

## PROGRAMMED CELL DEATH—III. NEURAL CONTROL OF THE BREAKDOWN OF THE INTERSEGMENTAL MUSCLES OF SILKMOTHS\*

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**Abstract**—The breakdown of the intersegmental muscles of the silkmoth abdomen is potentiated by exposure to ecdysone during the first few days of adult development. Three weeks later, after the completion of adult development, the actual breakdown is triggered by a neural mechanism. The latter consists of a sudden curtailing or cessation of the outflow of impulses in the motor nerves which innervate the abdominal muscles. The muscles attain the capacity to respond to this signal during the final 3 days prior to the moth's ecdysis. By chronic electrical stimulation of the nerves, the breakdown of the muscles can be opposed or prevented.

### INTRODUCTION

THE breakdown of the intersegmental muscles of the silkmoth abdomen is compounded of a series of carefully timed phenomena. The first of these is endocrinological in character and consists of a reaction of the muscle to the hormonal conditions obtaining during the first few days of adult development, namely the presence of ecdysone and the absence of juvenile hormone.

Exposure to ecdysone in the absence of juvenile hormone is prerequisite for the breakdown despite the fact that the actual dissolution of the muscle does not begin until 3 weeks later, after the moth is fully formed. In this sense the endocrine reaction is a precocious potentiation of the future histolysis (LOCKSHIN and WILLIAMS, 1964, 1965).

Once the 'death clock' (SAUNDERS *et al.*, 1962) has been set by the ecdysone reaction, the endocrine system seems to play no further role. The evidence points to some further signalling system which triggers the initiation of cell death in the endocrinologically potentiated muscles. The object of the present study is to identify this further mechanism.

### MATERIALS AND METHODS

#### 1. *Insect material*

The experiments were performed on five species of saturniids: *Antheraea pernyi*, *A. polyphemus*, *Hyalophora cecropia*, *Philosamia cynthia*, and *Rothschildia*

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*orizaba*. *Polyphemus* and *cecropia* were reared as described previously (LOCKSHIN and WILLIAMS, 1964, 1965). The other species were purchased from dealers. All pupae were chilled for at least 10 weeks prior to use—*pernyi* at 2°C, *cecropia* and *polyphemus* at 6°C, and *cynthia* and *orizaba* at 8°C. For the purposes of the investigation, the pupae were returned to 25°C, and either operated upon immediately or allowed to proceed to a certain stage in adult development.

## 2. *Surgical procedures*

The animals were anaesthetized with CO<sub>2</sub>, and the surgical manoeuvres performed under the magnification of a dissecting microscope. In experiments requiring the prolonged survival of the preparation, crystals of phenylthiourea (PTU) and streptomycin sulphate were placed in the wound (WILLIAMS, 1959).

Larvae and adults were slit along the mid-dorsal line and pinned to plasticine under Ringer's solution (EPHRUSSI and BEADLE 1936). Pupae were dissected after dorsal incision or frontal plane sections. The latter passed slightly dorsal to the spiracles. The viscera were removed, and the animal was washed thoroughly with Ringer and fixed, while still pinned, in 70% ethanol. *Minuten Nadeln* insect pins, mounted in wooden applicator rods, were employed as dissecting needles. As the dissection proceeded, the tissues were stained by drop-wise application of Delafield's haematoxylin (BECKEL, 1958), followed 1–3 min later by a thorough rinse with ethanol. Drawings were made with the aid of a camera lucida.

## 3. *Direct recording of spontaneous and induced nerve activity*

The abdomen of a *cecropia* moth was opened along the mid-dorsal line and pinned to plasticine under Ringer. The viscera were removed, the preparation was rinsed, and sufficient fresh Ringer was added to cover the preparation. A dorsal nerve (described below) was lifted out of the Ringer at the site indicated by the arrow in Fig. 3, on two uninsulated 34 B and S gauge chlorided silver electrodes. This location was chosen to minimize contamination by nerve impulses not concerned with the intersegmental muscles. Recordings were made from the pairs of nerves in all four abdominal segments.

The entire preparation was surrounded by a Faraday cage. The signals were fed as a push-pull input to a Grass P-8 preamplifier and then into a Tektronix Type RM122 low-level preamplifier. They were finally analysed on a Tektronix Type 36-oscilloscope with a Heathkit Signal Tracer as an audio monitor. The oscilloscope images were photographed directly with an Exacta 66 camera equipped with extension tubes. Records of approximately forty animals were made in this manner. For other purposes the signals were fed into a Magnacorder tape recorder over a period of 2 min, with an interruption after 1 min to moisten the nerve with saline. Play-back and recording at 1 mm/sec were made into a Grass Model 5 polygraph equipped with an integrator preamplifier.

## 4. *Ganglionectomy*

The tip of the abdomen was excised from an anaesthetized pupa. By gentle pressure, the blood was expressed into a vial containing crystals of the

PTU-streptomycin mixture. The animal was then positioned head down in the operating funnel. Through the terminal opening the nerve cord was grasped with watchmaker's forceps and the lateral nerves in each segment were cut with micro-scissors. The nerve cord was then severed between the third thoracic and first abdominal ganglia and removed. The haemolymph was returned to the haemocoel, and sterile Ringer added to fill any remaining air spaces. Finally, a plastic window was placed over the wound and sealed in place with melted wax, as described by WILLIAMS (1959).

In an alternative procedure the blood was not drained. Incisions were made in the mid-ventral line of the fourth and sixth abdominal segments. The first two abdominal ganglia, lying in the second and third abdominal segments, were removed through the anterior incision; the remaining three ganglia through the posterior incision. Both methods gave approximately equal survival until adult ecdysis. The second procedure had the advantage of leaving the rectum intact, thereby permitting the moth to void its meconium—an event prerequisite for the survival of the moth after emergence.

#### 5. *Chronic electrical stimulation*

With a single transverse cut with scissors the abdomens of moths were isolated just prior to ecdysis. The electrodes were implanted either directly into the central nerve cord or wrapped around it. Alternatively, one or both electrodes were placed directly upon the dorsal muscles. PTU-streptomycin was added, and the electrodes were sealed to the cuticle with melted wax. The stimuli consisted of a 60-cycle mains current, reduced by a transformer to 6–10 V and interrupted by a motor-driven disk for 55 sec in each min. In subsequent experiments a four-triode circuit was used which delivered seven or eight spike pulses, at 1 msec intervals, every 18 sec. The preparations were maintained under stimulation for 4 days and then dissected.

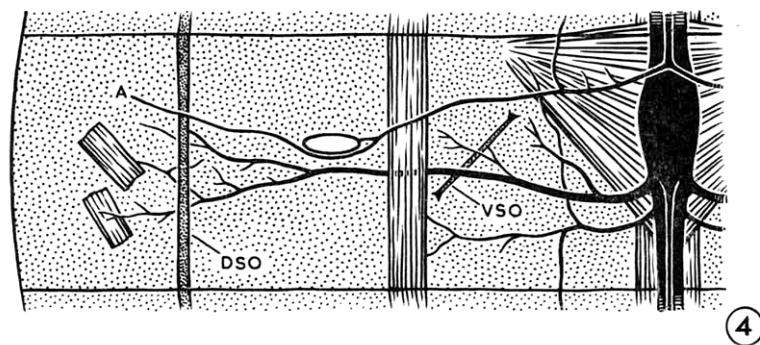
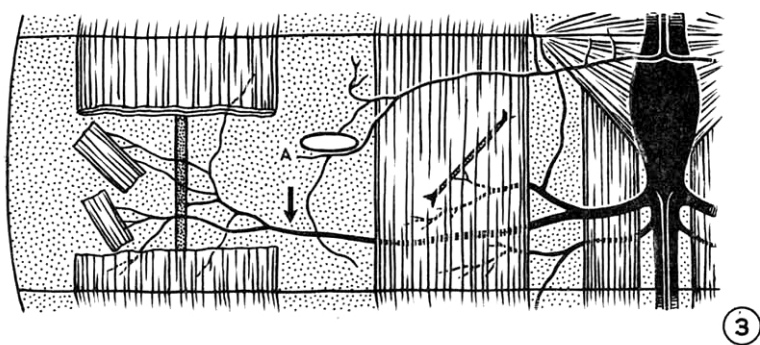
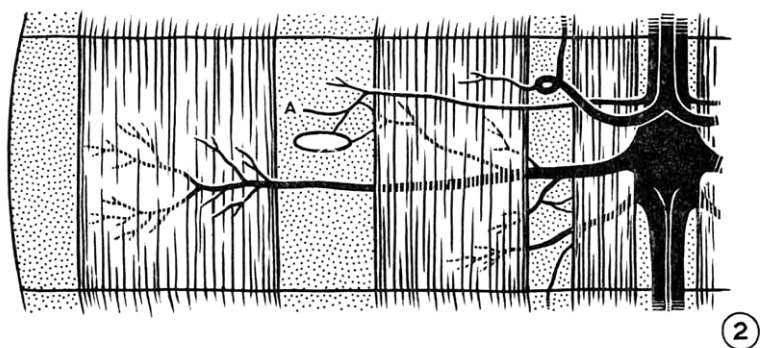
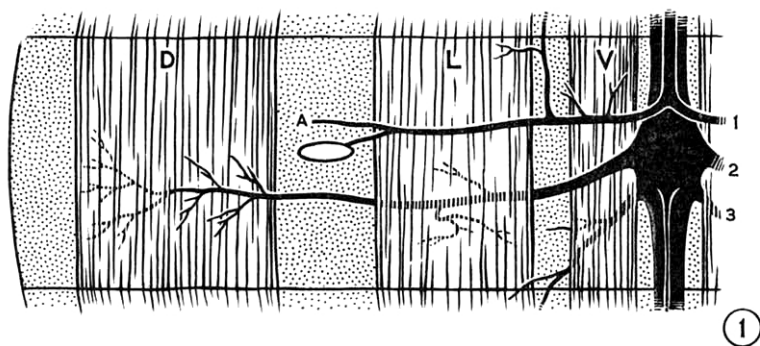
### RESULTS

#### 1. *Anatomy of the abdominal nervous system*

In the mature larva each of the first four abdominal ganglia gives rise to three major pairs of nerves. These are described according to the terminology of LIBBY (1959, 1961).

The anterior nerve, designated as (1) in Fig. 1, arises from the posterior portion of a ganglion and passes posteriorly along the nerve cord to the next ganglion, at which point it bifurcates. The branches pass laterally to the ventral portions of the body wall, the spiracles, and the dorsal heart. The paired dorsal nerves (2) innervate the lateral intersegmental muscles, the spiracles, the dorsal intersegmental, and other dorsal musculature. The ventral nerves (3) innervate the prolegs, the ventral intersegmental muscles, and other ventral musculature.

During the transformation of the larva into a pupa (Fig. 2) all three groups of intersegmental muscles degenerate in the more anterior and posterior segments but remain intact from the anterior margins of the third or fourth abdominal



segments to the posterior margins of the sixth segment. The dorsal nerves in the first three abdominal segments remain unchanged and eventually innervate newly formed muscles of the moth.

In the freshly emerged moth (Fig. 3) the central nervous system is heavily tracheated and furnished with newly formed alary muscles which pull it from side to side in an undulating motion. The innervation of the intersegmental muscles remains unchanged except that new nerves grow out from the main trunks to serve the newly formed adult muscles. These latter are seen clearly only after the intersegmental muscles have degenerated (Fig. 4).

Two days after ecdysis, the intersegmental muscles have totally disappeared. The nerves remain attached to a thin hyaline sheath which corresponds to the sarcolemma of the broken-down muscle fibres. Apparently intact neuromuscular junctions were observed when squashes of muscles from moths 24 hr after ecdysis were examined by phase microscopy (see LOCKSHIN and WILLIAMS, 1965). However, as we shall see, the nervous system undergoes substantial physiological alterations after the emergence of the moth.

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FIG. 1. Innervation of intersegmental muscle on the left side of the fourth abdominal segment of a fifth-instar *cecropia* larva. The insect has been opened along the mid-dorsal line and pinned with this line at the left-hand margin. The anterior end of the animal is at the top of the page, while the mid-ventral line lies beneath the nerve cord. Muscles other than the intersegmental muscles are not shown. The nerve marked 'A' innervates the alary muscles. The muscles shown are, from left to right: the dorsal (D), lateral (L), and ventral (V) intersegmental bands. The anterior, dorsal, and ventral nerves are indicated by the numerals 1, 2, and 3, respectively.

FIG. 2. Comparable dissection of a diapausing *pernyi* pupa. There are slight species differences, but the innervation is otherwise similar.

FIG. 3. Dissection of fourth abdominal segment of a freshly emerged *pernyi* moth. New structures seen in the abdomen are, from left to right: newly formed adult muscles, dorsal and ventral scolopophorous organs (stretch receptors), and ventral alary muscles attached to the ganglion. The nerve marked 'A' innervates the dorsal alary muscles which have been removed. The arrow indicates the point at which recordings of nervous activity were made.

FIG. 4. The same view of the fourth abdominal segment in a *pernyi* moth 4 days after its emergence from the cocoon. The intersegmental muscles have completely degenerated, leaving narrow bands of newly formed adult muscles, including narrow bands running in an antero-posterior direction just ventral to the spiracles and beside the nerve cord. These muscles are morphologically and cytologically distinguishable from the intersegmental muscles. Nerve A innervates the dorsal alary muscles. DSO: dorsal scolopophorous organ. VSO: ventral scolopophorous organ.

## 2. Spontaneous and induced electrical activity in the dorsal nerves

Records of spontaneous trains of impulses or of trains of impulses which occurred in response to mechanical stimulation indicated that the activity was predominantly motor in nature, and that in each nerve the signals of five or more

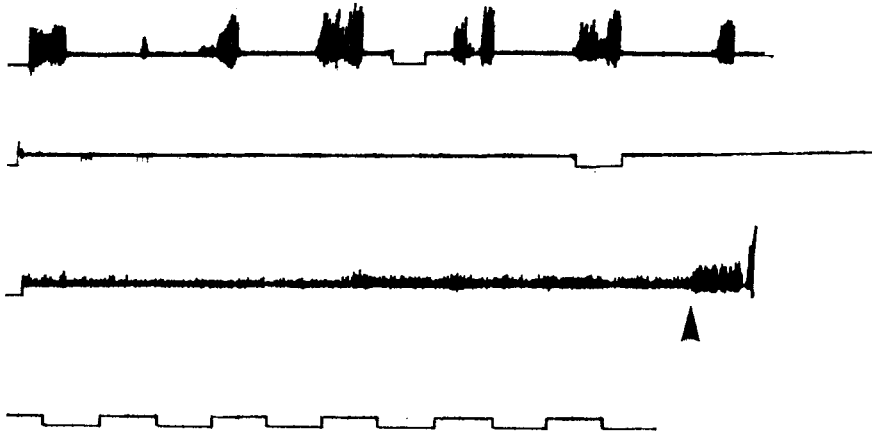


FIG. 5. Polygraph recordings from the dorsal nerves of the third abdominal ganglia of *cecropia* moths. The top trace, from a moth just prior to ecdysis, shows the typical bursts of activity recorded in preparations of this kind. The second trace is taken from a moth which had emerged  $3\frac{1}{2}$  hr prior to being dissected; very little activity is recorded. The third trace represents a nerve in a moth 2 days after ecdysis. Prodding of the ganglion at the point marked by an arrow elicited a short burst of motor activity. Time trace = 10 sec.

separate axons could be distinguished. The origins of the impulses were demonstrated by cutting the nerve either proximal or distal to the electrodes and by observing muscle contraction coincident with trains of impulses. The records also indicated that, even after the muscles had degenerated, large trains of motor impulses could be evoked by mechanical stimulation. In dissected preparations the activity of the stretch receptors was easily recognized and separated from other signals in terms of an extremely steady rate of firing, constant for a given degree of stretch.

Samples of the records of approximately 160 nerves analysed on the polygraph are given in Fig. 5. These results indicate that, although the motor fibres are capable of firing in response to a mechanical stimulus (*arrow*), they seldom fire spontaneously after the moth has spread its wings. The phenomenon was consistent in all specimens studied, except for some variability during the first few hours after the moth's ecdysis.

The cessation of motor nerve impulses occurs during the first 5 hours after ecdysis, i.e. at approximately the same time that the electron microscope reveals the first signs of cytolytic change in the muscles themselves (LOCKSHIN and WILLIAMS, 1965).

### 3. *The effect of ganglionectomy on the fate of the intersegmental muscles*

From one to five abdominal ganglia were removed from pupae and the latter then placed at 25°C and allowed to undergo adult development. The moths were sacrificed and dissected just after ecdysis, i.e. just after the notal ecdysial line had ruptured. As recorded in Table 1, sham-operated control individuals showed full and complete preservation of the intersegmental muscles at this stage. By contrast, ganglionectomy hastened the partial or complete breakdown of the denervated muscles in certain individuals of all five species which were studied. The species seem to differ among themselves in their reaction to denervation and, with the exception of *orizaba*, certain individuals failed to show precocious breakdown of the denervated muscles even in the absence of all five abdominal ganglia; this was particularly true of *polyphemus* (Table 1).

TABLE 1—EFFECTS OF PRIOR GANGLIONECTOMY ON THE RETENTION OF THE DENERVATED INTERSEGMENTAL MUSCLES IN FRESHLY EMERGED\* MOTHS

Number of abdominal ganglia excised	Species	Number of preparations	Percentage of animals with denervated muscles		
			Normal	Partially degenerated	Fully degenerate
None (controls)	<i>Cecropia</i>	15	100	0	0
	<i>Pernyi</i>	5	100	0	0
	<i>Polyphemus</i>	10	100	0	0
	<i>Orizaba</i>	2	100	0	0
1-4	<i>Cecropia</i>	4	25	75	0
	<i>Pernyi</i>	28	54	36	10
All five	<i>Cecropia</i>	22	14	9	77
	<i>Cynthia</i>	10	30	70	0
	<i>Polyphemus</i>	15	60	13	27
	<i>Orizaba</i>	2	0	0	100

\* Because of the immobility of their abdomens many moths could not actually escape from the cuticle. In these cases the preparations were considered to have 'emerged' when the old cuticle ruptured along the ecdysial lines.

Four *cecropia* pupae, from which all five abdominal ganglia had been removed, were dissected approximately 3 or 4 days prior to the completion of adult development. In all cases, intact though fibrillating denervated muscles were present. Evidently, complete denervation of *cecropia* muscles can accelerate breakdown by less than 3 or 4 days.

### 4. *The effects of chronic electrical stimulation*

The large current of the mechanically driven stimulator caused a high mortality (slightly over 50 per cent). Nevertheless, the results obtained on the surviving

preparations were similar to those obtained with the electronic stimulator. The data are combined in Table 2. It will be observed that about 50 per cent of the stimulated muscles failed to break down. In most of these cases one electrode had been wrapped around the central nerve cord slightly anterior to an abdominal ganglion, the other electrode being wrapped around the nerve cord posterior to the ganglion or implanted subcutaneously just exterior to the dorsal muscles of the segment in question. The preserved muscles were typically the dorsal and lateral muscles in the stimulated half of the segment, with occasional preservation of these bands one segment anterior or posterior to the stimulated region.

TABLE 2—EFFECT OF CHRONIC INTERMITTENT STIMULATION ON THE BREAKDOWN OF THE INTERSEGMENTAL MUSCLES

Experimental condition	Number of animals	Condition of muscles after 72-96 hr		
		Fully preserved	Partially preserved	Absent
Unstimulated muscles*	79	4 (5%)	1 (1%)	74 (94%)
Stimulated muscles†	50	22 (44%)	5 (10%)	23 (46%)

\* Combined results for unstimulated abdomens (twenty-nine preparations) and unstimulated regions of stimulated abdomens (fifty preparations).

† Electrodes were implanted into abdomens isolated from *cecropia*, *cynthia*, *orizaba*, *polyphemus*, or *pernyi* just prior to ecdysis. Electric stimuli were administered every 18 or 60 sec for 3 or 4 days.

In Table 2 attention is directed to the preservation of unstimulated muscles in about 6 per cent of the 'control' abdomens. We believe this was due to the desiccation and general poor condition of these particular abdomens—circumstances which sometimes prevent the normal breakdown of muscles in intact but feeble moths, such, for example, as develop from prolongedly chilled and desiccated pupae. In both experimental and control series moribund abdomens of this type were generally discarded; evidently, a few such preparations escaped detection.

##### 5. Influence of the higher nervous centres

Six groups of animals were subjected to several experimental regimens to test the influence of afferent and internuncial impulses. The following procedures failed to influence the timing of the muscle breakdown:

- Decapitation just prior to ecdysis. (Dissection 4 days later: five *cecropia*.)
- Decapitation 4 days prior to ecdysis. (Dissection 4 days later: two *cecropia*.)
- Transection of the ventral nerve cords of chilled pupae between the thoracic and abdominal ganglia. (Dissection just prior to ecdysis: ten *pernyi*.)
- Transection of the ventral nerve cords of chilled pupae between the thoracic and abdominal ganglia. (Dissection 4 days after ecdysis: five *pernyi*.)



- (e) Closure of the proctodeum of pharate adults with wax. (Dissection 4 days later.) This procedure prevented the expulsion of meconium and thereby maintained the abdomen in a distended condition. The rectal sac frequently ruptured and the animal died within 2 days. In the two *pernyi* moths which survived for 4 days, the intersegmental muscles had degenerated.
- (f) Removal of pharate adults from their pupal cuticles 2 or 3 days before ecdysis. This manoeuvre prevented the vigorous muscular activity associated with ecdysis and the unfurling of the wings. The animals were stored at 100 per cent humidity to prevent desiccation. Three *pernyi*, dissected on the day they would normally have emerged, contained intact, contractile intersegmental muscles; the muscles of three other *pernyi* dissected 4 days later were degenerate.

### DISCUSSION

The steps leading to the breakdown of the intersegmental muscles are, first, an endocrine potentiation occurring at the outset of adult development (LOCKSHIN and WILLIAMS, 1964) and, second, the formation and ultimate dissolution of lysosomes within the muscle itself (LOCKSHIN and WILLIAMS, 1965). The present study demonstrates the role of the central nervous system in timing the initiation of the breakdown.

Recordings of nervous activity after ecdysis demonstrate that the signal to the muscles consists of the cessation of efferent impulses to the muscles in question. Ordinarily, the impulses are curtailed within 1 hr after ecdysis, i.e. after the moth has emerged from the cocoon and expanded its wings. The electrical recordings document the striking change in the activity of the central nervous system at that time. Indeed, even under the stimulation presented by the dissection, the ganglia failed to generate motor impulses.

The curtailing of motor impulses evidently centres in the individual ganglia themselves. No interference with either the sensory input or the connexion of the ganglia to the higher nervous centres affected the eventual breakdown of the muscles. Furthermore, the ganglia could be provoked by direct prodding or electrical stimulation to fire motor impulses which were readily conducted by the nerve axons. Finally, we may note that the neuromuscular junctions remain unaltered until the dissolution of the muscles is far advanced (LOCKSHIN and WILLIAMS, 1965).

A second experimental approach indicated that, in certain species, such as *cecropia*, prior ganglionectomy can hasten the breakdown of the intersegmental muscles by 2 or 3 days. But, in other species, such as *cynthia* or *polyphemus*, the onset of histolysis was little affected. These observations recall earlier findings that, although innervation is required for the formation of insect muscles (WILLIAMS and SCHNEIDERMAN, 1952; NÜESCH, 1953, 1957), the viability and maintenance of the muscles is relatively independent of their innervation (FINLAYSON, 1956). During the last few days of adult development this autonomy is evidently lost, for the muscles then become sensitive to the withdrawal of motor innervation.

Thus, as we have seen, denervation can hasten the breakdown in certain species.

The intersegmental muscle acquires the capacity to break down only shortly before the signal is normally administered. In the case of *polyphemus* and *cynthia*, the muscle seems not to achieve this state until the day of ecdysis. The animal is thus equipped with a 'fail-safe' control which, while preparing the muscles for eventual dissolution, assures that they will not break down ahead of schedule. An analogous nerve-muscle relationship was demonstrated by FINLAYSON (1960) in pupating *cecropia* larvae.

The preservation of the muscles by direct stimulation is in many ways the most convincing of the experiments reported here. By this manoeuvre about half of the preparations were caused to retain muscles in one or more segments.

Thus, in summary, the evidence strongly argues that the final signal for the onset of histolysis consists of a curtailing of the outflow of motor impulses to the muscles in question. This conclusion will be further tested in the next paper.

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