J. exp. Biol. 108, 9–24 (1984)
Printed in Great Britain © The Company of Biologists Limited 1984

IN VITRO STUDIES ON THE ROLE OF THE BRAIN AND PROTHORACIC GLANDS IN THE PUPAL DIAPAUSE OF *MANDUCA SEXTA*

BY M. F. BOWEN, W. E. BOLLENBACHER AND L. I. GILBERT

Department of Biology, Wilson Hall 046A, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514, U.S.A.

Accepted 25 July 1983

SUMMARY

Pupal diapause in the tobacco hornworm, *Manduca sexta* (Johannson), is characterized by the absence of an increased ecdysteroid titre in the haemolymph during the first week of pupal life as measured by radioimmunoassay. This virtual absence of the steroid moulting hormone is thought to be responsible for the diapause state and it is apparently a consequence of the failure of the diapausing pupal prothoracic glands (PG) to synthesize ecdysone at an increased rate. In *Manduca*, this apparent failure of PG activation during diapause may be in response to two endocrinological circumstances: the curtailment of PTTH release as opposed to its synthesis and transport, and the development of refractoriness of the gland to stimulation by PTTH. The former was examined by measuring PTTH activity present in brains and brain-retrocerebral complexes of diapausing and non-diapausing pupae and the latter by assessing PG biosynthetic activity *in vitro* in the presence and absence of PTTH.

INTRODUCTION

The synchronization of biological events with environmental conditions is of utmost importance to virtually all organisms. This is particularly true of animals living in temperate latitudes where seasonal climatic conditions vary dramatically. Diapause is one strategy by which insects integrate their life cycle with seasonal change. By entering a state of diapause the insect circumvents climatic conditions that would otherwise be detrimental to the continuation of its life cycle. Diapause can be induced by various environmental cues, the most common being photoperiod. Such a 'neutral' environmental stimulus allows the insect to prepare in advance for a state of physiological dormancy and developmental arrest.

The physiological processes responsible for the transduction of environmental stimuli into a state of diapause appear to involve the insect's neuroendocrine system. In the case of pupal diapause in lepidopterous insects the accepted concept has been that environmental cues suppress the synthesis and/or release of the primary effector of insect postembryonic development, the cerebral neuropeptide, prothoracicotropic

Key words: Prothoracicotropic hormone, ecdysone, insect development.

hormone (PTTH) (Denlinger, 1984). The failure of PTTH to be synthesized and/or released results in the consequent failure of the prothoracic glands (PG) to be stimulated to synthesize ecdysone, the steroid prohormone that is subsequently hydroxylated to the moulting hormone 20-hydroxyecdysone in peripheral tissues (Bollenbacher, Smith, Wielgus & Gilbert, 1977). However, this concept of the neuroendocrine control of pupal diapause may not be applicable to all insects that diapause as pupae, since it appears that under certain experimental conditions some species are capable of terminating diapause after brain extirpation (Ozeki, 1954; McDaniel & Berry, 1967; Judy, 1972; Wilson & Larsen, 1974; Kind, 1978*a*,*b*). The fact that the brain is not always required for the termination of pupal diapause suggests that, depending on the insect, there may be varying degrees of temporal coupling between the brain and the PG (see Denlinger, 1984). For example, the brain is required for only a short period of time in *Heliothis zea*, a species in which pupal diapause is maintained by a temperature-evoked suppression of PG activity. Release of PTTH in this animal does not appear to be curtailed by the stimulus which induces diapause and occurs shortly after larval–pupal ecdysis even in diapause-destined individuals (Meola & Adkisson, 1977).

Most of the research conducted thus far on the endocrine control of pupal diapause has employed indirect approaches involving techniques such as ligation, extirpation and implantation, as well as exogenous hormone treatment, to elucidate the nature of the hormones involved. Although these approaches have provided some information and insight as to what endocrine systems may be involved in the control of pupal diapause, they have provided little biochemical information about the endocrinology of diapause. With the development of new methods (e.g. radioimmunoassays, *in vitro* incubation, *in vitro* bioassays, etc.) for examining the function of the endocrine system, it is now possible to investigate directly the endocrinology of diapause.

Pupal diapause in the tobacco hornworm, *Manduca sexta*, is induced photoperiodically (Rabb, 1966). The main advantage of using *Manduca* in the study of diapause is that its non-diapause developmental endocrinology is better understood than for any other insect species (see Gilbert, Bollenbacher & Granger, 1980; Bollenbacher & Gilbert, 1981). Not only does there exist basic information on the synthesis and titres of the juvenile hormones (B. J. Bergot, M. S. Hall, A. A. Furrer & D. A. Schooley, in preparation; Granger, Niemiec, Gilbert & Bollenbacher, 1982) and ecdysteroids (Bollenbacher, Vedeckis, Gilbert & O'Connor, 1975; Bollenbacher, Smith, Goodman & Gilbert, 1981) in this organism, but similar information is now available for PTTH as well (Agui, Granger, Gilbert & Bollenbacher, 1979; Agui, Bollenbacher, Granger & Gilbert, 1980; Gilbert *et al.* 1981; Bollenbacher & Gilbert, 1981). If the same basic information is generated for *Manduca* developing under diapause-inducing conditions as well as during diapause, it should be possible to assess whether there are significant photoperiod-induced differences in the endocrine system that could be causally related to diapause. The present study was undertaken to determine the nature of the endocrine events characterizing pupal diapause in *Manduca*. The resulting data suggest that diapause in *Manduca* is accompanied by specific functional changes in this endocrine axis, both at the level of PTTH release and PG sensitivity to PTTH.

MATERIALS AND METHODS

Animal rearing

Eggs from a strain of *Manduca sexta* with a high incidence of diapause were kindly supplied by Dr James Buckner, USDA, Fargo, North Dakota. Diapausing pupae were obtained by rearing larvae under a short-day photoperiod (LD 12:12) at 25 °C on an artificial diet (Bell & Joachim, 1976). Non-diapausing pupae were reared under a long-day photoperiod (LD 16: 8) on the same diet. At LD 12:12 essentially 100 % of the pupae entered diapause, the majority of which (approximately 75 %) remained in diapause for at least 2 months at 25 °C. Animals raised under long-day conditions at 25 °C emerged as adults within 3 weeks of larval–pupal ecdysis.

On day 7 of the fifth (final) larval instar, pharate pupae were transferred to wooden blocks and kept in constant dark at 25 °C where they were allowed to pupate. Day 0 pupae were obtained by selecting freshly-pupated animals and, depending on whether the animals were reared under long- or short-day photoperiods, they were referred to as non-diapausing day 0 (NDP_0) or diapausing day 0 pupae (DP_0). Older animals were referred to as NDP_1, NDP_2, etc. and DP_1, DP_2, etc.; the subscript denoting the number of days after pupation. Day 0 pupae were collected every afternoon, so day 0 was arbitrarily considered to begin at noon Eastern Standard Time.

Radioimmunoassay of haemolymph ecdysteroid titres

Haemolymph samples (0·05 ml) for analysis by the ecdysteroid radioimmunoassay (RIA) were obtained through a cut in the pupal proboscis. Ecdysteroids were extracted from the haemolymph with methanol (0·2 ml) and the extracts were stored at −20 °C until assayed. When fat body cells were present in the haemolymph they were removed before methanol extraction by centrifugation at $200\times g$ for 15 min. For RIA, extracts were thawed and centrifuged at $4000\times g$ for 10 min to pellet precipitated protein. Ecdysteroids were quantified by RIA using D-10 antiserum which exhibits equivalency of binding for ecdysone and 20-hydroxyecdysone (Bollenbacher *et al.* 1975). The labelled ligand used for the RIA was [23,24,^{3}H]-ecdysone at 45 Ci $mmol^{-1}$. Since the competing unlabelled ligand used as the standard was 20-hydroxyecdysone, RIA activity was expressed in 20-hydroxyecdysone equivalents. Each haemolymph extract was assayed in duplicate at two different concentrations and at least four animals were assayed individually for each datum point. These and all subsequent data were analysed using Student's *t*-test.

Gland dissection and PTTH extraction

Prothoracic glands were dissected from pupae in lepidopteran Ringers (Weevers, 1966) and then transferred to Grace's medium until used in an assay. The time between dissection and assay of the glands did not exceed 1 h.

Brains and brain-retrocerebral complexes (brains with attached corpora cardiaca and corpora allata or BR-CC-CA), were dissected in Grace's medium and transferred immediately to a defined volume of Grace's medium which varied depending upon the particular experimental design. The tissues were homogenized in this medium followed by heat treatment at 100 °C for 2 min. The extracts were centrifuged at

3000×*g* for 10 min and supernatants (PTTH extract) were frozen until assayed for PTTH activity. PTTH activity is not diminished by this procedure of sample preparation (Bollenbacher, Agui, Granger & Gilbert, 1979).

PTTH assay

Quantification of PTTH in brain extracts, BR-CC-CA extracts and culture medium was accomplished using the *in vitro* PG activation assay for PTTH (Bollenbacher *et al.* 1979). In this assay non-diapause day 0 pupal PGs are used, one gland from an individual serving as a control and the contralateral gland as an experimental. The control gland was incubated in Grace's medium alone and the experimental gland in Grace's medium containing PTTH extract. Activation of a PG by PTTH is expressed as an activation ratio (A_r) which is defined as the amount of ecdysone synthesized in 2 h by the experimental gland divided by that synthesized by the control gland. An RIA is used to quantify the ecdysone synthesized and this is accomplished by assaying an aliquot (0·01 ml) of the assay incubation medium (0·025 ml). The antibody to ecdysone used in this assay (H-3) has considerably greater affinity for ecdysone than for 20-hydroxyecdysone (Gilbert, Goodman & Bollenbacher, 1977). The labelled ligand was [23,24,^{3}H]-ecdysone at 4 Ci mmol^{-1} and the unlabelled competing ligand used as a standard was also ecdysone. Since ecdysone is the only ecdysteroid synthesized by the PG *in vitro* (King *et al.* 1974), RIA activity was expressed in nanograms of ecdysone. A dose-response protocol of PG activation was used to quantify relative PTTH activity by comparing the reciprocals of the amounts of each sample needed to half maximally activate the glands (ED_{50}) (Agui *et al.* 1979; Bollenbacher, Agui, Granger & Gilbert, 1980).

Time course of ecdysone synthesis

The kinetics of ecdysone synthesis by unactivated and activated PG were assessed using a previously established protocol (Bollenbacher *et al.* 1979). Glands were incubated individually in 0·025 ml of Grace's medium either with or without 0·5 brain equivalents of PTTH, and 0·01 ml aliquots were taken to measure the amount of ecdysone synthesized (each hour during a 6-h incubation). Immediately following removal of an aliquot, an equivalent volume of Grace's medium was added back to the incubation to maintain the 0·025 ml volume. Ecdysone present in the medium was then measured by the ecdysone RIA used in the *in vitro* PTTH assay. Ecdysone synthesis by the PG was expressed as the cumulative amount of hormone made during the incubation period.

RESULTS

Ecdysteroid titres

In probing the endocrine basis of diapause in *Manduca* the first requirement was to generate a quantitative haemolymph ecdysteroid titre during pupal–adult development for the North Dakota strain of *Manduca* reared under both long and short-day photoperiods. For non-diapausing pupae and pharate adults the temporal pattern and quantitative changes observed in the haemolymph ecdysteroid titre (Fig. 1) were

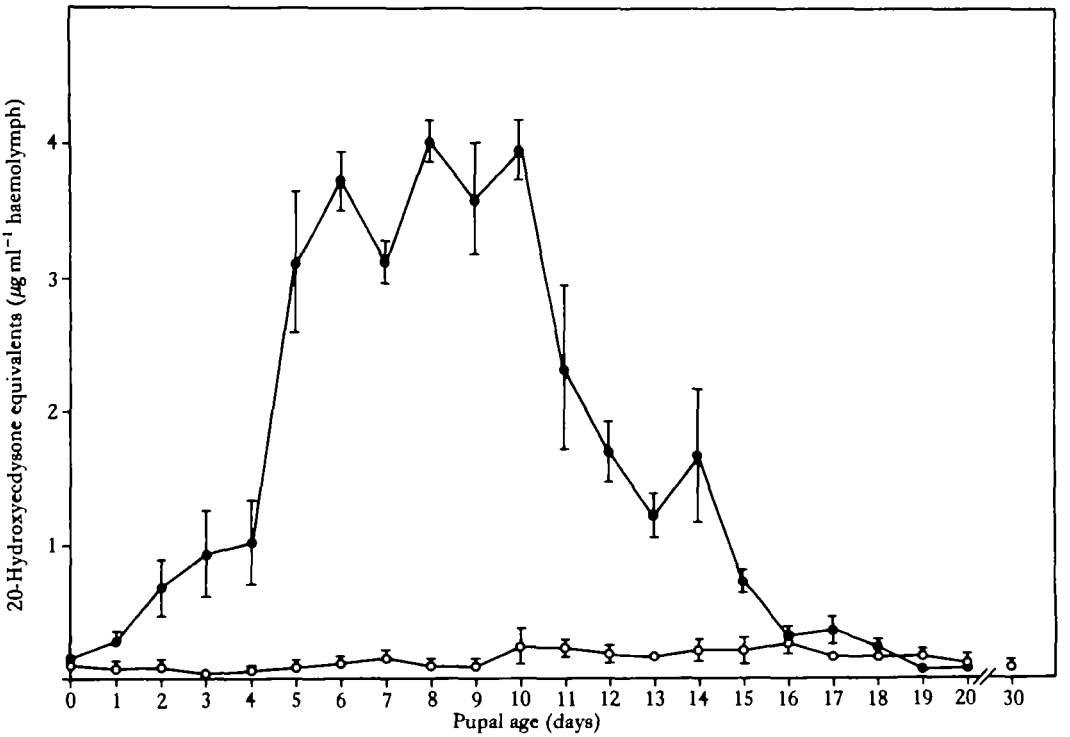

Fig. 1. Ecdysteroid titres in diapausing (○——○) and non-diapausing (●——●) *Manduca sexta* pupae. Day 0 refers to the day of larval–pupal ecdysis. Each datum point represents an average (±S.E.M.) of at least four animals, each assayed individually. Standard errors bars not shown were smaller than the size of the data points.

essentially the same as those reported previously for whole body extracts of *Manduca* maintained for 15 years under non-diapausing conditions (Bollenbacher *et al.* 1981). There was an initial increase in the ecdysteroid titre between days 1 and 2 after pupation (0·3 $\mu g\,ml^{-1}$ to 0·7 $\mu g\,ml^{-1}$) and this increase was followed by a second dramatic rise between days 4 and 5 to a level of approximately 3·1 $\mu g\,ml^{-1}$. From day 5 to 10 of pharate adult development the titre stayed relatively constant at approximately 3·5 $\mu g\,ml^{-1}$ until day 11 when it dropped rapidly to 2·3 $\mu g\,ml^{-1}$. Subsequently, the titre declined gradually to day 16 at which time it approached a basal level of 0·3 $\mu g\,ml^{-1}$. For pupae reared under LD 12: 12 the ecdysteroid titre was very low, remaining at a level of about 0·1 $\mu g\,ml^{-1}$ haemolymph for 30 days. These data demonstrated clearly that the ecdysteroid titres of short-day reared diapausing pupae are negligible, indicating that the absence of ecdysteroids is the principal factor contributing to the diapause state.

Prothoracic gland activity

The low ecdysteroid titre in diapausing *Manduca* pupae could have resulted from the decreased synthesis of ecdysone by the PG and/or an increased rate of ecdysone degradation (e.g. catabolism, excretion, conjugation). To determine if the biosynthetic activity of the PG played a major role in determining the ecdysteroid

titre, glands from diapausing and non-diapausing pupae at selected times during the pupal and pharate adult periods were incubated *in vitro* and their rates of ecdysone synthesis determined. The critical time chosen for these studies was the first 4 days after pupation, the period during which PTTH release is thought to occur in non-diapausing pupae (unpublished observations). For diapausing animals, PG activity was determined for the first 4 days of the pupal period as well as days 10, 15, and 28 after pupation. PG activity at these later times was assayed to determine if gland activity changed during diapause.

For non-diapausing pupae the activity of the PG from day 0 to day 2 was relatively constant, exhibiting a rate of ecdysone synthesis of approximately 3 ng 2 h^{-1} $gland^{-1}$ (Fig. 2). At day 2 plus 12 h there was a marked change in PG activity with the rate of ecdysone synthesis increasing to 7·7 ng 2 h^{-1} $gland^{-1}$, a level significantly different from that of day 2 PG ($P = 0{\cdot}04$). This increased synthesis is presumably in response to PTTH release since the brain critical period for pupal–adult development in *Manduca* ranges from day 2 to approximately day 4 (unpublished observations). From day 2 plus 12 h to day 4, PG activity remained in excess of 8 ng 2 h^{-1} $gland^{-1}$ and reached a high of 12 ng 2 h^{-1} $gland^{-1}$ on day 4. There was an overall 50 % increase

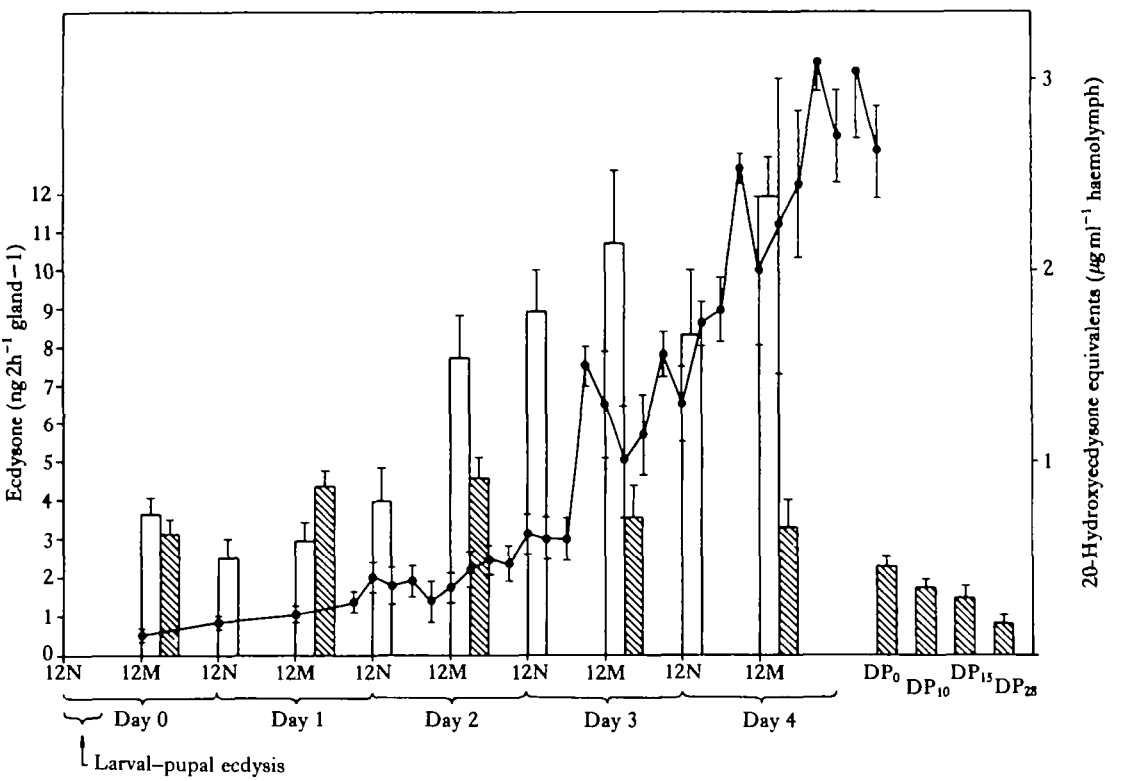

Fig. 2. *In vitro* activity of PG from diapausing (cross-hatched bars) and non-diapausing (open bars) pupae and haemolymph ecdysteroid titres of non-diapausing pupae (●——●) of *Manduca sexta*. Each bar and datum point represent the average (±S.E.M.) of at least four separately assayed individuals. DP denotes diapausing animals and the subscript refers to the number of days after pupation.

in gland activity during this time ($P = 0{\cdot}06$), which is unlikely to be due to chance alone but is probably a result of the exposure of the PG to PTTH.

That increased PG activity during the first 4 days of pupal–adult development can account for the increased ecdysteroid titre was demonstrated by comparing a detailed titre of haemolymph ecdysteroids to PG activity (Fig. 2). The titre was generated from the same population of animals used in the PG activity study. From day 0 plus 12 h to day 3 the titre increased gradually in a near linear fashion from $0{\cdot}1\,\mu g\,ml^{-1}$ haemolymph to $0{\cdot}6\,\mu g\,ml^{-1}$, a statistically significant increase ($P = 0{\cdot}01$). At day 3 plus 9 h the titre increased again, this time quite dramatically, to $1{\cdot}5\,\mu g\,ml^{-1}$ haemolymph (day 3 plus 6 h *vs* day 3 plus 9 h, $P = 0{\cdot}002$). For the next 1·5 days the titre increased in a linear manner to a maximum of approximately $3\,\mu g\,ml^{-1}$ haemolymph on day 5. These titre data are in agreement with those in Fig. 1. When compared to PG activity *in vitro* the haemolymph ecdysteroid titre increased as the rate of ecdysone synthesis increased with a lag of a few hours. This is the expected temporal relationship if synthesis by the PG were responsible for the increasing ecdysteroid titre. It is important to note that during the pupal period the ecdysteroid titre increased gradually as opposed to during larval and larval–pupal development where dramatic fluxes in the titre resulted in sharp, discrete peaks (Bollenbacher *et al.* 1981). These gradual, linear increases in ecdysteroid titre over several days may be critical for eliciting pupal–adult development.

When PG activity of diapausing pupae was assessed *in vitro* during the early pupal period it was observed that the rate of ecdysone synthesis during the first 4 days was comparable to that of non-diapausing pupal PG before the latter were activated by PTTH on day 2. The activity of diapausing pupal PG appeared to be different from non-diapausing pupal PG on day 2 ($P = 0{\cdot}06$) and was clearly significantly different on day 3 ($P = 0{\cdot}01$). The basal level of synthesis of approximately $4\,ng\,2\,h^{-1}\,gland^{-1}$ decreased to an even lower level of $1{\cdot}8\,ng\,2\,h^{-1}\,gland^{-1}$ by day 10; by day 28 the PG were almost inactive, synthesizing ecdysone at a rate of only $0{\cdot}8\,ng\,2\,h^{-1}\,gland^{-1}$. In summary, these data demonstrated that increased ecdysone synthesis by the PG is responsible, at least in part, for the increasing ecdysteroid titre during pupal–adult development and that the depressed titre in diapausing pupae was a result of the low rate of ecdysone biosynthesis by the PG. Presumably, this was due to the absence of PTTH in the haemolymph, although there were other possible causes, e.g. the inability of the PG to respond to PTTH stimulation (Meola & Adkisson, 1977).

PTTH in the brain and BR-CC-CA

That diapausing pupal PG had not been activated *in situ* by day 2 could have been a result of a failure to release PTTH from its neurohaemal organ, the CA (Agui *et al.* 1980), and/or a reduction in the quantity of PTTH synthesized and stored. It has been shown in *Hyalophora cecropia* that the level of PTTH in the early diapausing pupal brain was minimal, and this was suggested to be the basis for diapause in this saturniid (Williams, 1968). To determine if this were true for pupal diapause in *Manduca* as well, PTTH levels were determined in brains and BR-CC-CA of diapausing and non-diapausing pupae at selected stages using the *in vitro* PTTH assay and the quantitative dose-response protocol (Table 1). The quantity of PTTH in brains and BRCC of diapausing pupae fluctuated slightly during the first 20 days of

Table 1. *Relative PTTH titres in brains and brain complexes from diapausing and non-diapausing pupae*

Stage	BR-CC-CA	Mean ± S.E.M.	Brain	Mean ± S.E.M.	BR-CC-CA minus brain
NDP_1					
1	37·0	32·2 ± 7·3	34·5	26·5 ± 8·0	5·7
2	41·7		18·5		
3	17·9				
NPD_3	23·3		—		
DP_1					
1	31·3	27·3 ± 4·0	23·8	18·1 ± 3·3	9·2
2	23·3		17·9		
3	—		12·5		
DP_4	20·0		—		
DP_6	16·1		—		
DP_8	41·6		—		
DP_{10}					
1	34·5	46·8 ± 12·3	13·5	16·3 ± 3·2	30·8
2	71·4		12·7		
3	34·5		22·7		
DP_{20}					
1	18·5	20·1 ± 1·6	15·6	14·8 ± 0·85	5·3
2	21·7		13·9		
DP_{28}					
1	10·6	10·3 ± 0·3	10·6	8·4 ± 2·18	1·9
2	10·0		6·3		
DP_{34}	10·0		6·3		

Values expressed as $1/ED_{50}$, which is defined as the reciprocal of the tissue equivalents necessary to elicit half maximal activation of ecdysone synthesis by the PG.

diapause but these variations were not significant. The values were not significantly different from those for brains and BR-CC-CA from day 1 non-diapausing pupae (for all brain combinations $P > 0{\cdot}29$ and for all BR-CC-CA combinations $P > 0{\cdot}25$). This similarity in the amounts of PTTH in brains and BR-CC-CA from non-diapausing pupae and diapausing pupae for the first 20 days suggested that similar rates of synthesis and/or storage of PTTH occurred in diapausing and non-diapausing animals. For day 28 diapausing pupae, however, there was a significant decrease in the level of PTTH activity in the BR-CC-CA compared to day 1 diapausing pupal BR-CC-CA ($P = 0{\cdot}05$).

From these data it was also possible to determine if a difference existed in the amount of PTTH stored in the neurohaemal organ of diapausing and non-diapausing pupae. This was accomplished by computing the difference in PTTH activity between brain and BR-CC-CA at a given time since the activity remaining would represent PTTH present in the CC and CA (BR-CC-CA-BR in Table 1). The amount of PTTH present in day 1 diapausing pupal CC-CA was about equal to that present in day 1 non-diapausing CC-CA. On day 10, however, there appeared to be a substantial increase in CC-CA PTTH activity that decreased by day 20 of diapause, and dropped even further by day 28. The higher level of PTTH activity in diapausing pupal CC-CA on

day 10 could be a result of the accumulation of the neurohormone resulting from its failure to be released. In the light of these findings, which demonstrate that the BR-CC-CA of diapausing pupae have at least as much PTTH as the BR-CC-CA of non-diapausing pupae, it appears that the curtailment of PTTH release is the level at which diapause is controlled rather than its synthesis and storage.

PG activation

In addition to PTTH release as a control point in diapause induction in *Manduca*, the possibility existed that the PG were also involved, i.e. that their responsiveness to stimulation was altered. To ascertain if pupal PG change in their responsiveness to PTTH during diapause, time courses of ecdysone synthesis by unactivated and PTTH-activated PG were generated for glands from day 0 non-diapausing pupae and days 0, 15 and 28 of diapause.

For non-diapausing day 0 pupal PG, ecdysone kinetics for unactivated and PTTH activated glands (Fig. 3) were essentially the same as those previously reported (Bollenbacher *et al.* 1979). The biosynthetic activity of PG incubated without PTTH was linear over the 6-h incubation period at a rate of 0·9 ng h^{-1} $gland^{-1}$ with total synthesis being 5·6 ng ecdysone $gland^{-1}$. The contralateral PG incubated with PTTH exhibited a dramatic increase in the rate of ecdysone synthesis to 6·0 ng h^{-1} $gland^{-1}$ for the first 3 h followed by a levelling off, total synthesis being 23 ng $gland^{-1}$. The plateau may reflect depletion of the endogenous sterol precursor(s) for ecdysone. Day 0 diapausing pupal PG that were incubated without PTTH exhibited a synthetic rate of 0·8 ng h^{-1} $gland^{-1}$ with a total synthesis in 6 h of 5 ng $gland^{-1}$, values virtually identical to the kinetics data for non-diapausing day 0 pupal PG. In contrast, the time course of ecdysone synthesis by day 0 diapausing pupal PG incubated with PTTH differed from that of PG from day 0 non-diapausing pupae incubated with the hormone. For diapausing pupal PG, the rate of ecdysone synthesis and total hormone synthesized during the 6-h incubation period were only 3·5 ng h^{-1} $gland^{-1}$ and 9 ng $gland^{-1}$, respectively, values approximately half those of PTTH-activated non-diapausing day 0 pupal PG. The time course of synthesis of day 15 diapausing pupal PG was characterized by a basal rate slightly less than that of day 0 diapausing pupal PG and the rate of ecdysone synthesis for the PG incubated with PTTH was even more reduced than that noted for day 0 diapause PG. The rate of ecdysone synthesis by these glands was only 1 ng h^{-1} $gland^{-1}$, which was essentially the unactivated rate of ecdysone synthesis by the day 0 glands. By day 28 of diapause the activity for both control and experimental PG had dropped to approximately 0·25 ng h^{-1} $gland^{-1}$ with total synthesis in 6 h being only 1·5 ng $gland^{-1}$. These data indicate that day 28 glands are totally refractory to activation by PTTH.

These kinetics data reveal that at the time of pupation, PG from diapausing pupae are less capable of being activated by PTTH than non-diapausing pupal PG. As diapause progresses the glands continue to lose their ability to respond to PTTH, ultimately becoming refractory to activation by PTTH. The failure of the diapausing pupal PG to be activated by PTTH at a level comparable to that of non-diapausing pupal PG argues that responsiveness of the PG to PTTH may be another point at which diapause in *Manduca* is regulated. Thus, it appears that induction of pupal diapause in this insect may involve the shutdown of the brain-PG axis, resulting in the

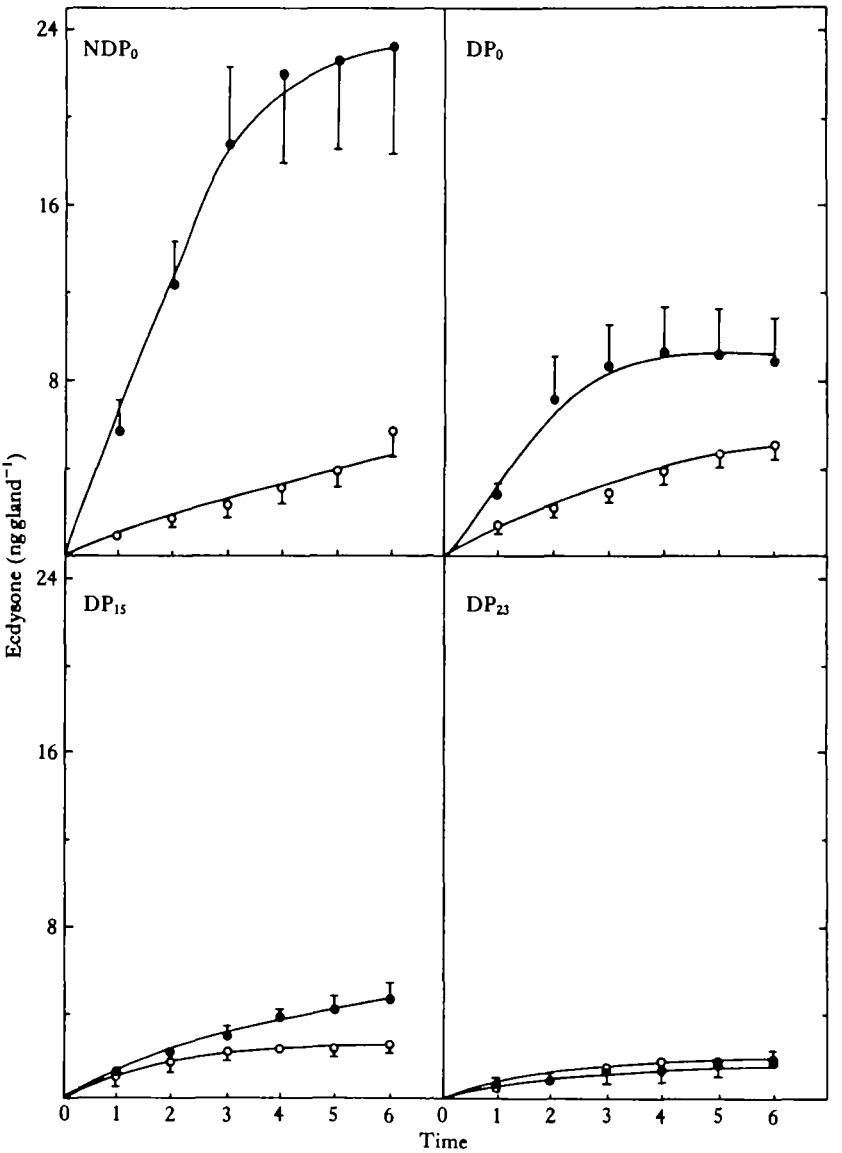

Fig. 3. Time course of ecdysone synthesis by PTTH-activated (●——●) and control PG (○——○) *in vitro*. Each datum point represents the average (±S.E.M.) of six individual determinations. NDP denotes non-diapausing pupae and DP denotes diapausing pupae, the subscript referring to the number of days after pupation.

concurrent suppression of PTTH release by the BR-CC-CA and the inability of PG to be activated by PTTH.

DISCUSSION

The present study has established that pupal diapause is a consequence of low ecdysteroid titres and that the latter are the direct result of suppressed PG synthetic

activity. There may be at least two mechanisms operating during diapause in this species that insure that ecdysteroid levels do not rise prematurely above the threshold necessary for pharate adult development: (1) the release of PTTH, rather than its synthesis or transport, appears to be inhibited; and (2) the PG actually become refractory to PTTH stimulation as early as the first day of pupal life.

Measurements of ecdysteroids in other insect species during pupal diapause have also revealed low levels of moulting hormone. Using the *Musca* bioassay, Walker & Denlinger (1980) found no detectable moulting hormone activity in the haemolymph of diapausing *Sarcophaga* pupae, while RIA of ecdysteroids in both *Hyalophora cecropia* (McDaniel, 1979) and *Heliothis virescens* (Loeb, 1982) revealed detectable but low hormone levels (5–60 pg μl^{-1} haemolymph). Thus, the moulting hormone may be present during pupal diapause but only at levels far below those necessary for the initiation of adult development. The haemolymph titre data generated in this study, in conjunction with the observation that diapause in *Manduca* can be terminated by injection of 20-hydroxyecdysone (Bradfield & Denlinger, 1980), confirm that low ecdysteroid titres are the immediate physiological cause of pupal diapause in this species.

While metabolic processes such as tissue uptake and/or catabolism of ecdysteroids could account for suppressed levels of the hormone, the biosynthetic activity of the PG, i.e. their rate of ecdysone synthesis, was the most likely underlying cause of the low ecdysteroid titres in diapausing animals. The importance of synthesis was demonstrated by the developmental study of PG activity *in vitro* which revealed that the synthetic capacity of diapausing PG never rose above a basal level during pupal life and, in fact, dropped as diapause continued. Non-diapausing pupal PG, on the other hand, exhibited an increased rate of activity by day 2, with activity increasing at days 3 and 4, presumably in response to PTTH release. Thus, as expected, the low basal level of activity of diapausing pupal PG correlated with low haemolymph ecdysteroid titres during diapause, and a high basal level of activity by PG from non-diapausing animals correlated with high haemolymph titres as pharate adult development was initiated.

Most evidence regarding the biosynthetic activity of the PG during diapause has been histological (e.g. Herman & Gilbert, 1966; Herman, 1967; McDaniel, Johnson, Saun & Berry, 1976). In *Hyalophora cecropia*, for example, PG from unchilled diapausing pupae at least 1 month old display cytological features characteristic of synthetic inactivity, i.e., compact nuclei, degeneration of the striated border and a paucity of cytoplasmic vacuolation. Pupae chilled for at least 3 months have PG cells that are larger in size with prominent striated borders, cytoplasmic vacuoles and less-compact nuclei, and older animals undergoing pharate adult development at room temperature display even more intense signs of cellular activity (Herman & Gilbert, 1966). The capacity of these glands to synthesize ecdysone was not investigated, but haemolymph ecdysteroid titres generated for diapausing pupae and developing pharate adults of *H. cecropia* (McDaniel, 1979) can be correlated with histological changes in the appearance of the PG during diapause and diapause break (Herman & Gilbert, 1966; McDaniel *et al.* 1976). These changes presumably reflect changes in the rate of ecdysone synthesis. In *Manduca* pupae there is a good temporal and quantitative correlation between PG production of ecdysone *in vitro* and endogenous

ecdysteroid titres during both diapause and non-diapause development. The data argue convincingly that the increase in the ecdysteroid titre in non-diapausing pupae is due to increased PG activity and strongly suggest that the PG are activated by PTTH during the first few days of pupal life in non-diapausing animals, i.e., during the head critical period.

Since PTTH is present in the brain and retrocerebral complex of diapausing pupae in quantities sufficient to elicit development, the most reasonable explanation for the failure of the diapausing pupal PG to be stimulated is that PTTH release is inhibited. This is evidently the case as well in *Antheraea pernyi* where pupal brain extracts are active in bioassay (Williams, 1968) and transplanted diapause brains can evoke development in diapausing recipients (McDaniel & Berry, 1967). Similarly, brains from diapausing larvae surgically implanted into diapausing recipients of *Ostrinia nubilatis* evoke diapause break and resumption of development (Cloutier, Beck, McLeod & Silhacek, 1962), and in *Mamestra* diapausing pupal brains of varying ages are capable of activating co-cultured PG *in vitro* (Agui, 1975). Brains from diapausing *Manduca* pupae behave quite differently when transplanted and do not induce development in brainless recipients; thus, they retain their diapause commitment, i.e. PTTH is not released, even when subjected to surgical manipulation (Safranek & Williams, 1980). Brains alone *in vitro* do not appear to release PTTH, nor do diapausing pupal brain complexes 4 days after pupation and thereafter (unpublished observations). In addition, non-diapausing *Manduca* brain-retrocerebral complexes incubated *in vitro* do not release significant amounts of PTTH-like activity spontaneously (Bollenbacher *et al.* 1980; Carrow, Calabrese & Williams, 1981; unpublished observations). Overall, the above examples suggest that release rather than synthesis of PTTH is controlled during diapause in *Manduca*.

Although the above studies argue for PTTH release as the level at which insect diapause is controlled, other studies suggest a diversity of endocrine regulatory schemes. In *Hyalophora cecropia*, for example, unchilled diapausing pupal brains, as well as brain extracts, are incapable of evoking the development of diapausing animals (Williams, 1947, 1956, 1968). In this case, apparently, diapause termination requires that PTTH be synthesized; only after this requirement is met can release of the neurohormone and subsequent PG activation occur. In *Heliothis*, the endocrine situation during pupal diapause differs from that of the other species discussed above. Here, PTTH is apparently released very early in pupal diapause; the PG remain inactive at low temperatures but resume activity as the temperature rises (Meola & Adkisson, 1977). In only one species described so far, therefore – *H. cecropia* – is PTTH absent in the diapausing pupa. In all the others, PTTH is present and in one genus – *Heliothis* – it appears to be released shortly after entry into diapause. Thus, no single endocrine mechanism seems to be solely responsible for controlling pupal diapause in all Lepidoptera; species-specific ecological requirements apparently demand various modes of endocrine regulation.

The observations in *Heliothis* (Meola & Adkisson, 1977; Browning, 1981) suggest that in some cases, diapause could be at least partially controlled at a level independent of the brain. Other examples support this conjecture. There have been numerous reports, for example, that animals can break diapause and complete development even in the absence of their brains. Under certain conditions, brain extirpation or wounding

can stimulate development and wounding alone can induce development even in brainless animals (Judy, 1972; McDaniel & Berry, 1967; Wilson & Larsen, 1974; Maslennikova, 1970; Kind, 1976). The effects of brain removal and trauma vary greatly, but appear to depend, at least in part, on both the species and the age of the animals used. Furthermore, there have been more direct demonstrations that the PG themselves are capable of responding directly to environmental stimuli. For example, debrained diapausing pupae of *Papilio* can develop directly in response to chilling (Ozeki, 1954). McDaniel *et al.* (1976) have observed histological changes in the appearance of the PG in *H. cecropia* which suggest that at least the initial stages of ecdysone secretion can be accomplished *via* a direct response to temperature by the glands themselves without the intervention of PTTH. These studies pose the possibility that the PG are capable of autonomously regulating diapause. More recently, it has been reported that the PG of *Philosamia* larvae retain a surprising degree of independence from the brain, as well as being inherently light-sensitive (Mizoguchi & Ishizaki, 1982).

The PG of *Manduca* may also be independently responsive to environmental stimuli, since diapause in this species is the result not only of curtailment of PTTH release from the CA but also of a diapause-programmed refractory state characteristic of the PG themselves. Since the glands are less sensitive to PTTH stimulation from the day of larval–pupal ecdysis, this condition appears to be at least partially independent of pupal brain prothoracicotropic activity and may be the result of events occurring late in larval life. This finding is in contrast to that of Safranek & Williams (1980) who reported that pupae less than 12 h old can respond to implanted, active, (non-diapause) brains *in vivo* by breaking diapause. These apparently contradictory observations may be related to the age of the animals used in the latter experiments, since PG from day 0 pupae are not totally refractory to PTTH. While the *in vitro* activation response of glands from diapausing pupae is only about one-third that of PG from non-diapausing pupae, this attenuated response may still have been sufficient to break diapause *in vivo*. To clarify this issue, one should implant 'active' brains into older diapausing recipients, at a time when the PG are completely refractory to PTTH stimulation *in vitro*.

A similar state of PG refractoriness has been observed in *Mamestra* where PG from older diapausing animals have been found to be more difficult to activate *in vitro* than PG from younger individuals (Agui, 1975). In contrast, diapausing pupal PG of *H. cecropia* respond readily to implanted active brains (Williams, 1947), indicating that in this species, PG from diapausing pupae are evidently not refractory to neurohormonal stimulation. These differences in PG responsiveness may reflect interspecific variations, but they also suggest that caution should be exercised in interpreting developmental responses observed with preparations from very young diapausing individuals, i.e. individuals whose entry into diapause may be incomplete.

Although the response of refractory PG to other factors besides PTTH *in vitro* was not investigated in this study, the possibility exists that the refractory condition of the glands is a general response to humoral stimuli. It has been suggested, for example, that ecdysone may exert a positive feedback effect on the PG (Williams, 1952; Siew & Gilbert, 1971; Kimura & Kobayashi, 1975). Since there are low levels of ecdysteroids present at all times in diapausing *Manduca* pupae, a general refractory

condition of the PG may be a physiological necessity to insure the absence of positive feedback during the period of dormancy.

The biochemical basis of PG refractoriness is conjectural at present. A depressed level of available PTTH receptors is one conceivable mechanism, although other possibilities exist. The levels of endogenous sterol precursors in the gland could be low, for example, or the mechanisms responsible for their uptake could be altered. The enzyme system responsible for ecdysone biosynthesis could also be repressed. Only with further studies can we begin to understand the molecular basis of the refractory state.

In conclusion, it appears that inhibition of PTTH release and PG refractoriness accompany diapause in *Manduca*. It must be noted, however, that this study has not demonstrated unequivocally the absence of PTTH release in diapausing animals. Results of this study only indirectly suggest that possibility. Since the PG are refractory to PTTH stimulation during diapause, it is possible that some release of this neuropeptide can occur during diapause. The possibility also arises that diapause termination does not come about merely because of a reversal of the inhibition of PTTH release; PTTH release may not be involved at all in this process.

Widely divergent mechanisms of endocrine control very probably reflect different ecological requirements for the environmental regulation of diapause. Ecological requirements may be considered to be a reflection of the particular environment to which the insect has adapted its overwintering strategy. Given the various adaptations of these different species, it is not surprising that the underlying endocrine mechanisms reflect this variety. The insect endocrine system is apparently characterized by a degree of plasticity quite adequate to encompass diverse evolutionary adaptations. Generalities, then, with respect to the endocrine basis of diapause may not be possible. An array of endocrine mechanisms may be operating even among those species which undergo diapause at identical stages of development.

The authors wish to thank Dr David Saunders for his critical reading of the manuscript and Sheila King for her assistance in preparing the manuscript. Supported by grants AM-30118 to L. I. Gilbert and NS-18791 and AM-31642 to W. E. Bollenbacher from the National Institutes of Health. M. F. Bowen is supported by National Research Service Award F32 AM06855 from N.I.H.

REFERENCES

Agui, N. (1975). Activation of prothoracic glands by brains *in vitro*. *J. Insect Physiol.* **21**, 903–913.

Agui, N., Bollenbacher, W. E., Granger, N. A. & Gilbert, L. I. (1980). Corpus allatum is release site for insect prothoracicotropic hormone. *Nature, Lond.* **285**, 669–670.

Agui, N., Granger, N. A., Gilbert, L. I. & Bollenbacher, W. E. (1979). Cellular localization of insect prothoracicotropic hormone: *In vitro* assay of a single neurosecretory cell. *Proc. natl Acad. Sci. U.S.A.* **76**, 5694–5690.

Bell, R. A. & Joachim, F. G. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. ent. Soc. Am.* **69**, 365–373.

Bollenbacher, W. E., Agui, N., Granger, N. A. & Gilbert, L. I. (1979). *In vitro* activation of insect prothoracic glands by the prothoracicotropic hormone. *Proc. natl Acad. Sci. U.S.A.* **76**, 5148–5152.

Bollenbacher, W. E., Agui, N., Granger, N. A. & Gilbert, L. I. (1980). Insect prothoracic glands *in vitro*. A system for studying the prothoracicotropic hormone. In *Invertebrate Systems* in vitro, (eds Kurstak, K. Maramorosch & A. Dübendorfer), pp. 253–271. Amsterdam: Elsevier/North-Holland.

BOLLENBACHER, W. E. & GILBERT, L. I. (1981). Neuroendocrine control of postembryonic development in insects: The prothoracicotropic hormone. In *Neurosecretion: Molecules, Cells, Systems*, (eds D. S. Farner & K. Lederis), pp. 361–370. New York: Plenum Press.

BOLLENBACHER, W. E., SMITH, S. L., GOODMAN, W. & GILBERT, L. I. (1981). Ecdysteroid titer during larval–pupal–adult development of the tobacco hornworm, *Manduca sexta*. *Gen. comp. Endocr.* **35**, 27–34.

BOLLENBACHER, W. E., SMITH, S. L., WIELGUS, J. J. & GILBERT, L. I. (1977). Evidence for an α-ecdysone cytochrome P-450 mixed function oxidase in insect fat body mitochondria. *Nature, Lond.* **268**, 660–663.

BOLLENBACHER, W. E., VEDECKIS, W. V., GILBERT, L. I. & O'CONNOR, J. D. (1975). Ecdysone titers and prothoracic gland activity during larval–pupal development of *Manduca sexta*. *Devl Biol.* **44**, 46–53.

BRADFIELD, J. Y., IV & DENLINGER, D. L. (1980). Diapause development in the tobacco hornworm: a role for ecdysone or juvenile hormone? *Gen. comp. Endocr.* **41**, 101–107.

BROWNING, T. O. (1981). Ecdysteroids and diapause in pupae of *Heliothis punctiger*. *J. Insect Physiol.* **27**, 715–721.

CARROW, G., CALABRESE, R. L. & WILLIAMS, C. M. (1981). Spontaneous and evoked release of prothoracicotropin from multiple neurohemal organs of the tobacco hornworm. *Proc. natl Acad. Sci. U.S.A.* **78**, 5866–5870.

CLOUTIER, E. J., BECK, S. D., MCLEOD, D. G. R. & SILHACEK, D. L. (1962). Neural transplants and insect diapause. *Nature, Lond.* **195**, 1222–1224.

DENLINGER, D. L. (1984). Hormonal control of diapause. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 8, (eds G. A. Kerkut & L. I. Gilbert), (in press). Oxford: Pergamon Press.

GILBERT, L. I., BOLLENBACHER, W. E., AGUI, N., GRANGER, N. A., SEDLAK, B. J., GIBBS, D. & BUYS, C. M. (1981). The prothoracicotropes: source of the prothoracicotropic hormone. *Am. Zool.* **21**, 641–653.

GILBERT, L. I., BOLLENBACHER, W. E. & GRANGER, N. A. (1980). Insect endocrinology: regulation of endocrine glands, hormone titer and hormone metabolism. *Ann. Rev. Physiol.* **42**, 493–510.

GILBERT, L. I., GOODMAN, W. & BOLLENBACHER, W. E. (1977). Biochemistry of regulatory lipids and sterols in insects. In *International Review of Biochemistry. Biochemistry of Lipids II*, Vol. 14, (ed. T. W. Goodwin), pp. 1–50. Baltimore: University Park Press.

GRANGER, N. A., NIEMIEC, S. M., GILBERT, L. I. & BOLLENBACHER, W. E. (1982). Juvenile hormone synthesis *in vitro* by larval and pupal corpora allata of *Manduca sexta*. *Mol. cell. Endocr.* **28**, 587–604.

HERMAN, W. S. (1967). The ecdysial glands of arthropods. *Int. Rev. Cytology* **22**, 269–347.

HERMAN, W. S. & GILBERT, L. I. (1966). The neuroendocrine system of *Hyalophora cecropia* (L.) (Lepidoptera: Saturniidae) I. The anatomy and histology of the ecdysial glands. *Gen. comp. Endocr.* **7**, 275–291.

JUDY, K. J. (1972). Diapause termination and metamorphosis in brainless tobacco hornworms (Lepidoptera). *Life Sci.* **11**, 605–611.

KIMURA, S. & KOBAYASHI, M. (1975). Prothoracicotropic action of ecdysone analogues in *Bombyx mori*. *J. Insect Physiol.* **21**, 417–421.

KIND, T. V. (1976). Endocrine regulation of the reactivation of diapausal pupae of the white Cabbage Butterfly, *Pieris brassicae L. Dokl. Akad. Nauk. SSSR* **229**, 1266–1267.

KIND, T. V. (1978*a*). A study of the reactivation of diapausing pupae of *Acronycta rumicis L.* (Lepidoptera, Noctuidae). I. Cold activation of the prothoracic glands in brainless pupae. *Ent. Rev.* **56**, 13–16.

KIND, T. V. (1978*b*). A study of the reactivation of diapausing pupae of *Acronycta rumicis L.* (Lepidoptera, Noctuidae). II. Reactivation of intact and brainless pupae at various temperatures. *Ent. Rev.* **57**, 163–167.

KING, D. S., BOLLENBACHER, W. E., BORST, D. W., VEDECKIS, W. V., O'CONNOR, J. D., ITTYCHERIAH, P. I. & GILBERT, L. I. (1974). The secretion of α-ecdysone by the prothoracic glands of *Manduca sexta in vitro*. *Proc. natl Acad. Sci. U.S.A.* **71**, 793–796.

LOEB, M. J. (1982). Diapause and development in the tobacco budworm, *Heliothis virescens*: a comparison of haemolymph ecdysteroid titers. *J. Insect Physiol.* **28**, 667–673.

MCDANIEL, C. N. (1979). Haemolymph ecdysone concentrations in *Hyalaphora cecropia* pupae, dauer pupae and adults. *J. Insect Physiol.* **25**, 143–145.

MCDANIEL, C. N. & BERRY, S. J. (1967). Activation of the prothoracic glands of *Antheraea polyphemus*. *Nature, Lond.* **214**, 1032–1033.

MCDANIEL, C. N., JOHNSON, E., SAUN, T. & BERRY, S. J. (1976). Ultrastructure of active and inhibited prothoracic glands. *J. Insect Physiol.* **22**, 473–481.

MASLENNIKOVA, V. A. (1970). Hormonal regulation of diapause in *Pieris brassicae L. Dokl. Akad. Nauk. SSSR* **192**, 942–945.

MEOLA, R. W. & ADKISSON, P. L. (1977). Release of prothoracicotropic hormone and potentiation of developmental ability during diapause in the bollworm, *Heliothis zea*. *J. Insect Physiol.* **23**, 683–688.

MIZOGUCHI, A. & ISHIZAKI, H. (1982). Prothoracic glands of the saturniid moth *Samia cynthia ricini* possess a circadian clock controlling gut purge timing. *Proc. natl Acad. Sci. U.S.A.* **79**, 2726–2730.

OZEKI, K. (1954). Experiments on the formation of imaginal structures in the pupae of the swallowtail, *Papilio xuthus Linnaeus*. *Scient. Pap. Coll. gen. Educ. Tokyo* **4**, 47–56.

RABB, R. L. (1966). Diapause in *Protoparce sexta* (Lepidoptera: Sphingidae). *Ann. ent. Soc. Am.* **59**, 160–165.

SAFRANEK, L. & WILLIAMS, C. M. (1980). Studies of the prothoracicotropic hormone in the tobacco hornworm, *Manduca sexta*. *Biol. Bull. mar. biol. Lab., Woods Hole* **158**, 141–153.

Siew, Y. C. & Gilbert, L. I. (1971). Effects of moulting hormone and juvenile hormone on insect endocrine gland activity. *J. Insect Physiol.* **17**, 2095–2104.

Walker, G. P. & Denlinger, D. L. (1980). Juvenile hormone and moulting hormone titres in diapause and non-diapause destined flesh flies. *J. Insect Physiol.* **26**, 661–664.

Weevers, R. D. (1966). A lepidopteran saline: effects of inorganic cation concentrations on sensory, reflex and motor responses in a herbivorous insect. *J. exp. Biol.* **44**, 163–175.

Williams, C. M. (1947). The physiology of insect diapause. II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia*. *Biol. Bull. mar. biol. Lab., Woods Hole* **93**, 89–98.

Williams, C. M. (1952). Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the cecropia silkworm. *Biol. Bull. mar. biol. Lab., Woods Hole* **103**, 120–138.

Williams, C. M. (1956). Physiology of insect diapause. X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the cecropia silkworm. *Biol. Bull. mar. biol. Lab., Woods Hole* **110**, 201–218.

Williams, C. M. (1968). The present status of the brain hormone. In *Insects and Physiology*, (eds J. W. L. Beaumont & J. E. Treherne), pp. 133–139. London: Oliver and Boyd.

Wilson, G. R. & Larsen, J. R. (1974). Debraining and diapause development in *Manduca sexta* pupae. *J. Insect Physiol.* **20**, 2459–2473.