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Comparative Inhibition of the Juvenile Hormone Esterases from *Trichoplusia ni, Tenebrio molitor,* and *Musca domestica*

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Twenty-seven compounds were screened as potential inhibitors of juvenile hormone esterases. Of these compounds *O*-ethyl-*S*-phenyl phosphoramidothiolate provided the best inhibition for the cabbage looper, *Trichoplusia ni* (Hubner), and the yellow mealworm, *Tenebrio molitor* L., while the juvenile hormone esterases of the house fly, *Musca domestica* L., were best inhibited by a juvenoid carbamate (1-(*m*-phenoxy-*N*-ethyl carbamate)-3,7-dimethyl-7-methoxy-2*E*-octene). The inhibition patterns of *T. ni* and *T. molitor* are similar, while those of *M. domestica* are relatively different. Further studies on the juvenile hormone and α-naphthyl acetate esterases of *T. ni* showed that they could be differentially inhibited. Diisopropyl phosphorofluoridate and an alkyl trifluoromethyl ketone selectively inhibit the hydrolysis of α-naphthyl acetate and juvenile hormone, respectively, while *O*-ethyl-*S*-phenyl phosporamidothiolate inhibits both enzymes. The juvenile hormone esterases of *T. ni* also appear to be unique enzymes that are selective for juvenile-hormone-like molecules. The *in vivo* inhibition of *T. ni* juvenile hormone esterases by *O*-ethyl-*S*-phenyl phosphoramidothiolate slows the *in vivo* hydrolysis of juvenile hormone and results in delayed pupation and malformed larvae that resemble larval–pupal intermediates. Thus, the esterases involved in juvenile hormone metabolism appear to be important in juvenile hormone regulation.

INTRODUCTION

The postembryonic development of an insect is regulated, in part, by juvenile hormone (JH)[2]. Classically, high hemolymph titers of JH maintain the larval or nymphal state, while low JH titers initiate pupal and/or adult development (1, 2). The JH titer in turn appears to be regulated by several mechanisms including synthesis, release, transport, and degradation (2, 3). Because the insect endocrine system, like the nervous system, is regulatory in function, the overstimulation or inhibition of any of the above processes could have profound and potentially lethal effects on an insect.

One of the primary routes of JH degradation is ester hydrolysis (4, 5). During the last instar, esterases that appear to be specific for JH (6–8) appear in the hemolymph. The activity profile of these JH esterases (JHEs) (9–14) seems to coincide with declines in the JH titer in other insects (2, 15–19). The production and/or release of these JHEs appears to be very closely tied to the JH titer (20, 21). Unfortunately the *in vivo* function of this enzyme has not been established. Thus, a survey was undertaken of compounds potentially capable of inhibiting these important enzymes in the cabbage looper, *Trichoplusia ni* (Hubner) (Lepidoptera); the yellow mealworm, *Tenebrio molitor* L. (Coleoptera); and the house fly, *Musca domestica* L. (Diptera). On the basis of the

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[2] Abbreviations used: JH(s), juvenile hormone(s); JHE(s), juvenile hormone esterases; α-NAE(s), α-naphthyl acetate esterases; EH(s), epoxide hydrolase(s); α-NA, α-naphthyl acetate; I_{50}, concentration which yields 50% inhibition; tlc, thin-layer chromatography; EPPAT, *O*-ethyl-*S*-phenyl phosphoramidothiolate; PMSF, phenylmethyl sulfenylfluoride; CMPS, *p*-chloromecuriphenyl sulfonic acid; EDTA, ethylenediaminetetraacetic acid; BEPAT, *S*-benzyl-*O*-ethyl phosphoramidothiolate; TFT, 1,1,1-trifluorotetradecan-2-one; PTTH, prothoracicotropic hormone; ACHE(s), acetylcholine esterase(s).

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survey, a potent inhibitor of *T. ni* JHE was found and then used to block the JHE of *T. ni in vivo* in an effort to better establish the function of the JHE and the effects of inhibiting JH catabolism.

METHODS AND MATERIALS

Insects. T. ni larvae were reared on an artificial diet (22) at 27 ± 2°C with a photoperiod of 14L:10D. Last-instar larvae (L5) containing either high JHE (Day 2) or high α-naphthyl acetate esterase (α-NAE) (Day 3) activity were selected on the basis of weight and time of molt to the last instar (14). Last-instar *T. molitor* larvae were obtained from California Mealworm Warehouse (Riverside, Calif.). Larvae were held in containers with untreated bran at 27 ± 2°C with a photoperiod of 14L:10D. Pupae were collected daily and used as the enzyme source. The S-NAIDM strain of *M. domestica* larvae used in this study were reared as previously described (23).

Enzyme preparation. T. ni hemolymph was collected from larvae at the time of the appropriate esterase peaks during the last larval instar as described previously (14). The hemolymph was diluted with 1:10 (α-NAE) or 1:100 (JHE) in cold (4°C) I = 0.2, pH 7.4 phosphate buffer containing 0.01% (w/v) phenylthiourea to inhibit the tyrosinases. *T. molitor* enzymes were prepared by homogenizing 10 pupae in 10 ml of the above buffer at 4°C. The resulting homogenate was centrifuged at 12,000*g* for 15 min at 4°C to remove the debris. The homogenate was removed via a Pasteur pipet, filtered through glass wool, and centrifuged at 100,000*g* for 70 min at 4°C. The resulting supernatant was retained as 100,000*g* soluble fraction. The 100,000*g* pellet was resuspended in the above buffer (1 pupal eq/ml) as the "microsomal" fraction. The *M. domestica* enzymes were prepared similarly to those of *T. molitor* except that 50 larvae (or pupae) were homogenized per 10 ml of buffer, and the "microsomal" fraction contained 5 larval (or pupal) eq/ml. For both *T. molitor* and *M. domestica,* the 100,000*g* fractions were vortexed vigorously just prior to use. All enzyme fractions were assayed immediately after preparation.

T. molitor pupae were examined for JHE and epoxide hydrolase (EH) activity 1, 3, 6, 12, 24 hr, and each day thereafter, following pupation. Likewise the mature larvae (last instar, 7 days old), white larvae (postfeeding last-instar larvae with cleared gut), white pupae (just pupated), and light brown pupae of *M. domestica* were also examined for JHE and EH activity.

Enzyme assays. JHE activity was monitored using *O*-methyl-labeled [^{3}H]JH III (*E,E;* 2.5 Ci/mmol) as substrate as previously described (8, 10). The inhibitor was added to the enzyme (100 μl) and preincubated for 10 min at 30°C, unless otherwise indicated. The substrate (JH III) was added in ethanol to give a concentration of 5 × 10^{-6} *M* and incubated at 30°C. Juvenoids and selected esters examined as potential competitive inhibitors (in 1 μl ethanol) were added to the enzyme just prior to the addition of the JH III substrate. The effects of selected inhibitors on α-NAE activity were determined by adding the inhibitor in ethanol (1 μl) to the enzyme (100 μl) and preincubating for 10 min at 30°C, and then proceeding as described previously (8, 10), using α-naphthyl acetate (α-NA) as substrate (2.5 × 10^{-4} *M*).

The respective enzymes were diluted so that conditions of substrate saturation were approached and for which the uninhibited reaction displayed a linear rate of hydrolysis throughout the time used for the analysis. Semilog plots of percentage inhibition vs inhibitor concentration provided the lines from which the reported I_{50} values (inhibitor concentration that results in 50% inhibition of the control enzyme activity) were taken.

All inhibitors were periodically checked for purity by thin-layer chromatography (tlc) and occasionally by their nuclear magnetic resonance spectra (8). All enzyme assays were run in triplicate on at least two

separate occasions. Ethanol-treated controls gave essentially the same response as untreated controls. EH activity was monitored as described previously (24).

In vivo JHE inhibition. Day 1, 2, and/or 3 last-instar *T. ni* larvae were treated topically with EPPAT (*O*-ethyl-*S*-phenyl phosphoramidothiolate) (22 μg) in 1 μl of ethanol. At selected intervals, these treated larvae and control (untreated or ethanol only) larvae (3, 4) were bled and the hemolymph assayed for JHE activity. Treated and control larvae were also examined for time of pupation, and behavioral and physical abnormalities. All experiments were run on three separate occasions.

The ability of EPPAT to actually slow or prevent the ester hydrolysis of JH *in vivo* was examined by topically treating early Day 2 last-instar *T. ni* larvae with 30 μg of JH I in 1 μl of ethanol mixed with radiolabeled JH I (13.5 Ci/mmol, ^{3}H at C10, New England Nuclear) and then either ethanol (1 μl) or EPPAT (22 μg in 1 μl of ethanol). Each treatment was composed of four groups, two larvae each. On the basis of preliminary experiments the larvae were assayed 6 hr after treatment. The hemolymph from each pair of larvae was pooled into a 6 × 50-mm culture tube from which 10 μl was removed and quantitated by liquid scintillation counting (lsc). An equal volume of ethanol was added to the remaining hemolymph, and after vortexing and centrifugation (5000*g* for 10 min), the JH I and metabolites present in the supernatant were separated via tlc (silica gel, F_{254}, 0.25-mm gel thickness, EM Laboratories). The tlc plates were briefly developed in methanol (1 cm), allowed to dry, and then run in hexane–ethyl acetate (3:2). Areas corresponding to JH, JH acid, JH diol, JH diol–acid, and polar metabolites were scraped from the plates and counted by lsc. No significant radioactivity appeared elsewhere on the plate. The cold standards used for the cochromatographic ultraviolet visualization of the metabolites included epofenonane (1-(4′-ethylphenoxy)-6,7-epoxy-3-ethyl-7-methylnonane), R-20458 (1-(4′-ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2*E*-octene), and their diols. Labeled JH I acid, also used as a tlc standard, was prepared as described previously (25).

Juvenoid effects on T. ni development. Early Day 1 last-instar larvae (n = 50) were treated topically with the juvenoid epofenonane (3 μg) in ethanol (1 μl), left untreated, or treated with ethanol only. The larvae were then examined for behavioral and physical abnormalities, as well as for the time of pupation.

RESULTS

JHE and EH Activity in T. molitor and M. domestica

Using the epoxide-containing juvenoid R-20458 as a substrate, all EH activity in the postmitochondrial supernatant of both *T. molitor* and *M. domestica* was found to be in the 100,000*g* "microsomal" pellet. JHE and EH activity was constant throughout the pupal stage of *T. molitor*, and no differences were detected between males and females. Thereafter 0- to 24-hr-old pupae were used for all enzyme preparations, for which the specific activities of the soluble and microsomal fractions for JHE were 1270 and 64 pmol/min-mg protein, respectively.

The JHE activity of the *M. domestica* soluble fraction ranged from 1.07 pmol/min-mg protein in mature larvae to 1.01, 0.74, and 0.82 pmol/min-mg protein in white larvae, white pupae, and light brown pupae, respectively. JHE activity in the microsomal fraction was higher, but followed a similar pattern with values of 4.74, 3.51, 3.89, and 4.61 pmol/min-mg protein for the mature larvae to light brown pupae, respectively. This pattern of microsomal JHE activity is similar to that reported by Yu and Terriere (26). Microsomal EH activity followed a pattern similar to that of the JHE. Although the white larval stage did not have the highest JHE or EH activity, it

was used for further studies because it could be precisely timed and was available in relatively large numbers.

Inhibitor Relationships

None of the carbamates, juvenoids (Table 1, Nos. 24, 25), PMSF (phenylmethyl sulfenylfluoride), CMPS (*p*-chloromecuriphenyl sulfonic acid), or EDTA (ethylenediaminetetracetic acid) provided good inhibition of the *T. ni* JHEs at the highest concentration tested (Table 1). Many of the organophosphates commonly used as esterase inhibitors (Table 1, Nos.

TABLE 1

In Vitro Inhibition of Juvenile Hormone Esterases from Three Insects[a]

No.	Compound				I_{50} ($\times 10^{-4}$ *M*)				
					T. ni	*T. molitor*		*M. domestica*	
					hemolymph	Sol.	Micro.	Sol.	Micro.
	Organophosphates $R(R')(R'')P{=}X$								
	R	R′	R″	X					
1	CH_3O	NH_2	CH_3S	O	0.07	>1	>1	>1	>1
2	CH_3CH_2O	NH_2	CH_3S	O	0.03	>1	>1	>1	>1
3	CH_3O	NH_2	CH_3CH_2S	O	0.05	>1	>1	0.01	>1
4	CH_3CH_2O	NH_2	CH_3CH_2S	O	1	>1	>1	>1	>1
5	CH_3CH_2O	$N(CH_3)_2$	CH_3CH_2S	O	>1	>1	>1	>1	>1
6	CH_3CH_2O	NH_2	ϕ-S	O	0.01	0.08	0.02	0.001	>1
7	CH_3CH_2O	CH_2CH_3	ϕ-S	O	0.1	0.2	0.1	0.0006	>1
8	CH_3CH_2O	CH_3CH_2O	ϕ-S	O	>1	>1	>1	>1	>1
9	CH_3CH_2O	CH_3CH_2O	*p*-O_2N-ϕ-O	O	0.02	>1	>1	>1	0.03
10	CH_3CH_2O	CH_3CH_2O	*p*-O_2N-ϕ-O	S	>1	>1	>1	>1	>1
11	CH_3O	CH_3O	$OCH{=}CCl_2$	O	0.2	0.6	0.6	1	0.3
12	CH_3CH_2O	CH_3CH_2O	$OP(O)(OCH_2CH_3)_2$	O	>1	>1	>1	>1	>1
13	i-prop-O	i-prop-O	F	O	>10	>10	>10	10	>10
14	*n*-but-S	*n*-but-S	*n*-but-S	O	>1	>1	>1	>1	>1
15	*o*-$CH_3\phi O$	*o*-$CH_3\phi O$	*o*-$CH_3\phi O$	O	>1	>1	>1	>1	>1
	Carbamates								
16	Propoxur				>1	>1	>1	>1	>1
17	Aldicarb				>1	>1	>1	>1	>1
18	Carbaryl				>1	>1	>1	1	0.05
19	1-naphthyl *N*-ethyl carbamate				>1	>1	>1	>1	0.003
20	Carbofuran				>1	>1	>1	>1	>1
21	Juvenoid ethyl carbamate[b]				>1	1	0.5	0.00004	0.003
22	Eserine sulfate				>10	>10	>10	>10	>10
	Miscellaneous compounds								
23	PMSF[c]				>1	>1	>1	0.4	0.2
24	Methoprene				>1	>1	>1	>1	>1
25	R-20458[d]				>1	>1	>1	>1	>1
26	CMPS				>1	>1	>1	>1	0.3
27	EDTA				>10	>10	>10	>10	>10

[a] Substrate concentration, 5×10^{-6} *M* JH III. Sources of the compounds are as follows: 1–11, 14–17, 20, T. R. Fukuto (University of California, Riverside); 12, 18 Chemical Service; 13, 22, 23, 26, 27, Sigma; 24, David Schooley (Zoecon Corp.); 19, 21, 25, were synthesized by published procedures.

[b] 1-(*m*-Phenoxy *N*-ethyl carbamate)-3,7-dimethyl-7-methoxy-2*E*-octene.

[c] PMSF, phenylmethyl sulfenylfluoride; CMPS, *p*-chloromercuriphenyl sulfonic acid; EDTA, ethylenediaminetetraacetic acid.

[d] 1-(4′-Ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2*E*-octene.

11–15) are either ineffective or display only moderate inhibitory activity. However, relatively good inhibition is demonstrated by some of the phosphoramidothiolates (Nos. 1–3, 6) and paraoxon (No. 9). As expected the sulfur atom of parathion (No. 10) has a deactivating effect on its ability to inhibit the JHEs compared to the oxygen analog paraoxon (No. 9). Among the phosphoramidothiolates, extension of either the *O*- or *S*-alkyl chain from methyl to ethyl results in increased activity (Nos. 2, 3), while extension of both alkyl chains to ethyl reduces activity (No. 4). However, the *O*-ethyl-*S*-phenyl analog, EPPAT (No. 6), displays the best inhibition of all the compounds assayed. Among the *S*-phenyl phosphates, *T. ni* JHE-inhibitory ability is phosphoramidate (No. 6) > phosphonate (No. 7) > phosphate (No. 8). The JHEs of *Blaberus giganteus* also show a similar pattern (8).

Inhibition patterns of the *T. molitor* soluble and microsomal fractions are virtually identical (Table 1). Although the overall inhibition pattern for *T. molitor* is similar to that of *T. ni*, the JHEs of *T. molitor* appear to be slightly more resistant to inhibition (Table 1). As with *T. ni*, the *T. molitor* JHEs are also only poorly inhibited by carbamates, juvenoids (Nos. 24, 25), PMSF, CMPS, and EDTA. For *T. molitor* EPPAT (No. 6) is somewhat less effective on the JHEs from the soluble fraction relative to those from the microsomal fraction. As with *T. ni* and *B. giganteus* (8), the JHE-inhibitory ability for the *S*-phenyl phosphates is also phosphoramidate (No. 6) > phosphonate (No. 7) > phosphate (No. 8). However, a notable difference between the JHEs of *T. ni* and *T. molitor* is the inactivity of paraoxon (No. 9) and most of the phosphoramidothiolates (Nos. 1–3) on the *T. molitor* JHEs (Table 1). A similar lack of paraoxon inhibitory activity has also been noted for *T. molitor* hemolymph esterases (27).

The JHEs of *M. domestica* larvae exhibit inhibition patterns that are quite different from those of either *T. ni* or *T. molitor* (Table 1). Although the overall inhibition pattern for the soluble fraction appears to be closer to those of *T. ni* and *T. molitor* than the microsomal fraction, the most active inhibitor of soluble JHEs for *M. domestica* is a juvenoid carbamate (No. 21). Interestingly, none of the other carbamates assayed (Nos. 16–20, 22) display any inhibitory activity against the soluble JHEs (Table 1). However, Mumby *et al.* (28) found certain *N*-alkyl carbamates of geranyl phenyl ether and 1-naphthol to be effective inhibitors of *M. domestica* soluble JHEs. Although not as potent as the juvenoid carbamate (No. 21), EPPAT (No. 6) is also a very effective inhibitor of JHEs from the soluble fraction.

The JHE activity of the microsomal fraction of *M. domestica* is not only distinct from the JHEs of *T. ni* and *T. molitor*, but also from those of the *M. domestica* soluble fraction (Table 1). Both PMSF and CMPS display slight inhibitory activity, while the juvenoids (Nos. 24, 25) and EDTA are inactive. Unlike the other JHEs, those of the *M. domestica* microsomal fraction are not inhibited by any of the phosphoramidothiolates (Nos. 1–6). In fact, the only organophosphates exhibiting any activity are paraoxon (No. 9) and dichlorovos (No. 11). The microsomal JHEs of *M. domestica* are also distinct in that several carbamates (Nos. 18, 19, 21) are good JHE inhibitors. The *N*-ethyl carbamates of 1-naphthol and geranyl phenyl ether (Nos. 19, 21) provide the best inhibition ($I_{50} = 3 \times 10^{-7} M$), while the *N*-methyl carbamate of 1-naphthol, carbaryl (No. 18), is somewhat less active. Other *N*-alkyl carbamates have also proven to be effective inhibitors of *M. domestica* microsomal JHEs (28). The *M. domestica* microsomal JHEs are also distinct from those of the soluble fraction in that they are very unstable, requiring assay immediately after preparation.

Inhibition of T. ni JHE vs α-NAE

A selected group of inhibitors was also evaluated for its ability to inhibit the α-NAEs of *T. ni* relative to the JHEs (Table

TABLE 2

In Vitro Inhibition of α-Naphthyl Acetate and Juvenile Hormone Esterases from Trichoplusia ni

No.	Compound	I_{50} ($\times 10^{-4}$ *M*)[a] JH III	α-NA	Ratio of inhibition (I_{50}) JH III/α-NA
1	Methamidophos	0.07	0.1	0.7
6	EPPAT	0.01	0.001	10
—	BEPAT[b,c]	0.06	0.003	20
7	Fonofos-oxon	0.1	0.0005	200
9	Paraoxon	0.02	0.001	20
11	Dichlorvos	0.2	0.02	10
13	DFP	>10	0.0004	>25,000
14	DEF	>1	~1	~1
15	TOCP	>1	1	~1
18	Carbaryl	>1	0.07	>14
22	Eserine sulfate	>10	>10	~1
26	CMPS	>1	>1	~1
—	TFT[c,d]	0.001	~1	~0.001
—	α-NA	>1	—	—
—	JH III	—	~1	—

[a] Substrate concentration: α-NA, 2.5×10^{-4} *M*; JH III, 5×10^{-6} *M*.

[b] *S*-Benzyl-*O*-ethyl phosphoramidothiolate.

[c] α-NA, JH III were from Sigma; TFT was synthesized by reaction of the corresponding bromide with sodium tetracarbonyl ferrate followed by the addition of trifluoracetic anhydride (51); Lovell and Hammock, unpublished); BEPAT was a gift from P. S. Magee (Chevron Chemical Co.).

[d] 1,1,1-Trifluorotetradecan-2-one.

2). DEF (*S,S,S*-tributyl phosphorotrithiolate), TOCP (tri-*o*-tolyl phosphate), eserine sulfate, and CMPS are equally poor inhibitors of both enzymes. Among the three phosphoramidothiolates, methamidophos is slightly less effective as an inhibitor of α-NAEs than the JHEs while both EPPAT and BEPAT (*S*-benzyl-*O*-ethyl phosphoramidothiolate) provide better inhibition of the α-NAEs than the JHEs. For *B. giganteus* the phosphoramidothiolates were found to be superior as inhibitors of JHEs relative to α-NAEs (8). Most of the compounds examined are much more effective in inhibiting the α-NAEs than the JHEs, and of these compounds fonofos-oxon and DFP (diisopropyl phosphorofluoridate) proved to be extremely potent α-NAE inhibitors. Unlike the other compounds examined the trifluoromethyl ketone, TFT (1,1,1-trifluorotetradecan-2-one), is inactive against the α-NAEs and yet is an extremely potent JHE inhibitor ($I_{50} = 1 \times 10^{-7}$ *M*). Thus, either enzyme, JHE or α-NAE, can be selectively inhibited by using the appropriate inhibitor. Both α-NA and JH III proved to be poor inhibitors for JHE and α-NAE, respectively.

Inhibition of T. ni JHE by Selected Esters

A selected group of juvenoids and methyl esters were also assayed for their ability to act as inhibitors of *T. ni* JHE. Relative to JH III all of the compounds examined, except for ZR-520, are poor inhibitors of the JHE even when present in a 20-fold excess (Table 3). Although hydroprene (ZR-512) is a poor inhibitor of the JHE, its 11-methoxy analog, ZR-520, appears to be a much better inhibitor.

In Vivo JHE Inhibition and Juvenoid Effects

EPPAT and paraoxon were found to be the best inhibitors of *T. ni* JHE *in vitro* (Table 1). Thus, it was hoped that one of these inhibitors could be used to block JHE activity *in vivo* and thereby help elucidate

TABLE 3
Inhibition of Trichoplusia ni Juvenile Hormone Esterases by Selected Esters[a]

Compound[b]	Ester	Percentage inhibition at 10^{-4} *M*	I_{50} ($\times 10^{-4}$ *M*)
JH III	Methyl	95	0.06
Hydroprene[c]	Ethyl	29	>1
ZR-520[d]	Ethyl	57	0.8
Methoprene[e]	Isopropyl	25	>1
Kinoprene[f]	Propynyl	32	>1
ETB[g]	Ethyl	29	>1
Methyl benzoate	Methyl	12	>1
Methyl cinnamate	Methyl	18	>1
Methyl crotonate	Methyl	13	>1
Methyl sorbate	Methyl	10	>1
Methyl hexanoate	Methyl	20	>1
Methyl decanoate	Methyl	21	>1

[a] Incubated 15 min with inhibitor and [^{3}H]JH III (5×10^{-6} *M*).
[b] Sources of the compounds were: hydroprene, ZR-520, methroprene, kinoprene, and ETB, D. A. Schooley and G. B. Staal (Zoecon Corp.); methyl sorbate, Frinton Laboratories; methyl benzoate, Mallincrodt; methyl hexanoate and decanoate, JH III, Sigma; methyl cinnamate and crotonate were synthesized by published procedures.
[c] Ethyl (*E,E*)-3,7,11-trimethyl-2,4-dodecadienotae.
[d] Ethyl (*E,E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate.
[e] Isopropyl (*E,E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate.
[f] 2-Propynyl (*E,E*)-3,7,11-trimethyl-2,4-dodecadienoate.
[g] Ethyl-4-(2-(*t*-butylcarbonyloxy)butoxy) benzoate.

the role of the JHE *in vivo*. When Day 1 last-instar *T. ni* larvae were treated topically with selected doses of either paraoxon or EPPAT, the 48-hr LD_{50}'s were 3 μg/larva and >200 μg/larvae, respectively. Thus, because it is much less toxic to *T. ni* larvae, EPPAT was used in an attempt to block JHE activity *in vivo*.

Application of a single dose of EPPAT (22 μg) during the time of the first JHE peak (early Day 1, last-instar larvae; (14)) quickly reduces the *in vivo* hemolymph JHE activity to about 10% of control (normal) and maintains this low level of JHE activity for several hours (Fig. 1). These results suggested that treatment of *T. ni* two or three times per day should maintain the JHE activity at a very low level.

Treatment of *T. ni* larvae twice a day for the first 2 days of the last instar (i.e., first JHE peak) with 22 μg of EPPAT reduces the hemolymph JHE to an average of 20% of control (normal) for the entire 2 days (Fig. 2). This level of JHE inhibition is without effect on larval behavior, or development. However, treatment with EPPAT three times per day during the first 2 days of the last instar, reduces the *in vivo* hemolymph JHE activity to an average of 15% of control (Fig. 2). Each data point in Fig. 2 for the EPPAT-treated larvae during the first JHE peak was determined just prior to the next EPPAT application and therefore represents maximal levels of JHE

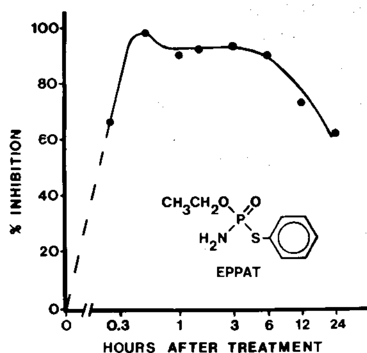

FIG. 1. *In vivo inhibition of JHE activity in the hemolymph of Day 1 larvae treated with a single dose of EPPAT (22 μg) relative to control larvae.*

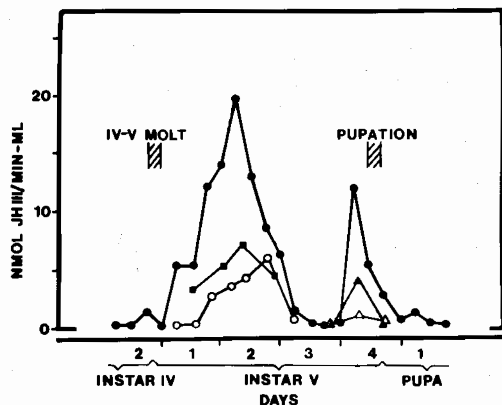

FIG. 2. *In vivo inhibition of the JHE activity peaks by EPPAT during the last larval instar of T. ni. Untreated JHE activity (●) data is adapted from Sparks et al. (14). For the first JHE peak, JHE activity resulting from EPPAT (22 µg) treatment for 2 (■) or 3 (○) times per day, and the JHE activity resulting from EPPAT treatment, 11 (▲) or 22 µg (△), prior to the second JHE peak. Each data point for the first JHE peak is the JHE activity measured just prior to the next EPPAT application and therefore represents maximum JHE activity.*

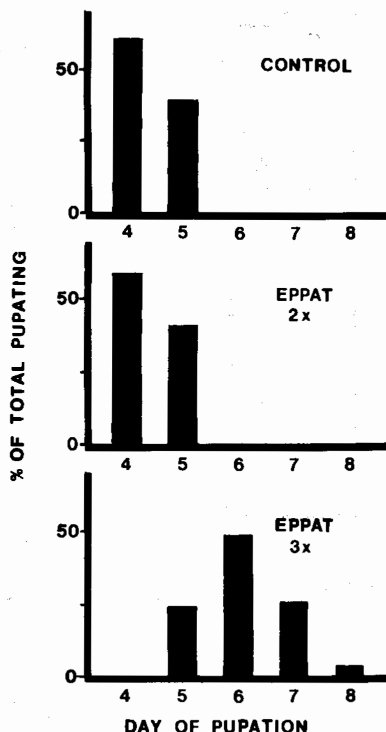

FIG. 3. *Effect of in vivo inhibition of the first JHE peak of T. ni by EPPAT on the length of the last instar (see text for details).*

activity. Furthermore, as demonstrated in Fig. 1, the JHE activity is rapidly inhibited following EPPAT application and then gradually increases so that the total amount of inhibition is much greater than is indicated in Fig. 2. At this level of inhibition the larvae remain in the prewandering phase longer than normal and pupation is delayed 1 to 3 days (Fig. 3). A similar delay in pupation also results when Day 1 last-instar *T. ni* larvae are treated with a single dose of epofenonane (Fig. 4).

The topical treatment of late last-instar larvae with a single 11-µg dose of EPPAT prior to the second JHE peak lowers the JHE activity at the time of the peak to about 40% of control (normal). This level of inhibition has no effect on behavior or development. Treatment of late last-instar larvae with 22 µg of EPPAT reduces the JHE activity to 10% of control (Fig. 2). Of the larvae so treated, 50% became malformed, resembling larval–pupal intermediates, after pupation.

Topical application of radiolabeled JH I to Day 2 *T. ni* larvae (high JHE; Fig. 2) results in about 0.1–0.2% (ca 1000 cpm) of the applied radioactivity, appearing in the hemolymph 6 hr post-treatment. The radioactivity present in ethanol-treated larvae is primarily JH-acid (59.9%) and lesser amounts of JH I (24.4%). However, larvae treated with EPPAT contain about 2.5-fold more JH I (61.8%) and much less JH acid (22.8%). Thus, EPPAT does slow the *in vivo* hydrolysis of JH by JHEs. Although the *in vivo* JHE inhibition by TFT is transitory relative to EPPAT, preliminary experiments with TFT also show a similar but less dramatic stabilization of JH I *in vivo* (Sparks, unpublished).

DISCUSSION

Compared to the JHEs of *M. domestica* (Table 1), *B. giganteus* (8), and *Schistocerca gregaria* (29), those of *T. ni* and especially *T. molitor* are relatively difficult to inhibit. Although the pupal JHEs of

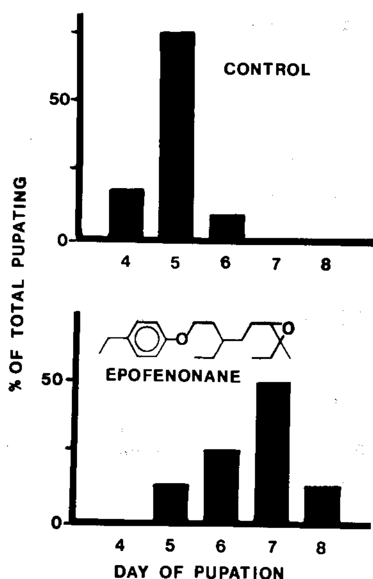

FIG. 4. *Effect of epofenonane treatment during the last instar of T. ni on the length of the last instar. Larvae were treated with epofenonane (3 μg) on Day 1 of the last instar.*

Manduca sexta were reported to be best inhibited by a group of carbamates and parathion (30), in general the JHEs of most insects so far examined, including *B. giganteus, S. gregaria, T. ni, T. molitor,* and *Galleria mellonella* (8, 27, 29, Table 1), as well as those of the salt marsh caterpillar (*Estigmene acrea*) and the Pacific spider mite (*Tetranychus pacificus*) (Sparks and Hammock, unpublished), are best inhibited by the oxon analogs of organophosphates, especially the phosphoramidothiolates. Furthermore, these JHEs are only poorly inhibited by the carbamates tested (8, 27, 28; Table 1; Sparks and Hammock, unpublished). The notable exceptions to the above observation are the JHEs of the Diptera. The JHEs of *Culex pipiens pipiens* were only poorly inhibited by any of the compounds (CMPS, eserine sulphate, juvenoids, organophosphates) assayed by Hooper (31) while those of *M. domestica* were best inhibited by the *N*-alkyl carbamates of geranyl phenyl ether or 1-naphthol (Table 1, 28). Thus, relative to other insects so far examined, the JHEs of the Diptera appear to be rather distinct. This observation is in keeping with other information available on dipteran endocrinology suggesting that the JHEs may not be as important in dipteran JH metabolism/regulation as they are in other insects (4, 5, 32). Inhibitors of JH metabolism, such as the ones reported here, may be useful tools for investigating insect endocrinology. For instance, the *N*-ethyl carbamate of 1-naphthol has proven to be useful as a means of stabilizing JH in studies on JH action in *Drosophila* cell lines (Chan, personal communication).

The JHE of *T. ni* is more sensitive to inhibition by the phosphoramidothiolates examined and less sensitive to the classical esterase inhibitors than are the α-NAEs. Similar results were also found for the JHEs and α-NAEs of *B. giganteus* (8). These different inhibition patterns suggest that the α-NAEs are not responsible to any large degree for the JHE activity observed in *T. ni*. Furthermore, α-NAE is a poor JHE inhibitor which suggests that the enzymes responsible for their hydrolysis are different. Thus, these results lend further support to earlier conclusions regarding α-NAE activity as being a poor indicator of the JHE activity in *T. ni* (14, 33) and other insects (8, 9, 34).

The trifluoromethyl ketone, TFT, appears to be a very potent and selective inhibitor of *T. ni* JHE (Table 2). TFT was specifically designed to act as a so-called transition-state analog for the JHE. Such analogs can be considered to mimic the transition state of the substrate and may, therefore, be more tightly bound than the substrate, making them very effective enzyme inhibitors (35). Besides TFT, other trifluoromethyl ketone juvenoids have also been found to be effective JHE inhibitors (Hammock *et al.*, in preparation). They are also known to inhibit acetylcholinesterases (36).

Except for JH III and ZR-520, none of the other ester compounds examined as potential competitive inhibitors were very effective in inhibiting the JHE of *T. ni*, even when present in a 20-fold excess. Studies of

JH I, JH III, hydroprene, and methoprene metabolism by crude hemolymph and partially purified JHE devoid of α-NAE activity, indicate that hydrolysis by α-NAE contributes very little to the metabolism of these compounds (Sparks and Shour, unpublished). These results suggest that when compounds of the juvenoid type do cause inhibition of JHE activity it is most likely by acting as alternate substrates. Thus, the ability of any of these compounds to inhibit the JHE can potentially be assumed to be a measure of their suitability as JHE substrates. On the basis of these assumptions the JHEs of *T. ni* appear to be specific for methyl, and to a lesser extent, ethyl esters of JH-like molecules. This apparent specificity may help explain the poor inhibition observed for organophosphates that have alkyl groups larger than ethyl anywhere other than the leaving group. Methyl esters of non-JH compounds and propyl esters are seemingly poor competitors with JH III for the JHE active site. Similar results have also been reported for the JHEs of *M. sexta* (6) and other insects (7, 8). It is of interest that even though the anti-JH ETB (ZR-2646) (37) seems to be a poor substrate for the JHE, it is able to function as a JH agonist/antagonist for what appear to be JH receptors in the fat body (38). Similarly, the juvenoid epofenonane readily induces JHE activity by interacting with these apparent fat body JH receptors, and yet epofenonane is a very poor JHE inhibitor (20, 38). These JH receptors seem to be responsible for the induction of JHE in last-instar *T. ni* larvae (20). Thus, it appears that the JHE and the supposed JH receptor(s) involved in JHE induction are rather different in their active sites. For the JHE of *T. ni,* not only does the size of the ester appear to be involved, but as implicated by the increased inhibition of the JHE by ZR-520 verses hydroprene, the overall fit or possibly a terminal electronegative center may also be involved.

The JHEs of *T. ni* are not affected by compounds which specifically inhibit metallo-(EDTA)- or sulfhydryl-(CMPS)-containing hydrolases. Similarly the carbamates are also poor *T. ni* JHE inhibitors. Although classical esterase inhibitors such as dichlorovos, TEPP, DFP, DEF, and TOCP (Nos. 11–15) are relatively poor JHE inhibitors, some of the phosphoramidothiolates, especially EPPAT (No. 6), are very potent. Based on an artificial classification system (39), the JH-hydrolyzing esterases of *Hyalophora gloveri* were classified as carboxylesterases (E.C. 3.1.1.1.) (40). Both systematic (41) and trivial (39) nomenclature are used to classify enzymes, and on the basis of systematic nomenclature it is clear that the JHE of *T. ni* is a carboxylic ester hydrolyase (E.C. 3.1.1.) (41). Although the *T. ni* JHE is not inhibited by the organophosphate inhibitors commonly used in enzymology, it is nevertheless inhibited by some organophosphates and therefore should probably be referred to as a carboxylesterase (E.C. 3.1.1.1.). Unfortunately the meaning of the term may be overinterpreted in the trivial sense. Clearly, α-NA is not an acceptable model substrate for JHE in the insects so far examined (9, 14, 33; Table 2). Thus, even based on our limited knowledge of *T. ni* JHE substrate and inhibitor specificity, it is apparent that these enzymes which can hydrolyze a very stable conjugated ester are unique.

On the basis of their apparent specificity for JH and their correlation with declines in the JH titer, the JHEs are thought to play a role in regulation of the JH titer (1, 3, 11); however, the *in vivo* function has not been proven. For *M. sexta* the JH titer may have already fallen to low levels before the JHEs appear in the hemolymph (42), which leads one to question the function of the JHEs.

The treatment of last-instar larvae of *T. ni* with JH or juvenoids results in delayed pupation and/or apparent larval–pupal intermediates (43, Fig. 4). Similar effects have also been observed for other lepidopterans (44, 45). In last-instar *M. sexta* larvae exogenous JH maintains higher than normal levels of JH in the hemolymph which causes a delay in the first burst of

prothoracicotropic hormone (PTTH) thereby delaying the initiation of pupation (45). The ability to EPPAT to cause a similar delay in pupation and the formation of malformed larvae that resemble larval–pupal intermediates, suggests that inhibition of JHE *in vivo* is maintaining a higher than normal hemolymph JH titer. The reduction in the *in vivo* ester hydrolysis of radiolabeled JH I by EPPAT lends further support to this hypothesis. Although EPPAT can also inhibit the esterases responsible for the hydrolysis of α-naphthyl acetate, this study and others (14, 33) have aptly demonstrated for *T. ni* that these other esterases contribute little if any to the ester hydrolysis of JH. However, while it is obvious that EPPAT does inhibit the *in vivo* JHE activity in late last-instar larvae, it is also possible that inhibition of other esterases such as acetylcholine esterase (ACHE) could in someway also contribute to the formation of the apparent larval–pupal intermediates. Such larvae are occasionally observed when late last-instar *T. ni* are treated with DFP or carbaryl, both relatively poor JHE inhibitors. Likewise EPPAT not only inhibits JHE *in vivo* (90% on early Day 4), but also α-NAE (85%) and to a lesser extent *T. ni* head ACHE (44%) (Sparks, unpublished results). However, EPPAT appears to be virtually nontoxic to *T. ni* and treatment of the late last-instar larvae with JH I or piporonyl butoxide also results in these apparent larval–pupal intermediates. Thus, the available evidence appears to favor endocrine, rather than nervous, disruption as the mechanism by which EPPAT acts to form the apparent larval–pupal intermediates. Therefore, for *T. ni,* it appears that the JHEs are indeed an important mechanism in the regulation of the hemolymph JH titer.

A sequence of events can now be envisioned for the action of EPPAT during the early last-instar of *T. ni*. Multiple treatments of EPPAT inhibit JHE activity resulting in a higher than normal hemolymph JH titer, thereby preventing the release of PTTH. Since PTTH is thought to be necessary for initiation of ecdysone production (45), the commencement of metamorphosis and, hence, pupation is also delayed. Presumably the termination of JH biosynthesis and release by the corpra attata as well as the continued production of JHE by the fat body eventually reduces the JH titer to levels sufficient to allow the release of PTTH and the consequent initiation of metamorphosis. The large amount of *in vivo* JHE inhibition required before any developmental effects are observed suggests that the JHE is being produced in excess to ensure complete removal of JH prior to the cellular reprogramming by ecdysone.

The results presented in our study also demonstrate that JH-like effects can be obtained by nonhormone compounds, such as insecticides, which act to stabilize endogenous JH by disrupting JH metabolism. This stabilization of JH has been suggested as one mechanism of action for some juvenoids (46). Thus, our results provide one possible explanation for the JH-like activity noted for some insecticide synergists (46) and for certain insecticides at sublethal doses (47–50).

It is obvious that JHE is but one of several interacting factors potentially responsible for regulating the JH titer. However, based on the results of this study, the hemolymph JHE of *T. ni* appears to be a unique enzyme that does play a physiological role in JH regulation and the larval–pupal transformation. The differences observed in the JHE inhibition patterns in this study and others (4, 5, 32) also suggest that there may be some fundamental differences in the endocrinology of the Diptera relative to the other insect orders. Thus, these findings point to the insect endocrine system as an excellent target site for the development of new and selective insect control agents.

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