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ERYTHROCYTE Li_i-Na_o COUNTERTRANSPORT SYSTEM

INHIBITION BY *N*-ETHYLMALEIMIDE PROBES FOR A CONFORMATIONAL CHANGE OF THE TRANSPORT SYSTEM

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Human erythrocytes were treated by a series of SH-reagents, including maleimides, iodo compounds, mercurials and oxidizing agents. Rates of Li efflux into Na-rich medium, Li leak and Li_i-Na_o countertransport were then determined. Of the 13 different reagents studied, only *N*-ethylmaleimide, iodoacetamide and iodoacetate inhibited selectively the countertransport activity. The effect of the various reagents indicates that the sensitive SH-groups of the countertransport system are not externally exposed. *N*-Ethylmaleimide was used to probe for changes elicited by substrate cations in Li_i-Na_o countertransport. In Na- and Li-free medium, inhibition of Li_i-Na_o countertransport by *N*-ethylmaleimide of 35% was reached within 2 s. In Na or Li medium, maximal inhibition was twice as great, but was attained much more slowly, within 10 min. Kinetic data and Hill plot analysis indicate the involvement of two classes of SH-groups: one expressed in the various media with and without substrate cations, and an additional one, which becomes specifically available to *N*-ethylmaleimide in the presence of external Na or Li. The affinity of Na to the site promoting inhibition by *N*-ethylmaleimide (apparent K_m = 12 mM) is higher than the affinity of Na to its external countertransport site (apparent K_m = 25 mM, as reported by Sarakadi, B., Alifimoff, J.K., Gunn, R.B. and Tosteson, D.C. (1978) J. Gen. Physiol. 72, 249–265). Reactivity of *N*-ethyl[^{14}C]maleimide was not modified by the media tested. It is concluded that external Na and Li cause a conformational change in the protein(s) of the countertransport system in human erythrocytes.

Introduction

Alterations in rates of Li_i-Na_o countertransport have been detected repeatedly in erythrocytes of patients with essential hypertension [1–8]. In view of the significance of hypertension [9] and the potential usefulness of this transport system for diagnosis and prediction of the disease [1,2], the detailed characterization of Li_i-Na_o countertransport is of interest and importance.

The Li_i-Na_o countertransport system of human erythrocytes mediates a one-to-one exchange of Li for Na and permits uphill or downhill Li transport in both directions across the membrane [10,11]. The system exhibits Michaelis-Menten type kinetics with a high affinity for Li and about 20-times lower affinity for Na [12,13]. It is not inhibited by

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Abbreviations: 1,5-AEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; 1,8-AEDANS, *N*-iodoacetyl-*N'*-(8-sulfo-1-naphthyl)ethylenediamine; Diamide, diazinedicarboxylic acid bisdimethylamide; MBTA, 4-(*N*-maleimido)benzyltrimethylammonium iodide; Mops, 4-morpholinepropanesulfonic acid; PCMB, *p*-chloromercuribenzoic acid; PCMBS, *p*-chloromercuribenzenesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

ouabain or ATP depletion [10,11], but is completely blocked by phloretin [12,14]. The thermodynamic properties of the Li_i-Na_o countertransport systems are intriguing, since there is a marked difference, of aout 10 °C, in the values of the 'break' in Arrhenius plots of the Li_i-Na_o exchange, between normotensives and most of the hypertensives [8,15].

Various SH-reagents have been used effectively to characterize membrane transport proteins [16–23]. This approach appears promising for further characterization of Li_i-Na_o countertransport in erythrocytes, as the system is sensitive to certain SH-reagents, most notably to *N*-ethylmaleimide [15,24–27]. We have already shown that Li_i-Na_o countertransport is resolved into *N*-ethylmaleimide-sensitive and -insensitive components, which differ in their thermodynamic properties [15]. *N*-Ethylmaleimide was used in the present study to probe for conformational changes in the Li_i-Na_o countertransport protein by the substrate cations. In agreement with an earlier study based on Li-uptake measurements [26], it is indeed demonstrated that the substrate cations modify the sensitivity of the transport system to *N*-ethylmaleimide, apparently by the conformational change. Differences in sensitivity to *N*-ethylmaleimide observed when Li uptake [26] or Li_i-Na_o fluxes are measured, suggest an asymmetry of the Li-Na countertransport system.

Methods

Solutions. (1) Na-rich medium contained 150 mM NaCl, 10 mM glucose, 0.1 mM ouabain and 10 mM Tris-Mops (pH 7.4); (2) Mg-sucrose medium ('Na-free medium') – same medium as (1) but NaCl is replaced by 75 mM $MgCl_2$ and 85 mM sucrose; (3) K medium – same medium as (1) but NaCl is replaced by 150 mM KCl; (4) Li medium – same medium as (1) with NaCl replaced by 150 mM LiCl; (5) washing solution contained 75 mM $MgCl_2$, 85 mM sucrose, 10 mM glucose and 10 mM Tris-Mops (pH 7.4).

Determination of lithium efflux. Blood, anticoagulated with heparin (25 units/ml), was drawn from healthy donors and processed within 30 min. Separation of red blood cells, loading the cells with Li and measurements of Li efflux were as described by Canessa et al. [1]. Efflux was performed simultaneously into Na-rich medium and into the Mg-sucrose medium at 32 °C. Lithium was determined by means of an atomic absorption spectrophotometer (Perkin Elmer Model 2380), and calibrated by standards corresponding to the medium used. The flux was computed from the linear regression of Li loss within 30 min. The differences between the rate of lithium efflux into sodium-rich medium and Mg-sucrose medium were taken as countertransport.

For the measurements of temperature dependence of the Li_i-Na_o countertransport, a graded temperature device was used to allow the simultaneous assays of Li efflux at the desired temperatures in the rane of 15–40 °C with 1 to 2 °C increments [8]. The pH of the medium varied from 7.38 (at 15 °C) to 7.24 (at 40 °C). The effect of this difference in pH on Li efflux rate when tested at constant temperature was found to be negligible, within the experimental error.

Reactivity of SH reagents. Stock solutions of SH-reagents were freshly prepared and their effective concentration was determined by a reaction with a defined quantity of glutathione. The complete assay mixture contained: 50 mM phosphate buffer (pH 7.4), 0.13 mM glutathione and the tested reagent at 50–100 μM. Following an incubation (10 min for *N*-ethylmaleimide, 30 min for the other reagents), 5,5′-dithiobis(2-nitrobenzoic acid) was added at 0.33 mM. The quantity of glutathione left unreactive was measured spectrophotometrically at 412 nm, based on $\epsilon_{mM} = 13.6$ for thionitrobenzoate [28].

Treatment with SH-reagents. Erythrocytes (5% hematocrit) were treated with *N*-ethylmaleimide for various durations at 4 °C in one of the following media: Na-rich medium, Mg-sucrose, K medium, or Li medium. Details about the media composition are described above, under 'solutions'. The treatment was stopped with 10-fold excess dithiothreitol and the cells were washed three times with the washing solution prior to the assay. The treatment with the other SH-reagents was similarly conducted, with several changes: Na-rich medium was used exclusively and the treatment lasted for 30 min. The treatment with mersalyl was for 5 min or more. PCMBS and PCMB were added directly to the assay mixture. After the addition of di-

thiothreitol, suspensions were centrifuged, the supernatant was removed and the cells were assayed.

Preparation of erythrocyte ghosts. Ghosts of human erythrocytes were prepared essentially according to the method of Dodge et al. [29]. The red cells were suspended in 0.1 M sodium phosphate buffer (pH 8) at a hematocrit of 20% and kept for 10 min in ice. The cells were then lysed with 15 vol. of the diluted buffer (10 mM) at 2–4°C. The suspensions were centrifuged at $20000 \times g$ for 20 min at 4°C and the pellets were washed twice more in cold 5 mM sodium phosphate buffer. Protein concentration was determined by the method of Lowry et al. [30] with bovine serum albumin as standard. Hemoglobin was determined according to the method of Drabkin [31]. The resultant ghost suspensions contained less than 1% hemoglobin on protein basis.

Statistical analysis. The differences in means were analyzed by the *t*-test. The plots were drawn as least square regression lines and tested by analysis of variance.

Materials. MBTA was a generous gift of Dr. A. Karlin, Columbia University, College of Physicians and Surgeons. Eosin-5-maleimide was obtained from Molecular Probes, Plano, TX. Monobromotrimethylammoniobimane and diamide (diazinedicarboxylic acid bisdimethylamide) were obtained from Calbiochem. All the other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Results

Effect of alkylating and oxidizing agents

Li_i-Na_o countertransport was determined by measurements of Li efflux in Na-rich medium and Na-free medium. In attempts to study the role of SH-groups in these Li efflux pathways, several alkylating and oxidizing agents have been examined. Altogether, 13 compounds were tested. Of the maleimides examined, only *N*-ethylmaleimide was inhibitory. At 1 mM, it inhibited 75% of Li_i-Na_o countertransport activity, while Li efflux in Na-free medium was not affected. Eosin-5-maleimide or MBTA at 1 mM were not inhibitory. The mercurials PCMB and mersalyl (0.1 mM) increased the Li leak in Na-free medium, but did not affect the net Li_i-Na_o countertransport. PCMBS (1 mM) did not affect Li efflux at all. Diamide at 1 mM increased both the passive permeability to Li and Li_i-Na_o countertransport. When the erythrocytes were first treated with *N*-ethylmaleimide for 10 min and then with diamide, the countertransport rate was equal to that of the control. The nonpenetrating agents 5,5′-dithiobis(2-nitrobenzoic acid) (1 mM), monobromotrimethylammoniobimane (1 mM), 1,5-AEDANS (1 mM) and 1,8-AEDANS (1 mM) were not inhibitory. Iodoacetate and iodoacetamide inhibited Li_i-Na_o countertransport selectively, like *N*-ethylmaleimide, with no effect on Li efflux in Na-free medium. Yet, maximal inhibition of Li efflux by *N*-ethylmaleimide was reached at a concentration of 1 mM (20 μmol/ml erythrocyte) within 10 min, whereas comparable maximal inhibition by iodoacetate and iodoacetamide required a longer period (30 min) and a higher concentration (10 mM), as shown in Fig. 1.

Fig. 2 compares the effects of iodoacetamide and iodoacetate in terms of the temperature dependence of the Li_i-Na_o countertransport. The countertransport can be resolved into components sensitive and insensitive to these alkylating agents, as already shown for the effect of *N*-ethylmaleimide [15]. The sensitive component exhibited a typical biphasic temperature response with a 'break' at 30°C as in the control Arrhenius plot.

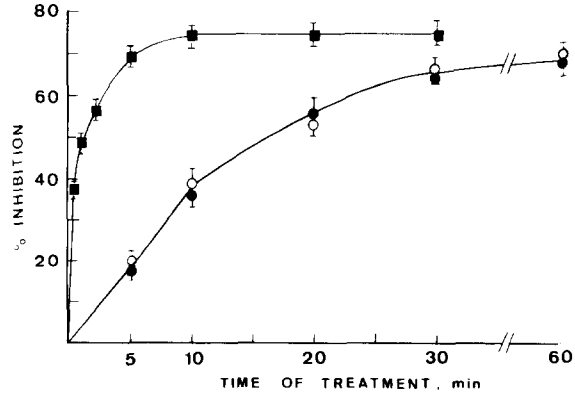

Fig. 1. Inhibition of Li_i-Na_o countertransport activity as affected by the duration of treatment with either 1 mM *N*-ethylmaleimide (■), 10 mM iodoacetamide (●) or 10 mM iodoacetate (○). The treatments were performed in Na-rich medium. The values are the average ± S.E. of ten experiments for the treatment with *N*-ethylmaleimide and of three experiments for the treatments with iodo compounds.

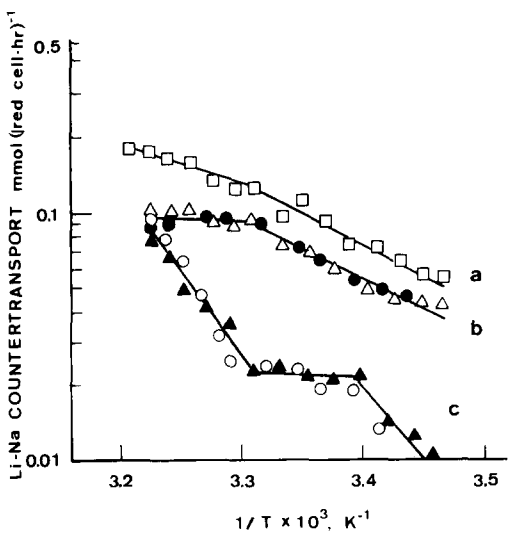

Fig. 2. Li_i-Na_o countertransport in erythrocytes as affected by treatment with iodoacetamide (△, ▲), iodoacetate (○, ●) and temperature. a, Untreated control (□); b, sensitive efflux; c, insensitive efflux. Average of three experiments is shown.

In contrast, the insensitive component exhibited a triphasic temperature dependence. Li efflux in Na-free medium was not affected by iodoacetate or iodoacetamide throughout the entire temperature range studied (not shown).

Since *N*-ethylmaleimide was a more potent inhibitor of Li efflux in human erythrocytes than any of the compounds tested, it was chosen for a detailed study.

Li efflux sensitivity to N-ethylmaleimide affected by external cations

Fig. 3 presents the inhibition of Li efflux in erythrocytes pretreated with *N*-ethylmaleimide in different media for various durations. After removal of *N*-ethylmaleimide, residual Li efflux was measured in Na medium (panel A) or as countertransport (panel B). When 1 mM *N*-ethylmaleimide was applied in the presence of isotonic NaCl or LiCl solutions, the inhibition proceeded gradually, reaching a maximum of 80% of the countertransport within 10 min. In contrast, the inhibition caused by *N*-ethylmaleimide in isotonic solutions of KCl or $MgCl_2$-sucrose reached a maximal level of only 33%, but more rapidly.

A comparison of *N*-ethylmaleimide treatment, applied for 10 min before or after the loading of Li, is presented in Table I. A similar inhibition of the efflux rates is evident.

Since Li efflux in Na-free medium was not modified at all by *N*-ethylmaleimide, the effect of *N*-ethylmaleimide on the efflux in Na medium and on Li_i-Na_o countertransport was qualitatively similar (Table I, Fig. 3). Therefore, the data presented henceforth will be restricted the to Li_i-Na_o countertransport.

Fig. 4 shows that the inhibition caused by treatment with *N*-ethylmaleimide in Mg-sucrose medium was enhanced by the addition of Na during treatment. The enhancement of the inhibitory effect of *N*-ethylmaleimide was similar when Na was added from the onset of the *N*-ethylmaleimide treatment or 5 min later. The same

TABLE I

EFFECT OF *N*-ETHYLMALEIMIDE APPLIED BEFORE OR AFTER Li LOADING ON ERYTHROCYTE EFFLUX

(a) *N*-Ethylmaleimide treatment applied before Li loading. The loading, with $LiCO_3^-$, was as described by Ibsen et al. [32], to a final cellular concentration of 8.5 mM Li. (b) *N*-Ethylmaleimide treatment applied after Li loading, as described in Methods. Rate in control (untreated cells), in mmol Li/l erythrocyte per h: (a) Li efflux in Na-rich medium: 0.68 ± 0.06, Li_i-Na_o countertransport: 0.56 ± 0.05; (b) Li efflux in Na-rich medium: 0.69 ± 0.06, Li_i-Na_o countertransport: 0.49 ± 0.07. The differences between (a) and (b) are not signifcant. The results presented are the average ± S.E. of four experiments, each in triplicate.

Medium for *N*-ethylmaleimide treatment	% Inhibition			
	Li efflux into Na-medium		Li_i-Na_o countertransport	
	(a)	(b)	(a)	(b)
Na-rich	59 ± 6	55 ± 6	74 ± 7	76 ± 5
Mg-sucrose	24 ± 7	22 ± 4	37 ± 8	32 ± 6

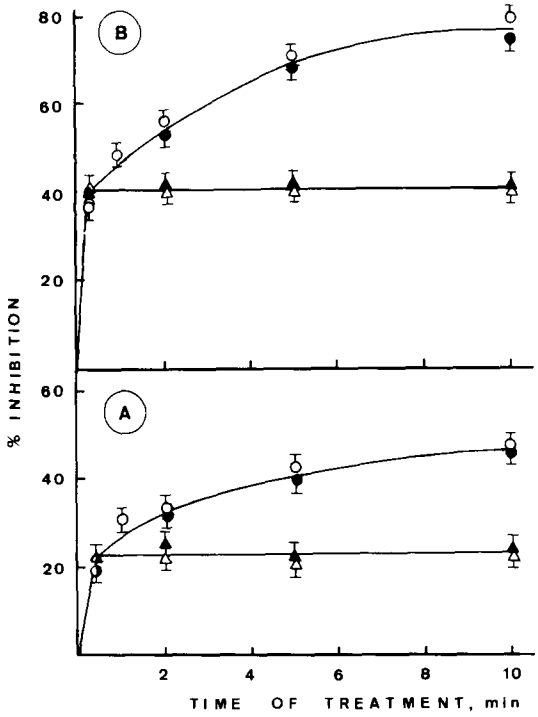

Fig. 3. The effect of time of treatment with 1 mM *N*-ethylmaleimide in different media on the inhibition of: (A) Li efflux into Na-rich medium; (B) Li_i-Na_o countertransport. Na medium (○), Li medium (●), Mg-sucrose medium (△), K medium (▲). The control rate, in mmol Li/l erythrocyte per h, for Li efflux into Na-rich medium: 0.71 ± 0.05, and for Li_i-Na_o countertransport: 0.49 ± 0.06. Average values ± S.E. of ten experiments are presented.

pattern of results was obtained when the treatment with *N*-ethylmaleimide was performed at low Na concentration, as presented in Table II. It is shown that the effects of *N*-ethylmaleimide in the presence and in the absence of Na are not additive.

Kinetic aspects

Fig. 5 demonstrates the effect of Na concentration on the inhibition of Li_i-Na_o countertransport by *N*-ethylmaleimide. The concentration of Na that caused half the enhancement of the inhibition by *N*-ethylmaleimide, the apparent K_m, was 12.0 mM. The inhibition of Li_i-Na_o countertransport in erythrocytes treated with *N*-ethylmaleimide in Na medium was increased, both in rate and in extent, as a function of the concentration of the reagent in the range of 10^{-4} to 10^{-3} M (Fig. 6). The approach used by Levy et al. [33] to analyze

TABLE II

INHIBITION OF Li_i-Na_o COUNTERTRANSPORT BY *N*-ETHYLMALEIMIDE AS AFFECTED BY THE ADDITION OF Na

Control rate of Li_i-Na_o countertransport: 0.52 ± 0.07 mmol Li/l erythrocyte per h. In treatments (3) and (4), the Mg-sucrose medium was supplemented with 2 or 4 mM Na, as indicated, prior to addition of *N*-ethylmaleimide. In treatments (5) and (6), Na was added 5 min after the addition of *N*-ethylmaleimide to the erythrocyte suspension in Mg-sucrose medium and the treatments then lasted for 5 additional minutes. Average values ± S.E. of two experiments, each in triplicate, are presented.

	Treatment		% Inhibition	
	medium	mM NaCl	5 min treatment	10 min treatment
1	Sodium-rich	150	58 ± 3	73 ± 3
2	Mg-sucrose	0	30 ± 4	30 ± 4
3	Mg-sucrose	2	35 ± 3	38 ± 3
4	Mg-sucrose	4	39 ± 4	41 ± 3
5	Mg-sucrose	0 → 2		37 ± 4
6	Mg-sucrose	0 → 4		40 ± 4

the order of myosin inactivation by 2,4-dinitrophenol was applied by us to evaluate the effect of *N*-ethylmaleimide on lithium efflux. Fig. 7 presents a plot of the inactivation process of Li_i-Na_o countertransport (expressed as the re-

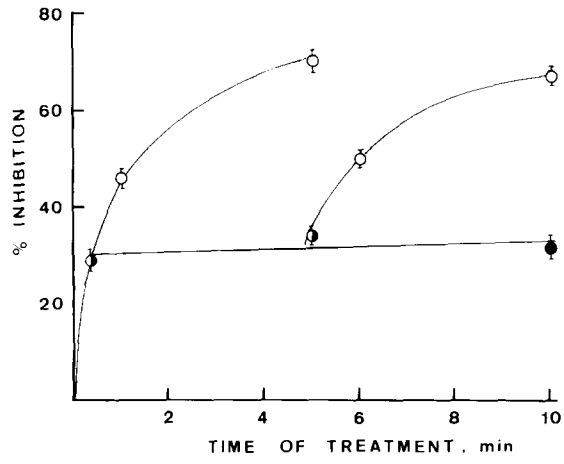

Fig. 4. Inhibition of Li_i-Na_o countertransport by *N*-ethylmaleimide as affected by addition of Na (75 mM final concentration) during treatment with *N*-ethylmaleimide in Mg-sucrose medium. Na was added either at the beginning of the treatment or 5 min later. Mg-sucrose medium (●), Na added (○). Control rate: 0.52 ± 0.04 mmol Li/l erythrocyte per h. The values are the average ± S.E. of three experiments.

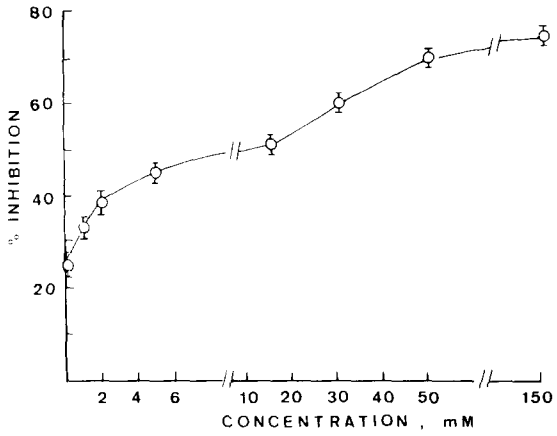

Fig. 5. The effect of Na concentration on the inhibition of Li_i-Na_o countertransport by *N*-ethylmaleimide. The treatment with *N*-ethylmaleimide was for 10 min. Control rate: 0.40 ± 0.04 mmol Li/l erythrocyte per h. Data presented are means $\pm$ S.E. of five experiments.

ciprocal of $t_{1/2}$, the time for half-maximal inactivation), as affected by *N*-ethylmaleimide concentration. The points, based on the data of Fig. 6, fit two straight lines, with slopes equal to 0.4 and 1.5 at *N*-ethylmaleimide concentration below and above 0.4 mM, respectively. According to Levy et al. [33], the slopes represent an average of the least number of *N*-ethylmaleimide molecules that bind

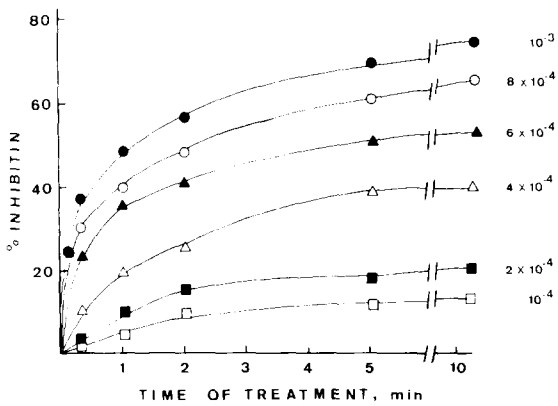

Fig. 6. Time and concentration dependence of the effect of treatment by *N*-ethylmaleimide in Na medium on Li_i-Na_o countertransport activity. Concentration of *N*-ethylmaleimide (molar units) are listed at the right side of each curve. The data present a representative experiment in triplicate. Three additional experiments showed similar results. Control rate of the countertransport activity was 0.46 ± 0.07 mmol Li/l erythrocyte per h.

to one molecule of the Li_i-Na_o countertransport protein when inactivation occurs.

When *N*-ethylmaleimide treatment was performed in a sodium-free medium, the extent of inhibition of Li_i-Na_o countertransport rose with increasing *N*-ethylmaleimide concentration, but maximal inhibition was established within 2 s. Since, due to this rapidity, the time dependence of the inhibition could not be conveniently measured, the interaction of *N*-ethylmaleimide with the countertransport was evaluated in terms of the maximal inhibition as a function of concentration. This interaction was analyzed by the modified Hill plot, obtained by plotting the log(percent residual activity/percent inhibition) against the log(*N*-ethylmaleimide concentration). It has been shown [34–36] that the interaction of irreversibly acting inhibitors may be analyzed in terms of such modified Hill plots. Fig. 8 compares the inactivation of Li_i-Na_o countertransport by *N*-ethylmaleimide in Na medium and in Na-free medium. The Hill plot for the inactivation in Na medium consisted of two linear portions with slopes of 1 and 1.8, at *N*-ethylmaleimide concentrations below and above 0.4 mM, respectively. The plot for the inactivation in Na-free medium was linear at the range of 10^{-4} to 10^{-3} M *N*-ethylmaleimide, with a slope of 0.9.

Effect of cations on the reactivity of N-ethylmaleimide

To test the possibility that the cation composition affects the reactivity of *N*-ethylmaleimide rather than the erythrocyte membrane, radioactive

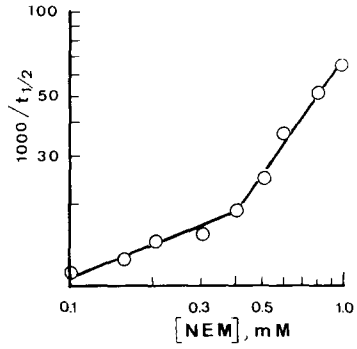

Fig. 7. Order of the inactivation process of the Li_i-Na_o countertransport with respect to concentration of *N*-ethylmaleimide (NEM). The values are the averages of three experiments, plotted according to Levy et al. [33].

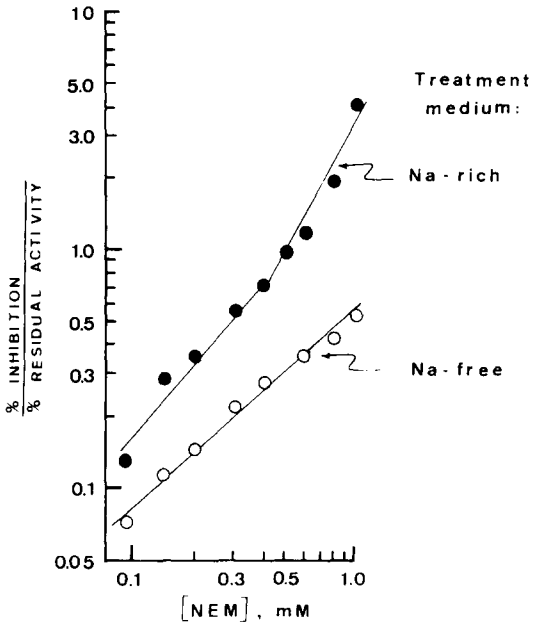

Fig. 8. Hill plot analysis of *N*-ethylmaleimide (NEM) inhibition was performed for 10 min in Na solution (●) or in Mg-sucrose medium (○). Plotted according to Godin and Schrier [35]. The values are the average of four experiments. The correlation coefficient is 0.92 for the lines shown for the treatment in Na solution, higher than all other alternatives.

N-ethylmaleimide was used. The inhibitory effect of *N*-ethyl[^{14}C]maleimide and the amount of label bound to the membrane, following treatment in Na medium and Na-free medium, were compared. Table III shows that, although the different media indeed lead to different degrees of inhibition, the binding of *N*-ethyl[^{14}C]maleimide in the two media was similar.

Discusison

The presence of the substrate cations, Na and Li, enhanced the inhibition of Li_i-Na_o countertransport by *N*-ethylmaleimide. When erythrocytes were treated in a medium free of Na or Li, *N*-ethylmaleimide caused maximal inhibition of 33% of the countertransport activity within 2 s, whereas in Na or Li medium, maximal inhibition was achieved in 10 min to over twice the extent. Since the reactivity of *N*-ethylmaleimide was not affected by these cations, it is likely that the cationic substrates induced conformational changes of the Li_i-Na_o countertransport protein. This conclusion corroborates the suggestion of an earlier study [26].

Under the experimental conditions used in the present study, *N*-ethylmaleimide interacts with SH-groups [26,37]. Several alternative explanations could be proposed for the role of SH-groups in the inhibitory effect of *N*-ethylmaleimide in response to external cations: (1) a single class of SH-groups is available to *N*-ethylmaleimide in the presence and the absence of Na and Li, but the conformation affected by Na and Li leads to modified location of the reactive SH resulting in a higher inhibition; (2) entirely different classes of SH-groups are present, one reacts with *N*-ethylmaleimide in the absence of Na and Li, and the other in their presence; (3) one common and one selective class of SH groups are present; one that reacts with *N*-ethylmaleimide, either in the presence or absence of Na and Li, and a second class that reacts only in the presence of Na and Li.

The first possibility appears unlikely, since the

TABLE III

Li_i-Na_o COUNTERTRANSPORT ACTIVITY AND *N*-ETHYL[^{14}C]MALEIMIDE BOUND TO THE MEMBRANES FOLLOWING TREATMENT OF ERYTHROCYTES IN DIFFERENT MEDIA

Control rate: 0.25 ± 0.01 mmol Li/l erythrocyte per h. Data presented are mean ± S.E. of three experiments, each in triplicate. n.s., not significant.

Medium *N*-ethylmaleimide treatment	Li_i-Na_o countertransport		*N*-ethyl[^{14}C]maleimide bound to the membrane (nmol/mg protein)
	rate mmol/erythrocyte per h	inhibition (%)	
Na-rich	0.092 ± 0.007	64 ± 3.5	9.18 ± 0.86
Mg-sucrose	0.182 ± 0.009	28 ± 3.2	10.22 ± 0.40
P	< 0.001	< 0.001	n.s.

assay, following the treatment, is conducted in the presence of Na so that the assumed conformational change takes place anyhow; nevertheless, the impact of the cationic substrate during the treatment is evident. It could be claimed, however, that the protein cannot undergo conformational changes after the reaction with *N*-ethylmaleimide in Na-free medium. Yet, a rise in inhibition does occur when Na is added 5 min after the beginning of the treatment with *N*-ethylmaleimide in Na-free medium (Fig. 4). If two different independent SH classes react in the different media, as proposed in the second possibility, then addition of Na during the treatment with *N*-ethylmaleimide in Mg-sucrose medium should lead to combined additive inhibition attained by the individual treatments. Hence, the nonadditive inhibition (Table III) indicates that this possibility is also unlikely.

It is concluded that in Na- and Li-rich medium, two classes of SH-groups are involved. The SH-groups are attacked by *N*-ethylmaleimide in both Na medium and Na-free medium with different kinetics due to their different exposure. The SH-groups of the second class are available only in the presence of Na or Li and cause the additional inhibition. Kinetic analysis of the effect of *N*-ethylmaleimide on Li_i-Na_o countertransport further supports this conclusion. The Hill plot of the inactivation by *N*-ethylmaleimide in Mg-sucrose medium suggests that the effect of *N*-ethylmaleimide results from the modification of a single class of sulfhydryls. In contrast, the Hill plot (Fig. 8) and the plot of the inactivation as a function of *N*-ethylmaleimide concentration (Fig. 7), both in Na-rich medium, are biphasic. This indicates that two different sulfhydryl classes react with *N*-ethylmaleimide.

Duhm and Becker [26] have already demonstrated that external Na or Li accelerates inhibition of Li uptake by *N*-ethylmaleimide and have suggested that the cations induce conformational changes of the Na_i-Li_o exchange protein in the membrane. Although we share the same general conclusion, our results differ in several aspects from those of Duhm and Becker. No inhibition of Li uptake was detected in their study when *N*-ethylmaleimide was applied in Na-free medium [26]. Yet we have observed about 33% inhibition of Li_i-Na_o countertransport when *N*-ethylmaleimide was added in Na-free medium. This difference is not related to the presence of intracellular Li when Li_i-Na_o countertransport is measured, since the same results were obtained when *N*-ethylmaleimide was applied before or after Li loading (Table I). There is also a marked difference between the two studies in the affinity of Na to the site promoting inhibition by *N*-ethylmaleimide: apparent K_m = 41 mM, when Li uptake was measured [26], but apparent K_m = 12 mM for the Li_i-Na_o countertransport system (this study, Fig. 5).

The different experimental approaches used in the two studies, namely, inward Li movement to measure Na_i-Li_o exchange on one hand, and outward Li movement to measure Li_i-Na_o countertransport, on the other hand, may indeed express inherent differences related to the direction of the measured flow. The direction of the flow is relevant to several documented parameters: (a) Affinity. There is a marked difference in the affinities of the external and internal sites of the exchange system [14]. (b) Flux. Phloretin-sensitive Li uptake is highly correlated with Li_i-Na_o countertransport, but the actual rate of Li_i-Na_o countertransport is 10-times higher than that of Li uptake [38]. (c) Clinical aspect. Significantly higher rates of Li_i-Na_o countertransport were detected in patients with essential hypertension by many laboratories [1–8]. However, when patients were analyzed on the basis of Li uptake, such differences could not be demonstrated [39]. These marked differences in Li flux possibly reflect an asymmetry of the membrane exchange system.

By the use of various reagents that react with SH-groups, it was possible to obtain information on the location of the sensitive sites of the Li_i-Na_o countertransport system in the erythrocyte membrane. Only permeable agents (*N*-ethylmaleimide, iodoacetamide and iodoacetate), which form alkyl substitutions [40], inhibited specifically the Li_i-Na_o countertransport. The impermeable compounds, 5,5′-dithiobis(2-nitrobenzoic acid) [41], monobromotrimethylammoniobimane [42], and the charged maleimide derivatives MBTA and eosin-5-maleimide [43–45], did not alter Li_i-Na_o countertransport at all. These observations cannot be explained by reactivity toward SH-groups, since in other membrane systems, MBTA was found more potent than *N*-ethylmaleimide [45]. PCMBS did

not inhibit the countertransport activity within 1 h [10,11,24]. However, inhibition was observed following 24 h of incubation with PCMBS [46], apparently due to its slow rate of penetration [47]. In agreement with previous reports [24,26,46], we conclude that the sensitive SH-groups are not externally exposed.

It has been suggested [35,48] that many of the effects caused by SH-reagents could relate to general changes in membrane structure and not necessarily to specific enzyme inhibition. However, *N*-ethylmaleimide, iodoacetate and iodoacetamide seem to react directly on the countertransport protein, according to several criteria: (a) Li_i-Na_o countertransport was inhibited, while Li efflux in Na-free medium was not changed; (b) the substrate cations Li and Na markedly affected the extent of inhibition; (c) the inhibition by *N*-ethylmaleimide was very rapid, $t_{1/2}$ within second; (d) *N*-ethylmaleimide, iodoacetate and iodoacetamide react with a limited number of SH-groups of the intact red cell membrane [50].

This study shows that the permeable compounds, PCMB [47] and mersalyl [49] do not alter the countertransport activity, but increase the passive membrane permeability. Jacob and Jandle [51] have demonstrated that PCMB induces an immediate and very pronounced swelling of the erythrocyte and thus disrupts the cation gradient across the membrane. These profound effects apparently overshadow a possible impact of PCMB on Li_i-Na_o countertransport, even if the countertransport is accessible to PCMB. Diamide is an SH-oxidizing agent, that crosslinks spectrin and perturbs the asymmetric phospholipid arrangement [52]. Under these conditions, the regular Li_i-Na_o countertransport activity is disturbed as well. Preincubation with *N*-ethylmaleimide prevented the effect of diamide on the membrane [52] and also on the Li_i-Na_o countertransport activity.

To sum up, several major conclusions are drawn from this study. The cationic substrates induce a conformational change of the Li_i-Na_o countertransport protein. SH-groups play a critical role in the operation of Li-Na exchange through the erythrocyte membrane. One class of SH-groups reacts with *N*-ethylmaleimide in the presence and in the absence of Li or Na, whereas a second class is reactive only in the presence of Li or Na. The SH-groups involved in the inhibitory process are not exposed at the exterior surface of the erythrocyte membrane. Differences associated with the direction of Li flow across the membrane possibly reflect an asymmetry of this membrane exchange system.

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