Comparative Study of Hen Yolk Phosvitin and Plasma Vitellogenin†

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ABSTRACT: Vitellogenin, the only phosphoprotein detectable in the plasma of laying hens, is present at an approximate concentration of 1 mg/mL and can be isolated by chromatography on diethylaminoethylcellulose. Vitellogenin has a molecular weight of 235 000–240 000 and contains approximately 3% phosphorus by weight. Evidence that this protein is the precursor of phosvitins includes its ability to act as an acceptor for phosphate with a phosvitin specific kinase, the generation of a peptide similar to phosvitin by trypsinization, and the presence of distinctive peptides of multiple clustered phosphoserine upon partial acid hydrolysis. This partial sequence similarity between phosvitins and vitellogenin has not been previously reported. The phosphorus content and amino acid composition of vitellogenin are consistent with a model which contains two phosvitins and one lipovitellin. The total molecular weights of these proteins (28 000 + 34 000 + 170 000 = 232 000) are close to that of vitellogenin.

Each hen's egg contains approximately 150 mg of phosphoglycoprotein, phosvitin. Two different hen yolk phosvitins, one (S_3S) of molecular weight 28 000 and another (S_1S) of 34 000, have been found (Clark, 1970). Both polypeptides contain a high proportion of phosphoserine but differ in amino acid composition. They are composed of approximately 50% clustered serine residues (Belitz, 1964; Williams and Sanger, 1959), 94–96% of which appear to be phosphorylated (Rosenstein and Taborsky, 1970). Phosvitin has been shown by detection of protein-bound phosphate to be synthesized in the liver and transported through the blood to the ovary and into the egg (Greengard et al., 1965; Heald and McLachlan, 1965). When laying one egg per day the liver of a hen is synthesizing massive amounts of phosvitins. As a result there should be a significant amount of phosvitin in the plasma. However, Heald and McLachlan (1965) and Beuving and Gruber (1971) have reported difficulty in isolating phosvitins from the plasma. They postulated that the difficulty of isolation resulted from phosvitin being complexed with lipid or protein in the blood. An alternative possibility that phosvitins are perhaps synthesized as a much larger precursor is supported by studies made by Bergink and Wallace (1974). They have isolated a precursor protein, vitellogenin, for phosvitin from the plasma of *Xenopus laevis*. Most recently Deeley et al. (1975) have demonstrated the synthesis of vitellogenin in estrogen-stimulated roosters. The relationship between vitellogenin and phosvitins in these studies was based primarily on the molecular weight estimations and the phosphorus contents. The present study was undertaken for the purpose of isolating and more rigorously characterizing vitellogenin and for obtaining more concrete evidence as to its precursor relationship with phosvitins.

Experimental Section

Methods

Isolation of Vitellogenin from Plasma. White leghorn chickens (Truslow Farms) were used exclusively. Laying hens and roosters were 27–36 weeks old. Non-laying hens were selected by Truslow Farms 17–22 weeks old. When roosters were treated with 17β-estradiol (Calbiochem), they were injected intramuscularly in the leg with 25 mg dissolved in 1 mL of propylene glycol and sacrificed 4 days later. Blood was removed by cardiac puncture from chloroform anesthesized animals. Plasma was prepared from blood by adding 15 mg/mL sodium citrate–0.1 mg/mL $PhCH_2SO_2F$[1] (Bergink and Wallace, 1974) and centrifuging at 2000 rpm for 30 min at 4 °C.

Samples of plasma were diluted with 2 volumes of buffer (0.1 M NaCl–0.1 M sodium citrate (pH 5.0)–0.1 mg/mL $PhCH_2SO_2F$; Beuving and Gruber, 1971), and were degassed and applied to a column of DEAE-cellulose 25 times the original plasma volume. Geometry of the column was chosen to permit a flow rate sufficient for completion within 4 h.

The column of DEAE-cellulose (DE 52 or DE 23 for higher flow rate without pumping) was equilibrated with column buffer (0.1 M NaCl–0.1 M sodium citrate (pH 5.5)–0.1 mg/mL $PhCH_2SO_2F$). After sample application the column was washed with this buffer until the absorbance at 280 nm was below 0.01. The column was then washed with excess 0.05 M Tris (pH 8.0)–0.10 M NaCl. The absorbance at 230 nm was monitored to follow the removal of citrate. The pH and conductivity of the effluent were measured until they matched the input buffer. The column was then eluted with a 0.1 to 0.5 M NaCl gradient buffered with 50 mM Tris, pH 8.0 (Connelly and Taborsky, 1961). Four fractions (I, II, III, and IV) were collected. Pooled fractions were concentrated either by dialysis and lyophilization or in an Amicon TCF 10 membrane pressure dialysis filtration apparatus.

Enzymatic Dephosphorylation. Isolated fraction IV, phosvitins, or casein were each dissolved at 1 mg/mL in 0.036 M Tris (pH 8.0) and 1/50 w/w of bacterial alkaline phosphatase (Worthington lot BAPC or Sigma) was added to them

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[1]Abbreviations used: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl; Pv, phosvitin; Tris, tris(hydroxymethyl)aminomethane.

(Shainkin and Perlmann, 1971). The reaction was carried out in a dialysis bag vs. 2.8 L of 0.036 M Tris, pH 8.0, at 37 °C. The dialysis was used to reduce enzyme inhibition by the phosphate released in the reaction. Phosvitins and fraction IV were incubated for 24 h, which removed 50–60% of the protein phosphorus. Longer incubation yields up to 90% dephosphorylation of phosvitins, but results in an insoluble gel which is a less effective acceptor for phosvitin kinase. Casein was incubated for 4 h to 50% dephosphorylation.

Isolation of Phosvitin Kinase. Phosvitin kinase was prepared from unstimulated (nonestrogenized) rooster liver according to Goldstein and Hasty (1973) with the following modifications. After homogenization and ammonium sulfate fractionation, the resuspended precipitate was dialyzed twice for 2 h, each vs. 10 volumes of Bio-Gel column buffer B (20 mM Tris (pH 7.4)–0.1 M NaCl–5 mM 2-mercaptoethanol–0.1 mM EDTA). This dialysis was necessary to prevent clogging of the Bio-Gel column with insoluble protein due to high salt after the ammonium sulfate fractionation. The fractions with activity obtained from the Bio-Gel column were pooled, and 0.08 volume of 5 mM NaCl and 0.033 volume of 1 M Tris (pH 7.4) were added prior to dialysis vs. the phosphocellulose column sample buffer (50 mM Tris (pH 7.4)–0.5 M NaCl–5 mM 2-mercaptoethanol–0.1 mM EDTA). The enzyme eluted at 0.9 M NaCl and was immediately diluted to 50 mM Tris (pH 7.4)–0.2 M NaCl–5 mM 2-mercaptoethanol–0.1 mM EDTA by the addition of 3.5 volumes of 50 mM Tris (pH 7.4)–5 mM 2-mercaptoethanol–0.1 mM EDTA. Ten milligrams of bovine serum albumin was added and then the enzyme concentrated to 2 mL by membrane pressure dialysis filtration, and stored in aliquots at 0–4 °C.

The yield of enzyme was 25% of that reported by Goldstein and Hasty (1973) with estrogen unstimulated rooster, and purification was 1500-fold compared with 8000-fold.

In Vitro Phosphorylation. Acceptors (0.6 mg/mL) were phosphorylated at 37 °C by the addition of 0.01 unit of enzyme and 10 μCi of [γ-^{32}P]ATP/mL (New England Nuclear; 5.81 Ci/mmol). The incubation was carried out in 1.0 mL of 0.050 M phosphate buffer (pH 6.6)–0.007 M $MgCl_2$. Incorporation of ^{32}P into protein as trichloroacetic acid precipitable counts was monitored by spotting on strips of Whatman No. 1 filter paper, rinsing twice in 5% Cl_3CCOOH–10 mM sodium pyrophosphate, twice in 5% Cl_3CCOOH, and twice in technical grade acetone. In some reactions, after the initial incubation, a 1000-fold excess of cold ATP and an equal amount of fresh enzyme were added. These reactions were incubated 100 min and dialyzed twice vs. 1 L of 10% NaCl at 4 °C, and repeated changes of deionized water until less than 10 cpm/mL were detectable in the dialysate.

Polyacrylamide Gel Electrophoresis. Samples were heated for 1 min at 100 °C in 2-mercaptoethanol and sodium dodecyl sulfate then applied to 0.6 × 12 cm (5 or 8%) gels prepared and run as described by Laemmli (1970). Some gels were bisected longitudinally and one-half stained for protein with Coomassie brillant blue and the other half stained for phosphoprotein by the method of Cutting and Roth (1973). Proteins of known molecular weight were run as standards on the same and parallel gels. Molecular weights were calculated by comparing the relative mobilities of these standards with vitellogenin, β-lipovitellin, and phosvitin as described by Weber and Osborn (1969).

Whole Coomassie stained gels of fraction IV from the DEAE-cellulose column were scanned on a Beckman DU spectrophotometer at 590 nm equipped with Gilford Model 2410 linear transport and Model 2000 recorder. The peaks were symmetrical and the areas under the peaks were estimated as width at half-height times height.

Acid Hydrolysis and Paper Electrophoresis. Partial hydrolysis was performed at 45 °C for 24 h in 6 N HCl. The HCl was diluted to 1 N and was removed by lyophilization through an NaOH trap. The hydrolysate was then resuspended in deionized water and relyophilized.

High-voltage paper electrophoresis was performed on the partial hydrolysates as by Williams and Sanger (1959). Staining was by immersion in ninhydrin solution (Dreyer and Bynum, 1967).

Analyses. Phosphorus content was assayed by the method of Chen et al. (1956) and protein by that of Lowry et al. (1951). Radioactivity was measured in a Packard liquid scintillation spectrometer in LSC fluor (Yorktown Research, 99% efficiency).

The amino acid analyses were performed by Dr. Sandra Graziano and Mr. P. O. Backman on a Beckman Model 121 amino acid analyzer and Technicon autoanalyzer TSM. Samples were hydrolyzed at 110 °C for 12, 24, 48, and 72 h. Values for amino acids were extrapolated to zero time to correct for destruction during hydrolysis. Tryptophan was estimated from the tyrosine/tryptophan ratio obtained by ultraviolet absorption (Bencze and Schmidt, 1957). To correct for destruction of phosphoserine during hydrolysis, equimolar amounts of D,L-*o*-phosphoserine and glycine were hydrolyzed for 10, 20, 45, and 90 min as well as 3, 6, 12, 24, 48, and 72 h. Destruction of free phosphoserine and appearance of serine were both calculated by amino acid analysis and corrected for recovery based on glycine.

Materials

Poly(L-serine), phosvitin, chick serum albumin, protamine, and β-galactosidase were purchased from Sigma. Trypsin (TRL) and α-casein were purchased from Worthington. Myosin was a gift of Dr. William Harrington. Crude histone was a gift of Dr. M. Mitchell Smith. Molecular weight markers ovalbumin, human γ-globulin, and bovine serum albumin were purchased from Schwarz/Mann. β-Lipovitellin was prepared from washed yolk granules by chromatography over Bio-Gel AG 1 × 2 400 mesh and hydroxylapatite as by Burley and Cook (1961) with 0.1 mg/mL $PhCH_2SO_2F$ present at all stages.

Results and Discussion

Isolation and Purification of Vitellogenin. A high-molecular-weight protein, vitellogenin, can be recovered from the plasma of laying hens (0.8–1.2 mg/mL plasma, 12 experiments). This protein is also present in the plasma of estrogen-stimulated roosters (1.6–2.2 mg/mL plasma, three experiments), but absent from the plasma of nonlaying hens or unstimulated roosters (three experiments each). Figure 1 shows the results of a typical DEAE-cellulose purification of vitellogenin from laying hen plasma. The protein of interest elutes as fraction IV at 0.2 M NaCl, a salt concentration lower than reported for yolk phosvitin (Connelly and Taborsky, 1961). If protein concentration is estimated by absorption at 280 nm, fraction IV represents 4% of the material recovered from the column at ~1 mg yield for each milliliter of plasma applied.

The sodium dodecyl sulfate–polyacrylamide gels in Figure 2 compare to whole plasmas the proteins present in fractions I and IV isolated by DEAE-cellulose chromatography. In each case two gels were run in parallel, the second gel stained for phosphorus. The phosphorus gel staining technique is capable of detecting 1 nmol of phosphate, but requires harsh treatment

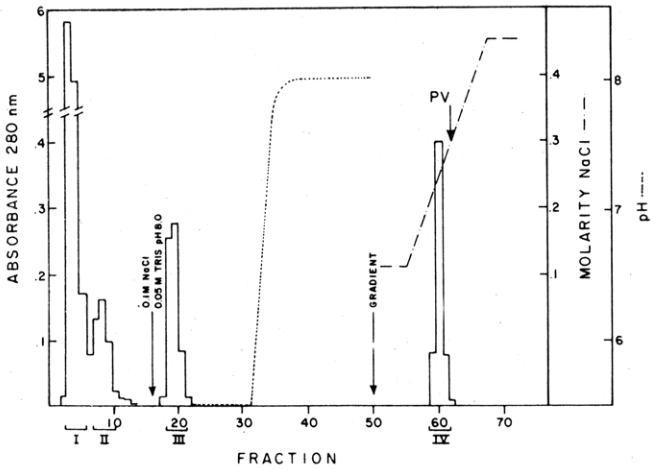

FIGURE 1: Purification of vitellogenin from laying hen plasma by DEAE-cellulose column chromatography. Eight milliliters of plasma diluted with 16 mL of 0.1 M NaCl–0.1 M citrate (pH 5.0) was degassed and applied to a 3 × 60 cm column of DE 52 which was degassed and equilibrated in 0.1 M NaCl–0.1 M citrate (pH 5.5). Fractions I–IV, pooled as indicated by brackets, were dialyzed and concentrated. $PhCH_2SO_2F$ (0.1 mg/mL) was present in all buffers and all steps were performed at 4 °C. The column was eluted at 200 mL/h and 24-mL fractions were collected. The elution of phosvitin (Pv) on such a column is indicated by the arrow. The salt gradient was determined by conductivity measurements.

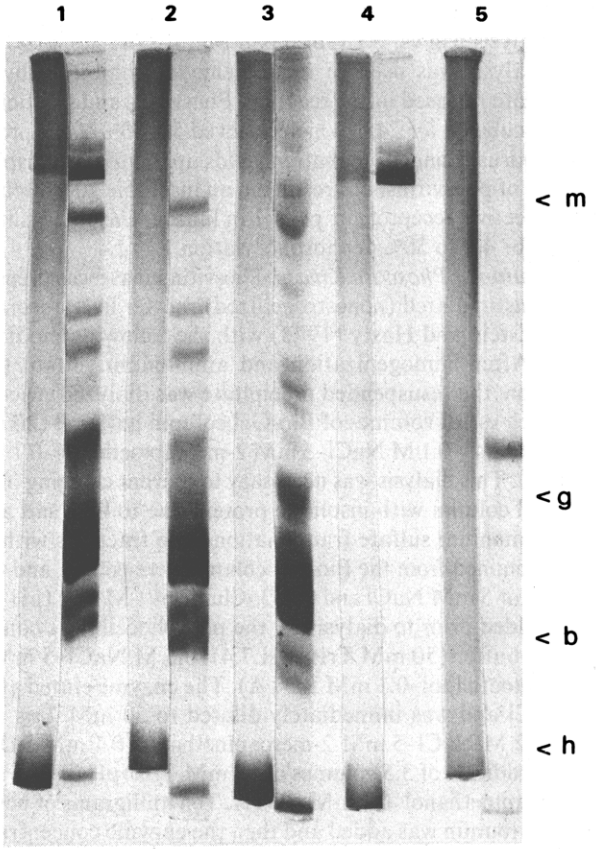

FIGURE 2: Five percent sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The left gel of each pair was stained for phosphoprotein; the right with Coomassie blue. Ten microliters of plasma from laying hens and roosters were applied to gels 1 and 2, respectively. Two hundred and fifty micrograms of fraction I, 50 μg of vitellogenin, and 20 μg of β-lipovitellin were applied respectively to gels 3, 4, and 5. The positions of molecular weight marker proteins are shown by arrows. m = myosin; g = β-galactosidase; b = bovine serum albumin; h = human γ-globulin.

of gels, resulting in approximately 10% shrinkage, and that all glassware be free of phosphorus. Still, in our hands, backgrounds were sometimes irregular and blemishes appeared. The only protein with detectable phosphate in hen's plasma, vitellogenin, migrates to the region of myosin. The protein is present originally in the plasma and can be purified by DEAE-cellulose chromatography (fraction IV).

Fraction IV is present only in laying hen plasma and estrogen-stimulated rooster plasma and contains largely vitellogenin (Figures 2–4). Plasma from nonlaying hens and unstimulated roosters yield no material which elutes in this position. The other fractions are present in the plasma from laying and nonlaying hens and roosters. The positions of fractions II and III vary slightly with the geometry of the column and at sufficiently lower loadings of laying hen plasma they become undetectable, while fraction IV is still detectable. When the DEAE column is overloaded, vitellogenin appears in fraction II. Since fractions II and III were not specific for laying hen, they were not investigated further. Fraction I elutes at the void volume of the column with greater than 90% of the plasma proteins.

Fraction I contains the bulk of the plasma proteins consisting largely of the two bands near the dye front. Fractions II and III contain residual amounts of the main bands of fraction I as well as a band near the center of the gel and a faint triplet of bands near the size of vitellogenin. The center band of this triplet in fraction II appears to be vitellogenin as it shows very faint phosphorus staining. The other two bands of the triplet show no phosphorus staining at three times the sample load. They do not appear to be related to vitellogenin and are minor components of the plasma compared with vitellogenin (Figure 3).

There was a marked reduction in the amount of material in fraction IV if the blood began to clot before addition of citrate (one experiment) or if $PhCH_2SO_2F$ was not added immediately upon withdrawal of blood from the animal (one experiment). This peak also contained a varying amount of degradation product if the above precautions were not observed, or if the plasma was not refrigerated and used immediately (one experiment). It was also important that the purification be completed as quickly as possible to avoid degradation. Figure 4 shows gels of vitellogenin degraded during isolation. Several protein and phosphorus staining bands can be seen. Some of these approximate the size of lipovitellin subunits but the pattern is complex and we have not sought to clarify the relationships.

Some minor protein contaminants occasionally become visible in fraction IV upon gel electrophoresis of grossly overloaded (>100 μg) gels. These were unimportant to the demonstration of the presence of phosvitin in vitellogenin since none of these contaminants contained significant phosphorus and could not have generated the phosphoserine peptides described later. Some of these contaminants appear in much larger proportions in partially degraded preparations and may be breakdown products of vitellogenin. Contamination in fraction IV was estimated by comparing areas under the peaks of gels loaded with greater than 100 μg of protein. This procedure assumes uniform staining efficiency of proteins with Coomassie blue and is not necessarily valid, especially since phosvitin does not stain with Coomassie blue. Therefore, one might expect a protein containing phosvitins to stain less efficiently than other proteins. Thus the relative staining of vitellogenin was compared with bovine serum albumin, β-galactosidase, and human γ-globulin heavy chain by scanning gels and comparing peak areas for known protein inputs. There was no detectable difference in the relative staining (Christmann, 1976). Vitel-

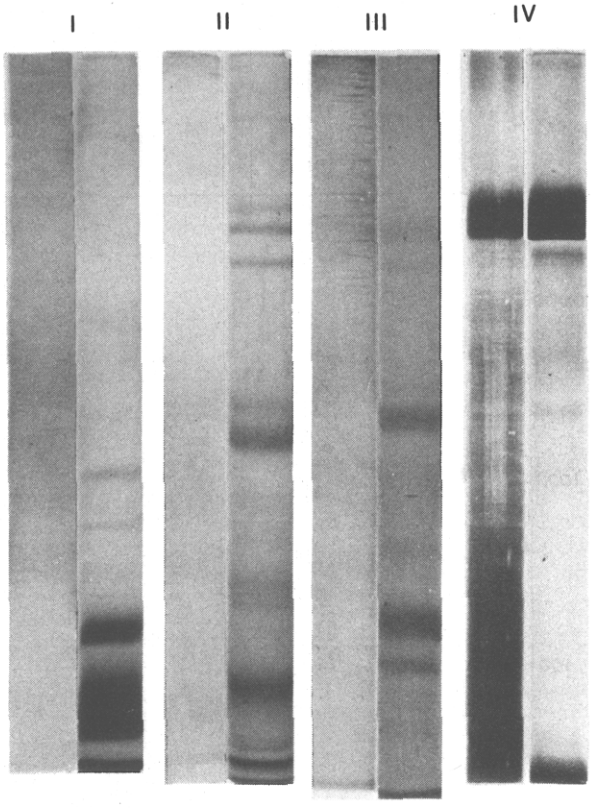

FIGURE 3: Five percent sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fractions pooled from DEAE-cellulose column chromatography. The gels were bisected longitudinally and the left gel of each pair was stained for phosphoprotein as described in Methods. The right gel was stained for protein with Coomassie blue. One hundred and fifty micrograms each of fractions I and IV were applied and 30 μg each of fractions II and III. For photography each gel was placed in a water-filled 1.1-cm diameter glass tube and a second tube with water only was stacked above it. The tubes function as a cylindrical lens focusing at the midplane of the gel. For comparison the gels were photographically enlarged to the same size.

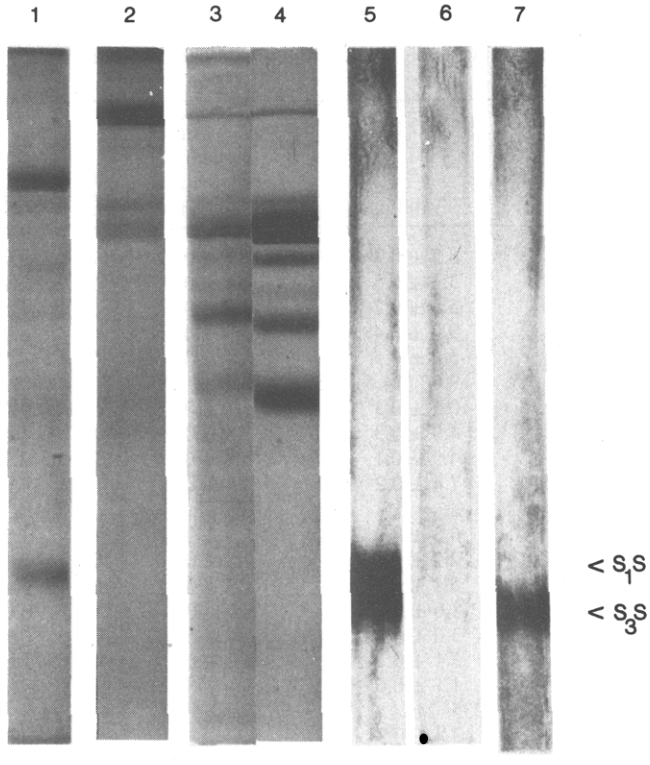

FIGURE 4: Eight percent sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels 1–3 photographed as in Figure 3. Gels 1, 2, and 4 stained with Coomassie blue, gels 3, 5, 6, and 7 stained for phosphoprotein. Gel 1, 30 μg of β-lipovitellin; gel 2, 30 μg of vitellogenin; gels 3 and 4, 90 μg of vitellogenin extensively degraded during isolation; gels 5, 6, and 7, 50 μg each of phosvitins, β-lipovitellin, and vitellogenin were digested 5 min at 37 °C with 1% w/w trypsin in 0.2 M NaCl, 0.05 M Tris (pH 8.0) and 0.1 mg/mL $PhCH_2SO_2F$ prior to electrophoresis. The positions of phosvitins S_1S and S_3S are indicated by arrows.

logenin was estimated to be greater than 95% pure by this technique. Preparations have been referred to as vitellogenin only when the contamination was less than 5% (Figures 2, 3 and 4).

The molecular weight protein standards for 5% sodium dodecyl sulfate–polyacrylamide gels were myosin, β-galactosidase, bovine serum albumin, and human γ-globulin heavy chain, and for the 8% gels they were bovine serum albumin, human γ-globulin heavy and light chains, and ovalbumin. The estimated molecular weights for the two major components of β-lipovitellin are 140 000 and 30 000, giving a combined protein molecular weight of 170 000. The estimates for phosvitins are 29 000 and 33 000 for S_3S and S_1S, respectively. The estimate for vitellogenin is 235 000. Since vitellogenin appears larger than the largest molecular weight standard, estimation by this method requires extrapolation rather than interpolation. Furthermore, with no marker slower than vitellogenin on electrophoresis, one cannot be absolutely certain migration in the gel is linear with the logarithm of molecular weight in this region of the gel. If this is the case, then the value calculated by this method (235 000) is a slight underestimate. Either error should not be large since the extrapolation is not far beyond the upper standard.

Since vitellogenin shows no dissociation on reducing sodium dodecyl sulfate–polyacrylamide gels, it is unlikely that bonds linking phosvitins with remainder of vitellogenin are hydrophobic, ionic, or disulfide. Furthermore, vitellogenin was prepared in excess citrate. It is unlikely that divalent cations are responsible for the large apparent molecular weight. Vitellogenin was also extensively delipidated, then reduced, and alkylated in 6 M guanidine hydrochloride with no evidence of dissociation detectable by gel filtration in 6 M guanidine hydrochloride (Christmann, 1976).

Characterization of Vitellogenin. 1. Amino Acid Compositions of Vitellogenin and a Fragment of Vitellogenin Generated by Trypsin Digestion. Amino acid analysis was not performed on any preparation of vitellogenin unless the contaminants represented less than 5% of the protein estimated as before. Four different preparations were analyzed. Variations between analyses of different preparations were no larger than variations on repeated analyses within preparations. The data in Table I are the average values corrected for amino acid destruction as described in Methods.

It was assumed that all phosphorus was in the form of phosphoserine, and the value shown is the corrected value based upon phosphoserine destruction (32.3%) and serine recovery.

To examine whether phosvitins can be generated from vitellogenin, undegraded vitellogenin was subjected to limited proteolysis with trypsin. Phosvitins might be expected as a partial limit digest as it is reportedly quite resistant to proteolysis unless dephosphorylated (Shainkin and Perlmann, 1971). The polyacrylamide gel electrophoresis pattern of the digest was compared with digested lipovitellin and phosvitins (Figure 4). It can be seen that a broad phosphoprotein band approaching the sizes of native phosvitin S_3S and S_1S is generated under these conditions. The digest was fractionated by

TABLE I: Amino Acid Compositions of Vitellogenin and Trypsin Resistant Fragments of Vitellogenin as Compared with Those of Phosvitins.[a]

	Vitellogenin	Trypsin resistant fragments of vitellogenin[c]	Phosvitin[d] S_1S	Phosvitin[d] S_3S
Lys	6.7 ± 0.3	5.9 ± 0.4	7.0	9.7
His	3.2 ± 0.5	4.1 ± 0.1	5.6	2.1
Arg	5.5 ± 0.2	4.2 ± 0.1	5.1	5.5
Asp	9.0 ± 0.4	6.3 ± 0.2	6.0	6.3
Thr	5.0 ± 0.5	2.1 ± 0.1	2.3	3.9
Ser	19.0[b] (14.5) ± 0.5	56.4[e] ± 3.1	56.4	49.9
Glu	10.3 ± 0.3	6.1 ± 0.1	5.1	9.6
Pro	4.9 ± 0.3	1.8 ± 0.5	1.4	1.5
Gly	4.9 ± 0.1	3.0 ± 0.3	2.1	2.7
Ala	7.4 ± 0.2	3.6 ± 0.2	3.2	4.0
Cys				
Val	6.3 ± 0.2	1.0 ± 0.1	1.2	1.5
Met	2.2 ± 0.1	0.7 ± 0.1	0.4	0
Ile	5.3 ± 0.2	1.3 ± 0.2	0.9	0.4
Leu	8.0 ± 0.3	1.6 ± 0.1	1.4	0.03
Tyr	3.0 ± 0.1	0.5 ± 0.1	0.2	0.9
Phe	3.1 ± 0.1	0.8 ± 0.1	0.4	1.8
Trp	1.1 ± 0.0			
Mol wt × 10^3	235-240	25-32	34	28
% P	3.4 ± 0.3		9.5	9.2

[a] Values expressed as mol % calculated from mol per 10^4 g of protein. [b] 19.0 is the value corrected for destruction of phosphoserine assuming all phosphate is on serine and there is a 32.3% loss of phosphoserine upon hydrolysis as there was for free phosphoserine; 14.5 is the corrected serine value based upon serine destruction alone extrapolated to zero hydrolysis time. [c] Isolation of trypsin resistant fragments of vitellogenin was described in the text and in Figure 4. [d] From Clark (1970). [e] Corrected as *b*.

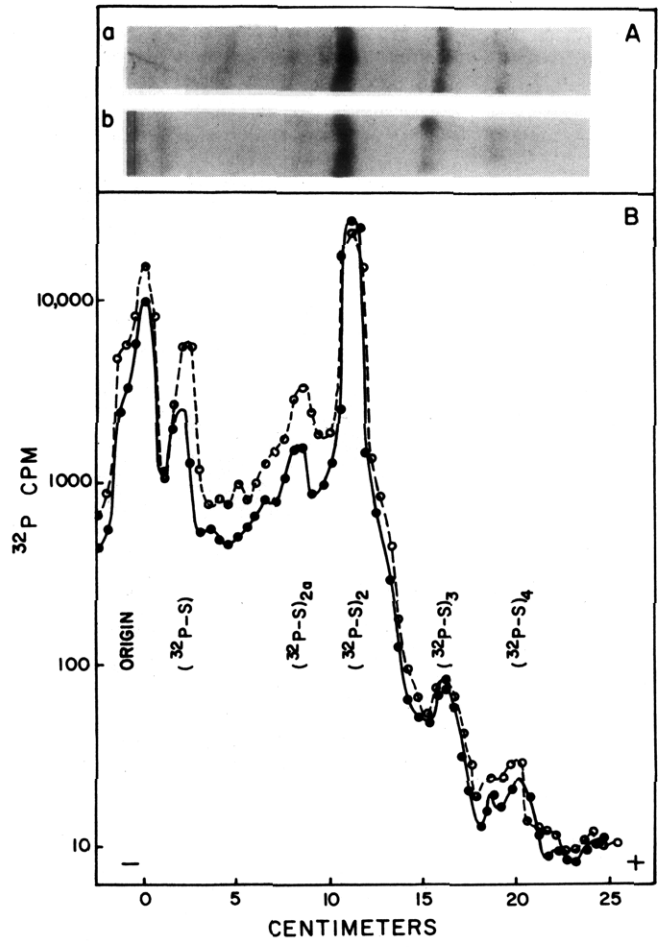

FIGURE 5: Partial hydrolysis was performed at 45 °C for 24 h in 6 N HCl. HCl was removed by lyophilization through a NaOH trap. The hydrolysate was resuspended in deionized water and relyophilized. High-voltage paper electrophoresis was for 2 h at 1200 V in 20% formic acid. Migration is to the anode. Electropherograms were compared relative to the movement of marker dyes (Brownlee, 1972). (A) Hydrolysates of 5 mg of phosvitin (a) and 10 mg of vitellogenin (b). Twenty percent of each hydrolysate was applied for electrophoresis and stained with ninhydrin. (B) Hydrolyzed phosvitin (0.6 mg) (dashed line, O) and vitellogenin (0.6 mg) (solid line, ●) after dephosphorylation and labeling with [γ-^{32}P]ATP via phosvitin kinase. Equal counts (300 000 cpm) were applied for each sample. Electropherograms were sliced in half longitudinally and one-half was cut into 0.5-cm strips for counting. The other half was exposed for autoradiography (not shown). (^{32}P-S) *o*-phosphoserine, (^{32}P-S)$_2$ dimer *o*-phosphoserine, (^{32}P-S)$_{2a}$ dimer *o*-phosphoserine, glutamic acid, (^{32}P-S)$_3$ trimer *o*-phosphoserine, (^{32}P-S)$_4$ tetramer *o*-phosphoserine. Phosphoserine and its clusters (A) migrated identically with those phosphorylated in vitro by phosvitin kinase (B).

DEAE-cellulose chromatography as by Connelly and Taborsky (1961). The material corresponding to the broad band in Figure 4 eluted at the position of phosvitin. After dialysis it was hydrolyzed and its amino acid composition determined. Comparison of the amino acid composition of this trypsinized vitellogenin with both phosvitins shows close agreement with published values of yolk isolated phosvitins (Table I).

Heald and McLachlan (1965) and Beuving and Gruber (1971) made a similar amino acid comparison with phosvitin isolated from estrogen-treated rooster blood. The latter suggested that proteolysis may be necessary for the isolation of phosvitin from the blood. Isolation of vitellogenin confirms this prediction. The major components of plasma isolated phosvitin or trypsinization of vitellogenin are probably not exactly the same as yolk phosvitin since in vivo vitellogenin would not likely be cleaved by these enzymes.

2. Phosphoserine Peptide Comparison of Phosvitin and Vitellogenin. Williams and Sanger (1959) reported the identification of peptides containing clusters of phosphoserine from partial acid hydrolysates of phosvitin and casein. The peptides were distinguished by their mobilities in high voltage paper electrophoresis at pH 1.5. At this pH phosphoserine has no net charge and remains at the origin. All other amino acids have net positive charge and move toward the cathode. Multimers of phosphoserine and phosphothreonine are the only peptides with net negative charge and move toward the anode generating a series of bands.

Partially hydrolyzed samples of chicken serum albumin, casein, phosvitins, vitellogenin, and fraction I were subjected to paper electrophoresis and the peptides visualized by ninhydrin. A comparison of phosphoserine peptides generated by partial acid hydrolysis of vitellogenin and phosvitins is shown in Figure 5A. Identical patterns of phosphoserine multimers were produced. These bands were shown to contain phosphorus by staining parallel electropherograms with the ammonium molybdate reagent used for phosphorus analysis (see Methods). Bands $(P\text{-}S)_2$ and $(P\text{-}S)_3$ were cut from other parallel electropherograms, eluted, hydrolyzed, and shown to yield greater than 90% serine upon amino acid analysis. Band $(P\text{-}S)_{2a}$ yielded approximately 75% serine and 15% glutamic acid. Chicken serum albumin and fraction I show no such peptides, and casein under these conditions yields only dimers and trimers (data not shown). There are two major difficulties in obtaining longer clusters of phosphorylated serine following partial hydrolysis: (a) the peptide bond between adjacent phosphoserines is only relatively more stable to acid than the

TABLE II: Phosvitin Kinase Catalyzed Incorporation of ^{32}P into Proteins.[a]

Substrate	Experiment[b] 1	2	3
Native phosvitin	2.58		
50% dephos. phosvitin[c]	0.01	0.01	0.01
50% dephos. phosvitin	42	85[d]	40
85–90% dephos. phosvitin	0.68		
Native vitellogenin	0.77		
50% dephos. vitellogenin[c]		0.01	0.01
50% dephos. vitellogenin		48[d]	9.0
Poly(L-serine)	0.01	0.01	0.05
15% dephos. β-lipovitellin			1.26
Dephos. α-casein[e]	1.88	1.36	2.01

[a] Reaction conditions as in Methods. Data reported as pmol of phosphate/mg of substrate. No incorporation (0.01 pmol) with bovine serum albumin, protamine, histones, or no substrate acceptor present. [b] Time of incubation was: experiment 1, 20 min; experiment 2, 40 min; experiment 3, 20 min. [c] No phosvitin kinase added. [d] At 140 min, 565 and 256 pmol respectively for phosvitin and vitellogenin. [e] α-Casein. Experiments 1 and 2, 50% dephosphorylated; experiment 3, 80% dephosphorylated.

TABLE III: Amino Acid Composition of Vitellogenin and Those from Phosvitins plus Lipovitellins.[a]

	Vitellogenin[b]	Phosvitin + lipovitellin[c] A[d]	B[e]
Lys	6.7 ± 0.3	7.0	7.0
His	3.2 ± 0.5	2.9	2.5
Arg	5.5 ± 0.2	5.9	6.3
Asp	9.0 ± 0.4	7.4	7.9
Thr	5.0 ± 0.5	4.2	4.2
Ser	19.0 (14.5) ± 0.5	19.5	19.1
Glu	10.3 ± 0.3	9.8	10.0
Pro	4.9 ± 0.3	4.6	4.5
Gly	4.9 ± 0.1	4.3	4.2
Ala	7.4 ± 0.2	7.0	6.7
Cys		(1.2)	(1.2)
Val	6.3 ± 0.2	5.8	6.1
Met	2.2 ± 0.1	1.9	2.0
Ile	5.3 ± 0.2	4.6	4.8
Leu	8.0 ± 0.3	7.0	7.1
Tyr	3.0 ± 0.1	2.3	2.5
Phe	3.1 ± 0.1	2.9	2.6
Trp	1.1 ± 0.0	(1.2)	(1.2)
Mol wt × 10^3	235–240	232	232
% P	3.4 ± 0.3	2.8	2.7

[a] Value expressed as mol % calculated from mol per 10^4 g of protein. [b] As in Table I. [c] Data of phosvitins (S_1S and S_3S) from Clark (1970). Data of lipovitellins (α and β) from Cook et al. (1962) and Burley and Cook (1961). [d] Calculated for each amino acid by: $[(MW_{S_1S} \times \text{mol \% } S_1S) + (MW_{S_3S} \times \text{mol \% } S_3S) + (MW_\alpha \times \text{mole \% } \alpha)]/(MW_{S_1S} + MW_{S_3S} + MW_\alpha)$. [e] Calculated for each amino acid by: $[(MW_{S_1S} \times \text{mol \% } S_1S) + (MW_{S_3S} \times \text{mol \% } S_3S) + (MW_\beta \times \text{mol \% } \beta)]/(MW_{S_1S} + MW_{S_3S} + MW_\beta)$.

other peptide bonds. (b) The in vitro labeling of vitellogenin and phosvitins (below) requires the dephosphorylation of the substrate by alkaline phosphatase prior to the radioactive labeling. Since only a very small proportion of the vacant sites are filled, the probability of ^{32}P label being in an intact cluster and surviving the partial hydrolysis is reduced. Other yolk phosphoproteins (lipovitellins) alone could not be the source since they contain 0.5 and 0.3% phosphorus respectively for α and β lipovitellin, and this is insufficient to account for the phosphoserine oligomer yield based upon phosphoserine oligomer recoveries of equal amounts of phosvitin.

3. Vitellogenin as Substrate for Phosvitin Kinase. Vitellogenin was tested for phosphorylation with phosvitin specific kinase to further confirm phosvitins as parts of the larger protein. Phosvitins and vitellogenin were partially dephosphorylated and then rephosphorylated with phosvitin kinase and [γ-^{32}P]ATP. The resultant incorporation of counts into Cl_3CCOOH precipitable protein (Table II) indicates that similar specific substrate sites are present in vitellogenin and phosvitin. The substrate specificities reported by Goldstein and Hasty (1973) have been confirmed for this preparation of phosvitin kinase.

Incorporation of counts was dependent upon enzyme and suitable substrate. Nondephosphorylated vitellogenin and phosvitins showed only slight incorporation. Poly(L-serine), however, is an unsuitable substrate so specificity must involve more than clustered serine. Highly dephosphorylated phosvitins (80–95%) are also poor acceptors for phosvitin kinase; they are insoluble at this degree of dephosphorylation and therefore may not be available and thus not directly comparable with poly(L-serine). Bovine serum albumin, protamine, and histone also failed to incorporate label. Partially dephosphorylated casein served as a less efficient acceptor for phosvitin kinase as expected. β-Lipovitellin also incorporated phosphate. It should be noted that the incorporation of phosphate into dephosphorylated vitellogenin and phosvitins is roughly proportional to the ratio of available serine sites in each. Only a small proportion (0.1%) of the dephosphorylated sites have been rephosphorylated.

The in vitro labeled vitellogenin and phosvitins from experiment 2, Table II, were each partially hydrolyzed and the hydrolysates subjected to paper electrophoresis as described previously. The electropherogram was sliced into 0.5-cm strips and counted. A series of ^{32}P phosphorylated serine clusters (Figure 5B) was found identical with the characteristic pattern of phosphoserine clusters of phosvitins and vitellogenin (Figure 5A). Thus phosvitin kinase is incorporating ^{32}P into similar clusters of phosphoserine in phosvitins and vitellogenin. In Experiment 3 all substrates were treated with alkaline phosphatase prior to phosphorylation. The higher background values for poly(L-serine) and protamine reflect correction of substrate concentration back to 1 mg/mL due to loss of these substrates upon dialysis.

Peptide maps of phosvitins cannot be easily constructed following the conventional methods such as enzymatic or cyanogen bromide cleavages. The reason is that, when phosphorylated, phosvitins are resistant to proteolysis and only one phosvitin contains a single methionine (Clark and Joubert, 1971). The phosphorylation also complicates the separation of peptides on the basis of charge since it has not been shown that phosvitins are identically phosphorylated. Extensive enzymatic dephosphorylation results in a product insoluble in aqueous media (Christmann, 1976). Short of direct primary sequence comparison, the three types of experiments reported in the present study seem to offer the strongest evidence that vitellogenin contains phosvitins. Our data are consistent with the model for vitellogenin structure proposed by Deeley et al. (1975) that vitellogenin contains two phosvitins (S_1S and S_3S) and either lipovitellin (α or β).

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Preparation and Properties of a New DNase from *Aspergillus oryzae*†

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ABSTRACT: A DNase present in commercial preparations of *Aspergillus oryzae* α-amylase was purified 1550-fold in 25% yield by acetone precipitation and by chromatography on diethylaminoethyl- and carboxymethylcellulose. The enzyme was isolated free of contaminating RNases and DNases. The molecular weight of the enzyme determined by gel filtration on Sephadex G-100 was 48 000, while a molecular weight of 58 000 was determined for the single band observed upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The isoelectric point of the DNase is 9.2. The enzyme hydrolyzed only DNA with a pH optimum of 8.2 and was activated by Co^{2+}, and to a lesser extent by Mg^{2+} and Mn^{2+}. Native DNA was a better substrate than heat-denatured DNA. Enzymatic digests of calf thymus and *E. coli* DNA yielded oligomers of chain lengths ranging from 10 to 200, with mono- and small oligonucleotides (chain length less than 5) detected only when large (100 mg) amounts of DNA were fractionated by column chromatography on diethylaminoethyl-Sephadex A-25 in 7 M urea. The digestion products contained 5′-terminal phosphate groups and mostly adenosine at the 3′ and guanosine and adenosine at the 5′ ends.

DNases are useful tools for the study of the structure of nucleic acids. Their application depends on the specificity of the particular nuclease. Thus, restriction enzymes cleave DNAs at a defined sequence of residues (Nathans and Smith, 1975). Conformation-specific enzymes such as S1 nuclease hydrolyze only single-stranded substrates (Ando, 1966; Vogt, 1973). Nonspecific nucleases may be used for a variety of purposes. For example, micrococcal nucleases, rodent liver endogenous endonuclease, DNase I, and DNase II have been used in many studies of chromatin structure (Clark and Felsenfeld, 1971; Gottesfeld et al., 1975; Hewish and Burgoyne, 1973; Noll, 1974a; Simpson and Whitlock, 1976a; Sollner-Webb et al., 1976). With such nonspecific nucleases, the influence of the physicochemical properties of the enzymes on their interaction with nucleoprotein substrates may also be studied.

As described here, we have purified a new DNase from commercial preparations of crude amylase from *Aspergillus oryzae*. The enzyme has catalytic properties similar to DNase I, but differs from it with respect to its molecular weight and isoelectric point of pH 9.2. The latter property facilitates the purification of the DNase by ion-exchange chromatography, giving a 1550-fold purification with a 25% yield.

This DNase differs from other enzymes (nucleases 0, S1, K1, and K2) derived from the same mold by one or more of the following parameters: molecular weight, isoelectric point, preferential hydrolysis of native rather than denatured DNA, or lack of activity toward RNA (Kato and Ikeda, 1968; Suzuki and Sakaguchi, 1974; Ando, 1966; Uozumi et al., 1968). The DNase can be used to generate oligonucleotides about 10 bases

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