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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*

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Summary. The reactivity of the SH-group essential for ouabain-resistant $Na^+ - Li^+$ (and $Na^+ - Na^+$) exchange and its location within the membrane are studied on human and beef erythrocytes and beef red cell ghosts.

N-ethylmaleimide (NEM), 1,6-hexane dimaleimide, and iodoacetamide can induce an irreversible, partial inhibition of $Na^+ - Li^+$ exchange in erythrocytes of the two species. The development of the inhibition due to the alkylating agents is greatly accelerated by external Na^+ and Li^+. The inhibition takes 3 min (NEM) and 60 min (iodoacetamide) to come to completion in isotonic Na^+ media, but is hardly detectable in choline$^+$, K^+ or Mg^{2+} media. The transport site of the exchange system and the site promoting NEM binding exhibit similar affinities for external Na^+. The impermeable, monofunctional glutathione derivative of 1,6-hexane dimaleimide does not inhibit $Na^+ - Li^+$ exchange.

The mercurials PCMBS, PCMB, and Hg^{2+} inhibit $Na^+ - Li^+$ exchange in beef, but not in human erythrocytes. The inhibitory action of PCMBS, being slightly accelerated by external Na^+, is fully reversed by penetrating thiols such as 2-mercaptoethanol, whilst glutathione, an impermeable thiol, is ineffective. Pretreatment with PCMBS affords partial protection from the irreversible inhibition caused by NEM. Oxidation with copper orthophenanthroline inhibits $Na^+ - Li^+$ exchange only when performed in the presence of penetrating thiols such as 2-mercaptoethanol.

It is concluded that the SH-reagents studied inhibit $Na^+ - Li^+$ exchange by modifying an essential SH-group of a membrane protein in such a way that the turnover number of the exchange system is reduced. This SH-group is separated from both the red cell exterior and interior by a penetration barrier and seems to be distinct from the cation binding site. The action of external Na^+ and Li^+ in promoting the reaction of alkylating inhibitors is interpreted to result from a conformational change of the transport protein induced by the binding of external Na^+ or Li^+.

In the foregoing paper evidence has been presented for a transport system in the membranes of human, sheep, rabbit and beef erythrocytes

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which can mediate a ouabain-resistant exchange of either Na^+ for Na^+, Na^+ for Li^+, or Li^+ for Li^+ (Duhm & Becker, 1979). The exchange system exhibits essentially identical transport characteristics in the four species, but shows marked interspecies variability in maximum transport capacity and some differences in susceptibility towards inhibitors. Human and beef erythrocytes, in addition, display a pronounced intraspecies variability in activity of the exchange.

A property of the exchange system common to the four species is its partial inhibition by N-ethylmaleimide (NEM), which is thought to arise from an interaction with an SH-group and implies that a protein is involved in the transport process. This SH-group has been first described by Motais and Sola (1973) in a detailed study on ouabain-resistant $Na^+ - Na^+$ exchange in beef red cells. These authors reported that NEM induces an irreversible, partial block of ouabain-resistant $Na^+ - Na^+$ exchange in beef and human erythrocytes. The SH-reagent *p*-chloromercuribenzene sulfonate (PCMBS) was found to be slightly more potent than NEM in beef red cells, but ineffective in human erythrocytes. The PCMBS effect could be relieved with dithiothreitol. We have obtained similar results with respect to the action of NEM and PCMBS on $Na^+ - Li^+$ exchange in the two species (Duhm & Becker, 1977, 1978*a*, 1979).

In an attempt to further characterize the transport protein with the aid of its essential SH-group, we observed that the irreversible inhibition of the $Na^+ - Li^+$ exchange system by NEM in human and bovine erythrocytes requires the presence of cations in the medium which are transported by the system, i.e., of Na^+ or Li^+ (Duhm & Becker, 1978*b*). This paper deals with the phenomenon of cation-specific reaction of the exchange system with SH-reagents. In addition, evidence is presented indicating that the SH-group considered is not superficially located, but rather embedded within the membrane, and that it is probably not identical with the cation binding site of the exchange system.

Materials and Methods

Human blood (anticoagulant heparin) was freshly drawn from the antecubital vein of the donor U.L., known to have erythrocytes of high Na^+-Li^+ exchange activity (Duhm & Becker, 1977). Beef blood was obtained from the local slaughter-house (anticoagulant citrate). The reagents phlorobenzophenone and 1,6-hexane dimaleimide were acquired from ICN/K & K Laboratories, Plainview, N-ethylmaleimide (NEM), *p*-chloromercuribenzene sulfonic acid (PCMBS), *p*-chlromercuribenzoic acid (PCMB), 1, 10-phenanthroline, phloretin, and iodoacetamide were from Sigma, St. Louis, 5,5′-dithiobis-(2-nitrobenzoic acid)

(DTNB), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and reduced glutathione were from SERVA, Heidelberg, and ouabain, monoiodoactetate (Na^+ salt), mercuric chloride, 2-mercaptoethanol, and all other substances were from Merck AG, Darmstadt. The mono-glutathione derivative of 1,6-hexane dimaleimide (1-maleimido-6-(2-glutathione-succinimido)-hexane) was prepared according to Abbot and Schachter (1976) and purified over a Sephadex G-15 column as described there. The lyophilized product was about 96% pure judging from the maleimide content determined with excess cysteine and DTNB (see Ellman, 1959).

$Na^+ - Li^+$ exchange in intact red cells was generally assessed by determining Li^+ net-uptake from isotonic choline chloride media containing 2 mM Li^+, as previously described (Duhm & Becker, 1977, 1979). Erythrocytes were washed three to four times in the prospective, Li^+-free, isotonic incubation medium, and suspended at 37 °C and an hematocrit of 3%, 0.1 mM ouabain being present in all incubation media. The pH was measured regularly during the incubation periods and adjusted to pH 7.4, if necessary. After an equilibration interval of 20–30 min, Li^+ uptake was initiated by adding an aliquot of isotonic LiCl stock solution. If inhibitors were to be present during the actual Li^+ uptake period, they were added to the red cell suspension in the equilibration interval, 15–20 min prior to initiation of Li^+ uptake. Phloretin and phlorobenzophenone were predissolved in ethanol, 1,6-hexane dimaleimide in dimethylsulfoxide, the other reagents in water. The final ethanol or dimethylsulfoxide concentration in the suspension (0.2%, vol/vol) had no influence on Li^+ transport. The same incubation protocol as described for Li^+ uptake was applied when erythrocytes were pretreated with various reagents and media (*see* Results), except that the Li^+ addition was omitted. For example, to reduce the number of nonspecific inhibitor binding sites in the experiments shown in Tables 2 and 3 and Figure 6, erythrocytes were preincubated with 0.5 mM NEM in choline chloride for 30 min at 37 °C, pH 7.4 and an hematocrit of 3%.

Beef erythrocyte ghosts were prepared by hypotonic hemolysis at 0 °C from cells either untreated or pretreated with 0.5 mM NEM or 25 μM PCMBS in isotonic NaCl solution for 15 min (pH 7.4, 37 °C, hematocrit 3%) according to a method modified from Bodemann & Passow (1972). The erythrocytes were first washed three times in isotonic NaCl medium containing 5 mM tris (hydroxymethyl)-aminomethane (Tris), 4 mM $MgCl_2$ and 1 mM $CaCl_2$, titrated to an extracellular pH of 6.4 at 0 °C in this medium and spun down. The cold cells (4.5 ml) were then added under stirring to 34 ml of ice-cold hemolyzing medium (5 mM Tris, 4 mM $MgCl_2$, 1 mM $CaCl_2$, pH 6.15 at 0 °C). After 5 min, isotonicity was restored with 2.3 ml ice-cold, 3 M NaCl, and the cells were allowed to stand for a further 5 min. The suspension was subsequently incubated at 37 °C for 1 hr to reseal the ghosts. Following sedimentation (10 min at $12,000 \times g$ and 0 °C), the ghosts were washed in the cold, once in isotonic NaCl (pH 6.4) and once in isotonic KCl (pH 7.4), both solutions containing 5 mM Tris, 4 mM $MgCl_2$ and 1 mM $CaCl_2$. The ghosts were ready for use after being decanted from a "pellet" which adhered to the centrifugation vessel. The $Na^+ - Li^+$ exchange activity of the ghosts was estimated from their capacity to establish an outwardly directed Li^+ gradient (*see* Duhm *et al.*, 1976). 2 min after being suspended in warm (37 °C), isotonic KCl solution containing 5 mM Tris, 4 mM $MgCl_2$ and 1 mM $CaCl_2$ (pH 7.4, ghostocrit 12–14%), the solution was made 1 mM with respect to Li^+. The hemoglobin content (about 0.2 μmol/ml packed ghosts, by tetramer) and the volume of the ghosts (as assessed from serial determinations of the ghostocrit) did not change throughout the incubation period.

Li^+ uptake was terminated at the times given in Results by cooling portions of the suspensions in an ice-bath. The cells were then washed three times in ice-cold, isotonic choline chloride, and finally packed by 4 min centrifugation at $10,000 \times g$. Cellular Li^+, Na^+ and K^+ contents and cation concentrations in the media were determined with a Perkin-Elmer atomic absorption spectrophotometer 420 after suitable dilution of the samples

with 6% *n*-butanol (vol/vol). The cellular hemoglobin content was determined simultaneously in the red cell sediment by the cyano-methemoglobin method. The Li^+ contents of intact cells given in Results are all corrected to a hemoglobin content (by tetramer) of 5.2 µmol/ml cells. Li^+ uptake by the ghosts was terminated as described above for intact cells, the ghosts, however, not being washed prior to packing for determination of cation content.

PCMBS was measured in the supernatant of cell suspensions according to Benesch and Benesch (1962), as modified by van Steveninck, Weed and Rothstein (1965), by determining the extinction at 235 nm with a Perkin Elmer spectrophotometer 124 D in the absence and presence of 2-mercaptoethanol.

Results

The effects of a number of "SH-reagents" on the Na^+-Li^+ exchange system of human and beef erythrocytes are listed in Table 1. Data on

Table 1. Effects of various sulfhydryl reagents and phloro-compounds on $Na^+ - Li^+$ exchange in human and bovine erythrocytes[a].

Agent	(mM)	% Inhibition of Li^+ Uptake[c]			
		Human	(*n*)	Beef	(*n*)
NEM	(0.5)	56±5	(13)	59±4	(20)
1,6-Hexane dimaleimide	(0.1)	50±2	(3)	47	(1)
1-Maleimido-6-(2-glutathionesuccinimido)-hexane	(0.1)	0	(1)	0	(3)
PCMBS	(0.005)	0	(4)	76±6	(28)
NEM+PCMBS	(0.5+0.005)	–[b]		83±3	(13)
PCMB	(0.025)	0	(5)	73±7	(5)
$HgCl_2$	(0.005)	0	(3)	47±9	(7)
Ag^+	(10^{-4}–10^{-1})	0	(2)	0	(1)
Monoiodoacetate	(5)	0	(3)	0	(3)
Iodoacetamide	(5)	0	(4)	0	(3)
Phloretin	(0.2)	75±3	(10)	68±9	(6)
Phlorobenzophenone	(0.5)	76±6	(3)	70±6	(5)

[a] $Na^+ - Li^+$ exchange was assessed by determining Li^+ net-uptake in isotonic choline chloride media containing 2 mM Li^+ (0.1 mM ouabain, pH 7.4, 37 °C, hematocrit 3%). The uptake period was 1 hr for human and 15 min for beef erythrocytes. When Ag^+ was being tested, the cells were either incubated in choline chloride media or repeatedly washed in potassium acetate and incubated in the same medium. The cells were preincubated with the inhibitors for 15 min. The studies on human erythrocytes were performed with red cells of the donor U.L. which have uninhibited uptake rates of about 0.2 µmol/ml cells × h (*see* Fig. 1). The percentages of inhibition determined with human red cells of lower $Na^+ - Li^+$ exchange activity were smaller than those listed (*see*, e.g., Table 3). The results for bovine erythrocytes were obtained on blood samples from 28 animals.

[b] Not determined.

[c] Mean values ± SD.

the action of phloretin and a chemically related derivative, phlorobenzophenone, are included. Whereas NEM, 1,6-hexane dimaleimide, phloretin, and phlorobenzophenone were inhibitory in both species, the mercurials PCMBS, PCMB, and $HgCl_2$ were effective only in beef erythrocytes. The inhibition by PCMBS in these cells slightly exceeded that induced by NEM, and combination of the two SH-reagents led to a small increase in inhibition above the value seen with PCMBS alone. In contrast to NEM and 1,6-hexane dimaleimide, the impermeant maleimide, 1-maleimido-6-(2-glutathionesuccinimido)-hexane (*see* Abbot & Schachter, 1976), was without effect on $Na^+ - Li^+$ exchange in the two species. 5 mM each of iodoacetamide and monoiodoacetate also did not inhibit the exchange system under the experimental conditions described in Table 1 (for inhibition by the two agents occurring after prolonged preincubation in Na^+ media, *see* below).

Cation-Dependence of $Na^+ - Li^+$ Exchange Inhibition by SH-Reagents

In studies concerned with the reversibility of the effects of NEM and PCMBS, it has been observed that the inhibition of the exchange system due to NEM does not develop in pure choline media (Duhm & Becker, 1978*b*). This is demonstrated for human erythrocytes in Fig. 1. The cells were preincubated either in choline or sodium chloride medium for various time intervals with 0.5 mM NEM (i.e., about 17 μmol NEM/ml cells), an amount known to cause the maximum inhibition to be achieved with NEM in human erythrocytes (Duhm & Becker, 1977, 1978*a*). The NEM not bound was removed by addition of a fourfold excess of 2-mercaptoethanol and repeated washing. The cells were then resuspended in choline chloride medium and Li^+ uptake was determined. Obviously, the uptake was not inhibited when the cells had been preincubated with NEM in choline chloride for as long as 60 min. Rather, the exchange system retained its activity under these conditions, and a second addition of NEM during the actual Li^+ uptake period induced the inhibition usually observed with the red cells of the donor studied (open squares in Fig. 1). In contrast, preincubation of the cells with NEM in sodium chloride medium led to an inhibition which could not be reversed by 2-mercaptoethanol and washing. The inhibition was already fully established after 3 min of contact with NEM in sodium chloride (Fig. 1), and a second addition of NEM had no further effect. At the high NEM concentration of 2 mM (64 μmol/ml cells), however, the system also

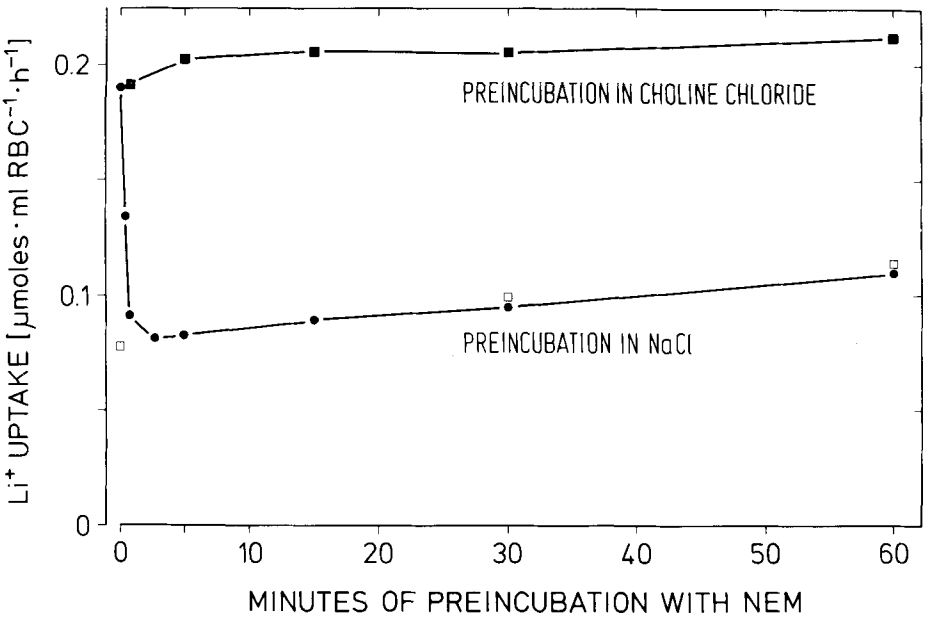

Fig. 1. Irreversible inhibition of Li^+ uptake by NEM – its dependence on external Na^+ and the duration of contact. Erythrocytes of the donor U.L. were suspended in isotonic choline (■, □) or sodium chloride media (●) containing 0.5 mM NEM (37 °C, pH 7.4, hematocrit 3%). At the time intervals shown on the abscissa the suspensions were made 2 mM with respect to 2-mercaptoethanol. After being washed four times in warm choline chloride, Li^+ uptake was determined as described in Materials and Methods (150 mM choline chloride, 2 mM LiCl, 0.1 mM ouabain, 37 °C, pH 7.4, hematocrit 3%). The open squares (□) refer to Li^+ uptake determined in the presence of 0.5 mM NEM with cells pretreated with NEM in choline chloride

seemed to become slowly inhibited in choline media. The increase in passive membrane permeability of cations induced by such high NEM concentrations (Giebel & Passow, 1961; Jacob & Jandl, 1962) made it difficult to quantify the effect on $Na^+ - Li^+$ exchange under this condition (results not shown).

To examine whether cations other than Na^+ stimulate the reaction of NEM with the exchange system, erythrocytes were pretreated with NEM in various media, as described in Fig. 2. Similar to choline medium, a 30-min pretreatment with NEM in potassium or magnesium medium did not markedly inhibit Li^+ uptake, whereas a second addition of NEM in the uptake period exerted the full inhibitory effect in the very same media. Again, when 150 mM Na^+ or even only 10 mM Na^+ were present during the preincubation, NEM blocked the system irreversibly and a

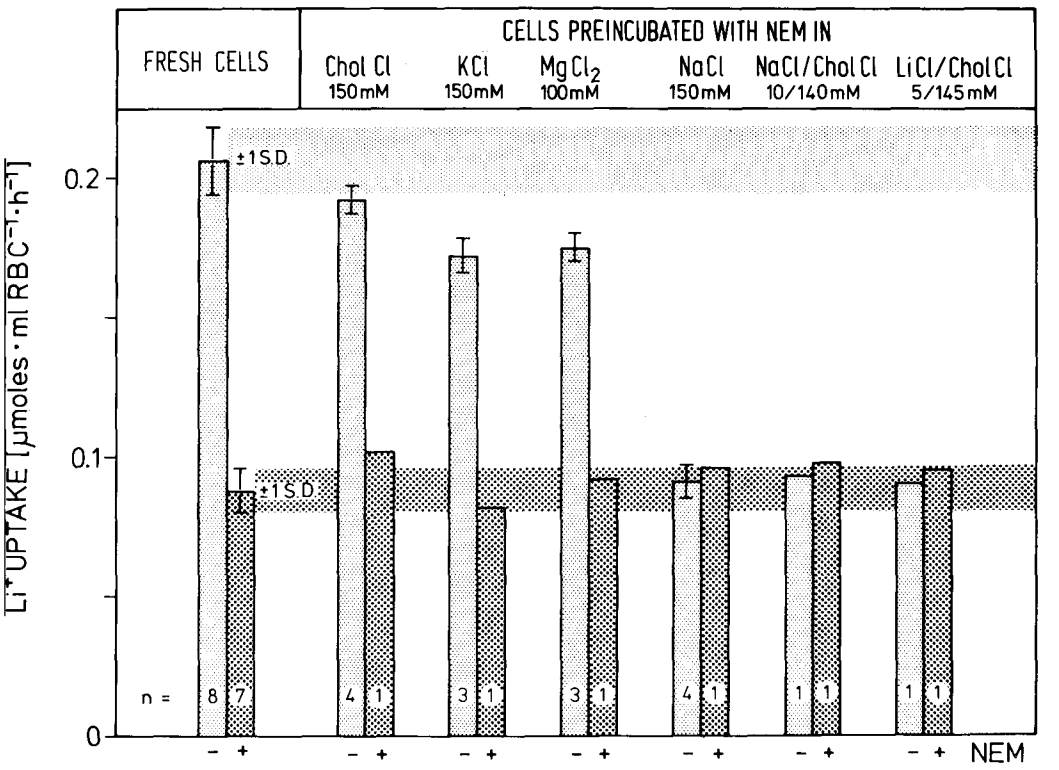

Fig. 2. Na^+- and Li^+-specific induction of irreversible inhibition of Li^+ uptake by NEM. Human erythrocytes were pretreated with 0.5 mM NEM for 30 min in isotonic solutions of choline, potassium, magnesium, and sodium chloride and in a choline chloride medium containing 10 mM NaCl, as well as for three minutes in a choline medium containing 5 mM LiCl (37 °C, pH 7.4, hematocrit 3%, donor U.L.). The preincubation was stopped by adding 2 mM 2-mercaptoethanol and four washings. Subsequently, Li^+ uptake was determined in isotonic KCl medium in the absence and presence of 0.5 mM NEM (*see* Methods). The numbers at the bottom of the columns refer to the number of the experiments conducted under each condition

second addition of NEM induced no further inactivation. The same result as with Na^+ was obtained when the cells were pretreated with NEM in a choline medium containing 5 mM Li^+ for a time as short as 3 min.

These experiments demonstrate that the presence of the transported cations Na^+ or Li^+ in the medium is a prerequisite for a rapid and irreversible inhibition of the $Na^+ - Li^+$ exchange system by NEM in human erythrocytes. A corresponding, specific requirement of external Na^+ or Li^+ is found for the reaction of NEM with the exchange system of beef, sheep and rabbit erythrocytes (results not shown).

Table 2. Na^+-dependence of the inhibition of $Na^+ - Li^+$ exchange induced by 1,6-hexane dimaleimide[a]

Preincubation medium	1,6-hexane dimaleimide (10 μM)	Li^+ uptake (μmol/ml cells × hr) 1,6-hexane dimaleimide (5 μM)	
		−	+
Na^+	−	0.19	0.10
Na^+	+	0.11	0.10
$Choline^+$	−	0.19	0.10
$Choline^+$	+	0.19	0.10

[a] Human erythrocytes (donor U.L.) were pretreated for 30 min with NEM (17 μmol/ml cells) in choline chloride to reduce the number of nonspecific, reactable SH-groups. The cells were then preincubated without and with 10 μM 1,6-hexane dimaleimide (330 nmol/ml cells) for 5 min, either in choline or in sodium chloride medium. These cells were washed 4 times in warm choline medium to remove residual amounts of the reagent. Subsequently, Li^+ uptake was measured in choline medium in the absence and presence of 5 μM 1,6-hexane dimaleimide (2 mM Li^+_e, 0.1 mM ouabain, pH 7.4, 37 °C, hematocrit 3%).

The inhibitory action of 1,6-hexane dimaleimide also depends on external Na^+. As shown in Table 2, a 5-min preincubation of erythrocytes with 10 μM (i.e., 330 nmol/ml cells) of the bifunctional maleimide sufficed to induce 85% of the inhibition maximally to be achieved with this reagent, provided Na^+ was the cation in the preincubation medium. If Na^+ was replaced by $choline^+$, no inhibition resulted from the pretreatment (Table 2). Exactly the same effect of Na^+ could be demonstrated on beef erythrocytes. The monofunctional, nonpenetrating glutathione derivative 1-maleimido-6-(2-glutathionesuccinimido)-hexane (0.1 mM) was not inhibitory in Na^+ or other media when examined under the conditions described in Table 2, even with contact times lasting up to one hour.

Iodoacetamide and iodoacetate, apparently inactive reagents (*see* Table 1), became capable of inhibiting the exchange system after prolonged incubation in Na^+ media. As demonstrated in Fig. 3, Li^+ uptake by human or beef erythrocytes was not affected by 10 mM iodoacetamide when the preincubation was performed in choline chloride. In Na^+ medium, however, iodoacetamide caused a slowly developing inhibition which reached its maximum after about one hour of contact, the inhibition then being comparable to that seen with phloretin (Fig. 3*a*) or PCMBS (Fig. 3*b*). In similar experiments performed with 10 mM monoiodoacetate, 1 hr preincubation in isotonic sodium chloride led to 40 and 50% inhibition with beef and human erythrocytes, respectively. Monoio-

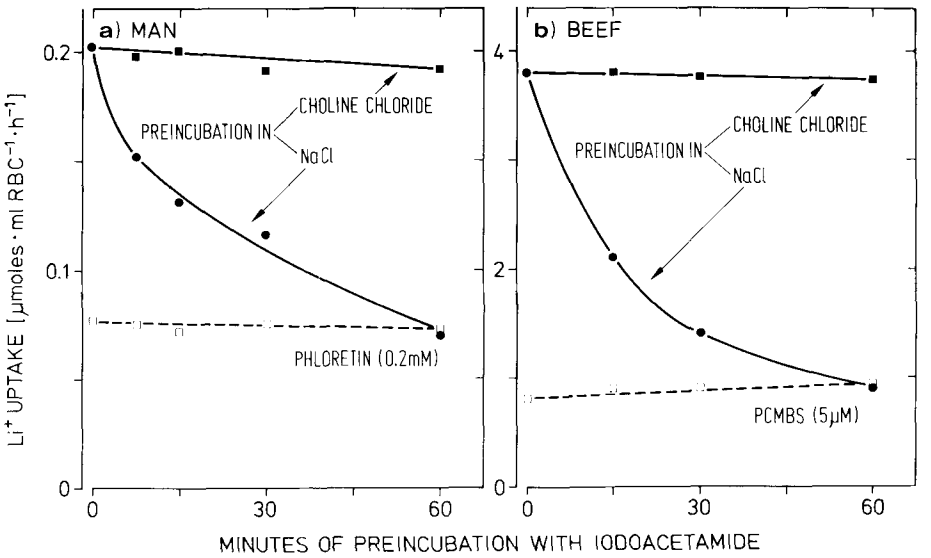

Fig. 3. Development of the Na^+-dependent inhibition of Li^+ uptake by iodoacetamide in human and beef erythrocytes. (*a*): Red blood cells of the donor U.L. were incubated in isotonic solutions of choline (■, □) or sodium chloride (●) with 10 mM iodoacetamide for the time intervals shown on the abscissa (37 °C, pH 7.4, hematocrit 3%). Li^+ uptake was measured as described in the legend of Fig. 1 after the cells had been washed 3 times in warm choline chloride. The open squares (□) indicate Li^+ uptake by cells pretreated in choline chloride, when determined in the presence of 0.2 mM phloretin. (*b*): Beef erythrocytes were treated as described under *a*. Li^+ uptake, determined over 15 min, is normalized to 1 hr values on the ordinate. The open squares (□) refer to Li^+ uptake by cells pretreated with 10 mM iodoacetamide in choline chloride, when determined in the presence of 5 μM PCMBS

doacetate had the additional effect of inducing some cation leakage, irrespective of the medium composition.

A Na^+-activating effect can also be detected in the interaction of PCMBS with the exchange system of beef red cells. In the experiments depicted in Fig. 4, beef erythrocytes were preincubated with 5 μM PCMBS (i.e., 160 nmol/ml cells) in either choline or sodium chloride medium, and the reaction was stopped after various time intervals by adding 1.5 times this amount of glutathione. The cells were then washed and the rate of Li^+ uptake was determined in choline chloride medium. Unlike NEM, PCMBS induced an inhibition in choline medium, too, but the inhibitory action developed more rapidly in the Na^+ medium (*see* Fig. 4). It is to be noted that the maximum effect was not yet achieved after 2 min.

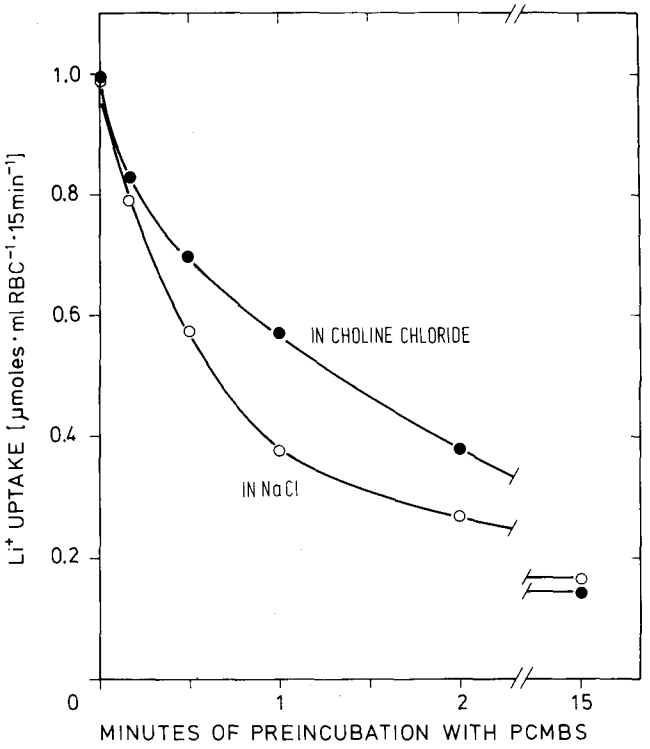

Fig. 4. The effect of extracellular Na^+ on the development of the inhibition of Li^+ uptake induced by PCMBS. Beef erythrocytes were suspended in isotonic choline or sodium chloride medium (37 °C, pH 7.4, hematocrit 3%). PCMBS (5 μM, 160 nmol/ml cells) was added and, at the time intervals given on the abscissa, the solutions were made 7.5 μM with respect to glutathione. The cells were washed four times in warm choline chloride and Li^+ uptake was determined as described in Materials and Methods

Na^+-Affinity of the Site Promoting NEM Binding

To study if Na^+ acts on the transport site itself to promote NEM binding, or on some other site, the dependence of the stimulation of NEM binding on the Na^+ concentration was determined. A difference in the affinity for Na^+ of the transport site and of the site promoting NEM binding would make an identity of the two sites unlikely.

Figure 5 depicts the development with time of the irreversible inhibition induced by NEM, as determined in media of various Na^+ concentration. In order to preclude the drop in NEM concentration during the experiment which would result from NEM binding to intracellular hemoglobin and glutathione, all cells were pretreated with NEM in choline

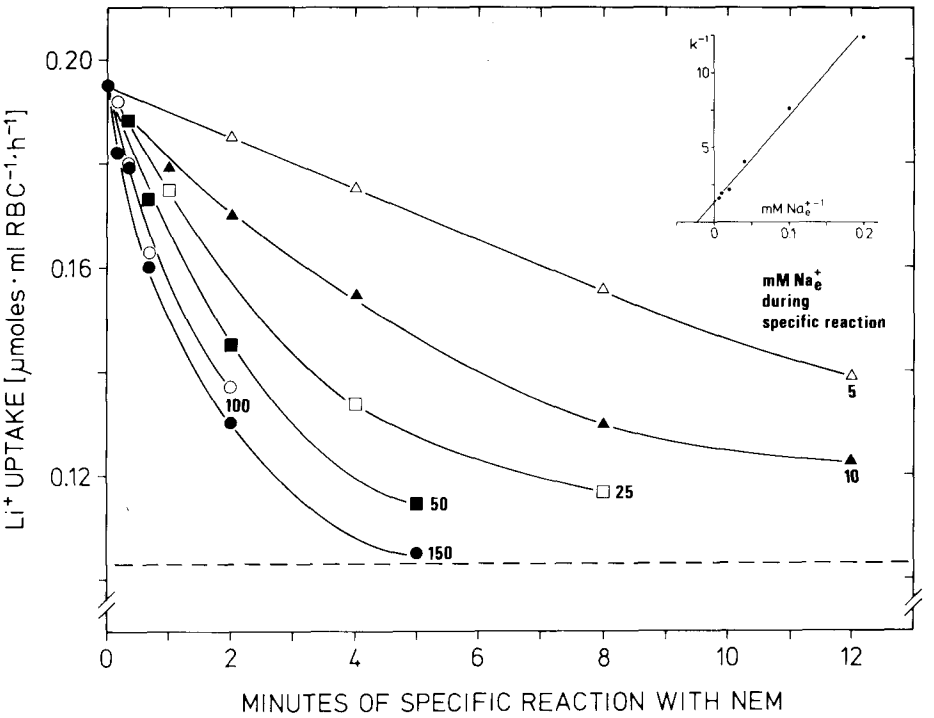

Fig. 5. Effect of the external Na^+ concentration on the development of the inhibition of Li^+ uptake induced by NEM in human erythrocytes. The red blood cells were "nonspecifically" reacted with NEM in choline chloride medium for 15 min (hematocrit 3%, 37 °C, pH 7.4, donor U.L.). After removing the residual NEM with 2-mercaptoethanol (2 mM) and washing, the "specific" reaction with NEM was conducted in media of the Na^+ concentrations given in the figure for the time intervals shown on the abscissa (choline chloride replaced by NaCl, 0.1 mM NEM, hematocrit 3%, 37 °C, pH 7.4). The "specific" reaction was stopped by 2-mercaptoethanol (2 mM) and washing, and Li^+ uptake was determined in KCl media as described in Materials and Methods. The broken line represents the inhibition attained when 0.1 mM NEM was additionally present during the Li^+ uptake period. The insert shows a double reciprocal plot of the rate constants (min^{-1}) for the development of inhibition *vs.* the external Na^+ concentration during the "specific" reaction. The rate constants were derived from semilogarithmic plots of the fractional inhibition *vs.* time of "specific" reaction. The apparent kinetic parameters obtained from the insert plot were a K_m value of 41 mM for Na^+ and a maximum rate of 0.72 min^{-1}

chloride. This "nonspecific" reaction was terminated with 2-mercaptoethanol. After washing, the cells were resuspended in mixtures of sodium and choline chloride and NEM was added to commence the "Na^+-specific" NEM binding. Following various contact times, the "specific" reaction was stopped by adding 2-mercaptoethanol again and washing. Finally, the remaining activity of the $Na^+ - Li^+$ exchange system was

assessed by measuring Li^+ uptake in potassium chloride medium. In a series of preliminary experiments the ratio of NEM to red cells had been determined (3,2 μmol/ml cells), which, during the "specific" reaction under the conditions specified in the legend of Fig. 5, induces optimum inhibition of the exchange system without opening a marked Li^+ leak (*see* Giebel & Passow, 1961).

Maximum inhibition (indicated by the broken line in Fig. 5) was obtained after 5 min in the presence of 150 mM Na^+. As the external Na^+ concentration was reduced, the rate of development of inhibition decreased, and at 5 mM Na^+, it already took about 10 min to achieve merely the half maximum effect. The insert in Fig. 5 shows a double reciprocal plot of the rate constants for the development of the inhibition *vs.* the external Na^+ concentration. The apparent K_m value of external Na^+ for the site promoting inhibition of the $Na^+ - Li^+$ exchange system by NEM derived from the plot is 41 mM. This value is in the range of the apparent K_m of 25–50 mM external Na^+ for the transport site (Duhm & Becker, 1977; Sarkadi *et al.*, 1978). It seems possible, therefore, that Na^+ promotes NEM binding by interacting with the transport site (*see* Discussion, p. 305).

Concentration Dependence of the Inhibitory Effect of PCMBS

To reduce the number of nonspecific PCMBS binding sites, beef erythrocytes were preincubated for 30 min with NEM in choline chloride (*see* Methods). The last 10 min of the preincubation were conducted in the presence of SITS (0.2 mM) to prevent subsequent access of PCMBS to the anion exchange system (Knauf & Rothstein, 1971 *b*). The unreacted reagents were removed by repeated washing. The cells were again suspended in choline chloride and PCMBS was added in the amounts indicated on the abscissa of Fig. 6. After 20 min of reaction, the suspensions were made 2 mM in Li^+ and the red cell Li^+ content was determined after a further 15 min of incubation. As demonstrated in the lower panel of Fig. 6, Li^+ uptake was not affected by PCMBS until an amount of 8 nmol/ml cells had been added (the PCMBS-binding capacity of the medium separated from the erythrocytes at the end of the uptake period was determined to correspond to about 10 nmol/ml cells). At higher PCMBS concentrations, the Li^+ uptake declined steeply, until a low value was reached at 26 nmol PCMBS/ml cells. With further addition of PCMBS, a slight additional drop in the rate of Li^+ uptake occurred.

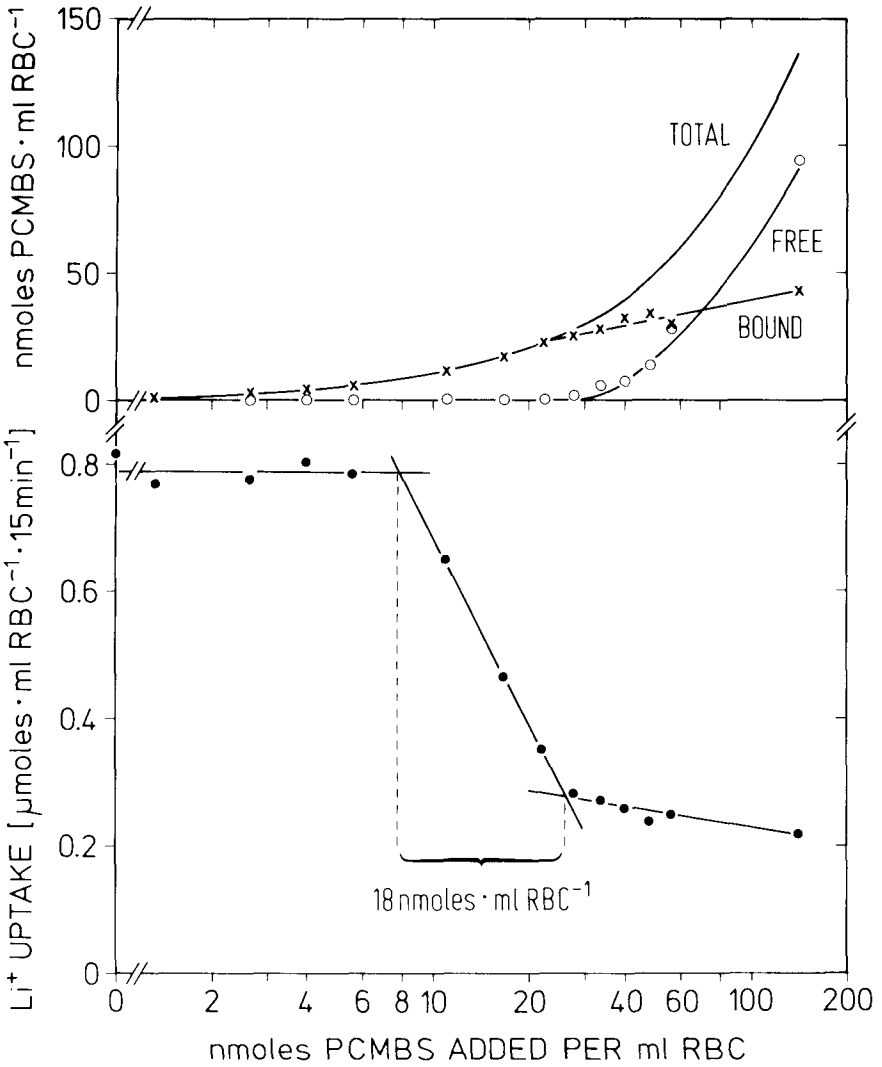

Fig. 6. *Lower panel:* Dependence of Li^+ uptake by beef erythrocytes on the amount of PCMBS added. The cells were "nonspecifically" reacted with 0.5 mM NEM for 30 min in choline chloride medium (37 °C, pH 7.4, hematocrit 3%). SITS (0.2 mM) was present during the last 10 min of the "nonspecific" reaction. The cells were then washed in warm choline chloride and resuspended in the same medium with the amounts of PCMBS shown on the abscissa. After 20 min of reaction with PCMBS, Li^+ uptake was initiated by adding LiCl to yield a concentration of 2 mM (0.05–5 μM PCMBS, hematocrit 3.6%, 37 °C, pH 7.4). *Upper panel:* Dependence of free and bound PCMBS on the amount of PCMBS added. The amount of free PCMBS was determined as described in Materials and Methods in the supernatant of the cell suspensions after termination of the Li^+ uptake shown in the lower panel. The amount of bound PCMBS was calculated by subtracting the free from the total PCMBS

Free PCMBS could be detected in the medium only when PCMBS was added in amounts exceeding 30 nmol/ml cells (upper panel of Fig. 6). Above this value, nearly all extra PCMBS appeared in the medium, whereas below this value the PCMBS was bound, either by the medium (about 10 nmol/ml cells; *see* van Steveninck *et al.*, 1965) or the membrane.

Protection from NEM Binding by PCMBS in Beef Erythrocytes

If NEM and PCMBS acted on the same group in inhibiting the $Na^+ - Li^+$ exchange system of beef erythrocytes, then PCMBS should afford some protection from the irreversible, Na^+-specific inhibition caused by NEM. Figure 7 illustrates this on beef red cells "specifically" reacted in NaCl with PCMBS and NEM alone, or successively with PCMBS and NEM. After removal of the bound and free PCMBS and the free NEM by a 1-hr incubation with an excess of 2-mercaptoethanol, the residual activity of the exchange system was assessed. Panel *I* of Fig. 7 shows Li^+ uptake by cells not specifically reacted in NaCl and its inhibition by PCMBS and NEM. Panel *II* demonstrates that the action of PCMBS is fully reversed by 2-mercaptoethanol. Glutathione, even when applied in an 100-fold excess for 1 hr, was not capable of relieving the inhibition induced by PCMBS (not shown). The action of NEM was irreversible (panel *III*). In cells first reacted with PCMBS and then with NEM, the access of NEM to the inhibitory site was partly occluded. The residual inhibition in such cells was substantially lower than that resulting from contact with NEM alone for the same length of time (*compare* the left hand columns in panels *IV* and *III* of Fig. 7).

Loss of Inhibitor Action Following Oxidation with Di (1,10-phenanthroline)-Copper (II)/2-Mercaptoethanol

The effect of inhibitors of $Na^+ - Li^+$ exchange was examined on human and beef erythrocytes oxidized for one hour with either di(1,10-phenanthroline)-copper (II) complex (CuP) alone, or in combination with 2-mercaptoethanol. The cells had been previously incubated with NEM in choline chloride to reduce membrane protein cross-linking and the subsequent rise in cation leak induced by the oxidizing agent. Neverthe-

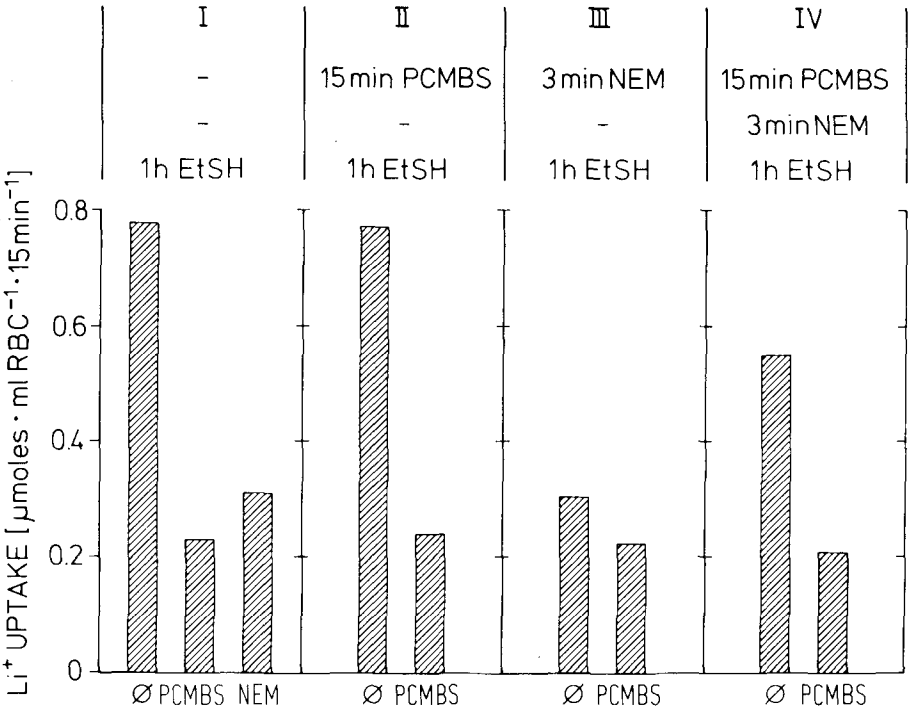

Fig. 7. Protection from NEM binding by PCMBS in beef erythrocytes. The red cells were nonspecifically reacted with 0.5 mM NEM in choline chloride medium for 30 min (hematocrit 3%). After 4 washings, the cells were resuspended in isotonic NaCl medium and treated with PCMBS (25 μM), NEM (0.2 mM) and 2-mercaptoethanol (EtSH, 2 mM) in the order shown at the top of the figure (hematocrit 3%). Subsequently, the cells were washed again 4 times in warm choline chloride. Finally, the erythrocytes were resuspended in choline chloride and the Li^+ uptake was determined in the absence (columns indicated by Ø) or presence of PCMBS (5 μM) or NEM (0.5 mM), as described in Materials and Methods. One of three similar experiments

less, the inhibition of $Na^+ - Li^+$ exchange eventually caused by the treatments could not be determined directly, because there was a residual increase in passive Li^+ leakage.

As can be seen from the data of Table 3, pretreatment with CuP alone did not lead to a marked reduction of the inhibitory action of NEM and phloretin in cells of two human donors, and PCMBS retained its effectivity in beef red cells. When the oxidation was carried out in the presence of 2-mercaptoethanol, however, all three inhibitors suffered a loss in potency.

Table 3. Loss of inhibitory action of NEM, phloretin, and PCMBS on $Na^+ - Li^+$ exchange in cells oxidized with di (1,10-phenanthroline)-copper II (CuP) in the presence of 2-mercaptoethanol[a]

Inhibitor	(mM)	Erythrocytes	Inhibitory effect (% of control) cells pretreated with	
			CuP	CuP + 2-Mercaptoethanol
NEM	(0.1)	Donor E.H.	100[b]	31
Phloretin	(0.1)	Donor U.L.	92[c]	65
PCMBS	(0.025)	Beef	95[d]	76

[a] The cells were first reacted nonspecifically with NEM in choline chloride (*see* Methods). Oxidation with 34 μM CuP complex (Huang & Richards, 1977) was performed in NaCl medium (1 hr, 37 °C, pH 7.4, hematocrit 3%) in the absence and presence of 2-mercaptoethanol (2 mM). After washing the cells three times in warm choline chloride, Li^+ uptake was determined in the absence and presence of the inhibitors, as described in Materials and Methods. Control cells were not reacted with CuP.

[b] 100% inhibitory effect (0.056 μmol Li^+ uptake/ml cells × hr) ≙ 42% of control uptake.

[c] 100% inhibitory effect (0.117 μmol Li^+ uptake/ml cells × hr) ≙ 55% of control uptake.

[d] 100% inhibitory effect (0.70 μmol Li^+ uptake/ml cells × 15 min) ≙ 68% of control uptake.

Location of the Reactive SH-Group in the Membrane

To examine whether the SH-group essential for $Na^+ - Li^+$ exchange is easily accessible from the inside of the membrane, experiments were conducted on beef red cell ghosts. The ghosts were loaded with Na^+ and suspended in K^+ media to which 1 mM Li^+ was added. According to the cellular Na^+ and K^+ contents and the Li^+ ratio, Li^+_i/Li^+_e, measured 3 min subsequent to Li^+ addition (about 55 and 100 μmol/ml ghosts and 0.6, respectively), only 30–40% of the ghosts obtained were tight towards the three cations. Nevertheless, in the control ghosts (curve *1* in Fig. 8), the Li^+ ratio increased up to 2.4 within 2 hr. This corresponds to a ratio of 4.5 in the tight ghosts when the ratio is one in the 60% leaky ghosts. If ghosts were prepared from erythrocytes pretreated for 15 min in Na^+ medium with 830 nmol PCMBS or 17 μmol NEM/ml cells, the establishment of the Li^+ gradient was markedly reduced (curves *6* and *7*, respectively). The PCMBS effect was abolished when 2 mM 2-mercaptoethanol was present during hemolysis and resealing (curve *3* in Fig. 8), whereas 2 mM glutathione was almost ineffective (*compare* curves *4* and *6*). Addition of 20 μM 1,6-hexane dimaleimide to the resealed ghosts reduced Li^+ uptake markedly (curve *5*), whilst its impermeant,

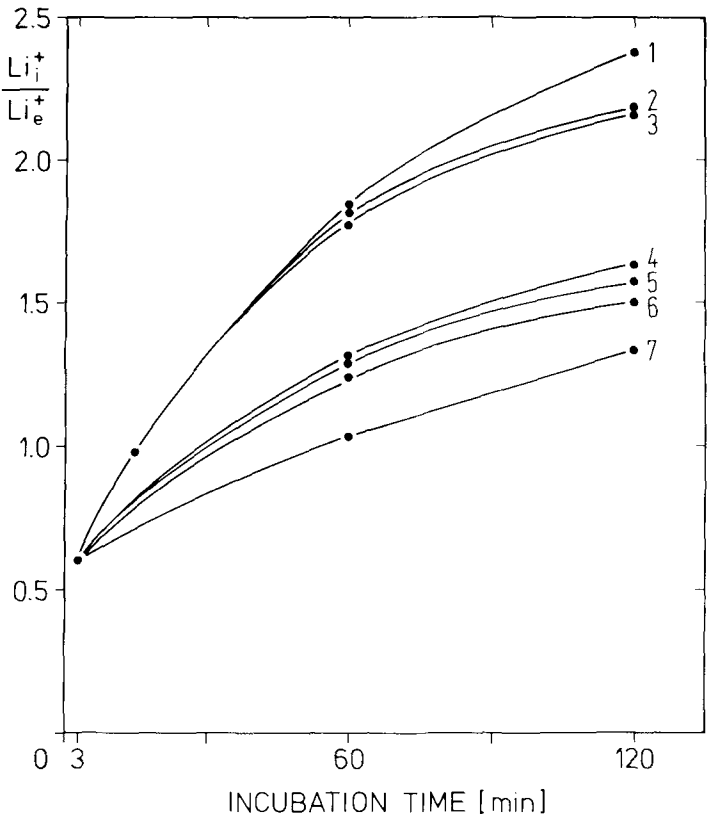

Fig. 8. The effect of various treatments on the accumulation of Li^+ by beef erythrocyte ghosts. Beef red cell ghosts, loaded with Na^+ as described in Materials and Methods, were suspended in isotonic KCl media containing 5 mM tris (hydroxymethyl)-amino methane, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM LiCl and 0.1 mM ouabain at 37 °C, pH 7.4 and a ghostocrit of 12–24%. The ratio of internal Li^+ (μmol/ml ghosts) to external Li^+ (mM) established by the ghosts is plotted *vs.* the incubation time. (*1*): Control ghosts. (*2*): Ghosts prepared and resealed in the presence of 1.3 mM 1-maleimido-6-(2-glutathionesuccinimido)-hexane. (*3*): Ghosts prepared from erythrocytes pretreated with 25 μM PCMBS for 15 min in NaCl (hematocrit 3%) and hemolyzed and resealed in the presence of 2 mM 2-mercaptoethanol. (*4*): Ghosts prepared from erythrocytes pretreated with 25 μM PCMBS for 15 min in NaCl (hematocrit 3%) and hemolyzed and resealed in the presence of 2 mM glutathione. (*5*) Control ghosts, Li^+ uptake determined in the presence of 20 μM 1,6-dimaleimido hexane. (*6*) Ghosts prepared from erythrocytes pretreated with 25 μM PCMBS for 15 min in NaCl (hematocrit 3%). (*7*) Ghosts prepared from erythrocytes pretreated with 0.5 mM NEM for 15 min in NaCl (hematocrit 3%)

monofunctional glutathione derivative did not inhibit Li^+ accumulation, even when present in 1.3 mM concentration during hemolysis and resealing (curve *2*). These results indicate that the SH-group is not located

superficially at the inside of the membrane and that it is only reactive towards reagents capable of reaching the interior of the membrane.

Discussion

The present experiments on the susceptibility of the $Na^+ - Li^+$ exchange system in human and beef erythrocytes towards sulfhydryl reagents indicate that a thiol group is essentially involved in the transport process. The same has been reported by Motais and Sola (1973) for ouabain-resistant $Na^+ - Na^+$ exchange in these erythrocytes. This similarity has lent further support to the idea that the systems mediating ouabain-resistant $Na^+ - Na^+$ and $Na^+ - Li^+$ exchange are identical (Duhm & Becker, 1979).

The transport of several molecular species across the red cell membrane can be inhibited by SH-reagents, e.g., the transport of glucose (Dawson & Widdas, 1963; van Steveninck *et al.*, 1965; Smith & Ellmann, 1973; Batt, Abbott & Schachter, 1976), choline (Edwards, 1973), and monocarboxylates (Deuticke, Rickert & Beyer, 1978), as well as the ouabain-sensitive $Na^+ - K^+$ pump (Rega, Rothstein & Weed, 1967; Chan & Rosenblum, 1969; Glynn, Hoffman & Lew, 1971; Richards, Rega & Garrahan, 1977). All these transport processes are commonly attributed to the action of membrane-spanning proteins. Thus, it appears reasonable to assume, at the present state of knowledge, that the ouabain-resistant $Na^+ - Li^+$ (and $Na^+ - Na^+$) exchange is mediated by a membrane protein, and that the inhibitory effects of the SH-reagents studied (*see* Table 1) are the direct result of a protein modification.

Among the SH-reagents inhibiting Na^+-Li^+ exchange, NEM and iodoacetamide are also capable of alkylating primary amino groups and imidazoles, but the reaction with SH-groups generally occurs much faster (Smith, Blumenfeld & Konningsberg, 1964; Webb, 1966; Brewer & Riehm, 1967). Furthermore, PCMB, PCMBS, and $HgCl_2$, at the low concentrations applied, can be considered as being specific reactants, forming mercaptides with free thiols (Benesch & Benesch, 1961; Webb, 1966). Taking also into account the protection afforded by PCMBS against the inhibition through NEM (Fig. 7), it is concluded that the effects of the "SH-reagents" result, in fact, from an interaction with an SH-group (or groups). Of additional support for this assumption is the observation that a loss of inhibitor action and a partial inactivation

of the exchange system could be attained by oxidation with copper orthophenanthroline in the presence of the thiol 2-mercaptoethanol (Table 3), a treatment expected to lead to the formation of mixed protein-mercaptoethanol disulfides (Huang & Richards, 1977). It would appear that the essential SH-group is not located in the immediate vicinity of other intrinsic thiols suited for cross-linking, because copper ortho-phenanthroline alone was without effect on $Na^+ - Li^+$ exchange (*see* Table 3).

The reactivity of the SH-group towards sulfhydryl reagents is greatly enhanced by the presence of Na^+ or Li^+ in the medium, i.e., by the cations which are specific "substrates" of the exchange system. Such a cation-induction has been observed for NEM (Figs. 1, 2 and 5), 1,6-hexane dimaleimide (Table 2), iodoacetamide (Fig. 3), and iodoacetate. The inhibition arising from PCMBS was also accelerated by external Na^+ (Fig. 4). This dependence of reactivity on substrate concentration is in accordance with the general phenomenon that enzymes or transport proteins undergo a conformational change upon substrate binding, and that these changes frequently alter the reactivity of functional groups, either at the substrate binding site itself, or at a remote site.

The observation that prolonged incubation in Na^+ medium induces inactivation of $Na^+ - Li^+$ (and hence, of ouabain-resistant $Na^+ - Na^+$) exchange by iodoacetamide (*see* Fig. 3) has an important inference to the practice of applying iodoacetamide to deplete red cells of ATP (*see*, e.g., Glynn *et al.*, 1971; Lew, 1971; Beaugé, 1975). At least in studies concerning the effect of ATP depletion on erythrocyte Na^+ and Li^+ movements, iodoacetamide (and mono-iodoacetate) should obviously not be indiscriminately employed, particularly not in high Na^+ media. Certain precautions may also be necessary in the interpretation of studies on Na^+ and Li^+ transport with erythrocytes, the cation content of which has been altered by pretreatment with PCMBS (Garrahan & Rega, 1967).

The inhibition arising from NEM reached a maximum value within 3–5 min of contact at each 150 mM Na^+ and 5 mM Li^+ (Figs. 1 and 2). The ratio of these equipotent cation concentrations corresponds to the ratio of the apparent K_m values of the transport site for external Na^+ and Li^+ (about 30; Duhm & Becker, 1977, Sarkadi *et al.*, 1978). Furthermore, the affinities of Na^+ to the external transport site (apparent $K_m = 25$–50 mM; Duhm & Becker, 1977, Sarkadi *et al.*, 1978) and to the site promoting NEM binding (apparent $K_m = 41$ mM external Na^+, *see* Fig. 5) are similar. It would thus appear that the transport site is identical with the site promoting the binding of SH-reagents. Alternatively, a modifier site with accidentally similar cation affinities must be assumed.

Experiments performed to evaluate if the action of SH-reagents is perhaps also influenced by internal Na^+ (or Li^+) revealed that raising the internal Na^+ concentration from 4 to 60 mM does not markedly alter the development of the inhibition due to NEM in choline media containing 25 mM Na^+ (results not shown). It can still not be decided, however, whether the action of external Na^+ and Li^+ permitting reaction of the SH-reagents requires a complete exchange cycle, or results alone from a conformational change associated with the binding of the cations at the externally oriented transport site (or at an external modifier site).

An attractive interpretation of the results would be, at first sight, that the SH-group reacting with the SH-reagents is the transport site itself. However, none of the SH-reagents tested was capable of inducing full inhibition of the exchange (Table 1). If the essential SH-group were part of the cation binding locus, there is no apparent reason why it should not react to 100% with the SH-reagents at the concentrations and exposure times applied. It is not feasible to postulate the existence of more than one population of transport sites (*see* Motais & Sola, 1973), because the apparent affinity of the exchange system for external Li^+ was unaltered in the presence of the inhibitory SH-reagents (Funder & Wieth, 1978; Duhm & Becker, 1979). Another argument concerns the pK value of the key binding group, which, according to the pH-dependence of the exchange, should lie close to pH 6.5 (*see* Motais, 1973; Duhm & Becker, 1979). This pK value appears too low for a free SH-group. The possibility that the pK is anomalously lowered, for example, due to the neighborhood of basic groups (*see* Polgár, 1975; Halász & Polgár, 1977), cannot be supported, since experimental evidence for the involvement of such groups has not been found. Furthermore, the charged mercurials PCMBS, PCMB, and Hg^{2+} inhibit $Na^+ - Li^+$ exchange in beef, but not in human red blood cells. This difference in reactivity is indicative of a more hydrophobic environment of the essential SH-group in human erythrocytes. If the SH-group were the cation binding site itself, then it would be difficult to understand why the group, located in a different environment in the beef and human erythrocyte membrane, would exhibit identical affinities for Na^+ (and Li^+) in the two species (Funder & Wieth, 1978; Duhm & Becker, 1979). According to these considerations, it appears more likely that the SH-group essential for $Na^+ - Li^+$ exchange is located at a site distinct from the cation binding locus, its reaction with SH-reagents reducing the turn-over number of the transport protein, but leaving the affinity of the transport site(s) for the cations unaltered.

Motais and Sola (1973) have suggested that the SH-group is superficially located at the outside of the beef red cell membrane. However, the reaction with PCMBS took several minutes to come to completion (Fig. 4), whilst a few seconds suffice for inhibition of the monocarboxylate carrier in mammalian erythrocytes by PCMBS, the interaction also being attributed to a superficially located SH-group (Deuticke *et al.*, 1978). The ready explanation would be that the reaction with PCMBS is slowed by diffusion barriers, such as have been postulated by Sutherland, Rothstein and Weed (1967) to exist for PCMBS between a compartment within the membrane and the medium and cell interior, respectively. Access of PCMBS to the cell interior is probably not rate limiting, since the presence of 0.2 mM SITS, which precludes the more rapid entry of PCMBS into the cells via the anion exchange pathway (Knauf & Rothstein, 1971*b*), did not slow the development of the inhibition of Li^+ uptake by PCMBS in experiments similar to those described in Figs. 4 and 6 (results not shown). Furthermore, the impermeable, monofunctional glutathione derivative of 1,6-hexane dimaleimide was unable to inhibit the exchange system, be it from the outside (Table 1) or from the inside of the membrane (Fig. 8), and neither internal nor external glutathione could relieve the inhibition induced by PCMBS, in strong contrast to the reversibility found with the penetrating thiols 2-mercaptoethanol and dithiothreitol (*see* Results and Figs. 7 and 8). The conclusion to be drawn is that the SH-group is not superficially located at the outside or inside of the membrane, a location within the membrane seeming more appropriate (*compare* van Steveninck *et al.*, 1965; Shapiro, Kollman & Martin, 1970; Knauf & Rothstein, 1971*a*; Naccache & Sha'afi, 1974; Grinstein & Rothstein, 1978).

The results shown in Fig. 6 allow for an upper estimate of the number of SH-groups per beef red cell involved in the PCMBS-induced inhibition of the $Na^+ - Li^+$ exchange system. Development of near maximum inhibition was associated with the disappearance of 18 nmol PCMBS per ml of cells. From the number of 1.72×10^{10} cells per ml of beef erythrocytes (estimated from data of M.M. Wintrobe (1933) cited by Rich *et al.*, 1967), one arrives at 6.3×10^5 groups per cell as the upper limit for the number of SH-groups partaking in the inhibition of the system (or the upper number of transport molecules, each carrying one essential SH-group). According to this value and the maximum activity of the exchange system of 7–15 μmoles/ml cells × hr (Motais, 1973; Funder & Wieth, 1978; Duhm & Becker, 1979), the turnover number of the system would be 0.1–0.2 per second per transport molecule. This

value is unreasonably low. The number of 6×10^5 sites per cell is, moreover, in the range of total PCMBS binding sites reported to be accessible in the membrane of intact human erythrocytes ($5–8 \times 10^5$, van Steveninck *et al.*, 1965; $14 \pm 2 \times 10^5$, Sutherland *et al.*, 1967). It appears more attractive to assume that the number of transport molecules is two to three orders of magnitude below the value of 6×10^5 per beef red cell and still even lower in human erythrocytes (*see* Duhm & Becker, 1979). Although the $Na^+ - Li^+$ exchange protein may then contribute with less than 0.1% to the total mass of membrane proteins, it might, nevertheless, be possible to identify it by differential labeling techniques, applying a Na^+- or Li^+-specific binding of SH-reagents as described in this paper.

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