ECDYSONE-MEDIATED STIMULATION OF DOPA DECARBOXYLASE ACTIVITY AND ITS RELATIONSHIP TO OVARIAN DEVELOPMENT IN *AEDES AEGYPTI*

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ABSTRACT

Very little dopa decarboxylase activity is detectable in adult female mosquitoes *Aedes aegypti* which have not been allowed to engorge blood. However, when such females are injected with the molting hormone β-ecdysone a marked stimulation of this enzyme's activity is observable. No stimulation is observed in males similarly injected, nor in females injected with cholesterol or a juvenile hormone mimic. In addition, ecdysone injection initiates ovarian development in these anautogenous non-blood-fed mosquitoes. The extent of stimulation in both cases is dependent upon the amount of β-ecdysone administered. These results suggested that ecdysone may play a role in ovarian development in *Aedes* and led us to hypothesize that a normal blood meal may trigger the synthesis, activation, or release of this hormone endogenously. Using the radioimmune assay for ecdysone developed by Borst and O'Connor (*Science* [*Wash. D. C.*] **178**:4–18.), we found that the titer of an antigenic-positive material, presumably ecdysone or a closely related analogue, substantially increased 24 h after blood feeding, thereby supporting our postulation.

INTRODUCTION

The chorions of both fertilized and unfertilized eggs of the mosquito *Aedes aegypti* are soft and white at the time oviposition. Within a short time, in air, they gradually darken and harden and after 4 h the egg is completely black. This process resembles the hardening and darkening of the cuticles of newly molted insects, referred to as sclerotization and/or tanning. Therefore, from this point of view it was not surprising to find that mature oocytes obtained from virgin *A. aegypti* contained high levels of dopa decarboxylase activity (1), an enzyme which in Diptera is necessary for the synthesis of the sclerotizing agent *N*-acetyldopamine (2). However, this in turn did suggest a possible correlation between this enzyme and ovarian development in *A. aegypti*. This postulation was confirmed when it was found that 5-day old non-blood-fed female adults exhibit very little dopa decarboxylase activity until after they are given a blood meal, a necessary requirement for subsequent normal ovarian development in this anautogenous species. A dramatic increase in enzymatic activity then ensued which paralleled oocyte maturation (3).

Removal of the resting ovaries before blood feeding resulted in no increase in dopa decarboxylase activity whatsoever, suggesting that the ovary itself may be the site of synthesis or activation of this enzyme (4).

The rise in dopa decarboxylase activity in adult female *A. aegypti* after a blood meal was reminiscent of an observation made by Karlson and co-workers who after injecting the molting hormone ecdysone into ligated *Calliphora* larvae found an increase in dopa decarboxylase activity (5). This led us to ask if injection of β-ecdysone into non-blood-fed adult females would also elicit an increase in enzymatic activity? Our preliminary observations were affirmative, i.e., injection of the molting hormone into 5-day old non-blood-fed females did result in a significant stimulation of the dopa decarboxylase activity (3, 6). The first part of this report will examine this enzymatic stimulation in more detail with reference to hormonal dosage effects and specificity.

Spielman et al. (7) reported that injection of β-ecdysone into non-blood fed, anautogenous adult female *A. aegypti* stimulated vitellogenesis and that prolonged feeding of the hormone resulted in the production of a few normal eggs. Thus, in the second part of this paper we will further address ourselves to the relationship between β-ecdysone, dopa decarboxylase activity, and ovarian development in these insects.

MATERIALS AND METHODS

Rearing of Mosquitoes

The wild type ROCK strain of *A. aegypti* (maintained at the Vector Biology Laboratory, University of Notre Dame) was used throughout the study. Mosquitoes were reared according to the generalized methods of Craig and VandeHey (8). All experiments were performed using 5-day old adult mosquitoes maintained on apple slices which had been commercially blanched, packed in sugar, and frozen. Adults were given apple at the time of emergence. They received fresh apple on day 3, were starved on day 4, and then utilized for experimentation on day 5. When required, a blood meal was administered using laboratory mice which had been anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, South Pasadena, Calif.). The time interval, post blood meal, was calculated from the midpoint of the feeding period. Only mosquitoes from the same rearing batch were used for any given experiment.

Injection

Adult mosquitoes were injected intrathoracically. The injection apparatus consisted of a mouthpiece, rubber tubing, a Leitz micromanipulator needle holder, and an injection needle made by drawing a capillary tube to a fine point. Routine injections involved a sample dose of 1.6 ± 0.1 μl. This volume distended the abdomen to approximately the same degree as does a normal blood meal. In the "ungorged" abdomen, the tergites and sternites slightly overlap, such that no portion of the intersegmental membrane is visible when viewed laterally. To avoid dilution of the injected sample, care was taken to inject only those individuals that met the requirement of the ungorged. For this reason, individuals were starved 1 day before injection. After injection, the mosquitoes were again given fresh apple slices.

The following substances were injected into *A. aegypti* adult mosquitoes to determine their effect on dopa decarboxylase activity: *Aedes* saline (9); 1% Tween 80 in saline; mouse blood:Alsevers (1:1); mouse serum; cholesterol (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio); synthetic juvenile hormone (Calbiochem, San Diego, Calif.); ecdysterone (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; Rohto Pharmaceutical Co., Ltd., Osaka, Japan). The cholesterol (2 μg/μl) and synthetic juvenile hormone (5 μg/μl) samples were prepared in 1% Tween 80 in saline, while saline alone was used for ecdysterone.

Dopa Decarboxylase Assay

The enzyme dopa decarboxylase (E.C. 4.1.1.28) converts 3,4-dihydroxyphenylalanine (dopa) to 3,4-dihydroxyphenylethylamine (dopamine). Its activity was assayed by measuring the rate of conversion of radioactive substrate [2-^{14}C]dopa to radioactive dopamine [1-^{14}C]dopamine. Separation of product and substrate, and subsequent monitoring of radioactivity was accomplished by using a modification of the radiochromatographic procedure of Lunan and Mitchell (10). Further details can be obtained elsewhere (3). Protein concentration was determined by the method of Lowry et al. (11) using a standard curve corrected for phenylthiourea (PTU) interference.

For each enzymatic assay, at least 50 but more usually 75 individuals were homogenized in order to obtain the extract. Each value reported herein represents the average of six replicates. The range of variability in all cases was less than ±5%.

Photomicroscopy

Transmitted-light photomicrographs were taken with a Zeiss Ultraphot II equipped with a Luminar

head. 4- by 5-inch Kodak Ektapan Professional film was used with this instrument.

Radioimmune Assay for Ecdysone

The radioimmune method of Borst and O'Connor (12) was used to assay for ecdysone in adult mosquitoes. The extraction and subsequent assay procedures were performed in Dr. O'Connor's laboratory at U.C.L.A. under the supervision of Dr. David Borst. Six samples of mosquitoes were tested for the presence of ecdysone: newly pupated females; 5-day old adult males; 5-day old pre-blood-fed adult females; and females 4-, 12-, and 24-h post blood feeding. The sample of newly emerged female pupae was included to serve as a control, testing the effectiveness of this assay in detecting the presence of ecdysone in mosquitoes. Approximately 1 g of each sample of mosquitoes of known number was respectively homogenized in approximately 5 vol (wt/vol) of 60% methanol for 2 min at room temperature using an Omnimixer. The homogenate was centrifuged at 5,000 *g* for 15 min, and the resulting supernate was filtered through paper. The precipitate was extracted two additional times in methanol as before; the filtrates were then combined and mixed with chloroform (for protein denaturation) to give a final concentration of 20%. After the resulting mixture was filtered into a round-bottom flask, the solvents were removed by flash evaporation at 48°C. The residue was dissolved in 5 ml of methanol; that which failed to dissolve was removed by centrifugation. About 10–50 μl of the methanol extract were assayed for the presence of ecdysone. An aliquot of this extract was pipetted into a 6- by 50-mm culture tube and evaporated to dryness by N_2 flushing. The residue was suspended in 100 μl of borate buffer, pH 8.4, to which was added 50 μl of anti-ecdysterone-bovine serum albumin (BSA) serum. The ecdysterone-BSA conjugate and the antiserum derived thereof has been previously prepared by Borst and O'Connor (12). After thorough mixing, the tube was incubated for 2 h at room temperature. It was then cooled to 4°C, and the haptene-antibody conjugate was precipitated by the addition of 200 μl of saturated $(NH_4)_2SO_4$. After 30 min at 4°C, the mixture was centrifuged at 5,000 *g* for 20 min. The supernate was removed by suction, and the precipitate was washed with 50% $(NH_4)_2SO_4$. The final pellet was dissolved in 25 μl of water and mixed with 600 μl of Aquasol (New England Nuclear, Boston, Mass.). This was monitored for radioactivity in a Beckman LS-233 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Ecdysone and Dopa Decarboxylase

An examination of Fig. 1 *a* and 1 *b* clearly reveals that β-ecdysone (also called 20-hydroxyecdysone or ecdysterone) does indeed stimulate the activity of the enzyme dopa decarboxylase when injected into unfed adult *A. aegypti* females. After a lag period of approximately 24 h, the amount

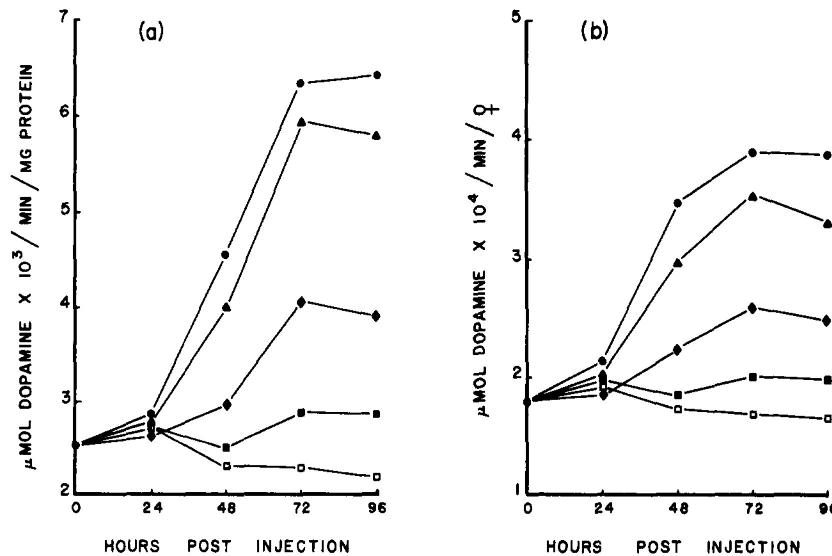

FIGURE 1 Dosage effect of β-ecdysone injection on dopa decarboxylase activity in unfed adult female *A. aegypti*. Activity after saline, □, and β-ecdysone injection: ■, 0.1 μg/μl; ◆, 1.0 μg/μl; ▲, 2.5 μg/μl; and ●, 5.0 μg/μl. Total volume injected in all cases was 1.6 ± 0.1 μl. Fig. 1 *a* depicts specific activity and Fig. 1 *b* activity per individual.

of activity increases dramatically until it begins to level off at approximately 72 h. Furthermore, although the amount of enzymatic activity observed is not directly proportional to the amount of hormone injected, a dose-dependent response is evident. This positive correlation between the amount of hormone injected and the amount of dopa decarboxylase activity observed is seen whether the specific activity (Fig. 1 *a*) or the enzymatic activity per female (Fig. 1 *b*) is monitored.

The increase in the specific activity of dopa decarboxylase suggests that β-ecdysone is not causing a general increase in protein synthesis but does suggest some specificity in the mode of action of the hormone. To gain further insight into this possibility two questions were asked: first, will β-ecdysone stimulate dopa decarboxylase activity in males and, secondly, will other materials injected into females mimic the hormonal effect observed? In order to answer the above questions critically, the enzymatic assay procedure was altered. The standard assay utilizes 10 μl of enzyme extract in which the reaction mixtures are allowed to incubate for 10 min. These conditions give one the true initial velocity of the reaction and serve as a measure of the amount of active enzyme present (3). However, to be absolutely certain that we would not miss small increases in dopa decarboxylase activity, the volume of the enzymatic extracts were increased to 40 μl and the reaction mixtures were incubated for 1 h before termination. Thus if any dopa were converted to dopamine enzymatically, the chances of it being undetected would be considerably diminished. This modified assay procedure for dopa decarboxylase activity was utilized for the experiments depicted in Figs. 2 and 3. Fig. 2 clearly demonstrates that adult males injected with β-ecdysone show no increase in dopa decarboxylase activity. Fig. 3

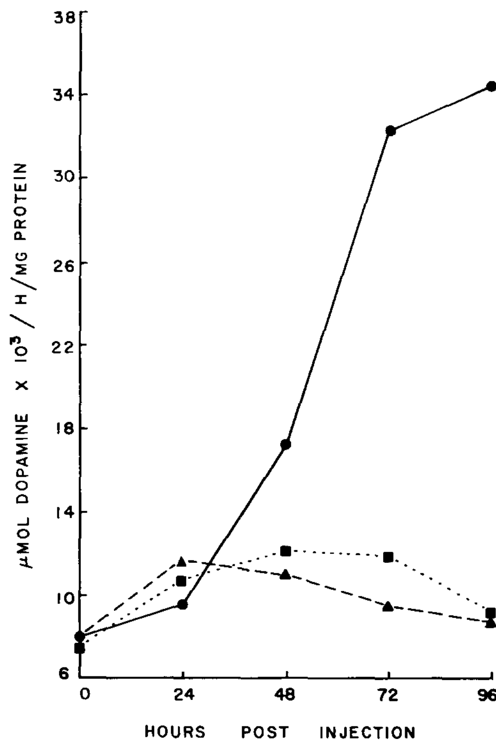

FIGURE 2 Effect of 5.0 μg/μl β-ecdysone injection on the adult levels of dopa decarboxylase activity in male and female *A. aegypti*. The volume injected in all cases was 1.6 ± 0.1 μl: —●—, specific activity of females; ····■····, specific activity of males; – –▲– –, specific activity of saline-injected female controls.

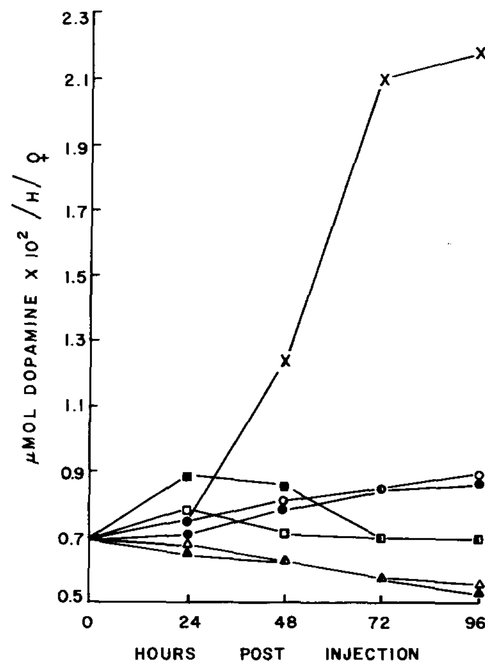

FIGURE 3 Activity of dopa decarboxylase exhibited by non-blood-fed adult female *A. aegypti* after injection of: ×, β-ecdysone; ●, mouse blood; ○, mouse serum; △, juvenile hormone mimic; ▲, uninjected females; ■, 1% Tween 80 in *Aedes* saline; and □, cholesterol. All females were 5-day old adults at the time of injection. In all cases 1.6 ± 0.1 μl per female of sample were injected in the following concentrations: β-ecdysone 5 mg/ml in *Aedes* physiological saline, juvenile hormone mimic, 5 mg/ml in 1% Tween 80 and *Aedes* saline and cholesterol, 2 mg/ml in the same solvent as the juvenile hormone mimic.

also shows that mouse blood, mouse serum, cholesterol, and a juvenile hormone mimic (Williams-Law mixture) do not by themselves stimulate dopa decarboxylase activity.

Ecdysone and Ovarian Development

Figs. 4–6 show the progression of ovarian development at 24, 48, and 72 h, respectively, after a normal blood meal and β-ecdysone injection. It appears that the 0.1 μg/μl β-ecdysone injection is below the threshold concentration required to elicit any significant yolk deposition. Likewise, this concentration failed to significantly enhance dopa decarboxylase activity (Fig. 1). At 24-h post injection there seems to be no real difference between the ovarian response to 2.5 μg/μl and to 5.0 μg/μl β-ecdysone. Much greater variation is observed among females injected with 1.0 μg/μl β-ecdysone. Some females show considerable development, others very little, while the majority exhibit a moderate degree of yolk deposition as seen in Fig. 4 *d*. The difference in ovarian development due to differences in ecdysone concentration become much more pronounced by 48-h post injection. While it appeared that ovarian development had been initiated in approximately equal numbers of follicles by 24-h post injection, we observe striking differences in the amount of yolk deposited and in the number of oocytes continuing to develop after 48-h post injection. There appears to be a correlation between the concentration of the injected ecdysone and the number of oocytes continuing to develop. The lower the ecdysone concentration, the fewer the oocytes that continue to develop, but the greater the yolk deposition in each oocyte. These same observations apply to the 72-h post injection stage. The fact that higher concentrations of injected ecdysone result in progressively greater amounts of dopa decarboxylase activity correlates exceedingly well with the morphological observations illustrated in Figs. 4–6.

The Radioimmune Assay for Detection of Endogenous Levels of Ecdysone

The data reported herein by us clearly confirm and extend the original observation of Spielman *et al.* (7) that β-ecdysone initiates yolk deposition, and, they are consistent with Fallon and Hagedorn's report (13) that β-ecdysone activates the synthesis of vitellogenin by mosquito fat bodies. Moreover, the ineffectiveness of β-ecdysone to stimulate dopa decarboxylase activity in males, and the unique effectiveness (of the substances tested) of β-ecdysone to stimulate dopa decarboxylase activity suggest that the responses elicited by this hormone in adult females are not artifactual. As a working hypothesis we propose that in anautogenous adult *A. aegypti* females, the engorgement of blood triggers the endogenous release, activation, or synthesis of ecdysone, which now assumes a gonadotropic role by activating or inducing the components necessary for normal ovarian development, including the enzyme dopa decarboxylase. Note that we are not suggesting that ecdysone is the only gonadotropic hormone, but only that it is a necessary one. A consequence of this postulation is the prediction that blood-fed females would exhibit a higher titer of ecdysone than non-fed individuals. Utilizing the Borst-O'Connor radioimmune assay for ecdysone (12), hormone levels were determined in newly emerged pupae (used as a guide), 5-day old adult non-blood fed males and females, and 5-day old females assayed at 4, 12, and 24 h after blood feeding.

Fig. 7 shows the levels of "ecdysone" detected in *A. aegypti* pupae (obtained within 10 min after the larval-pupal ecdysis) and adults. The data clearly establish the presence of ecdysone in the pupal control and also illustrate an increase in ecdysone levels in blood-fed females over the non-blood-fed control. Although some variation in the values obtained is indicated, there is a very obvious change in ecdysone titer by 4 h after a blood meal, and at 24-h post blood meal the ecdysone titer is comparable to that of newly emerged pupae. No other female samples were tested at this time. The 5-day old males show ecdysone levels comparable to that of 4–12-h blood-fed females. No detectable ecdysone-like antigenic activity was found in mouse blood.

DISCUSSION

The activation of egg development by ecdysone was first demonstrated by Spielman et al. (7) who found that injection of β-ecdysone into non-blood fed, anautogenous female *A. aegypti* stimulated vitellogenesis, and that prolonged feeding of the hormone resulted in the production of a few normal eggs. Development of oocytes was not initiated by three other steroids (cholesterol, hy-

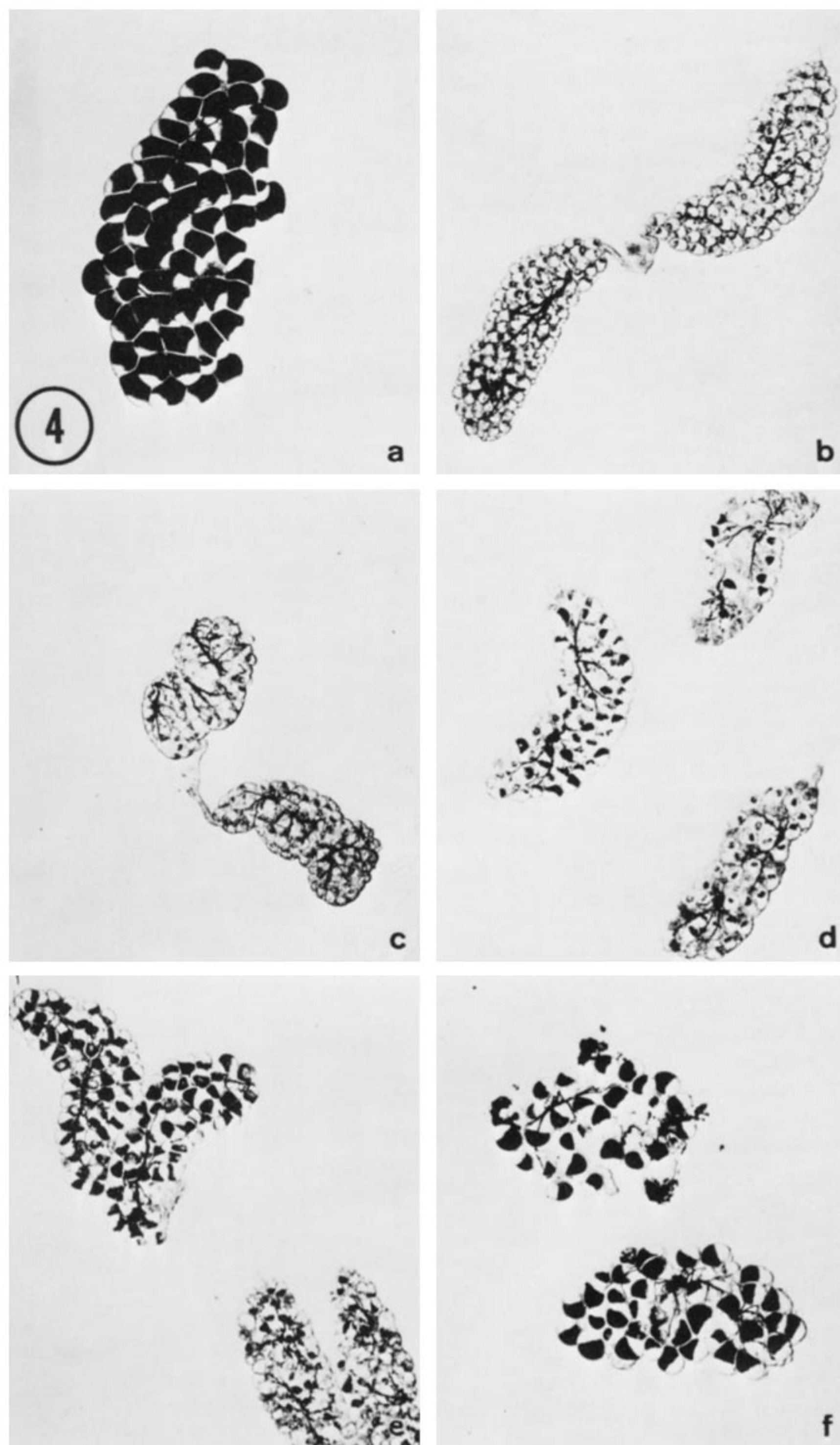

FIGURE 4 Comparison of normal ovarian development at 24-h post blood meal with that initiated by injection of β-ecdysone: (*a*) normal post blood meal ovary, (*b*) ovaries of a saline-injected control female; ovaries of β-ecdysone-injected females, (*c*) 0.1 $\mu g/\mu l$, (*d*) 1.0 $\mu g/\mu l$, (*e*) 2.5 $\mu g/\mu l$, and (*f*) 5.0 $\mu g/\mu l$, respectively. $\times$ 25.

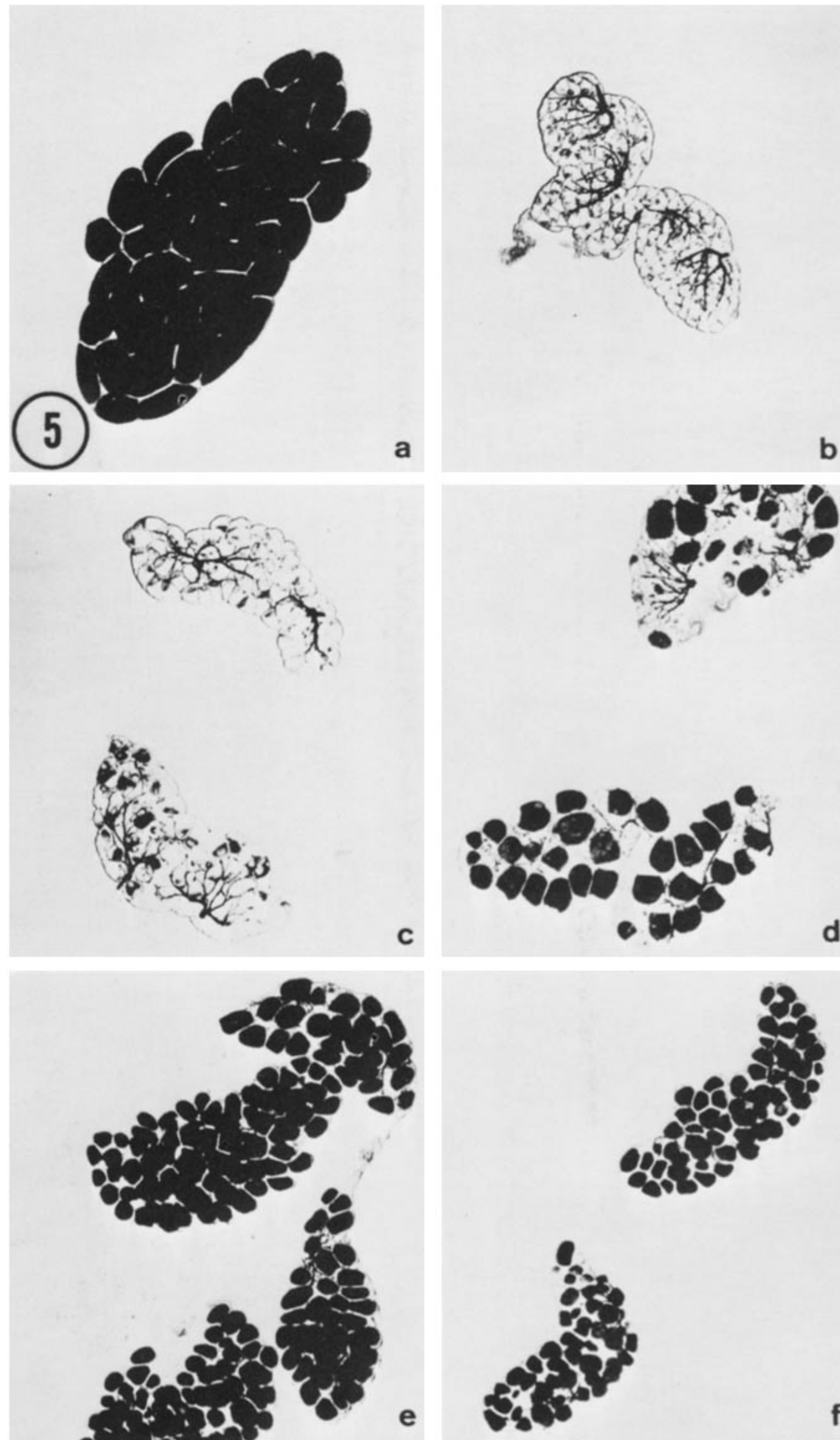

FIGURE 5 Comparison of normal ovarian development at 48-h post blood meal with that initiated by injection of β-ecdysone: (*a*) normal post blood meal ovary, (*b*) ovaries of a saline-injected control female; ovaries of β-ecdysone-injected females, (*c*) 0.1 $\mu g/\mu l$, (*d*) 1.0 $\mu g/\mu l$, (*e*) 2.5 $\mu g/\mu l$, and (*f*) 5.0 $\mu g/\mu l$, respectively. $\times$ 25. Note that in Fig. 5 *d* two pairs of ovaries are shown and in Fig. 5 *e* two complete pairs and part of a third pair can be seen.

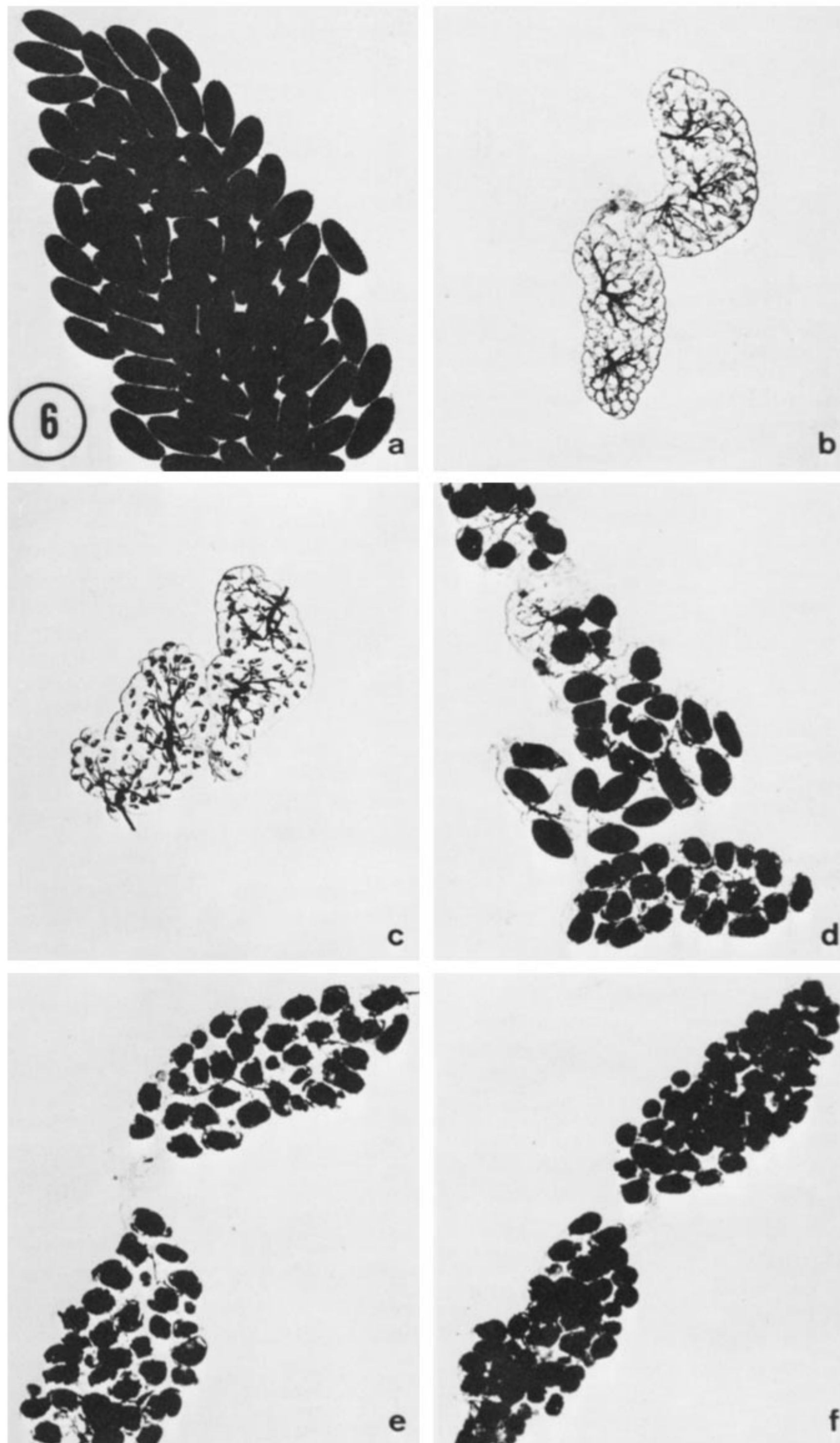

FIGURE 6 Comparison of normal ovarian development at 72-h post blood meal with that initiated by injection of β-ecdysone: (*a*) normal post blood meal ovary, (*b*) ovaries of a saline-injected control female; ovaries of β-ecdysone-injected females, (*c*) 0.1 μg/μl, (*d*) 1.0 μg/μl, (*e*) 2.5 μg/μl, and (*f*) 5.0 μg/μl, respectively. × 25. Note that Fig. 6 *d* depicts two complete pairs of ovaries.

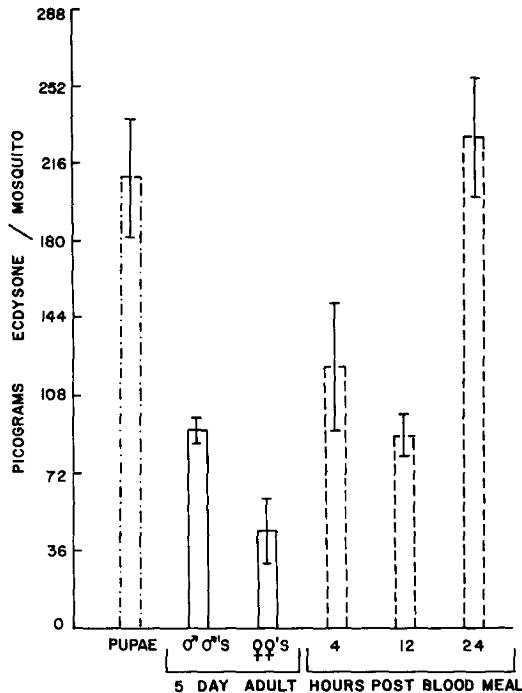

FIGURE 7 Levels of endogenous ecdysone in *A. aegypti* newly emerged pupae, 5-day old adults, and blood-fed females. Five replicates were performed for each stage shown. The variability indicated is the standard deviation.

drocortisone, and estriole), nor by phagostimulants normally present in vertebrate blood (ATP, serum, and hemoglobin). In addition, a juvenile hormone mimic (a terpenoid mixture) and synthetic *Cecropia* juvenile hormone had no discernible affect on ovarian development. Our work reported herein confirms their original observations in all aspects with regard to ovarian development and extends them to include the regulation of dopa decarboxylase by ecdysone. It was with this background that we were led to suggest that ecdysone is normally involved in ovarian development in *A. aegypti*. The results of our initial attempts to determine the endogenous levels of ecdysone in pre- and post-blood meal females support our hypothesis.

A major implication of our hypothesis is that active ecdysone is produced, activated, or released and subsequently functions in an adult insect. Until recently, it was believed that the prothoracic glands synthesized the active hormone, and since mosquitoes (14) as well as most other adult insects do not retain this gland (15), ecdysone was thought to have little if any normal function in adults. The fact that significant levels of ecdysone have been detected in several adult insects, such as the locust (16), the silkworm (17), and the milkweed bug (18), has been referred to as "a slightly puzzling fact" by so eminent an authority as Wigglesworth (15). β-Ecdysone is formed by enzymatic conversion from its precursor α-ecdysone (19), and there is now ample evidence indicating that β-ecdysone is the active form of the hormone (20). Nakanishi et al. (21) have shown that cholesterol is converted into both α- and β-ecdysone in isolated abdomens of silkworm larvae in the absence of the prothoracic gland, and Ellis et al. (22), working with locust prothoracic glands, concluded that this gland does not produce β-ecdysone. Thus, although there is not unanimous agreement among insect physiologists, more and more evidence is accumulating which strongly suggests that the presence of the prothoracic gland is not universally required for ecdysone activity. Conversely, the absence of these glands in adult insects does not necessarily mean, as had been previously assumed, that ecdysone is not produced and does not function in the adult.

The results of the Borst-O'Connor radioimmune assay for ecdysone are consistent with our hypothesis which supposes a normal gonadotropic function for ecdysone in adult mosquitoes, but by no means does it prove it. Our only firm conclusion from this assay can be that the concentration of a substance which is antigenically similar to β-ecdysone is present in low levels in non-blood-fed females and increases after blood feeding. Given that known β-ecdysone will initiate ovarian development, we suggest that the increase in antigenic specificity observed after a blood meal toward β-ecdysone antibody is due to an endogenous increase in an active ecdysone-like hormone. We are not prepared, at present, to specifically identify this material. This point is especially pertinent because Spielman et al. (7) found that 22-isoecdysone, an isomer of ecdysone which is devoid of molting hormone activity in other insect tissues, will also stimulate ovarian development in *A. aegypti*. Experiments to determine the biological activity of our antigenic-positive material as well as its chemical and physical properties in order to identify it are now being performed. Concurrently, the synthesis and function of this material in ovarian develop-

ment, whether it be bonified β-ecdysone or an analogue, are also being investigated.

Considerable literature has accumulated dealing with the relationship of the blood meal to the hormonal control of ovarian development in mosquitoes. Larsen and Bodenstein (23) reported that a blood meal is not obligatory for ovarian development in anautogenous mosquitoes. They sealed the anus with wax in a group of mosquitoes and fed them fruit juice. This resulted in a marked, prolonged distension of the gut, and by the 4th day, ovarian development had been initiated in a number of the mosquitoes. They concluded that distension of the abdomen for a sufficient period of time stimulates afferent nerve impulses leading directly or indirectly to the release of gonadotropic hormone. In mosquitoes, stretch receptors on the midgut or in the abdominal wall which send nerve impulses in order to initiate ovarian development have not been described. In fact, there is firm evidence that they do not exist (24, 25). Moreover, the suggestion that sustained gut stretching initiates egg development by anautogenous mosquitoes has not been confirmed by other workers (26). However, if gut distension does initiate a nervous impulse to the brain, it is possible that the pathway may proceed via the stomatogastric system (24).

Lea and co-workers (25, 27, 28), on the basis of electron microscopy and histological and ablation experiments, have concluded that in mosquitoes as in other Diptera, the corpus allatum (CA) and medial neurosecretory cells (MNC) each produce a hormone that is required for yolk deposition. The neurosecretory hormone (EDNH) appears to be stored in the corpus cardiacum which "meters" the release of EDNH for each batch of eggs after a blood meal. Parabiosis experiments indicated that a humoral factor was necessary for this release (25). The chemical nature of EDNH is unknown except that its presence in the cardiacum was found to be independent of positive paraldehyde-fuchsin staining material (29). The suggestion that a hemolymph-borne humoral factor is needed for ovarian development which is regulated by the blood meal is a viable alternative to the stretch receptor-nerve impulse idea (25). Other workers (30) have shown that the ovary is the source of a humoral factor which activates the fat body to synthesize vitellogenin in mosquitoes. They further state that injected ecdysone mimics the effect of the ovary. Obviously then, the ovary is a prime suspect as the likely anatomical site responsible for the increase of the ecdysone-like material we have observed after the blood meal. Experiments designed to determine the ecdysone titer of ovariectomized blood-fed females are now underway.

There are at least three major difficulties with our hypothesis: (*a*) Why do we observe so long a time lag between the ecdysone injections and the first indication of dopa decarboxylase activity? (*b*) Why is so high a concentration of ecdysone required to elicit a response? And, as corollaries to (*a*) and (*b*) above, (*c*) How can the injected ecdysone remain biologically active over a period of days? These are difficult questions and, frankly, require much more data than is presently available before definitive answers can be offered. However, we can offer what we think are reasonable speculations. Objections (*b*) and (*c*) are related in that exogenous ecdysone may be degraded in adult mosquitoes as has been shown in other adult insects (31). This fact may lend credibility to the large doses of ecdysone required to elicit a positive response. Even if ecdysone is not degraded, it is presumed that the injected material must go to some target tissue(s) in order to elicit a given response; therefore, it is possible that the large amount required for such a response is a reflection of random diffusion of the hormone in the insect. In reality, it may well be that only a very small amount of ecdysone actually reaches the appropriate site. The time lag question may not be debatable at all, since a similar lag is observed after a blood meal (3). The question should more probably focus on the action of ecdysone at the molecular level, and our work is proceeding along these lines.

It has been shown in other insects that high doses of exogenously introduced ecdysones result in abnormalities (32), and more specifically, it was found that high doses of ecdysone, when fed to adults, severely inhibited ovarian development in houseflies (33). Wright et al. (34) reported that β-ecdysone, when ingested by the stable fly, prevented vitellogenesis in developing oocytes. The opposite occurs in *A. aegypti* and, it is difficult to conceive that the positive biological responses we and others have observed after ecdysone treatment (i.e. initiation of ovarian development, stimulation of dopa decarboxylase activity, and vitellogenin synthesis by the fat body) are merely artifactual.

A most intriguing result was our recent finding that when cyclic AMP and β-ecdysone are injected simultaneously into non-blood-fed females, the dopa decarboxylase activity after 72 h is increased by approximately 50% (6). Cyclic AMP injected alone did not elicit any enzymatic activity above that of the saline control. Thus, the nucleotide is not a second messenger for ecdysone in this system. Further investigations to determine the endogenous levels of cyclic AMP (as well as other cyclic nucleotides) and the appropriate cyclases and their relationship to ecdysone are underway.

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