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Thiol-Dependent Passive K/Cl Transport in Sheep Red Cells: II. Loss of Cl^- and N-Ethylmaleimide Sensitivity in Maturing High K^+ Cells

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Summary. A fraction of the passive, ouabain-insensitive K^+ fluxes in mature low K^+ (LK) but not in high K^+ (HK) sheep red cells requires the presence of Cl^- anions and can be stimulated by volume expansion (Dunham, P.B., Ellory, J.C., *J. Physiol. (London)* **318**:511–530, 1981) or treatment with 2 mM N-ethylmaleimide (NEM) (Lauf, P.K., Theg., B.E., *Biochem. Biophys. Res. Commun.* **92**:1422–1428, 1980). In the present study it is shown that reticulocytes of both anemic LK and HK sheep possess the Cl^--dependent K^+ transport system which subsequently remains functional in mature LK but not in HK red cells. Kinetically, Cl^--mediated K^+ fluxes of reticulocytes of LK sheep are different from mature red cells only in their V_{max} values as measured in Na^+ or choline$^+$ media, while there is an apparently much lower affinity for external K^+ ions in reticulocytes of HK sheep. N-ethylmaleimide stimulated K^+ transport in the reticulocytes of both sheep genotypes suspended in Cl^- but failed to do so in NO_3^- media. The data are interpreted in terms of their biochemical, physiologic, and genetic implications for the HK/LK transition in sheep red cells.

Key Words passive K/Cl transport · sheep red cell maturation

Introduction

A substantial fraction of ouabain-insensitive, passive K^+ transport in low K^+ (LK) but not in high K^+ (HK) sheep erythrocytes is known to be Cl^- dependent and to increase further upon cell swelling in hyposmotic media [10] or after treatment with 2 mM N-ethylmaleimide (NEM) [21]. The action of NEM was interpreted as V_{max} stimulation of a temperature dependent, furosemide and Cl^--sensitive K^+ transport pathway genetically preserved in adult LK but turned off in HK sheep red cells [19]. 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^- anions [19].

The physiologic signifcance of the volume and NEM-sensitive K^+ transport pathway in LK sheep is not yet clear. The specific Cl^- requirement for K^+ translocation and the effect of loop diuretics remind one of similar cation transporters present in nucleated erythrocytes and a variety of epithelial cells. For example, nucleated red cells of ducks [16, 24], turkeys [5], flounder [7], and toadfish [18] when placed into hyposmotic Na^+ media down-regulate their volume by extrusion of K^+ plus water but only in the presence of Cl^-. While volume down-regulation may be a process physiologically important for the survival of birds and fish, it has to be established why mammalian nucleated cells such as mouse ascites tumor cells [3, 4, 13, 14], certain epithelial cells [1, 2], and even enucleate human red cells [8, 9, 18, 27] are also endowed with Cl^--dependent passive Na^+, K^+ cotransport.

Low K^+ sheep red cells derive from high K^+ reticulocytes by mechanisms still poorly understood. The relationship between the HK/LK transition and the Cl^--dependent K^+ transport that may be reactivated by volume expansion or NEM treatment is not clear. If the Cl^--dependent K^+ transport would be present in reticulocytes of both LK and HK type sheep, the loss of such a transport path during maturation of reticulocytes could be attributed to the action of the gene in control of the sheep HK status. Conversely and more in line with the fact that the LK gene is dominant, the preservation of Cl^- and NEM-sensitive K^+ fluxes in LK cells can be seen as a function of the LK gene activity.

In the present study, the changes in passive K^+ fluxes and cellular cation compositions of reticulocytes isolated by density gradient centrifugation from massively bled LK and HK sheep were analyzed. It was found that, independent of the adult red cell genotype, high K^+ reticulocytes possessed Cl^--specific K^+ fluxes which were markedly stimulated by NEM while mature HK erythrocytes completely lacked this system present, however, in

LK erythrocytes. Kinetic analysis revealed that the difference between reticulocytes and adult LK red cells lies in the V_{max} values for Cl^- and NEM sensitive K^+ transport. In reticulocytes from anemic HK animals, no saturation by external K^+ of Cl^--dependent K^+ flux was observed. A preliminary report of this work has appeared elsewhere [17].

Materials and Methods

Induction and Separation of Reticulocytes

Five healthy Dorset sheep, 3 with LK (LL #3, 15, & 16) and 2 with HK (MM #9 and 19) red cells were selected for successive bleeding experiments. A total blood volume of about 2% of the body weight was removed during two bleedings from the jugular vein. Appearance of reticulocytes thereafter was monitored on each day by vital staining of whole blood smears with methylene blue. Maximum levels of peripheral reticulocytosis were in the order of 11–14% as counted microscopically between days 5 and 8 after bleeding. In all animals studied, the appearance of reticulocytes followed in general a time course similar to that described earlier [15]. For isolation of sufficient reticulocytes, about 100 ml blood was collected into heparin on days 1, 5, 6, 7, 8, 13 and 35 in the LK, and on days 1, 5, 7, 8, 13, and 19 in the HK animals. Cell fractionation according to density [25] was performed as described previously [15]. In short, plasma and cells were first separated by centrifugation at 5000 rpm and 30 °C in a Sorval RC2B centrifuge (Dupont Instruments, Newton, Conn.) and the cells resuspended in plasma at a hematocrit of 90% prior to a second centrifugation at 15,000 rpm and 30 °C for 1 hr. The top 10% and bottom 10% cells were gently collected after removal of the tightly packed buffy coat. Reticulocytes were stained as 30% suspensions with new methylene blue and counted by light microscopy from a dried smear. There were more than 60% reticulocytes in the top 10% layer and none in the bottom 10% cells. Because of this striking separation the top 10% cells were also called the reticulocytes and the bottom 10% cells the erythrocytes. Separated and unfractionated cells were suspended in a 290 mOsM washing buffer at pH 7.4 and washed free of plasma.

Solutions and Treatment with N-ethylmaleimide (NEM)

The osmolarity of all Na^+ and choline$^+$ solutions used was 290 mOsM and hence isosmotic with that of sheep plasma (289 ± 3 SEM, $n=6$) as measured in a Wescor vapor pressure osmometer (Wescor, Model 5100 B, Logan, Utah). All Cl^- solutions used for effluxes were buffered with 10 mM Tris /Cl to pH 7.4 at 0 or 37 °C (Tris-buffered saline, TBS, or Tris-buffered choline-Cl, TBC) and with 10 mM Tris/NO_3^- (TBN) to pH 7.4 at the desired temperature.

Cation Fluxes

In the case of K^+ and Na^+ effluxes the respective flux media were either TBS or TBN, and TBC containing 10^{-4} M ouabain. These solutions were temperature equilibrated and added to the unseparated and fractionated packed cells to give a hematocrit of about 4%. Samples were taken in quadruplicates usually around 40–60 min after the beginning of the experiment, and cells and extracellular medium were separated by the dibutyl-phthalate ester method described earlier [20]. Processing of the samples and calculations of the K^+ efflux rate constants were done according to the methods detailed in the previous paper [19] with the exception that the rate constants were calculated from the mean $[K^+]_o$ values of the two time points taken instead by regression analysis. The accuracy of the technique was such that analysis at one time point would have sufficed to reach the same data (see Fig. 2, for example).

Measurement of K^+ influxes also followed the protocol and equations outlined in the previous paper [19]. At time 0 20 μCi ^{86}Rb (New England Nuclear Co., Boston, Mass.) were added to the cell suspension pre-equilibrated at 37 °C in the K^+ flux medium. A single time point in triplicates was taken after 30 min at which initial rates still prevailed. Processing of the sample was done as described earlier [19]. In the calculations of K^+ influxes, it was assumed that ^{86}Rb behaved indistinguishably from ^{42}K at $[K^+]_o$ between 1 and 14 mM/liter as validated in an earlier report [22]. All calculations were done on the basis of original cell volumes.

Results

Cellular Cations, and Basal K^+ Fluxes in LK Red Cells Seven Days after Hemorrhage

In a preliminary experiment a strong correlation was found between the appearance of reticulocytes, the erythrocyte cation changes and the behavior of ouabain-insensitive Cl^--dependent K^+ fluxes. Figure 1 shows the time-dependent changes of cellular cation concentrations in the LK (left panel) and the HK sheep (right panel) in unseparated cells, and the 10% top and bottom layer cells separated by density gradient centrifugation [15, 25]. The increase and decrease of $[K^+]_c$ and $[Na^+]_c$, respectively, seen in unseparated cells was due to the appearance of reticulocytes, which were harvested in the top 10% layer. As evidenced by methylene blue staining, these top 10% cells were at least 60% reticulocytes of the large and small type

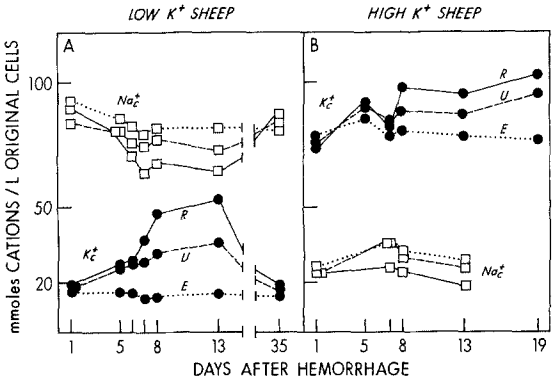

Fig. 1. Concentrations of K^+ (solid circles) and Na^+ (open squares) in unseparated red cells (*U*), reticulocytes (*R*) and mature erythrocytes (*E*) of (*A*) LK (LL #15) and (*B*) HK (MM #9) sheep at different time points after massive hemorrhage. In the LK experiment (Exp. 652/II/1981) the last sample point was on day 35 and in the HK experiment (Exp. 652/IV/1981) on day 19, respectively

Table 1. Evidence for Cl^--dependent K^+ transport in reticulocytes harvested from an LK sheep seven days after massive hemorrhage

Sample	Cells (LK LL #16) (% reticulocytes)	$(K^+)_c$ (mmol/liter orig. cells)	$(Na^+)_c$	$-{}^ok_K^{Cl}$ (hr^{-1})	$-{}^ok_K^{NO_3}$ (hr^{-1})	${}^iM_K^{Cl}$ (mmol/liter cells · hr)	${}^iM_K^{NO_3}$
Unbled	Unseparated (0.1–0.2)	16.7	90.0	0.085	0.076	0.117	0.071
7 days after bleeding	Unseparated (14)	24.1	78.8	0.138	0.080	0.227	0.160
	Top 10% layer (40–60)	54.0	40.0	0.229	0.074	0.403	0.238
	Bottom 10% layer (none)	10.9	76.9	0.067	0.061	0.164	0.106

$-{}^ok_K^{Cl}$ and $-{}^ok_K^{NO_3}$ are the rate constants for K^+ efflux into Cl^- or NO_3 equilibrated cells. ${}^iM_K^{Cl}$ and ${}^iM_K^{NO_3}$ denote K^+ influxes using ^{86}Rb in Cl^- or NO_3^- media containing 5 mM $[K^+]_o$.

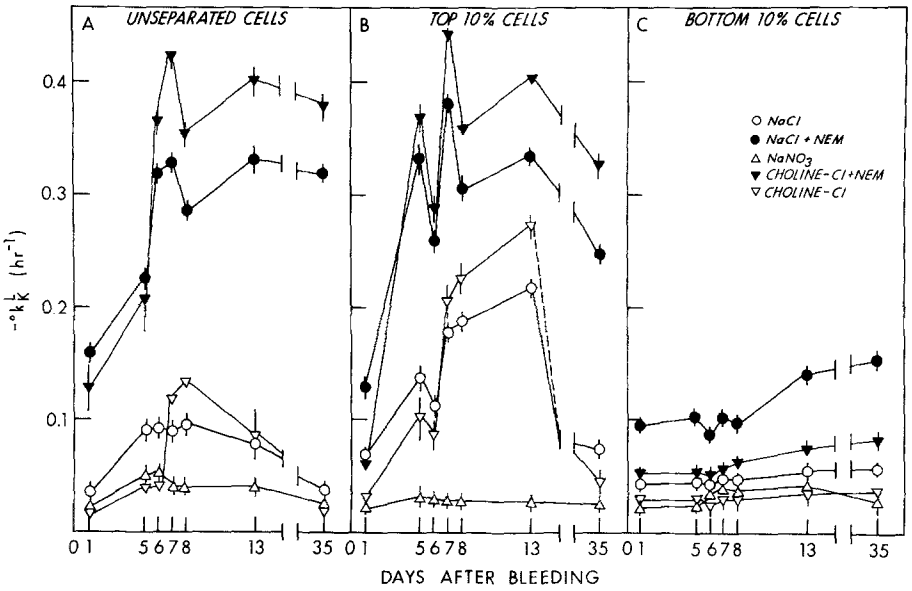

Fig. 2. Effect of NEM on the K^+ efflux rate constants of unseparated red cells (*A*), reticulocytes (*B*), and mature erythrocytes (*C*) of LK sheep (LL #15). K^+ fluxes of controls (open symbols) and NEM-treated cells filled symbols) were measured in 290 mOsM, buffered $NaNO_3$ (△, ▲), NaCl (○, ●), and choline Cl (▽, ▼) media, pH 7.4. Bars indicate + SEM for $n = 4$

reported earlier [23] with about equal $[K^+]_c$ and $[Na^+]_c$.

Note that the rise of $[K^+]_c$ observed after 5 days in unseparated cells of the LK sheep was mainly due to the appearance of high K^+ reticulocytes since $[K^+]_c$ remained constant in the older erythrocytes. That $[K^+]_c$ and $[Na^+]_c$ did not reach levels typical of high K^+ mammalian cells can be explained by the heterogeneity of the cells due to the presence of reticulocytes with lower $[K^+]_c$ values and some adult LK cells (*see* Discussion). In contrast, the 10% bottom red cells did not contain reticulocytes and were truly mature LK erythrocytes. In the HK sheep there was a slight upward trend of $[K^+]_c$ in the reticulocytes while all other cation profiles were uneventful as expected for these animals.

Table 1 compares cellular cation concentrations and K^+ fluxes from an unbled LK sheep with those obtained 7 days after bleeding. The observed changes in cation concentrations were similar to those described in Fig. 1 for the LK animal. The rate constant of ouabain-insensitive K^+ efflux from reticulocytes in Cl^- was about three times higher than in mature red cells and in reticulocytes analyzed in NO_3^- media. A similarly striking activation of Cl^--dependent K^+ influxes was seen on day 7 in reticulocytes and to a lesser degree in erythrocytes. Consistent with other reports on mature LK sheep red cells [10], the Cl^--sensitive component was about 40% in both reticulocytes and erythrocytes. In comparing K^+ efflux computed from the rate constant, ${}^ok_K$, and $[K^+]_c$ with K^+ influx it is readily apparent that, at about 5 mM $[K^+]_o$, K^+ influx was about an order of magnitude smaller than K^+ efflux measured under zero $[K^+]_o$. This observation is consistent with an earlier report that net K^+ efflux occurs below a $[K^+]_o/[K^+]_c$ ratio of 0.7 [19].

The Effect of NEM on Cl^- Dependent, Passive K^+ Effluxes in Reticulocytes and Erythrocytes of Anemic LK and HK Sheep

K^+ Effluxes in LK Sheep Red Cells. Figure 2 shows the rate constants of K^+ efflux into NaCl, $NaNO_3$

Table 2. Absence of NEM effect to stimulate ${}^{o}k_{Na}$ in LK sheep red cells measured in choline-Cl at various intervals after massive hemorrhage

Experiment	Cells ($n=4$)	2 mM NEM		${}^{o}k_{Na}$ (hr^{-1}) measured at					
				Day 1	Day 5	Day 6	Day 7	Day 8	Day 13
652/II/81	LK 15	−	$\bar{x}$	0.012	0.039	0.040	0.034	0.044	0.027
	U-cells		±	0.001	0.009	0.001	0.007		
		+	$\bar{x}$	0.013	0.038	0.030	0.037	0.034	0.027
			±	0.001	0.006	0.003	0.001		
	LK 15	−	$\bar{x}$	−	0.027	0.019	−	0.038	0.030
	R-cells		±						0.009
		+	$\bar{x}$	−	0.037	0.017	−	0.039	0.022
			±		0.004		0.001	0.002	0.001
	LK 15	−	$\bar{x}$	0.028	0.039	0.030	0.054	0.032	0.034
	E-cells		±	0.004	0.001	0.005	0.009		0.004
		+	$\bar{x}$	0.0024	0.035	0.025	0.040	0.030	0.026
			±	0.001	0.010	0.001	0.003	0.002	0.003
652/III/81	LK 13	−	$\bar{x}$	−	0.033	−	−	0.092	−
	R-cells		±		0.004				
		+	$\bar{x}$	−	0.021	−	−	0.122	−
			±		0.001				
	LK 13	−	$\bar{x}$	−	0.027	−	−	0.064	−
	E-Cells		±		0.002				
		+	$\bar{x}$	−	0.027	−	−	0.70	−
			±		0.002				

Missing $\bar{x}$ values were not measured, missing SEM values due to replicate determinations.

and choline-Cl media for control and NEM-treated cells of the LK sheep (cell cation changes were displayed in Fig. 1*A*) measured as a function of time after bleeding. Note that in NO_3^- media ${}^{o}k_K$ was between 0.025 and 0.05 hr^{-1} in unseparated cells (Fig. 2*A*), reticulocytes (Fig. 2*B*) and erythrocytes (Fig. 2*C*). As the volume/surface area ratio of reticulocytes is almost twice that of erythrocytes, the similarity of the ${}^{o}k_K$ values means that the apparent permeability to K^+ in NO_3^- media was equal or less in reticulocytes as compared to erythrocytes.

In unseparated cells the Cl^--supported K^+ fluxes rose around day 6 by about three- to fourfold in Na^+ or $choline^+$ media and gradually declined to base levels on day 35 after hemorrhage. From days 1 to 8 NEM treatment of unseparated cells enhanced their ${}^{o}k_K$ to about 0.3 and 0.4 hr^{-1} in NaCl and choline-Cl media, respectively, paralleling the trend of the Cl^--supported K^+ fluxes of the untreated cells. However, on days 13 and 35 relative stimulation of effluxes by NEM was greater due to a decline in the basal control fluxes.

Figure 2*B* and *C* shows that the time-dependent changes of ${}^{o}k_K$ seen for unseparated cells were entirely due to entrance into circulation of reticulocytes (days 7–13) with basal K^+ fluxes up to 10-fold larger in Cl^- than in NO_3^- media. The data reveal complex events. On the fifth day, NEM stimulated K^+ efflux four- and threefold in choline Cl and NaCl, respectively, but much less between days 8 and 13. Furthermore, like with the unseparated cells, there was a much higher relative stimulation by NEM on day 35 (Fig. 2*B*). Hence, the stimulation of K^+ transport by NEM was smallest in reticulocytes with highest basal Cl^--dependent K^+ fluxes and remained persistently high as the basal K^+ fluxes declined (Fig. 2*A*) in young cells maturing or disappearing from the top 10% layer.

The time course of ${}^{o}k_K$ measured in control and NEM-treated adult erythrocytes was monotonous in Cl^- media. The average NEM stimulation of K^+ flux determined up to day 8 was twofold in both NaCl and choline-Cl media with absolute ${}^{o}k_K$ values higher in NaCl than in choline-Cl in NEM-treated cells and in controls. Interestingly, this is a reverse situation to that found in reticulocytes, where both basal and NEM-augmented K^+ fluxes in NaCl were lower than in choline Cl. Note that in both control and NEM-treated erythrocytes the Cl^--dependent K^+ flux rose by day 35, a finding consistent with the interpretation that some of the newly formed reticulocytes, which by this time had reached the bottom 10% layer of the gradient [15], were still fully responding to the action of NEM.

Earlier it was reported that the Cl^--dependent

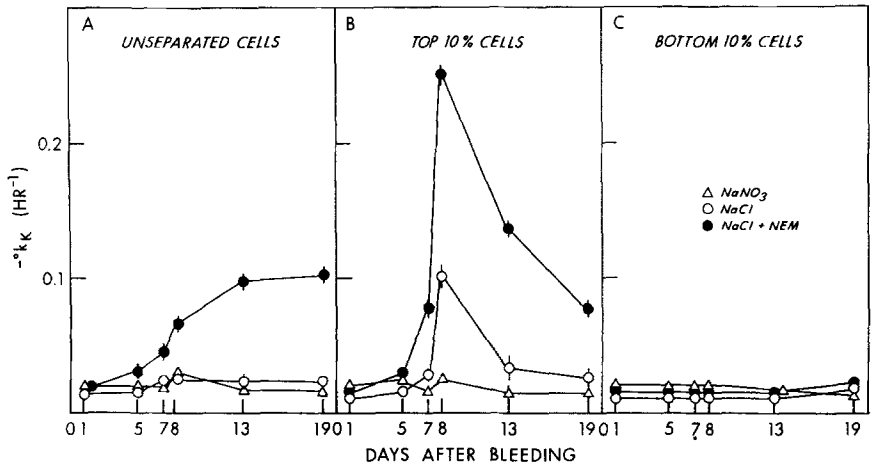

Fig. 3. Effect of NEM on K^+ efflux rate constants of unseparated (*A*), reticulocytes (*B*) and mature red cells (*C*) of HK (MM #9) sheep as function of time after massive hemorrhage. Filled symbols for NEM and open symbols for control cells. K^+ efflux was measured into 290 mOsM NaCl (○, ●) and $NaNO_3$ (△) buffered with Tris/Cl or Tris/NO_3 to pH 7.4. Bars indicate ±SEM for $n=4$

Table 3. Effect of N-ethylmaleimide on Rb^+ influxes in Cl^- or NO_3^- suspended reticulocytes of anemic LK and HK sheep

Sheep	Cells	2 mM NEM	Rb^+ Influx (mmol/liter orig. cells · hr)				Cl^--specific Rb^+ influx
			Cl^-	±SD	NO_3^-	±SD	
LK 15	Unseparated	−	0.67	0.02	0.41	0.01	0.26
		+	1.54	0.26	0.38	0.02	1.16
	Reticulocytes	−	1.41	0.11	0.28	0.03	1.03
		+	2.18	0.05	0.33	0.01	1.88
HK 19	Unseparated	−	0.33	0.03	0.28	0.03	0.05
		+	0.85	0.02	0.23	0.01	0.62
	Reticulocytes	−	0.56	0.04	0.28	0.02	0.28
		+	0.90	0.02	0.25	0.02	0.65

Exp 728/82. External Rb^+ Concentrations: 10 mM. $n=3$.

K^+ flux of LK red cells was unaccompanied by Na^+ movements and that NEM activated only the former [18, 19]. It was important to test whether this paradigma would hold for the newly formed reticulocytes of anemic LK sheep. Table 2 lists the Na^+ efflux rate constants for unseparated cells and fractionated cells in choline-Cl media. Note that $^{o}k_{Na}$ tended to be higher 5 days after (than before) bleeding in all cells. The longitudinal scattering of the data, however, precludes any firm statement at this time. Nevertheless, there was clearly no NEM effect on $^{o}k_{Na}$ in any of the samples tested. From these preliminary data on Na^+ fluxes it may be concluded that changes in apparent Na^+ permeability, if they occurred at this stage of the cell's development, were much less pronounced than those of the apparent K^+ permeability.

K^+ Effluxes in HK Sheep Red Cells. With exception of the duration of the experiment, the K^+ fluxes of unseparated cells, reticulocytes, and erythrocytes of the HK sheep shown in Fig. 3 were measured under identical conditions as those of the LK cells. Although no major change occurred in $[K^+]_c$, marked changes were found in the Cl^--supported K^+ efflux rate constants of control and NEM-treated reticulocytes. First, note that in NO_3^- the $^{o}k_K$ values were identical in unseparated and fractionated cells and less than half of those determined in the previous LK experiment. Second, NEM increased the $^{o}k_K$ values in unseparated cells after day 5 (Fig. 3*A*), but in comparison to the studies on the LK animal, the fluxes were generally lower. Third, on day 8 the K^+ flux in reticulocytes was four times that in NO_3^- media. In NEM-treated reticulocytes, a further 2.5-fold stimulation of Cl^--dependent K^+ flux was seen on day 8, which declined parallel to the basal K^+ fluxes in Cl^- at later time points, when the cells had left the top 10% layer due to maturational changes [15]. On the other hand, on day 19 there was still a marked NEM stimulation in unseparated HK red cells (Fig. 3*A*). This observation may be due to the persistence of the newly formed cells to

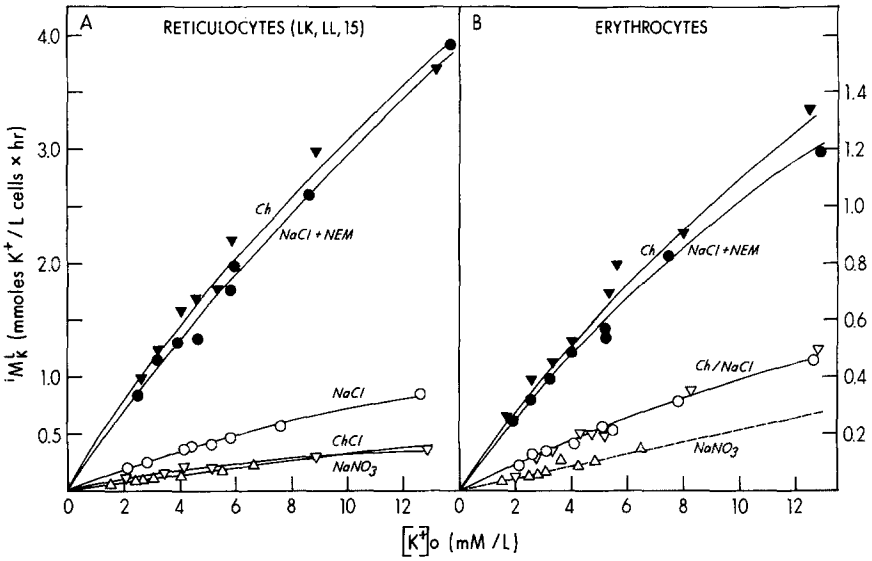

Fig. 4. Effect of external K^+ concentrations on passive K^+ influxes of reticulocytes (*A*) and erythrocytes (*B*) before (open symbols) and after (closed symbols) treatment with NEM. K^+ influxes were measured in 290 mOsM $NaNO_3$ (△) and NaCl (○, ●), and choline Cl (▽, ▼)

respond to NEM beyond their reticulocyte stage, because ^0k_K values measured in control and NEM-treated mature erythrocytes were identical at all time points whether measured in $NaNO_3$ or NaCl media (Fig. 3 *C*).

Absence of NEM Effect in NO_3^- Media. N-ethylmaleimide was shown to stimulate Cl^--dependent K^+ transport in reticulocytes of both LK and HK sheep, but only in mature red cells of LK sheep. However, both red cell types possess a basal K^+ permeability in the presence of NO_3^-. Hence, in regard to the hypothesis presented here that Cl^--mediated K^+ transport is turned off in maturing HK red cells, it is important to test the NEM effect on K^+ fluxes in both HK and LK reticulocytes in the presence of NO_3^-. Table 3 shows a Rb^+ influx experiment carried out with methods described elsewhere [19] on unseparated and top 10% (reticulocytes) cells harvested from LK 15 and HK 19 sheep 8 days after massive bleeding. In this experiment, all cells were NEM pretreated as usual and Rb^+ influx was measured in isosmotic Na^+ media in the presence of 10 mM $[Rb^+]_o$. Comparable to the data shown before in Figs. 2–4, Table 3 shows that Rb^+ influx was higher in reticulocytes than in unseparated red cells of both HK and LK sheep and that this increase was solely due to the appearance of Cl^--dependent K^+ transport in both cell types. N-ethylmaleimide stimulated only the Cl^--supported Rb^+ influx, without at all affecting the ouabain-insensitive Rb^+ influxes in NO_3^- media. A mirror image of this response pattern was obtained in K^+ efflux studies carried out on the same animals.

Kinetic Parameters of K^+ Fluxes

Earlier it was shown that half-maximum activation of Cl^--dependent and NEM-sensitive K^+ fluxes in LK cells occurred around 25 mM $[K^+]_o$ or $[Rb^+]_o$, while Cl^--independent K^+ fluxes of HK red cells failed to show any saturation [19]. Hence it was of interest to study this kinetic aspect in the newly formed reticulocytes of both LK and HK sheep. Figure 4 shows the NEM effect on ouabain-insensitive K^+ influxes in reticulocytes and erythrocytes of LK sheep (LL #13) measured in isosmotic $NaNO_3$, NaCl, and choline-Cl media as a function of $[K^+]_o$, and Fig. 5 shows the same in unseparated and fractionated cells of HK sheep (MM #9). For both animals the experiments were performed 6–7 days after bleeding, when in the peripheral blood maximum reticulocytosis was observed. The Cl^--supported K^+ fluxes were curvilinear in LK and linear in HK red cells. While NEM had no effect on K^+ influxes in $NaNO_3$ media, it stimulated markedly Cl^--dependent K^+ influxes in reticulocytes of both LK and HK sheep, and in erythrocytes of LK but not of HK sheep. These findings are consistent with the data shown in Figs. 2 and 3 that NEM- and Cl^--stimulated K^+ fluxes exist in reticulocytes of both sheep genotypes but in erythrocytes of only LK sheep. Note also that maximum K^+ influxes of NEM-treated HK cells were seen in choline-Cl media.

The effect of external Na^+ on K^+ fluxes seems to be of complex nature. In mature red cells of an unbled sheep it was shown that $[Na^+]_o$ did not affect K^+ influx in the presence or absence of NEM. Furthermore, NEM had no apparent effect

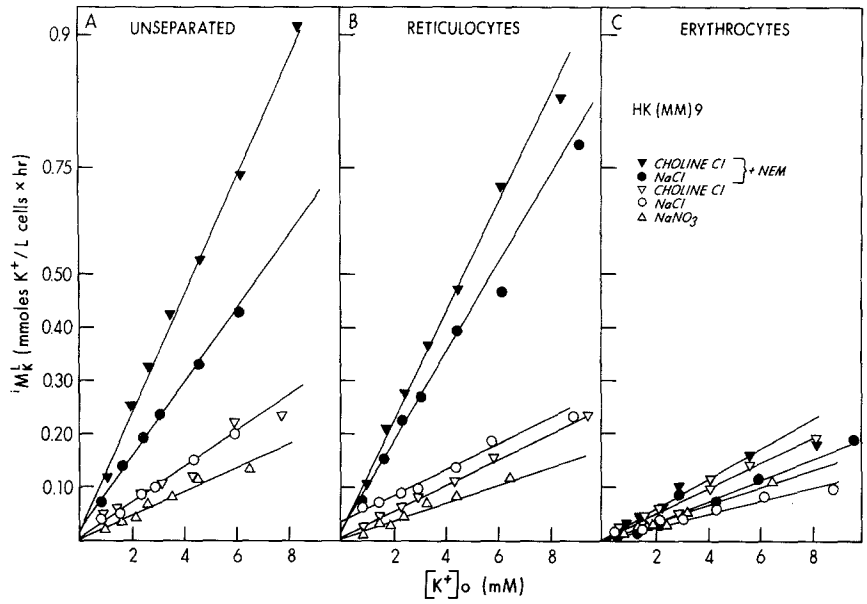

Fig. 5. Effect of external K^+ concentrations on passive K^+ influx on control (open symbols) and NEM-treated (closed symbols) unseparated cells (A), reticulocytes (B), and mature red cells (C) of HK sheep #9 in 290 mOsM $NaNO_3$ (△), choline Cl (△, ▽) and NaCl (○, ●) buffered at pH 7.4

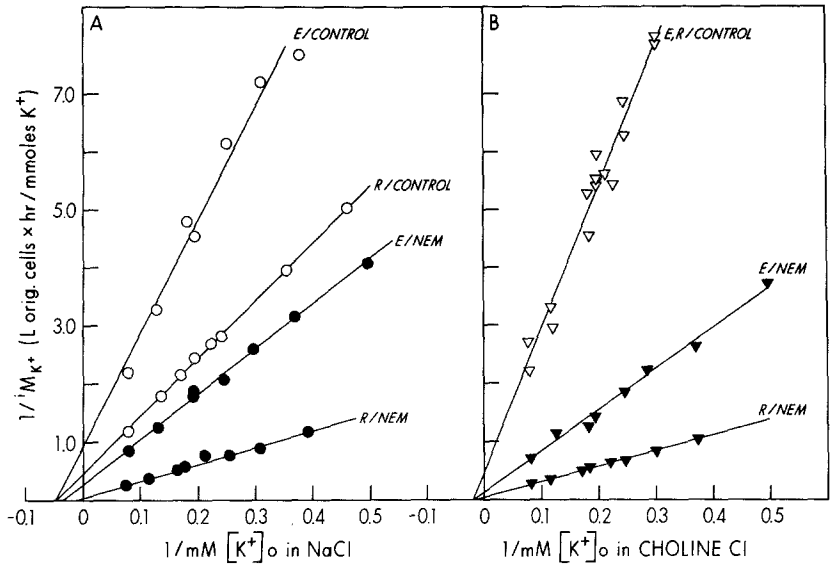

Fig. 6. Inverse plot of data from external K^+ activation experiment of Fig. 5. *E* and *R* stands for mature erythrocytes and reticulocytes, respectively. K^+ influxes in Na^+ (*A*) and in choline$^+$ (*B*) media

on ouabain-insensitive Na^+ effluxes in LK red cells suspended in choline-Cl media [19]. In cells from a bled LK sheep, the presence of external Na^+ had different effects on K^+ efflux and K^+ influx. Thus, in the top 10% cells, K^+ efflux was significantly lower in Na^+ than in choline media by day 7 after bleeding, while the reverse was observed in the bottom 10% cells, in both the presence and the absence of NEM (Fig. 2*B, C*). In contrast, K^+ influx in reticulocytes was higher in Na^+ and independent of Cl^- in the control cells, while no differences were found in cells treated with NEM in both cation media (Fig. 4*A*). In erythrocytes of the bled LK animal, the same characteristics were seen as in earlier studies [19]: that is, a chloride-dependent, Na^+-independent K^+ influx that appears only in the control cells (Fig. 4*B*). Taken together, these results suggest first that $[Na^+]_o$ affects differently potassium efflux and K^+ influx. Furthermore, the Na^+-dependence of K^+ influx in reticulocytes of the bled LK sheep is similar to the one observed in adult human red cells where a Na^+ K^+/Cl^- cotransport has been described [12, 27]. Although results of Fig. 4*A* raise the possibility of coupled Na^+ K^+/Cl^- cotransport in the immature cells, the complexity of the Na^+ effects demand further studies. In regard to the chloride dependence of K^+ fluxes, further experiments are

Table 4. Kinetic parameters for Cl^--dependent K^+ influxes in reticulocytes and erythrocytes of an anemic LK sheep and the effect of NEM

Cells	Treatment	Medium	V^*_{max}	$K^{**}_{0.5}$	n	r
Erythro-	None	NaCl	1.0	22	7	0.991
cytes	2mM NEM	NaCl	3.9	28	8	0.997
Reticulo-	None	NaCl	2.2	22	8	0.999
cytes	2mM NEM	NaCl	14.6	50	8	0.987
Erythro-	None	Choline Cl	2.5	50	8	0.991
cytes	2mM NEM	Choline Cl	9.5	50	8	0.964
Reticulo-	None	Choline Cl	2.5	50	8	0.991
cytes	2mM NEM	Choline Cl	17.4	50	8	0.998

* mmol K^+/liter cells · hr; ** mmol K^+/liter. r = correlation coefficient.

required to demonstrate if chloride is cotransported with K^+ or whether the enhanced K^+ fluxes are of electrogenic nature.

The nonlinear behavior of K^+ influxes in LK sheep red cells permitted calculation of V_{max} and $K_{0.5}$ values by using the regression lines of the inverse plot of all Cl^--supported K^+ influxes *vs.* $[K^+]_o$ as shown in Fig. 6. The parameters calculated from Fig. 6 are listed in Table 4. In erythrocytes suspended in NaCl media, there was the usual fourfold stimulation V_{max} of K^+ fluxes by NEM reported earlier [19]. The basal K^+ flux was higher in reticulocytes and stimulated 6.6-fold by NEM with a slight but nonsignificant change of $K_{0.5}$ for $[K^+]_o$. In choline-Cl, NEM stimulated K^+ flux 3.8 and 6.9-fold in erythrocytes and reticulocytes, respectively. The apparent affinities for external K^+ were similar and barely lower than in Na^+ media. The conclusion to be drawn from these data is that the NEM-stimulated and Cl^--dependent K^+ transport appeared to be very similar in reticulocytes and erythrocytes of LK sheep.

Discussion

The most important finding of this study was the observation that reticulocytes of both anemic LK and HK sheep possessed the Cl^--dependent (Table 1) and NEM-sensitive (Figs. 2 and 3) K^+ fluxes reported earlier for mature LK but not HK sheep erythrocytes [19, 21]. As before, the Cl^- sensitivity of these K^+ fluxes was established by the finding that in $NaNO_3$ the rate constants for K^+ efflux were similar in unseparated cells, reticulocytes, and erythrocytes (Figs. 2 and 3) and that NEM did not exert its effect in NO_3^- media (Table 3). The time course of the increase in Cl^- dependent K^+ fluxes (Figs. 2 and 3) paralleled that of $[K^+]_c$ changes in reticulocytes of LK but not of HK sheep (Fig. 1). Hence, Cl^--mediated K^+ fluxes are properties common to high K^+ reticulocytes of both LK and HK sheep. While the apparent Na^+ permeability was somewhat increased in reticulocytes derived from two LK sheep, no stimulatory or inhibitory effect of NEM was seen, a fact supporting further the hypothesis that Cl^-- and NEM-sensitive K^+ fluxes are not linked to Na^+ movements in sheep red cells (Table 2). The apparent affinities of the saturable Cl^--dependent K^+ influxes of reticulocytes from LK sheep were in the range of 22–50 mM $[K^+]_o$ (Figs. 4 and 6, Table 4) and hence close to those values reported earlier for mature LK sheep red cells [19]. In HK sheep, K^+ influxes of reticulocytes were a linear function of $[K^+]_o$ (Fig. 5).

Prior to examining the NEM-activated K^+ fluxes, it is imperative to consider the known properties of the reticulocytes produced by massive bleeding in both LK and HK sheep. There is considerable evidence that stress hematopoiesis produces at least two cell populations: small and large volume reticulocytes [23]. Small reticulocytes differ from the large ones by their final cation concentrations and K^+ fluxes, while large reticulocytes are emergency cells with higher $[K^+]_c$ levels and different K^+ pump and leak fluxes [23]. In hemoglobin A type sheep, these emergency reticulocytes carry in addition hemoglobin C. There is some evidence that the large reticulocytes do not undergo sufficient volume reduction to make them indistinguishable from normally produced erythrocytes. Hence, as monitored by their hemoglobin C content and their larger cell volume, these cells can be detected in the peripheral circulation for as long as 3 months [23]. The top 10% layer cells of the present study contained both small and large reticulocytes as determined by light microscopy. The kinetics of the appearance and disappearance of the reticulocytes in the top 10% layer is complex and involves volume reduction as well as buoyant density changes [15, 22]. As the large reticulocytes are not the normal precursors of the mature LK erythrocytes, any data obtained with these cells have to be interpreted with caution. This point is underscored by the finding of unabatedly high NEM stimulation of ${}^0k_K$ in unseparated HK cells tested more than 2 weeks after hemorrhage, indicating persistence of the response of these cells to NEM (Fig. 3*A*). A similar observation was made in unseparated LK red cells (Fig. 2*A*). Furthermore, one has to keep in mind that reticulocytes produced by hemorrhage in LK and HK sheep may exhibit differences which are not evident in the present analysis.

Whether or not the reticulocytes produced are emergency cells and direct precursors of LK cells, the presence of Cl^--dependent and NEM-stimulated K^+ transport in these cells signifies that in both LK and HK sheep the hematopoietic system is capable of producing cells with Cl^--mediated K^+ transport pathways. It must be then the special action of the LK (L)/HK (M) genes that leads to preservation of Cl^--and NEM-sensitive K^+ transport in LK erythrocytes and its absence in HK red cells. This conclusion may have a biochemical basis as well as physiologic and genetic implications.

Biochemically the action of NEM postulates the presence of SH-group containing Cl^--dependent K^+ transporters in reticulocytes of both LK and HK sheep. As NEM had no effect on mature HK red cells, and as there was also no Cl^--dependent K^+ flux, its loss in HK red cells may be accompanied by disappearance of those SH groups that responded to NEM in the reticulocyte. It is possible that in HK sheep the entire K^+/Cl^- transport molecule disappeared upon maturation of the reticulocytes. Alternately, it remained inserted in the membrane while, as manifestation of its inactivity, the NEM-susceptible SH groups have become inaccessible. Simple disulfide bond formation must be discounted since exposure of HK erythrocytes to dithiothreitol did not activate a Cl^--dependent K^+ flux in these cells (*unpublished findings*).

Physiologically, the presence of a K^+/Cl^- transporter in reticulocytes may be related to the process of volume involution in these cells (which may be as much as 20%, ref. 23) rather than to the HK/LK cation dimorphism or to the Ca^{++} activated K^+ channel [6]. This possibility is supported by the report in rabbits of a K^+ carrier which is 10 times more active than that of mature erythrocytes [26]. Interestingly, the K_m for $[Rb^+]_o$ of this K^+ carrier was about 12.5 and the V_{max} around 6–8 mmol/liter cells · hr [26], values quite in the range of those found in the present study. Moreover, the rabbit K^+ carrier was inhibited by furosemide and by external sucrose [26] which may have lowered the Cl^- concentration required for K^+ translocation. Preliminary experiments carried out in LK sheep reticulocytes also indicate that the Cl^--mediated K^+ transport is furosemide-sensitive and, moreover, that furosemide inhibits the NEM-induced stimulation of K^+ transport.

In the final development of the LK steady-state cell from the high K^+ reticulocyte or its precursor cell, a Cl^--dependent K^+ transport path is preserved and turned off during maturation of HK sheep red cells. At present, it is intriguing to test the hypothesis whether in sheep the K^+/Cl^+ transport system is under genetic control and perhaps involved in the HK/LK transition.

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