

CONTROL OF CUTICLE EXTENSIBILITY
IN THE WINGS OF ADULT *MANDUCA* AT THE TIME OF
ECLOSION: EFFECTS OF ECLOSION
HORMONE AND BURSICON

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SUMMARY

The wings of pharate adult tobacco hornworm moths, *Manduca sexta*, are relatively inextensible until 3 or 4 h before emergence from the pupal case. At this time the wing cuticle becomes plasticized, so that by the time of eclosion, the wings are readily extensible. This change in the mechanical properties of the wing cuticle is shown to be under the control of a factor from the head. This factor is present in the corpora cardiaca/corpora allata complex, and in the protocerebrum of the brain, being released into the blood prior to eclosion. It is able to act directly on isolated wings. The active principle was found to be indistinguishable in a number of ways from the hormone which triggers emergence from the pupal case, the eclosion hormone. Partial purification of the eclosion hormone failed to separate activity causing eclosion from activity causing wing cuticle plasticization. It is concluded that the same hormone is probably responsible for both effects. The cuticle plasticizing activity of the eclosion hormone forms the basis for a new, highly sensitive bioassay.

Another factor, distinct from the eclosion hormone, is able to cause wing cuticle plasticization. This factor is found in the abdominal nerve cord, and is only released into the blood after eclosion has occurred. It is probably identical with the tanning hormone, bursicon, which is released at this time. The factor in the nerve cord which causes cuticle plasticization is indistinguishable from bursicon in a number of ways, including partial purification by gel filtration. Bursicon evidently causes a further increase in wing cuticle extensibility after eclosion, at the time of wing inflation.

INTRODUCTION

Much of the body cuticle of the adult moth is tanned before it emerges from the pupal case. However, the wings are a notable exception. Before the moth assumes its final form, the wings must be inflated to their full size by the application of increased blood pressure. Just after eclosion, when this inflation takes place, the wing cuticle is relatively extensible. However, the wing cuticle only becomes extensible just before eclosion. It will be shown here that this change in the mechanical properties of the wing cuticle is under hormonal control. The hormone involved is probably the same one which triggers the stereotyped behaviour pattern which leads to the moth's emergence from the pupal case.

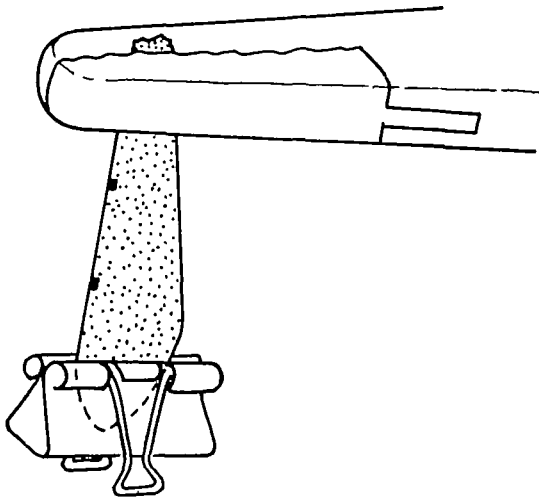


Fig. 1. To show the way in which wings from pharate adult *Manduca* were stretched. The load was 3 g.

Another hormone, bursicon, the tanning hormone, also causes plasticization of the wing cuticle. Here, release of hormone into the blood and the consequent change in wing cuticle extensibility occur only *after* eclosion.

MATERIALS AND METHODS

Tobacco hornworms, *Manduca sexta* (L) (Lepidoptera; Sphingidae), were reared in a large laboratory culture, being fed on an artificial diet (Bell & Joachim, 1976) and maintained as larvae under a 17L:7D photoperiod regimen at 25 °C. Shortly after pupal ecdysis, the pupae were transferred to a 12L:12D photoperiod (also 25 °C) where they completed their development. On the day of adult eclosion, however, the photophase was extended to allow for experimentation. Time used is arbitrary Zeitgeber time (AZT; Pittendrigh, 1965), where lights off is 24.00 AZT.

Diapausing pupae of the giant silkworm, *Hyalophora cecropia* (Lepidoptera; Saturniidae), were purchased from dealers. After chilling they were allowed to develop under a 17L:7D photoperiod at 25 °C.

Wing extensibility was measured by the simple technique of observing the increase in distance between two small paint marks on the leading edge of the wing as it stretched under an imposed load. The marks were set at 10 mm apart before testing began. The wing was suspended from the proximal end (at the 'shoulder') by a haemostat, and the load was applied at the distal end by means of a small 'bulldog' clamp (Fig. 1). As shown in Fig. 2, the increase in length of the wing on loading is highly time-dependent; that is, almost all of the ultimate extension is due to 'creep'. To compare the mechanical properties of a large number of test wings, the percentage extension at 3 min after the imposition of the load was taken as a measure of extensibility. The time allowed for extension was entirely arbitrary, and was chosen for convenience.

Isolated abdomens of *H. cecropia* were used in a bioassay for the presence of eclosion hormone activity. The movements of isolated abdomens following injection

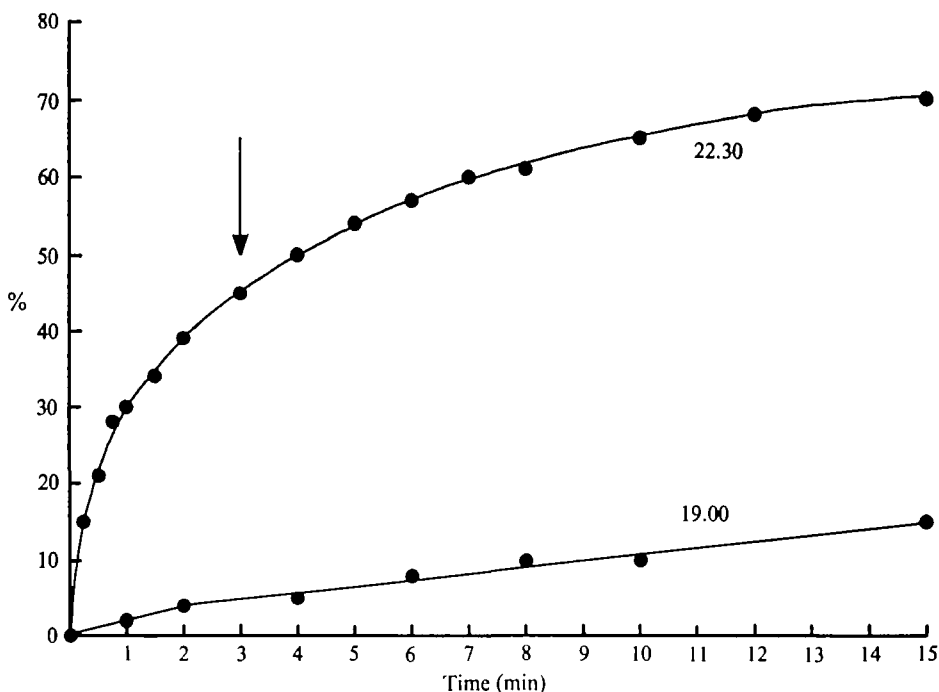


Fig. 2. Percentage extension of *Manduca* wings plotted against time after loading. The greater extensibility of the wing taken at 22.30 AZT is clearly seen. Arrow shows extension at 3 min, taken as a measure of extensibility.

test materials were recorded by a lever writing on a revolving smoked drum. The method for isolating *H. cecropia* abdomens, and their behaviour on injection of eclosion hormone have been described by Truman (1971). In the present work, each abdomen was scored as either showing typical eclosion behaviour on injection, or not. The degree of activity of test solutions was assessed by injection of a range of volumes of the test solution into a number of test abdomens. Each abdomen was used for only one test.

For some experiments, an assay for cuticle plasticizing activity was devised which used isolated wings of pharate adult *Manduca* taken a few hours before adult eclosion. The wings were isolated by cutting them from the donor insect at the wing base, at about 20.00 AZT. At this time the wings were just becoming dry, due to the resorption of moulting fluid. They were held in Petri dishes kept moist with damp filter paper. The wings were kept in pairs; the left wing, which was injected with saline solution, served as a control for the right wing, which was injected with the test solution. Injections, volume 10 μ l, were made from a Hamilton microsyringe into the costal vein. After 60–90 min the extensibility of the wing was measured as described above. The effect of the test solution was expressed as the ratio of the extensibility of the right wing to that of the left. The saline solution used was that of Ephrussi & Beadle (1936).

Eclosion hormone activity from corpora cardiaca/corpora allata (cc/ca) complexes was partially purified by gel filtration. The procedure was as follows. The supernatant fraction from a boiled (2 min), centrifuged (3 min, Beckman microfuge), homogenate

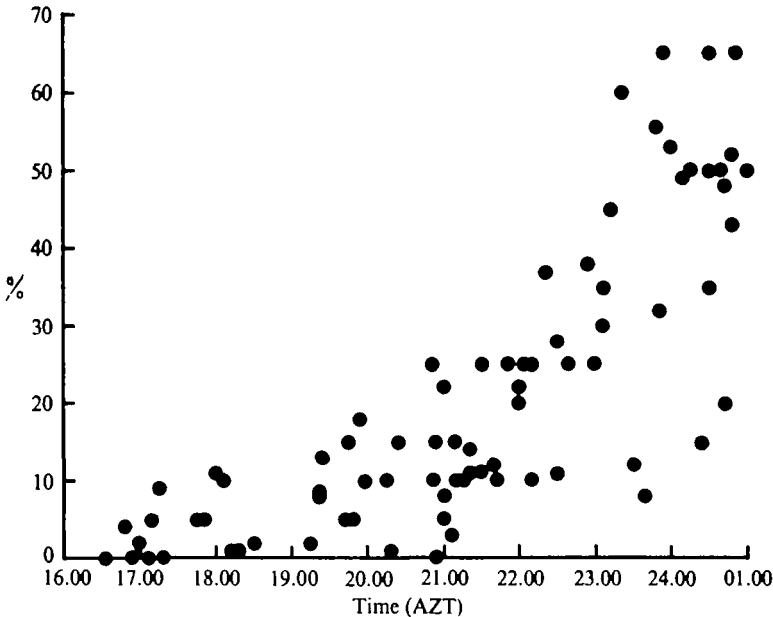


Fig. 3. The extensibility of *Manduca* wings tested at different times during the hours preceding adult eclosion. The ordinate shows the percentage extension of the wings 3 min after loading. The normal 'gate' for eclosion is from 23.00 AZT onward.

of 75 cc/ca complexes in 0.6 ml 0.1 M-NaCl solution was subjected to gel filtration on a column of Biogel P-10 (27 cm \times 0.8 cm), being eluted with 0.1 M-NaCl solution at room temperature (22 °C). The eluate was collected in 0.6 ml fractions which were assayed both for eclosion hormone activity, using isolated *H. cecropia* abdomens, and for plasticizing activity, using isolated *Manduca* wings, as above. Protein concentration was measured as absorbance at 280 nm. Bursicon activity from abdominal nerve cords was partially purified in essentially the same way, except that 30 nerve cords were used as a source of activity. These were homogenized, boiled and centrifuged as above. Activity was eluted from a Biogel P-60 (20 cm \times 0.8 cm) column using 0.1 M-NaCl, 0.1 mM-dithiothreitol (DTT, Cleland's reagent) as eluant. Temperature was 5 °C.

Bursicon activity was assayed using a modification of Truman's (1973a) method, which used isolated wings of pharate adult *Manduca*. The wings were isolated as described above and were injected with 10 μ l of test solution. They were scored as either positive or negative for tanning (yellow colouration on the wing veins) after 3 h, during which time they were kept at room temperature (22 °C) in a moistened Petri dish.

RESULTS

(a) *The eclosion hormone*

Pharate adult *Manduca* of both sexes were taken at various times during the last day prior to their eclosion from the pupal cuticle, and the mechanical properties of their wings compared as described above. The wings showed a progressive increase in extensibility (Fig. 3), beginning at about 20.00 AZT and continuing up to the beginning of the gate for eclosion, which under the conditions of our laboratory, was from about 23.00 AZT onward.

Table 1. *Effect of decapitation at various times on subsequent wing plasticization in M. sexta pharate adults*

Time decapitated (AZT)	Mean extensibility ± s.e.	(n)
18.00	10.3 ± 3.8	(4)
19.00	13.8 ± 2.7	(8)
20.00	13.8 ± 2.1	(10)
21.00	44.4 ± 4.4	(10)
22.00	46.6 ± 3.7	(10)
Control	43.2 ± 4.1	(18)

In all cases wings were taken and tested for extensibility between 23.00 and 24.00 AZT.

During this time there was a progressive change in the appearance of the pharate moths due to completion of the resorption of moulting fluid from the space between the pharate adult cuticle and the encasing pupal cuticle. The pharate adult moths, which were still quite damp at 18.00 AZT were fairly dry by about 20.00 AZT.

The plasticization of the wing cuticle observed from 20.00 AZT onward was dependent on the presence of the head up to that time. The head was removed from experimental insects by clamping the neck with a haemostat and cutting above it. Table 1 shows that insects losing their heads after 20.00 AZT plasticized their wings normally. Those decapitated at or before 20.00 AZT failed to plasticize their wing cuticle.

These experiments suggest that a factor from the head might be responsible for the increased wing extensibility seen prior to eclosion. Injections of homogenates of corpora cardiaca/corpora allata complexes (cc/ca) taken from pharate adult *Manduca* accelerated the normal plasticization process (Fig. 4). These injections also caused about 70% of the moths to eclose before the normal gate for eclosion. The amount of cc/ca extract required to produce premature eclosion was about the same as that required to produce wing cuticle plasticization (Fig. 5).

The cc of pharate adult *Manduca* are a potent source of the eclosion hormone (Truman, 1971), which is known to act on the central nervous system of silkmoths to elicit the characteristic motor programmes of pre-eclosion behaviour (Truman & Sokolove, 1972). Evidently *Manduca* can also respond to the eclosion hormone by eclosing prematurely. Presumably the 30% of insects which failed to eclose before the normal gate in these experiments (and also in those of Truman (1973a)) were in some way refractory to the injected hormone.

The eclosion hormone is, by definition, released before eclosion occurs. Truman's (1973b) data showing this in *Antheraea pernyi* are confirmed here for *Manduca* (Table 2), which also show that the cc/ca factor which causes wing cuticle plasticization is also released before eclosion. It is evident that not all of the active material in the cc/ca is released; cc/ca extracts from eclosing adults had significant, though much reduced, activity both in promoting premature eclosion and in causing cuticle plasticization. These results suggest that the eclosion hormone may be responsible for the increased extensibility of the wing cuticle prior to eclosion. The hypothesis is supported by data on the distribution of plasticizing activity within the nervous system (Table 3). This may be compared with the distribution of the eclosion hormone found

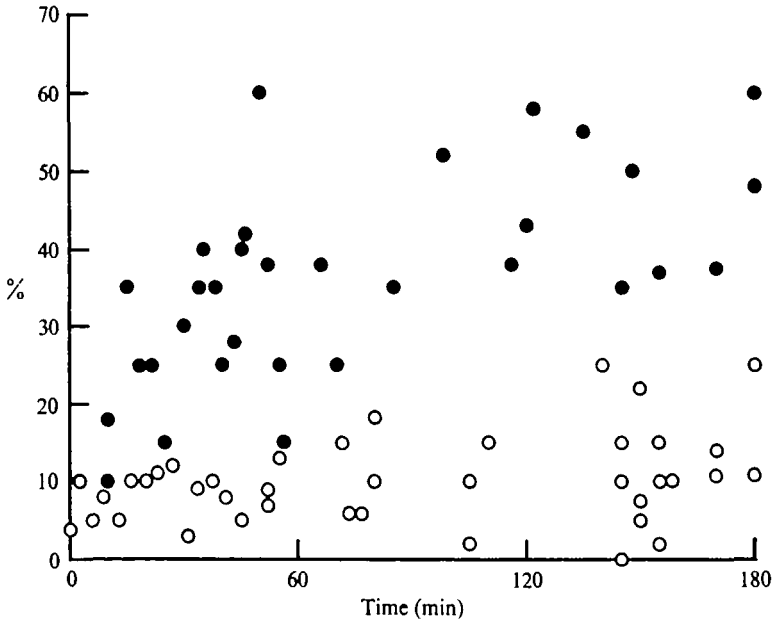


Fig. 4. The effect on wing extensibility of injections of cc/ca homogenates. The filled circles show the extensibility of wings from insects which were each injected at 18.30 AZT ($t = 0$) with one cc/ca complex, homogenized in 25 μ l of saline solution. \circ , Saline-injected controls.

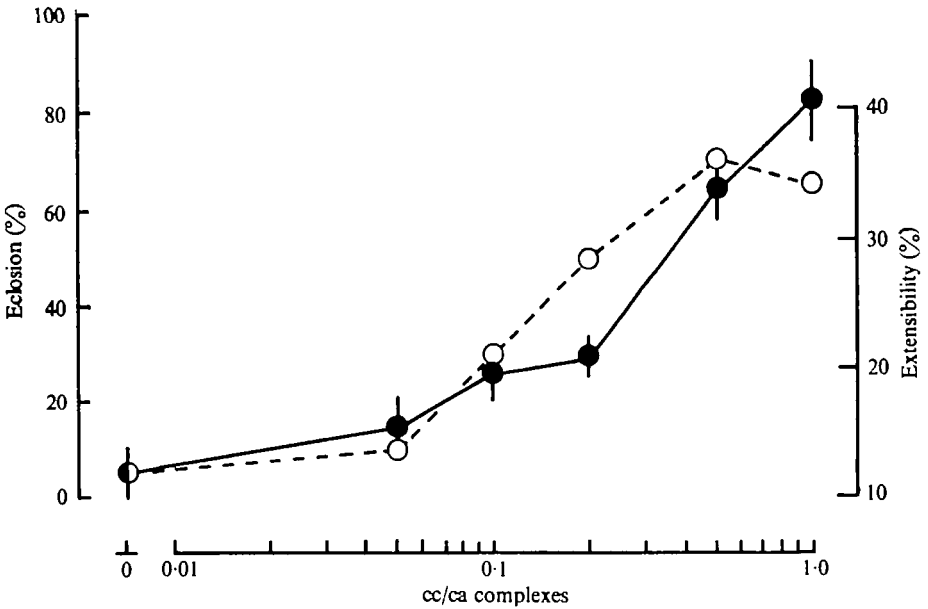


Fig. 5. Dose-response curve for the effects of injections of cc/ca complexes on wing cuticle extensibility (\bullet , means \pm S.E., $n = 5$ for each point), and in provoking premature eclosion (\circ , percentage response from at least 10 insects). Moths emerging before 23.00 AZT were classed as eclosing prematurely.

Table 2. Release of eclosion hormone, and of the cc/ca factor causing wing cuticle plasticization, from *Manduca cc/ca* before eclosion

Donors	Recipients		
	% eclosing prematurely (n)	Wing extensibility	
		Mean \pm S.E.	(n)
Pharate adults (last day, 16.00 AZT)	88 (8)	35.9 \pm 2.2	(11)
Eclosing adults	29 (7)	16.8 \pm 1.93	(14)
Saline control	0 (6)	11.0 \pm 1.45	(10)

Each recipient insect was injected with 25 μ l of a (pooled) cc/ca extract from the indicated donors, which was equivalent to a dose of one half of a cc/ca complex. This dose was chosen to give a submaximal response (see Fig. 5). Injections were at 17.30 AZT; wing extensibility was scored 1 h later. See Fig. 5 for criteria for premature eclosion.

Table 3. Plasticizing activities of various tissues on injection into *M. sexta pharate* adults

	Mean extensibility \pm S.E.	(n)
cc/ca complex (brain)*	40.8 \pm 3.2	(6)
Mid protocerebrum*	49.5 \pm 4.1	(6)
Lateral protocerebrum*	32.0 \pm 4.5	(6)
Suboesophageal ganglion	19.8 \pm 2.5	(6)
Frontal ganglion	11.0 \pm 1.5	(6)
Optic lobes	19.2 \pm 2.2	(5)
Abdominal nerve cord (controls)	4.2 \pm 0.5	(6)
Saline solution	16.0 \pm 3.5	(6)
Fat body	17.8 \pm 2.6	(5)
Malpighian tubules	14.3 \pm 0.9	(6)
Gut wall	13.3 \pm 3.4	(5)

Insects were injected with a homogenate of tissue in 25 μ l of saline solution. Each insect received either 1 cc/ca complex, 1 nerve cord, or in the case of brain tissue extracts, tissue from 1 brain. Control tissue extracts contained approximately the same amount of tissue as the brain extracts. The insects were injected at 18.00 AZT and their wings tested after 60–90 min. Tissues marked with an asterisk (*) contain eclosion hormone activity (Truman, 1973b).

by Truman (1973b). For both, activity is found only in the cc/ca complex, and in the protocerebrum of the brain. Within the latter, activity is greater in the median protocerebrum rather than in the lateral part, indicating the median neurosecretory cells as the probable source of activity.

Various control tissue extracts were without effect on wing extensibility, with the notable exception of the abdominal nerve cord. Under the conditions of the experiment reported in Table 3, abdominal nerve cord extracts caused the wings of injected insects to be *less* extensible than those of control insects. However, observing wing extensibility at earlier times after injection, it was found that the wings showed an initial plasticization of the cuticle, which then decayed rapidly, so that by 2 h after injection all were essentially inextensible (Fig. 6).

These effects of abdominal nerve cord extracts are clearly not due to eclosion hormone, which is not present in that part of the nervous system (Truman, 1973a). It will be shown in Section (b) that another hormone, the tanning hormone, bursicon, is probably responsible.

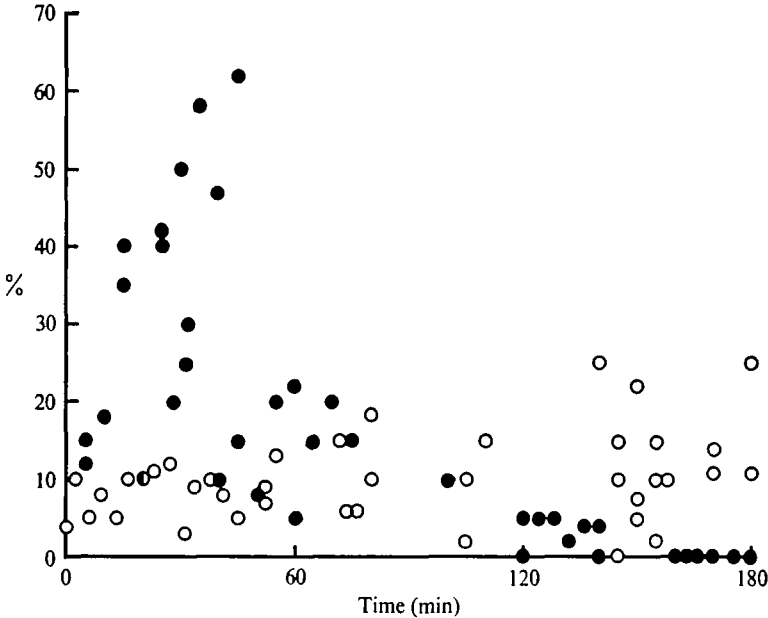


Fig. 6. The effect on wing extensibility of injections of abdominal nerve cord homogenates (●). Each insect received the equivalent of one cord, which was injected at 18.30 AZT ($t = 0$). The ordinate shows the percentage extension of the wings 3 min after loading. ○, Saline-injected controls.

Further confirmation of the identity between the eclosion hormone and the cc/ca factor causing cuticle plasticization was obtained by partial purification of the factors concerned by gel filtration. Both eclosion hormone and wing cuticle plasticizing activities eluted together in the partially included volume, the distribution of activities among the fractions eluted being essentially the same (Fig. 7). Since the cc/ca extract was boiled during the preparative procedure, both kinds of hormonal activity must be stable to boiling. The coincidence of the two kinds of activity in the material eluted from the column indicates a similar molecular weight. The eclosion hormone has a molecular weight of about 9000 daltons (S. E. Reynolds and J. W. Truman, unpublished data).

It seems reasonable to conclude that the eclosion hormone and the cc/ca factor which causes cuticle plasticization are one and the same, although, of course, a proper proof of such an identity would require complete chemical identification of the factors concerned.

(b) *Bursicon*

The abdominal nerve cord of *Manduca* contains no eclosion hormone activity (Truman, 1973a) so that the plasticizing effects of the nerve cord extracts mentioned above cannot have been due to that hormone. The abdominal nerve cord is, however, a potent source of the tanning hormone, bursicon (Truman, 1973a). An explanation of the transient plasticization caused by nerve cord extracts would be that bursicon causes cuticle plasticization in the wings of *Manduca*, as it is thought to do in the thoracic sclerites of newly emerged blowflies (Reynolds, 1976). Subsequently, bursicon

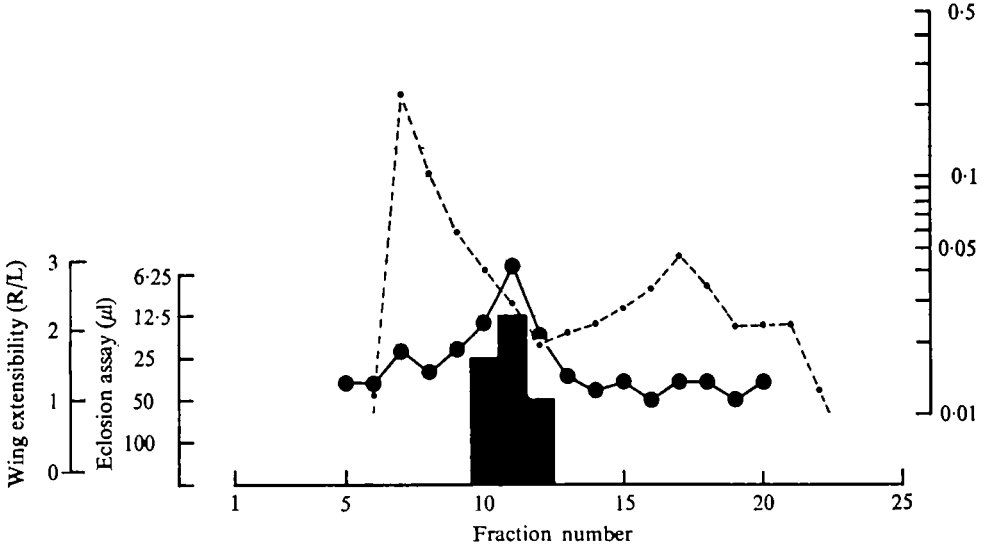


Fig. 7. Biogel P-10 column chromatography of a homogenate of cc/ca complexes (75 complexes in 0.6 µl 0.1 M-NaCl solution, boiled and centrifuged), eluted with 0.1 M-NaCl solution, collected in 0.6 µl fractions. ●---●, E_{100} ; ●—●, cuticle plasticizing activity assayed on isolated *Manduca* wings; histogram shows eclosion hormone activity assayed on isolated abdomens of *H. cecropia*. The height of the bar indicates the smallest volume which would elicit eclosion behaviour on injection. Fractions 5–20 were tested for eclosion hormone activity.

Table 4. Release of bursicon, and of the nerve cord factor causing wing cuticle plasticization, from the abdominal nerve cord of *Manduca* after eclosion

Donors	Recipients		
	% tanned (n)	Wing extensibility	
		Mean ± S.E.	(n)
Pharate adults (last day, 16.00 AZT)	60 (10)	31.9 ± 3.1	(8)
Eclosing adults (emerging into vials)	60 (10)	35.1 ± 3.5	(10)
Adults spreading wings (approx. 15 min after release from vials)	20 (10)	13.8 ± 1.6	(10)
One-day-old adults (free)	0 (10)	12.1 ± 1.3	(8)
Saline controls	0 (10)	11.3 ± 0.76	(10)

Each recipient insect was injected with 25 µl of a (pooled) nerve cord extract from the indicated donors, which was equivalent to a dose of one quarter of a nerve cord. This dose was chosen to give a submaximal response (see Truman, 1973a). Injections were at 17.30 AZT; wing extensibility was scored after 30 min, tanning after 3 h.

causes cuticle tanning and this decreases its extensibility, so that the plasticization would be short-lived.

Investigation of the time of release of the factor in the nerve cord which causes plasticization shows depletion only *after* the moths have eclosed and gained access to a suitable wing spreading site (Table 4), as is the case for bursicon. Thus the plasticizing factor in the nerve cord cannot be responsible for the increase in wing extensibility which occurs *before* eclosion. It is possible that the factor in the nerve cord is identical with bursicon.

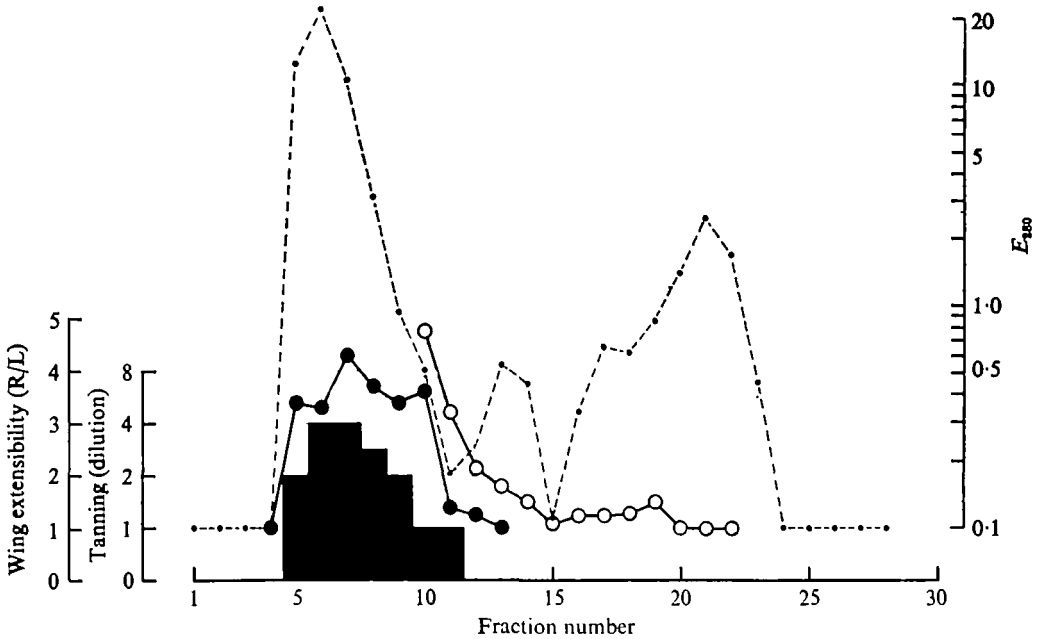


Fig. 8. Biogel P-60 column chromatography of a homogenate of abdominal nerve cords (30 cords in 0.6 ml 0.1 M-NaCl solution, boiled and centrifuged), eluted with 0.1 M-NaCl, 0.1 mM-DTT solution, collected in 0.6 ml fractions. ●—●, E_{280} . The continuous lines show cuticle plasticizing activity assayed on isolated *Manduca* wings, at two different dosages. Isolated wings were injected with 10 μ l of the undiluted eluate ○—○ or with 10 μ l of four-times diluted eluate (●—●). The histogram shows tanning (bursicon) activity, also assayed on isolated *Manduca* wings. The height of the bar indicates the extent to which the sample could be diluted while still causing tanning on injection. Fractions 4–22 were tested for tanning activity.

Further confirmation of this identity was obtained by partially purifying bursicon activity by gel filtration. Fig. 8 shows that both bursicon and wing cuticle plasticizing activities eluted together, just behind the void volume. The distribution among the eluted fractions of the two kinds of hormonal activity was essentially the same, indicating similar molecular weights, of slightly less than the exclusion limit of 60 000 daltons. Both kinds of activity were stable to boiling, at least as crude tissue extracts during the preparation procedure. Once subjected to gel filtration, however, both kinds of hormonal activity became somewhat labile, and it was necessary to work in the cold.

Again, it seems reasonable to conclude that bursicon and the nerve cord factor which causes cuticle plasticization are identical.

It is clear from the evidence presented above that bursicon can cause cuticle plasticization in wings which have not previously been exposed to the eclosion hormone. However, this is not the case *in vivo*, where bursicon release only occurs after the eclosion hormone has been present in the blood for some hours, and the wing cuticle is already plasticized. Does bursicon cause an additional increase in wing extensibility when it is released at the time of wing inflation? To answer this question, wing extensibility was compared in newly emerged *Manduca* which were confined in glass vials (this prevents bursicon release; see Table 4, also Truman, 1973a), and in those which were free and beginning to inflate their wings (when bursicon is

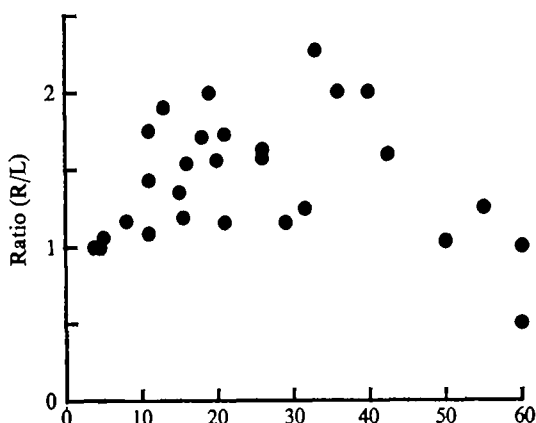


Fig. 9. Effect of injections of nerve cord extracts on the extensibility of isolated wings taken from moths newly emerged into vials. The wings were injected with $10 \mu\text{l}$ of saline solution containing the equivalent of $1/10$ cord. The ordinate shows the ratio of extension at 30 s after loading in the (R) injected wings as compared with extension in their paired (L) controls, which were injected with saline solution. The values of increased extensibility seen in these experiments are not comparable with other measurements reported here. For further explanation, see text.

released). The values obtained were 56.6 ± 1.98 ($n = 19$) and 57.8 ± 1.45 ($n = 10$) respectively (means \pm s.e.). These are not significantly different. However, once wing inflation commences in *Manduca*, the wings are very quickly stretched and can no longer be used for testing. It may be that these measurements of extensibility did not allow the released bursicon sufficient time to act. By injecting nerve cord extracts into *Manduca* wings which had been isolated from moths at the moment of eclosion, it was possible to show that bursicon can indeed cause a further increase in cuticle extensibility over and above that previously induced by the eclosion hormone (Fig. 9). Because the wings are already very extensible, even before bursicon injection, this effect was best seen by stretching the wings for a shorter period of time (30 s) than that previously employed (3 min). For this reason the percentage extensions recorded are not comparable with other data reported in this paper. Instead, increased extensibility is shown as the ratio of extension of the injected wings as compared to their (paired, saline-injected) controls. At times longer than 60 min after injection, the bursicon injected wings are less extensible than controls, presumably due to tanning, which reduces cuticle extensibility.

DISCUSSION

The increase in cuticle extensibility in the wings of *Manduca* which occurs in the last few hours before eclosion has been shown here to be under the control of a factor which is probably identical with the eclosion hormone. It has also been shown that a factor which is probably identical with the tanning hormone, bursicon, is likely to affect wing cuticle extensibility at the time of wing inflation, after eclosion. Final confirmation of the identity of these active factors with the eclosion hormone and with bursicon, respectively, must await more complete purification of these two hormones.

The ability of cc/ca extracts to cause cuticle plasticization in isolated wings of *Manduca* indicates that the effect of the active factor (probably the eclosion hormone)

is not mediated by the central nervous system, but is a direct effect on the wing epidermis. This is of considerable interest as the only previously known target tissue of the eclosion hormone has been the central nervous system.

The cuticle plasticizing effect of the eclosion hormone is useful as a bioassay. The procedure described in this paper, using the isolated wings of last-day pharate adult *Manduca*, is extremely sensitive, giving a full response to 0.01 cc/ca complexes per wing injected in 10 μ l of saline solution; less material can reliably be detected. However, because the assay also detects bursicon activity, it is necessary to confirm that bursicon is not present.

The experiments reported here suggest that the eclosion hormone is liberated into the haemolymph between 20.00 and 21.00 AZT, at least 3 h before the onset of the natural eclosion gate. Injections of cc/ca extracts produce eclosion in *Manduca* only after a delay of 3–4 h. It is evident that the effect of the eclosion hormone in triggering eclosion behaviour is much less rapid than in the silkmoths *Hyalophora cecropia* and *Antheraea pernyi* (Truman, 1971).

The finding that wing cuticle extensibility is increased by bursicon confirms the observation of Truman & Endo (1973), who noticed that abdominal nerve cord extracts appeared to cause increased wing stretching when injected into newly emerged *Manduca* adults confined inside glass vials. Bursicon is known to cause cuticle plasticization in newly emerged adult blowflies (Reynolds, 1976) and also in newly emerged adult *pernyi* silkmoths (author's unpublished data). It is clear how increased cuticle extensibility at the time of wing inflation may reduce the amount of energy expended by the moth in expanding its wings; however, it is not so clear why the function of cuticle plasticization (which occurs only *after* eclosion in blowflies and in *pernyi* silkmoths) should have been partly pre-empted by the eclosion hormone in *Manduca*, thus occurring to a considerable extent *before* eclosion. The adult moth must not only free itself of the pupal exuvium on emergence, but must also, under natural conditions, dig itself free of the soil in which it pupated. It might be imagined that it would be advantageous for the insect's wings to be *less* extensible at this time, rather than more so. However, it may be that the change in mechanical properties shown by the wing cuticle in response to the eclosion hormone is an incidental consequence of some other physiological process affecting the cuticle.

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