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ECLOSION HORMONE AND BURSICON TITRES AND THE ONSET OF HORMONAL RESPONSIVENESS DURING THE LAST DAY OF ADULT DEVELOPMENT IN *MANDUCA SEXTA* (L)

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SUMMARY

The blood titres of eclosion hormone in *Manduca sexta* indicate that the hormone is liberated into the blood during a span of about 20 min and then disappears with a half-life ($t_{\frac{1}{2}}$) of about 45 min. Eclosion normally follows 2·5 h after the appearance of the hormone in the blood.

Bursicon appears in the blood within 2 min after the newly emerged moth comes to rest at a wing spreading site. Hormone is apparently released during the following 10 min and then disappears with a $t_{\frac{1}{2}}$ of 40–50 min.

The target tissues for the two hormones become responsive during the last day of development. The wing epidermis and nervous system appear to become sensitive to the eclosion hormone at about the same time, approximately 4 h before the hormone's release. Wings become responsive to bursicon about 2 h earlier.

During the last day of adult development, *Manduca* show a precise onset of responsiveness to bursicon and eclosion hormone followed a few hours later by the gated release of the two hormones. This appearance of hormone sensitivity is likely to be due to a photoperiodically gated event which occurs late in adult development but prior to eclosion hormone release. The nature of this event is unknown.

INTRODUCTION

The emergence of large moths from their pupal case is known to be controlled by a blood-borne factor, the eclosion hormone (Truman & Riddiford, 1970; Truman, 1971). This hormone triggers the performance of a sequence of behaviour patterns which are pre-programmed into the central nervous system (CNS) and which have the function of extricating the pharate moth from its pupal case (Truman & Sokolove, 1972; Truman, 1978). After eclosion, another hormone, bursicon (Fraenkel & Hsiao, 1965) the tanning hormone, acts to cause the hardening and darkening of the cuticle of the newly-inflated wings (Truman, 1973*a*). This paper reports measurements of the titres of eclosion hormone and bursicon which occur around the time of eclosion

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and examines the timing of the onset of sensitivity to these two hormones in the tobacco hornworm, *Manduca sexta*.

MATERIALS AND METHODS

Tobacco hornworms, *Manduca sexta* (L) (Lepidoptera: Sphingidae) were reared individually in the laboratory on an artificial diet (Bell & Joachim, 1976). As larvae, they were exposed to 'long day' conditions (17L:7D) at 25 °C, in order to avert pupal diapause. At the start of the wandering stage, the larvae were transferred to chambers in wooden blocks, in which they pupated. Once adult development was initiated, they were subjected to a 12L:12D photoperiod at 25 °C. During the last half of development, the insects also experienced a low amplitude thermoperiod coincident with photoperiod (day 27 °C:night 25 °C). This resulted in greater synchrony of eclosion (Lockshin, Rosett & Srokose, 1975).

The bioassays for eclosion hormone used in this study were (*a*) triggering of precocious eclosion in *Manduca* (as described in the Results section) and (*b*) induction of cuticle plasticization in isolated wings of pharate adult *Manduca* (Reynolds, 1977). Briefly, the mesothoracic wings were isolated from pharate adults at various times of day as indicated in the Results. Wings were then injected with 10 μl of the appropriate material, held for 60–90 min in a moist atmosphere, and then tested for extensibility by measuring the percentage increase in length 3 min after a 3 g load had been imposed. Activity was expressed as the ratio of the increase in length of the test wing to that of the Ringer-injected contralateral wing. A ratio of two or greater was considered a positive indication of eclosion hormone activity.

The bioassay for bursicon also used isolated *Manduca* wings. The assay is a modification of the method of Truman (1973*a*) as described by Reynolds (1977). The isolated wings were injected with hormonally active material, and examined for tanning 3 h later. The tanning response was seen as a yellow colour in the cuticle of the wing veins.

The corpora cardiaca (CC) of pharate adult *Manduca* were used as a source of eclosion hormone (Truman, 1973*a*) being taken from insects no more than 1 day before eclosion. The CC were not dissected free of the corpora allata (CA) which are closely associated with them in the adult moth. The CC/CA complexes were homogenized in saline solution (Ephrussi & Beadle, 1936), boiled for 1 min, and centrifuged (Beckman microfuge) before use.

Bursicon was obtained from the medial nerves of the abdominal nervous system of pharate adult *Manduca* (Truman, 1973*a*). These were homogenized in saline solution, warmed to 70 °C to retard tyrosinase activity, and stored on ice until used.

Blood samples were collected either by decapitation (single samples) or by heart cannulation (sequential samples). In the latter case, a length of polyethylene tubing (PE-10; Clay Adams), with a bevelled point, was inserted through the cuticle of the metanotum into the heart, and held in place with Tackiwax. In most cases blood flowed freely through the cannula, or it was removed with a 50 μl Hamilton syringe inserted into the cannula. After sampling, the tip of the cannula was heat-sealed with hot forceps. It was reopened for the next sample by cutting off the tip.

All times are given as 'arbitrary zeitgeber time' (AZT; Pittendrigh, 1965), where lights-off is 24.00 AZT.

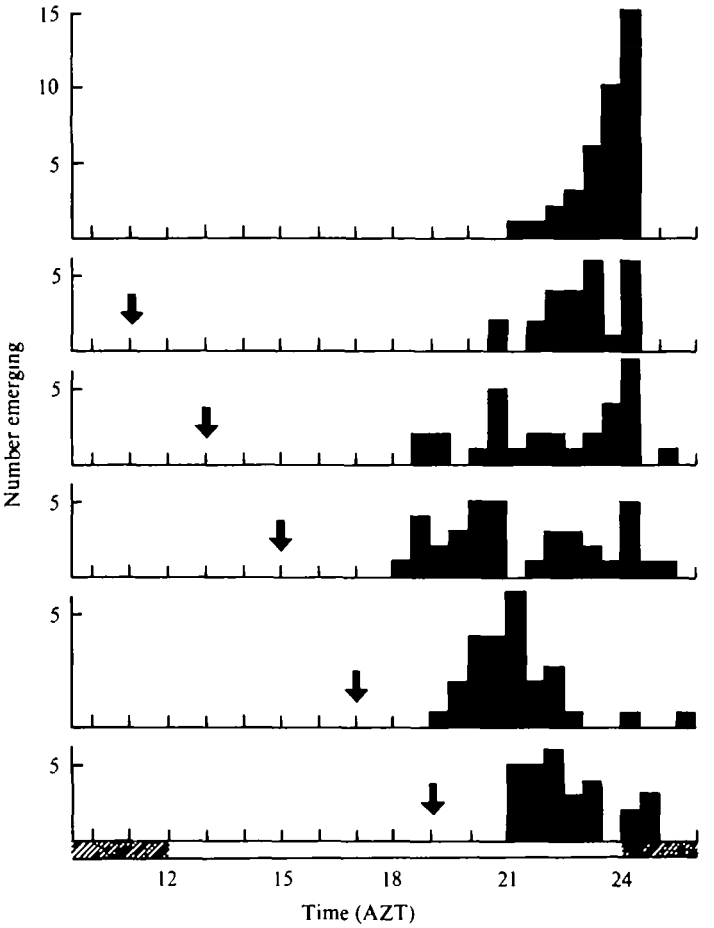

Fig. 1. Relationship of time of day to the effectiveness of eclosion hormone injections in triggering early eclosion of pharate adult *Manduca*. Each animal received the equivalent of 1 CC/CA complex. A, Uninjected controls; B, injected at 11.00; C, 13.00; D, 15.00; E, 17.00; F, 19.00. Arrows show time of injection.

RESULTS

The onset of responsiveness to the eclosion hormone

Under our photoperiod and temperature conditions, adult *Manduca* eclosed from about 22.00 to 24.30 h (Fig. 1 A). The emergence distribution showed a pronounced skewing with approximately 40% of the moths emerging during the half hour after the onset of darkness. The ability of CC/CA extracts to trigger precocious eclosion in *Manduca* was tested by challenging groups of moths with a standard dose of extract at various times during the last day of adult development. As seen in Fig. 1(B), injection of extract at 11.00 was ineffective since no moths emerged prior to their normal gate. After treatment at 13.00 (Fig. 1 C) a few moths were stimulated to eclose beginning $5\frac{1}{2}$ h after injection but most of the animals emerged during the gate. Injections at 15.00 (Fig. 1 D) were followed by the first moths eclosing after 3 h and the majority of animals eclosing before the gate. The best results were obtained after giving extracts at 17.00 and 19.00 (Fig. 1 E, F). In both cases the moths emerged in a relatively compact distribution that began about 2 h after injection. In the case

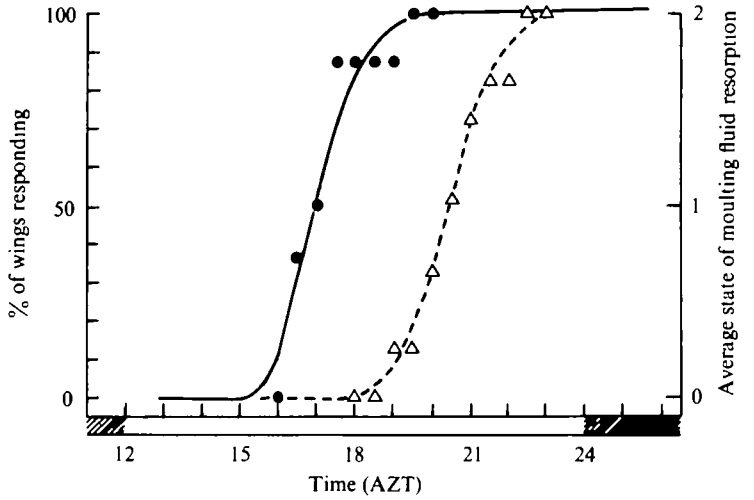

Fig. 2. The timing of the onset of responsiveness of the wing epidermis to the eclosion hormone. Wings were isolated and injected with a standard dose of CC/CA extract at the time indicated (filled circles), and tested for extensibility 60–70 min later. Each point represents eight wings. The progression of moulting fluid resorption for a group of intact animals (open triangles) is presented as an average score as described in the text.

of the last treatment, the injection was late enough that the distribution overlapped the normal eclosion gate.

Thus it appears that the stimulation of eclosion by CC/CA extracts in *Manduca* is less dramatic than that shown in the saturniid moths (Truman, 1973*b*). Only injection of extracts relatively late in the day consistently caused eclosion. Earlier treatments were considerably less effective and the responses occurred only after very long latencies.

In *Manduca* the wing epidermis is also a target tissue for the eclosion hormone (Reynolds, 1977). The onset of sensitivity to the eclosion hormone was examined by isolating wings at various times and challenging them with a standard dose of 1/10 CC/CA complex (about 10 times the amount of eclosion hormone needed to cause maximal wing cuticle plasticization). Wings first showed sensitivity to the hormone at 16.30 h and all had become sensitive by 20.00 h (Fig. 2). Also, the extent of plasticization caused by the hormone tended to increase with time, with the late positive scores showing more pronounced effects than the earlier ones.

Interestingly, the responsiveness of wings to the eclosion hormone seemed to be correlated with the extent to which the moulting fluid had been resorbed. Fig. 2 also shows the time course of the completion of moulting fluid resorption. Wings were subjectively scored as: wet (0), partially dry (1), or dry (2). This, of course, corresponds only to the final stages of a process begun some time before; but it is clear that sensitivity to the eclosion hormone develops before resorption is complete.

Timing of eclosion hormone release: titres in the blood

It was of interest to determine the length of time which elapsed between the acquisition of responsiveness to the eclosion hormone, and the hormone's normal time of release. To determine blood titres of eclosion hormone we used two techniques

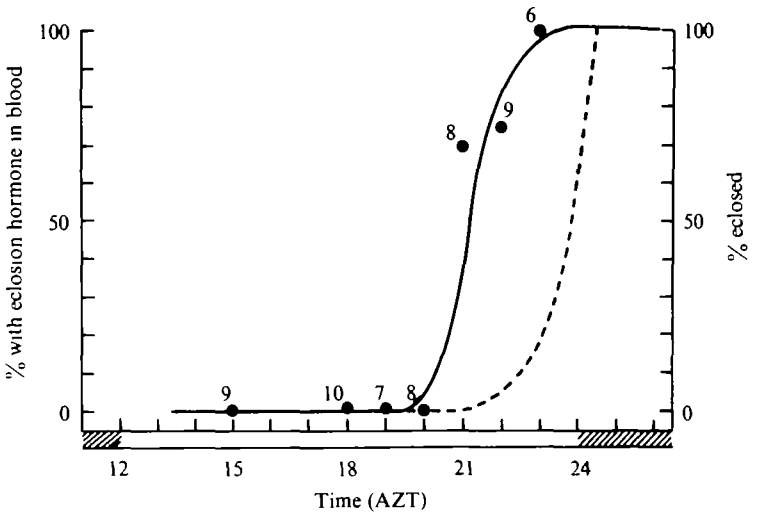

Fig. 3. The appearance of eclosion hormone activity in the blood of pharate *Manduca*. Moths were decapitated and their blood collected at the times indicated. Eclosion hormone activity was measured by the isolated wing assay. The number of animals used at each time point is indicated. Dashed line is the cumulative percent of moths eclosing from the data in Fig. 1 (A).

single samples from a population of moths and sequential samples from a smaller number of individual insects. The blood samples were tested for the eclosion hormone activity by bioassay on isolated *Manduca* wings. It should be noted that moths which were to be used for homone measurements after they had eclosed were placed in glass vials immediately at emergence to prevent wing spreading behaviour and bursicon release. This treatment was necessary because bursicon interferes with the isolated wing bioassay for the eclosion hormone (Reynolds, 1977).

The appearance of eclosion hormone activity in the blood of a population of *Manduca* is plotted in Fig. 3. No hormone was detectable in the blood of pharate moths up to and including 20.00 h, but by 23.00 AZT all of the animals sampled showed substantial hormone activity in their blood. Comparison of this data with those in Fig. 1, indicate a latency of approximately 2·5–3 h between eclosion hormone release and subsequent escape of the moth from the pupal cuticle.

Measurements of hormone titres in the blood of post-emergence moths indicated that eclosion hormone activity persisted for a relatively short period of time. In order to get a more precise indication of the time course of the eclosion hormone's appearance and subsequent disappearance, blood samples (each 25 μl) were taken every 20 min from individual pharate adult *Manduca* which had a cannula inserted into the heart. Each sample was progressively diluted until activity was lost. Hormone titres for three animals are shown in Fig. 4. In each case the titres of hormone rose abruptly and declined smoothly until it was undetectable by about 5 h after the peak. The shapes of the titre curves are consistent with the hypothesis that the eclosion hormone is released as a single pulse. The abruptness of the rise suggests that essentially all of the hormone release occurs within the first 20 min. The eclosion hormone does not persist very long in the blood once it has been released. Assuming that release is completed within 20 min, the half life in the blood is only about 45 min. Indeed, the

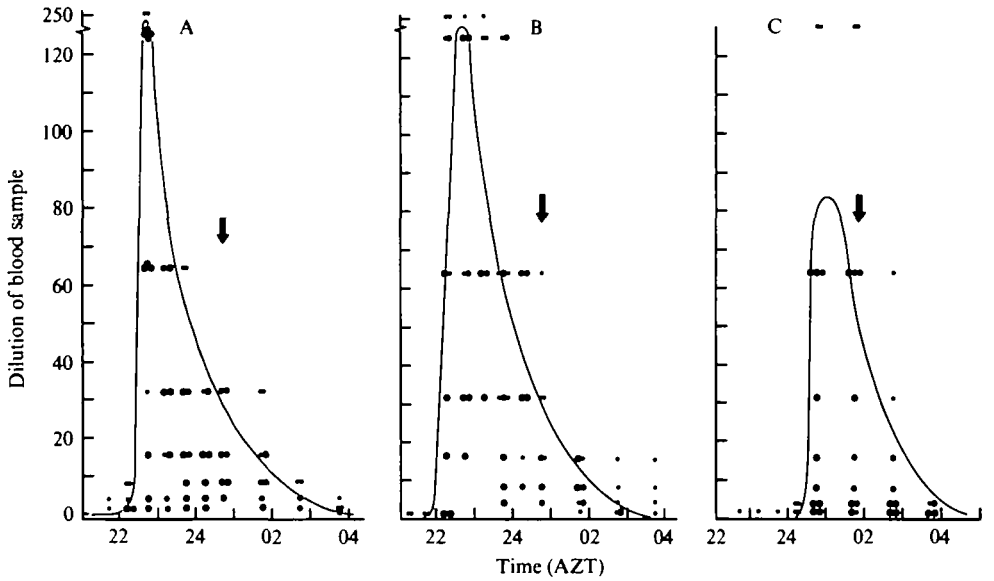

Fig. 4. Three examples of eclosion hormone titres in the blood of individual *Manduca* late on the last day of adult development. Blood samples taken at the times indicated were serially diluted and hormone activity measured by the isolated wing assay. Open circles, negative responses; half-filled circles, weak positive responses showing a test to control wing ratio of 1·9–2·4; filled circles, strong positive responses having a ratio of 2·5 or greater. Smooth curves have been drawn to approximate the boundary between positive and negative responses. Arrows identify time of eclosion.

titre of hormone in the blood has substantially decreased by the time the moth ecloses, and no activity is detectable by 3–4 h after eclosion.

Onset of sensitivity to bursicon

Just as the eclosion hormone is unable to elicit a response from its target tissues before a certain time on the last day before eclosion, so the tanning hormone, bursicon, also fails to induce tanning in the cuticle of the wings if it is injected before about 15.00 AZT (Fig. 5). In all these experiments, a supramaximal dose of bursicon was used ($\frac{1}{4}$ medial nerve, which is 50 times the dose needed to give a detectable tanning response). The time at which sensitivity to bursicon is acquired is some 2 h before responsiveness to the eclosion hormone develops. This sensitivity is acquired within a short period of time: none of the wings tested at 14.30 AZT tanned, whereas by 16.00 AZT, 100% of the wings were responsive.

Timing of bursicon release: titres in the blood

In *Manduca* bursicon release never occurs until after the release of eclosion hormone (Truman, 1973*a*). The release of bursicon is known to be associated with wing spreading behaviour, and if the newly-emerged moths are prevented from spreading their wings by confining them in glass vials, then bursicon release can be delayed for up to 24 h. Nevertheless, under normal circumstances, wing spreading follows eclosion rather quickly, usually beginning less than 15 min after escape from the pupal exuvium (Truman & Endo, 1974). It was of interest to determine how quickly bursicon appeared in the blood of moths which had begun to spread their wings.

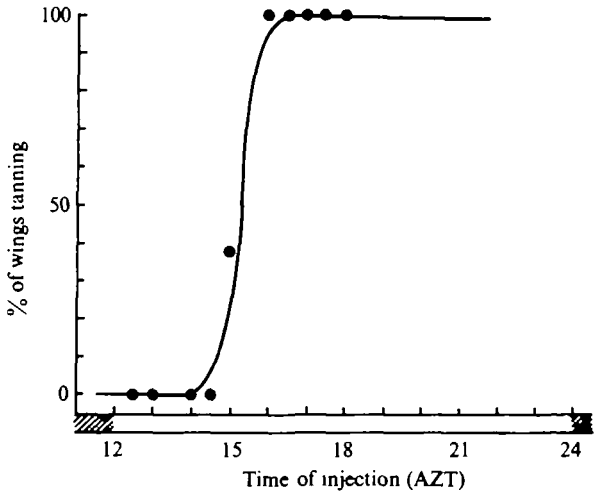

Fig. 5. The onset of responsiveness of the wing epidermis to bursicon. Wings were isolated and injected with a standard dose of perivisceral organ extract at the times indicated. Wings were examined for tanned cuticle about three hours later. At least 10 wings were tested at each point.

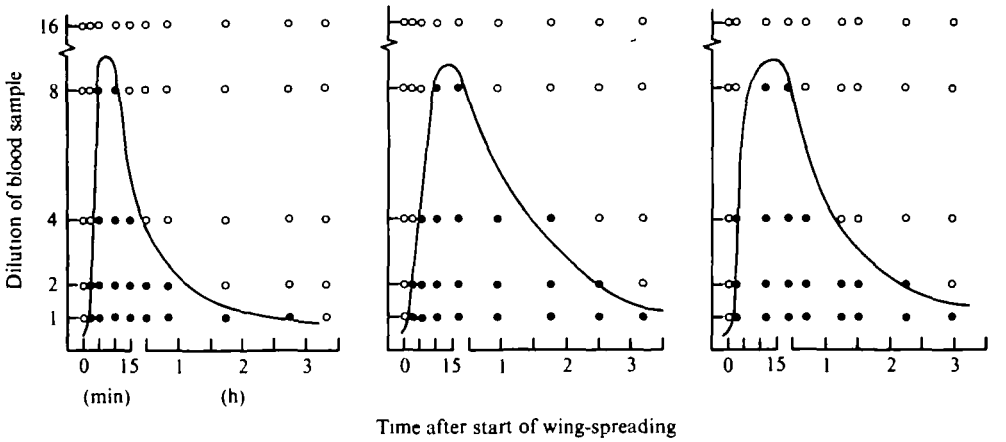

Fig. 6. Three examples of the time course of bursicon appearance in the blood of newly emerged moths after the onset of wing spreading behaviour. Blood samples were taken at the times indicated and serially diluted. Bursicon activity was indicated by the tanning of isolated *Manduca* wings. Open circles, negative assays; closed circles, positive assays. Smooth curves have been drawn to approximate the boundary between positive and negative responses. Note the change in scale along the abcissa.

Blood samples were taken sequentially from individual moths by the heart-cannulation technique described above, and the samples assayed for bursicon activity on isolated *Manduca* wings.

As seen in Fig. 6, bursicon activity appeared in the blood within 2 min after the newly emerged moth came to rest on a vertical support. By 4–12 min the blood titre approached a maximum and then declined steadily over the next 2 h. The half-life of bursicon in the blood appeared to be about 40–50 min.

DISCUSSION

Dynamics of the release of eclosion hormone and bursicon

The results from the serial blood samples taken from individual moths indicate that eclosion hormone release occurs over a span of about 20 min. During this period about 90% of the hormonal activity stored in the corpora cardiaca is released (Truman, unpublished). After this pulsed release, the hormone is subsequently removed from the blood with a half-life of about 45 min. Experiments with the isolated moth nervous system have shown that the eclosion hormone need be present in the bathing medium for only a few minutes in order to trigger the long neural programme that results in eclosion (Truman, 1978). The appearance of a single surge of hormone with its subsequent decay is also consistent with the role of the eclosion hormone as a trigger. One might expect that high blood titres would be maintained if the hormone was required continuously through the eclosion process or for some of the post-eclosion behaviours.

The data from the single blood samples taken from a group of *Manduca* through the day (Fig. 3) indicated that eclosion occurs about 2·5–3 h after hormone release. Similarly, the responses of *Manduca* injected with CC extracts (Fig. 1E, F) give a minimum latency of about 2–2·5 h. These estimates, however, are not in accord with the titres obtained from individual moths (e.g. Fig. 4) in which the latency ranged from 1 to 2·5 h with an average latency of 1·7 ($N = 7$). The discrepancy between the two methods of measurement appears to be related to the observation that very late in development relatively mild mechanical disturbances can trigger eclosion. The slight disturbance involved in taking the blood samples was presumably sufficient to cause some of the animals to emerge before the normal 2·5–3 h period had elapsed. Thus it appears that undisturbed moths usually emerge 2·5–3 h after hormone release but after the first hour other stimuli can cause an earlier eclosion. It should be noted that severe stimuli such as the removal of part of the pupal cuticle may cause eclosion behaviour in *Manduca* prior to eclosion hormone release (Kammer & Kinnamon, 1977) but in these cases the rest of the emergence sequence (i.e. spreading of the wings) does not usually occur until the normal time and is typically preceded by another bout of eclosion behaviour.

The onset of bursicon release can be more precisely identified than that of eclosion hormone because the former is associated with a behavioural marker – the start of wing inflation. Bursicon appears in the blood within 2 min after the moth comes to rest at a suitable wing spreading site. It is possible that release may even be more rapid than this; in *Rhodnius*, the diuretic hormone can be detected in the blood within 15 s of the start of feeding (Maddrell & Gardiner, 1977). Bursicon concentration in the blood reaches a peak by about 10 min which coincides with an 80% depletion of extractable activity from the ventral nerve cord (Truman, 1973*a*). Also, this time is marked behaviourally by the onset of wing expansion which is presumably aided by the additional plasticization of the wing cuticle caused by bursicon (Reynolds, 1977). By the time the wings are inflated and hardened (about 75 min) the blood titre of bursicon has dropped considerably. Thus, bursicon release also appears to be in a pulsed fashion as might be expected from its triggering role.

Onsets of hormone sensitivity

In the case of the moth *Antheraea pernyi*, the release of the eclosion hormone and, consequently, eclosion are gated events which are sensitive to photoperiod. In this species sensitivity to the eclosion hormone first appears on the day before eclosion in some of the population (Truman, 1976) and is found in nearly all animals by the morning of the day of emergence. The situation in *Manduca* is quite different in that individuals do not gain sensitivity to the eclosion hormone until midway through the final day of development, and all animals that will emerge on a given day become sensitive synchronously. Experiments with isolated wings clearly show that the wing epidermis begins to become sensitive at about 16.30 AZT (Fig. 2). However, injection of CC extracts into intact animals produced more complex results with regard to the stimulation of the eclosion behaviour. Injections after 16.00 were followed by a rather uniform latency and strong positive responses, but treatment before 16.00 produced mixed responses with only some of the moths emerging before their normal gate. An important feature of the latter treatment was that the latency became progressively longer as the injections were shifted to earlier times and no moths emerged prior to 18.00. This pattern of response coupled with the fact that the eclosion hormone can persist in the blood for a number of hours (Fig. 4) may indicate that the nervous system does not begin to become responsive until about 16.00. Those animals that emerged after earlier injections (e.g. 13.00 or 15.00) may represent cases in which sufficient hormone was still present at 16.00 to have an effect at that time. Emergence then started after 18.00 following an approximately normal latency. Therefore, it appears likely that the nervous system and the wing epidermis become responsive to the hormone at approximately the same time. The cellular events responsible for this rapid onset of sensitivity are unknown.

Sensitivity to bursicon also arises during a brief time period on the last day of development. The wings appear to become sensitive to bursicon about 1·5–2 h before they gain sensitivity to the eclosion hormone. In this case also, the events underlying this onset of responsiveness are unknown. Thus for both hormones it is clear that sensitivity first occurs in the target tissues only a few hours before the hormone is released – about 3·5 h in the case of the eclosion hormone and 7–8 h for bursicon.

While studying adult development in *Drosophila melanogaster*, Harker (1965*a*, *b*) claimed the existence of multiple 'gated' events during development. She argued that in this insect adult eclosion is not gated at all, but merely appears to be so because of the summed effects of the previous developmental gates. Pittendrigh & Skopik (1970) re-examined these findings in *D. pseudoobscura* and concluded that there was no evidence for any gated event other than eclosion. In the saturniids the release of the eclosion hormone is gated and can be influenced by shifts in photoperiod up to within a few hours of hormone release. In *Manduca* this study clearly shows that hormone release is synchronized by photoperiod (Fig. 3) but certain other developmental events such as the onset of hormone sensitivity are equally well timed but occur well before release of the hormone. This synchrony in the development of hormonal sensitivity must be due to another gated event that occurs earlier in development. It is clear that synchrony is not due to animals starting development at the same time and staying together through development since animals that pupate within the

same 2–3 h window will eventually be as many as 5 days apart at the end of development (J.W.T., unpublished). Thus, the synchrony is imposed at some time during adult development and, most likely, at a time near the end of development. At present the nature of this gated event or events is unknown.

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