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Vitellogenesis in *Hyalomma dromedarii* (Acari: Ixodidae): A Model for Analysis of Endocrine Regulation in Ixodid Ticks

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VITELLOGENESIS IN *HYALOMMA DROMEDARII* (ACARI: IXODIDAE): A MODEL FOR ANALYSIS OF ENDOCRINE REGULATION IN IXODID TICKS.

by

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ABSTRACT

Vitellogenesis in *Hyalomma dromedarii* (Acari: Ixodidae):

A Model for Analysis of Endocrine Regulation in Ixodid Ticks.

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The egg yolk proteins, vitellins, and their hemolymph precursors, vitellogenins, were characterized in the ixodid tick *Hyalomma dromedarii*. Three vitellins were identified. The high molecular weight vitellins, VN A and VN B, were composed of seven homologous subunit polypeptides, VN 1-7. The molecular weights of VN 1-7 ranged from 212 KD to 35.5 KD. VN A appears to be a dimeric form of VN B. A novel vitellin, VN C, was composed of a single polypeptide with an approximate molecular weight of 56 KD. Low levels of vitellogenin, were identified in western immunoblots utilizing antibody raised against purified vitellin. Adult protein and polypeptide profiles, including vitellin and vitellogenin, were characterized as a function of sex, tissue and reproductive development. An enzyme linked immunoabsorbant assay was developed for quantitative assessment of vitellin and vitellogenin. This is the first reported use of this type of an assay in the quantification of these tick proteins. By this method, a rise in the level of vitellogenin from 0.15 to 11.19% of the hemolymph protein between the stages of fed virgin and repletion in females was observed. Similarly, fat body levels rose from 0.15 to 3.0% during these physiological stages. The level of ovarian vitellin rose

rapidly from 0.39 to over 50% of the tissue protein during the four days after repletion. The level of vitellogenin in midgut, salivary gland and muscle, increased slowly during repletion, and never exceeded 1.1% of its respective tissue protein.

Tissue specific synthesis of vitellogenin and vitellin was monitored *in vitro*. Continuous synthesis of vitellogenin was demonstrated with replete fat body tissue over an eight day culture period. Although the ovary accumulated high levels of vitellin *in vivo*, this tissue did not display synthetic activity of vitellin in culture. Other tissues as well, including midgut, muscle and salivary gland, did not synthesize vitellogenin *in vitro*. These findings are consistent with reports suggesting the synthesis and secretion of vitellogenin by fat body in replete females followed by rapid uptake of the protein from the hemolymph by developing oocytes.

Stimulation of vitellogenin synthesis by various authentic hormones and tissue extracts was tested both *in vitro* and *in vivo*. Vitellogenin synthesis by cultured fed virgin fat body was stimulated 35 fold over controls by treatment with synganglion extracts from replete females. Stimulation of VG synthesis in tick tissues *in vitro* has not been previously reported. Similar stimulation of vitellogenesis with synganglion extracts was observed *in vivo*. Although Juvenile hormone bis-epoxide and 20 hydroxyecdysone treatment did not affect vitellogenin synthesis in cultured fed virgin fat body, they did demonstrate stimulatory activity *in vivo*. Multihormonal regulation of vitellogenesis, involving a synganglion factor, a juvenoid and 20 hydroxyecdysone is considered. The methods of monitoring vitellogenin synthesis activity *in vitro* and *in vivo* are proposed as model systems for continued investigation of endocrine regulation in ixodid ticks.

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CHAPTER 1. INTRODUCTION

Ticks rank above all other arthropods in the number and variety of pathogens which they transmit to man and animals; they are vectors of viral, bacterial, rickettsial, fungal and protozoan diseases. Moreover, they are second only to mosquitoes as vectors of important human disease (Harwood and James, 1979). In addition to their role as reservoirs and vectors of disease, ticks also cause direct harm to humans and other animals by their bites, including irritation, anemia, toxemia, allergic sensitization and paralysis. The annual world-wide cost of controlling ticks and tick-borne diseases combined with the loss of domestic animal productivity, is estimated over 9 billion dollars (Sonenshine, in press). During this century, an array of chemical acaricides, including arsenic salts, ogano-chlorines, organo-phosphorous compounds, carbamates, formamidines, synthetic pyrethroids and many others have been utilized in an attempt to control tick populations. The rapid development of resistance to these agents and a more recent awareness of the environmental effects of such poisons have mandated a search for rational alternative management programs. Considerable progress has been made in the development of vaccines against tick paralysis proteins (Stone and Binnington, 1986; Walladesen et al., 1990) and other tissue proteins (Ackerman, et al., 1980; Chinzei, 1988a), but commercial vaccines against ticks are not yet available. Similarly, practical technology for the use of hormone antagonists or other natural products, which has demonstrated substantial success in the control of insect pests (Staal, 1975, 1986), has not yet been developed for ticks. Indeed, compared to many groups of

arthropods, relatively few laboratories are investigating the basic endocrine mechanisms in ticks. In view of the tremendous reproductive potential of ticks, females of some species laying up to 20,000 eggs (Deihl et al., 1982), the manipulation of hormonally directed reproductive physiology would appear to offer a particularly effective means of managing these ectoparasites.

Much of what is known regarding arthropod endocrinology is based on work done in insects and crustaceans. Early pioneering discoveries of the hormonal regulation of molting, juvenoids, and ecdysteroids, led to a vast literature on the endocrines of insects (reviewed by Karlson, 1983). To a large extent, the popularity of insects as models for the investigation of hormonal regulation in arthropods, is based upon the anatomical organization of these invertebrates, especially the presence of a distinct head containing easily distinguished endocrine glands and neurohemal organs. Of equal importance is the availability of insects that grow to very large size, e.g., the giant Cecropia moths, tobacco horn worms, and others, which has facilitated gland and organ extirpation and ligation experiments. In contrast to these insects, the smaller size of ticks and their extreme body fusion present formidable obstacles to experimentation. There is no head. Moreover, the condensation of the central nervous system into a single compact synganglion, located anteriorly within the body near other vital organs, and the pervasive, easily ruptured midgut diverticula, present additional difficulties to the investigator. Thus, it is not surprising that, to date, no endocrine gland has been identified unambiguously or that the roles of tick neuroheamal organs are uncertain (Binnington and Obenchain, 1982; Binnington 1986). Additionally, interest in tick

endocrinology has been partially limited by the widely held assumption that the same hormones and neurotransmitters found in the better known insects must also occur in ticks and perform the same roles, despite the extreme evolutionary divergence of these taxa over geologic time. Thus, the investigator of tick endocrinology is confronted with a choice between of a convenient insect laboratory model which may offer insight into generally conserved mechanisms or a difficult, but authentic, model which may, nevertheless, contain unique and important regulatory mechanisms. Obviously, either of these approaches is scientifically worthy of pursuit, although an integrated experimental program seems most prudent.

Attempts to further our understanding of tick developmental endocrinology have pursued several lines of investigation. These include the regulation of mating, blood meal size and digestion, salivary gland activity, diapause, and cuticle synthesis (see reviews Solomon et al., 1982; Oliver, 1986). To date however, there has been a lack of investigation of hormonal regulation of gene expression at the molecular level within the acarines. One system that appears to be particularly amenable to analysis of hormonal regulation is that of vitellogenesis. Vitellogenin (VG), the serum precursor of the major egg yolk proteins, vitellin (VN) in invertebrates, and phosvitin and lipovitellin in oviparous vertebrates, is typically synthesized in extraovarial tissues, fatbody and liver, respectively. When needed, synthesis of VG mRNA and its translation are stimulated and the protein is secreted into the hemolymph (HL) or blood for uptake by the developing oocytes. Thus, vitellogenesis (in invertebrates) encompasses the synthesis, secretion and transport of VG and its ultimate uptake and concentration as egg yolk VN. VG synthesis is

regulated by juvenile hormone (JH) in most insects, by a combination of ecdysone and JH in some dipteran insects (Hagedorn and Kunkel, 1979), and by estrogen in vertebrates (Tata, 1978). Most VGs are large proteins (50,000 to several million daltons), relatively stable upon extraction, easily resolved, and produced in large quantities only by females. These properties make VG an excellent subject for the investigation of hormonal control of a specific gene product.

Recent reviews (Diehl et al., 1986; Connat et al., 1986; Oliver, 1986 and Coons et al., 1986; Chinzei, 1986) summarize our knowledge of vitellogenesis in ticks. As in most hematophagous insects, a blood meal is required for the induction of vitellogenesis. Mating is also required in most ticks although this is both a species-specific and intraspecific variable. As in other animal species, initiation of vitellogenesis probably requires more than one hormonal stimulus but the specific regulatory molecules remain to be identified.

Overall assessment of vitellogenesis in both argasid (soft) and ixodid (hard) ticks has often followed the scheme of ovum development proposed by Balashov (1972). Balashov's stage I - "the period of small cytoplasmic growth" - begins in the nymphal tick with the appearance of dividing primary oocytes. Balashov's stage II - "the period of great cytoplasmic growth", in the previtellogenic oocyte - is initiated with the adult blood meal and ends with the first appearance of cytoplasmic yolk granules. The vitellogenic phase of oocyte development includes stages III and IV. Mating and continued engorgement (repletion) are typically required for the completion of the vitellogenic stages. During stage III yolk granules appear to accumulate from both intracellular and extracellular sources and repeatedly fuse

form large homogeneous yolk granules (Brinton and Oliver, 1971). Oocyte pigmentation is largely the result of uptake of the pigmented VG and has been used to quantify the progress of vitellogenesis (Chinzei, 1988). The development of oocytes is however, asynchronous. The less developed oocytes, with scant accumulations of yolk remain localized near the longitudinal groove of the ovary while the most developed oocytes are typically located on the opposite side of the ovary. By this mechanism, vitellogenesis, ovulation and oviposition temporally overlap within a period of several days to weeks. Additionally, by widening the temporal window of VG uptake by the maturing oocytes, a gradual increase in the activation of VG synthetic machinery is facilitated. Egg shell development is also initiated during stage III and is completed by the end of stage IV when the most advanced population of eggs are ready for ovulation. Description of Balashov's stages of oocyte development serves to reiterate a number of requisites for VG production, namely; adult blood feeding, mating, and repletion. However, for the purpose of monitoring the regulation of VG synthesis, this developmental scheme lacks sufficient qualitative and quantitative resolution. In addition, analysis of the developing oocyte presumes that events prior to uptake of the serum precursor are not an issues of concern. In view of the required synthesis and secretion of vitellogenin by a presumed nonovarian tissue and subsequent hemolymph transport and ultimate uptake and concentration by the developing oocyte, simple ovarian analysis is an inadequate means of assessment.

The primary site of VG production in ticks, as in most other arthropods, is the fat body (Engelmann, 1979; Diehl et al. 1969, Coons et al., 1982; Chinzei and

Yano, 1985b); midgut and ovary may also contribute to a small degree (Coons et al., 1982; Rosell-Davis and Coons, 1989b). The fat body is a structurally heterogeneous tissue; present as pads, layers, chords and sheets. Additionally, its diffuse distribution is well adapted to provide maximal exposure to the hemolymph of an open, diffusion-facilitated type of circulatory system. While appearing superficially as an adipose storage tissue, the fat body is in reality a tissue of considerable metabolic activity. It's functional role in arthropods is analogous to the combined functions of liver and adipose tissue in mammals. It is the chief site of intermediary metabolism and detoxification and is the main source of hemolymph proteins, lipids and carbohydrates that serve as precursors of metabolism in other tissues (Dean, et al., 1985; Keeley, 1985). Because of its role in maintaining metabolic homeostasis and the correlation of its substrates and products with stage specific development, the fat body has become an increasingly popular model tissue in the study of endocrine metabolic regulation in arthropods (Kunkel, 1981).

Tick and insect fat body displays peripheral and central localizations. The central fat body of ticks forms a sheath of cells covering the reproductive tract in adults (Obenchain and Oliver, 1973). Differentiation of the central fat body occurs in male and female hard ticks in response to blood feeding and repletion respectively. The peripheral fat body of ticks is distributed as a network of single and branching strands, typically one, two or three cells thick. These strands are closely associated with tracheal trunks and hyaline connective tissue and thus are distributed throughout the body cavity. Additionally, a small amount of peripheral fat body lies below the layer of epidermal cells. The peripheral fat body

demonstrates extensive growth and differentiation during the single gonadotrophic period of the adult ixodid female. In contrast, differentiation is not observed in this tissue of argasid females or males of either family. This absence may be requisitely linked to multiple gonadotrophic cycles in these individuals.

Central and peripheral fat body also displays two histologically dissimilar cell types, trophocytes and nephrocytes. The trophocytes of adult females undergo a progressive differentiation which is temporally correlated with reproductive development. Trophocytes in unfed females have sparse rough endoplasmic reticulum and the most prominent cytoplasmic features of these cells are dense inclusions and free ribosomes. During feeding and oviposition the female fat body enlarges greatly and trophocytes display large arrays of RER, golgi bodies and secretory inclusions. These ultrastructural changes suggest stage specific functional roles; unfed trophocytes serve as storage cells whereas the trophocytes of feeding females are involved in the synthesis and secretion of protein (Obenchain and Oliver, 1973; Coons et al., 1986, 1990).

Nephrocytes are characterized by glycogen accumulations, smooth endoplasmic reticulum, coated and uncoated pits, coated vesicles and extracellular channels. Few, if any, alterations in nephrocyte ultrastructure are observed between unfed, fed and ovipositing females. Obenchain and Oliver (1973) observed that nephrocytes incorporated presumptive hemoglobin derivatives from the hemolymph. Although the function of nephrocytes is unknown, the presence of an array of organelles associated with receptor mediated endocytosis suggests that they may sequester substances from the hemolymph. The sparsity of nephrocyte RER in

feeding females, relative to trophocytes, suggests that these cells are not involved in the synthesis of large amounts of secretory protein.

Levels of VG in the hemolymph reflect a balance of the rates of synthesis and the rates of uptake by the oocytes. In insects these levels are highly variable and may constitute as much as 70% of circulating protein (Engelman, 1979). Based on electrophoretic comigration of egg VN and hemolymph proteins, stainable levels of VGs have been reported during the ovipositional period of several tick species (Boctor and Kamel, 1976; Chinzei, 1983a). In *O. moubata*, HL VG rises to a concentration of 65 μg/ul (80% of the total hemolymph protein) five days after repletion (Chinzei, 1983b). Production of such levels of protein greatly facilitates the analysis of synthetic activity. In addition, recent investigations have utilized immunoprobes to VN which have enabled selective and sensitive detection of not only VN but also VG (Coons et al., 1982; Chinzei et al., 1983b). This approach is particularly helpful in the analysis of trace amounts of VG or VN in complex protein mixtures and in culture systems where total amounts of synthetic activity may be low.

Egg yolk production in arthropods has been studied both *in vitro* and *in vivo*. In insects, many investigators have successfully extirpated endocrine glands, transplanted tissues or applied hormones and monitored VN accumulation in eggs or other markers of oocyte development. By such methods, identification of the source and physiological effects of many hormones has occurred prior to their chemical identification. Several groups of hormones which have been recognized and appear to be involved in the initiation and maintenance of vitellogenesis include

brain hormone (Prothoracicotrophic Hormone or ecdysiotropoin), molting hormone (ecdysteroids) and juvenile hormone. However, the assignment of endocrine mechanisms to these hormones must be carefully considered. It must be recalled that oocyte growth is a multifaceted process and is not necessarily directly related to the initiation and maintenance of VG synthesis. Additionally, VG uptake typically occurs concurrently with VG synthesis, thus one must be cognizant that circulating titers may not reflect synthetic rates. Moreover, the complexity of the whole animal model with multiple organ and tissue systems interacting at various levels of regulation, precludes a simple assessment. For example, an applied hormone may have its primary effect on additional endocrine glands which secondarily affect VG synthesis.

JH and ecdysteroid treatments have been conducted in both ixodid and argasid ticks. In one such study, topical application of 10 μg JH I or JH II to fed virgin *O. porcinus* induced oviposition in 5 of 18 and 8 of 28 females in two separate experiments. There was no oviposition in 19 solvent treated controls or 18 JH III treated females (Obenchain and Mango, 1980). In contrast, hemocoelic injection of JH II, but not JH I or JH III, induced oogenesis in the closely related *O. moubata* (Diehl et al., 1982). Another study found that topical application of precocene, an insect antiallatotropin, prevented oocyte development in *O. parkeri* (Pound and Oliver, 1979). This finding was of particular significance to tick endocrinology as a homolog of the insect corpora allata, in which the known juvenoids are synthesized, has not been identified in ticks. Furthermore, these authors reported that they were able to reverse the effects of precocene on oogenesis and oviposition by subsequent

application of JH III. Although only one of ten treated ticks in these studies went on to successfully oviposit, these results have spurred a substantial effort to verify the presence and functionality of insect JHs or very closely related molecules in ticks (Diehl et al., 1986; Connat et al., 1986, Venkatesh et al., 1990). Despite these efforts, a juvenoid has not been chemically identified in any tick system.

In contrast to suggestive evidence in argasid ticks, there is very little experimental evidence supporting a conserved function of the known insect juvenoids in ixodid ticks. Although Khalil et al. (1984) found a small reduction in *H. dromedarii* fertility as the result of topical JH I application to feeding adults, these authors and others (Dees et al, 1984) questioned whether the observed effects were not more indicative of toxicity than hormonally regulated development. The issues of toxicity and pharmacological concentrations of insect juvenoids and 20-OH ecdysone (ECD) has also been addressed *in vitro* with two ixodid cell lines (Kurtii and Munderloh, 1983). Cultures initiated from embryos of *Rhipicephalus appendiculatus* and *D. nitens* demonstrated concentration dependent adherence and mitogenic responses to these hormones and cytotoxicity was not observed with mM and nM concentrations of JH III and ECD.

In lieu of whole animal studies, several investigators have opted for partially isolated organ systems. This approach is readily achieved in many insect systems through ligature isolation of head, thorax and abdomen. By such methods specific endocrine glands are physically separated from tissues of interest and the investigator may hope to monitor the effects of withheld endocrines from these targets. Similar approaches have met some success in the soft ticks. Shanbaky and Khalil (1975)

were able to physically separate the synganglion from the ovary in *Argas arboreus* by ligation with a fine thread behind the second coxae. Ligation within one hour of the adult blood meal blocked ovarian development in 90% of the test animals. Injection of the posterior end with synganglia extracts or hemolymph (HL) from vitellogenic ticks induced ovarian development in over 75% of the recipients as compared to 14% in the saline treated controls. A more recent report of successful ligations in *O. parkeri* using standard office staples suggests that this procedure is relatively simple and affords a high survivorship ratio (Zhu et al., 1988). Similar ligations in hard ticks have not been reported.

Documentation of regulatory effects of endocrines on VG synthesis by cultured tissues has been accomplished in several insect model systems (reviewed by Bownes, 1986). Similar analyses of the regulation of vitellogenesis in ticks have not been reported. Nonetheless, several unique capabilities of this approach, which may be advantageous in the analysis of hormonally regulated systems, should be considered. Among these are included the ability to 1- select or mix cells/tissues of interest, 2-deliver quantified doses of test agents in a controlled environment (medium) and 3- avoid variable behavioral responses encountered in whole animal experimentation. Despite these desirable experimental features, the stimulation of vitellogenesis, under these conditions, by exogenous endocrines, or the lack thereof, must also be carefully interpreted. Although physically isolated, tissue and organ cultures are rarely composed of a single cell type and one must consider their interactive relationships when assigning regulatory functions to test agents. Additionally, cell, tissue or organ isolation for culture maintenance often requires a

series of stressful manipulations resulting in shocked cells and diminished yields. In order to overcome the trauma of such isolation, many cultures require a reconditioning period (hours to days) during which cells are unresponsive to many stimuli. Furthermore, the investigator must be aware that metabolic processes critical to the monitored event(s), may occur in tissues other than those selected for analysis. Kaufman (1988) suggests that the lack of a vitellogenic response in isolated tick ovaries to ecdysteroid treatment may have been due to the absence of yolk protein precursors under these conditions. Thus, the inability of a given endocrine to illicite a detectable stimulatory event, *in vitro* or *in vivo*, may be more reflective of the choice and means of analysis than the actual role of the test agent in the overall process. In summary, each approach, whole animal, partially isolated organ systems or tissue maintenance *in vitro*, offers its own advantages. Yet, no single approach is adequate. Thus, it appears most prudent to utilize a combination of these methods and to integrate the conclusions of these approaches when extrapolating to natural mechanisms.

As introduced, vitellogenesis offers many desirable features of a hormonally regulated model system and was thus chosen as the focus of the proposed investigations. The contained studies utilized the ixodid tick *Hyalomma dromedarii*, based on several characteristics of this species which facilitate its laboratory rearing. These include its willingness to feed on a single host (eg. rabbit) during all 3 of its developmental stages (ie., a one host tick), its simple environmental needs, and its ability to undergo a complete life cycle in approximately 3 months. Additionally, replete females of this species produce large egg clutches, thus providing a plenteous

source of VN.

In order to probe the regulatory nature of vitellogenesis, several preliminary research objectives were proposed. These included identification of VG and VN and the development of methods to quantify these proteins. Subsequently, the synthetic source of VG was investigated and tissue-specific levels of VG and VN (as a function of reproductive development) were analyzed. These baseline studies enabled investigation of the regulatory nature of VG synthesis *in vitro* and *in vivo* as a model systems through which our understanding of endocrine regulation in ticks could be further studied.

CHAPTER 2. MATERIALS AND METHODS

2.1 LABORATORY REARING OF TICKS.

Hyalomma dromedarii Koch; the camel tick, was colonized and reared from a stock obtained from the U.S. NAMRU-3, Cairo, Egypt (U.S. APHIS No. 9433). All life stages were fed on albino rabbits (*Oryctolagus cuniculus*). Nonfeeding ticks were held in an AMINCO-AIRE Climate Lab (American Instrument Co., Silver Spring, Md.) controlled environment chamber at 27 $\pm 1^{o}$C and 80 $\pm 2\%$ relative humidity.

2.2 DEVELOPMENTAL AND REPRODUCTIVE STAGES OF TICKS.

Tissues and hemolymph (HL) were collected from females of the following adult stages; freshly molted (FM), unfed (UF), fed virgins (FV), and replete (R). In addition, HL was collected from FM, UF and FV adult males. FM ticks were individuals collected within 24 hours of molting; UF adults were ticks kept in the environmental chamber for two weeks post-molting; FV females and males were ticks fed for seven to ten days (in the absence of the opposite sex) and forcibly detached from the host; R females (mated and fully fed) were collected and dated within 24 hours of voluntary detachment from the host. In the presence of mating adult males repletion required 7 to 12 days of feeding. The preoviposition period (R + X days) was monitored daily. Oviposition was initiated 6 to 8 days post repletion (R6-8) and continued for 12 to 20 days.

2.3 TISSUE AND HEMOLYMPH COLLECTION.

Ticks were collected at the developmental and reproductive stages described above and washed in Shen's saline solution (Oliver, 1972). Dissections were performed with the aid of a Wild MP-8 stereoscopic microscope (10.5-60X total magnification, Wild Heerbrugg Instruments, Inc., Farmingdale, NY.) and a Ehrenreich fiber optics lamp (Ehrenreich Photo Optical Co., Garden City, N.J.). Hemolymph was gently expressed from a small incision in the cuticle, collected with 10 μl Drummond capillary pipets (Scientific Products, McGaw Park, Il.) and transferred to microfuge tubes at 4°C. Samples contaminated with ruptured midgut or malpighian tubules were discarded. Ticks were transferred to cold Shen's solution (4°C) in watch glasses and dissected with a pair Braun microscissors (Fisher, Pittsburg, Pa.). The dorsal cuticle was removed and selected tissues, i.e., midgut (MG), ovary (OV), salivary glands (SG), muscle (MU) and synganglion were collected. Tracheal trunks, including spiracles, were isolated as the source of fat body (FB). Tissues were rinsed free of midgut with Shen's solution containing freshly prepared 1mM phenylmethylsulfonylfluoride (PMSF) and stored at -70°C.

2.4 PROTEIN ASSAY.

Collected tissues were sonicated in extraction buffer (see below) at 4°C. A Branson 200 sonicator (Branson Ultrasonic Corp., Danbury, CT.) was fitted with a microtip and utilized at 35% maximum output. Sonicated tissue was centrifuged

(Eppendorf Micro Centrifuge, Scientific Products, McGaw Park, Il.) at 2500 *g* to pellet insoluble material and the supernatant utilized for determination of protein concentration and further analysis. The BioRad protein assay kit (BioRad, Richmond, Ca.) was used to determine protein concentration in tissue extracts and hemolymph samples. Fatty acid free bovine serum albumin (BSA, SIGMA, St. Louis, Mo.) was used as the reference standard in both the micro and standard assays.

2.5 TISSUE PROTEIN EXTRACTION.

Freshly isolated and cultured tissues were sonicated in extraction buffer (0.05 M Tris-Cl, pH 8.5, 0.025 M NaCl, 1mM PMSF, 0.1% Triton X-100, 0.1% sodium deoxycholate) with a Branson 200 sonicator as described above in PROTEIN ASSAY.

2.6 EGG EXTRACT (EE).

Replete females were collected upon voluntary detachment from the host and held in petri dishes in the environmental chamber. Upon the initiation of oviposition eggs were collected twice daily and stored at -70°C. Pooled eggs were homogenized on wet ice in a glass/teflon homogenizer with 5 volumes of Shen's solution with 1mM PMSF. Insoluble debris was pelleted at 5000 *g*. The lipid layer was siphoned from the top of the spun extract and the aqueous protein layer was collected. Aqueous protein concentration was determined by BioRad protein assay and the EE was stored in 0.5 ml aliquots at -70°C.

2.7 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE).

Polypeptides and proteins were characterized on slab PAGE, under both denaturing and nondenaturing conditions, according to a modified protocol of Laemmli (1970). Gel recipes are recorded in Appendix B. Unless otherwise noted, resolving gels were linear 4-15% gradients formed with a Hoefer gradient maker and run on a Hoefer vertical PAGE apparatus (Hoefer Scientific Instruments, San Francisco, Ca.).

2.7.1 NONDENATURING PAGE. Nondenaturing gels were made in 0.187 M Tris-HCl buffer (pH 9.6) with a 3% stacking gel made in 0.350 M Tris-HCl (pH 6.9). Cathode buffer was 0.05 M Tris-glycine (pH 8.9) and anode buffer was 0.1 M Tris-HCl (pH 9.6). Samples were diluted with sample buffer (cathode buffer with 5% sucrose and 0.01% bromphenol blue). Gels were electrophoresed with 100 Volts (constant V) at 4-8°C.

2.7.2 DENATURING PAGE. Denaturing gels contained 1% sodium dodecyl sulfate (SDS) in 0.375 M Tris-HCl buffer (pH 8.8) with a 3% stacking gel in 0.625 M Tris-HCl buffer (pH 6.8). The running buffer was 0.5 M Tris-glycine (pH 8.4) and tank buffer was 0.025 M Tris pH 8.3, 0.192 M Glycine, 0.1% SDS. Protein samples were diluted 1:1 (v/v) in 2X denaturing buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated in a boiling water bath for 60 seconds. Gels were electrophoresed with 100 V (constant V) at 4-8°C.

2.7.3 TWO DIMENSIONAL PAGE. Native first dimension resolving gels (4%) were formed in glass tubes (100mm gels in 3mm ID by 150mm glass tubes). A 5mm, 4% stacking gel was poured on top of the gel resolving gel. Sample, gel, anode and cathode buffers were the same as those in NONDENATURING PAGE above. Proteins were resolved at 4°C with 10 mAmps constant current/gel until tracking dye reached the bottom of the gel. Resolved gels were rimmed with a fine wire and gels were gently extruded by positive pressure from an affixed two ml pipet-pump. The gels were stained (see below) or treated for 30 minutes in treatment buffer (0.0625 M tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) in preparation for second dimension PAGE. Treated gels were stored frozen in treatment buffer at -70°C or loaded directly onto second dimension gels. First dimension gels were sealed onto the tops of second dimension resolving SDS gels with molten agarose (0.125 M tris-HCl pH 6.8, 1% agarose, 1% SDS). Second dimension gels, without sample wells, were formed and electrophoresed as in DENATURING PAGE above.

2.8 MOLECULAR WEIGHT DETERMINATION OF NATIVE PROTEINS.

Molecular weights of native proteins were determined independently by column chromatography and Ferguson analysis of nondenaturing PAGE profiles. The determined molecular weights were based on the assumption that these proteins displayed conformational and hydration properties similar to those of standards utilized to generate the calibration curves.

2.8.1 COLUMN CHROMATOGRAPHY. 200 ml of Sepharose 6B-CL (SIGMA) was resuspended in 500 ml of buffered tick physiological saline (Shen's solution with 50 mM Tris-HCl, pH 7.5) and allowed to settle for 15 minutes. The supernatant and particles in suspension were siphoned off and the washing procedure was repeated twice. The washed resin was resuspended in 500 mls of buffer, degassed and poured to a height of 95 cm in a 1.5 by 100 cm glass column following manufacture's instructions. A gravity driven flow system of Shen's solution was adjusted to yield a rate of 24 ml/hour and run overnight prior to standard and sample analysis. All procedures were performed at 4°C. Samples and standard load volumes were one to two mls in buffered Shen's solution with 2.5% glycerol. Molecular weight standards (SIGMA) were reconstituted at the following concentrations; bovine albumin - 15 mg/ml, apoferritin - mg/ml, thyroglobulin - 8 mg/ml, and blue dextran - 2 mg/ml. Elution of standards and egg proteins was monitored at 280 *nm* (egg proteins were additionally monitored at 400 *nm*).

2.8.2 FERGUSON ANALYSIS OF NONDENATURING PAGE. A modification of Retamal and Babul (1988) was utilized. A nondenaturing 5-10% gradient resolving gel was generated and turned 90° on its side. A 3% stacking gel was poured on top of the transverse gradient. Standards and EE were placed together in a 10 cm well which traversed the 5-10% gradient. Additionally, the individual standards and EE were placed in separate 0.5 cm wells at the 5% end of the gel. Proteins were electrophoresed at 150 V constant voltage (4-8°C) until the tracking dye reached the bottom of the gel at the 5% end. The slope of the tracking dye was

recorded and the gel was stained with coomassie blue. Fergusen analysis of the relative mobilities of the standards and EE proteins, as a function of gel concentration, was used to determine molecular weight of EE proteins (Fergusen, 1964).

2.9 MOLECULAR WEIGHT DETERMINATION OF DENATURED POLYPEPTIDES. Linear gradient (5-15%) SDS PAGE was utilized to determine molecular weights of egg protein subunits and other polypeptides of interest. MW standards (SIGMA) and samples were treated as above for Denaturing PAGE. MW standards were used to generate a standard curve based on distance of polypeptide migration into the gel gradient ($\log_{10}$ polyacrylamide concentration) versus $\log_{10}$ MW (Lambin and Fine, 1979).

2.10 PREPARATIVE PAGE FOR VN PURIFICATION.

Crude EE (5 mg protein in nondenaturing sample buffer) was layered into a 10 cm wide well on a 3mm thick 4-10% nondenaturing gel. Constant power (150 V) was applied until the tracking dye reached the bottom of the gel. At this concentration of EE, the major protein constituent, VN B (EE II), was visible as a brown band. The glass plates were disassembled and VN B was cut from the gel with a razor blade (staining of representative portions of the gel was utilized to confirm protein location). The VN B gel strip was transferred to a 7mm ID by 15cm electrophoresis tube and sealed with 4% acrylamide. The pigmented band was electroeluted (16 hours at 200V) from the gel strip into an attached dialysis bag

(Fisher Scientific) and accumulated as a dense brown droplet. One quarter strength nondenaturing cathode and anode buffers were used for electroelution (see NONDENATURING PAGE, above). Total recovery, from the original egg homogenate to the final electroeluate, was 49% on a protein basis (BioRad protein assay). Purified VN B was re-electrophoresed to verify homogeneity.

2.11 PAGE STAINING.

Gels were fixed in 50% methanol, 10% acetic acid. For Coomassie blue staining, fixed gels were stained in fixative supplemented with 0.125% Coomassie Blue (G-250) for 2-12 hours. Destaining was done in 7% acetic acid, 5% methanol. Alternatively, fixed gels were silver stained using silver stain reagents and protocol (BioRad).

2.12 ANTIBODY (ANTI-VN) DEVELOPMENT AND PURIFICATION.

Approximately 1 mg of PAGE purified VN B (see above) was mixed with Freunds complete adjuvant (total volume 2 ml) in a 3 ml disposable syringe and emulsified by sonication. Each of two naive rabbits was injected subcutaneously at multiple sites along the back and flanks with half of the emulsion. Both rabbits were boosted twice with 0.2 - 0.4 mg of the PAGE purified VN in incomplete adjuvant at seven to ten day intervals. Three weeks after the original immunization, and at ten day intervals thereafter, blood was collected from ear veins (15-30 mls per individual collection). Blood was allowed to clot overnight at 4°C. The rimmed clot was pelleted at 1000 *g* and an aliquot of the serum was tested for anti-VN activity by

slide immunoprecipitation (Ouchterlony, 1949) at 4°C. Serum collections demonstrating visible slide immunoprecipitation of VN within 48 hours were pooled and stored at -70°C. Antiserum from each rabbit was kept separate.

Frozen anti-serum was thawed to room temperature, placed in a 50 ml conical centrifuge tube with a stir bar and saturated ammonium sulfate (AS) was added dropwise from a buret funnel to the stirring serum to affect 40% saturation. The precipitate was allowed to form overnight at 4°C with continuous stirring and centrifuged at 1500 *g* (4°C). The 1500 *g* pellet was reconstituted to the original volume in Tris buffered saline (TBS). AS precipitation and TBS reconstitution were repeated twice and the final suspension was dialyzed against TBS for 48 hours at 4°C. Anti-serum was retested for VN activity as described above and stored in 50 μl aliquots at -70°C.

2.13 WESTERN BLOTTING.

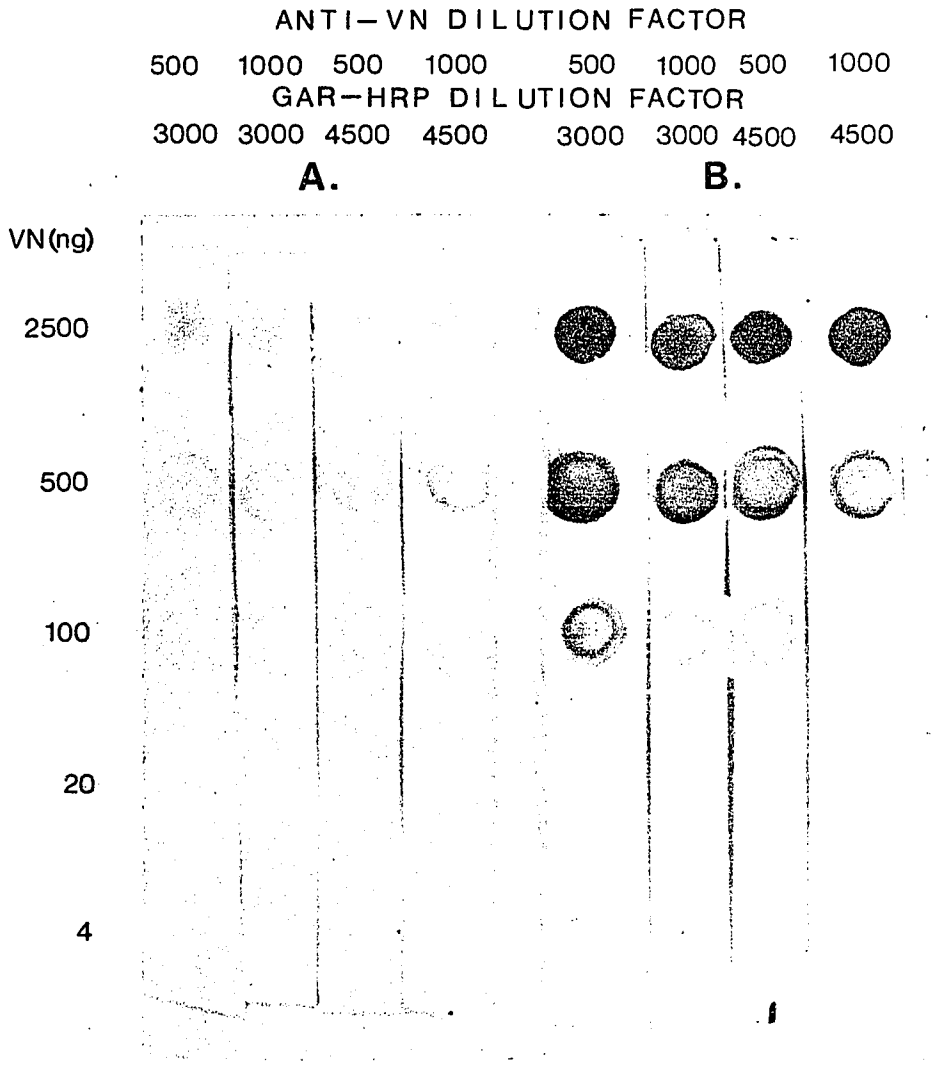
Gels were equilibrated for 15 minutes in Transfer Buffer (TB, see Appendix B). Nitrocellulose (Bio-Rad), cut to the size of the gel, was laid onto blotting paper in TB. The gel was placed on the nitrocellulose and an additional sheet of blotting paper was placed over the gel. The entire stack was placed in the transfer gel assembly with the nitrocellulose facing the anode relative to the gel. Electrophoresis was accomplished in TB with 25 V (constant V) for 12-14 hours at 4°C. Total transferred protein profiles were visualized by staining with amido black for 5 minutes (45% methanol, 8% acetic acid, 0.175% amido black). Blots were destained in the same solvent without amido black.

2.14 WESTERN IMMUNOPROBE.

Optimization of a western immunobloting system was initially approached by dot blot analysis of four variables. These were 1: limits of antigen detection, 2: primary antibody concentration, 3: secondary antibody concentration and 4: temporal length of exposure to color reaction substrate. These parameters were analyzed in a two dimensional array as depicted in *Fig. 1.* An 8mm grid was lightly drawn with a pencil onto nitrocellulose (BioRad) strips large enough to accommodate the number of samples to be analyzed. The nitrocellulose was immersed in TBS and allowed to air dry on filter paper. 2.5 μl aliquots delivering a range of 4 to 2500 ng of PAGE purified VN B were applied to squares as a drop with the aid of a 10 μl Hamilton syringe. Spotted nitrocellulose strips were blocked in 3% gelatin (BioRad) in TBS for 30 minutes and washed twice for 5 minutes in TBS with 0.05% Triton X-100 (TTBS). Primary antibody (rabbit anti-VN) was tested at dilutions of 1:500 and 1:1000 and similarly, secondary antibody (goat anti-rabbit horse radish peroxidase, GAR-HRP, BioRad) exposure was tested at dilutions of 1:3000 and 1:4500. Finally, the HRP color substrate (BioRad) was incubated with the immunoconjugate for 5 or 15 minutes.

Minimum detection limits were observed at approximately 100 ng. In addition, the length of the substrate reaction demonstrated a significant color intensity improvement at 15 minutes as compared to 5 minutes. At the concentrations tested, there was minimal loss in sensitivity with antibody dilution. Subsequent VN immunodetections were performed on nitrocellulose blotted SDS PAGE samples. Based on dot blot results above, primary and secondary antibodies

FIGURE 1. WESTERN IMMUNOBLOT: TITRATION OF REAGENTS. Dilution factors of primary (anti-VN) and secondary (GAR-HRP) antibodies are recorded above the blot columns. Nanograms of of spotted VN are recorded for each row. The HRP color reaction was allowed to proceed for 5 or 15 minutes (A and B respectively).



were utilized at dilutions of 1:800 and 1:4000 respectively.

Blotted nitrocellulose (see WESTERN BLOTTING, above) was blocked with 3% gelatin in TBS for 30 minutes. The membrane was washed two times for 5 minutes in TTBS and subsequently incubated for 45 minutes with a 1:800 dilution of rabbit anti-VN in antibody buffer. Unbound primary antibody was removed with two 5 minutes washes in TTBS. The membrane was transferred to a 1:4000 dilution of GAR-HRP in antibody buffer for 45 minutes. Unbound secondary antibody was removed with two washes in TTBS and a final wash in TBS (5 minutes each). The washed membrane was incubated with freshly prepared HRP Color Reagent until staining profile was evident. The color reaction was stopped with cold distilled H_2O. Buffers are described in Appendix B. All incubations and washes were performed at room temperature with gentle agitation.

2.15 ENZYME LINKED IMMUNOABSORBANT ASSAY (ELISA).

Coating buffer (CB), antibody buffer (AB) and wash buffer (WB) recipes are listed in APPENDIX B. Microtiter wells (Falcon, Fisher Scientific) were pre-coated with 100 μl of EE (1mg/ml) in CB overnight at 4°C or for three hours at 30°C. Unbound protein was removed by aspiration and the wells were washed three times with WB. All subsequent incubations were performed at room temperature with gentle agitation.

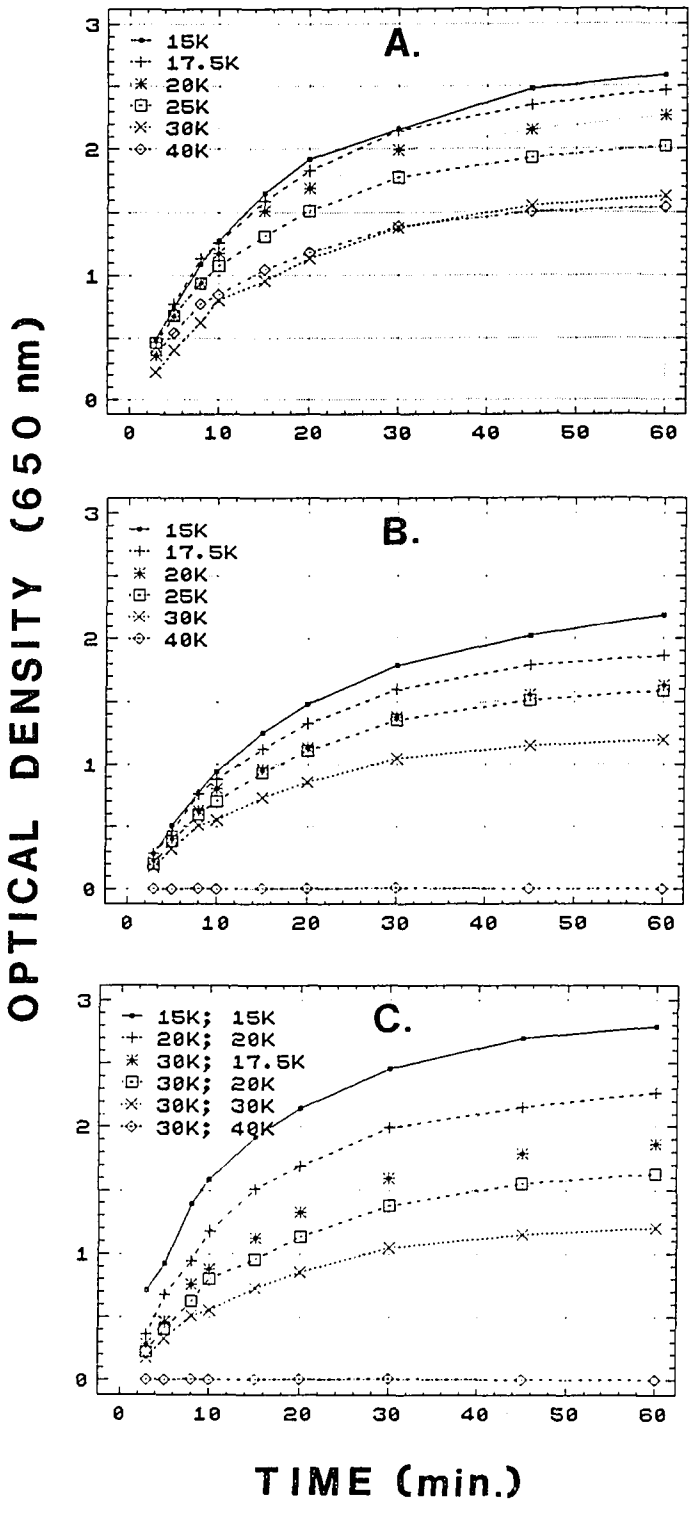
A standard inhibition ELISA for quantitative analysis of tissue and secreted VG and VN was developed. Initial testing was directed towards the effects of the following variables; 1- primary antibody concentration (rabbit anti-VN), 2- secondary

antibody concentration (GAR-HRP) and 3- pre-exposure of primary antibody to variable amounts of antigen (VN).

Primary and secondary antibody concentration effects were first tested without pre-exposure to VN. 100 μl of primary antibody was added to antigen coated microtiter wells and incubated for 45 minutes. The incubation mixture was aspirated from the solid phase and the solid phase was washed three times with WB. 100 μl of secondary antibody was added to each well and incubated for 45 minutes. The solution was aspirated from the solid phase and the solid phase was washed three times each with WB and distilled water. 100 μl of freshly prepared enzyme immunoassay substrate (EIA-HRP, BioRad) was added per well. The color reaction was monitored on a BioTek plate reader at 650 *nm* (BioTek Instruments, Winooski, Vt.). The kinetics of the color reaction as affected by increasing antibody dilutions are plotted (*Fig. 2A-C*). Reaction rates correspond directly to antibody concentrations and are approximately linear for the first 10 minutes. At a 1:40,000 dilution of the secondary antibody the final color reaction is completely abolished. Due to constraints of the spectrophotometric monitoring {optical density (OD) maximum = 3} and time efficiency, antibody dilutions were chosen to yield approximately 1.5 OD units within 30 minutes of incubation. These were 1:30,000 and 1:20,000 dilutions of primary and secondary antibodies respectively (see *Fig. 2C*).

The effects of pre-exposure of primary antibody to VN were subsequently tested. 100 μl of primary antibody (anti-VN, 1:30,000 in AB) was pre-incubated with known amounts of PAGE purified VN (1 to 500 ng) for 45 minutes in uncoated microtiter wells. An uninhibited control (ie., primary antibody pre-exposed to buffer

FIGURE 2. ELISA: TITRATION OF REAGENTS. Primary and secondary antibodies were titrated for the detection of VN by ELISA. The final color reaction was monitored for 60 minutes at 650 nm. (A) The effect of variable anti-VN and constant GAR-HRP (diluted 1:20,000) concentrations on the final color reaction ODs. (B) The effect constant anti-VN (diluted 1:30,000) and variable GAR-HRP concentrations on the final color reaction ODs. (C) Primary and secondary antibody concentrations are varied as shown. Variable antibody dilution factors are indicated in each figure legend.

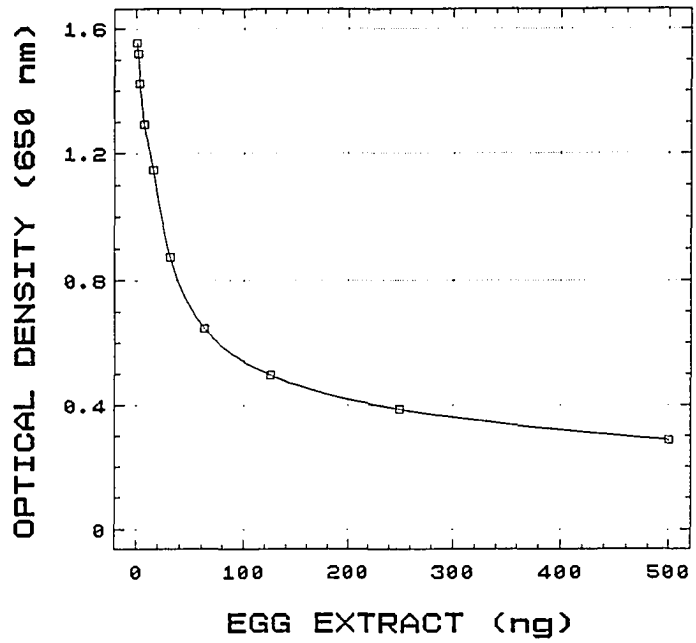


alone) was also included. The preincubation mixtures were transferred to antigen coated wells and unbound primary antibody was allowed to bind solid phase antigen for 45 minutes. Buffer washes, secondary antibody incubation (1:20,000) and color substrate incubation were performed as above. Inhibition of the final OD as affected by pre-incubation with VN was plotted in a linear and semi-log fashion (*Fig. 3*). A biphasic inhibition curve is observed when the VN preincubation amount is represented linearly (*Fig. 3A*). Inhibition of the color reaction is rapidly diminished with VN concentrations of <100 ng/assay. Logarithmic presentation of the VN dilution produces a sigmoidal inhibition curve (smoothed by the Q-splines software procedure, Statistical Graphics Corp., STSC, Inc., Rockville, Md. *Fig. 3B*). Minimum detection limits are observed with 3.9 ng of VN (OD value 3 standard deviations from control). A semi-linear region of absorbance inhibition is observed between 15 and 60 ng of added VN and a linear regression analysis (Statistical Graphics Corp.) for this range is plotted. This region of inhibition was utilized to compare the reference standard with sample plots of unknown VN content as described elsewhere (see below).

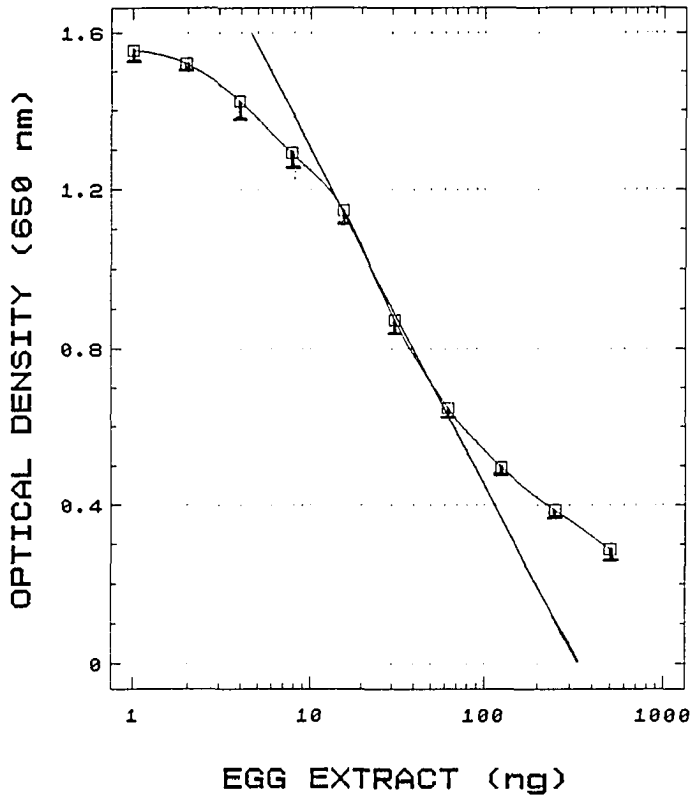
Replicate tissue extract or media aliquots, with unknown VG and VN concentrations, were diluted eight to twelve times (two fold dilutions in AB) and incubated as above in place of known amounts of VN (the protein concentration in tissue extracts was adujusted to 1 μg/ml prior to serial dilution). Reference standard inhibition controls were processed in parallel with each unknown. Unless otherwise noted, recorded VG and VN values represent the average of duplicate ELISA determinations from a single media or tissue sample (see figure legends and table

FIGURE 3. STANDARD INHIBITION ELISA FOR VN QUANTIFICATION. Primary antibody (diluted 1:30,000) was preincubated with 0-500 ng of PAGE purified VN B. Secondary antibody was utilized at a dilution of 1:20,000. The final color reaction was allowed to proceed for 30 minutes. Linear (A) and semi-logrithmic (B) plots of the effects of preincubation of primary antibody with known amounts of VN B on the final OD are shown. Error bars (shown in B) represent the standard error of duplicate samples. A regression plot of the final ODs resulting from preincubation with 15-60 ng of VN B is included in B (r^2 = 99.93%, multiplicative regression analysis).

A.



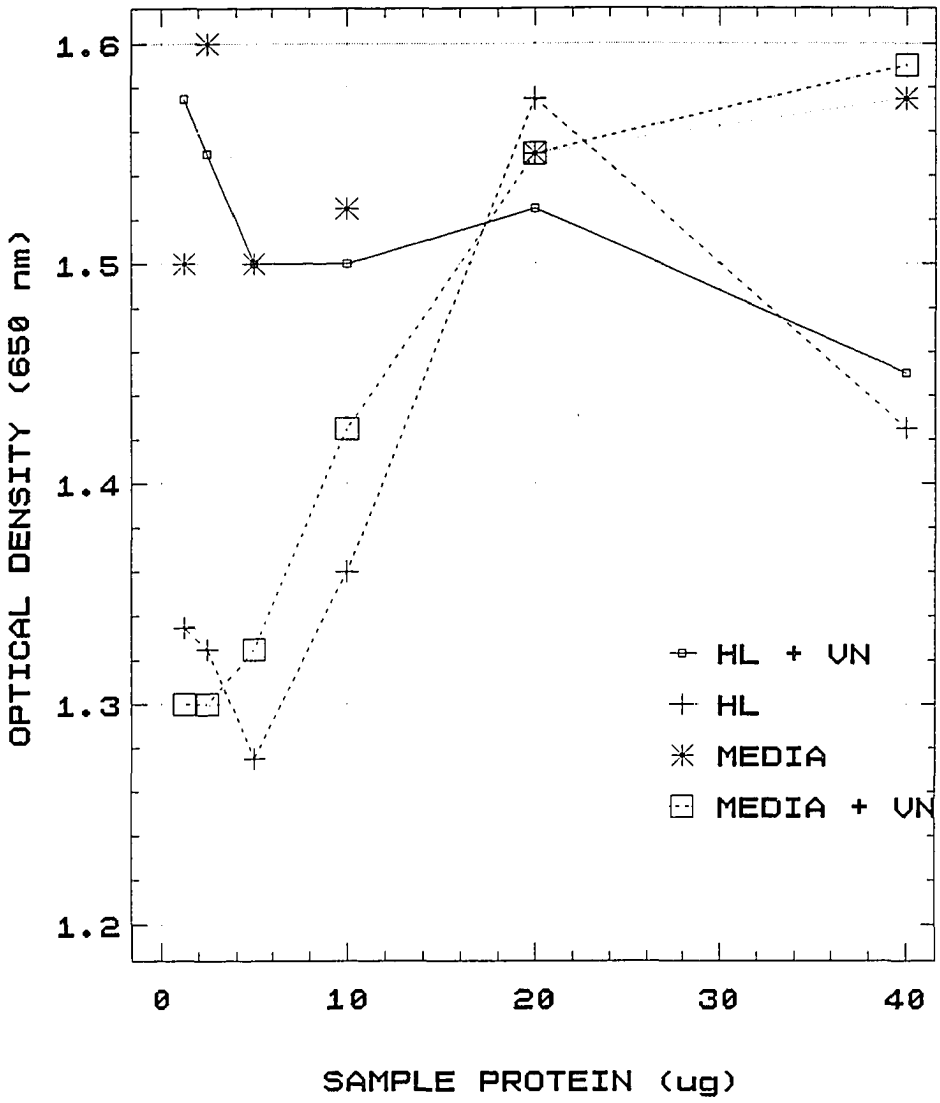
B.



footnotes). Final ELISA ODs were recorded when uninhibited reference standard wells reached an OD of 1.5 to 1.6 units. Reference standard and sample ODs were plotted against the log of sample dilutions. Comparison of absorbance values (corresponding to quantified protein amounts) from samples of unknown VG or VN content with those of known VN content from reference standard plots was utilized to quantify ELISA results. Coordinate comparisons (OD and sample protein) were accomplished through a interactive labeling facility in the graphics plotting software (Statistical Graphics Corp.).

The minimum detection limit of VN B by inhibition ELISA is approximately 3.9 ng. The affect of non-antigen protein concentrations on the detection limit of VN B by ELISA was assessed (*Fig. 4*). A constant amount of antigen (3.9 ng VN B) was added to increasing concentrations of non-antigen protein (UF female HL and culture media). Assay conditions were as described above for ELISA. Preincubation of primary antibody to 3.9 ng of VN yielded a final OD of 1.350 ($\pm$0.020). A final OD of 1.550 ($\pm$0.015) was obtained when primary antibody was pre-exposure to buffer alone (ie., uninhibited control). Addition of increasing amounts of media and UF HL protein to the VN sample resulted in diminished detection VN. Upon the addition of more than 10 μg of UF HL protein or 5 μl media to 3.9 ng VN, the final ODs were not significantly different from those of uninhibited controls. Based on these observations, ELISAs were performed on samples with no greater than 10 μg of tissue protein or 5 μl of culture media.

FIGURE 4. EFFECT OF SAMPLE PROTEIN CONCENTRATION ON ELISA DETECTION OF VN. Samples are indicated in figure legend. 3.9 ng of VN was added to indicated HL and media samples.



2.16 TISSUE CULTURE.

2.16.1 TISSUE MAINTENANCE. Ticks were surface sterilized by a series of one minute washes in 3% H_2O_2, 70% ethanol, and sterile water. Dissecting instruments were flame sterilized in 70% ethanol. Ticks were dissected and tissues isolated in a laminar flow hood at room temperature as described above. Tissues were transferred to Yunker Meibos Medium (YM, Yunker and Meibos, 1979) in 24 well culture plates (Scientific Products) or 500 μl microfuge tubes (Fisher) and maintained for up to two weeks at 27 to 30°C.

2.16.2 TISSUE STIMULATION. Tissue extracts and hormones were added to cultures (see above) of fat body and ovary. Hormones tested were Juvenile Hormone III (JH III, SIGMA), 20-hydroxyecdysone (ECD, SIGMA) and Juvenile Hormone III bis-epoxide (BIS, generously supplied by M. Roe, N.C. State University, Raleigh, N.C.). Stocks were reconstituted to 2 mM in dimethyl sulfoxide (DMSO) and stored at -20°C. Frozen stocks were thawed and diluted 2000 fold into YM media to yield 1 μM working hormone concentrations containing 0.05% DMSO. Control cultures contained 0.05% DMSO in YM media. Tissue extracts were prepared from synganglia and oviducts. These tissues were aseptically collected from R2 females and homogenized in Shen's saline solution at 0-4°C on wet ice. The 1000 *g* supernatant was diluted into YM media. Two to three female equivalents of synganglion or oviduct extract/50 μl YM were added to each test tissue. Additional tissue cultures were treated with 10 μl of HL aseptically collected from R2 females/50 μl YM.

Aliquots of the culture media and tissue were collected, as a function of time in culture, and analyzed by Western Immunoblot and ELISA (see above).

2.17 TREATMENT OF TICKS.

Adult females were treated with authentic hormones and tissue extracts. Samples (see TISSUE STIMULATION, above) were injected through a severed trochanter joint on the second leg with the aid of a finely drawn capillary pipet affixed to a drummond syringe. Injected tissues (oviduct and synganglion), aseptically collected from 2-3 females, were delivered in a total volume of 5 μl. Hemolymph injections consisted of a 10 μl total volume. For hormones, 5 μl of a 2mM stock (see above) were injected per tick. HL, FB and OV were collected from treated ticks and analyzed by western immunoblot and ELISA.

2.18 TISSUE ULTRASTRUCTURE.

Samples of tissues were examined by transmission electron microscopy for changes in ultrastructural morphology associated with development, reproduction and tissue culture maintenance. Dissected tissues (see above) were immersed in cacodylate buffered (0.1 M, pH 7.4) 2.5% glutaraldehyde/2% formaldehyde at 4^{o}C overnight. Fixed tissues were washed three times in 0.1 M cacodylate buffer and postfixed for two hours in 2% OsO_4 in cacodylate buffer (0.1M, pH 7.4). After two washes in cacodylate buffer (0.1M, pH 7.4), tissues were dehydrated in an ascending ethanol series and finally in acetone. Dried tissues were embedded in Polybed 812 and sectioned for light transmission microscopy (1 μm, stained with

methylene blue/azure II) and electron microscopy (~800 Å). Thin sections were picked up on naked copper grids and stained with uranyl acetate and lead citrate. The specimens were viewed and photographed using a Hitachi HU-11 B transmission electron microscope (TEM) operating at 60 KeV. For scanning electron microscopy (SEM), tissue were fixed and dehydrated as above and critical point dried with liquid CO_2 in a critical drying apparatus (Denton DCP, Denton Vacuum Systems, Cherry Hill, N.J.). Specimens were mounted on aluminum stubs with TV-corona dope (GC Electronics, Rockford, Ill.) and coated with 100-200 Å of gold/palladium in a sputter coater (Pollaron E-5200, Technics, Alexandria, Va.). Samples were viewed with a Cambridge S-100 SEM.

2.19 PROTEIN RADIOLABELING.

Tissue proteins were radiolabeled *in vitro* and *in vivo* by incorporation of radiolabeled amino acids (NEC 445, L amino acid mixture [^{14}C(U)], 0.1 mCi/ml, New England Nuclear, Boston, Mass.). Labeling conditions are described in appropriate table legends. Proteins were extracted (see TISSUE PROTEIN EXTRACTION, above) and aliquots were quantified for radioactivity by liquid scintillation spectroscopy.

2.20 IMMUNOPRECIPITATION OF RADIOLABELED VG.

Incorporation of radiolabeled amino acids (see above) into VG or VN secreted into media by cultured tissues was assessed by immunoprecipitation. 10 μg of EE protein and 50 μl of anti-VN was added to aliquots (up to 10 μl) of culture

media. The total volume was adjusted to 100 μl with antibody buffer and the mixture was incubated overnight at 4°C. The immune complex was pelleted at 1000 *g* for five minutes and the supernatant was siphoned off. The pellet was resuspended in 100 μl of antibody buffer and the incubation and pelleting were repeated twice. The final pellet was resuspended in 50 μl of PBS (containing 1% deoxycholate, 1% Triton X 100) by vortexing and quantified by liquid scintillation spectroscopy. Under these conditions 70-84% of radiolabeled VN was precipitated (tested VN sample range was 50 ng to 5 μg). The specificity of the immunoprecipitation was assessed by similar treatment of an adult male protein extract (radiolabeled *in vivo* prior to extraction). Less than 8% of the radioactivity associated with proteins in this sample were precipitated.

2.21 FLUOROGRAPHY.

Radiolabeled, coomassie blue stained gels were impregnated with EN^{3}HANCE (New England Nuclear) according to manufacturer's instructions and dried on 3mm paper (BioRad) in a slab gel dryer (Hoefer). Gels were placed on XOMAT AR film (Eastman Kodak, Rochester, N.Y.) in fluorography cassettes (Eastman Kodak) and exposed at -70°C. Exposed film was developed following manufacturer's instructions (Eastman Kodak).

CHAPTER 3. RESULTS

3.1 CHARACTERIZATION OF EGG YOLK PROTEINS

Initial investigation of vitellogenesis was directed towards identification and characterization of the major egg yolk protein, vitellin. Molecular weight determination of egg proteins was accomplished by column chromatography and native PAGE. Subsequently, tissue and yolk proteins were compared by native and denaturing PAGE.

3.1.1 MOLECULAR WEIGHTS OF EGG YOLK PROTEINS BY COLUMN CHROMATOGRAPHY. Egg extract (EE) was analyzed by Sepharose 6B column chromatography. The elution profile of the egg proteins at 280 nm and 400 nm is shown (Fig. 5). Three major peaks, identified as VN A, VN B and VN C, are resolved from the EE at both wavelengths. The coelution of these peaks at 280 nm and 400 nm suggests a direct relationship between the elution of proteins (280 nm) and heme-proteins (400 nm). Under the test conditions the absorption are not baseline resolved. The elution of VN A in the void volume (V_o) indicates that this egg protein is excluded by the gel pore size and therefor does not allow a molecular weight assignment. The elution volumes (V_es) of VN B and VN C correspond to molecular weights of 630 KD and 43 KD respectively (*Fig. 6*). Identification of VN C, is the first report of a native VN with a molecular weight below 300 KD in ticks. This is the first report of a small molecular weight VN (ie., VN C) in ticks. Selected column fractions were further resolved by native and SDS PAGE (*Fig. 7*). VN A, the excluded column fraction, was not detectable in native PAGE by silver staining

FIGURE 5. MOLECULAR WEIGHT OF EGG PROTEINS: SEPHAROSE 6B. Timed fractions (6.2 minutes) yielding 2.5 ml each were collected from a Sepharose 6B column. Column fractions of eluted egg extract are plotted against their absorbance values at 280 and 400 nm. Three absorbance peaks (VN A, VN B, VN C) are identified. Blue dextran, a non-included molecular weight standard, was utilized to determine the void volume (V_o). V_o = 21.5.

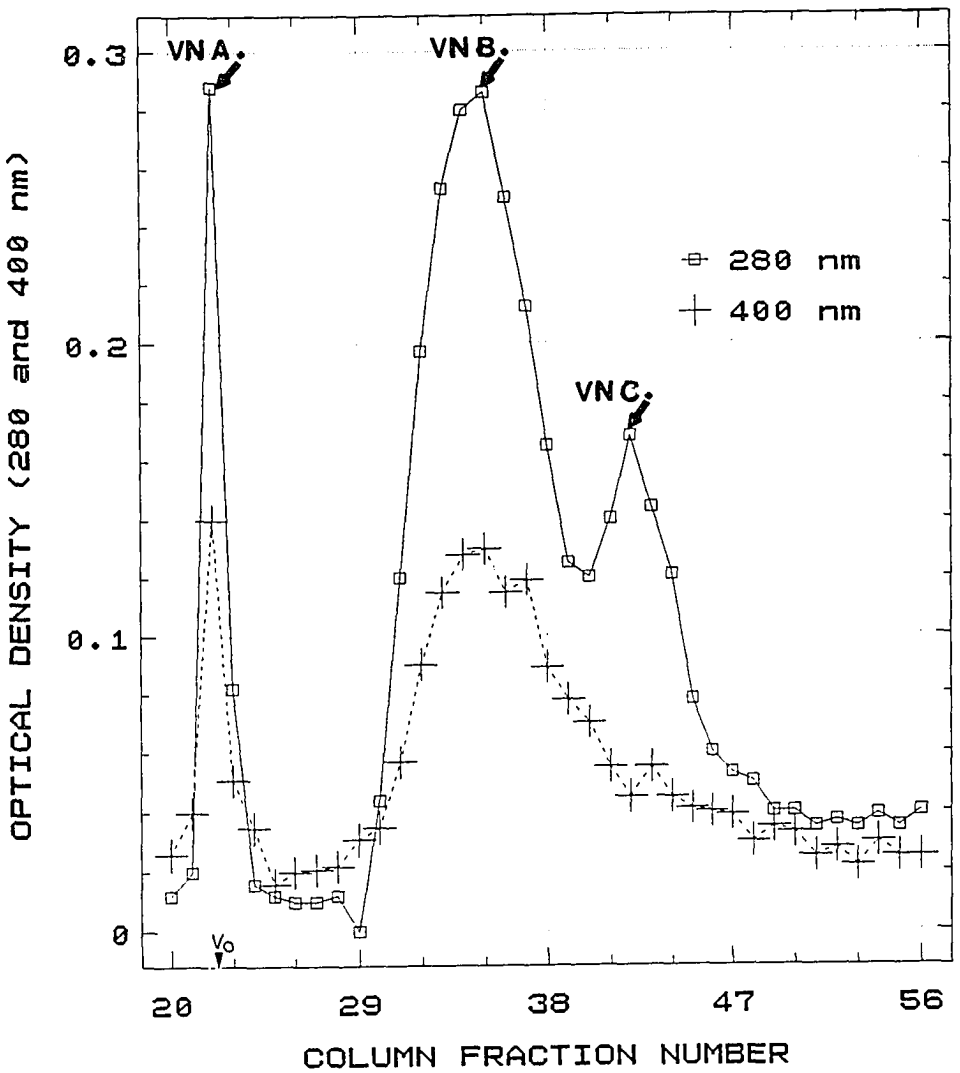


FIGURE 6. NATIVE PROTEIN MOLECULAR WEIGHT: SEPHAROSE 6B CALIBRATION CURVE. Elution volume (V_e) of each of the molecular weight standard and EE protein is equal to the number of fractions collected from the point of sample application to the maximum sample peak absorbance at 280 nm (see *Fig. 5*). V_o (blue dextran) = 21.5. Approximate molecular weights of standards are; 1. thryroglobulin (bovine) - 669 KD, 2. apoferritin (horse spleen) - 443 KD, 3. albumin (bovine serum) - 66 KD, 4. carbonic anhydrase (bovine erythrocyte) - 29 KD. The V_e/V_os of the egg extract proteins VN B and VN C are indicated.

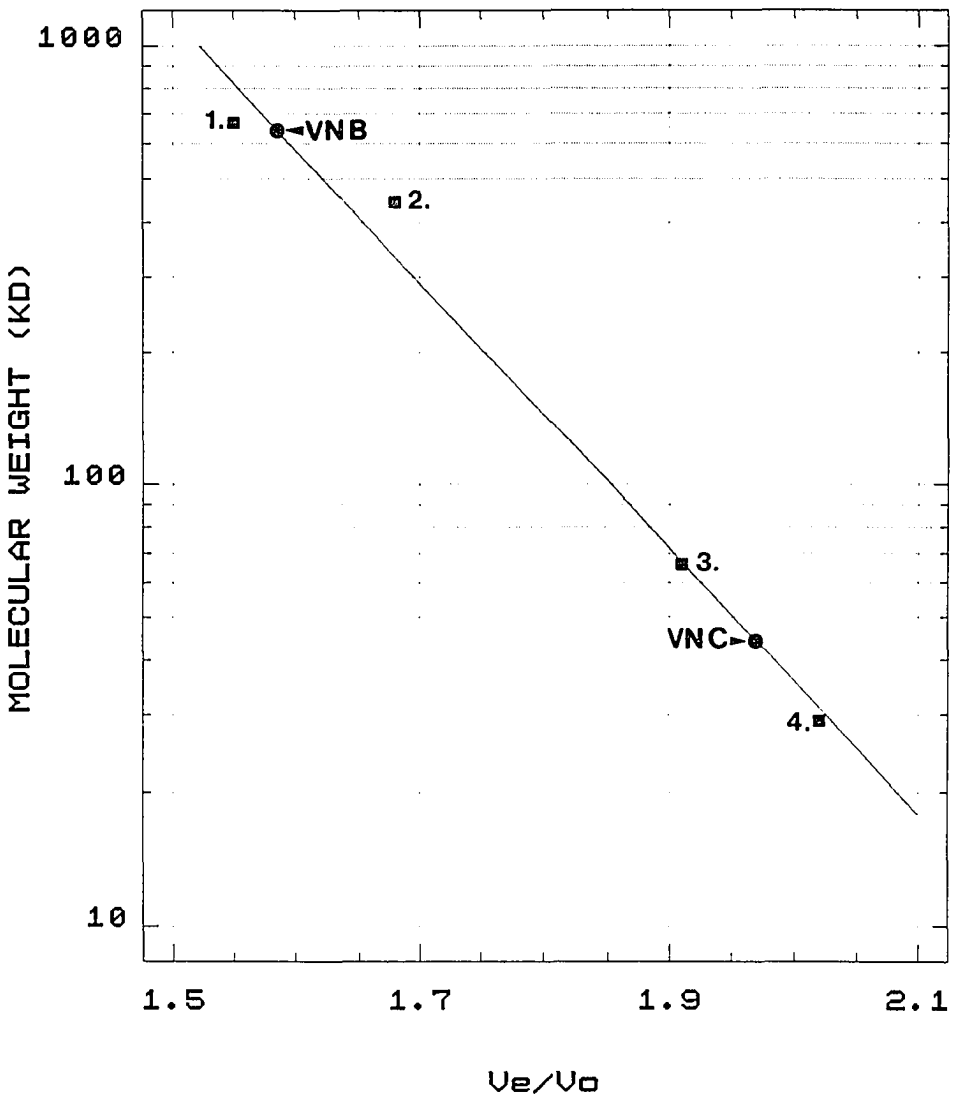
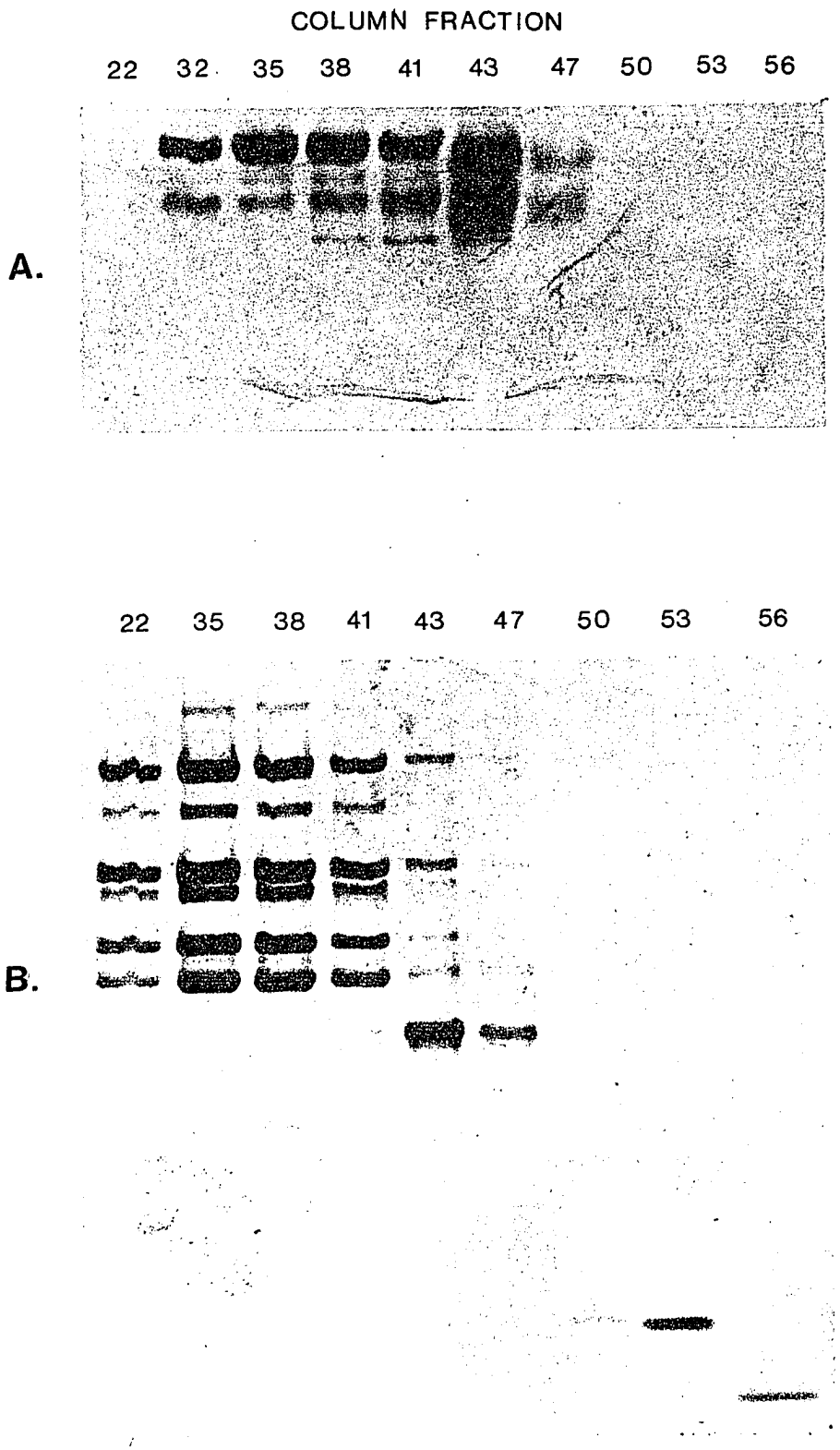


FIGURE 7. PAGE OF SEPHAROSE 6B RESOLVED EGG PROTEINS. Selected Sepharose 6B column eluate fractions of egg extract were further resolved by native (A) and SDS (B) PAGE. Column fraction numbers are indicated above appropriate PAGE sample lanes. VN A = fraction 22, VN B = fraction 32-38, VN C = fraction 43. Gels were stained with silver.



(*Fig. 7A*). The possibility that this protein did not enter the gel due to its large size can not be excluded. VN B resolves into two major bands in native PAGE. VN C resolves into four native PAGE bands, two of which comigrate with those of VN B. The presence of similarly migrating PAGE bands from VN B and VN C column fractions suggests an overlap in the elution of these proteins. The lack of baseline separation between the column eluted fractions is consistent with these overlaps. Proteins eluting from the column after VN C were not detectable in silver stained native gels. Upon denaturation, VN A and VN B give rise to replicate SDS PAGE patterns consisting of six bands of major staining intensity (*Fig. 7B*). The stained SDS PAGE of VN C displays a major low molecular weight band (50-56 KD) and a small amount of the same six bands observed from VN A and VN B. The 50-56 KD polypeptide is largely absent from the denatured profiles of VN A/VN B. Several lower molecular weight polypeptides are observed in silver stained SDS gels of fractions eluting after VN C. The absence of stainable bands from these fractions in native gels (see above) suggests an increased protein staining sensitivity in SDS gel.

3.1.2 MOLECULAR WEIGHT OF EGG PROTEINS BY NATIVE PAGE (TRANSVERSE GRADIENT). Molecular weight standards, ranging from 66,000 to 545,000 daltons, and EE were electrophoresed in a transverse gradient native gel (*Fig. 8*). Four protein bands (EE I-IV) were resolved from the egg sample. EE II was visible as a brown pigmented band without staining (during and after electrophoresis) and also constituted the major staining band. Ferguson plots for each MW standard and EE protein are shown (*Fig. 9*). It is observed that the

FIGURE 8. MOLECULAR WEIGHT OF EGG PROTEINS; TRANSVERSE GRADIENT PAGE. The gel percent gradient (10-5%) is recorded above the figure as are the boundaries of sample wells (A-D). A mixture of the molecular weight standards and egg extract (EE) was loaded in the large sample well, A. Additionally, the standards and EE were loaded in individual wells: B- urease trimer and hexamer (URt and URh), C- EE (EEI-IV), D- bovine serum albumin monomer and dimer (BSAm and BSAd). Each protein band is identified on the right side of the gel. The location of the tracking dye (TD) at the termination of electrophoresis is recorded. The gel was stained with coomassie blue.

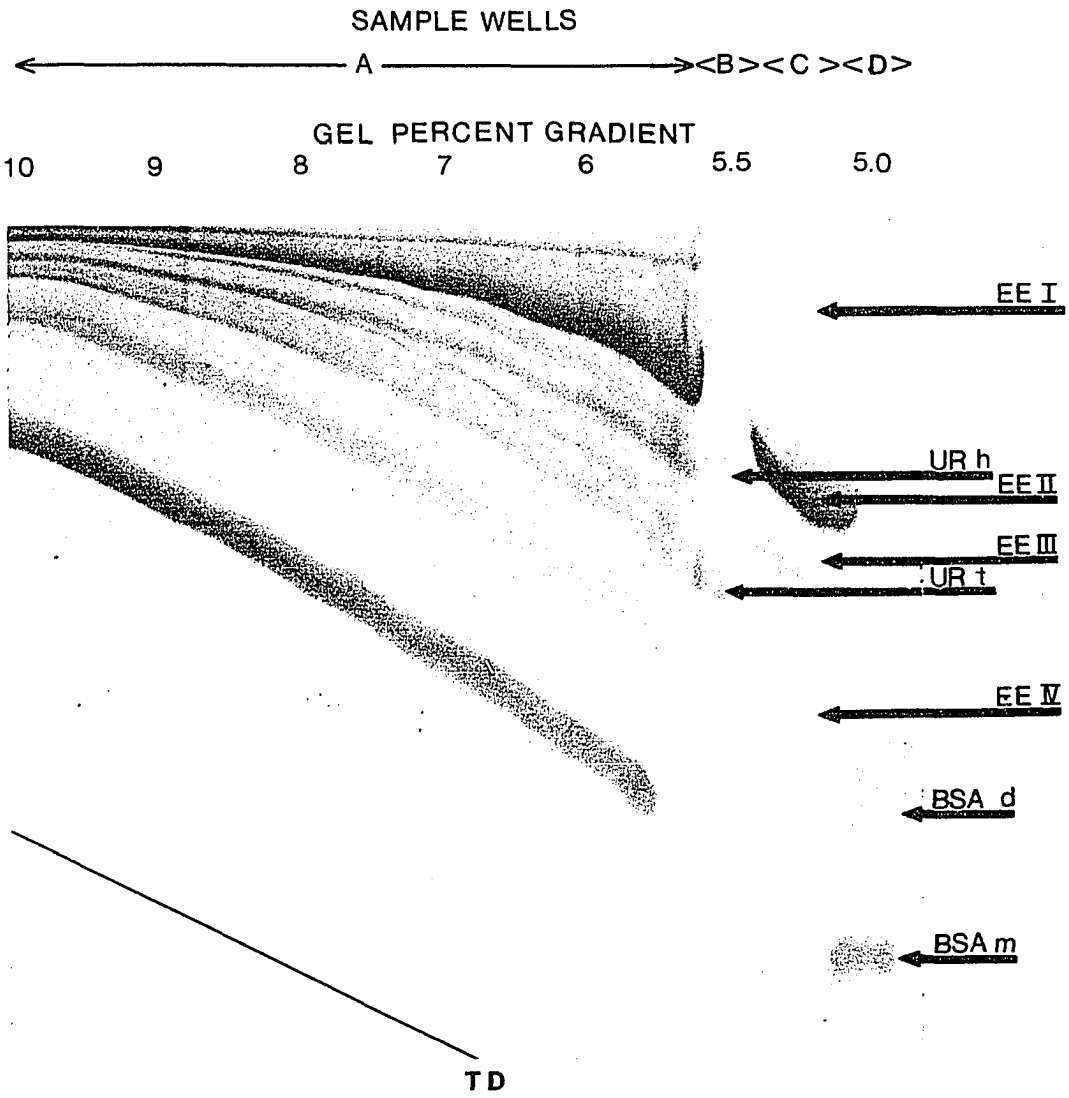
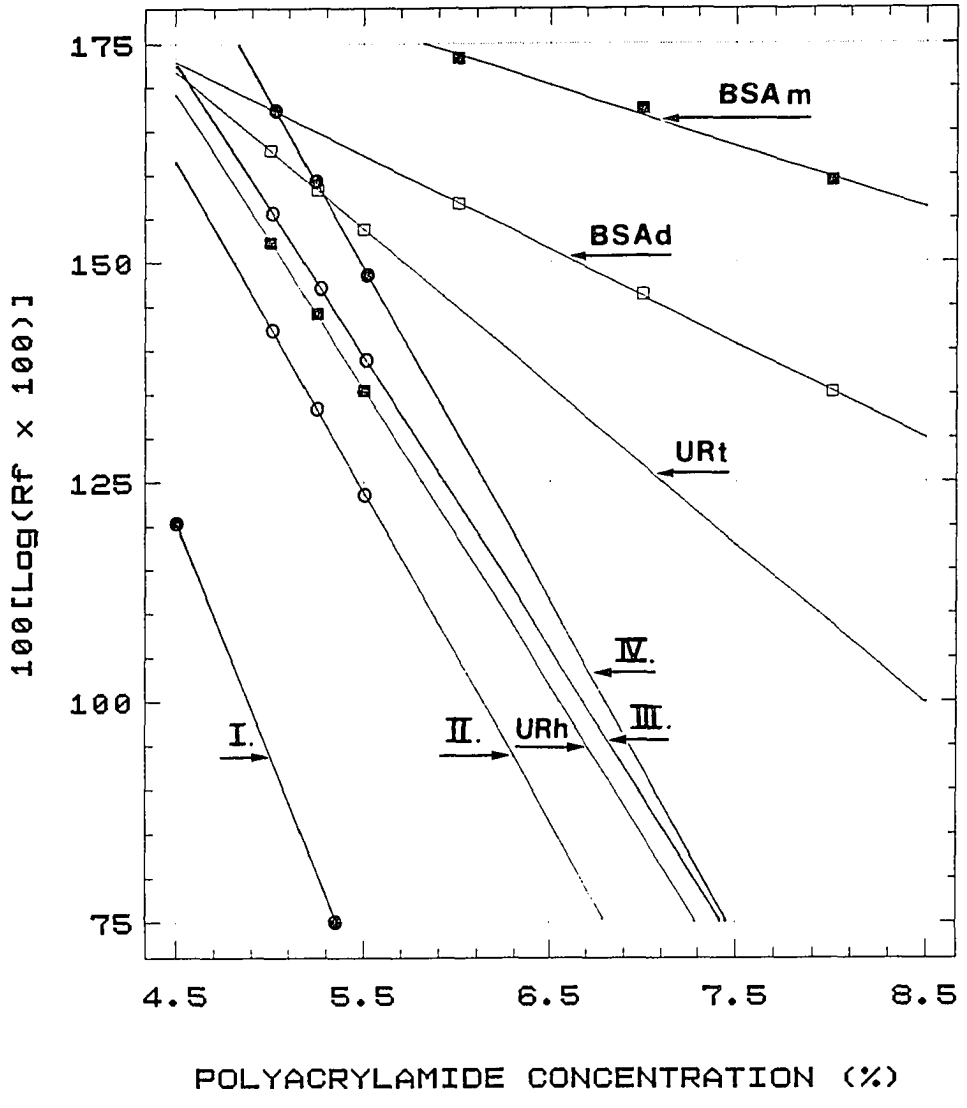


FIGURE 9. RELATIVE MOBILITY (R_f) OF MOLECULAR WEIGHT STANDARDS AND EGG PROTEINS AS A FUNCTION OF GEL % IN TRANSVERSE GRADIENT PAGE. The migration distance of each molecular weight standard and egg protein/distance of the tracking dye migration at a given gel concentration (from *Fig.8*) equals its R_f. R_f values are transformed (ie., $100[\text{Log}(R_f \times 100)]$) and plotted against % gel concentration. Linear regression analysis was utilized to determine best fit plots. MW standards are as in *Fig. 8*. I-IV = EE I-IV respectively.



steepness of these plots increases as a function of molecular weight. The slopes of these plots are utilized to generate a standard curve (*Fig. 10*). Based on their respective Ferguson plots, the molecular weights of egg proteins, EE I-IV, are 1060, 676, 572 and 681 KD. The close molecular weight estimates of EE II-IV suggests that these proteins may be charge isomers of a single size protein. Additionally, the molecular weight determinations EE II-IV approximate the size estimate of VN B, the major absorption peak, by column gel filtration (*Fig. 6*). EE I is approximatelytwice the size of EE II-IV and may correspond to the excluded column peak, VN A (see *Fig. 6*).

3.2 EGG SUBUNIT COMPOSITION

EE II, the most abundant egg protein, was purified from preparative native gels (see MATERIALS AND METHODS) and re-electrophoresed; two preparations are shown (*Fig. 11*). The major native staining band in both of the purified samples comigrates EE II in the crude egg extract. However, each purified sample also displays bands of reduced staining intensity which comigrate with the remaining egg proteins (EE I,III and IV). Gel purified EE II was also run in SDS PAGE (*Fig. 11B*). Under denaturing conditions EE II displays a total of seven bands, identified as VN 1-7. Subunits 1-6 correspond on a MW basis to the entire set of polypeptide subunits of VN A and VN B observed in *Fig. 7B*. The crude EE also displays an identical profile of VN 1-7 and an additional, poorly resolved, band(s) with a MW of 50 to 56 KD. This latter band corresponds, on a size basis, to VN C. Further clarification of the identities of EE I-IV was done by two dimensional PAGE (*Fig.*

FIGURE 10. NATIVE PROTEIN MOLECULAR WEIGHT: TRANSVERSE GRADIENT PAGE CALIBRATION CURVE. The negative slopes of linear regression plots for each standard in *Fig.9* are plotted against their known molecular weights {A. urease hexamer (Jack Bean) - 545 KD, B. urease trimer (Jack Bean) - 272 KD, C. albumin dimer (bovine serum) - 132 KD, D. albumin monomer (bovine serum) - 66 KD}. The determined molecular weights of the egg proteins, EEI-IV are 1,006 KD, 676 KD, 572 KD, and 681 KD, respectively.

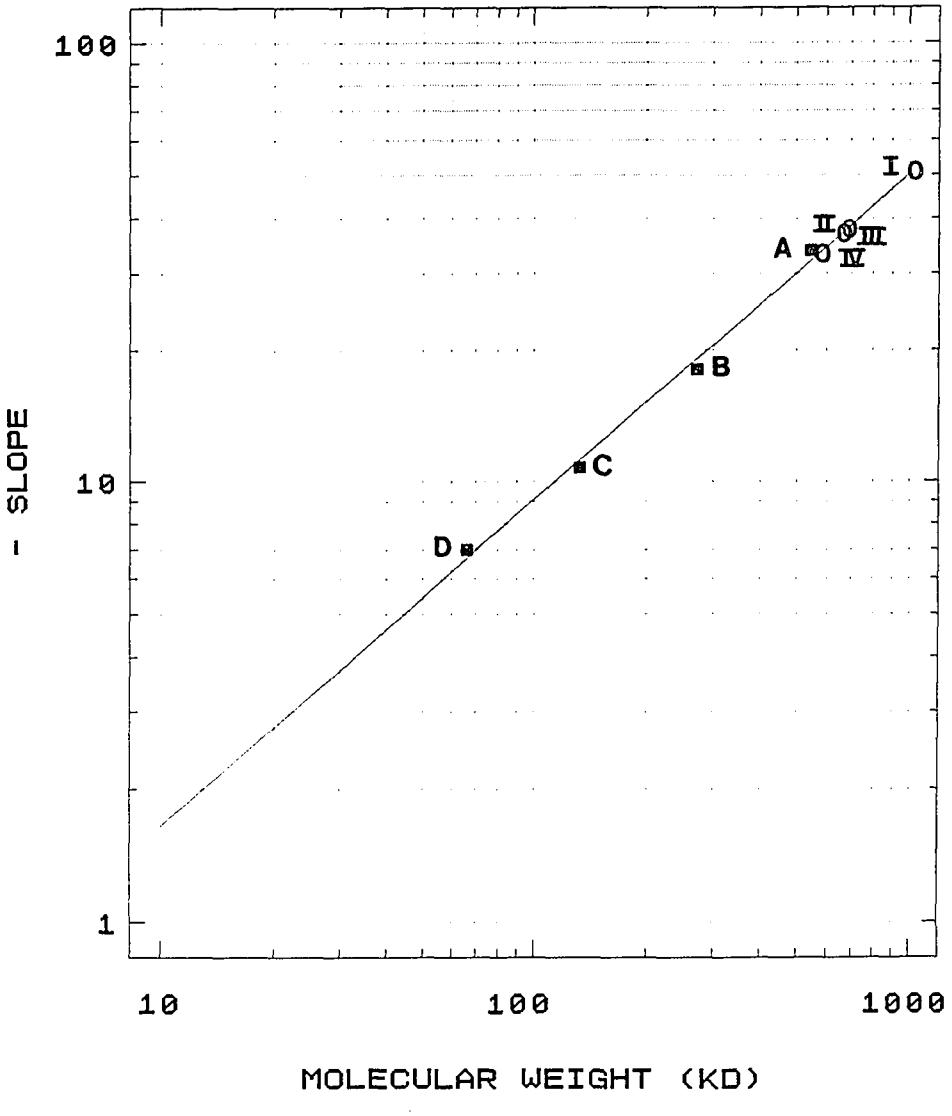
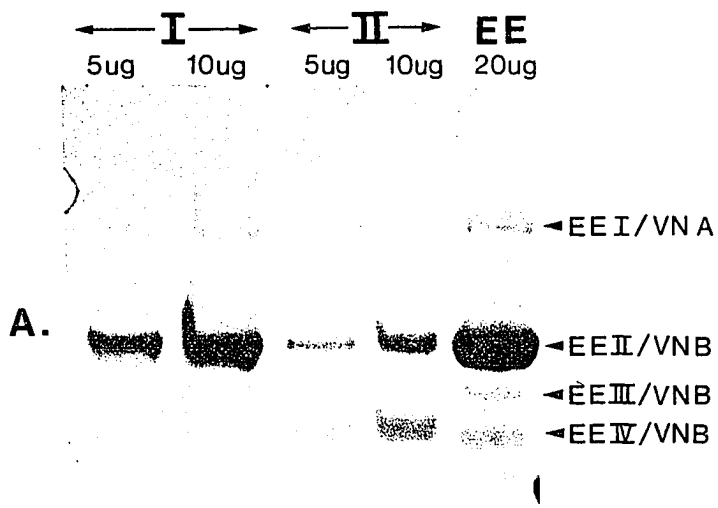


FIGURE 11. PAGE OF PURIFIED VITELLIN. PAGE purified VN B (EE II) from two seperate preparations (I and II) was re-eletrophoresed in nondenaturing (A) and SDS (B) PAGE. EE was run in parallel as indicated. Gels were stained with coomassie blue.



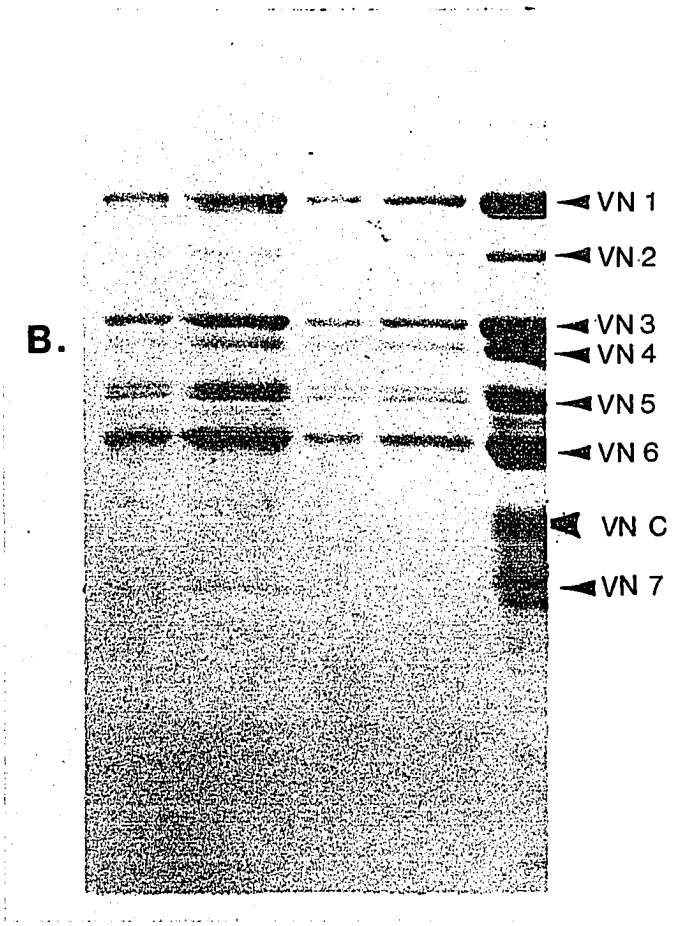
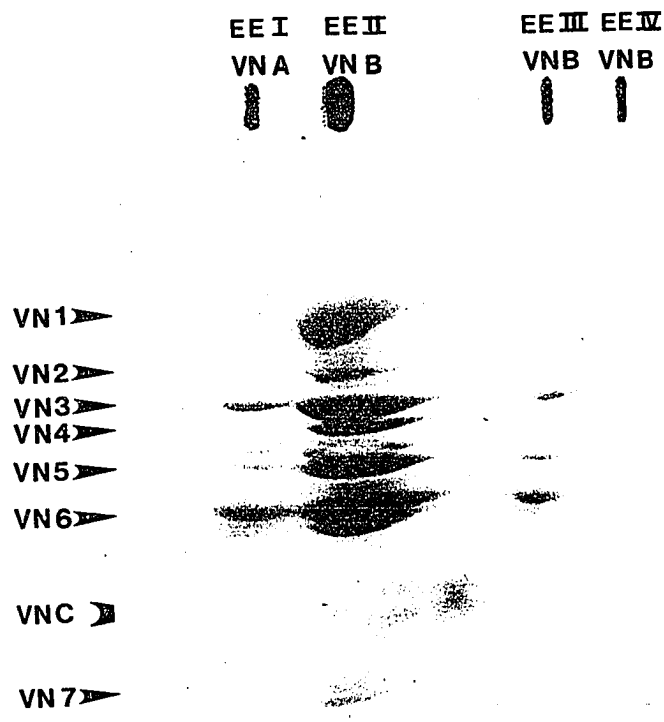


FIGURE 12. TWO DIMENSIONAL PAGE OF EGG EXTRACT PROTEINS. 150 μg of egg extract was resolved by two dimensional PAGE. Arrows are oriented towards the anode in the first (native) and second (SDS) dimensions. A replicate of the stained first dimension gel is shown aligned over the second dimension gel. VN A = EE I, the isomer forms of VN B are identified as EE II, EE III and EE IV.

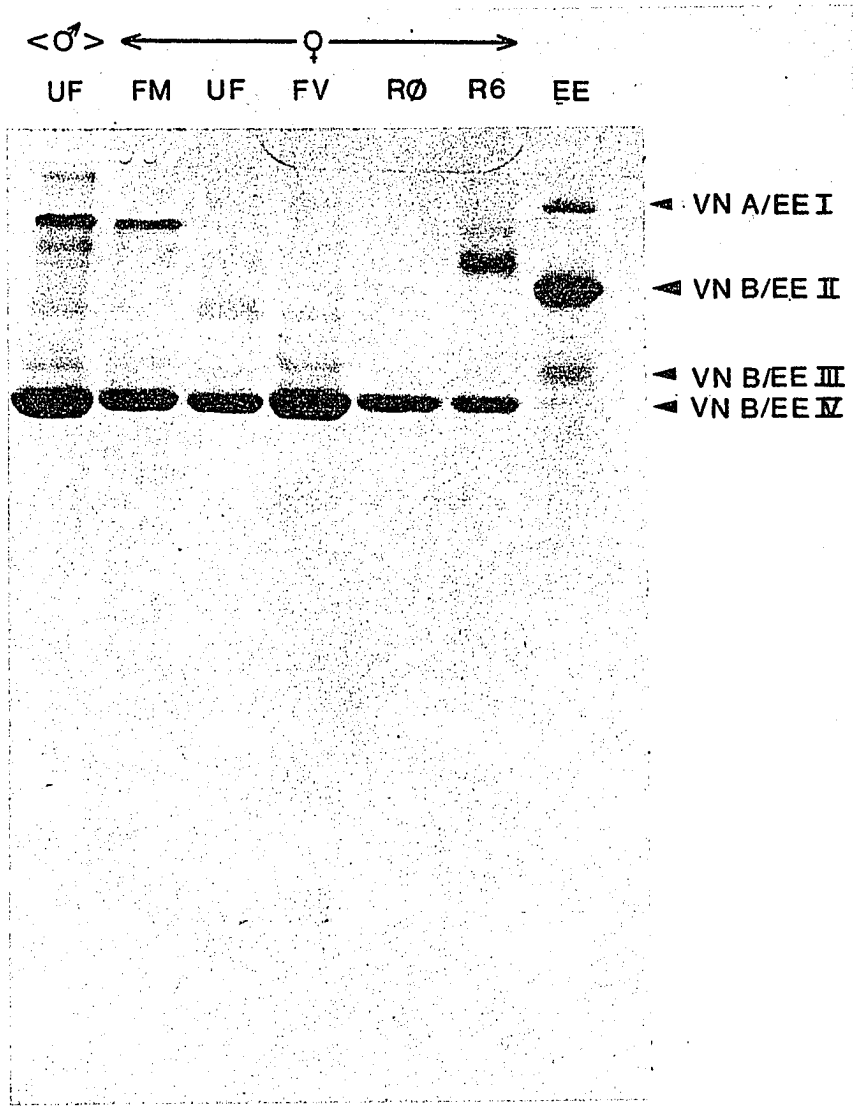


12). Egg homogenate was electrophoresed under native conditions in the first dimension. As previously observed, the egg proteins resolve into one dominant band (EE II) and three minor bands (EE I,III and IV) in the first dimension. Upon second dimension denaturation and electrophoresis, the subunit profiles of EE I, III and IV appear identical to that of EE II. Although the subunit electrophoretic patterns appear homologous, the relative contribution of each subunit to the native protein is variable, as established by their respective staining intensities. These results support previous evidence from transverse gradient PAGE suggesting compositional identity between egg proteins II-IV. Based on subunit comigration in SDS PAGE and native protein molecular weights, EE I was identified as VN A and EE II-IV were identified as VN B. Interestingly, the 50-56 KD egg polypeptide (VN C) observed in SDS PAGE dimension, migrates independently of the VN A and VN B proteins in the first dimension.

3.3 COMPARISON OF EGG AND TISSUE PROTEINS

Hemolymph samples from male and female adults in various stages of reproductive development were compared with egg VNs by native PAGE to determine the existence of protein electrophoretic homology (*Fig. 13*). Under nondenaturing conditions, egg extract displayed one band of major staining intensity and three lesser staining bands. As previously observed (*Figs. 11,12*), VN B/EE II is the dominant staining protein and is visibly pigmented without staining in wells loaded with $\geq$ 10 μg egg homogenate. One major protein band was also observed in all HL samples. This band demonstrated homologous electrophoretic mobility in male and female HL samples. Although the HL band did not comigrate with the

FIGURE 13. NONDENATURING PAGE OF EGG EXTRACT AND ADULT HEMOLYMPH PROTEINS. 20 μg of HL protein from each of the indicated male and female developmental stages, and EE, were resolved by native PAGE as indicated. The gel was stained with coomassie blue. Developmental stages, UF - unfed, FM - freshly molted, FV - fed virgin, R"X" - replete where "X" equals the number days post-repletion.



major form of VN B/EE II, it did however, appear to comigrate with a minor form of VN B, EE IV. HL bands of decreased staining intensity and electrophoretic mobility were also observed in all samples. No comigration between these bands and egg proteins was observed.

R2 and R8 tissue protein PAGE patterns are compared with that of EE (*Fig. 14*). Under native conditions, the major staining band in all R2 tissues migrates closely with one of the a minor VN B components (EE IV). This protein is most prominent in HL. No other proteins are observed in R2 tissues which comigrate with any of the egg proteins. In contrast to R2 tissues, R8 OV displays a major component which comigrates with EE II of VN B. As in R2 females, the major staining band in R8 FB and HL migrate closely with EE IV. R8 MG displays several protein bands which do not comigrate with any of the egg proteins.

3.4 COMPARISON OF EGG AND TISSUE POLYPEPTIDES.

Female HL samples from successive developmental adult stages were resolved by denaturing SDS PAGE (*Fig. 15*). Two intensely staining bands and several minor staining bands were resolved in all HL samples. R4 through R14 females presented two unique high molecular weight HL bands but these did not comigrate with any major egg subunits. Coelectrophoresis of VN 1 and a faint band in HL samples from all developmental stages was observed. VN 3 and VN 4 appeared to migrate slightly slower (above) the major doublet polypeptide characteristic of all HL samples. Several faint HL bands comigrate with the lower molecular weight VN C and VN 7 of EE. The latter were most apparent in R6 through R11 HL samples.

FIGURE 14. NONDENATURING PAGE OF EGG EXTRACT AND TISSUE PROTEINS. 10 μg of each tissue sample protein from R2 and R8 females were loaded per lane. 20 μg of EE protein was loaded in indicated well. Gel was stained with coomassie blue. Tissue samples, FB - fat body, HL - hemolymph, OV - ovary, MU - muscle, MG - midgut.

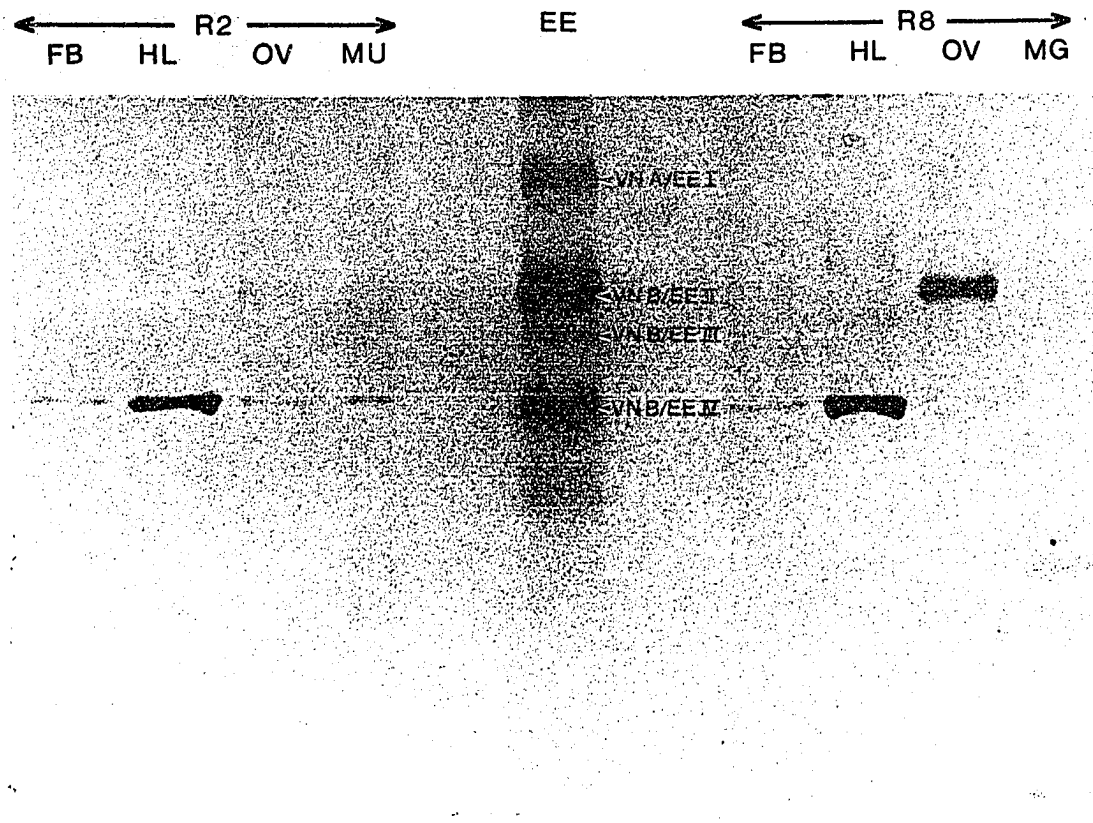
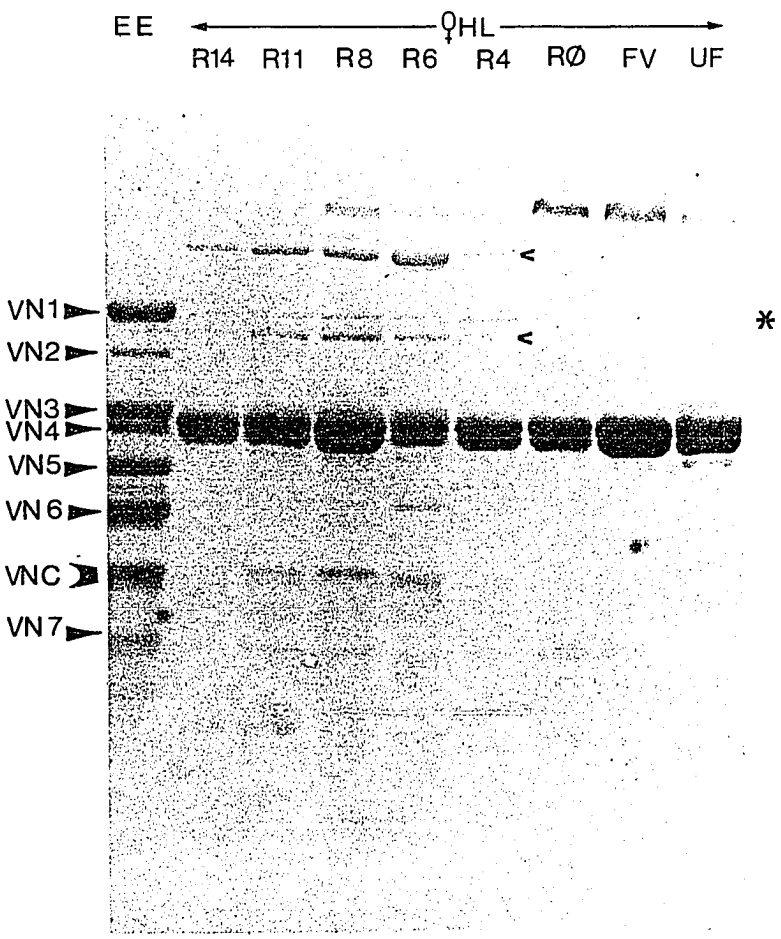


FIGURE 15. SDS PAGE OF EGG EXTRACT AND ADULT HEMOLYMPH POLYPEPTIDES. 25 μg of EE and HL from the indicated female developmental stages was electrophoresed/lane. * - polypeptide present in all HL samples which comigrates with VN 1. < - two high molecular weight bands present in R4 - R14 HL. The resolved gel was stained with coomassie blue. See *fig. 13* legend for description of developmental stages.



Tissue and egg polypeptide were also compared in denaturing SDS PAGE (*Fig. 16*). Each tissue type displays a unique banding pattern and these patterns are largely conserved from R2 to R8. Very little demonstrable electrophoretic homology is observed between HL, FB or MG polypeptide and the EE subunits. R2 OV displays a large number of polypeptide, some of which appear to migrate closely on a size basis with those of the EE. In contrast to these tissues, R8 ovary polypeptide displays a nearly identical electrophoretic pattern with that of the egg polypeptide.

3.5 IMMUNODETECTION OF VITELLIN AND VITELLOGENIN.

3.5.1 SLIDE IMMUNOPRECIPITATION. Detection of HL VG with anti-VN was demonstrated by slide immunoprecipitation. A continuous precipitin line was formed between the antiserum well and egg extract, PAGE purified VN B, R0 HL and R6 HL (*not shown*). These results indicated the presence of immunoreactive HL VG in R0 and R6 HL. In contrast, no precipitin line was formed between UF or FV HL and antiserum. Together, these results indicate the appearance of immunoreactive HL VG between FV and R0 stages of reproductive development. Further investigation of tissue VG was directed towards immunodetection in western blots.

3.5.2 WESTERN IMMUNOBLOT. Sensitive and selective staining of VN polypeptide is observed western immunoblots (*Fig. 17*). In part A, nonselective protein staining of all of the samples is observed. There is little or no polypeptide

FIGURE 16. SDS PAGE OF EGG EXTRACT AND ADULT TISSUE POLYPEPTIDES. 25 μg of protein from the indicated tissues of both R2 and R8 females was electrophoresed/lane. Additionally, 3 or 15 μg of EE was electrophoresed as indicated. The resolved gel was stained with coomassie blue. See *fig. 13 & 14* legends for a description of the tissues and developmental stages.

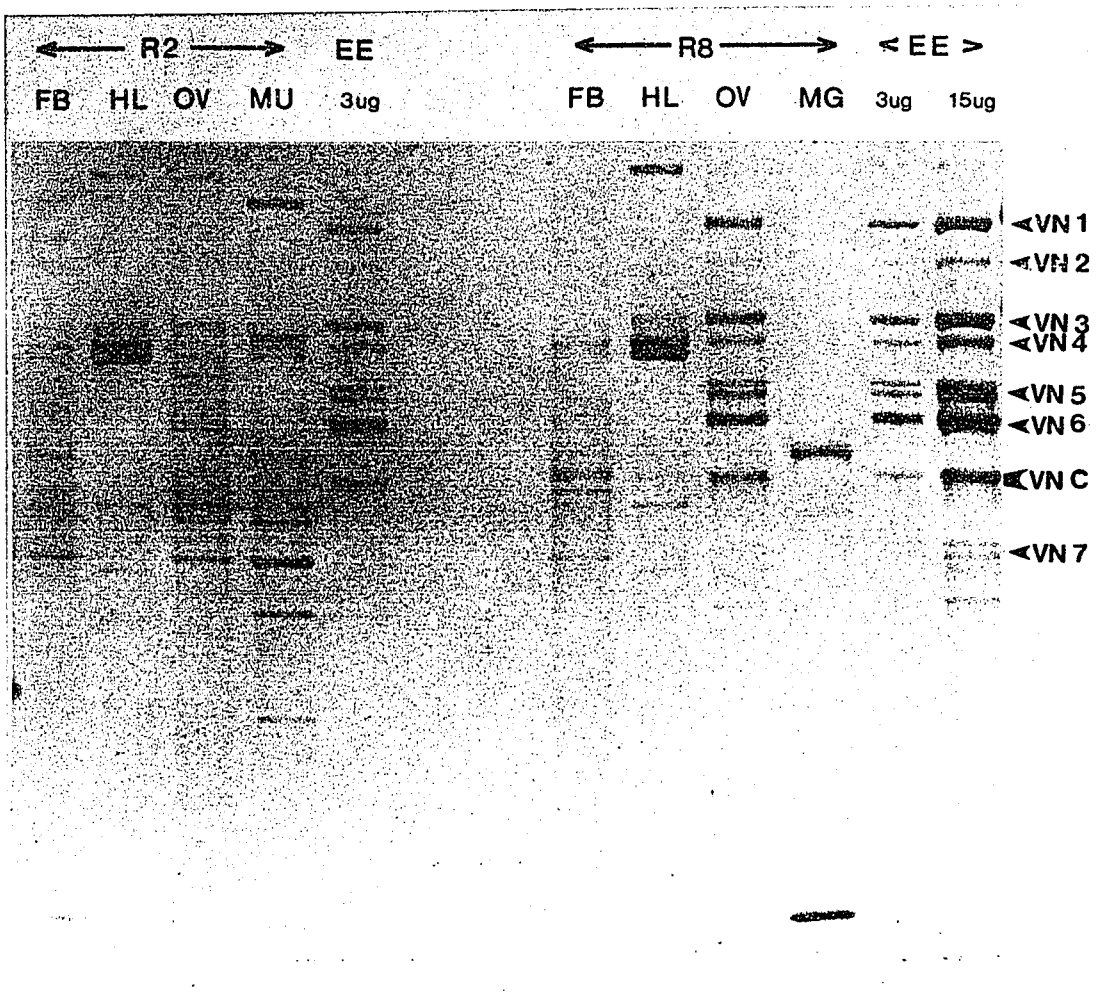
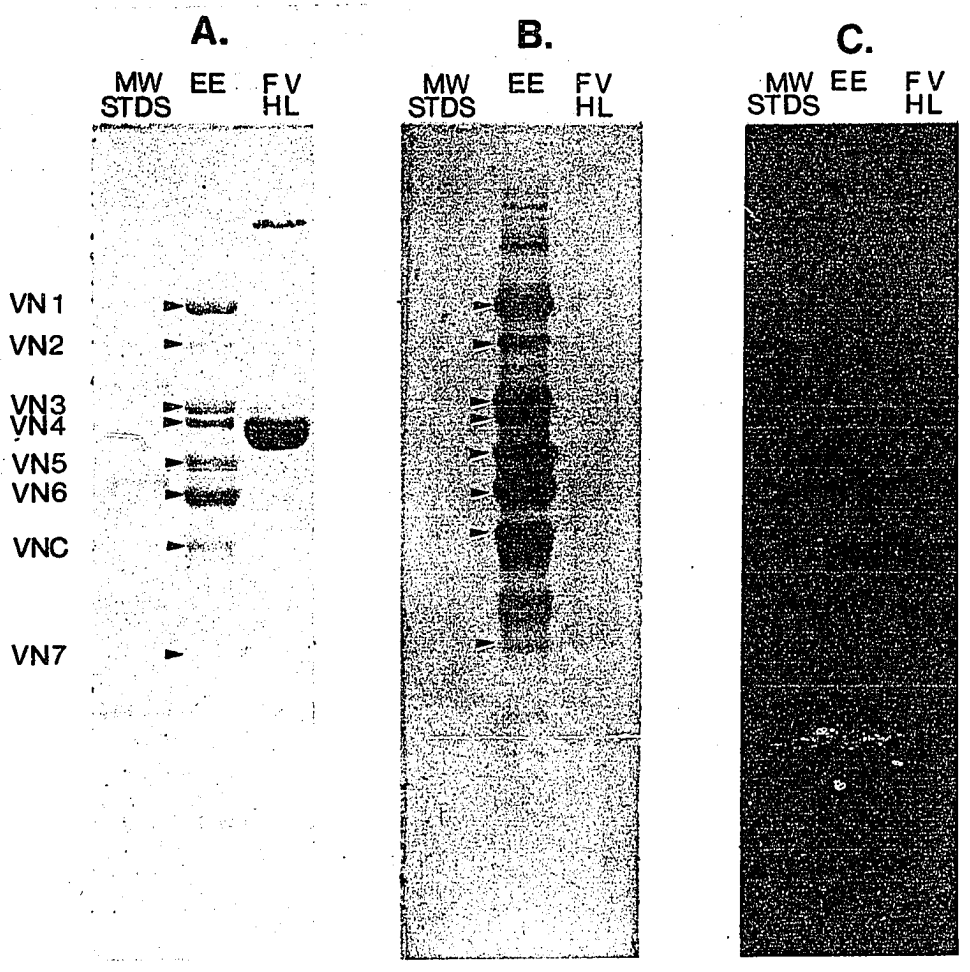


FIGURE 17. IMMUNODETECTION OF EGG EXTRACT POLYPEPTIDES IN WESTERN BLOT. 20 μg each, of molecular weight standards (MW STDS), egg extract (EE) and fed virgin hemolymph (FV HL) were resolved in three replicate SDS gels. The first gel was stained with coomassie blue (A). The remaining gels were blotted to nitrocellulose. The first blot (B) was probed with anti-VN, GAR-HRP, and peroxidase color substrate. The second blot (C) was treated as above (B) and subsequently stained with amido black.

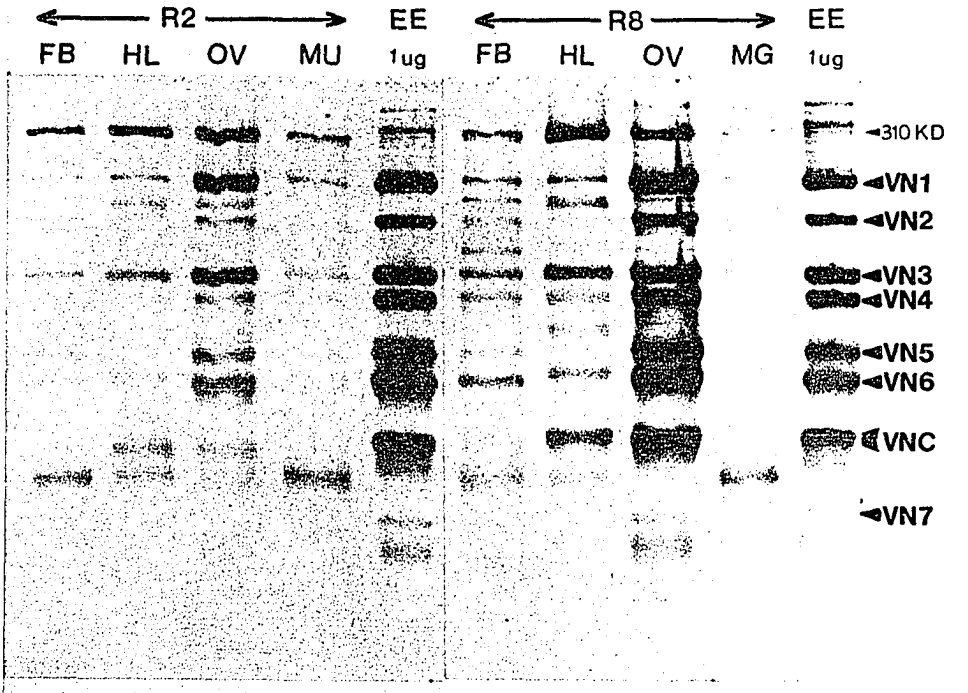


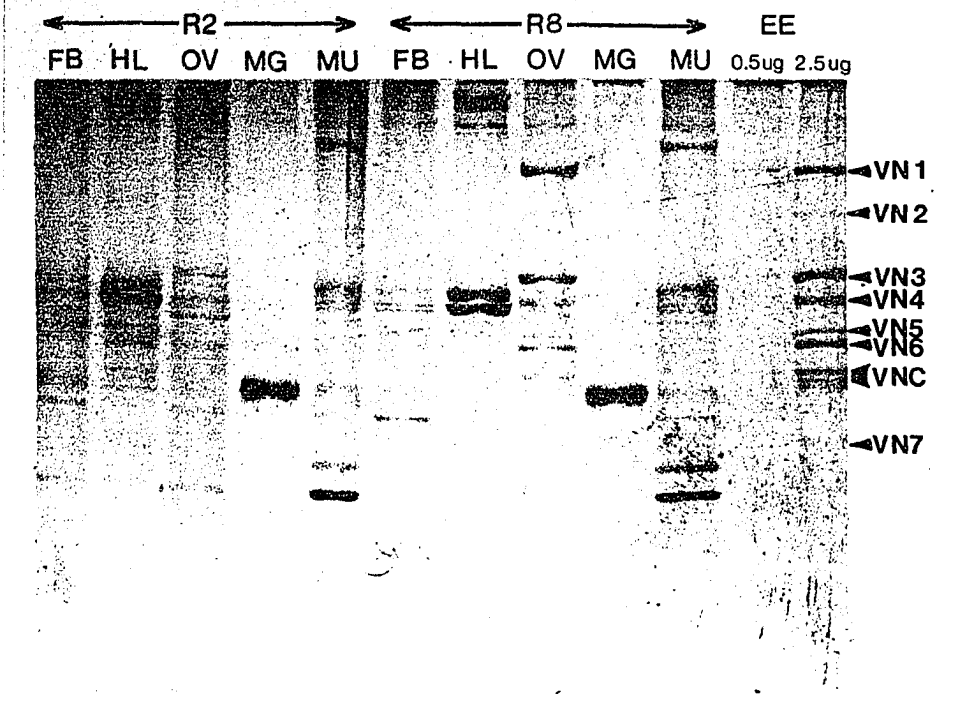
comigration between the three protein samples, with the possible exception of the upper band of the FV HL doublet and VN 4 (101 Kd). In part B, a replicate gel of A was blotted to nitrocellulose and immunoprobed; only egg polypeptide are immunostained. The FV HL bands with a mobilities close to that of the 101 KD egg polypeptide are not stained, indicating non-identity. Likewise, the additional HL polypeptide and those of the molecular weight standard sample are not identified by the immunoprobe. Interestingly, the immunoprobe demonstrates sensitivity to several egg polypeptide that were not detected in the coomassie stained gel (A). Efficient electrotransfer of all three sample polypeptide from gels to nitrocellulose is demonstrated by nonselective amido black staining in part C.

3.5.3 IMMUNOPROBE COMPARISONS OF EGG AND TISSUE PROTEINS.

SDS PAGE resolved tissue extracts from two mated female reproductive stages were immunoblotted and silver stained (*Fig. 18*). The tissue R2 and R8 polypeptide detected in the immmunoblot are not detectable by silver staining. Nevertheless, the immunopattern is well resolved and demonstrates a high degree of electrophoretic size homology (comigration) to that of the VN subunits. In contrast to FV HL in previous figure, HL and two tissues, FB and OV, from replete females demonstrate substantial immunoreactivity. Furthermore, VG immunoreactivity in these tissues increases as a function of reproductive development from R2 to R8. Muscle tissue demonstrates the lowest immunoreactivity of the R2 tissues assayed. Compared to other tissues, MG (R8) displays a greatly reduced immunoreactivity. A high molecular weight (310 KD) immunoreactive band is observed in both tissue and

FIGURE 18. WESTERN IMMUNODECTION OF VITELLIN AND VITELLOGENIN POLYPEPTIDES IN EGG AND TISSUE EXTRACTS. 10 μg of protein from the indicated R2 and R8 tissues was resolved in two replicate SDS PAGE gels. Egg extract was included as indicated in each figure. Gel A. was blotted and immunoprobed, gel B. was stained with silver. See *fig. 13 & 14* legends for description of tissues and developmental stages.



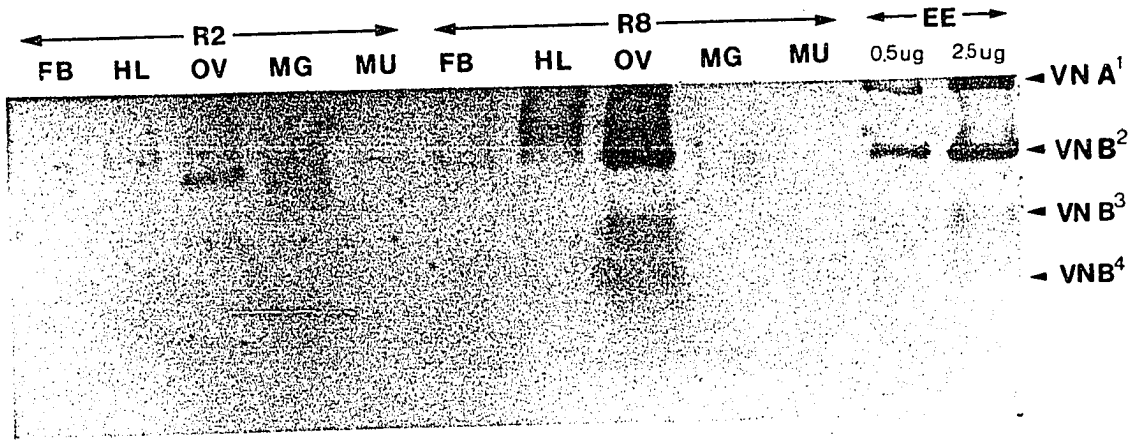


egg extracts. Curiously, the immunoreactive intensity of the 310 KD band in HL and FB (both R2 and R8) is noticeably greater than that of the lower molecular weight bands. In MG and EE, this band is a minor component. Although the resolution of the native PAGE immunoblot is not as high as that of the denatured replicate, similar results are demonstrated (*Fig. 19*). Namely, 1: HL, FB, and OV display increased immunoreactivity as a function of reproductive development, 2: low levels of immunoreactivity, which do not increase from R2 to R8, are displayed in MU and MG, 3: a high degree of electrophoretic homology between the immunoreactive tissue bands and the major VN bands is observed. A replicate native gel was silver stained (*Fig. 19B*). The major silver stained band of FB, HL, and MU (also present in lower amounts in OV and MG), which appears to comigrates with VN B/EE IV, is not detected by immunostaining. All egg bands (EE I-IV) are immunopositive.

HL samples from male and female adults during progressive developmental and reproductive stages were also analyzed by western immunoblot (*Fig. 20*). Interestingly, HL from freshly molted males demonstrates a low level of immunoreactivity which appears to be absent in fed-mated males. UF and FV female HL demonstrate trace levels of immunoreactivity. Female HL immunoreactivity increases slightly by R0 and dramatically by R4. Seemingly constant levels of immunoreactivity are observed in R4 through R8 HL. The major HL protein component in all adult samples (as previously demonstrated by coomassie staining, see *Fig. 15*), a doublet of 94 KD and 98 KD molecular weight, is not detected by immunoprobe. The location of this doublet is however, well delineated between the immunoreactive 102 KD and 86 KD bands (VN 4 and 5).

FIGURE 19. WESTERN IMMUNODETECTION OF NATIVE VITELLIN AND VITELLOGENIN IN EGG AND TISSUE EXTRACTS. 10 μg of protein from the indicated R2 and R8 tissues extracts was resolved in duplicate native gels. Egg extract samples were included as indicated. VN A^1 = EE I; VN B^2 = EE II; VN B^3 = EE III; VN B^4 = EE IV. The first gel was immunoblotted (A) and the second gel was silver stained (B). See *fig. 13 & 14* legends for description of tissues and developmental stages.

A.



B.

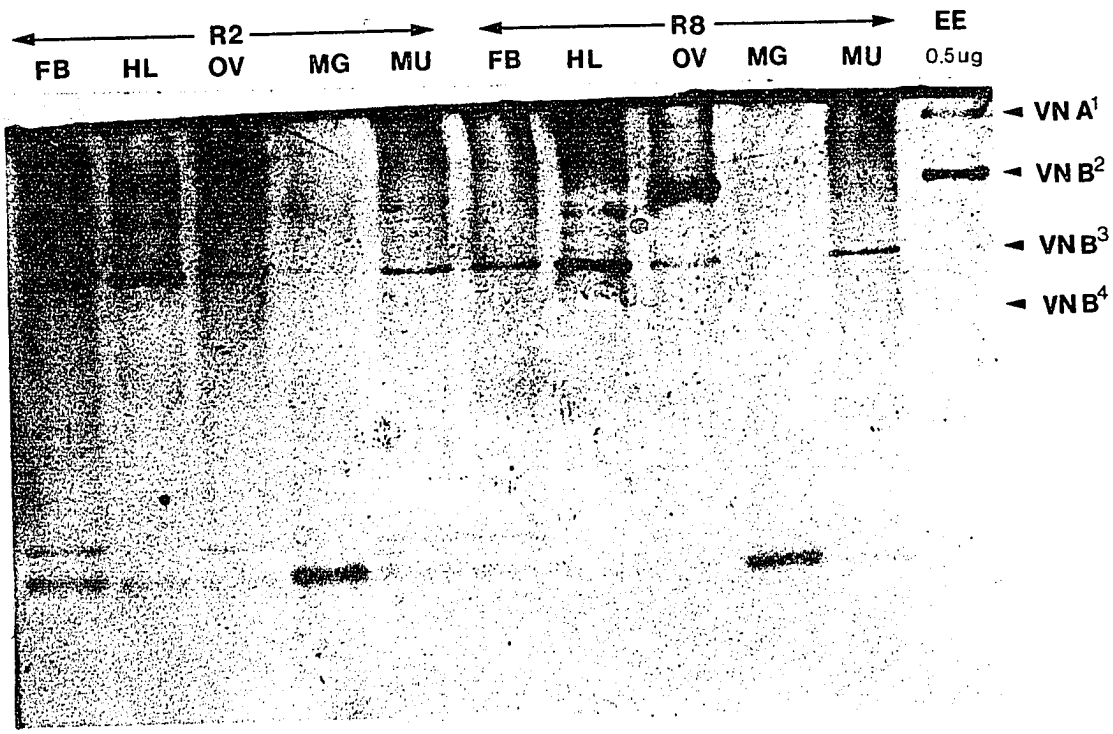
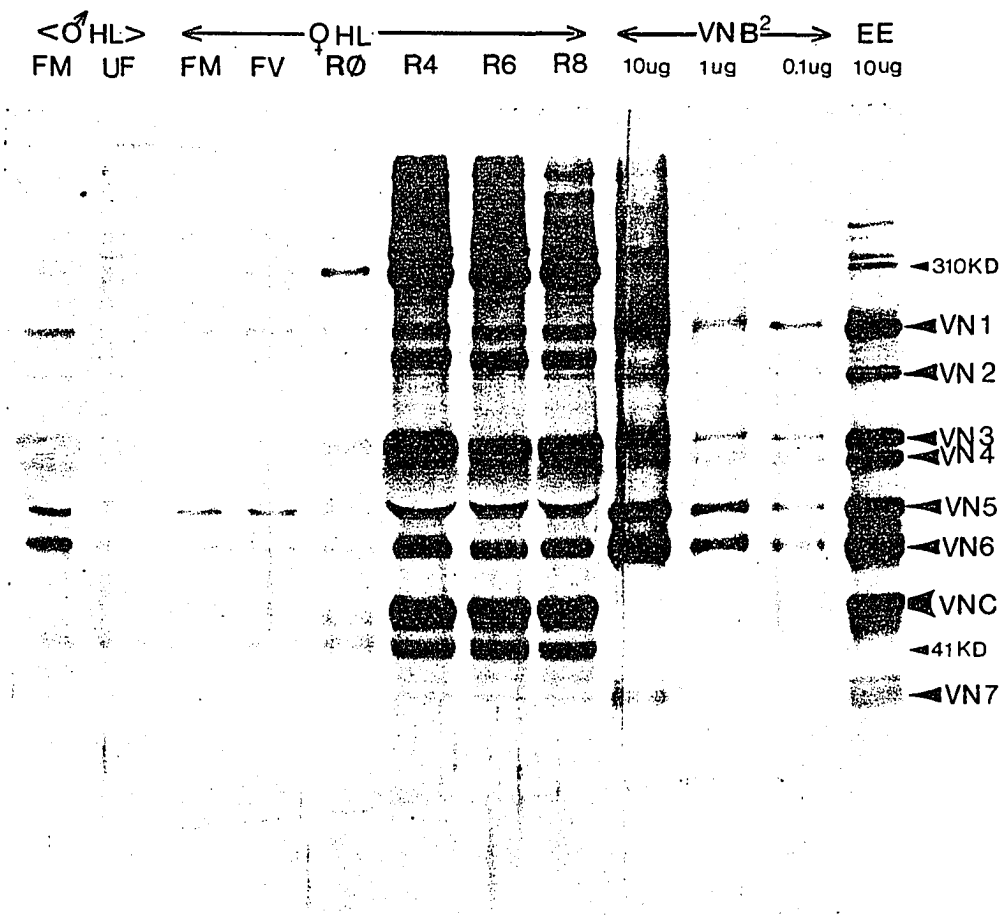


FIGURE 20. WESTERN INNUNODETECTION OF VITELLOGENIN IN HEMOLYMPH AS A FUNCTION OF REPRODUCTIVE DEVELOPMENT. 50 μg of HL protein from the indicated male and female stages was loaded/lane. VN B^2 and EE were loaded as indicated. Samples were resolved by SDS PAGE and immunoblotted. VN 1-7 and two additional HL VGs with MWs of 310 KD and 41 KD are also identified. VN B^2 = EE II. See *fig. 13* legend for description of developmental stages.



Also, as previously observed, all immunoreactive VN bands are represented by comigrating bands in replete HL samples. Curiously however, several additional immunoreactive bands are observed in HL samples. R4 through R8 HL display a series of high molecular weight bands, ranging from 400 KD to over 600 KD, and a band at 41 KD, which are absent or greatly diminished in egg and VN preparations. Additionally, as observed in FB and HL (*Fig. 18A*), the 310 KD band is intensely stained in R4, R6 and R8 HL, yet appears to be a minor component of VN B and crude egg homogenate. Also as observed previously, the 50-56 KD bands (VN C) of egg homogenate are not present in the VN B preparation. A VG band of this size is however, displayed in all replete HL samples.

3.6 MOLECULAR WEIGHTS OF DENATURED POLYPEPTIDES. Molecular weight analysis, under denaturing conditions, was performed in order to further characterize VN, VG, and tissue and hemolymph polypeptides (*Fig. 21*). PAGE resolved sample polypeptides were stained with coomassie blue or silver or blotted and immunostained. A standard curve for the determination of denatured polypeptide MWs is shown (*Fig. 22*). As previously described, VN B polypeptides are a subset of EE polypeptides. Electrophoresis and coomassie staining of ≤ 10 μg of PAGE purified VN A or VN B/sample well reveals seven major polypeptides (VN 1-7, *Table 1*). Increased sample loads results in a number of additional minor staining polypeptide. Under equivalent conditions (10 μg/well, coomassie stained), crude EE resolves these same seven polypeptides and VN C. When compared to coomassie or silver stained gels of EE, immunoprobed western blots demonstrate

FIGURE 21. MOLECULAR WEIGHT DETERMINATION OF VITELLIN AND TISSUE POLYPEPTIDES. Indicated samples were resolved on 4-15% linear gradient SDS gels. Gels were stained with coomassie blue (A). VG or VN polypeptides existing as minor components of complex proteins mixtures were identified by selective immunostaining (western immunoblot, B). The gel percent (%T) to which each MW standard and tissue polypeptide migrated was measured and recorded (*Table 1*). VN A/VN B subunits (VN 1-7) and VN C are identified. Additional immunoreactive VNs/VGs are identified by their MWs.

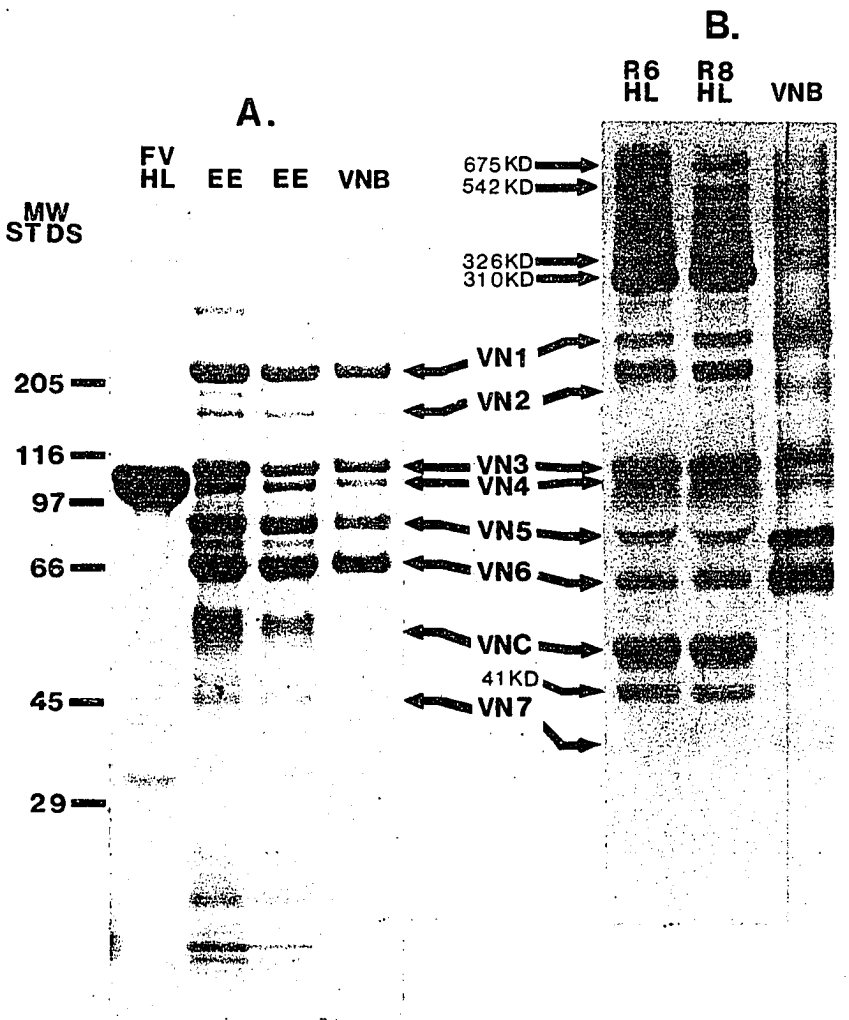


FIGURE 22. MOLECULAR WEIGHT DETERMINATION OF DENATURED POLYPEPTIDES: SDS PAGE CALIBRATION CURVE. The gel concentration (%T) to which each of the MW standards migrated (see *Fig. 21*) was transformed (Log_{10}%T) and plotted against the Log_{10}MW. A linear regression plot of these coordinates was derived and utilized to determine unknown tissue polypeptide MWs (*Table 1*). A - carbonic anhydrase (29 KD); B - egg albumin (45 KD); C - bovine albumin (66 KD); D - phosphorylase B (97.4 KD); E - galactosidase (116 KD); F - myosin (205 KD).

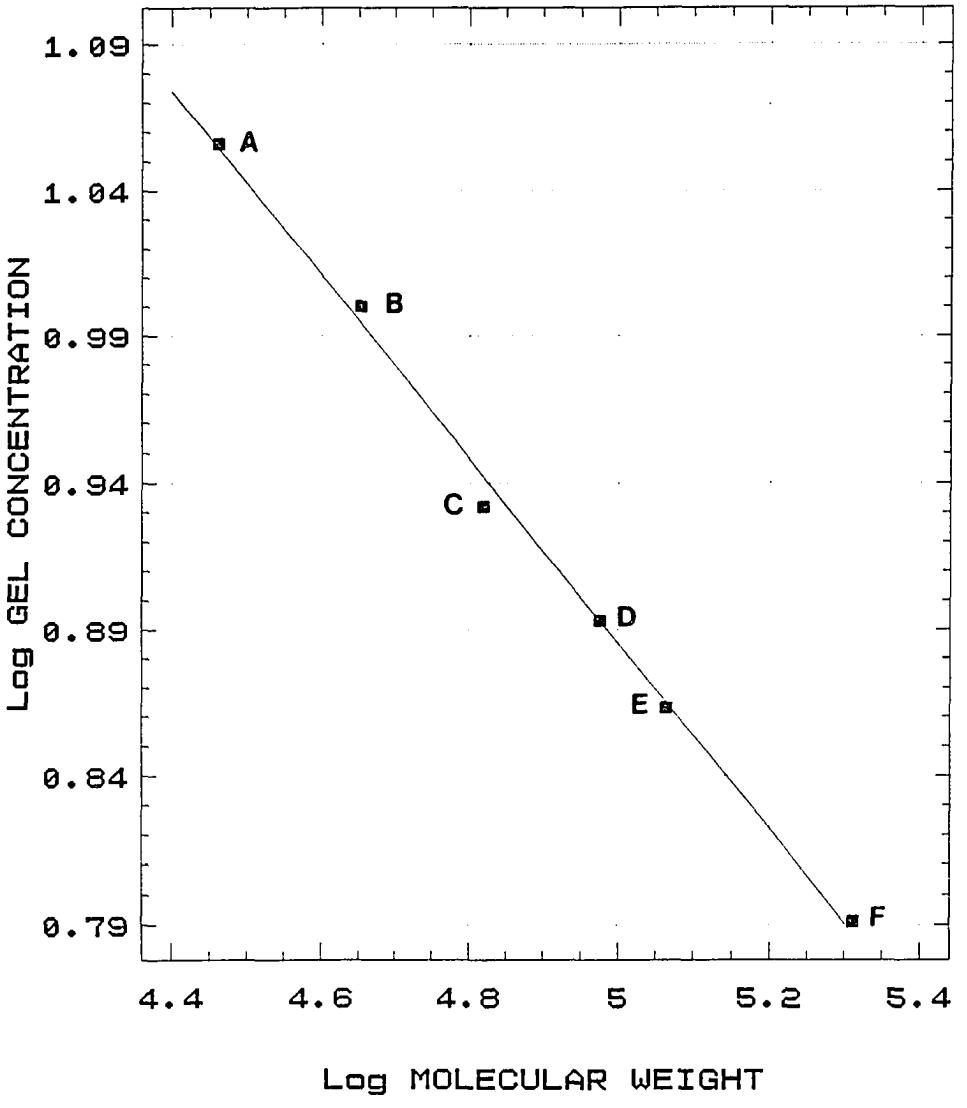


TABLE 1. MOLECULAR WEIGHTS OF TISSUE AND EGG POLYPEPTIDES.

SAMPLE	%T	Log_{10}%T	Log_{10}MW	MW
M.W. STANDARDS				
MYOSIN	6.18	0.791	5.312	205,000
β GALACTOSIDASE	7.29	0.863	5.064	116,000
PHOSPHORYLASE B	7.82	0.893	4.975	97,400
ALBUMIN (Bov.)	8.55	0.932	4.820	66,000
ALBUMIN (Chick)	10.00	1.000	4.653	45,000
CARBONIC ANHYD.	11.37	1.056	4.462	29,000
PAGE PURIFIED VN A/VN B SUBUNITS				
VN 1	6.05	0.782	5.326	212,000
VN 2	6.50	0.813	5.228	169,000
VN 3	7.36	0.867	5.057	114,000
VN 4	7.64	0.883	5.006	101,500
VN 5	8.05	0.906	4.933	86,000
VN 6	8.55	0.932	4.851	71,000
VN 7	10.67	1.027	4.550	35,500
ADDITIONAL EGG POLYPEPTIDES*				
463 KD	4.92	0.675	5.665	463,000
326 KD	5.28	0.723	5.513	326,000
310 KD	5.37	0.730	5.491	310,000
VN C	9.18	0.963	4.753	56,500
ADDITIONAL VG POLYPETIDES**				
675 KD	4.20	0.623	5.892	675,000
542 KD	4.50	0.653	5.734	542,000
506 KD	4.60	0.663	5.704	506,000
41 KD	10.00	1.008	4.753	41,000
NON-VITELLOGENIN HEMOLYMPH POLYPEPTIDES***				
HL 1	7.73	0.888	4.990	98,000
HL 2	7.83	0.894	4.971	93,500

%T values for MW standards and tissue polypeptides were determined from Fig. 21. Log_{10}%T values of MW standards are plotted against the Log_{10} of their respective MWs in a calibration curve (Fig. 22). MWs of tissue polypeptides were derived from their respective Log_{10}%T values. All VN SUBUNITS, ADDITIONAL EGG POLYPEPTIDES AND ADDITIONAL VG POLYPEPTIDES are detectable by immunostaining with anti-VN. ADDITIONAL EGG POLYPEPTIDES - Egg extract contains VN A/VN B SUBUNITS (VN1-7), the listed high molecular weight bands and VN C (The high MW bands are detected only with immunoprobe). ADDITIONAL VG POLYPEPTIDES** - HL and FB from replete females contain VN A/VN B SUBUNITS, ADDITIONAL EGG POLYPEPTIDES and the listed additional immunodetected polypeptides. NON-VITELLOGENIN HEMOLYMPH POLYPEPTIDES*** - HL 1 and HL 2 are the major coomassie staining hemolymph polypeptides in males and females. They are not however, detected by anti-VN.*

increased staining (sensitivity) of the VN A/VN B subunits and VN C. Additionally, several previously undetected high molecular weight bands were immunodetected. The molecular weights of the major immunostaining bands high molecular weight bands were also determined and are identified as ADDITIONAL EGG POLYPEPTIDES (*Table 1*). Immunoblots of FB and HL samples (see *Fig. 20*) displayed a set of VG polypeptides with molecular weight homology to VN C, subunits of VN A/VN B, and ADDITIONAL EGG POLYPEPTIDES. In addition, several very high molecular weight polypeptides, identified as ADDITIONAL VG POLYPEPTIDES, were observed in these samples. Two HL polypeptides which were not immunoreactive (NON-VG POLYPEPTIDES) are also included in the table. These polypeptides are the major protein constituents in all HL samples analyzed and serve as useful reference markers in PAGE resolved samples.

3.7 QUANTIFICATION OF VN AND VG BY ELISA.

Inhibition ELISA plots representative of quantitative tissue analysis of VG or VN content are shown (*Fig. 23*). Inhibition ODs for three tissues from R2 females and references standards are plotted against the log of sample protein amount/assay. The plots display similar slope features, suggesting that VG and VN display homologous immunorectivity on a unit protein basis. An OD of 1.0 unit corresponds to 19.8 ng of protein in the reference standard plot. Similarly, 1.0 OD unit corresponds to 207, 1190, and 3144 ng of OV, HL, and FB protein respectively. VG or VN percent of soluble tissue protein is summarized below (*Table 2*). In general, the level of VG and VN increases in all tested female tissues as a function of

FIGURE 23. QUANTIFICATION OF VITELLIN AND VITELLOGENIN: INHIBITION ELISA. ELISAs were performed with serial dilutions of tissue extracts (see Materials and Methods). Tissues were collected and pooled from 4 individuals of the appropriate developmental stage. Representative ELISAs of three tissues (OV, HL and FB) from R2 females are shown. Egg VN was run in parallel with tissue samples. Final ODs (mean values of replicate assays) are plotted against ng of sample protein. A final OD of 1.0 unit represents 19.8 ng of VN protein. The total amount of tissue protein (VG or VN plus non-antigenic protein) yielding an ELISA OD of 1.0 unit is indicated for each sample in lower portion of the figure.

TABLE 2. TISSUE LEVELS OF VITELLIN AND VITELLOGENIN

	TISSUES*					
	OV	HL	FB	MG	SL	MU
FM	0.10 (0.05)	0.10 (0.00)	0.05 (0.02)	N.D.	N.D.	N.D.
UF	0.22 (0.05)	0.05 (0.02)	0.05 (0.05)	<0.05	<0.05	<0.05
FV	0.30 (0.10)	0.15 (0.10)	0.15 (0.05)	0.10 (0.05)	0.10 (0.00)	0.10 (0.05)
R0	0.39 (0.10)	0.37 (0.10)	0.15 (0.05)	0.15 (0.10)	0.15 (0.05)	0.15 (0.07)
R2	9.94 (0.12)	1.54 (0.12)	0.70 (0.07)	0.25 (0.12)	0.35 (0.25)	0.25 (0.20)
R4	33.20 (4.30)	11.18 (1.42)	2.98 (0.01)	0.35 (0.10)	1.10 (0.50)	0.93 (0.20)
R6	56.20 (7.10)	11.00 (0.60)	3.00 (0.21)	N.D.	N.D.	N.D.
R8	46.50 (5.20)	11.30 (0.50)	2.90 (0.05)	0.42 (0.15)	N.D.	0.50 (0.35)

Tissues* - OV-ovary, HL-hemolymph, FB-fat body, MG-midgut, SL-salivary gland, MU-muscle - were collected and pooled from 4 adult females of the recorded reproductive stages (column 1). Ovarian VN and nonovarian (remaining tissues) VG were quantified by inhibition ELISA. Recorded values represent the VG or VN mean percent of the total soluble tissue protein as determined from duplicate aliquots of the pooled extract (range recorded in parenthesis). Detection limits (see Materials and Methods) are 0.05%. Tissues demonstrating non-detectable levels of VG or VN are recorded as <0.05. N.D. = not done.

feeding and reproductive development. At the time of molting (FM) low levels of VG or VN are present in HL, FB and OV. VG in MG, SG and MU was not detected until partial feeding had taken place (FV) and remains low throughout reproductive development. During the two days following repletion (R0 to R2) VG and VN levels increase over four fold in HL and FB and over 25 fold in ovary. HL and FB VG concentrations peak in R4 females and hold constant thereafter. Ovarian VN continues to rise until the R6 stage and drops slightly in R8 females. The ovarian peak and drop in VN concentration coincides temporally with the initiation of oviposition. MG, SG. and MU levels of VG remain low throughout the replete stages probably reflecting nonspecific uptake of HL VG. SG degeneration during the preoviposition stages did not allow analysis of SG VG in R6 and R8 females. Additionally, male and nymph HL were tested by ELISA. Low levels of VG were detected in both UF adult male HL and fed nymph HL (0.10 ± 0.05 and 0.10 ± 0.02 respectively) and may suggest constitutive VG production.

Although the VG and VN content of tissues listed in *Table 2* was determined from a single pool of the appropriate tissues, the consistency of ELISA determined VG and VN levels in tissues from seperate collections over an 8 month period supported the reliability of the single determinations (R2 HL VG mean = 1.48%, S.D. = 0.179, n = 5; R2 FB VG mean = 0.68%, S.D. = 0.072, n = 9).

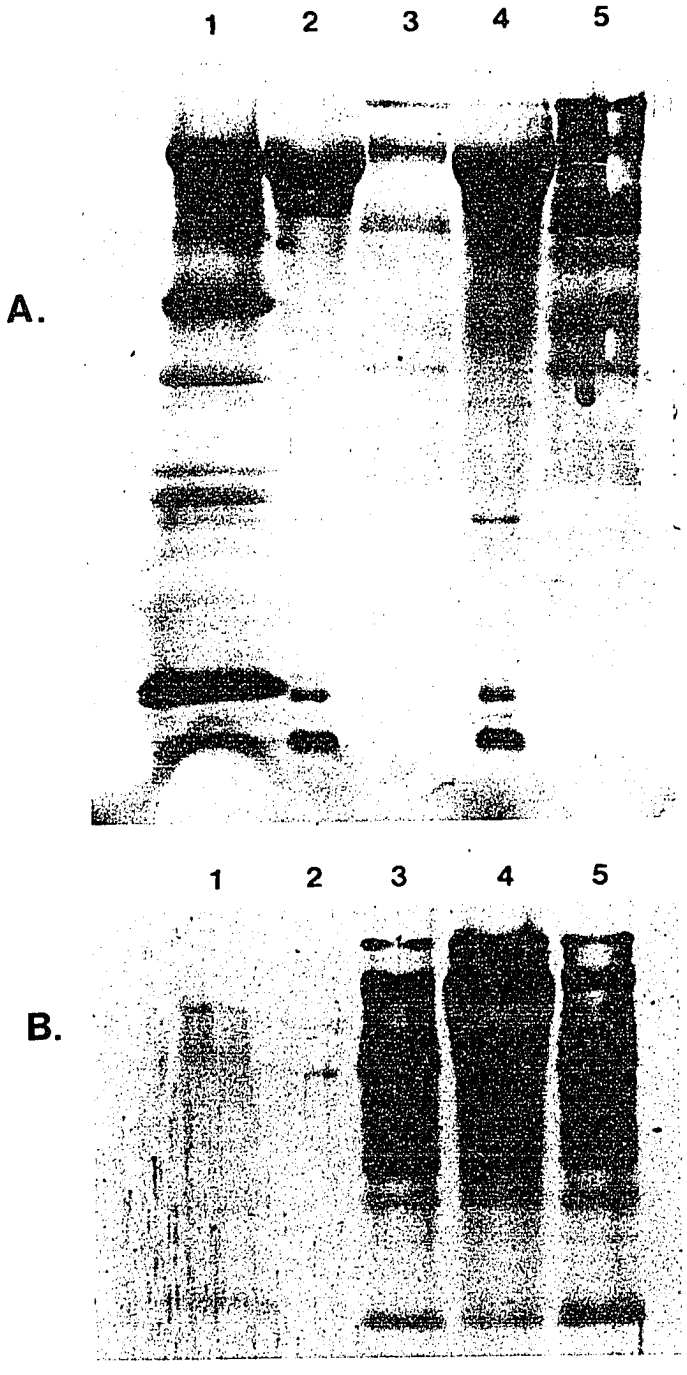
3.8 TESTS OF VN AND VG SYNTHESIS *IN VITRO*.

Results of studies to determine rates of incorporation of ^{14}C amino acids into VN and VG by OV and FB *in vitro* are summarized below. SDS PAGE of ammonium sulfate precipitated media and tissue proteins, which were exposed to

radiolabeled amino acids *in vitro*, was performed (*Fig. 24*). Polypeptides from OV maintained *in vitro* display an electrophoretic pattern homologous with freshly isolated OV polypeptides, suggesting that these polypeptides were not substantially altered or degraded under the culture conditions. Comparison of media alone and media from the OV culture suggests that small amounts of the OV proteins were released into the media by the cultured tissue. A replicate gel was immunoblotted (*Fig. 24B*). Molecular weight standards and media polypeptides were not immunodetected. The immunostaining patterns of culture maintained OV, media from cultured OV and freshly isolated OV all exhibited a high degree of homology, indicating that VN polypeptides are released from cultured OV. A third replicate gel was processed for fluoragraphy in order to visualize proteins synthesized *de novo*. No detectable radiolabeled polypeptides were observed in fluorograms of culture maintained OV or media from OV cultures (not shown, film was exposed for 4 weeks). Additionally, a fourth replicate gel was blotted to nitrocellulose and each sample lane was cut from top to bottom in 4mm slices. These slices were quantified for radioactivity by scintillation spectroscopy. The only sample which displayed radiolabel incorporation was OV from ticks radiolabeled *in vivo* (not shown). These results, which support those of the fluorogram, indicate that culture maintained OV was neither synthesizing or releasing newly synthesized VN protein. The viability of these tissues was strongly suggested by a normal cellular and subcellular morphology after 10 days of culture maintenance as revealed by TEM (not shown).

PAGE and immunoblot analysis identical to those of OV above were performed for culture maintained FB. Coomassie staining patterns reveal little or

FIGURE 24. SDS PAGE AND IMMUNOBLOT OF OVARY MAINTAINED *IN VITRO*. R6 OV was maintained for 3 days *in vitro*. YM media was supplemented C^{14} amino acids (0.5 μCi/100 μl YM). Tissue and media aliquots were collected daily, precipitated with 60% ammonium sulfate (AS) and resolved by SDS PAGE. 1 - MW standards; 2 - media control; 3 - OV after 72 hours of culture; 4 - media from 72 hour OV culture; 5 - OV radiolabeled *in vivo*. The resolved gel was stained with silver (A). A replicate gel was immunoblotted (B).



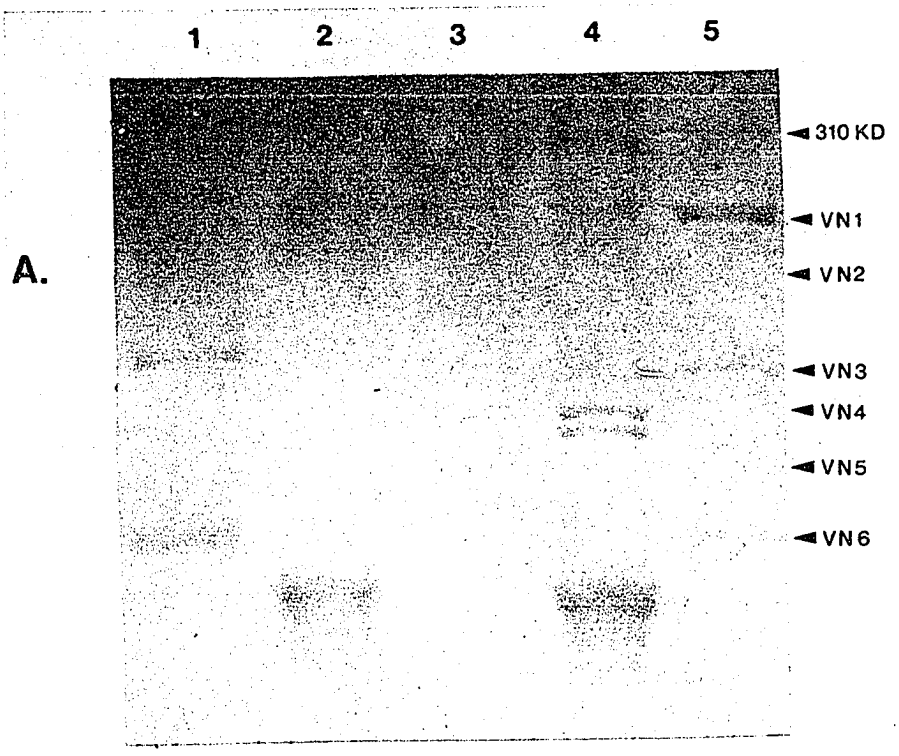
no electrophoretic homology between FB tissues or FB media and freshly isolated OV tissue (*Fig. 25A*). A replicate gel was processed for fluoragraphy in order to visualize *de novo* protein synthesis (*Fig. 25B*). Seven major bands are observed which increase in intensity (ie. radioactivity) as a function of time. Quantitative analysis of these bands was derived from sliced immunoblots (*Fig. 26*). On all three days of media sampling, a band of 310 KD MW displayed the highest level of radiolabel incorporation. Each of the radiolabeled bands (including the 310 KD band) corresponds closely on an electrophoretic basis to an immunopositive VN band as portrayed in the immunoblot above the figure. In contrast to radiolabeled proteins secreted by the FB, only a small amount of a single MW band (310 KD) was retained by the FB tissue.

The preceding results indicate synthesis of VG by FB over a three day culture period. OV cultures under identical conditions did not synthesize VN. Long term synthesis of VG by FB was further investigated. In a similar but separate experiment, R4 FB was maintained in culture for 24 hours and subsequently refurbished with fresh media supplemented with radiolabel. Radiolabel in immunoprecipitated media proteins was quantified by liquid scintillation spectroscopy (*Table 3*). As previously observed in R6 FB, FB from R4 females synthesized and secreted increasing amounts of VG as a function of time. ELISA quantified VG increases were paralleled by similar increases in immunoprecipitable radioactivity.

3.9 GROSS MORPHOLOGY AND ULTRASTRUCTURE OF ADULT FEMALE FAT BODY. In freshly molted, unfed and part-fed females the tracheae

FIGURE 25. SDS PAGE OF FB MAINTAINED *IN VITRO.*

R6 FB (1 tracheal trunk from each of 2 females) was aseptically collected and maintained in 100 μl of Y/M media containing 0.5 μCi of C^{14} amino acids. Media aliquots (25 μl) were collected daily and precipitated with ammonium sulfate (60% final concentration). Media aliquot samples were replenished with equal volumes of fresh radiolabel supplemented media. Ammonium sulfate precipitated samples were denatured in PAGE sample buffer and electrophoresed in 3 SDS gels. **A.** Commassie stained SDS PAGE; *lanes* - 1 = MW standards, 2 = media control (ie., media without fat body), 3 = FB after 72 hours of culture, 4 = media from 72 hour FB culture, 5 = OV radiolabeled *in vivo* (see materials and methods). **B.** Fluorogram of SDS PAGE; *lanes* - 1 = FB after 72 hours of culture, 2 = media from 24 hour FB culture, 3 = media from 48 hour FB culture, 4 = media from 72 hour FB culture, 5 = OV radiolabeled *in vivo*. The third SDS gel (see *Fig. 26*) was blotted to nitrocellulose and processed as described in figure legend.



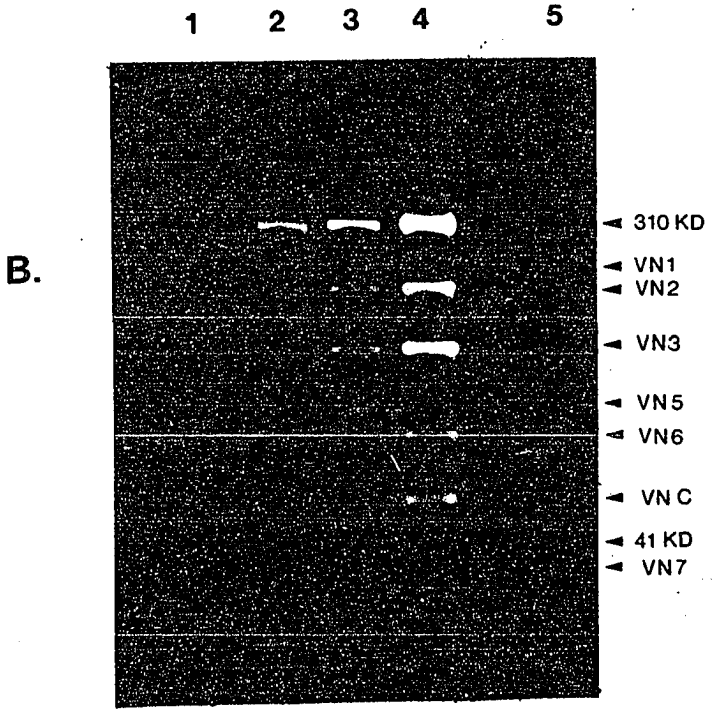


FIGURE 26. INCORPORATION OF RADIOLABELED AMINO ACIDS INTO VITELLOGENIN BY FAT BODY *IN VITRO*. R6 FB was maintained in vitro with ^{14}C amino acid supplemented media (described in *Fig. 25* legend). Daily media samples were precipitated with ammonium sulfate, resolved by SDS PAGE, and blotted to nitrocellulose. Individual sample lanes on the blot were sliced in 4 mm sections from top to bottom. Slices were air dried, dissolved in 300 ul tetrahydrofuran, and the radioactivity was quantified by liquid scintillation spectroscopy. CPM per sample lane slice are plotted against distance (cm) from top of gel blot. CPM are corrected for daily dilution resulting from sample aliqout removal and media replenishment. The western immunoblot profile of R6 HL is represented above the figure.

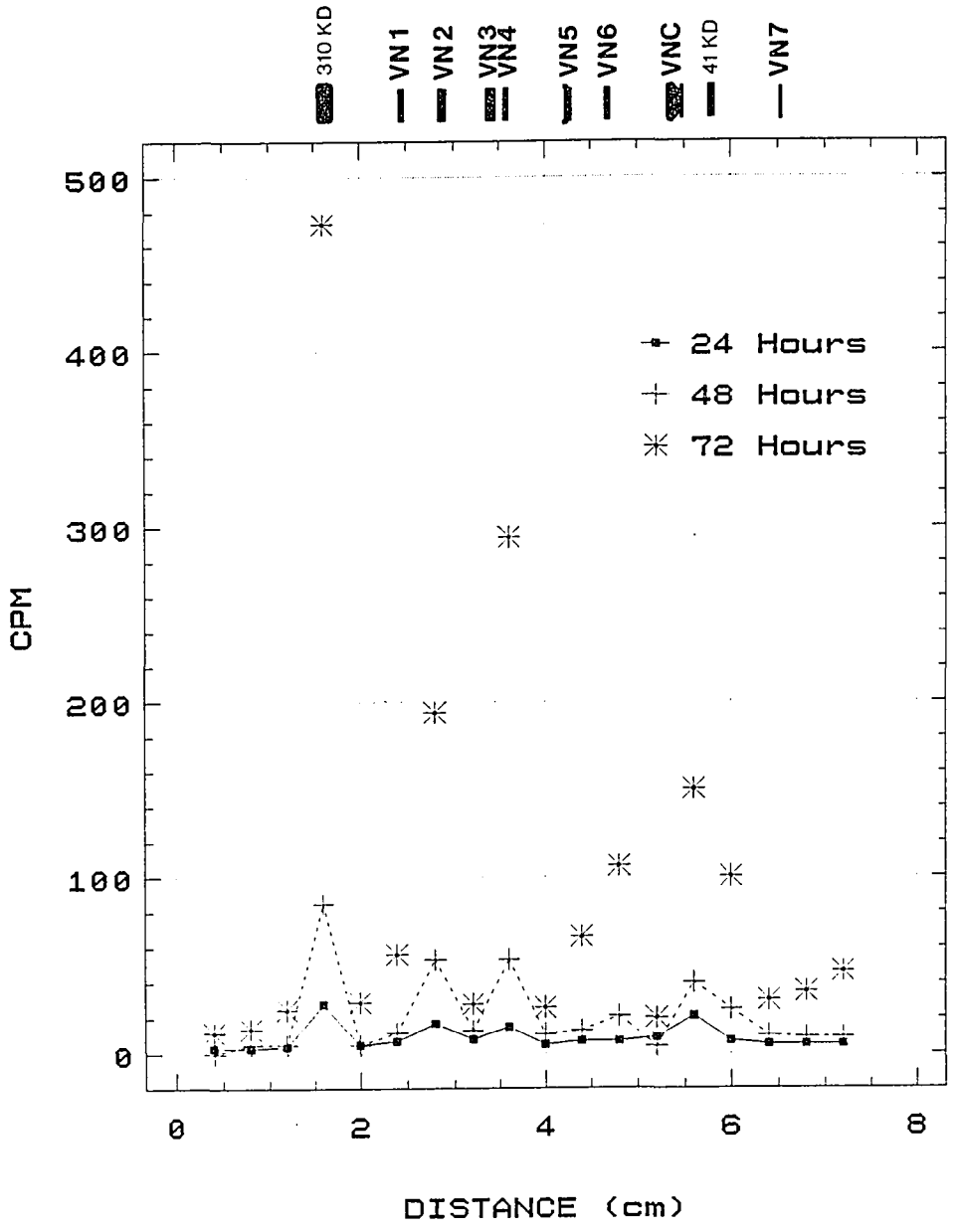


TABLE 3. VG SYNTHESIS AND SECRETION BY R4 FB

DAYS IN CULTURE	DAYS PULSED	VG CPM*	ug VG**
2	1	179	46.8
3	2	521	67.3
4	3	1084	75.9
5	4	1335	76.4
8	7	3242	121.2

R4 fat body (ie., tracheal trunks) from 2 females were aseptically collected and maintained in Y/M media (100 μl). After 24 hours the media was replaced with fresh media containing 1μCi of ^{14}C aminoacids. 20 μl aliquot samples were removed at the indicated times and replaced with fresh radiolabel supplemented media. 10 μl of each sample was immunoprecipitated and quantified for protein incorporated radiolabel by scintilation spectroscopy. Replicate 5 μl portions of the remaining sample were assayed for VG content by ELISA. Fat body protein associated with the tracheal trunks after 8 days in culture was quantified. VG CPM* and μg VG** are per mg of fat body protein. These values were adjusted as required to compensate for the dilution effect of sampling and media replenishment.

and their associated fat body appeared as discrete branches radiating from the spiracle. Upon repletion and during subsequent preovipositional development extensive development of a membranous connective tissue between tracheal stalks was observed. This membrane was largely absent in virgin females. *Fig. 27* exemplifies the association between chords of fat body cells and the tracheal trunks observed throughout the adult life stage. Ultrastructural analysis by TEM reveals the presence of two distinct cell types associated with the tracheal system (*Fig. 28A*). The fat body trophocytes appeared as epithelioid cells, frequently clustered as chords two or three cells thick and surrounded by a basement membrane. The nephrocytes typically appeared as oval or subcircular cells, and may be free floating or adjacent to trophocytes, attached along one edge.

In unfed and fed virgins, trophocytes contained abundant of lipid droplets, vacuoles and channels or canaliculi. Moderate amounts of rough endoplasmic reticulum (RER) and free ribosomes were also observed. In fed mated females the fat body trophocytes appeared larger than in virgin females. In some trophocytes the cytoplasm was largely filled with RER. Upon the initiation of oviposition approximately half the trophocytes displayed extensive and distended RER profiles (*Fig. 28B*).

The nephrocytes displayed a similar ultrastructure throughout the observed adult life stages. The cytoplasm of these cells was dominated by variable size vesicles. Large numbers of coated and uncoated pits and tube-like invaginations were observed.

FIGURE 27. SCANNING ELECTRON MICROGRAPH OF TRACHEA AND ASSOCIATED FAT BODY. Fat body cells (fb) adjacent to trachea (T) near the spiracle of a fed virgin female. Bar = 25 μm.

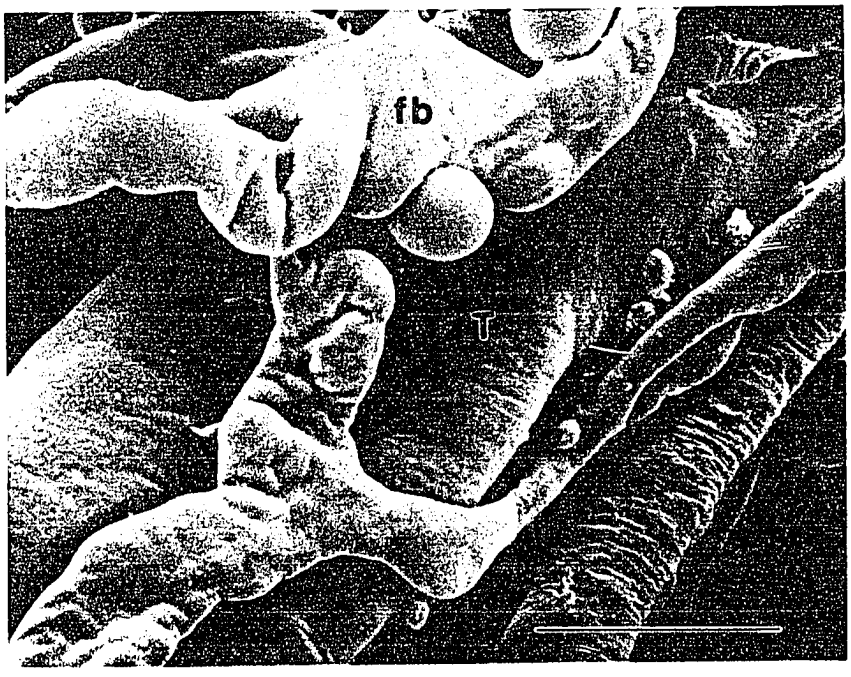
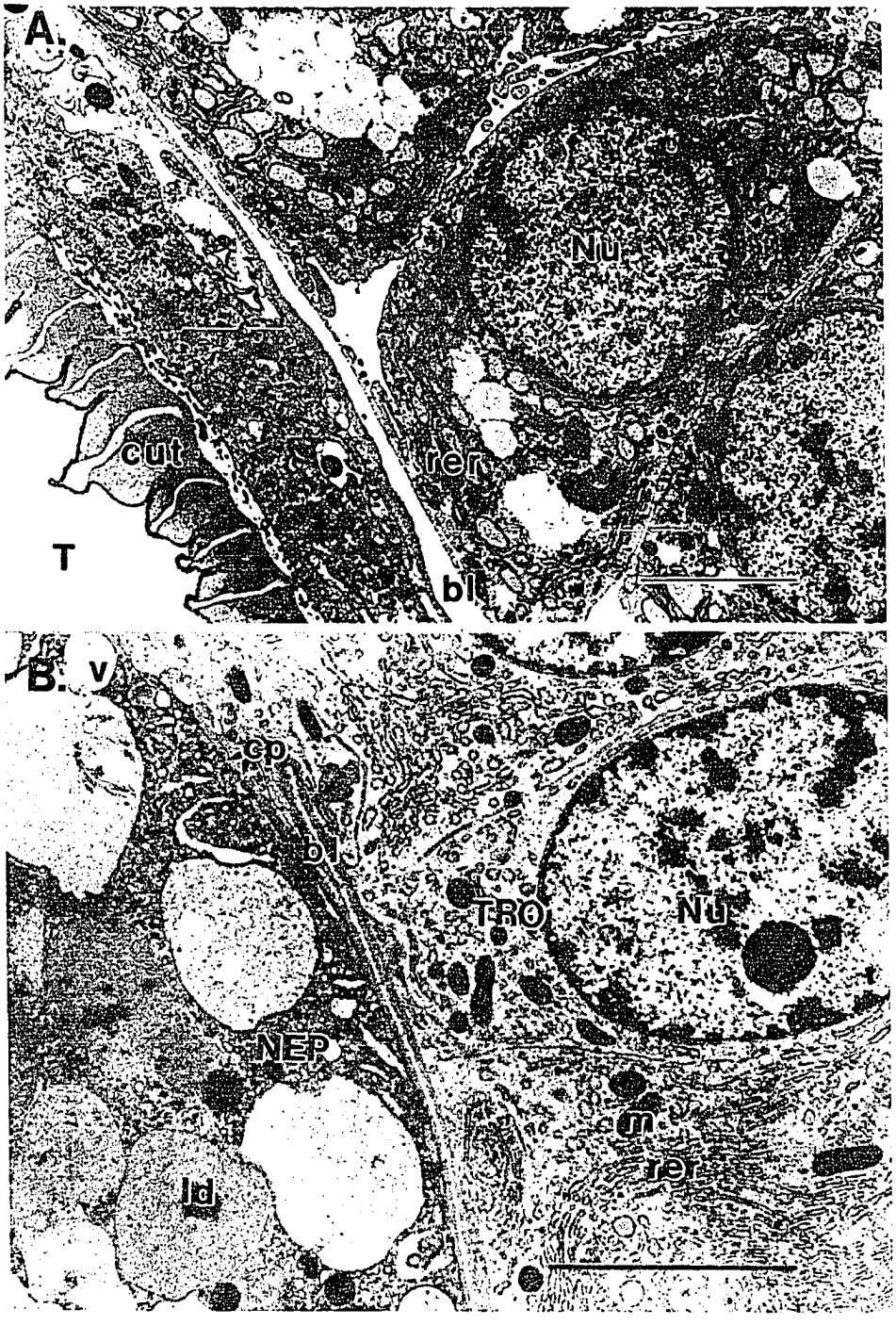


FIGURE 28. TRANSMISSION ELECTRON MICROSCOPY OF FAT BODY.

A. Fat body cells adjacent to a section of trachea from a fed virgin female. 4,580 X. B. Fat body trophocyte (TRO) and nephrocyte (NEP) from a replete (R6) female. 13,500 X. Nu = nucleus; cut = trachea cuticle; T = tracheal lumen; rer = rough endoplasmic reticulum; v = vacuoles; ld = lipid droplets; bl = basal lamina; m = mitochondria; cp = coated pits. Bar = 5 μm.



3.10 STIMULATION OF VG SYNTHESIS AND SECRETION IN FV FB.

3.10.1 TESTS *IN VITRO*. Tissue levels of VG in FV FB after 1-10 days in culture ranged from nondetectable (ie. <0.5 ng/μg) to 1.0 ng/μg of tissue protein (ie., 0.05-0.10%, *Table 4*). This range is slightly below that observed from freshly isolated FVFB (0.15% ±0.05, *Table 1*). None of the treatments, hormonal or tissue extract, caused significant increases in VG in the FB tissues compared to control cultures. Levels of VG secreted into the media in experiments I and II ranged from nondetectable to 0.75 ng/37 μg of FB protein. Treatments in these experiments did not result in significant increases in media VG as compared to controls, nor were there any obvious trends in secreted VG levels (treated or control) as a function of time in culture. Clearly, there is no evidence of biosynthesis of VG by FV FB *in vitro* in response to treatment with any of the agents used in experiments I and II. In contrast to these results, dramatic increases in secreted VG were observed FV FB cultures that were treated with extracts from R2 tissues (experiment III.). These increases were most pronounced (35 fold over control) in the cultures supplemented with synganglion. FB cultures treated with R2 HL also secreted greatly elevated levels of VG as compared to controls. Although media titers of VG were many-fold higher in both synganglion and HL treated cultures, the level of VG retained by the FB tissues of these cultures was not elevated. Further evidence of greatly stimulated VG synthesis in FB cultures treated with extracts from R2 tissues was supported by incorporation of ^{14}C amino acids into these proteins (*Table 5*). Incorporation of radiolabeled amino acids into immunoprecipitable protein was monitored in order to assess synthesis VG proteins *in vitro*. Low levels of VG

TABLE 4. STIMULATION OF VG SYNTHESIS IN FV FB *IN VITRO*.

TREATMENT	DAYS IN CULTURE	ug FV FB PROTEIN	ng VG/μg TISSUE PROTEIN MEDIA	TISSUE
EXPERIMENT I.				
CONTROL	1		0.75	
	2		0.50	
	3		0.10	
	4	450	N.D.	0.75
JHIII	1		0.50	
	2		0.25	
	3		0.25	
	4	438	N.D.	1.00
JHIII/ECD*	1		N.D.	
	2		0.50	
	3		0.25	
	4	818	N.D.	0.75
ECD	1		N.D.	
	2		N.D.	
	3		0.25	
	4	851	0.25	0.50
EXPERIMENT II.				
CONTROL	4		0.50	
	6		0.25	
	8		N.D.	
	10	504	N.D.	N.D.
BIS	4		0.10	
	6		0.10	
	8		0.25	
	10	636	N.D.	N.D.
BIS/ECD*	4		0.07	
	6		N.D.	
	8		N.D.	
	10	862	N.D.	0.75
FVHL,BIS/ECD*	4		0.10	
	6		0.10	
	8		N.D.	
	10	574	N.D.	1.00
EXPERIMENT III.				
CONTROL	5	364	1.25	1.00
OVIDUCT	5	210	2.56	0.50
SYNGANGLION	5	84	44.64	0.75
R2HL	5	156	32.05**	1.00

FV tracheal trunks and associated FB were maintained in YM media for up to 10 days. Hormone and tissue extracts treatments (column 1) were added to culture media (see Materials and Methods). The final media volume was 50 μl. On the indicated days in culture (column 2) a replicate media aliquots (7.5-10 μl) were collected and replaced with fresh media supplemented with the appropriate hormone or tissue extract. The amount of protein associated with tracheal trunks in each culture was determined at the termination of the experiment and is recorded in column 3. VG concentrations (mean value of replicate sample aliqouts) in tissues or media/μg tissue protein were determined by ELISA. Dilution effects on media VG due to sample aliquot collection and replenishment were compensated for as required. The minimum level of VG detectable in media was dependent upon the amount of tissue protein per culture and ranged from 0.06 to 0.11 ng/μg of tissue protein. The minimum level of VG detection in tissue was 0.5ng/μg tissue protein. ELISA determined VG levels below the detection limits are recorded as none detected (N.D.).

* - 20 hydroxyecdysone was added with the first media replenishment (ie., on days 1 and 4 of experiments I and II respectively).

** - The amount of VG transferred to the fat body culture media by the addition of 10 μl of hemolymph from R2 females (18.75 μg) was subtracted from the total observed on the sampling day.

TABLE 5. *DE NOVO* SYNTHESIS OF VG BY FV FB *IN VITRO.*

TREATMENT	DAYS IN CULTURE	VG CPM/MG PROTEIN	STIMULATION
CONTROL	3	346	0.0
	5	1,946	0.0
OVIDUCT	3	1,886	5.5
	5	2,919	1.5
SYNGANGLION	3	55,060	159.1
	5	31,491	16.2
R2HL*	3	23,923	69.1
	5	19,954	10.3

Incorporation of radiolabeled amino acids into VG by FV FB cultures (see *Table 4*, experiment III) was monitored in order to assess *de novo* synthesis. FV FB associated with tracheal trunks (4 tracheal trunks/culture) were incubated with ^{14}C amino acids (1 μCi) in YM media (50μl) and treated with extracts of R2 tissues (column 1). Two 5 μl aliquots of media were collected on day 3 and 5 for immunoprecipitation and quantification of VG associated radioactivity. The 3 day media samples were replaced with fresh radiolabel supplemented media (10 μl). Fat body protein in each culture was quantified at the termination of the experiment (recorded in *Table 4*). Immunoprecipitable VG CPM are standardized per mg of fat body protein (column 3). Stimulation (column 4) = immunoprecipitable CPM of extract Treatment/Control.
* - as a control for the R2HL treated fat body culture, a separate culture containing only R2HL (no fat body) was monitored. The immunoprecipitable radiolabel in this culture at days 3 and 5 was subtracted from those of observed in the fat body culture treated with R2HL.

synthesis and secretion are observed in control cultures on day three and increase on day five. Stimulated VG synthesis and secretion is demonstrated in all supplemented cultures, with dramatic increases, up to 159 fold compared to controls, in cultures

supplemented with synganglion. The level of media VG observed in the R2HL culture in *Table 4* is partially attributable to nonradiolabeled VG carried in the extract supplement. However, the increased level of radiolabeled VG in the FB culture supplemented R2HL, as compared to a culture of R2HL alone (see *Table 5* footnote), indicates that there is substantial *de novo* VG synthesis stimulated by a hemolymph factor(s). The small amount of VG synthesis stimulation in oviduct treated cultures may also be attributable to hemolymph factors transferred with this tissue extract. It was noted that the stimulation of VG synthesis by extracts from R2 females declined as a function of time in all supplemented cultures.

3.10.2 TESTS IN VIVO. Compared to normal FVs (seven day attachment), extended attachment (16 days) and supplement injections resulted in elevated levels of HL VG and OV VN (*Table 6*). HL levels of VG were slightly above normal in Control, HL and oviduct supplemented individuals. Greatly elevated HL VG concentrations were observed in FV injected with synganglion, ECD and BIS. Levels of HL VG in these treated virgins approximated those of the mated and replete females of the R4 stage of reproductive development (*Table 2*). Ovarian VN uptake mirrored HL VG supply with the exception of ECD injected females, which displayed only moderate VN accumulation.

TABLE 6. STIMULATION OF VG SYNTHESIS IN FV

TREATMENT	VG IN HEMOLYMPH	VN IN OVARY
CONTROL	0.77	0.55
HL	1.74	0.53
OVIDUCT	0.80	0.88
SYNGANGLION	4.34	44.00
ECD	4.83	4.20
BIS	4.50	33.33

Five pairs of 10 day part fed virgin females were injected with extracts from R2 females (HL, oviduct, or synganglion) or authentic hormones (ECD or BIS) and alowed to feed for 6 additional days. HL and OV from both individuals of each treatment group were collected. The VG and VN content, as determined by ELISA of pooled HL or OV, respectively, is recorded/mg of tissue protein. The VG and VN percent of total soluble tissue protein in untreated 7 day part fed virgin HL and OV are 0.15% and 0.30%, respectively (taken from *TABLE 2*).

3.11 STIMULATION OF VG SECRETION IN REPLETE FB IN VITRO.

Levels of secreted VG from R0 FB cultures range from 8.0 to 37.5 μg/mg tissue protein (*Table 7*). In general, all cultures demonstrate increasing amounts of VG as a function of time in culture. However, levels of media VG in the ECD treated culture are significantly greater Control, BIS or JH III treated cultures on each dayof sampling. The level of media VG in Control, BIS and JH III treated cultures are roughly equivalent to each other on any given day. ELISA estimates of VG synthesis are supported by radiolabel incorporation into immunoprecipitable material. Throughout the culture period, the level of immunoprecipitable C^{14} is highest in the ECD supplemented culture.

TABLE 7. STIMULATED SECRETION OF VG IN R2 FB CULTURE

TREATMENT	DAYS IN CULTURE			
	1	2	3	4
CONTROL	13.8(2.6)	18.6(0.6)	18.1(0.4)	21.6(3.7)
	699[a]		767[a]	
BIS	8.0(2.1)	12.8(2.1)	26.0(0.7)	22.4(0.1)
	342[a]		627[a]	
JHIII	12.8(2.1)	16.5(2.8)	21.8(7.8)	18.3(4.8)
	486[a]		845[a]	
ECD	19.1(9.7)[b]	29.6(1.9)[c]	35.8(1.4)[c]	37.4(4.2)[c]
	1006[a]		1430[a]	

Tracheal trunks and associated fat body were aseptically collected from R0 females and maintained in culture (two tracheal trunks/culture). Cultures (50 μl total volume) were treated with authentic hormones (column 1) for 4 days in two separate experiments. In the second experiment, ^{14}C radiolabeled amino acids (0.5 μCi) were also added to media. 20 μl media aliquots were collected on days 1-4 and replenished with fresh media ± radiolabel. Media aliquots from both experiments were processed for VG content by ELISA. The fat body protein of each culture was quantified after the 4 day sampling point. The mean values of VG (μg/mg tissue protein) from the two experiments are recorded (range is recorded in parenthesis).

[a] - portions of the radiolabeled culture media, from days 1 and 3, were immunoprecipitated (see Materials and Methods) and quantified by liquid scintillation spectroscopy (immunoprecipitable CPM/mg tissue protein).

[b] - VG values significantly greater than controls at the 0.025 level (T test).

[c] - VG values significantly greater than controls at the 0.01 level.

CHAPTER 4. DISCUSSION

The results of this study represent the first report documenting the ability of a soluble factor from synganglion homogenates to stimulate VG synthesis in fed virgin hard ticks both *in vitro* and *in vivo*. Moreover, a role for ecdysteroids and juvenoids in the regulation of this process is suggested in several experiments. These results, utilizing *H. dromedarii*, are consistent with several reports of stimulatory activity of vitellogenin synthesis by synganglion extracts in the soft ticks *A. hemanni* and *O. moubata* (Shanbaky, 1975, 1990; Chinzei, 1986).

To understand the regulatory events that control the process of vitellogenesis, it was necessary to determine what constitutes VG and VN and how these proteins could be identified and quantified. In addition, it was necessary to develop an experimental model in which the effects of natural, authentic or synthetic substances could be tested unambiguously. In the model developed for this purpose, a culture assay was created with FB tissues from fed virgin females, tissues which are capable of synthesizing VG in culture but do not normally do so until after mating. Thus, production of VG *in vitro* by these tissues after the deliberate introduction of known substances would be considered as strong evidence of stimulation. Similarly, production of VG by fed virgin females or enhancement of VG synthesis by cultured FB from replete females, following the introduction of test materials, would also support the role of such substances as stimulatory agents.

4.1 IDENTIFICATION AND CHARACTERIZATION OF VITELLIN.

Initial identification of VNs in the present study was based on: 1- their high concentration in eggs; 2- their specificity to females and; 3- their developmental dependence on mating and repletion. In spite of the fact that these features have been found to be largely conserved among the ixodid and argasid tick VNs which have been investigated, considerable controversy has occurred regarding their identification. These problems are in part due to the lack of any identifiable enzymatic activities which could serve as markers of protein purification. Further complicating this issue is the occurrence of MW microheterogeneity and the propensity of VN aggregation. For example, although in native PAGE *H. dromedarii* egg vitellins comprise 4 major protein bands, with MWs of 1060, 670, 570 and 680 KD, respectively, the close approximations of bands II-IV suggest that these bands are actually charge isomers of the same protein, herein identified as VN B. Similar electrophoretic behavior has been documented for a number of well studied protein charge isomers and is often the result of differential postranslational carbohydrate and lipid processing (Fishbein et al., 1977, and Dixon et al., 1980). Discrepancies in molecular weight estimates for native VNs in insects have been largely attributed to variation in the bound content of lipid and carbohydrate (Harnish and White, 1982). This issue has not been investigated in any tick system.

The largest MW egg protein band (band I, MW 1060 KD), herein identified as VN A, is approximately twice the size of the putative charge isomers and likely represents a dimeric form of VN B. This hypothesis was further supported by the subunit homology between VN A and VN B when resolved by SDS PAGE. Both

of the VNs gave rise to the same seven polypeptides (VN 1-7) under denaturing conditions. The ratios of these polypeptide subunits within the native aggregates appeared variable based on their individual staining intensities.

Aggregation of egg yolk proteins has been reported in both argasid and ixodid ticks. In *O. moubata*, native VG is present in monomer and dimer forms (VG 1 and VG 2) with respective MWs of 300 KD and 600 KD. In *D. andersoni* and *R. appendiculatis*, the tendency of VN to undergo self-aggregation has been correlated with the ionic strength of the suspending buffer; aggregation was promoted when the ionic strength of the buffer was lower than that of physiological saline (Boctor and Kamel, 1976, Diehl et al., 1982).

Further identification of *H.dromedarii* egg VNs and confirmation of their MWs was accomplished by gel filtration. Three fractions were resolved and displayed well defined absorption maxima at 280 and 400 nm, characteristic of protein and intact porphyrin moieties, respectively (Boctor and Kamel, 1976). The presence of heme-derivatives in tick yolk proteins was first detected by Wiggelsworth (1943). As in other hemoglobin ingesting arthropods, ticks utilize blood meal hematin in their development. Approximately 6-10% of the blood meal iron is passed to the eggs (Araman, 1979; Kitaoka, 1961). The coelution of column resolved egg absorption peaks at 280 and 400 nm further suggests the presence of heme-conjugates within each protein fraction. The first two of the three resolved column fractions have calculated MWs closely approximating those of VN A and VN B as determined in native PAGE (ie., over 1000 KD and 630 KD). The third of the column resolved fractions, VN C, has a calculated MW of 43 KD. This protein was

not detected by native PAGE, likely as a result of its low content in eggs relative to VN A and VN B. It was however, represented in stained SDS PAGE as a diffuse band with an approximate MW of 50-56 KD. The disparity in MW estimation of VN C between column filtration and SDS PAGE (43 KD vs 50-56 KD) is likely due to the differential effect of denaturation and reduction by SDS and mercaptoethanol in the PAGE resolving system. These MW estimations should therefor be considered as approximations. In summary, the results of both PAGE and column analysis suggest the presence of two VNs in *H. dromedarii* egg extract, one composed of seven subunit polypeptides (VN 1-7) and capable of aggregating in various forms (VN A and VN B) and the second composed of a single polypeptide with a heterogeneous MW between 43-56 KD (VN C). Similar analyses of native VN and its subunit polypeptides have been reported in both argasid and ixodid ticks. In *O. moubata*, the dimer size egg VN is the predominant form and is composed of 6 polypeptides with MWs ranging from 50 KD to 160 KD (Chinzei, 1983a). Two VNs, VN A and VN B, were characterized from eggs of the hard tick, *D. andersoni*, by two independent laboratories (Boctor and Kamel, 1976; Rosell-Davis and Coons, 1989a). The native MWs of VN A and VN B in this species are 480 and 370 KD, respectively, and both VNs dissociate into 7 or 8 comigrating polypeptides under denaturing conditions of SDS PAGE. The subunit polypeptides range in MW from 35 KD to 135 KD (Rosell-Davis and Coons, 1989a). Comparative analyses of egg VN from *H. dromedarii*, *D. variabilis* and *D. andersoni* indicates a high degree of subunit MW homology and immunologic cross-reactivity (see Appendix A). Thus, in addition to the sex, stage and tissue specific characteristics of VNs, several

physical features appear consistent among these proteins in both argasid and ixodid ticks; tick VNs are large proteins with MWs in excess of 300 KD, they are composed of a number (typically 6-8) of polypeptide subunits and they tend to form multiple aggregates. The current identification of an additional small MW VN, VN C, is unique among the tick VNs reported to date. Whether this protein is in fact a novel VN or more simply has not been previously characterized due to its low concentration in eggs, remains to be determined. Further discussion of the relationship of VN C to VN A/VN B is found elsewhere (see VG PROCESSING).

4.2 IDENTIFICATION OF VITELLOGENIN.

The need for a sensitive and selective immunologically based detection system of VG resulted from the inability to unambiguously identify VG with nonselective protein stains in SDS and native PAGE resolved tissue extracts. Initial attempts to identify the VN precursor(s) were based on the assumption that VG underwent little or no modification between its synthetic origin and its accumulation as egg yolk, and thus could be identified on the basis of electrophoretic comigration with VNs in various PAGE systems. Reports of HL VG in *O. moubata* reaching concentrations of 65μg/μl, or 80% of the total HL protein (Chinzei, 1983b), suggested that comparable levels in *H. dromedarii* would be easily detected by coomassie or silver staining. Although tested tissues (FB, HL, SG, MU and MG) displayed distinctive, well-resolved, and stage-specific staining patterns, conclusive electrophoretic homology patterns between VN and any of these tissues was not observed. The possibility that tissue levels of VG were below the limits of detection was considered

(for further discussion see below). These observations were contrasted by the demonstration of electrophoretic homology between VN and OV from R2 and all latter stages. The possibility that OV serves as a minor source of VN has been proposed. However, in view of the relative sparsity of protein synthetic machinery (i.e., ribosomes) in this tissue (Rosell-Davis and Coons, 1989b), the likelihood that OV plays a major role in the synthesis of VN appears remote. The failure of cultured OV to incorporate radiolabeled amino acids into VN (discussed below) cast further doubt on the importance of OV as a synthetic source of VN.

Regarding the inability to detect VG in stained gels of resolved tissue proteins, several possibilities were considered. First, it was possible that the VN precursor, VG, was modified upon uptake into the oocyte and thus presented an altered electrophoretic profile. Secondly, assuming VG was not modified upon oocyte uptake, tissue levels must have been below the level of detection (as suggested above) afforded by simple protein staining of gels. Following this logic and based on the sensitivity of silver staining ($\approx$ 0.5 μg/band) and sample loads ($\geq$ 25 μg/lane) in SDS PAGE, it was estimated that each of the VG polypeptides represented less than 2% of the total tissue protein from the developmental stages tested.

In order to address these issues an immunoprobe against egg VN was developed. Use of immunoprobes against VN to selectively detect trace levels of VG in gel immunoblots has been reported in several insect systems and in *D. variabilis* (Rosell-Davis and Coons, 1989a). Although it was considered that VN may represent a modified form of VG, it was assumed that a sufficient level of antigenic

epitopes would be retained by the egg protein to enable the development of a sensitive and selective probe. It was further assumed that yolk proteins constituted the vast majority of the aqueous soluble proteins in freshly oviposited eggs.

The usefulness of anti-VN in the identification of *H. dromedarii* VG was initially observed by slide immunoprecipitation. By this approach, proteins from both egg and HL, presumably VNs and VGs, respectively, were selectively precipitated. Furthermore, these results suggested that precursor VGs first appeared in female HL between the stages of virgin feeding and repletion. The antibody preparation also enabled selective detection of both native and reduced VN proteins in immunoblot formats. A high degree of MW homology between immunoreactive proteins and polypeptides from OV and nonovarian tissue was also observed. Three tissue samples, OV, HL, and FB, displayed increasing levels of immunoreactive proteins as a function of repletion and preovipositional development. In contrast, MU, MG and SG displayed low levels of VG which did not increase with reproductive development. These observations suggested that the immunoprobe identified a discreet set of proteins on the basis of MW and that production of these proteins correlated directly in a temporal fashion with the vitellogenic period in the female. Trace amounts of these proteins in all tested tissues prior to mating, further suggested that the immunoprobe was not reactive with proteins involved in normal cellular maintenance functions, as these proteins would be expected in all tissue types irrespective of the onset of vitellogenesis.

Based on the increasing levels of immunoreactive protein in HL, FB and OV as a function of reproductive development, these tissues were considered as potential

sources of VG or VN production. The low and consistent levels of immunoreactive proteins in other tissues are likely the result of non-specific uptake or adherence of circulating HL VG. Additionally, small amounts of trachea and its surrounding FB are observed associated with all tissues, and thus may serve as a background source of VG in each isolated tissue. Female specificity was suggested by the absence of immunodetectable VG or VN in native PAGE of male tissues (not shown). However, trace levels of VG were observed in SDS PAGE immunoblots of freshly molted adult male HL. Small amounts of VG in male HL have also been reported in *O. moubata* and *D. variabilis* and are postulated to serve a nutritive role during pre- and post-molting periods (Chinzei and Yano, 1985a; Rosell-Davis and Coons, 1989b). Thus, absolute female specificity is does not appear to be a characteristic of the tick VGs investigated to date. Interestingly, in addition to identifying VN 1-7 and VN C, several high MW polypeptide bands in egg and other tissue extracts were also stained with the immunoprobe. These additional immunopositive polypeptide bands were most prominent in non-ovarian tissue samples. The possibility that these high MW bands were precursors of lower MW VGs and VNs is addressed elsewhere (see VG PROCESSING).

Identification of VGs based upon immunological crossreactivity between VG and VN has been variably successful in other laboratories. Two egg proteins with hemolymph counterparts which were electrophoretically homologous and immunologically crossreactive were observed in *O. moubata* (Diehl, 1969,1970; Chinzei and Yano, 1983b). In contrast, Tatchell (1971) was unable to demonstrate immunological crossreactivity between two electrophoretically homologous

hemoglycoproteins from the hemolymph and egg of the hard tick *B. microplus*. However, two egg proteins of another hard tick, *D. andersoni*, were shown to have immunologically crossreactive components in hemolymph of ovipositing females (Boctor and Kamel, 1976; Coons et al., 1989).

4.3 QUANTIFICATION OF VG AND VN (ELISA).

ELISA based analysis of tissue VN and VG provided the first reports of quantitative assessment of these proteins in any hard tick species. This method corroborated the results visually presented in western immunoblots and, moreover, provides a sensitive and reproducible means by which VG synthesis can be assessed. Levels of HL VG were observed to increase steadily in the 4 days after repletion and plateau at a concentration of 13 μg/ul of HL or approximately 11% of the total HL protein. A similar temporal trend was observed in FB although VG reached a maximal level of only 3% of this tissue protein. The concentration of VN in OV increased from 0.4% at R0 to over 56% by R6. In contrast, the concentration of VG in other tissues, MG, SG and MU, never exceeded 1.1%. Of these tissues, SG displays the greatest level of VG; 1.1% at R4. However, this level is likely due to HL contamination of the large surface area of this tissue. Furthermore, the SG degenerates during the vitellogenic period and was not recognizable at or beyond the R6 stage, thus reducing the likelihood of its role as a source of VG. In summary, temporal correlation of tissue specific VG levels by ELISA supports the hypothesis presented from western immunoblot data; FB, HL and OV titers of VG or VN increase in a fashion consistent with synthetic activity. Contrary to reports of VG

synthesis by MG in *D. andersoni* (Coons et al., 1986; Rosell-Davis et al., 1989b), ELISA determined levels of VG in *H. dromedarii* MG do not support a vitellogenic role for this tissue.

In the soft tick *O.moubata*, VG and VN have been quantified by rocket immunoelectrophoresis (Chinzei, 1983b, 1989). The sensitivity of this method is substantially less than currently achieved by ELISA. VG in the HL of this tick was first detectable two days after repletion at a level of approximately 5μg/μl or 20% of the HL protein. VG concentrations increase rapidly over the next 3 days, reaching levels of 80% of the total HL protein. This level of HL VG is extremely high in comparison to those of *H. dromedarii* and may be reflective of several important differences between the argasid and ixodid reproductive strategies. In argasid females, feeding and oviposition are cyclic activities and may be repeated several times. Vitellogenesis in a new cohort of primary oocytes is activated by digestion of each subsequent blood meal. Blood meal volumes are independent of mating in argasids and 5-12 fold increases in meal dependent body weight are typical among members of this family of ticks. In the argasid tick, *O. moubata*, clutches of 35-340 eggs are laid (Walton, 1962). These features are in contrast to mating dependent engorgement weight increases of 80-120 fold and a single clutch of 5,000-18,000 eggs in ixodid ticks (Diehl et al., 1982). Thus, assuming comparable levels of VG synthesis and secretion and similar rates of uptake by individual oocytes, one would expect lower HL titers of VG in the ixodid tick due simply to the far greater number of oocytes drawing from the HL pool. Whether the lower tissue and HL levels of VG in *H. dromedarii* are representative of similar levels in other ixodids was

not determined by ELISA but was visually suggested by western immunoblot comparisons of *H. dromedarii*, *D. variabilis* and *D. andersoni* HL (Appendix A). Crossreactivity among the ixodid VGs tested combined with the sensitivity of the ELISA and western immunoblot systems should enable further comparative analyses of vitellogenic activity among these ticks.

4.4 SYNTHESIS OF VG BY REPLETE FAT BODY *IN VITRO*.

Based on tissue immunoblot and ELISA results as a function of reproductive development, two tissues, OV and FB, display features consistent with their potential as a source of VG. *De novo* synthesis of VG or VN, as assessed by incorporation of radiolabeled amino acids into immunoprecipitable protein, although tested in a variety of different tissues from replete females, was demonstrated only in FB cultures. A complete set of VN A/VN B and VN C polypeptides were demonstrated, suggesting a FB origin of all of the identified VNs. Interestingly, all but trace levels of VG synthesized by FB were secreted into the culture media. During the culture period, levels of VG synthesized *de novo* increased daily and when totaled, equated to 12.1% of the FB protein (121 μg VG/mg FB protein). These results indicate that FB from R4 and R6 females maintains its VG synthetic activity *in vitro*, that culture conditions are supportive of VG synthesis, and that VG is not stored in the FB, but is rapidly secreted. Although OV cultures also released immunoprecipitable protein, radiolabeled amino acids were not incorporated into this material. These results suggest that VN was not synthesized by the cultured OV tissues but rather released from preexisting accumulations (R4 OV containing

>300μg VN/mg protein), possibly due to cellular breakdown. Also considered, was the possibility that HL, more specifically, a cellular component thereof, was capable of VG synthesis. Indeed, on one occasion, a culture seeded with HL, did demonstrate low levels of VG synthesis. However, in view of the unavoidable presence of small amounts of tracheae and FB in expressed HL, the cellular source of VG in this sample was not identified. This issue may be clarified by hemolymph filtration. Nonetheless, despite several possible minor sources of VG the results of the present studies provide compelling evidence of VG synthetic activity only by FB preparations.

Another proposed source of VG is the MG. Studies utilizing two hard tick species, *R. sanguinius* and *D. variabilis*, suggest that MG, in addition to FB, actively synthesizes VG (Coons et al., 1986; Rosell-Davis et al., 1989b). These conclusions were based on immunocytochemical results. Utilizing gold labeled anti-VN, these authors reported localized immunoprobe in the ER lumen of dissected MG tissues. Radiolabel incorporation into VG cultures of these tissues was not reported. Attempts to verify these conclusions by detection of VG synthesis in isolated MG tissues from *H. dromedarii* were unsuccessful. Indeed, MG not only failed to synthesize immunoprecipitable protein but compared to other tissues (SG, MU, HL, FB, and OV), displayed the lowest synthetic activity of any protein (results not shown). Additionally, incorporation of radiolabeled methionine (^{35}S) into MG VG *in vivo* was not detectable. These results and conclusions support those of Chinzei (1985b) in *O. moubata* and Araman (1979) in *R. sanguineus*. However, the possibility that very small amounts of VG are synthesized and rapidly secreted by

MG *in vivo*, and that this tissue does not retain this activity *in vitro*, can not be ruled out.

Additional evidence consistent with the current observations of VG synthetic activity by replete FB *in vitro* has been reported (Araman, 1979; Chinzei, 1985b). In *R. sanguineus*, Araman reported radiolabel incorporation into VG proteins secreted by cultured FB over a 6 hour period. Similarly, Chinzei demonstrated incorporation of radiolabeled amino acids in VG proteins secreted by FB cultures from *O. moubata*. In the latter study, the level of VG synthesized *de novo* reached a plateau within 12 hours and remained constant under the test conditions to 18 hours. The present study in *H. dromedarii* is the first report of long term (8 days) culture synthesis of VG by tick tissues and provides a basis for further evaluation of putative modulators of VG synthetic activity *in vitro*.

4.5 REGULATION OF VG SYNTHESIS IN FAT BODY.

The utility of the anti-VN based immunoblot and ELISA systems in qualitative and quantitative analysis of VGs and the successful demonstration of VG synthesis by FB *in vitro* provide a basis by which the regulatory control of vitellogenesis can be tested. Interests in this regard were focused on the identification of initiation factors of VG synthesis. For this purpose, FB from fed virgin females, a reproductive stage prior to VG synthetic activity, was selected. Dramatic stimulation of VG synthesis was observed in cultures exposed to extracts of synganglia from replete females. Indeed, the levels of VG synthesized in synganglion extract treated FV FB cultures exceeded those produced by FB from

replete females. Whether the extent of this stimulation is due to the lack of inactivating systems normally encountered in the whole animal is not known. Evidence supporting of an endocrine nature of the synganglion factor was suggested by successful stimulation with HL from replete females. The existence of putative endocrine structures within the synganglion is discussed elsewhere (see SOURCE AND NATURE OF VG SYNTHESIS ACTIVATORS). Although the stimulatory activity of replete synganglia and HL extracts on VG synthesis by FV FB cultures were only tested once, these findings provide the first reports of exogenously stimulated VG synthesis in FV FB cultures.

Corroboration of the stimulatory effect of synganglion extract *in vitro* was provided by similar stimulation *in vivo*. Levels of HL VG in treated virgins approximated those normally observed in mated females 2-4 days after repletion. Additionally, results of treatments *in vivo* suggest that a synganglion factor may directly or indirectly initiate oocyte uptake of HL VG; ovarian VN was concentrated over HL titers and approximated those typically observed in ovipositing females. Similar stimulation of vitellogenesis with synganglion extracts have been observed in several soft tick studies (Aeshlimann, 1968; Shanbaky and Khalil, 1975; Chinzei, 1986).

In contrast to the stimulatory effect of synganglion, ECD, JH III, BIS, or various combinations thereof, failed to initiate VG production by FV FB *in vitro*. However, both ECD and BIS were capable of stimulating VG production *in vivo*. Reports of rising ECD titers between mating induced engorgement and the onset of vitellogenesis in the ixodid ticks *A. hebrareum* and *H. dromedarii* (Connat et al., 1985;

Dees et al., 1985) support a natural role of this hormone in vitellogenesis. The inconsistencies between stimulation of VG production *in vitro* and *in vivo* may be related to the functional concentrations of these agents or the absence of additional factors (eg. activating enzymes) under the culture conditions. In other laboratory trials, ecdysteroids also have a demonstrated modulatory action on oogenesis and molting, frequently reducing oviposition and inducing a supermolt in fed virgin argasid ticks (Kitaoka, 1972; Mango et al., 1976). However, combined exposure to ecdysone and high concentrations of JH III induced oviposition in 6 of 10 argasid females and no supermolting (Obenchain and Mango, 1980). These latter studies did not monitor the synthesis of VG, thus comparative analysis of these results with those of the present study are difficult. In contrast to the failure of ECD treatment to stimulate VG synthesis in FV FB cultures, similar treatment of replete female FB cultures resulted in significant enhancement of VG synthesis as compared to controls. Thus, in replete FB tissue, which demonstrate VG production *in vitro*, ECD appears to further stimulate synthetic activity. Recent evidence of VG synthetic activity in liver sections from *Xenopus laevis* indicates that estrogen acts to stabilize VG mRNA from cytoplasmic degradation (Blume and Shapiro, 1989). These results are representative of a number of systems in which steroids regulate the stability of specific mRNA (Ross, 1989). Whether a similar mechanism functions in tick FB, enabling increased translational rounds of existing copies VG mRNA in replete FB, remains to be tested.

The ability of juvenoids to affect vitellogenesis in ticks has been reported by many investigators (*see review* Connat et al, 1983). However, most of these reports

are limited to treatment of argasid ticks *in vivo*. Unfortunately, numerous species-specific and laboratory-specific variations of vitellogenesis stimulation are presented. To complicate matters, parthenogenesis occurs at a variable and frequently significant rate within this family of ticks. Whether differences in induction of oogenesis and oviposition represent true differences in the reproductive biology of these species, random variation within a species or improper controls, is difficult to discern. Nonetheless, collectively, these reports present a substantial amount of data suggesting that partial regulation of vitellogenesis in the FB is under control of a JH-like compound. Although BIS, a diepoxide derivative of JH III and a proposed juvenoid in higher diptera (Richard et al., 1989), stimulated VG synthesis and ovarian concentration of VN in *H. dromedarii*, interpretation of these results requires a great deal of caution. It must be remembered that neither this molecule nor any of the known insect juvenoids have been chemically identified in ticks. Additionally, the metabolic fate of BIS in ticks has not been determined. It may be speculated that this molecule is resistant to the esterase and epoxide hydrolase enzymes of ticks which are capable of inactivating insect juvenoids. Continuing studies in this laboratory of these issues may provide further insight into the nature of the JH-like substance in ticks and its role, if any, in vitellogenesis regulation.

The disparity between stimulated VG synthesis *in vitro* and *in vivo*, highlights the need for critical and integrated evaluation of experimental results. Evidence is presented suggesting that various aspects of vitellogenesis, i.e., VG synthesis and yolk accumulation, are independently regulated and amenable to separate investigation. However, the failure of BIS and ECD to stimulate VG production *in vitro* may

indicate that isolated FB cultures lack a full complement of molecules required in a cascade-type of control. This hypothesized multicomponent regulation of vitellogenesis is consistent with other well-known endocrine mechanisms of control (Callard et al., 1990). Under such conditions, treatment with anything short of a complete set of regulatory molecules may result in failure to induce the desired response, in this case, VG synthesis. Thus, the failure of a given agent to initiate VG synthesis does not rule out its role in the overall process. Indeed, based on stimulatory activity *in vivo*, further analysis *in vitro* with BIS and ECD seems warranted.

4.6 VG PROCESSING.

Since most of the reported arthropod VGs are secretory proteins, one would expect them to have a typical peptide recognition sequence that directs them to be synthesized on the RER. Identification of the signal sequence and its removal have been documented in several species of dipterans (Warren et al., 1979). Most arthropod VGs are further modified by glycosylation and lipidation. Addition of these moieties have been shown to be critical for the correct processing and secretion of VG in several species of cockroaches and mosquitoes (reviewed by Bownes, 1986). Ticks VGs are also glycolipoproteins. In the two tick species which have been investigated, VG lipid content ranges from 5.5 to 8.5% and carbohydrate content ranges from 4.5 to 12% (Boctor and Kamel, 1976; Chinzei et al., 1983a). Whether these modifications are required for proper post-translational processing of VG is unknown.

Once synthesized, many of the small MW insect VGs do not undergo any proteolytic modification until they are digested by the embryo. The larger VGs however, are reported to undergo extensive proteolytic cleavage. The function of these changes is not clear. In a cell free translation system, Bose and Raikhel (1988) demonstrated that two subunits of mosquito VG, 200 KD and 45 KD, originated from a common 220 KD precursor specific to mRNA of vitellogenic females. Similar postranslational proteolytic processing has been documented in locust VG. While still within the FB, the VG of *Locusta* undergo rapid but limited cleavage from the 250 KD precursor to several 100 KD and 50 KD peptides (Chen, 1980). Further cleavages occur within the oocyte after uptake of VG from the HL (Koppe and Offengand, 1976). Radiolabel pulse-chase studies of the primary product polypeptide(s) of VG mRNA were conducted for *O. moubata* (Chinzei, 1986). In these studies, radiolabeled amino acids were incorporated selectively into 2 VG proteins with MWs of 215 KD and 210 KD during a 15 minute pulse of replete FB (MW of the native VG monomer is 300 KD). As a function of chase time, the radioactivity shifted progressively to smaller polypeptide subunits of VG. A total of 6 subunits ranging in MW from 215 KD to 100 KD were radio-labeled within a 2 hour chase period. These results were interpreted to indicate that the two large subunits were primary products of VG gene(s) and that the smaller subunits were proteolytic products thereof.

Evidence of VG processing in *H. dromedarii* was also obtained. As discussed above, both VN A and VN B give rise to a subset of 7 electrophoretically homologous polypeptides upon denaturation suggesting that VN A is a multimeric

form of VN B. Additionally, two dimensional SDS PAGE analysis revealed that two smaller immunodetectable bands from FB, HL and egg (MWs of 326 KD and 310 KD) also gave rise to a complete subset of VN 1-7 when resolved in the second dimension (not shown). The sum total MWs of VN 1-7 (790 KD) is substantially in excess of the MW estimation of the latter aggregates or VN B (640 KD average of VN B charge isomers). These results suggest that individual VN molecule aggregates within a native PAGE resolved band are composed of a variable subset of polypeptides. However, it can not be concluded from the present experiments whether the polypeptide subunits are multiple gene products which have a high affinity for each other or are post-translational cleavage products of a single gene.

It is unlikely that the discrepancies between the native MW estimate and the sum total of the subunit polypeptides are artifactual due to incomplete SDS denaturation. The reproducible and well resolved VN electrophoretic pattern suggests that denaturation was maximized under the tested conditions. Indeed, increasing the standard denaturation conditions from one to ten minutes did not alter the polypeptide pattern. Chinzei (1983a) has also reported that the VG subunit polypeptide pattern of *O. moubata* is stable to repeated cycles freezing and thawing.

Identification of VN C in *H. dromedarii* is a novel finding among the reported tick VNs. Although this small MW VN (50-56 KD) is synthesized and secreted by the FB and is subsequently concentrated with VN A/VN B in the oocyte, it behaves as a separate protein in PAGE and column resolving systems. Nonetheless, antiserum raised against PAGE purified VN A/VN B also reacts with VN C, suggesting shared antigenic epitopes. The absence of comigration between VN C

and subunits of VN A/VN B indicates that VN C is not present as a complete subunit with the VN A/VN B native aggregate. Two possibilities may explain these findings. First, VN C is cleaved from a VN A and/or VN B precursor while still within the FB and subsequently dissociates and remains dissociated from the parent molecule. In this case, the immuno-crossreactivity observed is due to the presence of VN A and/or VN B proteins from which VN C was not processed or was differentially processed. The second possibility is that VN C represents a separate gene product from VN A/VN B which shares epitope(s) with these larger proteins, perhaps indicative of partial gene duplication.

4.7 FAT BODY ORGANIZATION AND ULTRASTRUCTURE.

The current studies strongly support the hypothesis that the fat body is the major source of VG in *H.dromedarii*. Morphological and ultrastructural analyses of this tissue are consistent with a massive gearing of cellular machinery for the synthesis and secretion of protein between the stages of mating induced engorgement and the initiation of oviposition.

In adult females, after an initial period of slow feeding, mating stimulates rapid engorgement which is temporally correlated with a period of FB autolysis. Autolysis is closely followed by the development of extensive sheets of connective tissue and associated fat body. Along with these morphological developments are the appearance of differentiated trophocytes (Obenchain and Oliver, 1973).
The ultrastructure of fat body cells, both trophocytes and nephrocytes, is reportedly conserved in unfed larvae, unfed nymphs, and unfed adults (Coons et al., 1990).

During adult feeding however, trophocytes undergo an extensive, albeit gradual, ultrastructural change. In contrast to the trophocytes in unmated females, replete females display extensive amounts of rough endoplasmic reticulum and mitochondria. RER regions with cisternal distensions containing electron dense material became increasingly prevalent during the preovipositional period. Numerous additional transformations e.g., increased cell size and numbers of mitochondria and distended extracellular spaces with electron dense deposits, were all consistent with cells synthesizing and exporting large amounts of protein. Although the identity of proteins synthesized specifically by trophocytes was not investigated, the gradual nature of the observed ultrastructural changes in these cells and the temporal correlation of these changes *in vivo* with the ability of isolated FB preparations (trophocytes and nephrocytes) to synthesize VG *in vitro*, suggests that the trophocytes are in fact the source of VG.

In contrast to changes in fat body trophocytes, nephrocytes displayed a conserved subcellular profile throughout adult development, characterized by large numbers of vacuoles, lacunae, free ribosomes and mitochondria. This profile is consistent with a cellular involvement in steroid synthesis and evidence of fat body metabolism and secretion of ECD has been reported (Schriefer et. al., 1987). It is tempting to hypothesize that ECD is secreted by nephrocytes of vitellogenic ticks *in vivo* and functions to stimulate VG synthesis or stabilize VG mRNA in neighboring trophocytes.

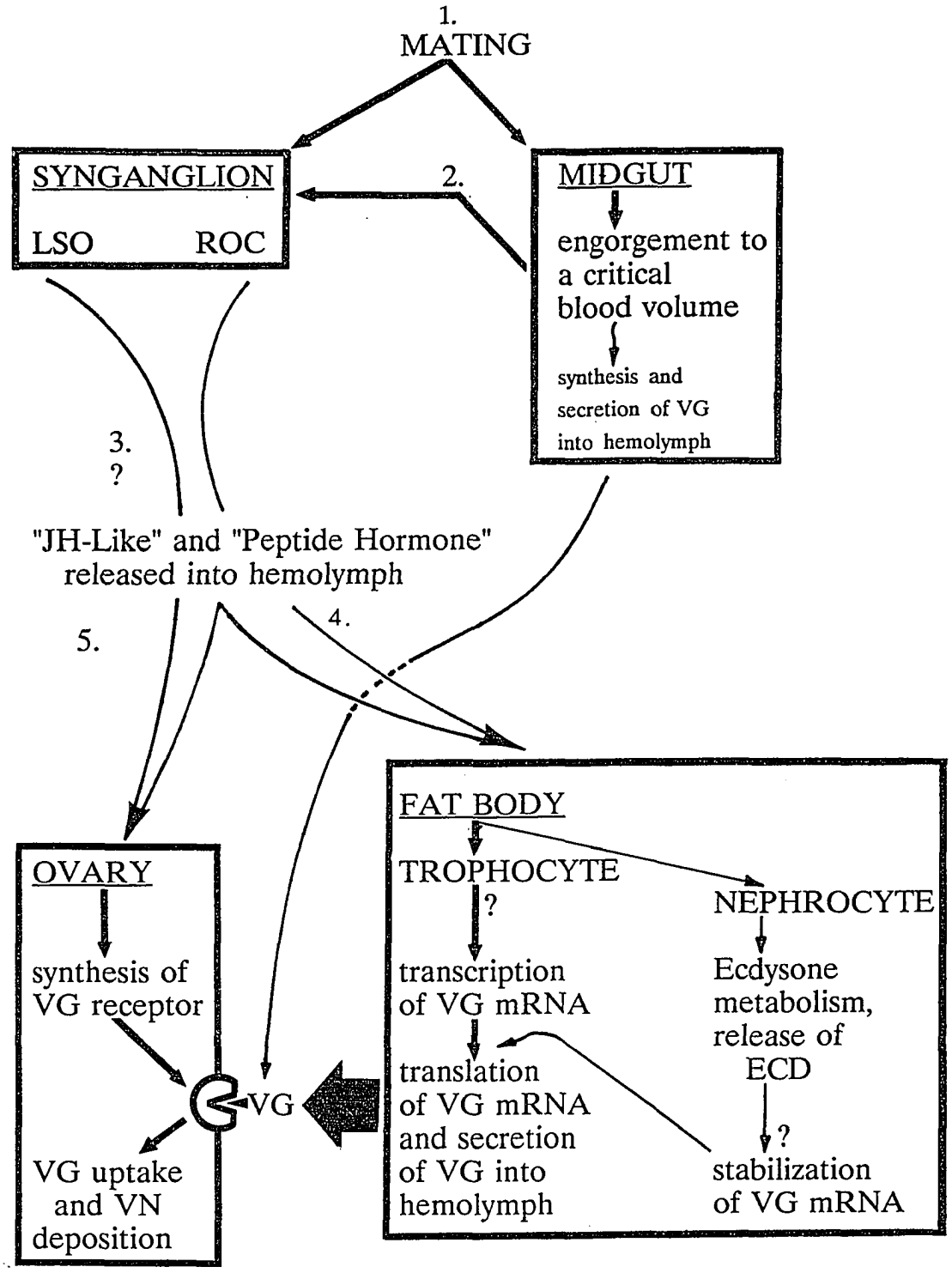
4.8 SOURCE AND NATURE OF VG SYNTHESIS STIMULATION.

The present studies indicate that the synganglion of replete females contain factors that stimulate FB VG synthesis and ovarian uptake of VG. The identity of these agents and how they reach their target tissues is unknown. However, based upon the current study and published reports, a model of the regulatory nature of tick vitellogenesis is considered (Fig. 29). A brief discussion of the tick synganglion and several postulated endocrine glands or tissues thereof will aid in the formation of this model.

In common with other acarines, the component ganglia of the ticks central nervous system are condensed into a central mass or synganlion. The synganglion is enclosed within a sinus of the circulatory system and is traversed by the oesophagus and the recurrent nerve. The neuropile, or central nerve fiber mass, is surrounded by several layers of glial cells, a cortex of nerve cell bodies and an acellular neural lamella. Electron microscopy studies have demonstrated an abundance of neurohaemal termini within the border of the neural lamella-glial cell perineural sheath. Thus, in contrast to insects and crustaceans, in which a peripheral neurohaemal sinus gland or an organ such as the corpus cardiacum is the site of neurohaemal release, ticks are thought to release neurohormones directly from the sheath of the central nervous system (Binnington, 1986).

Two structures of the tick synganlion, which have ultrastructures compatible with an endocrine functions, are the retrocerebral complex and the lateral segmental organs. Cells of the retrocerebral organ complex contain extensive networks of RER and secrete electron-dense granules which may contain peptide hormones. Recent

FIGURE 29. WORKING MODEL OF VITELLOGENESIS REGULATION IN IXODID TICKS. LSO - lateral segmental organ, ROC - retrocerebral organ complex. See text for description of regulatory mechanisms 1-5.



data indicates that peptides play a greater role in arthropod neurotransmission than previously suspected (Binnington, 1986). Further characterizations of these secretory products and the activity of the complex during vitellogenesis remains to be done. Since 1984 the number of fully characterized insect neuropeptides has increased from 4 to over 40 (Holman et al., 1990). Detection of these and similar neuropeptides by immunosurveys of tick samples would undoubtedly afford a means of rapid, preliminary and cost effective identification of putative neuroregulatory molecules.

The lateral segmental organs contain accumulations of SER which increase during feeding and vitellogenesis (Binnington, 1981). These accumulations are consistent with the production of lipophilic, perhaps JH-like, products. The ability of extracts of this tissue from vitellogenic females to stimulate VG synthesis in virgin fat body in culture is consistent with the proposed model of neuroendocrine regulation. Identification of these putative products has not been done although substantial efforts have been expended in an attempt to identify the production of known insect juvenoids. Fractions coeluting with known juvenoids (JHI, II and III) resolved by reversed phase HPLC have been quantified by radioimmunoassay (RIA). An immunoreactive substance corresponding to the elution of JH III was found in the hemolymph of *H. dromedarii* and *D. variabilis*. The levels of this immunoreactive compound ranged from 1 to 4 ng/ml of hemolymph (Sonenshine et al., 1989). The formidable task of chemical identification of this low level compound(s) by GC/MS has not yet been achieved.

Based on these observations the following model (Fig. 29) of regulation of vitellogenesis is proposed. 1)- Mating factor induces rapid engorgement, probably indirectly through the synganglion. 2)- Engorgement to a critical level signals the synganglion to synthesize a stimulator(s) of fat body activity. This may be in the form of a JH-like molecule (perhaps from the lateral organ complex) and/or a peptide hormone (possibly from the retrocerebral complex). 3)- The synganglion factors act on the fat body; the JH-like compound may act directly on the trophocytes to activate transcription of VG genes, the peptide hormone possibly activating synthesis of ECD in the nephrocytes. 4)- ECD enhances synthesis of VG in the fat body. 5)- The JH-like synganglion factor may also initiate the production of VG receptors in developing oocytes thus enabling the concentration of HL VG for deposition as a yolk VN.

Two additional models of vitellogenesis regulation in ticks have been proposed (Connat, et al., 1986; Chinzei, 1986). Although both of these earlier models pertain to the argasid tick *O. moubata*, many features are consistent with the present model. Roles for ecdysteroids, JH-like compounds, and unidentified neuropeptides are supported in each of these schemes. However, fundamental differences between argasid and ixodid ticks (eg., large differences in HL VG titers, multiple versus single gonadotrophic rounds, differential fat body development in adults) would suggest that unique regulatory mechanisms also exist.

The ability to stimulate VG production in virgin FB *in vitro* over extended periods of time suggests that this system may be particularly amendable to analysis on a molecular level. Additionally, evidence suggesting close identity between VGs

from several genera and species of hard ticks indicates that the present methodologies may be applicable to a variety of ixodids thus facilitating experimental interpretation between variable model systems. Further analysis of the active molecule(s) in synganglia extracts is of obvious importance as are additional experiments with variable hormone concentrations. Utilization of the current model system should enable rapid progress in our understanding of gene expression in ticks. Identification of specific tick hormones and their receptors will not only enhance our understanding of gene regulation but may enable identification of manipulable regulatory molecules of the reproductive development in these important disease vectors.

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APPENDIX A

COMPARISON OF VITELLOGENINS FROM *HYALOMMA DROMEDARII, DERMACENTOR ANDERSONI,* AND *DERMACENTOR VARIABILIS.*

FIGURE A1. SDS PAGE AND IMMUNOBLOT COMPARISON OF VITELLOGENIN FROM *HYALOMMA DROMEDARII, DERMACENTOR ANDERSONI* AND *DERMACENTOR VARIABILIS*. A. 50 μg of hemolymph from replete females was resolved by SDS PAGE and stained with Commassie blue. B. 5 μg of each hemolymph sample was resolved by SDS PAGE and immunobloted. 1. MW STDS. (25 μg), 2. *H. dromedarii*, 3. *D.andersoni*, 4. *D. variabilis*. The MW of the standards is recorded as are the the VG polypeptides of *H. dromedarii*.

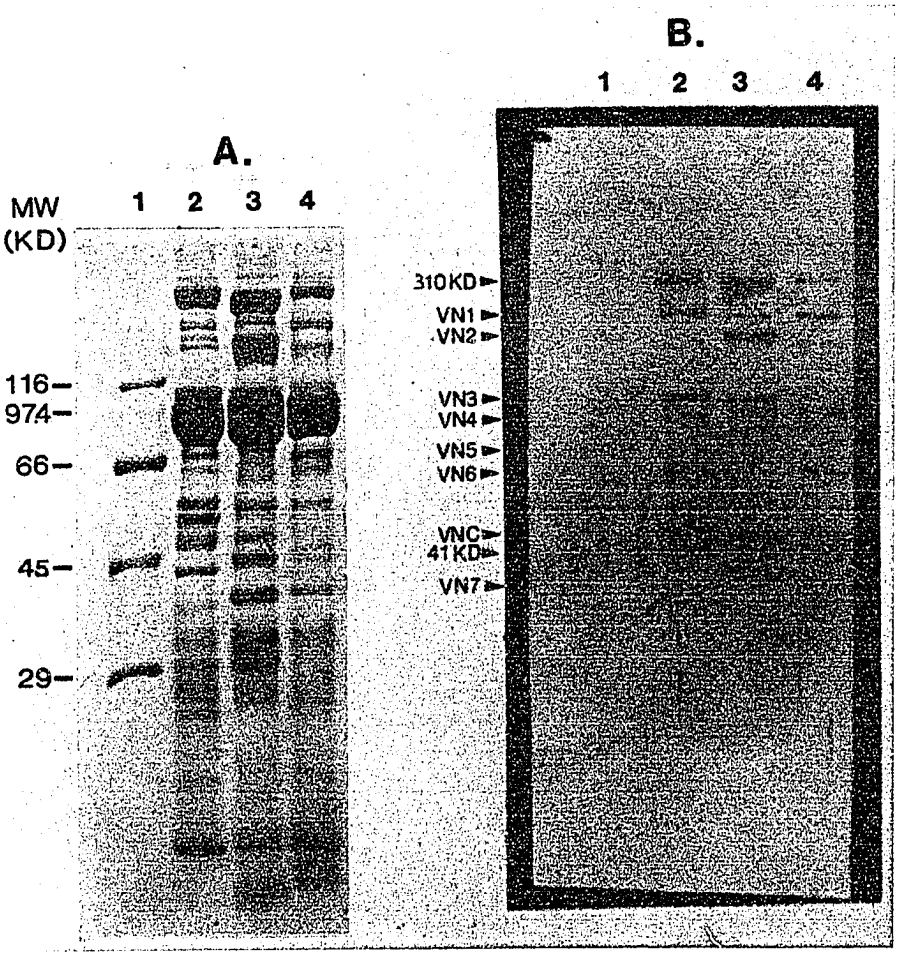

TABLE A1. MOLECULAR WEIGHT OF VITELLOGENIN POLYPEPTIDES IN TWO GENERA AND THREE SPECIES OF IXODIDS[a].

H.d.	D.a.	D.v.	D.v.[b]	Designation[c]
326	---	---	---	326 KD
310	305	308	---	310 KD
212	210	210	---	VN1
169	175	169	170	VN2
114	110	105	110	VN3
101	101	95	98	VN4
85	---	---	80	VN5
71	---	71	67	VN6
56	51	55	50	VNC
41	44	41	45	41 KD
35	---	---	35	VN7

[a] Polypeptides were identified in SDS PAGE immunoblots and MWs were determined by Ferguson analysis. H.d. = *Hyalomma dromedarii*, D.a. = *Dermacentor variabilis*, D.V. = *Dermacentor andersoni*.
[b] MWs of VG subunits as reported by Rosell-Davis and Coons, 1989a.
[c] designation used in this manuscript.

APPENIX B

REAGENTS AND RECIPES

REAGENTS AND RECIPES

PAGE REAGENT STOCKS:
Acrylamide - 30% acrylamide (%T), 2.7% N,N -Methylene-bis-Acrylamide (%C) (BioRad)
SDS - 10% Sodium Dodecyl Sulfate (BioRad)
Temed - N,N,N',N' - Tetramethylenediamine (BioRad)
Ammonium Persulfate (AMPS) - 10% (BioRad)
SDS Buffer A - 1.5 M tris-CL (pH 8.8). 2-Amino-2-hydroxymethylpropane-1,3-diol (Sigma)
SDS Buffer B - 0.5 M tris-Cl pH 8.8
Native Buffer A - 2 M tris-Cl, pH 9.6
Native Buffer B - 0.35 M tris-Cl, pH 6.9

For vertical slab gels, the above stock solutions were combined in the sequence and volumes indicated below for the respective percentage gels.

SDS PAGE

	RESOLVING GELS			STACKING GEL
	4%	10%	15%	4%
Acrylamide	2.26 ml	5.67 ml	8.5 ml	1.33 ml
Buffer A	4.25 ml	4.25 ml	4.25 ml	----
Buffer B	----	----	----	2.5 ml
SDS	0.17 ml	0.17 ml	0.17 ml	0.1 ml
Water	10.3 ml	6.9 ml	4.0 ml	6.6 ml
AMPS	37 ul	37 ul	37 ul	50 ul
TEMED	6 ul	6 ul	6 ul	5 ul

NONDENATURING PAGE

	RESOLVING GELS				STACKING GEL
	4%	8%	10%	15%	3%
Acrylamide	2.0 ml	4.0 ml	5.0 ml	7.5 ml	1.25 ml
Buffer A	2.8 ml	2.8 ml	2.8 ml	2.8 ml	----
Buffer B	----	----	----	----	1.0 ml
Water	9.9 ml	7.9 ml	6.9 ml	5.0 ml	6.55 ml
AMPS	75 ul	75 ul	75 ul	75 ul	30 ul
TEMED	6 ul	6 ul	6 ul	6 ul	3.5 ul

GRADIENT RESOLVING GELS. Linear gradients of nondenaturing and SDS PAGE were made using a BioRad gradient maker. Depending on range of gradient, appropriate light and heavy monomer solutions, without AMPS and Temed, were made to 0.5 volumes of the above recipes, degassed and pippeted into the gradient maker. AMPS and Temed were added to each solution and mixing affected with magnetic stirbars. The flow valves were opened and adjusted to deliver 3 ml/ minute.

BUFFERS:

Tris buffered saline (TBS) - 20mM Tris-HCl, 500mM NaCl (pH 7.5).

ELISA:
Coating buffer (CB) - 13.6 mM Na_2CO_3, 34.1 mM $NaHCO_3$ (pH 9.6).
Wash buffer (WB) - PBS,pH 7.2, 0.05% Tween 20
Antibody buffer (AB) - WB with 0.25% BSA

WESTERN BLOTTING:
Native gel transfer Buffer - 25 mM Tris (pH 8.3), 190 mM Glycine
Denatured gel transfer buffer - 25 mM Tris (pH 8.3), 190mM Glycine, 20% methanol

WESTERN IMMUNOBLOTTING:
TBS - 20 mM Tris, 500 nM NaCl, pH 7.5
TTBS - TBS plus 0.05% Tween-20
Blocking buffer - TBS plus 3% gelatin (BioRad)
Antibody buffer - TTBS plus 1% gelatin

APPENDIX C

LIST OF ABBREVIATIONS

ABBREVIATIONS:

AB	antibody buffer
anti-VN	antibody against VN
AS	ammonium sulfate
BIS	bis epoxide of JH
CB	coating buffer
DMSO	dimethyl sulfoxide
ECD	20-OH ecdysone
EE	egg extract
EIA-HRP	enzyme immunoassay-HRP
ELISA	enzyme linked immunoabsorbant assay
FB	fat body
FM	freshly molted
FV	fed virgin
GAR-HRP	goat anti-rabbit, HRP
HL	hemolymph
HRP	horse radish peroxidase
JH	juvenile hormone
MG	midgut
MU	muscle
OD	optical density
OV	ovary
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethyl sulfonyl fluoride
R	replete
R+"X"	replete plus X number of days
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SG	salivary gland
TB	transfer buffer
TBS	Tris buffered saline
TEM	transmission electron microscopy
TTBS	TBS with Triton X 100
UF	unfed
VG	vitellogenin
VN	vitellin
WB	wash buffer
YM	Yunker-Meibos media

AUTOBIOGRAPHICAL STATEMENT

Martin E. Schriefer was born in North Kingston, Rhode Island on November 6, 1952 the eldest of seven children to Walter A. and Ruth M. Schriefer. His father was a career naval aviator and Martin received his formal educational training in five states and Lima, Peru. After serving three years in the USN, including active duty in Vietnam, Martin received his Bachelors degree in Science from Old Dominion University in 1977. He was married to Carole J. Carter in 1975. In 1983 he received his Masters degree in Microbiology from the University of Kentucky. His wife graduated from Law School, also at the University of Kentucky, in 1982. Martin taught graduate and undergraduate courses in microbiology and immunology at the University of Kentucky and Lexington Community College from 1980 through 1985. In 1991 Martin completed his PhD in Biomedical Sciences at Old Dominion University/Eastern Virginia Medical School. Martin began a Post-Doctoral Research Fellowship in the Department of Microbiology and Immunology at the University of Maryland, Baltimore in 1990.

Memberships in Professional and Scientific Societies
Sigma XI
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Entomological Society of America
Virginia Academy of Science

Honors
1985-1990 Special Doctoral Research Assistantship, Eastern Virginia Medical School/ Old Dominion University

Publications
Articles

Schriefer, M., Stiener. S., (1984) Phosphatidylinositol (PI)-specific phospholipase C of chick and mouse myoblasts. Fed. Proc. 43:770.

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