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SPIN PROBE STUDY OF STRUCTURAL CHANGES IN ERYTHROCYTE MEMBRANES DURING COOLING

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The writers showed previously that disturbance of the barrier function of erythrocyte membranes during cooling takes place within the temperature range corresponding to the freezing out of spatial water [10]. The results formed a basis for a dehydration hypothesis, according to which the freezing out of water initiates structural transformations in lipid—lipid and lipid—protein complexes, leading to disturbance of the integrity of the bilayer.

According to data in the literature [3], freezing affects the structure of the lipid complexes first, and these may undergo thermotropic and lyotropic mesomorphism.

During freezing, however, the structure of the membrane protein also is significantly affected [2], and like phase transitions of lipids, this also leads to disturbance of protein—lipid interactions, which are evidently particularly sensitive to the action of low temperatures.

In this investigation the physical state of the lipids and of protein—lipid interactions were studied in erythrocyte membranes during cooling.

EXPERIMENTAL METHOD

Cells were washed free from plasma by centrifugation in 5 volumes of solution A: 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, at 3000-4000g in the cold. The procedure was repeated two or three times. White ghosts were obtained as described previously [14]. After separation the membranes were suspended in solution A. Lipids were extracted from the white ghosts by the method in [4]. The solvent was evaporated on a rotary evaporator, and the resulting lipids were treated with 1 ml of solution A, and liposomes were obtained by mechanical shaking [7].

The spin probe was the stearic acid derivative 5-doxylstearate (Fig. 1), from Syva (USA), and was added to a suspension of erythrocytes, white ghosts, and liposomes in the form of an alcoholic solution (final concentration in the sample 5×10^{-5} M). The final ethanol concentration was 2%. To remove the residue of ethanol and of probe not incorporated by the membranes, the test samples of erythrocytes, white ghosts, and liposomes were washed twice by centrifugation in the cold at 3000, 9000, and 18,000g respectively.

EPR spectra were recorded on an E-109 radiospectrometer (from Varian, USA), with a working frequency of 9 GHz, and with thermostatic control of the samples, within the temperature range from -50 to 20°C. The parameters of recording the EPR spectra were chosen, depending on the temperature conditions of the experiment, to abolish distortion of the spectrum due to saturation of superhighfrequency irradiation or rapid scanning. During lowering of the temperature of the sample the power of superhigh-frequency irradiation varied from 3 to 15 mW, the modulation frequency was 100 kHz, the amplitude 2 G, the range of scanning of the magnetic field 20 G, recording speed from 4 min to 1 h, and the filter 0.0128-1 sec. To characterize the orderliness of anisotropic movements of the hydrocarbon chains of the phospholipids in the membranes, the parameter $2A_{max}$ was used; this reflects the distance between the outer extrema

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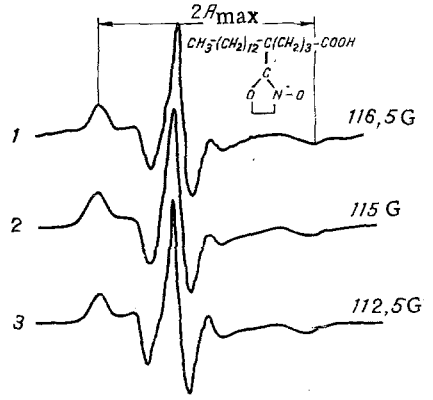

Fig. 1

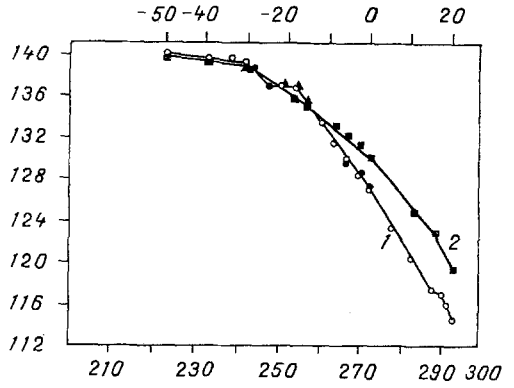

Fig. 2

Fig. 1. EPR spectra of 5-doxylstearic acid in erythrocyte membranes (1), white ghosts (2), and liposomes (3), recorded at 20°C.

Fig. 2. Dependence of parameter $2A_{max}$ on temperature in erythrocyte membranes. Empty circles — control (cooling), filled circles — supercooling, triangles — heating, squares — cooling in 30% glycerin. Here and in Fig. 3: ordinate, $2A_{max}$ (in G); abscissa, temperature: above in °C, below in °K.

of the EPR spectrum [16] (Fig. 1).

EXPERIMENTAL RESULTS

We know that the spin probe 5-doxylstearate, when introduced into biological membranes, positions itself in the phospholipid monolayer in such a way that the axis of the molecule remains perpendicular to its surface. The approximate depth of insertion of the spin fragment into the lipid phase is 0.8 nm [1]. Another characteristic feature of the probe used is its ability to establish itself both in the lipid phase of erythrocyte membranes and in a region of protein—lipid contacts [15], so that not only modifications of the lipid phase but also changes in protein—lipid interactions can be studied.

Spectra of 5-doxylstearic acid in native erythrocyte membranes, white ghosts, and liposomes, reconstituted from total lipids of erythrocyte membranes, are illustrated in Fig. 1a-c. The appearance of the spectra reflects the high orderliness of the microenvironment of the spin probe, for which only anisotropic movement is possible. Judging from the value of the parameter $2A_{max}$, the degree of immobilization of the probe decrease in the following order: native erythrocyte membranes > white ghosts > liposomes, probably due to the level of protein-lipid contacts in these objects [9]. However, the possibility cannot be ruled out that specific packing and distribution of the lipids in the bilayer also play an important role.

Significant differences in the physical state of the lipids in the test objects were discovered during a study of spectral parameters in the course of lowering of temperature. During cooling of the erythrocytes within the range from 20 to -50°C, four structural transitions could be distinctly recorded, as shown by marked disturbance of linearity of the change in the parameter $2A_{max}$, taking place at temperatures of 15, -19, -25, and -30°C respectively (Fig. 2). A small inflection at 0°C must also be noted.

The thermotropic structural transition observed at 15°C can evidently be identified with structural and functional changes in erythrocyte membranes described previously [6]. According to the authors cited, the nature of this transition is connected with cluster formation in the liquid bilayer or with modification of lipid-protein interactions. Structural changes taking place in the membranes at 0°C are most probably the result of a change in the physical properties of water. However, as the results of our experiments in which $2A_{max}$ was measured under supercooling conditions show, this was not connected with its crystallization.

The two next structural transitions were observed in the region of temperatures characterized by freezing out of spatial water below the critical level and dehydration of the membranes [10]. They could be recorded at temperatures of -19 and -25°C.

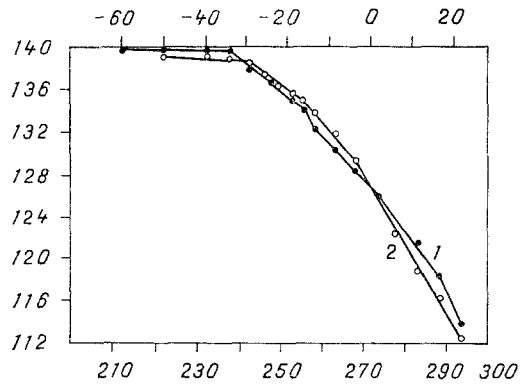

Fig. 3. Temperature dependence of parameter $2A_{max}$ in the membranes of erythrocyte ghosts (1) and liposomes (2) composed of total lipids of erythrocyte membranes.

The role of freezing out of water in the initiation of the structural transitions now being analyzed is shown by the results of experiments in which samples of native erythrocytes were frozen in the presence of 30% of glycerin, which is known to bind water and to hold it in an unfrozen state even at very low temperatures, and to enable a solution to be frozen in an amorphous state [5]. The presence of glycerin in the freezing medium smooths out the structural transitions at -19 and -25°C (Fig. 2). Comparison of the character of the changes in the parameter $2A_{max}$ in samples with glycerin and in the control revealed an ordering action of the cryoprotector on erythrocyte membranes.

Finally, with further lowering of the temperature to -28 and -30°C, yet another distinct structural transition took place, and it was observed in the presence of glycerin also. This indicates the thermotropic of structural changes in the membranes within the temperature range analyzed. The low-temperature structural changes which we found were recorded also at the stage of heating of the samples (Fig. 2), possible evidence of their phasic character [3]. To elucidate the nature of the structural transitions observed, we investigated changed in the parameter $2A_{max}$ of 5-doxylstearic acid, incorporated into the membranes of erythrocyte ghosts and liposomes composed of total lipids of the erythrocyte membranes. Analysis of the results (Fig. 3) shows that four principal structural transitions also were recorded during cooling of samples of white ghosts: at 15, -15, -17, and -30°C, with a small inflection in the region of 0°C. However, low-temperature structural transitions recorded in the region of freezing out of water were observed at higher temperatures (-15 and -17°C) than in membranes of native erythrocytes. Judging from data in the literature [9] and our previous data, this shift of the structural transitions now being analyzed may be due to differences in the character of protein-lipid interactions in the test objects, or the absence of hemoglobin in the ghosts, which supercools, binds water, and holds it in the unfrozen state down to very low temperatures [11].

When the results of the study of temperature dependence of the structural state of liposomal membranes are analyzed (Fig. 3) three temperature points may be noted at which the parameter $2A_{max}$ changes in a nonlinear manner: 0°C, -17°C, and -30°C, which indicates structural changes in the lipid component.

Structural transitions observed in erythrocyte membranes when the temperature falls from 20 to —50° are thus connected both with a change in the physical state of the lipids and with structural changes at sites of protein-lipid contacts.

An attempt was made previously [8, 12] to study structural changes in erythrocyte membranes by the x-ray diffraction method and by calorimetry. According to the results, when the temperature falls even to -50°C, no appreciable structural transitions could be found; in the opinion of the authors cited this was due to the high cholesterol content of these membranes. However, the possibility cannot be ruled out that the absence of changes in the experiments of the authors cited above was due to limitations of the methods used [3], for later [13], when the NMR method was used with deuterated fatty acids, incorporated into erythrocyte membranes, a number of structural transitions could be recorded within the temperature range from -5 to -50°C.

It can be concluded from the present investigation that one approach to the discovery of structural changes in erythrocyte membranes during deep cooling, accompanied by crystallization of water, may be to use a method of spin probes synthesized on the basis of fatty acids. Structural changes recorded under these circumstances in the lipid phase and in lipid-protein contacts are due both to lowering of temperature and to freezing out of water.

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