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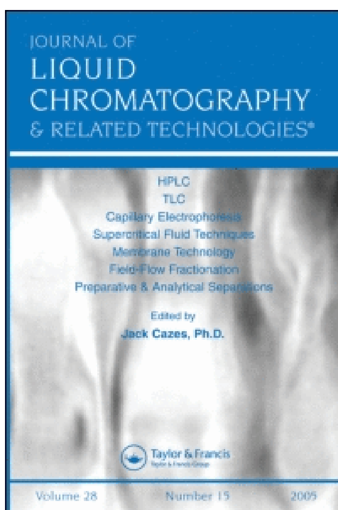

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A Developmental Analysis of Ecdysteroids During the Metamorphosis of *Drosophila Melanogaster*

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A DEVELOPMENTAL ANALYSIS OF ECDYSTEROIDS DURING THE METAMORPHOSIS OF *DROSOPHILA MELANOGASTER*

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ABSTRACT

A combination of ion suppression reverse phase high pressure liquid chromatography (RP-HPLC) and differential radioimmunoassay (RIA) was used to identify and quantify ecdysteroids from Drosophila melanogaster during the development of the white puparium to the adult. The whole body ecdysteroid titer revealed a large peak approximately 30 hours after pupariation. RP-HPLC-RIA analysis of the brain-ring gland complex secretion in vitro revealed the presence of: ecdysone, 20-deoxymakisterone A, and a yet uncharacterized low polarity ecdysteroid (LP-1). The metabolism of ecdysone and 20-deoxymakisterone A was followed by analysis of the ecdysteroids during development. The white puparium (0 hour) yielded a four-fold greater quantity of free 20-hydroxyecdysone and makisterone A than ecdysone and 20-deoxymakisterone A, respectively, indicating high 20-monooxygenase activity during this stage. Another metabolite of ecdysone, 20,26-dihydroxyecdysone, was present at a quantity intermediate between that of ecdysone and 20-hydroxyecdysone. Highly polar ecdysteroids (conjugates) treated with an esterase-phosphatase mixture yielded the free ecdysteroids, ecdysone, 20-deoxymakisterone A, 20-hydroxyecdysone, and makisterone A, suggesting the presence of novel ecdysteroid conjugates of 20-deoxymakisterone A and makisterone A. At 18 hours post-pupariation the levels of all free ecdysteroids were reduced when

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compared to the 0 hour white puparium with the highly polar ecdysteroids predominating. By 30 hour after pupariation the level of free ecdysteroids had increased, indicating net ecdysteroid synthesis. Both ecdysone and 20-deoxymakisterone A were in greater concentrations than 20-hydroxyecdysone and makisterone A, indicating a reduced level of 20-monooxygenase activity at this stage. At 48 hours post-pupariation *Drosophila* contained decreasing amounts of ecdysteroids except for 20,26-dihydroxyecdysone and low polar products. 20,26-Dihydroxyecdysone was the only known ecdysteroid to remain at a high level during the rest of adult development.

INTRODUCTION

As in the case of all insects studied thusfar, the molting process in *Drosophila melanogaster* is initiated by a polyhydroxylated steroid, 20-hydroxyecdysone, which is derived from ecdysone, the latter being a product of the insect's ring (prothoracic) gland (1). Whether ecdysone is strictly a prohormone or has an endocrinological role of its own is not known with certainty but both ecdysone and 20-hydroxyecdysone are examples of a large class of plant and insect steroids termed ecdysteroids (2,3). Notwithstanding the potential of *Drosophila* for solving endocrinological problems at the molecular level because of the possibility of using its potent genetic probes, this insect has been all but neglected in terms of the neuroendocrine control of molting, presumably because of its small size.

This laboratory has initiated a series of investigations on the endocrine control of *Drosophila* growth and metamorphosis with the prospect of cloning the genes for specific neuropeptides and studying the mode of action of several insect hormones in a variety of mutants. As a first step it was important to obtain basic information such as the titers of these hormones during

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development. Although several ecdysteroid titers have been obtained previously (4-7), all used a radioimmunoassay (RIA) procedure utilizing an antiserum derived from a single immunogen (ecdysone or 20-hydroxyecdysone derivative) which in most cases does not show high affinity for ecdysteroids with changes in the A-ring. Further, there have been few attempts to determine the nature of individual ecdysteroids. By the use of RP-HPLC and RIA using two antisera recognizing different portions of the ecdysteroid molecule, it has been possible to characterize the most prominent ecdysteroids in tissue extracts (8), and this strategy has been employed here to investigate the ecdysteroids of *Drosophila* during prepupal to adult development.

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MATERIALS AND METHODS

Chemicals

The acetonitrile, hexane, and methanol were HPLC grade, the ecdysone was obtained from Rohto while 20-hydroxyecdysone and makisterone-A were obtained from Sigma. The 20,26-dihydroxyecdysone was kindly supplied by Dr. M.J. Thompson and Dr. J. Svoboda (U.S.D.A.). Distilled water was deionized through ion exchange resins and activated charcoal organic adsorption columns, and filtered through a 0.2 μm filter (Millipore Milli-Q reagent water system). All other chemicals were reagent grade or higher.

Animal culture and collection

The *Drosophila melanogaster* (Ore-R) were reared on yeasted agar medium in plastic pint bottles at 25°C under a photoperiodic

regime of 12 hour light:12 hour dark. White puparium formation was selected as a convenient point in development for initial staging. The prepupae were placed in petri dishes lined with filter paper and maintained at 25°C under a 12 hour light:12 hour dark photoperiod. At specific developmental time points the animals were removed, weighed, and frozen or homogenized. The animals were stored in acetonitrile at -20°C until 3,000-5,000 animals were collected for HPLC sample preparation.

Sample preparation

For the whole body ecdysteroid titer, animals were collected at an appropriate stage and groups of 10 mixed-sex animals were counted, weighed, and homogenized in 0.5 ml methanol. The homogenate was transferred from the ground glass homogenizer to polypropylene Eppendorf tubes and the centrifuged pellet was extracted twice with 0.5 ml 70% methanol. The supernatants were pooled and concentrated to dryness in a Savant Speed-Vac Evaporator. The samples were resuspended in 70% methanol and aliquots were dried and subjected to RIA.

For the organ culture studies, brain-ring gland complexes (BrRG) were dissected from late third instar *Drosophila* larvae and incubated individually in a 10 μl hanging drop of Grace's medium (Gibco) at 25°C for 4 hr, at which time the tissue was removed and the culture medium pooled and stored at -20°C. The culture medium samples were purified by RP-HPLC using C_{18} Sep-pak cartridges (Waters Associates) (8,9). The medium sample was then loaded onto a solvent-primed Sep-pak, washed with 5 ml of water, and the

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ecdysteroids eluted with 10 ml of methanol. The sample ecdysteroids were dried and stored frozen until RP-HPLC analysis.

To characterize individual ecdysteroids whole body samples were thawed and homogenized in ACN (2 vol. x 5) using a polytron homogenizer (Brinkmann). After low speed centrifugation (5,000 x g) for 15 min, the pellets were re-extracted with 50% ACN (2 vol. x 5). The supernatant was pooled and non-polar lipids were removed by hexane partition (equal vol. x 5). The aqueous ACN layer was removed _in vacuo_, the residue resuspended in <10 ml of H_2O and the aqueous sample loaded onto the C_{18} Sep-pak (8). The column was washed with 5 ml H_2O and the ecdysteroids eluted with 10 ml of methanol. The eluant was evaporated off and the ecdysteroids resuspended in 1.5 ml of 0.02 M Tris/perchlorate buffer (pH 7.5). The sample was then subjected to ion suppression RP-HPLC and the chromatographic fractions collected and examined by RIA.

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Radioimmunoassay of ecdysteroids

The quantity of ecdysteroids in the culture medium, animal extract, and HPLC fractions was determined by RIA (8,10). The rabbit antisera were generated by immunization with two different antigens: 1) ecdysone-22-succinyl thyroglobulin amide (E-22-ST) and termed H-22 Ab (11); and 2) 20-hydroxyecdysone-2-succinyl thyroglobulin amide (20E-2-ST) and termed H-2 Ab (12). The antisera were diluted to a concentration that bound approximately 35% of the total [23,24-^{3}H]ecdysone (60 Ci/mmol, New England Nuclear). The RIAs were executed as described previously (8,10) and the concentrations of ecdysteroids initially expressed

as ng ecdysone equivalents per tube following data reduction using a logit-log RIA program.

High performance liquid chromatography (HPLC)

The ecdysteroids were separated by HPLC [Waters, 6000A pumps, 720/730 integrator/system controller, U6K universal injector; Schoeffel UV detector (242 nm), and an LKB fraction collector]. The sample extract was injected onto a C_{18} column (Waters, 3.9 mm x 15 cm, Resolve 5 μm spherical packing) and eluted in a gradient mode with an ACN/aqueous buffer (20 mM Tris/perchlorate, pH 7.5) (8,13). A pre-column filter (Waters, guard column) containing C_{18} packing material (Waters, 35-75 μm) was part of the column system. The gradient conditions were: initial condition, 1.0 ml/min 100% aqueous primary solvent (95% buffer/5% ACN) changed to 15% organic secondary solvent (5% H_2O/95% ACN) at 60 min in a linear gradient. The elution solvent was maintained in an isocratic mode of 15% organic secondary solvent/85% aqueous primary solvent for 5 min and then a final linear gradient to 100% of the organic secondary solvent was used during the following 10 min. The fraction size varied between 0.3 ml and 1.0 ml depending on the resolution required.

Hydrolysis of ecdysteroid conjugates

After aliquots of the IS-RP-HPLC fractions from the white puparium stage (0 hr) were analyzed by RIA, the fractions were dried in a Savant Speed Vac. The fractions were resuspended in 0.3 ml sodium acetate buffer solution (50 mM, pH 5.3) containing 0.1 mg H-1 glucuronidase/arylsulphatase from *Helix pomatia* (Sigma)

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and 0.1 mg potato acid phosphatase type II (Sigma) and incubated overnight at 37°C (8). The aliquots from the hydrolyzed fractions were assayed again by RIA. The HPLC fractions with retention times from 0-35 min were evaporated to dryness, and pre-purified on a C_{18} Sep-pak. The samples were subjected to RP-HPLC once again and aliquots of the collected fractions quantified by RIA to characterize the free ecdysteroids present.

RESULTS

Ecdysteroid titer during development

The population of sexed animals collected as white prepupae (WPP) developed and eclosed as adults approximately 95 hr after pupariation for the females while the eclosion of the males was delayed until 97 hr (Fig. 1). The whole body RIA ecdysteroid profile during the prepupal-adult development period obtained by use of the H-22 antiserum is depicted in Fig. 2. During this time frame, the animal is basically a closed system except for respiratory exchange until it ecloses as an adult fly. The average weight of the insect is approximately 1.2 mg so the titer curves are almost superimposable when the data are expressed either on a per animal or mg fresh weight basis. Although there is a statistically significant peak a few hours after pupariation and immediately after pupation which occurs at 12 hr post-pupariation, neither compares to the surge in ecdysteroid titer observed at 30 hr (from 120 to 280 pg/animal). This dramatic increase is probably responsible for eliciting the molting process that culminates in

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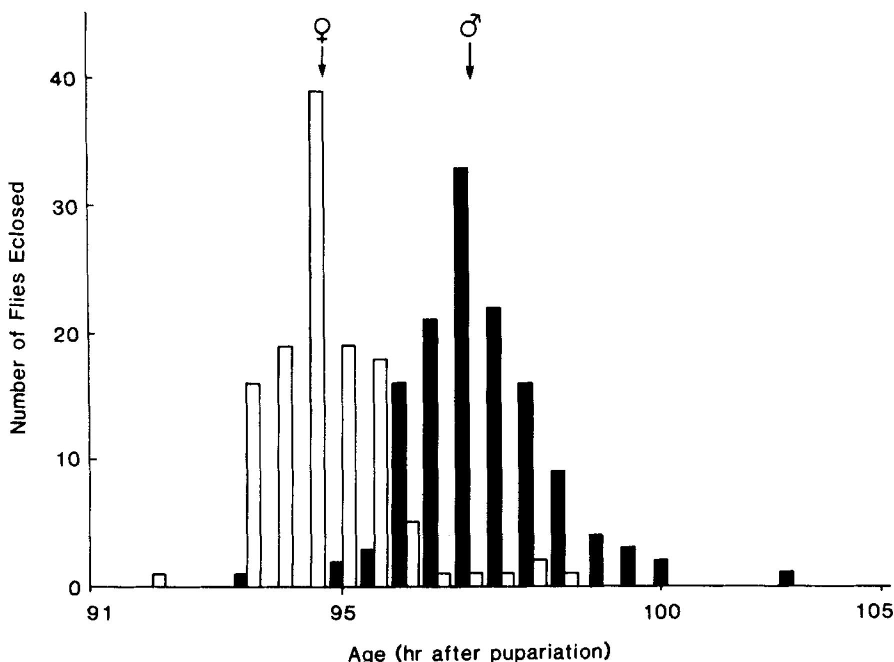

Figure 1. Sexual dimorphism in time of adult eclosion.

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adult eclosion. By 36 hr post-pupariation the titer has decreased to ∿200 pg/animal and decreases gradually thereafter until it reaches the basal level of ∿50 pg/animal at the red eye pigmentation (RE) stage. It then remains low until adult eclosion. Although such a titer provides some needed basic information on the gross changes in ecdysteroids recognized by the H-22 antiserum, it furnishes no data on individual ecdysteroids nor on the alterations in ecdysteroid composition during development. To accomplish the latter, we have utilized a combination of differential RIA and IS-RP-HPLC. Since the ultimate source of these

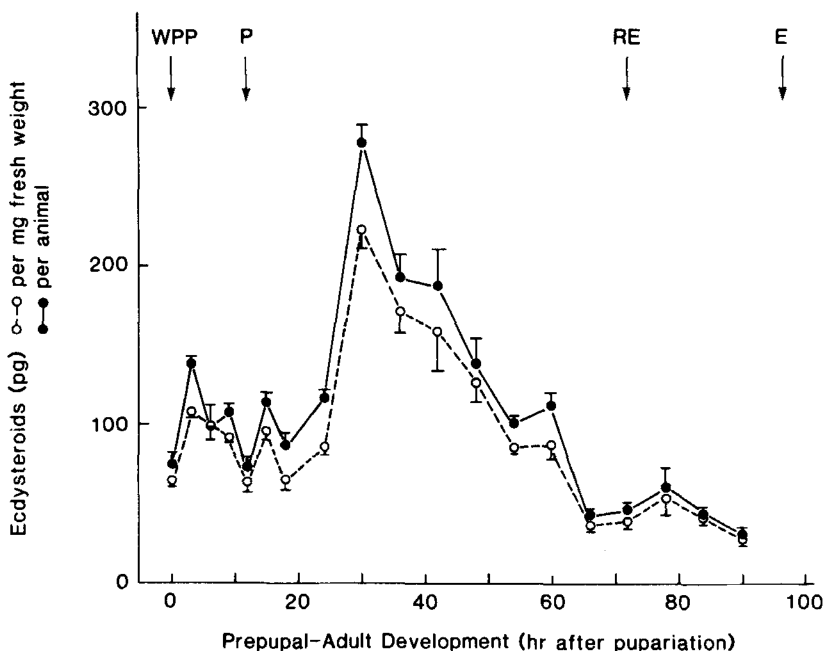

Figure 2. Ecdysteroid titer during prepupal-adult development. H-22 antiserum was utilized on whole body extracts. WPP = white puparium stage, P = time of pupation, RE = stage of red eye pigmentation, E = eclosion of the adult. Data are expressed in ecdysone equivalents.

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ecdysteroids is the ring gland, BrRG complexes were incubated in Grace's medium and the ecdysteroids analyzed. Dipteran tissue culture media such as those developed by Schneider and Robb showed no advantage over Grace's medium in these studies.

HPLC analysis of ecdysteroid synthesis by the ring gland

Before discussing the results of these studies, a few remarks on the differential RIA utilized are warranted (also see Materials and Methods). The H-22 and H-2 antisera are specific for different regions of the ecdysteroid molecule, the H-22 being

ecdysone nucleus specific and, therefore, able to recognize ecdysteroids with a modified side chain but whose A-ring is similar or identical to that of ecdysone. Thus, the H-22 antibodies can be utilized to identify metabolites of ecdysone where hydroxylation, conjugation or other covalent modification occurs on the side chain. On the other hand, the situation with the H-2 antiserum is the converse, being specific for the side chain of 20-hydroxyecdysone. Thus, this antiserum can be used to identify metabolites where epimerization or covalent modification is associated with the A-ring (8).

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To quantify the individual ecdysteroids it is important to know their cross-reactivity with the specific antiserum (8). With the H-22 antibodies the cross reactivities are: ecdysone, 1; 20-hydroxyecdysone, 4.5; 20,26-dihydroxyecdysone, 5; makisterone A, 5 (8,14). [Although no known standards for low polar-1 or 2 were available, it was assumed that their cross-reactivity was as reported previously (14). On the basis of the latter report and the retention time of low polar-2 (LP-2) in the present RP-HPLC system, LP-2 is tentatively identified as 20-deoxymakisterone A.] The H-2 antibodies show a cross-reactivity of 60 for 20,26-dihydroxyecdysone and 25 for makisterone A (8).

Utilizing the above regimen, BrRG complexes from late third instar larvae were found to secrete ∿265 pg ecdysteroids/4 hr, the products identified as ecdysone (30% of total RIA positive material), LP-2 (20-deoxymakisterone A, 51% of total RIA positive material) and LP-1 (19% of total RIA positive material) (Fig. 3).

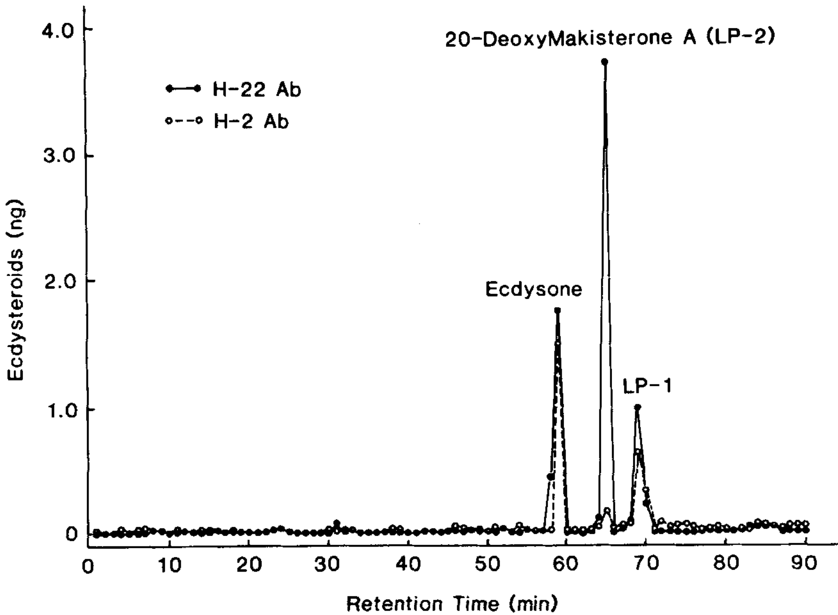

Figure 3. IS-RP-HPLC-RIA analysis of ecdysteroids secreted by the ring glands of late last instar larvae in vitro. LP = low polar product. Data are expressed in ecdysone equivalents.

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Ecdysone with a retention time of 59 min had equal affinity for both antisera, LP-2 eluting 5 min after ecdysone had extremely low affinity for the H-2 antiserum (indicating side chain modification), while LP-1 showed a retention time of 69 min and is recognized by both antisera indicating a basic structural similarity to ecdysone, but is much less polar.

Since the ring gland is assumed to be the ultimate source of all ecdysteroids in Drosophila, although there is some indirect evidence that this supposition may not be completely true (15), by analyzing the changes in the titer of individual ecdysteroids it

should be possible to trace the course of metabolism of the products of the ring gland.

Analysis of ecdysteroids during prepupal-adult development

About 3,000 white puparia (0 hr) were extracted and analyzed using the RP-HPLC-RIA regimen discussed above. Figure 4 reveals the presence of ecdysone (58.4 min), makisterone A (54.8 min), 20-hydroxyecdysone (44.9 min), 20,26-dihydroxyecdysone (32 min), a large amount of low polar ecdysteroids (predominantly H-2 Ab positive) and a signficant quantity of highly polar ecdysteroids (H-22 Ab positive). In addition, there are intermediate polar ecdysteroids eluting between 20-hydroxyecdysone and makisterone A and between makisterone A and ecdysone (both being predominantly H-2 Ab positive).

Since it is likely that some, or all of the highly polar ecdysteroids are conjugates (8), the material with a retention time between 0 and 35 min (Fig. 4) was incubated in the presence of an esterase-phosphatase mixture and the liberated free ecdysteroids once again subjected to RP-HPLC-RIA analysis. The data revealed the presence of significant quantities of free ecdysone, makisterone A, LP-2 (20-deoxymakisterone A), and 20-hydroxyecdysone (data not shown). Therefore, in addition to being present in the free form, these 4 ecdysteroids also exist as conjugates, presumably phosphates, at concentrations of 2 pg 20-hydroxyecdysone, 3.2 pg makisterone A, 0.45 pg ecdysone and 0.43 pg LP-2 (20-deoxymakisterone A), all expressed per mg fresh weight.

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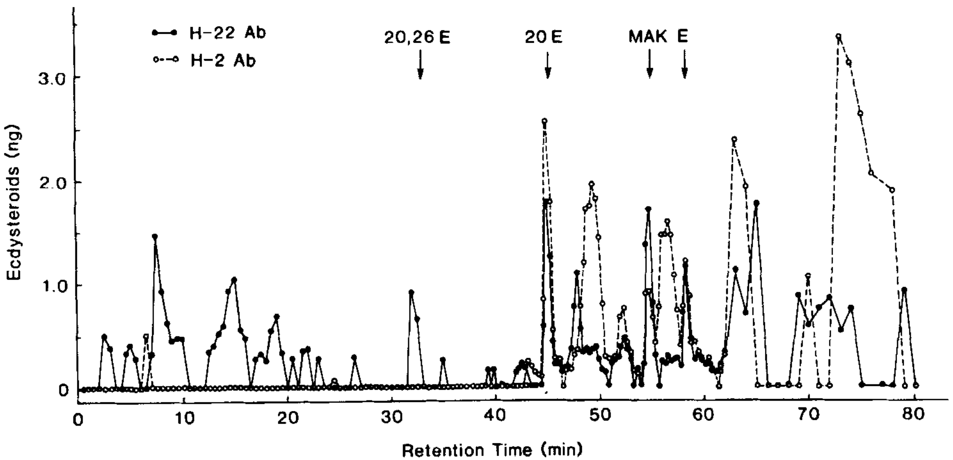

Figure 4. IS-RP-HPLC-RIA analysis of whole body extracts at the white puparium stage. The known standards are: 20,26E = 20,26-dihydroxyecdysone, 20E = 20-hydroxyecdysone, MAK = makisterone A, and E = ecdysone. Data are expressed in ecdysone equivalents.

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Free ecdysteroids were then analyzed during adult development (Fig. 5). The white puparia contained about a 5-fold greater concentration of free 20-hydroxyecdysone (4.2 pg/mg fresh weight) and makisterone A (5.3 pg/mg) than their immediate precursors ecdysone (0.87 pg/mg) and LP-2 (20-deoxymakisterone A, 0.60 pg/mg), respectively. This indicates the existence of an active 20-monooxygenase at this stage. 20,26-Dihydroxyecdysone, presumably derived from the hydroxylation of 20-hydroxyecdysone, is also present in a low but significant amount. When the as yet unidentified high (probably conjugates), intermediate and low polar ecdysteroids are taken into account and the data expressed on a percent basis, it is readily seen that these

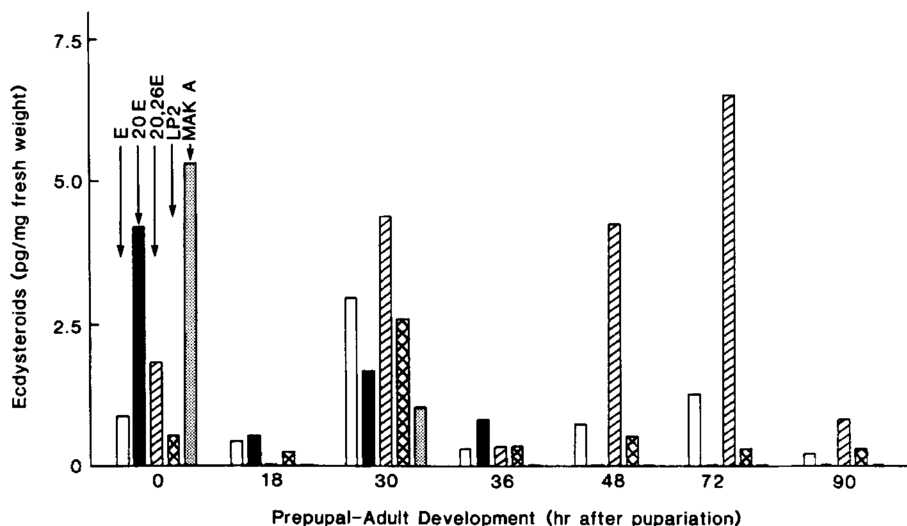

Figure 5. Titer of identified ecdysteroids during prepupal-adult development. E = ecdysone, 20E = 20-hydroxyecdysone, 20,26E = 20,26-dihydroxyecdysone, LP-2 = tentatively identified as 20-deoxymakisterone A, MAK A = makisterone A. LP-2 is expressed in ecdysone equivalents.

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unknown ecdysteroids account for the majority (∿ 70%) of the total ecdysteroids present (Fig. 6).

As development proceeds there is a drop in the concentration of the known ecdysteroids at 18 hr (Fig. 5) with the highly polar material being the predominant fraction (Fig. 6). The increase in ecdysteroids at 30 hr post-pupariation noted in Fig. 2 is reflected in increases in individual ecdysteroids (Fig. 5) with ecdysone and 20-deoxymakisterone A present in greater quantities than their products, 20-hydroxyecdysone and makisterone A, respectively. This suggested increased synthesis by the ring glands and/or decreased 20-monooxygenase activity. The highly

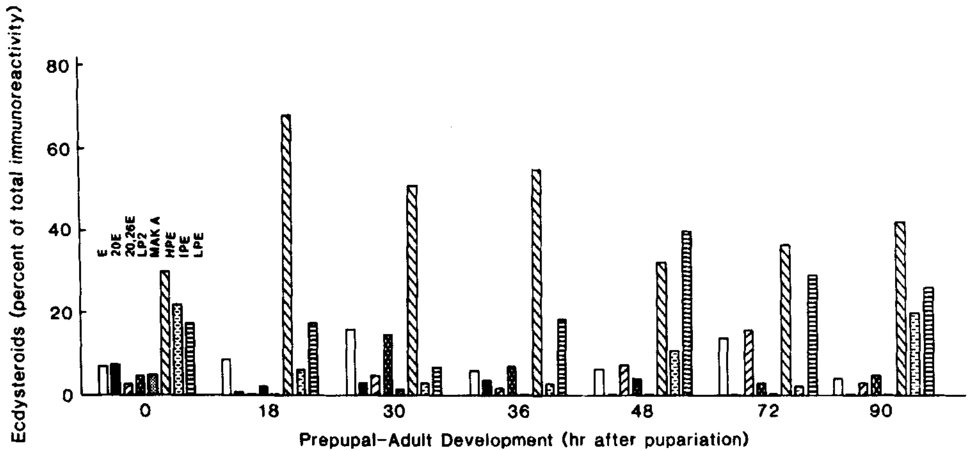

Figure 6. Titer of total ecdysteroids during prepupal-adult development. E = ecdysone, 20E = 20-hydroxyecdysone, 20,26E = 20,26-dihydroxyecdysone, LP-2 = tentatively identified as 20-deoxymakisterone A, MAK A = makisterone A, HPE = highly polar ecdysteroids, IPE = intermediate polar ecdysteroids (excluding those identified individually), LPE = low polar ecdysteroids.

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polar ecdysteroids are present at their highest level at this time reaching 9 pg ecdysone equivalents/mg fresh weight indicating conjugation and metabolism of free ecdysteroids (Figs. 5,6). Almost all of the ecdysone and 20-hydroxyecdysone is converted to 20,26-dihydroxyecdysone toward the end of adult development and makisterone A is absent by 36 hr. Prior to adult eclosion (90 hr), the total ecdysteroid titre has been reduced to its lowest level and is comprised almost entirely of unidentified high, intermediate and low polar products.

DISCUSSION

The whole body ecdysteroid titer noted here for prepupal-adult development confirms several previous studies on *Drosophila*

(4-7). The ecdysteroid peak at 30 hr after pupariation has been a consistent observation in the past and probably is responsible for the initiation of the molt to the adult (4,5). That the ring gland secretes ecdysone, LP-2 (20-deoxymakisterone A) and another low polar ecdysteroid is also an observation that confirms previous research (14).

The analysis of the white puparia and subsequent developmental stages shows the ability of the HPLC-differential RIA technique to elucidate possible ecdysteroid metabolites. The co-chromatography of ecdysteroid standards and the cross-reactivity of known ecdysteroids to the two antisera employed are requisite for the identification of ecdysteroids from animal or tissue extracts (8). It should be noted, however, that until NMR and/or mass spec. data are obtained, our identifications remain probable but not definitive. Previous HPLC analysis studies have indicated that whole body extracts of late third instar larvae contain ecdysone, 20-hydroxyecdysone and makisterone A (14). Our data revealed the presence of both ring gland ecdysteroids, ecdysone, and 20-deoxymakisterone A (LP-2) in extracts of white puparia, as well as the hydroxylated metabolites of these ecdysteroids, i.e. 20-hydroxyecdysone, 20,26-dihydroxyecdysone and makisterone A. Makisterone A and 20-hydroxyecdysone are present in large quantities relative to ecdysone and 20-deoxymakisterone A, indicating the presence of an active 20-monooxygenase. This supposition is consistent with the finding that just prior to pupariation there is potent P-450 20-monooxygenase activity (16).

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The high titer of 20-hydroxyecdysone relative to ecdysone seen here has been shown previously in late third instar larvae and prepupae (6,17). The relatively large amount of 20,26-dihydroxyecdysone noted later in development is also indicative of steroid hydroxylase activity in which 20-hydroxyecdysone is the immediate substrate.

The highly polar products isolated from adult Drosophila have been shown to be conjugates of ecdysone and 20-hydroxyecdysone (18), while the present study indicates the presence in white puparia of conjugates of these 2 ecdysteroids as well as conjugates of makisterone A and 20-deoxymakisterone A (LP-2). Although 20-deoxymakisterone A and makisterone A have been identified in insects previously (14,19), this is the first indication that these C-28 ecdysteroids exist in a conjugated form. The inability of the insect to dealkylate the C-24 methyl group of phytosterols does not appear to hinder the ability of the ring gland to synthesize 20-deoxymakisterone A as well as ecdysone, nor does the C-24 methyl group present a steric problem in the subsequent conjugation or hydroxylation reactions.

The free ecdysteroid level in the 18 hr post-pupariation animals is very low indicating either a low level of ecdysteroid synthesis or high degradative capacity. The 30 hr insect yielded high ecdysone:20-hydroxyecdysone and 20-deoxymakisterone A:makisterone A ratios indicating low 20-monooxygenase activity, while a previous HPLC analysis of this stage indicated equal quantities of these 2 ecdysteroids (6). Indeed, it has also been

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reported that the concentration of 20-hydroxyecdysone exceeds that of ecdysone at this developmental point (7). These discrepancies may be due to differences in staging and cross-reactivity of the antisera used.

The free ecdysteroids are present in decreasing amounts as development proceeds from 36 hr to 90 hr after pupariation except for 20,26-dihydroxyecdysone which peaks at 72 hr after pupariation. Also present at this time are basal levels of ecdysone and 20-deoxymakisterone A (LP-2). Since the prothoracic gland cells of the ring gland have probably degenerated by this time (15,20) it is possible that ecdysteroids are synthesized at sites other than the ring gland, a suggestion made previously (15). On the other hand, since ecdysone and 20-deoxymakisterone A may exist as conjugates, the presence of the free form of these ecdysteroids could be a result of deconjugation rather than *de novo* synthesis. The possibility of *de novo* synthesis is supported by the observation that mature ovaries synthesize ecdysone and other ecdysteroids (21). The present study is preliminary in the sense that more ecdysteroids remain to be identified in *Drosophila* (e.g. high and low polar products) than have been identified thusfar. It is believed that such studies along with metabolic analysis using [^{3}H] ecdysteroids and the utilization of ecdysteroid mutants will ultimately allow a greater understanding of the genetic and molecular control of molting hormone synthesis and degradation.

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