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Chemical Phosphorylation of Bovine β-Lactoglobulin

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Bovine β-lactoglobulin was phosphorylated with phosphorus oxychloride ($POCl_3$) at pH 8.5 to give a product that contained up to 14 mol of phosphorus/mol of protein. The acid lability of the phosphate residues and the ^{31}P NMR spectral data suggest that the protein lysine and histidine residues had been phosphorylated. Some dimerization of the protein also occurred. Circular dichroism showed that phosphorylation disrupted the native structure of the protein but did not denature it completely.

Whey, the principal byproduct of cheese making, is a fluid which contains 4–5% lactose, some mineral salts, and about 0.5% protein. The protein material can be separated from the whey and is a valuable source of essential amino acids such as lysine. It is soluble and stable between pH 2.5 and pH 10 and is able to form foams and gels under appropriate conditions (Marshall, 1979). It would be desirable, however, to extend the range of functional properties of whey protein, and this study was undertaken to explore the effects of phosphorylation on the major whey protein β-lactoglobulin as a possible prelude to the examination of phosphorylated whey protein.

Nonspecific protein phosphorylation can be accomplished with inexpensive reagents such as phosphoryl chloride ($POCl_3$) or phosphorus pentoxide dissolved in phosphoric acid, and several such artificial phosphoproteins have been studied (Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Ferrel et al., 1948; Salák et al., 1965; Willmitzer and Wagner, 1975). The earlier studies indicated that the phosphate could become attached to amino nitrogen such as in lysine or to hydroxyl oxygen, as in serine, and that the properties of the phosphoprotein were not simply an extrapolation of those of the original protein to take account of the increased negative charge.

EXPERIMENTAL SECTION

Phosphorylation. Purified β-lactoglobulin (3× crystallized; Sigma Chemical Co., St. Louis, MO) was dissolved to give a 1% solution. Phosphoryl chloride ($POCl_3$) was added as small aliquots over various periods of time with vigorous stirring at 25 °C while maintaining the pH at 8.5 by the dropwise addition of 5 M NaOH. The $POCl_3$ was added in three different forms: (1) dissolved in light mineral oil, (2) dissolved in CCl_4, and (3) neat with no dispersing solvent. After the addition of all the $POCl_3$, the aqueous phase was separated from the organic solvent, where necessary, and dialyzed against 0.9% (0.15 M) NaCl and against two changes of deionized water before being freeze-dried. Samples of this freeze-dried material were further purified by dissolving them in water and chromatographing them on a 2.5 × 30 cm column of Sephadex G-25 in 0.10 M NaCl at a flow rate of 0.7 mL/min. The column effluent was fractionated, and the 280-nm absorbances and phosphate contents of selected fractions were determined. The fractions containing the protein peak were bulked, dialyzed and freeze-dried, and examined further or frozen. A control reaction involved adding 10% $POCl_3$ dissolved in CCl_4 into the aqueous system without protein for 4 h to achieve hydrolysis and/or polymerization of the $POCl_3$. The protein was then added, and the mixture was allowed to stand overnight. Subsequently, the control protein was purified as above for the phosphorylated protein. This control measured any electrostatic interactions between any polyphosphates that might be formed and the protein.

Poly-L-lysine (VII-B; 40 000 molecular weight; Sigma, St. Louis, MO) was phosphorylated and purified by the above procedure using the direct addition method at a $POCl_3$:substrate molar ratio of 4000:1.

Phosphoprotein Hydrolysis in Solution. Purified phosphoprotein samples were dissolved in water (5 mg in 0.5 mL), and the pH was adjusted to 1.5, 4.5, 7.5, or 10.5 with 1 M NaOH or HCl. Each of these solutions was held at 37 °C for 24 h, the pH was then adjusted to 7.5, and they were dialyzed twice against 1-L changes of 0.05 M Tris buffer at pH 7.5 in 24 h. The samples then had their protein and phosphate contents determined by absorbance at 280 nm ($\epsilon_{1\%}$ = 9.4; Tanford and Nozaki, 1959) and sample digestion in 60% $HClO_4$ followed by phosphate analysis (King, 1952), respectively.

A second and third set of experiments included an intestinal alkaline phosphatase, EC 3.1.3.1 (Type VII, Sigma,

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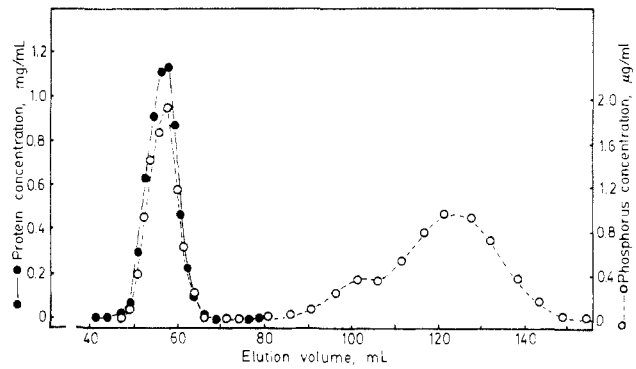

Figure 1. Gel filtration elution pattern of phosphorylated β-lactoglobulin.

St. Louis, MO), at a ratio of 16 μg of enzyme to 3.75 mg of phosphoprotein.

Nuclear Magnetic Resonance Measurements. Protein samples were dissolved at a concentration of 50–100 mg/mL in 0.05 M Tris buffer at pH 7.5 containing 0.002 M Na_2EDTA. Spectra were obtained from a Bruker WH-270 superconducting Fourier transform spectrometer operating at 109.29 MHz. The external standard was methyl phosphonate.

Circular Dichroism Measurements. Spectra were recorded with a Jasco (Japan Spectroscopic Co., Tokyo) Model J-41C spectropolarimeter with a spectral bandwidth of 2 nm, a scanning rate of 1.0 nm/min, and a time constant of 16 s. Between 190 and 250 nm a 1-mm cell was used and protein concentrations near 0.1 mg/mL were used. Molar ellipticity per residue, [θ], in deg cm^2 $decimol^{-1}$ was calculated from

$$[\theta] = 100dM_r n^{-1}C^{-1}$$

where d is the rotation in degrees per 1.0-cm path length of solution, M_r is the molecular weight of the protein [assumed to be 18 362 for β-lactoglobulin and phospho-β-lactoglobulin (Whitney et al., 1976)], n is the number of residues in the protein chain [162 for β-lactoglobulin (Braunitzer et al., 1973)], and C is the concentration of protein in mg/mL. Between 250 and 340 nm and 1-cm cell was used and protein concentrations near 0.6 mg/mL were used. The molar circular dichrosim, Δε, was calculated from

$$\Delta\epsilon = 0.0304dM_r C^{-1}$$

where the terms have the same meaning as for the earlier equation.

The spectropolarimeter sensitivity was measured by using a 60 mg/100 mL solution of camphor-10-*d*-sulfonic acid which has a positive circular dichroism, Δε, of 2.20 (a molar ellipticity of 7260 deg cm^2 $decimol^{-1}$), at 290.5 nm (Cassim and Yang, 1969).

Electrophoresis in polyacrylamide gels in 4.5 M urea- and $NaDodSO_4$-containing buffers (9% and 12% acrylamide, respectively) followed the general procedure of Laemmli (1970).

RESULTS

Preliminary data showed that $POCl_3$ in an organic solvent or alone could phosphorylate β-lactoglobulin and that higher pH favored a greater extent of phosphorylation. It was also found that any delays in the procedure, such as more extensive dialysis or holding samples overnight, allowed the samples to dephosphorylate. Thus the isolated and purified phospho-β-lactoglobulins varied in their phosphate content. After phosphorylation and dialysis to remove surplus low molecular weight material, the phosphoprotein was purified by gel filtration chromatography on Sephadex G-25. Figure 1 shows a typical elution pattern. It can be seen that the 280-nm absorbance to phosphate ratio is virtually constant across the main peak, centered on a 55-mL elution volume, and that there is a double phosphate peak at 100 and 125 mL. It seems likely that the leading shoulder of the second peak is caused by polyphosphates and that the second peak is orthophosphate. When control samples were eluted from the same column, no phosphorus was detectable in the protein fractions, indicating the absence of electrostatic interactions between polyphosphates and the protein.

Table I. Effect of $POCl_3$:Protein Ratio on Extent of Protein Phosphorylation[a]

$POCl_3$:protein ratio, mol/mol	P content, mol of P/mol of protein
500	5.2
1000	6.0
2000	11.3
3000	13.8
4000	13.6

[a] The $POCl_3$ was added directly to the reaction mixture over a 45-min period.

Table II. Effect of Organic Solvents on Extent of Protein Phosphorylation[a]

solvent	P content, mol of P/mol of protein
none	6.0
mineral oil	7.8
CCl_4	12.5

[a] The $POCl_3$ was added (a 10% v/v solution where a solvent was used) during 45 min at a ratio of 1000 mol of $POCl_3$/mol of protein.

Table III. Effect of Reaction Time on Extent of Protein Phosphorylation[a]

time, min	P content, mol of P/mol of protein
45	12.5
90	10.0
240	7.7

[a] The $POCl_3$ was dissolved in CCl_4 and the ratio was 1000 mol of $POCl_3$/mol of protein.

Effect of Varying the Reaction Conditions. The ratio of $POCl_3$ to protein was varied in the range 500:1 to 4000:1, all other conditions being held the same. The extent of phosphorylation increased at the higher ratios (Table I), but the limit appeared to be about 13.7 mol phosphate/mol of protein.

When phosphorylation was carried out in a two-phase system with the $POCl_3$ dissolved in CCl_4 or light mineral oil, the extent of phosphorylation was greater (Table II), and when the phosphorylation reaction was slowed by making the additions of reagent in smaller aliquots (Table III), the extent of phosphorylation decreased.

Thus the greatest phosphorylation would probably be achieved by using $POCl_3$ dissolved in CCl_4 at a high ratio (>2000:1) to protein and by rapidly intermixing the reagents so that the total reaction time was minimal.

Hydrolysis of Phospho-β-lactoglobulin. The lability of the phosphates was investigated at several pH values and as a result of enzyme action. When the protein was dissolved, titrated to a set pH, held at 37 °C for 24 h, readjusted to pH 7.5, and dialyzed against pH 7.5 0.05 M

Table IV. Acid and Base Hydrolysis of Phospho-β-lactoglobulin

treatment	P content, mol of P/mol of protein	hydrolysis,[a] %
freshly dissolved	7.65	
freshly dissolved and dialyzed (24 h; 2 °C; pH 7.5)	6.75	
held 24 h (pH 1.5; 37 °C); then dialyzed	1.30	80.7
held 24 h (pH 4.5; 37 °C); then dialyzed	1.68	75.1
held 24 h (pH 7.5, 37 °C); then dialyzed	3.53	47.7
held 24 h (pH 10.5; 37 °C); then dialyzed	3.50	48.1

[a] The sample dialyzed for 24 h was taken as the control and zero hydrolysis.

Table V. Enzymic Hydrolysis of Phospho-β-lactoglobulin

treatment	P content, mol of P/mol of protein		hydrolysis, %	
	sample 1	sample 2	sample 1	sample 2
freshly dissolved	7.76	11.02		
freshly dissolved and dialyzed (24 h; 2 °C; pH 7.5)	6.48	8.45		
dissolved in pH 10.4, 0.1 M glycine buffer; held 1 h, (37 °C); then dialyzed	4.48	5.96	30.9	29.5
dissolved as above; 16 μg of enzyme/3.75 mg of protein added; held 1 h; then dialyzed[a]	2.31	3.23	64.4	61.8

[a] The enzyme contained no measurable phosphate under the conditions of the analysis.

Tris buffer, it was found that the phosphate content of the protein decreased markedly at low pH (Table IV). Several controls were used. First, the protein was dissolved at pH 7.5 and dialyzed against the pH 7.5 buffer. This showed that about 11% of the phosphate was lost by the purification method. Second, the phosphate content of the system without phosphoprotein was measured and no phosphate was found.

The effect of an intestinal mucosa phosphatase was measured by comparing the extent of dephosphorylation of the protein in the presence and absence of the enzyme (Table V) at the optimum pH (10.4, following manufacturer's recommendation) for enzyme action. A second sample with a much higher phosphate content was found to hydrolyze at approximately the same rate as the first sample. However, the difference between the initial sample and the dialyzed sample (Table V) was greater for the second sample, indicating the greater lability of the more highly phosphorylated samples.

The combined sequential effect of enzyme hydrolysis and low pH hydrolysis was examined, and the results (Table VI) show that the combination is more effective than either treatment alone.

Gel Electrophoresis. A selection of the various preparation was examined by polyacrylamide gel electrophoresis in urea-containing buffers, and the resultant patterns are shown in Figure 2. It can be seen that in all instances phosphylation gives rise both to more and to less mobile material and that there is considerable band spreading, especially when the $POCl_3$ is added in a solvent. When these samples were electrophoresed in an $NaDodSO_4$ gel, the phosphoprotein bands were more spread than the β-lactoglobulin band (Figure 3). In addition, there were

Table VI. Sequential Enzyme and Acid Hydrolysis of Phospho-β-lactoglobulin

treatment	P content, mol of P/mol of protein	hydrolysis, %
freshly dissolved	7.08	
freshly dissolved and dialyzed (24 h; 2 °C; pH 7.5)	6.12	
dissolved in pH 10.4, 0.1 M glycine buffer; held 24 h (37 °C); then dialyzed	3.24	47.1
dissolved as above; 16 μg of enzyme/3.75 mg of protein added; held 24 h (37 °C); then dialyzed	0.96	84.3
enzyme treated (37 °C, 1 h); then pH 1.5 (23 h; 37 °C); then pH 7.5 and dialyzed	0.79	87.1
enzyme treated (37 °C; 3 h); then pH 1.5 (21 h; 37 °C); then pH 7.5 and dialyzed	0.66	89.2

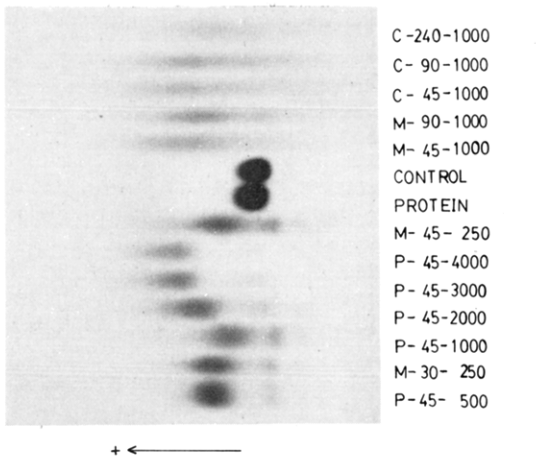

Figure 2. Electrophoresis pattern of phosphorylated β-lactoglobulins in a urea–polyacrylamide gel. First symbol of sample code: M, mineral oil solvent; C, CCl_4 solvent; P, no solvent. Second symbol of sample code: reaction time in minutes. Third symbol of sample code: molar ratio of $POCl_3$ to β-lactoglobulin, molecular weight 18362. Protein, freshly dissolved β-lactoglobulin; control, β-lactoglobulin dissolved in previously hydrolyzed $POCl_3$ reaction mixture and then purified according to the procedures used for the phosphorylated protein (see the text).

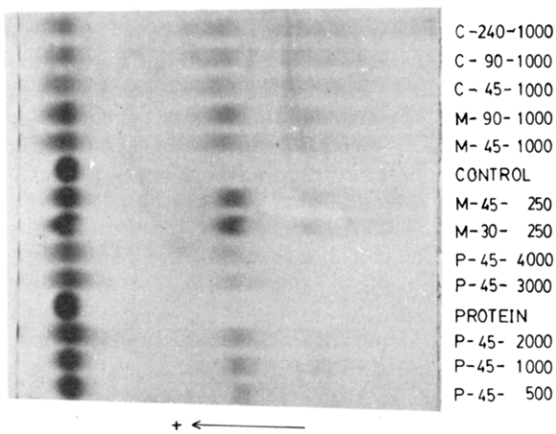

Figure 3. Electrophoresis pattern of phosphorylated β-lactoglobulins in $NaDodSO_4$–polyacrylamide gels. Sample codes are as shown in Figure 2 caption.

disperse bands of lower mobility, and in some instances a streak could be detected near the origin. A plot of log molecular weight vs. mobility by using the marker proteins, α-lactalbumin, β-lactoglobulin, trysinogen, ovalbumin, and

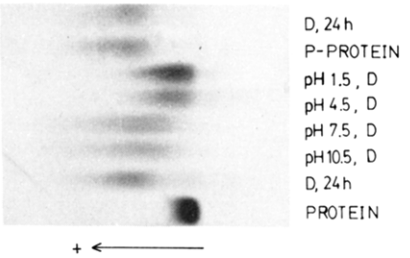

Figure 4. Electrophoresis pattern of acid- or base-treated phospho-β-lactoglobulin in a urea–polyacrylamide gel. Sample code: D, dialyzed for 24 h vs. 0.05 M Tris, pH 7.5; P, protein, freshly dissolved phosphorylated β-lactoglobulin; protein, freshly dissolved β-lactoglobulin.

bovine serum albumin, indicated that the disperse band of lower mobility was probably dimeric β-lactoglobulin. The quantity of this dimeric material was greater in the samples prepared with $POCl_3$ dispersed in a solvent.

The effect of holding samples of phospho-β-lactoglobulin at several pHs on electrophoretic mobility is shown in Figure 4.

It can also be seen that holding samples at pH 1.5 or 4.5 is more effective in reducing protein mobility than holding them at a higher pH. Alkaline phosphatase treatment followed by acid treatment also reduced the mobility of the proteins and decreased the band spreading (Figure 5). Electrophoresis of these latter samples in $NaDodSO_4$ buffers showed that dephosphorylation did not decrease band spreading (cf. Figure 3) markedly and the dimeric material was still present. These results suggest that $POCl_3$ causes reactions other than phosphorylation to take place.

^{31}P NMR Identification of Phosphorylation Sites in Phospho-β-lactoglobulin. Samples of phospho-β-lactoglobulin prepared by using $POCl_3$, $POCl_3$ in CCl_4 and $POCl_3$ in mineral oil were examined, and the spectra were indistinguishable. The spectrum of the protein prepared without solvents is shown in Figure 6. Each of these samples was held 1 week at 2 °C, pH 7.5, and the spectrum was redetermined both before and after dialysis against 0.05 M Tris buffer, pH 7.5. Hydrolysis of each compound caused loss of peak intensity for all peaks with a new peak appearing near 27.5 ppm. This peak was lost on dialysis of the protein, suggesting that it was caused by orthophosphate (Table VII). By comparison with reference compounds (Table VII), the major peak of phospho-β-lactoglobulin was likely to be *N*-phospholysine and/or *N*-phosphohistidine. (Both imidodiphosphate and phosphorylated poly-L-lysine have their major peaks near 30.5 ppm, while phospho-β-lactoglobulin has peaks at 30.15 and 31.65 ppm; Table VII and Figure 6.) It is quite clear that the protein does not contain any *O*-phospho-L-serine (27.4 ppm). The peaks between 32 and 55 ppm were not identified, but they could well arise from nitrogen di- or triphosphates, *O,O'*-phospho diesters, or *N,N'*-phosphodiamides.

CD of Phospho-β-lactoglobulin. The CD spectra of β-lactoglobulin in phosphate buffer or guanidine hydrochloride solution and of freshly dissolved and acid-hydrolyzed phospho-β-lactoglobulin were measured in the range 190–350 nm, and the distinguishably different spectra are shown in Figure 7. The unmodified protein in its native state gave a spectrum very similar to that reported earlied (Timasheff et al., 1967) over the whole wavelength range, although the present ellipticities were slightly greater. These earlier workers interpreted the far UV region of the spectrum to show that β-lactoglobulin was about 50% aperiodic, 25% α-helical, and 25% extended sheet. By use of the computer program of Siegel et al. (1980), the present spectrum was consistent with 17.9 ± 1.7% α-helical structure. Phosphorylation caused this to decrease to 16.1 ± 1.6% α-helical structure. The dephosphorylated phosphoprotein had a spectrum that was not distinguishable from that of the phosphoprotein, indicating that the structural changes brought about by phosphorylation were not reversed when the phosphate groups were removed.

The interpretation of near-UV CD spectra of globular proteins is complex because of the large number of absorption bands involved (Strickland, 1974). However, it seems likely that the deep sharp troughs at the highest wavelengths are caused by the ^1L_b transitions of one or both of the tryptophan residues of the protein. Ellipticity is normally a result of energy transfer to or from an asymmetric center, such as a segment of the polypeptide backbone or another aromatic residue. Treatment of β-lactoglobulin with dithiothreitol did not affect the spectrum, showing that the potent disulfide bond was not involved in the observed chiroptical effects. Guanidine hydrochloride, which completely disrupts periodic structure, gave an almost flat spectrum for β-lactoglobulin from 350 to 210 nm. (The absorbance by the guanidine itself precludes spectral measurement at lower wavelengths.) The results (near-UV CD spectra) show that the phosphoprotein has retained much of the original β-lactoglobulin structure.

DISCUSSION

The present results confirm and extend the earlier conclusions (Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Ferrel et al., 1948; Salák et al., 1965) regarding the chemical phosphorylation of globular proteins. These earlier studies showed that phosphorylation with $POCl_3$ in CCl_4 or P_2O_5 in H_3PO_4 gave products that gradually lost phosphate groups and this loss accelerated at lower pH. The phosphate residues seemed to be attached to protein ε-amino groups of lysine or the side-chain nitrogen groups of imidazole or arginine. However, the total number of phosphate groups bound, even under conditions of maximum phosphorylation, did not sum to the total number of possible sites. In some instances, there was evidence of serine or threonine phosphate formation. Ullman and Perlman (1975) found that they could produce a serine phosphate in unfractionated protamine by phosphorylation with H_3PO_4 and trichloroacetonitrile in Me_2SO, followed by 0.5 M H_2SO_4 treatment to remove acid-labile (nonserine) phosphates. Willmitzer and Wagner (1975) found that $POCl_3$ in trimethylphosphate gave serine phosphate and polyphosphate when a purified protamine, clupeine, was used in the capronate form as the substrate. In contrast, the present ^{31}P NMR results (Figure 6 and Table VII) show that no serine phosphate was formed in the $POCl_3$ phosphorylation of β-lactoglobulin and that the major phosphate species were likely to be *N*-phospholysine and/or *N*-phosphohistidine. The gel electrophoresis results (Figures 2 and 3) indicate that phosphorylation gives a multiplicity of components, including phosphorylated β-lactoglobulin dimers (molecular weight ~37 000). The band spreading shown in Figure 2 may have arisen from the differing extents of phosphorylation on the individual protein molecules. However, dephosphorylation (Figures 4 and 5) did not collapse the spread bands into bands of comparable width to those of the starting material, and this result, together with the realization that the dimerization that occurs most readily in the two-phase phosphorylating systems is not reversed on dephosphorylation and that the CD spectrum of dephosphorylated phos-

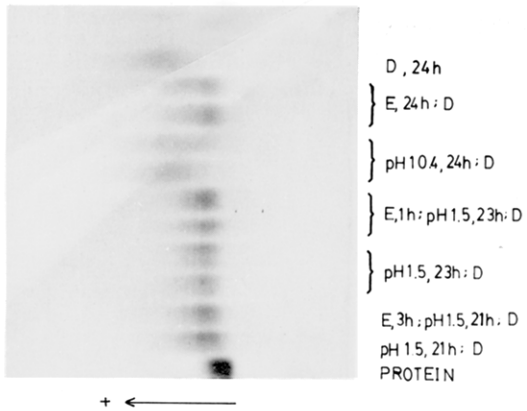

Figure 5. Electrophoresis pattern of enzyme- and acid-treated phospho-β-lactoglobulin in a urea–polyacrylamide gel. Sample code: E, treated with alkaline phosphatase; the rest of the sample codes are as described in the legend of Figure 4.

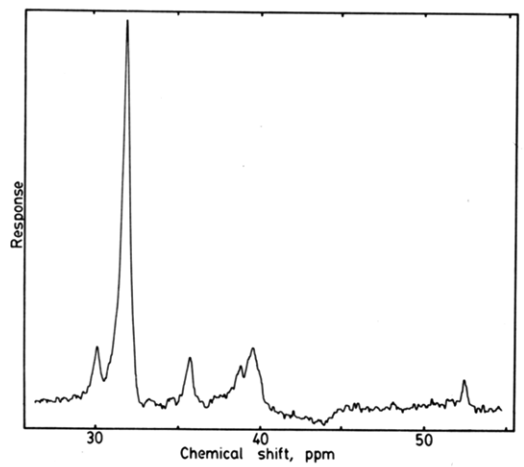

Figure 6. ^{31}P NMR spectrum of phospho-β-lactoglobulin.

Table VII. Chemical Shifts of Phosphorylated β-Lactoglobulin and Standard Compounds in 0.05 M Tris Buffer at pH 7.5[a]

compound	chemical shift, ppm
O-phosphoserine	27.40
0.1 M orthophosphate	27.80
85% orthophosphoric acid	31.79
imidodiphosphate	30.44
phosphocreatine	34.26
ATP: α	41.86
β	52.02
γ	36.70
phosphopolylysine	30.51, 36.32[b]
phospho-β-lactoglobulin[d]	30.15
	31.65
	35.80
	38.9, 39.6[c]
	52.65

[a] Chemical shift relative to methyl phosphonate. [b] Minor peak. [c] Doublet. [d] Average values for three solvent systems.

pho-β-lactoglobulin is similar to that of phospho-β-lactoglobulin, suggests that $POCl_3$ introduces covalent bonds other than phosphorus to nitrogen or oxygen bonds. One possibility might be the reaction sequence

$$R\text{–}NH_2 + POCl_3 \rightarrow P\text{–}NH\text{–}POCl_2 \xrightarrow{R'\text{–}CO_2^-} R\text{–}NH\text{–}CO\text{–}R'$$

thus forming an isopeptide linkage that could constrain the β-lactoglobulin molecules in some conformation other than the normal one. This would allow these modified β-lactoglobulin molecules to migrate through the polyacrylamide gel in urea or $NaDodSO_4$ solution differently from the unmodified protein.

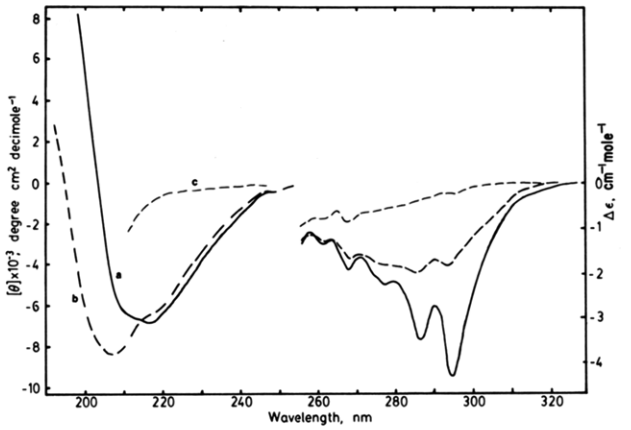

Figure 7. CD spectra of β-lactoglobulin (line a), phospho-β-lactoglobulin (line b), and β-lactoglobulin in 5 M guanidine hydrochloride (line c). Dephosphorylated phospho-β-lactoglobulin had a spectrum that was indistinguishable from that of the phospho-β-lactoglobulin.

Whey protein is used in the food industry partly because of its functional properties of whippability, solubility at acid pH, and heat denaturability to form stable foams and gels. Some of these properties depend on surface activity of the protein and its changes during denaturation processes. As a working hypothesis, it is likely that phosphorylation of whey protein will alter the net charge and the amphipathic nature of the proteins, and this may modify the emulsifying power of the protein, for example. It is our intention to explore these and other prospects for the incorporation of phosphorylated whey proteins into foods. The lability of the phosphate may be an advantage in a food system, and its enhancement at low pH means that the N–phosphate bonds will certainly be cleaved during digestion and hence the *N*-phospholysine should have the same biological availability as normal lysine.

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Effects of Formaldehyde on Protein Extraction and Quality of High- and Low-Tannin Sorghum

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Dilute solutions of formaldehyde were used to inactivate the tannins in high-tannin (bird-resistant) sorghum. By means of this treatment it was shown that high- and low-tannin sorghums contain similar proportions of the different classes of protein. The differences observed in protein yield, when high-tannin sorghums are extracted (without pretreatment) by using the Landry and Moureaux fractionation, are due to interactions between tannins and albumin, globulin, and prolamin proteins, the majority of these proteins being rendered insoluble in their usual solvents. In addition, electrophoresis indicated that those proteins which were extractable from high-tannin sorghum were bound to tannins. As formaldehyde facilitated the quantitative extraction of proteins from high-tannin sorghum, it is suggested that it may have a more general application in the study of proteins from plant material rich in polyphenols.

The proteins of high-tannin (bird-resistant) and low-tannin cultivars of sorghum have been examined by a number of workers (Jambunathan and Mertz, 1973; Chibber et al., 1978; Guiragossian et al., 1978) using the fractionation procedure of Landry and Moureaux (1970). These workers found that high-tannin cultivars contain a lower proportion of salt-soluble, albumin plus globulin, and alcohol-soluble, prolamin, proteins than low-tannin types. Conversely, the proportion of glutelins which are the proteins soluble in pH 10 borate buffer containing 2-mercaptoethanol and sodium dodecyl sulfate (NaDodSO$_4$) is greater in the high-tannin varieties.

Chibber et al. (1978) postulated that the lower proportion of albumins, globulins, and prolamins found in high-tannin sorghum is due to interactions between these proteins and the tannins, rendering them insoluble in their normal solvents but extractable by the glutelin solvent. Evidence to support this theory has been obtained by Fishman and Neucere (1980), who demonstrated the presence of salt-soluble protein–tannin complexes in the glutelin fraction obtained from a high-tannin sorghum cultivar.

Daiber (1976) patented a process in which high-tannin sorghum grain for use as malt in the brewing of sorghum beer is treated with dilute formaldehyde solution. The formaldehyde reacts with the tannins, thus preventing their subsequent complexing with and inactivation of enzymes formed during malting. The reaction between formaldehyde and tannins is probably similar to that which occurs between it and phenol in the formation of "Bakelite", a phenol–formaldehyde resin (Morrison and Boyd, 1966).

In this report which forms part of a larger investigation into sorghum polyphenols (Kaluza et al., 1980; Glennie, 1981), the effect of soaking grain in formaldehyde on the extraction and quality of salt- and alcohol-soluble proteins from high- and low-tannin varieties is described.

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Table I. Albumin plus Globulin and Prolamin Proteins Extracted from Sorghum Grain Treated with Different Concentrations of Formaldehyde

		% of total protein in grain	
	% formaldehyde	albumins + globulins	prolamins
SSK 52:[a]	0	0.6	10.4
	0.04	10.4	14.5
	0.08	10.4	38.5
	0.16	10.3	36.6
G 766 W:[b]	0	15.9	37.1
	0.04	17.2	43.3
	0.08	15.9	43.3
	0.16	14.8	41.9

[a] High tannin. [b] Low tannin.

EXPERIMENTAL SECTION

Grain (30 g) of sorghum cultivars SSK 52 (high tannin) and G 766 W (low tannin) was steeped for 6 h at room temperature in 30 mL of the following solutions: distilled water and 0.04, 0.08, and 0.16% formaldehyde. It was then blotted dry and dried further at 50 °C for 16 h.

The dried grain (20 g) was ground for 2 min in a Janke and Kunkel beater-type mill. Five grams of the resulting flour was extracted sequentially by stirring for three 1-h periods with 25-mL aliquots of 0.8 M NaCl at 4 °C and then with 55% (v/v) isopropyl alcohol plus 2% (v/v) 2-mercaptoethanol at 60 °C to extract the albumin plus globulin and prolamin proteins, respectively. The supernatants were dialyzed overnight against distilled water and freeze-dried.

Protein ($N \times 6.25$) was determined by the method of Thomas et al. (1967). So that nitrogen recovery could be checked, acetanilide was included with each batch of di-

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