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TEMPERATURE EFFECT ON THE INTERACTION OF POLYETHYLENE GLYCOL WITH MEMBRANES AND ORIENTATION ORDER OF CYTOSKELETAL PROTEINS IN HUMAN ERYTHROCYTES

The study identified discrepancies in the modification of erythrocyte membrane upon exposure to polyethylene glycol with a molecular weight of 1500 (PEG) at various temperatures (4 and 37 °C) using the fluorescent probe 4-(n-dimethylaminostyryl)-1-methylpyridinium-n-toluenesulfonate (DSM), as well as alterations in the orientation order of proteins in the membrane-cytoskeleton complex using polarization microscopy. Exposure of erythrocytes to PEG at 37 °C caused complete disappearance of the hydrophobic component from the DSM fluorescent spectrum due to competition between PEG and DSM molecules for binding sites. Lowering the exposure temperature of cells to a cryoprotective agent (CPA) to 4 °C reduced the possibility of its hydrophobic contacts with cell membranes attested by the presence of the hydrophobic component in the DSM spectrum, although at a lower level than in the control. The decrease in hydrophobic contacts of PEG to membranes upon a lowering temperature at the stage of erythrocyte exposure to CPA provided retaining to a large extent the orientation order of the membrane cytoskeleton molecules both under the incubation process with CPA and after freeze–thawing of cells.

Key words: *erythrocyte, membrane, polyethylene glycol, cryoprotective agent, hydrophobic interactions, orientation order of membrane proteins.*

The effect of cryoprotective agents (CPAs) on the solidification parameters of the liquid phase in cell suspensions under decreasing temperature is one of the most important factors of their protective mechanism. The inclusion of CPAs in the medium slows down the crystallization process, changes the size and shape of the crystals formed, thereby reducing the damaging effect of ice on cells. Concurrently, the cell survival under the freeze–thawing conditions depends on the features of the modification of individual subcellular components in response of the cryoprotectant effects. Endocellular CPAs, such as glycerol and DMSO, replace part of the intracellular water and can modulate the structural and functional parameters of macromolecular complexes and membrane systems in their immediate environment. Exocellular substances, such as polyethylene glycol, polyvinylpyrrolidone, and sucrose, initiate changes in subcellular elements via cell dehydration, which is accompanied by an increase in the concentration of cytosolic components and redistribution of ions, as well as via their effect on surface structures of plasma membrane. The modifications of subcellular components caused by CPA have ambiguous consequences for the cell stability under cryopreservation conditions.

Methods of cryopreservation of human erythrocytes, currently implemented in medical practice,

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are based on the using of glycerol but the need to remove it from cells after thawing makes these methods time-consuming and expensive. In this regard, the search for new CPAs, which enable to simplify or eliminate the washing step, remains relevant. Alternative methods can be developed using substances, which do not penetrate the cell membrane, *i. e*., exocellular CPAs, whose washing is not a mandatory part of biotechnology, given their low toxicity and biocompatibility. Exposure of human erythrocytes in a solution of polyethylene glycol (PEG) with a molecular weight of 1500 at room temperature provides a high level of cell preservation after freeze–thawing but such cells are unstable after returning to physiological conditions *in vitro* [19]. Increasing the stability of erythrocytes cryopreserved under PEG protection is achieved by reducing the temperature of cell exposure to CPA to 4 °C. Although this approach does not completely solve the problem of the stability of cryopreserved erythrocytes, comprehension of cell stabilization mechanisms under these experimental conditions can be used for the further development and improvement of cell cryopreservation methods.

The mechanisms of PEG action on cells are closely related to the physicochemical properties of this compound. At room temperature, PEGs with different molecular weights can be completely dissolved not only in water but also in some nonpolar organic solutions [6, 8] that reflects the manifestation of their hydrophilic and hydrophobic properties. In aqueous solutions, PEG binds 2—3 water molecules per monomer unit [6] that determines its dehydrating effect on various macromolecules and membranes. A change in the polarity and hydration of macromolecules in the membrane structure in response to PEG entering [5] induces modification of the surface properties of cells and can create conditions for their fusion.

The purpose of the study was to determine discrepancies in the modification of the erythrocyte membrane when exposed to polyethylene glycol with a molecular weight of 1500 (PEG) at various temperatures (4 and 37 °C) using the fluorescent probe DSM, as well as changes in the orientation order of proteins of the membrane-cytoskeleton complex using polarization microscopy.

MATERIALS AND METHODS

The following reagents were used in the work: fluorescent probe 4-(n-dimethylaminostyryl)-1-methylpyridinium-n-toluenesulfonate (DSM) (Zonde, Latvia), Tris (Sigma, USA), polyethylene glycol with a molecular weight of 1500 (PEG) (Fluka, USA), NaCl, HCl (chemical grade or special grade) of domestic production.

The study object was erythrocytes of donor blood collected using glucose-citrate solution at the Kharkiv Blood Service Center. The principles of the Declaration of Helsinki, adopted by the General Assembly of the World Medical Association, were observed in the experimental design when working with blood. The experimental design was approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. The erythrocytes were pelleted at 1200 g for 10 min at room temperature, the plasma and leukocyte components of the blood were removed. To the pelleted cells, a solution of 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), in an amount 5—7 times greater than the volume of cell mass, was added and residual plasma and leukocytes were washed away using three-stage centrifugation in a similar mode. An equal volume of a solution containing 0.2 M PEG, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) was added to the washed erythrocyte samples and incubated for 30 min at 4 and 37 °C. The control was erythrocytes incubated in a solution of 150 mM NaCl, 10 mM Tris-HCl (pH 7.4) at 37 °C. Determination of DSM fluorescence indices in erythrocyte suspensions, as well as the orientation order of the membrane cytoskeleton, was performed at room temperature (22—25 °C).

Freezing of erythrocytes exposed in PEG solution according to the above-described conditions was carried out by rapid immersion of the samples in liquid nitrogen (−196 °C). Thawing was carried out in a water bath at a temperature of about 40 °C until complete defrosting.

Study of DSM fluorescence parameters. An aliquot of the test sample was diluted in a cuvette with physiological solution (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) to a hematocrit of approximately 0.02%, after that 20 μl of DSM was added (final concentration 8 μM). Fluorescence spectra were recorded on a spectrofluorimeter "Signe-4M" (Biohims, Latvia) at an excitation wavelength of 460 nm and the width of the excitation and emission slits of 5.0 and 10 nm, respectively. The analysis was carried out according to the algorithm described in the studies by Dyubko T.S. and Gorbenko G.P. [8, 11] using the software "Signe" (Biohims, Latvia).

Determination of the orientation order in the membrane cytoskeleton of erythrocytes by polarization microscopy. Suspensions of control cells, as well as erythrocytes exposed to PEG at 4 and 37 °C and cryopreserved after the corresponding CPA treatment regimens, were applied to a glass slide. The characteristics of birefringence were studied on the instrument group consisting of a light source, a "UM-2" monochromator, a "MKU-1" polarizing microscope, and a "MIN-8" microphotographing device (Asma-device, Ukraine). The microscope was equipped with an additional rotary table with a limb, on which a compensator (a quarter-wave plate or a plate with a path difference of 10^{-2}—10^{-1} relative to the wavelength λ) was installed. The light sources were a mercury lamp "DRSH-250" and a projection incandescent lamp "K-30". The error in measuring the path difference did not exceed 5%. Light was supplied to the microscope through a monochromator or light filters.

Statistical processing of the results was performed using the software package "Statgraphics plus 2.1" (Statistical Graphics Corp., USA). Data are presented as M ± SE (mean ± standard error). The samples were paired; experiments were performed on blood from different donors (n = 4). The statistical significance of differences between experimental groups and control was assessed by the Wilcoxon test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Fluorescent probes enable to assess structural alterations of membrane systems caused by different types of impacts due to high sensitivity of their spectral parameters to physicochemical characteristics of the microenvironment of their molecules. The cationic probe DSM, which is a derivative of pyridine, has been shown to be effective for the study of different biological objects [9, 11]. Due to the presence of a flexible chain between the methylpyridine and aniline rings, the molecule has the properties of polyenes and easily undergoes conformational isomerization in polar environments with non-radiative conversion S1→So and a weak quantum yield. However, upon binding to biomembranes or proteins, as well as upon entering viscous environments, the fluorescence of DSM can increase by 10 or more times [9]. Depending on the microenvironment, the probe can fluoresce in the 'red' and 'green' parts of the spectrum. The position of the maximum in the spectrum at the region of about 600 nm indicates the surface localization of the bulk of probe molecules associated with cells, while the shoulder of the spectrum with a maximum of about 550 nm reflects the binding of a part of the probe molecules in nonpolar areas of the membranes. These characteristics of DSM enable to interpret experimentally obtained fluorescence spectra considering the different contributions of these components to the total spectrum. To isolate individual spectrum components, we used an approach based on its decomposing into two parts: a homogeneous component, which depends on the intramolecular properties of the probe, and an inhomogeneous broadening, the parameters of which are determined by the physicochemical characteristics of the probe molecule environment [9, 11]. The determination of spectral components corresponding to different types of probe localization and the parameters of the inhomogeneous broadening is based on the model of the formation of electronic vibrational spectra of polyatomic molecules. The mathematical algorithm for calculating parameters, implemented in the software for processing experimental fluorescence spectra and substantiated in the studies by Dyubko T.S. and Gorbenko G.P. [9, 11], enables to describe the spectrum using the equation:

$$S(\nu\{C_i, \nu_i^c, \sigma_i\}) = \sum_{i=1}^{n} \int_{\infty}^{-\infty} \frac{1}{\pi} \frac{\Gamma(\nu_s)}{(\nu-\nu_s^2)+\Gamma(\nu_s)} \frac{\mu^2(\nu_s) k_i C}{\sqrt{2\pi}\sigma} \times \exp\left\{\frac{1}{2}\left[\frac{\nu_i^c-\nu_s}{\sigma_i}\right]^2\right\} d\nu_s$$

where ν_i^c, σ_i are the parameters of the inhomogeneous broadening (the center and dispersion of the inhomogeneous broadening of *i* component, respectively); k_i is the fraction of probe molecules associated with centers of *i* type, S is the emitting transition of the fluorophore, Γ is the half-width of the homogeneous transition line between two electronic vibrational states of the probe, C is the normalization coefficient; μ is the electronic matrix element of the electric dipole moment of the emitting transition from the excited $_E$lectronic state (S_1) to the ground state (S_0); $\mu^2(\nu_s)$ is the probability of decay of the excited electronic state with emission; ν is the frequency of the light emitted by the molecule; ns is the transition frequency of the system. The value of *n* is chosen so that the equation describes the experimental spectra with accuracy up to the measurement error. It should also be added

that the parameter v_i^c reflects the hydrophobicity of the probe microenvironment, and the parameter σ_i enables to characterize the mobility of the dipoles in the environment directly related to the microviscosity.

Incubation of erythrocytes with CPA led to changes in the DSM spectrum characteristics relative to the parameters of control cells (Table 1) and concerned the fluorescence intensity and the wavelength value corresponding to the emission maximum (lmax). According to the above equation, the experimental DSM spectra in erythrocytes exposed to PEG at various temperature regimens were decomposed into constituent components (Table 2, Fig. 1) depending on the localization sites. Analysis of the inhomogeneous broadening parameters enabled to identify three spectral components: the first one (Table 2, Fig. 1) corresponded to the DSM characteristics in physiological solution; the second one reflected the hydrophilic interactions of DSM with erythrocyte membranes; and the third one was associated with hydrophobic interactions of the fluorophore with membranes. Comparison of the values of the inhomogeneous broadening parameters of DSM spectra in erythrocytes incubated with PEG at various temperatures showed that the only significant difference between the samples was the parameter k_3, which referred to the third component and characterized hydrophobic interactions, namely determined the proportion of probe molecules associated with centers of this type. Considering that for each experimental sample the total value of the three components of this parameter of inhomogeneous broadening of the spectrum was equal to 1, for the convenience of describing the interactions of CPA with erythrocyte membranes, we can proceed to the percentage expression of the values of this indicator. In particular, it was found that exposure of cells to CPA at 4 °C led to a decrease in the contribution of this component to the total spectrum to 3% compared to 5% in the control erythrocytes (Fig. 1, *a*, *b*, Table 2). Meanwhile, incubation of cells with PEG at 37 °C caused the complete disappearance of this component from the DSM fluorescence spectrum (Fig. 1, *c*, Table 2).

The obtained data on the decrease in the proportion of the hydrophobic component of the DSM spectrum after erythrocyte exposure to CPA could be stipulated by the competition between DSM and PEG molecules for binding sites in the plasma membrane of erythrocytes due to the commonality of their physicochemical characteristics. Pre-exposure of erythrocytes to PEG apparently promoted the occupation of hydrophobic binding sites by CPA molecules that prevented the fluorescent

Table 1. **Impact of PEG on DSM fluorescence parameters in erythrocytes**

Conditions for exposing erythrocytes	Fluorescence parameters	
	λ_{max}, nm	Fluorescence intensity F_{max}, relative units (r.u.)
Saline solution	602	4.9711
PEG solution	610	4.8159
Erythrocytes in saline solution, 37 °C	606	4.2923
Erythrocytes in PEG solution, 37 °C	608	5.0531
Erythrocytes in PEG solution, 4 °C	609	5.3517

Note: Representative data (typical results) are shown

Table 2. **Parameters of inhomogeneous broadening of DSM fluorescence spectra in erythrocyte suspensions**

Types of probe sorption centers	Variants for exposing erythrocytes			
		Saline solution, 37 °C	PEG solution, 37 °C	PEG solution, 4 °C
Component 1	v_1^c см$^{-1}$	15900 ± 48	15900 ± 36	15900 ± 40
	σ_1 см$^{-1}$	900 ± 24	900 ± 34	900 ± 28
	k_1	0.35 ± 0.006	0.40 ± 0,01	0.37 ± 0,01
Component 2	v_2^c см$^{-1}$	16100 ± 51	16180 ± 47	16100 ± 44
	σ_2 см$^{-1}$	700 ±	750 ±	700 ±
	k_2	0.60 ± 0.008	0.60 ± 0,005	0.60 ± 0,008
Component 3	v_3^c см$^{-1}$	17400 ± 65	—	17400 ± 72
	σ_3 см$^{-1}$	860 ± 36	—	860 ± 35
	k_3	0.05 ± 0.005	—	0.03 ± 0.005*

Note: representative data (typical results) on the isolation of the components of the DSM spectrum in erythrocytes according to design of experiment are shown.

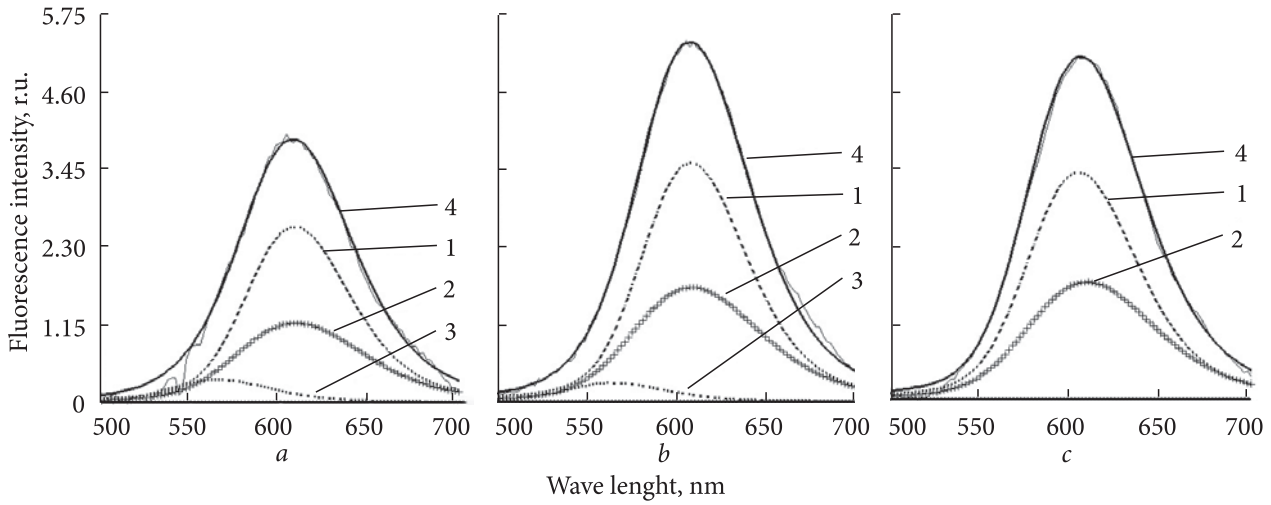

Fig. 1. Parameters of inhomogeneous broadening of DSM fluorescence spectra in erythrocytes. *a* — control cells, *b* — cells exposed to PEG at 4 °C, *c* — cells exposed to PEG at 37 °C: 1 — component of DSM spectrum in physiological solution; 2 — hydrophilic component of DSM spectrum in membrane; 3 — hydrophobic component of DSM spectrum in membrane; 4 — superimposition of experimentally recorded and theoretically calculated DSM spectra. Note: representative data (typical results) on the isolation of the components of the DSM spectrum in erythrocytes according to design of experiment are shown

probe from contacting the membrane via this type of interaction. It should be noted that a decrease in temperature reduces hydrophobic interactions between molecules that may explain the discrepancies between erythrocytes exposed to CPA at 4 and 37 °C in terms of hydrophobic component of the DSM spectrum. More specifically, the ability of PEG molecules to occupy hydrophobic sites in the erythrocyte membrane is reduced at 4 °C compared to their capability when cells are exposed to CPA at 37 °C. This enables DSM molecules to be absorbed into accessible hydrophobic sites on the membrane in cells exposed to PEG at 4 °C, although the number of such sites is reduced compared to the control. Meanwhile, at 37 °C, the hydrophobic sites for DSM binding to the membrane prove to be blocked by PEG molecules leading to the complete disappearance of the hydrophobic component from the fluorophore spectrum.

Considering the fact that erythrocytes exposed to PEG at 4 °C are more viable after freeze-thawing (–196 → 37 °C) compared to samples incubated with CPA at 37 °C [21], it can be assumed that, to a certain extent, this depends on the temperature regimens of the interaction of PEG with membranes, which are manifested in changes in the parameters of the inhomogeneous broadening of the DSM fluorescence spectrum.

The impact of CPA on erythrocyte membranes can affect the structural state of the protein network of the membrane-cytoskeleton complex, which largely determines the stability of cells under stressful conditions as well as in different erythrocyte pathologies.

Alterations in the orientation order of molecules can be assessed using polarization microscopy [4]. When studying anisotropic preparations, which include cell membranes, a polarizer is added to the usual microscope scheme before the illumination system, and an analyzer after the objective, both of which are located at a certain angle to each other. Light splits into two beams with perpendicular planes of oscillation when passing through the polarizer. The optical properties of anisotropic microobjects change depending on their orientation relative to the direction of the polarization plane. Therefore, polarized light changes its characteristics as it passes through an object when disturbances occur in it.

The investigation of the orientation order of different components of erythrocytes revealed that in polarized light a bright ring appears along the contour of the cell, parts of which are darkened in the directions parallel and perpendicular to the plane of polarization of the incident light, and the brightness is maximum when the beam falls at an angle of 45o [15] that is clearly visible in control erythrocytes (Fig. 2, *a*). The presence of the bright ring is stipulated by birefringence in the membranes and perimembrane layers of erythrocytes. Calculated

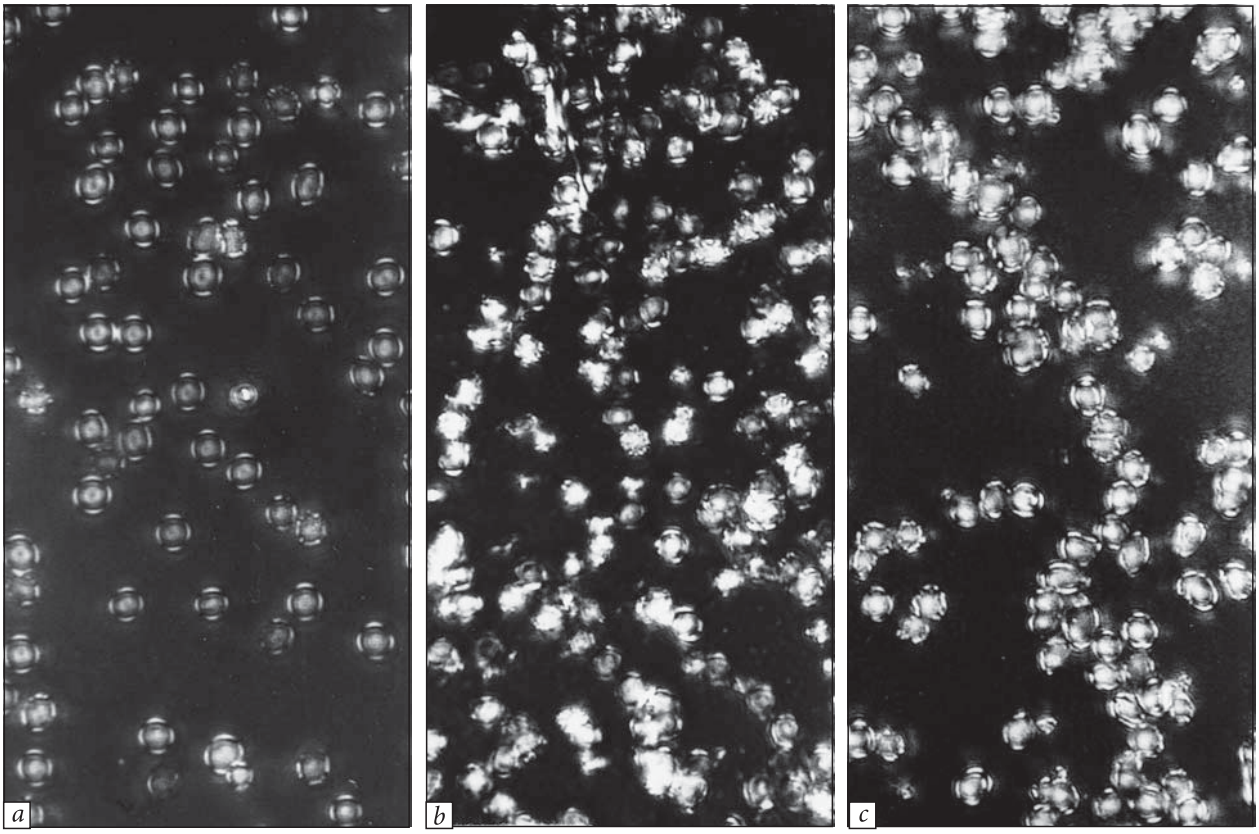

Fig. 2. Polarization microscopy of control erythrocytes (*a*) and erythrocytes exposed to PEG at 37 (*b*) and 4 °C (*c*). Note: (*a*) a bright ring is seen along the contour of the cells, parts of which are darkened in the directions parallel and perpendicular to the plane of polarization of the incident light, and the brightness is maximum when the beam falls at an angle of 45o; (*b*) the radiance is mainly observed as a multitude of individual discrete dots attesting alterations of birefringence and disturbances in the orientation order of molecules; (*c*) The radiance persists mainly in the form of a bright ring, although its contours are somewhat distorted; besides individual cells with a predominance of radiance as a multitude of individual discrete dots are revealed that indicates changes in the orientation order of the molecules. Representative data (typical results) are shown

data showed [15] that the specific optical anisotropy of proteins has a value close to the anisotropy of lipids, and the thickness of the birefringent layer is close to the thickness of the erythrocyte cytoskeleton (about 60 nm). This enables to conclude that birefringence is mainly caused by the cytoskeletal protein network, and the cytoskeletal molecules are oriented in such a way that their polarization perpendicular to the membrane is greater than in the direction parallel to its surface. The orientation order of the cytoskeleton molecules enables to determine the magnitude of birefringence, which changes in response to structural rearrangements in the cytoskeleton system.

Incubation of erythrocytes in a PEG-containing solution led to changes in the orientation order of molecules in the perimembrane zones of erythrocytes (Table 3), the level of which depended on the temperature regimens of cell exposure to CPA. In-

Table 3. **Birefringence indices in erythrocytes exposed and frozen in PEG presence**

Samples	The magnitude of birefringence (Δφ)
Erythrocytes in saline solution	6.330 ± 0.032
Erythrocytes exposed to PEG at 37 °C	birefringence is observed in individual cells
Erythrocytes exposed to PEG at 4 °C	6.037 ± 0.035 *
Frozen-thawed erythrocytes previously exposed to PEG at 37 °C	birefringence is virtually absent
Frozen-thawed erythrocytes previously exposed to PEG at 4 °C	5.530 ± 0.067 *

Note: Results are presented as M ± SE (mean ± standard error), $n = 4$. * Significant differences from control ($p < 0.05$).

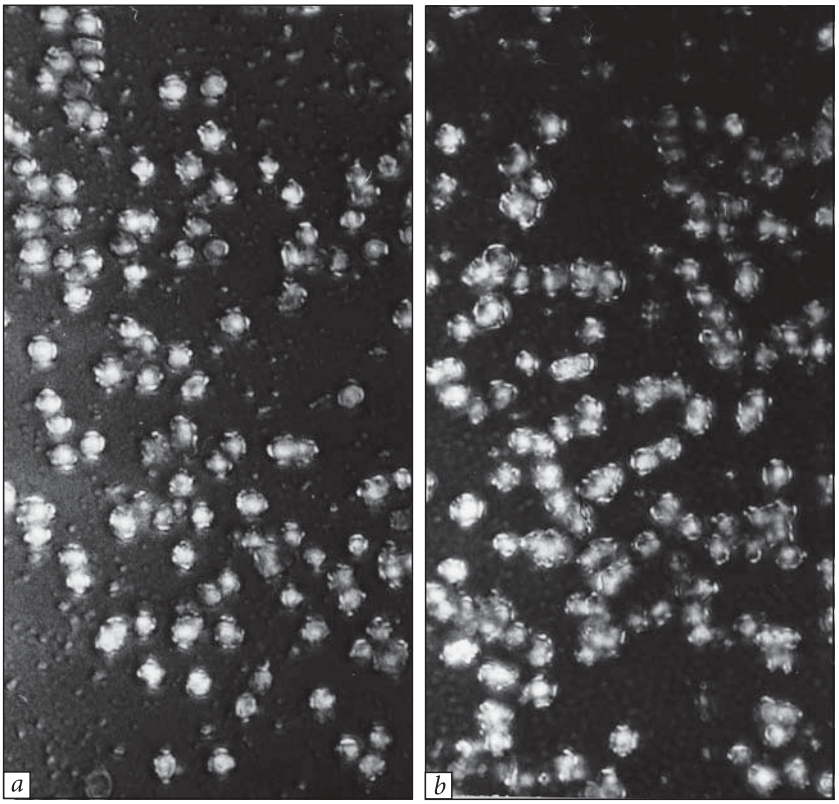

Fig. 3. Polarization microscopy of cryopreserved erythrocytes exposed to PEG at 4 (*a*) and 37 °C (*b*). Note: (*a*) the radiance is observed as a multitude of individual discrete dots attesting alterations of birefringence and disturbances in the orientation order of molecules; (*b*) the radiance in the cells is represented by a multitude of individual discrete dots with the preservation of a bright ring along the contour in a significant part of the cells, indicating disturbances in the order of molecular orientation but still allowing the magnitude of birefringence to be determined. Representative data (typical results) are shown. Note: Results are presented as M ± SE (mean ± ± standard error). For each experimental variant, mean values and corresponding standard errors were calculated for 9 repeated measurements (3 replicates from 3 donors). * Significant differences from control ($p < < 0.05$).

cubation of erythrocytes at 37 °C caused significant disturbances in the orientation order of molecules (Fig. 2, *b*) and determining the magnitude of birefringence was impossible (Table 3), since the radiance was observed in the form of many separate discrete dots. At 4 °C, the orientation of the cytoskeleton molecules was maintained at a sufficient level that enabled to measure the birefringence magnitude (Table 3). However, even in this case, in the cell images at polarized light, significant disturbances in the location of the light ring contours in the perimembrane zones of erythrocytes can be seen (Fig. 2, *c*).

More pronounced disturbances of the orientation order in the perimembrane zones of erythrocytes were observed after freeze–thawing of cells (Fig. 3, Table 3). In thawed erythrocytes previously exposed to PEG at 37 °C, there was no orientation ordering of molecules in the cytoskeleton, while in cells exposed to CPA at 4 °C before freezing, the orientation order of molecules was still preserved but the magnitude of birefringence was significantly reduced.

Thus, the temperature regimen of erythrocyte exposure to PEG significantly affects the nature of binding of exocellular CPA to different centers in the membrane and the orientation order of molecules in the membrane skeleton. The reduction of hydrophobic contacts of PEG with membranes upon a decrease in temperature at the stage of erythrocyte exposure to CPA enables to preserve, to a large extent, the orientation order of the membrane cytoskeleton molecules both during incubation with CPA and after freeze–thawing of cells.

DISCUSSION

One of the typically occurring features of the interaction of CPAs with macromolecules and membranes is their exclusion from the hydrated layers of the biological objects [9, 13, 15, 16]. Maintaining hydration provides the preservation of the native structure of macromolecules under stressful conditions. Nevertheless, even a small portion of the CPA molecules, which are capable of being incorporated into hydrate layers and interacting with individual areas of the biological object, induces certain changes in its structure or mode of operation [1]. In particular, NMR and molecular dynamics simulations using ubiquitin as an example have shown the variability of protein interactions with different substances including glycerol, PEG, and ficol, at high concentrations [1]. The effects of these substances on the protein structure were manifested as localized surface perturbations, dehydration,

changes in electrostatic characteristics, and dynamics of the macromolecule in general. With regard to PEG, it was found that its presence in the surrounding of protein provided the perturbation of hydrophobic sites on the surface of the protein macromolecule, and had virtually no effect on its polar areas. By interacting with membranes via a hydrophobic mechanism, PEG can change their structural and functional parameters, which undoubtedly affects the stability of cells to stress factors. The absence of a hydrophobic component in the DSM spectrum in erythrocytes exposed to PEG at 37 °C indicates the blocking of hydrophobic binding sites for the probe by CPA molecules. Meanwhile, exposure of cells to PEG at 4 °C enables DSM to contact membranes by this type of bonds, although in this case the hydrophobic component of the spectrum decreases.

Previously, in experiments using 14C-PEG, we found that some of the CPA molecules remain on the plasma membranes of erythrocytes even after 300—500-fold dilution of the cell suspension with subsequent 3-fold washing [18]. Moreover, the amount of PEG bound to the membranes significantly depended on the temperature of cell exposure to CPA. The level of radioactivity of cells exposed to CPA at 37 °C exceeded the value of this indicator by five times in cells incubated at 4 °C. The mechanism of such binding and the effect of temperature on this process remain largely unclear. However, the assessment of the spectral components of the DSM fluorescent probe showed that these differences are stipulated by changes in the intensity of hydrophobic interactions upon decreasing temperature. The limitations of hydrophobic contacts of PEG with erythrocyte membranes at 4 °C can be explained not only by a decrease in the intensity of hydrophobic interactions but also due to changes in the accessibility of sites for PEG binding via the hydrophobic mechanism due to temperature-dependent structural and functional rearrangements in the membrane. Hydrophobic contacts of CPA with membranes obviously negatively affect cell stability under stressful conditions, since, according to results obtained, a decrease in hydrophobic interactions at 4 °C contributes to an increase in the stability of erythrocytes during cryopreservation [19].

Similar results were observed for DMSO [2] when studying the temperature dependence of the stability of phospholipid bilayer structures. Experiments have shown that phospholipid membranes in the presence of DMSO are destabilized at higher temperatures due to the hydrophobic bond between CPA and the bilayer. In regard to ethylene glycol (EG), it has been found that CPA stabilizes α-lactalbumin (α-LA) at subzero temperatures and promotes its denaturation at high temperatures [13]. The authors suggested that at low temperatures EG reduced the amount of water around hydrophobic residues of α-LA and stabilized the protein against cold denaturation, while at high temperatures, preferential binding of EG shifted the folding equilibrium towards the denatured state. The important role of hydrophobic interactions in changing the stability of biological objects is demonstrated by model experiments with condensed arrays of hydrophobically modified polysaccharide, hydroxypropylcellulose [16]. The interaction of natural osmolytes, such as glycerol, sorbitol, and others, with hydroxypropylcellulose revealed that they were excluded from polysaccharide molecules. The energy shifts indicate that exclusion of osmolytes from hydrophobic sites significantly increases the stability of macromolecules. Furthermore, the temperature dependence of exclusion indicates a significant enthalpy contribution to the interaction energy, as opposed to a steric repulsion mechanism, suggesting that perturbations in the water structure or hydration forces underlie exclusion [16]. Even glycerol has been found to form hydrophobic contacts with proteins in some cases [14]. In particular, glycerol has shown the inhibition of calcineurin (CNN) activity through hydrophobic interactions. In this case, glycerol binding altered the secondary structure of CNN that could explain the inhibitory effect on its activity.

It should be noted that CPA–induced changes in the structure of proteins, in particular integral membrane proteins, can serve as a trigger for structural and functional rearrangements of supramolecular system elements in the membrane-cytoskeleton complex. Mismatches between the hydrophobic surface of the protein and the hydrophobic interior of the lipid bilayer induce bends or deformations of transmembrane protein segments [17], creating conditions, under which the lipid bilayer modulates the conformation of integral membrane proteins through interactions with hydrophobic transmembrane helices.

Findings of assessment of orientation order in erythrocyte membrane indicate a significant reor-

ganization in the membrane-cytoskeleton complex under the PEG impact, as evidenced by changes in the birefringence angle in erythrocytes exposed to CPAs at various temperature regimens. Lowering the temperature of cell exposure to PEG enables to determine this characteristic due to the preservation of a certain level of orientation order in the system, in contrast to erythrocytes incubated with CPA at 37 °C. The freeze-thawing cycle of erythrocytes exposed to CPA led to a more pronounced disorganization of the order in the membrane-cytoskeleton complex. However, in cryopreserved erythrocytes exposed to PEG at 4 °C, the possibility of determining the angle of birefringence still remained that distinguished them from cells incubated at 37 °C, where the radiance was observed in the form of numerous scattered dots. At the molecular level, the state of membranes in erythrocyte cryopreserved under PEG protection was previously assessed by changes in the electrophoretic profile of proteins in the membrane-cytoskeleton complex, which combined features of native erythrocytes and cells frozen in CPA-free medium [21]. PEG limited the structural rearrangements of the main proteins of the membrane-cytoskeleton complex under the impact of extreme factors, as evidenced by the comparable to the control amount of high-molecular polypeptide complexes induced by the protein-cross-linking reagent diamide. This data distinguished erythrocytes cryopreserved in the PEG presence from cells frozen without CPA protection, in which an increase in protein aggregation was revealed under the diamide effect due to the disruption of macromolecule folding. Concurrently, changes in the level of peroxiredoxin 2 (band 8 protein) in the protein profile of erythrocyte membranes were equally pronounced in cells frozen in the PEG presence and without CPA protection.

In addition, the effect of PEG on proteins of the membrane-cytoskeleton complex can be realized through the surface membrane constituent elements, in particular, changes in the expression of surface markers [20], which promote the further reorganization of the highly organized protein network and changes in the orientation order in the macromolecular system. Although the effect of PEG on the surface characteristics of the membrane and the electrophoretic profile of proteins has not been studied with consideration to temperature modulation, we can assume that it depends to some extent on hydrophobic component of PEG interaction with membranes.

Thus, hydrophobic interactions of PEG with membranes significantly depend on the temperature of erythrocyte exposure to CPA and significantly affect the cell stability under stressful conditions of cryopreservation, changing the structural ordering of proteins in the membrane-cytoskeleton complex.

CONCLUSIONS

1. Exposure of erythrocytes to PEG is accompanied by a decrease in hydrophobic component of fluorescence spectrum of the DSM probe that indicates the PEG impact on membranes via hydrophobic bonds as one of the constituent elements of the mechanism of cell interaction with this CPA. The complete absence of the hydrophobic component in the DSM spectrum in erythrocytes incubated with PEG at 37 °C indicates competitive occupation of binding sites by PEG molecules and their blocking for contact with the fluorescent probe. Lowering the exposure temperature to 4 °C reduces hydrophobic contacts of PEG with membrane that is confirmed by the presence of hydrophobic component in the DSM spectrum, although at a lower level than in the control.

2. Exposure of erythrocytes to PEG causes reorganization of the orientation order in membrane-cytoskeleton complex, as evidenced by the alteration of the anisotropic properties of the membrane according to polarization microscopy. The temperature regimen of cell exposure to CPA significantly impacts the orientation order of cytoskeleton macromolecules. At 4 °C exposure regimen, a decrease in the birefringence angle is observed in erythrocytes incubated with PEG compared to the control, while at 37 °C exposure regimen, the determination of this characteristic becomes virtually impossible. Freeze-thawing of erythrocytes previously exposed to PEG at various temperatures enhances these trends. The reducing in hydrophobic contacts of PEG with membranes at a lower temperature during the exposure stage provides better preservation of structural order in the membrane-cytoskeleton complex both during incubation with CPA and after freeze-thawing of cells.

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ВПЛИВ ТЕМПЕРАТУРИ НА ВЗАЄМОДІЮ ПОЛІЕТИЛЕНГЛІКОЛЮ З МЕМБРАНАМИ ТА ОРІЄНТАЦІЙНИЙ ПОРЯДОК БІЛКІВ ЦИТОСКЕЛЕТА ЕРИТРОЦИТІВ ЛЮДИНИ

У роботі визначено відмінності у характері модифікації мембран еритроцитів при експонуванні з поліетиленгліколем з молекулярною масою 1500 (ПЕГ) за різних температур (4 і 37 °С) із використанням флуо-

ресцентного зонда 4-(n-диметиламіностирил)-1-метилпіридиній-n-толуолсульфонату (ДСМ), а також змін орієнтаційного порядку білків мембрано-цитоскелетного комплексу методом поляризаційної мікроскопії. Експонування еритроцитів із ПЕГ при 37 °С спричиняє повне зникнення гідрофобного компонента флуоресцентного спектра ДСМ через конкурування молекул ПЕГ і зонда за ділянки зв›язування. Зниження температури експонування клітин з кріопротекторним агентом (КПА) до 4 °С зменшує можливість його гідрофобних контактів із мембранами клітин, що підтверджується присутністю гідрофобної складової спектра, хоча на нижчому рівні, ніж у контролі. Зменшення гідрофобних контактів ПЕГ із мембранами при зниженні температури на етапі експонування еритроцитів з КПА дає змогу значною мірою зберегти орієнтаційний порядок молекул мембранного цитоскелета як у процесі інкубування з КПА, так і після заморожування–відтавання клітин.

Ключові слова: еритроцит, мембрана, поліетиленгліколь, кріопротекторний агент, гідрофобні взаємодії, орієнтаційний порядок білків мембрани.