Soluble and Insoluble Ternary Complexes of Serum Proteins with Polyanions in the Presence of Cu^{2+} in Water

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SYNOPSIS

Complex formation between polyacrylic acid (PAA) and bovine serum albumin (BSA), human serum albumin (HSA), hemoglobin (Hb), globin (Gl), and, respectively, transferrin (Tr), were studied in neutral water. Water-soluble and insoluble complexes are formed upon addition of divalent copper ions to the solution. The contacts between proteins and PAA are achieved via chelate unit formation in which the copper ions are located at the center. The solubility of the polycomplexes depends on the nature of proteins and correlates with their isoelectric points (pI). In the mixtures of Hb–Cu^{2+}–PAA and Gl–Cu^{2+}–PAA, insoluble complexes are formed at pH = pI starting with very low concentrations of Cu^{2+} ($n_{Cu}/n_{AA} \leq 0.01$). On the other hand, these polycomplexes remain soluble at pH > pI. BSA, HSA, and Tr form soluble ternary polycomplexes at neutral water (pH 7). The formation of the polycomplexes in the mixture BSA–Cu^{2+}–PAA was intensively studied by titration, HPLC, electrophoretic, and spectrophotometric methods. The solubility, composition, and stability of these polycomplexes depend on metal/polymer and protein/polymer ratio. Insoluble polycomplexes are formed when concentration of Cu^{2+} reaches a critical value ($n_{Cu}/n_{AA} \geq 0.25$). At this concentration of Cu^{2+}, phase separation takes place, starting with very low concentration of protein in the system. Over the critical ratio of the protein/polymer, the mixture again exhibits water-soluble character. The pattern of distribution of Cu^{2+} between PAA coils and of protein globules between polymer–metal complex particules appeared to follow the self-assembly principle. A hypothetical structural scheme for the formation of soluble and insoluble ternary polycomplexes is proposed.

INTRODUCTION

Reactions of polyelectrolytes (PE) with proteins in aqueous solutions have attracted great attention in the last decades.[1-14] Polymer–protein complexes (PPC) form as a result of the polyion chains with the oppositely charged groups of the protein molecule during these reactions.

The extent of the interaction is found to be pH sensitive and dependent on the isoelectric points of the proteins. Such complexes represent a specific class of polymer–protein compounds that have important applications in various areas.[5]

Polyelectrolytes of synthetic origin have been found to increase immunoresponse to the immunizing antigen and to produce an adjuvant effect.[15-17] The use of PE as a carrier, which is firmly linked to microbial and viral antigens to form a stable complex (or conjugate), not only increased by several orders of magnitude the immune responsiveness of the organism but also afforded effective immune protection. This has, in turn, opened the way to the construction of artificial vaccines against yet uncontrolled infections.[17] Such systems include complexes stabilized by cooperative electrostatic and hydrophobic interactions between the fragments of PE and antigen molecules and conjugates in which the functional groups of the components are linked by covalent bonds. In those cases where PE macro-

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molecules do not contain the corresponding electrostatic or hydrophobic groups for antigen binding, it is necessary to modify the carrier polymer, which can give rise to changes in its effect(s) upon biological systems.

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 derivatization of the activated support, through formation of chelates.[18] Evidence has recently been presented for the existence of a ternary complex between proteins, Cu^{2+} ions, and aminoacids.[19] Some publications in the current literature are devoted to the construction of drugs based on such Me-mediated complexes of natural PE and antigens.[20-24]

Several investigators have studied the behavior of linear synthetic polyelectrolytes in such systems. In particular, insoluble complexes of bovine serum albumin (BSA) with nonfractionated poly(acrylic acid) (PAA) in the presence of Ba^{2+} have been described by Morawetz at al.[25] The soluble and insoluble ternary complexes of the positively charged poly-*N*-vinylpyridinium and poly-*N*-vinylimidazolium ions and BSA formed in the presence of Cu^{2+} have been studied recently.[9,26] Cu^{2+} induced complexes of the negatively charged polyampholytes–copolymers of acrylic acid with 2-methyl-5-vinylpyridine, *N*-vinylpyrrolidone, *N*-vinylimidazole, and maleic anhydride and of BSA have been reported to depend on the pH of solutions which may, in turn, give rise to the opposite or the same charged components.[4,27,28] The kinetics of the formation of insoluble ternary complexes between polyacrylamide, which contains a glycine end group and BSA were studied in neutral water.[14] Some of these complexes reveal high immunogenecity and provide high levels of immunological protection.[17,26-28]

However, the previously used PE were are characterized with higher compositional heterogenecity and molecular weight distribution. The composition and structure of such polycomplexes have not yet been investigated and, therefore, information concerning the relationship between the immunogenecity and physicochemical properties in the ternary PE–metal–antigen complexes is still absent.

The present study, which made use of fractionated homopolymers, for instance, polyacrylic acid fractions with a predetermined molecular mass, and investigated the mechanism of the formation of complexes of linear polyanions with serum proteins of different isoelectric points in the presence of copper ions, reports new data on the structures and transitions in these systems.

EXPERIMENTAL

Materials

PAA were synthesized and fractionated as explained in the literature.[29] Polyacrylic acid (PAA) was prepared by radical polymerization of acrylic acid in toluene with benzoilperoxide as an initiator. PAA was fractioned from 3–4% solution in methanol by fractional precipitation by ethyl acetate. The molecular weights of the fractions used in this study was 150 kDa. BSA, HSA, Tr, and Hb were purchased from the Sigma Chemical Company, St. Louis, MO. The Gl was prepared in our laboratory using hemolysate from adult human blood.[30] $CuSO_4 \cdot 5H_2O$ used was purchased from Merck (Darmstadt).

Some physicochemical properties of these proteins are listed in Table I.

Molecular weight and purity of the proteins used in this study were determined using HPLC with ion exchange and gel filtration columns, specrophotometry, and electrophoresis. The obtained values were found to be in agreement with the literature values.

Polymer–Metal–Protein Complexes

To produce a polymer–metal complex, various concentrations of the $CuSO_4 \cdot 5H_2O$, pH 4, solution were added to PAA, dissolved in phosphate buffer, pH 7.2. The pH values were adjusted with 1*M* NaOH to the desired pH.

The ternary complexes were, in turn, prepared by adding protein solutions to the polymer–metal complex (PMC) solution. Buffer systems were prepared using various concentrations of phosphate buffer.

Water-insoluble products of the complexes were investigated spectrophotometrically at 400 nm and by weighing dry amounts of pellet. The pellets were measured relative to H_2O.

Protein/polymer (n_{pr}/n_{PAA}) and Cu/AA (n_{Cu}/n_{AA}) ratios were calculated using the equation *n*

Table I Some Physicochemical 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

$= CN_A/M$, 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/100 mL.

Measurements

Gel Filtration HPLC

The molecular masses of proteins and the fraction compositions of the complexes were estimated by gel filtration chromatography using either Bio-Sil Sec 250 or Protein Pak 300 SWB column (7.8 mm × 30 cm). A Waters Model 501 HPLC was run in a buffer containing 100 m*M* potassium phosphate, pH 6.8, and 100 m*M* NaCl at a flow rate of 1.0 mL/min at room temperature. The eluate was monitored at 278 nm. The standards used to calibrate the column were thyroglobulin (670 kDa),immunoglobulin (155 kDa), ovalbumin (44 kDa), myoglobin (16.9 kDa), and vitamin B_{12} (1.35 kDa).

Anion-Exchange Chromatography

A water-soluble mixture of polymer–metal complexes and of ternary complexes (polymer–metal–protein) were separated on Bio Gel Sec DEAE 5 PW column (7.5 mm × 7.5 cm) using an anion-exchange chromotography system. Solvent A correspondeds to 10 m*M* potassium phosphate, pH 6.8, and solvent B contained, additionally, 100 m*M* NaCl. A linear gradient (solvent B/A) was run from 0–100% over a period of 30 min at a flow rate of 1.0 mL/min. The eluate was monitored at 278 nm.

Analytical Methods

Proteins and their mixtures with PE were analyzed by polyacrylamide gel electrophoresis (PAGE).[31] Proteins in gels were detected by Coomassie Blue staining. The band densities were determined using a Hoefer Scientific Instruments Scanning Densitometer and the areas of the peaks hereby obtained by weighing. The Cu^{2+} mixture was determined in a Zeeman Atomic Absorption Spectrophotometer (Hitachi-180–80). Protein amounts were determined by use of Folin phenol reagent method.[32]

Cyclic Voltammetry (CV)

For the cyclic voltammetric measurements a Wenking POS 73 Model potentiostat, which is interfaced with an enhanced Multi Labcard PCL-812, was used. Measurements were carried out in a conventional three-electrode system consisting of a platinum wire as a counter electrode, with a saturated calomel electrode (SCE) connected with electrolyte solution by a Luggin capillary. All measurements were carried out in deoxygenated medium, by passing nitrogen gas through the solution for 20 min.

RESULTS AND DISCUSSION

PAA–Cu^{2+} Complexes

The solutions of partially ionized polyanions, polyacrylic acid, or polymetacrylic acid, containing Cu^{2+} ions, were analyzed earlier by the titration shift method and measurements of the equilibrium dialysis, spectroscopy, and the like.[32] These data have implicated the presence of chelate with two carboxylate groups bound to a copper ion. Fast sedimentation and quenching of luminescence have been employed to study the binding Cu^{2+} by PAA recently.[33–35] 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 1. 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 involment, of 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 (V_e) corresponding to peak I does not change and remains equal to that of free PAA, but the values for the V_e 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 voltametry (see below under Fig. 5). Moreover, analysis by atomic absorbtion spectroscopy indicated an uneven distribution of Cu^{2+} among the peak fraction obtained by Sephadex G-100 filtration of PAA–Cu^{2+} mixture [Fig. 1(b)]. These findings indicated a nonrandom disribution of the copper ions between the polyanions under the

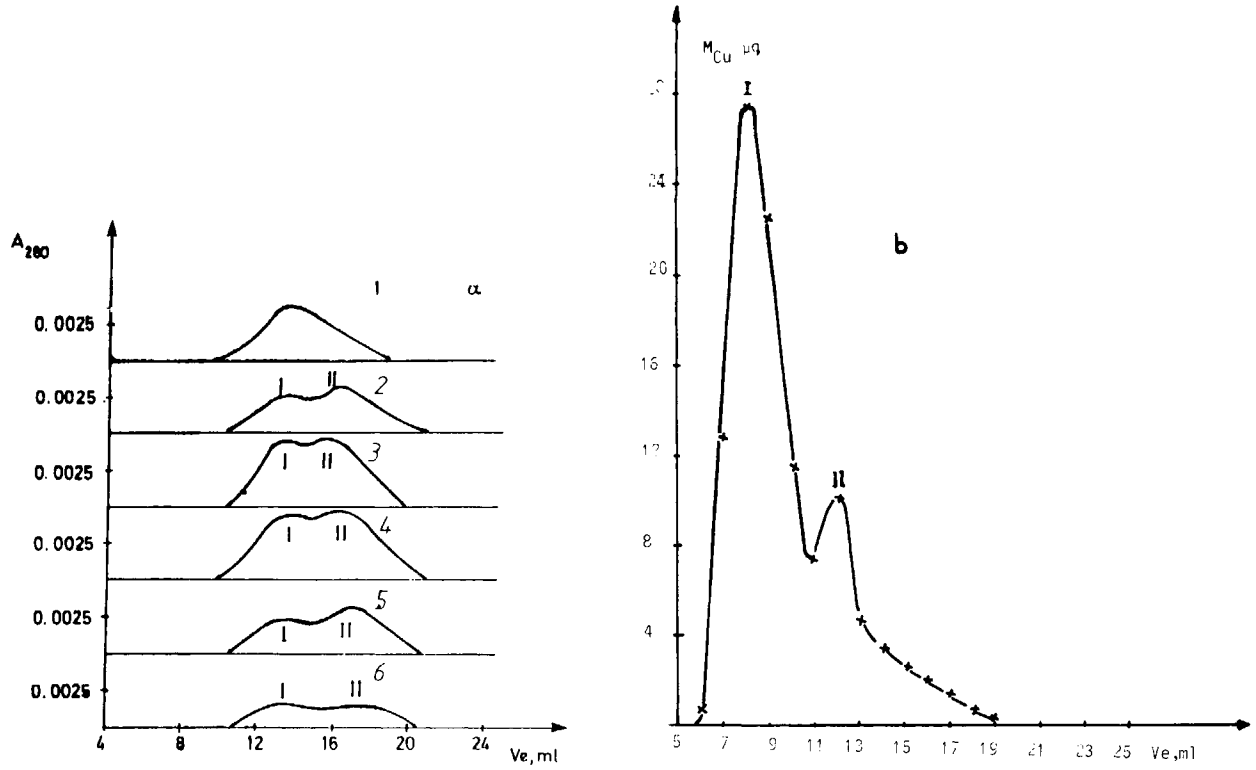

Figure 1 (a) Gel filtration HPL chromatograms of PAA (1) and of its mixtures with Cu^{2+} at different ratios of Cu^{2+} ions to acrilic acid monomers (AA) (n_{Cu}/n_{AA}): 2—0.08; 3—0.10; 4—0.15; 5—0.20; 6—0.25; (5-6)—the phase separation in the system takes plase. The concentration of PAA was 0.1 g/dL; pH 7. Bio-Sil Sac 25 column was used in the experiment. (b) Distribution of the amount of Cu^{2+} in the mixture PAA-Cu^{2+}.

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.

The Solubility of the Ternary PAA–Cu^{2+} Protein Systems

Figure 2(a) presents the dependence of the optical density (A_{400} nm) 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+}. It can be seen from these results that the solubility of mixture depends on the nature of the proteins and on pH of the solutions. Starting with very low values of ratio $n_{pr}/n_{PAA} = 0.25$, a phase separation took place in the systems Hb–Cu^{2+}–PAA, and GL–Cu^{2+}–PAA at pH 7. A_{400} value increased with further increase in the concentration of proteins. As for the systems BSA–Cu^{2+}–PAA, HSA–Cu^{2+}–PAA, and Tr–Cu^{2+}–PAA, observations provided a different picture. When the latter proteins were added to PAA–Cu^{2+} solution, pH 7, the ternary mixtures appeared to remain soluble in a range of $n_{pr}/n_{PAA} < 5.0$. Similarly, Hb and Gb solutions mixed with PAA–Cu^{2+} at higher values of pH (pH 10) gave rise to soluble ternary mixtures [Fig. 2(b)]. Figure 3 presents the results of turbidimetric titration of PAA–Cu^{2+} mixture with the solution of BSA at different concentrations of Cu^{2+}. 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 BSA added. However, there existed a critical concentration of Cu^{2+} in ternary mixtures ($n_{Cu}/n_{AA} \geq 0.25$), at which the system lost the homogeneity with formation of insoluble particles (Fig. 3). At $n_{Cu}/n_{AA} = 0.30$, the dependence of A_{400} on n_{BSA}/n_{PAA} passed through a maximum

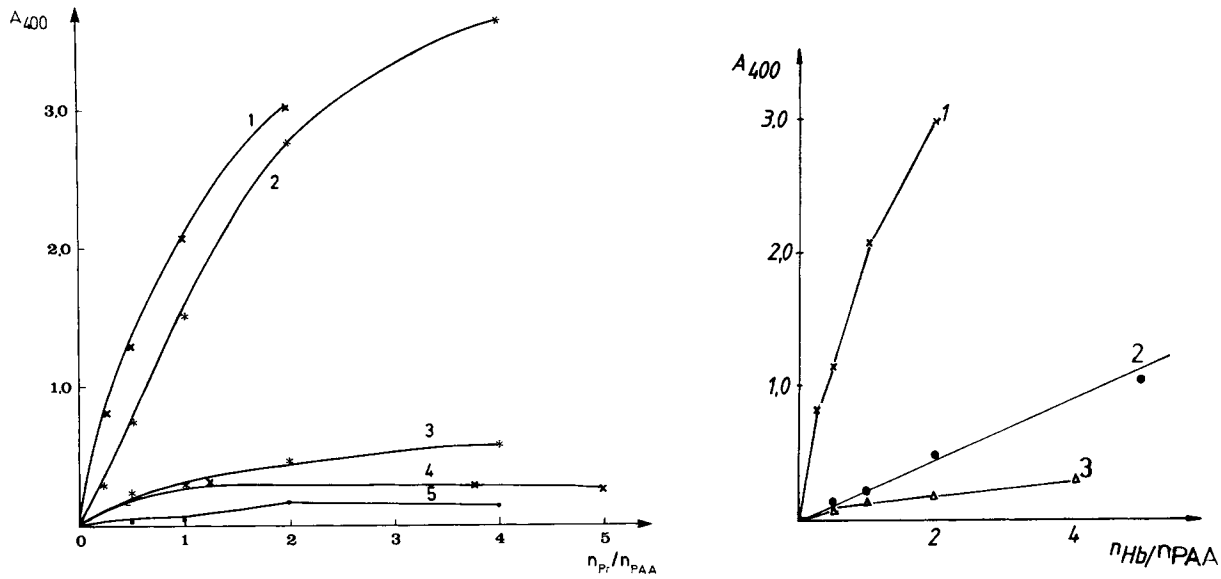

Figure 2 (a) Dependence of the optical density (A_{400}) of the ternary mixtures of different proteins with Cu^{2+} and PAA on n_{pr}/n_{PAA} at pH 7.0. (1) Hb—Cu^{2+}—PAA; (2) Gl—Cu^{2+}—PAA; (3) Tr—Cu^{2+}—PAA; (4) HSA—Cu^{2+}—PAA; (5) BSA—Cu^{2+}—PAA. C_{PAA} = 0.1 g/dL, n_{Cu}/n_{PAA} = 0.08. (b) 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.

(Fig. 3, curve 1). Similar to the systems Hb-Cu^{2+}-PAA and Gl-Cu^{2+}-PAA, starting with very low values of protein concentrations, phase separation took place. The amount of the precipitation increased with increase in n_{BSA}/n_{PAA} and then attained a limiting value. The pattern changed significantly with

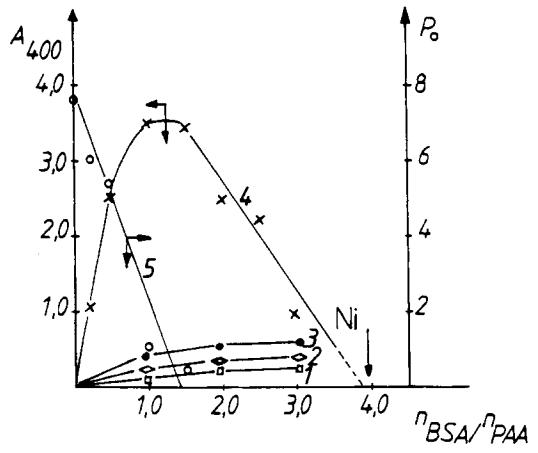

Figure 3 Turbidimetric titration of PAA—Cu^{2+} mixture with the solution of BSA. (1-4) Dependence of optical density (A_{400})of ternary mixture PAA—Cu^{2+}—BSA on n_{BSA}/n_{PAA} at difference n_{Cu}/n_{AA}: 0.08(1); 0.1(2); 0.2(3); 0.3 (4). (5) Dependence of the area of the free PMC peaks (P_0) on the ratio n_{BSA}/n_{PAA}. obtained in matrix solution of ternary mixture at n_{Cu}/n_{AA} = 0.3 C_{PAA} = 0.1 g/dL; pH 7; 25°C.

further increase in n_{BSA}/n_{PAA}. Depending on the protein concentration in the mixture, partial or complete prevention of phase separation than takes place. The minimum amount of protein (indicated by the arrow in Fig. 3), which must be introduced into the system to prevent precipitation corresponds to the point N_i, where only the homogenous mixture exists in the system. Therefore, depending on the concentration of BSA, transformation of insoluble protein–PAA mixture to soluble state took place.

In conclusion of the results presented in this section (as indicated, in particular, in Fig. 2 and Table I) it is suggested that the solubility of the ternary mixtures is closely correlated with the pI values of protein molecules and depends on the concentration of components in the mixture.

The Analysis of the Soluble Systems

The water-soluble mixtures of BSA-Cu^{2+}-PAA, HSA-Cu^{2+}-PAA, and Tr-Cu^{2+}-PAA were analyzed by spectrophotometric and HPLC methods under different experimental conditions. Figure 4 presents an example of HPLC for the soluble mixtures BSA-Cu^{2+}-PAA at different n_{BSA}/n_{PAA} and$_{Cu}/n_{AA}$. As suggested by the slight increase in peak I, the interaction between BSA and PAA at the pH 7.0 was weak, if not negligeable, in the absence of copper ions (B). The results are consistent with the results

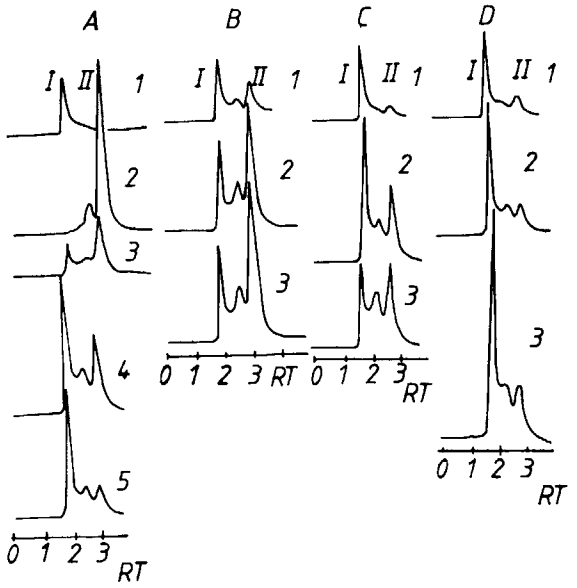

Figure 4 HPLC analysis of the formation of polymer–protein complexes in the presence of Cu^{2+}. Polymer–metal(Cu^{2+})–protein complexes were prepared and HPLC analysis on gel filtration column performed as described under Materials and Methods. (A) Dependence of the complex formation upon n_{Cu}/n_{AA}. (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).

obtained by sedimentation analysis of PAA–BSA systems.[5] 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 participitation of Cu^{2+} in the complex formation with PAA, BSA, and the ternary mixture was investigated by cyclic voltammetry anlysis 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 5. As it can be seen from the figure, for the Cu^{2+} solution a single cathodic peak was formed at about 100 mV and the reverse scan exibit 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_{PAA} = 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^0 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.

The pattern changes significantly on further increase in ratio, n_{BSA}/n_{PAA} > 1 [Fig. 4(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 [Fig. 4(C)]. The intensity of the peak II (monomer form) appeared to be at first essentially

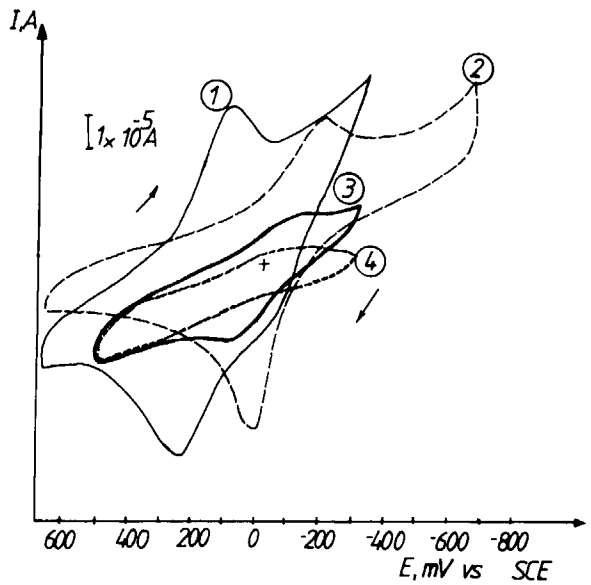

Figure 5 Cyclic voltammograms of $1.16 \times 10^{-3}M$ Cu (1); mixtures of PAA–Cu^{2+} (2), BSA–Cu^{2+} (3), and BSA–Cu^{2+}–PAA (4). The concentration of Cu^{2+} in all mixtures are constant and equal to concentration of Cu^{2+} in (1); n_{Cu}/n_{AA} = 0.15; working electrode = GCE; ionic strength = $0.1N$ $NaClO_4$; pH 7.

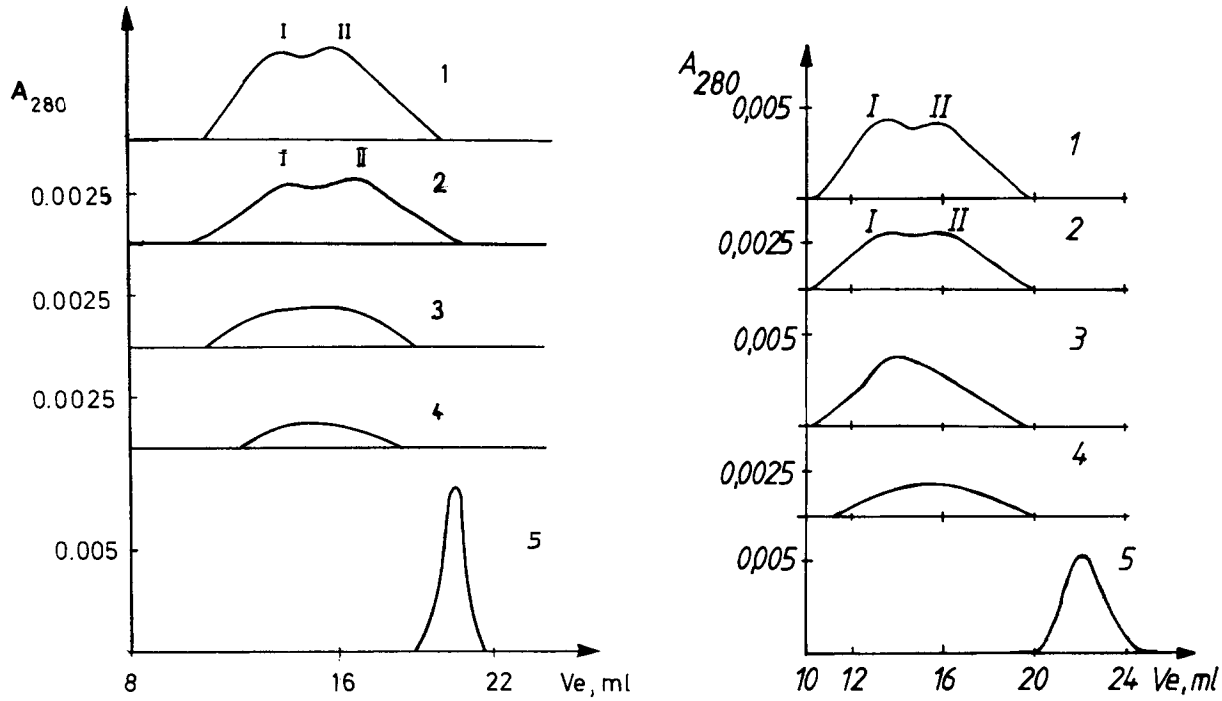

Figure 6 Gel filtration HPL chromatograms of free PMC and at the matrix solution of its ternary mixture (Hb–Cu^{2+}–PAA) (a) and (Gl–Cu^{2+}–PAA) (b) at different n_{Pr}/n_{PAA}. (a) (1) Free PMC; 2.8 (2); 6 (3); 10 (4) (5) free Hb. (b) (1) free PMC; 2.8 (2); 6 (3); 10 (4); (5) free Gl. pH = 7.0; C_{PAA} = 0.1 g/dL; n_{Cu}/n_{AA} = 0.08; T = 25°C. Bio Sil Sac 250 column was used in the experiment.

lower than the peak of the free BSA with equal concentration (diagram A,2 vs. C,2). 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 V_e 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^{2+} (or PAA):

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

As is known from the literature,[42] BSA form in the presence of Cu^{2+}-soluble protein–metal complexes with V_e corresponding to those of the monomer and dimer form of BSA. The higher capacity of BSA in complex formation with Cu^{2+} than PAA[37-42] is consistent with this proposal.

At higher Cu^{2+} concentrations ($n_{Cu}/n_{AA} > 0.15$) [Fig.4(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)].

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

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[34]:

$$\text{PAA*-Cu}^{2+} + \text{PAA} \rightarrow \text{PAA*} + \text{PAA-Cu}^{2+} \text{ and}$$

$$\text{PAA-Cu}^{2+} + \text{PAA*} \rightarrow \text{PAA} + \text{PAA*-Cu}^{2+}$$

where PAA* is an anthryl label containing poly(acrylic acid).

Analysis of the Insoluble Systems

Analysis of the supernatant of the insoluble ternary mixtures Hb–Cu^{2+}–PAA and GL–Cu^{2+}–PAA was carried out with HPLC–gel filtration methods (Fig. 6). 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}) increased also in proportion with the increase in n_{Pr}/n_{PAA} [Fig. 2(a)]. The peaks with V_e corresponding to elution volume of free Hb [Fig. 6(a), diagram 5] and Gl [Fig. 6(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+}–Pr. (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 (P_o) 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 7. 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 $P_o = 0$. This limit equals the number (N_i) of the protein molecules bound by a PAA of a given degree of polymerization under given conditions.

At the $n_{protein}/n_{PAA} > N_i$, only one peak was seen in chromatograms in both cases (Hb–Cu^{2+}–PAA, Gl–Cu^{2+}–PAA), corresponding to the free protein; therefore, after $n_{Pr}/n_{PAA} > N_i$, 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. Figure 3, curve 2, reveals the dependence of the concentration of free PMC on the ratio n_{BSA}/n_{PAA}in matrix solutions of mixture PAA–Cu^{2+}–BSA. It is seen that the increase of BSA content in polyelectrolyte mixture leads to a decrease of amount of free PMC and at about ratio of $n_{BSA}/n_{PAA} \cong 1$ the concentration of free PMC in mixture is equal to zero. This results show that in this case

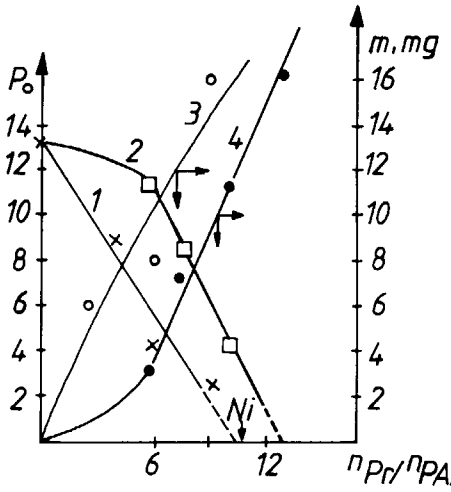

Figure 7 Dependence of the chromatogram peak area (Po) of free PMC and the amount (m) of the precipition in Hb—Cu^{2+}—PAA (1,3) and Gl—Cu^{2+}—PAA (2,4) on n_{Pr}/n_{PAA}; C_{PAA} = 0.1 g/dL; n_{Cu}/n_{AA} = 0.08; pH 7.

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 completly incorporated to an insoluble ternary comlex. 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 (Fig. 8). Therefore, in the ternary mixture at these concentrations of added metal ions, insoluble, and soluble ternary complexes appear to be formed simultaneously.

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,[37-43] 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 nitro-

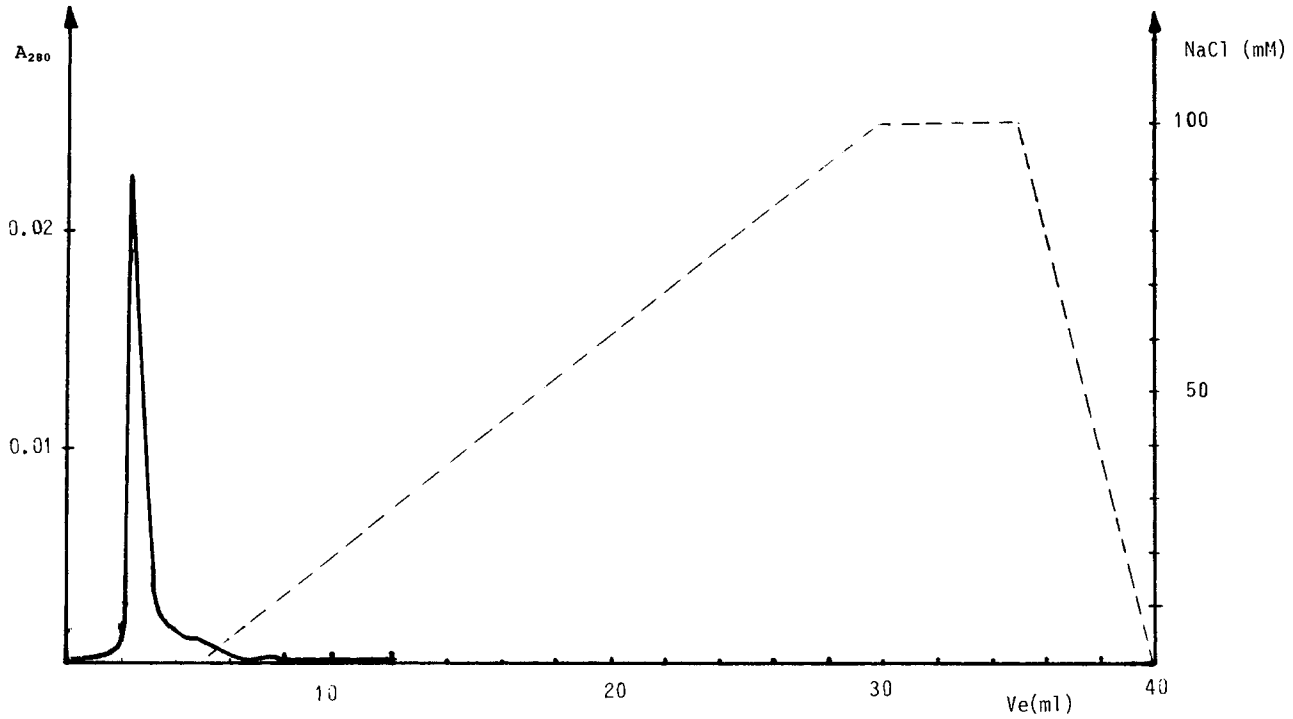

Figure 8 Anion-exchange chromatography of the matrix solution of the ternary mixture BSA—Cu^{2+}—PAA. (C_{PAA} = 0.1 g/dL): n_{Cu}/n_{AA} = 0.30 n_{BSA}/n_{PAA} = 1.0; pH = 7; 25°C. Broken line corresponds to (NaCl) concentration gradient.

gen), the β-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 Fig. 9).

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.

According to refs. 2–5, 44, and 45, 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

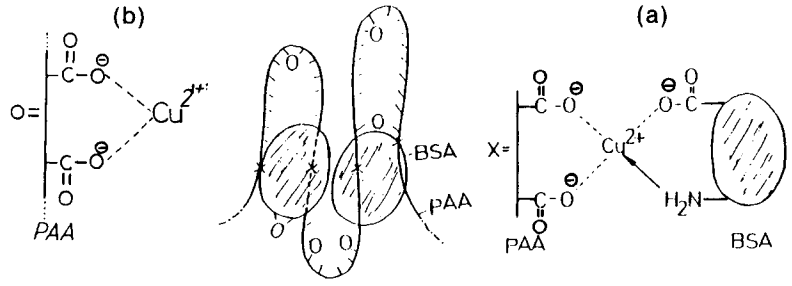

Figure 9 A schematic presentation of the formation of chelate units between the functional groups of the PAA and the protein globules with participation of copper ions (b) and structure of ternary PAA—Cu^{2+}—protein polycomplexes (a).

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.

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 (Fig. 9). 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 10, 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 8. 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

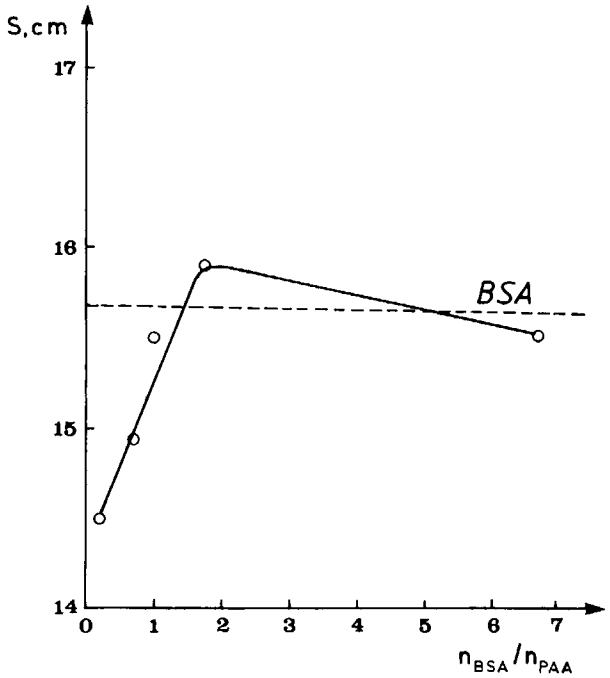

Figure 10 Dependence of the electrophoretic mobilities (distance-S) of the products of solution of the ternary mixture BSA—Cu^{2+}—PAA on n_{BSA}/n_{PAA}; C_{PAA} = 0.1 g/dL n_{Cu}/n_{AA} = 0.25; pH = 7; 25°C. Broken line represent free BSA.

and insoluble polycomplexes with a complicated structure, for instance, the existence of Cu^{2+}-induced crosslinking self-assembly of polycomplexes. 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.

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.

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