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PROTHORACICOTROPIC HORMONE IN *MANDUCA SEXTA*: LOCALIZATION BY A LARVAL ASSAY

BY DANIEL GIBBS* AND LYNN M. RIDDIFORD

Department of Zoology, University of Washington, Seattle, Washington 98195

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SUMMARY

1. A new method for the assay of insect prothoracicotropic hormone (PTTH) is described, using fourth instar larvae of *Manduca sexta*. Larvae neck-ligated at a critical time to prevent release of PTTH from the head fail to undergo the next larval moult. Such ligated larvae moult to fifth instar larvae or larval–pupal intermediates after injection of brain homogenates from *Manduca* larvae, pupae or pharate adults. The degree of response is proportional to the concentration of brain homogenate injected.

2. The source of PTTH in the pupal brain is the dorsal region of the protocerebrum containing the lateral neurosecretory cells. Microhomogennates of single pieces of brain showed activity with this method.

3. PTTH activity in partially purified extracts is water soluble, stable to boiling for 10 min, and is destroyed by Pronase or trypsin.

INTRODUCTION

The insect brain has for some years been known to contain a factor which promotes moulting, metamorphosis and adult development (Kopec, 1922; Wigglesworth, 1940; Williams, 1946). These processes occur in response to ecdysone, a steroid hormone liberated by the prothoracic glands (Wigglesworth, 1970). The role of the brain appears to be the synthesis and release of a prothoracicotropic hormone (PTTH) which activates these glands. Previous efforts to assay PTTH ('brain hormone', ecdysiotropin) from insect brains have made use of brainless lepidopteran pupae. Such pupae deprived of their brains before the initiation of adult development will fail to develop (Williams, 1946) and enter a state of diapause, even in species without a normal pupal diapause. Implantation of brains or injection of brain extracts into these 'dauer-pupae' results in adult development if the brains or extracts contain PTTH (Kobayashi & Yamazaki, 1966).

Identification of the neurosecretory cells which produce PTTH requires a sensitive bioassay which will detect the hormone in small bits of brain tissue. Fourth instar *Manduca sexta* larvae have been found to release PTTH during a certain well-defined interval or 'gate' on either the second or the third night following ecdysis (Truman, 1972). Experiments by Fain & Riddiford (1976) showed that larvae neck-ligatured

* Present address: Department of Biological Sciences, DePaul University, 1036 West Belden Avenue, Chicago, Illinois 60614, U.S.A.

just at the opening of this 'gate' did not moult to the next larval instar, but subsequently initiated metamorphosis to pupae within 9 days. When similar neck-ligatured larvae were immediately injected with ecdysone, they moulted to fifth instar larvae in 2–3 days. The difference in response was attributed to the different levels of juvenile hormone present at the time ecdysone initiated the moult. Thus, it seemed that these larvae might provide the sensitive PTTH bioassay sought since a larval response would indicate a rapid activation of the prothoracic glands. This paper reports the development of this relatively sensitive assay for PTTH and its application to determine the source of PTTH in the pupal brain of *Manduca*.

MATERIALS AND METHODS

Animals

Tobacco hornworms, *Manduca sexta*, were derived from a strain obtained originally from Dr R. A. Bell (Agricultural Research Service, U.S.D.A., Fargo, North Dakota) and reared according to the methods described by Truman (1972) and Bell & Joachim (1976) with slight modifications. A breeding culture was maintained under long-day conditions (17L:7D) at 25–26 °C. Experimental animals used for the assay or as a source of hormone were reared under a 12L:12D photoperiod at the same temperature. Times are given as arbitrary zeitgeber time (AZT) (Pittendrigh, 1965) with lights-off designated as 24.00 AZT.

Manduca larvae were selected on the day of ecdysis to the fourth instar. Routinely, such larvae initiate the moult to the fifth instar by releasing PTTH from the brain on either the second or third night after ecdysis to the fourth instar. PTTH release occurs only during a restricted portion of the night (Truman, 1972) and is thus 'gated' by the photoperiod (Skopik & Pittendrigh, 1967).

About an hour before lights-off on the second day after ecdysis to the fourth instar, larvae weighing 0·9 g or more were examined for head capsule slippage or spiracle apolysis (see Results for a description). Larvae with these characters were discarded since during the previous night they had released PTTH to initiate moulting. All the others (designated Gate II larvae) would release PTTH during this third night.

Ligations and injections

Larvae were anaesthetized with carbon dioxide and ligatured tightly about the neck with unwaxed dental floss at 23.30 AZT ± 15 min. Shortly thereafter, the ends of the ligature were trimmed and the ventral portion of the head cut away to ensure removal of the brain.

Within 2 h after ligation, test extracts were injected into relaxed anaesthetized larvae at the base of an anterior proleg using a 50 or 100 μl Hamilton syringe with a $\frac{1}{2}$ in, 30-gauge needle. After injection ligated larvae were kept in plastic Petri dishes on filter paper at 25·5° ± 0·5 °C.

Extracts

Brains and other ganglia or organs were routinely rinsed in insect saline (Ephrussi & Beadle, 1936) and stored frozen in sealed plastic wells (Linbro) at −20 °C until use. Frozen organs were thawed, homogenized in a small glass homogenizer with distilled water, and diluted to give the appropriate dose in an injection volume of 5–7 μl.

Extracts of single brain pieces containing neurosecretory cells were made by pinning the brain to a black wax dish under insect saline. The brain sheath was pulled back with fine forceps and the desired piece excised with electrolytically sharpened tungsten needles. The piece was transferred with a fine pipette to a microhomogenizer (a glass well slide roughened with carborundum), the saline removed, 10 μl distilled water added and the piece homogenized with a needle or glass pestle. Six to 10 μl of the homogenate were then taken up in a syringe and injected as usual.

Before heat treatment, homogenates were spun 3–4 min at 6200 *g* in a Beckman Microfuge. The supernatant was transferred to a glass tube, capped with foil and suspended in boiling water for a specified time. The resulting white precipitate was removed by centrifugation, leaving a clear supernatant to be used for injection or enzyme treatment.

Trypsin (Sigma, crystalline, Type III) and Pronase (Calbiochem, B grade) were dissolved in 0·01 M-$CaCl_2$, diluted in pH 7·4 phosphate buffer and added to boiled (5 min) supernatant of brain homogenate to give final concentrations of 10 μg/ml enzyme, $1{\cdot}6 \times 10^{-4}$ M-$CaCl_2$, and 0·045 M sodium phosphate buffer, pH 7·4. A control tube received $CaCl_2$ and buffer only. All were incubated 5 h at 26 °C, then boiled 5 min and stored frozen until assay.

RESULTS

Moulting response of ligated larvae after injection of pupal brain homogenates

Ligation between the head and thorax of Gate II fourth instar larvae at 23·30 AZT effectively prevented release of sufficient PTTH to activate the prothoracic glands and thus prevented the normal moult to the fifth larval instar, confirming earlier results of Truman (1972) and Fain & Riddiford (1976). Of 262 ligated, uninjected controls used in various experiments, only one moulted to a fifth instar larva. About 3% of these control animals moulted to larval–pupal intermediates as described below. Of 16 animals ligated and injected with 5 μl of distilled water, none showed any sign of larval moulting.

Typically, such uninjected controls, or those injected with inactive extracts formed precocious miniature pupae or at least initiated pupal development. Two to 4 days after ligation, these animals exposed the dorsal vessel and contracted along the anterior–posterior axis, signs of the normal onset of metamorphosis in the larger fifth instar larva (Truman & Riddiford, 1974). They often released a chalky fluid from the anus, comparable to the normal emptying of the gut which precedes metamorphosis. At 7–9 days after ligation, some animals showed the first signs of pupal tanning, with about 60% moulting to precocious miniature pupae by day 9.

In contrast, ligated larvae injected with potent extracts of pupal brains moulted much more rapidly and became headless fifth instar larvae or larval–pupal intermediates. The first sign of normal moulting to the fifth instar is spiracle apolysis, the apolysis of a membranous oval of old cuticle around each of the spiracles, outlining the new and larger fifth instar spiracle being formed (Truman, Riddiford & Safranek, 1973). Some ligated larvae injected with extracts of one half pupal brain equivalent or more per larva showed spiracle apolysis within 24 h. Most of the remaining instances of spiracle apolysis occurred within the next 24 h, and only a few individuals showed

this apolysis on the third or fourth day after injection. The most rapidly responding animals moulted to fifth instar larvae, complete with larger spiracles and crochets (rows of hooks on the abdominal prolegs) within 48 h. These larvae appeared black or greenish-black, a consequence of the removal of the corpora allata in the head and the subsequent decline of juvenile hormone in the haemolymph (Truman, Riddiford & Safranek, 1973; Fain & Riddiford, 1975).

Some of the animals which showed spiracle apolysis developed into larval–pupal intermediates showing almost every gradation between perfect larvae and miniature pupae. At one extreme, the animals still appeared as green larvae at 48 h, but by 72 h showed bits of tan pupal cuticle interspersed with larval cuticle on the dorsal abdomen and thorax. The greenish-blue epidermis was seen through the transparent larval cuticle and the pupal cuticle lacked any distinct features such as pock marks or gin traps (Truman, Riddiford & Safranek, 1974). The new dorsal 'horn' on the last abdominal segment in these animals was shorter, being about 1–2 mm long rather than the normal 4–6 mm. Other individuals had larger areas of tanned pupal cuticle with distinct outlines and pupal pock marks. Most of these were larval–pupal 'mosaics' in that portions of the cuticle showed no moulting and remained larval (Truman, Riddiford & Safranek, 1974). Individuals showing spiracle apolysis plus pupal contraction or in rare cases, spiracle apolysis plus dorsal vessel exposure, eventually moulted to more or less normal pupae with small, partly-formed crochet 'buttons'.

Relation between moulting response and dose of brain homogenate

When increasing doses of a pupal brain homogenate were injected into neck-ligatured larvae, the percentage of animals showing a larval moult increased. After considering a number of criteria for quantifying the moulting response, including total per cent spiracle apolysis and relative number of larvae vs. larval–pupal intermediates, the percentage of animals showing spiracle apolysis at 48 h after injection was selected as the response criterion. This single character gave results equivalent to more complicated scoring methods and eliminated some problems of false positives and questionable scores.

Homogenates of brains removed from short-day reared pupae 1 day after pupal ecdysis were diluted to give various doses in an injection volume of 5–6 μl. Such homogenates gave an approximately linear dose–response curve from $\frac{1}{16}$ to 1 brain-equivalent per larva as seen in Fig. 1. To eliminate possible variations due to differing brain contents of PTTH, dilutions of a single preparation of supernatant from boiled homogenate were injected. For this experiment, a mixture of day 1 brains plus brains after 15–33 days in pupal diapause was used. Diapausing brains of this age appear very similar to day 1 brains and contain substantial PTTH activity. Those pupae which fail to enter prolonged diapause after short-day rearing nearly always initiate adult development between 2 and 14 days after pupal ecdysis. Thus brains were taken before or after this period. This pooled homogenate also gave a linear dose–response curve as shown in Fig. 1, although it proved to be less active than the individual brain homogenates.

As the dose of brain homogenate was increased, an increasing number of recipients became fifth instar larvae as seen in Table 1. The remainder which showed spiracle apolysis usually formed larval–pupal intermediates.

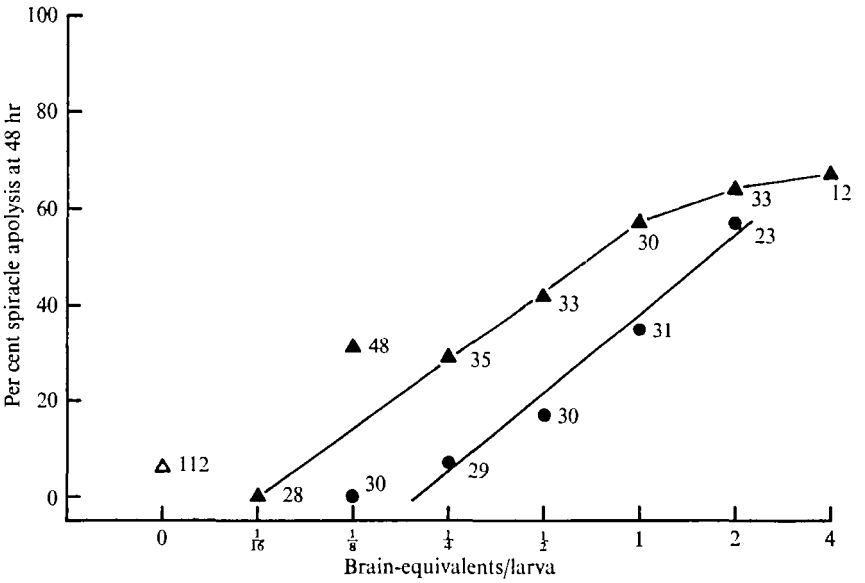


Fig. 1. Dose–response curves for injection of pupal brain homogenates or extract into neck-ligated fourth instar larvae. ▲, fresh homogenates prepared from brains removed 1 day after pupation and stored frozen until homogenization. ●, dilutions of a single preparation of supernatant of boiled (5 min) homogenate from day 1 brains plus brains after 15–33 days in pupal diapause. Dilutions stored frozen at −20 °C until injection.

Table 1. *Moulting response of neck-ligated Gate II fourth instar* Manduca *larvae to injection of pupal brain homogenates or extracts*

Dose in pupal brain-equivalents per test larva ...	0	1/8	1/4	1/2	1	2	4
(A) Fresh homogenate							
Number with spiracle apolysis at 48 h	6	15	10	14	17	21	8
Per cent becoming fifth instar larvae	17	20	30	50	59	57	100
(B) Supernatant of boiled homogenate							
Number with spiracle apolysis at 48 h	1	0	2	5	11	13	—
Per cent becoming fifth instar larvae	0	—	0	20	45	62	—

Data from Fig. 1 animals.

Specificity of the response

To test the specificity of the moulting response, homogenates of brains, ganglia, and other organs from various life stages were injected into ligated fourth instar larvae. Table 2 shows that brains of Gate II fourth instar larvae, removed during the 2 h period prior to the usual time of ligation, had substantial PTTH activity, and that the response was dose-dependent. Fifth instar larval brains and pharate adult brains (1 day before emergence) also had activity, as did corpora cardiaca and corpora allata from wandering stage fifth instar larvae.

A few animals showed spiracle apolysis at 48 h when injected with homogenates of subesophageal, thoracic, or abdominal ganglia. However, the response was not dose-dependent, and no response was obtained with doses of 5–10 ganglion equivalents per test larva. No activity was evident in fat body, Malpighian tubules, or abdominal muscle.

Table 2. *Per cent spiracle apolysis at 48 h in test larvae injected with organ or ganglion homogenates from* Manduca *larvae, pupae or pharate adults*

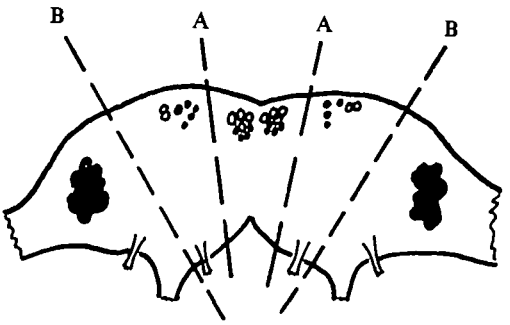
Stage, organ, dose per larva	No. test larvae	Per cent with spiracle apolysis
Fourth instar larva (0–2 h before ligation)		
1 brain	13	23
2 brains	12	67
4 brains	18	78*
6 brains	7	86
Fifth instar larva (feeding)		
1 brain	13	54
1 suboesophageal ganglion (SEG)	8	13
3 thoracic ganglia (TG)	7	0
fat body	11	0
Fifth instar larva (wandering)		
1 pair corpora cardiaca	12	25
1 pair corpora allata	11	55
Pupa		
1 SEG	8	0
2 SEG	12	8
5 SEG	6	0
4 TG	5	20
7 TG	7	0
2 abdominal ganglia (AG)	5	20
10 AG	6	0
fat body	7	0
Malpighian tubules	5	0
abdominal muscle	10	0
Pharate adult (1 day before eclosion)		
1 brain	11	82

* Scored at 64 h.

Source of PTTH in the pupal brain

As the first step in locating the PTTH-producing cells in the pupal brain, brains from day 1 pupae were cut into three pieces with microscissors. The cuts were made as shown in Fig. 2 with reference to the groups of medial and lateral neurosecretory cells which are visible with appropriate lighting (Tyndall effect) in the living pupal brain. Medial or lateral pieces from each cut were pooled and frozen until homogenization. Homogenate of the pooled pieces was injected at a dose of one brain-equivalent per test larva. Results presented in Fig. 2 indicate that the source of PTTH activity is lateral to cut A but medial to cut B, and thus in the region containing the lateral neurosecretory cells.

To localize further the source of PTTH, small pieces (0·1–0·2 mm) of the dorsal area of the protocerebrum were dissected out, homogenized and injected immediately into single test larvae. Each piece contained one group of either medial or lateral neurosecretory cells plus some surrounding tissue. Table 3 shows that the small region of the pupal brain containing the lateral neurosecretory cells had substantial PTTH activity, confirming the results shown in Fig. 2. A slight amount of activity was present in the area of the medial cells.



Cut	Per cent spiracle apolysis at 48 h			
	Medial piece		Lateral piece	
	N	%	N	%
A	20	10	20	70
B	11	82	11	0

Fig. 2. Per cent spiracle apolysis at 48 h in test larvae injected with homogenate of medial or lateral pieces of day 1 pupal brains. One brain-equivalent injected per test larva. Uninjected controls ($n = 13$) showed 8 % spiracle apolysis.

Table 3. *PTTH activity in microhomogenates of individual brain pieces containing neurosecretory cells*

	n	Per cent spiracle apolysis at 48 h
Medial neurosecretory cells	15	20
Lateral neurosecretory cells	15	67
Uninjected controls	64	2

Each piece contained $\frac{1}{2}$ brain-equivalent of neurosecretory tissue from pupal brains after 14–26 days in diapause.

Table 4. *Some properties of PTTH activity in* Manduca sexta

	n	Per cent spiracle apolysis at 48 h
Centrifugation and heat treatment		
First pellet	20	10
Unboiled supernatant	22	77
Boiled supernatant	24	83
Enzymatic digestion, 5 h at 26 °C		
Control	15	40
Trypsin	16	0
Pronase	15	0

Heat-treatment test used brains after 16–35 days in diapause. Enzyme test used day 1 pupal brains. First pellet was resuspended to original volume with distilled water. All injections contained approximately one brain-equivalent in 5 μl. Other conditions as in Materials and Methods.

Chemical properties of Manduca *PTTH*

As a preliminary characterization of PTTH from *Manduca* pupal brains, the homogenate was centrifuged then boiled and recentrifuged. As shown in Table 4, activity remained in the first supernatant. Boiling this supernatant for 10 min caused

a white precipitate, with PTTH activity again remaining in the supernatant. The hormonal activity in such partially purified preparations was destroyed by incubation with trypsin or Pronase for 5 h at 26 °C (Table 4).

Other species

Brains of four other species were tested for activity in the *Manduca* assay. Neither of the brains of the two lepidopterans tested, *Antheraea pernyi* or *Hyalophora cecropia* (diapausing pupae, which had been chilled 6 months, 1 brain-equivalent/test larva), showed activity. These injected larvae subsequently underwent metamorphosis. Larval brain homogenates of *Sarcophaga argyrostoma* (Diptera) and *Oncopeltus fasciatus* (Hemiptera) at a dose of 4 brain-equivalents/test larva resulted in rapid darkening of the larvae and death within 1–2 days. Lepidopteran brains did not produce this toxic reaction.

DISCUSSION

Moulting response after PTTH injection

By neck-ligating fourth instar *Manduca* larvae at various times relative to the light cycle, Truman (1972) found that prothoracicotropic activity was released from the head only during a restricted portion of the light cycle, or 'gate'. Larvae which moulted after ligation early in the gate were more likely to become pupae or larval–pupal intermediates, while those ligated later or after the gate became fifth-instar larvae. Fain & Riddiford (1976) concluded from ligation and ecdysone injection experiments that PTTH was being released during at least the first 3 h of the 'gate' and slowly activating the prothoracic glands to their full secretory capacity. When these larvae were neck-ligated at 23·30 AZT at the beginning of PTTH release and injected with ecdysone, they underwent a larval moult. Apparently, at this time in the absence of PTTH, the prothoracic glands can release only a low amount of ecdysone which eventually initiates a pupal moult due to the decline in the JH titre after ligation (Fain & Riddiford, 1975, 1976). By contrast, neck-ligature 3 h later results in a larval moult indicating full activation of the prothoracic glands by this time and hence the immediate initiation of the moult. Therefore, for this PTTH assay we chose to use larvae neck-ligatured at 23·30 AZT, a time when they would have a subthreshold amount of PTTH but would be primed to respond to it. If neck-ligated even 30 min later in the gate, a few animals would show spiracle apolysis without exogenous PTTH and therefore would give false positives in the assay.

In the present study (Table 1), these larvae neck-ligated at 23·30 AZT showed a moulting response which varied with the dose of brain homogenate or extract injected. High doses tended to produce larval moults and the more rapid onset of spiracle apolysis, whereas low doses yielded mainly larval–pupal intermediates. Such results support the hypothesis that high doses of PTTH provoke rapid activation of the prothoracic glands, while lower doses activate the glands more slowly.

The slow but apparently autonomous activation of the prothoracic glands which produced a pupal moult in these ligated larvae (see also Truman, 1972; Fain & Riddiford, 1976) is observed also in the fifth instar (Judy, 1972; Truman & Riddiford, 1974; Nijhout, 1976). Likewise adult development can occur in brainless pupae of

Manduca (Judy, 1972), *Bombyx mori* (Fukuda, 1944; Ishizaki, 1972), *Samia cynthia ricini* (Ishizaki & Ichikawa, 1967), and *Antheraea polyphemus* (McDaniel & Berry, 1967), which may indicate autonomous activation of the prothoracic glands.

Characteristics of the assay

Fain & Riddiford (1976) concluded that the prothoracic glands of neck-ligated larvae must be activated while JH or its effects are still present in order to achieve a larval moult. Spiracle apolysis is a larval character and thus can occur only within a limited time after neck-ligation and removal of the corpora allata. The dual constraints of a declining JH titre (Fain & Riddiford, 1975) and an early larval moulting character as the response criterion should limit the assay response to those substances which provoke rapid activation of the prothoracic glands.

Homogenates or boiled extracts of pupal brains gave essentially linear responses on a semi-log scale up to about 60 to 70 % spiracle apolysis at 48 h. Responses up to about 80 % were obtained with 4–6 fourth instar brains per larva but also appeared to level off. The cause of this levelling-off is unknown. It is possible that 15 to 35 % of the animals are simply unresponsive to PTTH at the doses tested. Alternatively, other substances in the homogenates may reduce the response (injection of 4 pupal brain-equivalents per larva resulted in high mortality at 3–5 days).

With a sufficient number of test larvae, the assay should detect about $\frac{1}{8}$ brain-equivalent of PTTH activity from day 1 pupal brains. Availability of assay larvae 9–10 days after hatching, a response time of 2 days, and brain removal by ligation make the assay faster and more convenient than the dauer-pupa methods.

The failure to find activity in brains of *A. pernyi* and *H. cecropia* does not rule out a trans-specific action of PTTH in the assay, since these brains may simply have had too low a titre of PTTH. Using brainless diapausing pupae of *H. cecropia*, Williams (1967) found no activity in saline extracts of chilled *H. cecropia* brains, but high activity in brain extracts from *A. pernyi*. Brainless *cecropia* pupae also developed after implantation of brains from *Samia cynthia walkeri*, *Callosomia promethea*, or *Telea* (*Antheraea*) *polyphemus* (Williams, 1946). Brainless pupae of *Samia cynthia ricini* are routinely used for assay of *Bombyx mori* brain hormone (Ishizaki & Ichikawa, 1967; Nishiitsutsuji-Uwo, 1972).

Localization of PTTH

The finding of PTTH activity in larval and pupal brains is in accord with the general theory that the insect brain controls moulting and metamorphosis in part through its ability to activate ecdysone-secreting glands. PTTH activity in pharate adult brains was unexpected, since the prothoracic glands have apparently degenerated by this stage. This activity is not due to the eclosion hormone which is present in high concentration in the pharate adult brain (Truman, 1973) since 10 μl of a partially purified eclosion hormone preparation (gift of Dr S. E. Reynolds) produced no spiracle apolysis in five injected larvae. Ishizaki (1969) and Nishiitsutsuji-Uwo (1972) also found substantial brain hormone activity in the brains of *Bombyx* pharate or newly emerged adults.

Results presented in Fig. 2 and Table 3 strongly suggest that the source of PTTH in the pupal brain is the small dorsal region containing the soma of the lateral neuro-

secretory cells. Using cobalt backfilling techniques, Nijhout (1975) has demonstrated two groups of soma in this area which fill from the *nervus corporis cardiaci* (NCC) I+II: a group of 5–7 cells filling on the ipsilateral side and a group of 2 on the contralateral side. Two groups comparable to these can sometimes be observed by Tyndall scattering in the freshly dissected pupal brain (Gibbs, unpublished observations), and by paraldehyde fuchsin staining of whole brains (Nijhout, 1975). The identity of cells visualized by these different methods has yet to be established. The finding of a slight amount of activity in brain pieces containing medial neurosecretory cells could be due to false positives, to a second hormone with weak PTTH activity or to the presence of axon collaterals from lateral neurosecretory cells. Axon collaterals containing neurosecretory material are present in the brains of *Leptinotarsa* adults (Schooneveld, 1974).

Through cutting and microcautery experiments, Williams (1948) and Van der Kloot (1961) concluded that both medial and lateral neurosecretory cells of the pupal brain were necessary to induce adult development in *H. cecropia*. An important difference between those experiments and the present ones, apart from species differences, was the requirement for an active, secreting brain fragment after implantation into a brainless pupa. A further caveat is that 'medial' or 'lateral' cell groups may contain different neurosecretory cells at different life stages, as in *Manduca* (Nijhout, 1975).

Another unexpected finding was the presence of substantial PTTH activity in an aqueous homogenate of the corpora allata (CA) of wandering fifth instar larvae. This stage occurs between the two peaks of ecdysone which promote the larval–pupal transformation (Truman & Riddiford, 1974; Bollenbacher *et al.* 1975), and it was expected that PTTH might be stored in neurohaemal areas at this time. Surprisingly, more activity per gland pair was observed in the allata than in the corpora cardiaca (CC). Likewise, Ishizaki (1969) found more brain hormone activity in larval *Bombyx* CA than in the CC, after pre-extraction with acetone to remove juvenile hormone. Fain & Riddiford (1976) found that injection of 10 μg C18 juvenile hormone into larvae ligated as in the PTTH assay promoted a larval response to injected ecdysone but had no apparent prothoracicotropic effect of its own. Ichikawa & Nishiitsutsuji-Uwo (1959) found that pupal CA of *Philosomia*, without apparent juvenile hormone activity, were nevertheless able to provoke adult development of brainless diapausing pupae. They concluded that PTTH from the brain might be stored in the CA in this species. Nijhout (1975) found that axons from the medial group and both groups of lateral neurosecretory cells innervate the CA. In conjunction with the present findings this suggests that the CA may be a site of release of PTTH, in addition to their role in synthesizing juvenile hormone. Proof of such a function requires the demonstration of hormone release from the presumptive neurohaemal area (Maddrell & Gee, 1974).

Properties of PTTH

The experiments reported in Table 4 indicate that *Manduca* PTTH activity is water-soluble, heat-stable, and sensitive to Pronase, properties similar to those reported for the brain hormone of *Bombyx* (Ichikawa & Ishizaki, 1963; Ishizaki & Ichikawa, 1967; Yamazaki & Kobayashi, 1969) and *Antheraea pernyi* (Williams, 1967) using dauer-pupa assays. *Manduca* PTTH activity is also destroyed by incubation with trypsin, as is purified *Bombyx* brain hormone (Yamazaki & Kobayashi, 1969). It should

be noted that results presented here for *Manduca* PTTH were obtained from relatively crude preparations, and that definitive statements about the properties of this PTTH will require purification of the active substance.

Of the three major insect developmental hormones (ecdysone, juvenile hormone, and PTTH), we know least about PTTH. Determination of the structure of PTTH, study of prothoracic gland activation *in vitro*, and development of a radioimmune assay for PTTH are possibilities which await the isolation and purification of this hormone. Additionally, positive identification of the PTTH-secreting neurosecretory cells may open the way to electrophysiological analysis of identified neurones involved in the control of moulting and diapause. The simple and sensitive bioassay for PTTH reported here should greatly facilitate these studies.

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