THE HORMONAL CONTROL OF DIURESIS IN THE CABBAGE WHITE BUTTERFLY *Pieris brassicae*

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**SUMMARY**

The diuresis which follows the pupal-adult ecdysis of *Pieris brassicae* is hormonally controlled. Use of the isolated Malpighian tubules as a bioassay shows the presence of substantial diuretic activity in homogenates of the brain and corpus cardiacum—corpus allatum complex. The hormone is probably produced in the brain and released from a storage site in the corpora cardiaca. The tubules of the butterfly are maximally responsive to the diuretic hormone at the time of eclosion.

**INTRODUCTION**

The pupal-adult ecdysis of the cabbage white butterfly *Pieris brassicae* (L.) is followed by a short but fast diuresis which drastically reduces the haemolymph volume (Nicolson, 1976a). Malpighian tubules isolated from the insect at the time of ecdysis secrete fluid at extremely fast rates when stimulated by adenosine 3',5'-monophosphate (cyclic AMP). It is now widely accepted that hormone action involves increases in the intracellular level of cyclic AMP (Robison, Butcher & Sutherland, 1971), and the ability of exogenous cyclic AMP to mimic the effect of a given hormone is evidence for its role as second messenger.

These observations suggest that tubule secretion in *Pieris* is stimulated by a diuretic hormone analogous to those already found in some other insect species. This paper reports an investigation of the control of diuresis.

**MATERIALS AND METHODS**

The culture of *Pieris brassicae* used in these experiments was as described previously (Nicolson, 1976a). A rough estimate of the stage of development of the pharate adults could be made from the degree of wing pigmentation and the extent of resorption of the moulting fluid visible through the pupal cuticle. After emergence butterflies were fed on 10% sucrose solution in artificial flowers (David & Gardiner, 1961).

The method of setting up Malpighian tubules as isolated preparations has been outlined in an earlier paper (Nicolson, 1976a). These preparations were used as a bioassay for the diuretic hormone (DH) activity in haemolymph, serum and tissues. Unless otherwise stated, the tubules were dissected only from adults near eclosion.

Haemolymph samples were collected by decapitation and gentle squeezing; the thin oesophagus of the adult prevented contamination by the gut contents. Serum was
Table 1. The occurrence of diuretic activity in the nervous systems of late pharate butterflies

(Tissue extracts assayed on isolated tubules at a concentration of 3 tissues per 100 μl.)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% increase in rate after addition of brei (mean ± S.E.)</th>
<th>No. of assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optic lobe</td>
<td>11 ± 10</td>
<td>6</td>
</tr>
<tr>
<td>Brain</td>
<td>278 ± 33</td>
<td>14</td>
</tr>
<tr>
<td>CC-CA complex</td>
<td>651 ± 166</td>
<td>5</td>
</tr>
<tr>
<td>Suboesophageal ganglion</td>
<td>69 ± 33</td>
<td>6</td>
</tr>
<tr>
<td>Prothoracic ganglion</td>
<td>68 ± 33</td>
<td>7</td>
</tr>
<tr>
<td>Mesothoracic ganglion</td>
<td>55 ± 31</td>
<td>6</td>
</tr>
<tr>
<td>Abdominal ganglion 3</td>
<td>11 ± 7</td>
<td>6</td>
</tr>
<tr>
<td>Abdominal ganglion 4</td>
<td>14 ± 9</td>
<td>6</td>
</tr>
<tr>
<td>Abdominal ganglion 5</td>
<td>38 ± 27</td>
<td>8</td>
</tr>
<tr>
<td>Abdominal ganglion 6–8</td>
<td>24 ± 12</td>
<td>6</td>
</tr>
</tbody>
</table>

prepared by holding a small test-tube of haemolymph in a boiling water-bath for 1 min, and centrifuging to separate the coagulated proteins from the clear supernatant.

The anatomy of the adult nervous system of Pieris has been described by Heywood (1965) and Ali (1973), and the relationship of the brain and retrocerebral endocrine system is illustrated in Cazal (1948). The corpora cardiaca can be distinguished from the corpora allata by their anterior position, smaller size and faint bluishness: unfortunately the two components are fused in Pieris (Hinton, 1951) and so could not be studied separately.

Tissue homogenates were prepared and tested as follows. Each portion of the nervous system was dissected from adults close to emergence and transferred on the tip of a dissecting needle to about 2 μl of distilled water in a small glass mortar. The tissue was homogenized with a ground-glass pestle. The resulting brei was then transferred to the liquid paraffin covering an isolated Malpighian tubule that was secreting in 30–40 μl of normal bathing medium. Secretion rates were measured for 30 min before and after the addition of the brei, and the percentage increase in rate provided a measure of its diuretic activity. Any change in the osmotic or ionic concentrations of the bathing medium caused by the use of distilled water for homogenization would be negligible.

Other methods are discussed below. The vertical lines in the figures represent ± the standard error of the mean.

RESULTS

Diuretic activity in haemolymph and serum

For a substance to qualify as a diuretic hormone, it should be found to occur naturally in the haemolymph at concentrations which affect the tubules (Maddrell, Pilcher & Gardiner, 1971). Drops of haemolymph removed from Pieris adults in diuresis were added to the medium bathing isolated tubules, causing their secretion rate (measured for 30 min) to increase from 9.3 ± 0.5 to 15.6 ± 2.0 nl/min (mean ± s.e.). In a control experiment, haemolymph collected from late pharate adults – those within a few hours of eclosion – had no effect on the secretion rate of isolated tubules.
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Fig. 1. Comparison of tubule response to breis of the brain (BR), CC-CA complex (CC) and suboesophageal ganglion (SOG). Breis were added to isolated tubules at 1 h (arrow), each tissue being at a final concentration of 3/100 μl in the bathing medium. The mean secretion rate is shown in nl/min.

In case diuretic activity had been obscured by deterioration of the haemolymph, tubules were isolated in samples of serum. Rates of 8.4 ± 0.9 nl/min in the serum from late pharate adults and 14.7 ± 1.0 nl/min in that from freshly emerged adults were sustained for an hour. As in the experiment with haemolymph, this is a relatively slight stimulation of secretion rates, but it is not due to the changes in the haemolymph composition after ecdysis, which would tend to decrease the rate of fluid secretion (Nicolson, 1976b). The results suggest that a diuretic factor is present in the circulation after emergence of the butterfly.

The source of the diuretic factor

Table 1 shows the distribution of diuretic activity in the nervous systems of late pharate butterflies. Significant amounts of activity are confined to the brain and corpus cardiacum–corpus allatum complex (CC–CA complex). The slight response produced by the other homogenates can perhaps be attributed to substances which would not normally be released from the tissues. Maddrell (1974) points out the need for caution in the interpretation of the apparent hormonal effects of crude homogenates of the nervous system: they may contain detectable quantities of neurotransmitters. Such compounds might be the reason for the reduced effect of the brain relative to the
Fig. 2. Dose/response curve for the diuretic hormone. CC-CA complex homogenates (dose expressed as number of complexes per 100 μl of bathing medium) were tested on isolated tubules. Secretion rates in nl/min each measured for 1 h.

CC–CA complex. 5-Hydroxytryptamine (5-HT), which accelerates fluid secretion in certain Malpighian tubules (Maddrell et al. 1971), has been identified in insect nervous systems (Colhoun, 1967). However, 5-HT is not the diuretic factor in the nervous tissue of *Pieris*, because the tubules are not responsive to this substance (Nicolson, 1975). Together with the hormonal activity demonstrated in the haemolymph, the marked stimulation of secretion rates by homogenates of the brain and the CC–CA complex provides reasonable evidence for the existence of a diuretic hormone in *Pieris*.

The time course of this diuretic effect on isolated tubules is shown in Fig. 1. Breis of the suboesophageal ganglion were tested as controls. As in the results in Table 1, the CC–CA complex seems to contain twice as much hormone as the brain, in spite of its comparatively minute size. The CC–CA complex was used for quantitative work on the DH because of its much higher concentration of hormone.

**The quantity of diuretic activity in the CC–CA complex**

The dose/response curve for the diuretic hormone (Fig. 2) was obtained by diluting breis of the CC–CA complex in varying amounts of bathing medium and assaying them on isolated tubules. Since the maximal response to DH can be slow, the rate of secretion of each tubule was measured for 1 h after addition of the brei. The tissue concentration is expressed as the number of complexes per 100 μl of bathing medium (CC/100 μl).

The considerable scatter in the rates of secretion probably reflects variation in both tubules and CC–CA complexes. The threshold for a diuretic response is approximately 0.15 CC/100 μl, and a maximal response is induced by a concentration of
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Fig. 3. Comparison of the effects of cyclic AMP and DH on fluid secretion by isolated adult tubules. (A) Response of a set of tubules to addition and removal of 2 successive doses of cyclic AMP. (B) Response of a set of tubules to stimulation with cyclic AMP and then with DH. Cyclic AMP (●) was used at a concentration of 5 × 10⁻⁴ M, DH (○) at a concentration of 1 CC/100 μl. Periods of stimulation are represented by horizontal bars (open for cyclic AMP, hatched for DH).

Externally applied cyclic AMP was compared with DH in its effect on secretion by *Pieris* tubules. A set of tubules was treated with 5 × 10⁻⁴ M cyclic AMP for 1 h, washed, and then treated with DH at a concentration of 1 CC/100 μl for another hour. The control experiment using two successive doses of cyclic AMP (Fig. 3A) shows that the tubules do not deteriorate in this time. From Fig. 3 (B) it is obvious that cyclic AMP is a far more effective stimulant of *Pieris* tubules: the dose/response curve does not, after all, reflect the maximal secretory capacity of the tubules. It seemed advisable to try alternative methods of obtaining samples of DH.
Table 2. The effect of the calcium ionophore A-23187 on tubule secretion in the presence and absence of CC-CA complexes

(Rates in nl/min, mean ± s.e., measured for 30 min.)

<table>
<thead>
<tr>
<th></th>
<th>Rate</th>
<th>N</th>
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<tbody>
<tr>
<td>Control</td>
<td>6.1 ± 0.4</td>
<td>8</td>
</tr>
<tr>
<td>Ionophore only</td>
<td>6.9 ± 0.5</td>
<td>8</td>
</tr>
<tr>
<td>CC+ionophore</td>
<td>21.5 ± 3.6</td>
<td>8</td>
</tr>
</tbody>
</table>

**DH extraction by high potassium and by calcium ionophore**

Maddrell & Gee (1974) have shown that exposure of neurohaemal areas to potassium-rich solutions causes rapid release of DH in *Rhodnius* and *Glossina*, probably by depolarizing the neurosecretory axon endings. One advantage of this technique is the release of hormones in an unbound form, free of other substances.

The potassium level in the haemolymph of a pharate butterfly is approximately 49 mM (Nicolson, 1976), and concentrations of 80 mM (Na-free medium) were used to evoke hormone release. CC-CA complexes were placed in 5 µl drops of 80 mM-K for 5–10 min; when these drops were added to 35 µl drops of normal bathing medium containing secreting tubules, the rate of secretion increased from 8.3 ± 1.2 to 23.0 ± 3.5 nl/min (mean ± s.e., 11 tubules). From the dose/response curve (Fig. 2) it can be calculated that this represents the release of only about 10% of the hormone available in tissue homogenates. Similarly, high K treatment releases 10% of the total extractable hormone in *Rhodnius* (Maddrell & Gee, 1974; Aston & White, 1974). It is possible, however, that higher K levels might increase the yield in *Pieris*.

The release of neurosecretory material by exocytosis is known to depend on the presence of calcium ions (Normann, 1974; Maddrell, 1974) and may be induced by certain ionophores which affect the calcium permeability of membranes (Cochrane & Douglas, 1974; Garcia, Kirpekar & Prat, 1975). The ionophore A-23187, supplied by Dr M. J. Berridge, was tested on CC-CA complexes of *Pieris*. The ionophore was diluted to a final concentration of 10⁻⁵ M in normal bathing medium, and tubules were isolated into 20 µl drops of this medium, some of which contained CC-CA complexes. The results in Table 2 show that the tubule assay is unaffected by the ionophore alone: this check was necessary because Prince, Rasmussen & Berridge (1973) found that this ionophore stimulates fluid secretion in the fly salivary gland by increasing the cellular calcium level. Treatment of the CC-CA complex with ionophore is successful in causing DH release, but this release corresponds to only 5% of the extractable hormone, and the preparation of tissue homogenates remains the most convenient method of extraction of *Pieris* DH.

**The fate of the hormone**

A breakdown mechanism is necessary to limit the time for which the DH is present in the haemolymph and thus the extent of diuresis. One possibility is the passive loss of the hormone through the tubule wall: however the DH of *Pieris* is not detectable in the secreted fluid. When isolated tubules were stimulated with DH at 20 times the threshold concentration the fluid they produced had no effect on the secretion rate of other tubules. A second mechanism is the spontaneous decay of the hormone: but
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Fig. 4. Change in the basal rate of secretion and the response to cyclic AMP and hormone after ecdysis. (A) Stimulation by $5 \times 10^{-4}$ M cyclic AMP. (B) Stimulation by DH at a concentration of 1.5 CC/100 μl. The insects from which tubules were removed for isolated preparations were late pharate adults (●), day 1 adults (○), day 2 adults (■) and day 3 adults (□). The stimulant was added at 60 min (vertical dashed line). Each point shows the mean rate of secretion of 10 tubules, with standard errors given by vertical bars.

*Pieris* DH is fairly stable. Tissue homogenates retained their diuretic activity when kept under liquid paraffin for 20 hours at 25 °C, and were not inactivated by 2 min of boiling.

Inactivation of the hormone by the tubules themselves does take place in other species (Maddrell, 1964; Berridge, 1966; Pilcher, 1970; Gee, 1975). This has usually been demonstrated by testing different tubules in succession in the same bathing drops.
This method is open to the criticism that the first tubules may alter the pH or deplete the medium of oxygen or other substances necessary for tubule function. A set of isolated tubules of *Pieris* was stimulated with CC-CA complex extract, and a second dose of hormone was added towards the end of the first diuresis. This resulted in a second, though smaller, diuresis, indicating that *Pieris* tubules are also able to destroy the DH.

*Changes in the diuretic response during development*

Initial observations showed some decline in the sensitivity of the tubules to hormonal stimulation after the pupal-adult ecdisis. The rates of secretion of 10 tubules from each of 4 age groups were measured for 1 h in control medium and 1 h in cyclic AMP. Both basal and stimulated rates vary greatly with age (Fig. 4A). The basal rate approximately doubles during the first day after ecdisis and then remains steady. This contrasts with the dramatic decline in the rate induced by cyclic AMP, which is highest at emergence.

The experiment was repeated with DH as stimulant. Fig. 4(B) shows a similar trend, with a diminished response of the tubules (cf. Fig. 3B). By day 3 there is no longer a discrepancy between the effects of the two stimulants; both increase the rate of secretion by only about 50%. The response does not disappear entirely. These physiological changes in the tubules can not be due to starvation or dehydration, because tubules dissected at the end of the third day from fed and unfed butterflies have identical basal and stimulated rates of secretion.
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The capacity to respond to cyclic AMP was also tested in tubules of butterflies several days before eclosion. The room temperature was 23 °C throughout this experiment, and the pupal instar lasted 11 days. Fig. 5 shows the changing rates of secretion as a function of age, with basal rates after eclosion included for comparison. The increase in stimulated rates before eclosion is as rapid as their decline after the event. The maximum sensitivity of the tubules to the hormone is apparently only a temporary change that is developed specially in preparation for hormone release at eclosion.

DISCUSSION

Diuretic hormones have been found in the brain and corpora cardiaca of several insects besides Pieris—Anisotarsus (Núñez, 1956), Dysdercus (Berridge, 1966), Schistocerca (Mordue, 1969) and Carausius (Pilcher, 1970). Conversely, extracts of the corpora cardiaca contain antidiuretic activity in, for example, Periplaneta, Locusta, Clitumnus and Gryllus (Wall & Ralph, 1964; Cazal & Girardie, 1968; de Bessé & Cazal, 1968). Since diuretic activity is present in both the brain and CC-CA complex of Pieris and since the corpora cardiaca are neurohaemal areas for neurosecretory products of the insect brain, it is likely that the brain is the site of synthesis of the DH and the corpora cardiaca store and release the hormone. In other species it is thought that the diuretic activity of the corpora cardiaca is due to material transported from the brain, rather than to secretion by their intrinsic glandular cells (Berridge, 1966; Mordue & Goldsworthy, 1969; Pilcher, 1970). Evidence that the CC-CA complex is the neurohaemal area for the hormone in Pieris comes from the treatment of this tissue with high potassium and the calcium ionophore; the rest of the nervous system would be protected from these agents by the perineurium.

The disadvantage of extraction by homogenization is the probable presence of unwanted substances in the sample, in addition to the physiologically active hormone (Maddrell, 1974). In Pieris this apparently results in a decreased response of the tubules to DH. Other techniques ought to provide purer samples of hormone – but not from the CC-CA complex, which is the neurohaemal area for a variety of hormones. None of the methods of hormone extraction used in the present study can produce a sample of DH free of the other hormones in the CC-CA complex.

The diuresis of an emerging butterfly must begin quickly, so that the insect may become mobile as soon as possible, like Rhodnius and Glossina after a blood meal. Diuresis in Pieris differs from that known in other insects in being independent of feeding. The stimulus causing the release of its DH might be a single event, one of the sequence of events occurring at eclosion (Truman, 1971). This idea is supported by the observation that cotton ligatures tied immediately after eclosion between head and thorax, or thorax and abdomen, fail to prevent diuresis. Perhaps the appropriate stimulus leads to the release of sufficient hormone to last for the whole period of diuresis. If this seems to lack the precision of the steady release in other species (especially bloodsucking insects), it must be remembered that Pieris is dealing with the excretion of a fixed volume of fluid, not a meal of variable size. The blood volume of a pharate butterfly will no doubt vary a little with the size of the insect and the relative humidity: the quantity of DH released at eclosion could be adjusted accordingly. It is known that the tsetse fly can control the extent of diuresis with respect to the degree of dehydration of its tissues as well as the size of the meal (Bursell, 1960).
A mechanism for removal or destruction of the DH is important in avoiding accidental desiccation in species which have a short, fast diuresis. Destruction of the hormone by the Malpighian tubules themselves is a general occurrence. Further control is achieved by the spontaneous decay of the DH in *Rhodnius* (Maddrell, 1964) and its inactivation by a specific breakdown enzyme in *Glossina* (Gee, 1975). Although the level of DH in *Pieris* might otherwise tend to rise continuously as the blood volume is lowered during diuresis, destruction of the hormone by the tubules and possibly other tissues is apparently enough to slow the rate of excretion quite soon after ecdisis.

Physiological changes in the Malpighian tubules control the extent of diuresis indirectly, by ensuring that it can take place only at the pupal-adult ecdisis. The growth in their responsiveness to stimulation before eclosion is not as exceptional as the dramatic loss of the hormone response during the three days following the ecdisis. Perhaps this decline is a safety mechanism to prevent dehydration of the insect if the DH remaining in the corpora cardiaca were abnormally released. The similar decline in sensitivity to both cyclic AMP and DH means that the change is not at the DH receptor site, which is probably located on the cell membrane facing the haemolymph (Maddrell *et al.* 1971), but rather in the intracellular action of cyclic AMP. Until the mechanism of involvement of cyclic AMP in tubule secretion is known, it is unwise to speculate further on this.

The doubling of the unstimulated rate of fluid secretion during the first day after ecdisis allows the tubules to cope with the fluid intake of the nectar-feeding butterfly. As in the continuously feeding larval stages, the tubules must maintain a slow but steady rate of secretion, and hormonal control is not necessary. Alteration in the intrinsic rate of tubule secretion with the physiological state of an insect has already been described for *Carausius* by Taylor (1973). Supplementary to direct hormonal effects, it is associated with structural changes in the tubule cells. Such long-term adaptation may also account for the seasonal variation observed in the larval tubules of *Pieris* (Nicolson, 1976a).

Other insects use their diuretic hormones almost continuously (e.g. *Carausius*) or at least repeatedly (e.g. *Rhodnius*). In contrast, the insensitivity to cyclic AMP of larval tubules (Nicolson, 1976a) and of adult tubules except around the time of eclosion provides good evidence that the diuretic hormone of *Pieris*, like the eclosion hormone (Truman, 1973), is used only once during the life cycle.

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REFERENCES


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