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HAEMOLYMPH JUVENILE HORMONE ESTERASE ACTIVITY IN SYNCHRONOUS LAST INSTAR LARVAE OF THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

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Abstract—Weight and time of moult during the last instar of the cabbage looper (*Trichoplusia ni*) were examined and used to select last instar larvae that had similar rates of development. Haemolymph protein content and titres of haemolymph esterases hydrolyzing juvenile hormone I, juvenile hormone III, and α-naphthyl acetate were monitored during the last instar using these closely timed larvae. Juvenile hormone I and juvenile hormone III esterase profiles were very similar and differed markedly from the α-naphthyl acetate esterase and protein content profiles. Two major peaks of juvenile hormone esterase activity were observed, one before ecdysone release and the other just prior to pupal ecdysis. Juvenile hormone I was hydrolyzed 15 times faster than juvenile hormone III when assayed at 5×10^{-6} M.

Key Word Index: *Trichoplusia ni*, Noctuidae, juvenile hormone, esterase, degradation, development

INTRODUCTION

THE JUVENILE hormones (JH's) of insects have two primary routes of degradation, ester hydrolysis and epoxide hydration. Ester hydrolysis is thought to be the major metabolic route for JH degradation in most insects and especially in the Lepidoptera (SLADE and ZIBITT, 1972; for review see HAMMOCK and QUISTAD, 1976). These JH esterases (JHE's) are differentially inhibited by certain organophosphates (SANBURG *et al.*, 1975a, b; HAMMOCK *et al.*, 1977) and seem to be selective for JH-like molecules (WEIRICH and WREN, 1973; HAMMOCK *et al.*, 1977). Haemolymph JHE's have been implicated as one mechanism in the regulation of JH titres (WEIRICH *et al.*, 1973; KRAMER *et al.*, 1976; NOWOCK and GILBERT, 1976). The JH titre in *Manduca sexta*, and several other lepidopterans, decreases in the early last instar (NIJHOUT and WILLIAMS, 1974a; VARJAS *et al.*, 1976; YAGI, 1976) and coincidental with this, in *M. sexta*, is a burst of haemolymph JHE activity (WEIRICH *et al.*, 1973; SANBURG *et al.*, 1975a, b; NOWOCK and GILBERT, 1976). Recent studies have also shown a rise in the JH titre (VARJAS *et al.*, 1976; YAGI, 1976) and in JHE activity (VINCE and GILBERT, 1977) just prior to pupation.

A detailed examination of the timing of endocrine and related events, which are often very short-lived, requires the use of closely timed insects. Moulting in *M. sexta* occurs at specific times of the day and the release of prothoracicotropic hormone (PTTH) appears to be the pacesetter for the endocrine events leading to ecdysis (TRUMAN, 1972). The *M. sexta* larvae are thus 'gated' by the endocrine events that start the moulting process, and this 'gating' can be determined on the basis of weight during the first few days of the last instar (NIJHOUT and WILLIAMS, 1974b).

Trichoplusia ni, a serious pest of agriculture, is relatively inexpensive to rear (SHOREY and HALE, 1965) and its biology and behaviour have been extensively studied (MCEWEN and HERVEY, 1960; SHOREY *et al.*, 1962; IGNOFFO, 1963; SMILOWITZ and SMITH, 1970). *Trichoplusia ni* is thus a promising subject for examining endocrine-behaviour interactions. However, little emphasis has been placed on the endocrinology or timing of larval development. Therefore, the timing of selected physiological events during the last instar has been examined and the information used to closely monitor esterase activity using JH I, JH III, and α-naphthyl acetate as substrates. A thorough search of the literature indicates that esterase activity has not yet been monitored using all three substrates on the same closely timed insects in the same laboratory. These conditions are important to make valid comparisons of esterase activity and/or endocrine events.

MATERIALS AND METHODS

Insects

Trichoplusia ni larvae were reared on an artificial diet at 27 ± 2°C with a photoperiod of 14L:10D (SHOREY and HALE, 1965). About 11 hr 'after lights on' (ALO), the fourth instar larvae (day 2: L4D2) that were ready moult to the fifth instar (day 0: L5D0) were removed from the stock culture and placed in individual 1 oz plastic cups which contained about 7.5 g of slightly modified media (the amount of agar was doubled to reduce mortality). Readiness of the fourth instar larvae to moult to the fifth instar is indicated by clearing of the gut, migration to the top of the rearing

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container, slippage of the head capsule and darkening of the mandibles. These larvae were kept under the same conditions as the stock culture.

Larval staging

The time of moult to the fifth instar and the weight to the nearest mg were recorded at selected intervals during the first 4 days. During this time physical and behavioural changes including cessation of feeding, colour change, migration (wandering), spinning and time of pupation were also noted.

Time of ecdysone release

Day three last (fifth) instar (L5D3) larvae were ligated just behind the thorax, every hour from 4 hr ALO to 12 hr ALO with unwaxed dental floss. Two days later the larvae were examined and those showing a response of 1 or more on a 0–3 point scale at each interval were recorded. The scale is as follows: 0—no sclerotization; 1—a single sclerotized band on dorsum of sixth abdominal segment; 2—2 bands or a patch of tanned cuticle on the dorsum, not covering more than two segments; 3—3 or more segments with tanned cuticle.

The data were analyzed on a response/no response basis by Probit Analysis (FINNEY, 1947) on an IBM computer.

Collection of haemolymph

Haemolymph was removed from the larvae by clipping one of the anal prolegs. The haemolymph from several larvae (15–20 for early fifth instar; 4–7 for late fifth instar) was collected in a culture tube (6 × 50 mm) at 4°C. From this pooled sample, 50 μl was withdrawn shortly after collection, using a 25-μl glass pipette and diluted 1:10 in 4°C 0.1 M sodium phosphate buffer pH 7.4 containing 10 mg of phenylthiourea (PTU) per 100 ml to inhibit tyrosinases. Pupae were bled by piercing the intersegmental line at the top of the head and the haemolymph was collected and treated as for the larvae.

JH esterase assays

JH I esterase activity was monitored by the method of HAMMOCK and SPARKS (1977) using chain labelled JH I (~10 Ci/mmole; ^{3}H at C-10, New England Nuclear Corp.) and cold Ayerst JH I (17% t, t, c). JH I in 1 μl of ethanol (to give a final concentration of 5×10^{-6} M) was added to a 100-μl sample of the diluted haemolymph (1:1000 in the phosphate buffer) in a siliclad- or carbowax-treated (to prevent JH I from binding to the glass) culture tube (10 × 75 mm). The substrate concentration utilized is well below the critical micelle concentration of JH I. This haemolymph concentration results in hydrolysis rates which are linear for the duration of the assay and which approach the maximal velocity. After incubating for 10 min at 30°C (unless otherwise noted), 50 μl of a methanol solution (10 ml reagent grade methanol, 9 ml distilled water and 1 ml of concentrated ammonium hydroxide) and 250 μl of isooctane were added. The tubes were vortexed and then centrifuged at 4°C for 10 min at 3000 *g*. JH I is in the organic phase, while JH acid is in the aqueous phase, so a 100-μl aliquot from each layer was removed and analyzed by liquid scintillation counting (lsc) to determine the amount of JH acid formed.

Esterase activity on JH III was assayed using methoxy labelled JH III (SANBURG *et al.*, 1975a; HAMMOCK *et al.*, 1977) and cold JH III (>95%, t, t). Labelled JH III in ethanol (1 μl) was added (to give a final concentration of 5×10^{-6} M) to 100 μl of diluted haemolymph (1:100 in buffer) in a culture tube (6 × 50 mm). This subtrate concentration was chosen to allow direct comparison of esterase action on JH I and JH III. After incubation for 15 min at 30°C (unless otherwise noted), 100 μl of a charcoal–dextran suspension (600 mg Norit A, 800 mg Dextran T-40, 0.5 ml glacial acetic acid, to 10 ml with distilled water) was added, vortexed, allowed to stand 10 min at 4°C and then centrifuged to precipitate the charcoal. The intact ester is adsorbed to the charcoal while [^{3}H]-methanol resulting from ester cleavage remains in solution. An aliquot of the supernatant (100 μl) was monitored by lsc. The concentration of 3×10^{-6} M and the apparent K_d's of *T. ni* binding protein of 4.2×10^{-7} M and 3×10^{-6} M for JH I and JH III, respectively (HAMMOCK *et al.*, 1977), indicate that the binding protein will have no effect on initial rates of JH hydrolysis at the substrate concentrations and haemolymph dilutions used. When diisopropyl phosphorofluoridate (DFP) was used with either of the JH esterase assays, it was added in 1 μl of ethanol, to give a final concentration of 1×10^{-3} M, and allowed to preincubate with the haemolymph solution (100 μl) for 10 min at 30°C before the JH substrate was added. Ethanol at these levels was not observed to affect the hydrolysis rate.

α-Naphthyl acetate esterase and protein assays

Esterase activity on α-naphthyl acetate (α-NA) was monitored as follows: 1 ml of 2.5×10^{-4} M α-NA was added to 100 μl of diluted haemolymph (1:10 in buffer) in a 13 × 100 mm test tube, and allowed to incubate at 30°C for 10 min. To stop the reaction, 500 μl of a solution containing 0.4% *o*-dianisidine diazotate and 3.4% sodium dodecyl sulphate in distilled water, was added, vortexed and allowed to stand at room temperature at least 10 min. Sodium dodecyl sulphate was found to quench the reaction immediately. This reaction mixture was then diluted to 3.0 ml with 0.05 M pH 7.2 phosphate buffer and absorbance read at 600 nm (Beckman Model 25) against reagent or enzyme blanks. The effects of DFP inhibition were determined by incubating the 1-μl of DFP in ethanol with 100 μl of the diluted haemolymph (to give a final concentration of 1×10^{-3} M) at 30°C for 10 min, then adding the α-NA and continuing as above. Haemolymph protein concentration was determined by the method of LOWRY *et al.* (1951), with and without precipitation by 25% trichloroacetic acid, absorbance at 260 and 280 nm (LAYNE, 1957) and fluorometry using fluorescamine (BÖHLEN *et al.*, 1973) with the standard curve generated from bovine serum albumin in each case.

Esterase and protein studies during development

Larvae were reared as described above and esterase activity on JH I, JH III, and α-NA as well as protein content of the haemolymph were monitored in synchronous gate I (GI) larvae. The optimal assay

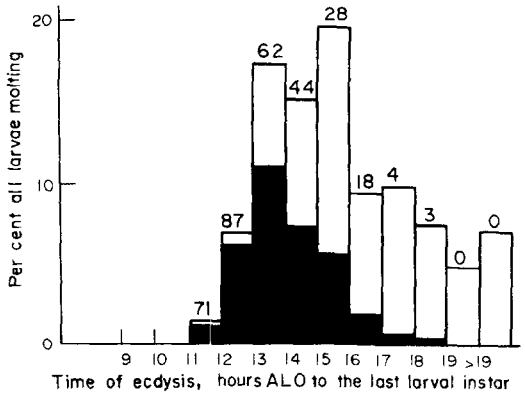

Fig. 1. Relationship between time of ecdysis to the last instar and the proportion of gate I larvae. Each bar represents the percentage of all larvae examined which moult between the specified times, while the shaded area of the bar represents only the gate I larvae. The number above each bar indicates the percentage of gate I larvae of only those larvae which moult during the hour indicated. Lights off at 14 hr ALO, $n = 529$.

conditions for JH I and JH III esterase activity were determined using haemolymph collected at 0600 hr L5D2 by investigating the time dependence of ester cleavage at several haemolymph concentrations. JH III was additionally assayed in triplicate at three incubation times (10, 15 and 30 min) every 6 hr during development (indicated below) to verify that the incubation conditions utilized gave linear rates through all of the larval stages examined. Assay conditions for α-NA esterase activity were similarly elucidated using haemolymph collected at 1200 hr L5D3. Assays were carried out every 6 hr; 0600 hr (1 hr ALO), 1200 hr (7 hr ALO), 1800 hr (13 hr ALO), and 2400 hr (19 hr ALO) as soon as the haemolymph was collected. The entire regime was repeated on three separate occasions, except for the early fourth instar which was only run once. The influence of 1×10^{-3} M DFP on JH I and JH III esterase activity was monitored during two separate developmental sequences as described above, while its influence on α-NA esterase activity was monitored during one sequence. Further assays with DFP were run on the individual JH and α-NA esterase peaks. For every time point in each developmental sequence the JH I assay was run in duplicate and the JH III assay in triplicate.

RESULTS

Preliminary observations on *T. ni* indicated a wide range in the number of last instar larvae pupating in 4 days (GI) vs 5 (gate II: GII) or more days. Last instar larvae selected and reared as described in the methods contained between 10 and 75% larvae that pupated in 4 days (GI) vs 5 (GII) days. During the time these studies were in progress, the GI larvae accounted for about 35% of the total population. GI larvae can be distinguished from GII larvae on L5D3 and L5D4 by physical and behavioural characteristics alone. Early on L5D3 (3 hr ALO) the larvae are entering the wandering stage. They have ceased feeding, cleared their guts, lost their stripes, and have become rotund and duller in colour. By the end of L5D3 (13 hr ALO) GI larvae have migrated to the top of their containers and started spinning. On the morning of L5D4 (3 hr ALO) the larvae have finished spinning, become smaller, and the segments are more pronounced. On the afternoon of L5D4, these larvae will pupate. During the first 2 days, however, no good physical criteria could be found to distinguish GI from GII larvae.

Time of moult vs gate

The first larvae that moult from the fourth to the fifth instar are more likely to be GI than GII larvae (Fig. 1). Selection of larvae that moult on or before 14 hr ALO will result in about 60% of the larvae being GI (Fig. 1). Larger proportions of GI larvae can be obtained by selecting larvae that moult at a time earlier than 14 hr ALO; however, this greatly reduces the number of larvae usable for experimental purposes (Fig. 1).

Weight vs gate

Larvae were weighed at 4 and 13 hr ALO on 4 successive days starting with L5DO. At each weighing period the heavier larvae contained a higher proportion of GI vs GII larvae. Unfortunately, very high ratios of GI to GII larvae, during L5D0 and L5D1, can only be obtained by using the heaviest larvae available which constitute only a small number of the total present (Fig. 2). As the larvae approach L5D2 higher GI to GII ratios can be obtained while still providing enough larvae to be of use (Fig. 2). GI larvae are heavier than GII larvae and the difference between their average weights increases with time (Fig. 3). Selecting larvae at specified times (4 and 13 hr ALO) and using only those that weigh at least the average weight of the GI's results in 55% GI larvae on L5D0, 75% on L5D1, and 85% on L5D2 (Fig. 3).

Time of ecdysone release and pupation

The results of ligations at selected intervals on L5D3 GI larvae indicate that the T_{50} (the ligation time at which 50% of the larvae respond) for ecdysone release is 6.5 hr ALO (Fig. 4). This time of ecdysone release agrees with the results of SMILOWITZ (1974). The use of ligation to monitor ecdysone release monitors a biological response which is composed of numerous biochemical events including ecdysone release, activation, distribution, and action. Pupation in GI larvae occurs on day 4. The time for 50% of the GI larvae to pupate (T_{50}) is 8.1 hr ALO (Fig. 4).

JH esterase activity during the last instar of gate I larvae

Ester hydrolysis of JH I was linearly dependent upon the concentration of haemolymph between 0.02 and 0.2% while the same was true of JH III between 0.1 and 1.3%. Under the assay conditions utilized, ester hydrolysis of JH I was linear for 20 min with 0600 hr L5D2 haemolymph, while JH III hydrolysis was linear for 15 min. The patterns for haemolymph esterase activity on JH I and JH III are very similar. For both substrates esterase activity peaked early on L5D2 (1 hr ALO) and early on L5D4 (1 hr ALO) (Fig. 5). Two smaller esterase peaks are also present. One occurs at the approximate time of the moult to the last instar

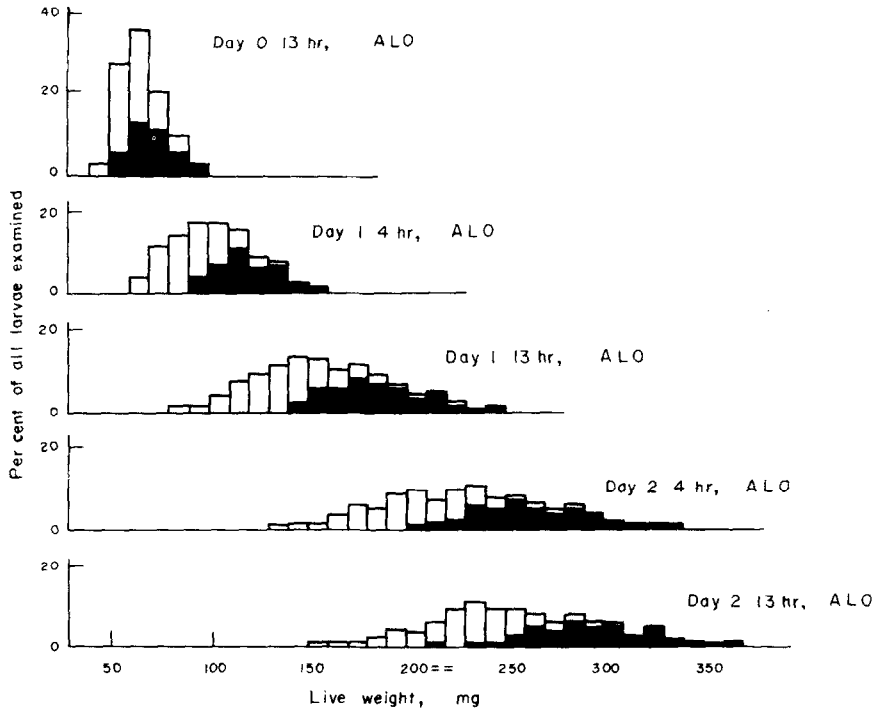

Fig. 2. Relationship between larval weight and the proportion of gate I last instar larvae. Each graph indicates a population of larvae weighed at the time indicated. Each bar represents the percentage of all larvae examined which had the specified weight range, while the shaded area of the bar represents only gate I larvae. For day 0 and day 1, 4 hr ALO, $n = 170$; for all others $n = 277$.

(L5D0: 13 ALO) and the other occurs at 1 ALO in 1-day-old pupae (Fig. 5). JH I appears to be a better substrate than JH III for the esterases present during the last instar of *T. ni* as indicated by the almost 15-fold greater rate of hydrolysis of JH I vs JH III (Fig. 5). No difference is found in the JHE activity between males and females for either of the major JHE peaks. DFP (10^{-3} M) has little or no effect on the esterase activity (< 30% inhibition) with either JH I or JH III as substrate.

α-NA esterase activity and haemolymph protein content

The esterase pattern using α-NA as substrate is different than the JH esterase patterns. Two peaks are present, one during the middle of L5D3 and the other early on L5D4 (Fig. 5). The second α-NA esterase peak appears to coincide with the second JH esterase peak. DFP (10^{-3} M) caused strong inhibition of α-NA esterase activity at all time periods assayed (> 80% inhibition with 1200 hr L5D3 haemolymph).

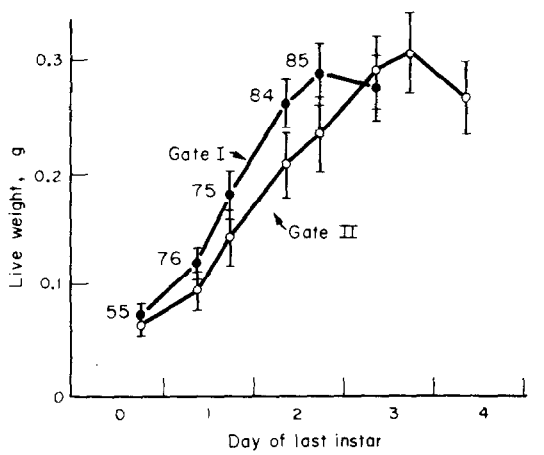

Fig. 3. Average weights of gate I (●) vs gate II (○) larvae during the last larval instar. Numbers next to the data points are the percent gate I larvae of all larvae at that weight or above. Points are means ± S.D. For each time >200 larvae were examined of which 35% were gate I.

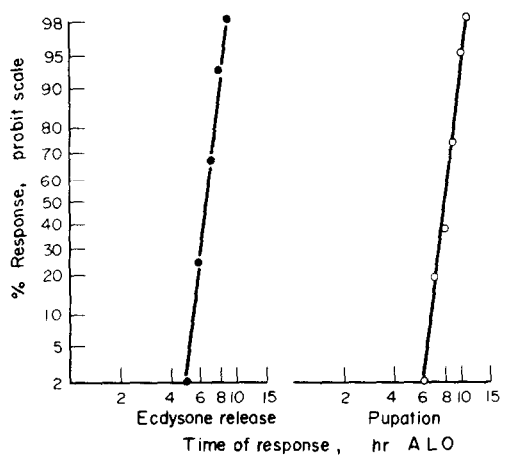

Fig. 4. Time of ecdysone release in L5D3 (●) and pupation in L5D4 (○) gate I last instar larvae. $n = 105$ and 100, respectively. T_{50} for ecdysone release is 6.5 hr ALO with upper and lower fiducial limits of 7.0 and 6.0 hr, and the T_{95} is 8.3 hr with limits of 7.6 and 10.0 hr. The T_{50} for pupation is 8.1 hr with limits of 7.5 and 8.7 hr, and the T_{95} is 10.5 hr with limits of 9.6 and 12.7 hr.

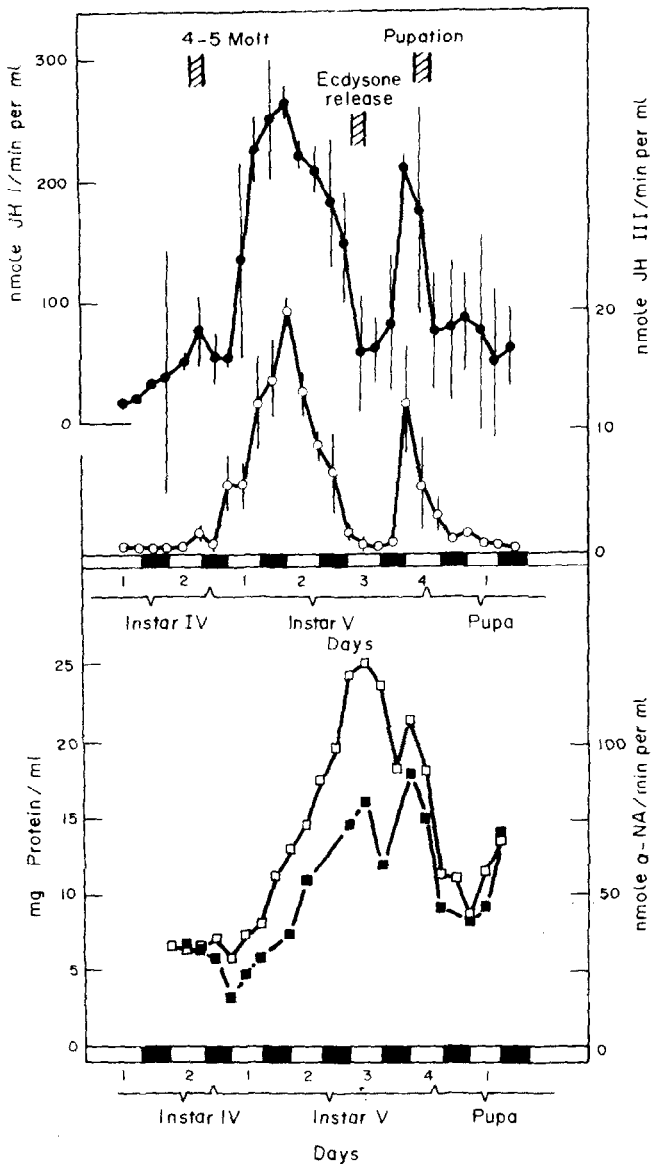

Fig. 5. Haemolymph esterase and protein titres during the last instar of gate I larvae: JH I (●), JH III (○), α-NA (■) and protein content (□). Shaded areas on abscissa represent lights off. For JH I and JH III points are means ± S.D. utilizing a single substrate concentration of 5×10^{-6} M.

The pattern of haemolymph protein content is very similar to the pattern of α-NA esterase activity (Fig. 5). Although the protein pattern does not change, the amount of protein present in the haemolymph varies considerably depending on the type of assay used, possibly due to interference by non-proteinaceous material in the haemolymph. Protein content on mid-day 3 larvae was about 200 mg/ml as monitored by absorbance at 260 and 280 nm; 164 mg/ml using fluorescamine; 60 mg/ml using Lowry's without precipitation and 24 mg/ml following precipitation by trichloroacetic acid and resolubilization. This last figure agrees most closely with the results of SMILOWITZ (1971), and this method was used to generate the data presented in Fig. 5. Centrifugation of the haemolymph samples at 10,000 *g* for 15 min before assaying for protein, α-NA esterase, or JHE did not affect the results.

DISCUSSION

Close synchronization of the last instar larvae of *T. ni* was considered a necessary prerequisite for the JHE activity patterns to be accurately monitored. A similar approach was taken in the examination of JHE in last instar *M. sexta* (VINCE and GILBERT, 1977). Synchronization of the last instar larvae involves optimization of rearing conditions and selection of groups of individuals that develop at similar rates. *Trichoplusia ni* as reared by the method of SHOREY and HALE (1965) has five larval instars, while four to seven instars have been reported for *T. ni* under different rearing regimes (MCKINNEY, 1944; SHOREY *et al.*, 1962). In the stock *T. ni* culture, larvae started from eggs at the same time will pupate over a period lasting about 1 week. Studies by VAN VORHIS KEY *et al.* (1978) indicate that larvae reared individually develop faster

and with less mortality than larvae reared at high densities (~30/cup). Therefore, rearing the larvae individually and selecting larvae preparing to moult to the last instar, as described in the Materials and Methods, results in larvae that will pupate in the early afternoon of either L5D4 (GI) or L5D5 (GII).

Criteria are needed for separating GI and GII larvae at an early stage. The weight of the larvae, as well as physical and behavioural characteristics have been used to determine the gate of last instar *M. sexta* (TRUMAN, 1972; NIJHOUT and WILLIAMS, 1974b). For *T. ni* the gate can be determined by physical characteristics alone after early L5D3. Selection of GI larvae before L5D3 requires the use of the time of moult and/or weight to bias the individual larvae selected for use toward GI or GII. Use of larvae moulting to the last instar early in the evening (11–14 hr ALO) results in higher proportions of GI larvae than are obtained in a normal population ($\geq 60\%$ vs $\leq 40\%$). The use of only the heaviest larvae during the first 2 days of the last instar also results in higher proportions of GI larvae ($\geq 75\%$). For this study the most efficient selection method in terms of number of usable insects and confidence in gate involved using larvae that weighed at least the average weight of GI larvae. The accuracy of this method is time dependent since the proportion of GI to GII larvae selected by this method increases with time. Although selection of larvae that were 100% L5D1 GI larvae was considered impractical in this study, proportions of GI larvae (75% at worst; L5D1) were high enough to allow consistent results. Subsequent studies on JHE titres in GII larvae have shown that JHE titres are practically identical in GI and GII larvae on L5D1 (SPARKS and HAMMOCK, unpublished observations). Other methods based on physical characteristics have been used to closely follow the development in *T. ni* (SMILOWITZ, 1973). Such methods were not applicable to this study because no indications of the gate or the length of each developmental phase were given.

The JHE activity pattern for the last instar of *T. ni* is similar to that reported for *M. sexta* (WEIRICH *et al.*, 1973; VINCE and GILBERT, 1977). Both *T. ni* and *M. sexta* display two major JHE peaks. The first and largest peak occurs just before the wandering stage and ecdysone release while the second smaller peak occurs immediately before pupation. Results of multiple assays near the times of peak JHE activity indicate that the second peak may be of much higher activity and of shorter duration than indicated in Fig. 5. Clarification of this observation awaits better methods of timing the insects' development. There is little if any difference in the JHE activity at the two major peaks between male and female larvae. The two JHE peaks in *T. ni* occur near times that correspond to reported decreases in the JH titre in other lepidopterans (VARJAS *et al.*, 1976; YAGI, 1976; YAGI and KURAMOCHI, 1976).

The JHE activity patterns in *T. ni*, as monitored by using JH I and JH III as substrates, are very similar, indicating that the two JH's are degraded by similar if not identical enzymes or enzymes regulated by similar mechanisms. JH I is a much better substrate for these enzymes than JH III as indicated by the more than 15-fold greater rate of JH I hydrolysis. These results are different from those obtained from the cockroach *Blaberus giganteus* for which the rates of JH I and JH III hydrolysis were nearly the same (HAMMOCK *et al.*, 1977).

The higher affinity of the binding protein (HAMMOCK *et al.*, 1977) and higher activity of JHE for JH I than JH III may correlate with JH I being a larval hormone as has been found for *M. sexta* (SCHOOLEY *et al.*, 1976; DAHM *et al.*, 1976). In light of our previous studies on the JH esterases of *Blaberus giganteus*, it is interesting that JH III is reported to predominate in the related species *Blaberus discoidalis* (DAHM *et al.*, 1976). It is thus likely that esterases can interact with binding proteins to vary the ratios of the three JH's. Such an interaction may help to explain the discrepancies between JH ratios found in corpora allata *in vitro* and ratios found *in vivo* in the haemolymph (DAHM *et al.*, 1976; SCHOOLEY *et al.*, 1976). It was earlier demonstrated under assay conditions which considered the influence of JH haemolymph binding protein, that the higher affinity of the JH binding protein in *T. ni* haemolymph for JH I over JH III led to much more rapid metabolism of JH III than JH I (HAMMOCK, unpublished observations) in mid-fourth instar *T. ni* haemolymph. The assay conditions used in this paper negate the influence of binding protein, and these conditions are biologically valid if the studies by SANBURG *et al.* (1975a, b) on *M. sexta* JH esterase-binding protein interactions hold true for *T. ni*. From previous results on *M. sexta*, one expects the binding protein to have little if any influence on direct JHE activity in the fifth instar since the JHE degrade JH rapidly regardless of the presence of binding protein. If JH III is present *in vivo* these studies therefore predict that it will be degraded more slowly than JH I even in undiluted fifth instar haemolymph.

There were two smaller JHE peaks additionally observed in this study, which apparently occur outside the range covered by previous reports (WEIRICH *et al.*, 1973; VINCE and GILBERT, 1977). The first of these additional peaks occurs at the time of the larval fourth to fifth instar moult (L5D0). In *M. sexta* a peak in the JH titre has been observed at this time (FAIN and RIDDIFORD, 1975), which suggests that JHE activity may be responsible for the hydrolysis of this small burst of JH.

The reproducible variations in protein measurements by four different accepted techniques of analysis well illustrate that all of these techniques are based upon the environment and properties of specific amino acids and each technique has its intrinsic sources of error. The α-NA activity and protein content patterns are somewhat similar in the timing of their peaks of activity. Both demonstrate a large peak during the middle of L5D3, about the time of ecdysone release. A second peak occurs on L5D4 and appears to coincide with the second large JHE peak. These results are different from those reported for *in vitro* assays on *M. sexta* (WEIRICH *et al.*, 1973; NOWOCK and GILBERT, 1976). These two groups found that the α-NA activity followed the same activity pattern as the first JHE peak. Conversely one of these groups (NOWOCK and GILBERT, 1976) also reported a difference in the α-NA and JHE patterns in isolated fatbody cultures.

JHE activity for both JH I and JH III was only slightly inhibited by 10^{-3} M DFP whereas the α-NA

activity was strongly inhibited. Similar selective inhibition has also been observed in other insects (SANBURG *et al.*, 1975a, b; KRAMER and DE KORT, 1976; HAMMOCK *et al.*, 1977; KRAMER and CHILDS, 1977). The different activity patterns between α-NA and JHE, especially on L5D3 when α-NA activity is highest and JHE activity is lowest, indicate that different enzymes are involved. Studies thus far indicate that α-NA hydrolysis is an unreliable indicator of JHE in *T. ni* as it is in other insects (KRAMER and DE KORT, 1976; HAMMOCK *et al.*, 1977). The presence of DFP causes little change in the *T. ni* JHE activity profiles. These studies indicate that 'general' esterases are relatively unimportant in JH hydrolysis compared to JH specific esterases during the developmental stages of *T. ni* studied. In contrast to *T. ni* KRAMER and CHILDS (1977) report that 50% of the JHE activity in a haemolymph fraction of *Plodia interpunctella* is inhibited by DFP. VINCE and GILBERT (1977) report on JHE in only DFP treated haemolymph of *M. sexta*. The definitions of 'general' and 'specific' JH esterases based on relative enzyme activity on JH and α-NA, enzyme activity in the presence of binding protein, and differential inhibition by DFP (SANBURG *et al.*, 1975a, b) are valuable in the context originally used, but, as in this study, their piecemeal application to other insects and developmental stages should be very cautious.

Numerous studies have suggested the involvement of JHE's and other enzymes in JH regulation based on a correlation with enzyme activity and a supposed drop in hormone titre (WEIRICH *et al.*, 1973; NIJHOUT, 1975; SANBURG *et al.*, 1975a, b; NOWOCK and GILBERT, 1976; VINCE and GILBERT, 1977), and this study is no exception. A more reasonable assumption is that, like all hormones, JH titre is determined by a dynamic equilibrium between synthesis and degradation and that this equilibrium is shifted toward lower JH titres as JH biosynthesis is halted (WILLIAMS, 1961).

In *M. sexta* (and possibly *T. ni*) it appears that the major peak of JHE activity in the mid-last instar may occur after JH titres have fallen to low levels (NIJHOUT, 1975; FAIN, personal communication; VINCE and GILBERT, 1977), although there is still some question about the timing of these events since the experiments were carried out in different laboratories. NIJHOUT (1975) thus questions the role of JH esterases in the maintenance of JH titres. After the moult of *M. sexta* from the fourth to the fifth instar the haemolymph JH titre rapidly falls below levels that can currently be detected, and this rate of reduction in JH titre must be concentration-dependent. Therefore, the rate will be greatly reduced as the titre falls below experimentally detectable concentrations. The JH specific binding protein (KRAMER *et al.*, 1974; HAMMOCK *et al.*, 1975; SANBURG *et al.*, 1975a, b; NOWOCK *et al.*, 1976; KRAMER and CHILDS, 1977) has been implicated as vital to the preservation of JH in solution. During the fifth instar it may have an additional role in conjunction with JHE in eliminating trace levels of JH still in the tissues. The relative JH concentration in the haemolymph and tissues is determined by the law of mass action cognizant of the fact that JH distribution is dependent upon differential solubility (JH is highly soluble in lipid depots) and both moderate and high affinity tissue binding sites. As JH titres drop, the contribution of binding sites to the total distribution of JH becomes much larger; so to remove the last traces of JH the insect needs to increase the rate of JH metabolism in target tissues and to shift the JH equilibrium as much as possible from tissue binding sites and lipid depots to the haemolymph. According to the model presented here, it is at this stage of development that insects use the interaction of a moderate affinity binding protein and a JHE with affinities allowing degradation of JH on the binding protein. These proteins thus work together by mass action to essentially pull JH residues from tissues. Data presented by NOWOCK *et al.* (1976) on JH efflux from the fatbody of *M. sexta* under *in vitro* conditions, preliminary studies by KRAMER and CHILDS (1977) indicating that JH binding protein levels increase during the last larval instar of *P. interpunctella*, and JHE levels reported here and by VINCE and GILBERT (1977) support this model.

Positive elucidation of the *in vivo* role of haemolymph JHE in insect development will require an understanding of the *in vivo* kinetics of JH distribution. This approach will require input from a number of disciplines allowing rapid assay of *in vivo* JH titres and specific inhibition of biosynthetic and degradative pathways.

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