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Occurrence of free ecdysteroids and yolk protein ecdysteroid conjugates in the embryo of the melon fly, *Dacus cucurbitae* Coquillet

Sanders, Deborra A., Ph.D.

University of Hawaii, 1990

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OCCURRENCE OF FREE ECDYSTEROIDS AND YOLK PROTEIN 
ECDYSTEROID CONJUGATES IN THE EMBRYO OF THE 
MELON FLY, DACUS CUCURBITAE COQUILLET 

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE 
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE 
REQUIREMENTS FOR THE DEGREE OF 

DOCTOR OF PHILOSOPHY 
IN 
ENTOMOLOGY 
AUGUST 1990 

By 
Deborra A. Sanders 

Dissertation Committee: 
Franklin Chang, Chairperson 
Marshal Johnson 
Wallace Mitchell 
Diane Ullman 
David Haymer
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and now it is...
ABSTRACT

The titer of ecdysteroids occurring in the embryos of the melon fly, *Dacus cucurbitae* Coquillet was determined at each developmental hour by radioimmunoassay. Free ecdysteroids were extracted from eggs in 60% methanol which showed one single peak at 18 h (i.e., time of germ band shortening). The maximal concentration of free ecdysteroids was 81 pg of 20 hydroxyecdysone equivalents per g. No observed morphological changes occurred during the 3 to 4 h peak hormone concentration. The involution of the head and the beginning of the dorsal closure of the midgut were observed during the declining concentration of free ecdysteroids. Embryonic cuticle was secreted ca. 5 to 6 h after the peak concentration of hormone. The major yolk protein (vitellin) was isolated and digested with several proteases to release ecdysteroid conjugates. The extracted conjugates became more immunopositive when hydrolyzed with *Helix* enzymes. Low amounts of ecdysteroid conjugates were detected in newly oviposited and young embryos. During late embryogenesis, ca. 18 h after oviposition, three peaks of ecdysteroid conjugates were detected. At 19 h, the first maximal concentration of 20 hydroxyecdysone equivalents was 85 ± 1.7 pg/ g vitellin. The second and third peaks occurred at 22 and 26 h with maximal concentrations of 20 hydroxyecdysone
equivalents $106.5 \pm 1.7$ and $102 \pm 2.3$ pg/g vitellin, respectively. The vitellin of the melon fly was composed of two polypeptides, having a molecular weight of 48,000 and 52,000 and an isoelectric point of 6.3.
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I. INTRODUCTION

The melon fly, *Dacus cucurbitae* Coquillet, is well established throughout the vegetable growing areas of the Hawaiian Islands. The origin of the melon fly occurs in the Indo-Malayan region (i.e., India, Philippine Islands, Ceylon, China, Japan) (Back and Pemberton 1919). Since its introduction to the Hawaiian Islands in 1897, its effect upon the production of cucurbits, tomatoes and many other vegetable crops has been devastating. In Hawaii, the melon fly oviposits in 36 fruit and vegetable varieties belonging to 12 plant families (Hardy 1949). The eggs are oviposited beneath the skin of the fruit which results in few available methods to control the melon fly during embryonic development. Elucidation of the presence of ecdysteroids during embryonic development may provide information on potential vulnerable periods during embryonic development.

Ecdysteroids, a generic term for molecules which compose the molting hormone, occurs in most insect embryos, ovaries and immature insects. In immature insects, ecdysteroids are biosynthesized by a prothoracic gland (ring or ventral glands) under the control of neurohormones (Mordue et al. 1980). The molting hormone is released into the hemolymph at certain periods of development to induce apolysis and ecdysis. Reproductively competent adult females synthesize ecdysteroids in ovaries, primarily in the follicular epithelium cells (Goltzene et al. 1978). The
ovarian ecdysteroids are passed from the epithelial cells into the ooplasm of the terminal oocyte. Comparatively, large amounts of ecdysteroids are deposited in the oocytes of insects (Hoffmann et al. 1985). Essentially all ovarian ecdysteroids synthesized are passed into the ooplasm but trace amounts are detected in the hemolymph and carcass tissue (Hoffmann et al. 1980).

Ovaries and eggs of various insect species contain large quantities of ecdysteroids in a conjugated form and trace amounts in a free active form (Hsiao and Hsiao 1979; Dinan and Rees 1981). Conjugates are formed by the ecdysteroids combining with sulfate or phosphate esters, glucuronides and glucosides (Koolman and Karlson 1985). The conjugated hormone is in a deactivated and stored form in the embryo. Most conjugates are highly polar with the exception of acetate esters (Hertu et al. 1985). Polar conjugates are extracted from crude egg homogenates with 25% methanol and water (v/v) and nonpolar conjugates with 100% methanol (Espig et al. 1989). Under in vitro conditions, the conjugate is removed with an aryl sulphatase (i.e., β-glucuronidase). The enzyme hydrolyzes the conjugate which liberates free ecdysteroids (Ohnishi et al. 1977). Conjugates of a lower polarity are more readily digestible with pig liver esterase (Dubendorfer and Maroy 1986).

Egg ecdysteroid conjugates of orthopterans are primarily phosphate esters such as, ecdysone 22-phosphate,
20-hydroxyecdysone 22-phosphate, 2-deoxyecdysone 22-phosphate and, 3 epi-2-deoxyecdysone 3-phosphate linked at the carbon 22 hydroxyl group of the hormone (Isaac et al. 1982; Isaac et al. 1983; Tsoupras et al. 1982). Thompson et al. (1985) identified 26-hydroxyecdysone 26-phosphate as the major conjugates in newly laid eggs of the tobacco hornworm, *Manduca sexta* (L.) (Fig. 1).

The occurrence of ecdysteroid conjugates during ovarian and embryonic development functions to regulate the hormone. Ecdysteroid conjugates occur at various concentrations in all stages of insects (Dubendorfer and Maroy 1986). However, conjugates in post embryonic stages will deactivate excess hormone but will not release active hormone (Koolman and Karlson 1985). In ovaries, the formation of conjugates promotes ecdysteroid synthesis by the removal of newly synthesized hormone (Isaac et al. 1983). During embryonic development, conjugates provide a source of ecdysteroids required during certain periods of embryogenesis (Koolman and Karlson 1985).

Ovarian and egg ecdysteroid conjugates are poorly immunoreactive and thus do not cross-react significantly with antisera raised against free ecdysteroids (Lagueux et al. 1984). The nonimmunopositive property is a result of the conjugate being tightly bound to the major yolk protein,
Fig 1. Conjugated ecdysteroids occurring during early embryogenesis of M. sexta embryo. a) 26 hydroxyecdysone 2-phosphate b) 26-hydroxyecdysone 26-phosphate c) 26-hydroxyecdysone 22-glucoside (Thompson et al. 1988)
vitellin (Lagueux et al. 1981). During embryonic development, the ecdysteroid conjugates are changed from poorly immunopositive to more immunopositive (Thompson et al. 1988). The change is caused by the use of the yolk protein by the embryo, causing the hormone to be more accessible (Bownes et al. 1988). The change of conjugates is linked to the end of the first and major phase of vitellin degradation in *Locusta migratoria* (L.) embryos (Lagueux et al. 1984).

The precursor to vitellin, vitellogenin, is synthesized in the fat body of adult females and transported via the hemolymph to the ovaries. It is then sequestered within the oocytes through receptor-mediated endocytosis (Engelman 1979) and packaged within yolk spheres (Telfer et al. 1982). It is undetermined if binding of ecdysteroid conjugates to vitellin occurs in the interfolliculo-oocytic space prior to incorporation of protein into the oocyte, or at a later phase of oocyte development (Hoffmann and Lagueux 1985).

Among the yolk proteins, vitellin is the predominate component, comprising 60-95% of total protein within insect eggs (Yamashita and Indrasith 1988; Engelman 1979). Vitellin of higher diptera have similar structure and polypeptide composition. Generally, vitellin isolated from diptera has a molecular weight \( m_r \) of 200,000 to 300,000. The number of small polypeptides of vitellin range from 1 to 5 with a \( m_r \) approximately of 40,000 to 54,000 (Huybrechts 5
and DeLoof 1982; Rina and Mintzas 1987; Levedakau and Sekeris 1987; Handler and Shirk 1988; Bownes and Hames 1977; DeBlanchi et al. 1985). The vitellin polypeptides isolated from *Dacus oleae* (Gmelin) was observed having an isoelectric point covering a narrow acidic range of pH 5.7 to 6.2 (Levedakou and Sekeris 1987).

Ecdysteroid conjugates bound to vitellin compose about 95% of ecdysteroids available to the embryo (Lageuex et al. 1984). It is assumed that release of free ecdysteroid controls embryonic cuticle formation in embryos. Hagett (1977) speculated that cycles of embryonic cuticulogenesis was controlled by the neuroendocrine system similar to that of immature insects. In contrast, Mueller (1963) had shown that under in vitro conditions, embryonic tissue containing a yolk supply and severed from head or thorax, would undergo cuticulogenesis. This research showed that the embryo could incorporate ecdysteroids maternally and perhaps synthesize them independently (i.e. without neuroendocrine control).

During the embryogenesis of *L. migratoria*, four distinct peaks of free ecdysteroids were detected which corresponded with events of embryonic cuticulogenesis (Lagueux et al. 1979). The first ecdysteroid peak occurred concomitantly with the onset of the deposition of the serosal cuticle (i.e., chitinous endocuticle). The latter three ecdysteroid peaks each occurred concomitantly with apolysis of the cuticle from the previous cycle of
cuticulogenesis, with the last embryonic cuticle functioning as the first-instar larval cuticle. Most orthopterans, lepidopterans, and hemipterans form three cuticles (excluding the serosa) during embryogenesis (Mueller 1963; Mueller and Bucklin 1965; Sharan and Shari 1960; Louvet 1974; Fournier and Radallah 1988). Embryos of the dipteran species *Calliphora erythrocephala* Macquart and *Drosophila melanogaster* Meigen secrete one cuticle after completion of the dorsal closure (Bordes-Alleaume and Sami 1987; Maroy et al. 1988). The onset of embryonic cuticulogenesis corresponds with a single peak of ecdysone (Wentworth and Roberts 1984; Garen et al. 1977).

Free ecdysteroid peak(s) are composed predominantly of ecdysone and to a lesser extent 20-hydroxyecdysone and 20,26-dihydroxyecdysone. The major function of ecdysone and 20-hydroxyecdysone during embryogenesis is speculated to be similar to the action of the molting hormone during post embryonic development (Koolman and Karlson 1985).

The origin of ecdysone and 20 hydroxyecdysone during development may or may not be limited to the hydrolysis of ecdysteroid conjugates. In some insects species (e.g., *L. migratoria*, *Schistocerca gregaria* Stal, *Bombyx mori* (L.)) it has been shown that the hydrolysis of conjugates could be responsible for the appearance of free ecdysteroid peaks in embryos (Hoffmann et al. 1985). Several species such as *C. erythrocephala* and *Pieris brassicae* (L.) have not been shown
to possess ecdysteroid conjugates (Beydon et al. 1989). However, ecdysteroid conjugates could exist having different physicochemical characteristics.

In embryos of *L. migratoria*, at least the first two peaks are maternal in origin but the last two peaks are presumed embryonic (i.e., prothoracic gland) (Lagueux et al. 1979). Late staged embryos are thought to be capable of synthesizing ecdysteroids *de novo* from cholesterol. However, this would require a functioning embryonic prothoracic gland or another type of ecdysteroid synthesizing tissue (Espig et al. 1989; Hoffmann and Lagueux 1985). *De novo* synthesis of ecdysteroids was demonstrated when radiolabelled cholesterol was injected into early staged embryos of *P. brassicae*. The radioactive material was incorporated into the extracted ecdysteroids at increasing rates during development (Beydon et al. 1989). It is assumed that *de novo* synthesis is the primary source of ecdysteroids during development because conjugates have not been isolated from this species.

In Diptera, conjugates are thought to be the prime source of ecdysteroids during embryonic development. When embryos of *C. erythrocephala* are mid-ligatured at the blastula, gastrulation or germ band extension the posterior embryonic half secretes cuticle (Bordes-Alleaume and Sami 1987). This indicates that cuticulogenesis of higher Diptera may not be dependent on the presence of an embryonic
ring gland. Difficulty still exists in determining the source of ecdysteroids occurring during embryonic development of certain insect species.

The objectives of this project are to (1) correlate ecdysteroid titer with events occurring during *D. cucurbitae* embryonic development; (2) correlate the concentration of protein (vitellin) associated ecdysteroid conjugates to events in embryonic development; and (3) characterize vitellin (i.e., molecular weight and isoelectric point).
II. MATERIAL AND METHODS

Melon fly eggs used in all experiments were obtained from the mass rearing facilities of the USDA Tropical Fruit and Vegetable Research Laboratory in Honolulu. The melon flies were allowed to oviposit in eggers (egg collecting devices) for one h. Eggs were rinsed and placed in Petri plates containing agar and were subjected to an average laboratory temperature of 25°C. At every hour, the desired amount of eggs were collected and frozen (-20°C) until needed.

A. Embryogenesis.

Wax Histology of Developing Embryos. Embryos were prepared for histology according to the method of Wieschaus and Nusslein-Volhard (1986), with minor modifications. About 500 mg of desired staged embryos were dechorionated for 5 min in 5 ml undiluted sodium hypochlorite, then rapidly rinsed in distilled water. Embryonic tissue was fixed by shaking embryos in 5 ml of the upper phase of a fixation solution (55% heptane, 10% formaldehyde and 35% ethanol) for 5 min. Fixed embryos were devitellinized by adding one ml of -20°C chilled methanol then rapidly swirled under warm tap water. To dehydrate the embryos, the fixation solution was removed and 10 ml methanol was added. After 30 min, methanol was replaced with toluene and cleared overnight.
Embryos were gradually warmed in toluene at $58^\circ$ C, for 30 min. Melted Paraplast wax (Sherwood Medical, St. Louis, MO) twice the volume of toluene was added to the embryos. The wax was changed five times during warming for 4 h. The wax infiltrated embryos were cooled in small plastic molds on ice. Wax blocks were sectioned on a microtome (American Optical, Buffalo, NY) at 10 microns. Sections were transferred to glass microscope slides coated with an adhesive, Tissue Tac (American Hospital Supply Corp., Miami, Fl) and dried overnight. Slides were mordant in 3% potassium dichromate for 24 h, to enhance staining with azocarmine G.

Embedded eggs were stained by a modified Azan method described by Humason (1979). The slides were deparaffinized twice for 5 min in xylene and hydrated through a series of ethanol dilutions (100%, 95%, 70%, 50% and distilled water) for 3 min. The tissue was stained for 30 s in 0.1% azocarmine G (Sigma Chem. Co., St. Louis, MO) (C.I. 50085) and rinsed with distilled water. The tissue was differentiated in a 0.1% aniline alcohol solution, until cells were visible under the microscope. Differentiation was stopped with 1% glacial acetic acid. Slides were mordant in a 5% phosphotungstic acid for 60 min and washed rapidly in distilled water. The sections were counterstained in 0.25% aniline blue W.S. (Sigma, C.I. 42780), 1% orange G (Sigma, C.I. 16230) and 4% acetic acid,
for 2 h. Sections were differentiated in 95% ethanol, passed through absolute alcohol and xylene. Slides were mounted with Pro-texx (American Scientific Products, McGraw Park, IL) and dried at laboratory temperature (25°C). The microphotographs of the developing embryos were taken with a Zeiss photomicroscope Model III (Carl Zeiss, West Germany).

B. Vitellin Isolation and Identification.

**Crude Egg Protein Extraction.** About 500 mg of the desired staged embryos were homogenized in 1 ml protein buffer (50 mM Tris-Cl, pH 7.5, 5 mM MgCl₂, 10 mM dithiothreitol (DTT), 1 mM phenylmethyl sulfonylfluoride (PMSF), 5 mM thiourea) on ice. The homogenate was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was diluted twice the volume with protein buffer and filtered through a 2 micron membrane. Crude egg protein was stored at -20°C until needed.

**Vitellin Extraction.** The isolation of vitellin was accomplished according to the method of Levedakou and Sekeris (1987). About 500 mg of the desired staged embryos were pulse-sonicated in a sonicator cell disruptor (Heat Systems-Utrasonics, Inc. Model W 200R, Plainview, NY), for 90 s in 50 μl of extraction buffer (20 mM sodium phosphate buffer, pH 6.0, containing 1 mM DTT, 0.1 mM PMSF and 0.4 M NaCl) on ice. The sonicated product was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was diluted twice its volume with 0.1 mM PMSF in distilled water.
After 60 min at 0\(^\circ\) C, the supernatant was centrifuged at 15,000 g for 15 min at 4\(^\circ\) C. The pellet containing the vitellin was suspended in 250 \(\mu\)l of extraction buffer and centrifuged at 15,000 g for 15 min at 4\(^\circ\) C. The final supernatant containing the vitellin was stored at -20\(^\circ\) C.

**Adult Hemolymph Extraction.** Hemolymph was collected by puncturing the thoracic area of the adult fly. A few crystals of glutathione (Sigma Chem. Co.) were added to pooled hemolymph to prevent melanization. The hemolymph was diluted twice its volume with 0.01 M phosphate buffered saline (0.01 M sodium phosphate in 0.85% NaCl, pH 7.8) and centrifuged at 15,000 g for 15 min and 4\(^\circ\) C. The supernatant was stored at -20\(^\circ\) C.

**Estimation of Protein Concentration.** Determination of protein concentration was performed by the bicinchoninic acid assay as described by Smith et al. (1985). Fifty parts of bicinchoninic acid (Sigma Chem. Co.) was combined with one part copper sulfate pentahydrate (Sigma Chem. Co.). Concentrations of bovine serum albumin between 20 to 200 \(\mu\)g were used as the protein standard. Absorbance was determined using a cuvette of 0.5 cm light path and Model 26 Beckman Spectrophotometer.

**Sample Preparation for Electrophoresis.** Equal parts of isolated vitellin or hemolymph and 2x treatment buffer (0.125 M tris Cl, pH 6.8, 4% sodium dodecyl sulfate (SDS),...
20% glycerol and 10% 2-mercaptoethanol) were boiled in water for 90 s. Samples were stored at -20\degree C.

**Molecular Weight Determination.** The molecular weight of vitellin peptides was determined on a 12% SDS polyacrylamide slab gel according to Laemmli (1970). Standard molecular weight markers used to plot the standard curve were myosin, B-galactosidase, phosphorylase, bovine plasma albumin, egg albumin and carbonic anhydrase. The gel was carried out at 300 volts for 4.5 h at 9\degree C. After electrophoresis, the gel was stained for 3 h with 0.125% (w/v) Coomassie blue (Sigma). After destaining in 50% methanol and 5% acetic acid, the polypeptide bands were visible. The bands were compared with the protein markers and the molecular weight determined.

**Vitellin Polypeptide Electrophoresis.** The vitellin polypeptide subunits were electrophoresed with the mini protean II dual slab cell (BioRad, Richmond, CA). The vitellin was electrophoresed on a 8-9 cm long 12% polyacrylamide gel with a 1 cm 4% stacking gel. Separation of the proteins were carried out at 200 volts for 50 min at 24\degree C. Gel was stained and destained as described in the molecular weight determination.

**Isoelectric Focusing.** The vitellin subunits were focused on Servalyt precote (3/10) (Serva, Westbury, NY) gels which were cut to a dimension of 125 x 70 mm. The vitellin was diluted in 1% glycine, to make a final protein
concentration of 5 μg/μl. A template was used to apply 2 μl of either the protein standard mix (Serva, protein standard 9) or vitellin. The samples were allowed to diffuse into the gel for 5 min and the template was removed. The gel was focused on a Model 111 Mini IEF cell (BioRad) at 24°C. The focusing was carried out under constant voltage conditions in a step-wise manner. Focusing was begun at 100 volts for 15 min, then increased to 200 volts for 15 min. The final voltage was increased to 450 volts for an additional 30 min. The gel was removed from the graphite electrodes and fixed in 20% trichloroacetic acid for overnight. The gel was stained (27% ethanol, 10% acetic acid, 0.04% coomassie blue and 0.5% copper sulfate) for 3 h. The gel was destained in 25% ethanol and 7% acetic acid for 2 h and air dried overnight. The isoelectric point of vitellin was determined from a standard curve by plotting the relative mobility (R_f) of the protein standard.

C. Determination of Ecdysteroid Titer.

Free Ecdysteroid Extract. About 250 mg of staged embryos were sonicated in 250 μl of 60% methanol with water (v/v). The embryo sonicate was centrifuged at 12,000 g for 15 min at 4°C. The supernatant was diluted to 500 μl with 0.166 M borate buffer (containing 0.19 M NaOH and 0.02 M NaCl, pH 8.2).
Enzymatic Hydrolysis of Vitellin and Conjugates.

Isolated vitellin (300 μg) from staged embryos was digested in an equal amount of a protease mixture. The protease mixture contained type XXI protease from *Streptomyces griseus* (4.5 mg/ml), protease type IV from *Streptomyces caespitosus* (1 mg/ml) and trypsin type III from bovine pancreas (1 mg/ml) (all from Sigma). All samples were digested in 200 μl of 50 mM sodium acetate, pH 5.3 and 100 ul of protease mixture (300 μg). Glucuronidase (1 mg/ml), isolated from Helix pomatia was added to the digestion solution to hydrolyse the conjugate on the ecdysteroid molecule. Experimental samples and controls were mixed and incubated overnight at 37°C. About 200 μl methanol was added and centrifuged at 12,000 g for 10 min at 4°C. The volume of the sample was reduced to 300 μl by nitrogen flow.

Titration of the Antiserum for the Radioimmunoassay.

The concentration of antiserum (H-22) required for 50% binding of the radiolabelled ecdysone was performed according to a modified procedure by Warren and Gilbert (1988). The H-22 ecdysteroid antiserum (received from L. Gilbert, University of North Carolina, Chapel Hill, NC) which was formed against ecdysone 22 succinyate throglobulin conjugate recognizes ecdysone, 20-hydroxyecdysone and or 26 hydroxyecdysone metabolites (Warren and Gilbert 1988). Ten mg of lyophilized H-22 antiserum was diluted into 1 ml of
0.166 M borate buffer, pH 8.2. The antiserum was diluted as follows: 1:5, 1:10, 1:50, 1:100, 1:150, 1:200 and 1:400. The radioactive tracer, \[^3\text{H}\]ecdysone (specific activity, 89 Ci/m mole, New England Nuclear) was first diluted in methanol and further diluted by 0.166 M borate buffer, pH 8.2. Each 50 μl solution had an average of 4,500 dpm (disintegrations per min). The antiserum dilutions (100 μl) were mixed with 4,500 dpm of \[^3\text{H}\]ecdysone in disposable 10 x 75 mm glass tubes. Normal rabbit serum (Calbiochem, San Diego, CA) diluted to a concentration of 1:10 in 0.166 M borate buffer was the precipitation agent used in the assay. One ml of 1:10 normal rabbit serum (NRS) was incubated with the diluted antiserum and radioactive tracer. The blank (control) contained NRS and 4,500 dpm of tracer. Each tube was vortexed and incubated at 4°C for 24 h.

The separation of bound from unbound radioactive tracer was achieved by a modification of the method of Farr (1971). The bound radiolabelled ecdysone was precipitated by adding 1 ml ammonium sulfate (saturated at 0°C) to each tube and incubated at 4°C for 30 min. The tubes were centrifuged at 2,500 g for 30 min at 2°C. The supernatant was removed and the pellet redissolved in 1 ml 50% ammonium sulfate solution (1:1 saturated ammonium sulfate and 0.166 M borate buffer pH 8.2 and chilled at 4°C). The pellet was washed twice with 50% ammonium sulfate solution to reduce non-specific binding.
below 10%. After each wash, the dissolved pellet was centrifuged at 2,500 g for 30 min at 2°C. The final pellet was dissolved with 200 μl of water and transferred into 15 ml Scintiverse II (Fisher Scientific, Fair Lawn, NJ) scintillation fluid. The pellet was counted for 10 min in a Beckman 8100 scintillation spectrometer. The results of the titration experiment were expressed as % of total tracer bound vs. percent of antiserum dilution (Fig. 2). The 50% binding of antiserum to the [3H] ecdysone occurred at 1:150 fold dilution. This was the dilution of antiserum used for the standard curve and unknown samples.

Assay. The radioimmunoassay was conducted similar to that described earlier in the antiserum titration section. [3H] ecdysone (4,5000 dpm) and 100 μl of 1:150 diluted antiserum were mixed with 200 μl of 1:10 NRS in 10 x 75mm glass tubes. The ecdysteroid standard curve was determined by adding 20 μl aliquots of an unlabelled 20-hydroxyecdysone solution, diluted in 0.166 M borate buffer, pH 8.2, to the antiserum. A concentration range of 6 to 25 ng of 20-hydroxyecdysone was used to plot the standard curve. Aliquots of the extracted residue (20 μl) or enzymatically hydrolyzed vitellin (20 μl) replaced the standard 20-hydroxyecdysone in other tubes. Maximum binding (zero standard or B₀) tubes contained only tracer, antiserum and 1:10 NRS which was included in each assay. The separation of bound from free radioactive tracer and processing of the
Fig. 2. Antibody dilution titer for H-22 antiserum. The antiserum dilution of 0.67% (1:150 fold dilution) was used in the radioimmunoassay.
pellet was accomplished in the same manner as that described for the antiserum titration.

The raw data (cpm) was analyzed by subtracting the blank (blk) from all antiserum-tracer bound (B) counts. The bound (B) counts were normalized to the maximum binding ($B_o$) of the antiserum (i.e., $B - Blk/ B_o - Blk \times 100$ ($\% B/B_o$)). A linear standard curve resulted from plotting $\% B/B_o$ vs. the concentration of 20-hydroxyecdysone. The unknown sample concentrations were obtained by interpolation. The unknown sample concentration values are expressed in 20-hydroxyecdysone equivalents because of its use in plotting the standard curve.
III. RESULTS

A. Embryogenesis.

A D. cucurbitae embryo required 27 to 28 h to develop from oviposition to eclosion at 25°C. A newly oviposited egg was approximately 1.2 - 1.25 mm in length with a diameter of 0.3 mm. The anterior region of the egg formed a pointed projection while the posterior was slightly rounded. The chorion was white and marked by regular hexagonal ridges (Figs. 3 and 4). Lying below the chorion was the vitelline membrane which was translucent and smooth. The vitelline membrane had hydrophobic properties which excluded the embryo from the aqueous exterior environment.

The duration of the developmental stages are means taken from several embryos. Therefore, the duration of a stage is an approximation, due to the difficulty of identifying the beginning and end of a developmental stage.

A newly oviposited egg was characterized by having a homogeneous cytoplasm (Fig. 5). Vacuoles were often visible and scattered at random in the cytoplasm. The periplasm, a thin layer of yolk-free cytoplasm, surrounded the periphery of the egg ca. 60 min after oviposition. The periplasm thickened at the anterior and posterior poles by the end of the first hour of development.
Fig. 3. The chorion, the outer egg shell of the melon fly embryo (6.3x).
Fig. 4. The hexagonal ridges of the chorion (10x).
Fig. 5. A newly oviposited egg, characterized by large vacuoles in the homogeneous cytoplasm (6.3x).
After 2 h, the cleavage nuclei (=cleavage energids) became visible migrating from the cytoplasm into the periplasm. The nuclei formed a cellular blastoderm which enclosed the central yolk mass. During the third hour of development, the number of visible nuclei in the periplasm greatly increased and became tightly packed in the periplasm (Fig. 6). During this time frame, pole cells formed at the posterior cap. The pole cells were located above the plasma membrane and were held in place by the vitelline membrane. Due to the removal of the vitelline membrane during preparation of the embryos, pole cells were sometimes not visible in histological sections.

During the 4th h, nuclei became spherical in a single layer at the periphery of the egg (Fig. 7). The surface layer at this point was termed a syncytial blastoderm. The following hour of development is marked by the transformation of the syncytial blastoderm into a cellular blastoderm (Fig. 8). Cellularization of the blastoderm was fairly rapid, requiring about one h to complete. After cellularization, blastoderm cells became elongated and columnar-shaped.
Fig. 6. A sagittal section of a three h embryo, illustrates the densely packed nuclei in the periplasm. At the posterior pole (right), pole cells are visible above the plasma membrane (10x).
Fig. 7. During the 4th h, the nuclei become spherical and occupy a single layer in the periplasm. The surface layer of the egg is referred as a syncytial blastoderm (6.3x).
Fig. 8. A sagittal section of a 5 h embryo after completing cellularization of the the blastoderm (6.3x).
Formation of the blastoderm resulted in a differentiated central yolk mass which contained yolk spheres of different sizes and color. Some yolk spheres were intensely stained with azocarmine and others with orange G (Fig. 9). However, differences in yolk spheres may have been due to artifacts from preparation of the embryos for sectioning.

Gastrulation (i.e., formation of germ layers by gradual invagination) began about 6 to 7 h after oviposition. Between 5 to 6 h, two conspicuous infoldings of the blastoderm appeared in the surface of the embryo. At 5 h, the cephalic fold (furrow) appeared as a deep lateral infolding of the blastoderm at the cephalic region. The second infolding, the ventral longitudinal furrow, appeared slightly after the formation of the cephalic furrow. The ventral furrow formed midventrally in the blastoderm. The cell thickness of the furrow at the anterior and posterior poles was about 1 to 2 cells deep. However, transverse histological sections through the midline showed that this region was more than 4 cells deep. At 6 h, the ventral furrow closes and forms a tubular mesodermal cavity (Fig. 10). The mesodermal cells will then change from a columnar to a cuboidal shape. The ectodermal layer is now known as a differentiation blastoderm.
Fig. 9. Different sized yolk spheres are dispersed in the central yolk mass of the embryo. Some yolk spheres take on an intense stain with azocarmine and others with orange G (40x).
Fig. 10. A sagittal section of a 6 h embryo completing the ventral furrow (bottom) which forms the mesodermal cavity (6.3x).
About 7 to 8 h, the amnioproctodeal invagination (i.e., posterior and anterior midgut primoridia) were formed by the inward movement of the germ band (=embryonic primordium) into the yolk mass. This invagination formed at the anterior region of the ventral furrow. The pole cells migrated toward and sunk into the amnioproctodeal invagination. By 10 h, the invagination extended farther into the yolk mass. The pole cells (i.e., gonad germ cells) started to migrate within the midgut primordium. The pole cells came to lie in the midgut lumen near the tip of the invagination (Fig. 11). Shortly after, the pole cells disappeared from view.

The germ band rapidly elongated during the formation of the two furrows and the fore and hindgut primordia. By the 12th h of development, the beginning of the slow phase of germ band elongation, the amnioserosa and the stomodeum (foregut) invagination, appeared. The amnioserosa (i.e., extraembryonic membrane) appeared anterior to the amnioproctodeal invagination as a narrow strip of cells derived from the germ band (Fig. 12). The extraembryonic membrane later unfolded and separated the exposed yolk mass from the vitelline membrane. The stomodeum invagination first appeared as a shallow gap (cell plate) at the anterior ventral tip of the embryo (Fig. 13). During the next few hours, the stomodeum invagination lengthens, forming the primordia of the foregut (Figs. 14 and 15). At this stage,
Fig. 11. At 10 h the pole cells are observed lying in the midgut primoridium separated from the yolk mass and the mesodermal cavity (40x).
Fig. 12. A sagittal section of a 12 hour embryo during germ band extension. The amnioserosa, the so-called extraembryonic membrane, appears anterior to the invagination (top left) (6.3x).
Fig. 13. The stomodeum first appears as a shallow gap at the anterior ventral tip of the embryo (bottom left). Segmentation of the ectoderm of the trunk region becomes defined (6.3x).
Fig. 14. About 15 to 16 h, the stomodeum invagination (top left) is clearly visible forming the foregut primoridium. The invagination of the posterior midgut primoridium (lower right) is also visible (40x).
Fig. 15. A sagittal section of an 18 h embryo, showing the stomodeum invagination (top left) and the posterior midgut invagination (bottom right) (6.3x).
the yolk mass showed a characteristic hook-shape form in the anterior region of the head. During the stomodeum invagination, the ectodermal segmentation of the trunk region of the embryo became clearly visible.

The continuous invagination of the stomodeum in the anterior region formed the gnathocephalon region. The procephalic lobe was found most anterior, followed by the mandibular, maxillary and labial buds (Fig. 16). These structures were visible on either side of the embryo ca. 15 to 16 h after oviposition.

The germ band began to shorten about 19 to 20 h, at which time the embryo took on a morphological similarity to the larval stage. At about 20 h, the contrast between cell layers and yolk became more difficult to distinguish. The ectoderm and the mesoderm of the germ band region differentiated into two clearly defined regions (Fig. 17). The yolk was eventually squeezed out of the anterior region and confined to the gut. Both the anterior and posterior midgut primordia become morphologically distinguishable from the foregut and the hindgut. At about 21 to 22 h, the anterior and posterior midgut primordia fused to form a concave-shaped yolk region (Fig. 18). After fusing, the buccal cavity and esophagus differentiated from the foregut.

Larvae of cyclorraphous Diptera are characteristically acephalic and apodous. The absence of an external larval
Fig. 16. A parasagittal section of a 15 h embryo, illustrating the segments of the gnathocephalon, which are the mandibular, maxillary and the labial buds (bottom left). The rounded cephalic region, the procephalic lobe, is visible (left) (6.3x).
Fig. 17. A horizontal section of a 20 h embryo, illustrating the differentiation of the ectoderm and the mesoderm of the germ band region into two cell layers (6.3x).
Fig. 18. A sagittal section of a 21 to 22 h embryo after the anterior and posterior midgut primordia has fused. The differentiation of the buccal cavity and the esophagus becomes visible at this hour of development (6.3x).
head is a result of the involution of the head region during embryonic development (Campos-Ortega and Hartenstein 1985). Approximately 20 to 21 h, the involution of the head was observed as the procephalic lobe was squeezed backward. This movement caused the formation of the pharynx and frontal sac. At the same time, the proventriculus (cardiac valve) formed at the junction of the fore and midgut (Fig. 19). The proventriculus was cone-shaped with the extremity of the foregut inserted into the base of the cone. The midgut was characteristically heart-like in shape at this developmental stage. The pharynx and esophagus were clearly defined within the foregut.

Within 22 h, the dorsal closure of the posterior midgut primordium began to seal the exposed yolk mass. The cells that formed the closure, covered the open yolk mass and sealed it. During this period, the protodeum reached its most anterior position and the anal slit became visible.

About 23 to 24 h and after the dorsal closure and the involution of the head were completed, the embryonic cuticle was secreted (Fig. 20). A secretion of cuticle was observed lining the ectodermis of the fore and hindgut. The lumen of the esophagus displayed secreted cuticle, but not the mesodermally-derived epithelium of the midgut.

The last major event was the conversion of the previous sac-like gut into convoluted tube. The gut in the last hours of development had three constrictions often appearing
Fig. 19. A sagittal section of a 23 to 24 h embryo, illustrating the proventriculus (cone-shaped) with the midgut inserted into the base of the cone (6.3x).
Fig. 20. A horizontal section of a 24 h embryo showing the embryonic cuticle (transparent blue) on the ectoderm and the esophagous region (40x).
as circles in the gut area (Fig. 21). The pharyngeal ridges and larval pattern of pharynx somatic musculature were well developed. During the final hour of development, the mouth hooks appeared and are quickly sclerotized (Fig. 22).

In living embryos, muscular activity was observed through the chorion prior to larval emergence. Waves of muscle contraction were visible moving forward along the trunk as the mouth hooks repeatedly protruded and withdrew. As development neared completion, activity increased, resulting in the rupture of the two outer membranes by the mouth hooks. The larvae escaped through longitudinal hatching lines (lines of weakness) of the chorion.

B. Vitellin Isolation and Identification.

1. Isolation.

The vitellin extraction method developed by Levedakou and Sekeris (1987) was the preferred procedure to isolate vitellin from embryos. This method permitted the extraction of vitellin from small egg samples (500 mg) and resulted in a relatively pure vitellin in a workable concentration. Several other vitellin extraction methods were explored such as, ammonium sulfate precipitation and protein electroelution from SDS polyacrylamide gel. Electroelution of vitellin subunits from polyacrylamide gels were often degraded during elution. This was a result of SDS, a heat absorber being omitted from the elution buffer because of its possible interference in antibody/antigen binding.
Fig. 21. A sagittal section of a 27 to 28 h fully developed embryo (6.3x).
Fig. 22. A sclerotized mouth hook of a 28 h embryo. Embryonic cuticle (transparent blue) is visible lining the ectoderm (40x).
in the radioimmunoassay. Both methods were considered labor intensive and often resulted in a low unusable concentration of vitellin.

In this procedure, *D. cucurbitae* eggs were sonicated rather than homogenized to extract the vitellin. Homogenization of the egg samples resulted in more contaminates and less of the vitellin in the final supernatant. Fig. 23 shows the electrophoretic profile of vitellin extracted on SDS polyacrylamide gel, using the method of Levedakou and Sekeris (1987). A marked loss of vitellin occurred during various steps of purification. The first centrifuge removed primarily cellular debris. The supernatant of the first centrifuge was similar to the protein bands of the crude egg extract (Fig. 23, lanes 3 and 4). Most egg proteins were precipitated in the second supernatant (Fig. 23, lanes 5 and 6). However, when the pellet was resuspended to the initial ionic strength, the vitellin was solublized, while the protein contaminates were precipitated (Fig. 23, lanes 7 to 9). The final vitellin extract did contain traces of other egg proteins. The purified vitellin from various developmental hours were electrophoresed on 12% SDS polyacrylamide gel. The isolated vitellin was estimated to have 5 to 10% of other egg proteins. This percentage of contaminates was considered to be acceptable.
Fig. 23. SDS polyacrylamide gel of yolk fractions during purification. Sonicated egg fractions (lanes 4-9) compared with lanes 1 and 2, male and female hemolymph (12 ug) and lane 3, crude egg extract. Lane 4, supernatant, after the first centrifugation. Lane 5, supernatant after the second centrifugation and the resulting pellet (lane 6). Lane 7, final pellet containing traces of vitellin and egg contaminates. Lanes 8 and 9, the isolated vitellin.
2. Identification.

Fig. 24 (lanes 1 - 10) shows the electrophoretic profiles of male hemolymph, female hemolymph, crude egg extract and isolated vitellin of *D. cucurbitae* on 12% SDS polyacrylamide gel. The two female specific polypeptides were identical to that of the two polypeptides in the crude egg extract and the isolated vitellin samples. Since these two major bands do not appear in male hemolymph protein (Fig. 24, lane 1) the bands were concluded to be vitellogenin and vitellin subunits, respectively. The molecular weight of subunit 1 and subunit 2 were estimated to be 48,000 and 52,000, respectively (Figs. 25 and 26). The isoelectric point of vitellin was determined to occur in an acidic region of 6.3 (Figs. 27 and 28).

C. Free Ecdysteroids and Vitellin Conjugated Ecdysteroid Titers.

1. Free Ecdysteroids.

Extraction of free ecdysteroids from developing embryos was accomplished by using 60% methanol in water (v/v). Changing the polarity of the extraction solution by increasing or decreasing the percent of methanol resulted in solubilization of apolar or polar ecdysteroid conjugates. A low concentration of soluble polar conjugates were thought to be extracted with the free ecdysteroids. Polar ecdysteroid conjugates in other insects
Fig. 24. Polypeptides from embryos of various developmental hours resolved on 12% SDS polyacrylamide gel (lanes 4 - 10). For comparison, lane 1 male hemolymph, lane 2 female hemolymph and lane 3 crude egg extract.
Fig. 25. Electrophoresis on polyacrylamide gel (12%) of standard molecular weight proteins (lane 6), crude egg extract (lanes 1 and 2), male hemolymph (lane 3) and female hemolymph (lanes 4 and 5).
Fig. 26. Molecular weight determination of *Dacus cucurbitae* vitellin subunits by gel electrophoresis. The arrows indicate the mobility of purified vitellin (50 μg). The subunits are: a = 48,000 and b = 52,000.
Fig. 27. Isoelectric focusing of *Dacus cucurbitae* vitellin on premade Serva gel. Vitellin (left) is compared with the isoelectric point of known standards (right). The acid pH is located on top and basic is on bottom of gel.
Fig. 28. Isoelectric point determination of *Dacus cucurbitae* vitellin by gel electrophoresis. The arrow indicates the isoelectric point of vitellin (10 μg) at 6.3.
species were found nonimmunopositive in the radioimmunoassay. However, uncertainty existed in the nonspecific binding of the ecdysteroid conjugates to the antiserum.

The analysis was conducted on two independent egg collections. The results were averaged and plotted as pg of 20 hydroxyecdysone (20-OH-ecdysone) equivalents per g fresh egg weight. The observed changes in the free ecdysteroid titer in developing embryos, as estimated by radioimmunoassay, is shown in Fig. 29. The measurement of the extracted ecdysteroids from developing embryos between 2 to 16 hours showed a very low ecdysteroid level. At these hours, the valves from the radioimmunoassay resulted from nonspecific background binding of the antiserum. Therefore, these values represent very low or zero ecdysteroid titer.

A single concentration peak of ecdysteroids became observable in the developing Dacus embryo prior to the germ band shortening. A conspicuous rise in free ecdysteroid titer began after the 16th hour of development. The maximal ecdysteroid peak occurred at 18 h representing a concentration of 20-OH-ecdysone equivalents of 8.1 ± 0.23 pg/g eggs. No observed morphological changes occurred
Fig. 29. Ecdysteroid titer occurring in developing embryos of *Dacus cucurbitae*. Results are expressed in 20 hydroxyecdysone (pg) per g wet weight. Each data point is the mean of 4 replications (means + SE).
during the 3 to 4 h of peak hormone concentration. However, two morphological events were observed during the declining concentration of free ecdysteroids. These events were the involution of the head and the dorsal closure of the midgut primordium. Both events began approximately in the same time frame.

A single embryonic cuticle appeared approximately 23 to 24 h after oviposition. This event occurred about 4 to 5 h after the appearance of the ecdysteroid peak. The ecdysteroid concentration dropped to zero during the completion of the dorsal closure and the secretion of the embryonic cuticle. Although Fig 29 indicates that a minimal concentration of ecdysteroids exist, these values may be a result of nonspecific background binding.

A slight increase of ecdysteroid concentration was observed two hours prior to eclosion. A titer of 20-OH-ecdysone equivalents of 2.4 ± 1.0 and 2.9 ± 0.5 pg/g were observed at 27 and 28 h, respectively. The appearance of embryonic mouthhooks appear during the final 2 h of embryonic development and become sclerotized before eclosion.

2. Ecdysteroid-Vitellin Conjugates.

Vitellin (300 μg) isolated from various developmental time periods, was digested with a protease mixture. The concentration of vitellin was determined before and after digestion to monitor the protease activity. The efficiency
of vitellin digestion by the protease was estimated to be ca. 92\%.

The supernatant of the vitellin digest after centrifugation was assayed for free ecdysteroids, but only a trace amount of immunopositive material was found. *Helix* enzymes (1 mg/ml) containing sulfatase and phosphatase activities, were incubated with the vitellin-protease mixture overnight at 37\°C, in order to hydrolyse the conjugates. *Helix* enzyme preparations often contain traces of ecdysteroids therefore, the enzymes were assayed alone to determine that ecdysteroid contaminants were not present.

The results of the protease-enzyme digestion of vitellin is shown (fig. 30). An appreciable amount of ecdysteroids were isolated from newly oviposited eggs. An approximate amounts of 20-OH-ecdysone equivalents were 21.2 ± 0.7 and 26.0 ± 0.6 pg/g of vitellin being detected during the first two h of embryonic development, respectively. The ecdysteroid titer then decreased after 2 h until a large peak appeared after the 16th h of embryonic development. The period between 6 to 16 h of embryonic development contained only traces of ecdysteroids. In order to ensure
Fig. 30. Ecdysteroids isolated from digested vitellin. The vitellin was digested with equal amounts of protease and Helix enzymes. Each point is the mean of 3 replications (mean ± SE).
that these ecdysteroid titers were indeed low, the level of Helix enzymes added to the vitellin-protease mixture was doubled. In spite of the addition of Helix enzymes, a further increase in ecdysteroid titer was not found.

During late embryogenesis, ca. 18 h after oviposition, a rise in ecdysteroid titer occurred. Three peaks of ecdysteroids were observed (Fig. 30). However, the three peaks appeared possibly being 3 independent releases of the hormone rather than one large pulse of ecdysteroid titer. At 19 h, the first maximal concentration of 20-OH-ecdysone equivalents was 85 ± 1.7 pg/g vitellin. The second and third peaks occurred at 22 and 26 h with maximal titer of 20-OH-ecdysone equivalents was 106.5 ± 1.72 and 102.5 ± 2.36 pg/g vitellin, respectively.

At 18 hours, the increase of ecdysteroids from digested vitellin corresponded with the appearance of the free ecdysteroids peak. During the rise of ecdysteroids, no observable morphological events occurred in the embryo. As the first peak of ecdysteroids gradually declined at 20 to 21 h, the beginning of the involution of the head and the dorsal closure were visible. During the appearance of the second peak of ecdysteroids, completion of the dorsal closure and the appearance secreted cuticle occurred. The third peak of ecdysteroid occurred at 26 h which coincides to a slight increase of free ecdysteroids observed prior to eclosion.
The source of the 3 peaks of ecdysteroids during late embryogenesis could be traced to the ecdysteroid conjugates that were hydrolyzed by Helix enzymes. The reason why the conjugates were not detected during early embryogenesis is not known. One can speculate that early in embryogenesis ecdysteroids change into unique forms of conjugates that resist digestion by the Helix enzymes. Somehow the conjugates are transformed during late embryogenesis which are amenable to hydrolysis by Helix enzymes. However, it cannot be ruled out that the three peaks of ecdysteroids occurring during late embryogenesis may be derived from de novo biosynthesis by embryonic tissue.
IV. DISCUSSION

The results obtained in this study show that concentrations of free and conjugated ecdysteroids occur in developing *D. cucurbitae* embryos. A large concentration of free ecdysteroids were detected in one prominent peak at the beginning of germ band shortening, at ca. 18 hours. The appearance of a single peak of free ecdysteroids is characteristic of other dipteran species including *D. melanogaster* (Maroy et al. 1988), *Sarcophaga bullata* Parker (Wentworth and Roberts 1984) and *C. erythrocephala* (Bordes-Alleaume and Sami 1987). Several studies showed that peak(s) of free ecdysteroids and the secretion of the embryonic cuticle(s) occurred concomitantly during embryonic development (Bulliere et al. 1979; Lagueux et al. 1979; Calvallin and Fournier 1981; Imboden and Lanzrein 1982; Bordes-Alleaume and Sami 1987; Maroy et al. 1988). The appearance of free ecdysteroids in *D. cucurbitae* embryos may have a similar role in controlling the cuticle secretion before the embryo has acquired the means for ecdysteroid synthesis. A single cuticle was secreted approximately 4 to 5 h after the appearance of a free ecdysteroid peak. A single cuticle has been shown to be secreted after the completion of the dorsal closure in other dipteran species (Maroy et al. 1988; Bordes-Alleaume and Sami 1987). This is common in embryos from other insect orders (e.g., *Nauphoeta*...
ciereca (Olivier) (Imboden and Lanzrein 1982) and *L. migratoria* (Lagueux et al. 1979).

A substantial increase of free ecdysteroids occurred at approximately 18 h and was thought to be derived from a large pool of maternal ecdysteroid conjugates which had undergone hydrolysis. Difficulty exists when comparing the results of other ecdysteroid conjugate studies because of the techniques used for extraction, purification, and quantitation of ecdysteroid conjugates. In most insect embryos studied, free polar conjugates (not bound to vitellin) were extracted in 25 to 70% methanol. Polar conjugates were not included in this study because of the difficulty in separating free ecdysteroids from these conjugates.

Even though the extraction method of ecdysteroid conjugates from embryos varied with different insect species, a similar pattern of ecdysteroid concentration was observed during embryonic development. In some insect species (e.g. *Locusta, Schistocerca, Bombyx*), newly laid eggs contain large amounts of conjugated ecdysteroids (Hoffmann et al. 1985). However, as the embryo develops, a gradual disappearance of the conjugates occurs. The ecdysteroid may be combined with other compounds which are not readily hydrolyzable with *Helix* enzymes or esterase. Later, ecdysteroids are released from these new conjugates when they reappear in development. Generally, conjugates
reappear before or at the time of free ecdysteroid peak(s) and secretion of the cuticle(s). The maximal concentration of the conjugate occurs several hours before eclosion.

Maternal conjugates were not detected in newly laid eggs of any dipteran species studied (Maroy et al. 1988; Borden-Alleaume and Sami 1987). However, Bownes et al. (1988) reported that ecdysteroid conjugates were indeed present in D. melanogaster, bound to yolk protein. This study showed that yolk protein extracted from embryos of D. cucurbitae and enzymatically digested, indicated that large amounts of ecdysteroid were bound to vitellin. The fluctuation in ecdysteroid titers isolated from conjugates in D. cucurbitae were similar to that found in embryos from other insect species. In N. cinerea, a small quantity of conjugated ecdysteroids were found bound to vitellin after enzymatic digestion. The majority of the ecdysteroid conjugates in the embryo of N. cinerea were found unbound (Zhu et al. 1983). All ovarian and most egg ecdysteroid conjugates of L. migratoria were found bound to vitellin (Lagueux et al. 1979). Other ecdysteroid conjugates may simply remain undetected because their physicochemical characteristics render them resistant to enzymatic hydrolysis.

The parallel appearance of free and conjugated (vitellin bound) ecdysteroids in embryo of D. cucurbitae at 18 hours indicate a transformation of the ecdysteroid
conjugates to a form easily hydrolyzed. The increase of ecdysteroid titers may be a result of hydroxylation of the conjugated maternal ecdysteroids stored in the oocyte before ovulation. This could result in a hormone pool available to the embryo after hydrolysis. The occurrence of immunopositive conjugates in newly oviposted and early developing eggs was quite puzzling. Since free ecdysteroids were not observed during the early hours of embryonic development of *D. cucurbitae*.

Eggs of most insect species have levels of conjugated ecdysteroids much greater than free hormones (Hoffmann et al. 1980). In *Locusta spp.*, ecdysteroid conjugates were 100-fold greater than free hormone. In *D. cucurbitae*, the concentration of ecdysteroids was much lower, about 10-to-15 folds greater than free ecdysteroids. As first proposed by Mizuno and Ohnishi (1975), ecdysteroid conjugates represent a storage form of the hormone which may yield free hormone during embryogenesis.

In summary, the normal processes of apolysis, ecdysis, and cuticle formation in the embryo of *D. cucurbitae* during development, appears to be initiated by a rise of ecdysteroids at ca. 18 h which is preceded by the appearance of the embryonic cuticle. The source of free ecdysteroid appears to be maternally derived since the ring gland (Weismann’s gland) was not differentiated at this time (i.e., at the completion of the dorsal closure).
Ecdysteroid conjugates, storage forms of the hormone, were hydrolyzed at critical developmental periods which released free hormone to initiate cuticle formation in the melon fly embryo. This study has correlated the appearance of free ecdysteroids with important developmental stages in *D. cucurbitae* embryo using the sensitive radioimmunoassay method.

The information derived from this study may prove valuable for future control methods of *D. cucurbitae* eggs oviposited in fruits of cultivated crops. Recent advances in genetics and protein bioengineering has made it possible to insert genes using vectors (i.e., *Agrobacterium spp.* ) into the plant’s genome and forming transgenic plants. Transgenic plants are manipulated to be resistant to a desired virus or bacteria. The most recent success occurring with transgenic plants was the transformation of tobacco plant cells to express mammalian antibodies (Hiatt 1990; Hiatt et al. 1989). It is believed that transgenic plants could be made to express ecdysteroid mimics or enzyme inhibitors (i.e., ecdysteroid conjugate hydrolysis enzyme). Disruption of the timed release of ecdysteroids in the embryo would effect the development as well as the secretion of the embryonic cuticle. Hormonal manipulation is an ideal method for insect control since it is insect specific and environmentally safe.
REFERENCES


