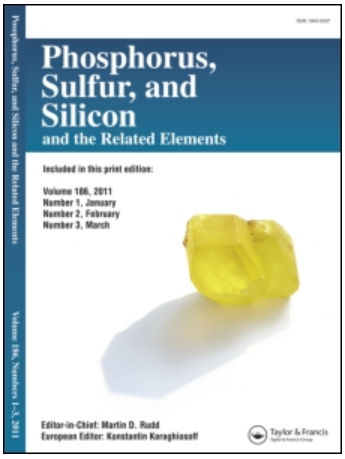


Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

NON-ENZYMIC METHODS FOR THE PHOSPHORYLATION OF PROTEINS[1]

Arlen W. Frank[a]
[a] U.S. Department of Agriculture, Southern Regional Research Center, Agricultural Research Service, New Orleans, Louisiana

To cite this Article Frank, Arlen W.(1987) 'NON-ENZYMIC METHODS FOR THE PHOSPHORYLATION OF PROTEINS[1]', Phosphorus, Sulfur, and Silicon and the Related Elements, 29: 2, 297 — 315
To link to this Article: DOI: 10.1080/03086648708080517
URL: http://dx.doi.org/10.1080/03086648708080517

Phosphorus and Sulfur, 1987, Vol. 29, pp. 297-315
Photocopying permitted by license only

NON-ENZYMIC METHODS FOR THE PHOSPHORYLATION OF PROTEINS[1]

ARLEN W. FRANK

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana 70179

(Received April 18, 1986)

Non-enzymic methods for the phosphorylation of proteins are reviewed with respect to type of reagent, protein source, reactive site (N—, O— or S—), extent of reaction, and effect on physical properties, functionality and biological properties. Experimental procedures are given to illustrate the most important methods. Some enzymic methods are included for comparison. The evidence for phosphorylation of specific amino acid residues, such as serine, threonine, histidine or lysine, is critically assessed. Conclusions are drawn regarding the status of phosphorylation as a synthetic method for modifying proteins.

I. INTRODUCTION

In view of the heightened awareness of the role played by phosphoproteins in enzyme regulation, interest is growing in the synthesis and characterization of proteins that have been purposely phosphorylated by chemical or enzymic methods. Enzymic methods were reviewed by Perlmann[2] in 1955, by Taborsky[3] in 1974 and by Krebs and Beavo[4] in 1979. Non-enzymic methods were briefly reviewed by the author[5] in 1984. In this review the non-enzymic methods are discussed in greater detail, particularly with respect to characterization of the products and comparison of their properties with those of the phosphorus-free proteins and the natural phosphoproteins.

II. SYNTHETIC METHODS

A. Non-Enzymic Methods

Egg albumin[6,7] and other proteins[8] combine with metaphosphoric acid to form insoluble precipitates that were later shown to be salts.[9] The first authentic chemical phosphorylation of a protein was reported in 1901 when Bechhold treated egg albumin with phosphorus oxychloride in the presence of sodium hydroxide and obtained a product regarded to be a true ester of phosphoric acid.[10] The method was improved by Neuberg[11,12] and Rimington[13] and has since been used extensively for the phosphorylation of proteins (Table I). The following procedure (Rimington, 1927) is still in use.

Method 1:

"A quantity of the protein, between 7 and 10 g, was · · · dissolved in water with the aid of the requisite quantity of sodium hydroxide, the total volume being

TABLE I

Phosphorylation of proteins by phosphorus oxychloride/ sodium hydroxide (method 1)

Protein	Percent P Before	After	Reference
Human			
Serum albumin	—	0.99	14, 15
Serum globulin	—	1.07	14
Serum protein	—	1.62	14
—, ^{32}P-labeled	—	1.36	16
Hemoglobin	—	0.92	15
Globin	—	0.68	15
Bovine			
Casein	0.85	1.76	12
Casein	0.80	1.77	13
Casein	0.83	1.83	17
—, dephosphorylated	—	1.75	13
Lactalbumin	—	1.01	14
β-Lactoglobulin	trace	1.28	11
Hemoglobin, type II	—	2.31	18, 19
	—	—	20
Horse			
Serum albumin	0	2.70	21
Serum globulin	—	0.71	13
Rabbit			
Serum protein	—	2.72	14
Plasma	—	2.72	14
Chicken			
Serum protein	—	—	14
Egg albumin	0.06	2.71	22
Egg albumin	—	—	9
Lysozyme	0	1.34	17
Silkworm			
Silk fibroin peptone	0	10.76	12
Legume			
Soy protein isolate	0.15	1.43	23, 24
Unspecified			
Blood globulin	trace	1.77	12
Witte peptone	—	3.61	12
Gelatin	—	—	14
See also poly-L-lysine.[18]			

brought up to 125–150 cc. Phosphorus oxychloride (20–25 g) dissolved in eight times its volume of carbon tetrachloride was allowed to drop into the ice-cold, rapidly stirring protein solution, to which had been added phenolphthalein to serve as an indicator, and the reaction was kept faintly alkaline by addition of 4*N* sodium hydroxide. The addition of the oxychloride extended over 6–8 hours and the temperature of the reactants never rose above 5°. After separating the aqueous layer from the carbon tetrachloride, the phosphorized protein could be

TABLE II

Phosphorylation of proteins by phosphorus oxychloride (method 2) or phenyl phosphorodichloridate (method 3) in the absence of base

Method	Protein	Percent P Before	Percent P After	Reference
2	*Herring Clupeine*			
	Unfractionated	—	3.7	25
	YI	—	3.2	25
	Z	—	1.9	25
	See also poly-L(or DL)-serine[26]			
3	*Unspecified*			
	Gelatin	<0.02	0.50	27
	Pepsin	0.1	0.7	27

precipitated by addition of 2*N* hydrochloric acid until maximum separation occurred. This corresponded to a pH between 3 and 4." (Quoted with permission from Reference 13. Copyright 1927 by The Biochemical Society.)

Two minor modifications of this method have been proposed (Table II). In method 2, the protein is dissolved in trimethyl phosphate and phosphorylated with phosphorus oxychloride in the absence of base for 2 to 6 days at room temperature; the reaction is terminated by pouring the mixture into twice the volume of water. This method was applied to herring sperm clupeine, converted to its capronate salt for enhanced solubility.[25] In method 3, the protein is phosphorylated with phenyl phosphorodichloridate—$C_6H_5OP(O)Cl_2$—either in the presence or absence of sodium hydroxide for 1.5 hour at 18–20°C. The phenyl group is cleaved off during the reaction, leaving the protein phosphate ester substituent-free. This method was applied to gelatin and pepsin.[27] Preliminary experiments showed that if the protein is treated with diphenyl phosphorochloridate—$(C_6H_5)_2P(O)Cl$—the second phenyl group is retained. Its removal could be troublesome.[28]

In 1948 Ferrel *et al.* reported that proteins can be phosphorylated by phosphoric acid containing phosphorus pentoxide (method 4).[29] The application of this method to a variety of proteins and model substances is summarized in Table III.

Method 4:

"The reagent was prepared by quickly weighing 75 g of phosphorus pentoxide into a beaker containing 100 g of 85% orthophosphoric acid and heating the mixture with stirring to dissolve. Ten grams of the cooled reagent was weighed into a small beaker and 100 mg of finely ground protein was dusted in with stirring to obtain a smooth dispersion. The beaker was then placed in a desiccator over phosphorus pentoxide to react for three days at room temperature. The reaction mixture was stirred several times during the first twenty-four hours to disperse any lumps formed. After seventy-two hours, the viscous reaction mixture was diluted by adding finely crushed ice with vigorous stirring. The diluted

TABLE III

Phosphorylation of proteins by phosphoric acid/phosphorus pentoxide (method 4)

Protein	Percent P Before	After	Reference
Bovine			
Serum albumin	—	2.97	29
α_{S1}-Casein	0.87	1.70	30
Chicken			
Egg albumin	—	4.80	29
Ovomucoid	—	5.17	29
Ovomucoid	—	5.57	31
Silkworm			
Silk fibroin	—	5.11	29
Sericin	—	11.09	29
Fish			
Salmine	—	9.41*	29
Isinglass	—	4.24	29
Cereal			
Gluten	—	4.89	29
Gliadin	—	2.29	29
Edestin	—	2.97	29
Bacterial			
Gramicidin	—	1.89	29
Unspecified			
Gelatin	—	4.83	29
Gelatin	—	—	32
γ-Globulin	—	4.40	29
Globin	—	1.86	29
Insulin	—	2.54	29
Insulin	—	2.35	33
See also poly(glutamic acid), polyglutamine, polyglycine and tyrosine-formaldehyde polymer.[29]			

* Salt-linked metaphosphate.

mixture was then poured over more cracked ice and neutralized (pH 7.5–8.0) with 10*N* sodium hydroxide, with stirring to prevent local overheating. The product was isolated by · · · dialyzing, first against ion-free water, then against 10% sodium chloride, and finally against distilled water until free of inorganic phosphates." (Quoted with permission from Reference 29. Copyright 1948 by the American Chemical Society.)

In method 5 (Table IV), the protein is incubated with cyclic sodium trimetaphosphate in aqueous solution for 3 hours at pH 11.5 and 35°C. The reagent is prepared by heating sodium dihydrogen phosphate for 5 hours at 530°C. This method has been applied only to soy protein isolate.[34,35]

Proteins, like many other substrates, do not react with phosphoric acid if the reagent is anhydride-free. Two methods have been developed in which the

TABLE IV

Phosphorylation of proteins by sodium trimetaphosphate (method 5), phosphoric acid/urea (method 6) or phosphoric acid/coupling reagent (method 7)

Method	Protein	Percent P Before	Percent P After	Reference
5	*Legume*			
	Soy protein isolate	—	0.48	34, 35
	See also poly-DL-serine[26]			
6	*Cereal*			
	Gluten	—	1.57	36, 37
6	*Legume*			
	Soy protein concentrate	—	0.73	38
7	*Bovine*			
	α_{S1}-Casein	0.88	1.74	39
	β-Casein	0.59	1.98	39
	κ-Casein	0.12	1.46	39
7	*Fish*			
	Salmon protamine	—	0.72	40
	—, ^{33}P-labeled	—	—	40

phosphoric acid is "activated" by another reagent (Table IV). In method 6, the protein is treated with phosphoric acid and urea, dried, ground to a powder and heated at 140°C for 30 minutes in a vacuum. This method has been applied to wheat gluten and to soy protein concentrate.[36–38] In method 7, the phosphoric acid is activated by a coupling reagent such as trichloroacetonitrile or dicyclohexyl carbodiimide. This method has been applied to salmon sperm protamine and to several casein fractions.[39,40]

Method 7:

"To 6 ml DMSO, containing 20 mg protamine chloride/ml, are added 15 μl 85% H_3PO_4 (14.7 M), 150 μl triethylamine (7.2 M), and 150 μl trichloroacetonitrile (10.0 M). Upon addition of the trichloroacetonitrile, the coupling reagent, the reaction mixture is incubated in a 40 ml conical glass tube for 30 minutes at 37°C, and is then rapidly chilled by the addition of 30 ml ice-cold ethanol. Phosphoprotamine is allowed to precipitate for 15 minutes at 0°C, and is then collected by centrifugation. The precipitate is dissolved in 5 ml 1*N* H_2SO_4, and incubated for 30 minutes at 30°C. The solution is again chilled, 20 ml of cold ethanol is added, and the precipitate again collected by centrifugation. The precipitated phosphoprotamine is · · · purified by passage over two 3 ml columns of Bio-Rad AG1-X8 Cl^- anion exchange resin, which was equilibrated and eluted with water." (Quoted with permission from Reference 40. Copyright 1975 by Academic Press, Inc.).

In 1956 Rathlev and Rosenberg introduced potasium hydrogen phosphoramidate—$NH_2P(O)(OH)OK$—as a phosphorylating agent for proteins

TABLE V

Phosphorylation of proteins by potassium phosphoramidate (method 8)

Protein	Percent P Before	After	Reference
Bovine			
Myelin basic protein	—	—	45
Histone H4	—	—	45, 46
—, (1–23)-peptide	—	—	46
—, (38–102)-peptide	—	—	46
—, ^{32}P-labeled	—	—	47
Ribonuclease			
—, unfractionated	0	0.35	43
—, A	0	0.20	44
Bacterial			
Protein HPr (*S. aureus*)	0	—	48
Phosphoramidate hexose transferase (*E. coli*), ^{32}P-labeled	0	0.026	49
Unspecified			
Insulin	—	0.24	41
Insulin	—	1	42

and related substances (method 8).[41,42] The reagent is prepared by the hydrolysis of either diphenyl phosphoramidate—$(C_6H_5O)_2P(O)NH_2$—or bis(2-cyanoethyl) phosphoramidate—$(NCCH_2CH_2O)_2P(O)NH_2$—with potassium hydroxide, and is stable only in neutral or alkaline solution. For ribonuclease, the preferred reagent is 1,3-diphosphoimidazole, which may be prepared from potassium phosphoramidate by reaction with imidazole. The reagent is used in the form of its sodium or calcium salt at pH 8.5.[43,44] The proteins that have been phosphorylated with these reagents are listed in Table V.

Method 8:

"One gram crystalline insulin and 0.22 g potassium PA [phosphoramidate] are dissolved at pH 7.3 in 20 ml water. After standing 24 hr, the insulin is precipitated by adjustment of pH to 5.2, centrifuged and again dissolved together with 0.22 g PA. The isoelectric precipitation and treatment with PA is repeated every day for about two weeks · · · . At the end of the phosphorylation period, the protein is completely soluble at pH 5.2 and is isolated from a solution of pH 4.4." (Quoted with permission from Reference 41. Copyright 1956 by Academic Press, Inc.)

For the record, mention should be made of the phosphorylation of proteins with organic phosphorus compounds of the nerve gas type, i.e. the cholinesterase inhibitors. These are not covered in this review for two reasons: (1) the reactions are not generally useful for synthetic purposes, and (2) the literature is voluminous. For a thorough recent review of these reagents and their mode of action, see Eto.[50]

B. *Enzymic Methods*

To place the foregoing methods in proper perspective, some of the more common enzymic methods are discussed here.

Although it was stated above (methods 6, 7) that proteins do not react with phosphoric acid unless the latter is "activated", the phosphatases are exceptions. Alkaline phosphatase, whether derived from animal (calf intestine, bovine liver)[51,52] or bacterial (*E. coli, S. marcescens*)[53–55] sources, binds up to one mol per mol of P_i at its active site, serine; acid phosphatase, whether derived from rat liver[56] or human prostate[57] (but not Baker's yeast),[58] binds up to one mol per mol of P_i at its active site, histidine. No uptake of P_i occurs in either case if the enzyme is denatured or inhibited.[51,53,57,59]

A more general method for the phosphorylation of enzymes *in vitro* is phosphorylation by ATP in the presence of a phosphorylase kinase. Because the phosphorus uptake is so low (<0.1%), special techniques for measuring the extent of reaction are necessary. The usual technique is a radioassay with ^{32}P-labeled ATP, as illustrated for glycogen phosphorylase (method 9).

Method 9:

"The following reaction mixture was used: (1) 100 ml of four times recrystallized AMP-free phosphorylase b solution containing 1.90 gm of the enzyme, (2) 2.5 ml of 2*M* Tris buffer adjusted so that the final reaction mixture would be at pH 7.8, (3) 2.0 ml of phosphorylase b kinase (6 mg) in neutral 0.3*M* cysteine, (4) 1.0 ml of 1.0*M* $Mg(Ac)_2$, (5) 4.0 ml of [β,γ-^{32}P-ATP] (31 μmoles). The labeled ATP was added as the last component, and the mixture was incubated at 25° [for 40 to 60 minutes]$\cdots$. After the reaction has reached completion, an equal volume of saturated ammonium sulfate, pH 7, is added, and the precipitated protein is collected by centrifugation, dissolved in a minimal amount of water, and dialyzed as described by Illingworth and Cori[61] for the crystallization of phosphorylase a.... [The] maximal phosphate incorporation in phosphorylase b [was] 8.3×10^{-3} μmoles P^{32} per mg [or] 4.1 moles P^{32} per mole." (Quoted with permission from Reference 60. Copyright 1958 by The American Society of Biological Chemists, Inc.)

By 1978 this method had been applied to some 21 different enzymes, and the number was expected to increase by two to three a year.[4] The ATP can sometimes by replaced by other phosphate donors such as GTP[62] or 1,3-diphosphoglycerate,[63,64] and the phosphorylase kinase by other protein kinases.[4]

A method that is described as non-enzymic but appears to be specific for a single enzyme, 6-phosphogluconic dehydrogenase (*C. utilis*), is phosphorylation by acyl phosphates such as acetyl phosphate—$CH_3C(O)OP(O)(OH)_2$—or carbamoyl phosphate—$NH_2C(O)OP(O)(OH)_2$. The enzyme, dissolved in triethanolamine buffer, pH 7.5, is treated with the acyl phosphate, incubated for 10 minutes at room temperature, and then passed through a Sephadex G-25 column to remove the excess reagents. No phosphorylation occurs when the phosphoryl donor is ATP, GTP or creatine phosphate. The method fails when applied to aldolase, glycerol-3-phosphate dehydrogenase or glutamic dehydrogenase.[64] Carbamoyl phosphate, in fact, acts as a carbamylating agent toward bovine glutamic

dehydrogenase, inactivating the enzyme by reaction with the ε-NH_2 group of lysine-97.[65]

These methods are distinguished from the non-enzymic methods by (1) their specificity for particular proteins and for particular sites within those proteins, (2) their low degree of substitution, being an order of magnitude lower and requiring special techniques for detection, and (3) the mildness of their reaction conditions.

III. REACTIVE SITES

The determination of which sites in proteins are phosphorylated by each of the foregoing methods has proven to be a formidable problem. Since few of the protein-phosphorus bonds are able to withstand the acid hydrolysis that is usually performed in amino acid analysis, other lines of evidence have been pursued to help solve the problem.

Table VI lists potential sites for phosphorylation in proteins. In general, any —NH, —OH or —SH group in an amino acid is capable of undergoing phosphorylation, and representatives of all three types are known.[5] Since the peptide —C(O)NH— group is inert, almost half of the commonly occurring amino acid residues are unreactive, except where they occur as end-groups; in effect, glycine, alanine, proline, valine, methionine, cystine, leucine, isoleucine and phenylalanine residues can all be considered to be inert.

The principal factors in the phosphorylation of amino acids are the substrate, the phosphorylating agent, and the reaction conditions.[5] In general, acid conditions favor O-substitution whereas alkaline conditions favor N- and S-substitution. Phosphorylation of aliphatic or aromatic hydroxyl groups is inferred if the phosphate groups are stable to acid and unstable to base. O-Phosphoserine and -threonine are stable in neutral or acid solution but undergo β-elimination in alkaline solution, giving pyruvic acid and α-ketobutyric acid, respectively. O-Phosphotyrosine exhibits the same behavior but regenerates tyrosine in alkaline solution. Conversely, phosphorylation of aliphatic or aromatic amino groups is inferred if the phosphoramidate groups are stable to base and unstable to acid. N-Phospho derivatives in which the phospho group is attached to a

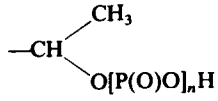
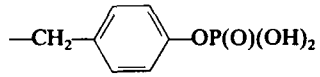
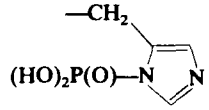
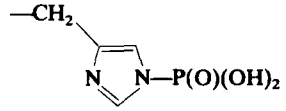
TABLE VI

Potential sites for phosphorylation in proteins

Site	Amino acid residue
—CO_2H	Aspartic acid, glutamic acid, any C-terminal residue
—NH_2	Lysine, hydroxylysine, any N-terminal residue
—CONH—	All residues
—CH_2OH	Serine
—CHOH (aliphatic)	Threonine, hydroxyproline, hydroxylysine
—COH (aromatic)	Tyrosine
—CH_2SH	Cysteine
—NH (aromatic)	Histidine, tryptophan
—$CONH_2$	Asparagine, glutamine
—$NHC(=NH)NH_2$	Arginine
—$P(O)(OH)_2$	All phosphorylated residues

TABLE VII

Structures of phosphorylated proteins

Amino acid residue	R in [—NHCHRC(O)—]$_x$
Serine	—$CH_2O[P(O)O]_nH$ $n = 1$, orthophosphate $n = 2$, di- or pyrophosphate $n > 2$, polyphosphate
Threonine	
Hydroxyproline	
Tyrosine	
Histidine, N_π	
Histidine, N_τ	
Lysine	—$(CH_2)_4NH[P(O)O]_nH$ $n = 3$, triphosphoramidate

nitrogen other than the α-amino group, such as N_π(or N_τ)-phosphohistidine, N_ε-phospholysine or N_ω-phosphoarginine, are more stable than the N_α derivatives, though still acid-sensitive.[5]

The structures of the phosphorylated amino acid residues are given in Table VII. The evidence for each assignment is as follows.

A. Serine

Phosphorylation of the aliphatic hydroxyl groups of serine residues is expected for method 4, where the reaction conditions are acidic throughout. The amount of bound phosphorus remaining after extensive dialysis against 10% sodium chloride correlates reasonably well with hydroxyl content for a series of proteins ranging from 4.6 (gliadin) to 37.7 (sericin) equivalents per 10^4 g. Titration, however, indicates that only 69–87% of the phosphate groups are in the orthophosphate form, compared with 100% for the naturally occurring phosphoprotein, phosvitin.[29] This matter is discussed further in Section IV. Phosphorylation of serine residues has also been suggested for methods 1,[17] 2,[25] 5[34,35] and 7,[39,40] though it was recently ruled out for method 1 on the basis of ^{31}P NMR evidence.[18] Proof was obtained for methods 2[25] and 7[40] by partial acid hydrolysis

of phosphorylated herring clupeine YI and salmon sperm protamine, respectively. O-Phosphoserine was identified in the hydrolysates by ion exchange chromatography[25,40] and confirmed by high voltage paper electrophoresis[25] or ^{33}P-radioactivity.[40]

B. Threonine

Phosphorylation of threonine residues has been inferred for methods 1,[17] 2[25] and 4,[29] based on the stability of the phosphoprotein to acid. Proof was obtained for method 2 by partial hydrolysis of phosphorylated clupeine YI, which contains two threonine residues per mole. O-Phosphothreonine was identified in the hydrolysate by ion exchange chromatography and confirmed by high voltage electrophoresis.[25]

C. Hydroxyproline

The amount of phosphorus introduced into the hydroxyproline-rich proteins gelatin and isinglass by method 4 suggests that the hydroxyl group of the hydroxyproline residue is available for phosphorylation, but this has not been confirmed.[29]

D. Tyrosine

The phenolic hydroxyl groups of tyrosine residues are phosphorylated to a limited extent, if at all, by methods 1[17] and 4.[29] A peak near 0 ppm in the ^{31}P NMR spectrum of phosphorylated lysozyme after treatment with acid could have been tyrosine-bound phosphate, but this was not confirmed.[17] Insulin and silk fibroin peptone both bind phosphorus in excess of their aliphatic hydroxyl content but their tyrosine content, measured colorimetrically (Folin), was only reduced from 9.0 to 8.5% and (—) to 6.0%, respectively.[29]

E. Histidine

Phosphorylation of histidine residues has been inferred for methods 1[17,18,20,23] and 8,[45,46,48] based on the stability of the phosphoprotein to base and instability to acid. Proof was obtained for method 8 by HPLC identification of N_τ-phosphohistidine in the potassium hydroxide digests of phosphorylated histone H4 and myelin basic protein.[45] Histone H4 contains two histidine residues per mole, and both are phosphorylated. The ^{31}P NMR spectrum of the protein at pH 7 shows a sharp peak for phosphorylated His-18 at 4.8 ppm and a broad peak for phosphorylated His-75 at 7.3 ppm. These assignments were confirmed by experiments in which peptide fragments H4(1–23) and H4(38–102) were phosphorylated separately, and further confirmed by chymotryptic cleavage of ^{32}P-labeled phosphorylated histone H4.[46] Protein HPr contains a single histidine residue, His-15, which is phosphorylated by method 8 to give a phosphoprotein whose ^{31}P resonance at pH 11.8 is 5.57 ppm; the phosphorylated protein, however, is unable to transfer phosphate and is therefore assigned the N_τ

structure to distinguish it from the enzymic phosphorylation product which is known to have the N_π structure.[48] The N-phosphohistidine isolated from phosphorylated bovine hemoglobin after alkaline hydrolysis was identified by ion exchange chromatography as the N_τ isomer.[20]

F. Lysine

Phosphorylation of the ε-NH_2 groups of lysine residues has been suggested for methods 1,[17,18,20,23] 5[34,35] and 8.[43] The ^{31}P NMR spectrum of phosphorylated β-lactoglobulin at pH 7.5 shows a strong signal at 0.14 ppm, close to the 1.28 ppm signal for phosphorylated poly-L-lysine.[18] Measurement of the acid liberated when phosphorylated ribonuclease is treated with Sanger's reagent (2,4-dinitrofluorobenzene) suggests that ε-NH_2 groups are blocked exclusively.[43] Proof was obtained for method 1 by partial hydrolysis of phosphorylated bovine hemoglobin. The N-phospholysine was identified by ion exchange chromatography and block zone electrophoresis, and confirmed to be the N_ε isomer by derivatization with Sanger's reagent.[20]

G. Cysteine

Few if any of the sulfhydryl groups of egg albumin are phosphorylated by method 4. Cysteine itself is unaffected by this reagent.[29]

H. Arginine

Phosphorylation of the guanidyl groups of arginine residues has been considered for methods 1[17] and 7,[41] but the acid-labile phosphate in the latter is probably serine polyphosphate rather than phosphoarginine.[25] Arginine itself requires a higher pH for N_ω-phosphorylation than those of methods 1–8.[66]

I. Tryptophan

The indole groups of gramicidin, which contains almost 40% tryptophan residues, are unreactive toward method 4.[29]

J. Aspartic and Glutamic Acids

Although aspartic acid and glutamic acid are both capable of forming O-phosphoanhydrides (acyl phosphates), no phosphoprotein derivatives of this type are known.

IV. FORMATION OF POLYPHOSPHATES

Several of the methods yield phosphoproteins that when first precipitated contain considerably more phosphorus than they do after work-up, particularly after dialysis. The presence of acid-labile phosphate in the phosphoproteins prepared

by methods 2 and 4 is indicative of the formation of polyphosphates. This was verified for phosphorylated clupeine, whose ^{31}P NMR spectrum before acid treatment showed signals whose pH dependence was consistent with ortho-, pyro- and polyphosphate residues of serine and threonine; upon treatment with acid, the pyro- and polyphosphate signals disappeared.[25]

Serious departures from the orthophosphate structure occur in the phosphorylation of the model compounds polyvinyl alcohol[29] and poly-L(or DL)-serine.[26] The by-products of the latter are now believed to be polyphosphate esters rather than N → O shift products.[25] Evidence of polyphosphates in phosphorylated proteins has also been obtained for method 1.[17]

In method 5, the ϵ-NH_2 groups of lysine residues in phosphorylated soy protein are believed to carry triphospho groups since no pyrophosphate is liberated when lysine itself is phosphorylated under the same conditions.[34]

V. PHYSICAL PROPERTIES

The procedures used to determine the physical properties of the phosphorylated proteins are listed in Table VIII, except for those related to functionality.

In most cases, sedimentation coefficients of the proteins are not affected by phosphorylation.[15,21,43] Phosphorylation does, however, alter the self-association and complex formation between bovine casein components, producing significant changes in their sedimentation coefficients.[39]

Optical properties of the proteins are altered only slightly by phosphorylation. Ribonuclease shows a small increase in optical rotation.[43] β-Lactoglobulin shows a small but irreversible change, with little loss of α-helical structure as measured by circular dichroism.[18] In clupeine, an increase in α-helical content was ascribed to the solvent.[25]

Phosphorylated gelatin, in contrast to gelatin itself, exhibits flow birefringence in acid solution.[27]

Nuclear magnetic resonance (NMR) spectra have been reported for several phosphorylated proteins, often with inconclusive results.[17,18] Protein HPr is an exception; this protein was proven to be phosphorylated at the N_τ position of its lone histidine residue (His-15) by ^{31}P NMR, confirmed by the behavior of the C-2 and C-4 imidazole protons in the ^{1}H NMR spectra.[48] ^{31}P NMR spectroscopy on histone H4 not only proves that both histidine residues (His-18 and His-75) are phosphorylated but also shows by the difference in line width that the former is much more mobile in solution than the latter.[46] The ^{31}P NMR spectra of phosphorylated clupeine provide evidence that the phosphorylated serine and threonine residues are mixtures of ortho-, pyro- and polyphosphates.[25]

Gel filtration chromatography on Sephadex C-25 or G-25 is a useful method for separating phosphoproteins from reagents and low molecular weight by-products. The eluant may be water[23,25,39] or 0.1*M* NaCl.[18] Gel filtration chromatography has been used to separate phosphorylated clupeine Z from peptide fragments, and for separating the former into singly, doubly and triply (fully) phosphorylated fractions.[25]

Ion exchange chromatography has been used to separate phosphorylated

TABLE VIII

Physical properties of phosphorylated proteins

Procedure	Reference
Sedimentation Coefficient	
Human serum albumin, hemoglobin and globin	15
Horse serum albumin	21
α_{S1}-, β-, and κ-Casein	39
Ribonuclease	43
Optical Rotation	
Ribonuclease	43
Circular Dichroism	
β-Lactoglobulin	18
Clupeine	25
Flow Birefringence	
Gelatin	27
^{1}H NMR *Spectroscopy*	
Protein HPr	48
^{31}P NMR *Spectroscopy*	
Casein and lysozyme	17
β-Lactoglobulin	18
Clupeine	25
Histone H4	46
Protein HPr	48
Gel Filtration Chromatography	
β-Lactoglobulin	18
Soy protein	23, 24
Clupeine YI and Z	25
α_{S1}-, β-, and κ-Casein	39
Salmon protamine	40
Ion Exchange Chromatography	
Salmon protamine	40
Ribonuclease	43, 44
Gel Electrophoresis	
Casein and lysozyme	17
β-Lactoglobulin	18
Soy protein	24, 34
Histone H4	46
Protein HPr	48
Electrophoresis	
Human serum albumin and hemoglobin	15
Horse serum albumin	21
Insulin	41
Ribonuclease	43

enzymes from the native enzymes and from each other. Phosphorylated ribonuclease can be separated into several distinct monophosphates, believed to be structural isomers, by chromatography on carboxymethyl cellulose buffered to pH 8 and eluted with a NaCl gradient.[44] This procedure is more suitable for phosphorylated ribonuclease than the Amberlite XE-64 procedure reported earlier.[43]

SDS polyacrylamide gel electrophoresis has been applied to a number of phosphorylated proteins.[17,18,24,34,46,48] Evidence of crosslinking by phosphoros oxychloride was obtained for casein,[17] lysozyme[17] and β-lactoglobulin,[18] and of dissociation by metaphosphate for soy protein.[34] The method has also been used to demonstrate the acid lability of phosphorylated histone H4.[46]

Electrophoretic mobilities have been measured for phosphorylated human serum albumin and hemoglobin,[15] horse serum albumin,[21] insulin[41] and ribonuclease.[43] In every case, as expected, there was a rise in anodic mobility upon phosphorylation, reflecting the introduction of negative charges into the proteins.

VI. FUNCTIONALITY

The physical properties related to functionality are listed in Table IX. The attachment of anionic phosphate groups to the proteins should increase their hydrophilicity, but the results are often unpredictable. Water solubility increases upon phosphorylation of soy protein[23,34] and globin,[15] but decreases upon phosphorylation of casein,[67] lysozyme,[67] human serum albumin[15] and hemoglobin.[15] Phosphorylation shifts the pI of soy protein by about 0.8 pH unit.[34,35]

Viscosity increases upon phosphorylation of casein,[17] horse serum albumin[21] and egg albumin,[22] but remains unchanged upon phosphorylation of gelatin[27] and lysozyme.[17]

Most of the phosphorylated proteins exhibit no tendency toward gelation, but there are exceptions. Human serum albumins containing 10 to 20 phosphoryl groups per mole form gels; those with more than 20 are insoluble at neutral pH whereas those with 5 or less are soluble at every pH.[15] Phosphorylated wheat gluten and many of its salts are gel forming.[29,36,37] Phosphorylated casein and soy protein both have a gel-like character even at neutral pH.[23,67] Phosphorylated β-lactoglobulin forms a gel upon dialysis against calcium ion.[19] The ability of gelatin to form gels is unchanged if the phosphorylation is done under acid conditions and decreases slightly if it is done under alkaline conditions.[27] The texture of phosphorylated soy protein gel resembles that of traditional Japanese cakes.[38]

Phosphorylation of bovine casein components increases their calcium binding ability.[30,39] The concentrations of calcium chloride required to precipitate the modified α_{S1}- and β-caseins are higher than those for the native caseins. κ-Casein is not precipitated, whether phosphorylated or not. Phosphorylation of α_{S1}- and β-casein does not impair their ability to form micelles with κ-casein, but the converse does; the phosphate content of the κ-casein must be kept low.[39]

TABLE IX

Functionality of phosphorylated proteins

Functionality	Reference
Water Solubility	
Human serum albumin, hemoglobin and globin	15
Soy protein	23, 24, 34, 35
Casein and lysozyme	67
Viscosity	
Casein and lysozyme	17
Horse serum albumin	21
Egg albumin	22
Gelatin	27
Gelation	
Human serum albumin	15
β-Lactoglobulin	19
Soy protein	23, 24, 38
Gelatin	27
Gluten	29, 36, 37
Casein	67
Coagulation	
α_{S1}-, β-, and κ-Casein	39
Water Absorption	
Casein and lysozyme	17
Gluten	29, 36, 37
Soy protein	34, 35, 38
Emulsification	
Casein	17
Soy protein	23, 24, 34, 35
Gelatin/acrylate copolymer	32
Whippability	
Soy protein	34, 35
Nitrogen Solubility Index	
Soy protein	34, 35

Water absorption almost doubles upon phosphorylation of lysozyme[17] and soy protein,[34,35] but casein unaccountably shows a slight decrease.[17] The hydration capacity of phosphorylated wheat gluten is about 200 g of water per g of product, compared to 200–300 for gluten sulfates.[29,36]

Emulsifying capacity and emulsion stability of soy protein both increase upon phosphorylation,[23,24,34,35] but the emulsifying activity of casein decreases significantly upon phosphorylation.[17] Emulsions of phosphorylated gelatin-acrylate or -methacrylate graft copolymers are useful for leather impregnation and finishing, giving leather products having good filling and tight grain in addition to good heat and flame resistance.[32]

Foam expansion and stability, which measure the whippability of soy protein isolate, are both improved by phosphorylation.[34,35] The nitrogen solubility index, which measures the extent of denaturation of the protein, is also improved.[34,35]

VII. BIOLOGICAL PROPERTIES

The procedures used to determine the biological properties of the phosphorylated proteins are listed in Table X. The properties of foremost interest are the digestibility of the phosphorylated proteins in comparison with the native proteins, and the behavior of the chemically phosphorylated proteins toward phosphatase enzymes in comparison with the naturally occurring phosphoproteins.

In general, the digestibility of the proteins is not adversely affected by phosphorylation. *In vitro* tests with pepsin, pancreatin, trypsin and α-chymotrypsin have been carried out on a number of phosphorylated proteins. Phosphorylation produces a significant decrease in the initial rate of hydrolysis of casein, though the extent of hydrolysis after 24 hours is the same.[17] For ribonuclease, the initial rate is faster but not significantly so.[43] Phosphorylation inactivates 90% of the trypsin inhibitor activity of ovomucoid.[31] In several cases it has been established that peptic, tryptic or chymotryptic digestion solubilizes the protein before any inorganic phosphate is liberated.[11–13,46]

In a bioassay of protein suitable for mammal use, the organism *Tetrahymena thermophili* was found to grow as well on phosphorylated casein as on Hammarsten casein.[17]

Acid phosphatase and bone phosphatase have no action on the phosphorylated proteins,[13,26,27] unless the proteins are first subjected to tryptic hydrolysis.[13] Alkaline phosphatase removes all of the phosphate from native or phosphorylated casein[39] but is only partially effective with phosphorylated β-lactoglobulin[18] and clupeine YI,[25] removing 25 or 34%, respectively, of the phosphorus as inorganic phosphate. It has no action on phosphorylated poly-DL-lysine.[26] The alkaline medium destroys phosphorylated trypsin.[27]

Phosphorylated salmon sperm protamine, in contrast, has been used as a substrate for the assay of phosphatase activity in rat tissue, using ^{33}P-labeled phosphoric acid as the phosphorylating agent. The distribution of phosphatase varies from tissue to tissue, but parallels that measured by enzymically phosphorylated protamine.[40]

The immunological properties of some of the phosphorylated proteins have been tested against rabbits. The specificity of egg albumin changes radically upon phosphorylation, without loss of antigenic properties.[20] The serological reactivity of horse serum albumin (SA) with anti-SA serum is reduced in proportion to the phosphorus content.[21] Anti-SA from phosphorylated human SA reacts strongly with the latter but not with phosphorylated rabbit or chicken serum proteins.[14] The ^{32}P-labeled proteins are quickly removed from the blood stream.[16]

Phosphorylated insulin is reported to be biologically active when injected into rabbits,[41] but in the mouse convulsion assay it loses 62% of its hormonal activity.[33]

The introduction of a single phosphate group into beef pancreatic ribonuclease is sufficient to bring about nearly complete inactivation of the enzyme.[44] Protein HPr, a component of the phosphotransferase system of *S. aureus*, is no longer able to function as a phosphocarrier when phosphorylated at the N_τ position of its lone histidine residue.[48]

TABLE X

Biological properties of phosphorylated proteins

Procedure	Reference
Pepsin Digestion	
Egg albumin	10
Lactalbumin	11
Blood globulin, silk fibroin peptone and Witte peptone	12
Casein	12, 13
Horse serum globulin	13
Soy protein	23, 24, 34
Pancreatin Digestion	
Lactalbumin	11
Soy protein	23, 24, 34, 35
Trypsin Digestion	
Blood globulin, silk fibroin peptone and Witte peptone	12
Casein	12, 13, 17
Horse serum globulin	13
Ovomucoid	31
Ribonuclease	43
α-Chymotrypsin	
Casein	17
^{32}P-Histone H4	46
Pronase E	
Soy protein	23, 24
Aminopeptidase-Prolidase	
Soy protein	23, 24
T. thermophili	
Casein	17
Bone Phosphatase	
Casein and horse serum globulin	13
Alkaline Phosphatase	
β-Lactoglobulin	18
Clupeine YI	25
Poly-DL-serine	26
Pepsin	27
α_{S1}-, β-, and κ-Casein	39
Acid Phosphatase	
Poly-DL-serine	26
Pepsin	27
Rat Tissue Phosphatases	
^{33}P-Salmon protamine	40
Immunological Properties	
Human serum protein	14, 16
Horse serum albumin	21
Egg albumin	22
Other Properties	
Insulin	33, 41
Ribonuclease	43, 44
Protein HPr	48

VIII. SUMMARY AND CONCLUSIONS

A variety of methods have been developed for the phosphorylation of proteins by chemical reagents. These methods have been applied to approximately fifty proteins and to a few related peptides and model compounds. The phosphorus contents of the products vary from 0.02 to 11.0%, but are usually of the order of 1 to 2%. Methods that employ acid conditions favor O-substitution whereas those that employ alkaline conditions favor N-substitution. The principal sites of reaction are the aliphatic OH of serine and threonine residues, the terminal NH_2 of lysine residues, and the aromatic NH of histidine residues, but they vary in unpredictable fashion from one protein to another.

The physical properties, functionality and biological properties of the proteins are altered in some respects, but these too are often unpredictable. In general, phosphorylation alters the solubility characteristics of the proteins without adversely affecting their digestibility. In some cases, the products differ in behavior from those phosphorylated by enzymic methods.

REFERENCES AND NOTES

1. Based on a paper presented at the 186th National Meeting of the American Chemical Society, Washington, DC, August 29, 1983. Names of companies or commercial products are given solely for the purpose of providing specific information. Their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.
2. G. E. Perlmann, *Adv. Protein Chem.*, **10,** 1 (1955).
3. G. Taborsky, *Adv. Protein Chem.*, **28,** 1 (1974).
4. E. G. Krebs and J. A. Beavo, *Ann. Rev. Biochem.*, **48,** 923 (1979).
5. A. W. Frank, *CRC Crit. Rev. Biochem.*, **16,** 51 (1984).
6. L. Liebermann, *Ber.*, **21,** 598 (1888).
7. J. Pohl, *Z. physiol. Chem.*, **13,** 292 (1889).
8. G. E. Perlmann, *J. Biol. Chem.*, **137,** 707 (1941).
9. G. E. Perlmann and H. Hermann, *Biochem. J.*, **32,** 926 (1938).
10. H. Bechhold, *Z. physiol. Chem.*, **34,** 122 (1901).
11. C. Neuberg and H. Pollak, *Biochem. Z.*, **26,** 529 (1910).
12. C. Neuberg and W. Oertel, *Biochem. Z.*, **60,** 491 (1914).
13. C. Rimington, *Biochem. J.*, **21,** 272 (1927).
14. J. C. Boursnell, H. M. Dewey and A. Wormall, *Biochem. J.*, **43,** 84 (1948).
15. J. Salak, Z. Vodrazka and J. Cejka, *Coll. Czech. Chem. Commun.*, **30,** 1036 (1965).
16. T. E. Banks, J. C. Boursnell, H. M. Dewey, G. E. Francis, R. Tupper and A. Wormall, *Biochem. J.*, **43,** 518 (1948).
17. G. Matheis, M. H. Penner, R. E. Feeney and J. R. Whitaker, *J. Agric. Food Chem.*, **31,** 379 (1983).
18. S. L. Woo, L. K. Creamer and T. Richardson, *J. Agric. Food Chem.*, **30,** 65 (1982).
19. T. Richardson and J. Kester, "Phosphorylation, Thiolation, Esterification and Amidation of Proteins to Modify their Interactions in Food Systems", presented at the 186th National Meeting, ACS, Washington, DC, August 29, 1983.
20. O. Zetterqvist and L. Engström, *Biochim. Biophys. Acta*, **141,** 523 (1967).
21. M. Mayer and M. Heidelberger, *J. Am. Chem. Soc.*, **68,** 18 (1946).
22. M. Heidelberger, B. Davis and H. P. Treffers, *J. Am. Chem. Soc.*, **63,** 498 (1941).
23. M. Hirotsuka, H. Taniguchi, H. Narita and M. Kito, *Agric. Biol. Chem.*, **48,** 93 (1984).
24. M. Hirotsuka and M. Kito [to Fuji Oil Co. Ltd.], *U.S. Patent*, 4,501,694 (Feb. 26, 1985).
25. L. Willmitzer and K. G. Wagner, *Eur. J. Biochem.*, **59,** 43 (1975).
26. U. Breyer, Y. Lapidot, J. Kurtz, Z. Bohak and E. Katchalski, *Monatsh. Chem.*, **98,** 1386 (1965).
27. P. Grabar, M. Pacht and E. Bourland, *Bull. Soc. Chim. Biol.*, **33,** 690 (1951).
28. E. Bourland and M. Pacht, *Bull. Soc. Chim. Biol.*, **33,** 681 (1951).
29. R. E. Ferrel, H. S. Olcott and H. Fraenkel-Conrat, *J. Am. Chem. Soc.*, **70,** 2101 (1948).
30. I. R. Dickson and D. J. Perkins, *Biochem. J.*, **124,** 235 (1971).

31. H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, **177,** 385 (1949).
32. K. P. Rao, K. T. Joseph, M. Santappa and Y. Nayudamma, *Leather Sci. (Madras)*, **22,** 140 (1975).
33. J. Fraenkel-Conrat and H. Fraenkel-Conrat, *Biochim. Biophys. Acta,* **5,** 89 (1950).
34. H. Y. Sung, H. J. Chen, T. Y. Liu and J. C. Su, *J. Food Sci.*, **48,** 716 (1983).
35. H. J. Chen, T. Y. Liu, H. Y. Sung and J. C. Su, *U.S. Patent,* 4,322,344 (Mar. 30, 1982).
36. A. Mohammad, D. K. Mecham and H. S. Olcott, *J. Agric. Food Chem.*, **2,** 136 (1954).
37. H. S. Olcott and A. Mohammad [to U.S. Dept. of Agriculture], *U.S. Patent,* 2,513,351 (July 4, 1950).
38. S. Ochiai-Yanagi, H. Miyauchi, K. Saio and T. Watanabe, *Cereal Chem.*, **55,** 157 (1978).
39. M. Yoshikawa, R. Sasaki and H. Chiba, *Agric. Biol. Chem.*, **45,** 909 (1981).
40. B. Ullman and R. L. Perlman, *Biochem. Biophys. Res. Commun.*, **63,** 424 (1975).
41. T. Rathlev and T. Rosenberg, *Arch. Biochem. Biophys.*, **65,** 319 (1956).
42. T. Müller, T. Rathlev and T. Rosenberg, *Biochim. Biophys. Acta,* **19,** 563 (1956).
43. G. Taborsky, *Compt. Rend. Lab. Carlsberg, Ser. Chim.*, **30,** 309 (1958).
44. G. Taborsky, *J. Biol. Chem.*, **234,** 2915 (1959).
45. A. W. Steiner, E. R. Helander, J. M. Fujitaki, L. S. Smith and R. A. Smith, *J. Chromatogr.*, **202,** 263 (1980).
46. J. M. Fujitaki, G. Fung, E. Y. Oh and R. A. Smith, *Biochemistry,* **20,** 3658 (1981).
47. Y. C. Lin and R. A. Smith, *J. Chin. Chem. Soc. (Taipei),* **28,** 213 (1981).
48. M. Gassner, D. Stehlik, O. Schrecker, W. Hengstenberg, W. Maurer and H. Rüterjans, *Eur. J. Biochem.*, **75,** 287 (1977).
49. J. R. Stevens-Clark, K. A. Concklin, A. Fujimoto and R. A. Smith, *J. Biol. Chem.*, **243,** 4474 (1968).
50. M. Eto, *Organophosphorus Pesticides: Organic and Biological Chemistry* (CRC Press, Inc., Cleveland, OH, 1974), 387 pp.
51. L. Engström, *Biochim. Biophys. Acta,* **52,** 49 (1961).
52. L. Engström, *Biochim. Biophys. Acta,* **92,** 71 (1964).
53. J. H. Schwartz and F. Lipmann, *Proc. Natl. Acad. Sci. U.S.*, **47,** 1996 (1961).
54. L. Engström, *Biochim. Biophys. Acta,* **56,** 606 (1962).
55. N. Zwaig and C. Milstein, *Biochem. J.*, **92,** 421 (1964).
56. M. Igarashi, H. Takahashi and N. Tsuyama, *Biochim. Biophys. Acta,* **220,** 85 (1970).
57. H. Greenberg and D. Nachmansohn, *J. Biol. Chem.*, **240,** 1639 (1965).
58. P. Boer and E. P. Steyn-Parvé, *Biochim. Biophys. Acta,* **206,** 281 (1970).
59. M. M. Pigretti and C. Milstein, *Biochem. J.*, **94,** 106 (1965).
60. E. G. Krebs, A. B. Kent and E. H. Fischer, *J. Biol. Chem.*, **231,** 73 (1958).
61. B. Illingworth and G. T. Cori, *Biochem. Preparations,* **3,** 1 (1953).
62. R. Rodnight and B. E. Lavin, *Biochem. J.*, **93,** 84 (1964).
63. J. B. Alpers and G. K. H. Lam, *J. Biol. Chem.*, **244,** 200 (1969).
64. F. Dallochio, M. Matteuzzi and T. Bellini, *Biochem. J.*, **203,** 401 (1982).
65. F. M. Veronese, D. Piszkiewicz and E. L. Smith, *J. Biol. Chem.*, **247,** 754 (1972).
66. J. M. Fujitaki, A. W. Steiner, S. E. Nichols, E. R. Helander, Y. C. Lin and R. A. Smith, *Prep. Biochem.*, **10,** 205 (1980).
67. G. Matheis, R. E. Feeney and J. R. Whitaker, *unpublished results,* 1981, cited in Reference 17.
68. M. Hirotsuka, H. Taniguchi, H. Narita and M. Kito, *J. Jap. Soc. Food Sci. Technol.*, **32,** 622 (1985).
69. M. Hirotsuka, H. Taniguchi, H. Narita and M. Kito, *J. Jap. Soc. Food Sci. Technol.*, **32,** 626 (1985).
70. J. R. Whitaker, M. Sitohy and J. M. Chobert, *J. Am. Oil Chem. Soc.*, **63,** 446 (1986).

Note added in proof:

Since this paper was written, Hirotsuka *et al*[68,69] have extended their investigation[23,24] of the phosphorylation of soy protein isolate and Whitaker *et al*[70] have reported the phosphorylation of zein by method 1.