CHOLINESTERASE AND THE SECRETION OF THE BRAIN HORMONE IN INSECTS

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Summary

Growth and moulting in insects are stimulated by a hormone from neurosecretory cells in the brain. In diapause these cells fail to secrete their hormone, and van der Kloot (1955) correlated this failure with the disappearance of cholinesterase and cholinergic substance from the brain. The experiments reported here show that eserine, which blocks the action of cholinesterase, will also retard the adult development of *Phalaenoides glycine* Lew. (Lepidoptera) if injected into the pupa before the brain has released its hormone. But in *Pieris rapae* L. and *Danaus plexippus* L. (Lepidoptera) the brain has usually secreted sufficient hormone before pupation and eserine does not delay adult development when it is injected into these pupae. Apparently cholinesterase is essential to the secretion of the brain hormone in non-diapausing insects, and by blocking it an artificial diapause may be induced.

I. Introduction

The influence of the brain on the growth and moulting of insects has been reviewed recently by Wigglesworth (1954). Briefly, the processes of growth and moulting require for their initiation, and possibly also for their maintenance, a hormone from the thoracic glands. The thoracic glands are themselves stimulated to produce this hormone by another hormone produced in neurosecretory centres of the brain. The mechanisms within the brain which lead to the production of the brain hormone are still largely unknown. In non-diapausing larval insects the activity of the neurosecretory cells appears to depend on adequate feeding (Wigglesworth 1934; Bounhiol 1938). Just after a moult and before feeding, such larval brains are quiescent but potentially active. By contrast the brain of a diapausing insect is called refractory because it does not become active, although the body of the diapausing insect will allow activity in a potentially active brain which has been transplanted into it (Williams 1952, 1956). A refractory brain may be converted into a potentially active one by chilling the diapausing insect for a sufficient time (Williams 1952). Clearly from such evidence the next step is to investigate the differences between refractory and potentially active brains.

At the biochemical level one piece of evidence has been reported. Van der Kloot (1955) found that the concentration of cholinesterase and cholinergic substance in the brain of the moth *Platysamia cecropia* fell to a low level at the onset of pupal diapause, and the electrical activity of the brain disappeared. During chilling and the completion of diapause development the concentration of cholinergic substance in the brain increased, and when the pupa was warmed

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the titre of cholinesterase also rose to a value close to that in the brain of a caterpillar in the final instar. In the ganglia of the ventral nerve cord, the concentration of cholinesterase and cholinergic substance remained at about the same level throughout. Van der Kloot suggested that diapause arises from a lack of cholinesterase and cholinergic substance in the brain, and that both these substances are necessary to neurosecretory activity.

In order to apply van der Kloot's idea to larval diapause, such as that of the codlin moth, *Cydia pomonella* (Andrewartha 1952), it is necessary to postulate a local deficiency of cholinesterase within the brain. Diapausing *Cydia* larvae are able to perform coordinated movements in walking and spinning new cocoons. Their brains are presumably electrically active and contain a functioning cholinesterase system. Yet they remain in diapause. Perhaps in them the deficiency of cholinesterase is confined to the vicinity of the neurosecretory cells. It may be that the lack of cholinesterase throughout the rest of the brain in *Platysamia* is associated with a rather inactive pupal life in which the coordinating activity of the brain is not called upon.

Van der Kloot's observations suggest that cholinesterase may also be essential for the neurosecretory activity of non-diapausing insects. Wigglesworth (1934) suggested that the secretory activity of the brain in *Rhodnius* is stimulated by nervous impulses through the ventral nerve cord. More recently Bounhiol (1952*a*, 1952*b*) has reported that in *Bombyx* also, the brain secreted only if its nervous connections with the ventral cord were intact. Perhaps cholinesterase functions merely in transmitting nervous stimuli which trigger the neurosecretory cells of the brain. On the other hand, in the moths *Platysamia* (Williams 1952) and *Phalaenoides* (Monro 1956), the secretory activity of the brain seems to be independent of its connections to the ventral cord. If the secretion of the brain hormone in these moths depends on cholinesterase, the latter probably acts within the brain itself.

The experiments recorded here were made in an attempt to demonstrate the influence of cholinesterase on the secretory activity of the brain by injecting eserine sulphate, an inhibitor of cholinesterase, into newly moulted pupae. For the purpose, comparisons were drawn between pupae of two different categories (Williams 1952). Williams distinguished between three types of lepidopterous pupae:

(*a*) *Non-diapausing.*—Those in which the brain is inactive while the thoracic glands go on secreting. Apparently the continued secretion of the thoracic glands is due to the presence of brain hormone carried over from the final larval instar (Bounhiol 1952*a*, 1952*b*).

(*b*) *Non-diapausing.*—Those in which the continued activity of the thoracic glands depends on further secretion by the brain soon after pupation.

(*c*) *Diapausing.*—Those in which both brain and thoracic glands are inactive for a long period after pupation.

If eserine suppresses or delays the secretory activity of the brain this could be shown by injecting it into a pupa of type (*b*) before the brain has secreted its

hormone and looking for a delay in adult development. In non-diapausing generations of *Phalaenoides glycine* Lew. the larvae pupate several days before the brain releases sufficient hormone to induce adult development (Monro 1956, 1957). This species therefore belongs to type (*b*). When eserine was injected into pupae of *Phalaenoides* a delay was observed but this may have been due to the temporary suppression of a stage of development after the brain had released its hormone (see Section III(*b*)). This could have been tested by injecting eserine into successively older batches of pupae. If the brain alone were influenced there would be a critical period corresponding to the time of release of the brain hormone. In pupae injected before this time there would probably be an increasing delay with later injections, and then no delay once the hormone was released. This would be true if eserine inhibits the release of the hormone, or if it returns the whole secretory cycle to its starting point. Unfortunately, sufficient numbers of *Phalaenoides* pupae were not available for such an experiment, so the possibility that eserine interfered with later stages of growth was tested on two other species of Lepidoptera, *Pieris rapae* L. and *Danaus plexippus* L. which belong to type (*a*) (see Sections III(*c*) and III(*d*)).

II. Material and Methods

In South Australia, the noctuid *Phalaenoides glycine* has three generations a year. In summer the pupae usually develop into adults without entering diapause. The pupal stage is about 3 weeks at 27°C. For these experiments non-diapausing pupae were reared from final instar larvae taken from the field and kept at 27±1°C in a photoperiod of 16 hr per day with grape-vine leaves for food.

In southern South Australia, *Pieris rapae* (Pieridae) has several non-diapausing generations and a winter diapause. The eggs of *Pieris* were collected in the field and the larvae reared on cabbage leaves at 27±1°C in a photoperiod of 16 hr per day. Under these conditions no diapausing pupae were found among several hundred reared over two years.

Danaus plexippus (Danaidae) is a non-diapausing species which feeds on *Asclepias* spp. Larvae of *Danaus* were taken from the field to the laboratory during the final instar and fed on leaves of *Asclepias* at 27±1°C in a 16 hr photoperiod per day until pupation.

All larvae were kept in colourless plastic dishes measuring 13·5 by 10 by 6·5 cm in a constant-temperature cabinet with white walls. Light was provided by an incandescent lamp (15-W, tungsten filament), attached to one wall of the cabinet. The length of the photoperiod was controlled by an electric "Venner" timing switch. The culture vessels were at a distance of 20–60 cm from the source of light. On pupation, the animals were transferred to glass tubes which were kept in a closed vessel over a saturated solution of potassium nitrate in water (93 per cent. R.H.). In the preliminary experiments, the ligatures were tied behind the head with silk thread. The parts in front of the ligature were cut away and the wound sealed with paraffin wax. When the brain of a pupa was removed (under CO_2 anaesthesia) the wound was covered with a window of glass sealed on with paraffin wax. Injections were made with standard hypodermic needles and an "Agla" micrometer syringe.

III. Experiments and Results

(a) *Preliminary Experiments*

Preliminary experiments were performed on non-diapausing pupae of *Pieris* to find out whether adult development could proceed when the brain was removed just before or just after pupation. The animals were reared at 27 ± 1°C with a photoperiod of 16 hr (diapause-preventing) and assigned at random to one of two treatments. The controls were allowed to pupate normally, while in the other group the final instar larvae were ligatured behind the head at a stage indicated by the withdrawal of the ocellar pigment.

Table 1

Influence of eserine sulphate on the duration of the pupal stage of phalaenoides glycine and danaus plexippus

Species	Group	Number of Animals	Mean Time of Development (days)	Range (days)
P. glycine	Controls	6	16·2	14–21
	Treated with eserine	5	23·4	20–28*
D. plexippus	Controls	13	8	7–9
	Treated with eserine	15	7·73	7–8†

*Significantly different from controls ($P < 0{\cdot}01$).
†Not significantly different from controls ($P = 0{\cdot}3$–$0{\cdot}4$).

Of 10 control animals all had developed black wing pigment in 4–5 days, while of the 10 which had been ligatured five died within 1–11 days of pupation, four developed black wing pigment in 10–11 days, and one showed no sign of adult development when dissected one month after pupation. A similar experiment in which the brain was removed during the first day after pupation, showed that it had ceased to influence the rate of adult development.

It would seem that the brain of *Pieris* has secreted sufficient hormone for the completion of adult development at, or about the time of, pupation. The newly moulted pupa of *Pieris* should, therefore, be a suitable subject for showing whether eserine can block steps in development later than the release of brain hormone.

Williams (1952) cited *Danaus plexippus* as an example of type (*a*). Pupae of this species were, therefore, used as well as those of *Pieris* to test the action of eserine on development subsequent to the release of brain hormone.

(b) *Influence of Eserine Sulphate on the Duration of the Pupal Stage in* Phalaenoides glycine

Non-diapausing pupae of *Phalaenoides* were assigned at random to one of two groups on the first day of this instar. In one group 0·01 ml of 0·1M eserine sulphate in physiological saline solution was injected into the thorax of each pupa.

The pupae of the other group each received an injection of 0·01 ml of unmodified saline solution. Both groups were kept at 27±1°C and 93 per cent. R.H. (saturated KNO_3) and inspected daily. The date on which orange and black pigment appeared in the wings was recorded. The behaviour of the moths which emerged was also observed.

The times required for adult development by each group are compared in Table 1. There was a significant delay in that group which received an injection of eserine compared with the group used as a control. The simplest explanation of this result seems to be that the release of brain hormone was delayed in the presence of eserine; the eserine was then slowly destroyed in the body, and the brain again

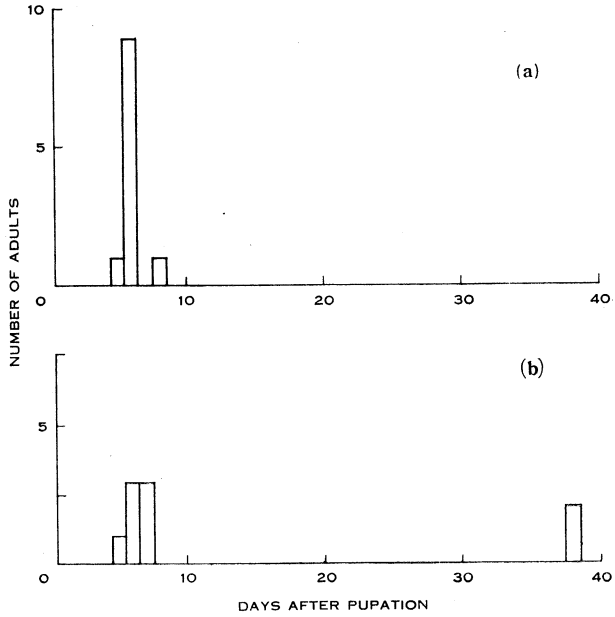

Fig. 1.—Duration of adult development from pupation to the appearance of wing pigment in *Pieris rapae*. (*a*) Pupae injected with 0·01 ml of saline solution during the first day after pupation; (*b*) Pupae injected with 0·01 ml of 0·1M eserine sulphate in saline solution during the first day after pupation.

became competent to secrete the hormone. Certainly the eserine appeared to be destroyed, or its effectiveness was reduced, because moths which emerged were able to walk or fly in a coordinated way.

(*c*) *Influence of Eserine Sulphate on the Duration of the Pupal Stage in* Pieris rapae

On the day of pupation pupae were assigned at random to two groups. In one group the pupae were given an injection of 0·01 ml of 0·1M eserine sulphate in physiological saline solution. Pupae in the other group received 0·01 ml of unmodified saline solution to serve as controls. Both sets of pupae were kept at 27±1°C and 93 per cent. R.H. (saturated KNO_3), and observed daily until the adults emerged, or until death. The time that the pupae in each group took to produce black wing

pigment was taken as the period needed for the development of the adult, because those treated with eserine were unable to emerge from the old pupal skin.

The periods needed for adult development are set out in Figure 1. Seven of the pupae that were treated with eserine reached the adult stage between the sixth and eighth day but two were delayed until the 38th day. It seems reasonable to exclude these two because a delay would be expected if, by chance, the eserine had been injected before the brain had secreted sufficient hormone to ensure the development of the adult. If these two are excluded the mean duration of adult development (up to the appearance of wing pigment) was 6·3 days for the seven treated pupae compared with 6·1 days for the 10 controls. The difference is non-significant.

(*d*) *Influence of Eserine Sulphate on the Duration of the Pupal Stage in* Danaus plexippus

Pupae of *Danaus* were assigned at random to one of two groups within 24 hr of pupation. In one group each pupa received 0·02 ml of 0·1M eserine sulphate in physiological saline solution and in the other 0·02 ml of unmodified saline solution. The pupae were inspected daily and the date of emergence of adults recorded. In *Danaus* the pupal cuticle was shed even by those treated with eserine.

The times required for development from pupation to emergence are set out in Table 1. There was no significant difference between the two groups ($P = 0{\cdot}3 - 0{\cdot}4$). The pupae treated with eserine gave rise to adults which were unable to walk or fly though a large proportion of them emerged and expanded their wings. As in *Pieris* these adults showed irregular twitching of the legs.

IV. Discussion

Eserine delayed development in *Phalaenoides* probably by inhibiting neurosecretory activity in the brain. In *Pieris* and *Danaus* pupae there was no delay if the brain had already secreted sufficient hormone and this will probably be found true of *Phalaenoides* also. The two *Pieris* pupae that were delayed by eserine probably had an insufficient titre of brain hormone in the blood at the time of injection. Later, the concentration of eserine was reduced, or the brain became less sensitive to it and was able to secrete once more.

Eserine not only inhibited the secretion of the brain hormone but it also interfered with the functioning of the voluntary muscles. The insects were not able to walk or fly and they showed an uncoordinated twitching of the legs. None of the *Pieris* was able to emerge and all those that were dissected out of the pupal cases showed these symptoms. But some of the *Phalaenoides* emerged and were able to walk and fly normally. The *Pieris* usually completed adult development within 5–8 days but the *Phalaenoides* took from 20–28 days. This suggests that the eserine gradually became inactive and that after 3 or 4 weeks the concentration had been reduced to the level at which it no longer interfered with the proper functioning of the voluntary muscles.

In *Pieris* either the muscles or nervous system were abnormal because they had differentiated in the presence of eserine, or eserine was still present. In dissections

of the thorax and legs no abnormality in the muscles was apparent at low magnification but this matter was not followed further. It is noteworthy that active cholinesterase does not seem to be necessary to the differentiation of thoracic muscles, though the extirpation of thoracic nerves and ganglia will prevent such differentiation in *Lymantria* (Kopec 1923), in *Telea* (Nuesch 1952), and in *Platysamia* (Williams and Schneiderman 1952).

An attempt was made to increase the proportion of *Pieris* pupae with an artifically induced diapause. Because the brain has secreted sufficient hormone for adult development at about the time of pupation, it ought to be possible to increase the proportion of pupae with delayed development by injecting eserine into final instar caterpillars nearing pupation. This was done but the experiment failed because all the eserine-treated animals died within the old larval skin at, or soon after, pupation.

The experiments on *Phalaenoides* and *Pieris* show that an artificial diapause may be induced by inhibiting cholinesterase. This result might have been predicted from van der Kloot's hypothesis and suggests that cholinesterase also plays an important part in the secretory activity of the brain in non-diapausing larvae and pupae. Possibly cholinesterase acts through its influence on nervous conduction between centres within the brain. In any event it is probably acting within the "inner mass" of the brain which, in *Platysamia*, is able to secrete the brain hormone when cut out of the brain of an adequately chilled pupa and implanted into a brainless pupa in diapause (Williams 1948). The inferred ativity of a large part of the brain in diapausing *Cydia* larvae is consistent with this hypothesis.

Williams (1952) described two sorts of non-diapausing pupae; one of which, like *Pieris* and *Danaus* had an inactive brain after pupation but still developed into an adult; while the other, like *Phalaenoides*, had an active brain in the pupal stage, and was unable to develop into an adult if the brain was removed. The second type of pupa may be converted into a "permanent" pupa by removing the brain just after pupation. Such "permanent" pupae are useful for testing the activity of implanted brains but in order to discover whether a pupa is of suitable type it has been necessary to remove the brain. The injection of eserine after pupation may possibly be used instead, as it is easier, and the subsequent mortality is much lower. Pupae which resemble *Pieris* in having already secreted sufficient brain hormone should develop into adults with little or no delay. Delay would indicate that the brain secreted its hormone after pupation, as in *Phalaenoides*. Other inhibitors of cholinesterase might also be useful, provided that they do not interfere with stages of growth which follow release of the brain hormone.

Williams (1951) has already described the action of pilocarpine in permanently preventing the development of diapausing pupae of *Platysamia*. He ascribed this action to the blocking of cytochrome synthesis but it may be simpler to regard it as due to the blocking of cholinesterase in the brain. Such an interpretation is strengthened by Williams' observation that pilocarpine was ineffective after the brain had secreted its hormone unless much larger quantities were injected.

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