Chromatographic Fractionation of Phosvitin*

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The presence of phosphorus-containing protein in the yolk of the hen egg was already recognized in 1900 (1). Since then, the egg yolk phosphoproteins received attention in several laboratories (cf. (2)). The first detailed study of the most highly phosphorylated fraction of yolk phosphoprotein, phosvitin, was reported by Mecham and Olcott (3) in 1949. They isolated a product containing 10% phosphorus, representing about 7% of the yolk protein and about 60% of the yolk protein-bound phosphorus. The protein, with a minimal particle weight of about 20,000, was reported to be homogeneous in the ultracentrifuge, but heterogeneous on electrophoresis under some conditions.

Results of later studies (e.g. (4-7)), mostly of a physicochemical nature, supported the suggestion (2) that phosvitin may be a fragment of a complex formed with other egg yolk components. It may also be identical with the vitellin fragments, vitellenic fragment of a complex formed with other egg yolk components.

In the course of a study of the chemistry of phosphoproteins, we found it desirable to investigate further the nature of phosvitin and to obtain additional information bearing on the chemical structure of this protein. In this paper, we describe the fractionation of phosvitin by ion exchange chromatography and some data on the relation and differences between the chromatographic fractions.

EXPERIMENTAL PROCEDURE

Phosvitin was prepared by the method of Mecham and Olcott (3) from large, grade A hen eggs, obtained from a local wholesale distributor. (One preparation was subjected to the ammonium sulfate precipitation step twice instead of only once as in the routine procedure without affecting the phosphorus and nitrogen content or the chromatographic behavior of the protein.) The dialyzed and lyophilized protein was stored over CaCl₂ at 0-4°. Further drying over P₂O₅ at 100° in a vacuum resulted in a weight loss of about 10%. Three moisture-free preparations contained, on the average, 9.9% phosphorus and 11.9% nitrogen (the values ranging between ±0.3 and ±0.4).

Elementary analysis was carried out for phosphorus according to Fiske and SubbaRow (10) or Sumner (11), by the micro-Kjeldahl procedure of Miller and Houghton (12) for nitrogen, and by the diethylidithiocarbamate method for copper, and the o-phenanthroline method for iron both described by Sandell (13). Hydrolysis of protein and O-phosphoserine (California Corporation for Biochemical Research) was performed with glass-distilled 6 N HCl in sealed evacuated tubes at 110° for 24 hours. Amino acid analyses were obtained by the method of Moore et al. (14-15).³ Tryptophan was determined according to Shaw and McFarlane (16).

Buffer solutions were prepared with analytical grade reagents adjusted with HCl or NaOH to the required pH measured with a glass electrode (Beckman model G pH meter) at room temperature. Measurements of the rate of acid liberation were performed with a Radiometer autotitrator at 25°. The instrument was standardized before and after each titration with pH 7 and 10 dilute buffer standards (Beckman) with readings obtained within 0.02 pH unit of the standard value. The surface of the solution was flushed with CO₂-free nitrogen saturated with water vapor and titration was carried out with standard NaOH solutions which had a carbonate content of about 1% or less.

DEAE-cellulose (Brown Company, reagent grade, 1.0 meq per g) was prepared for chromatography according to Peterson and Sober (17). Adsorption experiments were carried out in centrifuge tubes containing mixtures of the ion exchanger and protein in the appropriate buffer. Equilibration was obtained with intermittent shaking within 30 minutes at room temperature. Column chromatography was performed at 2° with a fraction collector. Columns were poured in 5-cm sections with pressures for packing not exceeding those used during subsequent elution. The length of each column was about 35 cm, the diameter 0.9, 2.5, or 4.4 cm. Flow rates of about 25 ml per hour per cm² were obtained with hydrostatic pressures of about 60 cm. Before the protein solution was introduced, each column was washed with the first eluting buffer until the O.D. (280 mp) of the effluent solution was less than 0.005. Elution was carried out with buffers of increasing ionic strength (constant buffer salt concentration), either in a stepwise fashion or with a continuous gradient. Theoretical gradient curves as obtained with one or two mixing chambers were calculated according to Alm et al. (18) and Svensson (19).

RESULTS AND DISCUSSION

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Preliminary Experiments—DEAE-cellulose equilibrated with 0.005 M Tris-HCl buffer, pH 8, containing 0.10 M NaCl, was

³ We are grateful to Dr. F. M. Richards for making his automatic amino acid analyzer available to us and to Mr. Guido Gordillo for performing the analyses.
capable of adsorbing phosvitin in a ratio of at least 1:4 (protein to exchanger). Maximal adsorption occurred at an ionic strength of about 0.1 M. As the ionic strength was increased, protein was released. At 0.4 M NaCl, all the protein was again free in solution. At lower pH values (down to 7), adsorption was slightly more extensive, whereas at higher pH values (up to 9), significantly less protein was adsorbed. At any pH between 7 and 9, the degree of adsorption decreased as the ionic strength was raised. Sodium diethylbarbiturate buffer gave essentially the same results but the use of sodium glycylglycinate buffer resulted in the over-all reduction of the adsorption (up to 50%) within the given pH and salt concentration range.

Gradient Elution—Chromatography of phosvitin on a small column eluted by the gradient technique resulted in the pattern shown in Fig. 1. The heterogeneity indicated by this pattern was further confirmed by the determination of the phosphorus content of some of the fractions. Expressed as a ratio of phosphorus concentration (microatoms per ml) to O.D. (280 mp), the results are also shown in Fig. 1. A constant ratio would be expected for a homogeneous preparation. Doubling the length of the column or extensive variations of the rate of the NaCl concentration increase during elution, including variations of the shape of the gradient curve, led to no significant improvement over the degree of resolution shown in Fig. 1.

Stepwise Elution—When the ionic strength of the eluting buffer was increased in a stepwise manner that portion of the eluted material which had a low P:O.D. ratio could be obtained as a discrete peak within one elution step (Fig. 2). (Tris-HCl or sodium diethylbarbiturate buffers at pH 8.0 gave closely agreeing results.) The ratio of the fast-moving to the slow-moving fraction (0.30:0.35) was 3.2 (based on O.D.). The P:O.D. ratios obtained in this experiment showed that 0.30-phosvitin was still heterogeneous. The symmetry of the peak is clearly not a satisfactory indication of homogeneity.

Although essentially all (95%) the applied phosphorus was recovered in the two fractions but only 59% of the original O.D., it is clear that either some phosphorus-poor, light absorbing material had remained on the column or the fractions had an absorbancy index different from that of the original protein. Inasmuch as the weight of the recovered, dialyzed, and lyophilized fractions amounted to 71% of the column charge, the average absorbancy index of the protein must have decreased upon chromatography. The 80% recovery based on weight is appreciably greater than the 59% based on O.D. This conclusion was confirmed after isolation of the fractions and the determination of their absorption indices.

Rechromatography of Fractions—The invariability of the behavior of a given chromatographic fraction was tested by rechromatography. When 0.35-phosvitin was rechromatographed, essentially all the eluted material emerged in the 0.35 M salt step (Fig. 3B). In a few experiments, such as the one shown, a small percentage of the total eluted material emerged in earlier elution

Pending a more definitive characterization of these fractions, we shall refer to them as 0.30-phosvitin and 0.35-phosvitin.
steps; in most cases, even these insignificant peaks were absent. Rechromatography of 0.30-phosvitin resulted at first in the elution of material with both 0.30 and 0.35 mM NaCl (not shown in the figure). The ratio of the two new fractions was about three times higher than the original 0.30:0.35 ratio of 2 to 3 (in several experiments). In other words, 0.30-phosvitin could give rise to 0.35-phosvitin, although to a lesser extent than unFractionated phosvitin. The chromatographic heterogeneity of 0.30-phosvitin was not unexpected in view of the observed inconstancy of the P:O.D. ratio across the width of the original 0.30-phosvitin peak (Fig. 2). Such results could arise either because of an incomplete resolution of the two fractions in the initial chromatographic experiment or because of a conversion of 0.30-phosvitin to 0.35-phosvitin during the chromatographic analysis. In the former case, it would be expected that a chromatographically homogeneous fraction could be obtained under the proper conditions, whereas in the latter case, chromatographic homogeneity could not be achieved. Table I shows that there is a correlation between the P:O.D. ratios of portions of a 0.30-phosvitin peak and the 0.30:0.35 ratio to which the same portions give rise on rechromatography. When only the portion corresponding to the effluent region with the highest P:O.D. ratio was rechromatographed, chromatographically homogeneous 0.30-phosvitin was obtained (Fig. 3A). Thus, under suitable conditions, both chromatographic fractions of phosvitin could be obtained without cross contamination, favoring the view that the initial experiments showed incompleteness of resolution rather than conversion of one fraction to the other.

Further Observations on Chromatography of Phosvitin—When the column was charged with an excess of phosvitin (5 times the usual amount), protein was eluted in every elution step (top curve, Fig. 4). The material eluted in the 0.20 and 0.25 mM salt steps (Peaks A and B) gave rise to 0.30- and 0.35-phosvitin on rechromatography (bottom curves, Fig. 4). The early breakthrough of material from an overloaded column is not unexpected (cf. (18)); but it is surprising that 0.35-phosvitin, the normally most strongly adsorbed fraction, was present in these early elution steps, and in successively decreasing amounts (relative to 0.30-phosvitin) (cf. ratios in Fig. 4). When 0.35-phosvitin alone was placed on a column in large excess (3 times the amount which could be held maximally by the column until the salt concentration was raised to 0.35 mM), all of the excess protein (two-thirds of the charge) emerged in the 0.30 mM salt step, and not in any of the earlier steps.

From experimental observations it has been inferred (20) that different sets of adsorption sites are present on a chromatographic column, each site exhibiting a characteristic degree of affinity for a given substance. When the substance is a polyfunctional macromolecule, this affinity is a sensitive function of such variables as pH or ionic strength (22). Thus, the repeated emergence from a column of the same protein may be explained if, under given conditions, some sites release the protein while others hold on to it. In protein mixtures, successive peaks may represent mixtures containing varying amounts of the components, possibly followed by the elution of peaks representing individual components. The ratio of components in a given peak will be a function of the relative shape of their adsorption isotherms. So long as these isotherms, as a function of the elution variable (pH, ionic strength, etc.), do not cross within the limits set for the elution variable in a given experiment, the less strongly adsorbed components will predominate in the early elution peaks whereas the more strongly adsorbed ones will do so in the peaks emerging later. Isotherms are known to exhibit maxima (cf. (21)). This or other features of their shape could result in their crossing. Then the elution of a given component could occur before and after the elution of another component. Another reason for such repeated emergence of a component before and after the elution of another component could be an interaction between the components, if such interaction is disrupted during the course of chromatography. The variables used for dissociating protein and adsorbent could readily cause dissociation between protein and protein also. Then, before protein-protein dissociation, elution of such a protein complex would be followed by the elution of the dissociated components in the order of their increasing affinity for the adsorbent.

4 Physical blocking of the elution of a normally less strongly adsorbed species by a more strongly adsorbed species, as suggested by one of the referees of this paper, could postpone the elution of the less strongly held species but it would then emerge together with the blocking species.
emerge in elution steps earlier than the one just preceding the "normal" elution step. Therefore, in the experiment in which unfractonated phosvitin was placed on the column in excess, crossover of adsorption isotherms could not be held responsible for the initially decreasing relative 0.35-phosvitin content of the earlier peaks. When the salt concentration of the eluting buffer was low (less than 0.30 M), however, interaction between 0.30- and 0.35-phosvitin could be responsible for the experimental observations. Such complications as might arise from combinations of protein and adsorptions sites at different levels of thermodynamic stability, subject to kinetic control, are of no practical concern with regard to this argument since variations of the elution rate (by a factor of 2) had no effect on the elution patterns.

Some Properties of Fractions of Phosvitin

Elementary and Amino Acid Analysis The nitrogen and phosphorus content of the chromatographic fractions did not differ significantly from the data obtained with unfractonated protein (Table II). In view of the report (3) that phosvitin contains significant quantities of metals, the copper and iron content of the preparations used in this investigation was determined and compared with the fractions (Table II). It was surprising to find significant differences which appeared to be rather specific since the variations in metal content for several preparations of a given fraction were relatively small. Of the two fractions, 0.35-phosvitin was the richer in both metals. It is clear that the copper content of the fractions could not be accounted for by the barely significant copper content of our unfractonated preparations but must have been introduced from the large volumes of solvent used in the fractionation or dialysis.

The chromatographic fractionation could not be simply a result of different degrees of metal content, perhaps of the same protein, since fractionation of phosvitin could be obtained even if phosvitin was rendered metal-free and buffer solutions were prepared with deionized water; or conversely, if the fractionation was performed with a copper salt added to the eluting buffers.

The apparent specificity of the metal content of the fractions (cf. Table II) is further supported by the finding that the copper and iron content of 0.30-phosvitin remained unchanged, within the limits given in Table II, when the fraction was rechromATOgraphed two more times under the same conditions as in the original preparation.

Except for a few significant differences, the two fractions gave approximately the same amino acid analysis, and the data were similar to those reported in the literature for unfractonated phosvitin (3, 23). 0.30-Phosvitin (chromatographed twice) was devoid of tyrosine but contained 1.25% (by weight) of phenylalanyl residues. 0.35-Phosvitin on the other hand contained 0.93% tyrosine but little phenylalanine (0.27%). The serine content was 32% for unfractonated protein, 32% for 0.30-phosvitin and 28% for 0.35-phosvitin.4

Effect of Alkaline pH—The two fractions differ markedly in their stability at pH values above pH 8.5 at 25°C. 0.35-Phosvitin released acid groups continuously over a period of several hours, whereas 0.30-phosvitin was quite stable under the same conditions (Fig. 5). Whereas 0.35-phosvitin released essentially no protons over a period of 6 hours, 1.5 equivalents of acid per 10⁴ g of protein were released by 0.35-phosvitin in 8 hours. Unfractonated phosvitin also liberates acid at alkaline pH values, but at a rate slower than that observed for an equal weight of the 0.35-fraction; such treatment of the unfractonated phosvitin did not change the chromatographic behavior under the conditions used in this study. It is premature at this stage to speculate on the nature of the acid release. It is not accompanied by the release of inorganic phosphate. Group migrations or changes in metal protein interactions or other structural alterations may be responsible.

SUMMARY

The hen egg yolk phosphoprotein, phosvitin, was resolved into two fractions by ion exchange chromatography. The fractions differ from each other in metal content, amino acid composition, and chemical stability at alkaline pH, although they are quite similar in terms of the gross aspects of their composition.

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4 These are values corrected for 32% loss on hydrolysis. A control experiment with O-phosphoserine showed that only 65% of the serine could be recovered.
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