Song, P.-S. (1980) in *Excited States of Biomolecules*, Academic Press, New York (in press).
Song, P.-S., & Moore, T. A. (1968) *J. Am. Chem. Soc. 90*, 6507.
Song, P.-S., & Sun, M. (1974) in *Chemical and Biochemical Reactivity* (Pullman, B., & Bergmann, E., Eds.) p 407, Israel Academy of Sciences, Jerusalem.
Song, P.-S., Choi, J. D., Fugate, R. D., & Yagi, K. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) p 381, Elsevier, Amsterdam.
Sun, M., & Song, P.-S. (1973) *Biochemistry 12*, 4663.
Sun, M., Moore, T. A., & Song, P.-S. (1972) *J. Am. Chem. Soc. 94*, 1730.
Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) p 405, Elsevier, Amsterdam.
Wyatt, B. A. H. (1976) *Adv. Phys. Org. Chem. 12*, 132.
Yagi, K., & Matsuoka, Y. (1956) *Biochem. Z. 328*, 138.
Yagi, K., Okuda, J., Dmitrovskii, A. A., Honda, R., & Matsubara, T. (1961) *J. Vitaminol. 7*, 276.
Yagi, K., Ohama, H., Takahashi, Y., & Okuda, J. (1967) *J. Vitaminol. 13*, 191.

Identification, Purification, and Characterization of Two Distinct Avian Vitellogenins†

Sho-Ya Wang and David L. Williams*

ABSTRACT: Chicken vitellogenin has been resolved into two species (VTG I and VTG II) by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Both vitellogenins are present in plasma from laying hens and estrogen-treated roosters, but neither is found in plasma from nonstimulated roosters. Amino acid and phosphorus analyses showed that VTG I and VTG II differed markedly in their content of serine, leucine, valine, and isoleucine but were indistinguishable in their phosphorus content. Comparison of VTG I and VTG II by limited proteolysis mapping yielded no evidence of similarity in either nonphosphorylated or phosphorylated peptides. Peptides generated through CNBr cleavage also showed no evidence of similarity. When tested by direct precipitin analysis or double-diffusion analysis, VTG I showed no evidence of reactivity with antibody raised against VTG II. We conclude from these data that VTG I and VTG II are physiologically relevant species which are products of distinct vitellogenin genes. The observation of VTG I and VTG II, without exception, in large numbers of birds from an inbred flock argues in favor of two vitellogenin genes within the haploid chromosome set. Comparison of lipovitellins and vitellogenins through limited proteolysis mapping yields evidence of a specific and complex relationship between these proteins. This analysis indicates that VTG II gives rise to polypeptides in both α-lipovitellin and β-lipovitellin while VTG I gives rise to only α-lipovitellin polypeptides. Several tentative assignments for specific polypeptides can be made. VTG II, for example, appears to be the precursor of the 125 000-dalton polypeptide common to both α-lipovitellin and β-lipovitellin. In addition, the intermediate molecular weight polypeptides which are unique to α-lipovitellin appear to derive from VTG I. Furthermore, calculation of vitellogenin phosphorus using amino acid analysis for the estimation of protein mass indicates that neither VTG I nor VTG II can give rise to both yolk phosvitins. The uncertainties in this type of calculation are discussed.

The hepatic synthesis of vitellogenin is regulated by estrogenic hormones in birds and amphibians as well as in other egg-laying vertebrates [for reviews, see Bergink et al. (1974) and Tata (1976)]. In the hen, vitellogenin is transported to the ovary and deposited in the yolk fluid after proteolytic cleavage to α-lipovitellin, β-lipovitellin, and phosvitins. Both lipovitellins contain two or more polypeptide chains which arise from regions of vitellogenin containing little or no phosphorus (Bergink et al., 1974; Bos, 1975; Jost et al., 1975; Deeley et al., 1975). The heavily phosphorylated regions of vitellogenin give rise to at least two phosvitins (Clark, 1970; Bergink et al., 1974; Christmann et al., 1977). It is of obvious importance for studies of vitellogenin regulation and vitellogenin structure to know whether vitellogenin is a unique protein. In the present study we report that chicken vitellogenin can be resolved into two species by $NaDodSO_4$[1]–polyacrylamide gel electrophoresis. The vitellogenins have been purified and compared on the basis of amino acid and phosphorus compositions, peptide maps, immunological reactivity, and their relationship to the yolk lipovitellins in order to evaluate the significance of this observation. The results of these comparisons lead us to conclude that the two vitellogenins are distinct gene products which serve as precursors to different lipovitellin polypeptides.

Experimental Procedures

Hormone Treatment, Isolation of Vitellogenin, and Lipovitellins. White leghorn roosters (SPAFAS, Norwich, CT) were injected intramuscularly with diethylstilbestrol (50 mg/kg) in propylene glycol on days 0 (primary), 4 (secondary), and 8 (tertiary) and bled 3 days after the indicated injection. Vitellogenin was isolated from plasma after secondary DES stimulation by the DEAE-cellulose procedure as described by Deeley et al. (1975) with three modifications. (1) Blood was drawn from the heart under pentobarbital anesthesia, and plasma was prepared as described (Williams, 1979). (2) The bulk of the plasma very low density lipoprotein was removed

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; VTG I, vitellogenin I; VTG II, vitellogenin II; diethylstilbestrol (DES), *trans*-α,α′-diethyl-4,4′-stilbenediol.

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by ultracentrifugal flotation for 2 h at 2 °C at 195000*g*. (3) Buffers contained 100 μg/mL phenylmethanesulfonyl fluoride. α-Lipovitellin and β-lipovitellin were purified through two cycles of triethylaminoethylcellulose chromatography as described by Wallace (1965) except that buffers contained 100 μg/mL phenylmethanesulfonyl fluoride.

Isolation of VTG I and VTG II. As described below, VTG I and VTG II were partially resolved by the DEAE-cellulose chromatography used for vitellogenin purification. As a result, the leading 15–25% of the vitellogenin eluate was used for VTG I purification while the middle and late fractions were used for VTG II purification. Appropriate fractions were pooled, dialyzed against sterile water, lyophilized, and dissolved in the sample buffer of electrophoretic system A (Williams, 1979) for fractionation by preparative $NaDodSO_4$–polyacrylamide gel electrophoresis using the discontinuous buffer system of Laemmli (1970). A 2.3-cm plug gel of 40% acrylamide in lower gel buffer was polymerized in a 10 × 14 cm vertical slab gel apparatus (Model 220, Bio-Rad Laboratories, Richmond, CA) using 3-mm preparative spacers. The plug gel was overlaid with 7 mm of 50% glycerol to form the elution chamber prior to application of a 4% acrylamide separation gel and a 3% stacking gel by using a 25-mm surface-forming plate. The acrylamide/bis(acrylamide)ratio was 30:0.8 for all gels. Electrophoresis was at 110 V with tap water circulated through the apparatus to maintain the temperature at 21–25 °C. Elution was carried out at 1 mL/min with lower gel buffer lacking $NaDodSO_4$. The vitellogenins eluted between 8 and 10 h. Fractions were assayed for VTG I and VTG II by analytical $NaDodSO_4$–polyacrylamide gel electrophoresis as described below. Appropriate fractions were pooled, dialyzed against sterile water, and lyophilized. Good resolution of VTG I and VTG II occurred with sample loads up to 1.2 mg. Recoveries averaged 20–30% with most of the loss due to electrophoresis onto the plug gel surface on the lower face of the elution chamber. Greater recoveries but extensive dilution resulted with faster elution rates. The slower elution rate was generally used since the eluate fractions could be analyzed by analytical $NaDodSO_4$–polyacrylamide gel electrophoresis without prior concentration. The yields from several runs were generally pooled to obtain sufficient material for analytical procedures. Prior to analysis of the vitellogenins, the sample was dissolved in water and chromatographed on Sephadex G-100 equilibrated with water. This step was essential to eliminate a nondialyzable nonprotein contaminant which interferes with amino acid, phosphorus, and protein measurements. The contaminant is probably polymeric acrylamide since it yielded large quantities of NH_3 upon acid hydrolysis. Radiolabeled VTG I and VTG II were prepared in the same fashion from roosters which had received an intraperitoneal injection of 1 mCi of [^{32}P]orthophosphate (carrier free, New England Nuclear, Boston, MA) 18 h prior to sacrifice.

Amino Acid and Phosphorus Analyses. Vitellogenins were dissolved in constant boiling HCl containing 0.1% phenol, vacuum sealed, and hydrolyzed for 22 h at 110 °C. Amino acids were measured with a two-column instrument (Beckman 120 C) by employing α-amino-β-guanidinopropionic acid (basics) and norleucine (acidics and neutrals) as internal standards to assess absolute recoveries. Serine was corrected for destruction as described (Richardson et al., 1978). Phosphorus was measured in acid hydrolysates prepared as above by the method of Chen et al. (1956) using a commercial phosphorus standard (Sigma Chemical Co., St. Louis, MO). For these analyses parallel samples of hydrolysate were also analyzed for neutral and acidic amino acids as an absolute measure of protein mass. Hydrolysate samples were dried, dissolved in 1 N NaOH, boiled for 30 min, reacidified, and assayed for phosphorus to ensure that acid hydrolysis completely released protein phosphorus. No additional phosphorus was released. The presence of free phosphorus in vitellogenin preparations was tested by assays of nonhydrolyzed vitellogenin and by measurement of the acid-soluble fraction after precipitation of vitellogenin with trichloroacetic acid. Lipid phosphorus was tested by assays of alkaline hydrolysates of vitellogenin before and after extraction with appropriate solvents. Neither free nor lipid phosphorus was detected. The reported values for amino acids and phosphorus represent means of duplicate determinations on three preparations of VTG I and VTG II.

Partial Proteolysis and CNBr Cleavage. Stock solutions of VTG I and VTG II were prepared, and protein concentrations were determined by amino acid analysis. Partial proteolysis (Cleveland et al., 1977) was performed with *Staphylococcus aureus* V8 protease (Miles Laboratories, Elkhart, IN) at a 1:10 enzyme/substrate ratio (w/w). Digestions were at 37 °C in 0.125 M tris(hydroxymethyl)aminomethane (pH 6.8), 2% $NaDodSO_4$, 2.5% β-mercaptoethanol, and 10% glycerol for the indicated times. Reactions were stopped by boiling, and samples containing ~20 μg of protein were analyzed by electrophoresis on $NaDodSO_4$–10% polyacrylamide gels. For CNBr cleavage vitellogenin solutions were lyophilized and dissolved in 70% formic acid. CNBr was added in dimethylformamide to a 3000:1 molar ratio to methionine, and the sample was shaken for 24 h at 25 °C. The sample was lyophilized, washed with water, lyophilized, dissolved in sample buffer as above, boiled, and run on a $NaDodSO_4$–10% polyacrylamide gel. Several preparations of VTG I and VTG II have been analyzed by partial proteolysis and CNBr cleavage. The reported peptide patterns are highly reproducible.

Electrophoresis. Slab gel (10 × 14 cm) electrophoresis was at 30 V with the discontinuous buffer system of Laemmli (1970) using either 5 or 10% acrylamide in the separation gel and 3% acrylamide in the stacking gel. Molecular weight standards for 10% gels were ribonuclease (R, M_r 14 000), deoxyribonuclease I (D, M_r 31 000), ovalbumin (OA, M_r 43 000), serum albumin (CSA, M_r 65 000), conalbumin (Con, M_r 77 000), and phosphorylase A (Phos, M_r 97 400). Standards for 5% gels were CSA, Phos, β-galactosidase (M_r 116 200), myosin heavy chain (M_r 200 000), and cross-linked oligomers of phosphorylase A assumed to be the dimer (M_r 194 800), trimer (M_r 292 200), and tetramer (M_r 389 600) as described (Williams, 1979). Gels were stained with 0.1% Coomassie blue in 50% trichloroacetic acid for 2 h at 37 °C and destained in 7% acetic acid. Radioactive peptides were visualized by autoradiography of the dried gel at −70 °C using preflashed Kodak XR-5 film (Laskey & Mills, 1975) and a Cronex intensifying screen (Du Pont).

Immunological Procedures. Antibody to VTG II was raised in a New Zealand white rabbit by subcutaneous administration of antigen (300–500 μg) in Freund's complete adjuvant with booster injections on days 14 and 74. The rabbit was bled on day 94, and a crude γ-globulin fraction was prepared by precipitation from 40% ammonium sulfate. The antibody fraction was dialyzed into 0.02 M sodium phosphate (pH 7.4), 0.15 M NaCl, 0.016 M ethylenediaminetetraacetic acid, and 0.02% NaN_3 and stored at −70 °C. Direct precipitin analysis was carried out with 250 μg of antibody in 80 μL of 0.02 M sodium phosphate (pH 7.4), 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 100 μg/mL phenylmethane-

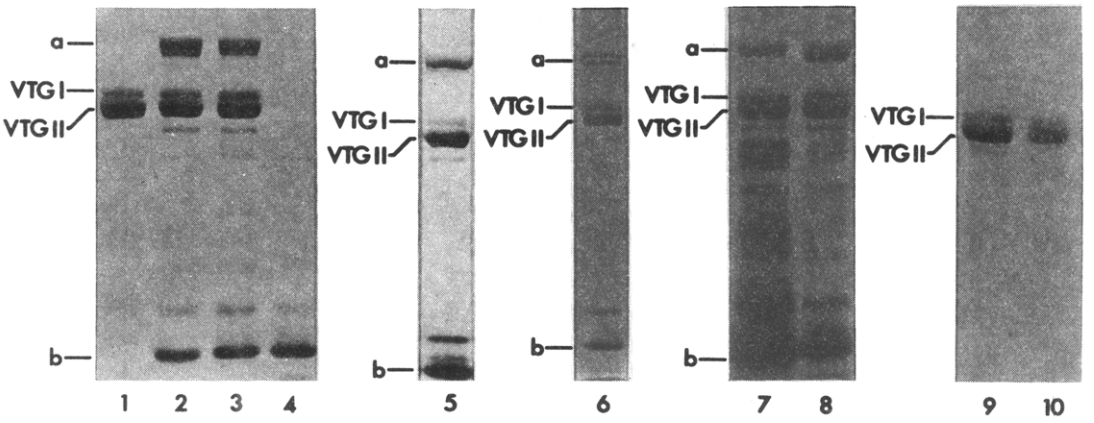

FIGURE 1: Occurrence of two vitellogenins. Purified vitellogenin and fresh plasma samples were run on $NaDodSO_4$–5% polyacrylamide slab gels. Gels 1–4 are adjacent lanes from one gel, gels 7 and 8 are adjacent lanes from a second gel, and gels 9 and 10 are adjacent lanes from a third gel. Gels 5 and 6 are separate gels. Gels 1–8 show Coomassie blue staining while gels 9 and 10 are autoradiographs. (Gel 1) Vitellogenin purified by DEAE-cellulose chromatography. This sample represents the entire phosphoprotein eluate corresponding to fractions 40–80 in Figure 2. (Gels 2, 3, and 8) 0.5 μL of plasma from three different roosters after secondary DES stimulation. (Gel 4) 0.5 μL of plasma from a nonstimulated rooster. (Gel 5) 1 μL of plasma after primary DES stimulation. (Gel 6) 0.25 μL of plasma from tertiary DES stimulation. (Gel 7) 8 μL of plasma from a laying hen. (Gel 9) [^{14}C]Vitellogenin marker. (Gel 10) 1 μL of plasma from a secondary stimulated rooster injected with [^{32}P]orthophosphate. Protein bands labeled a and b are apolipoprotein B of very low density lipoprotein and plasma albumin, respectively.

sulfonyl fluoride for 1 h at 0 °C. The precipitate was collected by centrifugation for 4 min at 10000*g*, washed 3 times with 1 mL of assay buffer, and dissolved in 0.1 N NaOH for measurement of protein (Lowry et al., 1951) with bovine albumin as the standard. Double-diffusion analyses were performed as described (Williams, 1979).

Results

Occurrence of Two Species of Vitellogenin. When purified vitellogenin was analyzed by electrophoresis in 5% polyacrylamide gels containing $NaDodSO_4$, vitellogenin was resolved into two species designated vitellogenin I (VTG I) and vitellogenin II (VTG II) (Figure 1, gel 1). Analysis of fresh plasma samples showed VTG I and VTG II in rooster plasma after primary (Figure 1, gel 5), secondary (Figure 1, gels 2, 3, and 8), and tertiary (Figure 1, gel 6) hormone stimulation as well as in plasma from the laying hen (Figure 1, gel 7). Neither VTG I nor VTG II was present in plasma from nonstimulated roosters (Figure 1, gel 4). Analysis of plasma from a diethylstilbestrol-stimulated rooster after in vivo labeling with [^{32}P]orthophosphate indicated that both VTG I and VTG II were heavily phosphorylated (Figure 1, gel 10). Visual comparison of the intensities of the protein stain and the autoradiograph suggested that VTG I and VTG II were phosphorylated to a similar extent.

The electrophoretic mobilities of VTG I and VTG II were highly reproducible in plasma samples from numerous animals. No differences in these mobilities were observed upon purification of the vitellogenins (Figure 1, gels 1–3). The molecular weights of VTG I and VTG II were estimated by comparison to the electrophoretic mobilities of serum albumin, β-galactosidase, myosin heavy chain, and the cross-linked oligomers of phosphorylase A as described (Williams, 1979). This analysis yielded apparent molecular weights of 260 000 and 246 000 for VTG I and VTG II, respectively.

Purification of Vitellogenin I and Vitellogenin II. Figure 2 shows the elution of bound phosphoprotein during the isolation of vitellogenin by DEAE-cellulose chromatography (Deeley et al., 1975). Electrophoretic analysis of the column eluate showed enrichment of VTG I in the lead fractions while VTG II predominated in the middle and late fractions (Figure 2). Rechromatography of early or late fractions did not yield further enrichment for VTG I or VTG II. Complete resolution of VTG I and VTG II was provided by preparative polyacrylamide gel electrophoresis. Figure 3 shows a typical preparative separation in which the sample load was taken from the VTG I enriched lead fractions of the DEAE eluate (Figure 3, lane 8). Analytical gels of the preparative gel eluate show only one fraction with detectable overlap (Figure 3, lane 4) while the early (Figure 3, lanes 1–3) and late (Figure 3, lanes 5–7) fractions are completely free of cross contamination with VTG I and VTG II, respectively.

Table I: Amino Acid Compositions of the Vitellogenins[a]

	VTG I	VTG II
Lys	8.4 ± 0.4	8.1 ± 0.2
His	3.4 ± 0.2	3.2 ± 0.1
Arg	6.3 ± 0.4	6.9 ± 0.2
Asp	9.6 ± 0.4	9.3 ± 0.4
Thr	5.1 ± 0.3	4.7 ± 0.2
Ser	14.2 ± 0.5	11.9 ± 0.4[b]
Glu	11.1 ± 0.5	10.8 ± 0.4
Pro	4.6 ± 0.3	4.8 ± 0.5
Gly	5.4 ± 0.5	5.0 ± 0.2
Ala	7.9 ± 0.4	7.7 ± 0.3
Val	7.2 ± 0.6	6.2 ± 0.5[b]
Met	1.7 ± 0.1	2.4 ± 0.5
Ile	4.5 ± 0.2	5.3 ± 0.4[b]
Leu	7.3 ± 0.4	8.3 ± 0.4[b]
Tyr	2.5 ± 0.2	2.8 ± 0.8
Phe	2.9 ± 0.3	2.6 ± 0.3

[a] Values shown represent mole percent ± SD from duplicate determinations on three preparations of VTG I and VTG II. Trp and Cys were not determined. [b] Statistical comparison of these residues between VTG I and VTG II showed Ser, Val, and Leu to differ at a probability level $P < 0.001$ and Ile at $P < 0.01$. Comparisons were made with a *t* test with arcsin transformation of percentages.

Amino Acid and Phosphorus Compositions of Vitellogenin I and Vitellogenin II. Table I shows the amino acid compositions of VTG I and VTG II as determined from 22-h hydrolysates. While this analysis reveals a considerable similarity in the overall compositions, several amino acids show striking differences. In particular, VTG I shows a greater content of serine and valine and a lesser content of isoleucine and leucine. These differences are highly significant (Table I). Comparison of phosphorus content was made by direct analysis of acid hydrolysates; a portion of the hydrolysate was assayed in

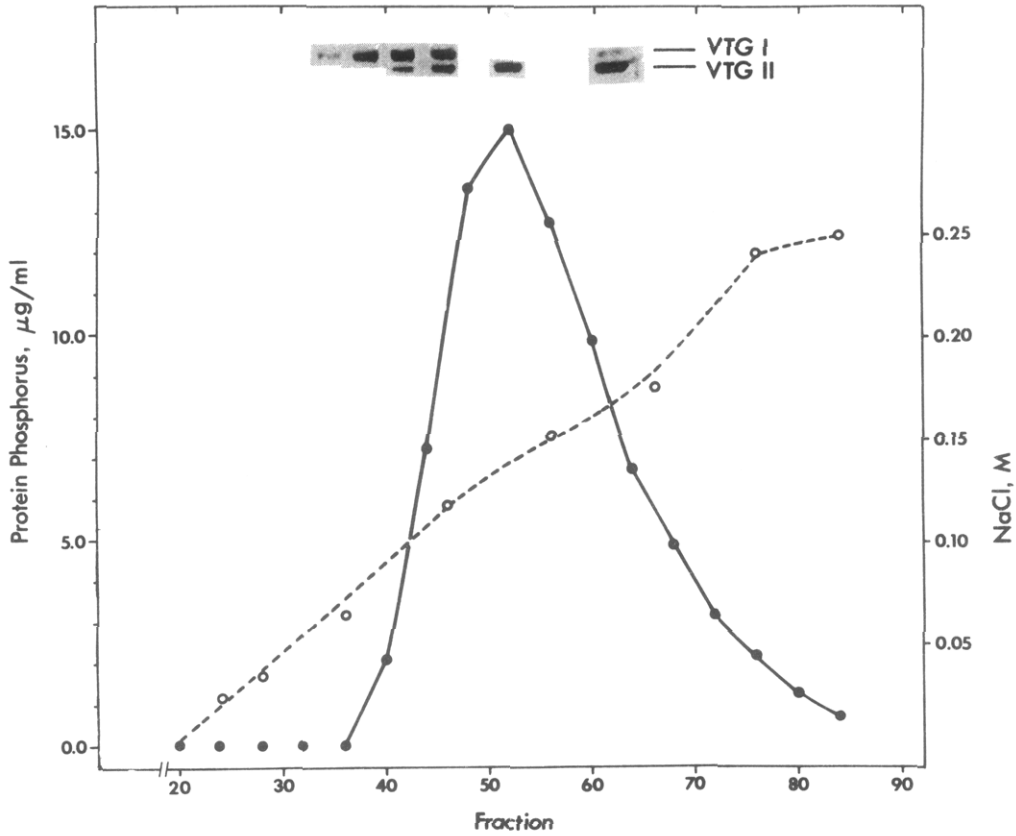

FIGURE 2: Partial resolution of two vitellogenins by DEAE-cellulose chromatography. Plasma from a secondary stimulated rooster was applied to the column, and unbound protein was washed through with load buffer. Vitellogenin was eluted with a shallow NaCl gradient (○), and column fractions were assayed for phosphorus (●) after hydrolysis with 1 N NaOH. Gels above the column profile show the distribution of VTG I and VTG II in different regions of the eluate. The gel shown in Figure 1 (gel 1) corresponds to the entire phosphoprotein eluate, fractions 40–80.

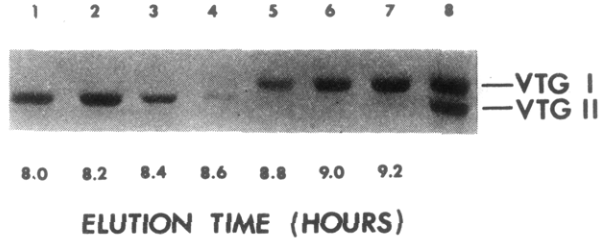

FIGURE 3: Complete resolution of two vitellogenins by preparative electrophoresis. The lead fractions from the DEAE eluate were run on a $NaDodSO_4$–4% polyacrylamide preparative gel as described under Experimental Procedures. Fractions from the preparative gel eluate were analyzed directly by $NaDodSO_4$–5% polyacrylamide slab gel electrophoresis (lanes 1–7). Lane 8 shows the sample load used for preparative electrophoresis. The gel was stained with Coomassie blue.

parallel for acidic and neutral amino acids as a measure of protein mass. Expression of the phosphorus measurements on the basis of three predominant amino acids which are equally common in VTG I and VTG II (aspartic acid, glutamic acid, and alanine, Table II) indicates no difference in the phosphorus content of VTG I and VTG II. The significantly lower phosphorus/serine ratio for VTG I (Table II) reflects the greater serine content of VTG I as compared to VTG II (Table I).

Partial Proteolysis of Vitellogenin I and Vitellogenin II. Digestion of VTG I and VTG II with V8 protease from *S. aureus* revealed virtually no similarities in intermediate or core peptides when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The kinetics of VTG II digestion indicate that products g and h are core peptides which are resistant to V8 protease (Figure 4, lanes 3–6). Peptides g and h are not

Table II: Phosphorus Content of the Vitellogenins[a]

	VTG I	VTG II
P/(Asp + Glu + Ala)	0.27 ± 0.04	0.28 ± 0.03
P/Ser	0.56 ± 0.07	0.69 ± 0.07

[a] Phosphorus content is shown as a molar ratio to Asp + Glu + Ala which are equally common in VTG I and VTG II and as a ratio to Ser which is not equally common in VTG I and VTG II. These values may be converted to weight percent phosphorus by summation of amino acid residue weights per 1000 mol of amino acid and expression of phosphorus as grams of phosphorus/(grams of amino acid + grams of phosphate). Mean residue weights are 112.8 and 112.0 for VTG I and VTG II, respectively. This calculation yields 2.0% phosphorus for VTG I and VTG II. This value is probably a slight overestimate since Trp, Cys, and carbohydrate are neglected. By use of molecular weights of 260 000 and 246 000, the maximum phosphorus contents of VTG I and VTG II are 167 and 159 mol/mol of protein, respectively. The uncertainties in this calculation are discussed in the text.

present in VTG I digests (Figure 4, lanes 1 and 2). Similarly, peptides c and d of VTG I (Figure 4, lanes 1 and 2) are not present in VTG II digests. Comparison of digestion intermediates shows similar differences. As examples, note that doublets e and f of VTG II (Figure 4, lanes 3–6) are absent from VTG I digests while peptides a and b of VTG I (Figure 4, lanes 1 and 2) are absent from VTG II digests. Numerous differences in less prominent peptides are also evident.

Analysis of [^{32}P]vitellogenin in this fashion shows that peptides e, f, g, and h of VTG II (Figure 5, lanes 3 and 4) and peptides c and d of VTG I (Figure 5, lanes 1 and 2) are not phosphorylated. Peptides a and b of VTG I (Figure 5, lanes 1 and 2) may contain traces of phosphorus. Comparison of phosphorylated products shows that peptides i, j, k, l, and m

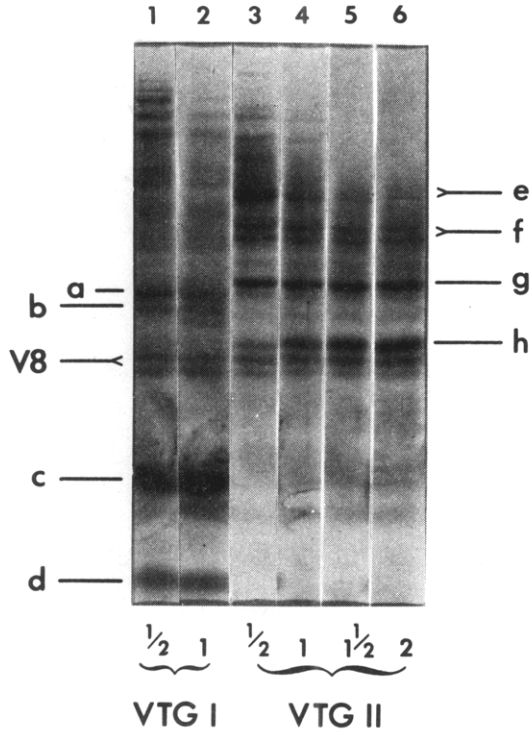

FIGURE 4: Limited proteolysis mapping of vitellogenin I and vitellogenin II. VTG I (lanes 1 and 2) and VTG II (lanes 3–6) were digested with V8 protease for the indicated times, and the digestion products were analyzed by $NaDodSO_4$–10% polyacrylamide slab gel electrophoresis. The gel was stained with Coomassie blue. The doublet labeled V8 is the protease used for digestion. Bands labeled a–h are described in the text.

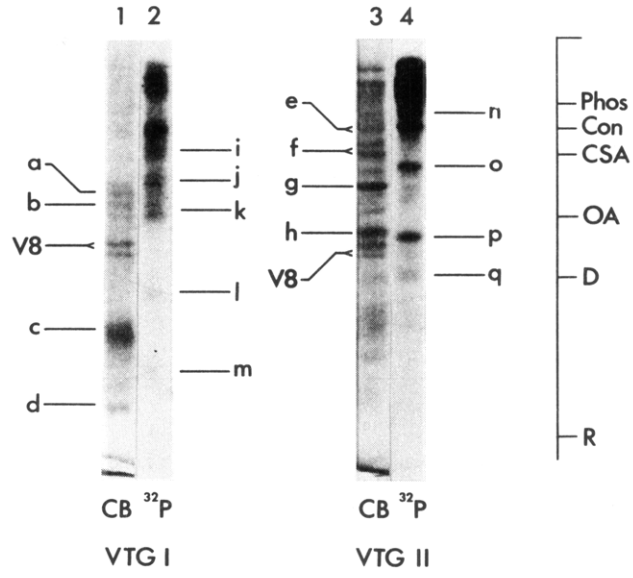

FIGURE 5: Comparison of phosphorylated and nonphosphorylated peptides generated through limited proteolysis. [^{32}P]VTG I (lanes 1 and 2) and [^{32}P]VTG II (lanes 3 and 4) were digested with V8 protease for 30 min, and the products were analyzed by $NaDodSO_4$–10% polyacrylamide slab gel electrophoresis. The gel was stained with Coomassie blue (CB), and an autoradiograph (^{32}P) was prepared. The gel was photographed in the dry state after autoradiography to ensure correct alignment of protein stain and radioactive bands. The doublet labeled V8 is the protease used for digestion. Bands labeled a–q are described in the text. The scale to the right shows the mobilities of molecular weight standards as described under Experimental Procedures.

of VTG I (Figure 5, lane 2) are not present in VTG II digests while peptides n, o, p, and q of VTG II (Figure 5, lane 4) are not present in VTG I digests. A point of interest from this analysis is that, in general, phosphorylated peptides do not stain or stain very faintly with Coomassie blue.

Cyanogen Bromide Cleavage of Vitellogenin I and Vitellogenin II. Analysis of CNBr cleavage products by $NaDodSO_4$–polyacrylamide gel electrophoresis revealed several distinct differences between VTG I and VTG II. In particular, VTG I yields a group of nonphosphorylated peptides designated region b (Figure 6, lanes 1 and 2) which are absent from the cleavage products of VTG II (Figure 6, lanes 3 and 4). Similarly, VTG II yields nonphosphorylated peptides designated region e (Figure 6, lanes 3 and 4) which are absent from the cleavage products of VTG I. Phosphorylated peptides a of VTG I (Figure 6, lane 1) and d of VTG II (Figure 6, lane 4) are also not common to both vitellogenins. The bulk of the phosphorylated peptides of VTG I and VTG II is present as a poorly resolved group in region c (Figure 6, lanes 1 and 4).

Immunological Comparison of Vitellogenin I and Vitellogenin II. Antibody raised against VTG II was tested for reactivity toward VTG I and VTG II by direct precipitin analysis. As shown in Figure 7, titration with VTG II yielded a precipitin curve with equivalence at 2 μg of VTG II. In contrast, anti-VTG II showed little or no reactivity toward VTG I. When tested by immunodiffusion analysis at an antigen/antibody ratio corresponding to VTG II equivalence, VTG I did not yield a precipitin line (data not shown).

Relationship of Vitellogenin I and Vitellogenin II to the Yolk Lipovitellins. Comparsion of VTG I, VTG II, α-lipovitellin, and β-lipovitellin by partial proteolysis with V8 protease revealed a specific and complex relationship between the plasma vitellogenins and the yolk lipovitellins. Peptides b, c, and d of VTG I (Figure 8, lane 1) are present in α-lipovitellin

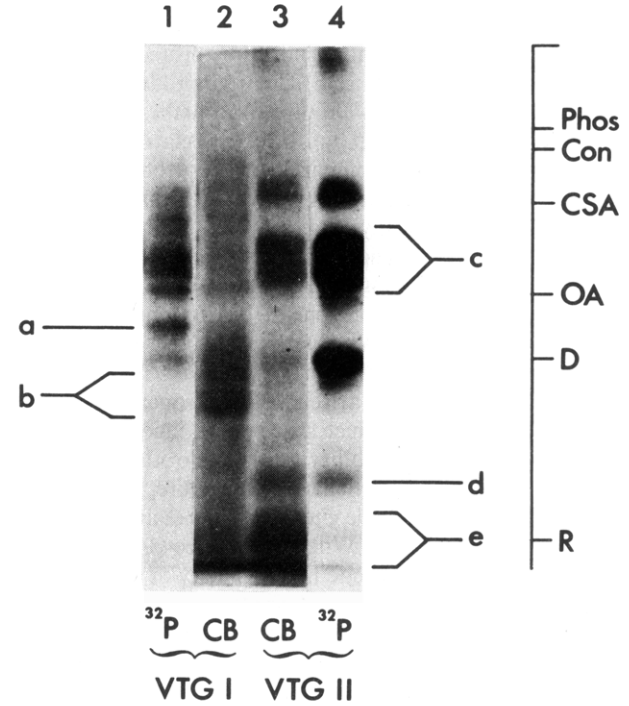

FIGURE 6: Comparison of vitellogenin I and vitellogenin II through CNBr cleavage. [^{32}P]VTG I (lanes 1 and 2) and [^{32}P]VTG II (lanes 3 and 4) were cleaved with CNBr, and the products were analyzed by $NaDodSO_4$–10% polyacrylamide slab gel electrophoresis. The gel was stained with Coomassie blue (CB), and an autoradiograph (^{32}P) was prepared. The gel was photographed in the dry state after autoradiography to ensure correct alignment. Bands labeled a–e are described in the text. Molecular weight standards are as in Figure 5.

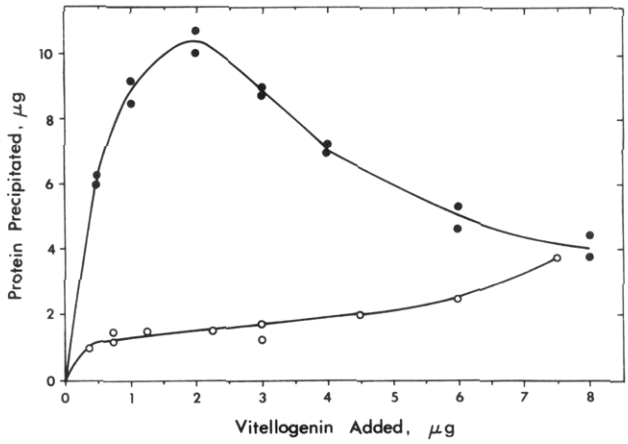

FIGURE 7: Immunological comparison of vitellogenin I and vitellogenin II. VTG I (○) and VTG II (●) were reacted with antibody against VTG II as described under Experimental Procedures.

(Figure 8, lanes 3 and 6) but are absent in digests of β-lipovitellin (Figure 8, lanes 4 and 7) and VTG II (Figure 8, lanes 2 and 5). Intermediate peptides e and f and core peptides g and h of VTG II (Figure 8, lanes 2 and 5) are present in both α-lipovitellin (Figure 8, lanes 3 and 6) and β-lipovitellin (Figure 2, lanes 4 and 7) but are absent in VTG I digests (Figure 8, lane 1).

Discussion

The results of this study document the occurrence of two distinct vitellogenins. It is clear from four lines of evidence that VTG I and VTG II are fundamentally different molecules. They differ not only in amino acid composition (Table I) but also are markedly different as judged by proteolytic (Figures 4 and 5) and CNBr (Figure 6) cleavage products as well as immunological properties (Figure 7). Furthermore, VTG I and VTG II show specific but different relationships to the yolk lipovitellins when compared by V8 protease mapping (Figure 8). We conclude from these data that VTG I and VTG II are physiologically relevant species which are products of distinct vitellogenin genes. Wahli et al. (1979) have recently reported multiple vitellogenin genes in the amphibian as a result of a direct analysis of cloned DNA fragments generated from vitellogenin mRNA. As noted by these workers, an uncertainty in their conclusion was that one main group of DNA clones could have arisen from estrogen-induced but nonvitellogenin mRNAs which share some sequence homology. They also noted the possibility that some of the vitellogenin mRNAs might be nonfunctional and, therefore, not translated. In the present study multiple vitellogenin genes are documented at the level of the protein. Wiley & Wallace (1978) have also demonstrated electrophoretic heterogeneity in amphibian vitellogenin. Estrogen-stimulated vitellogenin synthesis, therefore, appears to involve the regulation of multiple vitellogenin genes in both species. The occurrence of only two distinct vitellogenins in the chicken may reflect either heterozygosity or multiple genes within the haploid chromosome set. Since we have observed, without exception, both VTG I and VTG II in a large number of birds (>50) from an inbred flock, the latter possibility is more likely. Furthermore, our data by no means eliminate the possibility of other, yet undetected, avian vitellogenins.

A point of interest is the extent of difference between VTG I and VTG II. Mapping with V8 protease, for example, is particularly sensitive for detecting minor differences between proteins which are closely related, as through a precursor–product relationship (Cleveland et al., 1977). This technique

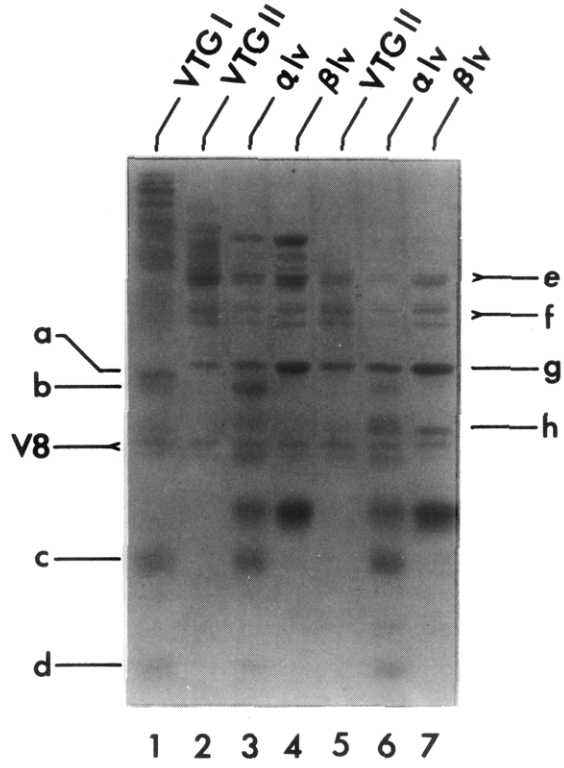

FIGURE 8: Comparison of vitellogenins and lipovitellins by limited proteolysis mapping. VTG I (lane 1), VTG II (lanes 2 and 5), α-lipovitellin (αlv, lanes 3 and 6), and β-lipovitellin (βlv, lanes 4 and 7) were digested with V8 protease for 30 min (lanes 1–4) or 60 min (lanes 5–7). The digestion products were analyzed by $NaDodSO_4$–10% polyacrylamide slab gel electrophoresis, and the gel was stained with Coomassie blue. The doublet labeled V8 is the protease used for digestion. Bands labeled a–h are the same as those illustrated in Figures 4 and 5.

not only shows extensive differences between VTG I and VTG II but also fails to yield evidence of any nonphosphorylated or phosphorylated peptides common to both vitellogenins (Figures 4, 5, and 8). Similarly, immunological data suggest little or no homology between VTG I and VTG II (Figure 7). Nevertheless, both proteins are clearly vitellogenins as judged by physical properties, regulation by estrogens, and relationships to yolk lipovitellins. Interestingly, the amphibian vitellogenin genes identified by Wahli et al. (1979) fell into two main groups which showed little homology as judged by annealing and melting properties. The occurrence of such divergent vitellogenins raises interesting questions about structure–function relationships in these proteins. Extensive divergence might be explained, for example, if the primary sequence in large portions of vitellogenin were not crucial for function. Such a situation may be suggested since the fate of vitellogenin is to undergo an apparently nonspecific hydrolysis to provide amino acids, phosphate, and phosphate-chelated metals for embryonic development. If this is the case, then any regions of significant homology between VTG I and VTG II may reflect sequences involved in hepatic processing, uptake into oocyte, and cleavage to lipovitellin and phosvitin polypeptides.

The V8 protease maps of the vitellogenins and lipovitellins indicate a specific and complex relationship between these proteins (Figure 8). The occurrence of V8 cleavage products b, c, and d in α-lipovitellin and VTG I but not in VTG II and β-lipovitellin suggests that some α-lipovitellin polypeptides are derived strictly from VTG I. Similarly, the occurrence of V8 cleavage products e, f, g, and h in VTG II, α-lipovitellin, and β-lipovitellin but not in VTG I suggests that some polypeptides

common to both lipovitellins are derived strictly from VTG II. This overall relationship is supported by immunological data which show that VTG I and VTG II both react with antibody against α-lipovitellin while only VTG II reacts with antibody against β-lipovitellin (unpublished experiments). Several tentative assignments for specific lipovitellin polypeptides may also be suggested from the data of Figure 8. Previous studies (Bergink et al., 1974; Bos, 1975; Jost et al., 1975) have shown that α-lipovitellin and β-lipovitellin both contain polypeptides of molecular weights 125 000 (125K) and 30 000 (30K) while α-lipovitellin also contains several polypeptides of intermediate molecular weights. The lipovitellins employed in the present study also show polypeptides of these molecular weights when analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (unpublished experiments). Since cleavage products e, f, g, and h of β-lipovitellin (Figure 8) exhibit molecular weights greater than the 30K polypeptide, these products must originate from the 125K polypeptide. The presence of these cleavage products in VTG II digests, therefore, indicates VTG II as the origin of the 125K polypeptide of β-lipovitellin. The occurrence of a 125K polypeptide in α-lipovitellin and the presence of cleavage products e, f, g, and h in α-lipovitellin digests similarly indicate VTG II as the origin of this polypeptide. Cleavage products b, c, and d of α-lipovitellin are not present in β-lipovitellin digests and, therefore, must arise from the α-lipovitellin polypeptides of intermediate molecular weight. These intermediate molecular weight polypeptides, therefore, must derive from VTG I since cleavage products b, c, and d are found only in this vitellogenin. While these assignments should be considered tentative, further application of appropriate mapping and immunological procedures should permit complete assignment of each lipovitellin polypeptide to the precursor vitellogenin from which it arose.

Gordon et al. (1977) constructed a model in which a single 240 000-dalton vitellogenin precursor yields either α-lipovitellin or β-lipovitellin through different sites of proteolytic cleavage in the precursor. In contrast, we interpret the present results to indicate that α-lipovitellin and β-lipovitellin contain different sets of polypeptides (some members of which are shared) which are derived from at least two vitellogenin precursors. The model of Gordon et al. (1977) also described both yolk phosvitins as arising from a single vitellogenin precursor. Measurements of vitellogenin phosphorus reported here are not consistent with the model. The maximum phosphorus content of VTG I and VTG II is 167 and 159 mol/mol of protein, respectively (Table II), by employing amino acid analysis to estimate protein mass. These calculations indicate that neither VTG I nor VTG II can accommodate the 210–220 mol of phosphorus present in both phosvitins plus either α-lipovitellin or β-lipovitellin (Burley & Cook, 1961; Wallace, 1965; Clark, 1970, 1973; Clark & Joubert, 1971; Gordon et al., 1977). It should be emphasized, however, that these calculations and those of others (Deeley et al., 1975; Gordon et al., 1977) are heavily dependent on the accuracy of $NaDodSO_4$–polyacrylamide gel electrophoresis for measurement of vitellogenin molecular weight. In addition to uncertainty in extrapolating outside the molecular weight range of available standards, the accuracy of these measurements is not well tested for a protein containing enormous numbers of phosphorylated residues. Estimates of the number of phosvitins per vitellogenin via phosphorus measurements, therefore, may be unreliable. Interestingly, VTG I and VTG II yield very different patterns of phosphorylated peptides when cleaved with V8 protease (Figure 5) or CNBr (Figure 6). These results suggest that VTG I and VTG II may contain different phosvitins. Additional studies will be required to determine if this is the case and whether VTG I and VTG II serve as precursors to the specific yolk phosvitins which have been identified (Clark, 1970).

References

Bergink, E. W., Wallace, R. A., Van de Berg, J. A., Bos, E. S., Gruber, M., & AB, G. (1974) *Am. Zool. 14*, 1177.

Bos, E. S. (1975) Ph.D. Thesis, The University of Groningen, Groningen, The Netherlands.

Burley, R. W., & Cook, W. H. (1961) *Can. J. Biochem. Physiol. 39*, 1295.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756.

Christmann, J. L., Grayson, M. J., & Huang, R. C. C. (1977) *Biochemistry 16*, 3250.

Clark, R. C. (1970) *Biochem. J. 118*, 537.

Clark, R. C. (1973) *Biochim. Biophys. Acta 310*, 174.

Clark, R. C., & Joubert, F. J. (1971) *FEBS Lett. 13*, 225.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102.

Deeley, R. G., Mullinix, K. P., Wetekam, W., Kronenberg, H. M., Meyers, M., Eldridge, J. D., & Goldberger, R. F. (1975) *J. Biol. Chem. 250*, 9060.

Gordon, J. I., Deeley, R. G., Burns, A. T. H., Paterson, B. M., Christmann, J. L., & Goldberger, R. F. (1977) *J. Biol. Chem. 252*, 8320.

Jost, J.-P., Pehling, G., & Baca, O. G. (1975) *Biochem. Biophys. Res. Commun. 62*, 957.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Richardson, W. S., Munksgaard, E. S., & Butler, W. T. (1978) *J. Biol. Chem. 253*, 8042.

Tata, J. R. (1976) *Cell 9*, 1.

Wahli, W., Dawid, I. B., Wyler, T., Jaggi, R. B., Weber, R., & Ryffel, G. U. (1979) *Cell 16*, 535.

Wallace, R. A. (1965) *Anal. Biochem. 11*, 297.

Wiley, H. S., & Wallace, R. A. (1978) *Biochem. Biophys. Res. Commun. 85*, 153.

Williams, D. L. (1979) *Biochemistry 18*, 1056.