

**Binding of Calcium to Casein: Influence of pH and Calcium
and Phosphate Concentrations**

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INTRODUCTION

Several reports have appeared (1, 2) on the binding of calcium ions to casein. One of these studies (1) covered only the range of calcium concentrations in which the casein is not visibly aggregated. It was desirable to amplify the binding studies in higher concentrations of calcium in which the casein is aggregated since the aggregation is time-dependent (3) and the effect of time had not previously been considered. Further, the aggregated casein is of additional interest because the casein occurs in milk in an aggregated form. The casein in milk occurs as a calcium caseinate-calcium phosphate complex, and for this reason, the influence of phosphate concentration on calcium binding has also been studied. The present studies include the effect of pH on the binding of calcium [reports on the influence of pH are contradictory (1, 2, 4)] as well as the effect of the binding on the isoelectric point (minimum solubility).

MATERIALS AND METHODS

Casein. The purification of the casein and the preparation of solutions has been described (3). For most of the experiments a stock 4% sodium caseinate solution, pH 6.5, was used, and final adjustment of the pH was made, after dilution and addition of reagents, with 0.1 *N* NaOH or HCl.

Analyses. Casein was determined from the ultraviolet absorption at 280 $m\mu$ (3). Calcium was determined by titration with Versene with Eriochrome Black T as the indicator (5). Casein in the concentrations used did not interfere. Chloride was determined by titration with mercuric nitrate with diphenylcarbazone as the

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indicator (5). Phosphorus was estimated as the blue phosphomolybdate complex with ferrous sulfate as the reducing agent.

Heating of Casein Solutions. The solutions were heated for 30 min. at 90° as described previously (3).

Determination of the Calcium Bound to Casein. (a) The casein is aggregated in the presence of calcium chloride. The aggregate is readily sedimented in a preparative ultracentrifuge in 45 min. at $105,000 \times g$. The clear supernatant solutions are analyzed for calcium and the binding determined by difference (5).

(b) The binding was also determined by equilibrium dialysis in order to have two methods for comparison, and this procedure could also be used for low calcium concentrations where little or none of the casein was aggregated. The use of this procedure, and correction for Donnan distribution from chloride analyses have been described (5).

RESULTS

The extent to which casein is aggregated in the presence of calcium chloride is shown in Fig. 1. Casein is composed of α , β , and γ components [75:22:3 (6)], but they are mutually aggregated in the presence of calcium ion, and at pH 5.0–6.0 almost all of the protein is obtained in the ultracentrifuge sediment. At pH 7.4, although only 82% of the protein is aggregated, electrophoretic examination by the Tiselius technique of the unaggregated portion at pH 8.6 showed that both α - and β -caseins are still present and the ratio is 69:31, almost that of the starting material.

The addition of phosphate (5 mmoles/l.) to the calcium–casein systems has little influence on the amount of sediment obtained with the ultracentrifuge. Only at pH's 7.4 and 8.5 with the higher calcium concentrations (10–15 mmoles/l.) is there a small increase in the amount of casein sedimented when phosphate is present.

In the above experiments, the calcium chloride–casein mixtures were at 25° for 2 hr. before centrifuging. In a series of experiments, the time before centrifuging was varied from 10 min. to 4 hr. Measurement of the calcium bound in the casein solutions permitted to aggregate for different time intervals showed that the amount bound was the same in each. Although the turbidity increased with time, as was observed previously (3), this was not reflected in an increase in the amount of sediment obtained in the ultracentrifuge. The increases in turbidity must be due to increases in amount (probably both size and number of particles) of aggregated casein, but apparently the centrifuging conditions (45 min. at $105,000 \times g$) sediment a range of particle sizes which prevents a time effect from being observed. Under certain conditions

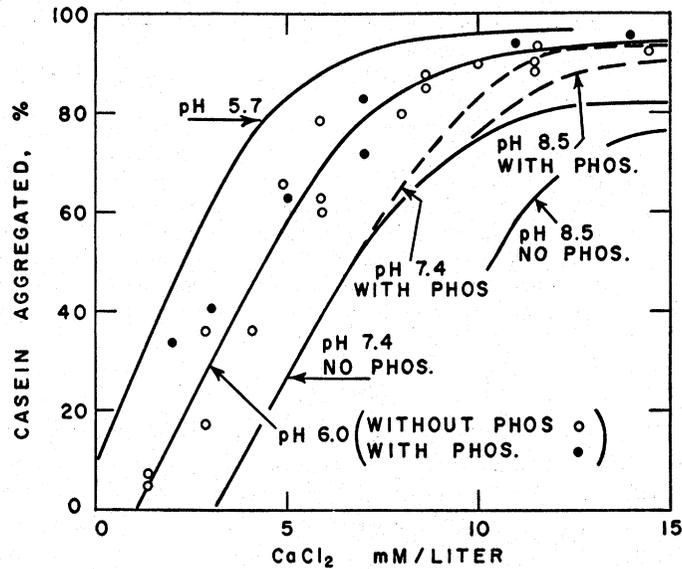


FIG. 1. Aggregation of casein (1.0%) by calcium chloride at pH 5.7-8.5. The solutions were held at 25° for 2 hr., and the aggregated casein was sedimented in the ultracentrifuge at 105,000 $\times g$ for 45 min. Also shown are results with similar mixtures containing 5 mmoles phosphate/l. Individual experimental data are shown only for pH 6.0 (without phosphate: \circ — \circ ; with phosphate: \bullet — \bullet). The number of experiments and range of the data at other pH values were about the same.

(low calcium, high pH, presence of NaCl) (unpublished studies) a time lag in the appearance of turbidity is observed. This probably means that intermediate aggregates are formed before the appearance of aggregates large enough to make the solution turbid. Sodium caseinate is not sedimented by the ultracentrifuge conditions used.

Since previous studies (3) had showed that the insoluble precipitates obtained at 90° in large part redissolved at lower temperature (25°), some binding measurements were made at different times after precipitation at 90°. The binding was measured immediately after cooling to 30°, and also after 1 hr. at 30°. The amount of precipitate was somewhat greater than obtained previously, because of the lower centrifuge speeds that had been used (3).

Typical experimental data follow: A 1% casein solution at pH 6.5, containing 10 mmoles CaCl₂/l., was divided into three portions. One portion

was held at 30° for 2 hr.; another at 90° for 30 min. then at 30° for 2 hr.; the third at 90° for 30 min. then at 30° for 2 min. All were ultra-centrifuged in the usual way. The respective amounts of aggregate were 75.8, 76.4, and 91.0%; the calcium bound was 34.5, 32.2, and 32.1 moles calcium/10⁵ g. casein. In a similar experiment with only 5 mmoles CaCl₂/l., the respective amounts of precipitate were 30.6, 38.0, and 48.6%; the calcium bound 32.8, 28.5, and 28.0 moles calcium/10⁵ g. casein. The 90° aggregates redissolved at 30°, but the binding per unit weight remained constant. The data suggest that the solutions not heated to 90° bind more calcium than those heated, but the difference is close to the variations between replicates.

The results in Fig. 2 show that the binding of calcium to casein is sharply influenced by pH. The course of the calcium binding at various pH values is shown in Fig. 3 for a free calcium concentration of 10 mmoles/l. Binding with 5 mmoles free calcium/l. follows the same course, and at pH 7.4 is 13% less; at lower pH values the differences are smaller. Equilibrium dialysis experiments (see later) gave the same change of binding with pH. Figure 3 shows also the way in which the net charge on the casein, obtained from titration data (7), changes, as

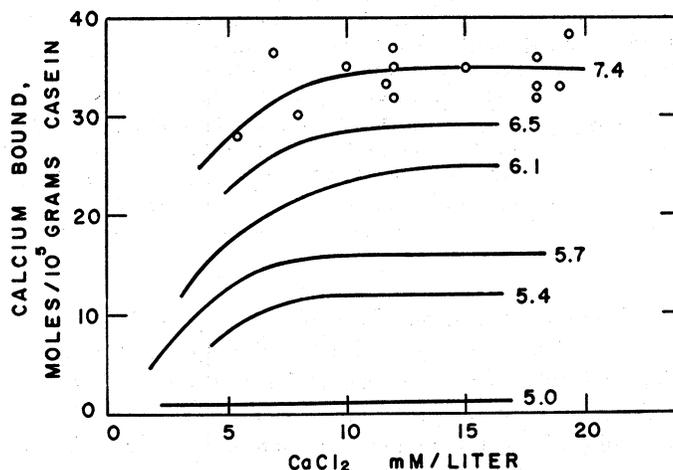


FIG. 2. Calcium bound to casein (1.0%) at several pH values and concentrations of calcium chloride, determined by analysis of the solutions from which the casein was sedimented (Fig. 1). Individual experimental data are shown only for pH 7.4. The number of experiments and range of the data at other pH values were about the same.

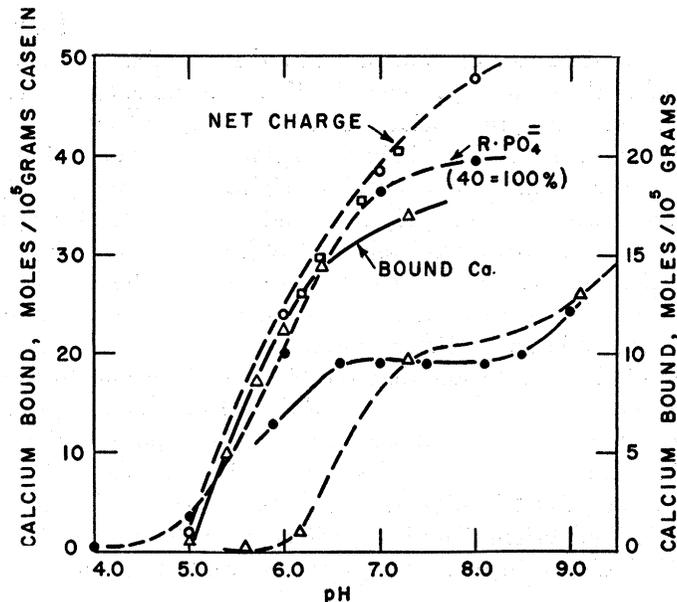


FIG. 3. Calcium bound to casein (1.0%) at pH 5-7.4 at a free calcium concentration of 10 mmoles/l. (Δ — Δ). Also shown is the net negative charge (\circ — \circ), divided by 2 for comparison with the bivalent calcium bound. These data are from the titration results of Hipp *et al.* (7) for α - and β -casein, and are calculated for a 75:22 mixture. The ionization of a substituted phosphoric acid (assumed pK of 6.0) is also shown (\bullet — \bullet) with 40 on the left-hand ordinate equal to 100% ionization. Also shown are the binding data of Ramsdell and Whittier (4) (\square). Included on the same graph (right-hand ordinate) are the binding of calcium at several pH values reported by Chanutin *et al.* (1) (4.1% casein, 5.6 mmoles CaCl_2 /l.) (\bullet — \bullet), and by Carr (2) (0.8% casein, 2.97 mmoles CaCl_2 /l.) (Δ — Δ).

well as the degree of ionization of a substituted phosphoric acid in this pH range. Both of these coincide with the binding curve up to pH 6.5.

Ramsdell and Whittier (4) determined the calcium bound to casein at several pH values by analyzing the sediments obtained with an ultracentrifuge. Points from their curve are included in Fig. 3 for comparison with the present data. Also shown are the results obtained by Chanutin *et al.* (1) and by Carr (2). Since their results are for different concentrations of casein and calcium, the scale is changed for a better comparison of the pH effect.

The binding values were also determined by equilibrium dialysis since this procedure can be done at lower concentrations of calcium

TABLE I

Equilibrium Dialysis Determination of Calcium Bound to Whole Casein (1% Concentration) at pH's 5.5 and 7.0 with Several Concentrations of Calcium

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

Calcium concentration initial	pH after dialysis	Calcium concentration after dialysis		Chloride concentration after dialysis		Calcium bound to casein	
		Inside sac (protein)	Outside	Inside	Outside	Uncorrected	Corrected ^a
<i>mmoles/l.</i>		<i>mmoles/l.</i>	<i>mmoles/l.</i>	<i>mmoles/l.</i>	<i>mmoles/l.</i>	<i>m/10⁵ g.</i>	<i>m/10⁵ g.</i>
10.0	5.53	11.0	9.0	21.5	22.0	20.0	15.5
5.0	5.55	5.88	4.65	12.1	12.0	12.3	12.3
2.5	5.60	3.20	2.10	7.5	7.4	11.0	11.0
7.5	6.95	8.95	5.75	14.1	14.9	32.0	25.3
5.0	6.92	6.34	3.40	9.5	10.2	29.4	24.6
2.5	6.88	3.80	1.18	4.93	5.70	26.2	22.5

^a Corrected for Donnan distribution of diffusible ions. Free calcium inside is calculated with the equation: $Ca_i = Ca_o (Cl_o)^2 / (Cl_i)^2$.

than in the aggregation experiments. Concentrations of 2.5–10 mmoles calcium chloride/l. were studied at pH values of 5.5 and 7.0. The results are illustrated with typical data in Table I, and given in full in Fig. 4. The calcium binding values at 5 mmoles free calcium for the respective

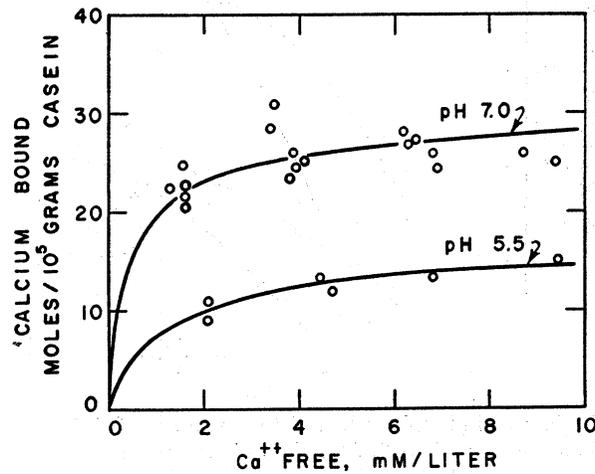


FIG. 4. Calcium bound to casein, determined by equilibrium dialysis at several pH values and concentrations of $CaCl_2$.

pH values were 13 and 26, in good agreement with the values obtained by sedimentation.

The association constant for the binding was determined from a plot (8) of the ratio of calcium bound/ 10^5 g. casein to the free calcium (r/A), against the calcium bound (r). 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. The equilibrium dialysis data at pH 7.0 handled in this way give an association constant of 2.2×10^8 , equivalent to a dissociation constant of 4.5×10^{-4} , or a pK of 3.34. This pK value is larger than those reported in the literature (1, 2), all of which were obtained with commercial samples of casein. Chanutin *et al.* (1) have obtained pK values of 2.39 and 2.73 with different preparations of casein. Carr (2) has recently reported a pK value of 2.66. Carr has generously determined the binding of calcium to the casein used in the present study with his membrane electrode technique (9). At pH 7.5 with free calcium concentrations of 3.2, 2.5, and 1.7 mmoles/l., the respective amounts of calcium bound were 32

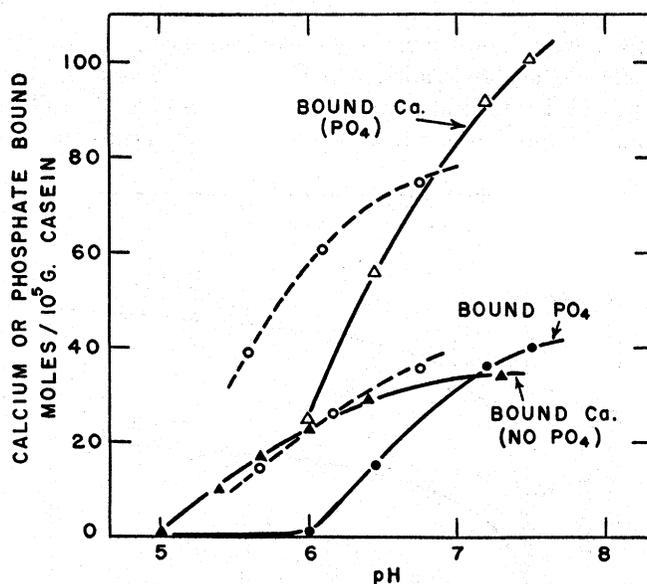


FIG. 5. Calcium and phosphate bound to casein at pH 5-7.5 with 14 mmoles CaCl_2 and 5 mmoles phosphate/l. Also shown is the calcium (upper dashed line) and phosphate (lower dashed line) in the natural casein complex in milk at several pH values reported by DeKadt and Van Minnen (10).

30, and 31 moles/ 10^5 g. casein. These values are in essential agreement with present values by sedimentation and equilibrium dialysis. The value of n , the maximum number of moles of calcium bound/ 10^5 g. casein obtained from the present studies is 29; the results of others give values of 48 (1) and 22 (2), all determined at pH 7.0–7.4.

The binding of calcium to casein was also studied with phosphate present. The aggregated casein was sedimented in the ultracentrifuge, and protein, calcium, and phosphorus were determined in the clear supernatant solutions. The effect of phosphate (5 mmoles/l.) on the amount of protein aggregated is shown in Fig. 1 at various concentrations of calcium and at several pH values. The amount of calcium and phosphate bound in the aggregate was determined, by difference, from the analytical data. The amount of these ions bound/ 10^5 g. casein is shown

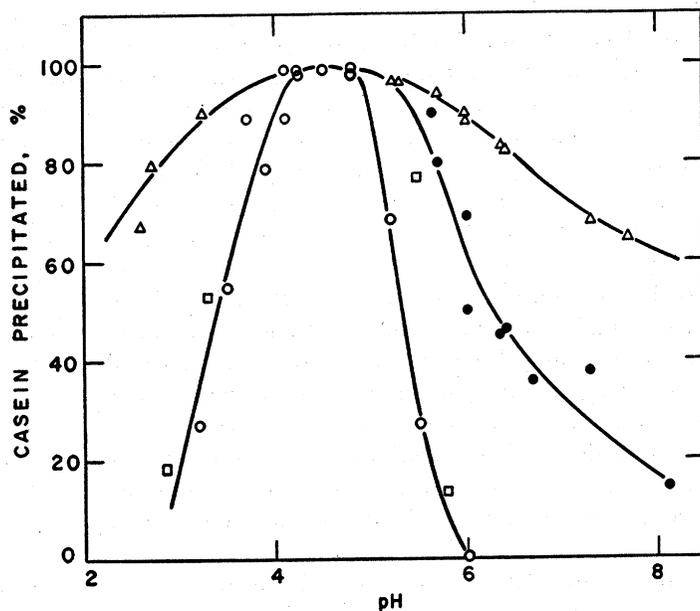


FIG. 6. Precipitation of casein from pH 2 to 8 without calcium (○, □) and with 5 (●) and 10 (Δ) mmoles CaCl_2 /l. For the experiments with CaCl_2 the mixtures stood for 2 hr. at 25° , then were ultracentrifuged at $105,000 \times g$ for 45 min. A few experiments without calcium (□) sedimented in the same way gave about the same results as experiments that stood only 30 min. and were centrifuged at $3000 \times g$ (○). Solutions containing 10 mmoles CaCl_2 /l. were also centrifuged at $3000 \times g$; a stable, opalescent colloid present in such solutions was not sedimented, and the precipitation curve is about that shown by the calcium-free solutions.

in Fig. 5 for a concentration of 14 mmoles calcium chloride/l. In the same figure is shown for comparison the calcium and phosphate composition of the natural calcium caseinate-calcium phosphate complex in milk at several pH values reported by DeKadt and Van Minnen (10).

Since calcium is bound to casein, it was of interest to determine whether the isoelectric point (minimum solubility) was raised by the concomitant reduction in charge. Casein was sedimented at various pH values, without calcium, and with 5 and 10 mmoles calcium chloride/l. The results are shown in Fig. 6.

DISCUSSION

A shift in the isoelectric point of casein in the presence of calcium chloride was expected because of the binding of the charged calcium ion. This has been observed in electrophoretic studies of β -lactoglobulin (1). Also, electrophoretic study (unpublished) of α -casein in Veronal buffer at pH 8.5, 0.1 μ has shown that whereas the mobility without calcium is 6.2×10^{-5} sq. cm./v./sec., in the presence of 5 mmoles CaCl_2 /l. at the same ionic strength the mobility decreases to 4.7 units. Our centrifugation studies show, however, that the isoelectric point (minimum solubility) is not shifted in the presence of calcium ions, but the region of aggregation is greatly broadened, particularly on the alkaline side where the casein is negatively charged. The explanation seems to lie in the results of the binding studies which show that no calcium is bound to casein at the isoelectric point, hence no change in the isoelectric point will occur. The binding at higher pH values leads to the expected reduction in charge with an increased tendency to aggregate. Michaelis and Szent-Györgyi observed (12) that calcium chloride at moderate concentrations did not shift the isoelectric point, and actually at high concentrations of calcium chloride a shift of the isoelectric point to more acid values was observed suggesting that chloride ion might be bound.

The effect of pH on the binding of calcium to casein observed in the present studies is somewhat different from previous reports (1, 2). Carr (2) reported that binding decreased to zero at pH 6.0; Chanutin *et al.* (1) observed binding at pH 6, but the slope of the binding curve was not very steep. These studies were with commercial samples of casein, but there is no explanation for these divergent results. The binding values of Ramsdell and Whittier (4) confirm the present studies at pH 6.2, but the pH-binding curve is somewhat steeper and at pH 7.4

their binding values are about 20% higher. The parallel between binding and net negative charge suggests that a favorable negative charge is an important factor in the binding of calcium to casein. The binding to β -lactoglobulin shows a similar parallel between binding and the net negative charge (5). The calcium may be bound, however, at specific sites. The ionization of substituted phosphoric acid in the pH region where the binding is increasing suggests that the phosphate in casein could provide the sites since it is present in the requisite amounts. The studies on the binding of phosphate within the calcium caseinate complex indicate that only the bivalent form is bound. This form might be required for binding calcium. Recent studies by Perlmann (13), however, indicate that a considerable part of the phosphate in casein is doubly bound; hence there would not be sufficient bivalent phosphate for it to be the only specific binding site.

No phosphate is bound to calcium caseinate up to pH 6 (Fig. 5). Above this pH, phosphate is bound and simultaneously there is a large increase in the amount of calcium bound. This is the pH region in which the phosphate becomes bivalent, and apparently this form must be present for complex formation to occur. The molar ratio of additional calcium bound to the phosphate is 1.5, the ratio in tricalcium phosphate. Thus, in the system of purified casein the calcium phosphate is bound as in the natural complex (4). DeKadt and Van Minnen (10) studied the influence of pH on the composition of the natural complex in milk and obtained the results shown in Fig. 5. The decreases in binding with decrease in pH are about the same as observed with purified casein, but the changes occur at lower pH values. A possible explanation for this is that milk contains much more salt than the purified system and, in the presence of salt, phosphate behaves like a considerably stronger acid. The total results emphasize the importance of pH in the formation of the calcium caseinate-calcium phosphate complex, an effect which probably arises principally from the requirement that the phosphate be bivalent for complex formation to occur.

The increase in the amount of aggregated casein in the presence of phosphate when the aggregates form at 25° is in contrast to similar systems heated at 90° (14). In the latter, the amount of casein precipitated is reduced by phosphate. The heat apparently dissociates the casein complex, forming the inert tricalcium phosphate which removes calcium from the system thereby reducing the amount of casein precipitated. The extra precipitate at 25°, on the other hand, may

result from extra cross links mediated by phosphate radicals between the protein-bound calcium ions.

The close agreement between the binding to the aggregates and the binding to the total solution by equilibrium dialysis suggests that the binding to the soluble and insoluble casein phases is the same. In contrast, with β -lactoglobulin heated in the presence of calcium ions (5), more calcium is bound to the precipitate than to the β -lactoglobulin in solution. The manner in which calcium ions contribute to the β -lactoglobulin denaturation probably accounts for the difference (5).

The association constant for calcium binding to casein is approximately the same as that for β -lactoglobulin (5). The total amount of calcium bound to casein is considerably greater, in parallel with the difference in the net negative charge of these two proteins (5, 7).

The aggregation of casein by calcium can be viewed, as in the case of β -lactoglobulin (5), as resulting from a reduction in the net negative charge by the binding of the positively charged calcium ions. This reduction in charge leads to the equivalent of isoelectric aggregation. Thus, the primary effect is the binding of calcium, and the observed aggregation results from secondary effects of cross bonding, changes in hydration, or other changes leading to a reduction in solubility. From this standpoint, the constancy of calcium binding through various periods of aggregation or during the reversal of aggregation following heating is understandable since only the secondary phenomena are changing.

SUMMARY

The binding of calcium to casein has been determined, both by analysis of the aggregated casein and by equilibrium dialysis. Values for maximum amount of calcium bound and dissociation pK values are reported for pH 7.0 and compared with values in the literature. Calcium is not bound to casein at pH 5, but binding does occur and increases as the pH is raised. The increase in binding parallels the increase in net negative charge of the casein, as well as the degree of ionization of a substituted phosphoric acid up to about pH 7. The time that the calcium caseinate aggregates are permitted to form does not affect the calcium binding. Aggregates formed at higher temperature in part redissolve at lower temperatures, but the binding of calcium per unit weight of aggregate is unchanged. In casein systems containing both calcium and phosphate, binding of the latter occurs only above pH 6,

and simultaneously the binding of calcium increases greatly. The isoelectric point (maximum precipitation) of casein is not changed in the presence of calcium chloride.

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