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Purification and Characterization of a Non-Vitellogenin, Estrogen-Induced Plasma Protein from the American Bullfrog *Rana catesbeiana*†

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ABSTRACT: A non-vitellogenin, estrogen-induced protein has been detected for the first time in the plasma of male *Rana catesbeiana*. A >90% purification of this plasma protein was achieved by salt fractionation with Mg(II) followed by ion-exchange chromatography on DEAE- and CM-cellulose. Immunoelectrophoretic analysis with various antisera showed no immunological cross-reactivity between this protein and vitellogenin. The molecular mass of the purified protein was determined to be 116 000 daltons by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and 105 000 daltons by analytical ultracentrifugation. Sedimentation studies indicate the protein is a nonaggregating spherical monomer with a sedimentation coefficient of 7.5 S. Amino acid analysis demonstrated a composition different from that of vitellogenin and lipovitellin A. Limited proteolysis with trypsin, chymotrypsin, and *Bacillus subtilis* protease revealed no common peptides on SDS-polyacrylamide gels. Phosphate analysis indicated that, on a molar basis, the non-vitellogen, estrogen-induced protein had ≤3% of the phosphate found in vitellogenin. Further studies of the structure, function, and metabolism of this protein may reveal information relating to the hormonal control of vitellogenesis.

Vitellogenin (Vg) is a plasma protein synthesized by the liver of female oviparous vertebrates in response to seasonal increases of estrogen. During transit through the hepatocyte, the Vg is glycosylated and acquires noncovalently bound lipid. The Vg secreted by the liver is removed from the bloodstream and subsequently processed by oocytes into the egg yolk proteins phosvitin and lipovitellin (Bergink & Wallace, 1974). The hormonal regulation of vitellogenesis has been widely studied in avian and amphibian species [e.g., see Tata & Smith (1979) and Banaszak et al. (1982)]. Amphibians offer specific advantages in that they undergo a metamorphosis during development and do not require calcium mobilization for egg shell production during the vitellogenic response, and being poikilotherms, the metabolic events associated with vitellogenesis occur over a longer period of time.

Wallace & Jared (1968) demonstrated that vitellogenesis can be induced in male frogs, which do not normally synthesize Vg, by a single estrogen injection. Such an approach allows one to measure the time sequence and magnitude of the molecular events associated with vitellogenesis against a zero background. For example, Baker & Shapiro (1977) have shown that the mRNA for Vg is undetectable in the liver of unstimulated male *Xenopus laevis*, but following estrogen administration, the mRNA Vg increases at a linear rate for 12 days to >30 000 copies per cell. As a consequence of the high levels of mRNA, large amounts of Vg are synthesized and secreted into the plasma. In male animals, Vg accumulates because it cannot be sequestered and processed by oocytes. Vg levels of 40-60 mg/mL of plasma have been demonstrated in male frogs 10-14 days after a single estrogen injection (Follett & Redshaw, 1968; Wallace, 1970; Hess, 1981).

Most studies of amphibian vitellogenesis have utilized the South African clawed toad *Xenopus laevis*. In our laboratory, we have used the American bullfrog *Rana catesbeiana* to investigate the metabolism of plasma proteins. We have purified bullfrog Vg by two independent methods and have shown that it contains two peptides having molecular masses of 185 000 and 195 000 daltons (Da) (Hess, 1981). Vitellogenin purified from *Xenopus* has a similar molecular mass (Wiley

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& Wallace, 1978; Hess, 1981).

In general, the purified Vg has been used to prepare antibodies which have then been used to investigate Vg synthesis and secretion [e.g., see Huber et al. (1979), Gottlieb & Wallace (1982), and Herbener et al. (1984)]. In addition to preparing antibodies to purified vitellogenin from *Rana catesbeiana*, we also immunized rabbits with plasma obtained from male frogs 2 weeks after estrogen induction. Antibodies cross-reacting with normal frog plasma proteins were removed by immunoaffinity chromatography. This approach allowed us to determine the presence of Vg and other estrogen-induced proteins in plasma. In this report, we describe the purification of a previously unknown, non-vitellogenin plasma protein obtained from estrogen-induced male *Rana catesbeiana*.

Materials and Methods

Agarose was purchased from Bio-Rad Laboratories (Richmond, CA). Sepharose and dextran 10000 were from Pharmacia (Piscataway, NJ). Whatman CM-52 and DE-52 celluloses and Sequanal-grade Edman reagents were obtained from Pierce Chemical Co. (Rockford, IL). Freund's complete adjuvant was purchased from Difco Laboratories (Detroit, MI). Trypsin, chymotrypsin, and *Bacillus subtilis* protease were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal Procedures. Male *Rana catesbeiana* bullfrogs, 5–7 in. in length and weighing 300–500 g, were obtained from the Charles D. Sullivan Co. (Nashville, TN). The frogs were injected in the thigh (intramuscular) with 17β-estradiol in propylene glycol (20 mg/mL) at a dosage of 40 μg/g body weight. The animals were maintained in fresh water at 23 °C. Fifteen days after estrogen injection, 5–7 mL of blood was removed from each frog by cardiac puncture. The blood was pooled in the presence of heparin (about 0.3 mg/mL) to prevent clotting. Plasma was obtained by centrifugation at 16000*g* for 10 min. The plasma from the first separation was subjected to a second centrifugation and used immediately or stored at −20 °C.

New Zealand white rabbits were immunized with an emulsion prepared by sonicating a mixture of 1.0 mL of bullfrog plasma and 1.5 mL of Freund's complete adjuvant. The rabbits were injected subcutaneously at 0, 4, and 6 weeks. Blood was obtained by cardiac puncture 2 weeks after the last injection and at 2–3-week intervals thereafter. Serum was prepared from clotted blood by centrifugation 2 times at 16000*g* for 10 min and stored at −20 °C until used. An antiserum against the plasma from estrogen-treated frogs was prepared in a similar manner by mixing 0.5 mL of plasma from male frogs 15 days after estrogen injection with 0.5 mL of deionized water and 1.5 mL of Freund's complete adjuvant.

Affinity Chromatography. Plasma proteins from male bullfrogs were coupled to cyanogen bromide activated Sepharose 4B according to the method of March et al. (1974). To prepare an antiserum specific for estrogen-induced plasma proteins, an aliquot (5 mL) of the antiserum against the plasma of estrogen-treated frogs was incubated with a normal frog plasma protein–agarose affinity resin in a column (1 × 20 cm) for 90 min at 23 °C. The unbound fraction, containing antibodies specific for estrogen-induced proteins, was eluted from the column with Dulbecco's phosphate-buffered saline (PBS) and concentrated by ultrafiltration. To ensure removal of all antibodies cross-reacting with normal plasma proteins, the ultrafiltrate was applied to a second identical column and the procedure repeated.

Immunoelectrophoresis. The specificity of all antisera and the relative purity of column fractions were determined by immunoelectrophoresis (Graber & Williams, 1953). Agarose gels were prepared on 10 cm^2 glass plates by using 1% agarose (w/v) and 2% dextran (w/v, M_r 10000) in barbital buffer (25 mM), pH 8.6. The samples were subjected to electrophoresis at 75 V for 75 min. After electrophoresis, 100 μL of antiserum was loaded into each trough. The plates were allowed to incubate for 24–30 h in a humidified chamber at 23 °C and photographed by using indirect illumination.

Purification of Vitellogenin. Vitellogenin was purified by Mg(II) precipitation using a modification of the method of Wiley et al. (1979). Fourteen milliliters of plasma from male bullfrogs, 14–15 days after estrogen injection, was diluted to 140 mL such that the final concentration was 15 mM $MgCl_2$ and 20 mM ethylenediaminetetraacetic acid (EDTA) at pH 7.7. The mixture was shaken for 90 min at 4 °C. Aliquots were layered over 10 mL of 0.5 M sucrose containing 20 mM EDTA–15 mM $MgCl_2$ and centrifuged in a Sorvall HB-4 swinging-bucket rotor at 16000*g* for 15 min. The vitellogenin precipitate was dissolved with 1 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 8.0. Buffer changes were accomplished by ultrafiltration. Vitellogenins from *Rana pipiens* and *Xenopus laevis* were similarly purified at 15 and 40 mM $MgCl_2$, respectively.

Polyacrylamide Gel Electrophoresis. Molecular weights were determined by using sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis according to the method of Weber & Osborn (1969). Protein samples were diluted to 1 μg/μL in 30% sucrose containing 2% β-mercaptoethanol and 1% SDS. Following electrophoresis at 40 V for 90 min, the gels were stained with Coomassie Blue R-250 and destained by diffusion. For densitometric analysis, the destained gels were scanned at 500 nm with a Gilford spectrophotometer interfaced with a linear-transport system. The area of each peak was quantitated by planimetry.

Peptide Mapping. Peptide mapping was carried out as described by Cleveland et al. (1977). Proteins were precipitated with 20% trichloroacetic acid, and pellets obtained after centrifugation were washed twice with diethyl ether. The precipitate was dissolved in 0.125 M Tris-HCl buffer, pH 6.8, containing 0.5% SDS, 10% glycerol, and bromphenol blue at a final protein concentration of 0.5 mg/mL. Trypsin and chymotrypsin were added at a 10:1 weight ratio of protein to protease. *B. subtilis* protease was added at a 100:1 ratio. After 30 min at 37 °C, the reactions were stopped by addition of SDS to a final concentration of 2% and β-mercaptoethanol to 10%. Electrophoresis was carried out at 20 mA for 4 h on 10% polyacrylamide slab gels (0.75 × 120 × 140 mm) followed by staining with Coomassie Blue R-250 (Laemmli, 1970).

Analytical Ultracentrifugation. Ultracentrifugation experiments were carried out in an ANF rotor (Beckman Instruments, Palo Alto, CA) at 20 °C using a Beckman L5-75 preparative ultracentrifuge equipped with a Prep UV optical scanning attachment to measure absorbance at 280 nm. Sedimentation velocity measurements of the estrogen-induced plasma protein were performed at a sample concentration of 0.34 g/L in PBS, ρ = 1.0071 g/mL and $\eta/\eta_{20,w}$ = 1.013. The sample was centrifuged at 50000 rpm. A value of 0.717 mL/g for the partial specific volume was calculated from the amino acid composition. Sedimentation equilibrium measurements were also conducted at a sample concentration of 0.34 g/L in PBS. After an overspeed centrifugation at 18000 rpm for 4 h, the sample was brought to sedimentation equilibrium by centrifugation at 12000 rpm for 22 h. The absorbance was determined as a function of the distance r from the axis of rotation by using the Prep UV scanning attachment to the ultracentrifuge.

Amino Acid Analysis. One milligram of purified vitellogenin from *Rana pipiens*, *Rana catesbeiana*, and *Xenopus laevis* was hydrolyzed with 6 N HCl at 110 °C for 24 h. Samples were analyzed on a Dionex D-300 amino acid analyzer using single column methodology and a ninhydrin detection system. Norleucine was added prior to hydrolysis to serve as an internal standard. The non-vitellogenin, estrogen-induced protein, at >90% purity, was analyzed in a similar manner. In addition, the peptide band corresponding to this protein was excised from replicate polyacrylamide gels. The gel slices, containing about 15 μg of the pure protein, were hydrolyzed in 2 mL of 6 N HCl with 5 μL of β-mercaptoethanol and 20 nmol of norleucine in sealed, evacuated ampules for 22 h at 110 °C. After hydrolysis, the sealed ampules were opened and centrifuged at 900*g* for 5 min. Supernatants were separated from the acrylamide pellet, transferred to clean ampules, dried over KOH, and dissolved in sodium citrate loading buffer. Aliquots were analyzed on a Dionex D-300 amino acid analyzer as described above.

For the gel electrophoresis samples, the basic amino acids were resolved from ammonia by using a second aliquot and a different buffer program. The column was equilibrated with 0.2 M sodium citrate, pH 3.26, and eluted at 45 °C by using a flow rate of 20 mL/h for 2 min (0.35 M sodium citrate, pH 5.68, for 61 min), then the temperature was raised to 65 °C, and the column was eluted for an additional 25 min with 1.0 M sodium citrate, pH 5.72 (Feldhoff et al., 1984).

Samples and standards were run in the range of 1–15 nmol of each amino acid. Individual components were quantitated by measurement of peak heights relative to those of the standards.

Edman Sequencing. A manual adaptation of the Edman procedure was used to assess N-terminal sequences. The protocol has been described in detail (Ledden & Feldhoff, 1983). In the present study, the PTH-amino acids were identified by high-performance liquid chromatography on a Zorbax (Du Pont, Wilmington, DE) C-18 reverse-phase column (0.46 × 25 cm). Elution was accomplished at 50 °C by using an acetonitrile gradient in 25 mM sodium acetate, pH 5.15. The PTH-amino acids were monitored at 254 nm.

Assays. Protein was determined with minor modifications of the method of Lowry et al. (1951) using bovine serum albumin as a standard. Covalently attached phosphate was measured, after chromatography and extensive ultrafiltration in the absence of phosphate, by the method of Bartlett (1959).

Results

We have prepared an antiserum against total plasma proteins from male bullfrogs, after estrogen treatment. This antiserum was subsequently made specific for estrogen-induced plasma proteins by removal of antibodies to normal plasma proteins on a normal plasma protein–agarose affinity column.

In preliminary experiments, we found that the antiserum specific for estrogen-induced plasma proteins formed immunoprecipitates with vitellogenin (Vg) and a second non-Vg protein which remained in the supernatant after Mg(II) precipitation of Vg. As shown in Figure 1A, the proteins in the supernatant were applied to a DEAE-cellulose column (1 × 20 cm) and eluted with 5 column volumes of 25 mM Tris-HCl, pH 8.0, followed by 0.6 M NaCl in the same buffer. Each peak was analyzed by immunoelectrophoresis. The estrogen-induced protein was not retained by DEAE-cellulose at 25 mM Tris-HCl, pH 8.0, and eluted in the first peak. The fractions indicated by shading were pooled, adjusted to 50 mM sodium acetate by ultrafiltration, and applied to a CM-cellulose column (1 × 18 cm). The proteins were eluted from the

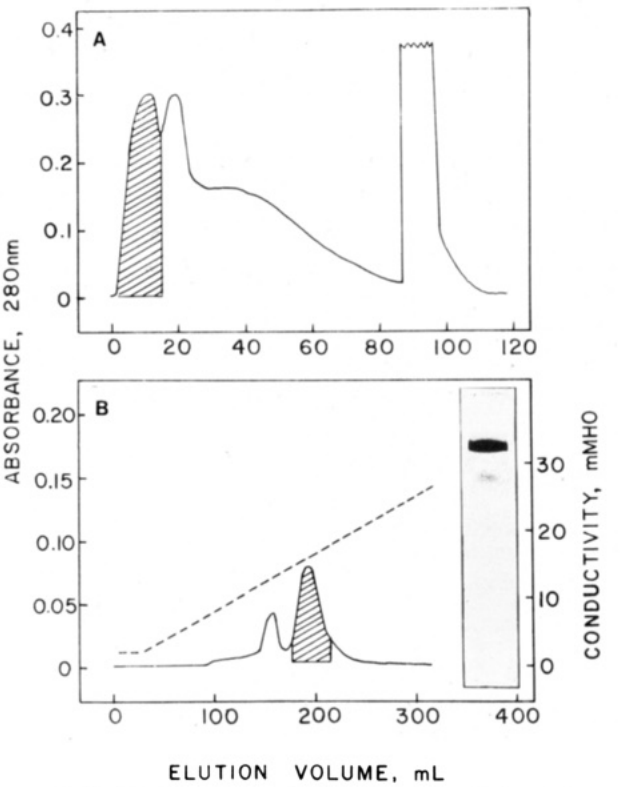

FIGURE 1: Purification of a non-vitellogenin, estrogen-induced plasma protein by ion-exchange chromatography. Plasma obtained from male frogs 15 days after estrogen administration was treated with Mg(II) to precipitate the vitellogenin as described under Materials and Methods. (A) The supernatant was adjusted to 25 mM Tris-HCl, pH 8.0, and eluted from a DEAE-cellulose column with the same buffer. (B) CM-cellulose chromatography of the shaded fraction from (A). The column was eluted with a NaCl gradient in 50 mM sodium acetate buffer, pH 5.0. The inset shows an aliquot of the shaded peak separated under reducing conditions on a 6% SDS–polyacrylamide gel.

CM-cellulose column with a linear gradient of 0.0–0.4 M NaCl in 50 mM sodium acetate, pH 5.0 (Figure 1B).

The shaded peak (Figure 1B), which eluted at 16–19 $m\Omega^{-1}$ (about 0.20–0.25 M NaCl), was analyzed by immunoelectrophoresis (Figure 2). The purified protein in wells e and f was reacted against antibodies to estrogen-induced proteins purified by affinity chromatography (trough 4), against antibodies to normal frog plasma proteins (trough 5), and vs. antibodies to total estrogen-induced plasma proteins (trough 6). The absence of a precipitin arc between wells e and f and trough 5 indicates the protein is not a constituent of control plasma.

In well d, the crossover of the precipitin arcs indicates there is no immunological cross-reactivity between Vg, which migrates on the anodic side of the sample well, and the novel estrogen-induced protein, which occurs as an arc centered around the sample well. Wells a and c contained plasma from control frogs, and wells b, d, and g contained plasma from estrogen-treated male bullfrogs. The precipitin arcs around wells a, b, c, and g demonstrate the specificity of the various antisera.

With minor variations, the ion-exchange chromatography experiments depicted in Figure 1 have been reproduced 5 times using different plasma preparations. In most cases, we have found it necessary to rerun the 16–19 $m\Omega^{-1}$ fraction from the CM-cellulose column under identical conditions. In other experiments, the proteins present in male frog plasma (±estrogen injection) and in the precipitates and supernatants following Mg(II) fractionation were also analyzed by

Table I: Amino Acid Composition of Estrogen-Induced Proteins[a]

amino acid	vitellogenin			lipovitellin A		non-Vg protein,
	Rana pipiens[b]	*Rana catesbeiana*[c]	*Xenopus laevis*[b]	*Xenopus laevis*[d]	*Xenopus laevis*[e]	*Rana catesbeiana*[c]
Asp	9.8	9.4	9.4	9.1	8.9	7.4
Thr	4.6	5.0	4.8	4.6	5.4	7.9
Ser	7.7	9.2	7.7	5.4	6.3	3.3
Glu	14.9	14.8	16.0	15.2	14.8	17.8
Pro	4.4	(4.9)[f]	4.4	3.9	4.6	(5.4)[f]
Gly	3.2	3.2	2.5	2.4	2.7	5.7
Ala	4.3	5.1	5.2	5.9	6.8	4.5
Cys	trace	(0.5)[f]	0.5	0.4	0.9	(8.0)[f]
Val	5.1	5.6	5.4	5.6	5.0	4.3
Met	1.1	1.3	1.2	3.4	2.7	1.2
Ile	6.0	4.7	4.8	5.9	4.5	4.0
Leu	8.3	7.4	8.2	10.4	8.8	4.7
Tyr	4.3	3.7	4.4	3.9	4.4	4.3
Phe	4.6	4.3	5.1	5.3	5.6	2.4
Lys	8.8	9.5	9.2	7.7	7.7	10.0
His	5.3	4.2	4.2	3.5	3.3	4.0
Arg	7.0	7.2	7.1	6.8	7.5	4.7

[a] Amino acid composition is presented as weight percent. [b] From acid hydrolysis of vitellogenin purified by Mg(II) precipitation. [c] From acid hydrolysis of the protein band obtained from polyacrylamide gels. [d] Calculated from the data of Ohlendorf et al. (1977). [e] Calculated from the data of Bergink & Wallace (1974). [f] Estimated from samples of >90% purity.

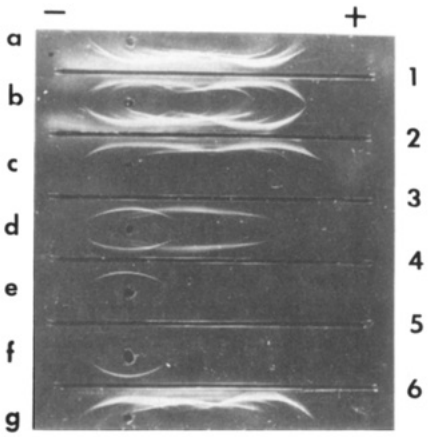

FIGURE 2: Immunoelectrophoretic analysis of plasma proteins from *Rana catesbeiana*. Samples were subjected to electrophoresis in agarose at pH 8.6. Wells a and c, normal frog plasma; wells b, d, and g, pooled plasma from estrogen-induced males; wells e and f, purified estrogen-induced protein from the CM-cellulose column as shown in Figure 1B. Troughs 1 and 5, antiserum to normal frog plasma proteins; troughs 2 and 6, antiserum to total plasma proteins from estrogen-induced male bullfrogs; troughs 3 and 4, antiserum to total plasma proteins from estrogen-treated male frogs following the removal of antibodies cross-reacting with normal frog plasma proteins. The precipitin arcs above well e and below well f show the purified non-vitellogenin, estrogen-induced plasma protein.

DEAE-cellulose chromatography and immunoelectrophoresis (Mitchell & Feldhoff, 1984). The concentration of the non-Vg, estrogen-induced protein does not appear to change as a consequence of experimental manipulations. In addition, we have observed no difference in the relative amount of the protein in freshly collected plasma and that which had been stored frozen for as long as 2 years.

The inset in Figure 1B shows an SDS gel of the non-vitellogenin, estrogen-induced protein in the presence of β-mercaptoethanol. The protein was shown to be >90% pure by densitometric analysis. The molecular mass of the peptide was determined to be 116 000 Da by comparison to a series of protein standards. The mobility of the purified peptide was similar in the absence of reducing agent, suggesting the absence of interchain disulfide bonds and that the native protein is composed of a single polypeptide chain.

Sedimentation velocity experiments indicate the non-vitellogenin, estrogen-induced protein has a sedimentation coefficient ($s_{20,w}$) of 7.5 S. The boundary formed during sedimentation indicated the presence of only one sedimenting species. Sedimentation equilibrium studies showed the protein to have a molecular mass of 105 000 Da and an f/f_{min} ratio of 1.08 (Tanford, 1961). The absence of curvature in the sedimentation equilibrium plot (data not shown) suggests the protein exists as a nonaggregating monomer in aqueous solution.

The amino acid compositions were determined for the non-Vg, estrogen-induced protein and for Vg purified from *Rana catesbeiana*, *Rana pipiens*, and *Xenopus laevis*. The data summarized in Table I show a similarity among vitellogenins from different species. However, the novel non-Vg, estrogen-induced protein has a composition very different from Vg. The composition of lipovitellin A is also included in Table I because it is known to be a cleavage product of *Xenopus laevis* vitellogenin and has a molecular weight (105 000) similar to that of the protein we have purified. The difference in composition observed between lipovitellin A and the estrogen-induced protein suggests that the latter protein is not a cleavage product of *Rana catesbeiana* Vg. By use of a manual Edman degradation procedure, the amino-terminal residue was found to be lysine.

The structures of Vg and the non-Vg, estrogen-induced protein were compared by carrying out peptide mapping according to Cleveland et al. (1977). As shown in Figure 3, no common peptides were found by using trypsin, chymotrypsin, and *B. subtilis* protease. In addition, on a molar basis, the non-Vg-induced protein contained ≤3% of the amount of phosphate as Vg (4.4 vs. 155 nmol of phosphate/nmol of protein).

Discussion

Vitellogenin has been purified from *Xenopus laevis*, *Rana catesbeiana*, and a number of other amphibian species (Follett & Redshaw, 1968; Wiley et al., 1979; Herbener et al., 1983). It has been shown that there are multiple genes coding for vitellogenin in *Xenopus* (Wahli et al., 1979) and that multiple forms of vitellogenin exist in *Xenopus* and *Rana* (Wiley & Wallace, 1978; Jaggi et al., 1980; Hess, 1981). For all species, the individual peptide chains which compose the native Vg have molecular masses in the range of 180 000–200 000 Da.

In amphibian systems, vitellogenin has thus far been the only plasma protein reported to be synthesized in response to es-

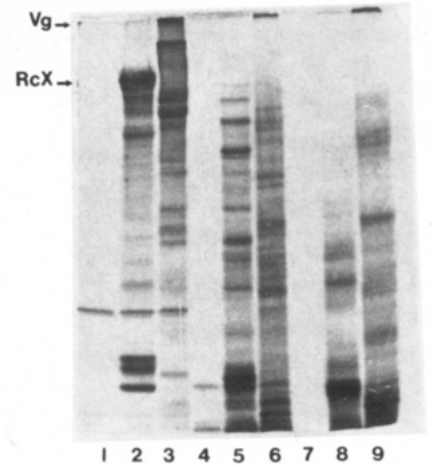

FIGURE 3: Peptide mapping of vitellogenin and the non-vitellogenin, estrogen-induced protein with trypsin, chymotrypsin, and *B. subtilis* protease. The proteins were denatured and exposed to proteases as described under Materials and Methods. Digests were submitted to electrophoresis on 10% acrylamide slab gels and were stained with Coomassie Blue. The electrophoretic mobilities of undigested vitellogenin (Vg) and the non-vitellogenin peptide (RcX) are indicated by arrows. Lane 1, 2 μg of trypsin after a 30-min incubation at 37 °,c; lane 2, 20 μg of RcX and 2 μg of Trypsin; lane 3, 20 μg of Vg and 2 μg of trypsin; lane 4, 2 μg of chymotrypsin treated as described in lane 1; lane 5, 20 μg of RcX and 2 μg of chymotrypsin; lane 6, 20 μg of Vg and 2 μg of chymotrypsin; lane 7, 0.2 μg of *B. subtilis* protease treated as in lane 1; lane 8, 20 μg of RcX and 0.2 μg of *B. subtilis* protease; lane 9, 20 μg of Vg and 0.2 μg of *B. subtilis* protease.

trogen induction. However, in the chicken, the synthesis of both vitellogenin and lipoprotein(s) can be induced with steroid hormones. Capony & Williams (1980) demonstrated a 500-fold increase in apolipoprotein B (350 000 Da) following estrogen treatment of roosters. To our knowledge, a relationship between apolipoprotein synthesis and estrogen has not been demonstrated in amphibia.

Most studies of Vg synthesis have been based upon assays using antibodies against purified Vg. However, such an approach does not allow one to ascertain whether or not other non-Vg plasma proteins are also induced by estrogen. One of our early objectives was to prepare antibodies against Vg from *Rana catesbeiana* using two independent approaches. Antisera were prepared against Vg purified by Mg(II) precipitation and against total plasma proteins from estrogen-treated bullfrogs. After the cross-reacting antibodies to normal frog plasma proteins were removed by immunoaffinity chromatography, we found that the latter antiserum contained antibodies to Vg and a non-Vg, estrogen-induced plasma protein. We subsequently purified the non-Vg protein by ion-exchange chromatography and determined the molecular mass to be 116 000 daltons by SDS gel electrophoresis. Until a function can be determined for this protein, we propose using the trivial nomenclature of RcX to indicate the species from which this protein has been derived (*Rana catesbeiana*).

As shown by immunoelectrophoresis, the crossover of the precipitin arcs indicates there is no immunological cross-reactivity between RcX and Vg. The different electrophoretic mobilities and molecular masses indicate RcX is not Vg, while the lack of common antigenic sites suggests RcX is not a breakdown product of Vg.

Sedimentation velocity studies show RcX to have a sedimentation coefficient ($s_{20,w}$) of 7.5 S. Sedimentation equilibrium experiments indicate RcX has a molecular mass of 105 000 Da and exists as a globular, nonaggregating monomer in aqueous solution. The difference in molecular mass observed by SDS–polyacrylamide gel electrophoresis and ultracentrifugation may be accounted for by the basic nature of the protein. Positively charged proteins, such as histones, have been shown to migrate with an anomalously high molecular weight on SDS gels (Panyim & Chalkley, 1971). The early elution of RcX from the DEAE-cellulose column and the migration position on the immunoelectrophoresis plate suggest the protein has a net positive charge at pH 8.0.

The amino acid analysis data summarized in Table I show that vitellogenins purified from *Rana catesbeiana*, *Rana pipiens*, and *Xenopus laevis* have similar amino acid compositions. It is noteworthy that the genera *Xenopus* and *Rana* have evolved independently for more than 200 million years and there is relatively little or no cross-reactivity between antibodies to their plasma proteins (Hess, 1981). Apparently, the general amino acid composition and the molecular mass of the Vg have been conserved, but not the antigenic sites. In contrast, RcX has an amino acid composition very different from Vg or lipovitellin A. Vitellogenin is known to be a glycoprotein containing oligosaccharides of the complex type (Gottlieb & Wallace, 1982). With our amino acid analyzer, the detection limit for amino sugars after acid hydrolysis is 300–500 pmol. Since we could not determine the presence of amino sugars in the acid hydrolysates, the data suggest that RcX contains little, if any, covalently bound carbohydrate.

Peptide mapping and phosphate analysis provide additional structural evidence that Vg and RcX are not related. With three different proteases, no common peptides were found. Furthermore, Vg possesses a 35-fold greater molar ratio of phosphate. From all of the structural, physicochemical, and immunochemical data, we have concluded that RcX is not derived from Vg.

After estrogen induction, Vg is present in the plasma of male frogs at levels of 40–60 mg/mL. On the basis of a recovery of 90% from ion-exchange columns and qualitative immunoelectrophoresis, we estimate that RcX is present in plasma at a level of about 0.5 mg/mL. The small quantities of RcX are, therefore, overshadowed by the large amounts of Vg. It is quite likely that the protein we have described in *Rana* may also exist in *Xenopus* but has not yet been detected.

It is not known if the presence of RcX in plasma is directly or indirectly related to the process of vitellogenesis. However, preliminary immunocytochemical experiments suggest that RcX is synthesized in the liver.[1] RcX did not appear to bind estrogens in a preliminary equilibrium dialysis experiment. Many aspects of the structure, function, and metabolism of this non-Vg, estrogen-induced protein must be investigated in order to determine its physiological significance.

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