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Phosphorylation of Casein and Lysozyme by Phosphorus Oxychloride

Günter Matheis, Michael H. Penner, Robert E. Feeney, and John R. Whitaker*

Casein and lysozyme were phosphorylated by phosphorus oxychloride at pH 6–8 and 3–20 °C. Up to 7.4 and 6.2 mol of phosphate/mol of protein were covalently attached to casein and lysozyme, respectively. ^{31}P NMR spectral data and pH stability studies of the phosphate residues indicated that in phosphorylated casein, the phosphate was exclusively bound to hydroxyl oxygen as monophosphate and diphosphate. The phosphate linkages were stable at pH 2.0–8.5. In contrast, the majority of the phosphate in phosphorylated lysozyme appeared to be bound to nitrogen. In addition to mono- and diphosphate, triphosphate bonds were present. Gel electrophoresis in the presence of sodium dodecyl sulfate and urea indicated that protein cross-linking occurred during phosphorylation. Although there was a considerable decrease in the initial rates of both trypsin- and α-chymotrypsin-catalyzed hydrolysis of phosphorylated casein, the extent of hydrolysis after 24 h was the same for control and phosphorylated casein. In the bioassay, *Tetrahymena thermophili* grew as well on the phosphorylated casein as on Hammarsten casein. Dispersions of phosphorylated casein had significantly higher viscosities than control casein. In contrast, the viscosity of lysozyme was not affected by phosphorylation. Both phosphorylated proteins adsorbed more moisture than the corresponding control proteins. The emulsifying capacity of phosphorylated casein was lower than that of control casein.

The feasibility of using alternative sources of proteins (e.g., trash fish, grain, microbes, and leaf) as food proteins is often limited due to their low biological value, undesirable organoleptic properties, toxic constituents, and poor functional properties. These problems may be overcome by physical or mechanical treatment or by microbial, enzymatic, or chemical modification. Modification of proteins by phosphorylation is examined in this paper as a means to improve functional properties.

Phosphorylation of proteins has been achieved by using a variety of chemicals: phosphorus oxychloride (Bechhold, 1901; Neuberg and Pollak, 1910; Neuberg and Oertel, 1914; Rimington, 1927; Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Boursnell et al., 1948; Salák et al., 1965; Willmitzer and Wagner, 1975; Woo et al., 1982), phosphorus pentoxide dissolved in phosphoric acid (Ferrel et al., 1948; Dickson and Perkins, 1971; Rao et al., 1975), phosphoric acid with trichloroacetonitrile as a coupling agent (Ullman and Perlman, 1975; Yoshikawa et al., 1981), monophenyl phosphodichloride (Bourland et al., 1949), phosphoramidate (Müller et al., 1956; Rathlev and Rosenberg, 1956), diphosphoimidazole (Taborsky, 1958), and trisodium trimetaphosphate (Sung, 1982). Changes in functional properties (Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Ferrel et al., 1948; Salák et al., 1965; Sung, 1982) and changes in in vitro digestibility (Neuberg and Oertel, 1914; Rimington, 1927; Taborsky, 1958; Sung, 1982) due to phosphorylation have been studied only occasionally.

According to previous studies, the phosphorus bound to proteins by chemical derivatization could be attached to the hydroxyl oxygen (Rimington, 1927; Ferrel et al.,

Department of Food Science and Technology, University of California, Davis, Davis, California 95616.

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1948; Willmitzer and Wagner, 1975; Ullman and Perlman, 1975) or to the amino and imidazole nitrogen (Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Rathlev and Rosenberg, 1956; Taborsky, 1958; Salák et al., 1965; Woo et al., 1982). Besides monophosphate esters, considerable amounts of di- and polyphosphate esters were found (Willmitzer and Wagner, 1975).

The purpose of this work was to conduct a fundamental study of the effects of phosphorylation of proteins on the in vivo and in vitro digestibility and on the functional properties of the modified proteins as well as to explore the nature of the phosphorus linkage(s). $POCl_3$ was used for the phosphorylation and casein and lysozyme were chosen as the model proteins.

MATERIALS AND METHODS

Materials. Hammarsten quality casein was obtained from Fluka, Buchs, Switzerland. Egg white lysozyme (grade I), bovine serum albumin, porcine trypsin (crystallized; type IX), bovine α-chymotrypsin (3× crystallized; type II), 2,4,6-trinitrobenzenesulfonic acid, phosphocreatine, and D_2O were obtained from Sigma, St. Louis, MO. Human serum transferrin was from Behring Diagnostics, Somerville, NJ. Soybean trypsin inhibitor was from Nutritional Biochemical Corp., Cleveland, OH. Cytochrome *c* was from Boehringer, Mannheim, West Germany. Ovalbumin was prepared in our laboratory. Mixed-bed resin AG 501-X8 (20–50 mesh) was obtained from Bio-Rad, Richmond, CA. 2,4-Diaminophenol dihydrochloride (amidol) was from Pfaltz and Bauer, Stanford, CO. Coomassie Brilliant Blue R-250 was from Eastman Kodak, Rochester, NY. The original inoculum of *Tetrahymena thermophili* was a gift of Professor Eduardo Orias, Department of Biological Sciences, University of California, Santa Barbara. All other reagents and chemicals were of analytical grade.

Phosphorylation. The procedure of Neuberg and Oertel (1914) was followed except that the molar ratio of $POCl_3$/protein was higher. To a solution of 2% protein in 0.1 M phosphate buffer, pH 7.0, a 20% (w/w) solution of $POCl_3$ in CCl_4 was added dropwise under continuous stirring over a period of 60–120 min. The molar ratio of $POCl_3$/protein was 2000. The temperature of the reaction mixture varied between 3 and 20 °C (maintained by an ice/water bath). The pH was kept between 6.0 and 8.0 by adding 5 N NaOH simultaneously. After addition of the $POCl_3/CCl_4$, stirring of the reaction mixture was continued until the pH remained constant at 7.0 (15–45 min). The CCl_4 was separated by centrifugation (25 °C; 20 min; 12 000*g*). The precipitate formed between the CCl_4 layer and aqueous layer (which was found to contain phosphate and protein) was discarded. The protein solution was dialyzed extensively against 0.1 M KCl and then against H_2O (phosphorylated casein, 25 °C; phosphorylated lysozyme, 2 °C). During dialysis, a precipitate formed in the phosphorylated lysozyme solution (this did not occur with phosphorylated casein). The precipitate was separated by centrifugation. The solutions and the precipitate were lyophilized. Control casein and control lysozyme were prepared in the same manner but in the absence of $POCl_3$. The following overall yields were obtained: phosphorylated caseins I–III and control casein, ~80%; phosphorylated lysozyme I and II (soluble fractions), ~40%; phosphorylated lysozyme III (precipitate), 17%; control lysozyme I (soluble fraction), 89%; control lysozyme II (precipitate), 1%. Operations using $POCl_3$ and CCl_4 were performed in ventilated hoods.

All phosphorylated protein samples contained inorganic phosphate (phosphorylated caseins, 0.17–0.37%; phosphorylated lysozymes, 0.37–0.49%). For removal of inorganic phosphate (P_i), phosphorylated casein II (0.37% P_i) was dispersed in H_2O, a mixed-bed resin (Bio-Rad AG 501-X8) was added, and the dispersion was stirred overnight at room temperature (23–25 °C). The pH of the dispersion dropped from 7.1 to 5.7 and part of the protein precipitated. After readjustment of the pH to 7.0 with 5 N NaOH, the precipitate dissolved. The resin was removed by passing through glass wool and the protein solution was lyophilized. The preparation (phosphorylated casein IV) still contained 0.12% P_i; i.e., 68% removal of P_i was achieved.

Phosphorus Determination. Total phosphorus was determined after sample digestion with $HClO_4$ according to Allen (1940) by a modification of the Fiske and Subbarow (1925) procedure. Inorganic phosphorus was determined after completely dissolving the protein so that the inorganic phosphate associated with protein may be released into solution but without causing any alkaline hydrolysis of the phosphate–ester bond (checked with casein) and then by precipitating the protein. This was done by adding 1 mL of 0.5 N NaOH to the protein solution or dispersion, keeping it for 30 s, and then precipitating the protein by making it 10% trichloroacetic acid. After centrifugation (25 °C; 10 min; 12 000*g*), the inorganic phosphorus was determined in the clear supernatant according to Allen (1940). Bound phosphorus was calculated from total phosphorus minus inorganic phosphorus.

Protein Determination. Protein determination was carried out according to Lowry et al. (1951) using casein and bovine serum albumin as reference proteins or by absorbance at 280 nm using $E^{1\%}_{280}$ = 26.35 for lysozyme (Osuga and Feeney, 1977).

^{31}P Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{31}P NMR spectroscopy was carried out on a Nicolet NT-200 NMR spectrometer operating at 81 MHz and 20 °C. H_3PO_4 (85%) was used as an external standard. Spectra at pH 8.0 (adjusted with 1 N NaOH) were obtained for the following compounds: 100 mg of phosphocreatine/mL of D_2O; 30 mg of control casein/mL of D_2O; 27 mg of phosphorylated casein III/mL of D_2O; 15 mg of control lysozyme/mL of D_2O [containing 0.7% sodium dodecyl sulfate ($NaDodSO_4$) and 0.7% urea]; 30 mg of phosphorylated lysozyme II/mL of D_2O (containing 0.7% $NaDodSO_4$ and 0.7% urea); 30 mg of phosphorylated lysozyme II/mL of D_2O after 24 h in 0.5 N HCl at 37 °C (containing 0.7% $NaDodSO_4$ and 0.7% urea). A spectrum at pH 3.5 (adjusted with 1 N HCl) was obtained for 100 mg of phosphocreatine/mL of D_2O. The number of scans were 100–200 for phosphocreatine, 2200–3000 for control proteins, and 3800–8600 for phosphorylated proteins. All spectra were obtained with an 80° tipping pulse and a 2-s repetition time. According to the IUPAC convention, chemical shifts are expressed as positive downfield.

Acid and Base Hydrolysis of Bound Phosphate. Protein (0.1–0.2%) in H_2O was adjusted to pH 1.5, 2.0, 3.0, 5.5, 7.0, 8.5, and 10.5 with 1 N HCl or 1 N NaOH and incubated at 37 °C for 24 h. In another set of experiments, 0.1–0.2% protein in 0.5 N HCl or 0.5 N NaOH (pH 0.8–1.1 or pH 12.2–13.2, respectively, depending on protein sample) was incubated under the same conditions. After incubation, total phosphorus and inorganic phosphorus were determined.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out in gels [7.5% acrylamide, 0.2% *N,N'*-methylenebis(acrylamide)] containing 0.1% $NaDodSO_4$ and 8 M urea. The gel and electrode buffer was 0.1 M phosphate, pH 7.2. The electrode buffer

Table I. Phosphorus Content of Phosphorylated Proteins

protein	phosphorus content: %	mol/mol of protein	mol of P/mol introduced
Hammarsten casein	0.83[a]	6.2	
control casein	0.84	6.2	
phosphorylated casein			
I[b]	1.71	12.8	6.6
II[b]	1.53	11.5	5.3
III[b]	1.83	13.6	7.4
IV[c]	1.52	11.3	5.1
phosphorylated lysozyme			
I[b,d]	1.13	5.2	5.2
II[b,d]	1.34	6.2	6.2
III[e]	0.61	2.9	2.9

[a] 0.83-0.88% according to Taborsky (1974). [b] Prepared by the same procedure. [c] Prepared from phosphorylated casein II by removing inorganic phosphate with ion exchanger. [d] Soluble fractions. [e] Insoluble fraction.

contained 0.1% $NaDodSO_4$ and 8 M urea. The sample buffer was 0.01 M phosphate, pH 7.2, containing 0.2% $NaDodSO_4$, 8 M urea, and 0.14 M mercaptoethanol. Samples were boiled in the sample buffer for 10 min prior to application. Bromophenol Blue was used for dye front. Electrophoresis was carried out at 1 mA/tube at room temperature (23–25 °C). Prior to staining, $NaDodSO_4$ was removed by washing the gels in 7.5% acetic acid/50% methanol. Proteins were stained with 0.25% Coomassie Brilliant Blue R-250 in 10% acetic acid/45% methanol. Gels were destained with 7.5% acetic acid/5% methanol (Weber and Osborn, 1975).

Digestibility. In vitro digestibility with α-chymotrypsin or trypsin was determined by measuring the amount of amino groups liberated with 2,4,6-trinitrobenzenesulfonic acid (Lin et al., 1969) as modified by Fields (1972). The reactions, containing 0.1% protein in 0.1 M phosphate buffer, pH 8.0, were performed at 38 °C. For determination of initial rates of hydrolysis, assays were done with 0.068 μM α-chymotrypsin or 0.070 μM trypsin. Aliquots removed at 5-min intervals for a period of 20 min were quenched by 0.05 N HCl. For determination of the extents of hydrolysis, assays were done for 24 h at 38 °C with 0.68 μM α-chymotrypsin or 0.70 μM trypsin. The reactions were quenched as above.

***Tetrahymena* Bioassay.** The *T. thermophili* parent culture was maintained in 2% proteose peptone containing 0.01 mM ferric chloride, penicillin G (250 μg/mL), and streptomycin (250 μg/mL) at 25 °C. Active cultures were maintained by weekly transfers into 2% proteose peptone as described above and kept at 25 °C. A 1-day-old culture grown at 30 °C in chemically defined medium (Rasmussen and Modeweg-Hansen, 1973) was used for the bioassays. Except for the defined medium, all amino acids were omitted and replaced with Hammarsten casein or phosphorylated casein II (final concentration 0.08% protein) for the bioassays. The experimental cultures were grown at 30 °C without shaking in 20 mL of the medium in 250-mL-capacity screw-capped Erlenmeyer flasks. They were inoculated with sufficient cells to give an initial absorbance at 600 nm of at least 0.060 but not more than 0.100. The increase in cell concentration was measured periodically at 600 nm, and the rate of growth was expressed as the slope of the line of log of absorbance plotted vs. time (minutes).

Viscosity. A Cannon-Ubbelohde (semimicro size 50) viscosimeter was used to measure the viscosity of 0.1% protein solutions or dispersions in 0.05 M borate buffer, pH 7.9 (caseins), or of 0.2% protein solutions or dispersions in 0.05 M ammonium formate buffer, pH 3.4 (lysozymes), at 25 °C (maintained in a water bath). Viscosities are expressed relative to that of the corresponding buffers.

Water Binding. Weighed samples of dried proteins (vacuum oven, 60 °C, 150 h) were kept in a desiccator equilibrated to 43.0% relative humidity (saturated potassium carbonate solution). After 150 h, the samples were reweighed.

Emulsifying Activity. Emulsifying activity was determined according to Sen et al. (1981) with 0.2% protein solutions or dispersions in 0.1 M phosphate buffer, pH 7.0.

RESULTS

Phosphorus Content of Phosphorylated Proteins. Table I shows the phosphorus content of the phosphorylated proteins. Up to 7.4 and 6.2 mol of phosphate/mol of protein could be covalently linked to casein and lysozyme, respectively. The phosphorylation of lysozyme resulted in a soluble and an insoluble fraction. The soluble fraction was phosphorylated to a higher degree than the insoluble fraction (Table I).

Nature of Phosphate Linkages. The nature of the phosphate linkages to the proteins was characterized by studies of the stability to pH (Table II) as well as by ^{31}P NMR spectroscopy (Figures 1–3). Most of the phosphate bound to phosphorylated casein is base labile (0.5 N NaOH, 24 h, 37 °C). In contrast, the majority of phosphate bound to phosphorylated lysozyme is acid labile (0.5 N HCl, 24 h, 37 °C). Phosphate linked to the hydroxyl groups of protein serine and threonine residues is known to be base labile and acid stable (Rimington and Kay, 1926; Anderson and Kelley, 1959). This was confirmed with Hammarsten casein (Table II), which contains only serine-

Table II. Stability of the Phosphate Bonds at Various pH Values[a]

pH	Hammarsten casein		phosphorylated casein III		phosphorylated casein IV		phosphorylated lysozyme II	
	mol of P/mol[b]	hydrolysis, %	mol of P/mol[b]	hydrolysis, %	mol of P/mol[b]	hydrolysis, %	mol of P/mol[b]	hydrolysis, %
untreated sample	6.2	0	13.6	0	11.3	0	6.2	0
0.8-1.1[c]	6.2	0.6	12.4	8.7	10.5	7.1	3.8	38.1
1.5[d]	6.2	0.6			10.5	7.1		
2.0-8.5[d]	6.2	0.6	13.4	1.4	10.9	3.6	5.6[f]	9.0[f]
10.5[d]	6.1	1.2	12.6	7.1	10.9	3.6		
12.2-13.2[e]	0.1	99.1	1.6	88.0	2.4	78.6	5.0	20.1

[a] 0.1-0.2% protein for 24 h at 37 °C. [b] Moles of P per mole of protein left after treatment at the indicated pH. [c] Protein in 0.5 N HCl; actual pH depended on the protein sample. [d] Protein in H_2O, adjusted to pH 1.5, 2.0, 3.0, 5.5, 7.0, 8.5, or 10.5 with 1 N HCl or 1 N NaOH. [e] Protein in 0.5 N NaOH; actual pH depended on protein sample. [f] Determined for pH 7.0 only.

Table III. Chemical Shifts (^{31}P NMR) of Phosphorylated Proteins and Reference Compounds at pH 8.0 Relative to 85% H_3PO_4

chemical shifts,[a] ppm			
phosphorylated casein III	phosphorylated lysozyme II	phosphorylated lysozyme II after 24 h in 0.5 N HCl	approximate chemical shifts of reference compounds
4.37	4.14	4.58	3.6 to 4.4 for phosphoserine[c-e]
			3.9 with a shoulder at 4.3 for control casein[f]
3.09	2.82	3.16	3.0 for inorganic phosphate[d,g-j]
	0.31/0.05[b]	0.71	1.4 for imidodiphosphate[e]
			1.3 for phosphopolylysine[e]
			0.5 to 1.0 for phosphodiesters such as 3-glycerophosphoserine[j]
			0.2 for α-naphthyl phosphate[k]
			0.0 for phenyl phosphate[k]
	−3.96		−2.4 to −3.2 for phosphocreatine and phosphoarginine[c,f,h,k,n]
−5.10	−5.81	−4.76/−4.79/ −4.99[b]	−4.9 to −6.0 for γ-P of ATP and β-P of ADP[e,g,h,n,o]
			−4.8 for terminal phosphates of polyphosphates larger than triphosphate[j]
	−6.43		−6.5 for inorganic diphosphate[i]
−9.61/−9.85[b]	−10.06/−10.23/ −10.31/−10.62[b]		−10.1 to −11.0 for α-P of ATP and ADP[e,g-i,o]
	−14.52	−13.65	not identified in literature
	−21.04		−20.2 to −23.0 for β-P of ATP and inorganic triphosphate[e,g-i,o]
			−17.4 to −22.5 for nonterminal phosphates of polyphosphates[l]

[a] Expressed as positive downfield. [b] Peaks not separated. [c] Willmitzer and Wagner (1975). [d] Coleman et al. (1979). [e] Woo et al. (1982) (pH 7.5). [f] Figure 4. [g] Moon and Richards (1973). [h] Gadian et al. (1979). [i] Salhany et al. (1975). [j] Navon et al. (1979). [k] Bock and Sheard (1975). [l] Chance et al. (1979). [m] Nageswara Rao (1979). [n] Burt et al. (1976). [o] Cohn and Hughes (1960).

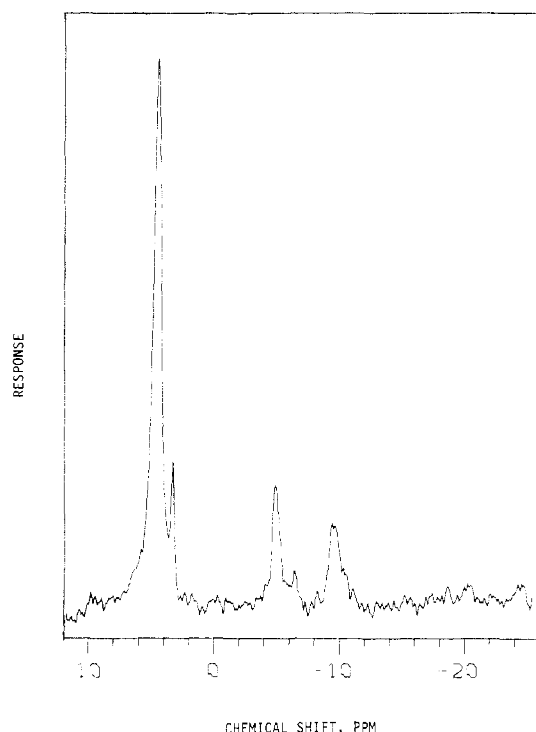

Figure 1. ^{31}P NMR spectrum of phosphorylated casein III at pH 8.0 and 20 °C. H_3PO_4 (85%) was the external standard. 8600 scans. Resonance signals downfield to H_3PO_4 are positive.

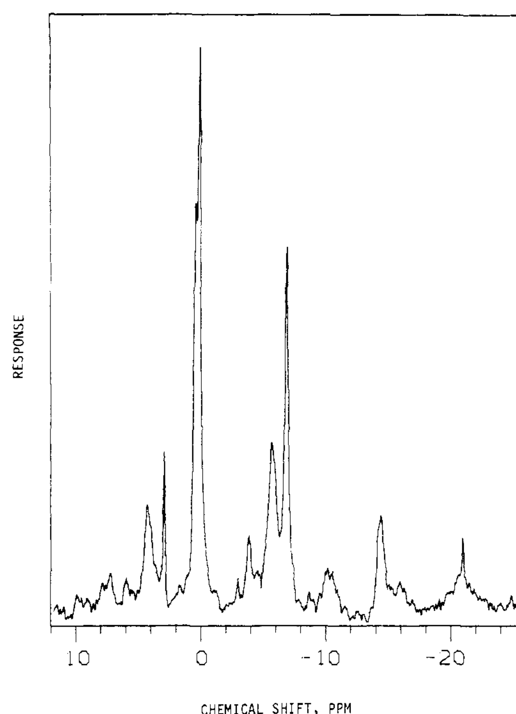

Figure 2. ^{31}P NMR spectrum of phosphorylated lysozyme II at pH 8.0 and 20 °C. H_3PO_4 (85%) was the external standard. 3800 scans. Resonance signals downfield to H_3PO_4 are positive.

and threonine-bound phosphorus (Bingham, 1976). Tyrosine-linked phosphorus is also base labile and acid stable (Matheis et al., 1981). In contrast, nitrogen-bound phosphorus and the ester bonds of di- and polyphosphates are acid labile and base stable (Rimington and Kay, 1926; Zervas and Katsoyannis, 1955; Lapidot and Halmann, 1958; Willmitzer and Wagner, 1975).

Figures 1 and 2 show the ^{31}P NMR spectra of phosphorylated casein III and phosphorylated lysozyme II at pH 8.0, respectively. The chemical shifts are related to 85% H_3PO_4. Attempts were made to relate the observed shifts to shifts of known reference compounds from the literature (Table III) and to control casein, control lysozyme, and phosphocreatine (Figure 4). Phosphorylated casein contains exclusively oxygen-bound phosphate. The peaks at −5.1 and near −10 ppm (Figure 1), which were not present in control casein (Figure 4A), probably represent diphosphate, since the chemical shifts are close to the signals of adenosine diphosphate (ADP) (Table III).

The spectrum of phosphorylated lysozyme (Figure 2) is much more complicated than that of phosphorylated casein. Only small amounts of oxygen-bound phosphorus are present. The majority of the phosphate appears near 0 ppm, where the signals of phosphodiesters (e.g., 3-glycerophosphoserine and 3-glycerophosphocholine) and

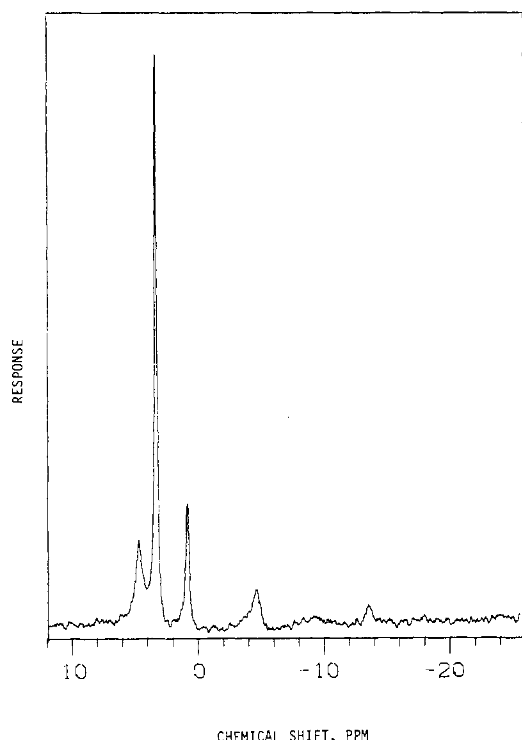

Figure 3. ^{31}P NMR spectrum of phosphorylated lysozyme II at pH 8.0 and 20 °C after 24 h in 0.5 N HCl at 37 °C. H_3PO_4 (85%) was the external standard. 5400 scans. Resonance signals downfield to H_3PO_4 are positive.

aryl phosphates appear (Table III). However, after acid treatment (0.5 N HCl, 24 h, 27 °C) of the phosphorylated lysozyme most of the phosphate near 0 ppm has disappeared (Figure 3). This makes it unlikely that it is a phosphodiester or an aryl phosphate, since the oxygen-bound phosphorus in these compounds should not be acid labile. Although the chemical shifts of low molecular weight compounds containing one nitrogen-bound phosphate, e.g., phospholysine, phosphocreatine, and phosphoarginine, are in the range of −2.4 to −3.2 ppm at pH 7.5–8.0 (Table III; Figure 4B), the chemical shift of imidodiphosphate, which contains two nitrogen-bound phosphates, is 1.4 ppm at pH 7.5 (Table III). The chemical shift of the high molecular weight compound phosphopolylysine is 1.3 ppm (with a minor peak at −4.5 ppm) at pH 7.5 relative to H_3PO_4 (Table III). It appears from this and from the acid instability that most of the phosphate near 0 ppm as well as the phosphate at −4 ppm, which is also acid labile (Figures 2 and 3), is nitrogen-bound phosphorus.

The peaks at −5.8, −10 to −10.6, and −21 ppm for phosphorylated lysozyme (Figure 2) probably represent tri- or polyphosphates, since the chemical shifts are close to the signals of adenosine triphosphate (ATP) and of polyphosphates larger than triphosphates (Table III). This is in agreement with the disappearance of the peaks after acid treatment (0.5 N HCl, 24 h, 37 °C) (Figure 3). While the peaks corresponding to α-P and β-P of ATP have disappeared completely, there is still some phosphate near the signal of γ-P of ATP at −4.8 to −5.0 ppm (Figure 3). This cannot be the terminal phosphate of a polyphosphate, and the nature of the phosphate linkage remains unidentified. The phosphorus at −14.5 ppm, which is acid labile (Figures 2 and 3), also remains unidentified.

In contrast to phosphorylated casein, phosphorylated lysozyme contained inorganic diphosphate (PP_i) in addition to inorganic monophosphate (P_i) (Figure 2; Table III). The narrow peak on top of the broader peak at −21 ppm

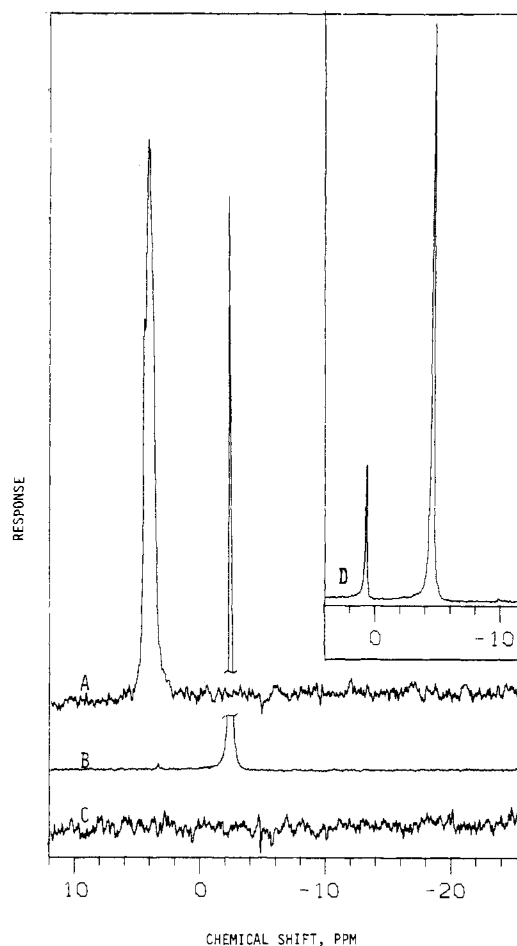

Figure 4. ^{31}P NMR spectra at pH 8.0 and 20 °C of (A) control casein, (B) phosphocreatine, (C) control lysozyme, and (D) phosphocreatine at pH 3.5 and 20 °C. H_3PO_4 (85%) was the external standard. 2200 (control casein), 100 (phosphocreatine at pH 8.0), 3000 (control lysozyme), and 200 (phosphocreatine at pH 3.5) scans. Resonance signals downfield to H_3PO_4 are positive.

even indicates the presence of small amounts of inorganic triphosphate (PPP_i). It should be pointed out that narrow line widths of peaks is indicative of phosphate bound to small molecules rather than bound to protein. The presence of PP_i in the sample was confirmed by thin-layer chromatography according to Seiler (1961) (results not shown).

Nitrogen-bound phosphorus is very acid labile (Zervas and Katsoyannis, 1955; Willmitzer and Wagner, 1975). We adjusted a phosphocreatine solution in D_2O to pH 3.5 and ran it immediately on the NMR spectrometer. Considerable amounts of P_i (at 0.56 ppm) were found (Figure 4D), although the whole procedure took less than 20 min. No P_i was present in the phosphocreatine sample at pH 8.0 (Figure 4B). Willmitzer and Wagner (1975) have shown that nitrogen-bound phosphate is much more acid labile than the diphosphate ester bond of ADP. Thus, the presence of PP_i and PPP_i in the phosphorylated lysozyme sample seems to arise from hydrolysis of nitrogen-bound di-, tri-, and/or polyphosphates. No NMR signal was obtained from control lysozyme (Figure 4C).

Attempts were made to quantify the ^{31}P NMR results. This was done by drawing a base line on the NMR spectrum, cutting out the peaks for the different phosphorus species, and weighing them. The percentage distribution of the different bound phosphorus species was compared to the results obtained by pH stability studies. The results for phosphorylated casein are in close agreement (Table

Table IV. Percentage Distribution of Different Phosphate Linkages in Phosphorylated Proteins

phosphate linkage	phosphorylated casein III		phosphorylated lysozyme II	
	pH stability studies	NMR	pH stability studies	NMR
$-O-PO_3^{2-}$	86.6[a]	85.5	11.1[b]	10.1
$-O-P_2O_6^{3-}$	14.6[c]	14.5	n.c.[d]	68.8
$-O-P_3O_9^{4-}$	not present			
$-NH-PO_3^{2-}$	not present			
$-NH-P_2O_6^{3-}$	not present			
$-NH-P_3O_9^{4-}$	not present			
unidentified	not present		n.c.[e]	21.1

[a] Base labile minus pH 2.0-8.5 labile; see Table II. [b] Base labile minus pH 7.0 labile; see Table II. [c] 8.7% (acid labile) minus 1.4% (pH 2.0-8.5 labile) = 7.3% was found (see Table II); since only the β-P of diphosphate is detected by pH stability studies (base-labile diphosphate is not detected by the Fiske-Subbarow method), 7.3 was multiplied by 2 to give the α- and β-P of diphosphate. [d] Cannot be calculated because the amounts of di-, tri-, or polyphosphates are not known. [e] Cannot be calculated.

IV). In the case of phosphorylated lysozyme, only the oxygen-bound monophosphate could be compared. Again, the results are in close agreement (Table IV).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis of phosphorylated lysozyme II and phosphorylated casein III in the presence of $NaDodSO_4$, mercaptoethanol, and 8 M urea is shown in Figure 5. Separation is based primarily on size. In addition to the monomer, the phosphorylated lysozome shows at least five bands of lower mobility and some material on top of the gel (Figure 5d). A plot of log of molecular weight vs. mobility by using the reference proteins (Figure 5a,e) indicated that the closest band to the monomer, which is also present in native and control lysozyme (Figure 5b,c), is probably dimeric lysozyme. The other bands of lower mobility fell into multiples of the monomer, indicating that some cross-linking occurred during phosphorylation of lysozyme.

Phosphorylated casein did not enter the gel. This was shown for both a dispersion of the protein (Figure 5h) and a supernatant of that dispersion after centrifugation (Figure 5i). This indicates that cross-linking also occurred during phosphorylation of casein.

The cross-links appear to be generated by $POCl_3$, since the corresponding native and control proteins show identical patterns (parts b and c and f and g of Figure 5).

The same results were obtained when electrophoresis was carried out in the presence of $NaDodSO_4$ and mercaptoethanol but without urea (not shown).

In Vitro Digestibility by Trypsin and α-Chymotrypsin. There was a significant decrease in the initial rates of both trypsin- and α-chymotrypsin-catalyzed hydrolysis of phosphorylated caseins (Table V). The extent of hydrolysis after 24 h of both trypsin- and α-chymotrypsin-catalyzed digestion was the same for control casein and phosphorylated casein but slightly decreased compared to that of Hammarsten casein (Table V). It appears that this slight decrease is due to solvent effects on the proteins rather than to the phosphorylation of the casein.

***Tetrahymena* Bioassay.** The use of *Tetrahymena* sp. for protein quality evaluation relies on its similarity to mammals in requirement for essential amino acids and in the presence of similar enzyme systems (Kidder and Dewey, 1951). The growth rates of *T. thermophili* on Hammarsten casein and on phosphorylated casein II are shown in Table VI. The organism grew at 79% the rate

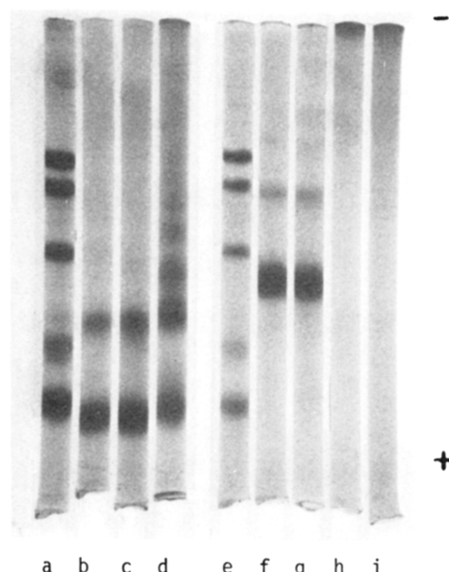

Figure 5. Polyacrylamide gel electrophoresis of proteins in the presence of $NaDodSO_4$, mercaptoethanol, and 8 M urea. (a) Reference proteins (from top to bottom) are human serum transferrin, bovine serum albumin, ovalbumin, soybean trypsin inhibitor, and cytochrome c; (b) native lysozyme; (c) control lysozyme; (d) phosphorylated lysozyme II; (e) reference proteins [see (a)]; (f) Hammarsten casein; (g) control casein; (h) phosphorylated casein III (dispersion); (i) phosphorylated casein III (supernatant).

Table V. Initial Rates of Hydrolysis and Extents of Hydrolysis after 24 h of Phosphorylated Caseins by Trypsin and α-Chymotrypsin[a]

protein	enzyme	rel initial rates	hydrolysis after 24 h: rel extent	hydrolysis after 24 h: degree of hydrolysis of susceptible bonds, %
Hammarsten casein	trypsin[b]	100	100	93
control casein		102	87	81
phosphorylated casein I		51		
phosphorylated casein III		40	86	80
Hammarsten casein	α-chymotrypsin[c]	100	100	94
control casein		96	96	91
phosphorylated casein I		30		
phosphorylated casein III		41	97	92

[a] 0.1% substrate in 0.1 M phosphate buffer, pH 8.0, 38 °C. [b] 0.07 μM for initial rates and 0.70 μM for extent of hydrolysis. [c] 0.068 μM for initial rates and 0.68 μM for extent of hydrolysis.

on Hammarsten casein as on the defined medium and at 95% the rate on phosphorylated casein II as on Hammarsten casein, i.e., *T. thermophili* grew as well on the phosphorylated casein as on the Hammarsten casein.

Stability of the Phosphorus Linkages at pH 2.0–8.5. The phosphate bonds of phosphorylated casein were stable through the whole pH range (Table II). The phosphate bonds of phosphorylated lysozyme were more labile at pH 7.0 than the phosphate bands in phosphorylated casein (Table II).

Functional Properties. *Viscosity.* Phosphorylated caseins had significantly higher relative viscosity than

Table VI. Growth Rate of *T. thermophili* on Phosphorylated Casein[a]

protein	growth rate,[b] $h^{-1} \times 10^2$	rel growth rate
defined medium	4.8	100.0
Hammarsten casein	3.8	79.2
phosphorylated casein II	3.6	75.0

[a] 0.08% protein in growth medium, 30 °C. [b] Slope of plot of log of absorbance vs. time.

Table VII. Functional Properties of Phosphorylated Proteins

protein	rel viscosity[a]	moisture adsorbed,[b] %	rel emulsifying activity[c]
Hammarsten casein	1.0169	7.88	100
control casein	1.0421	5.31	81
phosphorylated casein III	1.1714	6.00	27
phosphorylated casein IV	1.2354	7.44	25
lysozyme	1.0085	6.29	
control lysozyme I[d]	1.0042	4.34	
phosphorylated lysozyme I[d]	1.0127	11.19	
phosphorylated lysozyme III[e]	1.0085	12.00	

[a] 0.1% protein in 0.05 M borate buffer, pH 7.9 (caseins), or 0.2% protein in 0.05 M ammonium formate buffer, pH 3.4 (lysozymes), at 25 °C, relative to the corresponding buffer. [b] At 25 °C in 43% relative humidity. [c] 0.15% protein in 0.1 M phosphate buffer, pH 7.0, at 25 °C. [d] Soluble fractions. [e] Insoluble fraction.

Hammarsten casein and control casein (Table VII). In the case of lysozyme, however, phosphorylation did not affect the relative viscosity significantly (Table VII).

Water Binding. Phosphorylated lysozymes adsorbed more moisture than lysozyme or control lysozyme (Table VII). For example, phosphorylated lysozyme I (soluble fraction) bound 78% more water than lysozyme and 158% more water than control lysozyme at 43% relative humidity. Phosphorylated caseins adsorbed 13–40% more moisture than control casein but less moisture than Hammarsten casein (Table VII).

Emulsifying Activity. The phosphorylated caseins had significantly lower relative emulsifying activity than Hammarsten casein and control casein (Table VII).

DISCUSSION

Whole casein (Neuberg and Oertel, 1914; Rimington, 1927) as well as the casein fractions (Dickson and Perkins, 1971; Yoshikawa et al., 1981) has been chemically phosphorylated by various methods. The extent of phosphorylation of casein by $POCl_3$ reported here is in close agreement to the extents reported by the above authors.

According to the previous studies, the phosphorus in chemically phosphorylated proteins can be attached to the hydroxyl oxygen (Rimington, 1927; Ferrel et al., 1948; Ullman and Perlman, 1975; Willmitzer and Wagner, 1975) or to the amino and imidazole nitrogen (Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Rathlev and Rosenberg, 1956; Taborsky, 1958; Salák et al., 1965; Woo et al., 1982). Most of the information in the early work came from pH stability studies, determination of amino groups, and model studies with amino acids. Of the hydroxy amino acids, tyrosine was found to be either phosphorylated only to a limited extent (Ferrel et al., 1948) or not phosphorylated at all (Taborsky, 1958; Salák et al., 1965). No phosphorus bound to sulfur, carboxyl groups, or peptide bonds could be detected (Ferrel et al., 1948).

Recently, Willmitzer and Wagner (1975) and Woo et al. (1982) used ^{31}P NMR spectroscopy to determine the nature of phosphorus linkages in phosphorylated clupeine (a herring protamine) and in phosphorylated β-lactoglobulin, respectively. The NMR data indicated the presence of oxygen-bound mono-, di-, and polyphosphates in phosphorylated cluepeine. No nitrogen-bound phosphorus was detected. These findings agreed with the results of pH stability studies. The involvement of both serine and threonine residues in the phosphorylation was demonstrated by analyzing the protein hydrolysate. In contrast, the majority of phosphate in phosphorylated β-lactoglobulin was found by NMR and pH stability studies to be bound to nitrogen. No phosphoserine or phosphothreonine was found.

The results presented here (Figure 1; Tables II and III) confirm the early results of Rimington (1927) that in phosphorylated casein the phosphorus is exclusively bound to hydroxy amino acids. In an extension of Rimington's (1927) results, part of the phosphate was found to be bound as diphosphate.

In contrast to casein, the majority of the phosphate groups in phosphorylated lysozyme seems to be linked to nitrogen (Figures 2 and 3; Tables II and III) as was observed for β-lactoglobulin (Woo et al., 1982). The reason for this difference in reactivity to $POCl_3$ of groups in the three proteins is not known. The main peak on the NMR spectrum at 0.05 ppm is in close agreement to the main NMR peak (−0.14 ppm relative to 85% H_3PO_4) of phosphorylated β-lactoglobulin (Woo et al., 1982), which is believed to correspond to lysine- and/or histidine-bound phosphorus. The possibility remains that part of the phosphate near 0 ppm, which is still present after acid treatment (Figure 3), is tyrosine-bound phosphate and/or a phosphate linkage of the diester type. Only a small amount of the phosphate of phosphorylated lysozyme is bound to oxygen. Besides monophosphate bonds, polyphosphate bonds could be detected. The presence of inorganic polyphosphates in the sample suggests that at least part of the bound polyphosphates is linked to nitrogen, since the nitrogen–phosphorus linkage is the most labile linkage involved. At least two types of phosphate bonds in phosphorylated lysozyme remained unidentified. Unidentified NMR peaks are also reported for phosphorylated β-lactoglobulin (Woo et al., 1982). The authors suggest that they could well arise from nitrogen di- or triphosphates, *O,O′*-phosphodiesters, or *N,N′*-phosphodiamides.

Recently, Sung (1982) phosphorylated hydroxy and basic amino acids with trisodium trimetaphosphate. The reaction products of serine and lysine were phosphoserine and N^{ϵ}-lysinotriphophoramidate, respectively. A negligible amount of phospho derivatives was obtained with the other hydroxy and basic amino acids.

Breyer et al. (1967) obtained both base-labile and acid-labile phosphate bonds on phosphorylation of polyserine by chlorophosphoric acid. They proposed that the acid-labile phosphate was linked to α-amino groups after *N*–*O*-acyl migration. Willmitzer and Wagner (1975), in studying the occurrence of *N*–*O*-acyl migration in phosphorylated clupeine, concluded that the existence of N^{α}-phosphates "is very unlikely" when phosphorus oxychloride is the phosphorylating reagent.

In the present study, no attempts were made to determine whether the hydroxyl groups of all three hydroxy

amino acids (Ser, Thr, Tyr) in casein were phosphorylated or which nitrogen group in the side chain (Lys, His, Arg) of lysozyme is involved when chemical phosphorylation of proteins is done with $POCl_3$.

When β-lactoglobulin was phosphorylated by $POCl_3$, the gel electrophoresis results showed a number of components, including phosphorylated β-lactoglobulin dimers and protein near the origin of the gel (Woo et al., 1982). Similar results were obtained in the present work with lysozyme and casein (Figure 5). The nature of the cross-links generated by $POCl_3$ is not clear. Possible cross-links include phosphate bridges or isopeptide linkages as suggested by Woo et al. (1982).

The in vitro digestibility of chemically phosphorylated proteins by pepsin and pancreatic proteases has been studied previously (Neuberg and Oertel, 1914; Rimington, 1927; Taborsky, 1958; Sung, 1982). In the early work (Neuberg and Oertel, 1914; Rimington, 1927), the data were not compared to the hydrolysis of the unmodified proteins by the corresponding proteases. The results presented here (Table V) confirm and extend the previous findings that, although there was a considerable decrease in the initial rates of both trypsin- and α-chymotrypsin-catalyzed hydrolysis of phosphorylated casein, the extent of hydrolysis after 24 h was the same for control casein and phosphorylated casein. In the bioassay, *T. thermophili* grew as well on the phosphorylated casein as on Hammarsten casein, indicating that the phosphorylation either could be reversed in vivo or else did not affect the digestion, absorption, and utilization of the amino acids by the organism. It has been shown previously that both in tryptic hydrolysates of phosphorylated proteins (Rimington, 1927) and in intact phosphorylated proteins (Willmitzer and Wagner, 1975; Woo et al., 1982) the bound phosphate can be cleaved by phosphatases.

The viscosity of solutions or dispersions of the phosphorylated proteins increased only in the case of phosphorylated casein (Table VII). A possible explanation might be the greater extent of cross-linking in the case of casein (Figure 6). Increased viscosities were also reported for solutions of other phosphorylated proteins (Heidelberger et al., 1941; Mayer and Heidelberger, 1946). The dispersions of phosphorylated casein had a gel-like character, even at neutral pH (Matheis et al., 1981). These gel-forming properties need further investigation. Similar effects have been reported by other workers (Boursnell et al., 1948; Ferrel et al., 1948; Salák et al., 1965).

Both phosphorylated casein and lysozyme showed decreased water solubility (Matheis et al., 1981). Similar effects have been reported for other proteins phosphorylated by $POCl_3$ (Salák et al., 1965). This is unexpected in light of the hydrophilicity of the covalently attached phosphate groups. However, $POCl_3$ seems to introduce covalent bonds other than phosphate to oxygen or nitrogen and/or might cause the formation of *O,O′*-diesters or *N,N′*-diamines, leading to protein cross-linking (Figure 5). These cross-links may account for the decreased water solubility.

The greater amount of moisture adsorbed by the phosphorylated proteins (Table VII) is expected in light of the hydrophilicity of the phosphate groups which are capable of hydrogen bonding with water, but the reason that phosphorylated casein adsorbed less water than Hammarsten casein is not clear.

Covalent attachment of phosphate groups to proteins may be useful in order to change the functional properties of food proteins. The phosphate groups introduced by the chemical reaction with $POCl_3$ are stable in the pH range 2.0–8.5 for phosphorylated casein. The extent of in vitro hydrolysis of phosphorylated casein by trypsin and α-chymotrypsin and the digestion, absorption, and utilization of the amino acids of phosphorylated casein by *T. thermophili* were not significantly affected. The phosphorylated proteins showed increased viscosity (phosphorylated casein) and increased water binding (phosphorylated casein and phosphorylated lysozyme) but decreased water solubility when $POCl_3$ is the phosphorylating reagent. Recently, Sung (1982) phosphorylated soy protein with trisodium trimetaphosphate at pH 11–12 and 30–40 °C. The phosphorylated soy protein had better aqueous solubility, water hydration capacity, emulsifying capacity, emulsion stability, foam expansion, and foam stability compared to a commercial soy protein isolate and to sodium caseinate. The data reported in this paper and in the literature indicate that protein phosphorylation could be a promising tool for changing the functional properties of food proteins. Although phosphorus oxychloride might prove an economical and practical reagent for large-scale applications to foods, refinements and precautions in the procedures as well as other possible reagents should be investigated.

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Registry No. Lysozyme, 9001-63-2; phosphorus oxychloride, 10025-87-3; trypsin, 9002-07-7; α-chymotrypsin, 9004-07-3.

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High-Performance Liquid Chromatographic Determination of Glycyrrhizin in Licorice Products

W. Jeffrey Hurst,* J. Michael McKim, and Robert A. Martin, Jr.

A high-performance liquid chromatographic (HPLC) method is described for the analysis of glycyrrhizin in licorice products, determined as glycyrrhizic acid. The samples are extracted in a solution of NH_4OH with final HPLC determination using a reversed-phase column with detection at 254 nm. Precision studies indicate a % Cv of less than 2.5. Recoveries of added glycyrrhizic acid range from 93 to 105%, with data linear (r = 0.99) for a 20-fold range. The HPLC method was compared to a wet chemical spectrophotometric method, and good agreement was observed. The HPLC method is precise, accurate, and time conservative and was successfully applied to a wide variety of licorice-containing products.

The extract of the licorice root has many uses. It is used extensively in the tobacco, confectionery, and pharmaceutical industry (Hall, 1973). The root contains about 5–10% of a glycoside which contains two glucuronic acid units and glycyrrhetinic acid (Hall, 1973). The glycyrrhetinic acid has a structure (see Figure 1) similar to other steroids and a similar potential pharmacological effect (Strecher, 1976; Koster and David, 1968; Chamberlain, 1970). This glycoside usually occurs in a combined calcium and potassium salt form as glycyrrhizic acid. For a more detailed survey of the compound, one should refer to a treatise by Nieman et al. (1957).

The analysis of glycyrrhizin as glycyrrhizic acid has been accomplished by gas–liquid chromatography (GLC) (Larry, 1972), thin-layer chromatography (TLC) (Nour et al., 1976), and various wet chemical methods (Cundiff, 1964; Habib et al. 1979). High-performance liquid chromatography (HPLC) has been used for the analysis of glycyrrhizin (Chamberlain, 1970; Lunder and Neilsen, 1980; Beasley et al., 1979; Bricout, 1978). Additionally, it has been used for the determination of glycyrrhetinic acid after acid hydrolysis. The HPLC assay is accurate, precise, and time conservative. Other HPLC methods have analyzed licorice root while this method reports the analysis of glycyrrhizin in finished confectionery items containing licorice. In this method, HPLC is used for the analysis of glycyrrhizin in a wide variety of licorice products.

Hershey Foods Technical Center, Hershey, Pennsylvania 17033.

MATERIALS AND METHODS

High-Performance Liquid Chromatograph. The HPLC used in this study consisted of a Waters Model ALC/GPC 201. The unit consisted of a Model 440 absorbance detector with a 254-nm filter and an M6000A solvent delivery system. The injection system was a Rheodyne Model 7120 syringe injector. Data acquisition was accomplished with a Shimadzu E-1A data unit. The HPLC column used was an EM Laboratories RP-18 (25 cm × 4.5 mm i.d.). The HPLC mobile phase consisted of

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