THE CYTOLOGICAL EFFECT OF ECDYSTERONE ON THE MIDGUT CELLS OF THE FLESH-FLY SARCOPHAGA BULLATA

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ABSTRACT
Larvae of the flesh-fly, Sarcophaga bullata, were injected with the synthetic moulting hormone ecdysterone or saline at the beginning of the third and final larval instar. One group was left untreated. The ecdysterone-injected larvae showed an increase in number of secondary lysosomes in the midgut epithelial cells similar to that observed at the onset of metamorphosis, an event which would normally occur about 48 hr later in these larvae.

INTRODUCTION
The hormonal control of metamorphosis has been well documented for both hemimetabolous and holometabolous insects (11, 29, 32, 33). Two hormones, juvenile hormone and ecdysone, play important roles. The titer of ecdysone rises during each larval stage; when it reaches a certain level the insect moults. The titer of juvenile hormone is high in young larvae, decreasing with each successive instar until there is a low titer at the pupal moult. The low titer or absence of juvenile hormone in the presence of ecdysone causes the moult to be a metamorphic rather than another larval moult.

Metamorphosis in the larvae of the flesh fly Sarcophaga bullata is accompanied by conspicuous internal alterations: the Malpighian tubules discharge their contents to the hindgut, the proventriculus elongates, and the midgut epithelial cells shrink and become filled with lysosomes preparatory to degeneration and replacement by adult midgut cells (19). Sarcophaga larvae moult twice in 2 days to reach the third and final larval instar, which lasts approximately 3 days. Within 24 hr after the initiation of puparium formation the larvae undergo the pupal moult. The greatest increase in numbers of lysosomes in the midgut cells begins to occur 6–24 hr before puparium formation.

In the present study it was postulated that the increase in the numbers of lysosomes may be a result of the increased level of ecdysone activity in the presence of reduced juvenile hormone activity. We present evidence to show that proliferation of lysosomes in the midgut epithelial cells occurs in response to injection of the synthetic moulting hormone, ecdysterone, either by gene induction of increased hydrolase synthesis within the midgut cells themselves or via a pathway as yet unelucidated.

MATERIALS AND METHODS
Adults and larvae of Sarcophaga bullata were maintained on an artificial diet (9) supplemented with beef liver. Under these conditions the larvae usually pupated in 6–8 days at 30°C. To assure relative uniformity of development, the age span of larvae used in these experiments did not exceed 2 hr.

At the beginning of the third day of larval life, i.e., at the beginning of the third instar, the larvae were divided into three groups. The first group was injected into the body cavity through the cuticle with
50 µl of an ecdysterone solution in 0.65% NaCl containing 120 µg ecdysterone/ml. Each injection contained 6 µg of ecdysterone.¹,² There was a total of 32 larvae in this group. The second group (21 larvae) was injected with 50 µl of 0.65% NaCl. The injection site was plugged with a physiological adhesive.³ The third group was left untreated (27 larvae).

Samples of the midgut were taken for histochemistry and electron microscopy at 4, 9, and 22 hr after injection.

For histochemistry the anterior 2 or 3 mm of the midgut was fixed in Formol-calcium for 20–24 hr on ice, then allowed to soak in a gum-sucrose solution for 5–10 days in the refrigerator at approximately 5°C. The soaking solution was changed once after 24 hr.

The pieces of intestine were embedded in an agar block (0.75% agar in gum-sucrose solution) to hold them for Cryostat sectioning. The tissue and agar were frozen on a brass chuck placed on Dry Ice. To visualize the lysosome response a slight modification of the Barka and Anderson simultaneous coupling azo dye method for acid phosphatase (3) was used on 6–8 µm Cryostat sections. Naphthol AS-BI phosphate (5) was used as the substrate with a 40 min incubation time. Heat controls were incubated for 15 or 30 min in a moist chamber at 90–100°C before histochemistry. Incubations without substrate were also run.

For electron microscopy the 1 or 2 mm of intestine immediately posterior to the portion fixed for histochemistry were fixed for 1 hr at room temperature in 1% osmium tetroxide in 0.1 M Sorenson's phosphate buffer, pH 7.3, with 0.1 ml of 0.1 M CaCl₂ added per each 10 ml. After fixation the tissue was rinsed quickly in tap water, dehydrated in a series of alcohols, and embedded in Epon-Araldite mixture I or II (20). Sections were cut on a Porter-Blum MT-2 ultramicrotome with a diamond knife, stained with uranyl acetate (31) and lead tartrate (17), and examined with a Zeiss EM-9A electron microscope.

RESULTS

The appearance of the proventriculus was used as the marker for the approach of metamorphosis in these experiments since the proventriculus normally showed a change from a spherical to an elongate appearance on the fifth or sixth day of larval life. The proventriculus of the control larvae never showed elongation during the course of an experiment. Thus, all injections were made well before any of the events usually associated with metamorphosis took place.

Normal Appearance of the Midgut Epithelial Cells of 3-day-old Larvae

The midgut epithelial cells of 3-day-old Sarcophaga bullata (19) are large columnar cells which possess a striated border typical of epithelial absorbing cells (Fig. 7, a saline-injected control, shows the normal morphology). Beneath the border is a zone generally free of cytoplasmic organelles but containing vesicular profiles assumed to be the result of pinocytosis. There are numerous mitochondria throughout the cytoplasm, particularly in the basal region where the cell membrane is extensively infolded similar to the basal cell membrane in mammalian kidney tubules (27). There are relatively few lysosomes to be found at this stage, and those that are present are usually located in a supranuclear position and are about 1 µm in diameter. The cells at this time contain a moderate amount of rough endoplasmic reticulum distributed throughout the cytoplasm. The Golgi apparatus, if present, is inconspicuous in these cells. A body appearing to consist of concentric layers was observed infrequently. Bodies of similar appearance have recently been observed in midgut cells of a pseudoscorpion where they have been termed "urospherites" (2). The nucleus is spherical with regular contours. A basal complex containing a two-layered basement membrane, two tenuous muscle layers, and tracheoles lies subjacent to the epithelial cell layer.

Histochemistry

Figs. 1–6 show the cytological effect of injected ecdysterone. The untreated control animals were dissected at the same time as the saline- and ecdysterone-injected larvae. They were thus the same age, the only difference being that the experimental animals had been injected 4, 9, or 22 hr before dissection.

In these experiments the midgut epithelial cells of ecdysterone-injected larvae showed an increase in the numbers of lysosomes within 9 hr (Figs. 1–3). No observable increase was present after only 4 hr. By 22 hr after injection the cells were almost filled with lysosomes and had begun to shrink (Figs. 4–6). These events would normally occur at least 48 hr later. In conjunction with the

¹ Equivalent to 60 Calliphora units (14).
² Mann Chemical Corp., Louisville, Ky.
³ "Ethicon" (methyl 2-cyanoacrylate monomer), Ethicon, Inc., Somerville, N. J.
shrinkage of the cells the basal surface began to show a wavy contour.

Interestingly, although ecdysterone induced the lysosome response similar to that seen during histolysis of the midgut cells at metamorphosis, the injected larvae did not undergo pupation before the control larvae.

**Electron Microscopy**

By 9 hr after ecdysterone injection the appearance of the midgut cells had changed radically (Fig. 7), whereas saline injection had no effect on the general cytology (Fig. 8). The number of mitochondria was greatly reduced in the ecdyster-
one-injected larvae, and the number and size of lysosomes had greatly increased, especially in the apical portion of the cell. The basal region of the cells also showed an increase in the number of lysosomes, the number of mitochondria being reduced. The basal border of the cells had begun to show a distinctly wavy contour corresponding to its appearance under the light microscope (Fig. 9). The luminal margin contained beaded profiles, presumably indicating an increase in pinocytotic activity compared with controls (Fig. 10).
Figure 7: Apical portion of cytoplasm 9 hr after saline injection. The cytoplasm appears virtually the same as the cytoplasm of the untreated controls. Three lamellated bodies (LB) (urospherites?) are present near the brush border. The appearance of the nucleus is generally smooth and spherical. OsO₄. × 3500.

Figure 8: Apical portion of midgut epithelial cell 9 hr after ecdysterone injection. Lysosomes (L) fill the cytoplasm. Mitochondria (arrow) are found primarily in a narrow zone beneath the terminal web. OsO₄. × 3500. Insert: appearance of secondary lysosomes containing fragments of cytoplasmic organelles such as mitochondria (m) and endoplasmic reticulum (er). 9 hr after ecdysterone injection. OsO₄. × 18,200.
Figure 9 Basal portion of midgut epithelial cell 9 hr after ecdysterone injection. Large secondary lysosomes (L) are numerous. The basal border shows a distinctly wavy contour corresponding to its appearance under the light microscope. OsO₄. × 3500.

Figure 10 Apical portion of the cytoplasm 9 hr after ecdysterone injection. The terminal web contains numerous profiles of presumed pinocytotic vesicles (arrow). OsO₄. × 14,000.
Midgut epithelial cell 22 hr after ecdysterone injection. The cells have shrunk considerably since the 9-hr sample, (cf. Fig. 8, taken at same magnification). Lysosomes (L) fill the apical cytoplasm. Several lamellated bodies (LB) (urospherites?) appear scattered throughout the cell. The contour of the basal border has become more irregular since the 9-hr sample. OsO₄. X 3500.
The lysosomes in these cells appeared to be large secondary lysosomes (8), i.e., lysosomes containing fragments of other cytoplasmic organelles that are presumably being digested (Fig. 8, inset), and were 2–4 µ in size.

By 22 hr after ecdysterone injection (Fig. 11) the mitochondria were found closely packed in the cytoplasm just beneath the microvilli. The central cytoplasm was largely occupied by secondary lysosomes. The nucleus had an irregular wavy contour, sometimes appearing lobed. The basal part of the cells had become more irregular since the 9-hr sample, and the cells had shrunken considerably (cf. Figs. 8 and 11, both taken at the same magnification). The saline-injected and untreated controls had the same appearance after 22 hr as after 9 hr (Fig. 7).

There was no discernible difference between saline-injected and untreated controls at either 9 or 22 hr.

**DISCUSSION**

The present investigation has shown that the increased numbers of lysosomes normally associated with the onset of histolysis in *Sarcophaga bullata* may appear in response to the injection of a single high dose of the synthetic growth and metamorphosis hormone, ecdysterone. These lysosomes exhibit a strong acid phosphatase reaction as seen by the electron microscope, with mitochondria and portions of rough endoplasmic reticulum being clearly visible within them. The increased number of lysosomes appearing in response to ecdysterone injection, coupled with the decrease in the number of mitochondria, suggests that the onset of autolysis is marked by the early digestion of cytoplasmic organelles within lysosomes. Walker (30) observed a similar phenomenon at metamorphosis in the fat body cells of *Philosamia*. Whether mitochondria and endoplasmic reticulum are the primary targets for intracellular digestion at this time, or whether their engulfment is more evident merely because of their size and number, is not known.

It has been shown that natural ecdysone is rapidly inactivated in dipterous insects (15, 21) and that one injection can last only a few hours. We presume that the synthetic hormone used in our experiments is likewise inactivated, so that the continuing lysosome proliferation and cell degeneration seen 22 hr after the injection may indicate that the hormone has acted to trigger a chain of self-sustaining events leading eventually to the lysosome response.

This hypothesis seems not unreasonable in the light of work by Clever (6) on the salivary glands of *Chironomus tentans*, in which cells exposed to ecdysone showed evidence for activation of two gene loci within 3 hr following injection. Chromosomal puffing and RNA synthesis were taken as criteria of gene activity; the puffs subsided after the injected hormone was inactivated. Other gene loci in these chromosomes became active only 2 or 3 days following the single injection of ecdysone, and were interpreted to have been induced by gene products emanating from the earlier-reacting puff regions.

In the midgut epithelial cells of *Sarcophaga* there is an apparent increase in the amount of the enzyme acid phosphatase in response to injected ecdysterone, as seen by histochemistry. This increased enzymatic activity could similarly be the result of hormone-induced gene activation. However, the increased number of lysosomes could also be related to an altered cellular metabolism, itself induced by the injection of hormone. For instance, an increase in pinocytotic activity may influence the formation of lysosomes (7). We have at present no evidence to discriminate, in our experiments, between the effects of injected ecdysterone upon chromosomes, ribosomes and other cytoplasmic organelles, or the stimulation of general cellular metabolism.

Insect hormones may have diverse effects depending upon the particular cell type in which they act. Cells of imaginal disc tissue, for example, are stimulated to undergo rapid growth and progressive differentiation rather than histolysis under the influence of ecdysone. In reviewing the role of cell death during development throughout the animal kingdom, Saunders (25) has pointed out that in cases known to involve endocrine control of cell death, the hormone can also stimulate growth and development, the specificity residing of necessity within the cells of the target organ.

*Sarcophaga* larvae injected with ecdysterone in our experiments failed to show external signs of premature metamorphosis even though exhibiting a precocious lysosome response. Postlethwait and Schneiderman (22) have shown that imaginal disc cells in vitro require the continued presence of ecdysone analogues to maintain their course in...
development. Perhaps the same requirement holds for the larvae in our experiments, particularly since injected ecdysone is known to be rapidly inactivated.

The control of acid phosphatase level at metamorphosis (and presumably the level of other lysosomal hydrolases as well) through the action of ecdysone and related molecules is probably a general feature of the histolysis occurring at insect metamorphosis. It may be a characteristic of all histolytic events under endocrine control. We are led to these conclusions by the observations of others who have made a correlation between the onset of metamorphosis and increased hydrolytic enzyme activity. Hegdekar and Smallman (12) described an increase in acid phosphatase of whole-body homogenates in *Musca domestica*. They reported a rise in the level of lysosomal acid phosphatase as well as the appearance of what they interpreted to be a nonspecific lysosomal acid phosphatase during the peak of histolysis. Barker and Alexander (4) and Ashrati and Fisk (1) had earlier noted a correlation between increased acid phosphatase activity and the onset of pupation in *Musca* and in *Stomoxys calcitrans* (stable fly). Rasch and Gawlik (24) observed the accumulation of large acid phosphatase-positive secondary lysosomes in salivary gland and Malpighian tubule cells of *Sarcophaga bullata* (Parker). Stay (28) described patterns of the activity of phosphatases and other enzymes in paraffin-embedded tissues of the blowfly, *Phormia regina* (Meigen) during the larva-to-pupa transition. Przelecka et al. (23) reported intensified acid phosphatase activity and the onset of pupation in *Phormia* and in *Musca domestica* (stable fly).

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The reports cited above make it readily apparent that the histolysis which occurs during insect metamorphosis is associated with the increased activity of at least one lysosomal hydrolase. Considerable evidence is also at hand to indicate that cellular autophagy is associated with lysosome formation in a wide variety of cells and organisms (10). It is not certain whether the hydrolases are primarily responsible for initiating the histolytic action or whether they in turn depend upon prior alterations within the target cells. What is clear, however, is that the injection of ecdysone alone into mature larvae is sufficient to produce an early lysosome response identical, so far as we can tell, to that which would normally occur at a later time in untreated larvae.

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