monomer units ($\bar{l} = \overline{P_\eta}$ /N_i) or the average length of the section of macromolecules of polycation per one-protein globules in composition of polycomplex particle. It follows from this figure that the higher the % content of N-cetyl fragments in composition of polycation, the higher the "$\bar{l}$ " is (Figure 62).

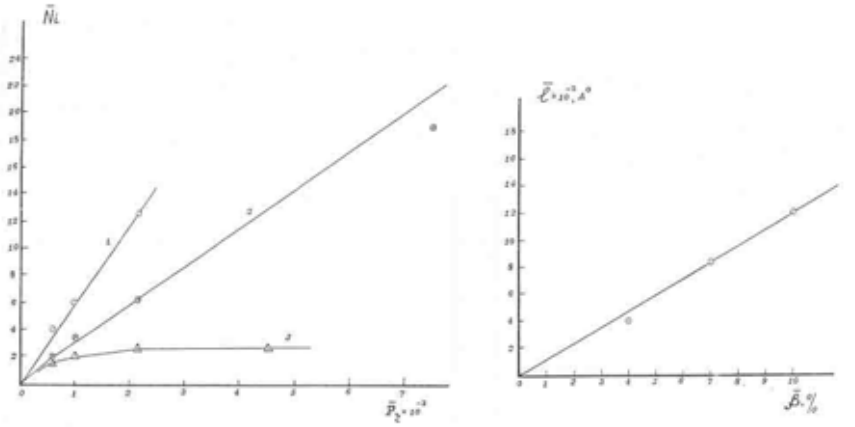


Figure 62A. Dependence of characteristic composition (N_i) of polycomplexes on degree of polymerization of PVP(R_0,R_{16}) at different β, %: 4 (1), 7 (2), 10 (3).

B. Dependence of the average value ($\bar{l}$) of the length of PVP(R_0, R_{16}) per one protein globule in polycomplex particles with composition of N_i on β (the results was obtained from Figure 62A)

The molecular characteristics of the particles of the soluble BSA-PVP(R_0, R_{16}) complexes with the composition $n_{BSA}/n_{PVP} = N_i$ were determined by light scattering measurements. For all investigated polycations over the whole studied range of concentrations the dependence of KxC/R_θ on the concentration was linear, indicating the absence of dissociation of the polycomplexes at dilution (Figure 63). In other words, the studied soluble BSA-PVP(R_0, R_{16}) polycomplexes are stable over a wide range of the solution concentrations and polycomplex composition.

Table 5.

Компоненты	dn/dc	$\bar{M}_w \cdot 10^{-3}$	$\bar{M}_z \cdot 10^{-3}$	$\bar{N}_c$	
				светорассеяние	седиментация
БСА	I,8	70	–	–	–
ПВП(R_0, R_{16})-I-6,5	2,2I	70	70	–	–
ПВП(R_0, R_{16})-II-7,0	2,I9	I20	II6	–	–
ПВП(R_0, R_{16})-II-8,0	2,I7	I25	II8	–	–
ПВП(R_0, R_{16})-II-I4,0	2,68	I30	I26	–	–
БСА+ПВП(R_0, R_{16})-I-6,5	2,I5	I20	I40	0,7	I,0
БСА+ПВП(R_0, R_{16})-II-7,0	I,85	320	336	3,5	3,0
БСА+ПВП(R_0, R_{16})-II-8,0	I,93	240	290	2,5	2,0
БСА+ПВП(R_0, R_{16})-II-I4,0	2,32	250	260	2,0	2,0

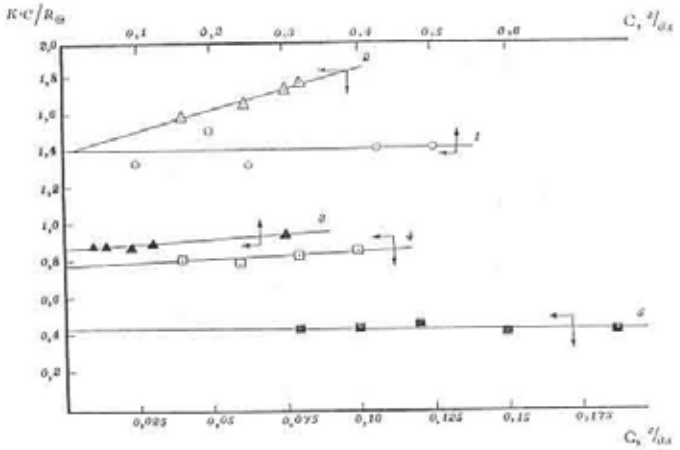


Figure 63. Dependence of K.c/R0 on C. 1-BSA; 2- PVP(R_0, R_{16}); (β=6,5), 3-BSA+PVP(R_0, R_{16}) (β=6,5); $n_{BSA}/_{PE}$=1 4-PVP(R_0, R_{16}); (β=8); 5-(BSA+PVP(R_0, R_{16}) (β=8) $n_{BSA}/_{PE}$=3; 0,5% CH_3COOH; pH 4.3 λ=4360A°

The results of the studies of BSA-PVP(Ro,R_{16}) complex solutions with different composition formed by polycations with a different molecular mass, Mw, and degree of quaternization " $\overline{\beta}$ " are listed in Table 5

As it is seen from the Table 5 the average values of molecular weights calculated from the sedimentation data, $M_s = (M)_{PVP} + N_i \times M_{BSA}$ are in a good agreement with the values Mw obtained by light scattering measurements. From these data it follows that only one polycation chain is contained in the particles of soluble BSA-PVP(R_0, R_{16}) complex.

Some information about the structure of the BSA-PVP(R_0, R_{16}) complexes are obtained from the comparison of the inherent viscosities and the sedimentation coefficients for these complexes formed by the polycations with different length at given degree of quaternization when $n_{BSA}/n_{PVP}=N_i$. It is seen in Figure 64 that the viscosity of the solutions sufficiently and strongly increases with increasing degree of polymerization of the polycation. At the same time the sedimentation coefficient changes insignificantly. The relation of this kind may exist for sufficiently extended particles at their elongation. The result obtained from the electron microscopy is quite conform with this conclusion (Figure 64): The separate particles of polycomplex with length 400-500 Å and thickness 100 Å are distinctly seen in the micrograph (the contour length of polycation is about $2.5x10^3A^o$, " $\overline{\beta}$ "=8 mol%).

Interaction between proteins and PVP(R_0, R_n) is not followed by any changes of the protein secondary structure (Figure 65)

Thus, the bindings of protein globules with polycations were realized by the results of introducing of some N-cetyl radicals (R_{16}) into hydrophobic parts of protein molecules. One can assume that, the molecules of protein in composition of polycomplex particles are in contact with each other and such positive interaction results in an additional decrease of the free energy exceeding; a free energy increase caused by the disturbance of the randomness of the distribution. In present situation, however, the last approval is not so evident, since non-uniform distribution may be also cause of necessity of partial destruction of initial compact structure of polycation-sorbent. Moreover, for the explanation of the correlation of protein binding capacity of PVP(R_0, R_n) with its conformation transition "coil-compact structure" one can suggest that the fragments

of initial compact structure of polyelectrolyte remain also in structure of polycomplex particles and is of importance of its stabilization. These fragments, probably, are the intramolecular clusters forming by the lateral hydrophobic R_n radicals, which are not directly involved in the complex formation (Figure 66).

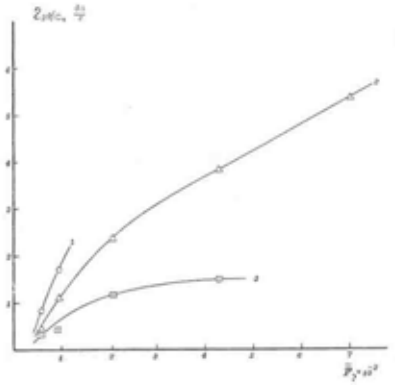


Figure 64. Dependence of inherent viscosity ($\eta_{Sp/C}$) of PVP(R_0,R_{16})-BSA complexes $n_{BSA}/_{PE}=N_i$ on Pη of PVP(R_0,R_{16}) at different β, %: 4 (1), 7 (2), 10 (3).

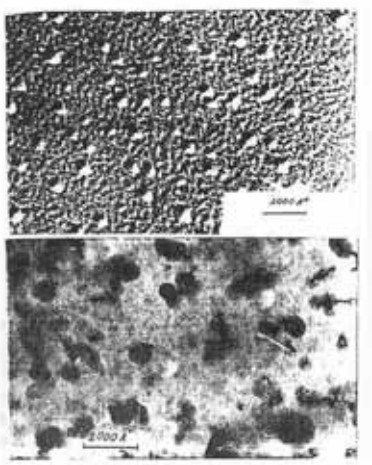


Figure 65 Electron micrographs of PVP(R_0, R_{16})-BSA complexes a-method freezing-etching; b-method thermal attachment. (0,01 g/dl). $n_{BSA/PE}=3$; $P\eta=10^3$; β~8%

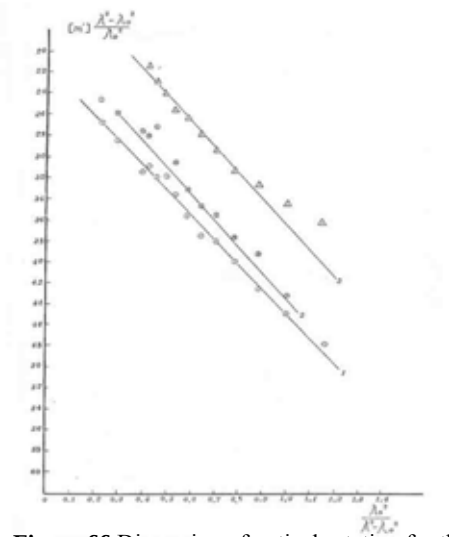


Figure 66 Dispersion of optical rotation for the solution of free BSA (1) and PVP(R_0, R_{16})-BSA complexes at different n_{BSA}/PE: 1 (2); 4 (3); processing with Moffite equation.

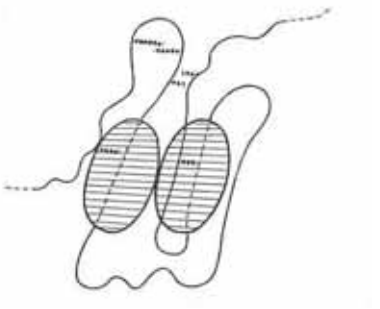


Figure 67 Proposed structure of BSA-PVP(R_0, R_n) complexes

It is clear that the self-organization of a whole structure requests the overcome of the electrostatic repulsion at the approach of the positive charged protein molecules and positive charged section of PVP(R_0, R_n) chains. It is only possible in the case when the R_n radicals are

characterized with sufficient length and amount, i.e. contribution of hydrophobic interaction energy must be predominating. The susceptibility of polycations to compactization is increased at the transition of the length of lateral radicals from R_{10} to R_{16} and at $\overline{\beta} \geq 3\%$: the number of protein globules sorbing by one polymer chain with given counter length is increased correspondingly (i.e. N_i).

An increase in protein content (at the ratios $n_{BSA}/n_{PVP} > N_i$) in mixtures results by the "polymerization" of polycomplex particles, which leads to the increasing of its molecular mass and asymmetry. Moreover, only a few of excess protein molecules were included to this process; the most part of them remains free and characterized as a separate peak on sedimentograms.

When NaCl solutions are added to polycomplex solutions at pH 4.3 above its critical concentrations (C_{NaCl} 0.154 M) and (or) pH of solutions were increased (pH $\geq$ 5) the water-soluble complex lost part of protein molecules and transform to insoluble state at what only one protein molecules bound by one polymer chains. These results are very important for the creation of stable polycomplexes in the conditions (ionic strength and pH) of living organism.

Modeling of nucleoprotein complexes

Complexes of proteins and linear natural polyions, which include chromatin, ribosomes and other cell components as well as viruses, are widely distributed in nature and fulfill an important function. It can be assumed that the basic contribution to the stabilization of the complexes is from cooperative electrostatic interactions involving ionogenic groups of proteins exposed on the surface of the globule. These interactions may result in soluble complex formation between nucleic acids and histones, complex coacervation, or the formation of amorphous precipitates. Such phenomena are also undoubtedly significant in the cell, where the Coulombic association of DNA with basic histones leads to the collapse of the nucleic acid and where basic polypeptides such as polylysine are thought to profoundly influence DNA behaviour. Similar electrostatic interactions between proteins and nucleic acid are likely to play a role in the transcription process.

It was found that protein interaction with poly-N-ethyl-4-vinylpyridinium bromide in aqueous solution results in self assembling of the asymmetrical aggregates [33-35,36-39]. Such aggregates may be considered as models of specific nucleoproteid complexes i.e. viruses, chromatin, ribosome strands and other cell components in complicated biological systems. It is quite probable that the principle laid in the basis of such nucleoproteid structure construction, is actually very general. To realize this principle it suffices to fulfill two thermodynamical conditions: 1) a possible cooperative coupling, i.e. "sticking" of a globule to an open linear chain; 2) sufficiently "strong" positive interaction of the protein adsorbed globules with each other. A necessary kinetic condition for self assembling is a possibility of slipping of the adsorbed globules along the chain-sorbent in the process of complex formation, in order to find optimal contacts among them, and using the trial and error method to permit the whole system to acquire the structure corresponding to the minimum of the free energy.

3. POLYELECTROLYTE-METAL-PROTEIN COMPLEXES

As it was mentioned above, polymer-protein complexes (PPC) is formed as a result of the interaction of polyion chains with the oppositely charged groups of the protein molecule during these reactions. The extent of the interaction is found to be pH and ionic strength sensitive and dependent on the isoelectric points of the proteins. Such systems include complexes stabilized by cooperative electrostatic and hydrophobic interactions between the fragments of PE and protein molecules and conjugates in which the functional groups of the components are linked by covalent bonds. In those cases where PE macromolecules do not contain the corresponding electrostatic or hydrophobic groups for protein binding, it is necessary to modify the carrier polymer (or protein), which can give rise to changes in its effect(s) upon biological systems. Moreover, covalent conjugation of polyelectrolytes with proteins may lead to partial changes in

the chemical structure of the antigenic (or catalytic, etc.) determinants as a result of their involvement in the formation of chemical covalent bonds. This approach is not technological, however, since the free components formed in the reaction system during the crosslinking procedure have to be separated from the main product, which requires additional labour expenditure and effort.

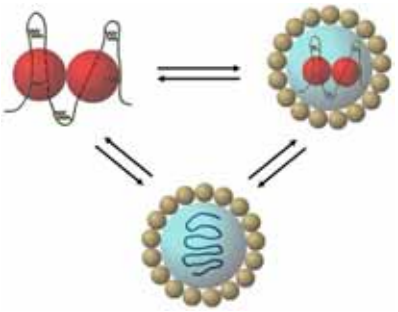
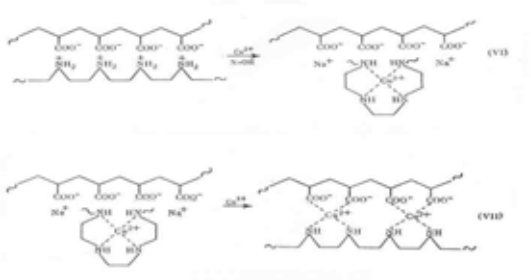


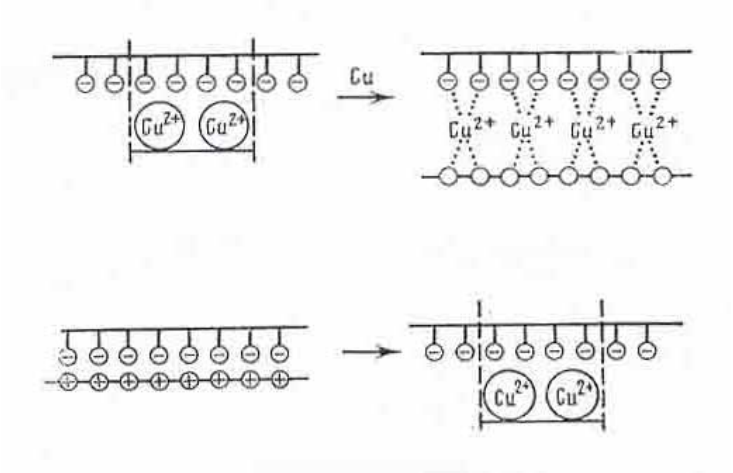
Figure 68. A schematic presentation of the structure formation in protein-polyelectrolyte mixtures at different protein/polyelectrolyte ratios (redistribution of protein and polyelectreolyte molecules in the structure formation in protein-polyelectrolyte complexes depending on the concentrations of protein globules).

A relatively new technique involves the use of transition metal (Me) compounds as means of activating the support surface and allowing direct coupling of proteins without prior to derivatization of the activated support, through formation of chelates [106,107]. Evidence has recently been presented for the existence of a ternary complex between proteins, Cu^{2+} ions, and amino acids [107]. Some publications in the current literature are devoted to the construction of drugs based on such Me-mediated complexes of natural PE and antigens [108-112]. These authors succeeded in demonstrating that polysaccharide-protein mixtures supplemented with metal ions are effective means of prophylaxis and treatment of some microbial infections in animal and human.

Metal chelate formation may proceed via binding with functional groups of two different macromolecules (usually one of them provides “acidic” functional groups, and the second “basic” groups). These studies are not numerous in number, although metal binding with mixed biopolymers, the process that is of great importance for biological reactions (replacement of histons from DNA, association of the ribosome components, etc.), is intensively investigated.
Both polymer-polymer compositions and interpolymer complexes are used as chelating agents for synthesis of such chelates. It has been shown by studying of the systems polyethylenimine (PEI)-PAA-Me (Me=Cu, Co, Ni) that Cu(II) ions give polycomplexes of two types [113-117]. The first ones contain four coordinated amino groups of PEI and cooperatively bind polyanions of PAA (at pH values above 10); other polycomplexes have two amino and two carboxylic groups in Cu(II) coordinative sphere (pH=7-9). Stabilization of these complexes is provided by formation of both salt-like and coordinative bonds:



Interpolymer complexes are usually formed within a short range of pH values. Thus, in the system PAA-P4VP-Cu(II) at equimolar ratio of pyridine and carboxylic groups only complexes of Cu(II) with P4VP are formed in acidic medium, whereas at pH 4.0-5.0 "mixed" complexes with chelate node $Cu(Py)_3(COO)$ are formed:



The effect of stability of restrictions of "triple" polycomplexes with regard to pH values can be used to carry out selective extraction of several metal ions (e.g., of Cu(II) ions by the systems PAA-PVP or by PEI-polyepichlorohydrin [115]).

Mixed ligand macromolecular metal complexes derived from a combination of synthetic and biopolymers are of special interest. Several investigators have studied the solubility of polyelectrolyte-metal-protein mixtures in water medium. In particular, insoluble complexes of BSA with nonfractionated polyacrylic acid (PAA) in the presence of Ba^{2+} have been described by Morawetz *et al*. [30]. The soluble and insoluble ternary complexes of the positively (or negatively) charged narrow fractions of polyelectrolytes with same charged proteins in the presence of different transient metal ions have been firstly reported and systematically studied by Mustafaev [118-129]. Such systems include the same charged polyelectrolyte and protein mixtures and do not possess the ability for complexation without metal ions. Poly-4-vinylpyridine (PVP), poly N-vinylimidazole (PVI), PAA, poly(N-isopropylacrylamide) (PNIPAAm), polyacrylamides (PAAm) which contain amino acid end groups and copolymers of acrylic acid with 4-vinylpyridine, 2-methyl-5-vinylpyridine, vinylimidazole, N-isopropylacrylamide, N-vinylpyrolidone, (maleic anhydride-N-vinylpyrrolidone copolymers) and some others with different composition were chosen as polyelectrolytes. Proteins with different pI such as BSA, HSA, BGG, hemoglobin (Hb), globin (GL), transferrin (Tr), (Table 6), ovalbumin (OA), superoxide dismutase (SOD), surface antigens of the influenza virus, protein fraction of the BCG Mycobacteria cell, and some others were chosen as biopolymers.

As it was mentioned above the routes of ternary PE-Me-Protein complex preparation are very simple. Thus, for example, polycomplex can be obtained by a direct mixing of the solutions of the polymer-metal complexes with the solutions of proteins.

Polyelectrolyte-Metal Complexes. The formation of PE-metal complexes in mixtures of P4VP-Cu^{2+}, PAA-Cu^{2+}, PNIPAAm-Cu^{2+} and in mixtures of different copolymers with different transient metal ions were analyzed by the titration shift method and measurements by HPLC, cyclic

votammetry, spectroscopy, equilibrium dialysis, and fast sedimentation methods [124,130-135]. It was shown that water-soluble and insoluble stable polyelectrolyte-metal complexes are formed in the wide range of pH, and the solubility of polycomplexes depends on the metal/polymer ratios and pH of solutions.

The solutions of partially ionized polyelectrolytes, containing Cu^{2+} ions, were analyzed by the titration shift method, cyclic votammetry and HPLC measurements recently [124]. These data have implicated the presence of chelate with four pyridine groups in P4VP-Cu^{2+} and two carboxylate groups in PAA-Cu^{2+} mixtures bound to a copper ion, correspondingly. Fast sedimentation and quenching of luminescence have been employed to study the binding Cu^{2+} by PAA recent1y [136]. It was shown that the metal ions are unevenly distributed between the macromolecules. However, these findings were obtained only by the rather indirect method of quenching of luminescence. Therefore, in this investigation, the interactions between Cu^{2+} and PAA were at first analyzed by HPLC, which allows study of the fraction composition at relatively low concentrations of mixture. Addition of copper ions did not affect the solubility of PAA within a certain range of n_{Cu}/n_{AA} values at pH 7. The phase separation in the system PAA-Cu^{2+} occurred only at some critical metal concentrations ($n_{Cu}/n_{AA} = 0.25$). A typical HPLC analysis of PAA and of its soluble mixture with Cu^{2+} ions at different ratios of their molecular concentrations is given in Figure 69. The mixture of PAA-Cu^{2+} was characterized in chromatograms by two peaks. Moreover, the presence of Cu^{2+} gave rise to an increase the optical density of the mixture. The increase in the optical density (A_{280}) of the solution may reflect the involvement of the part of PAA in polymer-metal complexes (peak II). The bimodal distribution of components may lie in the uneven distribution of Cu^{2+} between the polymer coils, which appears to move more slowly than free PAA (peak I). The elution volume *(Ve)* corresponding to peak I does not change and remains equal to that of free PAA, but the values for the *Ve* of peak II differed from those values of the individual PAA peak. Free Cu^{2+} ions were hereby absent in the PAA-Cu^{2+} mixture as indicated by cycle votammetry (see below under Figure 69). Moreover, analysis by atomic absorption spectroscopy indicated an uneven distribution of Cu^{2+} among the peak fraction obtained by Sephadex G-IOO filtration of PAA-Cu^{2+} mixture [Figure 69(b)]. These findings indicated a nonrandom distribution of the copper ions between the polyanions under the experimental conditions ($n_{Cu}/n_{AA} < 1$). PAA-Cu^{2+} mixtures are, thus, implicated to consist of two fractions: PMC-I (PAA-Cu^{2+} complexes with relatively low concentration of Cu^{2+} ions and/or free PAA) and PMC-II (PAA-Cu^{2+} complexes with relatively high concentration of Cu^{2+} ions). When the ratio n_{Cu}/n_{AA} is 0.25, a phase separation took place in the system and the area of the peaks in the matrix solution was decreased. Both peaks disappeared when the ratio of n_{Cu}/n_{AA} is 0.4. It follows from these data that, at relatively high concentrations ($n_{Cu}/n_{AA} \geq 0.25$), Cu^{2+} promotes the crosslinking of the macromolecule, as a result of which the system loses its solubility.

3.1. Ternary Polyanion-Metal-Protein Systems

Insoluble Complexes. To produce a polymer-metal complex, various concentrations of the metal salts (for example $CuSO_4x5H_2O$, pH: 4) solution were added to PE, dissolved in phosphate buffer. The pH values were adjusted with 1M NaOH to the desired pH. The ternary complexes were, in turn, prepared by adding protein solutions to the polymer-metal complex (PMC) solution. Water-insoluble products of the complexes were investigated spectrophotometrically at 400 nm and by weighing dry amounts of pellet. Protein/polymer (n_p/n_{AA}) and Cu^{2+}/AA (n_{Cu}/n_{AA}) ratios were calculated using the equation $n=CN_A/M$, where n is the number of the molecules in 1 ml; M is the

molecular weight of components; N_A is the Avogadro number; C is the concentration in g/100ml. Curves of the turbidimetric titration of a copolymers solution in the presence of metal ions with a solution of proteins are presented as the dependence of optical density (or amount of precipitate) of the solution on the n_P/n_{AA} and C_P/C_{CP} ratios (C_P and C_{CP} -weight concentration of protein and copolymer correspondingly).

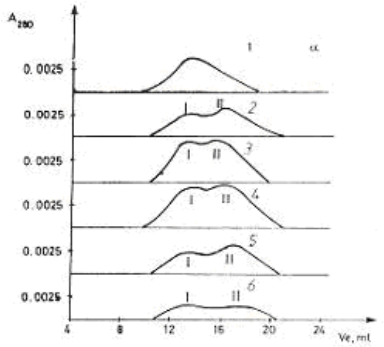


Figure 69. (a) Gel filtration HPL chromatograms of PAA (1) and of its mixtures with Cu^{2+} at different ratios of Cu^{+2} ions to acrilic acid monomers (AA) ((n_{Cu}/n_{AA}):2-0.08;3-0.10;4-0.15;5-0.20;6-0.25;(5-6)-the phase separation in the system takes plase. The concentration of PAA was 0.1 g/dL; PH7. Bio-Sil Sac 25 column was used in the experiment. (b) Distribution of the amount of Cu^{2+} in the mixtures PAA-Cu^{2+}

Table 6. Some physico-chemical properties of proteins

Protein	Origins	Molecular Weight (kDa)	Isoelectric Point (pI)
BSA	Bovine	67	4.7
HSA	Human	88	5.0
Tr	Human	88	5.9
Hb	Human	64	7.0
Gl	Human	16	6.8

Figure 71 presents the results of the turbidimetric titration for different ternary PE-M-Protein mixtures. As it follows from these figures in the general case the system is characterized by a three regions of reaction mixture composition and solubility; dependence of the optical density (A_{400} nm or precipitate amount) of the ternary mixtures on protein concentrations (at constant polymer-metal concentrations) passes through a maximum.

The system remains homogenous over a wide range of the protein/PMC ratios, i.e. the formed ternary complexes are soluble (region I) for the excess PMC case. The phase separation in the system occurs only at some critical protein concentrations depending on the degree of polymerization of PE, and metal concentrations. In this region II phase separation takes place, which indicates the formation of an insoluble triple complex. On further increase in C_p/C_{pe} the optical density increases and then attained a limiting value. The pattern changes significantly on further increase in protein concentrations. Depending on the protein concentration in the mixture, partial or complete prevention of phase separation then takes place and at some critical ratios of protein/PMC (region III) the system again becomes homogeneous.

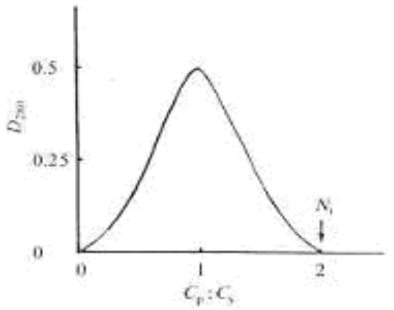


Figure 70. Dependence of the relative optical density (D) of the ternary mixture of protein (BSA), CP-1, and Cu^{2+} on the amount of protein (C_p) added at constant concentrations of CP-1 and Cu^{2+} (C_s).Point N_4 indicates the minimum weight of protein which must be introduced into the system to prevent precipitation.

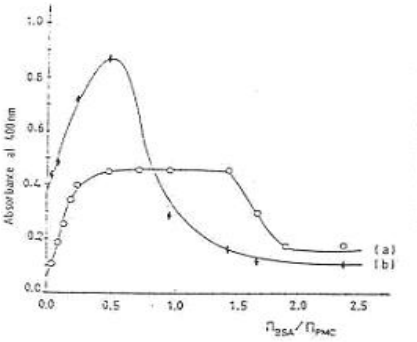


Figure 71. The Dependence of A_{400} on the ratio of components for the mixture PAAm-Cu^{2+}-BSA. The BSA solution at different concentration added to the solution of PMC at constant concentrations of PMC (n_{BSA}/n_{PMC}). Mw(PAAm)=20 000; 20 C°; pH 7; C_{PAAm}=0.15 g/dL;C_{CuSO4}-= 0.05 g/dL.(b) The PMC solution at different polymer concentrations of BSA (n_{PMC}/n_{BSA}) CBSA=0.5 g/dL; $n_{Cu^{2+}}/n_{PAAm}$ =0.1; ($C_{PAAm}\leq$3 g/dL;$C_{CuSO4}\leq$1.05 g/dL)

The solubility of the mixture depends on the nature (or pI) of the proteins and pH value of the solutions. Figure 70 presents the dependence of the optical density of the ternary mixture of different proteins, PAA and Cu^{2+}, on the amount of added proteins at constant concentrations of the PAA and Cu^{2+} (PMC). Starting with the ratio n_{Cu}/n_{AA} = 0.25, a phase separation took place in the systems Hb-Cu^{2+}-PAA and GL-Cu^{2+}-PAA at pH 7 even at very low concentrations of copper ions (n_{Cu}/n_{AA} =0.08). However, Hb and GL solutions mixed with PMC at higher values of pH gave rise to soluble ternary mixtures (Figure 71b). As for the systems BSA-Cu^{2+}-PAA, HSA-Cu^{2+}-PAA and Tr-Cu^{2+}-PAA, observations provided a different picture. The values of A_{400} did not change considerably over a wide range of metal concentration ($0 < n_{Cu}/n_{AA} < 0.25$) in the system with increase in the amount of protein added. However, there existed a critical concentration of Cu^{2+} ions in ternary mixtures ($n_{Cu}/n_{AA} \geq 0.25$), at which the system lost the homogeneity with formation of insoluble particles (see Figure 71). In conclusion of the results presented in this section it is suggested that the solubility of the ternary mixtures is closely correlated with the pI values of protein molecules (Table 6) and depends on the concentration of components in the mixture.

Studies of the role of different factors of reaction, environment permits to elucidate some important features characterizing ternary complex formation. The homogenous mixture of ternary systems shows some different behavior depending on the order of addition of protein to PMC or PMC to BSA (Figure 72). For mixture prepared by the addition of various protein concentrations to PMC solutions in the early stage, all systems were water soluble and homogeneous. However, depending on time and on the ratio of these mixtures, the homogeneity was lost and insoluble particles appeared in the system. For this case the absorbance of mixtures was measured after 24 hours. At the addition of PMC solutions to protein solution formation of the precipitate occurs on the same manner. However, as seen from Figure 74, the width of the curve of turbidimetric titration is narrower in last case. At the same time in both cases, independent of the method of preparation, the same character of the formation of soluble and insoluble ternary complexes versus ratios of components are observed. Thus the different ways of protein and PMC mixing differ by the intermediate states of ternary mixtures depending on the

component ratio in the mixture, but at a proper ratio the both lead to the formation of soluble polycomplexes.

It is critical that insoluble complexes, unlike soluble complexes which form instantaneously, take place depending on time. These results permit investigation of the velocity of the formation of the ternary insoluble complex by the spectrophotometric method at different reaction conditions (the effects of the preparing mixture, ratio of the components, low molecular salts, temperature and stirring, molecular weight of polyelectrolyte and Cu^{2+} concentration). Figure 75 shows the variations of the absorbance at 400 nm versus time for the mixtures, prepared by different methods and ratio of components. Obtained results showed that, the velocity of the formation of insoluble complexes in the case when BSA solution was added to PMC solution is faster. In this case the concentration of BSA was less than the concentration of PMC at the initial moment, in the system water soluble complex occurred. When PMC solution was added to BSA solution, because the concentration of BSA is small at the beginning of titration, insoluble ternary complex formed, therefore the reaction rate increased slowly, i.e. the diffusion process decreased. As can be seen with increasing ratios, the rate of reaction increases to reach a maximum value and then decreases. For a ratios which corresponds to soluble complexes, the value of dA/dt =0 (dA/dt-the rate of the reaction was obtained from the tangent to the curves in the early stages of the reaction).

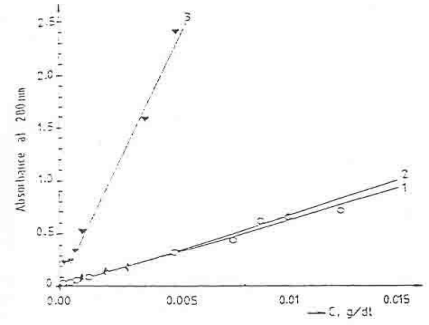


Figure 72. The dependence of λ_{280} of mixtures on concentration of BSA. The correlation coefficient (r)of straight lines are 0.9901 (1.BSA-PAAm), 0.9972 (2.BSA) and 0.9948 (3. BSA-Cu2+- PAAm), Mw=20 000; C_{PAAm} = 0.15 g/dL C_{CuSO4} = 0.05 g/dl)

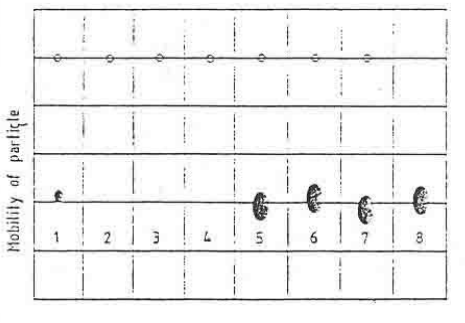


Figure 73. Electrophoresis of BSA and the mixtures of BSA-Cu^{2+}-PAAm (t=60 mins, V=300 volt). C_{BSA}=0.5g/dl (1); The concentrations of BSA in the mixtures of BSA in the mixtures of BSA-Cu^{2+}- PAAm are 0.075 g/dl (2), 0.125 g/dl (4), 0.375 g/dl (5), 0.5 g/dl (6), 0.875 g/dl (7) and 1.0 g/dl (8); C_{PAAm}=0.15g/dl; C_{CuSO4}=0.05 g/dl.

The mixture PMC-BSA was prepared at the different concentration of the sodium chloride to investigate the effect of the low molecular weight salts (Figure 76). With increasing NaCl concentration, the slopes of curves decrease and at higher concentration of the NaCl the ternary system becomes homogeneous and the absorbance against time practically does not change. Thus, the concentrations of NaCl affect not only a solubility of the system but also the velocity formation of the colloid particles.

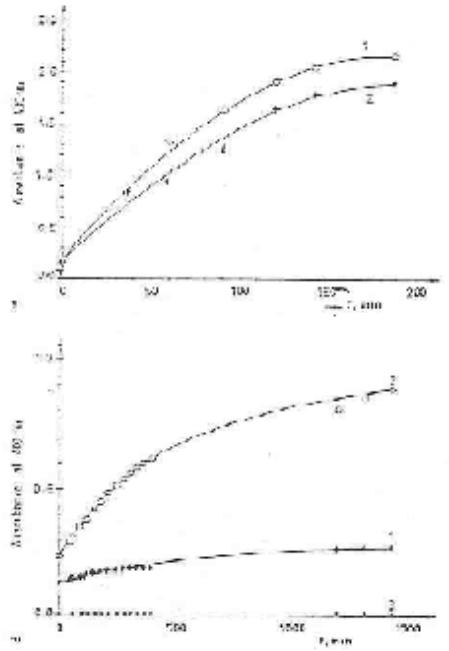


Figure 74. (a) The dependence of A_{400} of two mixtures BSA- Cu^{2+}-PAAm on times 1)BSA was added to the solution of PMC; 2)PMC was added to the solution of BSA; b)The dependence of the A_{400} of PAAm-Cu^{2+}-BSA mixtures on the time at three different (n_{BSA}/n_{PMC})=0.15 (1.dA/dt=0.15x10^{-3}); 1.0 (2. dA/dt=1x10^{-3}); 2.0 (3. dA/dt=0), (BSA was added to the solution of PMC) Mw (PAAm)=20 000.

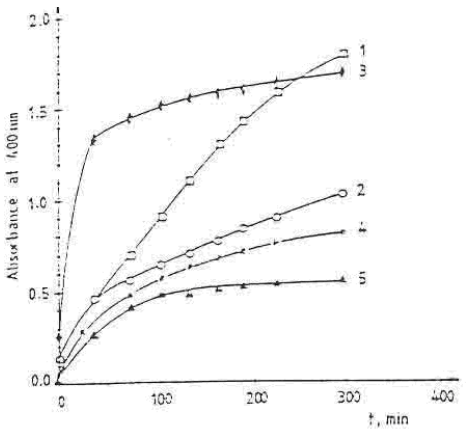


Figure 75. The dilution effect on the formation of the insoluble ternary mixture. The initial concentration of the mixtures of BSA-Cu^{2+}-PAAm; C_{BSA}=0.5 g/dl; C_{CuSO4}=0.05 g/dl; C_{PAAm}=0.15 g/dl; pH=7; 20 C°. C (1.dA/dt=0.67 x 10^{-3}); C/3 (2.dA/dt=0.89 x10^{-3}); C/5 (3.dA/dt=2.98 x10^{-3}); C/9 (4.dA/dt=1.02x10^{-3});C/17 (5.dA/dt=0.68x10^{-3})

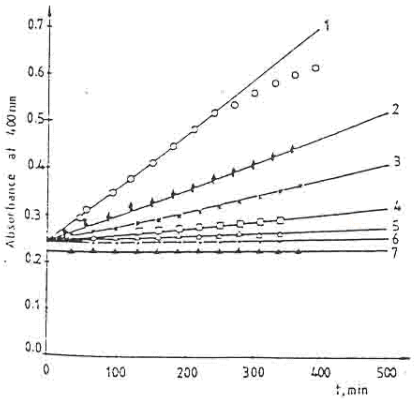


Figure 76. The dependence of A_{400} for the NaCl concentration in the mixture of BSA-Cu^{2+}-PAAm. pH = 7.0; 20°C; C_{PAAm} = 0.15 g/dl; C_{CuSo4} = 0.05 g/dl; C_{BSA}= 0.5 g/dl. 0 M NaCl (1.dA/dt = 117.7 x 10^{-5}); 0.01 M NaCl (2. dA/dt = 56 x 10^{-5}); 0.025 M NaCl (3. dA/dt = 42 x 10^{-5}); 0.05 M NaCl (4. dA/dt = 15.4 x 10^{-5}); 0.1 M NaCl (5. dA/dt = 6.25 x 10^{-5}); 0.125 M NaCl (6. dA/dt = 3.37x 10^{-5}); 0.5 M NaCl (7. dA/dt = 1.78 x 10^{-5}).

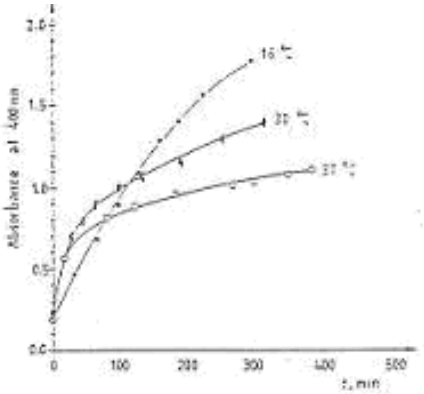


Figure 77. The effect of temperature on the formation of the insoluble ternary mixture pH 7; C_{PAAm}=0.15 g/dl; C_{BSA}=0.5 g/dl;C_{CuSO4}=0.05 g/dl

The formation rate of insoluble ternary complexes increases by increasing both the temperature and stirring of the mixture (Figure 77).

The effect of the molecular weight of polymer on the formation of soluble and insoluble ternary complexes has been studied at different molecular weight of P4VP (see below) and PAAm: 20000 (PMC1), 45000 (PMC2), and 230000 (PMC3). In all cases, the ratio of n_{Cu}/n_{AA} was kept constant at 0.1. The dependence of A_{400} of the ternary system on the ratio of components at different molecular weights of PAAm is shown in Figure 78.

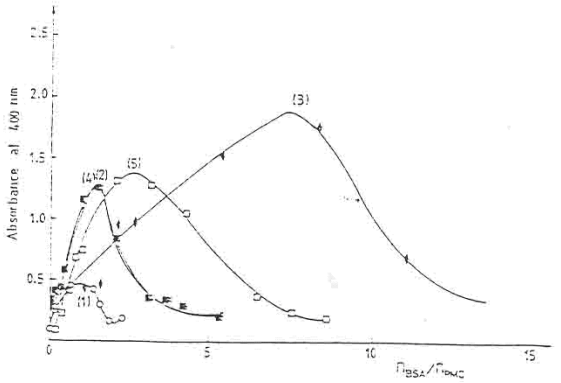


Figure 78. The dependence of A_{400} on molecular weight of PAAm and Cu^{2+} concentration for the mixture of BSA-Cu^{2+}-PAAm. pH = 7.0, 20°C. M_v = 20.000 (1), 45.000 (2) and 230.000 (3): C_{PAAm} = 0.15 g/dl; C_{CuSo4} = 0.05 g/dl (4); C_{CuSO4} = 0.1 g/dl (5).

The absorbance value of the ternary systems depends on the molecular weight of PAAm. At each of the three states, this dependence passes through a maximum. However with increasing of molecular weight of PAAm (Figure 78 curves 1-3), the maximum value of absorbance increases and soluble system takes place at higher values of n_{BSA}/n_{PMC}, i.e. it is necessary to use more BSA molecules to obtain soluble ternary system for high molecular weight of PAAm in ternary mixture. As it seen from this Figure 78 the increase of the Cu^{2+} concentration in ternary system at constant M=45000 is proportional to the amount of BSA on the ratio of n_{BSA}/n_{PMC} to obtain soluble ternary system. With increasing copper concentration in the system, the amount of Cu^{2+} which is bound to polymer increased, i.e. the capacity of binding of PMC molecule to protein increased. According to this idea, to obtain a soluble system in the case of greater Cu^{2+} concentration, it was necessary to add more BSA to system.

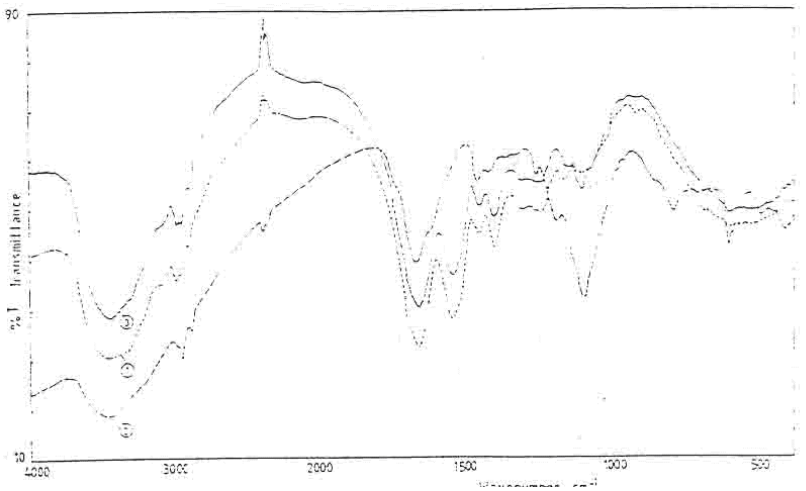


Figure 79. FT-IR spectra of PAAm (Mv =20.000) (1) and PMC1

The formation of insoluble ternary protein-metal-polymer complexes were investigated from FT-IR spectrophotometric measurements (Figure 79). As seen from the results in Figure 79 in ternary BSA-Cu^{2+}-PAAm mixture spectra the peaks at 592, 610 and 800 cm^{-1} corresponding to polymer-metal complexes are invisible. Also, the peak at 1100 cm^{-1} which is characteristic for the PAAm-Cu^{2+} strongly decreases. These results showed that the metal ions bound with PAAm after adding protein molecules partially bound with proteins. The FT-IR of the insoluble products of the ternary mixture of BSA with poly (N-isoporopylacrylamide-co-acrylic acid) copolymers in the presence of copper ions is shown in Figure 79. The copolymer spectrum has absorbances at 1240, 1500, 1580, 1650 and 1740 cm^{-1}, respectively. The BSA spectrum has absorbances at 1580 and 1650 cm^{-1}. In the CP-Cu^{2+}-BSA spectrum the band 1700 cm^{-1} disappeared and the 1580 and 1680 cm^{-1} bands shifted to 1650 and 1550 cm^{-1}, respectively. The intensity of the bands at the (1100-1200 cm^{-1}) strongly increased and changed their character. The bands 1200-1100 cm^{-1} correspond to the SO_4 counter ions of Cu^{2+} in solution. These results suggest that the copper ion is bound to the ---COOH, ---C=O, (--C—N) and (C—O) bands and mediated ternary complex formation between C_P and BSA.

The formation of the insoluble products in the mixture has influence on the surface tension of polymer solutions. (Table 7). Adding BSA molecules to the PAAm-Cu^{2+} solution initially (n_{BSA}/n_{PAAm} I) the surface tension of polymer solution decreases and at the n_{BSA}/n_{PAAm} I the surface tension takes a limiting value. These results indicate the formation of a new type colloid particle in systems; i.e., PAAm-Cu^{2+}-BSA complex.

Table 7. The dependence of surface tension to n_{BSA}/n_{PAAm}

n_{BSA}/n_{PAAm}	0.055	0.143	0.476	0.952	1.667	1.904
Surface tension (dyn/cm)	72.17	67.19	63.65	61.88	63.15	62.89

M_w(PAAm) = 20 000; 20 °C; pH = 7; C_{PAAm} = 0.15 g/dl; C_{BSA} = 0.05 g/dL

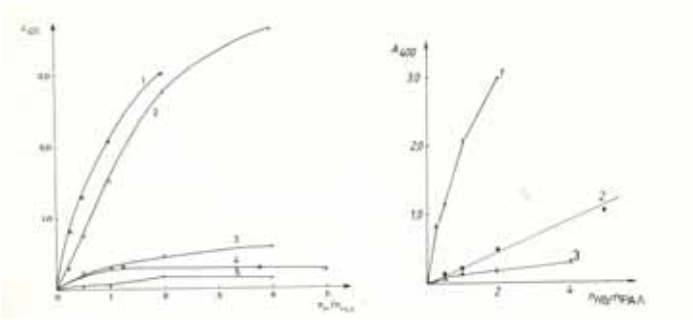


Figure 80. (a) Dependence of the optical density (A_{400}) of the ternary mixtures of different proteins with Cu^{2+} and PAA on n_p/n_{pAA} at pH 7.0. (1) Hb-Cu^{2+}-PAA; (2) Gl-Cu^{2+}-PAA; (3) Tr-Cu^{2+}-PAA; (4) HSA-Cu^{2+}-PAA; (5) BSA-Cu^{2+}-PAA. C_{PAA} = 0.1 g/dL, n_{Cu}/n_{pAA} = 0.08. **(b)** Dependence of the optical density (A_{400}) of the ternary mixture Hb-Cu^{2+} -PAA on n_{Hb}/n_{pAA} at different pH values. (1) pH 7.0; (2) pH 8.0; (3) pH 10. C_{PAA} = 0.1 g/dL; n_{Cu}/n_{AA} = 0.08; T = 25°C.

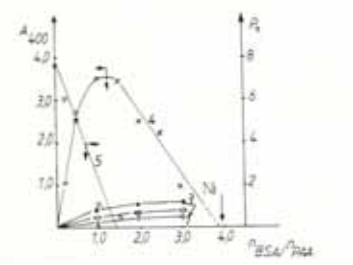


Figure 81. Turbidimetric titration of PAA-Cu^{2+} mixture with the solution of BSA. (1-4) Dependence of optical density (A_{400}) of ternary mixture PAA-Cu^{2+}-BSA on n_{BSA}/n_{pAA} at difference n_{Cu}/n_{AA}: 0.08(1); 0.1 (2); 0.2 (3); 0.3 (4). (5) Dependence of the area of the free PMC peaks (Po) on the ratio $n_{ESA}In_{FAA'}$ obtained in matrix solution of ternary mixture at n_{Cu}/n_{AA} = 0.3 C_{PAA} = 0.1 g/dL; pH 7; 25°C.

Water Soluble Complexes. The water-soluble mixtures of polyanion-metal complexes with negatively charged proteins were analyzed by different physico-chemical methods under different experimental conditions. For the analysis of the matrix solutions of the mixtures PAAm-PMC was used electrophoretic method (Figure 73). An electrophoretic study of this system showed that the proteins in the solutions are absent at the $n_{BSA}/n_{PMC\ I}$. At the $n_{BSA}/n_{PMC\ I}$ of mixture, the water soluble ternary complexes have been detected in the electrophoretic diagrams (at the same concentration, free BSA and ternary mixtures were shown to have different electrophoretic

mobility and optical density). The results plotted in this figure could be interpreted in terms of water soluble and insoluble ternary complexes formed simultaneously, at the $n_{BSA}/n_{PMC\ I}$. Further increasing the ratio n_{BSA}/n_{PMC} leads to the amount of the insoluble ternary complexes decreasing and soluble ternary complexes increasing.

The water soluble complex formation between proteins and polyanions in the presence of divalent copper ions were at first analyzed by HPLC method by Mustafaev [124]. Figure 82 presents an example of HPLC for the soluble mixtures BSA-Cu^{2+}-PAA at different n_{BSA}/n_{pAA} and n_{Cu}/n_{AA}. As suggested by the slight increase in peak I, the interaction between BSA and PAA at the pH 7.0 was weak, if not negligible, in the absence of copper ions (B). The results are consistent with the results obtained by sedimentation analysis of PAA-BSA systems.

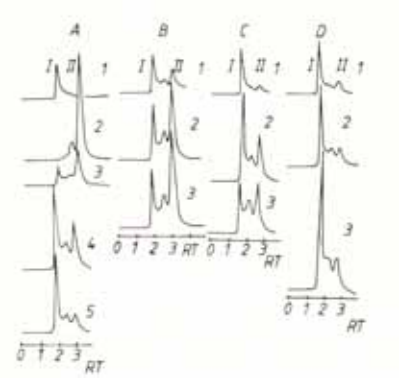


Figure 82. HPLC analysis of the formation of polymer-protein complexes in the presence of Cu^{2+}. Polymer-metal, (Cu^{2+})-protein complexes were prepared and HPLC analysis on gel filtration column performed as described in [124]. (A) Dependence of the complex formation upon n_{Cu}/n_{AA}: (1) 0.1 g/$_{100}$ mL PAA; (2) 0.1 g/$_{100}$ mL BSA; (3) (0.1 g/$_{100}$ mL PAA and 0.1 g/$_{100}$ mL BSA) plus Cu^{2+} (n_{Cu}/n_{AA} = 0.1); (4), as (3), with n_{Cu}/n_{AA} = 0.15; (5), as (3), with n_{Cu}/n_{AA} = 0.2. n_{BSA}/n_{pAA} = 1.0. (B) Chromatograms of PAA-BSA mixture in the absence of Cu^{2+}; (C) Chromatograms of PAA-Cu^{2+}-BSA mixtures at n_{Cu}/n_{AA} = 0.1. (D) Chromatograms of PAA-Cu^{2+}-BSA mixtures at n_{Cu}/n_{AA} = 0.2. For all (B, C, D) systems n_{BSA}/n_{pAA} = 0.5 (1); 1.0 (2); 3.0 (3). Diagrams represent normalized A_{280} values. (RT = retention time)

Stable complexation took place, however, upon addition of copper ions (A, C, D). The extent of complex formation was dependent on the amount of Cu^{2+} added and nearly quantitative under the experimental conditions at n_{Cu}/n_{AA} = 0.2. Thus, under conditions where both PAA and BSA have negative charges and are incapable of binding to one another, the divalent Cu^{2+} ions act as "fasteners", promoting the formation of fairly stable water-soluble ternary complex.

The participation of Cu^{2+} in the complex formation with PAA, BSA, and the ternary mixture was investigated by cyclic voltammetry analysis of soluble mixtures at pH 7. There were no peaks for PAA and BSA solution in the range of 700-800 mV.

Cyclic voltammograms of Cu^{2+} ions, PAA-Cu^{2+}, BSA-Cu^{2+}, and PAA-Cu^{2+}-BSA mixture were given in Figure 83. As it can be seen from the Figure 83, for the Cu^{2+} solution a single cathodic peak was formed at about 100 mV and the reverse scan exhibit an anodic peak at 250 mV. In the presence of PAA and BSA (PAA-Cu^{2+} and BSA-Cu^{2+}) the peak potentials correspond to reduction shift to more cathodic direction and peak currents decrease. When BSA in equal molar concentration with PAA (n_{BSA}/n_{AA} = 1)] was added to the PAA-Cu^{2+} mixture at pH= 7, the anodic and cathodic peaks disappeared practically completely. The cathodic peak was attributed to reduction of Cu^{2+} to Cu^{o} and reverse peak corresponds to its oxidation.

When the PMC solution is titrated with protein solution (n_{BSA}/n_{PAA} < 1), BSA is complexed with the polyion via copper ions. Some of the copper ion form intramolecular crosslinks in the free sections of the polyion and, thus, stabilize the structure as a whole.

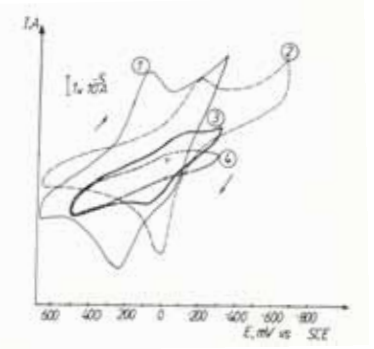


Figure 83. Cyclic voltammograms of 1.16 X ıo-3M Cu (I); mixtures of PAA-Cu^{2+} (2), BSA-CU^{2+} (3), and BSA-Cu^{2+} - PAA (4). The concentration of Cu^{2+} in all mixtures are constant and equal to concentration of Cu^{2+} in (1); n_{Cu}/n_{AA} = 0.15; working electrode = GCE; ionic strength = O.l *N* $NaClO_4$; pH 7.

The pattern changes significantly on further increase in ratio, n_{BSA}/n_{PAA} > 1 [Figure 84 (A) and (C)]. Under this condition, depending on the concentration of Cu^{2+}, the reaction between PMC and BSA may follow either of two different ways.

At low Cu^{2+} concentration (n_{Cu}/n_{AA} < 0.1), intensity of peak I first increased (n_{BSA}/n_{pAA} = 0.5 (1); 1 (2)) upon addition of BSA to the PMC solution. A further increase in BSA content (n_{BSA}/n_{pAA} = 2.0 (3)) led then to the decrease of the peak intensity nearly equal to that of free PAA at initial concentration [Figure 84 (C)]. The intensity of the peak II (monomer form) appeared to be at first essentially lower than the peak of the free BSA with equal concentration (diagram A,2 vs. C,2).

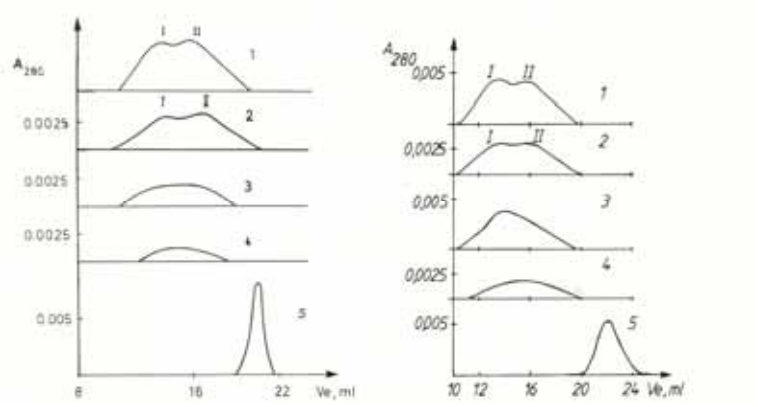


Figure 84. Gel filtration HPL chromatograms of free PMC and at the matrix solution of its ternary mixture (Hb-Cu^{2+}-PAA) (a) and (Gl-Cu^{2+}-PAA) (b) at different $n_p/n_{pAA'}$ (a) (1) Free PMC; 2.8 (2); 6 (3); 10 (4) (5) free Hb. (b) (1) free PMC; 2.8 (2); 6 (3); 10 (4); (5) free Gl. pH = 7.0; C_{PAA} = 0.1 g/dL; n_{Cu}/n_{AA} = 0.08; *T* = 25°C. Bio Sil Sac 250 column was used in the experiment.

At n_{BSA}/n_{PAA} = 2.0, the intensity of peak II corresponding to free (monomer form) of BSA increased only slightly. Notice that the intensity of peak II with *Ve* corresponding to the dimer form of BSA increased considerably. Thus, it can be proposed that a further increase in BSA content to the breakdown of the complex as in mechanism (1) by the formation of BSA-Cu^{2+}-BSA and BSA-Cu^{2+} complexes and free PAA-Cu(II) (or PAA):

$$[\text{BSA-Cu}^{2+}\text{-PAA}] \xrightarrow{\text{BSA}} [(\text{BSA})_2\text{-Cu}^{2+}] + \text{PAA}(\text{Cu}^{2+}) \quad \text{(mechanism 1)}$$

As is known from the literature [124], BSA form in the presence of Cu^{2+}-soluble protein-metal-complexes with *Ve* corresponding to those of the monomer and dimer form of BSA. The higher capacity of BSA in complex formation with Cu^{2+} than PAA is consistent with this proposal.

At higher Cu^{2+} concentrations (n_{Cu}/n_{AA} > 0.15) [Figure 84 (A) and (D)/ a further increase of BSA continued to increase in the area of peak I, and the area of peak II did not change particularly. Therefore, a further increase of BSA leads to the formation of nonstoichiometric polycomplexes, for instance, the number of BSA molecules bound per polyion chain exceeds 1 [mechanism (2)].

$$[\text{BSA-Cu}^{2+}\text{-PAA}] \xrightarrow{\text{BSA}} [(\text{BSA})_n\text{- Cu}^{2+}\text{-PAA}] \quad \text{(mechanism 2)}$$

A migration of Cu^{2+} from the free to protein bound sections of PAA may contribute to this letter process. This is explained by the fact that the filled and free PAA macromolecules may exchange Cu^{2+} ions:

$\text{PAA*- Cu}^{2+} + \text{PAA} \rightarrow \text{PAA*} + \text{PAA- Cu}^{2+}$ and $\text{PAA- Cu}^{2+} + \text{PAA*} \rightarrow \text{PAA} + \text{PAA*- Cu}^{2+}$ where PAA * is an anthryl label containing poly(acrylic acid).

Analysis of the supernatant of the insoluble ternary protein-metal-polyanion mixtures was carried out with HPLC-gel filtration methods (Figure 84). Two peaks were hereby seen in the chromatograms of the matrix solution in both cases of Protein-Cu^{2+}-PAA corresponding to PMC-I and PMC-II. The area of these peaks depended on the concentration of protein and decreased simultaneously with the increase in the ratio n_{pr}/n_{PAA}. The turbidity (A_{400} nm) increased also in proportion with the increase in n_{pr}/n_{pAA} (Figure 84 (a)). The peaks with *Ve* corresponding to elution volume of free Hb (Figure 84 (a), diagram 5) and Gl (Figure 84 (b), diagram 5) were absent in the chromatograms of the supernatants. The absence of free protein molecules in the matrix solution indicated that all the added protein molecules are strongly bound by the PMC, resulting in the formation of insoluble ternary complexes PAA- Cu^{2+}-protein. (Indeed, the absence of complexed protein in the matrix solution was also reflected in lack of reaction with the Folin phenol reagent method.)

It can be seen that, when the ratio n_{pr}/n_{pAA} > 1, protein-free fractions of PMC remain in the matrix solution. The existence of the PMC under these conditions unambiguously indicates a nonrandom distribution of the protein molecules between the coils of polyions.

An analysis of the formed insoluble polycomplex composition deserves some consideration. Dependence of the chromatography peak area *(Po)* of free PMC (the sum of the peaks corresponding both PMC-I and PMC-II) in the Protein-Cu^{2+}-PAA system on n_{pr}/n_{pAA} is shown in Figure 85. The intersection points obtained by the extrapolation of these plots to the zero area of the free PMC peak correspond to n_{pr}/n_{pAA} when all PMC macromolecules are bound to a complex with Hb and GL Taking into account the above-indicated fact of the quantitative binding of proteins to PMC, one may consider that lim (n_{pr}/n_{pAA}) = Ni, when P_0 = 0. This limit equals the number *(Ni)* of the protein molecules bound by a PAA of a given degree of polymerization under given conditions.

At the $n_{pr}/n_{pAA} > Ni$, only one peak was seen in chromatograms in both cases (Hb-Cu^{2+}-

PAA, GI-Cu^{2+} -PAA), corresponding to the free protein; therefore, after $n_{pr}/n_{pAA} > Ni$, all PMC molecules were trapped in the fraction of insoluble complexes and the surplus of protein molecules remained in the matrix solution.

Analysis of matrix solutions of BSA-Cu^{2+}-PAA mixture revealed the fact that only an individual free PMC component was observed at $0 < n_{BSA}/n_{pE} < 1$ in matrix solutions. The increase of BSA content in polyelectrolyte mixture leads to a decrease of amount of free PMC and at about ratio of $n_{BSA}/n_{pAA} = 1$ the concentration of free PMC in mixture is equal to zero. This results show that in this case:

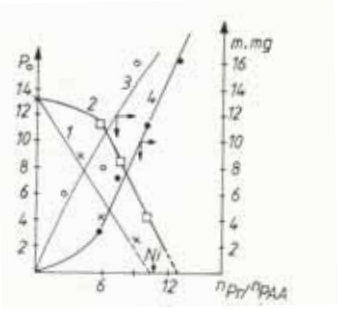
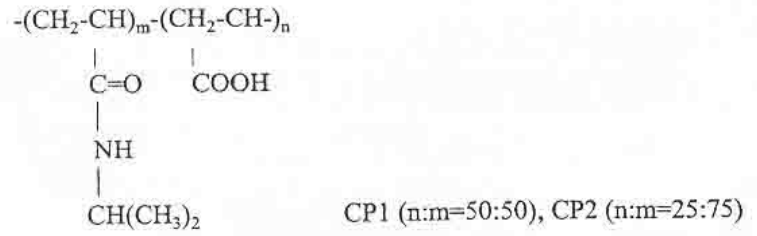


Figure 85. Dependence of the chromatogram peak area (Po) of free PMC and the amount (m) of the precipitation in Hb-Cu^{2+}-PAA (1,3) and GI-Cu^{2+}-PAA (2,4) on n_{pr}/n_{pAA}; C_{PAA} = 0.1 g/dL; n_{Cu}/n_{AA} = 0.08; pH 7.

the formation of a stoichiometric polycomplex (BSA : PMC = 1 : 1) insoluble in aqueous media has taken place. At maximum precipitation all the protein and PMC are completely incorporated to an insoluble ternary complex. When the ratio of the components in the solution is $n_{BSA}/n_{PE} > 1$, in matrix solution a transformation of insoluble complexes into soluble BSA-Cu^{2+}-PE complexes is observed.

The HPLC-ion exchange analysis of the matrix solutions of these ternary mixtures showed that the water-soluble products in the matrix solution were obtained as one peak in the free eluent volume (Figure 86). Therefore, in the ternary mixture at these concentrations of added metal ions, insoluble, and soluble ternary complexes appear to be formed simultaneously.

"Intelligent" polycomplexes. Cu^{2+}–mediated interpolyelectrolyte complex formation of BSA and water-born poly(N-isopropylacrylamide-co-acrylic acides) (CP) copolymers:



were studied by HPLC, UV-Visible spectrophotometer and fluorescence methods by Mustafaev [127,128]. HPLC analysis confirmed that at pH 7 BSA does not bind CP, since both protein and copolymer macromolecules are negatively charged at this pH and electrostatic repulsion forces prevent complex formation. However, complexes were formed following the addition of copper ions (Figure 86). The incorporation of BSA in the soluble complex with CP1-Cu^{2+} (PMC) was indicated by a peak shift corresponding to BSA on the chromatograms. The extend of complex formation was dependent on the composition of CP, the C_{BSA}/C_{CP} ratio and the amount of Cu^{2+}

added. Only one peak, corresponding to the stable water soluble ternary complexes, was observed for the C_{P1} at n_{Cu}/n_{AA} =0.2 and C_{bsa}/C_{cp}=1.0 in water and in 0.154 M NaCl. However, there is a critical concentration of Cu (n_{Cu}/n_{AA} =0.2) in ternary mixtures, (C_{BSA}/C_{CP}=1) at which the system lost homogeneity with the formation of insoluble ternary polycomplexes. CP2, which contains less acrylic acid comonomers, with n_{Cu}/n_{AA} 0.08, formed only insoluble ternary complexes. These results suggest that the hydrophobic N-isopropylacrylamide comonomers in composition of macromolecules decreased the solubility of their ternary complexes with proteins.

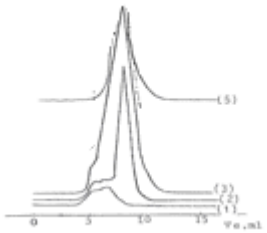


Figure 86. HPLC analysis of the soluble CP1-Cu^{2+} (1), CP1-BSA (2) and CP1-Cu^{2+}-BSA (3. 4) mixtures at different n_{cu}/n_{AA}: 0.1 (3); 0.18 (4); (5) corresponds to free BSA. CP at 280 nm does not absorb at pH 7.0. Solution is water without added NaCl. C_{BSA}/C_{CP} = 1.0; C_{CP} = 0.1 g/100 mL.

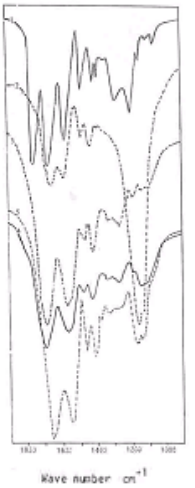


Figure 87. FT-IR spectra of (1)-CP1; (2)-CP1+Cu^{2+}; (3)-BSA; (4)-BSA+Cu^{2+} (5)-CP1 Cu^{2+}-BSA (n_{Cu}/n_{AA}=0.2)

Studies of thermal collapse property of CP-Cu^{2+}-BSA ternary mixtures in water and in the presence of low molecular weight salt permits to elucidate some important features characterizing ternary complex formation. The temperature dependence of OD_{500} values for the solutions of CP1-Cu^{2+}-BSA mixtures in water and in the presence 0.154 M NaCl, prepared at n_{Cu}/n_{AA} =0.2 and C_{BSA}/C_{CP}=1 are shown in Figure 86.

At pH = 7 the phase state of the ternary mixture prepared in water as CP and CP-Cu^{2+} complex was independent of temperature increases and remained soluble in water (they showed thermal collapse property at pH 3.0). In contrast, in solutions of 0.154 M NaCl the ternary mixture revealed increasingly higher OD_{500} values with increased temperatures. The ternary CP1-Cu^{2+}-BSA mixture in salt solution exhibited a negative temperature solubility coefficient and a phase transition on heating (near body temperature). The next series of experiments were devoted to analysis of the composition of the insoluble fraction of the CP1-Cu^{2+}-BSA mixture after transformation by temperature. HPLC results for the soluble mixture CP1-Cu^{2+}-BSA which was prepared by dissolution at room temperature of the precipitate obtained after heating the ternary mixture at 37° C are shown in Figure 87, 88, 89.

The single chromatogram peak showed that all of the reaction components after thermal collapse are composed of CP1-Cu^{2+}-BSA complexes. The absence of a soluble fraction in the matrix solution (no peaks were found in the chromatograms) suggest that the soluble CP1-Cu^{2+}-BSA complexes prepared in 0.154 M NaCl were transformed to the insoluble state by heating

while the insoluble complexes transformed to the soluble state. Therefore, water-soluble stable ternary complexes in salt solutions have a negative temperature solubility coefficient and show phase transition on heating. Dehydrated precipitate of ternary polycomplexes dissolved upon cooling, demonstrating their reversible properties.

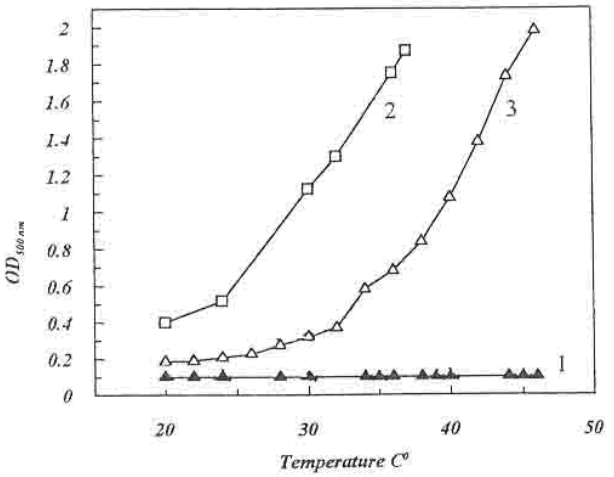


Figure 88. The temperature dependence of the optical density at 500 nm (OD_{500}) of CP-Cu^{2+}-BSA mixtures, prepared in water (1) and in 0.154 M NaCl: 2 (CP1-Cu^{2+}-BSA); 3 (CP2-Cu^{2+}-BSA); n_{Cu}/n_{AA}=0.2 (1,2) and 0.08 (3); pH 7.0; n_{BSA}/n_{CP}=1.0

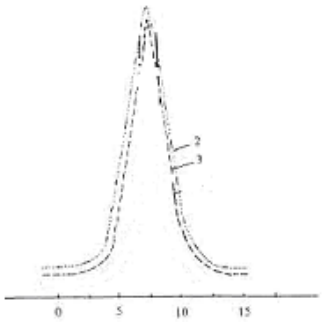


Figure 89. HPLC analysis of the soluble CPI-Cu^{2+}-BSA mixtures were prepared at different conditions: (1) ternary mixture prepared in water; (2) ternary mixture prepared in 0.154 M NaCl; (3) CPI-Cu^{2+}-BSA mixtures prepared in 0.154 M NaCl solution from the precipitate obtained after heating (37°C) the soluble ternary mixture in 0.154 M NaCl n_{cu}/n_{AA} = 0.2; n_{BSA}/n_{CP} = 1.0; pH = 7.0.

As seen in Figure 90, the UV spectrum of the ternary mixture in physiological salt solutions differed significantly from that of the polycomplexes in aqueous solution.

In the absorbance spectra of polycomplexes were prepared in physiological salt solution, two new supplementary peaks were observed at 324 and 364 nm. The intensity of these peaks increased and wavelength shifted from 360 to 376 nm with increased temperature. This alteration is essentially due to an exchange reaction, which is characterized by two processes. One is dehydration of the IPAAm/AA copolymers with increasing solution temperature. The other is a

cooperative hydrophobic interaction of dehydrated N-isopropyl groups, which increased the intensity upon addition of a low molecular weight salt to the system. These results suggest that the ---CONH— and ---COOH groups of the copolymers in salt solution may form ion-coordination complexes with copper ions which are observable in the UV at 320 and 360 nm.

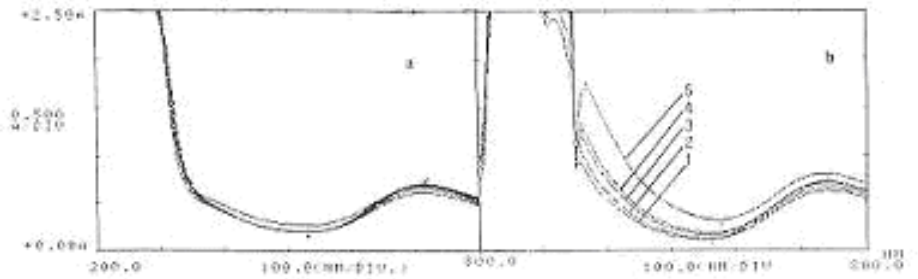


Figure 90. UV-Visible spectral results for the CP1-Cu^{2+}-BSA mixtures in water (a) and in 0.154 M NaCl (b) obtained at different temperatures °C: 15°(1), 18°(2), 30°(3), 35°(4), 45°(5), pH 7 n_{Cu}/n_{AA}=0.18; C_{BSA}/C_{CP}=1.0

Fluorescence Study. As it was mentioned above the formation of polycomplexes in the mixtures protein-metal-polyelectrolyte and their structure were intensively studied by titration, HPLC, electrophoretic, spectrophotometric (FT-IR, UV-Vis.), light scattering and hydrodynamic (viscosity and sedimentation) methods. These methods provide general information about the structure of polycomplex particles, while the information about the structure of protein globules in these particles is practically absent. Fluorescence techniques have recently been used to study protein-polyelectrolyte complexation (see above, section of protein-polyelectrolyte complexes). Previously we described the formation of water-soluble ternary polycomplexes between ovalbumin (OA) which was labeled with fluoresceinisotiocianate (F) (OAxF) and copolymers of acrylic acid with 2-methyl-5-vinylpyridine (CP) in the presence of copper ions [29]. Typical fluorescence spectra of pure OAxF and OAxF in mixtures with CP and CP-Cu(II) are given in Figure 91.

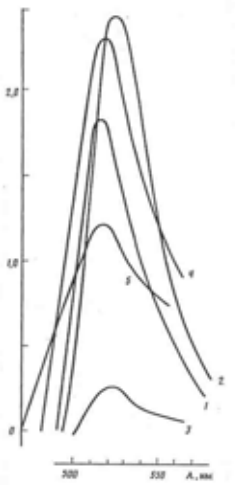


Figure 91. Fluorescence spectra of pure OAxF conjugate (I) and its complexes with PE:PVP(R_2, R_{16}) (2), PVP(R_2, R_{16}) (3), CP (4) and CP-Cu(II); β=4.6(2),13.2(3);

When CP solutions are added to solution OAxF at pH 7 the quantum yield of fluorescence of OAxF is increased that may testify to complex formation between protein and CP [29]. Hydrophobic interaction of protein molecules with methylvinylpyridine monomer units of copolymers and formation of hydrogen bonds between them provide the binding of the same (negative) charged macromolecules. The pattern is quite different the presence of copper ions; the fluorescence is quenched by adding of copper ions to solution of CP-OAxF mixtures. This results show to the including of copper ions into composition of ternary CP-Cu^{2+}-OAxF complexes.

It is known that from the fluorescent emission shift of tryptophan residues in proteins, it is possible to localize the interaction between proteins and polyelectrolyte at certain protein domains. Recently, BSA interactions with PAA, temperature sensitive poly(NIPAAm), and copolymers of acrylic acid with N-isopropylacrylamide in different composition at the presence of Cu^{2+} ions were investigated by fluorescence methods and HPLC analysis. The idea was to use Cu^{2+} ions both as an important component for the stabilization of the complexes and as a quencher. Effects of complexation conditions such as the sequence of mixing, metal/polymer and polymer/protein ratios, and hydrophobic-hydrophilic balance of polymers are discussed. Control experiments were made with a different quencher (succinimide, potassium iodide, and cesium chloride), which do not form complexes with the polymers used.

Poly(N-isopropylacrylamide) (PNIPAAm) and copolymers of *N-iso*propylacrylamide (NIPAAm) and acrylic acid (AA) (poly(NlPAAm-AA)) were prepared by a radical polymerization of NIP AAm and AA in distilled water in the presence of 2-oxoglutaric acid and UV irradiation (365 nm). The monomer compositions *(r)* of copolymers poly(NlPAAm-AA) used in this study were NlPAAm/AA = 3:1 (P25), 1:1 (P50), and 1:3 (P75), poly(NIPAAm) (PO), and PAA (PlOO) (Table 8).

Table 8. Characteristics of Polyelectrolytes

abbreviation	monomer composition NIPAAm:AA	η_{sp}/C,[a] dL/g	LCST, °C
P0	100:0	1.88	32
P25	75:25	2.95	32
P50	50:50	3.12	26
P75	25:75	13.25	34
P100	0:100	4.4	

[a] Reduced viscosity at 0.1 g/dL in PBS at 22 °C.

[Quencher]/[BSA] and [Cu^{2+}]/[BSA] ratios (n_q/n_{BSA}, n_{Cu}/n_{BSA}) were calculated using the equation $n = cN_A/M$, where n is the number of the molecules in 1 mL, M is the molecular weight of components, N_A is the Avogadro number, and c is the concentration in g/L. The heterogeneity of polymers and proteins and the fraction compositions of the mixtures were estimated by the HPLC system.

Fluorescence emission spectra were obtained using a Quanta Master spectrofluorimeter (Photon Technology International, Canada) operating in quanta counting mode. The slits of excitation and emission monochromators were adjusted to 2 or 3 nm. The excitation was at 280 nm. The fluorescence of proteins is widely used to study of their behavior depending on different influencing factors [129]. Binding of substrates, association reactions, denaturation, and interactions with other macromolecules may result in the changes of protein fluorescence spectra. In the present study we characterize them by the wavelength at the maximum of emission (A_{max}), fluorescence intensity in the maximum (I_{max}), and width at half-maximum level *(A)*.

From literature it is well-known that BSA interacts with Cu(II) in neutral water and water-soluble and insoluble complexes are formed [107]. However, these findings were obtained by the indirect methods. Therefore, in this investigation, the interactions between BSA and Cu(II)

were at first analyzed by HPLC, which allows study of the fraction composition at different metal/protein ratios. Addition of copper ions did not effect the solubility of BSA at pH 7 within a certain range of n_{Cu}/n_{BSA} values (Figure 92). The phase separation in the system BSA–Cu(II) occurred only at some critical metal concentration (n_{Cu}/n_{BSA} =10). Typical HPLC analysis of BSA and its soluble mixtures with Cu(II) ions at different ratios is given in Figure 93. Chromatograms of the mixture of BSA –Cu (II) are characterized by two peaks.

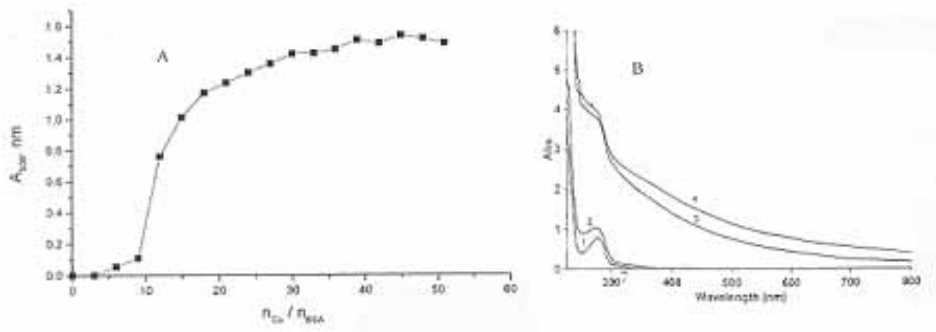


Figure 92. Turbidimetric titration of BSA solution with the solution of $CuSO_4$: (A) dependence of the optical density *(A_{500} nm)* of BSA-Cu^{2+} mixtures (n_{Cu}/n_{aSA}); (B) absorption spectra of BSA (1) and homogeneous solutions of BSA-Cu^{2+} mixtures at different n_{cu}/n_{asA} values (pH 7.0 and 25° C): 4 (2), 8 (3), and 40 (4). The concentration of BSA was 0.07 *g/dL*.

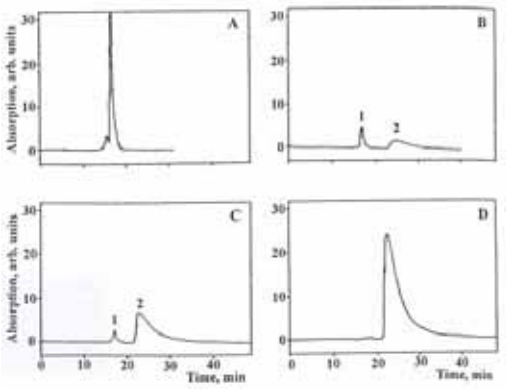


Figure 93. Gel filtration HPLC chromatograms of BSA (A) and of its mixtures with Cu^{2+} at different ratios of Cu^{2+} ions to BSA molecules (n_{Cu}/n_{aSA}): 4 (B); 8 (c); 40 (D).

The comparison of the retention time (RT) of the elution fractions corresponding to peaks 1 and 2 of the mixture and of the protein shows that the copper binding by BSA takes place: RT corresponding to peak 1 does not change and remains equal to that for free BSA, but RT of peak 2 differed from those values for a pure BSA peak. One may assume that peak 2 corresponds to the BSA–Cu(II) complex. HPLC analysis of homogeneous systems at different n_{Cu}/n_{BSA} permits the elucidation of some important features characterizing BSA–Cu(II) complex formation. Cu(II) increase in the mixture (at constant molar concentration of BSA) leads to a decrease of peak corresponding to free BSA molecules (peak 1) while the supposed peak of the complex (peak 2) increases (Figure 93 C). At the ratio [Cu(II)]:[BSA] = 10:1 the peak of free BSA disappears and there remains only the peak characterized by RT = 22.650 min. It is important to emphasize,

however, that free BSA still remains in the system over a rather wide range of the molar ratios [Cu(II)]/[BSA] even when n_{Cu}/n_{BSA} =10. These data indicate that the metal ions are unevenly distributed between the protein molecules: some protein globules may sorb the maximal quantity of Cu(II) ions, possible under given conditions, while the others remain practically unpopulated. Just this type of distribution was found previously at complexation of linear polyanionic and polycationic PE with Cu(II) ions [130,132]. The PE-Cu(II) complex exists as a chelate with carboxylate groups bound by copper ions. According to the literature [94] BSA has one specific binding site for Cu(II) at the NH_2-terminal tripeptide segment (Ala-Ala-His) involving the Asp-NH_2, His-N(l) imidazole, two deprotonated peptide nitrogens (Ala NH and His NH), and the Asp COO- group. At law concentrations of copper ions (n_{Cu}/n_{BSA} <10), the interaction can be considered to be only intramolecular: Cu(II) forms a complex with one protein globule. At high concentrations (n_{Cu}/n_{BSA} > 10) a phase separation takes place in the system (Figure 93 A). Analysis of the supernatant and sediment (by sediment dissolving in acetate buffer at pH 4.3) was carried out with HPLC gel filtration, UV-visible measurements, and a Zeeman atomic absorption spectrophotometer. The peaks with RT = 22.6 min, corresponding to the elution time of soluble BSA–Cu(II) complexes, were obtained in the chromatograms of the supernatants. The BSA fraction and Cu(II) ions coexist in sediment simultaneously. One can suggest that copper ions at n_{Cu}/n_{BSA} > 10 act more effectively as a crosslinking agent between two (or more) protein globules. This intermolecular interaction leads to the formation of soluble and insoluble protein-metal complexes with a complicated structure.

Solutions of mixtures of polyanions (PAA, poly(NIPAAm-AA) at different compositions) with BSA, in the absence and presence of Cu(II) ions, were analyzed recently by different physicochemical methods. It was shown that interaction between anionic PE and BSA at pH 7.0 at the absence of copper ions is weak, if not negligible. Stable complex formation took place, however, upon addition of copper ions. The extent of complex formation was dependent on the amount of Cu(II) added and nearly quantitative at n_{Cu}/n_{AA} = 0.2. Thus, under conditions where both PE and BSA have negative charges and are incapable of binding to one another, divalent Cu(II) ions act as "fasteners", promoting the formation of a fairly stable water-soluble ternary complex. The solubility, composition, and stability of these polycomplexes depend on composition of PE macromolecules and metal/PE (or protein) and protein/polymer ratios.

The values of optical density (A_{4OO}) did not change considerably over a wide range of metal concentrations ($0 < n_{Cu}/n_{AA} < 0.25$), when the system lost the homogeneity with formation of insoluble polycomplexes. At low concentrations of copper ions, their interactions may be considered as intramolecular (Cu(II) forms a complex with one polymer chain). At high concentrations the copper ion can act more effectively as a crosslinking agent between two (or more) polymer coils, which leads to the formation of soluble and insoluble ternary polycomplexes.

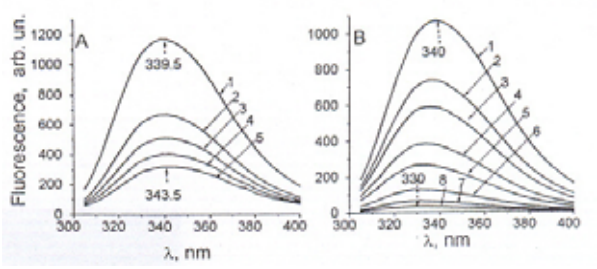


Figure 94. Fluorescence spectra of pure BSA (A) and BSA in mixtures of PE + BSA ([P50]/[BSA] = 3.52) (B) at different concentrations of Cu(II) ions in solution. BSA concentration was 0.71 mg/mL, phosphate buffer (pH 7). $CuSO_4$ concentration (in mM): 1, O; 2, 0.15; 3, 0.3; 4, 0.45; 5, 0.6; 6, 0.9; 7, 1.2; 8, 1.5.

Typical fluorescence spectra of pure BSA and BSA in mixtures PE (P50)-Cu(II)-BSA at different Cu(II) concentrations are given in Figure 94. It is well-known that tryptophan (Trp) fluorescence of proteins varies with their conformational changes resulting in changes of fluorescence parameters, such as the emission maximum (A_{max}), quantum yield, lifetime, an others. As shown in Figure 94 A the fluorescence intensity (I_{max}) of BSA at pH 7 and at the absence of polymer decreases (quenching) and A_{max} shows some red shift at Cu(II) concentration increase. BSA contains two Trp. One of them (spectral class 2 by Burstein with A_{max} = 340-342 nm and half width = 53-55 nm) is located on the bottom of BSA hydrophobic cleft. The second Trp of class 3 (A_{max} = 350-352 nm, half width = 59-61 nm) with law quantum yield (l/5 of the total BSA fluorescence) is superficial and completely accessible to aqueous solvent. The results in Figure 94 A indicate a much smaller efficiency of superficial Trp quenching at the background of very strong "cleft" Trp quenching, which suggests the presence of positively charged atoms in the vicinity of the former and negatively charged ones in the vicinity of the later. As a result at rather high [Cu^{2+}], the class 3 Trp becomes prevailing in BSA and the BSA emission spectrum shifts to longer wavelength. The situation is quite different at the same mainly conditions exert for polymer introduced into the solution. Figure 94 B shows that the BSA fluorescence spectrum, at the presence of polymer P50, shifts quite oppositely toward short wavelengths, which is indicative of the formation of a fairly stable water-soluble ternary polycomplex.

Study of a BSA tryptophan fluorescence for PE-Cu(II)-BSA mixtures in homogeneous systems at different component ratios and polymer compositions permits elucidation of some important features characterizing ternary polycomplex formation. Figures and present the dependence of A_{max} and l_{max} vs [Cu^{2+}] for BSA in the ternary mixture with different polymers at constant concentrations of the protein and different concentrations of polymers. These results clearly show that BSA fluorescence in mixtures depends on the chemical composition of polymers.

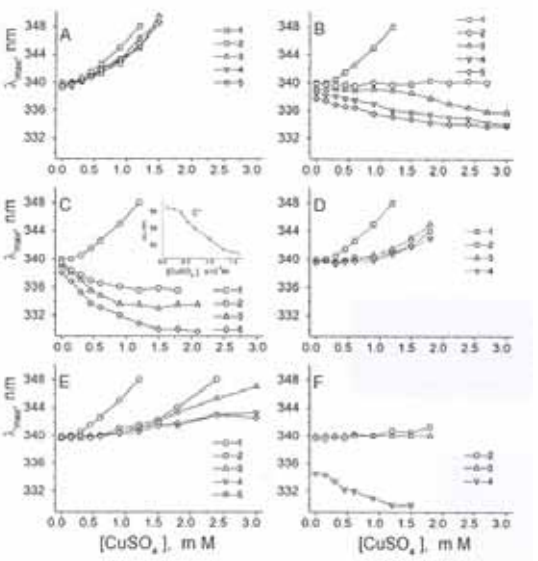


Figure 95. Position of fluorescence maximum of BSA in a mixture PE + Cu(II) + BSA vs Cu(II) concentration in solution (phosphate buffer, pH 7) at different polymers: A, O (PO); B, 25 (P25); C, 50 (P50); D,75 (P75); E, 100 (P100); F, the same as E but at pH 5. BSA concentration was 0.71 mg/mL. [P]/[BSA] (mg/mg) (A, B, C): 1, O; 2, 0.58; 3, 0.85; 4, 1.76; 5, 3.52. (D, E): 1, 0.58; 2, 0.85; 3, 1.76; 4, 3.52. Insert C*: half width of the spectrum vs [$CuSO_4$] for the data C, curve 5. F: 1, pH 7; 2, pH 5.8.

For the mixture PO + BSA the dependence of Amax vs [Cu(II)] for all PO concentrations is practically the same as that for pure BSA in solution (Figure 95 panel A), which witnesses for the absence of any interactions between BSA and PO through Cu(II) ions. This could be expected, because polymer PO contains no -COOH side groups; therefore the formation of coordination bonds between BSA and this polymer is impossible in principle.

The pattern is quite different with the presence of polymers P25 or P50: as the fluorescence is quenched by increasing [Cu(II)] (Figure 95, panels B and C), its maximum shifts toward the blue region (Figure 95, panels B and C). This indicates that BSA tryptophanyls become less accessible for water solution, which must be the result of increasingly tighter binding of the polymer through Cu(II) with the protein. Polymers P25 and P50 show some interactions with the protein even in the absence of copper in solution (panels B and C). Indeed at their maximal ratio to the BSA blue spectral shift of BSA fluorescence reaches 2 nm (from 340 to 338 nm), which suggests some screening of BSA tryptophanyls from water surrounding due to protein-polymers interactions. For other polymers we could not find such interactions with the absence of copper ions at neutral pH. But at pH 5 polymer P100 showed an even larger shift (about 5 nm) at a lesser polymer-to-protein ratio, R = 1.76 (Figure 95, panel F, curve 4).

The wavelength spectral shift is especially pronounced for BSA mixtures with P50. Indeed, even at a low ratio R = [P50]/[BSA] the increase of [Cu(II)] leads to the important blue spectral shift (Figure 95, panel C, curve 2).

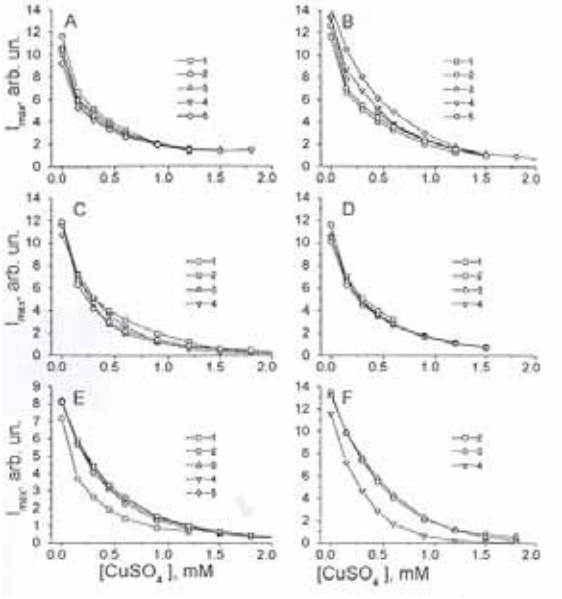


Figure 96. Intensity of BSA fluorescence in maxima for a mixture P + Cu(II) + BSA vs. Cu(II) concentration in solution (phosphate buffer, pH 7) at different polymers: A, O (PO); B, 25 (P25); C, 50 (P50); D, 75 (P75); E, 100 (P100); F, the same as E but at pH 5. BSA concentration was 0.71 mg/mL, [P]/[BSA] (mg/mg): 1,0.58; 2, 0.85; 3, 1.76; 4, 3.52.

At R = 3.52 (curve 5) this shift reaches a maximal value and the BSA fluorescence spectrum at [Cu(II)] = 1.5 mM becomes that of internal tryptophanyls, completely inaccessible for water surrounding (first spectral class) with A_{max} = 330 nm and half width of the spectrum 51 nm (see insert C*). This indicates that in the formed conjugate BSA + Cu(II) + P50 BSA tryptophanyls are completely isolated from water solution by the polymer, which is covering

seemingly all the BSA surface. It is worth noting that quenching of fluorescence for the system with P50 also reaches a maximal value compared with other polymers. Thus, at [Cu(II)] = 1.5 mM quenching for P50 is 20 times (Figure 96, panel C), when for other polymers it is about 10 times. This must indicate that in coordination bonds between BSA molecule and P50 there is utilized the largest quantity of copper ions as compared with other polymers used in the study.

In the case of P25 the spectral shifts are less pronounced (Figure 96, panel B). Maximal spectral shift at R = 3.52 and [Cu(II)] = 2.1 mM (B, curve 5) is 2-fold less (A_{max} = 334 nm) as compared with P50 (330 nm). So P25 covering of BSA in complex P25 + Cu(II) + BSA must be looser and, as a result, BSA tryptophanyls are not completely isolated from water solution (A_{max} = 334 nm is between the values characteristic for tryptophanyls of classes 1 and 2).

P75 and P100, which contain 75 and 100% of polar side chains, accordingly, must be a strong binder of Cu(II) ions. As one could see from Figure 96 at their presence in solution and [Cu(II)] increasing, the BSA spectrum shifts to longer wavelengths (panels D and E) as for pure BSA but less effectively. Comparison of curves (panels D and E) for pure BSA (curve 1) and BSA in mixtures with the polymers (curves 2-5) shows that there must be introduced into solution with the polymers 2.5 to 3 times higher concentration of Cu(II) ions to gain the same spectral shift as with pure BSA.

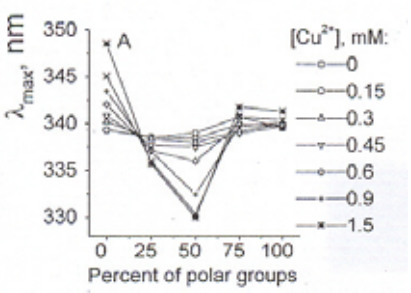


Figure 97. Position of fluorescence maximum (m) of BSA in mixtures P + BSA (1) and P + Cu(II) + BSA (2) vs percent of -COOH groups in the polyelectrolytes. [P]/[BSA] = 1.76; $[Cu^{2+}]$ = 0.5 mM.

This is the evidence that these polymers bind more then half of copper introduced into solution and therefore greatly decrease the concentration of free *Cu*(II) ions. It is their main effect. A *Cu(II)*-dependent red shift suggests that at neutral pH they do not form a covering around BSA molecules and the last behave mostly as those in pure BSA solution. But there is a peculiarity in this system behavior, which significantly distinguishes it from pure BSA solution. The matter is that the latter becomes turbid already at [Cu(II)] = 1.2 mM. So at this critical *Cu(II)* concentration coordinational bonds through *Cu(II)* ions are being established between BSA molecules, which leads to the formation of their aggregates. For the system BSA + Cu(II) + P100, the solution was transparent even at 3.9 mM *Cu(II)* , when the concentration of free *Cu(II)* in the solution was for certain higher than that of the critical one. This suggests that BSA molecules could not come together and form aggregates because they are anchored through Cu(II) ions on threads of P100.

The behavior of the system BSA + Cu(II)+P100 is quite different at low pH. As one can see from Figure 96 (panel F), polymer P100 at pH 5 and a rather high concentration even at the absence of *Cu(II)* ions produces a large blue shift (up to 335 nm) of BSA fluorescence, which points out its close interaction with the protein. At [Cu(II) increasing this shift reaches the value characteristic for first class tryptophanyls. Such a behavior of the system may be accounted for by the protonization of P 100 polar side group s (-COOH) and BSA amine groups at low pH, which leads to the formation of salt bonds between the protein and polymer. Further tightening of the formed cover is reached by the coordination bonds through Cu(II) ions.

To elucidate some important features characterizing the polymer-BSA complex

formation, we studied quenching of BSA fluorescence with standard quenchers that do not form coordination bonds with polymers or proteins. As such there were used [137,138] nonpolar succinimide, positively charged Cs^+ (CsCl), and negatively charged I^- (KI). Figure 98 shows dependence of I_{max} and A_{max} for pure BSA and BSA in mixtures P50- BSA and P AA - BSA vs. concentrations of added quenchers. As one can see, the quenchers used showed the same trends as Cu(II) ions in quenching BSA fluorescence but their effectiveness in the region we used for Cu(II) concentrations was about 10 times less.

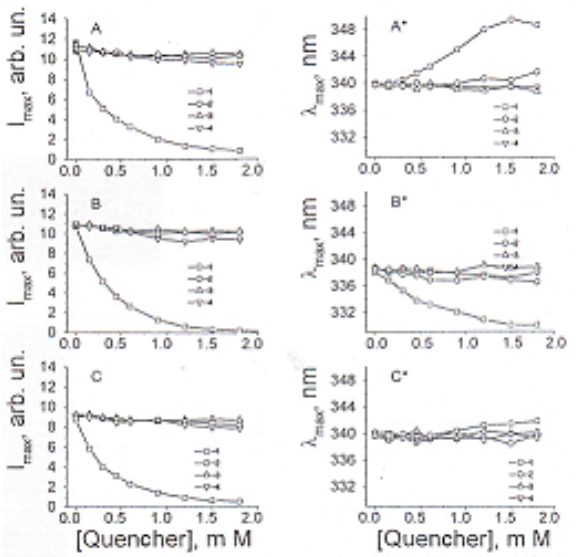


Figure 98. Intensity of fluorescence in maximum and position of the maximum for BSA solutions (0.71 mg/mL, phosphate buffer, pH 7) vs concentration of a quencher: A, A* for BSA solution without polymer; B, B*, at the addition of 2.5 mg/mL of P50 (copolymer 1:1 of acrylic acid with N-isopropylacrilamide); C, C*, at the addition of 2.5 mg/mL of P100 (polymer of acrylic acid). Fluorescence quenchers used: 1, $CuSO_4$; 2, CsCI; 3, succinimide; 4, KI.

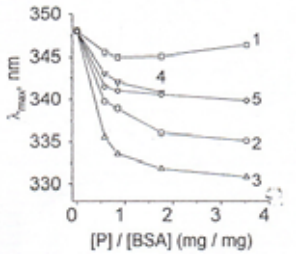


Figure 99. Florescence parameter λ_{max} for P + BSA mixtures at the presence of Cu(II) ions (1.2 mM) vs. ratio [P]/[BSA] (phosphate buffer, pH 7). P is polymer with percent of polar residues: 1, 0 (P0); 2, 25 (P25); 3, 50 (P50); 4, (P75); 5, 100 (P100).

We observed a small decrease in BSA fluorescence in the region (somewhat larger with the presence 75 of I^- ions) but no noticeable spectral shifts, which could be expected for such small quencher concentrations. These findings suggest that besides one site of Cu(II) binding , described in the literature [137] there is virtually an other one located in the vicinity of the "cleft"

Trp. *Cu(II)* binding in the former site, which is remote from both trpyptophanyls, has no influence on their fluorescence, but the emission of the latter Trp is strongly quenched at *Cu(II)* binding. At the absence of polymer this leads to prevailing in total fluorescence of Trp with A_{max} = 350 and low quantum yield. Just this Cu(II) bound in the BSA cleft must take part in coordination bond formation with polymers.

Figure 99, which presents data from Figures 98 with another scale on abscissa, shows some peculiarities of polymer-protein interactions. As can be seen from this figure, at rather high [Cu(II)] the maximal interaction of BSA with polymer, testified by spectral shift and quenching, is achieved already at rather low ratios;[P]/[BSA] (0.5-1), which indicates the maximal quantity of the polymer that can bind with the protein.

In this interval, the quenching in complex BSA + Cu(II)+ P50 reaches the maximal level and does not change with increasing the ratio of components, which suggests that in the formed tight cover the quenching is mainly static. Figure 98 shows that the ratio about 1:1 between polar and hydrophobic groups in the polymer is optimal for formation of a dense cover around protein. It seems that for smaller amounts of polar groups the polymer exists in the water solution as a coil, whereas at higher amounts the prevailing form is threadlike.

Kinetics of Complex Formation. To study transition at mixing to the equilibrium and the role of the sequence of different component mixing, the kinetics of fluorescence quenching was studied. The mixing was realized in such ways: (I) addition of Cu(II) in given concentration to the mixture of PE and BSA solutions (I), (2) addition of PE solution to the mixture BSA – Cu(II), or (3) addition of BSA solution to the mixture PE-Cu(II). Solutions were permanently stirred and the last component mixing took about 20 s. After that registration of fluorescence began immediately and continued for 15-20 min.

The kinetics curves of the PE + Cu(II) + BSA mixtures, prepared with different sequences of mixing for different polyelectrolytes are shown in Figure . One can see that the fluorescence quenching after 20 s reaches limiting values independent of the ways of mixing (1, 2, or 3) and chemical composition of the copolymers used. Somewhat lower quenching was achieved (5-7%) when Cu(II) ions were introduced into the mixture PE-BSA. An important conclusion, which follows from the data presented in Figure 100, is that the reaction of Cu(II)-induced ternary complex (PE –Cu(II) - BSA) formation is an equilibrium.

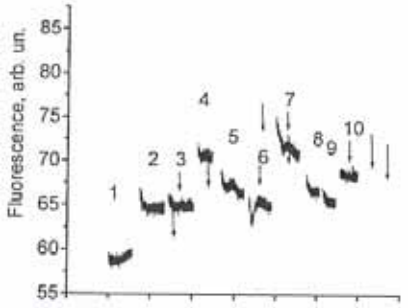


Figure 100. Quenching of BSA fluorescence by Cu(II) ions at the presence in solution of polymers depending on the order of different component mixing (polymers with percent of polar groups, 25 (P25), 50 (P50), and 75 (P75): 1, BSA + Cu(II) (control, after Cu(II) addition BSA fluorescence decreased 2-fold); 2, (P50 + Cu(II)) + BSA; 3, (BSA + Cu(II)) + P50; 4, (BSA + P50) + Cu(II); 5, (P75 + Cu(II)) + BSA; 6, (BSA + Cu(II)) + P75; 7, (P75 + BSA + Cu(II); 8, (P25 + Cu(II)) + BSA; 9, (BSA + Cu(II)) + P25; 10, (BSA + P25) + Cu(II). Fluorescence registration began immediately after mixing of the last component, which took about 20 s, and continued for 15-20 min. The concentrations of polymer, BSA, and Cu(II) in the resulting mixture were 2.5 mg/mL, 0.71 mg/mL, and 0.3 mM, accordingly.

Thus, the structure of ternary PE-Cu(II)-BSA complex at pH 7.0 depends on the monomer composition of copolymers. At low amounts of hydrophobic monomer ([COOH]/ [NIPAAm Upon] = 3/1) the forming polycomplex particles have friable structures with protein molecules practically exposed to the solution. Upon increase of hydrophobic monomer in the composition of copolymer, protein molecules in the ternary structure become densely covered as a shell by polymer chains and practically "fenced off' from the water environment. At low polymer concentrations, an intrapolymer complex is formed. This intrapolymer complex aggregates to interpolymer species upon increase in the polymer concentration.

Cu(II) ions quenches Trp fluorescence in the PE-Cu(II)-BSA complex by a static mechanism, suggesting that polymer-metal complexes interact preferentially with BSA tryptophan sites.

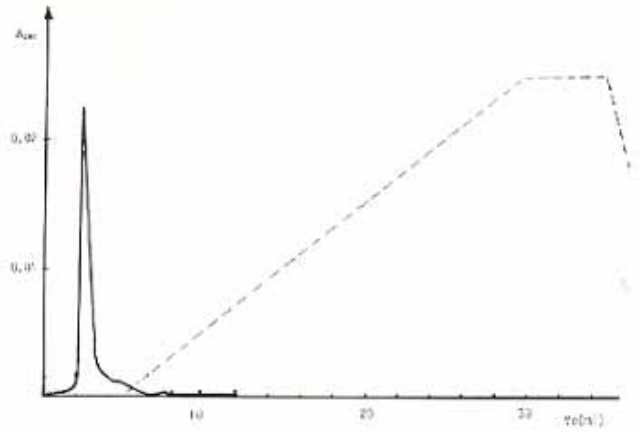


Figure 101. Anion-exchange chromatography of the matrix solution of the ternary mixture BSA-Cu^{2+}-PAA. (C_{PAA} = 0.1 g/dL): n_{Cu}/n_{AA} = 0.30 n_{BSA}/n_{pAA} = 1.0; pH = 7; 25°C. Broken line corresponds to (NaCl) concentration gradient.

3.2. Mechanism of Complex Formation

Our results indicate that water-soluble and insoluble stable ternary PAA-Cu^{2+} -protein complexes are formed at neutral pH. The preexisting electrostatic repulsive forces between PAA and proteins do not prevent the formation of polycomplexes in the presence of Cu^{2+} ions. Comparing these results with the corresponding results of copper-binding properties of proteins, we suggest that copper ions lead to the formation of chelate units. From what is known in the literature, [133-135,139,140,] the native sequence tripeptides, Asp-Ala-His-, Asp-Thr-His-, sequences represent the actual Cu^{2+} ions binding sites of HSA and BSA. NMR and, in particular, the ^{13}C technique results suggest that, in addition to the four nitrogen ligands (one amino, two peptides, and one imidazole nitrogen), the carboxyl side chain of aspartyl residue is involved in a pentacoordinated structure of the protein- Cu^{2+} complexes. Therefore, the carboxyl groups of PAA may compete with carboxyl group of aspartic residue and involve in Cu^{2+} binding (see Figure 101).

Hb-Cu^{2+}-PAA and Gl-Cu^{2+}-PAA systems at pH= 7 gives rise predominantly to insoluble ternary polycomplexes and the binding of protein molecules to a polymer is of a cooperative character, for instance, such binding lead to an irregular distribution of the protein between the macromolecules. The complex formation with BSA takes place in an analogous manner in relatively high concentration of Cu^{2+} to hemoglobin and globin, although, in some ratio n_{BSA}/n_{PAA}, in parallel to insoluble complexes, soluble triple complexes are simultaneously formed. Figure 102 A schematic presentation of the formation of chelate units between the functional

groups of the PAA and the protein globules with participation of copper ions (b) and structure of ternary PAA-Cu^{2+} -protein polycomplexes (a).

According to refs. [34,35], the reason for the demonstrated disturbance of the randomness of the distribution in the metal-containing triple systems Hb-Cu^{2+}-PAA and Gl-Cu^{2+}-PAA is probably a positive interaction of the protein globules adsorbed by one chain. In other words, the formation of contacts between protein globules "condensed" on the same polymer macromolecules results in an additional decrease of the free energy exceeding a free energy increase caused by the disturbance of the randomness of the distribution.

In our case, the interaction in the ternary mixtures was investigated at pH 7, which corresponds to isoelectric points of Hb and Gl. Therefore; these proteins at pH 7 show higher ability for intermolecular association in aqueous solutions.

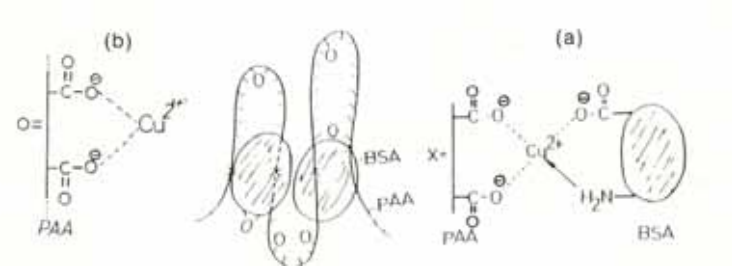


Figure 102. A schematic presentation of the formation of chelate units between the functional groups of the PAA and the protein globules with participation of copper ions (b) and structure of ternary PAA-Cu^{2+}-protein polycomplexes (a).

The pH of the reaction in the cases BSA-Cu^{2+}-PAA, HSA-Cu^{2+}-PAA and Tr-Cu^{2+}-PAA mixtures corresponds to the condition pH > pI The globules of these proteins being in this case negatively charged, their aggregation ability is low. One can see from the titration data as that these mixtures remain in a wide range of n_{pr}/n_{PAA} soluble. Phase separation in this system occurs at relatively high concentrations of metal ions. The results of physicochemical studies led us to propose a hypothetical structural scheme of ternary water soluble protein-metal-polyanion complexes (Figure 103).

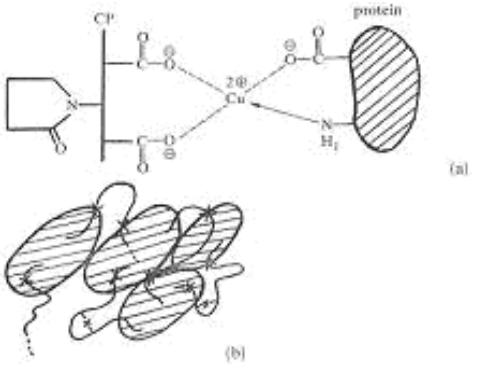


Figure 103. Schematic illustration of (a) the formation of chelate units between the functional groups of the copolymer (CP) and the protein antigen with participation of copper ions, and (b) the hypothetical structure of the triple polymer-metal complex of the protein antigen (crosses demote copper ions).

When PMC solution is titrated with protein solution ($n_{pr}/n_{PAA} < 1$), the protein globules are crosslinked with a linear polyion via copper ions. Some of the copper ions form intramolecular crosslinks in the free sections of polyions and, thus, stabilize the structure as a whole. The regions/sections of the polyion not directly involved in the complex formation (both with metal ions and without metal ions) exist in the form of free loops "dissolved" in water. The form of chelate units between protein globules and PAA and in the free sections of polyions leads to a change of charges of particles of ternary complex in dependence of the concentration of protein molecules. Therefore, as can be seen from the results in Figure 104, these substances migrate in electrophoretic field more slowly than the free protein molecules. On further increase in n_{pr}/n_{PAA}, the electronegativity increases and then attains a limiting value. Under this condition, the values of the electrophoretic mobilities of the triple complexes and free protein molecules are fairly close. The formation of nonstoichiometric polycomplex and redistribution of copper ions from the free sections to protein bound sections of PAA may contribute to this latter process. [We cannot analyze the distribution of protein molecules between polymer coils in soluble mixture because free PAA or (PMC) do not separate from polymer-protein complex in HPLC.] The formation of the water-soluble aggregates in the protein-PMC systems at the higher concentration of the Cu^{2+} ions is shown in Figure 103. At low concentration of copper ions, the interaction can be considered to be intramolecular only as Cu^{2+} forms a complex with one polymer chain. At high concentration one can speculate that the copper ion can act more effectively as a crosslinking agent between two (or more) polymer coils. This intermolecular coil interaction in the case of ternary systems leads to the formation of soluble and insoluble polycomplexes with a complicated structure, for instance, the existence of Cu^{2+}-induced crosslinking self-assembly of polycomplexes.

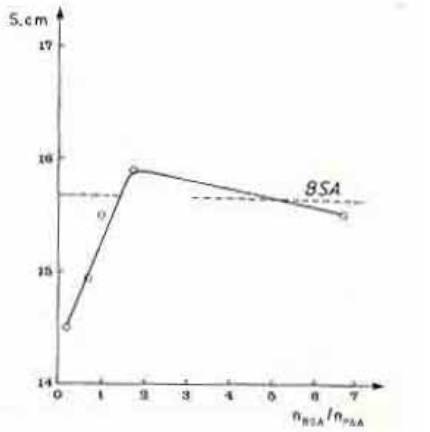


Figure 104. Dependence of the electrophoretic mobilities (distance-S) of the products of solution of the ternary mixture BSA-CU^{2+}-PAA on n_{esA}/n_{fAA}; C_{PAA} = 0.1 g/dL n_{cu}/n_{AA} = 0.25; pH = 7; 25°C. Broken line represents free BSA.

We can propose that in this case with the increase in concentration of protein molecules in ternary mixture, the mechanism (2) becomes more probable, resulting in phase separation and transformation of insoluble polycomplexes to a soluble state in the system.

3.3. Polycation-Metal-Protein Systems

The interaction between poly-4-vinylpyridine and bovine serum albumin with the participation of bivalent copper ions in acidic medium has been studied by Mustafaev [61,101].It is known that both P4VP and BSA (pI=4.9) carry the similar (positive) electrical charge in acidic media and

cannot react with each other without some intermediates. Interaction between cationic P4VP and BSA at pH=4.3 at the absence of copper ions is weak, if not negligible. At this pH the electrostatic repulsion forces between positive macromolecules prevent a stable complex formation. However, complexes were formed following the addition of copper ions. The ternary P4VP-Cu-BSA mixture was prepared by adding protein solutions to the polymer-metal complex (PMC) solution at pH 4.3. To produce a P4VP-Cu^{2+} complex, different concentrations of the $CuSO_4x5H_2O$ solution were added to P4VP solutions at pH 4.3. The ratio of copper ions per pyridine (n_{Cu}/n_{Py}) was 1:4, i.e. one Cu^{2+} ions per 4 pyridine groups. These numbers correspond to maximum capacity of P4VP to bind of copper ions. The fraction of P4VP with degree of polymerization (P) 950 (PVP-1), 2150 (PVP-2), 7400 (PVP-3) were used for the investigation. The mixing of P4VP and Cu^{2+} ions at this conditions results in formation of insoluble polymer-metal complexes with intensive blue color. The whole P4VP and Cu^{2+} were including into composition of insoluble complex. However, the situation changes completely in the presence of BSA molecules in this mixture, which is accompanied by partial (or full) elimination of the phase separation process depending on the protein concentration in the reaction mixture. The ultracentrifugation measurements of matrix solution of the PVP-Cu^{2+}-BSA mixtures show that only one peak with the sedimentation coefficients 20-30 sved and 50-80 sved. in the case of PVP-1 and PVP-2, respectively was observed on sedimentograms of mixtures (Figure 105).

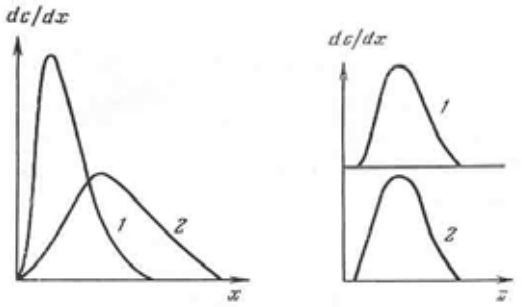


Figure 105. Sedimentograms of mixtures BSA-Cu(II)-PVPI (1) and BSA-Cu(II)-PVPII (2) at n_{BSA}/n_{PVP}=4(1),10(2);$P_w=10^3$ (PVPI) and $7.4x10^3$(PVPII) t=12 min. ώ =26000 min^{-1}.Right sedimentograms corresponds to BSA(1) and BSA-Cu(II) (2),t=60 min. ώ=56000 min^{-1}

From the results that the sedimentation coefficients of the reaction products were characterized by rather large value than Sc for free BSA (Sc for BSA equal 4.4 sved), and for BSA-Cu^{2+} complexes and the PVP-Cu^{2+} complex is not soluble at this reaction conditions, we can consider that these peaks corresponds to the soluble products of the binding of BSA with polycation. Thus, under conditions where both P4VP and BSA are positively charged and incapable of binding to one another that the divalent Cu^{2+} ions act as "fasteners", promoting the formation of relatively stables water soluble and insoluble ternary complexes.

Studies of solubility of ternary mixtures at different molecular weight of P4VP and protein concentrations permits to elucidate some important features characterizing Cu^{2+}-mediated protein-polycation complex formation. An increase of the number of protein molecules in the mixture (the weight concentration and Cu^{2+}/PVP ratio of PMC is kept constant) leads to a decrease of the amount of precipitate corresponding to free PMC insoluble complexes while the area of the peak of the water-soluble complex increases. When the BSA/PVP ratio (the minimum amount of BSA per one gram of PVP which is necessary for full prevention of phase separation in mixtures) is 2.8g BSA/1g PVP, the precipitate of PMC disappears and only soluble product remains. The described situation is typical for all studied fractions of PVP and equal 2.8g BSA 71 g PVP. At the same time the ratio of numbers of macromolecules at which one observes a

disappearance of the free PMC precipitate, depends on the degree of P4VP polymerization. From the sedimentation data obtained it fallows that the free protein is absent in the system over the whole studied range of the ratios n_{BSA}/n_{PMC}, i.e. all added BSA is strongly bound by the polycation-metal complexes. It was suggest that the complex formation is not dependence on the molecular weight of polycation in the range of degree of polymerization P_v =1000-7500. Taking into account the above proved fact of the quantitive binding of BSA with PMC one may consider that $\lim(n_{BSA}/n_{PMC}) = N_i$, when amount of precipitate "m" is 0. This limit equals the number of the protein molecules bound by polycation-metal complexes of a given degree of polymerization under given conditions. It follows from the results that the higher the degree of polymerization of PVP, the higher N_i is. Within the experimental error this dependence is linear. It means that the average the site of the polycation chain of the approximately constant definite length "l" is used for such globule binding. The average value of "l" is about 250 monomer units.

The molecular weight of the particle of soluble ternary BSA-Cu^{2+}-P4VP complex with the composition $n_{BSA}/n_{PMC} = N_i$ were determined by light scattering measurements (Figure 106). As seen from the sight scattering results the dependence of $K.c/R_0$ on the concentration was linear, indicating the absence of dissociation of the polycomplexes at dilution. In other words, the studied soluble ternary BSA-Cu^{2+}-PVP polycomplexes are stable over a wide range of the solution concentrations

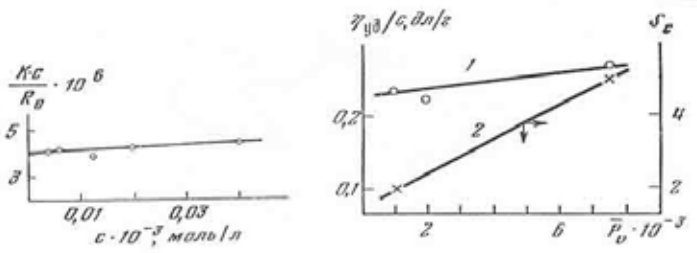
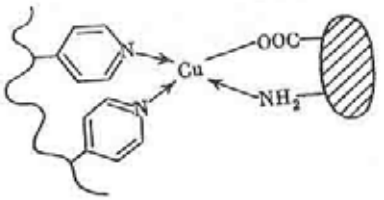


Figure 106. Dependence of $K.c/R_o$ on the concentration of BSA-Cu(II)-PVPI complexes, pH 4.25. Dependence of inherent viscosity (1) and sedimentation coefficient (Sc) of BSA-Cu(II)-PVP complexes at n_{BSA}/n_{PVP} corresponding to full homogenization of the systems on the degree of polymerization of PVP.

The average value of molecular weight (Mw) obtained from this dependence for the complexes of BSA with PVP-1 copper complexes is about 2.5x10. At the same time, the characteristic composition N_i for the polycomplexes in the case with PVP-1 was equal to 4 (N_i=4). The average value of molecular weight for this complexes calculated from $M_{sd} = M_w$ (PVP-1) + N_ixM(BSA) with taking into account of amount of copper ions was equal to $3.8x10^5$. Therefore the real particle of the triple polycomplex is an associate, which includes 6-7 polycations binded with protein globules via copper ions. One can assume that the contacts of protein and polycation are carrying out via chelate nodes in which the copper ions play a part as a central atom:



The further definition of the ternary BSA-PMC complex structure is obtained from the comparison of the inherent viscosities and sedimentation coefficients for these complexes formed

by the polycations with different length when $n_{BSA}/n_{PMC} = N_i$. It is seen in Figure 106 that the sedimentation coefficient of ternary complexes sharply increases with increasing degree of polymerization of the polycation. At the same time in this range of degree of polymerization of PVP the viscosity of the solutions was changed insignificantly. Remarkable that the values of η_{sp}/c were equal only 0.25 for such higher molecular weights of polycomplexes. The relation of this kind may exist only for particles with sufficiently compact structure. Thus, it is established the high compactness of particles of soluble triple complex by light scattering, sedimentation and viscosimetry methods.

In conclusion, the presented results show a wide variety to prepare polymer-protein complexes with desired physicochemical properties. A considerable interest exists for the establishment of the correlation between the structure of the polymer complexes of antigens and their immunological activity. Comparison of these results with the formation of complexes involving biopolymers, in particular, polynucleotides and nucleic acids, will contribute to the investigations on the roles of the multivalent ions in the regulation of these processes. Moreover, studies of the mechanisms of cooperative binding of proteins to synthetic PE will be of interest for the elucidation of the mechanism of action of PE in the organism, for example, in immobilization of enzymes and specific sorption of proteins on surfaces. In addition, such reactions may simulate, for instance, antigen-antibody reactions and processes of self-organization in biological systems.

4. COVALENT COMPLEXES (CONJUGATES)

Synthetic polyelectrolytes (PE) have been widely used to modify proteins via covalent attachment, increasing (or reducing) the immunoreactivity and/or immunogenecity of original antigenic proteins [1-11,141-149], and improving their in vivo stability with prolonged clearance times [19]. Besides, the PE conjugates with individual microbe antigens develop strong protective properties [9,9a] and they can be considered as a new generation of vaccinating compounds.

It is known that conventional methods of synthesis of protein-protein and protein-linear PE covalent conjugates are based on reactions between activated functional groups of macromolecules in aqueous solutions [150]. A whole series of well-known reactions can be used to activate functional groups of a polymer carrier or a protein molecule and to link them together. However, such reactions between macromolecules, carried out in solutions, meet some inherent difficu1ties and limitations with respect to their direction and yield control.

The basic reaction mechanism of carbodiimide-mediated modification of carboxyl group includes a two-step reaction sequence of condensation between carboxyl groups of polymer and amino groups of proteins. During the first step, the carboxyl group is activated by the carbodiimide an o-acylisourea intermediate, which can react in further with an amino group from a protein molecule. Reaction with an amino group from a protein will lead to a cross-link between the polymer and protein components. One can assume, that the formation of conjugates can involve several sequential steps. In our opinion, the first and the most important step is primary complex formation between the polymer activated by carbodiimide and the protein by electrostatic interactions and strengthening of the structure by hydrophobic interactions. Covalent cross-linking reaction is limited by structure formation and occurs in a slower time scale.

As it was mentioned above the complex formation between proteins and PE have been the subjects of numerous research efforts. Soluble polymer-protein complexes were studied by a wide range of methods that well known in colloid and polymer chemistry. The mode of binding proteins to PE has been found to depend on the ratio of components. The system exhibits characteristics of cooperative binding, so that the protein molecules are in homogeneously distributed among the polymer chains, and free PE molecules coexist with complex. Further increase in r leads to a secondary binding process along with an increase in the amount of free protein. In this approach however, the systematic analysis of the covalent binding mode in the

polymer-protein mixture is stilI absent and the mechanism is not completely understood. Fundamental questions, concerning the magnitude of the binding constant, the cooperativity of the binding and the effect of bulk mass ratio of protein to polymer on the binding mechanism, still remain to be answered.

Poly(N-isopropylacrylamide) (PNIPAAm) is a well known water-soluble polymer showing unique, reversible hydration-dehydration changes in response to small changes in temperature [151-153]. An aqueous solution of PNIPAAm demonstrates phase separation and the polymer precipitates at a certain temperature, which is called a lower critical solution temperature (LCST).

The objective of the present study is to examine the covalent binding mechanism of poly(N-isopropylacrylamide-co-acrylic acid) (poly(NIP AAm)) copolymers with BSA molecules depending upon the weight concentration ratio *(r)* of BSA to poly(NIP AAm-AA). The fraction composition of conjugates, degree of binding, thermal collapse property as revealed through a combination of HPLC, electrophoretic, fluorescence, UV - Visible spectroscopy and viscosity measurements are reported [153].

Temperature-responsive (N-isopropylacrylamide-coacrylic acid) (poly(NIPAAm-AA)) copolymers (CP) was synthesized from N-isopropylacrylamide (NIPAAm) and acrylic acid (AA) [153]. Compositions from mol feed ratios [NIPAAm]/[AA] = I/I were prepared. The viscosity (sp/c) in 0.154M NaCl solutions of the copolymers was 0.4434 dl/g. The aqueous polymer solutions were characterized by transformation from hydrophilic to hydrophobic states (LCST) at 26°C. Bovine serum albumin (BSA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from the Sigma Chemical Company, Sİ. Louis (USA) and used as received.

Activation of polymer acid group was carried out in water (pH 6.0) in a molar ratio [EDC]/[AA] = 1: 2; CP (50 mg) was dissolved in water, stirred at 4^oC and EDC (20 mg) was added. After 1 h, the pH values of mixture were adjusted to with 1 M NaOH and different concentrations of BSA (C_{BSA}/C_{CP} = 0.28; 0.59; 1.14; 1.70; C_{BSA} and C_{CP} weight concentration of BSA and CP; concentration of CP was kept constant and C_{CP} = 0.25 g/dl) were added to the reaction mixture and stirred 12 h. After removal of o-acylisourea intermediate by dialysis, the sample was lyophilized. The purified conjugates were redissolved in 50mM PBS at 4°C for the physico-chemical analysis.

The compositions of the soluble mixtures were estimated by gel filtration chromatography using a Bio-Sil Sec 250 column (7.8 x 30 cm). A Waters Model 501 HPLC was run with a 10 mM potassium phosphate buffer, pH 6.8 and 100mM NaCl at flow rate 1.0 ml/min at room temperature. The eluate was monitored by absorption at 278 nm. The standards used to calibrate the column were thyroglobulin (670kDa), immunoglobulin G (155 kDa), myoglobin (16.9 kDa), and vitamin B12(1.35 kDa).

Spectrophotometric titrations were performed both with a WTW 537 type pH meter and VV-Visible (Shimadzu VV-160 A) spectrometer with temperature control attachment. Reactions were monitored between 200 and 1100 nm. Optical transmittance of CP and conjugates in aqueous solution at various temperatures was measured at 500 nm using a spectrophotometer (Shimadzu VV-160 A). The cell was thermostated by a Cary Temperature Controller.
The viscosity measurements were carried out with an Ubbelohde-type viscometer. The temperature of the sample was controlled to 0.02^oC.

BSA and polymer-BSA mixtures were analyzed by polyacrylamide gel electrophoresis (PAGE) [154] and SDS-polyacrylamide gel electrophoresis [155]. Protein gels were detected by Coomassie Blue staining. The band densities were determined using a Hoefer Scientific Instruments Scanning Densitometer. SDS-polyacrylamide gel electrophoresis experiments were carried out in the presence of strongly anionic detergent, sodium dodecyl sulfate (SDS) in combination with a reducing agent and heat to dissociate the proteins before they are applied on the gel as already described [155]. The denatured proteins (polypeptides) bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the

molecular weight of the polypeptide, SDS-polypeptide complexes migrate through polyacrylamid gels in accordance with the size of the polypeptide.

Fluorescence emission spectra were obtained by using the Quanta Master spectrofluorometer (photon Technology International, Canada) operating in quanta counting mode. The slits of excitation and emission monochromators were adjusted to 2 or 3 nm. The excitation wavelength was 280 nm.

For the determination of amino groups of BSA, fluorescent measurements were performed in the presence of fluorescamine at the wavelengths of excitation 390 nm and fluorescence 475 nm. Conjugates and BSA were prepared in boric buffer with pH = 9. The boric buffer was made by dissolving the boric acid in water at concentration 1.24 g/100 ml. Fluorescamine was dissolved in acetonitrile and was added in the ratio one molecule of fluorescamine to one amino group of BSA.

Figure 107 presents the viscosity of reaction products and CP-BSA mixtures as a function of ratio of components, $r = C_{BSA}/C_{CP}$, in neutral water solution (pH = 7). The viscosity of solution of simple polymer-protein mixture practically does not change by the titration of polymer solution with BSA and is characterized by viscosity of polymer components because of the absence of stable complex formation between anionic polyions and similarly charged protein globules. In contrast to these mixtures, the viscosity behavior of the solution of the products of the reaction of carbodiimide activated CP with BSA depend strongly on the initial protein concentration in the mixture. By increasing of protein amount, viscosity of the systems first rises sharply ($C_{BSA}/C_{CP} < 1$) and then attains a maximum value ($C_{BSA}/C_{CP}=1$).

At this ratio, the viscosity of the modified CP-BSA mixtures is about three times of magnitude higher than the viscosity of the free CP or simple CP-BSA mixtures. As it follows from these viscosity results, the formation of the covalent conjugate CP-BSA occurs in these systems. It is important that the reaction products which are prepared over a wide range of the [BSA]/[CP] ratios are water-soluble.

The typical HPLC chromatograms of bioconjugates which were synthesized at different initial ratios (C_{BSA}/C_{CP}) of their macromolecule concentrations in the solution are given in Figure 108a. As it follows from this figure, the system is characterized by a bimodal distribution of elution components. A comparison of the values of the retention times (RT) corresponding to the peaks 1 and 2 of the solution of reaction mixtures and of the individual components shows that the rapidly eluting substance (peak 1) corresponds to a free CP and peak 2 corresponds to the polymer-protein conjugate.

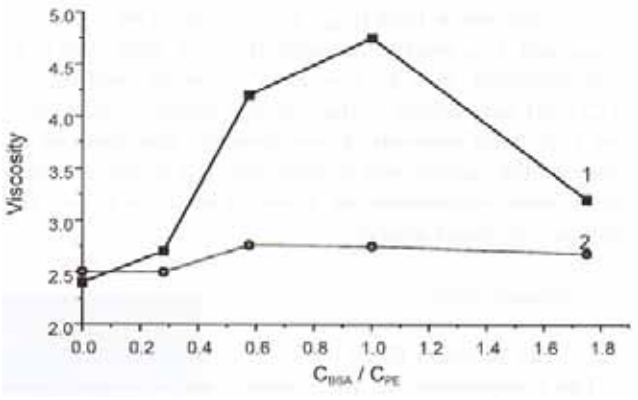


Figure 107. Dependence of viscosity (η_{sp}/C, dl/g) of reaction products (CP-EDC-BSA) (1) and mixtures (CP-BSA) (2) on the initial ratio BSA/CP in neutral water solution (PBS, pH 7). Molar ratio of [EDC]/[AA] = 1: 2; different BSA concentration; 23°C.

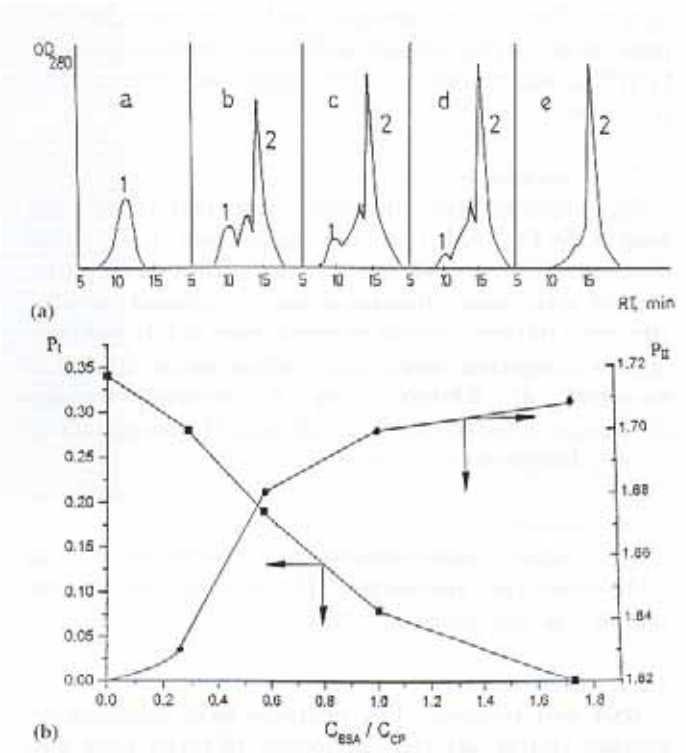


Figure 108. (a) Gel filtration HPLC chromatograms of free CP (1) and reaction products of CP-EDC-BSA prepared at different BSA/CP: 0.28 (a); 0.59 (b); 1.14 (c); 1.70 (d); free BSA (e) CBSA = 0.1 g/dl; different polymer concentration; 25°c. pH 7.2. RT- retention times. Diagrams represent normalized OD280 values. (b) Dependence of the chromatogram peak area (Po) of free CP (1 peaks I) and reaction products (2, peaks 2) (PII) on the BSA/CP'

The solution of the reaction products is characterized by one peak on the e1ectrophoregrams, obtained by the native and SDS-electrophoresis methods (Figure 109). The electrophoretic mobilities of the reaction products depend on the initial ratio of reaction components (Table 9).

The results obtained at the $C_{BSA}/C_{CP} < 1$ convinces the protein binding by CP and the absence of pure BSA in solution of the reaction products. Therefore, the peaks (2) in HPLC chromatograms (Figure 108) correspond to soluble covalent bioconjugates of CP-BSA. The character of the dependence of the studied parameters of the reaction products on the ratios C_{BSA}/C_{CP} shows that the mechanism of the binding and the composition (and structure) of the bioconjugates polyion-protein depends on the initial ratios of components.

It is remarkable that the above-described results, obtained by the analysis of the overall physico-chemical measurement data is confirmed by the data of fluorescence analysis. These conjugates, which had been prepared at different *r,* were studied by fluoremetric method. Figure 110 shows fluorescence spectra for solutions of free BSA and of conjugates with varying initial concentrations of CP. These results shows that the maximum yields of binding of BSA molecules to polymer chains occur at about $r = 0.28$ because of the maximum reduction of tryptophan fluorescence intensity. On the other hand, conjugation at $r < 1.0$ induces a marked blue shift of Amax (Table 9).

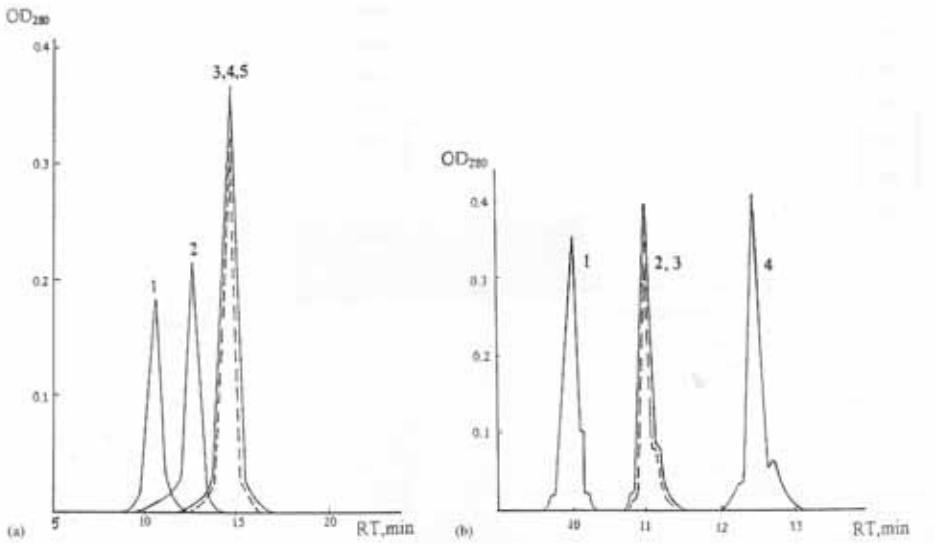


Figure 109. Electrophoregrams of BSA and reaction products of CP-EDC-BSA mixtures obtained at different BSA/CP ratios by two methods: native (a) and SDS-polyacrylamide gel electrophoresis (b). BSA/CP: 0.28 (1); 0.59 (2); 1.14 (3); 1.70. (4); free BSA (5 - native, 4 - SDS). Different polymer concentrations, RT - retention time. Diagrams represent normalized ODZ80 values.

Table 9. Characteristics of BSA-poly(NIPAAm-AA) conjugates

CBSA/CPE	β	RTn (min)	RTsDs (min)	in	Amax (nm)
0.28	48	10.5	10	4.4	338.0
0.59	39	13	11	6.5	339.9
1.14	35	15	11	9.2	340.9
1.70	35	15		9.5	341.0
BSA		15	12.5	10	341.0

aRTn and RTsDs - retention times in native and SDS-electrophoresis; in - intensity of fluorescence in maxima, Amax - robition of fluorescence maximum; β - average number of binding NHz -groups.

This indicates that in the CP-BSA conjugates formed, BSA tryptophanyls are complete1y isolated from water solution by the polymer, which cover apparently all the BSA surface. By increasing *r,* the position of the maximum of the spectrum approaches max of free BSA. Thus at $r > 1$ the polymer chains do not form covering around BSA molecules and the formed conjugate particles possess more friable structures in which protein molecules are practically open for the solution.

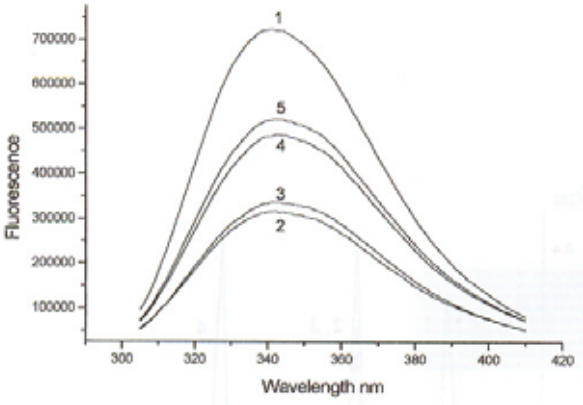


Figure 110. Fluorescence spectra of pure BSA (1) and conjugates, preparing at different initial BSA/CP ratios: 0.28 (2); 0.59 (3); 1.14 (4); 1.7 (5); BSA concentration 0.71 mg/ml, phosphate buffer (pH 7.2). Different polymer concentrations; 23 C.

The yield of conjugation should be directly proportional to the concentration of ε-aminolysil groups of protein molecules, assuming that they are all accessible for the reaction. The ε-aminolysil contents of the BSA and polymer-protein conjugates studied by fluorescamine interact with the primary amino groups of the samples. Figure 111 shows the fluorescence spectra of fluorescamine in its mixtures with BSA and conjugates, at different concentrations of fluorescamine *(*N_F/N_{BSA}). As the fluorescamine concentration in the solution of free BSA is increased, emission intensity of solution increases and reaches a maximum value at $N_F/N_{BSA} = 60$, which corresponds to total number of free ε -amino groups in one protein molecules. The fluorescence intensity in the bioconjugate solutions under the same conditions is lower than that in free BSA solutions and depends on the initial conjugation ratio *(r)* of components. Therefore, by analyzing the content of a free amino group in the reaction mixture, one can determine the number of covalent bonds formed by a protein globule with a polyelectrolyte macromolecule. The ε -aminolysil contents of BSA and the reaction products studied here are shown in Table. It is seen that an increase in BSA content in the mixture results at first, in an essential decrease in the number of free aminogroups *(Ni* = 12, *r* = 0.28). According to the scheme A (Figure 111), it shows an increase in the overall covalent bond number between the protein globules and the polyions. At $r > 0.3$ the number of covalent bonds between protein and polymer components make up about $N = 21\text{-}25$.

Thermal collapse property. In Figure 112 the reduced viscosity of the samples as a function of temperature is plotted. The reduced viscosity of solution of free polymer as well as bioconjugate solution decreases sharply with temperature and finally the reduced viscosity value becomes minimal. It seems that analogous to the pure polymer molecules, the bioconjugate coils collapse as the temperature increases buffer (pH 7.2) above the lower critical solution temperature (LCST) of the polymer, LCST = 24°C, i.e. in all the cases the coil-globule transition occurs. Nevertheless, between this, some differences are observed between these conjugates. The temperature which corresponds to minimal viscosity values of bioconjugates depends on the BSA/polymer ratio *(r)* and is decreased with increasing *r*. Figure 113 shows the temperature dependence of OD_{500} values for the solutions of CP and CP-BSA conjugates prepared at different concentrations.