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Reactions of Phosphoproteins in Alkaline Solutions

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Phosvitin, an egg yolk protein of mol wt 35 500 with 10% phosphate in the form of O-phosphoserine residues (reported value of 119 phosphoserine and one phosphothreonine residues), was used in model systems to investigate the effects of various experimental conditions on the rates of β elimination of phosphate from the phosphoserine residues and addition of indogenous nucleophiles to the double bond of the dehydroalanines formed. At low concentrations of phosvitin ($1–10 \times 10^{-6}$ M) the rates were independent of phosvitin concentration but directly dependent on the hydroxide ion concentration of the solution. The activation energies of the β-elimination and addition reactions were 20.2 and 25.0 kcal/mol, respectively. The rate of the β-elimination reaction was increased at increased ionic strengths and was markedly increased in the presence of $CaCl_2$. At 1.12×10^{-3} M $CaCl_2$, the rate of β elimination was 20 times faster than in the absence of $CaCl_2$. The rate of the addition reaction was not significantly affected by $CaCl_2$ or ionic strength.

Sodium hydroxide, on the GRAS (Generally Recognized as Safe) list of chemicals, is widely used in the food processing industry in the neutralizing, peeling, solubilizing, detoxifying (Screenivasamurthy, 1967), and texturizing (Gutcho, 1973) of foods. However, it is known that a number of reactions involving proteins occur in alkaline solution.

Alkali treatment of proteins has been shown to cause losses in serine, threonine, cystine, arginine, lysine, and an increase in ornithine (Pickering and Li, 1964; Geschwind and Li, 1964; Zeigler et al., 1967; Mellet, 1968; Blackburn, 1968; DeGroot and Slump, 1969; Parisot and Derminot, 1970; Whiting, 1971; Gottschalk, 1972; Provansal et al., 1975). Amino acid residues in proteins undergo racemization in alkaline solution more readily than do the free amino acids. These racemization reactions have been known for a long time (Dakin, 1912; Levene and Bass, 1928) and occur at different rates depending on the particular amino acid residue and experimental conditions (Neuberger, 1948; Hill and Leach, 1964; Pickering and Li, 1964; Pollock and Frommhagen, 1968; Tannenbaum et al., 1970).

New amino acids are formed during alkali treatment of proteins. Ornithine arises from the hydrolysis of the guanido group of arginine (Ziegler et al., 1967). Lysinoalanine residues have been reported in several proteins following alkali treatment (Patchornik and Sokolovsky, 1964; Bohak, 1964; Ziegler, 1964; Corfield et al., 1967; Robson and Zaidi, 1967; Whiting, 1971). Lanthionine has been identified in alkali-treated wool (Corfield et al., 1967; Asquith and García-Domínquez, 1968) as well as in other sulfur-containing proteins (Nashef et al., 1977). β-Aminoalanine has also been identified in an hydrolyzate of alkali-treated wool (Asquith et al., 1969) as has ornithinoalanine in alkali-treated proteins (Ziegler et al., 1967).

The new amino acids, lysinoalanine, lanthionine, ornithinoalanine, and β-aminoalanine, formed in proteins by alkali treatment can be explained by the initial formation of the common intermediate dehydroalanine (2-aminopropenic acid) from the decomposition of cystine, *O*-phosphoserine, and *O*-glycosylserine residues. The addition of the ϵ-amino group of a lysine residue, the δ-amino group of an ornithine residue, the sulfhydryl group of a cysteine residue, or ammonia to the double bond of the dehydroalanine forms the new amino acids. Direct evidence for dehydroalanine formation during alkali treatment of proteins is available (Asquith and Carthew, 1972; Miro and García-Domínquez, 1973). While there is evidence for the destruction and formation of new amino acids during the alkali treatment of proteins, there is very little information on the effect of various experimental parameters on the rates of destruction of cystine and O-substituted serine and threonine residues and the formation of new products.

Our laboratory has undertaken a systematic study of the reactions of proteins in alkaline solutions. Initially, we have chosen to study the behavior of model proteins and peptides and then to extend these data to a study of more complex food systems. We have reported on the behavior of cystine residues (Nashef et al., 1977) and *O*-glycosylthreonine residues (Lee et al., 1977) in proteins in alkaline solution.

In the present paper, we examine the effect of alkaline solution on the phosphoprotein, phosvitin. This protein is ideally suited for these studies since it contains a reported 119 phosphoserine residues, only one phosphothreonine residue, no cystine or cysteine, and the carbohydrate moiety is alkali stable as it is attached to the amide nitrogen of an asparagine residue (Taborsky, 1974). The amino acid sequence of phosvitin is nearly complete (Taborsky, 1974), facilitating the interpretation of the data,

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Table I. Change in Amino Acid Composition of Phosvitin in Alkaline Solution[a]

Time, min	Serine[c]	Lysine[c]	Dehydroalanine[b,c]	Lysinoalanine[c]	Lysinoalanine + lysine	Serine + dehydroalanine[b] + lysinoalanine
0	115	23.1	0	0	23.1	115
20	90.2	18.9	9.9	4.20	23.1	104
40	70.1	12.9	23.5	8.83	21.7	102
80	36.4	5.33	45.1	17.9	23.2	99

[a] The reactions were performed in KCl-NaOH buffer at 0.123 N NaOH, 60 °C, 1×10^{-5} M phosvitin, and ionic strength of 0.170. [b] Determined by increase in absorbance at 241 nm, using ϵ_M of 4200 M^{-1} cm^{-1}. [c] Mol/mol of phosvitin.

and other workers have also reported on the behavior of this protein in alkaline solution (Plimmer and Scott, 1908; Sundarajan et al., 1958; Taborsky, 1974; Bohak and Katchalski, 1975).

MATERIALS AND METHODS

Materials. Fresh chicken eggs not more than 2 days old were obtained from the University Farm. Lysinoalanine was a gift of Dr. John Finley, Western Regional Research Laboratory, Agricultural Research Service, Berkeley, Calif. All chemicals were reagent grade, and deionized water was used.

Methods. *Phosvitin Preparation.* Phosvitin was prepared from chicken egg yolk according to the combined procedures of Burley and Cook (1961) and Wallace et al. (1966).

The intact yolk was separated from the egg white, and the yolk content was obtained by puncturing the membrane. Care was taken to avoid membrane contamination of the yolk materials by removal of the membrane on cheese cloth. The yolk materials were diluted with an equal volume of 0.16 N NaCl and centrifuged for 10 min at 66 000*g*. The clear yellow supernatant liquid was decanted. The pellet of granules was dispersed in a volume of 0.16 N NaCl twice the original volume of the yolk. The granules were recovered by centrifuging at 13 000*g*. The granules (phosvitin–lipovitellin mixture) were then dissolved by stirring in five volumes of 1 N NaCl for at least 8 h. Lipovitellin was precipitated by adding two volumes of 100% saturated (at 0 °C) ammonium sulfate and collected by centrifuging at 66 000*g* for 15 min. The supernatant liquid was filtered to remove pieces of the lipovitellin pellet which broke away from the precipitate, then dialyzed extensively against water. The solution was further dialyzed against 0.04 N HCl to precipitate phosvitin. The precipitate was thoroughly washed sequentially with 0.04 N HCl, alcohol–ether (3:1), and ether and was finally air-dried. The protein contained 13.0% phosphorus and 12.1% nitrogen on a moisture-free basis.

All purification steps were done at 5 °C.

Phosphorus Determination. Phosphorus was determined according to the method of Fiske and SubbaRow (1925). Organic phosphate was released by heating the protein in 1 N NaOH at 100 °C for 15 min. Protein precipitated by the Fiske–SubbaRow reagent was removed after the reaction by centrifugation. Appropriate controls showed that this did not affect the results. Dibasic potassium phosphate was used as a standard.

Nitrogen Determination. Total nitrogen was determined by the method of Johnson (1941). Ammonium sulfate was used as the standard.

Amino Acid Analyses. Duplicate samples of protein were hydrolyzed in vacuo with 6 N HCl at 110 °C for 24, 48, and 72 h (Moore and Stein, 1963). The acid was removed under reduced pressure in a rotating evaporator at 25 °C. The residue was dissolved in 0.02 N citrate buffer, pH 2.2, containing 5 mL/L of bis(β-hydroxyethyl) sulfide. Amino acid analyses were performed on a Technicon Autoanalyzer by the procedure of Hamilton (1963) as modified by Technicon Corporation. Lysinoalanine was determined on the Technicon Autoanalyzer by the method of Williams and Woodhouse (1967). Dehydroalanine content was measured by two methods: (1) the difference between the amount of serine lost and the amount of lysinoalanine formed and (2) by the change in absorbance at 241 nm. An extinction coefficient of 4.20×10^3 M^{-1} cm^{-1} for dehydroalanine was used (Carter and Greenstein, 1946).

Alkali Treatment of Phosvitin. Buffers of NaOH–KCl were prepared just before use according to the method of Bates and Bower (1956) [see Bates and Bowers (1956) for the buffer capacity of these NaOH–KCl solutions]. Appropriate amounts of phosvitin were dissolved in 0.015 N NaOH to give stock solutions with pH of ~6. Reactions were done in glass-stoppered cuvettes in the thermostated chamber of a Beckman DU spectrophotometer equipped with Gilford attachments. The temperature in the spectrophotometer chamber was measured with a copper–constantan thermocouple. Reaction was started by adding 0.2 mL of temperature-equilibrated phosvitin to 2.8 mL of temperature-equilibrated buffer. The concentration of phosvitin in the reaction was checked by absorbance at 235 nm. Absorbance at 241 nm then was recorded continuously. Samples of equal concentration of phosvitin at pH 6.0 were used as controls during monitoring of the reaction.

In investigations of the effect of cations on the rates of β elimination and addition at 60 °C, the solvent used was 0.123 N NaOH with the appropriate concentration of $CaCl_2$.

All reactions were done at least in triplicate and generally at three to five different concentrations of phosvitin.

RESULTS

Changes in Amino Acid Composition. Treatment of phosvitin with 0.123 N NaOH at 60 °C resulted in a decrease in the serine and lysine content and an increase in dehydroalanine and lysinoalanine (Table I). In 80 min there was a reduction of serine from 115 (determined on our preparation of phosvitin) to 36 mol per mole of phosvitin with the production of 17.9 mol of lysinoalanine per mole of phosvitin. All the original lysine was accounted for as residual lysine or lysinoalanine. During this 80 min there was a loss of 17.8 mol of lysine per mole of phosvitin. Decrease in arginine content occurred only after an 80-min incubation period. As shown by the summation of serine, dehydroalanine, and lysinoalanine, >85% of the changes during the alkali treatment have been accounted for (Table I). The deviation from the expected sum of 115 could be due to formation of small amounts of unidentified derivatives, such as β-aminoalanine, or to the fact that the extinction coefficient of 4.20×10^3 M^{-1} cm^{-1} for dehydroalanine is in error by about 10–15%.

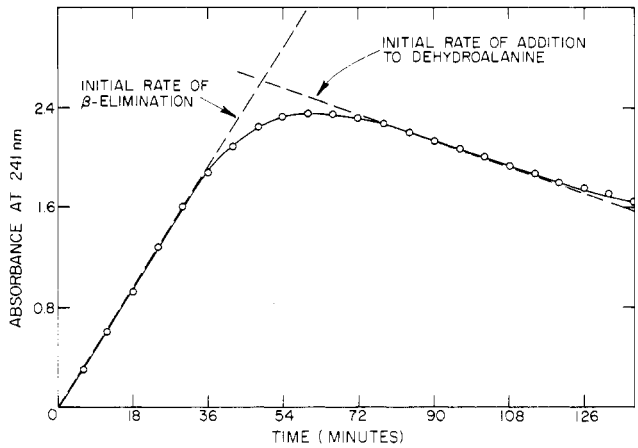


Figure 1. Change in absorbance at 241 nm of phosvitin in alkaline solution. The reaction was in KCl–NaOH buffer at 0.187 N NaOH, 60 °C, 1.09×10^{-5} M phosvitin, and ionic strength of 0.170. Absorbance was recorded continually on a Beckman DU spectrophotometer equipped with Gilford attachments.

Removal of Phosphate. In alkaline solution, phosphate groups are normally removed from phosphoserine (and phosphothreonine) by β elimination and by hydrolysis. In 0.123 N NaOH at 60 °C, 67% of the phosphate was removed from phosvitin in 80 min. Of the total phosphate removed, 87% could be accounted for by β elimination. How the remaining 13% of the phosphate was removed was not determined, but hydrolysis was considered the most likely mechanism. This removal of the 13% of phosphate groups was completed within the first 20 min of the reaction, indicating some uniquely reactive phosphoserine residues in phosvitin toward hydrolysis. The remaining 13% phosphate at 80 min referred to above was calculated as $[\text{phosphate}]_0 - ([\text{dehydroalanine}]_{80\text{ min}} + [\text{lysinoalanine}]_{80\text{min}})$.

Absorbance Change of Phosvitin in Alkaline Solution. Measurements of changes in amino acid composition during a reaction do not readily lend themselves to the many kinetic determinations needed to measure the effect of various experimental conditions on the effect of alkaline conditions on phosvitin. As earlier suggested by Mecham and Olcott (1949), there is a change in absorbance at 241 nm associated with the formation and loss of dehydroalanine (Figure 1). Initially, there is an increase in absorbance, followed by a decrease. The initial increase in absorbance was used in this work as a measure of the rate of β elimination. This is essentially so since initial rate data were used and the initial rate of dehydroalanine formation is at least six times (and probably much greater near zero time) that of lysinoalanine formation (Table I). We have used the rate of decrease in absorbance at 241 nm, determined as shown in Figure 1, as a measure of the rate of addition to dehydroalanine whether this be by lysine, ornithine, or other nucleophiles (such as ammonia produced by deamidation). There is some error in such a treatment because the β-elimination reaction has not been completed. The absorbance was converted to concentration of dehydroalanine by use of ϵ 4.20×10^3 M^{-1} cm^{-1} (Carter and Greenstein, 1946). Limited comparison between absorbance change and dehydroalanine concentration, measured as the difference between decrease in serine concentration and increase in lysinoalanine concentration by amino acid analyses, gave ϵ 3.96×10^3 M^{-1} cm^{-1} in this work. This method of calculating the extinction coefficient based on amino acid changes does not take into account addition of nucleophiles other than lysine to the dehydroalanine residues.

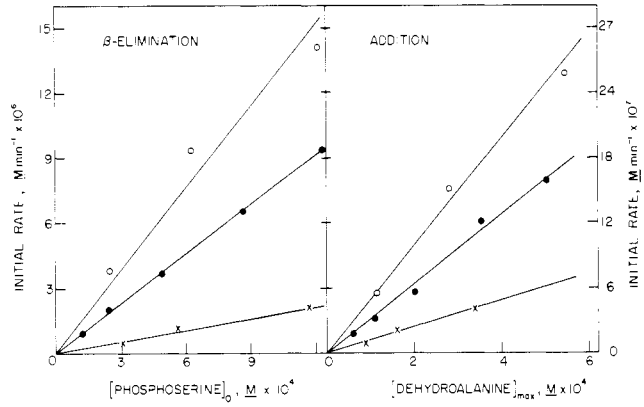


Figure 2. Effect of phosvitin and hydroxide ion concentrations on the initial rate of β elimination and rate of addition. The reactions were carried out in KCl–NaOH at 60 °C and the data corrected to an ionic strength of 0.170. The NaOH concentrations were: (○) 0.187 N; (●) 0.123 N; (X) 0.0541 N. Rate = [dehydroalanine]/min. For β elimination the phosvitin concentration is expressed in terms of phosphoserine concentration; for addition it is expressed in terms of maximum dehydroalanine concentration.

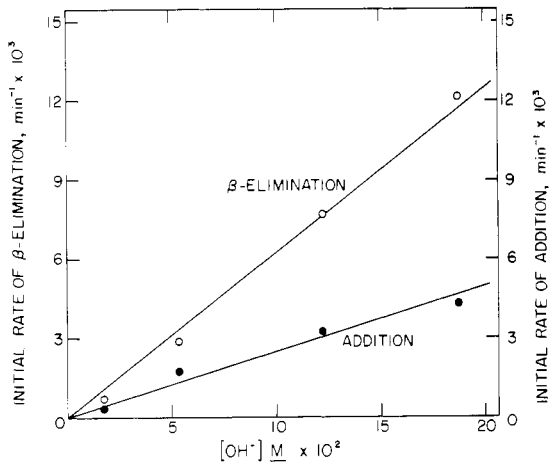


Figure 3. Effect of hydroxide ion concentration on initial rate of β elimination and rate of addition. Reactions were carried out as described in Figure 2 and the slopes of the lines in Figure 2 plotted on the y axis. The initial rate of β elimination is expressed as [dehydroalanine]/([phosphoserine]$_0$min). The rate of addition is expressed as [dehydroalanine]/([dehydroalanine]$_{max}$min).

The time to obtain a maximum change in absorbance at 241 nm (60 min in Figure 1) was independent of phosvitin concentration over the range of 1.04×10^{-6} to 1.11×10^{-5} M used in these studies but was dependent on hydroxide ion concentration, temperature, and ionic strength (see below).

Effect of Phosvitin and Hydroxide Ion Concentrations. The initial rate of increase (β elimination) and rate of decrease (addition) in absorbance at 241 nm were directly dependent on phosvitin concentration over the range of 1.04×10^{-6} to 1.11×10^{-5} M phosvitin as well as being dependent on the hydroxide ion concentration (Figure 2). As shown in Figure 3, there was a linear dependence of both the initial rate of β elimination and the rate of addition reactions on the hydroxide ion concentration. Therefore, the rates can be expressed as molar increases (or decreases) in dehydroalanine per minute per molar concentration of phosvitin per molar concentration of hydroxide ion (rate = Δ[dehydroalanine]/[phosvitin][OH^-]min) with units of M^{-1} min^{-1}). For β elimination

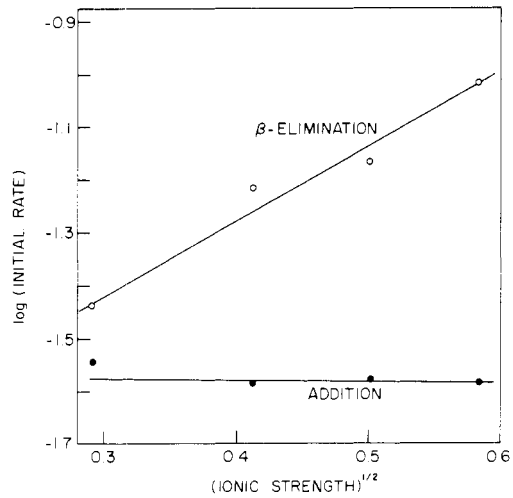


Figure 4. Effect of ionic strength on initial rate of β elimination and rate of addition. The reactions were carried out at 60 °C in KCl–NaOH buffer in which the ionic strength was adjusted with KCl. Reactions were performed in which hydroxide ion concentration was held constant as well as others in which it varied. Each initial rate is the average of at least three determinations done at 1.08×10^{-5} M phosvitin. Initial rate of β elimination is [dehydroalanine]/([phosphoserine]$_0$[OH$^-$]min). Rate of addition is [dehydroalanine]/([dehydroalanine]$_{max}$[OH$^-$]min).

the phosvitin concentration was expressed in terms of phosphoserine concentration (119 [phosvitin]), while for the addition reaction the phosvitin concentration was expressed in terms of the maximum concentration of dehydroalanine formed under a particular condition, [dehydroalanine]$_{max}$. From the data in Figure 3, the initial rate of β elimination and rate of addition at 60 °C were 6.25×10^{-2} and 2.48×10^{-2} M^{-1} min^{-1}, respectively.

Effect of Ionic Strength. We selected the KCl–NaOH buffers of Bates and Bower (1956) as a means of maintaining the hydroxide ion concentration and ionic strength constant during a reaction, as well as knowing the pH of the reaction. The effects of temperature and dilution on the pH of these buffers are well known. Unfortunately, the ionic strength of these buffers is not constant. As shown in Figure 4, only the initial rate of β elimination is dependent on ionic strength. All the data reported in this paper have been corrected to a constant ionic strength of 0.170 by use of the data in Figure 4.

The data in Figure 4 are plotted according to the Debye–Huckel equation:

$$\log k = \log k_0 + 1.02 Z_A Z_B \mu^{1/2} \quad (1)$$

where k and k_0 are the second-order rate constants at ionic strength (μ) and zero, respectively, and Z_A and Z_B are the charges on the reactants. While the Debye–Huckel equation is not strictly obeyed above ionic strengths of 0.01 (Frost and Pearson, 1965), its use gives a method of correcting for ionic strength variations among experiments and approximate values for determining effective charge on the reacting species. The reactants in β elimination are the hydroxide ion where Z_A is −1.0 and the phosphoserine residue. The slope of the line in Figure 4 for β elimination is 1.36, indicating that the effective charge on the phosphoserine residue is −1.36. Bohak and Katchalski (1975) obtained a Z_B value of −0.8 under somewhat different conditions (no KCl present). Since Z_B is lower than the expected value of −2 for the phosphoserine residue at pH 12, a part of the negative charge of the phosphate group may be masked by formation of ion pairs with the Na^+ and K^+ in the reaction.

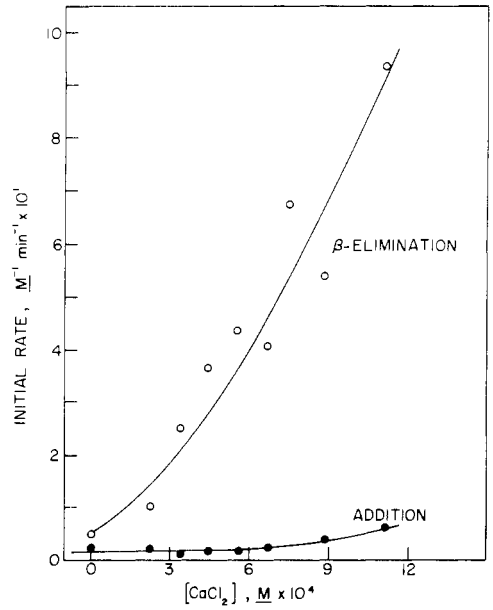


Figure 5. Effect of $CaCl_2$ concentration on the initial rate of β elimination and rate of addition. The reactions were performed at 60 °C in 0.123 N NaOH at 5.54×10^{-6} M phosvitin. Rates are expressed as described in Figure 4.

Table II. Thermodynamic Activation Parameters for the β Elimination and Addition Reactions of Phosvitin[a]

Reaction	E_a, kcal/mol	$\Delta H^{\ddagger}$, kcal/mol	$\Delta F^{\ddagger}$[b] kcal/mol	$\Delta S^{\ddagger}$[b] cal mol^{-1} deg^{-1} (esu)
β Elimination	20.2	19.5	24.1	−13.8
with $CaCl_2$[c]	20.8	20.1	22.5	−7.21
Addition	25.0	24.3	24.6	−0.90
with $CaCl_2$[c]	27.6	26.9	24.2	+8.11

[a] Rate data corrected to constant ionic strength of 0.170. [b] At 60.0 °C. [c] 7.47×10^{-4} M $CaCl_2$.

Z_B for the addition reaction is zero. Since the addition reaction involves reaction of the ethylene bond of dehydroalanine with the uncharged ϵ-amino group of lysine, it would be predicted there should be no effect of ionic strength on the reaction at pH 12.

Effect of Calcium Chloride. As shown above, the initial rate of the β-elimination reaction is dependent on the ionic strength of the reaction reflecting the reaction between the negatively charged hydroxide ion and negatively charged phosphoserine residue. Neutralization of the negative charges on the phosphoserine residue, by complexation with a divalent cation for example, should increase the rate of β elimination. This expectation is borne out by the data in Figure 5. The addition of 1.12×10^{-3} M $CaCl_2$ to the reaction in 0.123 N NaOH at 60 °C led to a 20-fold increase in the initial rate of β elimination, while there was little effect on the rate of the addition reaction. There was a small effect of $CaCl_2$ above 6.72×10^{-4} M on the rate of the addition reaction.

Effect of Temperature. The effect of temperature on the initial rate of β elimination and rate of addition of phosvitin in the absence and presence of $CaCl_2$ are shown in Figures 6 and 7, respectively. The effect of temperature on the two reactions are similar. The thermodynamic activation parameters are shown in Table II. E_a was found to be 20.2 and 25.0 kcal/mol for the β-elimination and addition reactions, respectively, in the absence of $CaCl_2$. In the presence of $CaCl_2$, E_a was 20.8 and 27.6 kcal/mol for the β-elimination and addition reactions, respectively. In the presence of $CaCl_2$, the increase in initial rate of β

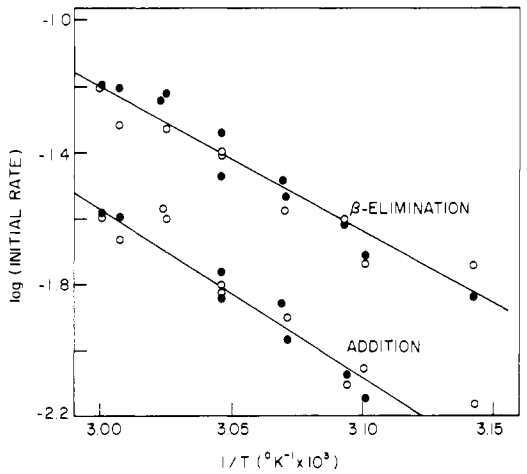


Figure 6. Effect of temperature on the initial rate of β elimination and rate of addition. The reactions were performed in KCl–NaOH buffers at several different concentrations of phosvitin. All reaction rates were corrected to an ionic strength of 0.170. (○) hydroxide ion concentration constant at 0.123 N at all temperatures; (●) hydroxide ion concentration was different at different temperatures. Rates are expressed as described in Figure 4.

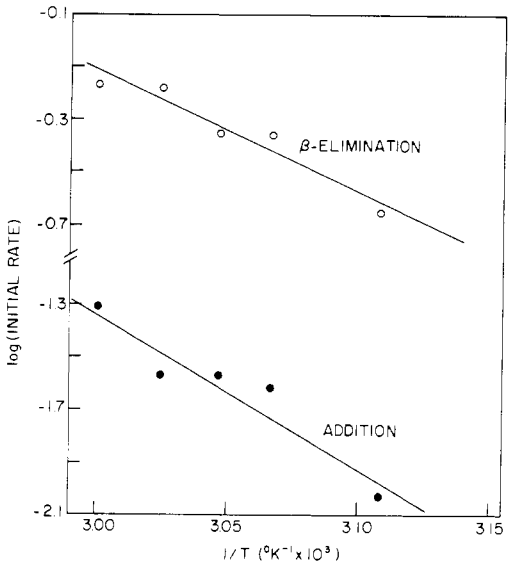


Figure 7. Effect of temperature on the initial rate of β elimination and rate of addition in the presence of 7.47×10^{-4} M $CaCl_2$. The reactions were performed in 0.123 N NaOH at a phosvitin concentration of 5.54×10^{-6} M. Rates are expressed as described in Figure 4.

elimination is primarily the result of an increase in $\Delta S^{\ddagger}$ as might be predicted from the expected complexation of Ca^{2+} with the negatively charged phosphoserine residue. In the presence of 7.74×10^{-4} M $CaCl_2$ $\Delta S^{\ddagger}$ for both the β-elimination and addition reactions increased by 7 to 8 entropy units. This marked effect on $\Delta S^{\ddagger}$ would indicate that $CaCl_2$ causes a large conformational change in both native and partially β-eliminated phosvitin. Grizzuti and Perlmann (1973, 1975) have shown that phosvitin undergoes a conformational change in the presence of divalent cations. Under the conditions used here, the phosvitin would be about 74% complexed with Ca^{2+} since the association constant, K_a, was reported to be 3.6×10^3 M^{-1} (Gruzzuti and Perlmann, 1975).

Maximum Observed Formation of Dehydroalanine. The maximum amount of dehydroalanine formed in a reaction will be determined by the relative rates of the β elimination and addition reactions (Figure 1). Over the concentration range of 1.04×10^{-6} to 1.11×10^{-5} M phosvitin, the maximum observed formation of dehydroalanine was independent of phosvitin concentration and temperature. On the other hand, the maximum observed formation of dehydroalanine was dependent on pH and ionic strength since these experimental parameters affect the initial rate of β elimination more than the rate of the addition reaction. Data illustrating these effects are shown in Table III. Less dehydroalanine accumulates at lower pH and lower ionic strength as might be expected from the data of Figures 3 and 4.

Table III. Effect of Hydroxide Ion Concentration, Temperature, Ionic Strength, and $CaCl_2$ on the Maximum Observed Dehydroalanine Formation[a]

Temperature, °C	(OH^-) M x 10^2	Ionic strength	Dehydroalanine, mol/mol of phosvitin
60.0	18.7	0.255	53.8
60.0	12.3	0.170	49.0
60.0	5.41	0.103	34.3
60.0	1.74	0.064	26.4
57.5	10.3	0.150	49.0
55.0	8.34	0.130	42.4
52.5	6.93	0.116	46.2
50.0	5.53	0.102	42.9
45.0	3.81	0.085	36.0
57.5	12.3	0.123	49.9
50.0	12.3	0.123	48.6
60.0	12.3	0.123	67.0[b]

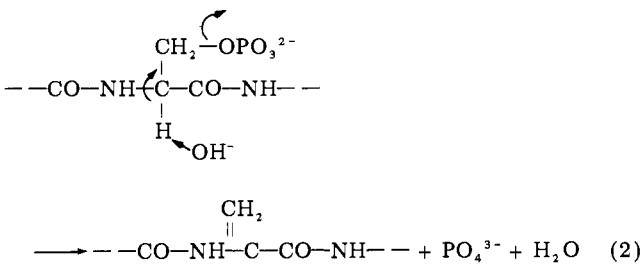
[a] Each value is the average of triplicate determinations on three to five different concentrations of phosvitin ranging from 1.04×10^{-6} to 1.12×10^{-5} M. [b] In presence of 1.12×10^{-3} M $CaCl_2$.

DISCUSSION

In alkaline solution, phosphate groups may be removed from *O*-phosphoserine-containing proteins by hydrolysis and by β elimination. Under the conditions used in this work, the majority, >85%, of the phosphate groups were removed by β elimination. The rate of β elimination can be followed by measuring the amount of serine left at various times by amino acid analyses or by the increase in absorbance at 241 nm due to formation of dehydroalanine (aminoacrylic acid) residues. Addition of nucleophiles to dehydroalanine may interfere with the latter method depending on the relative rates of the β-elimination and addition reactions.

β-Elimination of the phosphate group O–phosphoserine-containing proteins has been known for a long time (Plimmer and Scott, 1908) and is used as a diagnostic tool to distinguish O–P bonds from N–P bonds in phosphoproteins (Taborsky, 1974).

The mechanism of β elimination of the phosphate group in alkaline solution is thought to be that (Anderson and Kelley, 1959) shown in eq 2. The initial step is the ex-



(2)

traction of the α hydrogen from the amino acid residue by an hydroxide ion of the solvent. Rearrangement of a transient carbanion could lead to expulsion of the phos-

phate group and the formation of a dehydroalanine residue. The initial α-hydrogen extraction step is common for racemization of amino acid residues as well as for β elimination from cystine, cysteine, *O*-phosphoserine, *O*-glycosylserine, *O*-phosphothreonine, and *O*-glycosylthreonine residues. The carbanion mechanism is supported by this work since ionic strength and $CaCl_2$ affected the β-elimination reaction in the predicted fashion.

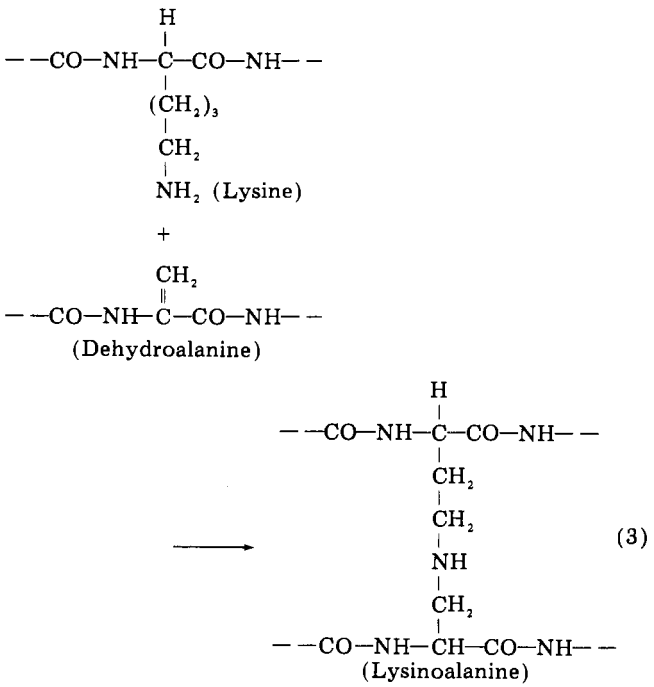
As shown in the present work, the rate of β elimination of phosphoserine residues in phosvitin is directly proportional to the hydroxide ion concentration, suggesting that the rate-determining step is the hydroxide ion extraction of the α hydrogen. This is likely to be the case with other types of compounds also. For example, Nashef et al. (1977) found the activation energy for the β elimination of cystine residues in lysozyme to be 24 kcal/mol, in agreement with values reported here. Donovan and White (1971) obtained an activation energy of 19 kcal/mol for the alkaline breakage of disulfide bonds in ovomucoid. They suggested the disulfide bonds were broken by hydrolysis, which does not appear to be supported by more recent data (Nashef et al., 1977). Gawron and Odstrchel (1967) reported the activation energy for β elimination of *N,N'*-dicarbobenzyloxy-L-cystinylglycine to be 21 kcal/mol. The activation energy for β elimination of *O*-glycosylthreonine residues in antifreeze protein has been found to be 10.3 kcal/mol (Lee et al., 1977).

In phosvitin, the rate of β elimination is accelerated by increase in hydroxide ion concentration, temperature, ionic strength, and Ca^{2+}. The marked enhancement in rate by Ca^{2+} is probably due to complexing of Ca^{2+} with the negatively charged phosphate group of the phosphoserine residue thus facilitating attack of the hydroxide ion on the α hydrogen of the phosphoserine residue. This is supported by the effect of Ca^{2+} on increasing the $\Delta S^{\ddagger}$ of the β-elimination reaction and effect of increased ionic strength in accelerating the rate.

The dehydroalanine residues formed by β elimination of phosphoserine residues are quite reactive and undergo Michael-type addition with various nucleophiles. Under the conditions used in our studies and measured as shown in Figure 1, addition to dehydroalanine residues occurs at about one-sixth the rate of the β-elimination reaction at 60 °C and in 0.123 N NaOH (Table I). The major addition product identified was lysinoalanine based on its similar chromatographic properties with an authentic sample of lysinoalanine. At the concentrations of phosvitin 1.04×10^{-6} to 1.11×10^{-5} M, addition of the ϵ-amino group of lysine to dehydroalanine was an intramolecular reaction as shown by the lack of dependence of the rate on phosvitin concentration and the failure to detect polymers of β-eliminated phosvitin by gel filtration. Formation of lysinoalanine in β-eliminated phosvitin may be facilitated in some cases by proximity of lysine and phosphoserine residues in the primary sequence of phosvitin (Belitz, 1965).

Reaction of the ϵ-amino group of a lysine residue with a dehydroalanine residue is shown in eq 3.

Formation of lysinoalanine (as well as ornithinoalanine and lanthionine) residues in proteins would be expected to change the physical and chemical properties of a protein (as is done in texturizing) as well as its digestibility with proteolytic enzymes, particularly trypsin. There are no known enzymes which can cleave the secondary amine linkages of lysinoalanine and ornithinoalanine. Thus, the β-elimination and addition reactions in alkaline solution result in loss of serine and lysine thereby affecting the nutritive quality of the protein. The toxicological properties of lysinoalanine residues in proteins determined by in vivo studies are not clear. Several workers have reported that rats fed severely alkali-treated soy protein showed renal lesions and nephrocalcinosis and that the treated proteins generally had reduced digestibility and nutritive value (DeGroot and Slump, 1969; Woodard and Short, 1973; Van Beek et al., 1974). With semipurified diets, Woodard and Short (1973) showed that feeding free lysinoalanine resulted in renal lesions, although DeGroot et al. (1976) were unable to show this to be the case with protein-bound lysinoalanine.



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Modification of the Physical Properties of Soy Protein Isolate by Acetylation

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The predictions of previously developed models for the hydration, gelation, and solubility of soy protein isolate regarding the effect of a chemical modification (acetylation) of the protein have been borne out by experiment. Acetylation decreased water binding, increased solubility in the pH 4.5–7 range, and decreased gel strength of soy protein isolate. A shift in molecular weight distribution of the protein to lower molecular weight species also occurred. This work shows that the functional properties (in a food use context) of soy proteins can be altered by chemical modification and that the effect of a particular chemical modification of these properties can be predicted.

In the fabrication of high protein foods, increasing use is made of soybean-derived material. It is possible to extract from soybeans the bulk of the intracellular protein in a form that contains no less than 90% protein. These protein isolates are of interest not only for their nutritional value, but because they possess physical properties that lend themselves to use in the fabrication of a variety of foods. Of particular interest is their ability, when dispersed in highly concentrated aqueous solutions (e.g., 20% protein), to form thermally irreversible, elastic gels upon heating (Wu and Inglett, 1974; Circle et al., 1964).

In addition to the ability of the protein to form gels, the water-binding ability of soy proteins is also of interest. It is well known that microbial growth in a food is dependent upon water activity (a_w) (Scott, 1957; Labuza et al., 1970; Troller, 1973). Binding of water to the protein and carbohydrate constituents of a food results in decreased a_w. Therefore, changes in water binding by food proteins could result in changes in microbial growth, depending upon the total water content of the food. These changes would be most apparent in the so-called intermediate moisture content regions ($0.6 \lesssim a_w \lesssim 0.9$).

Also of importance for the use of soy protein in foods is the solubility behavior as a function of pH (Wolf and Cowan, 1971). The solubility of typical soy protein isolates goes through a minimum at pH ~4.5 (Wolf and Cowan, 1971). This fact is useful in the preparation of most soy isolates, but also may restrict usefulness in many food applications. In applications where solubility is important it would be advantageous to be able to control solubility as a function of pH (e.g., increase solubility in the pH ~4.5–7 range without resorting to protein hydrolysis).

The purpose of the work presented here was to carry out a simple chemical modification of soy protein isolate and measure several properties of the modified protein relevant to its food use (e.g., gel behavior, solubility, water binding). Chemical modification of proteins has been used extensively for the purpose of determining functional groups of enzymes and the mode of action of drugs (Shaw, 1970). However, studies of the effect of chemical modification of proteins on their functional properties in a food use context have been more sparse. Acetylation was chosen as the chemical modification because of its specificity for free amino groups, principally lysine (Riordan and Vallee, 1971). Esterification of free amino groups with the neutral acetyl group will result in a reduction in the positive charge

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