Biochem. J. (1986) 240, 871–878 (Printed in Great Britain)

Chromatographic resolution of chicken phosvitin

Multiple macromolecular species in a classic vitellogenin-derived phosphoprotein

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Chicken phosvitin was prepared from egg yolk by a variety of published methods, including a modification of our own original procedure. Yolk granules and all phosvitin preparations have been previously found to contain major phosphoproteins at M_r 40000 and 33000 and minor satellite components when electrophoresed on polyacrylamide gradient gels and stained with Stains-all. However, only our current preparation contained three additional phosphoproteins (M_r 18000, 15000 and 13000) that are also present in yolk granules. Our current phosvitin preparation also appeared to have additional components when compared with other preparations by size-exclusion and anion-exchange chromatography. Particularly complex but entirely reproducible patterns were obtained by hydrophobic-interaction chromatography. However, a cross-referencing of fractions eluted by size-exclusion chromatography to the other procedures employed, including gel electrophoresis, reinforced the notion that unfractionated chicken phosvitin contains at least five major components, designated B, C, E_1, E_2 and F for the M_r 40000, 33000, 15000, 18000, and 13000 phosphoproteins, respectively. Stoichiometric considerations lead us to suggest that vitellogenin I gives rise to phosvitins C and F, vitellogenin II gives rise to phosvitin B, and vitellogenin III gives rise to either phosvitin E_1 or E_2, but not both. Thus, a fourth, as yet undetected, vitellogenin may exist for the chicken.

INTRODUCTION

Considerable evidence has accumulated over the last 20 years that in nonmammalian vertebrates a large, hepatically-derived lipophosphoprotein, vitellogenin, serves as the macromolecular precursor to the egg yolk proteins, lipovitellin and phosvitin (Wallace, 1978, 1985). In the chicken, lipovitellin and the highly phosphorylated phosvitin are found exclusively in the granule fraction of egg yolk (Burley & Cook, 1961; Wallace, 1965; MacKenzie & Martin, 1967). Three vitellogenins (I, II, and III) have thus far been isolated from chicken plasma and each can be distinguished by amino acid analyses, peptide cleavage patterns, and lack of immunological cross-reactivity (Wang & Williams, 1980; Wang *et al.*, 1983). Vitellogenins I and II contain about 2% protein-phosphorus, while the less abundant vitellogenin III contains only 0.76% protein-phosphorus, suggesting that a smaller or less phosphorylated phosvitin domain is present in this latter precursor. Thus far, two chicken phosvitin subpopulations with a similar protein-phosphorus content (10%) have been isolated and characterized (Connelly & Taborsky, 1961; Mok *et al.*, 1966; Taborsky & Mok, 1967; Clark, 1970), yet the available information implies that a third, less abundant, phosvitin may be present in the yolk granules. Further, the missing phosvitin is either smaller or less phosphorylated than the known phosvitins.

We have begun a search for the missing phosvitin (Wallace & Morgan, 1986). Yolk granules and phosvitin prepared by four different methods were found to contain two major phosphoproteins at M_r 40000 and 33000 together with a cluster of minor bands when electrophoresed on gradient polyacrylamide gels and stained with the cationic carbocyanine dye, Stains-all. However, only our current preparative procedure (Wallace & Morgan, 1986), which was developed to retain low-M_r species, also contained three additional phosphoproteins (M_r 18000, 15000 and 13000) that are also present in yolk granules. We here analyse these preparations further by a variety of chromatographic procedures and conclude that yolk granules contain at least five phosvitins. The relationship of the five phosvitins to the three known vitellogenins is also considered.

EXPERIMENTAL

Source materials

Chicken eggs were obtained from White Leghorn stock maintained at the University of Florida. Acrylamide and other electrophoresis-related chemicals were purchased from Bio-Rad. Phosvitin and Stains-all (1-ethyl-2-[3-(1-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-*d*]thiazolium bromide) were supplied by Sigma. Chemicals used for chromatography were 'Absolute Grade' reagents from Research Plus.

Phosvitin preparations

Phosvitin was prepared from egg yolk according to the procedures of Mecham & Olcott (1949), Joubert & Cook (1958) as modified by Clark (1970), and Wallace & Morgan (1986). Phosvitin purchased from Sigma was verified to have been prepared by the method of Sundararajan *et al.* (1960).

Gel electrophoresis

Protein samples and column effluents (after desalting) were electrophoresed on linear gradient slab gels and stained with Stains-all as previously indicated (Wallace & Morgan, 1986).

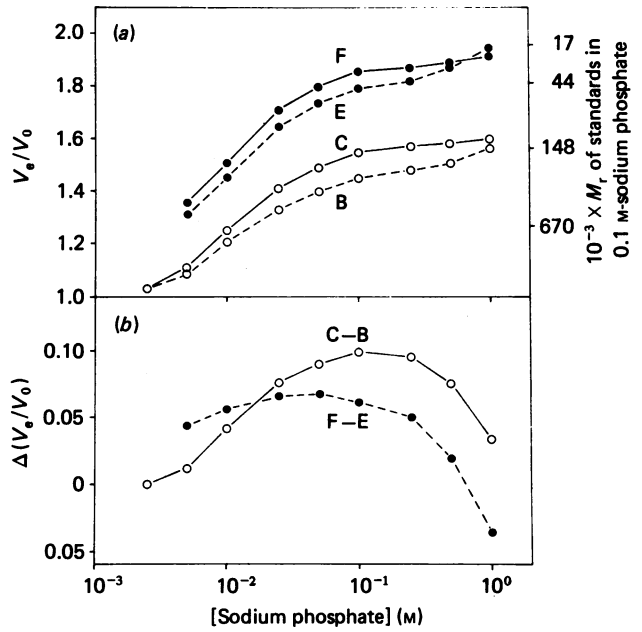

Fig. 1. Relative elution of phosvitin components by size-exclusion chromatography as a function of phosphate concentration

(*a*) Peak elution positions (V_e) for several phosvitin components in our current preparation are compared with the void volume (V_0; approx. 13 ml, as determined by low-density lipoprotein aggregates from egg yolk) at various concentrations of sodium phosphate, pH 6.8. (*b*) Resolution of components B from C, and E from F, is indicated as derived from the data in panel (*a*).

Chromatography

All chromatographic manipulations were performed on a Pharmacia FPLC system equipped with a dual chart recorder, 10-ml Superloop, monitors for 214 and 280 nm and containing high resolution flow cells, and a fraction collector. Two Pharmacia Superose 12 columns (10 mm × 300 mm each) were used in series for size-exclusion chromatography, an analytical Pharmacia Mono Q column (5 mm × 50 mm) for anion-exchange chromatography, and an analytical Bio-Rad Bio-Gel TSK Phenyl-5-PW column (7.5 mm × 75 mm) for hydrophobic-interaction chromatography. All solutions pumped onto the columns were filtered through 0.2 μm-pore filters and degassed by sonication. Protein samples were initially injected onto the column through 0.45 μm-pore nylon syringe filters (Micron Separations, Inc.). Sequential fractions eluted from the Superose columns were also either individually desalted on columns (25 mm × 90 mm) of Bio-Gel P-6DG equilibrated with water, evaporated to dryness and prepared for gel electrophoresis, or analysed via the Superloop by anion-exchange and hydrophobic-interaction chromatography.

RESULTS

Chromatographic resolution of phosvitin preparations

Phosvitin preparations were obtained from chicken yolk by the two most commonly used methods of Mecham & Olcott (1949) and Joubert & Cook (1958). Phosvitin, prepared by the method of Sundararajan *et al.* (1960), was also obtained commercially from Sigma Chemical Co. Finally, we modified our own general procedure for phosvitin purification (Wallace *et al.*, 1966) to obtain a fourth preparation (Wallace & Morgan, 1986).

Optimum conditions for size-exclusion chromatography of phosvitin were explored initially by eluting the preparation of Wallace & Morgan, which is the most complex (Wallace & Morgan, 1986), from Superose 12 columns in sodium phosphate buffers of various concentrations and pH values. At neutral pH, resolution of components B from C and components E from F (see Fig. 2*d* for nomenclature) was maximal at a phosphate concentration of around 0.1 M (Figs. 1*a* and 1*b*). Lowering of pH values below pH 5 resulted in later elution of all components without increased resolutions

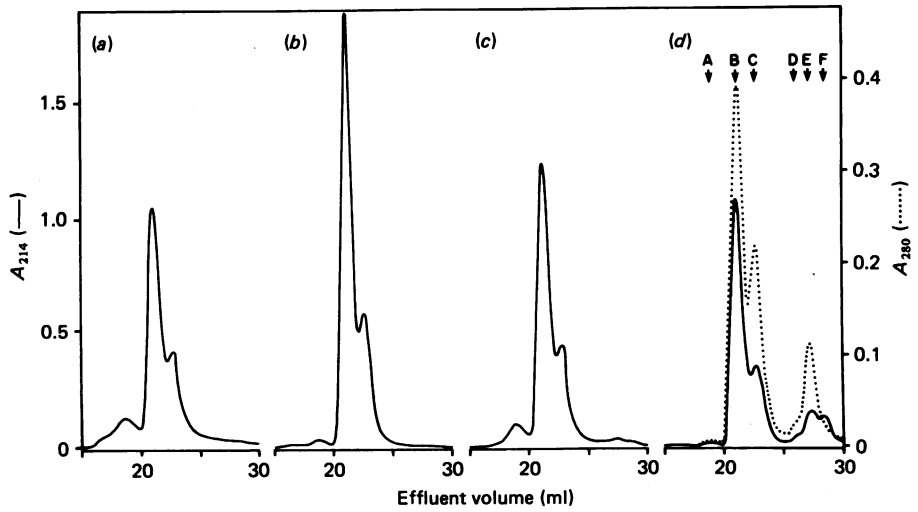

Fig. 2. Size-exclusion chromatography of several phosvitin preparations

Phosvitin was prepared according to (*a*) Mecham & Olcott (1949), (*b*) Joubert & Cook (1958), (*c*) Sundararajan *et al.* (1960) and (*d*) Wallace & Morgan (1986). Approx. 250–300 μg of protein was eluted with 0.1 M-sodium phosphate buffer, pH 6.8, at a flow rate of 0.5 ml/min.

(results not shown). Use of Tris-based buffers with variable NaCl concentrations led to the same conclusions: maximum resolution of components was achieved at neutral pH and at an ionic strength of around 0.2. Under these conditions, abnormally large M_r values were observed for all phosvitin components (Fig. 1*a*) compared with values determined by gel electrophoretic procedures (Wallace & Morgan, 1986). This effect was particularly pronounced at low ionic strength solutions and is never completely suppressed by phosphate buffer concentrations up to 1.0 M (Fig. 1*a*).

Size-exclusion chromatography of the four phosvitin preparations near optimum conditions is provided in Fig. 2. For our own preparation, six populations were indicated, designated A–F in order of their elution from the column (Fig. 2*d*). The six populations represented 1%, 61%, 22%, 1%, 8% and 7% of the total phosvitin applied, based on an integration of the areas under the 214 nm trace. Material eluting in the F-region did not appear to absorb light at 280 nm (Fig. 2*d*). Material eluting in the A-region was relatively more prominent in the Mecham & Olcott (Fig. 2*a*) and Sundararajan *et al.* (Fig. 2*c*) preparations. Traces of the E- and possibly F-populations were present in the Sundararajan *et al.* preparation (Fig. 2*c*), but these components were absent from both the Mecham & Olcott (Fig. 2*a*) and Joubert & Cook (Fig. 2*b*) preparations.

Anion-exchange chromatography yielded subpopulations eluting in three main regions, designated α, β, and γ for our preparation (Fig. 3*d*). The α-material was absent from the Mecham & Olcott (Fig. 3*a*) and Joubert & Cook (Fig. 3*b*) preparations, while a trace was present in the Sundararajan *et al.* preparation (Fig. 3*c*). The γ-material, however, was relatively more abundant in the Mecham & Olcott (Fig. 3*a*) and Sundararajan *et al.* (Fig. 3*c*) preparations. Attempts to resolve individual components of the α-subpopulation by use of a shallow salt gradient were relatively successful and indicated two peaks that absorbed light at 280 nm followed by several peaks that did not (Fig. 3*e*). A similar exercise for the β- and γ-subpopulations was unsuccessful, however, yielding an increased separation of the β- and γ-material from each other, but each subpopulation gave rise to irreproducibly jagged peaks (Fig. 3*f*). Nevertheless, heterogeneity could be indicated for at least the β-subpopulation by rechromatography of individual portions of the subpopulation indicated in Fig. 3(*d*) with

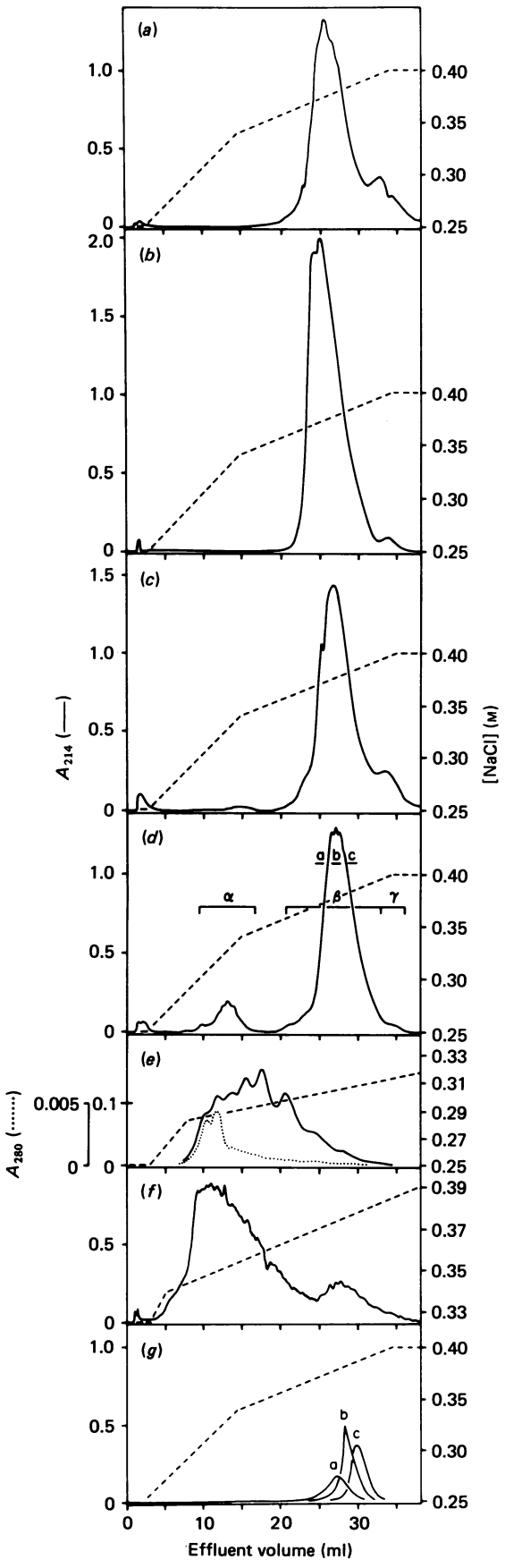

Fig. 3. Anion-exchange chromatography of several phosvitin preparations and subfractions

Phosvitin was prepared according to (*a*) Mecham & Olcott (1949), (*b*) Joubert & Cook (1958), (*c*) Sundararajan *et al.* (1960) and (*d*) Wallace & Morgan (1986). Shallow gradients were also used to resolve better the components present in (*e*) the α-region in our current preparation and (*f*) the β- and γ-regions in a Sundararajan *et al.* preparation. (*g*) The fractions indicated in (*d*) were diluted with an equal volume of water and rechromatographed with the original gradient. All samples were applied to a Mono Q column equilibrated with 50 mM-Tris/HCl, pH 7.5, containing 0.25 M-NaCl and were eluted with an increasing concentration of NaCl (indicated for the effluent) at a flow rate of 0.5 ml/min. Sample amounts: (*a*) 0.97 mg, (*b*) 1.25 mg, (*c*) 1.05 mg, (*d*) 0.98 mg, (*e*) 0.17 mg, (*f*) 1.26 mg.

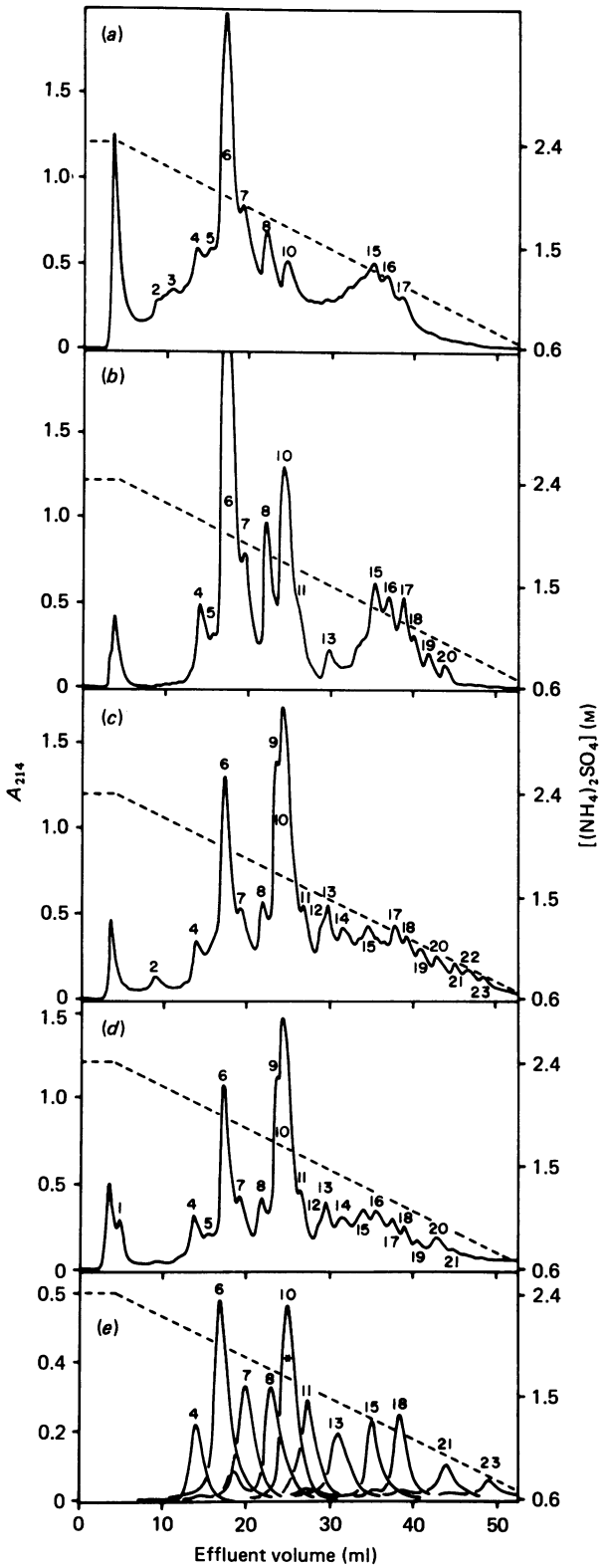

Fig. 4. Hydrophobic-interaction chromatography of several phosvitin preparations and subfractions

Phosvitin was prepared according to (*a*) Mecham & Olcott (1949), (*b*) Joubert & Cook (1958), (*c*) Sundararajan *et al.* (1960), and (*d*) Wallace & Morgan (1986). In addition, (*e*) eleven duplicate fractions from two runs of the Sundararajan *et al.* preparation were combined, adjusted to 2.4 M-$(NH_4)_2SO_4$, added to the column via the Superloop, and rechromatographed (the asterisk for peak 10 indicates that the absorbancy scale should be multiplied by two). All samples were applied to a Bio-Gel Phenyl-5-PW column equilibrated with 0.1 M-sodium phosphate, pH 6.8, containing 2.4 M-$(NH_4)_2SO_4$ and eluted with a decreasing gradient of $(NH_4)_2SO_4$ (indicated for the effluent) at a flow rate of 0.2 ml/min. The initial peak in each case is a procedural artifact. Approx. 2.0 mg of phosvitin was applied to the column in (*a*)–(*d*).

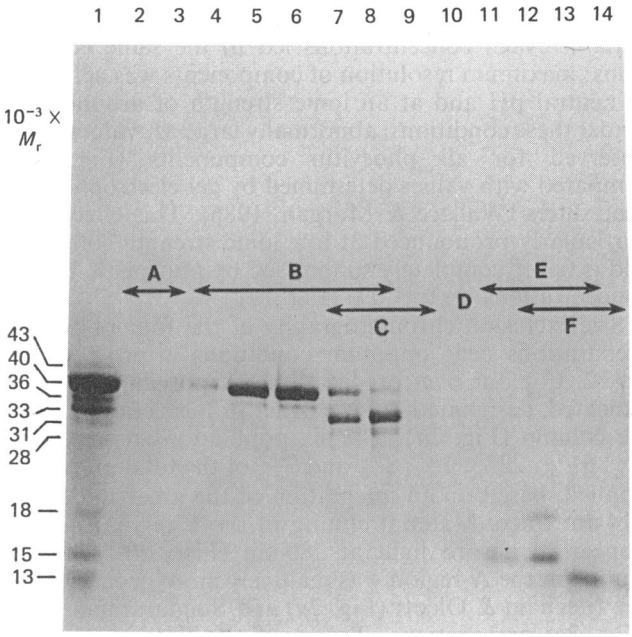

Fig. 5. Gradient gel electrophoresis of fractions obtained by size-exclusion chromatography

Approx. 1.0 mg of phosvitin prepared according to Wallace & Morgan (1986) was chromatographed as in Fig. 2(*d*) and individual 0.9 ml eluant fractions, representing components A–F, were desalted, dried on a vacuum centrifuge, and taken up with sample buffer. Lane 1 contained 20 μg of unfractionated phosvitin, lanes 2, 3 and 9–14 contained 27% of the original fractions, while lanes 4–8 contained 20%, 7%, 5%, 13% and 20% of the original fractions, respectively.

the original gradient: each fraction generally rechromatographed in the same position, albeit with considerable peak spreading (Fig. 3*g*).

Hydrophobic-interaction chromatography provided the most complex patterns, up to 23 different peaks being indicated in the various preparations (Fig. 4). Numerous individual differences in peak abundance could be found for the four preparations but, in general, the Mecham & Olcott (Fig. 4*a*) and Joubert & Cook (Fig. 4*b*) preparations, which were derived from whole yolk, and the Sundararajan *et al.* (Fig. 4*c*) and our own preparations (Fig. 4*d*), which were derived from isolated granules, most closely resembled one another, respectively. The complex patterns indicated were precisely reproducible, as determined over several dozen runs. Phosvitin prepared from individual eggs also provided the same patterns, indicating that the complexity was not due to genetic variation. Furthermore, individual peaks rechromatographed reproducibly, but when minor components were present, they always eluted earlier than the main peaks (Fig. 4*e*).

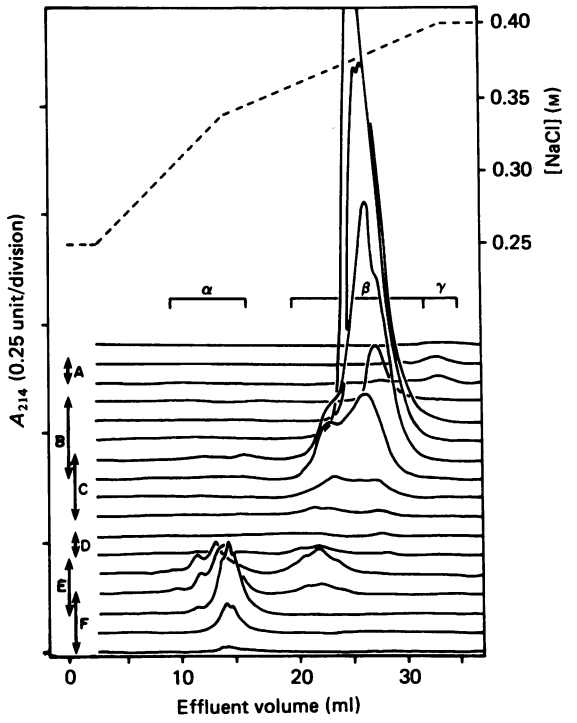

Fig. 6. Anion-exchange chromatography of fractions obtained by size-exclusion chromatography

Approx. 2.0 mg of phosvitin prepared according to Wallace & Morgan (1986) was chromatographed as in Fig. 2(*d*), but using 50 mM-Tris/HCl, pH 7.5, containing 0.25 M-NaCl, as the eluting buffer. Individual 0.7 ml eluant fractions, representing components A–F, were directly applied to a Mono Q column via the Superloop, and eluted with an increasing concentration of NaCl (indicated for the effluent) in 50 mM-Tris/HCl, pH 7.5, at a flow rate of 1.0 ml/min.

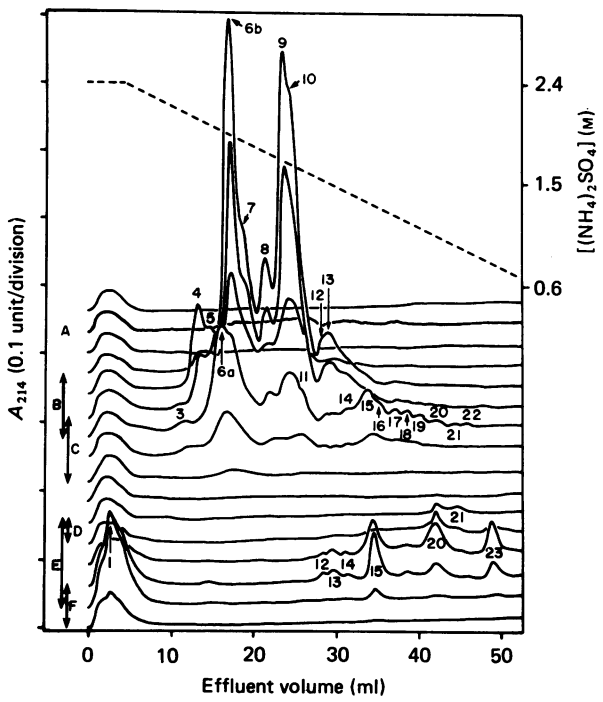

Fig. 7. Hydrophobic-interaction chromatography of fractions obtained by size-exclusion chromatography

Approx. 2.0 mg of phosvitin prepared according to Wallace & Morgan (1986) was chromatographed as in Fig. 2(*d*), but using 50 mM-Tris/HCl, pH 7.5, containing 0.25 M-NaCl, as the eluting buffer. Individual 0.7 ml eluant fractions, representing components A–F, were adjusted to 2.4 M-$(NH_4)_2SO_4$, applied to a Bio-Gel Phenyl-5-PW column previously equilibrated with 0.1 M-sodium phosphate, pH 6.8, containing 2.4 M-$(NH_4)_2SO_4$, and the column was eluted with a decreasing concentration of $(NH_4)_2SO_4$ (indicated for the effluent) in 0.1 M-sodium phosphate, pH 6.8, at a flow rate of 0.5 ml/min. The 'hump' at the beginning of each chromatograph represents Tris percolating through the column.

Some other attempts to resolve phosvitin components were unsuccessful. Reversed-phase chromatography with a gradient of acetonitrile in 0.1% trifluoroacetic acid or a gradient of methanol in 25 mM-triethylammonium acetate, pH 6.3, indicated complete retention or irreproducible elution of phosvitin components, respectively. Hydroxyapatite was also very retentive, and phosphate concentrations greater than 1.0 M were required to elute phosvitin as a single skewed peak.

Cross-reference of phosvitin components

In order to help elucidate the complexities revealed by our analytical methods, our own phosvitin preparations were subjected to size-exclusion chromatography (Fig. 2*d*) and the various fractions obtained were then analysed by the other procedures employed previously. As a first step, eluant fractions were individually desalted, lyophilized, and electrophoresed on gradient gels that were then fixed and incubated with Stains-all. The results (Fig. 5) indicated that the A-population contained a trace of the M_r 40000 component but, when overloaded, gave rise mostly to lane smearing (results not shown). The B-material was comprised of a major band at M_r 40000 and minor 43000 and 36000 components, C contained a major band at 33000 and minor 31000 and 28000 components, D could not be demonstrated, E contained the 18000 and 15000 components (with the latter eluting slightly before the former) and a trace of perhaps the 40000 component, while F gave rise only to the 13000 component.

Analysis of a similar set of fractions by anion-exchange chromatography (Fig. 6) revealed that the A-population exclusively gave rise to the γ-material. Early fractions of the B-population contained protein which eluted at 27 ml, but this was followed in later fractions by a large peak of protein at 25 ml that comprised the bulk of the β-material. As the B-population gave way to the C-population, a doublet emerged within the β-region. The small D-population also fell within the β-region and, as it gave way to the E-population, early-eluting components of first β- and then α-material emerged. The latter represents the 280 nm-associated peaks indicated in Fig. 3(*e*). Finally, the F-population emerged as that α-material indicated in Fig. 3(*e*) which was not associated with any absorbance at 280 nm.

Analysis of size-exclusion fractions by hydrophobic-interaction chromatography (Fig. 7) indicated no well-defined component within the A-population. For subsequent fractions, peak maxima emerged that were associated with each population. These were: for B, 4, 5, the later part of 6, 7–10, 12 and 13; for C, 3, the early part of 6, 11, and 14–22; for D, 21; for E, at first 20 and

Table 1. Relative abundance and cross-reference of chicken phosvitin components

The relative abundance of the six phosvitin components in the Wallace & Morgan (1986) preparation analyzed by size-exclusion chromatography was calculated by integrating the areas under the 214 nm trace in Fig. 2(*d*). The value (8%) found for component E was further subdivided into early- (E_1) and late-eluting (E_2) subfractions, which were evaluated from the 214 nm traces in Figs. 6 and 7 to be present in a 2:3 ratio, respectively. Each of the components indicated by size-exclusion chromatography was also correlated with entities found by other analytical procedures, including gel electrophoresis (Wallace & Morgan, 1986) and the other chromatographic methods described in this report.

Size-exclusion chromatography (Component)	Relative abundance (%)	Gel electrophoresis (M_r)	Anion-exchange chromatography (Region)	Hydrophobic-interaction chromatography (Peak)
A	1	–	γ	–
B	61	40000*	β(mid–late†)	4–10, 12, 13
C	22	33000‡	β(early–mid†)	3, 6, 11, 14–22
D	1	–	β(late)	21
E_1	3.2	15000	β(early§)	20, 23
E_2	4.8	18000	α(early‖)	12–15
F	7	13000	α(late¶)	1

* The major protein band indicated by Stains-all. Minor bands at M_r 36000 and 43000 are also present.
† At least two subpopulations are present (Fig. 6).
‡ The major protein band indicated by Stains-all. Minor bands at M_r 28000 and 31000 are also present.
§ At least three subpopulations are present (Fig. 6).
‖ At least two subpopulations associated with absorbance at 280 nm are present (Figs. 3*e* and 6).
¶ At least five subpopulations not associated with absorbance at 280 nm are present (Figs. 3*e* and 6).

23, and then 12–15; and finally for F, peak 1 which percolated through the column before the gradient started.

A summary of results found for phosvitin prepared by our current procedure is provided in Table 1, which cross-references the various phosvitin components we have observed by a variety of methods.

DISCUSSION

Each of the procedures we have used to analyse phosvitin subfractions has inherent advantages and limitations, especially with respect to phosvitin. Size-exclusion chromatography cannot be used in an absorptive mode and requires that the sample be applied as a high concentration in a small volume. It thus represents the ideal first procedure in a series of analyses. We have used size-exclusion chromatography here to separate phosvitin into groups of more or less decreasing size as they elute from the column. We have also varied the ionic strength of the buffer used from 0 to 2.0 mol/l and found that in water, all phosvitin components elute at the void volume (Fig. 1). As the ionic strength increased, the various components shifted to later elution positions and spread out in a complex manner, with optimum resolution being achieved at an ionic strength of about 0.2 mol/l. A plateau value for elution position was never reached for any of the components, however, so that we were never able to neutralize completely charge-repulsion phenomena by high salt concentrations. Thus, although size-exclusion chromatography has been used to estimate the size of some proteins, phosvitins elute anomalously early relative to standard proteins in reasonable buffer systems. As an example, an M_r of 299000 was calculated for the B-component in Fig. 2(*d*), a value considerably higher than the 40000 indicated for the major phosphoprotein in B after gel electrophoresis (Fig. 5). A similar problem was found when using Bio-Rad TSK size-exclusion columns. On the other hand, the bed materials used for size-exclusion chromatography, regardless of permeation properties, appear to be eminently suitable for desalting phosvitin preparations when equilibrated with water. As an aside, the E_1-component eluted earlier from the size-exclusion column than the E_2-component, even though it is somewhat smaller (Fig. 5). We attribute this to a greater degree of phosphorylation and hence charge-repulsion with the bed material. Elution of E_1 in the β-region and E_2 in the α-region during anion-exchange chromatography (Fig. 6) and the more pronounced staining of E_1 with Stains-all (Figs. 1 and 5), even though it is somewhat less abundant than E_2 (Figs. 6 and 7), are consistent with this interpretation.

A problem encountered during gel electrophoresis is that phosvitin behaves anomalously on straight-percentage gels, even in the presence of sodium dodecyl sulphate, yielding M_r estimates about half those determined by other methods (Ohlendorf *et al.*, 1977; Wiley & Wallace, 1981). However, we have found that the gradient-gel procedure introduced by Lambin (1978) for glycoproteins provided size estimates for *Xenopus* phosvitins which matched those determined by sedimentation equilibrium analysis (Wiley & Wallace, 1981), so we have used M_r values calculated by this procedure. However, a more stringent test can be applied here since the sequence of the major chicken phosvitin is now known (Byrne *et al.*, 1984). We have calculated a size for this sequence, added on the carbohydrate (Shainkin & Perlmann, 1971) and phosphate (Clark, 1970) associated with the 'major' component, and derived an M_r value of 32000. This value is 20% lower than that (40000) determined for the major band associated with the B component (Fig. 5), but is in excellent agreement with the value (31800; average of four determinations) obtained

by Clark (1970) using hydrodynamic procedures. We conclude that the absolute size estimates indicated here may be too large by as much as 20%, but that the relative sizes of the various phosvitin components may still hold, since these values would all be subject to approximately the same systematic error.

Anion-exchange chromatography separates substances according to their net negative charge. The Mono Q column we have used for this purpose provides separations similar to those achieved with DEAE-cellulose, using the same buffer systems (Richey, 1982). We have been able to achieve some satisfactory resolution of the smaller E_2- and F-components (Fig. 3*e*), but the B- and C-components are not adequately resolved, even with shallow salt gradients (Fig. 3*f*). A similar problem was noted by Connelly & Taborsky (1961) using DEAE-cellulose, and these authors as well as Belitz (1963) eventually resorted to a stepwise elution scheme in order to obtain phosvitin subfractions. The relatively poor resolution of phosvitin components experienced with anion-exchange columns may be inherent in phosvitin itself. Phosphorylation of protein, including the phosvitin precursor vitellogenin (Gottlieb & Wallace, 1981; Wang & Williams, 1982), is a post-translational process yielding a heterogeneous product. Since phosvitin would primarily bind to anion-exchange columns via its phosphate groups, some heterogeneity in binding characteristics would be expected and a resolution of the various phosvitin components into discrete peaks would be difficult if not impossible to achieve.

The most surprising and difficult to interpret finding in the present report is the numerous discrete peaks obtained with crude phosvitin by hydrophobic-interaction chromatography. This result was unexpected since earlier physical-chemical studies had indicated that unfractionated phosvitin behaves as a polyelectrolyte with an extensively unordered structure in benign solutions at neutral pH (Allgen & Norberg, 1959; Jirgensons, 1966; Perlmann & Allerton, 1966; Timasheff & Townend, 1967; Taborsky, 1968; Grizzuti & Perlmann, 1970; Perlmann & Grizzuti, 1971). However, a recent re-evaluation has revealed that component B contains an extensive secondary structure sensitive to environmental conditions (Renugopalakrishnan *et al.*, 1985). Multiple conformational states may be induced by the high concentration of $(NH_4)_2SO_4$ in which the samples are applied to the column (Ingraham *et al.*, 1985). Microenvironments experienced during hydrophobic interaction may also give rise to conformational changes similar to those observed during the freezing of phosvitin solutions (Taborsky, 1970) and which could subsequently specify elution conditions. Regardless of mechanism, the elution characteristics for a given phosvitin preparation are precisely reproducible, although there is some evidence that manipulation can generate new peaks. This evidence includes: (1) the generation of less hydrophobic components during rechromatography of isolated peaks (Fig. 4*e*), (2) the production of peak 23 material from component E_1 (Fig. 7) even though the original unfractionated sample does not give rise to peak 23 material (Fig. 4*d*), and (3) the generation of material in peaks 18–22 from component B (Fig. 7), while the Mecham & Olcott and the Joubert & Cook preparations (both of which contain component B) give rise to none or only some of these peaks, respectively (Figs. 4*a* and 4*b*). Future analyses of the various components resolved by hydrophobic-interaction chromatography will be needed to understand better the complex patterns generated.

In summation, we conclude that chicken phosvitin contains at least five major phosphoproteins that are present in unfractionated yolk granules. These are indicated on our gradient gels (Fig. 5) at M_r 40000 along with associated minor bands (component B), 30000 along with associated minor bands (component C), 18000 (component E_2), 15000 (component E_1), and 13000 (component F). Component A is most prominent in those preparations (Mecham & Olcott, Sundararajan *et al.*) that employ lipid extraction with organic solvents. Such preparations give rise to lane smearing during gel electrophoresis (Wallace & Morgan, 1986). Component A also cannot be resolved by either gel electrophoresis or hydrophobic-interaction chromatography. We therefore conclude that component A is a preparation artifact. Components B and C are essentially the only other components present in preparations other than our own. They thus correspond to the 'major' and 'minor' components, respectively, as defined by Clark (1970). Component D represents only 1% of our total phosvitin preparation (Fig. 2*d*, Table 1) and is completely absent from all other preparations (Figs. 2*a*–2*c*). We have not been able to resolve component D by gel electrophoresis (Fig. 5). Thus, there is insufficient evidence at present to indicate that it is a genuine component of chicken yolk granules. Components E and F are comprised of three small phosphopeptides that are present in yolk granules (Wallace & Morgan, 1986) and are only retained in our own preparations. They appear to be similar to the 'phosvettes' previously found to be present in amphibian yolk platelets (Wiley & Wallace, 1981).

Division of the relative amount of each major component (Table 1) by its relative size should yield an indication of molecular abundance, the ratios of which are 1.00:0.42:0.14:0.18:0.36 for components B, C, E_1, E_2 and F, respectively. A similar calculation for the data provided by Wang *et al.* (1983) indicates that vitellogenins I, II and III are present in the plasma in molar ratios of 0.31:1.00:0.09, respectively. Vitellogenin I and phosvitins C and F, vitellogenin II and phosvitin B, and vitellogenin III and phosvitins E_1 and E_2 are therefore present in roughly the same molar ratios in plasma and yolk, respectively. Vitellogenins I, II and III average 116, 116 and 44 phosphate groups/molecule, respectively (Wang *et al.*, 1983). Assuming the true size of each phosvitin is 80% of our values calculated from gradient gels and the average phosphorus content is 8.9% (Wallace & Morgan, 1986), the number of phosphate groups/molecule estimated for phosvitins B, C, E_1, E_2 and F is 92, 76, 41, 34 and 30, respectively. Thus vitellogenin I can accommodate both phosvitins C and F, vitellogenin II can accommodate phosvitin B, and vitellogenin III can accomodate phosvitin E_1 or E_2, but not both. We conclude from these considerations that a fourth, as yet undetected, vitellogenin may be present in the chicken at a level similar to that of vitellogenin III. A comparison of plasma vitellogenin and yolk phosvitin ratios assumes that all plasma vitellogenins are bound and sequestered by growing oocytes with the same avidity, which may not be the case. A further interesting complication arises from the recent indication that unfractionated phosvitin carries the ligand responsible

for receptor-mediated binding of unfractionated vitellogenin to oocyte membranes (Yusko *et al.*, 1981).

This work was supported by Grant GM 31606 from the National Institutes of Health, U.S.A.

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Received 10 June 1986/6 August 1986; accepted 9 September 1986

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