

Analysis of Modified Whole Casein with Different Phosphorus Contents Using Phosphorus-31 Nuclear Magnetic Resonance and Fourier Transform Infrared Spectroscopy¹

ABSTRACT

The different types of P in modified caseins and the role of P in casein interactions were studied using ³¹P nuclear magnetic resonance spectroscopy of whole casein with reduced or elevated P concentrations and using Fourier transform infrared spectroscopy of whole casein with elevated P concentrations. Bovine whole casein (5.7 mmol of P/mmol of casein, 100% P) was either enzymatically dephosphorylated or chemically superphosphorylated to obtain casein containing 0.4, 3.3, 9.1, 10.6, or 12.5 mmol of bound P/mmol of casein (7, 60, 160, 190, or 220% P, respectively). The nuclear magnetic resonance spectra showed that all casein samples contained serine monophosphates; the caseins containing 160, 190, and 220% P also contained additional diphosphates, and the 220% P casein contained inorganic phosphate and other di- and polyphosphates. When the concentration of the 160 and 190% P caseins increased, they gelled, yet the nuclear magnetic resonance spectra did not show any differences that were typical of changes in conformation. Analysis by Fourier transform infrared spectroscopy indicated that the addition of covalently bound P to whole casein in any amount did not change the distribution of extended strand and sheet, helix, loop, and turns in their secondary structures. Study of the phosphates that were bound to casein and the influence of phosphates on casein interactions improves the understanding of how casein interacts in food systems.

(**Key words:** casein, nuclear magnetic resonance, phosphorylation)

Abbreviation key: FTIR = Fourier transform infrared, HMPA = hexamethyl phosphoramide, NMR = nuclear magnetic resonance, P_i = inorganic P.

INTRODUCTION

The monophosphate groups that are covalently bound to the serine in casein, the major phosphoprotein of milk, have been extensively studied; these groups are essential for many of the functional interactions in food systems in which casein is involved. Whole casein has a P content of approximately 6 mol of P/mol of casein and is made up of four individual caseins containing from 1 to 13 monophosphates per molecule (6). These monophosphates are essential for the interactions among caseins to form submicelles and the binding of Ca²⁺ to form micelles (8, 25). Caseins that have had phosphate groups removed or added show dramatic changes in their functional properties (15, 17, 19, 22, 23). Chemical phosphorylation of casein using POCl₃ uses the hydroxyl groups of serine, threonine, and tyrosine as potential binding sites for P (17, 18, 19), although Matheis et al. (15) reported that additional phosphates were bound to the serine residue only.

Nuclear magnetic resonance (NMR) spectroscopy offers a nondestructive method of studying casein structure and interactions in solutions. Casein studies using ³¹P NMR have examined α_{S1}-CN and β-CN (20), superphosphorylated whole casein (15), and the complexes in milk that contain P (3, 4). The NMR spectroscopy has been used to evaluate the rigidity and mobility of β-CN and β-CN (f 1-25) peptide (7), to determine the effect of pH and casein concentration in skim milk and caseinate solutions (14), and to study the Ca²⁺-binding properties of phosphate groups (2) and their role in submicelle and micelle structures (8). Complex food systems, such as rennet-treated milk (13) and cheese (9), also have been examined by NMR spectroscopy.

Fourier transform infrared (FTIR) spectroscopy offers another way to study the global secondary structure of proteins. The use of the Fourier deconvolution, second-derivatives, and regression analysis results in accurate estimates of secondary structures for known globular proteins in aqueous solutions (12), and these methods have been used to study the

submicelle and colloidal micelle structures of whole casein in the presence of KCl and CaCl₂ (11).

Both NMR and FTIR analyses offer unique opportunities to enhance the knowledge of casein structure and interaction and to provide insight as to how modification can alter the functional properties of casein. Currently, very little NMR and FTIR data have been published about whole casein with different phosphate contents. This study used ³¹P NMR spectroscopy to determine phosphorylation sites and the types of P in whole casein containing 7 to 220% P compared with unmodified casein (100% P) and superphosphorylated caseins at increasing concentrations. Superphosphorylated caseins were examined using FTIR spectroscopy to determine how the additional phosphates influence the secondary structure of whole casein.

MATERIALS AND METHODS

Sample Preparation

Sodium caseinate was prepared from raw skim milk by isoelectric precipitation according to the procedure of Van Hekken and Strange (22); sodium caseinate had an estimated average monomer molecular mass of 23,000 Da and contained an average of 5.66 ± 0.20 mmol of P/mmol of casein (100% P). Protein content was calculated by multiplying total Kjeldahl N (1) by 6.34, the conversion factor for milk and dairy products (10). The total P content of the caseins was determined using the procedure of Sumner (21) in which the casein was digested in sulfuric acid, and the P content was colorimetrically assayed using ammonium molybdate, sulfuric acid, and ferrous sulfate. Free P was the amount of P left in supernatant after the 0.1% solution of casein in 0.15 M NaCl had been stirred for 24 h at 4°C, and casein had been precipitated with trichloroacetic acid treatment, and the precipitate had been centrifuged (23). Bound P was determined by subtracting the amount of free P from the total P.

Dephosphorylated caseins were prepared using potato acid phosphatase as described by Van Hekken and Strange (22). Modified casein contained either 0.38 or 3.34 mmol of bound P/mmol of casein (7 or 60% P).

Superphosphorylated caseins were prepared using a mixture of POCl₃ and CCl₄ and three different reaction pH as described by Van Hekken et al. (23). Casein that had been modified at pH 5, 7, and 9 had bound P contents of 9.09, 10.6, and 12.5 mmol of P/mmol of casein (160, 190, and 220% P, respectively).

³¹P NMR

All ³¹P NMR spectra were obtained using a Varian NMR spectrometer (Unity +400; Varian Associates, Palo Alto, CA), operating at 161.8 MHz. The spectra were obtained with a 5-mm broad band probe for a total sample volume of 1.0 ml. A 0.0012 mM hexamethyl phosphoramide (HMPA) reference capillary was used as an internal reference. The chemical shift of HMPA is 30.7 ppm with respect to 85% H₃PO₄. A pulse width of 45° and a recycle time of 2.5 s were used to collect most of the data, although recycle times of 2 and 10 s were used on one 220% P casein sample to determine whether P peaks were being overestimated or underestimated. Typically, 4098 or 5216 data points were obtained for each scan. The sweep width was 25000 Hz with a digital resolution of at least 6.1 Hz per point. The number of scans varied depending upon the concentration of the sample. Usually 10k scans were obtained for 2% samples and 50k scans for the 1% samples. Data were analyzed (NUTS-2D program; Acorn NMR, Fremont, CA), and each fit was exponentially multiplied with a 5-Hz line broadening before Fourier transformation. Unless otherwise noted, for each figure, the plot intensities were normalized to the HMPA peak that was set to 1.0. The peak areas were determined using the NUTS-2D line fit subroutine. The line fit subroutine is an interactive process that has an input of line width and peak height. The goodness of fit is used on the residual sum of the squares difference between the calculated spectrum and the actual spectrum.

Samples were prepared in D₂O (Cambridge Isotope Laboratories, Woburn, MA) without adjustment for pH (except for the 60% P casein sample, which was adjusted to pH 7 for solubility), and the spectra were obtained within 24 h. One percent solutions were prepared for each casein (7, 60, 100, 160, 190, and 220% P), and 0.2 and 2.0% solutions were prepared for the 100, 160, 190, and 220% P caseins.

FTIR

The 3% solutions for 100, 160, 190, and 220% P caseins were prepared in deionized water without pH adjustment and then were examined using an FTIR spectrometer (Nicolet 740; Nicolet Instrument Corp., Madison, WI). Spectra were obtained, and data were analyzed according to the methods described by Kumosinski and Unruh (12). The second derivatives of the original spectra were examined to identify the number and location of component bands. The unresolved spectra then were analyzed using Fourier deconvolution (nonlinear regression fitting proce-

dures) to enhance the resolution of the original spectra and to create the individual component peaks and the outer envelope peak. The integrated areas were calculated for the individual peaks that correlated with secondary protein structure, and the percentages of helices, sheets, turns, and loops were determined.

RESULTS AND DISCUSSION

^{31}P NMR

The ^{31}P chemical shift of the internal standard, HMPA, was 30.7 ppm (relative to 85% H_3PO_4). The HMPA reference was used instead of H_3PO_4 , which is located at 0.0 ppm, because we wanted the reference peak to be well removed from all other ^{31}P resonances found in our samples. Spectra that were obtained for modified samples without the HMPA reference showed no peaks near 30.7 ppm (data not shown) and, therefore, eliminated the possibility that the reference peak had masked any P peaks in our modified casein samples.

The pH of the casein solutions was not adjusted (except for 60% P casein) because we wanted to examine the casein without further modification and because all of the samples, except for the 60% P caseins, were soluble in D_2O . The unadjusted pH for the 1% casein solutions were 6.5 for 7% P, 4.3 for 60% P, 7.1 for 100% P, 7.4 for 160% P, 8.5 for 190% P, and 9.0 for 220% P caseins. The 60% P casein solution was adjusted to pH 7 because of poor solubility of the unadjusted pH. Chemical shifts for P compounds examined using ^{31}P NMR are pH dependent, and the inorganic P (P_i) was the most sensitive to pH differences (16). In our casein samples, the P_i peak was very sharp, easy to identify, and had chemical shifts that ranged from 3.4 to 2.3 ppm. Therefore, we expected the ^{31}P chemical shifts not to change much under the conditions used. To account for the pH variations, we grouped ranges of chemical shifts together, identified the ranges as A to H, and labeled the peaks in the ^{31}P NMR spectra found in Figures 1, 2, and 3 according to the chemical shift range in which they were located. The summary of the peak areas found within these chemical shift ranges is presented in Table 1.

Recycle time. To determine the quantitative reliability of our ^{31}P NMR data, two spectra were obtained using parameters that were identical except for recycle time (Figure 1). The spin lattice relaxation time of P_i in D_2O is approximately 50 s. The spin lattice relaxation time of the phosphates for native caseins are approximately 0.6 s for micelles and range from 0.2 to 1.0 s for submicelles (8). Using 45° ^{31}P pulse,

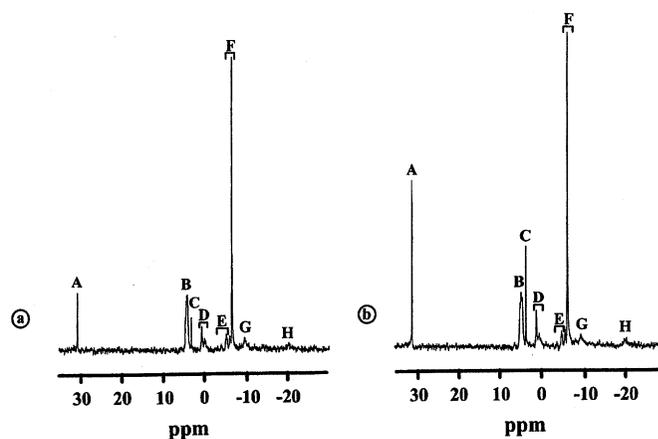


Figure 1. Effect of recycle time [2 s (a) or 10 s (b)] on the ^{31}P nuclear magnetic resonance spectra of 1% solutions of 220% P casein (relative to unmodified casein containing 100% P). Lettered peaks indicate chemical shift range in which the peaks were located: 30.7 ppm hexamethyl phosphoramide internal reference) (A), 3.6 to 4.7 ppm (B), 2.3 to 3.4 ppm (inorganic P) (C), 0.0 to 1.0 ppm (D), -4.0 to -5.7 ppm (E), -6.5 to -7.2 ppm (F), -9.7 to -10.6 ppm (G); and -20.2 to -20.8 ppm (H). See Table 1 for peak area data.

the intensity of the serine phosphates should be constant for both recycle times. Therefore, the spectra in Figure 1 were normalized to the serine phosphate peaks; thus, the noise levels in both spectra were comparable. In addition, differences in peak heights occurred only for the P_i (peak C) and the HMPA (peak A) reference, but the peak areas were the same. For all the other figures, the peak areas were normalized to the HMPA peak as the concentration of HMPA was constant.

Different P content. The spectra for whole casein containing 7 to 220% the amount of P found in unmodified casein are shown in Figure 2, and the areas for various peaks are given in Table 1. The ^{31}P NMR spectra of unmodified whole casein (Figure 2c) contained a single narrow peak between 3.6 and 4.7 ppm (peak B), which was assigned to the serine monophosphates. The chemical shift of the peak was well within the chemical shift range of 3 to 4.4 ppm that has been reported for serine monophosphate peaks in casein solutions (8, 15) and the 3.2 to 3.7 ppm reported for skim milk (3, 4). The serine monophosphate ^{31}P NMR peak is typically a broadly based triplet that is better resolved in the ^{31}P NMR spectra of casein submicelles (3.5, 3.8, and 4.2 ppm) than for casein micelles (3.6, 3.7, and 3.9 ppm) (8). The dispersion of the monophosphate peaks provides information about casein conformation and solution state. In ^{31}P NMR spectra obtained at low energy, the presence of paramagnetic species would have broadened the peaks (7). No peak broadening in the spec-

tra at 400 MHz was observed in this study. Dephosphorylation of whole casein using potato acid phosphatase is very specific in removing monophosphates; therefore, the reduction of the serine phosphate peak (B) and the appearance of the P_i peak (C) in the 60 and 7% P casein sample (Figure 2b and a) were expected. The spectrum for 7% P casein had a small sharp peak at 1.6 ppm (peak C), which was probably P_i . The peak for the remaining serine phosphate was hidden in the baseline noise.

Compared with the 100% P casein, the peaks for serine monophosphate in the 160 and 190% P caseins (Figure 2, d and e) had shifted upfield (4.7 vs. 3.5 and 4.3 ppm, respectively). Analysis of these spectra showed that the number of serine monophosphates did not change. When $POCl_3$ is used to phosphorylate whole casein, the hydroxyl groups of serine, threonine, and tyrosine are potential binding sites of the phosphates (17, 18, 19). The chemical shifts for the phosphorylated versions of these individual amino acids are relatively clustered: serine phosphate, 4.6 to 4.8 ppm; threonine phosphate, 3.7 to 4.0 ppm; and tyrosine phosphate, 0.9 ppm (5). Because the amount of serine monophosphates in the 160 and 190% P caseins did not increase, our study does not support the phosphorylation of threonine or tyrosine residues. Diphosphates have chemical shifts near -7 and -10 ppm (3, 15, 24), and the F and G peaks found in the 160 and 190% P caseins were identified as diphosphates.

The 220% P casein (Figure 2f) contained many different types of P. The serine monophosphate peak was located at 4.7 ppm (peak B) and had a greater peak area than those of the 100, 160, or 190% P caseins, which suggested that serine, threonine, or both had been phosphorylated during modification. The P_i peak was the sharp peak at 3.3 ppm (peak C). The peaks near 0.3, -4, -7, and -10 ppm (peaks D, E, F, and G) were tentatively identified as diphosphates. The peak near 0.3 ppm (peak D) may have been phosphorylated tyrosine (5) or may have reflected the phosphorylation of the carbohydrate moieties bound to κ -CN, because sugar phosphates have chemical shifts between 1 and 0 ppm (24). Terminal polyphosphates also have peaks near -7 and -10 ppm (24), and some of the F and G peaks in this range may have been polyphosphates rather than diphosphates. The peak near -20 ppm (peak H) was identified as nonterminal polyphosphates. This identification confirms the presence of polyphosphates in the sample.

Matheis et al. (15) modified whole casein using $POCl_3$ at pH 6 to 8 to bind an additional 7.4 mol of P/mol of casein and reported that the modified casein

was superphosphorylated exclusively through -O-O-bonds. Those researchers identified peaks at 4.4 ppm as serine monophosphate, at 3.1 ppm as P_i , and near -5 and -10 ppm as diphosphates. Although the casein used in that study had a higher content of bound P (similar to our 220% P casein), the ^{31}P NMR spectrum identified the same types of P (serine monophosphates, P_i , and diphosphates) that were found in our study for 160 and 190% P caseins. The main difference between spectra in the two studies

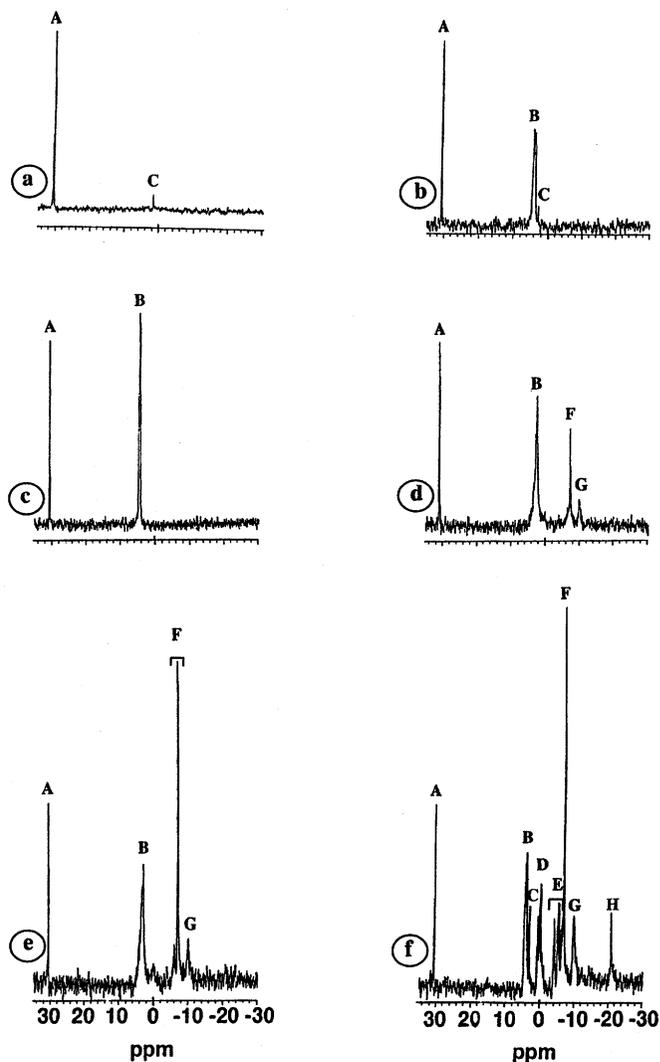


Figure 2. Effect of P content [7% (a), 60% (b), 100% (c), 160% (d), 190% (e), and 220% (f) P casein (relative to unmodified casein at 100% P)] on the ^{31}P nuclear magnetic resonance spectra of 1% solutions of casein. Lettered peaks indicate chemical shift range in which the peaks were located: 30.7 ppm (hexamethyl phosphoramide internal reference) (A), 3.6 to 4.7 ppm (B), 2.3 to 3.4 ppm (inorganic P) (C), 0.0 to 1.0 ppm (D), -4.0 to -5.7 ppm (E), -6.5 to -7.2 ppm (F), -9.7 to -10.6 ppm (G), and -20.2 to -20.8 ppm (H). See Table 1 for peak area data.

was that Matheis et al. (15) was able to separate the serine monophosphate and P_i peaks, and the area ratio of monophosphate to diphosphate was 6:1, but ratios in our study were 3:1 and 1:1 for the 160 and 190% P caseins, respectively.

Casein concentration. As the protein concentration increased from 0.2 to 2%, the area of the peaks for the different types of P increased (Figure 3 and Table 1). The 0.2% protein samples (Figure 3, a3, b3, c3, and d3) were not concentrated enough to give a

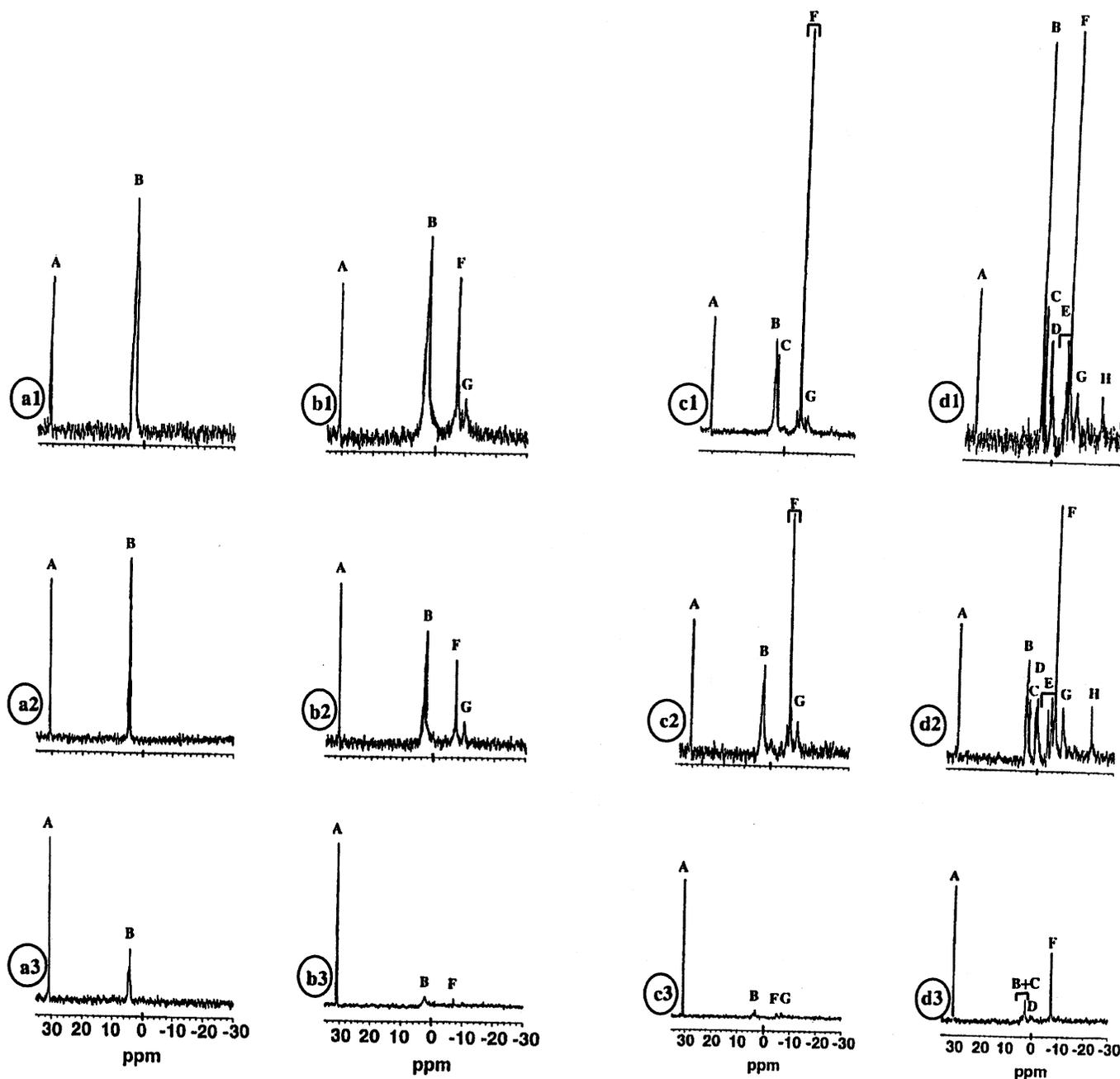


Figure 3. Effect of casein concentration [2.0% (1), 1.0% (2), and 0.2% (3)] and P content [100% (a), 160% (b), 190% (c), and 220% d P casein (relative to unmodified casein at 100% P)] on the ^{31}P nuclear magnetic resonance spectra of casein. Lettered peaks indicate chemical shift range in which the peaks were located: 30.7 ppm (hexamethyl phosphoramide internal reference) (A), 3.6 to 4.7 ppm (B), 2.3 to 3.4 ppm (inorganic P) (C), 0.0 to 1.0 ppm (D), -4.0 to -5.7 ppm (E), -6.5 to -7.2 ppm (F), -9.7 to -10.6 ppm (G), and -20.2 to -20.8 ppm (H). See Table 1 for peak area data.

good signal-to-noise ratio for ^{31}P NMR spectra. As the protein concentration increased, the solutions containing 160 and 190% P caseins (Figure 3, b and c) increased in viscosity and gelled; the 100 and 220% P caseins (Figure 3, a and d) remained fluid. Except for increases in peak area, the shape and location of the peaks in caseins containing 100, 160, and 190% P (Figure 3, a, b, and c) did not vary as concentrations increased, although the P_i peak (C) was visible in Figure 3, c1, d1, and d2. The spectrum for the 220% P casein showed the most variation as protein concentration increased from 0.2 to 1 to 2% (Figure 3, d3, d2, and d1). At $\geq 1\%$, the many different types of P could be identified, as discussed in the P content section. Increased protein concentration did not always increase the area for peaks, as occurred for the peaks at 3.3 and -10 ppm (peaks C and G), the doublet near 0 ppm (peak D), and the triplet between -4 and -5.7 ppm (peak E).

The viscosity of superphosphorylated caseins increased as the protein concentration increased, and some modified caseins formed gels at very low concentrations ($>0.5\%$ casein) (15, 17, 23). The 160 and 190% P casein samples of our study were prepared at concentrations at which the solutions were a liquid

(0.2%), a weak gel (1%), and a stronger gel (2%). The gelation property of the 160 and 190% P casein was attributed to the diphosphates that had ^{31}P NMR peaks at -7 and -10 ppm (peaks F and G). Because no other changes in the peak patterns were noted other than the increase in peak area, the increased protein concentration had not altered the P environment as monitored by the ^{31}P NMR. Electrophoretic studies have suggested that superphosphorylated caseins form complexes that cannot be disassociated with SDS, mercaptoethanol, or urea (15, 17, 23). The complexes contained very stable P interactions and associations that remained the same even as the samples gelled. This result was unexpected; Kakalis et al. (8) reported noticeable differences in the NMR spectra between casein samples in micellar and nonmicellar forms.

The 220% P casein also contained P components that had ^{31}P NMR peaks at -7 and -10 ppm (peaks F and G) as well as many other types of P, but had not increased in viscosity or gelled as the concentration increased. The sample also formed complexes that could not be disassociated with SDS, urea, or mercaptoethanol (23). This result suggests that the complexes contained very stable P associations, but the

TABLE 1. Summary of peak areas found within chemical shift ranges for ^{31}P nuclear magnetic resonance spectra for casein containing 7 to 220% P (relative to unmodified casein = 100% P) at various protein concentrations.¹

Casein	Concentration	Peak area for chemical shifts								Figure showing spectra
		A 30.7 ppm	B 3.6 to 4.7 ppm	C 2.3 to 3.4 ppm	D 0.0 to 1.0 ppm	E -4.0 to -5.7 ppm	F -6.5 to -7.2 ppm	G -9.7 to -10.6 ppm	H -20.0 to -20.8 ppm	
		(%)								
7% P	1.0	1.0	...	0.3	2a
60% P	1.0	1.0	4.3	0.1	2b
100% P	0.2	1.0	1.1	3a1
	1.0	1.0	4.7	2c, 3a2
	2.0	1.0	9.5	3a3
160% P	0.2	1.0	1.6	0.3	3b1
	1.0	1.0	4.5	1.0	0.6	...	2d, 3b2
	2.0	1.0	8.8	2.5	1.8	...	3b3
190% P	0.2	1.0	0.4	0.1	0.2	...	3c1
	1.0	1.0	4.8	2.9	1.7	...	2e, 3c2
	2.0	1.0	7.2	0.8	6.5	2.1	...	3c3
220% P	0.2	1.0	0.4	0.7	0.5	...	1.2	3d1
	1.0	1.0	5.9	0.6	3.4	3.6	4.0	3.0	1.3	2f, 3d2, 4a
	2.0	1.0	9.5	0.7	2.8	4.6	11.6	2.4	2.4	3d3
	1.0 ²	0.1	1.0	0.1	0.3	0.3	1.0	0.3	0.1	1a
	1.0 ³	0.1	1.0	0.1	0.3	0.5	0.8	0.3	0.1	1b

¹Chemical shift ranges were identified as A to H [hexamethyl phosphoramidate, internal reference (A), inorganic P (C)] and were used to label peaks in Figures 1 to 3. Data were collected at 2.5 s recycle time unless otherwise noted.

²Data collected at 2-s recycle time.

³Data collected at 10-s recycle time.

presence of polyphosphates prevented gelation. Further investigation is being conducted to understand this viscosity and gelation phenomenon.

FTIR

The FTIR data for 100, 160, 190, and 220% P caseins are presented in Figure 4, and the calculated distribution of secondary structure components is in Table 2. The original spectra and the second derivatives of the FTIR data appear to have slight differences among the casein samples, although the calcu-

lated distribution of secondary structure (extended strand and sheet, helix, loop, and turns) shows very little difference in the secondary structure.

The FTIR illustrated that the incorporation of different forms of the P bound to casein or the gelation of the superphosphorylated caseins did not alter the secondary structure of the whole casein. Any changes in the distribution of extended strand and sheet, helix, loop, or turn would have indicated that the addition of phosphate groups was either unfolding or compressing the proteins. The binding of significant amounts of negatively charged phosphate groups

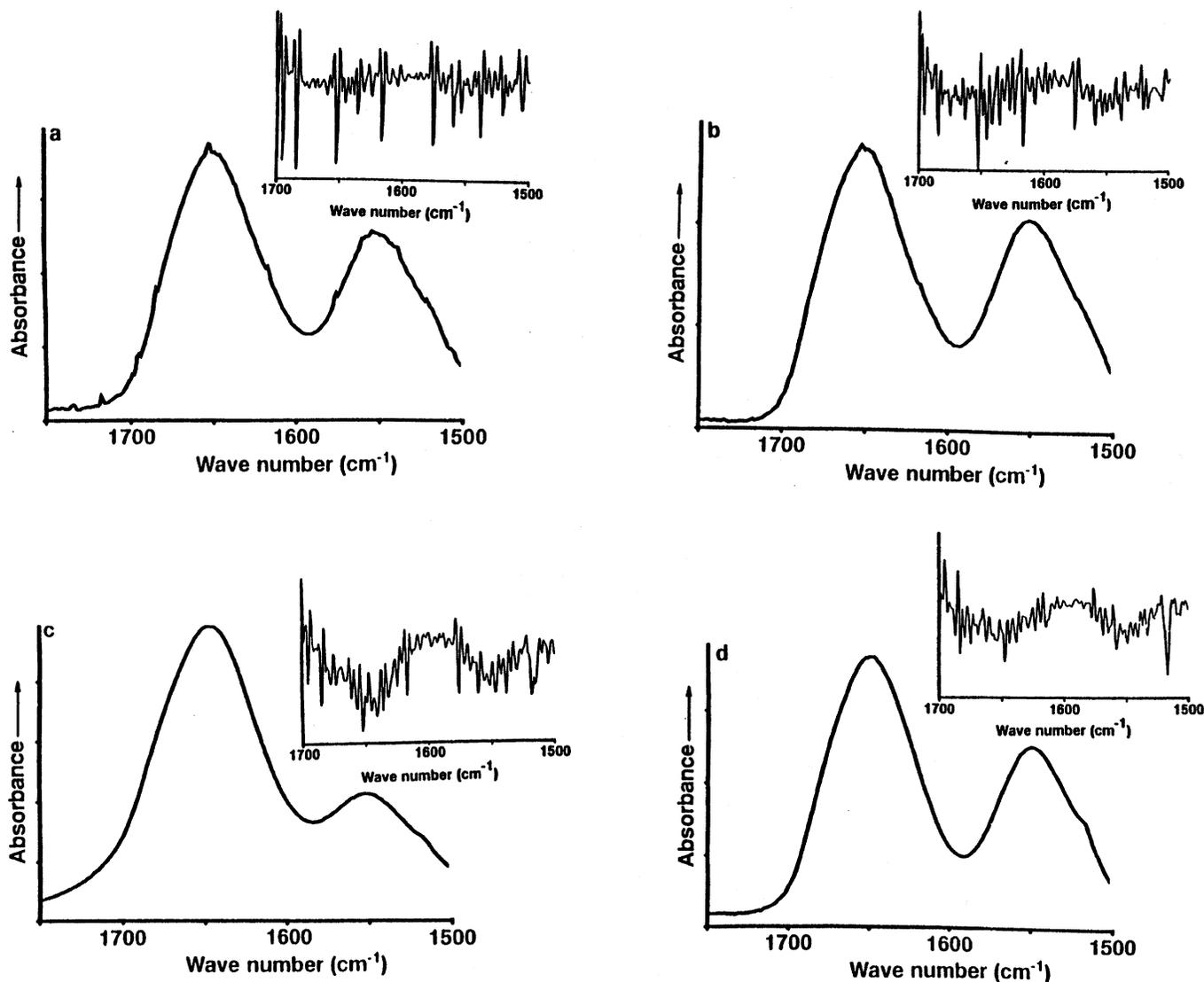


Figure 4. The Fourier transform infrared analysis of 3% solutions of casein with different P content [100% (a), 220% (b), 160% (c), and 190% (d) P casein (relative to unmodified casein = 100% P)]. The main figures are the original infrared spectra, and the insets are the second-derivative variation.

TABLE 2. Distribution of secondary structures in whole casein with P contents of 100 to 220% above normal P (percentage of P was relative to unmodified casein at 100% P) using Fourier transform infrared spectroscopy (11).

Structure	Casein			
	100% P	160% P	190% P	220% P
	(%)			
Extended strand and sheet	37.83	38.15	38.82	36.94
Helix	17.68	17.60	15.18	18.57
Loop, irregular	15.28	15.14	14.34	15.71
Turn	29.21	29.11	31.66	28.78

to whole casein would be expected to influence the secondary structure of the protein to a greater extent, especially in view of the electrophoretic studies discussed herein. The FTIR results suggested that tertiary or quaternary intra- or interactions were involved in the structural changes (formation of complexes or gelation) that were noted in superphosphorylated caseins.

CONCLUSIONS

The ^{31}P NMR spectra showed that the forms and amounts of P in dephosphorylated and superphosphorylated caseins were different from those in unmodified whole casein. All caseins contained serine monophosphates. Superphosphorylation up to 190% P resulted in the addition of diphosphates to whole casein; at 220% P, both di- and polyphosphates were formed. As protein concentrations increased, no changes in ^{31}P NMR spectra (outside of increased peak areas) were noted for the 160 and 190% P caseins, which gelled. The spectrum for 220% P casein showed more variability as concentration increased, but the sample did not gel. Therefore, the incorporation of diphosphates, but not polyphosphates, was conducive to the gelation of caseins that we noted in this study. The FTIR data showed that the superphosphorylation of caseins did not change the distribution of secondary structures and suggested that the structural changes noted in superphosphorylated caseins were due to tertiary or quaternary intra- and interactions. This research supplies baseline ^{31}P NMR spectra for whole casein at a wide range of P contents, identifies the type of P compounds present in superphosphorylated casein samples, and examines for the first time the distribution of secondary structures in superphosphorylated casein using FTIR. This study confirms the importance of phosphate groups in the interaction of casein with its environment and illustrates also why modification has such a dramatic influence on the physical and functional properties of a protein in a food system.

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