known. However, recent work has shown that a wide range of plant species released significant amounts of volatile sulfur compounds (0.1 to 2.0% of total sulfur) during oven drying (Grundon and Asher, 1972). Consequently, recoveries of plant sulfur are likely to be incomplete to this extent at least, regardless of the method used subsequently to estimate the sulfur content of the dry plant material.

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LITERATURE CITED

Asher, C. J., Grundon, N. J., *Proc. Int. Grassland Congr. 11th* Australia (1970).
Beaton, J. D., Burns, G. R., Platou, J., The Sulphur Inst. Tech. Bull. No. 14 (1968).
Benson, R. H., *Anal. Chem.* **38,** 1353 (1966).
Bruno, G. A., Christian, J. E., *Anal. Chem.* **33,** 1216 (1961).
Davidson, J. D., Oliverio, V. T., *Atomlight* **60,** 1 (1967).
Dobbs, H. E., *Anal. Chem.* **35,** 783 (1963).
Fox, B. W., *Int. J. Appl. Radiat. Isotopes* **19,** 717 (1968).
Greene, R. C., Patterson, M. S., Estes, A. H., *Anal. Chem.* **40,** 2035 (1968).
Grundon, N. J., Asher, C. J., unpublished data (1972).
Kalburer, R., Rutschmann, J., *Helv. Chem. Acta* **44,** 1956 (1961).
Lisk, D. J., J. AGR. FOOD CHEM. **8,** 119 (1960).
MacDonald, A. M. G., *Analyst* **86,** 3 (1961).
Martin, L. E., Harrison, C., *Biochem. J.* **82,** 18 (1962).
Patterson, M. S., Greene, R. C., *Anal. Chem.* **37,** 854 (1965).
Tanielian, C., Cochie, C., Deluyarchie, G., Laustrait, G., Maillard, D., *Int. J. Appl. Radiat. Isotopes* **15,** 11 (1964).
Turner, J. C., *Int. J. Appl. Radiat. Isotopes* **18,** 557 (1967).
van der Laarse, J. D., *Int. J. Appl. Radiat. Isotopes* **18,** 485 (1967).
Wagner, H., *Mikrochim. Acta* **19** (1957).
Williams, P. H., *Int. J. Appl. Radiat. Isotopes* **19,** 377 (1968).

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Interactions of β-Lactoglobulin with Polyphosphates

Nicholas Melachouris

β-Lactoglobulin aggregated in the presence of polyphosphates. The extent of aggregation was affected by the type of polyphosphate used. At pH 4 or lower, aggregation of β-lactoglobulin resulted in precipitation of the protein in the form of a β-lactoglobulin–polyphosphate complex. Precipitation of β-lactoglobulin and binding of polyphosphate increased with increasing polyphosphate chain length and polyphosphate concentration or decreasing pH. At high protein concentration, the binding became independent of polyphosphate chain length. Temperatures between 5 and 73.5°C did not affect the precipitation and binding markedly. Increased ionic strength reduced significantly β-lactoglobulin precipitation, although the reduction of polyphosphate binding was slight.

Polyphosphates are used extensively by the food processing industry to improve the quality of various food products. Polyphosphates have also been used to isolate proteins from different sources under acidic conditions. Gordon (1945) has utilized the interactions of polyphosphates and proteins to recover proteins from cheese whey by the addition of polyphosphates at pH 3. Hartman (1966) has reported good precipitation of proteins from acid whey after the addition of long-chain polyphosphates at pH 2.5. Sarcoplasmic fish proteins have been isolated from aqueous solutions by precipitation with polyphosphates (Spinelli and Koury, 1970). Differences in polyphosphate binding by the main components of casein have been observed and they have been used to isolate α_s-casein by a simple procedure (Melnychyn and Wolcott, 1967). The effect of polyphosphate chain length on the binding of polyphosphates by gelatin and egg white proteins has been reported (Lyons and Siebenthal, 1966) to be consistent with the law of mass action and dependent on polyphosphate chain length. The main factors determining precipitation of human plasma proteins with polyphosphates have been shown to be polyphosphate concentration, pH, and ionic strength (Nitschmann *et al.*, 1959). Briggs (1940) has contributed significantly to the elucidation of the mechanism of protein–polyphosphate interaction by his study on the binding of metaphosphate by serum albumin.

The objective of this investigation was to study the interactions of polyphosphates with the main protein component of cheese whey, namely β-lactoglobulin. More specifically, we were interested in the effects of various factors on the precipitation of β-lactoglobulin and the binding of polyphosphates by β-lactoglobulin. Finally, changes of the β-lactoglobulin molecule resulting from the treatment with polyphosphates were investigated.

EXPERIMENTAL PROCEDURE

Materials. Two polyphosphates were used in this investigation, sodium polyphosphate with a number-average chain length $\bar{n}$ = 10.3 (commercial sodium hexametaphosphate) and sodium polyphosphate, $\bar{n}$ = 28.1. Both compounds contained a distribution of linear polyphosphate species of varying chain length. The $\bar{n}$ values were determined titrimetrically according to the procedure of Van Wazer *et al.* (1954).

Five additional phosphates were used in one experiment. All the phosphates used in this investigation were available from this laboratory. Crystallized β-lactoglobulin (Pentex, Miles Laboratories, Elkhart, Ind.) was used throughout this investigation. The molecular weight of β-lactoglobulin was assumed to be 36,000.

Measurement of Polyphosphate Binding. Polyphosphate binding was determined by precipitating the protein–poly-

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phosphate complex and measuring the amount of phosphorus bound. To 4 ml of 1% β-lactoglobulin solution, pH 6.5, 0.6 ml of 1% freshly prepared polyphosphate solution was added. In this system, the concentration of β-lactoglobulin was 2.4×10^{-4} *M*, whereas the phosphorus concentration of sodium polyphosphate $\bar{n} = 10.3$ and sodium polyphosphate $\bar{n} = 28.1$ was 12.1×10^{-3} *M* and 12.5×10^{-3} *M*, respectively. After equilibration for 30 min, the pH of the mixture was adjusted to 4 with 0.1 *N* HCl. The mixture was left at room temperature for 1 hr, and then centrifuged (International Centrifuge, Model HT) at 12,500 rpm for 10 min. The precipitate was dispersed in water by adjusting the pH to 6.5 with 0.1 *N* NaOH and analyzed for protein and phosphorus content.

When the effect of pH on the polyphosphate–β-lactoglobulin interactions was determined, the pH of the protein–polyphosphate mixtures was adjusted to 4, 3, and 2, respectively.

When the effect of temperature was determined, the protein–polyphosphate mixtures were kept at 5, 25.5, 50.5, and 73.5°C, respectively, for 30 min. The pH was then adjusted to 4.0 and the samples were kept at the above temperatures for 1 hr before centrifuging.

For the determination of the effect of ionic strength, the protein was dissolved in sodium chloride solutions such as to give ionic strengths of 0.05, 0.2, and 0.6, respectively.

Analytical Methods. Protein concentrations were determined by the Biuret method (Layne, 1957).

Phosphorus was determined by the spectrophotometric vanadomolybdate method (AOAC, 1965) after digesting 40–70-mg samples with 3 ml of concentrated sulfuric acid for 10 min and oxidation with 30% hydrogen peroxide. Liquid samples were evaporated to dryness before digestion.

Gel Filtration. A Sephadex G-100 column (19.2 × 1.5 cm) equilibrated with 0.01 *M* NaCl solution was used for the gel filtration experiments. The amount of sample applied was approximately 6 mg of protein dispersed in 1 ml of eluent (0.01 *M* NaCl). The elution was performed under a hydrostatic pressure of 19 cm and a flow rate of approximately 20 ml per hr. The effluent was monitored at 280 mμ using a Beckman DB-G spectrophotometer equipped with a flow-through cell. A concentration converter was used to convert the absorption units continually to protein concentration.

In order to make the results independent of individual experimental conditions, the distribution coefficient K_{av} of the various protein components was determined using the equation

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

where V_e is the elution volume, V_0 is the void volume, and V_t is the total volume. Void volume determinations were made according to the procedure described by Granath and Kvist (1967). The protein components in the elution patterns were designated by their respective K_{av} values.

The following five samples were analyzed by gel filtration:

Sample a. 1% β-lactoglobulin solution adjusted to pH 5.7 and dialyzed against 0.01 *M* NaCl solution.

Sample b. To 4 ml of 1% β-lactoglobulin solution, 0.6 ml of 1% sodium polyphosphate, $\bar{n} = 10.3$, was added, the pH was adjusted to 5.7, and the sample dialyzed against 0.01 *M* NaCl solution.

Sample c. To 4 ml of 1% β-lactoglobulin solution, 0.6 ml of 1% sodium polyphosphate, $\bar{n} = 10.3$, was added and the pH was adjusted to 4.0. The precipitated β-lactoglobulin–polyphosphate complex was removed by centrifugation according to the procedure described earlier, dispersed in water by adjusting the pH to 5.7, and dialyzed against 0.01 *M* NaCl solution.

Sample d. To 4 ml of 1% β-lactoglobulin solution, 0.6 ml of 1% sodium polyphosphate, $\bar{n} = 28.1$, was added, the pH was adjusted to 5.7, and the sample was dialyzed against 0.01 *M* NaCl solution.

Sample e. To 4 ml of 1% β-lactoglobulin solution, 0.6 ml of 1% sodium polyphosphate, $\bar{n} = 28.1$, was added and the pH was adjusted to 4.0. The β-lactoglobulin–polyphosphate complex was isolated by centrifugation, dispersed in water by adjusting the pH to 5.7, and dialyzed against 0.01 *M* NaCl solution.

Table I. Precipitation of β-Lactoglobulin at Different pH Levels in the Presence of Phosphates

Phosphate	β-Lactoglobulin precipitated, pH 4	pH 3	pH 2
	%		
Disodium phosphate	0	0	0
Sodium acid pyrophosphate	4.0	0	0
Sodium tripolyphosphate	35.2	...	0
Sodium polyphosphate, $\bar{n} = 10.3$	99.0	98.1	98.1
Sodium polyphosphate, $\bar{n} = 17.1$	98.5	99.0	98.1
Sodium polyphosphate, $\bar{n} = 19.8$	100.0	98.5	98.5
Sodium polyphosphate, $\bar{n} = 28.1$	100.0	100.0	98.1

RESULTS AND DISCUSSION

Effect of pH and Polyphosphate Chain Length. The interactions of β-lactoglobulin with polyphosphates resulted in precipitation of β-lactoglobulin. The precipitation was influenced primarily by two factors, namely pH and the type of phosphate interacting with the protein. The data in Table I show that β-lactoglobulin did not precipitate in the presence of disodium phosphate at pH 4, 3, or 2. In the presence of pyrophosphate, β-lactoglobulin precipitated only at pH 4.

At pH 4 and in the presence of tripolyphosphate the amount of β-lactoglobulin precipitated was 35.2%. Because of experimental difficulties, precipitation at pH 3 could not be measured very well. No precipitate was formed at pH 2. With sodium polyphosphate ($\bar{n} = 10.3$ to 28.1), complete precipitation of β-lactoglobulin occurred at all pH levels.

The effect of polyphosphate chain length on the precipitation of β-lactoglobulin can be explained by the observation that below the isoelectric point β-lactoglobulin acts as a polycation and the polyphosphates act as polyanions. Precipitation of insoluble complexes formed as a result of polycation–polyanion interactions will increase as the size of either the polycation or polyanion is increased (Katchman and Van Wazer, 1954).

The binding of polyphosphates was also affected by the pH and the polyphosphate chain length, as is shown by the data in Figure 1. The binding was higher when sodium polyphosphate, $\bar{n} = 28.1$, rather than sodium polyphosphate, $\bar{n} = 10.3$,

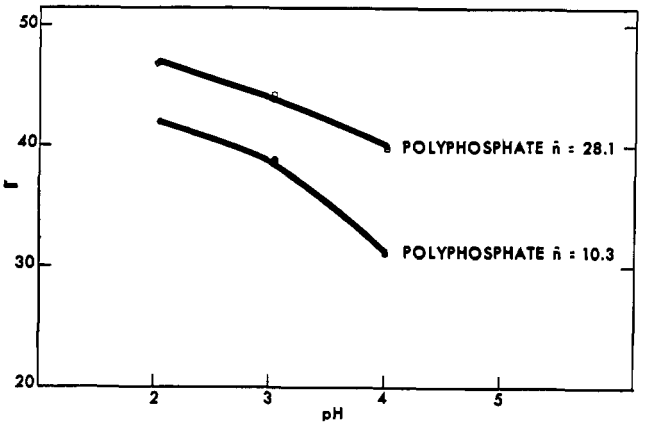

Figure 1. Effect of pH on the binding of polyphosphates of different chain length by β-lactoglobulin. r = moles of phosphorus bound per mole of β-lactoglobulin

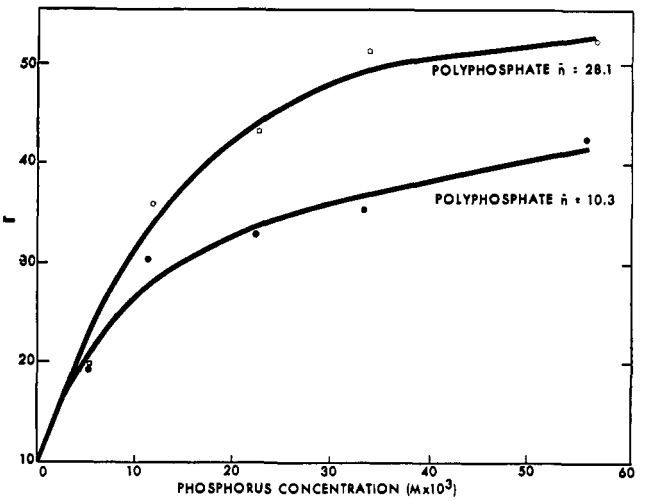

Figure 2. Binding of polyphosphates by β-lactoglobulin under conditions of constant protein concentration and varying concentrations of polyphosphates. r = moles of phosphorus bound per mole of β-lactoglobulin

was used. Moreover, the binding increased as the pH was lowered. The increased polyphosphate binding at lower pH may be due to unfolding or expansion of the protein molecule as the polyphosphate is bound, thus making available for binding more ionized groups, and also to a reduction in the repulsive electrostatic forces (Klotz, 1953).

Effect of Concentration of Polyphosphates and β-Lactoglobulin. Maximum binding of polyphosphate can theoretically be achieved when each free amino goup of β-lactoglobulin binds one polyphosphate molecule. There are 36 amino groups on each β-lactoglobulin molecule of 36,000 molecular weight; six guanidyl, 28 ε-amino, and two α-amino groups (McKenzie, 1967). For maximum binding, the polyphosphate concentration used should be such that an excess of polyphosphate is present in the system.

The data in Figure 2 show that increasing polyphosphate concentration increased the binding of polyphosphate very markedly. In agreement with the results of Briggs (1940), it was found that the degree of binding, that is, the change in binding per unit change in polyphosphate concentration, became lower at higher polyphosphate concentrations.

This limited increase in the degree of binding after the initial extensive binding may be due to changes in affinity of the binding sites.

Information regarding the number of binding sites on the β-lactoglobulin molecule and the affinity between β-lactoglobulin and polyphosphates can be obtained by the equation given by Klotz (1953):

$$\frac{r}{(A)} = kn - kr$$

where r is the moles of phosphorus bound per mole of protein at a given polyphosphate concentration, (A) is the molar concentration of free phosphorus, k is the binding constant, and n is the maximum number of moles of phosphorus which can be bound by a mole of β-lactoglobulin. A plot of $r/(A)$ *vs.* r would give an intercept on the abscissa equal to n and that on the ordinate equal to kn. Such a plot, shown in Figure 3, revealed that there was a deviation from linearity at high levels of polyphosphates, and therefore k and n could not be determined from this plot. The deviation indicated that there were significant interactions between binding sites. Such interactions are possible when the polyphosphate bound by one site affects the binding affinity of the other free sites on the protein molecule (Klotz, 1953). The presence of interactions between binding sites as well as the fact that a polyphosphate molecule can bind to a number of binding sites on the same protein molecule simultaneously does not make the estimation of the maximum polyphosphate binding by β-lactoglobulin feasible.

Differences were also observed between the binding of polyphosphate, $\bar{n}$ = 10.3, and polyphosphate, $\bar{n}$ = 28.1, by β-lactoglobulin (Figure 2). Polyphosphate, $\bar{n}$ = 28.1, at different concentrations was bound to a greater extent than polyphosphate, $\bar{n}$ = 10.3, at the corresponding concentrations. This was probably due to stronger Van der Waals' forces accompanying the larger polyphosphate molecule (Klotz, 1953).

On the other hand, when the polyphosphate concentration of the β-lactoglobulin–polyphosphate system was kept constant but the concentration of β-lactoglobulin was varied, the amount of polyphosphate bound per mole of β-lactoglobulin decreased, as is shown by the data in Figure 4. At lower protein concentrations the polyphosphates were bound to a higher degree than at higher concentrations. Polyphosphate, $\bar{n}$ = 28.1, was bound to a greater degree than polyphosphate, $\bar{n}$ = 10.3. However, the degree of binding of the longer polyphosphate decreased more than that of the shorter polyphosphate with increasing protein concentration. At a protein concentration of approximately 6×10^{-4} M, the polyphosphate bound by β-lactoglobulin became independent of polyphosphate chain length. The data suggest that differences in binding of polyphosphates by β-lactoglobulin were more pronounced when low concentrations of β-lactoglobulin were used.

During the course of this experiment it was noticed that the precipitation of β-lactoglobulin was complete when the protein concentration in the system was increased to approximately 4×10^{-4} M. When the protein concentration was higher than 4×10^{-4} M, the protein precipitation decreased. This decrease was due to the presence of bound polyphosphate at a level below the level required for complete precipitation.

Effect of Temperature and Ionic Strength. Interactions of β-lactoglobulin and polyphosphates are not markedly affected by the temperature at which the interactions take place. This is shown by the data in Table II. There was an in-

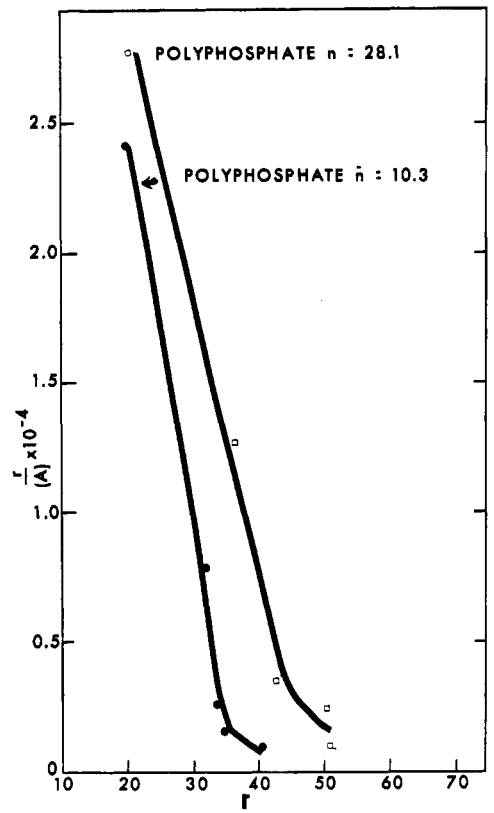

Figure 3. Binding of polyphosphates by β-lactoglobulin under conditions of varying concentrations of polyphosphates. r = moles of bound phosphorus per mole of β-lactoglobulin. (A) = molar concentration of free phosphorus in the system

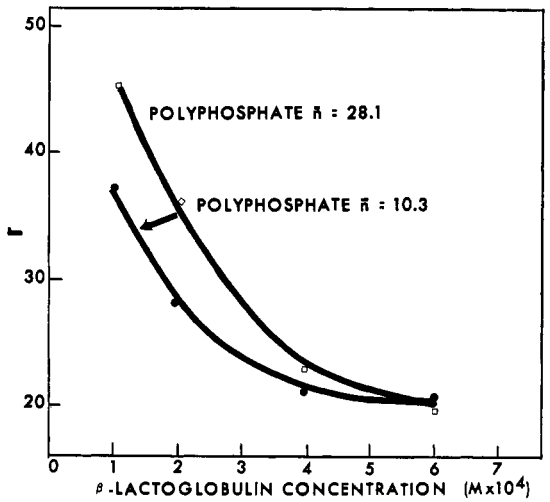

Figure 4. Binding of polyphosphates by varying amounts of β-lactoglobulin under constant polyphosphate concentration. r = moles of phosphorus bound per mole of β-lactoglobulin

creased binding of polyphosphate, $\bar{n}$ = 10.3, as the temperature was increased from 5 to 73.5°C. However, no trend could be observed for polyphosphate, $\bar{n}$ = 28.1.

Klotz (1953) has reviewed the effect of temperature on the binding of ions by proteins, mainly serum albumin, and he has concluded that temperature changes do not affect binding to a significant degree.

The effect of ionic strength on the binding of polyphosphate and precipitation of β-lactoglobulin is shown by the data in

Table II. Effect of Temperature on the Binding of Polyphosphates by β-Lactoglobulin

Temperature, °C	Polyphosphate	Phosphorus bound, mol/mol β-lactoglobulin
5	Sodium polyphosphate, $\bar{n}$ = 10.3	27.8
25.5		31.1
50.5		32.2
73.5		34.5
5	Sodium polyphosphate, $\bar{n}$ = 28.1	36.1
25.5		35.4
50.5		37.6
73.5		34.5

Table III. Effect of Ionic Strength on the Binding of Polyphosphates by β-Lactoglobulin

Ionic strength[a]	Polyphosphate	β-lactoglobulin precipitated, %	Phosphorus bound, mol/mol β-lactoglobulin
0	Sodium polyphosphate, $\bar{n}$ = 10.3	100.4	27.7
0.05		100.3	28.1
0.2		99.0	25.9
0.6		1.9	...
0	Sodium polyphosphate, $\bar{n}$ = 28.1	98.7	34.9
0.05		101.0	34.0
0.2		101.6	33.5
0.6		59.4	31.5

[a] It refers to NaCl present in the system.

Table III. There was a decrease in the binding of polyphosphate by β-lactoglobulin as the ionic strength of the system was increased. Moreover, the precipitation of β-lactoglobulin was significantly reduced. Thus, at ionic strength 0.6, only 1.9 and 59.4% of the protein precipitated in the presence of polyphosphate, $\bar{n}$ = 10.3, and polyphosphate, $\bar{n}$ = 28.1, respectively. When polyphosphate, $\bar{n}$ = 28.1, was used, the decrease in the percent of protein precipitated was not as large as that observed when polyphosphate, $\bar{n}$ = 10.3, was used. This can be attributed to the stronger interactions of β-lactoglobulin with polyphosphate, $\bar{n}$ = 28.1.

Increased ionic strength is expected to reduce protein–ion interactions. However, the possibility also exists that the effect of ionic strength on the precipitation of β-lactoglobulin and the binding of polyphosphates might be partially due to conformational changes of the polyphosphate molecule itself. The linear structure of sodium polyphosphate may be altered to form a coiled structure under conditions of increasing ionic strength. Such a change can reduce the cross-linking effect without a corresponding reduction in the amount of polyphosphate present in the complex.

Effect of Polyphosphates on β-Lactoglobulin. Gel filtration was used to determine changes of β-lactoglobulin resulting

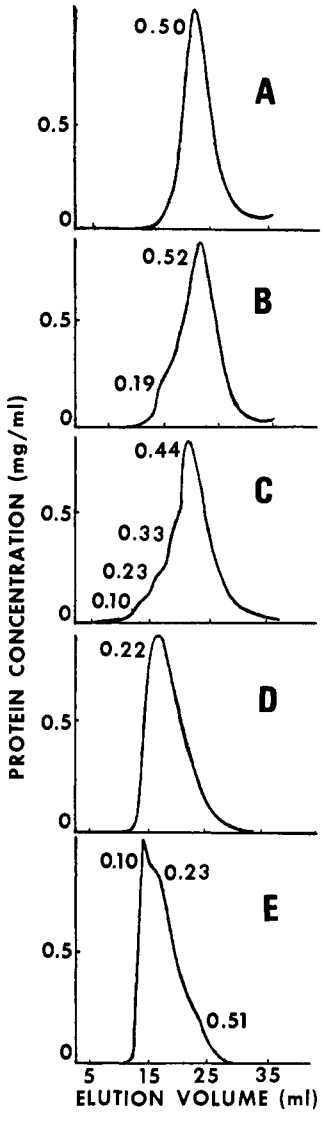

Figure 5. Gel filtration on Sephadex G-100 of β-lactoglobulin before and after treatment with polyphosphates. A, β-lactoglobulin, pH 5.7; B, β-lactoglobulin after the addition of polyphosphate, $\bar{n}$ = 10.3, pH 5.7; C, β-lactoglobulin precipitated at pH 4.0 in the presence of polyphosphate, $\bar{n}$ = 10.3. The precipitate was dispersed at pH 5.7; D, β-lactoglobulin after the addition of polyphosphate, $\bar{n}$ = 28.1, pH 5.7; E, β-lactoglobulin precipitated at pH 4.0 in the presence of polyphosphate, $\bar{n}$ = 28.1. The precipitate was dispersed at pH 5.7. The numbers designating the different protein components refer to distribution coefficients K_{av}. For chromatographic conditions, see text

from the treatment with polyphosphates. Five preparations were chromatographed, namely β-lactoglobulin, β-lactoglobulin in the presence of polyphosphate, $\bar{n}$ = 10.3, and in the presence of polyphosphate, $\bar{n}$ = 28.1, and β-lactoglobulin–polyphosphate complexes isolated at pH 4.0.

The gel filtration profiles of the various β-lactoglobulin–polyphosphate preparations provided information about the formation of aggregated species (Figure 5). The interactions of β-lactoglobulin with polyphosphates at pH 5.7 resulted in marked changes of the protein (Figure 5, B and D). A new component (0.19) of larger molecular size than β-lactoglobulin developed when β-lactoglobulin was treated with polyphosphate, $\bar{n}$ = 10.3. When polyphosphate, $\bar{n}$ = 28.1, was used, however, a much greater amount of β-lactoglobulin was converted to a larger molecular size product (0.22). These changes of β-lactoglobulin were more pronounced when the insoluble complexes of β-lactoglobulin and polyphosphates formed at pH 4.0 were analyzed (Figure 5, C and E). The elution patterns showed the complex to consist of a number of aggregates. Almost all β-lactoglobulin had been converted to heavier components (0.10, 0.23, 0.33, and 0.44). The complex of β-lactoglobulin and polyphosphate, $\bar{n}$ = 10.3, had a greater number of different aggregates than that formed between β-lactoglobulin and polyphosphate, $\bar{n}$ = 28.1. The latter complex, however, consisted primarily of the heaviest protein components 0.10 and 0.23. Since none of the heavy components was eluted with the void volume of the Sephadex G-100 column, the molecular weight of these components should have been less than 150,000.

The results of the gel filtration experiments clearly indicate that β-lactoglobulin aggregates after treatment with polyphosphates. The aggregates are formed through the cross-linking effect of the polyphosphates. The aggregated forms are present in the system, even though no insoluble β-lactoglobulin–polyphosphate complex is formed. The insoluble complex is formed at low pH as a result of charge suppression and extensive aggregation.

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LITERATURE CITED

Association of Official Agricultural Chemists, "Official Methods of Analysis," 10th ed., 2.016, 1965, p 11.
Briggs, D. R., *J. Biol. Chem.* **134,** 261 (1940).
Gordon, W. G., Smith Kline & French Laboratories, U.S. Patent 2,377,624 (June 5, 1945).
Granath, K. A., Kvist, B. E., *J. Chromatogr.* **28,** 69 (1967).
Hartman, G. H. Jr., Ph.D. Thesis, Univ. of Wisconsin (1966).
Katchman, B. J., Van Wazer, J. R., *Biochim. Biophys. Acta* **14,** 445 (1954).
Klotz, I. M., Protein Interactions in the "Proteins," Neurath, H., Bailey, K., Eds., Vol I, Part B, Academic Press, New York, N.Y., 1953, p 727.
Layne, E., Spectrophotometric and Turbidimetric Methods for Measuring Proteins, in "Methods in Enzymology," Colowick, S. P., Kaplan, N. O., Eds., Vol III, Academic Press, New York, N.Y., 1957, p 447.
Lyons, J. W., Siebenthal, C. D., *Biochim. Biophys. Acta* **120,** 174 (1966).
McKenzie, H. A., Milk Proteins, in "Advances in Protein Chemistry," Anfinsen, C. B., Jr., *et al.*, Eds., Vol 22, Academic Press, New York, N.Y., 1967, p 55.
Melnychyn, P., Wolcott, J. M., *J. Dairy Sci.* **50,** 1863 (1967).
Nitschmann, Hs., Rickli, E., Kistler, P., *Helv. Chim. Acta* **42,** 2198 (1959).
Spinelli, J., Koury, B., J. Agr. Food Chem. **18,** 284 (1970).
Van Wazer, J. R., Griffith, E. J., McCullough, J. F., *Anal. Chem.* **26,** 1755 (1954).

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