Phosphorus Nuclear Magnetic Resonance Study of Phosphoproteins

I. CHEMICAL NATURE OF PHOSPHORUS ATOMS IN BOVINE $\alpha_c$-CASEIN*

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SUMMARY

Phosphorus nuclear magnetic resonance spectroscopy has been used to investigate the chemical nature of the phosphorus atoms in bovine $\alpha_c$-casein. A satisfactory $^{31}$P nuclear magnetic resonance spectrum can be obtained in a sample containing 26% by weight of the protein in a 6.5 M urea. The average $^{31}$P chemical shift of $\alpha_c$-casein is 221 ppm from PCl$_3$ with a standard deviation of 1 ppm over the pH range 4 to 9. The variations with pH of the phosphorus chemical shift of model compounds adenosine 5'-triphosphate, O-phosphoserine, creatine phosphate, reduced diphosphopyridine nucleotide, and sodium pyrophosphate fall into two categories: (a) the chemical shifts in compounds with substituted pyrophosphate or phosphodiester linkages remain essentially constant in the pH range 4 to 9, whereas (b) for monoester compounds, there is a relatively large change in chemical shift (greater than 4 ppm) as the pH changes from 3 to 9, i.e. as the phosphate group goes from the singly ionized to the doubly ionized state. This difference (which is to be expected from a priori considerations) suggests that there may be phosphodiester or disubstituted pyrophosphate bonds, or both, in bovine $\alpha_c$-casein. The relation of this type of phosphate linkage to the structure of $\alpha_c$-casein and to its stability in aqueous solution is discussed.

Considerable controversy has centered around the nature of the organic phosphorus atoms in the caseins. The work of Perlmann (4) and Thoai, Roche, and Pin (5) indicated the presence of organic phosphomonoester, phosphodiester, and pyrophosphate groups. However, Sundararajan and Sarna (6), Hofman (7), Kalan and Telka (8), and Belec and Jenness (9) have given evidence suggesting exclusively the monoester form. Their conclusions (with the exception of that of Belec and Jenness, which was based on indirect calorimetric measurement) on the nature of the phosphorus atoms in caseins were based on the action of specific phosphatases toward the caseins compared with the action on simple model compounds. For example, on the basis of the action of prostate phosphatase, intestinal phosphatase, and potato phosphatase on $\alpha_c$-casein, Perlmann (4) concluded that $\alpha_c$-casein contained 60% of its phosphorus as monoester, $-\mathrm{O}-(\mathrm{PO})_3$; 40% as diester, $-\mathrm{OP}-(\mathrm{OH})_2$; and 20% as pyrophosphate, $-\mathrm{OP}-(\mathrm{OH})-(\mathrm{OP})-(\mathrm{OH})_2$. As Perlmann (4) and Hofman (7) pointed out, there were serious difficulties in interpreting these experiments. The main difficulty arose from the assumption that the specificity of a given phosphatase is the same whether it acts on phosphate links in small molecules or in proteins. Owing to the conflicting evidence from enzymic studies, Hofman (7) concluded that previous studies on the nature of the phosphorus atoms in caseins must be treated with reservation. Furthermore, purified components of the caseins were not available to these workers.

The purpose of this communication is to discuss a new physical-chemical technique, $^{31}$P nuclear magnetic resonance, to investigate the chemical nature of the phosphorus atoms in bovine $\alpha_c$-casein. The chief limitation in using $^{31}$P NMR arises from considerations of sensitivity. Fortunately, this limitation can be overcome to some extent by use of large sample tubes and also by the use of urea to increase the solubility of the protein and to reduce the viscosity of the aqueous protein solution. According to Waugh et al. (1), urea up to 6.5 M does not have any irreversible effects on bovine $\alpha_c$-casein. Indeed, Waugh et al. (1) were able to purify this protein in the presence of 4.5 M urea and to determine the molecular weight of the monomer in 6.5 M urea from pH 4.3 to 4.6.

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1 The abbreviation used is: NMR, nuclear magnetic resonance.
EXPERIMENTAL PROCEDURE

Materials—Purified bovine $\alpha_s$-casein was prepared according to the method of Waugh et al. (1). Fresh raw milk from a pure Guernsey breed and a pure Holstein breed was used. $\alpha_s$-Casein obtained from these two breeds belongs to the Type B genetic variant, according to the notation of Thompson and Kiddy (3). The protein solution was exhaustively dialyzed first against potassium citrate and potassium chloride, and then against deionized water, and was freeze-dried by the usual method. In order to increase the solubility of the protein, lyophilized $\alpha_s$-casein samples were dissolved to make a solution 26% by weight in 6.5 M urea, either in the presence of 0.1 to 0.54 M Tris in 0.02 M EDTA at pH 9 or in the presence of 0.1 to 0.54 M citric acid in 0.02 M EDTA at pH 4. Protein samples usually were prepared 10 to 16 hours before NMR measurements and were stored in a refrigerator until use unless stated otherwise. Urea (6.5 M, Baker reagent grade) was freshly prepared according to the method of Waugh et al. (1). O-Phosphoserine was purchased from Mann (Lot K1975). Reduced $\beta$-diphosphopyridine nucleotide was purchased from Sigma (Lot 458-6240). Adenosine 5'-triphosphate (disodium salt) was purchased from Sigma (Lot S37-7090). Creatine phosphate was purchased from Nutritional Biochemicals (Lot 5717). All other chemicals used were of analytical reagent grade and were used without further purification. Glass-distilled water was used in the preparation of solutions for NMR study. Compounds were dissolved in appropriate buffer solutions which had been adjusted to the desired pH. EDTA (final concentration, 0.003 to 0.02 M) was added to each solution to counteract the effect of possible contamination by paramagnetic metal ions. A Radiometer pH meter, model 4, in conjunction with a type C combination electrode, was used for pH measurements.

Methods—About 3 ml of the protein and other solutions were transferred to Pyrex test tubes (15 × 125 mm) for nuclear magnetic resonance measurements. NMR spectra were obtained from a Varian 4302 dual purpose spectrometer, operated at 15,085 mc per sec. In order to maximize the sensitivity for detecting the relatively weak $^3$P NMR signals from the protein, the following scheme was utilized. Audio side band detection was used at a modulation frequency of about 400 cps; the spectra were run in the dispersion mode, under rapid passage conditions similar to those often used for $^1$H NMR studies (10). Chemical shifts, $\delta$, were measured relative to an external reference of PCl$_3$.

RESULTS

The experimental results on the phosphorus chemical shifts as a function of pH for $\alpha_s$-casein, O-phosphoserine, creatine phosphate, reduced $\beta$-diphosphopyridine nucleotide, adenosine 5'-triphosphate, sodium pyrophosphate, and 85% phosphoric acid are summarized in Table I and Fig. 1. A typical $^3$P NMR spectrum of bovine $\alpha_s$-casein is given in Fig. 2. The first two peaks at the left (i.e., at low magnetic field) are the two side bands of the reference compound, PCl$_3$. The other two peaks correspond to the two side bands of $\alpha_s$-casein at pH 9. The radio frequency power used for the protein signal was about 2.5 times greater than that for the PCl$_3$ line. The signal to noise ratio of the protein signal was approximately 4:1. The concentration of total phosphorus in the $\alpha_s$-casein solution was about 0.086 M. Inasmuch as the protein solution at these concentrations was rather viscous, the natural line width for the protein-$^3$P NMR line was greater than that of the reference, PCl$_3$; the observed line width for the protein signal was about 80 cps, while that for the $^3$P signal in O-phosphoserine was, under the same modulation and radio frequency field conditions, about 37 cps. No change (to within approximately 25 cps, the accuracy of this measurement) in the line width of the protein signal could be detected over the pH range 4 to 9.

In order to determine what effects the medium might have on the $^3$P chemical shifts, the following experiments were carried out. It was found that the $^3$P chemical shifts for 0.1 M O-phosphoserine in the presence of 6.5 M urea at pH 3.81 and in the presence of 50% glycerol by volume (final concentration) at pH 3.87 were 223 ppm and 222 ppm from PCl$_3$, respectively; these shifts are essentially identical with that (222 ppm) of 0.1 M O-phosphoserine at pH 3.64. These results indicate that neither the presence of high concentration of urea nor the increase in viscosity of the medium affects the chemical shift of O-phosphoserine. There is no observable difference in $^3$P chemical shift for O-phosphoserine whether it had been dissolved in Tris buffer or citric acid buffer, provided these two buffer systems had been adjusted to the same final pH. There was a change of 6 ppm in $^3$P chemical shift for O-phosphoserine in going from the singly ionized phosphate state (222 to 223 ppm at pH 3) to the doubly very similar, the latter cannot be used as an external reference. Unfortunately, a more suitable reference, such as PCl$_3$, was not available to us. Even with a less favorable reference compound like PCl$_3$ (the chemical shift of PCl$_3$ is about 200 ppm downfield from $\alpha_s$-casein and other model compounds), accurate and reproducible chemical shift measurements could still be obtained. The experimental precision in this work, 2 ppm, represents the standard deviation of six or more measurements from the average for each value of the pH.

The modulation frequency was counted during the course of each run by a Beckman 5210 audio frequency counter.
A change of 4 ppm in phosphorus chemical shift was found in all ingredients previously having been immersed in ice water. Samples of 0.09 M creatine phosphate were freshly prepared just before measurement by solutions in buffers at pH greater than 4, all ingredients previously having been immersed in ice water. A change of 4 ppm in phosphorus chemical shift was found in going from pH 4.26 (228 ppm) to pH 8.85 to 9.3 (224 ppm). Samples of creatine phosphate at pH 4.26 and pH 9.30 were then each immersed in a boiling water bath for 10 min. After heating, the sample at pH 9.30 showed two phosphorus resonance signals, corresponding to chemical shifts of 223 ppm and 218 ppm from PCl₃. The final pH value for this sample (after being heated) was 8.84. The results suggest that creatine phosphate at pH 9.30 had been partially hydrolyzed after the heating. In the case of the 0.09 M creatine phosphate sample at pH 4.26, the phosphorus chemical shift changed from 228 ppm to 224 ppm and the pH of the solution increased to pH 5.21 after the sample was heated; the change in chemical shift here may be due to a change in pH or due to hydrolysis. These results indicate that the phosphorus chemical shifts for the original creatine phosphate solution (Table I) are those corresponding to the presumed molecule.

The phosphorus chemical shifts for 0.18 M adenosine triphosphate at pH 8.42 are 233, 243, and 227 ppm from PCl₃ for α- and β and γ-P atoms, respectively. In the case of the 0.06 M ATP at pH 4.90, the chemical shifts of the β-P and (α + γ)-P atoms are 244 and 232 ppm, respectively. There is a change of 5 ppm in the chemical shift of the γ-P atom in ATP from pH 4.90 to pH 8.42. This is to be expected because the γ-P phosphate group changes from the singly ionized state at pH 4.90 to the doubly ionized state at pH 8.42. There is a change of only 1 ppm for both α- and β-P atoms from pH 4.90 to pH 8.42, as would be expected for a phosphate group having 1 ionizable proton. Our experimental results on the variation of the ³¹P chemical shifts with pH for ATP are in good qualitative agreement with those of Cohn and Hughes (16); by converting our chemical shift data on ATP with respect to 85% H₃PO₄ as reference (219 ppm, see Table I), our data and those of Cohn and Hughes agree within 2 ppm (or within experimental accuracy). We might note that Cohn and Hughes found a small variation (less than 1 ppm) with pH in the chemical shifts of the α-P and β-P of ATP.

There is a change of 6 ppm for sodium pyrophosphate in going from the quadruply ionized state (pH 10.4) to the doubly ionized state (pH 9.4). Our data on pyrophosphate are in agreement with those of Callis et al. (17) and of Nielsen and Pustinger (18).

In the case of 0.1 M β-DPNH there is a change of only 1 ppm in phosphorus chemical shift found over the pH range 4.4 to 8.8. Since β-DPNH has a disubstituted pyrophosphate linkage, one would expect that the change of ³¹P chemical shift for β-DPNH should be very small or essentially independent of pH from 4 to 9. The average phosphorus chemical shift for bovine α-casein B₁ over the range of pH 4 to pH 9 is 221 ppm with a standard deviation of 9 ppm.

Unfortunately, no measurements on creatine phosphate at a pH of less than 4 were made, for fear the compound would be hydrolysed. Since the apparent dissociation constant for the second ionization step is given by a pK of 4.5 to 4.6 (12, 14), the full variation with pH of the ³¹P chemical shift of creatine phosphate was not obtained.

It is interesting to compare the chemical shifts of the protein with those of the model compounds. Within experimental error, the ³¹P chemical shift for α-casein over the pH range 4 to 9 is identical with that of O-phosphoserine at pH 3. Since Lipman (19) actually had isolated O-phosphoserine from casein, it is tempting to speculate that serine residues are linked to the phosphate groups in α-casein. This kind of speculation can be dangerous for a complicated molecule like a protein, because other environmental factors could affect the chemical shift (see "Discussion" and Bothner-By (20)).

### Table I

Phosphorus chemical shifts as function of pH with PCl₃ as external reference compound

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Concentration</th>
<th>³¹P chemical shift, δ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-Phosphoserine</td>
<td>3.04</td>
<td>0.045 M</td>
<td>223</td>
</tr>
<tr>
<td>O-Phosphoserine</td>
<td>3.27</td>
<td>0.089 M</td>
<td>223</td>
</tr>
<tr>
<td>O-Phosphoserinea</td>
<td>3.81</td>
<td>0.100 M</td>
<td>222</td>
</tr>
<tr>
<td>O-Phosphoserineb</td>
<td>3.87</td>
<td>0.100 M</td>
<td>222</td>
</tr>
<tr>
<td>O-Phosphoserine</td>
<td>8.49</td>
<td>0.089 M</td>
<td>217</td>
</tr>
<tr>
<td>O-Phosphoserine</td>
<td>8.76</td>
<td>0.045 M</td>
<td>216</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>4.26</td>
<td>0.000 M</td>
<td>228</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>8.84</td>
<td>0.000 M</td>
<td>224</td>
</tr>
<tr>
<td>Creatine phosphate</td>
<td>9.30</td>
<td>0.000 M</td>
<td>224</td>
</tr>
<tr>
<td>Adenosine 5′-triphosphate</td>
<td>4.90</td>
<td>0.000 M</td>
<td>223 (α + γ)-P</td>
</tr>
<tr>
<td>Adenosine 5′-triphosphate</td>
<td>8.42</td>
<td>0.180 M</td>
<td>244 α-P</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
<td>4.06</td>
<td>0.100 M</td>
<td>233</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
<td>9.67</td>
<td>0.180 M</td>
<td>227</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
<td>10.39</td>
<td>0.200 M</td>
<td>227</td>
</tr>
<tr>
<td>β-DPNH</td>
<td>4.40</td>
<td>0.100 M</td>
<td>233</td>
</tr>
<tr>
<td>β-DPNH</td>
<td>8.76</td>
<td>0.100 M</td>
<td>224</td>
</tr>
<tr>
<td>β-DPNH</td>
<td>8.76</td>
<td>0.100 M</td>
<td>224</td>
</tr>
<tr>
<td>α₁-Casein (Guernsey)</td>
<td>4.24</td>
<td>20%</td>
<td>222</td>
</tr>
<tr>
<td>α₁-Casein (Holstein)</td>
<td>5.27</td>
<td>20%</td>
<td>222</td>
</tr>
<tr>
<td>α₁-Casein (Guernsey)</td>
<td>5.40</td>
<td>20%</td>
<td>221</td>
</tr>
<tr>
<td>α₁-Casein (Holstein)</td>
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<td>α₁-Casein (Guernsey)</td>
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<td>20%</td>
<td>221</td>
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<tr>
<td>α₁-Casein (Guernsey)</td>
<td>9.17</td>
<td>20%</td>
<td>220</td>
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<tr>
<td>Phosphoric acid</td>
<td></td>
<td>85%</td>
<td>219</td>
</tr>
</tbody>
</table>

* a In the presence of 6.5 M urea.
* b In the presence of 50% glycerol by volume (final concentration).
* c The solution was prepared at room temperature and was allowed to remain at room temperature for 4 to 5 hours before measurement.
* d The solution was prepared about 12 hours before measurement and was stored in a refrigerator until measurement.
FIG. 1 (upper). $^3$P chemical shifts as a function of pH. A, summary of results for diprotic phosphate compounds; B, summary of results for monoprotic phosphate compounds. $\bigcirc$, O-phosphoserine; $\triangle$, creatine phosphate; $\Delta$, adenosine triphosphate; $\bigcirc$, sodium pyrophosphate; $\bigtriangledown$, $\beta$-DPNH; $\bullet$, $\alpha$-casein (these all represent experimental points). Solid lines in A are drawn from limiting values of the chemical shift in singly ionized state (pH approximately 3) and doubly ionized state (pH approximately 8) and from known values of the apparent dissociation constants of the phosphate groups, $pK'_2 = 6.13$ for O-phosphoserine (see Footnote 12); $pK'_2 = 4.50$ for creatine phosphate (12); $pK'_2 = 6.48$ for adenosine triphosphate (13); and $pK'_3 = 6.54$ and $pK'_4 = 8.44$ for sodium pyrophosphate (14). Solid lines in B are drawn from the average of the experimental points.

FIG. 2 (lower). Phosphorus resonance spectrum of 26% bovine $\alpha$-casein in 6.5 M urea in 0.54 M Tris in 0.02 M EDTA at pH 9.17. The modulation frequency is 407.9 cps.

After each measurement, the $\alpha$-casein sample was frozen. After 1 to 3 months of storage in a freezer, the protein samples were thawed at room temperature. It was found that the $^3$P chemical shifts of these samples were about 2 ppm higher than the original values (i.e. before the freeze-thaw process). The pH values of these protein solutions were then adjusted to the appropriate point by adding solid Tris or citric acid. However, the $^3$P chemical shifts of the protein solution were still about 1 to 2 ppm higher than the original values. For example, the $^3$P chemical shift of the $\alpha$-casein (Holstein) solution at pH 6.66 after the freeze-thaw process was 233 ppm, while the original value was 221 ppm. Upon addition of 0.1094 g of Tris to this sample, the pH of the solution increased to pH 8.75 and the corresponding $^3$P chemical shift was 222 ppm. The average $^3$P chemical shift of five samples after they had been frozen and thawed and the pH values had been readjusted was found to be 225 ppm with a standard deviation of 1 ppm in the pH range 4.47 to 8.75. It is possible that the freeze-thaw process had some irreversible effect on $\alpha$-casein. In any case, the conclusion reached in this paper, that the protein $^3$P chemical shift of $\alpha$-casein is independent of pH, would still hold, both for the freshly prepared samples and for those which had been frozen and thawed.

This upper limit is arrived at by an estimate of the limiting sensitivity of the NMR instrument: a viscous solution of 0.1 M O-phosphoserine at pH 3.87 in the presence of 50% glycerol (final concentration) gave (under the same experimental conditions as used for $\alpha$-casein) a signal with line width of 37 cps and a signal to noise ratio of 5.3:1; the 26% solution (by weight) of $\alpha$-casein gave a signal with line width of 80 cps and a signal to noise ratio...
firm conclusions can still be drawn concerning the nature of the phosphorus atoms giving rise to the observed signal (see "Discussion").

**DISCUSSION**

The application of nuclear magnetic resonance techniques to the determination of molecular structure has been discussed in detail by Pople, Schneider, and Bernstein (11). In general, the principal source of the chemical shift is the induced magnetic field arising from the electrons in the immediate environment of a nucleus. Chemical shift data for several hundred inorganic and organophosphorus compounds have been published, and attempts have been made to correlate these data with bond properties and molecular structure (17, 18, 21-24). Nevertheless, even with a large body of empirically based data available, one can still not make more than gross correlations between chemical shifts and molecular structure. For example, even though the distinction between phosphorus atoms in N—P—O and O—P—O linkages is not subtle, the observed range of chemical shifts (208 to 216 ppm relative to PC15 (18)) for the first type overlaps that for the second (213 to 243 ppm (18)). Thus, one could not, solely on the basis of chemical shift data, assign a phosphorus NMR line to an N—P—O linkage rather than to an O—P—O linkage.

The discussion above serves to emphasize that the conclusions derived from the present NMR study are based, not on an absolute correlation of the $^31$P chemical shift of the protein with those of model compounds, but rather on the variation of this chemical shift with pH.

Three properties of the $\alpha$-casein molecule enable $^31$P NMR techniques to be used with relative ease and with no ambiguity in the results; these properties have to do with the relatively high concentration of phosphorus atoms per molecule and with the stability of the molecule in the presence of urea and over a wide range of pH. There are 9 to 10 phosphorus atoms per molecule of $\alpha$-casein (1, 3). High concentrations of urea do not have any irreversible effects on this protein (1); consequently, the sensitivity of NMR measurements can be increased by dissolving high concentration of $\alpha$-casein in 6.5 M urea. In the presence of 6.5 M urea (over the range of pH 4 to pH 9), $\alpha$-casein is presumed in the form of monomer (1). Ho and Waugh (25) have found that all of the ionizable groups in $\alpha$-casein are completely accessible to hydrogen ions from pH 2 to pH 12 and that the titration curve is reversible.

In general, one should expect a titration curve-type change in the chemical shift of the $^31$P as the phosphate group goes from the doubly ionized state to the singly ionized state, with decreasing pH (Reference 16 and Fig. 1A). The fact that no large change in chemical shift ($>2$ ppm) was observed as the pH of the protein solution was varied from pH 9 to pH 4 suggests that there is only 1 ionizable proton attached to a phosphate group and that the phosphate is presumably a phosphodiester or a disubstituted pyrophosphate bond in bovine $\alpha$-casein. As shown in Fig. 1, the experimental results fall into two groups. The variation of $^31$P chemical shift as a function of pH for compounds which contain phosphate groups with 2 ionizable protons, i.e., diprotic phosphate groups, is shown in Fig. 1A and that for compounds which have phosphate groups with 1 ionizable proton, i.e., monoprotic phosphate groups, in Fig. 1B. The data of Fig. 1 indicate clearly that the variation of $^31$P chemical shifts as a function of pH for $\alpha$-casein is much closer to that of the disubstituted pyrophosphate or phosphodiester compounds than to that of the phosphomonoester compounds.

Ho and Waugh (25) reported that the agreement between the number of the charged groups of $\alpha$-casein as determined by titration and that determined by amino acid analysis is good (i.e., within two groups) with the exception of the side chain carboxyl groups, which are 40 ± 3 per molecule from titration (based on the assumption that all phosphorus atoms are in the form of monoester), whereas amino acid analysis gives a value of 46 ± 3. As pointed out by Ho and Waugh (25), this discrepancy is reduced or eliminated if some or all of the phosphorus atoms are in the form of phosphodiester or disubstituted pyrophosphate bonds, or both.11

The end group studies of Manson (27), Waugh et al. (1), and Kalan, Thompson, and Greenberg (28) indicated that there are two carboxyl-terminal groups (tryptophan and leucine) and only one amine-terminal group (arginine) per molecule of $\alpha$-casein. This apparent discrepancy between the end group studies may be resolved by the suggestion (1) that one of the NH2-terminal groups in $\alpha$-casein is blocked, like the acetyl derivatives in tobacco mosaic virus protein (29). Chemical analysis indicates that there are no disulfide bonds in $\alpha$-casein (1, 25). If there are indeed two polypeptide chains per $\alpha$-casein, other types of cross-linkage or some kind of forces (hydrogen bonding, electrostatic, or hydrophobic interactions), or both, must be responsible for holding or linking these two polypeptides chains together. The monomer of $\alpha$-casein can be obtained under the following experimental conditions: (a) in the presence of 6.5 M urea-0.1 M KC1 at pH 4.3 to pH 4.6 (1); (b) at pH 12, 0.3 to 1.2 ionic strength, and room temperature (30); and (c) in 0.01 M KC1 at pH 7 and 25° as determined by osmotic pressure.12 Furthermore, $\alpha$-casein combines reversibly with hydrogen ions over the pH range from pH 2 to pH 12 (25). All these results suggest that $\alpha$-casein is very stable over a wide range of extreme experimental conditions and that the molecule of 30,000 molecular weight does not dissociate any further even under extreme conditions. Consequently, if there are two polypeptide chains per monomer, the existence of phosphodiester or disubstituted pyrophosphate types of bonds as a linkage would be compatible with the experimental results.

The observed line width for a 26% $\alpha$-casein $^31$P NMR signal (in 6.5 M urea) is approximately 80 cps, which is about twice the

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11 Ho and Waugh (26) found from their infrared study that there is a weak and a broad absorption peak between 930 and 920 cm$^{-1}$, for $\alpha$-casein alone or in a mixture of $\alpha$- and $\varepsilon$-casein on dry film, which can be attributed to a pyrophosphate linkage. The present $^31$P NMR data on $\alpha$-casein are not inconsistent with those of the infrared results of Ho and Waugh, but supplement these results.

12 C. Ho and A. Chen, unpublished results.
of the $^{31}$P signal in 0.1 M O-phosphoserine (under the same modulation and radio frequency field conditions). The line width for the $^{31}$P protein signal might be much narrower once the corrections for viscosity and possible broadening by instrumental effects are made. The intrinsic viscosity for the monomer of $\alpha_\text{c-casein}$ is about 11 ml per g at 25°; this viscosity corresponds to the value for a flexible macromolecule (32).

As pointed out earlier, the small variation with pH of the chemical shift of the protein signal strongly suggests that the corresponding phosphate group has only 1 ionizable proton. We cannot ascertain, however, whether this phosphate group would be in a phosphodiester or in a disubstituted pyrophosphate linkage. The $^{31}$P chemical shift of either of these groups would be essentially independent of pH in the range 4 to 9. Furthermore, the possibility of a roughly 50:50 mixture of mono- and diprotic phosphate groups with superimposed lines is not precluded by our experimental results, although it is not likely since the line widths appeared approximately constant over the pH range 4 to 9. In any case the results indicate the presence of monoprotic phosphate (or disubstituted pyrophosphate) groups.

In conclusion, the experimental results on the $^{31}$P chemical shifts of $\alpha_\text{c-casein}$ and model compounds as a function of pH (as studied by us and as reported in the literature) support the conclusion that there are phosphodiester or disubstituted pyrophosphate bonds, or both, in bovine $\alpha_\text{c-casein}$. This conclusion is also supported by physicochemical evidence (25, 26) and is free of those limitations inherent in the enzyme method used by earlier workers. The present results confirm part of the conclusions reached by Perlmann (4) and Thosì et al. (5) and not those given by others, as discussed at the beginning of this paper.

We believe that this present communication is the first to report a phosphorus resonance in a protein. The demonstration of the existence of phosphodiester or disubstituted pyrophosphate bonds, or both, among the 10 phosphorus atoms in $\alpha_\text{c-casein}$ emphasizes once again the role of phosphate cross-linkages in phosphoproteins.

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REFERENCES

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