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VITELLOGENIN SYNTHESIS IN THE LADY BEETLE *COCCINELLA SEPTEMPUNCTATA*

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Abstract—To elucidate the hormonal mechanisms which regulate reproduction in a beneficial insect, we have begun to investigate the process of vitellogenesis in *Coccinella septempunctata*, the seven-spotted lady beetle. Vitellin (Vn) constitutes 60–70% of the total protein in *C. septempunctata* eggs and is composed of four polypeptides with molecular weights determined by electrophoresis in denaturing gels of 133,000, 130,000, 46,000 and 43,000. In the egg these polypeptides occur in a ratio of approx. 1:1:1:2. The two larger Vn polypeptides yielded similar peptide fragments upon partial proteolytic digestion which suggests that they are structurally related. The two smaller Vn polypeptides appear structurally distinct because they yielded unique proteolytic fragments. The Vn precursor, vitellogenin (Vg), was observed in the haemolymph of mature females, but was not detected in the haemolymph of immature females or males. The electrophoretic mobilities, antigenicity, and proteolytic fragmentation patterns of the Vg polypeptides were indistinguishable from those of their Vn counterparts. Thus the major processing of the Vn polypeptides appears to occur prior to their secretion into the haemolymph.

The synthesis of Vg was examined in whole animals and in organ cultures. Vg synthesis was observed in the fat body and to a smaller extent in the ovaries of mature females. The newly synthesized Vg was rapidly secreted. Vg synthesis was not detectable in brain or thoracic muscle of mature females or in the fat body of males or immature females. Very little vitellogenin synthesis occurred in female insects reared on artificial diets. The topical application of a juvenile hormone analogue induced Vg synthesis in non-fecund females but not in males.

Key Word Index: *Coccinella septempunctata*, vitellin, vitellogenin, vitellogenin synthesis, fat body, juvenile hormone

INTRODUCTION

Insect vitellogenins are yolk protein precursors synthesized in the fat bodies of mature female insects. After secretion into the haemolymph, the vitellogenins are selectively taken up by the developing oocytes and deposited as the major egg yolk proteins, the vitellins (Wyatt and Pan, 1978; Gong and Zhai, 1979; Hagedorn and Kunkel, 1979; Engelmann, 1979). In most insects, this process, known as vitellogenesis, is under the control of juvenile hormone from the corpora allata, although ecdysone may also play a role in some insects (Hagedorn and Kunkel, 1979). The process is analogous to estrogen-controlled vitellogenin synthesis in the liver and its deposition in the egg of oviparous vertebrates (Tata, 1978; Tata and Smith, 1979; Deeley and Goldberger, 1979; Shapiro and Baker, 1979).

In recent years, insect vitellogenins have been intensively studied not only because they are a prerequisite to reproduction of insects, but also because they provide an excellent experimental model for the study of mechanisms of insect hormone action at the molecular level. There are several comprehensive reviews dealing with the fundamental information and current progress of research on insect vitellogenin and vitellogenesis (Wyatt and Pan, 1978; Gong and Zhai, 1979; Hagedorn and Kunkle, 1979; Engelmann, 1979).

The seven-spotted lady beetle *Coccinella septempunctata* is employed in Chinese agriculture as an agent of biological control of the cotton aphid as an alternative to chemical insecticides (Adkisson *et al.*, 1982). Little is known of hormonal regulation of reproduction in predacious insects and we are investigating vitellogenin synthesis in *C. septempunctata* not only to define the molecular mechanisms of hormonally controlled gene expression in this insect but also to provide a basis for its artificial cultivation.

MATERIALS AND METHODS

Insects

C. septempunctata was collected from natural populations in New Jersey by personnel of the Beneficial Insect Research Laboratory of the USDA. They were kept in both the insect chamber and the laboratory. For the insect chamber cultures, green peach aphids (*Myzus persicae*) raised on cabbage seedlings in the green house were used to feed *C. septempunctata*. Laboratory cultures were maintained on an artificial diet (Gong *et al.*, 1980), composed of homogenized raw pig liver, honey and cane sugar (5:1:1, w/w/w).

Hormone treatments

C. septempunctata feeding on the artificial diet were treated with synthetic juvenile analogues (ZR-512 or ZR-

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Abbreviations used: Vn, vitellin; Vg, vitellogenin; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

515, a gift of Zoecon Corp.). These juvenile hormone analogues were dissolved in olive oil or acetone at a concentration of 100 μg/μl, and 1 μl was topically applied to the tergites of each insect.

Preparation of vitellin from C. septempunctata *eggs*

Fertilized eggs were harvested daily from a colony maintained on a diet of *M. persicae*. To prepare vitellin, eggs were washed and then homogenized in approx. 20 vol of ice-cold high-salt buffer (0.05 M Tris–HCl, pH, 7.2; 0.4 M NaCl). Following centrifugation (27,000 *g* for 20 min) to remove insoluble debris, and filtration through glass wool to remove lipid, the extract was diluted 10–20 times with ice-cold distilled water, a procedure which causes precipitation of vitellin. This precipitation was repeated two to three times and the material was lyophilized and stored at −20°C.

Preparation of vitellin antibodies

Partially purified vitellin, prepared as described above, containing 5 mg of protein in 0.5 ml of saline (prepared according to the inorganic cation content of the haemolymph of *C. septempunctata* as reported by Florkin and Jeuniaux (1974), was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into rabbits. Three further injections were given at weekly intervals. The rabbits were bled two weeks after the last injection. The antisera were prepared and stored in small aliquots at −20°C and showed a single line of precipitation on Ouchterlony plates (Gong *et al.*, 1980).

In vivo *labelling of vitellogenin*

In a typical experiment, 10 μCi of [^{35}S]methionine (> 800 Ci/mmol, New England Nuclear Corp.) in 5 μCi of *C. septempunctata* saline (see above) was injected with a fine capillary needle into the abdomen of an etherized insect. After the chosen incubation period, haemolymph was collected by a capillary tube from leg joints of five individual insects and combined. Fat body and ovary tissue from five individuals were obtained by dissection, combined and homogenized in 0.4 ml ice-cold saline. The homogenates were centrifuged (27,000 *g* for 20 min at 0°C) and the supernatant under the floating lipid layer was collected by a drawn out Pasteur pipette for analysis of labelled Vg.

Synthesis of vitellogenin in tissue culture

The fat body attached to the dorsal part of the abdominal body wall was carefully dissected in cold saline. After rinsing two to three times in saline, the fat body was transferred to 10 μl of modified Grace medium (Grace, 1962) (without methionine) supplemented with 15 μCi of [^{35}S]methionine and incubated at 25°C for various lengths of time. After incubation, the fat body was washed twice in saline and prepared as described above for analysis of intracellular vitellogenin. The culture medium was collected separate from the tissues for analysis of secreted vitellogenin. Ovary cultures were labelled and prepared under the same conditions.

SDS–PAGE and the quantitation of protein components

SDS–PAGE was performed in a system modified from Laemmli (1970) for the analysis of insect proteins. In a typical experiment a 5 to 15% linear polyacrylamide gradient gel was formed in a slab 30 × 25 or 14 × 16 cm and 1.5 mm thick. Polypeptides in gels were stained with Coomassie blue and scanned at 570 nm with a Gilford spectrophotometer. Areas under the peaks were measured. To detect labelled polypeptides, autoradiographs were scanned at 550 nm with a Gilford spectrophotometer and areas under the peaks quantified. In some cases, gels were cut into 2 mm slices. Each slice was incubated in 0.5 ml of 30% H_2O_2 at 60°C for 8–16 hr. After cooling, samples were counted in Aquasol II with a Beckman liquid scintillation counter.

Immunoprecipitation

The labelled vitellogenin was isolated by either of two methods of immunoprecipitation. Direct immunoprecipitation was accomplished by adding partially purified vitellin and vitellin antiserum to a radioactive sample followed by incubating at 30°C for 1 hr and then at 4°C for 8–16 hr. The immunoprecipitate was collected by centrifugation (5000 *g* for 15 min at 0°C) and washed by suspending it in 1 ml saline containing 1% Triton X-100 and 0.5% sodium deoxycholate followed by recentrifugation for 15 min at 5000 *g*. The washing was repeated two to four times. After washing, the immune precipitate was heated in electrophoresis sample buffer (Laemmli, 1970) and subjected to SDS–PAGE.

Immunoprecipitation using *Staphyloccocus aureus* Cowan Type I as an adsorbent was also employed (Ivarie and Jones, 1979).

Peptide mapping by limited proteolysis

Peptide mapping by limited proteolysis was performed by the method of Cleveland *et al.* (1977). Bands from SDS-gels were digested either without prior elution or after extraction from the gel slices (Sreekrishina *et al.*, 1980). Several common proteases including chymotrypsin, papain and proteases from *Staphylococcus aureus* V8 and *Streptomyces grieus* were used. The peptide maps were either developed by autoradiography or by the Silver staining methods of Oakley *et al.* (1980) or Goldman *et al.* (1981). Protein standards (see legend to Fig. 1) were obtained from Bio-Rad Laboratories Inc.

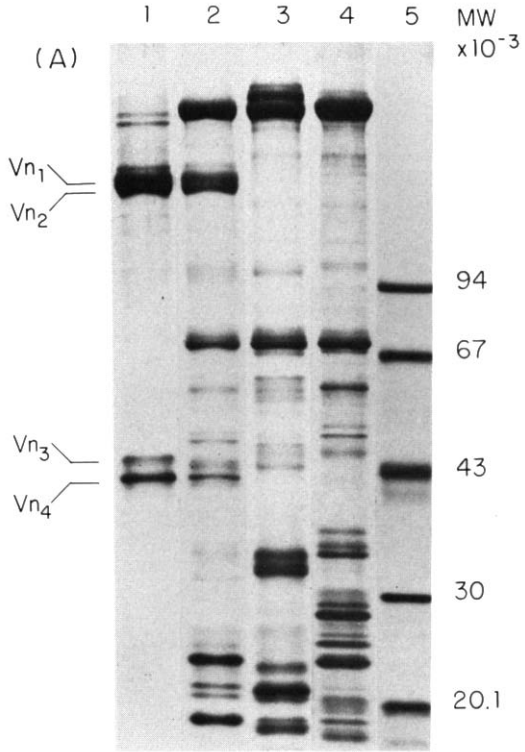
RESULTS

Nature of the vitellin and vitellogenin

The major yolk protein, vitellin (Vn), prepared from *C. septempunctata* eggs by precipitation at low ionic strength showed a single band on native polyacrylamide gels and constitutes about 60–70% of the total protein in the egg (Gong *et al.*, 1980). When analyzed under denaturing conditions by SDS–PAGE this single protein component yielded four polypeptides (Vn1, Vn2, Vn3 and Vn4; Fig. 1A, lane 1). The faint bands visible in Fig. 1A, lane 1 are not components of Vg because their quantity varied with different preparations and they were not always present (data not shown).

The approximate molecular weights of the four Vn polypeptides, determined by comparing migration rates with protein standards, were 133,000, 130,000, 46,000 and 43,000. The two larger polypeptides were similar in size, and could be separated into two distinct bands on SDS gels only when the protein concentration was low (Fig. 1B).

In order to quantitate the relative amounts of the four polypeptides in vitellin, stained gels were scanned and the area under the four peaks was integrated. Typical values were Vn1, 35%; Vn2, 35%; Vn3, 10%; Vn4, 20%. On the basis of the molecular weights of the individual polypeptides and assuming that the degree of staining was directly proportional to protein concentration, these values were converted to a molar ratio for the four vitellin polypeptides of about 1:1:1:2. This ratio of peptides if present in a single structure, would correspond to a molecular weight of 395,000. Indeed when analyzed by high performance gel permeation chromatography on TSK-3000 (Varian Associates, Inc.) all four polypeptides eluted together with an apparent molecular weight of about 4×10^5 (data not shown). These



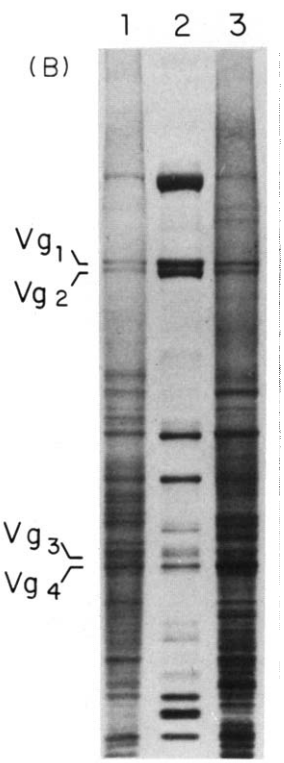


Fig. 1. SDS–PAGE analysis of *C. septempunctata* Vn and Vg. (A) (1) partially purified Vn, (2) mature female haemolymph, (3) immature female haemolymph, (4) male haemolymph, and (5) marker proteins. Protein markers used are phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100). (B) (1) female fat body extract, (2) female haemolymph, and (3) ovary extract. Proteins were detected by Coomassie blue staining.

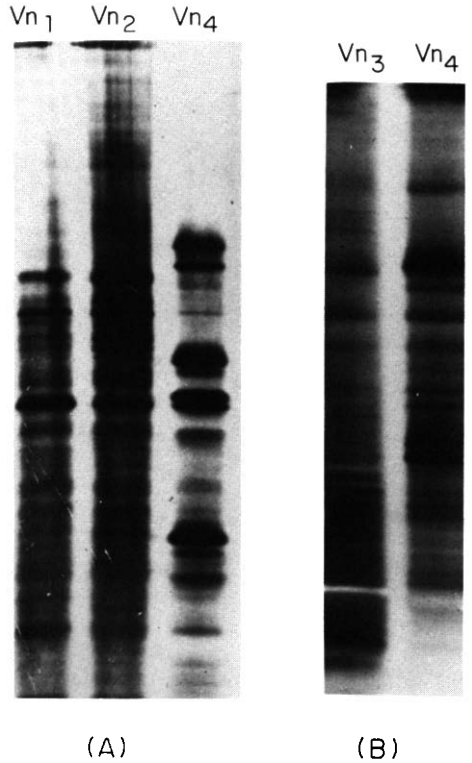


Fig. 2. Silver stained gel of peptide patterns generated by limited digestion of Vn by (A) chymotrypsin (10 μg), and (B) Sa V8 protease (5 μg).

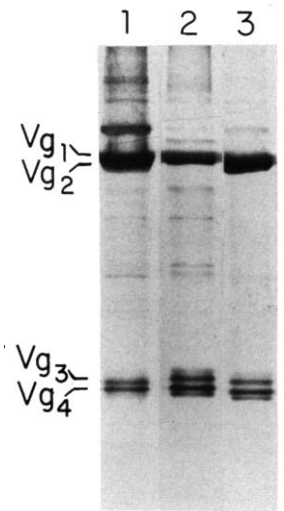


Fig. 3. Autoradiograms obtained from SDS–PAGE of *in vivo* labelled and antibody precipitated Vg and Vn. (1) fat body, (2) haemolymph, (3) ovary. Samples were from females labelled 2.5 hr *in vivo* with [^{35}S]methionine and prepared for direct immunoprecipitation as described under Materials and Methods.

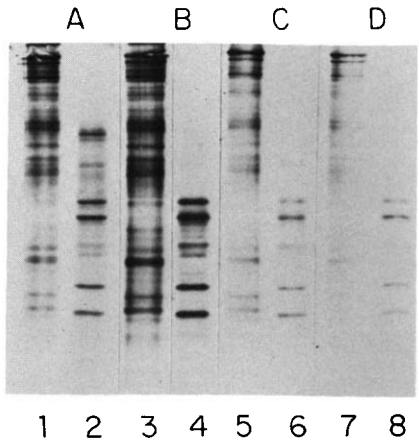


Fig. 4. Autoradiograms of peptide patterns generated by limited Sa V8 protease digestion. 1 and 2, 3 and 4, 5 and 6, 7 and 8 are peptide patterns of Vg 1 and Vg 4, respectively, from (A) female fat body labelled 4 hr *in vitro*, (B) female fat body culture medium, (C) female haemolymph labelled 4 hr *in vivo*, and (D) ovary labelled 4 hr *in vivo*.

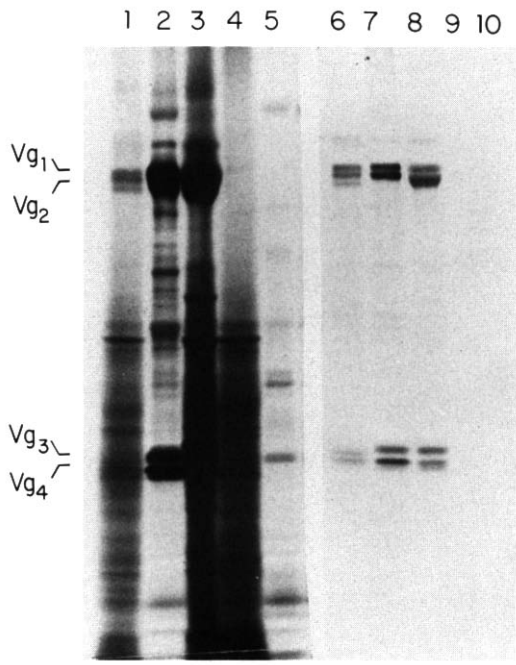


Fig. 5. *In vivo* synthesis of Vg (1–3). Autoradiograms obtained from SDS–PAGE of fat body, haemolymph, and ovary respectively, from a mature female 4 hr after injection of [^{35}S]methionine. (4–5) Autoradiograms of fat body and haemolymph, respectively, from a male labelled 4 hr *in vivo*. (6–10) Autoradiograms of corresponding samples of 1–5 after indirect antibody precipitation.

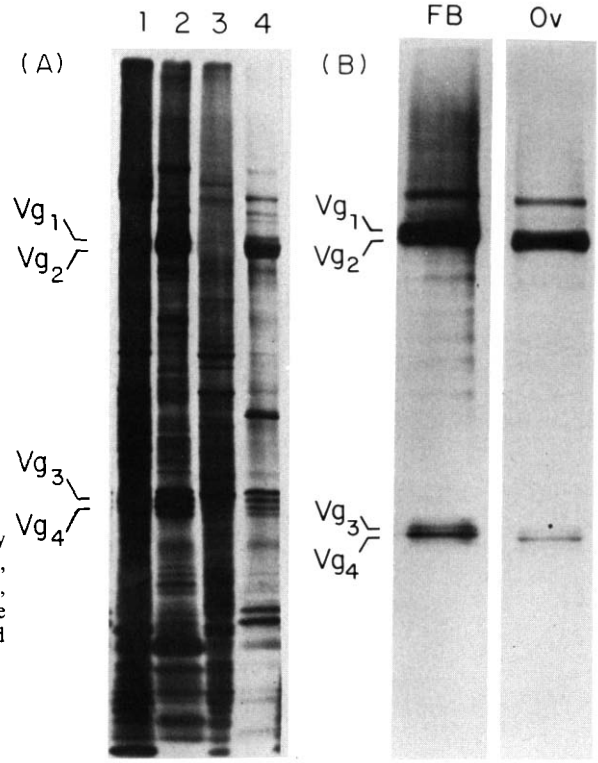


Fig. 7. Synthesis of Vg by fat body and ovary cultured in Grace's medium with [^{35}S]methionine. (A) Autoradiogram obtained from SDS–PAGE of (1) fat body, (2) fat body culture medium, (3) ovary, and (4) ovary culture medium. (B) Autoradiogram of direct antibody-precipitated Vg secreted into the culture medium by fat body ovary. Fat body and ovary were labelled 3 hr. Samples analyzed represent half the fat body and ovary from a female, and their corresponding media.

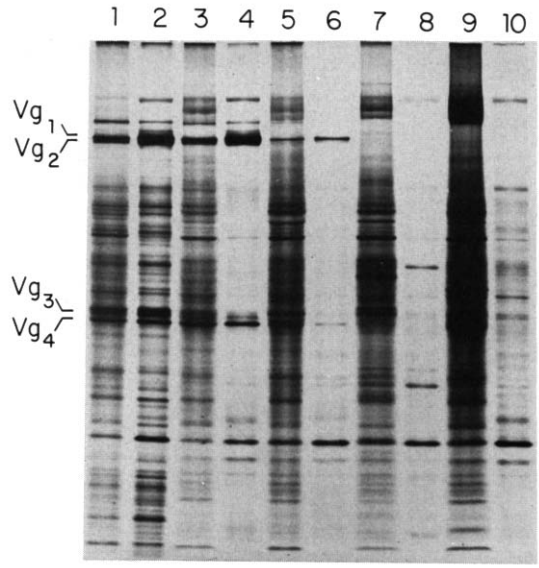


Fig. 8. Induction of Vg synthesis by juvenile hormone analogue ZR-512. 1 and 2, 3 and 4, 5 and 6, are *in vitro* cultured fat body tissue and its culture medium, respectively, from three ZR-512 treated females. 7 and 8 are corresponding samples from an untreated female. 9 and 10 are corresponding samples from a ZR-512 treated male. ZR-512 was topically applied at a dosage of 100 μg/insect. Fat bodies were collected 10 days after treatment.

findings are in accord with the possibility that vitellin occurs as a single multisubunit complex but do not rule out other alternatives.

In order to determine if the four Vns are structurally related, peptide maps generated by limited proteolysis were analyzed. Vn1 and Vn2 yielded similar fragments upon partial proteolytic digestion (Fig. 2), indicating similarity in chemical structure. Vn3 and Vn4 gave peptide fragmentation patterns which were distinct from each other and that of Vn1 and Vn2 (Fig. 2).

The yolk protein precursor, vitellogenin (Vg), present in the haemolymph of the mature females, also contained four polypeptides (Vg1, Vg2, Vg3 and Vg4) as shown by SDS–PAGE (Fig. 1). The four Vg polypeptides co-migrated with the four Vns of the egg* and also reacted with anti-Vn antibody. Therefore, the Vgs were electrophoretically and immunologically indistinguishable from their respective Vn counterparts (Fig. 3). When subjected to limited proteolysis, the Vg polypeptides yielded peptide fragmentation patterns indistinguishable from those of the corresponding Vns (Fig. 4).

The Vg polypeptides were also present, but usually in small amounts, in the fat body of mature females (Fig. 1B). They were absent from the haemolymph of immature females or males (Fig. 1A).

Synthesis of vitellogenin

In reproductively mature females, the injection of [^{35}S]methionine resulted in substantial radioactive incorporation into Vg of the fat body and haemolymph and Vn of the ovary (Fig. 5).

A semi-quantitative picture of the rate of Vg and Vn labelling in the intact mature female is provided by the data shown in Fig. 6A. Following injection of [^{35}S]methionine, radiolabelled Vg was first detectable in the fat body where it reached a maximum about 2 hr after injection of the isotope and then declined. The appearance of radiolabelled Vg in the haemolymph occurred after a brief delay and reached a maximum about 4 hr after injection of the isotope.

The synthesis and secretion of Vg was also observed in cultures of fat body tissue (Fig. 6B). In these cultures, the tissue level of radiolabelled Vg was still increasing at 1 hr of incubation with [^{35}S]methionine. The level of radiolabelled Vg in the culture medium rose sharply after a lag period. The specific nature of the elaboration of Vg by the fat body cultures is evident from the fact that the SDS–PAGE pattern of secreted proteins is very different from that of the proteins contained within the tissue (Fig. 7A). The peptide maps of Vg secreted in tissue culture were indistinguishable from those of the Vg obtained from haemolymph (Fig. 4).

No evidence of Vg synthesis was observed when thoracic muscle or brain tissue were cultured with [^{35}S]methionine (data not shown). In order to obtain this result, however, extreme care had to be taken in order to exclude invading fat body during the preparation of brain or muscle tissue samples. In contrast, significant Vg synthesis was detected in cultures of ovary tissue which did not appear to result from fat body contamination (Figs 7A and B). Although labelled Vg was clearly seen in the ovary culture medium the SDS–PAGE pattern of other labelled proteins in this sample was quite different from that observed with fat body culture medium and in the ovary culture very little newly synthesized Vg remained within the tissue (Fig. 7A). Vg newly synthesized by both cultures was readily precipitated by antibody prepared against Vn (Fig. 7B).

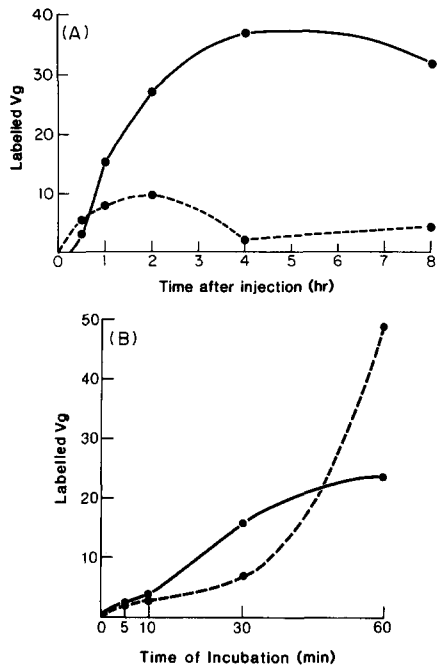


Fig. 6. Kinetics of Vg labelling *in vivo* and in fat body culture. (A) Vg labelling in fat body and haemolymph in females labelled for different periods of time with [^{35}S]methionine. (B) Labelled Vg in tissue and medium during the first hour of fat body culture in Grace's medium with [^{35}S]methionine. Sample uniformity was achieved by applying approx. 50 μg of protein to each well for SDS–PAGE. Autoradiograms obtained from SDS–PAGE were scanned and label incorporation into Vg was estimated by measuring the peak areas. The units of labelling are arbitrary and are expressed in the numbers measured by the polar planimeter. Fat body, dashed line; and haemolymph or medium, solid line.

Hormonal regulation of vitellogenin synthesis

In general, when immature *C. septempunctata* were fed an artificial diet the oocytes in most females developed only to the pre-vitellogenic stage and no Vg synthesis was observed. The ovaries of these individuals did not mature, no matter how long feeding was continued. However, topical application of the juvenile hormone analogues, ZR-512 or ZR-515, induced vitellogenesis in these non-fecund females. Vg synthesis in juvenile hormone-treated females was demonstrated both *in vivo* (data not shown) and in fat body culture (Fig. 8). Both *in vivo* and *in vitro* experiments showed that the fat bodies of male insects do not normally synthesize Vg (Fig.

*Occasionally, small differences in electrophoretic mobility appeared to distinguish Vn3 and Vg3 (cf. lanes 1 and 2 of Fig. 1A). But these differences were not always seen (cf. lanes 2 and 3 of Fig. 1B) so that their significance is unclear.

5), and juvenile hormone treatment of males failed to induce detectable Vg synthesis (Fig. 8).

DISCUSSION

The Vn isolated from eggs of *C. septempunctata* has an apparent mol.wt of approx. 4×10^5 which we have shown by denaturing gel analysis to be composed of four polypeptides of mol.wt = 133,000, 130,000, 46,000 and 43,000, respectively. Thus, the four polypeptides of *C. septempunctata* Vn fall into two size classes. This pattern fits that typical of most insect orders (Wyatt, 1980; Harnish and White, 1982). The quantity of the four polypeptides, as estimated from the intensity of their staining by Commassie blue, suggests that native Vn has the subunit structure, $ABCD_2$. The two larger polypeptides appear to be similar in sequence because they yield indistinguishable peptide maps upon partial proteolysis. By the same criterion, the two smaller polypeptides appear to have unique sequences.

The Vg precursor of Vn was readily detected in the haemolymph of mature females. In all respects this protein appears to have properties similar to those of Vn isolated from eggs. Indeed, we have thus far been unable to distinguish the two proteins. When subjected to denaturing gel electrophoresis, Vg yielded the same polypeptide pattern as Vn and the mobilities of the individual Vg and Vn polypeptides were indistinguishable. In addition, Vg was readily precipitated by Vn antibodies and the Vg polypeptides yielded peptide maps upon proteolysis which were indistinguishable from those of their Vn counterparts.

Most insect Vns are modified proteins containing covalently attached lipid and carbohydrate and proteolytic processing of Vg precursors has also been suggested (Hagedorn and Kunkel, 1979; Wyatt and Pan, 1978; Wyatt, 1980). The structural and functional significance of these modifications has not been elucidated, nor in most cases, has the cellular location of the modifying reactions been defined. In preliminary experiments, all four Vn polypeptides of *C. septempunctata* stained positively for carbohydrate and the two larger Vn polypeptides showed radiographic evidence for the presence of phosphorus when isolated from animals injected with [^{32}P]phosphate (Q. H. Zhai, unpublished observations). Because we have not been able to detect significant structural differences between Vg and Vn or observe any obvious precursor molecules, it would appear that the modifying reactions occur during or shortly after the assembly of Vg within the tissue(s) which produces it.

Vg synthesis in the fat body of mature females was readily detected in both the whole animal and in tissue culture. For example, when fat body cultures are incubated with [^{35}S]methionine, radiolabelled Vg rapidly appeared in the culture medium and this protein appeared to have the same characteristics as that found in the haemolymph of mature females. Thus, as with most insects (Engelmann, 1979), the fat body would appear to be the primary site of Vg production. However, as with *Drosophila* (Sridic *et al.*, 1979; Bownes, 1980; Jowett and Postlethwait, 1980; Postlethwait *et al.*, 1980), it would appear that *C. septempunctata* Vg is also produced in ovarian tissue. We have observed that ovaries, when carefully dissected to avoid contamination by fat body tissue, appear capable of incorporating [^{35}S]methionine into Vg in tissue culture. Most of the labelled Vg was found in the culture medium. From such labelling experiments, it is difficult to estimate the quantitative contribution of ovarian tissue to overall Vg production in the insect but under most circumstances this contribution is probably small. Early in development the mass of fat body tissue is very much greater than that of the ovary. Later in sexual maturation, ovarian production of Vg might become quantitatively significant. In *Drosophila*, Vg synthesis has been shown to occur in ovarian follicle cells (Brennan *et al.*, 1982). The site of Vg synthesis in ovaries of *C. septempunctata* has not been identified.

One of the major impediments to the large scale cultivation of *C. septempunctata* is the failure of the female to sexually mature when raised on artificial diets (Gong *et al.*, 1980). We have observed that very little Vg synthesis occurs in such females and that Vg synthesis can be induced in females but not in males by the topical application of juvenile hormone analogues. While this observation does not provide a practical solution to large-scale cultivation of the insect it does serve to implicate juvenile hormone as a controlling hormone in Vg production. In *Drosophila melanogaster* both juvenile hormone and ecdysterone have been shown to stimulate Vg synthesis in isolated female abdomens (Handler and Postlethwait, 1978; Postlethwait and Handler, 1979). The fat body responded to both hormones whereas Vg synthesis in the ovary was stimulated only by juvenile hormone (Postlethwait *et al.*, 1980). We have not yet performed comparable experiments on *C. septempunctata* with ecdysterone or attempted to hormonally stimulate isolated tissues. Nonetheless the present work demonstrates that the fat body is the primary site of Vg synthesis in *C. septempunctata* and suggests that juvenile hormone either directly or indirectly stimulates Vg synthesis in this organ.

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