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Ecdysteroid Titers during Pupal and Adult Development in *Drosophila melanogaster*

ALFRED M. HANDLER

Developmental Biology Center, University of California, Irvine, California 92717

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Ecdysteroid titers were determined by radioimmunoassay during prepupal, pupal, and adult development, and during adulthood in *Drosophila melanogaster* males and females synchronized at pupariation or adult eclosion. At several times in development ecdysteroids were identified using chromatographic techniques. After an ecdysteroid peak at pupariation, a small peak occurred after 10 hr at 25°C, with a subsequent rapid and large increase in activity at 30 hr. One, and possibly two more rapid peaks occurred during the mid-pupal period, with a gradual decline to basal levels in the pharate adult. During the first day of adulthood, the ecdysteroid level declined further and remained at approximately 10 pg/mg fresh weight for 2 weeks. Titers were not significantly different between males and females at any of the stages tested, with the biologically active 20-hydroxyecdysone hormone accounting for 70 to 90% of the ecdysteroids in both sexes. Ecdysteroids in adult females were localized to the hemolymph and abdominal tissue, but not to the ovaries. It is concluded that differences in the concentration of 20-hydroxyecdysone in males and females are not responsible for the sexually dimorphic expression of the yolk protein genes, and that a source other than the ovaries provides hormone in females for this purpose.

INTRODUCTION

In insects, the molting hormone, 20-hydroxyecdysone (20-OH-ecdysone), plays an important role during larval and pupal growth, and at molting, which advances the insect from one stage of development to the next. With the advent of radioimmunoassay and chromatographic techniques (Borst and O'Connor, 1972, 1974), the presence and activity of ecdysteroids in adult insects have also been discovered and studied with great interest. 20-Hydroxyecdysone exerts its effect by regulating gene activity (Ashburner, 1973) and it is therefore of particular interest to understand the various effects of 20-OH-ecdysone, and the time and extent of its activity, in a genetically well-characterized insect such as *Drosophila melanogaster*. For this reason the analysis of ecdysteroid activity in *Drosophila* has been quite extensive in recent years with attention concentrated on the larval and pupal periods (Borst *et al.*, 1974; DeReggi *et al.*, 1975; Garen *et al.*, 1977; Hodgetts *et al.*, 1977; Berreur *et al.*, 1979; Klose *et al.*, 1980; Maroy *et al.*, 1980; Richards, 1981). Analyses of ecdysteroids in adults have been less extensive, but have indicated that they do exist at physiologically active levels, with a transiently higher level in females (Hodgetts *et al.*, 1977). The majority of these ecdysteroids in females have been localized to the ovaries (Garen *et al.*, 1977). These results suggested a role for 20-OH-ecdysone during *Drosophila* oogenesis, especially considering the finding that 20-OH-ecdysone stimulates yolk protein synthesis in several insects, including the dipterans *Aedes aegypti* (Fallon *et al.*, 1974) and *Sarcophaga bullata* (Huybrechts and DeLoof, 1977). Subsequently, we found in *Drosophila* that 20-OH-ecdysone stimulates yolk protein synthesis in abdomens isolated from adult females (Handler and Postlethwait, 1978; Postlethwait and Handler, 1979) and in female adult fat body cultured *in vitro* (Jowett and Postlethwait, 1980). Our initial aim for the study reported here was therefore to continue the analysis of ecdysteroid activity in *Drosophila* adults to determine the extent to which differences in titers exist between males and females, and to determine whether the timing of such differences correlates with the sexually dimorphic production of yolk proteins.

Since yolk protein synthesis begins shortly before adult eclosion, an increase in 20-OH-ecdysone titer might be expected at some time during the latter part of metamorphosis in pharate adults, a time not previously assayed. This possibility led us to also carefully reanalyze the titers of ecdysteroids during metamorphosis in animals individually synchronized as white prepupae.

Since ecdysteroids have previously been detected in *Drosophila* ovaries (Garen *et al.*, 1977) and ecdysone is an ovarian hormone in *Aedes* (Hagedorn *et al.*, 1975), it was our second aim to discover whether the ovaries in *Drosophila* also secrete ecdysone to stimulate yolk protein synthesis, or if they accumulate it, possibly from an exogenous source, for embryonic development.

In addition to 20-OH-ecdysone, an analog of juvenile

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hormone (ZR-515) also stimulates yolk protein synthesis, and the effect is additive with 20-OH-ecdysone treatment (Postlethwait and Handler, 1979; Jowett and Postlethwait, 1981). While it is possible that both hormones independently perform the same function, experiments in tissue culture and isolated abdomens suggest the possibility that juvenile hormone, which is secreted by the corpus allatum in the thorax, stimulates the production or activity of 20-OH-ecdysone, which may then directly act upon the fat body. We therefore tested the influence of juvenile hormone on ecdysteroid titers in isolated abdomens.

METHODS

Culture and preparation of animals. *Oregon-R* wild-type *Drosophila* were raised on standard cornmeal–molasses–agar medium at either 20 or 25°C under a 12-hr light:12-hr dark photoperiod. For assays during metamorphosis, animals were individually selected subsequent to spiracle eversion as white prepupae early in the light cycle. This synchronizes animals to within 30 min of one another. White prepupae were washed in water and tanning prepupae selected out. For some experiments sexes were separated by distinguishing gonad size. Pupae were then placed on filter paper and kept under the above conditions.

Adult animals were collected at less than 2 hr after eclosion and raised on culture medium at 25°C. For adult stages of less than 1 day, animals were collected within 15 min of eclosion. Abdomens were isolated from newly eclosed females by tying a nylon ligature between the thorax and the first abdominal segment, and then cutting just anterior to the ligature. Isolated abdomens were treated with a 0.15-μl topical application of 10^{-3} *M* juvenile hormone analog, ZR-515 (a gift from Zoecon Corp.) dissolved in acetone from a 10-μl Hamilton syringe.

Sample preparation for radioimmunoassay. Animals to be assayed were counted, weighed, and homogenized vigorously in 1 ml 70% methanol containing 10^{-3} *M* phenylthiourea using a ground glass microtissue grinder. Final sample concentrations were 70 to 80 mg fresh wt (f.w.)/ml. Samples were vortexed and usually stored at −70°C until assayed. Just previous to the assay, samples were centrifuged at 12,000*g* for 15 min and aliquots taken from the supernatant. Samples of heads, thoracices, and abdomens were taken from the same animals by cutting body regions with an iridectomy scissor. Experiments in which abdominal body walls, immature ovaries, and internal tissues were assayed, tissues were dissected from the same animals in Robb's (1969) medium or *Drosophila* Ringer's (Chan and Gehring, 1971) solution and rinsed twice to wash away residual hemolymph. Tissues were collected together, excess medium was aspirated away and then homogenized in 70% methanol. For other assays, ovaries or ring glands were cultured in fresh medium, after which the tissue and medium were collected separately in 70% methanol, homogenized, and centrifuged. Hemolymph was collected from either white prepupae or newly eclosed adults by puncturing the pupal cuticle or integument with a drawn-out microcapillary coated with phenylthiourea. At least 15 μl of hemolymph was collected from approximately 150 animals (0.1 μl per animal), which was then extracted in 0.5 ml 70% methanol and centrifuged.

Radioimmunoassay. Radioimmunoassay was performed as described by Borst and O'Connor (1974) on 50- and 100-μl replicate aliquots of methanol extracts of each sample. The competition assay was performed with [23, 24-^{3}H]ecdysone (63 Ci/mmole; New England Nuclear), and antiserum to ecdysteroids was a kind gift from Dr. J. D. O'Connor. This antiserum (Horn I-2, 17-week) was prepared to the 22-hemisuccinate ester of ecdysone conjugated to thyroglobulin (Horn *et al.*, 1976) and has an approximate binding ratio of 2:1 for ecdysone to 20-OH-ecdysone.

Standard curves for the competition assay were obtained with unlabeled 20-OH-ecdysone at amounts ranging from 0 to 4000 pg. Therefore all titers, except for determination of activity in thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) fractions, are 20-OH-ecdysone equivalents which are expressed as "ecdysteroids" for convenience. For titering fractions from TLC and HPLC, standard curves for both 20-OH-ecdysone and ecdysone were obtained, allowing a direct measurement of activity in those fractions which comigrated with either the 20-OH-ecdysone or ecdysone standards.

Chromatographic techniques. For thin-layer chromatographic identification of ecdysteroids, 200 to 1000 animals were homogenized in 70% methanol, and centrifuged at 12,000*g* for 15 min. The extract was either dried down to 0.1 ml and chromatographed, or partially purified according to Chang *et al.* (1976) by initially partitioning against hexanes, redissolving in butanol, and partitioning against water. Combined butanol fractions were dried down and resuspended in 0.1 ml methanol. After centrifugation at 12,000*g* for 15 min the supernatant was streaked on a TLC silica gel plate (F-254, Merck Co.). Ecdysone (Simes) and 20-OH-ecdysone (Rohto Pharm.) standards were spotted separately on the plate which was developed in chloroform:96% ethanol (80:20). Bands, 1 or 0.5 cm were scraped from air-dried plates and the fractions eluted in methanol.

Fractions with ecdysteroid activity were determined by radioimmunoassay.

For high-pressure liquid chromatography ecdysteroid-active fractions from TLC were combined, dried down, resuspended in methanol, and centrifuged at 12,000*g* for 5 min. The supernatant was dried down and resuspended in 55% methanol (Nanograde, Mallinckrodt), and injected into an HPLC system using a Model 6000 solvent delivery system with a Model U6K universal injector (Waters Assoc.). The HPLC separation was performed on a reversed-phase Radial-PAK C-18 column (Waters Assoc.) at a flow rate of 2.5 ml/min. Ecdysone and 20-OH-ecdysone standards were also run through the column at the same rate and detected by uv absorption, and also collected as fractions. Fractions were collected at 30-sec intervals (1.25 ml) and ecdysteroid activity was determined by radioimmunoassay. Activity in fractions cochromatographing with the ecdysone or 20-OH-ecdysone standards was determined by radioimmunoassay, with an ecdysone or 20-OH-ecdysone standard curve, respectively.

RESULTS

Ecdysteroid activity during metamorphosis. For the first assay, animals of mixed sex were individually collected as white prepupae and allowed to develop under a 12-hr light:12-hr dark photoperiod at 25°C. Under these conditions adult eclosion occurs between 96 to 98 hr after pupariation (Handler and Postlethwait, 1977). During this period ecdysteroids were assayed at 3- to 5-hr intervals. The resulting developmental profile (Fig. 1) agrees qualitatively with profiles reported by Hodgetts *et al.* (1977) and Klose *et al.* (1980). A small peak (150 pg/mg f.w.) at 10 hr probably corresponds to the increase Klose *et al.* (1980) reported at 12 hr. After this minor peak, the amount of ecdysteroids increases sharply to a level of about 720 pg/mg f.w. at 30 hr, which was also previously observed. By 36 hr the titer decreases, with another subsequent sharp increase 40 hr after pupariation. A third, smaller mid-pupal peak is observed at 48 hr. The three mid-pupal peaks observed in this assay were not resolved by Hodgetts *et*

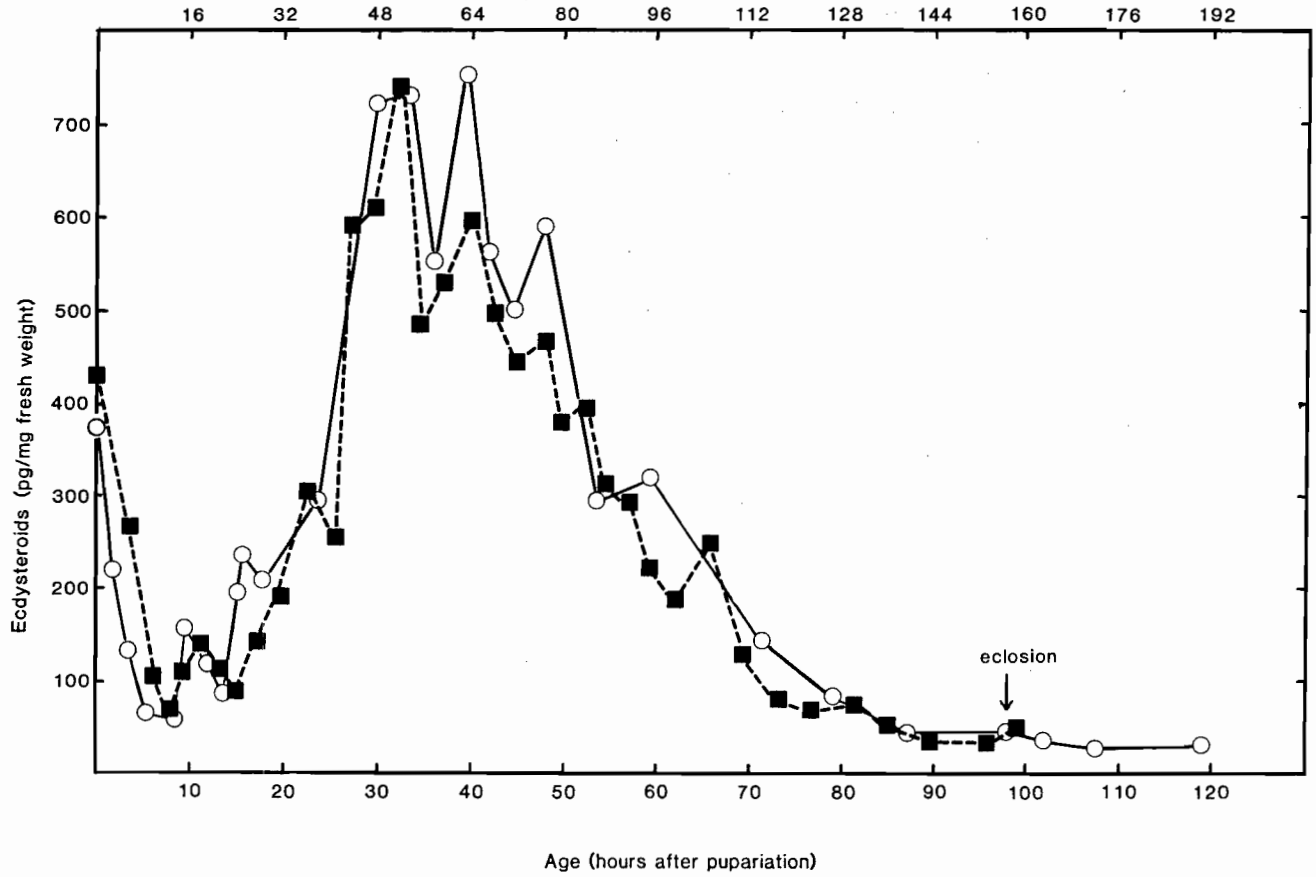

FIG. 1. Ecdysteroid titers during the prepupal, pupal, and pharate adult stages, in animals raised at either 20°C (■ - - - ■; upper time scale) or 25°C (○ —— ○; lower time scale). For each time point 70 animals were selected as white prepupae, raised at the indicated temperature, and weighed just prior to the time of extraction in 70% methanol. Titers at each temperature were determined in the same radioimmunoassay. Ecdysteroids represent 20-OH ecdysone equivalent values.

al. (1977), who observed a large broad peak at this time probably due to their longer time intervals between samples. In our assay at 25°C the rapid succession of the first two peaks could be due to quantitative variance since they are distinguished by only one time point at a lower titer. To resolve these peaks the developmental profile was repeated on animals raised at 20°C. At this temperature the time from pupariation to eclosion is extended from 98 to 160 hr, allowing titers at more time points for better resolution. This profile is also plotted, on an adjusted time scale, in Fig. 1. The profiles at 25 and 20°C are quite similar both qualitatively and quantitatively, with respect to the proportional changes in developmental time according to temperature. Again, a rapid decrease in the initial major midpupal peak at 52 hr is observed, with a second peak at 64 hr. The second peak decreases in a stepwise fashion, reaching a level of about 100 pg/mg f.w. by the last quarter of metamorphosis, during adult development. Ecdysteroid titers continue to fall at a decreasing rate until the end of the pharate adult stage. At this point previous to eclosion, titers vary between 30 and 50 pg/mg f.w., which are close to the basal levels observed between larval molts (Maroy *et al.*, 1980).

We have observed previously that under identical conditions females develop faster than males, and eclose as adults 4 to 5 hr before males when synchronized as white prepupae early in a photoperiod (Handler and Postlethwait, 1977). Since a difference of 1 hr in developmental age could obscure rapid fluctuations in ecdysteroid titer in a mixed sex population, we also assayed ecdysteroids in males and females separately at 6-hr intervals between pupariation and eclosion. In addition it was also necessary to titer ecdysteroids separately in male and female pharate adults since yolk protein synthesis begins at, or just before, adult eclosion. Therefore a surge of ecdysteroids might occur in females previous to this event as occurs at larval molting, or perhaps ecdysteroid levels increase in females relative to males during pharate adulthood. Figure 2 shows that while titers per milligram of fresh weight are slightly higher in males, there is no substantial difference in the titers between males and females, or in the timing of ecdysteroid fluctuations during the pupal and pharate adult stages. Notice the loss of resolution when time intervals are increased compared to Fig. 1.

TLC and HPLC chromatographic analyses of ecdysteroids (Table 1) at pupariation indicate that the ecdysone to 20-OH-ecdysone ratio at this time is 1:18. While 20-OH-ecdysone is clearly the predominant ecdysteroid, the presence of ecdysone at this time was not previously detected (Borst *et al.*, 1974). At the first major mid-pupal peak, 30 hr after pupariation, ecdysone and 20-OH-ecdysone are now present in equivalent amounts.

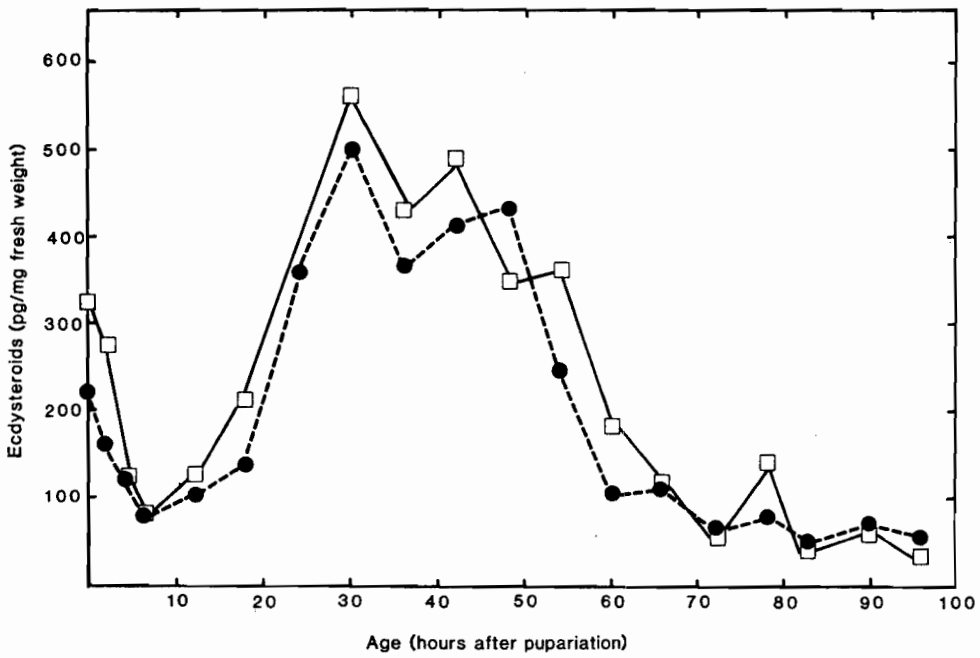

FIG. 2. Ecdysteroid titers of prepupal, pupal, and pharate adult females (● - - - ●) and males (□ —— □) raised at 25°C. Samples were prepared as described in Fig. 1 except that animals were sexed as white prepupae. Titers were all determined in the same radioimmunoassay. Ecdysteroids represent 20-OH-ecdysone equivalent values.

TABLE 1
MOLAR RATIO OF ECDYSTEROIDS DURING *Drosophila* DEVELOPMENT

Developmental stage	Ecdysone:20-hydroxyecdysone[a]
White prepupae	1:18
30-hr pupae (25°C)	1:1
Females at eclosion	1:9
Males at eclosion	1:13
Females at 2 days	1:2
Males at 2 days	1:7

[a] Ratios represent the mean of at least two determinations by TLC and HPLC.

Ecdysteroid activity during adulthood. At the time of adult eclosion the mean ecdysteroid titer in females is 35 pg/mg f.w. and 28 pg/mg f.w. in males (Fig. 3). These titers decrease coincidentally in both sexes to a level of less than 10 pg/mg f.w. within the first day after eclosion. In both sexes the ecdysteroid level rises to 15 pg/mg f.w. by 4 days after eclosion, and subsequently decreases. The titers in females fluctuate between 4 and 12 pg/mg f.w. during the next 10 days, while in males the titers are maintained closer to 5 pg/mg f.w.

Chromatographic analysis of ecdysteroids in newly eclosed females indicates an ecdysone to 20-OH-ecdysone molar ratio of 1:9, while in males the ratio is closer to 1:13 (Table 1). By 2 days after eclosion the ecdysone to 20-OH-ecdysone molar ratios are 1:2 and 1:7 in females and males, respectively. Thus, the level of 20-OH-ecdysone is slightly higher in males at these times given the same ecdysteroid titer in both sexes. Apparently adult males and females exhibit comparable levels of 20-OH-ecdysone, which can be extrapolated to a concentration of greater than 10^{-7} *M* in both sexes (assuming a hemolymph volume of 0.2 μl).

Localization of ecdysteroids in adult females. Previous reports indicate that ecdysteroids in adult *Drosophila* females are localized in the abdomen, and that most of these ecdysteroids are found in the ovaries (Garen *et al.*, 1977). Since 20-OH-ecdysone is important to the stimulation of yolk protein synthesis, we wanted to more clearly determine whether *Drosophila* ovaries have an endocrine function, or whether an alternative tissue might provide ecdysone for vitellogenesis. Therefore, we measured ecdysteroid activity in different adult body regions, and then in particular tissues, dissected from 100 animals.

Table 2 shows that more than 80% of total ecdysteroid activity is present in the abdomens of newly eclosed females, which agrees with the finding of Garen *et al.* (1977). However, only one-third of the expected abdominal ecdysteroids were recovered from dissected tissues after two assays. This activity was distributed between the hemolymph and other abdominal internal tissues, including the Malpighian tubules and gut. Ecdysteroids were not found in immature ovaries, and only in trace amounts in body walls which contain within them both fat body and oenocytes, as well as epidermis and muscle. Since ecdysteroids may accumulate in maturing oocytes of older females (Lagueux *et al.*, 1977), we also assayed mature yolky ovaries from 3- to 5-day-old females, and again found no activity. Assays on immature and mature ovaries were repeated four times using varying amounts of tissue, and in no instance were ecdysteroids detected. The possibility remains though, that ecdyste-

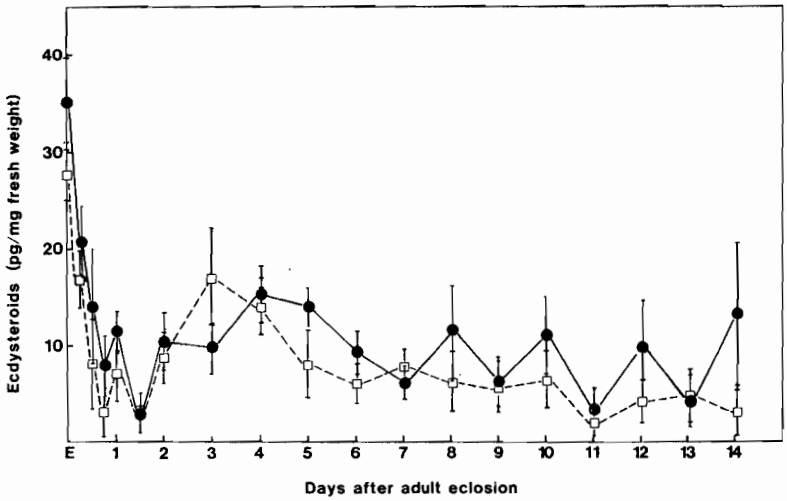

FIG. 3. Ecdysteroid titers of adult females (● —— ●) and males (□ - - - □) selected at eclosion and raised at 25°C. For each assay samples were prepared as described in Fig. 1. Titers are plotted as the mean value ± SEM of three to five separate assays for each time point. Ecdysteroids represent 20-OH-ecdysone equivalent values.

TABLE 2
ECDYSTEROID LEVELS IN *Drosophila* TISSUES

Tissue	Ecdysteroid content[a]
Body regions from females at eclosion	
Head	2 pg/head
Thorax	2 pg/thorax
Abdomen	20 pg/abdomen
Tissues from females at eclosion	
Abdominal body walls	<1 pg/body wall
Abdominal internal tissue	4 pg/gut
Immature ovaries	0 pg/ovary
Hemolymph	10 pg/μl (ca. 2 pg/animal)
Male hemolymph at eclosion	7 pg/μl (ca. 1.4 pg/animal)
Mature ovaries	0 pg/ovary
Mature ovaries in culture	0 pg/ovary
Culture media	0 pg/ovary
Immature ovaries in culture	0 pg/ovary
Culture media	0 pg/ovary
Late third larval instar ring glands	
Cultured glands (Robb's medium)	5 pg/gland
Culture media (Robb's medium)	221 pg/gland
Culture media (Ringer's medium)	450 pg/gland

[a] Mean titers of 20-hydroxyecdysone equivalents.

roids accumulate in a conjugated form not detected by our assay.

While our assays do not show an accumulation of ecdysteroids in ovaries, this does not preclude the possibility that the ovaries act as an endocrine gland, as do the ovaries in *Aedes aegypti* (Hagedorn *et al.*, 1975). Studies in other arthropods suggest that rapid secretion of ecdysone from the endocrine tissue may result in only a small, or even a nondetectable, accumulation in the gland itself (Chang *et al.*, 1976). We therefore dissected out 100 immature and mature ovaries and put them into tissue culture separately with Robb's (1969) medium for 24 hr, assaying the tissues and media separately after incubation. Ecdysteroid activity was not detected in either tissue or culture medium (Table 2). A possible cause for a lack of ovarian endocrine function could be inadequate culture conditions. As a test for these culture conditions, late-third-larval instar ring glands containing the prothoracic gland were also cultured in Robb's (1969) medium (15 glands), as well as in *Drosophila* Ringer's solution (Chan and Gehring, 1971; 20 glands). Table 2 shows that ring glands maintained activity for 24 hr under both conditions, although interestingly, twice as much activity was sustained in the Ringer's solution as by the more complex medium.

After three assays we observed a mean level of 10 pg/μl ecdysteroids in the hemolymph of newly eclosed females, while 7 pg/μl ecdysteroids was detected in male hemolymph. Our inability to recover all the abdominal ecdysteroid activity led us to wonder whether activity may have been lost from the hemolymph sample, during either collection or extraction in methanol. As a control test for our procedure, we also assayed hemolymph from white prepupae and observed a titer of 1600 pg/μl which corresponds to the levels found in whole organisms. To test for the possibility that hormone could be trapped in fat body or precipitated protein which was centrifuged out, we injected 0.1 μCi [^{3}H]ecdysone into a control sample of females before collecting their hemolymph. The hemolymph was collected in methanol and centrifuged in the normal fashion. Radioactivity in the supernatant and pellet was counted by liquid scintillation, and the results indicated that all of the [^{3}H]ecdysone was present in the supernatant.

Influence of juvenile hormone on ecdysteroid activity. To determine whether juvenile hormone stimulates yolk protein synthesis by stimulating an abdominal tissue to secrete ecdysone, we treated isolated abdomens from newly eclosed females with a juvenile hormone analog, ZR-515 and measured ecdysteroid activity at various times after treatment. Times beyond 6 hr were not tested, since the vitellogenic response to the juvenile hormone analog occurs before this time (Postlethwait and Handler, 1978). Figure 4 shows that juvenile hormone treatment does not increase ecdysteroid activity, and at some time points the activity is actually decreased.

DISCUSSION

Ecdysteroid activity in pupae. The profile of changes in ecdysteroid activity during *Drosophila* pupal and adult development observed in this study is comparable to profiles of activity detected by radioimmunoassay in previous analyses (see review by Richards, 1981), and shows a general similarity to changes in ecdysteroid

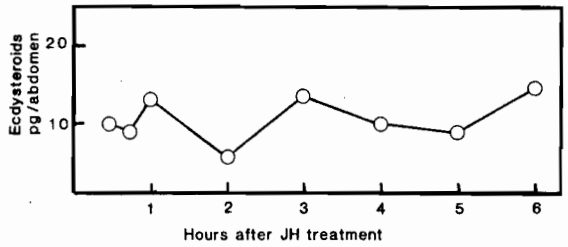

FIG. 4. Ecdysteroid titers of isolated female abdomens treated with juvenile hormone analog, ZR-515, and incubated for various lengths of time. For each time point abdomens from 70 newly eclosed females were ligated, treated with ZR515 after 24 hr, and then incubated at 25°C before extraction in 70% methanol. The ecdysteroid level in untreated abdomens is 13 ± 3 pg/abdomen at 24 hr after ligation.

levels seen in other dipterans during metamorphosis (Briers and DeLoof, 1980; Wentworth *et al.*, 1981). By assaying ecdysteroids at close time points, and extending the pupal period by decreasing temperature we were able in this study to discover two, and possibly three, rapid peaks during the mid-pupal period. This fluctuation in ecdysteroid titer compares quite well with changes in titer seen in *Sarcophaga* at a comparable time in development (Wentworth *et al.*, 1981), although this fluctuation was not detected in an earlier study with *Drosophila* due to the longer time intervals between samples (Hodgetts *et al.*, 1977). We also observed a smaller peak at 10 hr after pupariation which was also reported by Klose *et al.* (1980) and which coincides with the appearance of a 20-OH-ecdysone-dependent prepupal salivary gland puff (Richards, 1976).

Chromatographic analyses of ecdysteroids at the first major mid-pupal peak (30 hr at 25°C) reveal equivalent levels of ecdysone and 20-OH-ecdysone. This is in contrast to the clear predominance of 20-OH-ecdysone at pupariation. In this radioimmunoassay, the first two mid-pupal peaks have ecdysteroid activity (20-OH-ecdysone equivalents) at a threefold higher level than was detected previously (Hodgetts *et al.*, 1977). Since the antiserum used in this study has a twofold higher sensitivity to ecdysone than to 20-OH-ecdysone, the actual level of ecdysteroids at this time is closer to the amount reported by Hodgetts *et al.* (1977), whose antiserum was equally sensitive to ecdysone and 20-OH-ecdysone. The biological significance of fluctuations in ecdysteroid molar ratios is difficult to assess without knowing the turnover rates for each product. Clearly, ecdysteroid activity persists at higher than basal levels throughout most of the pupal period, and ecdysone is being produced and hydroxylated to 20-OH-ecdysone during this time.

Although a portion of this investigation is dedicated to finding the source of ecdysone in the *Drosophila* adult, it is of equal interest to consider the source of ecdysone in the pupae. We have shown here that indeed, a tissue within the larval ring gland, probably the prothoracic gland, secretes ecdysteroids. Yet, in other holometabolous insects the prothoracic gland degenerates early in the pupal period. It is possible that the prothoracic gland persists long enough to rapidly secrete the large amounts of ecdysone observed in pupae at 30 hr. The slow stepwise reduction in ecdysteroid titer during the remainder of the pupal period and pharate adulthood could then be the result of a regulated conversion of the accumulated ecdysone to 20-OH-ecdysone, which is then utilized by differentiating imaginal discs. One method of storage of ecdysone and 20-OH-ecdysone during this period may be through inactivation of the hormones by conjugation (Willig *et al.*, 1971). Ohtaki (1981) has shown in *Sarcophaga*, which has a similar developmental profile of pupal ecdysteroid activity (Wentworth *et al.*, 1981), that 20-OH-ecdysone injected into pupae may be initially inactivated by conjugation, and then subsequently liberated as active 20-OH-ecdysone at a later time (Moribayashi and Ohtaki, 1980). In lieu of an active endocrine system, biologically active ecdysteroid levels could be regulated enzymatically by reversible conjugation during the pupal period. Apparently, ecdysteroids become available to developing *Locusta* embryos in this fashion. Hydrolysis of maternally derived conjugated ecdysteroids make free ecdysone and its precursor, 2-deoxyecdysone, available to the embryo for cuticle deposition over a several-day period, before *de novo* ecdysone synthesis can begin (Lagueux *et al.*, 1981).

Activation of conjugated ecdysteroids would preclude the necessity for an active endocrine gland during metamorphosis. It would suggest that the high ecdysteroid content at this time is not just to stimulate particular developmental events (i.e., cuticle deposition), but may also reflect the accumulation of ecdysteroids to be utilized later in pupal and adult development.

Ecdysteroid activity in adults. In pharate adults 20 hr before eclosion ecdysteroid activity has reached low basal levels of 30 to 50 pg/mg f.w., which are maintained until eclosion. Within the first day after eclosion ecdysteroid titers decrease by about 70% in both males and females, and persist at close to that level for at least 2 weeks. In females yolk protein synthesis, which can be experimentally induced by 20-OH-ecdysone, begins at or just before eclosion (Handler and Postlethwait, 1977; Postlethwait and Kaschnitz, 1978). Clearly, in this assay there is no apparent surge in ecdysteroid titer coincident or previous to this event, nor is there a slow increase in titers in females relative to males. On the other hand, Hodgetts *et al.* (1977) report equivalent titers for males and females at eclosion, but they find that titers in females increase threefold relative to males after 2 days. While it is conceivable that our assay missed this ecdysteroid surge due to its low level or to differences in sample preparation or antisera, there are several reasons why such a possible difference may not be important to vitellogenesis in females. These are: (1) the increase in female titer begins several hours after yolk protein synthesis begins in the pharate adult; (2) the surge is discrete, with male and female titers becoming equivalent again after three to 4 days, while yolk protein synthesis continues in the female for several weeks (sequential radioimmunoassays, however, have not been carried out with older females); (3) the basal level of ecdysteroids found in both males and

females is sufficient to promote yolk protein synthesis, assuming that most of the ecdysteroid activity is 20-OH-ecdysone. Chromatographic analyses indicate that 90% of the ecdysteroid content of females at eclosion is 20-OH-ecdysone, while in males 93% is 20-OH-ecdysone. If the hemolymph volume of a newly eclosed female is about 0.2 μl and the majority of 20-OH-ecdysone is present here, then the 20-OH-ecdysone concentration would be about 4×10^{-7} *M*. We have previously shown that the half-maximal effect of 20-OH-ecdysone on yolk protein synthesis in isolated abdomens occurs at about 7×10^{-7} *M* (Postlethwait and Handler, 1979). The actual amount of ecdysteroids that we find in female hemolymph is 10 pg/μl (2×10^{-8} *M*) which is considerably less, but which correlates exactly with the concentration found in the hemolymph of *Sarcophaga* females, and has been shown to be sufficient to promote yolk protein synthesis in this insect (Briers and DeLoof, 1980). It is also possible that 20-OH-ecdysone within these organisms is compartmentalized, and exists at a higher concentration in between the endocrine tissue (or some storage site) and the fat body. In addition, an effect of 20-OH-ecdysone on *Drosophila* imaginal discs (Fristrom *et al.*, 1973), salivary glands (Ashburner, 1973), and embryonic cells (Cherbas *et al.*, 1977) in tissue culture can be detected at close to 10^{-8} *M*.

Localization of ecdysteroids in adults. Both ecdysone and 20-OH-ecdysone exist in *Drosophila* adults, but the source of these hormones has remained an enigma. In various insects such as *Aedes aegypti* (Hagedorn *et al.*, 1975), *Locusta* (Lagueux *et al.*, 1977), *Sarcophaga* (Briers and DeLoof, 1980), and *Drosophila* (Garen *et al.*, 1977), ecdysteroids have been found in the ovary, and in *Aedes*, the ovary has been shown to secrete ecdysone (Hagedorn *et al.*, 1975). While a similar function for *Drosophila* ovaries has been an attractive hypothesis (Hagedorn, 1980) most of the information up to this time argues that the ovary does not provide ecdysone for vitellogenesis in this insect. First of all, in this study we have not been able to detect ecdysteroids in immature or mature ovaries, nor have we observed the secretion of ecdysteroids from ovaries in culture. Garen *et al.* (1977) found the bulk of ecdysteroids in adult females within the ovary, with decreased levels found in mutant *ecd*^{1} females kept at 29°C. While these *ecd*^{1} females produce nonviable eggs, yolk protein synthesis is normal, as are yolk proteins sequestered by the oocyte at the restrictive temperature (Postlethwait and Jowett, 1981; Handler, unpublished). Furthermore, we have already shown that yolk protein synthesis is normal, if not higher, in ovariectomized and genetically agametic females (Postlethwait, *et al.*, 1980). Perhaps the most conclusive evidence for the lack of an endocrine role for ovarian tissue during vitellogenesis comes from experiments using a temperature-sensitive allele of the *transformer-2* (*tra-2*) locus (Handler and Belote, in preparation). At 29°C, a chromosomal female *tra-2*^{ts} mutant (X/X;*tra-2*^{ts}/*tra-2*^{ts}) develops in all respects as a phenotypic male. We have found that when these "pseudomales" are transferred to 16°C as adults, yolk protein synthesis commences. Therefore, an ovary is not necessary at any time for vitellogenesis in this mutant, and males must have a source of ecdysone that assumes the same function as in normal females. While ovaries may provide the bulk of ecdysone in some female insects, the presence of ecdysteroids in adult males indicates that males must also have an endocrine source for this hormone. None of our results suggests a source for ecdysone in *Drosophila* adults.

In several insects a large portion of the ovarian ecdysteroids are not biologically active, including conjugated forms of ecdysone and 20-OH-ecdysone which may be utilized during embryogenesis (see review by Hagedorn, 1980). The presence of these ecdysteroids in *Drosophila* has not been excluded by our studies, and indeed these molecules may represent the ovarian ecdysteroids detected by Garen *et al.* (1977) since the specificity of their antiserum was determined solely for ecdysone and 20-OH-ecdysone (L. Kauvar, personal communication). At this point, the ability or necessity for the ovary to act as an endocrine gland in *Drosophila* remains unproven.

Ecdysteroid activity occurs for the most part in the hemolymph and internal abdominal tissues in adults. A large portion of the abdominal tissue is made up of Malpighian tubules, which are a major site for hydroxylation of ecdysone to the biologically active 20-OH-ecdysone molecule in insects such as *Manduca* (Mayer *et al.*, 1978) and *Locusta* (Feyereisen and Durst, 1978). Fat body also has ecdysone hydroxylase activity, but body walls containing both fat body and oenocytes, which have also been suggested as a possible site for ecdysone secretion (Romer *et al.*, 1974), accumulate very small amounts of ecdysteroids. The possibility of testing oenocytes for ecdysone secretion *in vitro* is made difficult by the fact that these cells are distributed among the abdominal segments of the adult, and are enveloped by fat body which can metabolize ecdysone and 20-OH-ecdysone to degradation products. Another possible source of biologically active ecdysteroids is the reactivation of conjugated ecdysteroids, as was postulated previously for metamorphosis. If 20-OH-ecdysone is active throughout the several-week lifetime of the adult, it might seem unlikely that metabolism of conjugated ecdysteroids provides the sole source of hormone throughout this period without *de novo* synthesis.

Ecdysteroid activity and vitellogenesis. The sexually dimorphic production of yolk proteins in female animals is correlated with an increased titer of estrogen in the case of vertebrates (Lazier, 1978; Westley, 1979), or an increase in ecdysteroids in the case of several insects (Hagedorn *et al.*, 1975; Briers and DeLoof, 1980). In this study, we have shown that for *Drosophila*, ecdysteroid levels are nearly equivalent in males and females throughout the pharate adult, and adult stages. Apparently, a difference in the 20-OH-ecdysone concentration is not the reason for the female-specific synthesis of yolk proteins in *Drosophila*, nor has this been proved necessarily so for other insects where ecdysteroid titers do differ. Even if titers in *Drosophila* females are two to three times higher than those of males, previous experimental evidence suggests that males require at least a 1000-fold higher hormone concentration than is found in females, to elicit even a low level vitellogenic response (Jowett and Postlethwait, 1981; Postlethwait *et al.*, 1980). If, indeed, 20-OH-ecdysone activity results in the sexually dimorphic expression of the yolk protein genes in the female adult fat body, then it is unlikely that the concentration of 20-OH-ecdysone is the cause for this. It is more likely that control of reception or intepretation of the hormonal signal by the genome is more important to the synthesis of yolk proteins.

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