Denervation and Disuse Atrophy in Pigeon Breast Muscle

An Electron Microscopic and Biochemical Study

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A NUMBER of biochemical and electron microscopic investigations have conclusively shown that the contractile component of the skeletal fiber undergoes retrogressive changes upon sectioning of the motor nerve [1-5] and that these changes follow essentially a similar pattern in all types of muscle studied.[6-9] At variance with the rather uniform modifications of the contractile structure, the response of the sarcoplasmic components to denervation varies among the types of muscle. Both increase and diminution of mitochondria and sarcotubular constituents have been reported to occur in different muscles.[9-15] The wide variability of findings can only in part be accounted for by the different duration of denervation studied by the various authors, and by the animal species used. It has thus been considered fairly probable that the response of sarcoplasmic structures to denervation might depend on the metabolic specialization of the muscle studied.[16]

In order to comprehend in an univocal interpretation the differing, and even conflicting, results reported, the hypothesis has been advanced that, as a consequence of denervation, a regression occurs of the muscle fiber toward a less differentiated or "embryonic" form. This general proposition, formulated by Eccles [17] and others,[18,19] has been essentially based on the observation that embryonic or immature muscles exhibit neuroelectric and structural features somewhat different from those characteristic of the same muscles after innervation is completed.[20,21] However, only a few observations have been reported in relation to the forementioned hypothesis. In denervated rabbit gastrocnemius and soleus muscles, and in leg muscles from myodystrophic chicken, a variation in the lactic dehydrogenase contents and in the relative proportions of the main isozymes regressing toward levels characteristic of the embryonic stages of muscles has been observed.[22-24] The overdevelopment of the sarcoplasmic structures (mitochondria and sarcoplasmic reticulum), which occurs in frog semitendinosus muscle at the early stages

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This work has been supported by a Grant from the Italian National Research Council.
Accepted for publication Feb. 29, 1968.
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after nerve section, has been regarded as an adaptive phenomenon to a less differentiated metabolic situation of the muscle fiber.[9]

It seemed, therefore, of some interest to analyze the biochemical and ultrastructural changes that occur at the level of the sarcoplasmic constituents in a metabolically highly specialized muscle such as the pectoralis major of the pigeon. This muscle is well known to possess a high oxidative capacity which develops progressively in the course of maturation. In fact, striking biochemical modifications have been observed in mitochondria isolated from denervated pigeon breast muscle,[10,11] and a possible role of such functional injuries in the genesis of the retrogressive changes of the contractile material has been suggested.[11,25] It was of interest then to examine the biochemical lesions found in the isolated mitochondria and to determine if this was parallel with ultrastructural modifications of the mitochondria in the intact cell, and if these mitochondrial changes preceded modifications of the myofibrils.

In addition, in order to gain better insight into the problem of a possible long-term influence of nerve on muscle cell, a careful comparison was made of the changes induced by the section of the motor nerve with those due to simple disuse. This was accomplished by studying the effects of tenotomy in the same muscle.

Material and Methods

Animals

Adult pigeons (*Columba livia*) weighing 300–400 gm. were used. The animals were freely fed and kept in cages at room temperature. Operations were performed with ether anesthesia. Denervation of the breast muscle (pectoralis major) was obtained by incising and severing the wing plexus between the thoracic vertebrae and the scapula. Tenotomy was performed by cutting the insertion of the pectoralis major on the deltoid crest of the humerus.[26] The contralateral muscle was routinely used as a control.

Light and Electron Microscopy

For histologic examination, small bundles of tissue were fixed and treated according to the conventional procedures. Measurements of muscle fiber diameters were performed on cross sections of paraffin-embedded material, stained with hematoxylin and eosin. For electron microscopic studies, small bundles of fibers were carefully dissected and fixed at resting length in situ with a 2.5% glutaraldehyde solution buffered with 0.13 M phosphate, pH 7.4, and postfixed with a 1% osmium tetroxide solution in the same buffer. The specimens were fixed for 3 hr., rinsed in Tyrode's solution, and dehydrated in a graded series of acetone. All the operations were performed at 0° C. in an ice-cold bath. The material was then carefully dehydrated in absolute acetones, stained with a 0.3% uranyl acetate solution in absolute acetone, and embedded in Araldite. Sections were obtained with a Porter-Blum ultramicrotome and with a LKB 4800 Ultrotome ultramicrotone. Unstained sections and sections stained with lead,[27] were examined in a Siemens Elmiskop IA

and in a Akashi TRS 50 EI electron microscope. The primary magnifications varied from 2000 × to 40,000 ×. The measurements of the fiber and myofibrillar areas were made with a planimeter on the prints of cross sections.

Biochemistry

Muscle was quickly removed from the decapitated animals and collected in an ice-cold 0.25 M sucrose solution containing 5 mM ethylenediamine tetracetic acid (EDTA), pH 7.4. The muscle was freed from connective tissue, minced, and homogenized with a flat-bottomed Lucite homogenizer. The homogenization was performed in distilled water to a final concentration of 10% (w/v).

Cytochrome oxidase activity was measured under the conditions suggested by Schneider and Potter,[28] and assayed at 37° C.

Lactic dehydrogenase activity was measured spectrophotometrically according to the procedure described by Kornberg.[29] Aliquots of 10% homogenate were centrifuged at 105,000 *g* for 1 hr., and the activity was assayed on the supernatant fraction.[30] Aliquots corresponding to 0.5–1.0 mg. fresh tissue, were used. Enzyme assays were carried out in a Beckman Model D.U. spectrophotometer by determining the changes in optical density at 30-sec. intervals for 3 min. The degree of substrate inhibition was calculated by the ratio between the rate of activity with 3.3×10^{-4} M pyruvate and that observed with 1×10^{-2} M pyruvate.[30] From the degree of substrate inhibition, the relative proportion of the 2 main isozymes, LDH-1 and LDH-5, was derived according to the method suggested by Kaplan.[22,24]

Protein was determined by the biuret method. The chemicals, cytochrome C and NADH (Sigma Chemical Co., St. Louis, Mo.), and Na-pyruvate (Hoffmann-LaRoche, Basel, Switzerland) were of analytical grade.

Results

Denervation Atrophy

Light Microscopy. Table 1 illustrates the course of the atrophic process due to denervation in pigeon breast muscle. It is apparent that the mean diameter of the fibers is significantly changed from normal, even 1 day after nerve section, and it is diminished by about 30% after 5 days. From inspection of histologic specimens, there was evidence of pronounced degenerative modification, such as contracture and waxy degeneration, from the tenth day onward. Therefore, most of the work with the electron microscope was performed on material at the early stages of the atrophic process (1–10 days after denervation).

Table 1. Changes in Fiber Diameter of Pigeon Breast Muscle at Early Periods After Nerve Section

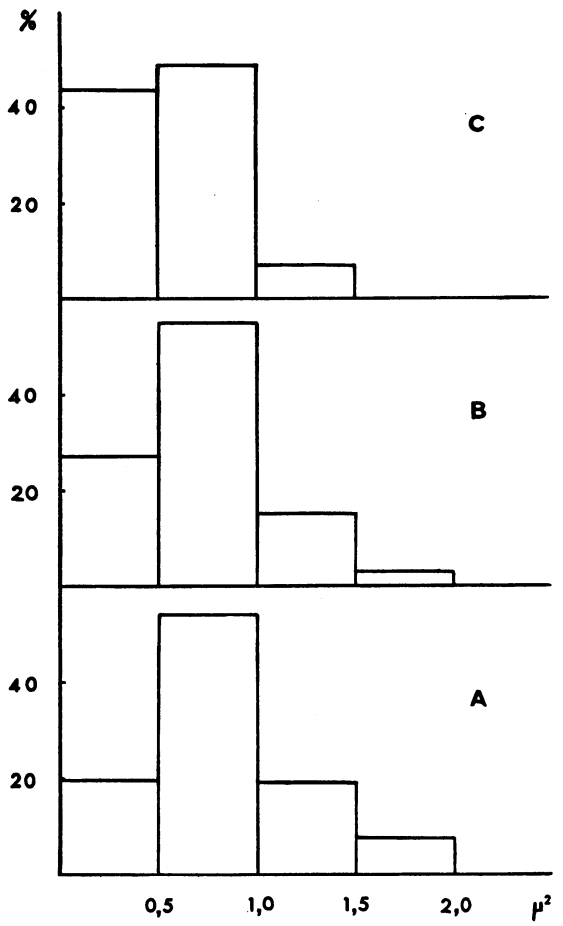
Days after denervation	Controls (μ)	Denervated (μ)	(%)
1	26.9 ± 0.57	25.7 ± 0.59	−5
2	26.8 ± 0.56	25.4 ± 0.68	−5
3	26.7 ± 0.57	24.6 ± 0.62	−8
5	26.8 ± 0.55	19.5 ± 0.53	−28

The differences between the two mean values of control and denervated muscles of individual groups were statistically significant ($p < 0.01$ in the *t* distribution test). The values for fiber diameter represent the mean ± standard error. The mean values of each individual group were obtained from about 300 measurements.

Electron Microscopy. Figure 1 illustrates a representative section through a normal fiber. The myofibrils show the ordinary cross striations seen in a somewhat relaxed state of the fiber. Very numerous mitochondria are visible in the intermyofibrillar spaces. They are organized as elongated columns and clusters, regularly distributed between the myofibrils. As already noted,[31] a characteristic feature of mitochondria of this muscle is the presence of a large number of inner membranes (cristae), indicating that they are particularly rich in cytochromes.[32] This has been substantiated by biochemical analysis of the isolated mitochondria.[11,25] The elementary structure of mitochondria, as well as their density per fiber, as revealed with the electron microscope, accounts for the high oxidative capacity of this muscle.[33] In close relation with mitochondria, lipid droplets are frequently observed. These are surrounded by a limiting membrane or layered material, and vary considerably in amount from fiber to fiber. In between the myofibrils, the sarcotubular system is seen as a regular array of tubular elements and vesicles. The system appears underdeveloped in pigeon breast muscle; however, its detailed structure conforms to the general pattern described in other types of skeletal muscles.[34] The triad units are located at the level of the Z band. Glycogen aggregates can also be seen in variable amounts between the myofibrils and myofilaments, most frequently at the level of the I band.

In Text-fig. 1, a histogram obtained from measurements of myofibrillar areas carried out on electron micrographs of cross-sectioned fibers, the frequency distribution of the myofibril areas values is illustrated. As may be noted, there is an increased frequency of smaller myofibrils 1 day after sectioning of the motor nerve and the diminution in diameter reaches a value of 17% in 3 days. The atrophic process conforms to the common pattern described in other types of muscles [6-9] (Fig. 2–4), and the residual structures do not show appreciable changes as far as their elementary geometry is concerned (Fig. 2 and 3).

The reduction in diameter of the myofibrils is paralleled by striking modifications of the level of mitochondria. As can be seen from Fig. 2—a representative section of a fiber 3 days after denervation—the mitochondria appear unusually spaced from each other and smaller than normal. This reduction of mitochondria proceeds very rapidly, and 10 days after denervation, large areas of the fiber appear devoid of the normal chondrioma (Fig. 3). It is noteworthy that the reduction in number and volume of mitochondria is paralleled by the appearance in the fiber of mitochondria significantly modified in their inner structure (Fig. 3 and 4). As is apparent in Fig. 4, mitochondria are observed



TEXT-FIG. 1. Histogram shows frequency distribution of myofibrillar areas from control (*A*) muscle and 1 day (*B*) and 3 days (*C*) after denervation. Mean values in control and denervated muscles were 1.02 sq. μ, 0.95 sq. μ, and 0.84 sq. μ, respectively. Abscissa shows myofibril area in square microns; ordinate, number of myofibrils of individual groups as percentage. Frequency distribution values were obtained from 500 measurements.

with a number of cristae greatly reduced, and unusually spaced in the matrix. The matrix appears increased in amount, but with a density significantly decreased as compared to that in the contralateral control. The spaces resulting from the reduction of the mitochondrial volume and from the diminution of the contractile material appear progressively filled up by unusually large aggregates of glycogen (Fig. 3) and by the membranes of the sarcoplasmic reticulum. The latter appears well preserved and organized, and apparently enlarged (Fig. 3). In the areas of pronounced myofibrillar atrophy, the reduction of the contractile material makes the reticulum even more evident. The T system is also preserved, although moderately enlarged, and its continuity along the whole fiber is maintained at the stages of atrophy studied. Little modification is noted at the level of the lipid bodies.

Biochemistry. In order to investigate whether changes occur at the

level of the fluid matrix of the sarcoplasmic reticulum as a consequence of nerve section, the lactic dehydrogenase activity of denervated muscle was compared with that of the control, for this enzyme is most probably located in the matrix of the reticulum.[35] The results are illustrated in Table 2. It can be seen that the specific activity is practically unchanged from normal up to the first week of denervation. It may be noted, however, from the same table that a variation occurs in the relative proportion of the two main forms of this enzyme as a consequence of the nerve section. This indicates that the lactic dehydrogenase which predominates in the adult muscle—*i.e.* the isozyme LDH-1 [36]—is first reduced after denervation. From the second week onward, there is a progressive decrease in enzyme content toward very low levels. Such variations in both the enzyme content and the isozyme ratio are in good agreement with previous observations by Kaplan and coworkers [22,23] in denervated soleus and gastrocnemius muscles of the rabbit.

Tenotomy Atrophy

Light Microscopy. In order to decide whether some of the observed changes were a specific consequence of the removal of the nerve supply, or were the results of damage from disuse associated with denervation, similar studies were carried out on tenotomized breast muscle of the pigeon. Table 3 illustrates the course of the atrophic process due to tenotomy. It is seen that there is a progressive decrease of the mean diameter of the fibers, and 7 days after tenotomy this is 10% less than the controls. By comparing the results reported in Tables 3 and 1, it is apparent that the atrophic process due to tenotomy is considerably slower than that subsequent to nerve section. In the latter, the mean diameter of the fibers showed the same reduction in 3–4 days. These results are in agreement with a number of observations where muscle sensitivity to either denervation or tenotomy was compared.[16]

Table 2. Changes in Lactic Dehydrogenase Activity of Pigeon Breast Muscle at Early Periods After Nerve Section

Days after denervation	Controls			Denervated		
	Spec. activ.	Substrate inhib.	LDH-1 (%)	Spec. activ.	Substrate inhib.	LDH-1 (%)
5	216	1.67	53.9	218	1.10	35.5
13	242	1.75	56.4	97	1.42	45.8

Specific activity was determined under the conditions described by Kornberg[39] on a 105,000 *g* supernatant fraction,[22] and it is given as units per gram fresh tissue. One unit of activity is the amount of enzyme preparation which causes an initial rate of oxidation of 1 μmole of NADH per minute with 3.3×10^{-4}M pyruvate. The substrate inhibition was obtained by determining the ratio between the rate with 3.3×10^{-4}M pyruvate and that observed with 1×10^{-2}M substrate. The percentage of LDH-1 isozyme was derived from substrate inhibition according to the method suggested by Kaplan.[24] The values reported are the results of 2 typical experiments.

Table 3. Changes in Fiber Diameter of Pigeon Breast Muscle at Early Periods After Tenotomy

Days after tenotomy	Controls (μ)	Tenotomized (μ)	%
4	26.8 ± 0.48	26.04 ± 0.54	−3
7	26.6 ± 0.49	24.04 ± 0.56	−10

The difference between the mean values of control and tenotomized muscles of individual groups were statistically significant ($p < 0.01$ in the t distribution test). The values for fiber diameter represent the mean ± standard error of the mean. The mean values of each individual group were obtained from about 300 measurements.

Electron Microscopy. Figure 5 illustrates a representative section through a fiber 8 days after tenotomy. The elementary structure of the myofibrils is well preserved and no variations are observed at the level of the sarcotubular system. Very numerous mitochondria are still seen in the intermyofibrillar spaces. They form clusters and are spaced from each other as in the controls. In some areas the mitochondria appear moderately smaller than normal; however, modifications of their inner structure such as those described in the denervated fibers were not observed.

Biochemistry. To evaluate quantitatively whether the mitochondrial complement of pigeon breast muscle was modified by tenotomy at the stages of atrophy studied, the specific cytochrome oxidase activity was assayed at different intervals from tenotomy. The results are reported in Table 4. It can be seen that no variations in enzyme content were found in tenotomized muscles as compared with contralateral control muscles, up to stages of atrophy corresponding to a 12% decrease of muscle fiber diameter.

Discussion

It is apparent from the *Results* that a peculiar feature of denervation atrophy in pigeon breast muscle is the high rate at which the atrophic process occurs. However, as far as the myoplasm is concerned, the nature of the process, as well as its detailed mechanism, is similar to that

Table 4. Changes in Cytochrome Oxidase Activity of Pigeon Breast Muscle at Early Periods After Tenotomy

Days after tenotomy	Specific activity (μl. O_2/gm. fresh tissue/hr.)	
	Control	Tenotomized
6	89,570	93,615
	(5)	(5)
8	94,320	92,240
	(6)	(6)

Cytochrome oxidase activity was tested at 37° C. on a distilled water homogenate under the conditions suggested by Schneider and Potter.[38] The degree of atrophy was about 8% and 11%, respectively, as estimated from the decrease of fiber diameter. Figures in parentheses are the numbers of experiments.

described in other types of muscles.[6-9] In fact, the atrophy is characterized, at the subcellular level, by a progressive loss of contractile material, as shown by the early and increasing reduction of myofibrillar diameters. The modifications of the myoplasm essentially follow a pattern similar to the atrophy consequent to tenotomy, although the rate of the retrogressive change due to disuse is considerably lower as compared to that of the alteration occurring upon sectioning of the motor nerve.

As far as the sarcoplasm is concerned, significant changes of mitochondria and sarcotubular system occur as a consequence of nerve section. A rapid and progressive diminution in number and volume of the mitochondria takes place in the fiber from the very early stages after denervation. The electron microscopic findings confirm in finer detail previous observations made with the phase-contrast microscope on the same material,[10] and are in agreement with a series of biochemical evidence obtained with differential centrifugation fractioning of muscle homogenate, indicating that there is a progressive decrease of the mitochondrial yield upon sectioning of the motor nerve in pigeon breast muscle.[11]

The diminution of the mitochondrial complement is paralleled by the appearance in the muscle cell of mitochondria greatly modified in their inner structure. As reported in *Results*, such mitochondria are characterized by cristae unusually spaced from each other, significantly reduced in number, and by a matrix of low electron density. It has been noted that although the relative proportions between matrix and membranes components in the same mitochondrion are greatly modified, the elementary structure of the membranes is unchanged from normal, at least as it can be judged from electron microscopic observations of sectioned material. This evidence may account for the biochemical finding that there is a considerable reduction in cytochrome oxidase activity, as well as in the ability to oxidase tricarboxylic acid cycle substrates, in mitochondria isolated from pigeon breast muscle undergoing denervation atrophy.[11] The fact that no modifications were found in the capacity of the same mitochondria to couple oxidation with phosphorylation points to the same conclusion. This process appears to be controlled by a phosphate acceptor similar to that found in the mitochondria from control muscle. In fact, such mitochondrial properties are known to require the maintenance of the normal organization of the membrane system.

It is noteworthy that similar changes, both morphologic and biochemical, are not found in mitochondria of pigeon breast muscle undergoing tenotomy atrophy, at comparable stages of the process. Consistent with this finding is the observation that there is an early impairment in the

turnover of the high-energy phosphates in denervated pigeon breast and rat gastrocnemius muscles that is not found in the same muscles after tenotomy.[37] The present results are also consistent with the biochemical evidence that denervation causes a marked decrease in the mitochondria-linked hexokinase, in contrast with the fact that tenotomy does not affect the hexokinase distribution pattern in the same muscles.[38] The presence of mitochondrial changes in denervated muscle that are not found in the same muscles after tenotomy may account for the observation that oxygen uptake is greatly reduced during denervation atrophy in rabbit tonic muscles, but it appears unchanged in the course of tenotomy in the same muscles.[39]

The absence of early changes in the mitochondria of tenotomized muscles seems to make unlikely the possibility that the mitochondrial damage may play a primary role in the loss of the contractile material. The hypothesis, in fact, has been considered that an impairment of protein biosynthesis might have occurred as a consequence of a reduced disposal of ATP of mitochondrial origin.[11,40]

Parallel to the mitochondrial changes, significant modifications also occur at the level of the sarcoplasmic reticulum. This appears decidedly more prominent in the atrophying fiber when compared to the contralateral control muscle, and moderate enlargements are visible along the whole tubular system. It seems not unreasonable to suppose that such structural changes may be related to metabolic modifications in the anaerobic utilization of carbohydrate. In this regard, it seems noteworthy that changes in both the quantity and the relative isozyme proportions of the lactic dehydrogenase—that is, of an enzyme most probably located in the matrix phase of the sarcoplasmic reticulum[35]—occur in pigeon breast muscle as a consequence of nerve section. Consistent with the above conclusion is also the reported observation that structural changes are seen in glycogen aggregates in denervated muscle. This morphologic finding may bear some relation to previous biochemical evidence that there is a change in the metabolism of this polysaccharyde induced by nerve section.[40]

As to the biologic meaning, the modifications observed seem to be consistent with the hypothesis that, consequent to the removal of the nerve supply, a regression occurs in the muscle cell toward a less differentiated structure and metabolism.[17-19] Several lines of evidence seem to support the conclusion that such dedifferentiation actually occurs at the level of the sarcoplasm in denervated pigeon breast muscle. The changes, both structural and biochemical, occurring in the mitochondria, indicate that the high levels of the aerobic metabolism that char-

acterize the adult pigeon breast are reduced to levels typical of the same muscle immediately after hatching.[41] At the same time, the inner structure of mitochondria tends to be of a less differentiated organization. The decrease of lactic dehydrogenase first involves the isozyme that predominates in the adult muscle, and thus the relative proportions of the main isozymes of lactic dehydrogenase tend to ratios similar to those found in the young muscles.[36] The absence of mitochondrial changes in muscles undergoing tenotomy atrophy points to the conclusion that the mitochondrial modifications should be considered not as a trivial damage associated with the atrophic process, but as most probably an adaptive phenomenon. It seems reasonable, therefore, to conclude that the maintenance of the structural relationships between nerve and muscle at the same time conditions the metabolic differentiation characteristic of the adult muscle.

Summary

Denervation atrophy is characterized, in pigeon breast muscle, by a very high rate of the atrophy process. Associated with the progressive loss of the contractile material, a series of modifications occur at the level of the sarcoplasmic substructures (mitochondria and sarcoplasmic reticulum).

From the very early stages of the process, mitochondria are decreased in number and volume, so that the mitochondrial complement per fiber is greatly reduced. This electron microscopic evidence accounts for the biochemical finding that denervation atrophy is associated in pigeon breast muscle with an early decrease of mitochondrial activities. The reduction of mitochondrial complement is paralleled by the appearance in the atrophying fiber of mitochondria of unusual organization as far as the cristae and the matrix are concerned. Similar modifications of both structure and number are not found in the same muscle undergoing tenotomy atrophy.

The mitochondrial modifications are associated with significant changes of the sarcoplasmic reticulum that appear, more prominent than the contralateral controls. The morphologic changes of the reticulum are most probably related with a series of changes occurring at the level of the anaerobic metabolism of carbohydrate, as shown by the variations in the relative proportions of the main isozymes of lactic dehydrogenase.

The above results are discussed in relation to the hypothesis that, as a consequence of nerve section, a regression occurs toward less differentiated levels or forms of structure and metabolism.

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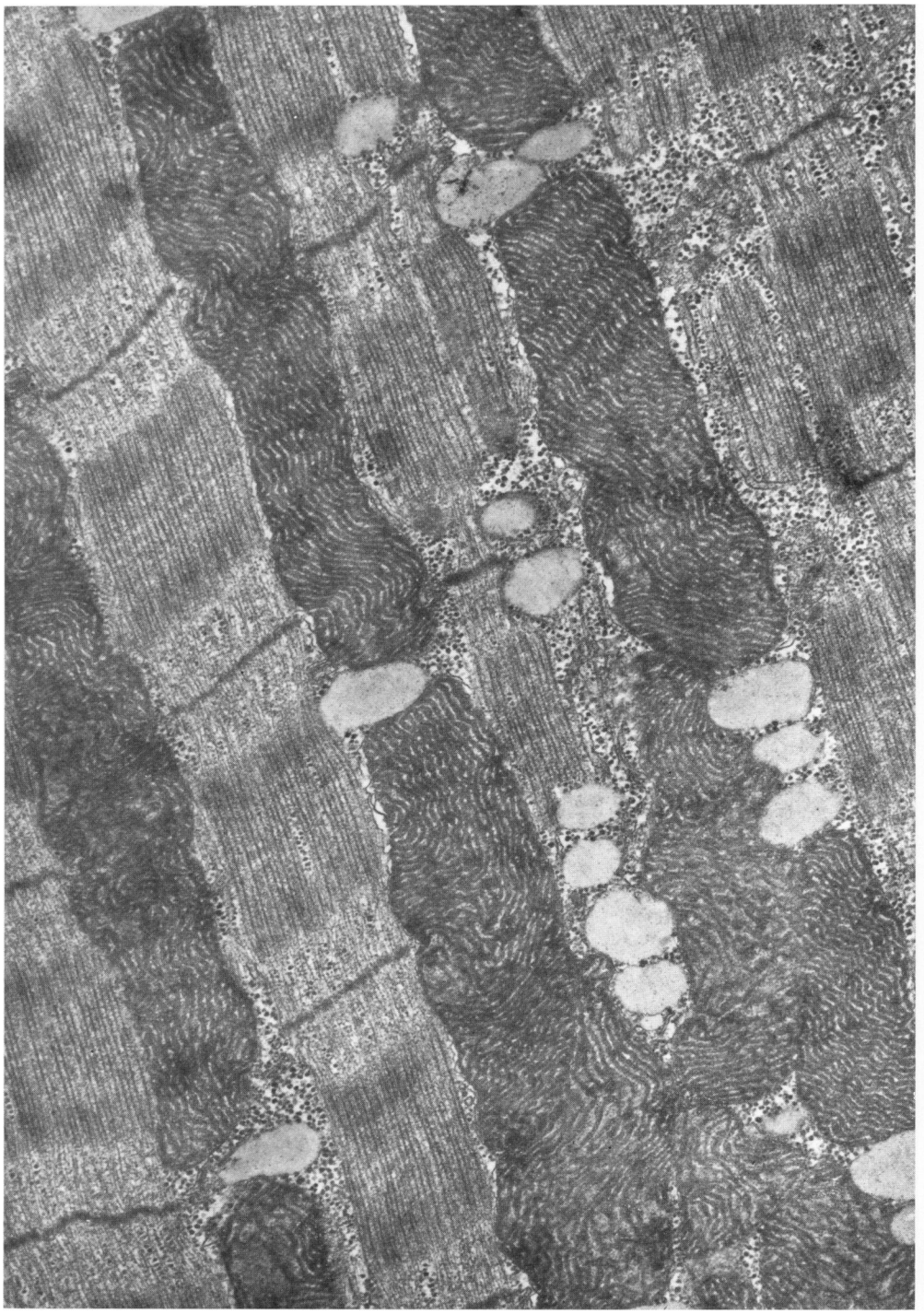
[*Illustrations follow*]

Legends for Figures

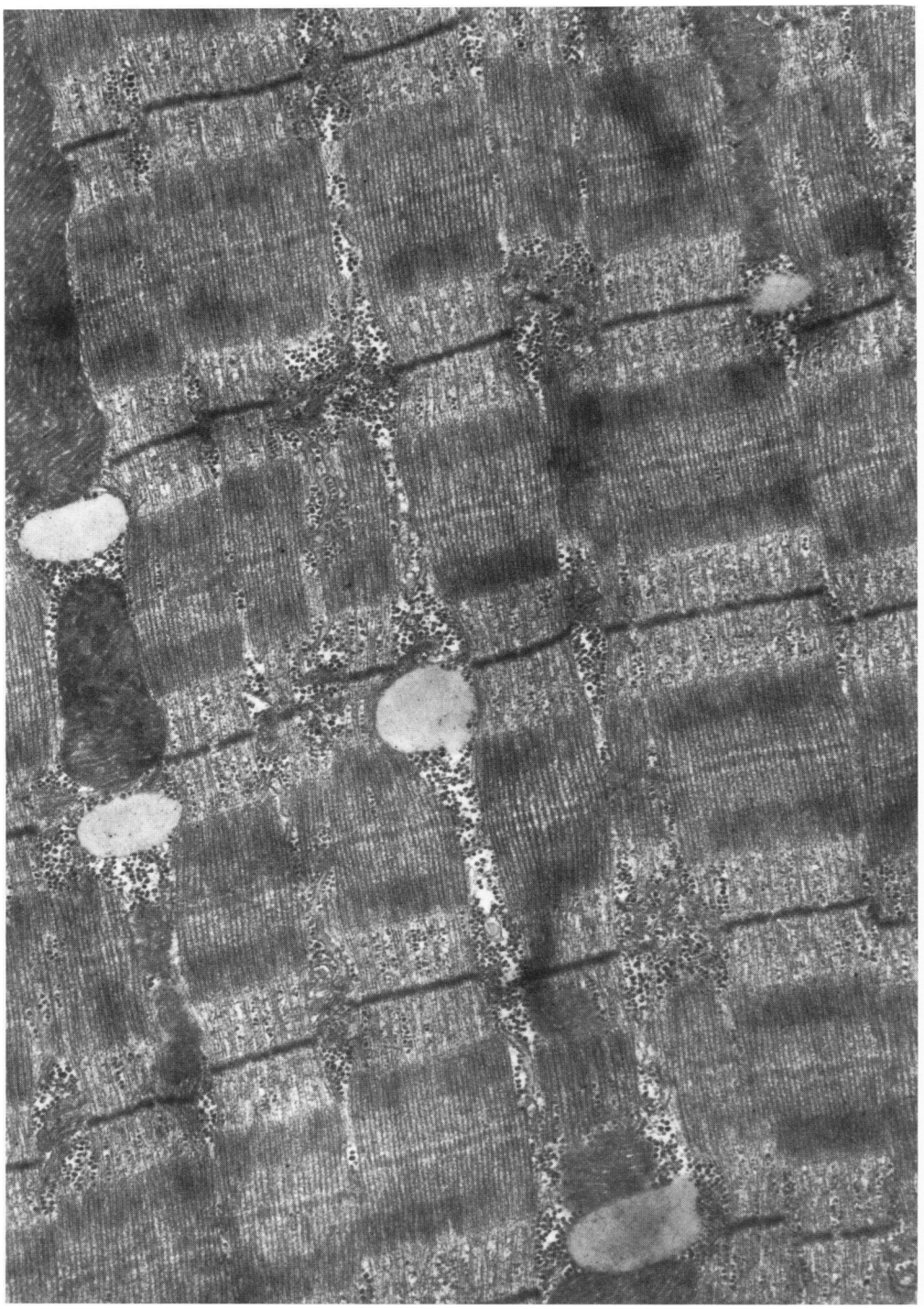
Specimens were fixed in glutaraldehyde, postfixed with osmium tetroxide, and embedded in epoxy resin. Sections were stained with uranyl acetate and Pb.

Fig. 1. Representative section through typical fiber from control pigeon breast muscle. Elongated columns and clusters of mitochondria are between myofibrils. Mitochondria are characterized by extraordinarily high number of inner membranes (cristae). Lipid bodies are also seen in close association with mitochondria. In intermyofibrillar spaces, glycogen is visible in variable amount. Membranes of sarcoplasmic reticulum can also be recognized, most clearly at level of I bands. × 24,000.



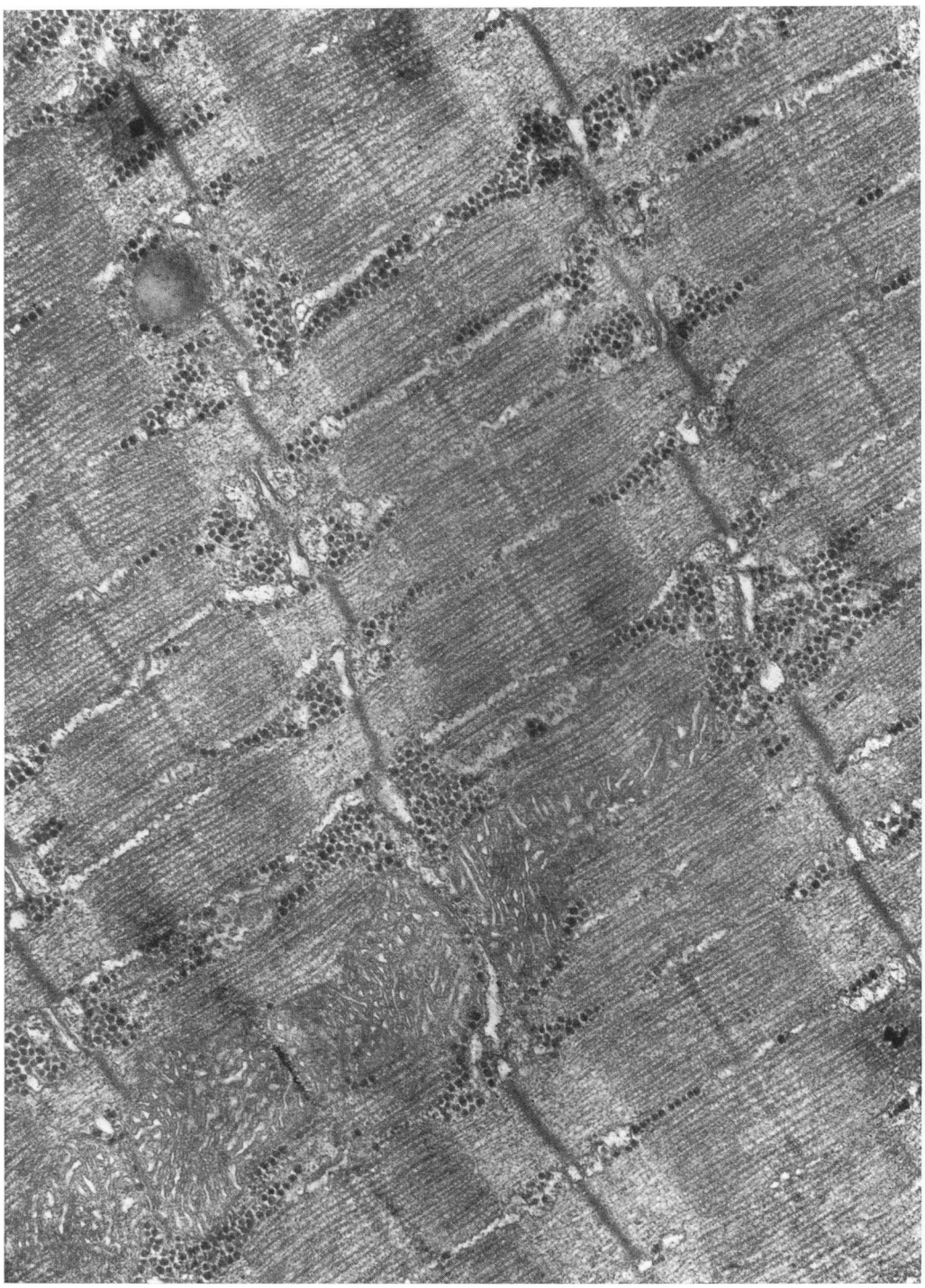
1

Fig. 2. Representative section through fiber 3 days after nerve section. Mitochondria appear reduced in volume and number, and in intermyofibrillar spaces, glycogen aggregates and reticular membranes appear more evident than in controls. Biochemical analysis showed a 38% decrease of cytochrome oxidase activity. × 24,000.



2

Fig. 3. Representative section through a fiber 10 days after nerve section. Advanced atrophy of contractile material is well evident. Very few mitochondria are seen, and inner structure appears greatly modified. The number of cristae per mitochondrion is in fact considerably reduced as compared to contralateral controls, and matrix shows decreased electron density (compare Fig. 1 and 3). Biochemical analysis showed a 62% decrease of the cytochrome oxidase activity of whole muscle homogenate as compared to contralateral control muscle. Also the elementary structure of glycogen aggregates appear changed from normal, as shown, by the larger diameters of the aggregates. × 32,000.



3

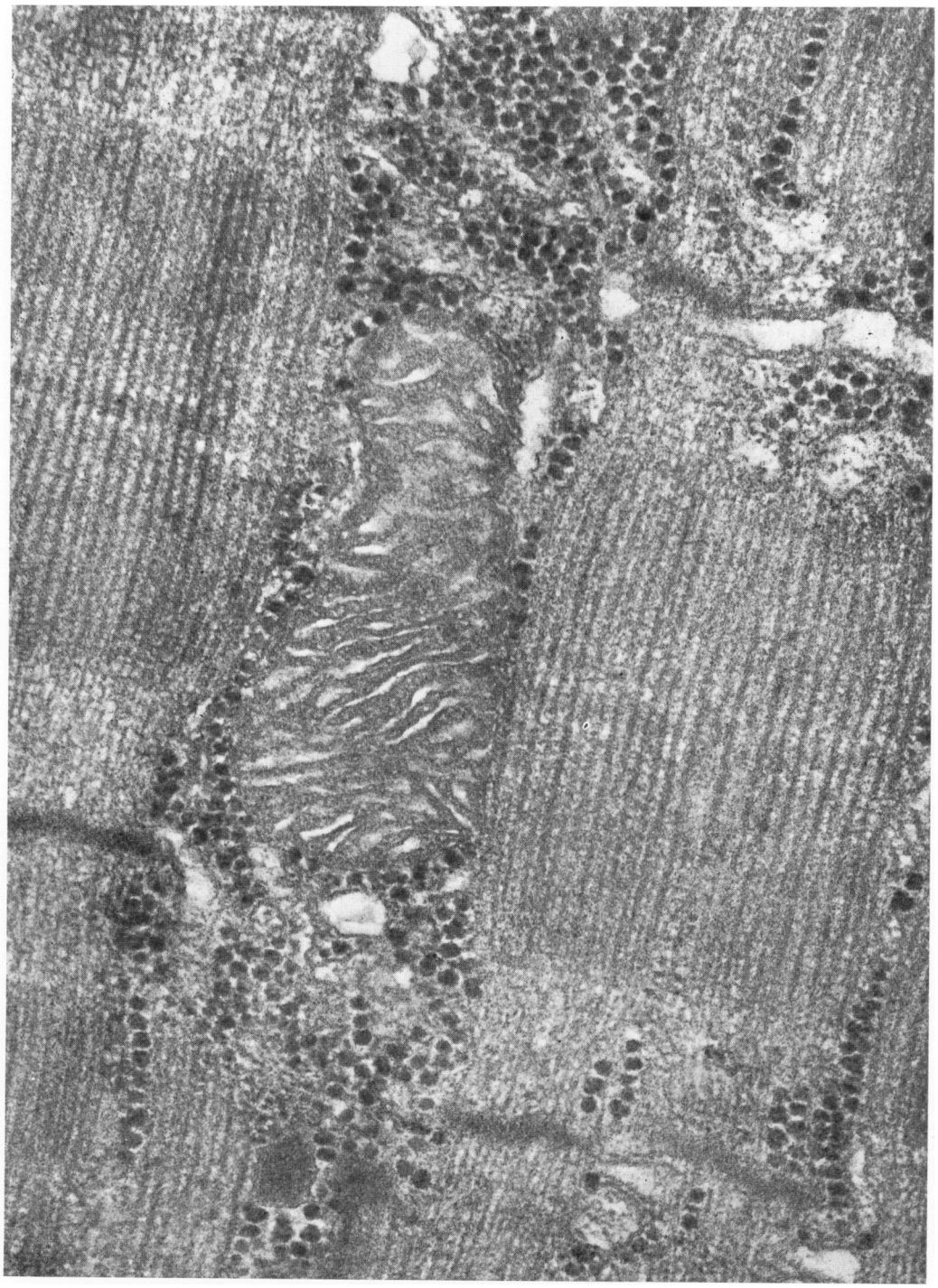


Fig. 4. Elementary structure of mitochondrion in a fiber 10 days after nerve section. Variation in proportion between matrix and membrane constituents of the mitochondrion is apparent, when comparison is made with mitochondria from contralateral control muscle. × 56,000.

5

Fig. 5. Representative section of a fiber 8 days after tenotomy. No modifications are seen at level of mitochondria. Biochemical analysis showed no variations in cytochrome oxidase activity in this muscle during period of atrophy studied. × 24,000.