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A Spin Label Study of Erythrocyte Membranes During Simulation of Freezing

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Summary. Human erythrocytes were labeled with stearic acid spin labels, and no change was detected in membrane fluidity under hyperosmotic stress, going from isotonicity to about 3000 mOsm. Intact erythrocytes labeled with an androstane spin label and submitted to simulation of freezing show the onset of irreversible structural breakdown occurring in a saline solution at 2,000 mOsm. Ghosts labeled with maleimide spin label (4-maleimide-2,2,6,6-tetramethylpiperidinooxyl) when submitted to solutions of increasing osmolalities (pH 7.4), exhibit protein conformational changes that are irreversible after a simulated freeze-thaw cycle. After sonication of maleimide spin-labeled ghosts, membrane buried sulfhydryl groups become exposed. Such preparations showed behavior similar to the unsonicated when in saline hyperosmolal medium (pH 7.4). Such results suggest the ionic strength of the medium as the determining factor of the detected conformational changes. Maleimide spin-labeled ghosts in 300 mOsm saline solution (pH 7.4) were treated with ascorbic acid (spin destruction of nitroxides), and the kinetic analysis indicates that 65% of the labeled sites are located at the external interface of the membrane or in hydrophilic channels. Deformation and rearrangements of membrane components in solutions of increasing osmolalities apparently are related to protein conformational changes, on the outside surface of erythrocyte membranes, with a significant amount being structurally dissociated of lipids.

Several theories have been suggested to explain the mechanism of cryoinjury. Lovelock [15] suggested that the damage suffered by red blood cells (RBC) due to freezing is caused by the high electrolyte concentration inside and outside the cells, when water is removed from the system by ice formation, and, apparently, such conditions could be simulated by using suitable concentrations of sodium chloride in the suspending medium. Mazur [17] suggested two factors to explain the mechanism of cryoinjury (intracellular freezing and "solution effect") and showed a maximum in the plot of survival rate *vs.* cooling rate. Meryman [18] proposed that the hyperosmotic stress was the factor responsible for the freezing damage. Litvan [14] suggested a thermodynamic approach to the problem and pointed to the vapor pressure difference as the primary cause of cryoinjury.

Up to the present, none of the theories proposed were able to account for all the experimental data; an example of this is the fact that the mechanism of action of extracellular cryoprotectants is unknown [2], and a multi-factor theory has been suggested [23].

Spin labeling techniques were introduced by McConnell and his co-workers [27], which seem useful for monitoring the molecular changes that occur in biological systems submitted to simulation of freezing. Under such conditions, artifacts introduced by the lowering of the temperature and the limitation imposed by the nonexistence of an adequate formalism for temperatures below +15 °C [11] can be avoided. Thus, to obtain information regarding changes induced by increasing the osmolality of the medium, on the molecular components of erythrocyte membrane, electron spin resonance spectroscopy was used, and data about the fluidity of phospholipids and protein conformational changes were obtained.

Materials and Methods

1. Erythrocytes and Ghosts

Human red cells were isolated from fresh blood, coagulation being prevented with citrate-phosphate-dextrose. The blood was centrifuged in a Sorvall RC-5B centrifuge at 1,478 *g* for 5 min, and plasma and buffy coat were removed by aspiration. The remaining cells were washed three times in isotonic solution, pH 7.4 (in mM):

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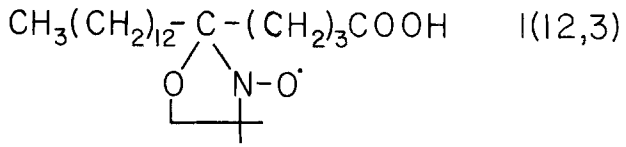
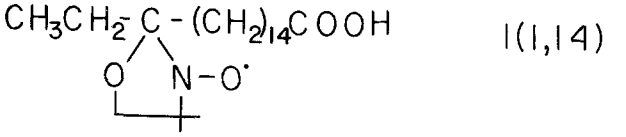
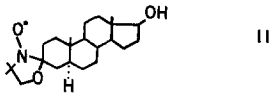


I(12,3)



I(1,14)



II

Fig. 1. Spin labels used: I(12,3): 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyoxyl; I(1,14): 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyoxyl; II(ASL): 17α-hidroxy-4′,4′-dimethylspiro(5β-androstane-3-2′-oxazolidin)-3′-yloxyl

NaCl, 138.6; NaH_2PO_4, 2.7; and Na_2HPO_4, 12.3. The washed RBC were resuspended in phosphate buffer of varying osmolality, obtained by varying the NaCl concentration alone. Human RBC ghosts were prepared by the method of Dodge et al. [4]. For ghosts, the washing procedure was performed in the cited centrifuge (rotor no. SS-34) at 27,138 × *g* at 4 °C, for 10 min.

2. Spin Labeling

a) Phospholipids. Whole RBC washed as described (with hematocrit about 80%) were labeled with (Fig. 1): I (*m,n*), stearic acid analogs with the nitroxide ring located at the hydrophilic end (*m,n* = 12,3), or at the hydrophobic end (*m,n* = 1,14); II, an androstane spin label (ASL).

For spin labeling [10], a methanolic solution of the label was dried as a thin film in the bottom of a small test tube under reduced pressure. The membrane preparation was added, and the mixture was carefully shaken until the label was taken up. Final concentration of the label was 10^{-5} M.

b) Proteins. RBC ghosts were labeled according to Holmes and Piette's method [8]. The spin label 4-maleimide-2,2,6,6-tetramethyl-piperidinooxyl (MSL) binds covalently primarily to free sulfhydryl groups but also to protein amino groups [3, 24]. In the literature we have a satisfactory quantitative treatment for spectra of maleimide spin-labeled ghosts, allowing an easy interpretation of the spectra [8, 24, 26].

3. ESR Spectroscopy

The ESR spectra were obtained with the preparation put in a flat quartz sample cell in a Varian E-4 spectrometer. Care was taken to avoid power saturation and overmodulation effects. All spectra were obtained at room temperature (23–25 °C).

The results will be presented as mean ± standard error of the mean(n), where n is the number of experiments. All spin labels were obtained from Syva Associates, Palo Alto, Calif.

4. Parameters Used in the Analysis of the ESR Spectra

a) Intact red blood cells. The position and the separation of the resonance lines are related with the ordering of the molecules, while the width of the resonance lines reflects the dynamical properties of the system [25]. Therefore, for the spin label I(12,3), with the nitroxide ring near the polar heads of the phospholipids, the outer hyperfine splitting ($2T'_{\parallel}$) was used and plotted as a function of the osmolality of the medium. The outer hyperfine splitting was measured to within ±0.5 G.

For the analysis of the spin label I(1,14), with the nitroxide ring in the core of the membrane, the "rotational correlation time" (τ_c) was used [11,27].

In the analysis of the ASL spectra, the appearance of a new spectral component indicates that new binding protein sites are exposed (strongly immobilized radicals).

b) RBC ghosts. The ESR spectra of maleimide spin-labeled ghosts have at least two components: one strongly immobilized (S) and one weakly immobilized (W). The ratio between the amplitudes of W_1 and S_1 was taken as a measure of the relative amount of these two types of states [26] (*see* Fig. 7*A*).

5. Hemolysis, Mean Cell Volumes, and Freezing Point Determinations

Hemolysis was estimated by converting hemoglobin to cyanmethemoglobin using Drabkin's reagent [5] and suitable dilutions read (15 min later) at 540 nm against standard solutions of hemoglobin, so that absolute amounts could be obtained. The percentage of hemolysis was expressed as the total amount of extracellular hemoglobin (Hb_s) compared to the total amount of hemoglobin (Hb_t) in the original aliquot of blood. The formula used was: Hemolysis (%) = (100-H_c) Hb_s/Hb_t.

For hematocrit(H_c) determinations, a small amount ot the suspension was placed in suitable capillaries, and one end was sealed. They were centrifuged in an Adams microhematocrit centrifuge.

The mean cell volume (MCV) of the cell population was determined using a Coulter counter, Model F, with mean cell volume computer (Coulter Electronics Inc., Hialeah, Fla.). The mean cell volume computer required only a few seconds for each determination. All MCV values were divided by that of the RBC volume in isotonic condition and plotted as percent of the isotonic volume in a Boyle-van't Hoff plot.

Osmolality was measured by freezing point depression in an osmometer (Model 68–31 WAS, Advanced Instruments, Newton Highlands, Mass.) calibrated by comparison with NaCl solutions of known osmolality.

Results

1. Fatty Acid Spin Labels

To gain information about the behavior of the polar head region of erythrocyte membranes during simulation of freezing, spectral observations were made with the stearic acid spin label I(12,3) incorporated into membranes submitted to increasing osmolality of the medium in the range of 300 to about 3,000 mOsm (pH 7.4). As can be seen in Fig. 2, there is no significant change in the outer hyperfine splitting by increasing the osmolality of the medium.

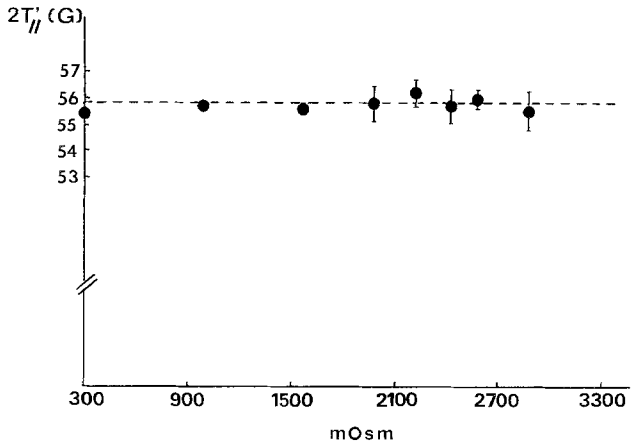


Fig. 2. Hyperfine splitting ($2T'_{//}$) of spin label *I*(12,3), incorporated into intact red blood cells, as a function of the osmolality of the medium

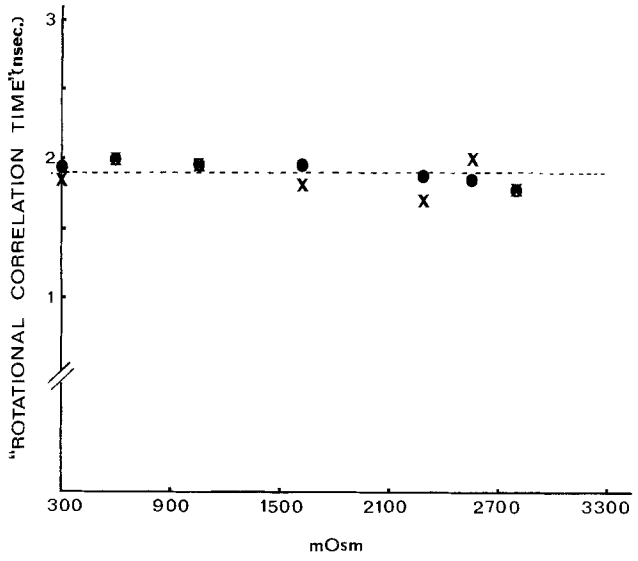


Fig. 3. "Rotational correlation time" (τ_c) of spin label I(1,14) incorporated into red blood cell membranes, as a function of the osmolality of the medium. X represent the values at 18 min after addition of spin labels to the suspension, and the filled circles represent the corresponding values 1 hr later

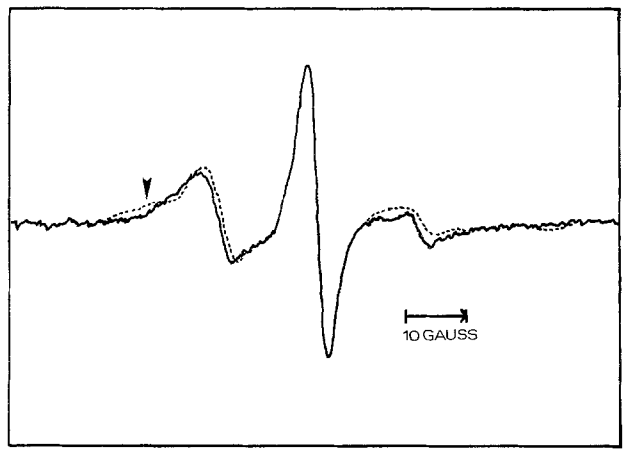


Fig. 4. Typical ESR spectra for spin label ASL incorporated into RBC membranes. The solid line represents the spectrum of the preparation when the medium is an isotonic saline solution, at pH 7.4. The dashed line represents the spectrum when the preparation is in a hypertonic medium (2,000 mOsm), at pH 7.4

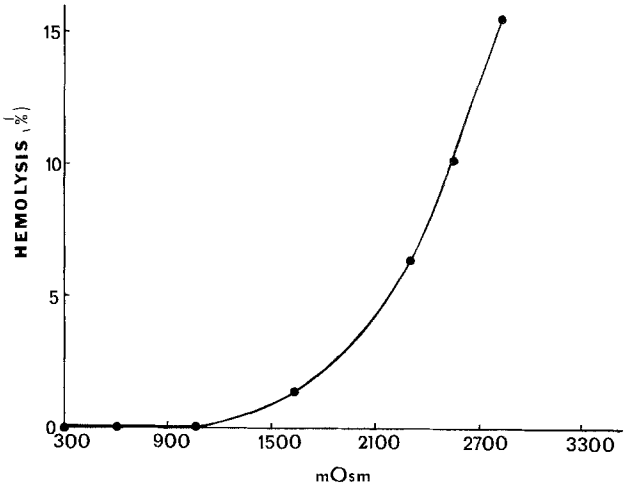


Fig. 5. Hemolysis of RBC exposed for 1 hr, at ambient temperature, to hypertonic solutions of sodium chloride, pH 7.4

Spin label I(1,14) was used to probe changes in the motion of the tail of phospholipids of intact RBC under a similar protocol. Again, no significant change in the "rotational correlation time" (τ_c) was observed from 300 to about 3,000 mOsm, using experimental times of 18 min and 1 hr (*see* Fig. 3).

2. Androstane Spin Label

The ASL has a resonance spectrum classified as "intermediate immobilization," being extremely sensitive to small changes in molecular motion. Its nitroxide group is located in the hydrophobic region of the membrane and the -OH group "anchored" in the aqueous phase [9]. With ASL it was possible to detect the onset of hemolysis in the system, evidenced by the appearance of a strongly immobilized component in the spectrum at 2,000 mOsm.

3. Hemolysis

Human RBC were exposed to hypertonic NaCl solutions for 1 hr at ambient temperature, and hemolysis (%) was measured as a function of the osmolality of the medium (*see* Fig. 5).

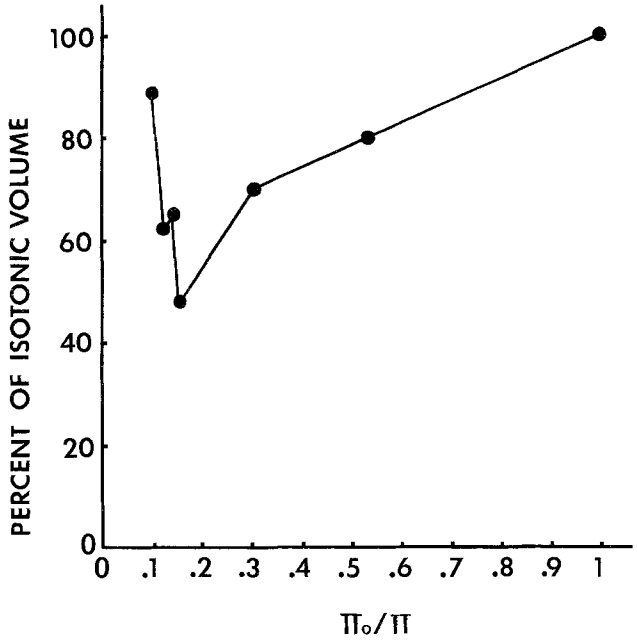


Fig. 6. Mean corpuscular volumes of RBC in hypertonic NaCl solutions, pH 7.4, at ambient temperature. π_o/π is the ratio of isotonic to experimental osmolalities of the suspending medium

Hemolysis begins at 1,100 mOsm. At 2150 mOsm there is about 5% hemolysis in the system, increasing more steeply for higher values of the osmolality of the medium.

4. Mean Cell Volume

The volume distribution was taken a few seconds after the addition of an aliquot of packed RBC in hypertonic solutions, since we were interested in the osmotic shrinkage that is almost intantaneous, in agreement with the results obtained by Kregenow [12]. As can be seen in Fig. 6, a linear reduction of the mean cell volume of the population of RBC occurs with the inverse of the osmolality of the medium, reaching the minimum volume at about 2,000 mOsm, after which the cells leak and swell.

5. Protein Spin Label

To detect protein conformational changes of MSL-ghosts when submitted to hyperosmolal solutions (pH 7.4) of electrolyte or nonelectrolyte solutes, aliquots of the labeled ghosts were placed in contact with each solution (0.1 ml packed membranes added to 0.9 ml solution). The spectrum for each one was recorded 10 min later (*see* Fig. 7*A–C*).

Fig. 7*A–C* shows a variation in noise level, since the intrumental parameters have been adjusted for spectral optimization. However, such procedures do not affect the amplitude ratios of MSL-spectra or the results.

A decrease in the amplitude ratios was detected when increasing the osmolality of the medium, as can be seen in Table 1, suggesting protein conformational changes, when membrane proteins are in hyperosmolal solutions (with NaCl or sorbitol as solutes).

6. Reversibility Tests

MSL-ghosts were washed three times in buffered NaCl solution (300 mOsm, pH 7.4), resuspended in the same solution (0.1 ml packed cells in 0.9 ml solution), and the spectra obtained.

In succession, these suspensions were submitted to a similar centrifugation procedure as used for membrane washing (*see* Materials and Methods, Section 1), and the pellets were resuspended (1:10) in 3,055 mOsm NaCl solution (pH 7.4). The spectra were obtained 10 min later. Finally, the membranes were returned to the initial medium. Again, after washing the membranes three times, the spectra were obtained. The mean amplitude ratios for all conditions are displayed in Table 2.

There is a significant increase in the amplitude ratio when comparing the final to initial (control) conditions (300 mOsm, pH 7.4), suggesting irreversible protein confomational changes during this cyclic process.

7. Sonication

When prolonged sonic irradiation is used there is the danger of the membrane being reduced to small vesicles that may not reflect the native state of membrane constituents or their organization. Thus, it is essential to adequately choose the best parameters of sonication since the preparation must reflect as much as possible the original state of organization of membrane components.

Maleimide spin-labeled ghosts in hypotonic phosphate buffer (20 mOsm, pH 7.4) were sonicated using the following parameters: the preparation in thermal equilibrium with an ice bath was sonicated for 2-sec periods, with a 2-min interval between sonications [20], using a sonifier Cell Disruptor, Model 140, Heat Systems, Ultrasonics, Inc., Plainview, N.Y., at an intensity setting of 65.

Typical spectra of unsonicated and sonicated maleimide spin-labeled ghosts is shown in Fig. 8. As can be seen, sonication causes protein conformational changes since, in contrast with the intact ghost membranes, the sonicated preparation contained only a small population of strongly immobilized labels.

This preparation, however, has a behavior similar to the unsonicated, e.g., still reacts with a decrease in the amplitude ratio, to increasing osmolality of the medium.

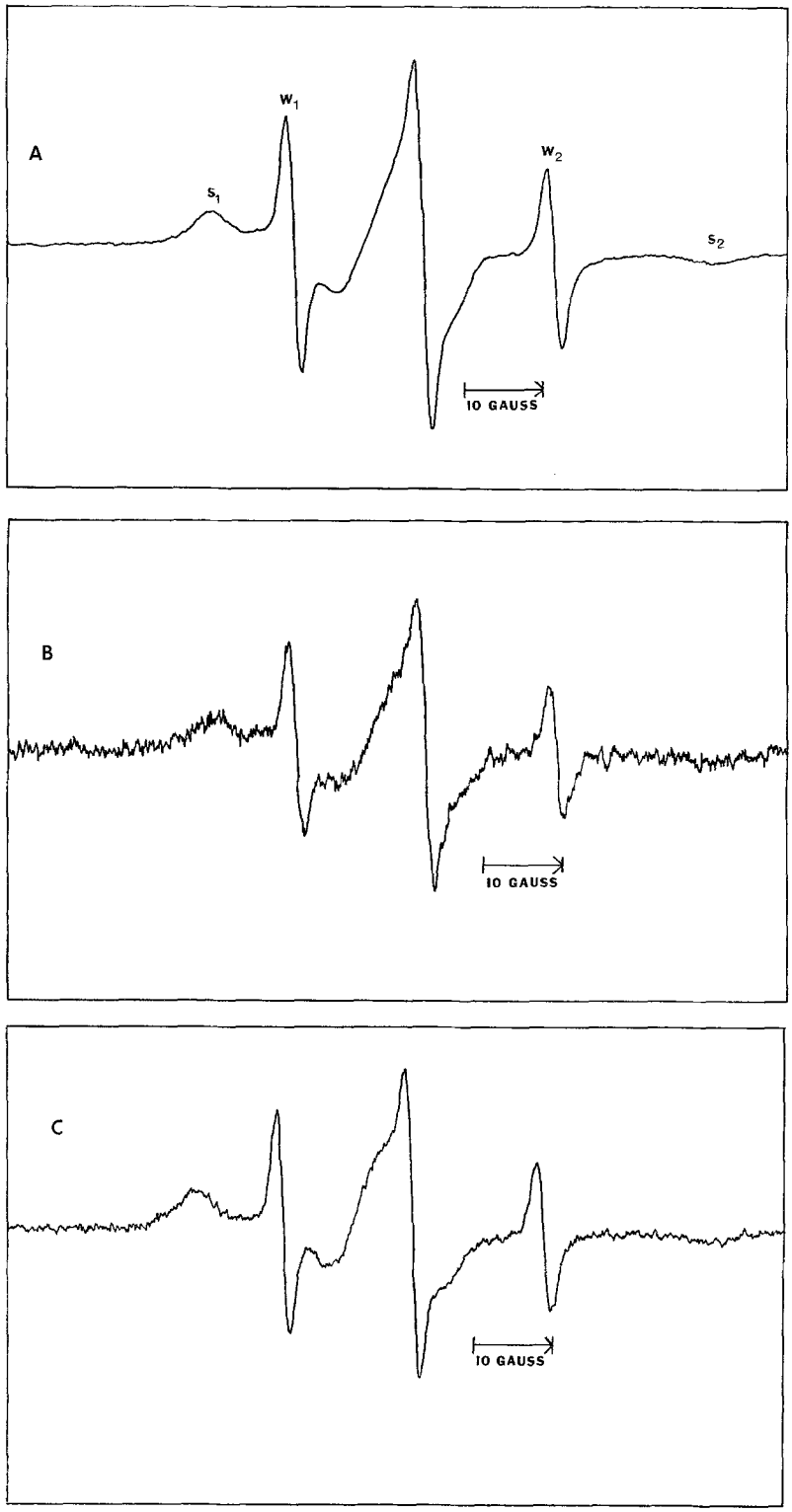


Fig. 7. ESR spectra of maleimide spin-labeled ghosts in: (*A*)300 mOsm NaCl solution, pH 7.4; (*B*) 3,055 mOsm NaCl solution, pH 7.4; (*C*) 3,055 mOsm sorbitol solution, pH 7.4

8. Ascorbic Acid Experiments

Ascorbic acid was used as a tool to obtain information regarding the localization of the labeled sites in erythrocyte membranes.

Maleimide spin-labeled ghosts in 300 mOsm NaCl solution (pH 7.4), were treated with ascorbic acid in the final concentration of 2.5 mM, at room temperature, and the kinetics of chemical reduction was measured as the ratio between amplitudes of the middle line of the resonance spectra at a given time t and the amplitude at time zero (before addition of ascor-

Table 1. Amplitude ratios for maleimide spin-labeled ghosts in phosphate buffer of different osmolalities obtained by increasing amounts of NaCl or sorbitol[a]

	Amplitude ratios		
	20 mOsm[b]	300 mOsm	3,055 mOsm
NaCl	4.73±0.28 (8)	3.81±0.12 (5)	3.33±0.28 (8)
Sorbitol		4.43±0.21 (3)	3.33±0.37 (5)

[a] All solutions at pH 7.4.
[b] Phosphate buffer only.

Table 2. Amplitude ratios for maleimide spin-labeled ghosts exposed to 300 mOsm saline solution, then to 3,055 mOsm, and back again to 300 mOsm[a]

	Amplitude ratios			
	300 mOsm	3,055 mOsm	300 mOsm	$\%\Delta^*_{300}$
NaCl	3.54±0.10 (4)	2.25±0.15 (4)	4.50±0.41 (4)	22

[a] All solutions at pH 7.4.
$\%\Delta^*_{300}$ is the percentual variation of the amplitude ratios between the final and initial conditions.

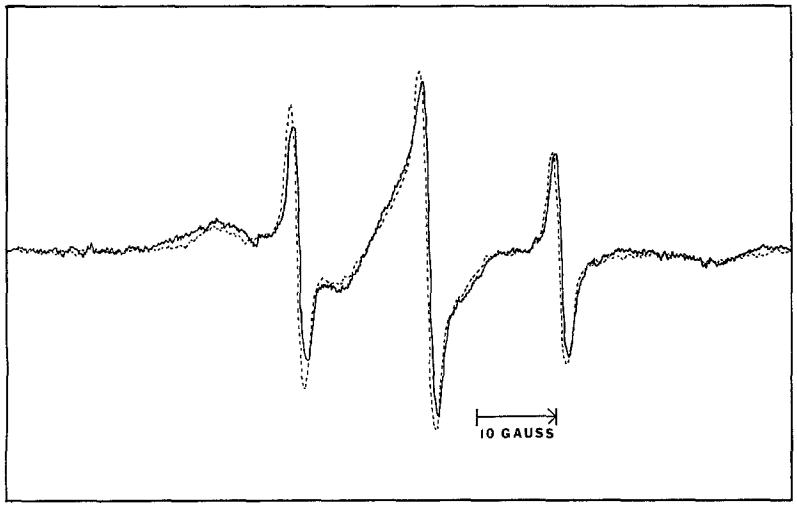


Fig. 8. ESR spectrum of maleimide spin-labeled ghost membranes (—) in hypotonic phosphate buffer (20 mOsm; pH 7.4). The dashed line represents sonicated MSL-ghost membranes at the same medium. Sonication parameters are presented in the text

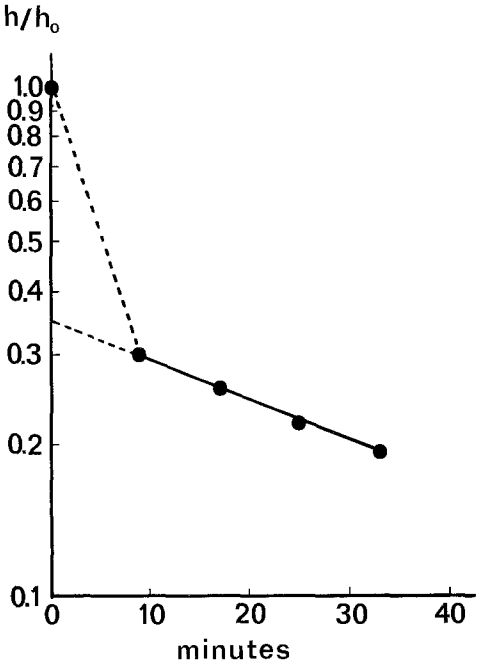


Fig. 9. Kinetics of reduction of maleimide spin-labeled ghosts after treatment with 2.5 mM ascorbic acid at ambient temperature. h_t/h_o is the amplitude ratio of the middle line of the resonance spectra at time t and 0

bic acid), plotted as a function of the experimental time. Figure 9 shows the evolution of the reduction, with a fast phase (within a few minutes after addition of vitamin C) corresponding to the sites easily accessible to the ascorbic acid molecules and a slow phase, first order reaction, probably corresponding to the labels at sites of difficult access to vitamin C molecules.

The extrapolation of the slow phase gives at the ordinate the value of 65% for h/h_0, suggesting that 65% of the labeled sites are easily available to the ascorbic acid molecules and, therefore, probably located at the external interface or in hydrophilic channels.

Discussion

Our results suggest no change in the RBC membrane fluidity when submitted to solutions of increasing osmolality of the medium in the range of 300 to about 3,000 mOsm. Such results reflect the stability of the phospholipid components of RBC membranes. There is information in the literature [24] that shows similar results for a small range of osmolalities. However,

we extended this range to about 3,000 mOsm, due to cryobiologic interest.

The detrimental effect of high "g" on RBC in hypertonic NaCl solutions is well known [6]. However, with ESR techniques such artifacts could be eliminated, allowing a more accurate detection of the value of osmolality that corresponds to the onset of irreversible structural breakdown in the membrane of RBC, which is a very controversial question in the literature [6,19]. The experiments performed using androstane spin label showed a strongly immobilized component in the spectrum at 2,000 mOsm. This can be explained as an exposition of new binding protein sites now available to the label as a consequence of hemolysis [9], since absence of the highly immobilized component of the spectrum indicates condition for structural integrity of the membrane.

It is interesting to compare such results with those obtained through conventional methods. As previously shown, hemolysis starts at 1100 mOsm, and at 2,150 mOsm there is about 5% hemolysis in the preparation, increasing faster for higher values of the osmolality of the medium. Probably, the manipulation of the preparation and the high "g" values used could account for such precocity, since it is well known that the RBC in hypertonic media are highly sensitive to mechanical and thermal stresses [15,16].

When erythrocytes are submitted to hypertonic medium of increasing osmolality, their volumes follow a gaussian distribution, and the reduction in cell volume is proportional to the increase in the tonicity, in the range of 300 to 1,000 mOsm. The decrease in cell volumes is less than one would expect if the cells behaved as "ideal osmometers." This behavior has been explained on the basis of a changing osmotic coefficient of hemoglobin with concentration (Adair & Adair [1]), the presence of the intracellular osmotically inactive "bound" water (Ponder [22]), and cooperative interactions between hemoglobin molecules (Gary-Bobo & Solomon [7]). At about 2,000 mOsm the cells leak and swell. These results are in agreement with those obtained with the androstane spin label. Both techniques indicate 2,000 mOsm as the value that corresponds to the onset of irreversible structural breakdown in the RBC membrane.

Kregenow [12] proposed two phases for duck erythrocytes when submitted to hyperosmotic stress: an initial phase of osmotic shrinkage that is instantaneous and a second more prolonged, volume regulatory phase. Our experiments with human erythrocytes showed a similar behavior, and even 30 min after the addition of aliquots of RBC in hypertonic solutions, persistent volume changes were observed.

The results obtained with ghosts labeled with maleimide spin label, showing a decrease in the amplitude ratio with increasing osmolality of the medium, suggest interaction at the charged sites of membrane proteins, causing a decrease in the tumbling of the spin label with loss of the narrow line intensity and broadening of the spectrum, if the narrow component of the spectrum arises from spin labels at that site [3,24].

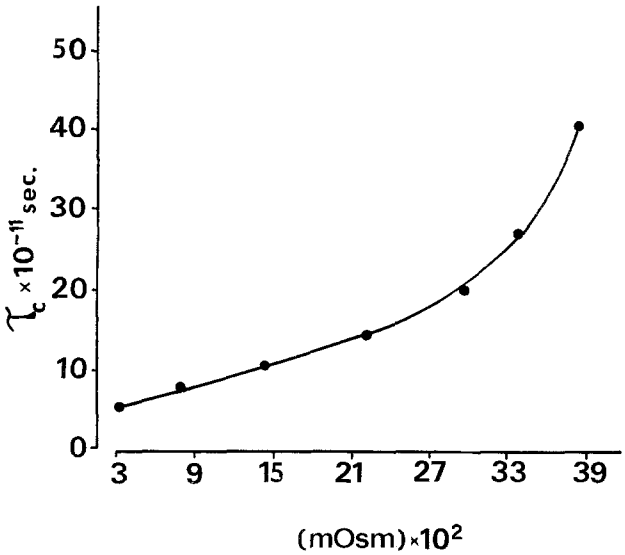


Fig. 10. Maleimide spin label was added to Sorbitol solutions of increasing osmolalities, there occuring a reduction in the spin label motion, as can be seen from the values of the rotational correlation time (τ_c). The values of τ_c were calculated from the ESR spectra, using equation proposed in reference [13]

There is evidence [21] that positively charged aminogroups are directly involved in the control of ion permeation through RBC, so the protein conformational changes detected could reflect a more specific action on the proteins responsible for ionic permeability.

Using Sorbitol at high osmolalities there is an increase in the viscosity of the medium. However, it is not possible to explain all the spectral changes in function of the viscosity of the medium, since the "rotational correlation time" would be different by at least one order of magnitude from the values obtained for maleimide spin-labeled ghosts at a given osmolality, as can be seen in Fig. 10.

The results of the reversibility tests, showing a variation in the amplitude ratio of 22% in a cycle simulating freezing and thawing, could be explained assuming that some of the labeled proteins were affected by the process and had a disrupted structure, with an increase in the population of the weakly immobilized component and a decrease in the strongly immobilized component of the ESR spectrum of maleimide spin-labeled ghosts.

Changes in the amplitude ratio of maleimide spin-labeled ghosts submitted to sonication are indicative

that this process causes protein conformational changes, suggesting that membrane-buried sulfhydryl groups have become exposed as a result of sonication. As sonicated maleimide spin-labeled ghosts have behavior similar to the unsonicated when submitted to simulation of freezing. such results strongly suggest the ionic strength of the medium as the determining factor of the detected conformational changes.

Since ascorbic acid is water soluble, its effect is reduced in hydrophobic zones, and this makes possible the selective destruction of signals from regions that contain water or have aqueous channels. In other words, since maleimide spin label is covalently bound to membrane proteins and there is no label free in solution (the preparation was washed three times in phosphate buffer), 65% of the labeled sites were in aqueous environment and of easy access to ascorbic acid molecules. Spectral analysis indicates that 65% of the strongly immobilized component was also in aqueous environment since it was reduced to 35% of its original amplitude after treatment with vitamin C.

In addition, all the modifications detected in the protein component seem not to reflect on the lipids (as evidenced by the results with labels I(12,3) and I(1,14), suggesting no strong interaction between lipids and proteins in human erythrocytes or, at least, that a significant amount of protein is structurally dissociated from lipids in RBC. Furthermore, there is additional evidence for the strong packing of phospholipids in RBC and that its deformation is closely related to conformational changes in the protein component of the membrane.

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