

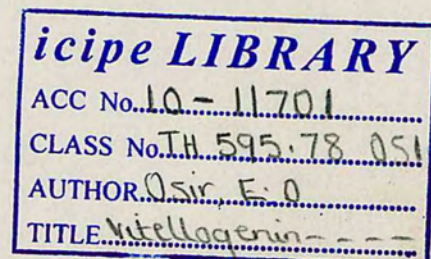
VITELLOGENIN OF THE TOBACCO HORNWORM, MANDUCA SEXTA: PROPERTIES AND
ENDOCYTOTIC INCORPORATION INTO FOLLICLES

by

Ellie Onyango Osir

A Dissertation submitted to the Faculty of the
DEPARTMENT OF BIOCHEMISTRY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

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GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read
the dissertation prepared by Ellie Onyango Osir
entitled Vitellogenin of the tobacco hornworm, *Manduca sexta*: Properties
and endocytotic incorporation into follicles

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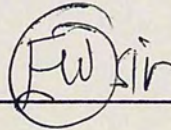
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A handwritten signature consisting of the letters 'F' and 'W' inside a hand-drawn circle, positioned above a horizontal line that extends to the right.

DEDICATION

This dissertation is dedicated to my parents, Gilbert M. Osir and Lydia A. Osir for their love, encouragement and prayers during the entire period of my education.

Also to my sisters, Clara, Eve and brothers, Alfred and George for their love, patience, support.

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LIST OF ABBREVIATIONS

Vg	Vitellogenin
ApoVg-I	Apovitellogenin-I
ApoVg-II	Apovitellogenin-II
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
Con A	Concanavalin A
FITC	Fluorescein isothiocyanate
PAS	Periodate-Schiff reagent
TLC	Thin layer chromatography
LDL	Low density lipoprotein
Endo-H	Endo- β -N-acetylglucosaminidase H
MES	2-(N Morpholino) Ethane Sulfonic Acid
BSA	Bovine serum albumin
NMR	Nuclear magnetic resonance
Man	Mannose
GlcNAc	N-acetylglucosamine

ABSTRACT

Manduca sexta vitellogenin is a phosphoglycolipoprotein ($M_r \sim 500,000$) that contains two copies of the apoproteins (apovitellogenin-I, M_r 180,000 and apovitellogenin-II, M_r 45,000), 13 percent lipids, 3 percent carbohydrates and 0.6 percent phosphorus. The two apoproteins are immunologically distinct polypeptides and apovitellogenin-II is not completely accessible to the aqueous environment in the intact molecule. The carbohydrate moiety located on apovitellogenin-I has a high mannose structure ($\text{Man}_9 \text{GlcNAc}_2$).

Sonicated follicle membranes bind ^{125}I -labeled vitellogenin with high affinity and specificity ($K_D \approx 1.3 \times 10^{-8} \text{ M}$). Total binding sites were estimated at 4×10^{14} sites/ g of follicle membrane protein. The binding was sensitive to pH and calcium. Competition studies showed that binding of vitellogenin was blocked by vitellin and deglycosylated vitellogenin but not by lipophorin, microvitellogenin or apovitellogenin-II. These results suggest that the uptake of vitellogenin involves binding to specific receptors on follicle membranes and the carbohydrate moiety and apovitellogenin-II are not involved in the interaction with the receptors.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

All insects begin their life as an egg. After hatching of the egg comes a series of complex morphological and physiological transformations that culminate in the mature reproducing form of the insect. These transformations, referred to as "metamorphosis", are typical of all insects except in the most primitive members of the group (Chapman, 1969). In general, growth and development in insects occurs by periodic shedding of the hard outer exoskeleton or cuticle. This process includes formation of a new cuticle, removal of the old one, and the actual change in form (Chapman, 1969). Depending on how an insect accomplishes its transformation into the adult form, two basic modes of development are recognized (Wigglesworth, 1972). In the first type, referred to as being complete or holometabolous, the caterpillars (larvae) molt into pupae which subsequently undergo a second molt into adults. In this two step development, juvenile forms differ remarkably from adults not only in structure but also in the types of food consumed, thus enabling the invasion of different habitats. This group of insects is exemplified by Lepidoptera, Coleoptera and Diptera. In the second mode of development, hemimetabolous, juvenile forms closely resemble adults and growth is achieved by a series of molts without the pupal stage. Examples of

insects in this group are Orthoptera, Hemiptera, and Homoptera. The process of molting in insects is under the control of two hormones, namely juvenile hormone (JH) and ecdysone (or growth and differentiation hormone) (Wigglesworth, 1972). Ecdysone is a steroid hormone produced by the prothoracic gland upon stimulation by prothoracicotrophic hormone, a small peptide hormone released by the brain neurosecretory cells and stored in corpora allata (Steele and Davey, 1985). In peripheral tissues, ecdysone is converted into 20-hydroxyecdysone (β -ecdysone) which is the active form of the hormone (Gilbert et al., 1980). On the other hand, JH (sesquiterpene derivatives) are produced by the corpora allata, small organs situated behind the brain. Between molts, JH exerts a "status quo" effect by inhibiting the formation of adult structures but at metamorphosis, JH titers decline thus allowing ecdysone to exert its molting effect.

Life cycle of Manduca sexta

The tobacco hornworm, M. sexta, is a holometabolous moth in the order Lepidoptera and family Sphingidae. The larvae of this insect are pests of tobacco and tomato plants but will also feed on other solanaceous plants (Hoofmann et al., 1966). After hatching of the eggs, the first instar larvae undergo four molts that give rise to the fifth and last larval instar. The larvae have powerful jaws which enable them to feed voraciously. Between the first and fifth larval instars, the larvae increase in weight 4000 fold in only about ten days (Shapiro and Law, unpublished observation). At the end of the

fifth instar, the larvae prepare to molt into the "resting stage" (pupae). But prior to pupation, a characteristic behavior called "wandering" ensues. The larvae first cease to feed, void their gut and then wander around as if they were burrowing in the soil. Although the pupae remain essentially immobile, they undergo internal morphological and physiological changes as they prepare for the next molt into adults after eighteen days. This final molt, termed adult eclosion, is generally preceded by darkening and softening of the cuticle. The entire development from the egg to the adult stage takes about thirty three days. The adult moths usually feed on nectar and live for 4-5 days. Mating between adult moths usually occurs at night and males must fly to find their female mating partners.

In many ways M. sexta can be considered as an ideal laboratory experimental animal. The insect can easily be cultured in the laboratory on an artificial diet that consists of agar, wheat germ, casein, vitamins and salt mix (Reinecke et al., 1980). Generation times are fairly short thus ensuring availability of different life stages at all times. Furthermore, the large size of insects permits single animal experiments.

Female reproductive system

The female reproductive system of insects consists of a pair of ovaries. Each ovary is composed of a series of egg tubes or ovarioles (Ross, 1965). The number of ovarioles vary from insect to insect, and in M. sexta the number is eight. In M. sexta, each

ovariole is approximately 6 cm long at the time of adult eclosion and consists of more than 200 follicles at different stages of development (Nijhout and Riddiford, 1979). The smallest and youngest follicles are found at the anterior tip of the ovariole and the follicles increase in size and age towards the posterior tip. Within the follicles are oocytes and a special group of cells referred to as "nurse cells". The whole complex is surrounded by follicle cells. The primary function of the nurse cells is to supply oocytes with ribosomes which serve as the machinery for protein synthesis during the early stages of embryogenesis or perhaps even for protein yolk synthesis in some insects such as Drosophila melanogaster (King and Buning, 1985).

Insect yolk proteins: synthesis and structure

In the cleidoic (closed system) eggs of insects, yolk serves as the most important source of nutrient during embryonic development. The predominant components of yolk are proteins, lipids and glycogen. Yolk proteins are primarily stored in storage vesicles called yolk granules (Anderson, 1970). Because yolk is an important component of insect eggs, understanding the processes involved in its formation is important from the standpoint of insect reproduction. In many insects, exogenous supply by the hemolymph is the most important source of yolk proteins. The major proteins which constitute up to 90 percent of all soluble yolk proteins are termed vitellins (Engelmann, 1979). Vitellins are derived from hemolymph precursors,

vitellogenins. Insect vitellogenins were first discovered in hemolymph of the silkworm, Hyalophora cecropia (Telfer, 1954). Since then, these proteins have been isolated and characterized from a wide variety of insects (Engelmann, 1979; Hagedorn and Kunkel, 1979; Kunkel and Nordin, 1985). Vitellogenins are usually distinguished from other hemolymph proteins by three main criteria: (i) they are largely female-specific (ii) they constitute between 60 to 90 percent of soluble yolk proteins and (iii) with a few exceptions, they are synthesized only in the fat body tissue. In many cases, the differences between vitellogenins and vitellins are very subtle. For example, lipid analysis of vitellin and vitellogenin in the cockroach, Blattella germanica showed only minor differences which suggested loss of lipids upon uptake (Oie et al., 1975). Heterogeneity of vitellogenins and vitellins has been reported for a few insects, for example, in M. sexta (Imboden and Law, 1983). M. sexta vitellogenin and vitellin each show three distinct bands when hemolymph or egg homogenate is subjected to electrophoresis under non-denaturing conditions. However, isolated vitellogenin or vitellin show only broad bands. Neither the differences between the three distinct vitellogenins nor the cause for their disappearance during purification is well understood. The most striking difference between vitellogenins and vitellins has been reported for Locusta migratoria and Leucophaea maderae (Chino et al., 1976; Koeppe and Ofengand, 1976). In both these cases, a substantial change in subunit structure apparently accompanies vitellogenin uptake into eggs. For example, in

L. maderae, vitellogenin is composed of three subunits while vitellin has four subunits. Proteolytic conversion of one or more of the vitellogenin subunits within the egg has been proposed as a possible cause for this observation. It would then appear that at least in a few well described cases, the use of the terms, vitellogenin and vitellin, is justified. In those insects for which no difference is apparent, the two terms are probably not appropriate.

In a wide variety of insects, vitellogenins are synthesized, processed, and secreted by the fat body tissue. However, Dipteran vitellogenins are also synthesized by the ovary (Brennan et al., 1982). For example, in D. melanogaster, ovarian cells supplement vitellogenin made by the fat body, and they secrete it into the space between follicle cells and oocyte (Jowett and Postlethwait, 1980). In M. sexta, cultured fat body tissue has been shown to secrete vitellogenin in vitro (Osir and Law, unpublished observation). There was no synthesis of vitellogenin by isolated follicles. In a wide variety of insects, vitellogenin synthesis is controlled by JH and ecdysone. For example, in Orthoptera, JH has been shown to have a direct effect on vitellogenin synthesis in vitro (Wyatt et al., 1976) while in the dipteran, D. melanogaster, synthesis is apparently controlled by both JH and ecdysone (Hagedorn and Kunkel, 1979). For many other species (including M. sexta), the control of vitellogenin synthesis is still not clear (Nijhout and Riddiford, 1974). As in other transported proteins, a number of post-translational modifications occur prior to secretion. Among these are

glycosylation, phosphorylation, sulfation and the addition of lipids. The resulting particles are usually complex proteins with molecular weights as high as M_r 600,000 (Harnish and White, 1982; Kunkel and Nordin, 1985).

Although most vitellogenins isolated contain lipids and are glycosylated, the presence of phosphate groups have been shown only in a few insects including L. maderae (Engelmann and Friedel, 1974), D. melanogaster (Brennan and Mahowald, 1982), Bombyx mori (Takahashi, 1983), Aedes aegypti (Borovsky and Van Handel, 1980), Culex pipiens fatigans (Atlas et al., 1978), Rynchosciara americana (Pereira and Bianchi, 1983), and M. sexta (Osir et al., 1986). In M. sexta vitellogenin, both apoproteins, apoVg-I and apoVg-II, contain covalently bound phosphate groups (Osir et al., 1986). As in other insects examined so far, all protein-bound phosphate groups in M. sexta vitellogenin are attached to serine. In general, insect vitellogenins contain lower amounts of phosphorus compared to vertebrate lipovitellins. For instance, avian or amphibian phosvitins contain approximately 10 percent serine-linked phosphorus compared to vitellogenins of L. maderae (0.14 percent) (Engelmann and Friedel, 1974) or M. sexta (0.6 percent) (Osir et al., 1986). Lipids are an integral part of insect vitellogenins constituting between 7-16 percent with the major components being phospholipids and diacylglycerol (Engelmann, 1979). A possible role of transporting diacylglycerols into eggs has been suggested for vitellogenin (Chino et al., 1977) although the major lipid transporting lipoprotein,

lipophorin, may be more involved in this function. For example, M. sexta egg lipophorin probably contains less than 10 percent lipids (Osir, Kawooya and Law, unpublished observation) compared to about 40 percent in hemolymph lipophorin (Pattnaik et al., 1979). Most of the lipids of lipophorin are probably released after uptake into the eggs. The function of lipids in vitellogenin is not well understood although they could be used to construct oocyte membranes or as an energy source during oocyte development.

All vitellogenins so far characterized contain covalently-bound carbohydrate moieties (Hagedorn and Kunkel, 1979). Indeed, many secretory proteins are also glycosylated (Hubbard and Ivatt, 1983). Interest in the study of carbohydrates in general is mainly due to the diverse biological functions which they perform. In many cases, the physiological and biological properties of glycoproteins have been directly related to the carbohydrate prosthetic groups that they contain (Ashwell and Morell, 1974). Although a few reports have assigned carbohydrate structures of insect vitellogenins based on chemical, enzymatic and lectin binding methods (Kunkel et al., 1980, Nordin et al., 1984), NMR spectroscopy has never been used to study these structures. In this dissertation, the primary structure of the carbohydrate moiety of M. sexta vitellogenin has been determined by high field proton NMR spectroscopy. NMR has an advantage over chemical methods in that it is non-destructive and complete information on primary structures can be obtained in a relatively short time. Furthermore, sequential modifications can be

made on the same sample which is recovered after each analysis. Current knowledge on insect hemolymph glycoproteins suggests that a similar glycosylation pathway is probably used throughout plant and animal kingdoms. The only difference is that the carbohydrate moieties of insect hemolymph proteins do not appear to be processed into more complex structures. In contrast to insect vitellogenin oligosaccharides, which are simple N-linked structures, vertebrate vitellogenins have complex carbohydrates. For example, the carbohydrate moiety of X. laevis vitellogenin is an N-linked complex structure containing GlcNAc, Man, galactose and sialic acid (Gottlieb and Wallace, 1982). The exact role(s) of these oligosaccharides (if any) with regard to vitellogenin synthesis, secretion and uptake still remains unresolved.

Uptake of vitellogenin into follicles

Different animal cells have complex transport mechanisms which enable them to sort out specific molecules from heterogeneous mixtures in their surroundings. As a result of these selective transport phenomena, the tissues are able to concentrate certain molecules. Vitellogenic blood protein sequestration by ovarian follicles of amphibians (Wallace and Jared, 1969) and insects (Telfer, 1960) are good examples of selective transport. In insect ovaries, the transport mechanism favors the female-specific protein, vitellogenin, which becomes concentrated by as much as thirty times the hemolymph concentration (Pan, 1971). Incorporation of



vitellogenin into oocytes has been demonstrated in vivo (Kunkel and Pan, 1976; Ferenz, 1978) and in vitro (Ferenz et al., 1981; Osir and Law, 1984; Telfer and Kulakosky, 1984). Vitellogenin uptake involves permeation of the intercellular spaces of the follicle (Telfer et al., 1981) followed by endocytosis at the oocyte surface. It should be noted that coated pits, the progenitors of coated vesicles, were first demonstrated in ovaries of the mosquito, A. aegypti, involved in vitellogenin uptake (Roth and Porter, 1964). Although the route of entry of vitellogenin appears to be well understood, the actual mechanism by which the protein is initially sorted out prior to enclosure within endocytotic vesicles is not entirely clear. An interesting question is whether vitellogenin binding sites should be called receptors. It is generally accepted that the key property of a binding site which defines it as a receptor is its association with a specific physiological response or function. Until any such function is found for vitellogenin, it might be safer to retain the term "specific binding site(s)" without overemphasizing receptor function.

Rationale of the study

In conducting these studies, the female insect has been chosen as the animal model. Specifically, some important aspects involved in egg production in insects have been studied. The processes involved in egg formation are interesting in that they could be used as models for studying other more complex systems.

Furthermore, a thorough understanding of some of these processes may eventually be useful in finding selective ways to control insect pests.

This dissertation is basically divided into two distinct sections. The first section (Chapter 2) presents detailed chemical and immunological studies of M. sexta vitellogenin. In particular, the immunological relationships between vitellogenin apoproteins have been explored. The primary structure of the carbohydrate moiety has also been determined. The information presented is deemed important in understanding how this class of insect lipoproteins are synthesized and later utilized by the developing embryo.

The second section deals with the first step in the utilization of vitellogenins, namely the uptake process. Although vitellogenin uptake is by and large a storage process, the mechanisms involved appear to be very complex. The process is highly selective for vitellogenin which becomes concentrated within the follicles. This suggests that there are specific binding sites on the oocyte surface that regulate the uptake process. There are three basic challenges in the search for vitellogenin binding sites. The first one is to design a binding assay using follicle membranes and the second is to find a method for solubilizing the receptor in an active state. The final part would be to identify and isolate the receptor itself. In this section, an in vitro system for the uptake of vitellogenin is presented. In addition, an assay for characterizing specific binding sites on the follicle membranes is described. This assay is potentially useful in understanding the structural basis for

the selectivity in vitellogenin uptake.

CHAPTER 2

CHEMICAL AND IMMUNOLOGICAL STUDIES ON MANDUCA SEXTA VITELLOGENIN

Introduction

Insect vitellogenins are glycolipoproteins that are synthesized by the fat body tissue, secreted into hemolymph and finally sequestered by the developing eggs or follicles (Engelmann, 1979; Hagedorn and Kunkel, 1979). The insect fat body which is generally diffuse and distributed all over the abdominal region may be compared to vertebrate liver and adipose tissue combined (Riddiford, 1980). The fat body stores lipids, synthesizes and secretes many hemolymph proteins. Although the follicles are constantly bathed by hemolymph that contains many proteins, only vitellogenin is selectively and rapidly taken up. Other hemolymph proteins are also taken up, albeit in only small amounts (Telfer 1960; Telfer, 1981). From these observations, two basic questions can be asked. First, what makes vitellogenin the preferred storage protein in the eggs . Second, what structural features of vitellogenin are responsible for its selective uptake . In order to address these questions, it is necessary to obtain complete information on the chemical structure of vitellogenins. Once this information is available, the next step is to search for any unique features that may confer selectivity in the uptake process. Although vitellogenins and vitellins have been

described from a number of insects, there is still very little detailed information on their native structures and on the arrangement of the apoproteins within the molecule.

This section of my dissertation describes detailed characterization of M. sexta vitellogenin. Vitellogenin apoproteins have been isolated and characterized. Rabbit antibodies against the isolated apoproteins were used to obtain structural information on the native vitellogenin molecule. The primary structure of the carbohydrate moiety was determined by high field proton NMR spectroscopy. It was also shown that vitellogenin apoproteins contain covalently-bound phosphate and that these groups are present only as phosphoserine.

Materials and Methods

Experimental animals

Adult female M. sexta used in all experiments were obtained from eggs supplied by Drs. J. P. Reinecke and J. S. Buckner, U. S. Department of Agriculture, Fargo, North Dakota. After hatching, the larvae were raised individually as previously described by Bell and Joachim (1976) on a high wheat germ diet (Reinecke et al., 1980).

Collection of hemolymph and preparative ultracentrifugation

Twenty to thirty adult insects, 1-2 days old, were bled as follows. The legs, wings, and heads were removed from the insects and the carcasses centrifuged head down into a solution (50 μ l) of phosphate buffered saline (PBS: 0.2 M NaCl, 0.02 M sodium phosphate, pH 6.7) containing 50 mM glutathione and 20 mM of the protease inhibitor, diisopropylphosphorofluoridate (DFP). The hemolymph was centrifuged (10,000 x g, 10 min, 4°C) in order to remove hemocytes. Potassium bromide was then added to the supernatant to give a final concentration of 44 percent (w/v). This solution was placed in a quick seal centrifuge tube (Beckman), overlaid with 0.9 percent NaCl and centrifuged (206,000 x g, 4 h, 10°C) using a vertical rotor (type VTi-50, Beckman) in a Beckman L8-70 ultracentrifuge. This centrifugation isolated lipophorin from the rest of the hemolymph proteins.

Gel permeation and cation-exchange chromatography

Subphase (15 ml) from the preparative ultracentrifugation tube was applied to Bio-Gel A 1.5m column (2.5 x 70 cm) equilibrated with PBS. Fractions (2.9 ml) were collected and absorbances measured at 280 nm. Tobacco mosaic virus and vitamin B₁₂ were used to obtain the void volume ($V_0 = 140$ ml) and total volume ($V_t = 430$ ml), respectively. Protein standards used for calibrating the column were thyroglobulin (M_r 670,000), γ -globulin (M_r 158,000), ovalbumin (M_r 45,000), and myoglobin (M_r 17,000) (Bio-Rad). Fractions that contained vitellogenin were concentrated by ultrafiltration, dialyzed (12 h, 4°C) against 46 mM sodium succinate buffer, pH 5.9 and applied to Cm Bio-Gel A column (1.5 x 14 cm) equilibrated in the same buffer. The column was then washed with 30 ml of starting buffer to remove non-adsorbed proteins and vitellogenin was eluted using 0.2 M NaCl. Isolated vitellogenin was stored at 4°C under nitrogen.

Density measurement

Potassium bromide was added to isolated vitellogenin to give a final concentration of 44 percent (w/v). The solution was overlaid with KBr solution (33 percent, w/v) and centrifuged for 20 h (Hauerland and Bowers, personal communication). Fractions (1 ml) were collected from the tube and refractive indices (R. I) measured. The absorbances of each fraction was measured at 280 nm. Densities (D) were calculated from the equation: $D = (R.I - 1.777) / 0.1561$.

Protein determination

Protein concentrations were determined using either the Folin reagent (Lowry et al., 1951) or the BCA protein assay reagent (Pierce) with BSA as protein standard.

Electrophoresis

Electrophoresis on SDS-polyacrylamide slab gels in 25 mM Tris, 192 mM glycine buffer, pH 8.3 was conducted as described by Laemmli (1970). Gradient slab gels were cast using a gradient maker (BRL). Gels were stained for protein with Coomassie Brilliant Blue (Pierce). High and low molecular weight markers for SDS-PAGE were obtained from Bio-Rad. Native electrophoresis was carried out on 4-20 percent gels at 4°C with a constant 140 V for 24 h. Protein standards used were: thyroglobulin (M_r 670,000); ferritin (M_r 440,000); catalase (M_r 232,000); lactate dehydrogenase (M_r 140,000) and BSA (M_r 67,000). Molecular weights were determined from the plot of log molecular weight versus relative migration of the protein standards.

Analytical ultracentrifugation

Sedimentation equilibrium was carried out at 6,000 rpm (20°C) using type AN-H rotor in a Spinco model E ultracentrifuge equipped with electronic speed control and a U.V. scanner. Vitellogenin was dialyzed against a buffer containing 0.1 M sodium chloride, 10 mM sodium phosphate, pH 7.0. Molecular weight was calculated using a partial specific volume (v) of 0.766 (Pan and

Wallace, 1974). The plot of $\ln (A_{280})$ versus r^2 (radial position) had a correlation coefficient of 0.921.

Lipid analysis

Vitellogenin (9 mg) was extracted with chloroform-methanol according to Bligh and Dyer (1959). The extract was assayed for phosphate (Bartlett, 1959) and an amount of sample containing 100 nmoles of phosphate was mixed with 10 μg each of ditridecanoin, tritridecanoin, stigmasterol, tetracosane and tetradecanoic acid as internal standards for diacylglycerols, triacylglycerols, cholesterol, hydrocarbons and free fatty acids, respectively. The lipids were separated by TLC and then quantitated by gas liquid chromatography (Fernando-Warnakulasuriya *et al.*, 1981), except that hydrocarbons were separated using a column (26 mm x 1.2 m) containing 2.5 percent SE-30 on Gas-chrom Q (Applied Sciences) and a temperature program of 130-300° at 4°/min. The injector port temperature was 235°C.

Limited trypsin digestion

Isolated vitellogenin (100 μg) was treated with trypsin (Worthington) for various times at 37°C in a water bath. A control incubation with no trypsin was included. The ratio of vitellogenin to trypsin was 100: 1. The reaction was stopped by boiling for 5 min and aliquots from each incubation (27 μg protein) were subjected to SDS-PAGE.

Isolation of apoproteins

Initial attempts to isolate vitellogenin apoproteins were carried out by the electroelution method after SDS-PAGE. However, this method was not successful since apoVg-II was always contaminated by apoVg-I. Furthermore, antibodies raised against these apoproteins showed cross-reactivity. For this reason, column chromatography was used to separate the apoproteins after dissociation in a suitable chaotrope. The apoproteins could easily be dissociated in 6 M guanidine HCl, 50 mM sodium phosphate, pH 7.0 and separated on a Sepharose CL-6B column (1.5 x 91 cm). Briefly, isolated vitellogenin (40 mg) was mixed with 1.1 g of guanidine HCl and incubated for 1 h at 50°C. After cooling, the sample was applied to the column which was eluted with the same buffer at 12 ml/h. Pooled apoproteins were dialyzed with four changes against deionized water. ApoVg-I which precipitated during dialysis, was collected by centrifugation (15,000 x g, 10 min) in a microcentrifuge (Fisher). The low solubility of apoVg-I compared to apoVg-II provided an alternative method for isolating the apoproteins. In this procedure, vitellogenin was first dissociated in guanidine HCl by boiling as described above and then dialyzed against water. After two changes, apoVg-II was still in solution while most of apoVg-I had precipitated out. The solution was centrifuged to separate the apoproteins. The remaining apoVg-I contaminating apoVg-II preparation was removed by affinity chromatography on Con A column since only apoVg-I is glycosylated.

High performance liquid chromatography

Gel permeation HPLC was carried out on a Varian 5000 liquid chromatograph with a UV-50 detector (set at 280 nm) using a TSK 250 column (Bio-Rad) in 0.15 M Na_2SO_4 , 0.02 M Na_2PO_4 , 0.02 percent NaN_3 , pH 7.0. Sample (50 μl) was injected and the flow rate maintained at 0.5 ml/min. Protein standards used were, thyroglobulin (M_r 670,000), IgG (M_r 158,000), ovalbumin (M_r 45,000) and cytochrome C (M_r 14,000) (Bio-Rad).

Scanning densitometry

In order to compare the staining intensity of the two apoproteins, the following experiment was conducted. Varying amounts of apoVg-I (7-30 μg) and apoVg-II (6-30 μg) were subjected to SDS-PAGE as described above. After staining with Coomassie Brilliant Blue for protein, the gel was scanned using model DU 8 Spectrodensitometer (Beckman) and the area corresponding to each concentration of protein determined. For each apoprotein, a plot of staining intensity versus the amount of protein applied to the gel was linear and the slopes of the two plots were also identical.

Phosphoprotein staining

Vitellogenin or vitellin was first delipidated (Bligh and Dyer, 1959) and then separated by SDS-PAGE as described above. The gel was fixed in 10 percent aqueous sulfosalicylic acid solution and stained with methyl green as described by Cutting and Roth (1973).

Amino acid composition

The amino acid composition of vitellogenin apoproteins were determined in duplicate samples hydrolyzed for 24, 48 and 72 h using 6 N HCl in vacuo at 110°C. The hydrolyzates were lyophilized, dissolved in 100 μ l 0.2 M sodium citrate, pH 2.2 and analyzed with a Dionex D-300 amino acid analyzer using a column supplied by the manufacturer. The buffer system was that described by Hirs (1983). Cysteine and cystine residues were oxidized to cysteic acid with performic acid using the method of Hirs (1967), hydrolyzed for 24 h in vacuo at 110°C in 6 N HCl and analyzed as above. Tryptophan was determined by amino acid analysis preceded by hydrolysis in 3 N mercaptoethanesulfonic acid (22 h, 110°C) (Penke et al., 1974). Amino acid peaks were integrated with a Hewlett-Packard 3388A integrator.

Phosphorus assay

Phosphorus determination on known quantities of isolated apoVg-I and apoVg-II was performed as described by Bartlett (1959). Prior to this determination, the samples were digested in perchloric acid according to Dittmer and Wells (1969).

Phosphoamino acid analysis

³²P-labeled vitellogenin was delipidated twice according to Bligh and Dyer (1959) and then dissolved in 6 M HCl. After evacuation, the sample tube was sealed under nitrogen and hydrolysis carried out at 100°C for 60 min (Sefton et al., 1981). The sample was

lyophilized and redissolved in 0.5 ml of 0.01 N HCl. Phosphoamino acids were separated from the rest of the amino acids by a Dowex 50 column equilibrated with 0.01 N HCl. Fractions were collected and radioactivity monitored by liquid scintillation counting. Under these conditions, most of the amino acids were retained by the column while phosphoamino acids came through. Separation of phosphoamino acids was achieved by 2-dimensional TLC using silica gel G 50 (Niederwieser, 1972). Solvents used were chloroform/methanol/17 percent w/w ammonia (2:2:1) and phenol/water (75:25, w/v) in the first and second dimensions, respectively. The following standards were used: phosphotyrosine, phospho-D-threonine, and phospho-L-serine (Calbiochem-Behring). Amino acids were located by spraying plates with ninhydrin in acetone (Offord, 1969). Radioactivity in each color spot was measured by scraping the silica gel from plates followed by liquid scintillation counting.

Immunological studies

Vitellogenin (1.5 mg), isolated apoVg-I (1.3 mg) and apoVg-II (1.0 mg) were completely emulsified in Freund's complete adjuvant (Miles) and injected intramuscularly into the limbs of New Zealand white rabbits (0.5 ml per limb). Four weeks later, booster injections (0.5 mg of each protein emulsified in Freund's incomplete adjuvant) were administered and after a further two weeks, the animals were bled through the main ear artery. Blood was allowed to clot in capped plastic tubes for 3 h at room temperature and then stored in the cold

(4°C) overnight. Serum was separated from the clots by centrifugation (7,000 x g, 15 min). After adding sodium azide (0.04 percent), the serum was stored at -70°C.

To test the binding properties of the antibodies, protein samples were separated by SDS-PAGE (4-15 percent) and electrophoretically transferred to nitrocellulose paper (Millipore). The papers were incubated with different antisera (anti-Vg, anti-apoVg-I and anti-apoVg-II) (Towbin *et al.*, 1979) and finally with ¹²⁵I-Staphylococcus aureus protein A (Sigma) (Burnette, 1981). Protein bands were visualized by autoradiography as described previously (Shapiro *et al.*, 1984). Antisera to Vg, apoVg-I and apoVg-II were also tested for their abilities to cross-react with vitellogenin of the giant silkworm (H. cecropia) using the immunoblotting technique. Female H. cecropia pupae and vitellogenin were the gifts of Dr. William Telfer (Department of Biology, University of Pennsylvania).

Double radial immunodiffusion (Ouchterlony, 1968) was performed on polystyrene plates (Miles) using 1 percent agarose (Seakem, ME) in PBS. Samples were allowed to diffuse for 12 h at room temperature and the plates soaked in PBS and then in water. After drying, the plates were stained with Coomassie Brilliant Blue. The amounts of samples used are detailed in the appropriate figure legends.

In vitro synthesis of vitellogenin

Fat body was dissected from two 1 day old female insects in lepidopteran saline (110 mM KCl, 4 mM NaCl, 15 mM MgCl₂, 4 mM CaCl₂, 5 mM KPO₄, pH 6.5) (Jungreis et al., 1973). The fat body tissue was then rinsed in saline and preincubated for 30 min in the same saline containing 0.1 percent (w/v) of sucrose. After this step, the tissue was transferred into a fresh medium (200 μ l) containing 100 μ Ci of [³⁵S]methionine (specific activity = 800 Ci/mmol, Amersham). After incubation (3 h, 27°C), the fat body was separated from the incubation medium by centrifugation (10,000 x g, 10 min) in a microcentrifuge (model 235 A, Fischer). After two washing steps with saline, a protease inhibitor, DFP, was added to the fat body which was homogenized. The homogenate was centrifuged as described above to remove the membranes and the supernatant solution saved.

Immunoprecipitation of the fat body homogenate and incubation medium was carried out as follows. The samples were incubated with rabbit antibodies against vitellogenin at 37°C. After 1 h, 20 μ l of Staphylococcus aureus cells (Pansorbin, Calbiochem-Behring) was added and incubation continued for another 1 h. Immunoprecipitates were then collected by centrifugation as described above and analyzed by SDS-PAGE. A similar experiment was also carried out using follicles isolated from 1 day old female insects except that immunoprecipitation was not carried out.

Labeling of vitellogenin in vivo

In order to study in vivo phosphorylation of vitellogenin, 10 μCi of [^{32}P]-inorganic phosphate (Amersham) was injected into the abdominal cavity of 1 day old female insects. The insects were left for about 12 h and vitellogenin was isolated from hemolymph as described above. Eggs dissected from the same insects were used to isolate vitellin as described (Chapter 3). Samples were delipidated (Bligh and Dyer, 1959) and separated by SDS-PAGE. The samples were then transferred to nitrocellulose paper as described above and autoradiography was carried out using a Kodak X-Omat AP film (4 h, -70°C).

Another group of adult female insects were each injected with 20 μCi of [^3H]Man (New England Nuclear) in PBS. After 12 h, vitellogenin in hemolymph was then isolated as described.

[^3H]-labeled vitellogenin sample was analyzed by SDS-PAGE. After drying, autoradiography was carried out for three days at -70°C .

Studies on the carbohydrate moiety

Isolated vitellogenin (20 μg) was dialyzed against endo-H buffer (0.1 M sodium citrate, pH 5.5) and then incubated with 42 milliunits of endo-H at 37°C for 6 h (Tarentino et al., 1978). The reaction was stopped by boiling for 3 min. A control sample was treated in exactly the same manner except that no enzyme was added. Aliquots from both samples were separated by SDS-PAGE (4-15 percent)

and stained with FITC-Con A (Furlan et al., 1979) and PAS (Kapitany et al., 1973).

Lyophilized delipidated vitellogenin (~ 100 mg) was suspended in 1.5 ml pronase buffer (0.1 M Tris-HCl, pH 8.0) and digested for three days with 0.05 percent solution of predigested pronase (Calbiochem-Behring) at 37°C. A few drops of toluene were added to prevent microbial growth. The digests were then centrifuged to remove particulate matter and the supernatant filtered using a 0.22 micron Millipore filter. The pronase digest was chromatographed on a Bio-Gel P-6 gel permeation column (1.5 x 175 cm). Elution was carried out using 0.2 M ammonium bicarbonate, pH 8.3 at the rate of 2.5 ml/h and fractions (2.5 ml) were collected. The amount of carbohydrate in each fraction was determined in 0.4 ml aliquots by the phenol-sulfuric acid assay (Dubois et al., 1956).

Glycopeptides isolated from Bio-Gel P-6 gel permeation chromatography were lyophilized and resuspended in 100 μ l of endo-H buffer. The glycopeptide solution was digested by addition of 164 milliunits of endo-H (Tarentino et al., 1978) for 4 h at 37°C. The digest was immediately rechromatographed over the same Bio-Gel P-6 column as above. Two fractions were isolated after detection by the phenol-sulfuric acid assay (Dubois et al., 1956).

[³H]Mannose-labeled vitellogenin was digested with pronase as described above and the digest separated on a Bio-Gel P-6 column. Fractions in the major peak were pooled, lyophilized and chromatographed on Con A-Sepharose and lentil lectin-Sepharose columns (bed volume = 8 ml) equilibrated with Tris buffered saline containing 0.15 M NaCl, 0.01 M Tris, pH 8.0, 1 mM CaCl₂ and 1 mM MgCl₂. The columns were washed to remove unbound material with the same buffer. Bound glycopeptides were eluted using 0.5 M α-methylmannoside and fractions (2.5 ml) were collected. Radioactivity was determined in 500 μl aliquots from each fraction by liquid scintillation.

The carbohydrate composition of vitellogenin was determined using alditol acetates (Grimes and Greeger, 1976). Gas-liquid chromatography was performed on a Hewlett Packard model 5700 A gas chromatograph equipped with a flame ionization detector. A glass column (6 ft x 1/8 in) containing 3 percent OV 225 on Supelcoport (Supelco Inc.) was used and programmed for 8 min at 170° followed by a 2°/min rise to 220° for 8 min. Carbohydrate standards of known retention times were used and the samples contained 2-deoxyglucose as the internal standard. Response and retention times were determined with a Hewlett-Packard 3370b integrator.

Fractions of the peak containing the major Bio-Gel P-6 vitellogenin pronase glycopeptide were pooled and lyophilized to remove ammonium bicarbonate. The sample was then exchanged in deuterium oxide for a period of five hours and re-lyophilized for a total of three exchanges. After the third exchange and prior to the

last drying, the sample was filtered with a 0.22 micron Millipore filter. The sample was placed in a plastic conical centrifuge tube in a desiccator containing phosphorus pentoxide and stored under vacuum for about 12 h. Prior to analysis, the sample was redissolved in 0.4 ml containing equimolar amounts of 99.996 atom percent deuterium oxide and acetone (which was used as the internal standard). The final glycopeptide concentration was approximately 20 mM. Proton magnetic resonance spectroscopy was performed on a Bruker 250 MHz instrument in the Department of Chemistry, University of Arizona. The spectrum was obtained by Fourier-transformation (FT mode) of accumulated free induction decay (FID) signals after 512 pulses, 16K data points and a delay between pulses of 4-12 sec. Spectral widths of 4000 Hz were examined at room temperature (300°K) (Atkinson et al., 1981). Chemical shifts were calculated by setting the resonance from internal acetone to 2.225 (relative to internal sodium 2, 2-dimethyl-2-sila-pentane-5-sulfonate). Resonance peaks were quantitated directly by computer integration.

Results

Isolation, physical and chemical properties

A comparison between adult male and female hemolymph and egg homogenate proteins on SDS-polyacrylamide gel is shown in Figure 1. The main difference observed is the presence of two proteins, namely vitellogenin (apoproteins represented by i and ii) and microvitellogenin (A) in the female hemolymph (lane 1) and egg homogenate (lane 3) and their absence in the male hemolymph (lane 2). Both proteins have been shown to be female-specific by immunological methods (Mundall and Law, 1979; Osir et al., 1986; Kawooya et al., 1986). On the other hand, lipophorin (apoproteins represented by I and II), is present in both males and females. In addition, a small amount of lipophorin is found in the eggs.

Vitellogenin and other hemolymph proteins were separated from lipophorin (the major hemolymph lipoprotein) by preparative ultracentrifugation in a KBr density gradient (1.03 to 1.30 g/ml) (Shapiro and Law, 1983). Under these conditions, lipophorin (density = 1.11 g/ml) floated in the upper part of the tube, while vitellogenin (density = 1.29 g/ml) and other hemolymph proteins remained in the lower half of the tube. Gel permeation chromatography of the subphase from ultracentrifugation yielded a major peak (A) of vitellogenin and a second peak (B) that contained a blue protein, insecticyanin (Riley et al., 1984), as well as other proteins (Fig. 2). Fractions that

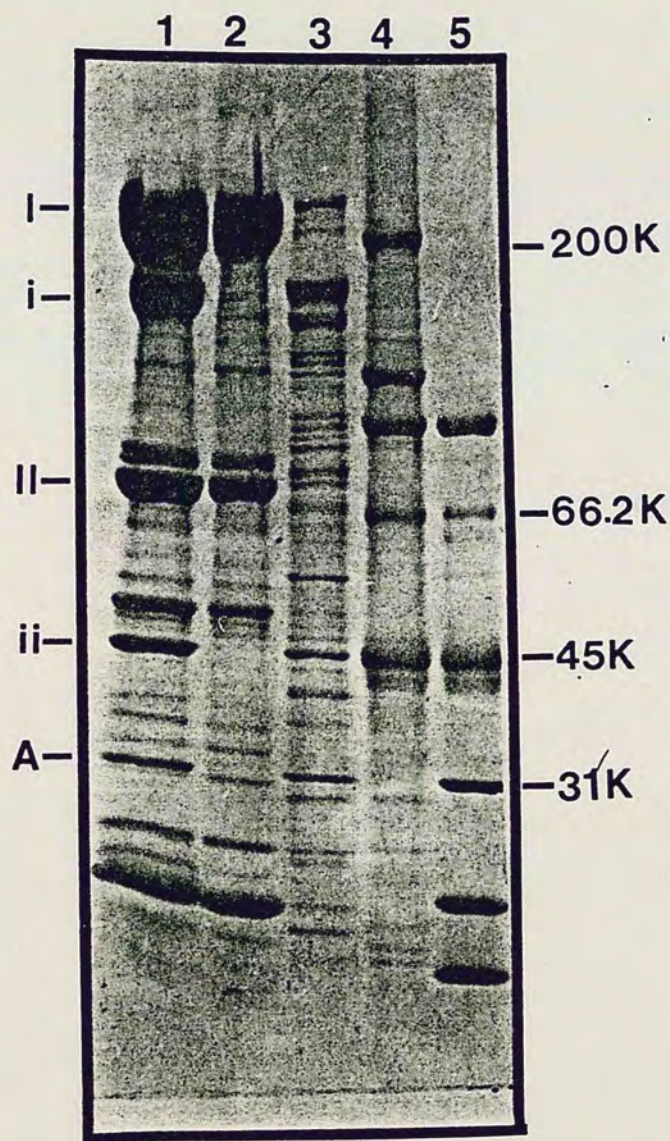


Figure 1. SDS-polyacrylamide gel electrophoresis of adult male and female hemolymph and egg homogenate of *M. sexta*—Samples were separated by SDS-PAGE (4-15 percent). 1. Female hemolymph (1:10 dil. 2 μ l); 2. Male hemolymph (1:10 dil. 1 μ l); 3. Egg homogenate (1:10 dil. 1 μ l); 4. High and 5. Low molecular weight standards (Bio-Rad). The gel was stained with Coomassie Brilliant Blue. I = apolipophorin-I; II = apolipohorin-II; i = apovitellogenin-I; ii = apovitellogenin-II; A = microvitellogenin.

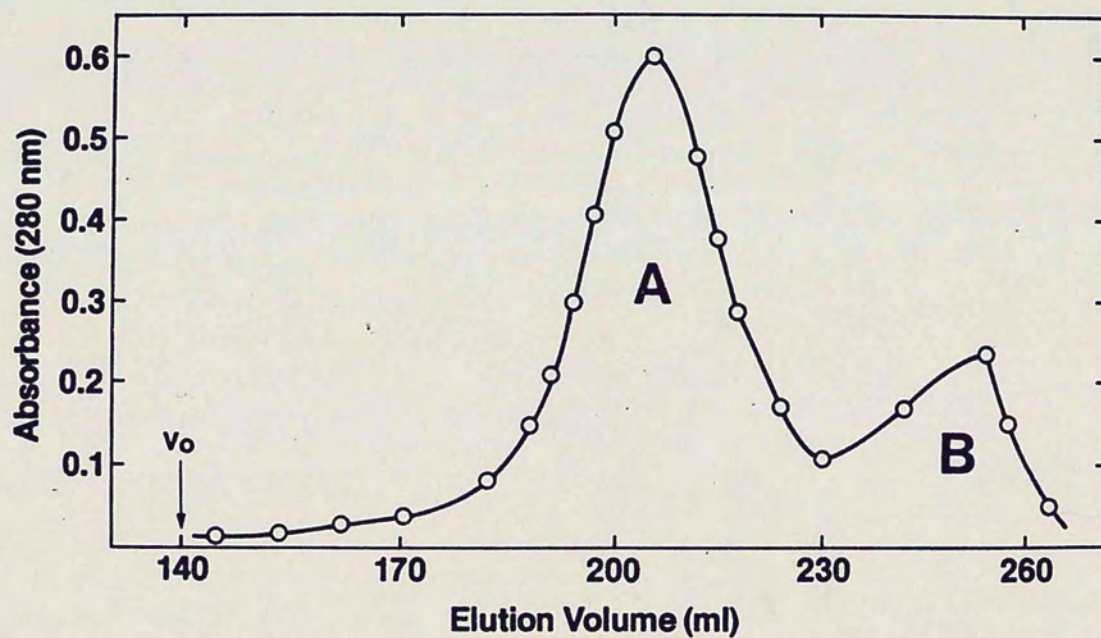


Figure 2. Gel permeation chromatography of subphase from preparative ultracentrifugation--The subphase (15 ml) from KBr density gradient ultracentrifugation was applied to Bio-Gel A 1.5m column (2.5 x 70 cm). The column was eluted using PBS at 9 ml/h and fractions (2.9 ml) were collected. The absorbances were measured at 280 nm.

contained vitellogenin (represented by A, Fig. 3) were pooled and concentrated. At this stage of purification, vitellogenin was still contaminated by arylphorin, a storage protein (native $M_r \sim 450,000$) (Kramer et al., 1980). Although it is generally considered that arylphorin is not found in the adult stage (Kramer et al., 1980), appreciable amounts have been found in the hemolymph of adult female insects. Cation exchange chromatography on Cm Bio-Gel A was used to remove this contaminant (Fig. 4). This isolation procedure (summarized in Fig. 5) yielded a pure sample as judged by both native and SDS PAGE.

A previous study had reported the molecular weight of M. sexta vitellogenin to be $M_r \sim 260,000$ based on gel permeation chromatography (Mundall and Law, 1979). However, as has been previously noted (Telfer et al., 1983), gel permeation often yields deceptively low molecular weight estimates for some insect proteins. In this study the molecular weight of vitellogenin was determined by two methods. A 4–20 percent PAGE conducted under non-denaturing conditions was standardized using molecular weight markers (Fig. 6). From a curve of log molecular weight versus relative mobility, the molecular weight was calculated as $M_r 5 \times 10^5$ (Fig. 7). In order to confirm this value, attempts were made to measure sedimentation velocity at 60,000 rpm. This technique was not successful since large aggregates were formed due to pressure effects. For this reason, low speed (6,000 rpm) sedimentation equilibrium was used. By this method, a molecular weight value of $M_r 491,000 \pm 9,000$ was obtained. This

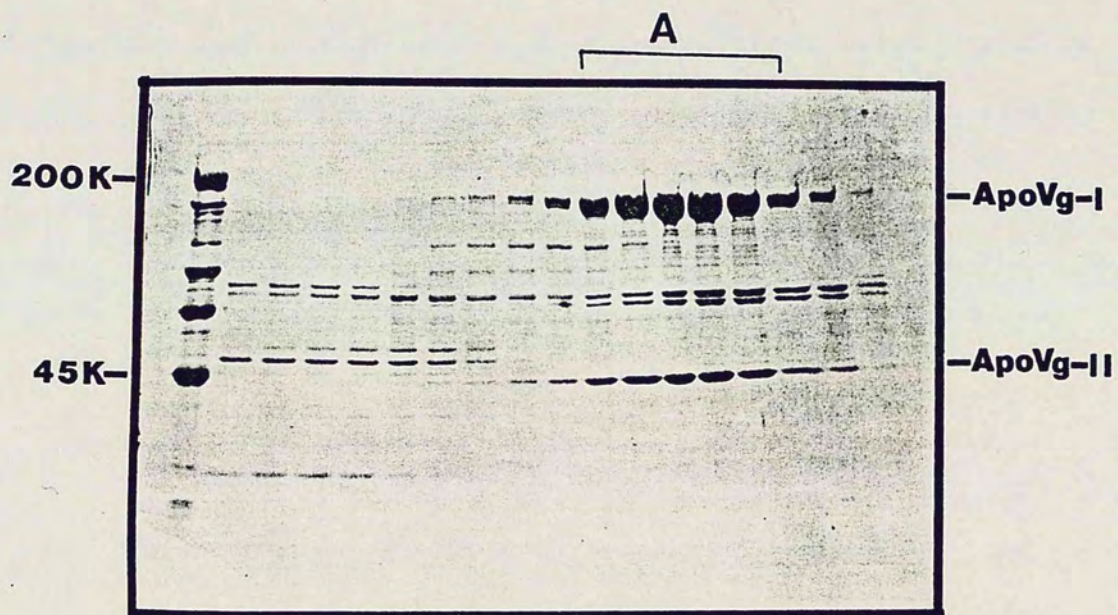


Figure 3. SDS-polyacrylamide gel of fractions from Bio-Gel A 1.5 m gel permeation column--Aliquots from each fraction were separated by SDS-PAGE (4-15 percent). Peak fractions (A) containing vitellogenin were pooled and concentrated (Fig. 2).

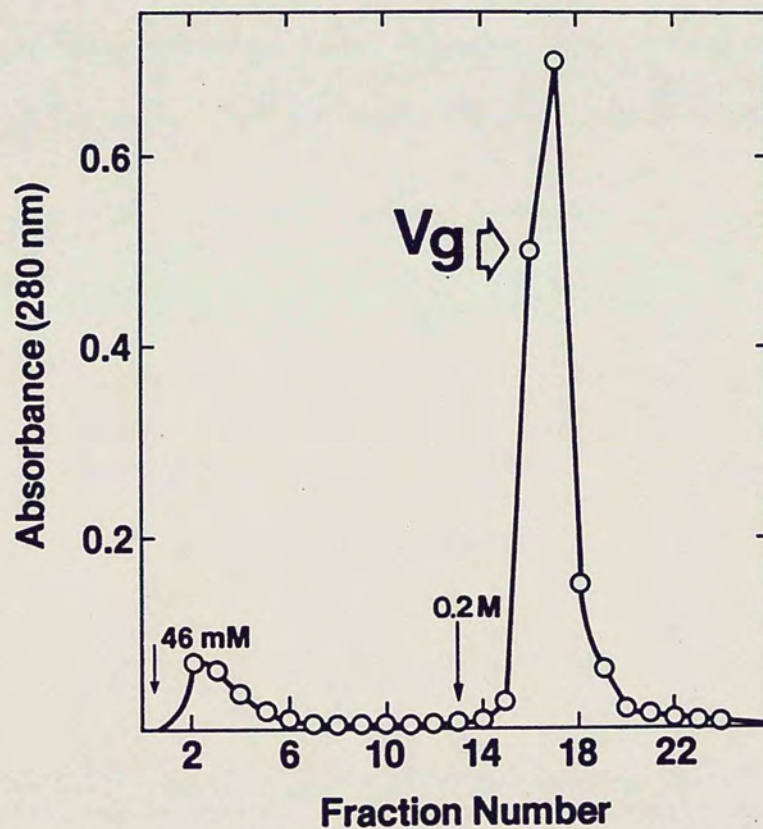


Figure 4. Cation-exchange chromatography—Pooled sample from Bio-Gel A 1.5m column (peak A) was dialyzed against 46 mM sodium succinate buffer, pH 5.9 and applied to a Cm Bio-Gel A column (1.5 x 14 cm) equilibrated with the same buffer. After washing the column to remove non-adsorbed proteins, vitellogenin (Vg) was eluted with 0.2 M NaCl. Fractions (2 ml) were collected and the absorbances measured at 280 nm.



Figure 5. Purification stages of vitellogenin-- Samples were separated by SDS-PAGE (4-15 percent). 1. Adult female hemolymph; 2. Sample after gel permeation ; 3. Arylphorin; 4. Vitellogenin (~ 15 µg protein); 5. Low and 6. High molecular weight standards (Bio-Rad).

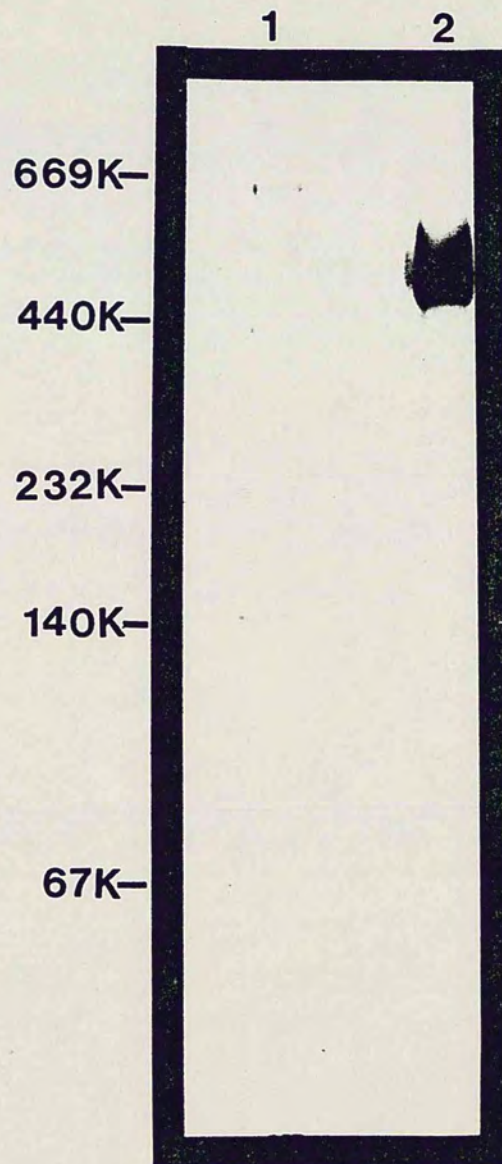


Figure 6. Polyacrylamide gel electrophoresis of vitellogenin—Isolated vitellogenin was separated by native PAGE (4–20 percent) for 24 h at a constant 140 V (4°C) 1. Molecular weight standards (Pharmacia) (see Materials and Methods); 2. Vitellogenin (~ 40 μ g).

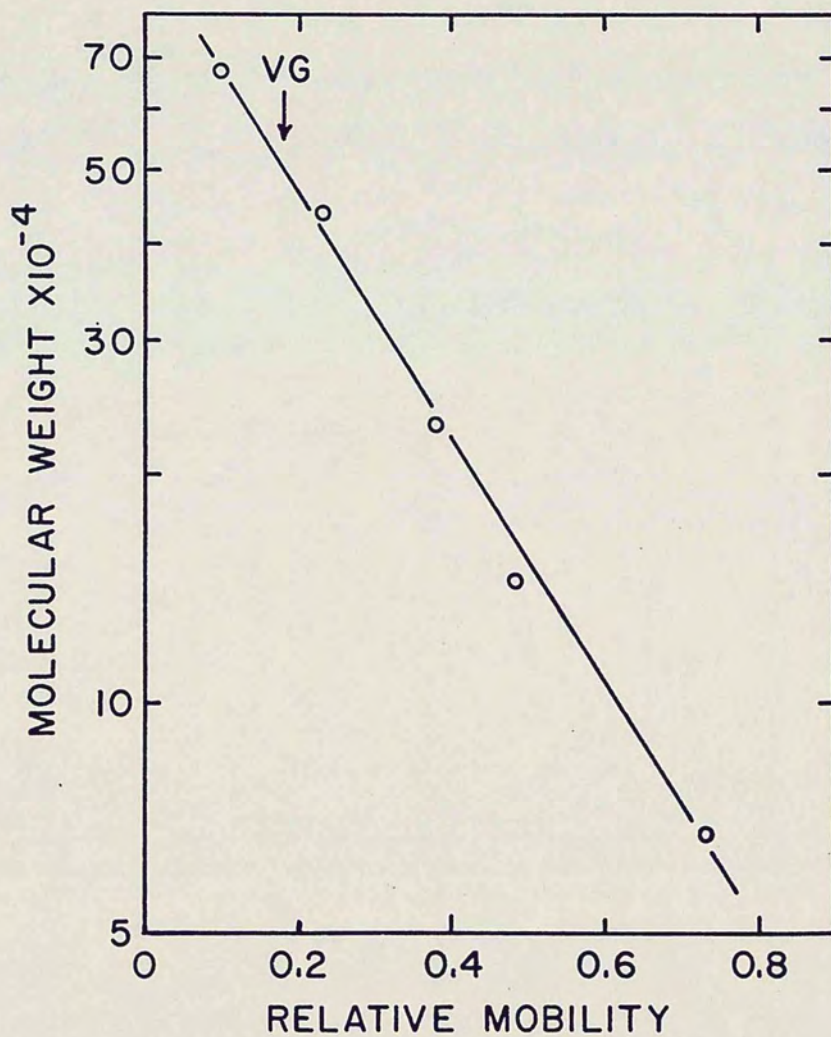


Figure 7. Molecular weight determination of vitellogenin—The gel was standardized using proteins of known molecular weights (Fig. 6).

Table 1. Composition of *M. sexta* Vitellogenin

Component	Percent (by weight) ^a
Proteins	84.5
Carbohydrates	3.0 ^b
Lipids	12.5
Phospholipids	7.2
Neutral lipids	5.3
	Percent (total lipids) ^a
Phospholipids	62.6
Diacylglycerol	15.8
Cholesterol	3.7
Triacylglycerol	6.9
Free fatty acids	4.3
Hydrocarbons	1.1

(a) Data represents average of two determinations (maximum range 2 percent).

(b) Determined by gas liquid chromatography as described under Materials and Methods.

value compares favorably with the molecular weight of H. cecropia vitellogenin (Pan and Wallace, 1974), a protein which has many similar characteristics with M. sexta vitellogenin, including identical chromatographic properties on gel permeation columns.

Isolated vitellogenin was also analyzed for lipid and carbohydrate compositions and the results are presented in Table 1. The molecule contains 85 percent protein, 13 percent lipids and 3 percent carbohydrates. The principal lipid components in vitellogenin are phospholipids (63 percent of total lipids) and diacylglycerol (16 percent of total lipids). The other lipid components were present only in small amounts. These observations are comparable to the results obtained with other vitellogenins (Peled and Tietz, 1975, Chino et al., 1977).

Limited trypsin digestion

Limited trypsin digestion of vitellogenin showed that apoVg-II is not accessible to the enzyme during the first 10 min of incubation (Fig. 8, lane 4). But as apoVg-I was progressively digested, apoVg-II became accessible to the enzyme (lanes 4-7). FITC Con A staining of the gel showed that the bands appearing below apoVg-II are degradation products of apoVg-I since they were also glycosylated.

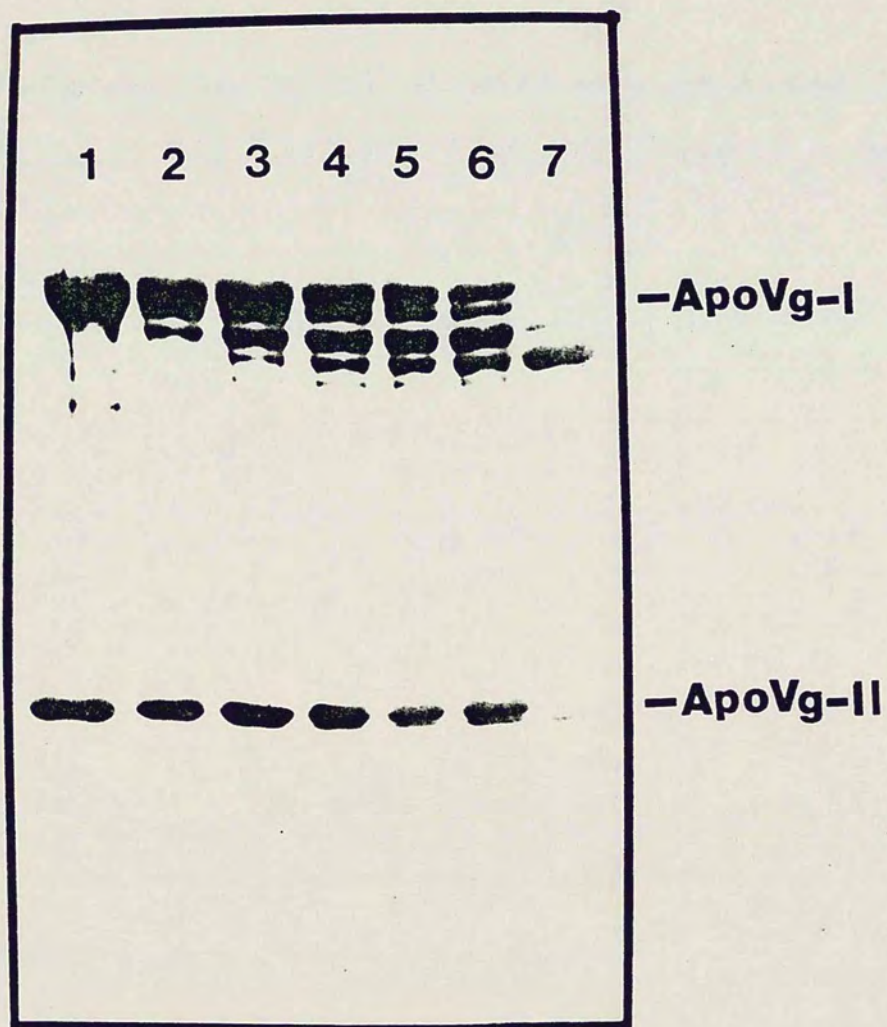


Figure 8. Limited trypsin treatment of vitellogenin—Samples treated with trypsin for various lengths of time were separated by SDS-PAGE (4-15 percent). 1. (1 min); 2. (2.5 min); 3. (5 min); 4. (10 min); 5. (15 min); 6. (20 min); 7. (60 min).

Isolation and properties of apoproteins

Vitellogenin was dissociated using 6 M guanidine HCl and the apoproteins separated by gel permeation chromatography on Sepharose CL-6B column (Fig. 9). After dialysis against deionized water, apoVg-I precipitated while apoVg-II remained in solution. Purity of the apoproteins was verified by SDS-PAGE (Fig. 10). The isolated apoproteins (apoVg-I and apoVg-II) were analyzed for amino acid composition and the results are presented (Table 2). The larger apoprotein (apoVg-I) contained 10 mol percent of proline compared to only 4 mol percent for apoVg-II. Tryptophan, glutamate and cysteine contents also showed some differences. By comparing the amino acid compositions, apoVg-II contained more basic residues than apoVg-I.

Earlier work reported the presence of one large (M_r 180,000) and one small (M_r 45,000) apoprotein and a native molecular weight of M_r 260,000 (Mundall and Law, 1979). However, the presence of two large apoproteins (apoVg-I a, I b) has been reported recently (Imboden and Law, 1983). Although two or more protein bands have occasionally been observed in this study, lack of reproducibility suggested that these extra bands may arise from proteolytic breakdown of apoVg-I. Furthermore, the extra apoVg-I was not seen on freshly prepared protein samples. It was noted that the degradation of most of the hemolymph proteins could be minimized if the insects were injected with DFP prior to bleeding. On the other hand, apoVg-II is very stable and appears on SDS-PAGE as a single protein band (M_r 45,000 \pm 5,000). This molecular weight value was confirmed by gel

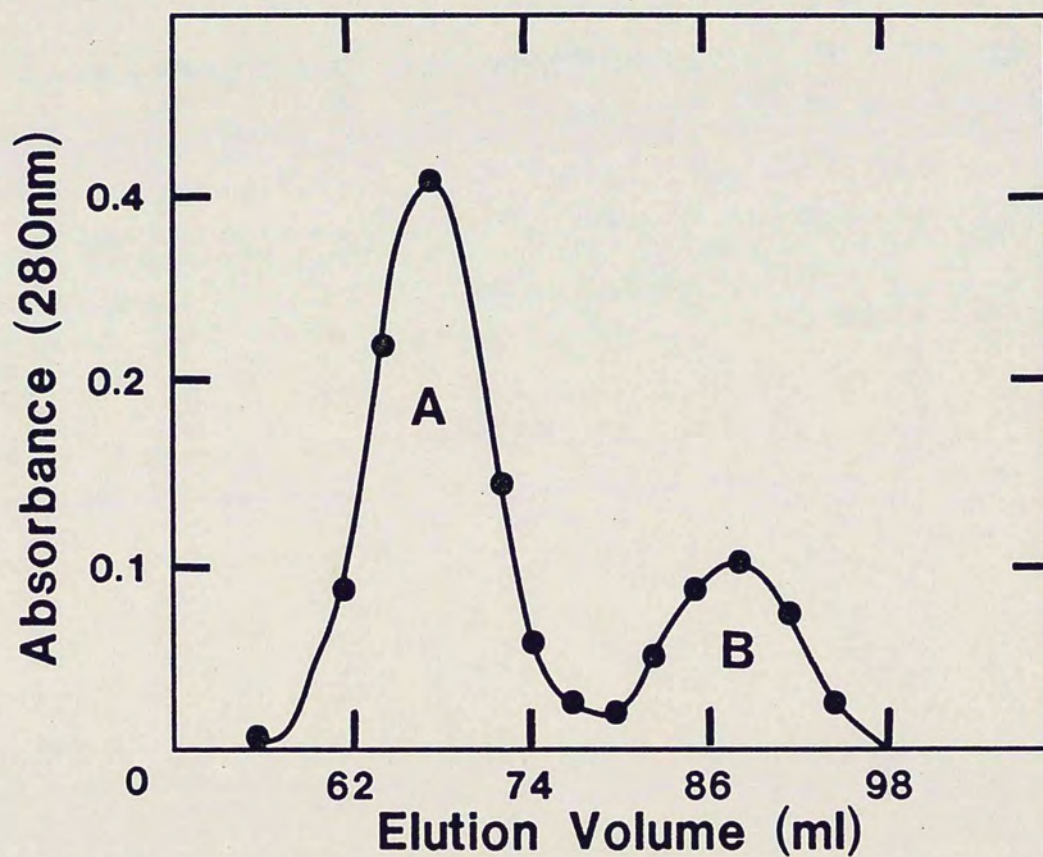


Figure 9. Isolation of vitellogenin apoproteins-- Vitellogenin was dissociated using 6 M guanidine HCl, 50 mM sodium phosphate, pH 7.0 and apoproteins separated on Sepharose CL-6B (1.5 x 91 cm). Fractions (2 ml) were collected and the absorbances measured at 280 nm. The apoproteins were identified by SDS-PAGE: A = apoVg-I; B = apoVg-II. $V_t = 180$ ml; $V_0 = 50$ ml.

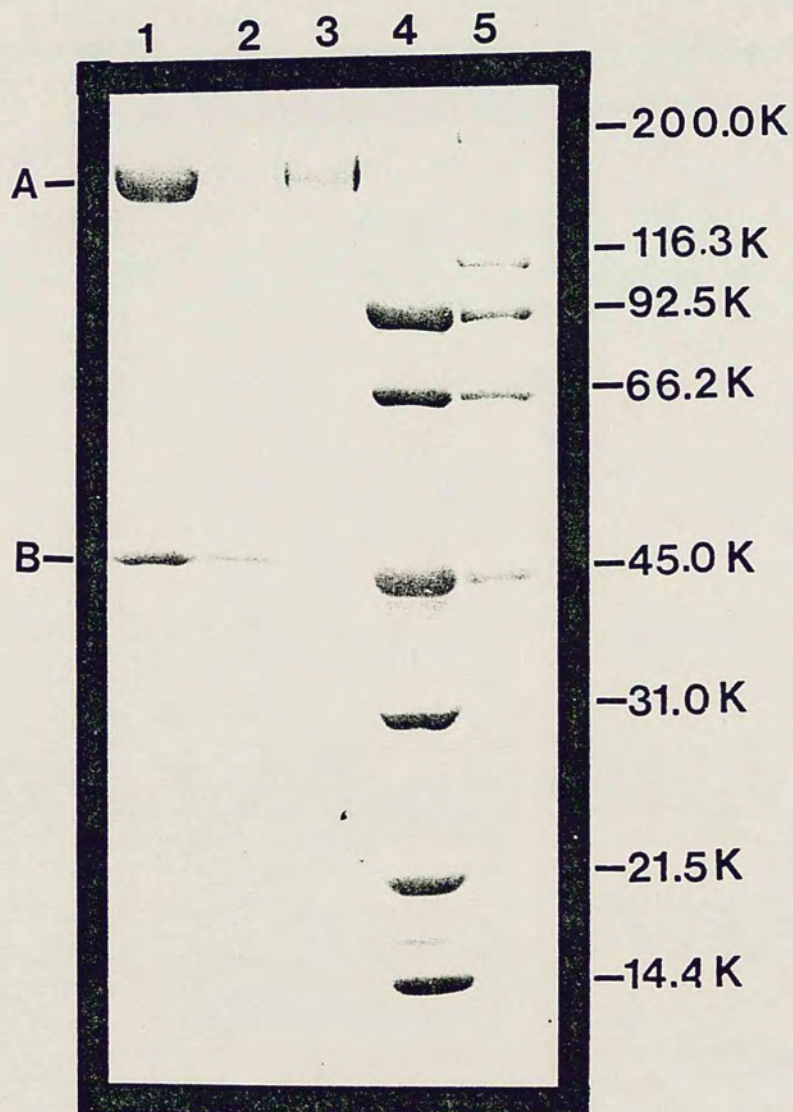


Figure 10. SDS-polyacrylamide gel electrophoresis of apoproteins--Samples were separated by SDS-PAGE (4-15 percent). 1. Isolated vitellogenin (15 μ g); 2. ApoVg-II (10 μ g); 3. ApoVg-II (13 μ g); 4. Low molecular weight standards; 5. High molecular weight standards. The gel was stained with Coomassie Brilliant Blue.

Table 2. Amino acid composition of vitellogenin apoproteins

Amino Acid	ApoVg-I		ApoVg-II	
	Mole percent	Residues/mol	Mole percent	Residues/mol
Aspartate ^a	9.94	160	10.35	42
Threonine	3.76	60	4.39	18
Serine	7.68	124	7.69	31
Glutamate ^a	17.22	277	12.99	53
Proline	9.95	160	4.37	18
Glycine	5.17	83	6.24	26
Alanine	8.21	132	8.96	37
Valine	5.75	93	6.45	26
Methionine	2.09	34	2.35	10
Isoleucine	4.24	68	4.33	18
Leucine	5.56	89	6.13	25
Tyrosine	3.94	63	4.49	18
Phenylalanine	2.58	42	2.97	12
Histidine	4.03	65	4.87	20
Lysine	6.86	110	8.13	33
Arginine	2.47	40	3.86	16
Cysteine ^b	0.27	4	0.90	4
Tryptophan ^c	0.27	4	0.43	2
Total	99.99	1608	99.90	408

(a) Includes acid + amide

(b) Determined as cysteic acid after performic acid oxidation
(Hirs, 1967)

(c) Determined directly after hydrolysis with 3 N
mercaptoethanesulfonic acid (Penke *et al.*, 1974).

permeation HPLC. The specific absorbances for apoVg-I and apoVg-II at 280 nm in 6 M guanidine HCl were $0.422 \text{ mg}^{-1}\text{ml}^{-1}$ and $0.624 \text{ mg}^{-1}\text{ml}^{-1}$, respectively.

The relationship between apoprotein concentrations and staining intensity was established by densitometric scanning. The results showed no significant difference in the staining behavior of the apoproteins. The ratio apoVg-I/apoVg-II was then determined by the scanning procedure to be 3.6:1.0 by weight or a molar ratio of approximately 0.90:1.0. The minimum molecular weight of intact vitellogenin assuming only one apoVg-I and apoVg-II per molecule is approximately 266,000. Since the actual molecular weight is $M_r \sim 500,000$, each vitellogenin molecule contains two copies of each apoprotein.

Immunological properties of the apoproteins

Immunoblotting experiments showed that rabbit antisera against apoVg-I and apoVg-II were specific for each apoprotein and antiserum against intact vitellogenin reacted only with apoVg-I (Fig. 11, lanes 3-8). These antisera also reacted similarly with the apoproteins of vitellin. In double radial immunodiffusion experiments (Fig. 12), anti-apoVg-I reacted with intact vitellogenin (B) while anti-apoVg-II did not (C).

Antisera against intact vitellogenin, apoVg-I and apoVg-II were used to examine immunological similarities between M. sexta and H. cecropia vitellogenins. H. cecropia vitellogenin has two kinds of

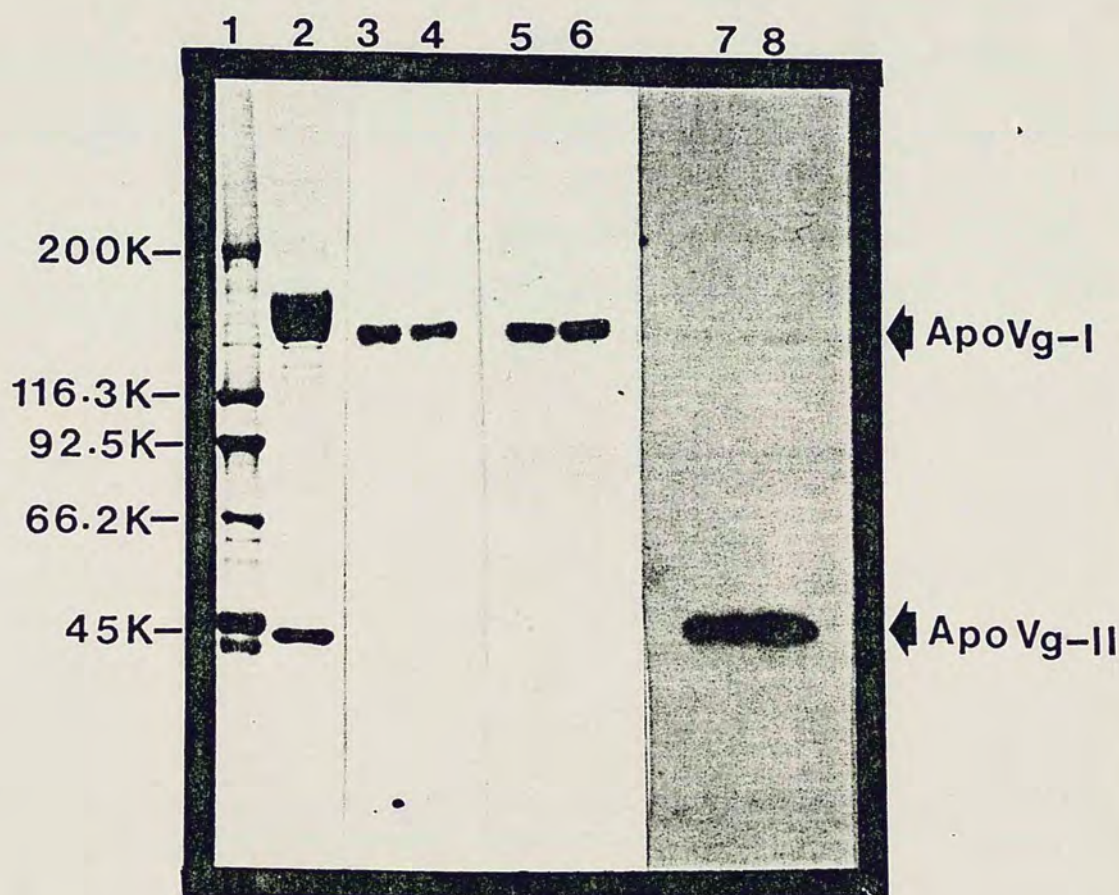


Figure 11. Immunological reactions of vitellogenin apoproteins—Adult female hemolymph samples (lanes 3, 5, 7) ; vitellogenin (lanes 4, 6, 8) and high molecular weight standards (Bio-Rad) (lane 1) were separated by SDS-PAGE (4–15 percent). Samples in lanes 3–8 were then transferred to nitrocellulose paper for 24 h at 16°C. Following the transfer, samples in lanes 3 and 4 were incubated with antibodies against intact vitellogenin; lanes 5 and 6 with anti-apoVg-I and lanes 7 and 8 with anti-apoVg-II. After several washing steps, the blots were then incubated with ^{125}I -*Staphylococcus aureus* protein A and the bands located by autoradiography. Vitellogenin (lane 2) and molecular weight standards (lane 1) were stained with Coomassie Brilliant Blue. Approximately 1 μg protein per lane was used in lanes 3–8 and 15 μg in lane 2.

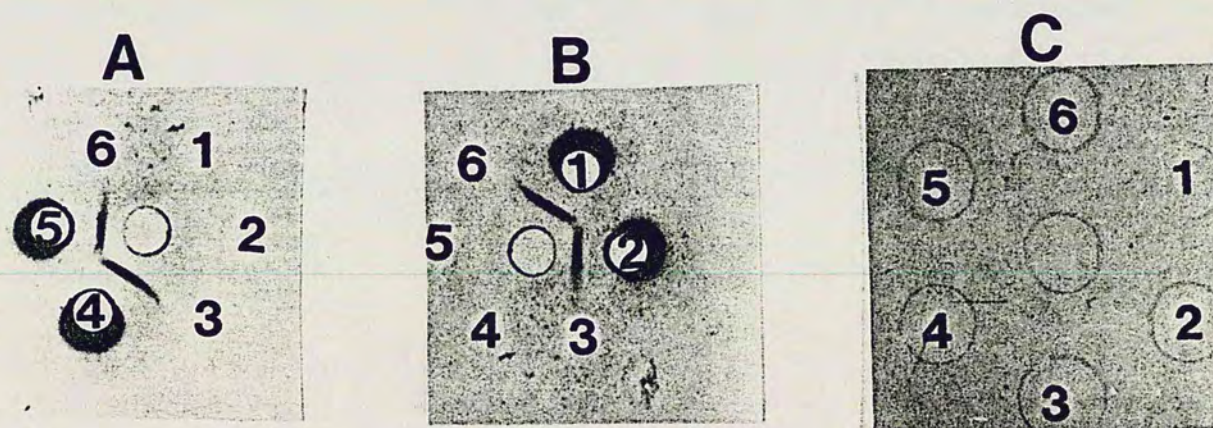


Figure 12. Double radial immunodiffusion--The center wells in A, B and C had 10 μ l each of anti-Vg, anti-apoVg-I and anti-apoVg-II, respectively. Peripheral wells had: (A) 3. Male *M. sexta* hemolymph (1:10 dil., 10 μ l); 4. Female hemolymph (1:10 dil., 10 μ l); 5. *M. sexta* vitellogenin (\sim 10 μ g); (B) 1. Female hemolymph (1:10 dil., 10 μ l); 2. *M. sexta* vitellogenin (\sim 10 μ g); 3. Male *M. sexta* hemolymph (10-fold dilution, 10 μ l). (C) 1. Female *M. sexta* hemolymph (1:10 dil., 10 μ l); 2. *M. sexta* vitellogenin (\sim 10 μ g). Diffusion was carried out for 24 h at room temperature. The plates were then dried and stained with Coomassie Brilliant Blue as described (Materials and Methods).

apoproteins, with molecular weights of M_r 180,000 and M_r 47,000, respectively (Harnish and White, 1982). Antiserum against M. sexta vitellogenin cross-reacted only with the larger apoprotein of H. cecropia vitellogenin. Antisera against apoVg-I and apoVg-II also cross-reacted specifically with the large and small apoproteins of H. cecropia vitellogenin, respectively.

Synthesis of vitellogenin in vitro

In order to define the site of synthesis of vitellogenin, the fat body tissue was incubated with [^{35}S]methionine in vitro. The incubation medium as well as the fat body tissue were then analyzed by SDS-PAGE and autoradiography. As shown in Figure 13 (lanes 1-4), vitellogenin apoproteins (represented by I and II) were labeled both in the fat body homogenate and the incubation medium. Identification of the apoproteins as those of vitellogenin was made by immunoprecipitation with anti-vitellogenin (Fig. 13, lanes 2 and 4). Labeled apoproteins were not detected in follicles that had been incubated with [^{35}S]methionine (lane 5).

Phosphorylation

Following injection of [^{32}P]-inorganic phosphate into one day old adult insects, hemolymph was collected and vitellogenin isolated. Vitellin was also isolated from eggs dissected from the same insects. The apoproteins were analyzed by SDS-PAGE and autoradiography. As shown (Fig. 14), apoVg-I and apoVg-II of both

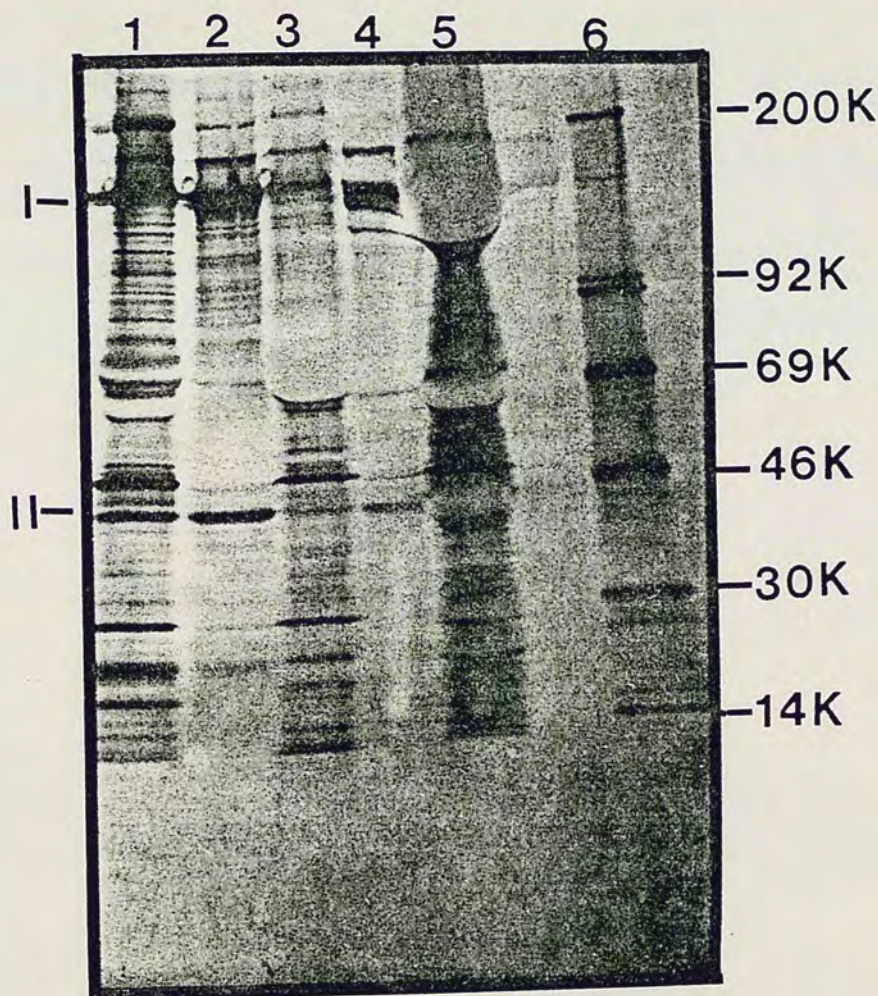


Figure 13. Synthesis of vitellogenin *in vitro*—The synthesis products were separated by SDS-PAGE, transferred onto nitrocellulose paper and the labeled proteins visualized by autoradiography as described under Materials and Methods. 1. Fat body homogenate; 2. Immunoprecipitate of fat body homogenate; 3. Secreted protein; 4. Immunoprecipitate of secreted protein; 5. Follicle homogenate; 6. [^{14}C]methylated protein standards (Amersham). I = apovitellogeni I; II = apovitellogenin I; arrow represents microvitellogenin.

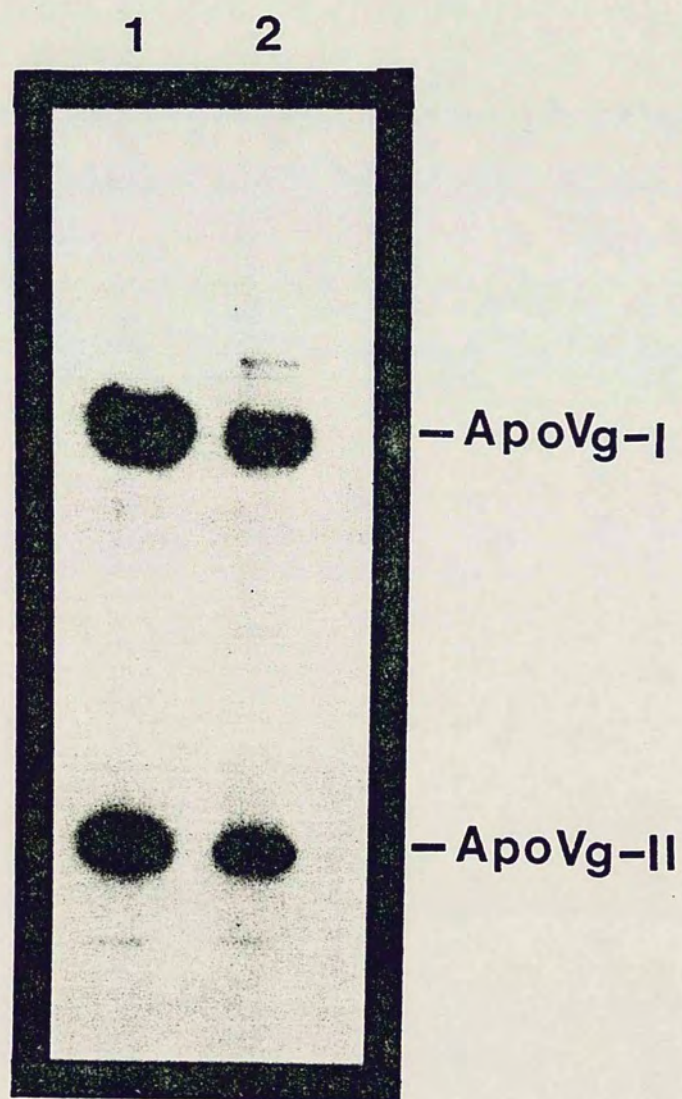


Figure 14. Phosphorylation of vitellogenin and vitellin apoproteins--Samples were separated by SDS-PAGE (3-8 percent) and transferred onto nitrocellulose paper as described (Materials and Methods). The bands were located by autoradiography at -70°C . 1. Vitellogenin ($\sim 20 \mu\text{g}$); 2. Egg homogenate ($\sim 50 \mu\text{g}$).

vitellogenin and vitellin contain labeled phosphate. Approximately 6 percent of the ^{32}P label in both vitellogenin and vitellin was removed by delipidation. Furthermore, vitellogenin apoproteins were found to be the only proteins of whole hemolymph that were significantly phosphorylated. Apoproteins prepared by gel permeation chromatography in guanidine HCl were used for measurement of phosphorus contents. There were no significant differences in the phosphorus contents of vitellogenin and vitellin apoproteins (Table 3). It was also determined whether some of the phosphate groups on apoVg-I were located on the carbohydrate moiety. The results showed that deglycosylation of apoVg-I with endo-H removed 5 percent of the phosphate label suggesting that the carbohydrate moiety may be partly phosphorylated. However, the possibility of contaminating phosphatases in the endo-H preparation could not be ruled out since only a small amount of ^{32}P label was removed. Vitellogenin was briefly hydrolyzed with 6 M HCl at 110°C and the hydrolyzates separated by 2 dimensional TLC (Fig. 15). Identification of the phosphorylated amino acid as serine was corroborated by comigration of the phosphate label with authentic phosphoserine. No phosphate label was found in either phosphotyrosine or phosphothreonine. Similar results were also obtained by exhaustive pronase digestion of vitellogenin instead of acid hydrolysis showing that the occurrence of Ser-P is not an artifact of acid hydrolysis.

Table 3. Phosphorus contents of apoproteins

	ApoVg-I	ApoVg-II
Vitellogenin	22.9 ± 0.41	15.8 ± 0.68
Vitellin	21.6 ± 0.08	16.7 ± 0.46

The values expressed in mol of phosphorus/ mol of protein are mean ± S.D. (n = 4).

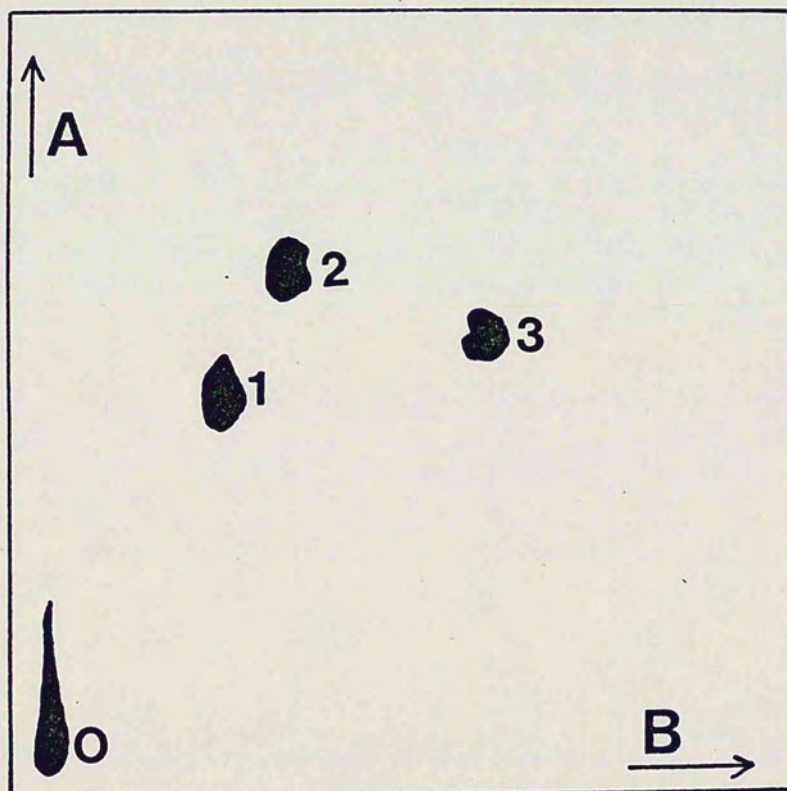


Figure 15. 2-dimensional TLC of phosphoamino acids—Phosphoamino acids were separated in the first (A) and second (B) dimensions by TLC as described under Materials and Methods. Amino acids were located using ninhydrin. 1. Phospho-D-serine; 2. Phospho-D-threonine; 3. Tyrosine

Carbohydrate composition

The carbohydrate composition of isolated vitellogenin as determined by gas liquid chromatography consisted mainly of Man and GlcNAc in addition to trace amounts of glucose. The ratio Man: GlcNAc was calculated as 11.6:2.0. This result is consistent with the previously determined values (Mundall and Law, 1979). From the composition data, it was calculated that each large apoprotein (apoVg-I) contains 4 Man₉GlcNAc₂ chains.

Location of the carbohydrate moiety

After separation of the apoproteins by SDS-PAGE, only apoVg-I reacted with both FITC-Con A (Fig. 16, lane 3) and PAS (results not shown). FITC-Con A binding was eliminated after deglycosylation of vitellogenin using endo-H (Fig. 16, lane 4). There was no detectable difference in the electrophoretic mobility between endo-H and non endo-H treated samples. Vitellogenin was also labeled in vivo to very high specific activity using [³H]Man. Analysis of this sample by SDS-PAGE followed by autoradiography showed that all the labeled Man present in vitellogenin was incorporated into apoVg-I thus confirming that only this apoprotein is glycosylated (results not shown). H. cecropia vitellogenin also has an endo-H sensitive carbohydrate moiety located on the larger apoprotein.

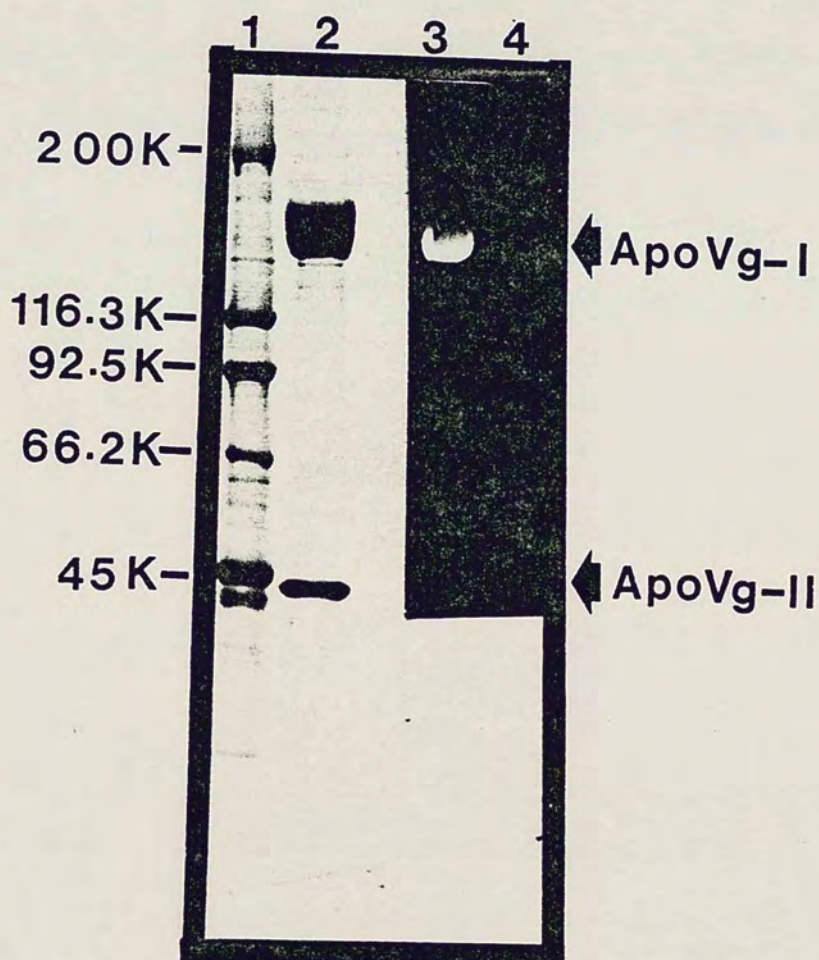


Figure 16. FITC Con A staining of vitellogenin— Samples were separated by SDS-PAGE (4–15 percent) 1. Molecular weight standards (Bio-Rad); 2 and 3. Isolated vitellogenin; 4. Endo-H treated vitellogenin. After electrophoresis, samples in lanes 1 and 2 were stained with Coomassie Brilliant Blue and samples in 3 and 4 with FITC-Con A as described (Materials and Methods).

Gel permeation chromatography of pronase glycopeptides

Isolated vitellogenin was first digested with pronase and then chromatographed on a Bio-Gel P-6 gel permeation column. The digest contained only one significant peak of carbohydrate as judged by the phenol-sulfuric acid assay (Fig. 17 A). A small amount of undigested material eluted in the void volume. Fractions of the major glycopeptide peak were pooled and lyophilized for analysis by high field NMR. The amounts of carbohydrate in the minor peaks were insufficient to permit analysis by NMR spectroscopy. After the initial spectrum was taken, the sample was digested with endo-H and rechromatographed over the same Bio-Gel P-6 column. The results showed that the entire glycopeptide was sensitive to the enzyme (Fig. 17 B). The digestion resulted in a shift of the peak by about thirteen fractions in the elution profile. In addition, a shoulder (arrow, Fig. 17 B) was partially resolved eluting with a slightly higher apparent molecular weight. This peak is roughly equivalent to the amounts quantitated for the unassigned signals in the NMR spectrum (see spectrum, page 60). The endo-H treated peak was isolated as two fractions (I and II, Fig. 17 B) in order to evaluate the contents of the minor peak. Both fractions were subsequently analyzed by high field NMR (see spectrum, page 66).

Lectin binding properties

[³H]Man-vitellogenin pronase glycopeptides were bound to Con A-Sepharose column and eluted using 0.5 M α -methylmannoside (Fig.

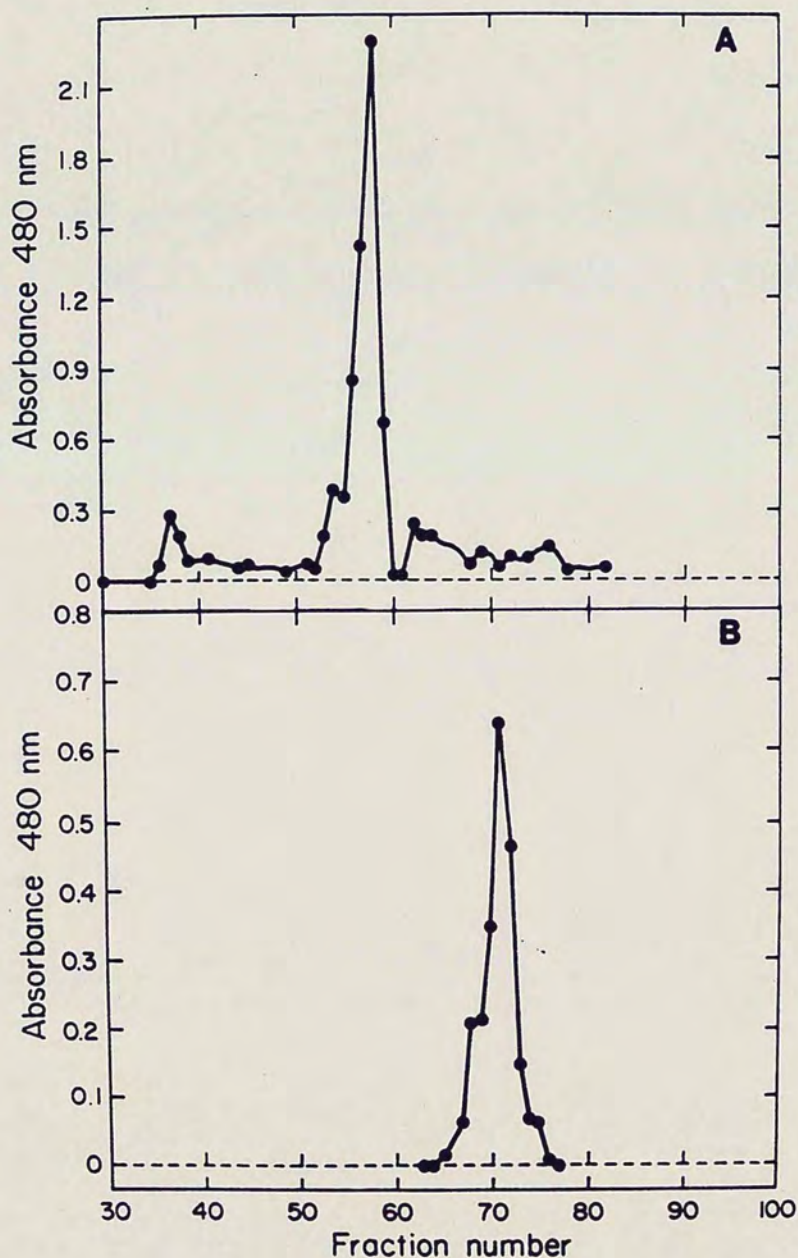


Figure 17. Bio-Gel P-6 profile of vitellogenin pronase glycopeptides--(A): Pronase digest of vitellogenin was applied to the column. Elution was carried out using 0.2 M ammonium bicarbonate buffer, pH 8.3 and fractions (2.5 ml) collected. Aliquots (0.4 ml) from each fraction were assayed for carbohydrate content by the phenol-sulfuric acid method and the absorbances measured at 480 nm. The major peak (51-60) was isolated and analyzed by NMR spectroscopy. (B): Elution profile of the same glycopeptide on Bio-Gel P-6 column after endo-H treatment. The column was eluted as in (A). Two fractions I (65-69) and II (70-76) were analyzed by NMR spectroscopy.

18 B). Conversely, the glycopeptides did not bind to a lentil lectin-Sepharose column (Fig. 18 A). Con A has been shown to bind mainly high Man oligosaccharides in addition to some biantennary complex and hybrid-type Asn-linked oligosaccharides. In contrast, lentil lectin does not bind high Man oligosaccharides (Kornfeld et al., 1981).

Proton NMR spectroscopy of glycopeptides

The 250 MHz NMR spectrum of vitellogenin glycopeptide, recorded in D_2O at 300°K, is presented (Fig. 19). Definitive features are shown (Table 4). Examination of the anomeric region of the spectrum revealed the presence of a relatively homogeneous high Man class glycopeptide. The spectrum is consistent with 9 Man and 2 GlcNAc residues (Fig. 20; see also Fig. 21). The chemical shifts of the C1-H protons of GlcNAc 1 and 2 together with the C1-H and C2-H protons of Man units 3, 4 and 5 indicate the presence of the core pentasaccharide Man 3 $\beta(1\rightarrow4)$ GlcNAc $\beta(1\rightarrow N)$ Asn substituted at C-3 and C-6 of Man 3 by α -linked Man units 4 and 5 (Vliegthart et al., 1981, 1983).

In addition to the β -anomeric signals, there are 8 α -linked C1-H proton signals belonging to Man units. Three of these protons at $\delta \cong 5.05$ ppm are assigned to 3 terminal $\alpha(1\rightarrow2)$ -linked Man residues located at the terminus of three branches in the glycan structure. The existence of these units is confirmed by the presence of the $\alpha(1\rightarrow3)$ -linked core Man chemical shift at $\delta = 5.337$ ppm, the α

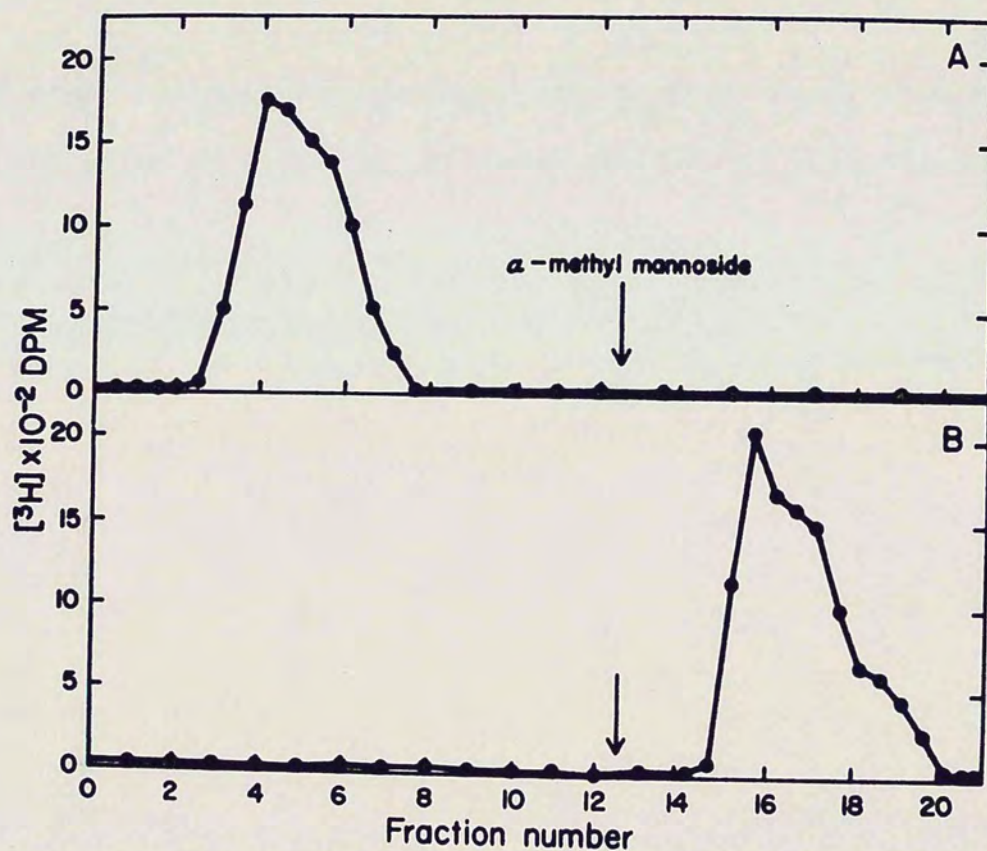


Figure 18. Profiles of labeled glycopeptides on lentil lectin and concanavalin-A columns— $[^3\text{H}]$ Man-vitellogenin pronase glycopeptides were chromatographed on lentil lectin-Sepharose (A) and Con A-Sepharose (B) columns equilibrated with Tris buffered saline containing 0.15 M NaCl, 0.01 M Tris, pH 8.0, 1 mM CaCl_2 and 1 mM MgCl_2 . Bound material was eluted with 0.5 M α -methyl mannoside and fractions (2.5 ml) were collected. Radioactivity was determined using 500 μl aliquots from each fraction.

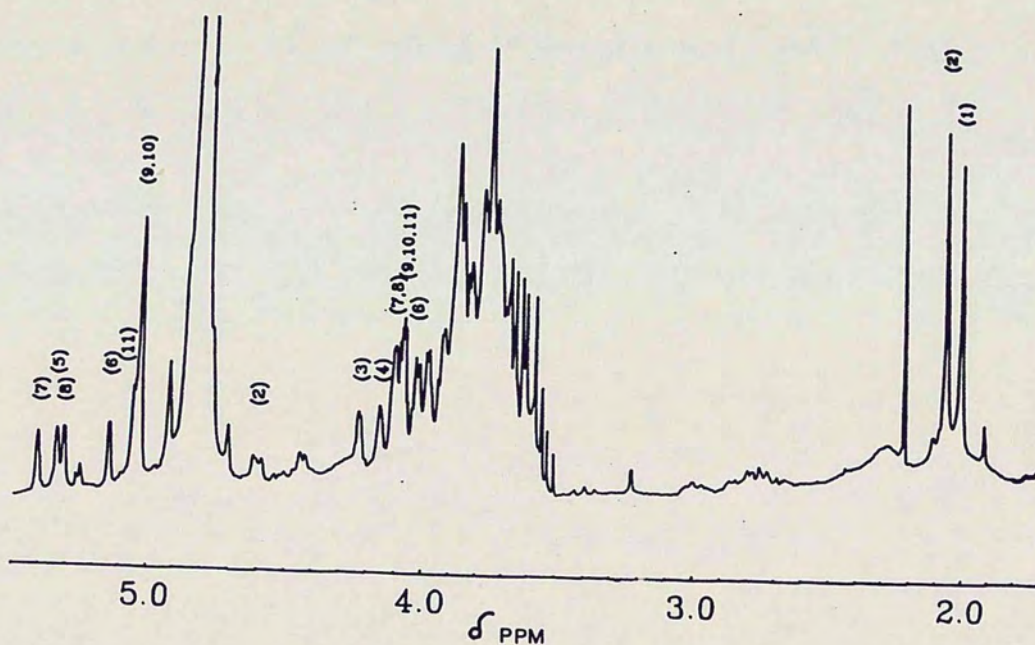


Figure 19. 250 MHz proton NMR spectrum of the major glycopeptide--The major identifiable reporter groups present in the H-1 and H-2 regions as well as the N-acetyl regions are designated by numbers as corresponding to known resonance peaks reported for previously defined structures. The numbers represent the monosaccharide units shown (Fig. 20).

Table 4. ¹H Chemical shifts of structural reporter groups
of constituent monosaccharides for the carbohydrate moiety

Monosaccharide Reporter groups	Unit No. ^a	Chemical Shifts (ppm) ^b	Anomeric Linkage
C1-H	1	5.088	β
C1-H	2	4.610	β
NAC-CH3	1	2.007	β
NAC-CH3	2	2.064	β
C1-H	3	4.773 ^c	β
C1-H	4	4.875 ^c	α
C1-H	5	5.337	α
C1-H	6	5.145	α
C1-H	7	5.408	α
C1-H	8	5.312	α
C1-H	9	5.052	α
C1-H	10	5.052	α
C1-H	11	5.060	α
C2-H	3	4.223	β
C2-H	4	4.158	α
C2-H	5	4.083	α
C2-H	6	4.025	α
C2-H	7	4.110	α
C2-H	8	4.110	α
C2-H	9	4.074	α
C2-H	10	4.068	α
C2-H	11	4.063	α

(a) Refer to Fig. 20.

(b) Data obtained at 250 MHz, T = 300°K.

(c) Partially obscured by HOD at 300°K.

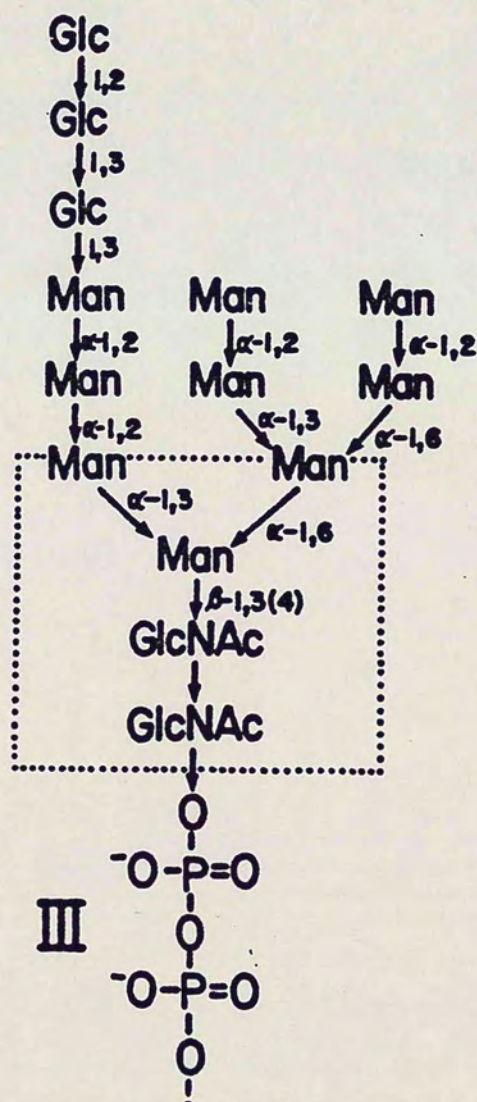


Figure 21. Schematic diagram of the lipid-linked intermediate—The diagram shows the structure that is transferred to an asparagine residue in the sequence: Asn—X—Serine or Threonine.

(1 \rightarrow 3)-linked arm Man at $\delta = 5.408$ ppm, and the internal α (1 \rightarrow 6)-linked arm Man at $\delta = 5.146$ ppm (units 5, 7 and 6, respectively) all of which reflect resonances of α (1 \rightarrow 2)-linked substituents (Vliegthart et al., 1981; 1983). In a similar manner unit 4 is assigned to the resonance peak at $\delta = 4.875$ which indicates a substitution on this residue at C-3 and C-6 by unit 7 and 6, respectively (Cohen et al., 1980; van Halbeek et al., 1981). The final H-1 residue located at $\delta = 5.312$ ppm is assigned to the internal α (1 \rightarrow 2)-linked Man (unit 8) located on the α (1 \rightarrow 3) arm and is present in equimolar amounts to all other H-1 resonances (Dorland et al., 1981). The assignment of the reporter groups found in the H-1 region follow very closely to those elements observed in the H-2 region. In particular, the presence of the β (1 \rightarrow 4) linked core Man proton signal at $\delta = 4.233$ ppm confirms the presence of the H-1 proton which is partially obscured by HOD at 300°K. Integration of this peak yields an area almost identical with the molar yields found from evaluation of other single unit protons. A total of 8 α -linked Man protons can be accounted for in this region. The combined expression of chemical shift values from these reporter groups given (Table 4) produce a glycopeptide structure that is almost identical to that reported for soybean agglutinin (Dorland et al., 1981).

Close examination of the spectrum showed the existence of two sets of resonance peaks which could not be assigned to the above mentioned structure. The resonances located at $\delta = 5.263$, 5.248, and $\delta = 4.430$ to 4.490 are not observed in structures of this type.

Quantitation by peak integration indicated that these unassigned resonances contribute a signal equal to about 5-10 percent of the total signals contributed by the other single unit protons. In order to find the cause of these unusual peaks, the original sample was treated with endo-H and reanalyzed by NMR. A partial spectrum of the region $\delta = 5.0-5.5$ is presented (Fig 22). Fraction I which was isolated as the shoulder from Bio-Gel P-6 chromatography (Fig. 17 B) contained a significant enrichment of the unassigned resonance seen at $\delta = 5.255$ and 5.257 (Fig. 22 A). On the other hand, very little of this signal is present in the main glycopeptide fraction II (Fig. 22 B). The other resonance peaks observed in the region of $\delta \approx 4.4-4.5$ ppm were present at approximately equal amounts in both glycopeptide fractions and thus appear to be independent of the peaks observed in the H-1 region.

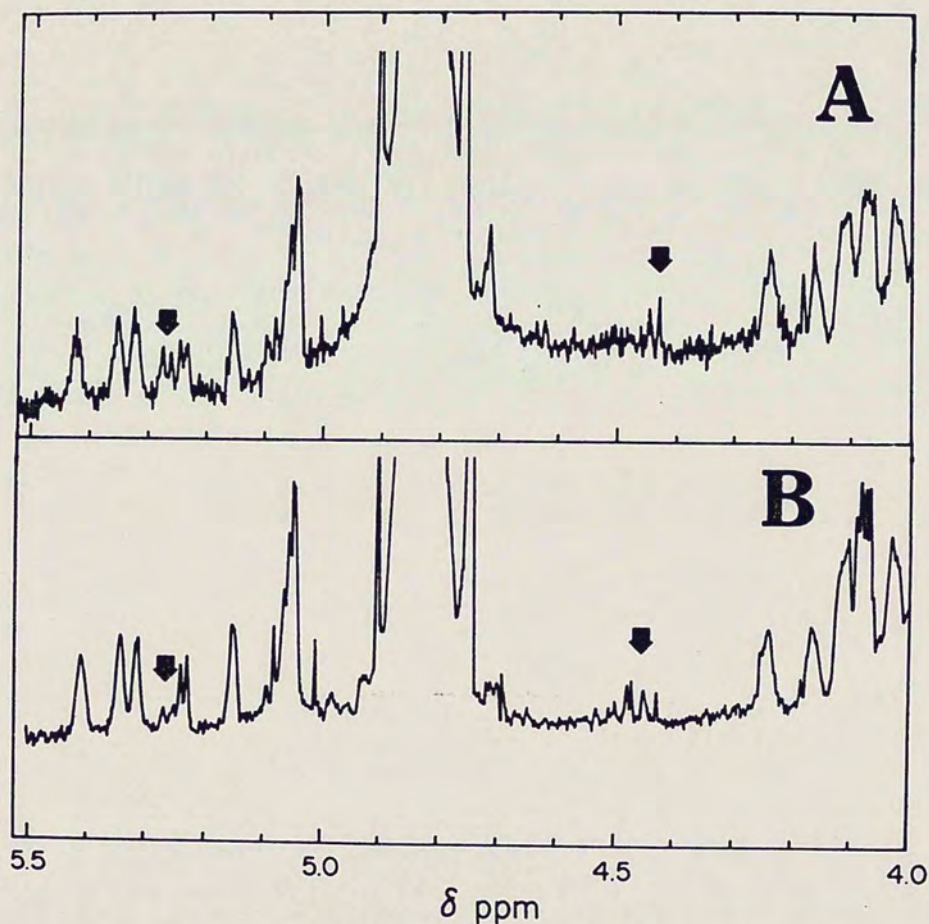


Figure 22. NMR spectrum of endo-H treated glycopeptide--250 MHz proton NMR spectroscopy of vitellogenin oligosaccharides isolated from Bio-Gel P-6 gel permeation after endo-H treatment. The arrows indicate the presence of unidentifiable resonance peaks located in the H-1 and H-2 regions of the spectrum $\delta = 4.0$ to 5.5 ppm. The presence of additional doublet peaks at $\delta = 5.22$ ppm not observed in the original spectrum are due to the removal of the single Asn-linked GlcNAc cleaved by the enzyme.

Discussion

Physical and chemical properties

As in the case of other Lepidoptera, M. sexta vitellogenin is composed of two apoproteins, namely apoVg-I (M_r 180,000) and apoVg-II (M_r 45,000). The protein falls into group 1 vitellogenins proposed by Harnish and White (1982). This group is distinguished by having two distinct apoprotein sizes (large and small). Also classified under this group are the orthopterans, ephemeropterans, and dermapterans. The numbers of large and small apoproteins vary in the different orders of insects in this group. Lepidopteran vitellogenins differ in this regard from a variety of other insects. For example, L. migratoria vitellogenin is composed of about eight separate polypeptides with molecular weights in the range M_r 52,000-140,000 (Chen et al., 1978) while D. melanogaster vitellogenin (group 3) has only three closely related small polypeptides (M_r 44,000, 45,000, 46,000) (Warren and Mahowald, 1979).

Using isolated M. sexta vitellogenin apoproteins, a complete amino acid analysis was carried out (Table 2). As in other insect proteins in general, both vitellogenin apoproteins contained relatively high percentages of aspartate and glutamate. The lower specific absorbance of apoVg-I at 280 nm compared to apoVg-II can be accounted for by the differences in tryptophan content of the apoproteins (0.27 mol percent in apoVg-I compared to 0.43 mol percent

in apoVg-II). However, the amino acid composition of the apoproteins did not differ significantly from the average values for other proteins in general (Dayhoff, 1973).

Synthesis of vitellogenin by the fat body

Vitellogenin was shown to be synthesized and secreted by the fat body in vitro. This tissue is the main site of vitellogenin synthesis in most insects so far examined (Hagedorn and Kunkel, 1979). Exceptions to this are found in D. melanogaster (Bownes, 1980) and Glossina austeni (Heubner et al., 1975) where vitellogenin synthesis also occurs within the ovary. In addition to vitellogenin, the fat body of adult females synthesizes many other other proteins. Notable among these is the newly discovered female specific protein, microvitellogenin ($M_r \approx 31,000$) (Fig. 12, arrow). A parallel experiment with follicles showed that no radioactivity was incorporated either into the apoproteins of vitellogenin or microvitellogenin. This indicated that in M. sexta, the follicles do not synthesize these proteins.

Phosphorylation

Both apoVg-I and apoVg-II are phosphoproteins as shown by methyl green staining (result not shown) and in vivo labeling with ^{32}P . The phosphate was found to be attached only to serine residues. The location of phosphate groups on the polypeptide backbone and their functions are questions that need to be addressed.

Since apoVg-I is also glycosylated, the possibility that some of the phosphate groups on this apoprotein may be located on the carbohydrate moiety was examined. The results showed that although most of the phosphate groups on apoVg-I are on the protein, a small amount was probably associated with the carbohydrate moiety. The phosphorus contents of M. sexta vitellogenin (0.6 percent) is considerably lower than that reported for R. americana vitellogenin (1.33 percent) (Pereira and Bianchi, 1983) but higher than in L. migratoria vitellin (0.3 percent) (Chen et al., 1978) or L. maderae vitellin (0.14 percent) (Engelmann and Friedel, 1974).

Immunological properties

Rabbit antisera against isolated vitellogenin apoproteins were used to show that apoVg-I and apoVg-II are distinct polypeptides since no immunological cross-reactivity was observed. Immunodiffusion studies using the same antisera provided some information about the arrangement of the apoproteins in the intact molecule. The fact that antiserum against vitellogenin and apoVg-I formed precipitin lines with vitellogenin while anti-apoVg-II did not, suggested that apoVg-II is not accessible to the aqueous environment in the intact molecule. Indeed, limited trypsinization of vitellogenin showed that apoVg-II was initially not accessible to the enzyme (Fig. 8). However, as more of apoVg-I was broken down, apoVg-II was degraded. The structural organization of M. sexta vitellogenin and lipophorin, the major lipid

transport protein, show some interesting parallels. As in vitellogenin, lipophorin apoproteins (apoLp-I and apoLp-II) are immunologically distinct from each other (Shapiro et al., 1984) and only the larger apoprotein (apoLp-I) is readily degraded by limited trypsin digestion (Pattnaik et al., 1979). More studies are needed to find out if this arrangement of the apoproteins is common to all insect lipoproteins. The similarities between vitellogenins of M. sexta and H. cecropia were examined by the immunoblotting technique. Antisera to M. sexta vitellogenin and its apoproteins showed the same binding specificities in H. cecropia vitellogenin as in M. sexta vitellogenin. It is evident from these results that vitellogenins of H. cecropia and M. sexta have similar structural features.

Carbohydrate moiety

This work has confirmed a previous report on the composition of the carbohydrate moiety of M. sexta (Mundall and Law, 1979). FITC-Con A and PAS staining was used to show that the carbohydrate moiety is located only on apoVg-I. Furthermore, all the [³H]Man in vitellogenin was found on apoVg-I. The ability of endo-H to remove the carbohydrate moiety provided evidence for presence of the chitobiosyl-Asn linkage (Yamashita et al., 1978). Since all the carbohydrate moiety was completely removed by endo-H, it is unlikely that any O-glycosidic linked carbohydrate structures exist in M. sexta vitellogenin.

This study reports the first complete primary structure of the carbohydrate moiety of an insect vitellogenin using high field proton NMR spectroscopy (see also Osir et al., 1986). The structure is essentially identical in all respects to that of N-glycosidically linked $\text{Man}_9\text{GlcNAc}_2$ oligosaccharide found in soybean agglutinin glycopeptide (Dorland, 1981) and in other mammalian glycoproteins such as porcine thyroglobulin (van Halbeek et al., 1980 a, b). The existence of the same structure in insect glycoproteins suggests that the method for synthesizing N-glycosidically linked oligosaccharides occurs in a general manner throughout the plant and animal kingdoms. The mechanism by which glycosylation occurs in insects appears to be similar and involves the transfer of the carbohydrate unit, $(\text{Glu})_3(\text{Man})_9(\text{GlcNAc})_2$, from a lipid intermediate to an Asn in the polypeptide backbone followed by a rapid removal of the terminal glucose residues on the α (1 \rightarrow 3) arm (Montreuil et al., 1980) (Fig. 21). This assumption is supported by the discovery of the lipid intermediate, dolichol phosphate, in the microsomes of the mediterranean fruit fly, Ceratitis capitata (Allue, 1980). The existence of two sets of unrecognizable chemical shifts in minor amounts suggests the presence of a small amount of heterogeneity in the structure. This finding contrasts with results reported in a recent paper on cockroach vitellins (Nordin et al., 1984). It was observed that the carbohydrate moieties of these vitellins showed a great deal of heterogeneity indicating extensive processing. In this dissertation, the minor peaks shown in the Bio-Gel P-6 profile of

pronase glycopeptides (Fig. 17 A) were not analyzed since their carbohydrate contents were very small. The primary structure presented is the "main structure" present. The minor peaks which were not analyzed may well represent some processed structures. It is also likely that some variations exist among different insects. Until the carbohydrate structures of vitellogenins from many insects are determined, attempts to make broad generalizations on the different structures that may exist should be avoided.

The minor chemical shifts in the area of $\delta \approx 4.4$ to 4.5 ppm are as yet unidentified. The second set of unidentifiable shifts at $\delta = 5.263$ and 5.248 are enriched in the higher molecular subfraction of the glycopeptide. This subfraction is only slightly resolved from the bulk of the major structure. It is present after release from the peptide portion by treatment with endo-H and is thus unlikely to be a result of interaction with polypeptide or other environmental effects. The presence of additional sugars such as glucose is unlikely because the difference in molecular weight would be expected to cause a more prominent shift in in the Bio-Gel P-6 column profile. Another possibility is the presence of phosphate which would account for the slight difference in molecular weight. A phosphate group would not in itself have a proton resonance signature but could cause an environmental effect on other protons of contributing sugars. Additional modification studies will be required to find the cause of these unusual peaks.

CHAPTER 3

STUDIES ON BINDING AND UPTAKE OF VITELLOGENIN.

Introduction

The major component of yolk in insect eggs is called vitellin. In most insects groups, vitellin is derived from a hemolymph precursor, vitellogenin (Engelmann, 1979). However, in a few cases, the ovary itself has the capacity to synthesize vitellogenin. For example, in D. melanogaster and other related species, follicle cells supplement vitellogenin made by the fat body (Postlewait et al., 1980). In another dipteran, Glossina austeni, yolk is presumably assembled primarily from follicle cell products (Heubner et al., 1975). In addition to vitellin, yolk contains other extra-ovarian proteins. An important example is the female specific protein, microvitellogenin (Kawooya and Law, 1983; Telfer and Kulakosky, 1984). Like vitellogenin, microvitellogenin is synthesized by the fat body, secreted into hemolymph and then taken up by the follicles. The hemolymph contains many other proteins that are not concentrated by the ovaries. For instance, the major hemolymph lipoprotein, lipophorin, is found only in trace amounts within the yolk (Chino et al., 1981; Kawooya, Osir and Law, unpublished observation). From the examples given above, it is apparent that insect eggs have a specific mechanism(s) for selectively extracting

proteins from the hemolymph. A likely candidate for such a mechanism is a receptor-mediated endocytotic process. The discovery of coated pits in mosquito oocytes by Roth and Porter (1964) was the first indication that vitellogenin uptake is indeed a receptor mediated process. Since this discovery, other endocytotic processes have been extensively investigated (Goldstein et al., 1979 a; Pastan and Willingham, 1981), but the insect ovary has been largely neglected as an appropriate object for investigations at the molecular level. The insect ovary is more complicated than simple systems like the fibroblast LDL system. Eggs develop in long strands or ovarioles with the oldest at the posterior end. In Lepidoptera, each egg is surrounded by a mass of follicle cells, which ultimately secrete the chorion, and nurse cells, which provide some proteins and RNA (Koch and King, 1966). A single follicle, at mid-development, has a monolayer of mixed follicle and nurse cells surrounding it (Wigglesworth, 1972). It is generally believed that the follicle cells separate to reveal the oocyte membrane, in which specific receptors bind vitellogenin from the hemolymph (Telfer et al., 1981). The very active endocytotic process necessarily entraps small amounts of other hemolymph or foreign proteins which are thereby coincidentally taken into the oocyte (Telfer, 1960).

Vitellogenin uptake may be conveniently divided into three intricately interrelated processes. The first step involves specific recognition and binding of vitellogenin to putative receptors on the oocyte surface. This is then followed by the actual endocytosis.

The transfer of vitellogenin, now called vitellin, into yolk granules might constitute the final process. Although some of the processes mentioned above have been examined independently, the overall picture of what happens during vitellogenin uptake cannot become clear until all the information becomes available.

Isolated labeled proteins have been used as physiological probes for studying the uptake process in vitro (Anderson, 1971; Ferenz et al., 1981; Kunkel and Pan, 1976; Lange and Loughton, 1980; Osir and Law, 1984; Rohrkasten and Ferenz, 1985 and Telfer and Kulakosky, 1984). However, no serious attempts have so far been made to use labeled vitellogenin as a ligand for identifying and characterizing specific binding sites on the egg membranes. In this chapter, an in vitro system for the uptake of ^{125}I -labeled vitellogenin into isolated follicles is described. Furthermore, a membrane binding assay for identifying specific binding sites on follicle membranes is presented. It is proposed that these binding sites are responsible for specifically recognizing vitellogenin in the hemolymph during uptake.

Materials and Methods

Isolation of vitellin

Eggs were dissected from freshly eclosed insects and homogenized in lepidopteran saline containing 50 mM glutathione and 20 mM of DFP. The egg homogenate was centrifuged (14,000 x g, 10 min) in a microcentrifuge (model 235 A, Fisher) in order to remove egg membranes. The homogenate was then dialyzed against 0.08 M sodium phosphate buffer, pH 7.0 and applied to a DEAE Bio-Gel A column (1.5 x 12 cm) equilibrated with the same buffer. After washing with two column volumes to remove non-adsorbed proteins, vitellin was eluted with a continuous NaCl gradient (0 to 0.2 M) using a gradient maker (BRL). Fractions (2.5 ml) were collected and the absorbance monitored at 280 nm. Fractions that contained purified vitellin were concentrated by ultrafiltration using Amicon YM 10 membranes (M_r cut off 10,000) and stored under nitrogen at 4°C. Freshly prepared samples were used for all experiments.

Deglycosylation

Vitellogenin (100 µg) was incubated with endo-H (42 unit/ml) in endo-H buffer containing 10 mM of DFP for 30 min at room temperature as described in chapter 2. After incubation, the sample was chromatographed on Con A Sepharose column in order to separate the deglycosylated protein from unreacted protein. Complete

deglycosylation was confirmed by FITC Con A binding as described in chapter 2.

Iodination of proteins

Vitellogenin, deglycosylated vitellogenin and vitellin were radioiodinated using Iodobeads (Pierce) as described by Markwell (1982). Approximately 50 μg of each protein in PBS was mixed with 0.3 mCi of $\text{Na}[^{125}\text{I}]$ (Amersham, specific activity $\approx 15 \mu\text{Ci}/\mu\text{g}$) and incubated with three Iodobeads for 15 min at room temperature. The reactions were stopped by pipetting the sample away from the Iodobeads. Free iodide was removed by passing the samples through Bio-Gel P-6DG desalting columns (1 x 21 cm) equilibrated and eluted with PBS. Prior to this, the column was washed with PBS containing 2 mg/ml BSA to reduce any binding of labeled proteins to the column. The specific activity of labeled vitellogenin or vitellin prepared in this way was approximately 5×10^5 cpm/ μg protein. These proteins had the same chromatographic and electrophoretic properties as the unlabeled samples. Lipid extraction by the method of Bligh and Dyer (1959) showed that approximately 4 percent of the radioactivity in either vitellogenin or vitellin was incorporated into lipids.

Incubation of follicles

Ovarioles containing developing follicles were carefully dissected from 1 day old female insects in lepidopteran saline. Under a dissecting microscope the muscular sheath covering the follicles was

carefully removed and the lengths of individual follicles measured using an ocular micrometer. Undamaged follicles (~ 0.8 mm in length) were transferred to plastic pipette tips sealed at one end. The incubation media had 200 μ l of lepidopteran saline containing 2 mg/ml BSA and either vitellogenin or vitellin (specific activity ~ 2×10^4 cpm/ μ g). After incubation (27°C or 4°C), the follicles were washed three times with 50 μ l of lepidopteran saline. Radioactivity associated with the follicles was determined in a gamma counter (model 4000 counting system, Beckman).

Trypsin treatment

Follicles were incubated with labeled vitellogenin as described above either at 4°C or 27°C. After incubation for 1 h, the follicles were washed with saline and transferred into a medium containing trypsin (1 mg/ml, Worthington). After further incubation for 1 h (4°C or 27°C), the follicles were washed and radioactivity determined. A control incubation with no trypsin was included.

Preparation of follicle membranes

Follicles of approximately 0.8 mm in length dissected from one day old female insects were used as the source of follicle membranes. The membrane complex used in this experiment consisted of follicle basement membrane, oocyte membranes and follicular cells. The follicles were first punctured to release yolk and membrane complex was washed 3-4 times with ice cold lepidopteran saline to

remove any adhering yolk. The membranes were thoroughly homogenized in a glass homogenizer and centrifuged (15,000 x g, 10 min, 4°C) in a microcentrifuge (model 235 A, Fisher). To obtain a uniform suspension the membrane pellet was resuspended in an ice cold buffer (20 mM MES , pH 8.0, 5 mg/ml BSA, for 30 min) and sonicated briefly (microprobe, setting No. 6, 8 sec.) on a sonifier cell disruptor (model 200, Branson Sonic Power Co.) on ice. Only freshly prepared membranes were used for the binding studies.

Binding of labeled vitellogenin to follicle membranes

Binding assays were carried out in polyethylene Eppendorf tubes (1.5 ml). In order to reduce non-specific binding of labeled protein to walls of the tubes, they were coated by soaking in polyethylene glycol (1 percent, w/v). The reaction mixture contained 70 μ l of the binding buffer (20 mM MES, pH 6.8, 5 mg/ml BSA, 5 mM CaCl_2), approximately 20 μ g of the homogenized follicle membrane preparation (protein content measured by the Pierce BCA protein assay reagent) and different amounts of ^{125}I -labeled vitellogenin, as indicated in the appropriate figure legends. Non-specific binding of labeled vitellogenin to the membranes was determined by carrying out binding assays in the presence of 150-fold molar excess of unlabeled vitellogenin. The specific activity of vitellogenin used in binding studies was approximately 3×10^5 cpm/ μ g protein. After incubation (70 min, 4°C), 300 μ l of the binding buffer was added to the reaction mixture and the tubes centrifuged (10,000 x g, 5 min, 4°C) in a

microcentrifuge (model 235 A, Fischer). The supernatant solutions were carefully removed with a Pasteur pipette and the pellets washed three times with the same buffer by recentrifugation as described above. Preliminary experiments showed that little or no radioactivity was released after three washing steps. The lower tip of the tubes were sliced in half and placed in gamma counting vials. Radioactivity was determined in a gamma counter. Since approximately 10 percent of the radiolabeled protein was found to bind to Eppendorf tubes, blank tubes were run exactly as in the experimental tubes except that membranes were omitted. The counts in the blanks were subtracted from the the total counts bound in each experimental tube.

For competition studies, excess unlabeled vitellin, deglycosylated vitellogenin, apoVg-II, microvitellogenin (gift of Dr. John Kawooya, Department Biochemistry, University of Arizona) and lipophorin were each tested for their abilities to compete with labeled vitellogenin for binding sites on the follicle membranes.

Results

Isolation of vitellin

Vitellin purification utilized a single step of anion-exchange chromatography on DEAE-Bio-Gel A column. The elution profile is shown in Figure 23. Vitellin (fraction II) was identified on the basis of its similarities with vitellogenin on SDS-PAGE. Purity of the samples was verified by SDS-PAGE (Fig. 24).

In vitro uptake of labeled vitellogenin by isolated follicles

Each M. sexta ovariole contains more than 200 follicles of varying lengths. The oldest follicle (~ 1.0 mm in length at the time of adult emergence, and reaching a maximum length of 1.9 mm when mature) is found at the posterior end of the ovariole and the lengths decrease towards the anterior tip (Nijhout and Riddiford, 1979). Insect egg follicles must achieve a certain critical size and age before becoming competent to incorporate vitellogenin in vitro (Telfer and Anderson, 1968). On the other hand, they lose this ability once they attain a larger size. Competent follicles were found by investigating the abilities of follicles with varying lengths to incorporate vitellogenin in vitro. The ability to incorporate labeled vitellogenin varied with the follicle length (Table 5). The greatest uptake was achieved with follicles between approximately 0.7 mm and 1.3 mm in length. Follicles less than 0.6 mm or larger than 1.6 mm

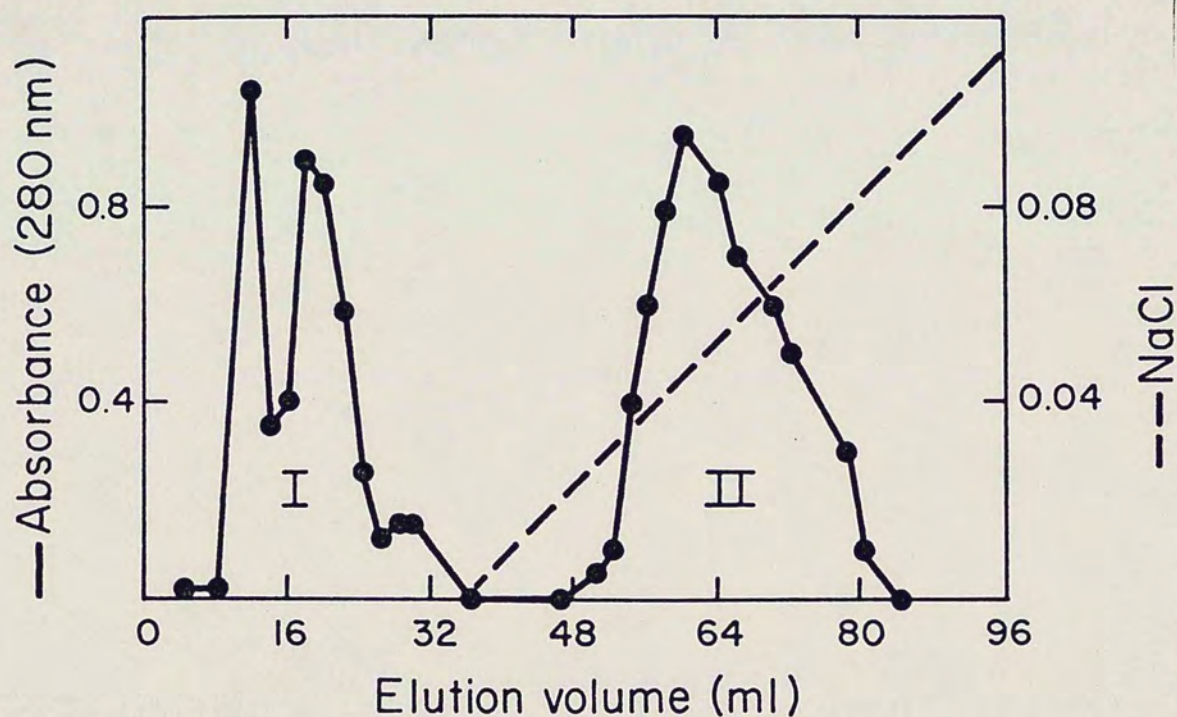


Figure 23. Elution profile of vitellin from DEAE-Bio-Gel A column—Egg homogenate was dialyzed against 0.08 M sodium phosphate buffer, pH 7.0 and applied to DEAE-Bio-Gel column (1.5 x 12 cm) equilibrated with the same buffer. After washing the column to remove non-adsorbed proteins, vitellin was eluted using a continuous gradient, 0.0–0.2 M NaCl (----). Samples containing vitellin (peak II) were pooled.

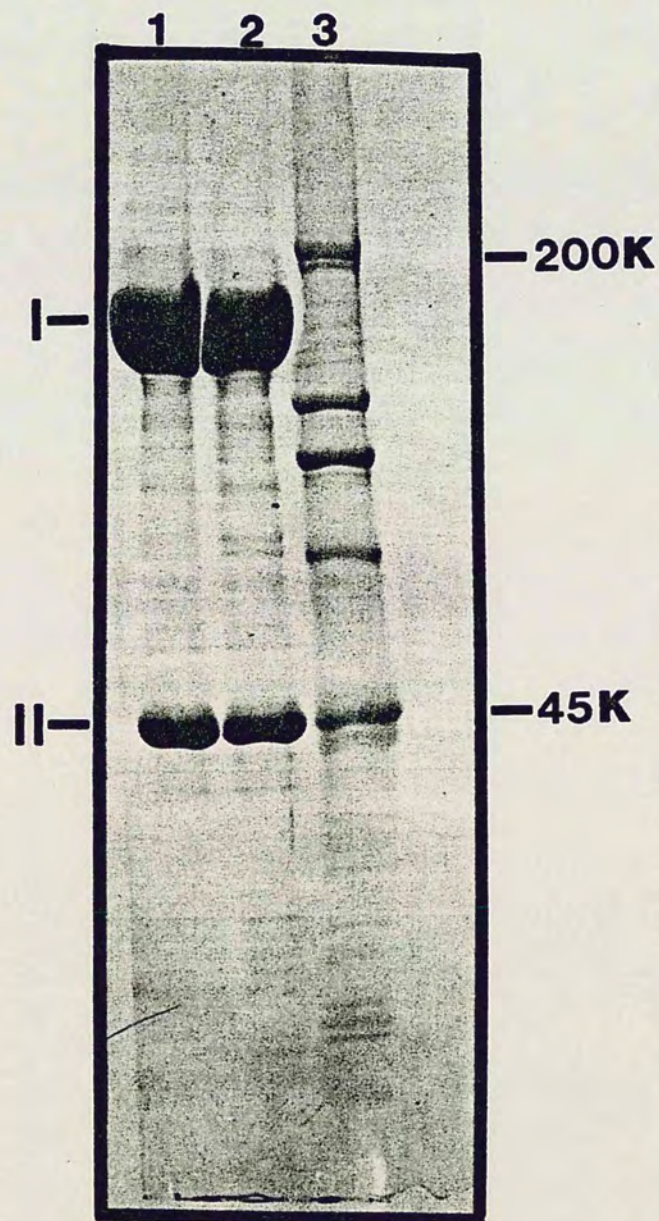


Figure 24. SDS-polyacrylamide gel of vitellin and vitellogenin--1. Vitellogenin (25 µg), 2. Vitellin (30 µg,) and 3. High molecular weight standards (lane 3) (Bio-Rad) were separated by SDS-PAGE (4-15 percent). The gel was stained using Coomassie Brilliant Blue. I = apovitellenin-I; II = apovitellenin-II.

Table 5. Relationship between follicle length and vitellogenin uptake in vitro

Follicle Length (mm)	^{125}I -VG Taken Up ($\mu\text{g protein follicle}^{-1} \text{ h}^{-1}$)
0.50	0.115 \pm 0.010
0.62	0.350 \pm 0.130
0.70	0.621 \pm 0.110
0.80	1.218 \pm 0.014
1.02	0.937 \pm 0.042
1.60	0.465 \pm 0.015
1.70	0.258 \pm 0.014

Follicles (0.5–1.7 mm) were incubated in lepidopteran saline (200 μl) containing ^{125}I -VG (150 $\mu\text{g/ml}$, specific activity = 2×10^4 cpm/ μg). After incubation (1 h, 27°C), the follicles were washed and radioactivity determined using a gamma counter as described (Materials and Methods). The values are mean \pm S.D (n = 4).

were less able to take up labeled vitellogenin. The uptake of labeled vitellogenin was also examined as function of the incubation time. As shown in Figure 25, uptake increased linearly during the entire incubation period at 27°C (~ 1.13 µg/follicle/h). Under these experimental conditions, uptake usually decreased after about 4 h. The uptake of ¹²⁵I-vitellin was found to be identical to that of vitellogenin under the same experimental conditions.

In order to assess the possible role of the carbohydrate moiety on uptake, the uptake of ¹²⁵I-endo-H treated vitellogenin was examined (Fig. 26). The results showed that there was no significant difference in uptake between the endo-H treated and the control sample, suggesting that the carbohydrate moiety is not required for the incorporation of vitellogenin into isolated follicles.

Distribution of labeled vitellogenin in isolated follicles

The distribution of labeled vitellogenin between the follicle membranes and yolk after incubation at 4°C and 27°C is shown in Table 6. Most of the labeled protein was localized on the follicle membranes after incubation for 1 h at 4°C. On the other hand, the yolk had most of the labeled protein at 27°C. Since most of the labeled vitellogenin was on the follicle membranes at 4°C, the effect of trypsin on membrane-bound vitellogenin was investigated. Trypsin treatment reduced the radioactive protein associated with the follicles at 4°C by 70 percent compared to a control incubation in

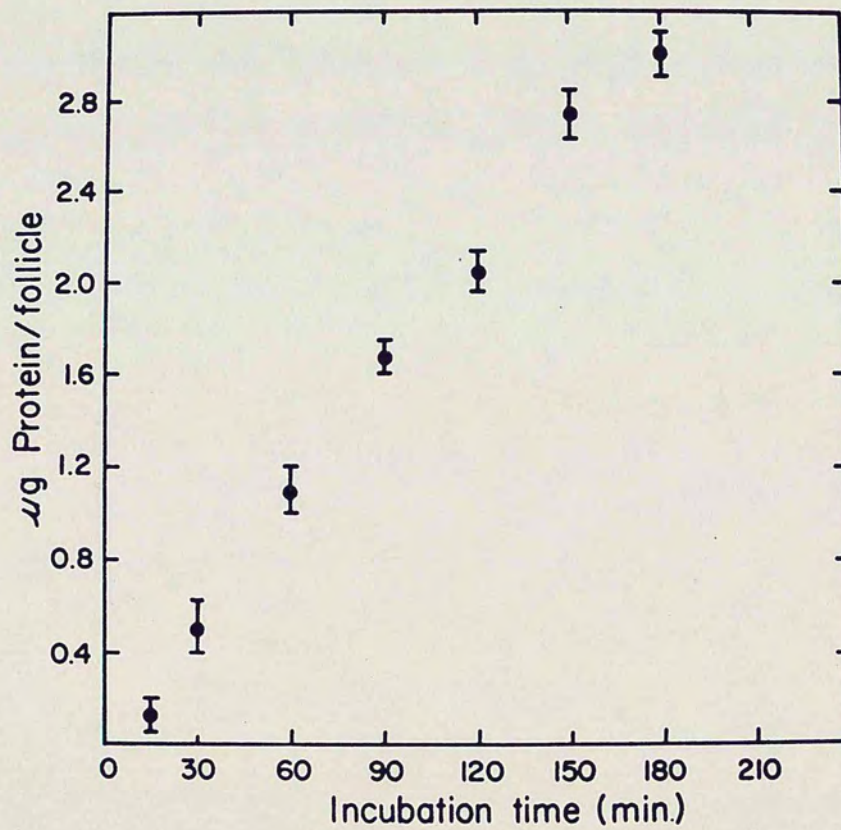


Figure 25. Uptake of vitellogenin by isolated follicles-- Pairs of follicles were incubated at 27°C with ^{125}I -vitellogenin. ($\sim 150 \mu\text{g/ml}$, $2 \times 10^4 \text{ cpm}/\mu\text{g}$) At the end of each incubation time, the follicles were washed and radioactivity determined as described (Material and Methods). The data shown are mean \pm S.D (n = 4).

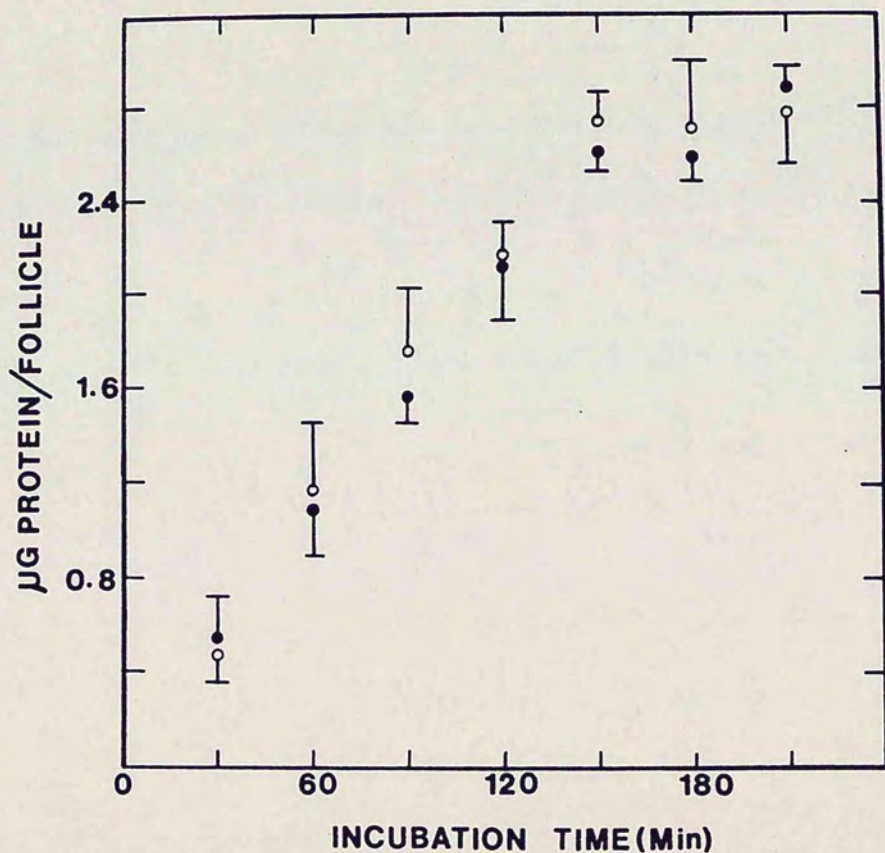


Figure 26. Uptake of endo-H (●) and non endo-H treated (○) vitellogenin by isolated follicles—Pairs of follicles were incubated with ^{125}I -VG samples ($\sim 75 \mu\text{g/ml}$) for up to 3 h at 27°C . After incubation, the follicles were washed and radioactivity determined in a gamma counter as described (Materials and Methods). Each data point represents mean \pm S.D (n = 4).

Table 6. Distribution of labeled vitellogenin in isolated follicles

Temperature (°C)	Radioactivity (cpm)			Percent	
	Total	Membranes	Yolk	Membranes	Yolk
4°	10,552	9,692	830	92.1	7.8
27°	26,888	2,150	24,466	7.9	90.0

Follicles were incubated with ^{125}I -VG for 1 h at 4°C or 27°C as described in Table 5. After incubation, follicles were washed and then punctured to release yolk contents. The follicle membranes were washed to remove any adhering yolk. Radioactivity associated with the follicle membranes and yolk including the washes were separately determined as described (Materials and Methods). Data are the averages of three incubations.

which trypsin was not added. On the other hand, most of the labeled vitellogenin was taken up into the follicles at 27°C and therefore not accessible to trypsin.

Characterization of specific binding to follicle membranes

Since vitellogenin is selectively incorporated by isolated follicles in vitro, we used ^{125}I -labeled vitellogenin to assay for specific binding sites on the homogenized follicle membranes prepared as described under Materials and Methods. Since receptor-mediated binding is a dynamic process, an experiment was carried out to determine the length of incubation time required for the binding reaction to reach equilibrium. The results showed that specific binding of ^{125}I -vitellogenin to the follicle membranes reached equilibrium after about 60 min of incubation at 4°C (Fig. 27) or 27°C (results not shown). All subsequent incubations were carried out for 70 min, sufficient time for the binding reactions to reach equilibrium. Incubations were carried out at 4°C because isolated vitellogenin is more stable at this temperature.

Binding of increasing amounts of vitellogenin to a fixed amount of follicle membrane preparation was studied at equilibrium. The results showed that specific binding increased to a saturation level (Fig. 28). In most experiments, non-specific binding was between 17 and 25 percent of specific binding. These results suggested the presence of specific saturable binding sites for vitellogenin on the follicle membranes. Analysis of the binding data

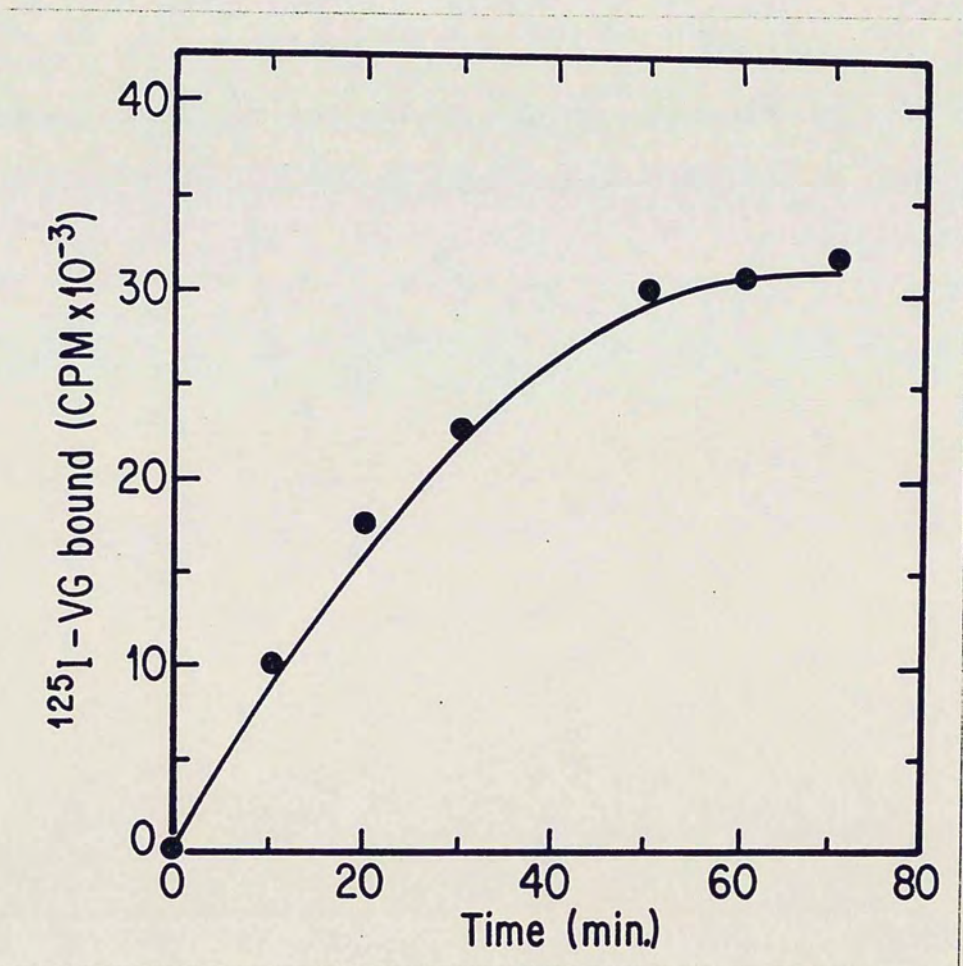


Figure 27. Time course of vitellogenin binding to follicle membranes—Membrane preparation ($\sim 20 \mu\text{g}$ protein) was incubated with $\sim 30 \mu\text{g}/\text{ml}$ of ^{125}I -VG at 4°C from 0-70 min. After incubation, bound vitellogenin was determined as described (Materials and Methods). In this experiment, incubation was considered to end when centrifugation began. All values were corrected for non-specific binding to the incubation tubes. Each point is the average of two determinations.

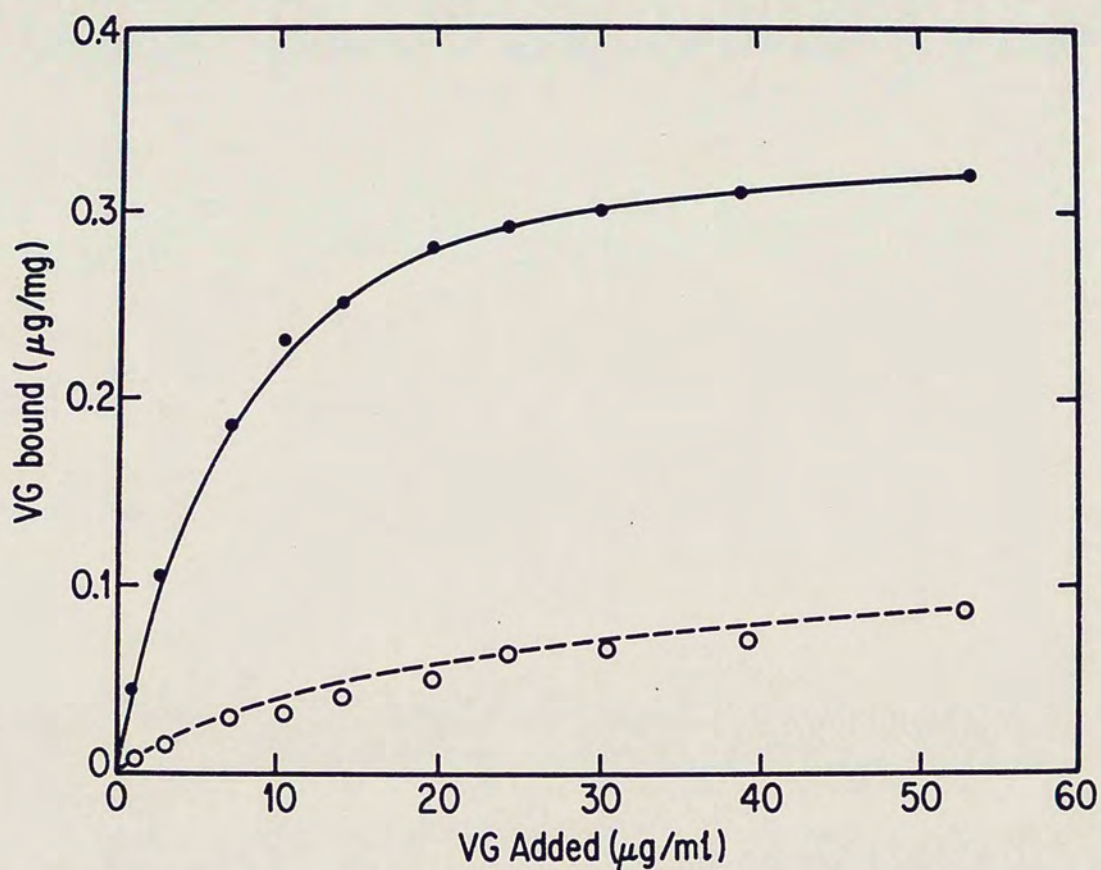


Figure 28. Concentration-dependent binding of vitellogenin to follicle membranes—A fixed amount of membranes ($\sim 20 \mu\text{g}$ protein) was incubated with increasing amounts of ^{125}I -VG in the presence or absence of excess unlabeled VG. After incubation (70 min, 4°C), bound VG was determined in duplicate aliquots as described (Materials and Methods). Specific binding (●) was calculated as the difference between total and non-specific binding (○). All values were also corrected for non-specific binding to the incubation tubes.

by the method of Scatchard (1949) yielded a linear plot indicating the presence of only one class of binding sites (Fig. 29). The apparent equilibrium dissociation constant (K_D) as calculated from the slope of the Scatchard plot ($1/K_D$) was $\sim 1.3 \times 10^{-8}$ M. The maximum amount of vitellogenin bound was deduced from the x-intercept of the same plot to be 0.36 ± 0.001 $\mu\text{g}/\text{mg}$ of membrane protein. Molar concentrations were based on a native molecular weight of M_r 5×10^5 for M. sexta vitellogenin (Chapter 2).

Binding of vitellogenin was found to be specific to the follicle membranes since the fat body and gut tissue membranes did not show any specific binding activity. The influence of pH on binding of labeled vitellogenin to the follicle membrane preparation was examined by varying the pH of the binding buffer from pH 3 to 8 with Tris-Malate. The apparent pH optimum was found to be between 6 and 7 (Fig. 30). The pH of M. sexta adult female hemolymph is approximately 7. As shown in Figure 31, binding to follicle membranes required calcium. The amount of protein bound increased as the amount of calcium in the incubation medium was increased from 0 to 6 mM. Further increase in the cation did not result in any increase in the amount of ligand bound. Under the same conditions, the influence of magnesium on vitellogenin binding was identical to that of calcium.

Competition studies

In order to determine the specificity of vitellogenin binding, the abilities of various hemolymph proteins to compete with

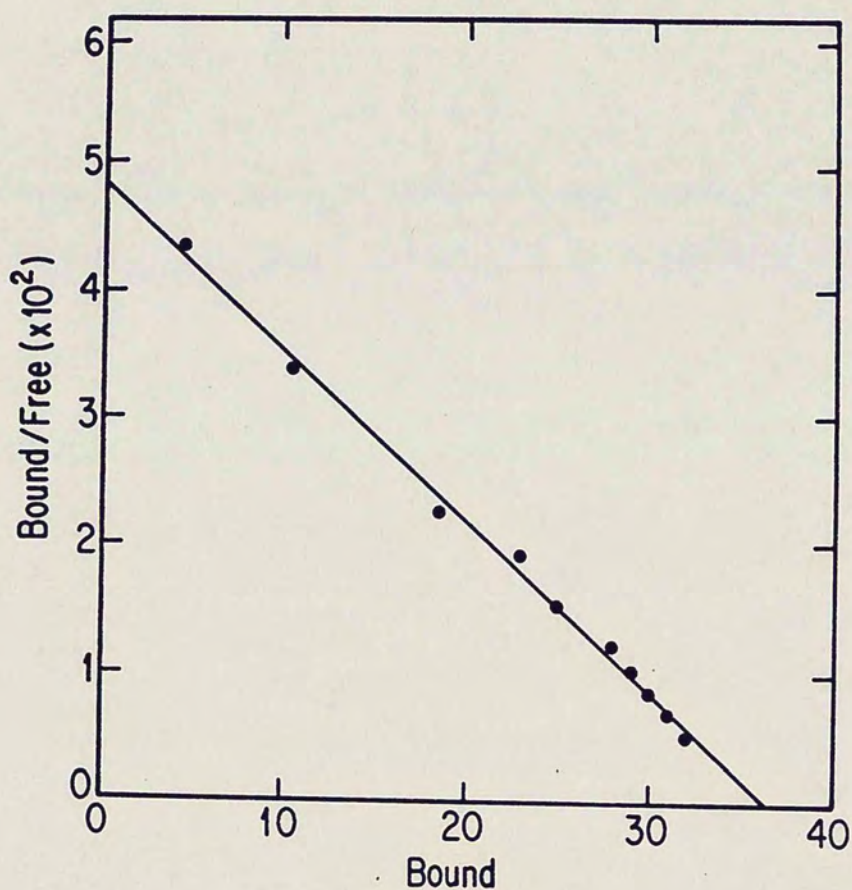


Figure 29. Scatchard analysis of binding data--The ordinate represents amount of vitellogenin (VG) bound ($\mu\text{g}/\mu\text{g}$ membrane protein) and the abscissa the amount of VG bound divided by VG free. The line was drawn by least squares analysis of the binding data. The slope ($1/K_D$) = $6.41 \pm 0.004 \mu\text{g}/\text{ml}$ (correlation coefficient = -0.997). The x-intercept (B_{max}) = $0.36 \pm 0.001 \mu\text{g}/\text{mg}$ of membrane protein.

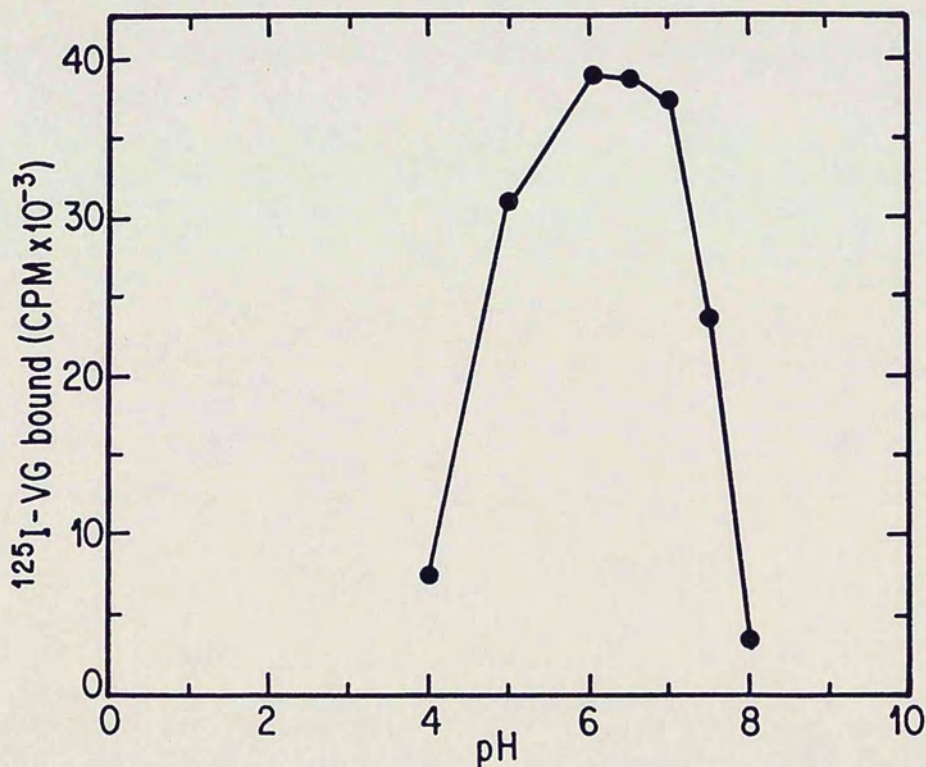


Figure 30. pH dependency of vitellogenin binding to follicle membranes—Binding of $^{125}\text{I-VG}$ ($30 \mu\text{g/ml}$) to a fixed amount of membranes ($\sim 20 \mu\text{g protein}$) was measured under different buffer pH conditions. The buffer pH was adjusted using Tris-Malate and bound VG was determined as described (Materials and Methods). The data was corrected for non-specific binding to the incubation tubes. Each point represents the average of two determinations.

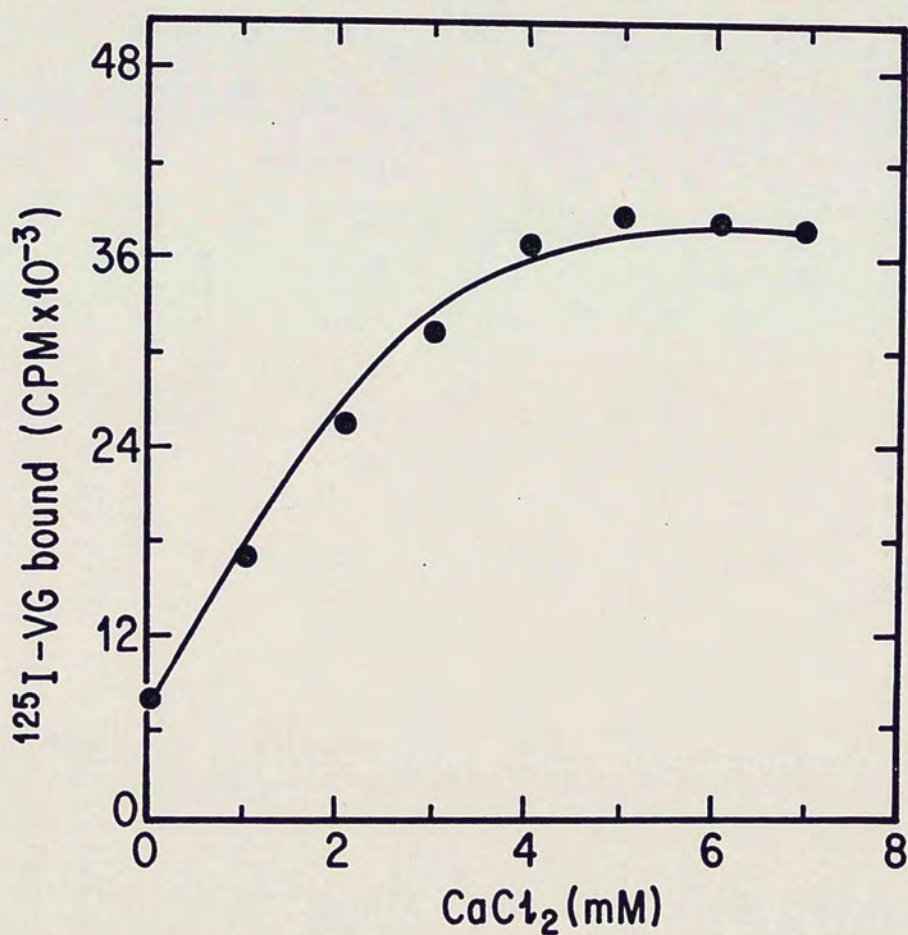


Figure 31. Calcium dependency of vitellogenin binding to follicle membranes—¹²⁵I-VG (30 $\mu\text{g}/\text{ml}$) was added to membranes (~ 20 μg protein) prepared as described (Materials and Methods) except that calcium was omitted from all buffers. To different reaction mixtures, increasing amounts of calcium (0–6 mM) was added and incubations carried out for 70 min at 4°C. Bound VG was determined as described (Materials and Methods). The data was corrected for non-specific binding to the incubation tubes. Each point is the average of two determinations.

labeled vitellogenin for binding to follicle membrane binding sites was examined. If these proteins bound to the same site as vitellogenin, then competition would result in a decrease in the labeled vitellogenin bound to follicle membranes. Lipophorin is the major insect hemolymph lipoprotein and a known constituent of insect eggs (Chino *et al.*, 1977), while microvitellogenin is a 31,000 dalton female-specific protein found in both hemolymph and eggs (Kawooya and Law, 1983; Telfer and Kulakosky, 1984). The results showed that binding could be significantly reduced by addition of unlabeled excess vitellin but not by apoVg-II, lipophorin or microvitellogenin (Table 7). Binding was also inhibited by deglycosylated vitellogenin suggesting that the carbohydrate moiety is not involved in the interaction with the binding sites.

Table 7. Effects of various proteins on binding of vitellogenin to follicle membranes

Addition to Incubation Medium	Percent Inhibition
Vitellin (6 mg/ml)	87.0 ± 0.31
Deglycosylated vitellogenin (7.5 mg/ml)	85.3 ± 0.67
Lipophorin (10 mg/ml)	2.5 ± 1.37
ApoVg-II (1.35 mg/ml)	3.1 ± 1.16
Microvitellogenin (0.9 mg/ml)	4.3 ± 2.60

Labeled vitellogenin (30 μ g/ml) was incubated with membranes (\sim 20 μ g protein) in the presence of each of the unlabeled proteins shown above. After incubation (70 min, 4°C), bound VG was quantitated as described (Materials and Methods). The data were corrected for non-specific binding to the incubation tubes. Values above (expressed as percent of control binding) are mean \pm S. D. (n = 5).

Discussion

In vitro uptake of vitellogenin

Incorporation of insect hemolymph proteins into eggs has been demonstrated in vivo (Kunkel and Pan, 1976; Ferenz, 1978) and in vitro (Lange and Loughton, 1980; Ferenz et al., 1981; Osir and Law, 1984; Telfer and Kulakosky, 1984). Foreign egg proteins have been tested for in vitro uptake and shown to be taken up by the host ovaries at lower rates than those of host proteins (Kunkel and Pan, 1976). Foreign non-egg proteins are also taken up at lower rates, probably by non-receptor mediated endocytosis. In approaching the study of vitellogenin uptake, we first established an in vitro system using isolated follicles. Individual follicles were isolated by carefully removing the muscular sheath around them. Since follicle size was found to be an important factor in vitellogenin uptake, all experiments were carried out using follicles of about 0.8 mm in length. The large size of the follicles permitted gross dissection, and thus enabled the assessment of the distribution of labeled vitellogenin between the yolk and follicle membranes after incubation at 4°C and 27°C. At 4°C, labeled vitellogenin was associated with the follicle membranes. This observation is consistent with the fact that, although ligands can bind to receptors at 4°C, only limited internalization occurs (Goldstein et al., 1979 a; Carpenter, 1979). Furthermore, approximately 70 percent of labeled vitellogenin bound at

4°C was removed by trypsin treatment. On the other hand, most of labeled vitellogenin was internalized at 27°C and therefore inaccessible to trypsin. These results are in close agreement with those reported for macrophages in which trypsin treatment removed two thirds of ^{125}I -LDL bound at 4°C (Goldstein et al., 1979 b).

Binding to follicle membranes

Binding of ^{125}I -vitellogenin to homogenized follicle membrane preparation showed the presence of specific binding sites. This binding not only exhibited high affinity and specificity for labeled vitellogenin but was found to be dependent on calcium and pH. The requirement for calcium makes the binding similar to that of the LDL receptor in human fibroblasts (Goldstein and Brown, 1977) or the storage protein receptor from Sarcophaga peregrina (Ueno et al., 1983). It is still unclear how calcium is involved in the interaction between vitellogenin and its binding site. The equilibrium dissociation constant (K_D) for vitellogenin-receptor complex was estimated to be 1.3×10^{-8} M. The linear plot obtained by Scatchard analysis indicated homogeneity of the follicle membrane binding sites. The fact that only follicle membranes showed specific binding suggested that only this tissue serves as the target tissue for vitellogenin. Once synthesized and released by the fat body, vitellogenin is not taken up again by the same tissue. This

observation is in contrast with M. sexta storage protein (SP-2) which is synthesized and sequestered by the fat body tissue (Ryan et al., 1984).

Competition for binding sites

Competition experiments with other proteins demonstrated that the binding sites for vitellogenin are indeed very specific; only vitellin and deglycosylated vitellogenin competed with labeled vitellogenin for binding. This result is in accord with the fact that vitellin is very similar to vitellogenin in M. sexta and that labeled vitellin is taken up by isolated follicle just as well as vitellogenin in vitro. Two other M. sexta hemolymph proteins, microvitellogenin and lipophorin were found unable to compete with labeled vitellogenin for binding sites, indicating that, if they are taken up by a receptor mediated process, then they must have different binding sites from those of vitellogenin. The ability of deglycosylated vitellogenin to compete for binding indicated that the carbohydrate moiety is not involved in the interaction with the binding site. Indeed, deglycosylated vitellogenin was taken up just as well as the untreated protein. ApoVg-II did not compete with vitellogenin for binding. ApoVg-I could not be tested in the competition studies since it was insoluble under the conditions used in the binding studies. Attempts to identify vitellogenin receptors by the ligand blotting technique have been unsuccessful.

SUMMARY AND FUTURE PROJECTIONS

This dissertation has presented detailed physicochemical and immunological studies on vitellogenin from the tobacco hornworm, M. sexta. The apoproteins of vitellogenin have been analyzed both for carbohydrate and phosphorus contents. The apoproteins have been shown to be immunologically distinct. Immunological techniques and limited trypsin digestion gave some insight into the arrangement of the apoproteins in the native molecule. The elucidation of the primary structure of the oligosaccharide is an important step towards understanding the role of this moiety in insect vitellogenins. I hope that NMR spectroscopic analysis of oligosaccharides of other vitellogens or vitellins will provide a better understanding of how synthesis and processing of the carbohydrate moiety occurs in insects. Finding the exact role of the carbohydrate moiety will be an interesting area for future research. This work has also described an in vitro assay for identifying specific binding sites for vitellogenin on the follicle membranes. The results suggest that these sites indeed meet the requirements for a receptor. This system is very useful in trying to understand the structural basis for selective transport of vitellogenin into the eggs. This aim might be achieved by carrying out various chemical modifications of vitellogenin. An interesting question is whether separate follicle receptors are involved in transport of the different proteins that are localized in

the eggs. Future efforts will undoubtedly focus on the isolation and characterization of the receptor for vitellogenin.

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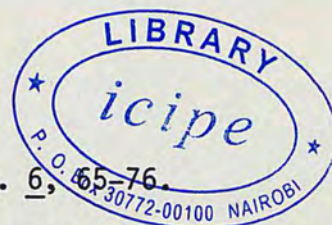
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