

## THE RELEASE OF THE PROTHORACICOTROPIC HORMONE IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*, IS CONTROLLED INTRINSICALLY BY JUVENILE HORMONE

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### SUMMARY

Pupal development is elicited early in the last larval instar of the tobacco hornworm, *Manduca sexta* (Johannson), by a precise temporal and quantitative increase in the haemolymph titre of 20-hydroxyecdysone. This increase in the titre is referred to as the pupal commitment peak, and it occurs once the titre of juvenile hormone (JH) has dropped. If the haemolymph titre of JH remains elevated at this time due to topical application of the hormone or of its analogue ZR512, commitment is delayed or inhibited in a dose-dependent manner. This delay or inhibition is due to the curtailment of the commitment peak in the ecdysteroid titre, which results from a failure of the prothoracic glands (PG) to increase the synthesis/secretion of the premoult hormone, ecdysone. Since the PG from ZR512- and JH 1-treated larvae are capable of being activated *in vitro* by the prothoracicotrophic hormone (PTTH), the effect of JH on the PG does not involve suppression of gland sensitivity to PTTH. The locus of the JH effect was determined to be the brain-retrocerebral complexes (Br-CC-CA), on the basis of experiments which tested the effect of implanted Br-CC-CA from pre-commitment larvae treated with JH on the occurrence of pupal commitment in head-ligated larval hosts. The implanted, JH-treated Br-CC-CA exhibited a delayed release of PTTH, and the effect was at concentrations of JH that were physiological. These results argue that JH functions to control the time during the last larval instar when pupal commitment occurs by dictating when PTTH will undergo gated release.

### INTRODUCTION

The basic roles of juvenile hormone (JH), the prothoracicotrophic hormone (PTTH) and ecdysteroids in the control of insect postembryonic development are well established (see Gilbert, Bollenbacher & Granger, 1980). By contrast, it is not known to what extent these hormones interact to regulate one another's synthesis and secretion, and thus to control the entire process of insect postembryonic development. Since interendocrine feedback is one of the fundamental properties of endocrine systems enabling a precise modulation of growth and development, this feedback is undoubtedly important in the regulation of insect development.

Key words: juvenile hormone, prothoracicotrophic hormone, prothoracic gland, *Manduca sexta*.

In the past decade, possible interendocrine interactions between JH and ecdysteroids in regulating their own and each other's synthesis have been investigated (see deKort & Granger, 1981; Tobe & Feyereisen, 1983; Bollenbacher & Granger, 1985) utilizing principally indirect, *in situ* approaches. These have yielded largely equivocal results (Cymborowski & Stolarz, 1979; Hiruma, 1980; Hiruma & Agui, 1982; Safranek, Cymborowski & Williams, 1980; Tobe & Feyereisen, 1983) that have limited our understanding of the extent to which interendocrine regulation contributes to the control of insect development. Recently, however, direct, physiological methods with both *in vitro* and *in situ* approaches, have been developed to investigate JH-ecdysteroid interactions in the control of pharate pupal development of the tobacco hornworm, *Manduca sexta* (Gruetzmacher, Gilbert & Bollenbacher, 1984a; Gruetzmacher *et al.* 1984b; Whisenton *et al.* 1985; Watson *et al.* 1985). These studies have established a regulatory link between the functioning of these endocrine systems and their control of metamorphosis.

Pharate pupal development in *Manduca* is the end result of a cascade of endocrine events set in motion by the release of PTTH from the corpora allata (CA) on day 3–4 of the instar (Truman & Riddiford, 1974; Bollenbacher & Gilbert, 1981; Bollenbacher & Granger, 1985). Release begins in the latter part of the scotophase of the third day, occurring in three bursts over a period of approximately 18 h (Bollenbacher & Gilbert, 1981; W. E. Bollenbacher, unpublished). Lights-off each night (the opening of a gate during larval development) is thought to be the signal that PTTH release can occur. Whether or not the hormone is released at this time depends on the developmental and/or physiological status of the larva. While it is not clear what constitutes the status necessary for PTTH secretion (see Bollenbacher & Granger, 1985), a drop in the JH titre has been implicated as an endocrine signal of its attainment (Nijhout & Williams, 1974b), and thus also as the trigger for PTTH release. Apparently, the decline in the haemolymph titre of JH, which drops to a critical level just before the scotophase of day 3 of the last instar, allows PTTH release when the gate opens at lights-out. Once released, PTTH activates the PG to increase the synthesis of ecdysone, which is then mono-oxygenated to 20-hydroxyecdysone (Bollenbacher, Agui, Granger & Gilbert, 1979), the ecdysteroid which elicits moulting.

How a high titre of JH can exert a prothoracicostatic effect is not known. Although the hormone could be acting at the level of the brain by inhibiting PTTH secretion (Nijhout & Williams, 1974b), it could also be acting on the PG, preventing them from being activated by PTTH (Cymborowski & Stolarz, 1979; Safranek *et al.* 1980), or on the target tissues of ecdysteroids, preventing them from responding to an elevated haemolymph ecdysteroid titre (see Riddiford, 1980). In this paper, *in vitro/in situ* methods are used to investigate the mechanism by which JH permissively controls pupal commitment in this holometabolous insect. The study has revealed that the principal action of JH occurs at the level of the brain-retrocerebral complex, preventing the gated release of PTTH. The relevance of this finding to the intriguing biological phenomenon of pupal commitment, i.e. covert developmental reprogramming of larval tissues to pupal synthesis, is discussed.

## MATERIALS AND METHODS

*Experimental animals*

Tobacco hornworms, *Manduca sexta*, were reared individually on an artificial diet (Bell & Joachim, 1976) under a long day photoperiod (L:D 16:8) at 26°C and high humidity (>60%). In a synchronous population, the moult to the fifth (last) larval instar occurs at 14.00h EST, 8h into the photophase, and this point is thus designated day 0 plus 0h of the instar. Gate II larvae, which release PTTH at the end of the scotophase of the third day of the instar, were used in this study. They were selected for use at day 3 plus 4h on the basis of their weight (Vince & Gilbert, 1977). This was approximately 12h before the release of PTTH that elicits pupal commitment, and thus these animals are termed pre-commitment larvae.

*Juvenile hormone and analogue application*

The application to larvae of juvenile hormone I (JHI) (CalBiochem, La Jolla, CA) or the analogues ZR512 (Hydroprene) and methyl farnesoate (gifts from Dr D. S. Schooley, Zoecon Corpn, Palo Alto, CA) was done topically, using analytical grade acetone as a vehicle for penetration of the compounds through the cuticle. A stock solution of JHI ( $98 \mu\text{g ml}^{-1}$ ), made in spectrograde methanol and determined spectrophotometrically, was stored at  $-20^\circ\text{C}$  until use. Before application to larvae, aliquots of the JHI stock were dried under  $\text{N}_2$  and then redissolved in acetone. Different concentrations of JH in a constant volume (0.01 ml) of acetone were applied to larvae along the dorsal midline of the thoracic and abdominal segments. Control larvae were treated with an equivalent volume of acetone alone. Immediately following treatment, larvae were returned to individual rearing cups and allowed to feed until use. The preparation of stock solutions of ZR512 and methyl farnesoate and the application of these substances to precommitment larvae, was the same as that for JHI.

*Permeability of larval cuticle*

The quantity of topically applied JHI reaching the haemolymph of day 3 larvae was determined from the topical application of  $50 \mu\text{g } [^3\text{H}]\text{JHI}$  at  $15.8 \text{ mCi mmol}^{-1}$  followed by an assessment of the amount of labelled material found in the haemolymph (Gruetzmacher *et al.* 1984a). A time course of  $[^3\text{H}]\text{JHI}$  uptake was determined by taking sequential aliquots of haemolymph (0.025 ml) from the prolegs of larvae at different times post-treatment. To prevent bleeding following removal of the haemolymph sample, the prolegs were ligated with dental floss. The amount of  $[^3\text{H}]\text{JHI}$  in the haemolymph was quantified by liquid scintillation counting of 0.01 ml aliquots of the haemolymph samples in 10 ml Aquasol (New England Nuclear, Boston, MA). Quenching of  $^3\text{H}$  by the haemolymph was negligible with this procedure. Since only the total amount of  $[^3\text{H}]$ -label was determined, the amount contributed by metabolites of  $[^3\text{H}]\text{JHI}$  could not be ascertained.

*Haemolymph ecdysteroid titre*

Ecdysteroid levels in haemolymph were determined by radioimmunoassay (RIA) (Bollenbacher, Smith, Goodman & Gilbert, 1981). Haemolymph (0.1 ml) was collected into glass culture tubes on dry ice from an incision in the anterior proleg of the larva. The tubes were sealed with parafilm and stored at  $-20^{\circ}\text{C}$  until assay. Following extraction with absolute methanol (0.3 ml, 3:1, v/v), the extracts were centrifuged at  $4000\times g$  for 10 min to remove precipitated proteins. The ecdysteroid content in an aliquot of the supernatant was then quantified by RIA using D16-20 antiserum, which binds ecdysone and 20-hydroxyecdysone equivalently (Bollenbacher *et al.* 1981). The radioligand was [ $^3\text{H}$ ]ecdysone ( $60\text{ Ci mmol}^{-1}$ ) (New England Nuclear) and the competing unlabelled ligand was also ecdysone (gift from Dr D. H. S. Horn, CSIRO, Melbourne, Australia). Thus the haemolymph ecdysteroid titre is expressed in ecdysone equivalents. Each datum point is the average of the ecdysteroid titre of at least six larvae, and since an equal number of males and females were used for each point, any sexual dimorphism in the ecdysteroid titre would not have been noted.

*Prothoracic gland activity in vitro*

Procedures for the dissection and incubation of larval prothoracic glands (PG) *in vitro* for determinations of their ecdysone biosynthetic capacity have been described in detail previously (Bollenbacher, O'Brien, Katahira & Gilbert, 1983; Bollenbacher *et al.* 1984). With this procedure, extirpated glands were incubated in 0.025 ml Grace's medium (GIBCO Laboratories, Grand Island, NY) at  $25^{\circ}\text{C}$  for 2 h in saturating humidity. Following incubation, 0.01 ml aliquots of the medium were assayed by RIA for ecdysone synthesized. The antiserum used for this RIA was H-3, which has an affinity for ecdysone that is approximately four times that for 20-hydroxyecdysone (Gilbert, Goodman & Bollenbacher, 1977). The labelled ligand was [ $^3\text{H}$ ]ecdysone ( $4\text{ Ci mmol}^{-1}$ ), and the unlabelled competing ligand was also ecdysone. Because ecdysone is the only ecdysteroid known to be synthesized by *Manduca* PG *in vitro*, synthesis was expressed in ng ecdysone gland $^{-1}$  2 h $^{-1}$ .

*PTTH activation of prothoracic glands in vitro*

The extent of PG activation by PTTH was assessed by the *in vitro* PG assay for the neurohormone (Bollenbacher *et al.* 1979, 1983, 1984). The PTTH preparation used for these assays was a heat-treated, Grace's medium extract of day 1 pupal brains which contained both forms of the neurohormone, i.e. big and small PTTH (Bollenbacher *et al.* 1983, 1984). PTTH stocks were stored at  $-20^{\circ}\text{C}$  until use. To ensure that the glands would be maximally activated, they were incubated in 0.25 units (brain equivalents) of PTTH, which is approximately four times the  $\text{ED}_{50}$  for activation of these PGs by PTTH (Bollenbacher *et al.* 1983). In the PG assay for PTTH, activation is expressed as an activation ratio ( $A_r$ ) (Bollenbacher *et al.* 1979, 1983), which is the quantity of ecdysone synthesized by the experimental gland (+PTTH) divided by the amount synthesized by a control gland (-PTTH). The

maximal activation achieved is expressed as an  $A_{\max}$ , which generally represents a four-fold increase in ecdysone synthesis by the experimental gland.

#### *Implantation of brain-retrocerebral complexes*

Brain-retrocerebral complexes (Br-CC-CA) were taken on day 3 plus 14 h (~2 h before PTTH release) from control, untreated, last instar larvae (acetone only), and from experimental larvae treated with ZR512 on day 3 plus 4 h. These complexes were then transplanted into day 3 plus 14 h head-ligated larvae. Extirpation of the Br-CC-CA was performed in such a way as to preserve the major tracheal trunks to the brain, maximizing the viability of the neural tissue *in vitro* in Grace's medium until it could be implanted (Bowen, Saunders, Bollenbacher & Gilbert, 1984). The tracheae were used as a 'handle' to implant the Br-CC-CA without damaging it. Transplant recipient larvae received no prior treatment, other than ligation behind the head with dental floss just before implantation of Br-CC-CA, to remove the endogenous source of PTTH and JH. A tracheated Br-CC-CA or a ventral ganglion from an experimental or control larva was then implanted into the haemocoel of a chilled, ligated larval host *via* a small incision a few mm dorsocaudal to the thoracic spiracle. The incision was sealed with Tackiwax (Cenco, Chicago, IL). Only Br-CC-CA with intact nervi corporis cardiaci I and II (NCC I and II) were implanted, and any recipient larva that bled profusely as a result of the implantation was discarded. Ligated larvae with Br-CC-CA implants were maintained in individual cups under standard rearing conditions. In all experiments involving transplantation of the Br-CC-CA, the surgical manipulations were completed approximately 2 h before the first of three bursts of PTTH (~day 3 plus 16 h) (Bollenbacher & Gilbert, 1981; Gilbert *et al.* 1981; W. E. Bollenbacher, unpublished).

## RESULTS

### *Dose-dependent delay of commitment by juvenile hormone*

Determining the level at which JH, or a JH analogue, controls the occurrence of pupal commitment in *Manduca* first requires a demonstration that the hormone's effect is dose dependent and that its effective concentration is physiological. From the dose-response results can also be obtained the concentration of JH necessary for a direct examination of the mechanism by which the hormone actually delays commitment. Therefore, a dose-response curve of JH I inhibition of pupal commitment, as evidenced by a delay of stereotypic wandering behaviour and of the overt morphological markers of this developmental event, i.e. dorsal vessel exposure, orange stripes and ommochrome pigmentation (cuticular markers) (Truman & Riddiford, 1974; Bollenbacher & Gilbert, 1981; W. E. Bollenbacher, unpublished), was generated. The results were then used to establish the developmental criteria with which JH effects on commitment could be scored in a bioassay.

For gate II last instar larvae, wandering behaviour and the morphological markers of pupal commitment are maximally evident at day 4 plus 20 h (Bollenbacher &

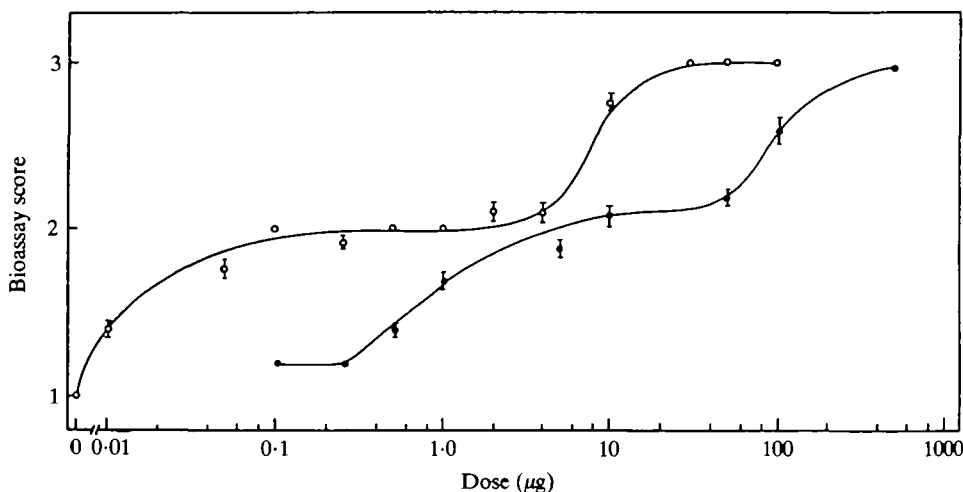


Fig. 1. Dose-response delay of pupal commitment in day 3 plus 4 h gate II, last instar larvae of *Manduca sexta* by topically applied JHI (●) and ZR512 (○). Bioassay score denotes the extent of delay and nature of the effect of JH on development: 1, no change in developmental programme; 2, pupal commitment delayed 1 or more days; 3, developmental programme disrupted, head cap slippage indicative of larval moulting occurs in 10 or more larvae per concentration assayed. Each datum point represents seven or more trials ( $\pm$ S.E.M.). Data points lacking S.E.M.s reflect an error smaller than the size of the point.

Bowen, 1983; Dominick & Truman, 1984; Bollenbacher & Granger, 1985). Treatment of day 3 larvae with JHI, or with ZR512, delayed commitment by one to several days (Fig. 1). The length of delay was concentration dependent and appeared to be gated, i.e. PTTH release began during the scotophase of subsequent days. To assess precisely the extent of the delay, a bioassay was developed which scored the time at which wandering and the morphological markers occurred: treated larvae that wandered and exhibited the overt markers in a physiological time were scored 1; those whose wandering and cuticular markers were delayed 1 to 3 days were scored 2; and larvae that failed to wander by 7 days (no pupal commitment), were scored 3. There was some variation in the bioassay, that is, the simultaneous occurrence of wandering and dorsal vessel exposure-ommochrome pigmentation in any given JHI-treated larva was inconsistent (Dominick & Truman, 1984). In these instances, wandering alone was considered to represent commitment.

Topical application of JHI, in the range from 0.25  $\mu$ g to approximately 10  $\mu$ g, evoked a dose-dependent delay in commitment. By contrast, 100  $\mu$ g or more of JHI curtailed commitment but did elicit head capsule slippage, which is a developmental marker for a supernumerary larval moult (Nijhout & Williams, 1974a). Since this high concentration of JH is undoubtedly pharmacological, the developmental response elicited was interpreted to be non-physiological. From the dose response curve, 50  $\mu$ g JHI, which delays commitment in 100% of the treated larvae, was selected for subsequent experiments.

There are two drawbacks to the use of JH I in routine studies investigating the endocrine function(s) of the hormone: it has a very short half-life *in situ* (Fain & Riddiford, 1975; Gruetzmacher *et al.* 1984a) and it is expensive. Thus analogues of JHs are generally used in such studies, and ZR512 is one analogue that has been found to mimic JH effectively in dictating the time at which pupal commitment occurs in *Manduca* (Safranek *et al.* 1980). ZR512 was tested for its ability to delay pupal commitment in the present system in order to assess its suitability as a substitute for JH I (Fig. 1). Topically applied ZR512, in amounts ranging from 0.01 to 0.1  $\mu\text{g}$ , delayed pupal commitment in a dose-dependent manner, and at amounts of 10  $\mu\text{g}$  and greater, commitment was curtailed in 100% of the treated larvae. Thus ZR512 ( $\text{EC}_{50}$  approximately 0.015  $\mu\text{g}$ ) was about 70-fold more effective than JH I ( $\text{EC}_{50}$  approximately 1.0  $\mu\text{g}$ ). The specificity of the bioassay response for JH I and ZR512 was demonstrated by the fact that acetone and methyl farnesoate (5–50  $\mu\text{g}$ ) failed to delay pupal commitment.

Because the larval bioassay response to ZR512 was temporally, behaviourally and morphologically identical to the JH I response, this analogue was deemed an effective substitute for JH I, and a dose of 5  $\mu\text{g}$  was selected for use. Nevertheless, the natural hormone was used periodically in experiments to validate the effects of ZR512.

#### *[<sup>3</sup>H]JH I penetration of the cuticle*

For a physiological analysis of the delay of commitment by JH I, the effective haemolymph concentration of applied hormone should fall within the physiological range and should increase and decrease in a manner consistent with normal concentration changes. The pre-commitment concentration of JH I in the haemolymph as a result of topical application of 50  $\mu\text{g}$  of the hormone was determined by a time course of uptake of 50  $\mu\text{g}$  [<sup>3</sup>H]JH I (Fig. 2). Disregarding the possible catabolism of the hormone, approximately  $10^{-6} \text{ mol l}^{-1}$  [<sup>3</sup>H]JH I was present in the haemolymph just 2 h after application. Between 2 h and 24 h, the apparent JH I titre dropped quickly to less than  $10^{-7} \text{ mol l}^{-1}$ . Since greater than 95% of the topically-applied JH I which gets into the haemolymph is metabolized in only a few hours (Gruetzmacher *et al.* 1984a), the actual titre of JH in the haemolymph of the treated, pre-commitment larvae is undoubtedly considerably lower. The titre probably ranges from an estimated concentration of no greater than about  $10^{-7} \text{ mol l}^{-1}$  just after application to less than  $10^{-9} \text{ mol l}^{-1}$  1 day later. This concentration range of JH I includes the apparent maximum amount of the hormone in haemolymph of day 2 last instar larvae (approximately  $10^{-8} \text{ mol l}^{-1}$ ) (D. Schooley, personal communication).

The fact that far less topically-applied ZR512 is needed to delay pupal commitment (Fig. 1) may reflect a higher effective molarity of the compound in the haemolymph as a result of its dramatically lower *in situ* rate of catabolism (Cymborowski & Stolarz, 1979). However, even with the minimum dose of ZR512 (0.1  $\mu\text{g}$ ) capable of delaying pupal commitment in 100% of the test population, the concentration of the analogue in the haemolymph would still be approximately  $10^{-9} \text{ mol l}^{-1}$ , a concentration again in the physiological range for JH I at this time.

*Effect of JH on the commitment peak of the haemolymph ecdysteroid titre*

The final event in the endocrine cascade eliciting pupal commitment which could be disrupted by JH is the response of larval tissues to the subtle peak in the ecdysteroid titre. To determine if this were the case, or if JH were having an effect upstream by curtailing ecdysone synthesis, haemolymph ecdysteroid titres in developing and in ZR512-treated larvae were compared from midday 3 to midday 4 of the instar (the period of PTTH release and resulting increase in the ecdysteroid titre) (Bollenbacher & Gilbert, 1981; Bollenbacher & Granger, 1985) (Fig. 3). Developing larvae exhibited the previously established commitment peak in the ecdysteroid titre (approximately  $100 \text{ ng ml}^{-1}$  haemolymph), but the ecdysteroid titre of ZR512-treated larvae, whose development is delayed more than 1 day, remained at a basal level of approximately  $16 \text{ ng ml}^{-1}$  haemolymph. JH I ( $50 \mu\text{g}$ ) treated day 3 plus 4 h larvae (three males and three females) similarly evoked a delay in the increase in the ecdysteroid titre [ $6.5 \pm 0.9$  and  $10.9 \pm 1.4 \text{ ng ml}^{-1}$  haemolymph were noted at day 3 plus 14 h and day 4 plus 8 h]. On this basis, JH appears to delay commitment

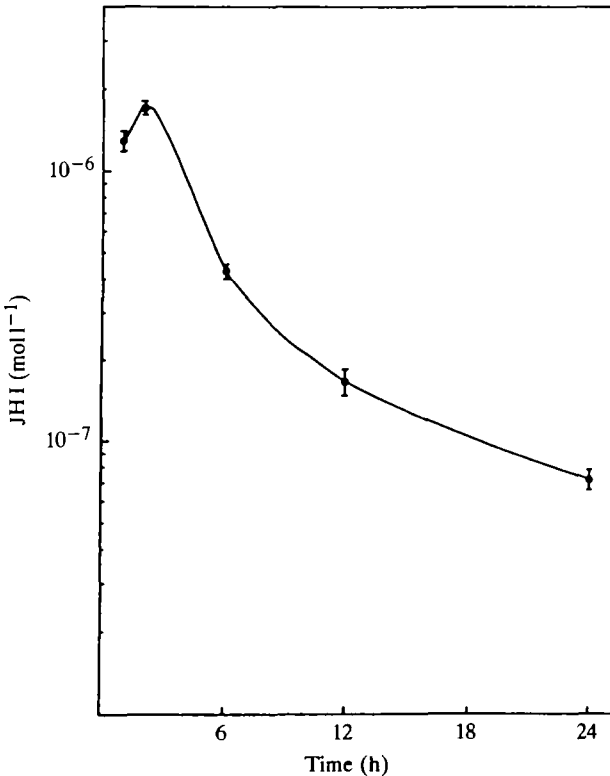


Fig. 2. Time course of uptake and turnover in the haemolymph of [ $^3\text{H}$ ]JH I topically applied to pre-commitment, last instar developing *Manduca sexta* larvae. JH I molarity was computed from total [ $^3\text{H}$ ] present in haemolymph, not accounting for its degradation to metabolites. Each datum point represents the mean ( $\pm$ S.E.M.) of [ $^3\text{H}$ ] in the haemolymph of five larvae bled sequentially over 24 h.



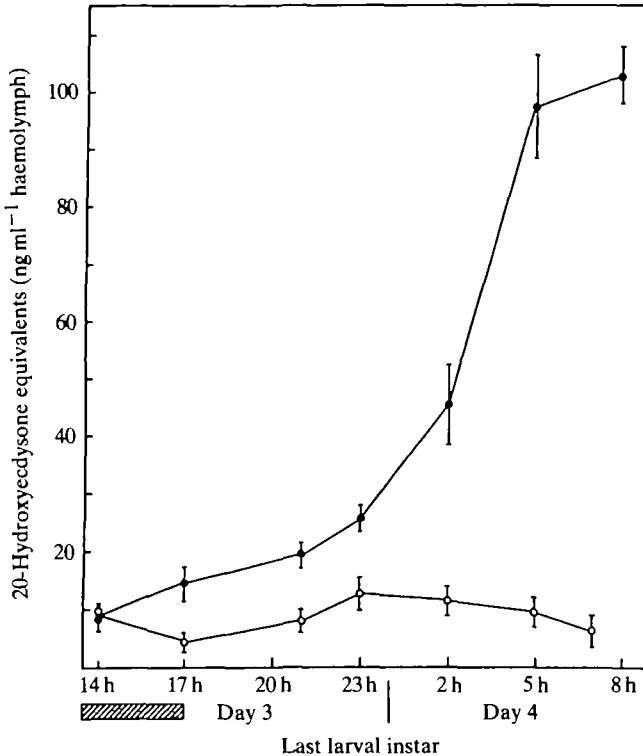


Fig. 3. The effect of ZR512 ( $5 \mu\text{g}$ ) on the occurrence of the pupal commitment haemolymph ecdysteroid titre peak in last instar *Manduca sexta* larvae. For both untreated, developing (●) and ZR512 (○) treated larvae each datum point represents the mean ( $\pm$ S.E.M.) of six animals, three males and three females. Hatched bar denotes scotophase.

by preventing an increase in the haemolymph ecdysteroid titre. While the hormone could also have a systemic role at the level of the target tissue, the ecdysteroid titre indicates that JH primarily controls commitment either by suppressing ecdysone synthesis/secretion by the PG and/or by dramatically accelerating its metabolism.

#### *Effect of JH on prothoracic gland activity*

To differentiate between the two remaining possible bases for the JH effect on the ecdysteroid titre, the effect of JH on the biosynthetic capacity of PG was determined before and during the commitment period (Fig. 4). If JH were inhibiting the PG, the glands would synthesize ecdysone *in vitro* at an unactivated rate in contrast to the increased rate of synthesis by PG of developing larvae which have been activated by PTTH *in situ* (Bollenbacher & Granger, 1985; W. E. Bollenbacher, unpublished). The anticipated increase in ecdysone synthesis by developing PG began at a basal level ( $2.5 \text{ ng gland}^{-1}$ ) before the first release of PTTH ( $\sim$ day 3 plus 16 h) and reached a maximum level ( $7.4 \text{ ng gland}^{-1}$ ) just after the last of the three pulsed releases of PTTH ( $\sim$ day 4 plus 8 h). However, the biosynthetic capacity of PG from

ZR512-treated larvae did not increase from the basal level ( $2.5 \text{ ng gland}^{-1}$ ) during this entire period. Although these results do not preclude accelerated ecdysteroid degradation as an additional effect of JH in delaying pupal commitment, the complete curtailment of increased PG activity in ZR512-treated larvae argues that JH delays commitment principally by preventing PG activation. The effect of JH I ( $50 \mu\text{g}$ ) treatment of day 3 plus 4 h larvae on the ecdysone biosynthetic capacity of the glands from these larvae at day 3 plus 14 h and day 4 plus 2 h ( $2.43 \pm 0.1$  and  $3.5 \pm 0.2 \text{ ng gland}^{-1}$ , respectively) was equivalent to that of ZR512.

*Activation of prothoracic glands by PTTH*

JH could affect ecdysone synthesis by the PG either by inhibiting activation of the gland by PTTH, or by curtailing PTTH secretion from the Br-CC-CA. To

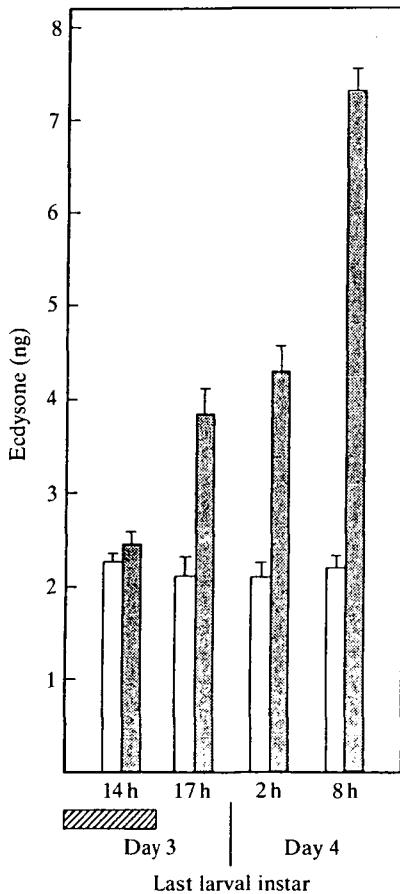


Fig. 4. Effect of ZR512 ( $5 \mu\text{g}$ ) on the capacity of last larval instar *Manduca sexta* prothoracic glands to synthesize ecdysone *in vitro* during pupal commitment. For both glands from untreated, developing larvae (closed bars) and glands from ZR512-treated larvae (open bars) each determination is the mean ( $\pm$ S.E.M.) of six separate prothoracic gland incubations. Hatched bar denotes scotophase.

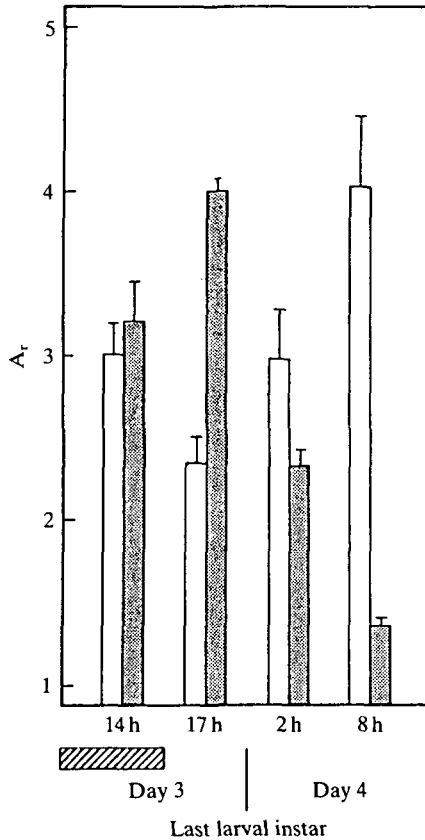


Fig. 5. The capacity *in vitro* of prothoracic glands from untreated, developing larvae and ZR512-treated larvae during pupal commitment to be activated by PTTH. Activation is expressed as an activation ratio ( $A_r$ ), which is the ecdysone synthesized by an experimental gland divided by synthesis by a control gland. For glands from both untreated, developing larvae (closed bars) and glands from ZR512-treated larvae (open bars) each determination is the mean ( $\pm$ S.E.M.) of six separate incubations. For all combinations of PG from ZR512-treated larvae ecdysone synthesis was not significantly different ( $P > 0.1$ ). Hatched bar denotes scotophase.

distinguish between these two possibilities, the capacities of PG from developing and ZR512-treated larvae to be activated by PTTH (Fig. 5) were compared before and during the commitment period. Glands taken from developing larvae just before PTTH release were activated by the neurohormone *in vitro* to the expected  $A_{max}$  of approximately 3.5 (Bollenbacher *et al.* 1983, 1984), indicating that they were competent to respond to the neurohormone. From day 3 plus 17 h to day 4 plus 8 h, however, the developing PG became progressively refractory to activation by PTTH *in vitro*, which suggested that they were being progressively activated by the neuropeptide *in situ*. By contrast, glands from ZR512-treated larvae taken at any time during the commitment period were activated by PTTH with values of  $A_r$  ranging from 2.4 to 4. The ranges of activation achieved at these different times were not significantly different (for all combinations  $P \geq 0.1$ ). If commitment were delayed by JH *via* direct inhibition of PTTH activation of the PG, an  $A_r$  value of 1

should have been obtained. JH I (50  $\mu\text{g}$ ) also had no effect on the capacity of the PG to be activated by PTTTH *in vitro* ( $A_r$  of  $3.4 \pm 0.6$  for day 3 plus 14 h and  $4.5 \pm 0.25$  for day 4 plus 8 h larvae). Since JH does not inhibit the PG from being activated by PTTTH, it appears that PTTTH release is the principle level at which JH affects commitment.

#### *Brain transplantation effects on commitment*

To demonstrate that JH acts on the Br-CC-CA to prevent PTTTH release and that the drop in the JH haemolymph titre permits the gated secretion of the neuropeptide, a method was developed to assess the affect of JH on the release of the neurohormone from the Br-CC-CA. This was accomplished by transplanting neural tissue (Br-CC-CA) from one insect to another without loss of its neuroendocrine function (Truman & Riddiford, 1970; Granger & Bollenbacher, 1981; Bowen *et al.* 1984; Bollenbacher & Granger, 1985).

Head ligation of last instar larvae (day 3 plus 14 h) just before PTTTH release prevents the ligated larvae from undergoing pupal commitment within the next 3 days, as shown by the absence of the stereotypic markers of commitment, i.e. cuticular markers and the normal increase in the haemolymph ecdysteroid titre.

Implantation of Br-CC-CA from developing larvae (day 3 plus 12 h) into these head-ligated hosts resulted in the appearance of the markers of commitment within a 'physiological' time frame, i.e. 14 h after implantation (Table 1; Fig. 6). This indicated that PTTTH release by the Br-CC-CA was not altered by transplantation

Table 1. *Effect of implanted brain-retrocerebral complexes from ZR512 (5  $\mu\text{g}$ ) topically treated day 3 plus 6 h last instar Manduca larvae on the occurrence of pupal commitment in day 3 plus 14 h untreated, head ligated recipient larvae*

Experimental treatment of larvae		Commitment bioassay score (hours after implantation) ( $\pm$ S.E.M.)	
		30	54
Control, no ligation	(10)	$1.5 \pm 0.06$	$2.2 \pm 0.08$
Control, ligated	(11)	$0.18 \pm 0.04$	$0.36 \pm 0.06$
ZR512, no ligation	(10)	$0.20 \pm 0.04$	$0.30 \pm 0.05$
ZR512, ligated	(10)	0.0	0.0
Ligated host, control ganglion implant	(19)	$0.25 \pm 0.03$	$0.50 \pm 0.1$
Ligated host, control brain implant	(40)	$1.2 \pm 0.03$	$2.3 \pm 0.08$
Ligated host, ZR512 brain implant	(38)	$0.20 \pm 0.03$	$1.7 \pm 0.07$

Numbers in parentheses are the number of animals per treatment.

A bioassay score of 1 denotes dorsal vessel exposure; a score of 2 denotes dorsal vessel exposure and orange stripes; a score of 3 denotes dorsal vessel exposure, orange stripes and ommochrome pigment.

The bioassay response at 30 h to ZR512 brain implants was not significantly different from the response to control ventral ganglion implants ( $P > 0.05$ ), while it was significantly different from the response at 30 h to control brain implants ( $P < 0.001$ ). The 54 h response to ZR512 brain implants was not significantly different from the 30 h control brain implant response ( $P > 0.05$ ), but was significantly different from the 54 h control brain implant bioassay response ( $P < 0.05$ ).

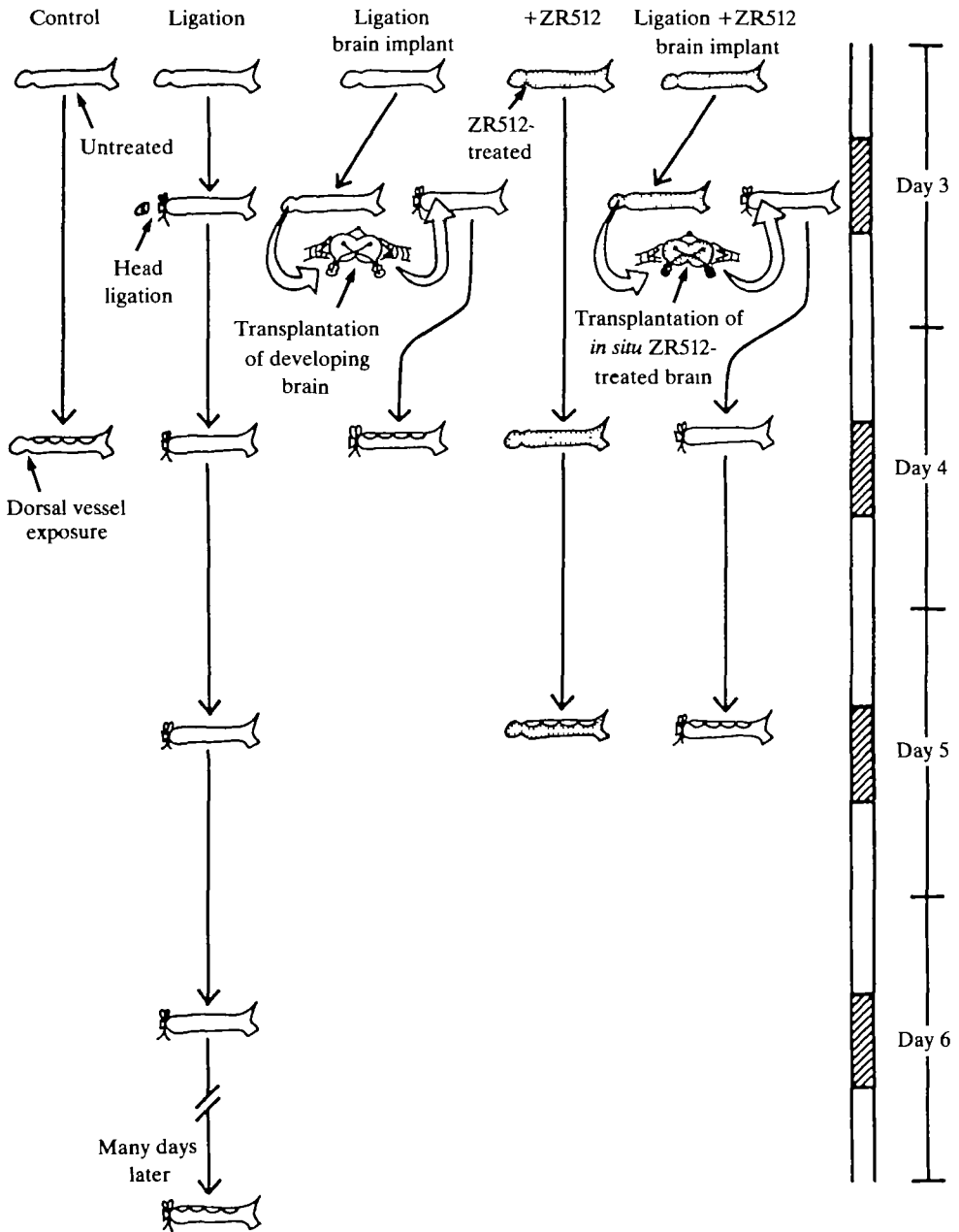


Fig. 6. Experimental method for demonstrating brain-centred juvenile hormone control of PTTH release in *Manduca sexta*. Hatched bars on day scale denote the scotophase of each day.

and that these markers could be used to assess the effect of JH on PTTH release from implanted Br-CC-CA. These experiments were conducted with Br-CC-CA rather than isolated brains in order to leave intact the connection between the corpora allata,

which is the neurohaemal organ for PTTH, and the brain. This eliminated the possibility of leakage of PTTH from the cut NCC I and II of implanted brains as a cause of a positive bioassay response. Since dorsal vessel exposure, orange stripes and ommochrome pigmentation are markers of commitment which normally occur in sequence, a hierarchical bioassay could be scored with 1 denoting dorsal vessel exposure, 2 representing dorsal vessel exposure and the appearance of orange stripes, and 3 being the appearance of all three markers.

The hypothesis that JH prevents the release of PTTH from the Br-CC-CA was tested by assessing the effect of ZR512 on the ability of transplanted Br-CC-CA to elicit commitment in pre-commitment, head-ligated larvae. Br-CC-CA from day 3 plus 12 h larvae treated with ZR512 on day 3 plus 6 h (pre-PTTH release) were implanted into ligated bioassay larvae of the same stage (Fig. 6). The occurrence of commitment was then scored (Table 1). In contrast to the results obtained with implantations of developing Br-CC-CA (bioassay score = 1.2 at 30 h), ZR512-treated Br-CC-CA did not elicit commitment by the next day (bioassay score = 0.22). However, larvae implanted with these treated complexes undergo dorsal vessel exposure 2 days after implantation (bioassay score = 1.7 at 54 h), a response essentially identical to that obtained with developing Br-CC-CA at 30 h. The fact that ZR512-treated Br-CC-CA eventually released PTTH is probably due to a critical drop in the titre of the analogue before the second scotophase following implantation, which is when the next gated release of the neurohormone would normally occur. The level of response elicited by the ZR512-treated Br-CC-CA at 30 h was not significantly different from that of ligated larvae implanted with ganglia. Therefore, ZR512 appears to delay commitment in *Manduca* by delaying the release of PTTH from the Br-CC-CA. Although not explicitly shown in the data presented, the eventual release of PTTH from ZR512-treated Br-CC-CA began during the scotophase of the second night, suggesting that the release of the neurohormone from the treated, transplanted brain was still gated.

Together these results demonstrate that the JH titre regulates PTTH release from the Br-CC-CA during larval development of *Manduca*. However, they have not resolved whether the effect of the hormone on the central nervous system is direct or indirect.

#### DISCUSSION

Evidence has accumulated in the last decade to suggest that the role of JH in regulating insect postembryonic development does not consist solely of its interaction with ecdysteroids at the level of target tissues to dictate the character of a moult. The hormone also appears to function as an interendocrine feedback regulator of the prothoracicotrope–prothoracic gland axis. In holometabola such as *Manduca sexta* (Nijhout & Williams, 1974b; Safranek *et al.* 1980; Gruetzmacher *et al.* 1984a,b), *Mamestra brassicae* (Hiruma, 1980; Hiruma & Agui, 1982) and *Spodoptera littoralis* (Cymborowski & Stolarz, 1979), the regulatory effect of JH on this axis is either stimulatory or inhibitory, depending on the stage of development. Artificially

elevated JH levels exert a prothoracicotropic-like effect in *Manduca* last instar larvae which have been debrained just after pupal commitment; the pupal development of these treated larvae is accelerated. This apparently pharmacological effect of JH may in fact reflect a physiological function of the hormone, since the JH haemolymph titre in last instar larvae increases a second time just after commitment to regulate metamorphosis to the pupa (Riddiford & Truman, 1978; Granger, Niemiec, Gilbert & Bollenbacher, 1982; D. Schooley, personal communication). Depending on the insect, JH may exert its effect on the PG either directly (Hiruma, 1982) or indirectly, the latter effect involving the JH-elicited release from the fat body of a factor which stimulates ecdysone synthesis (Gruetzmacher *et al.* 1984*a,b*; Watson *et al.* 1985). By contrast, JH treatment of these lepidopterans before pupal commitment has the opposite effect, delaying commitment and subsequent development by inhibiting PTTH release (Nijhout & Williams, 1974*b*) and/or ecdysone synthesis by the PG (Cymborowski & Stolarz, 1979; Safranek *et al.* 1980). Until the present report, the apparent prothoracostatic function of JH was supported only by results from pharmacological, *in situ* studies, which precluded a determination of the exact role of JH in delaying commitment.

This investigation has directly addressed the apparent pre-commitment, prothoracostatic action of JH at each of the levels in the cascade of neuroendocrine-endocrine events which ultimately elicit commitment. The results have shown that a drop in the haemolymph titre of JH below a threshold level before lights-out allows the gated release of PTTH from the Br-CC-CA. This finding supports the hypothesis that JH inhibits PTTH release in *Manduca* (Nijhout & Williams, 1974*b*) and argues strongly against an effect of the hormone on the PG (Cymborowski & Stolarz, 1979; Safranek *et al.* 1980). An effect on PTTH release is a logical focus of control by JH, since release of this peptide is the initial event in the hormonal cascade leading to commitment. Although JH may also be acting downstream in the cascade, e.g. by inhibiting the PG, by increasing rates of ecdysteroid turnover or by altering the capacity of the larval tissues to respond to the ecdysteroids, the results of the present study show that such effects are not readily apparent and would be secondary to the effect on PTTH release.

While it is clear that JH prevents PTTH release from pre-commitment Br-CC-CA of *Manduca*, the means by which this is accomplished, i.e. directly or indirectly, has not yet been established. Recently, Bowen *et al.* (1984) demonstrated that the *Manduca* larval Br-CC-CA could be maintained *in vitro* for 3 days and photo-periodically reprogrammed. The method involves the culture of Br-CC-CA (with an intact tracheal supply) for 3 days. With this system in mind, a similarly designed *in vitro* investigation has been initiated to demonstrate the possible direct effect of JH on PTTH secretion from pre-commitment Br-CC-CA in *Manduca*. Preliminary results indicate that the hormone does act directly to prevent PTTH release (D. B. Rountree & W. E. Bollenbacher, unpublished). If confirmed, these results would constitute the first demonstration of a direct action of JH on neural tissue, and the system could be an excellent model for probing mechanistic aspects of the endocrine control of cerebral neurosecretion in insects.

Although topically applied JH delays PTTH secretion in pre-commitment, last instar larvae of *Manduca*, this result does not prove that JH functions in this manner *in situ*. This can only be proved by the demonstration that the JH haemolymph titre in pre-commitment larvae fluctuates in the manner predicted by its permissive control of PTTH release. According to this hypothesis, the JH titre would have to drop dramatically in gate II, last instar larvae by the beginning of the scotophase on day 3, so that the gated release of PTTH from the CA could occur at its established time. Haemolymph titres of JH have been determined for early last instar *Manduca* larvae reared under short-day conditions (L:D 12:12) by both bioassay (see Riddiford & Truman, 1978) and physico-chemical methods (D. Schooley, personal communication). Although a complete titre has not been published, the data available indicate JH levels are elevated for the first 2 days of the instar and drop precipitously between days 2 and 3. Although this titre should be compared with caution to that in larvae reared under long-day conditions (L:D 16:8), it does appear to decrease just before the gated secretion of PTTH during pupal commitment (Nijhout & Williams, 1974b). It is not known whether JH affects PTTH release during larval moulting in the same way, but bioassay-derived, JH titre data for the fourth larval instar of *Manduca* (Fain & Riddiford, 1975) suggest that PTTH secretion may be regulated in a different way. JH does not appear to regulate PTTH release in the early pupa as well (Bowen *et al.* 1985).

Since JH plays an active, although permissive, role in the intrinsic regulation of PTTH release in early last instar *Manduca* larvae, the mechanisms by which synthesis and/or turnover of JH are controlled become the critical regulators of the developmental status of the larva. These mechanisms and the means by which developmental information is transduced to affect them become a central problem in understanding the endocrine regulation of insect postembryonic development. To date, it is not clear how the developmental programme can evoke synthesis of JH by the CA at one time and can curtail its synthesis at another, nor what specific cues are involved (see Nijhout, 1981). It is known, however, that several mechanisms may be involved in controlling the activity of the CA in Lepidoptera (see Bhaskaran, Jones & Jones, 1980; deKort & Granger, 1981; Granger, Mitchell, Janzen & Bollenbacher, 1984; Whisenton *et al.* 1985), and regulation has been proposed to involve either direct nervous control or regulation by neurohormones, or both. The critical role of JH as a primary interendocrine feedback regulator of larval-pupal development in *Manduca* underscores the importance of studies of the signals that are transduced to elicit a decrease in the JH titre and of the nature of the transductions.

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