Bioelectrochemistry 73 (2008) 23–29

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Effects of glycerol and sorbitol on the thermal dependence of the lysis of human erythrocytes by ethanol

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ARTICLE INFO

Article history:
Received 27 February 2007
Received in revised form 27 March 2008
Accepted 4 April 2008
Available online 13 April 2008

Keywords:
Erythrocytes
Ethanol
Membrane stability
Osmolytes
Temperature

ABSTRACT

In this work, the effects of 1 mol/L glycerol or sorbitol on the thermal dependence (27–47 °C) of the lysis of human erythrocytes by ethanol in saline solution (0.154 mol/L NaCl) have been evaluated. Lysis was monitored by measurement of the absorbance at 540 nm. Ethanol produced either lysis or protection against lysis depending on the conditions. These antagonistic effects are attributed to the existence of expanded (*R*) and compacted (*T*) erythrocytes, present under conditions of low and high osmolarity, respectively. The transitions of lysis of the *R* state and formation and lysis of the *T* state were all found to be sigmoidally defined. The ethanol concentration at the midpoint of the lysis transition of the *R* state (D_{50R}) was found to decrease with increasing temperature and osmolarity. In the presence of glycerol or sorbitol, an increase in temperature led to smaller decreases in D_{50R} and osmotic protection against lysis. The ethanol concentration at the midpoint of formation (S_{50T}) and lysis (D_{50T}) of the *T* state also decreased with increasing temperature and osmolarity. Lysis of *R* state erythrocytes is determined by the chaotropic action of ethanol, but the formation and lysis of *T* state erythrocytes are determined by osmotic pressure effects.

1. Introduction

The lipid bilayer that constitutes a biological membrane is primarily stabilized by the hydrophobic force, which compels the hydrophobic tails of phospholipids to join together. Secondary stabilization is provided by van der Waals attractive forces, which keep these groups gathered together in the anhydrous interior of the membrane, and by hydrogen bonds formed between the polar heads of the phospholipids and water, both within the cell and at its external surface [1,2]. The cytoplasmatic membrane constitutes the first cell target that suffers damage caused by external denaturing agents such as heat or ethanol. Heat increases the vibrational energy of chemical groups, favoring rupture of the non-covalent bonds that stabilize the native structure of the biological complex. Ethanol specifically decreases the intensity of the hydrophobic force by solvating the apolar groups that were hidden inside the native structure of the biological complex [3].

The mechanisms that living organisms use to combat denaturation include the controlling of osmotic pressure through the production and storage of osmostabilizing solutes, known simply as osmolytes, such as glycerol (1,2,3-propanetriol) and sorbitol (1,2,3,4,5,6-hexanehexol) [4,5]. The origin of the effects of glycerol and sorbitol on the stabilization of proteins has been extensively investigated, generally on the basis of thermodynamic considerations. The way in which these solutes stabilize a protein does not involve any direct interaction with it, merely the exclusion of other solutes from the hydration shells close to the macromolecule [6]. The equilibrium between the native (*N*) and unfolded (*U*) states of the protein is shifted in the direction of the more compact hydrated state (*N*). Hydration involves the expenditure of free energy; since the *N* state has a smaller external surface than the *U* state, the *N* state is stabilized relative to the *U* state [6–12]. However, the origin of the stabilizing effect of osmolytes on biological membranes does not seem to be presented in this way in the literature, although osmostabilization is a biotechnological procedure used in the cryopreservation of erythrocytes and other cells [13–18].

The shape of an erythrocyte is determined by a combination of factors, namely the membrane structure and conformation as well as the composition and physicochemical properties of the internal and external media. Shape alterations are associated with changes in the curvature of the lipid bilayer [19–21] and in the conformation of the

Abbreviations: *R*, population of erythrocytes in the expanded or relaxed state; *T*, population of erythrocytes in the tight or compacted state; D_{50R}, ethanol concentration that promoted 50% lysis of the *R* state erythrocytes; S_{50T}, concentration of ethanol that promoted 50% stabilization of the erythrocytes; D_{50T}, concentration of ethanol that promoted 50% lysis of *T* state erythrocytes; saline, 0.154 mol/L NaCl.

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doi:10.1016/j.bioelechem.2008.04.002

membrane skeleton network [22,23]. In a physiological medium, erythrocytes are stabilized in a discocyte shape, in which their membranes have the minimum conformational energy in relation to the constraints imposed by the external conditions, and the minimum cell volume and area [24,25]. Modifications in the internal medium are responsible for shape and property changes in erythrocytes [26]; sickle cell anemia and other hemoglobinopathies are living proof of this fact. Changes in the membrane composition also alter the area and the spontaneous curvature of the lipid bilayers [27–29]. The increase in stability in the membranes of erythrocytes with aging [30] may be a consequence of changes in their composition.

The composition and physicochemical properties of the external medium also affect the conformation [20,28,31] and properties [32–35] of the erythrocyte membrane. Glycerol converts biconcave erythrocytes into cells with membrane internalizations (stomatocytes). Ethanol converts biconcave erythrocytes into cells with externalizations (echinocytes). Both of these solutes decrease the surface area with the formation of smooth spheres [28].

Ethanol is also capable of promoting the lysis of erythrocytes, which has been attributed to the denaturing of membrane proteins [36]. The capacities of ethanol to generate stable erythrocytes and also to promote hemolysis constitute a paradox that can be explained in terms of its respective actions as an osmolyte and as a chaotropic agent. The action that prevails depends on several factors, such as the concentration of the ethanol, the presence of other osmolytes, and the temperature [37].

In an attempt to establish a correlation between the effects of stabilizing and denaturing agents on proteins and erythrocytes, a theoretical model was proposed whereby the shapes of erythrocytes under physiological conditions were considered as belonging to a morphological state named the relaxed state (R), whereas osmolarly contracted erythrocytes were considered as belonging to a tight state (T) [37]. In the present work, this model has been used in order to interpret the effects of glycerol and sorbitol on the lysis of erythrocytes induced by ethanol at different temperatures (27–47 °C).

2. Experimental

2.1. Chemicals

Absolute ethanol (PA, 99.3% v/v) and glycerol (ACS grade, 99.8%) were purchased from Quimex (São Paulo, SP, Brazil) and USB (Cleveland, Ohio, USA) and utilized without further purification.

2.2. Equipment

Reagents were weighed using a digital balance (AND, model 870) and volumes were determined using refractory glass burettes or automatic pipettes (Labsystems, model Finnpipette Digital). Thermal control of incubations was achieved by means of a thermostatted water bath (Marconi, model MA 184, Piracicaba, SP, Brazil). Absorbance measurements were made on a Micronal UV/Vis spectrophotometer (model B-442, São Paulo, SP, Brazil).

2.3. Blood sample collections

This work was previously approved by our institutional ethics committee. The blood samples (5 mL) were collected in evacuated flasks containing 50 μL of 25 mmol/L K_2EDTA from the antecubital vein of healthy young males after 8–12 h of fasting.

2.4. Stability of human erythrocytes

To evaluate the effects of glycerol and sorbitol on the stability of human erythrocytes in a saline medium, we used a set of Eppendorf flasks containing 1 mL aliquots of solutions containing between 0 and 5 mol/L glycerol or 0 and 1.5 mol/L sorbitol in 0.154 mol/L NaCl (saline solution), which were pre-incubated in a water bath (27, 32, 37, 42, and 47 °C) for 10 min. To evaluate the effects of the ethanol concentration (in a saline solution under physiological conditions) on the behavior of human erythrocytes, we prepared a set of Eppendorf flasks containing 1 mL aliquots of 0 to 6.8 mol/L ethanol in saline, which were pre-incubated in a water bath (27, 32, 37, 42, and 47 °C) for 10 min. To examine the influences of 1 mol/L glycerol and 1 mol/L sorbitol on the effect of ethanol on the human erythrocytes, we prepared two further sets of Eppendorf flasks containing 1 mL aliquots of 0 to 6.8 mol/L ethanol and 1 mol/L glycerol or 1 mol/L sorbitol in saline, which were also pre-incubated in a water bath (27, 32, 37, 42, and 47 °C) for 10 min. After pre-incubation, 25 μL aliquots of blood were added, and the mixtures were homogenized, incubated for 30 min, and centrifuged at the temperature of each experiment for 10 min at 1300 g. Thereafter, the absorbance of each supernatant was read at 540 nm (A_{540}) against respective individual control solutions with the same composition but without the blood sample. The Eppendorf flasks were kept hermetically sealed during the course of the experiments.

2.5. Analytical methods

Calculations, data manipulations, and statistical analyses were performed using the applicative OriginPro 7.5 software (Microcal Inc., Northampton, Massachusetts, USA). In assessing the lysis transitions, the dependences of the absorbances at 540 nm (A) on the ethanol concentrations (D) were adjusted to sigmoidal lines according to the Boltzmann equation:

$$A = \frac{A_1 - A_2}{1 + e^{(X - X_{50})/\Delta X}} + A_2 \quad (1)$$

where A_1 and A_2 represent the mean absorbance values at the first and second plateaus of the sigmoid, X is the ethanol concentration, X_{50} is the ethanol concentration at the midpoint of the sigmoid, and ΔX represents the amplitude of the sigmoidal transition between A_1 and A_2. For the lysis transitions of the R and T morphological states of the erythrocytes, X_{50} was defined as D_{50R} and D_{50T}, respectively. For the stabilization transition, X_{50} was defined as S_{50T}. The percentage of hemolysis in each tube was calculated by multiplying its absorbance value by 100 and dividing by the mean maximum absorbance in each experiment. The height of the stabilizing curve was designated as P_S, and was obtained from the difference between A_1 and A_2 for the stabilization transition.

2.6. Statistical methods

Each sigmoidal fitting was considered valid only for $p<0.05$. The thermal dependences of the transition midpoints (D_{50R}, S_{50T}, and D_{50T}), obtained in the absence or presence of 1 mol/L osmolyte (glycerol or sorbitol), were analyzed by linear regression, with $p<0.05$ indicating statistically significant fittings.

3. Results

The incubation of human erythrocytes for 30 min in 1 mol/L glycerol or sorbitol in a 0.154 mol/L NaCl solution (control) produced only negligible lyses at the temperatures employed. At 37 °C, A_{540} displayed a dependence on the glycerol concentration that could be described by the equation $A_{540} = 0.008 + 0.015$ [glycerol] (mol/L) up to 3.28 mol/L glycerol; thereafter, there was an acceleration in the lysis rate that could be described by the equation $A_{540} = -1.81 + 0.571$ [glycerol] (mol/L). At 37 °C, A_{540} varied with the sorbitol concentration according to the equation $A_{540} = 0.0209 + 0.077$ [sorbitol] (mol/L). Under our experimental conditions, the hemolysis induced by glycerol or sorbitol never exceeded 2% or 3%, respectively. In fact, incubation of

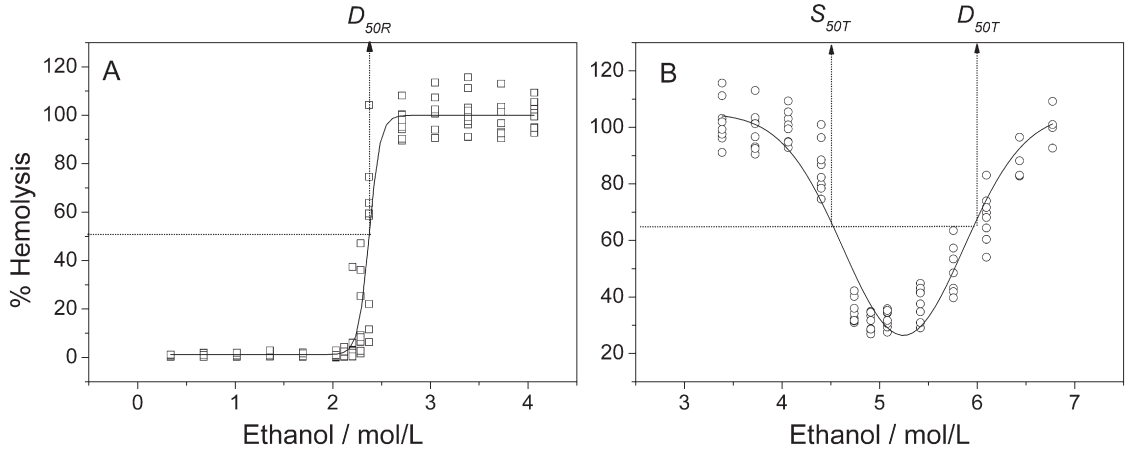

Fig. 1. Dependence of the percentage of hemolysis on the ethanol concentration in physiological-like saline solution at 37 °C. Plot A represents the ethanol-induced lysis of the morphological *R* state of the erythrocyte. This transition is characterized by the ethanol concentration that promotes 50% hemolysis (D_{50R}) in the *R* state of the erythrocytes. Plot B represents the ethanol-induced generation of a stabilized *T* state in the erythrocytes, characterized by its midpoint transition (S_{50T}), followed by an ethanol-induced lysis of this stabilized *T* state, characterized by its midpoint transition (D_{50T}).

erythrocytes with 7.53 mol/L glycerol for 30 min produced just 2% hemolysis at 20 °C [28].

The effect on human erythrocytes of saline solutions of ethanol was followed by monitoring the dependence of A_{540} on the ethanol concentration (Fig. 1). It is important to state that these results were obtained from fixed-time incubations (30 min) of human blood with solutions containing increasing concentrations of ethanol in 0.154 mol/L NaCl. They do not represent the effects of titrating erythrocytes with ethanol.

At low concentrations of ethanol (Fig. 1A), increasing the chaotropic concentration caused a sigmoidal increase in A_{540}. This sigmoid represents the transition of the ethanol-induced lysis for the erythrocyte population present in 0.154 mol/L NaCl, which was considered to exist in the so-called *R* state, in analogy to the expanded or relaxed state of proteins. This sigmoidal lysis transition was characterized by the parameter D_{50R}, which represents the ethanol concentration capable of producing 50% hemolysis.

At higher concentrations of ethanol (Fig. 1B), a decrease followed by another increase in A_{540} were observed, in a trend that constitutes an inverted Gaussian line. This behavior may be interpreted in terms of the occurrence of a stabilizing effect of the ethanol, giving rise to a decreasing sigmoid, followed by the lysis transition of the stabilized population of erythrocytes, giving rise to an increasing sigmoid. The Gaussian line (Fig. 1B) is the sum of these sigmoids. The erythrocyte population present at the base of the Gaussian line, under higher osmolarity conditions, was considered to be in a state referred to as the *T* state, in analogy with the tight or tense state of proteins. The sigmoidal stabilization transition of the erythrocytes (Fig. 1B) was characterized by the sigmoid midpoint, S_{50T}, which represents the concentration of ethanol capable of stabilizing 50% of the erythrocytes. The sigmoidal lysis transition of these stabilized erythrocytes was characterized by the sigmoid midpoint, D_{50T}, which represents the concentration of ethanol capable of promoting 50% lysis of the stabilized erythrocytes. These transition midpoints (D_{50R}, S_{50T}, and D_{50T}) were determined at 27, 32, 37, 42, and 47 °C in saline solution and in saline solutions of 1 mol/L glycerol and 1 mol/L sorbitol. The thermal dependences of D_{50R}, S_{50T}, and D_{50T} are shown in Figs. 2–4, respectively.

Within the considered thermal interval, the increase in temperature produced statistically significant decreases ($p<0.01$) in D_{50R} (Fig. 2), S_{50T} (Fig. 3), and D_{50T} (Fig. 4). The decrease in D_{50R} (Fig. 2) is probably a consequence of the synergism between the chaotropic effects of heat and ethanol. If the stabilizing effect of ethanol on erythrocytes were based on the osmolarity increase promoted by higher ethanol concentrations (Fig. 1B), then the observed temperature-dependent reduction in S_{50T} may be a result of the osmotic

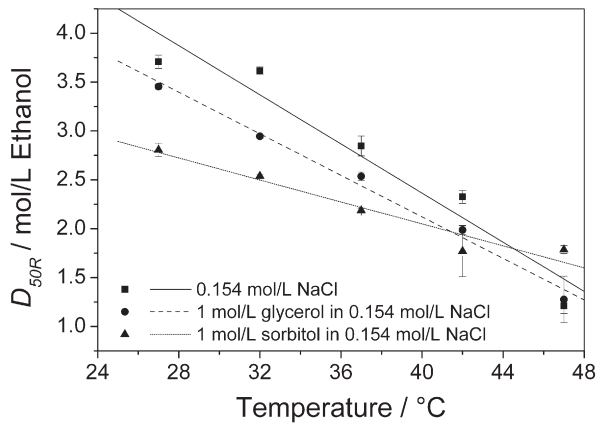

Fig. 2. Comparison of the effects of saline solutions of 1 mol/L glycerol and 1 mol/L sorbitol, in relation to a control saline solution, on the thermal dependence (27–47 °C) of the stability of the relaxed (*R*) state of human erythrocytes (D_{50R}) against lysis by saline solutions of ethanol. Values represent means ± SD ($n=6$).

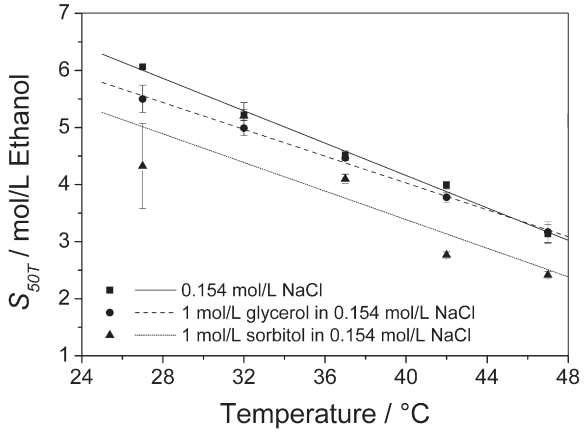

Fig. 3. Comparison of the effects of saline solutions of 1 mol/L glycerol and 1 mol/L sorbitol, in relation to a control saline solution, on the thermal dependence (27–47 °C) of the generation of the stabilized *T* state (S_{50T}) of human erythrocytes by saline solutions of ethanol. Values represent means ± SD ($n=6$).

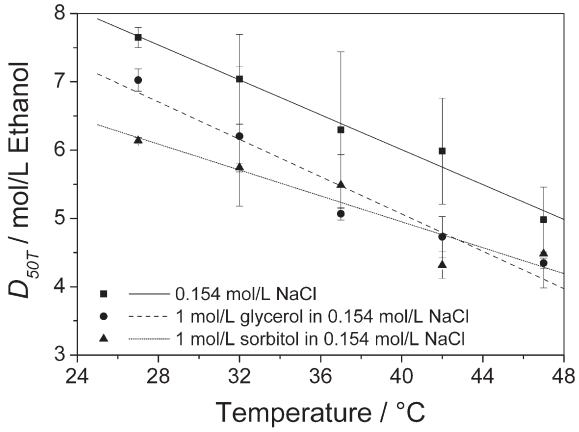

Fig. 4. Comparison of the effects of saline solutions of 1 mol/L glycerol and 1 mol/L sorbitol, in relation to a control saline solution, on the thermal dependence (27–47 °C) of the stability of the *T* state of human erythrocytes (D_{50T}) against lysis by saline solutions of ethanol. Values represent means ± SD (n=6).

pressure augmentation produced by heat (Fig. 3). Since the increase in temperature also leads to a decrease in D_{50T}, the osmotic pressure increase caused by the actions of heat and the ethanol osmolarity must also be an important factor in the lysis of the *T* erythrocytes. The incorporation of 1 mol/L glycerol or sorbitol also produced significant reductions in the values of D_{50R} (Fig. 2), S_{50T} (Fig. 3), and D_{50T} (Fig. 4) in relation to the control saline solution, indicating that these osmolytes enhance the effects induced by the increase in the ethanol concentration.

The optical density changes at 540 nm, allied to the stabilizing effect (P_S) of ethanol on the erythrocytes, were analyzed as a function of temperature (Fig. 5). Were the inverted Gaussian line in Fig. 1B to actually represent a stabilization effect, then P_S would represent the amount of erythrocytes that are stabilized by ethanol. In the absence of other osmolytes and at 37 °C (Fig. 1B), P_S corresponded to about 70% stabilization of the erythrocytes and displayed no dependence on temperature ($p>0.05$). In the presence of glycerol or sorbitol, P_S was directly related to the temperature ($p<0.01$), which indicates that the amount of stabilized erythrocytes was reduced with the decrease in temperature in the presence of these substances (Fig. 5).

4. Discussion

4.1. Model for the morphological stabilization of human erythrocytes by ethanol

We believe that the effects observed upon adjusting the variables considered in this work can best be explained in terms of a morphological stabilization model (Fig. 6) as was first described elsewhere [37]. According to this model, erythrocytes may exist in two basic morphological states, an expanded or relaxed state (*R*) and a contracted or tight state (*T*). Each state may be composed of several different shapes. Whereas the *R* state may comprise discoid and other related shapes of the erythrocytes, the *T* state may include solvent-induced contracted shapes [28].

The *R* and *T* states of the erythrocytes exist under conditions of low and high osmotic pressure, respectively. An increase in the osmotic pressure at the exterior of the erythrocytes would lead to the exclusion of inner water, with a consequent transition from the *R* to the *T* state. This transition would stabilize the erythrocyte against the action of chaotropic agents. The *T* state would be stable under these high osmotic pressure conditions due to a strengthening of the van der Waals attractive forces in the membrane lipid bilayer as a result of the erythrocyte tightening. This morphological state conversion is certainly related to the membrane microvesicles released, as reported elsewhere [20,28,31].

According to this model (Fig. 6), erythrocytes in the *R* state would suffer the chaotropic action of ethanol (route 1) or would be converted into the *T* state with increasing osmotic pressure up to a critical value (route 2). Route 2 would only be activated by an intermediate elevation in the ethanol concentration. More substantial elevations in the ethanol concentration would activate route 3, leading to *T* state lysis. Thus, the *R* erythrocytes would be lysed by the ethanol chaotropic action (route 1), while *T* cells would be vulnerable to a combination of the chaotropic and osmotic pressure effects of the solution (route 3).

Evidence in support of this model is discussed in the following.

4.2. Effects of ethanol on human erythrocytes

In aqueous solutions, ethanol decreases the dielectric constant and the intensity of the hydrophobic force, and also increases the osmotic pressure of the system. For a given concentration of ethanol, its net effect on the erythrocytes will be a consequence of the combination of these actions. The effect that prevails depends on several factors, such as the ethanol concentration, the presence of other solutes, and the temperature.

The action of ethanol, which is a chaotropic agent for proteins, is based on its capacity to attenuate the hydrophobic force and to better solvate the hydrophobic side chains of amino acids [2,12,38,39]. In the same way, ethanol lessens the hydrophobic force and weakens the van der Waals contacts that stabilize the membrane lipid bilayer, promoting escape of the phospholipids and their dispersion in the solvent.

At low ethanol concentrations in the interval studied (Fig. 1A), its net result is lysis of the *R* erythrocytes, as represented by route 1 in Fig. 6. The influence of the osmotic pressure in determining this chaotropic action of ethanol is of little consequence, since similar concentrations of glycerol and sorbitol, in the absence of ethanol, did not promote significant hemolysis under our experimental conditions.

On further increasing the ethanol concentration, the sigmoidal increase in lysis is followed by a decrease therein. A similar effect on the lysis of erythrocytes has been observed in response to increases in psoralen concentration (in ethanol solutions) [32]. Since this transition is dependent on an osmolarity gradient, it may be attributed to the increase in the osmotic pressure. The decline in lysis with increasing ethanol concentration was also described by a sigmoid, but with a less abrupt transition between the initial and final plateaus (Fig. 1B). This is a good indication that ethanol acts by other cooperative mechanisms in this case. These mechanisms may comprise processes such as the segregation of membrane lipids, with formation of membrane

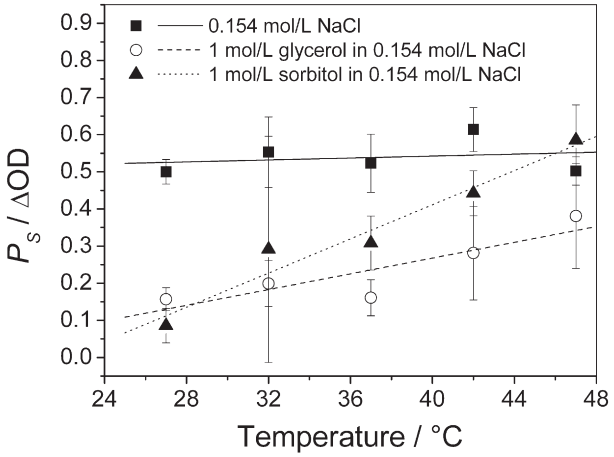

Fig. 5. Comparison of the effects of saline solutions of 1 mol/L glycerol and 1 mol/L sorbitol, in relation to a control saline solution, on the thermal dependence (27–47 °C) of variation in the optical density (P_S) associated with the stabilization of human erythrocytes by a saline solution of ethanol. Values represent means ± SD (n=6).

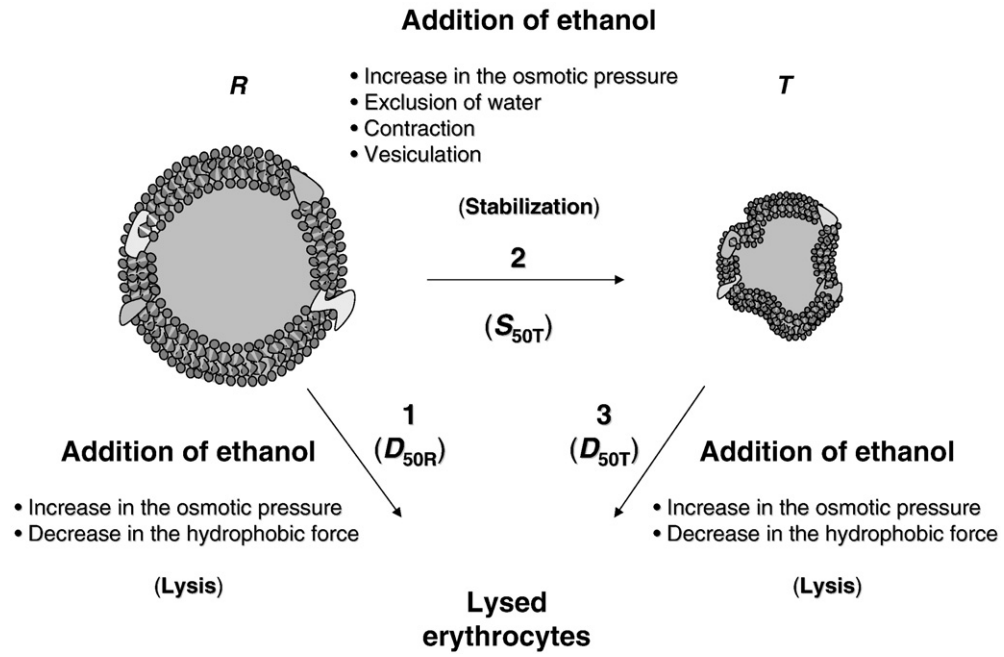

Fig. 6. Schematic representation of the actions of ethanol on erythrocytes in saline solution. Erythrocytes may exist in an expanded (*R*) or a compacted (*T*) morphological state. Each morphological state may represent an ensemble of different shapes. Low concentrations of ethanol promote lysis of the *R* state (route 1). Intermediate concentrations of ethanol promote conversion of the *R* erythrocytes to the *T* state (route 2) by increasing the osmotic pressure. High concentrations of ethanol promote lysis of the *T* state (route 3).

projections [40] or protrusions [29,41], followed by vesiculation and the generation of smooth spherocytes [28].

Hemolysis decline (Fig. 1B) displays a stabilization region at the bottom of the inverted Gaussian curve. The ethanol concentration in this region is around that at which erythrocytes have been described as smooth spheres with decreased surface areas [28]. Volume reduction has also been reported for the incubation of erythrocytes with osmolytes [15] or even with ethanol [42]. The erythrocytes would then pass from the *R* to the *T* state by the activation of route 2 (Fig. 6). This erythrocyte tightening will promote approximation of the membrane lipids, with intensification of the van der Waals attractive forces and stabilization of the erythrocytes under these conditions of high osmolarity.

Since such a decrease in the surface area is associated with an increase in the osmotic gradient, a reasonable reason to justify its occurrence is the exclusion of water from the cell interior. However, lipophilic solutes such as ethanol have a known capacity to suppress the permeability to diffusional water in red blood cells due to the net effect of two opposing actions: the reduction of channel-mediated permeability, associated with the denaturation of aquaporins, and the increase in lipid-mediated permeability, probably associated with the formation of aqueous leaks in the membrane hydrophobic barrier [33]. An increase in osmotic pressure will exacerbate the removal of water by this second action of ethanol. Since this process involves the compression of the erythrocyte membrane into a smaller volume, the formation and release of membrane microvesicles may occur as a natural consequence.

In the presence of higher ethanol concentrations, above a critical limit a combination of the chaotropic action with the osmotic pressure elevation will lead to *T* state lysis, as predicted in route 3 of Fig. 6.

4.3. Thermal dependence of the effects of ethanol on human erythrocytes

Since the osmotic pressure is directly dependent on the temperature, an increase in temperature will lead to an exacerbation of the effects determined by the osmotic pressure, in such a way as to favor routes 2 and 3 (Fig. 6). Indeed, an increase in temperature led to decreases in S_{50T} (Fig. 3) and D_{50T} (Fig. 4).

A part of the observed effect is also a consequence of the classical action of heat as a denaturing agent. Heat increases the vibrational energy of the chemical groups, which weakens the non-covalent attractive forces between them. The observed decrease in the D_{50R} values is a logical manifestation of this effect. It is perfectly reasonable to assume that the increase in temperature leads to a weakening of the non-covalent bonds of the membrane, thereby favoring lysis by the action of ethanol.

This means that heat also has a dual effect on this system. On the one hand, it increases the osmotic pressure, which favors the formation of the *T* state and the attraction of the groups in the lipid bilayer of the membrane. On the other hand, heat increases the vibration of the chemical groups, which weakens the attractive forces in the membrane. The net result of this complex system depends on the contribution of each individual factor.

In the absence of glycerol and sorbitol, and at low ethanol concentrations, an increase in temperature favors denaturation of erythrocytes by exacerbation of route 1 (Fig. 6), which is confirmed by the inverse dependence of D_{50R} on the temperature (Fig. 2). In this situation, the chaotropic actions on the system (ethanol and heat) prevail, with exacerbation of route 1 (Fig. 6).

4.4. Thermal dependence of the effects of ethanol on human erythrocytes in the presence of glycerol or sorbitol

At low concentrations of ethanol, glycerol and sorbitol potentiated the chaotropic action of ethanol on the *R* state erythrocytes, although this chaotropic synergism decreased as the temperature was increased (Fig. 2). In the presence of glycerol or sorbitol, the thermal dependence of D_{50R} is less pronounced than it is in the absence of these osmolytes. This is consistent with the fact that the presence of the osmolytes reduces the denaturation of the *R* state caused by the temperature increase (Fig. 2). At low ethanol concentrations, the incorporation of osmolytes makes the chaotropic effect of the system (ethanol and heat) less accentuated than the osmolarly based stabilization of the erythrocytes (route 2), thus disfavoring route 1.

Between the limits of 0 and 3.28 mol/L glycerol and 0 and 1.5 mol/L sorbitol, these osmolytes produced only negligible lysis in human erythrocytes under our experimental conditions. This behavior is in agreement with the stabilizing action on biological organization complexes described for these solutes [15,17,28,43–49]. The presence

of glycerol or sorbitol also decreased the thermal dependence of S_{50T} in relation to the control saline solution (Fig. 3). This indicates that glycerol and sorbitol favor the formation of the *T* state through route 2 (Fig. 6).

The dependence of D_{50R} on the glycerol concentration was found to follow a decreasing sigmoid [37]. This is a good indication that glycerol acts through cooperative mechanisms, which may comprise vesiculation and the generation of smooth spherocytes [21,28,29,31,50]. Whatever the nature of these mechanisms, they are strongly related to the osmotic pressure.

In the absence of glycerol or sorbitol, the amount of erythrocytes that was stabilized was around 70% (Fig. 1B), and this was not affected by the change in temperature since P_S displayed no dependence on temperature (Fig. 5). These results relate to fixed-time (30 min) incubations of human blood with pre-incubated experimental solutions at the temperature of each assay, in contrast to previous studies [28], in which erythrocytes were titrated with aliquots of the solvent at regular intervals to avoid osmotic injury.

If the protecting effect had been solely based on the action of osmotic pressure, one might have expected the amount of stabilized erythrocytes to decline with decreasing temperature. The absence of such a dependence of P_S on temperature means that ethanol must antagonize some membrane properties, such as fluidity or dielectric behavior, that change with the decrease in temperature. In fact, membrane dielectric behavior displays temperature dependence [34] and ethanol is capable of modifying the dielectric properties of the solution and also of the erythrocyte membranes [28].

In the presence of glycerol or sorbitol, however, P_S decreased significantly with the decrease in temperature (Fig. 5). This may mean that glycerol and sorbitol antagonize the mechanism by which ethanol stabilizes erythrocytes as the temperature is decreased. Conversely, an enhancement of P_S with temperature would indicate that glycerol or sorbitol favor the mechanism by which ethanol stabilizes these cells with increasing temperature (osmotic pressure).

The reason why the amount of ethanol-stabilized erythrocytes does not constitute 100% of the cell population may be due to competition between the chaotropic (route 1) and stabilizing effects (route 2) of the alcohol, probably due to kinetic differences between routes 1 and 2. This is a matter that requires further evaluation.

For the population of *T* erythrocytes, the presence of glycerol or sorbitol increases their denaturation. However, the slope of the plot of the thermal dependence of D_{50T} does not change substantially in relation to that of the control saline solution (Fig. 4). The stabilized erythrocytes (*T*) are already at the critical osmotic pressure, beyond which an increase in temperature will not stabilize them further. In this situation, an increase in temperature will increase the osmotic pressure, but with a net destabilizing effect on the erythrocytes and an exacerbation of route 3 (Fig. 6). Differences observed between the behaviors of glycerol and sorbitol (Figs. 2–4) may be attributed to the low permeability of the erythrocyte membranes to sorbitol [17,46].

The higher stability of the compacted *T* state in relation to the relaxed *R* state of the erythrocytes may be helpful in understanding the effect of osmolytes on proteins. According to the preferential hydration theory originally used to explain the stabilization of proteins by glycerol [5,7,8,12,51], the exclusion of glycerol from the inner hydration shell of proteins would demand a larger amount of free energy to promote hydration of the unfolded (*U*) state than of the folded (*N*) state. Thus, the presence of the osmolyte should stabilize the *N* state in relation to the *U* state by increasing the energy barrier to the interconversion of these states, making the transition $U \rightarrow N$ more probable than the transition $N \rightarrow U$. This explanation has also been used to rationalize the stabilizing effect of osmolytes on chromosomes [52].

As Nature uses the same osmolytes to stabilize different biological organization complexes (proteins, chromosomes, and membranes) in the same kind of hydrophilic environment, this suggests that a single explanation may be valid to rationalize the observed effects in all situations.

We believe that the exclusion of water followed by a tightening of the erythrocytes, promoted by an increase in the osmotic pressure, may be sustained by an increase in the magnitude of the van der Waals attractive forces in the membrane lipid bilayer. In the same way, it is possible that water exclusion promoted by an increase in the osmotic pressure in the external phase may enhance the van der Waals attractive forces inside a protein, which would surely stabilize its *N* state.

5. Conclusions

Depending on the osmolarity of the solution, erythrocytes are present in morphological states called expanded (*R*) and tight (*T*). Under conditions of low osmolarity, the erythrocytes are in the *R* state and suffer lysis by relatively low concentrations of ethanol. In the presence of higher concentrations of ethanol, the erythrocytes are in the *T* state. The addition of glycerol or sorbitol, or an increase in the temperature (27–47 °C), produce increments in the osmotic pressure of the solution, and act synergistically with ethanol in promoting the formation and lysis of the *T* state erythrocytes.

Acknowledgments

The authors manifest their gratitude to Laura Aguiar de Souza Penha for the initial language review of this text.

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