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Phosphorus Nuclear Magnetic Resonance Studies of Phosphoproteins and Phosphorylated Molecules. II. Chemical Nature of Phosphorus Atoms in α_s-Casein B and Phosvitin*

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance spectroscopy has been used to investigate the chemical nature of phosphorus atoms in hen's egg yolk phosvitin and bovine α_s-casein B. For concentrations as low as 0.05 M in phosphorus, phosphorus–proton coupling patterns of 40.5-MHz ^{31}P nmr spectra can be resolved with the use of a computer of average transients. Phosphorus–proton coupling constants for these two proteins, as well as for several other phosphorylated molecules of biological interest, fall in the range 5–8 Hz. The variation of ^{31}P chemical shifts with pH in the range 3–11, for model compounds such as *O*-phosphoserine, *O*-phosphothreonine, glucose 1-phosphate, diethyl phosphate, β-diphosphopyridine nucleotide, and sodium pyrophosphate, can be put into two categories: (i) the ^{31}P chemical shifts in compounds with phosphodiester and with symmetrically substituted pyrophosphate linkages remain essentially constant; (ii) for phosphomonoester compounds there are relatively large changes in ^{31}P chemical shifts (about 4 ppm) with change of pH from 3 to 9. From the nuclear spin–spin coupling pattern, the variation of the ^{31}P chemical shift with pH, and the values of ^{31}P chemical shift, and phosphorus–proton coupling constants, we conclude that most of the phosphate groups in both phosvitin and α_s-casein B are attached to seryl residues as monoesters. Reversible line-broadening phenomena, which are not yet fully explained, are observed for *O*-phosphothreonine, glucose 1-phosphate, phosvitin, and α_s-casein in certain pH ranges. In general it appears that the high-resolution ^{31}P nuclear magnetic resonance can yield explicit information about the nature and, in some cases, steric disposition, of groups neighboring phosphates in a protein, by way of the phosphorus–proton coupling patterns, ^{31}P line widths, and variations of ^{31}P chemical shift with pH.

In our previous communication, Ho and Kurland (1966) discussed the controversy surrounding the nature of the phosphate groups in bovine α_s-casein B, that is, whether these are in the form of monoesters, diesters, symmetrically substituted pyrophosphate, or some combination of the above. Chemical and enzymatic degradations have been used to study this problem in the past, but these methods suffer from inherent ambiguities, *viz.*, chemical degradation, usually involving partial acid hydrolysis in an attempt to isolate *O*-phosphoserine or other phosphorylated amino acids or peptides (Perlmann, 1955; Hofman, 1958), can give rise to O–N acyl shifts, particularly adjacent to seryl residues (Desuelle and Casal, 1948), and thus rearrange

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the structure of the protein. The ambiguities of the enzymatic studies are discussed adequately elsewhere (Ho and Kurland, 1966, and references therein). Hence, what was needed was a method which would allow us to analyze the intact protein under conditions as close to "native" as possible. Our first attempt at devising such a method is related in the previous communication (Ho and Kurland, 1966). In order to observe the ^{31}P nmr of bovine α_s-casein B at a concentration of about 26% by weight in 6.5 M urea with a Varian DP-60 nmr spectrometer operated at a frequency of 15.085 MHz, it was necessary to use dispersion mode, rapid passage conditions, similar to those often used for ^{13}C nmr studies (Lauterbur, 1957). Within the accuracy of these measurements, we were unable to detect any pH dependence of the ^{31}P chemical shift in α_s-casein B from pH 4 to 10 (Ho and Kurland, 1966). Such a pH dependence of the ^{31}P chemical shift would be expected for phosphomonoesters, ROP(=O)-$(OH)_2$, which undergo a change in ionization state in the pH range investigated, but not for the other two types of phosphoesters. We, therefore, concluded at that time that the phosphorus atoms in α_s-casein B are present primarily as phosphodiesters, RO(O=POH)-OR′, symmetrically substituted pyrophosphate esters, RO(O=POH)O(O=POH)OR′, or some combination of the two forms.

The purpose of the present communication is to describe improvements in instrumentation and techniques which have enabled us to obtain much better sensitivity and resolution in ^{31}P NMR studies of both α_s-casein B and of phosvitin. The line widths for α_s-casein B in the current work are an order of magnitude narrower than those previously reported.

Phosvitin, obtained from hen's egg yolks, is a protein which contains approximately 10% phosphorus (Allerton and Perlmann, 1965). The molecular weight is in dispute, and most preparations in which work has been done are probably heterogeneous (Taborsky and Mok, 1967) (see Figure 1 for a typical chromatogram of a commercial preparation). However, the minimal molecular weight, based on tyrosine, tryptophan, and methionine content is 45,000 (Allerton and Perlmann, 1965). Based on this figure, there are 121 seryl residues, 119 phosphate groups, and only 6 threonyl residues. This nearly 1:1 ratio of serine to phosphate leads to the assumption that most if not all of the phosphate is present as *O*-phosphoseryl residues. We therefore chose phosvitin as a model phosphoprotein to use in developing and refining our experimental techniques.

Experimental Procedures

Materials

Purified bovine α_s-casein B was prepared according to the method of Waugh *et al.* (1962). Fresh raw milk was obtained from cows which had been genetically typed to be homozygous for the B variant for α_s-casein according to the notation of Thompson and Kiddy (1964). The purified protein was exhaustively dialyzed against 0.05 M potassium citrate, 0.01 M KCl, and finally against distilled deionized water. After lyophilization, weighed samples were dissolved in 8 M urea containing 10^{-2} M EDTA, or in 6 M guanidine hydrochloride containing 10^{-2} M EDTA. The samples were adjusted to the appropriate pH by adding HCl or KOH. The final protein concentration was about 15–25% (g/100 ml). The molecular weight of bovine α_s-casein is about 27,300 (Waugh *et al.*, 1962).

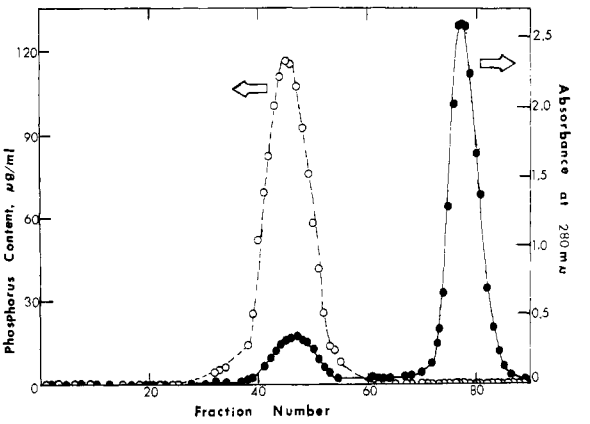

FIGURE 1: A typical chromatogram of a commercial sample of phosvitin by means of Sephadex G-200; 5 ml/fraction. (○—○) Phosphorus content in micrograms per milliliter and (●—●) absorbance at 280 mμ.

Phosvitin was prepared by gel filtration of commercial phosvitin, obtained from Nutritional Biochemicals, Inc. Aliquots of about 200 mg were applied to a 2.5 × 90 cm column of Sephadex G-200, operated in the upward flow mode, and eluted with 0.1 M potassium acetate buffer, containing 0.01 M EDTA (pH 5.5). Protein content in the effluent was monitored with a Gilson ultraviolet monitor at 280 mμ, and fractions of appropriate size were collected on a Gilson linear fractionator. Phosphorus concentration in appropriate fractions was determined by the method of Bartlett (1950), except that the digestion mixture contained 10 N sulfuric acid and 0.8 M perchloric acid, and the reducing agent used was freshly prepared 5% ascorbic acid. Figure 1 shows a typical elution pattern for commercial phosvitin. The elution pattern in the absence of EDTA was quite different and not amenable to a clean purification. A sample of phosvitin, prepared by the method of Jourbet and Cook (1958), kindly supplied by Dr. S. E. Allerton, showed a similar heterogeneity when subjected to gel filtration under these conditions. After the first pass over the column, the material in each peak was pooled, lyophilized, dialyzed against distilled water to desalt, and rechromatographed under the same conditions as before. Material from the first major peak was then again pooled, lyophilized, and dialyzed. This material was designated "purified phosvitin" and was used in the subsequent nmr experiments. Aliquots were dissolved in 0.5 M potassium citrate or 0.5 M Tris or 0.5 M imidazole buffer of appropriate pH, in some cases with 8 M urea, and in some cases with 0.02 M EDTA. The final phosvitin concentration was approximately 8–12% (g/100 ml).

DL-*O*-Phosphoserine and DL-*O*-phosphothreonine

TABLE I: ^{31}P Nuclear Magnetic Resonance Parameters for Phosphoproteins and Phosphorylated Molecules at 27°.

Compound	Concn M	pH	Scans	Splitting Patterns	^{31}P Chemical Shift, δ, ppm[a]	Coupling Constant, J_{HP}, Hz[b]
O-Phosphoserine	0.1	3.9	49	Triplet	112.4	5.3
	0.4	4.3	6	Triplet	112.1	5.5
	0.4	8.6	12	Triplet	108.5	6.4
O-Phosphoserine in 8 M urea	0.4	2.4	32	Triplet	112.6	5.5
	0.4	6.6	1	Triplet	109.1	6.7
	0.6	10.0	1	Triplet	108.3	6.1
O-Phosphothreonine	0.4	3.9	141	Broad	113.4	
	0.4	5.9	17	Broad	110.8	
	0.2	6.7	9	Broad	109.7	
	0.4	7.0	14	Broad	110.1	
	0.4	8.6	4	Doublet	109.3	8.0
	0.4	9.0	6	Doublet	109.3	8.1
O-Phosphothreonine in 8 M urea	0.4	6.7	112	Broad	110.4	
	0.4	9.2	4	Doublet	109.0	8.0
	0.4	10.0	1	Doublet	109.1	8.0
Glucose 1-phosphate	0.6	1.2	1	Broad doublet	113.9	6.0
	0.6	1.7	1	Broad doublet	11.39	6.0
	0.6	3.0	1	Broad doublet	113.8	6.0
	0.6	4.9	1	Broad doublet	113.4	6.0
	0.6	5.8	1	Doublet	111.9	6.5
	0.6	8.6	1	Doublet	110.1	7.0
Diethyl phosphate	1.5	3.8	16	Quintet	111.9	7.3
	1.5	10.2	6	Quintet	111.9	7.3
β-Diphosphopyridine nucleotide	0.2	4.3	9	Singlet	123.8	
	0.2	11.0	4	Singlet	123.6	
Sodium pyrophosphate	0.3	1.7	5	Singlet	122.8	
	0.3	5.5	9	Singlet	120.4	
	0.3	8.2	15	Singlet	118.2	
H_3PO	1.0		1	Singlet	112.5	
	0.1		1	Singlet	112.5	
α-Casein B in 8 M urea	25%	3.5	2100	Triplet	112.3	5.5
	25%	4.0	1362	Triplet	112.3	6.0
	25%	9.5	2021	Triplet	108.2	6.2
	25%	10.2	1910	Triplet	108.2	6.3
α-Casein B in 6 M guanidine hydrochloride	25%	10.1	334	Triplet	108.3	7.0
Phosvitin	12%	3.6	100	Triplet	112.2	6.5
	12%	7.1	129	Triplet	108.8	6.0
	12%	9.3	441	Broad	108.3	
Phosvitin in 8 M urea	12%	3.3	657	Triplet	112.2	6.3
	12%	11.9	281	Broad	108.2	
Phosvitin[c]	12%	10.1	10	Broad	108.4	
Phosvitin[d]	12%	3.7	196	Broad	112.4	
	12%	9.9	60	Broad	106.5	

[a] Chemical shift in parts per million upfield from P_4O_6; the precision of the measurement was ± 0.12 ppm or 5 Hz. [b] Absolute value of the phosphorus–proton coupling constant. The precision of the coupling constant is ± 0.5 Hz. [c] Prepared according to Joubert and Cook (1958) and kindly supplied by D. S. Allerton. [d] Commercial, unpurified phosvitin.

were obtained from Mann Research Laboratories and were used without further purification. Solutions were prepared in water, and appropriate buffer, or urea solution, containing 0.02 M EDTA in most cases. The concentration of *O*-phosphoserine and of *O*-phosphothreonine varied from 0.05 to 0.6 M. The final pH was adjusted to the desired point with HCl or KOH solution.

Diethyl phosphate, obtained from Eastman Organic Chemicals, was diluted to approximately 20%, and the pH was adjusted to 3.8 or 10.2.

Glucose 1-phosphate was obtained from Calbiochem as the dipotassium salt and was dissolved in water, approximately 25% solution, and the pH was adjusted to the desired values with 4 N HCl.

β-Diphosphopyridine nucleotide was obtained from Sigma as Grade III material and was dissolved in distilled water to make up about 0.2 M solution and the PH was adjusted to the desired value with KOH or HCl.

P_4O_6, used as an external reference, was obtained in sealed tubes from the Oldbury Division, Albright and Wilson, Ltd.

Urea was purchased from Calbiochem, A grade, and was used without further purification for preparing nmr samples.

Guanidine hydrochloride was purchased from J. T. Baker and was purified according to the method of Nozaki and Tanford (1967).

All other reagents and chemicals used were of reagent grade from major suppliers and were used without further purification.

A radiometer pH meter, Model 26, in conjunction with a Radiometer type C combination electrode or a Beckman Model 39036 frit junction combination electrode, was used for pH measurements.

Methods

Nmr spectra were obtained by means of a Varian HA-100 spectrometer operated at a frequency of 40.5 MHz, in the frequency-sweep, field-frequency controlled mode. The ambient temperature of the probe was 27°. Chemical shifts[1] were measured relative to an external reference, P_4O_6, contained in a coaxial capillary tube, immersed in a standard 5-mm sample tube containing about 0.5 ml of the solution under investigation. The lock frequency was obtained from a General Radio frequency synthesizer, Model 1161-A7C.[2] The precision of the ^{31}P chemical shift measurements is ±0.12 ppm or 5 Hz. In order to enhance the detection of the relatively weak ^{31}P signals, the output of the spectrometer was accumulated for a number of scans by means of a Varian C-1024 computer of average transients.

[1] The measured chemical shift, δ, is defined by $\delta = (H_{sample} - H_{ref})/H_{ref} \times 10^6$ and is given in parts per million, where H_{sample} is the resonance field of the signal being measured at a fixed frequency and H_{ref} is the corresponding field for the reference sample. The phosphorus chemical shifts were not corrected for the difference between bulk diamagnetic susceptibilities of the external reference (P_4O_6) and the sample. This correction would be relatively small (Pople *et al.*, 1959). Furthermore, changes in this correction would be even smaller in going from one sample to another, or in changing pH.

[2] The spectrometer was then locked on the upper side band of P_4O_6, and the frequency swept over the range of the lower side band of the sample; thus the chemical shift (in parts per million from P_4O_6) was measured as the sum of the lock frequency and the sweep frequency, divided by 40.5.

Results

The measured ^{31}P chemical shifts, δ, and phosphorus–proton coupling constants, J_{HP}, are given as a function of pH in Table I and Figure 2. Figure 2 is a graphical summary of the pH dependence of the ^{31}P chemical shifts for the compounds studied, compared with that of orthophosphate, taken from Crutchfield *et al.* (1963). Representative spectra for several of the compounds are shown in Figures 3–8. The line widths for the proteins were about 3–10 Hz for sharp spectra and 25–30 Hz for those in which the coupling was unresolved. For the model compounds, the line widths were about 3–10 Hz, except for *O*-phosphothreonine at low pH.

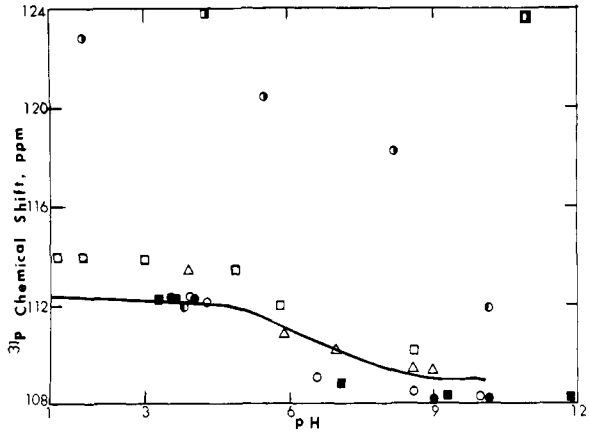

FIGURE 2: pH dependence of ^{31}P chemical shift for phosphoproteins and phosphorylated molecules. (○) *O*-Phosphoserine, (△) *O*-phosphothreonine, (□) glucose 1-phosphate, (●) α_s-casein B; (■) phosvitin, (◨) β-DPNH; (◑) sodium pyrophosphate, (◐) diethyl phosphate, and (—) orthophosphate.

The ^{31}P chemical shifts for DL-*O*-phosphoserine and DL-*O*-phosphothreonine show the expected change of about 4 ppm over the pH range 4–9. The presence or absence of urea made no significant qualitative or quantitative change in the spectra of either compound. Urea was used in some samples to check its effect, since it was necessary to dissolve α_s-casein B samples in 8 M urea in order to achieve the highest possible concentration of phosphorus. *O*-Phosphoserine spectra exhibit the expected triplet (Figure 3) under all conditions, reflecting the spin–spin coupling of the phosphorus with the two protons at the β-carbon atom of serine. There is no appreciable broadening of the *O*-phosphoserine nmr lines at all the pH values studied. However, there is a slight decrease in J_{HP}, from *ca.* 6.4 to *ca.* 5.5 Hz (just on the edge of the precision of our

measurements) in going from high pH 8–10 to low pH 2–4, and a very slight broadening at low pH (See Table I and Figure 3).

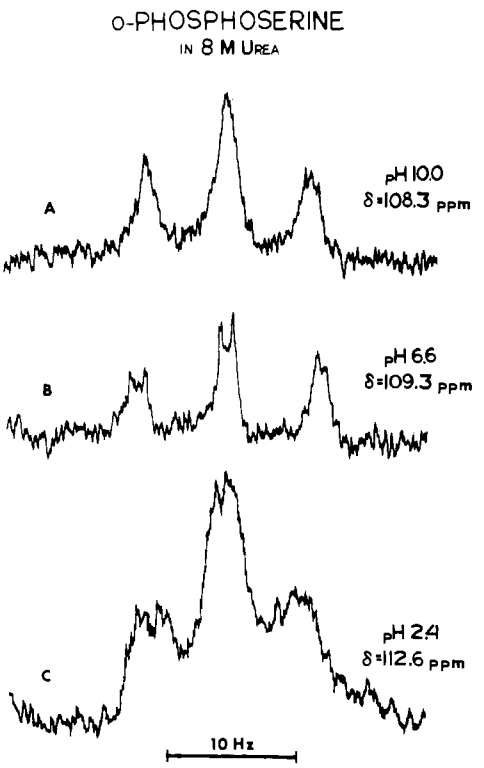

FIGURE 3: ^{31}P nuclear magnetic resonance spectra of *O*-phosphoserine in 8 M urea at (A) pH 10.0, (B) pH 6.6, and (C) pH 2.4.

O-Phosphothreonine at pH above 8 shows the expected doublet which would arise from spin–spin coupling between phosphorus and the single proton at the β-carbon atom of threonine (Figure 4). However, at lower pH values, the line broadens so much that the doublet is no longer resolved. This effect is the same with or without urea present. The effect was reversible; that is to say, when the pH of a sample was changed from 2.5 to 10 by the addition of concentrated NaOH, the doublet reappeared, which also happened when a pH 6.8 sample was changed to a pH 9.2 sample. The cause of this reversible broadening is evidently not due to instrumental factors or to trace metal contamination, since EDTA or Chelex-100 resin treatment did not improve the resolution. It should also be noted that the magnitude of the phosphorus–proton coupling constant for *O*-phosphothreonine is greater than that of *O*-phosphoserine (Table I).

Glucose 1-phosphate was also studied as a model compound for phosphomonoester types. Again, there is a change of about 3.7 ppm in the phosphorus chemical shift with change of pH from 3.0 to 8.6. The spectra showed a well-resolved doublet splitting pattern at pH values greater than 5 and a broadened doublet at lower pH values. The ^{31}P chemical shifts for *O*-phosphothreonine and glucose 1-phosphate are very similar and are about 1 ppm greater than the corresponding values for *O*-phosphoserine. This suggests that the ^{31}P chemical shift for the secondary phosphates is about 1 ppm higher than that of the corresponding primary phosphates.

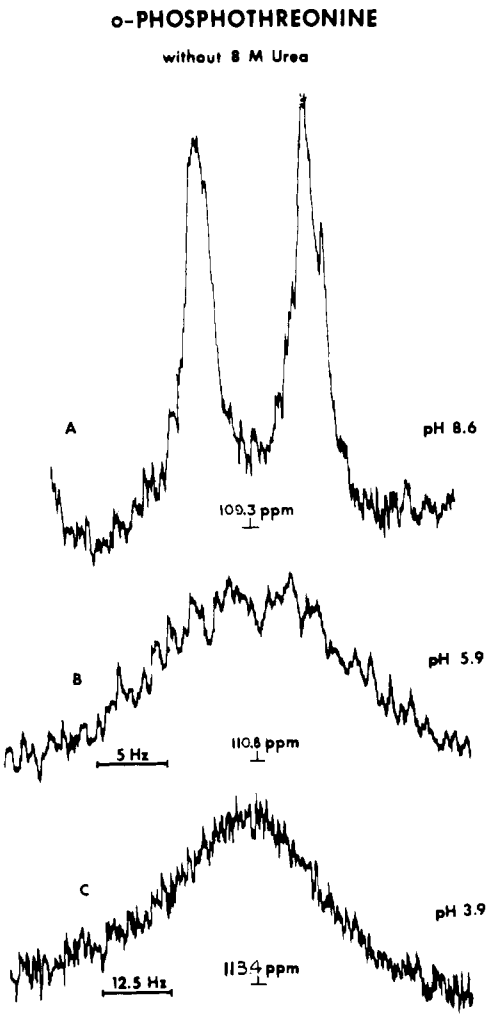

FIGURE 4: ^{31}P nuclear magnetic resonance spectra of *O*-phosphothreonine at (A) pH 8.6, (B) pH 5.9, and (C) pH 3.9.

Diethyl phosphate was used as model compounds for phosphodiester types. The ^{31}P nmr spectra of diethyl phosphate show a well-resolved quintet (Figure 5) arising from coupling with the four protons at the α-carbon atoms of the ethyl groups. Spectra of samples at pH 3.8 and 10.2 were essentially identical, with the same chemical shift and phosphorus–proton coupling constant.

^{31}P nmr spectra of β-diphosphopyridine nucleotide and sodium pyrophosphate were obtained as representative of the pyrophosphate-type compounds. As would be expected for a "symmetrically" substituted pyrophosphate, there was no observable change in the ^{31}P chemical shift for β-DPNH from pH 4.3 to 11.0. The chemical shift of sodium pyrophosphate

changes from 122.8 ppm at pH 1.70 to 120.4 ppm at pH 5.5 and to 118.2 ppm at pH 8.2, which correspond to the change in the state of ionization of the pyrophosphate group.

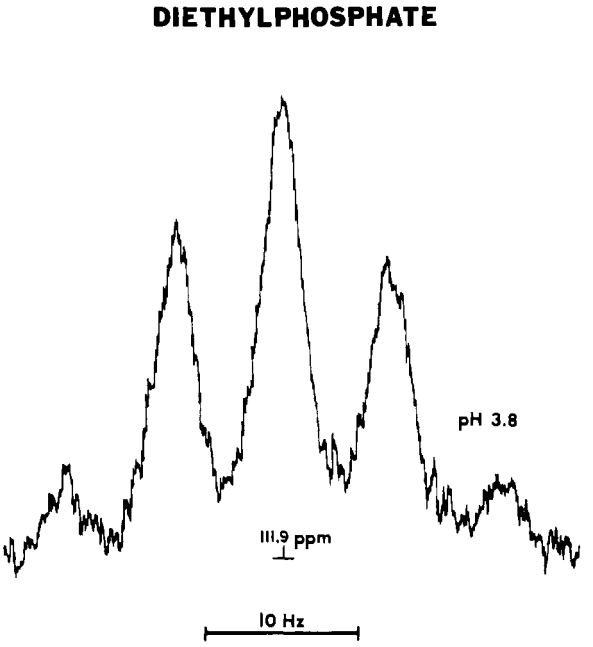

FIGURE 5: ^{31}P nuclear magnetic resonance spectra of diethyl phosphate at pH 3.8.

The ^{31}P chemical shift for both 0.1 and 1.0 M H_3PO_4 is 112.5 ppm from P_4O_6. This value is in agreement with that reported (112.5 ppm from P_4O_6) by Chapman *et al.* (1965).

The ^{31}P nmr spectrum of α_s-casein (Figure 6) is qualitatively similar to that of *O*-phosphoserine under the same conditions, although somewhat broader. The triplet structure, however, is evident, both at low and high pH. At pH 7, the lines were broadened to such an extent that no spectrum could be obtained even after prolonged signal averaging.

The ^{31}P nmr spectra of phosvitin in the absence and presence of 8 M urea (Figures 7 and 8) also exhibit a triplet structure (similar to those of *O*-phosphoserine) especially at low pH values. At alkaline pH values, the line broadened so much that the fine structure could not be resolved.

Discussion

The use of ^{31}P nmr to investigate the chemical nature of phosphorylated molecules is based on the interpretation of three spectral features: (i) the line positions, *i.e.*, chemical shifts, and their dependence upon pH; (ii) the nuclear spin–spin coupling patterns and the values derived from these for phosphorous–proton coupling constants; and (iii) line widths. In general, the chemical shifts give information about the local environments of the phosphorus nuclei; spin–spin coupling patterns and constants yield information about the identity of neighboring groups containing magnetic nuclei and, in some cases, clues as to the steric disposition of these groups; while line widths reveal the effect of dynamic processes, *e.g.*, exchange or molecular tumbling in solution, and indicate if there is a distribution of nonequivalent sites in the molecule (Pople *et al.*, 1959).

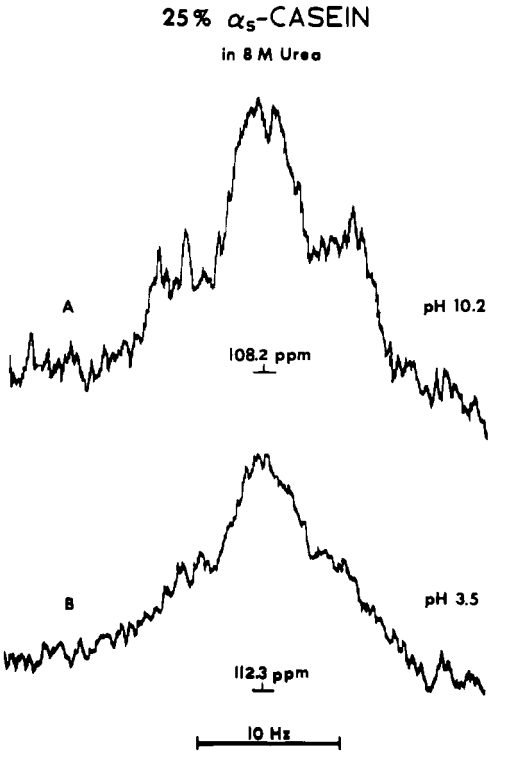

FIGURE 6: ^{31}P nuclear magnetic resonance spectra of 25% α_s-casein B in 8 M urea at (A) pH 10.2 and (B) pH 3.5.

In order to determine whether a phosphate linkage may be classified as phosphomonoester, phosphodiester, or symmetrically substituted pyrophosphate, the three major types which occur in phosphoproteins, one may determine the variation of the ^{31}P nmr chemical shift as a function of pH. One would expect that there should be a significant change in the ^{31}P chemical shift as the phosphate group goes from the singly ionized state to the doubly ionized state in a phosphomonoester compound as the pH is changed from pH 4 to 9. On the other hand, the ^{31}P chemical shift in phosphodiester or symmetrically substituted pyrophosphate-type compounds should be essentially independent of pH in this range, since no second ionization of the phosphate can occur. Our experimental results on model compounds are in accord with the considerations above (Table I and Figure 2, as well as Ho and Kurland, 1966).[3]

[3] Cohn and Hughes (1960) have studied the pH dependence of the ^{31}P chemical shift of ATP. Their results are consistent with the above.

From an analysis of the spin–spin coupling patterns in the ^{31}P nmr spectra, two sorts of information can be obtained. First, the form of the pattern indicates how many protons are bonded to neighboring or next nearest-neighbor atoms.[4] Thus, a P–O–C–H linkage should exhibit a doublet of 1:1 intensity ratio; a P–O–CH_2 grouping, a triplet of 1:2:1 intensity ratio, etc. Secondly, one would expect that the phosphorous–proton coupling constant, J_{HP}, might be a function of the time-averaged dihedral angle between the P–O–C and O–C–H planes in the P–O–C–H linkages.[5] Thus, the difference in J_{HP} for *O*-phosphoserine ($J_{HP} \sim 6$) and *O*-phosphothreonine ($J_{HP} \sim 8$) may reflect differences in the averaged conformation. We also note that the change in J_{HP} for *O*-phosphoserine from *ca* 6.4 to 5.5 Hz with a change in pH from *ca.* 9 to 2 could reflect a corresponding change in the time-averaged conformation.

Line broadening may be due to any of several mechanisms: intra- or intermolecular exchange between nonequivalent sites (Pople *et al.*, 1959) or, in the case of high molecular weight biopolymers, incomplete averaging of magnetic dipolar fields (McDonald *et al.*, 1964). The broadening that occurs for *O*-phosphothreonine at pH values less than 8 both in the presence and absence of 8 M urea could be due to a number of factors. Diastereomers are present as shown by the proton nmr spectrum of *O*-phosphothreonine (J. A. Magnuson, 1968, unpublished results). This suggests more than one ^{31}P chemical shift which may only become apparent at low pH. Another source of the broadening may come from some as yet undetermined chemical exchange processes which lead to an averaging of the chemical shifts of the various forms. Additional work, particularly on the variation of the phosphorus–proton coupling constants and chemical shifts with temperature and on the variation of vicinal proton–proton coupling constants in *O*-phosphothreonine will be needed to throw more light on this line broadening phenomenon.

Turning to the ^{31}P nmr of the two proteins, α_s-casein B and phosvitin, we note first that there is a change of about 4 ppm in ^{31}P chemical shift as the pH changes from 3 to 9, and second, that the form of the ^{31}P resonance is essentially a triplet of 1:2:1 intensity ratio. The first observation indicates that the phosphate groups in these compounds are of a monoester type; the second shows that a P–O–CH_2 linkage is present rather than a P–O–CH. We conclude, therefore, that phosphate groups in these proteins are esterified to seryl residues as monoesters. Additional evidence for this conclusion is the close correspondence of the chemical shifts and coupling constants for the proteins to those of *O*-phosphoserine (Table I and Figure 2).

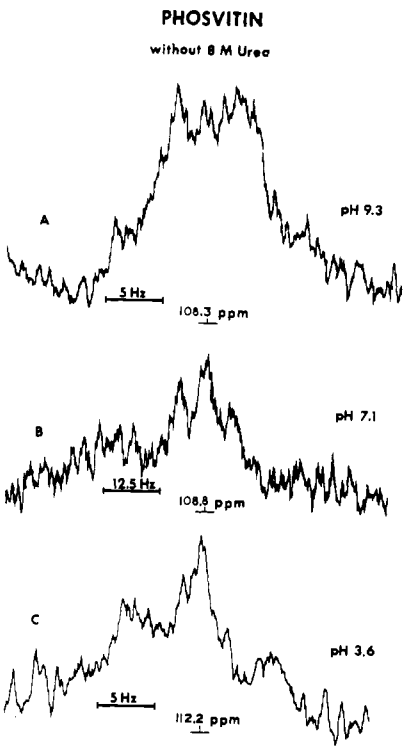

FIGURE 7: ^{31}P nuclear magnetic resonance spectra of 12% phosvitin at (A) pH 9.3, (B) pH 7.1, and (C) pH 3.6.

We cannot, at this stage, satisfactorily explain the line broadening for phosvitin at alkaline pH values or that for α_s-casein at pH values near 7. Chemical exchange processes may be one of the reasons for the broadening; a distribution of chemical shifts from nonequivalence of phosphorus atoms might be another source of line broadening. The well-resolved ^{31}P nmr spectra of the proteins may actually reflect accidental coincidence of the ^{31}P chemical shift. In the case of α_s-casein B, nmr measurements were carried out in the presence of 8 M urea at 27°. The measured intrinsic viscosity for α_s-casein B in 0.01 M KCl containing 8 M urea at pH 7 is 20.0 ml/g at 25° (J. B. Wilson, N. S. Magnuson, and C. Ho, unpublished results, 1968). This value is very close to the expected intrinsic viscosity if this protein is in the form of a random coil (Ho and Chen, 1967; Tanford *et al.*, 1966). In fact, the 100-MHz proton nmr spectrum of α_s-casein (C. Ho, R. H. Cox, J. B. Wilson, and J. A. Magnuson, unpublished results, 1967) in 0.01 M KCl containing 8 M urea at pH 7 and at 27° is very similar to the 220-MHz proton nmr spectra of denatured proteins, such as lysozyme, ribonuclease, etc., as studied by McDonald and Phillips (1967a,b). Hence, the line broadening of α_s-casein B at neutral pH is not likely due to a gross conformational change in the protein in going from pH 4 to 7 or from pH 9 to 7. In the case of phosvitin, the ^{31}P nmr spectra showed broad lines

[4] The nuclear spin–spin coupling between phosphorus and proton more than three bonds away is weak and may be manifested as a contribution to the line width (Pople *et al.*, 1959).

[5] It should be pointed out that the relationship between the phosphorus–proton coupling constant and the time-averaged dihedral angle is not founded on as sound a theoretical or empirical base as the corresponding case for proton–proton coupling constants discussed by Karplus (1959, 1960), Schug *et al.* (1960), and Conroy (1960).

at high pH both with 8 M urea present and without. Preliminary viscosity measurements of phosvitin in 8 M urea at 27° suggest that the *apparent* intrinsic viscosity at pH 10 is higher than that at pH 3.5 (N. S. Magnuson, J. B. Wilson, and C. Ho, unpublished results, 1968).[6] The increase in the *apparent* intrinsic viscosity at pH 10

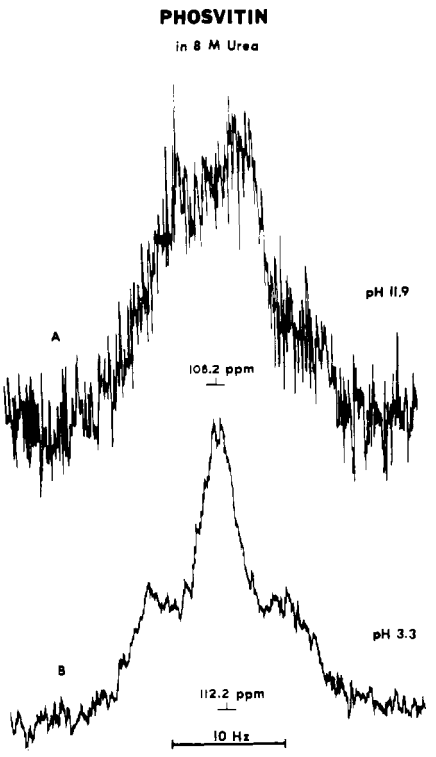

FIGURE 8: ^{31}P nuclear magnetic resonance spectra of 12% phosvitin in 8 M urea at (A) pH 11.9 and (B) pH 3.3.

could reflect that a portion of the phosvitin molecule may become rigid enough that the magnetic dipolar interactions are incompletely averaged out by molecular and intramolecular rotation in the solution. This might contribute to the line broadening at high pH for phosvitin. Further work on the proton nmr spectra of these two proteins and on the effects of temperature on ^{31}P nmr spectra and added electrolytes as well as denaturants is underway in order to elucidate the mechanism of this broadening.

[6] The *apparent* intrinsic viscosities, $[\eta]_{app}$, for phosvitin under various experimental conditions are: $[\eta]_{app}$ = 13.3 in 8 M urea and 0.02 M EDTA at pH 9.94; $[\eta]_{app}$ = 7.2 in 8 M urea and 0.02 M EDTA at pH 3.45; $[\eta]_{app}$ = 11.3 in 0.5 M Tris and in 0.02 M EDTA at pH 10.0; and $[\eta]_{app}$ = 6.2 in 0.5 M potassium citrate and in 0.02 M EDTA at pH 3.56. All the viscosity measurements were carried out at 27° and the concentrations were expressed in terms of arbitrary optical density units at 280 mμ. The extinction coefficient for phosvitin at 280 mμ is not known and apparently is a function of the degree of purification of the individual preparation (Taborsky and Mok, 1967).

In summary, we can say that two qualitative features of the ^{31}P nmr spectra of phosvitin and α_s-casein B, namely the variation of the ^{31}P chemical shift with pH and the character of the nuclear spin–spin coupling indicate that the phosphate groups giving rise to the ^{31}P nmr signals are present mainly as monoesters of the serine. The present results of α_s-casein B are not in agreement with our earlier work (Ho and Kurland, 1966), but the present results should supersede the earlier ones, we feel, because of advances in instrumentation. Potentially, additional information about the sterochemistry and environment of phosphate groups in phosphoproteins is available from the values of the phosphorus–proton coupling constants and analyses of the line-broadening effects. These considerations were qualitatively applied to the results of the simple model compounds studied in this work; however, before they can be applied to phosphoproteins, additional work on model compounds and correlations with proton nmr spectra will be necessary.

Perhaps the greatest difficulty in applying ^{31}P nmr spectroscopy to the study of biomolecules containing phosphorus is the problem of sensitivity. An approximate lower limit in concentration of equivalent phosphorus that can be studied is 0.04 M (with apparatus and techniques such as used in this work). With improvements in instrumentation, such as the use of larger diameter sample tubes and the use of spectrometers operating at superconducting fields, we hope to lower this limit by a factor of 10 or more in order to study other important phosphorylated biomolecules.

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