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I am submitting herewith a dissertation written by Henry Steven Wiley entitled "The Structural Relationship between Vitellogenin and the Yolk Proteins in Xenopus laevis." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Robin A. Wallace, Major Professor

We have read this dissertation and recommend its acceptance:

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THE STRUCTURAL RELATIONSHIP BETWEEN VITELLOGENIN AND THE YOLK PROTEINS IN XENOPUS LAEVIS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Henry Steven Wiley

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TO MY PARENTS

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ABSTRACT

The crystalline inclusions in amphibiam oocytes known as yolk platelets are formed by the incorporation and proteolytic processing of the soluble serum protein vitellogenin. The nature of this transformation was studied by isolating the yolk proteins and then reconstructing the parent molecule by the use of chemical and biological cleavage techniques. Several new methodologies had to be developed to achieve this goal.

Vitellogenin was isolated from the plasma of estrogen-treated animals by selective precipitation with Mg^{2+} in the presence of EDTA. This technique apparently relies on the specific complexing of the phosphate moieties of the protein by a Mg^{2+}:EDTA chelate. The vitellogenin thus obtained is greater than 99% pure and is relatively undegraded. This technique can also be used for determining the vitellogenin titer in the blood of estrogen-treated animals. Vitellogenin was also isolated by DEAE-cellulose chromatography. In combination, these two isolation procedures yield vitellogenin preparations of very high purity.

In order to study the processing of vitellogenin by the oocytes, a method was developed for labeling vitellogenin in vitro to a very high specific activity by using [γ-^{32}P]ATP and a phosphoprotein kinase isolated from Xenopus liver. The phosphoprotein kinase was purified 21,000-fold from liver cytosol by employing ammonium sulfate fractionation, DEAE-cellulose chromatography and phosphocellulose chromatography. It is specific for phosphoproteins such as casein and phosvitin and its K_m for ATP (2.2 μM) is one of the lowest yet reported for a protein

kinase. Using the phosphoprotein kinase, specific activities of over 5×10^8 cpm/mg have been obtained for milligram quantities of vitellogenin. The labeled vitellogenin was incorporated into oocytes at a high rate (approximately 300 ng/oocyte/hr) and was processed normally into the yolk proteins.

Vitellogenin was found to be a heterogeneous population of molecules that could be resolved into at least 3 different molecular weight forms by SDS gel electrophoresis. Native gel electrophoresis of vitellogenin resolves the population into 4 bands, three of which seem to differ in molecular weight and one of which seems to differ in net charge. Isoelectric focusing of vitellogenin confirmed that the protein population was heterogeneous with respect to charge. Chemical cleavage of vitellogenin at the cysteine residues with nitrothiocyanobenzoic acid demonstrated that there were two forms of molecules whose cysteine residues were at different positions with respect to the phosphorylated region of vitellogenin. Therefore, Xenopus vitellogenin seems to be the product of multiple genes.

The vitellogenin-derived yolk proteins were isolated from oocytes by ammonium sulfate fractionation, DEAE-cellulose chromatography and gel filtration. In addition to three previously described yolk proteins (lipovitellin 1, lipovitellin 2 and phosvitin), I found two additional populations of phosphoproteins: phosvette I, which contains 4.8% phosphorus and has a molecular weight of 19,000, and phosvette II, which contains 10.7% phosphorus and has a molecular weight ranging from 9,500 to 14,500. Amino acid analysis of the yolk proteins

demonstrated that phosvette I and phosvette II are not derived from the other yolk proteins. A comparison of the amino acid composition of vitellogenin and the yolk proteins revealed that all of the yolk proteins are not present in equimolar amounts in the platelets. The ratio between lipovitellin 2, phosvitin and the phosvettes was determined by protein phosphorus analysis and yielded a lipovitellin 2:phosvitin: phosvettes ratio of 1.00:0.70:0.27. The time course of the cleavage of vitellogenin into the yolk proteins was studied by incubating oocytes with [^{32}P]vitellogenin and examining the labeled cleavage products by SDS gel electrophoresis. This study showed that the phosvettes were indeed derived from vitellogenin and that the time course of their appearance was different from that of phosvitin. It thus appears that there are two classes of vitellogenin molecules that give rise to alternate cleavage products.

The placement of the yolk proteins in the vitellogenin molecules was investigated by N-terminal analysis and cyanogen bromide cleavage of vitellogenin and the yolk proteins. This study showed that the N-terminal of vitellogenin contains the future lipovitellin 1 molecule while the C-terminal region of vitellogenin gives rise to the lipovitellin 2 molecule. Phosvitin and the phosvettes seem to be derived from an internal region of vitellogenin. Since both the lipovitellin 1 and lipovitellin 2 region of vitellogenin are in tight association, and since phosvitin was found to be on the outside of the molecule, it is proposed that the cleavage of vitellogenin into the yolk proteins is a mechanism by which the phosvitin region of vitellogenin is removed, thus exposing the insoluble lipovitellin core proteins.

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CHAPTER I

GENERAL INTRODUCTION

The oocytes of nonmammalian vertebrates grow to a relatively enormous size primarily due to the accumulation of yolk (1). Until recently, the source of vertebrate yolk has been a controversy in which some investigators favored synthesis by the oocyte (autosynthetic) while others favored an extraoocyte origin (heterosynthetic) (2). Evidence for the heterosynthetic origin of yolk included the discovery of serum-like antigens associated with yolk in the chicken (3,4), and the demonstration by Glass (5) that proteins from the maternal bloodstream could be incorporated intact into growing amphibian oocytes. Additionally, Urist and Schjeide (6) demonstrated that estrogen treatment induced the appearance of yolk-like proteins in the blood of treated amphibians, an observation that had been made earlier in the case of birds (7). On the other hand, cytological studies had provided evidence for an intraoocyte mechanism of yolk formation (8,9). The possibility also existed that yolk formation occurred by a combination of the two processes. The relative roles of autosynthetic and heterosynthetic production of amphibian yolk was determined by Wallace et al. (2) who found that greater than 99% of the yolk proteins found in the amphibian yolk platelets were derived from material synthesized outside of the oocyte. Further studies have confirmed that amphibian oocytes grow primarily by the incorporation of exogenous protein (10).

The hypothesis that amphibian yolk was synthesized outside the oocyte led to a search for the yolk precursor protein(s) and its site of synthesis. It had been previously suggested that one of the main yolk proteins of laying hens, phosvitin, was apparently synthesized and secreted by the liver (11). In 1968, Wallace and Jared (12) showed that estrogens could induce the appearance of a new lipophosphoprotein in the serum of treated male Xenopus. This protein was shown to be synthesized by the liver (13) and when it was isolated in a radiolabeled form and injected into gonadotropin-treated female Xenopus, it was incorporated into the growing oocytes and converted into the yolk proteins lipovitellin and phosvitin (13). This yolk precursor protein is now known as vitellogenin (14).

At this point it might be helpful to compare what is known about the biochemical properties of vitellogen and the yolk proteins. Yolk in amphibians occurs as crystalline inclusions known as yolk platelets. These platelets are composed of two main proteins, lipovitellin and phosvitin. Lipovitellin is a large (approximately 420,000 MW) dimeric protein containing 18% lipid and 0.28% phosphorus (15), while phosvitin is a relatively small protein (approximately 32,000 MW) containing no lipid, 8.4% phosphorus and 0.5% carbohydrate (15). There are two phosvitin molecules for each lipovitellin dimer in the yolk. Each lipovitellin monomer can be further resolved into a large (approximately 120,000 MW) and a small (approximately 31,000 MW) subunit (16). When isolated, phosvitin is soluble under physiological conditions (neutral pH, approximately 0.1 M NaCl) while lipovitellin is insoluble (15).

When phosvitin is present together with lipovitellin, the two proteins form an insoluble complex under physiological conditions. Vitellogenin from Xenopus is a large (approximately 460,000 MW) phospholipoglycoprotein that contains 12% lipid and approximately 1.4% phosphorus. It is a dimer in aqueous solutions and in the blood contains one atom of calcium bound for every protein phosphorus group present (14). It is very water soluble and solutions of over 160 mg/ml can easily be prepared in normal saline (personal observation).

The nature of the conversion of Xenopus vitellogenin to the yolk proteins was investigated by Bergink and Wallace (16). These investigators found that after the incorporation of vitellogenin into the oocyte, it is proteolytically split at two sites yielding phosvitin and the two lipovitellin subunits which crystallize to form the main body of the yolk platelets. It should be noted that amphibian vitellogenin also seems to lose its bound calcium during this conversion process (6) (also personal observations). One of the most interesting aspects of this process is the conversion of the readily soluble serum protein, vitellogenin, into an insoluble yolk complex by the introduction of two cleavage sites into the molecule. Therefore, the structure of vitellogenin must allow for this ready conversion from the soluble state to the insoluble state. It has become apparent over the last few years that most, if not all, vertebrate yolk proteins arise from the proteolytic conversion of a soluble serum vitellogenin molecule (1). Hence this precursor molecule could be considered a vehicle by which the insoluble yolk proteins can be transported through the circulation

prior to their deposition into the oocytes. However, the exact nature of the macromolecular conversion of vitellogenin into the yolk proteins remains unknown.

My initial thesis project involved the study of the specific incorporation of vitellogenin by oocytes. For this project it was necessary to radioactively label vitellogenin in vitro to a high specific activity so that the binding of the vitellogenin to its oocyte receptor could be examined. Unfortunately, my initial attempts to label the vitellogenin molecule using previously published procedures were unsuccessful. Vitellogenin was iodinated to only a negligible extent (less than 1% of the labeling efficiency of other proteins such as bovine serum albumin). In addition, the iodinated vitellogenin was apparently degraded by the oocyte (17). Labeling of vitellogenin with ^{3}H via reductive methylation yielded preparations with a specific activity of 0.57 μCi/Mg, but the methylated vitellogenin was no longer recognized by the oocyte (17). Finally, an attempt to label vitellogenin with [γ-^{32}P]ATP using a protein kinase failed due to the precipitation of vitellogenin during the labeling reaction.

These preliminary efforts to label the molecule convinced me that the basic problem was a lack of understanding of the structure of vitellogenin. Without that understanding, I could not make an intelligent choice of a labeling procedure or know how to modify the reaction so that the vitellogenin was efficiently labeled and retained its ability to be specifically incorporated by oocytes. I thus undertook a project to understand the structural features of vitellogenin

that were modified during the conversion of the soluble vitellogenin into the insoluble yolk proteins.

Shortly after I initiated this project, it became obvious that there were very few methods available in the biochemical literature for examining large (200,000 MW) conjugated proteins. Therefore, a major part of this study was the development of new techniques for examining the vitellogenin molecule. The most fruitful approach was to take advantage of the normal processing of vitellogenin by the oocyte. I thus examined the structure of the yolk proteins and then "reconstructed" the vitellogenin molecule by establishing the regions of the vitellogenin molecule from which each of the yolk proteins were derived. In order to successfully complete this project, it was necessary for me to develop proficiencies in SDS gel electrophoresis, protein labeling, protein cleavage, intuitive reasoning and patience. As a direct result of these attempts I was able to develop new methods for vitellogenin isolation and labeling which are currently in use in the laboratory.

This thesis is arranged into chapters, each of which is organized to facilitate publication of the data. The chapters are not arranged chronologically, but are in an order I deemed most logical. The first two chapters describe the isolation of vitellogenin and the purification of the phosphoprotein kinase used to label the vitellogenin molecule. The next two chapters deal with the structure of vitellogenin and the yolk proteins. A single list of references follows. In addition, there are two appendices that deal with specific methodologies and data that could not be included gracefully in the main chapters. I feel

that this project has achieved its goals and that the groundwork has been laid to understand how oocytes reorganize the vitellogenin molecule and convert it into the crystalline yolk proteins.

CHAPTER II

THE PURIFICATION, ASSAY AND EVALUATION OF XENOPUS VITELLOGENIN

Summary

Two procedures are described for the isolation of vitellogenin from plasma. Vitellogenin was selectively precipitated from the plasma of estrogen-treated animals by Mg^{2+} in the presence of EDTA. The vitellogenin thus obtained was greater than 99% pure and was relatively undegraded. This method was also used for determining the vitellogenin titer in the blood of estrogen-treated animals. In the second procedure, vitellogenin was isolated by DEAE-cellulose chromatography, resulting in preparations that contained approximately 2% albumin. In combination, these two isolation procedures yielded vitellogenin preparations of very high purity.

Introduction

Vitellogenin is the sex limited yolk precursor protein in oviparous vertebrates (1). The hepatic synthesis of this large phospholipoglycoprotein can be induced *in vitro* (18) or *in vivo* (12) by estrogens and thus it has recently been used as a model steroid-induced protein (19). Vitellogenin has also been used to promote the growth and differentiation of Xenopus oocytes in culture (10). However, to date there has been no simple standardized method available for the isolation of vitellogenin free from other serum proteins.

The ultracentrifugal separation of Redshaw and Follett (20) is simple, but requires an ultracentrifuge as well as a very high titer of vitellogenin in the serum in order to achieve good yields. Preparations of vitellogenin obtained in this manner are also necessarily contaminated with other serum proteins. The dimethylformimide technique of Ansari et al. (21) is rapid, but it is very difficult to achieve quantitative precipitation of vitellogenin without an appreciable (approximately 5%) albumin contamination and with less than 10 ml of serum (21). In addition, dimethylformimide is highly toxic and the vitellogenin obtained with its use could not be used for cell culture.

The use of TEAE-cellulose (14) has proved to be the most satisfactory method for isolating relatively pure preparations of vitellogenin and this method has been extensively used in our laboratory. We have found that vitellogenin chromatographs very reproducibly when the same batch of TEAE-cellulose is used. Recently, I have purchased several new lots of TEAE-cellulose from Bio-Rad and have been unable to recover vitellogenin from columns of this new material. In these cases vitellogenin seemed to irreversibly bind to the column and was not eluted even under drastic conditions (greater than 1M NaCl, 0.5% Triton X-100). Considering the pronounced difference of different lots of TEAE-cellulose, I feel that this method is not as reliable as previously believed.

In view of the above considerations, I attempted to develop new procedures for vitellogenin purification. I describe here several new methods for the purification of vitellogenin in high yields that

meet the criteria of simplicity, reproducibility, and excellent purity. In addition, I also describe a very simple procedure for determining the vitellogenin concentration in the serum of estrogen treated animals.

Materials and Methods

General

The care and maintenance of animals, the injection of hormones and isotopes and the bleeding of animals was performed as previously described (22). Isotopes were purchased from Amersham. All chemicals and hormones were purchased from Sigma. Acrylamide and bis-acrylamide were recrystallized three times from acetone prior to use (23). 2-Amino,2-methyl-1,3-propanediol was recrystallized from ethanol and rinsed with acetone. SDS was reprecipitated from ethanol prior to use. All other chemicals were used without additional purification. Solutions were autoclaved prior to use and the operations were performed at 0° - 4°.

The starting material used in this study was Xenopus plasma, obtained by bleeding the animals into sterile 50 ml plastic centrifuge tubes (Falcon Plastics, Inc.) containing 1 ml of 50% phosphate buffered saline (calcium free) containing 0.07M sodium citrate. The blood was then centrifuged at 2500 x g for 15 minutes and the plasma was carefully removed and recentrifuged to remove any residual cells. The resulting plasma preparation was used immediately.

Selective Precipitation of Vitellogenin from Plasma

Plasma (5 ml) prepared as above was diluted with 20 ml of 20 mM EDTA, pH 7.7. After mixing, 1.6 ml of 0.5M $MgCl_2$ was added, and the contents of the tube were gently mixed by inversion. The resulting precipitate was collected by centrifugation at 2500 x g for 15 min. The supernatant was discarded and the pellet was redissolved in 3 ml of 1M NaCl, 50 mM Tris-Cl, pH 7.5 (buffer A) with the use of a stirring rod. Vigorous mixing was avoided to minimize denaturation of the vitellogenin. The tube was then centrifuged for 30 min at 2500 x g to remove any insoluble material and the green liquid was decanted into another tube and mixed with 25 ml of distilled water which precipitated the vitellogenin. The precipitate was collected by centrifugation at 2500 x g for 15 min and was redissolved in 3 ml of buffer A. The liquid was then placed in sterile dialysis tubing and dialyzed overnight against 100 volumes of buffer A. The efficiency of dialysis of the thick green liquid was improved by occasionally inverting the dialysis tube to mix the contents. The vitellogenin was next dialyzed against a desired buffer prior to storage at -20° (usually 50 mM Tris-Cl, pH 7.5).

Recovery of Vitellogenin

In order to monitor the recovery of vitellogenin from the plasma, estrogen-treated animals were injected with 2 mCi of [^{32}P] orthophosphate (carrier-free, 8 mCi/ml) 24 hours prior to bleeding. Acid insoluble ^{32}P in the plasma is a specific indicator of vitellogenin (14). The labeled plasma was collected and aliquots were mixed with various amounts

of unlabeled plasma from control male animals. Samples of this dilution series (0.2 ml) were mixed with 0.8 ml of a 20 mM EDTA solution (pH 7.7) and after mixing the magnesium concentration was adjusted by the addition of a 0.5 M $MgCl_2$ stock. The precipitates were collected by centrifugation at 2500 x g for 15 min and the supernatants were carefully removed and placed in 10 ml flasks. The precipitates were dissolved with 1 ml of buffer A and placed in separate 10 ml flasks; all flasks were then filled with buffer A. Duplicate 150 µl aliquots were spotted on Whatman 3 MM discs and processed to remove acid soluble counts (24). Discs were then counted in a Beckman Lo-Beta planchet counter. Recovery was expressed as the percentage of total counts at each dilution that was present in the precipitate.

DEAE-Cellulose Chromatography of Vitellogenin

The DEAE-cellulose used in most of these studies was Selectacel Type 40. Other types of DEAE-Cellulose were tested and gave very similar results. The DEAE-cellulose was prepared as described by Peterson (25) and then loaded into 32 × 1.9 cm columns (bed volume approximately 90 ml) as previously described (26). The columns were rinsed with 500 ml of 0.2 M sodium citrate-0.2% Triton X-100 (26) and then with at least 1 liter of starting buffer (50 mM Tris-Cl, pH7.5). Buffer was pumped through the column with a constant flow pump set at 150 ml/hr while the absorbance of the effluent from the column at 280 nm was monitored with an ISCO absorbance monitor. Either estrogenized Xenopus plasma or redissolved Mg^{2+}:EDTA precipitated vitellogenin was directly loaded onto the column and rinsed into

the bed with starting buffer. Vitellogenin was eluted using a 1000 ml linear salt gradient from 0.0-0.35 M KCl in 50 mM Tris-Cl, pH 7.5. Vitellogenin usually eluted at a salt concentration of 0.2M KCl while albumin eluted at 0.13-0.14 M KCl. In some cases the elution position of vitellogenin was monitored by labeling the plasma with ^{32}P prior to collection. In this case aliquots of column fractions were spotted on paper discs, then processed and counted as described above. The peak fractions corresponding to the vitellogenin were pooled and concentrated by vacuum dialysis (26).

Gel Electrophoresis

Native gel electrophoresis was performed by a modification of the procedures described by Chrambach et al. (23) using systems 1935 and 2860.0.X at pH 7.4 and 9.6 respectively. Gels containing 8% total acrylamide concentration (w/v) with 2% (of total acrylamide) bis-acrylamide crosslinking agent (8% T, 2% C gels) were polymerized at 20° in 5 × 120 mm tubes using 50 mg/100 ml potassium persulfate as the catalyst solution, 50 µl TEMED/100 ml gel solution as the initiator and water-saturated butanol as the overlay solution. Stacking gels were 6.25% T, 20% C, using the above modified catalyst solution. Vitellogenin was dissolved at a concentration of 2 mg/ml in upper reservoir buffer for system 1935, and in stacking gel buffer for system 2860.0.X. Both sample buffers contained 5 mM EDTA, 20% sucrose, and bromophenol blue as the tracking dye. Samples of 50 µl were applied to the gels which were run at 2.5 mA/gel at 4° until the tracking dye reached the bottom of the gel tubes. The gels were

then fixed and stained in 25% isopropanol, 10% acetic acid containing 0.025% Coomassie blue R250 for 5 hr at 20° and destained in 10% methanol, 10% acetic acid (27).

SDS gel electrophoresis was performed in 10% T, 2% C gels as described by Wyckoff et al. (27) except that the catalyst solution was modified as described above. Loads of 5 µg vitellogenin were applied to these gels.

Results

The addition of divalent cations to solutions of purified vitellogenin resulted in the precipitation of the protein (see Appendix A). This effect is due to the formation of a complex between the divalent cations and the phosphate moieties of vitellogenin. The cations also precipitate purified phosvitin, which contains most of the phosphorus present in vitellogenin (28, 16). This precipitation was dependent upon the concentration of both the protein and the divalent cation, with precipitation occurring within a very limited range. When I attempted to precipitate vitellogenin from diluted plasma by this procedure, I was unsuccessful (Table 1). However, the addition of EDTA equivalent to the divalent cations Ca^{2+}, Mg^{2+}, Mn^{2+}, or Fe^{2+}, resulted in the precipitation of vitellogenin (Table 1), whereas the addition of EGTA resulted in the precipitation of vitellogenin only in the presence of Ca^{2+}. The addition of EDTA or EGTA alone had no effect on the solubility of vitellogenin. Likewise, the addition of divalent cations and EDTA to diluted male plasma caused no precipitation of proteins.

TABLE I

PRECIPITATION OF VITELLOGENIN FROM PLASMA[a]

Additive	Cation[b]			
	Ca^{2+}	Mg^{2+}	Mn^{2+}	Fe^{2+}
None	-	-	-	-
50 mM EDTA	+	+	+	+
50 mM EGTA	+	-	-	-
50 mM EDTA + 1 M KCl	-	-	-	-

[a]Plasma containing approximately 140 mg/ml vitellogenin diluted 9:1 with distilled water.

[b]Cation present at a total concentration of 50 mM.

From these initial observations I attempted to selectively precipitate vitellogenin from plasma derived from estrogen-treated animals using calcium and EGTA. Redshaw and Follett also used high concentrations of calcium alone to precipitate vitellogenin from the plasma of laying hens (29). I was successful in obtaining a preparation of vitellogenin that migrated with authentic vitellogenin on native polyacrylamide gels. However, subsequent analysis of Ca^{2+}:EGTA precipitated vitellogenin by SDS gel electrophoresis revealed that the vitellogenin was extensively degraded (Figure 1) presumably by a Ca^{2+}-activated protease. This degradation occurred even in the presence of the serine-protease inhibitor PMSF. In contrast, vitellogenin prepared by using Mg^{2+} together with EDTA, or by DEAE-cellulose chromatography was relatively undegraded (Figure 1). I thus conclude that calcium should not be used to prepare vitellogenin as suggested by Redshaw and Follett (29).

I next attempted to define optimum conditions for the precipitation of vitellogenin from plasma utilizing Mg^{2+} together with EDTA. In order to monitor the extent of vitellogenin recovery, I prepared plasma from estrogen-treated animals injected with [^{32}P] orthophosphate prior to bleeding. The recovery of protein-bound ^{32}P was used to indicate the recovery of vitellogenin. Preliminary observations indicated that diluting the plasma with 4 volumes of 20 mM EDTA (pH 7.7) prior to adding the Mg^{2+} was the most satisfactory initial step. I diluted the ^{32}P-labeled plasma with control male plasma and then precipitated the vitellogenin with 20, 40, or 60 mM Mg^{2+}. The

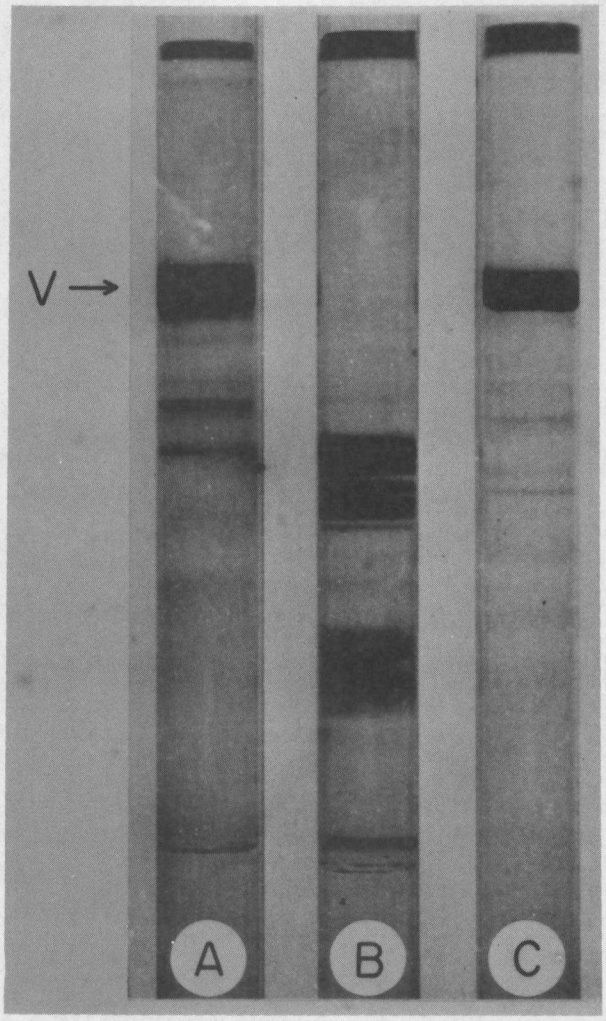

Figure 1. SDS-gel electrophoresis of vitellogenin prepared by (A) DEAE-cellulose chromatography, (B) Ca^{2+}:EGTA precipitation, and (C) Mg^{2+}:EDTA precipitation. The vitellogenin applied to gels B and C was isolated by use of a 2:1 ratio of cation to EDTA or EGTA. 10 µg of each preparation was electrophoresed on 5% T, 2% C gels as described in Materials and Methods. V indicates the migration position of authentic vitellogenin.

results are seen in Figure 2. All concentrations of Mg^{2+} had a similar effect. At high vitellogenin concentrations, recovery is nearly quantitative. However, I achieved less than 50% recovery of vitellogenin when the plasma concentration was below 10 mg/ml. The poorer recovery of low amounts of vitellogenin utilizing a Mg^{2+}:EDTA ratio of 1:1 was primarily due to the formation of a very fine precipitate that would not sediment under the centrifugation conditions used.

I attempted to improve the recovery of low levels of vitellogenin by various procedures, but without success. Dialyzing the plasma against 20 mM EDTA instead of diluting it resulted in poorer recoveries of vitellogenin. Raising or lowering the EDTA concentrations also resulted in lower yields. Thus the Mg^{2+}:EDTA precipitation technique is efficient only with plasma samples containing relatively high concentrations of vitellogenin. However, since the sera of estrogenized animals usually have titers of vitellogenin of over 50 mg/ml (14) this drawback does not seem serious.

One practical problem in any specific precipitation technique is the nonspecific trapping of proteins in the precipitate. To partially overcome this problem, I introduced a solubilization and reprecipitation step into the procedure. The pellets obtained from the first precipitation step can easily be dissolved in 1 M NaCl with the aid of a stirring rod. Any shaking or vortexing of the solution should be avoided as this will denature the vitellogenin. After the pellets are dissolved, the liquid is centrifuged to remove insoluble material, and then the vitellogenin is reprecipitated by simply diluting the solution down to

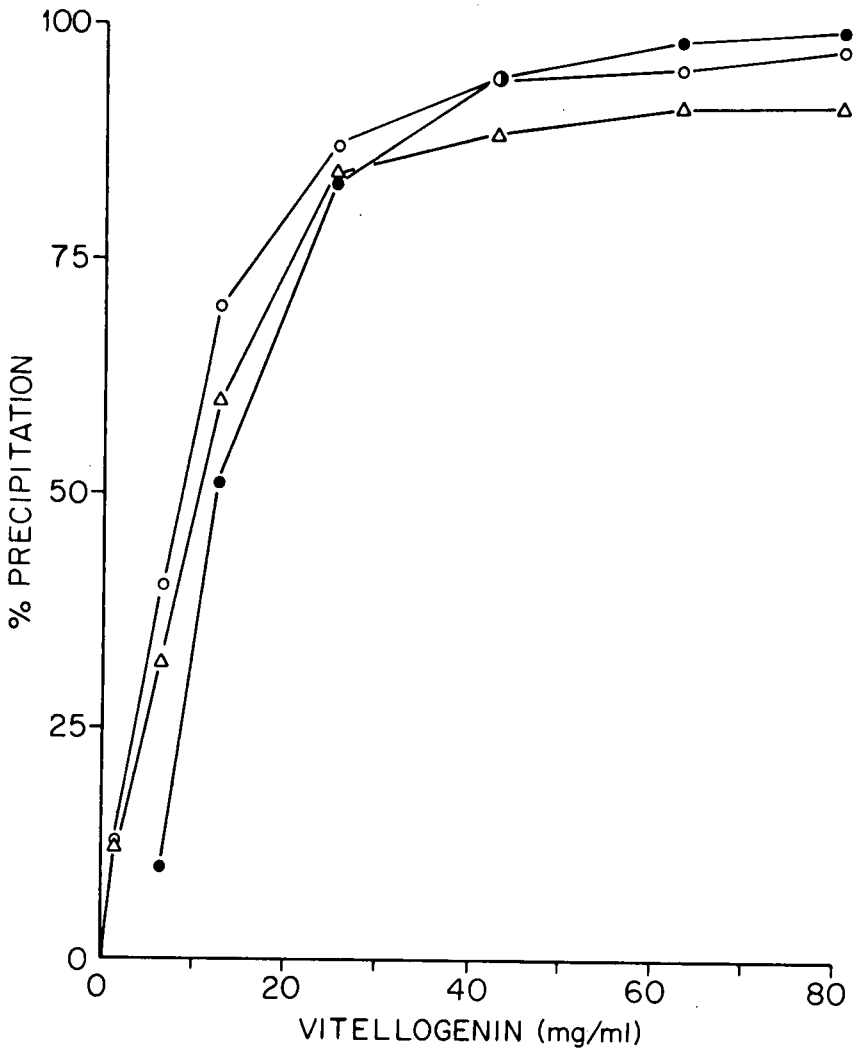

Figure 2. Precipitation of vitellogenin by use of different ratios of Mg^{2+}:EDTA. ^{32}P-labeled plasma from an estrogen-treated animal was diluted with unlabeled control male plasma, and four volumes of 20 mM EDTA (pH 7.7) was added. 0.5 M $MgCl_2$ was then added to give a 1:1 (●), 2:1 (O), or 3:1 (Δ) ratio of Mg^{2+}:EDTA. Percent precipitation was determined as described in Materials and Methods.

a lower ionic strength (less than 200 mM) with distilled water. The use of at least a 2:1 ratio of Mg^{2+}:EDTA in the first step was essential for quantitative reprecipitation.

After vitellogenin is precipitated a second time, it can be further purified on DEAE-cellulose. Alternatively, it can be dissolved in 1 M NaCl and dialyzed against a buffer containing 1 M NaCl to remove the Mg^{2+} and EDTA. After sufficient dialysis (usually overnight), the vitellogenin solution can be transferred to any desired low ionic strength buffer. Dialyzing the vitellogenin against a low ionic strength buffer prior to the removal of the Mg^{2+} and EDTA will result in the precipitation of the vitellogenin in the dialysis tubing.

I have also used the selective precipitation as a method for determining the concentration of vitellogenin in the serum of estrogen-treated animals. 200 µl of a serum sample is mixed with 0.8 ml of 20 mM EDTA (pH 7.7) and then with 80 µl of 0.4 M $MgCl_2$. The precipitate is pelleted in a centrifuge and dissolved in three ml of 50 mM Tris-Cl (pH 7.5) containing 1 M NaCl. The solution is transferred to a 10 ml volumetric flask which is then brought to 10 ml with the same buffer. The absorbance of the sample is read at 280 nm and the vitellogenin concentration is calculated using an absorptivity value of 0.75 liter/g•cm (14). Figure 3 shows the results of several experiments in which I assayed vitellogenic serum diluted with various amounts of male serum; an assay of purified vitellogenin diluted with normal saline is also shown. The assay was linear over its entire range. In a separate experiment I have verified that the assay remains linear up to a

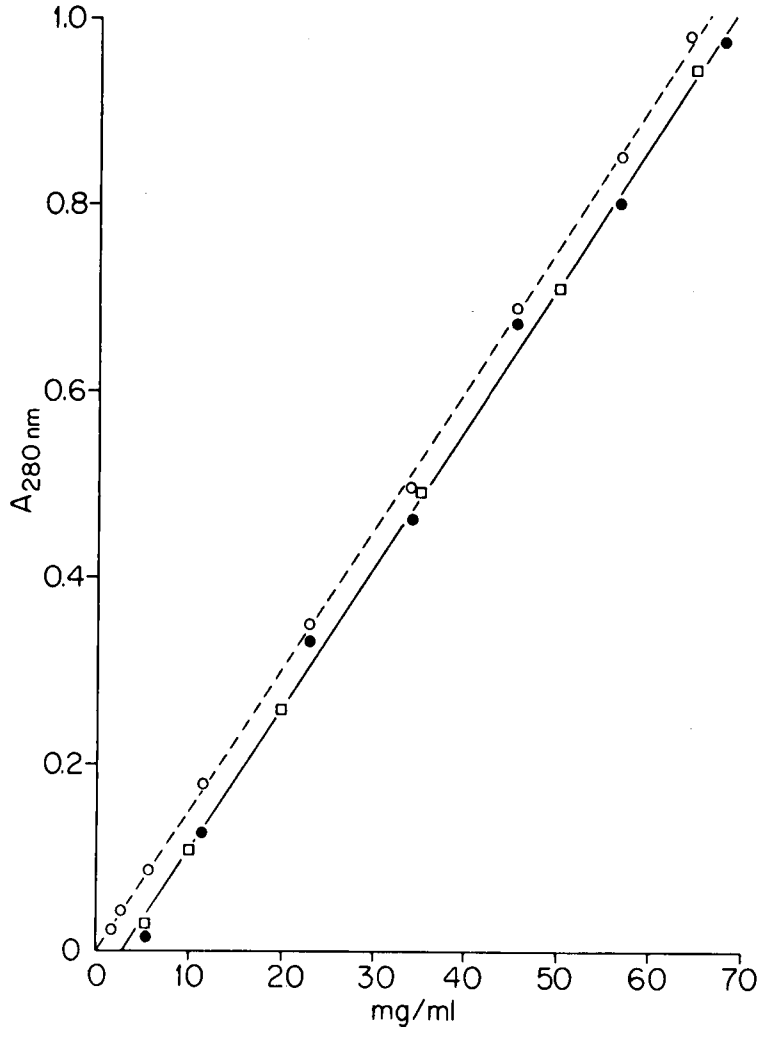

Figure 3. Assay of vitellogenin by Mg^{2+}:EDTA precipitation. Standard vitellogenic serum samples were diluted with normal male serum and aliquots (200 µl) were then assayed according to the method presented in the text. The absorbance at 280 nm of the final sample dilution is plotted against the known concentration of vitellogenin in the samples. The least-squares analysis of the data from two separate experiments (● , □) is indicated by the solid line. Also included is an experiment in which samples containing purified vitellogenin in normal saline were assayed (O); the line that should have been theoretically obtained is indicated by a dashed line.

concentration of 150 mg/ml (results not shown). Least-squares analysis of the data from the serum gives a linear correlation of 0.996. However, while the least-squares analysis of the serum data gives a line with the theoretical slope of 0.015 A_{280}·ml/mg, the line intercepts the X-axis at 2.6 mg/ml instead of at the origin. This phenomenon has been seen in several experiments and is apparently due to the presence of other serum proteins, since it is not seen in the case of purified vitellogenin (also see Appendix A).

Purification of Vitellogenin by DEAE-Cellulose Chromatography

It is sometimes desirable to further purify the Mg^{2+}:EDTA precipitated vitellogenin to remove all traces of other serum proteins. Alternately, it is also desirable to be able to isolate the vitellogenin present in low amounts in the blood. As an alternate method of purifying vitellogenin, I have developed a DEAE-cellulose procedure performed at neutral pH as described in Materials and Methods.

When I chromatographed vitellogenic plasma on DEAE-cellulose using a simple linear salt gradient, vitellogenin was the last protein eluted from the column. Chromatography of [^{32}P] vitellogenin confirmed the identity of the last peak as that of vitellogenin (Figure 4). During my initial attempts to chromatograph vitellogenin on DEAE-cellulose, I noted that the elution position of vitellogenin was dependent upon the amount of protein loaded onto the column (Figure 5). This is similar to the effect that our laboratory has observed with TEAE-cellulose (unpublished observations). At low protein loads, vitellogenin

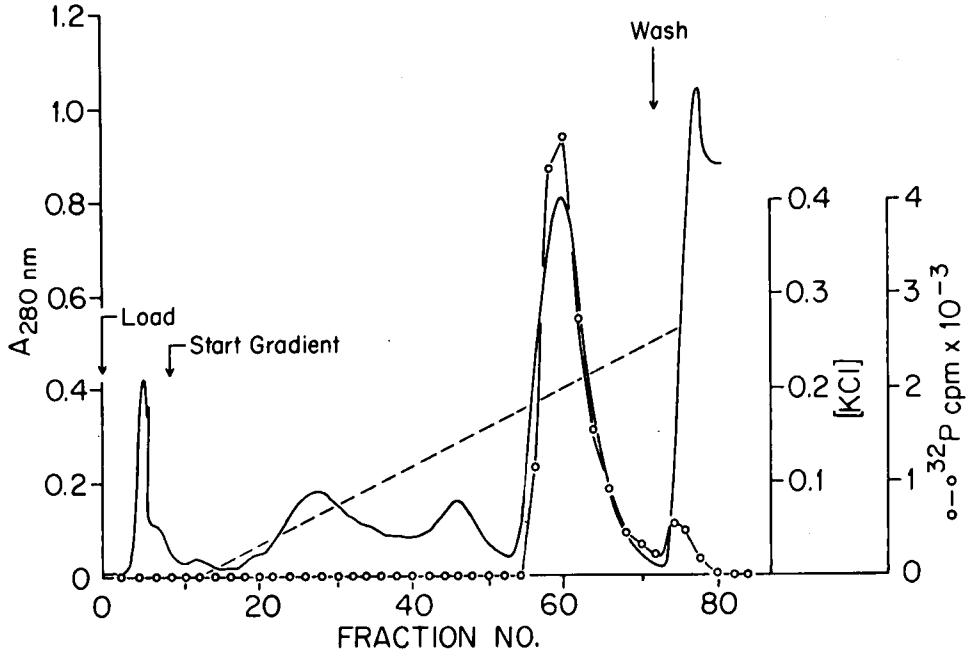

Figure 4. DEAE-cellulose chromatography of ^{32}P-labeled plasma from an estrogen-treated Xenopus female. Five ml of plasma from an animal injected with estradiol-17β 9 days previously was placed on a 1.9 × 32 cm column and eluted with a 1000-ml linear salt gradient from 0.0-0.35 M KCl. Acid-insoluble ^{32}P counts (o) were determined on 150-µl aliquots of each fraction.

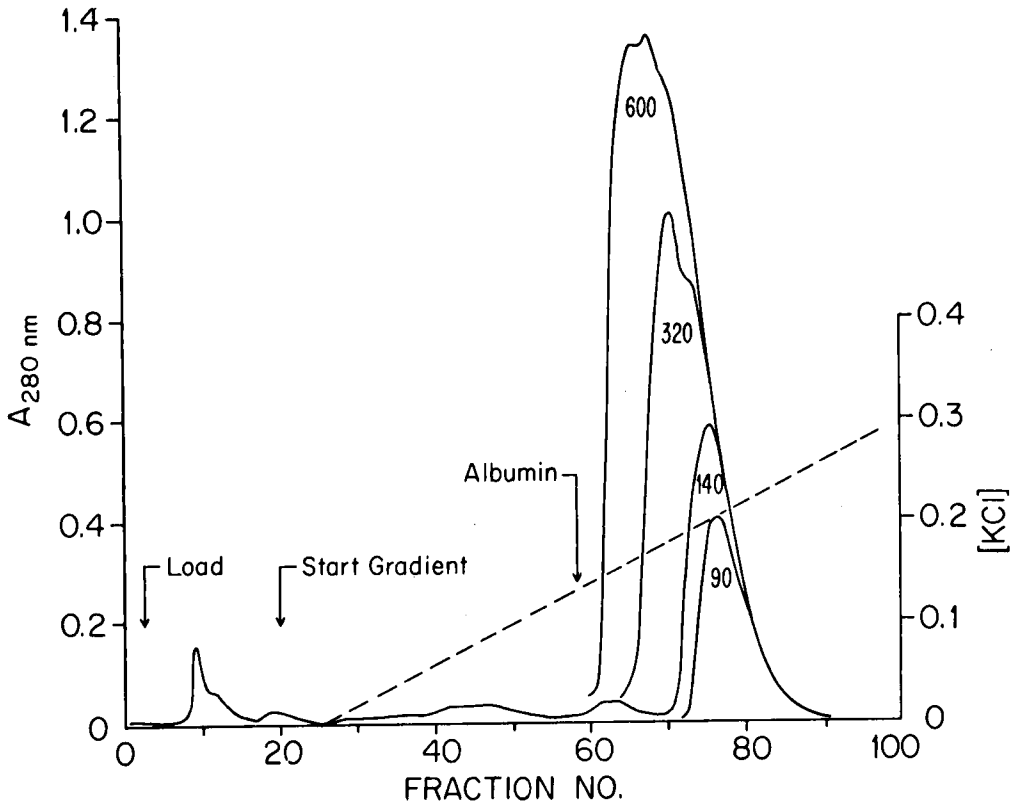

Figure 5. Effect of different loads of vitellogenin on its elution position from a DEAE-cellulose column. Plasma samples from estrogen-treated Xenopus females containing various amounts of vitellogenin as determined by the method described in the text were loaded on a 1.9 × 32 cm column of DEAE-cellulose and eluted with a 1000 ml salt gradient. The numbers under each curve indicate the amount (mg) of vitellogenin applied in each run.

elutes reproducibly at 0.205 M KCl as opposed to 0.135 M KCl for Xenopus albumin. With progressively higher loads of vitellogenin, the leading edge of the peak elutes progressively earlier, decreasing the resolution of the system. Thus, I recommend that loads on a 100 ml bed volume column do not exceed 250 mg vitellogenin in order to maintain adequate resolution in the system. This load is still higher than that feasible for TEAE-cellulose (25).

Vitellogenin that has been prepurified by Mg^{2+}:EDTA precipitation can be loaded directly onto a DEAE-cellulose column. The pellet obtained from the second precipitation of approximately 250 mg of vitellogenin (see previous section) is dissolved in 1 ml of 0.5 M KCL, 200 mM EDTA (pH 7.7). The green liquid is then diluted with 5 volumes of distilled water. If the liquid is opalescent after dilution, a 200 mM EDTA solution (pH 7.7) is added dropwise until the fluid becomes transparent. The solution is then directly loaded onto a DEAE-cellulose column and the vitellogenin is eluted as described above. It is very important to completely dissolve the vitellogenin with the EDTA solution prior to loading the protein on the column. Otherwise, the vitellogenin will not adsorb onto the DEAE-cellulose. Figure 6 is a chromatographic profile of vitellogenin prepurified by Mg^{2+}:EDTA precipitation. It can be seen that there was a small amount of protein that passed unadsorbed through the column. There is also a small amount of protein that eluted at 0.06 M and 0.14 M KCl. The latter corresponds to the elution position of Xenopus albumin. Vitellogenin eluted at its normal position (0.2 M KCl).

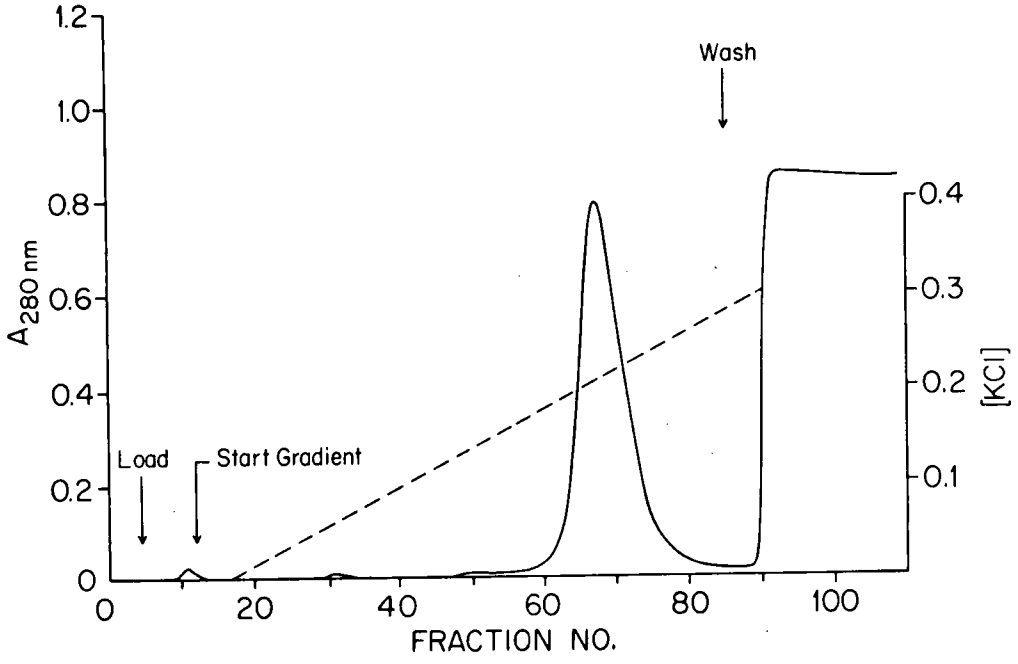

Figure 6. DEAE-cellulose chromatography of vitellogenin purified by Mg^{2+}:EDTA precipitation. The precipitate, containing 250 mg of vitellogenin, was dissolved in 200 mM EDTA as described in the text and directly loaded onto a 1.9 × 32 cm column of DEAE-cellulose. The vitellogenin was then eluted with a 1000 ml linear salt gradient.

In order to evaluate the purity of my preparations, I subjected large amounts of purified vitellogenin (100 µg) to gel electrophoresis using two different buffer systems: one at pH 7.4 and one at pH 9.6 (23). The results are seen in Figure 7. Both electrophoretic systems gave very similar results. The major protein found in plasma from estrogen-treated animals was vitellogenin. Vitellogenin purified by DEAE-cellulose chromatography was contaminated by albumin as was expected due to the close elution position of albumin. I estimated, by comparing the intensity of the albumin band with that obtained by electrophoresing known amounts of Xenopus albumin, that the albumin contamination accounted for less than 2% of the total protein. I have also observed a small albumin contaminant in TEAE-cellulose purified vitellogenin. Surprisingly, the vitellogenin obtained by Mg^{2+}:EDTA precipitation was nearly homogeneous. I was unable to estimate the degree of contamination due to the very small amount present. The contaminating proteins also seemed distributed throughout the gel, indicating that they probably arose through nonspecific adsorption to the precipitate and not through some specific precipitation. Vitellogenin purified by a combination of Mg^{2+}:EDTA precipitation and DEAE-cellulose chromatography also appeared nearly homogeneous. Again, no estimate of the degree of contamination was possible due to the very small amounts present. However, as stated previously, I observed that there are small amounts of serum protein removed when Mg^{2+}:EDTA precipitated vitellogenin is chromatographed on DEAE-cellulose (Figure 6). Therefore, the sequential purification of vitellogenin by both methods results in a more homogeneous preparation. Nevertheless,

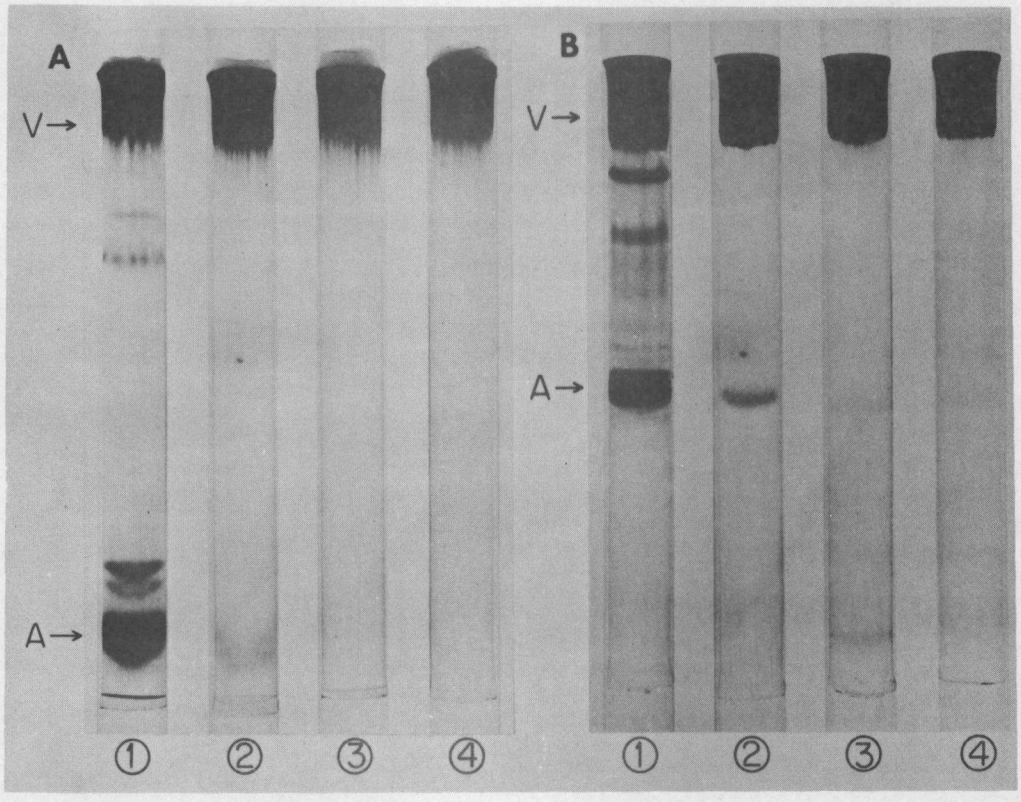

Figure 7. Native gel electrophoresis of different vitellogenin preparations. All gels were 8% T, 2% C in acrylamide. Set (A) is gel system 1935 (23) run at pH 7.4, and set (B) is gel system 28.60.0.X (10) run at pH 9.6. 1, Plasma from an estrogen-treated animal with a vitellogenin titer of 100 mg/ml. 2, DEAE-cellulose-purified vitellogenin. 3, Mg^{2+}:EDTA-purified vitellogenin. 4, Vitellogenin purified by Mg^{2+}:EDTA precipitation followed by DEAE-cellulose chromatography. All gels were loaded with samples containing 100 µg of vitellogenin and stained with Coomassie brilliant blue R-250. V corresponds to vitellogenin and A corresponds to albumin.

the purification of vitellogenin by Mg^{2+}:EDTA precipitation alone results in vitellogenin of greater purity than that prepared by any other single method to date.

Discussion

I have presented here two new methods for the purification of Xenopus vitellogenin. The methods represent a considerable improvement over previously described techniques. The Mg^{2+}:EDTA precipitation technique is remarkable in that it can specifically precipitate vitellogenin from a complex mixture of serum proteins. The exact mechanism whereby the Mg^{2+}:EDTA selectively complexes with vitellogenin is not known, but it presumably involves the attachment of the Mg^{2+} ions to the phosphate moeities of vitellogenin and a subsequent "cross-linking" of the magnesium-phosphate groups by the bifunctional chelating agent, EDTA. However, steric factors relating to the vitellogenin molecule are probably also involved since the Mg^{2+}:EDTA is not able to precipitate all phosphoproteins. As an example, I tried to precipitate goldfish vitellogenin with Mg^{2+}:EDTA, but was unsuccessful. Subsequent analysis of goldfish vitellogenin revealed that it has half of the protein phosphorus content of Xenopus vitellogenin (0.79% versus 1.4%). On the other hand, vitellogenins from the turtle (Chrysemys picta) the pigeon (Columba livia) and the chicken (Gallus domesticus) were all precipitated by Mg^{2+}:EDTA. The goldfish vitellogenin may therefore be the exception to the rule regarding the precipitability of vitellogenin by Mg^{2+}:EDTA, but it still indicates that the presence of protein phosphorus groups per se is not sufficient to account for the action of Mg^{2+}:EDTA.

The DEAE-cellulose chromatography technique was a straight forward application of the theory of ion-exchange columns. The presence of protein phosphorus groups on vitellogenin would be expected to give the protein a strong negative charge at neutral pH. I therefore predicted that vitellogenin should be the last protein eluted from the column when a simple salt gradient was used. Indeed, this proved to be the case (see Figure 4, page 22). There were several reasons for developing a DEAE-cellulose chromagraphic technique rather than using a TEAE-cellulose chromatographic procedure as previously described by our laboratory (26):

1. At neutral pH there is less chance of alkaline dephosphorylation of vitellogenin (30). The initial buffer of the TEAE-cellulose system is pH 9.9.

2. The load capacity of DEAE-cellulose is substantially higher at neutral pH than TEAE-cellulose at alkaline pH (25).

3. The DEAE-cellulose system requires only a two-bottle, linear gradient utilizing a readily obtainable Tris-buffer system instead of the three-bottle exponential gradient system and Monol buffers, which require redistillation prior to use (26).

In addition to the above, there is also the problem of extreme variability of batches of TEAE-cellulose as discussed in the introduction of this chapter. In view of these considerations, I feel that chromatography of vitellogenin on DEAE-cellulose is superior to the previously described TEAE-cellulose system (26).

The evaluation of the purity of isolated vitellogenin is somewhat difficult. Since over two-thirds of the serum protein in the blood of

estrogen-treated animals can be present as vitellogenin (14), partial purification can produce a single band on gels when small amounts are subjected to electrophoresis. Gel electrophoresis in the presence of SDS is not suitable because the generation of proteolytic cleavage fragments from vitellogenin during the isolation procedure can complicate the evaluation of purity (16). The electrophoresis of vitellogenin in native gels can lead to selective "stacking" or "unstacking" of contaminating proteins and hence lead to erroneous conclusions concerning the presence of contaminating serum proteins (23). I thus elected to subject large amounts (100 µg) of purified vitellogenin to native gel electrophoresis in two different buffer systems: one at pH 7.4 and one at pH 9.6 (23). The results (Figure 7, page 27) indicated that the Mg^{2+}:EDTA purified vitellogenin contained only trace amounts of other proteins. DEAE-cellulose chromatography of Mg^{2+}:EDTA purified vitellogenin (Figure 6, page 25) confirmed that only trace amounts of other proteins were present. Since DEAE-cellulose chromatography of Mg^{2+}:EDTA purified vitellogenin seems to remove these trace contaminants, vitellogenin purified by a combination of the two methods would seem to result in a preparation of greater purity than that prepared by any other methods to date. The availability of these simple methods for the purification of vitellogenin should greatly facilitate the study of the physical and chemical properties of this molecule.

CHAPTER III

THE PURIFICATION AND PROPERTIES OF A PHOSPHOPROTEIN KINASE ISOLATED FROM *XENOPUS LAEVIS* LIVER AND ITS USE IN LABELING VITELLOGENIN *IN VITRO*

Summary

A cytoplasmic phosphoprotein kinase (ATP:protein phosphotransferase; EC 2.7.1.37) was purified 21,000-fold from *Xenopus laevis* liver by employing ammonium sulfate fractionation, DEAE-cellulose chromatography and phosphocellulose chromatography. The enzyme requires Mg^{2+} for activity and is specific for highly phosphorylated proteins such as casein and phosvitin. The phosvitin precursor, vitellogenin, is also an excellent substrate for the enzyme. The kinase is neither stimulated by cyclic nucleotides nor inhibited by the heat-stable inhibitor protein. The enzyme is extremely stable and has an unusually high affinity for phosphocellulose. The K_m for ATP (2.2×10^{-6}M) is one of the lowest yet reported for a protein kinase. At pH 8.0, the enzyme displays an apparent negative cooperativity with regard to its protein substrate. However, at pH 9.5, this negative cooperativity is mostly abolished with a consequent increase in enzyme activity. At pH 9.5 the apparent K_m for vitellogenin is 4.3×10^{-6}M. The enzyme was used to label vitellogenin *in vitro* to a high specific activity (approximately 5×10^8 cpm/mg). The labeled vitellogenin was incorporated into oocytes at a high rate (approximately 300 ng/oocyte/hr) and was processed normally into the yolk proteins.

Introduction

The protein kinases (EC 2.7.1.37, ATP:protein phosphotransferase) are classified into two main groups, those whose activity is regulated by cyclic nucleotides and those whose activity is independent of such regulation (31). Among the cyclic nucleotide independent protein kinases (group III in the classification system of Walsh) (31) are included the phosvitin and casein kinases. This group of enzymes preferentially phosphosphorylate acidic phosphoproteins. The exact function of the acidic phosphoprotein kinases in the cell is not known since their endogenous substrates have never been positively identified. However, their widespread distribution with respect to species and cell type may indicate an important role of these enzymes in cellular function.

I have been studying the precursor-product relationship between vitellogenin and phosvitin in the amphibian Xenopus laevis. Vitellogenin is the yolk precursor protein synthesized by the liver of oviparous vertebrates under the influence of estrogens (1). Vitellogenin is incorporated into growing oocytes by endocytosis (32) where it is then proteolytically processed into phosvitin and lipovitellin (16). To investigate this process, I developed a method for labeling vitellogenin with [γ-^{32}P]ATP by using a phosphoprotein kinase that I isolated from Xenopus liver. I found that the enzyme is extremely stable and displays an apparent negative cooperativity with regard to its protein substrate. In addition, the K_m of its substrates are among the lowest ever reported for a protein kinase.

Materials and Methods

General

The care, maintenance and bleeding of Xenopus was performed as previously described (22). Isotopes were purchased from Amersham and the ammonium sulfate was ultrapure grade from Swartz-Mann. Most chemicals were from Sigma Chemical Company and included protein kinase inhibitor from rabbit muscle (200 units/mg), glyceraldehyde-3-phosphate dehydrogenase from yeast (lyophilized and sulfate-free powder), 3-phosphoglyceric phosphokinase (type X), adenosine 5'-triphosphate (Sigma grade), glyceraldehyde-3-phosphoric acid (free acid) and alpha-casein. Vitellogenin was isolated from the plasma of estrogen-treated animals by Mg^{2+}:EDTA precipitation (Chapter I) and phosvitin was isolated from amphibian ovaries through the ammonium sulfate step (33). Protein was determined by the method of Bradford (34) using bovine gamma globulin as a standard. DEAE-cellulose columns (Selectacel Type 40, Lot #D1391, 0.94 meq/g, Schleicher and Schuell Company) and phosphocellulose columns (Whatman P-11, 7.4 meq/g) were packed and washed as previously described (26). All columns were treated with serum proteins and washed (26) to minimize adsorption of proteins. All operations were performed at 0°-4°.

Preparation of [γ-^{32}P]ATP

The [γ-^{32}P]ATP was prepared by a modification of the procedure of Wallace (35). The reaction volume was 1 ml and contained 1 µmole EDTA, 1 µmole dithiothreitol, 0.1 µmole NAD, 0.01 µmole glyceraldehyde-3-phosphoric acid, 10 units of glyceraldehyde-3-phosphate dehydrogenase,

and 2 mCi of $^{32}P_i$, (carrier-free). After 30 min at room temperature, 5 µmole $MgCl_2$, 0.1 µmole ADP and 10 units of 3-phosphoglyceric phosphokinase were added. After an additional hour, the reaction mixture was placed over a 10 ml bed volume column of DEAE-cellulose equilibrated with 0.01 M NH_4HCO_3 (pH 8.6). The column was eluted with a 50 ml gradient ranging from 0.01 to 0.25 M NH_4HCO_3 (pH 8.6). [γ-^{32}]ATP emerged as the last peak (as determined by radioactivity measurements) at a concentration of 0.17-0.19 M NH_4HCO_3 and was concentrated by flash evaporation. The [γ-^{32}P]ATP was assayed by the luciferase assay (36) and was typically found to have a specific activity of 25-35 Ci/mmole. For the kinetic analysis of the enzyme, [γ-^{32}P]ATP (greater than 3000 Ci/mmole) was purchased from Amersham and diluted with unlabeled ATP to give preparations of exact specific activities.

Assay of Phosphoprotein Kinase Activity

Samples were assayed for phosphoprotein kinase activity in multiwell microtiter plates (with covers) using a total reaction volume of 100 µl. The reaction mixture was 50 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 10 µM [γ-^{32}P]ATP and contained 5 mg/ml of Xenopus vitellogenin. The reaction was initiated by adding 10 µl of enzyme sample and incubating at 20°. Under these conditions, the reaction was linear for 3 h and the rate was directly proportional to enzyme concentration (data not shown). There was no significant protein kinase activity in the absence of vitellogenin or the enzyme. Rate measurements were performed by taking aliquots (20 µl) of the reaction mixture at intervals and spotting them on Whatman 3 MM disks

which were then placed in 10% (w/v) trichloroacetic acid containing 5 mM sodium pyrophosphate. After processing (24), the disks were placed in 5 ml 0.6% 2,5-diphenyloxazole in toluene and counted in a scintillation counter. Alternatively, the disks were counted in a Beckman Lobeta II Planchet counter. One unit of enzyme activity is defined as that amount which transfers one pmole of ^{32}P into vitellogenin per min at 20° under the standard assay conditions.

Subcellular Fractionation

Xenopus livers were rinsed in 50% Dulbecco's phosphate-buffered-saline (Grand Island Biological Supplies) and finely diced with a razor blade. They were homogenized in 9 volumes of solution containing 100 mM sucrose, 50 mM KCl, 2 mM EDTA, 50 mM piperazine—N,N'-bis(2-ethane-sulfonic acid) (pH 7.0) (37) in a motor driven teflon homogenizer for 3 min at 1000 rpm. The homogenate was filtered through several layers of cheesecloth and sequentially centrifuged at 700 x g for 10 min to isolate the nuclei, 20,000 x g for 10 min to isolate the mitochondria, and at 105,000 x g for 1 h to isolate the microsomes (38). All fractions were reconstituted to the same volume with homogenization buffer and aliquots were tested for protein kinase activity.

Isolation of the protein kinase. Animals were bled by heart incision (39) and the livers were removed and rinsed in solution O-R2 (40) containing 0.07 M sodium citrate. Livers were then pooled and stored at -20° until needed. In a typical procedure, 88 g of frozen liver was broken up with a wooden mallet and pulverized in a Waring

Blendor prechilled to -20°. Buffer (440 ml) containing 100 mM sucrose, 50 mM KCl, 2 mM EDTA, 100 mM Tris-Cl (pH 8.0) was added and the mixture was blended for 3 min. The homogenate was centrifuged for 20 min at 10,000 x g and the dark pellet and lipid cap were discarded. Solid ammonium sulfate was slowly added to bring the concentration to 1.6 M (using the tables of Wood) (41). After equilibrating for 1 h, the solution was centrifuged at 10,000 x g for 25 min and the subnatant was carefully removed. The ammonium sulfate concentration was raised to 2.8 M and after 1 h, the pellet was collected at 10,000 x g for 25 min. The pellet was dissolved in 100 ml of 50 mM Tris-Cl, pH 7.5, and dialyzed against 2 changes of 3 liters of 50 mM Tris-Cl, pH 7.5. The insoluble material was removed by centrifugation and the liquid was pumped at 300 ml/h onto a 5 × 45 cm column of DEAE-cellulose equilibrated with 50 mM Tris-Cl, pH 7.5. The column was rinsed with 1 liter of 0.22 M KCl, 50 mM Tris-Cl (pH 7.5) and the phosphoprotein kinase was eluted with 1 liter 0.35 M KCl, 50 mM Tris-Cl (pH 7.5). The column elutate was then directly pumped at a rate of 50 ml/h onto a 1.2 × 10 cm column of phosphocellulose equilibrated with 50 mM Tris-Cl, 0.2 M KCl (pH 7.5). The enzyme was then eluted with a 150 ml linear gradient (0-0.5 M) of potassium phosphate buffer (pH 7.5) made up in 0.2 M KCl, 50 mM Tris-Cl (pH 7.5) at a flow rate of 38 ml/h. Fractions (6 min) were collected and assayed for enzymatic activity. Active fractions were pooled and concentrated by ultrafiltration using a PM-10 membrane (Amicon) or by dialysis against 15% polyethylene glycol. The final volume of 1.5 ml was dialyzed against 50 mM Tris-Cl (pH 7.5) prior to storage at -20°.

Results

Intracellular Localization of Phosphoprotein Kinase

Xenopus liver was homogenized and separated into a nuclear, mitochondrial, microsomal and cytosol fraction by differential centrifugation. Each fraction was assayed for phosphoprotein kinase activity with or without vitellogenin. The results are presented in Table II. The phosphorylation of endogenous substrates was very low in all fractions. This could be due to the presence of phosphatases, enzymatic inhibitors, or compartmentation of the enzymes in the fractions. With the addition of vitellogenin, however, there was substantial phosphorylation observed in the cytosol fraction with only minor activity observed in the nuclear and microsomal fractions. Thus, the vitellogenin-specific enzyme seems to be primarily localized in the cytoplasm of the liver cell.

Ammonium Sulfate Fractionation

The 105,000 x g supernatant from a liver homogenate was fractionated by ammonium sulfate precipitation. Each fraction was then assayed for phosphoprotein kinase activity and for total protein. The results are seen in Figure 8. Most of the kinase activity precipitated between 1.6-2.4 M ammonium sulfate, slightly ahead of the bulk of the cytoplasmic protein. While the increase in the specific activity of the enzyme is very small with this procedure (approximately 2-fold), I found that this step was essential since it removed some lipoproteins that interfered with subsequent purification steps.

TABLE II

PROTEIN KINASE ACTIVITY IN SUBCELLULAR FRACTIONS OF LIVER

Cell Fraction	Kinase Activity (pmol/min)	
	+Vitellogenin	-Vitellogenin
Nuclei	39	16
Mitochondria	14	3.3
Microsomes	35	4.5
Cytosol	2440	17

Liver (3.2 g) was fractioned by differential centrifugation as described in Materials and Methods and assayed for protein kinase activity with and without vitellogenin. The values given are the total amount of activity present in each fraction.

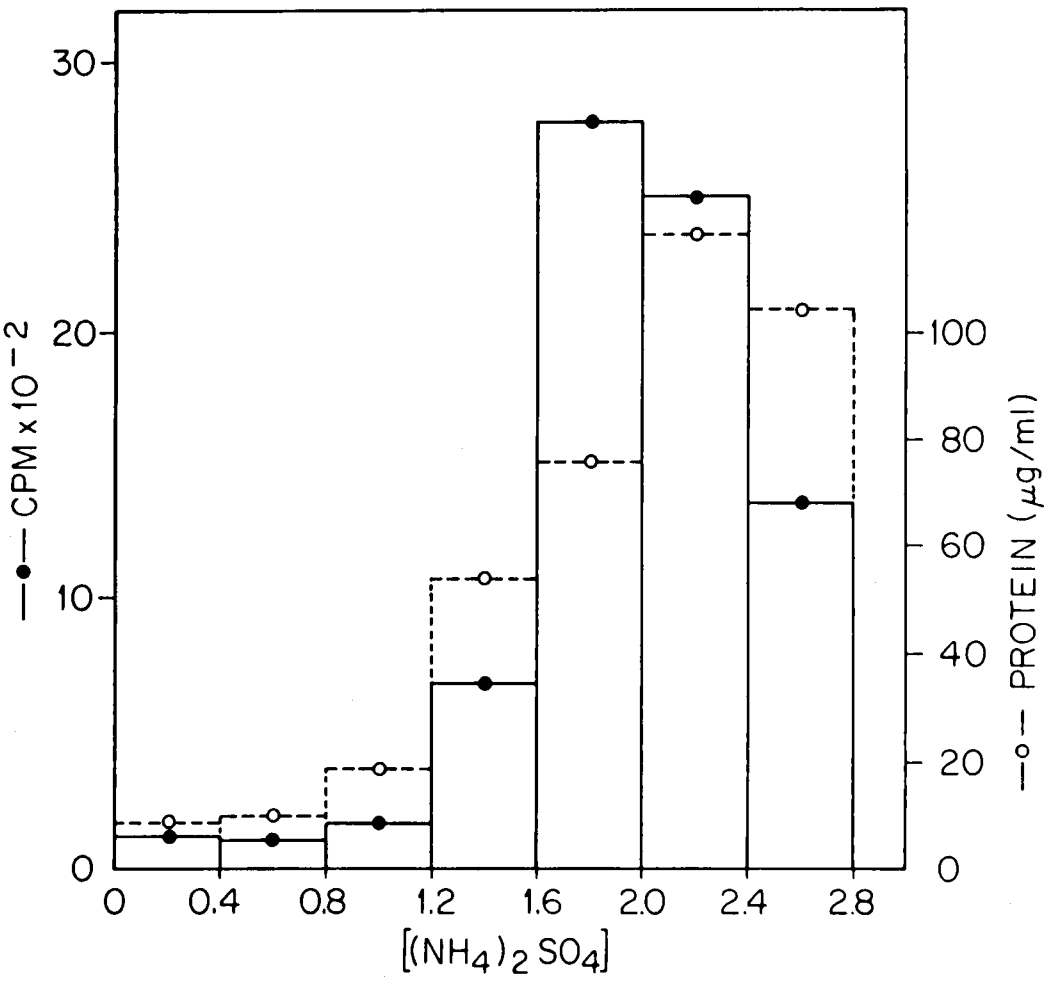

Figure 8. The precipitation of Xenopus phosphoprotein kinase by ammonium sulfate. A 105,000 x g supernatant from a 3.2 g liver was fractionated by ammonium sulfate and the precipitates were taken up and dialyzed against 0.1 M phosphate buffer, pH 7.3. After reconstituting all fractions to 4.0 ml, aliquots were assayed for protein (O) and kinase activity (●) using vitellogenin (5 mg/ml) as a substrate. All counts were corrected for activity in the absence of vitellogenin.

Agarose Gel Chromatography

The ammonium sulfate precipitate was further fractionated on a 5 × 90 cm column of Bio-gel A-1.5M. The results are seen in Figure 9. The majority of the activity eluted slightly before the position of hemoglobin. Activity also eluted between the void peak and the main enzymatic peak. When fractions from different elution positions were chromatographed on DEAE-cellulose (see subsequent section) a single peak eluting at 0.27 M KCl was always seen. The enzymatic activity eluting prior to the main peak is probably aggregates of the main phosphoprotein kinase. I tried gel chromatography in several high-ionic-strength buffers without any significant change in the elution pattern. A tendency to aggregate has also been observed for other phosphoprotein kinases. The gel chromatography resulted in a very small increase in specific activity (approximately 1.5 fold) and was thus not used in the routine preparation of the enzyme.

DEAE-Cellulose Chromatography

The protein fraction precipitating between 1.6-2.8 M ammonium sulfate was dialyzed against 50 mM Tris-Cl, pH 7.5, pumped onto a DEAE-cellulose column equilibrated with the same buffer, and the enzyme was then eluted with a linear salt gradient. The results are presented in Figure 10. Almost all of the phosphoprotein kinase activity eluted as a single peak at 0.27 M KCl, but occasionally, a minor second peak at 0.23 M KCl was also seen. The minor peak was not always present, but seemed to be present more often in liver preparations derived from estrogen-treated animals. The minor peak was not studied further.

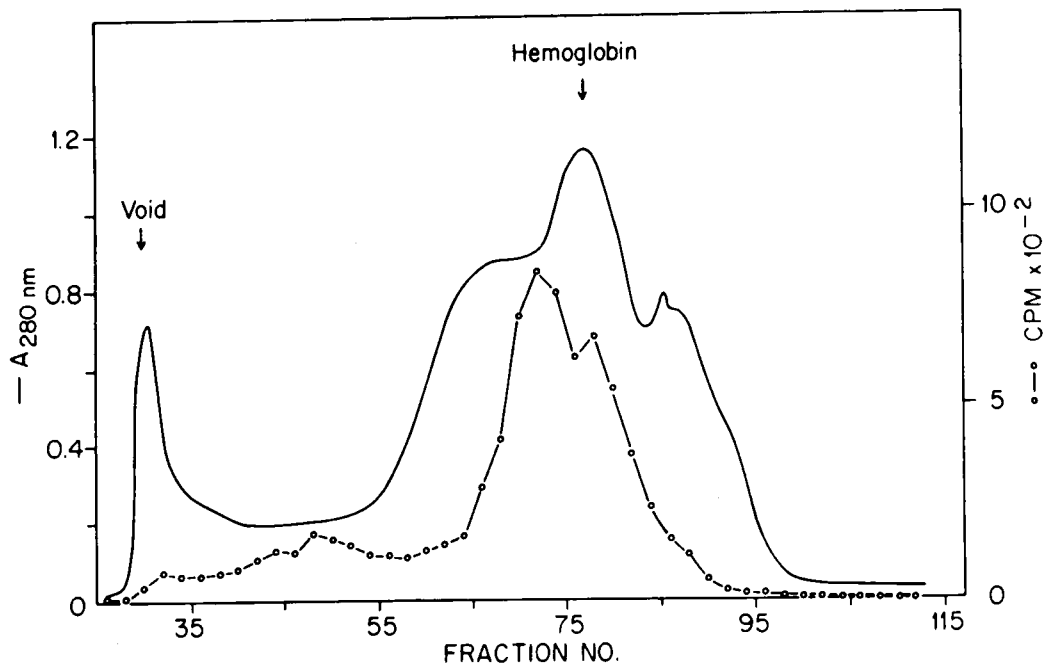

Figure 9. Fractionation of Xenopus phosphoprotein kinase by agarose chromatography. The protein from 49.2 g of liver 105,000 x g supernatant precipitating between 1.6-2.8 M ammonium sulfate was dialyzed against the running buffer (0.1 M potassium phosphate, pH 7.3) and 22 ml was placed on a 5 × 90 cm column of Biogel A-1.5 M and eluted at a rate of 100 ml/hr. Fractions (12 min) were collected and assayed for protein kinase activity using vitellogenin (5 mg/ml) as a substrate. The values were corrected for activity in the absence of vitellogenin.

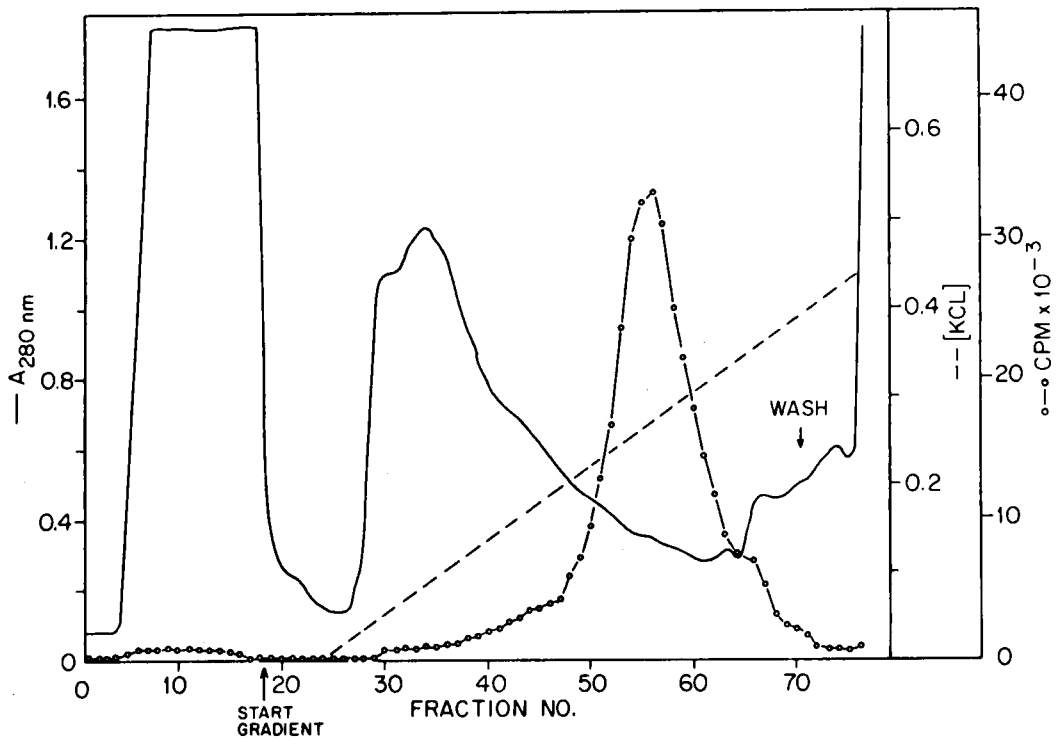

Figure 10. DEAE-cellulose chromatography of Xenopus phosphoprotein kinase. A postmitochondrial supernatant was obtained from 47 g of Xenopus liver and the fraction precipitating between 1.6-2.8 M ammonium sulfate was obtained. After dialysis against 50 mM Tris Cl (pH 7.5) the sample (150 ml) was pumped over a 200 ml bed volume column of DEAE-cellulose equilibrated with the same buffer at a rate of 290 ml/hr. The enzyme was eluted with a linear 1500 ml gradient from 0.0-0.6 M KCl and 4.5 min fractions were collected. The fractions were then assayed for protein kinase activity (o) using vitellogenin as the substrate.

Phosphocellulose Chromatography

The main peak of phosphoprotein kinase activity from the DEAE-cellulose column was collected and directly pumped onto a small column of phosphocellulose. I initially attempted to elute the enzyme from the phosphocellulose column with a KCl gradient, but no activity eluted even at a concentration of 1.4 M KCl. When 50 mM phosphate was included in the buffers, the enzyme eluted as a broad peak at an average KCl concentration of 0.8 M. However, the enzyme could be eluted as a well-defined peak with a linear phosphate gradient. As indicated in Figure 11, activity in the eluant was associated with a phosphate concentration of around 0.28 M and with relatively little UV-absorbing material. I therefore employed a phosphate gradient in my routine preparations.

Optimum Purification Procedure

The results from the above experiments were used to formulate an optimized procedure for purifying the phosphoprotein kinase from Xenopus liver as described in Materials and Methods. The analytical data for a typical preparation is given in Table III. The most noteworthy feature of the purification procedure is an increase in total enzyme activity after the DEAE-cellulose chromatography step. I am uncertain if this is due to the removal of an enzyme inhibitor (see below) or of a phophatase activity. A great increase in the specific activity of the enzyme is also achieved by the phosphocellulose chromatography step. My final preparation represents over a 21,000-fold increase in specific activity of the enzyme.

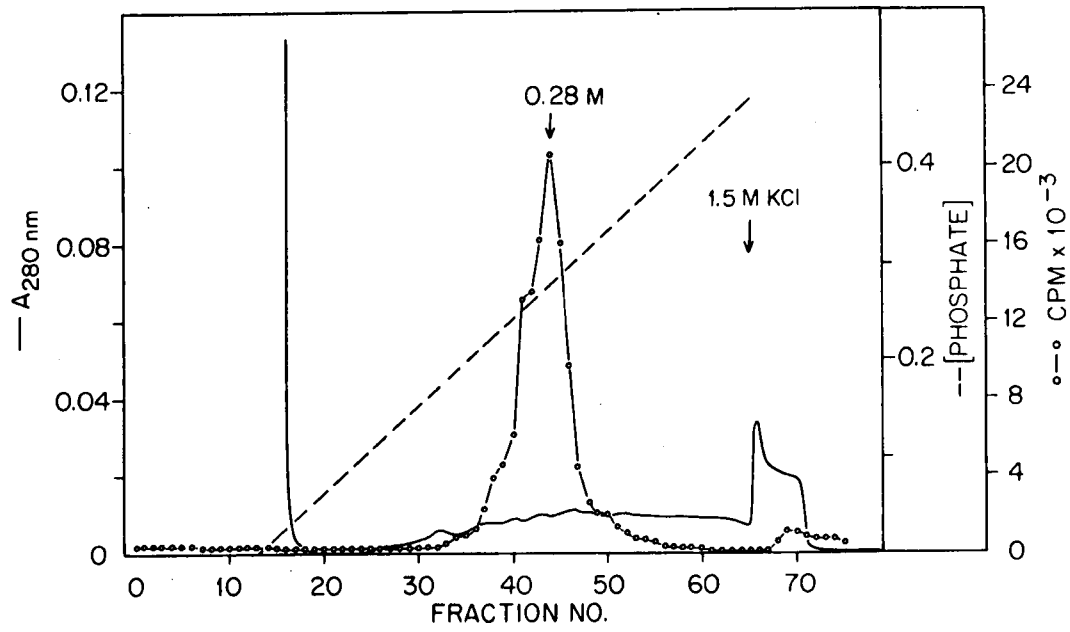

Figure 11. Phosphocellulose chromatography of Xenopus phosphoprotein kinase. The DEAE-cellulose elutate (1 Liter) from 110 g of Xenopus liver was directly pumped at 75 ml/hr onto a 30 ml bed volume column of phosphocellulose equilibrated with 0.2 M KCl, 50 mM Tris-Cl, pH 7.5. The column was then developed with a 390 ml gradient from 0-0.5 M potassium phosphate buffer (pH 7.5) made up in 0.2 M KCl, 50 mM Tris-Cl, pH 7.5. Fractions (12 min) were collected and assayed for protein kinase activity using vitellogenin (5 mg/ml) as the substrate. There was no activity in the absence of vitellogenin and no significant activity passed through the column during the loading of the sample.

TABLE III

PURIFICATION OF PHOSPHOPROTEIN KINASE FROM XENOPUS LIVER

	Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific Activity (units/mg protein)	Purification Factor	Percent Recovery
1.	Initial supernatant	425	1318	1700	1.27	---	100
2.	1.6-2.8 M $(NH_4)_2SO_4$ precipitate after dialysis	288	720	1300	1.82	1.43	76
3.	DEAE-cellulose elutate	988	32.6	7650	235	185	450
4.	Phosphocellulose elutate	32	0.103	2790	27,100	21,300	164

General Properties

The phosphoprotein kinase is very stable. Full activity was retained after several weeks of storage at 4° and after a year at -20° even with solutions as dilute as 3 µg/ml. The enzyme was stored in 50 mM Tris-Cl, pH 7.5, and no other additions were necessary. The enzyme was also stable to lyophilization, but the resultant solid was almost impossible to dissolve. Consequently, I concentrated the enzyme prior to storage by ultrafiltration with a Diaflo PM-10 membrane. Alternatively, I have successfully concentrated the enzyme by dialysis against 15% polyethylene glycol in 50 mM Tris buffer (pH 7.5). Even though I achieved a 21,000-fold purification, my preparation was not homogeneous as SDS gel electrophoresis revealed the presence of at least 6 bands. The elution position of the kinase on columns of Biogel A-1.5M (Figure 9, page 41) is consistent with a molecular weight of approximately 80,000, but this value is very uncertain due primarily to the tendency of the enzyme to aggregate.

pH Optimum and Salt and Divalent Cation Requirements

The Xenopus phosphoprotein kinase has an unusual pH optimum curve as seen in Figure 12A. Up to pH 9, the reaction was linear with time and showed a continual increase in rate. Above pH 9, the initial rate of phosphorylation was increased but the reaction slowed down with time and even reversed at pH 10 (Figure 12B). The latter is probably due to alkaline dephosphorylation of the protein (30). The unusual biphasic curve seems to be a property of the Xenopus phosphoprotein

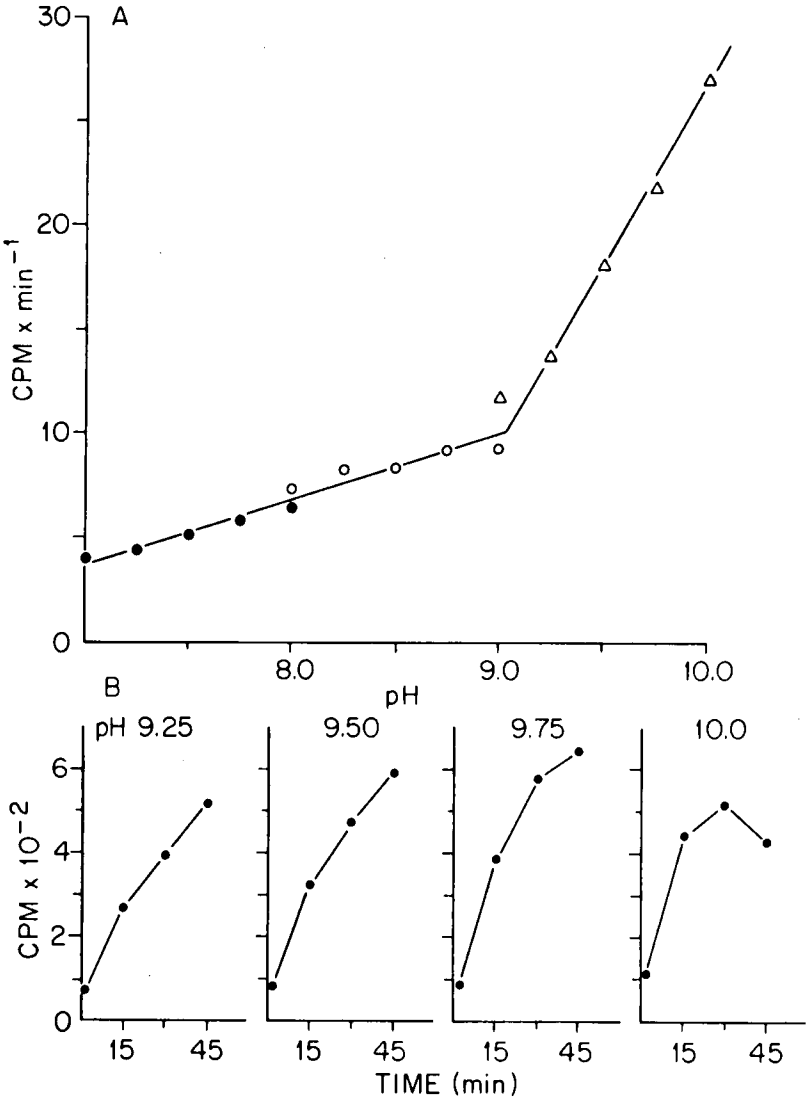

Figure 12. Activity of Xenopus phosphoprotein kinase as a function of pH. A. The phosphorylation of vitellogenin by 0.3 units of enzyme was examined under standard conditions except for the substitution of buffers (0.1 M) of different pHs. Indicated buffers are Hepes (●), Tris-Cl (o), and 2-amino-2-methyl-1-propanol (Δ). All rates were linear for 45 min except above pH 9. The points above pH 9 are the initial (15 min) rates of the reaction. B. The time courses of vitellogenin phosphorylation at high pHs. The buffer used was 0.1 M 2-amino-2-methyl-1-propanol. Counts are not corrected for background.

kinase and not the substrate since a phosphoprotein kinase isolated from bovine liver gave a "bell" shaped pH optimum curve centered at pH 8 using vitellogenin as a substrate (data not shown).

The Xenopus phosphoprotein kinase is stimulated by salt with maximum activity at 50 mM KCl (Figure 13). In the presence of 50 mM KCl, the enzyme has an absolute requirement for Mg^{2+}, with maximum activity at 5-25 mM (Figure 14). In the absence of KCl, the enzyme has a slightly higher requirement for Mg^{2+} (Figure 14), possibly due to Mg^{2+} substituting for KCl.

Substrate Specificity and Effects of Cyclic Nucleotides

The phosphoprotein kinase was found to be specific for highly phosphorylated proteins. Table IV shows the results of an experiment utilizing various substrates. Vitellogenin, alpha-casein and phosvitin were good substrates while the other proteins tested were phosphorylated to a negligible extent. The enzyme can thus be considered a phosphoprotein kinase. I also examined the effects of cAMP and cGMP on the enzyme. Neither compound had any effect on the enzyme (Table V). The protein kinase inhibitor protein, which specifically inhibits the catalytic subunit of cyclic nucleotide-dependent protein kinases (42), also had no effect on the Xenopus enzyme (Table V). Therefore, the enzyme can be classified as a cyclic nucleotide-independent protein kinase (31).

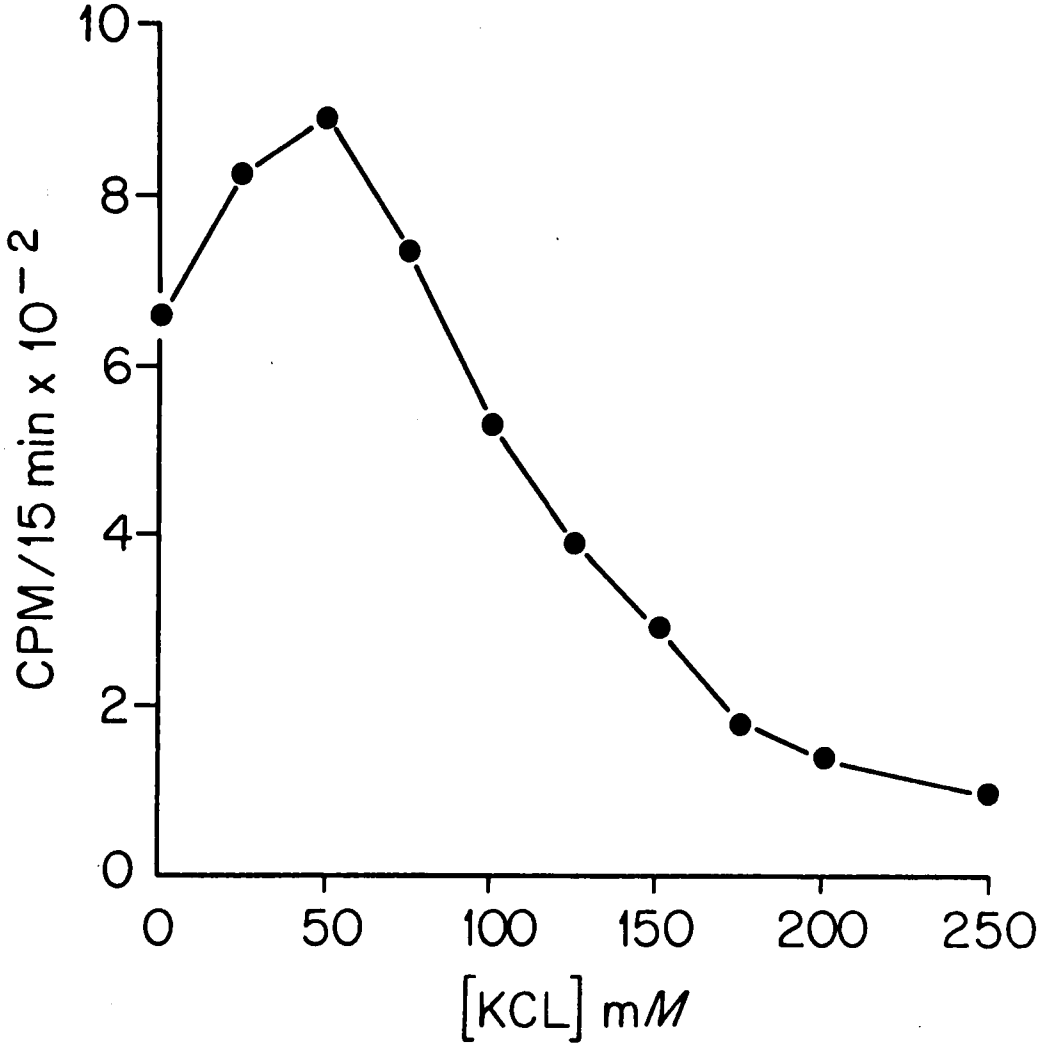

Figure 13. The effect of salt on vitellogenin phosphorylation. Vitellogenin was phosphorylated with 0.3 units of enzyme under standard conditions except for the inclusion of various concentrations of KCl. Points are the average of duplicate experiments.

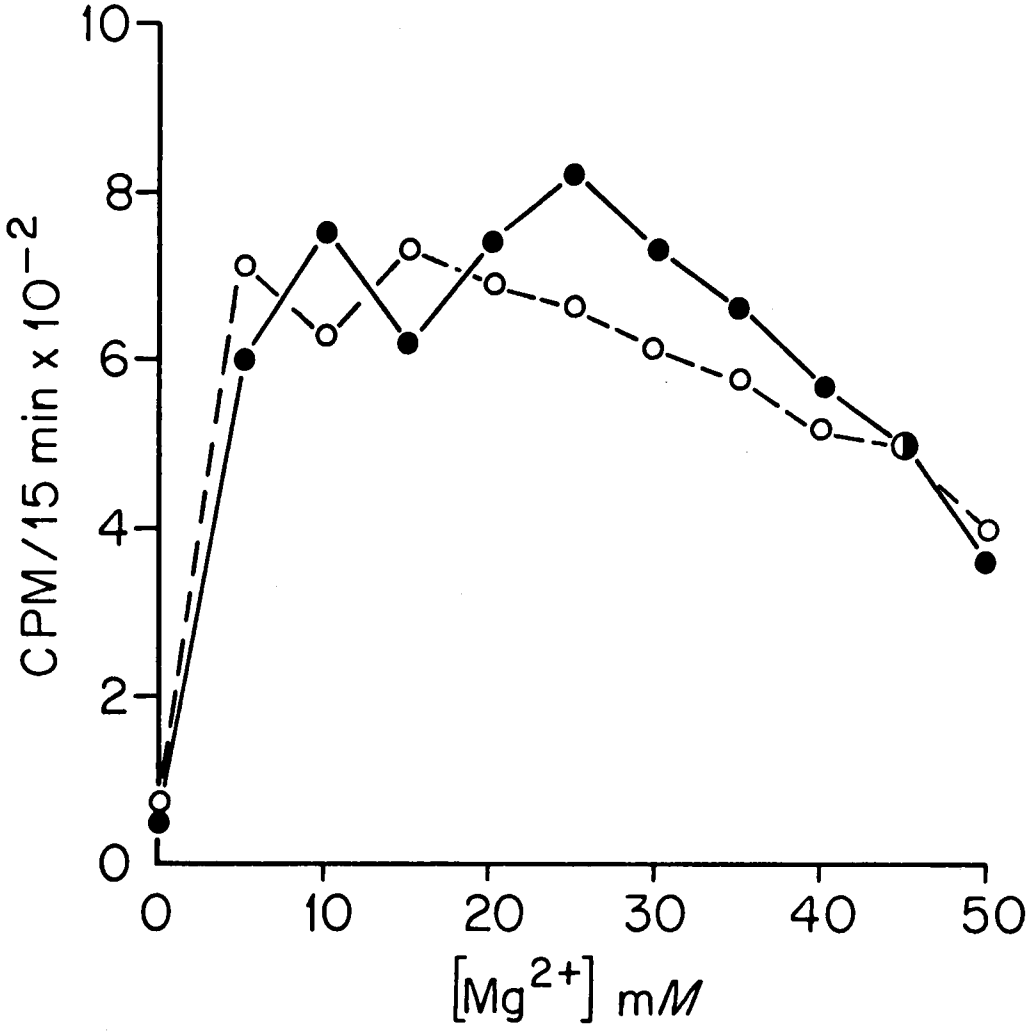

Figure 14. Requirement of Xenopus phosphoprotein kinase for Mg^{2+}. Vitellogenin was phosphorylated under standard conditions with 0.3 units of enzyme using different concentrations of $MgCl_2$ and in the presence (o) or absence (•) of 50 mM KCl. The points are the average of duplicate experiments.

TABLE IV

SPECIFICITY OF XENOPUS LIVER PHOSPHOPROTEIN KINASE

Substrate	cpm	Percent of Vitellogenin
Vitellogenin	9478	100
Albumin (BSA)	35	0.4
Aldolase	15	0.2
α-Casein	9016	95
Ovalbumin	0	--
Phosphorylase B	28	0.4
Phosvitin	13517	143
Ribonuclease A	0	--

The indicated substrates were incubated under standard conditions at a concentration of 1 mg/ml with 0.2 units of enzyme for 20 min at 21°. Indicated are the trichloroacetic acid-precipitable counts corrected for background.

TABLE V

EFFECTS OF CYCLIC NUCLEOTIDES AND HEAT-STABLE INHIBITOR PROTEIN ON XENOPUS LIVER PHOSPHOPROTEIN KINASE

Addition	cpm	Percent of Control
None	6196	100
10 µM cAMP	6313	102
10 µM cGMP	6382	103
5 units inhibitor protein	6419	104

0.1 units of kinase was incubated for 20 min at 21° using vitellogenin (1 mg/ml) as a substrate.

K_m of Substrates

The Xenopus enzyme was incubated with various ATP concentrations using vitellogenin at 5 mg/ml as a substrate and at pH 8.0. The results were plotted using the Woolf-Augustinsson-Hofstee plot and are seen in Figure 15. The plot is linear inclusive of all ATP concentrations and least squares analysis of the data gives an apparent K_m for ATP of 2.2×10^{-6}M. When the experiment was repeated using various concentrations of vitellogenin at pH 8.0 and an ATP concentration of 10^{-5}M, the Woolf-Augustinsson-Hofstee plot was nonlinear (Figure 16). However, when the reaction was run using different vitellogenin concentrations at pH 9.5, most of the nonlinearity of the plot was abolished (Figure 16) and at this pH the enzyme had an apparent K_m for vitellogenin of 2.0 mg/ml (4.3×10^{-6}M). Phosvitin also displayed the marked nonlinear Woolf-Augustinsson-Hofstee plot at pH 8.0 (data not shown). In addition, the plots for vitellogenin using bovine liver phosphoprotein kinase as the enzyme were linear at pH 8.0 (unpublished observations), indicating that this unusual behavior was a property of the Xenopus enzyme and not the substrate.

Labeling of Vitellogenin

Using the above observations, I defined a procedure for labeling vitellogenin to a high specific activity. I found that my final protein kinase preparation had no detectable protease contamination as determined by incubating vitellogenin with 15 units of the phosphoprotein kinase overnight at 20° and examining the protein by SDS gel electrophoresis. Vitellogenic sera was used as the substrate because

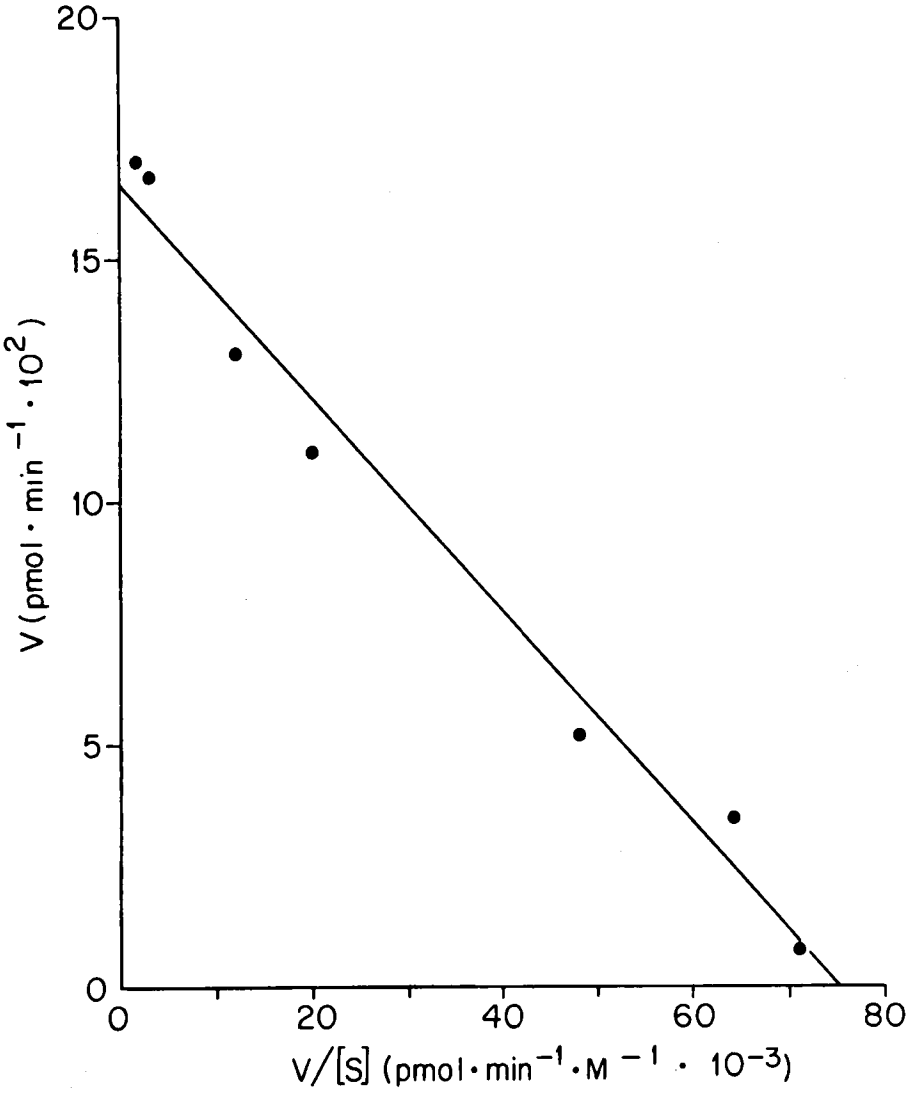

Figure 15. Woolf-Augustinsson-Hofstee plot of the rate of vitellogenin phosphorylation at different ATP concentrations. The velocity is expressed as pmol•min^{-1} using 0.13 units of enzyme at a concentration of 6.5 units/ml of reaction mixture. The points represent the average of duplicate experiments.

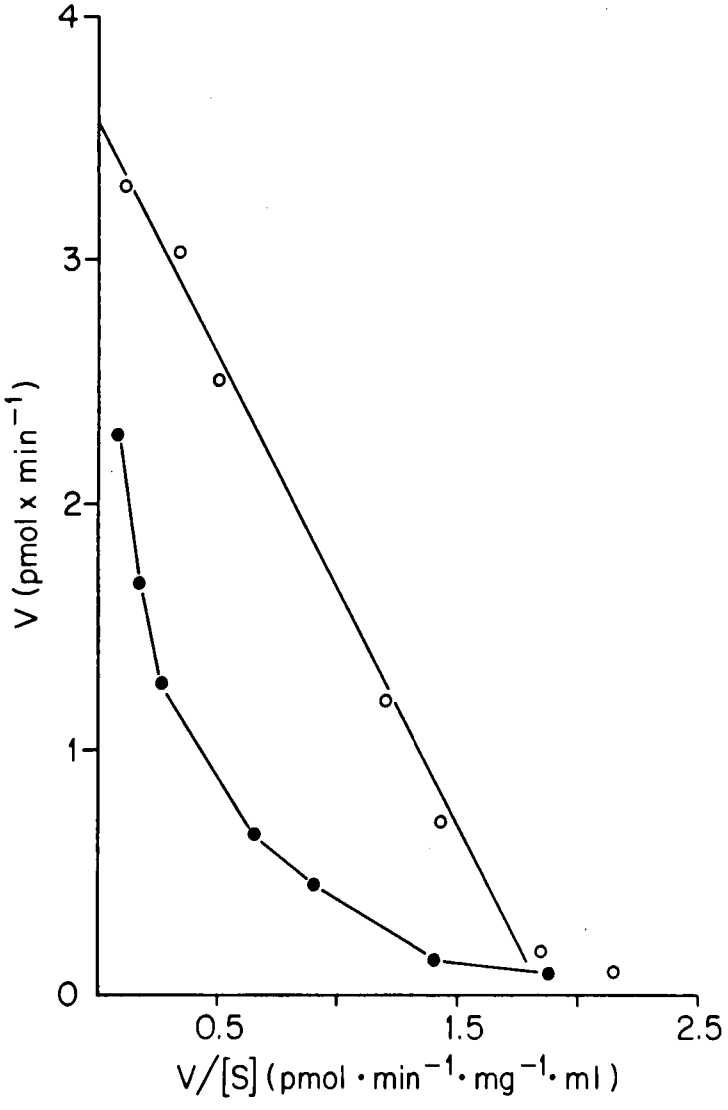

Figure 16. Rate of vitellogenin phosphorylation as a function of vitellogenin concentration and pH. The Woolf-Augustinsson-Hofstee plot of the data at pH 8.0 (•) and pH 9.5 (o). There was no significant phosphorylation in the absence of either vitellogenin or the enzyme.

it contains a component(s) that will keep vitellogenin soluble in the presence of divalent cations (see Appendix A). Because of the specificity of the phosphoprotein kinase, the serum proteins were labeled to a negligible extent. The [γ-^{32}P]ATP used was purchased from Amersham since it was available at a high specific activity (approximately 3000 Ci/mmol). It was evaporated to dryness with a stream of dry nitrogen and immediately reconstituted with 0.3 ml of water. The ^{32}P in this ATP preparation was transferred to vitellogenin with about a 50% efficiency as compared with a 95% efficiency for [γ-^{32}P]ATP that I generated myself. I also tried [γ-^{32}P]ATP purchased from New England Nuclear Company (which does not have to be evaporated down prior to use) and again obtained about 50% transfer of the ^{32}P after an 18 hr incubation period. The nature of the transfer problem remains unknown.

In order to label 3 mg of vitellogenin, 1 mCi of [γ-^{32}P]ATP in 0.3 ml of water is placed in a 1.5 ml microfuge tube and the following stock solutions are added: 10 µl of 2.5 M KCl, 10 µl of 0.5 M $MgCl_2$, 125 µl of 0.2 M Tris-Cl, pH 8.0. Serum containing 3 mg of vitellogenin (as determined by the assay described in Chapter II) is added together with 15 units of enzyme. The volume is made up to 0.5 ml and the reaction is allowed to proceed for 6-12 hrs. The time course of this reaction using an ATP concentration of 1.7×10^{-7}M is seen in Figure 17. The reaction was virtually completed at 5 hr and by 6.5 hr the transfer was approximately 95%. The reaction is terminated by passing the reaction mixture over a small (approximately 100 µl) column of phosphocellulose. This will remove the phosphoprotein kinase as well as most of the serum proteins. The [γ-^{32}P]ATP remaining can then be

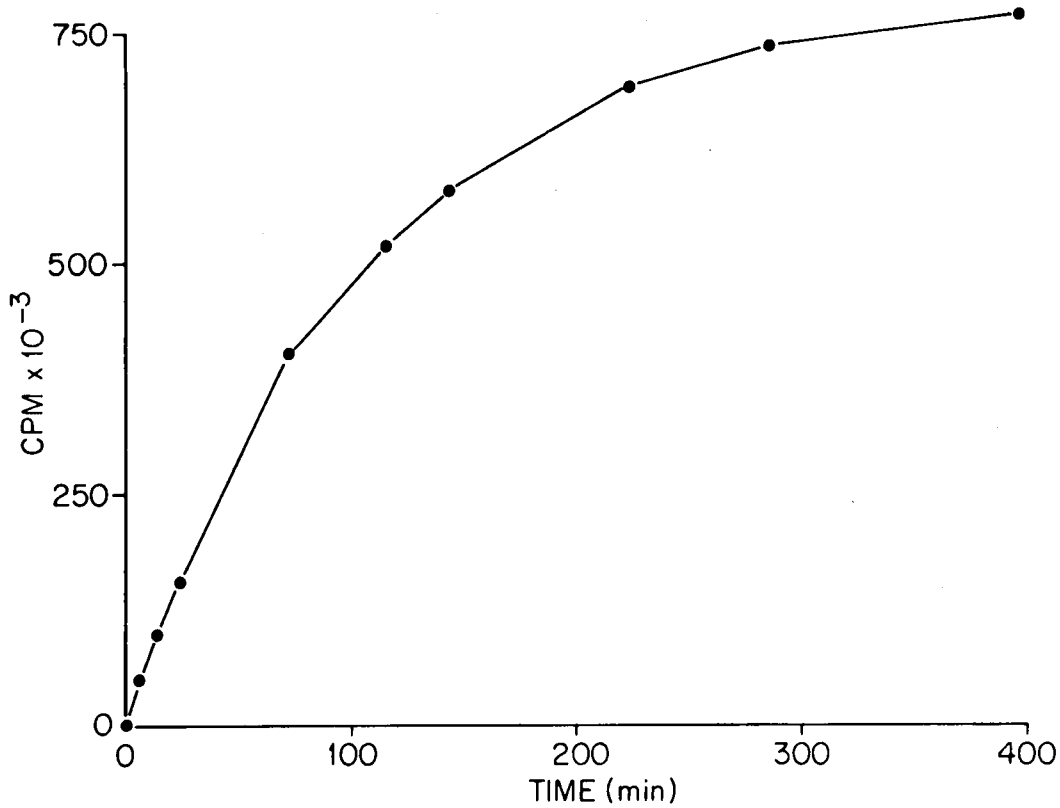

Figure 17. Time-course of labeling of vitellogenin via the phosphoprotein kinase reaction. Vitellogenin was labeled according to the procedure outlined in the text with a total ATP concentration of 1.7×10^{-7}M. Aliquots (20 µl) were removed at the indicated time points, spotted onto paper disks and processed to remove TCA-soluble counts. Total cpm/20 µl was approximately 780,000.

removed by dialysis. Using the above procedure, I have typically achieved specific activities of approximately 5×10^8 cpm/mg. The vitellogenin is relatively undegraded and when incorporated into oocytes, it is transformed into normal yolk proteins as shown in Figure 18. Note in Figure 18 that the lipovitellin 1 part of the vitellogenin molecule is labeled by the phosphoprotein kinase. Since lipovitellin 1 has very little protein phosphorus present (approximately 0.1%) (16) the ability of the phosphoprotein kinase to label it is puzzling.

Discussion

I describe here the purification and properties of a phosphoprotein kinase isolated from Xenopus liver. This enzyme shows characteristics similar to the casein kinases and phosvitin kinases isolated from other sources (43-51). Phosvitin kinase and casein kinase are trivial names given to any protein kinases that preferentially phosphorylate polyphosphoproteins. Since both casein and phosvitin are heterogenous substrates with regard to specific phosphorylation sites available, and since dephosphorylated casein and phosvitin are often used as the substrates, an enzyme's preference for either has no precise meaning. This point is illustrated by the study of Huang and Robinson (43) in which they found that a phosvitin kinase isolated from rabbit muscle had different K_ms for phosvitin and casein (1.6 mg/ml and 9.1 mg/ml, respectively), but had the same V_{max} (83-79 nmol P_i/min/mg enzyme). The term casein kinase should properly be reserved for the enzymes described by Bingham et al. (52) and Bingham and Groves (53). These seem to be the natural enzymes responsible for phosphorylating casein

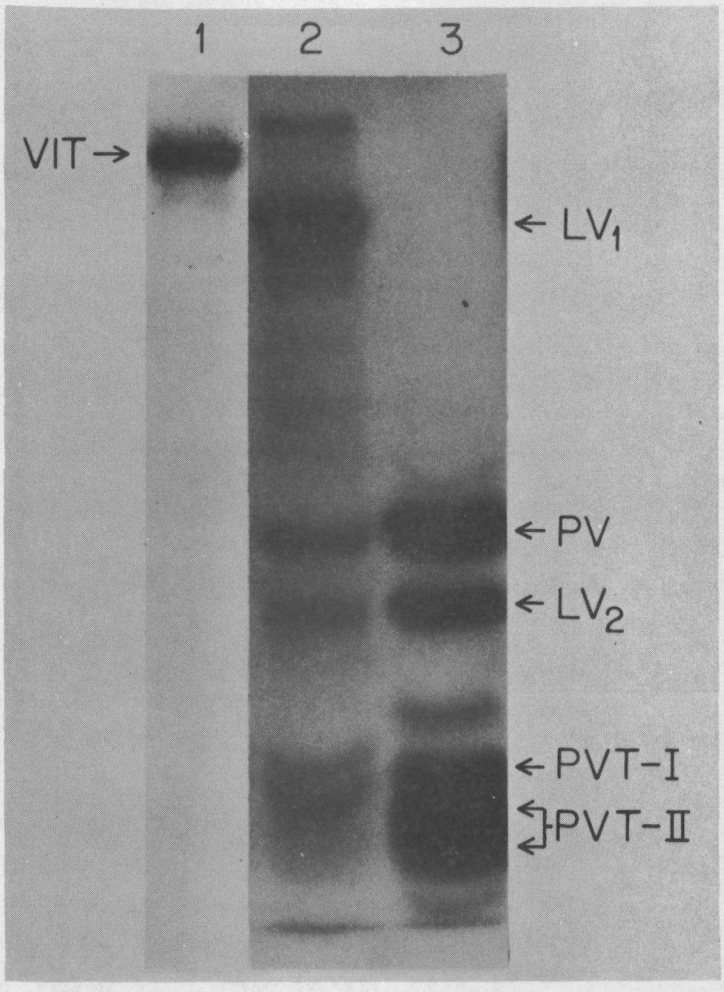

Figure 18. Conversion of in vitro labeled [^{32}P] vitellogenin into the yolk proteins. Presented is an autoradiogram of a slab gel containing the following samples: 1. In vitro labeled [^{32}P] vitellogenin. 2. Oocyte pulsed with [^{32}P] vitellogenin for 10 min and then chased with unlabeled vitellogenin for 24 hr. 3. Isolated in vivo labeled [^{32}P] yolk proteins. Indicated at the left is the mobility of unlabeled vitellogenin and at the right is the mobility of the yolk proteins identified in Chapter V.

in vivo. Since phosvitin never exists as a discrete entity outside of the ovary of oviparous vertebrates, but indeed is a part of the precursor molecule vitellogenin (16), a true phosvitin kinase cannot exist. The generic term "phosphoprotein kinase" is preferable for this class of enzymes since their preference for highly phosphorylated substrates is one of their most distinctive characteristics. I do not believe that the enzyme described here is responsible for the endogenous phosphorylation of vitellogenin in Xenopus liver primarily because of the enzyme's apparent cytoplasmic localization (Table II, page 38). I have also found that male Xenopus liver has approximately the same amount of vitellogenin-phosphorylating activity as estrogen-treated female liver. However, I sometimes observed a second peak of enzyme activity eluting at 0.23 *M* KCl from DEAE-cellulose columns when livers from estrogen-treated (hence vitellogenin-synthesizing) animals were used. Future studies will be necessary to determine if this "induced" enzyme may be the actual enzyme responsible for phosphorylating vitellogenin *in situ*.

The Xenopus liver phosphoprotein kinase seems to share several characteristics common to the class of phosphoprotein kinases. It is cyclic nucleotide-independent and has a strong affinity for phosphocellulose. Several workers have proposed that phosphocellulose acts as an affinity column for this class of enzymes (54-55). My observation that phosphate gradients are much more effective for eluting the enzyme from phosphocellulose and that a large increase in specific activity occurs after phosphocellulose chromatography are consistent with this suggestion. The observation that phosphoprotein kinases bind to columns

of phosphocellulose has become quite general and may be the single most useful criterion for classifying this group of enzymes.

There are several unusual properties of the Xenopus phosphoprotein kinase with respect to previously reported phosphoprotein kinases. The most notable is the biphasic pH-optimum curve (Figure 12, page 47). In order to understand this phenomenon, I determined the K_m of the enzyme at pH 8.0 and 9.5. The reaction was linear with time at both pHs. However, the Woolf-Augustinsson-Hofstee plot of the reaction at pH 8.0 was markedly nonlinear in contrast to the plot of the reaction at pH 9.5 (Figure 15, page 54). The kinetics of the reaction resemble those obtained with allosteric enzymes displaying negative cooperativity (56). The apparent negative cooperativity was not due to the use of vitellogenin as the substrate because phosvitin gave curves similar to those of vitellogenin and a phosphoprotein kinase isolated from bovine liver gave linear Woolf-Augustinsson-Hofstee plots with vitellogenin at a pH of 8.0. The bovine enzyme also displayed a bell-shaped pH optimum curve centered around pH 8.0 with vitellogenin as a substrate. These results also cannot be due to a phosphatase contaminant in the enzyme preparation which is active at pH 8.0 since at that pH I can achieve greater than 95% transfer of ^{32}P into TCA-insoluble counts using a 6.5 h incubation period and an ATP concentration of 1.7×10^{-7} M. It is unlikely that the biphasic pH curve and the apparent negative cooperativity of the enzyme could be explained by the presence of two distinct enzymes for the following reasons: the enzyme is highly purified (21,000-fold) and always elutes as a single peak of enzyme

activity during phosphocellulose chromatography. Since the Xenopus phosphoprotein kinase has an unusually high affinity for phosphocellulose, it elutes at a much higher concentration of phosphate (0.28 M) than other phosphoprotein kinases, such as bovine liver phosphoprotein kinase (0.17M) or rabbit erythrocyte phosphoprotein kinases (0.15 and 0.18 M) (46). I have rechromatographed the Xenopus enzyme on phosphocellulose and the single peak that reeluted at 0.28 M still fully displayed the biphasic pH optimum curve (data not shown). The K_m for ATP that I obtained for the Xenopus phosphoprotein kinase also argues against multiple enzymes in my preparation. The Woolf-Augustinsson-Hofstee plot of the reaction is linear (Figure 15, page 54) and the K_m value obtained (2.2 μM) is the lowest yet reported for a protein kinase. The above observations together with the findings on the unusual stability of the enzyme would tend to rule out the presence of multiple enzymes. However, since the enzyme preparation is not homogenous, I cannot totally exclude the presence of two distinct vitellogenin-phosphorylating enzymes. If I am indeed dealing with a single enzyme, then the biphasic pH curve I observe (Figure 12, page 47) can most easily be explained by an inactivation (dissociation?) of a regulatory (inhibitory) site on the enzyme by the high pH with a consequent increase in observed enzymatic activity.

This is the first reported example of a phosphoprotein kinase that has properties of an allosteric enzyme. My observations are different from those of Kumar and Tao (46) who observed inhibition of rabbit erythrocyte phosphoprotein kinases by high concentrations of phosvitin.

Those workers observed inhibition by phosvitin at concentrations considerably higher than the apparent K_m values for phosvitin (46). They also observed a reversal of the phosvitin inhibition by monovalent salts. Since my reactions were always run at an optimum salt concentration (50 mM, Figure 13, page 49), my results are clearly not analogous. It is possible that other isolated phosphoprotein kinases (43-51) may display allosteric behavior since the kinetic data available on these enzymes is very sparse. Unfortunately, in the studies on substrate versus velocity (45,46,48), narrow substrate concentrations were used and were analyzed by double-reciprocal plots. Since this type of analysis gives undue weight to the reaction velocities observed over a relatively narrow range of substrate concentrations, it would be very difficult to identify negative cooperativity in these experiments (56). Other phosphoprotein kinases, such as those isolated from rabbit reticulocytes (47), calf brain (50), and rat liver (51) have been shown to possess a subunit structure, one of which is capable of being autophosphorylated by the enzyme. However, these studies did not show an alteration in the activity of the phosphoprotein kinases due to the autophosphorylation. Since previous studies have not been directed towards identifying possible mechanisms of regulating the activity of phosphoprotein kinases, it remains possible that the apparent negative cooperativity shown by the Xenopus enzyme is shared by other members of this class of enzymes, although not by the bovine enzyme.

The role of phosphoprotein kinases in normal cellular function is not understood, primarily because the true intracellular substrate(s)

have not been identified. However, their widespread distribution in eucaryotic organisms implies that they may have some important regulatory role in cellular function. An understanding of how the activities of these enzymes can be regulated would be a step towards an understanding of their role in cells.

The phosphoprotein kinase described in these studies is a very stable, easily purified cytoplasmic enzyme. Its apparent allosteric behavior towards its protein substrate, but not the ATP substrate, implies that the activity of the enzyme may be regulated by either the level of available protein substrate or possibly by some other highly phosphorylated compound. An exact understanding of the mechanism(s) by which the Xenopus phosphoprotein kinase is regulated could help to reveal the importance of this class of enzymes in normal cellular function.

The particular utility of the Xenopus phosphoprotein kinase for labeling vitellogenin seems to be due to its unusually low K_m value. When the high-specific-activity [γ-^{32}P]ATP (approximately 3000 Ci/mmol) is used at a concentration of 2 mCi/ml, the ATP concentration is only about 7×10^{-7} M. Since the K_m of many phosphoprotein kinases is about 2×10^{-5}M (47,50), the efficiency of the reaction is relatively poor without the addition of large amounts of enzyme. However, this concentration of ATP is much closer to the K_m value of the Xenopus enzyme. Since the enzyme is free of vitellogenin protease activity and since the reaction runs very efficiently at room temperature, the labeling of vitellogenin via the Xenopus phosphoprotein kinase would

seem to be the method of choice for preparing radio-labeled vitellogenin. The specific activities routinely obtained (greater than 5×10^8 cpm/mg) are about 500 times the specific activities obtained by *in vivo* labeling methods. My observation that the *in vitro* labeled vitellogenin is also processed normally into the yolk proteins allows me to use the labeled vitellogenin as a probe for studying the events associated with the conversion of vitellogenin into the crystalline yolk proteins.

CHAPTER IV

VITELLOGENIN IS A HETEROGENEOUS POPULATION OF MOLECULES

Summary

Xenopus laevis vitellogenin was found to be a heterogeneous population of molecules. SDS gel electrophoresis at a pH of 9.6 resolves vitellogenin into 3 bands of molecular weight 197,000, 188,000, and 182,000. These three bands are present in a 2:2:1 ratio respectively as determined by counting the radioactivity in each of the bands derived from [^{3}H, ^{32}P]vitellogenin. Vitellogenin labeled *in vitro* with ^{32}P via the phosphoprotein kinase reaction can be resolved into 4 closely spaced bands when subjected to SDS gel electrophoresis at pH 8.0. The electrophoresis of vitellogenin under nondenaturing conditions also can resolve vitellogenin into at least 4 different bands, 3 of which have very similar if not identical free electrophoretic mobilities and one which appears to have a slightly higher free electrophoretic mobility. Isoelectric focusing of vitellogenin in the presence of 6 M urea demonstrated that the population of vitellogenin molecules is hetereogeneous with respect to charge. To show that vitellogenin is hetereogeneous with respect to amino acid sequence, [^{32}P]vitellogenin was cleaved with nitrothiocyanobenzoic acid at its cysteine residues. Partial fragments were removed by organomercurial-agarose chromatography. This experiment revealed that vitellogenin gives rise to two large (approximately 93,000 and approximately 78,000 MW) phosphopeptides

that are probably derived from the cleavage of flanking cysteine residues. Since the greater majority of the protein phosphorus of vitellogenin is derived from a single 35,000 MW segment of the molecule, this result indicates that the vitellogenin population contains at least two members that differ in amino acid sequence. Taken together, my results indicate that Xenopus vitellogenin is composed of at least two, and probably four, different molecules.

Introduction

Vitellogenin is the yolk precursor protein found in the blood of oviparous vertebrates (1). Estrogens can readily induce a pronounced synthesis and secretion of vitellogenin by the liver of Xenopus laevis either in vivo (12) or in vitro (18), and transcriptional, translational, and posttranslational events associated with vitellogenin synthesis are now being studied extensively in this amphibian model system (19). Vitellogenin can be readily isolated from the serum of females or estrogen-treated males (12) and has been found to migrate on sodium dodecyl sulfate (SDS) polyacrylamide gels as a single band having a molecular weight of approximately 200,000 (16). Thus, the product of the vitellogenin gene was thought to consist of a single polypeptide (16). In the ovary, vitellogenin is incorporated by growing oocytes within which it is proteolytically cleaved into phosvitin (35,000 MW) and the heavy (LV_1, 120,000 MW) and light (LV_2, 31,000 MW) subunits of lipovitellin (16). Recently, Ohlendorf et al. (57) have provided evidence that LV_2 consists of at least 3 peptides having molecular

weights of 35,500, 32,000, and 31,600. The origin of this heterogeneity in the vitellogenin cleavage-product is uncertain.

In a study on the cloning of vitellogenin mRNA, Wahli et al. (58) found that the vitellogenin messenger did not comprise a homogeneous population, but instead was composed of two distinct families of mRNA, with each family containing at least two members. Wahli et al. (58) also provided evidence that the genome of individual Xenopus contained all four distinct sequences encoding for the vitellogenin mRNAs. However, those researchers provided no evidence that the distinct messengers were actually translated into distinct vitellogenin molecules.

During SDS gel electrophoresis, I noted that vitellogenin resolved into three distinct bands that I termed α, β, and γ vitellogenin (59). However, I was uncertain that the three bands were distinct proteins in terms of their sequence rather than being posttranslational modifications of a common precursor. I thus attempted to isolate the three forms of vitellogenin and directly assess their sequence homology by chemical cleavage techniques. Unfortunately, all efforts to isolate the three forms of vitellogenin in a reasonable state of purity failed. The most successful effort was made with preparative gel electrophoresis, but this method only yielded microgram quantities of materials on the few occasions that it was successful. Therefore, I decided to use an indirect approach in determining if there were indeed multiple forms of vitellogenin. I used SDS gel electrophoresis at high and low pH, native gel electrophoresis and isoelectric focusing. All procedures revealed that vitellogenin was a heterogeneous population of molecules.

In addition, I developed a procedure for totally cleaving vitellogenin at the cysteine residues. This technique provided strong evidence that there are at least two types of vitellogenin that differ in their primary amino acid sequence.

Materials and Methods

The care of animals together with the injection of hormones and isotopes (22) and the bleeding and collection of plasma (16) were performed as previously described. Vitellogenin was labeled *in vivo* by injecting an animal with 1 mCi each of [^{3}H]leucine (1.0 Ci/mmol, 100 mCi/ml) and $^{32}P_i$ (carrier-free, 8 mCi/ml) 8 days after estrogen treatment (2) and 24 hr prior to bleeding. Vitellogenin was labeled *in vitro* using [γ-^{32}P]ATP as described in Chapter III. Vitellogenin was isolated by either Mg^{2+}:EDTA precipitation or DEAE-cellulose chromatography as described in Chapter II.

Acrylamide and N,N'-methylene-bisacrylamide were recrystallized three times from acetone prior to use (23). SDS was reprecipitated from ethanol or was purchased from Polysciences (electrophoresis grade). Ammediol (2-amino-2-methyl-1,3-propanediol) was recrystallized from ethanol, rinsed with acetone and dried under vacuum. Mono-tris (2-hydroxyethylamino-tris(hydroxymethyl)methane) was synthesized from Tris and ethylene oxide as described by Lewis (60) using a 1:1 molar ratio of the two reactants. It was recrystallized three times from ethanol and was found to have a pK_a of 7.38. Paper chromatography (60) gave a single spot with no sign of contamination with Tris or Bis-tris.

Nitrothiocyanobenzoic acid (NTCB) was synthesized by the method of Degani and Patchornik (61) using either KCN or [^{14}C]KCN (5 mCi/mmol, New England Nuclear). Organomercurial agarose was synthesized by the method of Cuatrecasas (62) using Biogel A-15M. The Biogel was reacted twice and had a final capacity of 1.3 µmol/ml as determined by DTNB titration (63).

SDS gel electrophoresis at high pH (9.58) was performed as described by Wyckoff et al. (27) with the substitution of potassium persulfate (1 mg/ml) as the catalyst solution. SDS gel electrophoresis at pH 8.0 was performed using a system calculated from the equations of Jovin (64) (also see Appendix B). The gels contained 0.09 M Mono-tris and 0.058 M HCl. The upper reservoir buffer contained 0.03% SDS, 0.045 M Bicine and 0.045 M Mono-tris while the lower reservoir buffer was 0.1 M Mono-tris-HCl, pH 8.0. Stacking gels were made in running gel buffer and contained 3.25% total acrylamide and 20% of the total acrylamide as Bis-acrylamide (3.25% T, 20% C). Gels contained 200 µg/ml of potassium persulfate as a catalyst and polymerization was initiated by the addition of 1 µl/ml of TEMED (for 5% T, 2% C gels).

Proteins for SDS gel electrophoresis were denatured at a concentration of 2 mg/ml in 1% SDS, 2 mM EDTA and 10 mM Tris-Cl, pH 8.0 at 100° for 5 min. Proteins were either reduced with 2% 2-mercaptoethanol prior to boiling or carboxyamidomethylated (65). All gels (10 cm) were cast in 0.6 × 13 cm pyrex tubes, contained 2% of the total acrylamide as Bis-acrylamide (2% C) and were overlayered with water-saturated butanol. Samples of the proteins were diluted with

20% sucrose, 10 mM Tris-Cl, 2 mM EDTA and 0.005% bromophenol blue (pH 8.0) to give a load volume per gel of 50 µl. Electrophoresis was conducted at 1.5 mA/gel until the dye reached the bottom of the tubes (approximately 3 hrs). In order to improve the resolution of the vitellogenin bands, some gels were run for an additional 3 hr after the dye had reached the bottom of the tubes. The gels were then fixed and stained with Coomassie blue R-250 as described by Wyckoff et al. (27). Gels were sliced into 1 mm sections with a Hoefer gel slicer and the sections were dissolved in 0.3 ml of H_2O_2 and 50 µl of NH_4OH at 60° for 2 hr. Aquasol was added (4.5 ml) and the radioactivity was counted in a scintillation counter. For autoradiography of gels, samples containing [^{32}P]vitellogenin or peptides were electrophoresed on 1.5 mm thick slab gels using the Mono-tris/Bicine system. After fixation and staining (27) the gels were dried on blotter paper using a Hoefer slab gel drier and exposed to Kodak X-Omat R film. A useful rule for exposure times for ^{32}P was found to be the length of time necessary for the radioactivity of the sample to drop approximately 1000 dpm.

Isoelectric Focusing

The isoelectric focusing of vitellogenin was performed in 5% T, 15% C gels using N,N'-diallytartardiamide (DATD) as the crosslinker (66). Gels were 0.35 × 10 cm and were cast in pyrex tubes prewashed in 5% HFl for 2 min, and coated with 1% Gelamide 250 (Polysciences) (67). The gel mixture (5 ml) was made by adding 1.25 ml of acrylamide solution (20% T, 15% C_{DATD}) to a 5 ml graduated cylinder containing

1.8 g urea, 250 µl of 40% Servalytes 2-11 (Accurate Chemical & Scientific Corporation), 1 ml of 50 mM phosphoserine, aspartic acid and glutamic acid and then bringing the solution to 4.95 ml. The solution was deaerated under house vacuum for 5 min and then 50 µl of 10% ammonium persulfate and 5 µl of TEMED were added. The tubes were immediately filled and overlayered with 5 M urea, 2% Servalytes 2-11. Upper buffer was 50 mM phosphoserine and the lower buffer was 50 mM arginine (68). Vitellogenin labeled in vivo with either $^{32}P_i$ or a mixture of [^{14}C] amino acids was dissolved at a concentration of 0.5 mg/ml in 8 M urea, 50 mM phosphoserine. Samples of 30 µl were placed onto the gels which were then overlaid with upper reservoir buffer. Focusing was carried out for 2 hr at 200 V, 2 hr at 300 V and an additional 5 hr at 400 V. The gels were removed from their tubes and the pH gradient was measured directly with a model MI-410 micro-combination pH probe (Microelectrodes, Inc.). The gel was sliced into 0.5 cm sections which were dissolved in 0.4 ml of 2% periodic acid (69). Aquasol (4 ml) was added and the samples were then counted in a scintillation counter.

Native Gel Electrophoresis

Native gel electrophoresis of vitellogenin was performed using a modification of the system of Ornstein (70) and Davis (71). The reservoir buffers contained 50 mM Tris and 50 mM glycine and the gels (containing 2% C) were polymerized using 0.25 mg/ml of potassium persulfate and 1 µg/ml of TEMED for 5% gels and 0.5 µl/ml of TEMED for 10% gels. Electrophoresis was conducted at 1.5 mA/gel at 20°

and the gels were fixed and stained with Coomassie blue G-250 (72). Molecular weight analysis was performed by Ferguson plot analysis (73) using as standards hemoglobin (64,000), bovine serum albumin monomer (68,000) and dimer (136,000), aldolase (160,000), catalase (232,000) and β-galactosidase (464,000).

Cleavage of Vitellogenin

Vitellogenin was cleaved at the cysteine groups using NTCB and a modification of the procedures of Jacobson et al. (74). Partial cleavage of vitellogenin was obtained by the following method. The protein was dissolved in 1% SDS, 100 mM Tris-Cl, 5 mM EDTA, pH 8.0 at a concentration of 1 mg/ml, and 10 mM of DTT was added. The solution was heated for 5 min at 100° and cooled prior to removing the DTT by centrifugal desalting (75) using Biogel P-2 equilibrated with 1% SDS, 100 mM Tris-Cl, 5 mM EDTA, pH 8.0. The total thiol concentration of the solution was determined by DTNB titration (76) and from 1 to 10 fold excess of [^{14}C]NTCB was added. After a 30 min reaction time at 20°, the extent of modification was determined by spotting aliquots of the solutions on Whatman 3 MM disks and processing them to remove TCA soluble counts (24). The extent of incorporation of the ^{14}C into the vitellogenin and hence the modification could be assessed from the known specific activity of the [^{14}C]NTCB. Cleavage was initiated by raising the pH of the solutions to 9.0 with 1 N NaOH. After an overnight incubation at 37°, the reaction was terminated by the addition of 2-mercaptoethanol to 1%. Samples were then subjected to SDS gel electrophoresis.

For the total cleavage of vitellogenin with NTCB, the protein was dissolved at 2 mg/ml in 6 M GuHCl, 0.375 M Tris-Cl, 1 mM EDTA, pH 8.0, 5 mM DTT, 2 mM PMSF. After reduction at 37° for 30 min, the solution was passed centrifugally over a column of Biogel P-2 equilibrated with 6 M GuHCl, 0.375 M Tris-Cl, 1 mM EDTA, pH 8.0. The thiol content was determined by DTNB titration (76) and a 3-fold excess of NTCB was then added. After 30 min at 20°, the pH was raised to 8.8 with 1 N NaOH and cleavage was allowed to proceed overnight at 37°. The reaction was then terminated by adding a 3-fold excess of DTT over the NTCB added. The protein solution was then desalted centrifugally (75) using Biogel P-2 equilibrated with 4 M urea, 50 mM phosphate buffer, 0.1 M KCl, 1 mM EDTA and 10% Me_2SO (pH 7.2). This solution was flushed with nitrogen prior to use. Any partial cleavage products of vitellogenin remaining were removed by passing the protein solution over a column of organomercurial agarose (1.3 µmol/ml capacity) equilibrated with the urea-Me_2SO solution. The vitellogenin peptides passing unretarded through the column were collected and desalted against 50% acetic acid. The solution was evaporated to dryness with nitrogen and stored overnight under a vacuum. The peptides were then dissolved in the SDS gel electrophoresis sample buffer and subjected to electrophoresis.

Results

When preparation of vitellogenin were electrophoresed on 10% T, 2% C SDS-polyacrylamide gels, only a single sharp band was seen (Figure 19). However, when I electrophoresed small amounts of vitellogenin (5 µg/gel) on 5% T, 2% C gels, I noted a lamellar structure

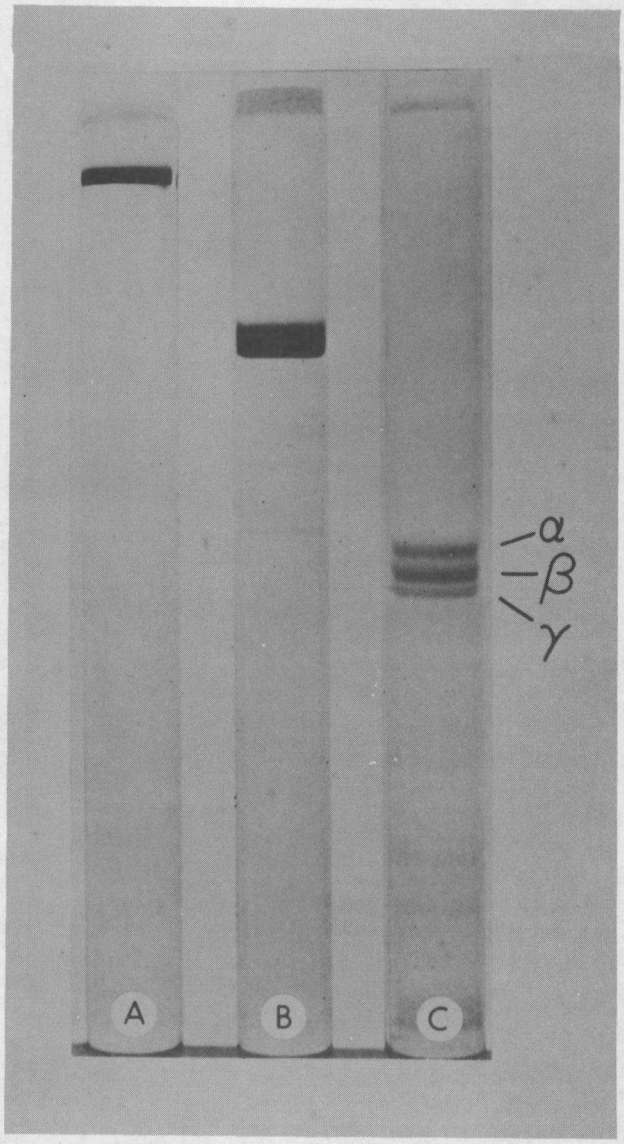

Figure 19. Electrophoresis of 5 µg reduced and carboxyamidomethylated vitellogenin on SDS-polyacrylamide gels. (A) 10% gel. (B) 5% gel. (C) Same as (B) but run twice as long.

in the vitellogenin band (Figure 19). The lamellar structure of the band was resolved further by running the gels for twice the length of time necessary for the tracking dye to reach the bottom of the gel. I preferred this approach over running gels with an even lower percentage acrylamide because of their fragility and also because of the higher resolving power of higher percentage acrylamide gels (77). When vitellogenin was run this extra distance on the 5% T, 2% C gels, the lamellar structure resolved into 3 distinct bands, designated as α-, β-, and γ-vitellogenin in the order of their increasing mobility (Figure 19C).

In order to ascertain whether the 3 bands of vitellogenin were a preparation artifact, I ran and compared SDS-polyacrylamide gels of plasma samples obtained from estrogen-treated animals as well as vitellogenin isolated by either Mg^{2+}:EDTA precipitation or DEAE-cellulose chromatography (78). As can be seen in Figure 20, the same pattern of 3 bands was obtained in all cases. I have also run vitellogenin obtained from 6 additional animals and have obtained in all cases the identical banding pattern. As a further check on the origin of the 3 vitellogenin bands, I have established that reduced vitellogenin, carboxymethylated vitellogenin (16), and carboxyamidomethylated (65) vitellogenin all show the identical pattern. Preparation of vitellogenin in the presence or absence of the protease inhibitor, phenylmethyl sulfonyl fluoride (16) likewise had no effect on the 3 bands. Finally, electrophoresis of small amounts of vitellogenin (5 μg/gel) in the SDS-polyacrylamide gel system of Laemmli (79) also resulted in a similar banding pattern.

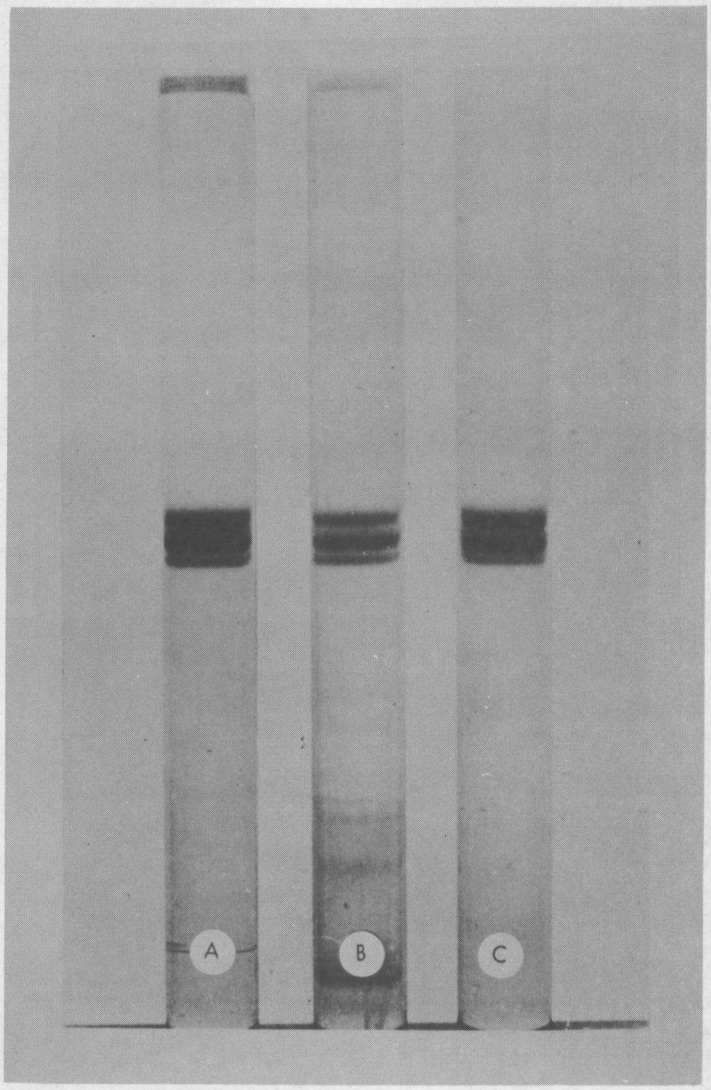

Figure 20. Electrophoresis of reduced and carboxyaminomethylated vitellogenin on 5% SDS-polyacrylamide gels. The vitellogenin samples were derived from 3 different animals and were prepared by either (A) DEAE-cellulose chromatography or (B) Mg^{++}-precipitation. Alternatively, (C) plasma containing vitellogenin was applied directly to the gel.

I thus conclude that the 3 bands on SDS-polyacrylamide gels probably reflect the presence of at least 3 different forms of vitellogenin present in the blood of the animals.

When the migration distances of the 3 vitellogenin bands were compared with molecular weight markers according to procedures previously described (16), sizes of 197,000, 188,000, and 182,000 daltons were obtained for α-, β-, and γ-vitellogenin, respectively. This agrees well with the molecular weight of approximately 200,000 reported previously for the unresolved vitellogenin polypeptide (16).

From a visual inspection of the 3 vitellogenin bands, α- and β-vitellogenin seem to be present in equal amounts while γ-vitellogenin appears to be present in a lesser amount. In order to quantitate the ratios of the 3 polypeptides, I doubly labeled vitellogenin with [^{3}H]leucine and [^{32}P]orthophosphate. The latter is a specific marker for delipidated vitellogenin in the blood (14). The labeled vitellogenin was electrophoresed on gels, the bands containing each of the 3 vitellogenin polypeptides excised, and the radioactivity measured. The results (Table VI) indicated that α-, β-, and γ-vitellogenin were present in an approximately 2:2:1 ratio. This was the case for both the ^{3}H and ^{32}P labels, the relative ratios of which were about the same for the 3 bands. The presence of ^{32}P in all 3 delipidated bands further confirms their identity as vitellogenin polypeptides.

The three different bands observed during SDS gel electrophoresis do not necessarily prove that the three observed bands of vitellogenin are different in molecular weight; it is only an implication of three

TABLE VI

RELATIVE LABELING IN THE THREE VITELLOGENIN BANDS

Band	^{3}H cpm	^{32}P cpm	$^{3}H/^{32}P$
α	182 ± 17	75 ± 7	2.4 ± 0.4
β	180 ± 6	85 ± 4	2.1 ± 0.2
γ	98 ± 13	44 ± 6	2.2 ± 0.5

The results are expressed as the average ± S.D.

forms of vitellogenin since conjugated proteins can behave anomalously during SDS gel electrophoresis (80) as has been observed for the vitellogenin derivative phosvitin (57). However, Bergink and Wallace have observed that phosvitin electrophoresed at pH 8.0 on continuous SDS gels apparently behaves normally with respect to molecular weight (16). Since continuous SDS gel systems do not have the resolving power of discontinuous gel systems (27), I designed a discontinuous Mono-tris/Bicine SDS gel electrophoresis system that runs at pH 8.0. When vitellogenin and selected proteins were run on this Mono-tris/Bicine system, the results in Figure 21 were seen. The standard proteins all yielded exceptionally sharp bands, and plots of log Rf versus molecular weight were linear for these proteins. Vitellogenin was labeled with ^{32}P via the protein kinase reaction (to increase the detection limit for the small amount of protein used) and then run on 5% T, 2% C Mono-tris/Bicine gels for twice the length of time necessary for the tracking dye to reach the bottom of the gel, after which the gels were sliced and counted for radioactivity. Under these conditions, four peaks of radioactivity were observed (Figure 22). The fastest migrating radioactive peak (designated peak IV in Figure 22) was seen in duplicate experiments and may represent either a minor form of vitellogenin or possibly a cleavage fragment of vitellogenin. The same preparation of labeled vitellogenin yielded only 3 bands of protein when examined by the previous method.

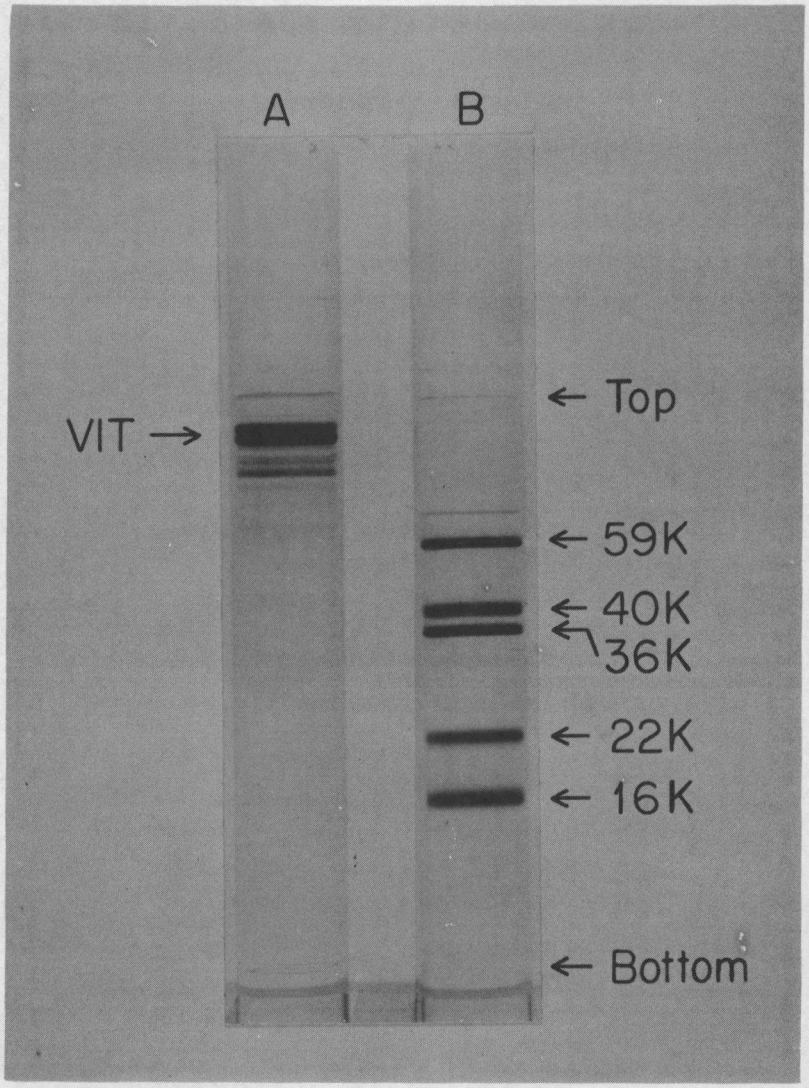

Figure 21. SDS gel electrophoresis using the Mono-tris/Bicine system. Gel A is 5 µg of vitellogenin and gel B is 5 µg of catalase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, soybean trypsin inhibitor and hemoglobin. Both gels are 10%T, 2%C and are stained with Coomassie blue.

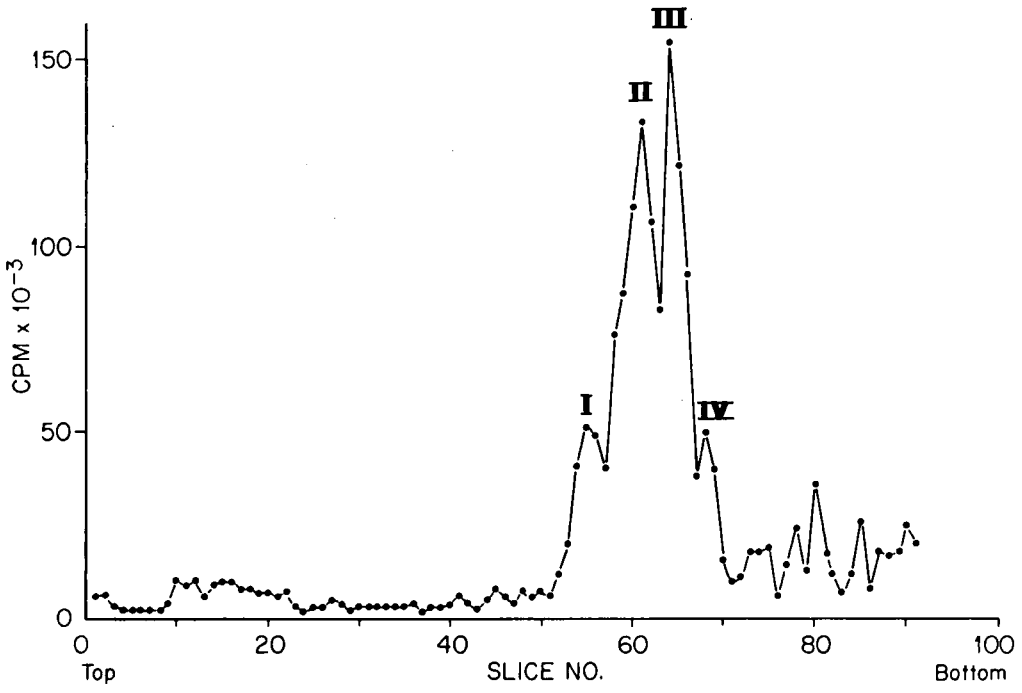

Figure 22. Electrophoresis of [^{32}P]vitellogenin on Mono-tris/Bicine SDS gels. Vitellogenin was labeled in vitro with [γ^{32}P]ATP via the phosphoprotein kinase reaction (see Chapter III) and then carboxymethylated prior to electrophoresis on 5%T, 2%C gels using the Mono-tris/Bicine buffer system described in Materials and Methods. After electrophoresis for twice the length of time necessary for the tracking dye to reach the bottom of the gel, the gel was sliced into 1mm segments which were dissolved and counted in a scintillation counter. Sample load was 5 µg.

Native Gel Electrophoresis of Vitellogenin

An alternative approach in examining the possible heterogeneity in vitellogenin is electrophoresing vitellogenin under nondenaturing conditions. When vitellogenin is electrophoresed in 5% T, 2% C gels (70, 71), it gives rise to at least four bands as seen in Figure 23. There are two very close middle bands flanked by two additional bands. The top band (band II in Figure 23) sometimes appears to resolve into two minor bands. Ferguson plot analysis of the vitellogenin bands is seen in Figure 24 and indicates that the vitellogenin bands represent the monomers of vitellogenin since when the Kds of the main bands are compared with those of standard proteins, the molecular weight of the bands are between 240,000-255,000 molecular weight. The faint top band (indicated as band I in Figure 23 and in Figure 24) has a molecular weight of approximately 470,000 and probably represents the vitellogenin dimer. The Ferguson plot analysis also shows that the top 3 vitellogenin monomer bands (II-IV) have approximately the same free electrophoretic mobility (the Y-intercept) and thus appear to differ only in molecular weight. The bottom band (band V) has a slightly different free electrophoretic mobility, but seems to have the same molecular weight as band IV. The data from the native gels suggests that there are three different molecular weight forms of monomer vitellogenin, but that one of the molecular weight forms may contain two forms of vitellogenin that differ in net charge.

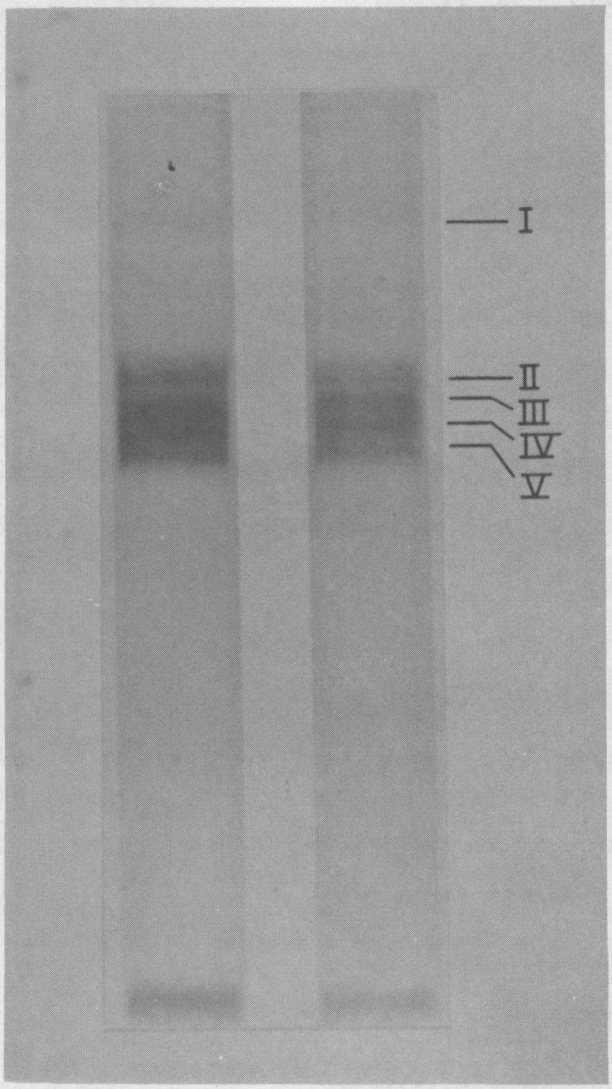

Figure 23. Native gel electrophoresis of vitellogenin. The gels are duplicates and were 5% T, 2% C; only the top 5 cm of the 10 cm gel is shown. At the right are the band designations. The bottom, undesignated band is bovine serum albumin used as an internal standard.

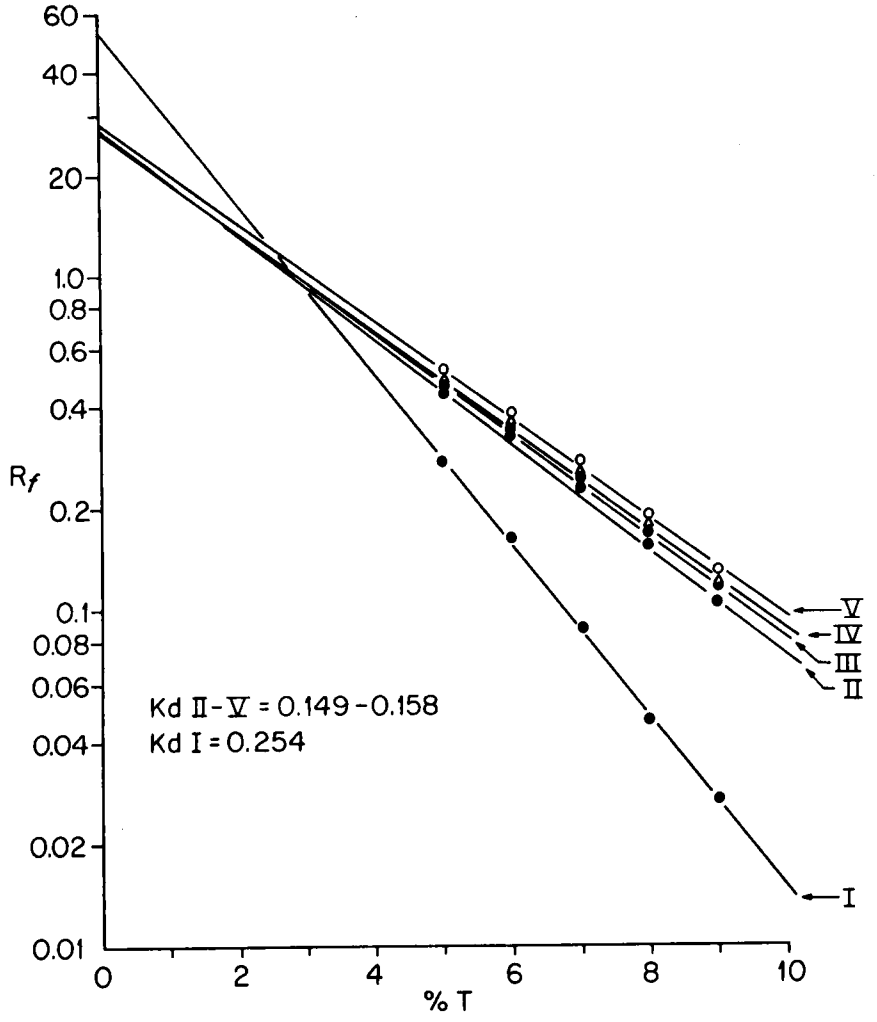

Figure 24. Ferguson plot analysis of Xenopus vitellogenin. Five µg of vitellogenin purified by Mg^{2+}:EDTA precipitation was electrophoresed on 5-9% T gels according to the procedure of Ornstein and Davis (70, 71). All gels were 10 cm in length and were 2% C in bis-acrylamide. Rf values were calculated from the mobility of the bromophenol blue tracking dye.

Isoelectric Focusing of Vitellogenin

In order to demonstrate that there are at least two forms of vitellogenin that differ in charge, isoelectric focusing of vitellogenin was attempted. At first results were disappointing because any bands that appeared were very broad. In addition, the presence of 6 M urea was necessary in order for vitellogenin to enter the gel. A rapid decay in the pH gradient in the acid and alkaline regions of the isoelectric gel was also a problem. The method that was finally used was to supplement the ampholines in the isoelectric focusing gel with several amino acids (68). The inclusion of the acidic amino acids phosphoserine, glutamic acid and aspartic acid was particularly helpful in extending the acidic region of the gel in the presence of urea (which suppresses the ionization of the acidic ampholines) (81). The gel was run the minimum length of time necessary for vitellogenin to reach its apparent isoelectric point (approximately 9 hr). The problem of staining vitellogenin in the gels was circumvented by using [^{14}C]-vitellogenin or [^{32}P]vitellogenin and then slicing and counting the gel. The results of one of these experiments is seen in Figure 25. [^{32}P]Vitellogenin focused as two broad peaks at pH values of approximately 4.6 and 4.4. This was also found to be the case with [^{14}C]-vitellogenin. Since the gels contain 6 M urea, the actual isoelectric points of vitellogenin are considerably lower (81). However, it seems that there are indeed at least two forms of vitellogenin that differ in net charge.

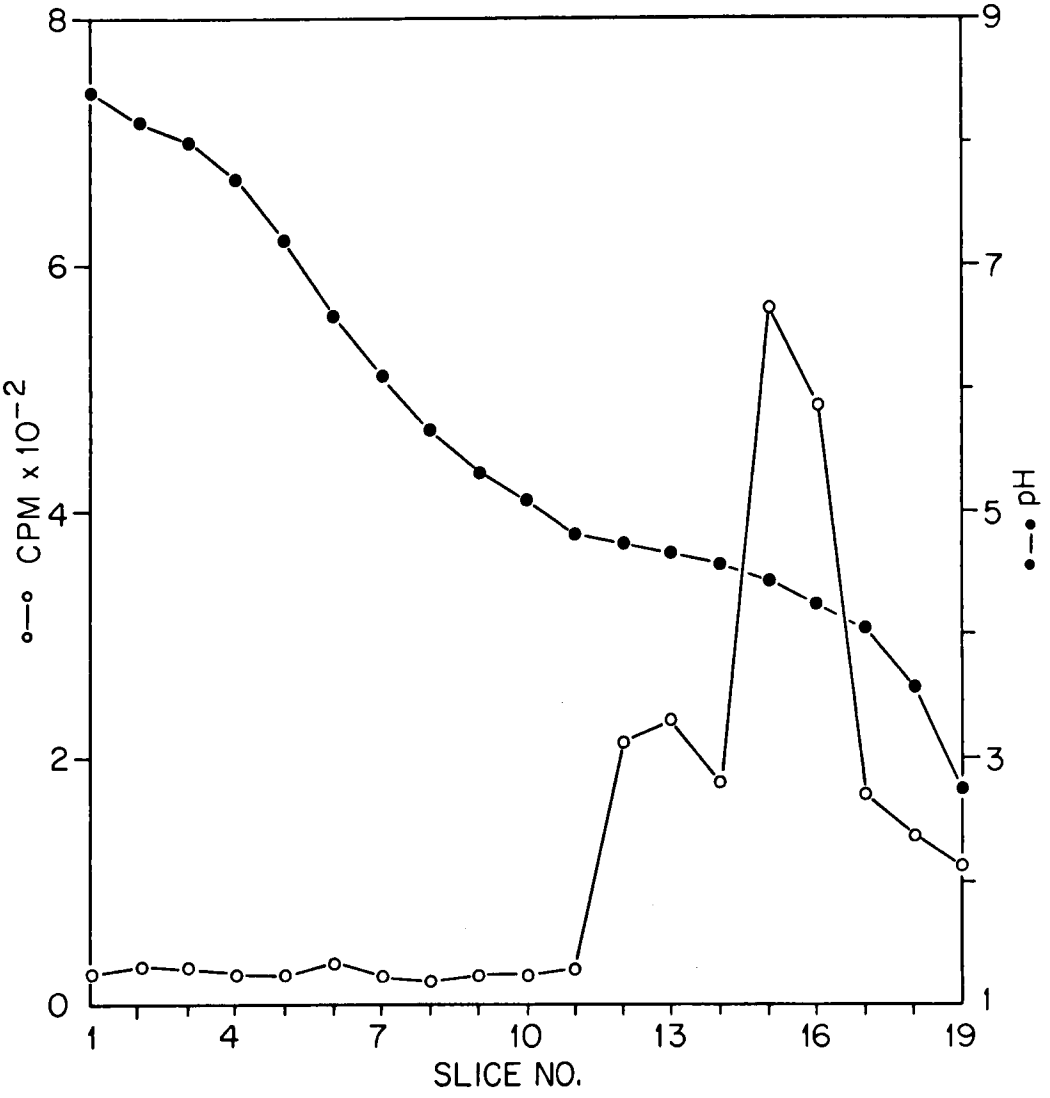

Figure 25. Isoelectric focusing of [^{32}P]vitellogenin. The gels contained 6 M urea, 2% Servalytes 2-11 and were 10 mM in phosphoserine, aspartic acid and glutamic acid. The anolyte was 50 mM phosphoserine and the catholyte was 50 mM arginine. Protein (15 µg) was placed at the anolyte end of the gel and focusing time was 9 hr. The pH gradient was measured across the gel and 0.5 cm segments were then dissolved and then counted for ^{32}P.

Heterogeneity at the Cysteine Residues of Vitellogenin

It is possible that some of the different forms of vitellogenin could differ in the primary amino acid sequence of the molecule. In an effort to see if this was the case, chemical cleavage of the molecule at the cysteine residue was attempted. It is known that the majority (greater than 60%) of the protein phosphorus of vitellogenin is present in the phosvitin region of the molecule (16). This region also contains no cysteine residues (57). If vitellogenin is cleaved at all of the cysteine residues, then a peptide(s) should be formed that is inclusive of the phosvitin region of the molecule. If complete cleavage of the cysteine residues is achieved, and different vitellogenin molecules have identical placement of the cysteine residues, then only a single major phosphopeptide should be obtained. Multiple phosphopeptides would indicate differences in the placement of the cysteine residues in the different vitellogenin molecules.

As an initial experiment in the cleavage of vitellogenin with the reagent, nitrothiocyanobenzoic acid (NTCB), I took [^{32}P]vitellogenin and modified it to various degrees with [^{14}C]NTCB in the presence of SDS. After cleavage at pH 9.0, the cleaved vitellogenin was subjected to SDS gel electrophoresis. The result of this experiment is seen in Figure 26. It can be seen that partial cleavage of vitellogenin gives rise to at least 10 different phosphopeptides. With an increase in the degree of modification with NTCB there is an increase in the lower molecular weight phosphopeptides and a corresponding reduction of the higher molecular weight phosphopeptides. When [^{14}C]NTCB is used

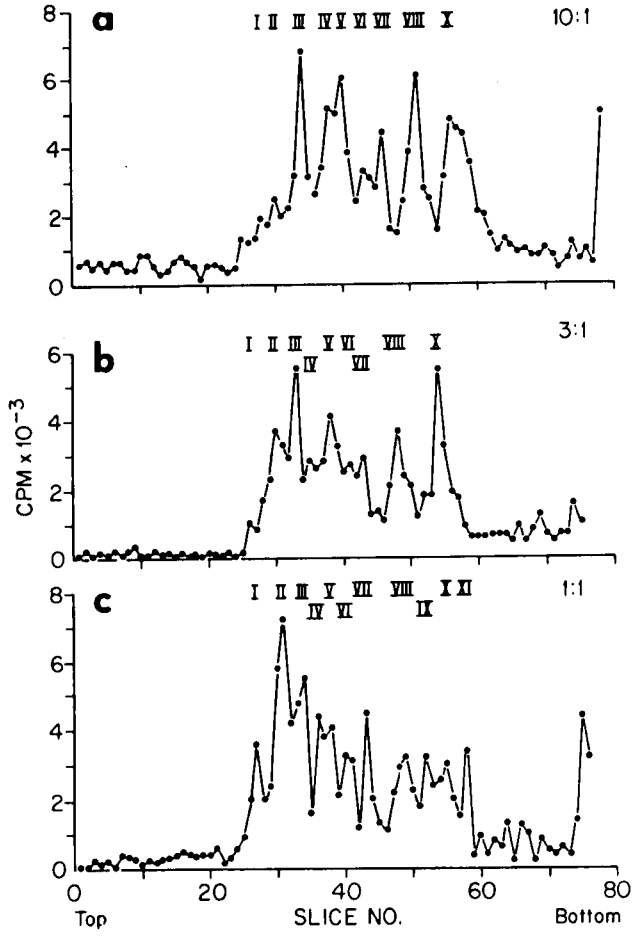

Figure 26. Partial NTCB cleavage of [^{32}P]vitellogenin. The protein was cleaved in a 1% SDS solution using different ratios of NTCB to total thiol groups present, as indicated in the right hand margin of each figure. The extent of vitellogenin modification was determined as outlined in Materials and Methods. Modification was 61%, 46% and 31% for figures A, B, and C, respectively. Cleaved vitellogenin was electrophoresed in 5% T gels and 1mm gel segments were each counted for the presence of ^{32}P. Uncleaved vitellogenin appeared in slice 22 on a duplicate gel.

as the cleavage reagent, the ^{14}C remains at the N-terminal of the cleavage site (74). Therefore, I examined for the presence of a ^{14}C peak corresponding to each phosphopeptide peak. The result of this experiment is seen in Figure 27. It can be seen that there is an exact correspondence for each peak and this indicates that not only is each peak a true cleavage fragment, but also that the lowest molecular weight phosphopeptide does not contain the N-terminal of the vitellogenin molecule (74). The lowest molecular weight phosphopeptide has a molecular weight of approximately 78,000, which is over twice the molecular weight of phosvitin (approximately 35,000). Thus the lowest molecular weight phosphopeptide contains not only phosvitin, but also a considerable portion of the lipovitellin region.

I next explored conditions for the total cleavage of the vitellogenin molecule. The most suitable solvent contained 6 M GuHCl, but even using this solvent system I was unable to achieve greater than 90% cleavage. I then experimented with organomercurial-agarose for removing partial cleavage fragments of vitellogenin. Since it has been shown that one can regenerate the sulfhydryl groups of uncleaved cysteine residues by the addition of DTT (74), and since organomercurial agarose will selectively bind cysteine-containing peptides (63), it would seem to be an easy matter to remove the partial cleavage fragments of NTCB cleavage. However, I found that there are only a few solvent systems that allow the selective binding of cysteine-containing peptides to organomercurial-agarose columns, and vitellogenin was not cleaved efficiently in these solvent systems. The method that was finally

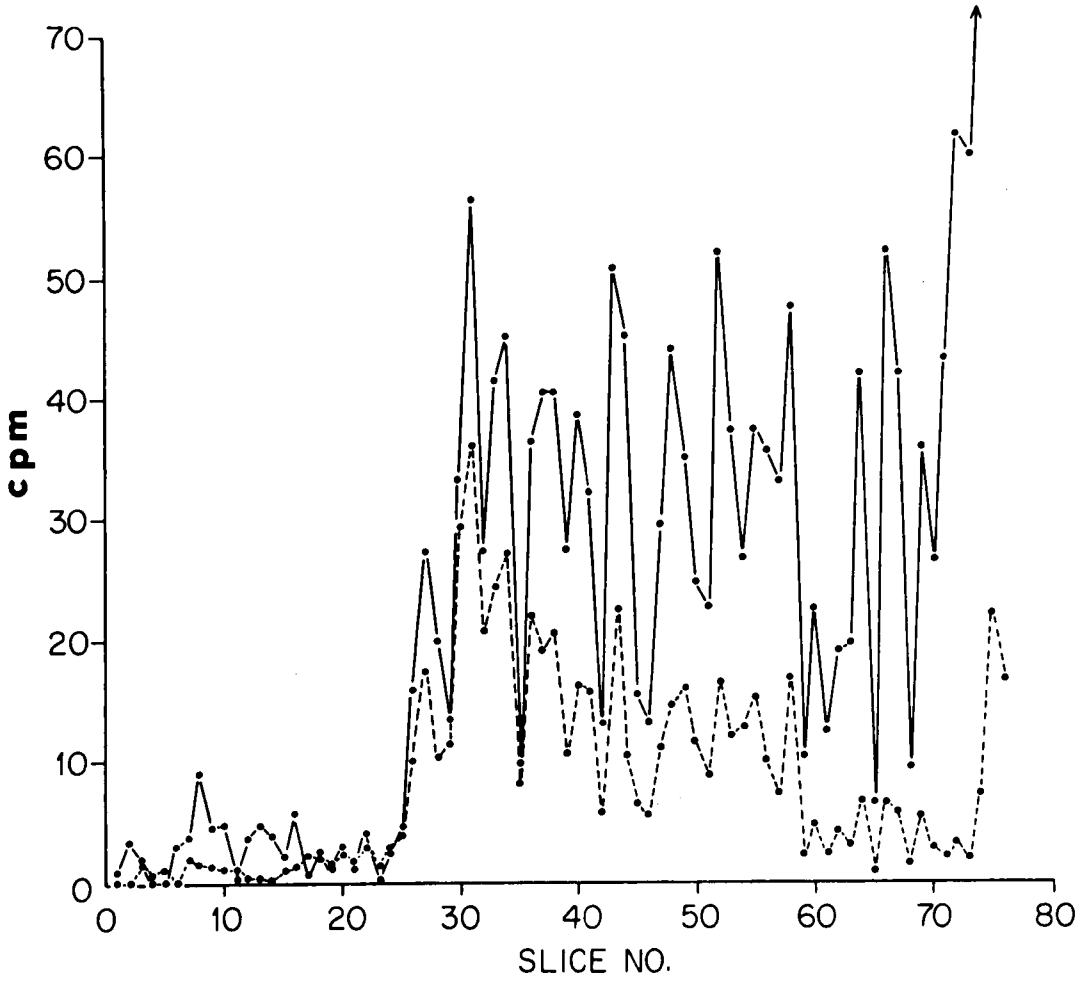

Figure 27. Cleavage of [^{32}P]vitellogenin with [^{14}C]NTCB. The solvent was 1% SDS and contained a 1:1 ratio of NTCB to total thiol groups present. After cleavage for 18 hr at 37°, the sample was electrophoresed in a 5%T, 2%C gel and 1mm slices were taken. After dissolving in 0.3 ml of H_2O_2, each fraction was counted for ^{14}C (•) and ^{32}P (o).

employed was to cleave the vitellogenin in 6 M GuHCl and then regenerate the uncleaved cysteine residues with DTT. The buffer was then exchanged centrifugally (75) with one containing 4 M urea and 10% Me_2SO. This then allowed the binding of the partial cleavage fragments to the organomercurial-agarose column. The result of one of these experiments is seen in Figure 28. Under the same solvent and elution conditions, uncleaved [^{32}P]vitellogenin is completely retained by the column. It can be seen that the majority of the cleaved [^{32}P]vitellogenin passed unretarded through the column. DTNB titration confirmed that this material contained no detectable cysteine residues. A small amount of partially cleaved vitellogenin was retained by the column and this material could be removed by the addition of 5 mM $HgCl_2$. The flow-through material was collected and desalted against 50% acetic acid after which it was dried down and reconstituted in SDS gel sample buffer. After electrophoresis on slab gels and autoradiography, the result seen in Figure 29 was obtained. The most striking result is that totally cleaved vitellogenin gives rise to two distinct phosphopeptide bands with molecular weights of 93,000 and 78,000 molecular weight. The material seen at the front in the autoradiogram does not form distinct bands when it is electrophoresed in a higher percentage (10%) gel and is probably derived from other parts of the vitellogenin molecule. It has been previously shown that the small subunit of lipovitellin (MW approximately 31,000) and phosvitin (MW approximately 35,000) are clustered on one side of the vitellogenin molecule (16). However, since the small subunit of

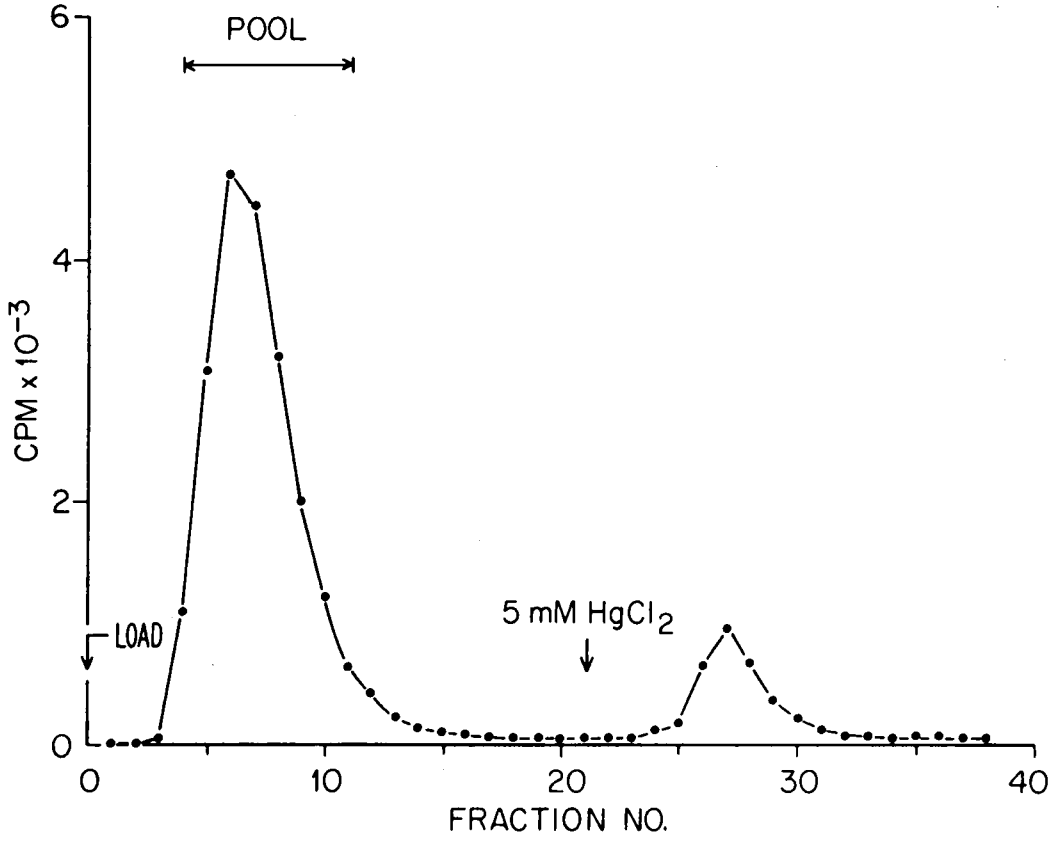

Figure 28. Elution of NTCB cleaved [^{32}P]vitellogenin from a column of organomercurial-Biogel A-15M. [^{32}P]vitellogenin was cleaved in 6M GuHCl as described in Materials and Methods. After exchanging the solvent for one containing 4 M urea, 250 µl of a sample containing 500 µg of protein was placed on a 1ml column of organomercurial-Biogel. The flow was stopped for 10 min and then the unbound vitellogenin was eluted with column buffer at a flow rate of 0.2ml/min. Three-drop fractions were collected and a 10 µl aliquot of each fraction was analyzed for radioactivity. The fractions marked "pool" were collected for further analysis by SDS gel electrophoresis.

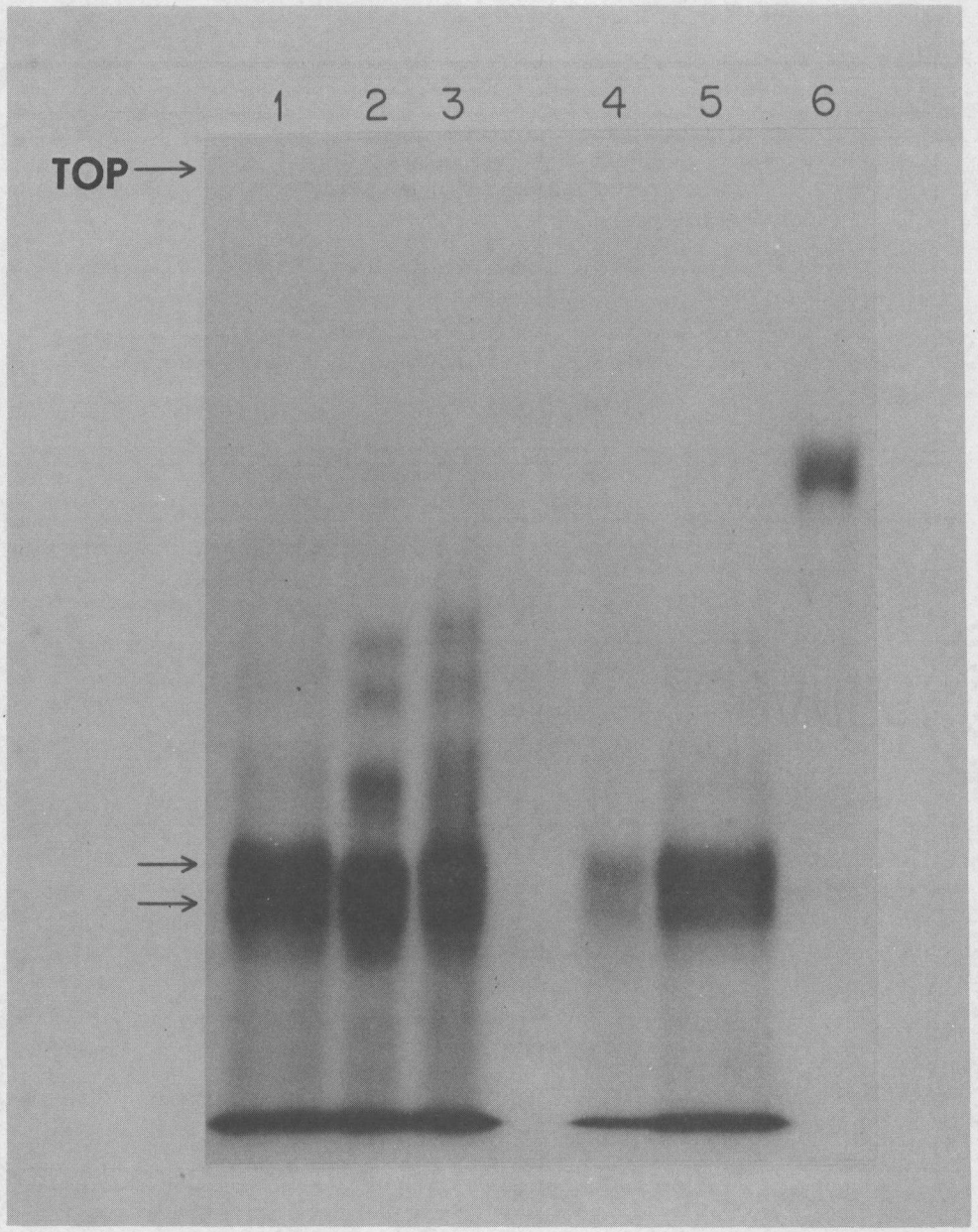

Figure 29. SDS slab gel (5% T, 2% C) electrophoresis of NTCB cleaved [^{32}P]vitellogenin. Shown is an autoradiogram of part of the gel. Slot 1 and 2 contain reduced [^{32}P]vitellogenin cleaved in 6M GuHCl and 1% SDS respectively, while slot 3 contains nonreduced [^{32}P]vitellogenin cleaved in 1% SDS. Slots 4 and 5 contain cleaved [^{32}P]vitellogenin filtered over a column of organomercurial-Biogel while slot 6 contains uncleaved [^{32}P]vitellogenin. The top of the gel is marked by the arrow.

lipovitellin contains at least 2 cysteine residues (57), the large (approximately 78,000 MW) limit phosphopeptide that I obtained cannot be derived from the C-terminal of the molecule, but instead must be derived from a position internal to the small lipovitellin subunit. Thus the large phosphopeptides that I obtained must be derived from the cleavage of cysteine residues flanking the phosvitin region of the vitellogenin molecule. The existence of two such large phosphopeptides indicates that there are at least two forms of the vitellogenin peptide that differ in their amino acid sequence.

Discussion

The purpose of this study was to show that Xenopus vitellogenin does not comprise a homogeneous population of molecules, but is instead a mixture of several different forms. I have shown by a number of different criteria that this is indeed the case. SDS gel electrophoresis at alkaline (9.6) pH resolves vitellogenin into 3 distinct forms termed α, β, and γ vitellogenin, while SDS gel electrophoresis at a lower pH (8.0) resolves vitellogenin into at least 3 and perhaps 4 different forms. Gel electrophoresis under nondenaturing conditions likewise resolves vitellogenin into at least 4 different bands. Isoelectric focusing of vitellogenin resolves the population into two main types, the major type (approximately 80% of the total) which has a more acidic isoelectric point than the minor type. As a final proof of the heterogeneity of vitellogenin, I have demonstrated that there are at least two populations of molecules that differ with respect to

the placement of the nearest cysteine groups to the phosvitin region of the molecule. Taken together, these results provide evidence that Xenopus vitellogenin is not a homogeneous propulation, but can be resolved into at least 3 size groups, 2 charge groups and 2 groups that differ in placement of their cysteine residues.

One major drawback to the present study is that there is no attempted correlation of the vitellogenins identified by each technique with those identified by other techniques. This was due to my inability to purify the different forms of vitellogenin and then study each by the various techniques. Vitellogenin is a very large protein (approximately 200,000 peptide MW) that is extensively conjugated with phosphate, carbohydrate and lipid (14). This places severe constraints on the methods available for isolating the various forms. Preparative gel electrophoresis was attempted on several occasions, but the very low load capacity of this method (several milligrams) yielded only microgram amounts of the purified proteins. Such amounts are not suitable for the characterization of the molecule without the use of sophisticated microanalytical techniques not available to the present investigator. Thus it was necessary to restrict myself to establishing the existence of multiple forms of vitellogenin.

A question that immediately arises is the nature of the heterogeneity in the vitellogenin population. My finding that there are two groups of vitellogenin that differ in their amino acid sequence implies that there are at least two vitellogenin genes, but the relationship between the multiple genes and the multiple vitellogenins is not clear. Since

vitellogenin is a highly conjugated molecule, differential posttranslational modifications of a parent molecule could give rise to multiple charged forms of the protein. Similarly, differential post-translational cleavage of one of the ends of the molecule could give rise to multiple molecular weight forms of the molecule irrespective of the exact amino acid sequence of the molecule. While such differential cleavage of a secretory protein that could give rise to a fairly constant ratio of the three forms has not been previously reported, it still remains a possibility.

The simplest explanation for the multiple forms of vitellogenin is that each form arises from a different vitellogenin gene. This is very plausible in light of the findings of Wahli et al. (58). Those investigators found that there were two families of vitellogenin mRNAs and that each family contained two members. Thus there are at least 4 distinct vitellogenin mRNA molecules. Native gel electrophoresis of vitellogenin does indeed give rise to at least 4 bands as does SDS gel electrophoresis in the Mono-tris/Bicine system. Thus I propose that while there are three different molecular weight forms of vitellogenin, one of the molecular weight forms contains an additional form that differs in net charge. This hypothesis would explain my observed results and is consistent with the data of Wahli et al. (58).

In conclusion, I find that vitellogenin is not a single distinct molecule, but is instead a mixture of at least three and possibly four different forms. While differential posttranslational modification of a common precursor cannot be ruled out as the source of some of the

observed heterogeneity, I feel that at least two of the different forms of vitellogenin can only have arisen due to the translation of two distinct gene products.

CHAPTER V

THE STRUCTURAL RELATIONSHIP BETWEEN VITELLOGENIN AND THE YOLK PROTEINS

Summary

The structure of vitellogenin was investigated by isolating the yolk proteins from the ovaries of Xenopus laevis and then reconstructing the precursor molecule from an analysis of these products. At least five different clases of yolk proteins were found, three of which (lipovitellin 1, lipovitellin 2 and phosvitin) correspond to previously described yolk proteins. Two new clases of yolk proteins, termed phosvette I and phosvette II, were also isolated and found to be phosphoproteins of 19,000 and 14,500-9,500 MW respectively. Phosvette I contained 4.8% phosphorus and was separated from phosvitin and phosvette II by DEAE-cellulose chromatography. Phosvette II contained 10.7% phosphorus and was separated from phosvitin by gel filtration. Amino acid analyses of phosvette I and II verify that these proteins cannot have been derived from the other yolk proteins. Lipovitellin 1 and 2 can each be resolved into 3 components by SDS gel electrophoresis, termed the α, β, and γ forms in the order of increasing electrophoretic mobility. Ferguson plot analysis of lipovitellin 1α, β, γ yields molecular weights of 121,000, 116,000 and 111,000 respectively. Lipovitellin 2α, β, γ have molecular weights of 34,000, 31,500 and 30,500 respectively. Phosvitin behaves very

anomalously during SDS gel electrophoresis and Ferguson plot analysis yields a value of 15,500 MW. However, when phosvitin is dephosphorylated, it shows no apparent anomalous behavior on SDS gels and resolves as two bands of 37,500 and 39,000 MW. Sedimentation equilibrium studies on phosvitin yield a molecular weight of 33,000.

A comparison of the sum of the amino acid content of each of the yolk proteins with that of vitellogenin indicates that the proteins cannot all be derived from the same parent molecule. The ratios of lipovitellin 2, phosvitin and the phosvettes was determined by protein phosphorus analysis and yielded a lipovitellin 2:phosvitin:phosvettes ratio of 1.00:0.70:0.27. It thus appears that there are two classes of vitellogenin molecules that give rise to alternate cleavage products. To verify that all of the observed yolk proteins were derived from vitellogenin, oocytes were incubated for 10-15 min with [^{32}P]vitellogenin and then chased for various lengths of time in unlabeled medium. The state of cleavage of vitellogenin was then assessed at each time point by SDS gel electrophoresis and autoradiography. This set of experiments demonstrated that phosvette I and II were indeed derived from the vitellogenin molecule and the time of their derivation was different from that of phosvitin.

The N-terminal amino acids of vitellogenin and the yolk proteins were determined by the dansyl chloride technique, which yielded aspartic acid for both vitellogenin and lipovitellin 1. Phenylalanine was the N-terminal of lipovitellin 2, but no residue was detected for phosvitin. Cyanogen bromide cleavage of vitellogenin and the yolk

proteins yielded a number of small lipovitellin peptides that had correspondence with peptides derived from vitellogenin. However, the phosvitin/phosvettes region of vitellogenin yielded predominantly a 65,000 MW peptide that contained little if any of the lipovitellin region of vitellogenin. These results indicate that the N-terminal of vitellogenin contains the future lipovitellin 1 molecule while the C-terminal region of vitellogenin gives rise to the lipovitellin 2 molecule. The phosvitin and the phosvettes seem to be derived from an internal region of vitellogenin. Titration of the cysteine groups of both native and reduced vitellogenin and the lipovitellins indicate that each of the lipovitellin regions of vitellogenin contain intrachain disulfide bonds. When these bonds are broken, the vitellogenin molecule takes on many of the solubility properties of the lipovitellins. Therefore, it is proposed that the cleavage of vitellogenin into the yolk proteins is a mechanism by which the phosvitin region of vitellogenin is removed, thus exposing the insoluble lipovitellin core protein.

Introduction

Vitellogenin is a very large protein (∿460,000 MW) which was thought to exist in the blood as a dimer containing two identical peptide chains with an approximate molecular weight of 200,000. Recently, however, it has been found that the mRNAs coding for vitellogenin do not comprise a homogeneous population, but instead are composed of two families, each having at least two distinct sets of sequences (58). Thus, the mRNA population in Xenopus liver would seem

to have the capacity to produce four different vitellogenin molecules. In the previous chapter, I have shown that vitellogenin is indeed comprised of a heterogenous population of molecules, some of which differ in their amino acid sequence.

The existence of several different vitellogenins was a complicating factor for my planned structural studies of vitellogenin. If one wishes to reconstruct a molecule from a mixture of its cleavage fragments, one must be able to tell which fragments overlap each other and which fragments are derived from different parts of the parent molecule. If the vitellogenin population contains members with different amino acid sequences, then cleavage of the population will yield corresponding peptides that would be impossible to classify. This is further complicated by the fact that even a single 200,000 MW peptide could yield an enormous number of cleavage peptides. Because of these considerations, examination of the intact vitellogenin molecule did not seem a viable approach; fortunately, however, another alternative existed.

Vitellogenin is the yolk-precursor protein from which is derived the smaller, more readily characterized yolk proteins. These proteins exist as an insoluble complex in the oocyte; however, the complex can be separated into its various components by column chromatography (26), or ammonium sulfate fractionation (82). Using these techniques, Bergink and Wallace (16) have shown that vitellogenin gives rise to three main yolk proteins. Lipovitellin 1 (LV_1) is a large (~115,000 MW) protein that contains little if any protein phosphorus. Lipovitellin 2

(LV_2) is a smaller protein (∿31,000 MW) that contains a significant amount of protein phosphorus and is normally tightly associated with the larger LV_1 protein in the yolk (16). The third main yolk protein, phosvitin (Pv; ∿35,000 MW) contains most of the yolk protein phosphorus. In addition, Bergink and Wallace (16) described what appeared to be several small phosphopeptides. All of these yolk proteins are entirely derived from vitellogenin. Thus my approach was to isolate these naturally occurring vitellogenin cleavage fragments and then "reconstruct" the parent molecule.

The heterogeneity observed in vitellogenin seems to be at least partially preserved during the formation of the yolk proteins. Ohlendorf et al. (57) have shown that the LV_2 peptide can be resolved into three different molecular weight forms that they designated lipovitellin B, C and D. Since the amino acid composition of these three different forms of LV_2 were very similar, it seems probable that they were derived from similar regions of the three molecular weight forms of vitellogenin. Thus an analysis of the yolk proteins would seem not only to eliminate the problem associated with identifying similar peptides derived from the heterogenous vitellogenin population, but also allow one to determine which regions of the vitellogenins are heterogenous with respect to each other.

Materials and Methods

The care of animals together with the injection of hormones and isotopes were performed as previously described (22). Acrylamide and

N, N'-methylene-bis acrylamide were recrystallized three times from acetone prior to use. SDS was purchased from Polysciences (electrophoresis grade). Ammediol (2-amino-2-methyl-1,3-propanediol) was recrystallized from ethanol, rinsed with acetone and dried under vacuum. Mono-tris was synthesized as described by Lewis (60) and was recrystallized from ethanol three times. Isotopes were purchased from Amersham and all other chemicals and reagents were the highest quality available.

SDS gel electrophoresis at high pH (9.58) was performed by a modification (59) of the procedure of Wyckoff et al. (27). SDS gel electrophoresis at pH 8.0 was performed using the Mono-tris/Bicine system described in Appendix B. Gels were fixed overnight in 45% methanol, 10% acetic acid and stained with 0.05% Coomassie blue R-250, 20 m$\underline{M}$ $AlCl_3$, 25% isopropanol and 10% acetic acid. The inclusion of the Al^{3+} in the staining solution was necessary for the visualization of the phosphoprotein bands (83).

Molecular weight estimations by SDS gel electrophoresis was performed using Ferguson plot analysis (84). Standard proteins were β-galactosidase (116,000), phosphorylase-b (96,000), bovine serum albumin (68,000), catalase (59,000), ovalbumin (45,000), aldolase (40,000), yeast glyceraldehyde-3-phosphate dehydrogenase (34,000), PMSF-inactivated trypsinogen (24,000), soybean trypsin inhibitor (21,500), β-lactoglobulin (18,400), hemoglobin (16,000) and cytochrome C (12,500).

SDS gel electrophoresis in slab gels employed 10% T, 2% C gels that were 1.5 mm thick. The separating gel was generally 13 cm long

while the stacking gels were 3 cm long and were 3% T, 20% C_{DATD} (66). Slots for the protein samples were 1 × 1 × 0.15 cm and load volumes of 50 µl were used. Slab gels were stained as described above and then dried on blotter paper using a Hoefer gel drier. For autoradiography, the gel was exposed to Kodak X-Omat R film at 20° for a length of time necessary for the activity of the ^{32}P in each slot to drop approximately 1500 dpm.

Radioactivity in tube gels was detected by slicing the gel into 1 mm sections using a Hoefer gel slicer. The slices were either directly counted in a Beckman Lobeta II planchet counter, or dissolved in 0.3 ml of 30% H_2O_2 and 50 µl of NH_4OH at 55° for 2 hr. Aqueous Counting Solution (Amersham) was then added (4.5 ml) and the samples were counted in a scintillation counter.

Protein Purification and Labeling

Unless otherwise stated, all isolation procedures were performed at 0-4°. Xenopus vitellogenin was isolated by Mg^{2+}:EDTA precipitation (Chapter II) in the presence of 2 mM PMSF. Vitellogenin was labeled *in vivo* by injecting 2 mCi [^{32}P]orthophosphate 24 hr prior to bleeding the animals.

^{32}P-labeled yolk proteins were isolated from animals that had been injected with 1000 IU hCG and 2 mCi [^{32}P]orthophosphate 72 hr and 24 hr respectively prior to sacrifice. The animals were first bled by heart incision (39) and the ovaries were then removed and rinsed in solution O-R2 (40). The ovaries were homogenized in 5 volumes of 0.25 M sucrose, 10 mM Hepes, 1 mM EDTA and 1% polyvinylpyrrolidone (pH 7.2) and the yolk platelets were isolated as described by Wallace (82). The platelets

were dissolved in 10 volumes of 1 M NaCl, 10 mM Hepes (pH 7.2) and any insoluble material was removed by centrifugation at 10,000 x g for 20 min.

Isolation of the Lipovitellin Subunits

Lipovitellin was separated from the other yolk proteins by the addition of 2 volumes of a saturated $(NH_4)_2SO_4$ solution dropwise over a period of 15 min to the dissolved yolk platelets (82). The precipitated lipovitellin was removed by centrifugation at 10,000 x g for 20 min and redissolved by the addition of an equal volume of water. After an overnight dialysis against 1M NaCl, 10 mM Hepes (pH 7.2), the lipovitellin was again precipitated with $(NH_4)_2SO_4$. The final pellet was dissolved in a small amount of water and then exhaustively dialyzed against distilled water continaing 2 mM PMSF. The lipovitellin was then delipidated (85) and 140 mg of the resulting powder was dissolved in 20 ml of 10% SDS, 10 mM Tris-Cl (pH 7.5) with or without 10 mM DTT at 100°. Any insoluble material was removed by centrifugation and the sample was applied to a 5 × 100 cm column of Biogel A-1.5M equilibrated with 0.1% SDS, 50 mM ammonium bicarbonate and 0.02% sodium azide. Elution was carried out at 20° at a flow rate of 59 ml/hr. The LV_1 and LV_2 subunits of lipovitellin were resolved by this procedure (57). Fractions containing either LV_1 or LV_2 (as determined by SDS gel electrophoresis) were pooled and lyophilized. The SDS was then removed by ion-pair extraction (86) and the proteins were then dried under vacuum. The sodium content of these preparations was approximately 1% as determined by atomic absorption spectroscopy.

Yolk Phosphoprotein Isolation

The soluble fraction of the yolk that remained after the $(NH_4)_2SO_4$ precipitation of the lipovitellin was exhaustively dialyzed against 50 mM Tris-Cl, 2 mM PMSF (pH 7.5) using Spectrapor-6 membrane tubing (MW cutoff ∿2000). Any insoluble material appearing during dialysis was removed by centrifugation. For the isolation of the [^{32}P]phosphoproteins, the sample was applied to a 1.5 × 18 cm column of DEAE-cellulose (Selectacel Type 40, Schleicher and Schuell Company, Lot No. D1391, 0.94 meq/g) prepared and packed as previously described (26). The phosphoproteins were then eluted with a 400 ml linear gradient from 0.0-0.35 M KCl in 50 mM Tris-Cl, pH 7.5, at a flow rate of 112 ml/hr. The protein load on these columns did not exceed 50 mg. For the large-scale isolation of the yolk phosphoproteins, the $(NH_4)_2SO_4$ supernatant from the yolk platelets was dialyzed against distilled water and lyophilized. Two grams of the resultant solid was dissolved in 50 ml of 50 mM Tris-Cl, pH 7.5, and applied to a 5 × 50 cm column of DEAE-cellulose equilibrated with the same buffer. The proteins were eluted from the column with a 5 liter linear gradient from 0.0-0.35 M KCl in 50 mM Tris-Cl, pH 7.5. The flow rate was 344 ml/hr and 3.5 min fractions were collected. Appropriate fractions were pooled and dialyzed against distilled water prior to lyophilization.

The phosphoproteins isolated by DEAE-cellulose chromatography were further purified by gel filtration. The lyophilized proteins were dissolved in 10 ml of 0.5 M NaCl, 50 mM Hepes-OH, 5 mM EDTA, pH 7.0, at a maximum concentration of 10 mg/ml. This solvent system was chosen so that the eluted phosphoproteins would exist entirely as the sodium salts. The dissolved proteins were applied to a 2.6 × 89 cm column

of Sephadex G-100 (fine) equilibrated with the above mentioned buffer, and eluted at 20° with a flow rate of 23 ml/hr. Peaks containing the desired protein fraction were collected and exhaustively dialyzed against distilled water prior to lyophilization.

Analytical Procedures

Amino acid analyses on samples were performed after acid hydrolysis in evacuated (<50 μm Hg) sealed tubes with 6 N HCl/0.01 M 2-mercaptoethanol at 110° for 21 hr. Samples were chromatographed on a Beckman 121M amino acid analyzer using Beckman's "3-hour-single-column" system and data was analyzed on a Beckman model 126 data system. The data was not corrected for hydrolytic destruction. The nonphosphorylated serine residues of phosvitin were quantitated by converting the phosphoserine to cysteic acid by β-elimination and sulfite addition as described by Simpson et al. (87). The remaining serine was then measured on the amino acid analyzer after hydrolysis. Hydrolysates containing high amounts of serine were analyzed at different dilutions, with the serine being measured with the higher dilutions and the other amino acids measured at the lower dilutions.

Cysteine was measured in the proteins by DTNB titration (76) with 50 mM Tris-Cl, 10 mM EDTA, pH 8.0, as the solvent for native proteins and 2% SDS, 50 mM Tris-Cl, 5 mM EDTA as the solvent for denatured proteins. Reduced vitellogenin was prepared by dissolving the protein in 50 mM Tris-Cl, 2 mM EDTA, pH 7.5 at a concentration of 2 mg/ml. DTT was added to 10 mM and the sample was then dialyzed 4 times against 1000 volumes of 0.02 M KCl at 4° for 24 hr. This solvent was autoclaved prior to use. The final dialyzate had no detectable thiol

content. Reduced lipovitellins were prepared by DTT reduction prior to their isolation by Biogel A-1.5 M chromatography (see above). All protein concentrations were determined by absorbance at 280nm using an $\mathcal{E}^{1\%}_{1cm}$ of 7.5 for vitellogenin (14), 1.96 for phosvitin (15), and 13.3 and 4.9 for LV_2 and LV_1 respectively (51).

N-terminal analyses were performed using dansyl chloride (88). The dansylated amino acids were separated on polyamide thin layer sheets (89) and compared to standards purchased from Sigma.

Elemental analyses were performed by Galbraith Laboratories, Incorporated, Knoxville, Tennessee. Protein samples were delipidated (85) and all reported values were corrected for water and sodium content. Sedimentation equilibrium of proteins was performed as described by Yphantis (90) at a rotor temperature of 20.0°. Proteins were equilibrated with the solvent (0.5 M NaCl, 50 mM Hepes-OH, 5 mM EDTA, pH 7.0) by gel filtration. The partial specific volumes were calculated from the amino acid composition of the samples (91) using a value for phosphoserine derived from the data of Joubert and Cook (92). This gave a value for Xenopus phosvitin of 0.58 and for phosvette II a value of 0.54. Sapphire windows were used on the double-sectored cells and the fringes were measured with a Nikon comparator.

Protein Cleavage

Proteins were cleaved with CNBr at the methionine resides in 70% formic acid (93). The proteins were dissolved at a concentration of 1 mg/ml and a 100-fold molar excess of CNBr was added over the

methionine groups present. After 6 hr at 20°, another 100-fold excess of CNBr was added. After 24 hr, the solutions were dried down with nitrogen and stored overnight under a high vacuum. In some experiments, the proteins were reduced and carboxymethylated (94) prior to cleavage with CNBr. This treatment did not alter the cleavage pattern of the proteins. Dried protein samples were dissolved in SDS gel electrophoresis sample buffer and subjected to electrophoresis as outlined above.

Intraoocyte cleavage of vitellogenin was accomplished by first labeling vitellogenin in vitro with [γ^{32}P]ATP using the Xenopus liver phosphoprotein kinase (see Chapter III). Specific activities were generally $\sim 5 \times 10^8$ cpm/mg. The vitellogenin was dissolved at a concentration of 2.5 mg/ml in solution O-R2 (40) containing 10% normal male Xenopus serum and 100 µg/ml of Gentamycin. Oocytes, 0.96 ± 0.04 mm in diameter, were manually dissected from the ovaries of hCG-treated donor animals (22), and maintained overnight in glutamine-supplement 50% L-15 medium containing 2.5 mg/ml vitellogenin (10). The following morning, all abnormal oocytes were discarded and groups of 30-45 oocytes were placed into the [^{32}P]vitellogenin-containing medium. After 15 min at 20°, the oocytes were rinsed in 4 changes of solution O-R2 containing ~0.5 mg/ml of lyophilized calf serum (to prevent sticking of the oocytes to the culture plate). They were then transferred to solution O-R2 containing 2.5 mg/ml unlabeled vitellogenin and 10% male serum at 20°. At different time intervals, groups of 3 oocytes were removed and dropped into 3 ml of acetone at 0°. The next day, the

acetone was removed and the oocytes were dried under a vacuum. Each group of 3 oocytes was placed in 250 µl microfuge tubes (Bel-Art Products) together with 150 µl of 5% SDS, 10 mM Tris-Cl, 2 mM EDTA, pH 8.0. The tubes were placed in a boiling water bath for 5 min and then stoppered and allowed to sit overnight at 4°. The solutions were then reheated to 100° for 10 min and the proteins were then reduced and alkylated according to the procedure of Lane (65). After removing any insoluble material by centrifugation, samples from each tube were removed and assayed for radioactivity (generally 500 cpm/µl). After adjusting all samples to the same specific activity with SDS sample buffer, ∿10 µl of each was mixed with 40 µl of 20% sucrose, 10 mM Tris-Cl, 2 mM EDTA, 0.005% bromophenol blue, pH 7.5, and electrophoresed on 10% T, 2% C slab gels as described above.

Results

Isolation of the Yolk Proteins

My approach for isolating the yolk proteins was partially dictated by the findings of previous investigators. These included the observation that phosphoproteins undergo a β-elimination reaction at alkaline pH values (30) and also that Xenopus yolk contains a small phosphopeptide(s) that is lost during dialysis (16). Therefore, all preparative steps were performed at a relatively neutral pH and Spectrapore-6 dialysis tubing (cutoff MW ∿2,000) was used throughout. Since some yolk phosphopeptides in fish do not absorb light at 280nm, my initial purification attempts employed yolk platelets derived from animals injected with [^{32}P]orthophosphate prior to sacrifice.

Yolk platelets were obtained by low speed centrifugation of ovarian homogenates; the resulting pellet was then dissolved in 1 M NaCl. The lipovitellins were isolated by several rounds of $(NH_4)_2SO_4$ precipitation and dialyzed against distilled water prior to lyophilization. The two lipovitellins were then separated by gel filtration in the presence of SDS. Figure 30 shows a typical elution profile of [^{32}P]lipovitellin from Biogel A-1.5 M. There are two main peaks of protein eluting from the column: peak A, a high molecular weight component containing little ^{32}P, and peak B, a lower molecular weight component containing most of the ^{32}P applied to the column. SDS gel electrophoresis identified peak A as LV_1 while peak B contained almost exclusively LV_2. The peak preceding LV_1 is probably an aggregate of LV_1 as determined by the A_{280}:^{32}P ratio and also by SDS gel electrophoresis. The peak designated "SDS" contains most of the SDS used to dissolve the lipovitellin prior to loading on the column, since it yielded several grams of material upon lyophilization that was completely dissolved in the SDS-extraction solvent used. Surprisingly, there was no evidence of contamination of my lipovitellin preparation with phosvitin, which elutes ahead of the LV_2 peak (57).

The material left in solution after the precipitation of the lipovitellins contains most of the yolk protein phosphorus (33). This material was exhaustively dialyzed against 50 mM Tris-Cl (pH 7.5) and then loaded on a column of DEAE-cellulose. When a linear salt gradient was applied to the column, the result in Figure 31 was obtained. Almost all of the UV-absorbing material and ^{32}P eluted as a single

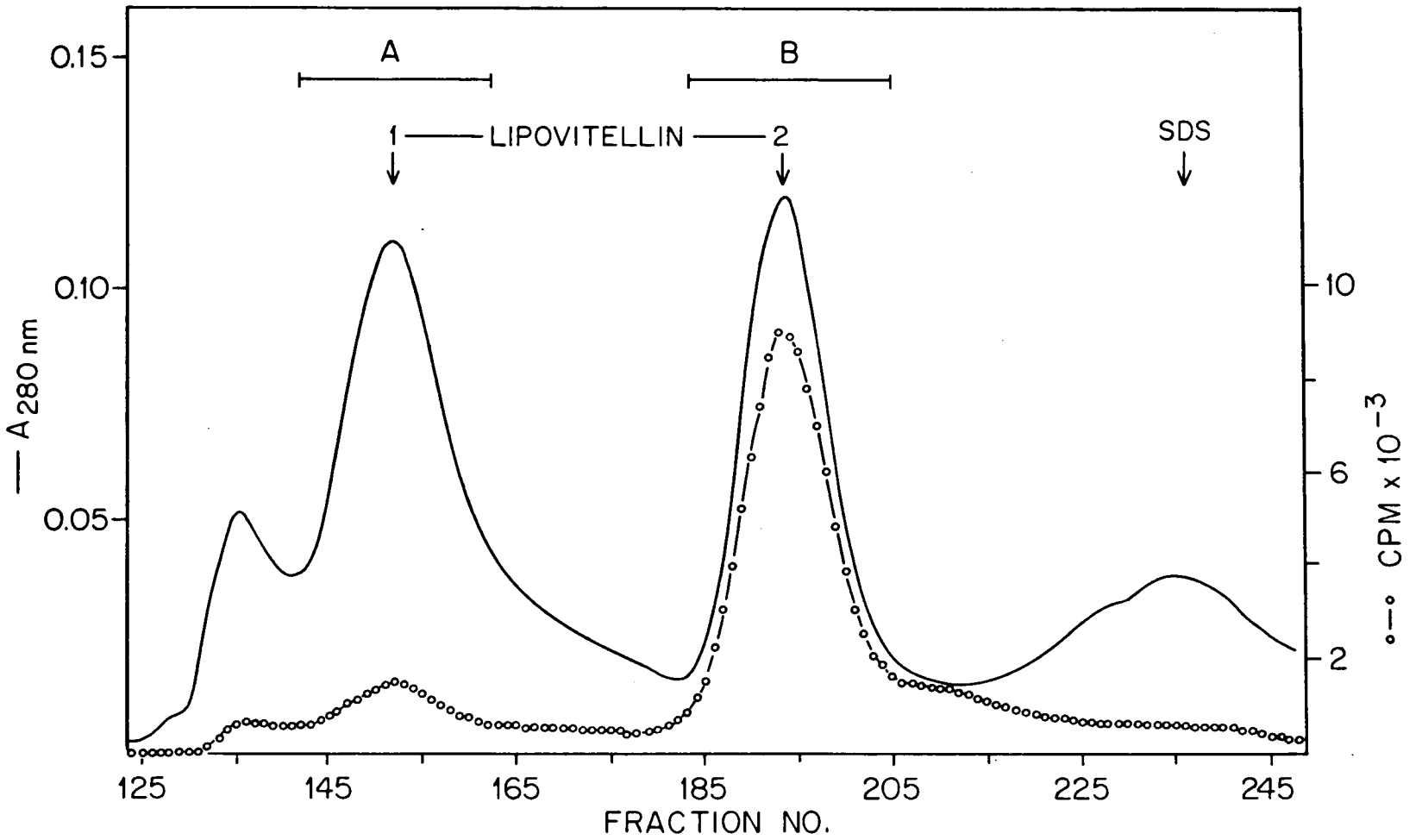

Figure 30. Gel filtration of Xenopus lipovitellin. 120 mg of delipidated [^{32}P]lipovitellin was dissolved in 20 ml of 10% SDS and applied to a 5 × 100 cm column of Biogel A-1.5 M equilibriated with 0.1% SDS, 50 mM ammonium bicarbonate and 0.02% sodium aside. Flow rate was 59 ml/hr and 12 min fractions were collected. Aliquots (100 µl) of each fraction were counted for the presence of ^{32}P.

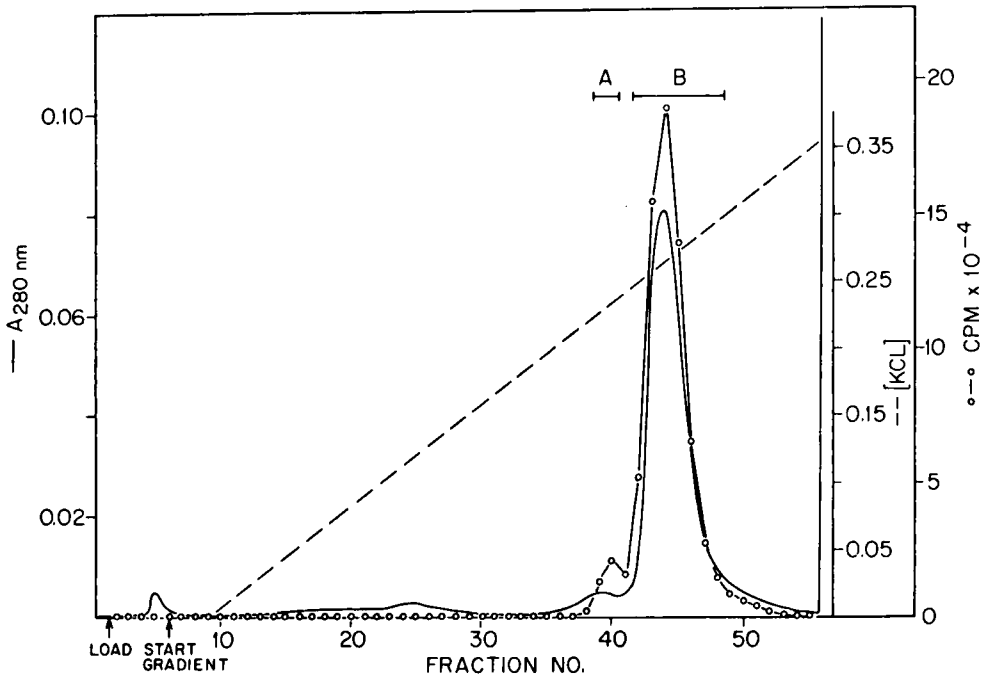

Figure 31. DEAE-cellulose chromatography of ^{32}P-labeled Xenopus yolk phosphoproteins. The material remaining in solution after the $(NH_4)_2\ SO_4$ precipitation step was dialyzed against 50 mM Tris-Cl (pH 7.5) and loaded on a 30 ml bed volume column of DEAE-cellulose equilibrated with 50 mM Tris-Cl, pH 7.5 and then eluted with a 400 ml linear salt gradient at a rate of 110 ml/hr. 5 Min fractions were collected and assayed for the presence of ^{32}P. The indicated fractions were pooled for further analysis.

peak at approximately 0.26 M KCl. However, there was a small peak of ^{32}P eluting prior to the main peak which seemed to be associated with a very small peak of UV-absorbing material. This small peak was seen in five separate runs. The main peak was collected, lyophilized and then chromatographed on Sephadex G-100 as shown in Figure 32. Two peaks of radioactivity were seen: peak A had an apparent molecular weight of ∿50,000 while the small peak behaved as though it had a molecular weight of approximately 20,000. The last peak eluting from the column apparently contained buffer components from the DEAE-cellulose chromatography step since prior dialysis would remove this material.

In an effort to investigate the minor peak seen during DEAE-cellulose chromatography, I took the $(NH_4)_2SO_4$ supernatant derived from ^{32}P-labeled yolk platelets, dialyzed and lyophilized it, and then subjected it to Sephadex G-75 chromatography. In this case (Figure 33), two main peaks of ^{32}P were again observed together with some other UV-absorbing material. The second peak was collected, dialyzed and then applied to the DEAE-cellulose column. The result seen in Figure 34 was then obtained. The main UV-absorbing and ^{32}P-containing peak again eluted at ∿0.26 M KCl. However, the minor peak was again observed, and this time it contained ∿23% of the total radioactivity applied to the column. Thus the minor DEAE-cellulose component seems to coelute with the major 20,000 MW component on gel filtration columns.

Purified fractions of each of the yolk platelet proteins were obtained and subjected to SDS gel electrophoresis. The results seen in Figure 35 were obtained. LV_1 and LV_2 migrate closely with their

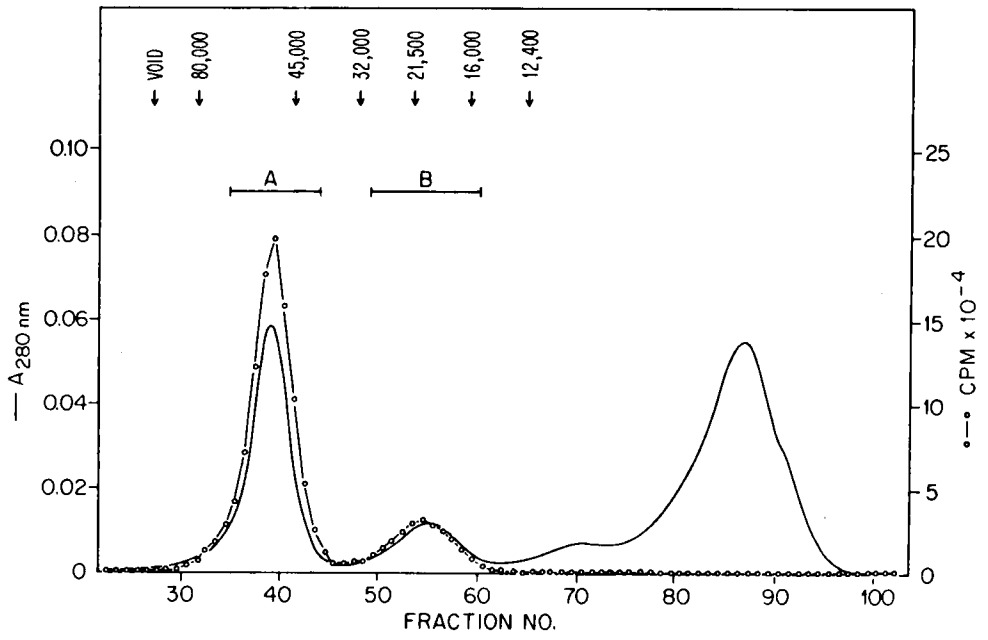

Figure 32. Gel filtration of the major yolk phosphoprotein peak isolated by DEAE-cellulose chromatography. The fractions designated "B" from Figure 31 were pooled and lyophilized prior to chromatography on a 2.6 × 89 cm column of Sephadex G-100 equilibrated with 0.5 M NaCl, 0.05 M Hepes-OH, and 5 mM EDTA (pH 7.0). The column was run at a flow rate of 23 ml/hr and 12 min fractions were collected. Each fraction was assayed for the presence of ^{32}P.

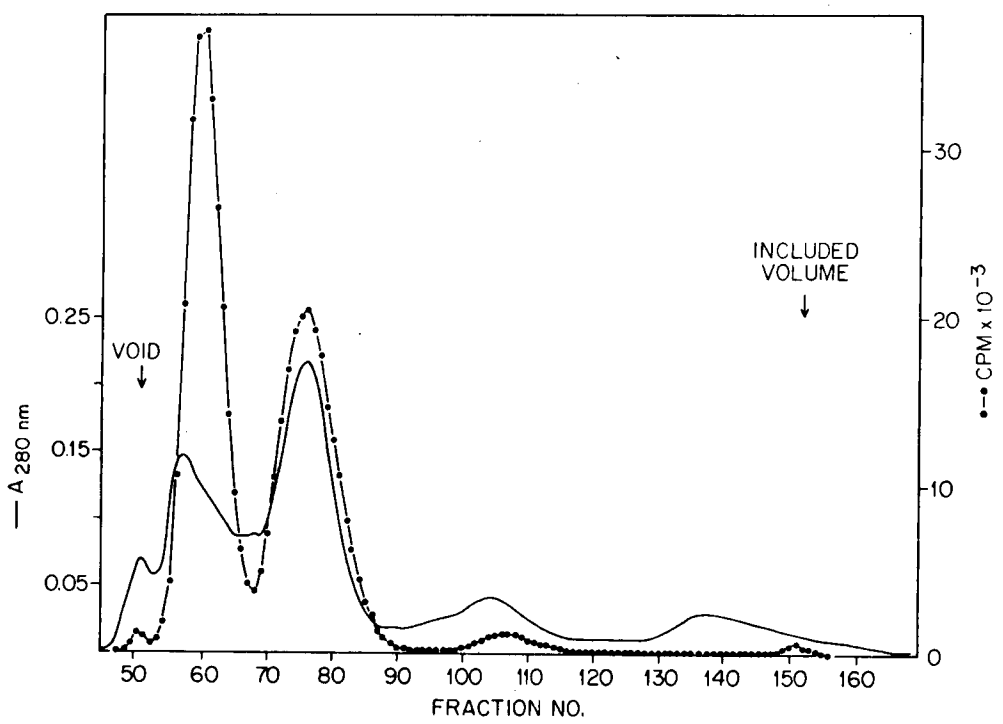

Figure 33. Gel filtration of ^{32}P-labeled phosphoproteins in Xenopus yolk. The yolk was dissolved in 1 M NaCl and the lipovitellins precipitated by $(NH_4)_2\ SO_4$. The supernatant remaining was dialyzed against water prior to lyophilization and loading on a 1.6 × 95 cm column of Sephadex G-75 (superfine) equilibrated with 0.5 M NaCl, 50 mM Hepes-OH, 5 mM EDTA (pH 7.0). The column was run at a flow rate of 12.7 ml/hr and 6 min fractions were collected and assayed for the presence of ^{32}P. The fractions designated "pool" were collected for further analysis. A total of 40 mg was applied to the column.

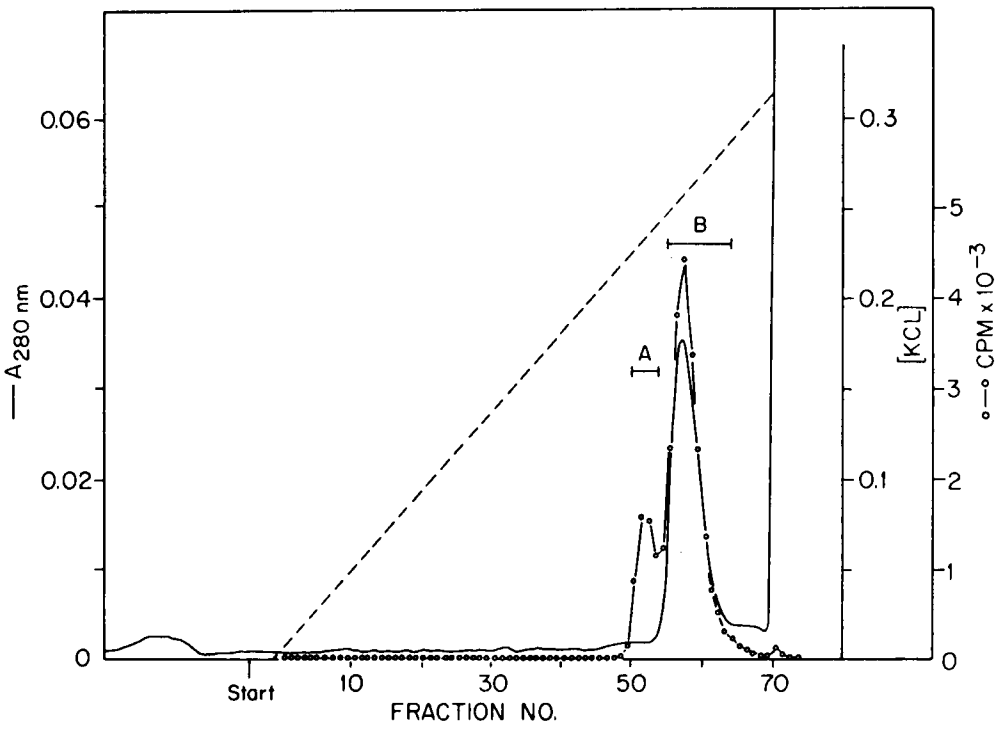

Figure 34. DEAE-cellulose chromatography of the second Sephadex G-75 peak derived from the yolk proteins. The ^{32}P-labeled material in fractions 70-84 in Figure 4, page 22, was dialyzed against 50 mM Tris-Cl (pH 7.5) and then loaded on a 30 ml bed volume column of DEAE-cellulose equilibrated with the same buffer. The protein was eluted with a 400 ml linear KCl gradient at a rate of 112 ml/hr. Fractions (3 min) were collected and 100 µl aliquots were assayed for the presence of ^{32}P.

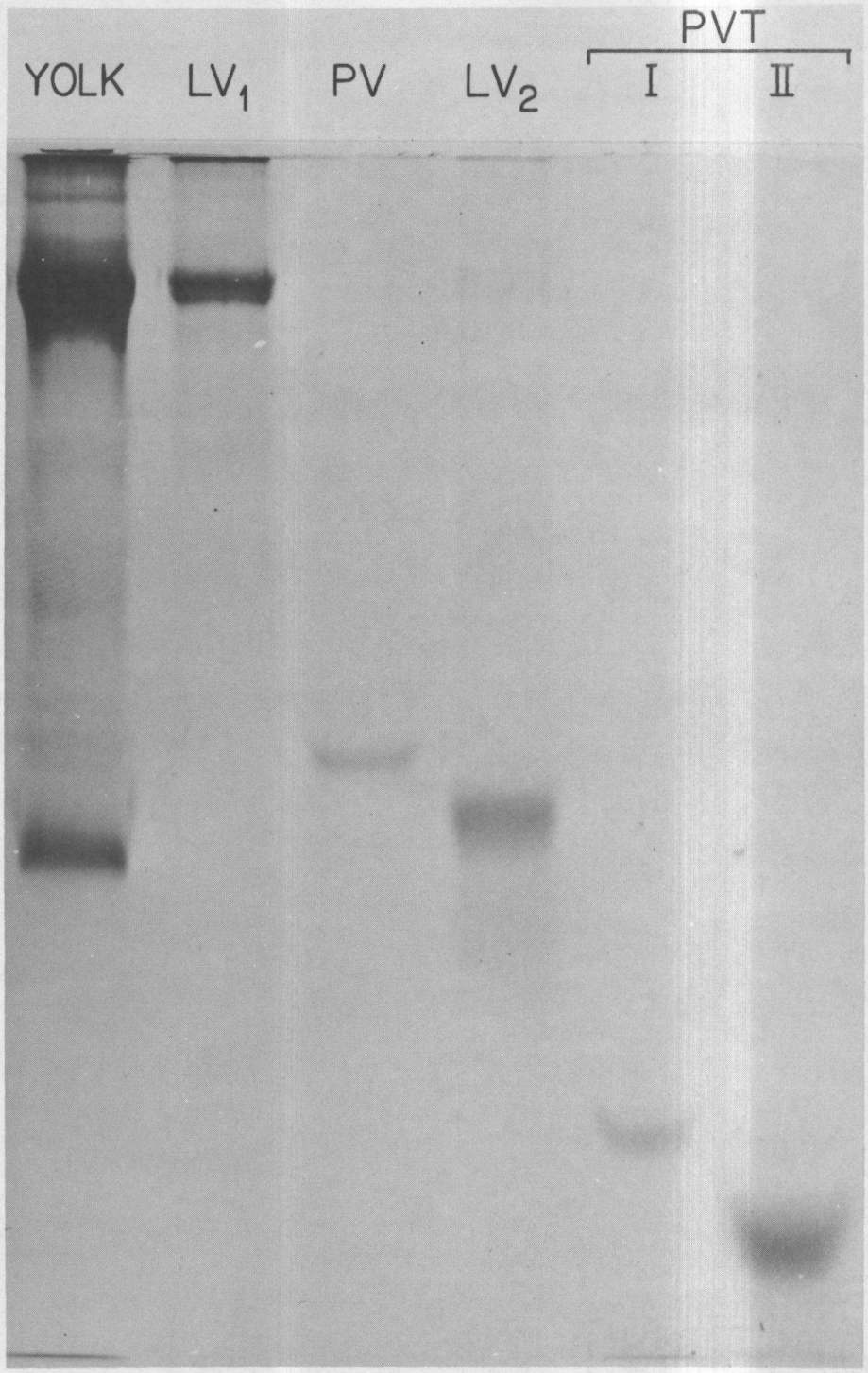

Figure 35. SDS gel electrophoresis of the purified yolk proteins. Each slot was loaded with purified samples of each yolk protein and electrophoresised in 1.5 mm thick slab gels until the tracking dye had traveled 13 cm. The gel was stained with a 0.05% Coomassie blue R-250 solution which contained 20 mM Al^{3+} to allow the phosphoproteins to stain. The two small-molecular-weight phosphoprotein peaks eluting off of the DEAE-cellulose column are designated PVT I and PVT II in order of their elution position.

counterparts seen during electrophoresis of total yolk platelets. The 50,000 MW component seen during gel filtration of the phosphoprotein fraction of yolk corresponds to what has been previously described as Xenopus phosvitin (Pv). However, the 20,000 MW phosphopeptide and the minor DEAE-cellulose component are low molecular weight compounds that seem to correspond to the "phosphopeptides" described by Bergink and Wallace (16). They are not seen in the total yolk platelet fractions except when the gels are grossly overloaded with respect to the highly molecular weight components, and thus seem to be minor components. These "phosphopeptides" were observed even when the protease inhibitor PMSF was included during the isolation procedures. I have named these two components phosvette I (Pvt-I) and phosvette II (Pvt-II) in order of their elution from columns of DEAE-cellulose. It should be noted that like Pv, Pvt-II does not stain with Coomassie blue R-250 except in the presence of Al^{3+}. However, Pvt-I does stain fairly well with Coomassie blue alone.

Molecular Weights of the Yolk Proteins

The molecular weights of the lipovitellins were determined by Ferguson plot analysis. As previously observed by Ohlendorf et al. (57), LV_2 gives rise to 3 separate bands during SDS gel electrophoresis. My determination of the molecular weight of these three bands (designated $LV_{2\alpha}$, $LV_{2\beta}$ and $LV_{2\gamma}$ in order of increasing electrophoretic mobility) is 34,000, 31,500 and 30,500 for $LV_{2\alpha}$, $LV_{2\beta}$ and $LV_{2\gamma}$ respectively. These values are in good agreement with the values of 35,500, 32,000 and 31,600 found by Ohlendorf et al. (57). However, while Ohlendorf and

co-workers observed only a single LV_1 band of molecular weight 105,500, I found that LV_1 also gives rise to three bands as seen in Figure 36. These three bands are designated $LV_{1\alpha}$, $LV_{1\beta}$ and $LV_{1\gamma}$ and have molecular weights of approximately 121,000, 116,000 and 111,000 respectively. I ascribe my finding of the three LV_1 bands to the low amounts of protein electrophoresized ($\sim$10 µg), since three bands of LV_1 are easily obtained using the exact electrophoretic conditions specified by Ohlendorf et al. (57). High loads as used by Ohlendorf et al. (57) give rise to a large single band of LV_1 (as seen in Figure 35, page 119).

SDS gel electrophoresis and Ferguson plot analysis can be used to determine the approximate molecular weights of the lipovitellins since their free electrophoretic mobilities as SDS-complexes are virtually identical to those of the standard proteins used [(57), also personal observation]. However, this is not true in the case of Pv (57). Ferguson plot analysis of Xenopus Pv led Ohlendorf et al. (57) to propose that the molecular weight of Pv was only about 17,000 instead of the value of 35,000 found by Bergink and Wallace (16) even though they had no theoretical basis on which to base their proposal. In an effort to resolve the question of the "true" molecular weight of Xenopus Pv, I decided to perform Ferguson plot analysis of Pv and dephosphorylated Pv. Dephosphorylation of Pv was accomplished with alkaline phosphatase (calf intestine, Sigma Type VII). [^{32}P]Pv was used as a substrate in order to follow the extent of dephosphorylation. The time course of Pv dephosphorylation was as seen in Figure 37. After a lag period of $\sim$1 hr, the reaction proceeded fairly rapidly

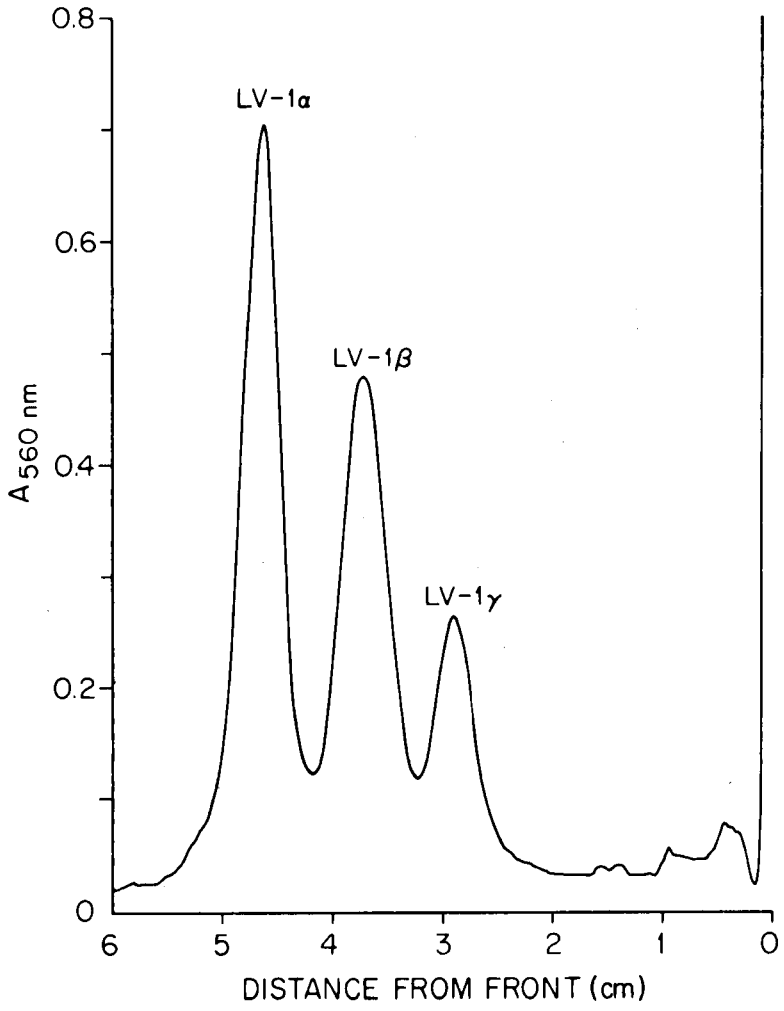

Figure 36. Absorbance scan of an SDS gel containing LV_1. A 5% T, 2% C gel containing 10 µg of LV_1 was run and stained with Coomassie blue R-250. The gel was then scanned at 560nm. The three LV_1 peaks are designated LV-1α, LV-1β and LV-1γ in the order of their increasing electrophoretic mobility. Only the bottom half of the 10cm gel was scanned.

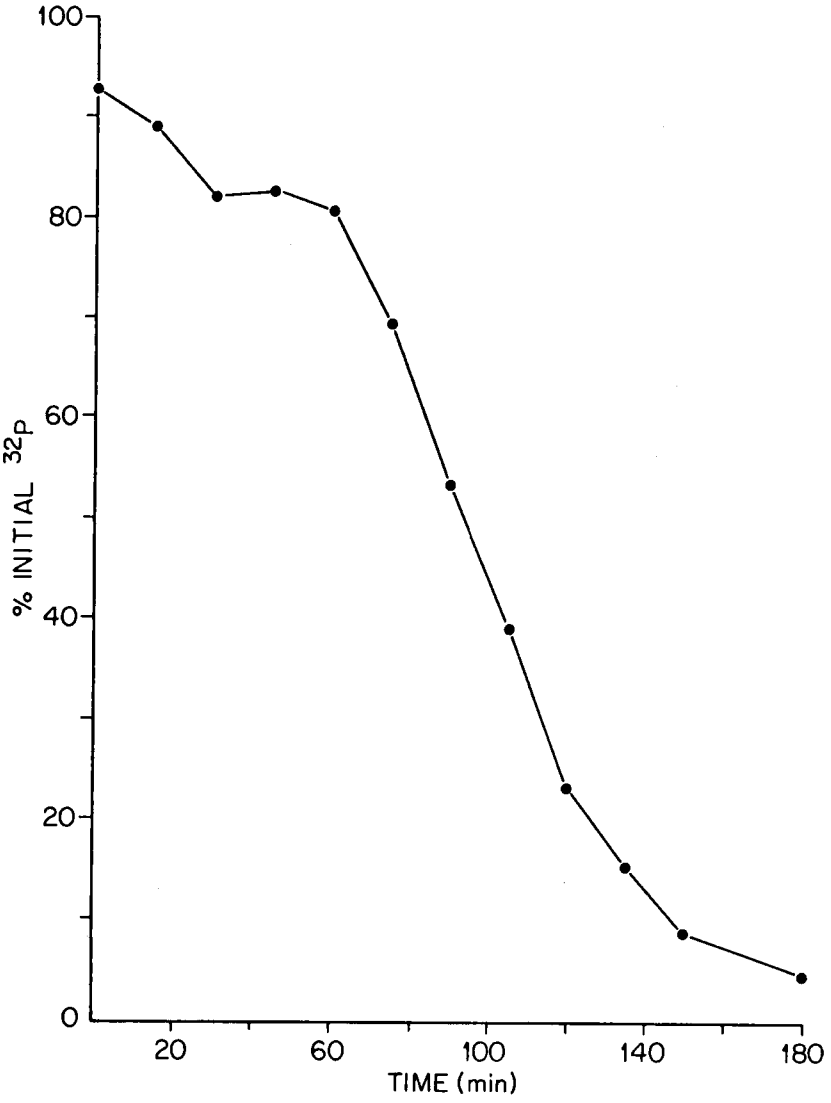

Figure 37. Dephosphorylation of [^{32}P]phosvitin with alkaline phosphatase. [^{32}P]phosvitin was incubated at a concentration of 1 mg/ml in 1 ml of 50 mM Mes-OH (pH 5.5) and 20 µg of alkaline phosphatase was added. At the different time points indicated, aliquots of the reaction mixture were removed and assayed for the presence of TCA-insoluble radioactivity.

until only about 5% of the initial ^{32}P remained. Aliquots of protein were removed at intervals and subjected to SDS gel electrophoresis using gels of various percentages of acrylamide. The results of this experiment are seen in Figure 38. The initial Pv preparation intercepted the Y-axis at a point considerably below that of the standard proteins. However, 95% dephosphorylated Pv apparently behaved normally with respect to its Y-intercept. Note that dephosphorylated Pv was resolved as two closely spaced bands. Partially dephosphorylated Pv (∿50%) resolved as a series of bands that were either very close to the position of the native phosvitin or to the position of dephosphorylated Pv. In Figure 39 is seen the slopes (Kd) from the Ferguson plot of my standard proteins plotted against their known molecular weights. Also indicated are the Kd values derived from Pv and dephosphorylated Pv. This analysis gives a molecular weight value of ∿15,500 for native Pv and ∿37,500 and 39,000 for dephosphorylated Pv. It is thus obvious that Ferguson plot analysis is useless for determining the molecular weight of some phosphoproteins. It should be noted that vitellogenin itself displays anomalous behavior during SDS gel electrophoresis and so molecular weight values for this protein derived by this procedure are also suspect.

The molecular weight values found for dephosphorylated Pv are also not necessarily correct since some proteins show normal Y-intercepts on Ferguson plots but still yield molecular weight values that are too large (80). Gel filtration also cannot be used for the determination of the approximate molecular weight of Pv or other highly phosphorylated peptides since these proteins assume an elongated random coil

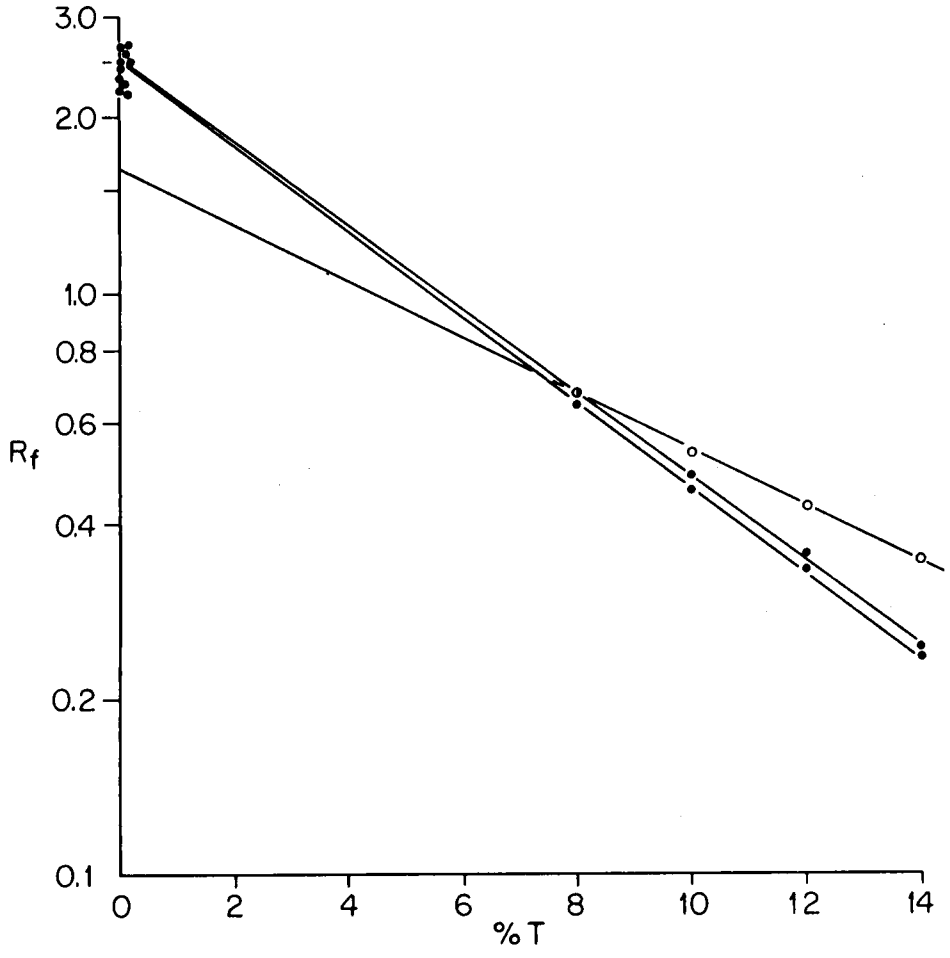

Figure 38. Ferguson plot analysis of phosvitin (o) and 95% dephosphorylated phosvitin (●). Dephosphorylated phosvitin was observed to form two closely spaced bands on these gels. Indicated as a series of solid points on the Y-axis are the Y-intercepts of the standard proteins used in this study.

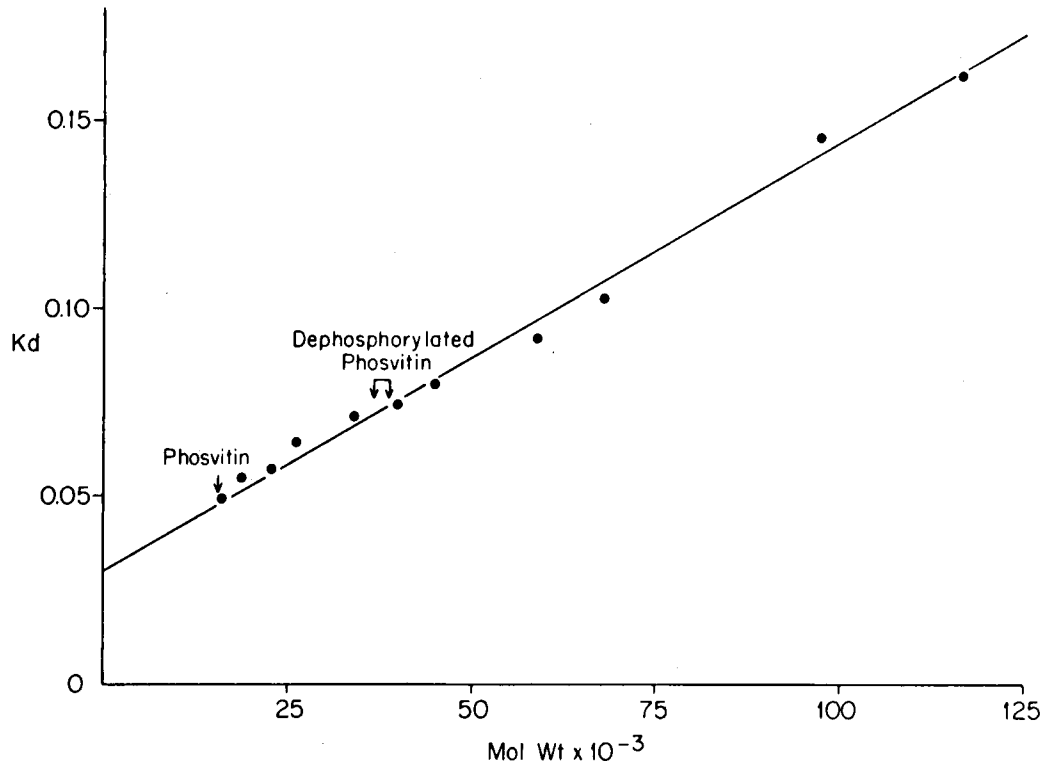

Figure 39. The Kd values of the standard proteins derived from the Ferguson analysis plotted against their molecular weights. The straight line through the points was derived by least-squares. Indicated is where the Kd values of phosvitin and dephosphorylated phosvitin fall on the graph.

configuration (92). I thus decided to use sedimentation equilibrium to derive the molecular weight of Pv. The result of this experiment is seen in Figure 40. The plot of log C versus r^2 is completely linear and shows no sign of aggregation. The molecular weight of the Pv sodium salt (92) derived by this method is 34,850, in fair agreement with the Ferguson plot analysis of dephosphorylated Pv. Subtracting the contribution of the sodium ions to the molecular weight of Pv gives a value for the molecular weight of native Pv of approximately 33,000.

Sedimentation equilibrium analysis of Pvt-II gave the results seen in Figure 41. The plot of log C versus r^2 is nonlinear and indicates a molecular weight range of between 9,500 and 14,500. This agrees fairly well with the values of 14,000-15,000 derived from SDS gel electrophoresis (personal observation). The molecular weight of Pvt-I was determined by SDS gel electrophoresis and found to be $\sim$19,000.

Analysis of the Yolk Proteins

The Xenopus yolk phosphoproteins were subjected to elemental analysis and the results are presented in Table VII. The values seen were corrected for moisture and sodium content. It is apparent that the proteins are quite different with respect to their phosphorus levels. The phosphorus level in Xenopus Pv is somewhat lower than the value found for Rana pipiens Pv (7.4 versus 9.3%), but the N/P ratio is lower than Rana Pv. Pvt-I has a considerably lower protein phosphorus content than Pv while Pvt-II has a somewhat higher level and also has a very low N/P ratio. LV_2 has a much lower protein phosphorus level than the other yolk phosphoproteins.

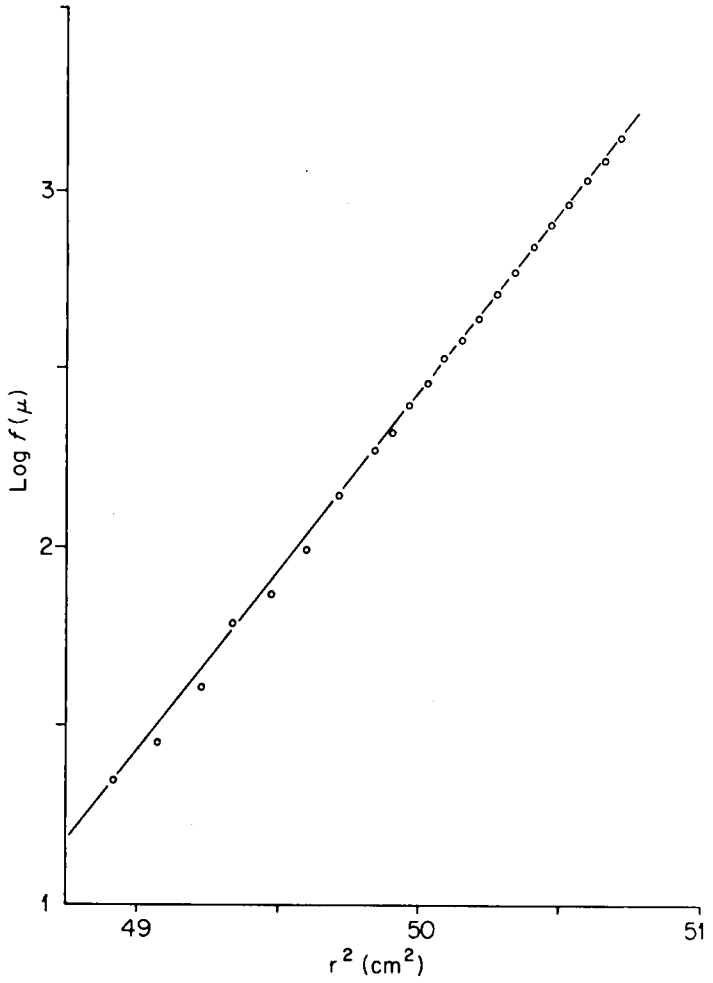

Figure 40. Sedimentation equilibrium analysis of Xenopus phosvitin. Phosvitin at an initial concentration of 0.2 mg/ml was dissolved in 0.5 M NaCl, 50 mM Hepes-OH, 5 mM EDTA. Equilibrium was achieved at 27,690 rpm and 20°.

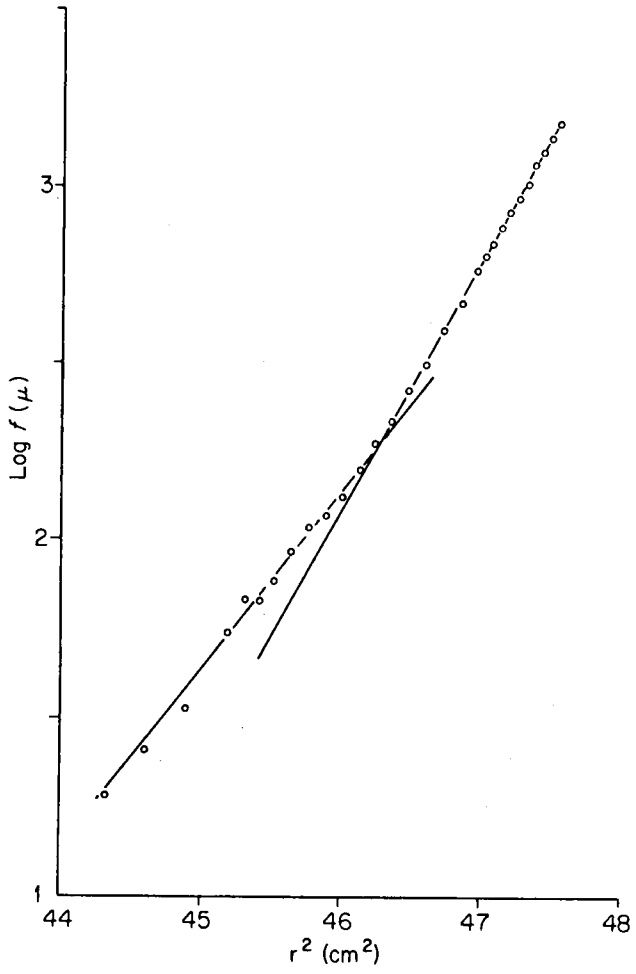

Figure 41. Sedimentation equilibrium analysis of Phosvette II. The protein was dissolved at an initial concentration of 0.2 mg/ml in 0.5 M NaCl, 50 mM Hepes-OH, 5 mM EDTA, pH 7.0. Equilibrium was achieved at 35,600 rpm at 20°C.

TABLE VII

CHEMICAL ANALYSIS OF YOLK PHOSPHOPROTEINS[a]

Protein	%C	%H	%N	%P	N/P[b]
PV	29.6	5.4	11.5	7.4	3.44
PVT-I	32	5.3	13.1	4.8	6.04
PVT-II	24.7	5.7	10.3	10.7	2.13
LV_2	---	---	---	1.2	---

[a]All values are corrected for the water and sodium content of the samples.

[b]The atomic ratio of Nitrogen to Phosphorus.

Amino acid analysis of the purified yolk proteins were performed as well as an analysis of several vitellogenin preparations. The results are seen in Table VIII. Also presented in Table VIII is the total of the amino acid contents of the different yolk proteins assuming that all are present in the yolk in an equimolar ratio. There are several noteworthy features of this analysis. My analysis of LV_1 and LV_2 agrees quite well with the values previously reported by Bergink and Wallace (16) and Ohlendorf et al. (57). However, my Pv preparation has a much higher methionine level than previously reported. Since my phosvitin preparation lacks 3 amino acids previously reported to occur in Xenopus Pv (threonine, valine and isoleucine), my preparations seem to be quite pure with respect to previous preparations. The high methionine level can best be ascribed to improved methodology in amino acid analysis. The amino acid analysis of Pvt-II is highly unusual since 6 amino acids are absent and also because of the very high serine and phosphoserine levels present. The presence of isoleucine in the preparation indicates the Pvt-II cannot be derived from Pv while the high phosphoserine level precludes its derivation from the other yolk proteins. The fractional amounts of isoleucine and valine observed is probably due to Pvt-II being a mixture of at least two proteins (Figure 41, page 129). Pvt-I also cannot be derived from Pv because it contains threonine, valine and isoleucine and cannot be derived from the other yolk proteins due to its high phosphoserine level. However, when the amino acid content of each of the yolk proteins are added together, they do not add up to the amounts found in vitellogenin.

TABLE VIII

AMINO ACID COMPOSITION OF VITELLOGENIN AND THE YOLK PROTEINS[a]

Amino Acid	LV_1	LV_2	PV	PVT I	PVT II	Sum of Yolk	VIT
ASX	78	24	16	12	5.1	135	156
THR	55	15	0	4.4	0	74	92
SER	66	14	13[b]	18	10	121	83
P-SER[c]	3	13	91	30	48	185	97
GLX	140	36	34	36	9.9	256	241
PRO	44	16	6.0	2.2	3.7	72	88
GLY	50	15	5.0	7.5	2.2	80	88
ALA	99	24	2.5	1.6	1.4	129	142
VAL	61	16	0	2.0	0.3	79	107
MET	28	8.2	3.9	2.6	0	43	44
ILE	59	12	0	1.4	0.5	73	85
LEU	99	18	2.9	1.1	0	121	148
TYR	30	12	2.1	0.5	0	45	53
PHE	45	11	1.9	1.7	0	60	65
HIS	30	7.2	13	11	1.5	63	63
LYS	74	22	25	20	6.4	147	140
ARG	55	17	19	12	7.0	110	91
CYS[d]	9	2	0	0	0	11	10

[a]The reported values are in grams per mole of protein using the following molecular weights: LV_1, 117,000. LV_2, 32,500. PV, 33,000. PVT-I, 19,000. PVT-II, 12,000. VIT, 200,000.

[b]Determined by the sulfite-addition technique (87).

[c]Calculated from the phosphorus content of each protein.

[d]Determined by DTNB titration (76).

The most obvious inconsistency is the values for serine and phosphoserine. This result implies that the yolk proteins are not all present at the same stoichiometric levels in the platelets.

Stoichiometry of the Yolk Proteins

In order to resolve the conflict in the amino acid data regarding the yolk proteins, I decided to determine the stoichiometry of the yolk proteins. It is known that LV_1 and LV_2 are present in a 1:1 ratio in the yolk platelets as determined by direct protein analysis (16). Since I have determined the phosphorus content of each of the yolk proteins and since I know approximately what the molecular weight of the proteins are, I can easily calculate the number of phosphate groups in each of the proteins. Now if I know what percentage of the total phosphorus in the yolk is associated with each of the proteins, I can calculate the ratio of the yolk proteins by the following representative equation:

$$\frac{LV_1}{Pv} = \frac{\%\ \text{total P in } LV_1}{MW_{LV_1} \times \%P_{LV_1}} \times \frac{MW_{Pv} \times \%P_{Pv}}{\%\ \text{total P in Pv}}$$

In order to determine the percentage of the total yolk protein phosphorus associated with each of the yolk proteins, I prepared ^{32}P-labeled yolk platelets and directly electrophoresed them on SDS gels at pH 8.0 in order to ensure that there was no alkaline dephosphorylation of the proteins during electrophoresis. The gels were then sliced and counted for ^{32}P. The result of this experiment is seen in Figure 42. I found that 61% of the protein-phosphorus of the platelets is associated with Pv while 14% and 21% of the radioactivity is associated with LV_2

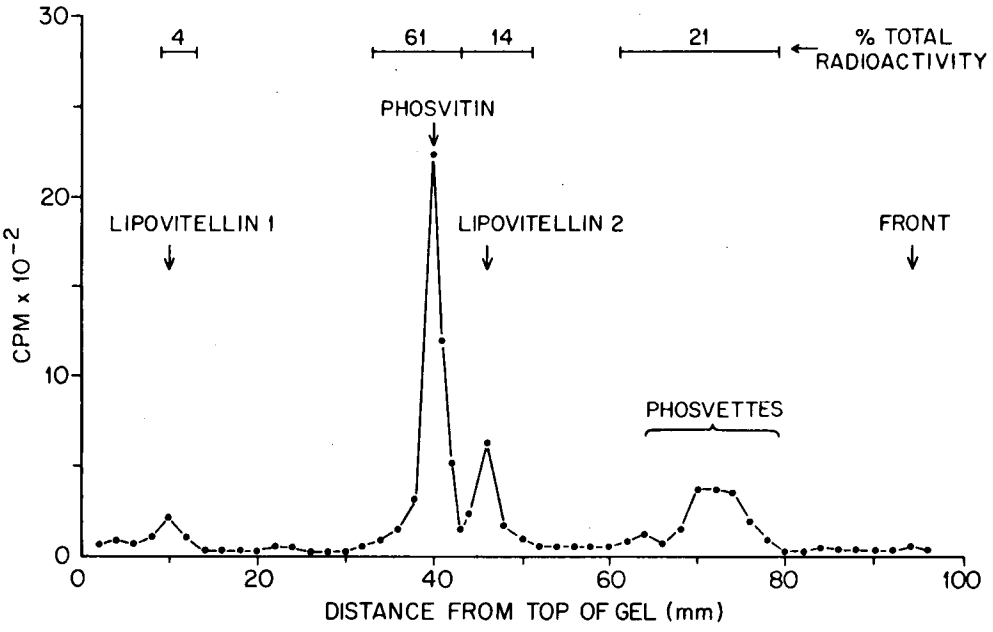

Figure 42. Ratio of protein phosphorus in the different yolk proteins. Yolk platelets labeled with ^{32}P in vivo were directly dissolved in 2% SDS, 10 mM Tris-Cl, 2 mM EDTA and 10 mM DTT at a concentration of ∿1 mg/ml. The proteins were electrophoresed using the Mono-Tris/Bicine gel system (pH 8.0). After fixation to remove the lipid, the gel was sliced and counted for radioactivity. Indicated are the migration position of the purified proteins on duplicate gels.

and the phosvettes respectively. Only ∿4% of the total is associated with LV_1. These values agree very well with those of Bergink and Wallace (16) who found that the ratio of ^{32}P in the yolk proteins was 1:0.22:0.35 for Pv:LV_2:phosphopeptides. This result confirms that the phosvettes are essentially the same small phosphopeptides described by Bergink and Wallace (16).

When the ratio of LV_2 to Pv was calculated, a ratio of ∿1:0.7 was obtained. This indicates that there is less than one Pv molecule for every LV_2 molecule. The ratio between the phosvettes and LV_2 is somewhat more difficult to ascertain since the stoichiometry between the phosvettes is not known. However, if the result in Figure 34, page 118, which shows that Pvt-I contains ∿23% of the total phosphorus is used then one can calculate that there is a Pvt-I:Pvt-II ratio of approximately 1:2 using the above equation. If the phosvettes are in a ratio of 1:2, then they have a combined molecular weight of ∿43,000 and an average phosphorus content of 8.1%. From these assumptions the ratio of LV_2 to phosvettes is 1:0.17. If one assumes that the phosvettes are present in a 1:1 ratio with respect to each other, then the ratio of LV_2 to phosvettes becomes 1:0.27. With either set of assumptions, it is apparent that Pv and the phosvettes are present in fractional amounts with respect to the lipovitellin molecules. Within the error associated with this type of analysis, the ratio of LV_2 to phosvitin plus the phosvettes is approximately 1:1. In view of the demonstrated heterogeneity of the vitellogenin precursor molecule (Chapter IV) and also considering the multiple forms of both LV_1 and LV_2, I propose

that one type of vitellogenin gives rise to LV_1, LV_2 and phosvitin while another type gives rise to LV_1, LV_2 and the phosvettes.

Cleavage of Vitellogenin in the Oocyte

An assumption implicit in the foregoing section was that all of the described yolk proteins were actually derived from vitellogenin. I decided to test this hypothesis directly by labeling vitellogenin in vitro to a high specific activity using [γ^{32}P]ATP and the Xenopus phosphoprotein kinase (see Chapter III). The labeled vitellogenin was isolated and then oocytes were incubated with the protein for 10 or 15 min after which they were transferred to unlabeled medium. After various time intervals the oocytes were fixed in acetone, dried and then dissolved in SDS prior to electrophoresis. It should be stated that there was no apparent loss of TCA-precipitable radioactivity during the chase. Figure 43 is an autoradiograph of the time course of cleavage of vitellogenin. Vitellogenin electrophoreses as a broad band at the top of the gel prior to the onset of cleavage. This broad band seems to be the result of the coelectrophoresis of the vitellogenin with large amounts of oocyte proteins since samples taken from the medium run as essentially a single sharp band (see Chapter III). There was no appreciable cleavage of vitellogenin until approximately 1.5 hr after its incorporation. In this particular experiment, the bands designated I, IV and V appear first with a very faint band preceding band V (see arrow). Band IV corresponds to the band position of LV_2. Between 2 and 4 hr the LV_1 band appears together with bands II and III. Band III corresponds to the position of Pv. Bands I, II and V persist until 6

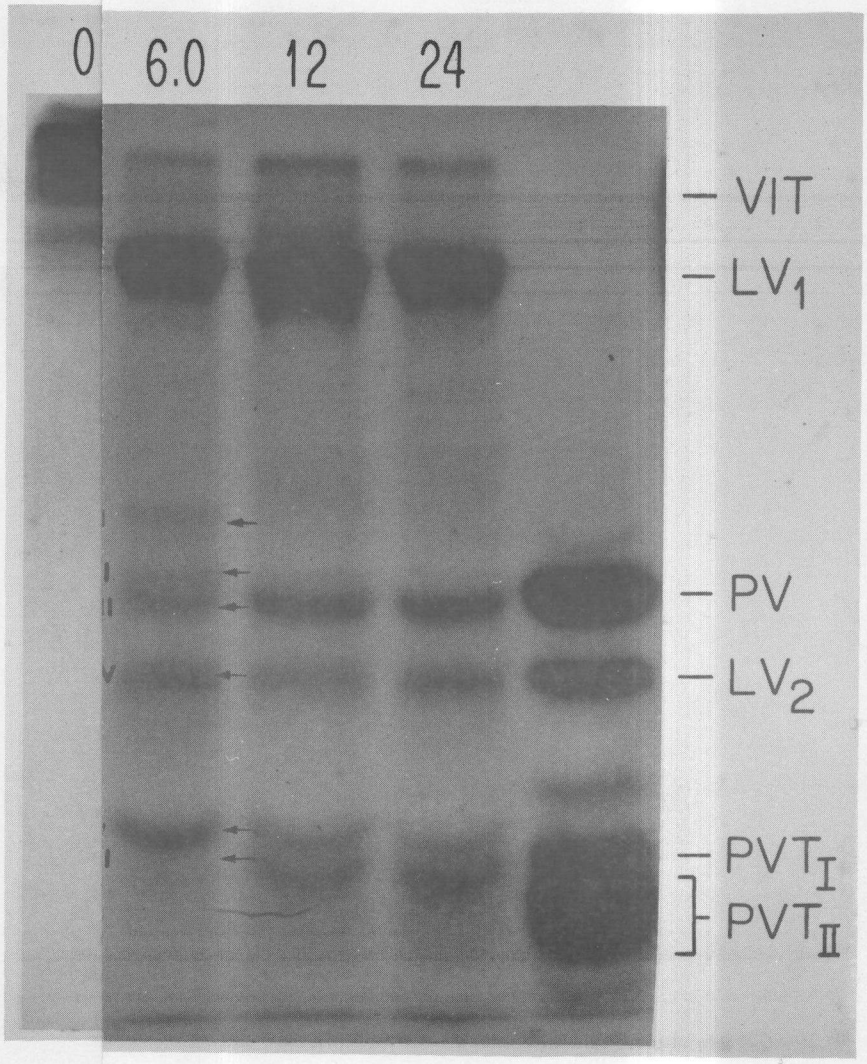

Figure 43. Time-course of cleavage of vitellogenin. Oocytes were pulsed for 10 min with [^{32}P]vitellogenin and chased for the number of hours indicated at the top of the figures. Oocytes were fixed in acetone and dissolved directly in SDS gel sample buffer. Each sample was diluted so that equal amounts of radioactivity was applied to each slot (∿3,000 cpm) and each lane contained approximately 1/10 of an oocyte. Shown is an autoradiogram of the gel. The last slot contains isolated [^{32}P] yolk proteins labeled in vivo. Also indicated is the migration position of the authentic yolk proteins determined by Coomassie blue staining of the gel.

hr after which they disappear. Note that the appearance of the last band (VI, which comigrates with Pvt-I) is correlated with the loss of band I. Also shown in the last slot at the right is a sample of *in vivo* labeled [^{32}P]yolk proteins.

The protein kinase does not label all vitellogenin preparations the same, probably due either to differences in batches of the phosphoprotein kinase or to differences in the vitellogenin. Likewise, the time course of appearance of the different bands is not always the same, but a comparison of a number of experiments has proved illuminating. One batch of vitellogenin was labeled almost exclusively in the Pv region of the molecule with minor labeling in band I. This experiment showed that band III was the only band to appear except for a faint band I. The disappearance of band I corresponded exactly with the appearance of band VI (the Pvt-I band). Other time-course-of-cleavage experiments showed that the LV_2 band never appeared after the Pv, and often preceded it. If oocytes are chased long enough, band V will completely disappear and Pvt-II then appears. The appearance of the LV_1 band is always correlated with the appearance of band II and the Pv. Band II always seems to disappear with time.

There are several conclusions that can be made from these studies. The most important one is that the phosvettes are indeed derived from vitellogenin. However, the time course of their appearance is different from that of Pv. This result strongly supports the suggestion that the phosvettes are derived from a different type of vitellogenin than Pv. Band I seems to be a precursor form of Pvt-I while band V seems to be

a precursor of Pvt-II. The faint band just above band V is somewhat of a mystery, for it is found in the in vivo labeled yolk proteins and copurifies with Pvt-II (see Figure 18, page 59); it may be another precursor form of Pvt-II. Band II is not always seen, but its very close position to Pv (∿2,000 MW higher) suggests that it may be a Pv precursor. Note that during the cleavage of vitellogenin a small amount of label appears at the front of the gel. This could represent small peptides split off the precursor molecules. Band I has a molecular weight of ∿46,000. If it is indeed a precursor of Pvt-I, which appears likely, then another molecule of ∿27,000 should appear. Since I have found that band I is not a precursor of Pv, the only other likely candidate is LV_2. In order to simplify the confusion of bands that appear in Figure 43, page 137, I have provided a hypothetical scheme in Figure 44 that accounts for the appearance of the various bands during vitellogenin processing.

The Placement of LV_2 in the Vitellogenin Molecule

In Chapter IV, I showed that cleavage of vitellogenin at the cysteine residues resulted in two large phosphopeptides that contained virtually all of the protein phosphorus of vitellogenin. I argued that it had to be an internal sequence of vitellogenin since both Pv and LV_2 are contiguous parts of vitellogenin (16) and the large phosphopeptides obtained could only have resulted from the placement of phosvitin between the LV_1 and LV_2 regions of vitellogenin. However, the direct assignment of the C-terminal and N-terminal to the different yolk proteins would be a more desirable approach. In an effort to

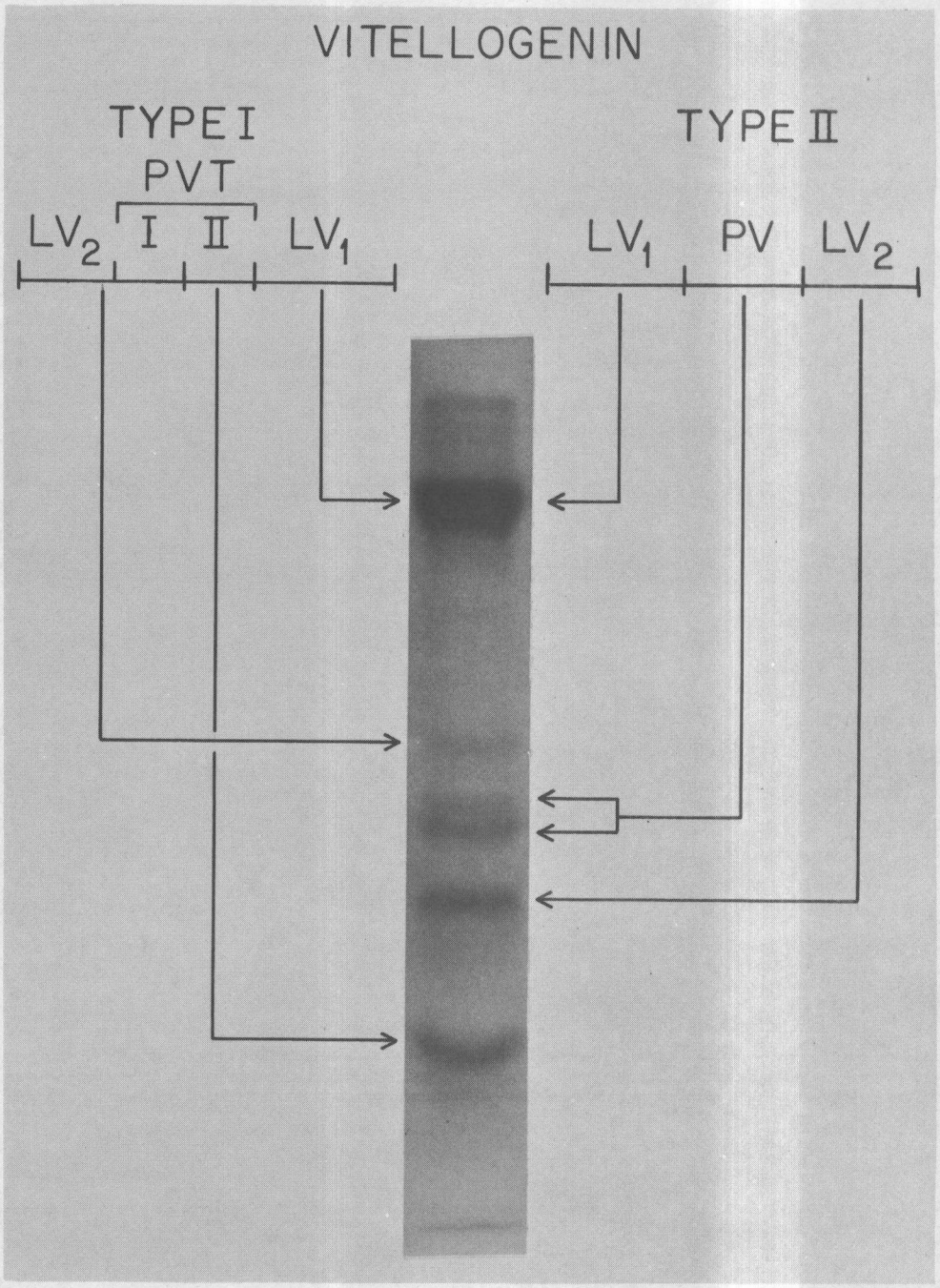

Figure 44. Hypothetical scheme to account for the appearance of intermediate peptides during cleavage of the yolk proteins.

resolve this problem, I determined the N-terminal amino acids of vitellogenin, the two lipovitellins and Pv by the dansyl chloride technique (88). The result of this analysis showed that the N-terminal amino acid of vitellogenin and LV_1 was aspartic acid while the N-terminal of LV_2 was phenylalanine. Pv did not yield an N-terminal amino acid by this technique. It thus seems that LV_1 is derived from the N-terminal region of the vitellogenin molecule.

Analysis of the C-terminals of vitellogenin and the yolk proteins proved to be more difficult than the N-terminal determinations. Direct analysis of the C-terminal was unsuccessful due to the extreme insolubility of the denatured proteins in the solvents that are required for carboxypeptidase digestion. This unfortunately forced me to use an indirect approach. The method that I used was to cleave vitellogenin and the yolk proteins at their methionine residues with cyanogen bromide (CNBr). Theoretically, CNBr cleavage of vitellogenin and the total yolk proteins should yield an identical set of peptides except where the yolk peptides were interrupted by a yolk protein cleavage site. In other words, every peptide derived from a CNBr digest of LV_2 should have an exact correspondence with peptides derived from CNBr cleavage of vitellogenin except for the terminal peptide that is unique to LV_2 due to its formation during the proteolytic processing of vitellogenin. If there is only one unique LV_2 peptide, then there was only one proteolytic event responsible for its derivation from vitellogenin. An internally derived yolk protein must of necessity give rise to at least two peptides after CNBr digestion that have no

correspondence with the peptides derived from CNBr digestion of vitellogenin. However, this type of analysis depends upon being able to distinguish vitellogenin peptides as being derived from a particular yolk protein. Since the yolk proteins vary considerably in their phosphorus content, this was the distinguishing criterion that I used.

^{32}P-labeled yolk proteins and [^{32}P]vitellogenin were isolated and cleaved with CNBr. The resulting peptides were then electrophoresed on a slab gel at pH 8.0 using the Mono-tris/Bicine SDS gel system. The autoradiogram of one of these gels is seen in Figure 45. [^{32}P]Pv gives rise to a characteristic doublet series on Mono-tris/Bicine gels (slot 3, Figure 45). The slightly slower mobility of the uncleaved Pv (in slot 3) has been seen in several experiments and may represent a second form of Pv that lacks cleavable methionine residues. Cleaved LV_2 (slots 1 and 7) gives rise to a series of closely spaced ^{32}P-containing bands. Vitellogenin does indeed give a series of labeled bands having the same mobility as most of the labeled LV_2 bands (slots 6 and 7). However, note in Figure 45 that there is a single part of the LV_2 pattern that is missing from the vitellogenin pattern (indicated by the arrow in slot 7). This may include the LV_2/Pv cleavage site. The Pv/phosvettes parts of the vitellogenin molecule (determined by their high ^{32}P content) migrate as a series of high molecular weight peptides (slots 2 and 6, Figure 45). The main ^{32}P-containing band has a molecular weight of $\sim$65,000 and stains well with Coomassie blue, indicating that it contains Pv and part of a lipovitellin molecule (probably LV_1). However, my results are complicated by the migration of a Pv cleavage fragment very close to the position

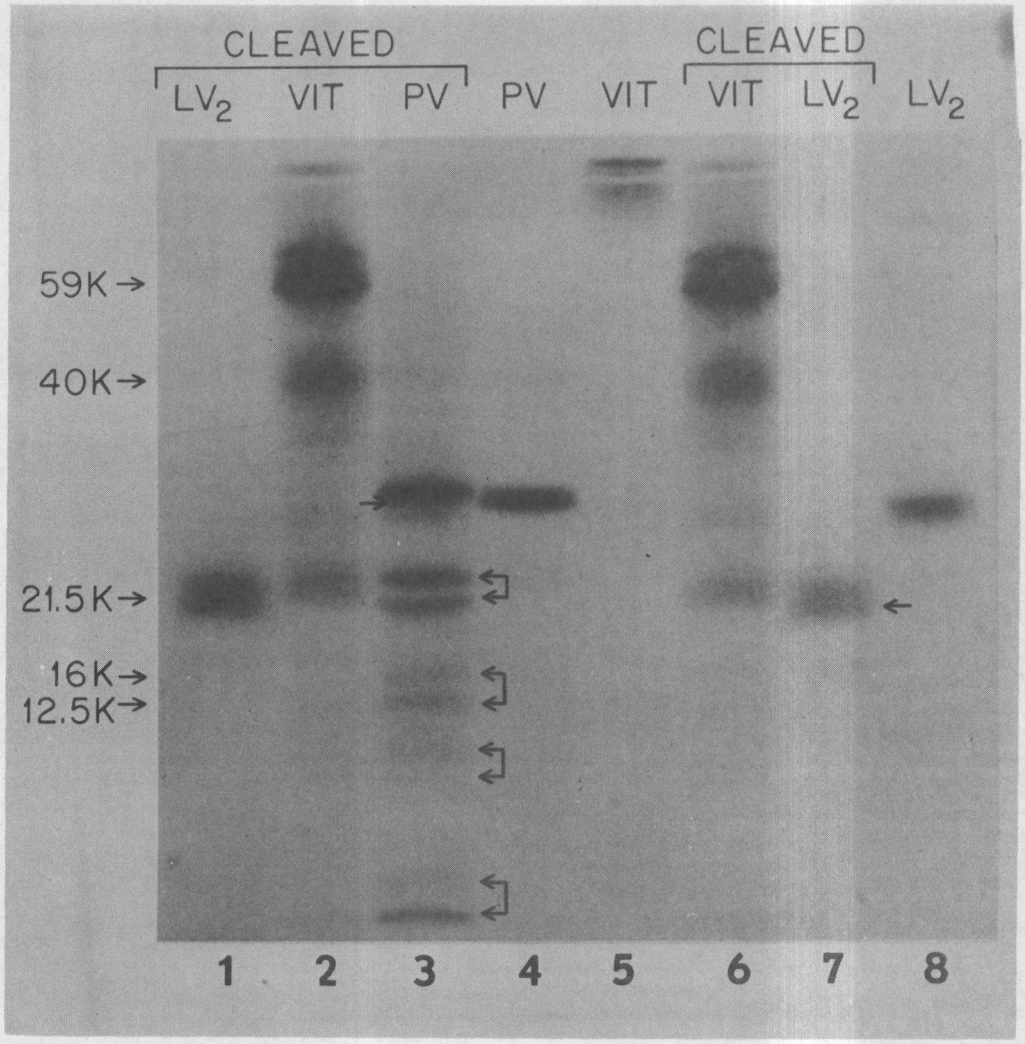

Figure 45. Cleavage of vitellogenin and the yolk proteins by CNBr. The ^{32}P-labeled proteins were isolated and carboxymethylated prior to cleavage with CNBr as outlined in Materials and Methods. The samples of cleaved (slots 1, 2, 3, 6, 7) and noncleaved proteins (slots 4, 5, 8) were electrophoresed on a slab gel using the Mono-tris/Bicine system. Note the faint band appearing below the CNBr cleaved phosvitin (Pv) band in slot 3 and the sets of doublets indicated by the arrows. At the left is indicated the migration position of the molecular weight markers.

of the LV_2 cleavage fragments (compare slots 1 and 3 in Figure 45). In order to ensure that the vitellogenin derived peptides that comigrate with the LV_2 derived peptides are indeed the same, I took advantage of the anomalous behavior of Pv on SDS gels at a high pH (9.58). By selecting a 12% gel, I was able to shift the Pv peptides away from the migration position of the LV_2 peptides. For this experiment I used tube gels and sliced and counted the gels to determine the position of the phosphopeptides derived from the CNBr cleavage of all of the yolk proteins. The results are presented in Figure 46. It can be seen that there are now no other phosphopeptides migrating at the position of the LV_2 phosphopeptides. CNBr-cleaved vitellogenin again gives rise to a peak of radioactivity that comigrates with the LV_2 peptides (designated IV_V in Figure 46). The amount of radioactivity associated with the apparent LV_2 peptides derived from vitellogenin is approximately 15% of the total vitellogenin radioactivity, in good agreement with the finding that $\sim$14% of the ^{32}P in vitellogenin is associated with the LV_2 portion of the molecule (see Figure 42, page 134). The Pv/phosphette region of the vitellogenin molecule again migrates as high molecular weight peptides, and thus must contain part of the LV_1 portion of the vitellogenin molecule. Thus the CNBr cleavage pattern of LV_2 fits the requirements of a molecule derived by a single cleavage event from vitellogenin, while the Pv/phosvettes cleavage pattern fits the requirements for molecules that are derived by multiple cleavages of vitellogenin. From this experiment I can conclude that LV_2 is indeed

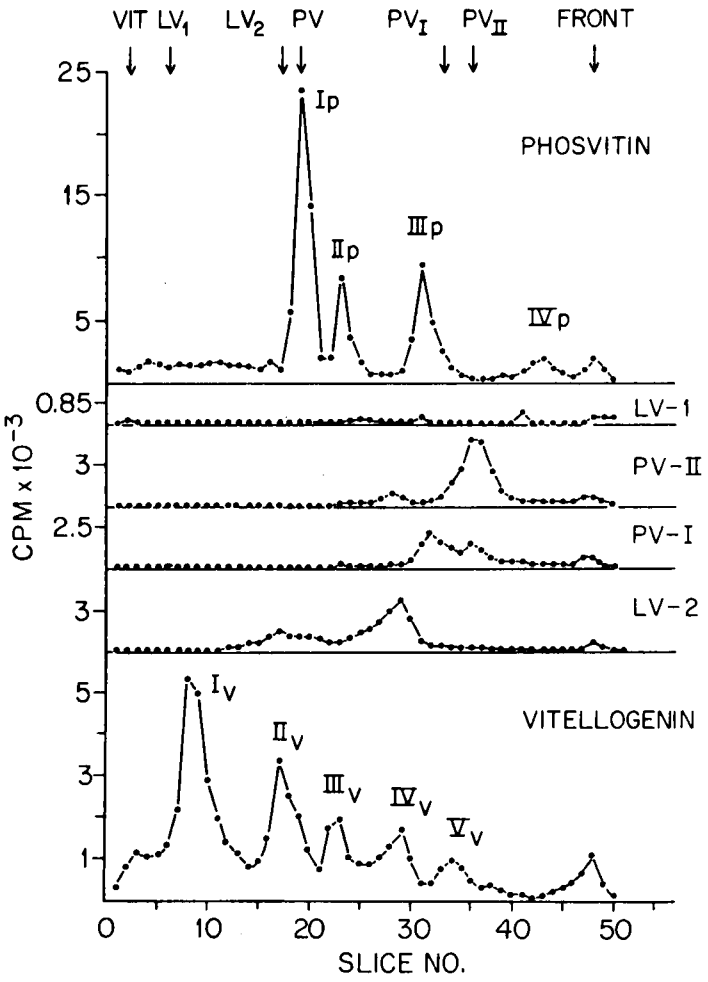

Figure 46. Cleavage of vitellogenin and all yolk proteins by CNBr. Purifed sample of each of the ^{32}P-labeled yolk proteins were cleaved with CNBr as described in Materials and Methods. They were then electrophoresed on 12% T, 2% C gels at pH 9.58 (27). The gels were sliced into 1 mm segments and counted for the presence of ^{32}P. The relative scales of the radioactivity in the different proteins have been adjusted so as to reflect the relative proportion of ^{32}P from vitellogenin that is associated with each of the yolk proteins. Indicated at the top of the figure is the migration position of the uncleaved proteins. All of the yolk proteins were cleaved to some extent with CNBr with the exception of phosvette II. Abbreviations used are VIT (vitellogenin), LV_1 (lipovitellin 1), LV_2 (lipovitellin 2), PV (phosvitin), PVI (phosvette I) and PVII (phosvette II).

the C-terminal region of the vitellogenin molecule and thus the Pv/phosvette region of vitellogenin is flanked by LV_1 on the N-terminal and LV_2 on the C-terminal.

Placement of Disulfide Bonds on Vitellogenin

During my attempts to determine the C-terminal amino acids of vitellogenin and the yolk proteins by carboxypeptidase digestion, I became acutely aware of the peculiar solubility properties of the different proteins. While vitellogenin is very water soluble when in a native state (up to 160 mg/ml), when it is denatured by delipidation or performic acid oxidation, it becomes extremely insoluble. At neutral pH, it is insoluble in 8 M urea and only slightly soluble in 1% SDS. LV_1 and LV_2 are also insoluble in 8 M urea and slightly soluble in 1% SDS solutions at a concentration of only 2 mg/ml). On the other hand, denatured Pv is readily soluble in water or other buffers at a neutral pH. It thus occurred to me that the position of the Pv moiety on the vitellogenin molecule may be involved in the maintenance of the solubility of the yolk precursor.

While I was developing a method for labeling vitellogenin in vitro with the phosphoprotein kinase, I noticed that the addition of the reducing agent DTT to solutions of vitellogenin resulted in the precipitation of the protein in 0.1 M KCl. The vitellogenin could be redissolved by dialysis against distilled water or the addition of high concentrations of salt (1 M KCl). Both of these treatments will also dissolve lipovitellin (15). Thus it seemed that the reduction of

the disulfide bridges on vitellogenin converted it to an insoluble analog of lipovitellin. Since the disulfide bonds of vitellogenin seemed to be such an important structural feature of the molecule, I decided to investigate the placement of these structures in vitellogenin.

The cysteine groups of both reduced and nonreduced vitellogenin were determined under denaturing and nondenaturing conditions by DTNB titration. The results are seen in Table IX. DTNB titration of vitellogenin revealed that there was less than one cysteine group exposed for each vitellogenin monomer. Denatured vitellogenin revealed the presence of about 6 residues for each vitellogenin monomer. Thus the free cysteine groups in vitellogenin are "buried." The titration of reduced-denatured vitellogenin gave a total value of approximately 10 or about 4 greater than unreduced vitellogenin. This indicates that there are two disulfide bridges in each vitellogenin peptide. An interesting result was obtained for reduced native vitellogenin. Instead of obtaining the value of 4 expected if just the cysteines involved in disulfide bond formation were exposed, a total of 8 was obtained. This indicates that breaking the disulfide bonds of vitellogenin not only exposes the cysteine groups involved in the bonds, but also "opens up" the molecule and allows the buried cysteine groups to be exposed. The disulfide bonds of vitellogenin are thus essential structural features of the molecule.

In an effort to determine what part of the vitellogenin molecule contains the disulfide bridges, reduced and nonreduced yolk proteins were prepared. Pv and the phosvettes contained no detectable cysteine

TABLE IX

DETERMINATION OF NUMBER OF SULFYLHYDRAL GROUPS ON VITELLOGENIN

Protein Treatment	Thiol/mole Peptide
Native	0.15
Denatured	5.8
Reduced-Native	8.0
Reduced-Denatured	10.2

Solvent was 50mM Tris-Cl, 10mM EDTA, pH 8.0. Two percent SDS was used as the denaturant and DTT was used as the reducing agent as described in Materials and Methods.

groups. Reduction of lipovitellin was not necessary to isolate the LV_1 and LV_2 subunits [also observed by Bergink and Wallace (16)] and so no disulfide bonds bridge the two. There was less than one free cysteine in LV_2 ($\sim$0.2) and 3 free cysteine groups in LV_1. However, reduced LV_2 had about 2 cysteines (1.8) while reduced LV_1 had 9 ($\sim$8.7). Thus LV_1 and LV_2 both contain disulfide bridges. I therefore conclude from this experiment that both the LV_1 and LV_2 regions of the vitellogenin molecule contain intrachain disulfide bonds.

Discussion

The purpose of this study was to examine the structure of vitellogenin by an analysis of its normal cleavage products, the yolk proteins. In the previous chapter (Chapter IV) I attempted to demonstrate that vitellogenin is a heterogeneous population of molecules. In this chapter, I have shown that the same can be said for the yolk proteins. The bewildering complexity observed in the yolk proteins is probably due to each vitellogenin giving rise to a distinct set of yolk proteins.

In order to make some sense out of my data, it was necessary to make several simplifying assumptions concerning the classification of the yolk proteins. Thus I assume that all of the LV_1 peptides and all of the LV_2 peptides are homologs of each other, differing only in their derivation from different parent vitellogenin molecules. The bulk properties of each of the subunit classes were compared with the bulk properties of the vitellogenin population even though this approach is somewhat unorthodox. However, each class of lipovitellin peptides

seems to share similar properties, such as molecular weight and the presence or absence of phosphate, so my assumptions seem reasonable. A different problem was encountered in assigning Pv and the phosvettes to the same class as was done with the lipovitellins, since their molecular weights were so different. When I first purified Pvt-I and Pvt-II, I assumed that they were derived from a different portion of the vitellogenin molecule than Pv and the lipovitellins. This scheme had each vitellogenin molecule giving rise to an LV_1 subunit, an LV_2 subunit, a Pv molecule and the phosvette molecules. The total molecular weight of all of these proteins was ∿225,000, which was somewhat larger than the maximum molecular weight of 200,000 for the vitellogenin monomer (16). However, a great inconsistency in the data was that vitellogenin would have to contain 2.8% phosphorus in order to accommodate all of these proteins in the same molecule, while the determined value of the phosphorus level in Xenopus vitellogenin is between 1.4-1.5% (14). Since the yolk proteins do not undergo phosphorylation during or after their deposition (2), Pv and the phosvettes clearly cannot be derived from the same parent molecule. My data on the stoichiometry between the LV_2 subunit and Pv and the phosvettes indicate that a minor fraction of the vitellogenin population (20-30%) gives rise to the phosvettes instead of Pv. This is evidence that at least one form of vitellogenin differs in the "Pv" region of the molecule. While it might be argued that the Pv region of a single vitellogenin molecule could be cleaved to form either Pv or the phosvettes, the amino acid analyses of these proteins show that the phosvettes are not simply

a Pv molecule that has been split at additional sites. The time course of cleavage of [^{32}P]vitellogenin in the oocytes (Figure 43, page 137) also shows that the time of appearance of the phosvettes and Pv are not the same. In addition, the phosvettes seem to first appear as precursor molecules that are further cleaved with time, while Pv always seems to appear in a mature or a very slightly higher molecular weight precursor. While only direct sequence analysis can completely rule out the derivation of Pv and the phosvettes from the same molecule, I feel that the likeliest explanation is that Pv and the phosvettes are derived from similar regions of distinct vitellogenin molecules.

The three molecular weight forms of LV_1 and LV_2 are also probably derived from distinct vitellogenin precursors, although there is little data to support this contention. However, the fact that the 3 molecular weight forms of vitellogenin give rise to 3 molecular weight forms of both LV_1 and LV_2 is illuminating. Since I have found that LV_1 is the N-terminal of the vitellogenin molecule and LV_2 contains the C-terminal region, it is plausible that the observed molecular weight heterogeneity of the lipovitellins is a result of differential cleavage of both ends of a common precursor molecule. However, since Wahli et al. (58) have demonstrated that there are at least 4 different mRNAs encoding for the vitellogenin molecule, the simplest explanation for the heterogeneity in both the lipovitellin and the Pv/phosvette region of the molecule is that it is simply a reflection of the processing of a number of related but distinct vitellogenin molecules.

In Chapter IV I presented data (NTCB cleavage) that indicated that Pv/phosvette was derived from a position in the vitellogenin molecule

between the LV_1 and LV_2 regions. My data on the CNBr cleavage of vitellogenin and the yolk proteins completely supports this hypothesis. The intercalation of a highly phosphorylated region between the two lipovitellin domains has interesting structural implications. LV_1 and LV_2 bind very tightly to each other and gel filtration in 6M GuHCl does not dissociate them (16). Pv complexes with the lipovitellins at neutral pH and low salt, but it is easily dissociated with high salt (33) or high pH (26). Thus one can envision the vitellogenin molecule as consisting of a core particle of the lipovitellins and a "loop" of phosphoprotein. It seems to be this loop of Pv/phosvette that is split off during the processing of vitellogenin in the oocyte. The conformation of the lipovitellin core particle is also important in maintaining the solubility of the vitellogenin, since the reduction of the disulfide bonds results in the precipitation of vitellogenin. Thus the conversion of soluble vitellogenin into the insoluble yolk proteins would seem to essentially consist of the exposure of the lipovitellin core particle by the removal of a phosphate shell or loop from the molecule.

CHAPTER VI

CONCLUDING REMARKS

The aim of this thesis project was to understand the structural features of vitellogenin that permit this readily soluble serum protein to be converted into the insoluble yolk proteins. The major complicating factor of the project was that vitellogenin is not a single molecule, but is instead a collection of related molecules. However, the heterogeneous vitellogenin population is converted into a heterogeneous yolk protein population, all of which share the same property of existing as an insoluble complex in the oocyte. Thus the structural feature(s) of vitellogenin that allows it to be transformed from a soluble form to an insoluble form seems to be common to all of the types of vitellogenin molecules. In seeking to understand this process, a realization of the similarities between the properties of vitellogenin and the yolk proteins is essential.

The bulk of the vitellogenin molecule is destined to become lipovitellin. Under physiological conditions, lipovitellin is a large (approximately 400,000 MW) protein that exists as a dimer. The vitellogenin molecule is somewhat larger (approximately 460,000 MW) and is also a dimer. It thus seems plausible that the two vitellogenin monomers bind in the same region as do the lipovitellin monomers. This would place the phosvitin part of the vitellogenin molecule on the "outside" of the dimer. Indeed there is reason to believe that phosvitin is on the outside of the vitellogenin molecule.

As an example, it has been known for many years that one can precipitate phosvitin from solution by the addition of divalent cations (92) due to the "crosslinking" of neighboring molecules. As I discussed in Chapter II, this is also true for vitellogenin. Native gel electrophoresis of vitellogenin also indicates that phosvitin is on the outside of the vitellogenin molecule. In Figure 24, page 85), I show a Ferguson plot of vitellogenin monomers and dimers. Note that the free electrophoretic mobility of the vitellogenin dimer (Y-intercept) is almost exactly twice that of the vitellogenin monomer. The free electrophoretic mobility of a protein is determined by the surface charge of that protein. If the negative surface charge of vitellogenin was evenly distributed, then the dimer should have some of the charged groups masked. For an ideal spherical protein, the surface area is a function of the square of the diameter while the volume is a function of the cube of its diameter. If it combines into an ideal dimer, its volume will be double the monomer volume, but its surface area will be increased by only a factor of 1.59. Dimers of some proteins, observed during gel electrophoresis do follow this rule (95). The fact that the vitellogenin dimer has twice the free electrophoretic mobility as the monomer indicates that the highly negatively charged region of vitellogenin (phosvitin) is as fully exposed in the dimer as in the monomer.

It is very tempting to speculate that the phosvitin on the outside of the vitellogenin imparts solubility to the molecule. It is certainly true that isolated phosvitin is a very soluble protein while lipovitellin is not. Since lipovitellin is a lipoprotein (approximately 20% lipid)

and phosvitin is a very hydrophillic protein, it is also tempting to speculate that phosvitin is a hydrophillic "shell" that surrounds the hydrophobic lipovitellin core protein. However, these are very simplistic models that do not take into account the exact reason that lipovitellin is insoluble in normal aqueous solutions.

Although lipovitellin contains a large amount of lipid, its interaction with other lipovitellin molecules does not seem to be of a hydrophobic nature. The yolk proteins, or purified lipovitellin, can be easily dissolved in 1 M NaCl (35), an observation clearly not compatible with a proposed hydrophobic interaction, but is instead indicative of an ionic interaction. High pH (greater than 9.5) or low pH (lesser than 4.0) also dissolve lipovitellin aggregates (26) (also personal observation), which again indicates an ionic interaction. When the yolk platelets are dissolved in high salt, the phosvitin disassociates from the lipovitellin (15). However, when the solution is dialyzed against water the phosvitin reassociates with lipovitellin (33). Since phosvitin is almost completely devoid of hydrophobic amino acid residues (Chapter V), this is clearly not a hydrophobic interaction. It seems that the two lipovitellin subunits (LV_1 and LV_2) do indeed associate via hydrophobic interactions since detergents, but not 6 M GuHCl, will disassociate them. On the other hand, the major aspect of lipovitellin that seems responsible for its relative insolubility is its ionic nature.

Where does this lead us regarding the role of phosvitin in maintaining the solubility of vitellogenin? Clearly the presence of

phosvitin associated with lipovitellin per se is not sufficient since a lipovitellin-phosvitin complex is virtually as insoluble as lipovitellin alone (26). It thus seems that it is the placement of the phosvitin region on the core lipovitellin region of vitellogenin that is of major importance. My structural studies on vitellogenin (Chapter V) indicated that the phosvitin region of vitellogenin was intercalated between the LV_1 region and the LV_2 region of the molecule. Since LV_1 and LV_2 are very tightly associated with each other (16), it seems evident that this would place severe constraints on the positioning of the phosvitin region. It is probably this exact placement of the phosvitin region that prevents the interaction and aggregation of vitellogenin molecules. This idea is supported by my finding that the reduction of the disulfide bonds of vitellogenin results in the vitellogenin molecule taking on many of the solubility properties of lipovitellin (Chapter V). Since the disulfide bonds are found only in the lipovitellin regions of vitellogenin, I envision that the reduction of these bonds allows the two "anchoring" points of phosvitin to shift to such that it assumes a more thermodynamically favorable position on the vitellogenin molecule. The now exposed lipovitellin association sites would then allow the vitellogenin molecules to aggregate and precipitate. I propose that a very similar event occurs during the processing of vitellogenin into the yolk proteins. In this scheme, the conversion process essentially consists of the splitting of the two phosvitin anchoring points by intraoocyte proteases. The "released" phosvitin would then shift to a more favorable association site on the lipovitellin molecule. This

hypothesis also accounts for the existence of two lipovitellin subunits. An interesting observation is that the chicken lipovitellins also possess large and small subunits (96) and I have recently found the same to be true for goldfish lipovitellin. This should be a common structural feature of all vertebrate lipovitellins.

The question I have tried to answer here is what are the structural features of vitellogenin that allow for its conversion into the yolk proteins? The answer seems to be that vitellogenin has on its surface a highly phosphorylated region (phosvitin/phosvettes) that is anchored into a specific conformation by two flanking regions (LV_1 and LV_2) that are in tight association. The phosphorylated region could thus mask the association sites of the core lipovitellin particle. The conversion of vitellogenin into the yolk proteins is a process whereby the placement on the surface of this phosphorylated region is proteolytically altered so as to allow the formation of the insoluble yolk complex.

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APPENDICES

APPENDIX A

APPENDIX A

THE INTERACTION OF VITELLOGENIN WITH DIVALENT CATIONS

Xenopus vitellogenin normally exists in the serum as the calcium salt. It has been shown that estrogen treatment of Xenopus results in an increase in the serum calcium level as well as the serum protein concentration. Direct evidence linking this increase in serum calcium levels to vitellogenin was provided by Redshaw and Follett (20) and Ansari et al. (21) who isolated vitellogenin from the serum of estrogen-treated animal by gentle methods and found that a sizeable amount of the calcium in the serum was associated with the vitellogenin molecule. Wallace (14) provided evidence that the increase in serum calcium levels due to estrogen treatment were exactly correlated with the increase in the vitellogenin concentration of the serum. He showed that there was an increase of 1.01 calcium ions for every additional protein phosphorus group contributed by vitellogenin in the serum. This result implied that the phosphate groups of vitellogenin existed in the serum almost exclusively as the calcium salt. However, the association of vitellogenin with calcium does not seem to be a strong one as gel filtration or cellulose chromatography of the serum removes the calcium from the vitellogenin (14).

These observations came to my attention as I attempted to label vitellogenin <u>in vitro</u> with [γ-^{32}P]ATP using the protein kinase I isolated from Xenopus liver (see Chapter III). The protein kinase requires Mg^{2+} as a cofactor, but I observed that the addition of Mg^{2+}

to solutions of purified vitellogenin almost always resulted in the precipitation of the vitellogenin. It seemed odd that although vitellogenin could exist as a soluble divalent cation salt in the serum, it was insoluble when in a purified form. Since the labeling efficiency of precipitated vitellogenin was rather low, I investigated the effects of divalent cations on the solubility of vitellogenin in an effort to circumvent this problem.

As a first step, I added various amounts of $CaCl_2$ to DEAE-cellulose purified vitellogenin. The results are seen in Figure 47. The added calcium had no effect on the solubility of vitellogenin until a molar ratio of Ca^{2+}:protein phosphorus of greater than 0.5 had been reached. Above this ratio vitellogenin became insoluble, but then redissolved as more calcium was added. Since the Ca^{2+}:protein phosphorus ratio in the serum from estrogen-treated animals is approximately 1:1 (14), vitellogenin should be insoluble in the serum.

I studied this phenomenon in more detail by performing a Scatchard analysis of $^{45}Ca^{2+}$ binding to vitellogenin using the method of Womack and Colowick (97). The results are seen in Figure 48. For an appreciation of this figure, it should be noted that vitellogenin binds approximately 180 calcium ions in the serum (14). Figure 48 shows that there is an initial high affinity binding of Ca^{2+} to vitellogenin (Kd approximately 6×10^{-5}M) until approximately 15 calcium ions are bound. The curve then levels off until ∿40 calcium ions are bound, after which the curve again slopes downward, indicating a higher association constant for Ca^{2+} and vitellogenin. At the point that the curve breaks downward,

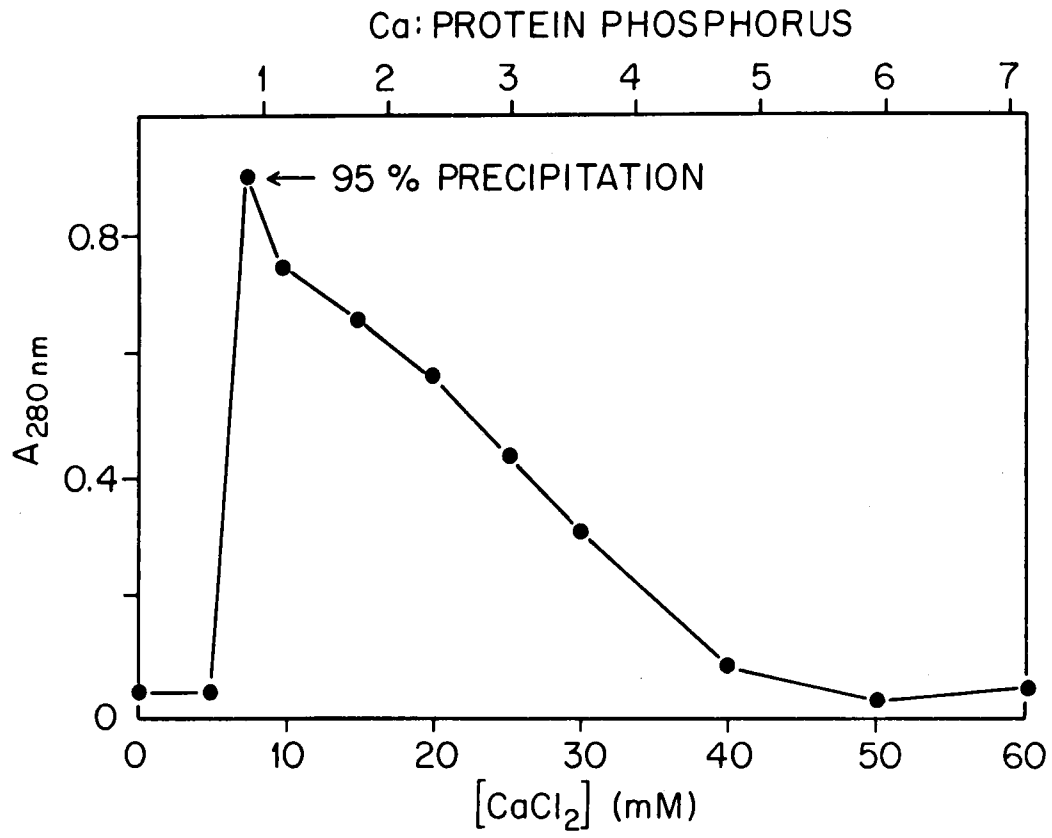

Figure 47. Precipitation of vitellogenin by $CaCl_2$. Vitellogenin was purified by DEAE-cellulose chromatography and was diluted to a concentration of 20 mg/ml in 50 mM Tris-Cl, pH 7.5. Different amounts of a 0.5 M stock solution of $CaCl_2$ was added to bring the concentration to the indicated levels. After 10 min incubation at 20°, any formed precipitate was removed by centrifugation, dissolved in 1 M NaCl and assayed for absorbance at 280nm. The Ca:protein phosphorus values were calculated using a protein phosphorus value for vitellogenin of 1.45%.

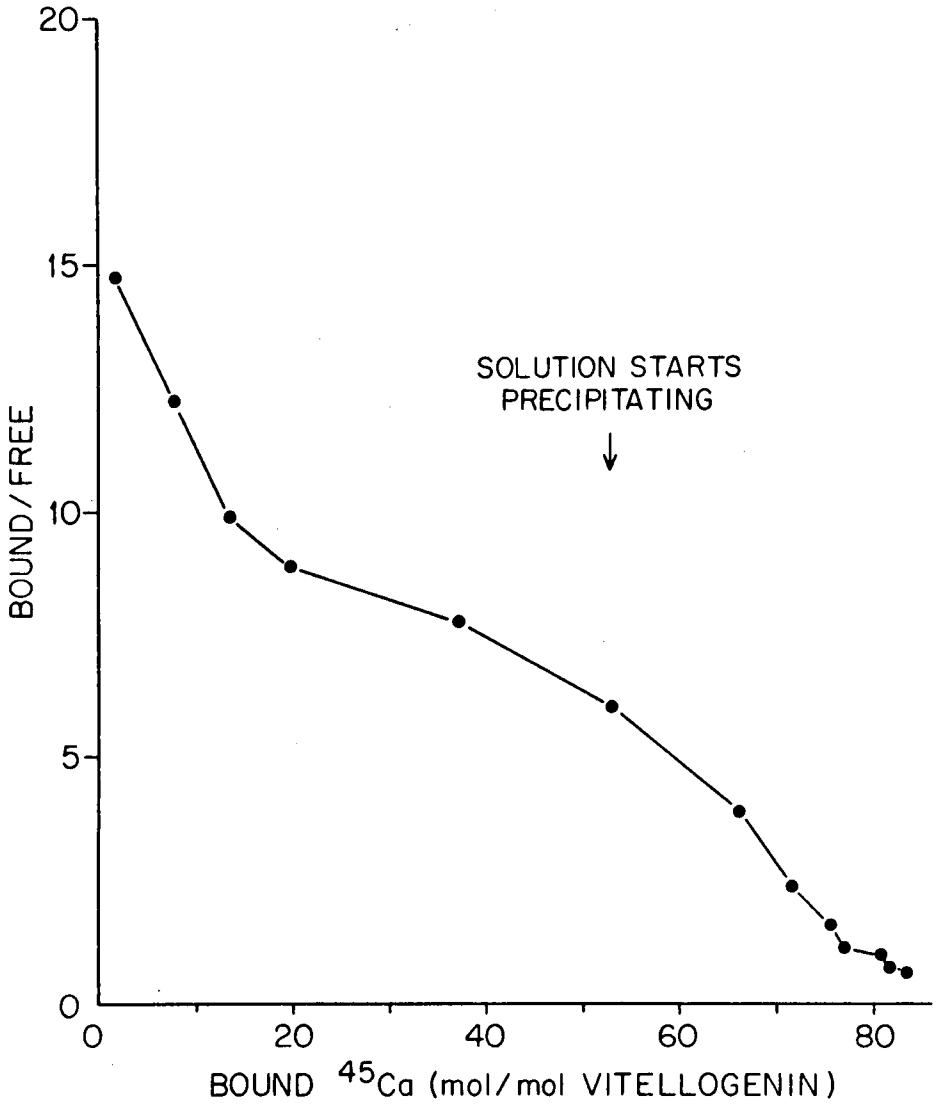

Figure 48. Scatchard analysis of the binding of ^{45}Ca to vitellogenin. DEAE-cellulose purified vitellogenin was dissolved at a concentration of 10mg/ml in 10 mM Tris-Cl, 50 mM KCl, pH 8.0. The initial data point was taken at a ^{45}Ca concentration of 1.6 mg/ml, the next three points at increments of 6.7 ug/ml, and the rest at increments of 20 ug/ml.

I observed that the vitellogenin solution became cloudy. This Scatchard analysis of the binding of $^{45}Ca^{2+}$ to vitellogenin is essentially identical to that obtained with the vitellogenin derivative phosvitin using Ca^{2+} and other divalent cations (28). The most plausible explanation of the data is that initially Ca^{2+} binds to the vitellogenin to form the calcium monophosphate salt of vitellogenin. As the concentration of Ca^{2+} is raised and a greater percentage of the phosphate groups in vitellogenin exist as the calcium monophosphate salt, the conditions become favorable for the interaction of vitellogenin molecules so as to form the calcium diphosphate salt with the phosphate groups of neighboring vitellogenin molecules. The "crosslinked" vitellogenin then precipitates. By adding a large excess of Ca^{2+}, the equilibrium would shift so that the calcium monophosphate salt would be again favored. The precipitation of vitellogenin by Ca^{2+} also implies that most of the protein phosphate groups are on the surface of the molecule.

Since my major interest was in maintaining the solubility of vitellogenin in the presence of Mg^{2+} so that in vitro labeling could be performed, I repeated some of these experiments using Mg^{2+} as the divalent cation. The results of one of these experiments is seen in Figure 49. Surprisingly, it took a much higher ratio of Mg^{2+}:protein phosphorus to cause vitellogenin to precipitate. Whereas it took a Ca^{2+}:protein phosphorus ratio of approximately 1:1 to affect approximately 95% precipitation of the vitellogenin, a Mg^{2+}:vitellogenin ratio of greater than 25:1 was required to affect the same extent of precipitation. Various amounts of salt inhibited the precipitation of vitellogenin by

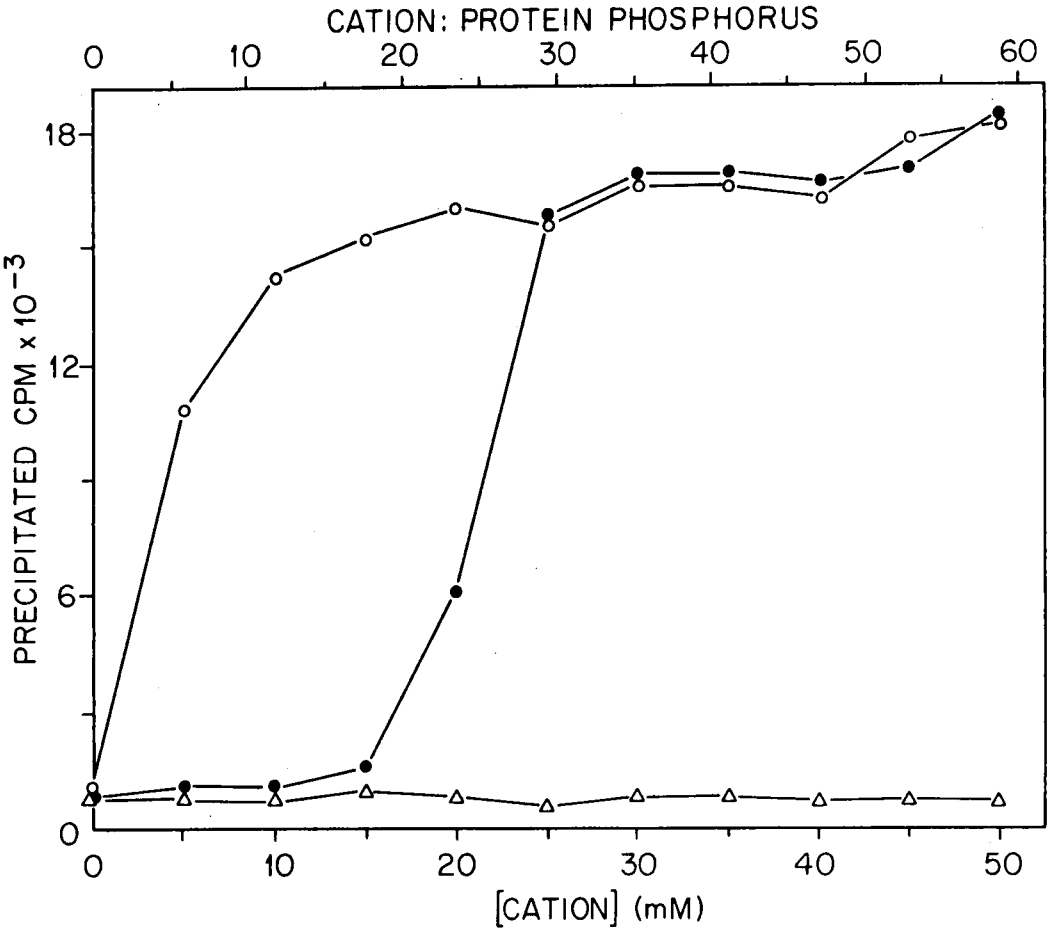

Figure 49. Precipitation of vitellogenin by $MgCl_2$. [^{32}P]-vitellogenin prepared by DEAE-cellulose chromatography was diluted to a concentration of 2 mg/ml with 50 mM Tris-Cl, pH 7.5 and the indicated concentrations of $CaCl_2$ (Δ) and $MgCl_2$ (○). After 10 min at 20°, the precipitate was removed by centrifugation and counted in a scintillation counter after dissolution in Protosol. Also shown is the effect of different concentrations of $MgCl_2$ on vitellogenin plus 5 mM $CaCl_2$ (●).

Mg^{2+} to a small degree (Figure 50). However, a low relative amount of Ca^{2+} (although high enough so that vitellogenin was soluble in its presence) strongly inhibited the precipitation of vitellogenin by Mg^{2+} (Figure 49). These results imply that vitellogenin preferentially binds Ca^{2+} over Mg^{2+} and monovalent salts have little effect on the divalent cation binding. This may explain why vitellogenin normally exists as the calcium salt in the serum.

It occurred to me that the addition of Mg^{2+} to vitellogenin-containing serum might provide one with a simple method of isolating vitellogenin. I therefore added $MgCl_2$ to some vitellogenin-containing serum but was unable to achieve precipitation at any $MgCl_2$ concentration. However, when the divalent cation chelator EDTA was added together with the $MgCl_2$, at a ratio of Mg^{2+}:EDTA of 2:1, I was able to achieve a nearly quantitative precipitation of the vitellogenin. This observation provided the basis of the vitellogenin isolation technique discussed in Chapter II of this thesis. Subsequently, I determined that complexes of Mg^{2+}:EDTA or Ca^{2+}:EGTA are more efficient in precipitating vitellogenin than Ca^{2+} or Mg^{2+} alone. A ratio of Mg^{2+}:EDTA:protein phosphorus of 1.6:0.8:1 is required for 95% precipitation of vitellogenin as opposed to 1:1 for Ca^{2+}:protein phosphorus or greater than 25:1 for Mg^{2+}:protein phosphorus. A model of the Mg^{2+}:EDTA:vitellogenin complex is presented in Figure 51. A surprising observation was that Mg^{2+}:EDTA is equally efficient in complexing vitellogenin as Ca^{2+}:EGTA. Thus the basis of the selectivity of vitellogenin for Ca^{2+} over Mg^{2+} is puzzling. A most useful property of Mg^{2+}:EDTA complexes in studies of

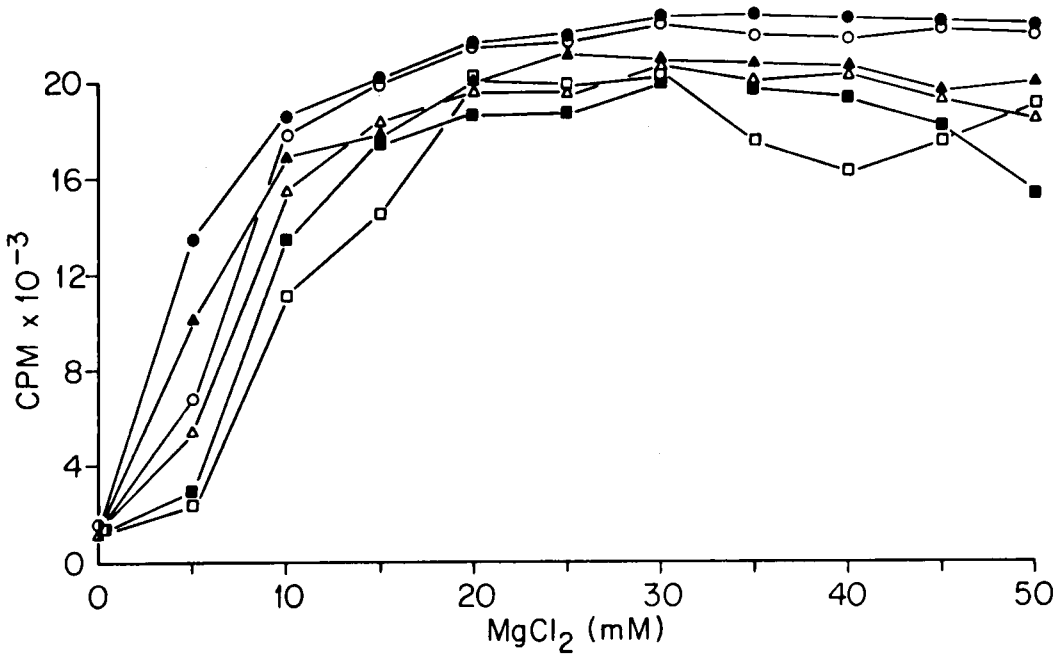

Figure 50. Inhibition of vitellogenin precipitation by monovalent salt. [^{32}P]vitellogenin (final concentration 2 mg/ml) was added to solutions containing the indicated final concentration of $MgCl_2$ and different amounts of KCl. Final concentrations of KCl were 20 mM (o), 40 mM (▲), 60mM (△), 80mM (■) and 100mM (□). The control (no KCl present) is indicated (●). Precipitates were removed centrifugally and dissolved in Protosol prior to counting in a scintillation counter.

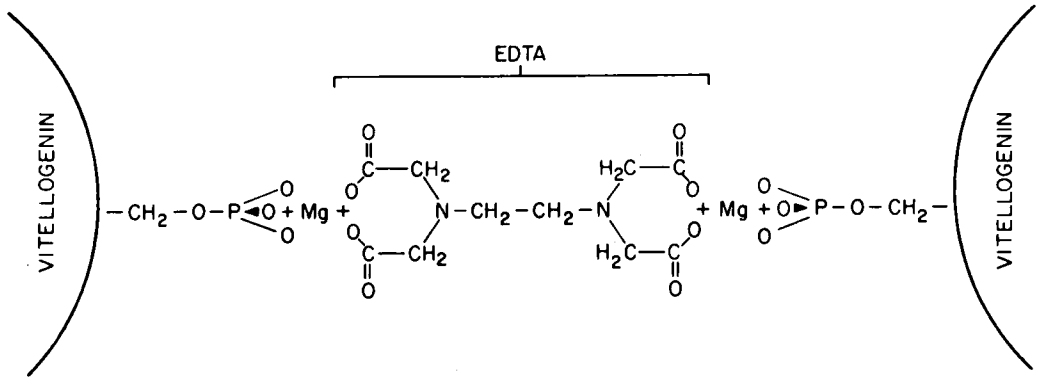

Figure 51. A model for the crosslinking of vitellogenin by Mg^{2+}: EDTA. The EDTA molecule is indicated by brackets.

the protein phosphate groups of vitellogenin is that very high ratios of Mg^{2+}:EDTA to vitellogenin do not result in the solubilization of the vitellogenin precipitate. Thus a solution of 40 mM $MgCl_2$ and 20 mM EDTA will quantitatively precipitate vitellogenin over a range of 1-150 mg/ml. However, this is only true when one starts with solutions of purified vitellogenin.

When I was attempting to quantitate the precipitation of vitellogenin from serum using a series of serum samples containing various concentrations of [^{32}P] vitellogenin, I noticed that the lower the amount of vitellogenin present in the serum, the lower the percent of recovery of the vitellogenin (see Figure 2 on page 18). By replotting my data so that the amount of vitellogenin in the serum was plotted against the amount of vitellogenin recovered, I derived the plot seen in Figure 3, page 20. It can be seen that the data derived from the vitellogenin-containing serum forms a straight line that is parallel to the line plotted from the data derived from the precipitation of purified vitellogenin (linear correlation is 0.996). However, the line intercepts the X-axis at a value of 2.6 mg/ml, indicating that at all vitellogenin concentrations used, 2.6 mg/ml of vitellogenin was nonprecipitable by the Mg^{2+}:EDTA. The most obvious explanation of the data was that there was a factor(s) present in the serum that was competing with the Mg^{2+}:EDTA for binding to the vitellogenin. I felt that it was unlikely to be other divalent cations since the ratio of Mg^{2+}:EDTA is not critical to the recovery of vitellogenin from serum (see Figure 2, page 18). If there is a serum component that competes with Mg^{2+}:EDTA for binding to

vitellogenin, then lowering the concentration of Mg^{2+}:EDTA used in precipitating vitellogenin should result in a greater amount of vitellogenin remaining soluble since the equilibrium between the Mg^{2+}:EDTA and the "vitellogenin binding factor" would shift so as to favor a greater amount of vitellogenin remaining soluble. I therefore repeated the previous experiment using a concentration of 8 m$\underline{M}$ $MgCl_2$ and 4 m$\underline{M}$ EDTA. These results are seen in Figure 52. It can be seen that the expected result was indeed obtained. The precipitation of vitellogenin from serum diluted with saline was quantitative until the ratio of Mg^{2+}:EDTA to vitellogenin dropped below 60:1. The curve derived from the vitellogenin-containing serum diluted with male serum shows the expected dropoff at the higher vitellogenin concentrations, but the lower part of the curve approaches and then parallels that obtained with saline-diluted serum. The X-intercept in this case is approximately 9 mg/ml. These data support the idea that there is a factor(s) in the serum that keeps the divalent cation salt of vitellogenin soluble and competes with Mg^{2+}:EDTA for binding to vitellogenin.

I have recently found additional evidence for a vitellogenin-solubility factor in Xenopus serum. When Xenopus serum is passed over a small column of phosphocellulose in 50 m$\underline{M}$ Tris-Cl buffer (pH 7.5), almost all of the serum proteins are retained (personal observation). Vitellogenin as well as a few unidentified proteins are not bound by phosphocellulose. When I passed a dilute vitellogenin-containing serum solution (2 mg vitellogenin/ml) over a small column of phosphocellulose equilibrated with 1 m$\underline{M}$ $CaCl_2$ (Ca^{2+}:protein phosphorous $\sim$1:1),

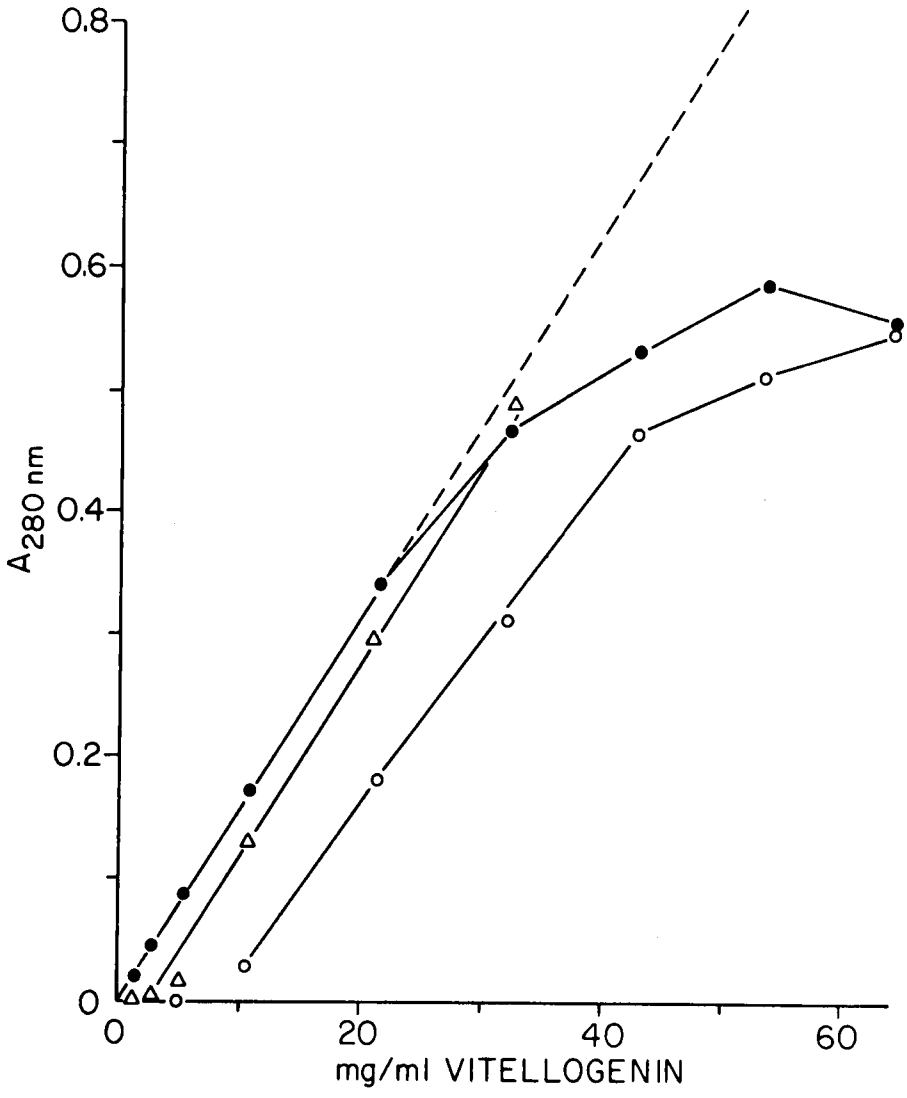

Figure 52. Precipitation of vitellogenin by Mg^{2+}:EDTA. Vitellogenin serum with a titer of 64 mg/ml was diluted with either normal saline (●) or normal male serum (0) in order to obtain the indicated vitellogenin concentrations. Aliquots (200 µl) were then diluted with 0.8 ml of 4 mM EDTA, 8 mM $MgCl_2$. The precipitates were collected, dissolved in 10 ml of 1 M NaCl, 50 mM Tris-Cl, pH 7.5 and the A_{280} was obtained. The dashed line indicates 100% recovery of the vitellogenin. Also indicated is the dilution series made with male serum, but then diluted with 0.8 ml of 20 mM EDTA, 40 mM $MgCl_2$ (Δ).

the vitellogenin eluted as a cloudy solution that precipitated upon standing. However, when a small amount of Xenopus serum was added to the filtrate the precipitate immediately dissolved. Thus it seems that phosphocellulose is capable of removing the factor(s) present in serum that serve to keep the calcium salt of vitellogenin soluble.

In summary, I have found that in contrast to the situation that apparently exists in vivo, purified vitellogenin is not soluble as the calcium salt. There apparently exists a factor(s) in the serum of the animal that serves to solubilize the vitellogenin:Ca^{2+} complex. The natural existence of vitellogenin as the calcium salt as opposed to the salt of some other divalent cation probably reflects the preferential binding of Ca^{2+} by the native vitellogenin molecule. The role of the unknown solubility factor(s) in the conversion of the soluble serum vitellogenin to the insoluble yolk proteins is obviously not known, but the existence of such "solubility" factors must be kept in mind in order to fully understand the mechanism of yolk formation.

APPENDIX B

APPENDIX B

CALCULATION OF PARAMETERS FOR ELECTROPHORESIS IN THE PRESENCE OF SDS

Gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS) is an empirical technique used to estimate the molecular weights of proteins (95). An assumption implicit in the technique is that the free electrophoretic mobility of all protein:SDS complexes are the same (95). For most proteins this is true, but a number of exceptions (such as histones and glycoproteins) are known (80). The reliability of these molecular weight estimates (in actuality average-chain-length measurements) can be evaluated by Ferguson plot analysis (73) which entails the electrophoresis of the unknown protein and standard proteins at various concentrations of acrylamide. Extrapolating the relative mobility of the proteins to a zero acrylamide concentration will yield a free electrophoretic mobility value relative to the mobility of the tracking dye used as the reference point in the system (95). If the unknown and standard proteins have the same relative free electrophoretic mobility, then the molecular weight can be estimated by comparing the relative mobility (R_f) of the unknown and the standard proteins with respect to the tracking dye (84).

Unfortunately, Ferguson plot analysis is restricted to proteins of limited molecular weights (20,000-100,000) since electrophoresis in tubes, which is a necessity for this type of analysis, is realistically restricted to an acrylamide concentration of between 5% and 12%. Gels

below a concentration of 5% are too soft to be readily handled while gels above this concentration range do not polymerize properly and are extremely difficult to remove from the electrophoresis tubes (27). This problem could be circumvented if one is able to modify the electrophoretic rate of the ion front (and hence the tracking dye) independently of the electrophoretic rate of the protein. In other words, if one slows down the dye front relative to the protein band, then the relative mobility of a large protein in a 5% gel can be made to be the same as that in a lower percentage gel. Conversely, the "pile up" of low molecular weight proteins at the dye front can be avoided by increasing the mobility of the dye front relative to the low molecular weight proteins.

Another problem inherent in SDS gel electrophoresis is the limited choice in the pH of the running gel. Discontinuous gel electrophoresis is generally recognized as being superior in resolving power to continuous gel systems (23), and the most commonly used discontinuous SDS gel system is Laemmli's modification (79) of the system of Ornstein (70) and Davis (71). This system has a running pH of approximately 9.4 (98) and this alkaline pH has the potential of dephosphorylating phosphoproteins during electrophoresis (30) or modifying other amino acids. It is thus desirable to be able to choose the running pH of the SDS gel system.

I will attempt to outline here a method by which one can easily change the mobility of the dye front relative to the protein. This method also allows one to choose the running pH of the gel system and

thus the experimenter can have control over all of the major parameters of SDS gel electrophoresis. The method is based on the theoretical calculations of Jovin (64) and also on the observation that the charge on SDS:protein complexes is independent of pH above a value of 7 (27).

Jovin (64) found that virtually all of the parameters of discontinuous gel electrophoresis systems were set by the choice of buffer constituents and the concentration of buffers and counter ions in the gel itself. In the case of the Laemmli gel system (79), the use of 0.375 M Tris-HCl buffer (pH 8.8) as the gel buffer and the use of glycine in the upper reservoir buffer automatically determines all parameters of the gel system. The concentration of glycine and Tris in the reservoir buffer theoretically has no effect on the electrophoresis. Thus one has the simple task of choosing the buffer constituents used and the electrophoretic parameters desired (i.e., pH, ionic strength, dye front mobility, etc.). For SDS gel electrophoresis, the gel buffer must contain a buffer base (such as Tris, Ammediol, Mono-tris) and a counter anion (usually Cl^-). The upper reservoir buffer must contain the buffer base and an acidic buffer (such as glycine, Bicine, Hepes). The lower reservoir buffer should contain the buffer base and any desired counter anion (such as $glycine^-$ or Cl^-). The running pH of the gel will always be between the pK_a of the buffer base and the acidic buffer in the upper reservoir and the buffer base always has to have a lower pK_a value (64). A difference of the pK_a values of the buffer base and the acidic buffer should be between 1-2 for maximum flexibility in the system. It is also necessary to know the relative

electrophoretic mobility of the buffer species relative to Na^+ (Rm). A useful table of buffers, their pK_a and Rm values is given in reference 23. As an example of the calculation of an SDS gel electrophoresis system, I will use the system I developed for electrophoresis of phosphoproteins at pH 8.0.

Because I chose a running pH of 8.0, the buffers I chose were Mono-tris (pK_a = 7.38, personal determination) and Bicine (pK_a = 8.3). The tables given in reference 23 give the following data:

Constituent	Rm	pK_a
1-Bicine	-0.670	8.3
2-Chloride	-1.552	---
3-Mono-tris	+0.440	7.38

The calculations for the system will give the composition of phase gamma (the gel prior to electrophoresis) and phase pi (the gel after electrophoresis). The terms used in the equations are as follows:

$\bar{C}_x^{pi}$ = concentration of constituent x in phase pi

$\bar{C}_x^{gamma}$ = concentration of constituent x in phase gamma

r_x = relative mobility (to Na^+) of constituent x

pK_x = pK_a of constituent x

pH_{pi} = pH of phase pi (running pH)

I = ionic strength during electrophoresis

Rm (1, pi) = relative mobility of constituent 1 (Bicine) in phase pi (speed of the tracking dye)

The equations needed were derived from Jovin's (64) and are as follows:

1. $$\bar{C}_1^{pi} = \frac{I}{10^{(pH_{pi} - pK_1)}} + I$$

2. $$Rm\ (1, pi) = \frac{r_1 \times I}{\bar{C}_1^{pi}}$$

3. $$\bar{C}_2^{gamma} = \bar{C}_1^{pi} \times \frac{1 - \frac{r_3}{r_1}}{1 - \frac{r_3}{r_2}}$$

4. $$\bar{C}_3^{pi} = -\bar{C}_1^{pi} \times \frac{1 + 10^{(pH_{pi} - pK_3)}}{1 + 10^{(pK_1 - pH_{pi})}}$$

5. $$\bar{C}_3^{gamma} = \bar{C}_3^{pi} - \bar{C}_2^{gamma} + \bar{C}_1^{pi}$$

Now for my system I chose a running pH (pH_{pi}) of 8.0 and an I of 0.015 (which is the minimum necessary for good resolution) (27). Using the buffer constituents I chose, the calculated parameters are:

$$\bar{C}_1^{pi} = 0.045\ \underline{M}$$

$$\bar{C}_3^{pi} = -0.078\ \underline{M}$$

$$\bar{C}_2^{gamma} = 0.0581\ \underline{M}$$

$$\bar{C}_3^{gamma} = -0.0906\ \underline{M}$$

Therefore, the gels I cast contained a final concentration of 0.0906 $\underline{M}$ Mono-tris and 0.0581 $\underline{M}$ HCl. The concentration of constituent 1 in the upper reservoir buffer was made equal to $\bar{C}_1^{pi}$, which is preferred for theoretical reasons (64). The concentration of Mono-tris in the upper reservoir buffer was set arbitrarily equal to that of Bicine

(0.045 M) and the lower reservoir buffer was arbitrarily 100 mM Mono-tris-HCl, pH = 7.6.

The relative mobility of the tracking dye [Rm (1, pi)] in my system was -0.224 which is slightly higher than the value of the system of Laemmli [-0.236 (98)]. This means that the dye front will run slightly slower in my system than in the system of Laemmli.

If it is desired to modify the existing parameters of a discontinuous gel system, such as modifying the Rm (1, pi) value, then the desired Rm (1, pi) value is inserted into equation 2, and a new value of $\overline{C}_1^{pi}$ can be calculated. When this value is substituted into equation 1, one will obtain a new running pH for the system. This will allow one to calculate the new gel buffer composition by the use of the other equations. It should be noted that if a value of Rm (1, pi) of less than -0.3 is desired for the system (such as -0.4), a stacking gel utilizing a buffer system with a Rm (1, pi) of greater than -0.3 must be used. Otherwise the SDS:protein complexes will not "stack" and thus will not form thin starting zones (27).

A change in the buffer composition of the gel necessitates the modification of the catalyst concentration used in polymerizing the gel. Optimum gel polymerization is achieved when polymerization (reappearance of a sharp refractile line between the gel solution and the overlaying solution) occurs approximately 10 minutes after the addition of TEMED (23). Since the polymerization time required is given by the relationship

$$\frac{1}{T} \propto [A] \times [P] \times [I] \qquad (23)$$

where T is time, A is total acrylamide, P is persulfate and I is TEMED, the time required to achieve polymerization in 10 min is easily achieved by adjusting the concentration of catalysts used. Also note that a 10% gel will polymerize in one half the time of a 5% gel when the concentrations of catalysts used remain the same.

The above equations allow one to modify the running parameters of gels so as to achieve optimum conditions for analyzing any particular protein. Thus SDS gel electrophoresis does not necessarily have to be an empirical method. The inherent flexibility in the method makes it a very valuable and powerful analytical tool.

VITA

Henry Steven Wiley was born in Memphis, Tennessee, on July 10, 1952 to Sidney J. and Joan Wiley. When he was five, his parents moved to Oak Ridge, Tennessee, where he attended elementary school and was graduated from Oak Ridge High School in June of 1970. After attending The University of Tennessee at Chattanooga for two years, he transferred to The University of Tennessee, Knoxville, and completed his undergraduate studies. In June of 1974 he received the Bachelor of Arts degree in Biology.

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