ROLE OF MEMBRANE PROTEINS *β*- AND *α*-STRUCTURES IN PLASMALEMM STRUCTURE CHANGE

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Abstract. Changes in the structure of plasma membranes affect the functions of membranes and cells. Some of these changes can lead to the development of pathologies of the body, which makes it actually to study the effect of changes in the structure of membranes on their functions. It has now been established that when stress hormones and androgens interact with plasma membranes, their structure changes. At the same time, interactions between proteins and lipids change in plasmalemmas, and a fixed quasi-periodic network of protein-lipid domains associated with the cytoskeleton is formed. The initiators of the formation of protein-lipid domains are membrane proteins, which have changed their secondary structure during the interaction of the membrane with hormones. However, it is still unclear exactly what changes in the secondary structure of membrane proteins contribute to the formation of protein-lipid domains around them. The aim of this work was to establish these secondary structures of membrane proteins. To achieve this goal, changes in the structure of membranes during their interaction with dehydroepiandrosterone, cortisol, androsterone, testosterone, and adrenaline were studied. In this work, a fluorescent method for measuring the relative microviscosity of membranes using a pyrene probe was used to study changes in the membrane structure. The change in the secondary structure of membrane proteins during structural transitions in membranes was studied by measuring the IR absorption spectra of membranes. It has been established that the initiators of the appearance of protein-lipid domains in plasma membranes are membrane proteins, in which, after interaction with hormones, the proportion of *β*-

structures increases. At the same time, the appearance of new α-helices in membrane proteins does not enhance the attraction between membrane proteins and protein-lipid domains are not formed. On the contrary, the appearance of a large number of α-helices in membrane proteins can lead to a decrease in the microviscosity of the lipid bilayer.

Keywords: *protein–protein and protein–lipid interactions in plasma membranes, protein-lipid domains in plasma membranes, β-structures, α-helices, structural changes of plasma membranes, apo A-1, Alzheimer's disease*

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INTRODUCTION

Changes in the structure of plasma membranes affect the functions of membranes and cells. Changes in the structure of plasma membranes are understood as changes in the secondary, tertiary and quaternary structures of membrane proteins, phases of the lipid bilayer, redistribution of proteins and lipids across the bilayer, and changes in membrane morphology. Some of these changes may lead to the development of pathologies of the organism. On the other hand, the development of certain pathologies may cause changes in the structure of plasmalemma. Thus, the study of the influence of changes in the structure of plasma membranes on their functions is relevant not only from the point of view of fundamental science, but also extremely important from the practical point of view.

A good model for studying the influence of changes in the structure of plasma membranes on their functions is the erythrocyte membrane, which can be easily isolated and has a structure similar to many other plasma membranes. Many works have been devoted to the study of this model. For example, the description of age-related changes in the structure of erythrocyte membranes is presented in reviews by Borovskaya et al. [1] and Boronikhina et al. [2]. The main factors damaging the structure of erythrocytes and impairing their vital activity are reactive oxygen species, to which erythrocytes are constantly exposed during 4-month functioning in the bloodstream. The consequences of oxidative stress are: disturbance of water-electrolyte balance of aging erythrocytes; decrease in their volume, surface area and area/volume ratio; increase in hemoglobin concentration and cytoplasm viscosity. All this leads to the destruction of the cytoskeleton and its connections with the plasmalemma, decreasing the ability of the membrane to deform in microcapillaries and the ability of the

red cell to perform its functions [1, 2].

Earlier studies of the influence of changes in the structure of plasma membranes on their functions were carried out on the example of the interaction of stress hormones and androgens with erythrocyte membranes, in which the structure of the latter changes [3-5]. In this case, interactions between proteins and lipids in plasmalemms are changed. A fixed quasi-periodic network of protein-lipid domains associated with the cytoskeleton is formed near membrane proteins that have changed their secondary structure [3-5]. In the formed domains, lipids are in liquid-ordered state (L_o), between domains lipids are in liquid-unordered state (L_d) [3-5].

As a result of such structural changes, the field of mechanical stresses in the plasma membrane changes, leading to the appearance of membrane folding [6-8], changes in the functions of membranes and red cells. For example, when the concentration of hormones in erythrocyte membrane suspension increases, there is an increase in microviscosity of membranes and an increase in mechanical compression stresses in them [9]. Because of this, the activity of Na^+ , K^+ -ATPase of these membranes with increasing concentration of hormones in suspension first increases, reaches a maximum, then begins to decrease [9]. As a result, the concentrations of Na^+ and K^+ ions on the inner and outer sides of the membrane change, the difference of electrochemical potentials on the inner and outer sides of the membrane and the zeta potential of the erythrocyte change, which affects its passage through the microcapillary channel [10, 11].

During the passage of erythrocytes through the microcapillary channel, when mechanical transverse compression forces and longitudinal tensile forces act simultaneously on its membrane, kinky solitons may appear in the fatty acid tails of phospholipids. As shown in Mokrushnikov et al. [12], these kinky solitons can enhance the transport of gas molecules across the membrane.

The quasiperiodic network of protein-lipid domains that appeared in the plasma membrane as a result of structural changes represents heterogeneities in which the lipid diffusion coefficient is lower than the membrane average. As a result, the pattern of lateral diffusion of lipids along the cytoplasmic membrane changes. In a homogeneous membrane, it represents simple Brownian diffusion. After the appearance of a network of protein-lipid domains, it becomes nonlinear, and time intervals may appear when advection exists along with diffusion [13].

The initiators of the formation of protein-lipid domains in the plasma membrane are membrane proteins that have changed their secondary structure when membranes interact with hormones [3 - 5]. However, it is still unclear which changes in the secondary structure

of membrane proteins contribute to the formation of protein-lipid domains around them. The aim of this work is to establish these changes in the secondary structure of membrane proteins.

To achieve this goal, the presented work in *vitro* investigates changes in *the* structure of rat erythrocyte membranes upon binding to dehydroepiandrosterone (DEA), cortisol, androsterone, testosterone, and adrenaline. The resulting changes in membrane protein structure were investigated by measuring the intrinsic fluorescence of membrane proteins. Changes in the structure of the lipid bilayer of membranes were studied using the fluorescence method of measuring membrane microviscosity with a pyrene probe. Changes in the secondary structure of membrane proteins were studied by measuring membrane absorption of infrared radiation.

By measuring the dependence of membrane microviscosity on the concentration of hormone in the membrane suspension, we determined the hormones whose interaction led to the formation of a network of protein-lipid domains in the membranes. We also determined the hormone, interaction with which did not lead to the formation of a network of protein-lipid domains in membranes. Further, analyzing the results of IR spectroscopy of erythrocyte membranes, we found differences in the changes in the secondary structure of membrane proteins at interaction of membranes with these hormones. Comparing the results obtained, it was possible to identify the secondary structure of membrane proteins, which contributes to the formation of protein-lipid domains in the plasma membrane.

MATERIALS AND METHODS

Erythrocyte membranes isolation. The main material for the experiments were rat erythrocyte membranes. In the presented work, the Dodge method was used for their isolation, which represents osmotic hemolysis of erythrocytes and washing of membranes in hypotonic buffer [14]. Rat erythrocyte membranes were obtained using the following methodology. Male Wistar rats were decapitated under mild nembutalol anesthesia. The rats were 3 months old and weighed 250-300 g. The freshly collected blood was diluted twofold with isotonic phosphate buffer (pH 7.4) containing 44 mM KH_2RO_4 and 136 mM Na_2NPO_4. Red cells were then precipitated by centrifugation for 10 min at 4° C and 330 *g* ($r_{(sr)}$ 6 cm). The supernatant was drained and the washing procedure was repeated 2 more times. Erythrocyte membranes were obtained after hemolysis of erythrocytes in hypotonic phosphate buffer (pH 7.4) containing 2.75 mM $KH_2RO_{(4)}$ and 8.5 mM Na_2NPO_4. Membranes

were precipitated by centrifugation for 10 min at 4 ° C and 5500 *g* ($r_{(sr)}$ 6 cm), and the supernatant was drained. The procedure was repeated 4 times. The membranes were obtained and stored at 4 C.°

Methods of membrane preparation study. A drop of the preparation was placed on the glass of a light microscope "Biomed-6" ("Biomed", Russia) for observation in transmitted light by the phase contrast method. After adding immersion oil to the center of the preparation, the sample was examined at a magnification of 1000× .

Extraction of lipids from erythrocyte membranes. To analyze the emission spectrum of membrane intrinsic fluorescence, data on the emission spectrum of membrane lipid intrinsic fluorescence are required. For this purpose, lipids were extracted from rat erythrocyte membranes according to the following procedure. A mixture of chloroform and membrane suspension was prepared in a volume ratio of 10/1. After shaking the contents of the test tube, the protein precipitate was removed by filtration. To remove residual proteins from the supernatant, distilled water was added in in a 1/10 ratio. After shaking for 5 min, the mixture was centrifuged for 10 min at 4° C and 870 *g* ($r_{(sr)}$ 6 cm). Centrifugation resulted in separation into two phases: an upper (aqueous) phase containing protein and a lower (organic) phase containing the lipid fraction. The upper phase together with the remaining protein precipitate was removed by pipette; the protein removal procedure was repeated 2 times. The resulting precipitate represented membrane lipids. The lipid suspension was injected from a syringe into phosphate hypotonic buffer, in which it formed liposomes. To this suspension of liposomes, hormones were added at the required concentration and the emission and excitation spectra of the intrinsic fluorescence of hormones and lipids were measured.

Methods of measuring intrinsic fluorescence of membrane proteins. In the presented work we measured the dependence of the intrinsic fluorescence intensity of membrane proteins (*F*) at a wavelength of 332 nm at an excitation wavelength of 281 nm on the specific concentration of hormones in a suspension of erythrocyte membranes. The specific concentration of hormone in erythrocyte membrane suspension is calculated by the formula:

$$c = C_h/C_{pep}, \quad (1)$$

where C_h is the molar concentration of hormone in the suspension, C_{rr} is the mass concentration of membrane proteins in the suspension [15].

The mass concentration of membrane proteins (C_{rr}) was determined by the method

of Warburg and Christian according to the change in the optical density of the suspension [16]. In different samples it varied from 0.050 mg/mL to 0.250 mg/mL. In the presented work, gross errors are absent in the experimental results, systematic errors are excluded, and the non-excluded systematic error (NSE) of the estimation of any measured quantity is negligible compared to the confidence limits of the random error of the estimation of the measured quantity. Three measurements of protein mass concentration ($C_{pep)}$ in the same suspension were made. The Student's distribution was used to calculate the estimate and confidence limits of the estimation error of C_{pep}. The confidence limits of the C_{pep} estimation error in relative form are ± 2% at a confidence level of 0.95.

Hormones produced by Sigma-Aldrich, USA, were used. Hormones, except for adrenaline, were pre-dissolved in dimethyl sulfoxide-ethanol mixture (1/1 by volume). The concentration of each hormone in stock mother liquor was 1 mM. If necessary, this solution was diluted with phosphate hypotonic buffer to the desired concentration. An adrenaline solution with a concentration of 1 μM was first prepared in hypotonic phosphate buffer. Then, this stock solution was added to the sample to obtain the desired concentration of hormone (c) in the sample. The confidence limits of the error in estimating the molar concentration of hormones ($C_{h)}$ in relative form are ± 2% at a confidence level of 0.95. In the presented work, the formula for calculating the confidence limits of the estimation error of the measured quantity A in indirect measurements (2) according to the recommendations of the State System for Ensuring Uniformity of Measurements [17] is used:

$$E_f = \frac{1}{\tilde{A}}\left(\sum_{i=1}^{m}\left(\frac{\partial f}{\partial a_i}\right)^2 \cdot \Delta^2\left(\tilde{a}_i\right)\right)^{0,5} \cdot 100\%, \quad (2)$$

where $\tilde{A} = f\left(\tilde{a}_1,...,\tilde{a}_m\right)$ is the measurement result $\tilde{A}$ at the point ; $\left(\tilde{a}_1,...,\tilde{a}_m\right)$ $f\left(a_1,...,a_m\right)$ is the nonlinear functional dependence of the measured quantity A on the measured arguments ; a_i $\frac{\partial f}{\partial a_i}$ is the first derivative of the function f on the argument a_i , computed at the point ; $\left(\tilde{a}_1,...,\tilde{a}_m\right)$ $\Delta\left(\tilde{a}_i\right)$ is the confidence limit of the error of the argument estimation . a_i

Using formulas (1) and (2), it is possible to calculate the confidence limits of the estimation error of the specific hormone concentration (c) measurement in relative form, which are equal to± 3%.

If membrane proteins interact with hormones, their conformation changes. The microenvironment near membrane protein radicals changes, and the excitation and emission spectra of membrane protein intrinsic fluorescence change. These spectra were measured on a SHIMADZU RF-5301(PC)SCE spectrofluorimeter ("SHIMADZU", Japan). 3 ml of

hypotonic phosphate buffer containing 2.75 mM $KH_{(2)}RO_{(4)}$and 8.5 mM $Na_{(2)}NPO_{(4)}$(pH 7.4), erythrocyte membranes and the required amount of hormones were introduced into a quartz cuvette of 1× 1× 4 cm. The sample cuvette was placed in the thermostat of the spectrofluorimeter for 10 min. The temperature in the cuvette was monitored by an electronic thermometer. In all experiments the temperature in the cuvette was (36± 0.1)° C. After the temperature in the cuvette reached steady-state, the emission spectrum was taken in the range of 300 nm≤ λ≤ 400 nm at an excitation wavelength of 281 nm. The maximum of the emission intensity occurred at a wavelength of 332 nm. The mean value of the intrinsic fluorescence intensity maximum of the membranes was obtained graphically after continuous measurement for 4 min; the spectral slit width was 1.5/10. Then, the fluorescence intensity of lipids with hormones at 332 nm (at the excitation wavelength of 281 nm) was subtracted from the maximum fluorescence intensity of membranes with hormones and the intrinsic fluorescence intensity of membrane proteins *F* incubated with hormones was obtained. The intrinsic fluorescence intensities of *F* at each value of hormone concentration (*c*) in the suspension were measured three times. Using Student's distribution, we calculated the estimate of *F* at each value of hormone concentration (*c*) in suspension and the confidence limits of the error in *F at* a confidence level of 0.95. The confidence limits of the *F* estimation error in relative form were found to be equal to± 3%.

We plotted the dependence of the extinction value of intrinsic fluorescence of membrane proteins, determined by the formula (3), on the specific concentration of hormones (*c*):

$$\Delta F = \frac{F_{\max} - F}{F_{\max}}, \quad (3)$$

where $F_{\max}$ is the intensity of intrinsic fluorescence of membrane proteins when no hormones are present in the suspension; ΔF is the value of the quenching of membrane protein fluorescence , expressed as a fraction of $F_{\max}$. The higher the ΔF, the greater the quenching of membrane protein fluorescence.

When adrenaline interacted with membranes, we measured the excitation intensity of intrinsic membrane fluorescence at λ= 228 nm and emission wavelength 332 nm. Next, the excitation intensity of lipid fluorescence with adrenaline at λ = 228 nm (emission wavelength - 332 nm) was subtracted from this membrane fluorescence excitation intensity and the fluorescence excitation intensity of membrane proteins (D_f) incubated with adrenaline was obtained. The intrinsic fluorescence excitation intensities of membrane

proteins (D_f) at each value of hormone concentration (c) in the suspension were measured three times. Using Student's distribution, we calculated the estimate of D_f at each value of hormone concentration (c) in suspension and confidence limits of D_f error with confidence probability 0.95. The confidence limits of the error of D_f estimation in relative form were equal to± 3%.

The formula (3) was used to find the value of quenching of fluorescence excitation of membrane proteins ($\Delta D_{(f)}$) by replacing F_{max} by $D_{(fmax)}$ and F by $D_{(f)}$, where $D_{(fmax)}$ is the intensity of fluorescence excitation of membrane proteins when there are no hormones in the suspension. We plotted the dependence of the magnitude of quenching of fluorescence excitation of membrane proteins on the specific concentration of hormones (c). Using the Student's distribution, using formulas (2) and (3), we calculated estimates and confidence limits of the estimation error (ΔF) and (ΔD_f), which are equal to± 4% at a confidence level of 0.95.

According to three independent measurements, using graphs of $\Delta F(c)$ and (ΔD_f) dependence, we graphically determined the estimation of maximum quenching of membrane proteins intrinsic fluorescence ΔF_{max}, determined *with* $_F$ - specific concentration of hormones, at which the maximum fluorescence quenching occurs; estimation of the maximum quenching of excitation of intrinsic fluorescence of membrane proteins ΔD_{fmax}, we determined c_D - specific concentration of hormones, at which the maximum quenching of excitation of fluorescence of membrane proteins occurs. Using Student's distribution, we obtained the confidence limits of the measurement error of these values at a confidence level of 0.95. The confidence limits of measurement error of ΔD_{fmax} and ΔF_{max} in relative form are equal to± 5%. Confidence limits of estimation error c_F and $\underline{c_D}$ in relative form are equal to± 6%. Random errors occurred because c_F and c_D were determined from the intersection of two straight lines in the plots of $\Delta F(c)$ and $\Delta D(c)$ approximating the experimental results. Since the experimental results are determined with some error, the slope angle of these straight lines is also determined with some error, and hence the values of c_F and c_D are also determined with some error. The differences between the samples were evaluated using the nonparametric Mann-Whitney U-criterion. Differences were considered statistically significant at $p < 0.05$.

Methodology for measuring the relative microviscosity of erythrocyte membranes by fluorescence method using a pyrene probe. Erythrocyte membrane microviscosity was measured according to the method described in Dembo et al. [18], on a SHIMADZU RF-5301(PC)SCE spectrofluorimeter. The experimental sample as a suspension was prepared as follows. 3 ml of hypotonic phosphate buffer (pH 7.4), containing

2.75 mM $KH_2RO_{(4)}$ and 8.5 mM $Na_{(2)}NPO_{(4)}$, erythrocyte membrane suspension, the required amount of hormones, and fluorescent probe pyrene were added to a quartz cuvette of 1× 1× 4 cm. Pyrene was pre-diluted in ethanol: its initial concentration was 1.5 mM and the concentration in the cuvette was 7.76 μM. All components of the suspension were stored at 4° C until use.

The cuvette with the sample was placed in the thermostat of the spectrofluorimeter for 10 min, after that its emission spectrum of intrinsic fluorescence was measured at a temperature of 36° C. Before putting the cuvette with the sample into the thermostat of the spectrofluorimeter, it was shaken vigorously for 1 min. To measure the fluorescence of membranes incubated with a different amount of hormones, a new sample was prepared each time. This is due to the fact that pyrene promotes rapid degradation of erythrocyte membranes.

Strictly speaking, microviscosity is defined through the diffusion coefficient in this case of the fluorescent probe pyrene in a biological membrane. The rate of progressive diffusion of the probe is determined by the microstructure of its immediate environment, which is why the term "microviscosity" is used. Practically, it is understood as some equivalent: it corresponds to the viscosity of a homogeneous medium in which the probe would move at the same speed as it moves in a real membrane [15]. In other words, microviscosity indicates the resistance encountered by the probe as it moves inside the membrane. Hence, it is clear that depending on the method of mobility measurement, the nature of the probe, and the depth of its localization in the membrane, the values of microviscosity may vary. Nevertheless, this parameter is crucial for the identification of structural changes in the biomembrane [15].

Before measuring the fluorescence emission spectrum of pyrene in membranes with hormones added to the membrane suspension, the fluorescence emission spectrum of membranes with hormones added to the membrane suspension but without pyrene added was obtained (excitation wavelength - 281 nm); spectral slit width - 1.5/5. As a result, the values of $I_{393}(c)$, $I_{(468)}(c)$, the fluorescence intensities of membranes incubated with hormones with specific concentration of c in suspension, were obtained at the emission wavelengths of 393 and 468 nm, respectively. $I_{(393)}(0)$, $I_{(468)}(0)$ are the fluorescence intensities of membranes when no hormones and pyrene are present in the membrane suspension, at 393 and 468 nm emission wavelength, respectively. To eliminate systematic error, the fluorescence intensities I_{393}, I_{468} were subtracted from the fluorescence intensities of samples $F_{(393)}$, F_{468} to improve the accuracy of measurement of relative membrane microviscosity (L). At an excitation wavelength of 337 nm and a spectral slit width of 1.5/3,

the fluorescence intensities of membranes and hormones at emission wavelengths of 393 and 468 nm were close to zero and could be neglected in calculations of membrane microviscosity.

Further, samples with pyrene were prepared and emission spectra of membranes with hormones and pyrene added were measured. Based on the measurement results, we plotted the dependences of the relative microviscosity (L) of the lipid bilayer according to the formula given in Dobretsov's monograph [15] on the specific concentration c of hormones in the suspension:

$$L = \eta(c)/\eta(0), \quad (4)$$

where $\eta(c)$, $\eta(0)$ are the membrane microviscosities when hormone with specific concentration c and without hormone added to the suspension, respectively.

For the lipid-lipid interaction region, the relative microviscosity of the lipid bilayer L was calculated using the formula given in Dobretsov's monograph [15]:

$$L = \eta(c)/\eta(0) = (F_{(393)}(c) \cdot F_{468}(0))/(F_{(468)}(c) \cdot F_{(393)}(0)), \quad (5)$$

where $F_{468}(c)$, $F_{468}(0)$ - fluorescence intensities of the sample at λ= 468 nm at specific concentration of hormone (c) in suspension and in the absence of hormone in suspension, respectively; $F_{393}(c)$, $F(393)(0)$ - fluorescence intensities of the sample at λ= 393 nm at specific concentration of hormone (c) in suspension and in the absence of hormone in suspension, respectively. Excitation wavelength is 337 nm; spectral slit width is 1.5/3.

The relative microviscosity L for the protein-lipid interaction region was calculated according to the formula:

$$L = \eta(c)/\eta(0) = (F_{(393)}(c) - I_{393}(c)) \cdot (F_{(468)}(0) - I_{(468)}(0))/[(F_{(468)}(c) - \cdot I_{(468)}(c)) \cdot (F_{(393)}(0) - I_{(393)}(0))], \quad (6),$$

where I_{393}, I_{468} are fluorescence intensities of membranes with hormone without pyrene at λ= 393 nm and λ= 468 nm, respectively. Excitation wavelength was 281 nm; spectral slit width was 1.5/5.

Measurements of lipid bilayer microviscosity at each value of hormone concentration in suspension were performed three times; using Student's distribution, we calculated the estimate and confidence limits of the error in estimating the relative lipid bilayer

microviscosity (L) at each value of hormone concentration in suspension with a confidence level of 0.95. Using formulas (5), (6), the formula for calculating the error in indirect measurements (2), we obtained the confidence limits of the error in estimating the relative microviscosity L of membranes in relative form, which are equal to± 6%, with a confidence level of 0.95. The confidence limits of the error of estimation error of c_{mah} in relative form are equal to± 10%. They were composed of errors in determining the concentrations of hormones in suspension and random errors in determining c_{mah}, similar to the error in determining c_F. The confidence limits of the estimation error L_{mah} are equal to± 6% The differences between samples were evaluated using the nonparametric Mann-Whitney U-criterion. Differences were considered statistically significant at $p< 0.05$.

Methodology for measuring membrane absorption of electromagnetic radiation by IR spectroscopy. To take IR spectra, a suspension of rat erythrocyte membranes was first obtained according to the method described above. Next, 60 µl of this membrane suspension was added to a cuvette with a fluorite substrate in 1 mM K,Na-phosphate buffer (pH 7.35). Another 30 µl of the same buffer and 1 µl of hormone mother liquor were then added to this volume. The concentration of hormone in the mother liquor was 1 mM. The resulting suspension was stirred and incubated for 10 min at 16° C. The suspension was dried to reduce the absorption of IR radiation by OH-groups of water. The cuvette was placed strictly horizontally on a special table of the vacuum unit. The film of erythrocyte membranes for taking their IR spectra was prepared in the cuvette by slow evaporation of water from the membrane suspension for 180 min in a weak vacuum at a pressure of about 0.1 atmosphere (~0.5 10^4 Pa) and a temperature of 4° C. Not all water molecules are removed from the film. Water molecules bound to membrane lipids and proteins remain in it, so that the film is not completely dry. As a result, the membranes in the film retain the structures they had in suspension.

After preparation of the film, the cuvette was placed in the optical chamber of the IR spectrometer, the chamber with the cuvette was purged with dry air at room temperature for 30 min, and then the scanning of the instrument was switched on. IR spectra of absorption (A) shadows of rat erythrocytes were taken on a Specord-M80 double-beam spectrometer ("Carl Zeiss", Germany). In all experiments, along with obtaining spectra of the experimental sample (membrane and hormone film), spectroscopy of the control sample (membrane film without hormones) was performed relative to the fluorite substrate. Three independent measurements of the IR spectrum of the same sample were performed.

RESEARCH RESULTS

Examination of the membrane preparation. Before making measurements, it is necessary to demonstrate what exactly is the preparation of membranes isolated according to this technique. It is necessary to find out what it is: vesicles or liposomes, where proteins embedded in membranes remained after isolation in approximately the same state as in the composition of the plasmalemma of erythrocytes? Or are they scraps of membranes? Or is it a mixture of lipid droplets and proteins in solution? For this purpose, a suspension of erythrocyte membranes obtained according to the method described above was diluted 10 times, and a drop of the obtained suspension was applied to the slide of a light microscope "Biomed-6" for observation in transmitted light by the phase contrast method. After adding immersion oil to the center of the preparation at a magnification of 1000× , ovals of regular shape with a size of 6-7 μm were visible on the slide, which corresponds to the size of rat erythrocytes. Thus, the obtained preparation is a suspension of rat erythrocytes from which hemoglobin has been removed. The majority of erythrocytes retained both internal cytoskeletons and plasma membranes attached to them.

Study of the dependence of intrinsic fluorescence intensity of membrane proteins on the specific concentration of hormones in suspension. Previously, group led by Yu.A. Vladimirov established that plasma membrane proteins have intrinsic fluorescence [15, 19]. However, in addition to proteins, plasma membranes also contain lipids. Therefore, we first measured the intrinsic fluorescence intensity of membrane lipids and hormones. A suspension of lipids isolated from membranes by the method described above was injected from a syringe into phosphate hypotonic buffer, in which they formed liposomes. The concentration of lipids corresponded to their concentration when the emission spectrum of the intrinsic fluorescence emission spectrum of membrane proteins was measured. To this suspension of liposomes, the hormones dehydroepiandrosterone, cortisol, androsterone, and testosterone were added separately in the same concentrations as when measuring the emission spectrum of intrinsic fluorescence of membranes incubated with these hormones. We measured the intrinsic fluorescence emission spectra of the lipid suspension with hormones at the same parameters as the measurements of the intrinsic fluorescence emission spectra of the membranes. The values of intrinsic fluorescence of lipid suspensions with hormones in the range 300 nm $\leq \lambda \leq$ 400 nm were constant for each hormone, did not depend on its concentration, were insignificant and did not exceed 4% of the value of membrane intrinsic fluorescence. In further calculations of the intrinsic fluorescence of membrane proteins, these constant values were subtracted from the intrinsic fluorescence of

membranes.

Fig. 1, *a* shows the dependence of the magnitude of quenching of intrinsic fluorescence of erythrocyte membrane proteins (*ΔF*) at a wavelength of 332 nm (excitation wavelength - 281 nm) on the specific concentration (*c*) of the hormone cortisol added to the cuvette. The maximum extinction of intrinsic fluorescence$\Delta_{F(max)}$= (0.110± 0.006) was observed at a hormone concentration of c_F= (4.60 ± 0.28)$10^{(-10)}$) mol/mg protein in the incubation medium (Fig. 1, *a*).

Fig. 1. Dependence of the magnitude of extinction of intrinsic fluorescence of erythrocyte membrane proteinsΔ F on the specific concentration c in the suspension of cortisol (*a*) and dehydroepiandrosterone (*b*). DEA - dehydroepiandrosterone

Fig. 1, *b* shows the dependence of erythrocyte membrane protein intrinsic fluorescence quenching (*ΔF*) at a wavelength of 332 nm (excitation wavelength - 281 nm) on the specific DEA concentration. The maximum extinction of intrinsic fluorescence ΔF_{max}= (0.103± 0.005) was observed at the concentration of DEA in the incubation medium *with*$_F$= (1.80 ± 0.11)$10^{(-8}$) mol/mg protein (Fig. 1, *b*). Fig. 1 shows that when cortisol and DEA were added to the erythrocyte membrane suspension, the quenching of intrinsic fluorescence of membrane proteins *ΔF* increased almost linearly, reached its maximum at the concentration of hormone c_F, and *ΔF* changed weakly with further increase in the concentration of hormone in the suspension Fig. 1, *a* and *b*).

Similar results were obtained when membrane proteins were incubated with testosterone and androsterone (Table 1). When membranes were incubated with adrenaline, the dependence of the magnitude of quenching of fluorescence excitation $\Delta D_{(f)\ of}$ membrane proteins at λ = 228 nm (emission wavelength - 332 nm) on the concentration of adrenaline was measured. When adrenaline was added to the erythrocyte membrane suspension, ΔD_f increased almost linearly up to the concentration of adrenaline *with*$_D$= (2.00 ± 0.12) $10^{(-10}$) mol/mg protein. The value of maximum quenching of fluorescence excitation intensity ΔD_{fmax}= (0.150± 0.008). With further increase of adrenaline concentration, the value of ΔD_f changed weakly. The results of measurements are presented in Table 1.

Table 1. Results of erythrocyte membrane fluorescence measurements during incubation with different hormones

Hormone	ΔF_{max}	c_F, mol/mg protein

DEA	0.103± 0.005	(1.80± 0.11) 10^{-8}
Testosterone	0.180± 0.009	(1.10± 0.10) 10^{-9}
Cortisol	0.110± 0.006	(4.60± 0.28) 10^{-10}
Adrenaline	0.150± 0.008*	(2.00± 0.12) 10^{-10}
Androsterone	0.060± 0.003	(1,000± 0.060) 10^{-10}

Note. Abbreviations used: ΔF_{max} - values of the maximum of extinction of intrinsic fluorescence of membrane proteins; c_F - specific concentrations of hormones at which extinction maxima occur; * - maximum extinction of fluorescence excitation intensity ($\Delta D_{(f)(max)}$) of membrane proteins was measured.

The values of $\Delta F_{(max)\ of}$ membrane proteins are statistically significantly different from ΔF *of* these proteins when there are no hormones in the membrane suspension. The magnitude of the maximum decrease in the relative intrinsic fluorescence intensity of membrane proteins ($\Delta F_{(max))}$ and the hormone concentration (c_F) at which the maximum quenching occurs are different for different hormones (Table 1). When membrane proteins interact with hormones, there is a change in their conformation. The microenvironment near the radicals of membrane proteins changes, and the emission and excitation intensities of fluorescence decrease. At the concentration of hormones in suspension higher than c_F or c_D, when emission or excitation of fluorescence almost ceases to change, the conformation of membrane proteins and microenvironment around them also ceases to change (Fig. 1; Table 1).

Results of membrane microviscosity measurements. One of the characteristics of membranes, which changes when its structure changes, is the microviscosity of its lipid bilayer [15]. Fig. 2, *a* and *b* show the dependences of relative microviscosity (*L*) of erythrocyte membranes on the specific concentration of cortisol and DEA, respectively. When membranes interacted with cortisol, a statistically significant increase in the microviscosity of the lipid bilayer in the protein-lipid interaction region reached 1.250± 0.075 relative to control samples when no hormone was added to the membrane suspension (Fig. 2, *a*; curve *2*). It increased until the hormone concentration c_{max}= (4.60 ± 0.46)-$10^{(-10)}$mol/mg (Fig. 2, *a*; curve *2*). The microviscosity curve then reached a plateau. For the region of lipid-lipid interactions, the microviscosity curve behaved similarly, but the increase was only up to 1.050± 0.063; the microviscosity values in this region were not statistically significantly different from the same values in the control samples (Fig. 2, *a*; curve *1*). The increase in microviscosity in the lipid-lipid interaction region began at a higher concentration

of cortisol (2.50± 0.25)-$10^{(-10)}$mol/mg protein and reached a lower value than in the protein-lipid interaction region (Fig. 2, *a*; curves *1* and *2*).

Fig. 2. Dependence of relative membrane microviscosity (*L*) on specific concentration of cortisol (*a*) and dehydroepiandrosterone (*b*). Curve *1* - change in relative microviscosity in the region of lipid-lipid interaction; curve *2* - change in relative microviscosity in the region of protein-lipid interaction. DEA - dehydroepiandrosterone

The measurements of lipid bilayer microviscosity in the protein-lipid interaction region shown in Fig. 2 are another confirmation that in the presented experiments pyrene was introduced into membranes that contained membrane proteins. At an excitation wavelength of 281 nm, no pyrene fluorescence was observed in lipid bilayers that did not contain proteins [15]. In membranes, proteins at an excitation wavelength of 281 nm fluoresce at a wavelength of 332 nm, which is close to the wavelength of the excitation maximum of pyrene fluorescence (336 nm). In the presented experiments, pyrene molecules fluoresced at an excitation wavelength of 281 nm, indicating the presence of membrane proteins in the isolated membranes (Fig. 2).

When comparing Fig. 1, *a* and Fig. 2, *a, it* can be seen that the range of cortisol concentration in the suspension, at which a change in the conformation of membrane proteins occurs, and the range of cortisol concentration, at which the microviscosity increases and reaches a constant value, coincide. This means that membrane proteins are the initiators of the structural transition in membranes during their interaction with cortisol. At the same time, the microviscosity of the lipid bilayer in the region of lipid-lipid interaction increased by a smaller value (Fig. 2, *a*; curve *1*) than near the membrane proteins that changed their conformation as a result of interaction with cortisol (Fig. 2, *a*; curve *2*). This means that lipids became more strongly attracted to these proteins and their surface density (the number of lipids per unit area of the membrane) increased. As a consequence, the structure of the lipid bilayer near these proteins changes, lipids from the liquid- disordered state to the liquid-ordered state ($L_d \rightarrow L_o$). Protein-lipid domains are formed. This change in structure leads to greater increase in the microviscosity of the lipid bilayer in the protein-lipid domains. Between the domains, the lipid bilayer remains in a liquid disordered state (L_d) and its microviscosity and surface density are lower than in the domains.

The interaction of membranes with DEA is qualitatively different. Under the influence of this hormone, the microviscosity of the lipid bilayer of membranes increased to

only 1.060± 0.064 compared with control samples to which no hormone was added (Fig. 2, *b*). The concentration at which the maximum microviscosity was reached was c_{max}= (1.70 ± 0.17)-10^{-8} mol/mg protein (Fig. 2, *b*). Curves *1* and *2* in Fig. 2, *b are* very close, indicating that the increase in microviscosity of the lipid bilayer occurs by the same amount in the protein-lipid and lipid-lipid interaction regions. Consequently, unlike the interaction of cortisol with membranes, the interaction of DEA with membranes does not lead to the formation of protein-lipid domains with different surface densities of lipids in the domains and between the domains.

When membranes interact with androsterone, testosterone, and adrenaline, the membrane structure changes similarly to the interaction of membranes with cortisol. Table 2 shows the results of measuring the microviscosity of erythrocyte membranes during their incubation with different hormones.

Table 2. Results of measuring the microviscosity of erythrocyte membranes during their incubation with different hormones

Hormone	Lipid-lipid interaction area		Protein-lipid interaction domain	
	L_{mah}	c_{max}, mol/mg protein	L_{mah}	c_{max}, mol/mg protein
DEA	1.060± 0.064	(1.70± 0.17)-10^{-8}	1.060± 0.064	(1.70± 0.17)-10^{-8}
Testosterone	1.150± 0.069	(2.00± 0.20)-10^{-9}	1,200± 0.072*	(1,10± 0,11)-10^{-9}
Cortisol	1.050± 0.063	(4.60± 0.46)-10^{-10}	1.250± 0.075*	(4.60± 0.46)-10^{-10}
Adrenaline	1,200± 0.072*	(2.00± 0.20)-10^{-10}	1,400± 0.084*	(2.00± 0.20)-10^{-10}
Androsterone	1.250± 0.075*	(3.00± 0.30)-10^{-10}	1,500± 0.090*	(2.50± 0.25)-10^{-10}

Note. Abbreviations used: L_{mah} - maximal increase of lipid bilayer microviscosity upon hormone addition; c_{mah} - specific concentration of hormones in erythrocyte suspension, at which membrane microviscosity reaches the maximum value; DEA - dehydroepiandrosterone. The values of L_{mah} and c_{mah} are given for lipid-lipid and protein-lipid interaction regions. * $p < 0.05$ - statistically significant differences compared with the corresponding control when no hormone was added to the membrane suspension.

When membranes interacted with androsterone, their microviscosity in the protein-lipid interaction region increased to L_{mah}= 1,500± 0.090 relative to the initial state ($p< 0.05$; Table 2). It increased up to a concentration of c_{max}= (2.50 ± 0.25)-10^{-10} mol/mg protein, after which it did not change. In the lipid-lipid interaction region, microviscosity increased to L_{mah}= 1.250± 0.075 relative to baseline ($p< 0.05$). The increase occurred up to a

concentration of c_{max}= (3.00 ± 0.30)-$10^{(-10)}$mol/mg protein (Table 2). The change in the conformation of membrane proteins occurred up to the concentration of androsterone (c_F) in the suspension (1.000 ± 0.060)-$10^{(-10)}$ mol/mg protein (Table 1), which is less than the concentration of c_{mah} androsterone in the suspension at which the relative membrane microviscosity reached its maximum value (Table 2). This means that when androsterone interacts with membranes, protein-lipid domains are formed in them, and it is membrane proteins that initiate this structural transition.

When testosterone was added, the microviscosity of membranes in the region of protein-lipid interactions increased almost to L_{mah}= (1.200± 0.072) (Table 2). The increase in microviscosity occurred up to a concentration of testosterone in suspension with membranes equal to c_{mah}= (1.10 ± 0.11)-10^{-9} mol/mg protein. In the lipid-lipid interaction region, the microviscosity increased up to a concentration of c_{mah}= (2.00 ± 0.20)-$10^{(-9)}$ mol/mg protein and increased to $L_{(mah)}$= (1.150± 0.069) relative to the initial state (Table 2). Hence, as in the case of cortisol and androsterone, protein-lipid domains are formed when testosterone interacts with membranes. Comparing the data of Tables 1 and 2, it can be seen that the range of testosterone concentrations at which changes in the conformation of membrane proteins (c_f) and the increase in the microviscosity of the lipid bilayer in the protein-lipid interaction region c_{mah} coincide. Thus, membrane proteins are initiators of the formation of protein-lipid domains in the membrane during the interaction of membranes with testosterone.

When membranes interacted with adrenaline in the region of protein-lipid interactions, there was an increase in relative membrane microviscosity (L) up to L_{mah}= 1.400± 0.084 (p< 0.05; Table 2). It increased until adrenaline concentration c_{mah}= (2.00 ± 0.20)-$10^{(-10)}$mol/mg protein, then the microviscosity curve reached a plateau (Table 2). In the lipid-lipid interaction region, the microviscosity also increased up to a concentration of c_{mah}= (2.00 ± 0.20)-$10^{(-10)}$mol/mg protein, but increased only to $L_{(mah)}$= 1.200± 0.072 relative to the initial state (p< 0.05; Table 2), indicating the formation of protein-lipid domains in the membrane. Comparing the data of Tables 1 and 2, it can be seen that the range of adrenaline concentrations at which changes in the conformation of membrane proteins occurred (c_D) and the increase in the microviscosity of the lipid bilayer in the protein-lipid interaction region (c_{mah}) coincide. Thus, membrane proteins are initiators of the formation of protein-lipid domains in the membrane during the interaction of membranes with adrenaline.

Based on the results obtained, it can be concluded that the interaction of cortisol, androsterone, testosterone, and adrenaline with membranes changes the structure of plasma

membranes, and a network of protein-lipid domains is formed in them. In contrast to the interaction with these hormones, the interaction of DEA with membranes does not lead to the formation of protein-lipid domains, and there is a uniform increase in the surface density of the lipid bilayer across the membrane. The reason for the different interaction of DEA and other hormones (cortisol, androsterone, testosterone, adrenaline) with membranes can be established by studying the changes in the secondary structure of membrane proteins during their interaction with hormones by IR spectroscopy. This is done in the following subsection.

Results of measuring the absorption of infrared radiation by membranes. The IR spectroscopy diagnosis is based on the Bouguer-Lambert-Bera law [20]. The absorbance of the sample is measured:

$$A = \ell g(I_0/I), \quad (7)$$

where I_0 is the intensity of electromagnetic radiation hitting the sample, I is the intensity of radiation missed by the sample.

By measuring the absorption of A, we can estimate the concentration of absorbing centers (c_0), which is related to A by a relation:

$$A = \varepsilon c_{(0)} \ell_{(0)}, \quad (8)$$

where ε is the molar absorption coefficient; $\ell_{(0)}$ is the thickness of the sample.

When hormones are added to membranes, interactions between functional groups of membrane protein and lipid molecules change. New hydrogen bonds are formed between them. All this leads to shifts in the frequencies at which the maxima of electromagnetic radiation absorption by functional groups of membrane proteins and lipids are observed. The intensity of these absorption bands changes according to formula (8). The separation of absorption bands corresponding to different functional groups of molecules was carried out according to the methods described in Yang et al. [20], Barth and Zscherp [21] and Nosenko et al. [22].

With three independent measurements of the IR spectrum of the same sample, the second derivative of the IR spectrum in the amide I region was calculated for each measurement using the OriginPro 2021 program. This second derivative was smoothed using a fast Fourier transform procedure. The second derivatives of the IR spectra were multiplied by (-1) for ease of decomposition. The smoothed second derivative of the IR spectrum was

then decomposed into individual bands. Each band is a Gaussian function with its standard deviation and mathematical expectation, which were selected so that the sum of these bands was equal to the second derivative of the IR spectrum. The areas of the individual bands in the decomposition of the second derivative of the absorption spectrum were used to determine the fractions of secondary structures of membrane proteins. Using Student's distribution, the estimation and confidence limits of of the error of estimation of the fractions of secondary structures of membrane proteins at the confidence level of 0.95 were determined. The differences between samples were evaluated using the nonparametric Mann-Whitney U-criterion. Differences were considered statistically significant at $p< 0.05$. The technique of spectra processing is described in Yang et al. [20], Nosenko et al. [22].

The absorption spectrum of control samples (membranes not incubated with hormones) is shown in Fig. 3, a. Fig. 3, *b* shows the decomposition of the second derivative of the absorption spectrum of control samples in the amide I frequency region. Fig. 4, *a* shows the absorption spectrum of membranes incubated with cortisol, its concentration in the film *c*= (2.100± 0.063)-$10^{(-)(10)}$ mol/mg protein. Fig. 4, *b* shows the decomposition of the second derivative of the absorption spectrum of membrane samples incubated with cortisol (*c* = (2,100± 0.063)-$10^{(-10)}$) mol/mg protein) in the amide I frequency region. In Fig. 5, *a* shows the absorption spectrum of membranes incubated with DEA, its concentration in the film *with*= (5.30± 0.16)-$10^{-)(11}$ mol/mg protein. In Fig. 5, *b* shows the decomposition of the second derivative of the absorption spectrum of membrane samples incubated with DEA (*from* = (5.30± 0.16)-$10^{(-11)}$) mol/mg protein), in the amide I frequency region. In these figures, numbers indicate the numbers of bands into which the second derivatives of the membrane absorption spectra are decomposed. The names and percentages of the secondary structures of membrane proteins corresponding to these bands are indicated.

Fig. 3. IR absorption spectrum of control (hormone-free) rat erythrocyte membrane samples in the range ν = 900-1800 cm^{-1} (*a*); decomposition of the second derivative of the IR absorption spectrum of control rat erythrocyte membrane samples in the amide I frequency region (*b*): *1* - β-sheet (30.3%); *2* - 3_{10} α-structure (18.2%); *3* - α-structure (37.1%); *4* disordered structure (14.4%)

Fig. 4. IR absorption spectrum of rat erythrocyte membrane samples incubated with cortisol (*c* = (2,100± 0.063)-10(**-10**) mol/mg protein) in the range ν = 900-1800 cm^{-1} (*a*); decomposition of the second derivative of the IR absorption spectrum of rat erythrocyte membrane samples incubated with cortisol (*c* = (2,100± 0.063)-10^{-10} mol/mg protein) in the

amide I frequency region (*b*): *1* - β-sheet (5.6%); *2* - β-turn (11%); *3* - 3_{10} α-structure (31.6%); *4* - α-structure (24.2%); 5 intermolecular β-sheets (27%)

Fig. 5. IR absorption spectrum of rat erythrocyte membrane samples incubated with DEA (with = (5.30± 0.16)-$10^{(-11)}$) mol/mg protein) in the range ν = 900-1800 cm^{-1} (*a*); decomposition of the second derivative of the IR absorption spectrum of rat erythrocyte membrane samples incubated with DEA (*with* = (5.30± 0.16)-10^{-11} mol/mg protein) in the amide I frequency region (*b*): *1* - *β* -sheet (15.6%); *2* $3_{(10)\,\alpha}$ structure (7.6%); *3* - *α* -structure (37.1%); *4* disordered structure (35.5%); 5 intermolecular β-sheets (4.2%). DEA - dehydroepiandrosterone

Table 3 shows characteristic frequencies of absorption spectra of rat erythrocyte membranes after their interaction with DEA, androsterone, testosterone, cortisol and adrenaline.

Table 3: Characteristic frequencies of absorption spectra of rat erythrocyte membranes after their interaction with steroid hormones

Hormone and its concentration *c*, mol/mg protein	Frequencies of valence vibrations of peptide C=O-group ν_{CO}, cm^{-1}	Frequencies of deformation vibrations of peptide NH-bonds ν_{NH}, cm^{-1}	Valence vibration frequencies of phospholipid C=O bonds $\nu_{C=O}$, cm^{-1}	Frequencies of valence asymmetric vibrations of phospholipid RO_2-bonds ν_{RO2}, cm^{-1}	Valence symmetric vibration frequencies of phospholipid RO_2-bonds ν_{RO2}, cm^{-1}	Valence vibration frequencies of phospholipid ROS bonds $\nu_{P-(O)(-)(C)}$, $cm^{(\ )(1)}$	Frequencies of valence vibrations of CH-bonds ν_{CH}, cm^{-1}
Control	1686 1655,4	1546,9	1748	1236	1080	1056	2948 2930,8 2848
DEA ($5{,}3\text{-}10^{-11}$)	1654,9	1546,9	1746	1247,6	1088	1070,7	2956,3 2925,8 2851,8
Androsterone ($5.5\text{-}10^{-11}$)	1656 1635 1620	1545		1260 1240	1098 1088		2958 2928 2848
Testosterone ($5.5\text{-}10^{-11}$)	1684 1657 1632	1545	1739,4	1247 1236	1100 1088	1065 1056	2956,4 2924 2850
Cortisol ($2{,}1\text{-}10^{-10}$)	1656,0 1620	1550 1540,2	1734	1239	1083,7	1044,0	2962 2925 2852
Adrenaline ($2\text{-}10^{-10}$)	1686 1656 1646	1544 1525	1740	1256 1248 1236	1096 1080	1070 1060 1044	2956 2924 2852

Fig. 3, *a* shows the absorption spectrum of control membrane samples (membranes not interacting with hormones). Analysis of the amide I band (Fig. 3, *b*) showed that, along with disordered structure, the proportion of which in membrane proteins is (14.4± 1.0)%, α-helixes are present in rat erythrocyte membrane proteins, the proportion of which in membrane proteins is (55.3± 2.6)%. There are also β-structures, the proportion of which in membrane proteins is (30.3± 1.4)%. There is a maximum at 1546.9 cm^{-1} in the amide II band produced by strain vibrations of the NH bond inherent in the *β-structure* Fig. 3, *a*; Table 3[)]. Membrane phospholipids also have a number of characteristic absorption bands, in particular for the valence vibrations of the C=O-bond at 1748 cm^{-1}, the asymmetric vibrations of the RO_2-bond at 1236 $cm^{(-)(1)}$), and the valence vibrations of the P-O-C-bond at 1056 $cm^{(-)(1)}$ (Fig. 3, *a*; Table 3). It should be noted that the absorption maximum band of the C=O-bond at 1748 cm^{-1} is rather narrow, which suggests that phospholipids are sufficiently ordered at the level of ester bonds of higher carboxylic acids and glycerol (Fig. 3, *a*).

Fig. 4, *a* shows the IR absorption spectrum of rat erythrocyte membranes incubated with cortisol. The specific concentration of the hormone in the sample is (2.100± 0.063)-10^{-10} mol/mg protein. Cortisol is known to be a hydrophobic compound, it is poorly soluble in water. However, its structure contains 3 OH-groups (in positions 11, 17 and 21) and 2 keto-groups in positions 3 and 20 [4], and it is these groups that take part in the formation of hydrogen bonds with CO- and NH-groups of membrane proteins and phospholipids. This is evident from the shift of the absorption maximum of the valence vibrations of the peptide CO-bond 1655.4→ 1656 cm^{-1}, which indicates structural transitions in membrane proteins. The appearance of the CO-bond valence vibrational absorption band 1620 cm^{-1} corresponds to the appearance of intermolecular β-sheets [20] (Fig. 4, *a* and *b*). The splitting of the NH-bond strain vibration band 1546.9→ 1550 and 1540.2 cm^{-1} also suggests the appearance of new hydrogen bonds between CO and NH peptide groups [21] and the appearance of new *β-structures* in membrane proteins (Fig. 4, *a* and *b*; Table 3). This conclusion is confirmed by the calculation of the fractions of secondary structures of membrane proteins in the interaction of membranes with hormones (Table 4).

Table 4. Effect of hormones on the content of elements of the secondary structure of membrane proteins

Hormone and its concentration (*c*), mol/mg protein	R - disordered structures, %	*α-helix*, %	*β-structures*, %

Control	14.4± 1.0	55.3± 2.6	30.3± 1.4
DAA ((5.30± 0.16)-10^{-11})	35.5± 1.6*	44.7± 2.1*	19.8± 1.0*
Cortisol ((2,100± 0.063)-$10^{-(10)}$)	< 0,1*	55.9± 2.6	44.1± 2.1*
Testosterone ((5.50± 0.17)-10^{-11})	9.8± 1.0*	55.9± 2.7	34.3± 1.6*
Adrenaline ((2,000± 0.060)-10^{-10})	< 0,1*	49.8± 2.2	50.2± 2.6*

Note. * $p < 0.05$ - statistically significant differences compared to the corresponding control.

Table 4 shows that when membranes interact with cortisol at its specific concentration in suspension (2.100± 0.063**)**-$10^{-(}$**10**$^{)}$mol/mg protein, the fraction of β-structures increases (from (30.3± 1.4) to (44.1± 2.1)%), compared to control measurements, and the fraction of disordered structures decreases from (14.4± 1.0)% to almost zero. The fraction of α-helixes does not change. Thus, there is a transition of disordered structure→ *β-structure* in the membranes. Compared to the control samples, there was an increase in the proportion of intermolecular β-sheets from 0 to (27.4± 2.0)% (Fig. 3, *b* and 4, *b*). This indicates the aggregation of membrane proteins, which promotes the formation of protein-lipid domains.

When cortisol interacts with membranes, the ordering of the lipid bilayer also increases. This is evidenced by the shift of the absorption band of the C=O-bond of the complex ester group of phospholipids 1748→ 1734 cm^{-1} (Fig. 4, *a*; Table 3). The shift of the absorption band of symmetric valence vibrations of the RO_2-bond of phospholipids 1080→ 1083.7 cm^{-1} and the shift of the band of asymmetric valence vibrations of the RO_2-bond 1236→ 1239 cm^{-1} Fig. 4, *a*) are associated with the process of dehydration of phospholipids as a result of an increase in their ordering [23]. At the same time, the relative microviscosity of the membrane in the protein-lipid interaction region increases, compared to the control, to 1.050± 0.063; in the lipid-lipid interaction region, the microviscosity does not change (Fig. 2, *a*). Thus, the formation of protein-lipid domains occurs in the membrane, in which the surface density of lipids is higher than between domains (Fig. 2, *a*) due to the formation of protein-protein cross-links and new protein-lipid bonds.

The hormones androsterone, testosterone, and adrenaline change the membrane structure in a similar way (Tables 3 and 4). Their interaction causes a shift of absorption bands in the amide I and amide II bands (Table 3), indicating a change in the secondary structure of membrane proteins. An increase in the intensity of the absorption band of CO-bond valence vibrations 1620 cm^{-1} is fixed, which corresponds to appearance of intermolecular β-sheets [22] (Table 3). Compared to the control, the proportion of *β-*

structures in membrane proteins increases as a result of the transition disordered structure→ β-structure (Table 4). The share of *α-structures* does not change statistically significantly (Table 4).

When androsterone, testosterone, and adrenaline interact with membranes, the ordering of the lipid bilayer increases due to increased protein-lipid and lipid-lipid interactions (Table 3). At the same time, the relative microviscosity of the membrane in the protein-lipid interaction region increases by a larger value than in the lipid-lipid interaction region (Table 2), which indicates the formation of protein-lipid domains in the membrane. Thus, the increase in the share of β-structures in the secondary structure of membrane proteins leads to the formation of protein-lipid domains in the membrane, in which the surface density of lipids is higher than between them.

In a different way, DEA interacts with membranes. Fig. 5, *a* shows the IR absorption spectrum of rat erythrocyte membranes incubated with DEA. The concentration of the hormone in the film is (5.30± 0.16)-10^{-11} mol/mg protein; it can be seen that there was no shift of the 1546.9 cm^{-1} band, which is intrinsic to the appearance of new *β-structures*proteins. Indeed, from Table 4 and Fig. 5, *b* shows that the proportion of *β-structures of* membrane proteins decreased (from (30.3± 1.4) to (19.8± 1.0)%) when membranes interacted with DEA. At the same time, the proportion of disordered structures increased (from (14.4± 1.0) to (35.5± 1.6)% compared to the control). The proportion of α-helixes decreased from (55.3± 2.6) to (44.7± 2.1)%. This means that during the interaction of DEA with membranes, unlike the interaction with cortisol, androsterone, testosterone, and adrenaline, there was no transition of disordered structure→ *β-structure* in membrane proteins, but instead, there were transitions β-structure→ disordered structure and α-helix→ disordered structure. The change in the conformation of membrane proteins in this case is confirmed by the decrease in the intensity of their intrinsic fluorescence (Fig. 1, *b*; Table 1).

The following results of the analysis of IR spectra of membranes indicate an increase in the ordering of the lipid bilayer during the interaction of DEA with membranes. A shift of the absorption band of asymmetric valence vibrations of the RO_2-bond 1236→ 1247.6 cm^{-1}, which reflects dehydration of phosphate groups of phospholipids, was observed (Fig. 5, *a*; Table 3). In addition, a shift in the C=O-bond frequency of phospholipids 1748→ 1732 cm^{-1} appeared, and the intensity of this band also increased (Fig. 5, *a*; Table 3). However, the increased ordering of the lipid bilayer upon its interaction with DEA led only to a slight increase in the microviscosity of the bilayer (Fig. 2, *b*; Table 2). Most importantly, the interaction of DEA with membranes did not lead to the formation of protein-lipid domains, and there was a uniform increase in the surface density of the lipid bilayer throughout the

membrane (Fig. 2, *b*).

Thus, when membranes interacted with androsterone, testosterone, cortisol, and adrenaline, new β-structures were formed in membrane proteins (Figs. 3, 4; Tables 3 and 4). With increasing concentrations of these hormones in the suspension, the increase in microviscosity in the protein-lipid interaction region was greater than the increase in microviscosity of the lipid bilayer in the lipid-lipid region (Fig. 2, *a*; Table 2). This means that the increase in the surface density of lipids near membrane proteins is greater than that in the lipid-lipid interaction region. The formation of protein-lipid domains occurred in the membrane, in which the lipid bilayer has a greater surface density than between the domains. When membranes interacted with DEA, the fractions of β-structures and α-helices decreased in membrane proteins (Fig. 5, *a*; Tables 3 and 4). At the same time, the increase in membrane microviscosity occurred uniformly throughout the membrane (Fig. 2, *b*; Table 2), and the formation of protein-lipid domains did not occur.

DISCUSSION OF RESULTS

It follows from the data described in the previous sections that, when hormones interact with membranes, the *β-structures of* membrane proteins formed by their interaction are responsible for the formation of protein-lipid domains in plasma membranes rather than *α-helices*. This is due to the fact that, first, the β-structures that appeared as a result of changes in the secondary structure of membrane proteins form intermolecular cross-links between membrane proteins (Fig. 3, *b* and 4, *b*). As a result of the increase in the number of hydrogen bonds between membrane proteins, their aggregation occurs [24-26]. Thus, clusters of proteins and surrounding lipids are created in the membrane. Secondly, in the β-structure of membrane proteins, aliphatic side chains of uncharged amino acids are located opposite to hydrocarbon chains of membrane lipids, aromatic groups of peptides prefer to interact with carbonyl groups of surrounding lipids, and polar groups of peptides interact with charged lipid head groups [27, 28]. This stimulates the attraction of lipids to proteins. As a result of these two mechanisms, protein-lipid domains are formed in which lipids near membrane proteins make the transition from a liquid disordered state to a liquid ordered state ($L_d \rightarrow L_o$). The consequence of the domains is an increase in the microviscosity and surface density of the lipid bilayer within the domains. Between domains, the lipid bilayer has lower microviscosity and surface density.

How do some hormones promote the formation of *β-structures* in membrane

proteins? Consider the interactions with the membrane of DEA and androsterone. Androsterone binding to the membrane leads to an increase in the proportion of *β-structures* in membrane proteins (Table 3) and the formation of protein-lipid domains in the membrane (Table 2). On the other hand, when DEA bound to membranes, these domains were not formed (Table 2) because the proportion of *β-structures* in membrane proteins decreased (Fig. 5, *a*; Table 4). The reason for this different interaction of DEA and androsterone with membranes should be sought in their structure. The only difference in the structure of these hormones is that androsterone has hydrogen attached to the carbon atom in the A-ring at position 5, while DEA does not [4]. This difference in the structures of DEA and androsterone allows us to formulate the mechanism of *β-turn* in the peptide molecule. It can be concluded that the presence of the H-group at the C5 atom in androsterone promotes the formation of such bonds with the protein molecule, the appearance of which leads to the formation of *β-turns* and the creation of *β-structures* in the molecule. Fig. 6 shows the mechanism of this β-turn formation. Here, the androsterone molecule is attached to the peptide molecule by means of hydrogen bonds (Fig. 6, left side). The carbon atom located in the A-ring of androsterone at position 5 forms a hydrogen bond with the CO group number 1 in the peptide chain through the attached H-group (Fig. 6, left part). In addition, the carbon atom in the A-ring of androsterone at position 3 forms a hydrogen bond with CO group number 3 in the peptide chain via an attached OH-group (Fig. 6, left part). As a result of the formation of these hydrogen bonds, the C-C bond between atoms 2 and 3 of the peptide molecule is rotated. This turn leads to a *β-turn of the* peptide molecule (Fig. 6, right part). In the DEA molecule, which lacks an H-group at the C5 atom in the A-ring, no hydrogen bond is formed between this C5 atom and CO group number 1 in the peptide chain. As a result, there is no C-C bond rotation between atoms 2 and 3 of the peptide molecule, no *β-turn of* the peptide molecule occurs, no secondary *β-structure of the* protein molecule is formed.

Fig. 6. Mechanism of *β-turn* and formation of a *β-structure* from a peptide strand with the help of androsterone molecule

Phenomena similar to the formation of protein-lipid domains in plasma membranes are also observed in other cases. It is known, for example, that protein structures called amyloid fibrils can appear in the tissues of an organism [29]. They represent stacked antiparallel β-structures directed perpendicularly to the fibril axis [30]. The process of formation of these amyloid fibrils begins with the formation of protofibrillar formations rich

in β-structures [29], similar to the formation of protein-lipid domains in membranes described above. The formation of insoluble β-amyloid fibrils in brain tissues accompanies many neurodegenerative diseases, such as Alzheimer's disease and Down syndrome [29].

The interaction between protein filaments coiled into *α-helixes* has a different character. On the basis of computer calculations of the free energy of spontaneous association of transmembrane *α-helixes*, it has been shown that lipids make a more significant contribution to the dimerization energy, whereas contacts of amino acid residues are often disadvantageous [31, 32]. This means that *α-helices* prevent protein aggregation and the formation of protein-lipid domains in the membrane. turn, this prevents an increase in the microviscosity of the lipid bilayer. Moreover, an increase in the proportion of α-helices in membrane proteins can lead to a decrease in the microviscosity of the lipid bilayer of membranes. Compounds are known which, when interacting with the membrane, reduce its microviscosity, i.e., loosen the lipid bilayer. Such a compound is, for example, apo A-1 transporter protein [33]. It was shown earlier that native apo A-1, being a part of high-density lipoprotein (HDL), has up to 85% of α-helices in its secondary structure [34]. HDL is used to reverse cholesterol uptake from cells and further transport it to the liver [35, 36]. This requires loosening of the lipid bilayer. This phenomenon was detected when native apo A-1 was incubated with erythrocyte membranes. The relative microviscosity of the membranes decreased by 15% upon interaction with native apo A-1 [33]. The decrease in microviscosity occurred almost equally in the lipid-lipid interaction and in the protein-lipid interaction [33]. This is due to the nonspecific interaction of apo A-1 *α-helices* with membrane proteins and lipids, resulting in the breakage of hydrogen bonds between membrane proteins and lipids.

The phenomenon of hydrogen bond breaking between molecules during their interaction with *α-helixes of* apo A-1 was discovered earlier by group led by Academician L.E. Panin using small-angle X-ray scattering [37]. It was shown that the interaction of tetrahydrocortisol-apo A-1 complex and eukaryotic DNA leads to the breakage of hydrogen bonds between pairs of nitrogenous bases and the formation of single-stranded DNA structures [37]. Similarly, in the cytoplasmic membrane apo A-1 breaks hydrogen bonds between membrane proteins and creates structural transitions β-structures→ disordered structures and α-helices→ disordered structures, which leads to a decrease in interactions between proteins and membrane lipids. As a consequence, the microviscosity of the lipid bilayer decreases [33].

Oxidatively modified apo A-1 does not change membrane microviscosity [33]. The reason for the existence of qualitatively different mechanisms of interactions between native and oxidatively modified apo A-1 lies in their different secondary structures. Earlier in the

work of Dudarev et al. [38] found that, compared to the native conformation, the proportion of *α-helices* in oxidatively modified apo A-1 decreases from 48% to 31% and the proportion of *β-structures* increases from 33% to 49%. The formed *β-structures of* oxidatively modified apo A-1 proteins strengthen the interaction between them and membrane lipids, create new hydrogen bonds with functional groups of phospholipids, compensating for the breakage of these bonds by *α-helixes of* apo A-1. As a result, the microviscosity of membranes does not change when oxidatively modified apo A-1 interacts with them [33].

It is known that promising drugs for the treatment of Alzheimer's disease are molecules that, when interacting with amyloid fibrils, are able to reduce the proportion of β-structures in them or prevent the formation of new β-structures in them [39, 40]. Since the nonspecific interaction of *α-helixes of* native apo A-1 with membrane proteins decreases the proportion of β- structures in membrane proteins by breaking hydrogen bonds between peptide filaments of membrane proteins, native apo A-1 may be one of the drug candidates for the treatment and prevention of Alzheimer's disease.

CONCLUSION

By fluorescence measurement of relative membrane microviscosity using a pyrene probe, it was found that when cortisol, androsterone, testosterone, and adrenaline interact with membranes, the structure of the plasmalemma changes and a network of protein-lipid domains is formed. In these fixed domains, the surface density of lipids is higher than between domains. At the same time, when DEA interacted with membranes, there was no formation of protein-lipid domains in the membranes. The reason for the different interaction of hormones with membranes was elucidated by IR spectroscopy of membranes. It turned out that when cortisol, androsterone, testosterone, and adrenaline interacted with membranes, there was an increase in the proportion of *β-structures* and a decrease in the proportion of disordered structures in membrane proteins. However, when DEA interacted with membranes, there was a decrease in the proportion of β-structures and *α-helices* in the secondary structures of membrane proteins. Only the fraction of disordered structures in the secondary structures of membrane proteins increased.

Thus, it has been established *in vitro* that the initiators of the appearance of protein-lipid domains in plasma membranes are membrane proteins in which the proportion of β-structures increases after interaction with hormones. At the same time, the appearance of new *α-helices* in membrane proteins does not increase the interaction between proteins,

protein-lipid domains are not formed. On the contrary, the appearance of a large number of *α-helices* in membrane proteins can lead to a decrease in the microviscosity of the lipid bilayer.

AUTHORS' CONTRIBUTION

V.Y. Rudyak, P.V. Mokrushnikov - concept and guidance of the work; P.V. Mokrushnikov - conducting experiments; V.Y. Rudyak, P.V. Mokrushnikov discussion of the research results; P.V. Mokrushnikov - writing the text; V.Y. Rudyak - editing the text of the article.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ETHICS DECLARATION

All procedures performed in studies involving animals complied with the ethical standards of the institution where the studies were conducted and with the approved legal acts of the Russian Federation and international organizations.

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