

Rheology and Microstructure of Chemically Superphosphorylated Whole Casein¹

ABSTRACT

Whole bovine casein was chemically modified using POCl_3 to contain 9.1, 10.6, and 12.5 mmol of bound P/mmol of casein [(160, 190, and 220% P, respectively) relative to the 5.6 mmol of P/mmol of unmodified casein (100% P)]. Superphosphorylation produced two types of modified caseins. Solutions made with 220% P casein had low viscosities, which remained constant between pH 5 and 9 and between protein concentrations of 0.2 and 0.7%; these solutions remained fluid when exposed to up to 30 mM Ca^{2+} . Solutions made with 160 or 190% P caseins increased in viscosity as protein concentration and pH increased; the solutions formed gels at 1% protein, which increased in elastic modulus, viscous modulus, and complex viscosity as the protein and Ca^{2+} concentrations increased. When exposed to Ca^{2+} , gels became more curd-like as protein aggregated and then underwent syneresis. Electron microscopy showed that the gel microstructure consisted of an open matrix of folded strands and sheets of casein in irregular sizes that condensed upon exposure to Ca^{2+} . These unique interactions among proteins and unique rheological properties suggest that superphosphorylation could be useful in creating novel dairy foods with added value and enhanced functionality.

(**Key words:** casein, gelation, phosphorylation, rheology)

Abbreviation key: G' = elastic modulus, G'' = viscous modulus, η^* = complex viscosity, **SEM** = scanning electron microscopy, $\tan \delta$ = ratio of viscous modulus to elastic modulus, **TEM** = transmission electron microscopy.

INTRODUCTION

The monophosphate groups that are covalently bound to casein are essential for many of the interac-

tions that make this major milk protein a desirable food ingredient. Studies of caseins with increased P content enhance the knowledge about the role of P in these interactions and suggest modifications that may improve the utilization of casein as a food ingredient.

Chemical phosphorylation techniques increase the P content of casein by the addition of mono-, di-, and polyphosphates (3, 11). Caseins with these additional negatively charged phosphates have lower isoelectric points (9, 12) but have the same distribution of secondary structures as unmodified whole casein, indicating no unfolding or compression of the protein (11). Compared with unmodified casein, superphosphorylated caseins have more flexible side chains (4), lower surface hydrophobicity (9), greater water-binding capabilities (3), and altered solubility, foaming, and emulsifying properties (3, 4, 8, 9, 12). The interaction between P and Ca^{2+} is the prime role of casein-bound phosphate. Caseins that have increased P content bind more Ca^{2+} but have impaired micelle-forming ability (8) and micelle stability (13). When high concentrations of POCl_3 are used for modification, the superphosphorylated caseins form complexes that cannot be disrupted with mercaptoethanol, SDS, or urea (3, 4, 12). However, if lower concentrations of POCl_3 are used, the caseins retain a monomeric form (9). Solutions of whole casein with elevated P content have higher viscosities than do unmodified caseins (3, 4), and, in addition, exhibit shear thinning instead of Newtonian behavior (4).

Solutions of superphosphorylated whole casein form gels at low concentrations (4, 12) and, as yet, no further information is available on these gels. Our laboratory has produced superphosphorylated caseins that contain 160, 190, or 220% the P content of unmodified whole casein (100% P) that have unusual gelation properties (12). The 160 and 190% P caseins form gels at low concentrations, but the 220% P casein remains fluid. This paper describes the viscosity of superphosphorylated caseins at different pH and low concentrations and the rheological properties and microstructure of casein gels exposed to different concentrations of Ca^{2+} .

MATERIALS AND METHODS

Modification of Casein

Superphosphorylated caseins were prepared as described by Van Hekken et al. (12). To determine bound P, the amount of free P in the sample (12) was subtracted from the total P in the sample (10). Protein content was calculated by multiplying total N (1) by 6.34, the conversion factor for milk and dairy products (2). Unmodified whole casein (100% P) had a P content of 5.6 mmol of P/mmol of casein and was prepared from raw skim milk by isoelectric precipitation. Superphosphorylated caseins were prepared using a molar ratio of POCl_3 to casein of 2000:1 and three reaction pH. Caseins modified at pH 5, 7, or 9 had bound P contents of 9.1 (160% P), 10.6 (190% P), or 12.5 (220% P) mmol of P/mmol of casein, respectively.

Viscosity

A response surface methodology was used to predict the change in viscosity because of the effect of degree of phosphorylation, protein concentration, and pH. Solutions were prepared containing 0.2, 0.27, 0.45, 0.63, or 0.7% of the 100, 160, 190, or 220% P caseins in 10 mM imidazole buffer at pH 5, 5.59, 7, 8.41, or 9. Solutions were mixed for 30 min at room temperature (25°C) before 16-g aliquots were placed in the Brookfield sample holder (Brookfield Engineering Labs, Inc., Stoughton, MA) and held at 22°C for 10 min. A Brookfield DV-III rheometer with a ULA spindle was used to collect data on torque, viscosity, and shear stress. Viscosity data were collected at 56 rpm for most of the samples, although data on a few samples with high viscosity were collected at lower revolutions per minute to keep the torque values within 10 to 90%. Samples were run in duplicate, and data were analyzed to generate regression models for response surface methodology (7).

Rheology

A split-plot experimental design was used to examine the effect of degree of phosphorylation, protein concentration, and calcium concentration on the rheological properties of casein gels. Samples were prepared containing 1, 2, or 4% of the 160 and 190% P caseins in 10 mM Tris·HCl buffer, pH 8.4. Duplicate samples of 160% P casein and triplicate samples of 190% P casein were tested. Because of the high viscosity, the samples were hand-stirred to ensure uniform dispersion of protein. Samples were transferred

into dialysis tubing (12,000 to 14,000 molecular mass cutoff; SpectraPor, Spectrum Medical Industries, Inc., Los Angeles) and dialyzed against Tris buffer containing 0, 5, 10, 20, or 30 mM CaCl_2 overnight at 4°C. Samples were brought to 20°C and carefully transferred with minimal physical manipulation from the dialysis tubing to parallel plates with radii of either 12.5 or 20 mm; the plates were closed to a 2.0-mm gap. Frequency sweeps (0.1 to 100 rad/s) were obtained at 10% strain and 20°C with a dynamic oscillatory shear spectrometer (RDA-700; Rheometric, Inc., Piscataway, NJ). Elastic modulus (G'), viscous modulus (G''), complex viscosity (η^*), and G''/G' ($\tan \delta$) at 10 rad/s were analyzed using the general linear models procedure, ANOVA, and Bonferroni t test ($P < 0.05$) (7).

Electron Microscopy

Gels containing 2% of the 160 or 190% P casein and either 0, 10, or 30 mM CaCl_2 were prepared as described in the section on rheology. After dialysis, samples were removed from the dialysis tubing and placed directly into 2% glutaraldehyde and 10 mM Tris·HCl, pH 8.4. Samples were divided in half and processed using standard scanning electron microscopy (SEM) or transmission electron microscopy (TEM) techniques. Samples that had not been exposed to Ca^{2+} first were centrifuged (Eppendorf microcentrifuge, Brinkman Instruments, Inc., Westbury, NY) to a pellet for TEM or transferred to microporous capsules for SEM. For 100 and 220% P caseins, 2% solutions were prepared in 100 mM imidazole, pH 7.2, and the protein was adsorbed on to glass coverslips for 1 min. The coverslips were placed in 1% glutaraldehyde in the imidazole buffer for 1 h and processed for SEM analysis.

Samples for SEM were washed in 100 mM imidazole·HCl (pH 7.2), dehydrated in 50 and 100% ethanol, dried to critical point from liquid CO_2 , mounted on SEM stubs, and coated with gold (LVC-76 Sputtering System, Plasma Sciences, Inc., Lorton, VA). Samples were examined using a JSM 840A scanning electron microscope (JEOL USA, Peabody, MA), and digital images were recorded at 1000 or 25,000× instrumental magnification.

Samples for TEM were rinsed in 100 mM imidazole·HCl, pH 7.2, with and then without 1% glutaraldehyde, postfixed for 2 h in 2% OsO_4 in the imidazole buffer, washed in distilled water, dehydrated in a graded series of ethanol (50 to 100%), rinsed in propylene oxide, cured in a propylene oxide epoxy resin mix for 48 h at 55°C, and embedded in epoxy resin. Thick sections were stained in 1% methylene blue and examined on a phase contrast light microscope (Olympus America Inc., Lake Success,

NY); digital images were recorded at 40× instrumental magnification. Thin sections were stained in uranyl acetate and lead citrate and then examined using a scanning-transmission electron microscope (CM12; Philips Electronic Instruments, Mahwah, NJ) and photographed at 10,000× instrumental magnification.

RESULTS

Viscosity

Regression models to determine the viscosity of the 100, 160, 190, and 220% P caseins at different pH and

low protein concentrations are shown in Figure 1, and the response surface regression coefficients are presented in Table 1. The models predicted that, as the pH and concentration increased, the viscosities of the 100 and 220% P caseins would remain constant (Figure 1, a and b), and the viscosities of the 160 and 190% P caseins would increase significantly (Figure 1, c and d). This prediction was based on the assumption that casein concentration increased from 0.2 to 0.7%. It is well established that the viscosity of sodium caseinate (our 100% P casein was a sodium caseinate) is dependent on pH and protein concentration and that 15% casein solutions are very viscous

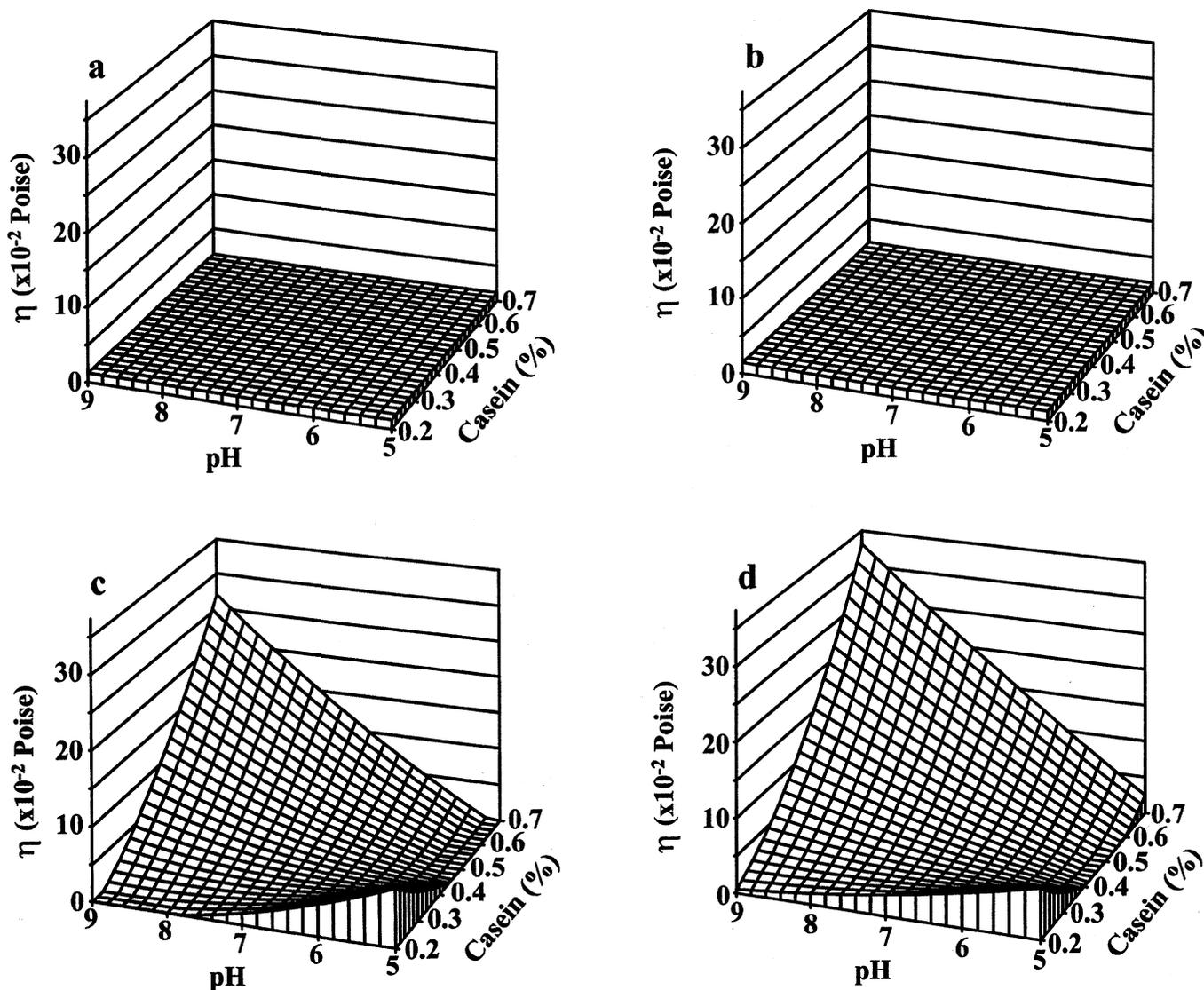


Figure 1. Response surface regression models for the viscosity of a) 100%, b) 220%, c) 160%, and d) 190% P caseins (P content relative to unmodified casein = 100% P) at different pH and low protein concentrations.

TABLE 1. Response surface regression equations used to predict the viscosities of caseins with 100, 160, 190, and 220% of the P content of unmodified whole casein (100% P) at different pH and low protein concentrations.

Casein	Response surface regression equations ¹	R ²
100% P	Viscosity = 1.02 - 0.05a + 0.05b + 0.05ab - 0.23a ² - 0.01b ²	0.39
160% P	Viscosity = 67.3 - 158a - 11.5b + 18.5ab - 53.1a ² + 0.42b ²	0.73
190% P	Viscosity = 55.1 - 173a - 8.11b + 18.4ab + 80.2a ² + 0.21b ²	0.89
220% P	Viscosity = 2.24 - 1.03a - 0.29b + 0.05ab + 1.23a ² + 0.02b ²	0.90

¹a = Casein concentration; b = pH.

(5). Solution viscosities were higher for the 190% P casein than for the 160% P casein. When the variability was partitioned into pH and concentration, the low R² value for the 100% P casein noted in Table 1 was largely the result of the large variability within samples. From this study, we determined that superphosphorylated casein gels prepared at pH 8.4 and containing more than 0.7% protein should be used in the rheology study.

Rheology

The 100 and 220% P caseins were very soluble, and solutions remained fluid even at 30 mM Ca²⁺. Because our primary interest was to study the gel properties and because the fluid samples could not generate sufficient torque to enable us to collect reliable data, the rheological properties of the 100 and 220% P caseins were not studied further.

The 160 and 190% P caseins were not very soluble and, at concentrations $\geq 1\%$ protein, formed clear gels

upon hydration. The 160 and 190% P caseins were hydrated with buffer before being dialyzed in buffer containing Ca²⁺. At 5 mM Ca²⁺, the caseins lost the clear gel-like appearance and formed small aggregates of protein that settled to the bottom of the dialysis tube. The dialysis tube was carefully drained, and the small amount of aggregated protein was collected. However, the amount of fluid in the 5 mM Ca²⁺ samples was variable, and the rheological data were not included in the statistical analysis. At and above 10 mM Ca²⁺, the caseins became opaque and formed curd-like mats of protein that expelled fluid and precipitated in the dialysis tubing.

The frequency sweep (0.1 to 100 rad/s) for 190% P casein exposed to 10 mM Ca²⁺ (Figure 2) was typical for this study. Frequency sweeps confirmed gelled samples when the G' was greater than the G'', the G' and G'' were parallel, the tan δ was <1.0, and the η^* decreased linearly as frequency increased.

The Ca²⁺ concentrations affected ($P < 0.05$) the rheological properties of 160 and 190% P caseins

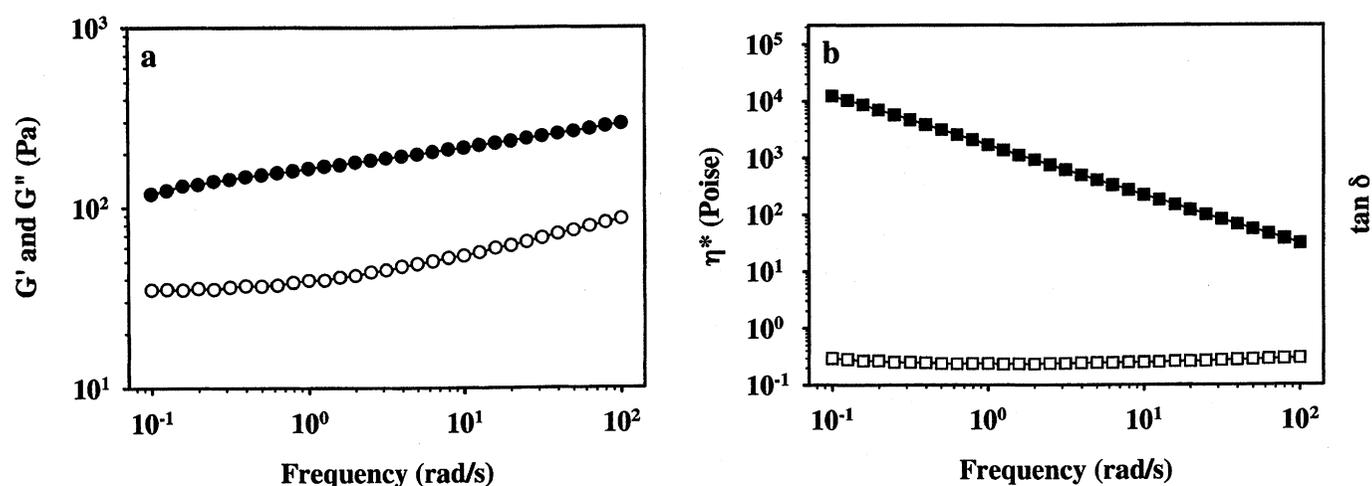


Figure 2. Frequency sweep of 190% P casein (P content relative to unmodified casein = 100% P) exposed to 10 mM CaCl₂ showing the effect of increasing frequency on the elastic modulus (G', ●), viscous modulus (G'', ○), complex viscosity (η^* , ■), and tan δ (G''/G', □). Results were typical of sweeps collected for the gelled superphosphorylated caseins in this study.

(Figure 3). Before the addition of Ca^{2+} , similar G' , G'' , or η^* values were obtained for all gels. The $\tan \delta$ values, which indicated a gel if <1.0 , were between 0.86 and 0.99 for the 160% P casein and between 0.66 to 0.88 for the 190% P casein. At 10 mM Ca^{2+} , the G' , G'' , and η^* values were significantly higher only in the gels made with 4% modified caseins. The $\tan \delta$ values for all gels had decreased from 0.66 to 0.99 for gels at 0 mM Ca^{2+} to 0.28 to 0.23 for gels at 10, 20, and 30 mM Ca^{2+} . At 20 mM Ca^{2+} , increases in G' , G'' , and η^* values were significant for the 4% solutions of 160% P casein and the 2 and 4% solutions of 190% caseins; the η^* value for the 2% solutions of 160% P casein also increased significantly. At 30 mM Ca^{2+} , only the 4% casein samples had significant increases

in G' , G'' , and η^* . Except for the 0 mM Ca^{2+} samples, the G' values (a measure of the elastic character of a gel) were fourfold higher than the G'' (a measure of the viscous character), which were twofold higher than the η^* . Also, the G' , G'' , and η^* values were higher for the 190% P caseins than for the 160% P caseins.

Protein concentration did not have as great an effect on gel strength as did the Ca^{2+} concentration. The general trend was that G' , G'' , and η^* increased as protein concentration increased, and gels made with 190% P casein had higher values than gels made with 160% P casein. Only at 20 and 30 mM Ca^{2+} were there significant increases in G' , G'' , and η^* as the concentration increased from 1 to 2 to 4% casein;

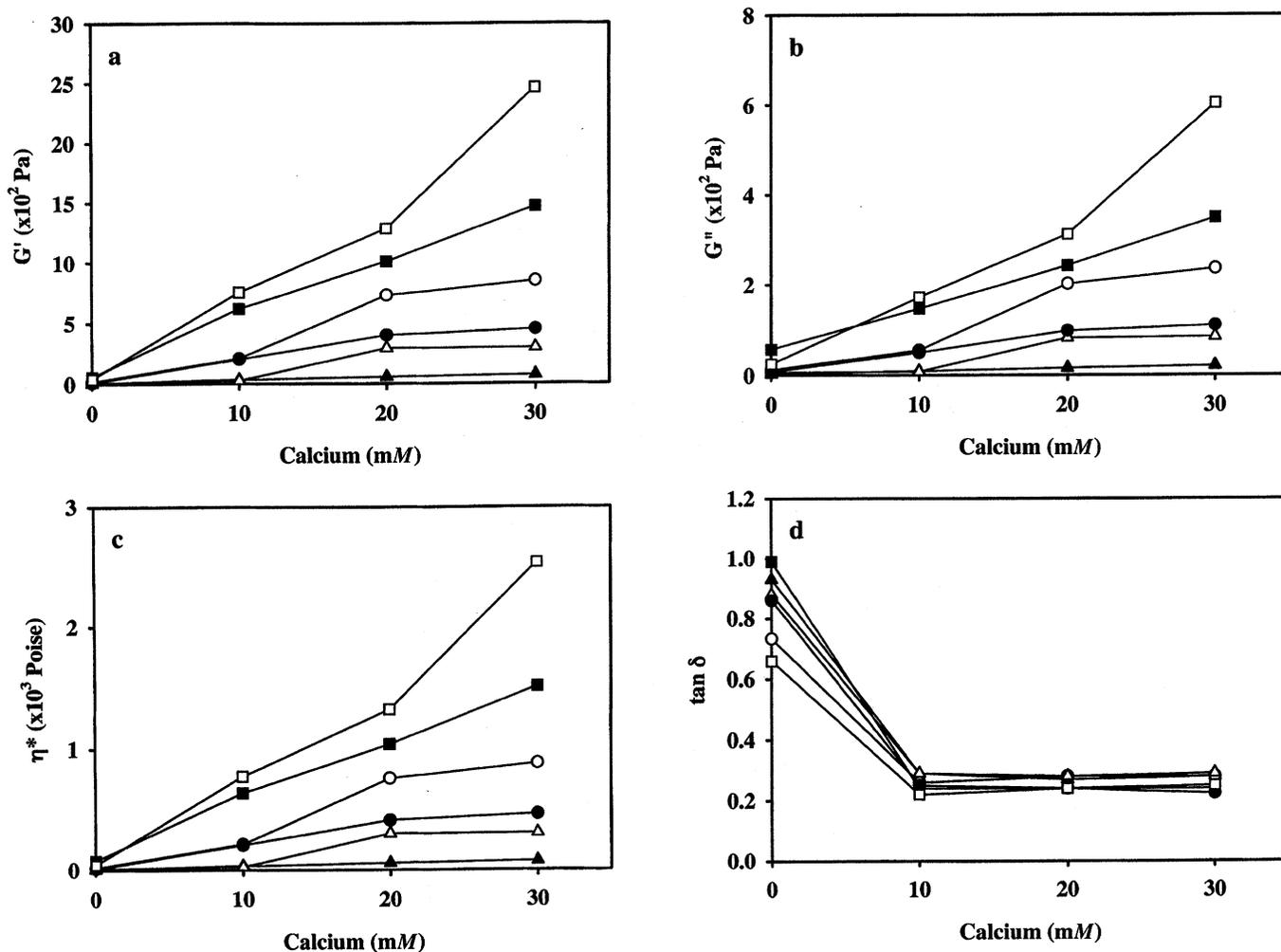


Figure 3. Effect of Ca^{2+} and protein concentration [1% (Δ , \blacktriangle), 2% (\circ , \bullet), and 4% (\square , \blacksquare)] on the a) elastic modulus (G'), b) viscous modulus (G''), c) complex viscosity (η^*), and d) $\tan \delta$ (G''/G') of gels made with 160% P casein (\blacksquare , \bullet , \blacktriangle) and 190% P caseins (\square , \circ , \triangle) (P content relative to unmodified casein = 100% P).

these increases probably reflected the increased number of Ca^{+2} binding sites in the denser gels. A significant increase in G' , G'' , and η^* was noted between the

2 and 4% casein samples at 10 mM Ca^{2+} . No significant differences in $\tan \delta$ values were found that were due to increases in protein concentration.

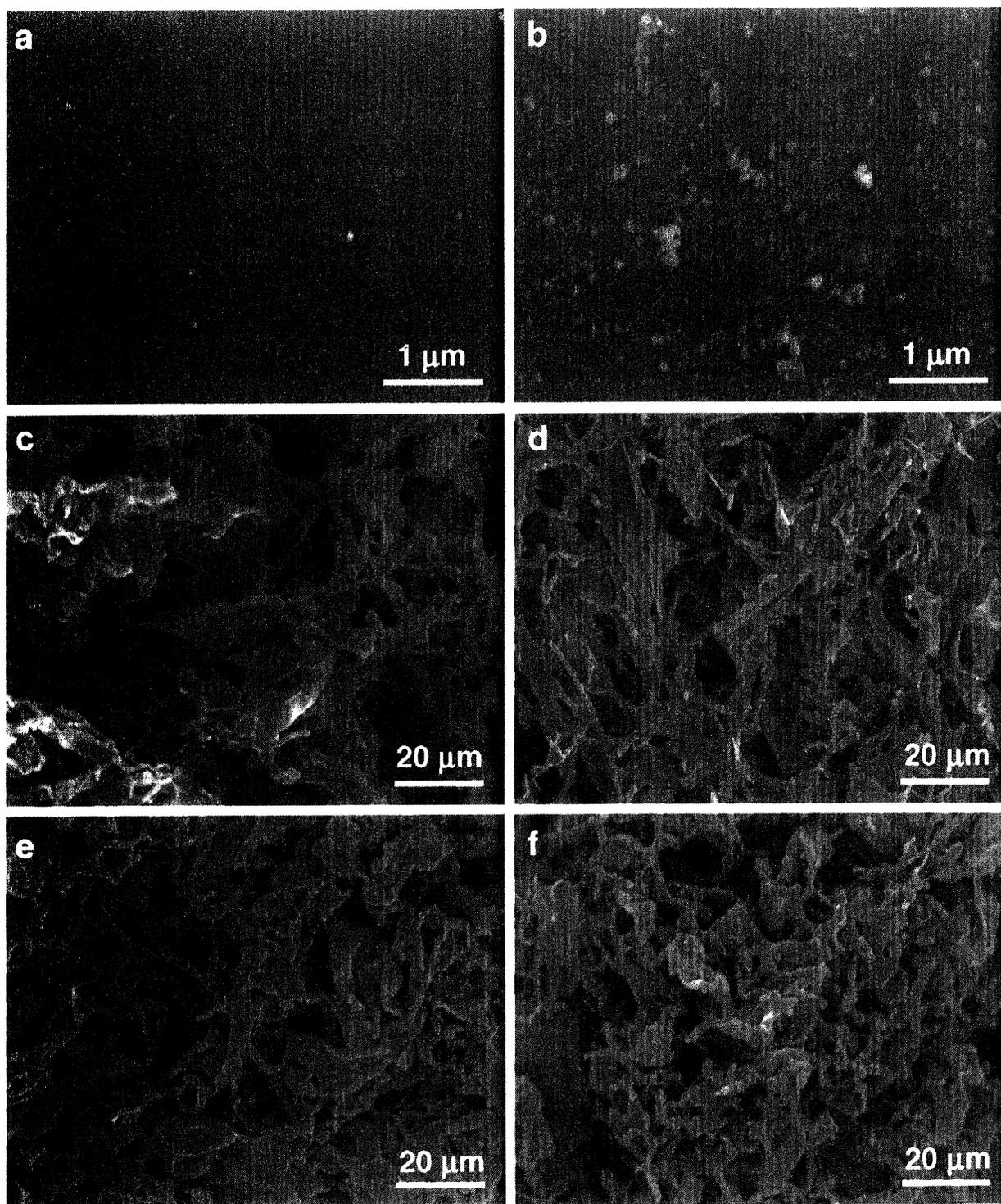


Figure 4. The scanning electron microscopy images of 2% whole casein samples containing a) 100% P (amount of P found in unmodified casein), b) 220% P, c) 160% P, d) 190% P, e) 160% P and 30 mM CaCl_2 , and f) 190% P and 30 mM CaCl_2 .

Electron Microscopy

The SEM images of the casein samples are in Figure 4. The 100 (Figure 4a) and 220% caseins (Figure 4b) were very soluble, and only at very high magnification were small particles of protein noticeable in solution. The particles that existed were

larger in the 220% P casein samples, but the overall comparison of the two solutions was very similar.

The SEM images of the 160 and 190% P casein samples showed an open continuous protein matrix of folded strands and sheets in irregular sizes and shapes (Figure 4, c, d, e, and f). As the Ca^{2+} concentration increased from 0 mM Ca^{2+} (Figure 4, c and d)

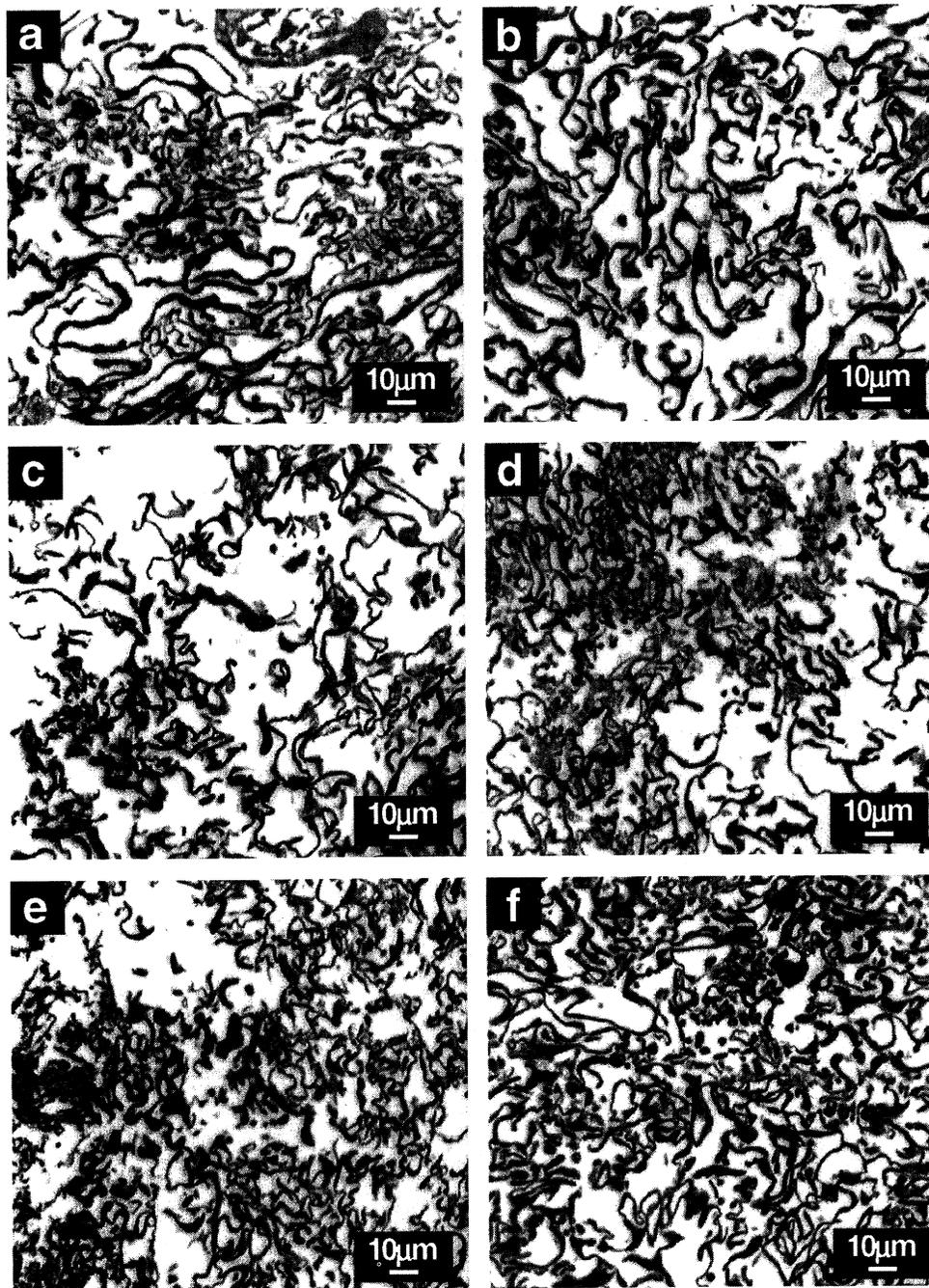


Figure 5. Light microscopy images of 2% whole casein samples containing a) 160% P, b) 190% P, c) 160% P and 10 mM Ca^{2+} , d) 190% P and 10 mM Ca^{2+} , e) 160% P and 30 mM Ca^{2+} , and f) 190% P and 30 mM Ca^{2+} (P content relative to unmodified casein = 100% P).

to 30 mM Ca²⁺ (Figure 4, e and f), the matrix was more compact without a thickening of the folded strands and sheets. This result agrees with our visual observations that, as the Ca²⁺ concentration increased, the samples went from a gel-like mass to a denser curd that expelled fluid.

Light microscopy confirmed that the overall structure of the gel consisted of folded strands and sheets of protein and that the density of the gel was greater at 30 mM Ca²⁺ (Figure 5, e and f) than at 10 mM Ca²⁺ (Figure 5, c and d). The changes between 0 and 10 or 30 mM Ca²⁺ gels could not be followed in either the light microscopy or TEM images because the 0 mM Ca²⁺ gel samples had been centrifuged during processing, which is also why the light microscopy images of the 0 mM Ca²⁺ (Figure 5, a and b) appeared similar, although visual observations noted significant differences between the two Ca²⁺ levels.

The internal structures of the protein matrixes of 160 and 190% P casein gels were revealed by TEM (Figure 6) and showed continuous strands of protein that had a grainy appearance but did not show any hint of the spherical submicelle or micelle structure. As the Ca²⁺ level increased from 0 mM Ca²⁺ (Figure 6, a and b) to 30 mM Ca²⁺ (Figure 6, c and d), more particles associated with the surfaces of the strands, giving them a fuzzy appearance. The 160% P casein gels (Figure 6c) accumulated more of these fuzzy particles within their matrixes than did the 190% P casein gels (Figure 6d).

DISCUSSION

In this study, we examined the superphosphorylated caseins that were first characterized by Van Hekken et al. (12), who mentioned their unusual gelation properties. In that earlier study, the 160 and 190% P caseins (referred to as Sup5 and Sup7, respectively) were different from the 220% P casein (referred to as Sup9) in that they were more sensitive to Ca²⁺, were less soluble in distilled water and 150 mM NaCl, and formed gels at low protein concentrations. Analysis of the phosphorus compounds in the samples using ³¹P nuclear magnetic resonance showed that the 160 and 190% P caseins contained serine monophosphates and diphosphates, and the 220% P casein contained mono-, di-, and polyphosphates (11). The 220% P casein, which had the same diphosphates as well as other di- and polyphosphates, did not gel. The presence of polyphosphates possibly provided sufficient electrostatic repulsion to inhibit protein aggregation and gelation. The diphosphates, without the polyphosphates

present, were the key to the interesting gelation properties of the 160 and 190% P caseins.

Other studies (3, 4) have noted the gelation properties of superphosphorylated caseins but declined to study the gelation further, opting instead to study the increase in viscosity noted at low concentrations. Matheis et al. (3) reported on superphosphorylated casein that had 5.1 mmol of added P/mmol of whole casein in mono- and diphosphate forms. The gel-like character of the modified casein was noted, but only the significant increase in relative viscosity was reported for 0.1% casein solutions in borate buffer at pH 7.9. Medina et al. (4) found between 3 and 14 mmol of bound P/mmol of casein but did not determine in which forms the P was attached. They studied the viscosity and flow properties of 0.2 and 0.5% solutions of caseins modified at pH 5.5, 7, and 9 and noted that flow properties could not be assessed at 0.5% concentrations because of gelation of the sample.

In our study, only the 160 and 190% P caseins showed an increase in viscosity as the concentration and the pH increased. Although unmodified casein showed no increase in viscosity under the conditions examined, the viscosity of casein is dependent on pH (enhanced amino function the farther from its isoelectric point) and increases as the protein concentration increases (5). We had selected the pH range of 5 to 9 because our modified caseins were shown to have consistent and highest solubility in that range (12). The method we used was not sensitive enough to detect the slight changes that occurred at these very low protein concentrations. We identified pH 8.4 for gelation (the highest viscosity value was obtained at this pH) and a starting concentration of 1.0% protein from the viscosity study and examined the gels that formed when the modified caseins were hydrated. The samples gelled as the tan δ became <1.0 (Figure 2d), but these gels did not fit the typical definition of a chemical gel, which require a lag time to form the covalent crosslinks for the protein matrix (6). Instead, these gels fit the definition of physical gels, which require noncovalent crosslinks and are dependent on concentrations.

Our whole caseins were modified chemically to bind phosphate groups covalently to the protein primary structure in mono- and diphosphate forms (11). High concentrations of POCl₃ were used to modify the casein, and protein complexes were formed that could not be disrupted with mercaptoethanol, SDS, or urea (12). This result suggests that during the modification reaction the superphosphorylated caseins formed a matrix that, when rehydrated, could form viscous or gel-like masses through noncovalent

interactions. In support, the findings presented in this study showed uniform distribution of casein in continuous strands and sheets. This result also agrees with earlier Fourier transform infrared spectroscopic analysis of 3% modified casein gels, which indicated that the distribution of the secondary structures in

the superphosphorylated caseins had not changed and that no compression or unfolding of the protein molecule had occurred because of the superphosphorylation (11), lending strong support to the theory that the gelation was a noncovalent interaction.

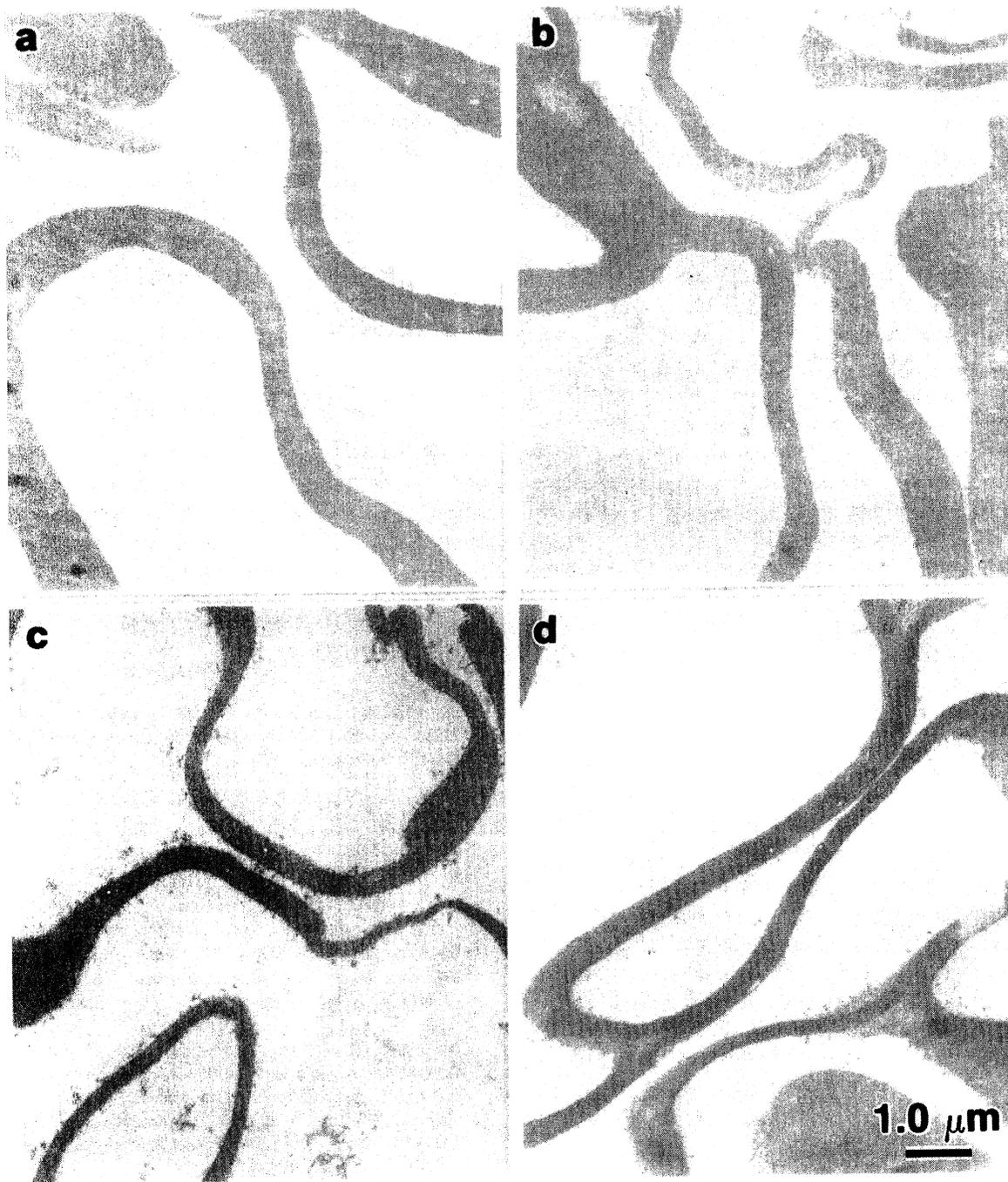


Figure 6. The transmission electron microscopy (TEM) images of 2% whole casein samples containing a) 160% P, b) 190% P, c) 160% P and 30 mM CaCl₂, and d) 190% P and 30 mM CaCl₂ (P content relative to unmodified casein = 100% P).

When exposed to increasing concentrations of Ca^{2+} , the hydrated 160 and 190% P caseins in the gels precipitated into aggregates that underwent syneresis to form curds that were stronger and had more elastic character. Theoretically, increasing the number of phosphate groups on casein should increase the sites that were available for binding Ca^{2+} . The binding of Ca^{2+} is an essential step for caseins to form micelles. Schmidt and Poll (8) found that phosphorylated caseins had increased binding of colloidal calcium phosphate, but the micelles that were formed were smaller and contained more Ca^{2+} , Mg^{2+} , and inorganic P. When Yoshikawa et al. (13) superphosphorylated the individual caseins, they found that all caseins required more Ca^{2+} to precipitate from solution and that only the superphosphorylation of κ -casein disrupted the ability of the caseins to form stable micelles. In an earlier study, the 160 and 190% P caseins were more sensitive to Ca^{2+} and precipitated from solution at lower levels (12). In the present study, Ca^{2+} concentrations as low as 5 mM caused visible disruption of the 160 and 190% P casein gels. Microscopy showed that the changes were not in the organization of the protein matrix, because the continuous strands and sheets remained intact, but in how tightly this matrix was held together. We did not observe any spherical submicelle or micelle structure in our samples before or after the exposure to Ca^{2+} , which indicated that the complexes that formed during the modification process were very stable and also that the binding of Ca^{2+} resulted in compacting the strands into a tighter matrix and caused syneresis.

CONCLUSIONS

Superphosphorylation of whole casein resulted in caseins with two types of rheological properties. The 220% P casein—which contained mono-, di-, and polyphosphates—resembled unmodified casein when in solution in that it retained a low viscosity between pH 5 and 9 and protein concentrations between 0.2 and 0.7%; the 220% P casein also remained fluid as the protein and Ca^{2+} concentrations increased. The 160 and 190% P caseins, which contained serine monophosphates and diphosphates, increased in viscosity as the pH and protein concentration increased and formed fairly clear gels at 1% protein, which increased in G' , G'' , and η^* as the concentration of protein and Ca^{2+} increased. When exposed to Ca^{2+} , the gels turned opaque and aggregated into curds

that underwent syneresis. Gel microstructure consisted of an open protein matrix of folded strands and sheets in irregular sizes that condensed upon exposure to Ca^{2+} .

The addition of phosphate groups to casein did not uniformly change the rheological properties of the modified caseins. The 220% P casein did not have gelation properties similar to those of the 160 and 190% P caseins, and we suggest that the presence of additional di- and polyphosphates on the 220% P casein may have been key factors in preventing gelation. Superphosphorylation studies are useful to enhance the understanding of the role of phosphate groups in casein interactions. The 160 and 190% P caseins demonstrated unique interactions among proteins and unique rheological properties, which suggest that controlled superphosphorylation could be useful in creating novel dairy foods with added value from unique microstructure.

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