DEGENERATIVE CHANGES IN THE MITOCHONDRIA OF FLIGHT MUSCLE FROM AGING BLOWFLIES

BERTRAM SACKTOR and YOSHIO SHIMADA

From the Laboratory of Molecular Aging, Gerontology Research Center, National Institute of Child Health and Human Development, National Institutes of Health, Baltimore City Hospitals, Baltimore, Maryland 21224

ABSTRACT

Mitochondria from flight muscle of aging blowflies, *Phormia regina*, were examined morphologically and biochemically with the electron microscope. An age-dependent degeneration of the mitochondria that is characterized, in part, by the reorganization of the inner membrane into myelin-like whorls has been found. The concentric rings increase in size and number, eventually replacing the normal cristal conformation. Glycogen rosettes are frequently seen in the center of the whorl and may represent the intrusion into the mitochondria of the glycogen in the cytoplasmic matrix of the muscle. The degenerating mitochondria are not associated with lysosomal activity, as indicated by the absence of acid phosphatase. An intense acid phosphatase activity is noted, however, in the dyad, comprising elements of the T system and sarcoplasmic reticulum. Cytochrome oxidase is active in the ultrastructurally intact portion of the mitochondrion but activity is not evident in that part of the mitochondrion that has undergone morphological change. Thus, the ultrastructural degradation of the mitochondria is correlated with a decrease in biochemical function. This suggests a correspondence between a decrease in the bioenergetic capacity of the flight muscle and a decline in the ability of the aged insect to fly.

In pioneering experiments, Williams et al. (1) reported that the flight ability of flies declines markedly with age. The mechanism of this deterioration with senescence remains essentially unknown. Our previous studies on the control of intermediary metabolism in flight muscle (2–4) prompted us to examine whether the bioenergetic processes in this horrendously active tissue undergo changes during aging. Notable differences in the ultrastructure and biochemical activity of the muscle from young and old blowflies, *Phormia regina*, have now been found. In this paper, degenerative alterations in mitochondria with age, as determined by electron microscopy, are reported.

METHODS

Blowflies were maintained in laboratory culture as reported previously (5). The flight muscles from female flies, 7–46 days old, were used. Mitochondria were isolated as described earlier (6); the isolation medium consisted of 0.15 M KCl, 0.01 M Tris chloride, 1 mM ethylenediaminetetraacetate (EDTA), and 0.5% bovine serum albumin, adjusted to pH 7.4.

Unless noted otherwise, flies were bisected by a medical cut with a razor blade in ice-cold fixative containing 2.5% glutaraldehyde, 0.05 M cacodylate buffer, pH 7.4, and 0.18 M sucrose (7). Samples of muscle, less than 1 mm^3, were kept in fixative, at 0–4°C, for 3 hr and washed overnight in the cold with 0.05 M cacodylate in 0.3 M sucrose. The tissue was transferred to cold 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 2 hr, dehydrated in an ethanol series, and embedded via propylene oxide in Epon 812. Sections were cut with a diamond knife on an LKB Ultrotome III and mounted on 300-mesh copper grids. The specimens were stained with saturated uranyl acetate and lead citrate. Grids were examined in a Hitachi HU-11D electron microscope.

Isolated mitochondria were fixed rapidly in 2% glutaraldehyde, 0.05 M cacodylate, pH 7.4, and 0.075 M sucrose. After 3 hr in fixative, the mitochondria were washed overnight in the cacodylate buffer containing 0.275 M sucrose. The mitochondria were transferred to cold osmium tetroxide and subsequently handled as described for the samples of muscle.

Tissues treated with α-amylase were fixed in glutaraldehyde and, after washing, incubated for 2 hr at 37°C with 0.5% α-amylase (2-times crystallized, from Worthington Biochemical Corp., Freehold, N. J.) in 10 mM phosphate buffer, pH 7.4, containing 20 mM NaCl (8). Control tissues were incubated in buffer without enzyme. The specimens were rinsed with 0.1 M phosphate buffer, pH 7.4, postfixed in osmium tetroxide, and prepared for electron microscopy as detailed above.

For demonstration of acid phosphatase activity, approximately 0.5 mm^3 of muscle was fixed in a medium containing 4% glutaraldehyde, 0.05 M cacodylate, pH 7.4, and 0.18 M sucrose for 2 hr at 0–4°C. After washing with the cacodylate buffer in 0.3 M sucrose, the tissue was incubated, according to Ogawa et al. (9), in 9 mM β-glycerophosphate, 0.05 M sodium acetate buffer, 1 mM lead nitrate, and 8% sucrose, adjusted to a final pH of 5.45, for 20 min at 37°C. The specimens were washed several times with 8% sucrose in the cold. Control tissues were incubated without β-glycerophosphate, or with substrate plus 0.01 M NaF. For demonstration of cytochrome oxidase activity, the muscle was fixed for 1 hr with ice-cold 4% paraformaldehyde in 0.05 M phosphate, pH 7.4, and 5% sucrose (10) and washed overnight with 0.05 M phosphate in 0.22 M sucrose. The cytochrome oxidase reaction was carried out with diaminobenzidine, as described by Seligman et al. (10), for 1 hr at room temperature, except that cytochrome *c* was not added exogenously. Control tissues were incubated identically, but in the presence of 0.01 M

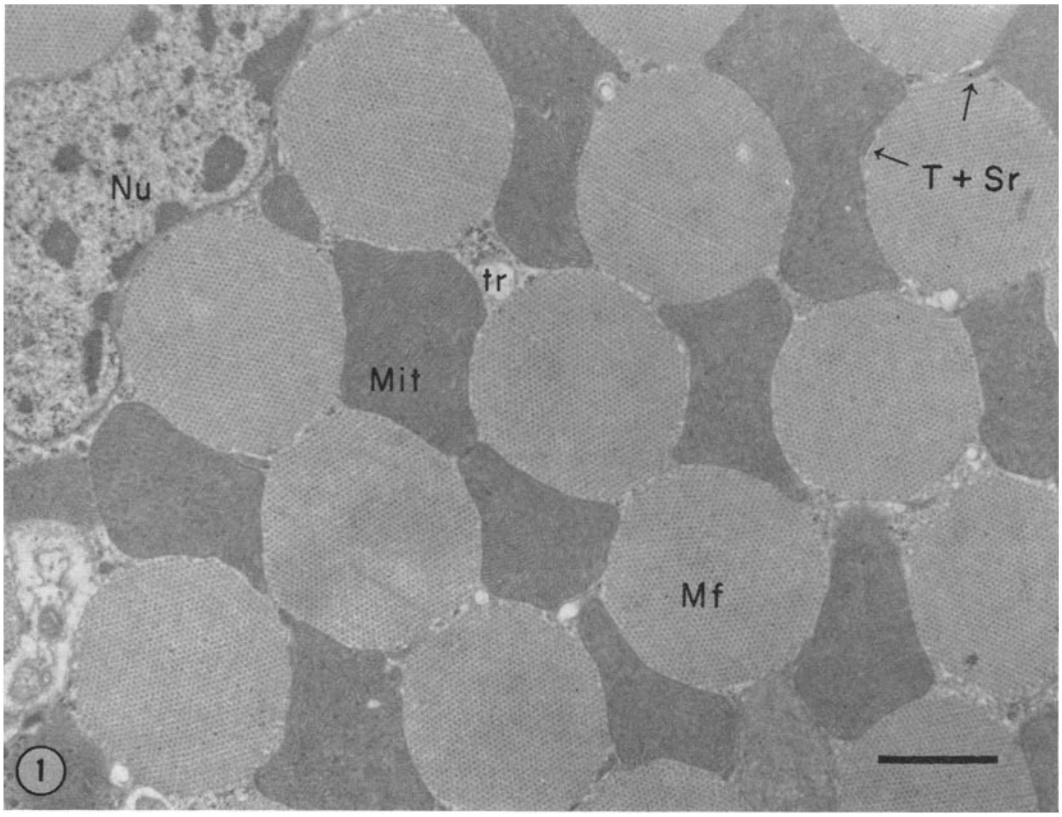


FIGURE 1 An electron micrograph of a transverse section of the dorsal longitudinal flight muscle of a 7-day-old, young adult blowfly. The cylindrical myofibrils (*Mf*) are surrounded by mitochondria (*Mit*). A nucleus (*Nu*) and tracheoles (*tr*) are noted. Dyads, comprising elements of the transverse tubular (*T*) system and sarcoplasmic reticulum (*Sr*) are evident adjacent to the fibril. The bar line in the lower right corner indicates 1 μ. × 16,300.

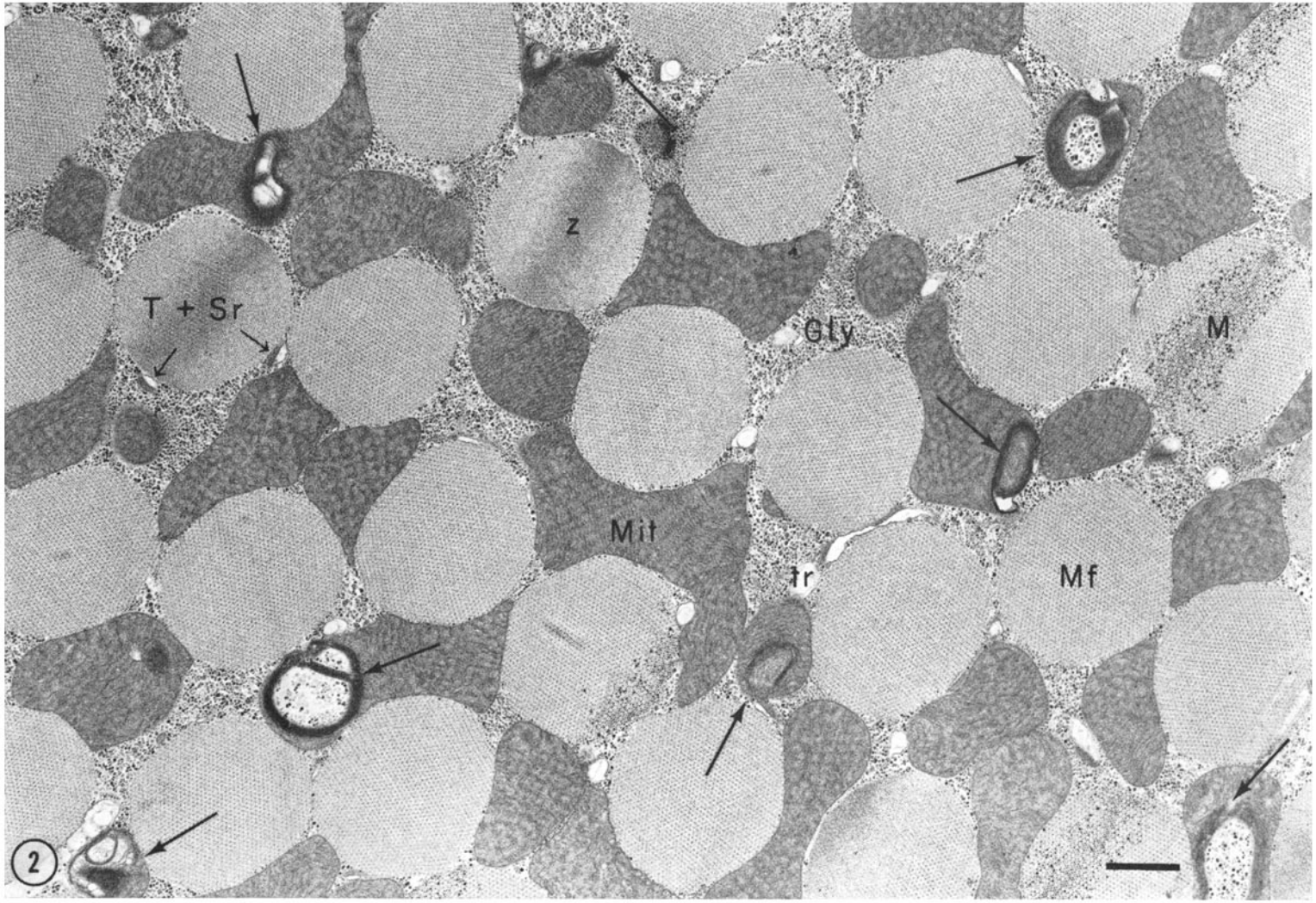


FIGURE 2 An electron micrograph of a transverse section of flight muscle of a 34-day-old blowfly. Note numerous whorls, indicated by arrows, in the mitochondria. Glycogen particles (*Gly*) are abundant in the cytoplasmic matrix, M band, and are evident in the center of the myelin-like figures. *Mf*, myofibrils; *Mit*, mitochondria, *T*, transverse tubular system; *tr*, tracheoles; *Sr*, sarcoplasmic reticulum. The bar line in the lower right corner indicates 1 μ. × 11,000.

KCN. After incubation, the muscle was rinsed several times in the cold with 0.05 M phosphate, pH 7.4, in 0.22 M sucrose, and transferred to 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, and postfixed for 1 hr at room temperature. Dehydration, embedding, and sectioning were carried out as described above.

RESULTS

A survey electron micrograph of the dorsal longitudinal flight muscle of a young adult blowfly, 7 days after emergence from the pupa, is illustrated in Fig. 1. As described previously (11–13), the cylindrical fibrils are very large, approximately 2 μ in diameter and 3 μ in length. The mitochondria are ovoid and irregular in shape, up to 4 μ in length, and are not precisely aligned with respect to the myofibrillar striations. The blowfly flight muscle mitochondrion has a distinct outer limiting membrane, and its inner membrane is arrayed in parallel fenestrated plates, 30–35 cristae per μ. Nuclei are few in number and are often located peripherally. The sarcoplasmic reticulum of this asynchronous muscle is markedly reduced and, together with elements of the T system, forms dyad associations adjoining the fibril surface (12). Glycogen is located prominently in the interfibrillar sarcoplasm, in the form of alpha particles or rosettes (5).

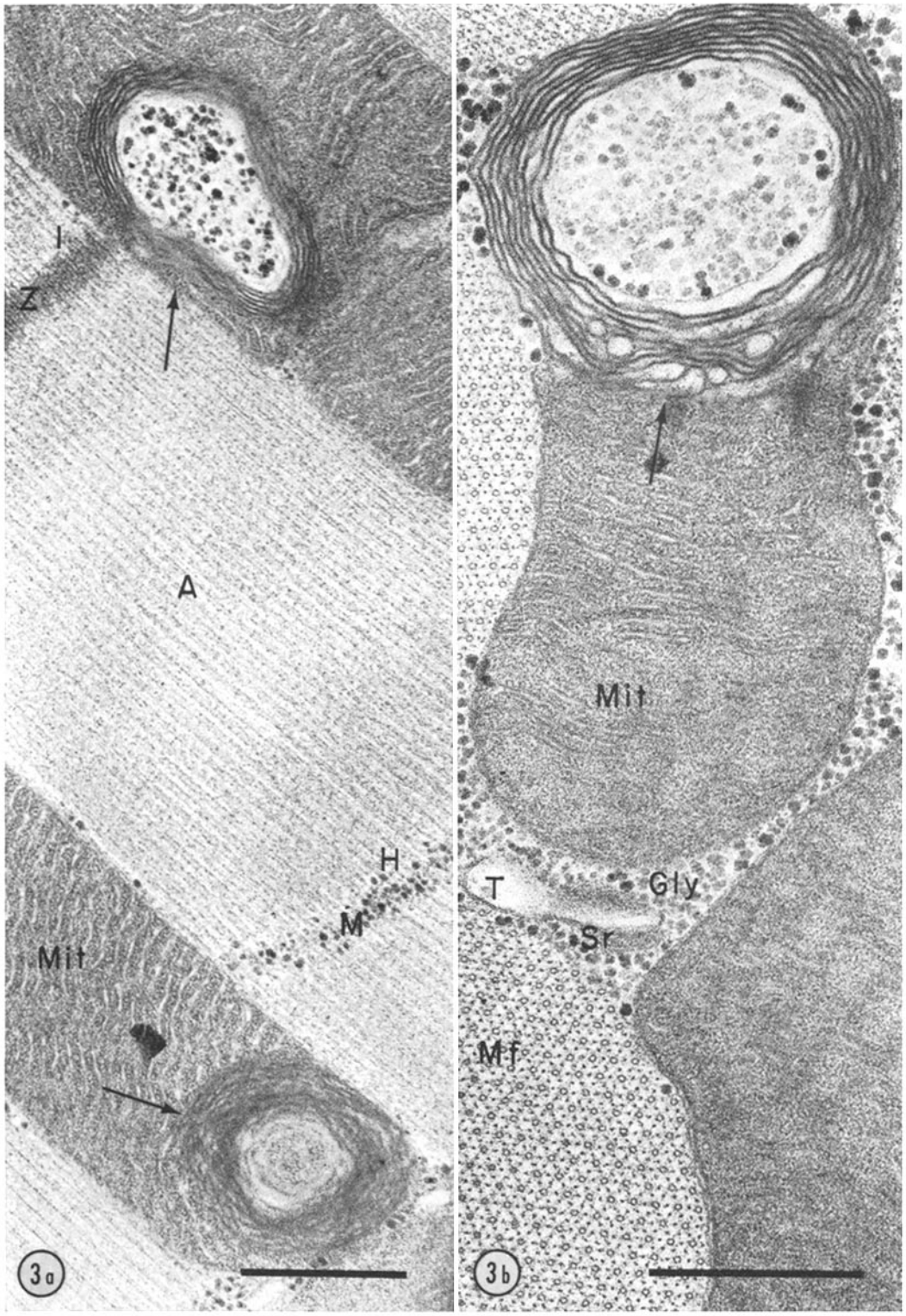
Fig. 2 shows the flight muscle of a 34-day–old blowfly. Membranous whorls in the mitochondria are abundantly evident. At higher magnifications (Figs. 3 and 4) the whorls appear as myelin-like figures. The concentric rings may be either loosely (Fig. 3 *b*) or tightly packed (Fig. 4 *a*, *b*), and seem to represent a reorganization of the inner mitochondrial membrane (also, cf. Fig. 9 *a*). Particles, resembling glycogen rosettes, are frequently seen in the center of the whorl. As shown in Fig. 3 *b*, a thin limiting membrane separates the particles from the membranes of the whorl.

Examination of numerous fiber profiles reveals mitochondria in different stages of ultrastructural degeneration. From representative profiles, four of which are illustrated in Fig. 4, a sequential pattern of mitochondrial deterioration is suggested. At the locus of the whorl the mitochondrion appears to have lost turgor, so that the cytoplasmic matrix, filled with glycogen particles, intrudes (Fig. 4 *a*, *b*). Such mitochondria, sectioned transversely to the whorl, would be depicted as comprising concentric rings filled with particles (Fig. 3 *a*). The whorls increase in size and number, eventually replacing the normal cristal conformation (Fig. 4 *c*). In a final stage the myelin-like membranes become compressed, the normal mitochondrial structure being almost completely obliterated and seemingly filled with glycogen-like particles (Fig. 4 *d*).

In the low magnification field shown in Fig. 2, which is representative of a 34-day–old blowfly, the myelin figures are seen in approximately 30% of the mitochondria. The whorls are occasionally found in the flight muscle of 20- to 25-day–old flies. The frequency of their appearance increases with the age of the insect; in the 45-day–old blowfly nearly 50% of the mitochondria have whorled configurations. Since serial sections were not made and other planes of the mitochondria are not seen, this value represents the minimum number of mitochondria undergoing deformation. In contrast, myelin-like figures have never been observed in the flight muscle of blowflies less than 20 days old. The initial appearance and subsequent increase in the frequency of the ultrastructural change with age is correlated with the life cycle of this species, the mean survival time of the female blowfly being 25–26 days (14).

Control studies have been carried out to pre-

FIGURE 3 Electron micrographs of flight muscles of 34-day-old blowflies. 3 a, Longitudinal section of muscle. The A, I, Z, M, and H bands of the myofibril are evident and appear structurally normal. Note glycogen rosettes in M line. Whorls in mitochondria, indicated by arrows, are not aligned with respect to myofibrillar striations. The concentric membranes of the whorl may be either tightly or loosely arranged. The appearance of glycogen particles is dependent on the plane of section. *Mit*, mitochondria. Bar line indicates 0.5 μ. × 50,000. 3 b, Transverse section of muscle. Note the loose arrangement of the membranes in the myelin-like figure. Glycogen rosettes, surrounded by a thin limiting membrane, are seen in the center of the whorl. A dyad, containing T system (*T*) and sarcoplasmic reticulum (*Sr*) elements, is adjacent to a myofibril and appears structurally normal. *Mf*, myofibrils, *Mit*, mitochondria; *Gly*, glycogen particles. Bar line indicates 0.5 μ. × 65,600.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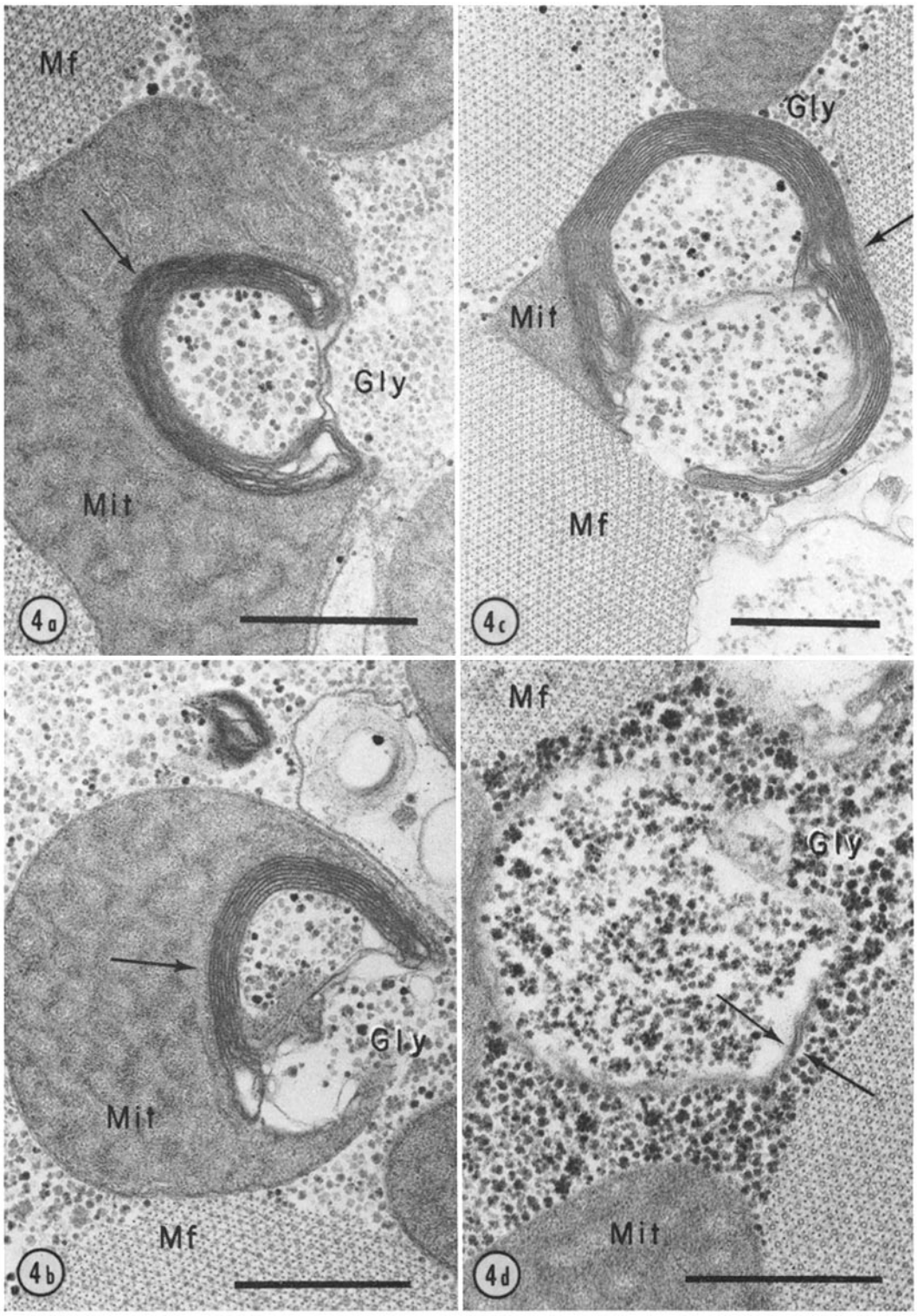

FIGURE 4 Electron micrographs of flight muscles of 34-day-old blowflies showing mitochondria in different stages of ultrastructural degeneration. Note the glycogen intruding into the mitochondria at the locus of the whorl (Figs. 4 *a*, *b*). In Fig. 4 *c* the membranous rings have become more numerous and the mitochondria are almost filled with glycogen. In a later stage of degeneration (Fig. 4 *d*), the normal structure of the mitochondrion is essentially completely obliterated. *Mf*, myofibrils; *Mit*, mitochondria; *Gly*, glycogen particles. Bar lines indicate 0.5 μ. Figs. 4 *a* and 4 *b*, × 52,500; Fig. 4 *c*, ×44,600; Fig. 4 *d*, × 59,500.

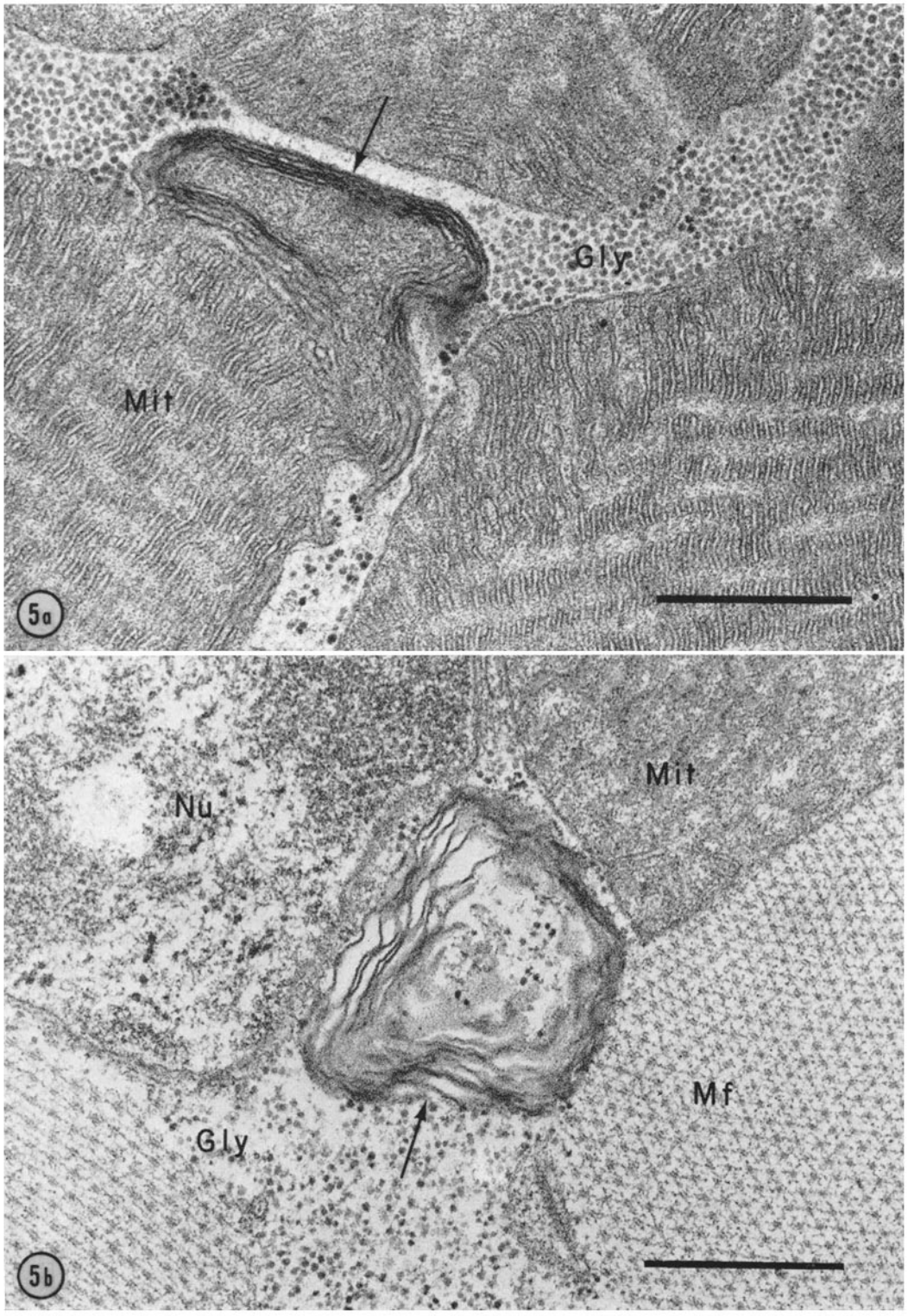

FIGURE 5 Electron micrographs of OsO_4-fixed flight muscle of a 36-day-old blowfly showing various stages of mitochondrial deterioration. Fig. *5 a*, Longitudinal section. Fig. *5 b*, Transverse section. Glycogen particles are evident in the mitochondria. *Mf*, myofibrils; *Mit*, mitochondria; *Gly*, glycogen particles; *Nu*, nucleus. Bar lines indicate 0.5 μ. × 60,400.

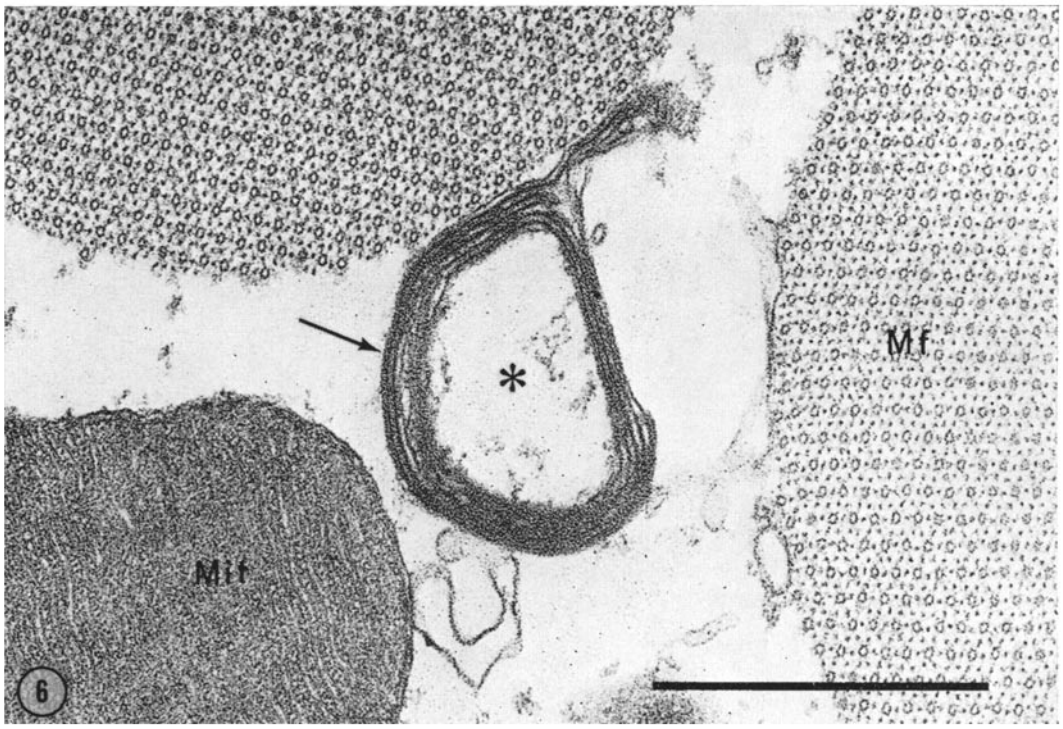

FIGURE 6 Electron micrograph of a flight muscle of a 29-day-old blowfly after incubation with α-amylase as described in the text. Note the absence of glycogen from the whorl (*) of a mitochondrion and from the cytoplasmic matrix between the myofibrils and mitochondria. *Mf*, myofibrils; *Mit*, mitochondria. Bar line indicates 0.5 μ. × 94,900.

clude the possibility that the reorganization of the mitochondrial membranes reported in this paper is an artifact of fixative procedures. As noted above, the whorls are found in the muscle of aged, never in young, blowflies and, as will be described below, mitochondria with myelin-like figures have been isolated from the flight muscle of old blowflies. The cacodylate buffer can be replaced by 0.1 M phosphate buffer, and different lots and suppliers of glutaraldehyde have been tested, all with identical results. Furthermore, as shown in Fig. 5, prominent whorls indicative of the various stages of mitochondrial degeneration are found when glutaraldehyde fixation is omitted entirely and the muscle is fixed directly with osmium tetroxide.

Incubation of the flight muscle with α-amylase results in the disappearance of the particles in the whorl, leaving only an amorphous network of opaque material (Fig. 6). This indicates that the "intramitochondrial" glycogen-like rosettes are, in fact, glycogen particles. Control tissues, incubated in buffer without α-amylase, retain their glycogen.

To determine whether the degenerating mitochondria have enzymatic activities usually considered to be associated with lysosomes, flight muscles of aged blowflies have been examined histochemically for acid phosphatase. As shown in Fig. 7, no evidence for acid phosphatase activity is found in the mitochondria or in the myelin-like figures. Quite unexpected, however, is the finding of intense acid phosphatase activity in the dyad, comprising elements of the T system and sarcoplasmic reticulum. Activity is specifically localized in the cisternae of the reticulum. Control experiments, in which NaF is added or β-glycerophosphate is deleted from the incubation reaction, show no activity. In other studies on the ATPases of this muscle, Dr. Lois Tice (personal communication) has not observed any artifactual deposition of lead phosphate at the dyad.

The ultrastructural degradation of the mitochondria is correlated with a decrease in biochemical function. As illustrated in Fig. 8, examination of the flight muscle, histochemically,

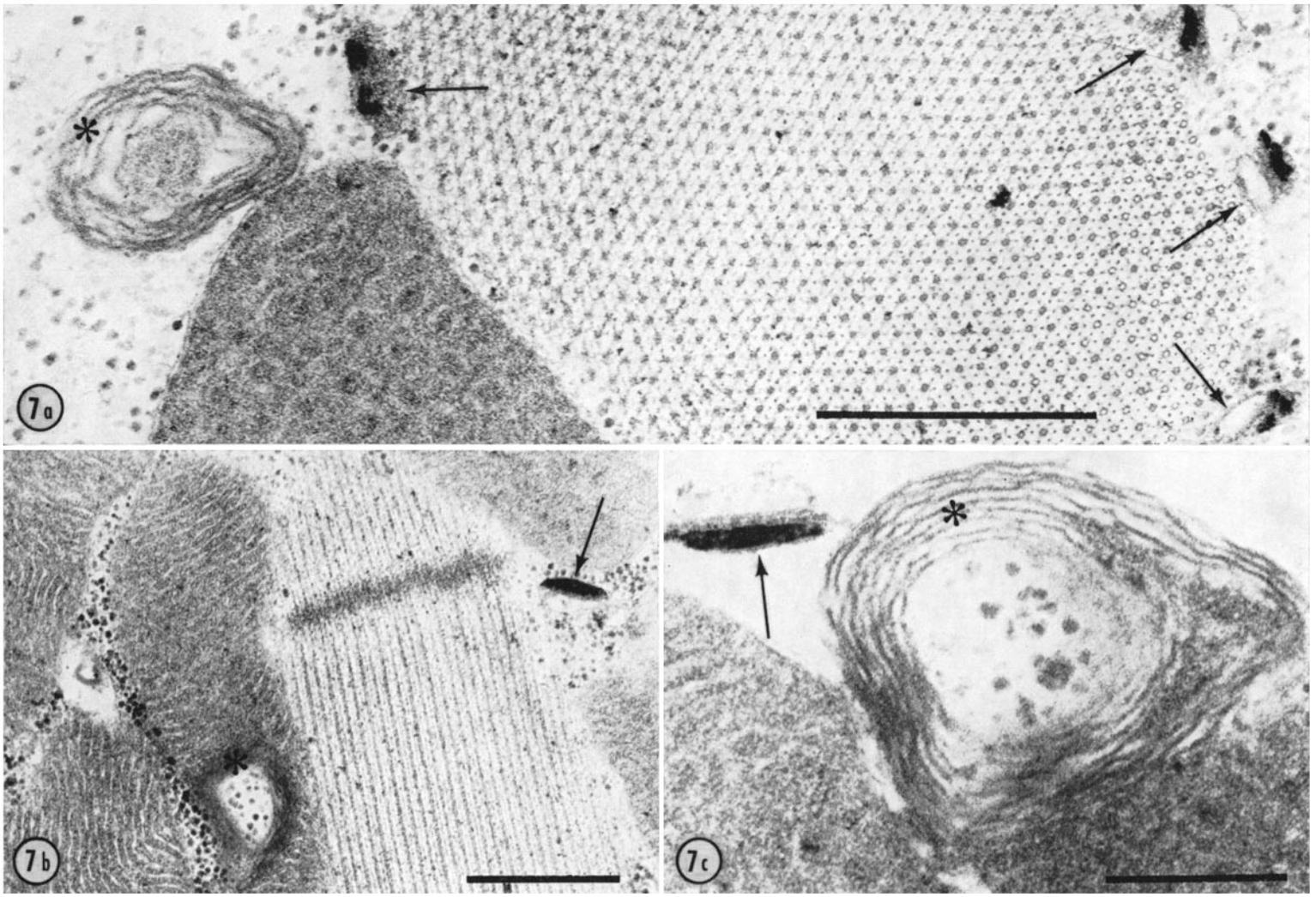

FIGURE 7 Electron micrographs of transverse and longitudinal sections of the flight muscles of 46-day-old blowflies examined histochemically for acid phosphatase activity. Enzymatic activity is absent from the degenerating mitochondria (*). Note the intense acid phosphatase activity in the cisternae of the sarcoplasmic reticulum at the dyad (indicated by the arrows). Fig. 7 *a*, Transverse section. Bar line indicates 0.5 μ. × 86,000. Fig. 7 *b*, Longitudinal section. Bar line indicates 0.5 μ. × 45,900. Fig. 7 *c*, Enlarged view of a longitudinal section. Bar line indicates 0.25 μ. × 110,300.

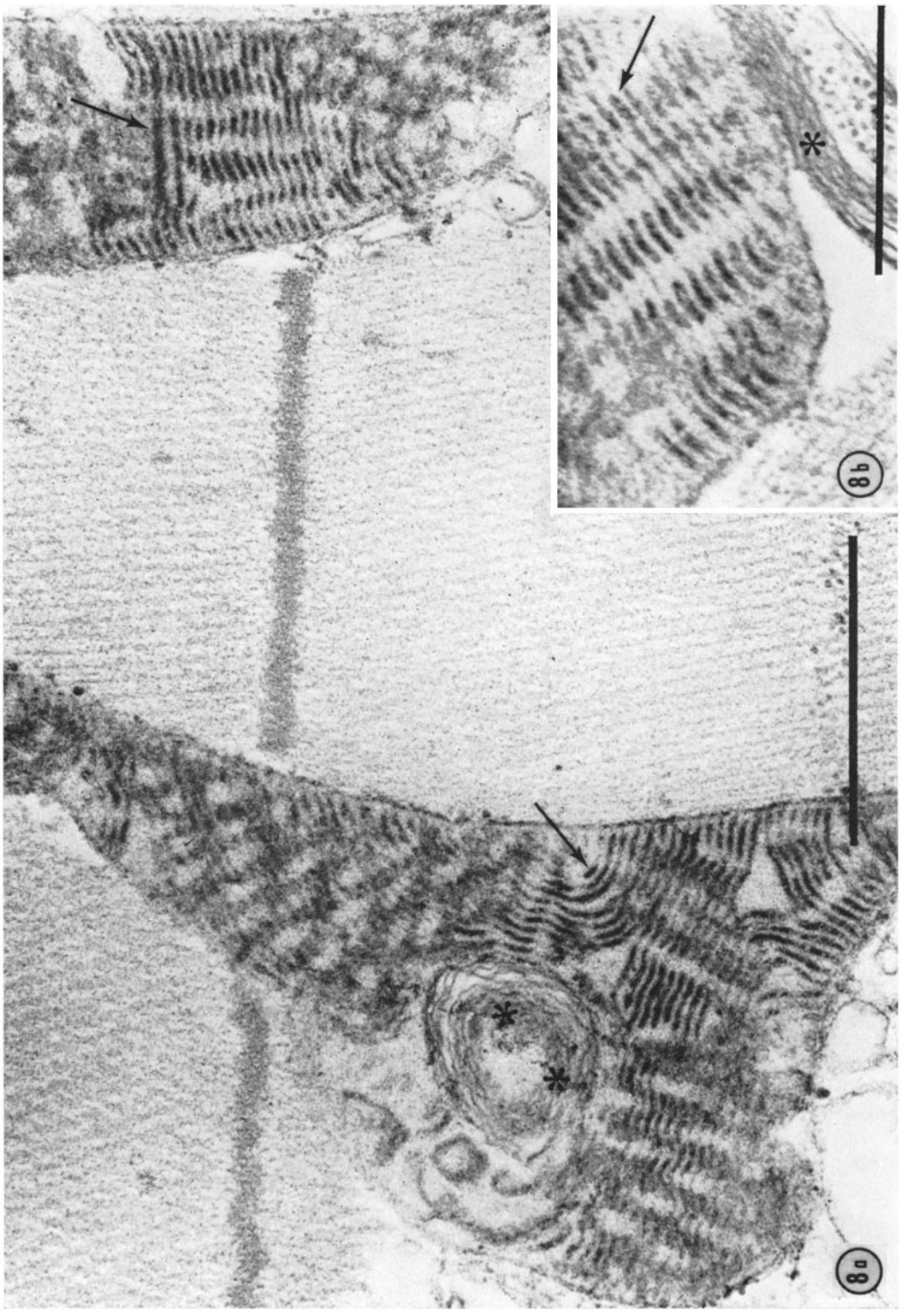

FIGURE 8 Electron micrographs of longitudinal (Fig. 8 *a*) and transverse (Fig. 8 *b*) sections of flight muscles of 38-day-old blowflies examined histochemically for cytochrome oxidase activity. Note the deposition of electron-opaque reaction product in the part of the mitochondrion that is structurally intact (shown by arrows) and the absence of enzyme reaction in the portion of the mitochondrion that has undergone degeneration (shown by asterisks). Reaction product is seen at the inner membrane and is lacking in the matrix. Fig. 8 *a*, Bar line indicates 1 μ. $\times$ 47,000. Fig. 8 *b*, Bar line indicates 0.5 μ. $\times$ 83,300.

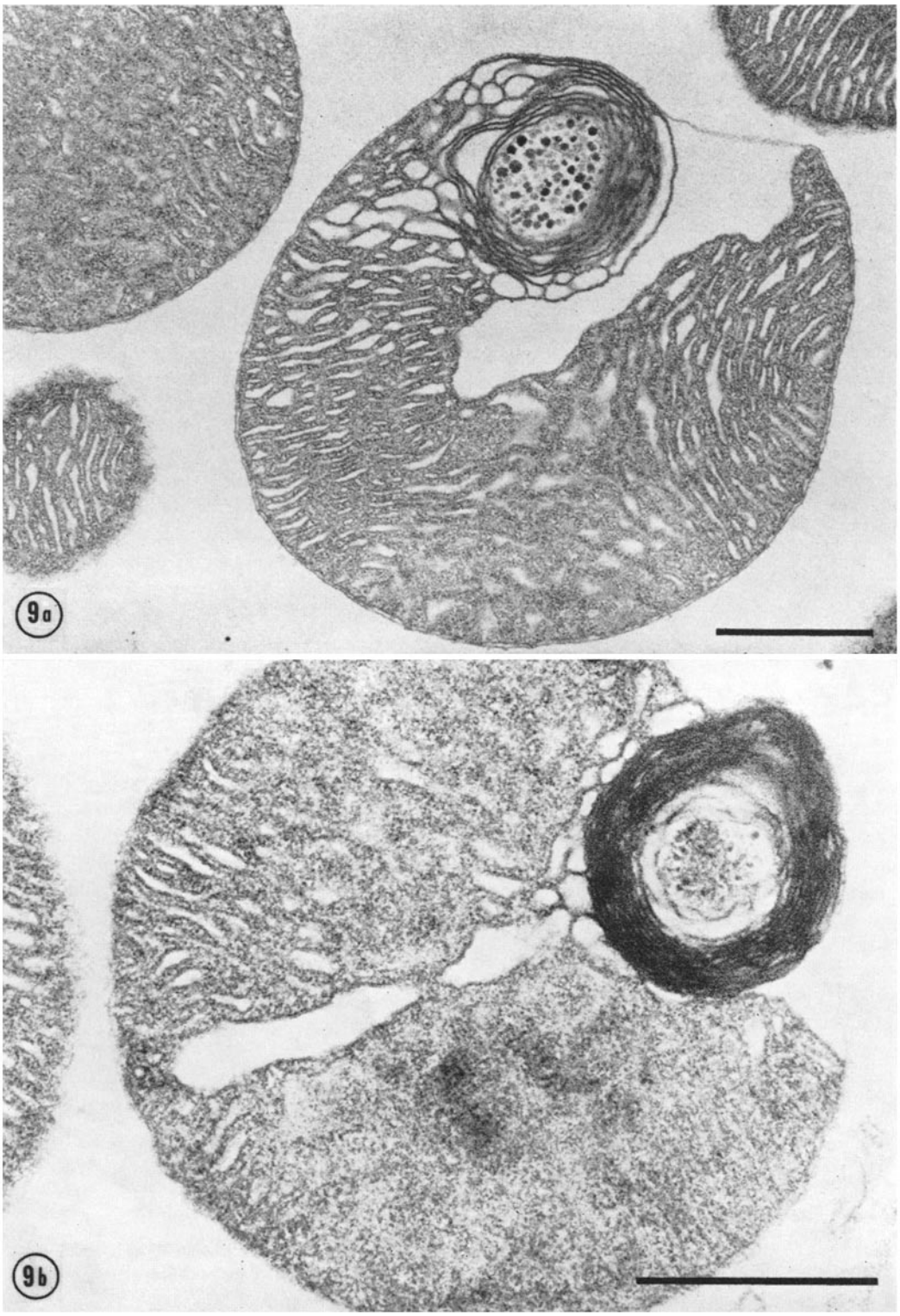

FIGURE 9 Electron micrographs of mitochondria isolated from the flight muscles of 32-day-old blowflies. Fig. 9 *a*, Note that the concentric membranous rings seem to be derived from the inner membrane. Glycogen particles and outer mitochondrial membrane are evident. Fig. 9 *b*, Reorganization of the inner membranes is further advanced. Bar lines indicate 0.5 μ. Fig. 9 *a*, $\times$ 47,300; Fig. 9 *b*, $\times$ 73,500.

for cytochrome oxidase reveals strong enzymatic activity in the structurally intact portion of the mitochondrion but no indication of enzymatic activity in that part of the mitochondrion that has undergone morphological change. Control experiments, carried out identically except for the addition of KCN to the incubation, show no reaction. It is also evident from Fig. 8 that the electron-opaque reaction product is deposited at the cristal membrane. The matrix of the mitochondrion, indicated by the pores in the fenestrated inner membrane, is free of deposit.

Whorls are found in mitochondria isolated from flight muscles of old blowflies (Fig. 9). The outer membranes and glycogen rosettes entrapped in the whorls are seen. That the concentric membranous rings are derived from the inner membranes is clearly suggested. It is also obvious that the myelin-like figures containing glycogen can not arise from fixation artifacts of the isolated mitochondria. Thus, the presence of these inclusions provides strong support for the view that the degenerating mitochondria are present in the muscle of aged blowflies, *in situ*.

DISCUSSION

The present study has described an age-dependent change in mitochondria from blowfly flight muscle. The alteration is characterized, in part, by the reorganization of the normal cristae into concentric myelin-like rings, and this ultrastructural degradation has been correlated with a loss of biochemical function. Thus, cytochrome oxidase activity is demonstrated in the intact cristae, whereas activity is not found in those areas of the same mitochondrion in which the inner membranes have become structurally aberrant. The morphological degeneracy in the mitochondria from old flies is also correlated with recent findings that mitochondria from aged blowflies, compared to mitochondria from young blowflies, have significantly lower adenosine diphosphate (ADP)-stimulated respiratory rates and respiratory control indices (14). A decrease of approximately 30% in the ability of flight muscle mitochondria isolated from 31–33 days old blowflies, as compared to 7–9 days old insects, to oxidize maximally (state 3) pyruvate + proline and α-glycerolphosphate (14) perhaps approximates the relative proportion of the cristae that has undergone ultrastructural reorganization. These changes in the bioenergetic systems with age may provide new insight into the mechanism of the decline in the capacity of the senescent insect to fly.

The age-related ultrastructural changes in flight muscle described in this paper are clearly distinct from degenerative changes in flight muscle of senescent flies previously noted (15, 16). The reported presence of two types of mitochondria in housefly (*Musca*) flight muscle, one of which degenerates early by swelling due to development of vacuoles in the intercristal space, whereas the other deteriorates later by disintegration of the dense matrix, leaving structureless areas (15), is not confirmed in this study with the blowfly. Nor do we find the complete disappearance of glycogen granules from flight muscle, as claimed by Simon et al. (15), for the old (12-day) housefly. The present observations also do not support the disintegration of mitochondria by densification and vesiculation, as reported in senescent *Drosophila* flight muscle (16). Greatly enlarged mitochondria containing granular material, presumed to be intramitochondrial glycogen, have been described in heart muscle of aging *Drosophila* (17).

Membranous whorls in mitochondria have been found in spinal ganglion neuroblasts (18) and in cases of familial myopathy (19, 20). Pannese (18) has suggested that the whorls may be related to the formation of new mitochondria. This hypothesis is not supported by our observations in flight muscle.

The significance of the finding of intense acid phosphatase activity in the dyad comprising the T system and sarcoplasmic reticular membranes in flight muscle remains to be clarified. Essner et al. (21) noted the dephosphorylation at pH 5 of cytidine monophosphate in the transverse sacs of the sarcoplasmic reticulum of rat cardiac muscle and speculated on the role of phosphatases in relation to the movement of calcium ions by the reticulum. As yet, no attempt has been made to determine whether the activities in rat heart and insect flight muscle are due to the action of a common enzyme or to explore further their possible functions.

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