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Novel Assay for Determining the Metabolic Fate of Juvenile Hormone III: A Study With *Drosophila melanogaster*

A thin-layer chromatographic assay was developed for the resolution of hydrolytic and conjugative catabolites of juvenile hormone (JH). A single-dimension, dual-development thin-layer system allowed complete resolution of the catabolites. Thus, this system provided a means for the rapid and economic analysis of JH hydrolysis even when different hydrolytic activities were present concurrently. Purified hydrolytic enzymes were found to be superior to chemical methods for the generation of small amounts of standards of JH catabolites. The relative levels of activities of an epoxide hydrolase and an esterase toward JH III were found to be similar in microsomal preparations from three lines of adult *Drosophila melanogaster* isolated from a field population. However, selection of flies by exposure to cut orange resulted in the elevation of levels of epoxide hydrolase activities, whereas esterase levels were not affected to the same extent. The formation of the JH acid-diol was not detected under the conditions of this study, suggesting that the JH acid and diol were not good substrates for epoxide hydrolase and juvenile hormone esterase, respectively.

Key words: juvenile hormone, epoxide hydrolase, JH esterase

The JHs* are terpenoid epoxides that are central regulators of insect biology [1–3]. The JHs are directly involved in the control of a variety of events critical for the survival of insects including metamorphosis [4], caste determination [5], diapause [6], and vitellogenesis [7–10].

Perturbations in JH levels at critical times during development have dramatic effects on both form and function in insects [11–14]. Thus, insecticides

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based on a strategy of disruption of JH homeostasis are attractive alternatives to the nerve poisons in current use [15].

Catabolism appears to be an important factor in the regulation of JH titer in some insects [16]. Hydrolysis of the methyl ester of JH by esterases and/or the 10,11-epoxide moiety by EHs constitute the predominant routes of JH catabolism in the insect species studied thus far [17–19]. In addition, conjugation [19] and oxidation [20,21] have been demonstrated in a limited number of species.

The generation of metabolites of varying polarity by these different enzymatic pathways makes a comprehensive study of JH catabolism formidable. The resolution, identification, and quantitation of all the possible metabolites of JH is further complicated by the use of equivocal internal standards and animals that are not staged carefully. Largely as a result of these difficulties, most reports on JH catabolism have concentrated on the study of one catabolic pathway (ester hydrolysis) and have neglected the possibility of complications by competing routes of catabolism. In this study, a simple and comprehensive assay for detecting the metabolites of JH was developed and utilized in the analysis of JH metabolism by *Drosophila melanogaster* females that differed in age, environmental influence, and selection regime.

MATERIALS AND METHODS

Chemicals

Labeled JH III (^{3}H at C-10; 11.9 Ci/mmol) was obtained from New England Nuclear Research Products (Boston). Unlabeled JH III was purchased from Calbiochem Biochemicals (San Diego). BBA was purchased from Aldrich Chemical Co. (Milwaukee). GSH (reduced form) was purchased from Sigma Chemical Company (St. Louis). Purified preparations of cytosolic epoxide hydrolase [22] and JH esterase [23] were generously provided by Roger Wixtrom and Yehia Abdel-Aal of this laboratory.

Preparation of Enzymes From *Drosophila melanogaster*

The Oregon R-C strain of *D. melanogaster* was reared as described previously [24], and the adults were assayed at 2 or 6 days. Previous work with the Oregon R-C strain [24] demonstrated an age-dependent difference in the metabolism of two epoxide substrates, TSO and CSO. In this study, the levels of EH activity toward JH were examined in flies of different ages to test the hypothesis that the regulation of an enzyme activity that metabolizes an intrinsic substrate (JH) is different from activities that metabolize xenobiotics (TSO and CSO).

Another stock was derived from a 1985 collection from a lemon grove near Kactus Korner, CA (Fresno Co.). The flies were mass-cultured on laboratory medium for 15 generations. At this point, the flies were divided into six groups: three that were reared on laboratory medium (Medium), and three groups that were reared and selected on freshly cut orange (Orange). The Orange flies were selected by holding them in 0.4-pint bottles with 10–15 g of freshly cut orange grown without pesticides. They were held at room tem-

perature (22–24°C) until ca. 50% of the flies had died. The survivors were transferred to bottles containing 30 g of the cut fruit over a bed of vermiculite ca. 2–3 cm deep. This regime was repeated for 20 generations at the time of this study as a part of a selection experiment (Harshman, Ottea and Hammock, in preparation).

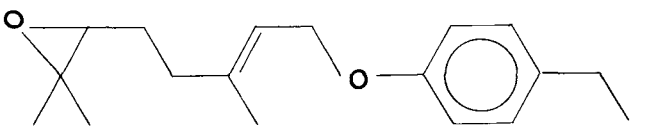
A preliminary study had shown that exposure to lemon induced EH activities toward TSO and CSO (unpublished data, this laboratory), and it was of interest to determine if EH activity toward JH was induced similarly. Females from the Medium line were held on laboratory medium for 5 days at 25°C. One-half of the flies were transferred to bottles with 30 g of freshly cut lemon on filter paper; the other half of the flies were transferred to bottles containing fresh medium. After 24 h flies from both treatments were assayed for JH metabolism as described below.

For enzyme preparation, 75–150 virgin female flies were anesthetized with CO_2 and homogenized in 0.25 M sucrose (final concentration of ca. 100 flies/ml sucrose) using 30 strokes of a Ten Broeck tissue grinder. This homogenate was filtered through glass wool and centrifuged at 10,000*g* for 10 min. The resulting pellet was discarded and the supernatant was centrifuged at 100,000*g* for 1 h. The pellet from this centrifugation was resuspended by gentle vortexing in 1.5 ml of 0.25 M sucrose; the supernatant was used as such. Enzyme homogenates were prepared and used on the day of assay.

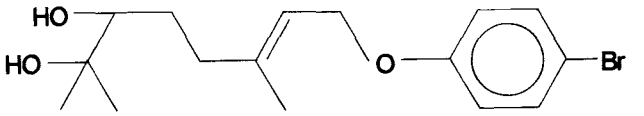
Preparation and Derivatization of Standards

Radiolabeled standards of the JH diol and acid were prepared enzymatically by adding 1 μl of a solution of radiolabeled JH III (ca. 6,000 cpm; diluted in unlabeled JH to a final concentration of 5 μM) to 1 mg/ml solutions (in potassium phosphate buffer, I = 0.2, pH 8.0) of either affinity-purified murine cytosolic EH (to produce the JH diol) or affinity-purified JH esterase from *Heliothis zea* (to produce the JH acid). The JH was incubated with EH and/or JHE for 2 h at 27°C. The acid-diol of JH was prepared in a similar fashion by incubating the enzymatically generated JH acid with purified EH. In addition, putative JH acid and diol standards were prepared by the chemical method described by Slade and Zibbit [17]. The epoxide was hydrolyzed by incubating radiolabeled JH III (ca. 2.2 ng) in 0.05 N sulfuric acid in 40% aqueous tetrahydrofuran for 4.5 h at 25°C. A similar quantity of JH III was mixed in 0.5 N sodium hydroxide in 50% aqueous ethanol for 23 h at 23°C to produce the JH acid.

The identities of the labeled standards were verified microchemically as described previously [25]. Solutions of labeled diol and acid-diol in ethyl acetate were mixed with an equal volume of BBA (1 mg/ml final concentration). BBA increases the Rf value of vicinal diols by derivatization to the corresponding boronic acid esters. The JH acid and acid-diol were converted to JH and JH diol, respectively, by treatment with diazomethane resulting in a shift to a higher Rf. R-20458 (1-[4'-ethylphenoxy]-3,7-dimethyl-6,7-epoxy-*trans*-2-octene; Stauffer Chemical Co., Mountain View, CA) and diol 1 (1-[4'-bromophenoxy]-6,7-dihydroxy-3,7-dimethyl-*trans*-2-octene) (Fig. 1) were used as unlabeled standards to visualize (under short-wave UV light) the position



R-20458



DIOL 1

Fig. 1. The structures of R-20458 (1-[4'-ethylphenoxy]-3,7-dimethyl-6,7-epoxy-*trans*-2-octene) and diol 1 (1-[4'-bromophenoxy]-6,7-dihydroxy-3,7-dimethyl-2-octene).

of the diol and JH, respectively, and as a positive control for the derivatization of the JH diol by BBA.

Assay Procedures

JH metabolism was monitored radiometrically by TLC by modifying the procedure of Slade and Zibitt [17]. The reaction between the enzyme solutions (in 100 μl of potassium phosphate buffer, pH 8.0; 0–60 μg protein/assay) and the radiolabeled JH (diluted in unlabeled JH to a final concentration of 5 μM) was terminated after 30 min of incubation at 27°C by adding 100 μl of ethyl acetate. To avoid decomposition of the epoxide by acidic contaminants sometimes present in ethyl acetate, pyridine was added (1 drop in 100 ml ethyl acetate). The tubes were vortexed vigorously and centrifuged (1,000*g* for 5 min) to break emulsions. This extraction procedure was repeated two additional times and routinely removed greater than 98% of the total radioactivity from the aqueous phase. In some cases when high levels of protein were used for assay, the percent of total radioactivity recovered from the aqueous phase by the ethyl acetate extraction was reduced to 94%. To ascertain the identity of the compounds that were not extracted into the ethyl acetate, the aqueous phase was spotted onto a Whatman LK5DF TLC plate containing a cellulose prelayer and developed as described below. The lanes of the plate were divided into 1-cm squares, which were scraped, added to 3 ml of ACS (Amersham, Inc.), and counted using an LKB 1270 scintillation counter. The detected radioactivity showed the same Rf as the labeled standards for the acid-diol, diol, and acid; and the ratios of the three materials were similar in the original ethyl acetate extract. Thus, the reduced extraction efficiency was probably due to entrapment of the compounds in a protein emulsion at the aqueous/organic interface and not to the selective extraction of the less polar metabolites.

For routine assays, the organic phase (containing metabolites and the parent JH) was removed carefully with a Pasteur pipette, pooled, and divided equally between two tubes (A and B). The volumes in the tubes were reduced to ca. 25 μl under a gentle stream of nitrogen, and the contents of tube A

were spotted directly onto a 20 × 20-cm, plastic-backed, silica gel TLC plate with a 254-nm fluorescent indicator (Aldrich Chemical Co., Milwaukee). The contents of tube B were mixed with 50 μl of a solution of BBA (1 mg/ml final concentration in ethyl acetate), incubated for 10 min, and spotted in a lane adjacent to the contents of tube A.

Four solvent systems were tested in this study: hexane:ethyl acetate (2:1, 3:1, and 7:4) and hexane:ethyl acetate:acetic acid (7:4:1). Prior to development, the TLC chamber was allowed to equilibrate with solvent for at least 1 h before inserting the plate. The plate was developed to about 15 cm, and the position of the unlabeled standards were observed and marked on the plate. In this study, when double development was used, the origin of the plate was cut and removed following the first development. By removing the origin at this stage, the acid-diol (and other polar metabolites if present) could be isolated from the acid, diol, and unmetabolized JH. In addition, the derivatization with BBA of the acid-diol caused this metabolite to leave the origin and migrate up the plate, enabling the acid-diol to be discriminated from radioactivity corresponding to other relatively polar metabolites that remain at the origin. In situations where no acid-diol or conjugates are formed, removal of the origin is not necessary. The plate was then redeveloped to 7.5 cm (ca. 1 cm below the position of the R-20458 spot), and the new position of the diol 1 standard was visualized under UV light. To determine the Rf values of the labeled standards, the plates were cut into 1-cm squares, added to 3 ml of ACS, and counted.

RESULTS

The migration patterns of the radiolabeled standards for the JH, JH acid, and JH diol are shown in Figures 2 and 3. Base hydrolysis of JH in this study yielded a product that had the same Rf as enzymatically generated JH acid (Fig. 2). However, there are cautions in the literature that hydrolysis of JH with sodium hydroxide causes rearrangement of the 2,3 double bond [26]. Also, it has been demonstrated that the epoxide of JH acid may be converted autocatalytically to the corresponding allylic alcohol, diol, and other products on storage [27]. Thus, the clean enzymatic production of the JH acid may offer advantages over chemical methods when the product will be used for further synthesis or biosynthesis. The enzymatic production of JH acid also facilitates the production of a small amount of standard, which can be used over a short period of time.

The migration of the products from the reaction of JH with mineral acid was markedly different from that of the metabolite produced by incubation with purified EH (Fig. 3). The diol produced by EH migrated to an Rf of 0.22–0.29 and was derivatized completely by BBA with >90% of the radioactivity migrating as a sharp band with an Rf of 0.71. The products from the acid hydrolysis of JH migrated with a wide range of Rfs and appeared to represent a number of compounds that were predominantly less polar than the actual diol. In addition, only 2% of the material produced chemically was shifted to a higher Rf by BBA. Thus, it appears that incubation in acid of nanogram quantities of JH yields very low levels of the JH diol, while

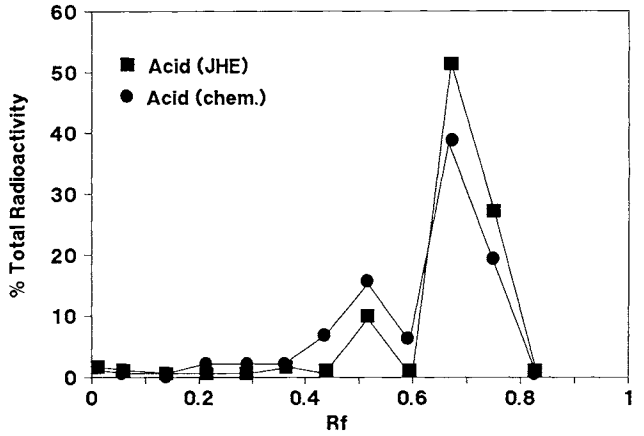


Fig. 2. Migration pattern of JH acid (Rf = 0.52) prepared by incubation of JH I with either JHE (squares) or 0.5 N NaOH in 50% aqueous ethanol (circles). The peak of radioactivity with Rf = 0.67 is unreacted JH III.

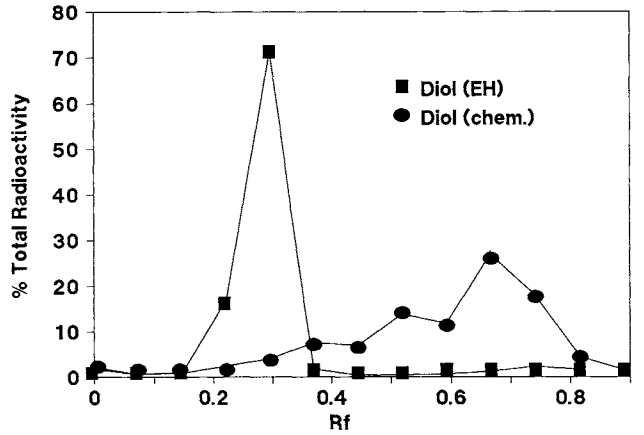


Fig. 3. Migration pattern of JH diol (Rf = 0.30) prepared by incubation of JH III with either EH (squares) or 0.05 N sulfuric acid in 40% aqueous tetrahydrofuran (circles).

enzymatic generation results in quantitative yields. This low yield of diol and production of materials that are less polar than the diol agrees with the results reported by Schooley [28] and Mumby and Hammock [29] who caution that the use of strong acid for the production of diols from submicrogram quantities of JH and other epoxides could result in the cyclization and rearrangement of the parent molecule. The use of dilute acetic acid [29], periodic acid [30], or carrier molecules (such as epoxycitronellyl acetate) can reduce some of the side reactions between JH and strong acid. However, even when the reaction is done on the milligram scale, the presence of trace amounts of metal ions can result in major contamination from side reactions [29].

The Rfs of the enzymatically generated standards and the nonradioactive standards in four different solvent systems are shown in Table 1. The values

TABLE 1. Rfs of JH, JH Metabolites, and Cold Standards Developed in Four Different Solvent Systems*

	Hexane:ethyl acetate:acetic acid			
	3:1:0	2:1:0	7:4:0	7:4:1
JH acid-diol	0	0	0	0.22
JH acid-diol + BBA	0.14	0.21	0.29	0.59
JH diol	0.07	0.07	0.14	0.50
JH diol + BBA	0.64	0.64	0.71	0.89
JH acid	0.14	0.29	0.29	0.79
JH	0.57	0.64	0.64	0.89[a]
Diol 1	0.07	0.10	0.17	0.47
R 20458	0.57	0.57	0.57	0.85

*Data represent the Rf values for the center of radioactive zones obtained by cutting TLC plates developed in various proportions of hexane:ethyl acetate:acetic acid into 1-cm squares and measuring the radioactivity by liquid scintillation counting.
[a]Under these conditions, over 75% of the JH added is degraded to more polar compounds.

shown represent the centers of the peaks of radioactivity (for the labeled standards) or the position of the UV-dense region (for the unlabeled standards). Although the positions of the JH metabolites appear to differ based on their Rf values, baseline separation was not obtained using any single solvent system tested and the analytical method described here. It also was noted that while the majority of the derivatized diol migrated in a sharp band with a higher Rf than JH, detectable levels (about 10%) of the radioactivity were also measured in a broad region of the plate above the position occupied by the underivatized diol and below that of JH. When a TLC plate spotted with the JH diol derivatized by BBA was developed in the second dimension using the same solvent system (hexane:ethyl acetate, 7:4), this radioactivity, as well as the radioactivity representing the BBA diester, comigrated with the labeled diol standard. It appeared, therefore, that breakdown of the boronic diester and consequent reformation of the JH diol was responsible for the minor, low Rf component of the BBA-treated diol.

Development of the radiolabeled standards with hexane:ethyl acetate:acetic acid (7:4:1) resulted in migration of the acid-diol away from the origin to an Rf of about 0.22. In addition, with this solvent mixture over 75% of the JH spotted migrated with an Rf corresponding to compounds that are more polar than JH. The unlabeled R-20458 standard did not appear to be affected by the acidity of this solvent system.

Complete resolution of the three metabolites was obtained by double-development of the plate in two different solvent systems in a single dimension (Fig. 4). Following development in the first solvent system (hexane:ethyl acetate, 7:4) 95% of the acid-diol remained at the origin, while the acid and diol migrated with Rfs of about 0.14 and 0.29, respectively. The radioactivity associated with the acid was distributed across 3 cm of the plate and comigrated with the leading edge of the diol peak. The acidic nature of the second solvent system (hexane:ethyl acetate:acetic acid, 7:4:1) protonated the acid, making it less polar, and allowed baseline resolution of the acid (Rf = 0.52) and the diol (Rf = 0.30). By cutting and removing the origin prior to the second development, the acid-diol was prevented from entering the plate

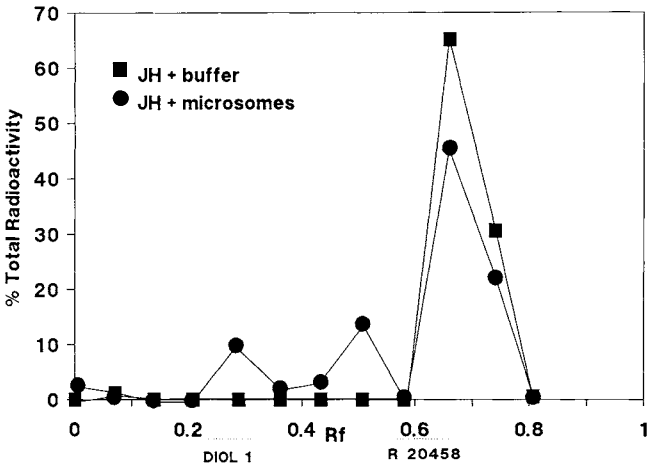


Fig. 4. Patterns of migration following TLC in one dimension (7:4:0 followed by 7:4:1, hexane:ethyl acetate:acetic acid) of ethyl acetate extractions of reaction mixtures in the presence or absence of microsomal preparations from *D. melanogaster*. The positions of the unlabeled standards are represented by dotted lines.

and interfering with the detection of the diol. The resolution of this technique may be improved by cutting the plate into smaller segments so that removal of the origin is not necessary for the separation of the diol and acid-diol following the second development. On a routine basis, the region of the TLC plate corresponding to the diol was located by counting the two 1-cm squares that preceded and cochromatographed with the diol 1 standard. JH migrated with or very near the R 20458 standard (Rf = 0.57) and was localized in two 1-cm squares. Radioactivity corresponding to JH, acid, diol, and acid-diol represented between 95% and 99% of the radioactivity recovered from the plate. Total recovery of the radioactivity spotted onto the plate was greater than 97%.

The formation of JH acid and diol was linear with respect to the concentration of microsomes from *D. melanogaster* (Fig. 5) and the incubation time (Fig. 6) used for these assays. Increasing the protein concentration in the reaction mixture caused a protein-dependent decrease in the extraction efficiency of the radiolabeled compounds from the aqueous medium, but even at the highest concentration tested (57 μg protein/assay) 94% of the radioactivity was extracted into the organic phase.

The specific activities of esterase and EH activities on JH from microsomal preparations of *D. melanogaster* are shown in Table 2. No conjugates (i.e., radioactivity remaining at the origin following derivatization with BBA) or acid-diol were detected during either the nonenzymatic or microsomal metabolism of JH. Further, the addition of GSH to the reaction mixture had no effect on the distribution of metabolites measured.

Levels of esterase activity were similar to those measured for EH in most of the assays (Table 2) and JHE activities did not differ significantly among the treatments. However, differences were measured for EH activities: the Orange (selected) flies had significantly higher levels of EH activity than did

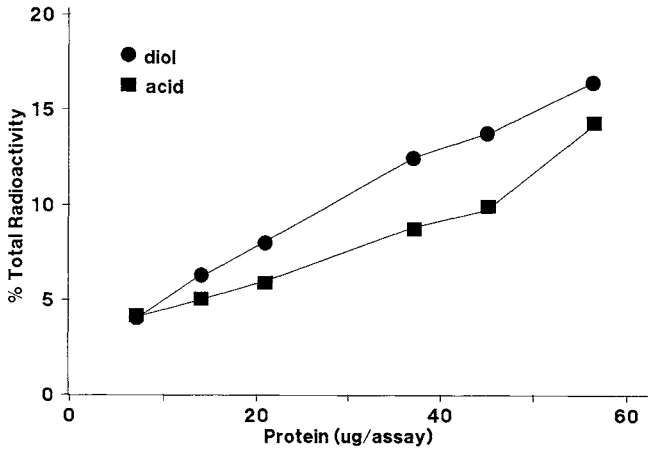


Fig. 5. Protein dependence of the formation of JH diol (circles) and acid (squares). Data represent the results of one experiment run in duplicate.

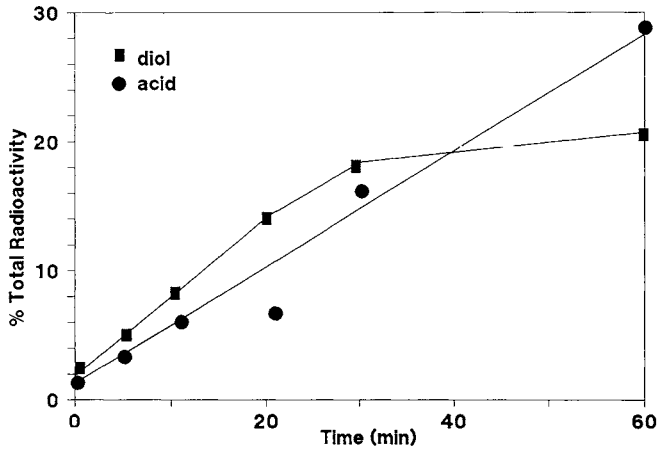


Fig. 6. Time dependence of the formation of JH diol (squares) and acid (circles). Data represent the results of one experiment run in duplicate.

the Medium (control) flies. In addition, the expression of EH activity in the Oregon R-C flies appeared to be age dependent and dropped from about 6 nmol JH metabolized/min·mg protein at day 2 to undetectable levels at day 6.

DISCUSSION

The partition assays for monitoring the catabolism of JH by JH esterase and EH are considerably less labor intensive than the TLC method described here [31,32]. However, when the possibility of competition for JH by EH and esterase exists, it is necessary to either block selectively the degradation of JH by one of the enzymes [33] or monitor the activities of the competing enzymes simultaneously. If inhibitors are to be used, it is important that the

TABLE 2. JH Metabolism by EH and Esterase From Microsomal Preparations of *D. melanogaster*

	Enzyme activity[a]	
Strain (n)	EH	Esterase
Medium (3)	4.3 ± 1.2	5.2 ± 0.6
Orange (3)	10 ± 2.9	8.6 ± 3.8
Oregon R-C		
−2 days old	4.9/7.5	4.8/7.5
−6 days old	ND[b]	5.3/8.9

[a]Activities are expressed as nmol JH metabolized/min·mg protein ± S.D. based on the No. of assays shown in parentheses. Where the experiment was only repeated once, the results from both assays are shown.
[b]Not detectable under assay conditions in which activity greater than 0.025 nmol metabolized/min was considered above background.

effects of these compounds on all the enzymatic pathways involved are examined. While it is possible to block esterase activities with highly active inhibitors [23,34], potent inhibitors of EH activity in insects are lacking [35]. Thus, at present, the simultaneous assay of JH metabolism by TLC and EH appears most prudent.

Important questions regarding the pharmacodynamics of JH may be addressed by using an assay that monitors JH degradation by different enzymatic pathways. For example, there are reports that JH acid may be methylated to JH, resulting in the recovery of bioactivity [36,37]. Thus, the hydrolysis of JH and/or the JH acid by EH may represent a critical metabolic step in removing the bioactivity of JH. For this reason, the kinetics of the JH catabolism by EH and JHE in conjunction warrants study.

The hydrolytic metabolites of JH can be resolved by two-dimensional TLC or high-pressure liquid chromatography, but these techniques limit the number of samples that can be analyzed. Single-dimension TLC followed by fluorography is a slow process requiring microcurie levels of radiotracers. An advantage of the TLC method described here over assays reported previously is that baseline resolution of JH and the predominant metabolites of JH hydrolysis was obtained using nanocurie levels of substrate and development in a single dimension. Thus, the assay may be performed using economical levels of commercially available JH. In addition, by derivatizing with BBA and removing the origin prior to the second development, radioactivity corresponding to the JH acid-diol may be discriminated from that of other relatively polar metabolites (e.g., conjugates). Finally, by using enzymes to generate the internal standards used, the possibility of contamination with impurities was minimized. When a new biological system is evaluated, the use of high-resolution techniques is essential to insure that only hydrolytic pathways of metabolism are important.

The results of the present study confirm previous findings [29] that hydrolysis of very small quantities of JH by strong acid results in poor yields of JH diol. Thus, care must be taken when generating a diol standard using this

conventional method, and consideration should be given to the use of either weak acid [38] or enzymes [39] for the production of diol standards from epoxides. While purified EH and JHE were used for this study, an earlier study has shown that the soluble fraction of mouse liver or kidney is able to convert JH to the JH diol without the formation of appreciable levels of JH acid [39].

Levels of activity for esterase and EH activity toward JH appeared similar in microsomal preparations of *D. melanogaster.* It is curious that both activities were present in the microsomes of this insect, yet no acid-diol formation was detectable. This finding suggests that, at least with the assay conditions used here, the JH diol and acid were not good substrates for esterase and EH, respectively. This pattern of activity is similar to that reported for the house fly *(Musca domestica),* blow fly *(Phormia regina)* and flesh fly *(Sarcophaga bullata)* [21], in which early in adult development microsomal fractions are reported to contain equivalent levels of esterase and EH activities (although the overall patterns of expression with respect to age was different for these activities). However, before conclusions may be drawn regarding the relative importance of these enzymes in the degradation of JH, it is important that a more detailed kinetic study be done with these activities.

No GSH conjugates of JH were detected in this study. Thus, the microsomal GST activity measured previously in *D. melanogaster* with different epoxide substrates [24] has no apparent effect on the degradation of JH III in *D. melanogaster* under the conditions used in the present study. In an earlier study, no conjugation was measured in assays of JH metabolism using microsomal preparations of *M. domestica* [20], an insect that also possesses a microsomal form of GST that is active in the hydration of epoxides [40].

In contrast, topical application of JH I to pupae [18] or adults [41] of *D. melanogaster* resulted in the formation of both acid-diol and metabolites that were presumed to be conjugates of JH. It is possible that the enzymes responsible for the conjugation of JH III with glutathione are extramicrosomal, and with the exception of glutathione none of the cofactors thought to be important for these reactions were added.

Epoxide hydrolase activity toward JH appears to be elevated early in adult development and to decline thereafter. In the present study, levels of EH activity in Oregon R-C flies was readily detectable in 2-day-old flies, but undetectable at 6 days. The timing of appearance measured for the EH activity active toward JH was similar to that seen previously in house flies [21] and in the same strain of *D. melanogaster* using a different epoxide as substrate [24]. Age dependence of both EH and esterase activities warrants further study and may enhance our understanding of the roles and interrelationship of these two enzymes in the metabolism of JH.

Differences were measured in EH and esterase activities in response to selection. EH activity on JH was 180% higher in the Orange selected flies, while esterase activities were higher but not significantly different from the Medium strain. In this study, Orange (selected) and Medium (control) flies were both reared under identical conditions. Therefore, the differences between them appear to be genetic changes resulting from selection. These results suggest that it is possible to genetically alter the pattern of JH metabolism.

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