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ABSTRACT

The functionality of caseins in food systems depends on their solubility. The solubilities of whole casein, α_{s1}-casein, β-casein, and dephosphorylated whole casein were measured as the pH was adjusted from 7 to 2 with HCl. When NaCl was added before pH adjustment, solubilities of whole casein at pH 2.5 decreased by 25, 40, 85, and 98% at NaCl concentrations of .01, .05, .1 and .2 *M*, respectively. Likewise, solubilities of dephosphorylated whole casein decreased by 50%, α_{s1}-casein by 30%, and β-casein by 90% in .1 *M* NaCl. When .01 and .05 *M* NaCl were added to whole casein solutions after pH adjustment, effects on solubility were slight; however, .1 and .2 *M* NaCl decreased solubility by 30 and 95%, respectively. When NaCl was added to casein solutions, pH decreased if the original solution was above the isoionic point, indicating Na ion binding; however, at pH between 5 and 3, the pH of the solution increased when NaCl was added. The pH shift upon addition of NaCl to dephosphorylated casein was less than with native caseins, suggesting that Na ion binding by phosphate and acid groups occurred in casein. Although NaCl is commonly used in food processing, these unusual solubility effects should be considered when NaCl is used with casein.

(**Key words**: casein, salt, solubility)

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INTRODUCTION

Functional properties of food proteins depend on their solubility in aqueous systems (14). The effect of Ca^{2+} ion on casein solubility has been intensively studied because of the importance of Ca^{2+} to the stability of the milk system (9). In formulated food systems, in which casein is used as a functional protein, NaCl is commonly used. Sharp and McInerney (15) showed that acid casein was insoluble at pH <4 in the presence of .1 *M* NaCl, NaF, and NaI. Ho and Waugh (10) noted that α_{s1}-casein was insoluble from pH 5.6 to 1.9 at an ionic strength of .4 but, at an ionic strength of .05, dissolved at pH <3.5. Cayot et al. (4) showed that the solubilities at pH 2 of α_{s1}-casein and β-casein at an ionic strength of .1 were about 25% of their solubilities in a salt-free system. However, Courthaudon et al. (5) showed that whole casein was only slightly less soluble in .1 to 1 *M* NaCl than in water at pH much greater or less (either acidic or basic) than that at the isoelectric point, pH 4.6; Medina et al. (11) showed that solubility of whole casein in .1 *M* NaCl was essentially unchanged from solubility in water at pH 2. These results are in sharp contrast to those reported for whole casein (15), for α_{s1}-casein (10), and for both α_{s1}-casein and β-casein (4) as just described. Ho and Waugh (10) showed that casein binds Na and K ions at neutral pH and that this binding occurs on the phosphoserine group of casein. Binding of Na ion to casein may be the cause of the anomalous solubility results discussed.

The objectives of this study were 1) to investigate the effect of pH ranging from 7 to 2 on the solubility of whole casein, β-casein, α_{s1}-casein, and dephosphorylated whole casein dissolved in NaCl at low concentration; 2) to evaluate the effect on solubility of addition of NaCl at pH from 7 to 2; and 3) to measure the effect on pH of the addition of NaCl to casein solutions at pH from 7 to 2 as an indicator of Na or Cl ion binding to casein.

MATERIALS AND METHODS

Preparation of Caseins

Whole casein was isolated by isoelectric precipitation at pH 4.6 from raw skim milk purchased from local dairies. The precipitation was carried out at 25°C using 1*N* HCl. The casein curd was washed with acidulated water, dissolved using minimal amounts of 1*N* NaOH at pH 7, filtered through Whatman number 541 paper (Whatman International, Ltd., Maidstone, England), and reprecipitated at 25°C with 1*N* HCl. The washing, dissolution with 1*N* NaOH, and filtering were repeated. The casein was freeze-dried and stored at –20°C until used. Individual caseins were isolated from the freeze-dried whole casein by the anion-exchange method of Thompson (18) as described by Strange et al. (16). Dephosphorylated whole casein was prepared by batch treatment with potato acid phosphatase (1) and was measured as being 96% phosphate-free by the Sumner (17) method.

Protein Content and pH

Protein concentration was measured by absorbance at 280 nm. Standard curves for 1- and .5-cm path length cells were prepared by measurement of the absorbance of serial dilutions of the starting casein solution from 360 to 200 nm with a Shimadzu UV/Visible recording spectrophotometer (Shimadzu Corp., Kyoto, Japan). The absorbance at 280 nm of a line extrapolated from the absorbance at 320 to 258 nm was subtracted from the absorbance at 280 nm, correcting for light scatter. This corrected absorbance was used to construct the standard curves from the serial dilution data and to determine the protein content of the casein solutions from absorbance data and the standard curves.

The pH was measured with a Radiometer model PHM 85 Precision pH meter (Radiometer America, Westlake, OH) equipped with a GK2401C combination electrode.

Solubility in Water and with NaCl Added After pH Adjustment

Solutions (.2%) of whole casein, α_{s1}-casein, β-casein, and dephosphorylated whole casein were prepared by dispersion of 200 mg of casein in 99.8 g of water. The pH of these solutions was decreased by the addition of .1*N* HCl with stirring, and 1.5-ml samples were removed after each addition; pH of the samples ranged from 7 to 2. As the pH approached 2, 1*N* HCl was used for acidification. Samples were centrifuged for 15 min at 8800 × *g*, and the protein content of the supernate was measured by spectrophotometry. Each sample was agitated to resuspend the casein, and pH was measured before the samples were adjusted to .1 *M* NaCl by addition of 75 μl of 2 *M* NaCl. The pH was again measured, and the change in pH was determined by subtraction of the original pH from that determined after the addition of NaCl. The samples were equilibrated overnight before centrifugation; the casein content of the supernates was determined by spectrophotometry. All operations were conducted at room temperature (22 to 25°C). Whole casein solutions were adjusted to .01, .05, and .2 *M* NaCl after pH adjustment. Protein content and change in pH were determined as described.

Solubility in NaCl

Solutions (.2%) of whole casein, α_{s1}-casein, β-casein, and dephosphorylated whole casein were prepared in .1 *M* NaCl. The pH was adjusted, and the protein concentration was determined on each of the supernates after centrifugation as described. Whole casein in .1 *M* NaCl was allowed to equilibrate, after pH adjustment, before centrifugation and protein determination. The protein contents of five additional 1.5-ml samples at pH 6.90 and 3.73 were determined to be .196 ± .002 and .0112 ± .0002% protein, respectively. Solutions (.2%) of whole casein were prepared in .01, .05, and .2 *M* NaCl before pH adjustment. Protein content was measured after centrifugation.

Solubility and change in pH were measured on a single original solution. The experimental design did not accommodate calculation of standard errors for the solubility curves. However, variability of the data is illustrated by triplicate solubility curves for whole casein versus pH in water, in water with .1 *M* NaCl added after pH adjustment, and in .1 *M* NaCl added before pH adjustment. (Figure 1, A, B, and C, respectively).

RESULTS

Processing behavior of many food ingredients can be affected by the sequence in which they are blended into the food system being prepared. To determine how whole casein solubility was affected by the acid milieu and ionic strength combinations, NaCl was added before or after pH adjustment. At the concentrations studied, whole casein was completely soluble in water, in .1 *M* NaCl, and in water to which .1 *M* NaCl was added after pH adjustment at pH ≥6 (Figure 2A). Results were similar when NaCl solutions at .01, .05, and .2 *M* were used (data not shown). When the pH of casein solutions in water was between 5.8 and 4.8, the casein began to precipi-

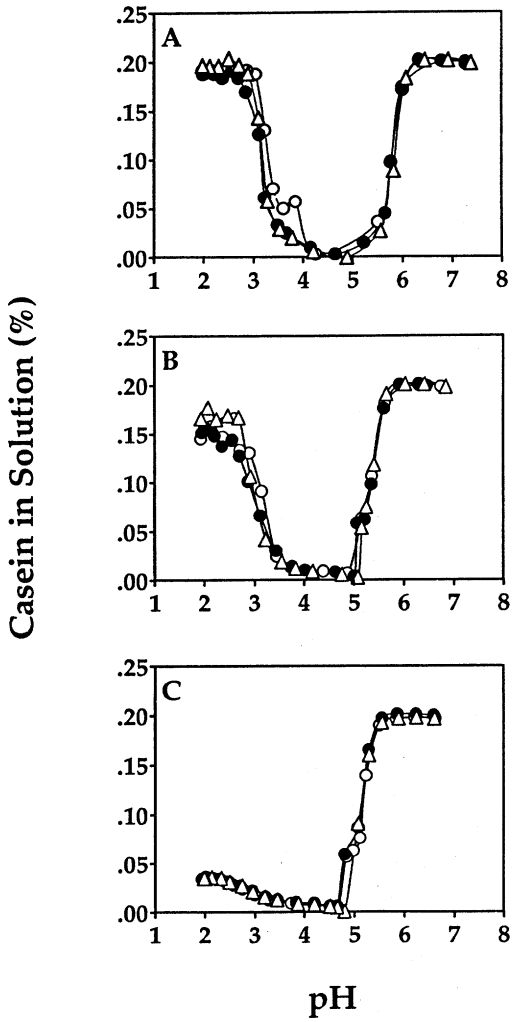

Figure 1. Triplicate curves for solubility versus pH of whole casein in A) water, B) water with .1 *M* NaCl added after pH adjustment, and C) .1 *M* NaCl: ○, ●, and ▽ represent a repetition of solubility versus pH experiments.

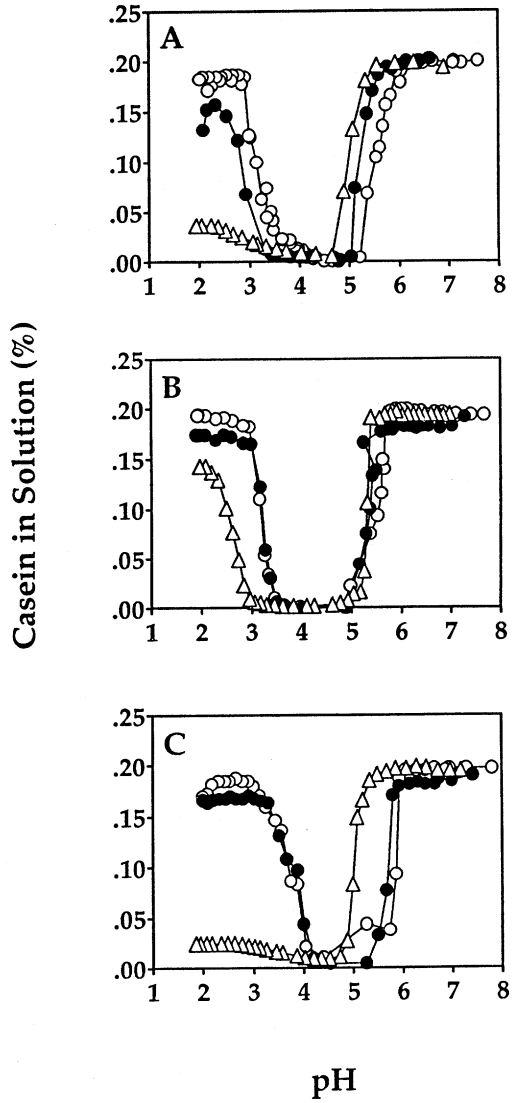

Figure 2. Effect of pH on solubility of caseins dissolved in water (○), dissolved in water and made to .1 *M* NaCl after pH adjustment (pH shown is the final pH attained) (●), and dissolved in .1 *M* NaCl (▽). A) Whole casein, B) α_{s1}-casein, and C) β-casein.

tate; casein was insoluble between pH 4.8 and 4; it began to redissolve between pH 4 and 3; and it was completely dissolved at pH <3. When casein solutions originally dissolved in .01, .05, .1, and .2 *M* NaCl had a pH between 5.8 and 5, casein was more soluble than when it was dissolved in water. At pH between 3 and 4, whole casein in .1 and .2 *M* NaCl was less soluble than casein in .05 or .01 *M* NaCl or in water. At pH <3, the higher the NaCl content, the lower the casein solubility was, and, at pH 2.5, the solubilities of whole casein in .01, .05, .1, and .2 *M* NaCl were 75, 60, 15, and 2% of its solubility in water at pH 2.5. Solubility curves for whole casein in .1 *M* NaCl with and without overnight equilibrium were very similar (data not shown).

The solubilities of whole casein when .01, .05, .1, and .2 *M* NaCl were added to the samples after pH adjustment were greater than when casein was dissolved in water at pH 5.8 to 4.8. From pH 5 to 2, samples that had .01 or .05 *M* NaCl added exhibited casein solubility behavior similar to that of casein in water (data not shown). In samples that had .1 *M* NaCl added at pH 4 to 2, casein was somewhat less soluble than casein dissolved in water at the same pH (Figure 2A). In samples with .2 *M* NaCl added, casein precipitated almost completely at pH <4.8 (data not shown).

When NaCl was added to casein dissolved in water and to previously acidified casein samples (Figure 3A), at pH above the isoionic point, the pH decreased upon addition of NaCl, indicating the release of H^+ ions. From pH 4.8 to 3, the solution became more basic, indicating the release of OH^- ion or, conversely, the uptake of H^+ ions. At pH <3, little change occurred. The magnitude of the change in pH was about the same when .05, .1, or .2 *M* NaCl was added; however, when .01 *M* NaCl was added, the change in pH was smaller, indicating that maximum replacement of H^+ ion by Na^+ ion at binding sites occurs at NaCl concentrations >.01 *M*.

Experiments with individual caseins were conducted only at .1 *M* NaCl. Although lower than the NaCl concentrations used in many food processing operations, this molarity is used for many functionality tests.

At pH above the isoionic point, α_{s1}-casein in .1 *M* NaCl remained in solution at slightly more acid pH than when it was dissolved in water or when .1 *M* NaCl was added after pH adjustment (Figure 2B). At pH between 5.0 and 3.5, no differences in solubility occurred among the α_{s1}-casein solutions tested; how-

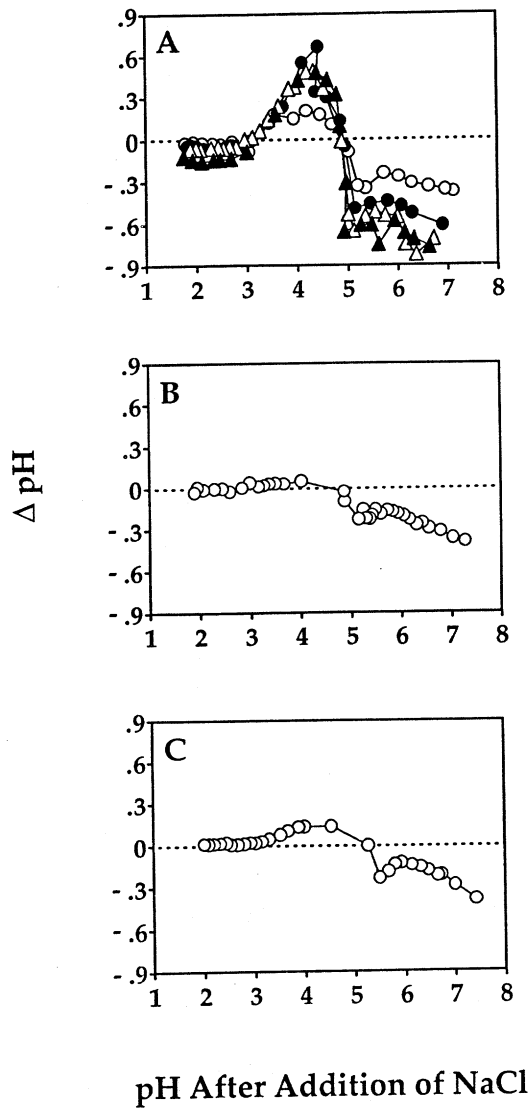

Figure 3. Effect of addition of NaCl on the pH of the casein solutions after the pH was adjusted with HCl. Δ pH = Change in pH occurring when NaCl was added; pH = the pH of the solution after addition of NaCl. A) Whole casein: .01 *M* NaCl (○), .05 *M* NaCl (●), .1 *M* NaCl (▽), .2 *M* NaCl (▼); B) α_{s1}-casein and .1 *M* NaCl (○); and C) β-casein and .1 *M* NaCl (○).

ever, at pH between 3.5 and 3, α_{s1}-casein in .1 *M* NaCl was much less soluble than when dissolved in water or when NaCl was added after pH adjustment. At pH <3, α_{s1}-casein was only partly soluble in .1 *M* NaCl, although it was almost completely soluble in the other two types of solutions.

The effect that addition of NaCl has on the pH of α_{s1}-casein dissolved in water or after pH adjustment with HCl was examined. Above the isoelectric point, the pH decreased upon addition of NaCl (Figure 3B). This shift was smaller than that for whole casein when .1 *M* NaCl was added. Around the isoelectric point, the change in pH was slightly positive, indicating release of OH^- ions or uptake of H^+ ions.

Changes in solubility for β-casein were more dramatic (Figure 2C). Above the isoionic point, β-casein in water or with .1 *M* NaCl added after pH adjustment had similar solubilities. However, β-casein that dissolved in .1 *M* NaCl before acidification remained in solution under conditions that were more acidic than did the β-casein in the other two solutions. At pH between 4.7 and 4, β-casein precipitated in all three solutions. At pH <4, β-casein in water or with .1 *M* NaCl added after pH adjustment was soluble, but β-casein in .1 *M* NaCl remained insoluble when acidulated.

At pH above the isoionic point of β-casein, the pH decreased, indicating release of H^+ (Figure 3C) upon the addition of .1 *M* NaCl. The change in pH was about the same as that for α_{s1}-casein and smaller than that for whole native casein. This difference may be the result of differing casein preparation techniques, because the actual number of H^+ ions released is extremely small. At pH between 5 and 3, the addition of NaCl distinctly increased pH, indicating a release of OH^- ions or uptake of H^+ ions. This increase is smaller than that for whole native casein (Figure 3A) but greater than that of α_{s1}-casein (Figure 3B).

Effects of NaCl and pH on the solubility of dephosphorylated whole casein were evaluated to determine the magnitude of the Na^+ binding that was unrelated to phosphate. Only minor differences occurred in the solubility of dephosphorylated casein in water or in .1 *M* NaCl added after pH adjustment over the entire pH range (Figure 4A). Dephosphorylated casein dispersed in .1 *M* NaCl before pH adjustment was more soluble at pH greater than

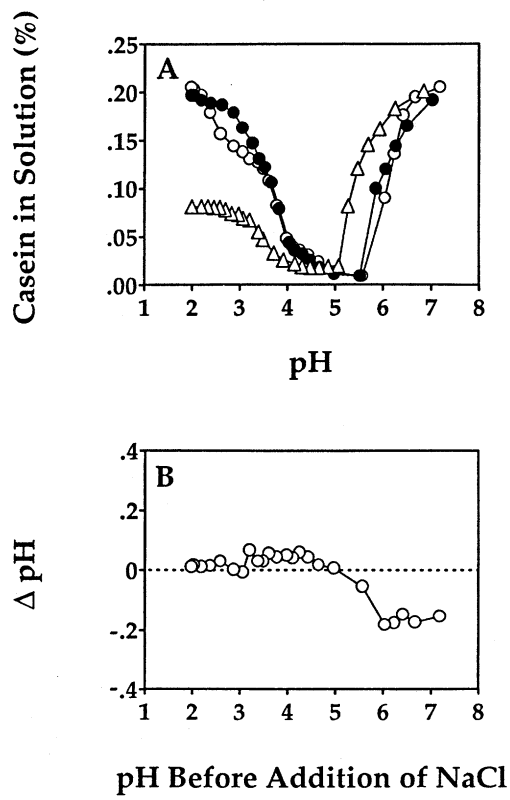

Figure 4. A) Effect of pH on the solubility of dephosphorylated whole casein: dissolved in water (○), dissolved in water and made to .1 *M* NaCl after pH adjustment (pH shown is the final pH attained) (●), and dissolved in .1 *M* NaCl (▽). B) Effect of .1 *M* NaCl on the pH of the dephosphorylated casein solution. Δ pH = Change occurring when NaCl was added after pH adjustment with HCl; pH = the pH of the original water solution.

the isoelectric point and, at pH below the isoelectric point, was less soluble than in water or in .1 *M* NaCl added after pH adjustment. Compared with β-casein and whole casein in .1 *M* NaCl, more dephosphorylated casein remained in solution at acid pH. Dephosphorylated casein in .1 *M* NaCl achieved its maximum acid solubility at pH 3, compared with α_{s1}-casein at pH 2.3.

The effect on pH of the addition of .1 *M* NaCl to dephosphorylated whole casein solutions was minimal (Figure 4B). The pH shift upon the addition of NaCl was much smaller for dephosphorylated casein than for any of the other caseins evaluated. At pH above the

isoionic point, the pH decreased, and, between pH 5 and 3, the pH increased slightly.

DISCUSSION

The increased solubility of casein in NaCl solutions at pH slightly above the isoionic points was noted by Sharp and McInerney (15) for whole casein, by Ho and Waugh (10) for α_{s1}-casein, and by Cayot et al. (4) for β-casein. Ho and Waugh (10) attributed this increased solubility to a salting-in effect, i.e., a decrease in the electrostatic interactions in a protein (6) that are maximal at the isoelectric point. Another possibility is that casein binds Na^+ to negatively charged groups, and the casein-Na complex is slightly more soluble than the unbound casein. Carr and Engelstad (3) found that whole casein bound 5 mol of Na^+/mol of casein at pH 7.5 and that an acidic peptide of casein bound measurable amounts of Na^+ at the lowest pH tested, 4.85. This binding was attributed to the presence of multiple, adjacent negatively charged groups. Such groups are present in β-casein, residues 15 to 19, and in α_{s1}-casein, residues 64 to 68 (13). Ho and Waugh (10) showed that α_{s1}-casein binds 4 to 5 Na^+ ions at pH of about 7 and that, for K^+ at least, pH at the isoionic point does not affect this binding. The Na^+ would be expected to bind more tightly to the casein than K^+ because of its smaller size (3).

We also observed that, at pH above the isoionic point, the pH of all casein solutions measured decreased upon the addition of NaCl, indicating a binding of Na^+ and a release of H^+. Ho and Waugh (10) also noted a decrease in pH when KCl was added to isoionic α_s-casein and attributed this decrease to the binding of K^+. However, this decrease in pH may also be attributed to replacement of H^+ from the diffuse, positively charged electronic layer, which can surround the negatively charged protein with Na^+ (2).

The pH range of minimum solubility of 4.8 to 4.2 corresponds to the isoelectric pH of whole casein. The NaCl concentrations studied were less than those concentrations associated with the increased solubility noted by Courthaudon et al. (5). The change in pH upon addition of NaCl in this range of minimum solubility was positive, possibly because of the substitution of Cl^- for OH^- bound to charged lysine and arginine residues.

The solubility of the caseins at acid pH showed gross differences that were due to concentration of NaCl, type of casein, and the pH of the casein solution when NaCl was added. Caseins showed decreased solubility in NaCl solutions (4, 10, 12, 15). However, Courthaudon et al. (5) and Medina et al. (11) showed little change at low pH in the solubility of whole casein that was due to concentration of NaCl. In an effort to resolve the differences in solubility, we determined the solubility of caseins at low pH when NaCl was added at low pH. For whole casein, a minimum of .1 *M* NaCl, added at low pH, was needed to initiate precipitation; casein was completely precipitated with .2 *M* NaCl. Little difference in solubility occurred when .1 *M* NaCl was added at low pH for α_{s1}-casein, β-casein, or dephosphorylated casein.

The decrease in solubility at pH <3 at NaCl concentrations above .05 *M* can be attributed to the tendency of hydrophobic compounds to be insoluble in water in the presence of salts (6), and caseins are among the most hydrophobic proteins (8). However, the salting-out effect should be unaffected by the order of addition of NaCl and acid. The formation of a pH-stable NaCl-casein complex at pH above the isoionic point that is insoluble at low pH explains the observation that casein dissolved in NaCl at high pH was insoluble at low pH. If this complex is not formed at low pH, the casein would remain soluble if NaCl were added at a low pH. Because solubility decreased when more NaCl was added, two different types of insoluble complexes may have formed: a complex that can be formed at low pH with high concentrations of NaCl and a complex that is formed at higher pH at NaCl concentration ≥.01 *M*. Ho and Waugh (10) noted that K^+ binds to casein at two sites, one with a dissociation constant of .8 m*M* K^+ and one with a dissociation constant of about 40 m*M* K^+. These dissociation constants are within the concentrations of Na^+ used in the present study.

The possible role of the phosphate groups of casein in the formation of Na-casein complexes was investigated by comparison of the solubility of dephosphorylated whole casein with whole casein. Dephosphorylated casein was soluble at low pH, to some extent, when dissolved in NaCl at high pH. This finding

suggests a role for the phosphate groups in the formation of Na-casein complexes at higher pH. The increased solubility of α_{s1}-casein at extremely acid pH (solubility maximum at pH 2.3) (Figure 3B) indicated that Na^+ may be bound to singly charged phosphate groups in α_{s1}-casein. The increased solubility may be due to the decrease in the number of negative charges at pH about the first pK_a, 2.65, of phosphoserine (7). These results also clearly indicate that sites other than serine-phosphates are involved in the solubility of casein in NaCl.

CONCLUSIONS

The variations in solubility of casein in NaCl profoundly affect the functionality of casein. Increased solubility of casein that is due to the presence of NaCl at pH slightly above the isoelectric point may be the reason that a minimum of .1 *M* NaCl was required to form an acid gel rather than a precipitate with Na caseinate (8). Many of the basic functionality tests, such as emulsion capacity and stability, are commonly performed in an NaCl solution. The results presented herein illustrate that order of addition of casein and NaCl may also be critical to the proper functioning of casein in products.

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