted line was calculated from the regression analysis of the ΔNEM $^iM^{OR}_{Rb}$ values and was not different from zero. Data in Panel *B* are the $Cl^-$-specific values derived by subtracting $^iM^{OR}_{Rb}$ in $NO_3^-$ from $^iM^{OR}_{Rb}$ in Cl (Panel *A*)

**Table 2.** Effect of metabolic manipulation on ouabain-insensitive $Rb^+$ influx and $K^-$ efflux in low $K^+$ sheep red cells assayed in iso- or hyposmotic media

| mOsmol/liter | Treatment | Anion | $[K]_c$ mmol/liter cells | $^iM_{Rb}$ mmol/(liter cells · hr) | $^ok_K$ ($hr^{-1}$) |
|---|---|---|---|---|---|
| 297 ± 2 | Controls | $Cl^-$ | 12.5 ± 0.5 | 0.27 ± 0.05 | 0.073 ± 0.011 |
| | | $NO_3^-$ | | 0.13 ± 0.02 | 0.034 ± 0.008 |
| | Starvation | $Cl^-$ | 12.3 ± 0.8 | 0.38 ± 0.05 | 0.058 ± 0.004 |
| | | $NO_3^-$ | | 0.09 ± 0.02 | 0.029 ± 0.003 |
| | Repletion | $Cl^-$ | 11.0 ± 0.5 | 0.53 ± 0.06 | 0.089 ± 0.018 |
| | | $NO_3^-$ | | 0.17 ± 0.03 | 0.044 ± 0.007 |
| 246 ± 4 | Controls | $Cl^-$ | 12.3 ± 0.6 | 0.48 ± 0.05 | 0.101 ± 0.009 |
| | | $NO_3^-$ | | 0.17 ± 0.03 | 0.045 ± 0.009 |
| | Starvation | $Cl^-$ | 12.1 ± 0.7 | 0.48 ± 0.07 | 0.068 ± 0.004 |
| | | $NO_3^-$ | | 0.09 ± 0.02 | 0.023 ± 0.006 |
| | Repletion | $Cl^-$ | 11.4 ± 0.6 | 0.60 ± 0.11 | 0.111 ± 0.012 |
| | | $NO_3^-$ | | 0.14 ± 0.05 | 0.036 ± 0.008 |

$n$ = 4 experiments on LK(LL) sheep 15 ± SEM.

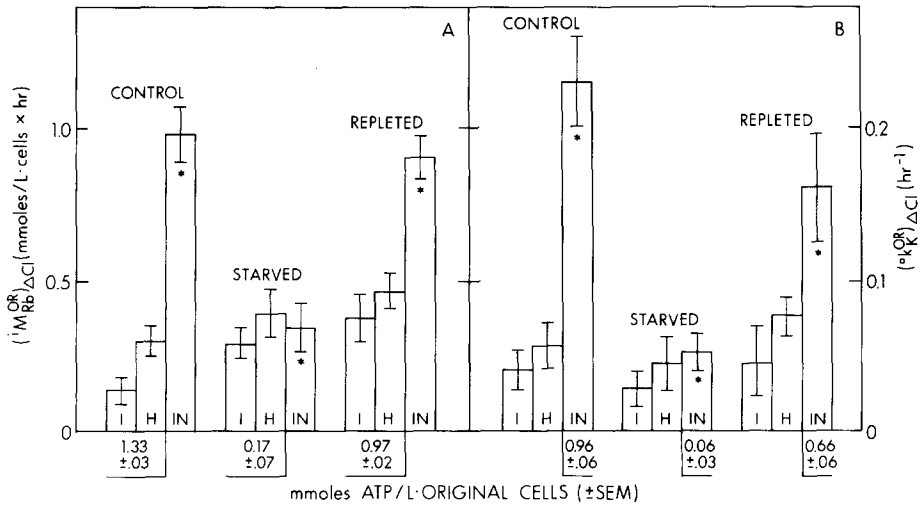



**Fig. 4.** Ouabain-resistant (OR) influx ($^iM^{OR}_{Rb}$, panel *A*) and rate constants of $K^+$ efflux ($^ok^{OR}_K$, panel *B*) of control, 16-hr starved, and repleted LK red cells, measured in isosmotic (I), hyposmotic (H, 250 mOsM media), or NEM-treated and measured in isosmotic media (IN). The numbers in the abscissa in *A* indicate the mmol ATP/liter original cells ± SEM for four determinations (*see also* bars). The stars on the IN columns with the ATP data (*see* panel *B*) indicate their replotting from Table 1 of ref. [20]

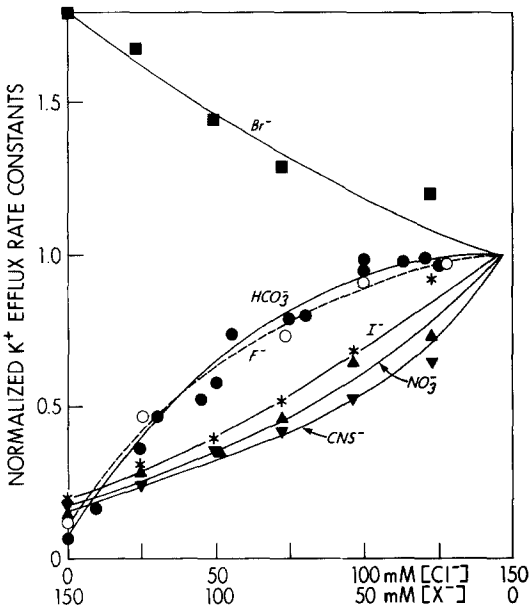



**Fig. 5.** The effect of $Cl^-$ replacement on ${}^{o}k_K^{OR}$ in NEM-treated LK red cells. For comparison, the data from the six replacement experiments were normalized with respect to the ${}^{o}k_K^{OR}$ in all $Cl^-$ media. [$X^-$] stands for the lyotropic anion concentration replacing [$Cl^-$]

$$Br^- > Cl^- \gg F^- > HCO_3^- \gg I^- > NO_3^- = CNS^-.$$

The data obtained with $HCO_3^-$ required a closer look as pH changes were involved. Figure 6 shows the rate constants of the NEM-activated $K^+$ efflux component as function of decreasing $Cl^-$ and increasing $HCO_3^-$ concentrations (bottom abscissa). For all points, excluding the star symbols, 10% $CO_2$ was used. Hence, the calculated pH increased with increasing $[HCO_3^-]_o$ (top abscissa). The continuous drop of ${}^{o}k_K^{OR}$ as $[HCO_3^-]_o$ increased, however, can not be ascribed to influences of increased pH, because when $CO_2$ was increased with $[HCO_3^-]_o$, the ${}^{o}k_K^{OR}$ values measured at 37.5 and 75 mM $[HCO_3^-]$ at $pH_o = 7.9$ fell well into the range of the other determinations performed at pH 7.6 and 7.4 (*see* star symbols). This finding also excluded the possibility of an effect due to pH-induced cell swelling. The saturability of the $HCO_3^-/Cl^-$ activation curve invited an analysis using the Hill equation. From the insert of Fig. 6 plotting the log of ${}^{o}k_K^{OR}$ remaining as a fraction of ${}^{o}k_K^{max}$, versus the log [$Cl^-$] a slope of 1.87 and an apparent $K_{0.5}$ for $Cl^-$ of 38 mM was calculated. A Hill coefficient of close to 2 may indicate molecular cooperativity or participation of more than one site at which $Cl^-$ and $HCO_3^-$ competed.

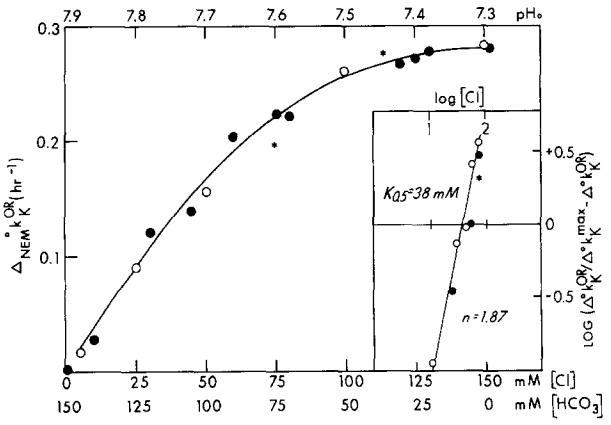



**Fig. 6.** Effect of $Cl^-$ replacement by $HCO_3^-$ on the NEM-specific ${}^{o}k_K^{OR}$ values as a function of [$Cl^-$] and $pH_o$. The open and closed dots are from two different experiments. Star symbols denote an experiment with the [$Cl^-$] and [$HCO_3^-$] as indicated but with pH adjusted to 7.9 by varying [$CO_2$] (*see* Materials and Methods). Insert is a Hill plot utilizing only the data between log + 1 and log − 1 on the abscissa

## Immunologic Identity of NEM- and Volume-Activated $K^+Cl^-$ Transport

Allo-immune anti-L is known to reduce the basal OR $K^+$ flux in LK red cells [7, 8] as well as the volume-activated [9, 11] and NEM-stimulated $K^+Cl^-$ fluxes [25, 27]. Hence, this immunologic tool should be a very sensitive probe to test the relationship between all three $K^+$ flux components. Because of limited quantities of anti-L available, and in order to avoid dilution effects caused by dialysis in different $Cl^-$ or $NO_3^-$ media, all immunologic experiments were carried out in $Cl^-$ media only but in the presence or absence of furosemide. It was previously shown that, in the presence of $Rb_o^+$, $10^{-3}$ M furosemide inhibits the OR $K^+(Rb^+)$ flux to the extent of $Cl^-$ replacement with $NO_3^-$ [19].

Table 3 shows that the effect of six antisera tested in 14 experiments on the furosemide-sensitive (FS) ${}^{i}M_{Rb}^{OR}$ of NEM-treated or hyposmotically swollen red cells varied between serum preparations, the lowest activity present in serum S41 and the highest in serum S33. Since by and large this variation affected both NEM-treated and swollen red cells in a parallel manner, and as the total percentage of inhibition was 51% of the FS ${}^{i}M_{Rb}^{OR}$ in both cell preparations, an apparently identical immunologic reactivity existed between the two FS $Rb^+$ flux activities. Anti-$L_I$ reduced only the FS and not the furosemide-insensitive ${}^{o}k_K^{OR}$ and ${}^{i}M_{Rb}^{OR}$ values of NEM-treated cells.

A stringent test for immunologic identity of the two $K^+Cl^-$ transport modes is to check whether

**Table 3.** Effect of various anti-L sera on furosemide-sensitive, ouabain-resistant $Rb^+$ influx in NEM-treated and swollen low $K^+$ sheep red cells

| Anti-L serum preparation | $Rb^+$ Influx (mmol/liter cells · hr) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NEM-treated (isosmotic) | | | | | Hyposmotic (250 mOsM) | | | | |
| | *n* | Total | F[a] | ΔF[b] | % Inhib | *n* | Total | F[a] | ΔF[b] | % Inhib |
| None | 5 | 1.36 | 0.33 | 1.03 | — | 6 | 1.33 | 0.34 | 1.00 | — |
| | | .13 | .04 | .10 | | | .07 | .01 | .06 | |
| S33 | 1 | 0.60 | 0.34 | 0.27 | 74 | 4 | 0.80 | 0.46 | 0.34 | 66 |
| | | | | | | | .04 | .03 | .03 | |
| S35 | 2 | 0.91 | 0.33 | 0.59 | 48 | 1 | 0.97 | 0.41 | 0.56 | 44 |
| S41 | 2 | 1.07 | 0.37 | 0.70 | 32 | 1 | 1.04 | 0.45 | 0.59 | 41 |
| S43 | 5 | 0.78 | 0.37 | 0.41 | 60 | 1 | 0.73 | 0.35 | 0.38 | 62 |
| | | .13 | .06 | .08 | | | | | | |
| S44 | 3 | 0.87 | 0.41 | 0.46 | 55 | 1 | 0.75 | 0.37 | 0.38 | 62 |
| | | .13 | .08 | .06 | | | | | | |
| S48 | 1 | 1.14 | 0.42 | 0.72 | 30 | 1 | 1.15 | 0.48 | 0.67 | 33 |
| 6 Sera | 14 | 0.90 | 0.37 | 0.53 | 51 | 9 | 0.91 | 0.42 | 0.48 | 51 |
| | | .08 | 0.01 | .07 | ±7 | | .07 | .02 | .06 | ±6 |

[a] Furosemide = $10^{-3}$ M.
[b] Furosemide-sensitive component.
*n* = number of determinations per anti-L preparation ± SEM.

NEM-treated and control cells have the same capacity to absorb anti-$L_l$-antibodies. Figure 7 *A,B* presents the data of two experiments. After absorption of anti-$L_l$ with an equal volume of either control or NEM-treated LK cells, the cell-free supernatants as well as the unabsorbed anti-$L_l$ were diluted and then tested for residual antibody activity on untreated hyposmotically (250 mOsM) swollen (*A*) or NEM-treated, isosmotically suspended (*B*) LK red cells (*see* Table 1). In contrast to unabsorbed serum which at highest concentrations reduced the control and the NEM-induced FS $^iM_{Rb}^{OR}$ influx by about 60% (*see* insert, Fig. 8*B*), the absorbed sera failed to affect $Rb^+$ influx in both test systems. These findings mean that 1) NEM did not destroy the $L_l$ antigen, 2) that the quantity of $L_l$ antigen/cell remained unchanged after NEM treatment, and 3) that the immunologic reactivity between the $L_l$ antibody and the $L_l$ antigen was identical for the two $K^+Cl^-$ transport modes (insert of Fig. 7*B*).

Before testing whether anti-$L_l$, once bound to the LK red cell membrane, would prevent also the action of NEM, the effect of NEM on the antibody itself was tested to exclude nonspecific effects. In Table 4, the effect of anti-$L_l$ on the hyposmotically stimulated FS $K^+$ flux was measured after the antiserum had been treated first with DTT (line 4), or with NEM ± DTT (lines 6, 8) as compared to controls without anti-$L_l$ (lines 3, 5, 7) and without chemicals (line 1). Note that DTT alone reduced FS $Rb^+$ influx by 30% (lines 3/1) and that the same effect was seen

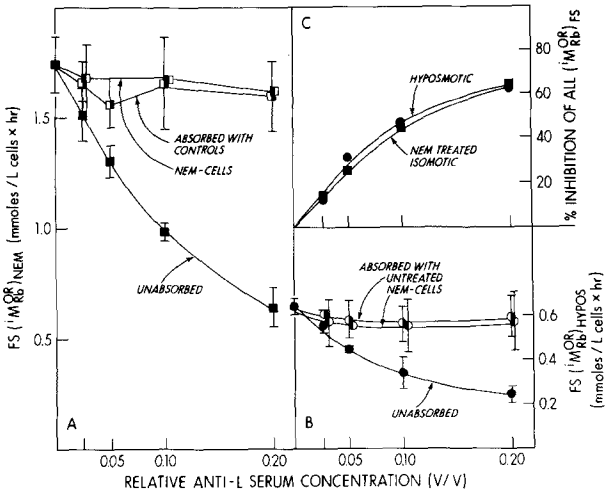



**Fig. 7.** Effect of unabsorbed anti-L on furosemide-sensitive (FS) $^iM_{Rb}^{OR}$ in NEM-treated (*A*, filled squares) and hyposmotically swollen (*B*, filled dots) LK cells. Data of *A* and *B* are plotted in *C* as percent inhibition of all FS ($^iM_{Rb}^{OR}$) points. Anti L absorbed with controls or NEM-treated cells was tested on NEM-treated (*A*, half-filled squares) or swollen (*B*, half-filled circles) LK cells

when DTT was used in excess of NEM (to inactivate the latter, lines 7/1). Consequently, while anti-$L_l$ without chemicals inhibited $Rb^+$ influx by 66% (lines 2/1), it inhibited by 73% (lines 4/1) when compared to controls with DTT (61%, lines 4/3). Pretreatment of anti-$L_l$ with NEM but without DTT

**Table 4.** Effect of NEM ± DTT on anti-L activity as tested on ouabain-resistant, furosemide-sensitive $Rb^+$ influx in swollen LK red cells

| # | Treatment order for anti-L serum | | | *n* | $Rb^+$ Influx (mmol/liter cells · hr) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Anti L 1st | NEM 2nd | DTT 3rd | | Total | + Furosemide[a] | $\Delta F^b$ | % Inhib. by anti-L | % Inhib. by DTT |
| 1 | − | − | − | 5 | 1.32 | 0.34 | 0.99 | | |
| | | | | | .09 | .02 | .08 | | |
| 2 | + | − | − | 4 | 0.80 | 0.46 | 0.34 | 66 | |
| | | | | | .04 | .02 | .02 | | |
| 3 | − | − | + | 4 | 1.03 | 0.34 | 0.69 | | 30 |
| | | | | | .02 | .04 | .04 | | |
| 4 | + | − | + | 3 | .67 | 0.40 | 0.27 | 61 | |
| | | | | | .05 | .01 | .04 | | |
| 5 | − | + | − | 5 | 2.28 | 0.37 | 1.91 | | |
| | | | | | .09 | .03 | .08 | | |
| 6 | + | + | − | 4 | 1.47 | 0.59 | 0.88 | 54 | |
| | | | | | .08 | .05 | .05 | | |
| 7 | − | + | + | 4 | 1.02 | 0.32 | 0.70 | | 30 |
| | | | | | .04 | .04 | .01 | | |
| 8 | + | + | + | 3 | 0.72 | 0.39 | 0.33 | 53 | |
| | | | | | .08 | .03 | .04 | | |

[a] $10^{-3}$ M.
[b] Furosemide-sensitive component.
*n* = number of determinations.

and subsequent additions to swollen LK cells decreased total and FS $Rb^+$ influx by 54% as compared to the respective control (lines 6/5). Although preventing totally the NEM effect, the presence of DTT did not alter the potency of the antibody further (53%, lines 8.7). Hence, NEM reduced the immunologic capacity of anti-$L_l$ to inhibit FS $^iM_{Rb}^{OR}$ influxes by 19 and 13% in the absence and presence of DTT, respectively.

With these data at hand, it was possible to test the effect of anti-$L_l$ pretreatment on the subsequent activation of FS $^iM_{Rb}^{OR}$ by NEM. Table 5 shows that anti-$L_l$ indeed prevented the full effect of NEM on $^iM_{Rb}^{OR}$ suggesting, as pointed out by others [27], that the $L_l$ antibody binding site is separate from the site reacting with NEM. This supposition is strengthened by the finding that the impermeant thiol reagent glutathione-maleimide-I had no significant effect on $Rb^+$ influx (*data not shown*) suggesting that the crucial SH groups reacting with NEM are either buried within the membrane or are on the cytoplasmic side.

## Discussion

The central problem emerging throughout these experiments on LK sheep red cells was the relationship between the chemically and the osmotically stimulated $Cl^-$-dependent $K^+$ fluxes, the latter being considered as a major pathway of OR passive $K^+$ transport [9]. In their response to volume changes and manipulation of cellular metabolism both activated fluxes behaved functionally different (Figs. 2–4, Table 2), but in their immunological reactivity they were similar (Fig. 7, Table 4). Prior to attempting a synthesis of the data and deriving a model combining all the features observed it is relevant to illuminate some of the details of the experiments with contradicting or supporting evidence for one and the same $K^+Cl^-$ transporter.

The data in Fig. 1 together with those of earlier findings [17] established that the furosemide-sensitive $K^+Cl^-$ transporter of controls and NEM-treated LK red cells is slightly asymmetric with respect to the apparent low affinities for cellular $K^+$ (13.2 and 10.8 mM $[K^+]_c$ for controls and NEM-treated cells, respectively) and to the external affinities for $Rb^+$ or $K^+$ of 17 versus 24 mM (*see* Fig. 7 in ref. 17). This transporter responds to the direction of the combined chemical gradients for $K^+$ and $Cl^-$ as proposed earlier [17].

An apparent functional dichotomy of $K^+Cl^-$ transport stimulated by NEM or osmotic swelling was documented first by the fact that, at all osmolarities tested, the NEM-activated component added on to the volume stimulated $K^+Cl^-$ flux. Hence, the $Cl^-$- and NEM-specific fraction of

**Table 5.** Effect of NEM on furosemide-sensitive $Rb^+$ influx in sheep red cells first exposed to alloimmune anti-L serum

| Treatment order for LK cells | | | $10^{-3}$ M furosemide | $^iM_{Rb}$ (mmol/liter cells · hr) | | Percent inhibition |
|---|---|---|---|---|---|---|
| 1st | 2nd | 3rd | | Total | F.S. | |
| Buffer | Buffer | DTT | − | 0.53 ± 0.04 | | |
| | | | + | 0.26 ± 0.02 | 0.27 | − |
| Anti-L | Buffer | DTT | − | 0.39 ± 0.03 | | |
| | | | + | 0.31 ± 0.02 | 0.08 | 70 |
| Buffer | NEM | DTT | − | 2.11 ± 0.04 | | |
| | | | + | 0.41 ± 0.02 | 1.70 | − |
| Anti-L | NEM | DTT | − | 1.34 ± 0.06 | | |
| | | | + | 0.48 ± 0.01 | 0.86 | 50 |

DTT = Dithiothreitol, 7.8 mM.
NEM = N-ethylmaleimide, 2 mM.
Anti-L: relative concentration 0.3 (vol/vol).
$n = 4 \pm$ SEM.

$K^+(Rb^+)$ transport was always of equal magnitude (ΔNEM, Figs. 2, 3). Second, the metabolic depletion studies revealed beyond doubt that $K^+Cl^-$ fluxes of depleted cells suspended in hyposmotic media were not decreased, which is in sharp contrast to the response of the NEM-activated component to metabolic manipulation (Table 2, Fig. 4). Perhaps longer times of depletion may be required to achieve a similar effect in the osmotically responsive as in the chemically activated system [20]. Alternatively to differences in affinities for the same metabolite, different metabolites may regulate the volume-sensitive $K^+Cl^-$ flux. The findings of a recent report [27] that NEM-treated LK red cells subsequently metabolically depleted did not show a reduction in NEM-stimulated $K^+Cl^-$ flux supports our view that metabolism is required for energization of the NEM-susceptible SH group or the stimulation of $K^+Cl^-$ transport itself.

Replacement of $Cl^-$ by other anions of the lyotropic series led, on the basis of inhibition or activation, to distinction of three anion subgroups for LK cells, but not for HK cells where the $^o k_K^{OR}$ values were statistically not different in media with anions other than $Cl^-$. The shape of the $Cl^-$ replacement curves suggest that $Br^-$ was preferred over $Cl^-$, that $HCO_3^-$ and $F^-$ competed comparatively weakly, and $I^-$, $NO_3^-$, and $CNS^-$ much more strongly with $Cl^-$ for its binding and/or transport sites (Fig. 5). For comparison, the anion sequence of the volume-sensitive $K^+/Cl^-$ pathway has been reported to be $Br^- > Cl^- > NO_3^-$ [27].

There are potential problems arising from studies replacing $Cl^-$ with anions of group 2. Firstly, the formation of $NaCO_3^-$ [3, 12] was certainly highest at pH 7.9 when the cells were equilibrated in $NaHCO_3$ only, a fact which could have caused volume increases and hence stimulation of $K^+$ efflux. The data in Fig. 6, however, exclude major volume effects on the basal $K^+$ flux in 150 mM $NaHCO_3$ solution. It cannot be ruled out that increase of cell volume due to $NaCO_3^-/HCO_3^-/CO_2$ equilibration actually inhibited $K^+$ transport by mechanisms different from those considered here. Secondly, the stimulating effects of $F^-$ on passive $K^+$ fluxes [10, 32] are well known as $F^-$ inhibits the enolase and hence lowers cellular ATP to cause the Gardos effect [28]. However, $F^-$ inhibited rather than activated $K^+$ fluxes and, furthermore, sheep red cells lack the Gardos phenomenon [4].

The Hill coefficient $n$ of the $HCO_3^-/Cl^-$ activation curve was found to be closer to two, hence, contrasting with an $n$-value of unity for $K^+$ or $Rb^+$ influxes as shown in Fig. 8. The apparent $K^+$ or $Rb^+$ affinity of 35 mM (Fig. 8), was well within the range of those reported earlier [17] and similar to that found for $Cl^-$ here. These data suggest that in contrast to $K^+$ there may be more than one group of sites for $Cl^-$, perhaps modifier sites with different affinities to which anions other than $Cl^-$, especially those of group 2 and 3 bind and thus inhibit $K^+Cl^-$ transport. This situation reminds one of the discussion of modifier sites in the $Cl^-/Cl^-$ self-exchange protein, kapnophorin [31], of human erythrocytes [14]. As recently reviewed [6], there is substantial evidence for lyotropic anion adsorption to macromolecules, a process which may offset the $H^+$ equilibrium due to a shift in the $pK_a$ of ionizable groups. The NEM effect is maximal when LK cells are pretreated at pH below 7 [2] indicating $H^+$ titration of

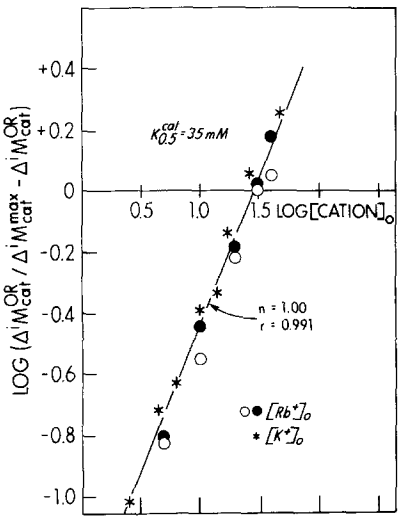



**Fig. 8.** Hill plot of $K^+(Rb^+)$ influx ($\log(\Delta\, {}^iM^{OR}_{cat}/\Delta\, {}^iM^{max}_{cat} - \Delta\, {}^iM^{OR}_{cat})$ from various $^{42}K^+$ and $Rb^+$ influx experiments on NEM-treated LK cells as a function of the log of $[cation]_o$: $n$ = Hill coefficient and $r$ = correlation coefficient. $\Delta\, {}^iM^{OR}_{cat}$ stands for the $Cl^-$-dependent $K^+(Rb^+)$ influx of NEM-treated LK cells

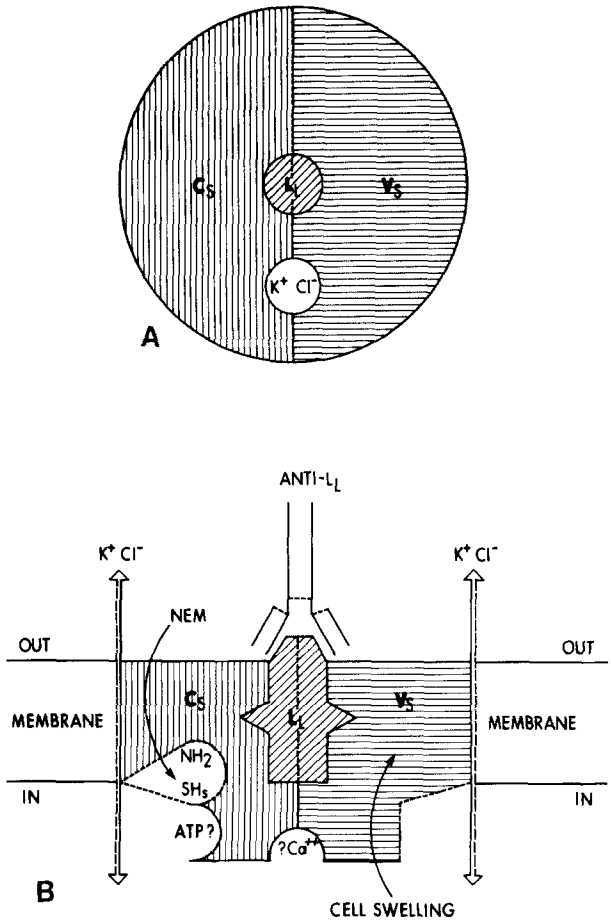



**Fig. 9.** A two-domain model accounting for the findings of operationally distinct but immunologically identical $K^+Cl^-$ transport in LK sheep red cells stimulated by N-ethylmaleimide (NEM) or cell swelling. *A* = the membrane exofacial aspect of the $K^+Cl^-$ transport system, *B* = membrane cross-section of the transporter. $C_S$ and $V_S$ = chemically and volume-stimulated functional domains or subunits of one component, respectively. In $C_S$, $SH_S$ is the SH group through which NEM stimulates $K^+Cl^-$ transport via one pathway (*A*) which in (*B*) is doubled to indicate also control by $V_S$. The apparent low $pK_a$ of the $SH_S$ group may be due to nearby $NH_2$ groups. ATP is placed into the metabolically controlled site of $C_S$ to indicate the role of metabolism, that has not been shown yet for $V_S$. The $L_l$-antigen ($L_L$), and the NEM reactive $SH_S$ groups of $C_S$ and $V_S$, are independent from each other while there is functional interdependency between the $SH_S$ groups and the metabolically controlled (ATP) site. The changes in $V_{max}$ induced by cell swelling or NEM are not yet specifically addressed in this model (*see* text). Also, no account is made for the documented presence of inhibitory SH groups [refs. 2 and 27] and for some complex effects of $Ca^{++}$ and its ionophore A23187 [9, 11, and Lauf, *unpublished observations*]

weak amino groups which may change their $pK_a$ values in the presence of inhibiting anions of group 2 and particularly of group 3. No conclusion can be made as to the mechanism by which $Cl^-$ together with $K^+$ is transported. It is, however, interesting to note that in swollen cells, $K^+$ fluxes increased also in $NO_3^-$ (Fig. 1), perhaps suggesting that even inhibitory anions, together with a $K^+$, slip occasionally across the system. Indeed, the nonlinearity of ${}^o k^{OR}_K$ versus $[K^+]_c$ in $NO_3^-$, in both LK (Fig. 1) and in HK red cells with $[K^+]_c$ artificially lowered to the levels of LK cells (*data not shown*) suggests a nondiffusional nature also of $K^+$ fluxes in $NO_3^-$, a supposition that needs to be addressed in the future.

The immunological studies strongly suggest that the two stimulated $K^+Cl^-$ transport modes are identical because the degree of inhibition by anti-L was indistinguishable and there was no evidence from the absorption studies for the presence of extra sites once the LK cells had been treated with NEM. Consistent with another report [27], the $L_l$ antigenic site cannot be close to the NEM target group as anti-$L_l$ reduced the $K^+Cl^-$ flux, i.e. anti-$L_l$ protected even when added before NEM treatment of the cells (Table 5). Furthermore, the NEM site is not external like the $L_l$ antigen, as the impermeant SH-reagent glutathione-maleimide-I failed to cause the typical stimulation of $K^+/Cl^-$ transport seen with NEM.

## A Two-Domain Model of $K^+/Cl^-$ Transport

Table 6 summarizes the differences and similarities of the basal, the volume- and NEM-stimulated $K^+$ fluxes. Based on the information now available the data presented here are consistent with a model (Fig. 9) of two operationally different subunits or domains $C_S$ and $V_S$, of the same transport molecule responsible for the basal $K^+Cl^-$ flux which may be coupled through the antigenic component, $L_l$ (Fig. 9*B*). The chemically activated domain $C_S$ responds to NEM and other SH-group specific reagents, an

**Table 6.** Some properties of $Na^+$-independent OR $K^+Cl^-$ fluxes in LK sheep red cells before and after exposure to hyposmotic media or to N-ethylmaleimide

| Parameter | Isosmotic controls | Hyposmotically swollen cells | NEM-treated cells |
|---|---|---|---|
| Approx. $V_{max}$ of $Rb^+(K^+)$ influx (mmol/liter cells · hr) | 0.6–1.0 [17] | 5–14 [9] | 4–7 [17] |
| Approx. $K_{0.5}$ for $Rb_o^+$ or $K_o^+$ (mM) | 20–50 [17, 18] | 34–47 [9] | 27–50 [17, 18] |
| Approx. $K_{0.5}$ for $K_c^+$ (mM) | ~13 [a] | unknown | ~10 [a] |
| Anion preference | $Br > Cl \gg SCN = I > NO_3$ | $Br > Cl \gg SCN = I > NO_3$ [9, 27] | $Br > Cl \gg HCO_3 = F \gg I = NO_3 = SCN$ [a] |
| Metabolic dependence | NO [20, a] | NO [a] | yes [20, a] |
| Inhibition by furosemide ($10^{-3}$ M) | partially [11, 27] | partially [27] | fully with $Rb_o^+$ [19] |
| Effect of $Ca_o^{++}$ + A23187 | – | No specific effect [9, 11] | – |
| Effect of anti-$L_L$ antibody | reduction [7, 9, 11, 21, 25] | reduction [9, 11] | reduction [21, 25, a] |

Numbers in [ ] indicate references; [a] = this study.

effect depending on the possible presence of titratable $NH_2$ groups [2]. Domain $C_S$ can only react with NEM when it is "energized" through metabolism, and only then will stimulation of the basal $K^+Cl^-$ flux occur. Domain $V_S$, affected by cell volume changes, apparently is independent of the metabolic state. The role of cellular $Ca^{++}$ has to be explored, since in human red cell ghosts NEM has been shown to significantly reduce the amount of tightly bound $Ca^{++}$ [29].The NEM- or volume-increased $V_{max}$ of $K^+Cl^-$ transport coupled with an apparent constancy in the number of L-antigenic sites requires further investigations. While features of this model are the interfaces between the $C_S$ and $V_S$ domains, the $L_I$ component and a $K^+Cl^-$ path (Fig. 9*B*), it is not excluded that dimeric oligomers exist containing either $C_S$ and $L_I$, and $V_S$ and $L_I$ (dotted line through $L_I$ in Fig. 9). From a speculatory point of view, the $C_S$ domain may act as a transducer between extracellular signals and cellular metabolism, and thus serve to regulate $K^+Cl^-$ transport during cellular maturation.

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