THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

pubs.acs.org/JPCB

Interaction Mechanism of Anabolic Steroid Hormones with Structural Components of Erythrocyte Membranes

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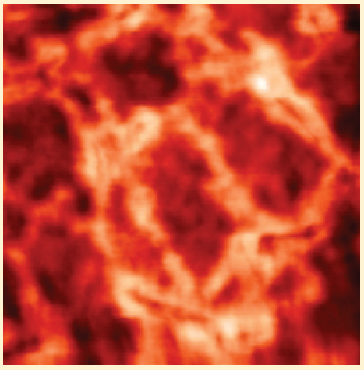
ABSTRACT: The interaction of testosterone, androsterone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS) with erythrocyte membranes was studied. It was shown that testosterone and androsterone have a high constant of binding to the membranes ($K_b \approx 10^6$ M^{-1}), whereas K_b's for DHEA and DHEAS are 2 orders of magnitude lower. Hydrogen bonds and hydrophobic interactions play an important role in binding of anabolic steroids. Hydrogen bonds form with CO and NH groups both of membrane proteins and phospholipids. This results in the formation of complex domains rising above the surface of membranes. Strengthening of hydrophobic interactions in the domains promotes the displacement of water dipoles to adjacent regions, thus loosening the phospholipid bilayer. Overall, microviscosity of erythrocyte membranes strongly increases, which decreases the plasticity of erythrocytes and hampers their motion in blood capillaries. This mechanism may underlie the development of diffusion myocardial hypoxia and hypoxic cardiac arrest.



INTRODUCTION

Anabolic steroid hormones have been used for many decades, finding their most extensive use in sports medicine. Nowadays, it is impossible to train as an international class athlete without anabolic hormones. A coach's aspiration for high sporting results prompts that coach to use an ever increasing amount of anabolic steroids. Lacking a profound knowledge of sports medicine, such a coach cannot imagine all of the negative effects of anabolics on the body of an athlete. Moreover, sports medicine itself has no comprehensive information on the subject. As a consequence, the number of sudden and unexpected deaths of atheletes during the competitions has drastically increased in recent years.[1,2]

Anabolic steroids are still in wide use as they allow athletes to build muscle mass fast by training. This occurs due to enhanced expression of genes that are responsible for synthesis of contractile proteins. Molecular mechanisms of this phenomenon are not quite clear. They are initiated not only in muscles but also in other tissues: liver, heart, red bone marrow, etc.[3,4] In this case, there occurs nonspecific enhancement of the protein biosynthesis rate in the cells of different tissues. Earlier we have shown that such enhancement can take place under the action of a biologically active complex steroid hormones: apolipoprotein A-1.[5]

Anabolic steroids include both natural and synthetic hormones: testosterone, androsterone, dehydroepiandrosterone, its sulfated form, retabolil, methyl androstenediol, methandrostenolone, etc.

Enhancement of protein biosynthesis in muscles under the action of anabolic steroid hormones certainly belongs to useful nonspecific effects. However, absolutely undesirable nonspecific effects are also possible, in particular, the ability of hormones to interact with cell membranes. The mechanism of this interaction is poorly understood. In this connection, most interesting are erythrocyte membranes. Interacting with such membranes, anabolic steroids may affect transmembrane diffusion of O_2 and CO_2 as well as microviscosity properties and ability of erythrocytes to move in capillary network.[6]

In this work, we studied the mechanism of testosterone, androsterone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS) interaction with structural components of erythrocyte membranes and changes in their microviscosity characteristics during the interaction. The results obtained could shed light on the causes of cardiovascular catastrophes, which are often observed in athletes taking anabolic steroid hormones for a long time.[7]

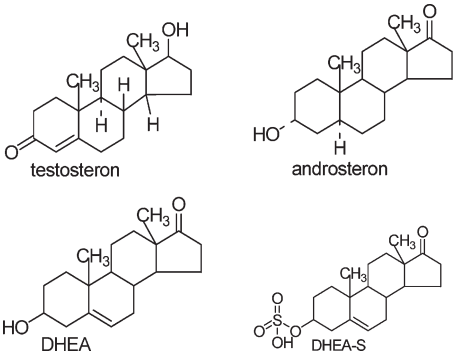
MATERIALS AND METHODS

The actions of four hormones testosterone, androsterone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone

Received: June 22, 2011
Revised: November 10, 2011
Published: November 11, 2011

dx.doi.org/10.1021/jp2058854 | J. Phys. Chem. B 2011, 115, 14969–14979

sulfate (DHEAS; Amersham) are analyzed in the work.



For this purpose, the following methods were used.

1. Atomic Force Microscopy (AFM) of Erythrocytes. Erythrocytes were obtained from fresh blood after decapitation of Vistar male rats under light nembutal narcosis. Their weight was 180−200 g, and the age was three months. Blood was diluted 2-fold by isotonic phosphate buffer (pH 7.35) containing 0.043 M of KH_2PO_4 and 0.136 M of Na_2HPO_4. After precipitation of cells by centrifuging at 330*g* for 10 min, the supernatant liquor was decanted, and the washing procedure was repeated twice more.

All of the procedures were performed at 4 °C. The resulting erythrocyte suspension of 20 mcl volume was deposited onto a glass slide as a thin smear. The smear was predried for 10 min in air at 24 °C and humidity of 40%. After evaporation of excessive surface moisture, the smear was observed under a Solver Bio atomic force microscope (NT-MDT, Russia) at 24 °C using a semicontact mode. An analogous procedure of obtaining red blood cells for the AFM examination was employed earlier by other authors.[8] In each experiment, we first tested a control specimen without hormones and then the experimental one. Silicon cantilevers NSG11 (NT-MDT, Russia) with a resonant frequency between 120 and 180 kHz and spring constant ~6 N/m were used (all of these probe parameters were offered by manufacturer). Images of the surface relief of erythrocyte membrane after absorption of hormones were obtained with the scan size $1 \times 1\ \mu m^2$ and $1.3 \times 1.3\ \mu m^2$.

2. IR Spectroscopy of Erythrocyte Shadows. Erythrocyte shadows were obtained after their hemolysis in hypotonic phosphate buffer (pH 7.35) containing 2.75 mM of KH_2PO_4 and 8.5 mM of Na_2HPO_4. Shadows were precipitated by centrifuging at 5500*g*, and the supernatant liquor was decanted. The washing procedure was repeated four more times.[9] All operations and further storage of shadows were performed at 4 °C.

A film for taking the IR spectra of erythrocyte shadows was prepared in a cuvette with fluorite backing via slow evaporation of water under weak vacuum at a pressure of ca. 0.1 atm (ca. 0.5×10^4 Pa) and temperature 4 ± 1 °C.[10,11] Drying lasted 180 min. A suspension of erythrocyte shadows in a 0.001 M phosphate buffer with pH 7.35 and volume 60 mcl was introduced into a cuvette. This was supplemented with 30 mcl of the same buffer and 1.0 mcl of the hormone solution with concentration 10^{-6} M. Stirring and incubation lasted 10 min at 16−17 °C. The cuvette was placed horizontally on a special table of a vacuum unit.

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

3. Fluorescence Analysis of Erythrocyte Shadows. Fluorescence measurements were performed with a Shimadzu spectrofluorophotometer RF-5301(PC)SCE. A total of 4 mL of hypotonic phosphate buffer containing 2.75 mM of KH_2PO_4 and 8.5 mM of Na_2HPO_4 (pH 7.35), and erythrocyte shadows were poured into a quartz cuvette of size $1 \times 1 \times 4\ cm^3$. The concentration of shadow proteins was determined by the Warburg−Christian method from changes in the optical density of suspension.[12] On the average, it varied in the range of 0.100−0.250 mg/mL.

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

A solution of hormones with the concentration 10^{-6} M was prepared in hypotonic phosphate buffer. The time of hormone incubation with shadows was one hour. Absorption and emission spectra were taken, the average value of emission and absorption intensity was measured. For each hormone (testosterone, androsterone, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS)), the binding constant K_b was calculated by the method as well as the stoichiometric concentration of a bound hormone B_{max} and a change in free energy of the system ΔG.[13] The interaction of hormone and erythrocyte membrane is described by the equation

$$B + nS = S_nB$$

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

$$K_b = \frac{[S_nB]}{[S][B]} \quad (1)$$

where $[S_nB]$ is the concentration of bound protein, $[B]$ is the concentration of free protein, and $[S]$ is the concentration of free

hormone. It is supposed that hormone, upon binding to protein, completely quenches its fluorescence. Thus, the fluorescence intensity F will be proportional to the concentration of free protein. Let us write C for total concentration of protein in the cuvette, and x for concentration of the bound protein. Then,

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

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

$$x = QC, \text{ where } Q = \frac{F_{\max} - F}{F_{\max}} \tag{3}$$

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

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

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

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

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

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

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

Relative error in measuring the F value was 3%. Relative measurement errors for K_b and B_{max} was equal to 10%.

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

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

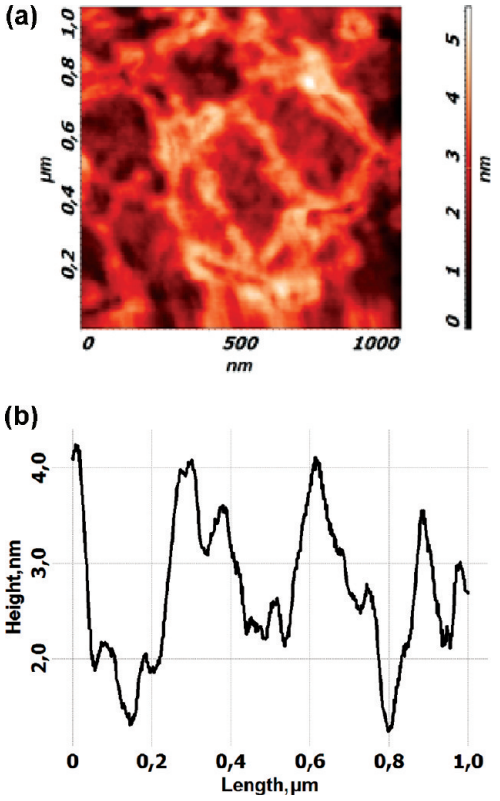


Figure 1. Control surface of rat erythrocyte. The erythrocyte suspension was supplemented with DMS and ethanol (0.25% of the mixture volume): (a) scan size 1×1 μm^2; (b) center section of the surface.

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

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

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

dx.doi.org/10.1021/jp2058854 |J. Phys. Chem. B 2011, 115, 14969–14979

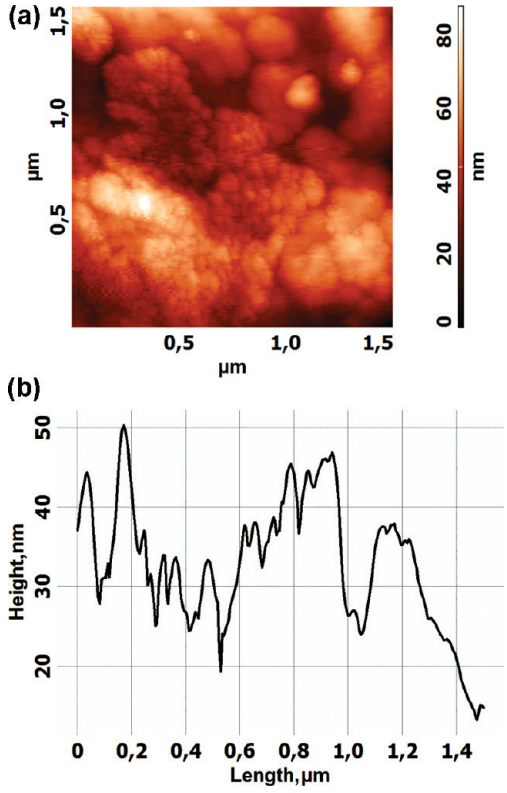


Figure 2. Surface of rat erythrocyte after adsorption of testosterone. Concentration of the hormone is 10^{-7} M: (a) scan size 1.5 × 1.5 μm^2; (b) center section of the surface.

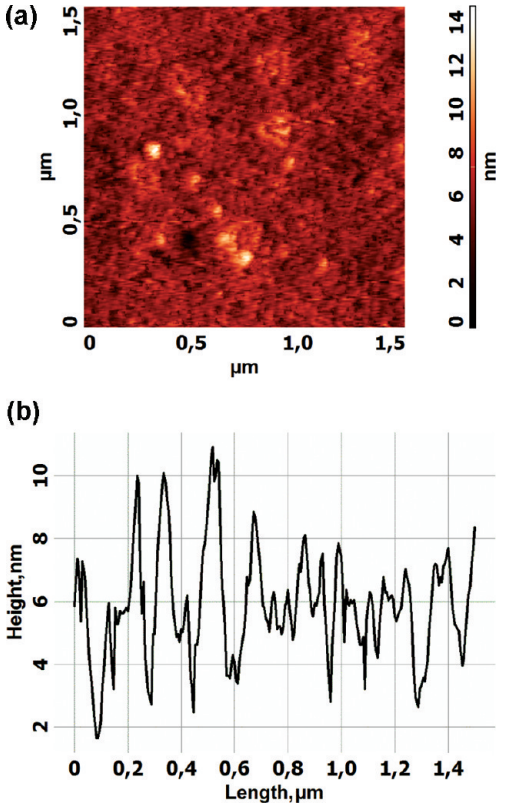


Figure 3. Surface of rat erythrocyte after adsorption of androsterone. Concentration of the hormone is 10^{-6} M: (a) scan size 1.5 × 1.5 μm^2; (b) center section of the surface.

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

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

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

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

RESULTS

Atomic Force Microscopy. Using atomic force microscopy, erythrocytes of healthy animals looked as large biconcave discs ca. 6 μm in diameter, which agrees with the results obtained by other authors.[8] At a higher magnification, their surface showed a slight nonuniformity caused most likely by the presence of membrane proteins. When the erythrocyte suspension was supplemented with DMS and ethanol (0.25% of the mixture volume), the surface nonuniformity increased, probably due to the denaturating effect of solvent on the surface structural proteins (Figure 1). Domains with the length 200−250 nm and height 2 nm are seen. The pattern changed upon addition of testosterone to erythrocyte suspension with the final concentration 10^{-7} M (Figure 2). The interaction of testosterone with erythrocyte membranes leads to their restructuring. The surface is tuberous, there are domains of size 400 × 400 nm^2 and height 20−25 nm, with smaller domains on the surface of large ones: size 50 × 50 nm^2 and height 10 nm. Between them, there are regions of loosened substance that form hollows. In this case, there are pronounced distortions in the primary structure of erythrocyte membranes.

Other structural changes of erythrocyte membranes were obtained in our study upon interaction with androsterone (Figure 3) with the final concentration 10^{-6} M. The surface is flat, there are domains of size 100 × 100 nm^2 and height 6−8 nm. In comparison with control specimens, domains decreased in area, but increased in height.

The surface of rat erythrocyte after adsorption of DHEA is depicted in Figure 4. The concentration of the hormone is 10^{-7} M. The surface is tuberous, and there are domains of size 220 × 220 nm^2 and height 20−25 nm. However, they are not separated into subdomains, as in the case of testosterone.

Of the four hormones, DHEAS has the weakest effect on the membrane morphology. The surface of rat erythrocyte after adsorption of DHEAS is shown in Figure 5. Concentration of the hormone is 10^{-7} M. The surface is flat, and there are domains of size 100 × 100 nm and height 3−4 nm. Changes are insignificant in comparison with control. It can be suggested that DHEA and DHEAS did not affect deep layers of the membranes, so the effect was much less pronounced.

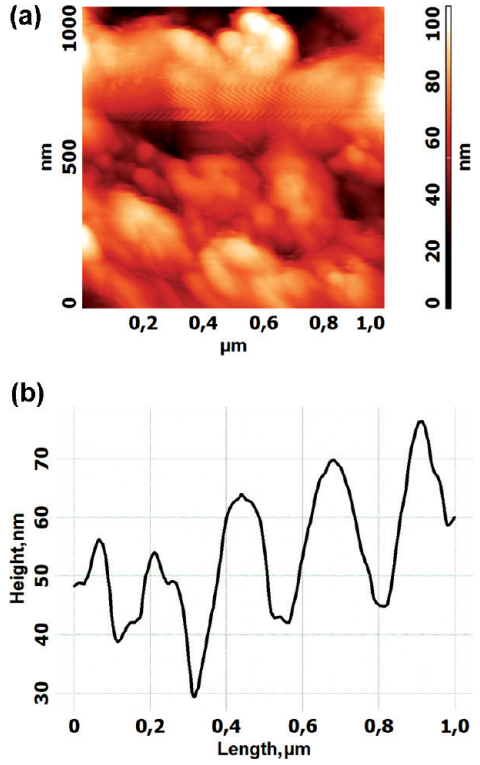


Figure 4. Surface of rat erythrocyte after adsorption of DHEA. Concentration of the hormone is 10^{-7} M; (a) scan size $1 \times 1\ \mu m^2$; (b) center section of the surface.

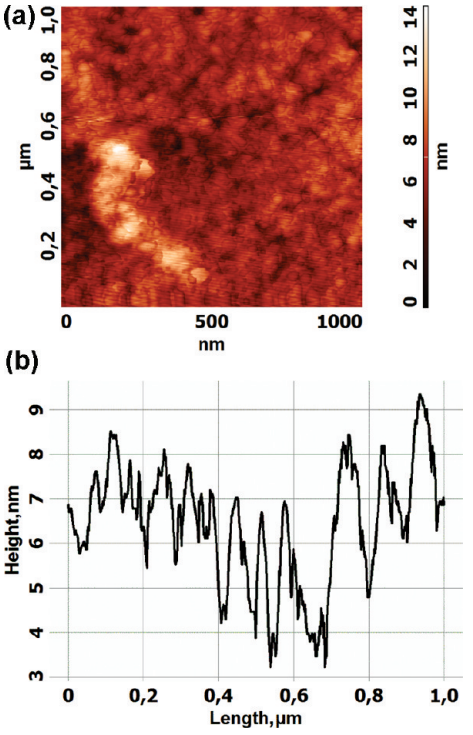


Figure 5. Surface of rat erythrocyte after adsorption of DHEAS. Concentration of the hormone is 10^{-7} M; (a) scan size $1 \times 1\ \mu m^2$; (b) center section of the surface.

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

2. IR Spectroscopy of Erythrocyte Shadows. Analysis of IR spectra of erythrocyte shadows obtained from rats with no hormone loading (Figure 6) revealed in membrane-bound proteins not only a disordered structure but also α-helix at 1650 and 1656 cm^{-1} and β-structure at 1686 and 1520 cm^{-1}. Besides, we recorded NH stretching vibrations in proteins (3308 cm^{-1}), CH stretching vibrations in proteins and phospholipids (2948, 2930, and 2848 cm^{-1}), and a set of bands corresponding to phospholipids, in particular, C═O bond (1748 cm^{-1}), P═O bond (1236 cm^{-1}), CH_2 deformation vibrations (1460 and 1386 cm^{-1}), $O_4C_4-C_5O_5$ bond (1048 cm^{-1}) and C−C bond of deformation vibrations (978 cm^{-1}). It should be noted that C═O band (1736 cm^{-1}) is quite narrow, which gives grounds to suggest that phospholipids in membranes of normal erythrocytes are well ordered at a level of ester bonds of higher carboxylic acids and glycerol.

Effect of Testosterone. Under the action of testosterone, intensity of absorption bands 1544, 1656, and 3292 cm^{-1} increased by 30% and more (Table 1, Figure 7). The absorption band of NH bond showed a 3308 → 3272 cm^{-1} shift ($\Delta\nu$ = 36 cm^{-1}). The bands 2852 and 2932 cm^{-1} increased in intensity; the ratio of band intensities 2852/2932 cm^{-1} changed. The enhancement of integral intensity of the indicated absorption bands indicates an increased ordering of membrane proteins and, in particular, an increased fraction of α-helices.

The fraction of α-helices grows due to structural transition tangle → α-helix. A 3308 → 3272 cm^{-1} band shift of the NH bond ($\Delta\nu$ = 36 cm^{-1}) is caused by the formation of a hydrogen bond between keto group (C_3═O) in testosterone A-ring and NH bond of peptide group in membrane protein or indole ring in tryptophan. An increased intensity of the 2932 and 2852 cm^{-1} bands together with a growing intensity ratio 2852/2932 cm^{-1} confirm the rising orderliness of the entire membrane. Absorption band 1740 cm^{-1} (C═O bond of the ester group in phospholipids) increased in intensity and shifted to the short-wave region. The enhanced intensity of C═O bond reflects an increased ordering of phospholipids within domains and an increased interdomain ordering. The short-wave shift of this band is caused by the formation of a hydrogen bond between the OH group at the C_{17} carbon atom in the testosterone D-ring and the C═O bond in phospholipids. Similar to segnetoelectrics a hysteresis phenomenon was observed in erythrocyte membranes.[14,15] The spectrin-actin-ankyrin meshwork, which is connected both with membrane proteins and phospholipids, also contributes to the ordering of phospholipids.[16] The 1088 → 1098 and 1236 → 1248 cm^{-1} shifts of absorption bands to the short-wave region result from dehydration of phospholipids due to increase in their orderliness, since the hydration process shifts these bands to the long-wave region.[17] An increased intensity of bands 1098 and 1247 cm^{-1} (P−O−C and P═O bonds of phospholipids, respectively) in comparison with control specimens confirms an enhanced ordering of phospholipids under the action of the hormone.

Thus, the formation of complex domains in erythrocyte membranes upon their interaction with testosterone is caused by simultaneous interaction of CO and OH groups of the hormone with CO and NH groups both of proteins and phospholipids. In the process, water is displaced to adjacent regions, which is accompanied by membrane loosening.

Effect of Androsterone. Incubation of rat erythrocyte shadows with androsterone ($C_C = 2.76 \times 10^{-8}$ M) results in shifting

the frequency of NH bonds (stretching vibrations of amide A) to the long-wave region by 38 cm^{-1} as well as shifting of NH bond of amide II ($\Delta\nu$ = 2 cm^{-1}; Table 1). Integral intensity of absorption bands of CO (1654 cm^{-1}) and NH groups (3280 cm^{-1}) increased by 30% and more. There appeared a band at 1635 cm^{-1} corresponding to the β-structure. An increase in intensity of stretching vibrations of CH bonds at 2848 and 2930 cm^{-1} was observed. The frequency shift of NH bond is related with the formation of hydrogen bond with C_{17}═O group of the hormone D-ring. Androsterone has a more flexible structure as compared to cholesterol: its A, B, and C rings can take a more favorable conformation during the interaction with membrane proteins. Only D-ring has a flat structure, due to the presence of carbon C_{17} with sp_2 hybridization. Hydrophobic interaction with the membrane surface should also be taken into account. High conformational mobility of the molecule creates more advantageous steric conditions for hydrophobic interaction both with tryptophan, which fluorescence quenching was observed in our study, and hydrophobic regions on the membrane surface. This increases the constant of their binding to hormone and leads to more pronounced structural changes in the membranes. An increase in intensity of CO-peptide bond is related with the growing fraction of α-helices due to transition tangle → α-helix. An increase in intensity of absorption band 1620−1635 cm^{-1} is caused by structural transition tangle → β-structure. Of interest is a hypothesis stating that the indicated transitions may take place in contractile proteins, since their removal from the membrane results in a decrease or disappearance of transitions.[14,15]

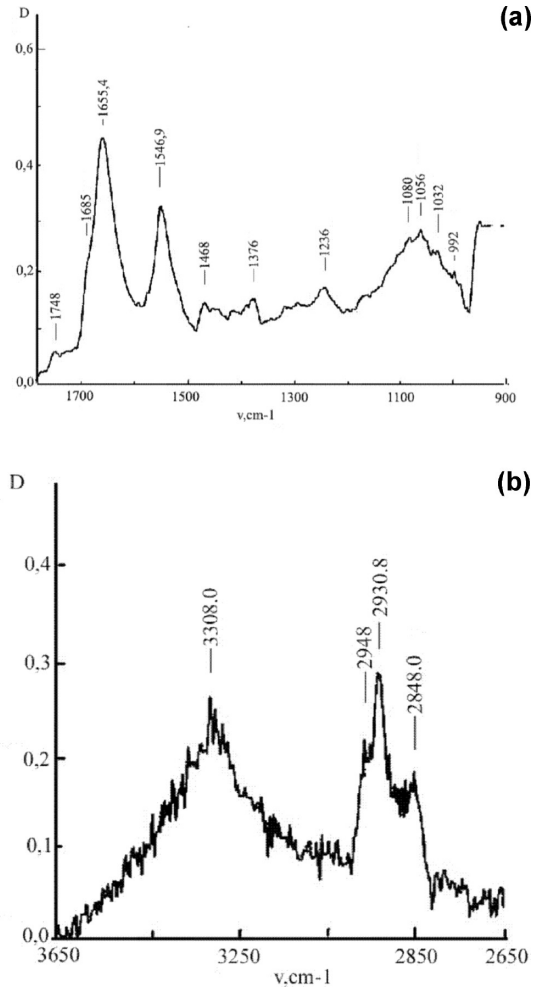


Figure 6. IR spectra of rat erythrocyte membranes (control) ($C_{phosph.buff.}$ = 0.01 M, pH 7.35, relative humidity 0%): (a) ν = 900−1800 cm^{-1}, (b) ν = 2600−3700 cm^{-1}.

Effect of Dehydroepiandrosterone. Incubation of DHEA with erythrocyte shadows showed that the frequency of stretching vibrations of the NH peptide bond shifted by 20 cm^{-1} to the long-wave region (3308 → 3288 cm^{-1}), whereas halfwidth of amide A decreased. An increase in the integral intensity of absorption bands at 1546, 1654.9, and 3288 cm^{-1} was observed (Table 1).

A 1236 → 1247.6 cm^{-1} band shift points to dehydration of phosphate groups in phospholipids. Shifting of the frequency of C═O bond in phospholipids (1748 → 1732 cm^{-1}) was observed; intensity of this band also increased. The 2930 → 2925.8 and 2848 → 2851 cm^{-1} shifts (CH stretching vibrations) took place, intensity of the bands increased. The intensity ratio 2852/2924 cm^{-1} changed.

Table 1. IR Spectroscopy[a]

compound	ν_{CO}	$\nu_{NH\ stretch.}$	$\nu_{C=O}$	$\nu_{P=O}$	ν_{P-O-C}	$\nu_{O5C4-C5O4}$	$\nu_{CH\ stretch.}$	A_{CO}
shadows (control)	1655.4	3308	1748	1236	1080	1056	2948	1.2150 × 10
	1686						2930	
							2848	
shadows + androsterone	1656	3270		1260	1098		2958	
	1635	329		1240	1088		2928	
(A = 2.76 × 10^{-8} M)	1620	2					2848	
shadows + testosterone	1657	3272	1739.4	1247	1098	1065	2956.4	2.2433 × 10
	1684	3298		1236	1088	1076	2924	
(A = 2.7x 10^{-8} M)	1632	3309					2850	
shadows + DHEA	1654.9	3288.0	1732	1247.6	1088	1070.7	2956.3	2.1266 × 10
(A = 2.64 x10^{-8} M)							2925.8	
							2851.8	
shadows + DHEAS	1656.0	3286	1738	1248.0	1084	1070	2952.0	1.2598 × 10
(A = 1.63 × 10^{-8} M)	1680	3300				1052.7	2926.4	
	1632.0	3312					2852.0	

[a] Frequency parameters of rat erythrocyte shadows after their interaction with hormones.

dx.doi.org/10.1021/jp2058854 |J. Phys. Chem. B 2011, 115, 14969–14979

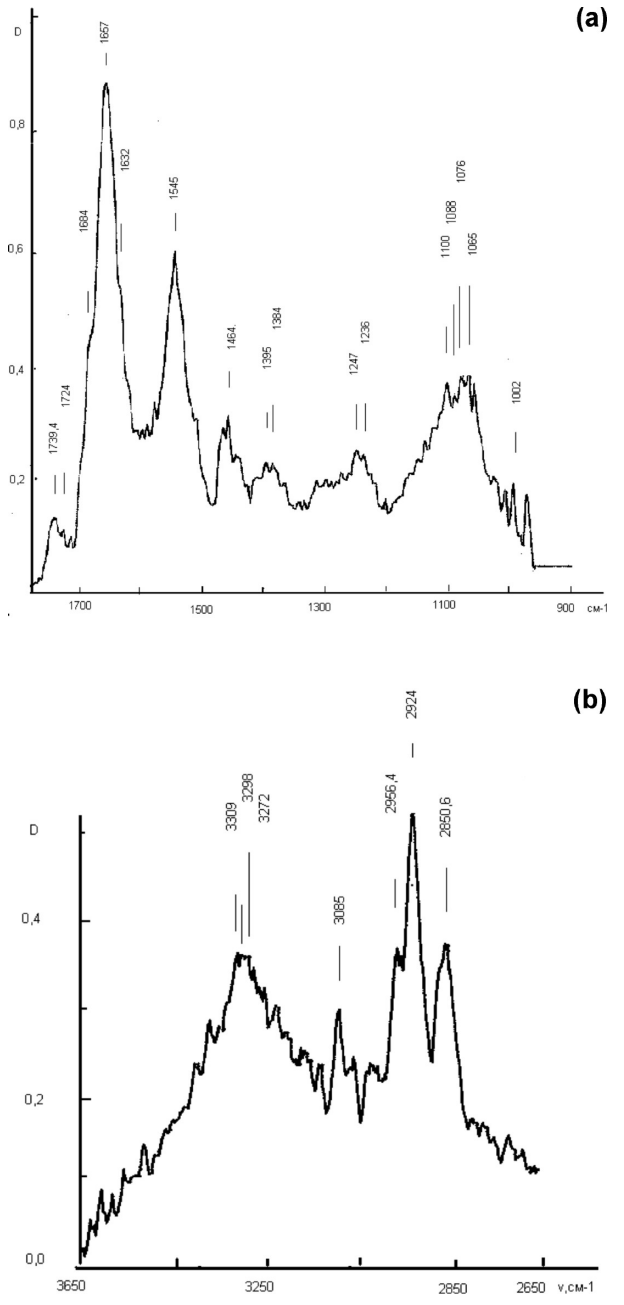


Figure 7. IR spectra of rat erythrocyte membranes incubated with testosterone (C_C = 2.7 × 10^{-8} M, $C_{phosph.buff.}$ = 0.001 M, pH 7.35, relative humidity 0%): (a) ν = 900−1800 cm^{-1}, (b) ν = 2600−3700 cm^{-1}.

Effect of Dehydroepiandrosterone Sulfate. Incubation of DHEAS with erythrocyte shadows resulted in the band shift 3308 → 3286 cm^{-1} (NH peptide bond) by 22 cm^{-1} (Table 1). Bands at 1548, 1656, and 3298 cm^{-1} increased in intensity with respect to control specimen; however, this was more pronounced upon addition of DHEA as compared to DHEAS. Absorption bands 1632 and 1684 cm^{-1} attributed to β-structure were observed. The band shift was recorded: 2930 → 2928 and 2848 → 2852 cm^{-1}, which was accompanied by a change in the 2852/2928 cm^{-1} ratio. The band at 1236 cm^{-1} (P═O bond) showed a strong splitting and had 3−4 bands in the region of 1236−1256 cm^{-1}. Bands at 1084 and 1100 cm^{-1} (P−O bond)

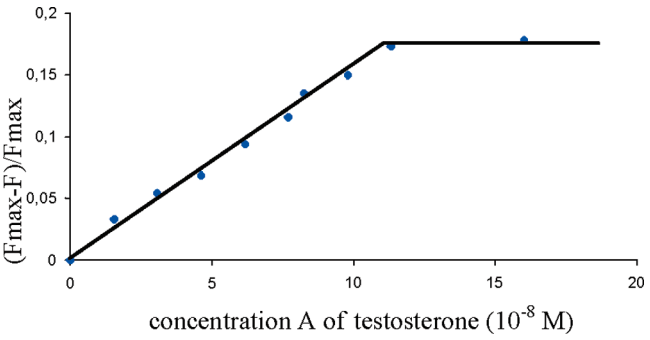


Figure 8. $Q = (F_{max} - F)/F_{max}$ versus the concentration A of testosterone hormone introduced into a cuvette. Concentration of membrane protein C = 0.101 mg/mL.

were observed. A 1748−1738 cm^{-1} shift was detected; however, it was less pronounced than in the case of DHEA addition. The DHEAS hormone has a stronger binding with hydrophilic heads of phospholipids as compared to DHEA and a weaker binding with membrane proteins. This suggests that DHEAS molecules cannot penetrate deep into the membrane due to their higher hydrophilicity with respect to DHEA.

Overall, it can be concluded that the interaction of DHEA and DHEAS with erythrocyte membranes is accompanied by the formation of hydrogen bonds between keto group (C_{17}═O) and NH group of proteins as well as between the OH group at C_3 in the A-ring of the hormones and the C═O group in biomembrane phospholipids. The formation of indicated hydrogen bonds leads to ordering of membrane proteins (transition tangle → α-helix) and phospholipids. Hydrophobic interactions of hormone with the surface of erythrocyte membranes also contribute to their structural rearrangement; however, they are much less pronounced for DHEAS as compared to DHEA. The reason is that substitution of OH group by SO_3 strongly diminishes the energy of hydrogen bond, since in the OH group the unshared pair of electrons is located on the oxygen atom, whereas in the SO_3 group it is delocalized over the entire π-conjugated bond.

3. Fluorescence Analysis. In the study, absorption intensity (D) and emission intensity (F) of tryptophan were estimated at different wavelengths. Corrections were made for dilution of erythrocyte shadow suspension after the introduction of a hormone solution, for tryptophan emission quenching by a solvent (DMS: ethanol), intrinsic fluorescence of hormones, and evaporation of water from a cuvette. To obtain a correction for solvent, the erythrocyte shadow suspension was titrated with solvent.

It was shown that the solvent decreases the intensity of tryptophan absorption at λ = 227.8 nm by 33% and results in its long-wave shift to λ = 230.2 nm. Absorption intensity at λ = 281 nm changed only by 1.3% without a long-wave shifting. A maximum of emission intensity differed from the control specimen also at λ = 332 nm. It did not shift upon addition of solvent, but its intensity decreased by 1.3%.

Upon addition of testosterone with the final concentration 3 × 10^{-6} M to erythrocyte shadows, the absorption intensity at 227 nm diminished by 19 au or by 2.8%; this was accompanied by an upward shift of λ to 230.4 nm. As the hormone concentration increased to 6.05 × 10^{-6} M, the absorption intensity at 227 nm decreased by 25 au, or 5.0%, which was accompanied by shifting the absorption maximum to 232 nm. In the region of 280 nm, addition of hormone caused only minor changes in absorption. Considerable changes in the spectrum were obtained upon

dx.doi.org/10.1021/jp2058854 |J. Phys. Chem. B 2011, 115, 14969–14979

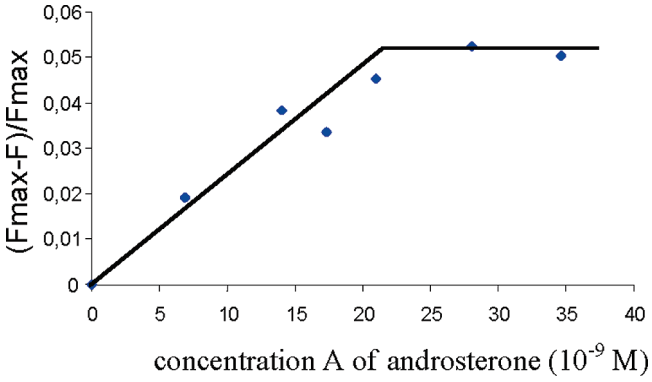


Figure 9. $Q = (F_{max} - F)/F_{max}$ versus the concentration A of androsterone hormone introduced into a cuvette. Concentration of membrane protein $C = 0.203$ mg/mL.

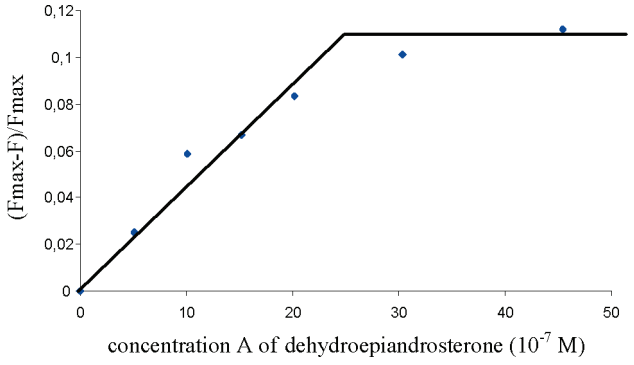


Figure 10. $Q = (F_{max} - F)/F_{max}$ versus the concentration A of dehydroepiandrosterone hormone introduced into a cuvette. Concentration of membrane protein $C = 0.139$ mg/mL.

addition of androsterone to the shadows. Even at a concentration of 6.92×10^{-8} M, which is 2 orders of magnitude lower compared to the case of testosterone, the absorption intensity at 227 nm decreased by 90 au, or 122%. It means that this hormone penetrates deeper into erythrocyte membranes than testosterone and enhances the tangle $\rightarrow$ α-helix transition in proteins, thus increasing their ordering. When erythrocyte shadows were supplemented with DHEA or DHEAS, the hypochromic effect was weak or entirely absent. A decrease in absorption intensity and a long-wave shift observed in our study can be attributed to the effect of solvent.

Analysis of the tryptophan fluorescence quenching spectra testifies that all four hormones interact with membrane-bound proteins, although a degree of this interaction differs (Figures 8−11), where $Q = (F_{max} - F)/F_{max}$ versus the concentration A of hormone introduced into a cuvette, where F_{max} and F are the intensities of tryptophan fluorescence in shadows without and with hormone, respectively. The excitation wavelength $\lambda = 281$ nm, and the emission wavelength $\lambda = 332$ nm.

The most pronounced quenching was observed in the case of androsterone (Figure 9). The maximum fluorescence quenching was observed at a concentration of 2.2×10^{-8} M. Testosterone showed a lower fluorescence quenching (Figure 8). The maximum quenching was observed at a concentration of 1.2×10^{-7} M, which is 5.5 times higher as compared to androsterone. Fluorescence quenching was even less pronounced with DHEA

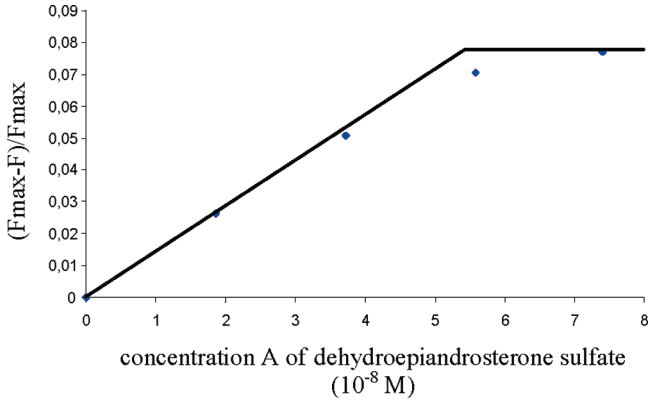


Figure 11. $Q = (F_{max} - F)/F_{max}$ versus the concentration A of dehydroepiandrosterone sulfate hormone introduced into a cuvette. Concentration of membrane protein $C = 0.139$ mg/mL.

(Figure 10). In this case, the maximum quenching occurred at a concentration of 2.4×10^{-6} M. And finally, the lowest fluorescence quenching was observed for DHEAS (Figure 11). The maximum quenching took place at a concentration of 5.3×10^{-6} M, which is 2.2 times higher as compared to DHEA.

According to the results obtained, testosterone and androsterone penetrate deeper into erythrocyte membrane and have a stronger effect on the structure of membrane-bound proteins toward their increased ordering. DHEA and DHEAS have some effect on erythrocyte membranes; these hormones adsorb on the membrane surface but do not penetrate deep into hydrophobic layer of the membranes. These hormones have a weaker binding with proteins via hydrogen bonds.

This conclusion is confirmed by the calculated values of hormone binding constant (K_b), total amount of bound hormone (B_{max}), and changes in free energy (ΔG) upon hormone transition from the free state to the membrane-bound one (Table 2). The highest values of K_b were obtained in our study for testosterone and androsterone, K_b for androsterone being higher by a factor of 4. The amount of the bound hormone (B_{max}) obeyed an inverse relationship: it was 2.4 times higher in the case of testosterone as compared to that of androsterone. Changes in free energy upon interaction of hormones with erythrocyte membranes were most pronounced.

All values for DHEA and DHEAS strongly differed from those listed above. Binding constants were nearly 2 orders of magnitude lower. Amount of the bound hormone (B_{max}) was much greater, indicating a low specificity of interaction with the membranes. Changes in free energy (ΔG) were low for both hormones (Table 2).

Thus, a comparison of two pairs of hormones demonstrated their considerable difference from each other. The higher is K_b, the greater is the binding specificity and the lower is the amount of bound hormone (B_{max}). Big negative values of ΔG for testosterone and androsterone testify that their interaction with erythrocyte membranes increases their ordering (negentropy). DHEA and DHEAS are characterized by a low specificity of binding to membranes. It shows up even when hormones are compared with each other. In DHEA, substitution of OH group by SO_3H in the third position of A-ring decreases K_b by a factor of 3.8 and increases B_{max} by a factor of 2.2. A decrease in ΔG is also pronounced. The reason is that the presence of the OH group and additionally of two keto groups and an S atom

Table 2. Parameters of Steroid Binding to Erythrocyte Membrane Based on Tryptophan Fluorescence Quenching of Membrane Proteins

steroid hormone	binding constant K_b (M^{-1})	amount of bound hormone B_{max} (mol/mg protein)	changes in free energy ΔG (kJ/mol)
testosterone	$2.24 \pm 0.22 \times 10^6$	$1.09 \pm 0.11 \times 10^{-9}$	−37.6
androsterone	$3.2 \pm 0.32 \times 10^6$	$4.46 \pm 0.45 \times 10^{-10}$	−38.5
DHEA	$5.99 \pm 0.60 \times 10^4$	$1.80 \pm 0.18 \times 10^{-8}$	−28.3
DHEAS	$1.56 \pm 0.16 \times 10^4$	$4.03 \pm 0.40 \times 10^{-8}$	−24.8

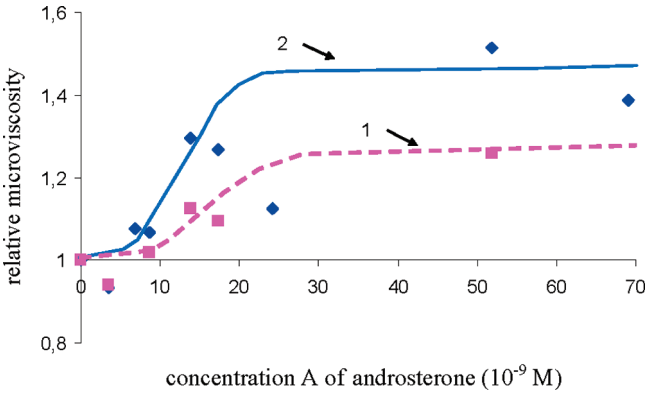


Figure 12. Changes in the relative microviscosity of membranes $\eta(A)/\eta(0)$ of erythrocyte shadows at the concentration A of androsterone hormone. Concentration of shadows C = 0.133 mg protein/mL.

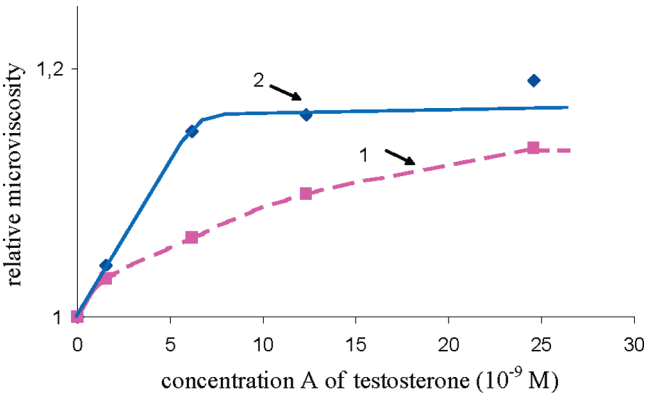


Figure 13. Changes in the relative microviscosity of membranes $\eta(A)/\eta(0)$ of erythrocyte shadows at the concentration A of testosterone hormone. Concentration of shadows C = 0.117 mg protein/mL.

enhances the interaction of DHEAS with hydrophilic CO and NH groups of the surface proteins. DHEA and DHEAS cannot bind to the proteins residing in hydrophobic layer of the membrane. These two hormones do not change the conformational state of spectrin-actin-ankyrin meshwork and have only a slight effect on the morphology of membrane surface. DHEAS, being most hydrophilic among the four hormones, has the weakest effect. As hormone hydrophilicity increases, the amount of membrane-bound hormone rises and K_b decreases. During the interaction of testosterone and androsterone with erythrocyte membranes, both hydrogen bonds and hydrophobic interactions may strongly contribute to the growth of K_b. This is explained by a deeper penetration of hormones into hydrophobic layer of erythrocyte membrane, which increases the specificity of their interaction. The accompanying structural transitions in membrane proteins, tangle → β-structure → α-helix, increase ordering of these proteins and substantially raise the ΔG value. Results obtained in the study agree well with changes in microviscosity of erythrocyte membranes.

4. Changes in Microviscosity. In erythrocyte membrane, a fluorescent pyrene probe is distributed in the lipid phase and can be a source of information on the state of its deeper layers. The rate of its migration and the ability to form excimers upon interaction with each other are estimated. This is the way to determine changes in microviscosity of the membranes. Changes in the relative microviscosity of membranes (Figures 12−15) $\eta(A)/\eta(0)$ of erythrocyte shadows at the concentration A of hormone, where $\eta(A)$ and $\eta(0)$ are microviscosities of the membranes, respectively, with hormone added to the shadows suspension and without hormone. Line 1 shows changes of relative microviscosity in the region of lipid−lipid interaction; line 2 shows changes of relative microviscosity in the region of protein−lipid interaction. The concentration of pyrene in the suspension is 5.9×10^{-6} M, the temperature of the specimens is 309.1 ± 0.1 K (36 °C), and the pH of the suspension is 7.35. The measured value of $\eta(A)/\eta(0)$ indicates an error of 6%.

In our study, an increase in microviscosity was most pronounced at the addition of androsterone to erythrocyte membranes. Microviscosity started to grow at a hormone concentration of 10^{-8} M, the growth proceeding up to 2.5×10^{-8} M with subsequent saturation (Figure 12). The S-shaped curve points to high cooperativity in changing the conformational state of the membrane. A microviscosity increment attained 50% with respect to the initial state. In the region of protein−lipid interactions it appeared earlier and reached a higher value as compared to the region of lipid−lipid interactions. The absorption intensity (D) and emission intensity (F) of tryptophan in membrane proteins started to decrease at the same concentrations and attained a maximum also at the same concentrations (Figure 9). Thus, our results revealed a cooperative nature of changes in erythrocyte membranes under the action of androsterone.

Addition of testosterone produced similar changes in membrane microviscosity. In the region of protein−lipid interactions, microviscosity increased at lower concentrations of hormone and attained higher values as compared to the region of lipid−lipid interactions (Figure 13). In both cases, the revealed structural changes were initiated in proteins and carried over to lipids by virtue of cooperativity.

The effect of DHEA and especially DHEAS on erythrocyte membranes is much less pronounced as compared to testosterone (Figures 14 and 15). DHEA and DHEAS increased microviscosity by 10% with respect to initial values. In these experiments, the concentration of DHEAS reached 8×10^{-6} M. For DHEA, the growth started at a hormone concentration of 5×10^{-7} M and attained its maximum at 1.5×10^{-6} M (Figure 14). Alteration of viscosity was described by S-curve and

dx.doi.org/10.1021/jp2058854 |J. Phys. Chem. B 2011, 115, 14969–14979

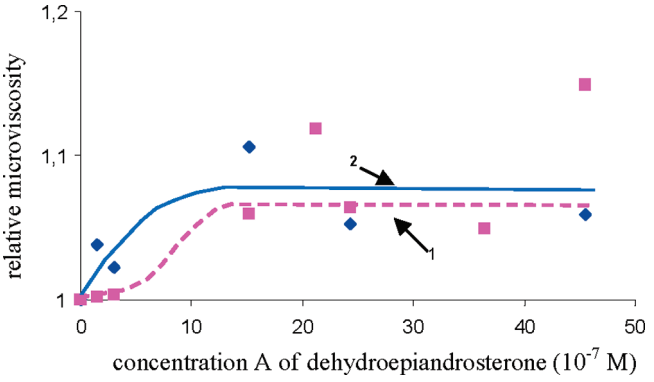


Figure 14. Changes in the relative microviscosity of membranes $\eta(A)/\eta(0)$ of erythrocyte shadows at the concentration A of hormone DHEA. Concentration of shadows C = 0.113 mg protein/mL.

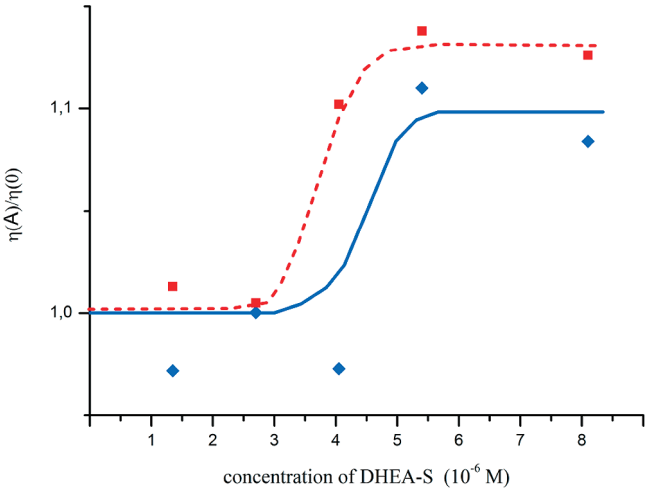


Figure 15. Changes in the relative microviscosity of membranes $\eta(A)/\eta(0)$ of erythrocyte shadows at the concentration A of DHEAS hormone. Concentration of shadows C = 0.290 mg protein/mL.

correlated with a decrease in fluorescence and absorption of tryptophan (Figure 10). The latter processes started at the same hormone concentrations and reached their minima also at the same concentrations. Microviscosity in the region of protein−lipid interactions increased earlier, at lower concentrations of hormone, and was more pronounced than in the region of lipid−lipid interactions. Structural changes were initiated in proteins and involved lipids due to cooperativity.

The mechanism of changes in membrane microviscosity under the action of a more hydrophilic hormone DHEAS is quite different. Microviscosity goes to a constant value at a higher concentration of DHEAS in suspension as compared to that of DHEA (5×10^{-6} M versus 1.5×10^{-6} M for DHEA). First changes of microviscosity appeared in the region of lipid−lipid interactions (Figure 15), which was followed by an increase of microviscosity in the region of protein−lipid interactions. DHEAS interacted with polar heads of phospholipids, then structural changes carried over to proteins due to cooperativity. Hydrophilic molecules of DHEAS cannot penetrate deep into hydrophobic layer of the membranes. There are only minor structural changes in the spectrin-actin-ankyrin meshwork and weak changes in membrane microviscosity.

DISCUSSION

Zona glomerulosa produces mineralocorticoids (aldosterone and deoxycorticosterone). They are responsible for regulation of mineral metabolism in the organism. Male sex hormones (testosterone, androsterone) are synthesized in testicles and are responsible for the development of male sexual characteristics. *Zona reticularis* produces androgenic hormones (DHEA and DHEAS). They regulate sexual functions and sexual behavior.[18] Their role in the organism is not completely understood.[19] However, along with main functions, these steroid hormones have numerous secondary functions. In particular, the enhancement of protein synthesis in various tissues, not only in the target organs. This function is known as an anabolic one.[20] It is widely used in the training of international class athletes.

The mechanism of anabolic action of steroid hormones is still unclear. We were the first to demonstrate that in a complex with apolipoprotein A-1 these hormones enhance gene expression and protein synthesis in many organs and tissues, i.e., exhibit nonspecific anabolic action.[3,4] Indeed, for this purpose they should possess a reduced Δ^4, 3-keto group of the hormone A-ring.[21] It comprises an active hydroxyl in the third position. This is exactly the hydroxyl that can rupture hydrogen bonds in GC pairs of promoter regions of the genes. The reduction process occurs in vivo, similar to the formation of a complex steroid hormone, apoA-1, with participation of macrophages. These cells are present in all organs and tissues, so the formation of biologically active complex may result in nonspecific enhancement of protein biosynthesis. The mechanism of this phenomenon was elucidated quite thoroughly.[5] An active OH group in the third position of hormone A-ring in GC pairs competitively ruptures the hydrogen bond and initiates local melting of DNA. Hydrophobic interaction between the rings of nitrous bases and steroid hormones leads to further rupture of hydrogen bonds in DNA. It becomes even more pronounced at the interaction of nitrous bases with hydrophobic regions of α-helices in apoA-1. RNA polymerase settles at the single-stranded DNA structures and initiates the process of gene expression.[21,22]

Sex hormones are transferred in the blood by sex hormone binding globulin (SHBG). However, its binding capacity is limited. As a result of long time consumption of anabolic steroids, the content of nonconjugated form hormones in the blood is greatly increased. It is known that normal blood content of testosterone in male varies within the range of $0.8-3.0 \times 10^{-8}$ M, free content of testosterone in male is 0.4×10^{-8} M.[23] This is much higher in athletes taking anabolic-androgenic steroids (AAS), $6.9 \pm 0.7 \times 10^{-8}$ M.[24] Accordingly, the level of free hormone is also higher. It should be taken into account that the binding ability of SHBG decreases in a long-term use of AAS.[24] Bodybuilders take 200−250 mg of testosterone twice a week.[25] Their blood level of the hormones can increase up to 10^{-5} M in 5 L of blood. The content of the free hormone rises proportionally. Cases of sudden death in athletes taking AAS in the training period or during competitions are reported quite often in the literature.[26,27] In our opinion, such cases are related to AAS interaction with the cell membranes of erythrocytes, which increases the membrane viscosity and decreases the velocity of capillary blood flow.

The present study demonstrated that functionally active groups of anabolic steroid hormones play an important role in the interaction with erythrocyte membranes. IR spectroscopy showed the involvement of O═ and OH groups of hormones,

CO and NH groups of proteins, and phospholipids of erythrocyte membranes in the formation of hydrogen bonds. Hydrophobic interactions strongly contribute to the formation of these bonds. Overall, this determines high values of the binding constants (Table 2). Especially high K_b values were found for androsterone and testosterone. Here, specific contribution of hydrophobic interactions is most essential. They determine also the ability of these hormones to penetrate deep into hydrophobic region of erythrocyte membranes. As the interaction involves simultaneously CO and NH groups both of proteins and phospholipids, this results in the formation of complex domains. They rise above the membrane surface (Figures 1−5). Due to enhancement of hydrophobic interactions, compressive stresses grow in the domains. Water dipoles are displaced to adjacent regions. This is accompanied by their loosening. When stresses in the membrane exceed a maximum value, the membrane loses its flatness and becomes tuberous (Figures 2 and 4). The use of fluorescent probes revealed a general increase in microviscosity of the membranes. It was much more pronounced in the region of protein−lipid interactions as compared to the region of lipid−lipid ones. This certainly deteriorates plasticity of erythrocytes, which can form numerous clots in blood capillaries and facilitate the development of diffusion hypoxia. In myocardium, such a process may result in cardiac arrest.[1,2]

Changes can occur in a small part of red blood cells, but if they fall into the coronary system of the heart, a myocardial infarction is imminent. That is what we see in healthy athletes during competition.[28]

For two other hormones (DHEA and DHEAS), the indicated mechanism is less dangerous. This is especially true for DHEAS. The presence of a large number of O═ and OH groups in the molecule determines their interaction with polar groups of phospholipids and proteins on the surface of erythrocyte membranes. They cannot penetrate deep into hydrophobic region of biomembranes to change the conformational state of the spectrin−actin−ankyrin meshwork. Their interaction with membranes produces no substantial changes in the membrane plasticity.

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