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PROGRAMMED CELL DEATH—IV. THE INFLUENCE OF DRUGS ON THE BREAKDOWN OF THE INTERSEGMENTAL MUSCLES OF SILKMOTHS*

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Abstract—The normal breakdown of the intersegmental muscles of the silkmoth abdomen can be opposed or prevented by the injection of the parasympathomimetic drugs, pilocarpine or physostigmine. Final concentrations of 1 μM/g live weight are fully effective provided that the injection is made before the initiation of histolysis—a process which normally begins about 6 hr after the moth's ecdysis. The protective effects of pilocarpine or physostigmine are annulled by the simultaneous injection of atropine, by the excision of the central nervous system, or by the denervation of the abdominal muscles. By diverse experiments it was possible to show that pilocarpine or physostigmine protect the muscle by an indirect mechanism. By their excitatory effects on the abdominal ganglia, they sustain and augment the outflow of motor-nerve impulses to the muscles in question. Under this circumstance, the muscles remain intact and contractile—a finding which further demonstrates that the normal signal for the initiation of histolysis is the cessation of motor-nerve impulses to the abdominal muscles.

INTRODUCTION

IN THE previous papers of this series we have demonstrated that, during the first few days of adult development, the endocrine system of silkmoths provokes in the intersegmental muscles a series of events which leads to their ultimate destruction (LOCKSHIN and WILLIAMS, 1964). Three weeks later, after the moth has emerged from its cocoon and expanded its wings, efferent nerve impulses to these muscles cease (LOCKSHIN and WILLIAMS, 1965b) and cytolysis immediately begins (LOCKSHIN and WILLIAMS, 1965a).

We have previously demonstrated that chronic electrical stimulation can block the breakdown of the muscles. Competence to respond to the cessation of nervous impulses is evidently acquired during the final few days of adult development, for denervation is inconsequential until a few days prior to adult ecdysis.

In the present study the neural control of muscle breakdown is examined in further detail by pharmacological means.

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MATERIALS AND METHODS

Pilocarpine hydrochloride and physostigmine sulphate were dissolved in sterile Ringer's solution (EPHRUSSI and BEADLE, 1936) at concentrations of 0·1 or 0·2 M and brought to approximately pH 6·5 by the addition of 0·1 N NaOH. Other water-soluble drugs were prepared in Ringer at 0·1 or 0·2 M, or to saturation. Caffeine was dissolved at 0·2 M in chloroform; this solution was suspended in two volumes of Ringer, and the suspension injected. DDT and parathion were dissolved in absolute ethanol in concentrations up to 10^{-1} M. These were mixed with 10 vol of Ringer immediately before injection. The solutions were stored for up to 2 months at – 20°C.

Animals of the following species were employed: *Antheraea pernyi*, *A. polyphemus*, *Hyalophora cecropia*, *Philosamia cynthia*, and *Rothschildia orizaba*.

Previously chilled pupae were placed at 25°C and allowed to develop to within 1–4 days of adult ecdysis. Each individual was then weighed, anaesthetized with carbon dioxide, and injected with a particular drug by means of a 27-gauge needle inserted through the mesothoracic tergum. The puncture was sealed with melted wax. The animals were then stored at 25°C in individual cardboard containers.

Dissections were performed either immediately after the moths emerged or 4 days after ecdysis. After the injection of physostigmine, recordings of nerve activity were made as described previously (LOCKSHIN and WILLIAMS, 1965b); the animals were injected a few hours before ecdysis with 3 μl of 0·1 M physostigmine and were maintained for 2 days at 25°C before being sacrificed.

In some instances the experiments were performed on isolated adult abdomens. To this end, male *pernyi* moths which had resorbed their moulting fluid were anaesthetized. The pupal cuticle was removed, and each individual was positioned posterior end down in a plasticine mould under continuous anaesthesia. With a single transverse scissor cut, the abdomen was severed from the thorax. The chain of abdominal ganglia was then cut free and removed through the opening. 0·3 μl of physostigmine in Ringer was introduced along with crystals of streptomycin and PTU. The wound was finally sealed with melted wax, and the abdomen returned to air at 25°C.

A less extensive series of experiments was performed on *cecropia* prepupae and on *cecropia* pupae less than 1 hr after ecdysis. The animals were anaesthetized, weighed, and injected with 10 μl of 0·2 M pilocarpine hydrochloride or with 2 μl of 0·1 M physostigmine sulphate in insect Ringer. Dissections were performed 7 days after pupal ecdysis.

RESULTS

Effects of autonomic drugs on muscle breakdown

1. *Ringer's solution.* As noted in Table 1, the injection of 0·05–0·3 ml of EPHRUSSI and BEADLE (1936) Ringer into pharate adults had no effect on the breakdown of the intersegmental muscles. All moths showed complete dissolution of the muscles when dissected 4 days after ecdysis.

2. *Pilocarpine hydrochloride.* Approximately one-third of the moths died within 4 days after injections of low doses of pilocarpine (Table 1). All species showed large variability among individuals, but among the various species the drug was most toxic to *pernyi.*

TABLE 1—EFFECTS OF AUTONOMIC DRUGS* ON THE BREAKDOWN OF INTERSEGMENTAL MUSCLES

Concentration μ M/g live wt.	Number of animals	Died (%)	Percentage of moths showing: Complete retention	Partial retention	No retention
Ringer's					
0·1–0·3 ml	48	0	0	0	100
Pilocarpine					
0·1–0·4 μM/g	12	33	33	17	17
0·5–0·9	16	38	31	31	0
1·0–1·4	52	29	63	4	4
1·5–4·0	37	78	19	3	0
Physostigmine					
0·1–0·5	12	8	17	17	58
1·0	12	42	58	0	0
1·5–2·0	15	27	73	0	0
Nicotine (*pernyi*)					
0·3–0·5	20	5	80	5	10
1·0–1·5	5	60	40	0	0
Nicotine (*cynthia*)					
0·25–1·0	5	0	0	0	100
1·0–1·5	7	100	0	0	0
Pilocarpine					
1–3·2	9	44	56	0	0
Pilocarpine 4 μM/g plus atropine, 4–8 μM/g	6	0	0	0	100

* Drugs were injected into the mesothoracic terga of pharate adults of *cecropia, cynthia,* or *pernyi* (1–2 days prior to ecdysis). Dissections were performed 4 days after ecdysis.

Among the vast majority of the surviving moths, the injection of pilocarpine blocked the breakdown of the intersegmental muscles for at least 4 days after ecdysis. The muscles were preserved in 89 per cent of the moths which had survived an injection of 1–4 μM of pilocarpine per g live weight. One *cecropia* moth survived for 8 days after injection; this individual retained all its intersegmental muscles.

3. *Physostigmine sulphate.* Physostigmine, at concentrations of 1–2 μM/g live weight, was uniformly effective in blocking the muscle breakdown. It was less toxic than pilocarpine for all species including *pernyi.* The relevant data are listed in Table 1.

The behavioural manifestations elicited by physostigmine were similar to those produced by pilocarpine. Both these drugs, at low doses, caused hypersensitivity to mechanical stimulation. Minimal disturbances provoked prolonged periods of

activity—wing fluttering, walking, or attempted flying. Higher concentrations of the drugs, in the range of 0·5–1 μM/g, produced continuous hyperactivity. Walking and flying motions were generally uncoordinated. Meconium was voided and fluid was ejected from the mouthparts. Males displayed copulatory behaviour, and females oviposited immediately but ordinarily failed to attach their eggs to the substratum. Still higher concentrations of pilocarpine or physostigmine caused first a spastic, and ultimately a flaccid paralysis.

4. *Nicotine.* As recorded in Table 1, nicotine was generally effective in blocking the breakdown of the muscles of *pernyi*; but, in *cynthia*, sublethal doses were ineffective. After the injection of nicotine the moths folded their legs against the bodies and continually 'shivered' their wings and bodies in a barely perceptible tremor.

5. *Other drugs.* A number of other drugs were tested and found ineffective in blocking muscle breakdown when injected into pharate moths. Neostigmine bromide, in doses greater than 0·2 μM/g, caused an initial hyperactivity which, over a period of several hours, progresses to flaccid paralysis and death. At lower doses no significant effects were observed on behaviour or muscle preservation. DDT and parathion produced behaviour similar to that of neostigmine; they were likewise ineffective at the highest doses permitting prolonged survival (0·01 μM/g). The following drugs caused flaccid paralysis but did not hasten or retard the normal muscle breakdown: caffeine (*pernyi*: 0·005 μM/g; *polyphemus* and *cecropia*: 0·015 μM/g), nicotinic acid diethylamide (2 μM/g), barbital (15 μM/g), and M-amino benzoic acid ethyl ester methane sulphonate (10 μM/g). The following agents were without detectable effects on either motor activity or muscle dissolution: D-tubocurarine (0·25 μM/g), magnesium chloride (12–24 μM/g), mescaline (1 μM/g), serotonin (0·1 μM/g) and reserpine (0·5 μM/g). Presumably these agents were either inert or failed to gain access to the nervous system.

6. *Antagonism of pilocarpine by atropine.* When injected into pharate pupae in doses of 1–4 μM/g, atropine caused a flaccid paralysis which persisted for a period dictated by the dose. After injection of the highest non-lethal concentration (4 μM/g), the paralysis persisted for 18–24 hr and the muscle broke down in the usual manner.

When injected with equimolar concentrations of pilocarpine, atropine antagonized all the behavioural and physiological effects of pilocarpine. One or 2 days after the simultaneous injection of both drugs into pharate *cecropia*, the moths became hypersensitive and somewhat hyperactive. Gradually the hyperactivity and lack of co-ordination increased until the moths were fully paralysed. This reappearance of the pilocarpine effect apparently was due to the breakdown of atropine and the persistence of pilocarpine.

If the moth remained under the influence of atropine for at least 1 day after ecdysis, the effect of pilocarpine was nullified and the muscles broke down on schedule.

7. *Delayed injection of pilocarpine.* In this series of experiments, pilocarpine was injected into pharate moths and at various times after ecdysis; the individuals were dissected 4 days later.

When pilocarpine was injected into pharate adults, 85 per cent of the individuals retained intersegmental muscles (Table 2). When the injection of the drug was postponed until the first 6 hr after ecdysis, fewer than 40 per cent of the moths retained fully intact intersegmental muscles when examined 4 days later; 37 per cent retained no intersegmental muscles whatever. This 6 hr period could not be further subdivided. Some moths injected immediately after ecdysis and dissected 4 days later retained no muscles. Others, injected 6 hr after ecdysis, retained a full set of contractile muscles. Injection of pilocarpine after the sixth hour failed in all cases to prevent muscle degeneration.

TABLE 2—EFFECTS OF PILOCARPINE ON THE DEGENERATION OF INTERSEGMENTAL MUSCLES

Stage at injection*	Number of surviving preparations	Percentage of moths showing: Complete retention	Partial retention	No retention
Pharate adult	75	85	13	1
0–6 hr after ecdysis	41	37	27	37
6–12 hr after ecdysis	10	0	0	100

* *Cecropia, cynthia, crizaba,* or *pernyi* were injected with 1–2 μM pilocarpine per g live weight at the time indicated, and were dissected 4 days after ecdysis.

TABLE 3—THE ROLE OF THE CENTRAL NERVOUS SYSTEM IN THE PROTECTION OF THE INTERSEGMENTAL MUSCLES BY PHYSOSTIGMINE*

Experimental condition CNS	Physostigmine	Number of surviving preparations	Percentage of moths showing: Complete retention	Partial retention	No retention
Present	0	31	0	0	100
Absent	0	4	0	0	100
Present	0·3 μM/g	7	71	0	29
Excised and reimplanted	0·3 μM/g	8	0	0	100
Absent	0·3 μM/g	10	0	0	100

* All operative procedures and injections were carried out on the isolated abdomens of adults just prior to ecdysis. The moths were dissected 4 days later. For further description see Methods.

8. *Effects of denervation.* As described previously (LOCKSHIN and WILLIAMS, 1965b) transection of the nerve cord was without any detectable effect on the breakdown of the intersegmental muscles. Likewise, as recorded in Table 3, the isolation of the abdomen failed to influence the normal breakdown or the preservation of the muscles by physostigmine.

There is convincing evidence that physostigmine acts by stimulating the central nervous system and sustaining the outflow of nerve impulses to the intersegmental muscles via the motor nerves. Thus, as summarized in Table 3, physostigmine was completely ineffective when injected into abdomens from which the central nervous system had been removed. Moreover, its effectiveness could not be re-established by reimplanting 'loose' nervous systems into denervated abdomens.

9. *Effects of drugs on the intersegmental muscles at pupation.* In the mature larva the intersegmental muscles extend throughout the abdominal and thoracic segments. The anterior and posterior extensions of the muscles degenerate immediately after the pupal ecdysis, leaving intersegmental musculature extending from the anterior end of the third or fourth abdominal segment to the posterior end of the sixth.

Cecropia prepupae were injected a few hours before ecdysis and dissected 7 days later. One prepupa, injected with 0·1 μM/g of pilocarpine, retained muscles from the first abdominal segment through the seventh; another, from the second through the eighth. Prepupae injected with 0·1 μM/g physostigmine retained muscles as far anterior as the mesothorax and as far posterior as the ninth abdominal segment. Moreover, several bands of transverse muscles which are ordinarily lost were retained. Evidently, the breakdown of the anterior and posterior extensions of the intersegmental muscles at pupation, like the breakdown of their abdominal homologues after adult ecdysis, can be prevented by the injection of these parasympathominetic drugs.

10. *Electrophysiological observations.* The effects of pilocarpine and physostigmine on nervous activity were studied as previously described (LOCKSHIN and WILLIAMS, 1965b). Each of ten moths received 0·3 μl of 0·1 M physostigmine just prior to ecdysis. Two days later, the individuals were dissected and their nervous activity recorded. Alternatively, freshly emerged moths were dissected under Ringer; a dorsal nerve was picked up on chlorided silver electrodes and a solution of physostigmine was applied locally to various regions.

The recordings showed that when a moth is injected with physostigmine prior to ecdysis, the central nervous system continues to generate efferent impulses for at least 2 days after ecdysis. The recorded activity contrasts sharply with the normal behaviour of the central nervous system in which efferent activity ceases during the first few hours after ecdysis (LOCKSHIN and WILLIAMS, 1965b).

Physostigmine applied directly to the central nerve cord elicits an immediate increase in motor-nerve impulses. But, when the drug was placed directly on the muscle, no response was detected in either nerve or muscle. Excessive amounts of physostigmine (approximately 0·2 μM/ml of Ringer's solution) silence the ganglia within seconds. These results further document the conclusion that physostigmine acts indirectly on the muscles by way of the central nervous system. Silencing of the ganglia by excessive amounts of physostigmine represents a form of pharmacological denervation. This fact was presumably responsible for the failure of the drugs to prevent breakdown of the muscles in 10 per cent of the experimental animals.

DISCUSSION

Previous electrophysiological studies have demonstrated a curtailing or cessation of motor-nerve impulses just before the initiation of muscle degeneration (LOCKSHIN and WILLIAMS, 1965b). Chronic electrical stimulation of the nerves or the ganglia was effective in opposing or preventing the breakdown. Evidently, the histolysis of the muscle is somehow opposed by motor-nerve impulses. Shortly after ecdysis, when these impulses are shut off, the muscles begin to break down.

The present study shows that the preservation of the muscles may also be achieved by pharmacological means. By stimulating the central nervous system, physostigmine and pilocarpine prevent the cessation of motor-nerve impulses. This was demonstrated by electrical recordings from the central nervous system and from the motor nerves in freshly emerged moths.

In order for the drugs to be effective in blocking the breakdown, the central nervous system must be present and the motor nerves intact. If the nerves are severed or the ganglia removed, the muscles degenerate at the normal time even in the presence of the drugs. Furthermore, the stimulating drug must be administered not later than the first few hours after adult ecdysis. These findings show that the drugs act *via* the central nervous system and the motor nerves which innervate the muscles. In the freshly emerged moth, even a brief interruption of the motor outflow can activate the agencies of breakdown.

The cessation of motor-nerve impulses is apparently the ultimate signal which initiates the histolytic reactions in the intersegmental muscles of these moths. The breakdown, itself, is the final episode in a programmed sequence of happenings, among which we have noted: first, an endocrine potentiation during the first few days of adult development; then, within the muscle itself, the synthesis and sequestering of hydrolytic enzymes; and, finally, the triggering of this latent mechanism by the cessation of motor-nerve impulses to the muscles in question.

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. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. *J. Ins. Physiol.* **10**, 643–649.

LOCKSHIN R. A. and WILLIAMS C. M. (1965a) Programmed cell death. I. Cytology of degeneration in the intersegmental muscles of the *pernyi* silkmoth. *J. Ins. Physiol.* **11**, 123–133.

LOCKSHIN R. A. and WILLIAMS C. M. (1965b) Programmed cell death. III. Neural control of the breakdown of the intersegmental muscles of silkmoths. *J. Ins. Physiol.* In press.