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THE ERYTHROCYTE MEMBRANE SITE FOR THE EFFECT OF TEMPERATURE ON OSMOTIC FRAGILITY

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Summary

The osmotic fragility of human erythrocytes is well known to decrease as the temperature is elevated. The cellular site for the temperature effect was studied by assessing possible roles of hemoglobin and of membrane lipids and by taking advantage of the unique response of camel erythrocytes to temperature. It is concluded that the erythrocyte membrane is the site for the temperature effect on osmotic fragility. The human erythrocyte is likely to rupture in protein-lipid boundary regions in the membrane, from which cholesterol is apparently excluded.

Introduction

The osmotic fragility of human erythrocytes is known for a long time to be temperature dependent: the higher the temperature during hemolysis in hypotonic solutions, the more stable the red cells appear to be [1—4]. The phenomenon has been attributed to different mechanisms. Jacobs and Parpart [1] concluded that the differences in osmotic fragility resulted from changes in the osmotic activities of the cells under the different temperatures, which in turn give rise to changes in the volume of the cells in isotonic media. In contrast, Murphy [2] demonstrated that the volume of red cells in serum was similar at 5—37°C, while the critical hemolytic volume was altered by temperature. Murphy concluded that the differences in osmotic fragility at different temperatures resulted from changes in the membrane. Seeman et al. [3] suggested that the increased K^+ leakage at elevated temperature, observed by Davson [5] may explain the reduced fragility at the higher temperature. However, Livne and Raz [4] measured essentially identical prelytic K^+ leakage at different temperatures (5—37°C), concluding that the effect of temperature on homolysis is associated not with a modified K^+ release, but with a direct effect on the cell membrane.

To sum up, two alternative explanations have been proposed; one, which relates the effect of temperature to intracellular phenomena and the other, which attributes it to the membrane. The present study re-examines the issue. It is illustrated that indeed the erythrocyte membrane is the site of the temperature effect and it is attempted to identify the membrane site.

Experimental

Fresh blood samples were obtained in heparin (20 units/ml). Stock suspensions (30%) of washed erythrocyte in a solution of 155 mM NaCl/2 mM sodium phosphate, pH 7.4, were prepared as described [6].

Osmotic fragility of erythrocytes. An aliquot (20 μl) of the erythrocyte stock suspension was rapidly mixed with 3 ml of hypotonic NaCl solution, buffered with 2 mM sodium phosphate, pH 7.4. Following an incubation for 10 min at a given temperature, the suspensions were centrifuged at 2000 $\times g$ for 3 min and the supernatant analyzed for hemoglobin content [6]. 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.

Preparation and osmotic fragility of resealed ghosts. Ghosts of camel and human erythrocytes, free of hemoglobin, were prepared as described [7], essentially according to Dodge et al. [8]. To obtain membrane-free hemolysate, packed erythrocytes were osmotically hemolysed at 4°C, by mixing with 3 volumes of distilled water, followed by centrifugation at 4°C for 20 min at 20 000 $\times g$. The hemolysate was recentrifuged to assure the removal of membranes and then concentrated several fold in a dialysis bag embedded in Sephadex G-200. Resealed ghosts were prepared according to Redman [9]. Membrane-free hemolysate, diluted as desired with distilled water, was augmented with 50 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$ and 10 mM Tris · HCl pH 7.2 (final concentrations). 10 volumes of the hemolysate were mixed with 1 volume of packed ghosts and incubated for 30 min at 37°C. The suspensions were then centrifuged at 20 000 $\times g$ for 20 min, the supernatant removed and the ghosts washed by resuspending in the isotonic medium followed by centrifugation. The packed resealed ghosts were suspended with an equal volume of the medium and kept at 4°C, to be analyzed within 2 h. Osmotic fragility of the resealed ghosts was essentially as described for erythrocytes, except for the aliquot sampled (0.2 ml) and the centrifugation applied (20 000 $\times g$ for 10 min).

Osmotic fragility of liposomes. Liposomes were prepared according to Bangham et al. [10] and Kleinschmidt et al. [11] as modified by Alhanaty and Livne [12] from erythrocyte lipids [13]. The osmotic fragility of liposomes was based on the release of 2,2'-dinitro-5,5'-dithiobenzoic acid (NbS_2) as described before [12]. Complete lysis was affected by Triton X-100 (0.3 mg/ml final concn.).

Determinations. Hemoglobin was determined according to the method of Drabkin [14]. The experiments described were repeated at least four times with 3—5 replicates.

Results

Species differences

Are human erythrocytes unique in exhibiting a temperature dependence of osmotic fragility? Fig. 1 presents hemolysis curves of erythrocytes from several species. In addition to those shown, canine and porcine erythrocytes were also

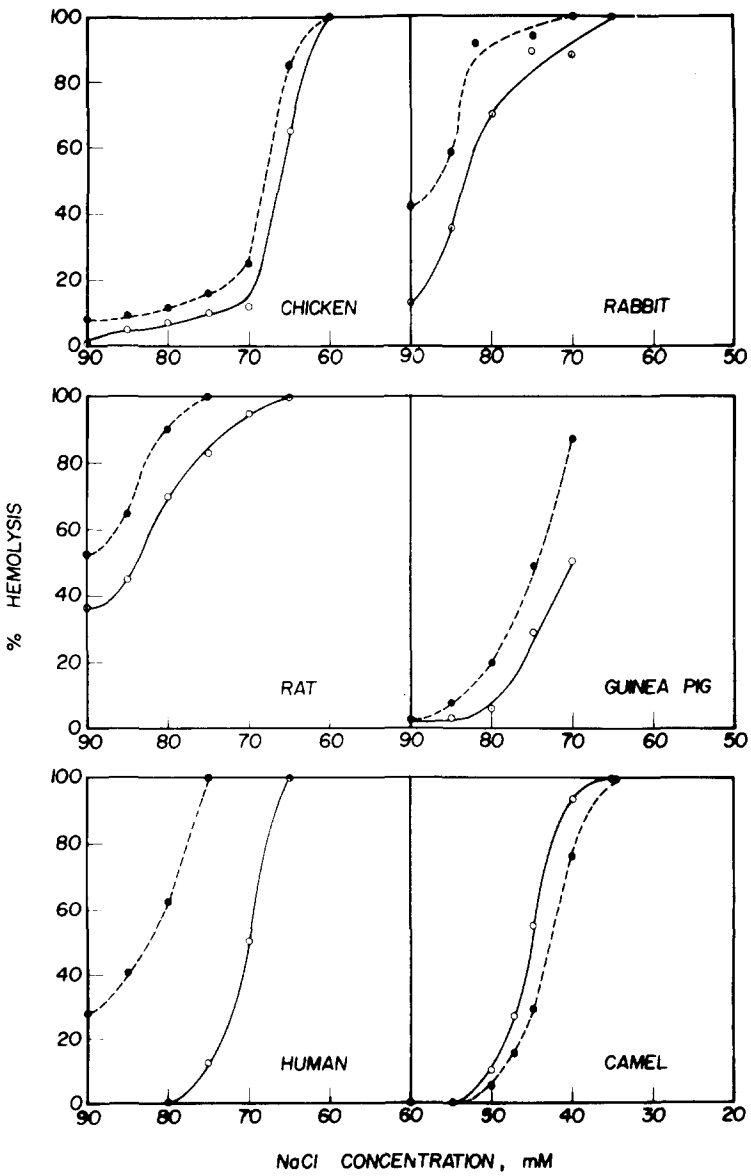


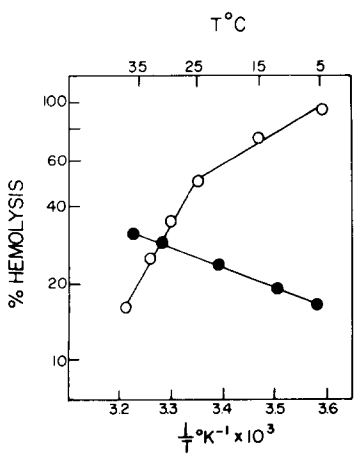
Fig. 1. Osmotic fragility of erythrocytes from several species at 0°C (dashed lines) or at 37°C (solid lines).

tested. The erythrocytes of all the species examined, with a single exception, were osmotically more stable at 37 than at 0° C. Camel erythrocytes, however, are not only particularly stable but are outstanding in exhibiting greater stability at the lower temperature. The contrasting effect of temperature on osmotic fragility of human and camel erythrocytes is further documented in Fig. 2.

Role of hemoglobin

Rehemolysis of resealed ghosts, containing defined fractions of their original hemoglobin, has been used by Hoffman to evaluate the role of hemoglobin in the mechanism of lytic hemolysis [15]. We have adopted this approach with certain modifications (note Experimental) to study the effect of temperature on hemolysis. If hemoglobin does indeed play a significant role in the sensitivity of osmotic fragility to temperature, then the temperature-dependent shift in hemolysis curves should correspond with the hemoglobin concentration. However, Table I shows that the shifts in hemolysis, caused by the change of temperature, are rather similar in both intact erythrocytes and resealed ghosts, despite the large differences in hemoglobin concentration. The resealed ghosts appear to be generally more stable osmotically probably due to an increased membrane permeability, compared to intact erythrocytes.

The presumed role played by hemoglobin, as advocated previously [1,15], should be considered with reference to the external ionic medium, either to sustain the "base-binding power of the hemoglobin" [1] or to allow for the Donnan swelling, which is fundamental for the colloid-osmotic mechanism of hemolysis [15]. Therefore we studied the effect of temperature on osmotic fragility in a non-ionic medium. Sucrose, to which the erythrocyte membrane is impermeable [16], was chosen for this experiment. Fig. 3 demonstrates



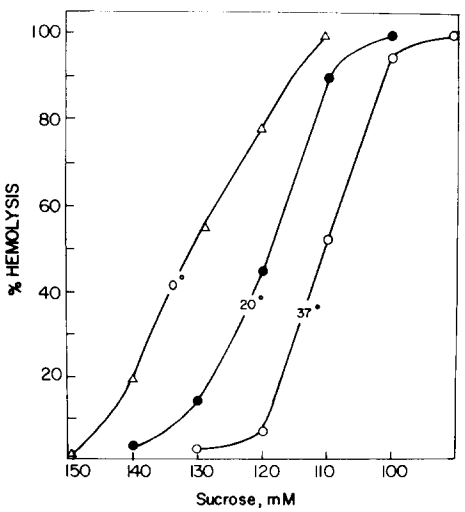


Fig. 2. Effect of temperature on osmotic fragility of human and camel erythrocytes. Human erythrocytes (○) were analyzed at 70 mM NaCl, while the camel cells (●) at 47 mM NaCl.

Fig. 3. Osmotic fragility of human erythrocytes in sucrose solutions as affected by temperature.

TABLE I

OSMOTIC FRAGILITY OF HUMAN ERYTHROCYTES AND RESEALED GHOSTS AT TWO TEMPERATURES

	Hemoglobin concentration (g/100 ml)	NaCl concn. of the medium for 50% hemolysis (mM)		Shift in hemolysis (mM NaCl)
		0°C	37°0	
Erythocytes	31.9	84	73	11
Resealed ghosts	13.2	55	45	10
Resealed ghosts	8.7	53	44	9
Resealed ghosts	5.0	53	42	11
Resealed ghosts	2.4	49	38	11

typical hemolysis curves in sucrose solutions. Evidently, erythrocytes in sucrose solutions are affected by temperature just as in NaCl solutions [4].

The marked differences in behavior of human and camel erythrocytes prompted us to conduct the following cross experiment. Hemoglobin-free ghosts, prepared from human or camel erythrocytes were resealed with hemolysate of the other species and the resealed ghosts were then tested for osmotic fragility at either 37 or 0°C. Fig. 4 shows that temperature differentially

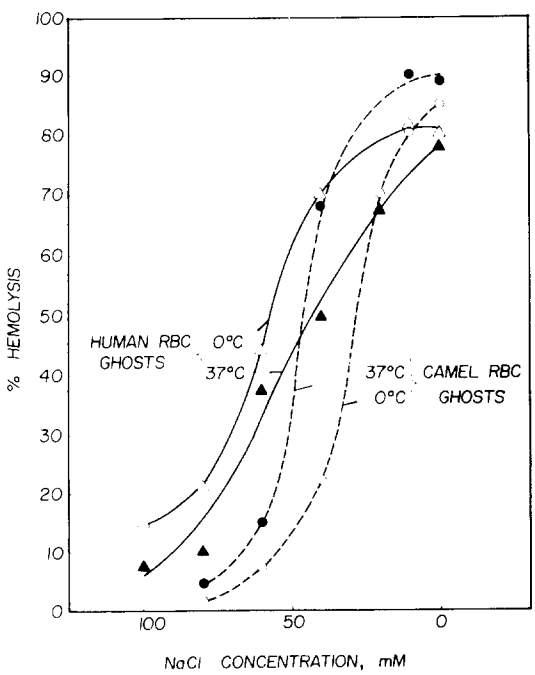


Fig. 4. Effect of temperature on the osmotic fragility of composite resealed ghosts, reflecting the origin of the membranes. Ghosts of camel red blood cells (RBC) were resealed with hemolysate from human erythrocytes and human RBC ghosts resealed with hemolysate from camel erythrocytes. Hemoglobin concentration: 4 g/100 ml. Similar results were obtained with more concentrated hemolysates (up to 14 g hemoglobin/100 ml).

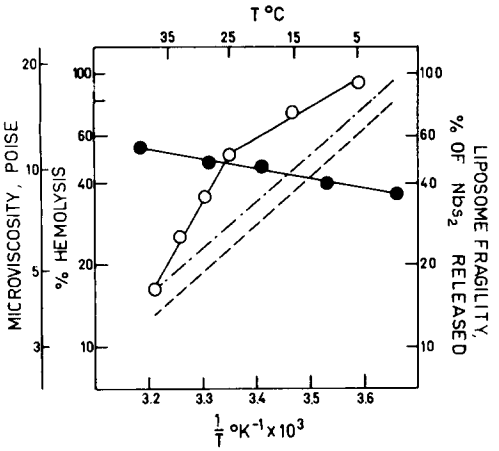


Fig. 5. Effect of temperature on the osmotic fragility of human erythrocytes (○) and of liposomes (●) as well as on the microviscosity in the hydrocarbon core of human erythrocyte membranes (– – –) and in liposomes (— · — · —). The viscosity data reproduced from Aloni et al. [17].

affected the fragility of the resealed ghosts, the pattern being distinctly dictated by the origin of the membranes and not by the origin of the hemolysate.

Role of membrane lipids

The capacity of the erythrocyte to withstand an expansion in a hypotonic medium as affected by temperature, may be entirely a property of the lipid matrix. In such a case, liposomes prepared from erythrocyte lipids, are expected to respond to temperature similarly to erythrocytes. Fig. 5 illustrates the effect of temperature on the osmotic fragility of human erythrocytes and of liposomes. The temperature-dependence of microviscosity of the two membrane systems [17] is reproduced in Fig. 5 to enable a close comparison of the two sets of data. Evidently, the behavior of liposomes and erythrocytes differ in several ways. Whereas the osmotic fragility of erythrocytes decreases as the temperature is elevated, the fragility of liposomes actually goes up, despite parallel changes in microviscosity in response to temperature. Furthermore, the logarithmic plot of liposome osmotic fragility is linear, while the temperature dependence plot of the erythrocyte system is characterized by two slopes with a break at 25° C.

Concerning the role of membrane lipids, it is also of interest that liposomes prepared from camel erythrocyte lipids and from human erythrocyte lipids show similar effects of temperature on osmotic fragility and on microviscosity.

Discussion

The conclusion [2,4] that the erythrocyte membrane is the site for the temperature effect on osmotic fragility, is further supported by several lines of evidence: (a) Irrespective of the hemoglobin concentration, osmotic fragility of erythrocytes and resealed ghosts were likewise modified by temperature (Table I); (b) resealed ghosts derived from human and camel erythrocytes typ-

ically responded to temperature according to the origin of the membrane (Fig. 4), and the membrane of camel erythrocytes is indeed unique [7,18,19]; (c) the great similarity in the effect of temperature on osmotic fragility in ionic [4] and in sucrose (Fig. 3) media is in harmony with a direct effect of temperature on the membrane, rather than with an indirect effect involving hemoglobin and ions [1,15].

As yet, a mechanism which fully explains the role of the membrane in determining the effect of temperature on osmotic fragility, is not available. One conceivable explanation is that with elevating temperature the erythrocyte membrane may expand, thus allowing the cell to swell to a larger hemolytic volume. However, this suggestion is unlikely as both the isotonic cell volume [2] and the critical hemolytic volume [3] are not modified by temperature.

We may assume that the erythrocyte lipids which constitute the continuous phase of the membrane are associated with the temperature effect. Liposomes indeed behave as osmometers [20,21] and erythrocyte membranes and liposomes show similar properties in term of thickness [22] and permeability [20, 23—25]. Both systems respond to pharmacological agents [26] and their osmotic fragility is similarly affected, qualitatively, by several antihemolytic compounds [12]. However, on the basis of the clear differences between liposomes and human erythrocytes in response to temperature, lipids cannot be solely equated with the site for the temperature effect, and membrane proteins must be involved. This conclusion is reinforced by the observation that liposomes made from human and camel erythrocytes responded in terms of osmotic fragility very similarly to temperature, despite the marked differences in behavior of the cells.

We propose that the human erythrocyte ruptures osmotically in regions of low cholesterol content. Indeed, cholesterol is known to promote membrane stability: removal of cholesterol from erythrocytes increases their osmotic fragility [27], while erythrocytes [28] and liposomes [12] enriched with cholesterol are osmotically more stable. Several recent studies indicate that protein-lipid boundary regions in the membrane are typically deficient in cholesterol. Warren et al. demonstrated that ATPase (from sacroplasmic reticulum) with a complete phospholipid annulus is not inhibited by cholesterol. However, as cholesterol replaces phospholipids in the annulus, ATPase activity is progressively inhibited. It was thus concluded that cholesterol is excluded from the first shell of the lipid bilayer surrounding the protein [29]. Using paramagnetic quenching of protein fluorescence by nitroxide lipid analogues, Bieri and Wallach concluded that much of the sterol in the erythrocyte ghost membrane is in clustered state, distributed away from penetrating proteins [30].

The differences in behavior of human and camel erythrocytes with respect to the temperature effect are apparently related to the dominant structural features typical for each system. In the human erythrocyte membrane, lipids comprise the continuous phase, whereas protein-protein interaction of integral proteins, presumably constituting an "integral skeleton", appears to be a dominant feature stabilizing the camel erythrocyte membrane [7]. Thus, while protein-protein electrostatic forces are of particular importance, hydrophobic interactions between lipids and proteins apparently play, relatively, a lesser role in stabilizing the camel erythrocyte membrane. Such a balance may have much in

common with the unique temperature dependence of osmotic fragility of camel erythrocytes (Figs. 1 and 2).

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