Interaction Mechanism of Cortisol and Catecholamines with Structural Components of Erythrocyte Membranes

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Received: December 17, 2009; Revised Manuscript Received: May 26, 2010

Nonspecific mechanisms of the stress hormones interaction with erythrocyte membranes were studied by means of atomic force microscopy, fluorescence analysis, and IR spectroscopy. It was shown that stress hormones (cortisol, adrenaline, noradrenaline) can bind to erythrocyte membranes with high affinity ($K_b \sim 10^6$ M^{-1}). The binding mechanism involves hydrogen bonds and hydrophobic and electrostatic interactions. Active groups of the hormones (NH_2, $NHCH_3$, keto, and hydroxy groups) interact simultaneously with CO and NH groups both of proteins and phospholipids. This leads to the formation of complex protein−lipid domains that distort the surface of the erythrocyte membrane. Water dipoles are displaced from the domains to adjacent regions and facilitate membrane loosening. The interaction of hormones with the membrane is accompanied by structural transitions of disorder → order (tangle → α-helix, tangle → β-structure) in membrane proteins and structural transitions of order → order in phospholipids. Formation of large domains (clusters) of the lipid−protein and lipid nature leads to distortion of membranes and deteriorates their elasticity and rheological properties.

Introduction

Physiological activity of the great majority of hormones is determined by the receptors located on plasmatic membranes of target cells. They all have high affinity for the hormone ligand. Several types of receptors may be related to the same hormone on cell membranes. In particular, four types of adrenoreceptors were found for catecholamines: α_1, α_2, β_1, and β_2.[1] By now, their subtypes have already been distinguished: α_{1A}, α_{1B}, α_{1C}, β_1, β_2, and β_3.[2] All α_1 receptors stimulate phospholipase that hydrolyzes phosphoinositides. All α_2 receptors inhibit adenylate cyclase. All β receptors activate this enzyme. Besides, α_{2A} and α_{2B} receptors inhibit Ca^{2+} channels, whereas β_1 receptors activate them. Noradrenaline receptors are located in synaptosomes, and adrenaline receptors reside on the membranes of various somatic cells (hepatocytes, muscle cells). Supposedly they are present also on plasmatic erythrocyte membranes and are related with the spectrin−actin−ankyrin meshwork.[3]

Receptors for glucocorticoids (cortisol, corticosterone, and their synthetic analogues) are understood much more poorly. In blood, these hormones are transferred by transcortin (corticosteroid-binding globulin). Besides, it is believed that 10% of glucocorticoids are bound to albumins and 6−8% to erythrocyte and leukocyte membranes, and 5−10% are unbound.[4] There is also an intracellular receptor for glucocorticoids with molecular weight 300 000 Da, which takes part in the induction of gluconeogenesis enzymes in the liver.[5] The existence of receptors for glucocorticoids on plasmatic cell membranes is discussed, but this issue is still unexplored.[4]

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It was supposed that membrane receptors play an important role in the action mechanism of any steroid hormones that are involved in the regulation of gene expression in the target organ cells.[6] Detection of a rather large amount of steroid hormones in the albumin fraction of blood and on plasmatic membranes of some cells (erythrocytes, leukocytes) points to the possibility of their nonspecific (nonreceptor) interaction with protein and lipid membrane structures. Fast effects of steroid hormones not related with their action via receptor apparatus of a cell were described earlier.[7] It was shown that they can be determined by direct interaction with a cell membrane (membrane-intercalation model).[8] This action mechanism of steroid hormones was verified with artificial phospholipid membranes. It manifests itself differently, depending on hydrophobic properties of the hormones.[9] The importance of studying this action mechanism is still under discussion in the literature.[10] The interaction for the cell is unclear. Here, of particular interest are glucocorticoids and catecholamines, for which blood content may increase several fold under stress. This will increase nonspecific interaction of these hormones with plasmatic membranes of blood cells.

The work is aimed at studying the mechanism of cortisol and catecholamine interaction with erythrocyte membranes and the mechanism of structural transitions that occur in membranes under the action of these hormones.

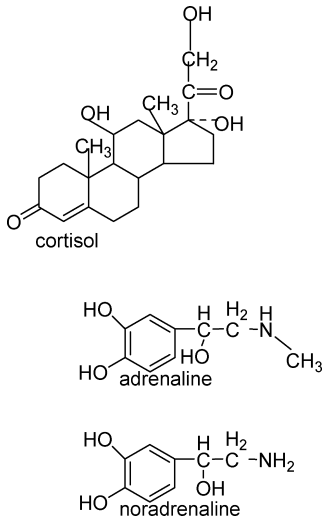
Materials and Methods

The action of three stress hormones, cortisol, adrenaline, and noradrenaline (Amersham), is analyzed in the work.

For this purpose, the following methods were used.

1. Atomic Force Microscopy (AFM) of Erythrocytes. Erythrocytes were obtained from fresh blood after decapitation of Wistar rats under light nembutal narcosis. Blood was diluted

10.1021/jp911917a
Published on Web 07/02/2010



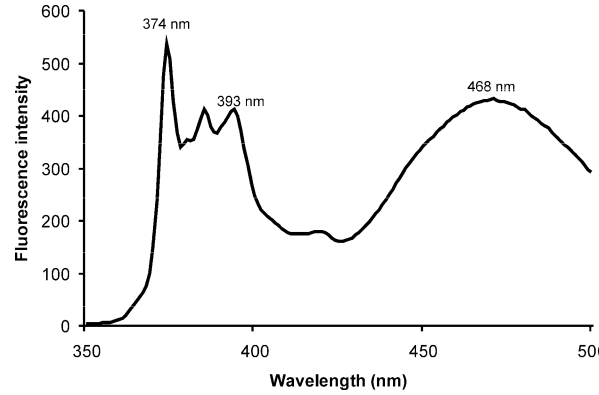


Figure 1. Emission spectrum of pyrene in the suspension of shadows. Excitation wavelength $\lambda = 337$ nm and spectral slit width 1.5/3. At that, the maxima of emission intensity were observed at $\lambda = 374$ nm and $\lambda = 393$ nm (the vibronic emission peaks of excited pyrene monomers) and $\lambda = 468$ nm (the emission maximum of excited pyrene dimer). Concentration of shadows $C = 0.128$ mg of protein/mL. Concentration of pyrene in the suspension is 7.7×10^{-6} M; the temperature of the specimens is 309.1 ± 0.1 K (36 °C); and the pH of the suspension is 7.35.

2-fold by isotonic phosphate buffer (pH 7.35) containing 0.16 M KH_2PO_4 and 0.18 M Na_2HPO_4. After precipitation of cells by centrifuging at 330*g* for 10 min, supernatant liquor was decanted, and the washing procedure was repeated twice more.

All the procedures were performed at 4 °C. The resulting erythrocyte suspension of 20 mcl volume was deposited onto a glass slide as a thin smear. The smear was predried for 10 min in air at 24 °C and humidity of 40%. After evaporation of excessive surface moisture, the smear was observed under a Solver Bio atomic force microscope (NT-MDT, Russia) at 24 °C using a semicontact mode. An analogous procedure of obtaining red blood cells for the AFM examination was employed earlier by other authors.[11] In each experiment, we first tested a control specimen without hormones and then the experimental one. Silicon cantilevers NSG11 (NT-MDT, Russia) with a resonant frequency between 120 and 180 kHz and spring constant ~6 N/m were used (all of these probe parameters were offered by the manufacturer). Images of the surface relief of the erythrocyte membrane after absorption of hormones were obtained with the scan sizes 1×1 μm^2 and 1.3×1.3 μm^2.

2. IR Spectroscopy of Erythrocyte Shadows. Erythrocyte shadows were obtained after their hemolysis in hypotonic phosphate buffer (pH 7.35) containing 2.75 mM KH_2PO_4 and 8.5 mM Na_2HPO_4. Shadows were precipitated by centrifuging at 5500*g*, and supernatant liquor was decanted. The washing procedure was repeated four more times.[8] All operations and further storage of shadows were performed at 4 °C.

A film for taking the IR spectra of erythrocyte shadows was prepared in a cuvette with fluorite backing via slow evaporation of water under weak vacuum at a pressure of ca. 0.1 atm (ca. 0.5×10^4 Pa) and temperature 4 ± 1 °C.[12,13] Drying lasted 180 min. A suspension of erythrocyte shadows in a 0.001 M phosphate buffer with pH 7.35 and volume 60 mcl was introduced into a cuvette. This was supplemented with 30 mcl of the same buffer and 1.0 mcl of the hormone solution with concentration 10^{-6} M. Stirring and incubation lasted 10 min at 16−17 °C. The cuvette was placed horizontally on a special table of a vacuum unit.

When the film was prepared, the cuvette was transferred into an optical chamber and blown with dry air for 30 min, and then the scanning unit was switched on. IR spectra were taken on a Specord-M80 spectrometer (Germany, Leipzig), sequentially experiment and control against the fluorite backing or experiment and control to obtain a difference spectrum. Integration, determination of the spectrum band frequency, and mathematical processing were performed with special programs enclosed to the spectrometer.

3. Fluorescence Analysis of Erythrocyte Shadows. Fluorescence measurements were performed with a Shimadzu spectrofluorophotometer RF-5301(PC)SCE. An amount of 4 mL of hypotonic phosphate buffer containing 2.75 mM KH_2PO_4 and 8.5 mM Na_2HPO_4 (pH 7.35) and erythrocyte shadows were poured into a quartz cuvette of size $1 \times 1 \times 4$ cm^3. The concentration of shadow proteins was determined by the Warburg−Christian method from changes in the optical density of suspension.[14] On the average, it varied in the range of 0.100−0.250 mg/mL.

A cuvette with the shadow suspension was placed into a spectrofluorimeter thermostat for 1 h. Getting a stationary temperature regime in the cuvette was controlled by an electronic thermometer. In all the experiments, the temperature in the cuvette was 36 °C. After establishing a stationary temperature in the cuvette, the intensity of the intrinsic fluorescence of tryptophan residues in protein membranes was measured. The tryptophan emission spectrum was taken in the range of 300 nm $\leq \lambda \leq$ 400 nm at the excitation wavelength 281 nm, with the maximum of emission intensity observed at 332 nm. The average value of maximum emission intensity was obtained graphically after its continuous measuring for 4 min. The intensity of tryptophan fluorescence fluctuated within 1%. The possible reasons include variation of temperature in the cuvette with suspension, instrumental error in determination of fluorescence intensity, and photochemical reactions occurring in the system. The spectral width of the slits was 1.5/10. The tryptophan absorption spectrum was recorded in the range of 220 nm $\leq \lambda \leq$ 300 nm at the emission wavelength $\lambda = 332$ nm. Cortisol was dissolved in a mixture of dimethyl sulfoxide (DMS) and ethanol (1:1, V/V). Concentration of the hormone in the initial mother liquor was 10^{-3} M. If necessary, the solution was diluted with hypotonic phosphate buffer to obtain a desired concentration.

A solution of adrenaline or noradrenaline with the concentration 10^{-6} M was prepared in hypotonic phosphate buffer. The time of hormone incubation with shadows was one hour. Absorption and emission spectra were taken, and the average value of emission and absorption intensity was measured. For each hormone (cortisol, adrenaline, or noradrenaline), the

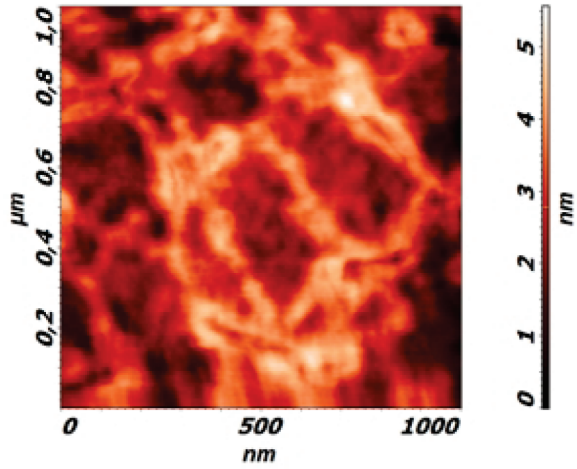


Figure 2. Control surface of rat erythrocyte. The erythrocyte suspension was supplemented with DMS and ethanol (0.25% of the mixture volume), and the surface nonuniformity increased, probably due to the denaturating effect of solvent on the surface structural proteins. Scan size: $1 \times 1\ \mu m^2$.

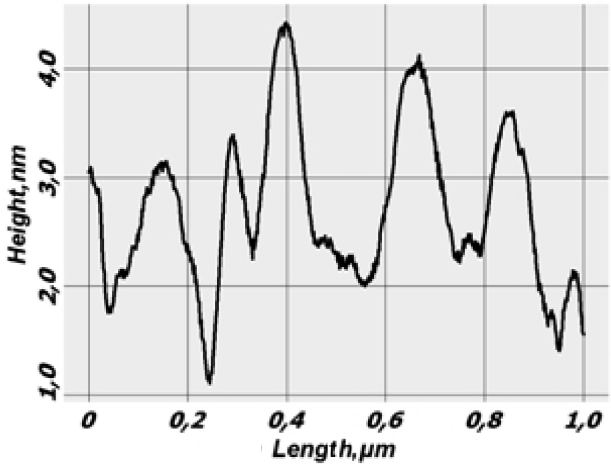


Figure 3. Center section of the control surface of rat erythrocyte. Section is made from left to right through the center of the scan in Figure 1. Domains with the length 200−250 nm and height 2 nm are seen.

binding constant K_b was calculated by the method[15] as well as the stoichiometric concentration of a bound hormone B_{max} and a change in free energy of the system ΔG. The interaction of cortisol and the erythrocyte membrane is described by the equation

$$B + nS = S_nB$$

where B is a membrane protein; S is the hormone; and n is the number of moles of hormone per mole of protein. The binding constant K_b was calculated by the formula

$$K_c = \frac{[S_nB]}{[S]\cdot[B]} \qquad (1)$$

where $[S_nB]$ is the concentration of bound protein; [B] is the concentration of free protein; and [S] is the concentration of free hormone. It is supposed that the hormone, upon binding to the protein, completely quenches its fluorescence. Thus, the fluorescence intensity F will be proportional to the concentration

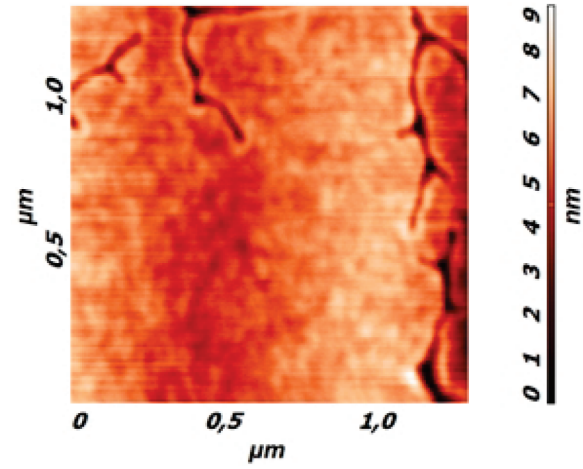


Figure 4. Surface of rat erythrocyte after adsorption of cortisol. Concentration of the hormone is 10^{-6} M. Scan size: $1.3 \times 1.3\ \mu m^2$. Deep meso-stripes with bifurcations are seen.

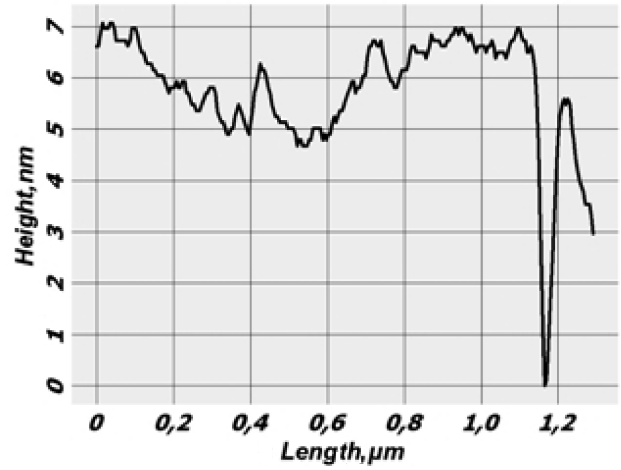


Figure 5. Center section of the surface of rat erythrocyte after adsorption of cortisol. Section is made from left to right through the center of the scan in Figure 3. The surface is flat, and there are hollows of 7 nm depth that divide the membrane into domains.

of the free protein. Let us write C for total concentration of protein in the cuvette and x for concentration of the bound protein. Then

$$\begin{aligned} F_{max} &= \beta C \\ F &= \beta(C - x) \end{aligned} \qquad (2)$$

where F is the intensity of tryptophan fluorescence at $\lambda = 332$ nm (the excitation wavelength $\lambda = 228$ nm); F_{max} is the intensity of tryptophan fluorescence in the absence of hormone (when the entire protein is free); β is the proportionality factor; and A_S is the stoichiometric concentration of the hormone. When the concentration of the hormone exceeds A_S, the fluorescence quenching does not increase. Dividing the first equation of set 2 by the second one gives

$$x = QC; \text{ where } Q = \frac{F_{max} - F}{F_{max}} \qquad (3)$$

$[S] = A - nx = A - nQC$, where A is the total concentration of hormone; $n = (A_S)/(C)$; $[B] = C - x = C(1 - Q)$. Substitution of 2 and 3 into the expression for binding constant

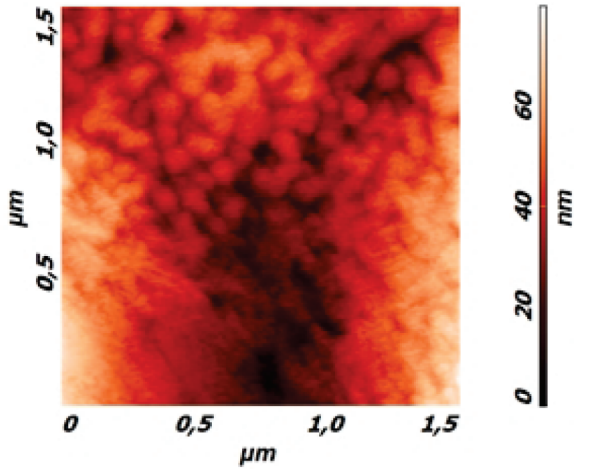


Figure 6. Surface of rat erythrocyte after adsorption of adrenaline. Concentration of the hormone is 10^{-6} M. Scan size: 1.5 × 1.5 μm^2.

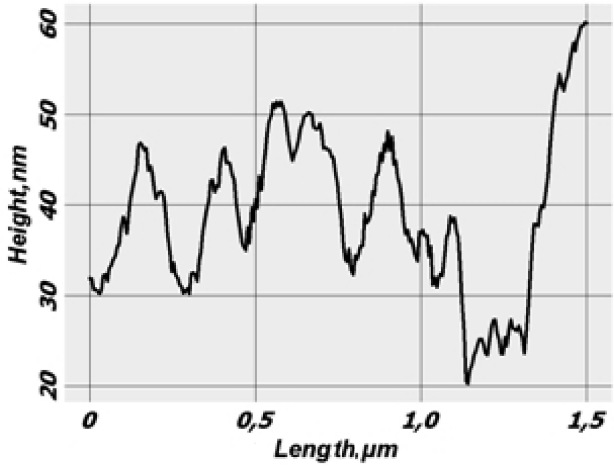


Figure 7. Center section of the surface of rat erythrocyte after adsorption of adrenaline. Section is made from left to right through the center of the scan in Figure 7. The surface is tuberous, and there are domains of size 250 × 250 nm, with smaller domains on the surface of large ones: size 50 × 50 μm^2 and height 5 nm.

1 gives

$$K_c = \frac{Q}{(1 - Q)(A - nQC)} \tag{4}$$

In our case, molar mass of membrane proteins is unknown, so the concentration of proteins in cuvette C is determined in milligrams/milliliter and the concentration of hormones A in moles/liter. The constant n is expressed in moles of molecules of hormone per milligram of protein (M/mg) and is a ratio of the maximum concentration of bound hormone to the concentration of membrane proteins. This can be written as

$$B_{max} = \frac{A_S}{C} \quad \text{[mol/mg of protein]} \tag{5}$$

Changes in Gibbs free energy ΔG of the system upon transition of the hormone from an aqueous medium to the erythrocyte membrane are calculated by the formula

$$\Delta G = -RT \ln(K_c) \quad \text{[J/mol]} \tag{6}$$

where $R = 8.314$ J/K mol and T is the absolute thermodynamic temperature.

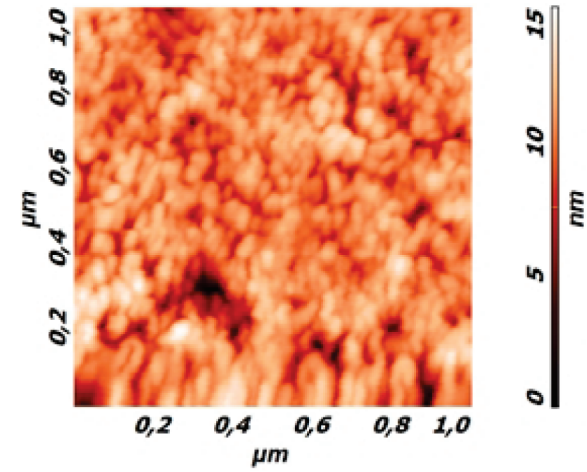


Figure 8. Surface of rat erythrocyte after adsorption of noradrenaline. Concentration of the hormone is 4 × 10^{-7} M. Scan size: 1 × 1 μm^2.

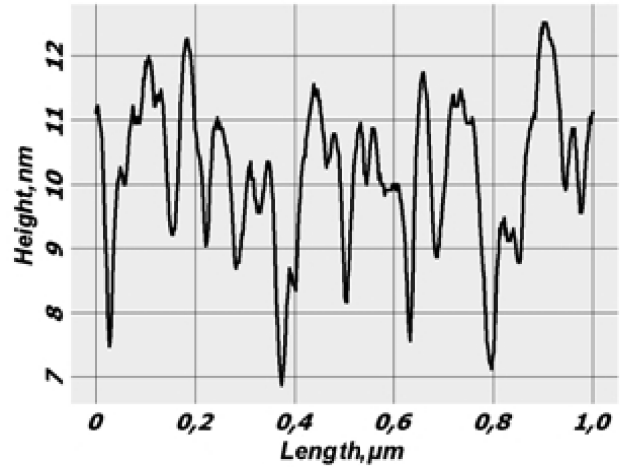


Figure 9. Center section of the surface of rat erythrocyte after adsorption of noradrenaline. Section is made from left to right through the center of the scan in Figure 5. The surface is flat, and there are domains of size 100 × 100 μm and height 2 nm.

For adrenaline and noradrenaline, in formulas 2 and 3 we use the dependence of $Q_1 = (D_{max} - D)/(D_{max})$ on concentration A of the hormone introduced into the cuvette. Here, D_{max} *and* D are the intensities of tryptophan absorption in shadows at $\lambda = 228$ nm, respectively, without addition of hormone and with hormone. The emission wavelength $\lambda = 332$ nm.

The measurement errors appeared due to inaccuracy in volumetric dosing of the shadow suspension specimens and their titration against hormones. Specimens were dosed using pipet dispensers DPAOF-1000 and DPAOF-50, their relative error at (20 ± 2) °C being 1% and 2%, respectively. An amount of 4 mL of the buffer was taken once with a DPAOF-1000 pipet, and suspension of erythrocytes and hormones was dosed twice using a DPAOF-50 pipet. The fluorescence intensity of erythrocyte shadows F is directly proportional to the concentrations of proteins C and hormones A in the specimen. Relative error E_F in measuring the F value can be estimated by the formula

$$E_F = \sqrt{(1\%)^2 + (2\%)^2 + (2\%)^2} = 3\%$$

Relative measurement errors for K_b and B_{max} can be obtained in the same way. They are equal to 10%.

TABLE 1: IR Spectroscopy: Frequency Parameters of Rat Erythrocyte Shadows after Their Interaction with Hormones [a]

compound	ν_{CO} (cm^{-1})	ν_{NH} stretch (cm^{-1})	$\nu_{C=O}$ (cm^{-1})	$\nu_{P=O}$ (cm^{-1})	ν_{P-O-C} (cm^{-1})	$NO_5C_4-C_5O_4$ (cm^{-1})	ν_{CH} stretch (cm^{-1})	A_{CO}
shadows (control)	1655.4 1686	3308	1748	1236	1080	1056	2948 2930 2848	*1.2150E+ 01*
shadows + cortisol ($A = 4.4 \times 10^{-8}$ M)	1655.2	3290.4 3308.0	1743	1236	1080	1051.2	2924.2 2848.9	*1.5169E+ 01*
shadows + cortisol ($A = 1.05 \times 10^{-7}$ M)	1656.0 1630	3280 3300	1740	1239	1083.7	–	2962 2925 2852	*1.5640E+ 01*
shadows + cortisol ($A = 2 \times 10^{-7}$ M)		3280 3296					2952.5 2931.4 2920.0 2853.8	
shadows + adrenaline ($A = 10^{-9}$ M)	1654	3276 3292 3316	1720 1734 1744	1220 1236 1244 1258	1080 1088 1094	1066	2958 2952 2928* 2852	
shadows + adrenaline ($A = 10^{-7}$ M)	1656 1646	3272 3288 3304 3320	1740	1220 1236 1248 1258	1080 1096	1070 1060 1044	2956 2924 2852	
shadows + noradrenaline ($A = 1.87 \times 10^{-7}$ M)	*1654*	*3271* *3300*	*1730*	*1246*	*1096*	*1072*	*2952* *2928* *2852*	

[a] Note. A_{CO} is the integral intensity of the ν_{CO} absorption band of the peptide bond on a semi-log scale.

In calculation, the values of fluorescence intensity F and absorption intensity D were corrected for dilution of suspension after the introduction of solution with hormone, for quenching of tryptophan emission by the solvent (a mixture of DMS and ethanol), for proper fluorescence of hormones, and for evaporation of water from the cuvette.

4. Measurement of Erythrocyte Membrane Microviscosity. Membrane microviscosity for translational diffusion of the pyrene probe was calculated as a ratio of fluorescence intensity of the pyrene dimer to fluorescence intensity of the pyrene monomer (Figure 1). Microviscosity of erythrocyte membranes was measured also on a Shimadzu RF-5301(PC)SCE spectrofluorimeter. The experimental specimen was prepared as follows: 4 mL of hypotonic phosphate buffer containing 2.75 mM KH_2PO_4 and 8.5 mM Na_2HPO_4 (pH 7.35), a fluorescent pyrene probe, erythrocyte shadows, and a specified amount of hormone were placed in a quartz cuvette of size $1 \times 1 \times 4$ cm^3. Before use, all the components were stored at 4 °C. The concentration of the shadow protein in the cuvette was 0.100−0.250 mg/mL; that of pyrene, 7.76×10^{-6} M. Pyrene was diluted in ethanol, its initial concentration being 1.5×10^{-3} M. The cuvette was placed into the spectrofluorimeter thermostat for 10 min, then the fluorescence measurements were performed at 36 °C. Before placing the specimen in the spectrofluorimeter thermostat, it was shaken vigorously for 1 min. For fluorescence measurements of shadows upon their loading with a different amount of hormones, each time a new specimen was prepared by the same procedure. Such a procedure is necessary because pyrene favors fast degradation of erythrocyte membranes.

To measure the microviscosity of a lipid bilayer near proteins (the region of protein−lipid interaction), we used the excitation wavelength $\lambda = 281$ nm and spectral slit width 1.5/5. Microviscosity of a lipid bilayer far from proteins (the region of lipid−lipid interaction) was measured with the excitation wavelength $\lambda = 337$ nm and spectral slit width 1.5/3. At that, the maxima of emission intensity were observed at $\lambda = 374$ nm and $\lambda = 393$ nm (the vibronic emission peaks of excited pyrene monomers) and $\lambda = 468$ nm (the emission maximum of excited pyrene dimer). Figure 1 shows the emission spectrum of pyrene in the shadows suspension.

The relative microviscosity of membranes was determined as a ratio $\eta(A)/\eta(0)$, where $\eta(A)$ and $\eta(0)$ are microviscosities of membranes, respectively, with and without hormone added to the shadow suspension. For the region of lipid−lipid interaction, relative microviscosity was calculated by the formula

$$\frac{\eta(A)}{\eta(0)} = \frac{F_{468}(0)}{F_{468}(A)}\frac{F_{393}(A)}{F_{393}(0)}$$

where $F_{468}(A)$ is the fluorescence intensity of pyrene at wavelength $\lambda = 468$ nm in a specimen at the hormone concentration A in suspension and $F_{468}(0)$ is the fluorescence intensity of pyrene at wavelength $\lambda = 468$ nm in a specimen with no hormone in suspension. $F_{393}(A)$ and $F_{393}(0)$ are the fluorescence intensities of pyrene at wavelength $\lambda = 393$ nm at the hormone concentration A in suspension and without hormone in suspension, respectively. The excitation wavelength is 337 nm.

For the region of protein−lipid interaction, relative microviscosity was calculated by the formula

$$\frac{\eta(A)}{\eta(0)} = \frac{F_{468}(0) - I_{468}}{F_{468}(A) - I_{468}}\frac{F_{393}(A) - I_{393}}{F_{393}(0) - I_{393}}$$

where I_{393} and I_{468} are the fluorescence intensities of tryptophan at wavelength $\lambda = 393$ nm and $\lambda = 468$ nm, respectively. The excitation wavelength is $\lambda = 281$ nm. A relative measurement error for relative microviscosity was equal to 6%.

Results

Atomic Force Microscopy. Under the atomic force microscope, erythrocytes of healthy animals looked like large

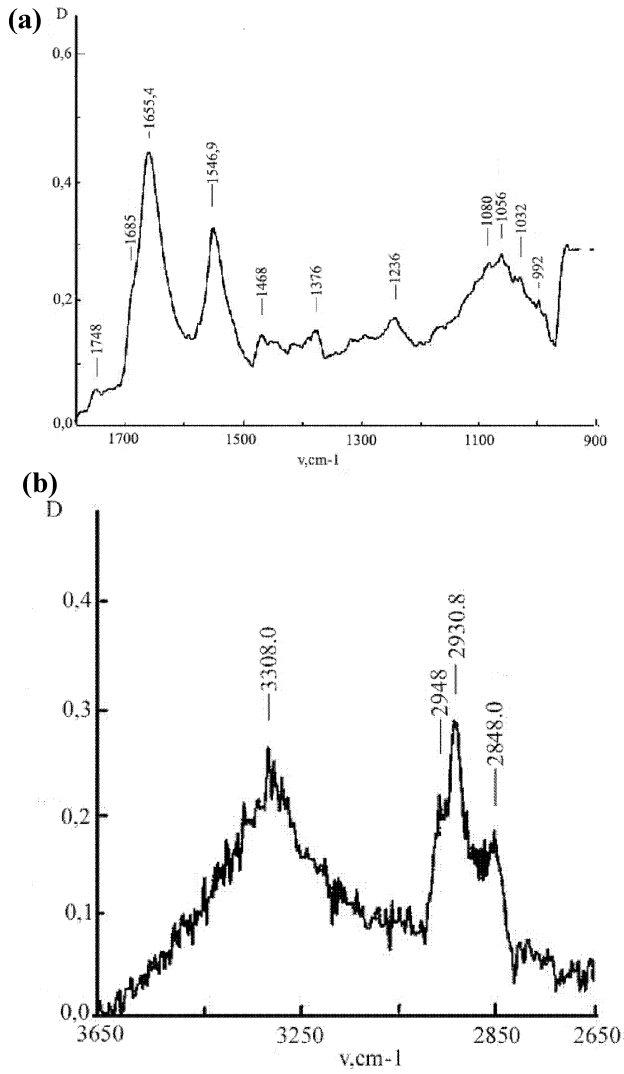


Figure 10. IR spectra of rat erythrocyte membranes (control) ($C_{\text{phosph. buff.}}$ = 0.01 M, pH 7.35, relative humidity 0%): (a) ν = 900−1800 cm^{-1}, (b) ν = 2600−3700 cm^{-1}.

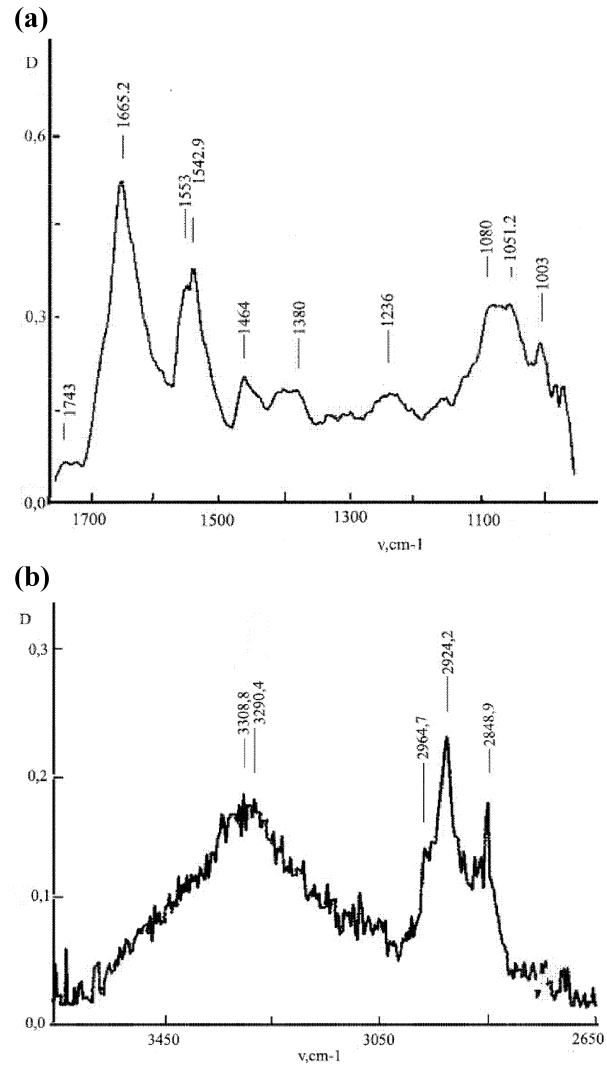


Figure 11. IR spectra of rat erythrocyte membranes incubated with cortisol ($C_C = 4.44 \times 10^{-8}$ M, $C_{\text{phosph. buff.}} = 0.001$ M, pH 7.35, relative humidity 0%): (a) ν = 900−1800 cm^{-1}, (b) ν = 2600−3700 cm^{-1}.

biconcave discs ca. 6 μm in diameter, which agrees with the results obtained by other authors.[11] At a higher magnification, their surface showed a slight nonuniformity caused most likely by the presence of membrane proteins. When the erythrocyte suspension was supplemented with DMS and ethanol (0.25% of the mixture volume), the surface nonuniformity increased, probably due to the denaturating effect of solvent on the surface structural proteins (Figures 2 and 3). The pattern changed drastically upon addition of cortisol to erythrocyte suspension with the final concentration of 10^{-6} M. On a smooth surface, there appeared numerous meso-stripes loosening the cell membrane structure (Figures 4 and 5). These meso-stripes are ca. 7 nm hollows in the erythrocyte membrane. They divide the erythrocyte membrane into flat domains.

Adrenaline produced more abrupt changes in the structure of erythrocyte membranes (Figures 6 and 7). The membrane surface lost its flatness, and there appeared convex domains with quasi-staggered arrangement alternating with considerable hollows on the surface. The addition of adrenaline led to domains of size 250 × 250 nm, with smaller domains on the surface of large ones: size 50 × 50 μm^2 and height 5 nm. Noradrenaline caused the formation of domains with the size 100 × 100 μm and height 2 nm (Figures 8 and 9).

IR spectroscopy allowed us to reveal the nature of these structural transformations.

IR Spectroscopy. The analysis of IR spectra of rat erythrocyte shadows upon cortisol addition showed an ca. 20% increase in the intensity of absorption bands of CO (1655.2 cm^{-1}) and NH bonds (1548 and 3290 cm^{-1}), the effect becoming more pronounced with increasing concentration of the hormone (Table 1, Figures 10 and 11). The increased intensity of the band at 1655.2 cm^{-1} points to a growing orderliness in membrane proteins caused by the structural transition tangle → α-helix. The appearance of the absorption band at 1630 cm^{-1} corresponds to β-structure (tangle → β-structure transition) at the cortisol concentration 10^{-7} M (Figure 12). The interaction of hormone ligand with protein enhances intermolecular interaction between membrane proteins and phospholipids and facilitates the formation of lipid−protein clusters.

A shift of stretching vibrations of the peptide bond (NH bond), 3308 → 3280 cm^{-1} ($\Delta\nu$ = 28 cm^{-1}), and an increase in its intensity were found, which are caused by the formation of a hydrogen bond between cortisol and the NH bond of proteins. It forms most likely between the keto group in the hormone A-ring (C_3 = 0) and the NH bond of the membrane protein, although both the keto group at C_{20} in the D-ring and the OH group at C_{11} in the C-ring may also participate in the formation

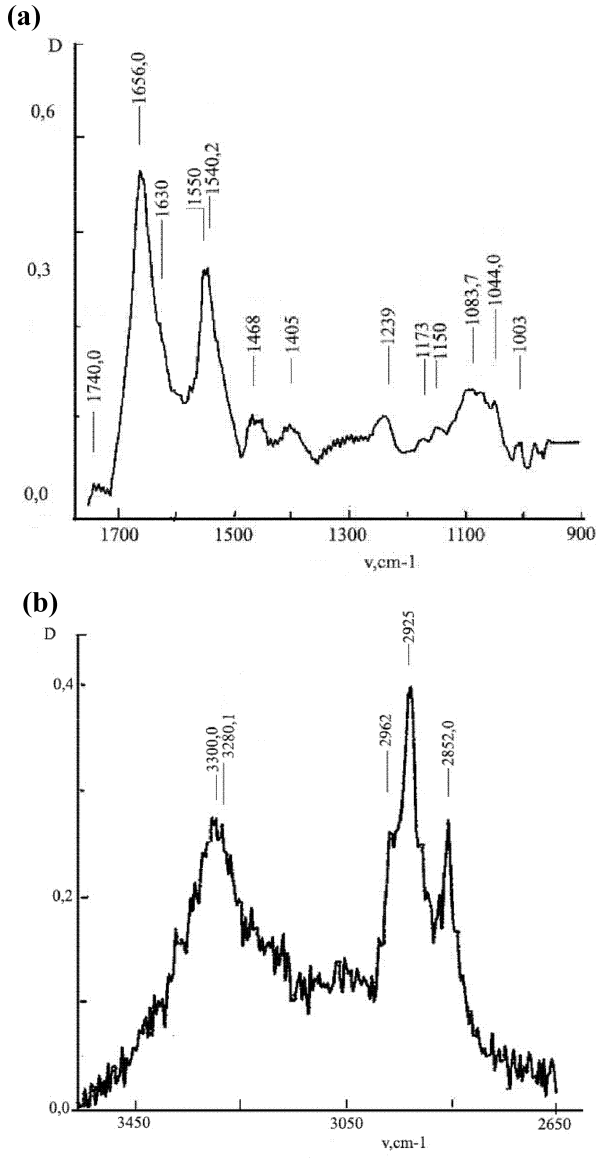


Figure 12. IR spectra of rat erythrocyte membranes incubated with cortisol ($C_C = 1.05 \times 10^{-7}$ M, $C_{phosph.\ buff.} = 0.001$ M, pH 7.35, relative humidity 0%): (a) $\nu = 900-1800$ cm^{-1}, (b) $\nu = 2600-3700$ cm^{-1}.

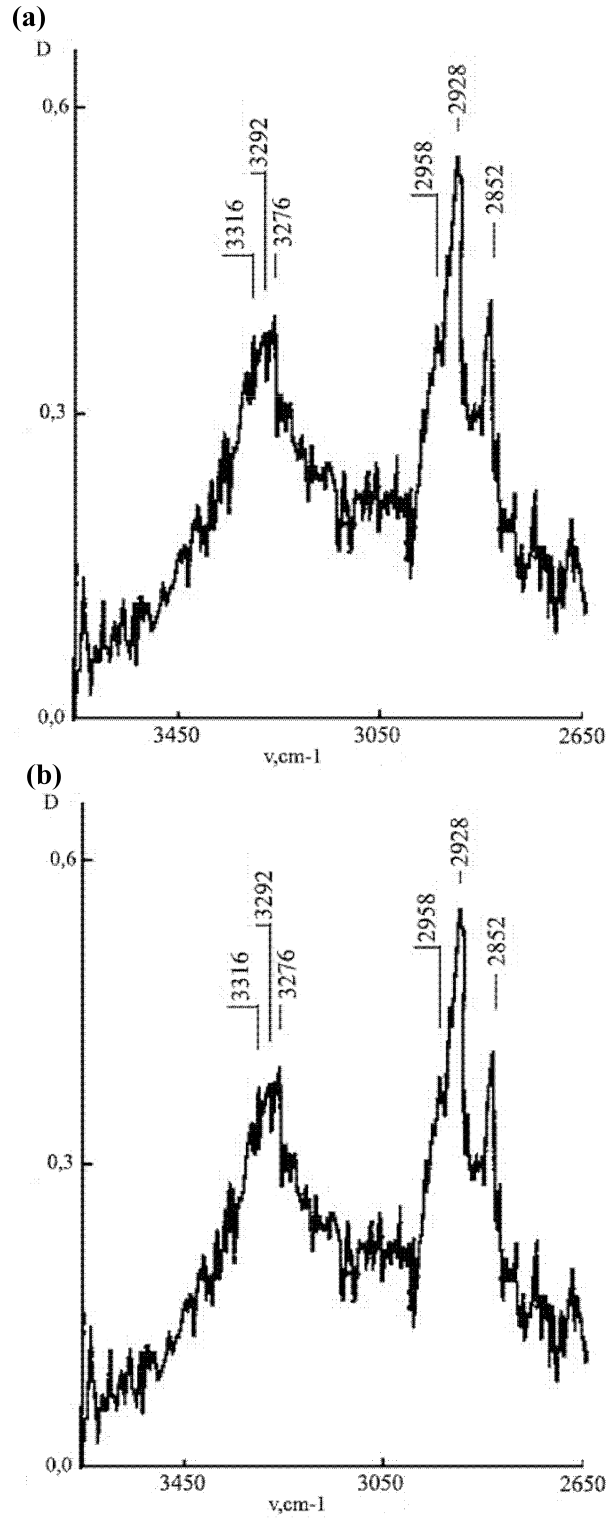


Figure 13. IR spectra of rat erythrocyte membranes incubated with adrenaline ($C_A = 10^{-9}$ M, $C_{phosph.\ buff.} = 0.001$ M, pH 7.35, relative humidity 0%): (a) $\nu = 900-1800$ cm^{-1}, (b) $\nu = 2600-3700$ cm^{-1}.

of hydrogen bonds. Cortisol is a cholesterol derivative; however, the presence of two keto and three hydroxy groups considerably changes its interaction with the surface of the erythrocyte membrane as compared to cholesterol.[16] The latter penetrates deep into the membrane and enhances the hydrophobic interactions between chains of fatty acids in phospholipids. Cortisol acts on the surface. Of interest are the shifts of CH bond stretching vibrations: 2848 → 2852 cm^{-1} ($\nu = 4$ cm^{-1}) and 2930 → 2925 cm^{-1} ($\nu = 5$ cm^{-1}). The latter increased also its intensity under the action of hormone. A change in the intensity of this bond confirms the occurrence of structural transition order → order under the action of cortisol but does not reveal where it takes place: in membrane proteins or in phospholipids, since the CH bond resides in both proteins and phospholipids. An increase in the intensity of the absorption band of the C═O bond in phospholipids and its shifting from 1748 to 1740 cm^{-1} were observed. This increase in the band intensity points to an enhanced orderliness of higher carboxylic acids and a decreased entropy in phospholipids. The band shift is caused by the formation of a hydrogen bond between the hormone, probably the OH group at C_{21}, and the CO bond in phospholipids. Such a concurrent interaction of the hormone with protein and phospholipids takes place at the interface of proteins and phospholipids, which enhances the lipid−protein interactions and leads to the formation of complex domains revealed by atomic force microscopy (Figures 2−9). A 3 cm^{-1} shift in the

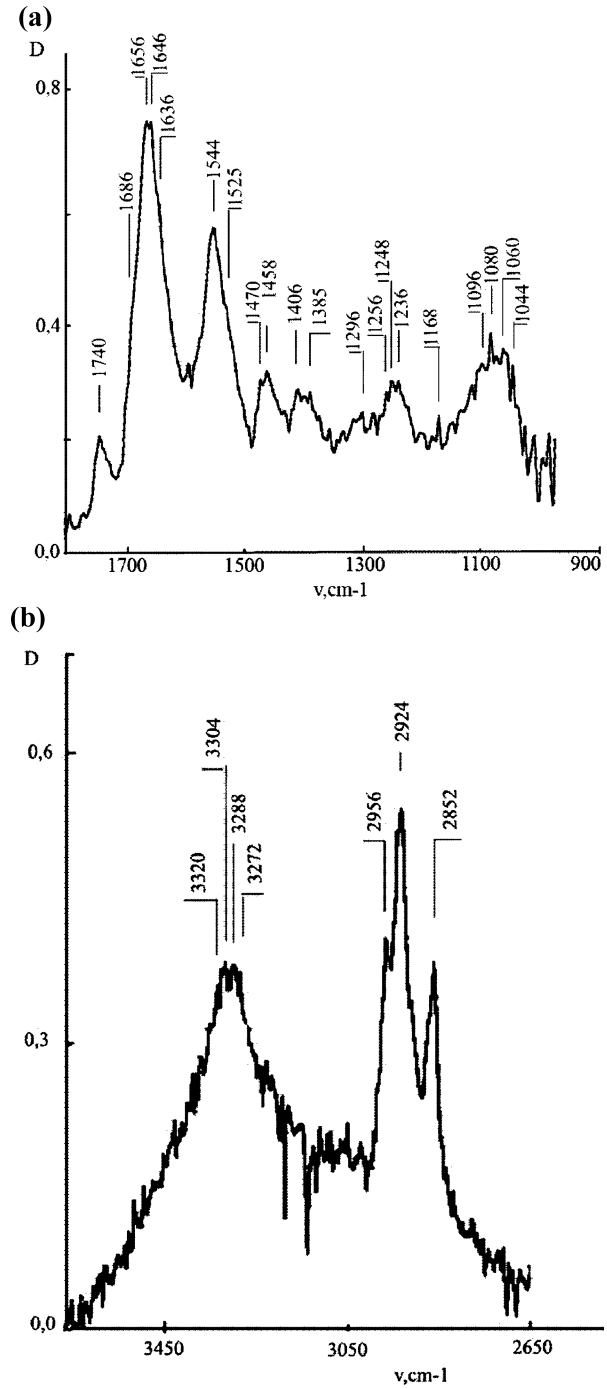


Figure 14. IR spectra of rat erythrocyte membranes incubated with adrenaline ($C_A = 10^{-7}$ M, $C_{phosph.\ buff.} = 0.001$ M, pH 7.35, relative humidity 0%): (a) $\nu = 900-1800$ cm^{-1}, (b) $\nu = 2600-3700$ cm^{-1}.

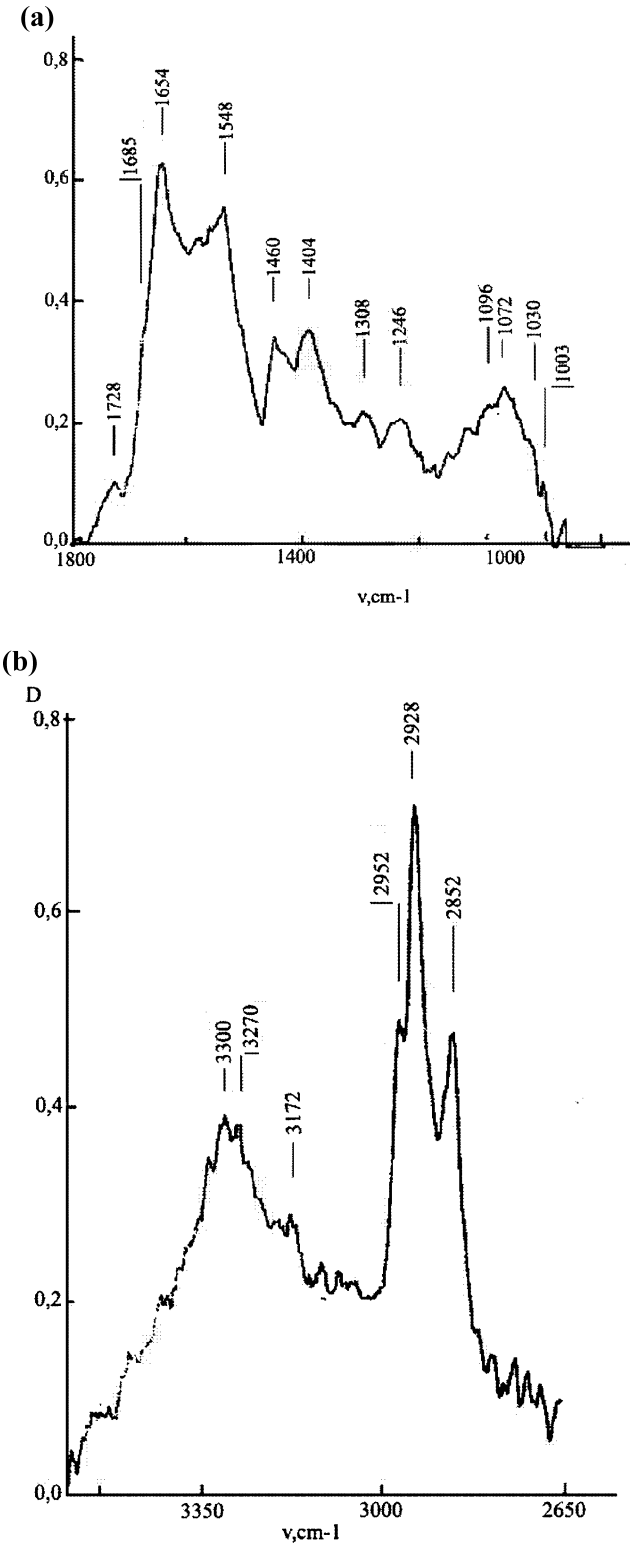


Figure 15. IR spectra of rat erythrocyte membranes incubated with noradrenaline ($C_{NA} = 1.87 \times 10^{-7}$ M, $C_{phosph.\ buff.} = 0.001$ M, pH 7.35, relative humidity 0%): (a) $\nu = 900-1800$ cm^{-1}, (b) $\nu = 2600-3700$ cm^{-1}.

frequency of the P═O bond to the short-wave region and a minor increase in its intensity were observed. This is attributed to dehydratation of membranes upon their deformation (compression) under the action of hormone. This is exactly the loss of bound water that increases the frequency of the P═O bond. Displacement of water dipoles from lipid−protein domains to adjacent regions results in the appearance of meso-stripes with tensile hydrodynamic stress (Figure 4).

It is known that spectrin accounts for 30% of membrane proteins.[17,18] It can be expected that additional deformation of membranes would occur due to the spectrin−actin−ankyrin meshwork capable of contraction, this meshwork residing on both the internal and external surfaces of the membrane.[18] This mechanism of erythrocyte membrane deformation plays a more essential role upon interaction with adrenaline and noradrenaline. Thus, adrenoreceptor and cholinoreceptor were found earlier on the surface of erythrocyte membranes.[3] The EPR spectroscopy study revealed that deformation (contraction) of erythrocyte membranes occurs in erythrocyte membranes (shadows) under the action of adrenaline or carbachol (the analogue of choline).

TABLE 2: Parameters of Cortisol Binding to the Erythrocyte Membrane Based on Tryptophan Fluorescence Quenching of Membrane Proteins and Parameters of Adrenaline and Noradrenaline Binding to Erythrocyte Membrane Based on the Hypochromic Effect of Tryptophan in Membrane Proteins

hormone	binding constant K_b (M^{-1})	amount of bound hormone B_{max} (mol/mg of protein)	changes in Gibbs free energy ΔG (kJ/mol)
cortisol	$(1.23 \pm 0.12) \times 10^6$	$(4.69 \pm 0.47) \times 10^{-10}$	−36.0
adrenaline	$(6.3 \pm 0.63) \times 10^6$	$(1.60 \pm 0.16) \times 10^{-11}$	−40.2
noradrenaline	$(1.70 \pm 0.17) \times 10^6$	$(8.10 \pm 0.81) \times 10^{-10}$	−36.9

The contraction effect was eliminated by cytochalasin, which can stabilize spectrin. Later, the IR spectroscopy study showed that carbachol and adrenaline produce changes in the secondary structure of these proteins. In the present study, we also observed a similar effect on erythrocyte membranes caused by the action of adrenaline and noradrenaline.

Upon incubation of adrenaline with concentration 10^{-9} M and shadows, we observed shifting of absorption bands of CO and NH groups of the peptide bonds. The CO band shifted from 1655.4 to 1654 cm^{-1}, whereas at $C_{adrenaline} = 10^{-7}$ M the shift was 1655.4 → 1656 cm^{-1}, accompanied by appearance of the band at 1646 cm^{-1}. In the first case, the absorption band of the NH bond shifted by 32 cm^{-1}, and in the second case, by 36 cm^{-1}. Splitting of the band of NH bonds was also recorded (Table 1, Figures 13 and 14).

Interestingly, the low concentration of adrenaline gave a 28 cm^{-1} shift of the band of phospholipid CO bonds, whereas with noradrenaline this value was only 18 cm^{-1}. Splitting of the band was also observed. At the same time, the absorption band of the PO bond showed strong splitting (Table 1, Figure 13). Its shift to the short-wave region was 22 cm^{-1} and to the long-wave region was 16 cm^{-1}. Upon splitting, the band shift between extreme bands attained 38 cm^{-1}.

The absorption band in the region of 2928 cm^{-1} (CH stretching vibrations) showed an additional splitting. Bands shifting was also recorded in this part of the spectrum (Table 2).

Incubation of noradrenaline with erythrocyte shadows was accompanied by shifting of absorption bands assigned to the peptide bond of membrane proteins, in particular, the 1655.4 → 1654 cm^{-1} shift of the CO bond ($\nu = 1.4$ cm^{-1}) and the 3308 → 3270 cm^{-1} shift of the NH bond (stretching vibrations) ($\nu = 38$ cm^{-1}) (Table 1, Figure 15). Shifting of the indicated absorption bands points to conformational transitions in membrane proteins: tangle → α-helix and tangle → β-structure. The bands at 1654 and 1685 cm^{-1} increased in intensity as well as the band of the NH bond (amide II) at 1548 cm^{-1}. In addition, splitting in the region of the NH bond (stretching vibrations) was observed. This splitting indicates the mutual interaction of intramolecular domains comprising α-helices or β-structures, which results from their ordering. However, the splitting may be related also with intermolecular interaction of proteins. Deformation or contraction of membrane under the action of noradrenaline takes place here, similar to the case of adrenaline,[3] thus leading to pronounced changes in the structure of membrane proteins. These changes may be caused by interaction of several functional groups of noradrenaline with the receptor, in particular, the amino group and OH groups. Hydrophobic interaction of the aromatic ring of noradrenaline with the hydrophobic region of its receptor is also possible.

The 1748 → 1730 cm^{-1} band shift of the phospholipid C═O bond was recorded as well as the 1236 → 1246 cm^{-1} shift of the phospholipid P═O bond. The band of the P−O−C bond also shifts by 16 cm^{-1} to the short-wave region of the spectrum, so strong shifting of these bands results from the increased ordering of phospholipids. However, this is caused also by the enhanced interaction with membrane proteins, in particular, integral proteins, protein of stripe 3, and finally with retraction proteins. Note that hydrogen bonds are formed with any of these proteins. Probably the receptor for adrenaline may have not only the protein but also the protein−lipid nature. If this is the case, OH groups of noradrenaline will interact with C═O or P═O bonds.

Shifting of the absorption band of $O_4C_4C_5O_5$ in monosaccharides was recorded (1056 → 1072 cm^{-1}, $\nu = 16$ cm^{-1}). This shift indicates the formation of hydrogen bonds of noradrenaline with the functional groups of monosaccharides as well as the ordering of monosaccharides relative to each other accompanied by changes in their conformation, such as boat → chair. The chair conformation is energetically more advantageous.

Thus, the comparison of adrenaline and noradrenaline effect shows that action of the first hormone on phospholipids is more pronounced as compared to the second hormone. This is related with the presence of the methyl radical at nitrogen. Due to higher electronegativity of the nitrogen atom, charge of the methyl group increases, thus enhancing the effect of adrenaline on the membrane. This gives ground to suggest that the amino group and the methylamino group interact with phospholipids by CO and PO bonds.

The total effect of all the hormones is presented in Table 1.

Fluorescence analysis allowed us to observe the contribution of proteins to initiation of structural transitions in erythrocyte membranes under the action of hormones. For this purpose, we used both the effect of light absorption and the tryptophan fluorescence quenching when determining the protein secondary structure.

Fluorescence Analysis. As was noted above, cortisol cannot penetrate deep into the phospholipid bilayer and interacts with proteins on the surface of the cell membrane. This leads to pronounced transformation of their secondary structure and is accompanied by a decrease in the absorption maximum at 228 nm. Addition of hormone to the incubation medium with the concentration of 1.82×10^{-7} M decreased the absorption intensity by 30 au, which made up 4.6% as compared to control (the hypochromic effect). The hypochromic effect is related with transition of the protein molecule from static tangle to α-helix,[19] i.e., with increased orderliness of its structure.

The absorption spectrum of macromolecules, which have an ordered structure in the excited state, reflects the interaction between excited monomeric units and strongly depends on their spatial arrangement in the molecule.

The structural transitions in proteins at cortisol interaction with erythrocyte membranes are evidenced also by the curves of tryptophan fluorescence quenching. A maximum of fluorescence decrease was observed at the hormone concentration in the incubation medium equal to 11.6×10^{-8} M and the protein content of 0.256 mg/mL, and ΔF was 34 au, which makes up 11% with respect to control (Figure 16).

The data on tryptophan fluorescence quenching were used to calculate the constants of hormone binding (K_b), the amount

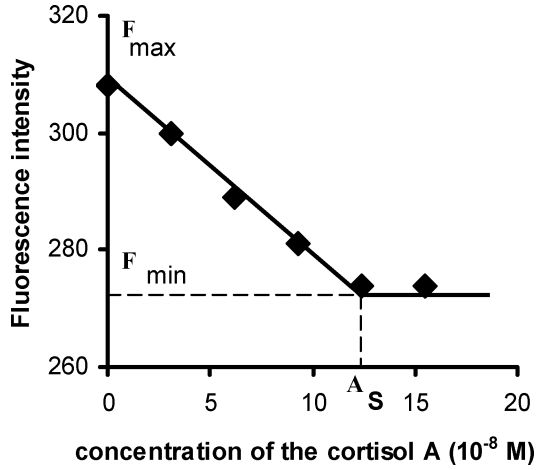


Figure 16. Dependence of fluorescence intensity of shadows on the concentration A of hormone cortisol introduced into the cuvette. A_S is the stoichiometric concentration of the hormone. Concentration of membrane protein: $C = 0.256$ mg/mL. The excitation wavelength $\lambda = 281$ nm, and the emission wavelength $\lambda = 332$ nm.

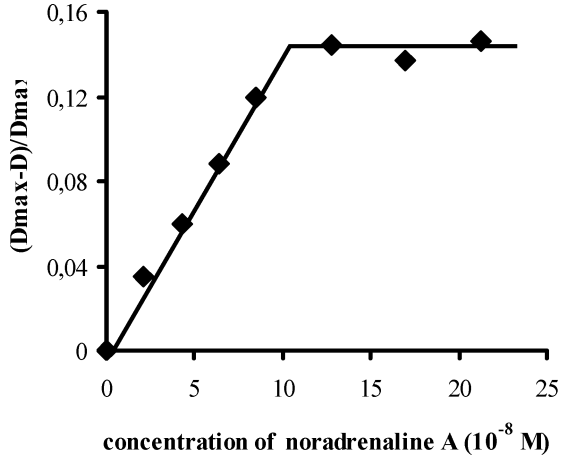


Figure 17. Dependence of $(D_{max} - D)/(D_{max})$ on the concentration A of hormone noradrenaline introduced into the cuvette. Here, D_{max} *and* D are the intensities of tryptophan absorption in shadows at $\lambda = 228$ nm, respectively, without addition of hormone and with hormone. Concentration of membrane protein $C = 0.124$ mg/mL. The emission wavelength $\lambda = 332$ nm.

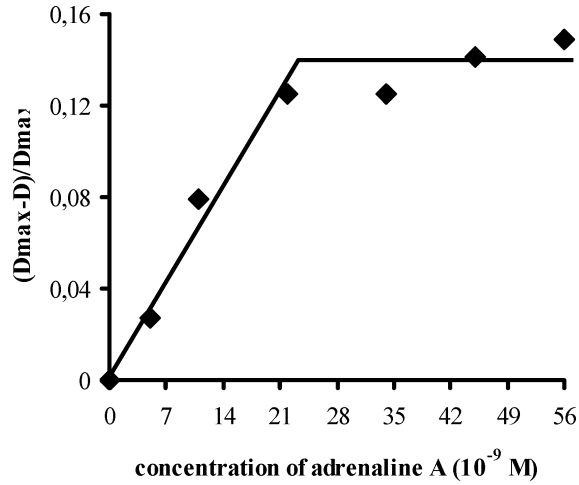


Figure 18. Dependence of $(D_{max} - D)/(D_{max})$ on the concentration A of hormone adrenaline introduced into the cuvette. Here, D_{max} *and* D are the intensities of tryptophan absorption in shadows at $\lambda = 228$ nm, respectively, without addition of hormone and with hormone. Concentration of membrane protein $C = 0.124$ mg/mL. The emission wavelength $\lambda = 332$ nm.

of bound hormone (B_{max}, mol/mg of protein), and a change in free energy (ΔG, kJ/mol) upon interaction of cortisol with the erythrocyte membrane (Table 2). It was found that K_b for cortisol is $(1.23 \pm 0.12) \cdot 10^6$ M^{-1}. A similar value for transcortin (a specific receptor) is 3×10^7 M^{-1},[20] which is an order of magnitude higher. However, even in the norm, 6−8% of the hormone is bound to blood cells. These are mainly erythrocytes. Under stress, this value may rise considerably. Our calculations showed that the maximal saturation of erythrocyte membranes with hormone (B_{max}) in vitro is $(4.7 \pm 0.47) \times 10^{-10}$ mol/mg of protein, which is quite a low value (Table 2). When cortisol interacts with erythrocyte membranes, the free energy of the entire system (ΔG) decreases by 30.0 kJ/mol. This indicates that under the action of hormone the orderliness of structural components in the erythrocyte membranes increases, whereas their entropy decreases.

Similar results were obtained in our study of two other stress hormones, adrenaline and noradrenaline. With both hormones, the hypochromic effect is even more pronounced than with cortisol (Table 2, Figures 17 and 18). This suggests an increased orderliness of the membrane proteins structure due to transition tangle → β-structure → α-helix. A decrease in absorption may be related with changes in the direction of dipole moments of quantum transitions of monomeric protein residues accompanying their transition into another conformation.

Due to the high intrinsic fluorescence of the hormones, their effect on tryptophan fluorescence quenching cannot be revealed. Nevertheless, the absorption measurements allowed us to calculate K_b, B_{max}, and ΔG for these hormones. Overall, they were of the same order of magnitude as those for cortisol. Adrenaline showed a higher value of K_b, a lower amount of bound hormone (B_{max}), and more pronounced changes of ΔG (−40.2 kJ/mol). The latter fact testifies that adrenaline increases the orderliness of structural components in erythrocyte membranes to a greater extent than other hormones. It provided also a stronger deformation of erythrocyte membranes, which agrees with the data of atomic force microscopy (Figures 8 and 9).

It was vital to relate structural transitions in erythrocyte membranes under the action of stress hormones with changes in their elasticity and viscosity.

Changes in Microviscosity of Erythrocyte Membranes under the Action of Cortisol and Catecholamine. The addition of cortisol, adrenaline, or noradrenaline to erythrocyte shadows increased microviscosity of erythrocyte membranes (Figures 19, 20, and 21). The effect was more pronounced in the presence of adrenaline (an increase by 40%) and less pronounced in the presence of noradrenaline (by 24%) and cortisol (by 25%). Adrenaline was able to increase the membrane microviscosity at a much lower concentration of hormone in the incubation medium. With adrenaline, a plateau was reached at the hormone concentration of 1.7×10^{-9} M and with noradrenaline and cortisol at 7×10^{-8} M. For all the hormones, in the region of lipid−protein interactions, microviscosity increased at lower concentrations and was more pronounced than in the region of lipid−lipid interactions (Figure 19−21). An increase in microviscosity of erythrocyte membranes correlated with a decrease of tryptophan adsorption in membrane proteins (Figure 16−18).

Presumably, structural transitions in erythrocyte membranes under the action of stress hormones are initiated to a greater extent in proteins and to a lesser extent in lipids.

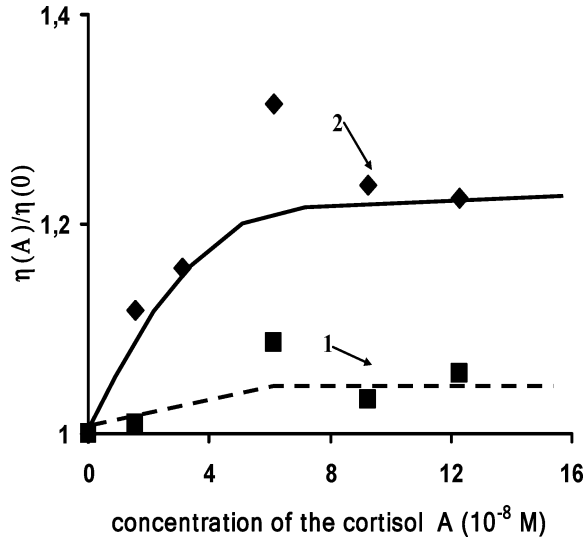


Figure 19. Changes in the relative microviscosity of membranes $\eta(A)/\eta(0)$ of erythrocyte shadows at the concentration A of hormone cortisol, where $\eta(A)$ and $\eta(0)$ are microviscosities of the membranes, respectively, with cortisol added to the shadow suspension and without hormone. Concentration of shadows $C = 0.128$ mg of protein/mL. Line 1: changes of relative microviscosity in the region of lipid−lipid interaction. Line 2: changes of relative microviscosity in the region of protein−lipid interaction. Concentration of pyrene in the suspension is 7.7×10^{-6} M; temperature of the specimens is 309.1 ± 0.1 K (36 °C); pH of the suspension is 7.35. The measured value of $\eta(A)/\eta(0)$ exhibits an error of 6%.

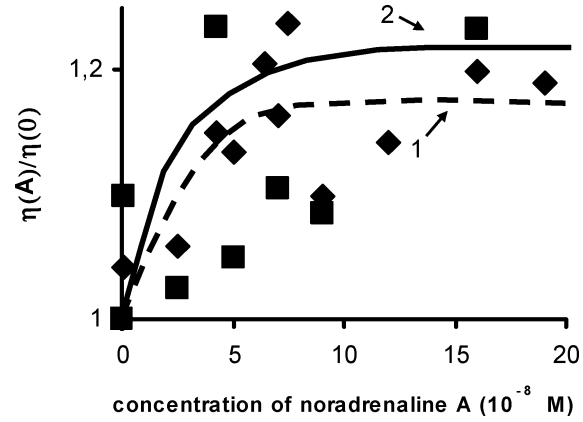


Figure 20. Changes in the relative microviscosity of membranes $\eta(A)/\eta(0)$ of erythrocyte shadows at the concentration A of hormone noradrenaline, where $\eta(A)$ and $\eta(0)$ are microviscosities of the membranes, respectively, with noradrenaline added to the shadow suspension and without hormone. Concentration of shadows $C = 0.128$ mg of protein/mL. Line 1: changes of relative microviscosity in the region of lipid−lipid interaction. Line 2: changes of relative microviscosity in the region of protein−lipid interaction. Concentration of pyrene in the suspension is 7.7×10^{-6} M; temperature of the specimens is 309.1 ± 0.1 K (36 °C); pH of the suspension is 7.35. The measured value of $\eta(A)/\eta(0)$ exhibits an error of 6%.

Discussion

It is known that the stress hormones (cortisol, adrenaline, noradrenaline) are involved in enhancement of energy exchange in the organism. This is their main role in implementation of various adaptation mechanisms under subextreme and extreme conditions of life.[21] This function of hormones is well understood now,[22] whereas numerous side effects of such hormones are still unclear. Their catabolic action in the organism is discussed quite thoroughly in the literature,[23,24] but nonspecific mechanisms of action remain obscure. As shown in this work, they include

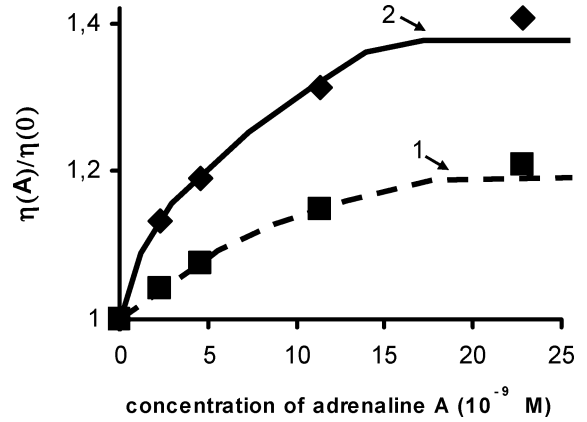


Figure 21. Changes in the relative microviscosity of membranes $\eta(A)/\eta(0)$ of erythrocyte shadows at the concentration A of hormone adrenaline, where $\eta(A)$ and $\eta(0)$ are microviscosities of the membranes, respectively, with adrenaline added to the shadow suspension and without hormone. Concentration of shadows $C = 0.128$ mg of protein/mL. Line 1: changes of relative microviscosity in the region of lipid−lipid interaction. Line 2: changes of relative microviscosity in the region of protein−lipid interaction. Concentration of pyrene in the suspension is 7.7×10^{-6} M; temperature of the specimens is 309.1 ± 0.1 K (36 °C); pH of the suspension is 7.35. The measured value of $\eta(A)/\eta(0)$ exhibits an error of 6%.

the effect of peripheral blood cells, primarily erythrocytes, on the structure of cytoplasmatic membranes. This problem becomes crucial in the case of chronic stress. In humans, of special importance is prolonged psychoemotional stress accompanied by developing anxiety, when the blood content of adaptive hormones rises several fold. Under such conditions, the extent of their interaction with erythrocyte membranes strongly increases and can lead to deep structural changes.

Nowadays, atomic force microscopy (AFM) is widely used for analysis of structural changes in erythrocyte membranes. AFM makes it possible to study the interaction of glycocalix with the cytoskeleton,[25] to estimate elastic properties of membranes[26] and interaction with biologically active compounds.[27] We employed this method to analyze the interaction of erythrocyte membranes with the stress hormones (cortisol, adrenaline, noradrenaline). The interaction was shown to result in the formation of domain structures under the action of all three hormones. The action of cortisol produced large domains and penetrating meso-stripes with bifurcations loosening the membrane (Figures 4 and 5). The action of adrenaline gave a similar pattern, but domains were larger and often aggregated into separate islets (Figures 6 and 7). Noradrenaline led to the formation of smaller domains with numerous penetrating pores between them. Further loosening of membranes occurred via these pores (Figures 7 and 8). We examined the mechanism of domain formation by means of IR spectroscopy and fluorescence analysis.

The IR spectroscopy study showed that hydrogen bonds and hydrophobic and electrostatic interactions play an essential role in this mechanism. Cortisol is known to be a hydrophobic compound, as it is poorly soluble in water. However, it comprises three OH groups (in positions 11, 17, and 19) and two keto groups (in positions 3 and 18). These are the groups that take part in the formation of hydrogen bonds with CO and NH groups of membrane proteins and phospholipids. As a result, protein−lipid domains form on the surface of erythrocyte membranes with participation of cortisol. The appearance of additional forces strengthens the "compressive" stresses in domains. Water dipoles are displaced to adjacent regions of the

membrane. This is facilitated by enhancement of hydrophobic interactions due to two additional CH_3 groups (in positions 10 and 11) and hydrophobic rings of the hormone. An excess content of water in adjacent regions gives rise to "tensile" stresses via the action of hydrostatic forces. Meso-stripes loosening the membrane are formed on the erythrocyte surface (Figure 4). However, overall the membrane stiffness rises, especially in the region of lipid−protein interactions. The membrane orderliness increases, while free energy of the system (ΔG) decreases (Table 2).

An increase in protein hydrophoby reinforces intermolecular interaction with hydrophobic regions of higher carboxylic acids of phospholipids, which is most pronounced in the annular layer. Due to enhanced intermolecular lipid−protein orderliness of the membranes and due to an rease in their microviscosity, phase separation is not observed; instead, there occur structural orientation transitions with the formation of large domains. Earlier we found similar anomalous changes of viscosity and specific conductivity in blood plasma lipoproteins.[13] The calculated enthalpy of the structural transition turned out to be quite low. Besides, we revealed changes of the secondary structure (transitions tangle → α-helix, tangle → β-structure) to occur in high density lipoproteins under the action of cortisol and observed splitting of some bands in phospholipids. These transitions are close to those we observed in erythrocyte membranes.

How is the action of the second stress hormone, adrenaline, related with this mechanism? Our studies revealed a higher value of its K_b, which means that adrenaline has an even greater affinity for erythrocyte membranes. However, the overall mechanism of structural transitions in membranes under the action of this hormone is virtually the same as with cortisol. This is confirmed by the IR spectroscopy data (Table 1). The molecule comprises three OH groups able to take part in the formation of hydrogen bonds, an NH group having a positive charge, and an adjacent CH_3 group enhancing the effect of this positive charge; thus, the same forces are acting here. However, they are supplemented with the action of spectrin−actin−ankyrin meshwork, which can be enhanced upon interaction of adrenaline with its membrane receptor. The atomic force microscopy of erythrocytes in the presence of adrenaline shows the formation of large domains rising above the erythrocyte surface, with the regions of very loose substance (hollows) between them. These are likely the regions with excess content of molecular water. The stretching tangential stresses are acting here. They seem to provide the "extrusion" of some domains, which are sometimes replaced with the through holes. A similar pattern was obtained with noradrenaline.

Thus, it can be supposed that cortisol and catecholamines have a unidirectional effect on erythrocyte membranes. Besides, there is also the additive effect. Such erythrocytes cannot provide full-scale functioning. Moreover, in the capillary network they are able to form numerous small clots, which may lead to hypoxia and even infarction. There are many cases of sudden death that occur in young sportsmen during competitions.[28,29] Coronary arteries of sportsmen are free of plaques, nevertheless, acute coronary deficiency develops somehow. Stress may be a possible reason. Under stress, the blood content of cortisol and especially adrenaline rises considerably. We believe that this mechanism may underlie the appearance of cardiac syndrome X often leading to cardiovascular catastrophes (infarctions and strokes).

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JP911917A