PHOSPHATE-PROTEIN RELATIONSHIPS OF CERTAIN PHOSPHOPROTEINS

by

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INTRODUCTION

Egg yolk proteins have been studied extensively, particularly the phosphoprotein fraction called phosvitin. Concurrently research has been carried out on blood serum proteins with some emphasis on those phosphoproteins that appear in the serum of laying hens or estrogenized birds. Large increases in protein bound phosphorus are noted in these sera. Attempts have been made to correlate this phosphoprotein fraction with yolk formation. Several researchers have come to the conclusion that the phosphoprotein fractions of both egg yolk and estrogenized chicken blood serum have a common source and in fact are the same phosphoprotein.

The purpose of this study is to investigate two phosphoprotein components, one from egg yolk and one from serum of diethylstilbestrol treated cockerels and to examine their protein-phosphorus linkages by the enzyme phosphatase.

REVIEW OF LITERATURE

The role of phosphorus in protein structure has been the subject of speculation for many years. Much of the work has been done on egg yolk and milk proteins, but in recent years the phosphorus containing proteins of the blood serum have been studied. All of this work has been handicapped by the difficulty of preparing samples pure enough for the determination of chemical and physical properties. Many of the published results have been determined on samples which have been proven subsequently to consist of more than one component.

Much of the work concerning the phosphorus containing proteins found in egg yolk and blood serum has recently been reviewed and will not be repeated here. DeGuzman (5) has summarized the work leading to separation of phosphoproteins from blood serum, and the development of the fractionation and properties of egg yolk proteins has been discussed in detail by Jambunathan (11).

Since many methods have been employed to fractionate these proteins and since the results depend on the methods used, it is difficult to make comparisons between our work and the data others have obtained. Hence the subsequent literature discussion will dwell on what has been carried out on our preparations, and comparison with other investigations will be reserved for the discussion.

Much of the work in our laboratories over the past 10-15 years has been concerned with the preparation of phosphoprotein components of high purity. In this regard Miller (18), Simlot (23), and Jambunathan (11) have been concerned with the purification of a phosphoprotein from egg yolk. Using $MgSO_4$ salt fractionation Miller (18) was able to precipitate a high phosphorus fraction from egg yolk. His preparation contained about 80% of the total

yolk phosphorus and included the fast moving high phosphorus electrophoretic fraction. Simlot (23) isolated an electrophoretically homogeneous phosphoprotein from egg yolk by means of ultracentrifugation and dextran-sulfate precipitation. The dextran-sulfate precipitated the lipoproteins leaving the phosphoprotein in solution. Simlot's phosphoprotein contained 4.2% phosphorus. He also did some preliminary separation of the dextran-sulfate precipitated fraction. Jambunathan (11) also used the ultracentrifuge to fractionate egg yolk. However, rather than dextran-sulfate, he employed ion exchange chromatography on a DEAE cellulose column to prepare his final samples. He eluted three protein fractions. The high phosphorus containing component had 5.4% phosphorus.

Concurrently others have been purifying phosphoproteins from the blood sera of diethylstilbestrol (DESB) treated cockerels, laying hens, and cholesterol fed birds. Clegg and Hein (3) observed that the fast moving electrophoretic Component I of such blood sera exhibited very high phosphorus activity. Ericson (6) evaluated the lipid and phosphorus contents of serum samples before and after ether extraction and decided that Component I may be a phospho-lipo-protein. Misra (19) discovered that Component I precipitated when blood sera of laying hens or DESB treated cockerels were dialyzed overnight against 0.005M sodium phosphate buffer pH 7.0. He then combined the use of the ultracentrifuge with the above technique and further dialysis against salt solutions to separate a precipitate containing Component I. Chen (2) isolated an electrophoretically pure Component I from DESB injected roosters sera. After ultracentrifugation at 40,000 rpm she used a combined technique of moving boundary electrophoresis, salt fractionation and preparative electrophoresis. Wu (25) pursued the preparation of this compound by subjecting the bottom fraction obtained from the ultracentrifuge to density gradient

separation on sucrose and subsequent passage through a DEAE cellulose column. She also indicated that Component I was probably not a lipoprotein. DeGuzman (5) used Wu's technique to prepare Component I and then performed compositional analysis as well as preliminary studies of its phosphorus linkages.

This study will employ enzymatic agents to examine the phosphorus relationships of both Component I of blood serum and the egg yolk phosphoprotein obtained by Jambunathan and will attempt to compare the two preparations.

METHODS AND MATERIALS

Preparation of the High Phosphorus Egg Yolk Component

Preparation of this component was done according to Jambunathan (11). The yolks of fresh eggs were separated and cleaned of the white and chalazae. The yolk membrane was punctured and the contents collected free of the membrane. The yolk was centrifuged in capped tubes at 30,000 rpm for 16 hours in a number 30 rotor at 0°C in a Spinco Model L ultracentrifuge (max. g's = 105,500). The surface of the bottom solid yellow zone was washed with a strong stream of distilled water; and the zone was further fractionated, as follows, according to Simlot (23). This ultracentrifuge fraction was dissolved in 10% NaCl (55 g./170 ml.) and centrifuged at 40,000 rpm in a Spinco rotor number 40 for 16½ hours at 0°C (max. g's = 144,700). The viscous yellow bottom layer was collected and was dialyzed against 0.067M bicarbonate buffer pH 9.8, then against 0.067M bicarbonate buffer pH 8.85 containing 0.1N NaCl and applied to a DEAE cellulose column. Stepwise pH elution was carried out and the fraction, eluted by the bicarbonate buffer of pH 9.8 containing 0.1M NaCl, was collected and lyophilized. The resulting high phosphorus precipitate was employed in subsequent experiments.

Preparation of High Phosphorus Component I from Blood Serum

This component was prepared according to the methods of DeGuzman (5). White Rock and White Leghorn cockerels were injected subcutaneously with 7.5 mg. of diethylstilbestrol dissolved in propylene glycol daily for seven days and were sacrificed on the eighth day. The blood was collected and the blood serum was prepared. The serum was then centrifuged in a number 40 Spinco rotor at 38,000 rpm for 16 hours at 0°C (max. g's = 127,200). The

resulting bottom fraction from three tubes was dissolved in approximately 23 ml. of borate chloride buffer[a] pH 8.6 and clarified by mild centrifugation. Five ml. of the clear supernatant was layered on top of a sucrose gradient consisting of 5 ml. portions each of 10%, 20%, 30%, 40%, and 50% sucrose (w/v) dissolved in the borate chloride buffer. The resulting gradient tubes were centrifuged in a "25-D", swinging bucket rotor for 16 hours at 15,000 rpm (max. g's = 32,000). After centrifugation, the pale yellow, semi-solid precipitate found in the bottom of the tube was dissolved in borate chloride buffer pH 8.6 and clarified by low speed centrifugation. The clarified supernatant was transferred to dialysis sacs and concentrated overnight at 5°C by means of Biodryex. The concentrated solution was dialyzed against distilled water for 24 hours and then lyophilized. The precipitate obtained was the Component I described by DeGuzman.

Total Phosphorus Determination

Total phosphorus was determined by the method of Fiske and Subbarow as described by Oser (21). Lyophilized samples were weighed into 30 ml. Kjeldahl flasks. Since the lyophilized samples were difficult to weigh, it was necessary to employ enough sample such that 1 ml. aliquots, taken after the final digestion mixtures were diluted to 5 ml., contained about 1 mg. of the egg yolk component or 2 mg. of serum Component I. Digestion and color development were carried out according to the standard procedure except for a modification of the sulfonic acid solution. In order to keep the sulfonic acid solution from precipitating out after a short time (several days) of standing, Jambunathan* proposed the following method for making up that reagent

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[a]This buffer consisted of 0.05M boric acid, 0.05M KCl, and 0.012M NaOH.
*Personal communication.

using sodium metabisulfite rather than sodium bisulfite:

Aminonaphtholsulfonic acid (0.25 gm) is added to 100 ml. of freshly prepared 15% sodium metabisulfite and mixed to form a suspension. Then 0.5 g. of anhydrous sodium sulfite is added and mixed with a magnetic stirrer. An additional amount of 20% sodium sulfite (2 ml.) is added until the sulfonic acid dissolves. The solution is filtered and stored in a brown bottle. The percentage of total phosphorus was calculated as follows:

$$\% \text{ Total Phosphorus} = \frac{\text{mg. standard X O.D. sample X 100}}{\text{O.D. standard X mg. sample}}$$

Dephosphorylation Experiments

Acid phosphatase procedure: This procedure is based on DeGuzman's (5) modifications of the procedure of Kalan and Telka (14). The samples were weighed so that aliquots of the reaction mixture (3-5 ml.) would contain either about 2 mg. of Component I or about 1 mg. of the yolk component. Some samples were suspended in 0.1M acetate buffer at pH 5.0, dissolved by addition of 1N NaOH, and the pH adjusted to between 5 and 6 with 0.1N HCl. Other samples were simply suspended in the acetate buffer. One tenth ml. of 0.1M $MgCl_2$ was added to each reaction mixture. Acid phosphatase from potatoes dissolved in the same acetate buffer in the ratio of 0.5-1.0 mg/ml. wad added in 1 ml. portions. In the beginning the incubations were carried out in covered test tubes, but in the latter experiments 50 ml. Erlenmeyer flasks were employed. In both cases the incubation was carried out at 37°C in a shaker.

Dephosphorylation with alkali (5): Samples were dissolved in 0.25N NaOH and incubated under toluene at 37°C in flasks placed in a shaker. Aliquots were taken at intervals to determine the phosphate liberated.

Determination of Phosphate Liberation: The amount of phosphate liberated

was detected by a modification of Fiske and Subbarow as developed by Kalan and Telka (14). A 1.0 ml. or a 2.0 ml. aliquot of the reaction mixture was added to 0.9 ml. of 20% trichloroacetic acid plus 0.8 ml. of 10N sulfuric acid kept in ice. Then 0.1 ml. of 10% sodium tungstate • $2H_2O$ was added and the precipitate centrifuged at 0°C at 5,000 rpm. The supernatant was pipetted in the amount of 2 ml. or 3 ml., depending on the volume of the reaction mixture originally taken, into 10 ml. volumetric flasks, and treated as follows for color development according to Fiske and Subbarow (7). To each flask 1 ml. of 2.5% ammonium molybdate and 0.4 ml. of aminonaphtholsulfonic acid reagent (prepared according to Jambunathan's modification) were added. This mixture was diluted to the mark with water and the color was read at 660 mμ using a red filter on a Bausch and Lomb Spectronic 20. A standard solution treated in the same fashion was also run as well as a blank. Calculations were performed as under total phosphorus determination.

Use of Proteolytic Enzymes

For Phosphate Determination

Trypsin procedure*: Samples suspended in 0.1M ammonium bicarbonate at pH 7.75 were treated with 5 mg. of trypsin dissolved in the same solution. The capped containers were incubated at 37°C for 24 hours at which time aliquots were taken for phosphate analysis and subsequently the pH of the remainder was adjusted to between 5 and 6 and acid phosphatase was added as previously described.

Pronase procedure: Samples were treated with this proteolytic enzyme using a modification of the procedure of Nomoto et al. (20). A 0.03M

* DeGuzman, unpublished data.

ammonium bicarbonate buffer (pH 7.4) rather than the suggested phosphate buffer was employed since we wished to determine phosphate release. The reaction mixture contained 3-5% ethanol as a preservative, 1 mg. of the enzyme, and incubation was carried out at 37°C for about 45 hours. Aliquots were taken and the remaining solution was adjusted to pH 5 to 6 before addition of the phosphatase as above.

For Amino Acid Analysis

Papain procedure: Papain hydrolysis procedure was based on the method of Johnston (12). About 15 mg. of the egg yolk component in 5 ml. of 0.05M citrate buffer at pH 5 was reacted with about 1 mg. of papain in the same buffer and incubated at 37°C for about 15 hours. At the end of this time, aliquots of 1 ml. were taken to determine the phosphate released. The rest of the enzyme mixture was treated according to Block et al. (1) as follows: 4 ml. of the reaction mixture were placed in a centrifuge tube and 2 ml. of 20% sulfosalicylic acid and 2 ml. of water were added. The resulting mixture was centrifuged and the supernatant was filtered through a sintered glass funnel. An enzyme blank (no sample) was also treated the same way. An aliquot of each of these was used to determine the free amino acids present employing a Beckman Model 120 C Amino Acid Analyzer using 0.3N lithium citrate buffers of pH 2.8 and pH 4.16.

Pronase procedure: Pronase hydrolysis was used following the method of Nomoto et.al. (20). The egg yolk component was treated as described under use of proteolytic enzymes for phosphate determination but this time using 0.03M sodium phosphate buffer at pH 7.4. The incubation was carried out for about 45 hours. At the end of this time no phosphate analysis was done but the reaction mixture was treated with sulfosalicylic acid as above and an amino

acid analysis performed.

Progressive Dephosphorylation with Acid Phosphatase

Three flasks of each component were treated with acid phosphatase as described before and allowed to begin incubation. After $1\frac{1}{2}$ hours, one flask of each was stopped by placing the necessary aliquot in the TCA-H_2SO_4 mixture as previously described. To the other two flasks of each component another 1 ml. portion of the phosphatase enzyme was added and incubation was continued. After 5 hours of incubation one of each set of flasks was stopped and to the other a third portion of enzyme was added and incubation was continued as usual until about 24 hours had passed. Free phosphate determination was then run on aliquots of the six samples and calculations were again performed as under total phosphate determination.

RESULTS AND DISCUSSION

The reason for this investigation was twofold: to extend our knowledge of the type of phosphorus-protein linkage present and to compare two phosphoproteins, one of which was isolated from blood serum and the other from egg yolk. Both of these proteins are relatively high in phosphorus. Component I, which is the high phosphorus-containing protein present in the blood sera of diethylstilbestrol (DESB) treated cockerels (6) has been shown to contain 2.13% phosphorus on a dry, ash free basis (5). The protein isolated from egg yolk was shown by Jambunathan to have a phosphorus content of 5.4% (7.47% on a dry, ash free basis) (11). Simlot employing a different method of preparation indicated a phosphorus content of 4.2% (23). It should be emphasized at this point that various investigators have obtained phosphorus percentages of the egg yolk fraction depending on the method of preparation. For example, the high phosphorus egg yolk component prepared recently in this laboratory contains approximately 4.8% phosphorus. Other laboratories using other methods of preparation obtain 9-10% phosphorus. Since the sample prepared for this investigation has been intensively purified by several techniques, the chances of phospholipid and other phosphorus-containing impurities have been minimized. Thus our egg yolk preparation may contain less phosphorus than reported by other preparative methods.

Various investigations have indicated that the high phosphorus-containing protein of egg yolk and the high phosphorus-containing protein of estrogenized chicken blood serum have the same origin and are in fact the same protein. Scheide and Urist (22) link a serum phosphoprotein from such birds with an egg yolk phosphoprotein on the basis of phosphorus percentage and amino acid composition. Heald and McLachlan (10) conducted a similar study and arrived

at the same conclusions. The work done by Greengard et al. (9) lent further credence to the theory that the phosphoprotein produced in the sera of DESB treated cockerels was identical to the high phosphorus component of egg yolk or phosvitin. Many of these comparisons were carried out with relatively impure samples and therefore there is ample reason to compare the two proteins isolated for this investigation, both of which are the high phosphorus-containing components from their source.

Jambunathan ran an amino acid analysis of the egg yolk phosphoprotein and DeGuzman conducted a similar analysis of the blood serum protein. The egg yolk sample contained nearly 23% by weight of serine while the blood serum sample contained less than 9%. A quick survey of the analyses shows that in general the egg yolk component contained smaller amounts of the neutral amino acids but greater amounts of the basic ones than the blood serum component. There appears to be little basis for comparison of the two proteins with regard to amino acid analysis.

On the basis of phosphorus percentage, the two proteins are quite different also. The egg yolk phosphoprotein has 4.84% phosphorus which is significantly more than the blood serum phosphoprotein which is only 1.86% phosphorus. Both of these values are reported for the air-equilibrated samples. No corrections have been made for moisture or ash content.

DeGuzman (5) ran some preliminary studies on Component I to investigate how the phosphorus may be bound to that protein. She used a variety of enzymes to dephosphorylate the protein but was unable to hydrolyze off more than 45% of the total phosphorus with any procedure. These experiments enlarge on her work and do so by concentrating on the use of acid phosphatase.

Hydrolysis with alkali is a method for determining the presence of ortho-phosphate links and this was run first. DeGuzman (5) using 0.25N NaOH as

described in the section on methods and materials to hydrolyze Component I, was able to liberate only 48% of the phosphorus in the sample. An identical experiment on the egg yolk sample released approximately 90% of its phosphorus. Figure I illustrates a representative run on both proteins. While the egg yolk component has most of its phosphate in the ortho form, Component I either has a large part of it in some other form or else the protein is structured so that a large part of the phosphorus is blocked from contact with the alkali. DeGuzman (5) ran other experiments using acid and other enzymatic hydrolyses and had eliminated the possibilities of P-P and N-P linkages.

In the next series enzymatic hydrolysis using acid phosphatase from potato was employed. The egg yolk protein generally liberated about 70% of its total phosphorus but the blood serum component released only about 50%. DeGuzman (5) had been able to release only 35% but in our case a much greater enzyme-substrate ratio produced a release of more of the phosphorus. It was assumed as above that a possible reason for this incomplete hydrolysis is that part of the phosphoryl groups might be inaccessible to the enzyme for steric reasons.

In order to compensate for the possible steric effects of the protein structure, these proteins were digested with digestive enzymes before adding the acid phosphatase. In this way the proteins would be broken up and hopefully more of the phosphorus would then be accessible to the phosphatase.

Trypsin was the first proteolytic enzyme used. Results appear on Table I. As described in Methods and Materials the trypsin experiments were run in ammonium bicarbonate buffer. After predigestion of the egg yolk component with trypsin and reaction with phosphatase about 79% of the phosphorus was released as opposed to the 70% released without reacting with trypsin. Component I revealed a significant increase in phosphorus release from 55%

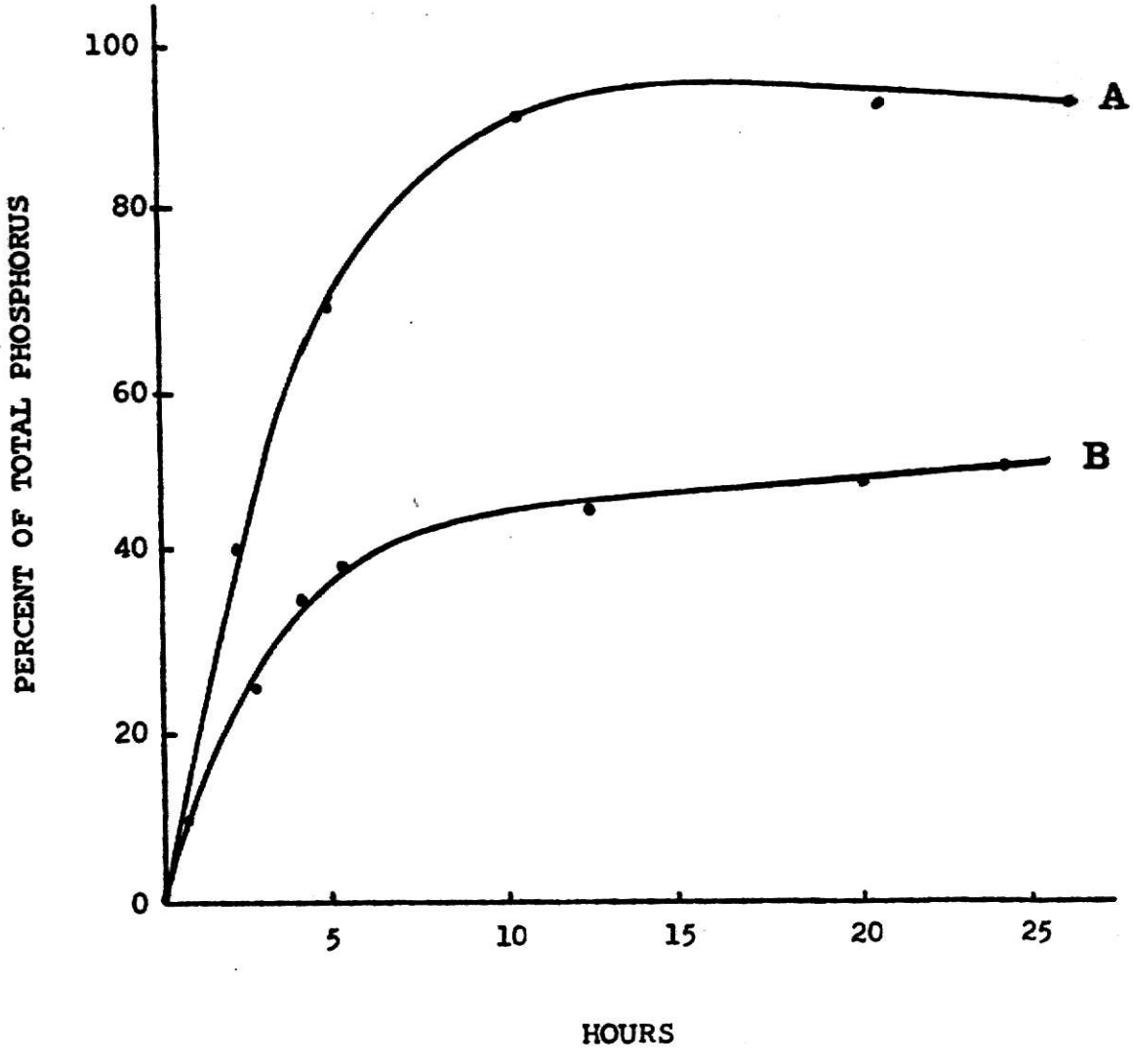

Fig. 1. Dephosphorylation of the two components with alkali.
The samples were dissolved in 0.25N sodium hydroxide and incubated at 37°C.
A. Egg Yolk component.
B. Component I.

TABLE I

% Total Phosphorus Released with and without predigestion with Trypsin[a]

	Egg Yolk Component[b]	Component I[b]
Buffer	3% ± 1%	11% ± 1%
Buffer plus trypsin	3% ± 1%	11% ± 1%
Phosphatase	70% ± 5%	55% ± 2%
Trypsin plus phosphatase	79% ± 5%	77% ± 1%

[a] Average of 2-4 runs.

[b] These percentages have been rounded off to the nearest whole number.

without the trypsin to 77% with the predigestion. A release of 11% of the total phosphorus was also noted in the presence of the ammonium bicarbonate buffer alone or the buffer plus trypsin.

Since trypsin is an enzyme for specific links, it does not cleave the protein into many small pieces. Therefore pronase, a non-specific proteolytic enzyme, which can hydrolyze a protein fairly completely to amino acids was employed. Results are shown in Table II. It appeared that the combination of the pronase plus phosphatase yielded about 65% of the phosphorus of the egg yolk component which is about the same as without the pronase.

Component I released about 61% of its phosphorus with the combination of enzymes, only 8% more than with phosphatase alone. The most interesting aspect of this experiment was the apparent ability of the pronase enzyme itself to liberate phosphorus. The egg yolk protein released 18% of its total phosphorus in the presence of pronase alone in the buffer, while Component I from blood serum liberated 53% of its phosphorus.

In order to form a better picture of what products are being formed on incubation with digestive enzymes, the remaining protein was precipitated with sulfosalicylic acid and the amino acid analyses of the supernatants were determined. Only the egg yolk sample was used in this part.

A sample digested with papain, a non-specific proteolytic enzyme, was treated according to the description under Methods and Materials. The amino acid analysis showed that release of amino acids had occurred only to a small degree. However, a selective release of phosphoserine was observed and it was the only amino acid that was identifiable in the run. (See Table III.)

The same treatment was applied to a pronase hydrolysate. Again hydrolysis to amino acids was far from complete but a wide range of amino acids including phosphoserine was released demonstrating the non-specific nature of pronase.

TABLE II

% Total Phosphorus Released with and without Predigestion with Pronase[a]

	Egg Yolk Component[b]	Component I[b]
Buffer	4% ± 1%	5% ± 1%
Buffer plus pronase	18% ± 4%	53% ± 3%
Phosphatase	65% ± 12%	53% ± 4%
Pronase plus phosphatase	65% ± 4%	61% ± 6%

[a] Average of 3-6 runs.

[b] These percentages have been rounded off to the nearest whole number.

TABLE III

Amino Acids present in Protein Hydrolysates
Acidic and Neutral Column

Papain		Pronase
X	Phosphoserine	X
	Aspartic Acid	X
	Threonine	X
	Serine	X
	Asparagine	X
	Glutamic Acid	X
	Glutamine	X
	Proline	-
	Glycine	X
	Alanine	X
	Cystine	-
	Valine	X
	Methionine	X
	Isoleucine	X
	Leucine	X
	Tyrosine	X
	Phenylalanine	X

(See Table III.) Apparently both components do contain some phosphorus bound to serine.

Because of the inability of the phosphatase even in combination with digestive enzymes to release all of the phosphorus from the phosphoproteins a different phosphatase procedure was used. On the theory that the phosphatase enzyme might be becoming denatured over the incubation period and unable to function properly after a while, progressive hydrolysis, i.e., repeated addition of fresh enzyme at intervals during the incubation, was employed. The procedure is described in the section on methods and materials.

The yolk component is extremely insoluble even with addition of 1N NaOH so a suspension of it was used for this experiment. The results are shown in Figure II. Eighty three percent of the phosphorus was released within the first 2 hours and by 22 hours 88% had been removed.

The blood serum component dissolved with the addition of base so both dissolved and suspended samples were run and the results are in Figure II. Both samples of Component I were markedly more slow in releasing their phosphorus than was the egg yolk component. However, after 22 hours the suspended sample had released 77% and the dissolved sample close to 90%. An explanation for this difference may be the denaturing effect of dissolving the protein in base before running the experiment. The base may change the structure of the protein enough to render the phosphate more available to the phosphatase.

On the basis of the above information, the two phosphoproteins used for this investigation seem not to be the same protein. The amino acid analyses reported by Jambunathan (11) and DeGuzman (5) seem to agree for no amino acid and they indicate a marked difference in the serine content of the two proteins. In addition, the phosphorus content of the two components is quite

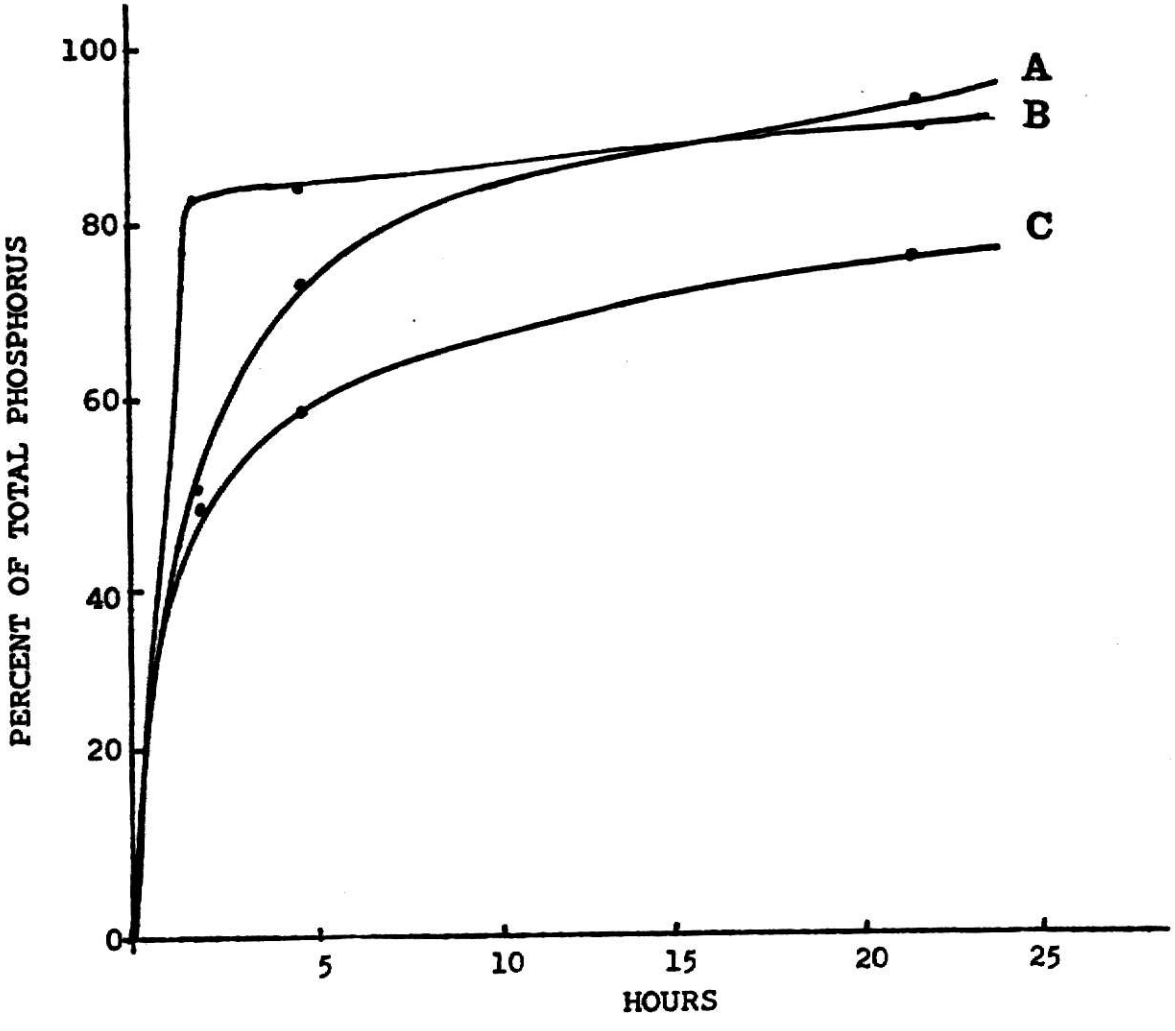

Fig. 2. Progressive hydrolysis of the two components with acid phosphatase. Usual phosphatase procedure was used but fresh enzyme was added at 1½ and 5 hours of incubation.

A. Component I dissolved.
B. Egg yolk component.
C. Component I suspended.

different. The egg yolk component has the much higher phosphorus and serine content.

The dephosphorylation experiments also point out differences between the two phosphoproteins. The use of alkali showed that the egg yolk component has more alkali labile phosphorus than Component I from blood serum. In addition the egg yolk component is also more sensitive to the action of acid phosphatase.

The use of digestive enzymes indicated that the phosphorus of the Component I is protected from the action of dephosphorylating agents somewhat more than the egg yolk component. Component I also demonstrated a remarkable sensitivity to pronase as it released half its phosphorus on reaction with it.

Progressive hydrolysis on the two samples using acid phosphatase indicated that the egg yolk component released its phosphorus quite quickly and then leveled off while Component I showed a much more gradual release of its phosphorus.

Thus the two proteins do not have the same phosphorus and amino acid content and they do not react in the same way to any of the procedures employed on them. The conclusion is that the two phosphoproteins are different entities.

A variety of methods have been used to isolate phosvitin, the high phosphorus-containing protein from egg yolk. All employ some use of $MgSO_4$ precipitation. Mecham and Olcott (16) further extracted with ether and precipitated with $(NH_4)_2SO_4$. Joubert and Cook (13) dissolved the $MgSO_4$ precipitate in 10% NaCl and freed the preparation of lipovitellin by diluting with water and dialyzing against pH 4 acetate. Sundararajan (24) and Greengard (9) diluted yolk with two volumes of water and the resulting precipitate was dissolved in 10% NaCl, extracted with butanol and ether, and

precipitated with $MgSO_4$. All obtained phosphoprotein fractions containing about 10% phosphorus.

Blood serum phosphoproteins have also been prepared by a variety of methods. Common and Mok (4) adjusted the serum to pH 5 and obtained a precipitate by diluting the serum with water. They further isolated the phosphoprotein by following the procedure of Joubert and Cook (13). Heald and McLachlan (10) obtained their phosphoprotein fraction by precipitation with $CaCl_2$ and then used other typical methods of phosphoprotein isolation with final DEAE cellulose fractionation. These researchers also obtained phosphoprotein fractions containing about 10% phosphorus.

This laboratory has been unable to obtain a homogeneous component containing such a high percentage of phosphorus. Attempts to reproduce any of the above researchers' work led to heterogeneous fractions. The methods used in this laboratory are relatively mild ones. No salt fractionation or organic solvents were used and the resulting preparations are therefore less likely to be denatured. They have also been found to be fairly homogeneous.

In order to further assess the reasons for the inability to remove the phosphorus from these proteins by means of hydrolysis with acid phosphatase, a study of the specificity of this enzyme is suggested. Folsch (8) has described a method for preparing various di- and tripeptides containing O-phosphoryl-serine. Koehn and Kind (15) report that acid phosphatase will partially hydrolyze a variety of these peptides but only ran the enzymatic hydrolysis for a very short period of time and therefore it is not possible to determine from their data if the phosphorus would be completely removed from these various di- and tripeptides if the hydrolysis was allowed to go to completion.

When these individuals were performing these experiments, the Merrifield (17) polypeptide synthesis was not available; and since this technique provides a good source of peptides of known sequence, it may be possible to determine if

the difficulty encountered in the action of phosphatase on the original phosphoproteins may be caused by the effect of the sequential arrangement of the amino acids in these proteins on the activity of the phosphatase. It has already been shown that the proteolytic enzymes utilized in this study have removed serine, phosphoserine, and phosphorus from these phosphoproteins when they were employed to fragment the original molecule. Therefore it would not appear practical to employ enzymatically prepared fragments of these phosphoproteins to study the specificity of the phosphatase enzyme, especially since it would be necessary to separate and do sequential analysis on these fragments before they could be used. The only feasible way of approaching this problem by this means is by the use of synthetically prepared substrates.

SUMMARY

Two phosphoproteins have been isolated - one from egg yolk was obtained by the method of Jambunathan, and the other from the blood serum of diethylstilbestrol treated cockerels was obtained by DeGuzman's procedure. The two phosphoproteins were subjected to a variety of analyses and hydrolyses in order to compare the two, especially with regard to their phosphate-protein relationships.

Previously determined amino acid analyses were compared and indicated substantial differences in the amino acid content of the two phosphoproteins. In addition total phosphorus analyses and dephosphorylation with alkali and with acid phosphatase also revealed differences between the two components. Several proteolytic enzymes were employed in conjunction with acid phosphatase in an attempt to release more of the phosphorus from the preparations. Progressive hydrolysis using acid phosphatase was found to be the most effective method thus far for dephosphorylating the phosphoproteins. Here, too, the proteins revealed different properties and are assumed to be different proteins.

Due to problems associated with the use of acid phosphatase, a method for determining the specificity of that enzyme using synthetic polypeptides was proposed.

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PHOSPHATE-PROTEIN RELATIONSHIPS OF CERTAIN PHOSPHOPROTEINS

by

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ABSTRACT

Two phosphoproteins have been isolated. The phosphoprotein from egg yolk was prepared by the technique of Jambunathan using ultracentrifugation and column chromatography. The other from blood serum of diethylstilbestrol treated cockerels was obtained by DeGuzman's procedure of ultracentrifugation and density gradient centrifugation. It has been proposed that the high phosphorus fractions of both egg yolk and estrogenized chicken blood serum have the same origin and are in fact the same phosphoprotein, so several experiments were run on the two components in order to compare them.

Previously determined amino acid analyses were compared and indicated substantial differences in the amino acid content of the two phosphoproteins. In addition total phosphorus analyses and dephosphorylation with alkali and with acid phosphatase also revealed differences between the two components. Several proteolytic enzymes were employed in conjunction with acid phosphatase in an attempt to release more of the phosphorus from the preparations. Progressive hydrolysis using acid phosphatase was found to be the most effective method thus far for dephosphorylating the phosphoproteins. Here, too, the components revealed different properties and are assumed to be different proteins.

Due to problems associated with the use of acid phosphatase, a method for studying the specificity of that enzyme using synthetic polypeptides was proposed.