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Thiol-Dependent Passive K/Cl Transport in Sheep Red Cells: III. Differential Reactivity of Membrane SH Groups with N-Ethylmaleimide and Iodoacetamide

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Summary. Treatment with N-ethylmaleimide (NEM) is known to stimulate ouabain-insensitive, Cl^--dependent K^+ transport in low K^+ (LK) but not in high K^+ (HK) sheep red cells (Lauf, P.K., and Theg, B.E., 1980, *Biophys. Biochem. Res. Commun.* **92**:1422–1428). The dependence of this effect on the pH of pretreatment with NEM and/or iodoacetamide (IAA) was studied. Maximum stimulation of Cl^--dependent K^+ transport in LK red cells was produced by prior treatment with 1–5 mM NEM at pH 6 at which only about 30–40% of the 10^7 SH groups present per membrane reacted. At pH 6 no NEM effect was seen on $Na^+ + K^+$ fluxes in HK red cells. Treatment with NEM below pH 6 enhanced Cl^--independent K^+ transport in both LK and HK red cells. At higher pH values or higher concentrations the NEM-stimulation of K^+ transport was reduced and absent at pH 8.7. Exposure of LK cells to 5 mM IAA prior to NEM abolished the stimulatory effect of NEM on K^+ transport. Hence at least two different chemical groups were reacting with NEM: acidic SH groups responsible for the stimulatory action of NEM, and more alkaline SH or NH_2 groups whose reaction with NEM leads to an inhibition of the NEM effect brought about at pH 6.

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

Introduction

In ruminant erythrocytes N-ethylmaleimide (NEM) is known to affect passive Na^+ and K^+ permeabilites in different ways: Ouabain-resistant $Na^+ - Na^+$ exchange is inhibited in beef and sheep red cells by NEM (and organomercurials [19]) only when Na^+ ions are present [2]. In genetically low K^+ (LK) but not high K^+ (HK) sheep and goat red cells, 1–2 mM NEM selectively stimulates Cl^--dependent K^+ transport [13–17]. In the preceding paper [15] it was shown that at physiologic pH NEM altered V_{max} of furosemide and Cl^--sensitive K^+ transport and that, common to both LK and HK sheep red cells, there was a basal K^+ flux in Cl^- or NO_3^- media not affected by NEM at physiologic pH values. The specific effect of NEM in LK sheep and goat red cells raises the possibility that particular SH groups are involved in the cation permeability changes of the LK cell. The present study aims at a further characterization of the chemical groups reacting with NEM in LK red cell membranes. Preliminary reports have appeared elsewhere [1, 14].

Materials and Methods

Buffers

Tris(hydroxymethyl)aminomethane buffered isosmotic solutions contained either 155 mM $NaNO_3$ (TBN) or 155 mM NaCl (TBS), respectively. The pH was adjusted to the desired value either with HNO_3 (TBN) or with HCl (TBS). The temperature coefficients of Tris were always considered for TBN or TBS used at 0 °C or 37 °C. Isosmotic nitrate solutions of pH values ranging from 5.5–7.0 contained 140 mM $NaNO_3$ buffered with 25 mM citric acid/phosphate mixtures (CPBN). At pH values below 7.0, 30 mM sucrose was added to prevent cell lysis [11]. Sucrose was present only during NEM treatment to offset colloid osmotic swelling at low pH. Prior to the flux experiment, the cells were re-equilibrated in isosmotic media (*see* below). All buffers contained 10^{-4} M ouabain.

Red Cells

Blood was obtained by jugular venipuncture into heparinized vials from healthy Dorset sheep with red cells of known cation and antigenic genotypes. The cells were washed three times in TBN to remove plasma and buffy coat.

Adjustment of pH and Treatment with Thiol Reagents

About 1.2 ml packed cells were resuspended and washed twice in 35 ml of either TBN or hyperosmotic CPBN, followed by incubation for 15 min at 37 °C to allow complete H^+ equilibration. After two additional washes in the equilibration media, the cells were resuspended in 15 ml of their respective nitrate solutions, and 30 µl dimethylsulfoxide (DMSO, Sigma Chemicals, St. Louis, Mo.) ±1 M N-ethylmaleimide (NEM, Sigma Chemicals), or 30 µl buffer ±1 M iodoacetamide (IAA, Sigma Chemicals) were added. The samples were mixed and incubated for 15 (NEM) or 30 min (IAA), respectively, at 37 °C. After

NEM or IAA treatment, the cells of all samples were washed three times with 35 ml TBN, pH 7.4, and incubated for 15 min at 37 °C, to readjust the pH to 7.4.

K^+ Efflux Measurements

K^+ efflux measurements were performed as described before [15]. Calculation of the rate constant of passive K^+ efflux was done by regression analysis of the fractional K^+ release at 45, 60, 70, and 90 min as described in the preceding paper [15].

SH Group Determination

Hemoglobin-free membranes were prepared from NEM-treated or untreated cells by one step osmotic hemolysis [7] in 10 mM Tris/HNO_3, pH 7.6. Membrane proteins were quantitated by the Lowry method [18]. The SH groups were determined photometrically with 5,5-dithiobis(2-nitrobenzoate) (DTNB, Sigma Chemical Co., St. Louis, Mo., *c.f.* ref. 12). From the difference between the DTNB bound/mg of protein of untreated and NEM-treated cells, the percentage of SH groups blocked by NEM was calculated. Correction for nonmembranous SH groups (mainly hemoglobin) was done by subtraction of hemoglobin-SH groups from the basal SH groups detected per sample.

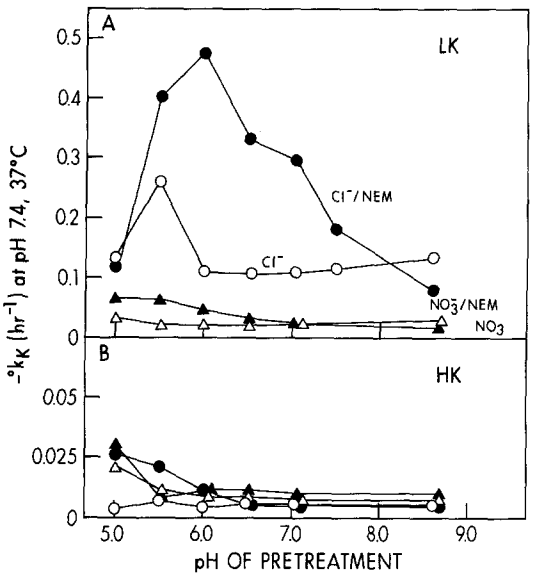

Fig. 1. Effect of treatment with NEM at various pH values on K^+ efflux rate constants measured in Cl^- or NO_3^- media at pH 7.4. (*A*): LK (LL) cells; (*B*): HK (MM) cells. Open symbols: Controls not treated with NEM. Closed symbols: treatment with 2 mM NEM for 15 min at 37 °C. K^+ fluxes measured in Cl^- (○, ●) or NO_3^- (△, ▲)

Results

Figure 1*A* shows the K^+ efflux rate constants ($^o k_K$) of LK sheep red cells, measured at pH 7.4 in either Cl^- or NO_3^- media after pretreatment for 15 min at 37 °C with 2 mM NEM at different pH values. The $^o k_K$ values, measured in Cl^- media revealed a bell shape dependence on the pH values of NEM pretreatment. At pH 6 maximum stimulation was observed, decreasing at lower or higher pH values. Cells incubated without NEM showed little change in the basal K^+ fluxes with the exception of pH 5.5. After pretreatment at this pH, the basic K^+ flux was also increased 1.7-fold as compared to that at pH 7.4. In NO_3^- media, only cells pretreated with NEM below pH 6.0 exhibited a small stimulation of $^o k_K$.

High K^+ cells in either Cl^- or NO_3^- behaved very much like LK cells in NO_3^-. Figure 1*B* shows that over a wide pH range, the rate of K^+ efflux from control HK cells in Cl^- medium was lower than that from LK cells in NO_3^- (note the expanded y axis). Furthermore, no effect of Cl^- replacement by NO_3^- was observed on the efflux rate constants of HK cells. However, after NEM treatment of HK cells at pH 5.0 or 5.5 K^+ efflux was enhanced in both Cl^- and NO_3^- media. Hence, NO_3^--supported K^+ flux was stimulated by NEM in both LK and HK red cells at low pH values although the absolute values of $^o k_K$ where about 1/20 of those seen in Cl^- media.

From earlier work it was known that NEM treatment at physiologic pH stimulated Cl^--dependent K^+ transport without affecting the ouabain-insensitive Na^+ permeability [13, 14, 17]. Table 1 shows an experiment in which Na^+ efflux was measured in choline Cl or choline iodide (recrystallized, Aldrich Chemicals, Milwaukee, Wisc.) media (pH 7.4) after LK red cells had been treated with or without NEM at pH 6.0. The data indicate that under these conditions NEM did not significantly

Table 1. Na^+ efflux rate constants of LK and HK sheep red cells pretreated with NEM at pH 6

Cells	$n=4$	Choline-chloride[a]			Choline-iodide[a]		
		None	pH 6	pH 6+NEM	None	pH 6	pH 6+NEM
LK	$\bar{x}$	0.021	0.015	0.019	0.025	0.027	0.028
	±SD	0.002	0.001	0.001	0.002	0.002	0.003
HK	$\bar{x}$	0.014	0.010	0.022	0.018	0.020	0.024
	±SD	0.001	0.001	0.004	0.001	0.003	0.001

[a] Flux media.

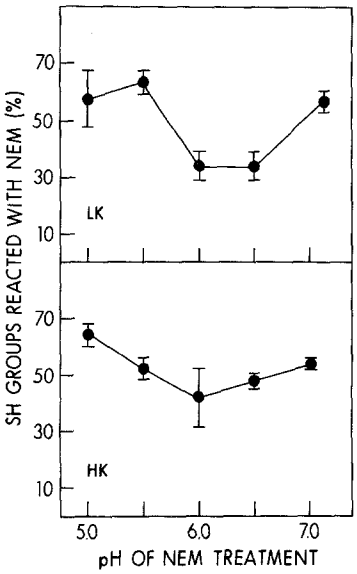

Fig. 2. Fraction of membrane SH groups reacted with NEM at various pH values in LK and HK red cells. Bars indicate ±SD for $n=4$

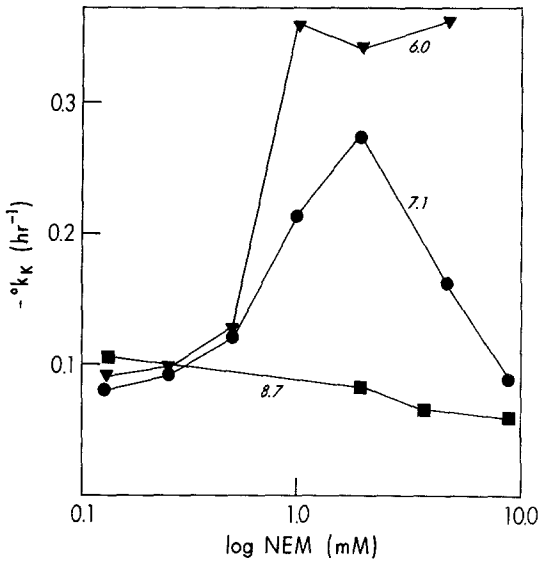

Fig. 3. Effect of increasing NEM concentrations at three different pH values on the K^+ efflux rate constant of LK sheep red cells. Cells were pretreated with the NEM concentration and at the pH values indicated and then analyzed for K^+ efflux at pH 7.4 as described in Materials and Methods

Table 2. Effect of pretreatment with iodoacetamide and N-ethylmaleimide on the K^+ efflux rate constant of LK sheep red cells in Cl^- media

First treatment	Second treatment	
	pH 6	pH 6+2 mM NEM
Controls	0.055	0.193
5 mM IAA	0.095	0.119

After the first treatment at pH 7.4 with and without IAA (left column) each sample was divided into two parts and treated a second time at pH 6 with or without NEM.

alter Na^+ fluxes at the pH where maximum stimulation of Cl^--dependent K^+ stimulation was seen.

The higher stimulation of Cl^--mediated K^+ efflux by NEM at pH 6.0 was surprising, because a more alkaline pH favors the NEM reaction with SH groups [8]. Therefore, we measured the fraction of membrane SH groups which had reacted with NEM (2 mM) during 15 min incubation at 37 °C. There were 9.40 ($\pm$0.99) $\times 10^{-6}$ ($n=12$) and 9.06 ($\pm$1.27) $\times 10^{-6}$ ($n=12$) SH groups per LK and HK membrane, respectively. On a protein basis this amount was comparable to that reported for human red cells [20]. Figure 2 shows that in both LK and HK cell membranes about 40% of all SH groups were alkylated by NEM at pH 6.0, while 55–60% reacted at pH 7.1 and 5.0, respectively. The data suggest that maximum stimulation of Cl^--dependent K^+ transport by NEM correlates with minimum reactivity of membrane SH groups with NEM.

We investigated whether IAA, which also reacts with SH groups, would alter the NEM effect. Cells were exposed to iodoacetamide prior to NEM, and then the Cl^--dependent K^+ efflux was measured. Table 2 shows that 5 mM iodoacetamide reduced the NEM effect, but also affected somewhat the basal Cl^--dependent K^+ efflux. A concentration of 5 mM and a reaction time of 30 min were required to obtain significant inhibition of the NEM effect by IAA. The results suggest that NEM binds to SH groups and that the stimulation of Cl^--mediated K^+ efflux appears to be dependent on specific properties of the bound NEM molecule.

The dependence of K^+ flux stimulation on the NEM concentration during pretreatment is shown in Fig. 3. At pH 7.1, the Cl^--dependent K^+ efflux was maximally stimulated in cells treated with 1 to 2 mM NEM. Only 20% of the maximal effect was observed, and none was found in LK cells treated with 0.5 mM and 0.25 mM NEM, respectively. After treatment with either 5 or 10 mM NEM, only 40 and 10% of the maximal stimulatory effect was observed, respectively. At pH 6, however, NEM stimulated maximally at all concentrations above 1 mM. At pH 8.7 no NEM stimulation was observed. Figure 4 shows that the NEM effect was identical after exposure to 2 and 5 mM NEM at pH 6.0. However, when below or above pH 6.0 the cells were treated with 5 mM NEM the effect was reduced as compared to treatment with 2 mM

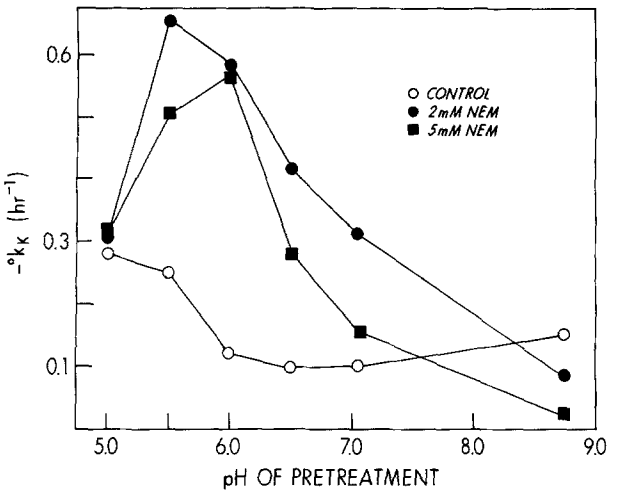

Fig. 4. Effect of two different NEM concentrations during pretreatment of LK sheep red cells at different pH values on the K^+ efflux rate constants. Fluxes were measured at pH 7.4 in isosmotic Cl^- media

Table 3. Effect of successive treatments with N-ethylmaleimide and varying pH on the K^+ efflux rate constants of LK sheep red cells in Cl^- media

First treatment	Second treatment			
	pH 6.0	pH 6.0 +2 mM NEM	pH 8.7	pH 8.7 +2 mM NEM
Controls	0.070	0.238	0.070	0.112
2 mM NEM	0.370	0.352	0.241	0.082

After the first treatment of pH 6.0 in the presence or absence of NEM (left-hand column) each sample was divided into 4 aliquots and treated a second time, as shown above. Each treatment was performed during 15 min at 37 °C.

NEM. The Cl^--dependent K^+ fluxes of cells pretreated with 5 mM NEM at pH 8.7 were lower than those of controls.

To test for the possible presence of two kinds of membrane SH groups differing in their reactivity to NEM, LK cells were treated successively with 2 mM NEM at two different pH values as shown in Table 3. K^+ efflux of LK cells treated twice with NEM at pH 6.0 was fully stimulated. However, if after the first NEM treatment at pH 6.0 a second was performed at pH 8.7, the stimulation was lost. In contrast, incubation at pH 8.7 without NEM did not reduce the NEM effect already produced at pH 6.0. Hence, there are two population of NEM target groups in the membrane of LK red cells. The first population, responsible for stimulation of Cl^--mediated K^+ transport, consists of probably acidic SH groups readily reacting with NEM at pH 6.0. The second population may be either SH or NH_2 groups, binding NEM mainly at pH 8.7. Once these latter groups were blocked by NEM, reaction of NEM with the more acidic SH groups and hence stimulation of Cl^--dependent K^+ flux were prevented.

Discussion

The following important findings were made: (i). Maximum stimulation of ouabain-insensitive and Cl^--dependent K^+ transport was seen after pretreatment of LK sheep red cells with NEM concentrations of 1 mM or higher at pH 6 (Fig. 1 *A* and *B*). No such effect was seen in HK sheep red cells, nor was there any significant change in Na^+ effluxes in either LK or HK cells (Table 1). (ii) Treatment of both LK and HK sheep red cells with NEM at pH values below 6 caused an increase of K^+ efflux which was Cl^--independent. (iii) The maximum stimulation for Cl^--dependent K^+ flux correlated with a 40% reduction of the total membrane SH-groups by NEM (Fig. 2). (iv) At pH 6, the effect of NEM was independent of its concentration above 1 mM. However, treatment of LK cells with both higher pH and NEM concentrations caused less stimulation of Cl^--dependent K^+ transport (Figs. 3 and 4). (v) Pretreatment of LK red cells at pH 8.7 with 2 mM NEM or at pH 7.4 with 5 mM IAA prior to NEM exposure at pH 6 abolished the stimulation of Cl^--mediated K^+ transport (Tables 2 and 3).

N-ethylmaleimide is known to react at pH 6.0 only with sulfhydryl groups of proteins [3]. At higher pH values and NEM concentrations, however, an increasing reactivity of NEM with NH_2 groups was found [5]. Although the intramembranous pH values are unknown, it can be assumed that NEM reacted at pH 6 specifically with membrane SH groups, causing a more than fourfold stimulation of Cl^--dependent K^+ transport in LK red cells. The finding that at pH 6 NEM binding was lowest may be explained by the lower degree of SH dissociation. That after NEM treatment at pH 5.0 and 5.5 (Fig. 2) higher numbers of SH groups are blocked is difficult to understand, because at pH 5.0 the rate constant of the NEM reaction with SH groups is about 10% of that at pH 7.0 [8]. Perhaps this result may be due to conformational changes of certain membrane proteins [9].

The observation that NEM treatment at pH values above 6 caused less or no (pH 8.7) stimulation of Cl^--dependent K^+ transport suggests blocking of chemical groups different from those available for adduct formation at pH 6. Most likely these blocking groups are also SH residues since iodoacetamide, a specific SH reagent [3] also

diminished the NEM effect. Participation of some NH_2 groups in the modulation of the NEM reactivity at pH values above 6 cannot be ruled out, as NH_2 groups modifying passive Na^+ permeability via SH groups have been reported for dog red cells [6].

Iodoacetamide only slightly stimulated Cl^--dependent K^+ flux and inhibited NEM stimulation, suggesting that IAA bound to those SH groups which specifically participated in the NEM-mediated stimulation of K^+ transport. Hence some properties of the NEM-SH group adduct may augment K^+ flux more than that of IAA alkylated SH groups. Interestingly, NEM-SH group adducts are known to form red complexes with alkali ions at pH values above 10 [4]. This phenomenon, however, was independent of the type of anions present. Although it is known that NEM reacts mainly with SH groups of spectrin, ankyrin, band 3, and band 4.2 proteins of human red cell membranes [4*a*, 10, 12, 20], studies to show specific binding of NEM to the Na^+/Li^+ countertransport system have met with difficulties [2].

The present study adds further weight to the hypothesis that there is a Cl^--independent K^+ transport path common to both LK and HK sheep red cells [15] because in the presence of NO_3^- and at pH values below 6, NEM to some extent stimulated K^+ flux in both LK and HK sheep red cells. Hence, the Cl^--independent K^+ movements appear to be different from the Cl^--specific K^+ transport system. In HK cells no NEM effect was seen when the cells were treated above pH 6, but the K^+ efflux was significantly enhanced after NEM treatment below pH 6. The stimulation in the presence of Cl^- seemed to be higher than in NO_3^-. However, the K^+ efflux rate constants were so small that further work is required to clarify this point and to assess whether the protein involved in Cl^--dependent K^+ ion translocation disappears during maturation of reticulocytes to high K^+ sheep red cells [16].

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References

1. Bauer, J., Lauf, P.K. 1982. Evidence for differential reactivity of sulfhydryl groups in the N-ethylmaleimide activated Cl^- specific K^+ transport system of sheep erythrocytes. *J. Gen. Physiol.* **80:**10*a*
2. Becker, F., Duhm, J. 1979. Studies on lithium transport across the red cell membrane. VI. Properties of a sulfhydryl group involved in ouabain-resistant $Na^+ - Li^+$ (and $Na^+ - Na^+$) exchange in human and bovine erythrocytes. *J. Membrane Biol.* **51:**287–310
3. Benesch, R., Benesch, R.E. 1957. Determination of sulfhydryl and disulfide groups by specific proton displacement. *Biochem. Biophys. Acta* **23:**643
4. Benesch, R., Benesch, R.E., Gutcho, M., Laufer, L. 1956. New color test for thiols and thiolesters. *Science* **123:**981–982

4*a*. Bennett, V. 1978. Purification of an active proteolytic fragment of membrane attachment site for human erythrocyte spectrin. *J. Biol. Chem.* **253:**2292–2299

5. Brewer, C.F., Riehm, J.P. 1967. Evidence for a possible non-specific reaction between N-ethylmaleimide and proteins. *Anal. Biochem.* **18:**248–255
6. Castranova, V., Mills, P.R. 1977. Study of amino and sulfhydryl sites in the sodium pathway in dog red blood cell membranes. *J. Membrane Biol.* **33:**263–279
7. Dodge, J.T., Hanahan, D.J., Mitchell, C. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100:**119–130
8. Goorin, G., Mastic, P.A., Doughty, G. 1966. Kinetics of the reaction of N-ethylmaleimide with cystein and some congeners. *Arch. Biochem. Biophys.* **166:**593–597
9. Green, F.A. 1967. Erythrocyte membrane sulfhydryl groups and Rh antigen activity. *Immunochemistry* **4:**247–257
10. Grinstein, S., Rothstein, A. 1978. Chemically-induced cation permeability in red cell membrane vesicles: The sidedness of the response and the proteins involved. *Biochim. Biophys. Acta* **508:**236–245
11. Gunn, R.B., Dalmark, M., Tosteson, D.C., Wieth, J.O.. 1973. Characteristics of chloride transport in human red blood cells. *J. Gen. Physiol* **61:**185–206
12. Haest, C.W.M., Plasa, G., Kamp, D., Deuticke, B. 1978. Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane. *Biochim. Biophys. Acta* **509:**21–32
13. Lauf, P.K. 1981. A chemically unmasked, chloride dependent K^+ transport in low K^+ sheep red cells: Genetic and evolutionary apsects. *In:* Erythrocyte membranes 1: Recent Clinical and Experimental Advances. W.C. Kruckeberg, T.W. Eaton, and G.T. Brewer, editors. pp. 13–30. Alan R. Liss, New York
14. Lauf, P.K. 1982. Evidence for specific SH group participation of Cl^- dependent K^+ fluxes in LK sheep red cells. *Biophys. J.* **37:**336*a*
15. Lauf, P.K. 1983. Thiol-dependent passive K/Cl transport in sheep red cells. I. Effect of Cl^- replacement and external $K^+[Rb^+]$ ions. *J. Membrane Biol.* **73:**237–246
16. Lauf, P.K. 1983. Thiol-dependent passive K/Cl transport in sheep red cells. II. Loss of Cl^- and N-ethylmaleimide sensitivity in maturing high K^+ cells. *J. Membrane Biol.* **73:**247–256
17. Lauf, P.K., Theg, B.E. 1980. A chloride dependent K^+ flux induced by N-ethylmaleimide in genetically low K^+ sheep and ghost erythrocytes. *Biophys. Biochem. Res. Commun.* **92:**1422–1428
18. Lowry, O.H., Rosebrough, N.J., Farr, A., Randall, R. 1951. Protein measurement with the Folin reagent. *J. Biol. Chem.* **193:**265–275
19. Motais, R., Sola, F. 1973. Characteristics of a sulphydryl group essential for sodium exchange diffusion in beef erythrocytes. *J. Physiol (London)* **233:**423–438
20. Van Steveninck, J., Weed, R.I., Rothstein, A. 1965. Localization of erythrocyte membrane sulfhydryl groups essential for glucose transport. *J. Gen. Physiol.* **48:**617–632

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