

Juvenile hormone bisepoxide biosynthesis in vitro by the ring gland of *Drosophila melanogaster*: A putative juvenile hormone in the higher Diptera

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Richard, D.S., S.W. Applebaum, T.J. Sliter, F.C. Baker, D.A. Schooley, C.C. Reuter, [V.C. Henrich](#), and L.I. Gilbert (1989) Juvenile hormone bisepoxide biosynthesis in vitro by the ring gland of *Drosophila melanogaster*: A putative juvenile hormone in higher Diptera. *Proc. Natl. Acad. Sci. USA* 86: 1421-1425.

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Abstract:

The *in vitro* production of juvenile hormone (JH) was investigated by using isolated ring glands from third instar *Drosophila melanogaster*. A JH-like molecule is secreted that comigrates with a synthetic sample of methyl 6,7;10,11-bisepoxy-3,7,11-trimethyl-(2E)-dodecenoate (JHB3) during TLC, liquid chromatography, and GC analysis. Purified product from farnesoic acid-stimulated ring glands was analyzed by electron impact GC/MS and gave a mass spectrum identical to synthetic JHB3. Additional structure confirmation was obtained following conversion of product from unstimulated biosynthesis to a derivative that comigrated on liquid chromatography with the derivative prepared from synthetic JHB3. Physiological studies revealed that JHB3 is produced solely by the corpus allatum portion of the ring gland *in vitro*. Isolated ring glands from other cyclorrhaphous dipteran larvae also produce JHB3 almost exclusively *in vitro*. Corpora allata from mosquito larvae, however, produce only JH III, indicating that JHB3 production may be restricted to the higher Diptera. Topically applied synthetic JHB3 caused developmental responses in newly formed *D. melanogaster* white puparia similar to those obtained with JH III. The data suggest that JHB3 is a fly juvenile hormone.

Keywords: methyl 6,7;10,11-bisepoxyfarnesoate (RN25506-84-7)/corpora allata/radiochemical assay/insect development/fly juvenile hormone

Abbreviations: FA, farnesoic acid; JH, juvenile hormone; EI, electron impact; LC, liquid chromatography; RPLC, reversed-phase LC; RT, retention time.

Article:

In many adult insects, juvenile hormone (JH) plays an important role in reproduction, whereas in larvae JHs are modulators of morphogenetic processes (1, 2). To date, JH III is the only homolog unequivocally identified in larval and adult insects in orders other than the Lepidoptera (3). In several species of the higher Diptera (Cyclorrhapha), JH III appears to have a role in the control of adult female vitellogenesis (4) and has been detected by GC/MS analyses in larval and adult stages of *Drosophila hydei* and *Drosophila melanogaster* (5-7); however, the role for JH III in the larval or larval—pupal development of flies is unclear (8, 9).

The larval corpus allatum of cyclorrhaphous Diptera is situated in the apical portion of the complex endocrine-neurohaemal organ, the ring gland, which includes the two lateral prothoracic glands and the ventral corpus cardiacum (10). Although ecdysteroid synthesis by the prothoracic gland portion of the *D. melanogaster* ring gland occurs *in vitro* (11), no studies have been reported on JH biosynthesis by the corpus allatum. Considering the importance of this species in research, the lack of knowledge regarding regulation of the JH titer is surprising. Data presented here indicate that one possible reason for this lack of progress is that ring glands from *D. melanogaster* larvae synthesize an unusual JH.

MATERIALS AND METHODS

Animals. Ring glands were excised from third-instar post-feeding larvae or puparia (timed from formation of white puparia) of the Canton-S wild-type strain of *D. melanogaster* reared at 25°C under a 16-hr light/8-hr dark photoperiod. Other dipterans (*Sarcophaga bullata*, *Musca domestica*, and *Calliphora vicina*) were reared under a non-diapause-inducing 16-hr light/8-hr dark photoperiod at 25°C on a beef muscle/artificial medium diet (12). Final instar mosquito larvae, *Toxorhynchites brevipalpis*, were kindly supplied by L. Whisenton (Millersville University).

In Vitro Radiochemical Assay (Adapted from Ref. 13). Ring glands from larvae or puparia were incubated in Eagle's minimum essential medium with Earl's salts, without methionine and bicarbonate (Lineberger Tissue Culture Facility, Univ. of North Carolina, Chapel Hill), supplemented with 20 mM Hepes buffer (Sigma), 20 mM Ficoll 400 (Sigma), and 25 µg of ampicillin per ml (United States Biochemical). The pH was adjusted with 0.1 M NaOH and the medium was sterilized by filtration through a 0.2-µm pore size Acrodisc (Gelman) and refrigerated. The medium was supplemented prior to use with 0.5 µM 3-octylthio-1,1,1-trifluoro-2-propanone, a JH esterase inhibitor (14), a gift of R. M. Roe (North Carolina State University), and with L-[methyl-³H]-methionine (New England Nuclear; specific activity, 80 Ci/mmol; 1 Ci = 37 GBq). In some experiments, the labeled methionine was diluted with L-methionine to a specific activity of 8 Ci/mmol.

Incubation parameters such as optimal pH, time, methionine, and (2*E*,6*E*)-farnesoic acid (FA) concentrations were examined. Experiments typically lasted 2 hr at 25°C with one ring gland in 5 µl of medium placed in a well of a 24-well dish (Falcon 3047). Incubations were terminated by adding 50 µl of water to each well and extracting with 150 µl of cold hexane. Aliquots of the organic phase were either radioassayed in ScintiVerse H (Fisher), or pooled and analyzed by TLC or liquid chromatography (LC).

Chromatography. TLC was performed on 20 x 20 cm plastic backed silica gel F plates (Kodak), developed with hexane/ethyl acetate (3:2). Standards of JH III (Calbiochem) and methyl 6,7;10,11-bisepoxyfarnesoate were applied as hexane solutions and visualized under UV light. Bands (1 cm) were cut from the plates and radioassayed or eluted with ethyl acetate for reversed-phase LC (RPLC) analysis (Waters 6000A pump, 720/730 integrator/system controller, Schoeffel UV detector at 230 nm). Samples were injected as solutions in 55% acetonitrile in water onto a C₁₈ column (5-µm resolve; 3.9 mm x 15 cm; Waters) and eluted with 55% acetonitrile. The Waters guard column (3.9 x 23 mm) was packed with Bondapak C₁₈/Corasil (37-53 µm). Silica LC was conducted using a Haskel Model MCP-36-C pump and Kratos Model 773 UV detector.

Methyl 6,7;10,11-Bisepoxy-3,7,11-Trimethyl-(2*E*)-Dodecenoate (Methyl 6,7;10,11-bisepoxyfarnesoate)

Synthesis. Synthetic racemic JH III (20 mg, Fluka: 1 in Fig. 1 shows natural 10*R* isomer) in 4 ml of dry benzene was oxidized with ~10% molar excess 80% *m*-chloroperbenzoic acid for 20 hr at 21°C. The reaction mixture was extracted two times with 2 ml of 5% aqueous NH₃ and two times with 2 ml of saturated NaCl, concentrated *in vacuo*, dissolved in dichloromethane, dried with Na₂SO₄, filtered, and evaporated. The crude bisepoxide (JHB₃) (epoxidized JH III = methyl 6,7;10,11-bisepoxyfarnesoate; 2 in Fig. 1) was purified by preparative TLC (silica gel; Merck) developed as described above. The purified bisepoxide (assumed to contain equal amounts of four stereo-isomers) was analyzed by NMR and GC/MS to confirm its structure.

Proton NMR spectra were determined on a Varian FT-80A instrument. The spectrum of JH III (in C²HCl₃) contained two broad multiplets, corresponding to vinylic protons at C-6 and C-2 (at δ 5.09 and 5.64). After epoxidation, the resonance at 5.09 disappeared. The ratio between quaternary and vinylic methyl groups (2:2 in JH III) was now 3:1 in the bisepoxide, confirming that JH III is converted to methyl 6,7;10,11-bisepoxyfarnesoate. The NMR analysis was repeated with a Bruker AM-300 instrument with the same results.

Further confirmation of the identity of synthetic JHB₃ was gained by using a Finnigan 4610 GC/MS [electron impact (EI) mode] with a Nova 4 data system. GC was conducted with 30-m capillary columns (SE 45 and DB 5; J & W Scientific, Rancho Cordova, CA), temperature was programmed from 150°C to 240°C at 6°C/min

using helium at 3 ml/min. JH III and JHB₃ were well resolved; their mass spectra revealed molecular ions (266 and 282, respectively) with the appropriate fragmentation.

Methyl [10-³H]6,7;10,11-bisepoxyfarnesoate was prepared by oxidation of methyl (2E,6E)-[10-³H]farnesoate with two equivalents of *m*-chloroperbenzoic acid in dichloromethane at 0°C. The product was purified by LC on a 22 x 0.8 cm ZorbaxSil column (DuPont) using 25% diethyl ether in pentane (half water saturated); flow rate was 5.0 ml/min.

Chemical Characterization of Biosynthesized Methyl 6,7;10,11-Bisepoxyfarnesoate (JHB₃). *GC/MS evidence.* A sample of ≈1.9 μCi (≈230 pmol) of [³H]JHB₃ was obtained by culturing 264 *D. melanogaster* ring glands in medium containing 20 μM L-[methyl-³H]methionine (specific activity, 8 Ci/mmol), 20 μM (2E,6E)-farnesoic acid, and 0.5 μM 3-octylthio-1,1,1-trifluoro-2-propanone. The product was purified on a 25 x 0.46 cm ZorbaxSil column using 5% diethyl ether in dichloromethane (half water saturated). Great care was taken to avoid contamination with synthetic JHB₃. The fraction eluting from 9-12 min [similar retention time (R_T) to that of JHB₃] was collected, concentrated, and repurified using the same column with 20% diethyl ether in pentane (half water saturated). The fraction eluting from 18-20 min (same R_T as synthetic [³H]JHB₃) was collected, concentrated, redissolved in acetonitrile, and subjected to EI GC/MS analysis using an HP 5996 instrument. Major fragments from the biosynthetic product were as follows: m/z (relative intensity) 282 (M⁺, 0.5), 281 (1.4), 253 (1.4), 233 (0.9), 207 (2.2), 191 (1.6), 181 (1.1), 165 (2.2), 155 (5.1), 147 (4.7), 135 (3.4), 125 (12), 111 (24), 108 (23), 93 (30), 83 (22), 81 (18), 71 (34), 69 (27), 59 (19), 55 (43), 43 (100), 42 (6.4), 41 (46).

Conversion to tetraol and related products. An aliquot of [³H]JHB₃ (from FA-stimulated biosynthesis) was diluted with 50 μg of synthetic JHB₃ and dissolved in 100 μl of tetrahydrofuran plus 50 μl of 0.1 M H₂SO₄. After 15 min, the reaction mixture was diluted with brine and extracted with ethyl acetate. Aliquots were analyzed by RPLC (Spectra-Physics 8700) on a 25 x 0.46 cm RP8 column using a linear gradient from 20% CH₃CN to 60% CH₃CN over 20 min at 1.5 ml/min. Fractions were collected for liquid scintillation counting and the results were compared with those from an identical hydrolysis of synthetic [10-³H]JHB₃.

Conversion to methyl 6,10-bis(thiophenyl)-7,11-dihydroxyfarnesoate. About 30 pmol of biosynthetic [³H]JHB₃ was obtained by incubating 144 larval *D. melanogaster* ring glands in medium containing L-[methyl-³H]methionine (specific activity, 8 Ci/mmol), but lacking FA. An aliquot of purified sample was diluted with 100 μg of synthetic JHB₃ and treated at 21°C overnight with 100 μl of 5 M thiophenol plus 4.8 M KOH in methanol containing 2% water. After dilution with saturated NH₄Cl, the product was extracted with hexane/dichloromethane (1:1) and applied to a silica Sep-Pak (Waters), which was then washed with 4 ml of dichloromethane. The product was eluted with 2 ml of ethyl acetate and further purified by LC on a 25 x 0.46 cm ZorbaxSil column with 10% diethyl ether in dichloromethane (half water saturated). The pure product was analyzed further by EI GC/MS and direct chemical ionization MS. GC/MS was conducted on an HP 5996 instrument. Direct chemical ionization MS (HP 5985) used ammonia or methane with a source temperature of 200°C or 250°C, respectively.

NMR (¹H, ¹³C) of synthetic methyl 6,10-bis(thiophenyl)7,11-dihydroxyfarnesoate was conducted with a Varian VX 400 instrument. Proton and ¹³C NMR yielded similar chemical shifts for the slow and fast eluting derivatives. Fast eluting derivative, ¹H-NMR (C²HCl₃): 1.22 (s, CH₃); 1.23 (s, CH₃); 1.31 (s, CH₃); 1.55 (m, 2CH₂); 1.90 (m, CH₂); 2.15, 2.50 (m, CH₂); 2.08 (d, CH₃); 2.44 (s, OH); 2.63 (s, OH); 2.9-3.0 (t, 2CH); 3.6 (s, CH₃); 5.45 (s, CH); 7.1-7.5 (m, 10 CH). ¹³C-NMR (C²HCl₃): 18.76 (CH₃); 24.13 (CH₃); 26.30 (CH₂); 26.34 (CH₃); 27.00 (CH₃); 30.12 (CH₂); 37.03 (CH₂); 39.25 (CH₂); 50.77 (CH₃); 64.18 (CH); 65.97 (CH); 73.24 (C); 74.51 (C); 115.9 (CH); 126.7-131.2 (aryl CH); 137.0, 137.4 (SC-aryl).

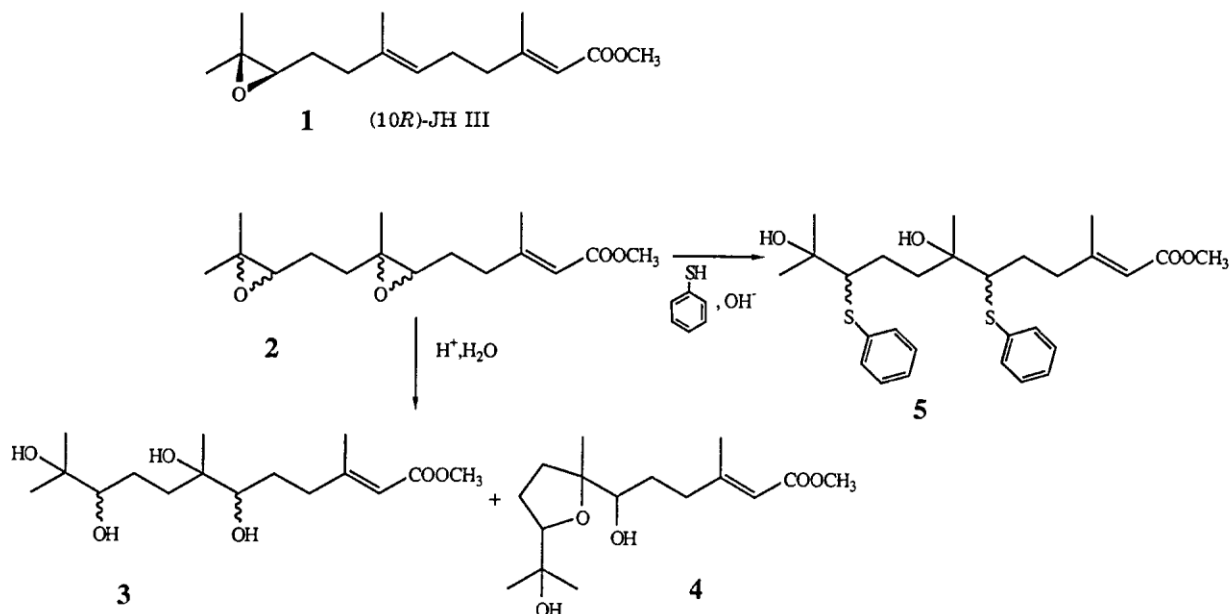


FIG. 1. Chemical structures of JH III (1), JHB₃ (2), tetraol derivative of JHB₃ (3), 6,11-dihydroxy-7,10-tetrahydrofuranyl derivative of JHB₃ (15) (4), and methyl 6,10-bis(thiophenyl)-7,11-dihydroxyfarnesoate (5).

Table 1. JHB₃ biosynthesis *in vitro* by various tissues of *D. melanogaster* larvae and portions of ring glands from *M. domestica* larvae

Tissue and source	JHB ₃ biosynthesis, pmol/hr
<i>Drosophila</i>	
Ring gland	0.15
Ring gland and farnesoic acid	0.62
Other tissues	ND
<i>Musca</i>	
Ring gland	0.42
Prothoracic gland and corpus cardiacum	
portion of ring gland	0.002
Corpus allatum portion of ring gland	0.22

Tissue and gland incubation were as described in *Materials and Methods*. The predominant product was JHB₃. Other tissues of *Drosophila* tested were brain-ventral ganglion (without ring gland), epidermis, gut, salivary gland, fat body, Malpighian tubules, and wing imaginal disks. ND, not detected.

Bioassay. The potency of JH III and JHB₃ was tested by standard dipteran bioassays (8, 9). JH III or synthetic JHB₃ was dissolved in acetone. These solutions were applied as 0.2- μ l drops on the dorsal abdominal surface of newly formed white puparia of *Drosophila*. Treated puparia were maintained at 25°C in humid chambers.

RESULTS

JH Produced *in Vitro*. We assumed originally that JH III (57) would be the only product of the corpora allata in the ring gland of flies, but it was soon apparent that JH III is only a minor product of the larval ring gland. The time course of radiolabel incorporation into the hexane-soluble extract of incubation medium was linear for 2 hr followed by a decline at 4 hr. The system was tolerant to a wide range of pH (tested between 5.8 and 7.8). [³H]Methionine concentrations in the range of 25 nM to 400 μ M were examined; incorporation of ³H occurred in a dose-dependent manner up to 20 μ M, although 100 μ M was used subsequently. The JH III precursor FA (16) was used for estimating maximal ring gland JH biosynthetic capacity (17) (Table 1) and for demonstrating the structural relationship of the assay product to known JHs. Its concentration for optimal activity was 20 μ M. Standard assay conditions were a 2-hr incubation in medium at pH 7.1-7.5 supplemented with 100 μ M [³H]methionine, with or without 20 μ M FA.

TLC analysis of the hexane extract of incubation medium of *D. melanogaster* ring glands revealed that 95% of the radiolabel migrated as a spot more polar ($R_f = 0.55$) than JH III ($R_f = 0.66$) but less polar than JH III diol ($R_f = 0.33$). RPLC analysis demonstrated that the ring gland product coeluted not with JH III but with synthetic JHB₃. The material produced by ring glands stimulated with FA cochromatographs on both TLC and RPLC with that produced by nonstimulated glands. The possibility that ring gland JHB₃ was a metabolite of JH III after its release into the medium was excluded by incubating *Drosophila* ring glands in medium supplemented with [³H]JH III. RPLC analysis of the hexane extract indicated that only a small percentage of the exogenous JH III was metabolized to JHB₃.

Whole-body homogenates of higher Diptera metabolize exogenous JH I (originally thought to be the JH of Diptera) to its bisepoxide (18) via NADPH-dependent microsomal oxidases (19). In our investigation, when [³H]JH III was incubated with the postmitochondrial supernatant of ring gland homogenates containing NADPH, much of the radio-label was converted to a product similar in TLC and LC mobility to the ring gland JHB₃. Therefore, JH III may be a precursor and the ring gland may contain an NADPH-dependent enzyme system capable of effecting the conversion. In contrast, cell-free preparations of fat body and brain were ineffective.

These data suggested strongly that the major JH of the *Drosophila* larval ring gland *in vitro* was JHB₃. Similar chromatographic profiles were obtained when larval ring glands from other cyclorrhaphous Diptera (*S. bullata*, *M. domestica*, and *C. vicina*) were examined. Isolated larval corpora allata from the mosquito *T. brevipalpis* (Nematocera), however, only produced JH III, suggesting that JHB₃ production may be specific for the higher Diptera. Interestingly, adult *D. melanogaster* corpora allata produced both JHB₃ and JH III, the former predominating (M. Altaratz, D. Segal, D.S.R., L.I.G., and S.W.A., unpublished data).

Chemical Characterization of Biosynthesized JHB₃. Biosynthetic [³H]JHB₃ from *D. melanogaster* ring glands (incubated with 20 μ M FA) migrated with a k' value similar to that of synthetic standard during sequential LC analysis using two different solvent systems. The absorbance of the product was similar to that calculated for the mass anticipated (based on ³H specific activity). GC/MS analysis of this product showed several components, one having an identical R_T (7.41 min) and mass spectrum to those of synthetic JHB₃. The other components observed by GC/MS were also present in a process blank analyzed similarly.

Attempts to convert JHB₃ to its tetraol derivative (3 in Fig. 1) by acid hydrolysis resulted in several products, most likely including 6,11-dihydroxy-7,10-tetrahydrofuran derivatives (4 in Fig. 1) and lesser amounts of isomers of the tetraol (15). However, when [³H]JHB₃ from FA-stimulated biosynthesis was reacted with dilute H₂SO₄ and was analyzed by RPLC, the profile of labeled products was essentially identical to that observed when synthetic 10(*RS*)-[10-³H]JHB₃ was similarly acidified and analyzed. This indicates that the biosynthetic product and synthetic JHB₃ are chemically similar if not identical.

Silica LC analysis (5% diethyl ether in dichloromethane) of synthetic JHB₃ prepared from 10(*RS*)-JH III showed only slight separation of the two pairs of enantiomers. However, methyl 6,10-bis(thiophenyl)-7,11-dihydroxyfarnesoate (5 in Fig. 1) prepared from synthetic 10(*RS*)-JHB₃, was readily separable by silica LC into two diastereomers, each of which is a DL pair (Fig. 2). The two derivatives were analyzed by EI GC/MS and their mass spectra were similar but not diagnostic. Direct chemical ionization MS using either ammonia or methane gave ions supporting the molecular weight (M_r , 502) of the expected product. The structural assignment of these derivatives of synthetic JHB₃ was confirmed by NMR analysis (proton and ¹³C). These data specifically rule out alternative structures resulting from attack by thiophenol at carbons 7 and 11 vs. carbons 6 and 10. [³H]JHB₃ from unstimulated biosynthesis was diluted with unlabeled racemic material and converted to methyl 6,10-bis(thiophenyl)-7,11-dihydroxyfarnesoate. This was analyzed by silica LC; ³H was detected only in the fast-eluting peak (Fig. 2). Therefore, a maximum of 2 of the 4 possible isomers are biosynthesized by the *D. melanogaster* ring glands.

Table 2. Bioassay of methyl 6,7;10,11-bisepoxyfarnesoate (JHB₃)

Quantity applied, μg per animal	Effect, %		
	Noneclosion	Decreased bristle number	Abnormal rotation of genitalia
0.01	0	0	0
0.10	0	0	0
0.50	60	100	100
1.00	60	100	100

JHB₃ was applied in acetone to white puparia and animals were examined when controls eclosed (see *Materials and Methods*). Application of JH III at a dose of 0.04 μg per animal yielded results equivalent to the 0.50 μg per animal dose of the JHB₃.

Physiological Studies. Having determined that JHB₃ is the primary secretory product of the ring gland, it was critical to establish the corpus allatum portion as the sole source of JHB₃ if it is to be considered the predominant JH of fly larvae. No *Drosophila* tissue other than the ring gland produced JHB₃ or any other JH-like material under the same assay conditions (Table 1). Next, *Musca* ring glands, which are much larger than *Drosophila* ring glands, were surgically divided into corpus allatum and prothoracic gland/corpus cardiacum portions and the portions were incubated and analyzed as described above. Only the corpus allatum portion produced JHB₃ *in vitro* (Table 1), but its rate of JHB₃ biosynthesis was only ≈53% that of whole ring glands. This is probably a result of damage during the microsurgery.

In the cyclorrhaphous Diptera, unlike the situation in most other insects, the role of JH during larval life and metamorphosis is conjectural. This may be related to the formation of puparia and the unique role of imaginal disks and abdominal histoblasts (20). The absence of a clearcut response to JH in immature Diptera has resulted in a lack of specific and quantitative larval bioassays such as have been available for other orders for several decades (21). We have, therefore, used previously reported bioassays involving pupal–adult development—i.e., inability to initiate adult eclosion (8), decrease in adult bristle number (9), and abnormal rotation of the male genitalia (9).

Test solutions were applied to timed *Drosophila* white prepupae, which were examined for developmental abnormalities at the time control groups subsequently eclosed as adults. Application of 0.5 μg of the JHB₃ prevented eclosion of 60% of the animals (Table 2), while this dose was 100% effective in interfering with development of a normal bristle pattern and induction of abnormal rotation of the male genitalia. This is about 10-fold the amount of JH III necessary to achieve similar effects. Recent data reveal that JHB₃ can also inhibit ring gland ecdysteroid synthesis *in vitro* (D.S.R., S.W.A., and L.I.G., unpublished data) and can stimulate vitellogenin synthesis (D. Saunders, D.S.R., S.W.A., M. Ma, and L.I.G., unpublished data).

We report the identification of a newly discovered JH, methyl 6,7;10,11-bisepoxyfarnesoate (JHB₃) produced by the ring gland and isolated corpora allata of higher Diptera. JH titers have been determined for *D. melanogaster* during development by two different GC/MS assays (6, 7) but JHB₃ would not have been detected by either. Recently, another group has reported the biosynthesis of an unidentified compound by adult *Phormia regina* (Diptera) corpora allata *in vitro* (22) with chromatographic properties similar to JHB₃.

The major difference between the ring gland system and all others studied is that the predominant end product of biosynthesis by the larval corpus allatum *in vitro* is JHB₃. Although few comparative studies have been conducted, the release of JHB₃ from the corpus allatum appears to be characteristic of the higher Diptera. The identification of JHB₃ as a secretory product was difficult because of its relatively low rate of production and extreme chemical lability. The former problem was surmounted by first isolating the product of FA-stimulated biosynthesis. Once this was identified by GC/MS and microchemical derivatization as authentic JHB₃, we showed that the secretory product of unstimulated glands was identical by chromatographic characterization of JHB₃ and one derivative. JHB₃ is extremely labile under acidic conditions, as protonation of the epoxide rings leads to cyclization reactions (15). We also observed partial decomposition during LC separation. The only

derivative that we could form in high yield, of several nucleophilic derivatizations of the epoxide rings attempted, was the product of the base-catalyzed reaction with thiophenol. Unfortunately, the properties of this derivative make it a poor candidate for developing a GC/MS assay for determining titers *in vivo*.

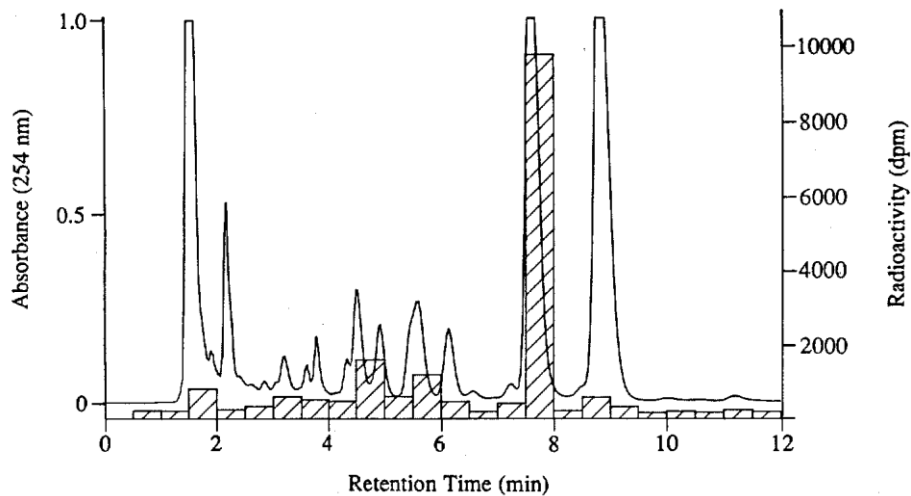


FIG. 2. Silica LC separation of synthetic methyl 6,10-bis(thiophenyl)-7,11-dihydroxyfarnesoate into two diastereomers (each of which is a pair of enantiomers) and coelution of [^3H]JHB₃ derivative from intrinsic nonstimulated biosynthesis with the fast-eluting diastereomer.

Data presented here suggest that the efficacy of JHB₃ in dipteran bioassays is lower than that of JH III in eliciting abnormal adult development. However, relative hormonal potency is a poor indicator of endogenous JH identity since JH I and JH II are more potent than JH III in virtually all JH assays. One example is the *Tenebrio* assay, where the respective potencies of JH I/JH II/JH III are 16,000:600:1 (23), although JH III is the native hormone (24). The larval and pupal development of the higher Diptera are quite different than that of hemimetabolous and other holometabolous insects (25). In the absence of an established dipteran larval bioassay for JH III or JHB₃, our results are only indicative that JHB₃ is a "new" JH and has a direct role in modulating morphogenesis. The role for JH in larval insects other than flies has been largely elucidated by microsurgical (e.g., allatectomy or implantation of corpora allata) studies, resulting in precocious metamorphosis or supernumerary molts, respectively. No such studies have been reported for *Drosophila* or related flies, perhaps because ring gland extirpation would be most difficult and even if accomplished would deprive the larva of its prothoracic glands and corpus cardiacum. In essence, there is no evidence in these dipterans for a role of JH in morphogenesis. Thus, it is currently not possible to delineate a role for JHB₃, although the changes in the ring gland's synthetic capability for this JH during development is indirect evidence for a regulatory role (D.S.R., S.W.A., and L.I.G., unpublished observation).

Acknowledgements:

This paper is dedicated to the memory of Dr. Alex Goldman (Hebrew University, Rehovot, Israel), chemist and humanist, who conducted the initial characterization and synthesis of JHB₃ early in our studies and died so suddenly at an early age. We thank Nikhil Shah for conducting direct chemical ionization MS, Renee Susnea-Litman for NMR of the JHB₃ derivative, and Shelia King for manuscript preparation. This work was supported by Grant DK35347 and MERIT award DK30118 from the National Institutes of Health and National Science Foundation Grant DMB-8518299.

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