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PROGRAMMED CELL DEATH—V. CYTOLYTIC ENZYMES IN RELATION TO THE BREAKDOWN OF THE INTERSEGMENTAL MUSCLES OF SILKMOTHS*

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Abstract—During the 3 week period of adult development cytolytic enzymes are synthesized and sequestered in lysosome-like particles in the intersegmental muscles of the silkmoth abdomen. A cathepsin begins to increase in titre during the first few days of adult development and continues to increase slowly during the first 2 weeks of this period. Then, during the final week of adult development, it increases at a logarithmic rate of about 30 per cent per day. In centrifuged homogenates prepared in sucrose, most of the enzymatic activity accompanies the mitochondrial 'fraction', from which a substantial proportion can be released by osmotic shock or by detergents. In sucrose homogenates of degenerating muscle, a lesser fraction of the enzyme is sedimentable, and a greater percentage of the total enzymatic activity is 'free' prior to the addition of detergent. An acid phosphatase follows a similar pattern but is more readily solubilized by the homogenization procedure. These enzymes are considered to be directly responsible for the destruction of the intersegmental muscles.

INTRODUCTION

In freshly emerged moths the intersegmental muscles of the abdomen are intact, contractile, and seemingly unchanged from their condition in the pupa (Lockshin and Williams, 1965a). One could easily get the impression that during the 3 week period of adult development these muscles have been unaffected by the hormones which have provoked conspicuous changes in most other tissues.

We have previously demonstrated that such is not the case (Lockshin and Williams, 1964). The endocrine situation at the outset of adult development (ecdysone acting in the absence of juvenile hormone) promotes a biochemical metamorphosis of the intersegmental muscles which culminates in their swift and complete cytolysis within 48 hr after adult ecdysis. The present investigation has inquired into the nature of this hormonally induced, neurologically activated, cytolytic mechanism which is built up in latent form and then released a few hours after adult ecdysis.

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MATERIALS AND METHODS

1. *Experimental material*

Developing pupae and moths of *Antheraea pernyi* and *Hyalophora cecropia* were maintained at 25°C until they attained the desired stage of development. They were then placed at 2°C for approximately 0·5 hr prior to dissection. The latter was carried out under cold 0·44 M sucrose which had been brought to neutrality by the addition of a few drops of 0·1 M pH 7·0 phosphate buffer.

2. *Differential centrifugation*

Dissections were performed as previously described (LOCKSHIN and WILLIAMS, 1965a) and preparative procedures were carried out at 0–2°C. The intersegmental muscles from one or more animals at the same developmental stage were ground in cold 0·44 M sucrose in a motor-driven Teflon-glass homogenizer and filtered through organdy muslin. The fibrous material remaining on the filter was collected, rehomogenized, and refiltered. The filtrates were combined, and sucrose was added to yield a final volume of 0·5 ml filtered homogenate per animal. The preparation was separated into two aliquots which were centrifuged for 15 min at 20,000 × g in an International PR-2 centrifuge. This preliminary centrifugation was termed 'I', as indicated in the generalized flow chart illustrated in Fig. 1. The supernatants (S_1) were sucked off and stored at 0°C.

The pellets P_1, as shown in Fig. 1, were resuspended either in 0·44 M sucrose or in a lysing solution consisting of distilled water or a 0·44 M sucrose solution containing 0·1% (w/v) Triton X-100 or Na desoxycholate. These suspensions were allowed to stand for 15 min at 0–2°C. In many experiments they were then subjected to differential centrifugation at 2°C as outlined under II in Fig. 1.

The first differential centrifugation was at 600 g for 5 min (II_a). The pellet was then resuspended in 0·5 ml of the same medium as before and the centrifugation repeated (II_b). In certain experiments, the operation was repeated until the resulting supernatant was clear ($II_{c,d,e}$). The nuclear pellet (P_2) resulting from these centrifugations was stored at 0–2°C. The supernatants (S_2) were combined and centrifuged at 15,000 g for 15 min (centrifugation III) to yield the 'mitochondrial' fraction P_3. The supernatants (S_3) were decanted and diluted to 1 or 1·5 ml with buffer. Meanwhile, the P_2 and P_3 pellets were each suspended in 0·5 ml of distilled water or 0·1 M buffer at the desired pH.

3. *Cathepsin assay*

Assays for cathepsin were performed in 0·1 M universal 'ABC' buffer (BERTHET and DE DUVE, 1951; GIANETTO and DE DUVE, 1955) or in 0·1 M phosphate-citrate buffer adjusted to pH 3·9. Occasionally 0·1 M acetate buffer was employed. The substrate was bovine haemoglobin or serum albumin, denatured and cleared of peptides by repeated precipitation with 5% trichloroacetic acid (TCA). The

protein was washed and dissolved in distilled water or buffer as a 2% (w/v) solution; merthiolate was added as a preservative in a final concentration of 0·0001%.

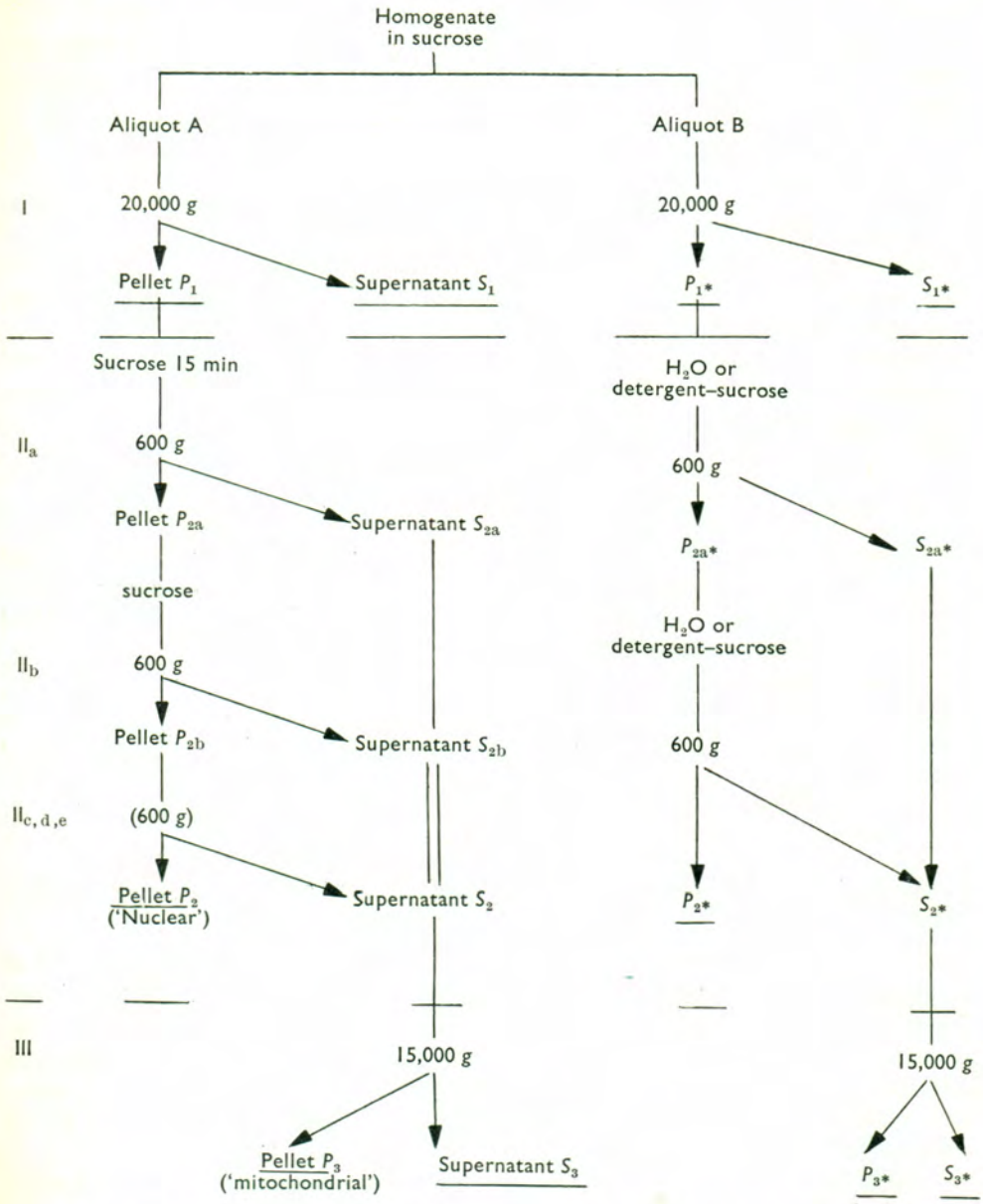

FIG. 1. Generalized schedule of centrifugation. In some experiments only centrifugation I was carried out. In certain other experiments, washes $II_{c,d,e}$ were omitted. Underlined fractions were analysed for enzymatic activity.

One-tenth ml of the suspensions (P_1, P_2, or P_3) and the two supernatants (S_1 or S_3) were placed in separate tubes containing 0·5 ml of the protein substrate solution. Tenth molar acetate, phosphate-citrate, or ABC buffer was then added to a final volume of 1 ml. After incubation at 25°C for 0·5 or 1 hr, 1 ml of 10% TCA was added to stop the reaction and precipitate the protein. The precipitate was removed by centrifugation (10 min at 800 *b*), followed by suction of the supernatant through a coarse sintered funnel.

TCA-soluble tyrosine, a measure of the amount of protein digested, was determined by the use of a Lowry reagent (LOWRY *et al.*, 1951) modified by the use of his reagent D (2% Na_2CO_3) in place of reagent A (2% Na_2CO_3 in 0·1 M NaOH). The modification yields a slower-forming but more stable colour. Colour formation is complete 40 min after the addition of reagent 'B'. It attains 95 per cent of maximum within 15 min and does not decrease below this level for at least 3 hr. Readings were taken between 30 and 40 min after the addition of reagent 'B'.

In most cases catheptic activity was also assayed by the digestion of exposed Kodak Panatomic-X 120 roll film (GORDON *et al.*, 1952; ADAMS and TUQUAN, 1961). Adox KB-14 was equally satisfactory, but other types of films digested far more slowly.

4. *Acid phosphatase assay*

Phosphatases were assayed in ABC buffer or in an 0·1 M acetate buffer adjusted to pH 4·5. The substrate, 0·1 M Na-β-glycerophosphate, was prepared daily in distilled water. The inorganic phosphate liberated by the enzyme was measured by the FISKE–SUBBAROW (1925) technique.

All enzymatic activities were calculated in terms of an 'activity ratio' (WEBER, 1963), i.e. μmoles of phosphate or tyrosine liberated per μmole of protein tyrosine in the homogenate. The latter was determined by means of the modified Lowry technique.

RESULTS

1. *Cathepsin activity in sucrose homogenates of the intersegmental muscles of pharate adults*

This series of experiments was performed on the intersegmental muscles of pharate moths showing generalized wing pigmentation (1–2 days prior to ecdysis). The muscles were homogenized in sucrose and centrifuged at 20,000 *g*. The resulting supernatant (S_1) and pellet (P_1) were analysed directly for catheptic activity, the pellet being resuspended in sucrose or in buffer. Various substrates and buffers were employed to identify some of the salient characteristics of the enzyme. All experiments were confirmed by the film-digestion method.

These studies revealed the presence in the intersegmental muscles of an enzyme capable of digesting denatured haemoglobin, bovine serum albumin, and gelatin (Table 1). The pH optimum was 3·9–4·0 (Fig. 2). As shown in Table 1, the enzyme is inhibited by silver ions in excess of 10^{-4} M and by heat in excess of 50°C.

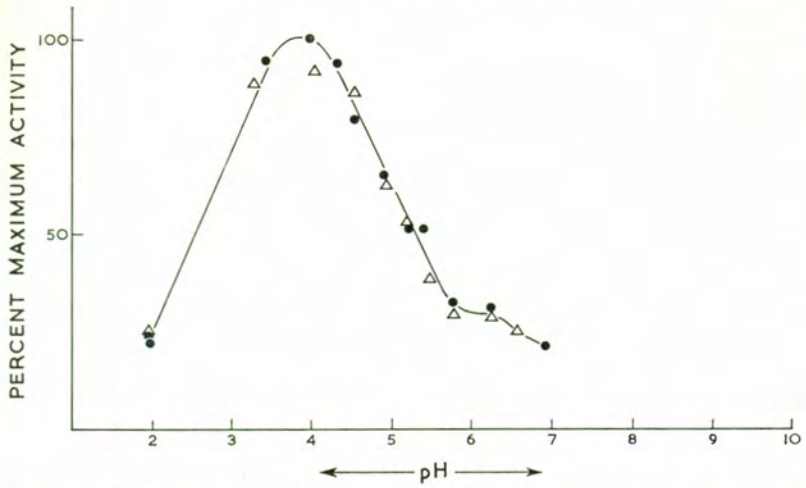

FIG. 2. The pH optimum for the digestion of denatured bovine haemoglobin by sucrose homogenates of intersegmental muscles during 0·5 hr incubation at 25°C. Two series of experiments are recorded.

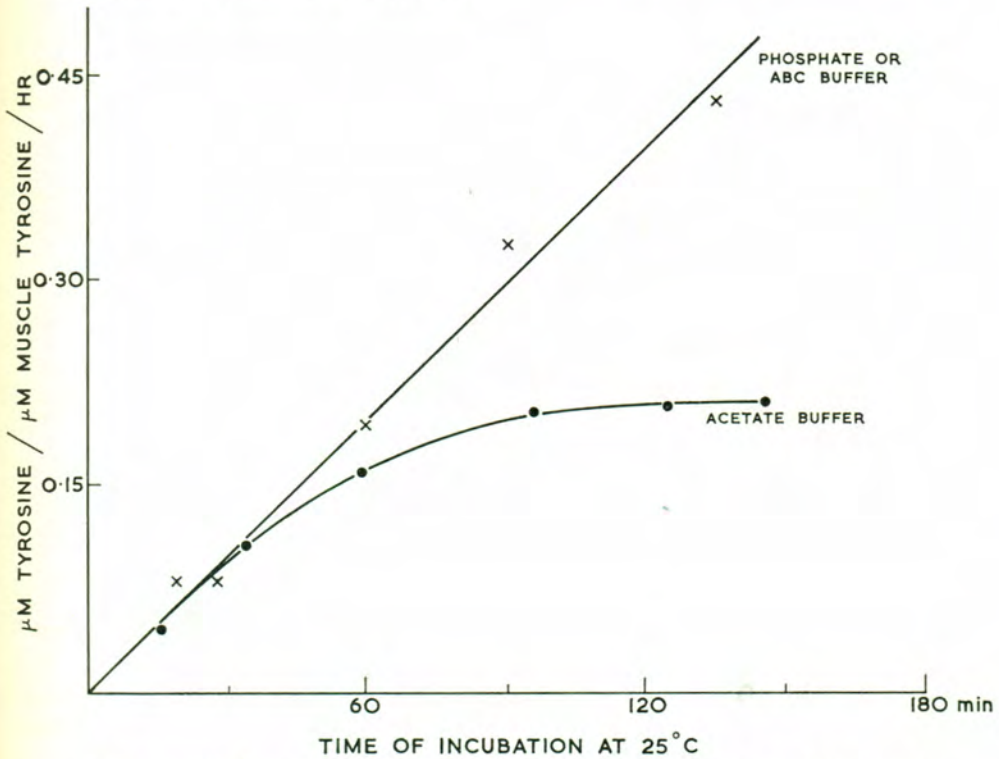

FIG. 3. Proteolysis as a function of time of incubation at pH 3·9 and 25°C. Homogenates of muscles from pharate adults were prepared in 0·44 M sucrose. The total preparations were centrifuged at 20,000 *g* and the pellets resuspended in 0·1 M buffers as described in Methods (section 3).

Enzymatic activity also rapidly declined when the preparation was incubated at 25°C in 0·1 M acetate buffer, but remained constant for at least 1 hr in the presence of phosphate-citrate or 'universal' buffers at pH 3·9 (Fig. 3). Enzymatic activity increased linearly with increasing concentration of homogenate, and no inhibition was noted at the 5 per cent level—the highest concentration tested.

TABLE 1—PROTEOLYTIC ACTIVITY OF SUCROSE HOMOGENATES OF THE INTERSEGMENTAL MUSCLES OF PHARATE ADULTS

Substrate	Activity ratio (μM tyrosine solubilized/μM muscle tyrosine/hour at 25°C)
None (self-digestion of undiluted homogenate at pH 3·9)	0·11
Bovine haemoglobin (1%)	0·17
Serum albumin (1%)	0·14
Gelatin (photographic film)	equivalent to 2·5 μg/ml trypsin at pH 7.0
Haemoglobin + 10^{-4} M $AgNO_3$ at 25°C	$<0·007$
Haemoglobin at 60°C	$<0·007$

In all cases homogenates were prepared in 0·44 M sucrose to which 0·1% Triton X-100 had been added. Incubations were carried out at pH 3·9 and 25°C except where noted otherwise.

TABLE 2—SEDIMENTABILITY OF CATHEPTIC AND PHOSPHATIDIC ACTIVITY IN SUCROSE HOMOGENATES OF MUSCLES OF PHARATE ADULTS

Centrifugation schedule*	% of activity sedimenting at			Final
	20,000 *g*	600 *g*	15,000 *g*	
Cathepsin				
I	97	—	—	3
I at 25°C	65	—	—	35
I, $II_{a,b}$, III	—	68	26	6
I, $II_{a,b,c,d,e}$, III	—	32	50	18
Acid phosphatase				
I	50	—	—	50
I, after pre-incubation in Triton X-100†	30	—	—	70
I, $II_{a,b}$, III	—	7	23	70

* The roman numerals are centrifugations indicated in Fig. 1. All centrifugations were performed at 0°C except where otherwise noted.

† The addition of Triton X-100 resulted in a net loss of 10–20 per cent of acid phosphatase activity.

2. *Sedimentability of catheptic activity in homogenates of intersegmental muscles of pharate adults*

Homogenates prepared in 0·44 M sucrose were subjected to differential centrifugation as outlined in Fig. 1, 'aliquot A'. The results, summarized in Table 2, show that 97 per cent of the enzymatic activity was sedimentable when maintained at 0°C. When the preparation was exposed to 25°C, the activity was partially released to the supernatant.

The enzyme sediments predominantly in the 600 *g* ('nuclear') fraction. When this fraction was repeatedly resuspended and recentrifuged, the majority of the enzyme accompanied the 15,000 *g* ('mitochondrial') fraction, while 18 per cent became unsedimentable at this speed.

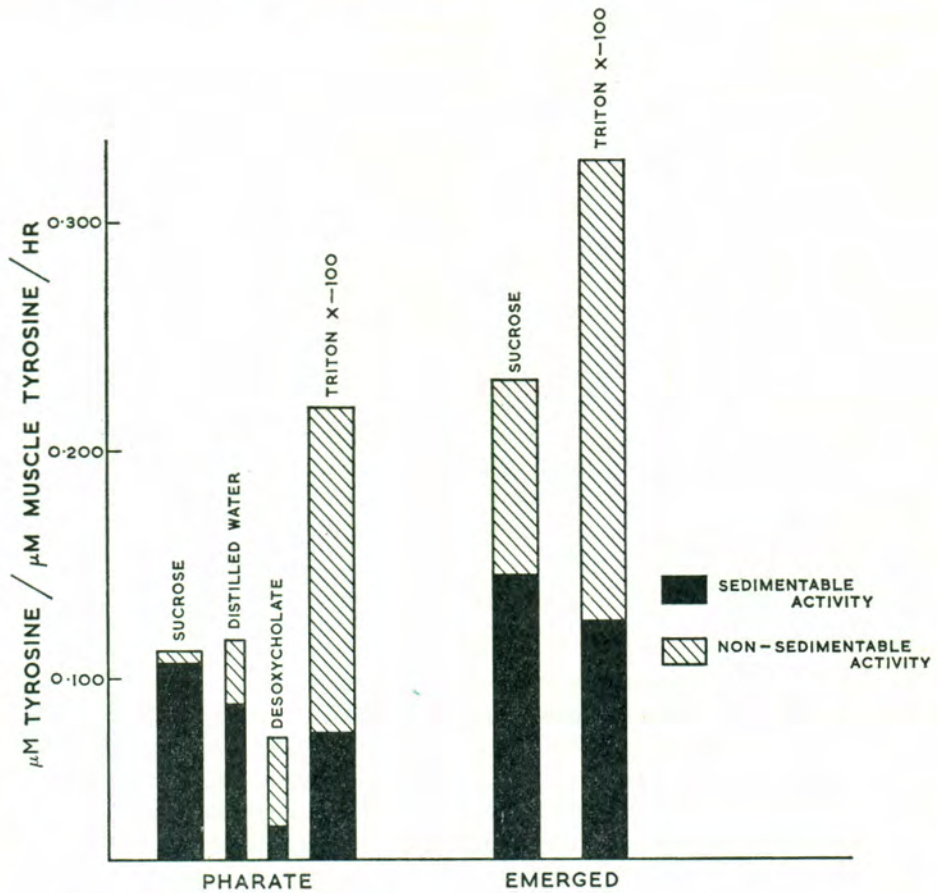

FIG. 4. Sedimentable and non-sedimentable catheptic activity in homogenates of intersegmental muscles before and after ecdysis. The activity is recorded in terms of an 'activity ratio,' or μM tyrosine solubilized per μM muscle tyrosine per hour. Emerged animals were assayed 5–10 hr after ecdysis.

3. *Sedimentability of cathepsin in homogenates prepared from moths before and after ecdysis*

Sucrose homogenates were prepared from muscles of pharate and freshly emerged moths, respectively. Each homogenate was divided into equal aliquots, and centrifuged at 20,000 *g* (centrifugation I in Fig. 1). One pellet was suspended in 0·44 M sucrose; the other in distilled water or sucrose solution containing Triton X-100 or Na desoxycholate. After 15 min at 0–2°C the preparations were recentrifuged at 20,000 *g*.

The supernatants and pellets were separated and, in order to unmask any latent activity in the pellet fractions, detergent was added to those fractions lacking it. When distilled water was used as a lysing agent, sucrose was added to the fractions prior to incubation. Thus, the centrifugations were carried out under different conditions but incubation of the enzymes was at all times carried out in the presence of detergent and sucrose. The data therefore indicate the sedimentability of total enzyme activity after exposure to lytic agents (distilled water or detergent).

The results, summarized in Fig. 4, show that in sucrose homogenates of the muscles of the pharate moth, nearly all (94 per cent) of the catheptic activity is sedimentable and that this fraction decreases to 63 per cent after adult ecdysis. Moreover, we note in Fig. 4 that a substantial proportion of the catheptic activity is released to the 20,000 *g* supernatant, with an increase in total activity, after exposure to lytic agents. Thus, after the addition of Triton X-100, the proportion of solubilized cathepsin increased twelvefold (to 75 per cent) for the pharate moth and less than onefold (to 64 per cent) for the moth after ecdysis. The detergent furthermore unmasks a considerable latent activity, augmenting the total activity by 82 per cent in the pharate moth and 41 per cent in the emerged moths.

Fig. 4 also illustrates that the activity ratio increases as the muscle begins to break down. This increase, which was variable from one experiment to another, is apparently due to the decrease in muscle protein, for the total activity per animal remained constant. Assays of muscle from moths later than 15 hr after ecdysis were impractical owing to the extremely fragile condition of the muscle after this time.

4. *Ontogeny of catheptic activity in intersegmental muscles*

In this series of experiments the intersegmental muscles were removed from animals at different stages of adult development and homogenized in sucrose. Triton X-100 was added to a final concentration of 0·1%, and the preparations were allowed to stand 15 min at 0–2°C. After vigorous stirring, aliquots of the suspension were taken for immediate analysis without any centrifugation. Thus, the results represent maximum enzymatic activity in the entire muscle homogenate.

As indicated in Fig. 5, little proteolytic activity (0·01 units) was detected in the intersegmental muscles of chilled diapausing pupae. The activity increased about fourfold by the second day of adult development. During the first 2 weeks of adult development at 25°C, the level remained nearly constant. Then during the final week of development, catheptic activity increased rapidly at about 30 per cent per

day. In pharate Pernyi moths just prior to ecdysis, the activity reached 0·17 units—a sixteenfold increase over the initial level in the chilled pupa. This final level appeared to be maintained during the first few hours after ecdysis and then increased, as noted in the preceding section.

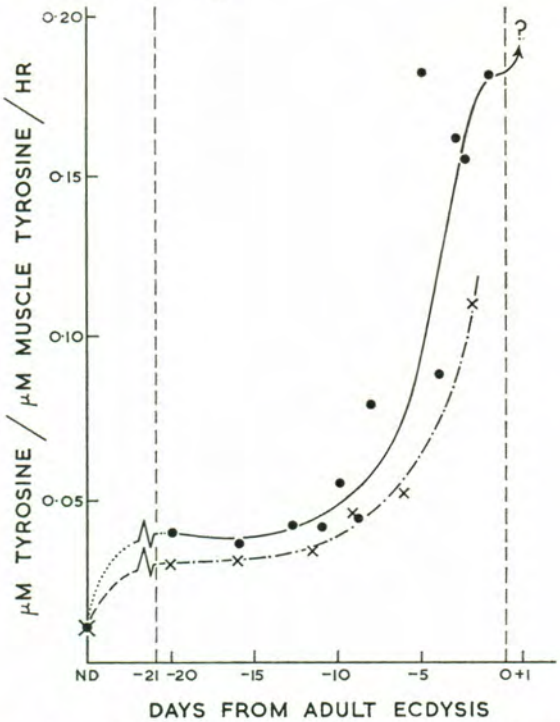

FIG. 5. Change in catheptic activity as a function of adult development at 25°C. Ordinate: catheptic activity in μmoles of tyrosine solubilized per μmole of muscle tyrosine per hour. Abscissa: days before or after adult ecdysis. The point marked ND represents diapausing pupae which showed no visible signs of development after prolonged storage at 25°C. The upper curve (●——●) represents activity in *A. pernyi*; the lower (× ·—·— ×), in *H. cecropia*.

5. *Catheptic activity of the blood, fat body, and thoracic muscles of pupae and adults*

These several tissues were analysed in the presence of detergent by the methods outlined in section 4. The results, listed in Table 3, demonstrate that the blood contains only a trace of cathepsin; the same is true for the thoracic muscles of moths. By contrast, the fat body contains about the same catheptic activity as the intersegmental muscles and, like the latter, shows a large increase during adult development.

6. *Effects of juvenile hormone on catheptic activity*

When juvenile hormone is injected into pupae just prior to the initiation of adult development, then, to varying degrees as dictated by the concentration of juvenile

TABLE 3—CATHEPTIC ACTIVITY IN SUCROSE AND DETERGENT HOMOGENATES OF BLOOD, FAT BODY, AND MUSCLES OF PUPAE AND ADULTS*

Tissue	Catheptic activity (μM tyrosine released/μM tissue tyrosine/hr)
Chilled pupae	
Blood	<0·007
Fat body	0·013
Intersegmental muscle	0·010
Pharate adults	
Blood	<0·007
Fat body	0·18
Intersegmental muscles	0·17
Thoracic muscles	<0·007

* The tissues were homogenized in 0·44 M sucrose in the presence of 0·1% Triton X-100. Aliquots of these total homogenates were incubated in the medium described in section 3 of the Methods.

hormone, the formation of the imago is blocked and the pupa transforms into a creature which may be little short of a second pupal instar. Meanwhile, the intersegmental muscles are preserved to a degree proportional to the preservation of other specialized pupal tissues (LOCKSHIN and WILLIAMS, 1964).

TABLE 4—CATHEPTIC ACTIVITY IN SUCROSE + DETERGENT HOMOGENATES OF MUSCLES AND FAT BODY OF PUPAL–ADULT INTERMEDIATES

Animal number	Developmental status	Catheptic activity (μM tyrosine released/μM tissue tyrosine/hr)	
		Intersegmental muscles	Fat body
A	Normal chilled pupa	0·010	0·013
B	Normal pharate moth	0·17	0·18
1	+2*	0·062	0·052
2	+2	0·045	0·046
3	$+3\frac{1}{2}$	0·044	0·050
4	$+3\frac{1}{2}$	0·027	0·050
5	+5	0·023	0·010
6	+5	0·015	0·025

* Preservation of pupal characters scored according to WILLIAMS (1961).

Catheptic activity was studied in the homogenates of abdominal muscles and the fat body of six individuals which had been injected with juvenile hormone. Two of the six had formed essentially second pupae; the other four showed a mixture of pupal and adult characters.

The assays were carried out, in each case, on aliquots of total homogenates to which Triton X-100 had been added as described above in section 4.

The results are recorded in Table 4. It is clear that juvenile hormone curtailed the increase in catheptic activity which takes place during the normal metamorphosis of both the intersegmental muscles and the fat body. The activity was roughly proportional to the amount of adult differentiation accomplished by the epidermis; in no case did it exceed one-third of the activity found in normal adult moths.

7. *Ontogeny of acid phosphatase activity in the intersegmental muscles*

The intersegmental muscles and fat body were analysed for acid phosphatase as described in section 4 of methods. A phosphatase with pH optimum of 4·5 was demonstrated in both these tissues of diapausing pupae (Table 5). The titre declines slightly during the first few days of adult development and then increases markedly during the final 2 weeks of development.

TABLE 5—TITRE OF ACID PHOSPHATASE IN THE INTERSEGMENTAL MUSCLES AND FAT BODY OF PUPAE AND DEVELOPING ADULTS*

Age of animal	Activity (μmoles phosphate released/μmole tissue tyrosine/hr)		Ratio: muscle/fat body
	Muscle	Fat body	
Diapausing pupa	1·37	0·49	2·80
Second day of adult development	0·72	0·31	2·32
Twentieth day of adult development	2·80	2·65	1·05

* Total homogenates of intersegmental muscle and fat body were incubated in the presence of Na-β-glycerophosphate in ABC buffer, pH 4·5, for 30 min at 25°C.

8. *Characteristics of acid phosphatase in the muscles of pharate adults*

In sucrose homogenates half the acid phosphatase activity was soluble (Table 2). Differential centrifugation resulted in 70 per cent of the enzyme appearing in the supernatant. As noted in the table, the addition of Triton X-100 also increased the soluble fraction from 50 to 70% in sucrose homogenates of pharate adults and emerged moths. The detergent partially inhibited the enzyme.

DISCUSSION

1. *Lysosomes and latent cathepsin*

The synthesis and ultimate activation of a latent cathepsin appears to play a major role in enforcing the biological death of the intersegmental muscles. Prior to the initiation of cytolysis the enzyme is apparently bound to or sequestered by small particles. These are probably equivalent to lysosome-like bodies which are

visible in electron micrographs of intersegmental muscles during the final week of adult development (LOCKSHIN and WILLIAMS, 1965a).

In homogenates prepared in cold hypertonic sucrose, nearly all the latent enzyme is sedimentable, predominantly in the mitochondrial fraction (Table 2). Active enzyme is released to the supernatant when the particulate fraction is exposed to elevated temperatures or to lytic agents such as distilled water or detergents (Tables 2 and 3).

By suitable measurements, it was possible to show that the latent enzyme undergoes a remarkable increase in titre during the final (third) week of adult development (Fig. 5). It is of particular interest and importance to note that when pupae were injected with juvenile hormone just prior to the initiation of development, this large increase in cathepsin did not occur and the muscles failed to break down after ecdysis (Table 4).

2. *Activation of cathepsin*

In electron micrographs prepared from muscle at successive stages in adult development, the cytolytic reaction begins about 5 hr after adult ecdysis. At this time the lysosome-like bodies showed distinctive changes suggestive of a release of sequestered enzymes (LOCKSHIN and WILLIAMS, 1965a). Moreover, as demonstrated in the present study, these cytological changes are synchronized with the activation of latent cathepsin (Fig. 4).

3. *Acid phosphatase*

The titre of an acid phosphatase follows the same general pattern as that of cathepsin, with the exception that acid phosphatase is apparently contained in extremely fragile particles. Because of this complication, it was not possible to follow its activation after ecdysis. This suggests either that acid phosphatase can readily escape from the particles or that the enzymes may be distributed among different lysosomes.

4. *Enzymes and nervous system*

We have seen that the cytolytic mechanism is fully established with the intersegmental muscles during the final week of adult development. Yet the fact remains that a further signal is required for the activation of the latent mechanism. This signal consists of a curtailing of the flow of the motor nerve impulses to the individual muscle fibres within the first 5 hr after adult ecdysis (LOCKSHIN and WILLIAMS, 1965b). If this signal is blocked by electrical or pharmacological excitation of the nervous system, the muscles fail to break down (LOCKSHIN and WILLIAMS, 1965c).

This same signal can, of course, be administered precociously by excising the ganglia which supply motor innervation to the intersegmental muscles. Operations of this type are inconsequential when performed on pupae; indeed, the entire

central nervous system can be excised from pupae without provoking the breakdown of the intersegmental muscles (WILLIAMS and SCHNEIDERMAN, 1952; FINLAYSON, 1956). The same is true until adult development is virtually complete and the latent cytolytic mechanism has been fully established within the intersegmental muscles (LOCKSHIN and WILLIAMS, 1965c).

5. *Nerve impulses and lysosomes*

At the moment of adult ecdysis, the cytolytic mechanism is present and poised within the individual cells of the intersegmental muscles. Yet, as we have seen, this latent, lethal mechanism fails to 'go off' if, by experimental manœuvres, the flow of motor impulses is sustained in the nerves.

We are therefore persuaded that, by some direct or indirect mechanism, the neuromuscular transmitter preserves the integrity or the impermeability of the lysosomes of the intersegmental muscles. A more direct test of this proposition should be possible after the neuromuscular transmitter of insects has been identified.

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