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THE INTERACTIONS MELAFEN AND IHFANs WITH ANIMAL'S SOLUBLE PROTEIN

Key words: Melafen; hybrid antioxidant; bovine serum albumin; fluorescence.

The main goal of this work was the investigation of the action of two types of biological active substances to the soluble proteins that enriched the animal's blood serum. The first task was the test of the influence of plant growth regulator, applied in agriculture, - Melafen (melamine salt of bis (oximethyl) phosphinic acid), to the structural properties of soluble protein - bovine serum albumin (BSA). Melafen was used as the aqua solutions in the wide region of concentration (10^{-21} – 10^{-3} M). The Melafen interactions with BSA were tested by the spectral methods: BSA absorptions and intrinsic fluorescence of BSA tryptophane residues. We did not reveal any noticeable structural changes of the BSA molecules under the concentrations of Melafen that used at crop production. Melafen has not any destruction's influences to the soluble proteins structure under the concentrations that activate the plant growth (10^{-18} - 10^{-10} M). The second task was the investigation of the influence of hybrid antioxidants IHFANs that was expected to use as neuroprotector, to the BSA structure properties. IHFANs were used as the aqua-ethanol suspensions in the wide region of concentration (10^{-21} – 10^{-3} M). IHFANs protect from the destruction of BSA molecule, when the ultra low and low concentrations (10^{-18} - 10^{-10} M), by absorbing its alkyl tails at albumin surface. But the "loosening" of molecule BSA structure occurred, when the large concentrations (10^{-5} - 10^{-3} M) of Melafen were presented at medium for intrinsic BSA fluorescence registration. But IHFANs, when the large concentrations (10^{-5} - 10^{-3} M), not only decreased the intrinsic BSA fluorescence, but IHFANs shifted the fluorescence maximum to more short-wavelength. This indicates the initiation of more polar state of environment for BSA tryptophane residues.

Ключевые слова: Мелафен; гибридные антиоксиданты; бычий сывороточный альбумин, флуоресценция.

Основная цель работы - исследование действия двух типов синтетических биологически активных веществ на растворимые белки сыворотки крови млекопитающих. Первая задача – тестирование с помощью спектральных методов влияния регулятора роста растений – Мелафена (меламиновая соль бис (оксиметил) фосфиновой кислоты), на структурные свойства бычьего сывороточного альбумина БСА. Исследование действия водных растворов Мелафена в широком концентрационном диапазоне (10^{-21} – 10^{-3} М) не выявило значительных изменений структуры БСА при концентрациях Мелафена, применяемых в сельском хозяйстве (10^{-18} - 10^{-10} М). Второй задачей было тестирование действия потенциальных нейропротекторов – гибридных антиоксидантов ИХФАНов, созданных на базе фенозана с добавлением холина и алкилгалогенидов, в широком концентрационном диапазоне (10^{-21} – 10^{-3} М) на структуру БСА. ИХФАНы защищали молекулы БСА от деструкции при низких и сверхнизких концентрациях (10^{-18} - 10^{-10} М), по-видимому, в результате адсорбции алкилгалогенидных «хвостов». Но большие концентрации как ИХФАНов, так и Мелафена вызывали «разрыхление» молекулы БСА, что выражалось в тушении собственной флуоресценции БСА. При этом максимум флуоресценции БСА в присутствии ИХФАНов сдвигался в более коротковолновую область, что свидетельствует о конформационных перестройках молекулы БСА.

Introduction

The main goal of this work was the investigation of the actions of two types of synthetics biological active substances to the soluble proteins that enriched the animal's blood serum. The first task was the test of the influence of plant growth regulator, applied in agriculture, - Melafen, to the structural properties of soluble protein - bovine serum albumin (BSA). The second task was the investigation of the influence of hybrid antioxidants IHFANs that was expected to use as neuroprotectors, to the BSA structure properties.

The first targets are the blood cells and the components of blood plasma also, when biological active substances appeared into the blood-vascular system. This is why we carried out the serum albumin as the test for investigations of biological active substances actions. Serum albumins are water soluble globular proteins. BSA is a simple model of primary serum targets. The serum albumin has small size and, as the component that enriched blood plasma up to 50%, plays the essential role in the maintaining of osmotic balance. As albumin enriches the blood sera, it facilitates the correct distribution of tissue liquid at many cases. The total area of all surfaces of albumins is biggest thanks to large amount of molecules and little molecular size of albumin. Besides this, the molecule may adsorb as hydrophilic, and the hydrophobe materials. These is why, the albumins are high effective carriers for most different molecules in blood plasma. And BSA takes essential part in transport of fatty acid, ions, vitamins, hormones and other materials that are needed to animal's organism good functioning.

So, the main purpose of our work was to determine how the aqueous solutions or emulsions of two types of synthetic biological active substances: Melafen and IHFANs, in the wide region of concentration (10^{-21} – 10^{-3} M) influence to the structure of soluble proteins with animals originated. Due to the present work the Melafen and IHFANs, action on soluble proteins has been examined. By this obtained data we may suppose or even predict how the biological active substances will be influenced on the protein's structure as well as protein-lipid interactions. Both these parameters are very important for a number of biological functions of

albumins at animal body. So, if Melafen or IHFANs have some successful effects on albumin molecular structure, which may be followed by any changing of albumin functional properties, too. At this case, the transport function, or ability to support the osmotic balance will be changed, too.

Thus the selection of BSA as of experimental object was determined by the number of causes. Albumins have the famous structural and functional properties. The serum albumin is 50% from mass of all containing in blood sera of proteins. This is one of the first targets for biological active substances in blood serum composition. BSA is the perfect carrier for a numerous materials: endogenous ones, like some free fatty acids, hormones, metal ions, bilirubin and etc., and exogenous ones (for example, materials that we want to test). Its structure is labile, and varies very easily. The molecular interaction of serum albumins with transported materials is determined of albumin's structural mobility, conditioned by the loop's stowage of one polypeptide chain, composed of 582 amino acid residues. Polypeptide chain forms 9 loops that are fixed by 17 disulfide bonds. It is assumed that the polypeptide chain is laid in three more or less independent cooperative domains. One free SH-group exists in albumin molecule, which can take part in education of disulfides. Disulfides are at the core of trigger assembly of denaturation of this protein.

Some changes of serum albumins conformation were registered on change of extent of quenching its intrinsic fluorescence. The numerous works is performed by this time that using of this approach for the test of actions of any biological active substances on albumins [1]. The albumin's binding with the exogenous synthetics materials we tested by using the registration of the intensity of intrinsic fluorescence of the BSA. BSA contains 2 tryptophane residues in hydrophobic regions of its molecule. There is the fluorescent emission of 2 tryptophane residues in hydrophobic regions of molecule BSA after excitation of tryptophane. First residue is located with close to a surface, second residue located at the deep inside of the protein globule. When the BSA molecule loosening, or unfolding, the availability of tryptophane residues for quencher - oxygen, witch was dissolute in water, increase greatly. The quenching of tryptophane fluorescence was observed at this case. These changes of BSA tryptophane fluorescence intensity we registered with or without Melafen or IHFANs, when the wide concentration's region. And on the base of these data we may conclude, what Melafen- or IHFANs-aqua solutions or emulsion, under the certain concentration's region, can influence to the BSA structure. Than we may suppose how our tested materials and under what region of concentration may have any influences to the functional properties of BSA.

The first task was the test of the influence Melafen to the structural properties of soluble proteins - bovine serum albumin (BSA). Melafen is a plant growth regulator - heterocyclic organophosphor compound, synthesized at the A. E. Arbuzov Institute of Organic and Physical Chemistry of RAS. Melafen is the melamine salt bis (oximethyl) phosphinic acid. It was acquired by one stage with high stepping out of industrially available products [2]. Melafen is a hydrophilic poly functional substance.

Melafen raises the plants stress-resistance in the conditions of overcooling and drought, increasing the effectiveness of energy metabolism. In this case Melafen causes the change of the fatty acid composition and the microviscosity of microsome and mitochondrial membranes in vegetable cells [4, 5]. Melafen is the strong regulator of plants stress tolerance under the bed environment. Aqueous solutions of Melafen at concentration 10^{-11} - 10^{-9} M increased the plant growth, but the raising of concentration of Melafen up to 10^{-8}, 10^{-7} M leads to plant's seeds dye. Therefore, our studies were carried out in the wide region of concentration (10^{-21} – 10^{-3} M).

Taking into account the close interdependence of plant's and animal's bodies in nature, it was necessary to investigate the action of plant growth regulator at any objects of animal origin. The primary targets for biological active substances in animal's cells are membrane and their components. Performed analysis of actions of aqueous solutions of Melafen to the structural and functional characterizations of lipids and protein that built into the cellular membranes [6-8], was complemented by the testing of Melafen influences on free soluble proteins unbounded with membranes. As such model the protein of bovine serum albumin (BSA) was used.

The second task was the investigation of the influence of hybrid antioxidants IHFANs to the BSA structure properties. IHFANs were expected to use as neuroprotectors. For the experiments IHFANs were used as the aqua-ethanol suspensions in the wide region of concentration (10^{-21} – 10^{-3} M). IHFANs are the derivatives of antioxidant phenozan (β - (4-hydroxy-3, 5-di-tert-butylphenyl) propionic acid). Phenozan was created for stabilization of polymer at Institute of Chemical Physics of RAS Moscow [9]. It is known that the antioxidants often shall be used for the therapy of any pathological states. The derivative of phenozan – its potassium salt, was tested as biological active materials. It was turned out that potassium salt of phenozan exhibited the property of strong antioxidant and structural effectors on enzymes and on biomembranes [10]. However, the phenozan didn't have the certain targets for its actions at membrane. As, the amphiphilic agent, phenozan acts primarily at all regions of surface layers of biomembrane, as in exterior and in internal sheets of bilayer. It was appeared, that phenozan penetrated through biomembrane defects to internal surface of bilayer plasma membrane. These phenomenons were discovered by the ESR (electron spin resonance) on erythrocytes when using of spin-labelled probes [11]. For substances ingression into deeper layers of membrane without defects, it was necessary synthesized the more hydrophobic antioxidant. For that purpose the choline esters was added to phenozan that was quaternized by long chain alkyl halogenides with number of carbon atoms from 8 up to 16 - ((β - 4-hydroxy-3, 5-di- tert- butyl phenyl) propionyl butyl] ammonia halogenides that were named IHFANs [12].The series of hybrid multitarget antioxidants – IHFANs for orientation of antioxidant action, had been synthesized in

IBHF RAS. Constructions were biological active. IHFANs were based on phenozan with conservation of screened phenol. And one choline residue and one alkyl residues of different lengths (C8-C16) were inserted in that the complex molecules. So that molecules received antioxidant activity, and bought the new activities: the anticholinesterase activity. And IHFANs received the ability to penetrate bilayer by introducing of alkyl residues of different length (C8-C16) into the hydrophobic regions [13].

The hybrid antioxidants – IHFANs have a charged onium group and a lipophilic long-chain alkyl tail. These structures of complex molecules allowed them to interact effectively with a charged lipid bilayer, insert to hydrophobic regions of cell membranes and maintain the antioxidant status. These molecules are bounded on membrane surface by the positive charge on quaternary nitrogen (the anchor), and the alkyl residue (the float) is introduced in bilayer being disposed among fatty acid's residues of phospholipids. And alkyl halogenides with variable length, that were added to phenozan: R-C8H17; C10H21; C12H23; C16H33, were implemented to the membrane bilayer on the different deeps. Thus, ICHFANs localized in anion heads regions and in fatty acids tails. This phenomenon fortifies the membrane structure so much. And the membrane became resistant to any bad environment actions.

Performed analysis of actions of aqueous suspension of IHFAN-C-10 to the structural and functional characterizations of protein, that built into the cellular membranes – the erythrocytes and its ghost [14, 15], were complemented by the influence testing of ICHFANs on free soluble proteins unbounded with membranes. The protein of bovine serum albumin (BSA) was used, as such model. These experiments of IHFANs influencing to BSA structure, were similar as experiments of Melafen testing that were performed with aid of the method of quenching its intrinsic fluorescence.

Materials and methods

The materials: BSA (Sigma). Melafen (melamine salt bis (oximethyl) phosphinic acid) was synthesized at the A. E. Arbuzov Institute of Organic and Physical Chemistry of RAS, Kazan. IHFANs ((β - 4-hydroxy-3, 5-di- tert- butyl phenyl) propionyl butyl] ammonia halogenides were synthesized at Institute of Biochemical Physics of RAS, Moscow.

BSA was used as the aqua solutions. Melafen was used as the aqua solutions in the wide region of concentration (10^{-21} – 10^{-3} M). IHFANs were used as the aqua-ethanol suspensions in the wide region of concentration (10^{-21} – 10^{-3} M).

The standard methods with the standard conditions had been used for measurements of fluorescence quenching: 1mkM of BSA protein aqua solution, 20^{o} C. The quartz cell (1 sm.) was used for BSA (with or without Melafen or IHFANs) fluorescent intensity registrations by fluorescent spectrophotometer "Perkin-Elmer MPF-44B". The spectrophotometer "Specord M 40" was used for measurements of optical density of BSA protein aqua solutions when low concentrations.

Results and discussion

The first task of this work was the supporting of the Melafen influence to the albumin structure. Melafen at this case was the one of the factor of the certain variable bed environments. It is clear that the probability of albumin molecules to release and absorb the fatty acids and another absorbed substances was under the strong influence of environment. Melafen increase the crop producing power of vegetables and seeds. The plant cells drastically increased its metabolism under the treatment by small doses of Melafen. This treatment leads to high magnification of plant's resistance to difficult environment. But Melafen's treatment leads to great oppression of plant development and even death when high Melafen concentrations. The Melafen-BSA interactions were tested in the wide region of concentration (10^{-21} – 10^{-3} M). For determining of the Melafen-BSA interaction we provided the spectral analysis. Data of the dependence of optical density from varied concentrations of Melafen supported that the effect of aqueous solutions of Melafen on absorption spectrum patterns of BSA was negligible when varied concentrations. The shape of BSA absorption spectrums, the locations of maximums of absorption didn't was changed drastically, when concentrations of Melafen were varied. Maximum of absorption spectrum BSA didn't not shift, however occurred the some change of the absorption degree and of spectrum shapes. Data of the dependence of correlation of BSA optical density with Melafen to BSA optical density without Melafen (D_{mel}/D) from Melafen concentrations were presented at fig. 1. The correlation (D_{mel}/D) was under the polymodal small changing, when Melafen concentrations varied.

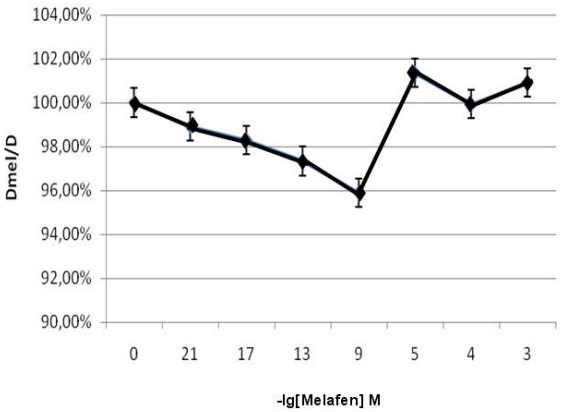

Fig. 1 - The correlation of BSA optical density with Melafen to without Melafen dependence on Melafen concentrations

Data of Melafen influence to the BSA absorption spectrums provides the evidence about absence of the covalent linkage between molecule Melafen and protein BSA. However, when registration of fluorescence spectrum, the great influence of aqueous solution of Melafen in the wide region of concentration (10^{-21} – 10^{-3} M) to conformation of BSA molecule existed. All solutions under the different concentrations of Melafen were not displaced the wavelength of fluorescence maximum. However the fluorescence intensity had undergone a change. The greatest quenching of

tryptophan fluorescence was found, when 10^{-4} M of Melafen. And the burst of fluorescence intensity was found too, when 10^{-17} - 10^{-10} M (fig. 2).

The data that fluorescence of BSA tryptophane residues was quenched by Melafen under the wide region of concentration were presented at fig.2.

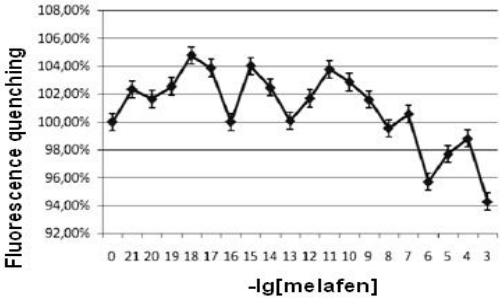

Fig. 2 - The Melafen influence to the fluorescence intensity of BSA. The control samples in absentia of Melafen have been adopted the fluorescence intensity as 100%. More 100% - was some burst of fluorescence intensity, less 100% – was the quenching of fluorescence intensity

The shapes of emission spectra were similar, only the maximal value of fluorescence intensity was change in dependence from the Melafen concentrations. Than, we build a curve of the dependence of value at the maximum of tryptophan emission from Melafen concentration (fig. 2). We obtained the noticeable tendency of the fluorescence quenching by Melafen, when large concentrations. And the some increasing of fluorescence intensity was occurred when small and ultra small concentrations of Melafen presented at the experimental medium. The dose-dependence was polymodal, which is representative for biological active substances, effectual in small and ultra small doses [16]. Evidently, conformational rearrangements occurred in BSA molecules. These rearrangements were small, and had the different direction.

The second task of our work was the investigation of the influence of hybrid antioxidants IHFANs to the BSA structure properties. IHFANs were expected as protectors of neuropathology. IHFANs were used as the aqua-ethanol suspensions in the wide region of concentration (10^{-21} – 10^{-3} M). The spectral analysis of BSA-IHFANs interactions was occurred as well as of Melafen–BSA interactions testing.

The soluble protein the serum albumin (BSA) in the presence of large concentrations of testable materials was turning, and becomes greatly accessible to water introduction. In the presence of low concentrations, on the contrary, the protein structure appears to much be getting stronger. The alkyl-halogenides tails of IHFANs are adsorbed on protein and protect it from molecular untwisting and the water penetration into deep disposed BSA molecule locus. The degree of protection was depended on length of alkyl tail directly in proportion. The maximum of protection has been found for IHFAN-C16 interaction with BSA (fig.3).

The proteins, which tryptophanes are contained, absorb the light near 280 nm, and the fluorescence spectrums are shifted to the short wavelength in contrast with tryptophane spectrums in water. Respectively importance their maximums may change from 343 nm (the serum albumin). Primary cause of this is the processes presence of orientation interaction, in which the spectrum regulation is determined by the polarity and microenvironment rigidity of chromophore. The shifts of tryptophan's emission maximums to the short wavelength is common to no polar environment, to long-wave - for polar environment. The emitting maximums of proteins reflect average the availability their tryptophane residues in aqueous phase.

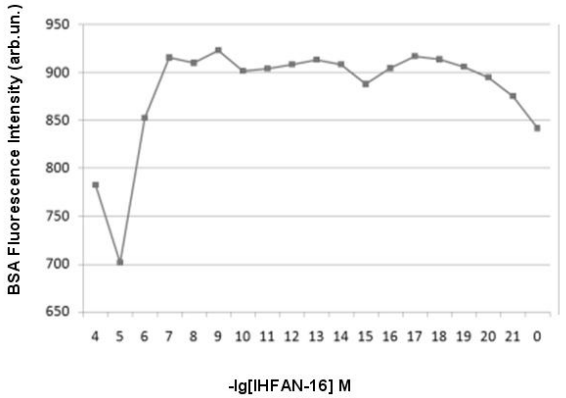

Fig. 3 - The IHFAN-C16 influence to the tryptophan fluorescence intensity of BSA. The fluorescence intensity BSA dependence on concentration IHFAN-C16 (10^{-21}-10^{-3} M)

As was shown at fig. 4 the emitting maximums of BSA were shifted under the varied IHFAN-C16 concentrations. This shifts were bigger when IHFAN concentrations were 10^{-6} - 10^{-3} M. It was likely that IHFANs "stick all over" the molecule of BSA and as result the environment of tryptophane residues becomes more hydrophobe, than at IHFANs absences or when low their concentrations exist.

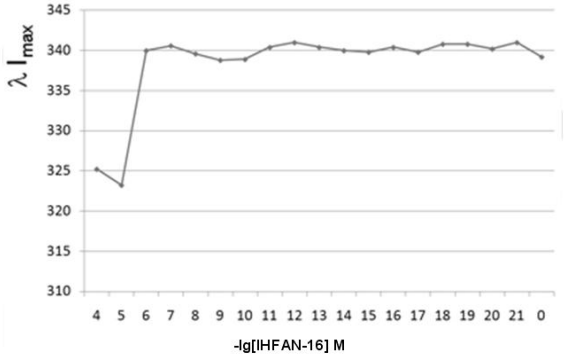

Fig. 4 - The influence of IHFAN-C16 (10^{-21}-10^{-3} M) to the tryptophane fluorescence intensity of BSA. The dependence of changes of wavelength of BSA fluorescence maximum from concentration IHFAN-C16

When we test the IHFANs actions at the soluble protein BSA, we found the decreasing of tryptophan fluorescence when 10^{-4} M and the raising of tryptophan fluorescence when 10^{-17} - 10^{-7} M. While for IHFAN-C16, which have the greatest alkyl-halogenides tail, the inflammability had been greatest. It was likely, IHFANs

adsorbed at BSA surface, when low IHFAN's concentrations. So, IHFANs, when low and ultra low concentrations, defend tryptophan residues from contacts with water. And, IHFANs change the BSA structure, when large concentrations. And, it was likely this phenomenon increased the availability of tryptophane for water. Some oxygen, which was solute at water, quenched the intrinsic tryptophane fluorescence of BSA molecules. But IHFANs, when the large concentrations (10^{-5} - 10^{-3} M), not only decreased the intrinsic BSA fluorescence, but IHFANs shifted the BSA fluorescence maximum to more short-wavelength. This indicates the initiation of more polar environment for BSA tryptophane residues.

Conclusion

Evidently, Melafen molecules affected to the albumin so that under the small and ultra small concentrations there was the preserving of the protein tryptophane residues from quenching from oxygen, dissolved in water. And under the large Melafen concentrations the change of protein conformation became so essential. At this case the tryptophane residues that lying at deep molecule locus became more available to water (and oxygen, respectively), on that indicated the fluorescence quenching. Occurs "the loosening" of BSA molecule structure. We may conclude that the soluble proteins that unhardened of the membrane lipids were under the essential Melafen actions. Taking into account that Melafen is the hydrophilic substance, and it can change the water environment. At this case we may suppose that Melafen influence to BSA by two ways: mediated through the water, or directly to the influence to hydrophilic sites of BSA molecules. Mechanism was unknown. These influences were mainly changed in dependence on Melafen concentration present in surrounding solution. There were not clear evidences of BSA-Melafen linkage existence. However mediated action through the change of water medium appears to occur surrounding the protein's molecules.

Also the water solutions of Melafen may be the regulator of transporting function of albumins, as its will be introduced to the animal's body. And it may be take part in extracting fatty acids from any molecules, or bounding of free fatty acids by albumins. As its known the water solutions of Melafen change the fatty acid's content of membranes [5].

The IHFANs actions on the BSA structure were different from Melafen actions. Evidently, because the Melafen – is the simple neutral hydrophilic substances, but the IHFANs are the complex substances with hydrophobic part - long chain alkyl halogenides, and hydrophilic part - positive charge on quaternary nitrogen. By this molecules of IHFANs are bounded on BSA surface by the positive charge and introduced to the deep locus of BSA by chains alkyl halogenides. As was shown at our work, the IHFANs protect BSA molecule from "loosening" of molecule BSA structure. And the region of this nondestructive IHFANs concentrations was extended more (10^{-21}-10^{-7} M), than for Melafen nondestructive concentration's region (10^{-21}-10^{-9} M). The degree of protection was depended on length of alkyl tail directly in proportion. The maximum of preserving has been found for IHFAN-C16. It may be supposed that alkyl halogenides tails what having occupied all hydrophobic regions in albumin molecule, in practice came to form the containment shell from water encroachment to tryptophane residues. Respectively and the oxygen, which was dissolute in water, was failed to penetrate to tryptophane residues. Thus the mechanisms of BSA-Melafen or BSA-IHFANs interactions were different. There were not the covalent binding, but the grade of absorption, points for absorptions were different. The "loosening" of molecule BSA structure occurred, when the large concentrations (10^{-5} - 10^{-3} M) of Melafen were presented at medium for intrinsic BSA fluorescence registration. And IHFANs, when the large concentrations (10^{-5} - 10^{-3} M), not only decreased the intrinsic BSA fluorescence. IHFANs shifted the fluorescence maximum to more short-wavelength. This indicates the initiation by IHFANs of more polar environment for BSA tryptophane residues.

The data, obtained at this work, suggest to the fact that albumin may be one of the first target in blood, in particular, in blood plasma, was exposed to as Melafen, and IHFANs, used in the wide region of concentration (10^{-21} M – 10^{-3} M). The albumin structure was varied under the presence of Melafen or IHFANs. Respectively, the presupposition was arising that if some properties of albumin change, as carrier of biologically active substance and as osmotically active substance. Albumins maintain the colloid-osmotic pressure at blood plasma and at other fluids (in cerebrospinal fluid, for example). It can be assumed that the transport effectiveness will be decreased and can be unbalanced of osmotic pressure in compartments with biological fluids. This is why the application these materials demands the great cares and the observances of concentration limitations, because the soluble protein of animal origin - bovine serum albumin, changes his structural properties in their attendance so much.

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