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Fenoxycarb levels and their effects on general and juvenile hormone esterase activity in the hemolymph of the silkworm, *Bombyx mori*

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Abstract

We studied the relationship between fenoxycarb levels and general esterase (GE) activity, juvenile hormone esterase (JHE) activity, and induction of permanent fifth instar (dauer) larvae in *Bombyx mori*. Fenoxycarb (1 μg/animal) was topically applied to *B. mori* at 0 h of the fifth instar. Hemolymph fenoxycarb levels, as determined by an ELISA, reached a peak within 1 h, and then decreased to undetectably low at 42 h. After 48 h, the feces of these larvae contained 99% of the applied fenoxycarb dose. The above fenoxycarb treatment induces dauer larvae with persistently increased hemolymph GE and JHE activities for the rest of the fifth instar. Application of fenoxycarb later in the fifth instar (48 h), when it still could induce dauer larvae, resulted in a similar pattern of elimination from the hemolymph and produced steadily elevated hemolymph GE and JHE activities for the rest of the fifth instar. Application of fenoxycarb late in the fifth instar (132 h) did not prevent pupal ecdysis; it increased hemolymph JHE activity, but not GE activity. The latter treatment prevented the JHE activity decrease observed in control larvae before pupal ecdysis. In vivo, in control animals, the highest hemolymph JHE activity was observed 12 h after pupal ecdysis. At that developmental time, fenoxycarb inhibited this enzyme in vitro ($IC_{50} \approx 2\,\mu M$). The com-

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bined results suggest: (1) fenoxycarb is rapidly excreted from the larval body; (2) induction of dauer larvae and increased hemolymph GE and JHE activities are not directly associated with its presence in the larval body of *B. mori*.
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Keywords: Fenoxycarb; *Bombyx mori*; General esterase; Juvenile hormone esterase; Dauer larvae

1. Introduction

Sesquiterpenoid juvenile hormones (JHs) play a crucial role in the development, metamorphosis, and reproduction of insects [1]. Understanding of the importance of JHs as regulators of insect development led to the developmevnt of JH analogues (JHAs), a class of synthetic insect growth regulators (IGRs) with JH activity. JHAs can induce developmental and functional disturbances in insects [2]. Another approach to the disruption of regulatory development of insects is interference with JH esterase (JHE), one of the JH degrading enzymes. The resulting anti-JH effect causes reduction of feeding [3]. Juvenile hormone esterase appears to play an essential role in moulting in early instars [4] and in the actual ecdysis to the pupal stage of at least noctuid larvae [5,6].

The application of potent JHAs such as fenoxycarb [7,8] in insect control has led to the need of monitoring their levels in environmental samples as well as in target and non-target organisms [9,10]. Such monitoring efforts and also studies on details of the JHAs' mode of action require simple, inexpensive, and high sample-throughput analytical methods for these IGRs. To this end, an enzyme-linked immunosorbent assay (ELISA) was developed for fenoxycarb ([9,10]; Székács, unpublished data). We previously reported on the fenoxycarb-induced morphological alterations in the development of the fifth instar larvae of *Bombyx mori* [11]. In the present study, we have used the above ELISA to determine levels of fenoxycarb after its application on to *B. mori* larvae. Our aim is to determine the relationships between fenoxycarb levels and the induction of permanent fifth instar larvae (dauer larvae) in *B. mori* [11]. Furthermore, we have investigated whether fenoxycarb is perceived by the fifth instar larvae as a JH mimic, thereby leading to increases in JHE or general esterase (GE) activity in the larval hemolymph.

2. Materials and methods

2.1. Insects

All experiments used larvae from the hybrid J106xDAIZO. The larvae were reared on mulberry leaves under a 12:12–L:D photoperiod at 25 ± 1 °C and 60% relative humidity [11]. Larvae were staged after every larval ecdysis, and the day of each ecdysis was designated as day 0. Since the larvae mainly moult to the final (fifth) instar during the scotophase, all the larvae that ecdysed during the scotophase were segregated just after the photophase. This time was designated as 0 h of the fifth instar. The onset of larval-pupal commitment occurs at 60 h (day 2), and the onset of wandering behaviour occurs 144 h (day 6) after the final larval ecdysis. In this study, as 0 h of the pupal stage we defined the time of pupal ecdysis and animals that ecdysed to pupa at the 204 h of the fifth instar were selectively used. Female larvae were exclusively used in this study.

2.2. Reagents

Fenoxycarb (ethyl 2-(4-phenoxyphenoxy)ethylcarbamate, $C_{17}H_{19}O_4N$) of analytical grade (99% purity) was a gift from Novartis (Basel, Switzerland). In in vivo experiments, treatments were done by topical applications of 5 µl of 0.2 µg/µl fenoxycarb solution in acetone to each larva. Control larvae received 5 µl of acetone. For in vitro experiments, a fenoxycarb stock solution (1 mM) was prepared in ethanol. Biochemicals and immunochemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Horseradish peroxidase conjugated to goat anti-rabbit IgG was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). Methyl 1-heptylthioacetothioate (HEPTAT; [12]) was prepared as described [13].

2.3. ELISA in the immobilized antigen format for fenoxycarb

A competitive ELISA ([9,10]; Székács, unpublished data) was adapted to determine the concentration of fenoxycarb in the hemolymph of fenoxycarb-treated and control animals. The hapten-heterologous ELISA, based on a polyclonal anti-fenoxycarb antibody and a BSA-fenoxycarb conjugate as coating antigen, was similar to that described previously [10]. A solution of the coating antigen (2.5 μg/ml in 0.1 M carbonate buffer, pH 9.6, 100 μl/well) was dispensed into the wells of a 96-well microplate (Iwaki, Tokyo, Japan) then incubated at 4 °C overnight. After washing three times with 15 mM phosphate buffered saline (pH 7.4, PBS) plus 0.2% Tween 20 (PBST (0.2), 150 μl/well), a 3% solution of gelatin in PBS (150 μl/well) was added to the wells, and the plate was incubated at 37 °C for 90 min. After washing three times with 150 μl of PBST (0.2), 50 μl of anti-fenoxycarb antibody solution in PBS plus 0.05% Tween 20 (PBST (0.05), 1:2000 dilution) and 50 μl of hemolymph solution in PBST (0.05) (1:10 or 1:50 dilution) were added to each well. The plate was incubated at 37 °C for 2 h. After washing three times with PBST (0.2) (150 μl), 100 μl of goat anti-rabbit antibody-horseradish peroxidase conjugate solution in PBST (0.05) (1:2000 dilution) was added to each well. The plate was then incubated at 37 °C for 1 h. After washing four times with PBST (0.2) (150 μl well), 200 μl of a chromogenic substrate solution containing 0.025 mg/ml 3, 3′, 5, 5′-tetramethylbenzidine dihydrochloride (TMB, made from Sigma's TMB tablets) and 0.06 mg/ml hydrogen peroxide in substrate buffer (50 mM phosphate-citrate buffer, pH 5.0, prepared according to Sigma's TMB protocol) was added to each well. The plate was incubated at 37 °C for 30 min. The reaction was stopped by addition of 50 μl of 2 M H_2SO_4 to each well, optical density (OD) at 450 nm was then measured on a model 550 Bio-Rad microplate reader (Bio-Rad, Hercules, CA, USA). To construct standard curves, a stock solution of fenoxycarb in methanol was serially diluted with PBST (0.05). The lower limit of detection (LOD, IC_{80}) for this ELISA was found to be about 73 pg/well based on a standard curve generated using fenoxycarb solution in PBST (0.05) (Fig. 1). In control runs, the ELISA assay buffer (PBST (0.05)) was supplemented with the same amounts of hemolymph of control animals as used above in the experiments with treated animals, but the characteristics of the standard curve did not change significantly.

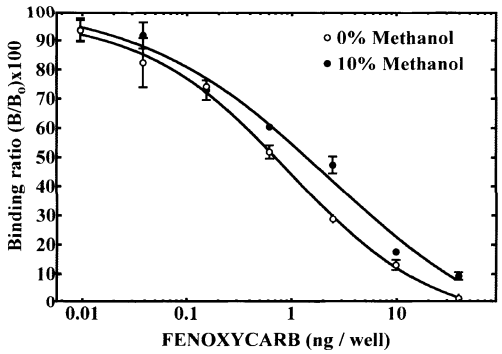

Fig. 1. Standard curves obtained by the competitive ELISA for fenoxycarb. Indicated amounts of fenoxycarb were incubated with polyclonal anti-fenoxycarb antibody in the wells covered with the coating antigen (see Section 2.3 for details). The ELISA assay buffer contained either 0% methanol (curve A) or 10% methanol (curve B). Each point represents the mean ± SEM of the OD values at 450 nm, which were from three replicate wells on the same microplate.

Feces samples from fenoxycarb-treated or control animals were collected in Eppendorf tubes, homogenized in methanol (1 g/2 ml) using a glass-glass homogenizer, then centrifuged at 20,000*g* at 4 °C for 10 min. The supernatants were removed and saved, then the pellets extracted twice with methanol (2 ml/1 g). The combined supernatants from each sample were evaporated in vacuo and re-dissolved to 200 μl with methanol. Each methanolic concentrate was diluted with nine times its volume of PBST (0.05) then assayed by ELISA. In these experiments, a fenoxycarb standard curve, determined in assay buffer containing 10% methanol (Fig. 1), was used to measure the fenoxycarb levels in the samples. The final pellets were further homogenized in PBST (0.05) (1 g/ml) and centrifuged at 20,000*g* at 4 °C for 10 min. The supernatants were removed and assayed by ELISA.

2.4. Determination of juvenile hormone esterase and general esterase activity in the hemolymph of *B. mori*

Juvenile hormone esterase (JHE) activity in the hemolymph of *B. mori* was quantified by a colorimetric assay based on HEPTAT [12,14], a surrogate substrate, as described earlier [13]. Hemolymph from control and fenoxycarb-treated

animals was collected in chilled Eppendorf tubes. An aliquot was withdrawn and immediately diluted with an equal volume of phenylthiourea (PTU) solution in 0.1 M phosphate buffered saline plus 10% sucrose (pH: 7.4) to give a final concentration of 0.05% PTU. Then each sample was centrifuged at 1000*g* at 4 °C for 5 min. The supernatant was removed and stored at −80 °C until assayed. To measure hemolymph JHE activity, each hemolymph sample (2 μl) was added to 296 μl of PBS containing 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB; 0.015% final concentration) in an individual microplate well, 2 μl well of HEPTAT solution in ethanol (0.2 mM final concentration in the wells) was then immediately added. Hydrolysis was monitored at 405 nm at 20 s intervals for a total of 2 min. The conditions for initial hydrolytic reactions were determined to be linear with time and hemolymph volume under these conditions. The resulting hydrolysis reactions were carried out at 25 °C and measured in a continuous assay using 96-well microtiter plates (Iwaki, Tokyo, Japan) by an SME 3400 microplate reader (Iwaki, Tokyo, Japan) at 405 nm. Using the integrated software, the results were expressed in mOD/min units, then converted into mmol/min/ml units. To determine the in vitro effects of fenoxycarb on the hydrolysis of HEPTAT by hemolymph JHE, the above colorimetric assay was only slightly changed as follows. Each hemolymph sample (2 μl) was added to 294 μl of PBS containing DTNB (0.015% final concentration) in an individual microplate well. Fenoxycarb solution (2 μl well, various concentrations) in ethanol was then added, and the microplate was incubated for 10 min. HEPTAT solution (2 μl well, 0.2 mM final concentration) was then added, and the hydrolysis product was monitored at 405 nm at 20 s intervals for a total of 2 min.

General esterase (GE) activity in the hemolymph of *B. mori* was measured with 1-naphthyl acetate (also known as α-naphthyl acetate, NapAc) and 4-nitrophenyl acetate (also known as *p*-nitrophenyl acetate, pNPAc) as substrates following literature methods [14–16]. The hydrolysis of either NapAc or pNPAc was measured by the procedure as follows. Each hemolymph sample (5 μl was mixed with 293 μl of PBS (containing 2.5 mg/ml of Fast Blue RR in the case of NapAc) in an individual microplate well. Two μl of substrate (either NapAc or pNPAc) solution in ethanol (0.2 mM final concentration) was immediately added. Using the microtiter plates and microplate reader described above, hydrolysis at 25 °C was monitored at 450 nm for NapAc and at 405 nm for pNPAc at 20 s intervals for a total of 2 min. Using the integrated software, the results were expressed in mOD/min units, then converted into mmol/min/ml units. The conditions for initial hydrolytic reactions were determined to be linear with time and hemolymph volume under these conditions.

2.5. Statistical analyses

Statistical significance of the results was determined by analysis of variance (ANOVA) or Student's *t* test. For some experiments, ANOVA was followed by Tukey multiple comparisons test. Test results (significance) are shown in the figure legends. The statistical analyses were done with the computer software GraphPad Prism 2.0.

3. Results

3.1. Development of an indirect ELISA for fenoxycarb

A standard curve (Fig. 1) was generated using the immunoassay in the coating antigen ELISA format similar to that described previously [10]. The assay displayed high sensitivity for fenoxycarb with an LOD value (analyte concentration at 80% assay signal, B/B_{o80}) of 73 pg/well and an IC_{50} value (analyte concentration at 50% assay signal, B/B_{o50}) of 683 pg/well of fenoxycarb (Fig. 1, curve A). The antibody showed a good tolerance to the presence of methanol. The standard curve was only slightly shifted towards higher analyte when the assay buffer contained 10% of methanol. The LOD was not dramatically altered by addition of up to 10% methanol to the assay buffer (LOD: 110 pg/well with 10% methanol; Fig. 1, curve B).

This ELISA is highly selective for fenoxycarb, but displays some cross-reactivities for compounds structurally similar to the target compound (10; Dedos and Fugo, unpublished data; Székács, unpublished data). In the methanolic feces extracts (see Fig. 3), there might be some fenoxycarb-metabolites. However, only early metabolites with structures close to the parent compound are expected to have high cross-reactivity ($CR[\%] = 100 \times IC_{50}$ fenoxycarb/IC_{50}; [10]), while terminal metabolites, including only structural fragments of the entire fenoxycarb molecule, do not cross-react significantly. For instance, 4-phenoxyphenol, a metabolite in the feces of *He-*

liothis virescens [17], a likely terminal metabolite in the feces of *B. mori*, exhibits only 0.2% CR in this ELISA (Dedos and Fugo, unpublished data). Thus, Fig. 3 (see below) appears to represent mainly the amounts of fenoxycarb and possibly some early (highly cross-reacting) metabolite(s) eliminated in feces. Terminal metabolites may have also added to the calculated amounts, but their contribution is expected to be only marginal due to their low cross-reactivities (CR 0.2% or less) (Székács, unpublished data).

3.2. Hemolymph content of fenoxycarb after application at 0 h of the fifth instar

Fenoxycarb (1 μg/animal) was topically applied at the onset of photophase after the last larval ecdysis (0 h of the fifth instar). Then hemolymph was collected from each animal, and its fenoxycarb content was determined by the ELISA. Two approaches were adopted. In the first approach (long term; Fig. 2), samples were collected until late in the fifth instar. In the second approach (short term; inset of Fig. 2) samples were collected until 8 h of the fifth instar. Using the long-term approach, the highest levels of fenoxycarb were observed at the first collected samples, 6 h after its application (Fig. 2). The fenoxycarb titer significantly dropped thereafter, and it could not be detected in the hemolymph 42 h after its application (Fig. 2). Using the short-term approach, it was found that fenoxycarb was very quickly detectable in the hemolymph of the animals. Within 5 min of its application, about 17 ng fenoxycarb/100 μl hemolymph was detected (inset of Fig. 2). Its titer peaked within 90 min and stayed at high levels until 2 h after the application, then decreased considerably (inset of Fig. 2). Assuming that the hemolymph volume (134 μl represents 28% of the body weight [18], a calculation, based on body weight of 0.48 g, showed that only 39% of the applied dose was present in the hemolymph at 1.5 h after application and this amount dropped to 19% at 6 h after application.

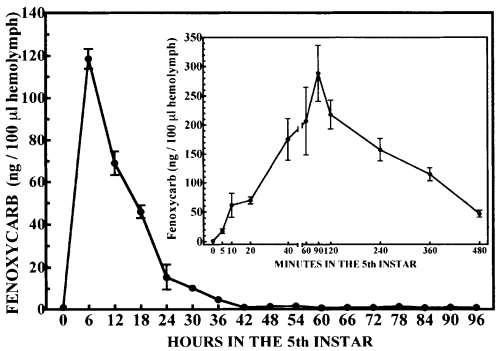

Fig. 2. Fenoxycarb content in the hemolymph after topical application of 1 μg of fenoxycarb at 0 h of the fifth instar. Each value represents fenoxycarb equivalents in the hemolymph and it is the mean ± SEM from three animals. Results of Tukey multiple comparisons tests (pairs of means enclosed by the range of a bracket are not significantly different, $P > 0.05$): [0.70–4.70], [4.70–15.40]. *Inset*. Fenoxycarb content in the hemolymph as a function of minutes after its topical application. The peak hemolymph level corresponds to about 7.2 μM. Each value represents fenoxycarb equivalents in the hemolymph and it is the mean ± SEM from three animals. Results of Tukey multiple comparisons tests (pairs of means enclosed by the range of a bracket are not significantly different, $P > 0.05$): [17.5–69.4], [46.5–177.3], [114.6–206.7].

3.3. Fenoxycarb content in feces after application at 0 h of the fifth instar

The rapid elimination of fenoxycarb from the hemolymph made necessary the determination of the amounts of fenoxycarb in the feces of the animals. Thus, the same application as described above was done and feces were collected from individually reared animals at 12 h intervals. Extraction of feces with methanol and analysis of fenoxycarb in the extract by ELISA revealed that about 58% of the applied dose (1 μg/animal, 0 h of the fifth instar) was in the feces within 12 h of the application of fenoxycarb (Fig. 3). The amounts of fenoxycarb in the feces sharply decreased thereafter, but the cumulative amount of excreted immunoreactive fenoxycarb equivalents from each animal reached 97.8% 48 h after its application (Fig. 3). After removal of methanol, a final homogenization of the collected feces samples with ELISA assay buffer revealed that highly polar metabolites of fenoxycarb can be traced by ELISA in the feces only at 12 and 24 h after its application (inset of Fig. 3). These metabolites represented only a very small fraction of the applied fenoxycarb (inset of Fig. 3). Feces from control animals were processed in the same way as in the case of the treated animals. Control methanolic extracts did not considerably affect the ELISA standard curve for fenoxycarb, when were mixed with the assay buffer up to 10% v/v concentration. Our results with the feces extracts are in good agreement with the undetectable levels of

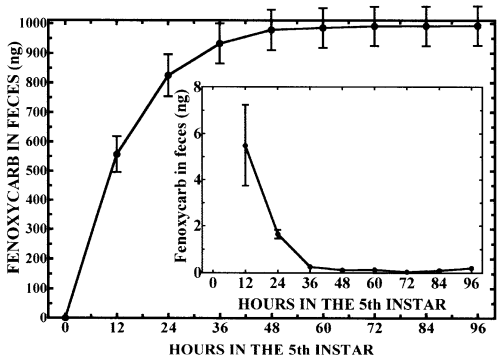

Fig. 3. Excretion of fenoxycarb in feces after topical application of 1 μg at 0 h of the fifth instar. The cumulative amount of fenoxycarb in the feces is shown. Each value represents fenoxycarb equivalents in the feces and it is the mean ± SEM from four animals. Methanolic extracts (up to 10% v/v) of feces did not significantly alter the standard curve. *Inset*. The amounts of highly polar metabolites of fenoxycarb in feces are expressed in fenoxycarb equivalents. Each value represents fenoxycarb equivalents in the feces and it is the mean ± SEM from four animals. Results of Tukey multiple comparisons tests (pairs of means enclosed by the range of a bracket are not significantly different, $P > 0.05$): [0.4–1.64].

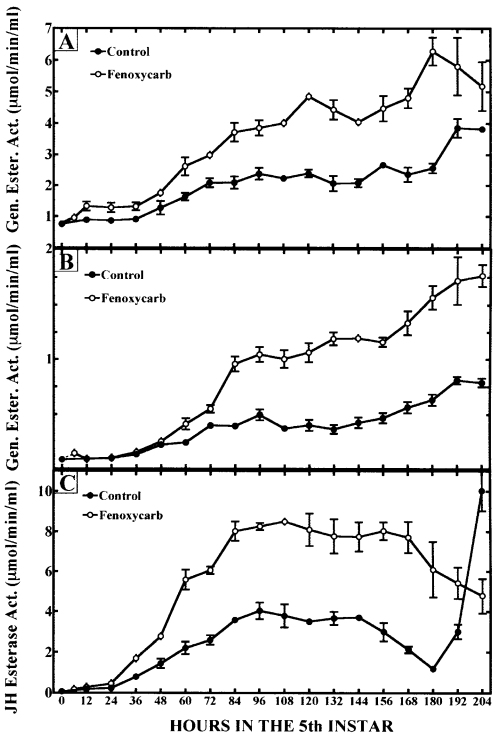

Fig. 4. Effect of fenoxycarb on general and juvenile hormone esterase activity after its topical application (1 μg/animal) at 0 h of the fifth instar. GE activity (Gen. Ester. Act.) was measured in control and fenoxycarb-treated animals using 1-naphthyl acetate (A) or 4-nitrophenyl acetate (B) as substrates. JHE activity (JHE Act.; C) was measured using HEPTAT, a surrogate substrate, as described in Section 2. Each value is the mean ± SEM of three animals. Tests of the difference between control and experimental values (unpaired *t* tests) revealed the means were not significantly different ($P > 0.05$): (1) 1-naphthyl acetate: 24, 48, 192, and 204 h of the fifth instar; (2) 4-nitrophenyl acetate: 12, 24, 36, and 48 h of the fifth instar; (3) HEPTAT: 12 h of the fifth instar.

fenoxycarb in the hemolymph 42 h after its application.

3.4. General esterase and JH esterase-like activity in the hemolymph after application of fenoxycarb at 0 h of the fifth instar

Application of fenoxycarb (1 μg/animal, 0 h of the fifth instar) produces dauer larvae that do not initiate the larval–pupal differentiation and remain in the fifth instar for up to 20 days in some cases. Since fenoxycarb behaves as a JH mimic, we examined whether its presence in the hemolymph is perceived by the animal as JH thereby leading to induction of increased hemolymph JHE activity. This investigation was coupled with determination of hemolymph GE activity of control and dauer larvae to differentiate between the probable effects on JHE activity and those on GE activity. In control animals, JHE-like activity, measured with a colorimetric assay, gradually increased in the fifth instar and reached a prolonged plateau from 84 h (day 4) until 156 h (day 6) of this instar. It gradually decreased to a low level by 180 h of the fifth instar, then rose sharply at the time of pupal ecdysis (Fig. 4C). In the fenoxycarb-treated dauer larvae, the pattern of JHE-like activity was similar to the control only until the 12 h of the fifth instar. Then it rose sharply and reached a prolonged plateau, decreasing only at the time when the last samples were collected.

Similar fluctuation patterns were recorded for hemolymph GE activity. Using either substrate (NapAc; Fig. 4A or pNPAc; Fig. 4B), it was found that GE activity gradually increased in the fifth instar and reached peak levels shortly before the pupal ecdysis in control animals. GE activity in fenoxycarb-treated dauer larvae was considerably different from control. Using NapAc as

substrate, GE activity was significantly higher than control almost throughout the investigated period (Fig. 4A). However, with pNPAc as substrate, the GE activity was found to be significantly higher than control only after the 60 h of the fifth instar and remained steadily higher than control throughout the rest of the investigated period (Fig. 4B).

3.5. Hemolymph content of fenoxycarb after application at 48 h of the fifth instar

In a previous study [19], we also showed that application of fenoxycarb (1 μg/animal) at 48 h of the fifth instar induces dauer larvae that do not undergo larval–pupal differentiation. The same treatment was done in this study to determine if the previously observed rate of elimination of fenoxycarb from the hemolymph (see Fig. 2) was similar to that when fenoxycarb was applied at 48 h of the fifth instar. The results revealed that the rate of incorporation and elimination were slower at this treatment (Fig. 5). The amount of fenoxycarb in the hemolymph gradually increased and reached a peak 18 h after its application (Fig. 5). Using the method above [18], it was assessed (body weight: 1.58 g) that only 4.4% of the applied dose was present in the hemolymph at 6 h and this amount increased to 8.8% at 18 h (body weight: 1.85 g) after application. No fenoxycarb could be detected in the hemolymph at 66 h after treatment (114 h of the fifth instar; Fig. 5), which period was almost twice the time required for elimination of fenoxycarb after application at 0 h of the fifth instar.

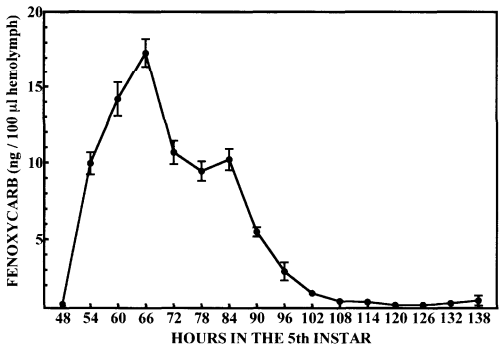

Fig. 5. Fenoxycarb content in the hemolymph after topical application of 1 μg of fenoxycarb at 48 h of the fifth instar. Each value represents fenoxycarb equivalents in the hemolymph and it is the mean ± SEM from three animals. Results of Tukey multiple comparisons tests (pairs of means enclosed by the range of a bracket are not significantly different, $P > 0.05$): [0.7–2.9], [2.9–5.5], [9.4–10.6], [14.7–17.3].

3.6. Effect of fenoxycarb on general esterase and JH esterase-like activity in the hemolymph after application at 48 h of the fifth instar

Treatment with fenoxycarb (1 μg/animal, 48 h of the fifth instar) caused an immediate increase in the hemolymph GE and JHE-like activity (Fig. 6). Within 12 h (60 h of the fifth instar) of fenoxycarb application, the levels of hemolymph GE activity (NapAc; Fig. 6A or pNPAc; Fig. 6B) increased to levels much higher than control (see Fig. 4) and were similar to those observed when treatment

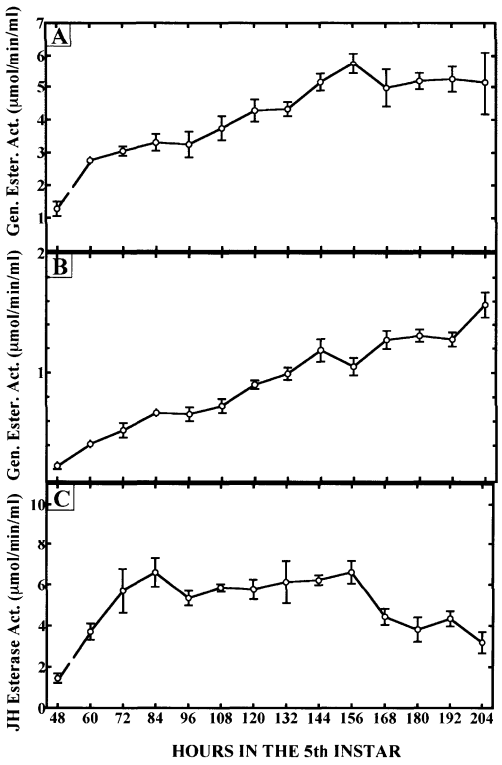

Fig. 6. Effect of fenoxycarb on general and juvenile hormone esterase activity after its topical application (1 μg/animal) at 48 h of the fifth instar. Each value is the mean ± SEM of three animals. Tests of the difference between control (see Fig. 4) and experimental values (unpaired t tests) revealed the means were not significantly different ($P > 0.05$): (1) 1-naphthyl acetate (A) 96 and 204 h of the fifth instar; (2) 4-nitrophenyl acetate (B) 72 and 96 h of the fifth instar; (3) JHE surrogate substrate (C) 96, 132, and 192 h of the fifth instar.

was done at 0 h of the fifth instar (see Fig. 4). Thereafter, the GE activity exhibited fluctuation patterns and reached levels similar to that of the previous treatment (compare with Fig. 4). Comparable results were obtained for JHE-like activity (Fig. 6C) which exhibited a fluctuation pattern remarkably similar to that of the treatment done at 0 h of the fifth instar (see Fig. 4C).

3.7. Effect of fenoxycarb on general esterase and JH esterase-like activity in the hemolymph after application late in the fifth instar

The results presented above demonstrated persistently increased GE and JHE-like activities that did not correlate with the concentration of fenoxycarb in the hemolymph (Figs. 2, 4, and 6). To further investigate whether the presence of fenoxycarb is related to any increases in GE or JHE-like activity, fenoxycarb was applied late in the fifth instar (132 h) and samples were collected until late in the pupal stage. Treatment with fenoxycarb at 132 h of the fifth instar did not produce any significant difference in hemolymph GE activity between control and fenoxycarb-treated animals for the rest of the investigated period (NapAc; Fig. 7A or pNPAc; Fig. 7B). Such a treatment prevented, however, the transient decrease in hemolymph JHE-like activity, which trend was observed in control animals late in the fifth instar (Fig. 7C). However, hemolymph JHE-like activity in the fenoxycarb-treated animals was not significantly different from control at 0 and 12 h of the pupal stage at the time, when the highest hemolymph JHE-like activity was observed in control animals (Fig. 7C). The hemolymph JHE-like activity then gradually decreased in both groups and reached low levels at 96 h of the pupal stage (Fig. 7C), while the hemolymph GE activity did not fluctuate substantially in this developmental period (Figs. 7A and B).

JHE-like activity was persistently increased in the hemolymph after application of fenoxycarb (Figs. 4, 6, and 7). Thus, it was important to determine whether fenoxycarb is a stimulator of JHE-like activity in vivo or exhibits positive interference with the assay in vitro. Thus, we determined the in vitro effects of fenoxycarb on hemolymph JHE-like activity. For these experiments, we selected a stage at which JHE-like activity was the highest, that is, at 12 h after pupal ecdysis (see Fig. 7C). The colorimetric assay results demonstrated that fenoxycarb actually inhibited the JHE-like activity in vitro in a dose-

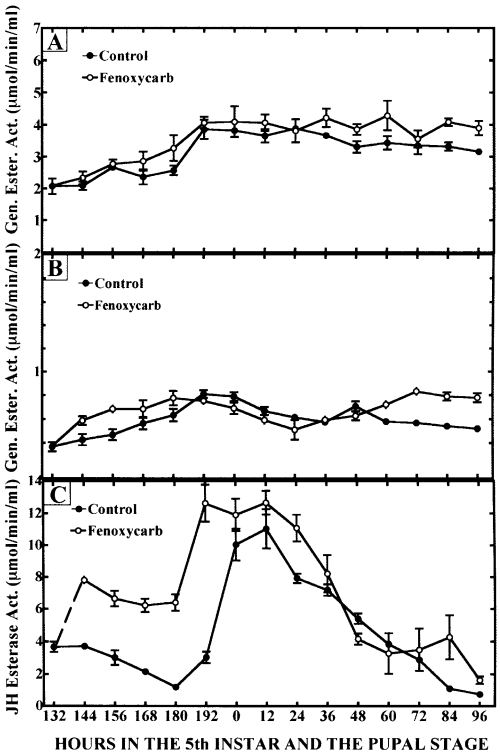

Fig. 7. Effect of fenoxycarb on general and juvenile hormone esterase activity after its topical application (1 μg/animal) at 132 h of the fifth instar. Each value is the mean ± SEM of three animals. Tests of the difference between control and experimental values (unpaired t tests) revealed the means were significantly different ($P < 0.05$): (1) 1-naphthyl acetate (A) 84 and 96 h of the pupal stage; (2) 4-nitrophenyl acetate (B) 156 h of the fifth instar and 60, 72, 84, and 96 h of the pupal stage. (3) JHE surrogate substrate (C) 144, 156, 168, 180, 192 h of the fifth instar and at 24 and 96 h of the pupal stage.

dependent manner with an IC_{50} of approximately 2 μM (Fig. 8). The peak hemolymph concentration of fenoxycarb immunoreactive material was approximately 3 μM at this particular developmental stage (data not shown). Therefore, if we assume that fenoxycarb acts as a competitive inhibitor of JHE, it may be responsible for some decrease in the JHE-like activity (Fig. 8). However, the K_M value of the surrogate substrate HEPTAT is approximately 300 times higher than that of JH, and the V_{max} values of the enzymatic reactions are also approximately 35 times higher for HEPTAT than for JH [12,13]. Therefore, the specificity of the surrogate substrate (characterized by the V_{max}/K_M ratio) is only 8.7–8.9 times lower than that of JH in this assay system [12,13].

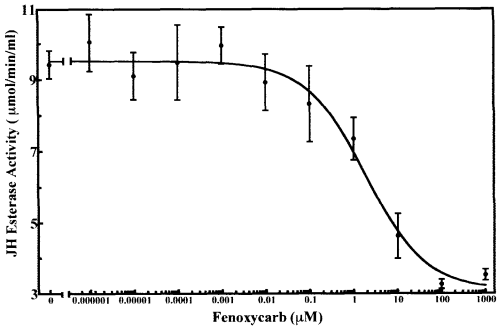

Fig. 8. In vitro effect of fenoxycarb on juvenile hormone esterase activity. Juvenile hormone esterase activity was measured in the presence of the indicated concentrations of fenoxycarb using a surrogate substrate (HEPTAT) as described in Section 2. Each value is the mean ± SEM from hemolymph of three animals at the 12 h of the pupal stage. Generation of a dose-response curve revealed an $IC_{50} \approx 2\,\mu M$. Results of Tukey multiple comparisons tests (pairs of means enclosed by the range of a bracket are not significantly different, $P > 0.05$): [10.0–7.3], [7.3–4.6], [4.6–3.2].

This means that the surrogate substrate is utilized with lesser specificity than JH. Nevertheless, its interaction with JHE will be less affected by competitive JHE inhibitors than in the case of JH being used as a substrate. Thus, it is unlikely that fenoxycarb will significantly inhibit the hydrolysis of JH by JHE at these concentrations.

4. Discussion

In a previous study, we showed that when fenoxycarb was topically applied until the 48 h of the fifth instar of *B. mori*, the animals became unable to initiate the larval–pupal differentiation and remained as permanent fifth instar larvae (dauer larvae; [11]). What the previous work did not investigate was the actual link between the fenoxycarb-mediated induction of dauer larvae and the presence of fenoxycarb in the larval hemolymph. Development of an ELISA for determination of fenoxycarb at trace-levels ([9,10]; Székács, unpublished data) facilitated this study on fenoxycarb concentrations in the hemolymph of *B. mori*. The ELISA employed in this work displayed some cross-reactivity toward only compounds with chemical structures closely related to that of fenoxycarb (10; Székács, unpublished data). Furthermore, some JH-related compounds such as methoprene did not cross-react with this ELISA (Dedos and Fugo, unpublished data). The hemolymph of *B. mori* showed only minor matrix effects with this ELISA ([20]; Székács, unpublished data).

The use of the ELISA for fenoxycarb enabled us to investigate how the presence of fenoxycarb in the hemolymph is involved in the fenoxycarb-mediated induction of dauer larvae. Our results demonstrate that the continued presence of detectable levels of fenoxycarb in the larval body of *B. mori* is not directly related to the subsequent induction of dauer larvae (Figs. 2 and 3). Apparently, the fenoxycarb-mediated induction of dauer larvae in *B. mori* is a developmentally delivered effect that is imprinted when fenoxycarb enters the larval haemocoel at sufficiently high amounts for the first time [21–23]. This induction is manifested at a certain time of the fifth instar [19]. This effect has been shown to be associated with the ability of this JH mimic to interfere with the steroidogenic competence of the prothoracic glands at about the 72 h of the fifth instar [19]. In other words, the presence of fenoxycarb at sufficiently high amounts [22,23] even in previous instars [21] and before that developmentally critical period results in induction of dauer larvae without the requirement of further presence of fenoxycarb. These authors [23] showed that administration of only 1 ng of fenoxycarb per larva early in the fifth instar induces dauer larvae, which also supports this conclusion. Furthermore, fenoxycarb could not be traced in the hemolymph 48 h after its application (0 h of the fifth instar), yet these larvae never initiated the larval–pupal commitment (Fig. 2). Application at 48 h resulted in the same induction of dauer larvae. Although fenoxycarb was present in the hemolymph at 72 h (Fig. 5), such larvae did not have residual fenoxycarb in their hemolymph much later in the fifth instar (Fig. 5).

Elimination of fenoxycarb from the hemolymph (Fig. 2) correlated well with the detection of residual fenoxycarb in the larval feces (Fig. 3). About 58% of the applied fenoxycarb could be traced in the feces 12 h after its application, and this level reached a cumulative amount of 99% at 48 h after fenoxycarb application (Fig. 3). These results show that very little fenoxycarb is present in the hemolymph and that all the applied amount has been excreted (Figs. 2 and 3) by the critical time of induction of dauer larvae [19]. Similarly low rates of incorporation in and high rates of elimination from *B. mori* hemolymph have been reported for methoprene [24]. These authors found that topical application of 1 μg/animal of

^{14}C-methoprene at 0 h of the fifth instar resulted in the presence of only 22% of the applied dose at 6 h and absence of any radioactivity in the hemolymph within 24 h of application [24]. Furthermore, research on the metabolism of orally administered ^{14}C-fenoxycarb in fifth instar of *Heliothis virescens* showed that 95% of the administered dose was excreted within 5 days and no radioactivity was observed in fifth instar larvae after treatment in the fifth instar [17]. Such data show that the high potency of these JHAs is due in part to the metabolic stability of the molecules.

Using 4-nitrophenyl acetate, we detected no differences in the hemolymph GE activity between the treated and control groups (Fig. 4B) until the time, when all the fenoxycarb was cleared from the hemolymph (Figs. 2 and 3). This trend, however, was not noticed, when 1-naphthyl acetate was employed in a similar experiment (Fig. 4A). This delayed increase in the hemolymph GE activity of dauer larvae that we observed at treatments at 0 h of the fifth instar (Fig. 4B) did not occur when fenoxycarb was applied at 48 h of the fifth instar (Fig. 6B) even though the same physiological effect was produced. In the latter case (application at 48 h), hemolymph GE activity increased within 12 h of application and remained at high levels exhibiting fluctuation patterns and levels similar to those seen in the former case (compare Figs. 4 and 6). To determine whether fenoxycarb-mediated increases in hemolymph GE activity are directly produced by fenoxycarb or are related to the induction of dauer larvae, treatments were done later in the fifth instar (132 h), when fenoxycarb can no longer disturb larval–pupal differentiation [11]. This treatment showed no fenoxycarb-mediated increase in hemolymph GE activity until late in the fifth instar (Fig. 7), which is an indication that the increases in hemolymph GE activity are closely related to the induction of dauer larvae (Figs. 4 and 6) and not to the presence of fenoxycarb in the hemolymph (Fig. 2).

The relationship among the presence of fenoxycarb in the hemolymph, the JH titer and hemolymph JHE-like activity early in the fifth instar is clarified by results [23], which showed that fenoxycarb could induce dauer larvae without having any effect on *B. mori* hemolymph JH titers. Fenoxycarb stimulated the hemolymph JHE-like activity 24 h after its application early in the fifth instar (Fig. 4C) and immediately did so in the middle (Fig. 6C) and late fifth instar (Fig. 7C). Application at 48 h caused the hemolymph JHE-like activity to reach levels and exhibit a fluctuation pattern (Fig. 6C) similar to that of application at 0 h (Fig. 4C). High hemolymph JHE-like activity, however, was observed even well after the clearance of fenoxycarb from the hemolymph (Figs. 4 and 6), but no significant differences were observed in the pupal stage (Fig. 7C) even though fenoxycarb can be traced at that stage in the hemolymph (Dedos and Fugo, unpublished data). These results and those presented earlier [23] seem to suggest that only application of fenoxycarb late in the fifth instar stimulates JH biosynthesis and release from the corpora allata (CA) of *B. mori*. The resulting elevated JH level in turn induces higher JHE activity in the hemolymph. There is, however, conflicting evidence in the literature about this issue. Hemolymph JH levels are high very early in the fifth instar of *B. mori* [23,25,26], and so is JH secretory activity from the CA at that stage [26]. The hemolymph JH titer decreases quickly and reaches a basal level by day 2 of the fifth instar and remains at such low levels until the onset of wandering behaviour [25,26], while the JHE activity is continuously increasing during that period [14]. However, it was shown [23] that there is no difference in JH titers between control and fenoxycarb-treated larvae, thus discrediting any interpretation of our data (Figs. 4 and 6) in terms that fenoxycarb stimulates JH biosynthesis/release early in the fifth instar, when the JH secretory activity is high. Such assumption, moreover, would be in contrast to data [27], which showed that topical application of fenoxycarb in *Manduca sexta* actually inhibits the in vitro JH secretory activity of CA. Similar results have been reported for other externally applied JHAs and compounds with anti-JH activity [28], who suggested that excess JH (or JHA) induces a negative feedback mechanism which depresses JH biosynthesis by the CA while the externally applied JHA takes over the role of endogenous JH [27,28]. If we adopt the notion put forth by these authors, the increases in hemolymph JHE-like activity of fenoxycarb-treated animals in the early and middle stage of the fifth instar (Figs. 4 and 6) are not directly related to the presence of endogenous JH and/or fenoxycarb. These increases rather seem to be the overt effect of a mechanism sensing the absence of an imminent larval–pupal differentiation. This assumption gains credibility, if we consider that fenoxycarb is quickly excreted from the hemolymph (Figs. 2, 3, and 5), but hemolymph JHE-like activity remains high for several days after the clearance of fenoxycarb. Further

evidence to discredit any fenoxycarb-mediated stimulation of endogenous JH biosynthesis/secretion early in the fifth instar is provided [29]. This study has shown that a decrease in the ecdysteroid titer early in the fifth instar of *B. mori* brings about an inhibition of JH secretory activity of the CA [29]. Such decreased hemolymph ecdysteroid titer is characteristic of the fenoxycarb-induced dauer larvae [22,23], which again suggests that fenoxycarb does not increase the endogenous JH titer early in the fifth instar in *B. mori*. Based on allatectomy experiments, it was shown that the fenoxycarb-mediated induction of dauer larvae is not related to the presence of JH [22]. However, high JH titer in the hemolymph of fenoxycarb-treated larvae of *B. mori* have been reported [21].

The hemolymph JHE-like activity sharply increased after application of fenoxycarb at 132 h of the fifth instar (Fig. 7C), but this increase only lasted until the time of pupal ecdysis, and no major differences were observed between the two groups in the pupal stage (Fig. 7C). At 12 h after the pupal ecdysis, the JHE-like activity reached its highest level in the hemolymph of *B. mori* (Fig. 7C). At that time, fenoxycarb did not stimulate the hemolymph JHE-like activity in vivo and inhibited the surrogate substrate hydrolysis in vitro ($IC_{50} \approx 2\,\mu M$, see Fig. 8). A similar in vitro inhibition of hemolymph JHE activity by fenoxycarb was reported in the tortrix moth, *Adoxophyes reticulana* [30]. Using the JHE partition assay [31], these authors measured a 79% inhibition of hemolymph JHE activity in vitro in the presence of 5 μM fenoxycarb [30]. Using a colorimetric assay for the hemolymph JHE-like activity, we observed a 79% inhibition in the presence of approximately 16 μM fenoxycarb in this study (Fig. 8).

Fenoxycarb is a carbamate and, in theory, could act as a carbamoylating agent and covalently react with JHEs. Most of the carbamates screened on JHEs have little or no inhibitory potency. However, some inhibition of JHE by carbamates with good leaving groups has been seen in vitro [5,30]. With its bonding pair of electrons, a good leaving group easily cleaves from a carbon atom. Good leaving groups include aryloxy moieties, where the cleaved arylate ion is stabilized by the delocalized aromatic electron system. Thus, carbaryl with the excellent 1-naphthyloxy leaving group inhibited microsomal JHE from *Musca domestica* [5]. On the other hand, alkoxy groups that lack stabilization by a delocalized electron structure are considered poor leaving groups. Fenoxycarb contains a rather poor ethoxy leaving group that renders it quite stable against non-enzymatic alkaline hydrolysis. Its 2-(4-phenoxyphenoxy) ethyl moiety, however, appears to be a mimetic for the sesquiterpene chain in JHs. Therefore, the compound may bind to biological molecules that recognize the JH backbone, but it is unlikely to act as a carbamoylating agent. The lack of any significant difference in the JHE-like activity between the two groups at 12 h of the pupal stage (Fig. 7C) could be due to the presence of fenoxycarb in the hemolymph of the treated animals. Our results (Fig. 8) appear to indicate that fenoxycarb is recognized by and can bind to the esterase molecule; although, it clearly is not a potent inhibitor of JHE. Studies in other insect species have reached similar conclusions and have shown that the carbethoxy group of fenoxycarb is not cleaved by JHE(s) [17,30]. Therefore, because fenoxycarb has a very poor leaving group, we speculate that it may be a competitive and not an irreversible inhibitor.

In our studies, JHE activity in the hemolymph was assessed by a HEPTAT-based colorimetric assay [12–14]. JH itself is the best substrate for monitoring JHE activity. However, the high cost of the radiolabeled JH-based assays forced us to use HEPTAT as surrogate JHE substrate. In the literature, there are numerous cautions regarding the use of HEPTAT in whole hemolymph [12,13]. Nevertheless, a study [14] showed that HEPTAT could be useful for JHE activity measurements in *B. mori* hemolymph. This method provided developmental activity patterns similar to those observed with radiolabeled JH in *B. mori* hemolymph [14]. We performed several control experiments, similar to those described [14], to confirm that the HEPTAT-based JHE activity assay was suitable in our case. Our results (data not shown) were in general agreement with those reported [14]. Furthermore, the pattern of JHE-like activity in the hemolymph was consistent with physiological expectations and distinct from GE activity profiles seen with the assays based on 1-naphthyl acetate and 4-nitrophenyl acetate (Figs. 4, 6 and 7). Our results taken together support the hypothesis that the JHE-like activity, monitored with the HEPTAT-based assay, reflects largely endogenous JHE activity (see also [14]).

The hemolymph JHE-like activity in control larvae exhibited an interesting fluctuation pattern late in the fifth instar and pupal stage. It decreased to low levels at 180 h of the fifth instar, then increased sharply and reached its highest peak at the time of pupal ecdysis, and then declined by the

96 h of the pupal stage to a very low level (Fig. 7C). This fluctuation pattern suggests that an increase in endogenous JH titer occurs at the pharate pupal stage of *B. mori* and the subsequent increase in JHE activity serves to degrade JH to its acid. Such increases in JH and JH acid titers accompanied by increases in hemolymph JHE activity have been reported for *M. sexta* [32]. In *B. mori*, elevated JH titer at the prepupal stage has been reported [25,26]. These authors [26] have also reported increases in JH acid titer at the prepupal stage of *B. mori*, which is an indication of increased JHE activity at that stage [26]. We assume that JHE has a dual role at this stage of development: (1) it reduces JH titers; (2) it synthesizes JH acid that may have hormonal functions other than those of JH [33,34]. If this is the case, it is difficult to determine whether the fenoxycarb-mediated increase in hemolymph JHE activity in the pharate pupal stage is a direct effect of fenoxycarb on the levels of protein expression and secretion of JHE from the silkworm fat body [35] or its action is indirect. The latter mechanism means that fenoxycarb stimulates JH biosynthesis/secretion specifically at that stage. To complicate matters further, fenoxycarb produces high increases in the ecdysteroid titer at the pharate pupal stage (Dedos and Fugo, unpublished data), which may exert a positive feedback effect on the JH secretion rate. Such a feedback mechanism was suggested for *M. sexta* [36]. The high levels of fenoxycarb needed to inhibit JHE in vitro make it unlikely that direct inhibition of the enzyme in vivo has a major role (Fig. 8). Thus we hypothesize that the increase in hemolymph JHE-like activity (Fig. 7C) is an indirect effect and occurs possibly through a JH-mediated increase in synthesis and secretion of JHE from the larval fat body late in the fifth instar.

In conclusion, the results in this study suggest that fenoxycarb is rapidly eliminated and excreted from the larval body of *B. mori* after its topical application. The fenoxycarb-mediated induction of dauer larvae in this insect occurs even in the absence of this JH mimic once it was present in the larval body at sufficiently high amounts. The induction of higher hemolymph GE activity in fenoxycarb-treated animals is related to the induction of a certain physiological state (dauer larval state) and not directly associated with the presence of fenoxycarb. Similarly, the induction of higher hemolymph JHE-like activity late in the fifth instar does not suggest a clear association with the presence of fenoxycarb and/or with its effect on the endogenous JH titer. To resolve this issue, direct measurements of hemolymph JH titer and JH secretory activity of the CA of fenoxycarb-treated animals late in the fifth instar will have to be carried out. It also remains to be determined whether this JH mimic or its metabolites have any influence on JH biosynthesis and/or secretion.

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