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Role of Membrane Thermotropic Properties on Hypotonic Hemolysis and Hypertonic Cryohemolysis of Human Red Blood Cells

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The hypothesis of a correlation between the effects of temperature on red blood cells hypotonic hemolysis and hypertonic cryohemolysis and two thermotropic structural transitions evidenced by EPR studies has been tested. Hypertonic cryohemolysis of red blood cells shows critical temperatures at 7°C and 19°C. In hypotonic solution, the osmotic resistance increases near 10°C and levels off above 20°C. EPR studies of red blood cell membrane of a 16-dinyloxyl stearic acid spin label show, in the 0–50°C range, the presence of three thermotropic transitions at 8, 20, and 40°C. Treatments of red blood cells with acidic or alkaline pH, glutaraldehyde, and chlorpromazine abolish hypertonic cryohemolysis and reduce the effect of temperature on hypotonic hemolysis. 16-Dinyloxyl stearic acid spectra of red blood cells treated with glutaraldehyde and chlorpromazine show the disappearance of the 8°C transition. Both the 8°C and the 20°C transitions were abolished by acidic pH treatment. The correlation between the temperature dependence of red blood cell lysis and thermotropic breaks might be indicative of the presence of structural transitions producing areas of mismatching between differently ordered membrane components where the osmotic resistance is decreased.

Key words: hypertonic cryohemolysis, hypotonic hemolysis, thermotropic membrane processes, membrane structure–function relationship, erythrocyte membrane treatments

Evidence of a role of thermotropic membrane properties in the resealing process of human red blood cells (RBC) has been previously reported [1]. RBC hemolysis appears to be influenced by temperature [2] and may be a process opposite to resealing. It is therefore conceivable that the hemolysis may be somehow dependent upon thermotropic membrane properties. Moreover results recently obtained by

Abbreviations used: 16-DSA, 2-(14-carboxy-tetradecyl)-2-ethyl-4,4-dimethyl-3-oxazoli-dinyloxyl; EPR, electron paramagnetic resonance; PBS, phosphate-buffered saline.

This paper is dedicated to the memory of Sergio M. Sanfilippo, who provided skillful technical assistance.

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Lieber and Steck [3] show that only one hole, dependent on temperature and salt concentration, is produced during the lysis of resealed ghosts. These results may indicate that localized membrane areas are especially relevant in RBC hemolysis.

In this paper the effect of temperature on two types of hemolysis has been studied: the hypotonic hemolysis and the hypertonic cryohemolysis. The latter is defined as the lysis that occurs when cells are incubated in hypertonic solution at temperatures above 20°C with subsequent cooling at 0°C [4]. The mechanism, at molecular level, of this type of hemolysis is still unclear, although it has been recently suggested that cryohemolysis might be regulated by protein–membrane interactions [5]. Were this hypothesis correct, the hypertonic cryohemolysis could be mediated by a mechanism similar to that suggested by us for ghost resealing [1].

Data reported show that both hemolytic processes are temperature-dependent with critical temperatures close to those at which a spin-labeled stearic acid (16-dinyloxyl stearic acid; 16-DSA) detects a change in the freedom of motion monitored in the membrane. The temperature dependence of both hemolytic processes can be reduced or eliminated by treatments of RBC with acidic pH, glutaraldehyde, chlorpromazine, and chymotrypsin digestion of cytoplasmic proteins. The same membrane treatments also produce evident changes in the thermotropic properties of membrane as monitored by the spin probe. These correlations might be indicative that the role of temperature on RBC hemolysis could be due to the presence of two membrane structural transitions (the term "structural transition" is used in a general sense; see discussion in the accompanying paper [6]). As a consequence of these, at low temperature hemolysis is induced or increased in areas with reduced osmotic resistance.

MATERIALS AND METHODS

Materials

Chlorpromazine was obtained from CIBA (Saronno,VA, Italy). Glutaraldehyde was obtained from Merck (Darmstadt, Germany), chymotrypsin from Boehringer (Mannheim, Germany), and 2-(14-carboxy-tetradecyl)-2-ethyl-4,4-dimethyl-3-oxazoli-dinyloxyl (16 DSA) from Syva (Palo Alto, CA).

Hypotonic Hemolysis

Human RBC were washed three times in phosphate-buffered saline (PBS) (5 mM sodium phosphate, 150 mM NaCl, pH 7.8). After each centrifugation (5 min, 2,000g) the buffy coat was removed by aspiration. To minimize individual variability all experiments were performed using a pool of RBC from 5–12 donors. Before lysis, RBC and phosphate buffers were equilibrated at the chosen temperature; 10 μl of packed RBC was then mixed with 1 ml of phosphate buffers with the indicated NaCl concentration. After 10 min incubation at the indicated temperatures the samples were centrifuged at 15,000g for 2 min. The percent lysis calculated by the absorbance of supernatants at 543 nm was compared with that of control RBC totally lysed in 5 mM sodium phosphate, pH 7.8.

Hypertonic Cryohemolysis

The cryohemolysis assay was performed essentially as described by Dubbelman et al [7]. Ten microliters of PBS-washed RBC was suspended at the indicated

temperatures in 1 ml of 1 M NaCl, 5 mM sodium phosphate, pH 7.8, and, after 10 min incubation, cooled at 0°C for 10 min. To study the temperature dependence of the cooling step 10 μl of RBC was incubated for 10 min at 27°C in 1 ml of 1 M NaCl buffer and subsequently transferred for 10 min at the indicated temperatures. In both cases after the cooling step the samples were centrifuged 2 min at 15,000g, and percentage of hemolysis was calculated as for hypotonic hemolysis.

pH Treatment

To study the effects of pH on hemolysis, RBC were suspended overnight at 4°C with the following buffers: pH 5–6, 140 mM NaCl–10 mM acetate; pH 6.5–8.5, 145 mM NaCl–5 mM sodium phosphate; pH 9–10, 83 mM NaCl–67 mM glycine-NaOH. The cell/buffer ratio was 1:100 (v/v). Intracellular pH values were measured in the acidic region by ^{31}P NMR method [8], and in the alkaline region the method of Wessels and Veerkamp [9] was employed.

Glutaraldehyde Treatment

RBC were incubated for 1 hr at room temperature with glutaraldehyde-PBS in a cell/buffer ratio of 1:100 (v/v). The cells were then washed with PBS, containing 0.01 mM lysine.

Chlorpromazine Treatment

RBC or RBC digested by chymotrypsin as reported in [6] were incubated for 1 hr at 37°C in PBS, containing the indicated chlorpromazine concentrations. The cell/buffer ratio was 1:100 (v/v). After centrifugation chlorpromazine-treated RBC were utilized for EPR studies and the hypo- and hypertonic assays were performed with buffers containing the same chlorpromazine concentration.

EPR Measurements

16-DSA incorporation into RBC membranes was performed as reported in [6]. The EPR spectra were recorded on a Varian E-4 spectrometer (Milan, Italy) equipped with a variable temperature accessory. Temperature was monitored by a digital thermometer set above the cavity. At each temperature four spectra were recorded and the average value of peak heights used for calculation. Computer analysis of data has been performed as described in [6].

RESULTS

Effect of Temperature of Hypertonic Cryohemolysis and Hypotonic Hemolysis

The effect of temperature on hypertonic cryohemolysis is reported in Figure 1. When RBC were preincubated at the indicated temperatures in 1 M NaCl and then cooled at 0°C (Fig. 1a), the usual pattern of cryohemolysis was observed. According to previously reported experiments [7], cryohemolysis, which is detectable above 19°C, reaches a maximum value around 30°C and decreases thereafter (Fig. 1a). In Figure 1b RBC were preincubated in 1 M NaCl at a fixed temperature—ie, 27°C—and then cooled at the indicated temperatures. It is evident that hypertonic cryohemolysis becomes visible only when RBC are preincubated above 19°C and cooled

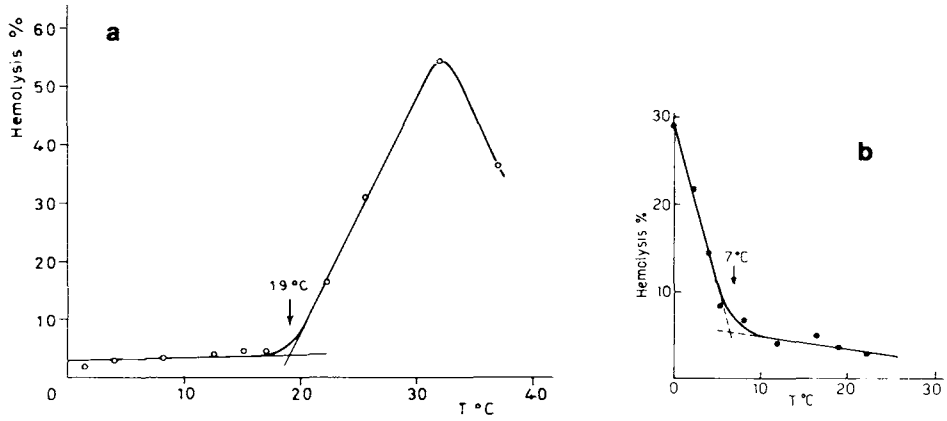

Fig. 1. Cryohemolysis of RBC in 1 M NaCl as a function of temperature. a) RBC preincubated 10 min at the indicated temperatures and cooled 10 min at 0°C; b) RBC preincubted 10 min at 27°C and cooled 10 min at the indicated temperatures.

below 7°C. Therefore, these two are the temperatures that appear to be critical for this process and not only one, as previously reported [4,5,7].

As for hypotonic hemolysis, it is known that it depends on temperature (see [2] and references reported therein). Data in Figure 2 show that, by decreasing NaCl concentration, the percentage of hemolysis follows, at both 0°C and 37°C, sigmoidal curves. It is also evident, however, that at 0°C the hemolysis begins at a salt concentration higher than at 37°C. The salt concentration necessary to obtain 50% of

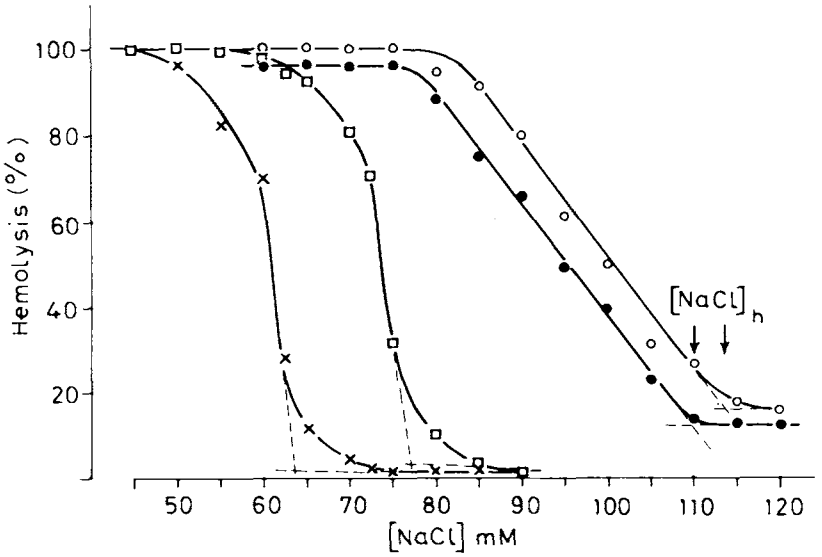

Fig. 2. Hypotonic hemolysis of RBC. (□—□), pH 7.8 at 0°C; (X—X), pH 7.8, at 37°C; (○—○) pH 5.4 at 0°C; (●—●), pH 5.4 at 37°C. Arrows show the extrapolation of the lowest nonhemolysing NaCl concentration $(NaCl)_h$.

hemolysis, $(NaCl)_{50\%}$, ranges from 74 to 61 mM between 0°C and 37°C, respectively (Fig. 2).

The values of $(NaCl)_{50\%}$ obtained at different temperatures are plotted in Figure 3. Two inflection points are present near 10°C and 20°C (Fig. 3). They indicate that 1) the osmotic fragility of RBC is unmodified between 0°C and 10°C; 2) some thermotropic membrane changes occur above 10°C, bringing about an increased osmotic resistance that persists until 20°C; and 3) at around 20°C, a second change seems to occur so that RBC resistance keeps its maximum value.

Effects of pH, Glutaraldehyde, Chlorpromazine, and Chymotrypsin Treatment of RBC on Hypertonic Cryohemolysis

As reported by Jung and Green [4] acidic pH is able to decrease hypertonic cryohemolysis. In this respect, both acidic and alkaline pH inhibit hypertonic cryohemolysis (Fig. 4). However, RBC must be preincubated at acidic or alkaline pH, respectively, and then transferred to a hypertonic solution at the same pH to obtain cryohemolysis inhibition (data not shown). On the contrary, the cryohemolysis of RBC suspended at neutral pH and then exposed to acidic hypertonic solutions is not inhibited. This may indicate that the inhibition is due to equilibration of pH inside the cells. To measure intracellular pH of intact cells we used the ^{31}P NMR technique that is based on pH dependence of inorganic phosphate (Pi) chemical shift [8]. The measurement of Pi chemical shift of RBC shows that the modification of pH inside the cell is a slow process. Therefore, we used RBC equilibrated overnight at 4°C with different pH buffers. After this incubation period, the Pi NMR signal increases owing to metabolic depletion [10], and a complete equilibration of pH inside and outside the cells is obtained. In the alkaline region the chemical shift of Pi does not change along with increasing pH values. To measure intracellular pH at pH $\geqslant$ 8,

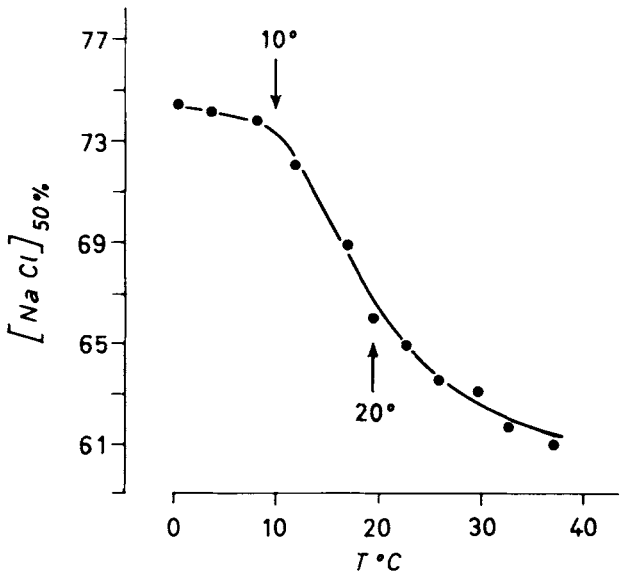

Fig. 3. NaCl concentration that produces 50% hemolysis $(NaCl)_{50\%}$ as a function of temperature. Hemolysis was measured in 5 mM phosphate buffer, pH 7.8, with the indicated salt concentrations.

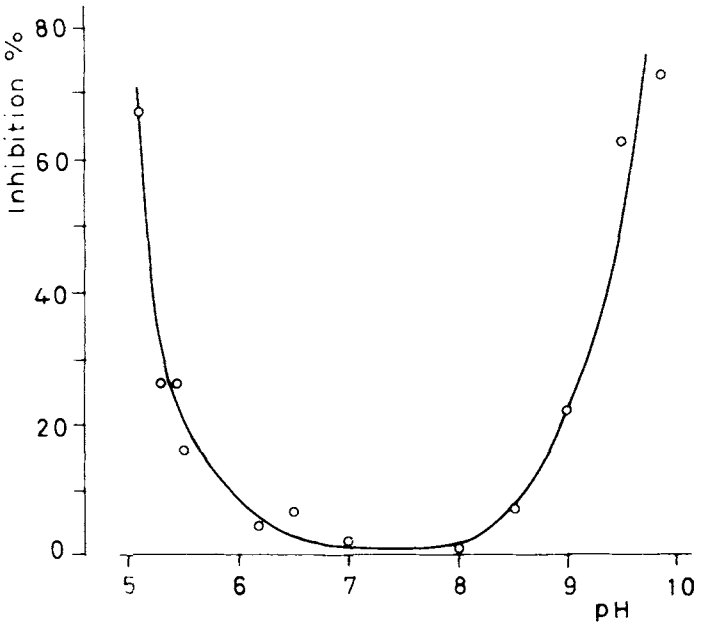

Fig. 4. Inhibition of hypertonic cryohemolysis as a function of pH. RBC were incubated overnight at 4°C at varying pH values and then suspended in hypertonic buffers at the same pH. Cryohemolysis was measured after 10 min incubation at 27°C and subsequent cooling at 0°C for 10 min.

therefore, we used the hemolysis method of Wessels and Veerkamp [9]. pH values reported hereafter are thus taken to represent actual pH values inside the cells. Figure 4 shows the effect of pH on hypertonic cryohemolysis. Below pH 6 and above pH 8 a consistent inhibition of cryohemolysis (maximal at pH 5.1 and 9.8) is observed. More acidic or alkaline pH produced RBC hemolysis during overnight equilibration.

The effects of glutaraldehyde treatment on RBC membrane have been extensively studied [11]. This agent produces cytoplasmic protein cross linking by interacting mainly with protein amino groups [12]. EPR studies of the effects of glutaraldehyde on RBC membrane suggest a modification of protein-lipid interactions [13]. Very low millimolar concentrations of glutaraldehyde inhibit hypertonic cryohemolysis (Fig. 5). Glutaraldehyde (1 mM) produced the known fixative effect of this agent. In presence of 0.4 mM glutaraldehyde, in fact, the amount of hemolysis caused by subsequent addition of 5 mM sodium phosphate is only 50–60% that observed in control samples.

Chlorpromazine is a local anesthetic that influences RBC hemolysis [14]. Moreover, this drug is known to decrease the temperature of the thermotropic transition at 25°C in human erythrocyte ghosts [15]. We observed that 0.12 mM chlorpromazine inhibits by 50% hypertonic cryohemolysis, as previously reported [7]. These effects occur only if the drug is present during the entire incubation period [7], thus indicating that they are not due to a permanent membrane modification. This is at variance with all the described treatments, the effects of which are irreversible.

Chymotrypsin digestion of intact RBC did not modify the critical temperatures of hypertonic cryohemolysis, whereas chymotrypsin-digested RBC after an hemolytic

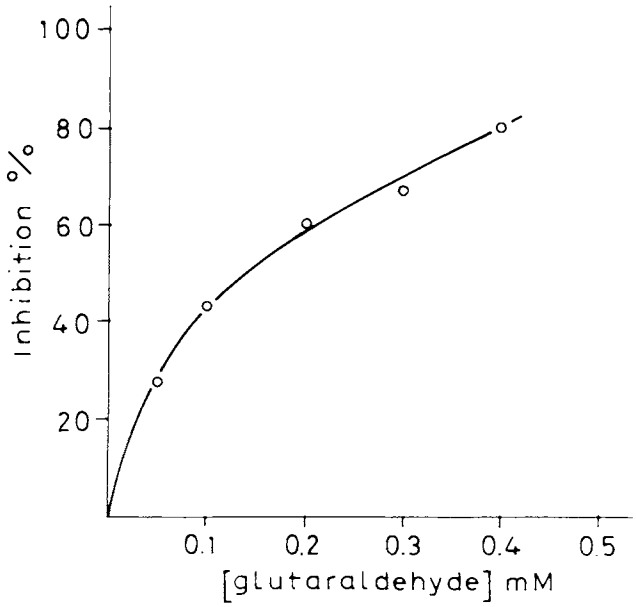

Fig. 5. Inhibition of hypertonic cryohemolysis by glutaraldehyde. RBC were incubated for 1 hr at room temperature. After RBC washings, cryohemolysis was performed as in Figure 4.

shock to allow enzyme penetration inside the cell (hereafter indicated as cytoplasmic chymotrypsin-treated RBC) produced a complete inhibition of hypertonic cryohemolysis (data not shown).

Effect of pH, Glutaraldehyde, and Chlorpromazine Treatment of RBC on Hypotonic Hemolysis

As reported by Lepke and Passow [16], hypotonic hemolysis is affected by pH. Moreover, at pH 5.4 the effect of temperature on hypotonic hemolysis is reduced: $(NaCl)_{50\%}$ ranges from 100 to 95 mM between 0°C and 37°C, respectively (Fig. 2). The closeness of $(NaCl)_{50\%}$ values at 0°C and 37°C hinders the possible detection of this value at intermediate temperatures. A similar result was obtained at pH 9.5 (data not shown). At acidic pH a flattening of the osmotic curve is observed (Fig. 2). To estimate this flattening we report in Table I both the $(NaCl)_{50\%}$ values and the lowest nonhemolysing NaCl concentration, $(NaCl)_h$ (see extrapolation in Fig. 2). The flattening of the osmotic curve can be inferred, in fact, by the difference between $(NaCl)_h$ and $(NaCl)_{50\%}$ values.

The effects of glutaraldehyde and chlorpromazine treatment on the hypotonic hemolysis are reported in Table I. These treatments reduce by 40–60% the temperature dependence of hypotonic hemolysis (Table I). Interestingly, by comparison with untreated RBC, and at variance with all the other RBC treatments, glutaraldehyde produces an increase in the osmotic resistance [compare $(NaCl)_h$] and $(NaCl)_{50\%}$ in Table I). This suggests that low concentrations of glutaraldehyde affect thermotropic membrane properties; nevertheless the osmotic resistance of RBC is increased.

TABLE I. Effects of Membrane Treatments on Hypotonic Hemolysis

RBC treatment	$(NaCl)_h^a$ (mM) 0°C	$(NaCl)_h^a$ (mM) 37°C	$(NaCl)_{50\%}^b$ (mM) 0°C	$(NaCl)_{50\%}^b$ (mM) 37°C	$(NaCl)_{50\%}^{0°C} - (NaCl)_{50\%}^{37°C}$ (mM)
Control	77	63.5	74	61	13
pH 5.4	114	110	100	95	5
pH 9.5	103	96	87	81	6
Glutaraldehyde (0.4 mM)[c]	68	63.5	61	56	5
Chlorpromazine (0.04 mM)	92	85	77	69	8

[a]$(NaCl)_h$ = Lowest nonhemolysing NaCl concentration (see Fig. 2).
[b]$(NaCl)_{50\%}$ = NaCl concentration that produces 50% of hemolysis.
[c]The lysis of 0.4 mM glutaraldehyde-treated RBC in 5 mM sodium phosphate was 50% of control RBC.

Effect of pH, Glutaraldehyde, and Chlorpromazine Treatment of RBC on EPR Spectra

By using several different physicochemical techniques many reports have been published showing the presence of thermotropic structural transitions in RBC membrane between 0°C and 50°C [17–28]. The presence of these transitions suggests a heterogeneous distribution of membrane components, but ^2H NMR results seem to exclude the presence of extended lateral phase separations between membrane components (see [29] and discussion of the accompanying paper [6]). The physical changes involved in the thermotropic breaks are unknown, but there is general agreement that they arise from thermotropic structural changes at the level of lipid or protein-lipid areas [6,17–28].

The spectra of 16-DSA in anisotropic membranes, can be evaluated by measuring the amplitude ratio of lines h_0 and h_{-1} as an empirical fluidity index (see results and discussion of the accompanying paper [6]). In Figure 6 the plot of log (h_0/h_{-1}) against the reciprocal of temperature for RBC and for RBC submitted to different treatments is shown. In control RBC the 16-DSA spin probe identifies three abrupt changes in its freedom of motion at 8, 20, and 40 ± 1.5°C (Fig. 6), and identical thermotropic breaks are obtained with RBC digested externally with chymotrypsin (data not shown).

As for the 40°C transition, acidic pH and chlorpromazine treatments decrease this temperature to 34–35°C, whereas glutaraldehyde completely effaces this break (Fig. 6). Similar results where obtained with 0.25 mM glutaraldehyde (data not shown).

In the temperature range between 0°C and 20°C, the treatment with acidic pH effaces all breaks whereas glutaraldehyde and chlorpromazine efface only one thermotropic break (Fig. 6). In glutaraldehyde-treated RBC the 20°C transition seems to be preserved, whereas chlorpromazine treatment produces a break at 15°C (Fig. 6). This last transition could be attributed to a lowering of the 20°C transition. To check with more accuracy the effect of chlorpromazine on the 20°C break, we used cytoplasmic chymotrypsin-digested RBC. As reported in Figure 2 of the accompanying paper [6], this treatment effaces the protein-dependent 8°C break but maintains the 20°C break virtually unmodified. These membranes, after chlorpromazine treatment, show one break at 15°C (data not shown). This result allows the identification

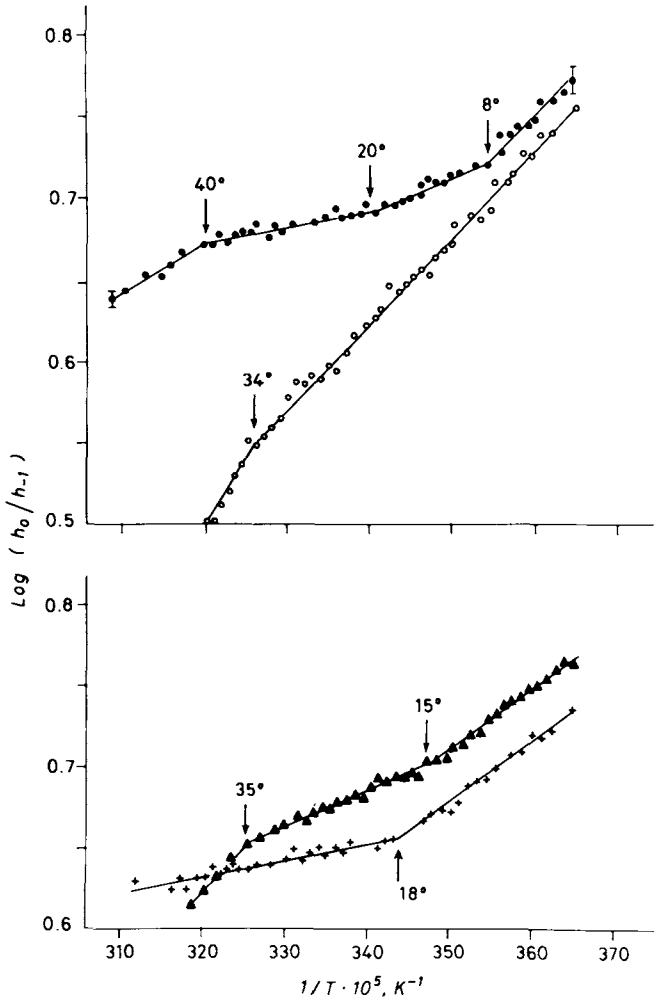

Fig. 6. Effect of different treatments on the plot of log (h_0/h_{-1}) vs 1/T for 16-DSA-labeled RBC. All treatments were performed before the spin label incorporation. (●—●), control RBC; (○—○), pH 5.1-treated RBC; (+—+), 0.4 mM glutaraldehyde-treated RBC; (▲—▲), 0.12 mM chlorpromazine-treated RBC. Points are average values of four spectra. Experimental errors usually decrease with temperature increasing: typical ± SEM are indicated. Break temperatures were determined by computer analysis.

of this transition and suggests that the 15°C break of chlorpromazine-treated RBC is a drug-induced lowering of the 20°C transition. In this respect our data are in accordance with published results obtained with a different spin label [15] and indicate a drug-induced perturbation of protein–lipid interactions [6].

DISCUSSION

Data reported show that both hypertonic cryohemolysis and hypotonic hemolysis present two critical temperatures at about the same values (Figs. 1, 3). Thus the

peculiar temperature dependence known up to now only for hypertonic cryohemolysis seems to be present also in hypotonic hemolysis. The hypothesis of structural thermotropic transitions in RBC membrane at temperatures of the above reported critical values seems to be plausible. To test this hypothesis the following two conditions should be fulfilled as a prerequisite: 1) The influence of temperature on RBC hemolysis must show critical values at temperatures identical or similar to those of thermotropic transitions detected by spectroscopic techniques; 2) membrane modifications that change the influence of temperature on RBC hemolysis must also change thermotropic transitions.

The first condition is supported by Figures 1, 3, and 6. At approximately the same temperatures of thermotropic transitions detected by 16-DSA a change in the hypo- or hypertonic hemolysis properties of RBC is observed. The transitions at both 8°C and 20°C appear to be relevant to RBC hemolysis. In fact, at temperatures below that of the first transition (8°C), RBC are easily lysed in both hypotonic and hypertonic media, whereas above 20°C the RBC membrane is more resistant to osmotic shocks (Figs. 1, 3).

To prove the second condition we studied the effects of some treatments on both hemolysis and thermotropic transitions. RBC treatments inhibit hypertonic cryohemolysis (Figs. 4, 5) and decrease the influence of temperature on hypotonic hemolysis (Table I). Moreover, looking at thermotropic transitions detected by the spin label, the effect common to all membrane treatments is the disappearance of the 8°C thermotropic break (Fig. 6).

A role for the 20°C transition in RBC hemolysis is suggested by the critical hemolytic behavior of RBC around 20°C (Figs. 1, 3). Nevertheless there is no membrane treatment, among those reported, that effaces the 20°C transition and preserves the 8°C transition, and we cannot say whether or not the lack of only the 20°C transition is sufficient to affect RBC hemolysis.

A possible hypothesis to explain the correlation between thermotropic transitions and hypertonic cryohemolysis is that lysis occurs when the membrane structural organization present above 20°C jumps to the structural organization present at temperatures lower than 8°C. In accordance with the hypothesis we suggested for ghost resealing process [1], different membrane structural organizations responsible for RBC cryohemolysis may be thermotropic lateral phase separations between membrane components (see also discussion in the accompanying paper [6]). Temperature-dependent membrane structures can produce, in fact, non-ideal mixing between membrane components (phase boundaries). In hypotonic buffers the decreased osmotic resistance below 10°C may be due to the presence of "boundary effects" between differently ordered membrane components, and above 20°C improved mixing of membrane components may be responsible for the increased osmotic resistance.

However, this hypothesis requires, in order to be proved definitively, that the time scale of the biological phenomena (ie, loss of permeability barrier) and the time scale of different membrane structural reorganization be comparable. Or in other words, it is required that the thermotropic structural changes be present in the membrane for a period of time sufficient to allow hemoglobin release. This conclusion cannot be reached by EPR results essentially because 1) the physical changes involved in the thermotropic breaks are unknown and 2) the time scale of the EPR technique is too short ($\sim 10^{-8}$sec) to prove the existence of spatially distinct domains but do not exclude this possibility (see also the discussion of the accompanying paper [6]).

In addition to hemolysis, different RBC membrane phenomena present thermotropic discontinuities at temperatures similar to or coincident with thermotropic transitions detected by spectroscopic techniques (ie, water exchange [30], ion and sugar transport [31,32], Sendai virus–RBC interaction [33], lateral diffusion of intrinsic proteins [25], cell viscosity [17], ghost resealing [1,3], membrane-associated enzymatic activity [15]). The hypothesis of a correlation between physicochemical and biological phenomena implies, therefore, that thermotropic structural changes occur on a relatively low time scale compatible with above mentioned membrane processes.

The experiments designed to investigate a relationship between RBC hemolysis and membrane thermotropic transitions allow some considerations about the effects of different treatments. The effect in common to all treatments seems to be a modification of cytoplasmic exposed proteins. In fact, besides effects on the spin label [34] or on other proteins [35] or lipids [36], acidic pH strongly modifies cytoplasmic exposed proteins [37] and, further, these proteins are preferentially cross-linked by glutaraldehyde [6,12]. Chlorpromazine is a RBC cup-former [38] and, according to the bilayer-couple hypothesis [39], its effects on shape may be due to a drug partition into the inner monolayer. The effects of these treatments on both hemolysis and membrane thermotropic transitions suggest the presence of cytoplasmic localized targets especially critical with respect to these phenomena. The possibility of developing more specific agents to modify these targets, together with the understanding of physical changes involved in thermotropic transitions, may be crucial to obtain a conclusive picture of thermotropic structural and functional membrane phenomena.

ACKNOWLEDGMENTS

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