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Water-Insoluble Triple Complexes: Bovine Serum Albumin–Bivalent Metal Cation–Alginate

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ABSTRACT

Formation of water-insoluble complexes between bovine serum albumin (BSA) and sodium alginate in the presence of bivalent metal cations was studied. Complexing was observed to take place close to the BSA isoelectric point and above only for cations having a high affinity for BSA (Cu(II), Cd(II) and Zn(II)) and not other divalent ions (Mg(II), Mn(II) and Ca(II)). Addition of NaCl suppressed precipitation. The quantity and composition of the precipitate formed in the system BSA–Cu(II)–alginate was analysed with reference to the initial protein/polysaccharide weight concentration ratio (C_P/C_M) *and Cu(II) concentration. In the precipitate the Cu(II)/alginate monosaccharide molar ratio (Cu/A) is constant and equal to* $0{\cdot}7 \pm 0{\cdot}1$. *When* $C_P/C_M > 8$, *the Cu/BSA molar ratio is also practically constant, amounting to 18–20 and being equal to the number of binding sites for copper cations (II) in the BSA molecule.*

It is believed that insoluble triple complexes BSA–Cu(II)–alginate form due to bridging between copper-binding sites of BSA and alginate carboxyl groups via Cu(II) cations. At $[Cu]/[A] > 1$ *and* $C_P/C_M > 8$, *the coordination sphere of Cu(II) cations is probably characterized by a constant structure. The change in this structure at* $[Cu]/[A] < 1$ *and/or* $C_P/C_M < 8$ *is accompanied by a decrease in the yield of the precipitated complex and also the protein content of the complex.*

INTRODUCTION

The complexing of proteins with anionic polysaccharides is of practical interest for the separation and fractionation of proteins as well as for the control of their functional properties. In dilute solutions a precipitate of the protein-containing insoluble complex usually forms at pH values

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below the protein isoelectric point, i.e. the process takes place when polyelectrolytes have nett charges of opposite sign and complex formation results mainly from electrostatic interactions. However, the presence of polyvalent metal cations in the solution may lead to complex formation at pHs above the protein isoelectric point. In particular, in the presence of Ca(II) ions and at pH values above the protein isoelectric point, coprecipitation was observed in the systems: casein–carboxymethylcellulose (Gluskey *et al.*, 1969) and whey proteins–carboxymethylcellulose (Hill & Zadow, 1978). It is believed that in this case Ca(II) ions play the role of bridges, and this results in the formation of the triple complex protein–divalent metal ion–polysaccharide. It is also known that at pHs above the protein isoelectric point complexes between α_{s1}-casein and κ-carrageenan form only in the presence of calcium ions (Skura & Nakai, 1980). Nevertheless, the mechanism of formation of a triple complex is still unknown. For example, we do not know what role is played by metal ion–protein and metal ion–polyanion interactions in the complexes. In an attempt to clear up this question, precipitation of complexes formed by divalent metal ions with BSA/alginate was studied.

MATERIALS AND METHODS

Sodium alginate was purchased from British Drug Houses, England. The preparation was purified by reprecipitation with a mixture of isopropyl alcohol and hydrochloric acid. To remove polyvalent metal impurities, the alginate was dissolved in 0·01 M EDTA and then separated on Sephadex G-25. The contents of blocks were: MM(30%), GG(20%), and MG(50%), where M and G are mannuronic and guluronic acid residues, respectively; the mannuronic acid residue content was 57%.

Bovine serum albumin (BSA) was obtained from the Bacterial Preparation Enterprise of the Research Institute of Epidemiology and Microbiology, Academy of Sciences of the USSR, and used without further purification. The protein content in the dry preparation was 99%. The denaturation temperature was 57·8°C (pH 7·0; 0·05 M phosphate buffer); according to gel-permeation chromatography data the dimer content is approximately 15%.

The amounts of protein and polysaccharide in the triple complex precipitate were determined according to Itzhaki & Gill (1964) and Dubois *et al.* (1956) respectively after it had been dissolved in 0·1 M NaOH. The amount of copper in the precipitate was found from absorption of the copper–rhodanide complex at 354 nm after the precipitate had been dissolved in 0·1 M KCNS. The nephelometric titration was carried out on a spectrocolorimeter Spekol (Karl Zeiss, Jena) provided with a special adapter 'T_i' used to measure light scattering at 90°. The wavelength was 436 nm; the cell volume was equal to 30 ml.

RESULTS

The nephelometric titration of BSA–sodium alginate solutions with bivalent metal salts

BSA and sodium alginate form an insoluble complex only at pHs below the protein isoelectric point (Fig. 1, curve 1). Addition of copper ions to the solution causes precipitation in neutral and alkaline media (curve 2). In the latter case the pH range allowing precipitation of the BSA–Cu(II)–alginate mixture is much wider than that of BSA precipitation with Cu(II) ions (curve 3). A similar effect is caused by some other transition metal ions, in particular, by Cd(II) and Zn(II). The titration of the BSA–sodium alginate mixture with Cu(II), Cd(II) and Zn(II) salts at pH 5·5 (Fig. 2) shows that in the presence of alginate the precipitating

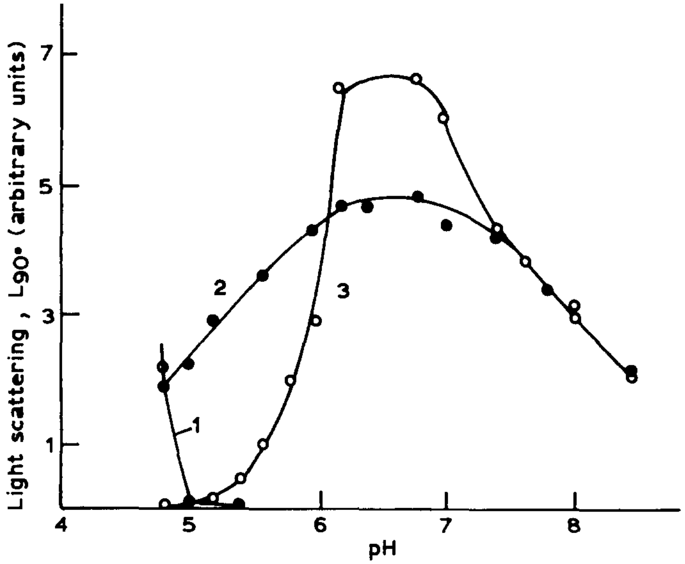

Fig. 1. Dependence of light scattering on pH in aqueous solutions of the mixtures: 1, BSA and alginate; 2, BSA, Cu(II) and alginate; 3, BSA and Cu(II). BSA concentration: 0·095 mg/ml. Alginate concentration: 0·005 mg/ml ($C_P/C_M = 19$). Copper concentration: 0·3 mM.

ability of divalent metal ions is much higher. The titration curves are S-shaped, and this points to the cooperative character of the formation of the insoluble complex. No alginate precipitation was observed in the absence of BSA (over the divalent metal ion concentration range used). In Fig. 2 the curves show cations precipitate BSA in the same order in the presence and absence of alginate. A similar titration of BSA and BSA-sodium alginate mixtures with salts of Ca(II), Mg(II) and Mn(II) cations does not result in an increase in solution turbidity except at high concentrations of Ca(II) in the alginate-BSA solutions when a precipitate of calcium alginate forms. Thus, above the BSA isoelectric point in the BSA-divalent metal ion-alginate solutions, precipitation is observed to take place readily only in the case of high cation affinity for serum albumin, i.e. when Cu(II), Cd(II) and Zn(II) are used (Martins & Drakenberg, 1982); no precipitation is observed in the absence of such affinity, i.e. when Mg(II), Mn(II) and Ca(II) are used (Saroff & Lewis, 1963; Hirsh-Kolb *et al.*, 1970; Frye *et al.*, 1974). It is quite possible that this correlation is not incidental. It may be because the protein-cation interaction plays an important part in the formation of the triple complex as well as being due to the fact that that protein aggregation under the

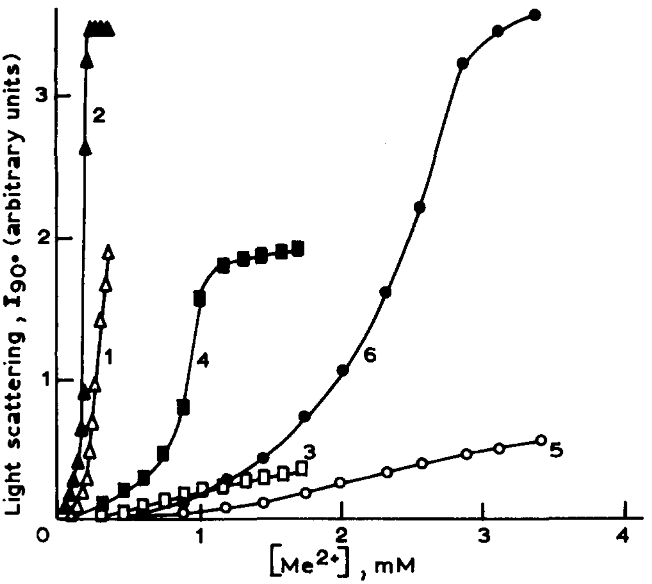

Fig. 2. Changes in light scattering when BSA solutions (curves 1, 3 and 5) and BSA-sodium alginate mixtures (curves 2, 4 and 6) are titrated with ions of Cu(II) (1 and 2), Cd(II) (3 and 4) and Zn(II) (5 and 6). BSA concentration, 0·17 mg/ml; alginate concentration, 0·03 mg/ml ($C_P/C_M = 5{\cdot}6$); pH, 5·5; 0·01 M acetate buffer.

influence of metal cations is related to aggregation of the triple complexes. The titration curves of the BSA–sodium alginate solutions with different Cd(II) salts (sulphate and chloride) suggest that the nature of the anion has little effect upon the precipitation character (Fig. 3). Figure 3 shows that addition of NaCl depresses insoluble complex formation.

The chemical analysis of the insoluble complexes BSA–Cu(II)–alginate

Using the system BSA–Cu(II)–alginate as a model we studied the dependence of the yields of protein, polysaccharide and copper, as well as precipitate composition, on the initial BSA/alginate ratio (C_P/C_M) at constant total concentrations of the macromolecules and different concentrations of Cu(II) ions. The experimental conditions were chosen on the basis of the nephelometric measurements reported above (Fig. 1). The insoluble complex was obtained by titration of the BSA–alginate solutions with $CuCl_2$ solution or by mixing alginate and BSA solutions containing varying concentrations of Cu(II). The majority of the results were obtained by the second method.

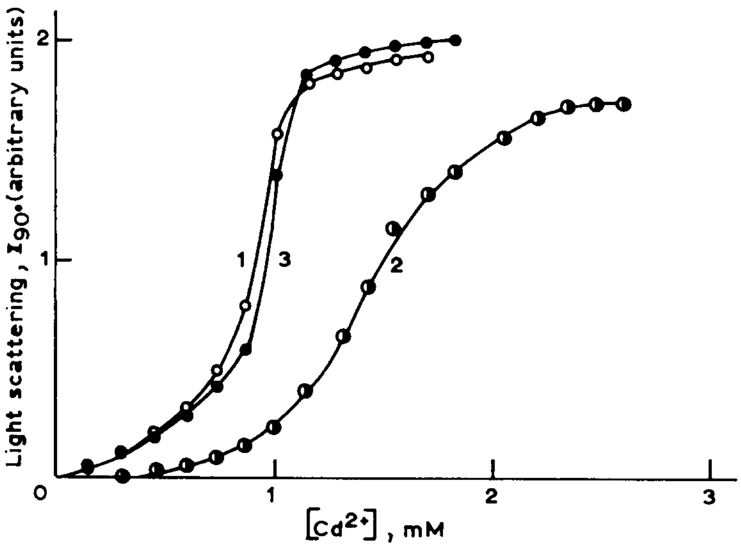

Fig. 3. Titration of the BSA–sodium alginate solution with $CdCl_2$ (curve 1 — in the absence of NaCl; curve 2 — NaCl concentration: 0·01 M) and $CdSO_4$ (curve 3). BSA concentration, 0·17 mg/ml; alginate concentration, 0·03 mg/ml ($C_P/C_M = 5·6$); pH, 5·5; 0·01 M acetate buffer.

The results of the analysis were unaffected by the method of mixing of the macromolecular solutions; this points to the fact that a precipitate forms as a result of equilibrium complexing reactions. Since a precipitate forms only in the presence of all three components (BSA, Cu(II) and alginate), this proves that precipitation is caused by formation of the triple complex.

The precipitation of the triple complexes takes place over a narrow range of copper ion concentrations (Fig. 4) showing that there exists a threshold ion concentration at a given concentration ratio of protein to polysaccharide. The data can also be plotted in the form of a phase diagram (Fig. 5). The upper boundary in Fig. 5 shows the conditions for maximum protein yield at a given macromolecular concentration ratio. In particular, we see that at [Cu] $\leqslant$ 0·1 mM, no precipitation (and, probably, no formation) of the triple complexes takes place.

The BSA, Cu(II) and alginate yields in the precipitate are shown in Figs 6(a) and 6(b).

In Fig. 7 data on the yield of the insoluble triple complexes are presented according to the method of continuous variation (Beck, 1973). This method has been successfully applied to determine the composition of some insoluble macromolecular complexes (Tolstoguzov & Wainermann, 1975; Nakajima & Shinoda, 1976). As seen in Fig. 7, the

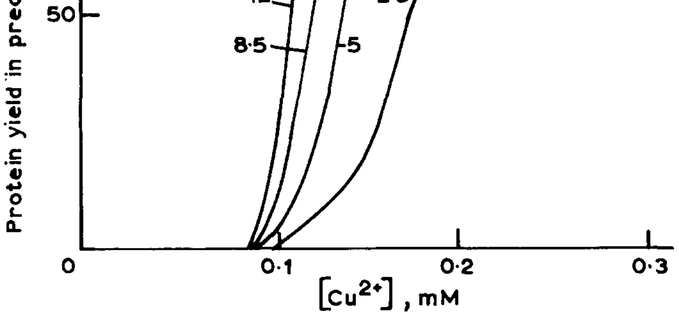

Fig. 4. Dependence of BSA yield in the precipitate on concentration of copper ions. C_P/C_M is indicated for each curve. Total concentration of macromolecules: 0·22 mg/ml; pH 5·2; 0·004 M acetate buffer.

macromolecular composition of the insoluble complexes, found by the continuous variation method, depends on the copper concentration in the solution: the higher it is, the lower the BSA content in the complex at the maximum yield points on the curves in Fig. 7. Thus, an increase in Cu(II) concentration is similar to the increase in the positive charge on the protein which results when protein–polysaccharide complexing takes place at pHs below the protein isoelectric point. In other words, formation of the BSA–Cu(II)–alginate complexes and changes in their composition with increasing copper ion concentration can be explained by the fact that copper ion sorption on BSA makes the latter positively charged. The value of this charge can be found from the relationship

$$Z_p = \left(\frac{[A]}{[BSA]}\right)^*$$

where Z_p is the charge created by copper ions on a BSA molecule, and $([A]/[BSA])^*$ is the alginate/BSA molar concentration ratio corresponding to the peaks on the curves in Fig. 7. This equality proceeds from the assumption that in the insoluble complexes the positive charge created

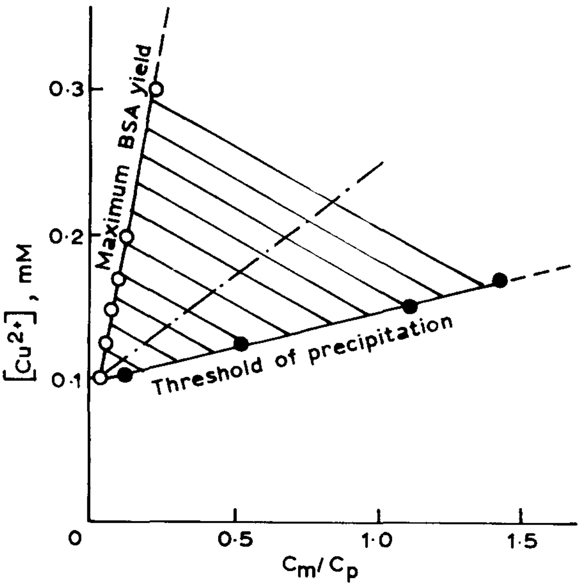

Fig. 5. A phase diagram for the system BSA–Cu–alginate. ●, dependence of the C_P/C_M threshold ratio on copper concentration; ○, maximum BSA yield points; — · — · —, 50% precipitation of BSA. For experimental conditions see Fig. 4.

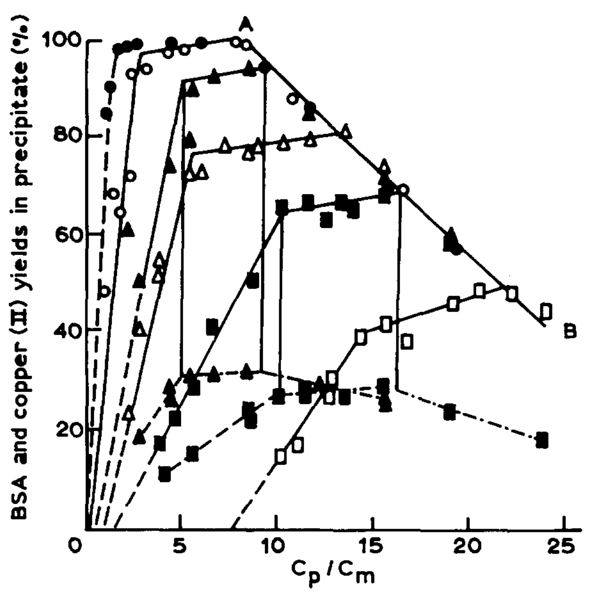

Fig. 6(a). Dependence of BSA yield (r_P, full lines) and copper yield (dotted lines) in the precipitate on the initial macroagent concentrations (C_P/C_M) at different Cu(II) concentrations, mM: ●, 0·3; ○, 0·2; ▲, 0·175; △, 0·15; ■, 0·125; □, 0·1.

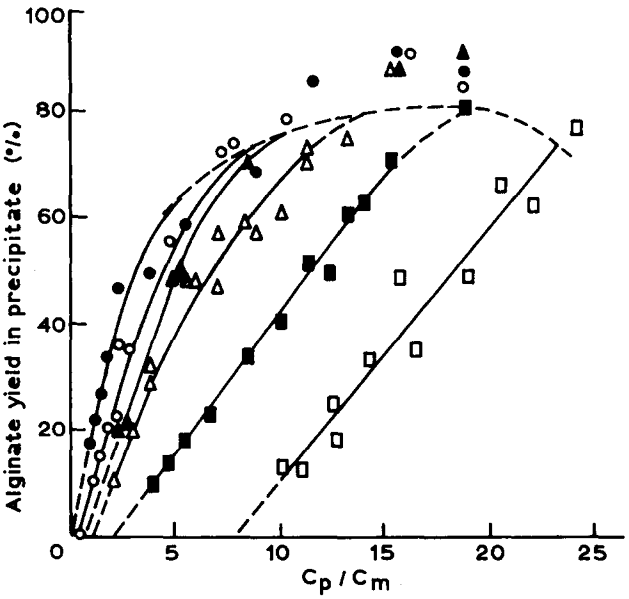

Fig. 6(b). Dependence of alginate yield (r_M) in the precipitate on C_P/C_M at different Cu(II) concentrations (for symbols see Fig. 6a). (The dashed line shows the maximum alginate yield calculated from r_P and the precipitate composition average for different copper concentrations. For experimental conditions see Fig. 4.

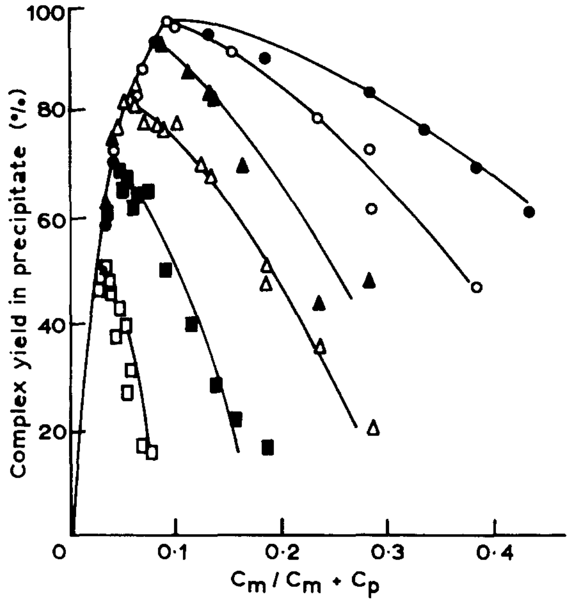

Fig. 7. Dependence of total macromolecule yield on the initial ratio (plotted according to the method of continuous variation). For symbols see Fig. 6a.

by copper ions on BSA is fully compensated by the negative alginate carboxyl groups. The ([A]/[BSA])* ratio is given in Table 1 (the q_2-value which correlates with the composition found by the continuous variation method). However, the complex composition determined by this method is inconsistent with its determination (Fig. 8).

The strong dependence of the precipitate composition on the initial weight concentrations of macromolecules (C_P/C_M) also points to the fact that in this case it is not advisable to apply the method of continuous variation.

In the insoluble complexes the BSA/alginate weight ratio varies over the range from 4–5 to 15–16 (from 75–80 monosaccharide units per BSA molecule to 20–22) with increasing C_P/C_M from 0 to 10–12 (Fig. 8). However, as this takes place the insoluble complex composition is characterized by constant Cu/A= 0·7 ± 0·1 over a wide range of C_P/C_M variation (Table 2). It may be noted that the stoichiometric ratio Cu/A= 0·5 corresponds to coordination of each copper atom to two alginate carboxyl groups.

At $C_P/C_M > 8$, the A/BSA ratio, and consequently the Cu/BSA ratio, in the precipitate is also constant (Fig. 8 and Table 2). The latter is equal to 18 and 20 at copper ion concentrations in the solution of 0·125 and

TABLE 1
Initial Ratios of Component Concentrations Corresponding to Break Points on Curves in Fig. 6a

[Cu], *mM*	$C_P/C_M=q_1$			$C_P/C_M=q_2$		
	A/BSA	*Cu/A*	*Cu/BSA*	*A/BSA*	*Cu/A*	*Cu/BSA*
0·30	222	0·7	150	44	2·3	102
0·20	111	0·7	80	43	1·6	68
0·17	69	0·9	61	37	1·5	57
0·15	60	0·9	53	26	1·9	49
0·125	33	1·2	41	20	2·0	40
0·10	23	1·4	32	15	2·1	32
Mean value ± standard deviation	—	1·0 (±0·3)	—	—	1·9 (±0·3)	—

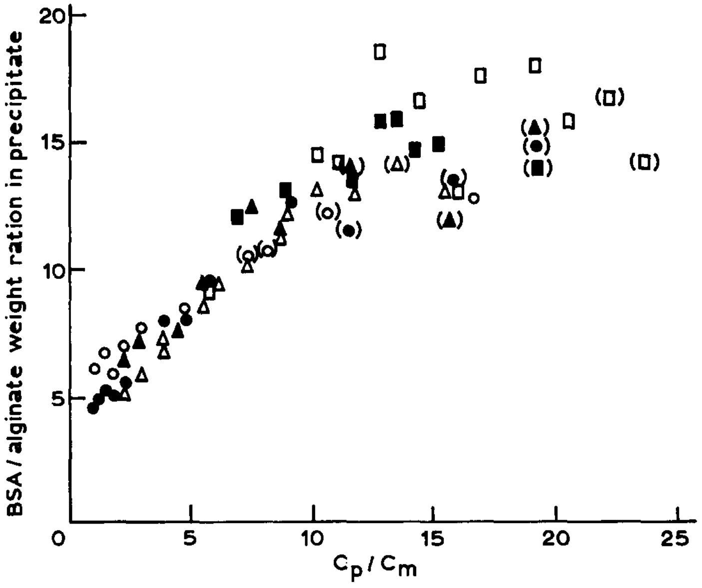

Fig. 8. Dependence of the BSA/alginate weight ratio in the BSA-Cu-alginate precipitate on the initial ratio of macroreagent concentrations (C_P/C_M) at different concentrations of copper ions (for symbols see Fig. 6a). For experimental conditions see Fig. 4.

0·17, respectively. It is known that the number of active BSA sites adsorbing Cu(II) ions is 16–19 (Mohanakrishnan & Chignell, 1982). So the general stoichiometric relation between precipitate components of the triple complexes correlates with such copper coordination where

TABLE 2
Molar Ratios of Components in the Precipitate of the Insoluble Complex BSA–Cu(II)–Alginate

C_P/C_M	*[Cu] = 0·125 mM*			*[Cu] = 0·17 mM*			
	A/BSA	*Cu/A*	*Cu/BSA*	C_P/C_M	*A/BSA*	*Cu/A*	*Cu/BSA*
4·0	46	0·7	30	3·0	45	0·6	27
5·6	36	0·7	24	4·6	23	0·6	25
		0·5	18			0·5	22
8·6	25	0·8	19	5·6	33	0·6	21
		0·8	18	6·7	27	0·7	20
10·1	20	0·9	18	8·6	28	0·7	20
11·5	23	0·7	17	12·3	23	0·8	19
15·6	22	0·8	17	15·6	28	0·7	20
19·0	23	0·7	17			0·7	19
Mean value ± standard deviation		0·7 (±0·1)	17·7 (±0·8)			0·66 (±0·09)	19·8 (±0·8)

both (or, less probably, one) alginate carboxyl groups and one active BSA site take part. It can be seen that the insoluble complexes obtained with excess copper ions are characterized by a certain type of Cu(II) ion coordination which over a wide range does not vary with the initial composition of the system.

Figures 6a and 6b demonstrate the change in BSA and alginate yield depending on C_P/C_M. Since in most cases the BSA concentration was much higher than the alginate one, the data presented in Fig. 6a are more reliable than those of Fig. 6b. As seen, the dependence of BSA yield on C_P/C_M at different copper concentrations may be interpreted as three straight lines with two break points corresponding to $C_P/C_M = q_1$ and $q_2 (q_1 < q_2)$. Figure 6a also shows that, at a relative excess of copper ions when $C_P/C_M \leqslant 8$, the maximum BSA yield (r_P^*) in the precipitate is equal to 100%. At $C_P/C_M > 8$, r_P^* decreases with increasing C_P/C_M. The yield of macromolecules (r^*) and the macromolecular composition of the insoluble complexes

$$\left(\frac{\mathrm{A}}{\mathrm{BSA}}\right)_{\mathrm{AB}}$$

represented by the straight line AB in Fig. 6a, are given in Table 3. As seen from this Table and Fig. 8,

$$\left(\frac{\mathrm{A}}{\mathrm{BSA}}\right)_{\mathrm{AB}}$$

TABLE 3
Yield of Macroreagents and their Molar Ratio in the Complex Precipitate, Corresponding to the Straight Line AB in Fig. 6a

C_P/C_M	r_P^*	r_M^*	A/BSA[a]
8·0	100	74	31
9·1	95	68	26
10·6	89	79	28
11·5	86	70	23
11·6	83	86	30
13·5	82	74	23
15·6	74	95	28
15·6	76	88	25
15·8	71	76	23
16·5	72	91	26
19·0	60	91	21
19·0	60	80	23
19·2	58	75	22
22·1	49	63	20
24·0	45	77	24

[a] Mean value ± standard deviation = 25 ± 3.

is only slightly dependent on C_P/C_M. (In Fig. 8 the points corresponding to the straight line AB are in brackets.) Thus, at $C_P/C_M \geqslant 8$ and a relative excess of copper ions, the precipitate composition varies only slightly if at all, and the quantity of the precipitate is controlled solely by the initial ratio of the macromolecular concentrations (at a given total concentration). Accordingly, the alginate yield corresponding to the straight line AB in Fig. 6a reaches a maximum value of only 79 ± 5% (Table 3). The copper/alginate molar concentration ratio correlating with the straight line AB is constant, whereas the copper/BSA ratio varies (Table 1). The fact that an average of 20% of alginate is not incorporated into the insoluble complex in spite of protein and copper excess (this fact was confirmed once again by analysis of the supernatant after concentration by 12–13 times at $C_P/C_M = 11{\cdot}5$ and 15·6) can hardly be explained by the formation of soluble complexes in addition to insoluble ones. It is known that proteins and oppositely charged polyelectrolytes form soluble complexes at ratios which are very different from those corresponding to zero nett charge (Tolstoguzov & Wainermann, 1975). However, in the case of soluble complex formation the yield of both components must go down with decreasing C_P/C_M. Figures 6a and 6b show that at $C_P/C_M < q_1$ this is in fact the case. However, at $C_P/C_M > q_2$, the yield of alginate stays practically the same with varying solution composition. Besides, if at $C_P/C_M > q_2$ a decrease in the quantity of protein in the precipitate was caused by soluble complex formation, then probably the r_P^* value would depend on copper concentration. So, irrespective of C_P/C_M, the question of incomplete precipitation of alginate still remains unclear. It is felt that at all macromolecular concentration ratios a fraction of alginate stays in the solution and coordinates copper ions with protein not being involved. This is connected with alginate heterogeneity.

As seen from Fig. 6a, at $C_P/C_M > q_2$, an increase in the proportion of the alginate fraction in the solution (at constant concentration of copper ions) is accompanied by increasing protein yield. At $C_P/C_M = q_2$ the protein yield stops increasing. As mentioned above, the q_2 ratio corresponds to a constant [Cu]/[A] ratio in the solution at all copper concentrations. From this it follows that cessation of the growth of the BSA yield at $C_P/C_M = q_2$ is caused by a change in the ratio between concentrations of Cu(II) ions and alginate. At $q_1 < C_P/C_M < q_2$ we observed a decrease in protein yield unaccompanied by a change in composition of the insoluble complex only when $C_P/C_M \geqslant 8$. Further growth in the alginate fraction in the solution (at $C_P/C_M = q_1$) is accompanied by a rapid decrease in BSA yield (Fig. 6a). The beginning of the section representing BSA yield decrease (the break point at $C_P/C_M = q_1$) corresponds to the copper/alginate molar concentration ratio $[Cu]/[A] = 0{\cdot}7$–$1{\cdot}4$. The BSA yield decrease is accompanied by a decrease in the alginate yield too (Fig. 6b). However, unlike when $C_P/C_M > 8$ (the straight line AB), at $C_P/C_M < 8$, the complex yield can be raised up to 100% through a growth in protein concentration. Returning to the above-mentioned analogy with paired protein–polyelectrolyte complexes, we can suppose that at $C_P/C_M < q_1$ additional adsorption of alginate leads to an increase in the complex charge, and consequently to an increase in its solubility. It can also be supposed that at $C_P/C_M < q_1$ a shortage of copper ions, compared with the amount necessary to create the maximal number of active sites for BSA adsorption onto alginate, leads to a change in the Cu(II) coordination type. This second type of Cu(II) coordination is not beneficial for forming insoluble complexes. At a certain C_P/C_M value (Fig. 6a), only the second type of copper ion coordination takes place, and the insoluble complexes (and also, probably, soluble complexes) do not form at all.

DISCUSSION

These results are insufficient to draw a definite conclusion concerning the structure of the coordination sphere of copper ions in the insoluble complexes. Different types of coordination can probably take place including coordination on the active sites of only one macromolecule. The type of coordination depends on both the ratio of the macromolecular concentrations and the copper ion concentration. It is important to keep in mind that the results were obtained at low total concentrations of the macromolecules. For example, an increase of the total concentration up to 9 mg/ml, at $C_P/C_M = 5$ (copper ion concentration up to 3 mM), did not bring about the formation of insoluble complexes. Under these conditions we observed the formation of an alginate gel. After the gel had been removed, by analytical centrifugation, the solution contained only BSA. A further increase in the concentration of copper ions resulted in BSA precipitation. All this can be explained by copper binding to the separate macromolecules without triple complex formation.

Our results can be described in terms of simple paired complexing reactions.

(1) Formation of a positively-charged metal–protein complex BSA–$(Cu)_n$:

$$BSA + nCu \rightleftharpoons (BSA)(Cu)_n \tag{1}$$

where n is the number of Cu(II)-binding sites in a BSA molecule.

(2) Formation of a triple complex:

$$(BSA)(Cu)_n + mA \rightleftharpoons (BSA)(Cu)_nA_m\downarrow \tag{2}$$

A is an alginate monosaccharide unit.

Each of the two reactions is a sum of a number of intermediate reactions and therefore can be considered in terms of the theories of multiple equilibria. However, we feel that the amount of the intermediate metal–protein complexes is very small and they do not form insoluble complexes with alginate. The experimental data (Fig. 8 and Table 3) point to the fact that at $C_P/C_M \geqslant 8$ the stoichiometric coefficient m is also constant. So we suggest that stoichiometry of the triple complex BSA–Cu(II)–alginate is predominantly controlled by the BSA–$(Cu)_n$ complex composition, and the BSA content in the insoluble triple complex depends mainly on BSA–metal binding, i.e. on the extent of BSA saturation with Cu(II) ions. This extent is probably dependent on both the [Cu]/[BSA] ratio and BSA concentration in the solution. The lower this concentration is, the higher the concentration of copper ions required to achieve a certain degree of BSA saturation. This explains growth of the [Cu]/[BSA] ratio corresponding to q_1 and q_2 with decreasing C_P/C_M (Table 1). At $C_P/C_M \geqslant 8$, the concentration of copper ions seems to be high enough for all the BSA to be involved in the BSA–$(Cu)_n$

complexes. In this case the precipitate yield (as well as the BSA content in the precipitate) is controlled solely by the C_P/C_M ratio. For example, on the straight line AB (Fig. 6), at $C_P/C_M \geqslant 8$, the amount of alginate is not sufficient to incorporate the whole amount of BSA into the (BSA) $(Cu)_nA_m$ complex. As copper concentration decreases, the concentration of the BSA-$(Cu)_n$ complex drops with diminishing yield of the insoluble complex. The q_2 ratio (on the straight line AB in Fig. 6) therefore corresponds to complete binding of the BSA-$(Cu)_n$ complex by alginate and to the equivalent A/BSA-$(Cu)_n$ ratio. Addition of Cu(II) does not result in an increasing precipitate yield due to complete precipitation of the 'active' alginate. Similarly, growth of alginate fraction at the same copper ion concentration ($q_2 > C_P/C_M > q_1$), is not accompanied by an increasing BSA yield in the precipitate. This is similar to excessive addition of a macromolecule to the protein–polyelectrolyte complexes of equivalent composition discussed in the results section. Such an explanation is confirmed by the fact that concurrently with a decrease of the alginate yield in the precipitate, the composition of the latter remains the same (at $C_P/C_M \gtrsim 8$).

A third reaction involving coordination of copper ions to alginate only:

$$m\text{A} + n\text{Cu} \rightleftharpoons \text{A}_m\text{Cu}_n \tag{3}$$

can also take place in the system.

The importance of reaction (3) probably increases with the alginate fraction in the system (i.e. with decreasing C_P/C_M), though it is also possible that reaction (3) takes place at any C_P/C_M, which always results in incomplete precipitation of alginate (Fig. 6b). The swing to copper coordination by alginate explains the rapidly decreasing yield of both macroagents at $C_P/C_M < q_1$ (Fig. 6) as well as a change in the precipitate composition at $C_P/C_M \lesssim 8$ (Fig. 8). Coordination of copper ions to some monosaccharide units of alginate without BSA participation is equivalent to a decrease in alginate charge. This is accompanied by a growth of the alginate fraction in the complex and increasing Cu/BSA ratio with the same Cu/A ratio in the precipitate. At C_P/C_M below the threshold value (Fig. 6a), copper is probably coordinated solely with alginate and the triple complexes do not form.

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