

Mechanism of Erythrocyte Deformation under the Action of Stress Hormones

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Abstract Optical methods (interferometry, UV, FTIR and IR spectroscopy) were used to study erythrocyte deformation occurring under the action of adrenaline and cortisol. These hormones give rise to mechanical stresses (contraction) on erythrocytes ghosts, which show up as an increased structural orderliness of membrane proteins, in particular, of the protein domains belonging to contraction and integral proteins. An increasing orderliness of protein domains is accompanied by splitting the absorption band of NH peptide bond (stretching vibrations). In this case, changes of domain orderliness occur mainly in contraction proteins due to their high concentration with respect to other membrane proteins. In addition, adrenaline β -structure and raise the intensity of absorption bands at 1630, 1686 or 1696 cm^{-1} . Cortisol increase the fraction of α -helices. Finally, along with alteration of the protein structure, we observed an increase of phospholipid orderliness in domains and that of interdomain orderliness, which show up as splitting of absorption bands of the stretching and deformation vibrations of CH bonds as well as P=O and POC bonds. The effects produced by the hormones in erythrocyte ghosts are shown to persist on intact erythrocytes. FTIR spectroscopy study using the Frustrated Total Internal Reflection method revealed splitting of absorption bands of the stretching vibrations of NH, CH and PO₂ bonds and an increase in the fraction of β -structure and α -helices. We found also the hypochromic effect of absorption band at 418 nm (UV spectrometer), which corresponds to the band of heme. The hypochromic effect of this band is caused by increasing orderliness of hemoglobin and heme, it indicates a growing affinity for oxygen. Thus, stress hormones alter the oxygen transport properties of erythrocytes. Besides, interferometry allowed us to observe an abrupt change of refraction factor, and UV spectroscopy detected a non-monotonic increase of light diffusion in erythrocyte suspensions with the hormones. These data reflect the deformation of erythrocytes under the action of hormones. In our opinion, a possible mechanism of structural transitions in hemoglobin produced by stress hormones may be related with changes in elastic free energy of a cell and subsequent changes in osmotic, oncotic and hydrodynamic pressure. Changes in elastic free energy of a cell are caused mainly by the contraction network. Deformation of erythrocytes under the action of stress hormones occurs as adiabatic process. Taking into account the structural transitions in erythrocytes under the influence of adrenaline we might suggest that the change of β -structure (α -helix \rightarrow β -structure transition) occurs to adrenoreceptor at first spreads to the contractile net-in whole, that leads to generalized transition in membrane and cell.

Keywords FTIR, UV-Spectroscopy, Deformation of Cell, Mechanism, Protein, Structure, Heme, Stress Hormones

1. Introduction

Changes in blood concentration of stress hormones affect the rheological properties of erythrocytes [1, 2]. Since the hormones give rise to structural transitions in membrane proteins, glycoproteins, phospholipids and glycolipids [3, 4], they are expected to change also the rate of mass transfer through the membranes, in particular, the diffusion rate of oxygen, carbon dioxide, H₂O, hydrogen ions, etc. This is an interesting scope of questions directly related with erythrocyte deformation.

Adsorption of hormones on hematocytes can proceed either specifically or nonspecifically. ESR spectroscopy studies revealed the presence of adrenaline receptors on the surface of erythrocytes and demonstrated the ability of adrenaline to increase the orderliness of phospholipids, which raised membrane microviscosity. Meanwhile, preincubation of erythrocytes with cytochalasin B inhibits a response to adrenaline. This is caused by specific binding of cytochalasin B to a contraction protein, actin, which results in cleavage of actin-spectrin bond and disappearance of contraction properties in the network [5]. Later, in a work with erythrocyte ghosts, we revealed changes in the secondary structure of membrane proteins to occur under the action of adrenaline and carbachol (an analog of acetylcholine) [4, 6]. Such changes in the secondary structure of membrane proteins can be related with

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contraction proteins. Cortisol, similar to adrenaline, binds to erythrocyte membranes. Binding constant for the cortisol is $1.23 \cdot 10^6 \text{ M}^{-1}$ [2]. The existence of receptors to cortisol on erythrocyte membranes remains to be proved.

The mechanism of erythrocyte deformation and structural transformation of membranes and hemoglobin by the action of stress hormones is still scantily investigated. The interaction of hemoglobin with contraction proteins and band 3 protein of erythrocytes is reported in the literature [7-9]. These works imply that the disturbance and deformation of erythrocyte membrane caused by stress hormones or other external factors can be transferred to hemoglobin by means of band 3 integral protein or contraction proteins. According to modern ideas, contraction proteins reside at the inner side of membrane. Within this concept, a reverse response is also possible, i.e., the disturbance can be transferred from hemoglobin to the cell membrane. We understand the functional importance of these interrelations and do not deny them; however, an alternative way of transferring the disturbance from membrane to hemoglobin is also possible. In the present work we consider the mechanism of erythrocyte deformation using the theory of elasticity, classic electrodynamics as well as structural transitions in membrane proteins, phospholipids and hemoglobin caused by the action of stress hormones.

Aim of the work: to elucidate the molecular mechanism of erythrocyte deformation and structural transitions in membrane proteins, phospholipids and hemoglobin under the action of stress hormones.

2. Materials and Methods

2.1. Erythrocyte suspensions were examined upon addition of stress hormones — adrenaline and cortisol using UV (Evolution 300, Thermo Scientific, USA) and FTIR spectrometry (Nicolet 6700, Thermo Scientific, USA) and interferometry (LIR-1, Russia). Merk or Sigma reagents were used in the work.

Blood was taken by a micropipette from human finger into isotonic K^+ , Na^+ phosphate buffer at a temperature of $4.0\text{-}5.0^\circ\text{C}$. Volume of a blood sample did not exceed 0.1 ml; volume of the buffer was 5.0 ml. Erythrocyte suspensions were prepared by triple washing of serum in the isotonic buffer at pH 7.4 and $T = 4.0\text{-}5.0^\circ\text{C}$. The washed erythrocytes were introduced into the buffer and subjected to photometric testing. After photometry, the erythrocyte suspension was supplemented with solutions of adrenaline or cortisol, their ultimate concentration being $10^{-9}\text{-}6 \cdot 10^{-9} \text{ M}$ and $10^{-8}\text{-}6 \cdot 10^{-8} \text{ M}$, respectively. Volume of a hormone solution was 1500 smaller as compared to that of suspension, which prevented a substantial dilution of suspensions upon addition of the hormone. Incubation was performed for 5 min at 25°C . Scanning time was ~ 2.0 min at a 200-700 nm wavelength. The suspension was stirred before scanning.

2.2. Some experiments were carried out with of rat erythrocyte ghosts that were obtained by a technique

reported in [10]. A suspension of erythrocyte ghosts in a 0.001 M K^+ , Na^+ phosphate buffer with pH 7.35 and volume 60 μl was placed in a cuvette with fluorite backing. This was followed by adding 30 μl of the same buffer and 1.0 μl of adrenaline or cortisol solution with a concentration of $10^{-6}\text{-}10^{-7} \text{ M}$. Final concentration of the hormones in suspension was $10^{-8}\text{-}10^{-9} \text{ M}$. The suspension was stirred and incubated for 10 min at $16\text{-}17^\circ\text{C}$. To take the IR spectra, a film of erythrocyte ghosts was prepared in the cuvette by slow evaporation of water under weak vacuum at a pressure of 0.1 atm ($\sim 0.5 \cdot 10^4 \text{ n/m}^2$) and temperature of $4 \pm 1^\circ\text{C}$ [11, 12]. The cuvette was placed strictly horizontally on a special table of a vacuum unit. Drying lasted 180 min. After preparation of the film, the cuvette was transferred to an optical chamber and blown with dry air for 30 min; then scanning was switched on. IR spectra of erythrocyte ghost films were taken on a Specord-M80 spectrometer (Germany, Leipzig) at a resolution of 0.5 cm^{-1} , sequentially experiment and control against fluorite backing, or experiment and control to obtain a difference spectrum. Integration, determination of the spectral band frequency, and mathematical processing were made using special programs enclosed to spectrometer. This allowed us to obtain a fine structure of the membrane spectrum in the frequency range of $900\text{-}4000 \text{ cm}^{-1}$.

2.3. Spectra of erythrocytes were taken on a FTIR spectrometer (Nicolet 6700, Thermo Scientific, USA) by means of Frustrated Total Internal Reflection using a diamond attachment, at a resolution of 4 cm^{-1} in the frequency range of $900\text{-}4000 \text{ cm}^{-1}$, both against the isotonic K^+ , Na^+ phosphate buffer upon addition of salts (pH 7.4) and the difference spectra of erythrocyte / erythrocyte + hormone. Spectra of erythrocyte suspension were recorded without hormone (control) and with sequential addition of the hormone. Suspension with a ligand was incubated for 10 min at 25°C . Volume of the suspension was 15 μl . The suspension was covered with a fluoroplastic cap that was clamped to the cuvette. The time of spectra recording ranged from 30 s to 1 min. Recording and mathematical processing of the spectra were made with a special OMNIC software enclosed to spectrometer.

For the quantitative definition of the elements of secondary structure in membrane proteins we used the method of separation of wide FTIR bands to their composites. After using the second and the fourth derivatives in the region $1600\text{-}1700 \text{ cm}^{-1}$ we got the set of narrow bands which corresponded elements of the secondary structure ($1645\text{-}1660\text{-}\alpha$ -helix, $1660\text{-}1670$ -tangle and $1625\text{-}1640$, 1680 , $1690\text{-}1700 \text{ cm}^{-1}$ $-\beta$ -structure). The bands of adsorption 1686 and $1630\text{-}1640 \text{ cm}^{-1}$ correspond to antiparallel β -structure. This band has two times less width than α -helix, and 3-4 times less width than unordered structure [13-16].

We took the area of the absorption band in the region $1600\text{-}1700 \text{ cm}^{-1}$ for 100%. Then we calculated the area of each band and its percentage.

Deformation of erythrocytes was investigated using a LIR-1 interferometer (Russia). Changes in the refraction

factor were calculated by the formula

$$\Delta n = k\lambda/l \quad (1)$$

where k is the shift of interference band measured by a micrometer mechanism, λ is the wavelength, and l is the length of cuvette equal to 2.0 cm.

3. Results

3.1. During sequential introduction of adrenaline, which concentration ranged from 10^{-9} to $6 \cdot 10^{-9}$ M, into erythrocyte suspension, a UV spectrometer showed a decreasing intensity of the absorption band with $\lambda = 418$ nm. This band characterizes the state of heme in the iron-porphyrin complex. The measurement gave a set of curves showing that the band intensity decreased by 17.4% when attaining a minimum (Fig. 1). This points to the hypochromic effect occurring in hemoglobin [17]. The revealed effect is caused by increasing orderliness of hemoglobin itself and its prosthetic group — heme due to cell deformation. Changes in other parts of the spectrum were less pronounced; in particular, we observed an increase in the optical density at 600-700 nm, its value being equal to 0.03-0.04 optical units (~1%). The measurement error in this part of the spectrum did not exceed 0.5%. This region of the spectrum demonstrates an increase in diffusion of light due to increasing cell density.

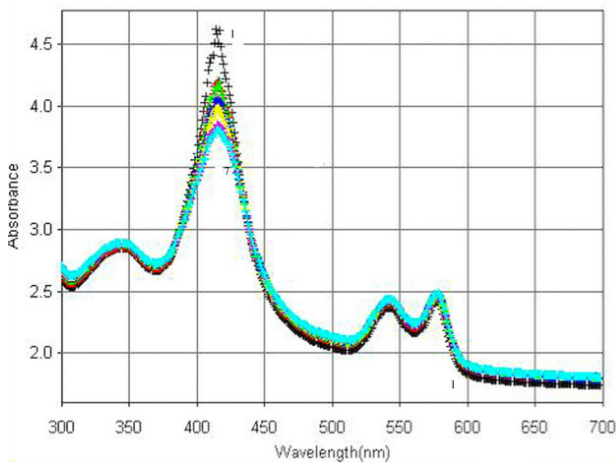


Figure 1. UV spectra of human erythrocyte suspensions at the addition of adrenaline ($C = 10^{-9}$ - $6 \cdot 10^{-9}$ M), (1-control, 2- $C_1=10^{-9}$ M, 3- $C_2=2 \cdot 10^{-9}$ M, 4- $C_3=3 \cdot 10^{-9}$ M, 5- $C_4=4 \cdot 10^{-9}$ M, 6- $C_5=5 \cdot 10^{-9}$ M, 7- $C_6=6 \cdot 10^{-9}$ M)

The addition of cortisol to erythrocyte suspension with the hormone concentration of 10^{-8} to $6 \cdot 10^{-8}$ M also produced a set of UV spectral curves. The measurement conditions were similar to those of experiments with adrenaline. A maximum of absorption band at 418 nm was shown to decrease with increasing the hormone concentration. A decrease in the optical density was 22% as compared to erythrocyte suspension without hormone (control) (Fig. 2). The resulting set of curves was used to plot the dependences of optical density for band 418 nm on the concentration of hormones. Erythrocytes incubated adrenaline are characterized by the

S-shaped plot of intensity (Fig. 3), which indicates a cooperative transformation of hemoglobin structure during cell deformation. Regression analysis allowed us to determine form of the function that reflects changes in the optical density of hemoglobin caused by the action of adrenaline and cortisol on erythrocytes. In both cases, the function had the same form. In the first case, coefficients at x were greater as compared to the second case, thus indicating the faster changes in optical density of hemoglobin versus adrenaline concentration (Figs. 3 and 4).

We observed also an increase in the optical density in the regions of 600-700 and 310 nm, which points to increasing diffusion of light. In these regions, optical density changed by ~5.5%, which considerably exceeds the measurement error (0.5%) (Fig. 2). It should be noted that absorption band at 418 nm may shift spontaneously by $\pm 2-3$ nm in different runs, generally to the short-wave region. Shifting of this band occurs either due to fluctuations in the structure of hemoglobin itself [18] or by the action of fluctuations in the structure of membrane and cell as a whole [12, 19].

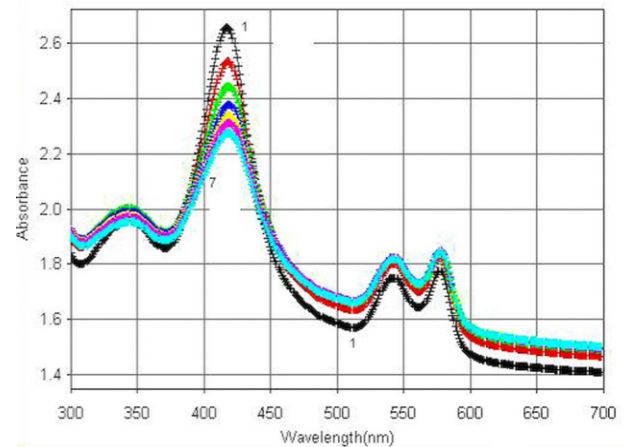


Figure 2. UV spectra of human erythrocyte suspensions at the addition of cortisol ($C = 10^{-8}$ - $6 \cdot 10^{-8}$ M), (1-control, 2- $C_1=10^{-8}$ M, 3- $C_2=2 \cdot 10^{-8}$ M, 4- $C_3=3 \cdot 10^{-8}$ M, 5- $C_4=4 \cdot 10^{-8}$ M, 6- $C_5=5 \cdot 10^{-8}$ M, 7- $C_6=6 \cdot 10^{-8}$ M)

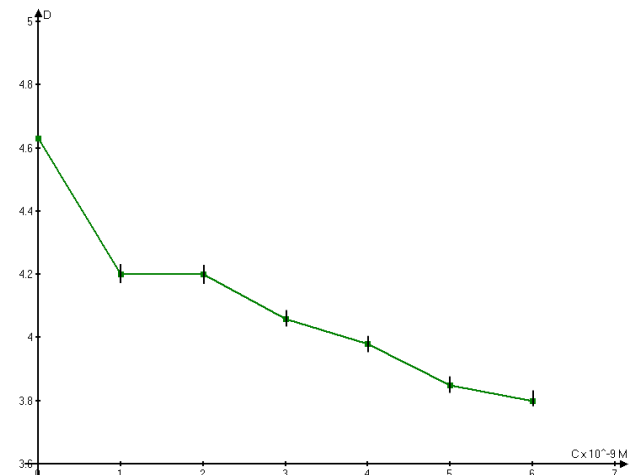


Figure 3. Changes in the optical density of absorption band 418 nm at the addition of adrenaline to human erythrocyte suspensions ($C = 10^{-9}$ - $6 \cdot 10^{-9}$ M), $p \leq 0,5$

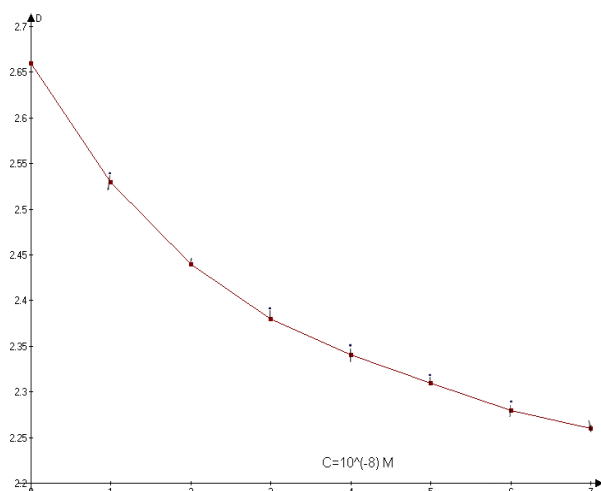


Figure 4. Changes in the optical density of absorption band 418 nm at the addition of cortisol to human erythrocyte suspensions ($C = 10^{-8} \cdot 6 \cdot 10^{-8}$ M), function $D(x) = (3,968254 \cdot 10^6) x^7 - (8,333 \cdot 10^5) x^6 + (6,544 \cdot 10^4) x^5 - 0,0029167 x^4 + 0,004778 x^3 + 0,018 x^2 - 0,1504762 x + 2,66$, ($x = C$), $p \leq 0,5$

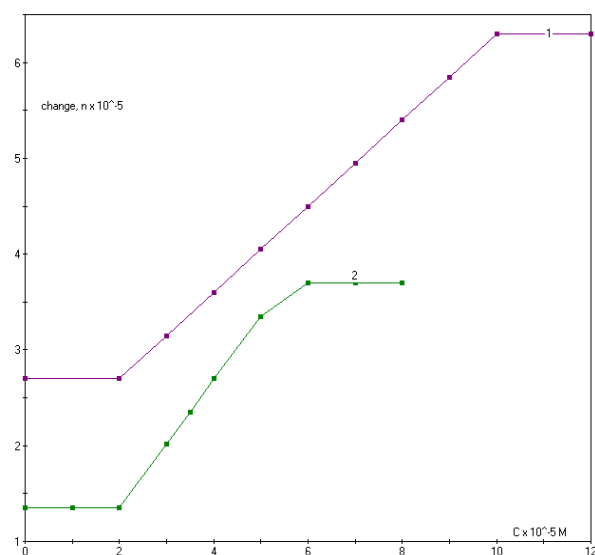


Figure 5. Changes in refraction factor of erythrocyte suspensions at the addition of adrenaline (1) or carbachol (2), pH 7,3

3.3. Besides, interferometry showed an increase in refraction factor after addition of adrenaline, carbachol (Fig. 5) or digitonin to cell suspension; digitonin can specifically bind to cholesterol in equimolar ratio [5]. As pH of the medium shifted from 6,0 to 8,0, this also increased the refraction factor, which testifies a growing cell density. Cell examination with an interference microscope (Zeiss, Germany) revealed a deformation at pH 8,0, which means that in an alkaline medium erythrocytes take the form of echinocytes [6]. According to [20], refraction factor varies in proportion to the density of gas under examination or concentration of a solute in the liquid:

$$(n-1)/\rho = \text{const} \quad (2)$$

where n is the refraction factor, and ρ is the concentration of substance.

In a coarsely dispersed colloidal solution of erythrocyte suspensions, refraction factor grows when density of the particles increases at a constant concentration of suspensions. An increase in light diffusion in the regions of 600-700 and 310 nm [21] under the action of hormones points to an increased density of the particles (erythrocytes). Both the addition of hormones and the shifts in pH of the medium gave S-shaped plots [6], which reflects the cooperative nature of structural changes in erythrocyte membrane and whole cells.

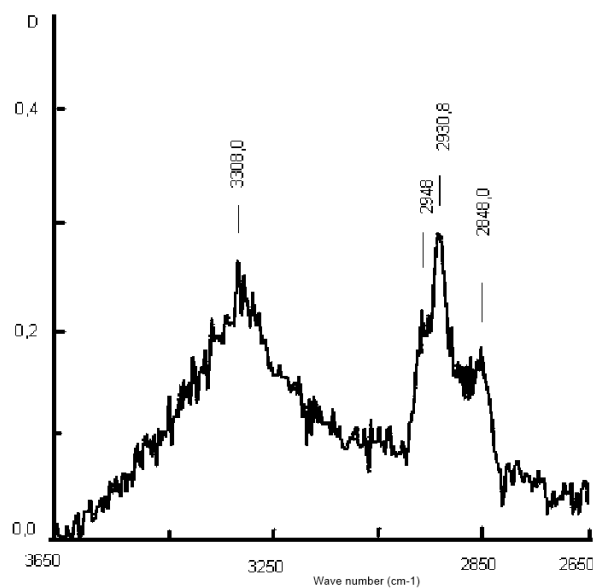
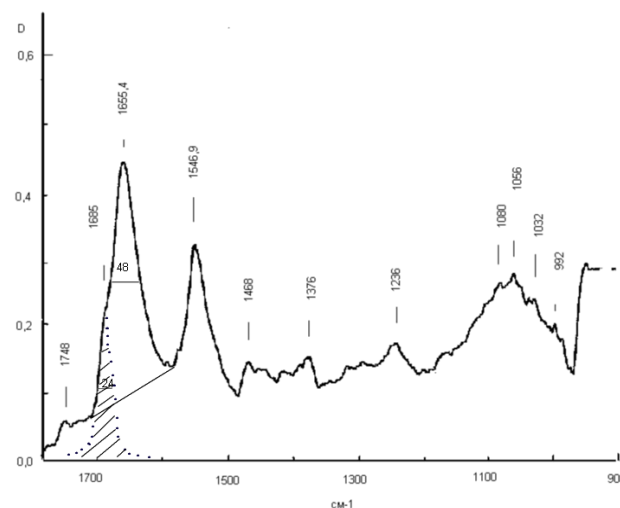


Figure 6. IR spectra of rat erythrocyte ghosts (control): a) $\nu = 1000-1800 \text{ cm}^{-1}$, b) $\nu = 2600-3400 \text{ cm}^{-1}$, $C_{\text{bur}} = 0,001 \text{ M}$, pH 7,2

3.4. The analysis of IR spectra of rat erythrocyte ghosts not loaded with hormones (control, Fig. 6) revealed not only a disordered structure, but also the presence of α -helix $1645-1660 \text{ cm}^{-1}$ and β -structure (1686 and 1530 cm^{-1}) in the proteins of rat erythrocyte ghosts [3, 5, 6, 11, 16]. We recorded NH stretching vibrations of proteins (3308 cm^{-1}), CH stretching vibrations of hydrocarbon chains in proteins and phospholipids (2948 , 2930 and 2848 cm^{-1}) as well as some bands typical of phospholipids, in particular, C=O

bond (1748 cm^{-1}), P=O bond (1236 cm^{-1}), CH_2 deformation vibrations (1460 and 1386 cm^{-1}) of hydrocarbon chains, $\text{O}_4\text{-C}_4\text{-C}_5\text{-O}_5$ bond (1048 cm^{-1}) of monosaccharides in glycolipids and glycoproteins, and C-C deformation vibrations (978 cm^{-1}). Note that the C=O band (1748 cm^{-1}) is quite narrow; hence it follows that phospholipids are well ordered at the level of ester bonds in higher carboxylic acids and glycerol.

Study of the interaction between hormones and erythrocyte ghosts showed that adrenaline produces substantial changes in the membrane structure even at a concentration of 10^{-9} and 10^{-7} M , which shows up as splitting of absorption band of NH peptide bond in comparison with ghosts not loaded with adrenaline (Fig. 7). Splitting of the absorption band of NH bond points to intramolecular interaction between elements of the secondary structure, in particular, α -helices [22] and protein domains as well as β -structure and tangle, due to their ordering. Protein domains were found in contraction and integral proteins [23]. Besides, splitting may indicate also a strengthening of intermolecular interaction between contraction and integral proteins due to ordering of the membrane structure. We observed also changes in the ratio of secondary structures, for example, tangle \rightarrow α -helix or tangle \rightarrow β -structure transitions.

The graphical analysis of the band of adsorption 1686 cm^{-1} in the control probe and after incubation with adrenaline has shown the area of the band had increased in 1,3 times which meant the growth of its integral intensity. Besides, the band 1636 cm^{-1} appeared in the probe with adrenaline. So that in this case we observe the effect of addition of the bands 1636 and 1686 cm^{-1} which is determined by enlargement of β -structural part in erythrocyte membrane proteins under the influence of adrenaline (Fig. 6a, 7a).

At a 10^{-7} M concentration of adrenaline, there appears a band at 1646 cm^{-1} indicating an increase in the fraction of α -helices (Table 1).

Along with structural transformation of membrane proteins, changes in the lipid part of the spectrum were also recorded. In particular, incubation of erythrocyte ghosts with adrenaline resulted in splitting of the absorption band 1236 cm^{-1} (PO bond) into the bands 1220 , 1236 and 1258 cm^{-1} at adrenaline concentration 10^{-9} M . As the hormone concentration increased to 10^{-7} M , a $1236 \rightarrow 1248 \rightarrow 1256\text{ cm}^{-1}$ shift was observed for one of these bands. The absorption band 1080 cm^{-1} (POC bond) also split. The same was observed for the band of C=O bond in the region of 1740 cm^{-1} . This was accompanied by additional splitting in the region of CH bond stretching vibrations (2948 cm^{-1}) and an increase in their integral absorption intensity (Table 1, Fig. 6). Splitting of the indicated bands testifies the ordering of phospholipids and their domains by the action of hormone. A shift of absorption band 1236 cm^{-1} to the short-wave region reflects the formation of hydrogen bond between PO group and OH group of adrenaline, $C = 10^{-7}\text{ M}$. This is an evidence of nonspecific (nonreceptor) action of adrenaline

during structural transformation of erythrocyte membranes (Table 1).

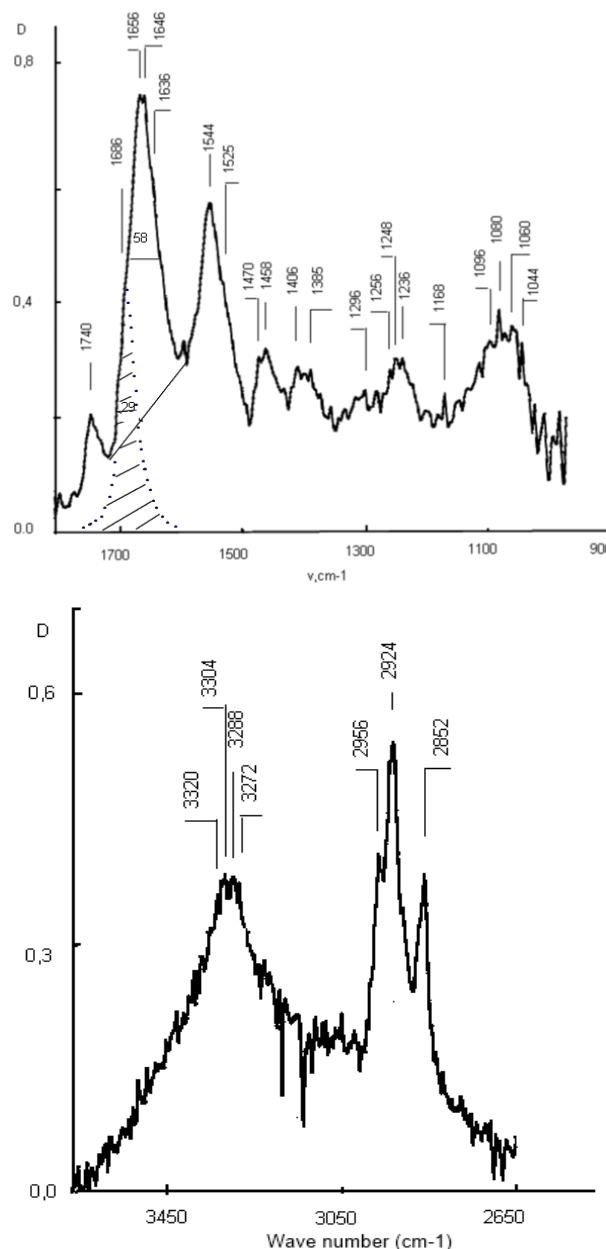


Figure 7. IR spectra of rat erythrocyte ghosts at the addition of adrenaline ($C = 2 \cdot 10^{-9}\text{ M}$): a) $\nu = 1000\text{-}1800\text{ cm}^{-1}$, b) $\nu = 2600\text{-}3400\text{ cm}^{-1}$, $C_{\text{buf.}} = 0,001\text{ M}$, pH 7,2

3.5. Analysis of the IR spectra of rat erythrocyte ghosts upon incubation with cortisol at its concentration of $4.4 \cdot 10^{-8}\text{ M}$ revealed a ca. 20% increase in intensity of the absorption bands of CO (1656 cm^{-1}) and NH bonds (1550 and 3296 cm^{-1}), the effect building up with an increase in the hormone concentration (Table 1, Fig. 8). A growing intensity of the band 1656 cm^{-1} testifies an increase in the fraction of α -helix [11, 12]. The increasing fraction of α -helices in membrane proteins is related with the structural transition tangle \rightarrow α -helix.

Table 1. Frequency characteristics of human erythrocytes and rat erythrocyte ghosts before and after their interaction with hormones

№	Compound	ν_{CO}	ν_{NH} бал.	$\nu_{C=O}$	$\nu_{P=O}$	ν_{P-O-C}	$\nu_{O5C4-C5O4}$	ν_{CH} бал.	A_{CO}
1.	Ghosts (control)	1655,4 1686	3308	1748	1236	1080	1056	2948 2930 2848	1,2150E + 01
2.	Ghosts + cortisol ($C = 4,4 \times 10^{-8}M$)	1656,0	3296,0 3280,0	1740,0	1239,0	1083,7	1044,0	2962,5 2931,4 2920,0 2853,8	1,5169E + 01
3.	Ghosts + cortisol ($C = 10^{-7}M$)	1656,0 1630	3300 3280	1740	1239	1083,7	-	2962 2925 2852	1,5640E + 01
4.	Ghosts + adrenaline ($C = 10^{-9}M$)	1656 1646 1686 1636	3316 3292 3276	1740	1256 1248 1236	1096 1080	1060 1044	2956 2924 2852	1,5830E + 01
5.	Ghosts+adrenaline ($C = 10^{-7}M$)	1656 1646	3320 3304 3288 3272	1740	1258 1248 1236 1220	1096 1080	1070 1066 1050	2956 2924 2852	1,6108E +0,1
6.	Erythrocytes (control)	1649,9	3282,1 3272,0		1245,7	1106,2		2956,8 2935,8 2871,7 3030,2	
7.	Erythrocytes + cortisol ($C = 3 \times 10^{-8}M$)	1650,5 1636,3	3285,3 3270,2 3247,3	1741,1 1707,5	1242,0	1105,5		2956,6 2937,5 2872,3 3028,4 3052,4	
8.	Erythrocytes + adrenaline ($C = 10^{-9}M$)	1649,9 1640,3	3286,5 3277,2 3266,0	1757,1 1724,9	1243,4	1126,1 1108,4 1087,9	1072,1	2963,8 2957,3 2872,4 3029,8 3043,0	
9.	Erythrocytes + adrenaline ($C = 2 \times 10^{-9}M$)	1652,0 1637,5	3292,8 3278,9 3270,5 3263,8 3254,3	1734,1 1713,7	1237,9	1104,9 1084,2	1053,5	2957,1 2926,6 2871,8 2851,8 3026,8	

Note. A_{CO} is the integral intensity of absorption band ν_{CO} of the peptide bond in semilogarithmic form.

A shift of NH bond (stretching vibrations of peptide bond, $3308 \rightarrow 3280 \text{ cm}^{-1}$, $\Delta\nu = 28 \text{ cm}^{-1}$) was accompanied by a growth of its intensity, which is related with the formation of hydrogen bond between cortisol and NH group. Hydrogen bond is likely to form between keto group of A-ring ($C_3=O$) and NH group of the membrane protein. Meanwhile, keto group ($C_{20}=O$) of D-ring and OH group at C_{11} in C-ring could also be involved in the formation of hydrogen bonds. The presence of several hydrophilic groups strongly changes the biological activity of cortisol and other steroid hormones, in distinction to cholesterol. Cholesterol binds to phospholipids mainly due to hydrophobic interaction (Van der Waals forces) with fatty acid residues [11]. Shifting of CH bond stretching vibrations $2848 \rightarrow 2852 \text{ cm}^{-1}$ ($\Delta\nu = 4$

cm^{-1}) and $2930 \rightarrow 2925 \text{ cm}^{-1}$ ($\Delta\nu = 5 \text{ cm}^{-1}$) were observed. The latter increased in intensity under the action of hormone. Changes in intensity of this band confirm the presence of structural transition, but cannot differentiate the place where the transition occurs — in membrane proteins or in phospholipids, as CH bond is present both in proteins and phospholipids. However, as seen from our experimental data, this band reflects mainly the changes in phospholipid orderliness.

An increase in intensity of the absorption band of phospholipid C=O bond and its shift $1748 \rightarrow 1740 \text{ cm}^{-1}$ were observed. This increase of the band intensity indicates a growing orderliness of higher carboxylic acids and a decreasing entropy in phospholipids. Shift of the band is

related with the formation of hydrogen bond between hormone, for example OH group at C₂₁, and CO bond of phospholipids. Such interaction of the hormone simultaneously with protein and phospholipids can occur at the interface between protein and phospholipids, i.e., in a near-boundary or annular layer of the band 3 integral protein, glycophorin and other proteins.

P=O bond shifted in frequency by 3 cm⁻¹ to the short-wave region and increased in intensity. Shifting of P=O bond to the short-wave region is attributed to dehydration of membranes during their deformation under the action of hormone. A loss of bound water increases the frequency of P=O bond [2]. Deformation (contraction) occurs due to spectrin-actin and spectrin-ankyrin networks [4, 5, 12], since the extraction of spectrin from membrane relieves the deformation caused by hormones. It should be noted that 30% of membrane proteins is represented by spectrin [24]. Overall, contraction proteins

constitute 55-60% of all membrane proteins [25]. Steroids can attack either the spectrin-actin-ankyrin network located both on internal and external surfaces of the membrane [26] or the integral proteins associated with contraction proteins [27].

3.6. Our FTIR spectroscopy study of the hormones effect on intact erythrocytes revealed considerable changes of the spectra in absorption regions both of proteins and phospholipids. In particular, adrenaline and cortisol gave rise to absorption band 1636 cm⁻¹ corresponding to β -structure of membrane proteins, which indicates a transformation in the secondary structure of membrane proteins (α -helix \rightarrow β -structure and tangle \rightarrow α -helix transition) involving also the contraction proteins. Shifting of some other absorption bands attributed both to proteins and phospholipids was observed too (Figs. 9 and 10).

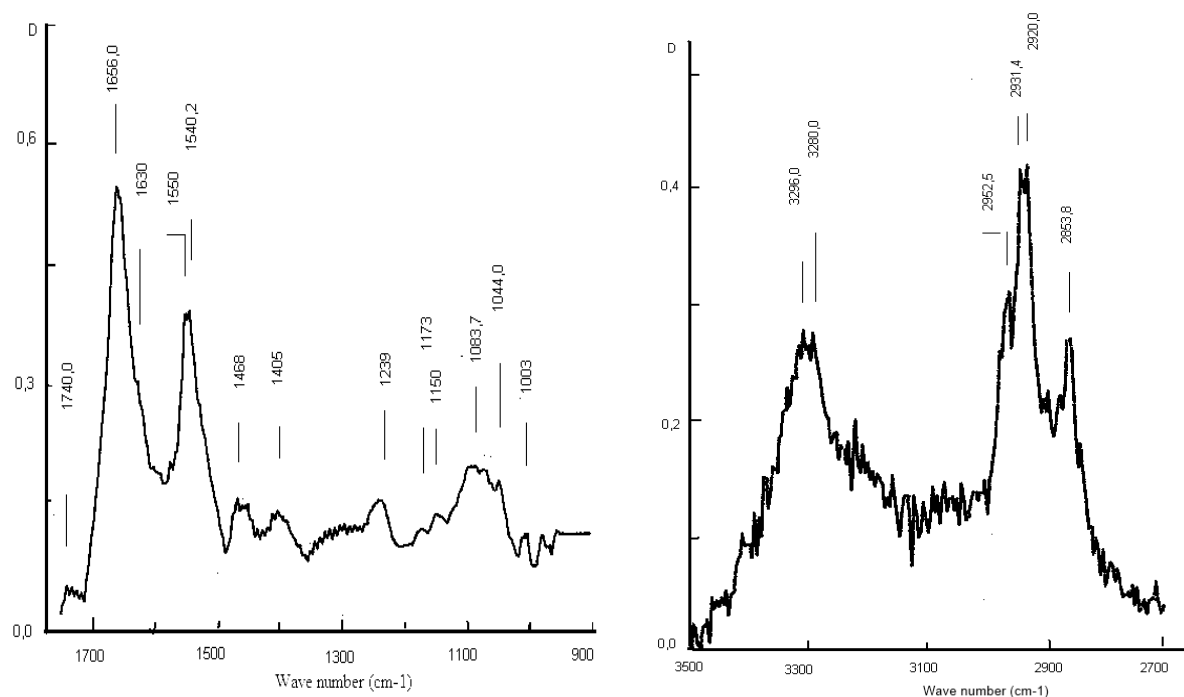
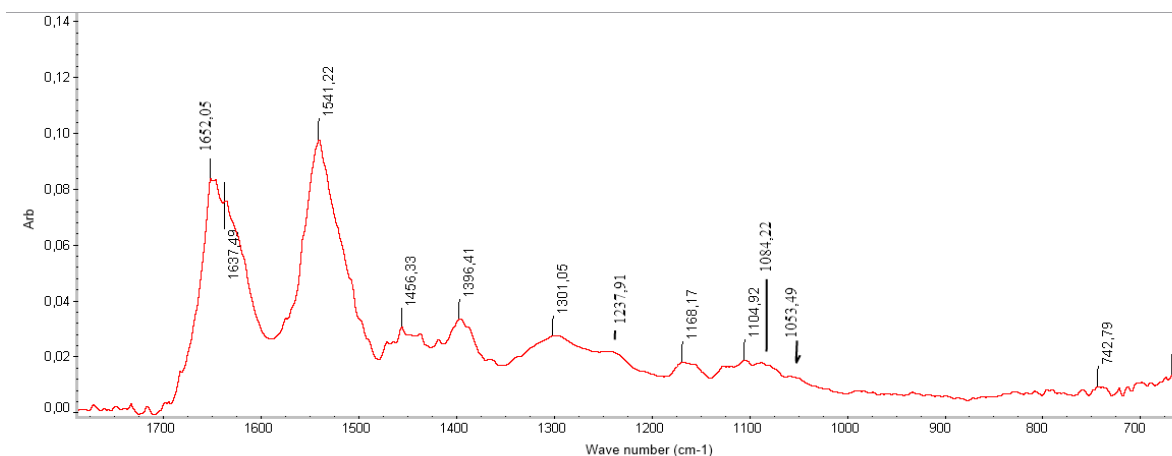


Figure 8. IR spectra of rat erythrocyte ghosts at the addition of cortisol ($C = 4.4 \cdot 10^{-8}$ M): a) $\nu = 1000-1800$ cm⁻¹, b) $\nu = 2600-3400$ cm⁻¹, $C_{\text{buf}} = 0.001$ M, pH 7.2



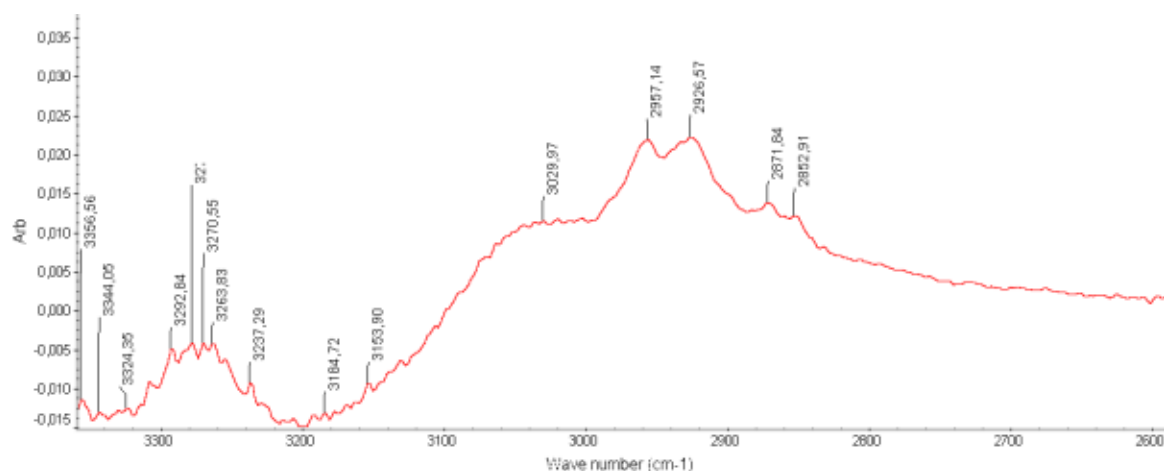


Figure 9. FTIR spectra of human erythrocytes at the addition of adrenaline ($C = 2 \cdot 10^{-9}$ M): a) $\nu = 1000\text{--}1800\text{ cm}^{-1}$, b) $\nu = 2600\text{--}3400\text{ cm}^{-1}$, pH 7,3

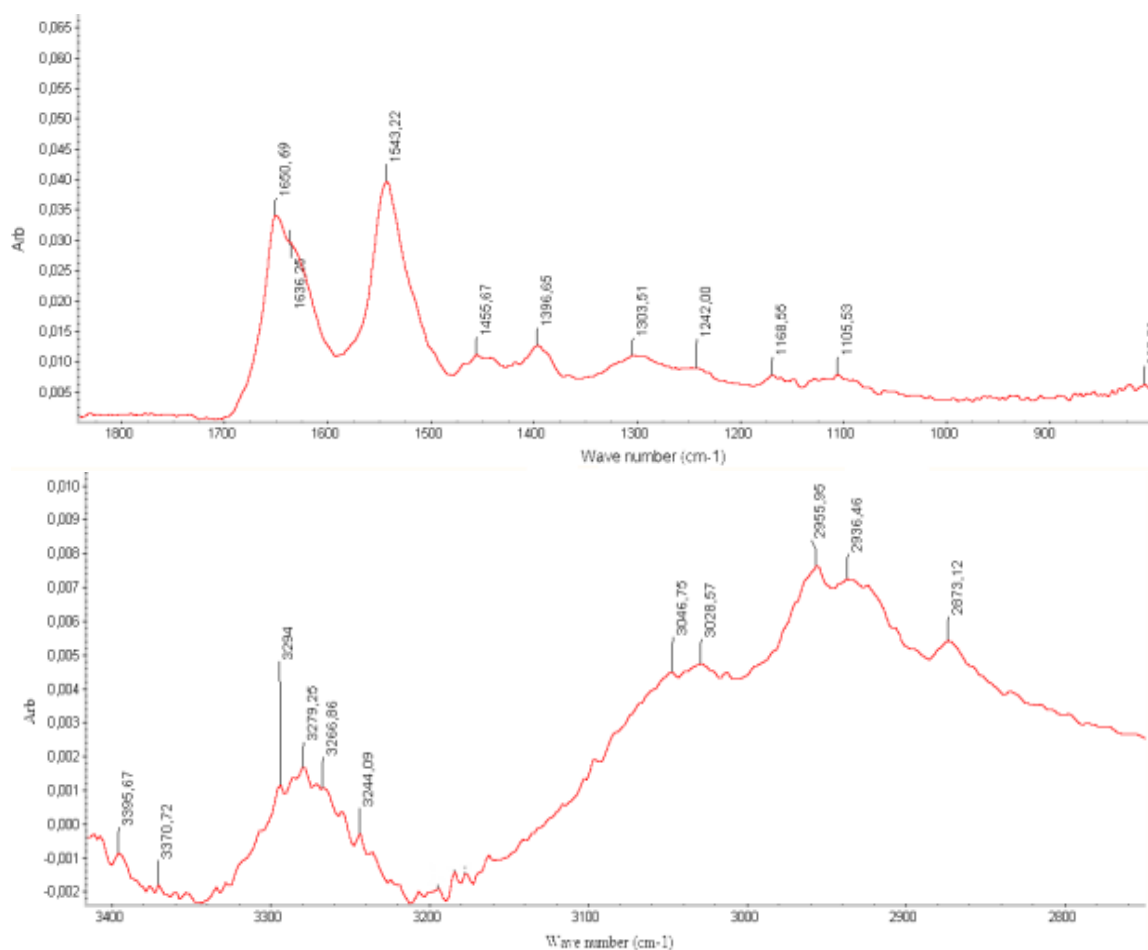


Figure 10. FTIR spectra of human erythrocytes at the addition of cortisol ($C = 3 \cdot 10^{-8}$ M): a) $\nu = 1000\text{--}1800\text{ cm}^{-1}$, b) $\nu = 2600\text{--}3400\text{ cm}^{-1}$, pH 7,3

Noteworthy are the shift of absorption band 2870 cm^{-1} corresponding to stretching vibrations of CH_3 bond in hemoglobin [28], and a more pronounced splitting in the region of stretching and deformation vibrations of phospholipid CH orderliness in and between the domains. A stronger splitting of CH bonds testifies the formation of new lipid-protein clusters as a result of intermolecular interaction, due to compaction of membrane elements caused by structural transformation of the contraction proteins network.

In our earlier studies of high density lipoproteins (HDL), when calculating the enthalpy of structural transitions from experimental data, we suggested the occurrence of smectic A \rightarrow smectic C transition in HDL phospholipids [11, 12]. Such a transitions may occur here, since it has a low enthalpy [11, 12, 29, 30].

Of interest is the appearance of the absorption band $2851,8\text{ cm}^{-1}$, which is assigned to stretching vibrations of CH bond in phospholipids [11]. This band results from structural

transition in membrane phospholipids.

Splitting in the region of 1088 (POC bond) and 3282 cm^{-1} (NH bond) was observed. Splitting of these bands indicates an increasing orderliness in phospholipids and membrane proteins, respectively. An increase in the fraction of β -structure points to the α -helix \rightarrow β -structure transitions, due to redistribution of intensity between absorption bands at 1650 and 1638 cm^{-1} . The first band corresponds to α -helices, the second one to β -structure [3, 12, 13].

Using the fourth derivative of the absorption band 1600-1700 cm^{-1} we calculated maintenance of elements of the secondary structure in the erythrocyte membranes and their ghosts. The results are given in Tables 2, 3. This table shows the considerable increase of β -structure under the action of adrenaline. In this case we observe the structural transition α -helix \rightarrow β -structure. However, under the action of cortisol we see the increase of α -helices. So it can be concluded the structural transition α -helix \rightarrow α -helix took place (Fig. 11-13).

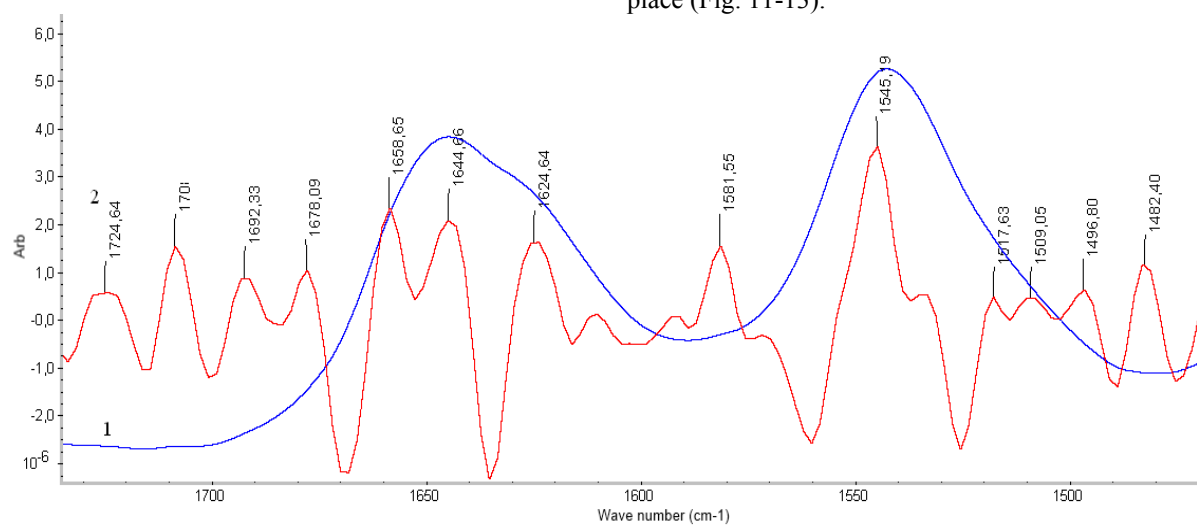


Figure 11. FTIR spectra of human erythrocytes: 1) Difference spectrum generated after subtraction of the spectrum of buffer, 2) fourth derivative of (1), pH 7,2

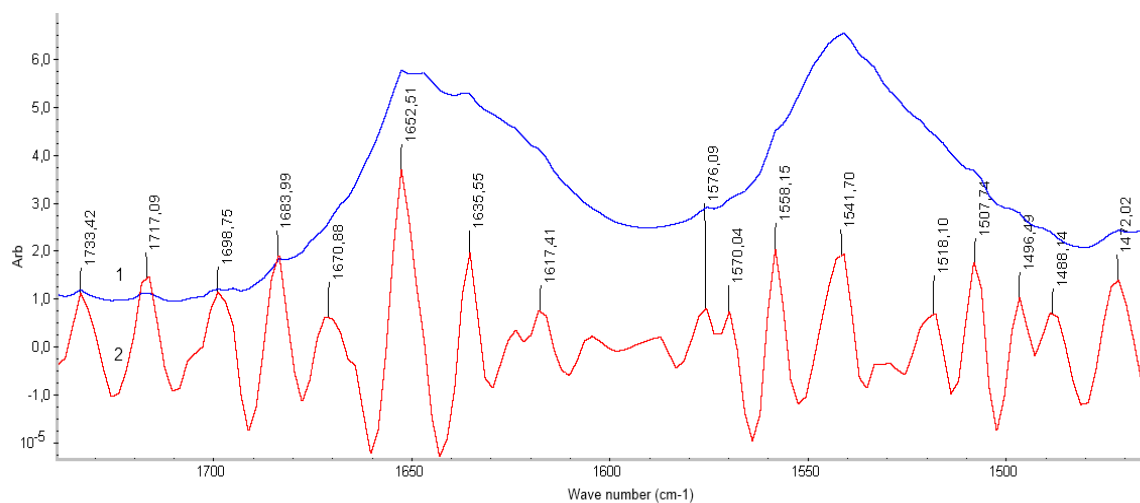


Figure 12. 1) FTIR spectra of human erythrocytes + adrenaline ($C=2 \cdot 10^{-9}\text{M}$), 2) fourth derivative of (1)

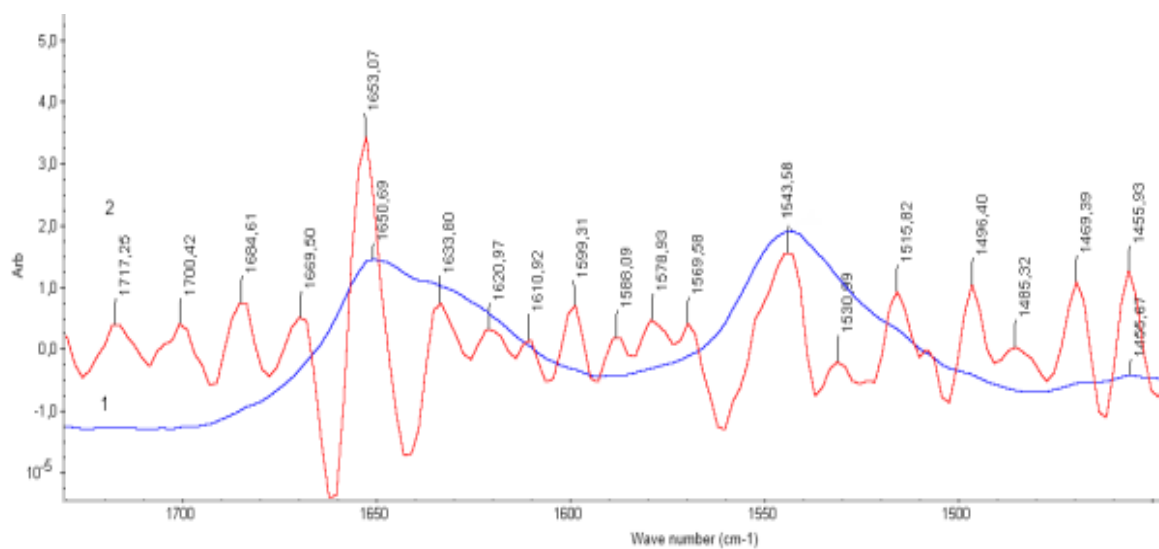


Figure 13. 1) FTIR spectra of human erythrocytes + cortisol ($C = 3 \cdot 10^{-8}$ M), 2) fourth derivation of (1)

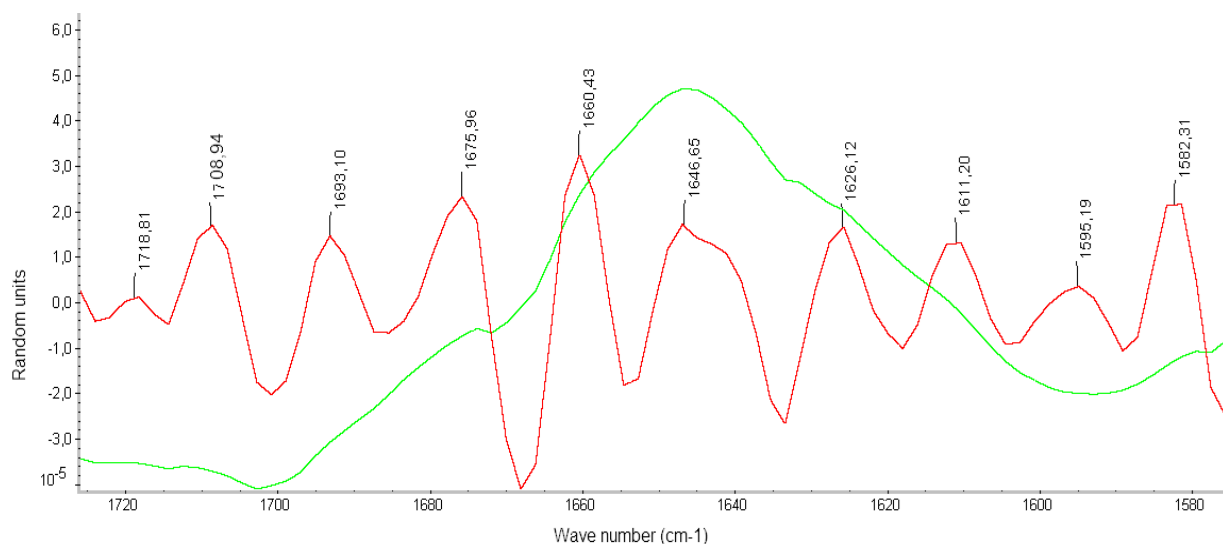


Figure 14. 1) FTIR spectra of human ghost erythrocytes, 2) fourth derivation of (1), $C_{\text{buf.}} = 0.001$ M, pH 7.2

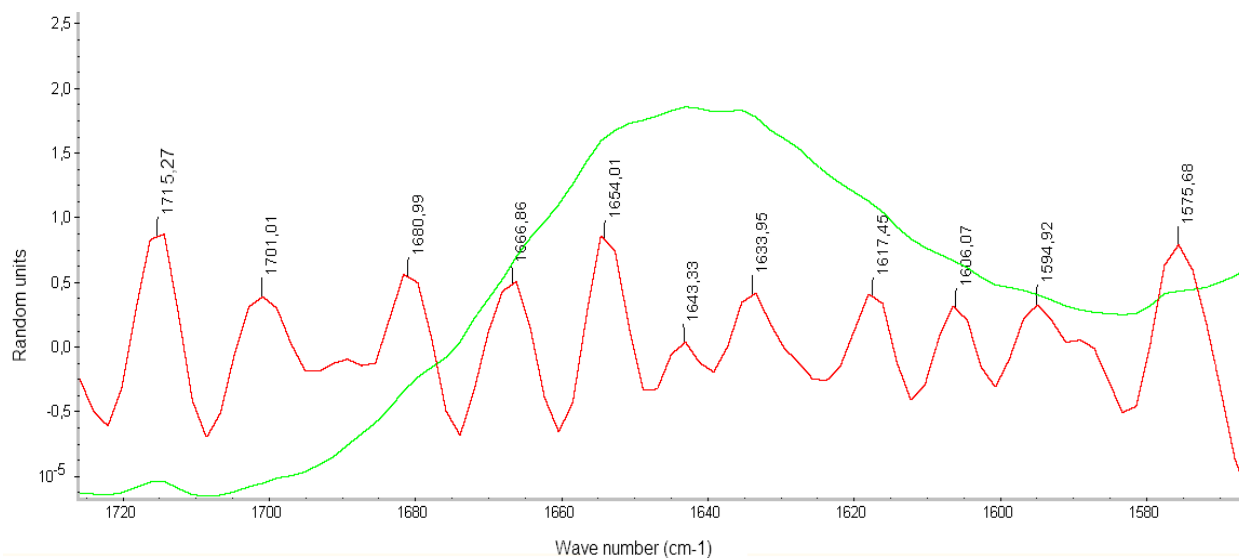


Figure 15. 1) FTIR spectra of human ghost erythrocytes + adrenaline ($C = 1 \cdot 10^{-9}$ M), 2) fourth derivation of (1), $C_{\text{buf.}} = 0.001$ M, pH 7.2

Table 2. The quantitative definition of the elements of secondary structure in membrane proteins of the whole red blood cells

Conformation	Erythrocyte (control)	Erythrocyte + adrenaline $C_{adren.}=2 \cdot 10^{-9}$ M	Erythrocyte+corti-sol $C_{corti.}=3 \cdot 10^{-8}$ M	S Dispersion	Σ Standard deviation
α -helix	$47,7 \pm 6,4 \%$	33 %	53,85 %	$\pm 6,0$	$\pm 6,4$
β -structure	$26,2 \pm 4,2 \%$	56 %	30,8 %	$\pm 3,8$	$\pm 4,2$
Random coil	$14,7 \pm 3,0 \%$	11 %	15,4 %	$\pm 2,7$	$\pm 3,0$

Table 3. The quantitative definition of the elements of secondary structure in membrane proteins of the erythrocyte ghosts

Conformation	Ghost erythrocytes (control)	Ghost erythrocytes + adrenaline ($C=1 \cdot 10^{-9}$ M)
α -helix	43,2 %	33,3 %
β -structure	27,3 %	41,7 %
Random coil	20,4 %	16,7 %

A comparison of IR spectra obtained from ghosts and intact erythrocytes revealed some general regularities: 1) splitting of absorption bands of NH peptide bonds, 2) an increased intensity of absorption bands corresponding to α -helix and β -structure, 3) splitting of absorption bands corresponding to CH bonds of phospholipids, 4) a frequency shift of some bands (Table 1). However, there is also a distinction related with the appearance of absorption bands at 2870 and 1108 cm^{-1} corresponding to hemoglobin [28, 31]. These bands are shifting when erythrocytes are subjected to the action of stress hormones.

4. Discussion

Intensity (I) of light diffusion depends on $I = I(n_1, n_2, \lambda, c_d, v, I^0)$, where n_1 is the refraction factor of disperse phase, n_2 — that of dispersive medium, c_d is the concentration of substance, I^0 is the intensity of incident light, v is the volume of particles, and λ is the wavelength. According to Rowley,

$$I = 24\pi^3 c_d v (n_1^2 - n_0^2 / n_1^2 + 2n_0^2) I^0 / \lambda^4 d \quad (3)$$

Equation (3) is the basis of optical methods for investigating the diffusion of light in colloidal solutions [32], where d is the particle diameter.

From equation (3) it follows that light diffusion is proportional to the particles volume (v). In our case, compressive deformation of erythrocytes implies a decrease in the volume. Hence, diffusion of light is expected to decrease. However, one should take account of the second component, the refraction factor ($n_1 - n_0$), which grows faster as compared to changes in the volume. An increment in refraction factor is related with the growing erythrocyte density and dielectric permittivity (ϵ):

$$n = \sqrt{\epsilon \mu} \quad (4) [20]$$

where magnetic permeability μ is ~ 1 ; as hemoglobin is a paramagnetic, its magnetic permeability is slightly above 1, this value seeming to remain constant in our experiments. Dielectric permittivity in the near-membrane layer can increase due to formation of new ordered domains under the action of stress hormones [2].

Interferometry allowed us to demonstrate a growth of refraction factor for erythrocyte suspensions with raising the concentration of adrenaline, carbachol and other biologically active substances. However, particles with a size strongly exceeding the light wave length are characterized also by reflection [32]. In this case, a partial spherulation of erythrocytes by the action of hormones certainly increased the reflection factor. This is another component contributing to an increase of optical density in the region of 600 - 700 nm . Thus, the growth of refraction factor, reflection and light diffusion caused by increasing density of erythrocytes under the action of hormones raises the intensity of light passing through the samples in the regions of 600 - 700 and 310 nm [32].

Due to decreasing the mean corpuscular volume, volumetric cell deformation increases the molar concentration of ions and low-molecular compounds, and hence the osmotic pressure:

$$\pi = cRT \quad (5)$$

where c is the molar concentration of ions, R is the gas constant, and T is the Kelvin temperature. There is also an increase in oncotic pressure caused by hemoglobin itself [32]. Osmotic energy is known to be an essential component of the cell energy [33]. In addition, as shown by experiments, cell deformation by stress hormones proceeds rapidly, it is not accompanied by heat exchange with the environment; so, this is an adiabatic process. In this case, according to the first law of thermodynamics, equation

$$\delta A = \delta Q - dU \quad (6)$$

is reduced to the form

$$\delta A = -dU \quad (7)$$

This indicates that the work (δA) related with cell deformation should change its internal energy ($-dU$). Here, internal energy of a cell implies the kinetic energy of all cytoplasmic microparticles (electrolyte ions and molecules) plus the potential energy of hemoglobin, which changes with its orderliness. Internal energy includes also the fluctuation energy [34].

According to theory of elasticity, full Helmholtz free

energy of a coarsely dispersed particle can be determined by the relationship

$$F(\{xn\}) = F_{\text{in-plane}} + F_{\text{bending}} + F_{\text{surface stress}} + F_{\text{volumetric stress}} \quad (8) \quad [35]$$

where $F_{\text{in-plane}}$ is the free energy in a plane, F_{bending} is the free bending energy, $F_{\text{surface stress}}$ is the free energy of surface stress, and $F_{\text{volumetric stress}}$ is the free energy of volumetric stress.

We think that the impact of hormones on a cell results in the uniform compression deformation; in this case, all components of free energy (7) will change. Nevertheless, one should take account of the free energy of volumetric stress, which depends on changes in the relative volume and length of spectrin filaments (L).

$$F_{\text{volumetric stress}} = k_{\text{volume}}(V_{\text{total}} - V_{\text{total desired}})^2 k_B T / 2L_0^3 V_{\text{total desired}} \quad (9) \quad [36]$$

where k is the elastic ratio, $\Delta V/V$ is a change of the relative volume, $L_0 = 75$ nm (the length of spectrin filament). Under deformation, the volume of erythrocyte and the length of spectrin filament will decrease.

In terms of classic electrodynamics, energy of a cell is determined by three components: electric, osmotic and chemical.

Electric energy (W_{el}) referred to 1 mol of a substance is expressed as

$$W_{\text{el}} = zF(\varphi_2 - \varphi_1) \quad (10)$$

where z is the charge of ion, F is the Faraday constant, φ_2 is the electric potential on the external surface of membrane, and φ_1 is the electric potential on the internal surface.

Transmembrane potential in erythrocytes is ca. 10^{-13} mV [37], which is nearly five times lower as compared to that in excited membranes. So, a potential difference in erythrocytes is expected to change only slightly under the action of adrenaline. It means that changes in the average electric energy in erythrocytes after incubation with hormones are minor. However, account should be taken of the potential jumps occurring in membrane due to structural transitions under the action of stress hormones and due to surface inhomogeneity of erythrocyte membrane. Equipotential surfaces determined for the outer and inner sides of membrane have a complicated shape. Local structural transitions and jumps of transmembrane potentials may lead to electric break-down [38].

Chemical potential (μ) is responsible for the substance synthesis. As such synthesis does not occur in erythrocytes, it can be assumed that

$$\mu_{01} - \mu_{02} = 0 \quad (11)$$

Osmotic energy (W_{osm}) referred to a mole of substance is determined as

$$W_{\text{osm}} = RT \ln(c_2/c_1) \quad (12)$$

where R is the gas constant, c_1 and c_2 are the concentrations of substance in moles outside an inside the cell, respectively.

Thus, compressive deformation of erythrocytes increases the full free elastic energy F , which raises the molar concentration of ions in a cell and hence the osmotic pressure. There is also an increase in oncotic pressure, which is

proportional to hemoglobin concentration. Such a growth of osmotic, oncotic and hydrodynamic pressure under volumetric cell deformation caused by the hormones leads to conformational changes and increases the orderliness of hemoglobin and heme. Hemoglobin is highly sensitive to external action; this may be related with its high (35%) concentration in erythrocyte [39]. Thus, an increased orderliness of hemoglobin raises the internal energy of a cell ($-dU$), which was noted above. We consider this as a universal and biologically important mechanism of structural transformations in hemoglobin and heme, and hence as a mechanism of altering the oxygen transport functions.

Adrenaline and cortisol are known to have different interaction mechanisms with the membrane and its contraction network. Adrenaline transfers a disturbance to membrane through β -adrenoreceptor and then through adenylatecyclase. Cortisol is adsorbed nonspecifically on the membrane surface; local disturbances, due to cooperative properties of membrane, are transferred to contraction proteins, which bring membrane to a new ordered state. Thus, the mechanism of interaction with membrane is different for adrenaline and cortisol, whereas their effect on hemoglobin is unidirectional. It can be supposed that other biologically active substances or physicochemical factors leading to deformation in erythrocytes would also produce conformational transitions in hemoglobin.

At present time we don't have any data about the quantitative characteristics of the erythrocyte volume changes under the action of stress hormones, so that we've decided to make preliminary calculations of the size of free energy produced by the deformation under the action of stress hormones relatively to the energy of volume fluctuation.

We supposed that fluctuational change volume of erythrocyte consist $1 (\mu\text{m})^3$, volume stress under effect gormons change on $10 (\mu\text{m})^3$ and full volume consist $90 (\mu\text{m})^3$, $L_0 = 75$ nm (length of spectrin filaments) [27, 35].

$$F_{\text{vol.fluctuation}} = k_{\text{vol}} k_B T \cdot (\Delta V)^2 / 2 \cdot L_0^3 \cdot (V_0 - \Delta V) \cdot 10^{-18} \text{ m}^3 = k_{\text{vol}} k_B T \cdot (1 \cdot 10^{-18} \text{ m}^3)^2 / 2 \cdot (75 \cdot 10^{-9} \text{ m})^3 \cdot 89 \cdot 10^{-18} \text{ m}^3 = k_{\text{vol}} k_B T \cdot (1 \cdot 10^{-18})^2 / 2 \cdot (75)^3 \cdot 10^{-27} \text{ m}^3 \cdot 89 \cdot 10^{-18} \text{ m}^3 = k_{\text{vol}} k_B T / 7,51 \cdot 10^7 \cdot 10^{-9} = k_{\text{vol}} k_B T / 7,51 \cdot 10^{-2}, \text{ if } k_{\text{vol}} = 600 \quad [36], F_{\text{vol.fluctuation}} = 0,8 \cdot 10^4 k_B T, (T=295 \text{ K}).$$

$$F_{\text{vol.stress}} = k_{\text{vol}} k_B T \cdot (\Delta V)^2 / 2 \cdot L_0^3 \cdot (\Delta V) \cdot 10^{-18} \text{ m}^3 = k_{\text{vol}} k_B T \cdot (10 \cdot 10^{-18} \text{ m}^3)^2 / 2 \cdot (75 \cdot 10^{-9} \text{ m})^3 \cdot (80 \cdot 10^{-18} \text{ m}^3) = k_{\text{vol}} k_B T \cdot (10^{-34} \text{ m}^6) / 2 \cdot (75)^3 \cdot 10^{-27} \text{ m}^3 \cdot (80 \cdot 10^{-18} \text{ m}^3) = k_{\text{vol}} k_B T \cdot \text{m}^6 / 2 \cdot 421875 \cdot 8 \cdot 10^{-10} \text{ m}^6 = k_{\text{vol}} k_B T \cdot / 6750000 \cdot 10^{-10}, \text{ if } k_{\text{vol}} = 600 \quad [36], F_{\text{vol.stress}} = 0,89 \cdot 10^6 k_B T, (T=295 \text{ K}).$$

So that, while decreasing the erythrocyte volume by 10-11 % under the influence of stress hormones the free energy of erythrocyte volume deformation increased in 111 times in compartion with its free energy of volume fluctuation.

Thus, the UV spectroscopy, interferometry and FTIR spectroscopy study of erythrocyte suspensions subjected to the action of stress hormones revealed an abrupt change of light diffusion at 600-700 nm and light absorption at 418 nm (the band of heme), an S-shaped change of refraction factor,

and splitting of some absorption bands in the FTIR spectra of phospholipids and proteins. The data obtained indicate cell contraction, which leads to hypochromic effect both in heme and hemoglobin. The contraction was initiated by transformations in the secondary structure of membrane proteins, in particular the contraction proteins, and by phospholipid ordering. Deformation (contraction) of erythrocytes under the action of adrenaline or cortisol abruptly alters the structure of membrane and hemoglobin. The alteration of hemoglobin conformation caused by adrenaline or cortisol has a strong effect on the heme structure and hence on the affinity for oxygen. In our opinion, a possible mechanism of structural transformations in hemoglobin produced by stress hormones may be related with changes in elastic free energy of a cell and subsequent changes in osmotic, oncotic and hydrodynamic pressure. Changes in elastic free energy of a cell are caused mainly by the contraction network. Deformation of erythrocytes under the action of stress hormones occurs as adiabatic process.

IR spectroscopy allowed us to elucidate the interaction mechanism of steroid hormones with erythrocyte ghosts. The hormones were shown to interact both with membrane proteins and phospholipids via the formation of hydrogen bonds with NH bond of proteins and C=O bond of phospholipids. The hydrophobic interaction also takes place here; it depends on conformational mobility and flexibility of perhydrocyclo-phenanthrene nucleus of cortisol. The hormones initiate structural transitions in erythrocyte membrane involving membrane proteins and phospholipids. In membrane proteins, there occur changes of the secondary structure, i.e., structural transitions $\text{tangle} \rightarrow \alpha\text{-helix}$, $\text{tangle} \rightarrow \beta\text{-structure}$ and $\alpha\text{-helix} \rightarrow \beta\text{-structure}$. In membrane phospholipids, the order \rightarrow order structural transition was found. The indicated transitions caused by the hormones lead to deformation (contraction) of membranes and thus to cell deformation.

5. Conclusions

Stress is a frequent phenomenon in the human life. Stress is one of the factors causing the infarction of myocardium [1, 2]. The level of stress hormones grows up in several times, therefore it is important to study their influence on the structure of blood cells. We have chosen erythrocytes as much as red blood cells determine rheological and oxygen-transporting functions of blood. Under the action of mechanical factors, pH and temperature shifts erythrocytes and hemoglobin undergo different structural transformations [12, 27].

Hypochromic effect occurring in haemoglobin under the action of physiological levels of the stress hormones deserves special attention, in our opinion, because of significant changes in the oxygen-transporting function of erythrocytes. In whole, structural transitions in erythrocyte membrane affect the rheological properties of blood [1, 2, 12].

At last, modification of secondary structure of membrane proteins under the action of hormones is no less important result of our research, since it necessarily affects the contractile proteins such as actin, spectrin and ankyrin. Also, it is known for the impact on the activity of Na^+ , K^+ -AT Phase, acetylcholinesterase and rate of glycolysis [12]. The local changes of membrane structure in the area of hormonal adsorption spread widely in the whole cell through the contractile net.

Taking into account the structural transitions in erythrocytes under the influence of adrenaline we might suggest that the change of β -structure (α -helix \rightarrow β -structure transition) occurs to adrenoreceptor at first spreads to the contractile net-in whole, that leads to generalized transition in membrane and cell.

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