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ULTRASTRUCTURAL STUDIES ON MUSCULAR
ATROPHY IN MAREK'S DISEASE
I. DENERVATION ATROPHY IN CHICKEN
SKELETAL MUSCLE
A LIGHT AND ELECTRON MICROSCOPIC STUDY

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When denervation was performed by nerve-cutting and nerve-crushing in chickens, three phases could be observed morphologically.

The first phase was characterized by foregoing degenerative changes of the neuro-muscular junctions (axon terminals), followed by atrophic changes of the muscle fibers.

The second phase was characterized by irregular arrangement of the myofilaments in atrophic muscle fibers and appearance of regenerative muscular changes by 20 days after denervation. By this time, no morphological differences could be discerned between the nerve-cut and the nerve-crushed specimens.

The third phase was characterized by distinct morphological differences between the nerve-cut and the nerve-crushed specimens. This phase began from the 30-day after denervation. These differences became more marked day by day. On the 60-day after denervation, almost all muscle fibers showed normal structures of the myofibrils in the nerve-crushed specimens, whereas, muscle fibers were still atrophic in the nerve-cut specimens.

Key words: denervation, neuro-muscular junctions, myofilaments

INTRODUCTION

Marek's disease (MD) is a lymphoproliferative and neuropathic disease of domestic chickens caused by MD virus and belonging to the family Herpetoviridae. Clinical signs of the disease are inability to stand or leg-weakness due to progressive paresis, or complete paralysis of the extremities; therefore, it has been called "fowl paralysis"

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for a long time. The peripheral nerves are frequently affected, and at the same time, the skeletal muscles are often involved. Previous accounts of muscle lesions in MD refer to macroscopic tumorous nodules or microscopic infiltrations of neoplastic cells, except the classification by WIGHT (1966) and OKADA (1970). They classified the lesions into 3 categories or types independently. Category I (neurogenic atrophy) by Wight and type II (neurogenic and disuse atrophy) by Okada both indicated the atrophic changes of the muscle. During the survey of the muscular lesions of spontaneous cases of MD, the authors unexpectedly encountered many cases of myoatrophic changes, which are considered as neurogenic.

In order to clarify the pathogenesis of such atrophic changes and to supplement the findings of the denervation experiment by FUJIMOTO et al. (1970), we conducted a further experimental denervation atrophy study. There are almost no reports on electron microscopic studies of muscular lesions of MD, so we also conducted such studies. The results will be reported in the next report. Therefore, considering the muscular lesions of spontaneous cases of MD, experimental denervation was performed by two methods: one was by nerve-cutting to obtain specimens without reinnervation, and the other was by nerve-crushing to get some grade of reinnervated specimens. These changes were chronologically observed by the light and electron microscopy.

MATERIALS AND METHODS

Young White Leghorn chickens of 5 or 6 weighing 1kg were used in each experiment. They were kept in cages at room temperature and allowed to feed and drink *ad libitum*. Surgery was performed under nembutal anesthesia administered intravenously and was conducted by two methods: 1) nerve-cutting and 2) nerve-crushing.

1) Nerve-cutting: The common trunk of the brachial plexus was ligated and cut off to about 1cm in length, and then the central nerve stump was moved in the opposite direction and glued to the muscle to prevent reinnervation.

2) Nerve-crushing: The common trunk of the brachial plexus was exposed and crushed with a clamp for about 3 minutes.

In each case, care was taken not to damage the blood vessels. The muscles (*M. pectorales superficiales*) were obtained on the 1-, 2-, 4-, 10-, 20-, 30-, 40-, and 60-days after the operations mentioned above.

Light microscopy

The birds were killed, then the muscles (*M. pectorales superficiales*) were removed and immediately fixed in 10% formalin, embedded in paraffin, and sectioned at 4 μ m. Sections for routine examination were stained with hematoxylin and eosin.

Electron microscopy

To prevent artificial contraction, the dissected specimens were stretched and fixed

in 2.5% cacodilate-buffered glutaraldehyde for 30 minutes. Then, they were cut into small pieces (1mm) and fixed again. After that, they were postfixed in 1% phosphate-buffered osmium tetroxide and dehydrated in graded ethanol series and embedded in Epon 812. Blocks were cut by a glass knife with a PORTER-BLUM MT-2B ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate. Observations were made at 75kV with an HU-12A HITACHI electron microscope.

RESULTS

Light microscopic findings

On the 1-, 2-, and 4-day after denervation, the muscle fibers appeared almost unaltered. On the 10-day after denervation, apparent atrophic changes appeared both in the nerve-cut and the-crushed specimens for the first time. Particularly, in the nerve-cut specimens, atrophic myofibers were found around the groups of the normal muscular fibers and situated in the peripheral regions of the muscle bundles (fig. 1). The atrophic muscle fibers had central nuclei which apparently had increased in number. There were open spaces around the individual muscle fibers. The endomy-sium and perimysium were widened and loosened.

On the 20-day after denervation, no morphological differences were discernible between the nerve-cut and the nerve-crushed specimens, and small groups of atrophic fibers with apparently increased nuclei were observed around the normal muscle bundles.

On the 30-day after denervation, atrophic changes of the nerve-cut specimens were more marked than those of the nerve-crushed ones, and group atrophy was prominent (fig. 2).

On the 40-day after denervation of the nerve-cut specimens, numerous atrophic fibers, which were angulated and extensively eosinophilic stained, were observed among the normal or swollen muscle fibers. The interstitium was widened and accompanied by loosening and proliferation of the connective tissues (fig.3). In contrast to the nerve-cut specimens, the muscle fibers in the nerve-crushed specimens began to show regenerative process.

On the 60-day after denervation, the muscle fibers in the nerve-cut specimens were atrophic in general (fig. 4). But the muscle fibers in the nerve-crushed specimens recovered almost to the normal state.

Electron microscopic findings

On the 1- and 2-day after denervation the muscle fibers appeared almost unaltered. But in the axon terminals, morphological changes had already appeared as early as 1-day after denervation. The mitochondria showed swelling and the synaptic vesicles were reduced in number (fig. 5). On the 4-day after denervation, the degenerated axon terminal was completely replaced by Schwann cells. The space between the muscle fiber and the axon terminal was slightly enlarged, and the electron

opaque basal lamina appeared more widened (fig. 6).

On the 10-day after denervation, morphological changes were variable in severity and distribution, but almost all muscle fibers were accompanied by atrophic changes. There were no morphological differences between the nerve-cut and the nerve-crushed specimens. The most prominent changes were observed in the myofibrils. Focal disorganization and disappearance of the Z-bands and irregularity in the stream of the myofilaments were found (fig. 7). The diameter of the myofibrils was reduced in width because of the loss of the peripheral contractile myofilaments. The intermyofibrillar spaces also showed enlargement (fig. 8). By this time, the changes mentioned above already showed diffuse distribution, but they were more severe in the periphery of the muscle fibers. Moreover, typical changes were noticed by the 10-day: very small-diameter muscle fibers occasionally appeared between the normal fibers (fig. 9).

They did not group into clusters. At higher magnification, these very small-diameter muscle fibers consisted of matured myofibrils with a single and smooth basal lamina. Some of them showed loss of myofibrils in the peripheral area.

On the 20-day after denervation, atrophic changes of the muscle fibers were more severe and extensive. Myonuclei became irregular in shape and deeply indented. Intermyofibrillar spaces became enlarged because of the decrease in diameter of the irregularly shaped myofibrils (fig. 10). Particularly in the severe atrophic parts of the muscle fibers, disorganization and breakdown of the myofibrils and the Z-bands, dilatation of the sarcoplasmic reticulum, appearance of honeycomb-like structures, and autophagosomes were more prominent (figs. 11 & 12). Occasionally, there were multicentric lesions consisting of myofilaments crossed with each other which were accompanied by dark spotted Z-band materials. These myofilaments were often irregularly arranged, and some showed a whorled appearance (fig. 13). These changes were most prominent on the 20-day after denervation. Besides these atrophic changes, regenerative changes had already appeared by this time. Myoblasts, myotubes and immature muscle fibers were observed. These regenerative muscle fibers were small in diameter. They had multiple ranged nuclei containing dispersed chromatin and prominent nucleoli, irregular streams of myofilaments, spotted Z-bands and dilated sarcoplasmic reticulums (fig. 14). But at the same time, these regenerative muscle fibers had apparent degenerative changes. For example, they had swollen mitochondria, dilated sarcoplasmic reticulums, appearance of autophagosomes, and honeycomb-like structures (figs. 11 & 15). Furthermore satellite cells were found between the sarcolemma and the basal lamina of muscle fibers, and they were prominent on the 20-day after denervation. According to the above-mentioned findings, there were no clear morphological differences between the nerve-cut and the nerve-crushed specimens by the 20-day after denervation.

But after 30 days of the operations, morphological differences were found between nerve-cut and the nerve-crushed specimens. In the nerve-cut specimens, myonuclei

were centrally located and increased in number (fig. 16). Disorganization and breakdown of the myofibrils and dilatation of intermyofibrillar spaces were prominent (fig. 16). The regenerative muscle fibers showed irregular indentation of the sarcolemma, dilatation of the T canals and sarcoplasmic reticulum and appearance of honeycomb-like structures (figs. 11, 12 & 15). On the other hand, in the nerve-crushed specimens, many myofibrils recovered their normal diameter and regular arrangement, and the intermyofibrillar spaces became normal width in some muscle fibers. Adjoining these muscle fibers, apparent atrophic muscle fibers were often noticed. Therefore, atrophic and regenerative changes were simultaneously present by the 30-day after denervation.

The morphological differences became more marked between the nerve-cut and the nerve-crushed specimens on the 40-day and the 60-day after denervation. In other words, after the 40-day of denervation, the nerve-cut specimens showed continuous atrophic changes, while the nerve-crushed specimens almost normal structures of the myofibers (fig. 17). In the nerve-crushed specimens, the recovery of the myofibrils progressed, and by the 60-day after the denervation, almost all muscle fibers showed normal structures (fig. 18). Whereas, in the nerve-cut specimens, muscle fibers were still atrophic.

DISCUSSION

When denervation was performed by nerve-cutting or nerve-crushing, 3 phases could be chronologically observed.

The first phase consisted of atrophic changes of the muscle fibers, which was apparent by the 10-day after denervation light and electron microscopically. Especially in the nerve-cut specimens, muscle fibers were continuously atrophic until 60 days after the operations. Light microscopically, these atrophic changes started focally and progressed day by day. Finally, the changes became extensive. Ultrastructurally, the degenerative changes of the axon terminals began initially, followed by replacement by Schwann cells. Then, atrophic changes of the muscle fibers occurred, namely, disorganization and disappearance of the Z-bands, breakdown and loss of the myofibrils and enlargement of the intermyofibrillar spaces. The present observed atrophic changes in the muscle fibers were almost similar to those seen in the other reports in mammals^{1,15,26,27)} and in birds.^{9,10,17)} Changes in the axon terminals were also the same as those of the other reports.^{14,16,20,21)}

The second phase was represented by regenerative changes. In other words, in this phase the satellite cells became active and regenerative muscle fibers appeared in the interstitium, and furthermore, whorl-like structures of the myofilaments were found in the atrophic muscle fibers. These changes were most prominent at 20 days after operations, and morphological differences were not discernible between the nerve-cut and the nerve-crushed specimens. Some authors suggested that the satel-

lite cells became active after denervation.^{7,19)} Other investigators suggested that the satellite cells were concerned with muscle regeneration and had myoblastic potentials.^{8,13,22,24)} Among these authors, SCHULTZ (1978) proposed that the small-diameter immature myofibers were derived from the satellite cells and that they were observable within 2 to 3 weeks after denervation. In our experiments, immature fibers were observed in the interstitial spaces by the 20-day after denervation, and by this time, the satellite cells were clearly activated. This finding, therefore, suggests that the satellite cells are intimately connected with muscle regeneration. But these immature fibers also showed prominent degenerative changes at the same time. Concerning this point, ONTELL (1975) and SCHULTZ (1978) also indicated that immature fibers degenerated in the absence of nerve supplies.

As to other changes, some authors referred to the "target" or "targetoid fibers" as the typical initial changes after denervation.^{2,4,12,23,25)} DUBOWITZ (1967) observed other architectural changes such as "ring fibers" and "coiled fibers". The whorl-like structures of myofilaments in our experiments were similar to those of the coiled fibers. DUBOWITZ (1967) reported that the coiled fibers represented disordered morphogenesis, and they were confined to the early phases of reinnervation, after which they became normal. But they are known to be non-specific, and they have not been reported in other reinnervation experiments. So, it is doubtful whether they are indeed reinnervation phenomena.¹¹⁾ In the present experiments, the whorl-like structures of myofilaments were observed both in the nerve-cut and in the nerve-crushed specimens. According to our findings, these abnormal structures were considered as ephemeral phenomena which occurred under a condition of incomplete reinnervation.

The third phase consisted of changes observed on the 30-, 40-, and 60-day after denervation. In this phase, clear morphological differences were depicted. In the nerve-crushed specimens, the atrophic changes were reduced after the 30-day. By the 60-day, muscle fibers recovered almost to normal. In contrast to these specimens, muscle fibers of the nerve-cut specimens had atrophied severely, by the 60-day after the operation. In order to elucidate the differences between them, it was important whether muscle fibers were reinnervated or not. Unfortunately, in our present experiments, we could not find the neuromuscular junctions in this phase. But in similar previous experiments, GONZENBACH and WASER (1973) observed the endplates. They found almost completely normal endplates after 60 days, when the nerve was dissected and glued to a collagenous splint. Considering these findings, reinnervation of muscle fibers seems to be established mostly in nerve-crushed specimens. In this way, reinnervation may reveal the morphological differences between them.

Finally, TROUT et al. (1982) observed unusually small fibers of less than 10 μ m in diameter after 5 days of chloroquine administration in chickens. These fibers were very similar to the small-diameter muscle fibers in our experiment, but their origin

was not clear.

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EXPLANATION OF PLATES

PLATE I

- Fig. 1 The nerve-cut specimen on 10-day after denervation. *M. pectorales superficiales*. The atrophic myofibers are seen around the groups of the normal muscle and situate in the peripheral regions of the muscle bundles. H. & E. stain $\times 60$
- Fig. 2 The nerve-cut specimen on 30-day after denervation. Marked eosinophilic stained atrophied muscle with apparently increased numbers of central nuclei are seen in the peripheral regions of the muscle bundles. H. & E. stain $\times 150$
- Fig. 3 The nerve-cut specimen on 40-day after denervation. Angulated and highly eosinophilic atrophied muscle fibers are seen among the normal sized or swollen fibers. Some connective tissue proliferation is seen in the interstitium. H. & E. stain $\times 150$
- Fig. 4 The nerve-cut specimen on 60-day after denervation. Almost all muscle fibers are atrophic H. & E. stain $\times 60$

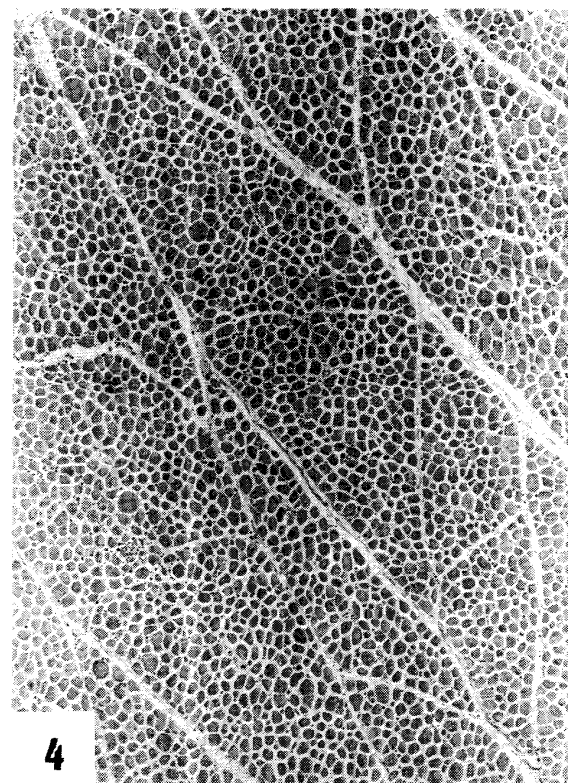
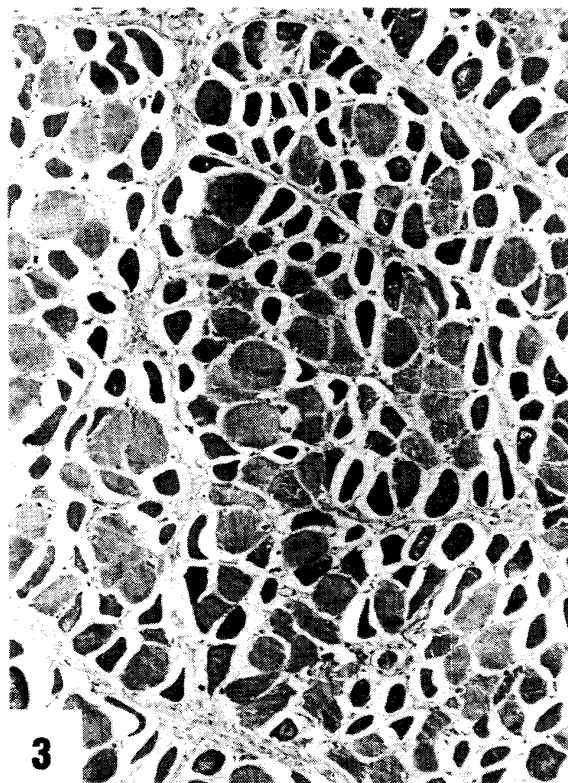
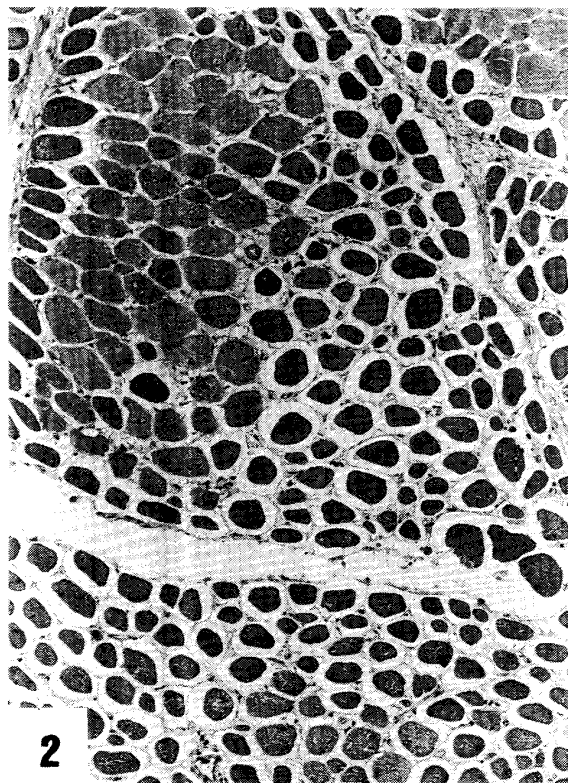
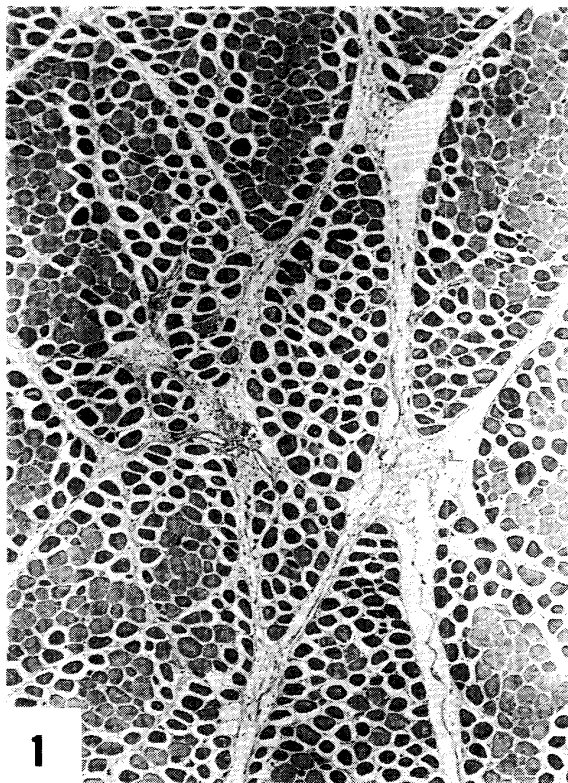


PLATE II

- Fig. 5 The nerve-cut specimen on 1-day after denervation. Axon terminal. There are some swollen mitochondria and apparent loss of synaptic vesicles. $\times 25,000$
- Fig. 6 The nerve-crushed specimen on 4-day after denervation. Axon terminal. The neuromuscular junction is replaced by the cytoplasm of the Schwann cell. $\times 33,000$

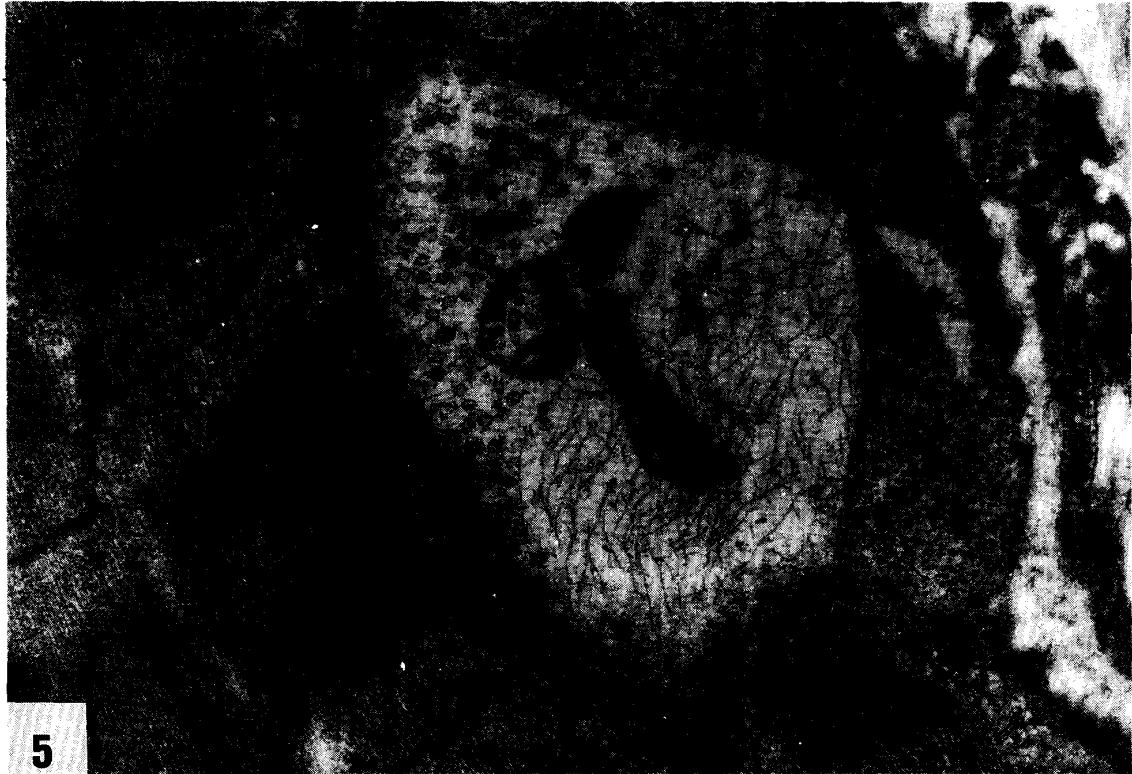


PLATE III

- Fig. 7 The nerve-cut specimen on 10-day after denervation. The Z-bands disappear and the diameter of the myofibrils is reduced because of the breakdown and disappearance of the myofilaments. The streams of the myofilaments are irregular and the intermyofibrillar spaces are enlarged. $\times 13,000$
- Fig. 8 The nerve-crushed specimen on 10-day after denervation. The diameter of the myofibrils is reduced, and the intermyofibrillar spaces are enlarged. $\times 13,000$

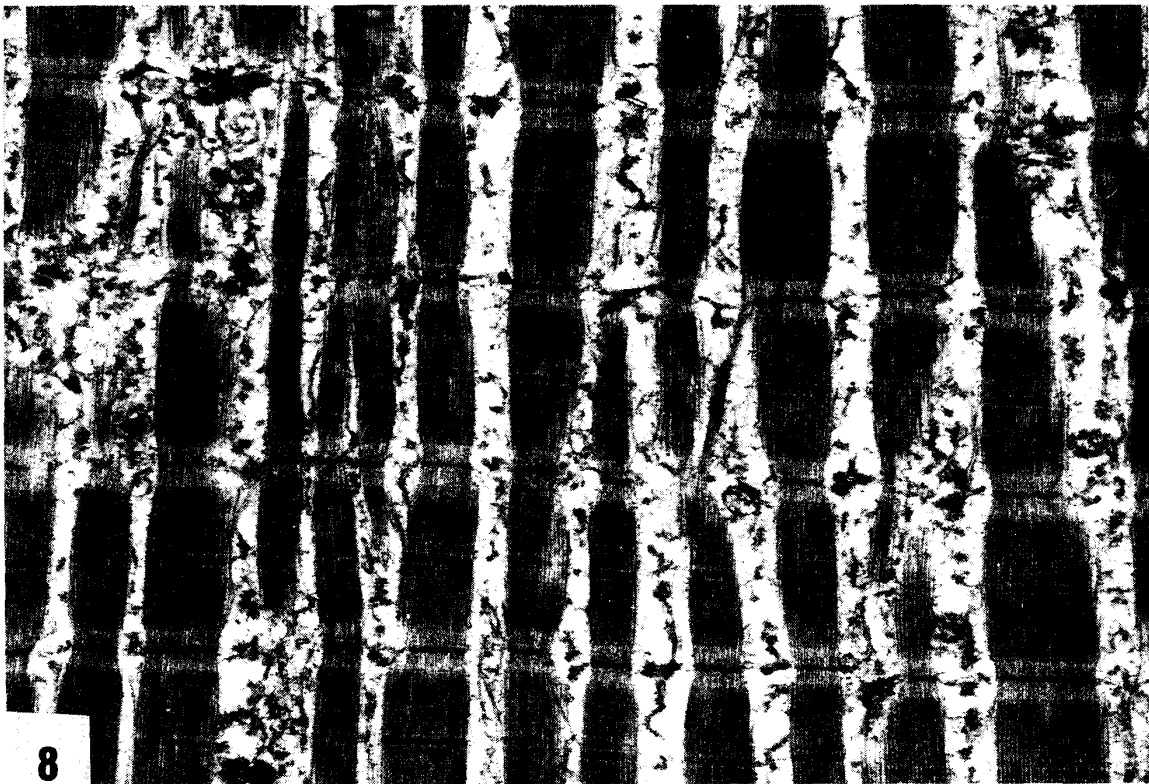
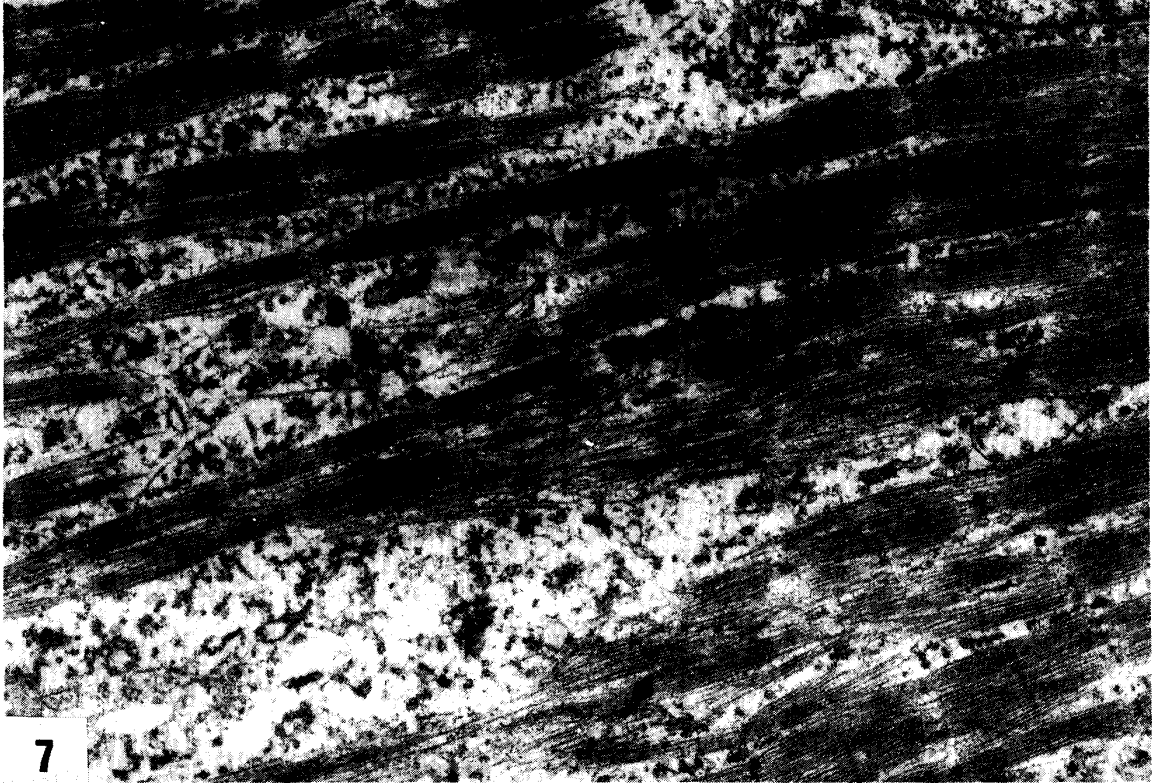


PLATE IV

Fig. 9 The nerve-cut specimen on 10-day after denervation. A myofiber of the small diameter is located among the normal sized muscular fibers. $\times 3,300$

Fig. 10 The nerve-cut specimen on 20-day after denervation. Loss of myofibrils and widened intermyofibrillar spaces are clear. Irregular shape of the myonucleus is seen. $\times 6,000$

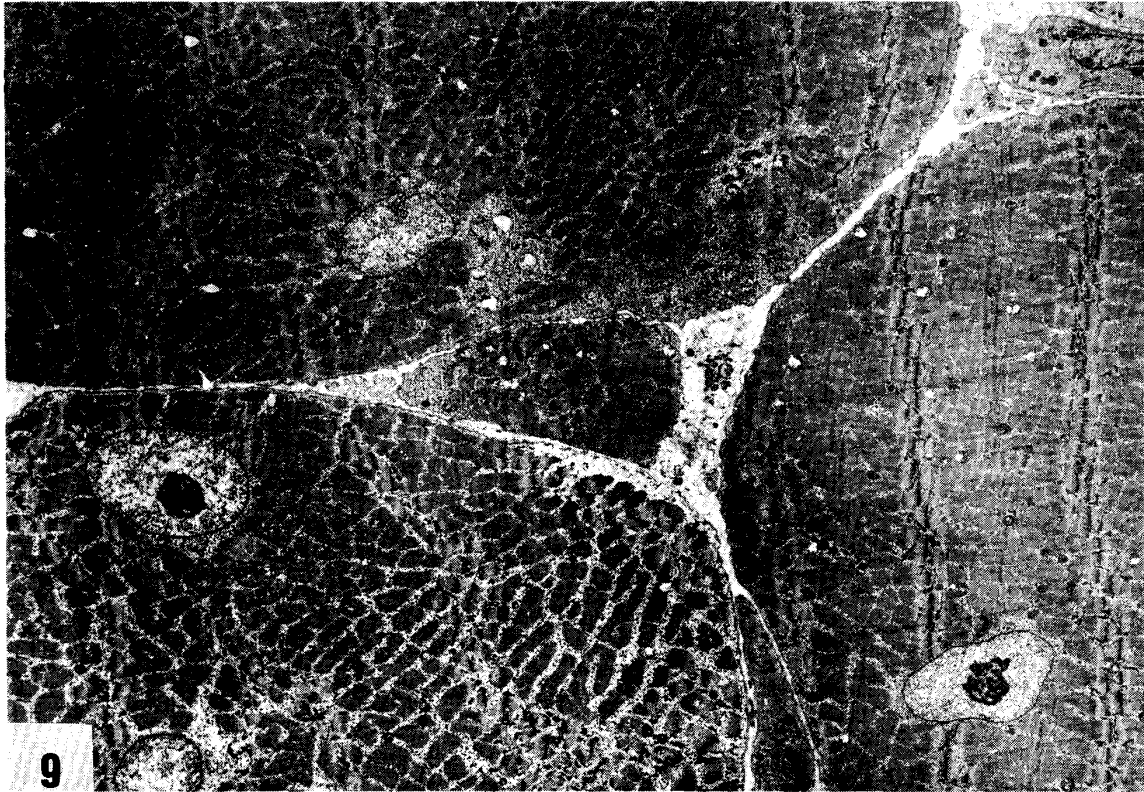


PLATE V

- Fig. 11 The nerve-cut specimen on 20-day after denervation. A centrally located muscle fiber shows severe disorganization and breakdown of myofilaments, dilatation of sarcoplasmic reticulum, spotted appearance of Z-bands, honeycomb-like structures, and irregular shape of the myonuclei. $\times 10,000$
- Fig. 12 The nerve-crushed specimen on 20-day after denervation. The atrophic muscle fibers show multifocal disorganization of the myofilaments. Irregular contour of the sarcolemma is also seen. $\times 8,400$

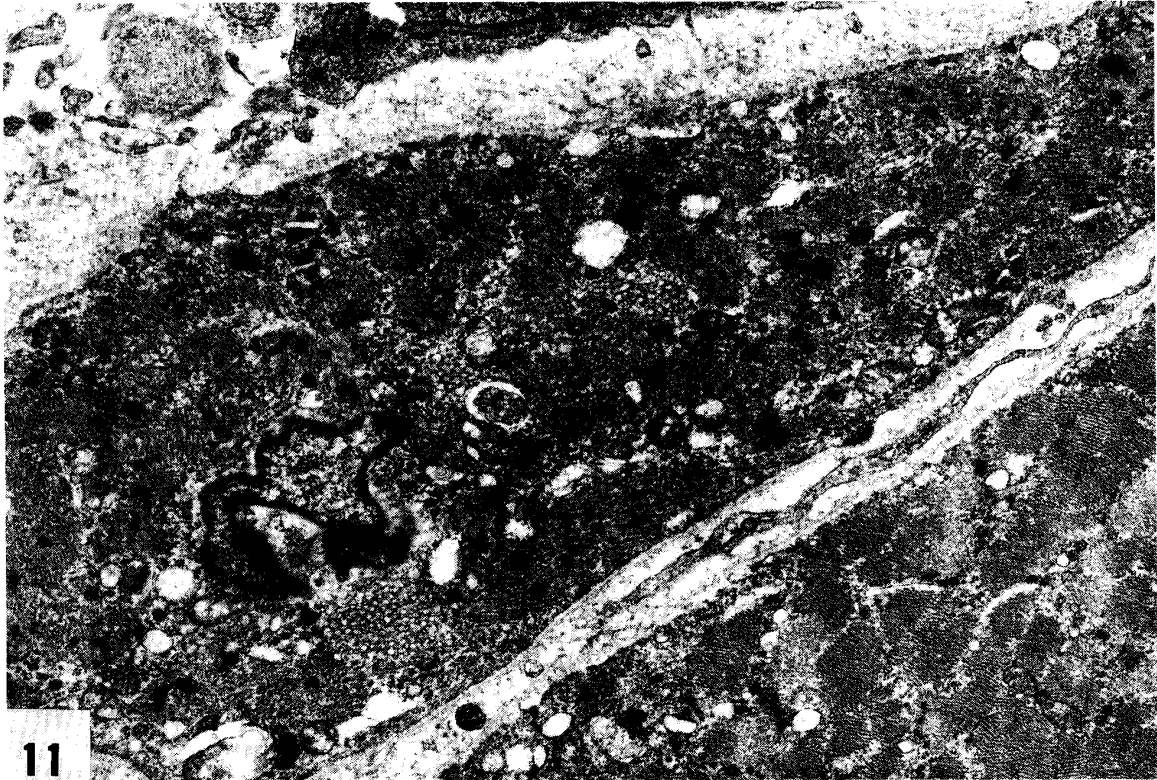


PLATE VI

- Fig. 13 The nerve-cut specimen on 20-day after denervation. The focal whorl-like structures of the myofilaments with spotted appearance of Z-bands are seen. $\times 10,000$
- Fig. 14 The nerve-cut specimen on 20-day after denervation. Myotube has a row of the large myonuclei with large nucleoli and dispersed chromatin. $\times 10,000$

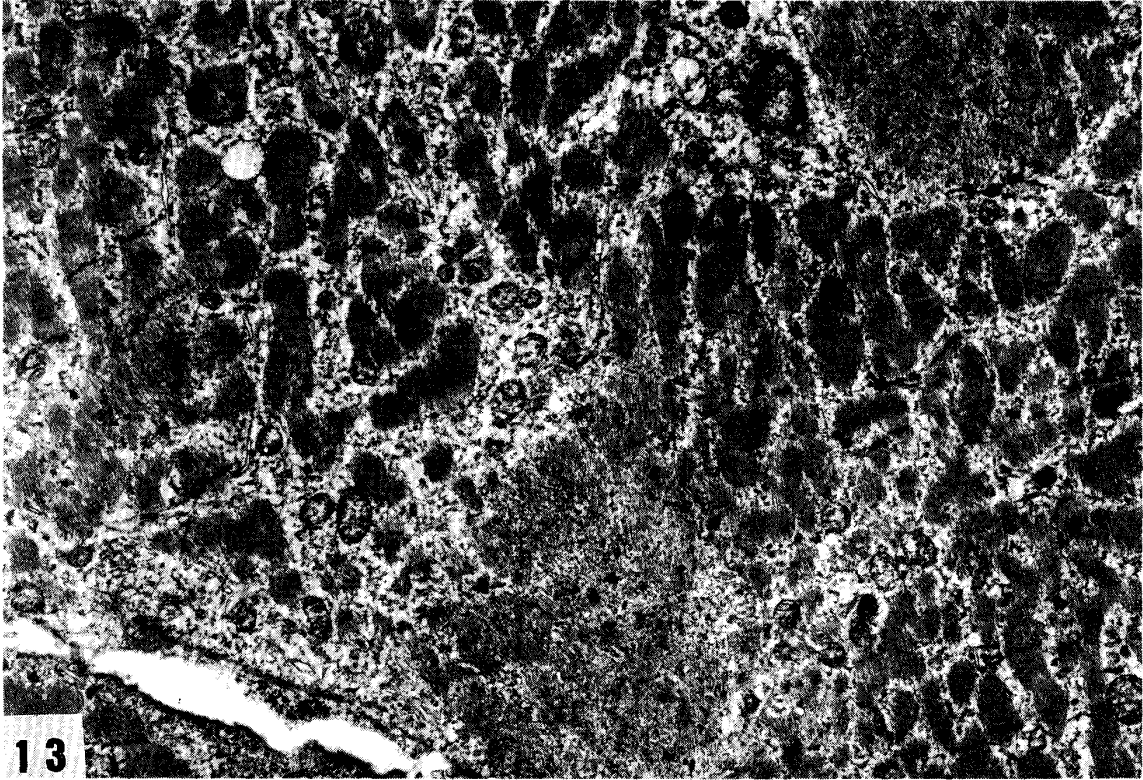


PLATE VI

- Fig. 15 The nerve-cut specimen on 30-day after denervation. The atrophic muscle fibers have apparent degenerative changes such as disorganization of the Z-bands, honeycomb-like structures and loss of myofibrils. $\times 5,000$
- Fig. 16 The nerve-cut specimen on 30-day after denervation. A row of 4 myonuclei is seen in the atrophied myofiber. $\times 5,000$

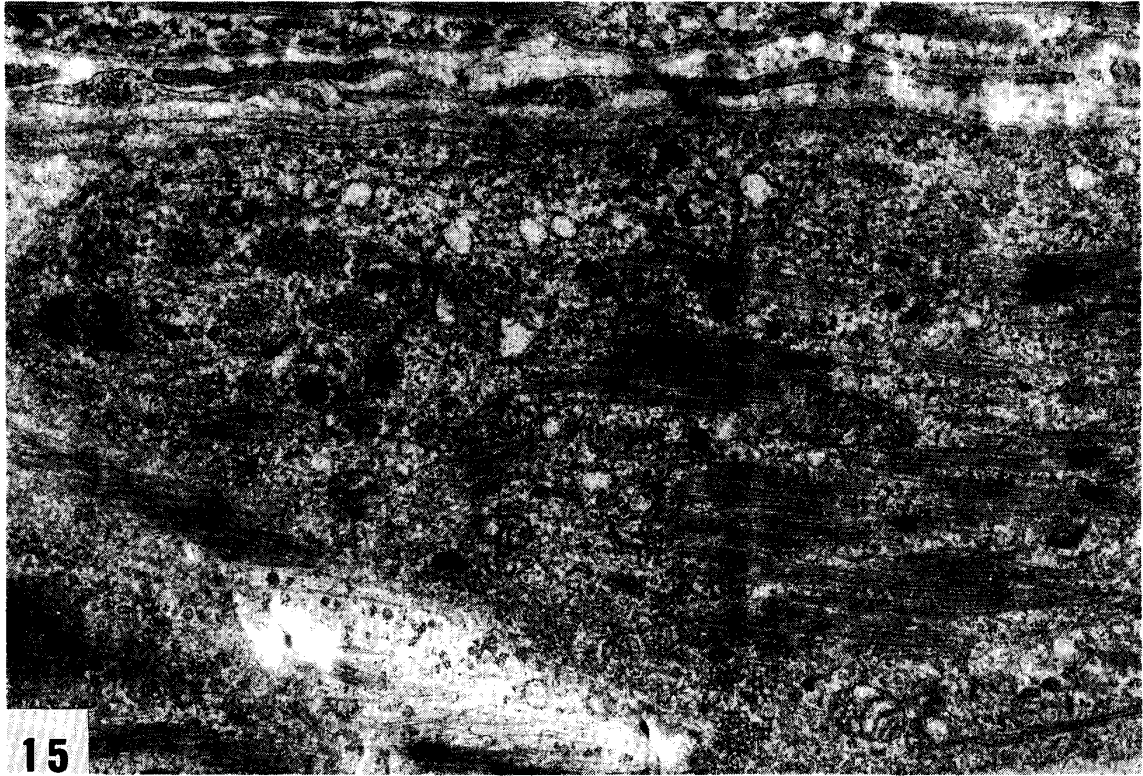


PLATE VIII

Fig. 17 The nerve-crushed specimen on 40-day after denervation. The muscle fibers have almost normal appearances. But some myofibrils are slender. $\times 10,000$

Fig. 18 The nerve-crushed specimen on 60-day after denervation. The muscle fibers have almost normal structures. $\times 10,000$

