J. exp. Biol. (1980), **88**, 327–337
With 7 figures
Printed in Great Britain

PHYSIOLOGY OF PUPAL ECDYSIS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

I. EVIDENCE FOR CONTROL BY ECLOSION HORMONE

BY JAMES W. TRUMAN, PAUL H. TAGHERT AND STUART E. REYNOLDS*

Department of Zoology, University of Washington, Seattle, WA 98195, *U.S.A.*

(*Received* 20 *March* 1980)

SUMMARY

The time of pupal ecdysis in *Manduca sexta* can be accurately predicted by the appearance of external markers late in pupal development. Eclosion hormone injections resulted in premature ecdysis of staged pharate pupae. The latency between injection and initiation of ecdysis movements was inversely correlated with dosage and served as the basis for a sensitive, rapid, and quantitative bioassay for the hormone.

Behavioural responsiveness to the hormone arose about 8 h before normal ecdysis at 23 °C. When insects were injected at these early times the larval cuticle was not sufficiently digested to allow its rupture and these animals remained permanently trapped inside the larval cuticle.

Measurement of eclosion hormone in the blood of pharate pupae showed the appearance of biological activity in the blood about 30 min before the onset of ecdysis.

It is concluded that pupal ecdysis is under endocrine control and that the hormone involved is likely to be the eclosion hormone.

INTRODUCTION

Ecdysis in insects is a complex event requiring the coordination of a series of behavioural, physiological and developmental processes (Reynolds, 1980). The adult eclosion of saturniid and sphingid moths has been particularly suitable for the experimental investigation of ecdysis because its time of occurrence is restricted to a period of only a few hours each day. The time of this 'gate' is dictated by a circadian clock in the brain (Truman, 1972*a*) and is highly predictable. Moreover, the time at which the insect becomes competent to eclose may be separated by a number of hours from the eclosion gate thereby presenting a period during which one may experimentally attempt to evoke early ecdysis. By means of this approach it was found that a peptide hormone, the eclosion hormone, triggers eclosion and associated behaviours (Truman & Riddiford, 1970; Truman, 1971) and integrates these with

* Permanent address: School of Biological Sciences, University of Bath, Claverton Down, Bath, BA2 7AY, U.K.

the physiological events characteristic of eclosion (for a review see Reynolds, 1980).

The ecdyses of earlier stages in the life history of the moth do not show circadian gating but rather occur as the last step in the developmental programme that is played out at each moult (Truman, 1972*b*; Truman & Riddiford, 1974). However, by using certain developmental markers, we have been able to predict the time at which ecdysis will occur in these earlier stages with some accuracy. This has allowed us to investigate the role of the neuroendocrine system in controlling the behavioural and physiological changes that occur during pupal ecdysis of the tobacco hornworm, *Manduca sexta*.

MATERIALS AND METHODS

Experimental animals

Tobacco hornworms, *Manduca sexta* (L), were reared individually on an artificial diet (Bell & Joachim, 1978) under long-day conditions (17L:7D) at 26 °C. Following the start of the wandering stage, the larvae were stored in holes bored in wooden blocks. Four to 12 h before pupal ecdysis the pharate pupae were transferred to glass vials so that the terminal stages of pupal development could be readily observed.

Staging of pharate pupae

Development to the pupal stage in *Manduca* begins with the release of prothoracicotropic hormone and ecdysone by the wandering larva and ends approximately 60 h later with the shedding of the larval cuticle (Truman & Riddiford, 1974). The late stages of pupal development can be characterized by features of the forming pupal cuticle visible through the larval skin. The first pupal structures to appear are two heavily sclerotized bars placed on either side of the dorsal midline on the metathorax. These 'dorsal bars' become visible under the larval cuticle about a day prior to ecdysis. During the next hours, various pupal abdominal structures such as the lateral gin traps and the small depressions ('pocks') on the anterior half of each segment appear and gradually darken. The gradual appearance of these structures made them poor markers to define the progression of development. Discrete characters only became available late in pupal development and these are listed in Table 1.

During the last 4 h of development, animals were staged according to the extent of digestion of the larval cuticle and the state of tanning of the dorsal abdominal cuticle in the area between the pocks. Selection of animals of earlier ages was accomplished by timing them relative to the appearance of the dorsal bars.

Determination of eclosion hormone titres in blood

Blood was collected into ice-chilled tubes which were then transferred to an 80 °C water bath for 5 min. This heat-treated blood was centrifuged at 1000 *g* for 15 min and the clear supernatant was frozen at −20 °C until assayed. Eclosion hormone activity in the samples was measured using the *Manduca* wing assay devised by Reynolds (1977) as modified by Reynolds & Truman (1980). Pairs of mesothoracic wings were collected from suitably staged pharate adults and stored

Table 1. *Timetable of the appearance of morphological characters in pharate pupae of* Manduca sexta *at* 23 °C

Stage	Characteristics	Time before ecdysis (h)
Dorsal bar	First appearance of paired, transverse, sclerotized bars on the dorsum of metathorax	23
Anterior shrinkage (AS)	Molting fluid resorbtion in anterior segments; lateral aspect of first abdominal segment shows a sharp transverse fold in larval cuticle	3·5
Posterior shrinkage (PS)	Distinct folds in cuticle covering the posterior segments; most evident on dorsum of last segment	2·5
A_1 tanned	Anterior dorsal region of first abdominal segment uniformly tanned in area between pocks	1·5
A_2 tanned	Anterior dorsal region of second abdominal segment as above	0·5

together on moist filterpaper. One wing of each pair was injected with 10 μl of heat-treated serum and the other wing served as a control. After incubation at room temperature for about 1½ h the extensibility of each wing was estimated by hanging a 3 g load from the wing for 2 min and measuring the amount the wing had stretched during that period. Scores were calculated as the increase in length of the test wing minus that of the control wing. Differences greater than 1 mm indicated eclosion hormone activity.

Preparation of hormonal material

Extracts containing eclosion hormone were obtained from corpora cardiaca-corpora allata (CC–CA) complexes that were dissected from pharate adult *Manduca sexta*. Glands were homogenized in distilled water. Homogenates were heat-treated at 80 °C for 5 min, centrifuged at 1000 *g* for 15 min, and the supernatant frozen until needed.

A few experiments utilized eclosion hormone that had been purified from *Manduca* CC–CA complexes by the method of Mumby, Truman & Reynolds (in preparation) which involved fractionation of similar extracts through a Sephadex G-50 column followed by narrow band electrofocusing on polyacrylamide gel.

Materials were injected into the posterior segments of pharate pupae by means of a 50 μl Hamilton syringe.

RESULTS

Pupal ecdysis

The morphological changes that occur during pupal ecdysis are depicted in Fig. 1. Prior to ecdysis the pharate pupae maintained a dorsal-side-up posture and were usually slightly flexed to one side. The first sign of impending ecdysis was a straightening of the animal. Ecdysis began with the periodic retraction of the terminal segment into the penultimate one. Initially these contractions involved only the

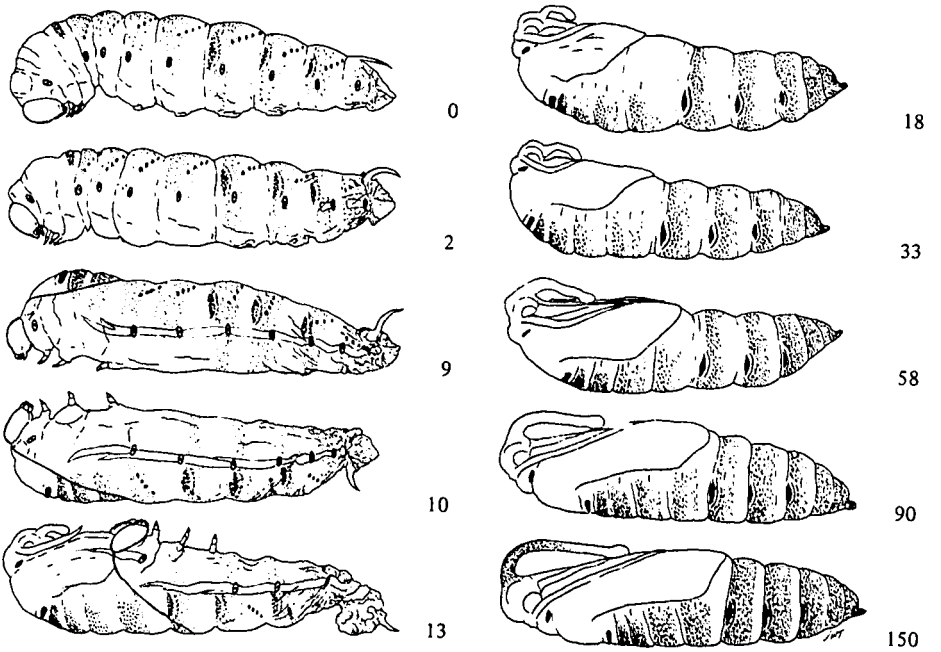

Fig. 1. Pupal ecdysis and postecdysial expansion in *Manduca sexta*. Numbers refer to the time in minutes from the start of ecdysis behaviour. Traced from photographic prints.

last two segments but gradually more segments were recruited until peristaltic waves of contraction moved anteriorly along the entire length of the abdomen. These contractions served to move the larval cuticle posteriorly, drawing first on the cuticle of the most posterior segments (Fig. 1, 2 min), and, as this moved backwards, tension was transmitted to more anterior segments. The main trachael linings in each segment were withdrawn along with the cuticle and appeared as a white band along the side of the animal (Fig. 1, 9 min).

The increasing tension in the larval cuticle eventually caused it to rupture along the dorsal midline at a point between the dorsal bars. These sclerotized structures apparently act as 'cuticle bursters'. At approximately the time when the widening ecdysial seam reached the old larval head capsule, the animal rolled over on to its back (Fig. 1, 10 min), the larval head capsule split open, and the tension generated by the abdominal movements drew the head capsule away from the pupal head appendages (Fig. 1, 13 min). The freeing of the legs and wings followed shortly thereafter. The larval cuticle was cast off about 20 min after the inception of the behaviour.

As seen in Fig. 1 (18–150 min), ecdysis was followed by the expansion of head and thoracic structures to their normal pupal size. This was accomplished by blood forced up from the abdomen to expand these appendages. Expansion was generally completed by 2 h after the end of ecdysis.

The movements that generated the force for the shedding of the larval skin were the rhythmically occurring peristaltic waves. Fig. 2 shows that the course of ecdysis was marked by stereotyped changes in the periodicity of these waves. Initially, the waves occurred with a relatively short periodicity and involved only the terminal

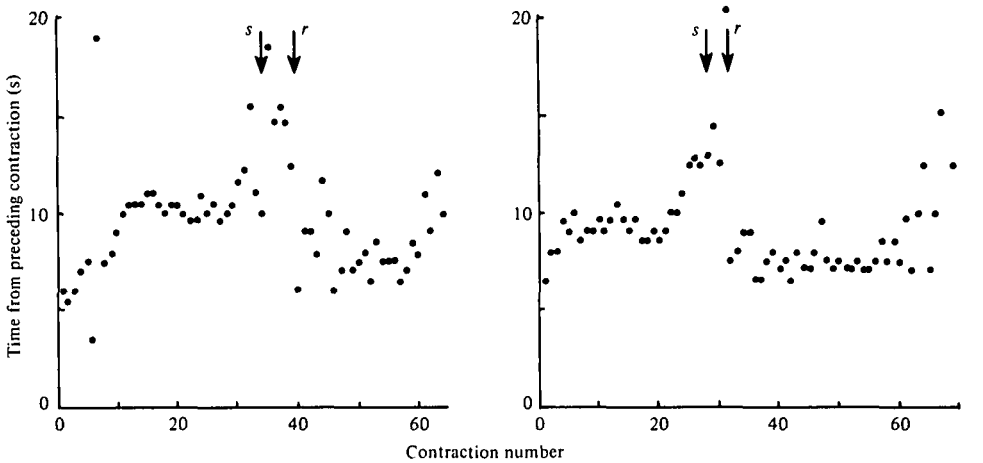

Fig. 2. The periods between successive contractions of the sixth abdominal segment during pupal ecdysis of two individuals. *s*, Splitting of the dorsal exuvial seam; *r*, the insect rolls on to its back.

segments. As more segments were recruited, the period of the movements increased and stabilized at about 10 s. A few cycles before the split of the larval skin, as the cuticle was being stretched taut over the anterior end, the period again lengthened and became somewhat erratic. The longest intervals between waves were those of the cycles between the initial rupture of the cuticle and the animal rolling onto its back. With the pupa ventral-side-up, the period length abruptly shortened, this time to an average value of about 7 s and stayed relatively constant while the larval cuticle was being moved along the abdomen. The terminal phases of casting off the cuticle were marked by occasional rotary movements and very variable cycle lengths.

Release of ecdysis behaviour by eclosion hormone

The corpora cardiaca of pharate adult moths provide the most concentrated known source of eclosion hormone (Truman, 1973). Extracts of pharate adult CC–CA were injected into pharate pupae at the anterior shrinkage stage (AS; Table 1) which was about 3·5 h before the expected time of ecdysis. As seen in Fig. 3, these extracts caused early ecdysis of the test animals whereas extracts prepared from muscle had no such effect. Moreover, the latency between injection and the onset of ecdysis movements appeared to be a function of the dose of CC–CA extract that was administered. Pharate pupae also responded to 0·1 CC equivalents of eclosion hormone (Fig. 3) that had been purified from pharate adult *Manduca* CC–CA complexes as described in the Materials and Methods. The response of the pharate pupae to the hormone was intermediate between the responses of test animals to 0·12 and 0·06 CC equivalents of the crude extract respectively. Thus it appears that all of the pupal ecdysis stimulating activity in crude extracts from pharate adult CC–CA can be accounted for by their known content of eclosion hormone.

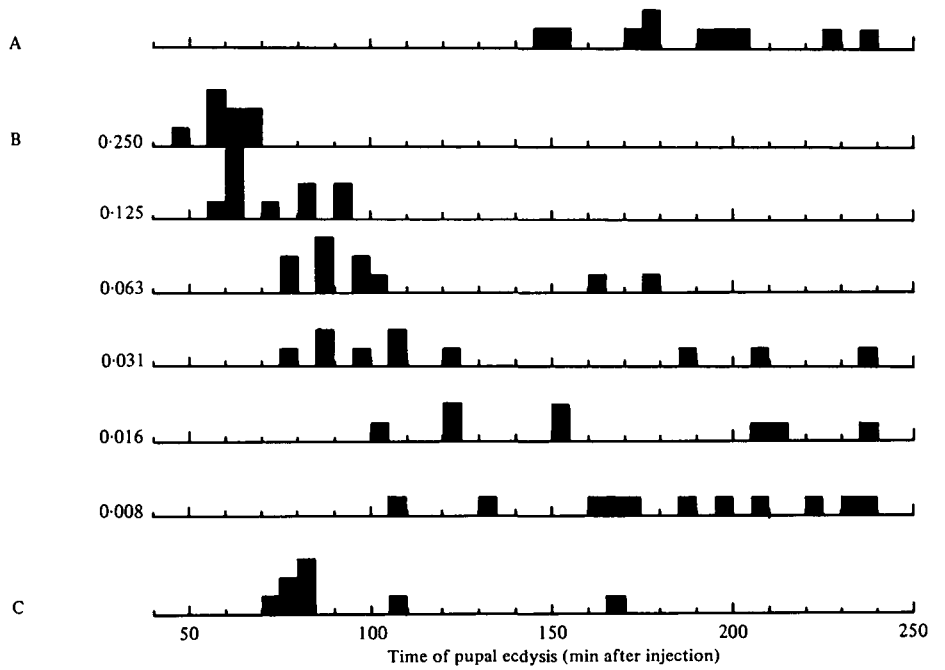

Fig. 3. Time of initiation of ecdysis by AS stage animals after injection with (A) muscle extract; (B) various dilutions of a pharate adult CC-CA extract; (C) 0·1 CC-CA equivalents of purified eclosion hormone. Temperature was 23 °C.

The pupal ecdysis bioassay for eclosion hormone

The variation in the response latency with the dosage of hormone injected was used as the basis for a quantitative bioassay for eclosion hormone. AS stage pharate pupae were injected with test solutions, and the latency between the injection and the start of ecdysis was recorded. This latency was converted to a numerical score as follows:

$$\text{bioassay score} = 10^4/\text{latency (min)}.$$

In order to allow for the fact that pharate pupae selected at stage AS will undergo ecdysis at times ranging from 2·5–4·0 h later, even if they received no exogenous eclosion hormone, we routinely assigned a score of 51 to negative assays (insects that had not begun ecdysis by 130 min after injection). This score was selected because the mean score for all the negative responses which were measured in the course of this study was $51{\cdot}4 \pm 0{\cdot}6$ (S.E.) ($N = 304$).

This scoring system was used to score the response of AS stage pharate pupae to various dosages of pharate CC–CA extract. As seen in Fig. 4, positive responses were obtained with doses as small as 0·02 CC–CA pair, the response being linear over more than one log unit increase in concentration. Thus the latency of the response of prepupae to extracts that contain eclosion hormone appears to provide a sensitive and quantitative estimate of the amount of eclosion hormone that is present.

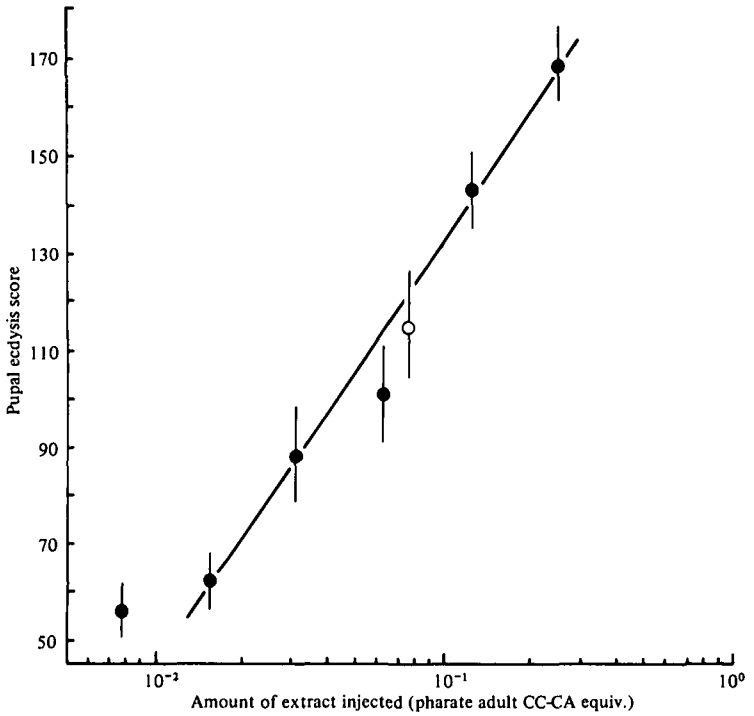

Fig. 4. The response of AS stage pharate pupae to various dilutions of a pharate adult CC-CA extract (solid circles) or purified eclosion hormone (open circle). Each represents the mean ± S.E. of approximately ten determinations. Scores were calculated as described in the text. The line is eye-fitted through the solid circles.

Development of behavioural sensitivity to eclosion hormone

The above section shows that pharate pupae at stage AS are fully sensitive to eclosion hormone and undergo successful precocious ecdysis in response to hormone injection. To determine at which time this sensitivity developed, we challenged pharate pupae with CC–CA extracts at various times after the appearance of the dorsal bars. The normal interval between this marker and ecdysis was temperature-dependent, ranging from 17·5 h at 26 °C to about 28 h at 21 °C. Since we experienced some changes in holding temperatures during the course of this study, we expressed the time of injection as the percentage of the normal time between the onset of dorsal bars and ecdysis. As seen in Fig. 5, a behavioural response to the hormone first appeared about 60–70% of the way through this last phase of development (about 8 h before ecdysis at 23 °C).

Although hormone treatment could evoke very early ecdysis behaviour, in many cases the larval cuticle was not sufficiently digested to allow for its shedding. For example, of the 14 animals that attempted ecdysis after treatment at 63%, only 5 managed to shed the larval cuticle. For these insects, cuticle shedding was not accomplished within the normal 15–20 min period; instead 2–3 h elapsed between the start of ecdysis movements and the eventual casting off of the old skin. The remainder of the insects initiated the peristaltic waves typical of ecdysis, continued for a number of hours, and then terminated the behaviour without rupturing the

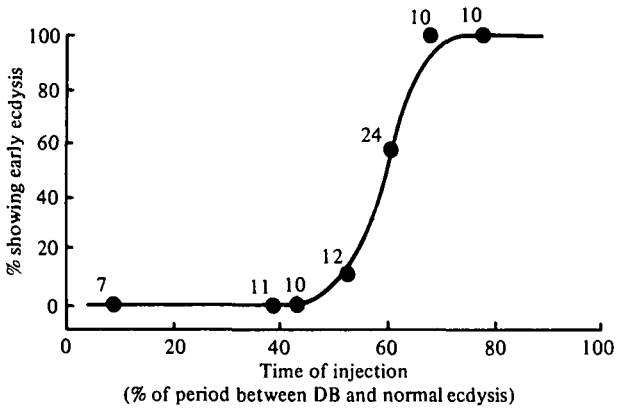

Fig. 5. The percentage of pharate pupae that showed early ecdysis behaviour after injection with CC-CA extract at various times during the period between the appearance of dorsal bars (DB) and ecdyses. Numbers refer to the number of animals injected at each time.

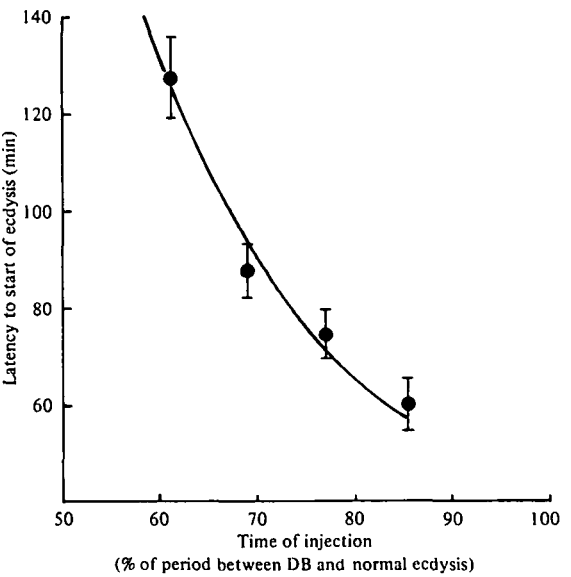

Fig. 6. Relationship between the time of injection of one pharate adult CC-CA equivalent into pharate pupae at various times in development and the subsequent average latency (±S.E.) to the onset of ecdysis behaviour. Animals were held at 23 °C after the injections.

larval cuticle. Interestingly, most of the animals assumed the ventral-side-up posture characteristic of the pupa by the time that they stopped their ecdysis movements.

The response latencies of these insects were typically longer than those shown by pharate pupae injected with the same dosage of hormone at the AS stage. For example, pharate pupae injected at 63% showed an average response latency of 140 min as compared to the 60 min latency seen after treatment of AS stage pharate pupae (85% completion of the last stage in development). As seen in Fig. 6, there was a systematic reduction in response latency as the insects progressed toward their normal time of ecdysis. Obviously, in terms of obtaining consistent bioassay responses, one should be as precise as possible in staging the test animals.

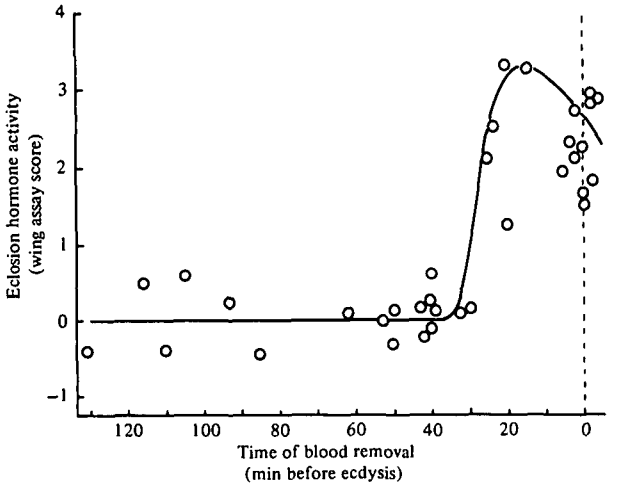

Fig. 7. Eclosion hormone activity in the blood of pharate pupae at various times before the onset of pupal ecdysis. Each dot represents an average of two assays performed on the blood of an individual insect. Positive scores are greater than 1.

Table 2. *Response of AS stage pharate pupae to injections of blood from pre-ecdysis and ecdysing animals*

Stage of donor	Vol. injected (μl)	Number	Ecdysis score ($\bar{x} \pm$ S.D.)
Posterior shrinkage	100	8	51 ± 0
Start of ecdysis	100	11	172 ± 48

Blood titres of eclosion hormone in Manduca *pharate pupae*

Insects were selected late in pupal development, the side of the head was nicked and 5–10 drops of blood were collected into ice-chilled tubes. The blood samples were processed and assayed for eclosion hormone activity using the adult *Manduca* wing test (see Materials and Methods). All of the donors subsequently initiated ecdysis and the time at which ecdysis behaviour began was recorded relative to the time of blood removal. The eclosion hormone activity in the blood of these animals is shown in Fig. 7. The hormone appeared abruptly in the blood about 30 min before ecdysis.

The data in Fig. 7 clearly show that a substance having activity in an adult eclosion hormone assay appears in the blood prior to the onset of ecdysis behaviour. To establish that this activity was related to stimulation of pupal ecdysis, prepupae were selected at 2 times, at the PS stage (see Table 1) and at the start of ecdysis. Blood was collected from animals in each group, treated as described above, and then injected into AS stage prepupae. As seen in Table 2 the blood from PS stage animals failed to show any ecdysis stimulating activity whereas the blood from ecdysing animals was very active.

DISCUSSION

The results reported in this paper suggest that the eclosion hormone does not function only in adult eclosion as has previously been suggested (Truman, 1973). During pupal ecdysis an active factor appears in the blood just prior to ecdysis

which evokes this behaviour when injected into other prepupae. This blood-borne material is active in eclosion hormone assays, and eclosion hormone itself, either in crude CC–CA extracts from pharate moths or in purified form, can also trigger pupal ecdysis behaviour. Accompanying this suggestive evidence are data in the following paper (Taghert, Truman & Reynolds, 1980) that show that the CNS of prepupae stores a hormonally active substance that is identical to the eclosion hormone in apparent molecular weight and isoelectric point. Moreover, this store of hormonal activity is depleted at the same time that ecdysis-stimulating activity appears in the blood. These various lines of evidence strongly argue that the eclosion hormone serves to control pupal ecdysis as well as adult eclosion.

During the past 7 years a number of bioassays have been developed for the eclosion hormone. The stimulation of adult eclosion in *Antheraea pernyi* showed a half maximal response at a dose of about 0·9 CC equivalents/animal (Truman, 1973). Release of eclosion behaviour in isolated abdomens from pharate *Hyalophora cecropia* proved slightly more sensitive showing a similar response at about 0·6 CC equivalents/abdomen (Mumby, Truman & Reynolds, in preparation). The pupal ecdysis assay in *Manduca* is about 10 times more sensitive than the above assays since the half maximal response dosage is 0·06 CC equivalents/animal. Thus, the prepupal assay is the most sensitive behavioural assay for the hormone that has yet been reported. However, it should be noted that it is still 10-fold less sensitive than the isolated *Manduca* wing assay which exploits the action of the hormone in increasing cuticular extensibility (Reynolds, 1977; Reynolds & Truman, 1980).

In the pharate adult moth, the onset of behavioural sensitivity to the eclosion hormone occurs at the time at which the animal can successfully break out of the pupal cuticle (Reynolds, Taghert & Truman, 1979). Interestingly, in the case of pupal ecdysis, behavioural sensitivity is acquired prior to complete cuticle breakdown, so that when eclosion hormone is injected too early, ecdysis behaviour may fail to rupture the larval cuticle. Animals that attempted such a premature ecdysis did not then terminate their behaviour after the normal time of 20 min but continued for a number of hours. Apparently, sensory feedback associated with the successful shedding of the cuticle provides the usual stimulus for the end of the program. It is also noteworthy that after these insects failed in their precocious attempt, they did not then reinitiate the behaviour with the arrival of their normal ecdysis time. They remained trapped inside the old larval cuticle. Seven similarly trapped insects were injected with a second dose of hormone at a stage of larval cuticle digestion that corresponded to AS. Four subsequently reinitiated ecdysis behaviour after a latency of 60–80 min and, of these, two successfully shed their larval skins. These results indicated that under some conditions the pupal ecdysis programme can be activated multiple times with successive hormone treatments. The fact that insects that are stimulated to show an early, unsuccessful bout of ecdysis behaviour do not then initiate a second bout on their own after the larval cuticle has been digested, suggests that they do not then release their own store of hormone at the usual time in pupal development. This failure is presumably due to the action of eclosion hormone in turning off these centers. A similar type of negative feedback action of the eclosion hormone on its release centers has been shown to occur in the adult (Truman, 197[illegible]

The data in Fig. 7 show that eclosion hormone appears in the blood about 30 min

fore ecdysis begins. However, in our hormone injection experiments we have never seen latencies between injection and behavioural response which were that short. The dose–response curve for the pupal ecdysis bioassay reached a plateau at an average latency of about 60 min although occasional individuals showed latencies as short as 39 min. A reason for this discrepancy may be related to the fact that the response latency to a given dose of hormone markedly declined during the few hours preceding the AS stage (Fig. 6). A continuation of this trend would result in a latency that was considerably shorter than 60 min by a time late in the A_1 stage, the apparent time of hormone secretion. Consequently, we believe that 30 min provides the best estimate of the normal latency between the appearance of hormone in the blood and the start of ecdysis behaviour.

These studies were supported by grants from NSF (PCM 77–24878) and from NIH (RO1 NS 13079 and KO4 NS 00193). P. H. T. was supported by a NIH training grant (GM 0710805) and by an NSF Predoctoral Fellowship (63–6665).

REFERENCES

BELL, R. A. & JOACHIM, F. A. (1978). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Ent. Soc. Am.* **69**, 365–373.

REYNOLDS, S. E. (1977). Control of cuticle extensibility in the wings of adult *Manduca* at the time of eclosion: effects of eclosion hormone and bursicon. *J. exp. Biol.* **54**, 805–814.

REYNOLDS, S. E. (1980). Integration of behaviour and physiology in ecdysis. *Adv. Insect Physiol.* **15** (in the Press).

REYNOLDS, S. E. & TRUMAN, J. W. (1980). Eclosion hormone. In *Insect Neurohormones* (ed. T. A. Miller). New York: Springer-Verlag (in the Press).

REYNOLDS, S. E., TAGHERT, P. H. & TRUMAN, J. W. (1979). Eclosion hormone and bursicon titres and the onset of hormonal responsiveness during the last day of adult development in *Manduca sexta* (L). *J. exp. Biol.* **78**, 77–86.

TAGHERT, P. H., TRUMAN, J. W. & REYNOLDS, S. E. (1980). Physiology of pupal ecdysis in the tobacco hornworm, *Manduca sexta*. II. Chemistry, distribution, and release of eclosion hormone at pupal ecdysis. *J. exp. Biol.* **88**, 339–349.

TRUMAN, J. W. (1971). Physiology of insect ecdysis. 1. The eclosion behaviour of saturniid moths and its hormonal release. *J. exp. Biol.* **54**, 805–814.

TRUMAN, J. W. (1972*a*). Physiology of insect rhythms I. Circadian organization of the endocrine events underlying the moulting cycle of larval tobacco hornworms. *J. exp. Biol.* **57**, 805–820.

TRUMAN, J. W. (1972*b*). Physiology of insect rhythms. II. The silkmoth brain as the location of the biological clock controlling eclosion. *J. comp. Physiol.* **81**, 99–114.

TRUMAN, J. W. (1973). Physiology of insect ecdysis. II. The assay and occurrence of the eclosion hormone in the Chinese Oak Silkmoth, *Antheraea pernyi*. *Biol. Bull. mar. biol. Lab., Woods Hole* **144**, 200–211.

TRUMAN, J. W. (1978). Rhythmic control over endocrine activity in insects. In *Comparative Endocrinology* (ed. P. J. Gaillard and H. H. Boer), pp. 123–148. Amsterdam: Elsevier/North Holland.

TRUMAN, J. W. & RIDDIFORD, L. M. (1970). Neuroendocrine control of ecdysis in silkmoths. *Science, N.Y.* **167**, 1624–1626.

TRUMAN, J. W. & RIDDIFORD, L. M. (1974). Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. exp. Biol.* **60**, 371–382.