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

J. Cassis,†† L. G. Harshman,† A. Messeguer,† E. Kuwano,‡ and B. D. Hammock§

*Department of Entomology, University of California, Davis, California 95616; †Department of Biological Organic Chemistry, C.I.D., C.S.I.C., A. Girona 19-21, 08034 Barcelona, Spain; ‡Laboratory of Postecile Chemistry, Department of Agricultural Chemistry, Kyushu University, Fukuoka, 812-Japan; and §Department of Environmental Toxicology, University of California, Davis, California 95616

Received July 18, 1980, and is revised from November 13, 1980

In vitro metabolism of juvenile hormone III (JH III) and juvenile hormone III bisepoxide was investigated using purified mouse liver cytosolic epoxide hydrolase (eEH) 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 eEH. After incubation with JH III bisepoxide, all cell fractions and eEH produced epoxy-diol, cis- and trans-trans-tetrahydrodiol, and tetraol as metabolites. An increase in the concentration of eEH 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.© 1981 Academic Press, Inc.

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). Estereases acting on juve
dine hormone are widespread and in some insects a spe
cific 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 eEH 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 artificial 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 de
dscribed in a previous study (5). They include the acid, diol, and epo
diol metabolites. For analysis of JH III bisepoxide metabolism, cis- and trans-trans-tetrahydrodiol diols, epoxy-diol, and tetraol (Scheme 1) were a sufficient set of standards because esterase activity was low in D. mel
anogaster larvae. A % (w/v) solution of trans-JH III bisepoxide in tet
rahydrofuran was added with added HClO4 (0.7%) for 3 h at room temperature and then extracted. Product cis- and trans-trans-tetrahydrofuran diols were separated on silica gel using benzene-ethylacetate (3:1) 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 bio
gical activity of it and the tetrahydrofuran diols is described in Cassis et al. (7). Since the starting compound (JH III) was a racemic mixture, all these compounds are mixtures of stereoisomers which were not re
collected with the preparative procedure used in this study.

Tritiated JHIII was purchased from New England Nuclear Research Products (Boston). For assays, it was diluted with unlabeled JH III (5 × 10−5 m or 5 × 10−6 m, final concentration) synthesized by the method of Anderson et al. (8) with slight modification. The specific activity of the diluted substances ranged from 3-4 mCi/g and 50-80 mCi/g.
The three trichodermin substances used in this study were JH III, JH III biopside, and 3,5,5-tri-epoxy-6,7-epoxide derived from the biopside. Labeled biopside was prepared by storing trichodermin JH III in solution: benzene (4:1) with an excess of dry dimethylformamide (70-80 ml in 20 ml of acetonitrile) for 2 min at room temperature (48). Resin extract and solvents were removed under nitrogen and the expected product was isolated by comparison with unlabeled biopside on TLC. Likewise, labeled 6,7-epoxide was prepared by dimethylformamide reaction with the trichodermin 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 desoxo- and epoxy-desoxo were mixed with unlabeled acetone to give a final concentration of 5 x 10^-4 M with a specific activity of 5 mCi/mmol.

Enzyme inhibitors were used in this study to protect insect homogenates from proteases or tyrosinase activity (diphenylfluorophosphate (DFP), and phenyl thiohydrazide (PTU), respectively. DPF and PTU were purchased from Sigma Chemical Co. (88. Lewis, MO). 3-Octylthio-1,1-trichloroacrylonitrile (OFTV) was used to inhibit JH esterase activity (10) and 4-fuoroacetoacetoacetic acid was used to inhibit EBH.

ISOmers: The C-8 isomer of C = 1,1,2 was used in a manner similar to that described in Oita et al. (53). Essentially, the 9s and 9R fractions were homogenized with a Ten Broek air-plug tissue grinder in 0.25 M acrylamide (1%) plus 0.5 mM DFP and 0.1% PTU. After homogenaton, debris was removed by centrifugation for 10 min at 6000g. The post-tube supernatant was centrifuged for 10 min at 20,000g and the pellet was retained. The 20,000g pellet was re-suspended in 20 ml of 0.25 M sucrose and centrifuged again at 20,000g for 20 min. The supernatant from the first 20,000g spin was centrifuged at 105,000g for 90 min. The supernatant was removed and the pellet resuspended at 0.25 M sucrose plus 0.5 mM DFP and 0.1% PTU in 10-20% of the cytosol volume. The protein concentration of each fraction was determined using the method of Bradford (111). Bovine serum albumin (BSA, Fraction V, Sigma Chemical Ca.) was employed as a protein standard.

**TABLE I**

<table>
<thead>
<tr>
<th></th>
<th>JH III (5 x 10^-4M)</th>
<th>JH III (5 x 10^-4M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EH</td>
<td>ES</td>
</tr>
<tr>
<td>100,000ug</td>
<td></td>
<td></td>
</tr>
<tr>
<td>superimt</td>
<td>1.65 (0.04)</td>
<td>0.05 (0.03)</td>
</tr>
<tr>
<td>100,000ug</td>
<td>1.40 (0.08)</td>
<td>0.02 (0.04)</td>
</tr>
<tr>
<td>20,000ug</td>
<td>2.00 (0.15)</td>
<td>0.40 (0.14)</td>
</tr>
</tbody>
</table>

*Every activity expressed in terms of the amount of JH III hydrolyzed/mg/mg protein. Activities determined from the proportion of diol and acid metabolites separated on TLC after in vitro incubation of cell fractions with JH III at two different substrate concentrations. 4 Not detected where the limit of detection is 0.02 mmol/mg protein.

**TABLE II**

<table>
<thead>
<tr>
<th></th>
<th>JH Activity (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laval</td>
<td>Adults</td>
</tr>
<tr>
<td>100,000ug superimt</td>
<td>0.03 (0.02)</td>
</tr>
<tr>
<td>100,000ug pellet</td>
<td>1.03 (0.05)</td>
</tr>
<tr>
<td>20,000ug pellet</td>
<td>2.40 (0.34)</td>
</tr>
</tbody>
</table>

*Activity determined from the proportion of the diol metabolite in vivo incubation of cell fractions from third instar larvae of 7-day-old adults. (Standard deviations) are based on four to six replicate determinations.

**Note:** No detectable activity.

Maternal EBH is isolated from cholesterol-induced mouse liver by affinity chromatography (12). Enzyme dilutions were made in buffer containing 1% BSA.

Assays: Subcellular fractions from D. melanogaster and C. rubra were utilized with phosphatase (50 units), pH 6.0 and 7.0, and 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 (0.05% maximum total hydrolysis) of JH II and JH III biopside. Thereafter, assays were performed within this range with the exception of experiments designed to evaluate the relative abundance of biopside metabolites at higher levels of hydrolysis. For the assays of 100 ul of enzyme assay buffer added to 10 x 7.5 mm borosilicate tubes. Substrate was dissolved in ethan and added to 1:1 aliquots. For routine specific assays incubation was 1:1 shaker water bath at 37°C for 30 min and EBH was then incubated at 37°C for 20 min.

After incubation, the tubes were placed one in ice and 100 ul of saturated brine was added to each for extraction of 200 ul of ethyl acetate was added followed by vigorous vortexing and centrifugation at 2500g to break the emulsion. The solvent was removed carefully to avoid taking any of the substrate, 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 ul or less with a stream of nitrogen. Pts. to the spotting of TLC plates at 30 A of chloroform was added for each. In some tubes a mixture of cold standards was also added before the contents were applied to precoat silica gel TLC plates (2 x 25 cm, Aldrich Chemical Co.). Samples were spotted at 5-10 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 benzene and ethyl acetate (7:3 or 5:5) for 60-70 minutes. After development the positions of the unlabeled standards were marked under u.v. light and the plates automatically counted with a TLC scanner (System 200 Imaging Scanner, Hoeicon, Washington D.C.). Each lane was counted for 40-60 minutes. Coomassie blue and with desorption in 1% sulfuric acid. The dashed lines indicate the position of the unlabeled standards on the TLC plate. Plate was counted by integrating the peak using the scanner software package (NISCAN). The fidelity of the scanner readings was checked by scintillation counting 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 spotlighted onto TLC paper and then allowed to dry. The areas were counted immediately and compared to the first plate. All experiments were performed in a manner similar to that described in Oita et al. (53). 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 (mol/min mg protein). Metabolites were separated by TLC and quantified with a TLC plate scanner.

RESULTS

Table 1 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^{-5} 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 100,000g 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 100,000g supernatant. For both substrates the specific activity was higher in the 20,000g pellet and 100,000g pellet than in the 100,000g supernatant.

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

SCHEME 1.
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 tetrad became the predominant metabolite as cEH protein concentration increased (Fig. 3). At lower levels of cEH concentration the relative abundance of the epoxy-diol 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 biopoxide than on JH III (Fig. 5).

Figure 6 shows the abundance of each metabolite after incubation of the biopoxide 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 epoxy-diol 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 tetrad versus other JH III biopoxide metabolites as a function of mammalian cEH concentration (from 0.9 to 45 μg per incubation).

FIG. 4. Percentage of epoxy-diol, co-furan and trans-furan JH III biopoxide metabolites as a function of mammalian cEH concentration (from 0.2 to 0.9 μg per incubation).
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 vivo 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 (6). 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 in glands from *D. melanogaster* produce the bisphosphate of JH III as did cell-free preparations after incubation with NADPH and radiolabeled precursor. The bisphosphate was not produced after precursor incubation with fat body or organs of the brain outside the ring gland (1). The bisphosphate 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-disopae oxide compounds have been previously generated both chemically and enzymatically. For instance, using JH I and ethyphenoxy analogs of JH III it was possible to chemically produce disopae and tetrahydrofuran derivatives (21, 22). Horsely microsomes were capable of converting an epoxidiized form of the ethyphenoxy JH analogs to a disopae end to tetrahydrofuran diols (23). Injection of radiolabeled ethyphenoxy epoxide into adult *Periplaneta americana* yielded a metabolite that comigrated with an ethyphenoxytetraol standard on TLC (23). Ajami and Riddiford.

![Diagram](image-url)

**FIG. 5.** Metabolism of JH III and JH III bisphosphate 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 bisphosphate.

**FIG. 6.** Incubation Conditions

![Diagram](image-url)

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

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

Is the present study we found that mammalian cEH and cell fractions from D. melanogaster produced cis- and trans-tetrahydrofuran diols, tetrol, and epoxy-diol after incubation with JH bisepoxide. There was an indication of differences in the pattern of metabolites 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 at or > -20°C when traces of acid were present. The results of the incubated 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 tetrol did not tend to increase in D. melanogaster cell fractions and actually dropped sharply in the 100,000g supernatant. The decrease in tetrol 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 epoxide to a second incubation and 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 tetrol 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 bisepoxide is the predominat biosynthetic product in D. melanogaster larvae and adults it suggests that this form of the hormone may play an important physiological role.

ACKNOWLEDGMENTS

This work was supported by NSF Grant DCB-8518097, NIH Grant ES-01710 and a grant from the Superfund Basic Research Program (PB2-04899). J.C. was supported by a Fulbright-Spanish Ministry of Education Fellowship. B.M. was supported by a Burschiesen Welfaumew Toxicolem Scholar. We thank I. Segel, Department of Biochemistry, University of California at Davis, for helpful discussions.

REFERENCES