The influence of weakly polar solvent chloroform on the state of water adsorbed by serum albumin

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The hydration shell structure of human serum albumin (HSA) and HSA adsorbed on a surface of highly disperse silica in a weakly polar solvent (chloroform) or with various addition of this solvent was studied by ^{1}H NMR spectroscopy and layer-by-layer freezing-out of a liquid phase. The influence of chloroform can result in changes in the ratio of volumes of internal regions of protein globule characterized by different hydration levels and filled up with structured water unfrozen at T < 273.

Key words: *human serum albumin, highly disperse silica, chloroform, ^{1}H NMR spectroscopy*

Introduction Human serum albumin (HSA) is one of the main and the most studied blood plasma proteins [1-5]. In aqueous environment the albumin molecules are of deformed spheroid shape, the size of which depends on pH value. Cavities in globules are filled with water [6]. Being close to the surface of a protein molecule, almost all the water inside the globule is subject to the influence of polypeptide groups and amino acid residues [6]; therefore, it is bound water, which is proven by the decrease of freezing temperature. Water may be in either hydrophobic or hydrophilic environment in the cavities of a protein molecule [7]. HSA hydration occurs due to the formation of hydrogen bindings of water molecule to polar functional protein groups and Van der Waals forces,

responsible for hydrophobic interactions while contacting non-polar groups. The issues of both hydrophobic and hydrophilic hydration of proteins are presented in the literature [8-11]. The influence of hydrophobic fragments on bound water may be revealed in specific properties of the system. Thus, the curve of albumin solution density dependence on its concentration in the range of small protein content shows a density minimum which is thought to be connected with the hydrophobic effect [12].

Recent studies of protein macromolecules interaction with components of aqueous-organic mixtures have attracted significant attention due to intensive development of a new scientific trend *i.e.* fermentative catalysis in organic environments at low water content [13]. Water has been determined to play a key role in the formation of proteins structure and their

functioning both in aqueous [14] and low-water organic environment [13, 15, 16]. The study on mechanisms of protein-water interaction in organic environment may stimulate the creation of novel effective biocatalytic systems.

Weakly polar organic solvents may be expected to have strong effect on HSA hydration [17, 18]. Protein drying results in slight changes in its conformation [19] in comparison with the native state; however, the addition of weakly polar molecules may cause the change in the ratio of volumes of internal regions of protein globule hydrated by both hydrophobic and hydrophilic mechanisms [7-9].

Placing of proteins into the environment with the organic solvent results in the formation of heterogeneous systems which are hard to be investigated using optical methods; therefore, it is reasonable to study such HSA systems by calorimetric methods [16, 20], FTIR [16] and ^{1}H NMR spectroscopy.

Current work presents the study on hydration shell structure of human serum albumin and albumin adsorbed on a surface of highly disperse silica in a weakly polar solvent (chloroform) or on various addition of this solvent.

Materials and Methods Dry HSA (*Biopharma*, Ukraine) with over 95% content of monomer form was used in the work. Deuterated chloroform (CDCl$_3$) contained 99.9% of deuterium isotope. The samples were prepared adding a fixed portion of distilled water (5-10 mg) to HSA powder (70-100 mg) at intense stirring for 30 min at room temperature (on subsequent addition of 10-300 mg of deuterated organic solvent on the NMR measurements). On obtaining the equilibrium (15 min) the sample was placed in the NMR spectrometer. Deuterated chloroform was used for the signal of organic addition protons not to change the signal intensity of bound water protons.

Highly disperse silica (HDS) A-300 (Experimental plant at the Institute of Surface Chemistry, NAS of Ukraine) at the specific surface area S = 300 m^2/g was used. 2 g of HDS were added to 200 ml of 0.5% HSA solution. The adsorption was carried out for 2 hr at constant stirring with subsequent centrifugation at 8000 rpm for 20 min at 20±1°C. To remove free protein the precipitate was washed with distilled water until absorption band of the protein in the electronic spectra disappeared. The suspension was centrifuged again and obtained residue was dried at room temperature. The amount of adsorbed albumin was calculated by the difference of initial and equilibrated HSA concentrations in the solution after contacting HDS. Protein concentration in both initial and equilibrated solutions was calculated using the corresponding spectral data (absorption at = 280 nm which is HSA characteristic band) obtained with a Specord M-40 (*Karl Zeiss*, Germany) spectrophotometer. Silica samples were obtained with the content of 60 mg protein per 1 g of HDS and studied in different media (different ratio of air, water and chloroform).

NMR spectra were registered using a Bruker WP-100 SY spectrometer. The probing 90° pulses were used to obtain ^{1}H NMR spectra. The temperature of the sensor was regulated with Bruker VT-1000 thermoadapter. The reproducibility of ^{1}H NMR signal intensity was ±10% for ±1 K. The spectra of unfrozen water were measured after cooling the sample to 190-200 K [21-25]. The signal of water in ice (as well as macromolecule protons) did not contribute to the signal registered by the ^{1}H NMR method due to short time (~ 10^{-6} sec) of proton transverse relaxation in solids.

The measurements were performed using standard NMR ampoules (5 mm diameter), containing 70-300 mg of the substance. The value of chemical shifts of proton resonance of bound water was determined relative to the signal of CHCl$_3$ protons at $_H$ = 7.26 ppm. Measured temperature dependences of integral signal intensities (*I*) of unfrozen water were used to calculate the dependence of unfrozen water concentration (C$_{uw}$) on temperature (*T*), transformed into the dependence of changes in Gibbs free energy (G) of water (as a result of its interaction with protein and silica) on C$_{uw}$. Since the equilibrium of free energies of adsorbed water and ice is the prerequisite of water freezing on interphase protein/water boundary [26], the decrease of freezing temperature (273 - *T*) of adsorbed water determines the decrease of water free energy, caused by adsorption interactions (G = G$_0$ – G, where G$_0$ – free energy of ice at *T* = 273 K) [5]. As free energy of ice changes with temperature

decrease according to the linear law, the ΔG value may be calculated by the formula [26]

$$G = 0.036 \,(273 - T) \qquad (1)$$

The area under the ΔG (C_{uw}) curve determines the value of interfacial energy (γ_s) of the investigated sample in aqueous environment:

$$\gamma_s = K \int_{o}^{C_{uw}^{max}} \Delta G dC_{uw} \qquad (2)$$

In this equation C_{uw}^{max} is the quantity of water unfrozen at T $\to$ 273 K; K is a constant depending on the type of units used [23]. The parameters of strongly and weakly bound water (concentration, Gibbs free energy, and interphase energy) were calculated relative to ΔG(C_{uw}) dependences. Weakly bound water is thought to be a part of unfrozen water, free energy of which is only slightly decreased due to intermolecular interactions with solid surface and adsorbed organic molecules. It freezes at about 273 K. Strongly bound water may be unfrozen even at significant cooling of the system. Its free energy is greatly decreased after interaction with protein and silica [22, 23].

The content of each water type (C_{uw}^{s} and C_{uw}^{w} for strongly and weakly bound water, respectively) and maximum values of decreasing free energy for these types of water (ΔG_s and ΔG_w) may be obtained using linear extrapolation of linear portions of the ΔG(C_{uw}) graphs to axes. It is noteworthy that there are two types of water, namely, unfrozen fraction (C_{uw}) and total amount (C_{H_2O}). A part of unfrozen water with ΔG < -0.8 kJ/mol may be related to weakly bound water [21-25].

In narrower pores water freezes at lower temperatures which is illustrated by Gibbs-Thompson equation [27]:

$$\Delta T_m = T_m(R) - T_{m,} = \frac{2 \sigma_{sl} T_m}{\Delta H_f \, R}, \qquad (3)$$

where $T_m(R)$ is melting temperature of ice localized in pores of R radius; $T_{m,}$ – melting temperature of bulk ice; ρ – the density of solid phase; σ_{si} – the energy of solid-liquid interaction; ΔH_f – bulk enthalpy of fusion. This equation may be used to calculate pore size distribution according to temperature dependence of C_{uw} value obtained using ^{1}H NMR spectroscopy and

layer-by-layer freezing-out of water for aqueous suspensions of solids or hydrated biological objects [28], when the usage of other methods of porous structure analysis is complicated.

Results and Discussion Fig. 1 presents spectra of ^{1}H NMR water bound to HSA at different deuterochloroform content in the range of 0-46% masses at various temperatures. Water concentration was 193 mg/g for all samples. Chloroform being absent, spectra have the shape of a singlet whose width increases from 1 to 20 kHz with temperature decrease, while the degree of chemical shift in the point of maximum intensity is close to 5 ppm (signal *1*), which coincides with the chemical shift of bulk water [26]. Larger signal width of adsorbed water is conditioned by low molecular mobility of water bound to HSA molecules [29]. A narrower signal with the chemical shift δ_H = 1.3 ppm (signal *2*) is revealed on the background of a wide signal on the addition of chloroform. Its intensity increases with increasing chloroform concentration (C_{CDCl_3}). Intensity of signal *2* remains steady with lowering temperature.

While C_{CDCl_3} value is sufficient for chloroform isolation into a separate phase (Fig. 2) (80% masses in suspension), signal *1* intensity at T < 275 K becomes considerably lower than that of the same temperature in samples with less chloroform content (Fig. 1), and the intensity of signals *1* and *2* is close. Spectrum characteristics of signal *2* depend on temperature slightly. Data in Fig. *2* show signal 2 even in case of freezing the main part of liquid chloroform.

The magnitude of water chemical shift is defined by two main factors, namely, the strength of hydrogen bonds and the average number of hydrogen bonds per water molecule [30, 31]. Calculations reveal the strength of hydrogen bonds to depend slightly on the number of water molecules [25]. Water molecules which do not participate in the formation of hydrogen-bound complexes have a chemical shift of δ_0 = 1.3 – 1.7 ppm [26]. This value is characteristic of water dissolved in chloroform, benzene, and other weakly polar solvents [32]. Chemical shift of proton resonance for ice is δ_2 = 7 ppm when all water molecules have four hydrogen bonds each [33]. Therefore, data in Fig. 1, 2 may be interpreted as follows: signal *1* relates to water in the composition of
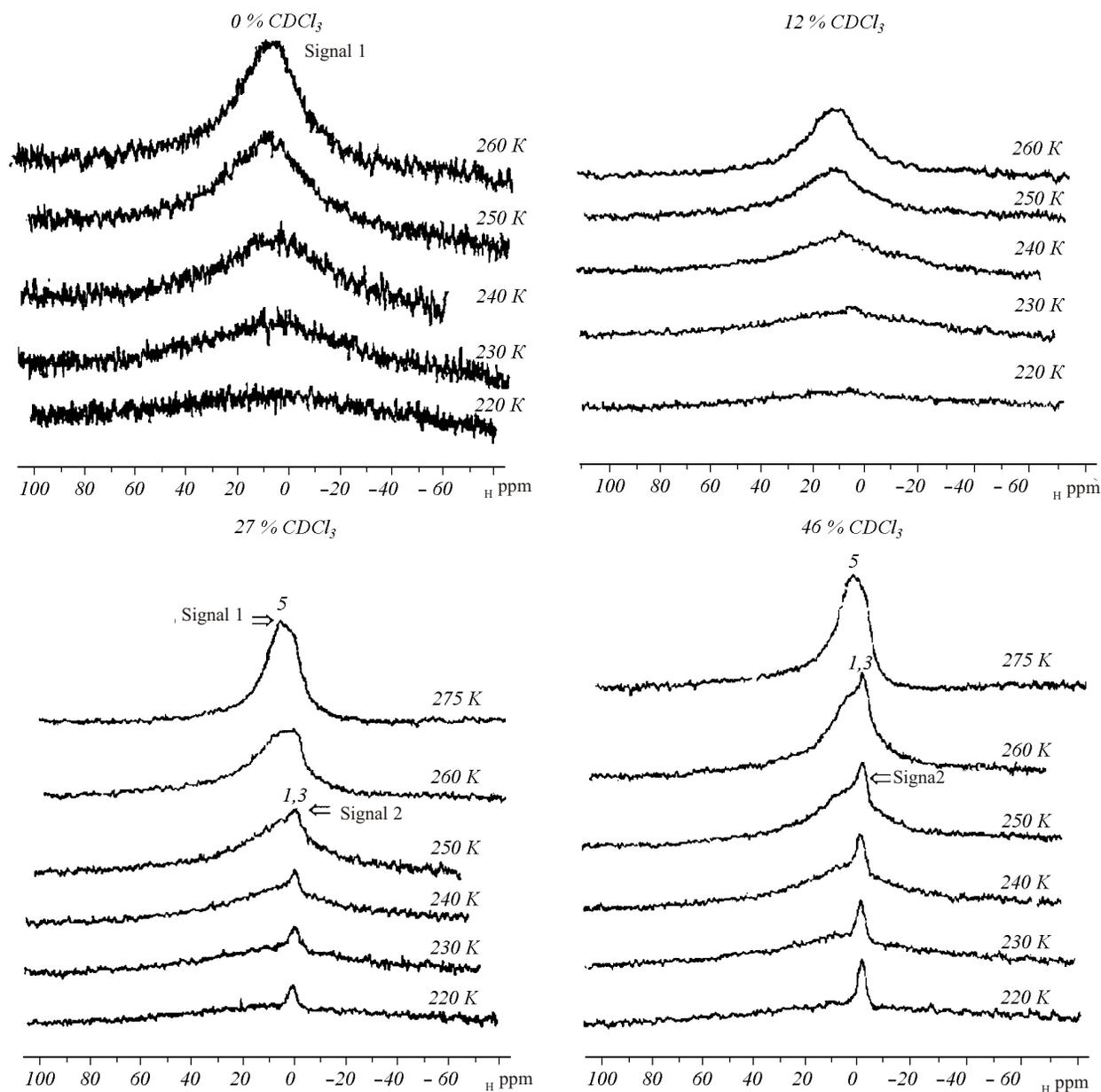
Fig.1. Effect of chloroform addition on the form of ^1H NMR spectra of water, bound with albumin (C_{H_2O} =193 mg/g)

polyassociates with the network of hydrogen bonds, which is similar to that of bulk water, and signal 2 corresponds to water molecules in weakly associated state. Previously weakly associated forms of water at the interfaces of hydrated solids and hydrophobic environment were registered for Aerosil mixtures with methylaerosil [34] and trimethylsilylated Aerosil in which the degree of substituted hydroxyl groups for trimethylsilyl ones did not exceed 50% [35].

Fig. 3 shows temperature dependences of unfrozen water concentration (*a*) and calculated dependences $G(C_{uw})$ (*c*) for hydrated HSA with 0-80% chloroform masses content. Vertical fragments of the graphs linked to unfrozen water at T > 250 K relate to HSA powders in the range of G < -1 kJ/mol on $G(C_{uw})$ dependences. According to abovementioned criteria of strongly and weakly bound water, hydrated HSA contains only strongly bound water. Chloroform
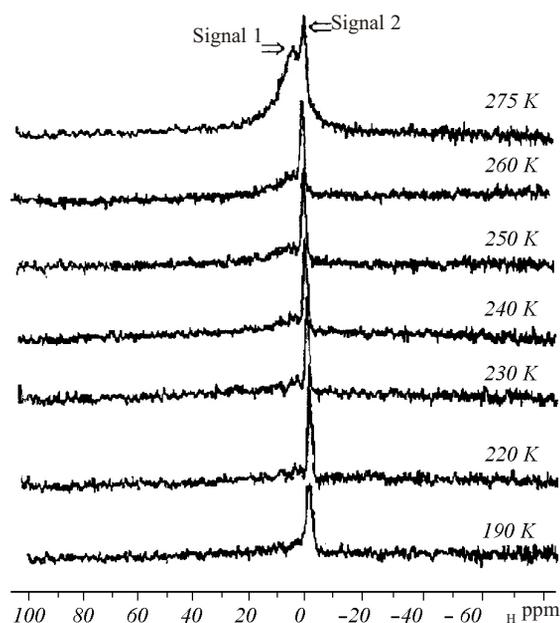
Fig.2. Temperature changes in ^{1}H NMR spectra of water (C_{H_2O} = 193 mg/g) adsorbed on HSA at C_{CDCl_3} = 80 wt.%.

environment reveals sharp decrease in bound water concentration at T > 220 K and transformation of some water from the strongly bound state into weakly bound one (freezing at about 273 K). Fig. 3, *b, c* shows analogous results for HSA adsorbed on HDS A-300 surface. The transfer of some strongly bound water into weakly bound state in chloroform environment is not seen.

Weakly associated water of hydrated HSA molecules is supposed to be in structural cavities restricted by hydrophobic groups. Due to structural restrictions or peculiarities of intermolecular interactions in these cavities, water molecules have thermodynamic advantage while existing in a weakly associated state.

It is noteworthy that chemical shift of weakly associated water coincides with that of water dissolved in chloroform [25]. However, water solubility in chloroform does not exceed 0.6% at room temperature, and it decreases with decreasing temperature. The concentration of weakly associated water in the system albumin/water/chloroform in Fig. 2 is 1.7% which is much higher than the maximum concentration of dissolved water in chloroform at T = 190 K [35]. Taking into account that the main part of chloroform freezes at this temperature, the conclusion

is made that weakly bound water concentration in chloroform unfrozen due to the interaction with HSA molecules would equal several dozens percent.

Having been freeze-dried, the majority of HSA molecules retain their native globular structure in which air borders mainly hydrophilic sites easily adsorbing water and forming hydrate shell of protein molecules in both air and aqueous environment [7]. While adding chloroform, its molecules are mainly concentrated on hydrophobic sites of the protein, not bound to water (or in hydrophobic cavities). Both hydrophobic and hydrophilic constituents of powders are spatially divided and to some extent chloroform presence has insignificant effect on hydrate features of protein molecules. Stepwise change of water characteristics bound to HSA at the change in chloroform concentration (Fig. 3, *a, c*) may be conditioned by such a change in native conformation of protein molecules which provides minimum of free energy of the system on transferring from air to weakly polar CDCl$_3$ environment. In this case hydrophobic areas of HSA molecules become more accessible for chloroform, while hydrophilic centres change their spatial location to provide their minimum free energy in nonpolar environment. This process is accompanied by total decrease in HSA molecules hydration (Fig. 3, *a, c*) and, probably, by the formation of additional intramolecular hydrogen bonds between functional HSA groups. HSA adsorbed on HDS surface (Fig. 3, *b, c*) does not have the same effect which is probably conditioned by weak conformational flexibility of adsorbed albumin.

According to the data of Fig. 2 there is only signal *2* at T < 240 K in ^{1}H NMR spectra for HSA suspension in CDCl$_3$. Bound water concentration, responsible for this signal, is 80 mg/g (Fig. 3, *a*). Since chloroform molecules are capable of solvating hydrophobic groups of HSA molecules, hydrophobic cavities of HSA molecules contain both water and chloroform molecules. Taking into account a large amount of weakly associated water, water and chloroform inside hydrophobic areas of protein globule are expected to be capable of forming concentrated solution of water in chloroform *i.e.* the properties of water participating in hydrophobic hydration are considerably different
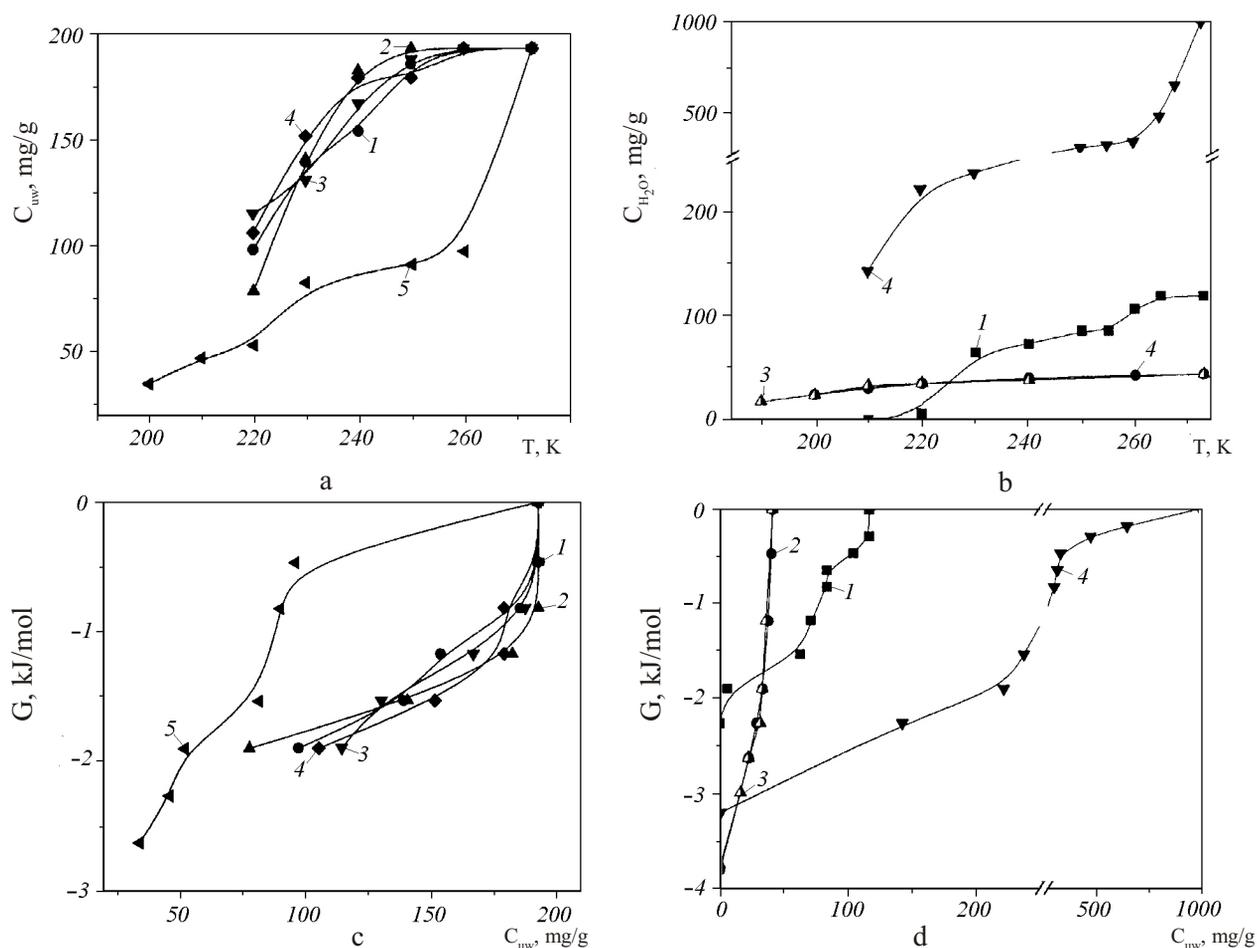
Fig.3. Temperature dependences of unfrozen water concentration and dependence of Gibbs free energy on unfrozen water concentration in the system HSA/water/chloroform (*a, c: 1 – 0% CDCl₃, 2 – 12% CDCl₃; 3 – 27% CDCl₃; 4 – 46% CDCl₃; 5 – 80% CDCl₃*) and (HSA-HDS)/water/chloroform (*b, d: 1-117.67 mg/g in CDCl₃; 2 – 41.9 mg/g in CDCl₃; 3 – 41.9 mg/g in air; 4 – in water*) (C_{H_2O} = 193 mg/g) at CDCl₃ content 0-80 wt.%.

from the properties of volume water, structured by the network of hydrogen bonds.

Table presents characteristics of bound water layers (concentration, Gibbs free energy, value of interfacial energy) and chloroform concentration for all the systems studied. For hydrated albumin with added $CDCl_3$ the value of interfacial energy is slightly dependent on $CDCl_3$ concentration until complete chloroform environment is formed, then it decreases sharply due to the transformation of a portion of strongly bound water into weakly bound state. In case if albumin is adsorbed on silica surface, interfacial energies in air and chloroform environment coincide. Weakly bound water is not registered in chloroform environment even in case if C_{H_2O} increases two-fold.

Fig. 4 presents distribution of unfrozen water concentration (internal cavities filled up with unfrozen water) according to pores radius (PSD_{uw}) for hydrate HSA in the presence of $CDCl_3$. Two maxima are registered in initial HSA on PSD_{uw} (r) dependence. The first maximum corresponds to pore radius $r = 0.35$ nm, the second maximum is $r = 1$ nm. The former increases with chloroform concentration growth and shifts toward high r values. Stepwise change of distribution curve shape occurs in chloroform environment. Maximum intensity at $r = 0.35$ nm decreases several times and new maxima appear at $r = 0.8$ and 8 nm. In case if hydrated albumin is in initial state or at relatively small chloroform additives, the main part of water is likely to be concentrated in microcavities with the radius $r = 0.35$ nm, formed by
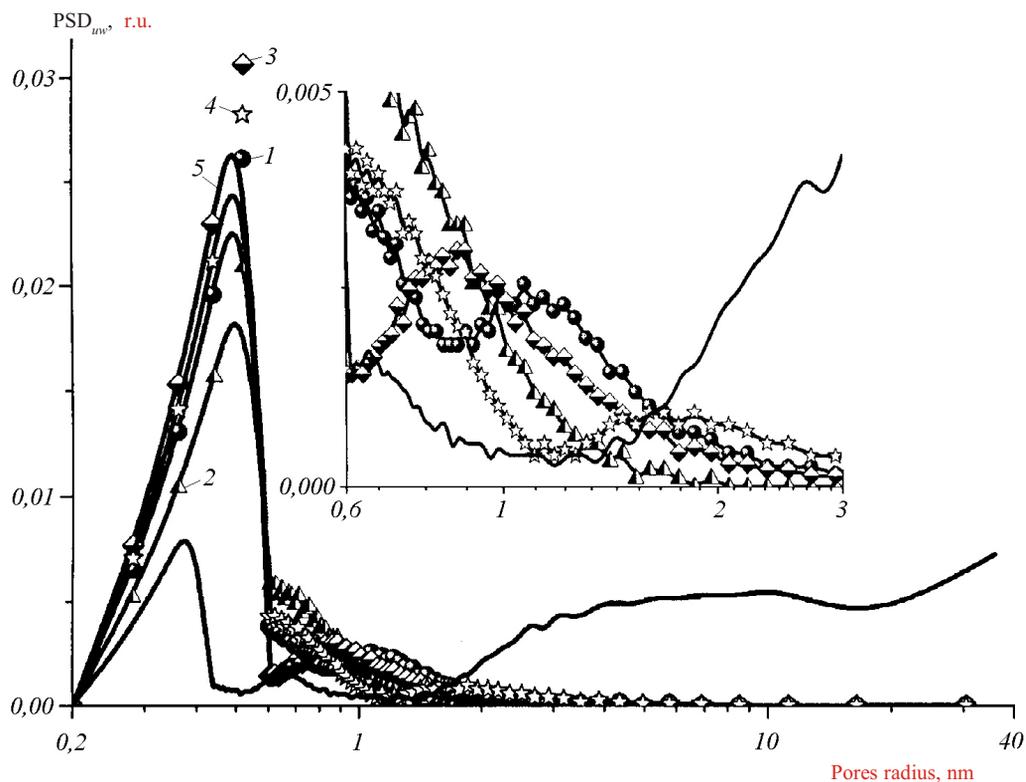
Fig.4. Dependence of distribution by sizes of cavities, filed up with not freezing water on chloroform content in hydrate powders of HSA and its suspensions: *1* – 0% CDCl$_3$; *2* – 12% CDCl$_3$; *3* – 36% CDCl$_3$; *4* – 72% CDCl$_3$; *5* – 80% CDCl$_3$)

hydrophilic sites of protein molecule, with which water molecules may form stable hydrogen bonds. The size of these cavities increases because of the effect of chloroform. It is probably caused by conformational changes in the protein molecules stabilized by growth of hydrophobic areas, where water is in weakly associated state which is revealed in ^{1}H NMR spectra in signal *2*.

In case if chloroform forms continuous phase, conformation changes in the protein molecules cause water removal from narrow hydrophilic areas into bigger cavities, formed anew with smaller amount of hydrophilic centers. This water becomes weakly bound (in the current case with HSA), since its interaction with interfacial boundary decreases, but it remains strongly associated as it forms a net of hydrogen bonds with other water molecules. Some water remains in hydrophobic areas with $r = 0.35$ nm, therefore, it is strongly bound (having strong perturbation influence from the surface) though weakly associated (having chemical shift $_H = 1.4$ ppm). This special state of water is stabilized by weakly polar deuterochloroform environment.

Probable condition for water transfer into weakly associated state is both presence of a sufficient number of hydrophobic areas, interacting with water molecules only according to disperse mechanism, either on the surface or in the environment, and spatial restrictions, hindering from the formation of three-dimensional network of hydrogen bonds. HSA adsorption on silica surface diminishes sharply conformational flexibility of HSA molecules and internal cavity volume, where hydrophobic hydration of protein molecule is possible in weakly polar environment.

Conclusions Hydrated HSA contains only strongly associated water at chemical shift $_H = 5$ ppm, adsorbed according to the mechanism of hydrophilic hydration. Signals of water adsorbed by the mechanisms of hydrophobic and hydrophilic hydration are separate in ^{1}H NMR spectra in the presence of weakly polar chloroform molecules. The concentration of weakly associated water ($_H = 1.3$ ppm) is much higher than maximum concentration of water dissolved in chloroform at T = 190 K. It increases with increasing chloroform concentration.

Characteristics of bound water layers (concentration ($C_{н2o}$), Gibbs free energy (ДГ) and the value of interfacial energy (г) in hydrated albumin (HSA) and albumin, adsorbed on silica surface (HDS))

Sample	C_{CDCl_3}, wt.%	G^s, kJ/mol	C_{H_2O}, mg/g	C_{uw}^w, mg/g	S, J/g
HSA hydrated powder	0	-2.7	193	0	19.6
	12	-2.25	193	0	19
	27	-3.2	193	0	22
	46	-2.7	193	0	21
	80	-3.5	193	90	13
HSA/HDS	0	-2.25	42	0	6
	80	-3.7	42	0	7
	80	-3.7	118	0	8
	0	-3.25	5000	500	45

The change in native conformation of protein globule is shown to occur under the influence of weakly polar chloroform environment which is supported by partial dehydration of surface and considerable decrease of internal cavity volume, binding water according to the mechanism of hydrophilic hydration.

Adsorptive fastening of protein molecules on silica surface decreases their conformation mobility due to which weakly associated form of water is not registered in the ^{1}H NMR spectra for system (HSA-HDS)/water/chloroform.

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Влияние хлороформа на состояние воды, адсорбированной сывороточным альбумином

Резюме

Методом [1]Н ЯМР спектроскопии с вымораживанием жидкой фазы изучено строение гидратной оболочки лиофилизированного и сорбированного на поверхности высокодисперсного кремнезема сывороточного альбумина человека, находящегося в слабополярном растворителе (хлороформе) или при его добавлении к гидратированной системе. Показано, что влияние хлороформа может приводить к изменению объемов внутренних областей белковой глобулы, характеризующихся различной гидрофильностью и заполненных структурированной водой, которая не замерзает при T<273 K.

Ключевые слова: вода, сывороточный альбумин человека, высокодисперсный кремнезем, хлороформ, [1]Н ЯМР спектроскопия.

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