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In Vitro Metabolism of Juvenile Hormone III and Juvenile Hormone III Bisepoxide by *Drosophila melanogaster* and Mammalian Cytosolic Epoxide Hydrolase

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***In vitro* metabolism of juvenile hormone III (JH III) and juvenile hormone III bisepoxide was investigated using purified mouse liver cytosolic epoxide hydrolase (cEH) and cell fractions from *Drosophila melanogaster*. JH III was metabolized faster than JH III bisepoxide by epoxide hydrolase activity in *D. melanogaster* cell fractions and by cEH. After incubation with JH III bisepoxide, all cell fractions and cEH produced epoxy-diol, *cis*- and *trans*-tetrahydrofuran-diols, and tetraol as metabolites. An increase in the concentration of cEH resulted in an increase in the proportion of tetraol as a JH III bisepoxide metabolite but this trend was not observed in the *D. melanogaster* cell fractions. Differences between cell fractions in the metabolism of JH III and JH III bisepoxide suggests the presence of juvenile hormone epoxide hydrolase isozymes.**

Juvenile hormone III (JH III) bisepoxide may be a form of juvenile hormone (JH) in *Drosophila melanogaster* (1). Evidence for this hypothesis includes the observation that *in vitro* incubation of ring gland tissue with labeled JH precursor results in the synthesis of more JH III bisepoxide than JH III and the bisepoxide has JH activity (1). Metabolism could partially define the physiological role of both forms of JH. Thus, we have investigated the *in vitro* metabolism of JH III bisepoxide and JH III in *D. melanogaster* cell fractions.

A variety of enzymes contribute to the metabolism and disposition of insect JHs (2, 3). Esterases acting on juvenile hormone are widespread and in some insects a specific juvenile hormone esterase helps to clear JH from the hemolymph (3). EH activity on juvenile hormone is also widespread, but relatively little is known about this class of enzymes, which is surprising considering their potential role in development and as physiological regulators (3).

Mammalian cEH was used for comparative purposes in this study because it is a purified enzyme with relatively high activity on JH (4). Efficient enzymatic conversion of the epoxide to diol minimizes the chance of artifactual chemical rearrangement, and it is informative to compare metabolism by a mammalian enzyme with insect cell fractions.

MATERIALS AND METHODS

Compounds. Standards for JH III metabolism were prepared as described in a previous study (5). These include the acid, diol, and acid-diol metabolites. For analysis of JH III bisepoxide metabolism, *cis*- and *trans*-tetrahydrofuran diols, epoxy-diol, and tetraol (Scheme 1) were a sufficient set of standards because esterase activity was low in *D. melanogaster* larvae. A 7% (w/v) solution of *trans*-JH III bisepoxide in tetrahydrofuran was stirred with added $HCLO_4$ (0.7%) for 4 h at room temperature and ether extracted. Product *cis*- and *trans*-tetrahydrofuran diols were separated on silica gel using hexane-ethylacetate (2:1) and (1:1). A comprehensive analysis of these compounds and a description of the synthesis and characterization of the epoxy-diol and tetraol is reported elsewhere (6). The synthesis of JH III bisepoxide and the biological activity of it and the tetrahydrofuran diols is described in Casas *et al.* (7). Since the starting compound (JH III) was a racemic mixture, all these compounds are mixtures of stereoisomers which were not resolved with the separative procedures used in this study.

Tritiated JH III was purchased from New England Nuclear Research Products (Boston). For assays it was diluted with unlabeled JH III (5×10^{-3} M or 5×10^{-4} M, final concentration) synthesized by the method of Anderson *et al.* 1972 (8) with slight modification. The specific activity of the diluted substrates ranged from 5–8 mCi/mol to 50–80 mCi/mol.

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[2] Abbreviations used: JH, juvenile hormone; DFP, diisopropylfluorophosphate; PTU, phenyl thiourea; OTFP, 3-octylthio-1,1,1-trifluoropropan-2-one; BSA, bovine serum albumin.

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The three tritiated substrates used in this study were JH III, JH III bisepoxide, and 10,11-diol-6,7-epoxide derived from the bisepoxide. Labeled bisepoxide was prepared by stirring tritiated JH III in toluene: hexane (4:1) with an excess of dry dimethyldioxirane (70–90 mM in acetone) for 2 min at room temperature (9). Reagent excess and solvents were removed under nitrogen and the expected product was isolated by comigration with unlabeled bisepoxide on TLC. Likewise, labeled epoxy-diol was prepared by dimethyldioxirane reaction with the tritiated 10,11-diol derivative of JH III, which was obtained by acid hydrolysis of JH III. For metabolism studies the compound was purified by TLC. For assays the labeled diepoxide and epoxy-diol were mixed with unlabeled substrate to give a final concentration of 5×10^{-3} M with a specific activity of 5 mCi/mol.

Enzyme inhibitors were used in this study to protect insect homogenates from protease or tyrosinase activity [diisopropylfluorophosphate (DFP), and phenyl thiourea (PTU), respectively]. DFP and PTU were purchased from Sigma Chemical Co. (St. Louis, MO). 3-Octylthio-1,1,1-trifluoropropan-2-one [OTFP] was used to inhibit JH esterase activity (10) and 4-fluorochalcone oxide was used to inhibit cEH.

Insects. The Canton-S strain of *D. melanogaster* was used in this study. Flies were reared at 23°C in 0.5-pint bottles on 40 ml of a standard medium (cornmeal, 6.2%; semolina, 3.1%; sucrose, 3.6%; dextrose, 7.1%; agar, 1.1%; and dead yeast, 1.5%). Density was controlled by initiating each bottle with approximately 200 adults which were cleared after 48 h of egg laying. Assays were performed on mixed-sex homogenates of third instar larvae or 5- to 7-day-old adults.

Enzyme sources. *D. melanogaster* homogenates and subcellular fractions were prepared in a manner similar to that described in Ottea *et al.* (5). Essentially, flies were homogenized with a Ten Broeck all-glass tissue grinder in 0.25 M sucrose (4:1, v/w) plus 0.5 mM DFP and 0.1% PTU. After homogenization debris was removed by centrifugation for 10 min at 6000*g*. The post-debris supernatant was centrifuged for 20 min at 20,000*g* and the pellet was retained. The 20,000*g* pellet was resuspended in 20 ml of 0.25 M sucrose and centrifuged again at 20,000*g* for 20 min. The supernatant from the first 20,000*g* spin was centrifuged at 100,000*g* for 1 h. The supernatant was removed and the pellet resuspended to the supernatant volume in 0.25 M sucrose. Both 100,000*g* supernatant and resuspended 100,000*g* pellet were centrifuged again at 100,000*g* for 1 h. The final 20,000*g* and 100,000*g* pellets were resuspended in 0.25 M sucrose plus 0.5 mM DEP and 0.1% PTU to 10–20% of the cytosol volume. The protein concentration of each fraction was determined using the method of Bradford (11). Bovine serum albumin (BSA, Fraction V, Sigma Chemical Co.) was employed as a protein standard.

TABLE I

Epoxide Hydrolase (EH) and Esterase (ES) Activity in Larval Cell Fractions from *Drosophila melanogaster*[a]

	JH III (5×10^{-5} M)		JH III (5×10^{-6} M)	
	EH	ES	EH	ES
100,000*g* supernatant	1.00 (0.35)	0.05 (0.03)	0.10 (0.05)	ND
100,000*g* pellet	1.83 (0.81)	0.08 (0.04)	0.42 (0.09)	0.03 (0.01)
20,000*g* pellet	2.33 (0.43)	ND[b]	0.46 (0.13)	ND

[a] Enzyme activities expressed in terms of the amount of JH III hydrolyzed (nmol/min mg protein). Activities were determined from the proportion of diol and acid metabolities separated on TLC after *in vitro* incubation of cell fractions with JH III at two different substrate concentrations.

[b] Not detected where the limit of detection is 0.02 nmol/min/mg protein.

TABLE II

Larval and Adult EH Activity on JH III in Cell Fractions from *Drosophila melanogaster*[a]

	EH activity (nmol/min mg protein)	
	Larvae	Adults
100,000*g* supernatant	0.93 (0.27)	ND[b]
100,000*g* pellet	1.53 (0.56)	0.37 (0.09)
20,000*g* pellet	2.40 (0.34)	0.13 (0.10)

[a] Activity determined from the proportion of the diol metabolite after *in vitro* incubation with cell fractions from third instar larvae or 5- to 7-day-old adults. Standard deviations ( ) are based on four to six replicate determinations.

[b] No detectable activity.

Mammalian cEH was isolated from clofibrate-induced mouse liver by affinity chromatography (12). Enzyme dilutions were made in buffer containing 0.1% BSA.

Assays. Subcellular fractions from *D. melanogaster* and cEH were diluted with phosphate buffer (50 mM, pH 8.0 and 76 mM, pH 7.4, respectively) just prior to the assay. An initial series of dilutions was performed on each fraction to determine the range of protein concentration which yielded linear hydrolysis (30% maximum total hydrolysis) of JH III and JH III bisepoxide. Thereafter, assays were performed within this range with the exception of experiments designed to evaluate the relative abundance of bisepoxide metabolites at higher levels of hydrolysis. For the assays 100 μl of enzyme source was added to 10 × 75 mm borosilicate tubes. Substrate was dissolved in ethanol and added in 1-μl aliquots. For routine studies fractions were incubated in a shaking water bath at 30°C for 30 min and cEH was incubated at 37°C for 20 min.

After incubation, the tubes were placed on ice and 100 μl of saturated brine was added to each. For extraction 200 μl of ethyl acetate was added followed by vigorous vortexing and centrifugation at 2500*g* to break the emulsion. The solvent was removed carefully to avoid taking any of the interface, and the extraction was repeated. Liquid scintillation counts of the extracted aqueous phase indicated that this procedure recovered approximately 98% of the total radioactivity. The extracted ethyl acetate was reduced to 5 μl or less with a stream of nitrogen. Prior to the spotting of TLC plates 30 μl of chloroform was added to each tube. In some tubes a mixture of cold standards was also added before the contents were applied to precoated silica gel TLC plates (20 × 20 cm, Aldrich Chemical Co.). Samples were spotted at 2.5-cm intervals under a gentle stream of air to reduce diffusion at the point of application. TLC plates were developed in equilibrated tanks containing a mixture of hexane and ethyl acetate (7:4 or 5:5) for 60–75 minutes.

After development the positions of the unlabeled standards were marked under uv light and the plates automatically counted with a TLC scanner (System 200 Imaging Scanner, Bioscan, Washington DC). Each lane was counted for 10–20 min. Cochromatography with uv-dense unlabeled standards was the basis for tentative identification of metabolites. Peak area was quantified by integrating the peaks using the scanner software package (NSCAN). The fidelity of the scanner readings was checked by scintillation counts of silica scraped from 1-cm subdivisions of TLC plate lanes.

Experiments were performed to evaluate the stability of the epoxy-diol. Labeled epoxy-diol stored in chloroform was spotted onto TLC plates which were developed and scanned. In another experiment JH III bisepoxide was incubated with cEH. Some reactions were stopped after 5 min by extraction while others were stopped by the addition of 4-fluorochalcone oxide, an inhibitor of cEH (13). The incubation mix-

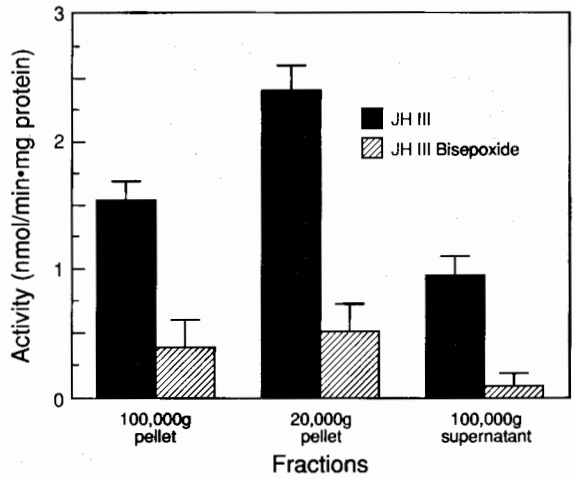


FIG. 1. EH activity on JH III and JH III bisepoxide in larval cell fractions from *D. melanogaster*. Activity is expressed as total hydrolysis of the substrates (nmol/min mg protein). Metabolites were separated by TLC and quantified with a TLC plate scanner.

tures with added 4-fluorochalcone oxide were left at 37°C for the remainder of the 20-min incubation period to assess the stability of the epoxy-diol.

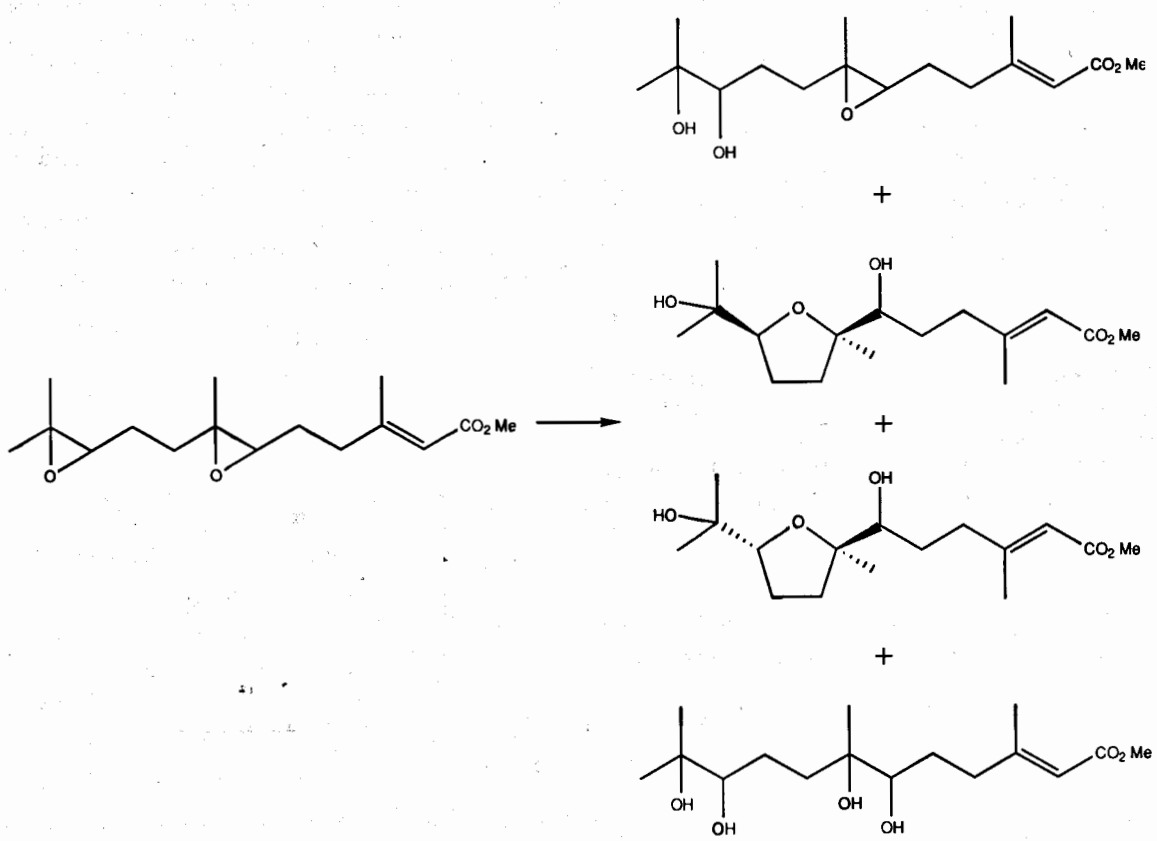
RESULTS

Table I presents the EH and esterase activity in larvae determined at two concentrations of JH III (EH and esterase activity were calculated from the proportion of the diol and acid metabolites, respectively). Esterase activity on JH III was low in third instar larvae, and the ability to detect it appeared to increase as the substrate concentration increased. In larvae, EH activity was observed in all cell fractions at both substrate concentrations.

In this study OTFP was not used routinely since JH esterase activity was low in the larval cell fractions employed for *in vitro* metabolism. OTFP partially inhibited EH activity at 10^{-3} M (at both JH III concentrations) and at 10^{-4} M (at the lower JH III concentration). This inhibition may have been a result of the surfactant properties of OTFP.

Table II shows that the activity of EH was generally higher in larvae than adults and was found in every fraction except the adult 100,000*g* supernatant. Figure 1 presents a comparison of the specific activity of larval EH on JH III and JH III bisepoxide. In each cell fraction there was more EH activity on JH III than JH III bisepoxide and this difference was especially pronounced in the 100,000*g* supernatant. For both substrates the specific activity was higher in the 20,000*g* pellet and 100,000*g* pellet than in the 100,000*g* supernatant.

Incubation of JH III bisepoxide with *D. melanogaster* fractions resulted in the formation of epoxy-diol, tetraol, *cis*- and *trans*-tetrahydrofuran diols (Scheme 1). Figure



SCHEME 1.

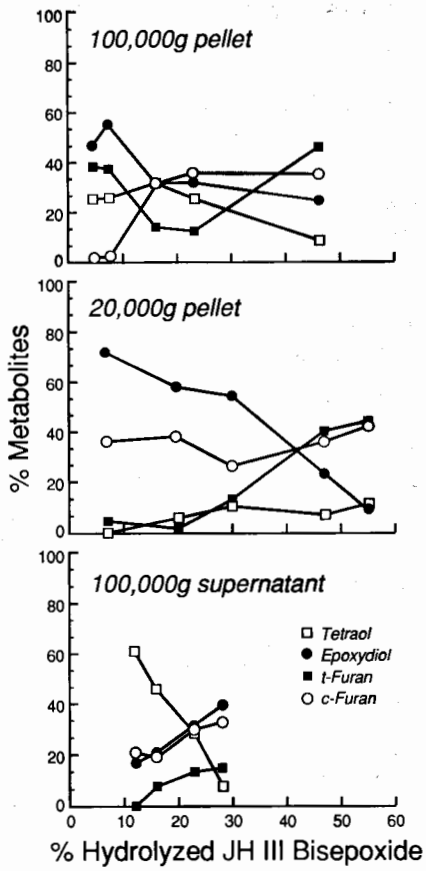


FIG. 2. Relative abundance of JH III bisepoxide metabolites in relation to percentage hydrolysis of JH III bisepoxide derived by varying protein concentration from *D. melanogaster* cell fractions. From the 20,000*g* pellet 30, 50, and 150 μg of total protein was used in the incubations. From the 100,000*g* pellet 75, 100, and 300 μg total protein was used. From the 100,000*g* supernatant 150, 350, and 580 μg total protein was used. The data presented are based on two or three independent replicates.

2 shows the abundance of these metabolites as a function of percentage substrate hydrolysis by *D. melanogaster* cell fractions. In contrast to *D. melanogaster* cell fractions the tetraol became the predominant metabolite as cEH protein concentration increased (Fig. 3). At lower levels of cEH concentration the relative abundance of the epoxydiol peaked at approximately 0.5 μg cEH per incubation followed by an increase in the proportion of furans (Fig. 4). There was less cEH activity on JH III bisepoxide than on JH III (Fig. 5).

Figure 6 shows the abundance of each metabolite after incubation of the bisepoxide with cEH. The percentage of the epoxy diol was similar after 5- and 20-min incubations and was similar in both 5-min treatments (one terminated by extraction and the other terminated by addition of an inhibitor), which suggests that the epoxydiol was not highly unstable in the incubation solution.

A scraped TLC plate was compared to a scanned plate developed under the same conditions. The position of JH

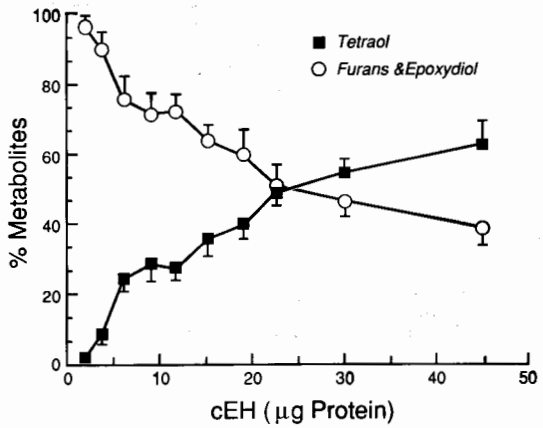


FIG. 3. Percentage tetraol versus other JH III bisepoxide metabolites as a function of mammalian cEH concentration (from 0.9 to 45 μg per incubation).

III bisepoxide and metabolites was determined to be the same by both quantification methods, but the scanner gave better peak resolution (Fig. 7). The previously described partition methods for the analysis of EH activity on JH are rapid (14, 15). However, due to the incomplete separation of JH and JH diol with the partition characteristics described, the assay is not adequate for careful kinetic studies. As cautioned previously, interpretation of the partition assay is questionable if there is the possibility of competing reaction pathways (15). The resolution of high performance liquid chromatography presents an attractive alternative, but analyses are necessarily sequential. In contrast the use of TLC plates allows many reactions to be run quickly and in parallel. Newer TLC scanners such as the Bioscan described here have been shown to yield highly quantitative data useful in monitoring EH activity in mammals (16), and this observation also extends to JH as illustrated by the data in

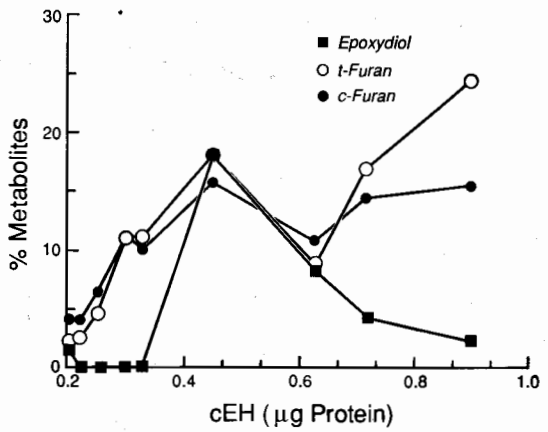


FIG. 4. Percentage of epoxy-diol, *cis*-furan and *trans*-furan JH III bisepoxide metabolites as a function of mammalian cEH concentration (from 0.2 to 0.9 μg per incubation).

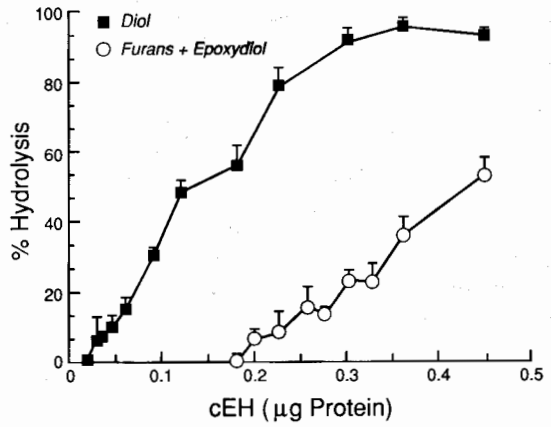


FIG. 5. Metabolism of JH III and JH III bisepoxide by mammalian cEH. This range of cEH concentrations encompasses the linear range of activity on both substrates. The specific activity of cEH is 600 nmol/min mg protein on JH III and 147 nmol/min mg protein on JH III bisepoxide.

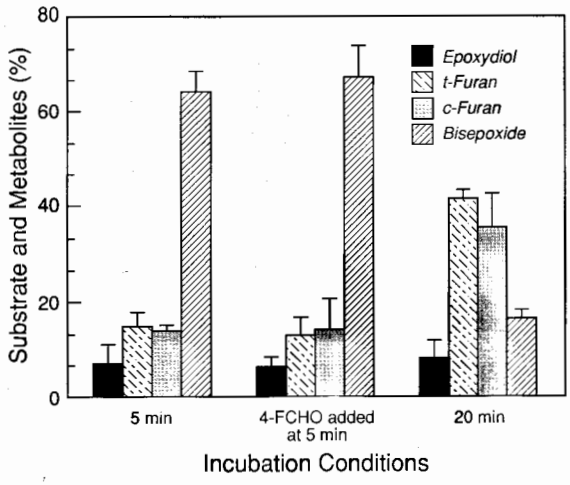


FIG. 6. Interrupted incubation by extraction or addition of 4-fluorochalcone oxide (4-FCHO) and percentage of JH III bisepoxide metabolites (0.9 µg of mammalian cEH).

this report. At this time the major drawback is that one must use large amounts of the weak β emitter, tritium. By labeling JH in chain or even the methyl ester with ^{14}C, a very inexpensive, rapid, and quantitative assay for JH metabolism could be developed.

DISCUSSION

JH metabolism studies have previously been conducted on *Drosophila.* An *in vitro* investigation using *D. hydei* demonstrated that the fat body had a high level of esterase and EH activity whereas the body wall was characterized largely by esterase activity (17). *D. melanogaster* imaginal disks readily metabolize JH but the specific enzymes involved were not identified (18). *In vivo* metabolism studies with *D. melanogaster* suggested that JH conjugates can be formed (18, 20). An *in vitro* metabolism study using microsomes from *D. melanogaster* noted the lack of an acid-diol metabolite (5). In the present study we also failed to convincingly identify the acid-diol which may be expected given the relatively low level of esterase activity.

Richard *et al.* (1) report that ring glands from *D. melanogaster* produce the bisepoxide of JH III as did cell-free preparations after incubation with NADPH and radiolabeled precursor. The bisepoxide was not produced after precursor incubation with fat body or regions of the brain outside the ring gland (1). The bisepoxide may be characteristic of higher Diptera since it was generated by *S. bullata, M. domestica,* and *C. vicina,* but not by a mosquito species (1).

Metabolites of terpenoid-diepoxide compounds have been previously generated both chemically and enzymatically. For instance, using JH I and ethylphenoxy analogs of JH III it was possible to chemically produce diepoxide and tetrahydrofuran derivatives (21, 22). Housefly microsomes were capable of converting an epoxidized form of the ethylphenoxy JH analogs to a diepoxide and to tetrahydrofuran diols (23). Injection of radiolabeled ethylphenoxy epoxide into adult *Periplaneta americana* yielded a metabolite that comigrated with an ethylphenoxytetraol standard on TLC (23). Ajami and Riddiford

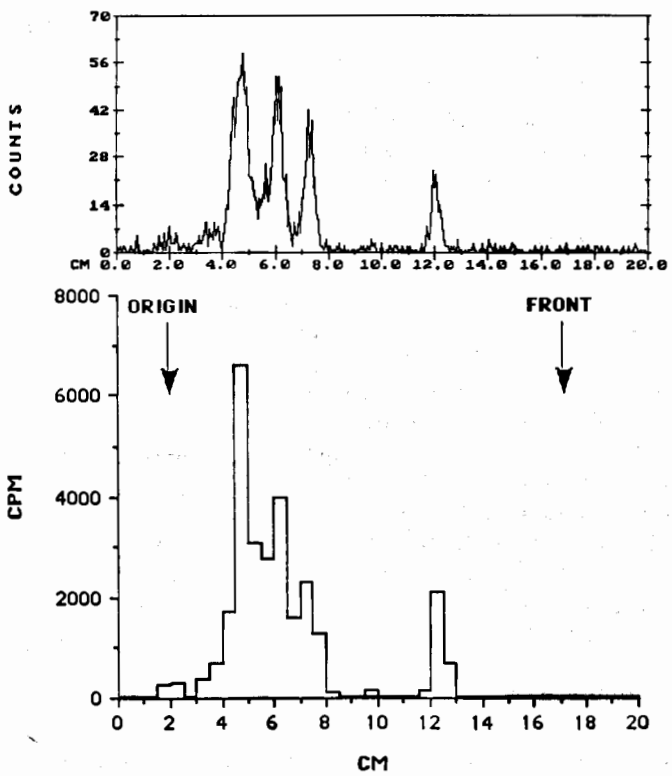


FIG. 7. Comparison of scanned (top) and scraped TLC plates with JH III bisepoxide (12 cm) followed by the tetrahydrofuran diols and epoxy-diol.

(19) had earlier reported the tetraol to be a metabolite but this observation has been questioned because the migration pattern suggested it was a cyclic derivitive (3). Injection of *P. americana* by Hammock *et al.* (23) also resulted in *cis*- and *trans*-ethylphenoxytetrahydrofuran diols which were hypothesized to be derived from an epoxy-diol intermediate.

In the present study we found that mammalian cEH and cell fractions from *D. melanogaster* produced *cis*- and *trans*-tetrahydrofuran diols, tetraol, and epoxy-diol after incubation with JH bisepoxide. There was an indication of differences in the pattern of metabolites produced by cell fractions which may indicate the presence of EH isozymes. It was not clear to what extent tetrahydrofuran diols were a result of spontaneous hydrolysis or resulted from enzymatic cyclization. The epoxy-diol partially cyclized on TLC plates or at −20°C when traces of acid were present. The results of the interruped incubation experiment (Fig. 6) suggest the epoxy-diol is not highly unstable in solution under these conditions. In *D. melanogaster,* cyclic metabolites usually increased in abundance as protein concentration increased (Fig. 2), which indicated that the transition from epoxy-diol may have been partially enzyme-mediated. It is noteworthy that in contrast to cEH incubations the proportion of tetraol did not tend to increase in *D. melanogaster* cell fractions and actually dropped sharply in the 100,000*g* supernatant. The decrease in tetraol may be another indication of enzymatic cyclization in *D. melanogaster.* Additional evidence for enzyme-mediated cyclization was suggested by the observation that the relative abundance of *cis*- and *trans*-tetrahydrofuran diols varied depending on the cell fraction tested and protein concentration employed (Fig. 2).

The role of the epoxy-diol as a JH bisepoxide metabolic intermediate could be partially evaluated by adding excess epoxy-diol as substrate and subsequently determining whether EH activity is suppressed by feedback inhibition. If metabolism of the bisepoxide proceeds by successive steps this might be a unique kinetic process in which an intermediate metabolite is produced and then further modified to generate two product classes, i.e., the tetraol and tetrahydrofuran diols.

Differential *in vitro* susceptibility to enzymatic degradation indicates endogenous differences in JH III and JH III bisepoxide persistence. When this observation is coupled with the finding of Richard *et al.* (1) that the bisepoxide is the predominate biosynthetic product in *D. melanogaster* larvae and adults it suggests that this form of the hormone may play an important physiological role.

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