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The ovary as a source of α-ecdysone in an adult mosquito

(steroids/vitellogenesis/organ culture/radioimmunoassay/*Aedes aegypti*)

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ABSTRACT **The ovaries of the mosquito *Aedes aegypti* cultured *in vitro* secrete material that behaves like ecdysone in a radioimmunoassay. The material was identified as α-ecdysone by high-resolution liquid and gas-liquid chromatography. Secretion reached a maximum 16 hr after a blood meal as shown by bioassay and direct determination. Ovariectomy reduced the concentration of ecdysone in the adult after a blood meal. Qualitative analysis of whole-body extracts indicated β-ecdysone to be the principal species present. Thus the ovaries appear to secrete a prohormone, α-ecdysone, which is converted to β-ecdysone. β-ecdysone plays a significant role in stimulating egg development in the adult mosquito and may have reproductive roles in other insects.**

In mosquitoes the blood meal triggers egg development. This process, which has been found to be quite complex (1), involves the elaboration of yolk for later use by the developing embryo. The major proteins which become yolk are synthesized by the fat body, secreted into the hemolymph, and selectively taken up by the developing oocyte (2, 3). These proteins have been termed vitellogenins (4). Recent investigations into the control of this process showed that, in contrast with most insects, the mosquito ovary was the source of a hormonal factor which activated and maintained vitellogenin synthesis by the fat body (5). Thus synthesis by the fat body was abolished by ovariectomy and restored by reimplantation. Further, when ovaries from blood-fed females were incubated *in vitro* with the inactive fat body from unfed females, synthesis of vitellogenin was activated. This ovarian hormone was designated the vitellogenin stimulating hormone, VSH (1).

The discovery that injected β-ecdysone stimulates egg development (6) and dopa decarboxylase activity (7) in the absence of a blood meal led us to investigate the possibility that VSH might be β-ecdysone. Our results indicated that β-ecdysone mimicked VSH *in vivo* and *in vitro* (8) and that material with ecdysone-like activity could be detected in mosquitoes after a blood meal (9). These lines of evidence strongly suggested that VSH was β-ecdysone or a closely related steroid. We here describe experiments designed to identify the secretory product of the ovary.

METHODS

Mosquitoes (*Aedes aegypti*) were reared at 27 ± 0.5° using standard techniques (8, 9). The assay for vitellogenin synthesis has been described in detail (1, 8). The radioimmunoassay (RIA) was performed as previously described (10, 11).

Abbreviations: RIA, radioimmunoassay for ecdysone; HRLC, high-resolution, reverse-phase, liquid-solid adsorption chromatography; VSH, vitellogenin stimulating hormone.

Ovaries were removed from females 15 hr after a rabbit blood meal and incubated at 25° with gentle shaking in groups of 100 in 100 μl of a defined medium (8). After 24 hr the medium was removed and the ovaries were rinsed with saline. An equal volume of methanol was added to the combined medium and rinse. Particulates were removed by filtration. The extract was evaporated to dryness under reduced pressure, redissolved in chloroform:methanol (2:1) and partitioned against water according to Folch *et al.* (12). The epiphase was dried under vacuum and partitioned between butanol and water. These extracts were assayed for RIA activity and the values obtained were converted to pg of β-ecdysone equivalents per female.

Whole adult females were blood fed on mice and were subsequently extracted in 60% methanol. The extracts were handled as described above. Ovariectomies were performed as described previously (13).

Components active in the RIA assay were separated by continuous flow thin-layer chromatography (11, 29) using chloroform: 97% ethanol (80:20) on methanol-washed silica gel plates containing a fluorescent indicator. After development the plates were divided into 0.5 cm or 1 cm bands which were scraped from the plate, eluted with ethanol, and subjected to RIA. Active fractions were developed using a reversed-phase high-resolution liquid chromatography column (HRLC) [1.2 m × 9.5 mm; Poragel PN, 37–75 μm; 60% methanol, 40% water; 2 ml/min]. Detection was by ultraviolet absorbance at 254 nm.

The RIA active fractions from the reversed phase column were derivatized by heating (96°, 30 min) in the presence of trimethylsilylimidazole (Applied Science Labs). The trimethylsilyl ether derivatives were extracted with *n*-hexane (10, 17) and analyzed on a 1.2 m × 2 mm (inside diameter) 1.5% OV-101 glass column. Other conditions were: N_2 flow at 65 ml/min; injector: 300°. β-ecdysone was purchased from Rhoto Pharmaceutical Co., Osaka, Japan; α-ecdysone was a gift of Dr. J. B. Siddall, Zoecon Corp., Palo Alto, Calif.

RESULTS

The best evidence that β-ecdysone directly activates vitellogenin synthesis comes from experiments where fat body from unfed females was incubated *in vitro* with the hormone (8). We can now confirm and extend these observations by showing a direct relationship between hormone concentration and the response of the fat body (Fig. 1). The response is half-maximal at 10^{-7} M β-ecdysone.

The response of the fat body from unfed females can now be used as an assay for ecdysones. In this assay, fat body from unfed females is incubated with ecdysone, or a sus-

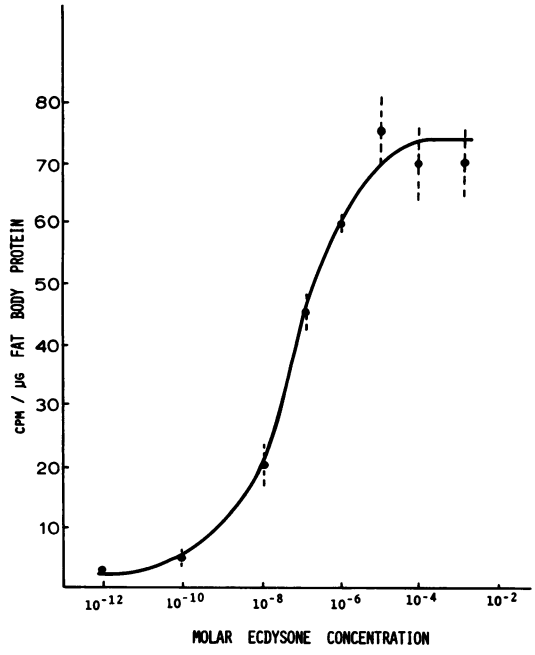

FIG. 1. Response of the fat body from unfed females to β-ecdysone *in vitro*. Fat bodies were incubated in 0.1 ml of medium (8) containing various concentrations of β-ecdysone for 18 hr. The medium was then replaced with 0.1 ml of medium containing 5 µCi of [^{3}H]phenylalanine and the incubation was continued for 3 hr. The medium was then withdrawn and the tissues were washed twice with 0.05 ml of medium. The fat body was removed and frozen. The medium was then assayed for labeled vitellogenin (1, 8). The fat body tissues were homogenized and analyzed for protein by the method of Bramhall (28). Data are expressed as counts per minute antibody precipitate (vitellogenin) per microgram of fat body protein (not a specific activity), and are shown with the SEM.

pected source of ecdysone, for 18 hr.¶[^{3}H]Phenylalanine is then added for a further 3 hr and the amount of vitellogenin secreted during the 3 hr is determined using a specific antibody. We have taken advantage of this assay to determine when the ovary releases VSH after the blood meal. The main peak of hormone release occurs about 16 hr after a blood meal (Fig. 2, solid line) and maximum vitellogenin synthesis (broken line) occurs about 12 hr later. These data correlate nicely with results from radioimmunoassay (RIA) of whole-body extracts which document the increasing concentration of ecdysone(s) following a blood meal (Fig. 3). In both assays a reproducible minor peak of activity is found at 4 hr. Although the values obtained from the RIA assay at 2–10 hr do not appear to be statistically different, the constancy of the result suggests a possible biological significance to the observation.

The 3-day-old unfed mosquito has a total water content of about 1.9 µl (T. Flanagan, unpublished observations) which is the maximum volume in which the ecdysone could be distributed. Assuming that the fed mosquito, excluding gut contents, has a similar water content, the concentration of ecdysone at 16 hr (about 275 pg per female, Fig. 3) is about 3×10^{-7} M. This compares remarkably well with the concentration found effective *in vitro* (Fig. 1), and is comparable to other ecdysone-activated insect systems (15, 16).

To determine the nature of VSH we incubated ovaries removed from blood-fed and unfed females in a defined medium (8), for 24 hr. The medium was then analyzed for ma-

¶ Under assay conditions, synthesis is maximal 12–18 hr after hormone stimulation (Bohm, unpublished observation).

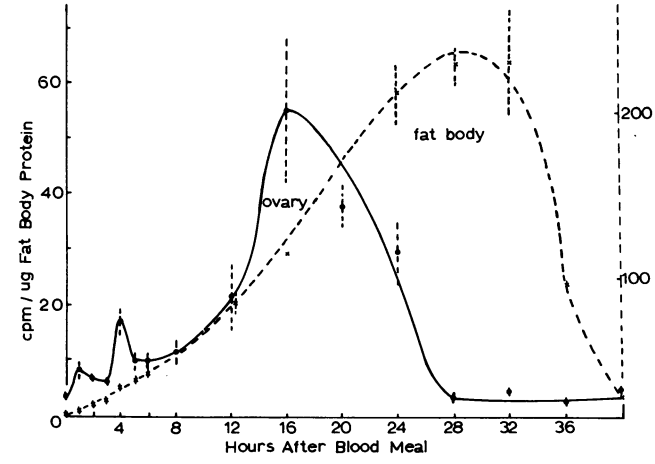

FIG. 2. Bioassay for the timing of ecdysone release by the ovary. The ovaries and fat bodies were dissected from mosquitoes at various times after a blood meal. The ovaries were assayed by incubating them with fat body from unfed females for 18 hr. [^{3}H]Phenylalanine was then added for a further 3 hr and synthesized vitellogenin was assayed as described in Fig. 1. The fat body from the fed females was assayed by incubating them for 3 hr with [^{3}H]phenylalanine, followed by the vitellogenin assay. The graph, therefore, shows when the ovary is maximally capable of activating fat body *in vitro* (solid line), and when fat body is maximally active *in vivo* (broken line). Both are expressed as labeled vitellogenin synthesized per unit fat body.

terial with ecdysone-like activity by RIA. Only medium in which ovaries from fed females was incubated contained RIA activity (Table 1). A methanolic extract of media in which ovaries removed from 2000 females 15 hr after the blood meal had been incubated contained approximately 87 ng of material which is immunologically similar to ecdysone. Following partition between water and butanol, 63 ng of active material were recovered in the butanol phase. Subsequent chromatography using a reversed-phase packing in a high-resolution liquid (HRLC) system demonstrated that the RIA activity migrated as α-ecdysone (Fig. 4). A trimethylsilyl derivative of this fraction from HRLC had the same retention time in gas-liquid chromatography as a derivatized α-ecdoysone standard (Fig. 5A). This chromatographic behavior of both the derivatized and underivatized fractions leads us to believe that α-ecdysone is released under the *in*

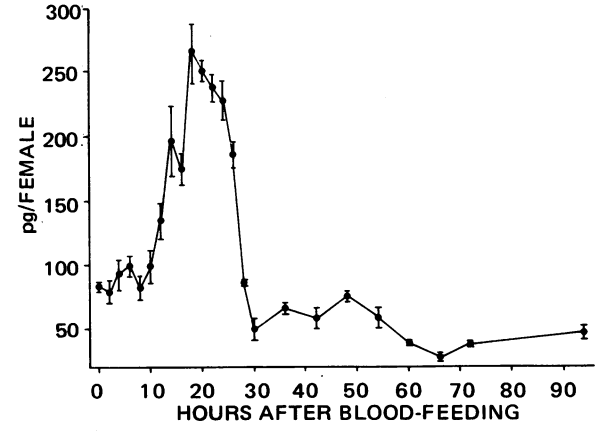

FIG. 3. Endogenous ecdysone levels of adult blood-fed females as determined by the radioimmunoassay. For each time period 65 to 125 animals (average = 89) were extracted. Mouse blood showed no detectable RIA activity. The data are expressed as pg of β-ecdysone equivalents. The variation shown represents the SEM for four replicates.

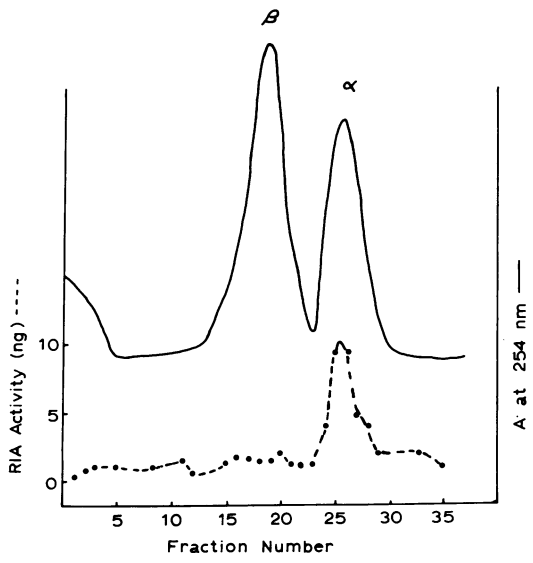

FIG. 4. Isocratic elution of RIA activity, and α- and β-ecdysone standards, from a reversed phase HRLC column. ——, *A* at 254 nm of ecdysone standards; ● - - ●, radioimmunoassay activity of extracted medium partially purified using thin-layer chromatography.

vitro conditions described from the ovaries of blood-fed mosquitoes. However, qualitative analysis of ecdysones in whole adult females indicates β-ecdysone to be the principal species present (Fig. 5B).

The involvement of ovarian tissue in the elevation of ecdysone titers following blood feeding is supported by the observation that ovariectomy reduces the peak of RIA activity (Fig. 6).

DISCUSSION

These data demonstrate that the ovary of an adult insect can be a source of ecdysone. In the adult female mosquito, β-ecdysone has been shown to play a significant role in stimulating vitellogenin synthesis by the fat body (1) and the appearance of dopa decarboxylase activity after the blood meal (9). We have shown previously that the ovary secretes a hormone (VSH) which stimulates vitellogenin synthesis by the fat body (5). These data strongly suggest that VSH is ecdysone. It remains to be determined, however, whether the ovary actually synthesizes ecdysone. Also, while we have shown that β-ecdysone mimics VSH, the ovary actually releases α-ecdysone. The active hormone is not yet known.

The high titer of ecdysone in mosquitoes before the blood meal (Figs. 3 and 6) poses an interesting problem. It can be calculated from Fig. 3 that the concentration of ecdysone in a mosquito before the blood meal is about 1×10^{-7} M. The increase in titer after the blood meal raises the concentration

Table 1. RIA activity recovered from medium incubated with ovaries

	β-ecdysone equivalent (ng)
Medium control	4.1
Ovaries from unfed females	4.1
Ovaries from fed females	22.8

Ovaries were removed from 50 fed mosquitoes 18 hr after the blood meal, or from 50 unfed mosquitoes, and incubated for 5 hr in a defined medium (8). The medium was then removed and treated as described in *Methods*.

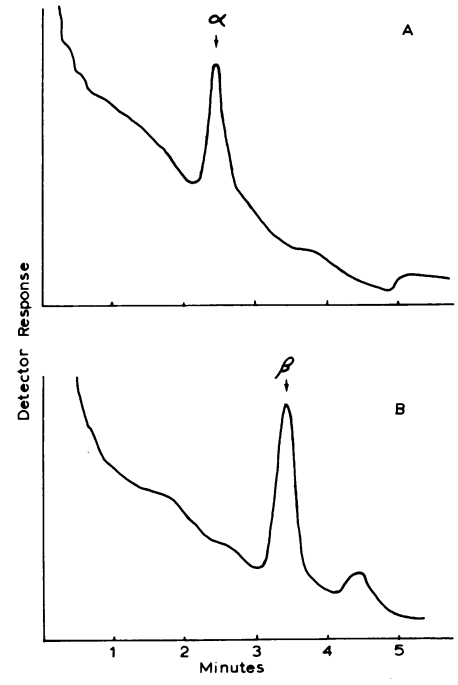

FIG. 5. Gas-liquid chromatography of the RIA active material. Arrows indicate the time of elution of standard, α- and β-ecdysone. (A) RIA active fraction from reversed-phase column chromatography of ovarian incubation media. (B) RIA active fraction from continuous flow thin-layer development (28) of methanolic extract of whole mosquitoes 14–26 hr after blood meal. Peak at 4:14 represents incompletely derivatized β-ecdysone.

to 3×10^{-7} M. We have reason to suspect that the fat body does not respond to the ecdysone before the blood meal because of an inhibiting factor which disappears after the blood meal (Hagedorn, in preparation). The source of the ecdysone in the unfed female is not known. The ovariectomy data do not eliminate the ovary as the source since the operation was done 4 days after emergence. By this time the ovary could have produced the ecdysone seen.

The presence of vanishingly small quantities of α-ecdysone in the extract of whole mosquitoes suggests its rapid conversion to β-ecdysone following its release from the ovaries. Such a conversion is known to take place in several insect tissues (14). Indeed it has been recently shown in a number of insects that the prothoracic gland releases α-ecdysone which can subsequently be converted to β-ecdysone by hydroxylation (17–20). Thus, in both larval and adult insects, and whether involved in molting or ovarian development, α-ecdysone appears to be secreted from the respective endocrine glands. Yet β-ecdysone is more active on many target tissues.

The presence of ecdysone in an adult insect is not without precedent. In *Shistocerca, Bombyx, Oncopeltus,* and *Drosophila* (21, 22), significant levels of ecdysone have been found in the adult. We have shown that the increase in ecdysone after the blood meal plays an important role in egg development. There are some recent indications that this may also be so in other insects: In *Leucophaea maderae* β-ecdysone in adults is found only in reproducing adult females (ref. 19, and E. P. Marks, personal communication). In female *Bombyx mori* there are two increases in the ecdysone titers following the larval-pupal ecdysis. The second of these increases, which occurs approximately 3 days prior to adult eclosion, is abolished by ovariectomy (23). In the tent caterpillar, *Malacosoma pluviale,* isolated abdomens treated with

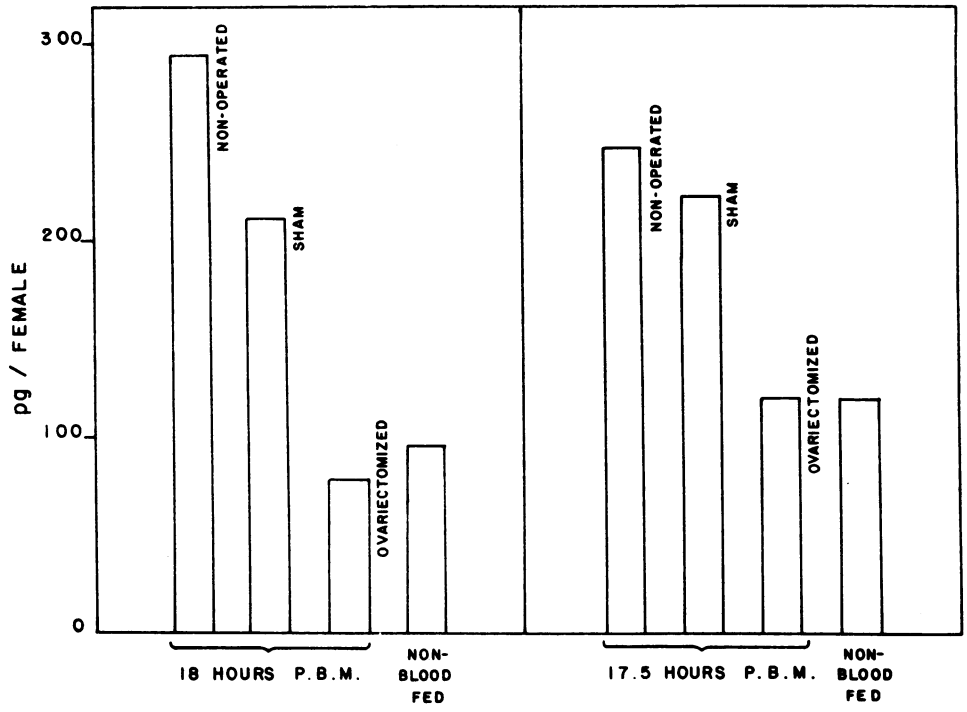

FIG. 6. The effect of ovariectomy on endogenous ecdysone levels in blood fed females. Four days after adult emergence females were ovariectomized as previously described (13) and allowed to recover for 36 hr. The sham operation consisted of making an incision in the intersegmental membrane in the region of the ovary. They were then blood fed and extracted in methanol and chloroform 18 and 17.5 hr later (P.B.M. = post blood meal). The extract was then assayed for RIA activity. The data are expressed as pg of β-ecdysone equivalents. In experiment 1 (18 hr) 58 operated and 71 shams were used, in experiment 2 (17.5 hr) 48 operated and 57 shams. All the females were the same age in each experiment.

β-ecdysone had greater numbers of oocytes, although their growth was not stimulated (24). In *Tenebrio molitor*, isolated nymphal ovaries could be stimulated to grow and differentiate in the presence of ecdysone (25). In *Periplaneta americana*, competence of the ovary to undergo vitellogenesis is promoted by β-ecdysone (26). In the latter case the events described are seen late in the last larval stage, before or during adult ecdysis, and involve previtellogenic development of the ovary. In contrast, ecdysone in mosquitoes is involved in controlling vitellogenic growth.

It appears therefore that the ecdysones, which play a central role in the molting process in immature insects, also have important functions in ovarian development in mosquitoes and perhaps other insects. A similar situation is found with another insect hormone, the juvenile hormone, which is involved in the molting process and also has a central role in ovarian development in adults (2, 27).

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