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DEGENERATIVE CHANGES IN THE MUSCLE FIBERS OF *MANDUCA SEXTA* DURING METAMORPHOSIS

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Summary

The ultrastructural changes associated with the early stages of degeneration of the larval mesothoracic muscle fibers of *Manduca sexta* were examined during the prepupal period and on the first day after ecdysis. Over this 5 day period, the muscle fibers decrease in cross-sectional area but increase in apparent surface area compared to the dimensions of early fifth-instar fibers. Large numbers of electron-dense granules or droplets are formed and extruded from the muscle cytoplasm into the hemolymph; this process may account for some of the decrease in muscle fiber mass and may represent a developmental mechanism for recycling nutrients. As the fibers shrink, the thick basal lamina is thrown into folds. Phagocytic hemocytes (granulocytes) congregate in clusters over the surface of the degenerating fibers and appear to remove specifically the basal lamina. The timely removal of the thick larval basal lamina may be essential for subsequent fusion of myoblasts to the residual larval myofibers. The contractile elements within the degenerating muscle fibers become disorganized but are not dysfunctional at the end of the first 12 h after the pupal ecdysis. Tracheoles withdraw from intimate contact with each muscle fiber in its clefts and T-tubules and associate in groups adjacent to it. Mitochondria appear to be degenerating. These structural changes are concurrent with a previously observed decline in resting potential and suggest that a significant change in the electrical properties of the muscle fibers should be expected as well.

Introduction

The programmed cell deterioration, de-differentiation, and remodeling that occur during insect metamorphosis include many interesting processes fundamental to muscle degeneration in general as well as to development. In lepidopterans and other insects undergoing holometabolous development, the transformation includes all of the modifications necessary to change from a crawling and feeding lifestyle to one designed for flight. Many of the larval muscles, which have structures typical of tonic muscle and slow contraction times, involute or degenerate. Some disappear completely. Others, which persist, re-differentiate

Key words: muscle fiber, degeneration, ultrastructure, basal lamina, *Manduca sexta*, insect development.

with entirely different structures and physiological properties as fast-contracting phasic and fibrillar muscles (Crossley, 1965, 1978; Rheuben and Kammer, 1980).

The control of processes underlying the programmed cell deterioration and remodeling may rest both within the cell itself and with circulating hormones. Insect muscle appears to be directly responsive to the influences of fluctuating levels of hormones at the times of ecdysis and metamorphosis. For example, the normal degeneration of the abdominal intersegmental muscles of the moth *Antheraea polyphemus* is modulated by both 20-hydroxyecdysone and eclosion hormone prior to and after the adult ecdysis (Schwartz and Truman, 1984). Cultures derived from *Drosophila melanogaster* embryos include adult muscle fiber types (instead of only larval fiber types) if ecdysone is added at the second week of culture (Dubendorfer *et al.* 1978). In cultured muscles removed from larval *Calliphora erythrocephala* prior to metamorphosis, Zachary and Hoffmann (1980) found that the responses to 20-hydroxyecdysone depended upon the expected final fate of the particular muscle: those destined to persist *in vivo* in the imago exhibited only an involution of contractile structures rather than a complete degeneration and those destined to degenerate *in vivo* underwent a complete degeneration in culture under identical hormonal regimes.

Even amongst the muscles destined for degeneration during metamorphosis, the process may not always proceed uniformly. Finlayson (1956) noticed that degeneration of the abdominal muscles in Lepidoptera appeared to occur in two waves, one travelling rostral to caudal and a second initiated in the posterior segments. In the proleg retractor muscle of *Galleria mellonella* degenerative changes began at the cuticular origin and insertion and spread towards the middle of individual fibers (Randall and Pipa, 1969). Consequently, the exact timing and course of changes within any given muscle are likely to be highly specific to the muscle in question.

The precise constellation of structural changes occurring in association with metamorphic degeneration seems to vary considerably from muscle to muscle and insect to insect and frequently differs from degenerative changes induced by injury or experimental manipulation. A few of these features seem to occur consistently in degenerating muscle, regardless of whether it is occurring as part of a normal physiological event or is due to experimental manipulation such as denervation or tenotomy. These include the presence of abnormal mitochondria (condensed, darkened, enclosed in separate membranes) and swollen T-tubules, changes in the amount of sarcoplasmic reticulum, disorganization and loss of contractile elements, the presence of phagocytic hemocytes and atrophy (Randall and Pipa, 1969; Lockshin and Beaulaton, 1979). The last change, atrophy or a decrease in mass of the fibers, although seen in both metamorphic muscle and experimentally manipulated muscle, seems to occur very slowly in denervated or tenotomized muscle, requiring 30–40 days to develop (Wood and Usherwood, 1979; Rees and Usherwood, 1972; Jahromi and Bloom, 1979).

Other distinctive degenerative changes have been noted only in particular situations. An infiltration of fat cells with lipid droplets was only observed in

tenotomized muscle (Jahromi and Bloom, 1979). The presence of liposomes (lipid droplets or granules) in the sarcoplasm and the collapse of tracheoles supplying the muscle fibers has been noted in several muscles degenerating under some kind of hormonal control at either the beginning or end of metamorphosis (Randall and Pipa, 1969; Beaulaton and Lockshin, 1977; Crossley, 1972*a*), but not in denervated muscle. However, in juvenile-hormone-induced degeneration of the adult flight muscles in the bark beetle, lipid droplets appear in the treated animals, but the tracheole system does not deteriorate (Unnithan and Nair, 1977).

This study was made in conjunction with an electrophysiological assessment of the functional changes in the larval and early pupal mesothoracic muscles and neuromuscular junctions of *Manduca sexta* (Sonea and Rheuben, 1992; I. M. Sonea, M. B. Rheuben and C. Young, in preparation). Some, but probably not all, of the dozen or so dorsal mesothoracic muscles contribute to the formation of the adult dorsal longitudinal flight muscle (Rheuben and Kammer, 1980; Casaday, 1975; Heinertz, 1976) and to the other mesothoracic muscles. The specific contributions of all the different larval muscles to the adult flight muscles have not yet been determined. We examined the ultrastructure of the AB and C muscle fibers, which probably do contribute to the adult musculature, in the regions from which physiological recordings were obtained for those structural changes that might be correlated with the physiological changes we observed and for those changes that would lead to further understanding of the developmental processes. In addition to some of the predictable degenerative processes such as atrophy, involution of the contractile apparatus and degeneration of mitochondria, we obtained evidence for the formation and massive exocytosis of droplets from the muscle cytoplasm and for the specific removal of the larval basal lamina by phagocytic hemocytes. These changes may relate to the processes of re-development to follow.

Materials and methods

Experimental animals

Tobacco hornworms [*Manduca sexta* (Linnaeus)] were raised from eggs kindly provided by the Insect Hormone Laboratory, Department of Agriculture, Beltsville, MD. Larvae were fed an artificial diet (Yamamoto, 1969) and kept at 26°C on a 16h:8h L:D cycle.

Dissection and preparation

The detailed methods of anesthesia, dissection and mounting of those animals studied electrophysiologically or subjected to prewashes prior to fixation are described in Sonea and Rheuben (1992). Otherwise larvae were anesthetized by chilling, bisected and pinned out flat with the gut ligated, and submerged in fixative at room temperature for 30 min. Intact pupae were injected with 5-10 ml of fixative and left for 30 min. After this initial period of fixation, individual muscle

bundles were dissected free, preferably including the cuticular attachments at both ends, and submerged in vials of fresh fixative for an additional 1.5 h.

Two different fixative protocols were used. The first protocol included primary fixation with 4 % paraformaldehyde, 1 % glutaraldehyde and 0.2 $mmol\,l^{-1}$ $CaCl_2$, in 0.1 $mol\,l^{-1}$ phosphate buffer (Millonig's pH 7.2), followed by rinsing in 0.1 $mol\,l^{-1}$ phosphate buffer and secondary fixation in 1 % osmium tetroxide in phosphate buffer with 0.2 $mmol\,l^{-1}$ $CaCl_2$ for 2 h. After block staining in aqueous uranyl acetate, the tissue pieces were dehydrated, infiltrated and embedded in English Araldite.

For the second protocol, the muscle was first exposed to an oxygenated presoak containing 60 $mmol\,l^{-1}$ $MgCl_2$, 25 $mmol\,l^{-1}$ KH_2PO_4 and 100 $mmol\,l^{-1}$ glucose, pH 6.6 for 10 min. The first primary fix, containing 60 $mmol\,l^{-1}$ $MgCl_2$, 5 $mmol\,l^{-1}$ EGTA, 15 $mmol\,l^{-1}$ KH_2PO_4, 28 $mmol\,l^{-1}$ glucose, 3 % glutaraldehyde and 1.5 % paraformaldehyde, pH 6.6, was pipetted vigorously over the muscle and left to soak for 5 min. A second aldehyde fixative containing 10 $mmol\,l^{-1}$ $MgCl_2$, 79 $mmol\,l^{-1}$ KH_2PO_4, 20 $mmol\,l^{-1}$ glucose, 0.1 $mmol\,l^{-1}$ $CaCl_2$, 3 % glutaraldehyde and 1.5 % paraformaldehyde was then applied, and individual muscle bundles were dissected out after 30 min and placed in vials containing the same solution for an additional 1.5 h. The buffer wash was 0.125 $mol\,l^{-1}$ phosphate with 0.1 $mmol\,l^{-1}$ $CaCl_2$, and the same vehicle was used for the 2 h secondary fixation with 1 % osmium tetroxide. The tissue was then dehydrated and embedded without block staining. This second protocol gave somewhat better preservation than the first, and most of the illustrated sectioned material was prepared using this method.

Thin sections were stained for 2 h with 10 % uranyl acetate in 50 % methanol, followed by 5 min in Reynold's lead citrate.

Specimens to be examined with the scanning electron microscope were fixed using the aldehyde mixture described in the first protocol. They were then slowly transferred from phosphate buffer through a graded buffer:water series to distilled water, impregnated with osmium using the osmium thiocarbohydrazide method, or (larvae) treated with hydrochloric acid and collagenase to remove the basal lamina prior to osmication, as described previously (Schaner and Rheuben, 1985).

In this part of our investigations on the development of the dorsal longitudinal muscles, 12 fifth-instar larvae, 15 prepupae and 29 pupae were examined ultrastructurally.

Identification of muscle fibers

For simplicity, and because of two potentially confusing errors in the figures of the larval mesothoracic muscles in a recent morphological treatise describing *Manduca sexta* (Eaton, 1982), we have continued to use the older nomenclature of Lyonet (1762), which accurately represents the muscles studied. The muscles primarily examined in this study, A, B and C, probably correspond respectively to muscles DL_1 (A and B combined) and DP_1 (Eaton, 1982); or dil_{1b}, dil_{1a} and dio (Tsujimura, 1983). These muscles are of particular interest because they partici-

pate to some extent in the formation of the anlagen of the dorsal longitudinal flight muscles of the adult.

Muscles were first carefully drawn *in situ*; if electrophysiology had been performed on them, the recording sites were indicated. The portions of the muscles removed from the animal were also indicated and, after embedding, the groups of fibers in the block were again drawn. After preparing the block face, thick sections were drawn using a drawing tube and individual fibers were assigned numbers. These drawings were used to identify the fibers and specific neuromuscular junctions during photographic sessions on the electron microscope.

Data analysis

For measurement of the cross-sectional areas and circumferences of the larval and pupal muscle fibers, low-magnification electron micrographs were taken of individual fibers from various stages using a JEOL 100CX transmission electron microscope. It was found that the calibration of the low-magnification mode was not stable from picture to picture, so the magnifications were checked by measuring identified structures both on the micrographs and from the thick sections using a Bioquant II computer-interfaced morphometric digitizing system. The micrographs were measured using a Joyce Loebel Magiscan with a program written by Debra Trytton that allowed us to subtract the many holes and fissures in the degenerating fibers to derive relatively accurate estimates of cytoplasmic areas and of the length of the infolded muscle membranes.

Results

The dorsal longitudinal muscles examined in this study (muscles A, B and C) were from the second (mesothoracic) segment of the larvae of *Manduca*. Fig. 1 in a companion paper (Sonea and Rheuben, 1992) illustrates the location of the muscles within the segment; they occupy much of the dorsal third of the body wall musculature. Fig. 1 (this paper) shows a scanning microscope view of the dorsal mesothoracic musculature from a third-instar larva of *Manduca* and indicates the nomenclature being used and the specific regions examined in this study. The precise location of the separation between the dorsal fibers of muscle A and the more ventral fibers of muscle B has not been clearly ascertained; consequently, the two parts have been collectively referred to as muscle AB for the purposes of this study. Although there are distinct physiological differences between the C muscle fibers and the AB fibers (Sonea and Rheuben, 1992; I. M. Sonea, M. B. Rheuben and C. Young, in preparation), no differences were detected between the properties of fibers in the dorsal A region and those in the ventral B region. No obvious ultrastructural differences have been detected between A, B or C muscle fibers. All muscles in this study appear to be innervated by single motor neurons; sections through nerve twigs supplying either a particular muscle or a single fiber contain only one large axon plus small neurosecretory axons running outside the

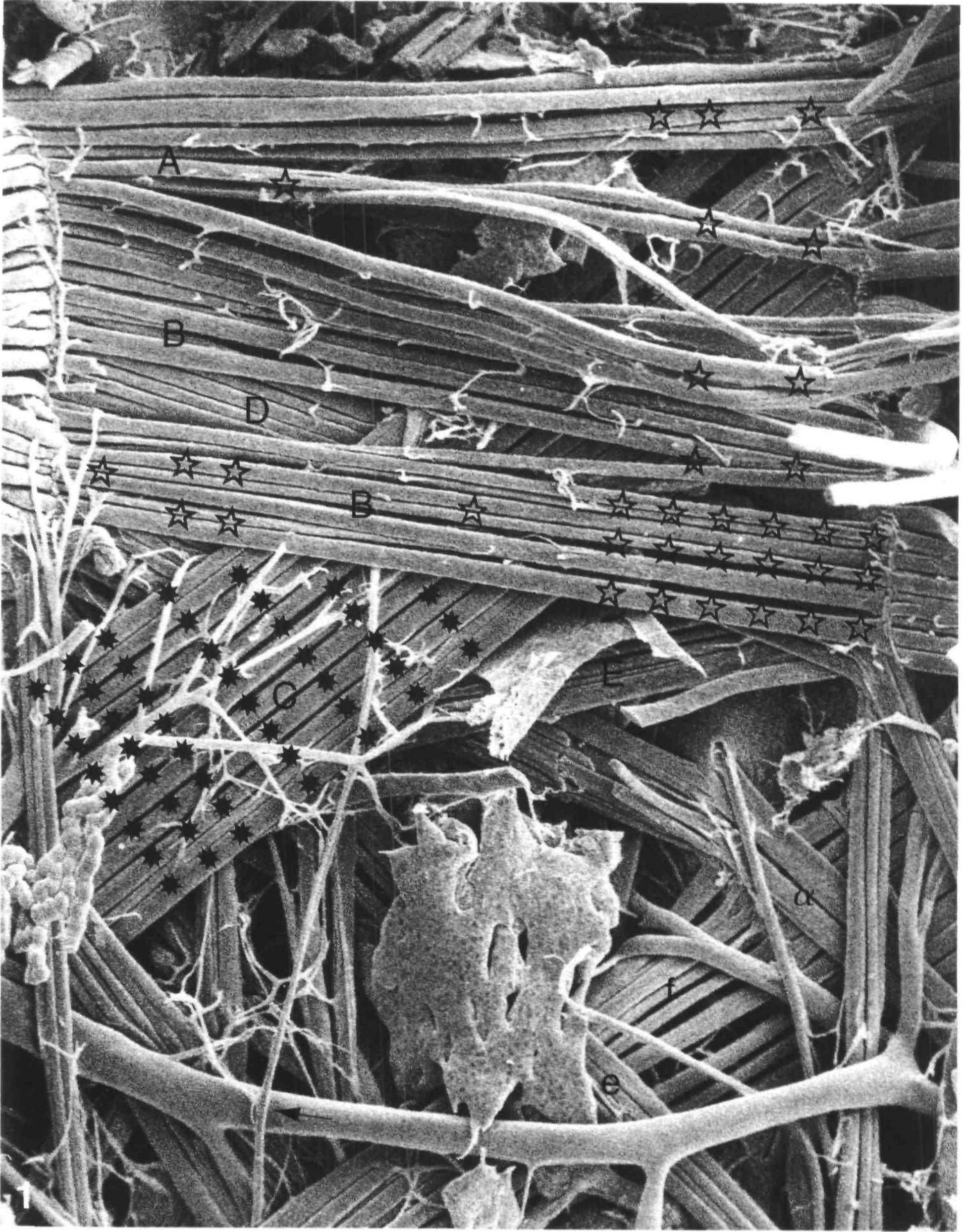

Fig. 1. Scanning electron micrograph of the dorsal region of the right mesothoracic segment of a third-instar *Manduca sexta* larva. Medial view. The dorsal midline is at the top, and the rostral and caudal boundaries of the segment are just at the left and right borders of the photograph, respectively. The nerve IIN1 (running dorsally) is indicated with an arrow where it crosses over a main tracheal trunk. The distribution and density of stars (AB fibers) and asterisks (C fibers) serve to identify the muscles and reflect the frequency that a given region was sampled. Muscle(s) AB include all of the superficial horizontally running fibers. Small regions of the more lateral muscles D and E are also visible. See also Fig. 1 in Sonea and Rheuben (1992) for comparison. 68×.

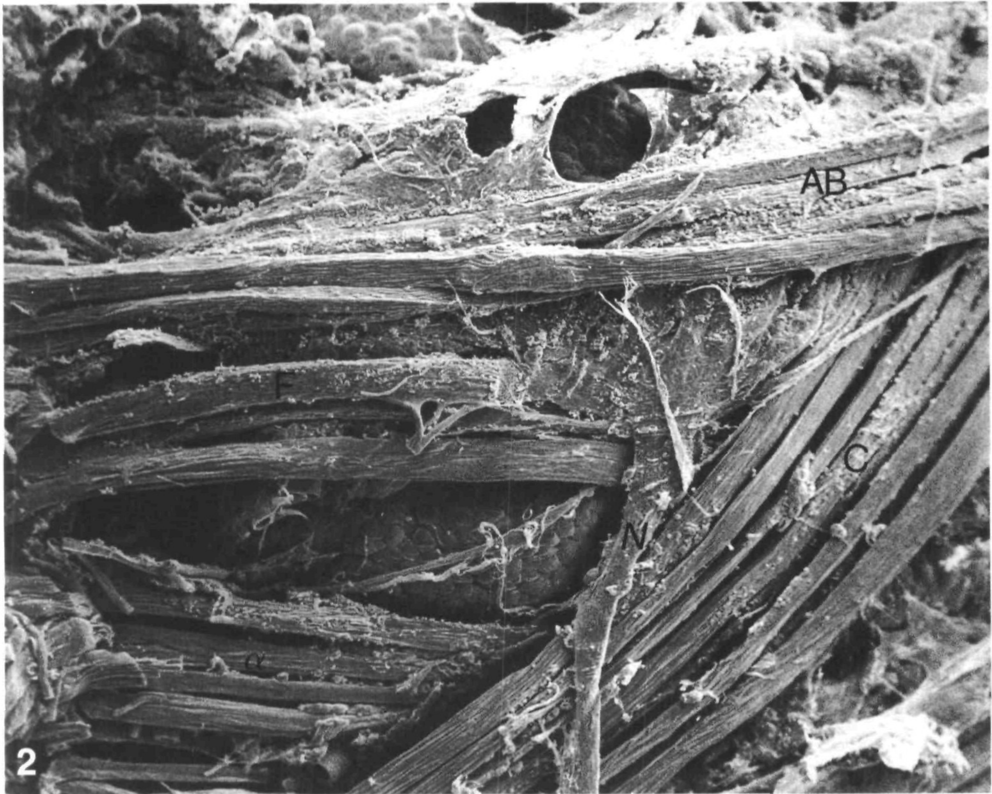

Fig. 2. Scanning electron micrograph of the dorsal region of the right mesothoracic segment from a day 8 fifth-instar (prepupal) larva. All but five of the AB fibers have been removed to show the anlagen forming in the central region around the nerve (*N*). Muscle D is not exposed but lies just dorsal to muscle F under AB. 36×.

perineurial sheath. Graded stimulation of the main nerve typically evokes an all-or-nothing electrical response from individual muscle fibers.

The nerve trunk supplying the dorsal mesothoracic musculature, nerve IIN1, crosses a large longitudinal tracheal trunk medially. It innervates muscle C on its medial surface and then passes between muscles AB and C to innervate AB and other dorsal muscles from the lateral side. During metamorphosis, the anlagen of the adult dorsal longitudinal flight muscle forms around the nerve where it is sandwiched between muscle AB, muscle C and the more lateral muscles D and F. As the anlagen grows, it spreads to infiltrate and incorporate some fibers from all of the above muscles and possibly additional ones (Fig. 2, and see also Casaday, 1975).

The fifth instar in *Manduca* under our culture conditions encompasses about 8 days; the first 4 days are characterized by feeding and growth. After feeding ceases and the larvae begin to wander (termed the prepupal period), degenerative changes can be detected in the muscle fibers and the anlagen begins to form

around the nerve. The ultrastructural observations described in this paper and the electrophysiological observations described in an additional paper in this series (Sonea and Rheuben, 1992) were made on the same or comparable regions of fibers from muscles AB and C as they degenerated from the prepupal period through the first 13 h after ecdysis.

Thin sections taken from early fifth-instar larvae through the prepupal period and on the first day after pupation indicate that the mass of individual muscle fibers decreases drastically in conjunction with degeneration beginning in the prepupal period. Significant decline has already occurred by the pupal ecdysis, but further shrinkage is evident when 0- to 2-h pupae and 12- to 13-h pupae are compared (Figs 3, 4, 5 and 6).

Cross sections, similar to those shown in Figs 3 and 4, of five fibers from early fifth-instar larval AB and C muscles and five from 6-h pupal AB and C muscles were carefully digitized from low-magnification electron micrographs and the cross-sectional areas and the circumferences, including all the infoldings, were measured. Each fiber was digitized three times; the average values are listed in Table 1. The cross-sectional areas of pupal fibers were reduced to 0.31 (AB) and 0.46 (C) of the larval values. The apparent circumferences of pupal fibers, however, increased by 1.46 (AB) and 1.92 (C) times the larval values.

The cytoplasm of degenerating muscle fibers is characterized by what appear to be dilations of the T-tubules and by mitochondria which increasingly take on a darker, more condensed appearance. Some mitochondria have disorganized cristae or appear only as electron-dense remnants occupying part of a mitochondrion-sized, membrane-bound space (Figs 7 and 8). Whether these images correspond to the digestion of mitochondria by lysosomes as described by Lockshin and Beaulaton (1974) cannot be determined, but they are structurally similar. Contractile elements become increasingly disorganized throughout the prepupal period, but are still clearly present through the first day of pupation. The Z-band, instead of having a reticular appearance in cross section, is present as tufts of electron-dense material. Groups of myofilaments are no longer clearly partitioned into myofibrils (Fig. 8).

Large numbers of electron-opaque granules or droplets were found within the sarcoplasm early in the prepupal period (as early as the sixth day of the fifth instar) and were present in varying densities in some fibers as late as we sampled (on

Table 1. *Dimensions of AB and C muscle fibers from early fifth-instar larvae and 6 h pupae*

	AB fibers		C fibers	
	Larva	Pupa	Larva	Pupa
Area (μm^2)	7703.34±2941.44	2419.59±740.90	5970.74±1766.93	2773.91±481.88
Circumference (μm)	849.92±137.55	1237.52±439.80	667.21±236.18	1283.42±244.75

Values are mean±S.D. (N=5).

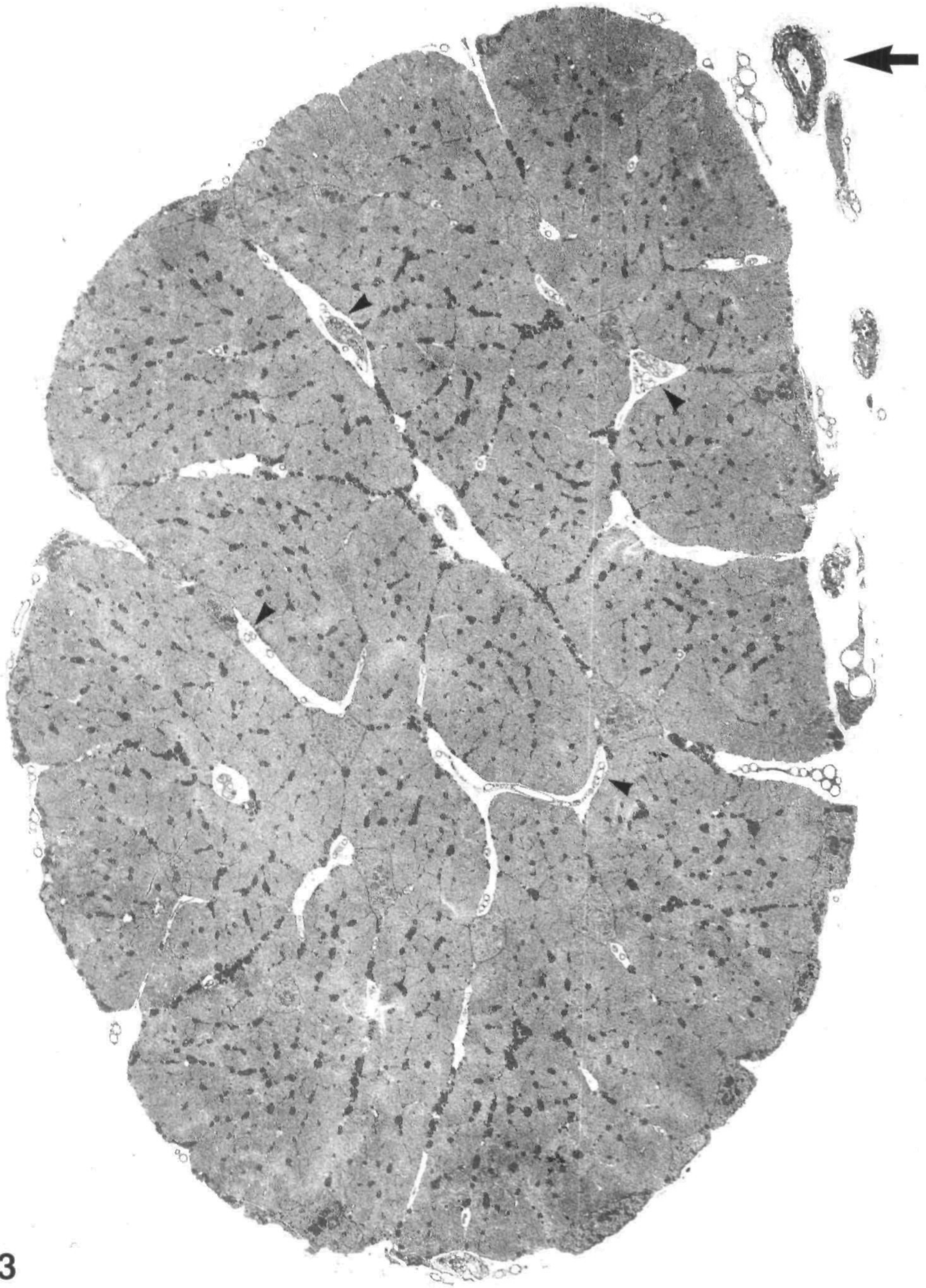

Fig. 3. Transmission electron micrograph cross section through a single larval fiber from muscle C. The innervating nerve twig with a single large motor axon is visible (arrow). Tracheoles (arrowheads) are present within the deep clefts. 1920×.

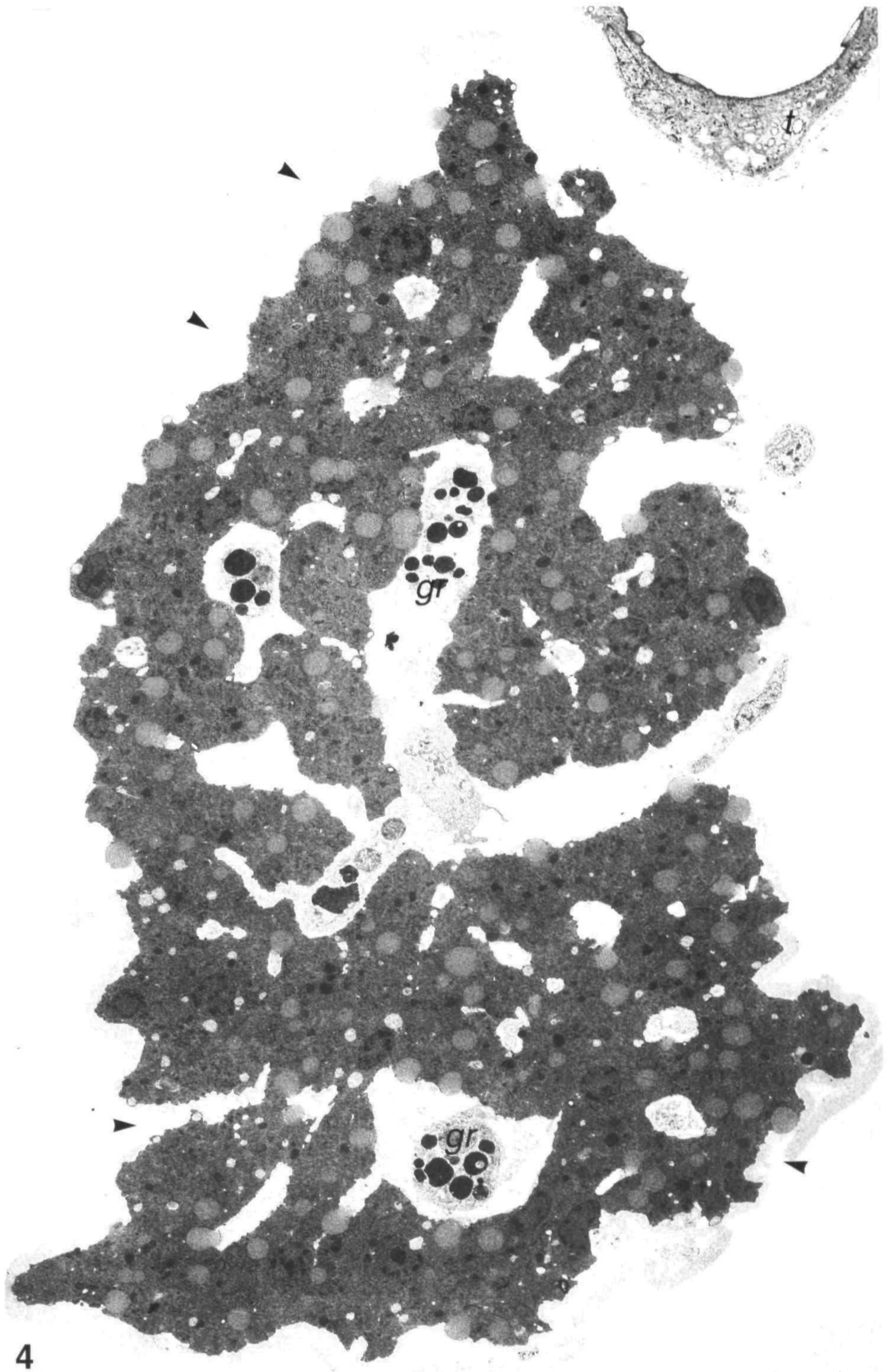
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Fig. 4. Transmission electron micrograph cross section through a single pupal AB muscle fiber, 2 h post ecdysis. The basal lamina is interrupted in several places (arrowheads) and granulocytes (*gr*) are present within the clefts in the fiber. A cluster of tracheoles (*t*) is to the upper right. Many large round inclusions, presumptive liposome granules, are evident within the cytoplasm. 1980×.

pupal day 1) (Figs 4, 7 and 8). The mean diameter of these droplets was $1.09 \pm 0.18\,\mu m$, $N=20$ (TEM measurements) or $1.55 \pm 0.20\,\mu m$, $N=20$ (SEM measurements). Many of these granules appeared to be in the process of exocytosis from the muscle fiber as seen both in transmission and scanning

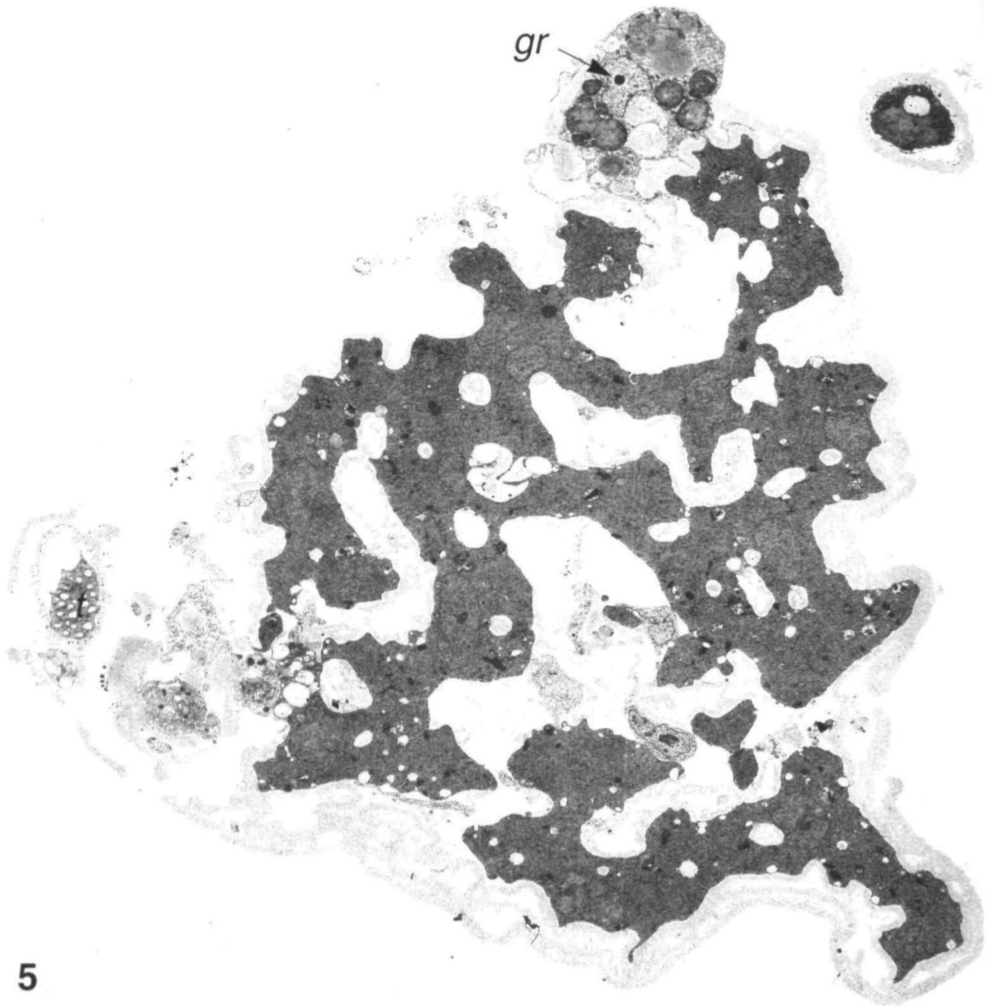
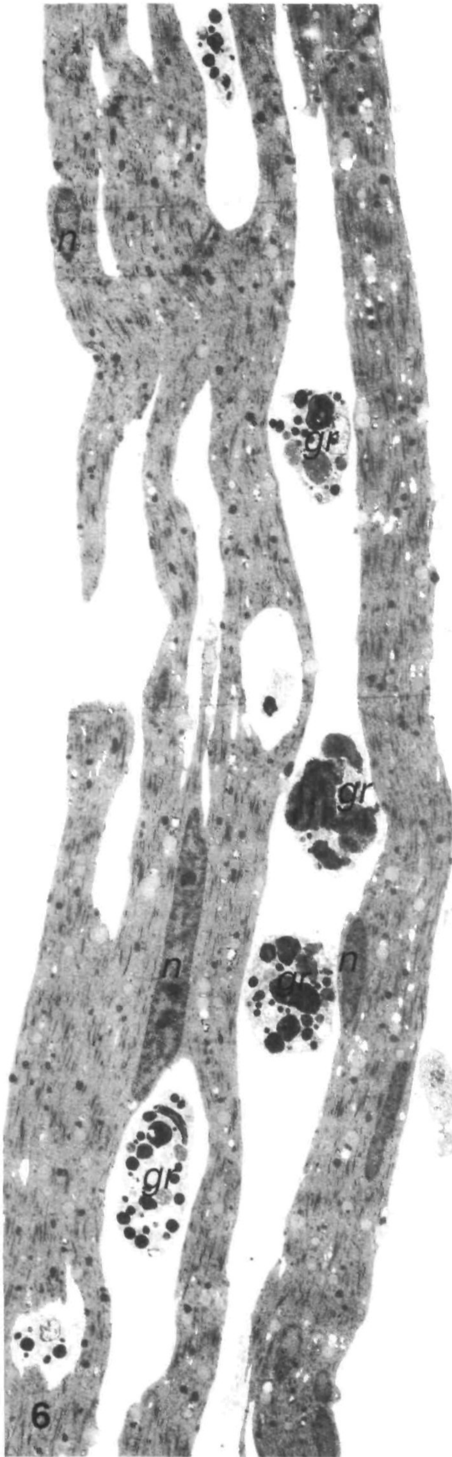


Fig. 5. Transmission electron micrograph cross section through a C muscle fiber from a 12 h pupa (compare with Fig. 4), illustrating the further shrinkage that occurs rapidly after the pupal ecdysis. Fewer liposome granules are present. Granulocyte, *gr*; cluster of tracheoles, *t*. 2200×.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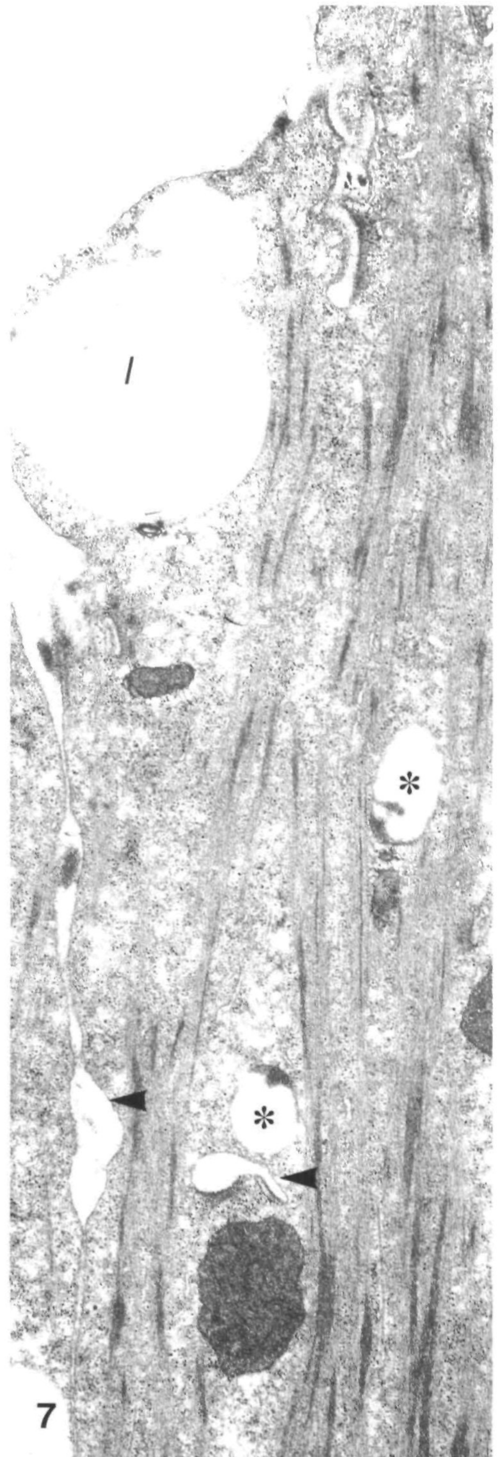
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Fig. 6. Low-magnification transmission electron micrograph longitudinal section through an AB fiber from an 8 h pupa, illustrating the extent to which deep clefts divide the fiber. Numerous granulocytes (*gr*) are present and much of the basal lamina is gone. Nuclei (*n*) are fairly evenly distributed. 1200×.

Fig. 7. Longitudinal section through an AB fiber from a 2 h pupa. Dilations in the T-tubules are indicated by arrowheads. Two holes containing electron-dense debris (asterisks) may represent remnants of mitochondria. *l*, liposome granule. 18 600×.

electron micrographs (Figs 8 and 9). Some had clear cores in TEM sections, possibly reflecting the solubility of the contents in the various solvents used in tissue preparation.

We obtained the impression that the AB fibers had a greater density of these granules than did C fibers from day 1 pupae. We also had the impression that their frequency of occurrence reached a maximum at about 2 h post ecdysis. However, there was appreciable variability in granule frequency within fibers from the same muscle or within the same fiber at different levels of the block, making it impossible without a larger sample size to determine statistically if the two muscles differed in the time course of the droplet formation and discharge. These large granules closely resemble structures termed liposome granules by Lockshin and Beaulaton (1974) in ultrastructural studies of degenerating intersegmental muscles of adult Lepidoptera.

Several authors have observed blebbing and separation of membrane-bound portions of cytoplasm from insect muscle fibers, either with or without an included nucleus, as part of metamorphic degeneration (pycnotic nuclei, final stage of degeneration, Beaulaton and Lockshin, 1977; blebs without nuclei, Crossley, 1972*a*; portions of the cytoplasm, aphid, Johnson, 1980). In the development of the dorsal longitudinal flight muscle of the lepidopteran *Antheraea*, extruded larval nuclei and the adherent cytoplasm were suggested to become the myoblasts, which increase in number and fuse to form the adult muscle (Eigenmann, 1965). In muscles AB and C through the first day of pupation we saw no clear indications of blebbing of either the cytoplasm or the nuclei. The extreme shrivelling of the fibers (Fig. 5) gave rise to images in which portions of a single fiber appeared separate, but connections to the rest of the fiber were often observed in other planes of section. Nuclear profiles were counted in low-magnification micrographs of cross sections of entire fibers from AB and C muscles comparing fifth instars with various ages of day 1 pupae (Table 2). No decline in numbers of nuclei during this

Table 2. *Mean number of nuclear profiles per muscle fiber cross section*

	AB fibers	C fibers
Fifth instar	19.5±11.5 (10)	14.6±7.4 (10)
Ecdysis+2–4 h	17.1±5.4 (10)	23.1±7.5 (9)
Ecdysis+6–8 h	18.0±5.4 (10)	13.1±4.1 (9)
Ecdysis+12–13 h	15.0±4.9 (10)	21.6±8.7 (10)

Values are mean±S.D. (*N*).

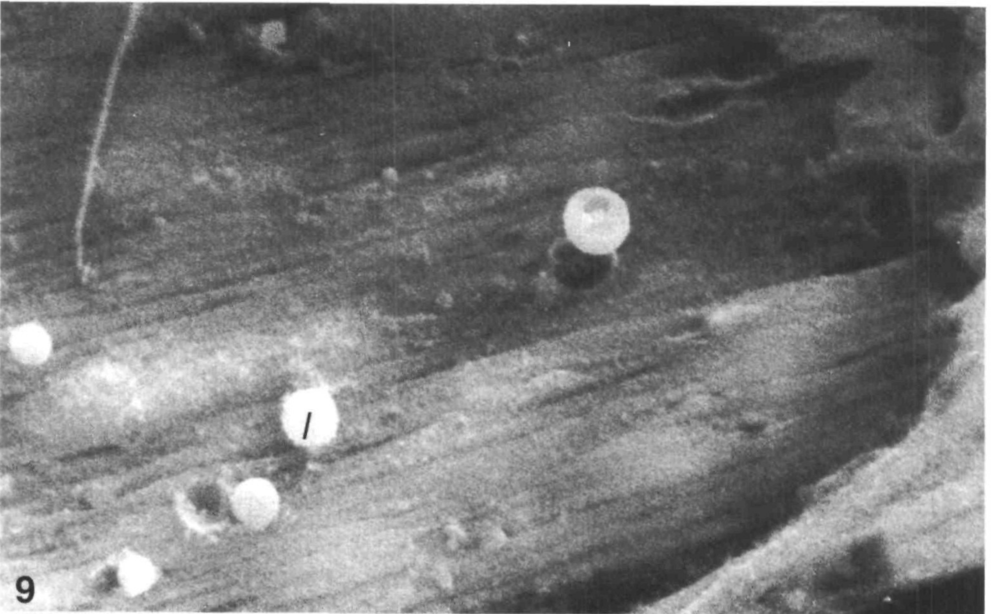
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Fig. 8. Cross section through an AB fiber from a 2 h pupa, illustrating the apparent discharge of a liposome granule (*l*). T-tubules are dilated (arrowheads), and thick and thin filaments are disorganized into smaller than usual clusters. 36800×.

Fig. 9. Scanning electron micrograph of the surface of an AB fiber from a day 7 fifth-instar (prepupal) larva. What appear to be liposome granules (*l*) on the basis of their size can be seen adjacent to craters on the surface membrane. The tissue was treated with hydrochloric acid and collagenase, prior to osmication, to remove the basal lamina. The granules appear to maintain structural integrity after being extruded into the extracellular fluid, at least in the presence of fixative. 460×.

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period was apparent, although such processes could very well occur either later in pupation or in regions of the muscle that we did not sample.

The tracheole system supplying oxygen to the muscle fibers underwent distinctive degenerative changes during the prepupal and early pupal period. The fine tracheole branches, which are ordinarily intimately associated with the muscle fiber, lying deep within the clefts and penetrating the T-tubules, instead clustered in groups adjacent to it (Figs 5 and 10). Some groups of tracheoles were surrounded by a common cytoplasm, and some appeared to be collapsing. Dysfunctional tracheole systems have been observed in the early stages of degeneration in several insect muscles (Randall and Pipa, 1969; Beaulaton and Lockshin, 1977; Lockshin and Beaulaton, 1979).

Various additional cell types were associated with the degenerating muscle fibers. The most prominent were large granulocytes (also referred to as adipo-hemocytes or phagocytic hemocytes). These were primarily spherical cells with the capability of forming a halo of numerous thin processes and which characteristically contained round inclusions of variable electron-density. As early as day 4 of the fifth instar they congregated outside the basal lamina of the main nerve trunk where it entered the muscle, and they increased in numbers particularly in the region of the anlagen and over the degenerating fibers on subsequent days (Figs 2, 4, 5, 6 and 11). They were found outside the basal lamina as well as underneath it within clefts in the muscle fiber. The granulocytes ranged in diameter from 7 to 12 μm as seen in SEM and TEM micrographs (mean 8.9±2.5 μm, N=16, from sectioned material).

The thick basal lamina around the degenerating muscle fibers was thrown into folds because of their extensive shrinkage, and images suggesting that the granulocytes participated in, or were very closely associated with, its removal were common. Figs 13 and 14 illustrate the association of a cluster of granulocytes with a region in which the basal lamina was missing from a muscle fiber. Processes of the granulocytes frequently appeared to be surrounding portions of basal lamina (Fig. 11). Vacuole-like structures within the granulocyte contained either material with the same appearance as the basal lamina or, occasionally, as the droplets seen exocytosing from the muscle fiber. Coated pits and dilated cisternae of rough endoplasmic reticulum were numerous within their cytoplasm (Fig. 12). Cells of this description have been described as participating in the phagocytosis of the neural lamella from degenerating ventral nerve cords of *Manduca* (McLaughlin,

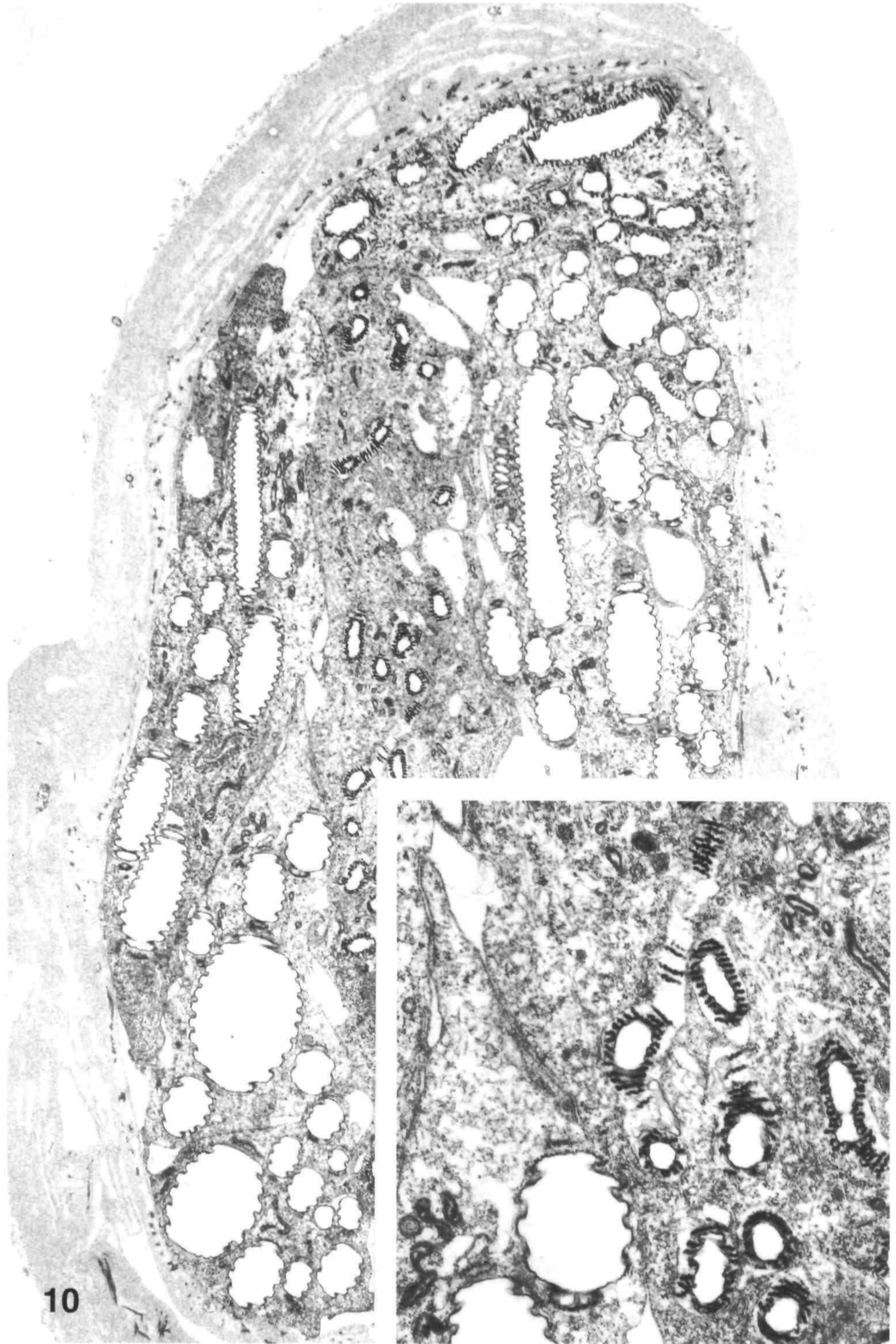
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Fig. 10. Tracheole cluster from a 12 h pupa, 6650×. The inset shows two relatively normal tracheoles (left) and some in which the chitinous rings appear to be unravelling. 17 900×.

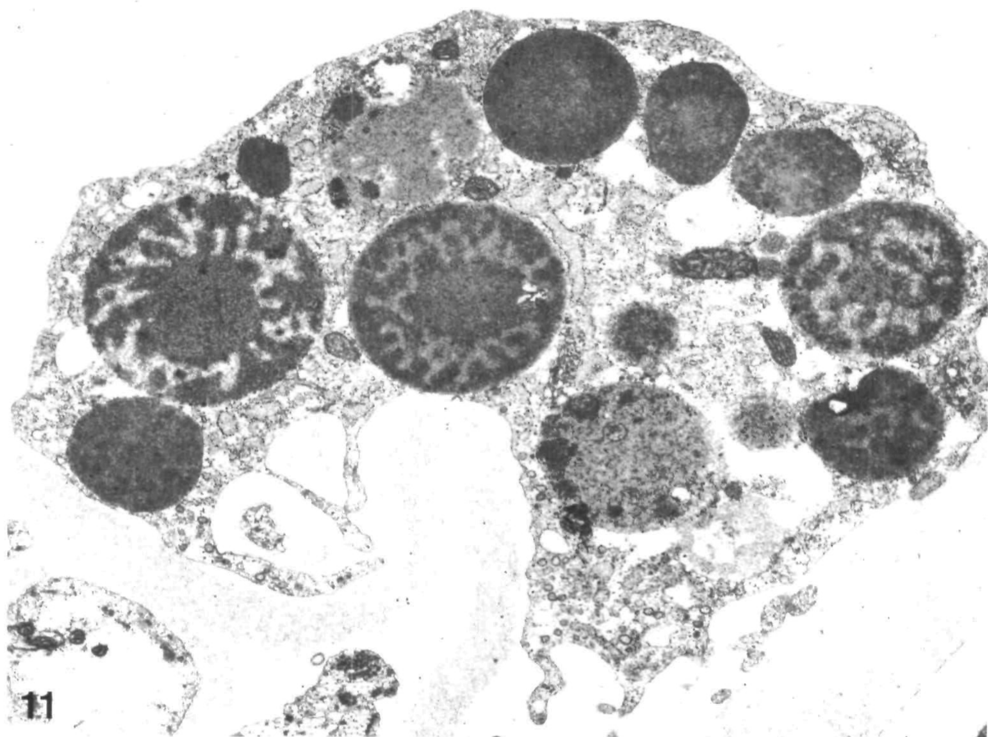
1974) and to increase in number during metamorphosis of silkworms (Walters, 1970).

Discussion

Several striking ultrastructural changes occur in the larval muscles of *Manduca sexta* as metamorphosis begins. The tracheoles withdraw from their intimate association with the muscle fibers and may be found clumped together in a common cytoplasm adjacent to them. The muscle fibers themselves shrivel, throwing the basal lamina into folds, and large numbers of hemocytes congregate in and around the basal lamina in association with its removal. The contractile elements degenerate, and the sarcoplasm is characterized by swollen T-tubules and degenerating mitochondria. What appears to be lipid material coalesces into large numbers of droplets or granules in the cytoplasm, which are then apparently exocytosed; there is a resultant change in dimensions of the muscle fiber.

The formation of both large electron-lucent granules and electron-dense granules in degenerating insect muscle fibers has been noted in several studies of developmental changes, and it is not always clear which are comparable to the granules we see, since the appearance is likely to be dependent upon the method of preparation, and the contents of the granules have not been identified in our studies or most of those reported elsewhere. Crossley (1972*a*) describes the presence of both electron-lucent 'droplets' and electron-dense granules within the cytoplasm of larval intersegmental muscles shortly after puparium formation in *Calliphora erythrocephala*. Granules similar to the 'electron-lucent' type are found within the cytoplasm of phagocytic hemocytes and floating in the hemolymph adjacent to degenerating muscle, and have been identified as 'lipid droplets' on the basis of light microscopic histochemical results (Crossley, 1968). The figures of Zachary and Hoffmann (1980) illustrating the thoracic muscles from *Calliphora* in culture show similar electron-lucent granules in the muscles that are destined to participate in the formation of adult flight muscles. Unnithan and Nair (1977) report that 'lipid droplets' are more common in the muscle fibers undergoing degeneration in response to application of juvenile hormone in the adult bark beetle, *Ips paraconfusus*, but do not illustrate them or describe them further.

Lockshin and Beaulaton, in a series of papers describing the degeneration of the abdominal intersegmental muscle after the imaginal ecdysis of the moth (Lockshin and Beaulaton, 1974; Beaulaton and Lockshin, 1977; Beaulaton, 1986; Beaulaton *et al.* 1986), illustrate both an electron-lucent granule similar to the one we see in the degenerating muscle of *Manduca*, which they term a liposome or liposome granule, as well as structures which they variously call osmiophilic bodies or lipoid droplets. In a more recent study (Beaulaton *et al.* 1986) they rename these latter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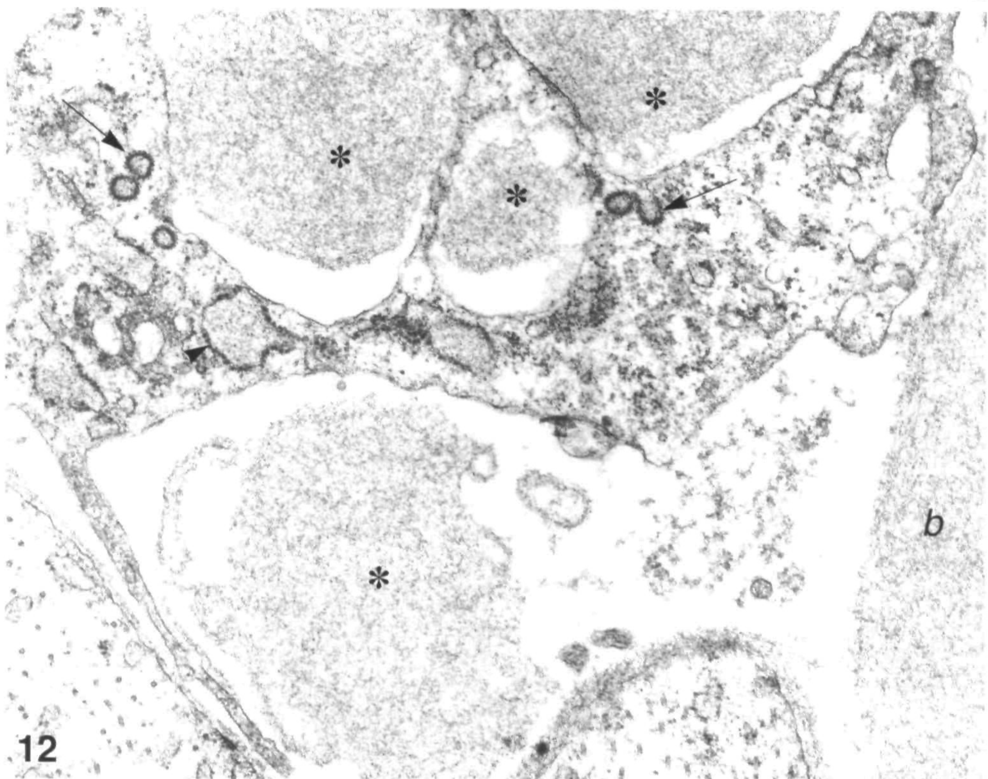
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Fig. 11. Granulocyte at the surface of an AB fiber from an 8 h pupa. The texture of the granular inclusions is quite variable. These cells often form processes which appear to engulf parts of the basal lamina. 12200×.
Fig. 12. Portion of a granulocyte illustrating encircled regions (asterisks) which have the same appearance as adjacent basal lamina (*b*). The cytoplasm of the granulocyte has dilated cisternae of rough endoplasmic reticulum (arrowhead), coated pits and coated vesicles (arrows). 30850×.

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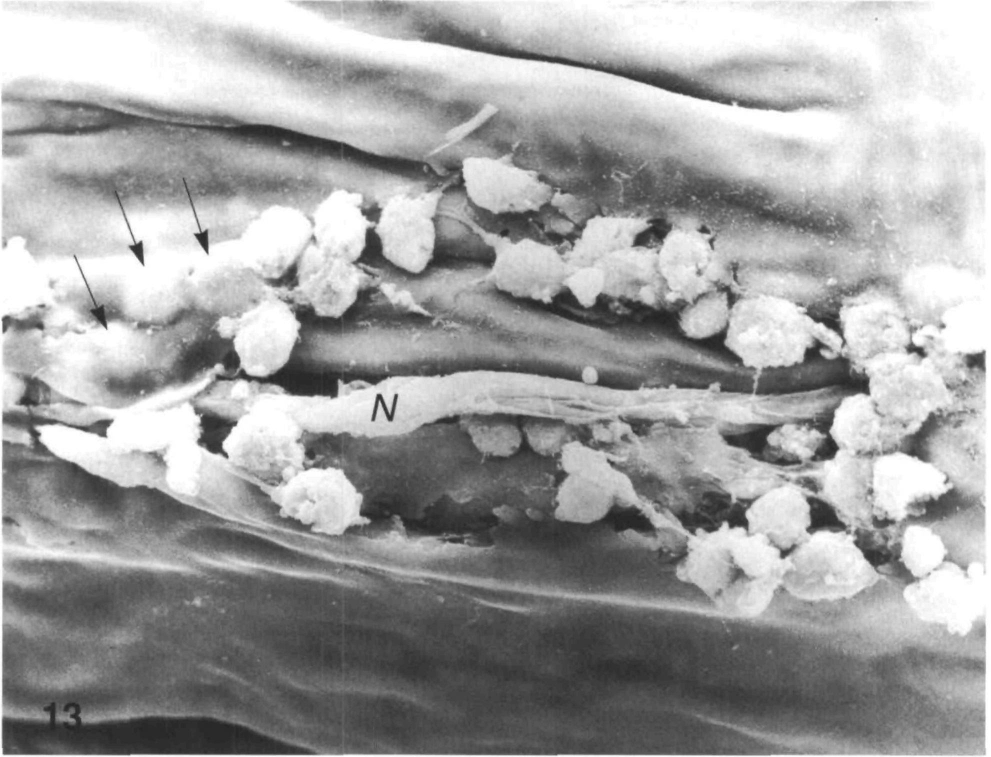
structures 'electron dense droplets' after noting that they are not osmiophilic. The 'electron dense droplets' may be distinguished from the liposome granules by the fact that the former occur within the lumen of the T-tubules, whereas the latter seem to be formed within the cytoplasm.

Thus far, electron-lucent droplets with the appearance of the structures termed liposome granules have been reported only from the cytoplasm of muscles whose degeneration is likely to be hormonally regulated and is part of a normal process of development. They have not been reported in studies of degeneration associated with nerve transection (Rees and Usherwood, 1972; Wood and Usherwood, 1979) or caused by tenotomy and joint immobilization (Jahromi and Bloom, 1979). However, the formation of the liposome granules was not described in the metamorphosis of the proleg plantar retractor muscle in a lepidopteran (Randall and Pipa, 1969) or in the irreversible breakdown of the flight muscle of aphids (Johnson, 1980). These observations make it impossible to derive clear generalizations, but do suggest that the formation of liposome granules may occur only during a relatively narrow window in time (which may have been missed in some studies) and/or may occur only in certain muscles during hormonally controlled degeneration.

A massive exocytosis of these liposome granules has not been described previously, although Beaulaton and Lockshin (1977) did suggest that exocytosis may occur. This is an interesting observation from the point of view of explaining the significant decrease in mass of the muscle fibers which occurs before there is any evidence for phagocytosis by hemocytes. The appearance of the granules also suggests this may be a way of recycling materials for further development by extracting them selectively from the cytoplasm and making them available in the hemolymph. The exact composition of the droplets and the mechanisms by which they are formed within the cytoplasm are of interest from a cell biological point of view, since the droplets appear to begin forming within the cytoplasm without an obvious limiting membrane. The borders of the granules are more electron-dense than the rest of the granule, but a clear membrane was usually lacking under the present fixation protocols. Presumably one has formed or is created at the time of exocytosis to preserve the integrity of the cell, but concrete evidence of the process is presently unavailable.

The exocytosis of the granules or droplets is associated not only with a decrease in volume of the pupal muscle fiber but also with an apparent increase in surface area compared with the day 2 fifth-instar stage examined in these studies. The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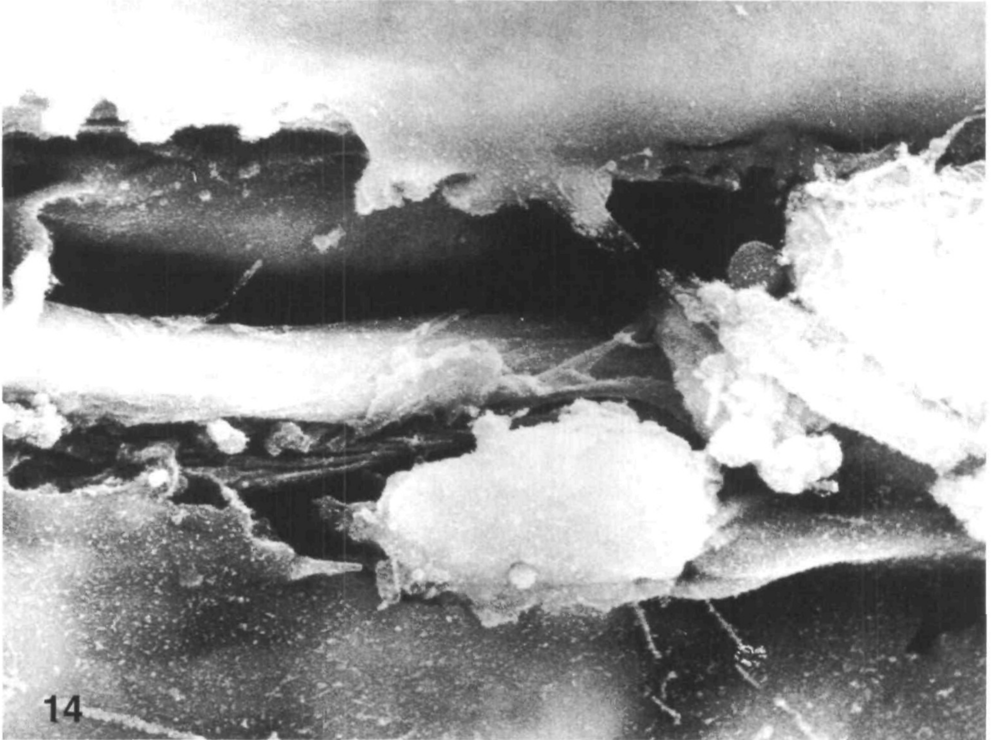
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Fig. 13. Scanning electron micrograph of a muscle fiber from a day 8 fifth-instar (prepupal) larva. A cluster of granulocytes appears to be removing the basal lamina. A nerve twig (*N*) is running along the surface of the fiber. Note the hazy outlines of granulocytes which are underneath the basal lamina (arrows). 670×.

Fig. 14. Scanning electron micrograph at higher magnification of an adjacent region of the fiber shown in Fig. 13 to illustrate the appearance of the edge of the basal lamina and the irregular profiles of the granulocytes. 2970×.

increase in surface area may arise from the addition of membrane to the surface during exocytosis of the granules. However, shrivelling of the fiber might also expose membrane which was formerly part of the T-tubular system and not included in the measurements of the larval fibers. It is also possible that the fibers may have increased further in both cross-sectional area and perimeter on days 3 and 4, before beginning to shrivel during the prepupal period, leaving a greater relative perimeter in the degenerating fibers, depending upon the degree to which the cytoplasmic volume and membrane surface area can decrease independently of each other.

The reduction in cross-sectional area and the increase in surface membrane of the degenerating fibers would be expected to affect the cable properties when the pupal fibers and the day 2 fifth-instar larval fibres are compared. One would predict an effect on the input resistance and the time constant of the muscle fiber, and subsequently on the electrical response to synaptic input. Lockshin (1973) reported an initial threefold increase and then a decrease in effective membrane resistance during degeneration of the intersegmental muscles from silkmoths. This occurred at roughly the same time that their volumes were decreasing, with volume being estimated from the diameter measured with an ocular micrometer and assuming a circular cylindrical shape.

The degenerating muscle fibers that we have examined are not circular in cross section, so that it may not be appropriate to estimate their expected input resistance from the usual versions of the linear cable equations, where input resistance varies as the $-3/2$ power of the fiber radius (Jack *et al.* 1975). Instead, input resistance, R_{in}, can be better estimated by going back to the original form of the cable equations and using actual values for muscle fiber perimeter and cross-sectional areas. If one assumes that the specific membrane resistance and cytoplasmic resistivity remain constant (not necessarily a valid assumption), the input resistance of degenerating fibers would be expected to increase by only 1.25 times that of larval fibers on the basis of the dimensions in Table 1. In these calculations, the effect of including the increase in surface membrane is to minimize the effect of the decrease in the cross-sectional area on the input resistance.

These predicted values are substantially less than the threefold increase measured electrophysiologically from degenerating fibers by Lockshin (1973), but we have no direct measurement of input resistance for comparison. The discrepancy could either arise from the stage examined (the fibers he measured

could have degenerated to a greater degree) or because specific membrane resistance may not be constant between the two stages. In any case, an increase in input resistance would result in an increase in the effectiveness of the existing synapses; this question is explored in a subsequent paper (Rheuben, 1992; M. B. Rheuben and S. M. Baer, in preparation).

The importance of the composition of the basal lamina to various developmental processes has been pointed out in numerous preparations. Regulation of the development, organization and position of the presynaptic active zone has been attributed to the basal lamina enveloping the muscle fiber (Sanes *et al.* 1978; Nystrom and Ko, 1988; Ko and Folsom, 1989). In grasshopper embryos, the removal of basal lamina by elastase is associated with retraction of axonal growth cones, suggesting an adhesive interaction between growth cone and lamina (Condic and Bentley, 1989). In the wing imaginal disc of *Manduca*, concomitant with a rise in ecdysteroid levels, tracheae begin to migrate through an extracellular matrix of changing histochemical affinities. At the same time, a basal lamina appears on the surface of epithelial cells and sensory neurones send processes along this surface (Nardi *et al.* 1985); a guiding function is proposed for the lamina. Anderson and Tucker (1988, 1989) provide evidence for variation in structural organization of the basal lamina with development and for the many filopodial contacts of pioneer neuronal growth cones with the basal lamina; they also interpret their results as supporting the idea that the basal lamina has a role in guiding the outgrowth of developing axons. Lebart-Pedebas (1984) observed that myoblasts appear to be restricted in their migration towards larval muscles to a path beneath the basal lamina of the motor nerve supplying the muscle. Consequently, it is particularly interesting, but perhaps not surprising, that we should observe what appears to be a highly organized method of removing the basal lamina from the larval muscle fibers at a point in development just preceding major changes.

Both thin section and scanning electron microscopic images provide circumstantial evidence that the basal lamina is being removed specifically, by a process of phagocytosis by granulocytes or phagocytic hemocytes, from the degenerating larval muscle fibers prior to their losing function and structural integrity. This process appears to be quite similar to that used by human neutrophils to degrade extracellular matrix in tightly localized regions in inflammatory disease states (Rice and Weiss, 1990).

The participation of hemocytes in the removal of basal lamina from the neural sheath during metamorphosis in *Manduca* has been suggested previously, on the basis of essentially similar electron microscopic observations, by McLaughlin (1974). More recent direct evidence for the process has been provided by using monoclonal antibodies. In developing locusts, an antibody to components of the basal laminae over muscle fibers and nerve also labels compartments within hemocytes, implying that they participate in their formation (Ball *et al.* 1987). Similarly, Nardi and Miklasz (1989) found that antibodies raised against wing tissues of *Manduca* recognized epitopes seen in the basal lamina and, at certain

times, within the hemocytes. Their ultrastructural observations suggested that hemocytes participated both in the laying down and in the phagocytosis of the basal lamina. Our observations are consistent with a special population of hemocytes having the function of removing the basal lamina from the larval AB and C mesothoracic muscle fibers studied here. Whether these hemocytes go on to engulf fragments of the muscle itself remains to be determined.

The mesothoracic larval AB and C muscle fibers eventually participate either directly or indirectly as a scaffolding in the formation of the adult muscle. Myoblasts appear to fuse with each other and with the degenerating larval muscles at a later stage (M. B. Rheuben, unpublished results) in a way similar to the processes seen in flies (Crossley, 1972*a*) and in the lepidopteran *Antheraea polyphemus* (Stocker, 1974). The removal of the basal lamina might be a key step to allow this fusion. In addition, the motor nerves must regrow to form new junctions and may rely upon guidance cues from a newly secreted basal lamina. In the dorsal longitudinal muscle, the adult basal lamina is much thinner than that produced around larval fibers (M. B. Rheuben, unpublished results); this difference in thickness is also found as development proceeds in the wing, where the adult and larval basal laminae are also found to bind antibodies to different degrees (Nardi and Miklasz, 1989). The different composition of adult and larval basal laminae suggests the opportunity for the incorporation of different guidance mechanisms during development; other functional differences between adult and larval laminae remain unexplored.

During the prepupal and early pupal period, the tracheoles supplying the larval muscles withdraw from intimate association with the fibers to form clusters adjacent to them. In addition, mitochondria appear to be degenerating. At this time, the most notable physiological change in the functioning of the muscle fiber is a decline in the amplitude of the resting membrane potential (Sonea and Rheuben, 1992). The structural observations and physiological changes may be related. A large part of the muscle fiber resting potential in Lepidoptera is dependent upon oxidative metabolism (Huddart and Wood, 1966; Rheuben, 1972; Djamgoz, 1986). Experimentally, maintenance of a normal resting potential is dependent upon adequate oxygenation directly *via* the tracheoles in some larval (Yamaoka and Ikeda, 1988) and adult insect muscles (Rheuben, 1972; Djamgoz, 1986). Although the time course is not quite the same, Lockshin and Beaulaton (1979) have noted a correlation between the withdrawal and collapse of tracheoles in the intersegmental muscles of *Antheraea* and the relatively abrupt reduction in the resting potential of the fibers. Bidlack and Lockshin (1976) measured a loss of respiratory enzymes as degeneration of the intersegmental muscle began. Consequently, the decline in resting potential observed (Sonea and Rheuben, 1992) in these fibers over the prepupal and early pupal period may result from the combined effects of the withdrawal of the respiratory tracheoles from the larval fibers and of impairment of the metabolic function in these muscle cells, as signalled by the degeneration of the mitochondria.

Although the anlagen forms among these muscles, and there is subsequently a

massive fusion of myoblasts to and around them, the precise fate of all the cellular components of the larval fibers is not yet known. The ultimate fate of the larval muscle nuclei is of interest because of the various reports regarding their participation in the adult muscle fiber. Eigenmann (1965) thought that some *Antheraea* larval muscle nuclei with adherent cytoplasm separated from the muscle fibers and became the myoblasts, subsequently fusing in large numbers to form the adult fiber. Crossley (1972*a*,*b*) observed fusion of myoblasts, possibly originating from imaginal discs, to residual larval fibers and the consequent presence of two distinct populations of nuclei that remained throughout further development of the adult muscle of *Calliphora*. At this early stage we did not find any decrease in the number of larval nuclei per muscle fiber or any evidence for a contribution of larval nuclei and adherent cytoplasm from these muscles to the population of myoblasts that later appear in great numbers, but we cannot preclude that it may happen in other muscles or at a later time. In *Manduca* the origin of the myoblasts has not yet been determined.

The processes by which specific muscles degenerate during metamorphosis appear to be highly variable, as described above. Since some muscles in insects degenerate and disappear completely during metamorphosis and others involute and participate in the formation of the adult muscle, structural and physiological differences are to be expected. Zachary and Hoffmann (1980) found that cultured larval muscles from *Calliphora* differed in their responses to 20-hydroxyecdysone depending upon whether they were removed from the animal before a critical period 16 h before pupariation and upon whether they belonged to the category destined to disappear or to the one destined to form adult muscle. Those destined to persist *in vivo*, if excised after the critical period, underwent involution in the presence of 20-hydroxyecdysone but did not degenerate. This suggests that innate characteristics of specific muscles as well as external timing cues are important. The results of examining a given muscle during metamorphic degeneration are likely to be quite specific to the individual muscle and to the insect species; this complexity necessitates both caution in comparing the data from different muscles as well as providing the opportunity to examine interesting developmental mechanisms.

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