

JUVENILE HORMONE ESTERASE IS A BIOCHEMICAL ANTI-JUVENILE HORMONE AGENT

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Abstract—Juvenile hormone esterase, purified by affinity chromatography from the larval hemolymph of *Manduca sexta* in the fifth stadium, was injected into larvae of the same species in the earlier stadia resulting in a blackening of the cuticle following ecdysis to the next larval stadium. This anti-juvenile hormone response was dose-dependent for an injection in the second, third or fourth stadium. Cuticular blackening was prevented by treating larvae with the juvenoid epifenonane. Larval response to injected juvenile hormone esterase also varied with the time of injection within a single stadium, having a maximum effect for injections at the time of head capsule slippage. Juvenile hormone esterase activity measured from the hemolymph after injection of larvae in the second stadium decreased over an 11 h time-course. Because the anti-juvenile hormone effects resulting from a single injection of juvenile hormone esterase were dependent on the time of injection, it appears that when juvenile hormone biosynthesis is active in the insect, the duration of enzyme activity limits the anti-juvenile effects that can be induced.

Key Word Index: juvenile hormone esterase, anti-juvenile hormone agent, *Manduca sexta*, affinity chromatography of esterase, biological role of esterase

INTRODUCTION

Anti-juvenile hormone agents (anti-JH agents) have been of interest as chemicals to control insect development. Various chemical agents with different biochemical actions and targets have been developed to block JH biosynthesis and JH–receptor interactions as effective anti-JH agents (Staal, 1986). In the present study, a novel approach to achieve anti-JH effects is demonstrated in *Manduca sexta*, the tobacco hornworm, using JH catabolism to reduce the circulating titer of JH.

It is known that in at least three orders of insects, there exist esterases with a high degree of specificity towards JH (Hammock, 1985). These juvenile hormone esterases (JHEs) hydrolyze JH to the biologically inactive acid (Weirich *et al.*, 1973). In the Lepidoptera, JHE appears in the final larval stadium as two distinct peaks of hydrolytic activity, contributing to the rapid decline in the JH titer that signal the onset of pupation (Hammock, 1985). In the early instars, JHE activity is usually very low (Bühler *et al.*, 1985; Hanzlick and Hammock, 1988) while the JH titer remains relatively high (de Kort and Granger, 1981). The high affinity for JH (low K_m) and relatively rapid turnover number (k_{cat}) of the enzyme indicate that JHE should be extremely efficient at hydrolyzing the hormone under biological conditions (Abdel-Aal and Hammock, 1985, 1988; Hanzlick and Hammock, 1988). Therefore, if JHE were introduced prematurely into young larvae, the enzyme should induce physiological and morphological anti-JH effects by

degrading JH at a time when JH biosynthesis is active. Such a demonstration would support the hypothesis that the JH titer in lepidoterans is regulated, in part, by increased degradation in addition to fluctuations in biosynthesis of the hormone. Such a demonstration would also indicate that JHE, as a novel anti-JH agent, will be potentially useful both for insect control and as a biochemical tool for insect physiologists.

Cuticular blackening is recognized as a sensitive indicator of JH deficiency in the tobacco hornworm and has been used to screen the biological activity of chemical anti-JH agents (Staal, 1986). When the JH titer is reduced sufficiently during a critical window of time before a larval molt, the newly forming cuticle will blacken from the deposition and darkening of premelanin granules in the cuticle (Truman *et al.*, 1973; Curtis *et al.*, 1984; Hiruma *et al.*, 1985). Larvae will blacken when they are ligated 4–6 h before head capsule apolysis (Kiguchi and Riddiford, 1978) but not 6–7 h after this event (Truman *et al.*, 1973). This same cuticular blackening occurs in individuals of a black mutant strain of tobacco hornworm that naturally have a low titer of JH throughout larval growth (Safranek and Riddiford, 1975). As confirmation that blackening of the cuticle is, the fact, due to a deficient JH titer, the blackening response can be prevented by treating larvae with JH or a JH analog before the subsequent molt (Truman *et al.*, 1973; Safranek and Riddiford, 1975; Fain and Riddiford, 1975; Curtis *et al.*, 1984; Hiruma *et al.*, 1985). In the present study, cuticular blackening was used as a sensitive, categorical biological assay for measuring the anti-JH effects of JHE in larvae of *M. sexta*.

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MATERIALS AND METHODS

Insects

Wild type larvae of *Manduca sexta* were supplied as eggs by M. Johnson, Sandoz Corp. (Palo Alto, Calif.) and reared at 28°C on a 14L:10D photoperiod and fed an artificial diet (Bell and Joachim, 1976). Black mutant larvae (Safranek and Riddiford, 1975) were supplied by L. M. Riddiford and were reared as described above. All times are referenced to "lights on" and are reported as times "after lights on" (ALO). Larval ecdysis to the third stadium is designated L3D0, with head capsule slippage (HCS) occurring for first gate larvae on L3D2. Ecdysis to the fourth stadium (L4D0) occurs 3 days after ecdysis to the third stadium. Abbreviations for all other stadia follow these same guidelines.

JHE purification

A few crystals of phenylthiourea were dissolved in ethanol and used to coat the inside of a 50 ml centrifuge tube to prevent the action of tyrosinases in larval hemolymph. Larvae of *M. sexta* (Gate I) were chilled in ice on the third day of the ultimate stadium (L5D3). Hemolymph was collected by clipping the prolegs of larvae and pooled. To remove cellular matter, the pooled hemolymph was centrifuged at 10,000 g for 10 min at 2°C. The resultant plasma was frozen at -70°C until used for the purification of JHE.

JHE was purified by a modification of the affinity chromatography procedure described by Abdel-Aal and Hammock (1986) and is elaborated in Philpott *et al.* (1990). The integrity of the purified JHE was determined by assaying its catalytic activity and by SDS-PAGE. The enzyme was stored in 10 µl aliquots at -70°C for up to 9 months without a measurable loss of activity. The maximal specific activity of the enzyme used for injection was determined *in vitro* and was 1400 nmol JH III hydrolyzed min⁻¹ mg⁻¹ protein (± 85SE, n = 16). All JHE activity was measured in sodium phosphate buffer (pH 7.4 I = 0.2 M containing 0.0025% Triton X-100, 0.02% sodium azide and 5% sucrose) using the partition method: a 10 min incubation assay at 30°C using [³H]JH III as the substrate at 5 × 10⁻⁶ M final concentration (Hammock and Sparks, 1977). Racemic JH III (2E, 6E) was purchased from Chemical Dynamics (South Plainfield, N.J.) and [³H]JH III was purchased from New England Nuclear (Boston, Mass.). The enzyme was diluted in buffer to be within the linear region of the partition assay and the percentage of JH III metabolism was expressed as nmol JH III hydrolyzed min⁻¹ mg⁻¹ protein (or ml⁻¹, for activity measured from larval hemolymph).

JHE injections and bioassay

Larvae were selected for injection in each stadium within 1 h of head capsule slippage (HCS ± 1 h) before the molt. In all cases, those larvae selected before HCS were obvious, showing clear slippage within the hour following injection. Larvae (8–12 per treatment group, 1–4 replications) were anesthetized with CO₂ and injected in the first abdominal proleg with the appropriate amount of JHE diluted in bovine serum albumin (BSA) (6.6 mg/ml in buffer) using a drawn-out microcapillary pipet (20 µl) attached to a rubber tube and mouth adaptor: for injections in the second stadium, 49–164 pmol (3–10 µg) in 1.5 µl and for injections in the third and fourth stadia, 49–360 pmol (3–22 µg) in 4 µl were used. Control injections made in the second stadium (2 µl) and in the third and fourth stadia (4 µl) routinely contained BSA buffer (6.6 mg/ml). A high concentration of BSA (84 µg/µl) was also injected into larvae as a control (second stadium, 2 µl and third–fourth stadia, 4 µl) to emphasize that large amounts of protein alone do not cause any ill-effects or anti-JH effects unrelated to the action of JHE. Larval hemolymph (L5D3, 6 µg/µl), from which the

JHE had been removed by the affinity purification procedure, was injected as a control (second stadium, 2 µl and third–fourth stadia, 4 µl) to establish that no other proteins from the L5D3 hemolymph affected the cuticular blackening response. The inhibitor, 3-octylthio-1,1,1-trifluoropropanon-2-one (OTFP), that was used to elute JHE from the affinity gel was injected in buffer (10⁻⁴M) as a control (second stadium, 2 µl and third–fourth stadia, 4 µl) to demonstrate that no side-effects are seen from the trace amounts of OTFP remaining in the JHE preparations used for injection. On the day following the injections of JHE, all larvae ecdysed to the subsequent stadium. Because melanization often increased for a day or two after ecdysis (Fain and Riddiford, 1975), larvae were scored visually for cuticular blackening 1 day after ecdysis.

In order to prevent the cuticular blackening response in larvae injected with JHE, the larvae were treated topically with the juvenoid epofenonane, 1-(4'-ethylphenoxy)-6,7-epoxy-3-ethyl-7-methylnonane (MW = 301), immediately after they were injected with JHE in the second stadium. Epofenonane lacks an ester bond and is not metabolized by JHE. Larvae were injected with 148 pmol (9 µg) of JHE, then topically treated with varied concentrations of epofenonane in 1 µl of methanol. Larval black mutants in the second stadium were also treated with epofenonane to compare the effective dose-range for preventing the blackening response with the dose-range necessary for preventing the response after injection of JHE.

To determine how the actual level of JHE enzymatic activity in the injected larvae correlates with the biological activity that results from injection of the enzyme, the esterase present in the hemolymph after injection of larvae in the second stadium was measured for two doses of the enzyme, 82 and 115 pmol (5 and 7 µg). A sample of blood (4 µl) was removed at hourly time-points (1–11 h) from 4 or 5 larvae by clipping off the horn and collecting the hemolymph with a capillary pipet. Hemolymph samples were diluted in buffer and stored at -70°C for later analysis as described earlier.

To determine how the biological activity of JHE varies with the specific time of injection within a single stadium, larvae were injected once with 131 pmol of JHE at a time-point between 0 h ALO on day 2 of the third stadium (L3D2) and 2 h ALO on the day of ecdysis to the fourth stadium (L4D0). HCS occurs about 10 h ALO on L3D2 with the first larvae ecdysing to L4 about 0 h ALO and the last by 10 h ALO. The injections made at 2 h ALO on L4D0 were given only to larvae that had not yet molted.

Scoring system

Scoring systems that have been reported for the cuticular blackening anti-JH response in *M. sexta* provide clear means to distinguish degrees of cuticular melanization after treatments of JH or a JH analog (Truman *et al.*, 1973; Fain and Riddiford, 1975; Hiruma *et al.*, 1985). Because the bioassay in the present study involved increases in cuticular blackening and because there were no localized effects, such as a black spot at the point of injection, as is seen with the application of juvenoids, it was necessary to define our own scoring system empirically. No localized effects were seen in this study presumably because the enzyme is injected directly into the hemocoel, resulting in a systematic response to the cuticle. In this study, larvae scored for cuticular blackening in one larval stadium (e.g. fourth stadium) were injected with JHE in the previous stadium (e.g. third stadium).

Negative response (no visible darkening of the cuticle)

Normal—blue-green with light transverse and oblique abdominal stripes, not visually distinct from the control injected larvae.

Positive response (visible increase in the darkening of the cuticle)

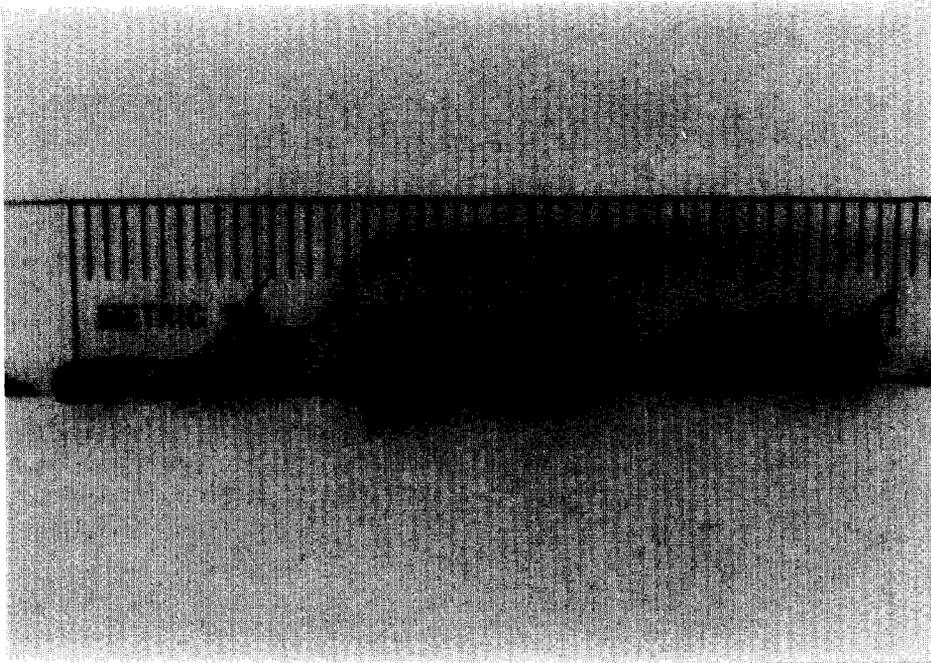


Fig. 1. Cuticular blackening response of *M. sexta* in the third larval stadium after injection with 148 pmol of JHE (9 µg) at 10 h ALO, L2D1. Following injection of the JHE, larvae were treated topically with 0.5 µg epofenonane (MW = 301) in 1 µl methanol (left), 0.005 µg epofenonane (middle) or 1 µl methanol (right). The intermediate dose of epofenonane (0.005 µg) was not sufficient to prevent blackening of the cuticle completely, but instead produced a localized effect, giving blackened larvae the appearance of wearing a green saddle. Larval weight at the time of injection was 33 ± 0.55 mg (\pm SE).

Intermediate—larvae predominantly blue-green with heavy darkening of the transverse and oblique abdominal stripes. Often, the head capsule, anal proleg (plates) and dorsum of the prothorax also were darkened.

Complete—larvae mottled black with patches of green or larvae entirely black without mottling. These two categories were grouped together for ease of data interpretation. Separating the responses into additional categories did not improve the interpretation of the results.

Larvae treated with epofenonane to prevent cuticular blackening were scored using the above system to provide a consistent interpretation of the results. In addition to our scoring system, we analyzed the data on epofenonane-treated larvae using a numerical scoring system that included localized effects on the cuticle, such as a green spot at the point of application (Fig. 1), to enable a comparison of our results with those of Fain and Riddiford (1975).

Multiple injections of JHE

In an attempt to assure the continued presence of JHE activity in larvae, we administered JHE to six larvae in multiple injections during a 24 h period spanning the presumed release of the prothoracicotrophic hormone, PTTH (Truman, 1972). The rationale for this study was based on the general understanding that the absence of JH during the release of PTTH is, in part, responsible for the reprogramming of larval tissues to become pupal at the molt (Truman, 1972; Fain and Riddiford, 1976). The injection technique used was the same as that described earlier. The first injection (141 pmol JHE) was given at 10 h ALO, L3D1, followed by 4 more injections (141 or 211 pmol JHE) given approx. 6 h apart through 10 h ALO, L3D2. Control injections into 10 larvae were given at each time-point and contained 336 µg BSA in buffer (4 µl). Uninjected larvae were used as an additional control to compare the weight gain of injected larvae and the time of normal ecdysis to the fourth stadium.

RESULTS

Cuticular blackening of the larval cuticle in the third stadium

Cuticle blackening of the larval cuticle in the third stadium was dose-dependent following injections of JHE in the second stadium (Fig. 2). Larvae remained darker than normal throughout the stadium, and the cuticle returned to normal coloration after the subsequent molt to the fourth stadium unless larvae were reinjected with JHE. In order to provide a working approximation of the dose of JHE necessary to blacken the cuticle in 50% of the larvae, ED₅₀, we calculated a linear regression line of best-fit for doses 49–164 pmol. Using these calculations the ED₅₀ was 121 pmol (224 ng JHE/mg wet weight of the larvae injected in the second stadium). No ill-effects or positive anti-JH effects were seen in the larvae for any of the control injections given in the third stadium: 2 and 4 µl of BSA in buffer (6.6 µg/µl), 168 µg of BSA in buffer (2 µl of 84 µg/µl), 2 µl of larval hemolymph, 2 µl of OTFP (10⁻⁴ M) or 4 µl of buffer.

Effects of treatments with epofenonane

Treatment of larvae with epofenonane following an injection of JHE in the second stadium prevented cuticular blackening of larvae in the third stadium. This response was dose-dependent over a nanogram range of epofenonane concentrations (5–500 ng)

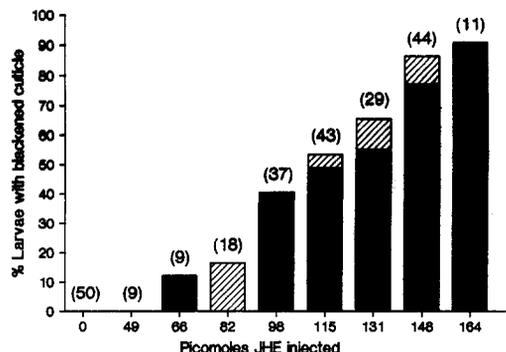


Fig. 2. Cuticular blackening response of *M. sexta* in the third larval stadium after injection with JHE at 10 h ALO, L2D1. Controls were injected with 2 µl BSA in buffer cocktail (6.6 µg/µl). Other control injections are noted in the text. Larval weight at the time of injection = 33 ± 1.1 mg (± SE, n = 250). Solid bars: the percentage of black or mottled larvae. Cross-hatched bars: the percentage of larvae with intermediate blackening. The number of larvae injected for each dose is indicated in parentheses above the respective bar. Linear regression of % larvae with a black or mottled cuticle onto dose (pmol JHE) was calculated (slope = 0.8, r² = 0.92, P < 0.0001).

[Fig. 3(A)]. As the dose of epofenonane was increased, the amount of black pigmentation in the newly formed larval cuticle decreased: from entirely black, to mottled and intermediate coloration with a green spot or band at the point of application (Fig. 1), to a normal blue-green coloration, to pale green and finally to white (50 µg epofenonane, data not shown). For the *black* mutant larvae treated with epofenonane in the second stadium, the degree of

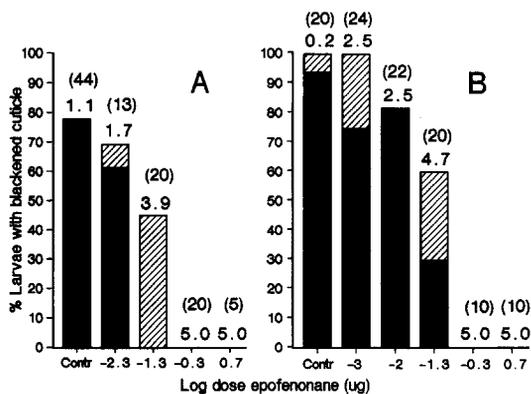


Fig. 3. Cuticular blackening response of *M. sexta* in the third larval stadium after injection with 148 pmol of JHE at 10 h ALO, L2D1 (A). Following injection of the JHE, larvae were treated topically with epofenonane in 1 µl methanol. Larval weight at the time of injection = 33 ± 0.55 mg (± SE, n = 208). Larval *black* mutants of *M. sexta* were also treated with epofenonane to compare the effective dose-range necessary to prevent cuticular blackening (B). Solid bars: the percentage of black or mottled larvae. Cross-hatched bars: the percentage of larvae with intermediate blackening. The number of larvae injected for each dose is indicated in parentheses. Using a numerical scoring system (Fain and Riddiford, 1975), the mean score (0–5) was calculated for each dose and is indicated above each bar. Linear regression of mean score onto log dose (µg) was calculated for the JHE injected (slope = 1.65, r² = 0.86, P < 0.2).

cuticle blackening in the third stadium also decreased within the same range of epofenonane concentrations (1–500 ng) [Fig. 3(B)].

We evaluated the data in the present study using a numerical scoring system such as the one described in Fain and Riddiford (1975). Linear regression of the mean score onto log dose epofenonane was generated for the JHE injected larvae (slope = 1.65 and $r^2 = 0.96$) and for the *black* mutant larvae (slope = 0.97 and $r^2 = 0.86$). The doses of epofenonane necessary to generate the median score (2.5) were 10 ng for the JHE injected larvae and 1.0 ng for the *black* mutant larvae of the same age.

Measurement of JHE activity after injection

When 115 or 82 pmol of JHE was injected into larvae in the second stadium, the JHE activity recovered from the hemolymph was similar at 1 and 3 h after injection for each dose, then declined steadily through 11 h (Fig. 4). The maximum JHE activity recovered for each injection was 133 and 88 nmol JH III hydrolyzed $\text{min}^{-1}\text{ml}^{-1}$ hemolymph, for the 115 and 82 pmol injections, respectively. Using the line of best to measure the decline in hemolymph JHE activity from 3 to 11 h, 50% of the maximal activity disappeared by 8.0 and 7.9 h after injection, for the 115 and 82 pmol injections respectively.

Cuticular blackening in the fourth stadium

Cuticular blackening was induced in the fourth stadium larval cuticle with the highest degree of sensitivity for all three stadia examined (Fig. 5). At the lowest dose of JHE injected, 49 pmol, 26% of the larvae showed an increased blackening of the cuticle. For 82–360 pmol doses of JHE, the number of larvae with a blackened cuticle increased dramatically and remained high, with 80–100% of the larvae showing a blackened cuticle. Using the linear regression line of

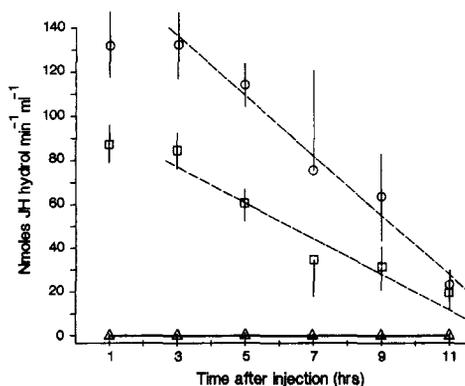


Fig. 4. JHE activity in the hemolymph of second stadium larvae after injection of 115 pmol (○) or 82 pmol (□) of JHE. Controls were injected with 9 μg of BSA (△). Larval weight at the time of injection was 33 ± 0.87 mg (\pm SE, $n = 78$). The *in vitro* specific activity of JHE incubated in larval hemolymph from L4D3 did not decrease over the 11 h time course (723 nmol JH III hydrolyzed $\text{min}^{-1}\text{mg}^{-1}$ JHE). Each value represents the mean \pm SE from 4 to 5 larvae. Linear regression of nmol JH III hydrolyzed $\text{min}^{-1}\text{ml}^{-1}$ hemolymph onto time after injection was calculated for the decline in activity from 3 to 11 h for the 115 injection (slope = -13.6 and $r^2 = 0.98$, $P < 0.001$) and for the 82 pmol injection (slope = -8.0 and $r^2 = 0.93$, $P < 0.08$).

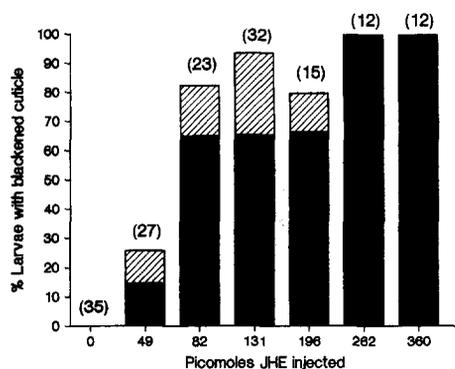


Fig. 5. Cuticular blackening response of *M. sexta* in the fourth larval stadium after injection with JHE at 10 h ALO, L3D2. Controls were injected with 4 μl BSA in buffer cocktail ($6.6 \mu\text{g}/\mu\text{l}$). Other control injections are noted in the text. Larval weight at the time of injection = 189 ± 40 mg (\pm SE, $n = 156$). Solid bars: the percentage of black or mottled larvae. Cross-hatched bars: the percentage of larvae with intermediate blackening. The number of larvae injected for each dose is indicated in parentheses above the respective bar. Linear regression of % larvae with a black or mottled cuticle onto dose was calculated for doses 0–262 pmol JHE (slope = 0.35, $r^2 = 0.78$, $P < 0.02$).

best-fit for doses 49–262 pmol, the ED_{50} was 109 pmol (35 ng JHE/mg wet weight of the larvae injected in the third stadium). No ill-effects or positive anti-JH effects were seen in larvae after ecdysis to the fourth stadium for any of the control injections: 4 μl of BSA in buffer ($6.6 \mu\text{g}/\mu\text{l}$), 336 μg of BSA in buffer (4 μl of $84 \mu\text{g}/\mu\text{l}$), 4 μl of larval hemolymph or 4 μl of OTFP in buffer (10^{-4} M).

Injections at different time-points in the third stadium

Blackening of the larval cuticle in the fourth stadium varied for injections of JHE given at different times within the last 24 h of the third stadium (Fig. 6). The number of larvae that displayed cuticular blackening in the fourth stadium was low for the JHE

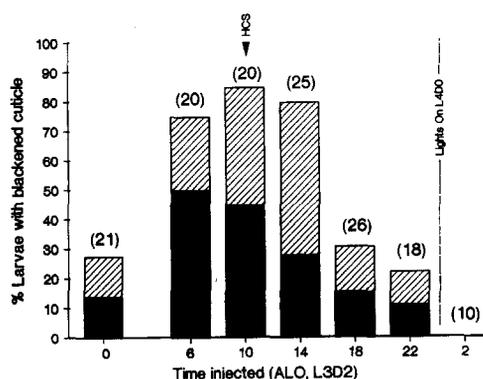


Fig. 6. Cuticular blackening response of *M. sexta* in the fourth stadium after injection of JHE at different times in the third stadium. Control injections contained 4 μl BSA ($84 \mu\text{g}/\mu\text{l}$) and did not cause blackening for any of the injections (data not shown). Head capsule slippage began within 1 h the time indicated by the arrow. Solid bars: the percentage of black or mottled larvae. Cross-hatched bars: the percentage of larvae with intermediate blackening. The number of larvae injected for each dose is indicated in parentheses above the respective bar.

injection given at 0 h ALO (L3D2). The blackening response in larvae after ecdysis to the fourth stadium reached a maximum for injections given between 6 and 14 h ALO in the third stadium (L3D2), and then declined dramatically for injections given at 18 h ALO (L3D2). By 2 h ALO on the day of ecdysis to the fourth stadium (L4D0), injection of the JHE had no effect on the fourth stadium cuticle. Larvae injected with 4 μ l of BSA (6.6 mg/ml) at each time-point did not show any ill-effects or blackening of the cuticle in the fourth stadium.

Cuticular blackening in the fifth stadium

For the range of doses used, cuticular blackening of the fifth stadium larval cuticle after injection in the fourth stadium was not as complete as for the two previous stadia (Fig. 7). The cuticular blackening response was low for doses of 82 and 131 pmol (23 and 20% of the larvae with any darkening of the cuticle). The number of larvae responding to the injected JHE increased to 50% at the 196 pmol injection, and finally 90% for the 262 pmol injection. Due to the large amount of JHE required to induce blackening of the cuticle in these larvae, we did not use higher doses. Using the available data, we calculated a linear regression of the overall blackening response to dose ($ED_{50} = 178$ pmol JHE, or 14 ng JHE/mg wet weight of larvae injected in the fourth stadium). No ill-effects or positive anti-JH effects were seen in the larvae after ecdysis to the fifth stadium for any of the control injections: 4 μ l of BSA in buffer (6.6 μ g/ μ l), 336 μ g of BSA in buffer (4 μ l of 84 μ g/ μ l) of larval hemolymph or 4 μ l of OTFP (10^{-4} M).

Multiple injections of JHE in the third stadium

The larvae that were injected at five consecutive time-points in the third stadium with a total of 846 pmol of JHE did not show any visible signs of precocious development. In addition, many of the

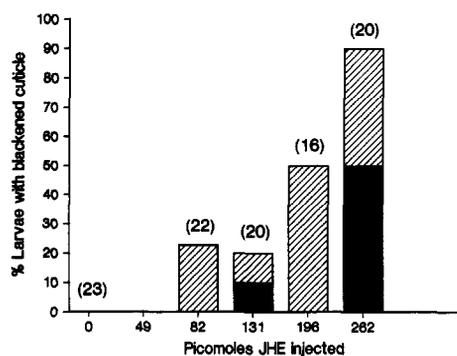


Fig. 7. Cuticular blackening response of *M. sexta* in the fifth larval stadium after injection with JHE at 10 h ALO, L4D3. Controls were injected with 4 μ l BSA in buffer cocktail (6.6 μ g/ μ l). Control injections are noted in the text. Larval weight at the time of injection = 0.8 ± 0.062 g (\pm SE). Solid bars: the percentage of black or mottled larvae. Cross-hatched bars: the percentage of larvae with intermediate blackening. The number of larvae injected for each dose is indicated in parentheses above the respective bar. Linear regression of % larvae with any increased blackening of the cuticle (total positive response) onto dose was calculated for dose 49–262 pmol JHE (slope = 0.39, $r^2 = 0.93$, $P < 0.008$).

larvae appeared to be traumatized by the repeated injections, which themselves probably contributed to a disruption of normal development. By 12 h ALO on L3D2, most of the uninjected control larvae showed at least the first signs of HCS. In contrast, the BSA injected larvae initiated HCS over 3 days, 50% of the larvae showing HCS on L3D3 and the remainder on L3D4 and D5. The larvae injected with JHE did not initiate HCS until 6 days after ecdysis to the third stadium. Some of the larvae from both the JHE and BSA injected groups did not gain as much weight as did the uninjected control larvae by ecdysis to the fourth stadium [139 ± 29 vs 200 ± 20 mg (\pm SE, $n = 6$) for the injected and uninjected larvae, respectively] indicating that the repeated injections may have been responsible for causing a reduction in feeding. A blackening of the fourth stadium cuticle was not observed in any of the larvae injected with JHE. This absence of cuticular blackening was probably due to delay between the last JHE injection and the eventual molt to the next stadium caused by the trauma associated with the injections, allowing sufficient time before the molt for the JHE activity to be cleared from the hemolymph and the JH titer to increase.

DISCUSSION

The results of this study demonstrate that even at a time with normally high levels of JH biosynthetic activity, juvenile hormone esterase injected into the hemolymph can induce an anti-juvenile hormone response, indicating that both the circulating titer and residual JH in the integument were metabolized. For the three larval stadia examined, juvenile hormone esterase induced a blackening of the cuticle that was dependent on a narrow dose-range of JHE protein.

The later stadia (fourth and fifth) appeared to be more sensitive to the injections of JHE when the ED_{50} values for the blackening response in each stadium were expressed as JHE (ng) per wet weight of larvae injected in the stadium prior to the blackening response: 224 ng JHE/mg for the third stadium, 35 ng JHE/mg for the fourth stadium and 14 ng JHE/mg for the fifth stadium. This observation is interesting because one would expect the opposite trend. We are currently testing the hypothesis that the enzyme is more stable in later stadia. It is also possible that the biosynthesis of JH or sensitivity of tissues to JH is lower in later stadia.

The amount of JHE activity present in the hemolymph after an injection into larvae in the second stadium was measured to compare the actual titer of JHE activity to the induced blackening of the larval cuticle in the third stadium. For an injected dose of 115 pmol JHE, roughly equal to the ED_{50} value, the maximal activity recovered was 133 nmol JH hydrolyzed $\text{min}^{-1} \text{ml}^{-1}$ hemolymph. This titer of JHE activity in larvae of *M. sexta* is higher than the endogenous titer of JHE activity in the fifth stadium measured at the prewandering and prepupal peaks of JHE activity [45 and 8 nmol JH III hydrolyzed $\text{min}^{-1} \text{ml}^{-1}$, respectively (Vince and Gilbert, 1977)]. However, JH biosynthesis has ceased when JHE is active in the fifth stadium; whereas, JH biosynthesis is active in the early larval stadia and represents a

significant barrier which must be overcome to induce an anti-JH effect. Consequently, it is interesting that the titer of JHE activity in the second stadium that was required to induce cuticular blackening in the third stadium is only 3-fold higher than the endogenous titer in the fifth stadium. Although the titer of JHE activity was not measured in this study for larvae injected in the third or fourth stadium, it is clear from ED₅₀ values expressed per mg larval weight (35 and 14 ng JHE/mg, respectively) that the actual titer of JHE activity resulting from these injections must be considerably lower than for the injections in the second stadium.

In the study, the nanogram dose-range of epofenonane required to prevent cuticular blackening in the third stadium was similar to the dose-range of other reports for reversing the blackening effect in black mutants and ligated *M. sexta* (Truman *et al.*, 1973; Fain and Riddiford, 1975; Safranek and Riddiford, 1975; Hiruma *et al.*, 1985). For the JHE injected larvae, the slope (1.65) and ED₅₀ (10 ng) calculated from the linear regression of the mean score onto log dose epofenonane was similar to those for treatment of larval black mutants with epofenonane in this study (slope = 0.97, ED₅₀ = 1 ng) and with JH I (slope = 1.50, ED₅₀ = 1 ng) (Fain and Riddiford, 1975). The similarity of the doses of epofenonane needed for preventing cuticular blackening indicates that the reduction in the JH titer generated by a single injection of JHE may be similar to or perhaps greater than the reduction in black mutant and ligated larvae.

The low picomole amounts of JHE required to elicit this anti-JH effect demonstrate that JHE is a powerful anti-JH agent. However, the half-life of the enzyme under the conditions of this study appears to be relatively short (8 h) and the anti-JH effects of a single injection of JHE appear to be limited to the duration of enzyme activity. Consequently, if an injection of JHE is made too early relative to a critical period of JH sensitivity at a time when JH biosynthesis is still active, the JH titer would increase following the decrease in JHE activity and prevent the anti-JH response. The critical window of JH sensitivity for the developing cuticle has been defined as an 8 h period following head capsule apolysis (Truman *et al.*, 1973; Hori *et al.*, 1984). For the present study, the rapid drop-off in biological activity for injections administered after 14 h ALO (L3D2) confirmed the time when the critical window is expected to close (HCS + 8 h). The low biological activity seen for injections at 0 h ALO (L3D2) indicated that as the JHE active-life declined, the titer of JH was allowed to increase within the critical window to prevent an anti-JH response (HCS - 10 h). The approximate half-life of JHE activity following a single injection of the enzyme (8 h) provides an interesting comparison with the half-life estimated from the natural decline in the prewandering peak of JHE activity seen in other studies, 6.8 h (Vince and Gilbert, 1977) and 8.6 h (Sparks *et al.*, 1983). In a different species of Lepidoptera, *Trichoplusia ni*, the estimated half-life of JHE activity (purified from *M. sexta*) was 3.6 h for an injection of 9.8 pmol JHE into L5D2 larvae (Philpott, 1989). Interestingly, the decline in hemolymph activity per pmol JHE injected (slope/pmol JHE) was similar for *T. ni* (0.14 for 9.8 pmol injection)

and *M. sexta* (0.12 for 115 pmol injected and 0.10 for 82 pmol injection). It will be of interest to learn whether the rapid decline in JHE activity in both of these species is due to a specific mechanism for inactivating the enzyme or clearing it from the hemolymph.

Induction of precocious development in *M. sexta* larvae was not observed as a result of multiple injections of JHE given during the presumed release of PTTH. Although the cuticular blackening anti-JH assay in *M. sexta* is highly sensitive to reductions in the JH titer, it is generally believed that induction of precocious metamorphosis requires a more complete reduction of the JH titer over a longer duration of time than required to induce a black cuticle (Staal, 1986). There was an indication that the multiple injections may have caused a delay in normal development (perhaps due to an anti-feeding response in the larvae), but additional studies are necessary to alleviate effects caused by trauma from the repeated injections.

From a fundamental standpoint, the results of this study are interesting because they demonstrate that the specific catabolic activity of juvenile hormone esterase can influence the normal regulation of the epithelial juvenile hormone titer. The clear anti-JH activity of JHE observed in this study indicates that as a biochemical anti-JH agent, JHE provides a promising complement to the chemical anti-JH's and surgical allatectomy in the study of JH dynamics and the role of this hormone in insect development. From a practical standpoint, it is hoped that the development can be disrupted by sustaining a high level of JHE activity over a long period of time. To this end, the anti-JH effects of JHE identifies this enzyme as an exciting target for engineering its gene into insect specific baculoviruses to provide a continuous expression system for JHE in pest insects. JHE introduced into insects in this manner may prove to be an effective anti-JH agent for insect control.

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