Polyphosphate Binding Interactions with Bovine Serum Albumin in Protein-Polyphosphate Precipitates

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Bovine serum albumin (BSA) solutions were incubated in the presence of polyphosphate under various pH conditions using sodium polyphosphate with number-average chain lengths of 14 or 22 phosphorus atoms. The polyphosphates caused aggregation and quantitative precipitation of BSA at pH 4.5 or lower, under the conditions studied. The BSA-polyphosphate precipitates contained increased numbers of moles of phosphorus bound per mole of BSA when polyphosphate of longer chain length was used or if lower pH was used to form the precipitates. At high polyphosphate to protein molar ratios, the binding of the polyphosphates was shown by Scatchard analysis to deviate from linearity. The degree of aggregation of resuspended BSA-polyphosphate complexes was studied by using column chromatography. The degree of aggregation of the complexes was influenced by pH and the chain length of the polyphosphate used.

Polyphosphates react under acidic conditions with a variety of soluble proteins to form protein-polyphosphate precipitates. The protein precipitating ability of polyphosphates has been utilized by the food industry to produce products which display increased water-holding capacity as well as other interesting properties (Grettie, 1940). Recently, sodium polyphosphate has been utilized to quantitatively remove protein from industrial animal blood, and polyphosphates have been suggested for use in the isolation of high-quality blood proteins for the production of animal feed supplements (Vandegrift and Ratermann, 1979).

In the laboratory, the nature of the binding of sodium polyphosphate to protein has been studied for several proteins. Spinelli and Koury (1970) have studied the interaction of polyphosphates with sacroplasmic fish proteins, and Melachouris (1972) has investigated the interaction of polyphosphate with β-lactoglobulin. Lyons and Siebenthal (1966) have studied the effect of polyphosphate chain length on the binding of polyphosphate by gelatin and egg white proteins. However, not since the work of Briggs (1940) has the binding of polyphosphate to bovine serum albumin been extensively investigated.

The objective of the research reported here was to expand and broaden the observations made by Briggs (1940) by investigating the interaction of sodium polyphosphates of known chain length with bovine serum albumin under various conditions of protein and polyphosphate concentration and pH. For a number of polyphosphate-albumin complexes, the number of moles of phosphorus (as PO_3) bound per mole of BSA has been determined, and the degree of aggregation of the polyphosphate-protein complexes has been studied by means of column chromatography. Our results indicate that the binding of polyphosphates to BSA is similar to that of β-lactoglobulin (Melachouris, 1972) but that qualitative and quantitative differences do exist.

EXPERIMENTAL SECTION

Purified fatty acid free bovine serum albumin, obtained from Sigma Chemical Co. (St. Louis, MO), was dissolved in and dialyzed against 0.005 M Mes [2-(*N*-morpholino)-ethanesulfonic acid] buffer prior to polyphosphate binding. Polyphosphate binding to bovine serum albumin was effected by precipitation of the protein-polyphosphate complex, followed by determination of the amounts of phosphorus and protein in the precipitates. In experiments where concentrations of polyphosphate were varied, bovine serum albumin solutions were 5.27 g/L (7.95×10^{-5} M) in protein. To 20 mL of protein solution at pH 6.0, solid sodium polyphosphate of number-average chain lengths of $\bar{n} = 14$ or $\bar{n} = 22$ phosphorus atoms (Sigma Chemical Co., St. Louis, MO) was added while stirring. Sodium polyphosphate concentrations varied from 1 g/L (9.29×10^{-3} M in phosphorus for $\bar{n} = 14$; 9.47×10^{-3} M in phosphorus for $\bar{n} = 22$) to 7 g/L. In experiments where pH was varied, the sodium polyphosphate concentrations were held constant at 1.25 g/L and the albumin concentrations were held at 5.27 g/L. In experiments where protein concentration was varied, aliquots of the stock solution of bovine serum albumin were diluted with pH 6.0 Mes buffer to achieve the desired protein concentration, which varied from 1.05 to 5.27 g/L bovine serum albumin. Next, 1.25 g/L sodium polyphosphate (1.16×10^{-2} M in phosphorus for $\bar{n} = 14$; 1.18×10^{-2} M in phosphorus for $\bar{n} = 22$) was added as a solid white stirring. After an incubation of 30 min, the pH of the solutions was adjusted to pH 3.0 (for all experiments, except when pH was varied) by using 1 M H_2SO_4 while stirring, and the solutions were allowed to stand for 1 h and then centrifuged at 12000*g* for 15 min. Precipitates were dispersed overnight in enough 0.005 M Mes buffer to achieve a total volume of 20 mL. Portions of the resuspended precipitates were used for phosphorus analysis, protein determination, and molecular exclusion chromatography experiments. Protein concentrations were determined by absorbance at 280 nm by using a calculated molar absorptivity of 43 500 in 0.005 M Mes buffer, pH 6.0. The calculated molar absorptivity at pH 6.0 was not significantly affected by the presence of polyphosphate in the concentration range used in these experiments. Protein concentrations were checked periodically by dye binding analysis (Bradford, 1976). Phosphate analyses were performed by the vanadomolybdophosphoric acid method (American Public Health Association, 1971). Samples were heated with acid (33% H_2SO_4; 0.4% HNO_3) at 250 °C, 15 psi, in an autoclave for 30 min. After cooling and neutralization, aliquots were reacted with the vanadate-molybdate reagent. The reagent was allowed to react for 10 min, and the absorbance at 400 nm was taken before a time of 30 min had lapsed.

Molecular exclusion column chromatography experiments were performed by utilizing Sephacryl S-200 Superfine (Pharmacia Fine Chemicals, Piscataway, NJ). An analytical column (1.5 × 60 cm) was packed to a final bed volume of 97 cm^3 with an operational flow rate maintained at 20 mL/h by a Buchler Multistaltic pump (Buchler Instruments, Fort Lee, NJ). Fractions were monitored at

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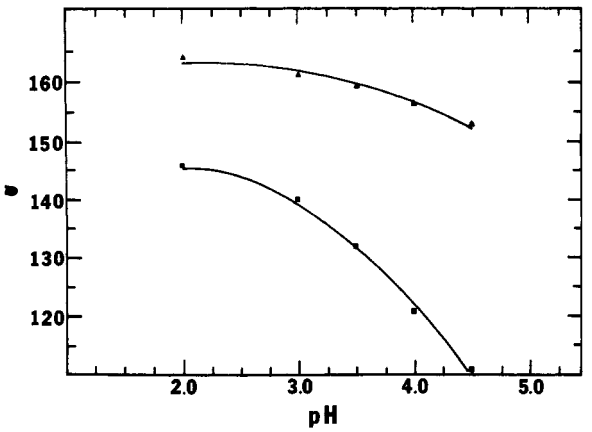

Figure 1. υ [moles of phosphorus (PO_3) per mole of serum albumin] vs. pH for protein–polyphosphate precipitates prepared by using sodium polyphosphates of $\bar{n}$ = 14 (■) or $\bar{n}$ = 22 (▲).

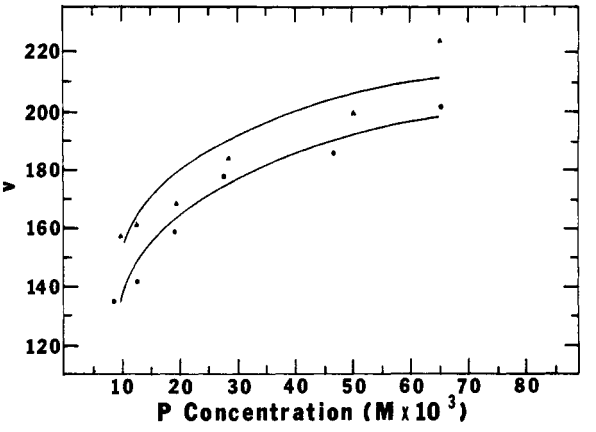

Figure 2. υ [moles of phosphorus (PO_3) per mole of serum albumin] vs. input phosphorus concentration for the precipitates prepared at pH 3.0 by using sodium polyphosphate of $\bar{n}$ = 14 (●) or $\bar{n}$ = 22 (▲).

280 nm by using a Cary Model 14 spectrophotometer. Void volume (36 mL) and salt volume (77 mL) determinations were made with Blue Dextran 2000 ($M_r = 2.0 \times 10^6$) and disodium phosphate, respectively. Column equilibration was achieved by using 0.005 M Mes, pH 6.0.

In order to make the results of the column chromatography experiments independent of individual experimental conditions, the distribution coefficient, K_{av}, of the various protein complexes was determined by

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_0 is the void volume, V_e is the elution volume, and V_t is the total volume. Protein complexes in the elution profile are designated by their respective K_{av} values.

RESULTS AND DISCUSSION

Sodium polyphosphates of average chain length $\bar{n}$ = 14 phosphorus atoms and $\bar{n}$ = 22 phosphorus atoms are effective precipitants of bovine serum albumin (BSA) from aqueous solution. Polyphosphates in solution with BSA bind to the protein in sufficient quantities to cause turbidity beginning at pH 5.2, followed by precipitation at lower pH values (Briggs, 1940). At pH 4.5 or lower, the removal of bovine serum albumin from solution in the presence of either of the two polyphosphates tested was essentially quantitative. No significant differences were observed between the relative precipitating abilities of the two polyphosphates at the concentrations tested. In all experiments conducted, the number of moles of protein in the precipitate was 96 ± 2% of input as determined by protein analysis of the resuspended precipitate.

Although the precipitation of protein did not vary from pH 2.0 to pH 4.5, the ratio of the number of moles of phosphorus per mole of protein in the precipitates did vary, as is shown by the data displayed in Figure 1. When the ratio of the number of moles of phosphorus to the number of moles of BSA (υ) is plotted vs. pH, the binding was shown to be greater when sodium polyphosphate of $\bar{n}$ = 22 was used as compared to sodium polyphosphate of $\bar{n}$ = 14. Therefore, the measured binding is a function of chain length. Moreover, the binding increased as the pH was lowered. The pH effect was also observed by Briggs (1940), using sodium polyphosphate of unspecified chain length, in experiments where polyphosphate binding was determined by plotting the amount of polyphosphate required to produce an isoelectric complex of protein and polyphosphate. In experiments similar to ours using polyphosphate and β-lactoglobulin (Melachouris, 1972), an increased polyphosphate binding to protein at lower pH levels was attributed to protein unfolding as polyphosphate is bound, making available more interacting groups and lowering electrostatic repulsion. As the pH is lowered, aspartic acid and glutamic acid repulsive groups on protein are effectively neutralized, enhancing polyphosphate binding. Thus, the greatest changes in binding of polyphosphate to BSA occur in the pK ranges of these groups, whereas the change in polyphosphate binding is less between pH 2 and pH 3.5 (Figure 1). If unfolding does occur, denaturation of the protein must be essentially reversible, since removal of the polyphosphate from the protein by dialysis regenerates the original properties of the protein (Briggs, 1940).

Maximum binding of polyphosphate to bovine serum albumin can theoretically be achieved when each free positive group of the protein binds one polyphosphate molecule (Klotz, 1953). Bovine serum albumin contains 17 histidine residues, 59 lysine residues, 23 arginine residues, and 1 free ε-amino group per molecule of 66 210 molecular weight (Peters, 1975). Thus, there is a maximum of 100 positive groups present on BSA at low pH levels. In each of the precipitation experiments performed, the concentration of polyphosphates was chosen to ensure quantitative precipitation of the protein. Thus, an excess of polyphosphate is present in all experiments. The observed binding of the polyphosphate to the protein under these conditions is quite high, even at the lower concentration of phosphorus used.

When increasing amounts of polyphosphate are added to BSA to form complexes at pH 3.0, a plot of υ vs. input polyphosphate (in moles of phosphorus) reveals that increasing polyphosphate concentration increases the amount of polyphosphate present in the precipitate (Figure 2). In agreement with the results of Briggs (1940), it was observed that the change in binding per unit change in polyphosphate concentration diminished at higher polyphosphate concentrations. The limited increase in the degree of binding after initial extensive binding is to be expected since phosphate initially bound to the protein should reduce the affinity of protein for subsequent phosphate binding due to electrostatic repulsion between species of like charge. Difference between the binding of $\bar{n}$ = 22 and $\bar{n}$ = 14 polyphosphate molecules may be explained by stronger van der Waals forces accompanying the larger polyphosphate molecule (Klotz, 1953).

Information regarding the number of binding sites for the PO_3 groups in the polyphosphate chain and the affinity between polyphosphates and bovine serum albumin can

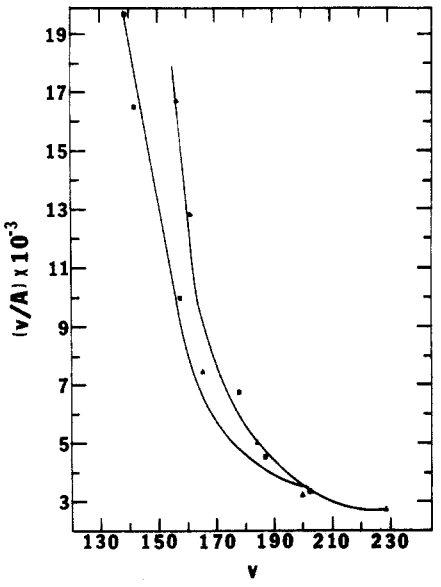

Figure 3. Scatchard plot ($v/[A] \times 10^{-3}$ vs. v) for BSA–polyphosphate complexes prepared at pH 3.0 by using sodium polyphosphate of $\bar{n}$ = 14 (■) or $\bar{n}$ = 22 (▲).

be obtained from a Scatchard plot. The Scatchard equation is

$$v/[A] = K(N - v)$$

where v is the number of moles of phosphorus bound per mole of protein at a given polyphosphate concentration, [A] is the molar concentration of free phosphorus (which is the difference between the input phosphorus concentration and the amount bound), K is the binding constant, and N is the number of moles of phosphorus which can be bound by a mole of bovine serum albumin. A plot of $v/[A]$ vs. v should yield an intercept on the abscissa equal to N with the intercept on the ordinate equal to NK and the slope equal to $-K$. Such a plot, shown in Figure 3, reveals a deviation from linearity at high levels of polyphosphate, and therefore K and N cannot be determined from this plot. Such deviation from linearity suggests that the binding sites on the protein are not equivalent or that interaction between the binding sites occurs. When the slope of the Scatchard plot becomes less negative with increasing v, as in this case, either negative interactions are observed such that the binding of one ligand decreases the affinity for the binding of successive ligands or stronger binding sites are becoming occupied first. Negative interactions resulting from electrostatic repulsion often are observed when large numbers of ions are bound to protein (Haschemeyer and Haschemeyer, 1973). Thus, the data could be taken to suggest that all sites on the protein have equal affinity for the polyphosphate, but repulsions become significant at a critical degree of saturation. The deviation from linearity in the Scatchard plot occurs at a concentration of ~2–3 g/L polyphosphate at a BSA concentration of 5.27 g/L, regardless of the chain length ($\bar{n}$ = 14 or $\bar{n}$ = 22).

The binding of polyphosphate to bovine serum albumin in these experiments resembles an adsorption process. The variation of adsorption with concentration at constant temperature can often be represented by an empirical equation known as the Freundlich isotherm:

$$A = KC^{1/n}$$

where A is the number of grams of phosphorus adsorbed per gram of protein, C is the concentration of phosphorus grams per liter, and K and n are constants. The log–log

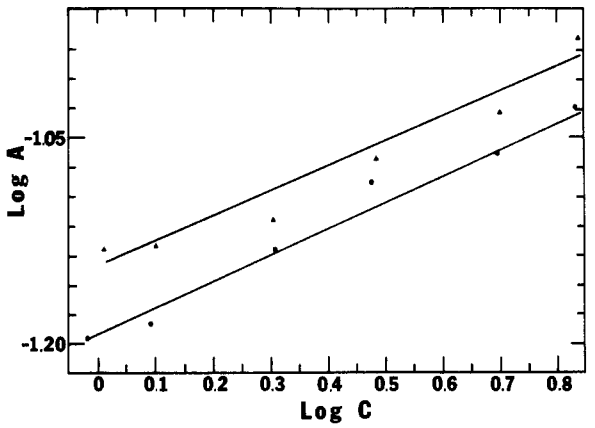

Figure 4. Freundlich isotherm plot (log A vs. log C) for polyphosphate–BSA complexes prepared at pH 3.0 by using sodium polyphosphate of $\bar{n}$ = 14 (●) or $\bar{n}$ = 22 (▲).

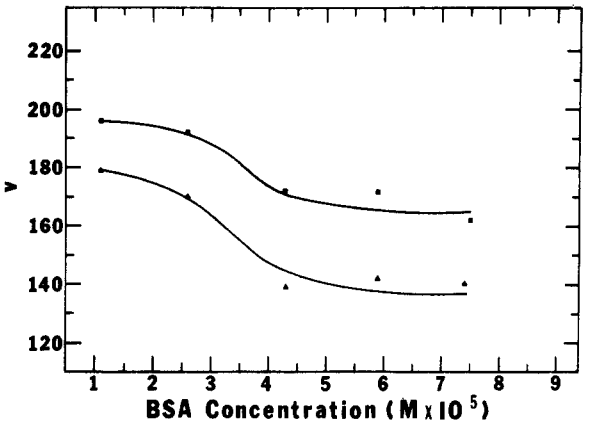

Figure 5. v [moles of phosphorus (PO_3) per mole of serum albumin] vs. molar concentration of BSA for BSA–polyphosphate complexes prepared at pH 3.0 by using sodium polyphosphate of $\bar{n}$ = 14 (▲) or $\bar{n}$ = 22 (■).

curve of the weight to weight ratio (A) vs. concentration in grams per liter (C) (Figure 4) is a straight line with a slope of ~0.2. The slope is independent of the polyphosphate chain length. This result suggests that the amount of phosphorus (as PO_3) bound by weight of the protein varies according to the equation $A = K^{0.2}$. This result indicates that the theoretical limit of one polyphosphate polymer binding for each amino group on the protein is not observed. This deviation from theory was also observed by Briggs (1940).

When the polyphosphate concentration of the protein–polyphosphate system was held constant but the concentration of the protein was lowered, the binding per mole of protein varied as is shown in Figure 5. At lower protein concentrations the polyphosphates were bound to a higher degree than at higher concentrations. Under the conditions of these experiments, where all protein is quantitatively precipitated, the number of moles of phosphate bound in the reconstituted complexes was dependent on chain length. Sodium polyphosphate of $\bar{n}$ = 22 exhibited a greater number of moles of PO_3 bound than polyphosphate of $\bar{n}$ = 14. It is of interest to note that the curve in the graph shown as Figure 5 occurs at about the same ratio of polyphosphate (1.25 g/L) to BSA (2.65 g/L) as does the deviation from linearity observed in the Scatchard plot, described previously. Such a curve is qualitatively different from that reported by Melachouris (1972) for polyphosphate–β-lactoglobulin precipitates.

Molecular exclusion chromatography may be used to determine changes in bovine serum albumin aggregation

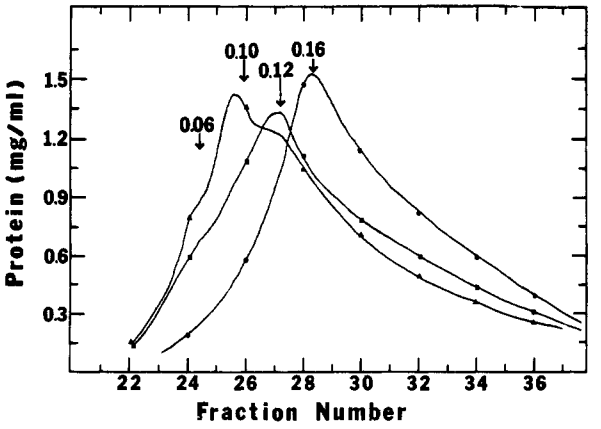

Figure 6. Bovine serum albumin protein (milligrams per milliliter) vs. fraction number (1.5 mL/fraction) for BSA prepared in the presence of sodium monophosphate (●) at pH 6.0, BSA in the presence of sodium polyphosphate ($\bar{n}$ = 14) at pH 6.0 (■), and BSA–sodium polyphosphate complexes (▲) ($\bar{n}$ = 14) prepared at pH 2.0 and resuspended at pH 6.0. All samples were chromatographed on a column packed with Sephacryl S-200 and equilibrated with 0.005 M Mes buffer.

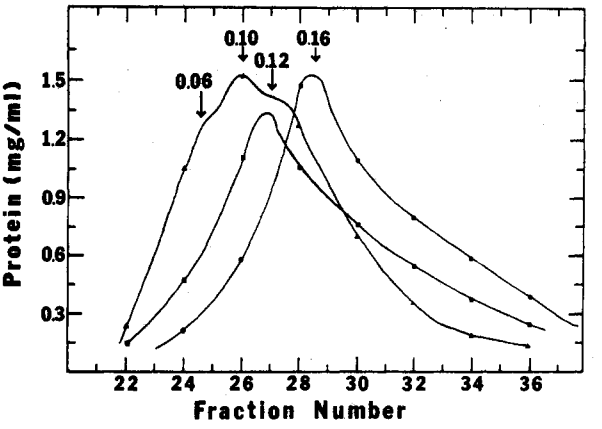

Figure 7. Bovine serum albumin protein (milligrams per milliliter) vs. fraction number (1.5 mL/fraction) for BSA prepared in the presence of sodium monophosphate (●) at pH 6.0, BSA in the presence of sodium polyphosphate ($\bar{n}$ = 22) at pH 6.0 (■), and BSA–sodium polyphosphate complexes ($\bar{n}$ = 22) (▲) prepared at pH 2.0 and resuspended at pH 6.0. All samples were chromatographed on a column packed with Sephacryl S-200 and equilibrated with 0.005 M Mes buffer. See the text for experimental details.

resulting from the treatment with polyphosphates. Three preparations were chromatographed for each chain length, namely, BSA (5.27 g/L) in the presence of 1.25 g/L sodium monophosphate at pH 6.0, BSA (5.27 g/L) in the presence of 1.25 g/L of sodium polyphosphate of $\bar{n}$ = 14 or $\bar{n}$ = 22 phosphorus atoms at pH 6.0, and BSA–polyphosphate complexes prepared from (5.27 g/L) BSA and 1.25 g/L sodium polyphosphate ($\bar{n}$ = 14 or $\bar{n}$ = 22), isolated at pH 2.0 and resuspended at pH 6.0 (Figure 6).

Interaction of bovine serum albumin at pH 6.0 in Mes buffer with both $\bar{n}$ = 14 and $\bar{n}$ = 22 polyphosphate compounds resulted in marked changes in the elution profile of the protein as compared to that of bovine serum albumin in the presence of orthophosphate at pH 6.0 (K_{av} = 0.16). As displayed in Figures 6 and 7, a new component (K_{av} = 0.12) of greater molecular weight appears in the elution profile in the presence of both polyphosphates, even though the complexes have not been precipitated. The changes in the elution profile were more pronounced when the insoluble complexes of bovine serum albumin and polyphosphates formed at pH 2.0 were prepared and resuspended at pH 6.0 prior to chromatography. These elution profiles suggest that the complexes consist of a number of aggregates. The bovine serum albumin complexes contain a number of components, especially compounds with K_{av} values of 0.10 and 0.06. A greater amount of the higher molecular weight aggregates is indicated in the presence of $\bar{n}$ = 22 vs. $\bar{n}$ = 14 polyphosphates. In addition, in experiments where the chain length of the polyphosphate is held constant, a greater proportion of higher molecular weight aggregates is produced when precipitates are formed at pH 2 vs. pH 3 or 4 (data not shown). The aggregates observed in these experiments did not elute in the void volume of the Sephacryl S-200 column, suggesting that the molecular weights of these components are less than 250 000. These results indicate that bovine serum albumin aggregates after treatment with polyphosphate. These aggregates are presumably formed through the cross-linking effect of the polyphosphate. Although the number of bovine serum albumin molecules in the aggregates cannot be determined by this method, the number should not exceed four, since aggregates did not elute with the void volume. Aggregated forms present at pH 6.0 occur even though no precipitation is observed. The insoluble complex formed at low pH is presumably a result of extreme aggregation.

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