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Selective Inhibition of JH Esterases from Cockroach Hemolymph[1]

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Juvenile hormone III was tritium labeled on the methyl ester and utilized with other substrates in an investigation of inhibition and substrate specificity of hemolymph esterases from the cockroach, *Blaberus giganteus*. The structure of labeled juvenile hormone III was supported both chemically and biochemically. Forty-two potential inhibitors were examined, and the best inhibitors included phosphoramidothiolates and *S*-phenylphosphates. One of these inhibitors was found useful in hormone biosynthesis studies dealing with the enzymatic conversion of methyl farnesoate to juvenile hormone in corpora allata homogenates. Several commonly used inhibitors of carboxyesterases caused only weak inhibition of JH esterases. Gel filtration elution patterns, inhibitor relationships, and specific activities of the hemolymph esterases indicate that juvenile hormones I and III are degraded by similar if not identical enzymes. In some cases, α-naphthyl acetate and juvenile hormone esterase activity could be differentially inhibited. Hemolymph esterases were not capable of degrading ethyl or isopropyl conjugated esters of two juvenoids or three model substrates.

INTRODUCTION

The juvenile hormones (JHs)[2] (Fig. 1) of insects regulate insect development and in some cases reproduction (1). JH titer is, in turn, regulated by a variety of factors including synthesis, release, specific and nonspecific binding, tissue uptake, degradative metabolism, and excretion (2). In most insects examined hemolymph esterases are important in JH degradation, while tissue localized esterases and epoxide hydratases are of secondary importance (3; for other references, see 4). Both high and low molecular weight hemolymph proteins are involved in JH binding in various insects (see 5 for review), and there is evidence in some insects that these proteins may protect JH from degradative metabolism and nonspecific tissue uptake (2, 6, 7). Thus, changes in JH hemolymph binding proteins or esterases may drastically alter the titers and distributions of the JHs in an insect.

Because of its regulatory function, the insect endocrine system offers promise as a site for the development of insect control agents. Small changes in a regulatory system may be amplified as major changes

[1] A summary of these results was presented at the 172nd American Chemical Society National Meeting, San Francisco, California, August 31, 1976 as paper No. 41 in the Pesticide Chemistry Division.

[2] Abbreviations used are as follows: JH(s), juvenile hormone(s); tlc, thin-layer chromatography; hrlc, high-resolution liquid chromatography; glc, gas–liquid chromatography; HE, hexane–ether (6:1); HEt, hexane–ethylacetate (3:1 or 2:1); nmr, nuclear magnetic resonance; NBP, 4-(*p*-nitrobenzyl)-pyridine; uv, ultraviolet light; AChE, acetylcholinesterase; CA, copora allata; I_{50}, concentration which yields 50% inhibition.

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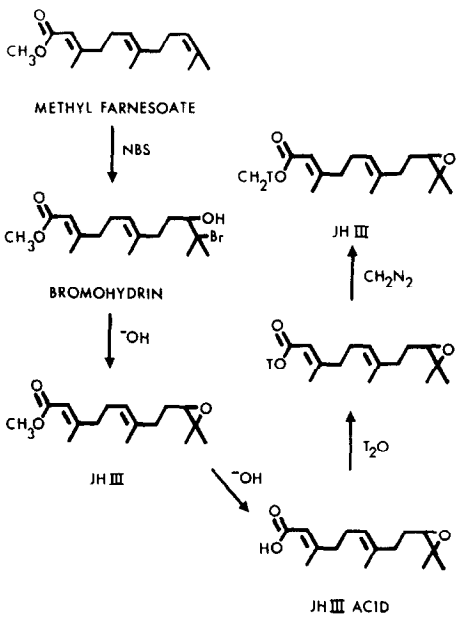


FIG. 1. *Synthesis route for [^3H]methyl-labeled JH III.*

in the physiology or survival capacity of an insect. Juvenoids or JH mimics offer promise as insect control agents, and several such compounds are on or near the market. In addition to the development of juvenoids, the development of agents which disrupt the biosynthesis, transport, or degradative metabolism of insect hormones also offer potential for insect control. Thus, a survey of compounds which may be capable of inhibiting JH degradation by hemolymph esterases was undertaken.

MATERIALS AND METHODS

Radiosynthesis of Juvenile Hormone III

Methyl farnesoate (from natural farnesol via farnesoic acid) was purified by thin-layer chromatography (tlc) on precoated silica gel 60 F-254 chromatoplates (EM Laboratories) of 0.25-mm thickness developed in hexane–ether, 6:1 (HE) (Fig. 1). The lower ($\Delta 2E$ or 2,3-*trans*) methyl farnesoate band was further purified by high-resolution liquid chromatography (hrlc) on a 10 × 250-mm tapered inlet column of slurry-packed 10μ Spherosorb (Spectra Physics) using 0.5% ether in hexane. The final product showed a single peak on analytical hrlc and on gas–liquid chromatography (glc). A Varian Aerograph 1400 with a flame ionization detector was used for glc with a glass column (6 ft. × $\frac{1}{8}$ in. i.d.) packed with Carbowax on Gas Chrom W prepared by the method of Auc *et al.* (8). This column is capable of giving baseline separation of the four geometrical isomers of synthetic methyl farnesoate as well as dihydromethyl farnesoate.

To the 2*E*, 6*E* methyl farnesoate (40 mg in 1 ml of 80% aqueous *t*-butanol), equimolar *N*-bromosuccinamide (in 6 ml of 80% aqueous *t*-butanol) was added dropwise (9; D. A. Schooley and W. Hafferl, personal communication). The bromohydrin was purified by tlc developed in hexane–ethyl-acetate, 3:1 (HEt), then cyclized to its epoxide in methanolic potassium carbonate followed by tlc purification in HEt (3:1) to give a 40% yield of juvenile hormone (JH III) from methyl farnesoate. The structure was confirmed by nuclear magnetic resonance (nmr) and comparison with an authentic standard. JH III acid was prepared by incubating JH III in a silylated tube with 1 *N* sodium hydroxide in 50% aqueous ethanol (2 hr, 30°C). At the end of this time no JH was found to remain by tlc, and the major product cochromatographed with 2*E*, 6*E* JH III acid in HEt (2:1) (which separates the 2*E* and 2*Z* isomers) and had an nmr appropriate for the assigned structure. The product was found to react with 4-(*p*-nitrobenzyl)-pyridine (NBP) (10), indicating the presence of an epoxide both before and after the reaction of an aliquot with diazomethane to yield JH III. Approximately 40% of the JH was lost during the formation of the acid. This loss could possibly be caused by poor tlc extraction or isomerization to non-uv (ultraviolet light)-absorbing compounds (D. A. Schooley, personal communication). If the JH acid is not used immediately, it rearranges to give

the corresponding allylic alcohol, and this procedure seems to be hastened by the addition of pyridine which is sometimes used to stabilize epoxides. The allylic alcohol does not react with NBP and has a lower R_f on tlc than JH III following methylation.

The JH III acid was immediately exchanged in ether with carrier-free tritium water (60 Ci), generated outside of the reaction flask to avoid chain labeling, followed by exposure to diazomethane by Dr. J. Leak (ICN Pharmaceuticals) using a modification of the method of Trautmann *et al.* (11). The material was purified by tlc in HE. The mass of the recovered material was determined by its absorbence at 213 nm in spectral quality methanol and comparison with known concentrations of an authentic standard before and after hrlc. The radioactive JH III was also mixed with cold standards and analyzed by analytical hrlc on a 3×1000-mm column which had been slurry-packed in 250-mm sections with 5μ Spherosorb, then joined with drilled-out 0.25 in. Swageloc unions. Using a solvent system of 7% ether in hexane, only 10% of the tlc pure JH III radioactivity was found to cochromatograph with authentic JH III, with the majority of the radioactivity chromatographing in the region of JH II. Rechromatography of the hrlc-purified radioactive material by both normal and reversed phase (5μ ODS, 2×250-mm column, Dupont, 25% aqueous methanol solvent) hrlc indicated that >95% of the remaining radioactivity cochromatographed with 2*E*, 6*E* JH III in each case. The specific activity of the JH III is 2.5 Ci/mmol (approximately one-tenth the theoretical limit). The yield of radioactivity cochromatographing with JH III on tlc was 50% based on JH III acid, but the yield was only 5% for material cochromatographing with JH III on hrlc. After several months storage in benzene–hexane solution under N_2, a second minor radioactive tlc spot was detected, but this decomposition product has not been identified.

The radioactive material was found to cochromatograph with JH III in several tlc solvent systems, and added proof of structure was obtained by reactions to form JH III diol and its *n*-butylboronic acid diester followed by chromatography with standards in each case (12). JH esterases from *Heliothis virescens* and *Blaberus giganteus* were found to release >98% of the radioactivity in a water-soluble form. As in the case of *Manduca sexta* JH esterase (13), *H. virescens* JH esterase rapidly metabolizes 2*E* but not 2*Z* conjugated esters (Mumby and Hammock, unpublished observations).

The structure of the radiolabled JH III was additionally supported by its binding to *Trichoplusia ni* mid-last instar hemolymph treated with compounds 15 or 27 (1×10^{-4} *M*) (Table 1). Binding was analyzed by the method discussed by Kramer *et al.* (14) utilizing a charcoal assay (6). The concentration of binding sites was estimated as 2.9×10^{-6} *M* for JH III and 3.0×10^{-6} *M* for JH I, while the dissociation constants were 3.7×10^{-6} and 4.2×10^{-7} *M*, respectively. These values compare favorably with the concentration of 7.7×10^{-6} *M* binding sites and a dissociation constant of 3×10^{-7} *M* found for JH I binding in diisopropylfluorophosphate-treated *M. sexta* hemolymph (14). The lower binding affinity of JH III is consistent with competition assays run by a number of workers (5; 15; Hammock and Gilbert, unpublished).

A cold synthesis was performed utilizing deuterated water and conditions similar to those described by Trautmann *et al.* (11). The extent of deuterium incorporation was monitored by the decrease in the nmr integral amplitude of the methyl ester proton peak (3.7 ppm δ) compared to the epoxide and two olefin proton peaks (2.7, 5.2, and 5.7 ppm δ). The nmr spectrum indicated an 85% incorporation with a 60% overall yield following workup. Hrlc showed

only a peak for JH III with about 3% of the material cochromatographing with 2*Z* JH III.

Other radiolabeled materials used for esterase assays include methoprene (^{3}H, 10 position, 97% 2*E*, 1960 mCi/mmol) (Zoecon Corporation), JH I (^{3}H, 10 position, >98% 2*E*, 6*E*, 10*Z*, 10 Ci/mmol) (New England Nuclear Corporation), and Ro-8-4314 (^{14}C, isomer mixture, 11 mCi/mmol) (Hoffman-La Roche & Co.).

Model Substrate Synthesis

The ethyl ester of 3,3-dimethyl acrylic acid was prepared by adding the appropriate acid chloride (Aldrich, 0.1 mol) to dry ethanol (50 ml, 0°C) containing sodium hydroxide (0.15 mol). Following work-up, a large excess of toluene was added (to aid in removal of excess ethanol) and the product was distilled (106–115°C, 200 mmHg). No ethanol could be detected in the product enzymatically or by nmr. The ethyl ester of JH III was prepared by potassium cyanide-catalyzed transesterification of methyl farnesoate (2*E*, 6*E*) with ethanol (16), which resulted in a final product containing only 3% methyl ester (nmr). Alternatively, the material was prepared by reacting farnesoic acid with diazoethane. In each case, the epoxide was prepared as described earlier. Cinnamic acid (2*E*, Aldrich), also yielded its ethyl ester when reacted with diazoethane.

Esterase Preparation

B. giganteus were held at 27°C on a 18:6 photoperiod with Purina Laboratory Chow and water provided *ad libitum*. Last instar nymphs were chilled, the mouth and anus were sealed with bee's wax, the legs were removed, and the nymphs were centrifuged (480*g*, 10 min, 3–5°C) to remove hemolymph. The hemolymph was centrifuged (12,000*g*, 10 min), and the supernatant was placed on an upward flow Sephadex G 150 column (2.5 × 90 cm, 5°C) and eluted with phosphate buffer (0.1 *M*, pH 7.4). The transmission of the effluent was monitored at 280 nm by an LKB Uvicord II, and 6-ml fractions were collected and stored in an ice bath. Each fraction was monitored for esterase activity on JH I, JH III, α-naphthyl acetate, and *p*-nitrophenyl acetate as well as the fraction's ability to bind JH I and JH III. The three major JH esterase peaks were assayed separately (Fig. 2).

Esterase and Binding Assays

The assay for esterase activity on methyl-labeled JH III is similar to one reported earlier (7). Enzyme (100 μl) was placed in a Carbowaxed or silylated glass tube (5 × 50 mm) (6), and JH III was added in 1 μl of 95% ethanol to give a final concentration of 5×10^{-6} *M*. After incubation at 30°C for 30 min (unless otherwise noted), a charcoal–dextran suspension (100 μl) (600 mg of Norit A, 800 mg of dextran T-40, 0.5 ml glacial acetic acid, to a final volume of 10 ml with distilled water) was added, and the tube was vortexed and centrifuged to precipitate the charcoal. The intact ester was precipitated with the charcoal, while the [^{3}H]methanol resulting from ester cleavage remained in solution, and an aliquot (100 μl) was monitored by liquid scintillation counting (lsc). Subsequent studies indicated that Carbowaxed or silylated tubes were not needed to prevent JH III binding to glass.

Esterase activity on JH I, methoprene, and Ro-8-4314 (ethyl ester of 10-methyl-10,11-epoxyfarnesoic acid) was monitored in a similar manner, except that chain-labeled substrates were utilized. The reaction was terminated by the addition of ammonium or sodium chloride and ethyl acetate containing a trace of pyridine. An aliquot of the organic phase was co-spotted with cold standards on tlc, developed in HEt (3:2), and the appropriate spots were scraped and analyzed by lsc.

The esterase activity on α-nathphyl acetate (2.5×10^{-4} *M*) was monitored at

600 nm measuring the absorbence after the reaction was terminated by the addition of 0.4% *o*-dianisidine diazotate–3.4% sodium dodecyl sulfate in distilled water. The release of *p*-nitrophenol from 5×10^{-4} *M* *p*-nitrophenyl acetate was observed spectrophotometrically at 345 nm.

Ethyl ester model substrates were assayed at 5 and 1×10^{-4} *M* final concentrations in solutions containing 100–500 μl of undiluted homolymph made up to 2 ml with phosphate buffer (0.1 *M*, pH 7.4). The reaction mixture additionally contained nicotinamide adenine dinucleotide (NAD, 0.05 m*M*) and yeast alcohol dehydrogenese (Calbiochem, 30 μg, 250 units/mg). The absorbence (340 nm) of the NADH (reduced NAD) produced in the reaction was monitored continually on a Beckman 24/25 spectrophotometer for time periods up to 1 hr. A standard curve indicates that these conditions can detect less than 0.1 μmol of ethanol released.

JH binding activity was observed by adding distilled water containing radiolabeled JH I or JH III (100 μl) to a 5 × 50-mm Carbowaxed tube containing the appropriate column fraction (100 μl). Following incubation (20 min), a charcoal–dextran suspension (100 μl) (without acetic acid) was added to adsorb unbound JH, and the tube was vortexed and centrifuged to precipitate the charcoal. The radioactivity, still in solution, was taken as indicative of binding activity. Binding studies were also run with column fractions pretreated with compounds to inhibit JH esterases. This method does not determine specificity, affinity, or quantity of binding activity; rather, it is a method designed to detect the relative JH binding of various fractions. Modifications of this method have been used by a number of workers (2, 5, 6, 14, 15)

Inhibition Studies

Candidate inhibitors were added to the enzyme (100 μl) in ethanol (1 μl) unless otherwise noted and allowed to preincubate (10 min, 30°C) before the addition of the substrate in ethanol (1 μl) to give a final concentration of 5×10^{-6} *M*. Appropriate incubation times (usually 30 min) were determined by running a time course to insure that the rate of radiolabeled methanol release was linear at the time chosen. All inhibitor concentrations were run at least three times on at least two different enzyme preparations. The percentage of inhibition caused by each compound was plotted against the logarithm of the inhibitor concentration, and the concentration required to give 50% inhibition was determined (I_{50}).

JH Epoxidase

Corpora allata (CA) were removed through the neck membrane of adult female *B. giganteus*, and extraneous tissue was dissected away. The CA were held in phosphate buffer (0.1 *M*, pH 7.4, 1% bovine serum albumin, 2–5°C) with or without inhibitor (1×10^{-4} *M*) until the dissections were completed, then washed and homogenized in fresh buffer at 2 CA/100 μl. The CA homogenates were incubated (2 hr, 30°C) with or without NADPH (reduced nicotinamide adenine dinucleotide phosphate) (1×10^{-5} *M*), last instar hemolymph (10 μl, 1:3 dilution), and an inhibitor (compound 3, Table 1; 1×10^{-4} *M*) with radiolabled methyl farnesoate (5×10^{-8} *M*). The reaction was terminated by the addition of cold standards, ammonium chloride, and ethyl acetate, and the products were analyzed by tlc and lsc (17).

RESULTS AND DISCUSSION

Radiosynthesis

A lower specific activity was obtained for the ^{3}H-labeled JH III than expected, based on the previous deuterium oxide experiment, possibly due to a trace of moisture in the tritiation reaction mixture.

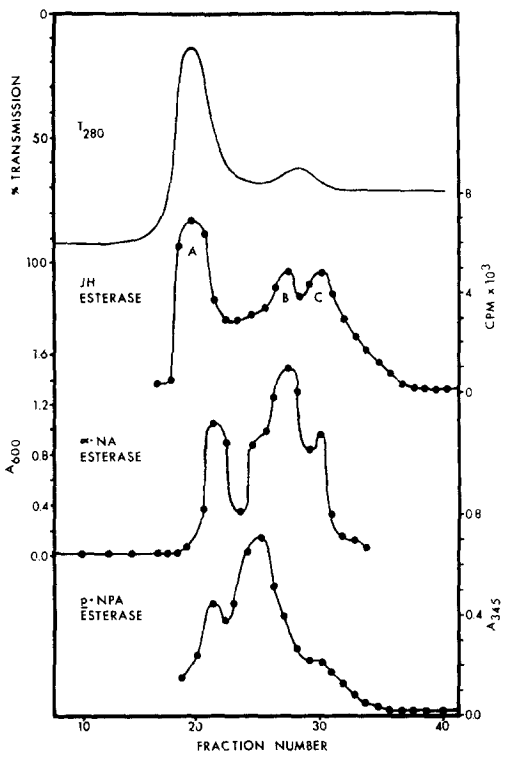


FIG. 2. *Sephadex G 150 elution pattern for uv-absorbing materials (T_{280}), JH I and JH III esterases (identical patterns) (cpm × 10^3 of [^3H]methanol released or JH I acid formed out of 10,000 cpm of JH III or JH I), α-naphthyl acetate esterases (A_{600}), and p-nitrophenyl acetate esterases (A_{345}) from B. giganteus hemolymph. All JH I and JH III binding appeared in the exclusion volume.*

Although the yield of tritiated material cochromatographing with JH on tlc was reasonable, the yield of hrlc pure tritiated JH was very low.

The purity and yield of the JH III from the deuterium exchange reaction on hrlc indicate that the intense radiation in the tritiation process or a trace of copper oxide catalyst could have led to anomalous reactions of diazomethane with the terpene chain of JH. Such reactions are known to occur under intense uv radiation or with metal catalysts. This tritiation method should be very suitable for producing moderate specific activity methyl-labeled hormones which are adequate for many endocrinology studies.

Gel Filtration Patterns

Gel filtration resolved three major JH esterase peaks (Fig. 2), and JH I and JH III esterase activity demonstrated the same elution profiles on three separate runs. Esterase activity monitored on α-naphthyl acetate demonstrated an elution profile similar but not identical to the JH esterases while *p*-nitrophenyl acetate esterase activity demonstrated a distinct elution profile. JH I and JH III binding activity was found only in the exclusion volume with no low molecular weight binding protein analogous to that of *M. sexta* (2, 5, 6, 14), and the situation appears similar to that found in *Locusta migratoria* (18). The specificity of the JH binding was not further investigated.

Inhibitor Relationships

The I_{50}s presented in Table I for JH esterases were not run under conditions of substrate saturation of the enzyme so that esterase activity on JH I and JH III could be compared. Although high substrate levels of JH I have sometimes been used to monitor JH esterase activity, this is a questionable procedure. Surface tension and other studies have indicated that JH I, methoprene, and Ro-8-4314 are no longer in true solution above about 1.2×10^{-5}, 1.3×10^{-5}, and 5×10^{-5} *M*, respectively (19). This problem of solubility has complicated kinetic studies because incubations cannot easily be run at enzyme saturation of JH I without using very dilute esterase preparations (20, 21).

Neither of the two juvenoids tested, one with and one without an ester moiety, (methoprene No. 38 and R-20458 No. 39, respectively), seemed to compete with JH as evidenced by their lack of inhibition of JH metabolism at 1×10^{-4} *M* (Table 1). DDT (No. 40), examined because of its lipophilicity, the antiallatotropin precocine II (No. 37) (22), and the ethyl esters Nos. 41 and 42, were found to cause no inhibition. None of the carbamates (Nos. 30–35)

or methyl phenylsulfonylfluoride (No. 36) led to significant inhibition of JH esterases.

As expected from the deactivating effect of the thiono sulfur atom, parathion (No. 23) is a poorer inhibitor than paraoxon (No. 24) (thiono effect). The weak inhibition of parathion is possibly due to some oxon included in the sample, and oxon impurities may also explain the inhibition of *M. sexta* JH esterases reported for some thiono organophosphorus pesticides by Ajami (23). Commonly used esterase inhibitors such as compounds 21, 22, 24, 26, and 27 demonstrated only weak inhibition of JH esterases, although conditions are not appropriate for the metabolic activation of compound 22 (24).

Several *S*-phenyl phosphates and phosphoramidothiolates demonstrated good inhibition. Among the *S*-phenyl inhibitors examined, the JH esterase inhibition activity was phosphoramidate (No. 15) > phosphate (No. 19) > phosphonate (No. 20). As with inhibition of acetylcholinesterase (AChE), the addition of an *N*-substituent greatly decreases the inhibition of JH esterase while only slightly decreasing the rate of alkaline hydrolysis. *N,N*-Disubstituted phosphoramidates are inactive as inhibitors and are refractory to alkaline hydrolysis, although *N*-acetyl-substituted compounds may be activated by amidases.

Eto (25) reports a positive correlation between the presence of an *S*-alkyl group in an organophosphate compound and its inhibition of house fly head AChE (thiolo effect). This thiolo effect is not observed for phosphoramidothiolates for house fly head AChE (25, 26), but a strong thiolo effect is evident in their inhibition of JH esterases (No. 13 and 18). The phosphonamidothiolates examined are poorer inhibitors than the corresponding phosphoramidothiolates (No. 1 > No. 6, No. 3 > No. 11). There appears to be little correlation between inhibition of *Musca domestica* head AChE and *B. giganteus* JH esterase, since among other compounds paraoxon (No. 24) and DDVP (No. 27) inhibit (I_{50}) house fly head AChE at 1.1×10^{-7} (25) and 8×10^{-9} *M* (27), respectively, whereas the best two JH esterase inhibitors (Nos. 15 and 3) only inhibit AChE at 3.3×10^{-6} (calculated from 28) and 4.1×10^{-5} *M* (26), respectively. Phosphoramidothiolate compounds that have very similar AChE I_{50}s and alkaline hydrolysis constants (Nos. 1, 2, 3, 13) (26) have different JH esterase I_{50}s.

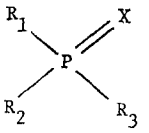
Substrate and Inhibitor Specificity

With the exception of two compounds (Nos. 12 and 24), and to a lesser extent four others (Nos. 14, 17, 21, and 34), the I_{50}s are similar for each inhibitor on the three JH esterase peaks, indicating that the influence of JH binding on the I_{50}s in diluted hemolymph is probably negligible (Table 1). The inhibition of JH esterase activity in peak C but not in peak A by compound 12 may be due to amidases in the former peak releasing the active phosphoramidothiolate by the removal of the acetate moiety. The approximately 10-fold decrease in the inhibition of JH esterase by compounds 21 and 24 in peak A compared to peaks B and C could possibly be due to the DEF and paraoxon reacting with other components in the exclusion volume. Minor differences in the I_{50}s for compounds 14, 17, and 34 could not be accounted for.

The rates of hydrolysis of JH I and JH III for each of the three esterase fractions are very similar (Table 2). Inhibition studies on each of the three JH esterase peaks were carried out using three different substrates, (α-naphthyl acetate, JH I, JH III) and selected inhibitors (Table 2). Using either JH I or JH III as the substrate, all three esterase peaks demonstrated similar inhibition. The I_{50}s for α-naphthyl acetate esterases in peak A were similar to JH I and JH III esterase I_{50}s with the exception of compound 15, where a fivefold higher concentration was required to give

TABLE 1

In Vitro Inhibition (I_{50}, M) of Three Blaberus giganteus JH Esterase Fractions[a]



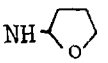
	R_1	R_2	R_3	X	Esterase peaks (I_{50}, M) A	B	C
			Organophosphates				
1.	CH_3S	CH_3O	NH_2	O	4×10^{-7}	5×10^{-7}	4×10^{-7}
2.	CH_3S	C_2H_5O	NH_2	O	3×10^{-7}	1×10^{-7}	2×10^{-7}
3.	C_2H_5S	CH_3O	NH_2	O	3×10^{-8}	2×10^{-8}	2×10^{-8}
4.	CH_3S	CH_3O	$N{=}C(CH_3)NH_2$	O	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
5.	CH_3S	CH_3	$NHCH_3$	O	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
6.	CH_3S	CH_3	NH_2	O	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
7.	CH_3S	CH_3	NH_2	S	4×10^{-5}	$>10^{-4}$	$>10^{-4}$
8.	CH_3	CH_3O	NH_2	S	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
9.	CH_3O	CH_3O	$NHCH_3$	O	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
10.	CH_3S	n-C_3H_7	NH_2	O	9×10^{-6}	2×10^{-5}	1.5×10^{-5}
11.	C_2H_5S	CH_3	NH_2	O	5×10^{-5}	5×10^{-5}	5×10^{-5}
12.	C_2H_5S	C_2H_5	$NHC(O)C_2H_5$	O	$>10^{-4}$	5×10^{-6}	7×10^{-6}
13.	C_2H_5S	C_2H_5O	NH_2	O	2.5×10^{-6}	2×10^{-6}	2×10^{-6}
14.	C_2H_5S	C_2H_5O	$N(CH_3)_2$	O	$>10^{-4}$	4×10^{-5}	2.5×10^{-5}
15.	C_6H_5S	C_2H_5O	NH_2	O	2×10^{-9}	1×10^{-9}	8×10^{-10}
16.	i-C_3H_7S	i-C_3H_7S	NH_2	O	$>10^{-4}$	$>10^{-4}$	$\sim 10^{-4}$
17.	C_2H_5S	C_2H_5S		O	$>10^{-4}$	2×10^{-5}	$>10^{-4}$
18.	C_2H_5O	C_2H_5O	NH_2	O	$>10^{-4}$	$>10^{-4}$	$\sim 10^{-4}$
19.	C_2H_5O	C_2H_5O	C_6H_5S-	O	5×10^{-8}	4×10^{-8}	7×10^{-8}
20.	C_2H_5	C_2H_5O	C_6H_5S-	O	1×10^{-7}	2×10^{-7}	1×10^{-7}
21.	n-C_4H_9S	n-C_4H_9S	n-C_4H_9S-	O	$>10^{-4}$	3×10^{-5}	3×10^{-5}
22.	o-$CH_3C_6H_4O$	o-$CH_3C_6H_4O$	o-$CH_3C_6H_4O$-	O	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
23.	C_2H_5O	C_2H_5O	p-$NO_2C_6H_4O$-	S	3×10^{-5}	2×10^{-5}	1.5×10^{-5}
24.	C_2H_5O	C_2H_5O	p-$NO_2C_6H_4O$-	O	2×10^{-5}	2×10^{-6}	2×10^{-6}
25.	C_2H_5O	C_2H_5O	$ON{=}C(CH_3)$-m-$C_6H_4NO_2$	O	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
26.	C_2H_5O	C_2H_5O	$OP(O)(OC_2H_5)_2$	O	3×10^{-6}	1.5×10^{-6}	1.5×10^{-6}
27.	CH_3O	CH_3O	$OCH{=}C(Cl)_2$	O	4×10^{-6}	4×10^{-6}	3×10^{-6}
28.	CH_3O	CH_3O	$OC(CH_3){=}CH$-$C(O)OCH_3$	O	8×10^{-6}	3×10^{-6}	4×10^{-6}
29.	CH_3O	CH_3O	$OC(CH_3){=}CH$-$C(O)NHCH_3$	O	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
			Carbamates				
30.	2,3-dichlorophenyl *N*-methylcarbamate				7×10^{-5}	8×10^{-5}	$>10^{-4}$
31.	*o*-isopropoxyphenyl methylcarbamate (propoxur)				$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
32.	2-methyl-2-(methylthio)-propionaldehyde-*O*-methylcarbamoyl oxime (aldicarb)				$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
33.	α,α,α-trifluoroacetophenone-*O*-methylcarbamoyl oxime				$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
34.	α-napthyl *N*-methylcarbamate (carbaryl)				$>10^{-4}$	7×10^{-5}	2.5×10^{-5}
35.	α-napthyl *N*-ethylcarbamate				$>10^{-4}$	$>10^{-4}$	$>10^{-4}$

TABLE 1—*Continued*

		Esterase peaks (I_{50}, M)		
		A	B	C
	Miscellaneous compounds			
36.	methylphenylsulfonylfluoride	3×10^{-6}	1.5×10^{-6}	4×10^{-6}
37.	Precocine II	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
38.	Methoprene (ZR 515)	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
39.	R-20458	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
40.	DDT	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
41.	ethyl-α-(4-chlorophenoxy)-α-methyl propionate	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
42.	ethyl-α-(4-chlorophenoxy)butyrate	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$

[a] Substrate concentration: 5×10^{-6} M JH III. Sources of compounds are as follows: 1–25, 27, 30–33, T. R. Fukuto (this Division); 37, W. S. Bowers (Cornell University); 36, Calbiochem; 38, Gary Quistad (Zoecon Corp.); compounds 35, 39, 40, 41, and 42 were synthesized in this laboratory by published procedures; and 26, 28, 29, and 34, Chem. Service.

a similar inhibition of α-naphthyl acetate esterases.

Large differences were noted on peaks B and C for inhibition of JH esterases using α-naphthyl acetate compared to JH I and JH III. The phosphoramidothiolates (Nos. 1, 3, 15) were poorer inhibitors (1000×) as was compound 27 (10×) with α-naphthyl acetate as substrate rather than JH. The carbamates (Nos. 31, 34) were equally poor inhibitors with all three substrates. Paraoxon (No. 24) was more effective (10×) when α-naphthyl acetate was the substrate, as one would expect for a general carboxyesterase inhibitor.

The model substrates ethyl 3,3-dimethylacrylate, ethyl cinnamate, and ethyl 10,11-epoxyfarnesoate (pure ethyl ester) were examined in hopes of finding a useful tool for the detection of JH esterases on gels and in solution as monitored by the release of ethanol. Ethanol can be detected by a number of reagents based on its conversion to acetaldehyde by alcohol dehydrogenase while reducing NAD or another redox cofactor. In the case of hemolymph from *B. giganteus*, no release of ethanol could be detected with any of the three substrates utilized. Lack of ester cleavage of the conjugated ethyl ester Ro-8-4314 and the conjugated dienoate isopropyl ester methoprene under concentrations of whole *B. giganteus* homolymph resulting in >80% ester cleavage of JH III in 30 min supports the premise that *B. giganteus* JH esterases are specific for methyl esters. Kramer and de Kort (21) also report lack of ester cleavage of 2,3–4,5-dienoate and isopropyl ester juvenoids. This lack of metabolism does not imply that *B. giganteus* hemolymph esterases cannot metabolize other ethyl or higher esters. All of the model compounds used in this study are stable, conjugated esters in contrast to nonconjugated model substrates reported to be metabolished by mosquito esterases by Hooper (29). Since slow hydrolysis of JH or juvenoid ethyl esters has been reported in another insect (13), these model substrates may find some use, but this study indicates that any results obtained from them must be interpreted with caution.

The determination of the precise substrate specificity of *B. giganteus* JH esterases awaits esterase purification and the use of high resolution techniques such as electrophoresis and isoelectric focusing, but the differential inhibition of α-naphthyl acetate esterases and JH esterases in the case of peaks B and C indicates that α-naphthyl acetate may be in some cases misleading in studying JH esterase activity.

TABLE 2

In vitro Inhibition of B. giganteus Esterases Utilizing Three Different Substrates

Inhibitor number	Esterase peaks (I_{50})[a]								
	A			B			C		
	Substrate								
	α-NA	JH I	JH III	α-NA	JH I	JH III	α-NA	JH I	JH III
1	4×10^{-7}	2×10^{-7}	4×10^{-7}	$>10^{-4}$	3×10^{-7}	5×10^{-7}	1×10^{-4}	1×10^{-7}	4×10^{-7}
3	3×10^{-8}	3×10^{-8}	3×10^{-8}	2×10^{-5}	3×10^{-8}	2×10^{-8}	1×10^{-5}	8×10^{-9}	2×10^{-8}
15	1×10^{-8}	4×10^{-9}	2×10^{-9}	1×10^{-6}	9×10^{-10}	1×10^{-9}	3×10^{-6}	8×10^{-10}	8×10^{-10}
24	2×10^{-6}	1.5×10^{-6}	2×10^{-5}	3×10^{-7}	2×10^{-6}	2×10^{-6}	3×10^{-7}	1×10^{-6}	2×10^{-6}
27	4×10^{-6}	3×10^{-6}	4×10^{-6}	2×10^{-5}	2×10^{-6}	4×10^{-6}	2×10^{-5}	1.5×10^{-6}	3×10^{-6}
31	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
34	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$	7×10^{-5}	7×10^{-5}	5×10^{-5}	$>10^{-4}$	2.5×10^{-5}
Specific activity (mmol of JH acid produced/mg of protein/min)	—	2.5×10^{-6}	2.3×10^{-6}	—	1.9×10^{-7}	2.2×10^{-7}	—	1.7×10^{-7}	1.7×10^{-7}
Protein content (mg/ml)		41.7			4.2			4.5	

[a] Substrate concentrations: 5×10^{-4} *M* (α-NA) and 5×10^{-6} *M* (JH I and JH III).

TABLE 3

Effect of Organophosphorus Treatment on the Metabolism of Methyl Farnesoate by Corpora Allata Homogenates

	Incubation conditions[a]													
CA Epoxidase	+	+	+	+	+	+	+	+	+	+	+	−	−	−
NADPH	−	+	+	+	−	−	−	−	+	+	+	−	−	−
Hemolymph[b]	−	−	−	−	−	−	+	+	+	+	+	−	+	+
Organophosphate[c]			A	B	A	B		A		A	B			A
Metabolites	Percentage of metabolites													
Methyl farnesoate	95	52	51	52	97	97	69	96	34	49	53	96	71	97
JH III	1	45	41	44	1	1	1	1	17	32	45	1	1	1
Farnesoic acid	1	1	1	1	1	1	28	1	10	3	1	1	24	1
JH acid	2	2	6	2	1	1	2	1	36	13	2	1	3	1
Other	1	0	1	1	0	0	0	1	3	3	0	1	1	0
Total oxidase[d]	3	47	47	46	2	2	3	2	53	45	47	2	4	2
Total esterase[d]	3	3	7	3	2	2	30	2	46	16	3	2	27	2

[a] The data shown are averages of at least three experiments performed on at least 2 separate days. All percentages show $< \pm 5\%$ variation within the same preparation and $< \pm 10\%$ in different preparations. Incubations (30°C, 2 hr) contained 2 CA equivalents in the 120 μl of buffer.

[b] Hemolymph from last instar *B. giganteus* was diluted 1:3 with buffer, and 10 μl was added to the incubation medium or 10% final volume to the CA wash buffer.

[c] The *O*-methyl *S*-ethyl phosphoramidothiolate (No. 3, 1×10^{-4} *M* final concentration) was added directly to the incubation medium (A) or to the CA wash buffer (B), then NADPH (10 μl of water, 1×10^{-5} *M*) and methyl farnesoate (1 μl of ethanol, 5×10^{-8} *M*) were added after a 10-min preincubation.

[d] Total oxidase = JH III + JH acid and Total esterase = farnesoic acid + JH acid.

In other insects, α-naphthyl acetate hydrolysis may be a better indicator of JH esterase activity (7, 20). Electrophoretic studies of *Leptinotarsa decemlineata* and *M. sexta* hemolymph esterases have shown lack of coincidence between α-naphthol acetate straining and JH esterase activity (21; Nowock and Gilbert, unpublished information).

Effect of a Phosphoramidate on CA Homogenates

In studies on the biosynthesis of JH by organs or enzymes *in vitro*, one is faced with the situation that competing reactions may degrade the JH produced. In adult female *B. giganteus*, this problem may be largely overcome by very careful dissection of the CA and washing the CA several times, presumably to remove hemolymph, before use, but this appears not to be the case with some insects (30).

JH epoxidase is microsomal and converts methyl farnesoate to JH III in a NADPH-dependent reaction (17, 31). Either holding the CA in buffer containing compound 3 (1×10^{-4} *M*) or actually incubating the inhibitor with the CA homogenates resulted in no apparent decrease in epoxidase activity. This inhibitor was able to block the esterase activity when a small volume of hemolymph was added to the CA or to their homogenates (Table 3). The data in Table 3 indicate that addition of the esterase inhibitor directly to the incubation medium with NADPH slightly increases the apparent esterase activity when compared to carefully dissected glands. This phenomenon is not adequately explained. The inhibitor in all cases greatly reduces the esterase activity of added hemolymph. The addition of hemolymph slightly but consistently increases total apparent oxidase activity which may be explained by

rapid epoxidation of farnesoic to JH acid. The addition of compound 3 to the CA homogenate caused little change in the epoxidase activity, while, in contrast to the data of Pratt (32) on *Schistocerca gregaria* CA *in vitro*, we found that the high levels of paraoxon or diisopropyl paraoxon necessary to inhibit esterases in hemolymph-contaminated CA homogenates gave a decrease in JH epoxidase activity.

CONCLUSIONS

The data presented in this paper should be taken as directly applicable only to hemolymph JH esterases from *B. giganteus*. Our findings are different in some respects from those of Ajami (23), Pratt (32), and Hooper (29) on inhibitors of *M. sexta* pupal, *S. gregaria*, and *Culex pipiens* JH esterases, respectively. This laboratory has also found variation in the activity of inhibitors on JH esterases from various tissues of several different insects.

Gel filtration elution patterns, inhibitor relationships, and specific activities of *B. giganteus* hemolymph esterases demonstrate that JH I and JH III are degraded at similar rates and probably by similar enzymes. At least in *B. giganteus*, it is thus unlikely that JH esterases significantly vary the relative titers of the JH isomers, although differential binding may expose one substrate preferentially. Surface tension studies (19) indicate that JH III exists in monomeric form in aqueous solutions at concentrations greater than 1×10^{-4} *M*, so JH III can be used in the future for kinetic investigations of JH esterases and their inhibitors.

The JHs are conjugated esters and as such they are much more stable to base hydrolysis than nonconjugated esters (4, 7). Several papers in the field give the impression that the JHs may be degraded by nonspecific carboxyesterases. The stability of JH to hydrolysis by several carboxyesterases from various sources (4) indicates that JH esterases are somewhat unique in their ability to hydrolyze conjugated esters, and the differential inhibition of JH esterases and α-naphthyl acetate esterases further supports the premise that JH esterases should not be considered as "general" carboxyesterases. One can further conclude that the JH esterases show high specificity for the ester portion of the molecule, while structural requirements for the rest of the molecule are somewhat less rigorous.

The lack of inhibition of JH esterase by two juvenoids fails to support the hypothesis that juvenoids may act by preventing the degradation of intrinsic JH (33). It is interesting that several phosphoramidothiolates and *S*-phenyl phosphates are good inhibitors of the JH esterases studied, since some of them are rather potent insecticides; yet they are, in some cases, only mediocre inhibitors of house fly head acetylcholinesterase (25, 26, 28).

Eto (25) has suggested that the phosphoramidothiolates undergo oxidation which convert these compounds to potent inhibitors of AChE. Since hemolymph fractions should not contain oxidases, the inhibition of these JH esterases by phosphoramidothiolates (Nos. 1, 2, 3, 13) demonstrates that an oxidative activation step is not obligatory for strong inhibition of at least some esterases.

Some workers have indicated that JH esterase inhibitors may be effective juvenoid synergists, and the demonstration that some JH esterases can be specifically inhibited supports this premise (although there are no conjugated methyl ester juvenoids on the market). Additionally, the utility of specific JH esterase inhibitors in biosynthetic studies has been demonstrated, and these compounds may additionally be useful in assessing the *in vivo* role of JH esterases or investigating other *in vitro* or *in vivo* systems. Interpretations of the use of general carboxyesterase inhibitors (such as compounds 21, 22, or 24) as potential synergists in elucidating the role of ester cleavage in JH or juvenoid metabolism should be cautious. This con-

clusion is evidenced by other workers who report that diisopropylfluorophosphate is a poor inhibitor for some JH esterases (7, 21). Our study further indicates that selective pesticides based on degradative metabolism of insect hormones may be developed.

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