

EFFECT OF CERTAIN SALTS ON PRECIPITATION OF CASEIN BY CALCIUM CHLORIDE AND HEAT

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Precipitation of casein by calcium chloride and heat and the subsequent re-resolution of the precipitate at lower temperature has been reported (13). The possible relationship of this phenomenon to the drop in viscosity of evaporated milk in storage was discussed (13). To relate the observations more directly to the heating of milk, the effects of sodium chloride, citrate, and phosphate on this precipitation reaction have been studied, because of the presence of these salts in milk and also because of the use of the latter two as milk stabilizers.

MATERIALS AND METHODS

Casein was prepared and the solutions were made as described previously (13). The pH values were adjusted after the salts or other reagents were added.

Heating of the samples, centrifuging, sampling, and determination of the casein by light absorption at 280 m μ have been described (13). Viscosity was determined in a Bingham type viscometer. Details of the measurement have been reported (12). Calcium was determined by the murexide (ammonium purpurate) method (11); phosphate in the concentrations used in these experiments did not interfere. Phosphorus was estimated with the ammonium molybdate reagent after reduction with ferrous sulfate.

RESULTS

Sodium chloride. The ability of increasing quantities of sodium chloride to reduce the precipitation of calcium and sodium caseinates by calcium chloride and heat is shown (Figures 1 and 1A). For the calcium caseinate experiments, calcium chloride was added to 2% calcium caseinate to give the total calcium

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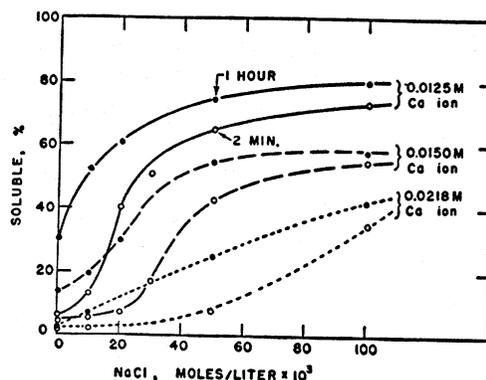


FIG. 1. Precipitation of 2% calcium caseinate heated at 90° C. for one hour in the presence of sodium chloride. The experiments were done at pH 6.5 with indicated concentrations (total) of calcium. Casein in solution at 25° C. two minutes (O O) and one hour (● ●) after heating.

concentrations indicated in Figure 1 (0.0068 *M* calcium being contributed by the calcium caseinate). For the sodium caseinate experiments, corresponding concentrations of calcium were provided wholly by the calcium chloride added, while corresponding total sodium concentrations were achieved by the sodium chloride and the 0.012 *M* sodium already present in the 2% sodium caseinate solutions. In all cases, duplicate 4.0-ml. samples at pH 6.5 were heated for one hour at 90° C., after which they were centrifuged at 25° C., one two minutes after heating and the other one hour after heating.

It is evident that sodium chloride kept the caseinates in solution; that is, it lessened the precipitation of casein when the solutions were heated. Extent of the solubilization was determined by the total sodium and total calcium and was less marked with the higher concentration of calcium. It had previously been assumed (13) that sodium caseinate-calcium chloride is approximately

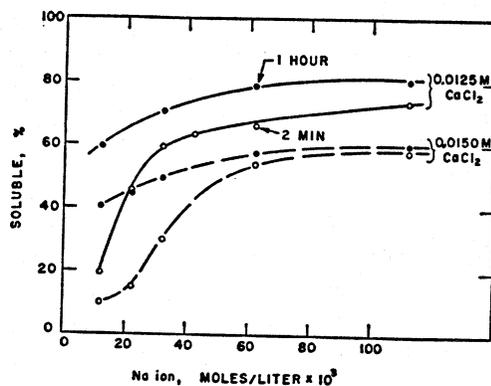


FIG. 1A. Precipitation of 2% sodium caseinate heated at 90° C. for one hour in the presence of sodium chloride. The experiments were done at pH 6.5 with indicated concentrations of calcium chloride. Curves show casein in solution at 25° C. two minutes (O O) and 1 hour (● ●) after heating.

equivalent to calcium caseinate if the effect of the sodium ion is considered. The data (Figures 1 and 1A) confirm this equivalence for calcium precipitation in the presence of sodium ion. The curves for both caseinates are almost identical for 0.0125 *M* calcium ion, and the small deviation at 0.015 *M* calcium ion is probably within the accuracy of the method. Re-solution of the precipitate at 25° C., indicated by the higher level of the "one-hour" curves as compared to the "two-minute" curves, took place, although modified quantitatively by the sodium chloride.

Sodium citrate. Effect of sodium citrate on the precipitation of 2% sodium caseinate containing 0.0125 *M* calcium chloride, pH 6.6, is shown (Figure 2). This compound was very effective at low concentrations in preventing the precipitation of casein by calcium chloride and heat. This would be expected of a compound like citrate, which has a strong binding affinity for calcium ions (5).

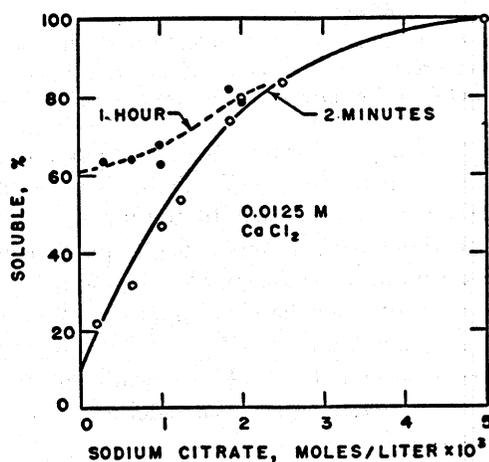


FIG. 2. Precipitation of 2% sodium caseinate containing 0.0125 *M* calcium chloride at pH 6.6 heated at 90° C. for one hour in the presence of sodium citrate. Casein in solution at 25° C. two minutes (O O) and one hour (● ●) after heating.

Sodium phosphate. Effect of sodium phosphate, (a mixture of mono- and di-sodium salts to give the designated pH values), on the precipitation of 2% sodium caseinate containing 0.0125 *M* calcium chloride is shown (Figure 3). Results are given for both pH 6.1 and 6.6. With sodium phosphate present, a re-solution of the casein precipitates was not obtained; hence, only one curve is shown at each pH value. The sodium chloride and citrate curves ("two minutes") are added for comparison, to show the relative effectiveness of each compound in preventing casein precipitation.

At pH 6.1 the phosphate was much less effective in preventing casein precipitation than it was at pH 6.6. At the higher pH, the solution contained additional sodium ion, added as NaOH, but that this was not responsible for the difference was shown when the same amount of sodium ion (0.0043 *M*) was added as NaCl at pH 6.1 without any change in precipitation. Other possible

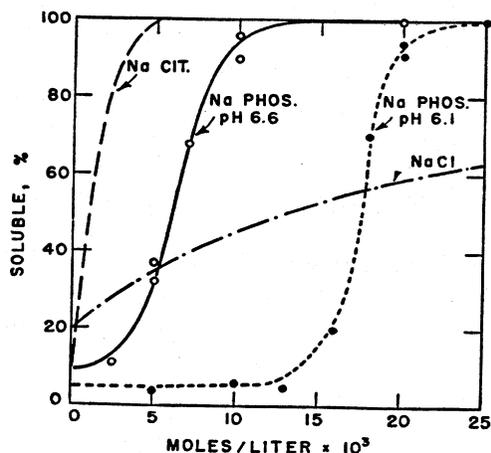


FIG. 3. Precipitation of 2% sodium caseinate containing 0.0125 *M* calcium chloride heated at 90° C. for one hour in the presence of sodium phosphate at pH 6.6 and 6.1. The "two-minute" curves for sodium chloride and citrate at pH 6.6 are also shown for comparison.

explanations are the greater tendency of casein to precipitate, or the lesser ability of phosphate to bind calcium, at the lower pH. To investigate this further, varying amounts of calcium chloride were added to sodium caseinate samples containing no phosphate and to those with a constant phosphate concentration of 0.005 *M*, and their solubilities were measured at pH 6.2, 6.6, and 7.4. As shown in Figure 4, phosphate at this concentration had no apparent effect on the precipitation of calcium caseinate at pH 6.2, but at pH 6.6 it decreased the precipitation considerably, and markedly so at pH 7.4. These results indicate that, as might be expected, phosphate has a greater affinity for calcium at the higher pH values of the limited pH range studied.

When phosphate was added to calcium-containing solutions in a concentration sufficient to prevent the precipitation of casein, the solutions were quite clear, although a small amount of sediment was apparent after centrifugation. It was of interest to know the composition of the sediment in calcium and phosphorus,

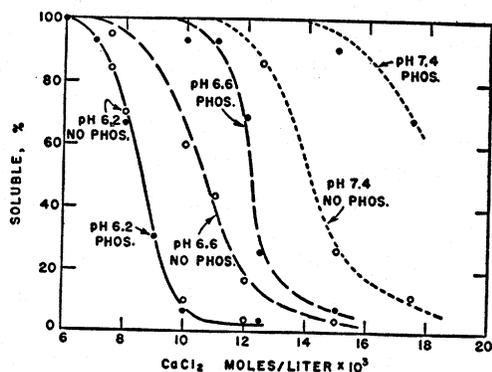


FIG. 4. Precipitation of 2% sodium caseinate with calcium chloride and heat for one hour at 90° C., without (O O) and with (● ●) 0.005 *M* sodium phosphate, at pH 6.2, 6.6, and 7.4.

and to determine whether the amount of a calcium phosphate formed was sufficient to account for the prevention of casein precipitation. In these experiments, the sample volumes were 4.0 ml., and the concentration of calcium chloride was 0.0125 *M* (calcium in 4.0 ml. = 2.00 mg.). Experiments were conducted at pH 6.1 and 6.6 with several concentrations of sodium phosphate ranging from 0.0110 *M* (P in 4.0 ml. = 1.36 mg.) to 0.0318 *M* (P = 3.94 mg.). Although at pH 6.1, and with the lower concentrations of phosphate at pH 6.6, some casein was sedimented, the casein was colloidal and could be decanted to leave the dense sediment of calcium phosphate for analysis. Results (Figure 5) are given in milligrams

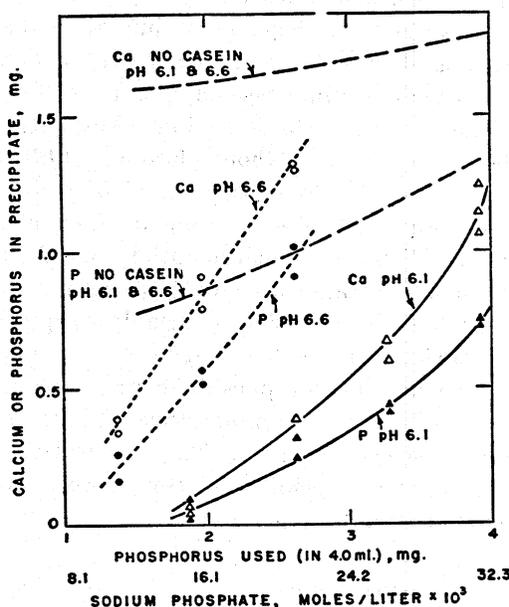


FIG. 5. Calcium and phosphorus precipitated when precipitation of casein by 0.0125 *M* calcium chloride and heat at 90° C. for one hour is prevented by excess of sodium phosphate. Also shown are curves for the precipitation of calcium phosphate (Ca and P) when no casein is present.

of Ca or P in the calcium phosphate precipitate, in relation to the milligrams of phosphate present. Each point represents a separate experiment.

Curves are also shown (Figure 5) for the precipitation of Ca and P from similar solutions when no casein is present. With these solutions the pH adjustment with NaOH was continued until the pH ceased to drop. The drop in pH on adding alkali to calcium phosphate solutions in this pH range is due to the transformation of dicalcium to tricalcium and the soluble acidic monocalcium phosphates (10). Even though the pH was thus carefully adjusted, the pH dropped almost one unit when the solutions were heated. This drop in pH did not occur when casein was present.

From Figure 5 can also be calculated, by subtraction from the amount originally present in the sample, the percentage of Ca and P in the supernatant fluid.

These calculations show that the formation of insoluble calcium phosphate at pH 6.6 decreased the calcium concentration 19 to 67%, depending on the phosphate concentration. It is apparent that such a formation is sufficient to account for the nonprecipitation of the casein. Other aspects of this reaction are dealt with in the discussion.

The ability of sodium phosphate to prevent the re-solution of calcium caseinate precipitates was investigated in some detail. It was found that as little as 0.00063 *M* sodium phosphate in a 2% casein solution at pH 6.6 containing 0.0125 *M* calcium chloride prevented the re-solution of the precipitate obtained by heating. Without the phosphate, when samples were heated for one hour at 90° C., the casein remaining in solution was about 12%; but after they stood for another hour at 25° C., it increased to 60% (13). Changes in viscosity obtained on the addition of calcium chloride to sodium caseinate, and the effect of heat on viscosity (13) were also explored with 0.005 *M* sodium phosphate present. In general, the results were about the same as without phosphate (13) up to a concentration of 0.010 *M* calcium chloride. That is, the viscosity dropped as the calcium concentration was increased and there was some decrease in the viscosity when the solutions were heated. With higher concentrations of calcium, however, the viscosity increased, presumably because a colloidal precipitate was formed. When these solutions were heated, the viscosity invariably dropped. Also, the calcium-casein precipitates appeared to be more dense when phosphate was present. In every instance, the presence of phosphate prevented the viscosity increase on heating, and the reversal at lower temperature that was obtained with sodium caseinate-calcium chloride systems (13). The observations were made as long as 18 hours after heating. The possibility of slow re-solution of the precipitates can not be excluded at present.

DISCUSSION

The solubilizing effect of sodium chloride on calcium caseinate precipitates is probably due to a reduction in the activity of the calcium ion, and in part to the displacement of the calcium ion by the sodium ion. Van Slyke and Bosworth (9) observed that acid caseinates insoluble in water were readily soluble in 5% sodium chloride. They showed by dialysis that an exchange of bases had occurred. Chanutin, Ludewig, and Masket (3) have reported that the affinity of calcium ion for casein in unheated solutions was decreased by the presence of sodium chloride, and Carr and Topol (2) have observed that sodium ions are bound to casein. It also has been reported (4a) that the number of micelles in skimmilk is decreased by the addition of sodium chloride, presumably by dissociation of calcium-casein complexes and formation of nonmicellar sodium caseinate.

The solubilizing effect of sodium citrate on calcium caseinate is expected, because of its strong affinity for calcium ion. According to Hastings *et al.* (5) the association constant, *K*, for the formation of calcium citrate, expressed by the ratio of the equilibrium concentrations $[Ca^{++}] [Citrate=] / [CaCitrate]$,

is 6.0×10^{-4} (pK of 3.22). Chanutin, Ludewig, and Masket (3) have investigated the influence of citrate on unheated calcium-casein solutions and found that the reduction in the amount of calcium bound to casein conformed to this association constant. On the basis of this K value, with a concentration of 0.0125 M total calcium and for the formation of 0.005 M calcium citrate, only 0.0004 M free citrate would be present at equilibrium. Reduction of the calcium concentration by the amount bound to citrate, together with the sodium ion effect, is sufficient to prevent the precipitation of the casein completely. Sommer and Hart (6) have reported qualitative tests showing the solubilizing effect of citrate on calcium citrate precipitates.

The ability of sodium phosphate to prevent the precipitation of calcium caseinate was observed by Sommer and Hart (6). Their qualitative studies suggested that the results were independent of heating. In the present studies, the extent of precipitation was greatly increased by heating, and all the results reported are for heated casein solutions. The magnitude of the effect of heat is shown by specific examples; solutions used for the data in Figure 4 containing 15 and 12.5×10^{-3} M calcium chloride per liter, together with phosphate, at pH 6.6, were completely soluble before heating. After heating to 90° C. for one hour the solubility had decreased to 7% and 26%, respectively.

It was of interest to know whether excess phosphate prevented the precipitation of casein by calcium by forming a soluble complex with the calcium caseinate, or whether the solubilization occurred because of the removal of calcium by formation of insoluble calcium phosphate. It is apparent from the data that the latter provides the explanation. The increased effectiveness of sodium phosphate at higher pH values in solubilizing calcium-caseinate precipitates lies in the formation of additional insoluble calcium phosphate; however, the reason more is formed at pH 6.6 than at pH 6.1 is not apparent. It was surmised that this difference reflected the greater affinity of calcium and phosphate at higher pH values, as shown by the greater tendency to form insoluble calcium phosphates. Experiments with calcium and phosphate without casein present indicate that this is probably not the explanation, with the small difference in pH involved. Also, the greater formation of a calcium phosphate at pH 6.6 can not be governed by the binding of calcium to the casein, for this binding is less at lower pH values (1, 3).

Composition of the calcium phosphate sediment is of interest; at pH 6.6 the molar ratio of Ca:P in the sediment from the low phosphate solution is 1.46, corresponding to tricalcium phosphate, decreasing to 1.08 from the high phosphate solution, corresponding to dicalcium phosphate. Titration studies (10) indicate that dicalcium phosphate is unstable, having a tendency to form tricalcium phosphate and the soluble monocalcium phosphate. The excess of phosphate may, however, stabilize the dicalcium phosphate, or alternately the excess phosphate might be adsorbed to the tricalcium phosphate (7). The molar ratio of the Ca to PO_4 remaining in solution varies from 1.08 in the low phosphate solution to 0.3 in the high phosphate solution. This ratio probably has little relation to

complex formation with casein because of the presence of free phosphate. The influence of the phosphate concentration on the composition of the sediment, however, suggests that a variable phosphate concentration in milk might equally well cause a variation in the composition of the calcium phosphate associated with casein. This might account for the variation in the composition of the casein complex from individual milk samples (8). A reduction in the Ca:P ratio in the casein complex of milk has been obtained in the presence of added phosphate (4).

There appears to be general agreement (4a) that in the casein complex of milk the tricalcium phosphate is bound to the calcium caseinate by chemical bonds and not by physical forces such as adsorption. In the case of destabilization of this casein complex by heat, however, it has been suggested that the calcium caseinate coprecipitates or is adsorbed to the precipitate of tricalcium phosphate. Also, it has been suggested that whereas calcium caseinate is most unstable in acid solution, in the presence of tricalcium phosphate it would be more unstable at higher pH values, where there is a greater tendency of tricalcium phosphate to precipitate. The present studies, however, indicate that little or no calcium caseinate coprecipitates or is adsorbed to the calcium phosphate. It has also been found (unpublished studies) that freshly precipitated tricalcium phosphate added to calcium caseinate did not remove any significant amount of casein. From this it appears that the stability of calcium caseinate, either alone or complexed with tricalcium phosphate, will be determined principally by the available calcium concentration and the pH (13). In the case of the calcium-caseinate-calcium-phosphate complex an excess of calcium precipitates both the tricalcium phosphate and calcium caseinate, but the sequence has not been determined. The influence of pH on the heat stability of the casein complex will be twofold; as the pH is raised more calcium is required to precipitate casein, and at higher pH values phosphate will bind calcium more strongly. The total effect will be that the heat stability of the casein will be greatly enhanced in a system of constant composition by raising the pH.

The prevention by phosphate of the re-resolution of heated casein precipitates when stored at a lower temperature makes less likely the suggestion (13) that this phenomenon of re-resolution might explain the drop in viscosity on the storage of evaporated milk. The re-resolution of the casein precipitates can not be completely excluded as an explanation for the drop in viscosity, because other components of milk might make it possible for heated colloidal casein to redissolve on storage at lower temperature. Furthermore, studies at longer time intervals might reveal that a slow re-resolution does occur. The ability of phosphate to prevent re-resolution of casein, compared with citrate which does not interfere, emphasizes the differences between these two substances as stabilizing agents and might provide a clue to their total stabilizing effect.

SUMMARY

The effect of sodium chloride, citrate, and phosphate on the precipitation of casein by calcium chloride and heat, and the reversal of the precipitation at lower

temperature, has been investigated. The re-resolution of the casein precipitate occurs in the presence of sodium chloride and citrate but not in the presence of phosphate. With excess of citrate or phosphate, the precipitation of casein by calcium chloride and heat is prevented; with sodium chloride, a large reduction in the amount of precipitate occurs. Both citrate and phosphate act by binding calcium, a reaction that in the case of phosphate leads to the formation of insoluble calcium phosphate. Sodium phosphate is more effective in preventing casein precipitation by calcium chloride and heat at pH 6.6 than at pH 6.1, because more calcium phosphate is precipitated at the higher pH.

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