can cause a decrease in bioavailable lysine.

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Chemical Phosphorylation of Food Proteins: An Overview and a Prospectus

Günter Matheis and John R. Whitaker*

Chemical phosphorylation of proteins may be useful for changing the functional properties of food proteins. The use of various reagents to phosphorylate proteins is reviewed. Attention is also given to covalent attachment of low molecular weight organophospho compounds to proteins. The nature of the phosphate linkages involved and the effects of phosphorylation on the functional properties, as well as on the in vitro and in vivo digestion of the proteins, are discussed. Phosphorylation of proteins with phosphorus oxychloride ($POCl_3$) improved the gel-forming properties, particularly in the presence of Ca^{2+}. Incubation of soybean proteins with sodium trimetaphosphate (STMP) improved a number of functional properties, including water solubility, emulsifying activity, and foaming properties. Conflicting data exist as to whether or not STMP is covalently bound to the soybean proteins. In vitro and in vivo digestibility studies of phosphorylated proteins indicate that the nutritional value of the proteins was not reduced to a significant extent by the phosphorylation. Of the phosphorylating reagents tested so far, only $POCl_3$ and STMP might prove economical and practical reagents for large-scale application.

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 in large part due to insolubility. These problems may be overcome by physical or mechanical treatment or by microbial, enzymatic, or chemical modification.

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Table I. Amount of Phosphorus Covalently Bound to Chemically Phosphorylated Proteins

phosphorylated protein	phosphorylating agent	maximum phosphorus introduced: %	maximum phosphorus introduced: mol/mol of protein	reference
casein[a]	$POCl_3$	1.0	7.4	Neuberg and Oertel (1914); Rimington (1927); Matheis et al. (1983b)
α_{s1}-, β-, and κ-casein[b]	H_3PO_4/Cl_3CCN	1.5	11.2	Yoshikawa et al. (1981)
α_{s1}-casein[b]	P_2O_5/H_3PO_4	0.8	6.3	Dickson and Perkins (1971)
dephosphorylated casein	$POCl_3$	1.8	13.5	Rimington (1927); Boursnell et al. (1948)
lactalbumin	$POCl_3$	1.3	7.1	Neuberg and Pollack (1910)
β-lactoglobulin	$POCl_3$	1.2	13.8	Woo et al. (1982)
ovalbumin	$POCl_3$	0.6	7.8	Heidelberger et al. (1941)
lysozyme	$POCl_3$	1.3	6.2	Matheis et al. (1983b)
serum albumins	$POCl_3$	2.4	50	Mayer and Heidelberger (1946); Boursnell et al. (1948); Salák et al. (1965)
serum globulins	$POCl_3$	1.8	84	Neuberg and Oertel (1914); Rimington (1927)
hemoglobin	$POCl_3$	0.75	15	Salák et al. (1965)
clupeine	$POCl_3$	3.9	5	Willmitzer and Wagner (1975)
protamine	H_3PO_4/Cl_3CCN	0.44	1.1	Ullman and Perlman (1975)
gelatin	monophenyl phosphodichloride	0.5	15.6[c]	Bourland et al. (1949)
insulin	phosphoramidate	1	1.9	Müller et al. (1956); Rathlev and Rosenberg (1956)
ribonuclease	diphosphoimidazole	1.5	6	Taborsky (1958)
6-phosphogluconate dehydrogenase	acetyl phosphate, carbamoyl phosphate, and 1,3-bisphosphoglycerate	0.03	1	Dalocchio et al. (1982)

[a] Native casein contains 0.83–0.88% P (Taborsky, 1974). [b] Native α_{s1}-, β-, and κ-caseins contain 8, 5, and 1 mol of P/mol of protein, respectively (Brunner, 1977). [c] Molecular weight taken as 100 000.

Chemical phosphorylation of proteins may be useful in order to change the functional properties of food proteins. In his excellent review on current methods of phosphorylation of biological molecules, Slotin (1977) gives only two examples for the phosphorylation of proteins.

The purpose of the present review is to summarize the scattered data on chemical phosphorylation of proteins, the nature of the phosphate linkages involved, and the effects of phosphorylation on the functional properties and the in vitro and in vivo digestion of the proteins.

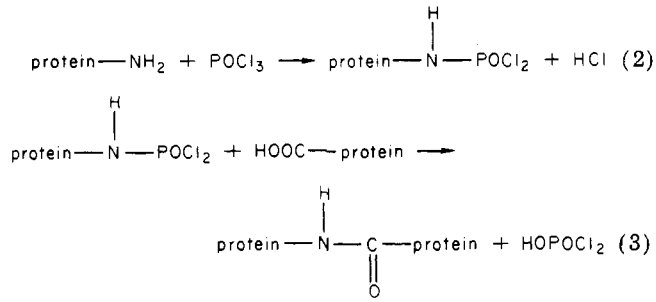
Treatment of enzymatic phosphorylation of proteins is beyond the scope of this review. The reader is referred to several excellent reviews on this topic (Nimmo and Cohen, 1977; Krebs and Beavo, 1979; Weller, 1979, England, 1980; Wold, 1981).

Phosphorylating Reagents. *Phosphorus Oxychloride.* Although phosphorus oxychloride ($POCl_3$) is a very reactive reagent, it is frequently used for protein phosphorylation. $POCl_3$ can be used in an aqueous or a nonaqueous system. In an aqueous system, $POCl_3$ reacts rapidly with H_2O in an exothermic reaction (eq 1). When $POCl_3$ is

$$POCl_3 + 3H_2O \rightarrow H_3PO_4 + 3HCl \quad (1)$$

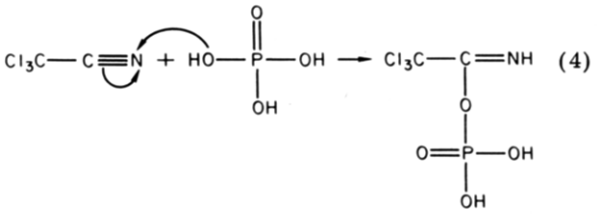
added directly to an aqueous protein solution, the low pH (eq 1) and heat produced leads to denaturation of the protein. In order to minimize these problems, $POCl_3$ is often dissolved in an organic solvent (usually carbon tetrachloride) and added in small portions to aqueous protein solutions. The pH is kept constant by adding sodium hydroxide and the temperature is controlled (usually in an ice bath). The following proteins have been phosphorylated with $POCl_3$ in an aqueous system: casein (Neuberg and Oertel, 1914; Rimington, 1927; Boursnell et al., 1948; Matheis et al., 1983b), lactalbumin (Neuberg and Pollack, 1910), β-lactoglobulin (Woo et al., 1982), ovalbumin (Heidelberger et al., 1941), serum albumins (Mayer and Heidelberger, 1946; Boursnell et al., 1948; Salák et al., 1965), serum globulins (Neuberg and Oertel, 1914; Rimington, 1927; Boursnell et al., 1948), and hemoglobin (Salák et al., 1965). The amount of phosphorus introduced is shown in Table I.

$POCl_3$ can cause intermolecular cross-linking of proteins as was shown by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) and mercaptoethanol (Woo et al., 1982; Matheis et al., 1983b). The nature of these cross-links is not known. Possibilities include *O,O'*-phosphodiester and/or *N,N'*-phosphodiamidate bridges as well as isopeptide bonds. The latter are thought to be formed according to eq 2 and 3 (Woo et al., 1982) where the $-NH_2$ is the ϵ-amino group of lysyl residues.



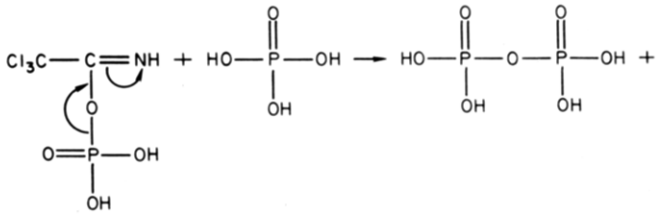
$POCl_3$ has also been used in a totally nonaqueous system (Willmitzer and Wagner, 1975). In this case, clupeine (a protamine from herring) was converted to clupeine capronate and suspended in trimethyl phosphate. When $POCl_3$ was added, the suspension became a clear solution. The reaction was terminated by pouring the solution into water. Up to five phosphate residues were introduced per mol of clupeine (Table I).

Phosphoric Acid. Trichloroacetonitrile reacts with phosphoric acid to give trichloroacetimidoyl phosphate (eq 4) (Cramer and Weimann, 1961). Trichloroacetimidoyl phosphate can react further with phosphoric acid and with



(4)

carboxyl groups and hydroxyl groups (eq 5–7) (Cramer and Weimann, 1961).



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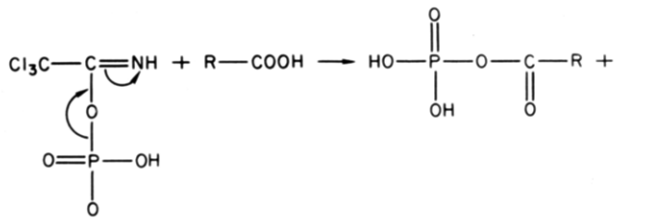

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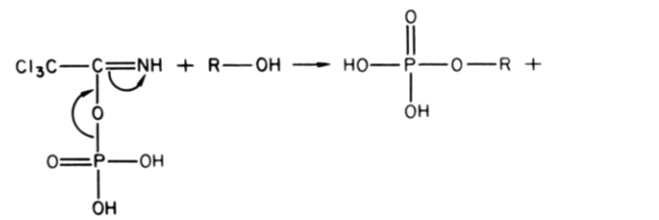


(7)

When ROH (eq 7) is protein-bound serine or threonine, phosphorylation of a protein by phosphoric acid with trichloroacetonitrile as a coupling reagent occurs. This was done with protamine (Ullman and Perlman, 1975) and with casein fractions (Yoshikawa et al., 1981). The reaction was carried out in dimethyl sulfoxide (Me_2SO) as the solvent. The amount of phosphorus covalently bound by this procedure is shown in Table I.

Phosphorus Pentoxide/Phosphoric Acid. Phosphorus pentoxide (P_2O_5) dissolved in H_3PO_4 has been used for the phosphorylation of a number of proteins (Ferrel et al., 1948; Dickson and Perkins, 1971; Rao et al., 1975) (Table I). The proteins were kept in the reaction mixture (P_2O_5 in H_3PO_4) for several days at room temperature. For example, α_{s1}-casein was phosphorylated with P_2O_5 in H_3PO_4 for 48 h at room temperature. The phosphorylating reagent was prepared by mixing 75 g of P_2O_5 with 100 g of 85% phosphoric acid. One-hundred milligrams of dry protein was dispersed in 10 g of reagent, and the reaction was placed in a desiccator over P_2O_5 and held at room temperature for 48 h. The solution was diluted with ice–ice water, neutralized with 10 N NaOH while maintaining the temperature below 10 °C, and dialyzed. This is a rather harsh treatment and does not seem to be suitable for the phosphorylation of food proteins. Gelatin phosphorylated by P_2O_5/H_3PO_4 has been used for leather impregnation and finishing (Rao et al., 1975).

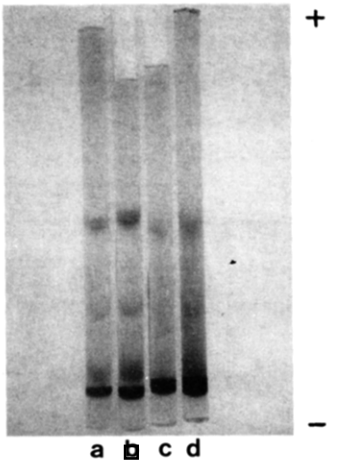


Figure 1. Polyacrylamide gel electrophoresis (PAGE) of water-soluble soybean proteins incubated with sodium trimetaphosphate (STMP). (a and b) 40 and 80 μg of control soybean proteins, respectively. (c and d) 40 and 80 μg of STMP-treated soybean proteins, respectively. Water-soluble soybean proteins were prepared by aqueous extraction of soybean protein grade I (obtained from United States Biochemical Corp., Cleveland, OH). Incubation of the proteins with STMP at pH 12 and 40 °C for 3 h was carried out according to Sung et al. (1983). The samples were then dialyzed extensively against 0.1 M KCl and then against H_2O. The proteins were recovered by lyophilization. Control soybean proteins were prepared in the same manner but in the absence of STMP. PAGE was carried out in acidic gels according to Nagai et al. (1964). No stacking gel was used. Proteins were stained with Coomassie Brilliant Blue R-250 (Matheis et al., 1983b). Data are taken from Matheis et al. (1983a).

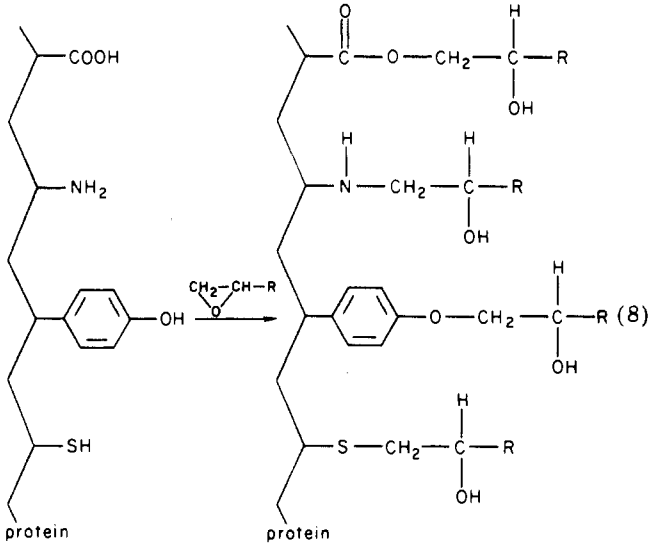
Sodium Trimetaphosphate. Sung et al. (1983) used cyclic sodium trimetaphosphate (STMP) for the phosphorylation of soybean protein under alkaline conditions (pH 10.5–12.5, 25–45 °C, 3 h). The reaction was found to be dependent on pH, temperature, and STMP concentration. At pH 11.5, 35 °C, and 1% STMP, 30% of the serine residues in soybean protein were reported to be phosphorylated.

Matheis et al. (1983a), however, failed to detect any covalently bound phosphate (by means of polyacrylamide gel electrophoresis) when soybean and lysozyme were treated with STMP as described by Sung et al. (1983). It was found that STMP was very strongly adsorbed to the proteins (as is known for orthophosphates) and that dialysis against 0.1 M KCl and then against H_2O was necessary to remove free STMP. Dialysis against water alone did not remove much of the phosphate. Figure 1 shows that there was no change in the electrophoretic behavior of the proteins, an unexpected result if modification had occurred.

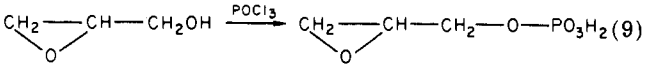
Miscellaneous Reagents. Monophenyl phosphodichloride has been used to phosphorylate gelatin (Bourland et al., 1949). Phosphoramidate (Müller et al., 1956; Rathlev and Rosenberg, 1956) and diphosphoimidazole (Taborsky, 1958) have been used for the phosphorylation of insulin and ribonuclease, respectively. 6-Phosphogluconate dehydrogenase was phosphorylated by acetyl phosphate, carbamoyl phosphate, and 1,3-bisphosphoglycerate (Dallocchio et al., 1982). This seems rather specific for 6-phosphogluconate dehydrogenase, since other proteins were not phosphorylated under the same conditions (Dallocchio et al., 1982). The amount of phosphorus covalently bound to proteins by these methods is shown in Table I.

Covalent Attachment of Phospho Compounds. Although the covalent attachment of low molecular weight phospho compounds to proteins is not a phosphorylation (attachment of phosphate) in the strict sense, we shall include it here.

Phospho Epoxides. It was recognized long ago that 1,2-epoxides can be used for the modification of proteins (Fraenkel-Conrat, 1944). Depending on the reaction conditions, the 1,2-epoxides can react with carboxyl, amino, sulfhydryl, and tyrosyl groups of proteins (eq 8). Rose



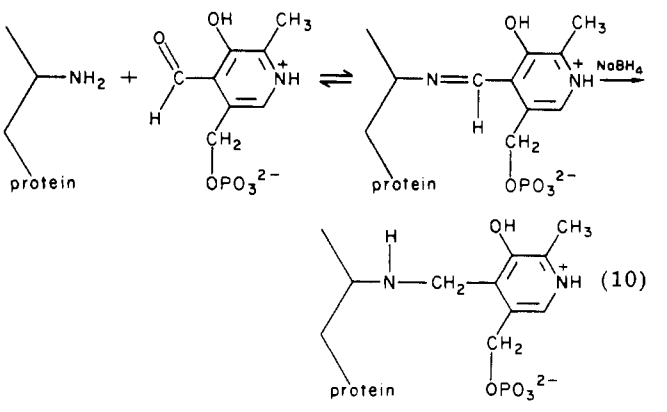
and O'Connell (1969) prepared glycidol phosphate (1,2-epoxypropanol 3-phosphate) from glycidol (1,2-epoxypropanol) in the presence of *N,N*-dimethylaniline to prevent ring opening by chloride ions (eq 9). They then



incubated triosephosphate isomerase and enolase with glycidol phosphate at pH 7.4 and 15–37 °C up to 7 h. The modified proteins contained ~0.5% of glycidol phosphate (~1 mol per catalytic subunit of the enzymes).

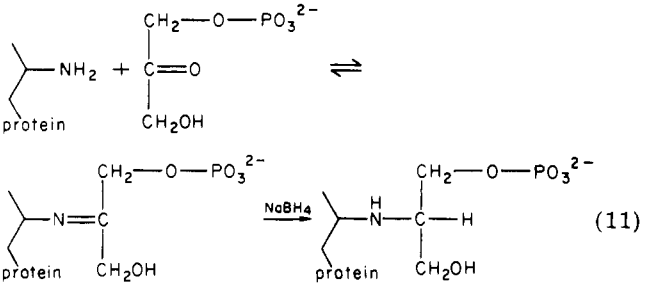
Phospho Aldehydes and Phospho Ketones. Reductive alkylation of amino groups in proteins with aliphatic aldehydes and ketones is a rather mild procedure (0–25 °C, pH 9) and generally proceeds more readily with aldehydes than with ketones (Means and Feeney, 1968; Means, 1977). Reversible reductive alkylation of proteins is possible by using α-hydroxycarbonyl compounds (e.g., glycolaldehyde, acetol) followed by periodate treatment at pH 7–9 and room temperature (Geoghegan et al., 1979).

Pyridoxal phosphate (PLP) has been used to label the PLP binding site of many enzymes in which it occurs (Means and Feeney, 1971) (eq 10). Many proteins that



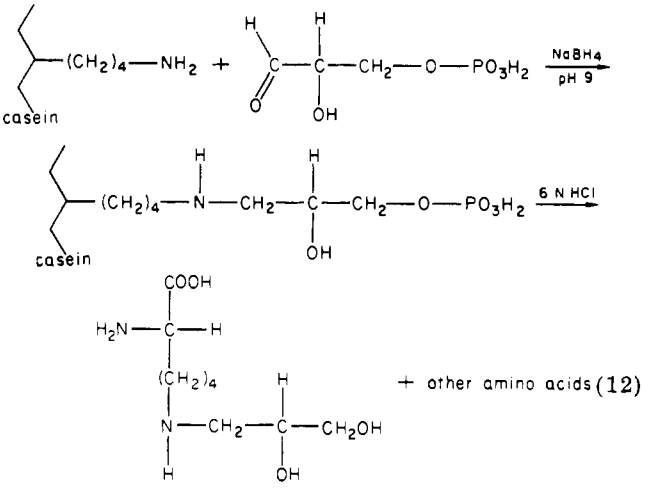
do not normally contain PLP nevertheless can be labeled with it (Means and Feeney, 1971). It appears that the specifically labeled lysine residues are in or near phosphate binding sites of the proteins.

Lai et al. (1967) used dihydroxyacetone phosphate (DHAP) to label the lysine residue at the active site of aldolase (eq 11).



Hartman (1968) synthesized 1-hydroxy-3-iodo-2-propanone phosphate (ICH_2–CO-CH_2–O–PO_3H_2), a compound structurally similar to DHAP. This compound was used as an active site reagent for triosephosphate isomerase (Hartmann, 1968); 1.7 mol of 1-hydroxy-3-iodo-2-propane phosphate/mol of enzyme was covalently bound.

Reductive alkylation of amino groups in casein with glyceraldehyde 3-phosphate (G-3-P) was studied by Matheis et al. (1983a). The reaction conditions are described by Geoghegan et al. (1979). $NaBH_4$ was the reducing agent. The molar ratio of G-3-P:protein amino groups was 84. After hydrolysis of the modified casein (6 N HCl, 22 h), only one lysine derivative was found, which chromatographed between phenylalanine and lysine on the amino acid analyzer (Figure 2) using the buffer system described by Fretheim et al. (1979). The derivative is most likely N^{ϵ}-(2,3-dihydroxypropyl)lysine (eq 12).



N^{ϵ}-(2,3-dihydroxypropyl)lysine

Thirty percent of the amino groups in casein were modified under the conditions used. This corresponds to ~4 mol of G-3-P/mol of protein. The reason for the relatively poor reactivity of G-3-P may lie in the ionic binding of its phosphate group to the protein, thus making G-3-P less available for reductive alkylation. Polyacrylamide gel electrophoresis (PAGE) of Hammarsten, control, and modified caseins is shown in Figure 3. Only the β-fraction of casein was more negatively charged due to modification by G-3-P. The reason why the other casein fractions did not react with G-3-P to an appreciable extent is not clear. The modified casein was readily water soluble, in contrast to casein and other proteins phosphorylated by $POCl_3$.

Table II. Nature of Phosphate Linkages in Chemically Phosphorylated Proteins

phosphorylated protein	method of analysis[a]	nature of phosphate linkage	reference
casein	A, B	Ser(Thr)–O–PO_3^{2-} and Ser(Thr)–O–$P_2O_6^{3-}$	Rimington (1927); Matheis et al. (1983b)
β-lactoglobulin	A, B	–NH–PO_3^{2-}	Woo et al. (1982)
ovalbumin	A	Ser(Thr)–O–PO_3^{2-} and –NH–PO_3^{2-}	Heidelberger et al. (1941)
lysozyme	A, C	Ser(Thr)–O–PO_3^{2-}, –NH–O–PO_3^{2-}, –O(NH)–$P_2O_6^{3-}$, and –O(NH)–$P_3O_9^{4-}$	Matheis et al. (1983b)
serum albumins	A, C	–NH–PO_3^{2-}	Mayer and Heidelberger (1946); Salák et al. (1965)
serum globulin	A	Ser(Thr)–O–PO_3^{2-}	Rimington (1927)
hemoglobin	C	–NH–PO_3^{2-}	Salák et al. (1965)
clupeine	A, B, D	Ser(Thr)–O–PO_3^{2-}, Ser(Thr)–O–$P_2O_6^{3-}$ and Ser(Thr)–O–$P_3O_9^{4-}$	Willmitzer and Wagner (1975)
protamine	A, D	Ser–O–PO_3^{2-} and –NH–PO_3^{2-}	Ullman and Perlman (1975)
insulin	A	–NH–PO_3^{2-}	Müller et al. (1956); Rathlev and Rosenberg (1956)
ribonuclease	A, C	–NH–PO_3^{2-}	Taborsky (1958)
6-phosphogluconate dehydrogenase	A, C	His–PO_3^{2-}	Dallocchio et al. (1982)

[a] A: pH stability studies. B: ^{31}P NMR studies. C: Analysis of reactive protein groups. D: Digestion of protein and analysis for phospho amino acid.

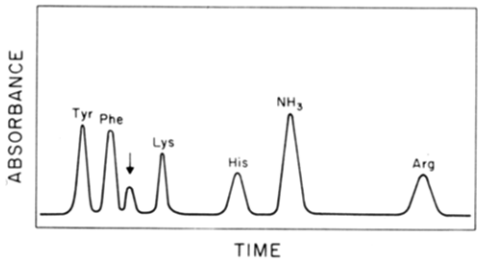


Figure 2. Portion of a chart from amino acid analysis of casein reductively alkylated with glyceraldehyde 3-phosphate (G-3-P). Casein was reductively alkylated with G-3-P and $NaBH_4$ as the reducing reagent according to Geoghegan et al. (1979). The molar ratio of G-3-P:protein amino groups was 84. Modified casein was hydrolyzed in sealed tubes (6 N HCl, 22 h, 110 °C). Amino acid analysis was carried out according to Fretheim et al. (1979). Data are taken from Matheis et al. (1983a).

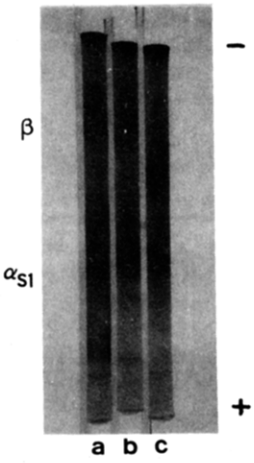


Figure 3. Polyacrylamide gel electrophoresis (PAGE) of (a) Hammarsten casein, (b) control casein, and (c) casein reductively alkylated with glyceraldehyde 3-phosphate (G-3-P). Reductive alkylation was carried out as described in the legend to Figure 2. PAGE was carried out in basic gels [6% acrylamide, 0.16% *N,N'*-methylenebis(acrylamide)]. The gel buffer was Tris-HCl, pH 8.9; the electrode buffer was Tris/glycine, pH 8.6. No stacking gel was used. 40 μg of each protein was applied. Electrophoresis was carried out at 2.5 mA/tube at room temperature (~25 °C) for ~1 h. Proteins were stained with Coomassie Brilliant Blue R-250 (Matheis et al., 1983b), containing 0.1 M $AlK(SO_4)_2$ (Hegenauer et al., 1977). Data are taken from Matheis et al. (1983a).

G-3-P is the only phospho aldehyde and DHAP is the only phospho ketone we are aware of being commercially available. Both compounds are very expensive, and their use to change the functional properties of food proteins is unlikely to be of commercial significance at this time.

Nature of Phosphate Linkages. The nature of the phosphate linkages to proteins has been studied by (a) pH stability of the phosphate bonds, (b) analysis of loss of reactive protein groups (hydroxyl, tyrosyl, amino, imidazole, guanidino, carboxyl, and sulfhydryl groups as well as peptide bonds), (c) digestion of protein and analysis for phospho amino acids, and (d) ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Using these methods, it has been established that the phosphorus can be attached to hydroxyl oxygen, amino nitrogen, and imidazole nitrogen (Table II). Tyrosyl groups were found to be either phosphorylated only to a limited extent (Mayer and Heidelberger, 1946; Ferrel et al., 1948) or not phosphorylated at all (Taborsky, 1958; Salák et al., 1965). Phosphorus bound to sulfhydryl groups, carboxyl groups, or peptide bonds could not be detected by Ferrel et al. (1948). Heidelberger et al. (1941) speculated that part of the phosphate bound to ovalbumin could be linked to carboxyl groups. The presence of diphosphate and tri(or poly)-phosphate residues in chemically phosphorylated proteins was found only recently through ^{31}P NMR studies (Table II).

Nitrogen-bound phosphate is very acid labile (Zervas and Katosyannis, 1955; Willmitzer and Wagner, 1975; Matheis et al., 1983b). Even at neutral pH, phosphorylated proteins containing nitrogen-bound phosphate lose part of their phosphate in aqueous solution at 2–37 °C (Rathlev and Rosenberg, 1956; Taborsky, 1958; Salák et al., 1965; Woo et al., 1982; Matheis et al., 1983b). Therefore, phosphorylated proteins containing acid-stable oxygen-bound phosphate are more desirable for incorporation into foods.

Phosphorylation of proteins with phosphoamidate, diphosphoimidazole, acetyl phosphate, carbamoyl phosphate, and 1,3-bisphosphoglycerate leads to nitrogen-bound phosphate (Tables I and II). With the exception of diphosphoimidazole, no more than 1 mol of phosphate/mol of protein could be covalently bound by using these reagents (Table I). Since 1 mol of phosphate/mol of protein is not likely to change the functional properties of proteins very much, and since nitrogen-bound phosphate is very acid labile, the above reagents are not recommended

Table III. Changes in Functional Properties of Chemically Phosphorylated Proteins

change in functional properties	phosphorylated protein	reference
increased water solubility	soybean protein	Sung et al. (1983)
decreased water solubility	casein, lysozyme, serum albumin,[a] hemoglobin	Matheis et al. (1983b); Salák et al. (1965)
increased viscosity	casein, ovalbumin, serum albumin	Matheis et al. (1983b); Heidelberger et al. (1941); Mayer and Heidelberger (1946)
increased water binding	casein, lysozyme, soybean protein	Matheis et al. (1983b); Sung et al. (1983)
increased buffering capacity	ovalbumin, serum albumin	Heidelberger et al. (1941); Mayer and Heidelberger (1946)
increased emulsifying activity	β-lactoglobulin, soybean protein	Woo and Richardson (1983); Sung et al. (1983)
decreased emulsifying activity	casein	Matheis et al. (1983b)
increased foam expansion and stability	soybean protein	Sung et al. (1983)
loss of heat coagulability	ovalbumin	Heidelberger et al. (1941)
increased calcium binding ability	α_{s1}-, β-, and κ-caseins, β-lactoglobulin	Yoshikawa et al. (1981); Woo and Richardson (1983)
gel-forming properties	casein, serum globulin, gluten, serum albumin[b]	Matheis et al. (1983b); Boursnell et al. (1948); Ferrel et al. (1948); Salák et al. (1965)
gel-forming properties in the presence of Ca^{2+}	β-lactoglobulin	Woo and Richardson (1983)

[a] Containing more than 20 mol of phosphate groups/mol of protein. [b] Containing 10–20 mol of phosphate groups/mol of protein.

for the phosphorylation of food proteins.

Phosphorylation of proteins with $POCl_3$ can lead to the covalent linkage of up to 50 mol of phosphate/mol of protein (Table I). In some proteins (casein, serum globulin, clupeine) the phosphate reacts exclusively with hydroxyl oxygen; in other proteins (β-lactoglobulin, serum albumin, hemoglobin) the phosphate reacts exclusively with nitrogen; in still others (ovalbumin, lysozyme) it reacts with both oxygen and nitrogen (Table II) when $POCl_3$ is the phosphorylating reagent. The reason for this difference in reactivity of groups in the different proteins to $POCl_3$ is not known.

Functional Properties of Phosphorylated Proteins. Functional properties have been studied for a number of chemically phosphorylated proteins (Table III). In light of the hydrophilicity of the covalently attached phosphate groups, phosphorylation should result in increased water solubility. This was only reported for soybean protein that was phosphorylated by sodium trimetaphosphate (STMP) (Sung et al., 1983). When $POCl_3$ was the phosphorylation reagent, decreased water solubility of the phosphorylated proteins was found (Salák et al., 1965; Matheis et al., 1983b). Phosphorylation with $POCl_3$ leads to protein cross-linking (Woo et al., 1982; Matheis et al., 1983b). These cross-links may account for the decreased water solubility.

Proteins phosphorylated with $POCl_3$ have increased viscosity and gel-like properties (Table III). The cross-links may also account for this. Both increased and decreased emulsifying activity has been reported for proteins phosphorylated with $POCl_3$ (Table III). The reason for this difference is not clear.

The increased calcium binding ability of phosphorylated casein fractions and β-lactoglobulin (Table III) is apparently due to the increase of phosphate groups. When solutions of phosphorylated β-lactoglobulin at pH 5 or 7 were dialyzed against a Ca^{2+} solution, gelation occurred rapidly (Woo and Richardson, 1983). Emulsions prepared in the presence of Ca^{2+} (1–5 mM) at pH 5–7 with phosphorylated β-lactoglobulin as the emulsifier varied in their creaming stabilities. A Ca^{2+} concentration of 1 mM decreased creaming stability, and 5 mM Ca^{2+} increased creaming stability. The viscosity of an emulsion prepared with phosphorylated β-lactoglobulin in the presence of Ca^{2+} was about double that prepared with untreated β-lactoglobulin; the consistency was similar to that of a commercial mayonnaise.

Digestibility of Phosphorylated Proteins. The in vitro digestibility of chemically phosphorylated proteins by pepsin and pancreatic proteases has been studied for a number of proteins (Neuberg and Oertel, 1914; Rimington, 1927; Taborsky, 1958; Matheis et al., 1983b; Sung et al., 1983). 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.

Taborsky (1958) studied the enzymatic hydrolysis of phosphorylated ribonuclease by trypsin. He found that the phosphorylated protein was only a little better as a substrate for trypsin than was native ribonuclease, indicating that the protein structure was not strongly affected. Matheis et al. (1983b) studied the enzymatic hydrolysis of phosphorylated casein by trypsin and α-chymotrypsin. 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. Sung et al. (1983) studied the enzymatic hydrolysis of phosphorylated soybean protein by pepsin and pancreatin (trypsin, α-chymotrypsin, carboxypeptidases A and B). The results indicated that the digestibility value of soybean protein was not reduced to a significant extent by the phosphorylation.

The phosphate bonds in phosphorylated proteins can be cleaved by phosphatases. This was shown for tryptic hydrolysates of phosphorylated proteins (Rimington, 1927) and for intact phosphorylated proteins (Willmitzer and Wagner, 1975; Woo et al., 1982).

The in vivo digestibility of phosphorylated proteins was only studied in the case of phosphorylated casein (Matheis et al., 1983b). In the bioassay, *Tetrahymena thermophili* grew as well on phosphorylated casein as on untreated casein, indicating that the phosphorylation did not affect the digestion, absorption, and utilization of the amino acids by the organism. The use of *Tetrahymena* sp. for protein quality evaluation relies on its similarity to mammals in the requirement for essential amino acids and in the presence of similar enzyme systems (Kidder and Dewey, 1951).

Conclusions. Protein phosphorylation could be a promising tool for changing the functional properties of food proteins (Table III). The extent of in vivo hydrolysis

of phosphorylated proteins by pepsin and pancreatic proteases does not seem to be significantly affected. Of the phosphorylating reagents tested so far (Table I), only $POCl_3$ and sodium trimetaphosphate (STMP) might prove economical and practical reagents for large-scale applications to foods.

Phosphorylation with STMP has been reported to improve a number of functional properties of soybean protein, including water solubility (Table III). Conflicting data exist as to whether or not STMP is covalently bound to soybean protein (Matheis et al., 1983a; Sung et al., 1983). Phosphorylation with STMP requires alkaline pH. In a typical experiment, soybean protein was incubated with STMP at pH 11.5, 35 °C, and 3 h (Sung et al, 1983). Proteins can undergo many changes in alkaline solutions, including formation of lysinoalanine and racemization of amino acids (Masters and Friedman, 1980; Whitaker, 1980). It is important to study the possible formation of lysinoalanine and D-amino acids in proteins that are phosphorylated by STMP.

Phosphorylation of proteins with $POCl_3$ can be done at 3–25 °C and pH 6–8.5 (Woo et al., 1982; Matheis et al., 1983b). In spite of these rather mild conditions, protein cross-linking occurs (Woo et al., 1982; Matheis et al., 1983b), which leads to dereased water solubility (Salâk et al., 1965; Matheis et al., 1983b). However, phosphorylation of proteins with $POCl_3$ improves the gel forming properties (Table III), particularly in the presence of Ca^{2+} (Woo and Richardson, 1983). Water binding capacity is also improved (Table III).

In vivo digestibility of phosphorylated proteins has only been studied in the case of phosphorylated casein; the modified protein was fed to *T. thermophili* (Matheis et al., 1983b). Experiments with animals are necessary to determine the digestion, absorption, and utilization of the amino acids of phosphorylated proteins by mammals and to study the introduction of potentially toxic residues into the proteins that may prevent their direct utilization in foods.

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Registry No. STMP, 7785-84-4; $POCl_3$, 10025-87-3.

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