The Binding of Calcium Ions by β-Lactoglobulin Both before and after Aggregation by Heating in the Presence of Calcium Ions

The heating of β-lactoglobulin in the presence of calcium ions results in aggregation, but does not affect the amount of calcium bound in the *p*H region of 6.2 to 7.5. In the heated calcium-containing solutions aggregation appears to be determined by the net charge on the protein resulting from the binding of the positive calcium ions and is analogous to isoelectric aggregation. Thus, isoelectric precipitation of heated β-lactoglobulin solutions can be obtained by lowering the *p*H to the isolectric region, or at a constant alkaline *p*H by the addition of calcium. Further, although the calcium in the aggregated, heated β-lactoglobulin is physically less accessible, it is in equilibrium with the calcium in solution and can be removed by suitable procedures. The amount of calcium bound to β-lactoglobulin is equivalent to the net negative charge in the *p*H range of 6 to 8.

It is of theoretical and practical interest to know whether the binding of ions to proteins is changed when the proteins are denatured, particularly when the denaturation is performed in the presence of the ions. In the present report the binding of calcium ions to unheated and heated β-lactoglobulin in the *p*H range from 6 to 8 is investigated. Binding of calcium ions to unheated β-lactoglobulin was studied by equilibrium dialysis[2] and by the ultracentrifuge technique of Chanutin, *et al.*[3] Binding of calcium to heated β-lactoglobulin was studied by taking advantage of the fact that β-lactoglobulin aggregated when heated in the presence of calcium ions.[4] The aggregated protein was sedimented in the ultracentrifuge and the calcium bound in the sediment was determined.

Methods

Determination of Calcium.—Two procedures were used for the determination of calcium: the colorimetric murexide method[5] which requires that the protein-containing solutions be ashed, and a Versene titrimetric procedure[6] with Eriochrome Black T as the indicator. The latter method was somewhat more satisfactory since protein-containing solutions could be titrated directly, and reproducibility was better than that of the colorimetric method. The solutions to be titrated, containing about 8 γ of calcium, were made alkaline with NaOH, and $MgCl_2$ was added to obtain a sharper end-point.[7] Titrations were performed in triplicate; analytical error was about ±0.5%.

Determination of Chloride.—Chloride was determined by titration with mercuric nitrate with diphenylcarbazone as the indicator.[8] When β-lactoglobulin-containing solutions were titrated an initial, faint pink color was obtained which disappeared, but the final pink end-point was satisfactory. Chloride values in solutions containing 1% β-lactoglobulin were about 5% high. For this reason, the standard chloride curve was prepared with β-lactoglobulin present. Even with the protein correction, occasional high values for chloride were obtained in β-lactoglobulin-containing solutions which could not be attributed to binding (see later).

Determination of Total Protein.—Protein concentrations were determined by ultraviolet absorption at 280 mμ. An absorption of 0.93 in a 1-cm. cell was equivalent to 1 mg. of β-lactoglobulin per ml.

Preparation of Solutions.—β-Lactoglobulin solutions containing calcium chloride were prepared from separate solutions of the salt and protein. The crystalline, isoelectric β-lactoglobulin was dissolved and stock solutions were prepared by neutralization with sodium hydroxide to *p*H 7.0. Measured volumes of protein and salt solutions were mixed and the final *p*H and volume adjustment made by addition of 0.1 *N* NaOH or HCl and water. The calcium chloride solution was prepared from reagent grade salt. The heated β-lactoglobulin solutions were prepared by holding 10-ml. volumes for 30 minutes at 90° as described previously.[4] All *p*H measurements were at 25°.

Determination of the Amount of Calcium Bound to Unheated β-Lactoglobulin. (a).—The ultracentrifuge technique of Chanutin, *et al.*,[3] was used. A Spinco Model L preparative ultracentrifuge[9] was used to obtain the desired protein gradient. Unheated protein–calcium chloride solutions were centrifuged for 3 hours at 40,000 r.p.m. (105,000 × *g*) with the No. 40 rotor, and 12-ml. plastic tubes. The centrifuged solution was sampled in three equal portions by removing successive layers with a supported syringe and needle. Total protein and total calcium were determined in each portion.

(b).—Equilibrium dialysis[2] was also employed to determine the amount of bound calcium. To facilitate equilibrium calcium chloride was placed in the dialysis bag with the protein as well as in the outer solution. Dialysis was carried out at 7°; equilibrium was attained in 48 hours when the solutions were stirred. The total calcium concentration was determined in and outside the bag. The chloride concentrations were determined also.

Donnan membrane distribution of the calcium chloride in this system must be considered. Following the treatment given by Wagner,[10] the distribution equation was derived for calcium chloride as: $\Delta CaCl_2 = n_p C_p C_s/2(n_p C_p + 6C_s)$, where C_p is the moles of protein with the effective net charge n_p, and C_s is the moles of calcium chloride. A $\Delta CaCl_2$ value of 0.29 mmole per liter was calculated for a 1% β-lactoglobulin solution at *p*H 7.5 containing 7.5 mmoles of calcium chloride per liter. For this calculation a molecular weight of 35,000 was used for β-lactoglobulin, and the net charge on the protein was taken to be 13.6 from the titration data of Cannan, *et al.*[11] (87.5% of the published value, which was for a molecular weight of 40,000). Thus in this system without binding, the calcium chloride concentration outside the bag would be 5.29 mmoles per liter, inside 4.71 mmoles per liter. To the calcium chloride concentration inside the bag must be added the equivalents of calcium required to neutralize the charge on the β-lactoglobulin, 3.89 milliequivalents per liter or 1.95 mmoles of calcium per liter. (This value will be reduced by about 17% by the sodium ions in the stock β-lactoglobulin solution.) Thus at a maximum the total calcium inside is 6.66 mmoles per liter, ex-

(1) A Laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

(2) T. R. Hughes and I. M. Klotz, *Methods Biochem. Anal.*, **3**, 265 (1956).

(3) A. Chanutin, S. Ludewig and A. V. Masket, *J. Biol. Chem.*, **143**, 737 (1942).

(4) C. A. Zittle and E. S. DellaMonica, *J. Dairy Sci.*, **39**, 514 (1956).

(5) M. B. Williams and J. H. Moser, *Anal. Chem.*, **25**, 1414 (1953).

(6) A. E. Sobel and A. Hanok, *Proc. Soc. Exp. Biol. Med.*, **77**, 737 (1951).

(7) R. L. Griswold and N. Pace, *Anal. Chem.*, **28**, 1035 (1956).

(8) O. Schales and S. S. Schales, *J. Biol. Chem.*, **140**, 879 (1941).

(9) Mention of trade name does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

(10) R. H. Wagner in "Physical Methods of Organic Chemistry," Vol. I, Part 1, 2nd Ed., Interscience Publishers, Inc., New York, N. Y., 1949, p. 494.

(11) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

ceeding that outside by 25.9%. This Donnan distribution theoretically could be reduced by lowering the *p*H or concentrating the protein, but from an analytical standpoint both of these changes were infeasible, particularly the latter. Accordingly, the chloride concentrations inside and outside were determined, and the free calcium inside was calculated from the equation[10]: $Ca_i^{++}.(Cl_i^-)^2 = Ca_o^{++}.(Cl_o^-)^2$. This equation is valid only if chloride is not bound. The evidence that chloride is not bound is presented later.

Determination of the Amount of Calcium Bound to Heated β-Lactoglobulin.—The heated β-lactoglobulin–calcium chloride solutions were centrifuged for 45 minutes at 40,000 r.p.m. in the preparative ultracentrifuge with the same rotor used for the unheated samples. This was sufficient time to sediment the white, aggregated protein leaving a water-clear supernatant solution. The supernatant solution was decanted and analyzed for protein and calcium content. The calcium bound to the aggregated, sedimented protein was determined by difference.

When precipitation of the protein is complete the accompanying calcium represents total bound calcium. When precipitation is incomplete, however, as with lower calcium concentrations and higher *p*H values, the calcium with the protein aggregate may represent a maximum binding value and not be representative of the total solution. The calcium bound in the precipitate may represent the upper range of a distribution curve of the calcium bound to individual protein molecules in the total solution. The results support this suggestion. In the precipitation of human mercaptalbumin by Pb^{++} it has been concluded[12] that the equilibria in solution and in amorphous precipitates are very similar; however in this system heat is not involved in the precipitation.

Results

The distribution of calcium and β-lactoglobulin in the top, middle and bottom portions of solutions containing both components was determined after ultracentrifugation for 3 hours. This was done with solutions at *p*H 7.5 containing 2, 4, 5 and 10 mmoles of calcium per liter. The total protein in each portion in % was plotted against total calcium in mmoles per liter following the method of Chanutin, *et al.*[3] The free calcium concentration is obtained from the extrapolation to zero protein concentration. Since the system is in equilibrium this is also the free calcium concentration in the protein-containing solutions. Straight lines were obtained for the plots and the respective free calcium concentrations for the solutions studied were 0.30, 0.80, 1.50 and 5.8 mmoles per liter, and the slopes 0.91, 1.72, 1.94 and 2.33. A plot of the slopes against the reciprocals of the free calcium concentrations, following Chanutin, *et al.*,[3] gives a linear plot from which a dissociation pK of 3.3 was calculated.

Perhaps a more informative plot is obtained by the method of Klotz[13] in which the ratio of calcium bound per mole of protein to the free calcium (r/A) is plotted against the calcium bound (r). In this case the intercept on the r-axis gives kn, where k is the association constant and n is the maximum number of moles bound. The intercept on the r/A axis gives n directly (9.5 moles per mole of β-lactoglobulin).

The moles of calcium bound per mole of β-lactoglobulin, calculated from the ultracentrifuge analytical data, are shown in Fig. 1 (●), together with the more extensive equilibrium dialysis data. The limitation in this procedure with the use of the Spinco preparative centrifuge was the inability to get a sufficient spread between the highest and lowest concentrations of β-lactoglobulin for complete reliability of the extrapolation to zero protein concentration. Little increase in spread was obtained even when centrifugation was extended to 5 hours. This difficulty was attributed to the stir-back which occurs when the centrifuge is stopped, and could be demonstrated with a formed-boundary using a colored protein like hemoglobin. Because of this difficulty subsequent binding studies with the unheated protein were done by equilibrium dialysis.

The binding of calcium to native β-lactoglobulin was determined by equilibrium dialysis with several concentrations of calcium at *p*H 6.2, 6.8 and 7.5. Representative data from which the binding values were calculated are given in Table I, and the binding values in full are given in Fig. 1. The maximum binding extended to low concentrations of calcium. The binding at lower concentrations of calcium where binding is less than maximal was not determined because of the large effect of titration errors (> ± 0.5%) on the small difference in concentration between inside and outside solutions.

TABLE I

EQUILIBRIUM DIALYSIS DETERMINATION OF CALCIUM BOUND TO β-LACTOGLOBULIN (1.0% CONCENTRATION)[a]

Ca concn., orig., mmoles/l.	*p*H Initial	*p*H After dialysis	Ca concn. after dialysis (mmoles/l.) Inside sac (Protein)	Ca concn. after dialysis (mmoles/l.) Outside	Cl concn. after dialysis (mmoles/l.) Inside	Cl concn. after dialysis (mmoles/l.) Outside	Ca bound to β-lactoglobulin, moles/mole
1.92	6.0	6.22	2.48	1.64	5.34	4.85	3.13
5.76	6.0	6.13	6.05	5.15	12.6	12.3	3.35
1.92	7.1	6.79	2.42	1.35	4.80	4.50	4.22
5.76	7.1	6.96	6.45	5.25	11.8	11.7	4.47
1.92	7.8	7.51	2.70	1.24	4.52	4.54	5.68
5.76	7.8	7.48	6.35	4.75	11.6	11.8	6.22

[a] Period of dialysis, 48 hr. with stirring; volumes on each side of membrane, 10.0 ml.

The concentration of chloride inside and outside the dialysis membrane was also determined to estimate the influence of Donnan distribution on the calcium concentrations. The chloride distribution was always 1:1 within the limits of the method (± 0.5%), except for an occasional high value in the presence of protein. It was important to know whether the high values were due to chloride binding or to analytical errors. Carr has shown[14] that no chloride is bound to β-lactoglobulin above *p*H 4.5 when added as sodium chloride. The possibility remained that chloride might be bound in the presence of calcium ions. This could not be determined in our dialysis system, but measurements in our heated systems at *p*H 6, where the β-lactoglobulin is aggregated, showed that no chloride was bound. Since the chloride ratio is 1:1 the excess calcium inside the membrane must be all bound calcium. Actually, from the calculation under Methods for 1% β-lactoglobulin, *p*H 7.5, 5 mmoles of calcium chloride per liter, a 12.3% excess of chloride would be expected outside on the basis of the Donnan distribution. The fact that this is not observed is evidence for the binding of the positive

(12) F. R. N. Gurd and P. E. Wilcox, *Advances in Protein Chem.*, **11**, 311 (1956).

(13) I. M. Klotz in "The Proteins," Vol. I, Part B, Academic Press, Inc., New York, N. Y., 1953, p. 773.

(14) C. W. Carr, *Arch. Biochem. Biophys.*, **46**, 417 (1953).

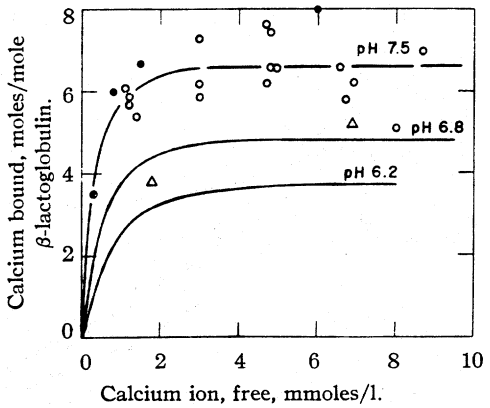

Fig. 1.—Binding of calcium to β-lactoglobulin (0.9%), determined by equilibrium dialysis at 2 to 10 mmoles of $CaCl_2$ per liter (initial concentration inside and outside dialysis sac). Individual experimental points (O) are shown only for *p*H 7.5. The curve for *p*H 6.8 is based on 16 experiments, the curve for *p*H 6.2 is based on 12 experiments in the same range of $CaCl_2$ concentrations. Also shown are two values (△) reported by Carr[15] for *p*H 7.4, and four values (●) at *p*H 7.5 obtained from the ultracentrifuge experiments.

calcium ion so that the negative charge on the β-lactoglobulin is reduced and repulsion of the chloride across the membrane becomes negligible.

The aggregation obtained by heating solutions of β-lactoglobulin (0.9 and 1.8% concentration) containing calcium chloride is shown in Fig. 2 for

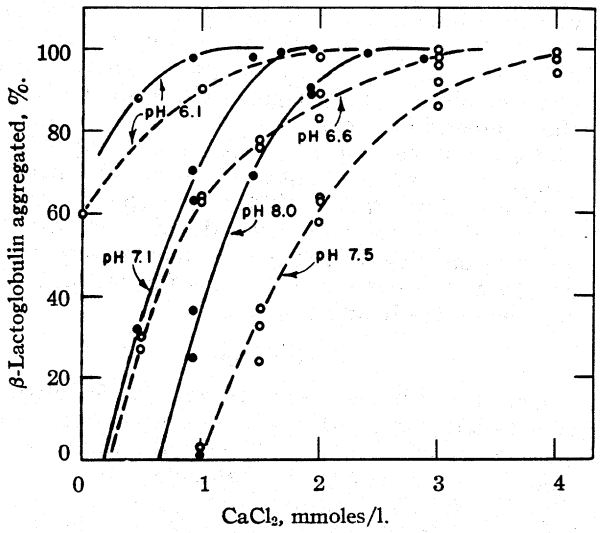

Fig. 2.—Aggregation of β-lactoglobulin when heated to 90° for 30 minutes in the presence of calcium chloride at several *p*H values. The aggregates were sedimented in the ultracentrifuge at 105,000 × *g* for 45 minutes. Solid lines (●——●) are the results with 0.9% β-lactoglobulin, the dashed lines (O——O) with 1.8% β-lactoglobulin.

several *p*H values. These solutions were ultracentrifuged for 45 minutes which was sufficient to sediment the white, aggregated β-lactoglobulin. Much of this aggregated β-lactoglobulin can be sedimented at low centrifuge speeds,[4] but clear supernatant solutions are obtained more readily with the ultracentrifuge. These experiments provided the first indication that calcium ion aggregation and hydrogen ion aggregation were of similar nature. This was provided by the data for *p*H 6.1 with 1.8% β-lactoglobulin which shows that the amount of aggregate with no calcium falls on the extended calcium aggregation curve.

The calcium bound in the sediment of the aggregated β-lactoglobulin is presented in Fig. 3A (0.9% β-lactoglobulin), and 3B (1.8% β-lacto-

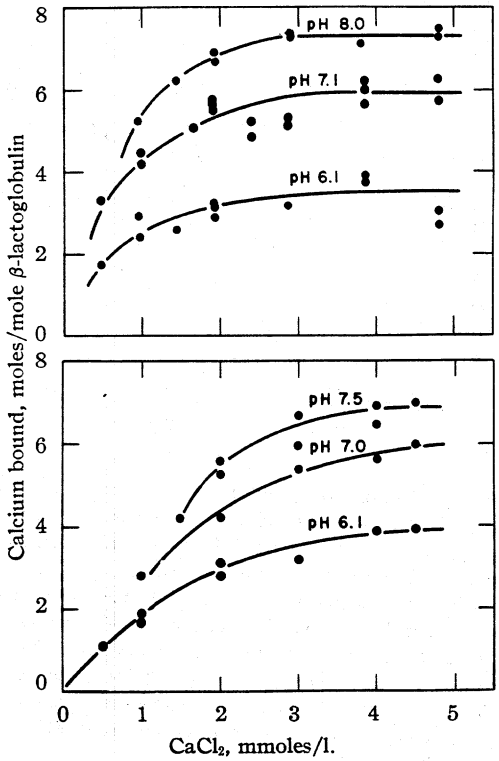

Fig. 3A.—(Top). Calcium bound to β-lactoglobulin (0.9%) in aggregates obtained by heating at 90° for 45 minutes. Fig. 3B.—(Bottom). Calcium bound to β-lactoglobulin (1.8%) in aggregates obtained by heating at 90° for 30 minutes. The aggregated β-lactoglobulin was sedimented at 105,000 × *g* for 45 minutes in the ultracentrifuge. Protein and calcium were determined in the clear supernatant solutions. The initial calcium concentrations are given.

globulin). The binding is stoichiometric since the amount of calcium bound per mole of β-lactoglobulin is the same for both concentrations of protein. The calcium bound when 100% of the β-lactoglobulin was sedimented can be compared with values obtained with other procedures. It was anticipated that the calcium bound to the precipitate in the region of incomplete precipitation would be higher than the value for the total solution because of the distribution of binding mentioned earlier. This was found to be the case and is most apparent at high *p*H and low calcium values with the 1.8% β-lactoglobulin solutions. The calcium bound to the β-lactoglobulin precipitate, calculated in terms of total β-lactoglobulin per liter, exceeds the original concentration of calcium per liter. This apparent paradox indicates that the calcium bound to the β-lactoglobulin still in solution is less than that bound to the precipitate.

The effect of the pH on the binding of calcium to β-lactoglobulin is shown in Fig. 4 for both the unheated and heated solutions. The calcium binding parallels the change in the binding of hydrogen ions, or the net charge in this pH region,[11] which is also shown in Fig. 4. In the same figure the effect of

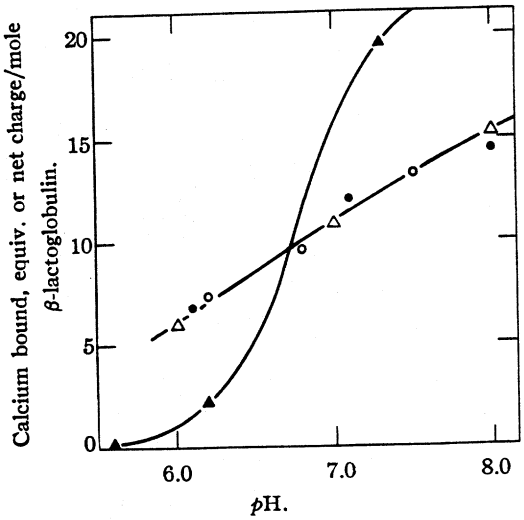

Fig. 4.—Binding of calcium in equivalents to heated and unheated β-lactoglobulin (0.9%) at several pH values with 4 mmoles of free calcium per liter, and net charge on the β-lactoglobulin molecule from titration data: O, calcium bound to unheated β-lactoglobulin; ●, calcium bound to β-lactoglobulin heated with calcium chloride present; △, net charge on the β-lactoglobulin molecule (hydrogen association values, h,[11] at 0.010 μ reduced by 12.5% for molecular weight of 35,000 instead of 40,000). Also shown (▲) are the equivalents of calcium bound to 10^5 g. casein at three pH values obtained by Carr.[15]

pH on the binding of calcium ions to casein, reported by Carr,[15] is shown for comparison.

Although β-lactoglobulin bound the same maximum amount of calcium whether unheated or aggregated by heating, it was possible that the calcium was more firmly bound to the aggregated β-lactoglobulin. Dialysis was used as a means of gaining information on this point. The loss of calcium from cellophane bags containing the protein solutions was determined, and the results are shown in Fig. 5. When the β-lactoglobulin aggregate (stirred) was dialyzed the loss of calcium was slower than from a solution of unheated β-lactoglobulin. To determine whether the calcium was being physically entrapped in the aggregates, they were dissolved in urea containing some mercaptoethanol. These solutions when dialyzed lost calcium at about the same rate as unheated solutions containing the same reagents. Thus, if it is assumed that the non-ionic urea and mercaptoethanol did not change the binding of the ionic calcium to β-lactoglobulin, it can be concluded that the binding to unheated and to heated β-lactoglobulin is equally reversible.

Since the aggregation of β-lactoglobulin heated in the presence of Ca^{++} or H^+ appeared to be analogous, the effect of pH alone on the aggregation

(15) C. W. Carr, *Arch. Biochem. Biophys.*, **46**, 424 (1953).

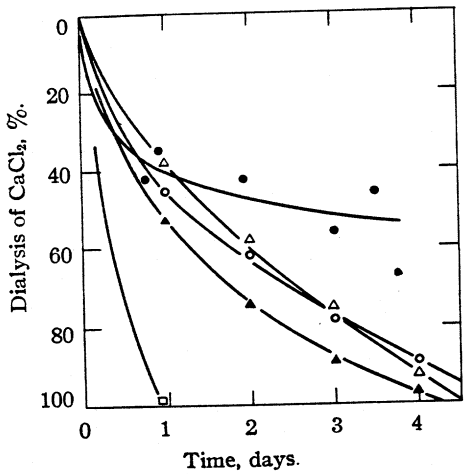

Fig. 5.—Dialysis of calcium from 1.8% β-lactoglobulin solutions under various conditions at 7°: ●———●, suspension of β-lactoglobulin aggregated at 90° for 30 minutes with 4.5 mmoles of $CaCl_2$ per liter present, at pH 7.5; O———O, unheated β-lactoglobulin solution containing 4.5 mmoles of $CaCl_2$ per liter at pH 7.5; △———△, β-lactoglobulin aggregated by heat like above, then dissolved with 8 M urea and 0.4 M mercaptoethanol; ▲———▲, unheated β-lactoglobulin containing 4.5 mmoles of $CaCl_2$ per liter, urea and mercaptoethanol; □———□, calcium chloride (4.5 mmoles per liter) with no β-lactoglobulin present. The dialysis was performed at 7° with a volume of 10.0 ml. in a cellophane sac against occasionally renewed 2-liter portions of distilled water.

was investigated for comparison with the calcium data. The results are shown in Fig. 6 for a pH range of 5.2, the isoelectric point where the β-lactoglobulin is totally aggregated, to pH 6.4 where the aggregation begins to occur. The results are plotted, not against pH, but against the charge per mole of protein obtained from the titration data of Cannan, *et al.*[11] The aggregation, at a constant pH, but with added calcium is shown for comparison with the net charge calculated from the charge at the particular pH less the amount neutralized by the bound calcium.

Discussion

Calcium is bound very strongly to β-lactoglobulin as indicated by the low concentrations needed to give maximal binding. The equilibrium dialysis data with unheated β-lactoglobulin (Table I and Fig. 1), and the data with aggregates obtained by heating (Fig. 3) are in agreement on this point. Two values reported by Carr[15] for β-lactoglobulin are shown in Fig. 2. These values, obtained by a membrane electrode procedure, are lower than the present values although a value at a higher concentration of calcium (20 mmoles/per liter) is identical.[15] The reason for the difference at low calcium concentration is not clear. The present experiments were done with β-lactoglobulin neutralized with sodium hydroxide, whereas Carr used calcium hydroxide. Since Carr has shown[16] that sodium ion is bound to β-lactoglobulin also, the present results would be expected to be somewhat below values obtained with calcium as the sole cat-

(16) C. W. Carr, *ibid.*, **62**, 476 (1956).

ion. A few experiments done at *p*H 6.8 with β-lactoglobulin neutralized with calcium hydroxide, however, gave values that fell within those shown in Fig. 1. The results in Fig. 1 show that the binding values obtained by use of the ultracentrifuge are about the same as those obtained by dialysis. No association constants have been calculated from the equilibrium dialysis data since there are too few results in the region of incomplete binding of calcium to the protein. It is obvious, however, from the shape of the curves, that the dissociation *pK* must be about the same as the value of 3.3 calculated from the ultracentrifuge data.

Unheated β-lactoglobulin, and β-lactoglobulin heated at 90° with calcium ions present both bind the same amount of calcium in the *p*H range of 6 to 8. The first was measured by equilibrium dialysis at 7°, the heated aggregates were centrifuged at 25°; however, the few studies on the temperature coefficient of binding indicate that it is slight.[17] The temperature span from 25 to 90° is large, but either the temperature coefficient is negligible over this large span or a new equilibrium could have been attained at the lower temperature. The close relation between charge and aggregation shown in Fig. 6 suggests that the binding at 90° is probably not very different from that at 25°. Klotz and Urquhart[18] have discussed the effect of denaturation on the binding ability of proteins. Both increases and decreases have been reported. It appeared in general that the change in binding might be explained by chemical changes in the protein molecule like loss of amide groups or transformation of the ε-amino groups of lysine. It is unlikely that such changes have occurred in β-lactoglobulin with the heating conditions (90° for 30 minutes) that were used for aggregation.

The *p*H values were measured both before and after heating the calcium chloride–β-lactoglobulin solutions. At the higher *p*H values there was a small drop in *p*H (7.9–8.1 to 7.3–7.5, and 7.45–7.6 to 7.0–7.3); however, in the region of *p*H 6 a rise in *p*H took place (6.0–6.1 to 6.2–6.6). The initial *p*H values were used in reporting the data since *p*H changes accompanying binding would be fast and would have occurred when the first *p*H measurement was made, and the binding at these *p*H values would determine the amount of aggregation. The change in *p*H from the heating probably reflects denaturation changes.

β-Lactoglobulin that has been previously denatured by heat or alkali does not precipitate as readily with calcium chloride as when solutions of β-lactoglobulin are heated with calcium chloride present. The concentration of calcium chloride at *p*H 7 required to give a maximal precipitation of denatured β-lactoglobulin is about 0.010 *M*. Native β-lactoglobulin is kept in solution by small concentrations of salts even at its isoelectric point, and is not precipitated by calcium chloride (concentrations up to 0.05 *M* were used). Addition of calcium chloride (0.05 *M*) to β-lactoglobulin solutions provides a simple test for the presence of the

(17) I. M. Klotz in "The Proteins," Vol. I, Part B, Academic Press, Inc., New York, N. Y., 1953, p. 777.

(18) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

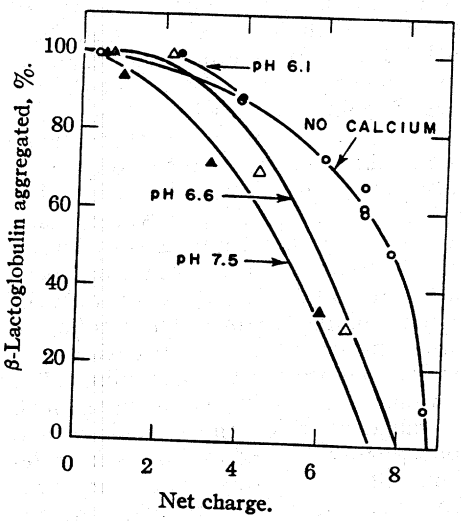

Fig. 6.—Aggregation of 0.9% β-lactoglobulin by heat (90° for 30 minutes), at low *p*H values (5.2 to 6.4) without calcium, and at several constant *p*H values (6.1, 6.6, 7.5) with calcium present, in relation to the net charge on the β-lactoglobulin molecule. The net charge on the β-lactoglobulin without calcium is obtained from titration data.[11] These values are given in Fig. 4 shown earlier. The net charge on the β-lactoglobulin in the calcium-containing solutions is calculated from the charge at the particular *p*H less the amount of charge neutralized by the bound calcium. For example, at *p*H 6.6 the net charge is 9.0 per mole of β-lactoglobulin. In the presence of 1.0 mmole of $CaCl_2$ per liter, in which 70% of the β-lactoglobulin is aggregated when heated, the calcium bound is 2.6 moles per mole of β-lactoglobulin. This value, when multiplied by 2 to give equivalence, is subtracted from 9.0 to give the calculated net charge of 3.8.

denatured form. The calcium ion is bound, making the protein isoelectric, and the denatured form precipitates without the need for a careful adjustment of the *p*H to the isoelectric point. The different effects of calcium on denatured β-lactoglobulin, whether present when heated or added later, might be due to the type of cross bonds formed between the protein molecules. The precipitate obtained by adding calcium chloride to denatured β-lactoglobulin is soluble in urea, indicating hydrogen cross bonds. However, the precipitate obtained with calcium present during the heating can be dissolved with urea only if a reducing agent like mercaptoethanol is added. This suggests that disulfide cross bonds are formed when the isoelectric β-lactoglobulin is heated at a temperature as high as 90° whereas these do not form to the same extent at 25°.

The binding of calcium to β-lactoglobulin parallels the net charge on the protein molecule as illustrated in Fig. 4. This also has been observed for the binding of calcium to bovine serum albumin.[19] This, however, is not observed with all proteins. Carr[15] has investigated the binding of calcium to a number of proteins and has found that the binding varies greatly. For example, the proteins casein, serum albumin and egg albumin with about the same isoelectric point show a wide varia-

(19) C. W. Carr, *Arch. Biochem. Biophys.*, **43**, 147 (1953).

tion in calcium binding (17, 9 and 4 M per 10^5 grams, respectively, with 10 mmoles of free Ca^{++} per liter at pH 7.4). Thus, factors other than net charge also must be important. Study[4] of the viscosity of β-lactoglobulin heated with calcium chloride at various pH values suggested that certain groups ionizing in the pH range of 6 to 7.5, perhaps α-amino or histidine groups, might be involved in calcium binding. These groups, however, as a rule coördinate poorly with calcium, and carboxyl–hydroxyl (serine) chelation with calcium has been suggested[12] as specific binding sites, with net charge an important determining factor. The binding of calcium to casein measured by Carr[15] is shown in Fig. 4 for comparison with β-lactoglobulin. The pH region in which the binding is changing most rapidly is the range in which the secondary acid group of the phosphate esterified to the casein molecule is ionizing. Studies in this Laboratory (unpublished) show that the binding parallels the net charge as well.

The dialysis results in Fig. 5 show that although the calcium in the aggregate is apparently mechanically trapped, this calcium is freely dialyzable when the aggregates are dissolved. The results also illustrate the long periods of dialysis required to free a protein of ions that have an affinity for it.

The calculations shown in Fig. 6 support the conclusion that the aggregation of β-lactoglobulin in the presence of calcium is an isoelectric aggregation. Thus, isoelectric precipitation of β-lactoglobulin solutions can be obtained by lowering the pH to the isoelectric region, or at constant pH by the addition of calcium or any other cation that is bound to it to a similar degree. Actually an even better agreement than shown is likely since electrophoretic studies[20] of β-lactoglobulin in the presence of calcium have shown that the charge is not reduced to the extent expected from the calcium binding. It was surmised that simultaneously with the binding of calcium some other positive ion, perhaps hydrogen ion, might be dissociated from the molecule to some extent. These conclusions suggest that calcium ion, or any other cation that is bound, would be expected to precipitate a negatively charged protein if the protein is insoluble in dilute salt solution at its isoelectric point. Casein is insoluble at its isoelectric point in its native state and it is aggregated or precipitated by calcium ions. Myosin, too, is an example of such a protein and it too is precipitated by cations.[21] The precipitation of proteins by metal ions has recently been discussed.[12] The importance of the net charge was emphasized although with certain metals other factors are important as well.

(20) C. A. Zittle and J. H. Custer, *Arch. Biochem. Biophys.*, in press.
(21) N. K. Sarkar, *Enzymologia*, **14**, 237 (1950).

PHILADELPHIA, PA.