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THE ERYTHROCYTE MEMBRANE IN ESSENTIAL HYPERTENSION

CHARACTERIZATION OF THE TEMPERATURE DEPENDENCE OF LITHIUM EFFLUX

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Erythrocytes of most patients with essential hypertension are distinguished by a typical pattern of temperature-dependence of Li efflux. In the present study we have attempted to characterize this unique temperature response. Measurements of Li efflux into Na medium and Li_i-Na_o countertransport were conducted simultaneously at finely spaced temperature intervals with increments of 1 to 2°C in the range of 10–40°C. The Arrhenius plots for the efflux in Na medium and for Li_i-Na_o countertransport in erythrocytes of both normotensives and hypertensives were biphasic with slopes representing apparent energies of activation of about 28 and 8 kcal/mol below and above the 'break', respectively. However, the 'break' in the Arrhenius plot appeared at distinctly different temperatures: 30°C for normotensives and 20°C for hypertensives. The Li efflux was resolved into *N*-ethylmaleimide-sensitive and -insensitive components. The sensitive component exhibited a typical biphasic temperature response, with the characteristic 'break': at 30°C for normotensives and at 20°C for hypertensives. In contrast, the *N*-ethylmaleimide-insensitive component was alike in normotensives and hypertensives. It is concluded that: (a) the unique temperature dependence of Li efflux in erythrocytes of hypertensives results from a localized modification in the membrane; (b) the *N*-ethylmaleimide-sensitive component represents a protein moiety which distinguishes between the erythrocyte membrane of normotensives and hypertensives; (c) the expression of the temperature dependence as judged by the sharp transition in slope (within 1 to 2°C), apparently reflects the cooperative involvement of membrane lipids, associated with the Li efflux system.

Introduction

Alterations in cellular properties, mainly of blood cells, have been described in man and animal models in association with essential hypertension [1–7]. Two erythrocyte transport systems, Li_i-Na_o countertransport [8] and Na + K cotransport [9], attract particular interest, since they show familial aggregation in essential hypertension and may serve to investigate gene-environment interaction of this disease. Many [10–15] but not all [16] studies indicate that rates of Li_i-Na_o countertransport are elevated in erythrocytes of patients with essential hypertension. The ouabain-insensitive Li_i-Na_o countertransport has been characterized with respect to kinetic parameters, sensitivity to inhibitors and possible physical relationships to other transport systems [17–19], but further studies are required to define this transport system on the molecular level.

Investigation of temperature dependence of a membrane parameter is a useful tool to probe for the involvement of membrane proteins and lipids. In an earlier study [20] we have detected an intri-

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

guing difference in the temperature dependence of Li efflux in erythrocytes of normotensive and hypertensive persons. In a more detailed study [21], it was found that among 52 patients with essential hypertension, 75% exhibited unique Arrhenius plots of Li efflux with a change in slope ('break') at about 20°C. In contrast, the corresponding 'break' for all the normotensives was about 30°C. Furthermore, analysis of normotensive offspring of hypertensives and of patients with secondary hypertension indicate that the temperature dependence of Li efflux may serve as a genetic marker for essential hypertension.

In the present communication we report attempts to characterize the temperature dependence of lithium efflux in sodium medium and Li_i-Na_o countertransport. The study deals with erythrocytes from hypertensives showing the unique temperature dependence of the flux [21] in comparison with erythrocytes from normotensives. It is demonstrated that the membrane changes expressed by the Li efflux of hypertensive erythrocytes are likely to be localized, apparently involving a protein moiety and associated lipids.

Materials and Methods

Blood donors. Blood was obtained by consent, either from healthy normotensive persons (both parents were normotensive and so were all living relatives) or from age- and sex-matched patients, whose sole disease was essential hypertension. The patients had an established family history of hypertension. They did not receive medication at all or at least for a year. Unless indicated otherwise, the data refer to blood from a normotensive person.

Determination of lithium efflux. Blood was drawn into heparin solution (25 units/ml) 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. [8]. Efflux was performed simultaneously into sodium-rich medium (150 mM NaCl/10 mM glucose/0.1 mM ouabain/10 mM Tris-Mops, and into sodium-free medium (same medium but NaCl was replaced by 75 mM $MgCl_2$ and 85 mM sucrose), at different temperatures. A graded temperature device allowed the simultaneous assays of Li efflux at the desired temperatures in the range of 10–40°C with 1–2°C increments. The pH of the medium varied from 7.44 (at 10°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. Lithium was determined by means of an atomic absorption spectrophotometer (Varian Techtron Model AA6), 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 sodium-free medium was taken as countertransport [8].

N-Ethylmaleimide was dissolved at 70% (v/v) ethanol and added, when a treatment is indicated, to a 5% suspension of the lithium-loaded erythrocytes, for 2 min at 4°C. Thereafter the cells were washed twice with a washing solution [8]. Equivalent ethanol (up to 2 μl/ml) was added to control. Alternately, when specified, *N*-ethylmaleimide was added to the assay medium.

ATPase activity. Erythrocytes were separated and suspended in 155 mM NaCl, washed three times with this solution and then hemolysed by the addition of 4 ml erythrocyte suspension (15% hematocrit, about $1.5 \cdot 10^9$ cells/ml) to 15.2 ml H_2O at 4°C. ATPase activity was determined immediately thereafter as described [22]. The assay mixture contained, in 1 ml: 50 mM Tris-HCl, pH 7.6/1.5 mM ATP/6 mM $MgCl_2$/20 mM KCl/100 mM NaCl/$1.5 \cdot 10^8$ hemolysed erythrocytes. Ouabain, at 0.1 mM, was added for the determination of $(Na^+ + K^+)$-ATPase activity. Following an incubation for 30 min at a given temperature, the reaction was stopped by the addition of 1 ml 10% (w/v) trichloroacetic acid. Inorganic phosphate liberated was determined by the color produced with ammonium molybdate and ferrous sulfate as described [23].

Osmotic fragility of erythrocytes. Stock suspensions (30%) of washed erythrocyte in a solution of 155 mM NaCl/2 mM sodium phosphate, pH 7.4, were prepared as described [24]. An aliquot (20 μl) of the erythrocyte stock suspension was rapidly mixed with 3 ml of 70 mM NaCl solution, buffered with 2 mM sodium phosphate, pH 7.4. After 10 min at a given temperature, the suspensions were centrifuged at $2000 \times g$ for 3 min and the super-

natant analyzed for hemoglobin content [24]. To control the temperature during hemolysis, the solutions and the stock suspensions were equilibrated at the desired temperature for 4 min prior to the fragility test. To obtain 100% hemolysis, Triton X-100 was added, at a final concentration of 0.3 mg/ml.

Statistical analysis. The differences in means were tested with the *t*-statistic. The plots were drawn as least-squares regression lines and tested by analysis of variance.

Materials. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Temperature dependence of Li efflux

A comparison of Li efflux in erythrocytes from representative normotensive and hypertensive individuals is given in Fig. 1. The rates of Li efflux, both in sodium medium and countertransport, are plotted on a log scale, as a function of the reciprocal of absolute temperature, K^{-1}. The Arrhenius plots of Li efflux in erythrocytes of both normotensives and hypertensives are nonlinear [21]. In order to clarify the sharpness of the temperature 'break' and to calculate the energy of activation more precisely than possible earlier [20], over 20 experimental points were recorded for each curve, at 1–2 deg. C intervals. Indeed, the two groups of persons differ significantly in the temperature 'break': as much as 10°C difference was recorded, either for Li efflux in sodium medium or countertransport (Table I). While the apparent energy of activation was markedly different for temperature ranges below and above the 'break', Table I shows that the corresponding values of E_a for normotensive and hypertensives were similar. The activation energies recorded are in the range predicted by Parsegian [25] for the energy barrier in a carrier-mediated transport model.

Fig. 2 indicates that the temperature effect is reversible. In shifting the temperatures from 14 to 42°C or vice versa, rate adjustment took place

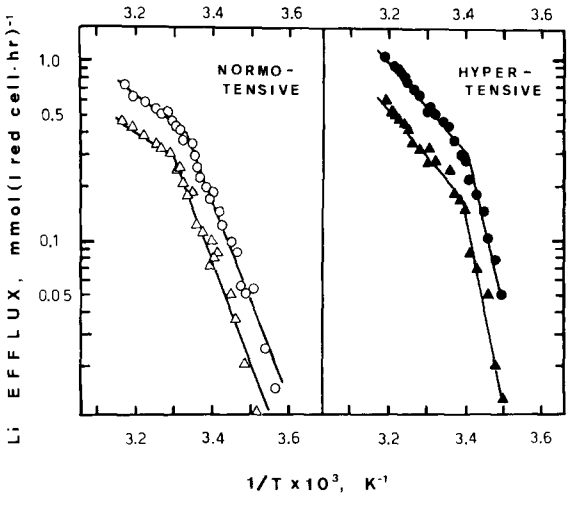


Fig. 1. Temperature dependence of lithium efflux in erythrocytes of a normotensive and a hypertensive individual. Symbols: circles (○, ●), Li efflux in sodium-rich medium; triangles (△, ▲), countertransport.

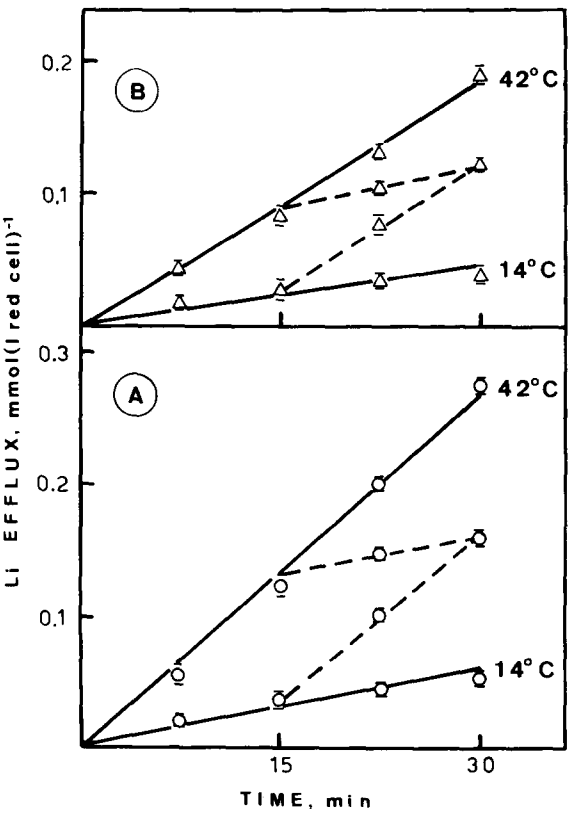


Fig. 2. Reversibility of temperature-dependence of lithium efflux in erythrocytes. At 15 min, assay suspensions were transferred (broken lines) from 14 to 42°C and vice versa. A. Li efflux in sodium medium; B. countertransport. A single experiment is presented in duplicate, with erythrocytes from a normotensive donor. Three additional experiments yielded similar results.

TABLE I

PARAMETERS OF ARRHENIUS PLOTS OF Li EFFLUX IN ERYTHROCYTES FROM NORMOTENSIVE AND HYPERTENSIVE DONORS

Data presented are average values ± S.E. for three normotensive and three hypertensives, calculated from multipoint Arrhenius plots, such as presented in Fig. 1. The results are in full agreement with additional measurements, for 25 normotensives and 39 hypertensives [21].

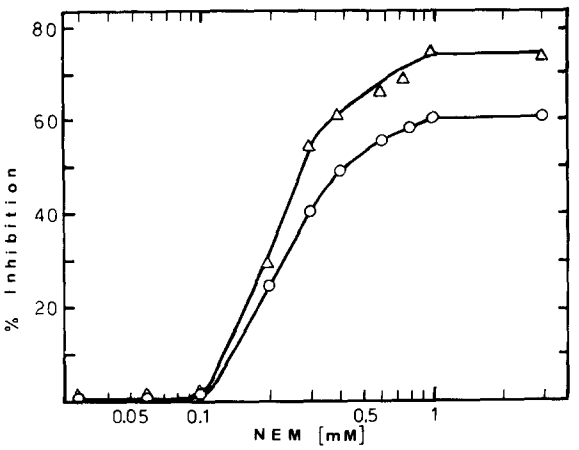
Parameter		Normotensive	Hypertensive	P
Temperature 'break' (T_b) (°C)				
Li efflux in Na medium		29.7 ± 0.5	18.6 ± 0.5	< 0.001
Countertransport		30.7 ± 0.8	20.0 ± 0.7	< 0.001
Apparent energy of activation (kcal/mol)				
Li efflux in Na medium,	Temp. < T_b	29.6 ± 2.9	24.4 ± 1.2	> 0.25
	Temp. > T_b	7.9 ± 1.0	8.6 ± 0.9	> 0.38
Countertransport	Temp. < T_b	31.5 ± 3.5	26.3 ± 2.1	> 0.29
	Temp. > T_b	7.7 ± 1.4	9.5 ± 1.0	> 0.20

readily, with no measurable time lag and with no indication for an irreversible denaturation. The reversibility suggests that the process is under thermodynamic control [26].

Effect of N-ethylmaleimide

Fig. 3 presents the inhibitory effect of *N*-ethylmaleimide on Li efflux in human erythrocytes. Li efflux in sodium medium and countertransport were maximally inhibited to the extent of 60 and 73%, respectively, by 1 mM *N*-ethylmaleimide. Consequently, this concentration was applied to further experiments.

Several procedures for the treatment of erythrocytes with *N*-ethylmaleimide were compared (see Materials and Methods): (a) The inhibitor was added to the efflux medium, (b) the cells were pretreated prior to the efflux measurement,



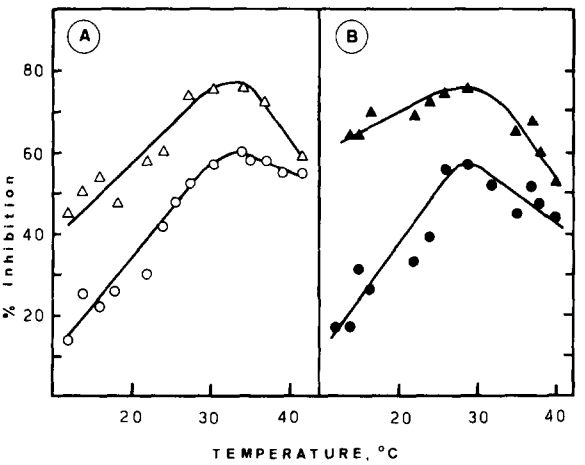


Fig. 3. Effect of *N*-ethylmaleimide (NEM) concentration on lithium efflux in erythrocytes. Symbols: circles (○), Li efflux in sodium-rich medium; triangles (△), countertransport. Erythrocytes from normotensive persons were treated by NEM as indicated for treatment b in Table II. The values are the average of three experiments.

Fig. 4. Inhibition by *N*-ethylmaleimide of Li efflux in erythrocytes as affected by temperature. A. Normotensives (average of three), B. Hypertensives (average of three). Symbols as in Fig. 1. Erythrocytes were treated by NEM as indicated for treatment b in Table II.

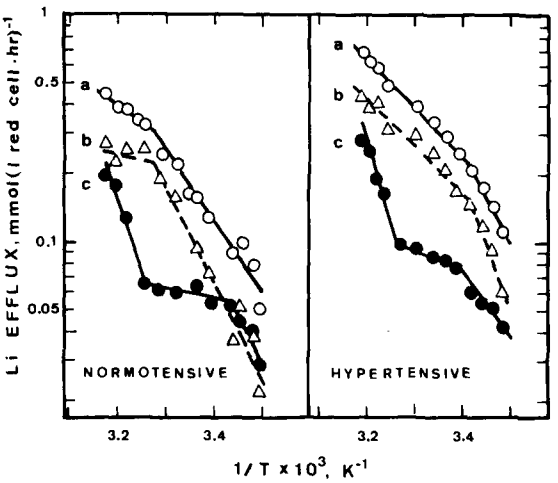


Fig. 5. $Li_i - Na_o$ countertransport in erythrocytes as affected by *N*-ethylmaleimide and temperature. Symbols: a. control, b. *N*-ethylmaleimide-sensitive efflux, c. *N*-ethylmaleimide-insensitive efflux. Average for three normotensives and three hypertensives are shown. The treatment by *N*-ethylmaleimide was as indicated for treatment b in Table II.

TABLE II

EFFECT OF *N*-ETHYLMALEIMIDE TREATMENTS ON Li EFFLUX IN ERYTHROCYTES

The treatments by *N*-ethylmaleimide: a. *N*-ethylmaleimide (1 mM) added to the efflux medium; b. *N*-ethylmaleimide (1 mM) added for 2 min at 4°C to Li-loaded erythrocytes, then washed twice as described. c. a and b combined. A single experiment, run in duplicates, with erythrocytes from a normotensive donor, is presented. Three addition experiments yielded similar results. Results are given as mmol Li per liter red cell per h.

Li efflux	Control	*N*-Ethylmaleimide treatment		
		a	b	c
In sodium-rich medium	0.60	0.29	0.28	0.27
In sodium-free medium	0.20	0.19	0.185	0.17
Countertransport	0.40	0.10	0.095	0.10

and (c) these two treatments were combined. Table II shows that the three procedures yielded a similar inhibitory effect. Furthermore, *N*-ethylmaleimide, introduced by either of these procedures, did not inhibit Li efflux in Na-free medium.

Erythrocytes pretreated with *N*-ethylmaleimide (procedure b above), and assayed at different temperatures, exhibited a non-uniform extent of inhibition of Li efflux: maximal inhibition was observed at about 30°C in erythrocytes from normotensives and hypertensives (Fig. 4).

Since Li efflux can be resolved into *N*-ethylmaleimide-sensitive and -insensitive components, the effect of temperature on these components was further analyzed. Fig. 5 shows that the *N*-ethylmaleimide-sensitive component of the countertransport exhibited a typical biphasic temperature

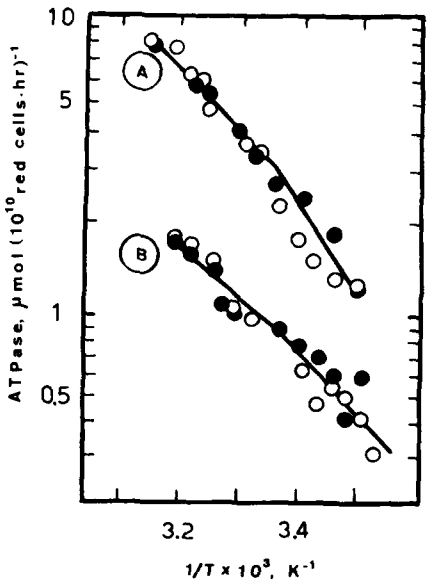


Fig. 6. Effect of temperature on ATPase activity in erythrocytes. A. ATPase ('total'), B. ouabain-sensitive (Na+K)-ATPase. Open circles, normotensives; solid circles, hypertensives. Average values for 10 individuals are presented.

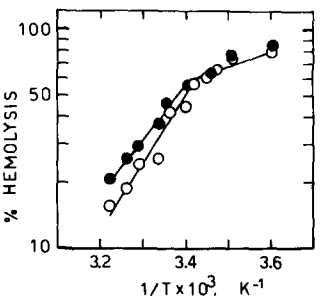


Fig. 7. Effect of temperature on osmotic fragility of erythrocytes. Symbols as in Fig. 6.

response with a characteristic 'break', namely at 30°C for normotensives and 20°C for hypertensives. In contrast, the *N*-ethylmaleimide-insensitive component exhibited a triphasic temperature dependence. Significantly, this pattern of the *N*-ethylmaleimide-insensitive component was very similar in erythrocytes from normotensives and hypertensives.

Temperature dependence of other membrane parameters

The erythrocyte membrane modification in hypertensives, reflected by the temperature dependence of Li efflux, may be a widespread membrane property or a localized phenomenon. Additional membrane parameters were therefore assayed. Fig. 6 shows that erythrocytes from normotensives and hypertensives exhibit similar Arrhenius plots of ATPase and ouabain-sensitive $(Na^+ + K^+)$-ATPase. Furthermore, the osmotic fragility of erythrocytes from the two groups of persons was similarly affected by temperature (Fig. 7); the difference between the slopes was statistically insignificant.

Discussion

The human erythrocyte membrane exhibits a spectrum of discontinuities in temperature dependence of functional and physical parameters. Thus, for example, 'breaks' in Arrhenius plots were recorded for Cl^-/Cl^- and HCO_3^-/Cl^- exchange at 15 [27] and 17°C, respectively [28], for structural transitions at 15 and 37°C [29], for viscosity at 18–19°C [30], for transport of glucose [31,32] and lactate [33] at 18°C, for acetylcholinesterase activity [34,35] and osmotic fragility [36] at 25°C. In the present study it is demonstrated that Arrhenius plots of Li efflux in sodium-rich medium and Li_i-Na_o countertransport consist of two straight lines intersecting at 30° and 20°C in erythrocytes from normotensives and hypertensives, respectively. The present study differs significantly from an earlier one [20] in the pattern of the Arrhenius plots. We attribute the difference in Arrhenius plots to experimental aspects. The present work is distinguished by a wider temperature range than used before and particularly in the use of a device which permits simultaneous measurements at finely spaced temperature intervals, with increments of only 1–2 deg. C. In the earlier study, the assays were conducted in just eight different temperatures. Errors in the plot of temperature dependence that might be derived from limited experimental points have already been demonstrated. The Arrhenius plot for the activity of membrane-bound ATPase in *Acholeplasma laidlawii* B was reported to be bilinear [37], but was subsequently proved by the same authors to be curvilinear when more temperature points were collected [38]. The temperature dependence of β-galactoside transport in *Escherichia coli* membrane was considered biphasic [39], while further measurements in a wider temperature range indicated a triphasic dependence [40].

Several mechanisms have been offered to explain the nonlinear plots for processes in biological membranes. The suggested mechanisms include lipid-protein interaction related to lipid phase transition [39–41], protein thermotropic response [26,42], finite heat capacity of activation and multiple reaction steps [42]. We are not able to offer an exact mechanism for the nonlinear Arrhenius plot of lithium efflux, but several relevant conclusions may be suggested.

The marked sensitivity of lithium efflux to *N*-ethylmaleimide (Ref. 43, and this study) and *p*-chloromercuribenzene sulfonate [19] indicates that certain protein -SH groups are essential for the transport. The Arrhenius plots of Li_i-Na_o countertransport and of the *N*-ethylmaleimide-sensitive component of the flux exhibit a characteristic pattern, with a break at 30°C for normotensives and 20°C for hypertensives (Fig. 5). Thus, the *N*-ethylmaleimide-sensitive component represents a protein moiety which distinguishes between the erythrocyte membrane of normotensives and hypertensives, as expressed by the typical difference in the temperature response of lithium efflux. Further study is required to elucidate whether the *N*-ethylmaleimide efflux indeed represents the involvement of a certain protein or merely reflects secondary changes, due to the role of the -SH group in membrane structure, as may be deduced from other studies [44,45].

The 'break' in the Arrhenius plots of lithium efflux (Fig. 1) is typically sharp: the change in slopes is apparent within 1–2 deg. C. Based on

thermodynamic considerations, Barnett [41] suggested that the sharpness of the 'break' may aid in evaluating the possible involvement of membrane proteins and lipids. Assuming a simple model of a membrane enzyme existing in two conformational forms, each predominant (~ 90%) below or above the temperature 'break' and assuming a sharp 'break' within 1 deg. C, the associated enthalpy and the entropy change are calculated to be 379 kcal/mol and 1290 e.u., respectively. It is unlikely, according to Barnett, that such enormous enthalpy and entropy changes can be accounted for by a temperature-dependent conformational change alone; it is more likely that a protein conformation change is coupled to a much larger cooperative process, possibly contributed by lipids [41]. Indeed, the enthalpy for lipid crystal to gel phase transition is 5–10 kcal/mol lipid [46] and 50–100 phospholipid molecules are associated with a protein [47,48]. Silvius and McElhaney, on the other hand, proposed that a sharp 'break' of a nonlinear Arrhenius plot is predicted by assuming either of the following: the lipid environment of the enzyme changes with temperature and influences the reaction rate, or the protein undergoes a thermotropic conformation change which alters the activation parameters of the reaction.

We suggest that the Li_i-Na_o countertransport is composed of two, or more, functional components tightly associated: (1) a protein, with essential SH groups, (2) additional component(s), possibly lipids or another protein. Several considerations are compatible with this rationale. (a) The countertransport is not fully inhibited by *N*-ethylmaleimide, (b) The *N*-ethylmaleimide-insensitive portion of the efflux exhibits a triphasic Arrhenius plot. A plot with two breaks has been shown to involve lipids [40] but other explanations are available [42]. (c) The sharp 'break' indicates a cooperative process [42]. (d) The pattern of inhibition of the efflux by *N*-ethylmaleimide as affected by temperature is bell-shaped (Fig. 4). Such a pattern is best explained by assuming that the transport system contains more than one component, with different temperature responses.

Elevated $(Na^+ + K^+)$-ATPase activity was reported in erythrocytes of hypertensive patients [49,50]. However, no difference between normotensives and hypertensives in $(Na^+ + K^+)$-ATPase activity was reported by others (Ref. 51 and Fig. 6, in the present study). In support of the latter reports, an equal number of ouabain-binding sites was found on erythrocytes from normotensive and hypertensive persons [52]. Erythrocytes of normotensive and hypertensive persons exhibit similar temperature dependence of acetylcholinesterase [20,34], ATPase and $(Na^+ + K^+)$-ATPase (Fig. 6) as well as osmotic fragility (Fig. 7). It is of interest that the human erythrocyte is considered to rupture osmotically in protein-lipid boundary regions in the membrane [36].

In conclusion, it is unlikely that the temperature dependence, typical for lithium efflux, is widespread in the erythrocyte membrane of hypertensives. Rather, it represents a unique, localized modification in a membrane protein, while its expression may involve associated lipids.

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