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PROGRAMMED CELL DEATH—II. ENDOCRINE POTENTIATION OF THE BREAKDOWN OF THE INTERSEGMENTAL MUSCLES OF SILKMOTHS*

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Abstract—The abdominal intersegmental muscles of saturniid pupae are well preserved during the entire pupal period and throughout the three weeks required for the development of the adult moth. Then, within 48 hr after the emergence of the moth, the muscles break down and disappear. The breakdown is a result of developmental reactions within the muscles themselves; their dissolution, in this sense, is a carefully timed, final episode in their own metamorphosis. Thus, the endocrine conditions obtaining at the onset of adult development (the presence of ecdysone and the absence of juvenile hormone) dictate both the formation of all adult structures, including muscles, and the eventual death of the intersegmental muscles. If juvenile hormone is injected, the breakdown does not occur in the intersegmental muscles or in any of the other tissues which are normally destroyed during the pupal–adult transformation. The final signal for the initiation of breakdown does not appear to be endocrine in character. Thus, when pairs of pupae were joined in parabiosis so that they shared the same blood, the breakdown of muscle in one individual did not hasten or retard the breakdown in the other individual. This finding points to some further signalling system which triggers the initiation of cell death in the endocrinologically potentiated muscles.

INTRODUCTION

THE previous paper in this series considered the histology and cytology of the breakdown of the abdominal intersegmental muscles of saturniid moths. The process was interpreted as a form of autonomous cell death in that the agencies of the breakdown apparently reside within the muscles themselves in the form of lysosome-like particles.

The breakdown is carefully timed and ordinarily takes place only after the moth has emerged from the pupal exuviae and spread its wings. Moreover, the histolysis is sharply localized within the muscles in question; it does not spread to other muscles in the same insect. These findings direct attention to mechanisms, not only for programming the autonomous death of the intersegmental muscles, but also for controlling and timing the implementation of this latent lethal programme.

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In the present investigation we have sought to clarify the role of the endocrine system in potentiating and controlling the muscle breakdown.

MATERIALS AND METHODS

1. *Experimental animals*

The study was performed on pupae of the following species of 'wild' silkworms: *Antheraea pernyi*, *Antheraea polyphemus*, *Hyalophora cecropia*, and *Samia cynthia*. In order to promote the termination of diapause, the cocoons were stored at low temperatures for at least 10 weeks prior to use—*pernyi* at 2–3°C, *cecropia* and *polyphemus* at 6°C, and *cynthia* at 8°C. The pupae were then returned to 25°C, removed from their cocoons, and used in the experimental procedures. The second day of adult development was recognized by the detachment and retraction of the leg epidermis from the overlying pupal cuticle; development was timed from this point. In *cecropia*, the timetable described by SCHNEIDERMAN and WILLIAMS (1954) was helpful in recognizing successive stages of adult development at 25°C.

The time of emergence of the moth from the pupal exuviae was scored as the zero day of adult life, and the age of the moth was counted from this day. When animals were sacrificed before adult ecdysis, the stage of development was evaluated by morphological criteria for each species and estimated as days prior to adult ecdysis.

2. *Experimental procedures*

Juvenile hormone was prepared by diethyl ether extraction of the abdomens of male *cecropia* moths (WILLIAMS, 1956). The ethereal extract was washed three times with water, the aqueous phases being discarded. The ether was evaporated and the resulting oil extracted seven times with anhydrous methanol. The methanolic phases were pooled and the solvent evaporated *in vacuo* to obtain a golden oil of high juvenile hormone activity. By means of a hypodermic syringe equipped with a 27-gauge needle, the oil was injected in volumes of 0·01–0·1 ml into the mesothoracic tergum of anaesthetized previously chilled pupae, the perforation being sealed with melted wax. The pupae were then placed at 25°C to await the initiation of development.

In experiments where animals were caused to share the same blood, pairs of pupae were joined in parabiosis by previously described methods (WILLIAMS, 1946, 1959, 1963). Before sacrificing the animals, the patency of the blood connexion was confirmed by injecting 0·5 ml of 0·1% methylene blue in EPHRUSSI and BEADLE (1936) Ringer into one of the individuals.

In other experiments individual pupae were exposed to longitudinal temperature gradients and caused to develop at dissimilar rates at the two ends. The procedure was that described by WILLIAMS (1942).

At the termination of each experiment the individual animals were dissected in order to ascertain the condition of the abdominal intersegmental muscles. Animals younger than 4 days prior to adult ecdysis were cut longitudinally with scissors and both halves pinned to plasticine under insect Ringer's solution. In the case of

more mature individuals, the abdomens were excised, cut down the mid-dorsal line, and spread and pinned to plasticine under Ringer. The preparations were rinsed in a stream of Ringer and the internal organs and most of the fat body removed. After a further rinse in Ringer, the state of the intersegmental muscles was ascertained by means of a dissecting microscope. If the muscles were present, a few fibres were cut with microscissors to determine their contractility. The status of the muscles was scored as follows:

+++ Muscles completely intact and contractile (as in a freshly emerged moth).
++ Dorsal muscles degenerating; lateral muscles intact and contractile (as in a moth 15 hr after ecdysis).
+ Lateral muscles still present but degenerating (as in a moth 25 hr after ecdysis).
0 All parts of muscles degenerate and absent (as in a moth 48 hr after ecdysis).

RESULTS

1. *The effects of juvenile hormone*

A highly active extract of juvenile hormone was injected into each of a series of ten previously chilled *polyphemus* pupae. Within 2–3 weeks at 25°C, the pupae transformed into moths which retained pupal characteristics. After the completion of development, as signalled by the breakdown of the pupal endocuticle and the full or partial resorption of the moulting fluid, the pupal exuviae were removed and the animal rinsed briefly in water. The intensity of the juvenile hormone effect was scored from 0 (no pupal characters) to V (full preservation of pupal characters) according to the diagnostic criteria previously described (WILLIAMS, 1961, p. 575).

Each animal was stored at 25°C for at least 2 days and then dissected. The results may be summarized as follows: Individuals showing low-grade response to juvenile hormone (I and II) contained fully or partially degenerate intersegmental muscles. By contrast, all individuals with high-grade reactions (IV and V) showed complete preservation of the muscles. Manifestly, the breakdown of the muscles is strongly opposed by juvenile hormone.

The ability of juvenile hormone to block the realization of adult characters rapidly declines when the injection is postponed until after the initiation of adult development (WILLIAMS, 1961). The same is true for its ability to prevent the breakdown of the intersegmental muscles. Thus, when a large injection was made on the second day of adult development, the individual transformed into a nearly normal moth (I reaction) which, after 4 days at 25°C, showed complete degeneration of the muscles in question.

2. *Effects of parabiosis*

According to the results just considered, the breakdown of the muscles is the culmination of developmental reactions set in motion by the endocrine conditions at the outset of adult development. These conditions consist of the presence of a high titre of ecdysone and the absence of juvenile hormone. But, as we have seen,

the actual breakdown of the muscles occurs only 3 weeks later—after the completion of adult development and the emergence of the moth. We therefore questioned the role of the endocrine system in the timing of the breakdown itself.

Answer to this question was sought in a direct manner; namely, by joining pupae in parabiosis so that they shared the same blood. Under this circumstance one of the partners ordinarily reaches maturity a day or two before the other (WILLIAMS, 1952). In order to amplify this difference, *cecropia* pupae (requiring 21 days for adult development at 25°C) were joined to *cynthia* pupae (requiring only 17 days) or to *polyphemus* or *pernyi* pupae (requiring 18 to 20 days). The brain of the more slowly developing partner was excised in order to ensure that it did not initiate development independently of its partner.

TABLE 1—THE STATUS OF THE INTERSEGMENTAL MUSCLES IN PARABIOTIC PREPARATIONS

No. of preparations	Partner 1			Partner 2		
	Species	Stage*	Condition of muscle†	Species	Stage*	Condition of muscle†
4	*Cecropia*	−>20	+++	*Cecropia*	+2 to +5	0
3	*Cecropia*	0	+++	*Cecropia*	0	+++
1	*Cecropia*	+1 to +4	0	*Cecropia*	+2 to +5	0
1	*Cecropia*	−1	+++	*Cynthia*	+3	+
2	*Cecropia*	0	+++	*Cynthia*	0 to +1	+
3	*Cecropia*	0	+++	*Cynthia*	+2 to +4	0
2	*Cecropia*	+2	+	*Cynthia*	+3	0
3	*Cecropia*	+2 to +4	0	*Cynthia*	+3 to +5	0
1	*Cecropia*	−2	+++	*Polyphemus*	+4	+
1	*Cecropia*	+2	+	*Polyphemus*	−1	+++
2	*Cecropia*	+3	0	*Polyphemus*	+3	0
2	*Cecropia*	0	+++	*Pernyi*	+1	++
1	*Cecropia*	0	+++	*Pernyi*	+2	+
1	*Cecropia*	0	+++	*Pernyi*	+2	0
2	*Cecropia*	+2	0	*Pernyi*	+2	0
1	*Cynthia*	−26	+++	*Cynthia*	+4	+
2	*Cynthia*	0	+++	*Cynthia*	+2 to +4	0
2	*Cynthia*	+2	0	*Cynthia*	+2	0

* Zero is recorded as the day of adult ecdysis; days prior to ecdysis are estimated and scored as minus and days after ecdysis, as plus.

† For description, see text (p. 645).

The results of these experiments are summarized in Table 1. In all except two preparations the condition of the muscles corresponded to the developmental stage of the animal in which they were found and showed no relation to the developmental stage of the partner; i.e. the breakdown of the muscles in one individual neither accelerated nor retarded the breakdown in its partner. In the two exceptional

cases the older partner was noticeably desiccated. The delayed degeneration of muscles in such animals is a common phenomenon.

Three preparations were examined but are not listed in the table. One was a chilled *polyphemus* pupa parabiosed to a freshly emerged headless *cecropia* moth. After 17 days, the *polyphemus* had progressed to approximately stage — 10 and retained its longitudinal muscles. The *cecropia* moth retained no intersegmental muscles, but the newly formed adult muscles were perfectly normal. An equivalent preparation, between a headless male *cecropia* moth and an unchilled male *cecropia* pupa, survived 10 weeks without apparent change. Even after this long period, the pupa retained normal intersegmental muscles, and the moth retained normal adult muscles.

The third preparation consisted of the parabiosis of a headless male *cecropia* moth to a chilled female *cecropia* pupa. The pupa initiated development and, under the influence of juvenile hormone from the male moth, formed a second pupa. The musculature of this animal was that of a normal pupa. The moth showed a normal complement of adult muscles. Its intersegmental muscles had not re-formed.

3. *Effects of temperature gradients*

By exposing individual pupae to a longitudinal gradient in temperature, one end was caused to reach maturity before the opposite end (WILLIAMS, 1942). Fourteen previously chilled *cecropia* pupae were treated in this manner, as noted under Methods.

TABLE 2—THE STATUS OF THE INTERSEGMENTAL MUSCLES IN ANIMALS EXPOSED TO LONGITUDINAL TEMPERATURE GRADIENTS

Number of animals	Stage* of head	Stage* of abdomen	Condition of muscles†
Abdomen chilled			
5	+2 to +4	−2 to 0	+++
1	+4	+2	+
2	+4	+2	0
Abdomen warm			
1	−2	0	+++
2	−1 to 0	+1	+
1	0	+2	+
1	0	+2	0
1	0	+5	0

* Zero is recorded as the day when an animal at this stage of development would ordinarily emerge; days prior to this stage are estimated and scored as minus and days after this stage, as plus.

† For description, see text (p. 645).

The results, summarized in Table 2, show that the condition of the intersegmental muscles of the abdomen was dictated by the developmental stage of the

abdomen itself and was independent of the developmental stage attained by the anterior end of the same animal.

DISCUSSION

In the absence of ecdysone, the intersegmental abdominal muscles of the pupa remain viable and contractile throughout the 8 months of pupal diapause. Ecdysone is then secreted by the prothoracic glands and acts throughout the pupa to provoke the termination of diapause and the initiation of adult development. According to the present investigation, it is this normal endocrinological situation—ecdysone acting in the absence of juvenile hormone—which is responsible for potentiating the intersegmental muscles to break down 3 weeks later.

It is worth noting that this same endocrine stimulus, the presence of ecdysone and the absence of juvenile hormone, simultaneously provokes the formation of the new muscles of the moth—for example, the entire assembly of flight muscles which differentiate from the thoracic myoblasts during the 3 week period of adult development. Consequently, it is a single endocrine agent, ecdysone, which promotes the genesis of certain muscles and the death of other muscles in the same individual. This fact gives assurance that the two types of muscles have been cast and programmed for dissimilar roles in the insect as a whole, and that the death of the one, as well as the formation of the other, are both to be regarded as genuine developmental reactions. Because the metamorphosing insect is a cleidoic system, the control and interlacing of synthesis and degradation are clearly necessary.

Attention has previously been directed to the ability of juvenile hormone to oppose the acting-out of the life-plan and thereby to prevent the programmed cell death implicit in metamorphosis (WILLIAMS, 1961). Confirmation of this finding is seen in the experiments reported here. Thus, when juvenile hormone is injected to act alongside ecdysone at the initiation of adult development, then, to varying degrees as dictated by the concentration of juvenile hormone, the formation of the imago is blocked and the pupa transforms into a creature which may, in the extreme instance, be little short of a second pupal instar (WILLIAMS, 1959, 1961, 1963). Meanwhile the intersegmental muscles are preserved to a degree proportional to the preservation of other specialized pupal tissues.

Among the various tissues which break down during a normal metamorphosis, the intersegmental muscles are peculiar in that their dissolution is postponed until adult development is complete—until, in fact, the moth has emerged from its exuviae and spread its wings. The survival value of this delay is self-evident: the contraction of the abdominal muscles is responsible for the compression of the haemolymph which, in turn, is prerequisite for the spreading and unfurling of the wings of the newly emerged moth. The delay in the breakdown of the intersegmental muscles is therefore a biological necessity which has been enforced in the evolutionary history of the species.

Once the endocrine signal has been administered, the intersegmental muscles are potentiated to degenerate and the role of the endocrine system is at an end. As we have emphasized, the muscles remain intact and functional for 3 further weeks.

We have been unable to detect any influence of the endocrine system in initiating the actual breakdown of the muscles after the ecdysis of the moth. Pairs of animals united in parabiosis show a timing of breakdown which is independent in the two individuals—a finding which discounts any blood-borne 'go-ahead' signal. The same conclusion was reached in experiments performed on individuals exposed to longitudinal temperature gradients and thereby forced to mature at different rates at the two ends.

We are therefore persuaded that the actual breakdown of the intersegmental muscles requires some further signal which is not endocrinological in character. This aspect of the matter will be examined in the next paper in this series.

REFERENCES

EPHRUSSI B. and BEADLE G. W. (1936) A technique of transplantation for *Drosophila*. *Amer. Nat.* **70**, 218–225.

LOCKSHIN R. A. and WILLIAMS C. M. (1964) Programmed cell death in an insect. I. The histology and cytology of cell death (in preparation).

SCHNEIDERMAN H. A. and WILLIAMS C. M. (1954) The physiology of insect diapause. II. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bull., Woods Hole* **106**, 238–252.

WILLIAMS C. M. (1942) The effects of temperature gradients on the pupal–adult transformation of silkworms. *Biol. Bull., Woods Hole* **82**, 347–355.

WILLIAMS C. M. (1946) Physiology of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull., Woods Hole* **90**, 234–243.

WILLIAMS C. M. (1952) Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull., Woods Hole* **103**, 120–128.

WILLIAMS C. M. (1956) The juvenile hormone of insects. *Nature, Lond.* **178**, 212–213.

WILLIAMS C. M. (1959) The juvenile hormone. I. Endocrine activity of the corpora allata of the adult Cecropia silkworm. *Biol. Bull., Woods Hole* **116**, 323–338.

WILLIAMS C. M. (1961) The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. *Biol. Bull., Woods Hole* **121**, 572–585.

WILLIAMS C. M. (1963) The juvenile hormone. III. Its accumulation and storage in the abdomens of certain male moths. *Biol. Bull., Woods Hole* **124**, 355–367.