REGULATION OF ECDYSONE BIOSYNTHESIS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*: TITRE OF THE HAEMOLYMPH STIMULATORY FACTOR DURING THE LAST LARVAL INSTAR

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SUMMARY

A recently isolated haemolymph protein appears to be an important regulator of ecdysone biosynthesis by prothoracic glands in *Manduca sexta*. Using a dose–response titration protocol, the haemolymph titre of this stimulatory factor was determined during the last larval instar. The titre was high ($>2 \cdot 0\,\mathrm{U\,ml}^{-1}$) on days 0 and 1, then dropped significantly to $0 \cdot 55\,\mathrm{U\,ml}^{-1}$ on day 2, and remained depressed until day 4. The titre of the stimulatory factor then increased to a peak of $1 \cdot 62\,\mathrm{U\,ml}^{-1}$ on day 7, and remained elevated (approx. $1 \cdot 1\,\mathrm{U\,ml}^{-1}$) until the end of the instar. A set of physical and biochemical criteria was used to confirm that the stimulatory activity present in haemolymph on different days of the instar represented the presence of the factor. The data are consistent with the hypothesis that fluctuations in the titre of the haemolymph stimulatory factor play a critical role in regulating ecdysone biosynthesis during larval–pupal development.

INTRODUCTION

Ecdysteroids are steroid hormones that elicit moulting and metamorphosis in insects as a result of their stage-specific effects on target tissues (see Riddiford, 1980a; Koolman & Spindler, 1983). Precise regulation of the haemolymph ecdysteroid titre is therefore requisite for the normal progression of insect development. In the tobacco hornworm (*Manduca sexta*) the ecdysteroid titre is regulated, in large part, by controlling the rate at which ecdysone is synthesized and secreted by the prothoracic glands (see Smith, 1985).

The principal regulator of the prothoracic glands is the neuropeptide prothoracicotropic hormone (PTTH) (see Bollenbacher & Bowen, 1983; Bollenbacher & Granger, 1985). At specific times during development, PTTH is released from the corpora allata into the haemolymph (Agui, Bollenbacher, Granger & Gilbert, 1980). PTTH then acts *via* a Ca^{2+}-dependent cyclic AMP second messenger to activate the prothoracic glands (Smith, Gilbert & Bollenbacher, 1984, 1985; Smith & Gilbert,

1986), increasing the rate of ecdysone biosynthesis (Bollenbacher, Agui, Granger & Gilbert, 1979; Bollenbacher, O'Brien, Katahira & Gilbert, 1983).

While PTTH is generally recognized as the primary regulator of the prothoracic glands, recent research has revealed the regulatory process is more complex than previously considered. It has become increasingly apparent that gland activity is also influenced by a number of secondary regulators; these include environmental cues (Meola & Adkisson, 1977; Mizoguchi & Ishizaki, 1982), direct neural input (see Richter & Gersch, 1983), juvenile hormone (Hiruma, Shimada & Yagi, 1978; Hiruma, 1980; Safranek, Cymborowski & Williams, 1980; Cymborowski & Zimowska, 1984), ecdysteroids (Beydon & Lafont, 1983) and non-cerebral humoral factors (Meola & Gray, 1984; Gruetzmacher et al. 1984a; Gruetzmacher, Gilbert & Bollenbacher, 1984b).

In *Manduca*, it appears that the most important of these secondary regulators is a factor recently isolated from larval haemolymph (Watson et al. 1985; Watson, Whisenton, Bollenbacher & Granger, 1986). This heat-labile, low M_r (approx. $30 kD = 30\,000$) protein stimulates ecdysone synthesis *in vitro* by about five-fold (Watson et al. 1985). Further, its steroidogenic effects are additive with those of PTTH. This is a clear indication that the stimulatory factor and PTTH enhance steroidogenesis *via* different cellular mechanisms (Smith, Watson, Gilbert & Bollenbacher, 1986). Although the precise chemical nature of the stimulatory factor is not known, it is hypothesized that the molecule transports to the prothoracic glands a sterol precursor from which ecdysone is synthesized (Watson et al. 1985).

The immediate stimulus for the present study was our preliminary finding that the titre of the stimulatory factor increases between day 3 and day 6 of the last larval instar (Watson et al. 1985, 1986), a pattern that suggested the molecule may be a limiting factor in ecdysone biosynthesis and, consequently, an important regulator of the ecdysteroid titre. During the last larval instar of *Manduca*, there are two peaks in the ecdysteroid titre (Bollenbacher, Smith, Goodman & Gilbert, 1981). The initial peak (day 4–5) is small in magnitude (approx. $60\,\mathrm{ng\,ml^{-1}}$ haemolymph); it elicits a change in the developmental commitment of target tissues, i.e. it reprogrammes the tissues from larval to pupal macromolecular syntheses. The second peak (day 7–8) is considerably larger (approx. $1{\cdot}5\,\mu\mathrm{g\,ml^{-1}}$ haemolymph); it stimulates moulting, but because of the change in commitment, the moult is metamorphic, i.e. to a pupa. Thus, our preliminary results suggested that the titre of the stimulatory factor was low just prior to the small peak in the ecdysteroid titre, and much higher prior to the large increase in the ecdysteroid titre. We hypothesized that the relative size of the ecdysteroid peaks is determined by the amount of stimulatory factor present in the haemolymph at times when PTTH is released to activate the prothoracic glands.

To define more clearly the role of the stimulatory factor in regulating prothoracic gland biosynthetic activity, we report here a complete haemolymph titre for the molecule during the last larval instar. The results support the hypothesis that fluctuations in the level of the stimulatory factor play a critical role in regulating the ecdysteroid titre during larval–pupal development in *Manduca*.

MATERIALS AND METHODS

Animals

Manduca sexta larvae were reared individually on an artificial diet under a non-diapausing photoperiod (L:D 16:8) at 26°C, and were staged as described previously (Vince & Gilbert, 1977; Rountree & Bollenbacher, 1986).

Haemolymph stimulatory factor

Isolation

Haemolymph was collected from larvae through slits in the prolegs. After the addition of glutathione (approx. 1.5 mg ml^{-1}) to inhibit oxidation, the haemolymph was centrifuged ($12\,000\,g$ for 20 min) to remove haemocytes. A 2-ml sample of the resulting supernatant was fractionated by gel filtration chromatography on Sephadex G-100 as described previously (Watson *et al.* 1985). Column fractions containing the factor were pooled and concentrated to 0.5 ml by ultrafiltration (Multi-Micro system with YM-10 filters; Amicon Corporation, Danvers, MA). Once prepared, the haemolymph factor was diluted and used immediately.

In vitro *assay*

Pairs of day 7 fifth larval instar prothoracic glands were dissected in lepidopteran saline (Weevers, 1966), transferred to Grace's tissue culture medium (GIBCO, Grand Island, NY), and held no longer than 1 h prior to use. The standard assay for haemolymph stimulatory factor was as previously described (Watson *et al.* 1985). Briefly, one gland of a pair was incubated in a 0.025 ml standing drop of culture medium containing a test sample of the haemolymph factor; the contralateral gland was incubated in 0.025 ml of culture medium alone. The glands were maintained for 2 h at 25°C, after which a 0.005 ml sample of medium was removed from each incubation well for assay of ecdysone content using the macro-radioimmunoassay (RIA) (see Bollenbacher *et al.* 1983). The ecdysteroid antibody used (H-3) was generated in rabbits against an ecdysone-22-succinyl thyroglobulin conjugate synthesized by Dr D. H. S. Horn (CSIRO, Melbourne, Australia); its antigenic specificity has been described previously (Gilbert, Goodman & Bollenbacher, 1977). [23,24-^{3}H]Ecdysone (60 Ci mmol^{-1}; New England Nuclear Corporation, Boston, MA) adjusted to 4 Ci mmol^{-1} was the labelled ligand; ecdysone (standard range: 0.25–32.0 ng) was the competing unlabelled ligand. Bound and free ligand were separated using staphylococcal protein A (Warren, Smith & Gilbert, 1984). In certain instances, the effect of the haemolymph factor on ecdysone biosynthesis was expressed as a stimulation ratio (S_r), which is the amount of ecdysone synthesized by the factor-stimulated prothoracic gland divided by the amount synthesized by the control gland.

Titration

The dose–response method used to titrate the stimulatory factor was essentially that used previously to titrate PTTH levels in *Manduca* tissues and haemolymph

(Agui, Granger, Gilbert & Bollenbacher, 1979; Agui *et al.* 1980; Bollenbacher & Gilbert, 1982; O'Brien *et al.* 1986; Bollenbacher, Granger, Katahira & O'Brien, 1987). Briefly, serum from 10–12 precisely staged larvae was pooled and processed according to the isolation protocol described above. Once isolated, stimulatory factor was diluted serially with Grace's medium and assayed at doses equivalent to 2·0, 1·0, 0·5, 0·25 and 0·125 times the concentration of factor in haemolymph. Using this dose–response protocol, the amount of stimulatory factor required to achieve half-maximal stimulation (the ED_{50}) is a measure of how much factor is present in a sample. Variation between assays in the maximum level of stimulation (S_{max}) is an inherent property of such *in vitro* bioassays. However, this did not alter the ED_{50} obtained for a given sample, nor did it significantly affect ED_{50} values between samples from the same developmental stage. Thus, comparison between samples of the reciprocal of ED_{50} values allowed a determination of the relative amount of factor in each sample. The level of stimulatory factor in a sample was expressed in units (U), 1 U being the stimulatory factor activity present in 1 ml of day 6 larval haemolymph. Loss of activity during sample preparation was assumed to be constant between samples.

Determination of heat stability

The heat stability of stimulatory factor from day 0, day 6 and day 9 haemolymph was determined by heating a partially purified preparation of each for 2 min at 100 °C. The heated samples were then centrifuged (5000 *g* for 10 min), and the resulting supernatants assayed for stimulatory factor activity.

Quantification of cyclic nucleotides

The accumulation of cyclic AMP in prothoracic glands was assayed by the method of Shimizu, Daly & Creveling (1969), as modified by Meeker & Harden (1982). Glands were preincubated individually in 0·025 ml of Grace's medium containing 1 μCi [^{3}H]adenine (29 Ci mmol^{-1}; ICN, Irvine, CA) for 90 min, rinsed in fresh Grace's medium, and placed in 0·025 ml of medium containing partially purified big PTTH (0·2 U, a saturating dose; see Bollenbacher *et al.* 1984), stimulatory factor (1 U ml^{-1}) or no treatment. Following incubation for 20 min, a time previously found to coincide with enhanced levels of both cyclic AMP and ecdysone synthesis (Smith *et al.* 1984), glands were placed in 0·2 ml of ice-cold trichloroacetic acid (TCA) and maintained at 4 °C overnight. Chromatographic separation of the [^{3}H]cyclic AMP and [^{3}H]ATP extracted from glands was accomplished by the method of Salamon, Londos & Rodbell (1974), as described previously (Smith *et al.* 1984). Accumulation of cyclic AMP was expressed as a percentage of conversion of [^{3}H]ATP to [^{3}H]cyclic AMP.

High performance liquid chromatography (HPLC) of ecdysteroids

Media from incubations of day 7 prothoracic glands were pooled, then extracted for ecdysteroids using Sep-Pak C18 cartridges (Waters Associates, Milford, MA) as described by Watson & Spaziani (1982). An LKB (Gaithersburg, MD) 2150 HPLC

pump, 2152 controller and 2140 Rapid Spectral Detector were used for HPLC analyses. Samples were fractionated by normal phase HPLC using a 0.46×25 cm Zorbax Sil column (DuPont, Wilmington, DE) and a solvent system of HPLC-grade methylene chloride/isopropanol/water ($125:25:2$) pumped at 1 ml min^{-1}. Fractions (1 ml) were collected at 1-min intervals. A 0.1 ml sample of each fraction was dried and assayed for ecdysteroids using the macro-RIA described above. Samples of ecdysone standard were run in parallel to allow an estimation of percentage recovery.

Statistical analyses

The statistical significance of differences among means was determined using a single classification analysis of variance and Student–Newman–Keuls (SNK) test procedure. By convention, such data are arrayed in order of magnitude, and any pair of means enclosed by the range of a bracket is not significantly different (95 % confidence level) (Sokal & Rohlf, 1969). Test results are shown in the appropriate figure legends.

RESULTS

Titration of the haemolymph stimulatory factor

Before the haemolymph titre of the stimulatory factor could be determined, it was necessary to establish an accurate and reproducible quantification protocol. The method employed was to titrate the biosynthetic response of prothoracic glands to different doses of stimulatory factor. The ED_{50} from such a dose–response titration is a measure of how much factor is in a sample.

To illustrate the range of responses obtained, three representative dose–response titration curves are shown in Fig. 1. The dose–response curve in the upper panel was generated using stimulatory factor from day 0 fifth instar haemolymph (V_0). The S_{max} was 3.8 and the ED_{50} was 0.16 haemolymph equivalents. The reciprocal of the ED_{50} for this titration is 6.25. When normalized to $1/ED_{50}$ for day 6 haemolymph (1 U of stimulatory factor), this is equivalent to 2.54 U ml^{-1}. The reproducibility of this quantification protocol was demonstrated by the fact that four separate titrations of day 0 haemolymph yielded S_{max} values ranging from 2.5 to 5.0, ED_{50} values from 0.15 to 0.40 haemolymph equivalents, and units of stimulatory factor activity from 1.02 to 2.71.

In contrast to the relatively high level of factor detected in day 0 haemolymph, a titration of day 3 haemolymph (V_3) (Fig. 1, middle panel) revealed that the level of stimulatory factor had dropped below the lower limit of detection of the assay. In two additional titrations of day 3 haemolymph, the titre of stimulatory factor was similarly low, requiring at least 2.0 haemolymph equivalents to achieve S_{max}.

For day 7 haemolymph (V_7), the dose–response titration of stimulatory factor again revealed a relatively high level of activity (Fig. 1, bottom panel). The ED_{50} for the representative curve shown was 0.18 haemolymph equivalents, and the S_{max} was 6.0. In four separate titrations, the S_{max} values ranged from 2.8 to 6.0, and the ED_{50} values from 0.18 to 0.43 haemolymph equivalents, the latter representing
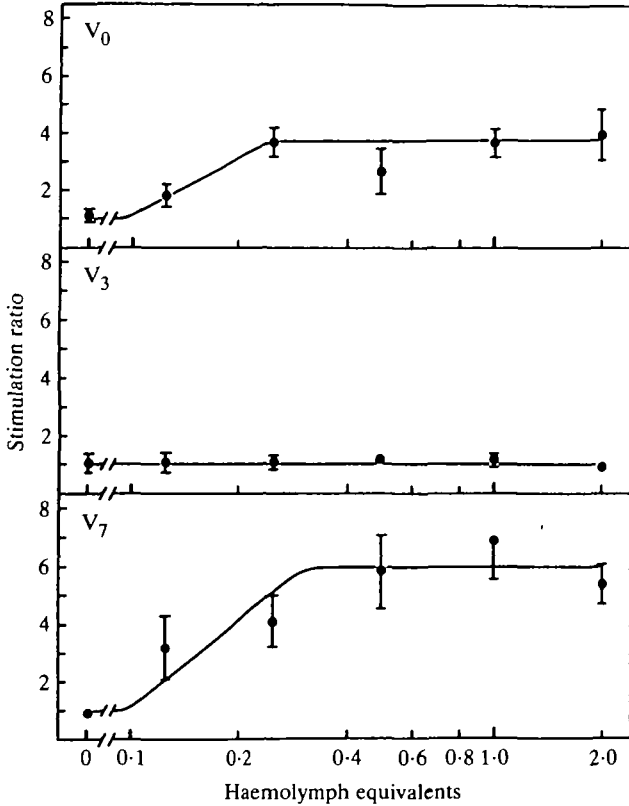
Fig. 1. Dose–response titration of the amount of stimulatory factor present in the haemolymph of day 0 (V_0), day 3 (V_3) and day 7 (V_7) last instar *Manduca sexta* larvae. Prothoracic glands were incubated for 2 h in control medium or medium containing a test sample of stimulatory factor. The response is expressed as a stimulation ratio. The dose of haemolymph factor is expressed in haemolymph equivalents, with 1·0 haemolymph equivalent being the concentration of factor found in normal haemolymph. Each point is the mean ± s.e.m. of 3–4 separate determinations.

0·95–2·56 U of activity. In each determination, the concentration of stimulatory factor present in day 7 haemolymph was greater than that required to activate the glands maximally.

The results indicated the dose–response titration protocol could be used to detect and quantify fluctuations in the level of the haemolymph stimulatory factor during larval–pupal development in *Manduca*.

Titre of the haemolymph stimulatory factor

The dose–response titration protocol was used to determine the amount of stimulatory factor present in *Manduca* haemolymph on each day of the last larval instar (Fig. 2). At the time of ecdysis to the fifth larval instar, the titre of stimulatory factor was high ($2·05 ± 0·44$ U ml^{-1}). The titre remained elevated through day 1, then dropped sharply to $0·55 ± 0·33$ U ml^{-1} on day 2 ($P < 0·05$). The amount of stimulatory factor present in haemolymph remained low from day 2 to day 4, then

increased to a peak of $1 \cdot 62 \pm 0 \cdot 38$ U ml^{-1} on day 7, a level which was not significantly different from that on days 0 and 1 ($P > 0 \cdot 05$). The apparent decrease in the titre on days 8 and 9 was not statistically significant ($P > 0 \cdot 05$).

In summary, the level of the stimulatory factor in *Manduca* haemolymph fluctuates significantly during the last larval instar, and those fluctuations occur at times which suggest the molecule may play a critical role in regulating the ecdysteroid titre.

Verification of the titre of the stimulatory factor

Since the titration protocol employed to measure the amount of stimulatory factor in haemolymph was indirect (i.e. it measured the biosynthetic response of a target gland rather than measuring a physical property of the factor), it was conceivable that the activity detected on different days of the instar was due to agents other than the stimulatory factor. It was therefore necessary to demonstrate that the activity
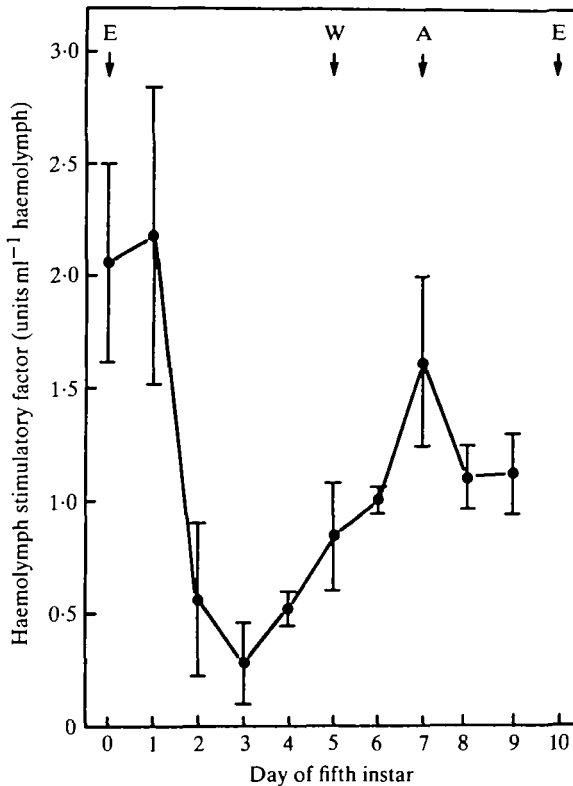

Fig. 2. Titre of the haemolymph stimulatory factor during the last larval instar of *Manduca sexta*. The amount of stimulatory factor present on each day was determined by dose–response titration. Ecdysis, wandering and cuticle apolysis are denoted by E, W and A, respectively. Each point is the mean $\pm$ S.E.M. of 3–4 separate determinations. SNK test results (any pair of means enclosed by the range of a bracket is not significantly different, $P \leqslant 0 \cdot 05$): ($0 \cdot 27$–$1 \cdot 62$), ($0 \cdot 51$–$2 \cdot 05$), ($0 \cdot 84$–$2 \cdot 17$).

Table 1. *Effect of heat treatment on the activity of stimulatory factor isolated from the haemolymph of last instar* Manduca sexta *larvae*

Source of stimulatory factor	Stimulation ratio		Percentage activity lost
	Before heat treatment	After heat treatment	
Day 0	4·24 ± 0·35	1·56 ± 0·32	82·7*
Day 6	3·26 ± 0·66	1·39 ± 0·39	82·7**
Day 9	4·18 ± 0·98	1·20 ± 0·13	93·7**

Stimulatory factor was isolated from the haemolymph of day 0, day 6 or day 9 last instar larvae. Glands were incubated for 2 h with untreated stimulatory factor or with factor that had been heat-treated at 100°C for 2 min. Each stimulation ratio is the mean ± S.E.M. of three incubations. *$P < 0·01$, **$P < 0·05$.

detected throughout the instar was due to the presence of the factor. The experimental strategy was to determine whether samples isolated during critical times of the instar exhibited properties which define the haemolymph stimulatory factor (Watson *et al.* 1985). The properties used to characterize the factor were as follows: (1) its apparent relative molecular mass, (2) the kinetics by which it stimulated steroidogenesis, (3) its heat lability, (4) its cellular mechanism of action, and (5) the identity of the ecdysteroid homologue secreted in response to the factor.

Apparent relative molecular mass

Using gel filtration chromatography, the apparent M_r of stimulatory factor in day 6 haemolymph was estimated previously to be 30 kD (Watson *et al.* 1985). The efficiency (i.e. peak sharpness) of the liquid chromatographic method employed was such that fractions eluting between approx. 20 and 40 kD contained activity. In each titration reported here, stimulatory factor activity eluted in fractions encompassing that same range of relative molecular mass.

Kinetics of stimulation

As illustrated in Fig. 1, when stimulatory factor was detectable in the haemolymph, its steroidogenic effects on prothoracic glands were dose-dependent, saturable, and occurred over a narrow concentration range. That the kinetics of prothoracic gland stimulation are similar for factor isolated on different days of the instar (see Watson *et al.* 1985) provides further evidence that the biological activity assayed is the same throughout the instar.

Heat lability

Heat treatment destroys the biological activity of stimulatory factor isolated from day 6 haemolymph (Watson *et al.* 1985). Similarly, exposure of stimulatory factor isolated on other days of the instar to 100°C for 2 min resulted in an almost complete loss of biological activity (Table 1).

Cellular mechanism of action

Stimulatory factor isolated from day 6 haemolymph enhances steroidogenesis by a cyclic AMP-independent mechanism (Smith *et al.* 1986). To determine whether this property is common to stimulatory factor isolated from haemolymph on other days of the instar, formation of cyclic AMP and secretion of ecdysone were monitored in control (unstimulated) prothoracic glands and glands incubated in the presence of stimulatory factor isolated from day 1, day 6 or day 9 haemolymph. Since cyclic AMP is a known second messenger in the stimulation of steroidogenesis by PTTH (Smith *et al.* 1984, 1985; Smith & Gilbert, 1986), PTTH-stimulated glands were included as a control. The effects of stimulatory factor and PTTH on ecdysone biosynthesis were determined after 20 min, the length of the standard cyclic AMP assay.

Incubation of glands with PTTH resulted in a >50-fold increase in cyclic AMP formation ($P < 0.05$) (Fig. 3B). The apparent doubling in ecdysone synthesis by PTTH-stimulated glands was not statistically significant ($P > 0.05$) (Fig. 3A), a finding consistent with previous results showing a 10- to 20-min lag between the time the glands are exposed to PTTH and the onset of ecdysone synthesis (Smith *et al.* 1984, 1986). In contrast, cyclic AMP levels were not enhanced in glands incubated in the presence of haemolymph stimulatory factor ($P > 0.05$) (Fig. 3B), even though each test sample of stimulatory factor effected a significant increase in ecdysone synthesis ($P < 0.05$) (Fig. 3A). The observation that each of the test samples of factor stimulated steroidogenesis by a cyclic AMP-independent mechanism provided further evidence that the activity assayed on different days of the instar was the stimulatory factor.

Stimulation of ecdysone synthesis

The final criterion used to demonstrate that the activity detected on different days of the fifth larval instar reflected the presence of stimulatory factor was the identity of the ecdysteroid homologue secreted in response to the factor.

A previous report indicated that unstimulated *Manduca* prothoracic glands secrete only ecdysone (King *et al.* 1974). Our results confirm this. When 12 prothoracic glands were incubated individually for 4 h in Grace's medium, the pooled media contained 396 ng of ecdysone RIA equivalents; HPLC analysis revealed that the only RIA-detectable ecdysteroid present in that media eluted with ecdysone standard (data not shown). Similarly, ecdysone was the only ecdysteroid secreted by factor-stimulated prothoracic glands. When 12 prothoracic glands were incubated individually for 4 h in the presence of day 6 stimulatory factor, the pooled media contained 966 ng of ecdysone RIA equivalents, a 2·4-fold increase over synthesis in control medium. After HPLC, 94·1 % of that activity was recovered in fractions having a retention time identical to that of standard ecdysone (Fig. 4). Since recovery of standard ecdysone in parallel experiments was 90–95 %, the results indicate the increase in steroidogenesis elicited by day 6 stimulatory factor is accounted for by an increase in ecdysone biosynthesis. Similar results were obtained when prothoracic glands were incubated with stimulatory factor from day 1 or day 9

haemolymph: in each case there was an increase in ecdysteroid biosynthesis, and essentially all of that increase was detectable in a single peak that eluted with ecdysone standard.

Given the lower limit of detection of the macro-RIA (0·25 ng), and the fact that only a single antiserum was used in this study, it is conceivable that the haemolymph factor also stimulated the synthesis of ecdysteroids other than ecdysone. However, the data indicate that ecdysteroids other than ecdysone could constitute <5 % of the total ecdysteroids secreted, indicating that ecdysone is the primary secretory product of factor-stimulated prothoracic glands regardless of the haemolymph source of the stimulatory factor.

The results indicate that the stimulatory activity titrated during the last larval instar was due to the haemolymph stimulatory factor. Further, the data validate the
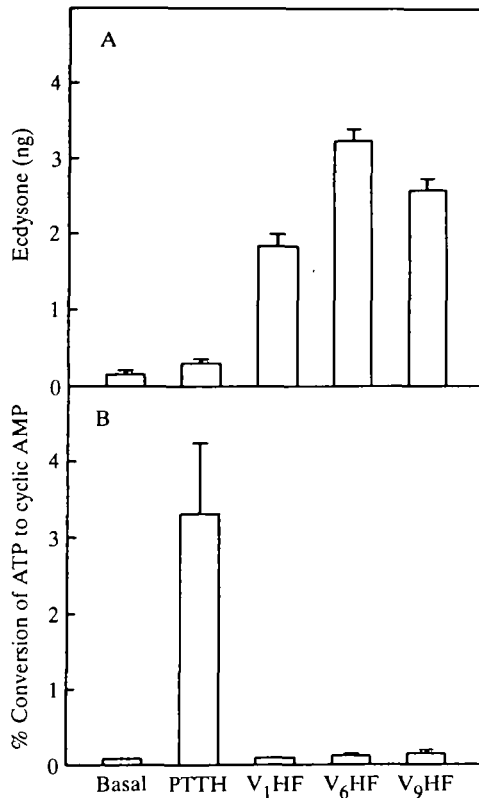

Fig. 3. Effects of the haemolymph stimulatory factor and of prothoracicotropic hormone (PTTH) on the secretion of ecdysone (A) and accumulation of cyclic AMP (B) by day 3 last larval instar *Manduca sexta* prothoracic glands *in vitro*. Glands were incubated for 20 min with PTTH (0·2 U), or with stimulatory factor (1·0 U ml^{-1}) from day 1 (V$_1$HF), day 6 (V$_6$HF) or day 9 (V$_9$HF) haemolymph. Bars represent mean ± S.E.M. for eight glands. SNK test results for ecdysone secretion (any pair of means enclosed by the range of a bracket is not significantly different, $P \leqslant 0·05$): (0·14–0·31), (1·83–2·58), (2·58–3·26). SNK test results for cyclic AMP accumulation ($P \leqslant 0·05$): (0·06–0·13), (3·30).
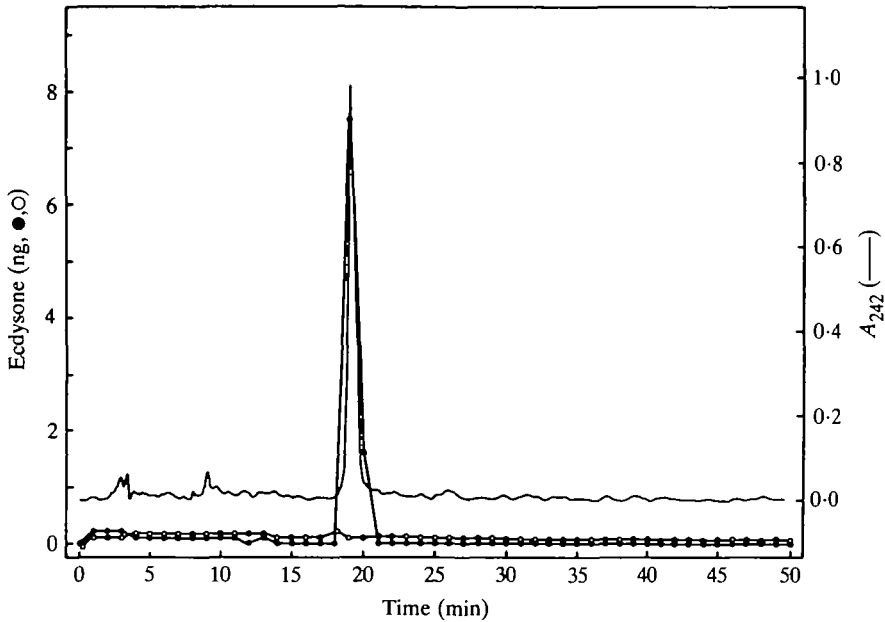
Fig. 4. High performance liquid chromatography (HPLC) separation of the ecdysteroid synthesized by prothoracic glands incubated with day 6 haemolymph stimulatory factor. Open circles represent the ecdysone radioimmunoassay activity in the medium before incubation, and closed circles the activity in medium after incubation. Ecdysone standard (A_{242}) eluted at 19·5 min. HPLC separations were performed on a normal phase Zorbax Sil column using a solvent system of methylene chloride/isopropanol/water, 125:25:2.

finding that the haemolymph titre of the molecule fluctuates significantly during larval–pupal development.

DISCUSSION

An increasing body of evidence suggests that the haemolymph stimulatory factor plays a critical role in regulating ecdysone biosynthesis during *Manduca* development (Watson *et al.* 1985, 1986; Smith *et al.* 1986). Our working hypothesis is that the relative size of the two peaks in the ecdysteroid titre during the last larval instar is determined by the amount of stimulatory factor present in the haemolymph when PTTH is released. The data reported here are consistent with that hypothesis.

PTTH is released during two periods in the last larval instar of *Manduca* (see Bollenbacher & Gilbert, 1982). The initial release of PTTH, which appears to occur in three distinct bursts, begins on day 3 and spans approximately 18 h. During this period, the titre of the stimulatory factor is at its lowest level for the instar, and is below that required for the stimulation of ecdysone biosynthesis. Consequently, the glands are activated solely by PTTH, and the resulting increase in the ecdysteroid titre (the pupal commitment peak) is small in magnitude (approx. $60 \, \text{ng ml}^{-1}$). PTTH is released again on day 7, this time in a single burst. By day 7, the titre of the stimulatory factor has risen to a saturating level for ecdysone synthesis. As a result,

the glands are maximally stimulated by the combined effects of the factor and PTTH, and the consequent peak in the ecdysteroid titre (the moult-stimulating peak) is large ($>1 \cdot 5 \, \mu g \, ml^{-1}$). Thus, although it is conceivable that the mechanism of PTTH release (pulsatile vs a single burst), or the preferential release of a specific molecular form of PTTH (Bollenbacher et al. 1984), could account for the dramatic quantitative differences in the two peaks in the ecdysteroid titre, the present results suggest those differences are dictated by the titre of the haemolymph stimulatory factor.

Paradoxically, however, the amount of stimulatory factor present in haemolymph is also high on days 0 and 1, a time when the ecdysteroid titre is low. This seeming contradiction – a low ecdysteroid titre in the presence of high levels of stimulatory factor – is apparently explained by the finding that prothoracic glands are not competent to respond to the stimulatory factor for the first several days of the last larval instar (Ciancio, Watson & Bollenbacher, 1986). Thus, even though the titre of the stimulatory factor is high on days 0 and 1, the ecdysteroid titre remains depressed because the prothoracic glands have not yet achieved the functional maturity required for a significant biosynthetic response. While the incompetence of the prothoracic glands on days 0–1 appears to explain why the ecdysteroid titre stays low on those days, the fact remains that the stimulatory factor titre is high during that period, and thus the moiety could conceivably have an alternative function at this time in the instar.

The finding of significant fluctuations in the level of stimulatory factor suggests that the titre of the molecule is regulated. There are several indications that the titre may be regulated by juvenile hormone (JH). First, the titre of the stimulatory factor is high whenever the JH titre is high (see Riddiford, 1980b; Baker, Tsai, Reuter & Schooley, 1987) and whenever the corpora allata are actively synthesizing JH (Granger, Niemiec, Gilbert & Bollenbacher, 1982). Second, the haemolymph stimulatory factor appears to be identical to a JH-regulated protein released in vitro from Manduca fat body (Gruetzmacher et al. 1984b). And finally, data from our laboratory indicate that the haemolymph titre of the stimulatory factor can be altered by perturbing the JH titre (Watson, Agui, Haire & Bollenbacher, 1987).

As stated above, the precise chemical nature of the haemolymph stimulatory factor is not known. Our hypothesis is that the molecule transports a sterol substrate utilized by the prothoracic glands in ecdysone biosynthesis. In comparable vertebrate systems, cholesterol is the primary sterol substrate utilized by steroidogenic endocrine glands; cholesterol is transported in the blood of vertebrates bound to high M_r lipoprotein molecules (see Brown, Kovanen & Goldstein, 1979). The situation appears to be similar in the silkworm, Bombyx mori, where high M_r lipoproteins (lipophorins) apparently transport the ecdysteroid precursor cholesterol to prothoracic glands (Chino et al. 1974). However, in Manduca the high M_r lipoproteins found in haemolymph do not stimulate steroidogenesis (Watson et al. 1985). Further, cholesterol may not be the immediate sterol precursor utilized by Manduca prothoracic glands (Bollenbacher, Galbraith, Gilbert & Horn, 1977; Gilbert et al. 1977). If the substrate carrier hypothesis is borne out by future

experimentation, the finding of fluctuations in the level of such a molecule as a means of regulating steroidogenesis would be, to our knowledge, unique. When vertebrate steroid-secreting endocrine glands are chronically stimulated, the amount of sterol substrate available for steroidogenesis is increased by enhancing the capacity of the glands to bind and take up lipoprotein molecules (see Brown *et al.* 1979; Gwynne & Strauss, 1982) which exist in the blood at a relatively constant level (Brown, Kovanen & Goldstein, 1981).

In summary, the results of this study support the hypothesis that the haemolymph stimulatory factor plays an important role in regulating the synthesis of ecdysone by prothoracic glands. Specifically, the data suggest it is the relationship between the titre of the stimulatory factor and the release of PTTH that accounts for the precise regulation of the ecdysteroid titre during larval–pupal development of *Manduca*.

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