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DEVELOPMENTAL ENDOCRINOLOGY OF LARVAL MOULTING IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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SUMMARY

A larval moult in the tobacco hornworm, *Manduca sexta*, involves an endocrine cascade that begins with the release of a cerebral peptide hormone, the prothoracicotropic hormone (PTTH). The release of PTTH is gated, occurs during the scotophase and appears to be developmentally cued. In fourth instar *Manduca* larvae, PTTH release into the haemolymph occurs as a single burst over a few hours during the head critical period, i.e. the time during which the head (brain) is needed for the initiation of the moult to the fifth (last) instar. Released PTTH activates the prothoracic glands (PGs), and within a few hours the cumulative effect of this event results in a dramatic increase in the haemolymph ecdysteroid titre, which then elicits the moult. An assessment of the capacity of the corpora allata (CA) to synthesize juvenile hormone (JH) *in vitro* indicates that the above sequence of endocrine events begins only when JH synthesis has reached a nadir for the instar. Since CA activity is an indirect measure of the haemolymph titre of the hormone, it is conceivable that the developmentally cued release of PTTH is permissively controlled by a decreasing haemolymph titre of JH. With the increase in the ecdysteroid titre which marks the end of this endocrine cascade, the CA again become active, presumably to cause the increase in the JH haemolymph titre which directs the larval moult. This investigation has thus established the temporal and quantitative dynamics of the PTTH–PG axis that drive larval moulting and provides insight into the inter-endocrine regulatory relationships that may exist between the ecdysteroids and JHs. These possible relationships and the role of the brain in their regulation are discussed.

INTRODUCTION

For over half a century, research on moulting and metamorphosis in insects has centred on the demonstration that these developmental phenomena are regulated by the dynamic interactions and actions of three hormones: the prothoracicotropic hormone (PTTH), ecdysone and juvenile hormone (JH) (see Granger &

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Bollenbacher, 1981). From this research a general regulatory scheme has evolved, according to which, at precise times during development, the cerebral peptide PTTH is released to activate the prothoracic glands (PGs) to synthesize/secrete ecdysone. Ecdysone is then hydroxylated to 20-hydroxyecdysone, which elicits a moult. The nature of the moult is then determined by the JH titre at a critical time prior to PTTH release (Riddiford, 1980; Granger & Bollenbacher, 1981).

Advanced techniques for measuring and identifying these developmental hormones have provided information indicating that the endocrine control of a moult, regardless of whether it is larval or pupal, involves precise fluctuations in their circulating titres. This is achieved by developmentally precise changes in the biosynthesis, release and catabolism of the hormones (see Feyereisen, 1985; Hammock, 1985; Smith, 1985; Koolman & Karlson, 1985; Bollenbacher & Granger, 1985). This information also suggests that different homologues of JH, different ecdysteroids, and possibly even different PTTHs, may be involved (Granger & Bollenbacher, 1981; Bollenbacher *et al.* 1984*b*). Adding to these qualitative and quantitative dimensions of endocrine control are the proposed roles of secondary effectors of these hormones and of interendocrine regulation (Nijhout & Williams, 1974; Chippendale, 1977; Whisenton *et al.* 1985; Whisenton, Granger & Bollenbacher, 1986; Watson *et al.* 1985; Watson, Whisenton, Bollenbacher & Granger, 1986; Rountree & Bollenbacher, 1986). These, together with temporal factors affecting the duration of hormone synthesis/release, the mode of release (continuously or in bursts) and the half-lives of the hormones, would direct development.

Although aspects of the synthesis, release, titres and degradation of the ecdysteroids, JHs and PTTH have been investigated in many insect species (see Kerkut & Gilbert, 1985), the relationships between these hormones in temporal, quantitative and qualitative terms have not been assessed in a single insect species. Such information is critical to the eventual understanding of the interhormonal dynamics that direct moulting and metamorphosis.

This report is the first of two that investigate temporal relationships in the synthesis, release and titres of the ecdysteroids, JHs and PTTH during moulting and metamorphosis in a holometabolous insect, the tobacco hornworm, *Manduca sexta*. In this report, the endocrinology of the moult from the fourth to the fifth larval instars is described. The findings of this study provide considerable information about the interrelationships of these hormones in driving the larval moulting process.

MATERIALS AND METHODS

Experimental animals

Larvae of the tobacco hornworm, *Manduca sexta*, were reared on an artificial diet (Bell & Joachim, 1976) at 26°C and 70% relative humidity under non-diapause photoperiodic conditions (L:D 16:18). Fourth instar larvae were selected at the time of the moult from the third instar and were gated by weight (Truman, 1972; W. E. Bollenbacher & N. A. Granger, unpublished results). Only gate I larvae were

used for this study because their development was more synchronous than that of gate II or gate III larvae. The moult to the fourth larval instar in a synchronous population occurs at approx. 14.00 h eastern standard time (EST), 8 h into the photophase, and this point is thus designated day 0 (0 h) of the fourth instar.

Juvenile hormones and ecdysteroids

Juvenile hormone I (JH I) and juvenile hormone III (JH III) were obtained from CalBiochem (La Jolla, CA) and [10-^{3}H(N)]JH I (11·3 Ci mmol^{-1}) and [10-^{3}H(N)] JH III (11·6 Ci mmol^{-1}) were purchased from New England Nuclear Corporation (Wilmington, DL). Ecdysone was a generous gift from Dr D. H. S. Horn (CSIRO, Melbourne, Australia) and 20-hydroxyecdysone was obtained from CalBiochem. [23,24-^{3}H]Ecdysone (57 Ci mmol^{-1}) was obtained from New England Nuclear Corporation. The purities of the JHs and ecdysteroids, determined by thin-layer chromatography (Vince & Gilbert, 1977; Smith *et al.* 1979), were greater than 96 % and 98 %, respectively.

Ecdysteroid and juvenile hormone radioimmunoassays

The D-10 and H-3 antisera used in the ecdysteroid radioimmunoassay (RIA) were generated against carboxymethoxyamine (Borst & O'Connor, 1972) and 22-hemisuccinate (Gilbert, Goodman & Bollenbacher, 1977) derivatives of ecdysone, respectively. The D-10 antiserum binds to ecdysone and 20-hydroxyecdysone with equal affinity, while the H-3 antiserum binds ecdysone with an affinity approximately four times greater than that for 20-hydroxyecdysone.

Two ecdysteroid RIAs were used, a macro-RIA and a micro-RIA (Bollenbacher, O'Brien, Katahira & Gilbert, 1983). The macro-RIA, which utilizes the H-3 antiserum and [^{3}H]ecdysone at 4·0 Ci mmol^{-1} as the labelled ligand, has a standard curve ranging from 0·25 to 32 ng ecdysone, and this assay was used to quantify ecdysone biosynthesis by the PGs *in vitro*. The micro-RIA, which utilizes the D-10 antiserum and [^{3}H]ecdysone at 57 Ci mmol^{-1}, has a standard curve ranging from 0·01 to 4·0 ng ecdysone, and this assay was used to quantify ecdysteroids in the haemolymph. The protocols for both RIAs have been described previously (Bollenbacher *et al.* 1983; Warren, Smith & Gilbert, 1984).

Two JH RIAs were used to quantify JH I and JH III synthesis (see Granger *et al.* 1979; Granger, Niemiec, Gilbert & Bollenbacher, 1982*b*). The antisera to JH I and JH III (DB-195 and DB-3394, respectively) for these RIAs were generously provided by Dr F. Dray and Dr J.-C. Baehr (Pasteur Institute, Paris, France). The labelled ligand for the JH I RIA was [^{3}H]JH I and for the JH III RIA was [^{3}H]JH III. The protocols for both RIAs have been described previously (Granger *et al.* 1979, 1982*b*; Granger, Bollenbacher & Gilbert, 1981). The JH I RIA, with a lower limit of sensitivity of 50 pg JH I, has considerable specificity for this homologue, exhibiting only 12 % cross-reactivity with JH II and essentially none with JH III. The RIA for JH III has a high specificity for this homologue and essentially no affinity for JH I or JH II. The acids of JH I and JH III cross-react equally with their respective homologues in the RIA; thus the levels of both the JH

homologue and its acid are measured with these assays (Granger *et al.* 1979, 1981, 1982*b*).

Head critical period for PTTH release

From a population of staged, fourth instar larvae, individuals weighing between 0·7 and 0·9 g at 8 p.m. of day 1 were selected. These larvae were >98 % gate I. At 3-h intervals from the time the larvae were weighed, groups of 15 larvae were neck-ligated as described previously (Truman, 1972; Bollenbacher, Agui, Granger & Gilbert, 1979). The time and duration of PTTH release, i.e. the head critical period (HCP), was determined by scoring spiracle apolysis plus subsequent black larval moult in the ligated population (Fain & Riddiford, 1976; Gibbs & Riddiford, 1977). The bioassay was scored at the time when spiracle apolysis and larval moulting normally occurred in an untreated population of gate I, fourth instar larvae. Larvae ligated prior to PTTH release did not exhibit these biological responses, but instead eventually underwent gut purging and dorsal vessel exposure, markers indicative of a larval–pupal moult (Truman & Riddiford, 1974). When determined in this way, the HCP for PTTH release was defined as the period during which a linearly increasing percentage of ligated larvae exhibited spiracle apolysis and larval moulting.

In vitro *prothoracic gland assay for PTTH*

PTTH activity in haemolymph was detected and quantified with the *in vitro* prothoracic gland (PG) assay which has been described previously for the neurohormone (Bollenbacher *et al.* 1979, 1983, 1984*a*,*b*). Briefly, day 0 pupal PGs of *Manduca* were incubated in 0·025 ml standing drops of Grace's culture medium (GIBCO, Grand Island, NY) and phosphate buffer (0·05 mol l^{-1}, pH 6·8), 1:1 (v/v), or in medium containing an extract of haemolymph. After a 2-h incubation at 25 °C, the ecdysone synthesized by a gland was measured by the standard macro-ecdysone RIA of a 0·01-ml sample of medium (Bollenbacher *et al.* 1979, 1983). Since ecdysone is the only ecdysteroid synthesized by *Manduca* PGs, RIA activity was expressed in ng ecdysone PG^{-1} 2 h^{-1}. RIA data were analysed using an IBM-PC computer program for a log–logit transformation.

Extraction of PTTH from haemolymph

Haemolymph (2 ml) was collected by pooling equal samples from each of 30–40 larvae and was partially purified for PTTH according to the following procedure. Haemolymph was collected on ice and then immediately heated at 100 °C for 5 min. The sample was then centrifuged at 8000 ***g*** for 10 min to remove precipitated protein. The resulting supernatant was chromatographed on a Biogel P-10 column (10 cm × 1 cm) in a sodium phosphate buffer (0·05 mol l^{-1}, pH 6·8). For this separation, the P-10 column was calibrated with [^{3}H]ecdysone, blue dextran, insulin B-chain and PTTH from day 1 *Manduca* pupal brains. This step thus removed from the PTTH preparation any endogenous ecdysteroids that, because of their cross-reactivity in the ecdysone RIA, could have interfered with the measurement of PTTH by the *in vitro* PG assay. PTTH activity eluted between the blue dextran and the insulin B-chain at

a yield of approximately 90%, while the ecdysteroids eluted just after the insulin B-chain. In addition to removing ecdysteroids from the PTTH fraction, this protocol eliminated other low molecular weight haemolymph constituents. Next, the post-gel filtration PTTH fraction was concentrated by ultrafiltration to 0·25 ml in phosphate buffer, thus yielding an eight-fold concentration of the activity in the original haemolymph sample. This preparation was then assayed for PTTH with the *in vitro* PG assay and dose–response protocol, an approach yielding an ED_{50} value from which the PTTH activity in a sample can be measured in relative terms (Agui, Bollenbacher, Granger & Gilbert, 1980; Bollenbacher *et al.* 1984*a*,*b*; O'Brien *et al.* 1986). For the dose–response protocol, the PTTH extract was serially diluted with Grace's medium: phosphate buffer (1:1) to generate a range of concentrations from two to 0·125 times the original haemolymph volume. Each concentration was assayed in triplicate, and the presence of PTTH in an extract was demonstrated by its activation of ecdysone synthesis by the PG. Activation was expressed as an activation ratio (A_r), which is the quantity of ecdysone synthesized by an experimental gland (+ extract) divided by that synthesized by a control gland (+ Grace's medium: phosphate buffer, 1:1).

The relative amounts of PTTH in haemolymph extracts from different stages were determined from the reciprocals of the ED_{50} values for each extract, i.e. the amount of PTTH needed to activate a PG half-maximally (Agui *et al.* 1980; Bollenbacher *et al.* 1984*a*,*b*; O'Brien *et al.* 1986). PTTH activity measured by this procedure is expressed in PTTH units, with one unit (U) equalling the neurohormone activity in a day 1 pupal brain of *Manduca* and thus equal to the reciprocal of the ED_{50} value of 0·06 brain equivalents (16·7).

Prothoracic gland activity in vitro

The ecdysone biosynthetic activity of the PG during the fourth larval instar was assessed by determining, at different times, the capacity of glands to synthesize ecdysone *in vitro*. Pairs of larval PGs were dissected in lepidopteran saline (Weevers, 1966) and incubated *in vitro* in 0·05 ml of Grace's medium for 2 h at 25 °C. Ecdysone synthesis per gland pair was determined by micro-RIA of duplicate samples (0·01 ml) of the incubation medium.

Ecdysteroid titre

Haemolymph (0·05 ml) was taken from the dorsal horn of each of eight larvae, and each sample was extracted separately with methanol (0·95 ml). Following centrifugation of the methanolic extract at 8000 ***g*** for 10 min, the ecdysteroids in the resulting supernatant were measured in duplicate at three different concentrations by the micro-RIA (Bollenbacher, Smith, Goodman & Gilbert, 1981). Since in this assay 20-hydroxyecdysone was the unlabelled, standard ligand and [^{3}H]ecdysone was the labelled ligand, RIA activity was expressed in 20-hydroxyecdysone equivalents.

In vitro *incubation of brain–corpora cardiaca–corpora allata complexes*

Brain-corpora cardiaca-corpora allata complexes (Br–CC–CA) from fourth instar larvae were dissected in Grace's medium. Four Br–CC–CA were transferred to 0·21 ml standing drops of Marks 19AB medium (Granger & Borg, 1976) and maintained *in vitro* for 6 h at 25°C. This incubation medium contains macromolecules which, together with carbowax treatment of the incubation vessels, maintain JH in solution (>95 %) for up to 12 h (Granger & Borg, 1976; Granger *et al.* 1979). The amounts of JH I and JH III synthesized by the Br–CC–CA in an incubation were determined by JH I and JH III RIAs of duplicate 0·05-ml samples of the medium (Granger *et al.* 1979, 1981, 1982*a*,*b*). For each stage of the fourth instar, rates of JH I and JH III synthesis were assayed in 4–6 replicate incubations, and 3–4 such rate determinations were used to establish a mean rate of synthesis. Since the standard ligands for the respective RIAs were JH I and JH III, JH activity in an incubation was expressed as ng JH I or JH III equivalents complex^{-1} 6 h^{-1}.

RESULTS

Fourth instar head critical period for PTTH release

The head critical period (HCP) of an instar is the period when the brain becomes progressively unnecessary for a moult. This is presumed to be the time of PTTH release and thus the time of the interactions of juvenile hormone, ecdysteroids and PTTH which regulate a moult. Therefore, the first step in this study was to establish the HCP in fourth instar, gate I larvae. This was accomplished by neck-ligating larvae at approximately 3-h intervals from day 1 plus 6 h (8 p.m.) of the instar to about day 2, and scoring the occurrence of spiracle apolysis and larval moulting in each population of ligated larvae (see Materials and Methods). Larvae that did not moult remained as larvae, eventually exhibiting dorsal vessel exposure approximately 6–11 days later. The HCP for the fourth instar began at day 1 plus 10 h (12 a.m.) and ended just before day 1 plus 17 h (7 a.m.) (Fig. 1); during this time, the percentage of larval moults increased linearly. The 7 h duration of the HCP agreed well with that previously reported (Truman, 1972) for gate II larvae reared under short day conditions (L:D 12:12). However, the HCP occurred later during the scotophase (50 % response at about 3 a.m., which is 5 h after lights-out) than for gate II larvae (Truman, 1972; W. E. Bollenbacher, unpublished results). The period of linear (0–100 %) moulting response of the ligated larvae presumably reflected the time of PTTH release in the population, and the 100 % response at day 1 plus 17 h presumably represented the maximum haemolymph titre of the neurohormone. Since PTTH release occurred over a short time in a large experimental population (>200 larvae), its release in individual larvae probably occurs over only a few hours.

Determination of the HCP for the fourth larval instar established when the cascade of endocrine events leading to the larval moult began and thus when the inter-endocrine regulation of the moult would occur. Therefore, the period of development between day 1 plus 6 h (8 p.m.) and day 2 plus 4 h (6 p.m.) of the fourth instar was the focus of the rest of this study.

Titre of PTTH activity in the haemolymph

While it is generally accepted that the HCP represents the time of PTTH release into the haemolymph, this event has never been directly demonstrated. To do this, a haemolymph titre of PTTH activity was generated for the fourth instar HCP. For each time point, which corresponded to a certain larval weight within the 0·74–0·9 g range for the HCP, 3–4 haemolymph extracts were assayed for PTTH by the *in vitro* PG assay, using a dose–response protocol. A PTTH titre was derived from the $1/ED_{50}$ value for each dose–response curve of PG activation. Representative dose–response curves generated for different times before, during and after the HCP (Fig. 2) revealed apparently dramatic changes in the PTTH titre which corresponded temporally with the HCP. PTTH activity was detectable in the haemolymph by day 1 plus 10 h (0·74 g), and by day 1 plus 15 h (0·82 g) it had reached a level capable of maximally activating the PG. The titre then declined to a basal level by day 1 plus 22 h. Thus, sufficient PTTH activity to activate the PG *in situ* was apparently present in the haemolymph by day 1 plus 15 h, a time which coincided with the approximately 100 % moulting response for the HCP (Fig. 1).

Since these preliminary titre data indicated that haemolymph PTTH activity could be detected and measured, a complete titre of its activity (Fig. 3) was

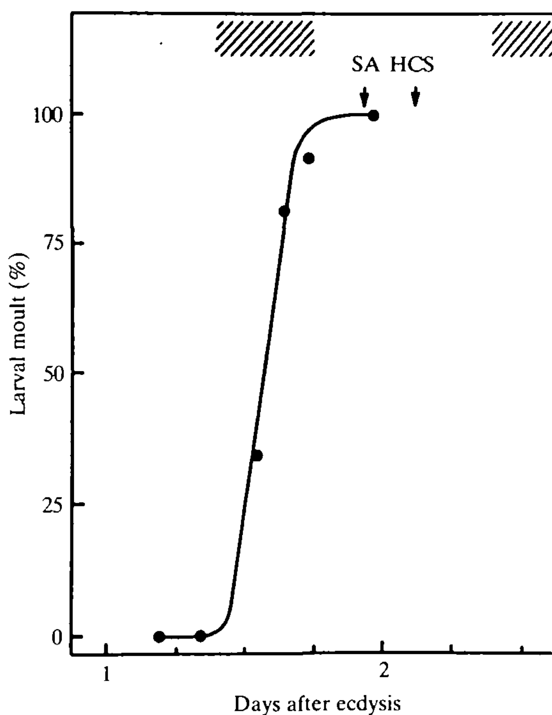

Fig. 1. Determination of the head critical period for prothoracicotropic hormone release during the fourth larval instar of *Manduca sexta*. Gate I larvae were used and the percentage moult denotes the percentage of individuals in a test population that exhibited spiracle apolysis (SA) after ligation at each time point. Hatched bars denote the scotophase of each day of the instar. HCS, head capsule slippage.

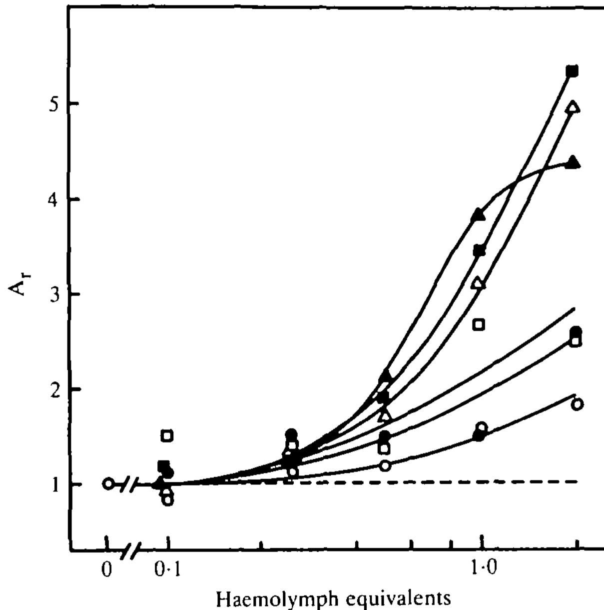

Fig. 2. Dose–response analysis of the prothoracicotropic hormone (PTTH) activity present in the haemolymph of gate I, fourth instar *Manduca* larvae before, during and after the head critical period for PTTH release. Haemolymph equivalents denote the PTTH activity present in haemolymph assuming 100% recovery of activity after partial purification. A_r denotes the activation ratio for ecdysone synthesis by the prothoracic glands in response to haemolymph extracts. The symbols show haemolymph from larvae at different times of the fourth instar: ○, day 1 plus 10 h; ●, day 1 plus 11 h; △, day 1 plus 13 h; ▲, day 1 plus 15 h; ■, day 1 plus 17 h; □, day 1 plus 20 h. The dashed line represents the PTTH activity in the haemolymph at times up to 6 h before, and more than 6 h after, the times given above.

generated. From day 1 plus 4 h to day 1 plus 9 h, PTTH activity was not detected in the haemolymph. By day 1 plus 11 h, however, approximately 0·04 U ml^{-1} haemolymph of activity was present, and by day 1 plus 15 h, the titre peaked at approximately 0·18 U ml^{-1} haemolymph, a time coincident with the HCP. Between this time and day 1 plus 22 h the titre dropped quickly to a negligible level and remained there until day 2 plus 9 h. The rise and fall of the titre exhibited remarkable symmetry, with about 5 h elapsing before the titre peaked, and a comparable amount of time passing before it returned to a basal level. This suggested that the release and catabolism of PTTH is under precise control and that the action of the neuropeptide would depend on the length of time its titre is above a threshold level. Since fourth instar Br–CC–CA possess approximately 0·2 U of PTTH (Agui *et al.* 1980; O'Brien *et al.* 1986) and since larvae at the HCP contain about 0·25 ml haemolymph, the amount of PTTH activity present in the haemolymph at the peak would represent about 25 % of that activity.

While it is likely that PTTH levels were being measured, it is possible that haemolymph ecdysteroids in the PTTH samples were contributing to this titre. This possibility was precluded by the fact that micro-ecdysteroid RIA of the haemolymph

extracts revealed the absence of any ecdysteroids. This result, together with the finding that partially purified PTTH from fourth instar haemolymph elicited a dose-dependent response in the *in situ* moulting bioassay for PTTH (Gibbs & Riddiford, 1977; M. A. O'Brien & W. E. Bollenbacher, unpublished results), provided convincing evidence that PTTH levels in the haemolymph were being measured and that the fluctuations observed really reflected PTTH release during the HCP.

In vitro *capacity of the PG to synthesize ecdysone*

In the endocrine cascade that elicits larval moulting, the event following PTTH release is its activation of the PGs. To confirm that PTTH release had actually occurred and to assess the dynamics of gland activation and of the decay of activation relative to the PTTH haemolymph titre, the biosynthetic capacity *in vitro* of fourth instar larval PGs was determined around the time of PTTH release. If PTTH release occurred between day 1 plus 9 h and day 1 plus 15 h, then the capacity of the PGs to synthesize ecdysone should increase substantially in response. The capacity of PGs to synthesize ecdysone increased substantially during the HCP (Fig. 4), from a basal

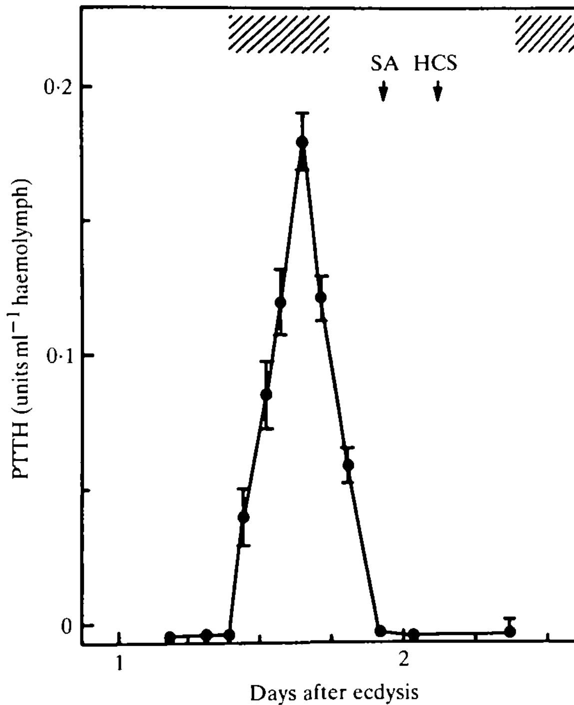

Fig. 3. Titre of prothoracicotropic hormone (PTTH) activity in the haemolymph of gate I, fourth instar *Manduca* larvae during the head critical period for this instar. PTTH activity was determined using the *in vitro* prothoracic gland assay for the neurohormone and PTTH activity is expressed in units, with one unit equal to the amount of hormone in a day 1 pupal brain of *Manduca*. Each point is the mean ± S.E.M. of 3–4 separate determinations. SA and HCS denote spiracle apolysis and head capsule slippage, respectively, and the hatched bars denote the scotophase of each day of the instar.

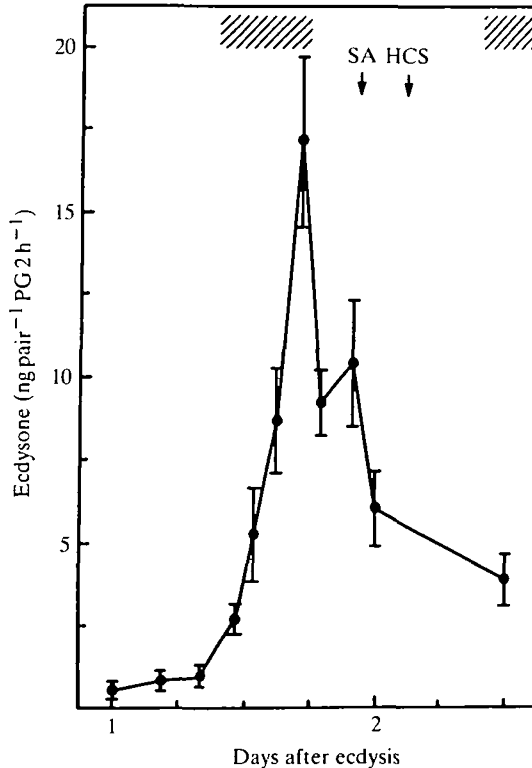

Fig. 4. The capacity of gate I, fourth instar *Manduca* prothoracic glands (PG) to biosynthesize ecdysone *in vitro* during days 1–2 of the instar. Each point is the mean ± S.E.M. of six separate determinations. SA and HCS denote spiracle apolysis and head capsule slippage, respectively, and the hatched bars denote the scotophase of each day of the instar.

level of approximately 1 ng $pair^{-1}$ 2 h^{-1} between day 1 and day 1 plus 8 h to a peak level of 17 ng $pair^{-1}$ 2 h^{-1} by day 1 plus 18 h. Synthesis levels then decreased to a stable intermediate level of about 5 ng $pair^{-1}$ 2 h^{-1} by day 1 plus 22 h. The increase in the biosynthetic capacity of the glands followed the increase in the PTTH haemolymph titre by 2 h. Considering that the threshold concentration of PTTH necessary to activate the PGs half-maximally is 0·064 units, the delay between PTTH release and PG activation would actually be less than 2 h. A tightly coupled temporal relationship between PTTH release and an increase of PG activity was expected since: (1) the neurohormone activates the glands by a mechanism involving Ca^{2+}-dependent cyclic AMP second messenger (Smith, Gilbert & Bollenbacher, 1985), and (2) delay is consistent with the kinetics of gland activation by PTTH *in vitro* (Bollenbacher *et al.* 1983). The subsequent decrease in PG activity occurred a few hours after the decrease in the PTTH titre, consistent with the kinetics of the decay of the PG response to PTTH *in vitro* (Bollenbacher *et al.* 1983). However, the biosynthetic capacity of the glands failed to return to pre-HCP levels, but instead levelled off at about 10 times higher, suggesting that other factors may be regulating

the glands. Regardless of the reason for the gradual drop in PG activity, the increased synthesis of ecdysone by the PGs *in vitro* indicated the glands were activated *in vivo* by PTTH released during the HCP.

Haemolymph ecdysteroid titre during the fourth larval instar

Activation of the PG by PTTH *in vivo* should result in an increase in the haemolymph ecdysteroid titre. This would be expected to follow PG activation by some hours, since the accumulation of a hormone is not only a function of an increased rate of synthesis but is also affected by changes in its excretion, sequestration and metabolism. A lag in the temporal relationship between PG activity and the titre would confirm this and also reveal times of possible inter-endocrine feedback regulation between the ecdysteroids and JHs. The fourth instar ecdysteroid titre (Fig. 5) determined from day 1 to day 2 plus 12 h showed one large increase. From day 1 to approximately day 1 plus 11 h, the titre increased gradually from 0·1 μg ml^{-1} haemolymph to 0·3 μg ml^{-1}, began to rise quickly at day 1 plus 15 h and then rose steeply to a peak of 2 μg ml^{-1} on day 1 plus 22 h. Since the increase in

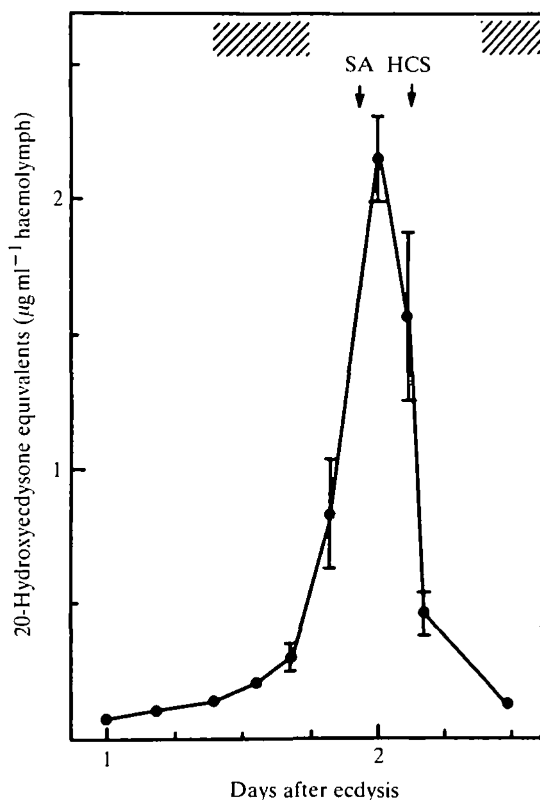

Fig. 5. Haemolymph titre of ecdysteroids in gate I, fourth instar *Manduca* larvae during days 1–2 of the instar. Each point is the mean ± S.E.M. of the titre in six larvae. SA and HCS denote spiracle apolysis and head capsule slippage, respectively, and the hatched bars denote the scotophase of each day of the instar.

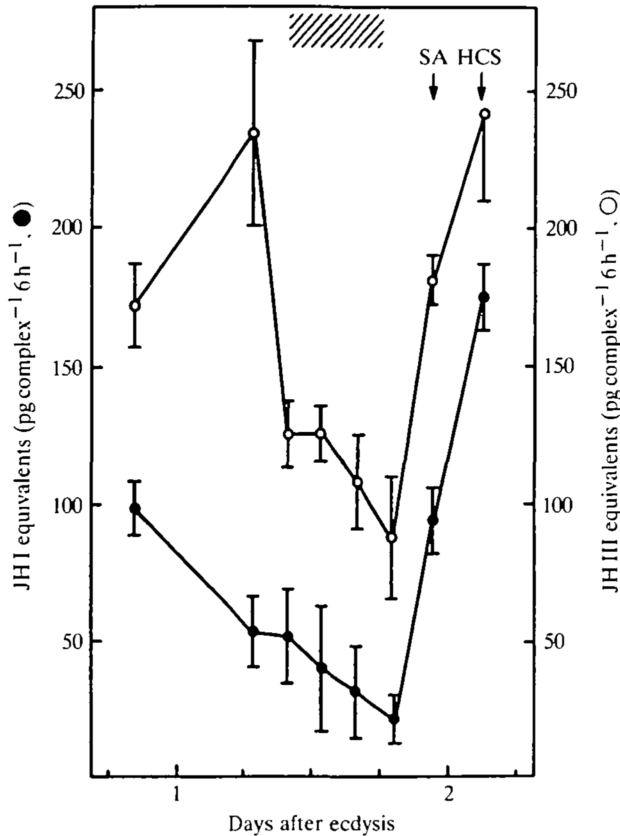

Fig. 6. Capacity of the brain–corpora cardiaca–corpora allata complex from gate I, fourth instar *Manduca* larvae to synthesize JH I and JH III *in vitro* during days 1–2 of the instar. Each point is the mean ± S.E.M. of 6–8 separate determinations, with each determination run in triplicate. SA and HCS denote spiracle apolysis and head capsule slippage, respectively, and the hatched bars denote the scotophase of each day of the instar.

the ecdysteroid titre followed the increase in the PTTH titre by 7 h and the increase in PG activity by 4 h (reasonable delays given that synthesized hormone must accumulate) the predicted order of endocrine events that ultimately evoke a larval moult was confirmed. That both the ecdysteroid titre and the biosynthetic capacity of the PG were characterized by one peak agrees with the PTTH titre data which revealed a single burst of PTTH release, occurring within a period of only a few hours.

Corpora allata activity in relation to the head critical period

To determine if an interendocrine regulatory link exists between the PTTHs, JHs and ecdysteroids in fourth instar *Manduca*, as has been suggested to occur in the fifth instar of this insect (Whisenton *et al.* 1985, 1986; Watson *et al.* 1986; Rountree & Bollenbacher, 1986), the JH biosynthetic capacity *in vitro* of corpora allata (CA) in complex with the brain–corpora cardiaca (Br–CC) was assessed before, during and

after the HCP. While this approach is an indirect measure of the haemolymph titre of the JHs (Granger *et al.* 1982*a*), it was necessary since: (1) a titre of JHs for fourth instar *Manduca* larvae determined by direct physicochemical methods has not yet been published; (2) this study used gate I larvae, with a 3-day fourth instar, which have not been previously measured by any method; and (3) critical decreases and increases in hormone titres of endocrine gland activities in this study occurred within a few hours, necessitating direct analysis of these events within a single population of animals rather than extrapolative analysis of data from colonies reared under different conditions and/or at different developmental gates. The capacity of the CA to synthesize JH I RIA activity (JH I and/or its acid) (Fig. 6) decreased steadily from a rate of approximately 100 pg complex^{-1} 6 h^{-1} on day 0 plus 21 h to less than 25 pg complex^{-1} 6 h^{-1} on day 1 plus 20 h. After this, and coincident with the dramatic increase in the haemolymph ecdysteroid titre, synthesis of JH I increased sharply, reaching approximately 175 pg complex^{-1} 6 h^{-1} by day 2 plus 4 h. The same Br–CC–CA biosynthesized JH III RIA activity (JH III and/or its acid) in a pattern very similar to JH I synthesis (Fig. 6), although the levels of synthesis were higher. Synthesis of JH III reached its nadir of 90 pg complex^{-1} 6 h^{-1} at the same time as JH I synthesis reached its lowest level, then increased essentially in parallel with JH I synthesis. Thus JH I and JH III biosynthesis decreased to a basal level at the beginning of the scotophase of day 1, just before PTTH release, and did not increase again until late in the instar, when the haemolymph ecdysteroid increased (Whisenton *et al.* 1986).

Although CA activity *in vitro* is not a direct measure of the haemolymph titre of JH, it does compare favourably (Granger *et al.* 1982*a*) with titres of JH determined by bioassay (see Riddiford & Truman, 1978) and physicochemical methods (D. Schooley, personal communication) given differences in animal rearing and assay methods. Thus the temporal relationships between fluctuations in the titres of ecdysteroids and in CA activity suggest that these hormones may be regulating each other's synthesis.

DISCUSSION

This investigation of fourth instar *Manduca* larvae has defined for the first time the dynamics of the neuroendocrine/endocrine cascade that regulates larval development in a holometabolous insect. The results obtained, together with information from a comparable study of the endocrinology of pupal commitment in this insect (W. E. Bollenbacher, N. A. Granger, M. A. O'Brien & E. J. Katahira, in preparation), permits the development of hypotheses concerning the relationships of the temporal, quantitative and possibly qualitative endocrine fluctuations in the regulation of moulting and metamorphosis by interendocrine feedback.

In fourth instar *Manduca* larvae, a temporally precise drop in the JH haemolymph titre (Fain & Riddiford, 1976; Riddiford & Truman, 1978) marks the beginning of the brain-initiated endocrine cascade which drives the moult to the last instar. A comparison of this JH titre data with the time of PTTH release noted in the present

study – the middle of the scotophase of day 1 – shows that by this time the JH titre is dropping to its lowest level during the instar. By the time 50% of the fourth instar larvae have released sufficient PTTH to elicit a moult (3 a.m. of day 1), JH I bioassay activity is approaching a level that is about half the level during the rest of the instar. During this time, the synthesis of JHs by the CA has decreased to a level of about half that earlier and later in the instar, suggesting that the JH haemolymph titre is decreasing in response to a comparable decrease in CA activity. Additionally, the CA activity data revealed that the synthesis of at least two JH homologues (and/or their acids), JH I and JH III, fluctuated in a comparable way, raising the possibility that both hormones function to control larval moulting.

Although not demonstrated here, the drop in the JH titre as a result of decreased CA activity that occurs at the time of gated release of PTTH from the CA may be permissive for the commitment period of the last instar of *Manduca* (Nijhout & Williams, 1974; Rountree & Bollenbacher, 1986). Given that comparable relationships between decreasing CA activity/JH titre and the gated release of PTTH exist for both larval moulting and commitment, it is possible in *Manduca* that JH also permissively regulates PTTH secretion in the fourth larval instar. This hypothesis contrasts with the results of an indirect analysis of the effect of JH on PTTH release during larval moulting in the silk moth, *Bombyx mori* (Sakurai, 1983), which showed that JH had no effect on PTTH release. A current investigation in this laboratory should show whether JH is involved in the control of PTTH release during the larval moult in *Manduca*.

Whether JH permissively controls PTTH release or not, a decrease in the JH titre is fundamental for the initiation of moulting and metamorphosis. At present, it appears that regulation of JH synthesis by the CA is the most important level at which the JH haemolymph titre is controlled (deKort & Granger, 1981; Tobe & Feyereisen, 1983). Regulation appears to be exerted principally by neural elements emanating from the brain, e.g. direct nervous and/or neuroendocrine (paracrine) innervation of the glands (Ferenz & Diehl, 1983; Granger, Mitchell, Janzen & Bollenbacher, 1984; Gadot & Applebaum, 1985; Rankin, Stay, Aucoin & Tobe, 1986; N. A. Granger & W. P. Janzen, in preparation). The potential significance of this control, which undoubtedly affects the decrease in CA activity noted in this study, is that the brain now assumes a central role in the interendocrine dynamics regulating development.

The measurement of PTTH activity in fourth instar haemolymph marks the first time this neurohormone has been measured in haemolymph. While this activity has not been chemically identified as one of the PTTH peptides present in a *Manduca* pupal brain (Bollenbacher *et al.* 1984*a*,*b*), it nevertheless is a peptide which has activity in the *in situ* bioassay for PTTH (M. A. O'Brien & W. E. Bollenbacher, unpublished results). Two observations provide indirect proof that the activity measured in the haemolymph is PTTH: (1) the time during which PTTH activity was present in the haemolymph was identical to the HCP for the neurohormone's release; and (2) the capacity of PGs to synthesize ecdysone during and after the HCP fluctuated in a manner consistent with gland activation by PTTH *in situ*, i.e

increased ecdysone synthesis lagged behind the increased PTTH titre by about 2 h. This short lag in PG response was expected, given the nature of the kinetics of PG activation by PTTH *in vitro* (Bollenbacher *et al.* 1983). A similar temporal correlation between PTTH and PG biosynthetic capacity *in vitro* has been made for the larval moult in *Bombyx* (Sakurai, 1983). The fact that PG activity *in vitro* began to decline just after the fall in the haemolymph PTTH titre further supports the conclusion that the PTTH activity detected reflects the presence of the hormone (Bollenbacher *et al.* 1983).

An important property of the PTTH haemolymph titre during the fourth larval instar was that release occurred as a single burst over a period of only a few hours. Thus if a high ecdysteroid titre were to be maintained for a longer period during this instar, a prolonged release of PTTH would be required to sustain activation of the PG. This has been shown to occur during the pupal commitment period of the fifth instar in *Manduca* (W. E. Bollenbacher, N. A. Granger, M. A. O'Brien & E. J. Katahira, in preparation), when PTTH release occurs in a pulsatile manner; three bursts of release occur over an approximately 18 h period, with each burst lasting as long as the single PTTH release during the fourth instar. Thus it appears that the mode of PTTH release, single *vs* multiple bursts, is also important in regulating PG activity and the ecdysteroid titre.

Since PTTH release during the fourth larval instar occurs in only one burst over a few hours, the delayed decrease in both PG activity and the ecdysteroid titre late in the instar must occur in response to trophic factors other than the neurohormone. One of these factors could be a recently discovered haemolymph protein which stimulates the PGs and which acts with PTTH to effect different rates of ecdysteroid biosynthesis (Watson *et al.* 1985, 1986). Sustained PG activity might also be a result of the developmentally modulated intrinsic competency of the glands to respond to different trophic effectors (Ciancio, Watson & Bollenbacher, 1986). The regulation of the PGs by factors and mechanisms in addition to PTTH would explain the numerous discrepancies existing between actual fluctuations in the ecdysteroid titre and the classical mechanisms by which these fluctuations are thought to be achieved.

Just as a temporally precise decrease in the JH titre may initiate the endocrine cascade which elicits larval moulting, a precise increase in the JH titre may terminate the cascade and ensure that a larval moult occurs. Although little is known about how the drop in the JH titre is regulated, the subsequent increase in the titre late in the fourth instar has been shown to involve an interendocrine feedback loop between 20-hydroxyecdysone (increasing haemolymph ecdysteroid titre) and the CA (Watson *et al.* 1986; Whisenton *et al.* 1986). The exact nature of this relationship, demonstrated *in vitro*, involves 20-hydroxyecdysone stimulation of JH synthesis by the CA *via* the brain–corpora cardiaca. In a similar fashion, during the fifth larval instar in *Manduca*, the post-commitment increase in the JH titre occurs in response to 20-hydroxyecdysone stimulation of the CA (Whisenton *et al.* 1985; Watson *et al.* 1986). Essentially nothing is known of the mechanism by which 20-hydroxyecdysone regulates the CA, but it could involve either a stimulation or a removal of an inhibition, or both, exerted *via* nervous and/or neuroendocrine effectors.

The 20-hydroxyecdysone-evoked increase in the JH titre may play one further role in the fourth instar, and that is to terminate the endocrine cascade possibly initiated by the drop in the JH titre. Here, the rising JH titre would prevent another gated release of PTTH before the moult to the fifth instar took place. The fact that JH biosynthesis increases by lights-out on the second day of the fourth instar supports this idea. An inhibitory effect of JH on PTTH release from the brain would then apparently be maintained until some 6 days later in development, when a declining JH titre before pupal commitment in the fifth larval instar again permits the gated release of PTTH (Nijhout & Williams, 1974; Rountree & Bollenbacher, 1986).

This fundamental investigation of the endocrine regulation of larval moulting serves to emphasize the precise nature of the temporal and quantitative changes in the endocrinology of *Manduca* that drive moulting and metamorphosis. Perhaps more importantly, the findings of this investigation have provided a basis for formulating testable hypotheses of the interendocrine relationships between ecdysteroids, JHs and PTTH. It is our present belief that the effects of each hormone on the synthesis and titres of the others are basic components of the endocrine control of moulting and metamorphosis and that these effects are mediated by the brain. Thus cerebral peptides like PTTH, and those that regulate JH biosynthesis by the CA (allatotropins and allatostatins), are the primary participants in this inter-regulatory scheme. With new insights into the hormonal regulation of insect development derived from the endocrine data of this study, we are now in a position to begin investigating the cerebral neural mechanisms by which this interendocrine regulation is mediated.

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