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Thiol-Dependent Passive K/Cl Transport in Sheep Red Cells: I. Dependence on Chloride and External K^+ [Rb^+] Ions

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Summary. Treatment with 2 mM N-ethylmaleimide (NEM) caused a marked increase in K^+ permeability of low K^+ but not of high K^+ sheep red cells suspended in isosmotic Cl^- media with 10^{-4} M ouabain. The Na^+ permeability was unaltered. Kinetic analysis by K^+ efflux and K^+ or Rb^+ influx measurements suggests that NEM primarily increased the bidirectional fluxes of K^+ and Rb^+, since (a) no significant change in the apparent external affinities of these ions was found, and (*b*) below unity, the observed flux ratios were close to those calculated from the Ussing relationship. Replacement of Cl^- by NO_3^- abolished the NEM-stimulated and reduced the basal K^+ flux rates. Similarly, 10^{-3} M furosemide inhibited Cl^--dependent K^+ fluxes in both control and NEM-treated LK red cells. Exposure of LK cells to hyposmotic but not to hyperosmotic salt solutions increased the basal Cl^- dependent K^+ flux twofold as reported by Dunham and Ellory (*J. Physiol. (London)* **318**:511–530, 1981) but did not affect its fractional stimulation by NEM. The action of NEM is interpreted as a stimulation of a temperature-dependent and Cl^--requiring K^+ transport pathway genetically preserved in adult LK but turned off in HK sheep red cells. In addition, common to both LK and HK sheep red cells was a basal K^+ flux that operated in the presence of either Cl^- or NO_3^-.

Key Words sheep erythrocytes · passive K^+Cl^- cotransport · sulfhydryl (SH) groups

Introduction

Genetically low K^+ (LK) and high K^+ (HK) sheep red cells maintain their cation steady states by differences in their active and passive Na^+ and K^+ fluxes: LK red cells have a lower absolute Na^+K^+ pump activity and higher passive K^+ fluxes than HK red cells [12, 24, 45]. A remarkable genetic association exists between the two cation steady states and two membrane surface antigens: The L antigens are located on LK and the M antigens on HK red cells [34, 39, 40, 46]. Much attention has been paid to explain the mechanism by which anti-L serum activates the kinetically different Na^+, K^+ pumps in LK sheep and goat red cells [15, 30, 32], but progress in the area of passive cation permeabilities of the two cell types is only of very recent nature [9, 10].

Two novel discoveries have shed new light on the nature of the passive Na^+, K^+ fluxes in cation dimorphic ruminant red cells. First, cell swelling was shown to enhance ouabain-insensitive K^+ fluxes in LK but not in HK sheep red cells [11, 14], and anti-L serum, known to reduce specifically passive K^+ leak fluxes [9, 33], only partially prevented this effect [11, 14]. Second, in two preliminary studies it was reported that treatment with the sulfhydryl (SH) group reagent N-ethylmaleimide (NEM) stimulated passive K^+ and not Na^+ fluxes in LK but not in HK sheep red cells, respectively [35, 36]. As in case of volume manipulation [11, 14], the NEM enhanced K^+ flux was detected only in Cl^- or Br^- media, but not in solutions with NO_3^-, I^-, SO_4^{2-}, PO_4^{3-}, or SCN^- [28, 35, 36], anions which also equilibrate across the red cell membrane via the anion exchange pathway.

In sheep red cells Ca^{++} ions apparently do not induce K^+ movements by the Gardos effect [6]. Rather, the Cl^- specificity of the observed K^+ fluxes augmented after cell swelling or NEM treatment implies that passive transport of this cation may be more complex and not solely explained by electrodiffusion. Consistent with this interpretation are recent reports of the existence of passive K^+ Cl^- cotransport or Cl^--dependent Na^+, K^+ cotransport in human red cells [7, 8, 13, 17, 48], in avian and fish red cells [5, 21, 27, 29], in lymphocytes [19], ascites tumor cells [4, 18, 23], and in epithelial cells [1, 2, 20, 37]. There is evidence that transport of these ions is electroneutral, since no effects of membrane potential changes were seen.

The purpose of this study was to further characterize the properties of ouabain-insensitive K^+ fluxes stimulated by NEM in LK sheep red cells. Data will be presented on the kinetics of trans-

membraneous K^+ or Rb^+ fluxes, on the Cl^- dependence of K^+ fluxes (NO_3^- replacement), and on the relationship between K^+ fluxes induced by cell volume expansion and NEM treatment. The bulk of the results support the hypothesis that adult LK red cells possess Cl^--dependent K^+ transport which is specifically stimulated by NEM and may be related to a volume-sensitive K^+ transport [11, 14]. Although the primary effect of this SH reagent appears on the maximum velocity, V_{max}, of ouabain-insensitive K^+ transport, subtle kinetic changes cannot be ruled out at this time. In addition, a basal K^+ transport common to both LK and HK sheep red cells exists in the presence of NO_3^- and is principally unaffected by NEM under physiologic conditions. A preliminary report has appeared elsewhere [31].

Materials and Methods

Red Cells, Treatments, and Chemicals

Blood was drawn into heparin (10 IU/ml blood) from healthy Dorset sheep with known red cell K^+ and Na^+ composition and centrifuged within 2 hr to separate cells from plasma. As standard procedure, the cells were washed in isosmotic (290 mOsm) $NaNO_3$ solutions buffered with 10 mM Tris/NO_3 (TBN), pH 7.1 at 0 °C and containing 10^{-4} M ouabain, which was present throughout all subsequent steps. For treatment with NEM, cells were suspended at a hematocrit of 10% (vol/vol) in TBN, pH 7.1 at 37 °C to which an aliquot of 1 M N-ethylmaleimide (NEM, Sigma Chemicals, St. Louis, Mo.) dissolved in dimethylsulfoxide (DMSO, Sigma Chemicals, St. Louis, Mo.) was added to give a final NEM concentration of 2 mM; controls received DMSO only. The use of the lower pH value for treatment with NEM was justified on grounds of preliminary findings that NEM exerted its maximum effect at lower pH values. After 15 min incubation in a shakerbath, aliquots of 1 M dithiothreitol (DTT, Sigma Chemicals, St. Louis, Mo.) dissolved in TBN, pH 7.1, 37 °C, were added to all samples sufficient to remove on a molar basis the unreacted NEM. Cells were then washed once or twice in TBN, pH 7.4, at 0 °C, resuspended to 10% (vol/vol) in TBN, pH 7.4, at 37 °C for equilibration prior to the flux experiments. In the experiments involving the loop diuretic furosemide, control or NEM-treated red cells were suspended at a hematocrit of 10% in TBN, pH 7.4, at 37 °C, containing 10^{-3} M furosemide (Lot 0842, Hoechst-Roussel Pharmaceuticals, Sommerville, N.J.) and maintained at that temperature for 30 min. Furosemide was also present during the actual flux experiment. Tracer K^+ was obtained as $^{42}K_2CO_3$ from Burlington Nuclear Research Facilities, Raleigh, N.C., with a specific activity of about 15 Ci/mol. Ultrapure RbCl or $RbNO_3$ salts were obtained from Aldrich Chemical Co., and from Johnson Matthey Chemicals, Hertfordshire, England, respectively. K^+ contaminations were less than 1/10,000. Cell water was determined by the wet wt/dry wt method used earlier [29]. Osmolarities were measured using the Wescor (Logan, Utah) vapor pressure osmometer model 5100 B. Furosemide was a gift of Hoechst-Roussel Pharmaceuticals, Sommerville, N.J.

K^+ Effluxes

Aliquots of anion equilibrated cell suspensions were transferred into 40-ml polyethylene centrifuge tubes and spun at 37 °C and 15,000 rpm in a Sorval RC5B centrifuge to obtain sharp pellets of about 0.8 ml cells/tube. At $t=0$, 20 ml of temperature equilibrated flux medium (isosmotic with sheep plasma [290 ± 2 SEM = 6] and with varying K^+ or Rb^+ concentrations replacing Na^+ or choline on a molar basis) was added to each tube subsequently covered with parafilm and kept at 37 °C in a shakerbath. At 4–6 time intervals, about 3-ml aliquots were removed and placed on a 2-ml dibutylphthalate-ester cushion in 10-ml polypropylene centrifuge tubes kept on ice. Cells were collected beneath and cell free supernatants above the organic phase by centrifugation at 12,000 *g* for 2 min in a Sorval RC2B centrifuge. For K^+ analysis (Perkin Elmer atomic absorption spectrophotometer, model 460) and hemoglobin determinations ($OD^{527\,nm}_{sample}$), measured aliquots of supernatants were removed, mixed with 1/100 volume of 0.4 M CsCl required for removal of ionization interference and/or diluted into 0.004 M CsCl in acationic detergent. The measured extracellular K^+ concentration, $[K^+]_o$, was corrected for K^+ released by hemolysis (less than 1–2%) using the ratio of $OD^{527\,nm}_{sample}$ to the calculated optical density at 527 nm of 1 ml hemolyzed packed red cells, $OD^{527\,nm}_{pc}$. An aliquot of each suspension was hemolyzed with detergent to determine the concentration of hemoglobin ($OD^{527\,nm}_{sus}$), and the hematocrit ($OD^{527\,nm}_{sus}/OD^{527\,nm}_{pc}$). The equilibrium K^+ concentration, $[K^+]_e^{t=\infty}$ approximates $[K^+]_o^{t=\infty}$, the concentration in the extracellular medium [42]. Hence, for K^+ release the true K^+ efflux rate constant, $^{o}k_K$, was computed from the ratio of $[K^+]_o^t/[K^+]_o^{t=\infty}$ using the first order rate equation:

$$-\ln\left(1-\frac{[K^+]_o^t}{[K^+]_o^{t=\infty}}\right)={}^{o}k_K t. \tag{1}$$

In most experiments $^{o}k_K$ was estimated by regression line analysis of

$$-\ln\left(1-\frac{[K^+]_o^t}{[K^+]_o^{t=\infty}}\right)$$

as a function of the 4–6 measured time points. Data with correlation coefficients, *r*, less than 0.95 were rejected. Equation (1) was also used when ^{42}K efflux was measured in tracer preloaded red cells. For this purpose, 12 ml packed sheep red cells were preloaded with 2.5 mCi $^{42}KNO_3$ in a total volume of 90 ml for 3 hr at 37 °C in phosphate buffered isosmotic NaCl media, pH 7.4, in the presence of 5 mM glucose and without ouabain. Once preloaded, the cells were treated with NEM as described above, and ^{42}K release was monitored by counting quantitatively transferred supernatants in an Intertechnique counter. All other parameters were obtained and treated as in experiments determining net K^+ fluxes.

K^+/Rb^+ Influxes

Experiments were performed with ^{42}K as tracer or with ultrapure Rb^+ salts. The protocol of ^{42}K influx was similar to those published [25, 26]. At $t=0$ about 20 μCi $^{42}KNO_3$ (about 1 μM/25 μl) was added to 1 ml 10% cell suspension in isosmotic NaCl, $NaNO_3$ or choline-Cl media, containing 10^{-4} M ouabain, and buffered with 10 mM Tris/Cl or Tris/NO_3 to pH 7.4 at 37 °C. Influx was stopped after 1 hr by pipetting 0.2-ml cell suspension samples into 7 ml ice-cold $MgCl_2$ layered on top of 2-ml dibutylphthalate cushions and followed by subsequent centrifugation at 10,000 rpm. The cells collected below the phthalate cushion were hemolyzed for hemoglobin determinations at 527 nm and for ^{42}K counting. The volume of cells was calculated from the ratio $OD^{527\,nm}_{sample}/OD^{527\,nm}_{pc}$ multiplied by the total sample volume. Ouabain-insensitive K^+ influx was computed from Eq. (2):

$$^{i}M_K = \frac{[^{42}K]/\text{ml cells}}{[^{42}K]/\text{ml supernate}} \times [K^+]_o \times t^{-1} \quad (2)$$

from which the inward rate coefficient, $^{i}k_K = {}^{i}M_K/[K^+]_o$ was obtained. In case of Rb^+ influxes, RbCl or $RbNO_3$ was added at $t=0$ to 4% cell suspensions pretreated as described above. Aliquots of 1 ml were taken at indicated time intervals. The cells collected under the phthalate cushion were hemolyzed in detergent containing CsCl medium. Cellular Rb^+ was read at 780.1 nm by flame photometry using the Perkin Elmer 460 model with emission chopper. For calculations of Rb^+ influxes, the following equation was used:

$$^{i}M_{Rb} = \frac{E^{780\,nm}_{sample}}{e^{780\,nm}_{standard}} \times \frac{OD^{527\,nm}_{pc} \times DF_{Rb}}{OD^{527\,nm}_{sample} \times DF_{Hb}} - [Rb^+]^{cell}_{native} \quad (3)$$

where

$E^{780\,nm}_{sample}$ = Rb emission from the sample.

$e^{780\,nm}_{standard}$ = Emission of 1 mM Rb standard, usually 8–10/mM,

$DF_{Rb,\,Hb}$ = dilution factors necessary for sample reading by flame or hemoglobinometry at 527 nm,

$[Rb^+]^{cell}_{native}$ = (About 40–60 μM Rb^+/liter cell) natively occurring in sheep red cells.

For calculation of the expected flux ratio, the Ussing flux ratio equation [47]

$$^{i}M_{cat} + {}^{o}M_{cat} = [Cat^+]_o/[Cat^+]_i \exp(-EF/RT) \quad (4)$$

was used with assumption $E=-0.01$ mV (50). Implicit in the use of this equation is the fact that $E_m = E_{Cl}$ and $P_{Cl} \gg P_{K+} = P_{Rb+}$. The terms '$Cat^+_{o,i}$' stand for K^+ or Rb^+ ions, while $\exp(-EF/RT)$ has the usual meaning.

Results

K^+ Specificity of the NEM Effect

It was previously shown that exposure of homozygous LK (LL) sheep red cells to 2 mM NEM increased the ouabain-insensitive K^+ efflux almost immediately, leading to a three- to fourfold higher efflux rate constant than in the control. In contrast, NEM had no apparent effect on ouabain-insensitive Na^+ effluxes in LK red cells suspended in choline-Cl media [28, 35, 36]. Figure 1 shows that K^+ influx was independent of extracellular Na^+ concentrations, $[Na^+]_o$, between 0 and 100 mM $[Na^+]_o$ using choline as replacement cation and that NEM stimulated V_{max} by fourfold without affecting the apparent affinity for $[K^+]_o$, which was 25 mM in both control and NEM-treated cells. However, the inference that passive Na^+ fluxes were neither affected by NEM treatment nor that Na^+ ions were participating in the control or NEM-stimulated K^+ fluxes has to be made with caution, because in other experiments (*unpublished data*) the K^+ efflux rate constants were frequently smaller when measured in choline-Cl as compared to NaCl, without affecting the fractional stimulation by NEM. The possibility of *cis*-effects of Na^+ or of *trans*-effects of Na^+ and/or choline (*see also* reference 11) on $^{o}k_K$ needs further experimental exploration.

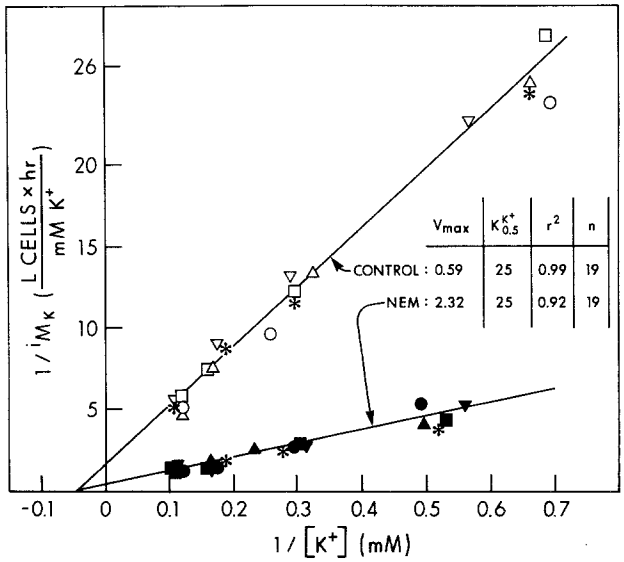

Fig. 1. K^+ influx in control (open symbols) and NEM-treated filled symbols) LK (LL) sheep red cells suspended in Cl^- media of varying Na^+ and increasing K^+ concentrations using choline-Cl as replacement salt. Inverse plot of $^{i}M_K$ versus $1/[K]_o$ ○● = zero, △▲ = 25, ▽▼ = 50, □■ = 75, and * = 100 mM NaCl

Chloride Dependence of NEM-Induced K^+ Flux

Earlier we reported that NEM-stimulated K^+ efflux was not observed when Cl^- was replaced by NO_3^-, SO_4^{2-}, or PO_4^{3-} anions, but not by Br^- [35] and that chloride replacement had only a very small effect on the basal K^+ flux of controls, an observation contradicted by others [11, 14]. It is possible that this discrepancy rested on a volume effect, since it is known that the Cl^--sensitive K^+ flux component is highly dependent on cell volume [11, 14]. Subsequent experiments consistently revealed K^+ fluxes which were reduced in NO_3^- equilibrated red cells as compared to Cl^- controls. Table 1 summarizes the rate coefficients of K^+ influx at $[K^+]_o$ varying from 2–8 mM/liter for all three red cell genotypes suspended in Cl^- or NO_3^- media. In NO_3^-, ouabain-insensitive K^+ influx was inhibited by 51% in homozygous LK (LL) and by 30% in heterozygous LK (LM) red cells, while HK red cells did not discriminate between Cl^- and NO_3^- anions. It is also noteworthy that $^{o}k_K$ was larger in LK red cells with lower $[K^+]_c$ values, suggesting that the magnitude of $^{o}k_K$ is somehow related to $[K^+]_c$. Analysis of a larger sample may reveal whether the trend of the Cl^--dependent K^+

Table 1. K^+ Influx rate coefficients in LK and HK red cells suspended in isosmotic NaCl or $NaNO_3$, pH 7.4

Cells	Antigen	$[K^+]_c$ mM/l. cells	n	$^ik_K(hr^{-1})$ in NaCl	$^ik_K(hr^{-1})$ in $NaNO_3$
LK 4	LL	10.8	7	0.094±0.008	0.046±0.002
LK 15	LL	16.8	7	0.058±0.004	0.028±0.001
LK	LL	13.8	Mean	0.076	0.037
LK 6	LM	19.0	8	0.050±0.004	0.032±0.001
LK 18	LM	21.7	8	0.037±0.002	0.029±0.001
LK	LM	20.3	Mean	0.044	0.031
HK 5	MM	81.7	8	0.034±0.003	0.028±0.001
HK 5*	MM	81.7	7	0.025±0.001	0.022±0.001
HK 21	MM	87.4	7	0.020±0.001	0.022±0.001
HK	MM	84.5	Mean	0.026	0.024

Data ± SEM from Exp 644/1980 and * from Exp 642/1980. n refers to the number of determinations at $[K^+]_o$ between 1 and 8 mM/liter.

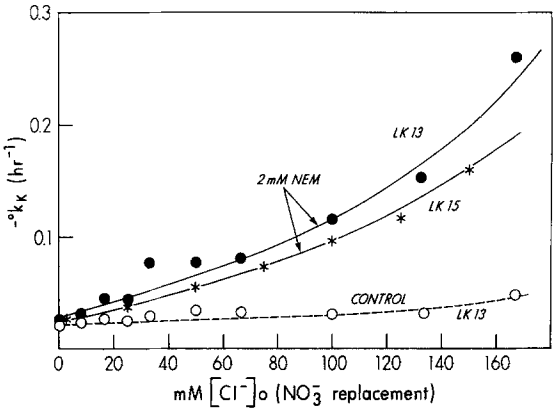

Fig. 2. Effect of Cl^- replacement by NO_3^- on K^+ efflux rate constants of control LK 13 (o) and NEM- treated LK 13 (•) or LK 15 (*) red cells. For NEM treatment, erythrocytes were suspended in isotonic NO_3^- and then analyzed for K^+ efflux in media with increasing proportions of Cl^- replacing NO_3^-

flux activities in the three red cell genotypes indicates a gene-dose effect.

The influence of gradually replacing NO_3^- by Cl^- anions on ^ok_K was investigated further in NEM-treated LK cells of two different homozygous sheep. Figure 2 shows that $^ok_K$was an upward shallow and curvilinear function of extracellular Cl^- concentrations, $[Cl^-]_o$, in both controls and most clearly in NEM-treated LK red cells. Earlier, a linear relationship was reported to exist between the inward rate coefficients of passive K^+ transport and the fractional $[Cl^-]_o$ of the medium [28]. The data support the hypothesis that in LK red

Table 2. Effect of cell volume changes and NEM treatment on K^+ influxes in LK sheep red cells

Medium osmolarity (mOsm/liter)	Cell water (kg H_2O/kg DCS[a])	Relative K^+ efflux rate constants[b]		Ratio of NEM/Control
		Control	NEM	
246±16	2.06±0.04	1.5 ±0.3	4.2±1.3	2.9±0.8
312±21	1.74±0.06	1.0 ±0.0	3.0±0.4	3.0±0.4
383± 5	2.60±0.00	0.83±0.03	2.6±0.6	3.2±0.8

[a] DCS = dry cell solids.

[b] Data from four different experiments were normalized with respect to control K^+ effluxes at 312 mOsm/liter and are expressed as relative rate constants.

$\bar{x}$ ± SD; $n=4$.

cells, NEM stimulated a Cl^--dependent K^+ transport system that was already present at low activity under isosmotic conditions. Nitrate anions may either inhibit Cl^--sensitive K^+ transport or may not be transported or both. In HK sheep red cells such a study was not performed because the ouabain-insensitive K^+ transport was insensitive to Cl^- replacement by NO_3^-, and NEM appeared to be ineffective [35].

Cell Volume and Cl-Dependent K^+ Flux Stimulated by NEM

Recently it has been reported that ouabain-insensitive K^+ fluxes increased by two- to eightfold when measured in LK red cells suspended in hyposmotic Cl^- media [11, 14]. Hence, it was of interest to investigate whether NEM still would elicit its effect in swollen LK red cells. Table 2 shows the rate coefficients of K^+ effluxes measured in LK cells suspended in 383, 312, and 242 mOsm Cl^- media. Prior to the flux measurements, all cells were exposed for 15 min to NO_3^- media of identical osmolarities ±NEM, pH 7.1. After exchange of NO_3^- by Cl^- and at the beginning of the flux experiment, samples were removed for cell water analysis. Note that cell swelling or shrinkage by 18–20% led to a change in cell water commensurate with the well-known Ponder relationship between cell water and the inverse of the medium osmolarity. In shrunken LK red cells ^ok_K was about 17% lower and in swollen cells about 50% higher than the basal K^+ flux rate of isosmotically suspended cells, a finding consistent with other reports [11, 14]. However, in the presence of NEM, ^ok_K was stimulated threefold and hence, the fractional stimulation was similar in all media. This finding suggests no overlap between volume-dependent and NEM-stimulated K^+ fluxes. In additional experiments it was shown

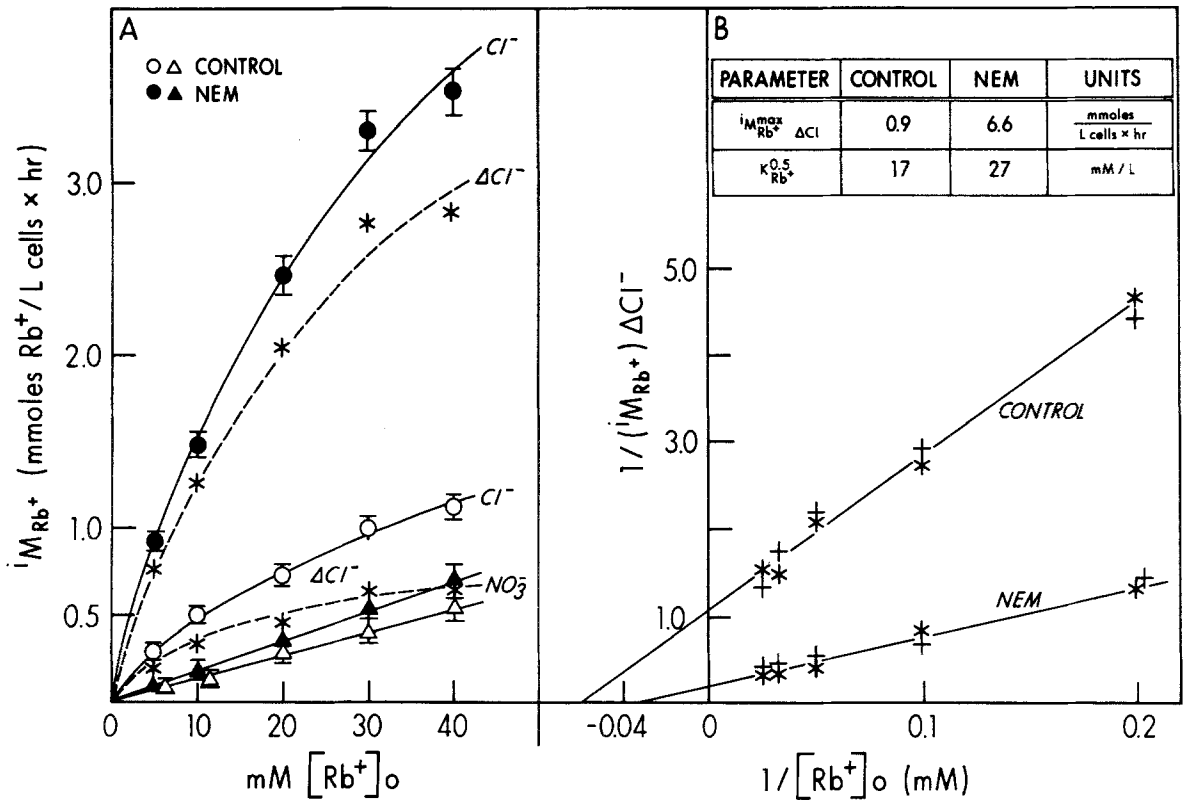

Fig. 3. Rb^+ influx as a function of $[Rb^+]_o$ and the effect of Cl^- replacement in controls and NEM-treated LK 15 red cells. (A): Open symbols = controls, filled symbols = NEM treated cells. (*B*): Inverse plot of the Cl^--specific Rb^+ influx component versus $1/[Rb^+]_o$. *, Data from *A*; and $^+$ from an earlier experiment on the same cells

that the alteration of Cl^- dependent K^+ fluxes presumably caused by volume expansion or contraction was of reversible nature and that the chemical action of NEM on its target group in the membrane was cell volume independent.

Effect of NEM on Simultaneous K^+ Effluxes and K^+ [Rb^+] Influxes in Cl^- and NO_3^- Media

The transmembranous fluxes of K^+ ions in Cl^- or in NO_3^- media were further investigated as a function of various extracellular Rb^+ or K^+ concentrations in order to decide whether NEM activated an obligatory K^+/K^+ exchange. Efflux of K^+ was measured within the same sample by K^+ analysis of the supernatants and cellular Rb^+ influx by atomic absorption spectrophotometry of the cell pellets collected separately beneath the dibutylphthalate cushion (*see* Methods). Figure 3*A* shows that the relationship between $^{i}M_{Rb}$ and $[Rb^+]_o$ was hyperbolic in control and NEM-treated LK red cells when suspended in Cl^- but linear in NO_3^- medium. Figure 3*B* shows the Lineweaver-Burke plot of the Cl^- specific Rb^+ influx component (i.e., the total Rb^+ influx in Cl^- minus Rb^+ influx in NO_3^-) versus $[Rb^+]_o$ yielding apparent affinities for $[Rb^+]_o$ of 27 and 17 mM, and V_{max} values of 6.6 and 0.9 moles/liter cells × hour for NEM-treated and control red cells, respectively. Note that the Cl^--sensitive Rb^+ influx was more than threefold greater than the NO_3^--supported Rb^+ influx. Figure 4 contains an identical experiment except that ^{42}K tracer was used to measure K^+ influx in Cl^- or NO_3^- media. Both Rb^+ and K^+ influxes were indistinguishable, validating the use of Rb^+ ions as effective K^+ analogues for these studies. Again there was a fourfold difference between $^{i}M_K$ measured in Cl^- versus NO_3^-. No such differences were found in HK red cells as shown in Fig. 5. Since in HK red cells $^{i}M_K$ appeared to be a rather linear function of $[K^+]_o$ their $K_{0.5}$ for $[K^+]_o$ seems to be much larger than in LK red cells.

The magnitude of K^+ or Rb^+ influxes was comparable to that of the K^+ effluxes, a finding compatible with obligatory exchange characteristics. To test this hypothesis, $^{o}k_K$ was measured in NEM-treated LK red cells suspended in NaCl or $NaNO_3$ containing increasing $[Rb^+]_o$ or $[K^+]_o$.

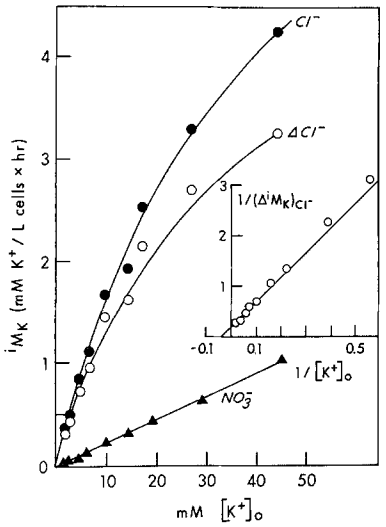

Fig. 4. Ouabain-insensitive K^+ influx in NEM-treated LK 15 red cells at varying $[K^+]_o$ and the effect of Cl^- substitution by NO_3^-. The experiment was carried out under conditions identical to those of Rb^+ influx in Fig. 3. The Cl^--specific component (o) was calculated from the difference between Rb^+ influx in Cl^- (●) and NO_3^- (▲). *Insert:* Inverse presentation of data yields a V_{max} of 5.1 mmol/liter cells · hr and $K_{0.5}$ at 25 mM $[K^+]_o$

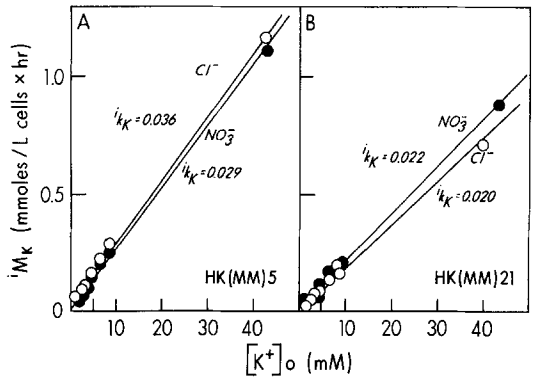

Fig. 5. Ouabain-insensitive ^{42}K influx in HK red cells of two sheep as function of $[K^+]_o$ and the effect of Cl^- replacement. The rate coefficients (^ik_K) were derived by dividing $^iM_K/[K^+]_o$

Figure 6 depicts an experiment measuring ^ok_k in NEM-treated LK red cells suspended in Cl^- or NO_3^- media with 0, 5, 10, 20, 30, and 40 mM $[Rb^+]_o$. The slopes of the straight lines represent the rate constants for K^+ efflux. Note that in NO_3^- media there was little effect of external Rb^+ on ^ok_K. However, in Cl^- media, ^ok_K fell progressively, levelling off at about 30–40 mM $[Rb^+]_o$. At these concentrations external K^+ or Rb^+ ions exert apparent *trans*-effects on K^+ effluxes in Cl^- but not in NO_3^- media, and in NEM-treated cells more than in controls. This is evident in the plot of the

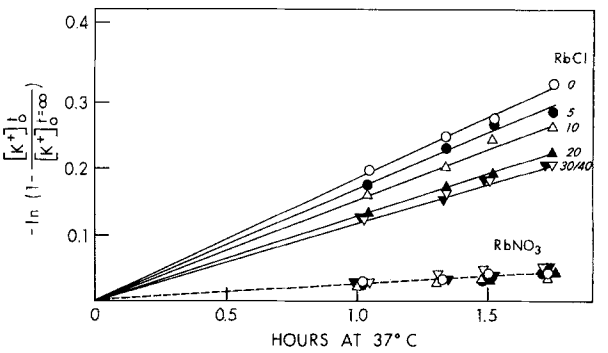

Fig. 6. Ouabain-insensitive K^+ efflux rate constants in LK sheep red cells treated with NEM and analyzed in the presence of various RbCl or $RbNO_3$ concentrations

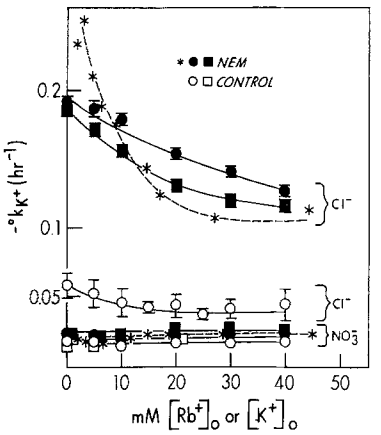

Fig. 7. Transinhibition of K^+ efflux in NEM-treated LK red cells. Data from three experiments. Solid symbols for NEM-treated cells, open symbols for controls. Presence of Cl^- and NO_3^- as indicated. Note the similarity of the efflux rate constants measured in the presence of external Rb^+ (●, ▲) and external K^+ ions (*)

K^+ efflux rate constants as a function of $[Rb^+]_o$ or $[K^+]_o$ shown in Fig. 7. Note that ^ok_K was independent of $[K^+]_o$ or $[Rb^+]_o$ in controls and in NEM-treated cells suspended in NO_3^- media. However, in NaCl media with increasing $[K^+]_o$ or $[Rb^+]_o$ there was a progressive reduction of ^ok_K which was greater in cells treated with NEM than in controls. It is concluded that the apparent inhibitory effects of K_o^+ or Rb_o^+ as well as the observed net effluxes in the absence of K_o^+ or Rb_o^+ exclude obligatory K^+/K^+ exchange.

In order to elucidate the nature of the driving forces involved in the K^+ transport stimulated by NEM, I have calculated from the data in Figs. 3, 4, 6, and 7, the net outward and inward fluxes at various $[K^+]_o$ or $[Rb^+]_o$ in Cl^- suspended LK sheep red cells, assuming no discrimination for K^+ and Rb^+ ions. Figure 8 shows that in both controls

Table 3. Ouabain-insensitive K^+ fluxes with Cl^- or NO_3^- at equilibrium and the effect of furosemide (10^{-3} M)

Experiment	Condition	K^+ efflux (mM/liter cells · hr)				Rb^+ influx (mM/liter cells · hr)	
		Control		NEM		Control *B*	NEM *B*
		A	*B*	*A*	*B*		
717 *A/B*	Cl^-	0.62	0.92	1.76	1.81	1.83±0.05	3.68±0.43
(LK 15)	NO_3^-	0.27	0.32	0.25	0.40	1.18±0.13	1.53±0.24
	Cl^- + furosemide	0.18	0.40	0.22	0.60	0.83±0.15	1.03±0.14
	NO_3^- + furosemide	–	0.23	0.12	0.23	0.86±0.16	0.58±0.09

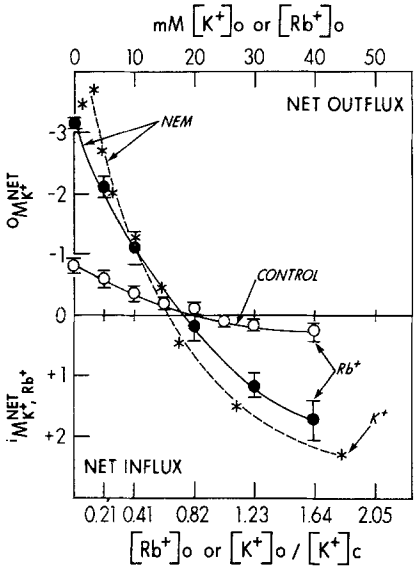

Fig. 8. Net fluxes of K^+ out and K^+ (*) or Rb^+ (o●) into control and NEM-treated LK sheep red cells as function of $[K^+]_o$ or $[Rb^+]_o$ (upper abscissa) or of the concentration ratio of cellular K^+ to extracellular K^+ or Rb^+ ions (lower abscissa). Results from two experiments involving the use of external Rb^+ are plotted for two NEM experiments (solid curves with filled symbols) and four control experiments (open circles). The interrupted curve is data from a tracer flux experiment performed on the same cells under the same conditions but at different times, indicated by *

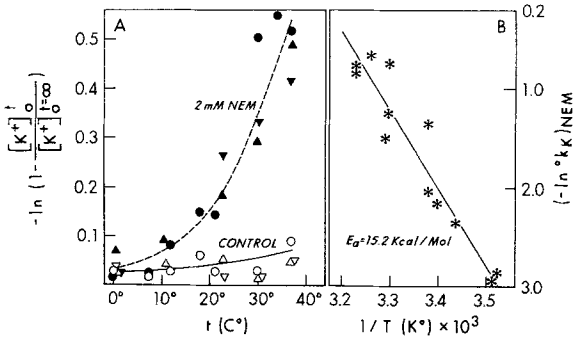

Fig. 9. Temperature dependence of NEM-induced and Cl^--dependent K^+ flux. (*A*): Efflux data were plotted for controls and NEM-treated LK cells of three experiments. (*B*): The NEM-specific component is depicted in an Arrhenius plot

and NEM-treated cells, net K^+ efflux occurred up to about 17 mM $[K^+]_o$ or $[Rb^+]_o$, while at higher values there was net influx of the two cations. The cross over at the *x* axis, i.e., when ${}^iM^{NET}_{K,Rb} = {}^oM^{NET}_K$, was about 0.7 $[K^+]_o/[K^+]_c$ or $[Rb^+]_o/[K^+]_c$, and hence close to the Donnan ratio of chloride ions.

Effect of Furosemide and Temperature

In our preliminary note we reported very little effect of the loop diuretic furosemide on Cl^--mediated K^+ transport stimulated by NEM. In light of recent reports that the effect of loop diuretics may be modified by the type of anions and cations present [21], the effect of 10^{-3} M furosemide was again tested in LK red cells suspended in isosmotic NaCl or $NaNO_3$ media in the presence of 40 mM $[Rb^+]_o$. Table 3 clearly shows that furosemide inhibited the NEM effect on K^+ efflux as well as reduced the control K^+ efflux in Cl^- to levels close to those seen in NO_3^- media. There appeared to be also a further augmentation of the furosemide effect when K^+ efflux was measured in the presence of NO_3^-. There was a similar effect of furosemide on Rb^+ influx. Further work is necessary to clarify the interaction between extracellular Rb^+ ions, anions, and furosemide on K^+ fluxes in LK red cells.

Furosemide-sensitive K^+ transport in human red cells is highly temperature dependent [44]. However, in the data set summarized in Fig. 8, no temperature effect was taken into account. In further experiments there was a striking temperature dependence of K^+ efflux in NEM-treated cells (Fig. 9*A*) measured in Cl^- media. Figure 9*B* represents an Arrhenius diagram of ${}^ok_K$ calculated for the NEM-specific component corrected for the background flux *vs.* the inverse of the temperature. The computed activation energy was about 15 cal/mol, suggesting a complex temperature dependence of the translocation process of K^+ ions.

Discussion

N-ethylmaleimide penetrates the red cell membrane within minutes, and at physiologic pH it reacts preferentially with SH-groups ubiquitously present in cytoplasm and membranes of biological cells [38]. However, NEM may also bind to other chemical groups, rendering its use as a probe in the study of membrane cation transport of limited value. This fact is best illustrated by the observation that, under the conditions chosen for the experiments presented here, only about 40% of some 10^7 SH groups/LK membrane (determined according to ref. 22) had reacted with NEM. Thus it was a surprise that NEM specifically amplified severalfold the passive K^+ permeability in LK but not in HK sheep red cells, and without apparent alterations of passive Na^+ fluxes. Although the precise physiological significance of a thiol and Cl^--dependent K^+ transport in LK red cells remains to be established, the findings bear upon the genetics of the HK/LK cation dimorphism.

Germane to the interpretation of the NEM effect on passive K^+ transport are the following properties common to control and NEM-stimulated K^+ fluxes. (i) Ouabain-insensitive K^+ transport, apparently unaccompanied by Na^+ movements, showed half maximum saturation around 25 mM $[K^+]_o$ (Fig. 1.). When Rb^+ was used instead of K^+ identical results were obtained (Figs. 3 and 4). (ii) Chloride replacement by NO_3^- led to a progressive loss of K^+ flux (Table 1, Fig. 2), an effect exclusively seen in LK red cells, but not in HK erythrocytes (Table 1, Fig. 5). (iii) Manipulations of cell volume did not alter the fractional stimulation of K^+ flux by NEM. (iv) In both control and NEM-treated LK cells zero net flux of K^+ or Rb^+ ions occurred at a point between 15–20 mM $[K^+]_o$ or $[Rb^+]_o$, i.e., at $[Rb^+]_o$ or $[K^+]_o$ to $[K^+]_c$ ratios of 0.6–0.8 (Fig. 8). There was net K^+ efflux above and net K^+ or Rb^+ influx below this range, with larger values in NEM-treated cells. (v) The loop diuretic furosemide inhibited both control and NEM-stimulated K^+ fluxes in Cl^--suspended LK cells (Table 3).

Saturation behavior has been previously ascribed to ouabain-insensitive K^+ influxes of isosmotically suspended [9] or hyposmotically swollen LK red cells [11, 14] with $K_{0.5}$ values for $[K^+]_o$ ranging from 7 [9] to 34 mM in Na^+ and 47 mM in choline media [11, 14, 28]. In this study, $K_{0.5}$ for $[K^+]_o$ was found to be about 25 mM in control or NEM-treated LK red cells suspended in Na^+ media or in media where Na^+ was partially or completely replaced by choline, suggesting that NEM increased only the number or the turnover of the existing K^+ transport loci. The reported $K_{0.5}$ values for $[K^+]_o$ are much different from those of $Na^+/K^+/2\ Cl^-$ cotransport of human [13, 48] and avian [21] red cells but close to those of volume-down-regulatory K^+/Cl^- cotransport of duck erythrocytes [21]. The external loading sites of control and NEM-treated cells did not distinguish between K^+ and Rb^+ ions as similar kinetic values were obtained, ranging from 17 to 27 mM $[Rb^+]_o$, respectively.

The fact that in LK cells, the NEM-stimulated K^+ flux was abolished by Cl^- replacement with NO_3^-. precludes Ca^{++}-induced K^+ flux, which in human red cells is accelerated in NO_3^- [41]. Also, the Gardos effect was found to be absent in sheep red cells [6]. Rather, the apparent requirement for Cl^- places this system closer to those described for human erythrocytes [7, 8, 13, 17, 48], nucleated avian and fish red cells [21, 27, 29], lymphocytes [19], tumor cells [4, 18, 23], and certain epithelial cell lines [1, 2, 20, 37, 43]. The reduction by NO_3^- of K^+ flux in control and NEM-stimulated LK red cells (Fig. 3) is certainly complex, suggesting a combination of effects, which require detailed consideration beyond assuming lack of transport [14]. Upward concave or linear relationships between K^+ flux and fractional Cl^- concentration have also been reported for Na^+/K^+ cotransport in human red cells [7, 8] and cultured epithelial cell lines [1, 2]. One cannot ascribe the inhibitory effect of NO_3^- to the finding of slightly smaller cells volumes, because no K^+ flux activation was seen in LK red cells suspended in 125 mM (about 230 mOsm) $NaNO_3$.

It is important to note that in HK red cells replacement of Cl^- by NO_3^- anions did not affect passive fluxes (Table 1, Fig. 5). In fact, HK red cells behaved somewhat like shrunken LK sheep red cells for which an extremely low $K_{0.5}$ value of 85 mM $[K^+]_o$ has been claimed for Cl^- dependent K^+ flux [14]. Furthermore, under the conditions of this study, NEM did not alter passive K^+ flux in HK red cells. These results then suggest that both LK and HK sheep red cells share a transport path which permits passage of KNO_3 as well as KCl, while LK sheep red cells possess the additional feature of NEM-stimulated K^+ transport where anions more hydrophobic than Cl^- or Br^- [49] become severely rate limiting.

The response of the K^+ influx rate coefficients to swelling and shrinking in control LK red cells suspended in Cl^- confirmed the report by others [11, 14]. Exposure of control and NEM-treated LK cells to anisosmotic media reduced $^o k_K$ to a

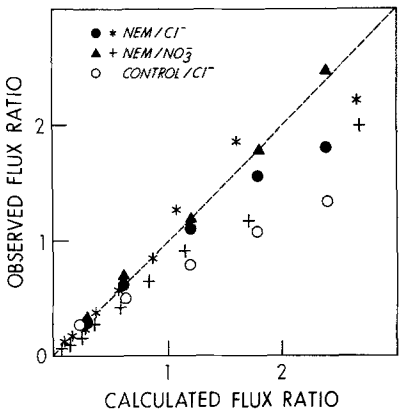

Fig. 10. Correlation between observed and calculated flux ratios. NEM-treated LK cells in Cl^- (●, *) and NO_3^- (▲+). Cl^- controls (○). Data from Rb^+/K^+ (●▲○) and from $^{42}K/^{42}K$ exchange fluxes (*, +)

similar extent, and hence no change in the fractional NEM stimulation occurred. This finding does not exclude that NEM acts on the same molecule that responds to volume manipulations. One also cannot rule out that the flux changes observed were not influenced by slight membrane potential changes which occurred while the cells were in the respective salt media. Yet there is evidence that volume changes by different methods led to similar K^+ flux modifications, hence excluding membrane potential effects [11, 14].

The observation that no net flux of K^+ ions occurred at $[K^+]_o/[K^+]_c = 0.7$ indicates that the relation between the observed and calculated flux ratios [47] may be close to unity. Figure 10 reveals that the flux ratios observed for control cells in Cl^- or NO_3^- are rather close to the calculated values below unity and tend to deviate somewhat at higher values. This finding is certainly different from earlier reports of passive cation fluxes in human red cells, where only in the presence of anions other than Cl^- or Br^-, the ratio between measured K^+ fluxes agreed well with the calculated flux ratios for passive, independent K^+ movements [16]. Further work is necessary to clarify the scatter of the data at higher calculated flux ratios.

From Fig. 10, important conclusions may be drawn concerning the effect of NEM on the Cl^--dependent K^+ transport system in LK sheep red cells. By reaction with membrane SH groups, NEM most probably opens pre-existing K^+ transport pathways, as suggested by the V_{max} effects and the absence of significant $K_{0.5}$ changes. The fact that observed and calculated flux ratios did agree fairly well does not exclude K^+ translocation through a K^+/Cl^- symport mechanism that is inhibited by furosemide and has a high energy of activation, but it also does not allow one to assume separate electrochemical diffusion of K^+ and Cl^-. Regardless of the mode of translocation, the data have clearly established that NEM activated a K^+ transport system to which only Cl^- (or Br^-) anions, but not NO_3^- and other anions of the lyotropic series, have access. In HK red cells, this system is absent as passive K^+ transport is of similar magnitude in Cl^- and NO_3^- media.

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