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Fine Structure of Degenerating Abdominal Motor Neurons after Eclosion in the Sphingid Moth, *Manduca sexta*

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Summary. Ultrastructural aspects of the natural degeneration of a group of six motor neurons in the fourth abdominal ganglion of *Manduca sexta* are described. These motor neurons innervate intersegmental muscles that degenerate and disappear immediately after adult eclosion. The first detectable changes in the cell bodies appear 12 h after eclosion and include disruption of the endoplasmic reticulum and an increase in the size and number of lamellar bodies. At 32 h the nuclear membranes rupture, and the membranous and granular cytoorganelles segregate in different parts of the cell. At that stage the surrounding glial cells participate in the digestion of material from the degenerating neurons. From 72 h onward the remaining neuronal structures become disrupted, and are finally transformed into a single, large lamellar body (residual body) within the glial profile. The degeneration pattern differs significantly from that of embryonic vertebrate neurons.

Key words: Programmed cell death – Motor neurons – Neuro-glial interaction – Ultrastructure – *Manduca* (Lepidoptera).

Introduction

The emergence of an adult insect from the investing pupal cuticle, a process known as eclosion, is the final major event of post-embryonic development, in the wake of which certain muscles and neurons degenerate. In moths, where the process of eclosion has been most studied, an eclosion hormone triggers a complex sequence of behaviors that accomplish the escape of the adult from the pupal cuticle (Truman, 1973). Shortly after emergence the intersegmental muscles of the abdomen begin to degenerate and they have disappeared within three days (Finlayson, 1956; Lockshin and Williams, 1965). The eclosion hormone serves as the signal to initiate the breakdown of these muscle groups in saturniid moths (Truman 1970; Schwartz and

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Truman, unpublished). Similarly, in the sphinx moth *Manduca sexta*, adult eclosion is triggered by the eclosion hormone and is accompanied by degeneration of the intersegmental muscles of the abdomen and of the motor neurons and interneurons that presumably control these muscles (Taylor and Truman, 1974).

By analogy with the post-eclosion breakdown of the intersegmental muscles in silkmoths it has been proposed that the degeneration of motor neurons and interneurons in *Manduca* may also be signaled by the eclosion hormone (Truman, 1973). Since only specific muscles and neurons are involved, this type of cell death is an intrinsic, preprogrammed cellular characteristic (Lockshin and Beaulaton, 1974).

This study was undertaken in order to determine whether the proposed action of eclosion hormone in triggering the degeneration of the abdominal motor neurons might be detected in early ultrastructural changes within cell bodies of the motor neurons in question. In this context the role of glial cells as possible agents of neuronal degeneration is of special interest. Comparisons with other reports of cell death in insects and in vertebrates have been made in an attempt to elucidate aspects of causal relationships in neuronal degeneration.

Materials and Methods

Tobacco hornworms (*Manduca sexta*) were reared on an artificial diet according to the method described by Truman (1972). Imagines were kept at room temperature for various times after eclosion up to 96 h. After immobilization by cooling, heads and thoraces were removed and the abdomens were opened dorsally. Abdominal ganglia 3 to 5 were dissected, prefixed over night in Karnovsky's fixative (paraformaldehyde 2%, glutaraldehyde 0.5%; see Edwards, 1971), postfixed in 2% OsO_4 and embedded in epon.

Only the fourth abdominal ganglion (A_4) of male pharate and imaginal moths was used in this study. Complete series of transverse thick sections (1.0 or 1.5 μm) were made of some A_4 ganglia and stained with Azure II/Methylene Blue (Richardson et al., 1960). In ganglia chosen for thin sectioning, the relevant neuron group was located in 1 μm sections. Transverse thin sections were then made at that level in the ganglion with a Porter-Blum MT-2 ultramicrotome. Thin sections were mounted on carbon-coated copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Philips EM 300 at 60 kV.

Results

The degeneration studies were performed with an easily recognizable group of six motor neurons in the fourth abdominal ganglion of *Manduca* (the D-IV neurons of Taylor and Truman, 1974). In whole mounts of ganglia from pharate moths (stained with toluidine-blue) these neurons are readily identified by their large size and their position at the antero-dorsal end, along the midline of the ganglion (Fig. 1A). They can also be recognized in 1 μm sections by their size and location. In later stages D-IV neurons can be identified further by their degeneration features which distinguish them from motor neurons 18 and 19 situated in the same area (Taylor and Truman, 1974) as well as from non-degenerating interneurons (Fig. 1B). Although a number of interneurons in the A_4 ganglion degenerate after eclosion, it

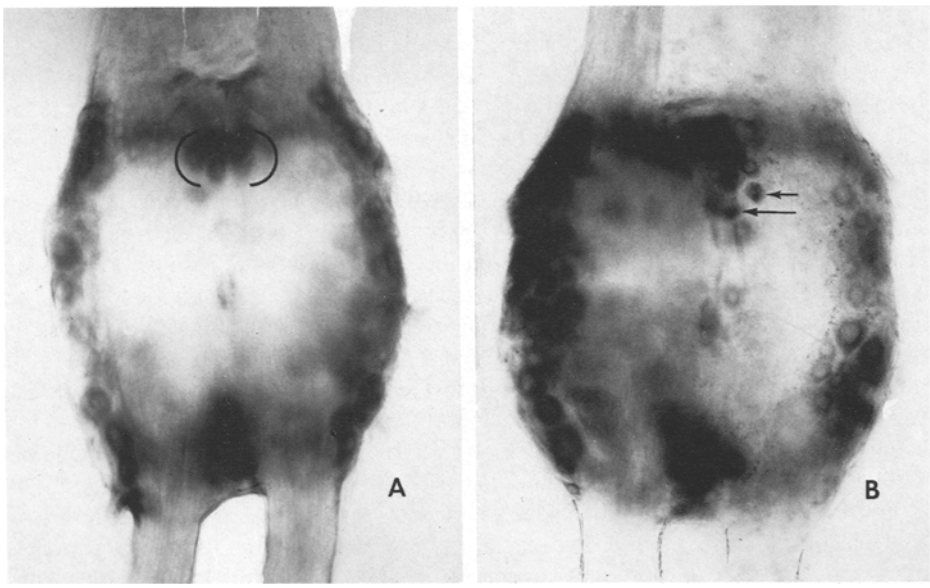


Fig. 1A and B. Toluidine-blue stained whole mounts of the dorsal surface of the fourth abdominal ganglion of *M. sexta*. **A** From a newly emerged moth. The D-IV neuron group lies between the brackets. **B** From a moth 24 h after eclosion. Arrows point to degenerating neurons

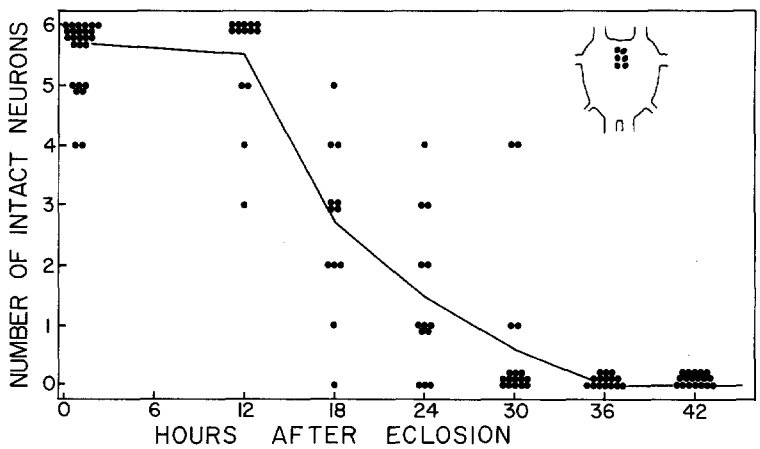


Fig. 2. The number of intact neurons in the D-IV group as a function of time after eclosion. Each dot represents a count from one abdominal ganglion. The line follows the average values

is believed, on the basis of size, that the following observations are restricted to the D-IV motor neurons.

The time course for the loss of neurons from the D-IV group was first determined using toluidine-blue stained whole mounts. Intact neurons were characterized by a distinct, light staining nucleus surrounded by a darker staining

cytoplasm. The number of cells in the D-IV group remained unchanged through the first 12 h after eclosion (Fig. 2). During the next 24 h, the number rapidly decreased so that by 36 h after eclosion no intact neurons remained in the group. The cellular remains were then removed during the ensuing days. In accord with the observations on whole mounts, the first ultrastructural signs of degeneration appeared 12 h after eclosion and the last remnants had been removed by 96 h.

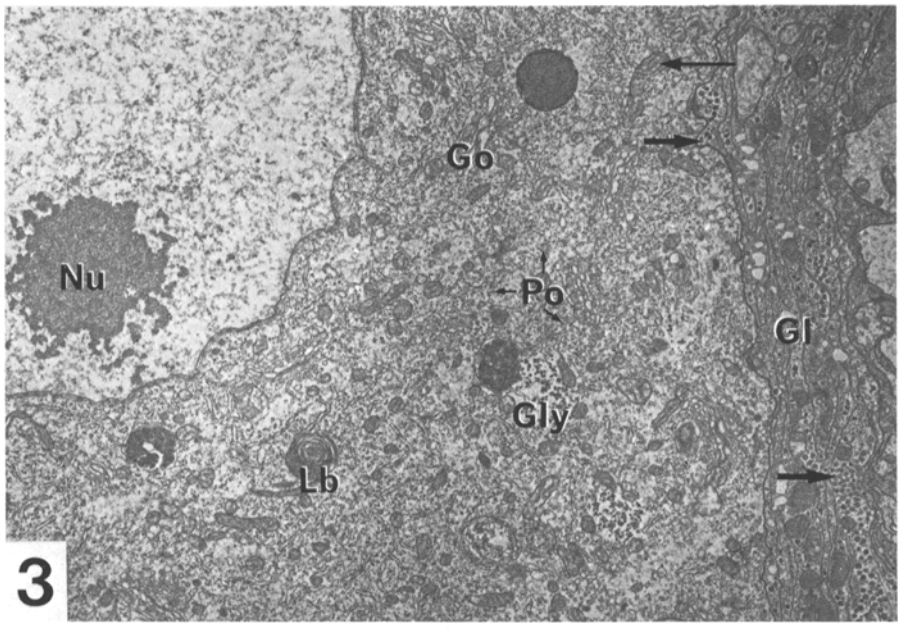
In pharate moths prior to the exposure to eclosion hormone the cell bodies of the D-IV group may measure up to 40 μm in diameter (Fig. 1A). Their nuclei have an ovoid, or in rare cases an irregular, outline. One or two very distinct nucleoli may be observed in the same section. The cytoplasm shows characteristics common to many insect neurons (Figs. 3, 4): the greater part is occupied by elongate mitochondria, a well-developed granular endoplasmic reticulum, and a large number of ribosomes which occur almost exclusively in clusters (polysomes). Cristae mitochondriales in general are longitudinally oriented. They contain many ribosome-like particles within the matrix. RER-channels as well as much less conspicuous microtubules are randomly oriented. Up to 20 Golgi bodies may be present throughout the cytoplasm in a single section. Less frequent structures in the cytoplasm, which are not seen in every section, are membrane-bound dark granules, lysosome-like lamellar bodies of various shape and size (autophagic vacuoles), and clusters of dark granules, larger than ribosomes, which are presumed to be glycogen deposits. Lamellar bodies and glycogen granules are preferentially located in the periphery of the perikaryon.

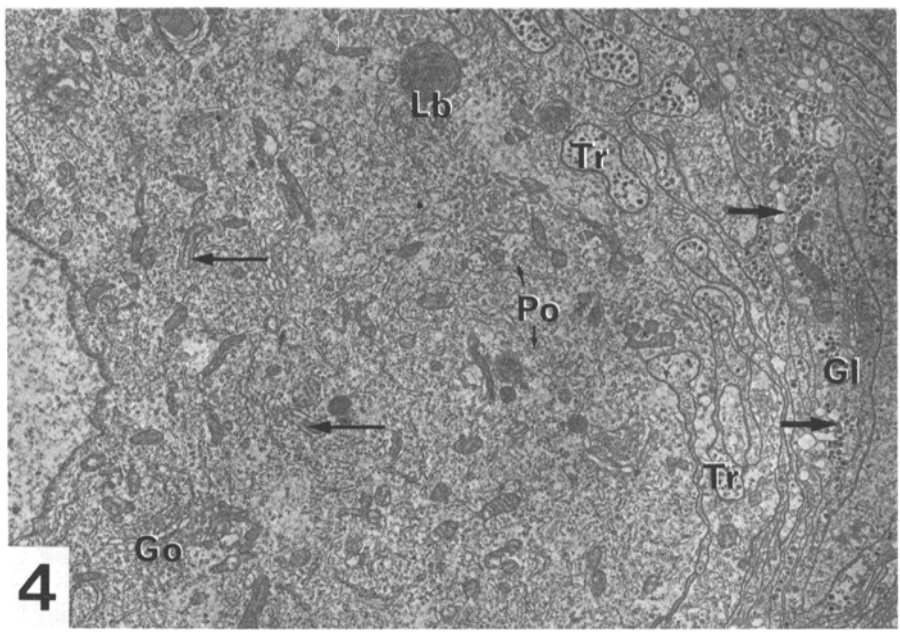
The neuron cell bodies are enveloped by multiple layers of glia, which in some areas may measure as little as 0.05 μm in thickness (Figs. 3, 4). The glial cells form deep invaginations into the nerve cell cytoplasm, the "trophospongium" described by light microscopists (see Smith, 1967). Single indentations may be formed by one or more glial sheaths. Within the neuronal cytoplasm channels of RER are frequently associated with the distal end of these fingers, as similarly reported from the stick insect brain (Smith, 1967) (Fig. 4).

The most prominent characteristic of the glial cytoplasm is the arrangement of glycogen granules arranged, unlike those of the neuronal cytoplasm, in rosettes of 0.1 μm diameter (Figs. 3, 4). The number of mitochondria, RER, ribosomes, and microtubules in glial cytoplasm is similar to that of the neuronal cytoplasm, but cristae mitochondriales are preferentially oriented in the transverse plane and rearely studded with ribosome-like particles. An additional feature of glial profiles is the presence of spherical vacuoles of 1 μm diameter containing a concentrically layered, moderately electron-dense substance. They may be identical to the lipid droplets reported from other insect glial and neural cells (Smith, 1967). Lamellar bodies of diverse shape are present in small numbers in glial profiles surrounding D-IV neurons and non-degenerating motor neurons.

The content and distribution of cytoplasmic components remains stable in both D-IV neurons and the surrounding glial profiles until 12 h after eclosion. At that time initial changes are observed in some of the D-IV neurons. However, other D-IV cells start degeneration as late as 24 h.

Changes in the neuron cell bodies are first seen in the cytoplasm, the nucleus remaining structurally intact for some time. Mitochondria change shape from elongate to ovoid and the electron-density of the matrix increases (Fig. 5). RER-





Figs. 3 and 4. D-IV neuron in the fourth abdominal ganglion of pharate adult. The neuron cell body is surrounded by multiple glial sheaths (*Gl*), which form indentations into the perikaryon (the "trophospongium": *Tr*). The neuronal cytoplasm is characterized by mitochondria with cristae bearing ribosome-like particles (thin arrows). The glial cytoplasm, in contrast, is readily identified by the presence of glycogen deposits arranged in rosettes (thick arrows). *Gly* glycogen granules? *Go* Golgi bodies. *Lb* lamellar bodies. *Nu* nucleolus. *Po* polysome clusters. × 8500

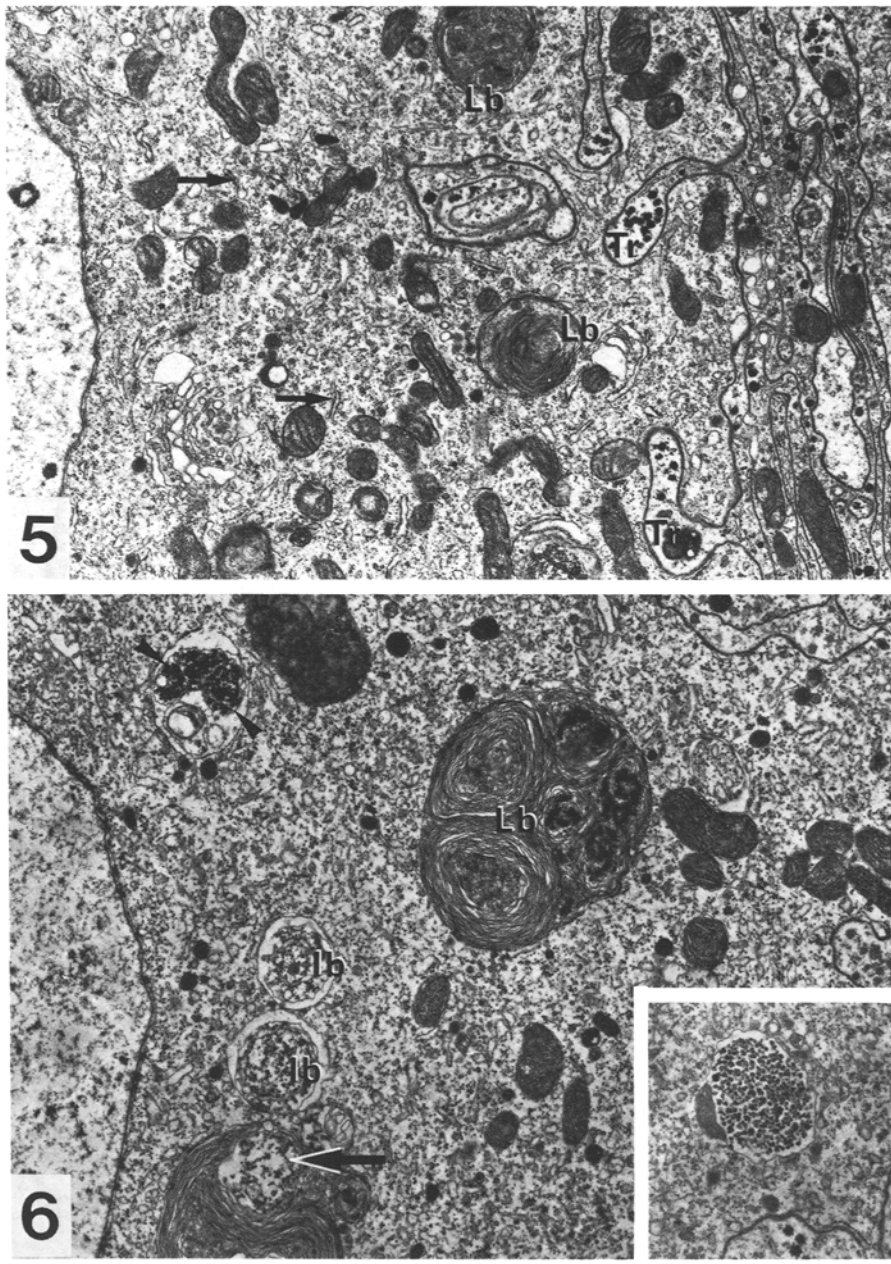


Fig. 5. D-IV neuron of a moth 12 h after eclosion showing first degenerative changes: Mitochondria have become ovoid and exhibit increasing electron-density; RER-channels are shorter than before (arrows). *Lb* lamellar bodies. *Tr* glial "trophospongium". ×11,000

Fig. 6. Degenerating D-IV neuron 18 h after eclosion. Mitochondria are rounded off, RER-channels are disrupted and ribosomes released. Isolation bodies (*Ib*) either increase in electron-density (arrowheads) or become incorporated into lamellar bodies (*Lb*) before condensation starts (arrow). The inset shows a membrane-bound neuronal glycogen deposit. ×11,000

channels become disrupted and are transformed into short, sometimes swollen bodies no more than 0.5 μm in diameter (Fig. 6). Ribosomes are apparently released from the RER and from polysome clusters. Golgi bodies decrease in number. Glycogen deposits may be enveloped by a single membrane (Fig. 6 inset).

After eclosion the most prominent changes occur in the perikarya of neurons where lamellar bodies increase both in size and number (Figs. 6, 7). At least some of them originate from isolation bodies, structures commonly observed in D-IV neurons of adults 12 h and older. Isolation bodies, which are separated from the surrounding cytoplasm by two membranes of ER, include random cytoplasmic areas containing RER, ribosomes and even mitochondria (Figs. 6, 7 inset). As they shrink their electron-density is increased and some of them become surrounded with additional membranes. Such structures represent an initial, simple type of lamellar body. However, most isolation bodies observed do not develop into separate lamellar bodies, but aggregate with pre-existing ones to form compound structures. These may contain up to ten or more elements, each of which consists of a more or less electron-dense core derived from the original cytoplasmic area and a sheath of 20 to 30 membranes.

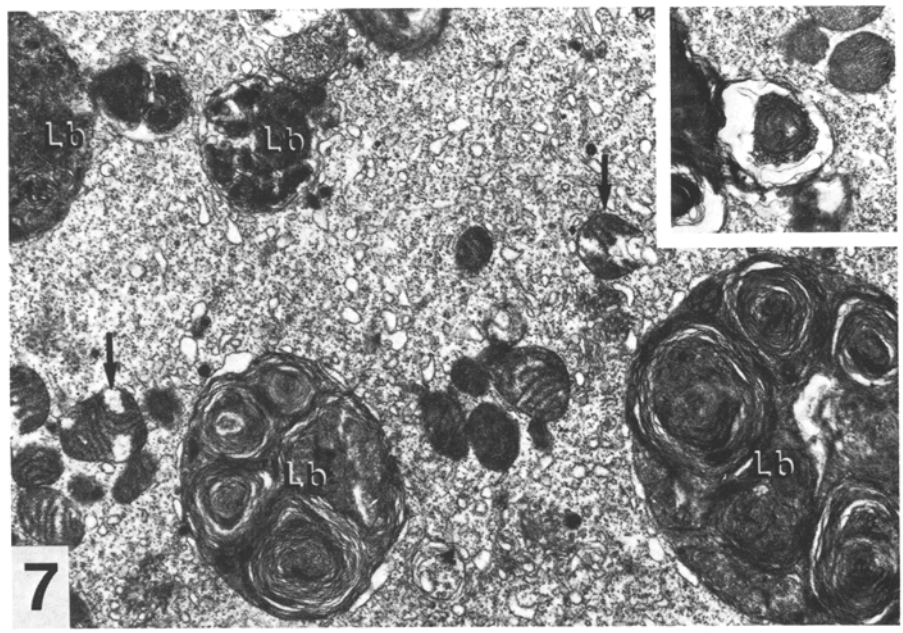
In all 18–24 h animals observed the neuro-glial "trophospongium" and the cytoplasmic content of glial cells remained unchanged.

By 32 to 48 h after eclosion further significant changes take place in the D-IV neurons. Their nuclei rupture and release their contents into the cytoplasm (Fig. 8). The karyoplasm and the cytoplasm intermingle homogeneously but the nucleolus remains as one or several patches of dark granular material within the cell body. The broken nuclear membrane consists of several randomly arranged pieces. Frequently observed parallel pairs of fragmentary nuclear membranes result from the collapse of cytoplasmic invaginations into the nucleus.

Mitochondria at 32–48 h post-eclosion tend to aggregate at the periphery of the cell where they change further in shape from ovoid to polygonal (Fig. 9). Vesicles of ER and ribosomes begin to segregate and to accumulate in separate areas (Fig. 8). The remaining Golgi bodies are disrupted and are assembled in areas rich in ER. At an advanced stage of degeneration the cell body may be divided into two distinct parts, one consisting of ribosomes and nuclear remnants, and the other containing the remainder: mitochondria, ER, Golgi vesicles and lamellar bodies.

At 32 h initial changes are observed in the neuro-glial "trophospongium" and the composition of glial cytoplasm. Glial fingers are by now composed of a single cellular layer. In general the network of neuro-glial invaginations is deepened (Fig. 9). Isolated neuronal fragments engulfed by glia or isolated glial profiles within neuronal cytoplasm are abundant. The glial cytoplasm contains numerous irregular and inhomogeneous bodies about 1.5 μm in diameter, resembling lysosomes. At 48 h these structures are frequently observed in close association with degenerating mitochondria and other cytoplasmic elements enveloped by double membranes (Fig. 10). The characteristic shape and the presence of studded cristae indicate the neuronal origin of the mitochondria. It thus seems very likely that part of the neuronal cytoplasm is taken up by the glial sheath, and digested by glial lysosomes.

The most dramatic step in the degeneration of D-IV neurons occurs at 72 h after eclosion, at which time all remaining cytoplasmic structures are disrupted. The



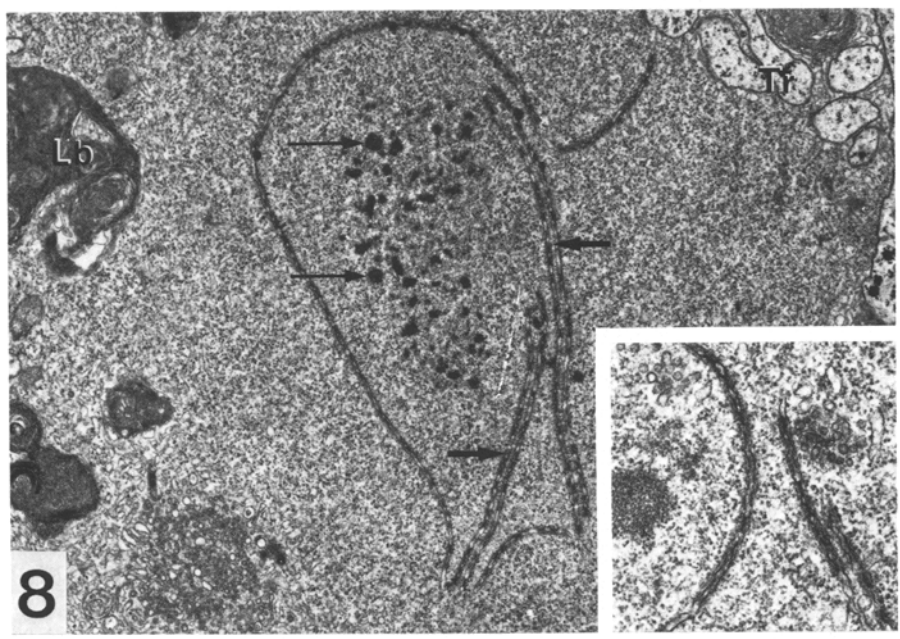
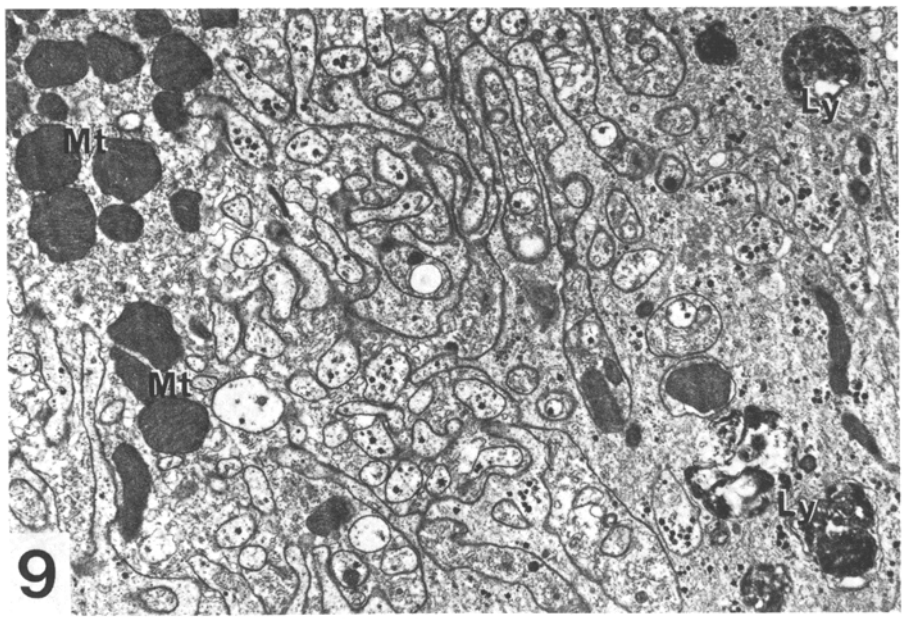


Fig. 7. $19^1/_2$ h after eclosion. The number of lamellar bodies of various types (*Lb*) is still increasing. Mitochondria show clear signs of breakdown (arrows). Inset: isolation body containing a mitochondrium. ×11,000

Fig. 8. 48 h after eclosion. The nucleus of a D-IV cell has ruptured. Karyo- and cytoplasm are intermingled. Remnants of the nucleolus are still visible (thin arrows). Parallel pairs of nuclear membranes result from collapsing cytoplasmic indentations (thick arrows). Mitochondria, lamellar and Golgi bodies (in the left corner of the micrograph) tend to segregate from ribosomes and nuclear remnants. *Lb* lamellar body. *Tr* glial "trophospongium". ×11,000. Inset shows details of the broken membranes of a nucleus collapsed at 32 h. ×20,500



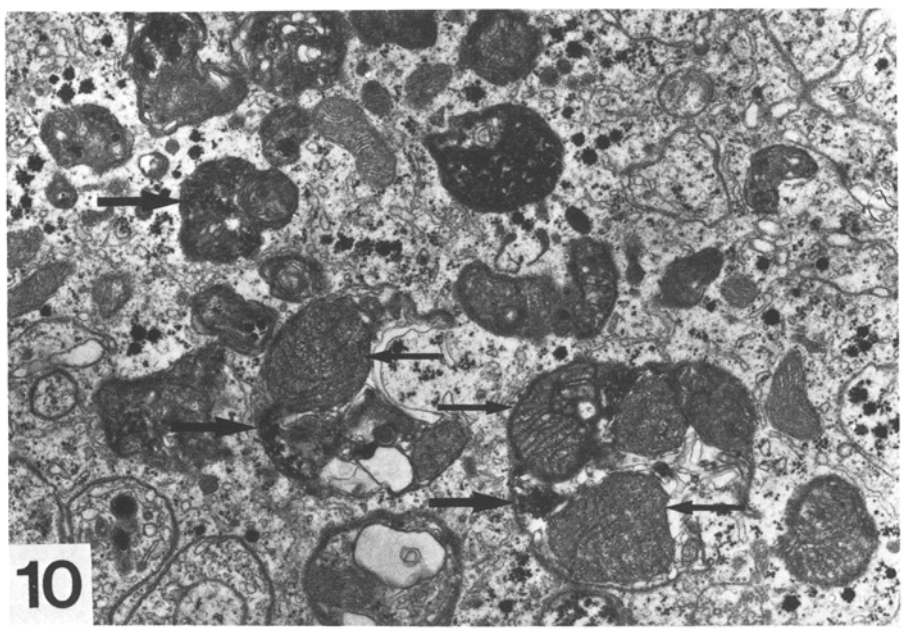


Fig. 9. 32 h after eclosion. The neuro-glial "trophospongium" is intensified. Neuronal mitochondria have become polygonal (*Mt*). The glial cytoplasm contains large lysosome-like bodies (*Ly*). × 7700

Fig. 10. 48 h after eclosion. Lysosome-like structures in the glial sheath of D-IV neurons are frequently associated with degenerating mitochondria (thick arrows). As judged by the studding of their cristae these mitochondria are of neuronal origin (thin arrows). × 17,000

remains of the cell body is then entirely filled with distorted vesicles which are presumably former ER-channels together with mitochondria, multivesicular and lamellar bodies, all of which show clear signs of swelling (Fig. 11). In the mitochondria swelling separates cristae from the surface membrane, whereas in the lamellar bodies the previously electron-dense core is expanded so that structural details (e.g. distorted vesicles) reappear. In comparison with previous stages the numbers of ribosomes within the cytoplasm and of ribosome-like particles in mitochondria are very low. Instead, certain cellular areas and mitochondria contain spots of opaque material, presumed to be ribosomal remnants. The cross sectional area of the cell body has not yet decreased at this stage.

There are no further signs in glial profiles of participation in the process of degeneration (Fig. 11). Glial invaginations are retracted and the lysosome-like particles observed at 32 to 48 h have disappeared. There is no evidence of disintegration of the neuro-glial membranes.

Later stages of breakdown involve the complete disappearance of the remaining ribosomal granular and opaque material (Fig. 12). Mitochondria are no longer discernible since their surface membranes and cristae have apparently been transformed into a number of small vesicles. Lamellar bodies are disrupted and their stacks of membranes become straightened over long distances. As judged by the average size of cell profiles observed and the density of structures contained within them, it seems that the cell bodies are shrinking at this stage.

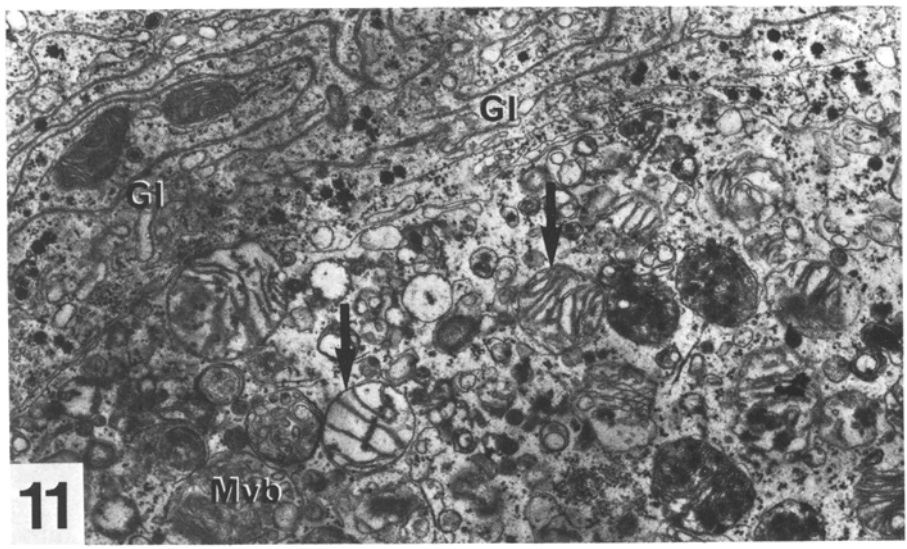
By 96 h the decrease in cellular volume has become obvious (Figs. 13, 14). Profiles of degenerating cell bodies appear as giant opaque lamellar bodies within a single glial cell. The glial cytoplasm shows no structural sign of phagocytosis of such remnants.

Discussion

The Degeneration Process

To our knowledge this study is the first description of the fine structural events during degeneration of insect motor neurons. A similar study performed on the embryonic chick spinal cord (O'Connor and Wyttenbach, 1974) demonstrated that the onset of degeneration in a particular motor neuron is marked by alterations in nuclear structure such as the condensation of chromatin and decrease in nuclear size. Nuclei of *Manduca* abdominal motor neurons, in contrast, remain morphologically intact well into the lytic phase, as has been observed in most studies of programmed cell death (Lockshin and Beaulaton, 1974). The onset of degeneration is marked by changes in shape of mitochondria similar to those observed in isolated motor axons (Rees and Usherwood, 1972; Nüesch and Stocker, 1975). This is followed by the liberation of RER-bound and polysome-bound ribosomes, a process which in chick neurons has been interpreted as a decrease in the level of transcription (O'Connor and Wyttenbach, 1974).

Lamellar bodies are frequently observed in degenerating insect neurons (Tung and Pipa, 1971; Rees and Usherwood, 1972; Nüesch and Stocker, 1975) but they are also found in intact nerve and other cells (Spencer and Thomas, 1974; Lockshin and Beaulaton, 1974), and probably reflect the normal turnover of materials within the cytoplasm. In *Manduca,* too, solitary lamellar bodies occur in both non-degenerating neurons, and in early D-IV neurons before the onset of degeneration.



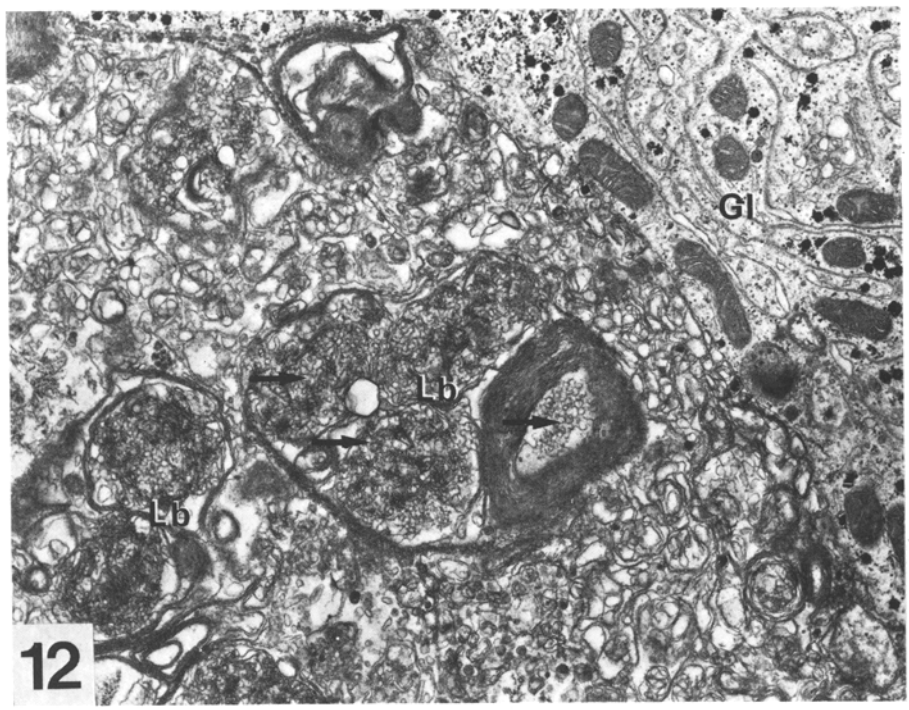


Fig. 11. 72 h after eclosion. Part of D-IV neuron during final degeneration phase. Mitochondrial remnants show discrete signs of swelling (arrows). The glial sheath (*Gl*) is apparently no longer involved in digesting neuronal material. *Mvb* multivesicular body. ×17,000

Fig. 12. 72 h after eclosion. Same as Fig. 11. Lamellar bodies (*Lb*) become disrupted (presumably by swelling) which expands their previously electron-dense cores (arrows). Mitochondria are no longer discernible in this section. *Gl* glia. ×13,600

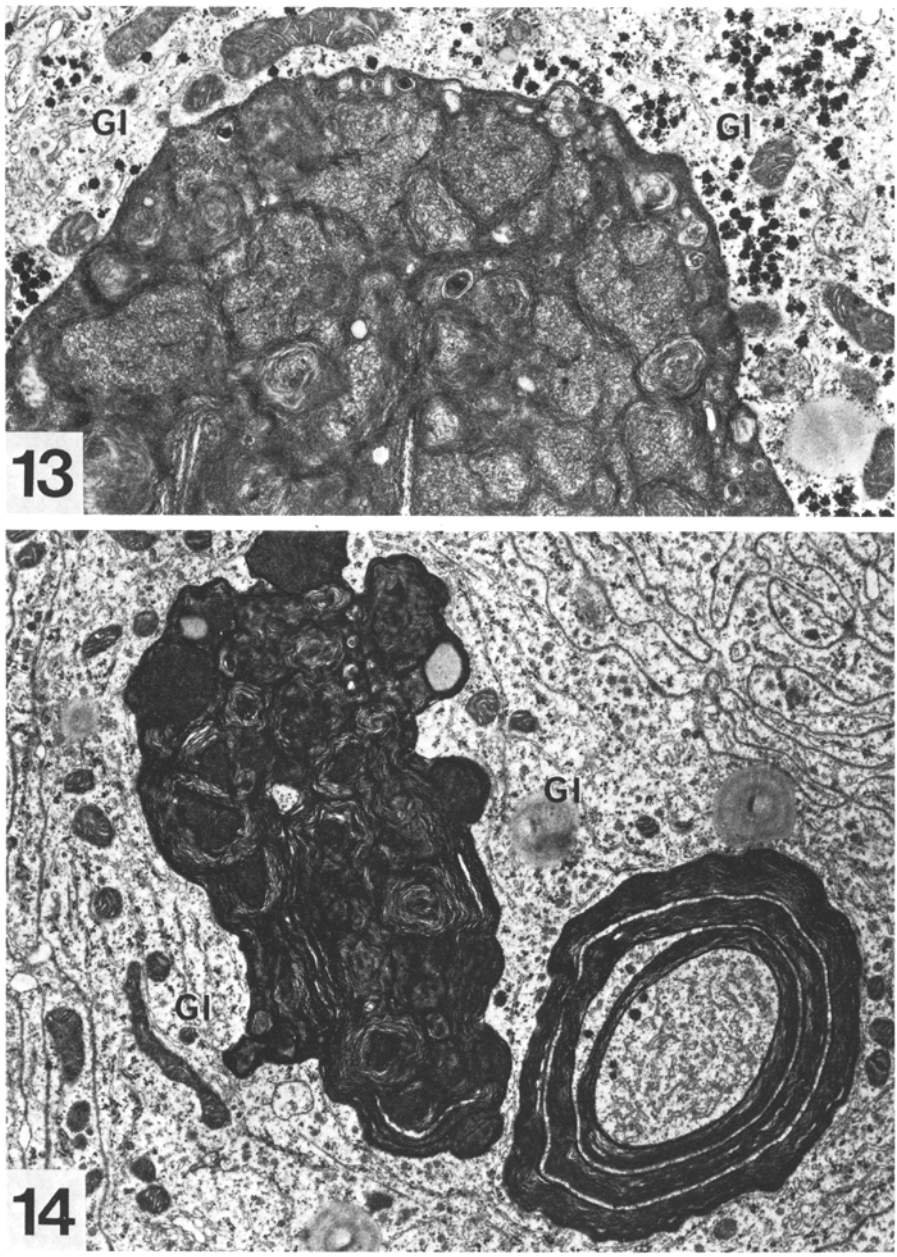


Fig. 13. 96 h after eclosion. The whole neuronal cytoplasm has shrunk and become electron-dense. It is contained within a single glial profile (*Gl*). ×13,600

Fig. 14. 96 h after eclosion. A single glial profile (*Gl*) contains large degeneration figures (residual bodies) presumably derived from a degenerating neuron. ×11,000

Lamellar bodies increase numerically and quantitatively in D-IV neurons only 12 h after eclosion and thus indicate a gradual prevalence of catabolic processes in the soma. Lamellar bodies, which presumably represent a type of lysosomal structure, are very likely to be the remnants of membranous organelles, such as mitochondria (Tung and Pipa, 1971; Rees and Usherwood, 1972; Nüesch and Stocker, 1975) or ER. This view is also supported by the present study, but the lamellar bodies formed in D-IV neurons are much more complex structures than hitherto reported. Although the mode of formation of lamellar bodies is described in this investigation, the factors that govern their assembly process remain unknown, e.g., the generation of isolation bodies (autophagic vacuoles?) within the cytoplasm, the rearrangement of membranes in stacks, and the assembly of subunits to extremely intricate complexes. The significance of the segregation process in degenerating D-IV neurons, resulting in an accumulation of ribosomes in one cellular region and of membranous structures (ER, vesicles, mitochondria and lamellar bodies) in another region, remains obscure.

The disintegration of the nucleus, a process that begins about 32 h after eclosion, is very characteristic and differs from that of vertebrate neurons (O'Connor and Wyttenbach, 1974). Instead of a shrinkage of the nucleus and a condensation of the karyoplasm the nuclear membrane breaks without previous changes of the nuclear architecture; cytoplasm and karyoplasm intermingle homogeneously. The ultrastructural data thus suggest that the degenerative forces are operating from the cytoplasm.

The extreme structural changes observed in D-IV neurons 72 h after eclosion are probably produced by changes in osmolarity (cf. Anderson and Westrum, 1972; Nüesch and Stocker, 1975). Vesicles, mitochondria and lamellar bodies initially become swollen and eventually rupture, probably as a result of decreasing external osmolarity. The neuronal profiles then gradually shrink, and become increasingly electron-dense. They finally take the appearance of huge and extremely complex lamellar bodies surrounded by a single glial profile. By this time they are, strictly speaking, intracellular glial structures. According to the lysosome concept, such structures should be termed residual bodies (DeDuve, 1963). The process of increasing electron-density is a well-known observation that has been reported from isolated axons (Lamparter et al., 1967; Rees and Usherwood, 1972; Nüesch and Stocker, 1975; Stocker, in preparation) as well as from degenerating neuron perikarya (O'Connor and Wyttenbach, 1974).

Initial changes in glial cells first appear about 36 h after those of D-IV neurons. They include the appearance of lysosome-like particles associated with structures of neuronal origin. This observation suggests that glial cells act as phagocytes of neuronal debris. It should be noted, however, that only part of the neuronal cytoplasm seems to be digested in this way, the majority being removed by condensation into a single, huge residual body (see above). The glial profiles surrounding residual bodies do not show any fine structural signs that would indicate participation in the disintegration process. This observation stands in sharp contrast to the evidently active glial profiles in the vicinity of degenerating chick motor neurons (O'Connor and Wyttenbach, 1974). Although the morphological data alone are insufficient to evaluate physiological changes, the observations on the behavior of glial profiles in *Manduca* suggest that glial cells do not participate in the first steps of disintegration of D-IV neurons, whereas at 48 h

they may act to engulf and digest neuronal debris and later may become hosts of neuronal residual bodies.

Factors Affecting the Degeneration Process

It is very clear that the degeneration of the D-IV neurons represents "programmed cell death" (Lockshin and Beaulaton, 1974). This is demonstrated by the fact that (a) other neurons in the immediate vicinity remain intact, and (b) major disorganization of the cell occurs very late during the degeneration process, indicating that "the intracellular milieu, as well as the extracellular, is under control, and that the cells, although beating a retreat, are doing so in an organized and physiological manner" (Lockshin and Beaulaton, 1974). Nüesch and Stocker (1975) came to the same conclusion when comparing normal, metamorphic degeneration of the larval muscle innervation in *Antheraea* with Wallerian breakdown of the adult innervation after lesioning.

Two questions remain unresolved as to the triggering of motoneuron degeneration in *Manduca*. The first relates to the nature of triggering signal for the breakdown. By analogy to the role of the eclosion hormone in the degeneration of the intersegmental muscles of silkmoths (Truman, 1970; Schwartz and Truman, unpublished), it would seem reasonable to suppose that the hormone also triggers the post-emergence death of the motoneurons in *Manduca*. The relatively strict temporal relationship of neuron degeneration with time after eclosion (Fig. 2) suggests that the agent which triggers eclosion also initiated motoneuron degeneration. However, it has recently been found that in *Manduca* the release of eclosion hormone occurs at a rather fixed interval after some earlier developmental events including the onset of CNS sensitivity to the hormone itself (Reynolds, Taghert, and Truman, 1978). Consequently, it is not clear whether eclosion hormone release or a slightly earlier event is the trigger for neuron degeneration.

The second unresolved question is whether the degeneration response of the motoneurons is an autonomous response to an external signal. The D-IV motoneurons do not show ultrastructural signs of degeneration until about 12 h after eclosion. But by this time breakdown of selected groups of interneurons is well under way (Truman, unpublished). Thus, the loss of the motor cells may be related to the degeneration of the neurons which normally drive them. Alternatively both types of neurons may show an independent programmed response to the same signal.

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