

## **Involvement of Erythrocyte Membrane Proteins in Temperature-Induced Disturbances of the Permeability Barrier**

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The ion permeability of human erythrocyte membrane displays two maxima at 48–52°C and 62–67°C [6, 8]. Both these independent maxima were investigated in modified membranes in order to elucidate the participation of the main types of membrane proteins. The modification protocols included the bilateral proteolytic digestion of membranes with 2–20 µg/ml trypsin, denaturation of the peripheral protein spectrin by exposing the membranes to 50°C for 4 min or 1.5 M urea for 20 h, and preparation of the inside-out vesicles depleted of main peripheral proteins. Only the second maximum was registered in these membranes. Also, both maxima were absent in the unilamellar liposomes prepared from lipids extracted from intact membranes. The results indicate that different types of proteins were involved in the two disturbances: peripheral proteins (mainly spectrin) – in the first one and part of integral proteins – in the second. The different sensitivities of the disturbances to local anesthetics, protein thermostabilizers, *n*-alcohols, and detergents correlated with this conclusion. A correlation between the peak temperature of the second disturbance and the sphingomyelin content in the membrane of mammalian erythrocytes was also shown.

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More and more works indicate that a significant role in the mechanism of cell thermal death is played by the disturbance of the barrier function of the cell membrane [1–5]. For mammalian erythrocytes as well as for nuclear erythrocytes of some other species the disturbance of the membrane barrier function at high temperatures was established to occur in two stages differing in both the magnitude of the inducing temperature and the strength of the evoked increase in permeability [6]. Apparently, these disturbances occur independently of each other and are due to the thermoinduced changes of different groups of membrane proteins.

In human erythrocytes, the first disturbance of permeability is within the temperature range in which there occurs denaturation of spectrin [6] – the main protein of the membrane peripheral network [19]. The enzymatic and chemical destruction of the peripheral network increases the permeability of the membrane for non-electrolytes [7]. Thus, it appears to be realistic that heat denaturation of spectrin is responsible for this permeability disturbance.

At the second barrier disturbance the ionic permeability sharply increases by about three orders of magnitude as compared with the norm. This is due to the emergence of pores in the membrane, through which first inorganic ions and then sucrose move along the concentration gradient [6, 8]. The influx of osmotically active particles increases the osmotic pressure of the cytoplasm, due to which water starts to come inside the cell. This leads to the swelling of erythrocytes or their closed ghosts and can evoke their hemolysis [8]. It has been established that at a temperature higher than 48°C thermal hemolysis is also related to the colloid-osmotic effect. The temperature dependence of the ionic permeability of the membranes is of the same form in intact erythrocytes and their ghosts and obeys the Arrhenius equation. This made it possible to determine the activation energy of the permeability (250 kJ/mol) and inducing temperature  $T_m$ , at which the permeability of the membrane is disturbed (61–62°C) [9]. In a large group of mammals the magnitude of  $T_m$  varied within a broad range, but it always coincided with the magnitude of the peak temperature of the second barrier disturbance extrapolated to the zero heating rate [5]. The magnitude of the inducing temperature  $T_m$  in cells of these species closely correlates with their thermoresistance, which in turn increases as the content of sphingomyelin in membranes goes up [5]. These results indicate that thermal hemolysis of erythrocytes is related to the thermoinduced disturbance of membrane permeability [5, 9–12].

The purpose of this work is to investigate and, if possible, discriminate the participation of the main groups of membrane proteins – peripheral and integral proteins – in two observed disturbances of the barrier function of erythrocyte membranes.

## EXPERIMENTAL

We used trypsin, chlorpromazine, toluenesulfonyl fluoride, thermoprotectors glycerol and erythritol, detergents Triton X-100 and sodium dodecyl sulfate, oleic acid, nitrobenzylpyridine (Sigma, USA), maximally purified aromatic alcohols.

White closed ghosts were obtained as follows [13]. Erythrocytes were taken from the fresh blood and washed three times with excess physiological saline. The lysis was carried out in a 3 mM Tris buffer (pH 8.0) containing 0.5 mM  $MgCl_2$ , at a cell and buffer solution ratio of 1:30. After a 5 min incubation at 2°C, 50 mM NaCl was added. The ghosts were separated by

centrifugation. The procedure of washing the ghosts was repeated three times, with each washing the tonicity of the saline being gradually increased. At the last stage, 150 mM NaCl was used. The ghosts were "cured" by incubation at 37°C for 15 min.

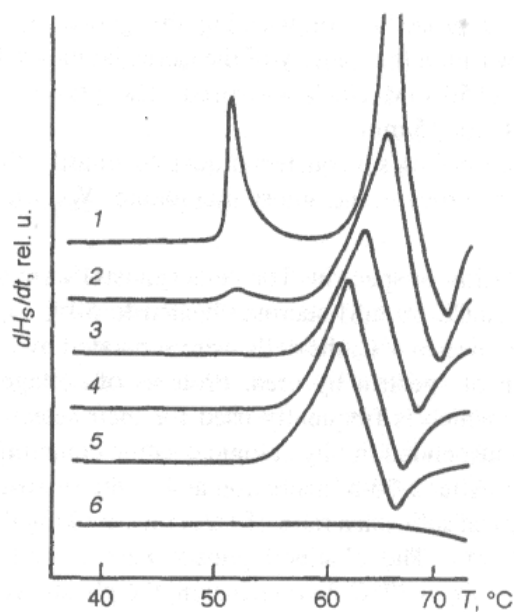
The literature describes several techniques to modify the submembrane network of the erythrocyte and ghost membrane. We used the following techniques.

1. Heat denaturation of spectrin. The cells (ghosts) were resuspended in a buffer (120 mM NaCl, 60 mM sucrose) heated to 50°C [14]. In 4 min the suspension was cooled to 4°C; the cells were separated by centrifugation.

2. Denaturation of spectrin by urea. Proteins of collagen type are very sensitive to urea, which is frequently used for their selective denaturation. The cells were resuspended in physiological saline containing 125 mM urea (hematocrit 0.20). After a 20-h incubation at 4°C the suspension was diluted by cold physiological saline at a ratio of 1:20, thus evoking the posthypotonic hemolysis for 5 min. The obtained ghosts were closed by keeping the suspension for 20 min at 37°C. In conclusion, the ghosts were twice washed with physiological saline.

3. Bilateral proteolytic digestion of erythrocyte membrane [15]. The residue formed from closed white ghosts was supplemented with 5 mM Tris buffer containing 2–20 µg/ml trypsin (pH 7.4, hematocrit 0.10). The suspension obtained was kept at 4°C for 30 min. The proteolysis was arrested by adding 1 mM toluenesulfonyl fluoride, the membranes were separated and washed twice with the buffer (150 mM NaCl). In conclusion, the membranes were closed by incubation at 37°C for 15 min. The proteolytic splitting of spectrin network proteins was determined by gel electrophoresis.

Inside-out vesicles were obtained by incubation of white ghosts at 37°C for 1 h in 1 mM borate buffer (pH 9.0) containing 0.5 mM EDTA and 0.2 mM toluenesulfonyl fluoride [7]. During the incubation, the suspension was passed three times through an ophthalmologic needle, which decreased its viscosity and turbidity. The vesicles were isolated by differential centrifugation. First the suspension was subjected to low-revolution centrifugation (5000 rpm, 10 min) to sediment intact membranes. The collected supernatant was centrifuged at 25 000 g for 30 min. The obtained sediment consisted of vesicles. The test for the change of the acetyl esterase activity after solubilization of vesicles with 1% solution of sodium dodecyl sulfate showed that about 80% of them were inside-out vesicles. A solution (0.8 M NaCl, 2 mM MgCl<sub>2</sub>) was poured into the sedimented vesicles at a volume ratio of 1:4. The suspension was incubated for 20 min at 25°C to close the membranes. Prior to use, the suspension was diluted fivefold by an isotonic sucrose solution to establish a transmembrane gradient of NaCl. Extraction of the peripheral network proteins – spectrin and actin – and the emergence of spectrin in the medium was determined by gel electrophoresis [7].



**Figure 1.** Thermograms of the conductance derivative of the native and modified human erythrocyte membranes, recorded in the presence of NaCl transmembrane gradient, directed inside the cell. Hematocrit and the heating rate are, respectively, 0.10–0.20 and 2°C/min. 1, white ghosts, inside which the buffer is 150 mM NaCl, and outside, 40 mM NaCl + sucrose. The first peak at 51°C conforms to the increase in the ohmic conductance of the membrane, the second peak at 65°C characterizes the rate of equilibrating the transmembrane gradient of NaCl, and the subsequent (negative) peak describes the dissipation of the sucrose gradient [6, 8]; 2, white ghosts with spectrin, denatured by 1.5 M urea (20 h, 4°C); 3, white ghosts with thermally denatured spectrin; 4, closed white ghosts treated with trypsin (2–20 µg/ml); 5, spectrin- and actin-free inside-out vesicles; 6, unilamellar liposomes from membrane lipids (the total concentration of lipid, 1 mg/ml).

The concentration of the alcohols in the erythrocyte and ghost membrane,  $C_m$ , was determined by the formula:

$$C_m = K \cdot C_0, \quad (1)$$

where  $C_0$  is the concentration of an alcohol in the suspension medium;  $K$  is the membrane/medium partitioning coefficient for the alcohol (the values are taken from [25]). The rated labilizing capacity of the alcohol,  $L$ , was calculated by the formula:

$$L = \Delta T_m / (T_m \cdot C_m), \quad (2)$$

where  $T_m$  is the peak temperature of the second barrier disturbance;  $\Delta T_m$  is the decrease of this temperature in the presence of an alcohol in the membrane.

The cytosol-independent thermoinduced barrier disturbances in the erythrocyte membrane were registered as described [6, 16]. The method consisted in the registration of the time (temperature) derivative of the conductance of the suspension of closed erythrocyte ghosts under conditions of NaCl transmembrane gradient and gradual heating. The isotonicity at both sides of the membrane was provided by sucrose. When the temperature interval, within which the membrane permeability is disturbed, was reached, diffusion of ions began as well as subsequent changes in the volume, the rate of which was reflected by a peak in the thermogram. The increase of membrane conductance was also recorded as a peak not depending on the NaCl gradient. The method is insensitive to membrane vesiculation. The reproducibility of the peak temperatures is within  $\pm 0.2^\circ\text{C}$ .

The erythrocyte membrane lipids were extracted according to the method described in [17], using white ghosts. After evaporation of the solvent on a rotor evaporator, the physiological saline was added to the lipid film and the suspension obtained was sonicated (50 W, 3 min). The transmembrane gradient of NaCl was established by diluting the suspension by the isotonic sucrose solution at a ratio of 1:5. The final concentration of the lipids was 1 mg/ml.

Triton X-100, nitrobenzylpyridine, sodium dodecyl sulfate and oleic acid were incorporated into the erythrocyte membrane by incubation of the cells in a medium containing a respective detergent for 1 h at  $20^\circ\text{C}$ , hematocrit 0.10 at constant stirring. Oleic acid and nitrobenzylpyridine, diluted in ethanol, were added to the cell suspension. Prior to use, the cells were twice washed with physiological saline.

## RESULTS AND DISCUSSION

As it is known, the erythrocyte membrane is equipped with the submembrane network of peripheral proteins, consisting predominantly of spectrin and actin. This network can be selectively broken down by various effects: denaturation by heating, urea, splitting by trypsin, extraction under conditions of low ionic strength and in the absence of divalent cations [19]. Figure 1 presents a thermogram of the conductance derivative of a suspension of intact human erythrocytes and their ghosts (curve 1) as compared with the thermograms of modified membranes: the membranes with the spectrin network broken down using urea (curve 2) and heat (curve 3); the membranes treated with trypsin (curve 4); inside-out vesicles (curve 5). The thermogram of the sonicated liposomes prepared from extracted membrane lipids shows that the membrane proteins are directly involved in both thermally induced barrier disturbances (curve 6).

As seen, any influence on the peripheral protein network affects the first barrier disturbance. In the cases when the extent of the effect increased (a more concentrated solution of urea or trypsin), the amplitude of the first peak decreased up to the total disappearance, whereas the peak temperature virtually did not change (data not shown). These results indicate that the preliminary decomposition or modification of the peripheral network affecting predominantly spectrin "extinguishes" the processes evoking the disturbance of the barrier functions of the native membranes at a spectrin denaturation temperature. Based on that, it can be concluded that the heat denaturation of spectrin is responsible for the first barrier disturbance.

In contrast to this, the above modifications of the membranes had virtually no effect on the amplitude of the second disturbance. However, the peak temperature of this process decreased significantly after some of the above effects (Fig. 1). In inside-out vesicles the peak temperature depended also on temperature and healing time of the membranes (data not shown). These facts indicate that the second disturbance, probably, involves thermally labile proteins the stability of which depends on their environment. These proteins can be of integral nature, because the absence of peripheral proteins affects only the first disturbance. At the same time, the absence of integral proteins in the liposome suspension, prepared from extracted lipids, also eliminates the second disturbance (Fig. 1, curve 6). It is known that the peripheral proteins are totally accessible for digestion by proteolytic enzymes, in particular, by trypsin, while the integral proteins are decomposed by them only partially [15]. The results indicate (Fig. 1, curve 4) that the second disturbance involves the membrane proteins little accessible for trypsin, which is consistent with the suggestion of their integral character.

The information on the nature of the proteins, involved in both membrane processes, was obtained also using membranotropic agents – cell thermosensitizers [3] (Table 1). The presence of a local anesthetic in the medium linearly decreased the peak temperatures of both disturbances in intact erythrocytes and their white ghosts. This allowed us to determine the sensitivity of each process as a ratio of the decrease of its temperature,  $\Delta T_m$ , to the concentration  $C_0$  of the agent in the aqueous medium. The used anesthetics lidocain and tetracain have close values of protein binding coefficients –

**Table 1.** A decrease of the peak temperatures of both barrier disturbances ( $\Delta T_m$ ) in the presence of local anesthetics in the medium.

Anesthetic	Sensitivity $\Delta T_m/C_0$ , °C/10 mM	
	first disturbance	second disturbance
Tetracain	-2.20	-13.7
Lidocain	-1.25	-0.20

respectively, 64 and 76 – but strongly differ by their solubility in a lipid medium – respectively, 2.9 and 80 [20, 21]. As seen in Table 1, the given anesthetics had almost the same effects on the first disturbance, whereas the second defect strongly depended on the solubility of the agent in the lipid media. This indicates that the thermolabilization of the protein involved in the first disturbance depends primarily on the capability of the anesthetic molecules to be bound to it, whereas the thermolabilization of the protein involved in the second disturbance is determined mainly by the solubility of the anesthetic in the lipid matrix. Thus, it could be suggested that the first protein has the surface largely facing the aqueous medium, and the second protein predominantly contacts the lipid bilayer.

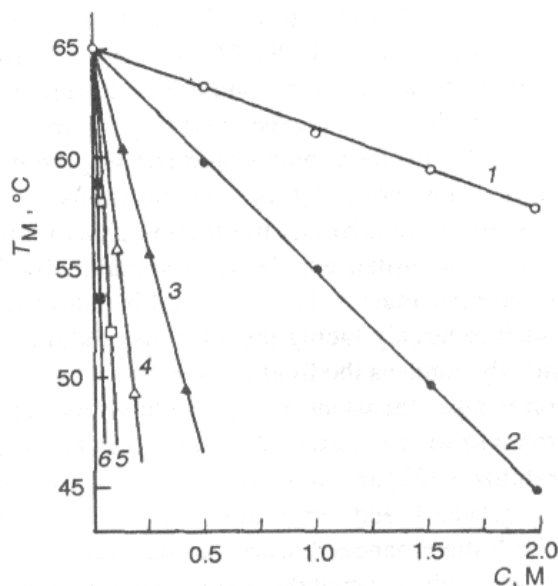
This conclusion is also consistent with the data on the sensitivity of both disturbances (proteins) to the presence of water-soluble agents which are protein thermostabilizers [22] and at the same time cell thermoprotectors [3]. The presence of glycerol and erythritol (up to 30%) shifts the peak temperatures of both disturbances linearly to the right-hand side along the temperature axis. This indicates that the membrane proteins involved in both processes are stabilized by these agents. Both agents demonstrated almost equal stabilizing capabilities which, however, proved to be almost three times higher for the first protein as compared with the second one (Table 2). According to the current views of the mechanism of action of thermostabilizers [23], these results can be explained by assuming that the molecular surface contacting the aqueous medium is much larger in the first protein than in the second one.

The presence of a series of aliphatic and aromatic proteins in the medium as well as of some other aliphatic agents also decreased the temperatures of both disturbances linearly, at least at low temperatures (Fig. 2), which is consistent with the results of the interaction of proteins and alcohols [24]. Removal of the alcohol after incubation with cells and their ghosts totally restores the normal temperature.

The incorporation of a series of amphiphilic agents into the erythrocyte membrane also decreased the thermostability of the proteins (Fig. 3). To compare the labilizing capability of various compounds, it is necessary first of all to create their equal concentrations in the erythrocyte membrane. For

**Table 2.** An increase of the peak temperatures,  $\Delta T_m$ , of both barrier disturbances in the presence of protein thermostabilizers in the medium.

Polyatomic alcohol	Sensitivity of $\Delta T_m/C_0$ , °C/10 weight %	
	first disturbance	second disturbance
Glycerol	+2.20	+0.7
Erythritol	+1.8	+0.7



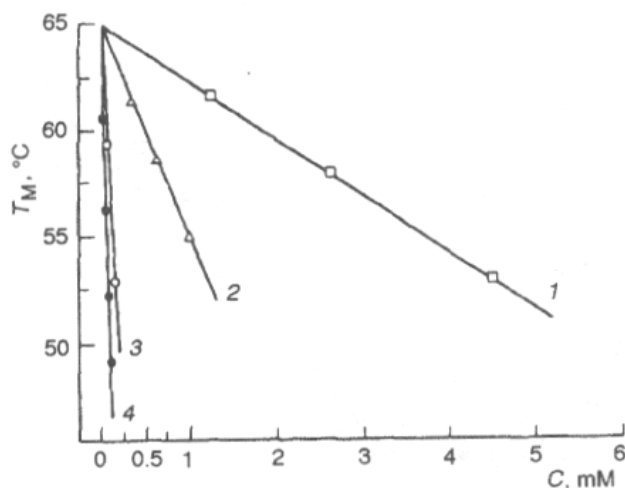
**Figure 2.** A dependence of the peak temperature  $T_m$  of the second barrier disturbance on concentration ( $C$ ) in a medium of normal aliphatic alcohols. 1, methanol; 2, ethanol; 3, propanol; 4, butanol; 5, pentanol; 6, hexanol.

this, one should know the coefficient of partitioning between the lipid and aqueous phases for each agent [25]. As shown in Table 3, at 1 M concentration in the membrane, normal alcohols of the methanol–octanol series have virtually the same labilizing effect,  $\Delta T_m/T_m$ , for proteins of the

**Table 3.** A decrease of the peak temperature,  $\Delta T_m$ , of the second disturbance (labilization of the second membrane protein) by a number of amphiphilic agents on condition of their equal concentrations in the membrane,  $C_m$ .

Amphiphilic agent	Membrane/medium distribution coefficient [25]	Rated labilizing capacity $L$ , $m^{-1}$ *	Amphiphilic agent	Membrane/medium distribution coefficient [25]	Rated labilizing capacity $L$ , $m^{-1}$ *
Methanol	0.045	$-1.15 \pm 0.03$	1-Heptanol	39.6	$-0.93 \pm 0.07$
Ethanol	0.14	$-1.15 \pm 0.03$	1-Octanol	151.8	$-0.85 \pm 0.07$
1-Propanol	0.45	$-1.10 \pm 0.03$	Phenol	8.5	$-0.64 \pm 0.05$
1-Butanol	1.50	$-1.10 \pm 0.03$	Thymol	400	$-0.37 \pm 0.05$
1-Pentanol	3.60	$-1.05 \pm 0.05$	Pyridine	0.9	$-0.70 \pm 0.05$
1-Hexanol	13.0	$-1.00 \pm 0.05$	Chlorpromazine	1400	$-0.105 \pm 0.03$

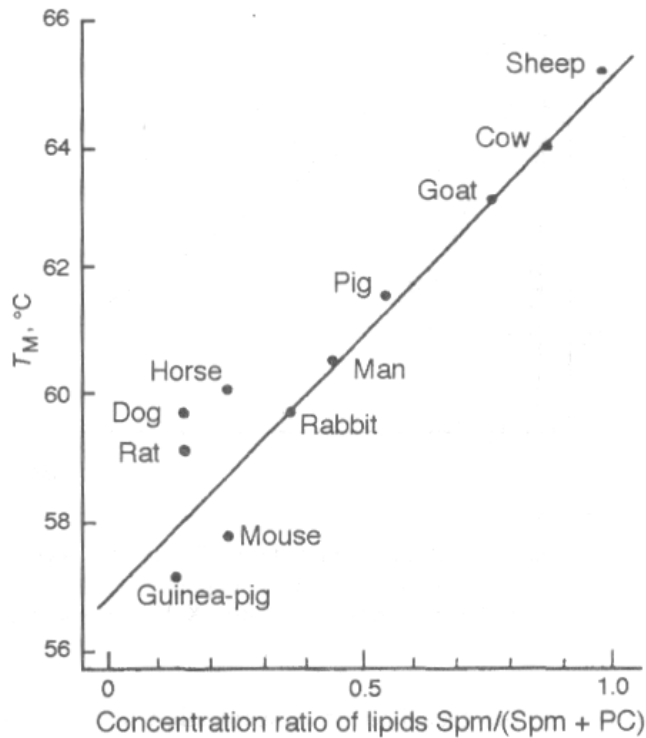
\* $L = \Delta T_m / (T_m \cdot C_m)$ .



**Figure 3.** A dependence of the temperature ( $T_m$ ) of the second barrier disturbance on the concentration ( $C$ ) of some amphiphilic agents in erythrocyte membrane. 1, nitrobenzylpyridine; 2, Triton X-100; 3, sodium dodecyl sulfate; 4, oleic acid.

second group despite the fact that the concentration of the alcohols in the aqueous medium differs 3200-fold. Thus, the labilizing effect of this agent on second-group proteins is determined predominantly by its ability to be dissolved in the lipid bilayer, which is, evidently, the environment for these proteins. This conclusion is consistent with the observed effect of the labilization of these proteins after incorporation of the detergents or oleic acid into the erythrocyte membrane (Fig. 3). Earlier, it was shown that the value of  $\Delta T_m/T_m$  for proteins of the second group is proportional to the increase of the fluidity,  $\Delta\eta/\eta$ , and to the decrease of the organization of the lipid bilayer measured using the fluorescent probe diphenylhexatriene [26]. It was found that the rated labilizing capacity of aromatic alcohols and chlorpromazine is obviously lower than that of aliphatic alcohols, which, probably, corresponds to their ability to "liquefy" the lipid bilayer (Table 3).

The above agents also decreased the temperature of the first defect but to a lower extent. As compared with the labilization of second-group proteins, the relative decrease of this temperature was 2.5 times smaller in methanol, 3.5 times smaller in ethanol, 4.3 times smaller in propanol and about 5.5 times smaller in butanol. At the same time, the detergents and oleic acid had virtually no effect on the temperature of the first disturbance. These data indicate that, as the lipophilicity of the agent rises, its ability to labilize the (integral) proteins of the second group increases to a larger extent than those of the first group (peripheral proteins). Using the calorimetric method, the authors established [29] that ethanol predominantly decreased the



**Figure 4.** A dependence of the peak temperature ( $T_m$ ) [6] on the content of sphingomyelin (Spm) in the mammalian erythrocyte membrane [27]. The ratio of the main groups of the membrane lipids, including the group of choline-containing lipids, in the erythrocyte membranes of the above species is approximately the same [27]. PC, phosphatidylcholine.

temperature of denaturation of a typical integral protein (the anionic channel) as compared with the thermolabilization of spectrin, which is consistent with the conclusions of the present paper.

The lipid composition of the mammalian erythrocyte membrane is characterized by the following features: the percentage of the main lipid fractions (cholesterol, phosphatidylserine, phosphatidylethanolamine and choline-containing fractions) is approximately the same in different species; however, the ratio inside the group of choline-containing lipids (phosphatidylcholine and sphingomyelin) varies within a wide range [27]. Figure 4 shows an experimental dependence of the peak temperature  $T_m$  of the second disturbance on the content of sphingomyelin in erythrocyte membranes in various mammals. The magnitudes of  $T_m$  were published earlier [6], and the lipid composition was taken from [27]. As seen, the replacement of phosphatidylcholine by sphingomyelin in some species, with other lipids remaining the same, leads to the increase in the magnitude of  $T_m$ . This implies that the

thermostability of the second group proteins depends on the lipid composition of the membrane, especially on the content of sphingomyelin. This can be explained by the larger hydrophobicity of this lipid as compared with phosphatidylcholine, because its degree of unsaturation is smaller and the average length of the tail is larger [27]. In contrast with this, the temperature of the first peak does not depend on the lipid composition of the membrane [6]. These results are consistent with the view that, unlike the first group proteins, the thermal stability of the second group proteins depends on their contact with the hydrophobic moiety of the lipid bilayer of the membrane.

Thus, various groups of proteins are involved in the first and second barrier disturbances. The first disturbance is determined by the temperature denaturation of spectrin – the main structural protein of the membrane. The second disturbance is related to structural changes of at least part of the integral proteins, the thermal stability of which depends on the state of the lipid bilayer, in particular, its composition and fluidity. As this process is involved in thermogenesis [5], a further investigation of its nature is required. Tsybmal and Moiseyev [28] found that in the temperature interval of the second defect the microviscosity of the bilayer increased, and they explain it by the irreversible violations of the membrane structuredness. At present, the character of these structural changes and the type of the proteins involved are investigated.

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