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The University of Michigan, Ph.D., 1975
Anatomy

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HISTOLOGICAL AND HISTOCHEMICAL STUDIES ON THE NERVOUS INFLUENCE ON MINCED MUSCLE REGENERATION OF TRICEPS SURAE OF THE RAT

by
Franz Sheng-Fu Mong

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Anatomy)
in The University of Michigan
1975

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INTRODUCTION

Nerve-muscle relationships can be studied *in vitro* (Kano *et al.*, '72; Mendell *et al.*, '72; Crain, '70) and *in vivo* (Close, '71; Jansen *et al.*, '73). The *in vivo* studies can be approached from the standpoint of either normal development or the regeneration of muscle fibers. The purpose of this investigation was to determine the extent to which the nerve is necessary in order for minced muscle fragments to regenerate. The following paragraphs will provide the rationale for this investigation.

One characteristic of the nerve-muscle relationship is that nerves seem to influence the muscle fibers, causing the normal muscle activities intimately associated with the presence of nerve fibers. Factors influenced, for example, include the speed of muscle contraction (Buller *et al.*, '60a; Close, '64; Eccles *et al.*, '62), sensitivity of muscle fibers to acetylcholine (ACh) (Miledi, '63; Axelsson and Thesleff, '59; Thesleff, '63, '74, Fambrough, '70), integrity and development of sole plate components of motor end plates (MEP's) (Lentz, '69, '72; Kelly and Zacks, '69; Hsu and Lentz, '73), integrity of muscle morphology (Gutmann and Zelena, '62), and finally, histochemical characteristics (Burke *et al.*, '71; Guth *et al.*, '70; Peter *et al.*, '72). For the purpose of the present study, muscle morphology and histochemical differentiation of muscle fiber types were selected as end-points of nervous

influence.

Muscle fibers are by no means homogeneous. Early workers in the last century had already noticed that some fibers were red and some fibers were white (see Dubowitz, '65a and Hudgson and Field, '73 for review) and observed that red fibers contracted slowly but were capable of prolonged activity while white fibers contracted quickly but fatigued easily. Since then, many workers have studied skeletal muscle fiber characteristics and have agreed that there are at least three types of fibers in muscle although the nomenclature differs widely (Table 1). Finally, Peter et al. ('72), using a combination of speed of contraction and intensity of histochemical staining activity [succinate dehydrogenase (SDH) and myofibrilar ATPase], devised a useful way of classifying the fibers as follows:

1. F.O.G.--Fast-twitch-oxidative-glycolytic fibers
2. F.G. --Fast-twitch-glycolytic fibers
3. S.O. --Slow-twitch-oxidative fibers

(Table 2).

The best illustration of a nervous influence on muscle fiber types is given by cross-innervation experiments. Buller et al. ('60b) crossed the nerves of a fast contracting muscle (e.g., extensor digitorum longus, EDL) to a slow contracting muscle (e.g., soleus, SOL) and vice versa. They found that the speed of contraction of cross-innervated muscles was reversed; that is, the SOL contracted more quickly whereas EDL contracted more slowly than their respective normal muscles. Also, Mommaerts ('68) and Fex and Sonesson ('70)

TABLE 1: Nomenclatures of three fiber types.

	FIBER TYPES		
Peter et al. (1972)	F.O.G.	F.G.	S.O.
Romanul (1964)	II	I	III
Yellin & Guth (1970)	αβ	α	β
Stein & Padykula (1962)	C	A	B
Padykula & Gauthier (1967)	Red	White	Intermediate
Burke et al. (1971)	FR	FF	S

TABLE 2: Enzyme contents and staining intensities of three fiber types according to Peter et al. (1972).

	SDH	ATPase
F.O.G.	High	High
F.G.	Low	High
S.O.	High	Low

revealed that the enzymatic profiles of cross-innervated muscles were converted to those of the other type. If the nerves were allowed to return to their original muscle, the speed of contraction and enzymatic profiles were returned to normal.

Despite the emphasis on the dependence of muscles on nerves, the intrinsic developmental capacity of muscle had largely been neglected. Steward ('68) cut the nerves to the leg muscles of new-born rats and observed that weights of the muscles still increased, although he did not determine whether it was due to muscle fibers or to connective tissue fibers. Zelena ('62) denervated the tibialis anterior muscle of fetal rats three days before term and observed that the weight increased until two months afterward. She carried out biochemical analysis and found that total protein content increased but the actomyosin did not. Nevertheless, she did notice that in the denervated condition myotubes (precursors of muscle fibers) could undergo morphological differentiation into mature fibers, although the rate was slowed. Engel and Karpati ('68) found that in neurectomized neonatal rats 65% of the myogenic tissues remained in the myotube stage while the rest of the myogenic elements had differentiated into mature fibers. This was also confirmed by Shafiq et al. ('72). Thus, it seems that muscle fibers might have independent activity, at least during the ontogenic development.

One alternative to investigating the intrinsic ability

of muscle fibers to differentiate in vivo during normal embryonic and postnatal development is through regeneration studies since muscle regeneration is essentially a recapitulation of ontogeny (Carlson, '73). It is relatively easy in regeneration studies to separate muscles from nerve fibers or to change their relationships.

The ability of muscle to regenerate after trauma was known more than a century ago (see Hudgson and Field, '73 for review), but was emphasized only during the last two or three decades. Le Gros Clark et al. ('45, '46, '47) observed a substantial degree of regeneration after devascularization and crushing of muscle fibers. Allbrook ('62) also noticed the regeneration of muscle fibers after necrosis and ischemia and gave detailed electron microscopic descriptions. Mauro ('61) described satellite cells and proposed them as the origin of myoblasts for muscle regeneration. This stimulated intensive research in this area.

The injuries most commonly used to cause muscle regeneration are those in which the proximal - distal continuity of muscle fibers are more or less maintained. These include heat (Denny-Brown, '51; Shafiq and Gorycki, '65), cold (Price et al., '64a, '64b), ischemia (Allbrook and Aitkin, '51; Le Gros Clark et al., '45; Le Gros Clark, '46; Reznik, '69), physical injury (Gay and Hunt, '54; Lash et al., '57; Bintliff, '60; Allbrook et al., '66), chemical agents (Jirmanova and Thesleff, '72; Hall-Craggs, '74) and local

irradiation (Reznik, '70), etc. The mincing procedure is one in which the orientation and organization of muscle fibers are completely disrupted. This method was initiated in Russia by Studitsky (Studitsky et al., '63) in the early 1950's and introduced to the western hemisphere by Carlson ('68, '70).

In the minced muscle model the operative procedures are simple and straightforward. One simply removes a muscle [triceps surae (gastrocnemius, soleus, and plantaris) in the present study], minces it into fragments of one mm^3 in size and replaces them back into the original muscle bed. The implanted fragments first degenerate and from these degenerating muscles myogenic elements (myoblasts) emerge. These myoblasts then fuse to form multinucleated structures called myotubes which eventually differentiate into mature muscle fibers. Carlson and Gutmann ('72) found that the mature regenerates proved to be functional upon direct stimulation and behaved like fast contracting muscle. However, specific fiber types were not determined histochemically.

Many investigators in the field of nerve-muscle studies have questioned whether or not muscle can regenerate independently without nerves. For instance, Studitsky (Studitsky et al., '63) first proposed that muscle could not regenerate without nerve since, according to him, the fusion of myoblasts to form myotubes would not occur. Zhenevskaya ('62) maintained, however, that in the absence of nerves, myoblasts and myotubes developed intensively, but further differentiation of muscle fibers was retarded. Jirmanova and Thesleff ('72)

also observed fusion of myoblasts in the absence of nerve fibers after the muscle was injured by methyl-bupivacaine, a toxic agent. Allbrook and Aitkin ('51) and Wildbolz ('70) studied post-ischemic muscle regeneration in the rabbit and found few histological differences between normal and denervated regenerates; however, they did observe degenerative changes subsequently in the denervated regenerates. Hsu ('71), studying the minced muscle regeneration of frogs, observed occasional morphologically mature fibers (cross-striations and peripheral nuclei) in the denervated condition. As in the previous studies, these fibers eventually degenerated. Thus, by 25 to 30 days after the operation, she rarely saw any fibers except those of the connective tissues. None of these investigators determined the degree of maturation of muscle fibers in the denervated regenerates in terms of histochemical fiber types.

Motor end plates (MEP's) are unique structures interposed between muscle and nerve fibers. Their roles in the mediation of nervous (neurotrophic) influence are also the subject of debate (see discussion). In their regeneration studies, Allbrook and Aitkin ('51) observed regenerating nerves around 12 days after trauma and MEP's around 21 days. Zhenevskaya ('62) in the minced system, observed regenerating nerves at the end of the first week and MEP-like structures around the third week. Both of these studies were based on the silver staining techniques rather than direct acetylcholinesterase (AChE) staining.

The present investigator therefore concluded that the following needed to be investigated: (1) direct evidence of MEP's in the regenerates, (2) fiber types, if any, in the regenerates, and (3) the degree of muscle fiber dependence on nerves for regeneration. Thus, the objectives of the present investigation were two-fold:

I. To determine if and when MEP's and distinct fiber types would return, if at all, in minced muscle regenerates.

II. To investigate whether or not nerve is necessary for the regeneration of minced muscle and, if so, the extent to which muscle fiber types, size, number, and morphology depend on the presence of nerve fibers.

MATERIALS AND METHODS

Male Sprague Dawley rats, 70-100 grams in weight (1-1 1/2 months old), were used in the entire experiment. All animals were operated upon according to the technique of Studitsky et al. ('63) and Carlson ('68, '70a). The detailed procedures are described in Appendix I. The experiments can be divided into the following three parts:

I. Normal control--Normal regenerates

II. Experimental----Denervated regenerates

III. Quantitative comparison between normal and denervated regenerates

SERIES I. NORMAL CONTROL---NORMAL MINCED MUSCLE REGENERATION WITH INNERVATION PRESENT

In this series, all the nerves were preserved after the operation. It's purpose was to serve as a control for the ensuing denervation experiments. The points of focus were centered on:

A. Morphological development and characteristics,

B. Time and distribution of nerve return,

C. Time and distribution of motor end plate return, and

D. Time and sequence of events of histochemical fiber type differentiation.

A. Morphological Development

Sixty-three animals were used, and they were examined from day 1 to day 270 after the operation. Paraffin sections (9 um), both longitudinal and cross sections, and H and E staining were used in the entire series. Specimens of early regenerates (day 1 to day 25) were from those originally designed for the detection of nerve regrowth and specimens of long-term regenerates were from those originally intended for histochemical fiber type detection.

B. Return of Nerve Fibers

Twenty-three animals were used. They ranged in age from day 1 to day 25. Longitudinally cut paraffin sections (9 um) stained with Hsu's ('71) modification of the Palmgren ('60) silver stain were used for the purpose. Regenerates from five more rats were prepared with Namba's ('67) silver stain which called for thick (ca 50 um) frozen sections. In both cases, sections of normal gastrocnemius muscle were used for control. The detailed staining procedures can be seen in Appendix II.

C. Return of Motor End Plates (MEP's)

For observation of motor end plates (MEP's), the AChE method of Koelle and Friedenwald ('49) was first tried on 25 regenerates ranging from day 5 to day 45. Then the Koelle method was abandoned

in favor of Henderson's modification of Gomori's acetylthiocholinesterase method (Pearse '72, p. 1312, see Appendix II) because of the ease with which it can be done. Fourteen regenerates from day 5 to day 45 were examined.

Freshly isolated regenerates were immersed in isopentane which had been cooled by dry ice. The whole regenerates were then mounted on chucks which contained nearly hardened embedding medium [International Equipment Company (IEC) or Lipshaw]. Caution had to be exercised to prevent the thawing of tissues. Longitudinal sections (10 um) were cut in the cryostat (IEC). The sections were picked up with cover slips, air dried (10 to 20 min.), and incubated according to Pearse ('72). Some sections were counter-stained with H and E to show muscle fibers. Thick sections (ca 50 um) in some regenerates were also additionally stained with Namba's silver stain. Normal muscle fiber sections were used for control.

D. Differentiation of Muscle Fiber Types

Forty-three regenerates were examined from day 10 to day 270 after the operation. For each scheduled date two regenerates were used except for day 270 for which only one regenerate was used.

Each regenerate was quickly frozen dry-ice-cooled isopentane. It was then divided into proximal, middle, and distal segments. In the latter case terminal tendon was discarded. Cross sections (10 um) were cut from the

distal end of each segment. They were air dried and incubated, together with normal muscle sections which served as controls. For SDH staining, the modified method of Nachlas et al. ('57) and for ATPase stain, a modification of Padykula's method (Padykula and Herman, '55a,b) was used (see Appendix II for both methods). Serial sections were used so that consecutive sections could be stained for both SDH and ATPase. Some sections were fixed in 10% formalin for H and E staining to facilitate the identification of muscle morphology.

After sectioning, the remaining tissues were quickly transferred to Bouin's solution for paraffin embedding. The paraffin sections were used in the morphological studies and for the quantitative analysis which will be discussed later. To aid in identification of the proximal-distal orientation of each segment, a short (2-3mm) No. 6 silk suture was attached to the proximal end of each segment before fixation.

SERIES II. DENERVATION EXPERIMENT

In this series, the sciatic nerve was cut immediately before the muscle mincing operation. This was accomplished in manner described below.

An incision of about 1 cm in length was made in the gluteal region closely parallel to the midline. The gluteal muscles were exposed and artificially separated so that the sciatic nerve could be located proximal to

the level of acetabulum. The nerve was then tied with No. 6 silk suture and a cut was made distal to the knot. The proximal nerve stump was pulled superficial to the gluteal muscles before they were sutured. The nerve stump lay then between the skin and gluteal muscles. The skin was eventually sutured before the mincing of triceps surae during which all the nerves distal to the cut were completely removed down to the level of ankle joint.

A. Morphological Development

Sixty-three animals were used; they were examined from day 1 to day 270 after the operation.

As in the control series, sections of early regenerates (day 1 to day 35) were taken from regenerates originally designed for nerve detection and sections of long-term regenerates (day 10 to day 270) were taken from those originally designed for fiber type detection. Treatment and staining procedures were identical to those used for normal regenerates.

B. Return of Nerve Fibers

Longitudinal sections of denervated regenerates were checked with Hsu's modified Palmgren silver stain to ensure that these regenerates were devoid of nerve fibers. This included the short-term (day 1 to day 35, 20 animals) and long-term (day 10 to day 270, 43 animals) regenerates. Sections were always stained along with sections

of normal regenerates and of normal muscles. Treatment and staining procedures were identical to those used for normal regenerates. Namba's method was not used in this series.

C. Differentiation of Muscle Fiber Types

Forty-three regenerates were examined from day 10 to day 270 after the operation. The number of regenerates for each specific date, the treatment, the staining procedure, and the subsequent handling of the tissues were identical to those described for the normal regeneration series. Sections of denervated regenerates were always incubated simultaneously with sections of normal muscles.

SERIES III. QUANTITATIVE COMPARISON BETWEEN NORMAL AND DENERVATED REGENERATES

Since abnormal fibers were encountered in both normal and denervated regenerates (see Appendix III for the description of abnormal fibers), it was decided to quantify the data in order to see if there were statistical differences or similarities between the two. Also, because the fiber distribution in the regenerate was not known, all three segments of each regenerate were included in the fiber counting.

Paraffin blocks were derived from the remaining groups of tissues originally designed for detection of fiber types.

Cross sections (9μ m) were cut from the distal end of each segment of the regenerate and mounted. Thus, three slides (proximal, middle and distal) could be made from each regenerate and each slide contained 25 to 30 serial cross sections.

After the cross sectioning, the paraffin blocks were trimmed so that longitudinal sections could be cut. All three segments of each regenerate were serially sectioned and the ribbons of each segment were divided into six equal portions. Thirty to thirty-five sections from adjacent portions were selected so that five slides could be made (No. 1 to No. 5). The purpose of this maneuver was to make certain that a random sample could be selected throughout the regenerate. Since slide No. 1 and No. 5 represented the peripheral fibers of the regenerate only and since the peripheral fibers, especially in the early phases, were in morphologically more advanced stage than the central fibers, these slides were not included in the fiber counting. About 5 or 6 sections from each portion of the ribbons were mounted on a single slide for silver stain to demonstrate that in the normal regenerates nerves could be seen and that in the denervated regenerates no nerve fibers had returned. Thus, for each segment of a regenerate, seven slides were made, i.e., 1 cross section, 5 longitudinal sections and 1 for silver stain; this gave a total of 21 slides for each regenerate. For each scheduled date 2 normal and 2 denervated regenerates were

used.

A. Measurements from Cross Sections

In cross sections, the following parameters were estimated.

1. Cross sectional area
2. Number of muscle fibers in the regenerate
3. Number of muscle fibers with peripheral or central nuclei per 100 fibers counted.

1. Cross Sectional-Area

Sections were projected under high power to a paper on screen. The distance between the screen and the projector (microscope) was kept constant so that under high power a final magnification of 1000x could be achieved. On the screen (paper), a rectangular area of 25 x 20 cm was outlined, and those fibers falling into this rectangle were traced individually along the contour of the fibers. The fibers in one rectangle were considered to be one sample and there were four samples for each regenerate, three of them from the peripheral area and one from central area.

After the fibers were traced, a planimeter was used to measure the cross sectional area of every fiber in cm^2. The actual fiber area was then converted to μm^2 and divided by 1000 (Maxwell et al., '73).

Only the sections from proximal segments of regenerates were measured. The normal regenerates were measured on days 10, 20, 30, 40, 65, 90, 150, and 270 and the denervated regenerates on day 10, 20, 30, 40, 65 and 90. Except for days 90, 150 and 270, two regenerates were measured at each scheduled date.

2. Number of Muscle Fibers

The purpose of this estimation was not to show statistical differences but rather to give a numerical appreciation of the amount of muscle fibers, especially in the denervated regenerates.

A grid (100 divisions, 10 x 10) was inserted into the eyepiece. Under high power (40x), the grid was moved, stepwise and successively, through the entire section. Fibers included in the grid were counted and added together. Since only the total number was of interest, no distinction was made between normal and abnormal fibers.

Two normal and 2 denervated regenerates were analyzed on days 10, 20, 30, 40 and 65. Again, only the proximal segments were used.

3. Numbers of Muscle Fibers with Peripheral or Central Nuclei Per 100 Fibers Counted

A line was set up to divide the field of microscope into two halves. Under high power (40x) this line was

swept across the entire section, passing through its center. Muscle fibers touched by the line were observed and those with nuclei were tabulated as central or peripheral. Sweeps were made across the section until one sample (100 nucleated fibers counted) was collected. Successive sections, about 50 to 60 μm apart, were used so that six samples were collected from each segment (proximal, middle and distal) of the regenerate.

The determination of the position of nuclei was sometimes difficult. This was especially true in the thin fibers in which the nuclei occupied almost the entire cross sectional area of the fibers. In such cases, the fibers were usually neglected in the counting. Other situations occurred, such as nuclei in the periphery but with a rim of cytoplasm to be seen or some fibers with two nuclei with one in the peripheral area and the other in the non-peripheral position. These were counted as nuclei in the intermediate position. As will be seen in the results section, both central and intermediate nucleated fibers were less frequently encountered than peripherally nucleated muscle fibers.

Day 10, 20, 30, and 40 regenerates were counted and, for each date, 2 normal and 2 denervated, were used.

B. Measurements from Longitudinal Sections

In longitudinal sections, the following parameters

were measured.

1. Number of normal and degenerating muscle fibers per 50 fibers counted (day 10, 20, 30, and 40)
2. Number of normal and degenerating myotubes per 50 fibers counted (day 10 only).

As mentioned earlier, only slides No. 2, 3, and 4 were included in the fiber counting. Based on the fact that adult rat muscle fibers average 100 μm in diameter, sections were chosen at 90 to 100 um (10 to 11 sections) intervals to prevent counting the same fibers twice.

As in the cross-sections, under high power, the line swept through the entire section about 250 to 500 um above the distal end of each segment (proximal, middle and distal) of the regenerates. Muscle fibers touched by the line were counted and tabulated as normal fibers, degenerating fibers, normal myotubes and degenerating yotubes, etc., until 50 fibers were counted. Occasionally, the line was moved up and down to follow muscle fibers which had lost their continuity during the sectioning.

The method of statistical analysis will be discussed in Appendix IV.

RESULTS

SERIES I. NORMAL MINCED MUSCLE REGENERATION

A. Morphological Development

The events of normal minced muscle regeneration are consistent with those described in the early works of Carlson ('68,'70a) and have already been reported in detail. For simplicity, only a general outline is given below.

One day after mincing, the major part of the regenerate still consisted of fragmented muscle fibers (Fig. 4). By the second or third day, the peripheral rim of the regenerate was devoid of intact muscle fibers, while the remainder was occupied by ischemic eosinophilic muscle fragments (Fig. 5). The myogenic tissues were located in the peripheral rim. Around the second or third day, myotubes could be seen in the periphery (Fig. 5, Fig. 6 a,b). By 5 or 6 days, the distal end of the regenerate was connected to the original calcaneal tendon. At this stage, a regenerate can be divided histologically into three zones of activity (Fig. 7): (1) a peripheral basophilic regenerating zone, (2) a central eosinophilic ischemic zone and (3) an intermediate area of active degeneration. With passage of time, the peripheral area increased in thickness at the expense of the central ischemic zone. The intermediate zone, situated between the peripheral and central zone, migrated inwardly toward the center and gradually disappeared along with the central ischemic area. In this intermediate

zone myoblasts could first be identified. They were under the basement membranes (Fig. 8) of the original muscle fibers which were now in the terminal stage of degeneration. These myoblasts fused to form myotubes (Fig. 6 a,b) which were located in the periphery of the regenerates. By 10 to 12 days the central eosinophilic area was completely gone. During the same time-span, the number of myotubes became abundant and some of them became larger. The first sign of cross striation could be seen as early as 7 or 8 days, especially in the large myotubes (Fig. 9). While the rest of the myotubes followed this pattern of development, those myotubes already possessing striations simply increased in size. Almost immediately the nuclei of the most highly developed myotubes began moving toward the periphery. In time, additional myotubes became striated and by about day 15, practically no myotubes could be seen. After this time, maturation of the fibers was reflected by an increase in diameter. Pictures of 40 and 68 day old regenerates are shown in Fig. 10 and Fig. 11 respectively.

The regenerate is smaller in size than the normal control muscle and it contains more connective tissue than normal muscles. The latter is especially true in those denervated regenerates of advanced age (see later) and in the distal end of each regenerate. The more than usual amount of connective tissue in the regenerate renders it difficult to cut frozen sections in cryostat. Occasionally degenerating myotubes (Fig. 12 a, Appendix III) and

degenerating muscle fibers (Fig. 13 a, Appendix III) were intermingled among normal muscle fibers of the regenerate.

B. Return of Nerve Fibers

Nerve staining revealed that from day one to day six or seven, only the nerve trunk could be stained. The first sign of nerve sprouting from the nerve trunk appeared at around day 7 or 8 (Fig. 14). However, nerve fibers had not yet reached to the regenerating muscle fibers (Fig. 15). Around day 14 or 15 nerve fibers could be seen dispersed among the regenerating muscle fibers consistently, although typical neuromuscular junctions (motor end plates) were not established until about a week later (see next section). Fig. 16 shows a thirty-day-old regenerate with nerves spreading among the muscle fibers.

C. Return of Motor End Plates (MEP's)

In early regenerates (e.g. day 5 or 6), MEP's were observed in the remnants of muscle fragments located in the central area. They were degenerating, with round rather than claw-shaped structures. No positive reaction was seen in the peripheral regenerate. With passage of time, both the H & E staining intensity and the positive AchE reaction of the central zone became diminished. In the peripheral zone, a diffuse reaction gradually began to appear around day 10 or 11. The reaction products were distributed along the myotubes or early muscle fibers. The

AChE was positive but not in a circumscribed area (Fig. 17). By about 19 days, some fibers started to have localized staining (Fig. 18). It was around this time that the first MEP-like structures were seen. They were in the periphery of the muscle fibers and exhibited a claw-shaped appearance. With increasing age, diffuse staining reactions were no longer seen and all reactions were clear and concrete (Fig. 19).

Generally speaking, the MEP's in the regenerates were randomly distributed among the muscle fibers rather than arranged in bands as in normal muscles. Thus, they appear to be more numerous than those of normal muscles.

D. Differentiation of Muscle Fiber Types

It was clear from this series of experiments that muscle can not only regenerate after trauma, but will also differentiate into three fiber types.

In early regenerates (day 10 to day 30), there was no sign of fiber type differentiation, although the nerve returned to the regenerating muscle fibers around 15 days and MEP's returned around 20 days after the operation. A positive reaction was indeed observed, but the intensity was weak and uniform (Fig. 20a), except for some large fibers. Intracellularly the sarcoplasmic deposits of diformazan granules were sparse. In large fibers, heavy lumpy precipitates was seen in the cytoplasm and along the cell membrane (Fig. 21a). The cells were round instead of

polygonal in shape as in the mature types and their diameters were small. By 35 or 40 days various intensities of SDH could be differentiated (Fig. 22b), but the muscle fibers were still thin. Usually smaller fibers were darkly stained whereas larger fibers were lightly stained. Regardless of whether or not the fibers were lightly or darkly stained, the distribution of the diformazan granules was even, as in the case of adult fibers. Beyond 40 days, the fibers simply increased in size and changed in shape (toward the polygonal mature type), but the histochemical fiber pattern remained the same.

The intensity of the ATPase reaction was dark and uniform throughout the early phases of regeneration (day 10 to 30, Fig. 23a). Light and dark fibers were first seen around day 35, but differences in intensity were not prominent. The light fibers were fewer and sporadic. This situation remained the same through the next few days. By around day 40, the full differentiation of light and dark staining fibers was seen, although the muscle fibers were still thin (Fig. 24a).

The pattern of fiber types (fiber grouping) in the regenerate was different from that of normal muscles. Instead of the checkerboard pattern of normal muscle fibers, the same types of muscle fibers tended to group together in the regenerate (Fig. 22a, Fig. 24a). Only occasionally was the checkerboard pattern seen. An illustration of three fiber types of normal gastrocnemius and normal

regenerate is shown in Fig. 25a, Fig. 25b.

SERIES II. THE REGENERATION OF MUSCLE IN DENERVATED LIMBS

A. Morphological Development

Grossly the denervated limbs remained permanently fixed. Ocassionally, the toes (4th and/or 5th) were chewed. Pinching of the foot did not cause any response. All these indicated that the legs were indeed denervated as was also demonstrated by silver staining. Upon palpation before removal from the rat, the regenerates usually felt hard. This was especially true in old regenerates (65 days or older). After the regenerate was removed and divided into three segments, the distal tendon always had the appearance and texture of cartilage. Again this was more so in old regenerates.

The early events and time sequence of regeneration resemble very closely those of the normal controls. The myoblasts also arose from the minced fragments. They fused to form myotubes, which synthesized myofibrils and eventually became fibers with clear striations. Denervated regenerates also established connections with the original calcaneal (Achilles) tendon and during early regeneration showed three zones of differential activity (peripheral, central and intermediate) that were seen in normal regenerates. Myotubes could be seen two or three days after the mincing and striations could also be identified around 7 or 8 days. As in the controls, no

myotubes could be seen beyond 14 or 15 days. The number of muscle fibers seemed comparable to that of normal regenerates (see Table 5). Degenerating myotubes (Fig. 12b) and degenerating muscle fibers (Fig. 13b) were also encounted as in the case of normal regenerates. They are few in number and the quantities are comparable to that of normal regenerates up to 30 days, as will be shown in the sections of quantitative studies. Fig. 26a,b are illustrations of 25 and 35 day old denervated regenerates in longitudinal section.

The close similarity of denervated to normal regenerates persisted until around 40 days. At that time the abnormal fibers could be seen rather easily. There seemed to be two kinds of fiber degeneration. The first and most rapid type was fiber swelling with deeply stained sarcoplasm. The nuclei were pycnotic. This type of degenerating fiber disappeared rather quickly. A second type was simple reduction in fiber size. No detectable signs of abnormality in sarcoplasm and nuclei were observed. The fibers simply underwent gradual atrophy. Regardless of the type of degeneration, connective tissues and adipose tissues gradually replaced the muscle fibers (Fig. 27 a,b). The time for complete disappearance of muscle fibers varied with individual regenerates (Fig. 28). In some cases, it occurred around 90 days and, in others, traces of fibers could still be seen as late as 150 days postoperatively.

B. Return of Nerve Fibers

Throughout the studies, no nerve fibers were detected among the denervated regenerating muscle fibers.

C. Differentiation of Muscle Fiber Types

Both the SDH (Fig. 20b) and ATPase (Fig. 23b) reactions were very close to those of normal regenerates during the early stages of regeneration (day 10 to day 30), i.e., both were positively stained. With SDH reaction, not only the intensity and shape of muscle fibers but also the distribution of diformazan granules inside the fibers were similar (Fig. 21b). Essentially no difference could be found between normal and denervated regenerates in the early phases (before 30 days).

By 35 to 40 days, the denervated fibers remained in an undifferentiated primitive stage (Fig. 22b, Fig. 24b) while those of normal regenerates had begun to differentiate. As time went on, the enzymatic activity of denervated fibers became progressively weaker. With SDH stain, the cell boundaries became unidentifiable. With ATPase, although individual fibers were still positively stained, they were shrinking gradually. There was no indication throughout the studies of any fiber type differentiation.

It is thus clear that nerves are needed for fiber type differentiation and for the maintenance of structural integrity of the regenerated muscle fibers.

SERIES III. QUANTITATIVE COMPARISON BETWEEN NORMAL AND DENERVATED REGENERATES

A. Cross-Sectional Areas

The mean cross-sectional areas of regenerating muscle fibers are shown in Table 3 and in Fig. 29. The frequency distribution of different fiber sizes is shown in Table 4 and Fig. 30. It is clear that the fibers increased in cross-sectional area in normal regenerates and, in the early phases also in denervated regenerates. In the normal regenerates, the mean area increased from 80 μm^2 at 10 days to 1523 μm^2 at 270 days. The major increase was in the period between day 30 and day 150 (ca 1100 μm^2 increase within a 120-day interval). From day 150 to day 270, the rate of increase was less (only 61 μm^2 increased within another 120-day interval). In denervated regenerates, the mean cross-sectional area increased from 46 μm^2 at 10 days to a maximum of 161 μm^2 at 30 days. The fibers then decreased in size to 35 μm^2 at 90 days.

Note that there was nearly a four-fold increase from 10 to 30 days in the fiber areas in both normal (80 μm^2 to 299 μm^2) and denervated (46 μm^2 to 161 μm^2) regenerates. The rate of decrease of fiber size in denervated regenerates was slow and gradual. In fact, it took 20 days (day 10 to 30) for the cross-sectional area to increase 4 times (to maximum) but it took 60 days (day 30 to 90) to decrease 4 times (from maximum).

In regard to frequency distribution of different fiber sizes, it could be seen that there was a high percentage of small fibers (less than 90 μm^2) in 10 and 20 day regenerates, both normal and denervated. By 30 days, in both normal and denervated regenerates, the percentage of small fibers became minimal and the percentages of fibers within the range of 100-190 um^2 were fairly close to each other. After 30 days, most fibers in normal regenerates increased in diameter and moved to the larger fiber ranges. On the other hand, most of the fibers in the denervated regenerates became smaller and fell into 100-190 μm^2 or less than 90 μm^2 range. In the 90 day denervated regenerate, almost 96% of the fibers were smaller than 90 μm^2. Note that even in the 65 or 90 day denervated regenerates a few fibers remained at a size of 200-390 μm^2.

B. <u>The Number of Muscle Fibers in Normal and Denervated Regenerates</u>

As mentioned earlier, the present effort was only intended to give a numerical appreciation of the number of muscle fibers per regenerate. Table 5 is the summary of the number of muscle fibers found in normal and denervated regenerates of various ages.

It indicates clearly the following:

1. The number of muscle fibers varied considerably among the regenerates. This was not only among

regenerates of different age groups but also among regenerates in the same age group (e.g., day 30, between normal and between denervated; day 40, between denervated; and day 65, between normal).

2. Substantial numbers of muscle fibers persisted even in regenerates deprived of nerves for 65 days (day 65, D_1 2623, D_2 3231. Compare this with a day 65 normal regenerate N_1 3476).

3. In the early regenerates, day 10 day 20, the numbers of fibers in normal and denervated regenerates were close to each other.

C. Statistical Analysis of Normal and Denervated Regenerates

The parameters analysed are as follows

(1). Number of muscle fibers with peripheral nuclei per 100 fibers counted (P%).

(2). Number of normal muscle fibers per 50 fibers counted (NM/50).

(3). Number of normal myotubes and degenerating myotubes per 50 fibers counted (N.Mt/50 and D.Mt/50).

Table 6 presents the data of mean P%, while table 7 shows the data of mean NM/50. In either case, "A" represents TREATMENT (A_1:normal, A_2:denervated), "B" represents AGE (B_1:days, B_2:20 days, B_3:30 days, B_4:40 days), and "C"

represents LOCATION (C_1:proximal, C_2:middle, C_3:distal).

The results of statistical analysis ("Three-Way Analysis of Variance", see Appendix IV) are presented in table 8 for P% and table 9 for NM/50. On all tests α= 0.05 level of significance was used. From table 8 and table 9, the following can be seen:

1. The interactions between TREATMENT, AGE and LOCATION (ABC), between AGE and LOCATION (BC), and between TREATMENT and LOCATION (AC) are insignificant.

2. The interaction between TREATMENT and AGE (AB) is significant ($p<0.005$ for P%, $p<0.005$ for NM/50).

3. Factor C is significant ($p<0.001$ for P%, $p<0.005$ for NM/50). It means that P% and NM/50 are not the same between proximal, middle, and distal parts.

4. Factor A (TREATMENT) and factor B (AGE) are significant. However, the significant difference of TREATMENT (A) is conditioned on AGE (B) because the interaction between TREATMENT and AGE (AB) is significant and thus further analysis (Multiple Comparison, see Appendix IV) is necessary to find out exactly when (AGE) the TREATMENT becomes significant.

The results of "Multiple Comparison" between TREATMENT and AGE (see Appendix IV) are shown in table 10 for P% and in table 11 for NM/50. No statistically significant difference is indicated between TREATMENT A_1 (normal) and TREATMENT A_2 (denervated) under the conditions of B_1 (10 days), B_2 (20 days), and B_3

(30 days). However, a statistically significant difference exists between A_1 (normal) and A_2 (denervated) under the condition of B_4 (40 days). Therefore, there are no differences between normal and denervated regenerates from 10 to 30 days. At 40 days significant differences become evident.

The data of P% and NM/50 (mean $\pm$ S.E.) from the proximal parts are presented in table 12 and shown in Figure 31 and Figure 32 respectively. Note the comparison is proximal to proximal, rather than proximal to middle or proximal to distal or any of the other combinations, since the fiber distribution was not the same in the three parts of the regenerate, as mentioned previously.

Lastly, data on parameter (3) are presented in table 13. Again the comparison is restricted to proximal parts only and $\alpha = 0.05$ level of significance was used. These data indicate clearly that no differences in terms of N.Mt/50 or D.Mt/50 exist between normal and denervated regenerates.

DISCUSSION

The results of the present investigation support the early observations of Zhenevskaya ('62) and Jirmanova and Thesleff ('72) that in regeneration myoblasts can fuse to form myotubes in the absence of nerves. In vitro studies (Strohman et al., '74; Doering and Fischman, '74; Harris et al., '73) also support this view. Further, these data confirm the findings of Allbrook and Aitkin ('51) and Hsu ('71) that, in the denervated state, muscle can regenerate into cross-striated fibers with peripheral nuclei. Although the muscle fibers in the denervated regenerates eventually degenerated due to the lack of nerve fibers, there were essentially no differences between the innervated and denervated regenerates during the early stages.

NORMAL AND DENERVATED REGENERATION IN THE EARLY PHASES--BEFORE 30 DAYS

The intrinsic ability of myogenic cells to develop in the absence of nerves can be clearly demonstrated in the early phases of denervated minced muscle regeneration.

In normal regeneration, nerves started sprouting from the nerve trunk at around 7 or 8 days, but did not come close to the regenerating muscle fibers until around 14 days after the operation. In fact, the nerve itself was undergoing degeneration and then regeneration during the first week, and it is doubtful that nerve fibers could have exerted any influence. In this sense, during the first

week, the conditions of normal and denervated regenerates were essentially the same: that is, there was a lack of nerve influence. Nevertheless, morphological development of the newly emerged myogenic cells went smoothly and no differences could be detected between the two groups. In both cases, the first cross-striated muscle fibers could be seen around 7 or 8 days. From this time on, the denervated regenerates remained nerveless while, in the normal regenerates, nerve fibers could gradually be seen among the regenerating muscle fibers until the establishment of typical motor end plates at around 20 days. Although it is debatable whether these nerves could have released any neurotrophic factors before the motor end plates established it is nevertheless clear that the myogenic tissues did not require these factors for continuing early development. This can be revealed by the following evidence:

(1) Ten-day-old denervated regenerates were similar to normal regenerates of the same age in terms of numbers of normal and degenerating myotubes per 50 fibers counted.

(2) After approximately 15 days, no myotubes could be found in either case and all myogenic tissues had developed to the stage of early muscle fibers at least, indicating the rates of development were similar for normal and denervated regenerates.

(3) Up to 30 days, denervated and normal regenerates were comparable in regard to the percentage of muscle

fibers with peripheral nuclei and the percentage of normal muscle fibers per 50 fibers counted.

(4) The two types of regenerates were also comparable in the numbers of muscle fibers.

(5) The regenerated muscle fibers in both cases showed clear cross-striations.

The only consistent difference was that the normal regenerates had larger muscle fiber cross-sectional areas than the denervated, although the rates of increase of the diameters were the same (nearly four times, see table 3). The possible explanations for this will be discussed shortly.

Histochemically, reactions for both SDH and myofibrillar ATPase of denervated regenerates were positive as were those of the normal innervated ones. In SDH staining, the two types of regenerates were similar not only in the intensity but also in the distribution of diformazan granules inside the fibers. Occasional coarse, lumpy deposits could be seen in the sarcoplasm in both cases. In the ATPase reaction, the fibers stained darkly and their intensity was uniform throughout the early phases of both types of regenerates. During the early periods of minced muscle regeneration, there was no sign of light or dark fiber type differentiation regardless of whether the nerves were present or absent.

The intrinsic activities of myogenic tissues have also been observed in other studies. Hsu ('71) studied

denervated minced muscle regeneration of frogs and reported similar findings to those in the present study. Although no quantitative comparisons or histochemical staining was carried out, she observed cross-striations and peripherally located nuclei--the signs of morphological maturity--in the denervated regenerates. Allbrook and Aitkin ('51) studied muscle regeneration after ischemic injury and claimed they observed very few differences between normal and denervated regenerates.

Conflicting evidence, however, was presented by Studitsky et al. ('63) and Zhenevskaya ('62). The former stated that, without nerves, minced muscle regeneration could not go beyond the myoblast stage while the latter maintained that denervation would retard minced muscle regeneration only after the myotube stage.

In a denervated sliced muscle (extensor digitorum longus of rats) transplantation experiment, Carlson and Gutmann ('75, to be published) observed that, in early phases, the denervated muscle transplant underwent degeneration and regeneration successfully without any difficulties. Their morphological, histochemical and contractile characteristics in the early phases were essentially the same as those of innervated transplants. Hall-Craggs and Seyan ('75) in the Methyl-Bupivacaine induced degeneration and regeneration of gastrocnemeius muscle observed also that nerves were not needed for the initial stage of morphological and histochemical

development.

In normal muscle development, the myogenic tissues exhibit the same potential. In amphibians, the aneurogenic limb development is a good example (Harrison, '04; Hamburger, '28; Yntema, '43). Tweedle et al. ('72) observed fully developed muscle fibers in the aneurogenic limbs of frogs ultrastructurally. Although these fibers eventually degenerated, they were no different from those of normal limbs before degeneration set in. In mammals, denervation did not retard development either. For example, when the nerves to the muscles were cut 3 days before term (Zelena, '62) or immediately after birth (Engel and Karpati, '68; Shafiq et al., '72), fibers with cross-striations could always be seen. However, other results of these investigations were not always consistent with the above observations. Thus Zelena ('62) claimed that, while the total protein content of muscles increased, the muscle fiber protein, (actomyosin) content did not. Engel and Karpati ('68) claimed that 65% of myogenic tissues remained in the myotube stage whereas Shafiq et al., ('72) maintained there were no ultrastructural differences between the developing muscles of normal and neo-natally neurectomized rats.

In the histochemical reactions, it was anticipated that SDH and ATPase would be positive in normal innervated regenerates since regeneration is a recapitulation of ontogeny and normally developing muscles were positively stained before their eventual fiber type differentiation.

(Wirsen and Larsson, '64; Dubowitz, '65 a,b; Fenichel, '66 Kamieniecka, '68; Guth et al., '64). It was not expected, however, that the SDH and ATPase reactions would also be positive in the denervated regenerates and that they would be indistinguishable from the normal ones in the early stages. This is because the present investigator was not sure denervated muscle would regenerate at all although Askanas et al. ('72) cultured embryonic myogenic tissues in the absence of nerves and observed positive SDH and ATPase reactions along with morphological development, and in in vivo studies, Engel and Karpati ('68) obtained similar results in neo-natally neurectomized rats. Certainly, these results (Askanas et al., '72; Engel and Karpati, '68) should be carefully interpreted since it is not known whether their embryonic myogenic cells had been "informed" by the nervous tissues. In fact, the nerves were present in the myotomes before the formation of myotubes (Filogamo and Gabelle, '67) even though functional connections were not established. In this regard, regeneration might not necessarily have been the same as the ontogenic development because the newly emerged myoblasts in regeneration would have had no previous exposure to nerve fibers.

As mentioned earlier, the only consistent difference between normal and denervated regenerates in the early stages was the smaller fiber cross-sectional area in the denervated regenerates, although the rate of fiber

cross-sectional area increase was similar in the two cases (fourfold, between 10 and 30 days, see table 3). First, the small fiber diameter might not necessarily imply abnormality. Second, it could well be due to the lack of use of the denervated legs rather than to the lack of nerves.

Use and disuse of muscles has been shown to affect muscle fiber sizes. For instance, excessive use of muscles (tread mill running, swimming, etc.) has caused the muscles to increase in weight and the fiber to enlarge in diameter (Maxwell et al., '73; Syrovy et al., '72; Edgerton et al., '72). On the other hand, no exercise (e.g., limb immobilization, denervation of antagonistic muscles, etc.) has caused muscle atrophy (Riley et al., '73) and reduced fiber sizes (Tomanek and Lund, '74; Edgerton et al., '75 Maier et al., '73). In this regard, it is interesting to note that Yellin ('74), who denervated the rat's diaphragm on one side while stimulating the phrenic nerve on the other side, caused the whole diaphragm to contract and thus exercise the muscles of the denervated side. As a result, the denervated muscle fibers, rather than undergoing denervation atrophy, actually became hypertrophied for roughly 1 month.

In the case of denervated regenerates, the leg was permanently flexed and not used. Thus, the smaller fiber sizes could be due to the lack of exercise. In fact, Le Gros Clark and Wajda ('47) reported that immobilization of leg muscles by tenotomy retarded the repair of muscles injured

by ischemia. In contrast, Hsu ('71) claimed that femurectomy (reducing the exercise of legs) did not affect the minced muscle regeneration in frogs. Thus, control experiments, such as spinal isolation of normal regenerates, or direct stimulation of denervated regenerates (which exercises the muscle) have to be carried out to differentiate the effects of the lack of nerves and the lack of exercise.

In this study the contractile properties of denervated regenerates were not studied although those of normal regenerates have been investigated by Carlson and Gutmann ('72) by direct stimulation. Thus, it is not known whether the denervated regenerates were capable of contraction. However, relevant information tends to support the speculation that they are able to contract upon direct stimulation. That information is as follows:

(1) In the present study, the denervated regenerates showed positive SDH and ATPase reactions and also showed cross-striations. These are the morphological and histochemical bases for muscle contractions.

(2) In the normally innervated regenerates, Carlson and Gutmann ('72) observed contractile activity upon direct stimulation as early as 8 days postoperatively. At that time nerves had not yet reached to the muscle fibers.

(3) In the nerveless crossed sliced muscle transplantation experiment (Carlson and Gutmann, '75, to be published), the transplants were seen to be capable of contraction

upon direct stimulation. These nerveless transplants were morphologically and histochemically similar to those of innervated transplants and to the innervated and denervated minced muscle regenerates.

(4) Peterson and Crain ('70) observed fibrillation contractions in cultured aneural myotubes.

Thus, from all the above mentioned evidence it is clear that myogenic tissues retain considerable potential to develop independently. The present investigation demonstrated that muscle can regenerate in the absence of of nerves into the early stages of maturation, that is, cross-striation and peripheral nuclei.

NORMAL AND DENERVATED REGENERATION IN THE LATE PHASES--AFTER 30 DAYS

The close similarities between normal and denervated regenerates did not last permanently, however. Starting from 30 or 35 days, differences between the two became evident.

Morphologically, the normally innervated regenerates became highly matured, although non-peripheral nuclei could still be seen in a few instances. Degenerating fibers could no longer be seen in longitudinal sections. Most fibers increased in diameter rapidly and only a few fibers remained thin (see table 3 and table 4). Histochemically, fully differentiated fiber types could be detected in 40-day-old regenerates, with predominantly fast fibers.

Quite contrary to the normal regenerates, degenerative changes gradually set in in the denervated regenerates. The fiber diameters gradually decreased. The cross-striations of most fibers disappeared and only a few fibers retained them for a long period. A full spectrum of degenerating nuclei, from healthy to pycnotic, could be seen. Most nuclei remained in the peripheral position (see table 6 and figure 31), at least at the onset of degeneration. Sarcoplasm of some fibers became retracted and, in the late period, their continuity became interrupted. Almost invariably connective tissues and adipose tissues became the main constituents of the regenerates. A few very small fibers in some regenerates persisted for a long time (ca. 150 days).

Histochemically, the SDH and ATPase reactions remained positive but primitive. There were no indications of any fiber type differentiation. With decreasing fiber diameter, the individual fiber could only be recognized through ATPase staining. In SDH reactions, cell boundaries could no longer be seen and gradually even the diformazan granules were hardly discernable.

These drastic differences between normal and denervated regenerates in the late phases indicate the necessity for nerve action. Thus, nerves are needed for differentiation of fiber types and maintenance of fiber structural integrity.

Other studies supported this view. Hsu ('71) in her amphibian studies stated "Muscle differentiation in minced muscle regeneration is independent of innervation. Nerves appear to be significant in maintaining the integrity of

the differentiated muscle fiber and in influencing the process of its maturation and growth." Peterson and Crain ('70) observed in vitro initial development of myotubes in the absence of nervous tissue, but the muscle fibers eventually degenerated due to the lack of nerves. Askanas et al. ('72) studied aneural embryonic muscle cultures with SDH or ATPase and found positive reactions of both enzymes. However, they observed no muscle fiber type differentiation at all. In vivo study results were similar. Whether the study was of the aneurogenic amphibian limb formation (Yntema, '43; Tweedle et al., '74) or of the development of neonatally neurectomized mammalian muscle (Shafiq et al., '72), the muscle fibers developed into cross-striation and peripheral nuclei, but eventually they were replaced by connective tissue fibers. Engel and Karpati ('68) investigated the histochemical development (SDH and ATPase) of rats' triceps surae following sciatic denervation immediately after birth and found no fiber type differentiation.

The process of complete degeneration of denervated regenerating muscle fibers was long and gradual in rats while that in frogs (Hsu '71) was short and sudden. The precise reason for this is not known, although it could be due to the intensity of metabolism of muscle and/or the life span of various animals as was demonstrated in experiments of denervation atrophy of normal muscles (Hnik and Skorpil, '62). That the ability and events of

minced regeneration vary with different species of animals was further indicated by the fact that myotubes could be seen around 8 to 10 days in axolotls (Carlson, '70b), around 6 or 7 days in mice (Mufti and Sultana, '72) and around 2 or 3 days in rats in the present investigation. Cross striations and peripheral nuclei in minced muscle regenerates could be seen around 34 days in axolotls (Carlson, '70b), around 17 days in mice (Mufti and Sultana, '72) and around 6 or 7 days in rats. In the denervated condition, the minced muscle regenerates of mice (Mufti, unpublished) exhibited different events from those of rats. In mice, regeneration proceeded slowly and the regenerated muscle fibers showed barely discernable striations. Only in a few instances were truely peripheral nuclei seen. As in the case of frogs (Hsu, '71), around 20 days postoperatively, Mufti (unpublished) found no muscle elements except connective tissues. Thus, in assessing the regenerating ability of muscle in the absence of nerves, species difference has to be taken into consideration.

In normally innervated regenerates the dark ATPase stained fibers are more numerous than the light ones. Classifying these darkly ATPase stained fibers requires caution since Guth ('73) and Samaha and Yunis ('73) claimed earlier that at pH = 9.4 mitochondrial ATPase was also active and the phosphate released from the mitochondria was deposited on the myofibrils and contributed to the dark stain of the fibers. However, this contradicts the findings of

Burke et al. ('71). Certainly, the present investigator feels that these dark fibers were fast fibers since physiological studies (Carlson and Gutmann, '72) have shown that the speed of contraction of the minced muscle regenerates is indeed fast.

MOTOR END PLATES IN THE REGENERATES

The development of MEP's in the regenerates also resembled that of normal muscle ontogeny. In the initial phase (7 or 8 days postoperatively), the MEP's of the original muscle fibers were seen degenerating in the central ischemia area. Early in the second week, traces of positive AChE staining could be seen on the myotubes and young muscle fibers and, with the increase in time, they could be seen spreading around the entire regenerate except for the distal tendon and where connective tissue predominates.

The diffuse reaction of AChE in young muscle fibers in the regenerates was similar to that of normal developing fibers (Kupfer and Koelle, '51; Mumenthaler and Engel, '61) and was shown to be due to the non-specific ChE along the entire surface of the developing muscle fibers. Tennyson et al. ('71, '73) also observed randomly distributed AChE in the myotubes and maintained that there was soluble AChE from the mononucleated cells in the vicinity of the developing junctions. These cells might also have contributed to the diffuse reactions. As the nerves gradually came in contact with the muscle fibers, the diffuse reaction disappeared.

During the course of this investigation, Bennett et al. ('74) reported experiments with similar methods. They also observed degenerating MEP's in the central ischemic area and diffuse AChE reactions on the myotubes and young muscle fibers in the peripheral zone.

As Bennett et al. ('74) pointed out, the difference in MEP formation between the regeneration and the ontogenesis of mammalian muscle was that nerves entered the regenerate relatively late during myofiber formation whereas during ontogeny the nerves were present in myotomes before myotube formation.

The time of MEP return was around the 3rd week as also indicated by Studitsky et al. ('63) and Allbrook and Aitkin ('51) based on silver stains. This time was well within the interval of 18-26 days observed by Bennett et al. ('74).

The MEP's are unique structures and there are conflicting views of their role in the literature. Zhenevskaya ('62) denervated the minced muscle regenerates 10 days after the operations (that is, 10 or 11 days before the return of MEP's) and observed continued growth and differentiation. She claimed that the eventual atrophy came much later than in case of muscles denervated at the time of mincing. This indicates that nerves can exert influences on the muscle fibers before the formation of MEP's. However, in the present study, there were no differences between normal and denervated regenerates before 30 days (10 days after the 1st sign of MEP formation) indicating no nervous influence

before MEP's were formed. Furthermore, the fact that the fiber types would not differentiate until 10 to 15 days after MEP return strongly suggests that MEP's must be present so that nervous influences can be transferred to muscle fibers.

Controversies also surround other studies. Fex ('69; Fex and Sonesson, '70) inserted fast nerves (nerves to EDL) to normally innervated slow muscle (soleus). He observed no new functional MEPs in the soleus. However, the contraction speed and histochemical fiber types were changed to a fast muscle type. He concluded, then, that MEP's were not needed in order for nerves to influence muscle. In minced muscle regeneration studies, Carlson and Gutmann ('72) observed the greatest change in speed of contraction before and during the time of appearance of MEPs. Novoselova ('71), studying the effects of the repeated denervation and reinnervation of rat's gastrocnemius, stated that axons exerted an "inductive" effect on muscle fibers before functional activity of the neuromuscular junction was restored. Other evidence was obtained from <u>in vitro</u> studies and the innervation of muscles from the sympathetic nerves. When muscles were innervated by splanchnic nerves where no MEPs were found, they were 40% atrophied as opposed to denervation atrophy which is 60%. Splanchnic nerves caused five times less sensitivity to ACh than denervation (Guth, '74). <u>In vitro</u>, if the spinal cord was removed and replaced by the splanchic nerve, the muscle fibers were

maintained (Guth, '74).

Contrary to the above, however, Guth and Wells ('72) and Crockett and Edgerton ('74) claimed that they could not repeat the experiment originally performed by Fex ('69) and maintained there were no trophic effects on the muscle from the implanted non-functional nerves. Zelena ('62) stated that the "nervous system does not exert a trophic influence on the muscle until nerve endings are found in the muscle tissue," although she did not supply concrete evidence.

The conflicting conclusions on the role of MEP's is probably due to the model systems used (in vivo or in vitro; normal development or regeneration) and the end points measured (enzyme specificity, speed of contraction, fiber types, morphology, etc.). It is certainly worthwhile to study this area further, for example, to study the morphology and fiber type changes and/or differentiation by constant denervation before MEP formation or by inserting Millipore^{R} filters between nerves and muscles.

MUSCLE SPINDLES IN THE REGENERATES

In the present study, no muscle spindles were found in the regenerates, and information in the literature on the return of muscle spindles to the regenerates is limited. Zhenevskaya ('62) found no muscle spindles in the regenerates and was supported by Zelena and Sobotkova ('71) and Zhenevskaya et al. ('65, see Carlson '73), although

Studitsky ('51, in Carlson '73) claimed earlier that muscle spindle-like structures were seen in minced regenerates.

INFLUENCE OF NERVES ON MUSCLE FIBERS

Thus, it is clear that nerves are not needed for the initial stages of muscle development or regeneration, but they are needed for the subsequent development of muscle fibers. Therefore, it is pertinent at this stage to review briefly the activities of muscle fibers that are influenced by nerves and to probe the characteristics of the nervous influence.

(1) Structural integrity:

Nerves maintain the structural integrity of muscle fibers: denervation causes the muscles to atrophy. With denervation the muscle fibers gradually became reduced in diameter and the nuclei apparently increased in number. They were often localized centrally or in clumps. With the passage of time, the muscle fibers were gradually replaced by connective tissue. Denervation atrophy has been extensively discussed by Gutmann and Zelena ('62).

(2) Speed of muscle contraction and muscle fiber types:

The speed of muscle contraction and histochemical fiber types are also nerve influenced phenomenon. Buller et al. ('60a, '60b) studied closely the development of contraction speed in the new-born kitten and found that all muscles contracted slowly and with the same

speed. During development, the fast muscles (e.g., EDL) gradually increased in speed of contraction to assume the adult pattern. The slow muscles (e.g., soleus), however, first increased their speed until 7 weeks and then slowed down gradually to reach the adult pattern. When the nerves of fast muscles are crossed to innervate the slow muscles and vice versa, the speed of contraction is reversed.

Efforts have been made to correlate the speed of contraction with the enzyme content and thus established the different fiber types (Edstrom and Kugelberg, '68; Kugelberg and Edstrom, '68; Barnard et al., '71; Burke et al., '71). Thus, fast contracting muscles contain high ATPase but fatigue easily because the oxidative enzyme SDH is low while the slow contracting muscles, due to their low content of ATPase, contract slowly, but they are fatigue resistant because of the constant energy supply due to the high content of the oxidative enzyme SDH. In cross-innervation experiments (Barany and Close, '71; Fex and Sonesson, '70; Karpati et al., '67) and cross muscle transplantation experiments (Carlson and Gutmann, '74; Gutmann and Carlson, '75) not only was the speed of contraction reversed but also the enzyme content. It is therefore clear that nerves can change the genetic activities of muscle fibers with subsequent protein synthesis (low ATPase to

high ATPase content) which is manifested in physiological activities (change of contraction speed).

The characteristics of the nervous influence itself have been studied by a number of investigators. Vrbova ('63) suggested it was the nerve impulse conduction pattern that influenced the muscle activities, especially the speed of contraction. Thus, by stimulating the muscles electrically with a frequency similar to the impulse conduction pattern of nerves to fast (phasic pattern) and slow (tonic pattern) muscles, the contractile characteristics of mammalian muscles could be altered. For example, Salmons and Vrbova ('69) demonstrated that the time course of the contraction of the slow muscle became faster when the continuous activity at 10/sec that was normally received was reduced, and a fast muscle was made to contract more slowly when it was subjected to activity at a slow frequency. Further, Pette et al. ('72, '73) studied the effect of long-term stimulation of rabbit fast muscles (EDL) with a frequency of normal soleus muscle (10/sec) and observed biochemical, histochemical, and physiological conversion of EDL toward soleus type. In light of these findings, it would be worthwhile to see whether long-term direct stimulation with tonic or phasic frequency could cause the denervated regenerates of the present study to differentiate in terms of histochemical fiber types.

The impulse pattern of nerves explains very well the

contractile activities and fiber types of muscles, but it does not imitate all the muscle activities influenced by nerves, especially the maintenance of structural integrity and sensitivity to ACh. In fact, vinblastine or colchicine, which block the axoplasmic flow of nerve axons but have no effect on the conduction of electrical impulse, caused similar denervation changes in muscles when applied to sciatic nerves (Hoffman and Thesleff, '72; Albuquerque et al., '72; Hoffman et al., '75). This implies that humoral substances are transported through the axons and that they are responsible for the muscle activities.

Buller et al. ('60b) proposed a chemical hypothesis of neural influence exerted by nerve cells on muscle fibers. He proposed "neural influence would be exerted by a specific chemical substance that courses along the motor nerve fibers, then traverses the neuromuscular junction and finally passes thence along the whole length of muscle fibers, inducing therein changes that alter the speed of muscle contraction." Direct evidence supporting this view was obtained when Korr and his co-workers ('67, '74) applied H^3-leucine to the hypoglossal nuclei and claimed that they observed radioactive substances in the hypoglossal nerves and tongue muscles. Although more sophisticated control experiments have to be carried out to verify these results, it is nevertheless known that axoplasmic flow is active in conducting neurotrophic materials (Hofmann et al., '75) and that different materials are transported (Korr et al.,

'67; Korr and Appeltauer, '74; Appeltauer and Korr, '75). In dystrophic nerves the waves, speed, and amount of axoplasmic flow are different from those of normal (Komiya and Austin, '74; Tang et al., '74) and this further supports the hypothesis that humoral substances are involved in neurotrophism.

The actual material of the neurotrophic substance remains unknown and the work in this area is limited. Drachman ('64,'67, '74) proposed that acetylcholine (ACh) was the actual substance. He applied botulinum toxin, which prevents the release of ACh, to the nerves and observed morphological changes in the muscle almost identical to those of denervation. However, gross application of ACh to denervated muscle does not prevent the muscle from atrophy (Miledi, '60). Further, it is not conceivable that "ACh" can make one muscle contract faster while making the other contract slower.

Lentz ('72b,'74), in a culture system, found that brain extract can mimic the trophic effect. This brain extract was heat-labile, not retained in dialysis tubing and inactivated by protease. He thus proposed it was a small protein which probably acted through cyclic AMP (Lentz, '72b). This was supported by Oh and Johnson ('72) and Oh ('75) who proposed that a protein factor (Mol. wt. 300,000 dalton), found in the nerve extract, could stimulate morphological development, AChE activity and protein synthesis of muscles. Both Lentz ('74) and Oh ('75), however, did not

include "ACh" as a trophic factor. Since the blockade of ACh release mimics so closely the effects of denervation morphologically, it is tempting to speculate that ACh is chemically linked or part of the protein substance and removal of ACh could either change or destroy the activity of the trophic influence. This is because Nachmansohn ('59) proposed that in the resting condition, ACh was bound to a storage protein and this "bound" ACh concept was further suggested by Hubbard ('70) and Hoskin ('72). Intensive research in this area would be fruitful in elucidating neurotrophism and its role in the neuromuscular interactions.

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Table 3. Cross-sectional area (μm^2) of muscle fibers in normal and denervated regenerates. Samples were collected only from proximal parts of the regenerates. For each scheduled date two animals were used except for days 90, 150, and 270. Each number is a mean $\pm$ S.E.

Table 3.

AGE	DENERVATED	NORMAL
10 DAYS	46 ± 1.35	80 ± 3.37
20 DAYS	88 ± 2.79	208 ± 4.19
30 DAYS	161 ± 3.20	299 ± 8.70
40 DAYS	127 ± 2.64	515 ±20.29
65 DAYS	84 ± 2.46	801 ±32.01
90 DAYS	35 ± 1.48	1238 ±76.02
150 DAYS	____	1462 ±98.99
270 DAYS	____	1523 ±103.10

Table 4. Frequency distributions of various fiber cross sectional areas (μm^2) of normal and denervated regenerates. Samples were collected only from proximal parts of the regenerates. For each scheduled date two animals were used except for day 90.

Table 4.

AGE	AREA(μ^2)	NORMAL 1	NORMAL 2	DENERVATED 1	DENERVATED2
10 DAYS	200+	28 (7.35%)	18 (4.36%)	29 (6.12%)	1 (0.23%)
	100-190	88 (23.10%)	72 (17.43%)	76 (16.03%)	21(4.92%)
	0-90	265(69.56%)	323(78.23%)	369 (77.85%)	405(94.86%)
	Total	381	413	474	427
20 DAYS	400+	25 (0.56%)	30 (9.93%)	13 (5.06%)	0 (0%)
	200-390	119(33.52%)	135(44.75%)	36 (14.00%)	5 (1.33%)
	100-190	161(45.35%)	109(36.09%)	69 (26.85%)	53(14.13%)
	0-90	59 (16.62%)	28 (9.27%)	139 (54.09%)	317(84.53%)
	Total	355	302	257	375
30 DAYS	400+	85 (33.73%)	54 (21.51%)	2 (0.79%)	2 (0.68%)
	200-390	128(50.79%)	106(42.23%)	102 (40.48%)	81(27.93%)
	100-190	21 (8.33%)	78 (31.08%)	125 (49.60%)	151(52.07%)
	0-90	20 (7.93%)	13 (5.18%)	23 (9.13%)	56(19.31%)
	Total	252	251	252	290
40 DAYS	1000+	22 (10.89%)	14 (6.39%)	0 (0%)	0 (0%)
	400-990	74 (36.63%)	116(52.97%)	0 (0%)	7 (1.39%)
	200-390	48 (23.76%)	53 (24.20%)	12 (4.88%)	102(20.24%)
	100-190	40 (19.80%)	24 (10.96%)	116 (47.97%)	251(49.80%)
	0-90	18 (8.91%)	12 (5.48%)	118 (47.97%)	144(28.57%)
	Total	202	219	246	504
65 DAYS	1600+	13 (7.64%)	17 (10.18%)	0 (0%)	0 (0%)
	1000-1590	27 (15.88%)	32 (19.16%)	0 (0%)	0 (0%)
	400-990	85 (50.00%)	63 (37.72%)	0 (0%)	1 (0%)
	200-390	33 (19.41%)	40 (23.95%)	1 (0.63%)	22 (6.27%)
	100-190	8 (4.70%)	13 (7.78%)	48 (30.38%)	98 (27.92%)
	0-90	4 (2.35%)	1 (0.60%)	109 (68.99%)	230(65.53%)
	Total	170	167	158	351
90 DAYS	1600+	28 (27.18%)		0 (0%)	
	1000-1590	28 (27.18%)		0 (0%)	
	400-990	39 (37.86%)		0 (0%)	
	200-390	6 (5.83%)		3. (0.90%)	
	100-190	1 (0.97%)		11 (3.33%)	
	0-90	1 (0.97%)		316 (95.75%)	
	Total	103		330	

Table 5. Total number of muscle fibers in normal and denervated regenerates. Fibers were counted from the cross sections of only the proximal parts of regenerates.

Table 5.

AGE	NORMAL	DENERVATED
10 DAYS	N 1=4763	D 1=4870
	N 2=4148	D 2=4572
20 DAYS	N 1=6921	D 1=7848
	N 2=5961	D 2=7608
30 DAYS	N 1=8709	D 1=2653
	N 2=3993	D 2=5118
40 DAYS	N 1=9018	D 1=3456
	N 2=9333	D 2=6876
65 DAYS	N 1=6451	D 1=2623
	N 2=3476	D 2=3231

Table 6. Number of muscle fibers with peripheral nuclei per 100 fibers counted in cross-sections (P%). Each number is a percentage and is the mean of six samples (one sample = 100 fibers counted). The data are arranged to fit the "Three-Way Analysis of Variance" (see Appendix IV). N denotes the number of the animal for each scheduled AGE; 16 animals were used for the entire experiment. A represents TREATMENT (A_1 = normal, A_2 = denervated), B represnets AGE (B_1 = 10 days, B_2 = 20 days, B_3 = 30 days, B_4 = 40 days), and C represents LOCATION of the regenerates (C_1 = proximal, C_2 = middle, C_3 = distal). A_1B_1, A_2B_1, A_1B_2, $\cdots\cdots A_2B_4$ are the subtotal (out of a possible 600) from all LOCATIONS of both animals for each specific AGE and they are used in Table 10 for "Multiple Comparisons" (see Appendix IV) between the interactions of TREATMENT and AGE.

Table 6.

A (TREATMENT)		B (Age)	N	C (Location) C_1 Proximal	C_2 Middle	C_3 Distal	Total
A_1	Normal	B_1	1st	81.00	74.83	74.67	A_1B_1=445.33
		(10 days)	2nd	69.00	75.00	70.83	
		B_2	1st	88.33	85.67	74.00	A_1B_2=514.43
		(20 days)	2nd	93.50	87.60	85.33	
		B_3	1st	93.33	91.50	87.00	A_1B_3=534.32
		(30 days)	2nd	90.83	87.33	84.33	
		B_4	1st	97.33	97.67	96.17	A_1B_4=579.50
		(40 days)	2nd	97.50	98.33	92.50	
A_2	Denervated	B_1	1st	76.16	74.33	69.67	A_2B_1=432.31
		(10 days)	2nd	72.82	73.00	66.33	
		B_2	1st	84.33	82.60	85.20	A_2B_2=503.46
		(20 days)	2nd	86.00	85.33	80.00	
		B_3	1st	97.00	88.00	83.83	A_2B_3=516.69
		(30 days)	2nd	90.83	85.83	81.20	
		B_4	1st	79.83	76.00	64.00	A_2B_4=448.49
		(40 days)	2nd	82.66	76.67	69.33	

Table 7. Number of normal muscle fibers per 50 fibers counted in longitudinal sections (NM/50). Each number is a ratio out of a possible 50 and is the mean of six samples (one sample=50 fibers counted). The data are arranged to fit the "Three-Way Analysis of Variance" (see Appendix IV). N denotes the number of animals at each scheduled AGE; 16 animals were used for the entire experiment. A represents TREATMENT (A_1=normal, A_2=denervated), B represents AGE (B_1=10 days, B_2=20 days, B_3=30 days, B_4= 40 days), and C represents LOCATION of the regenerates C_1=proximal, C_2=middle, C_3=distal). A_1B_1, A_1B_2, ...A_2B_4 are the subtotal (out of a possible 300) from all LOCATIONS of both animals for each specific AGE and they are used in Table 11. for "Multiple Comparisons" (see Appendix IV) between the interactions of TREATMENT and AGE.

Table 7.

A (TREATMENT)		B (Age)	N	C (Location) C_1 Proximal	C_2 Middle	C_3 Distal	Total
	A_1 Normal	B_1	1st	35.33	39.66	25.83	A_1B_1=203.48
		(10 days)	2nd	31.83	36.50	34.33	
		B_2	1st	46.17	46.33	44.75	A_1B_2=272.75
		(20 days)	2nd	48.17	45.83	41.50	
		B_3	1st	48.50	49.00	47.50	A_1B_3=276.50
		(30 days)	2nd	47.17	46.00	38.33	
		B_4	1st	50.00	50.00	50.00	A_1B_4=300.00
		(40 days)	2nd	50.00	50.00	50.00	
	A_2 Denervated	B_1	1st	38.33	34.50	36.00	A_2B_1=205.13
		(10 days)	2nd	34.00	33.83	28.67	
		B_2	1st	44.00	45.60	45.00	A_2B_2=275.01
		(20 days)	2nd	48.00	46.83	45.25	
		B_3	1st	45.33	46.00	43.67	A_2B_3=268.16
		(30 days)	2nd	45.00	44.33	43.83	
		B_4	1st	33.50	31.00	7.83	A_2B_4=129.16
		(40 days)	2nd	17.50	31.00	8.33	

Table 8. Summary of "Three-Way Analysis Variance" of P% (P%: number of muscle fibers with peripheral nuclei per 100 fibers counted). A, B, and C represent TREATMENT, AGE, and LOCATION, respectively. AB represents the interactions between TREATMENT and AGE: AC, the interactions between TREATMENT and LOCATION; BC, the interactions between AGE and LOCATION; ABC, the interactions between TREATMENT, AGE and LOCATION.

SS represents sum square; df, degree of freedom; MS, mean square; F, F-test. Note, in the right column F, $\alpha = 0.05$ level of significance was used and it indicates that A, B, AB, and factor C are significant.

Table 8.

Source of variation	SS	df	MS	F	P
Between Subjects	3160.69	12			
A (Treatment)	620.86	1	620.86	22.60*	P=0.005
B (Age)	1542.99	3	514.33	18.72*	P<0.005
AB	859.49	3	286.50	10.43*	P<0.005
Subject w group [error (between)]	137.35	5	27.47		
Within Subject	617.03	32			
C (Location)	372.19	2	186.10	28.10*	P<0.001
AC	24.38	6	4.06	0.61	P>0.05
BC	6.37	2	3.19	0.48	P>0.05
ABC	108.12	6	18.02	2.72	P>0.05
C x Subject w group [error (within)]	105.97	16	6.62		

Table 9. Summary of "Three-Way Analysis of Variance" of NM/50 (NM/50: Number of normal muscle fibers per 50 fibers counted). See legend of Table 8. for detailed explanations. Note: in the right column F, α =0.05 level of significance was used and it indicates that A, B, AB, and factor C are significant.

Table 9.

Source of Variation	SS	df	MS	F	p
Between Subjects	3912.25	15			
A (Treatment)	638.53	1	260.82	29.84*	p< 0.005
B (Age)	1366.51	3	455.50	21.29*	p< 0.005
AB	1800.21	3	600.07	28.04*	p< 0.005
Subject w groups [error (between)]	106.40	5	21.40		
Within Subject	887.60	32			
C (Location)	265.30	2	132.65	7.84*	P< 0.005
AC	113.10	6	18.85	1.11	p> 0.05
BC	21.98	2	10.99	0.64	p> 0.05
ABC	216.36	6	36.06	2.13	p> 0.05
C x subject w groups [error (within)]	270.86	16	16.93		

Table 10. Test of the interactions between TREATMENT and AGE in terms of p%. Each number represents the subtotal (out of a possible 600) of all the percentages from all LOCATIONs of both animals of each specific AGE (see Table 6. also). The method used was "Multiple Comparisons" (see Appendix IV), to determine whether differences exist between normal (N) and denervated (D) regenerates on day 10, 20, 30, and 40 respectively. $\alpha=0.05$ level of significance was used and $F_{(3,5)\ \alpha=0.05}=5.41$ ($^{*}p<0.0001$).

Table 11. Test of the interactions between TREATMENT and AGE in terms of NM/50. Each number represents the subtotal LOCATIONS of both animals of each specific AGE (see Table 7. also). The method used was "Multiple Comparisons" (see Appendix IV), to determine whether differences exist between normal and denervated regenerates of day 10, 20, 30 and 40 respectively. $\alpha=0.05$ level of significance was used and $F_{(3,5)\ \alpha=0.05}=5.41$ ($^{*}p<0.0001$).

Table 10.

	B_1 (10 days)	B_2(20 days)	B_3(30 days)	B_4(40 days)
A_1 (N)	A_1B_1=445.33	A_1B_2=514.43	A_1B_3=534.32	A_1B_4=579.50
A_2 (D)	A_2B_1=432.31	A_2B_2=503.46	A_2B_3=516.69	A_2B_4=448.49
F	0.5142	0.3650	0.9424	52.07*
p	p >0. 25	p >0. 25	p >0. 25	p< 0.0001

Table 11.

	B_1 (10 days)	B_2(20 days)	B_3(30 days)	B_4(40 days)
A_1	A_1B_1=203.48	A_1B_2=272.75	A_1B_3=276.50	A_1B_4=300.00
A_2	A_2B_1=205.33	A_2B_2=275.01	A_2B_3=268.16	A_2B_4-129.16
F	0.0133	0.0198	0.2708	113.65*
p	p >0. 25	p >0. 25	p >0. 25	p <0.0001

Table 12. Number of muscle fibers with peripheral nuclei per 100 fibers counted (P%, mean ± S.E.) and Number of normal muscle fibers per 50 fibers counted (NM/50, mean ± S.E.) of normal and denervated regenerates from 10 to 40 days postoperatively. In P% of the left column, each number is a percentage and in NM/50 of the right column, each number is a ratio out of 50. Data are only from proximal parts of regenerates and are shown in Fig. 31 and Fig. 32.

Table 13. t-test of number of normal myotubes per 50 fibers counted (N.M t/50) and number of degenerating myotubes per 50 fibers counted (D.Mt/50) between 10 day old normal and denervated regenerates. Each number (mean ±S.E.) is a ratio out of 50. The data are only from proximal parts of the regenerates. $\alpha=0.05$ level of signicance was used. * $t_{0.05}=2.074$.

Table 12.

	P %		NM/50	
	Normal	Denervated	Normal	Denervated
10 days	75.17±2.24	74.50±1.45	36.08±2.52	36.17±1.58
20 days	90.92±0.96	85.17±0.84	47.17±0.74	46.17±1.13
30 days	92.08±0.67	88.92±1.22	47.83±0.44	45.17±0.65
40 days	97.42 ±0.51	81.25±1.29	50.00±0.00	25.50±2.99

Table 13.

	Normal (mean ±S.E.)	Denervated (mean ±S.E.)	t-test	p
N.Mt/50	13.25±1.39	10.75±1.27	0.20*	p >0.40
D. Mt/50	3.17±0.63	3.08±0.47	0.91*	p >0.10

Figure 1.

Right leg of a rat after removal of the plantaris, soleus, and gastrocnemius muscles. The sural nerve (S), tibial nerve (P) were left in situ. (Reproduced with permission of Dr. B. M. Carlson and Wistar Press.)


S
T
1

Figure 2.

Right leg of rat after minced muscle fragments were autotransplanted back to original site. The biceps femoris was held by forceps. M: minced muscle fragment. (Reproduced with permission of Dr. B. M. Carlson and Wistar Press.)


2

Figure 3.

Right leg of rat with part of biceps femoris (B) sutured over the minced muscle fragments (M). The skin was eventually closed after the entire minced mass was covered by biceps femoris. (Reproduced with permission of Dr. B. M. Carlson and Wistar Press.)

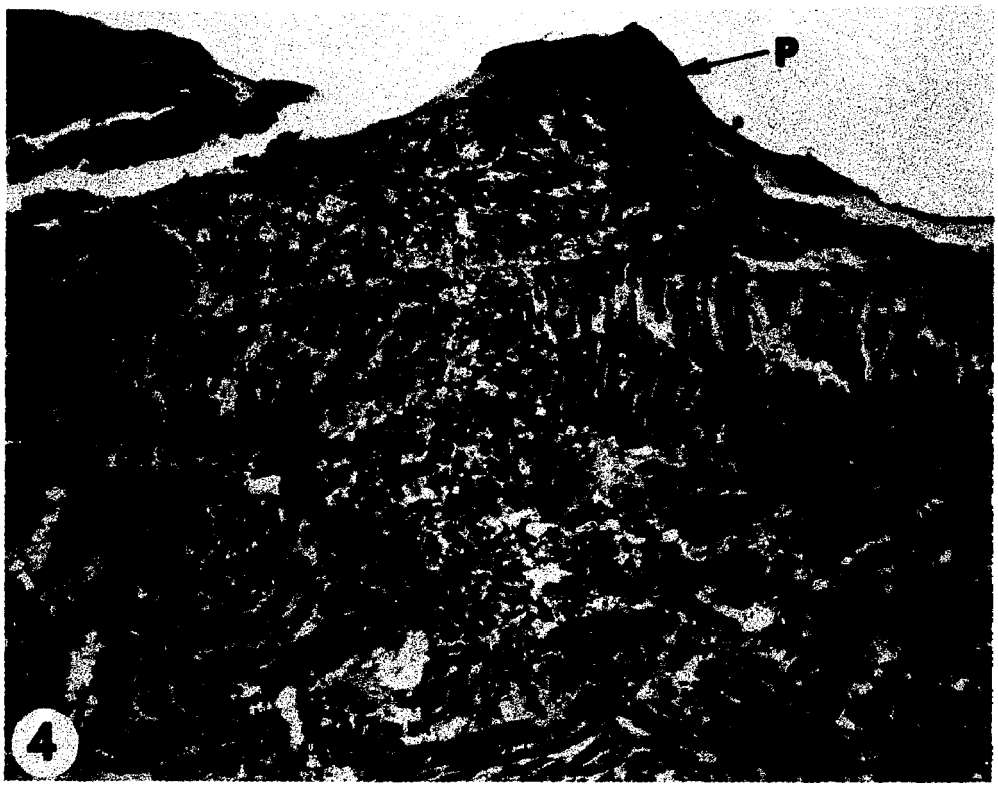

3

Figure 4.

One-day-old regenerate. Almost the entire regenerate consists of minced muscle fragments (M). Only the peripheral margin (P) shows early regeneration activity. (9 μm thick section, H & E staining, 10X)

Figure 5.

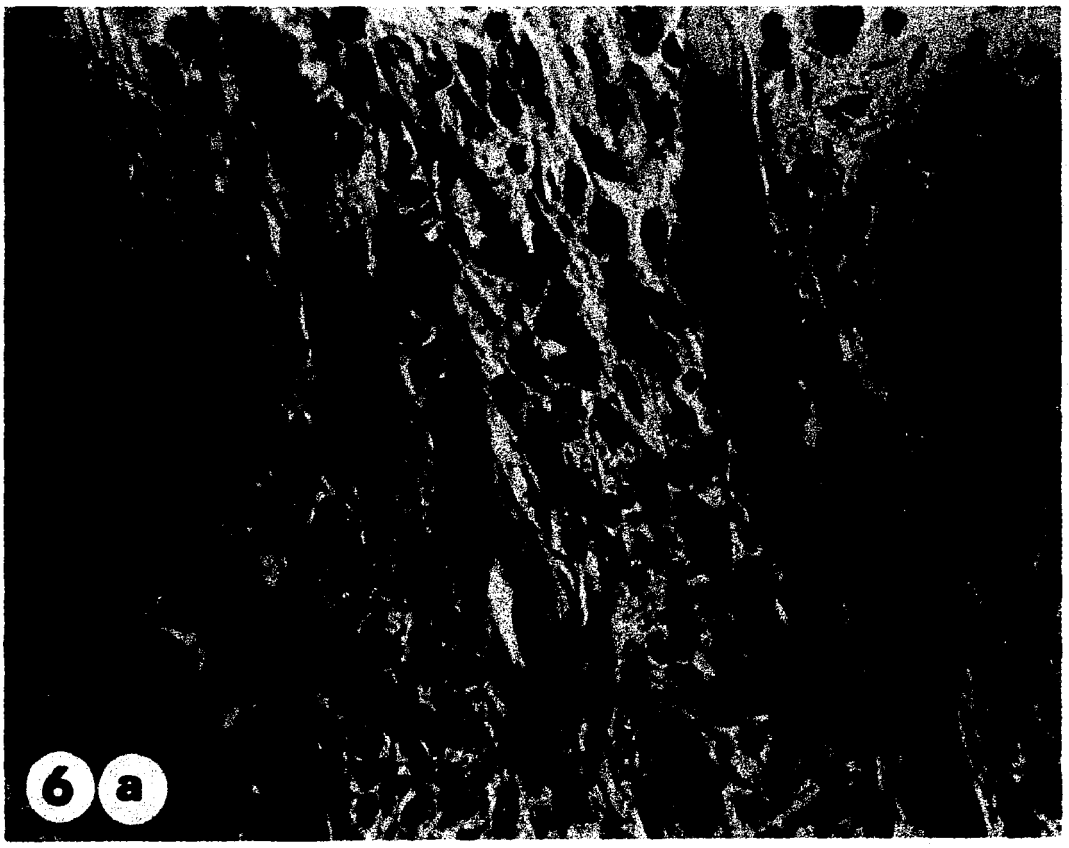
Three-day-old regenerate. The peripheral zone (P) is devoid of original minced muscle fragments, which occupy the central area (C). In the peripheral zone, myotubes (arrows) can be seen. (9 μm thick section, H & E staining, 25X)


P
4


5

Figure 6 a, b.

Two different views (a and b) of typical myotubes (arrows). The nuclei are situated in the center of the myotubes, close to each other, and lined up in a row. Note the prominent nucleoli in the nuclei. (9 μ m thick section, H & E staining, 400X)


6a

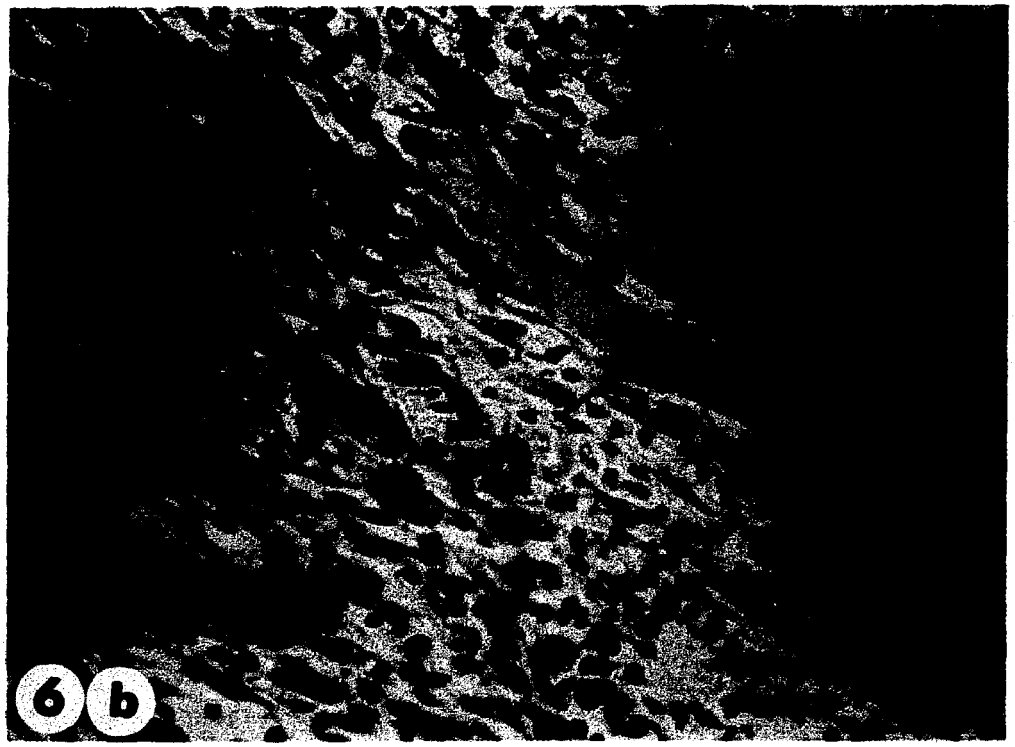

6b

Figure 7.

Five-day-old regenerate. Three zones of differential activity can be seen. P: peripheral basophilic regenerating zone; C: central eosinophilic ischemic zone; and I: intermediate zone. At this early stage, the central zone is thicker than the peripheral zone. The intermediate zone is sandwiched between the central and peripheral zones. With an increase in time, the intermediate zone gradually shifts toward the center. (9 μm thick section, H & E staining, 10X)

Figure 8.

High power view of the intermediate zone. The original minced muscle fibers are undergoing degeneration as revealed by their fragmented appearance. However, under the basement membranes (B) of the original muscle fibers, the myogenic myoblasts (M) will emerge. These myoblasts will fuse to form myotubes. (9 μm thick section, H & E staining, 400X)


7

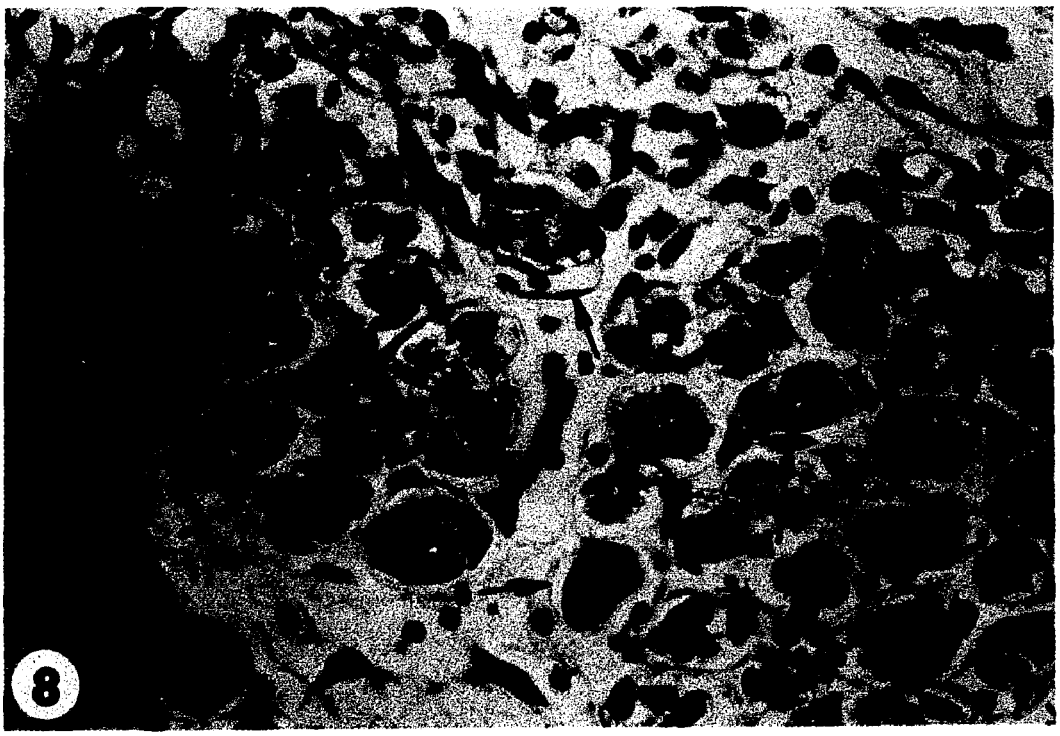

8

Figure 9.

Ten-day-old regenerate. Striations can be seen clearly in these young muscle fibers, although first signs of striation could just barely be seen as early as 7 or 8 days postoperatively. Note that the nuclei have already migrated to the periphery of the fibers. (9 μm thick section, H & E staining, 100X)

Figure 10.

Cross section of 40-day-old regenerate. Most fibers, except for a few (arrow), are running parallel to the axis of the regenerate. N: nerve trunk. (9 μm thick section, H & E staining, 10X)


9

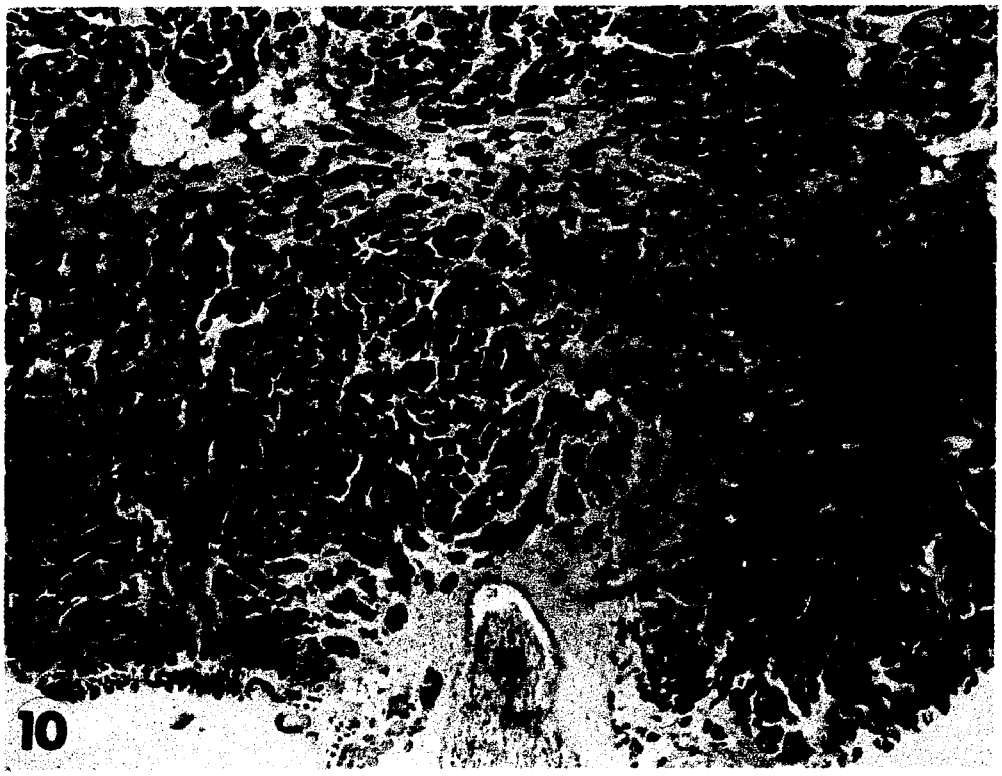

10

Figure 11.

Sixty-eight-day-old minced muscle regenerate. The distal end is connected to the calcaneal (Achilles) tendon. (Reproduced with permission of Dr. B.M. Carlson and S. Karger Press, Switzerland)

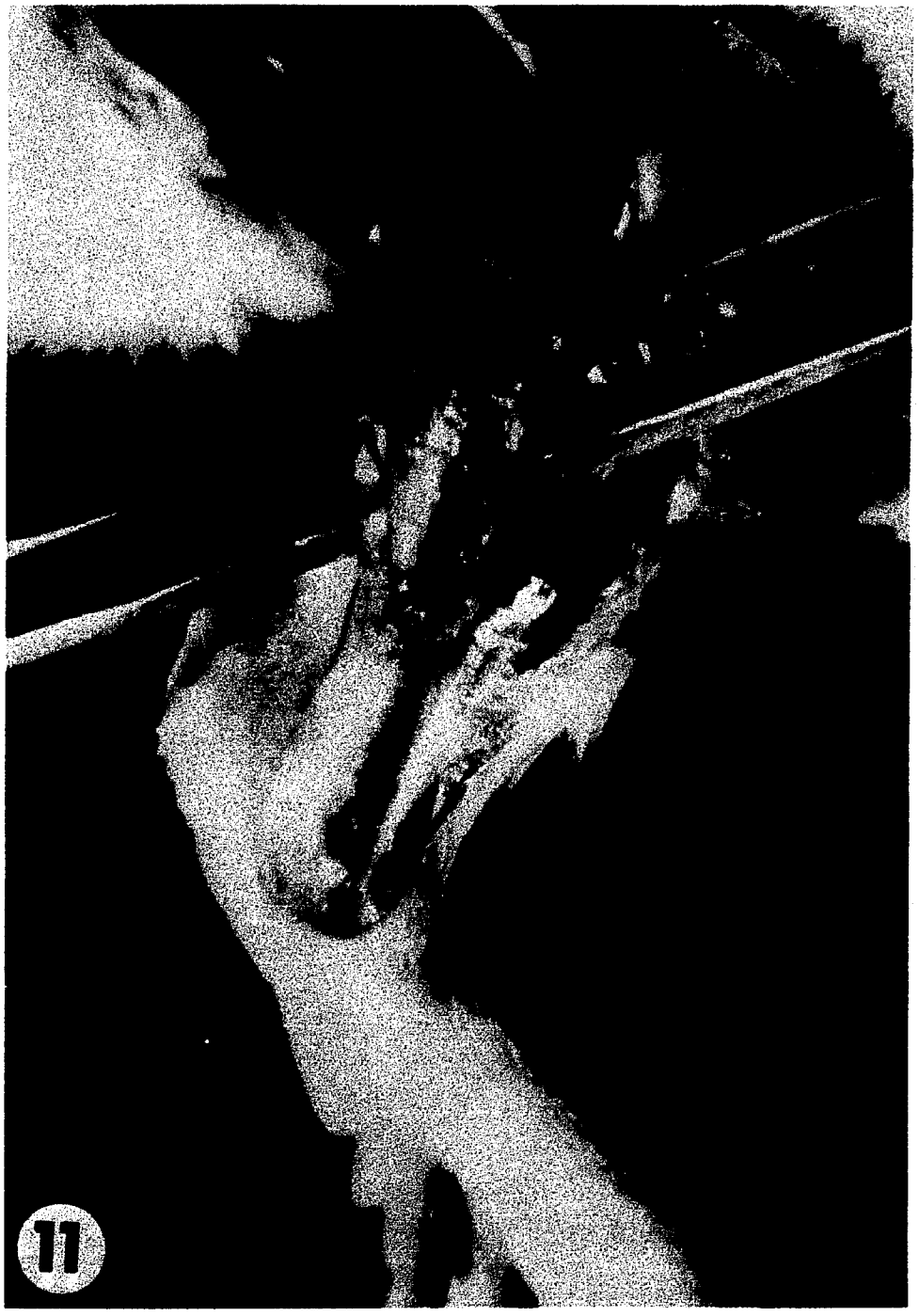
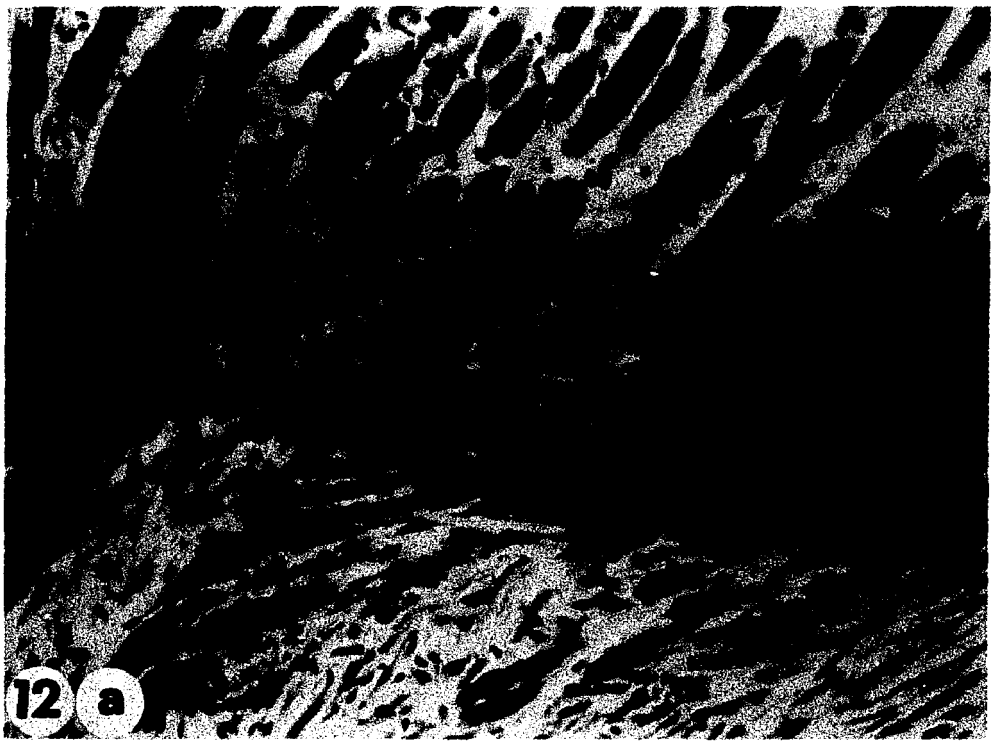

11

Figure 12 a, b.

Degenerating myotubes (black arrow) of normal (a) and denervated (b) regenerates. The nuclei are dark and shrunken and stacked up like the profiles of RBC's in the capillary. There are empty spaces (white arrows) immediately surrounding the nuclei of some degenerating myotubes. The cytoplasm is usually more deeply stained than those of normal myotubes. (9 µm thick section, H & E staining, 64X)


12 a

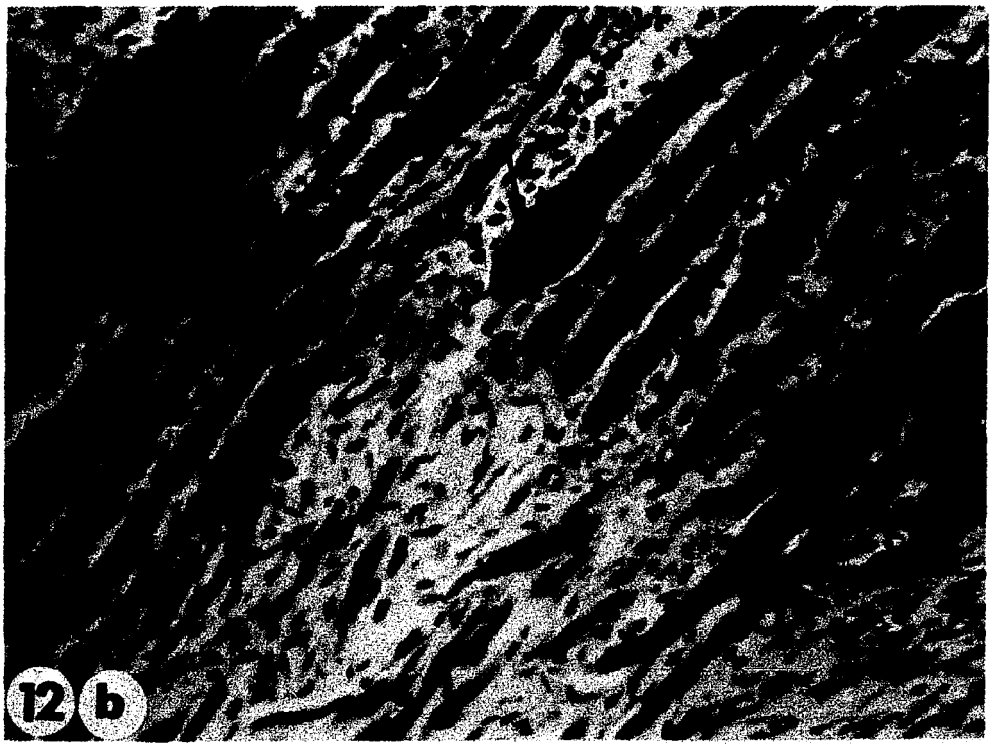
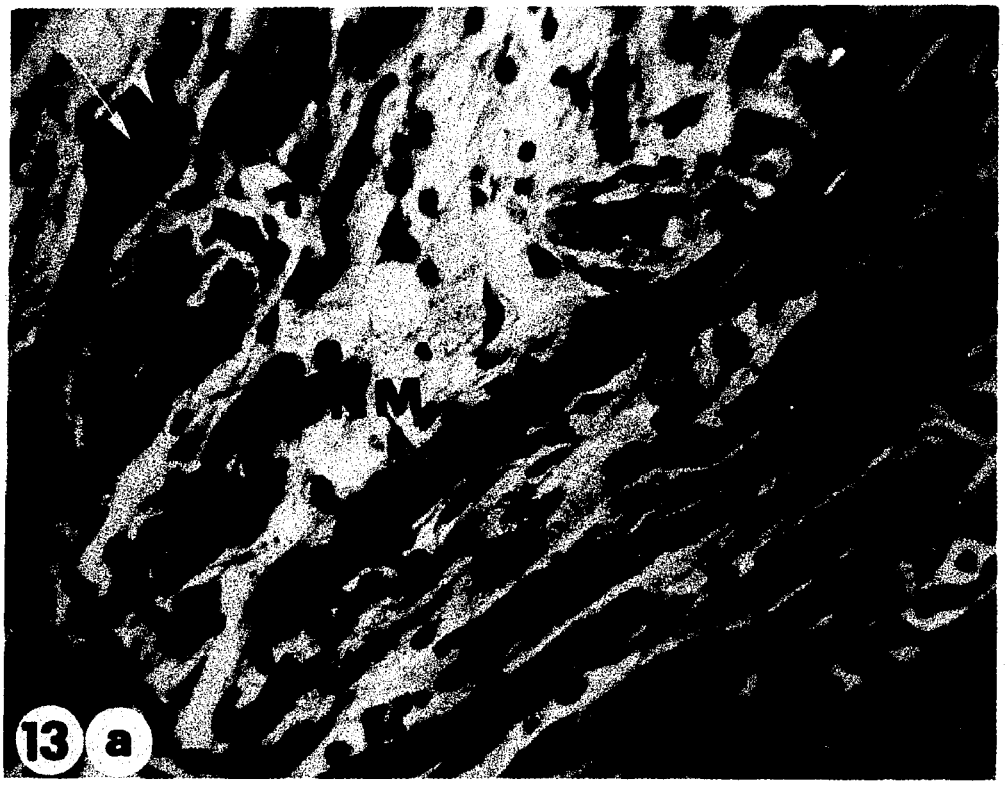

12 b

Figure 13 a, b.

Degenerating muscle fibers (DM) of normal (a) and denervated (b) regenerates. Their sarcoplasms are deeply stained and swollen (white arrow); occasionally a retraction cap and clot (dark arrow) can be seen. The nuclei are usually shrunken and pyknotic (white arrowhead). NM: normal muscle fibers. (9 μm thick section, H & E staining, 100X)


13 a


13 b

Figure 14.

Seven-day-old regenerate. Sections were stained with Hsu's modification of Palmgren's method. Arrowheads show the nerve trunks and arrow shows that the nerve fibers are sprouting and breaking away from the confinement of epineurium. (9 µm thick section, 10X)

Figure 15.

Eleven-day-old regenerate. Sections were stained with Namba's silver method. Nerve fibers are branching away from the nerve trunk (lower left corner) and approaching the regenerating muscle fibers (upper right corner). No nerve fibers can be seen among the muscle fibers until 3 or 4 days later. (ca. 50µ m frozen section, 100X)


14


N
15

Figure 16.

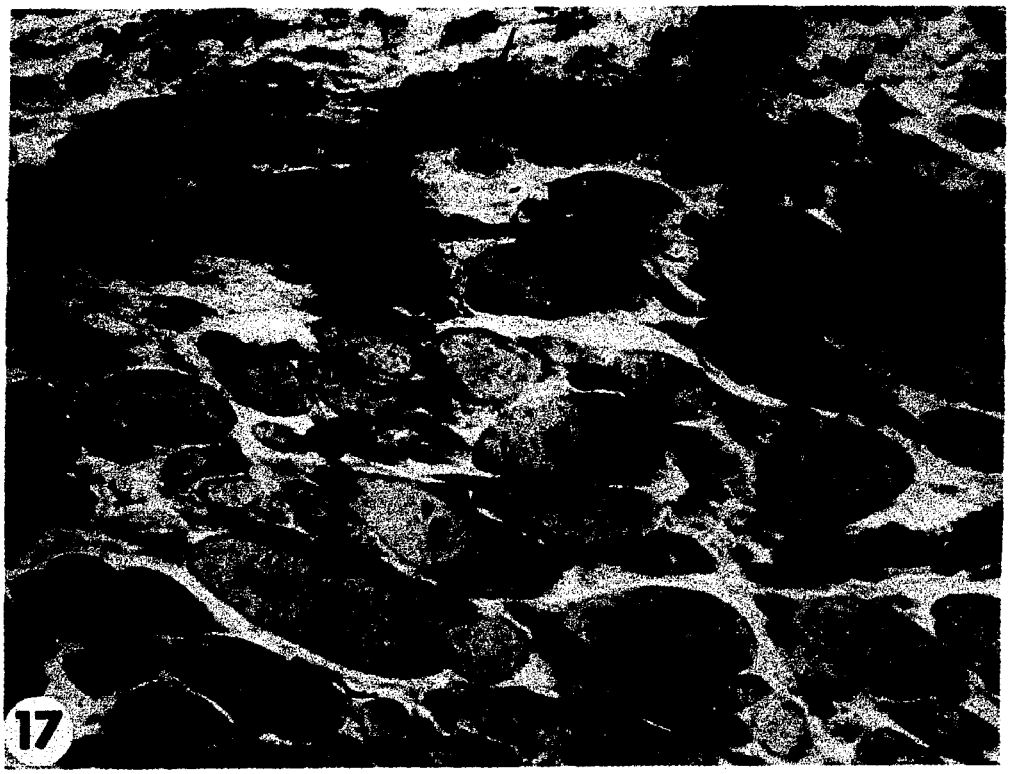
Thirty-day-old regenerate. Sections stained with Hsu's modified Palmgren silver method. It shows nerve fibers (arrows) spreading in between the regenerating muscle fibers. (9 µm thick section, 100X)

Figure 17.

Thirteen-day-old regenerate. Sections were incubated to show AChE according to Gomori's method (see Appendix II). The staining is positive in the peripheral area of the regenerate. The reaction product is diffusely distributed (arrows) along the regenerating muscle fibers. F: muscle fibers. (10 µm frozen section, 400X)



16



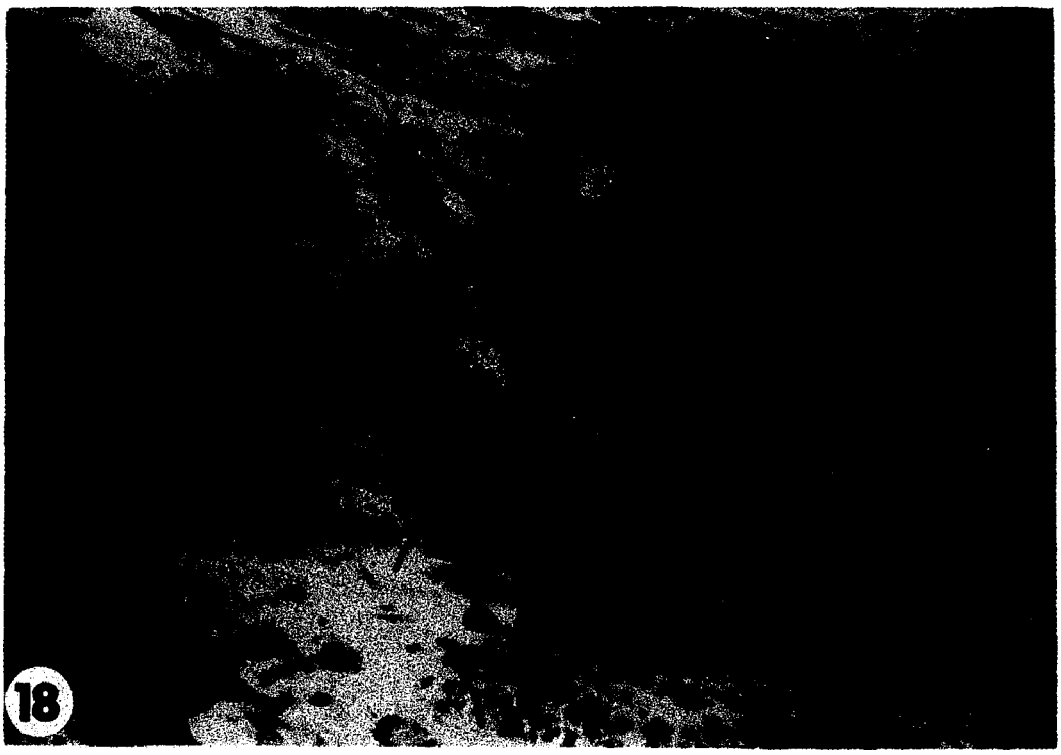
17

Figure 18.

Nineteen-day-old regenerate. Section was incubated to show AChE according to Gomori's method (see Appendix II). The diffuse reaction has gradually disappeared and some localized staining (arrows) can now be seen. (10 µm frozen section, 100X)

Figure 19.

Forty-five-day-old regenerate. Section was incubated to show AChE (MEP) according to Gomori's method (see Appendix II). The reaction is localized and typical MEP's (arrows) can be seen. (10 µm frozen section, 400X)


18

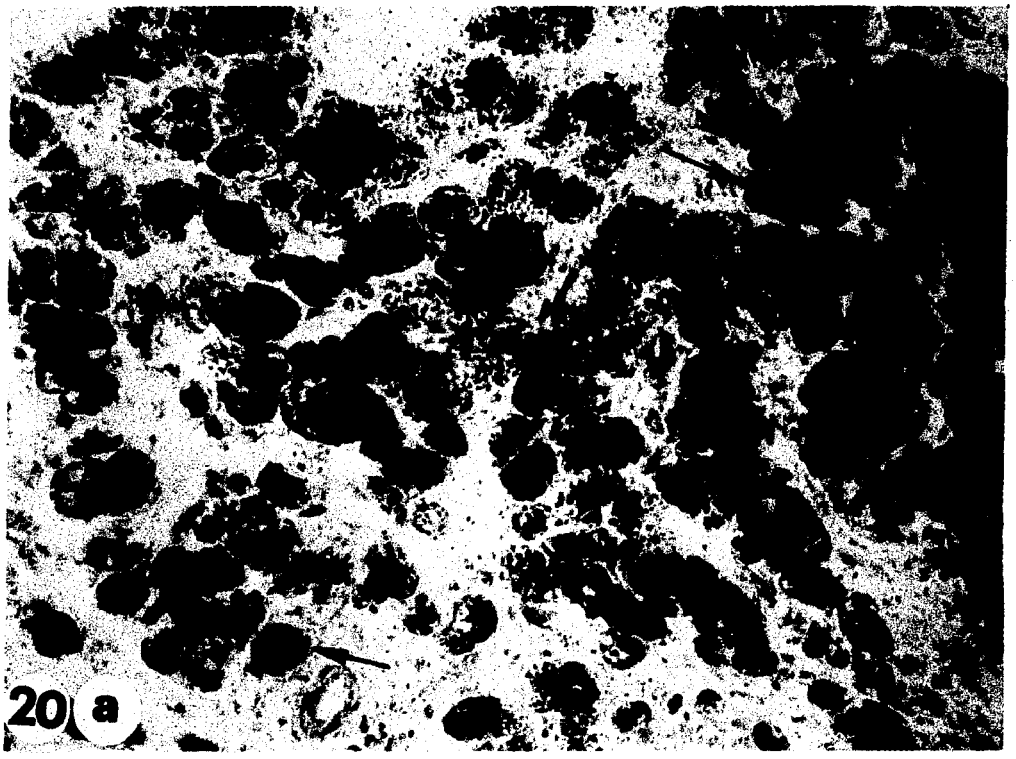

19

Figure 20 a.

Twenty-day-old normal regenerate. Section was stained with SDH (see Appendix II). The reaction is positive. Except for a few darkly stained fibers (arrows), the staining intensity is uniform. There is no indication of fiber types. (10 μ m frozen section, 100X)

Figure 20 b.

Twenty-day-old denervated regenerate. Section was stained with SDH (see Appendix II). The reaction is positive and the staining intensity is uniform. There is no indication of fiber types. (10 μm frozen section, 100X)


20 a

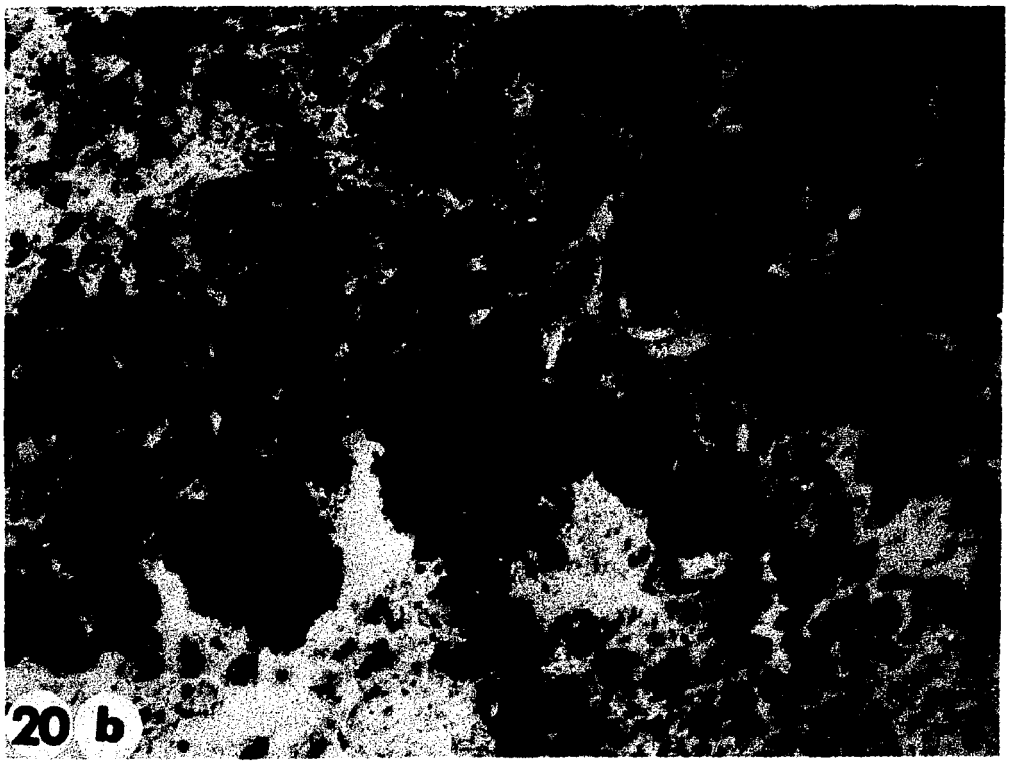
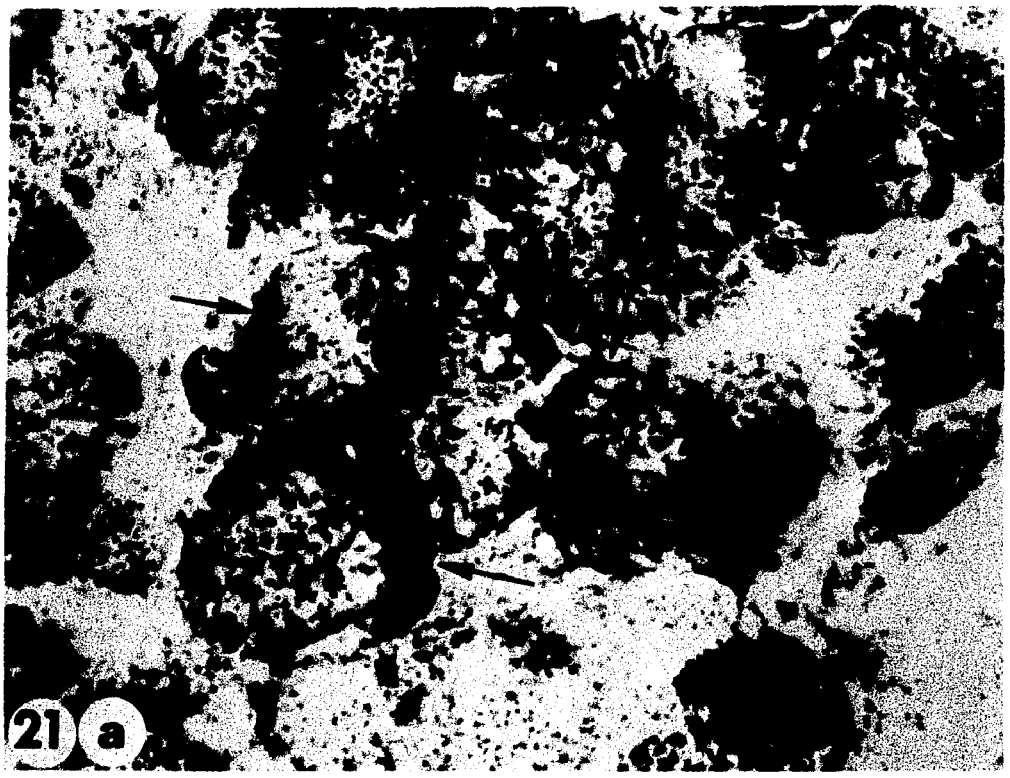

20 b

Figure 21 a.

Thirty-day-old normal regenerate with SDH reaction (see Appendix II). Diformazan granules can be seen in the cytoplasm and heavy precipitate can be seen in some fibers along the cell membrane (arrows). There is no indication of fiber types. (10 µm frozen section, 400X)

Figure 21 b.

Thirty-day-old denervated regenerate with SDH reaction (see Appendix II). Diformazan granules and heavy precipitate along cell membrane can also be seen. Essentially no difference can be observed between normal and denervated regenerates at this stage. (10 µm frozen section, 400X)


21 a

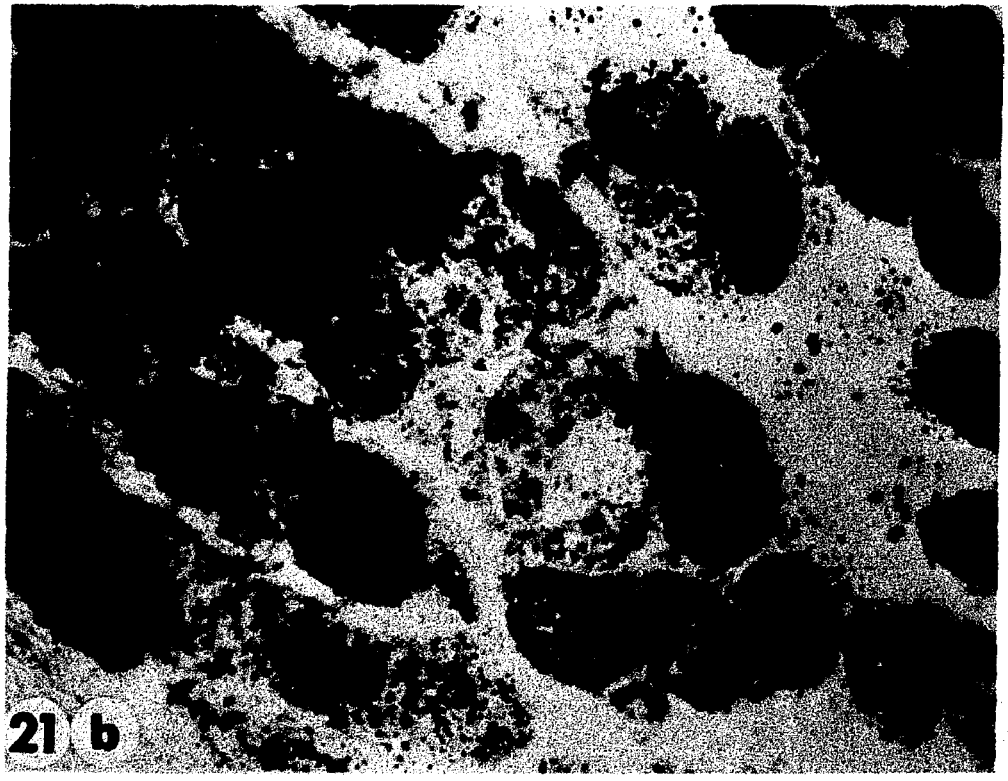
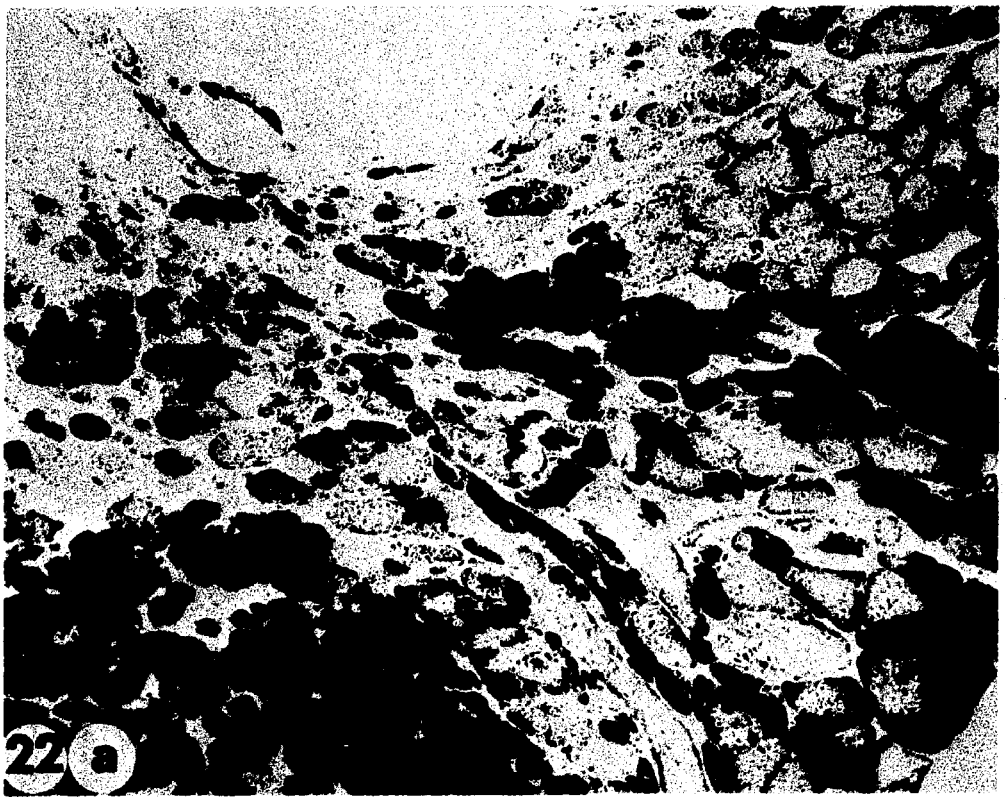

21 b

Figure 22 a.

Forty-day-old normal regenerate with SDH reaction (see Appendix II). Dark staining fibers are in the lower left corner, while light staining fibers are in the upper right corner. Thus, fiber type differentiation can be seen. Note: Muscle fibers of the same type tend to group together. (10 μm frozen section, 100X)

Figure 22 b.

Forty-day-old denervated regenerate with SDH reaction. No indication of fiber type differentiation can be seen. The reaction remains positive but primitive. (10 μm frozen section, 100X)


22 a

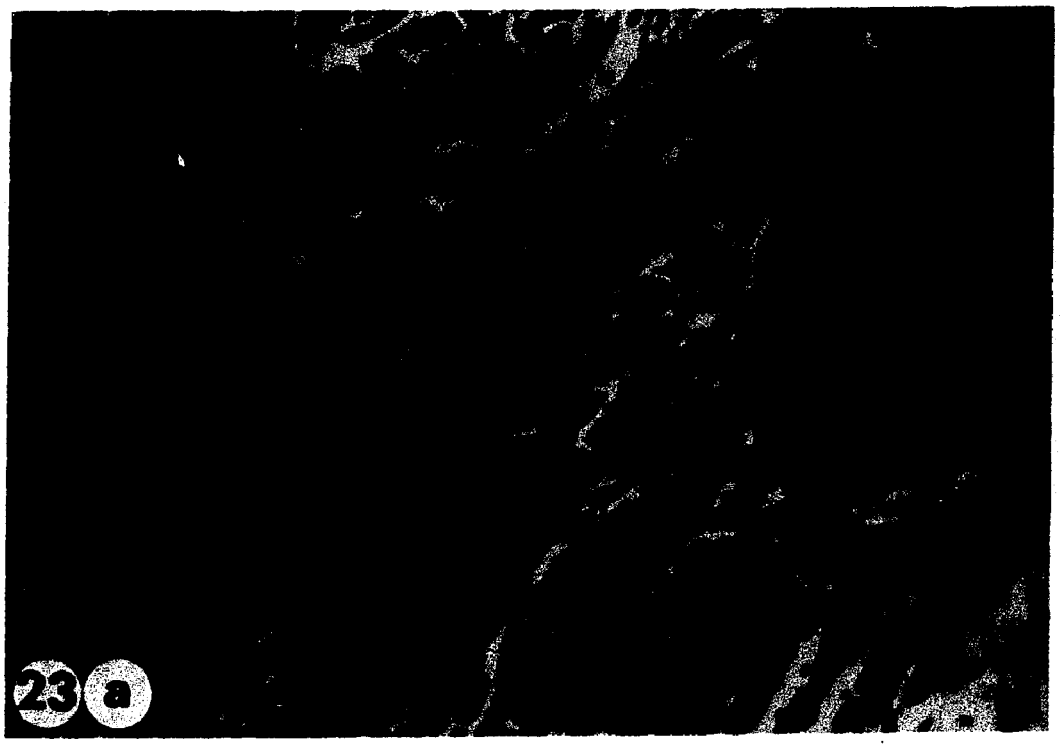

22 b

Figure 23 a.

Thirty-day-old normal regenerate with myofibrillar ATPase reaction. The fibers are positively stained, but the intensity of different fibers is uniform. Typical fiber types cannot be seen at this stage. (10 µm frozen section, 100X)

Figure 23 b.

Thirty-day-old denervated regenerate with myofibrillar ATPase reaction (see Appendix II). The reaction and the staining intensity are similar to those of the normal regenerate of the same age. No fiber types can be seen. (10 µm frozen section, 64X)


23 a

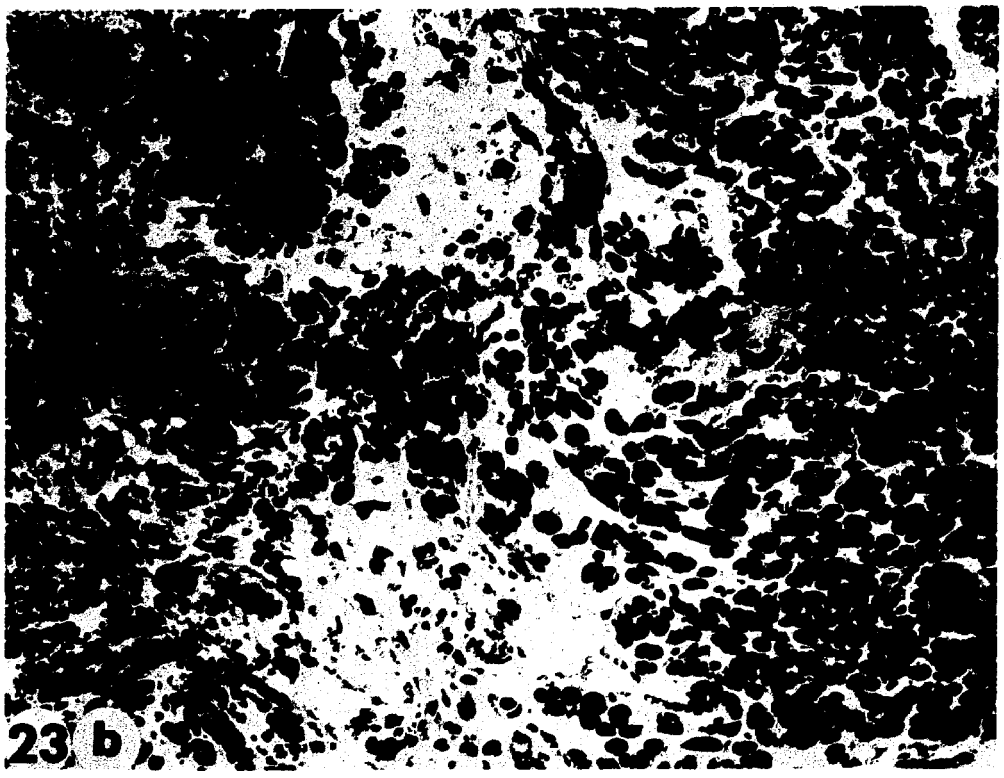
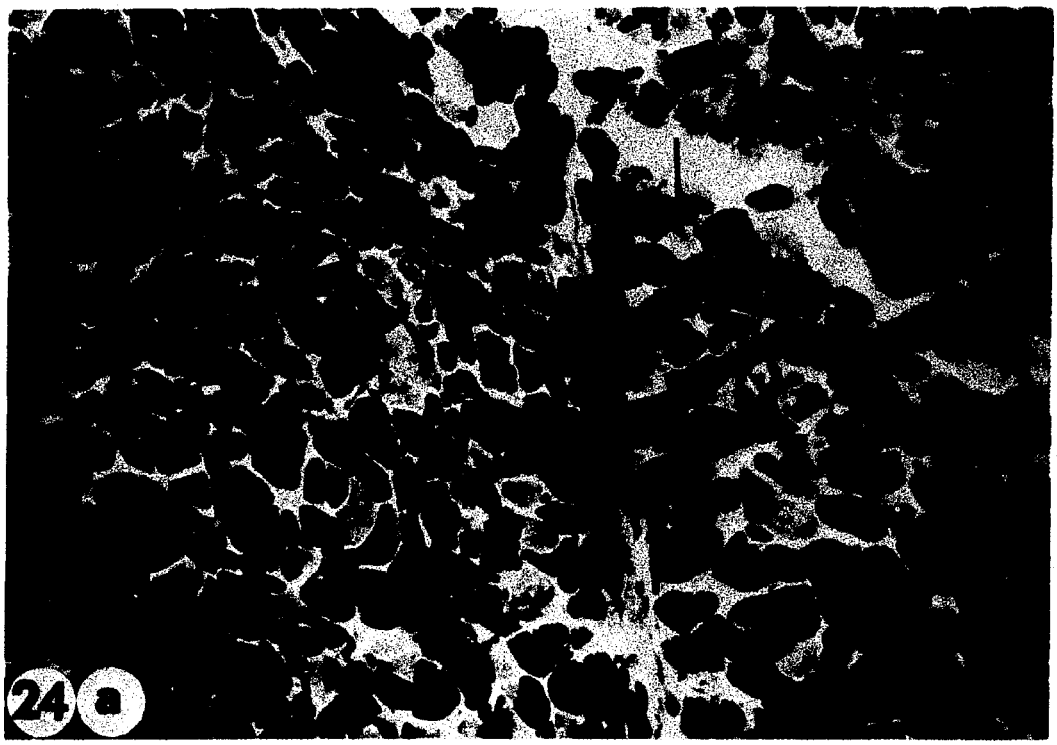

23 b

Figure 24 a.

Forty-day-old normal regenerate with myofibrillar ATPase reaction (see Appendix II). Lightly stained fibers (dark arrows) and darkly stained fibers (white arrow) can be seen clearly at this stage. (10 μm frozen section, 100X)

Figure 24 b.

Forty-day-old denervated regenerate with myofibrillar ATPase (see Appendix II). The reaction remains positive, but fiber type differentiation has not occurred. (10 μm frozen section, 64X)


24a

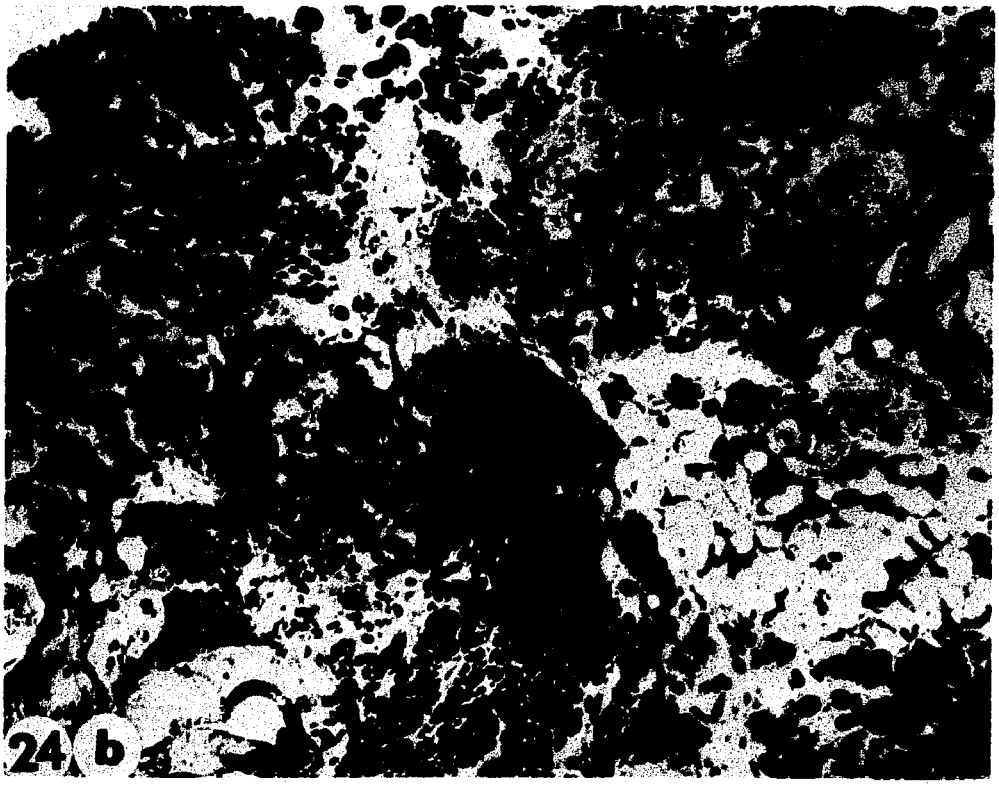

24b

Figure 25 a.

Normal gastrocnemius serial sections showing 3 fiber types. SDH reaction is on the left and myofibrillar ATPase reaction is on the right. FOG: fast-twitch-oxidative-glycolytic fibers; FG: fast-twitch-glycolytic fibers; SO: slow-twitch-oxidative fibers. (10 μm thick frozen section, 400X)

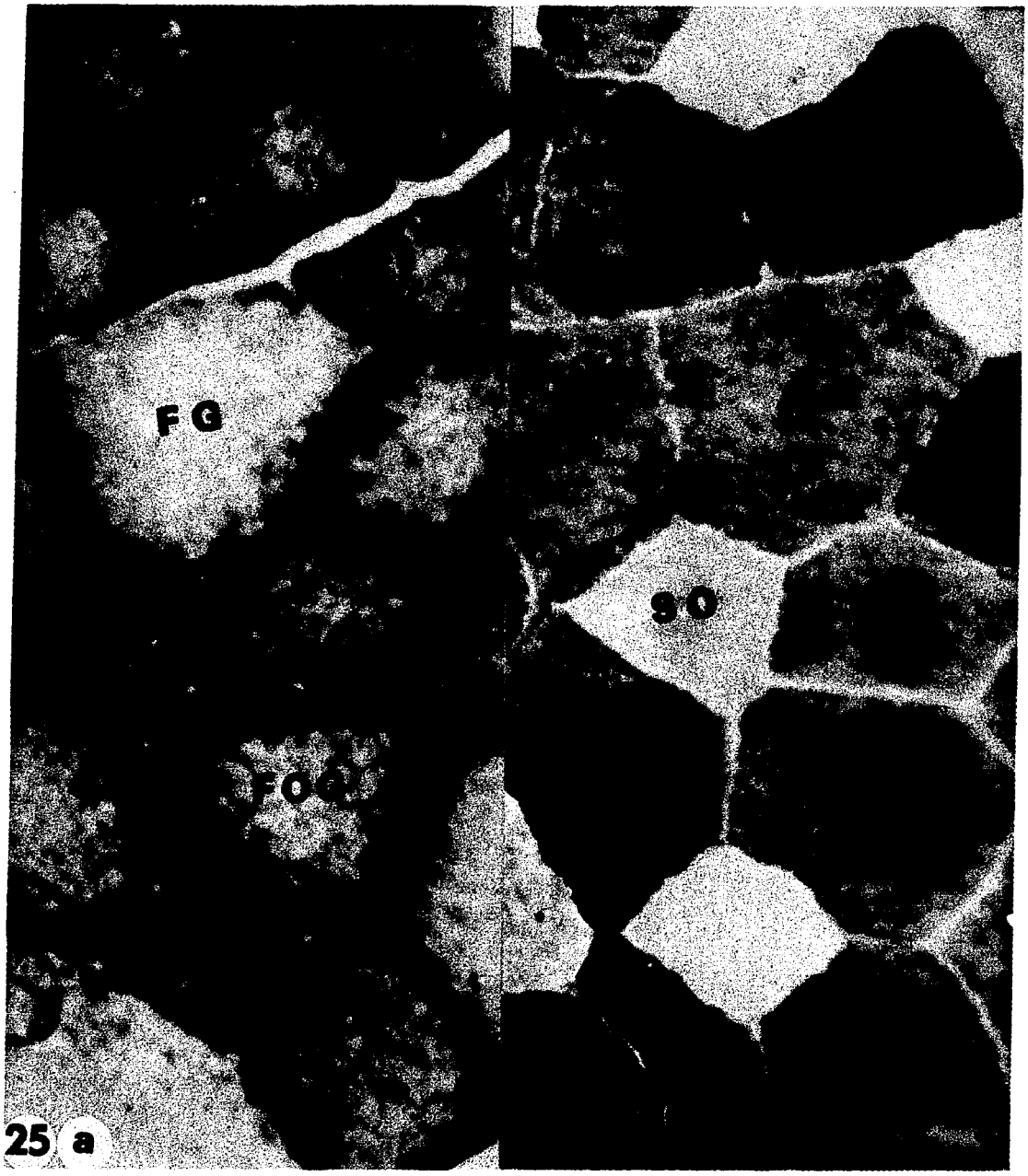

FG
SO
FOC
25 a

Figure 25 b.

Normal regenerate serial sections showing 3 fiber types. SDH reaction is on the bottom and myofibrillar ATPase reaction is on the top. FOG: fast-twitch-oxidative-glycolytic fibers; FG: fast-twitch-glycolytic fibers; SO: slow-twitch-oxidative fibers. (10 μm thick frozen section, 400X)

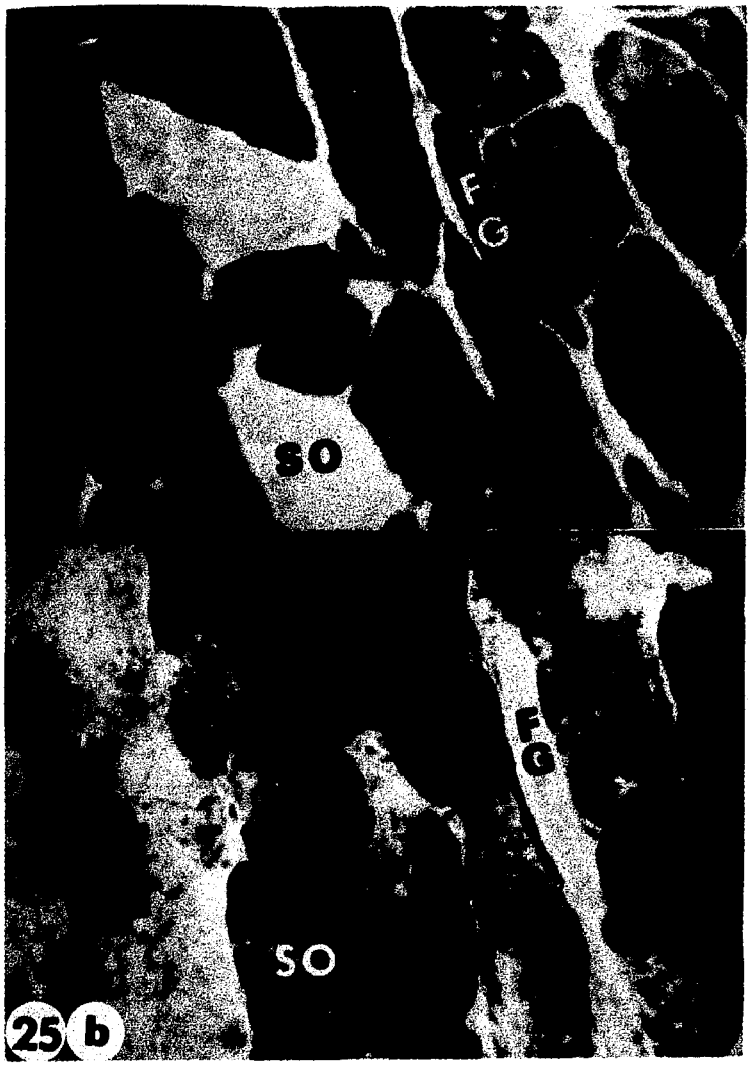

FG
SO
FG
SO
25 b

Figure 26 a, b.

Longitudinal sections of 25-day-old (a) and 35-day-old (b) denervated regenerate. Cross striations and peripherally located nuclei can be seen clearly. [9 µm thick paraffin section, H & E staining, (a) 100X, (b) 400X]


26 a

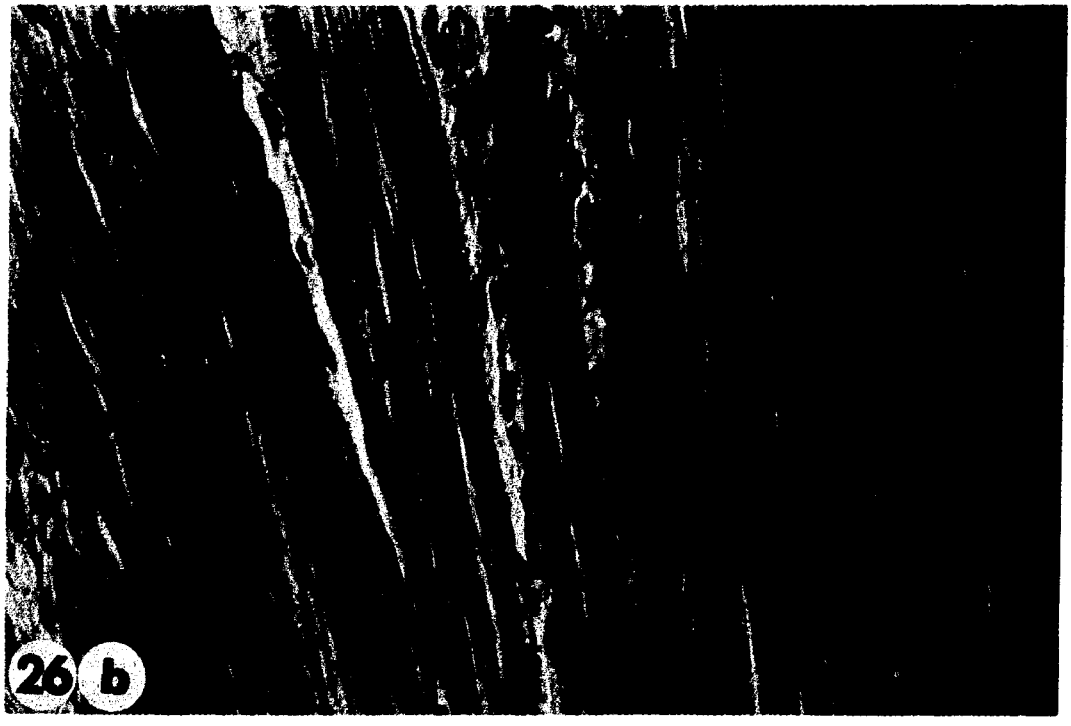
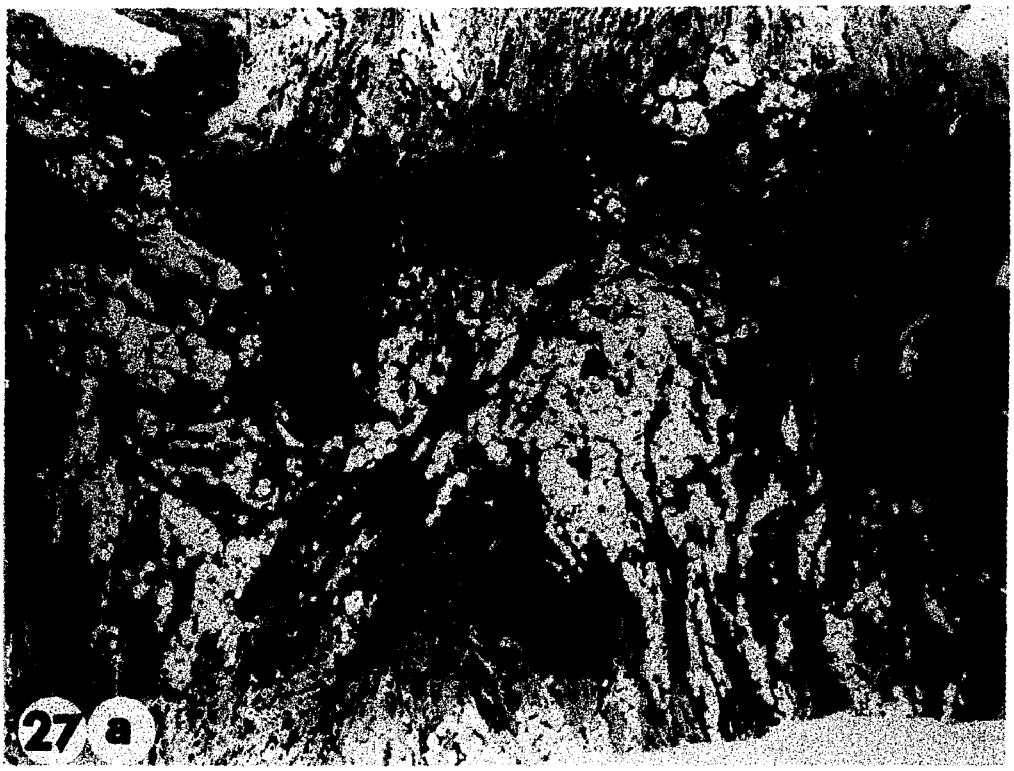

26 b

Figure 27 a, b.

Longitudinal sections of 65-day-old denervated regenerate. (a) low power view (10X), A: adipose tissue, C: connective tissue. (b) higher power view (100X), the connective tissue (dark arrows) is replacing the degenerating muscle fibers (white arrows). (9 μm paraffin section, H & E staining)


27 a

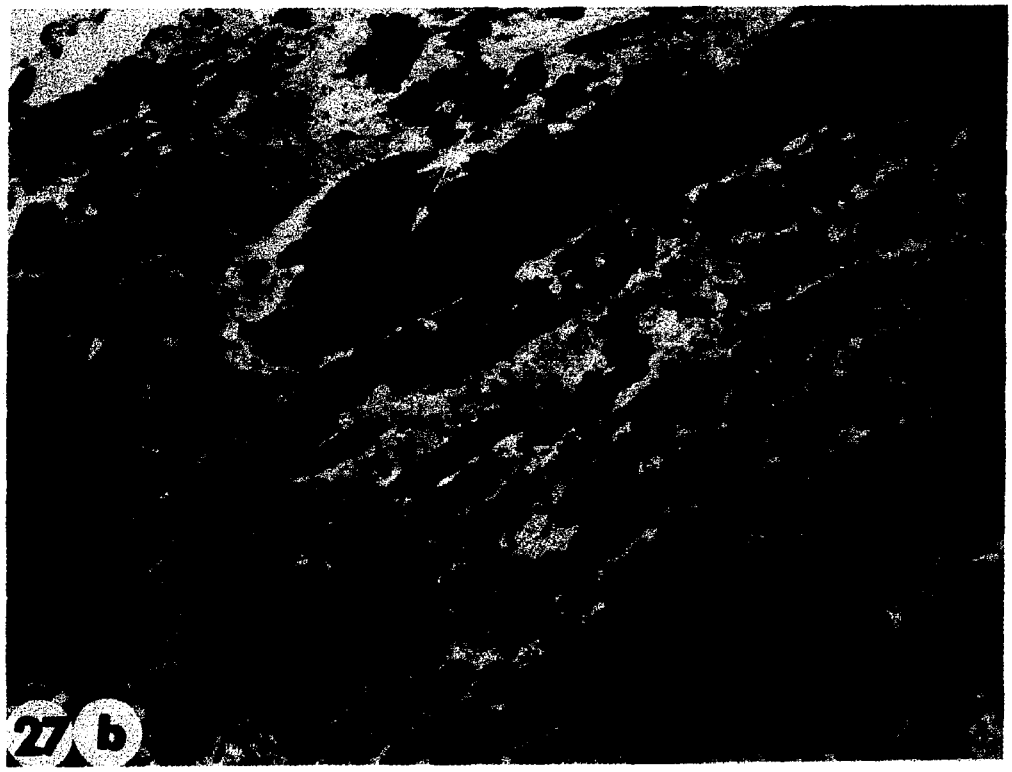

27 b

Figure 28 a, b.

Cross section of 90-day-old denervated regenerate. (a) low power view (10X). Connective tissue (C) and adipose tissue (A) become the main constituents of the regenerate. In the central area (encircled by arrows), however, a number of small degenerating fibers can still be seen. (b) high power view (400X) showing the degenerating muscle fibers in the central area. F: muscle fibers, C: connective tissue. (9 μm paraffin section, H & E staining)


28.C

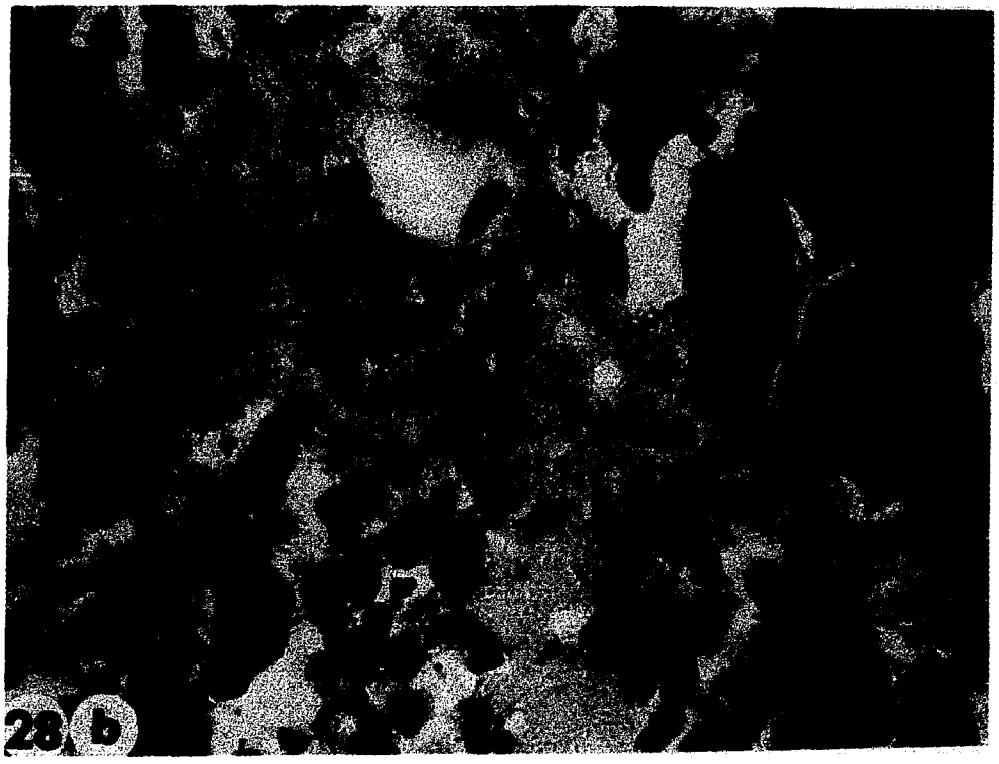
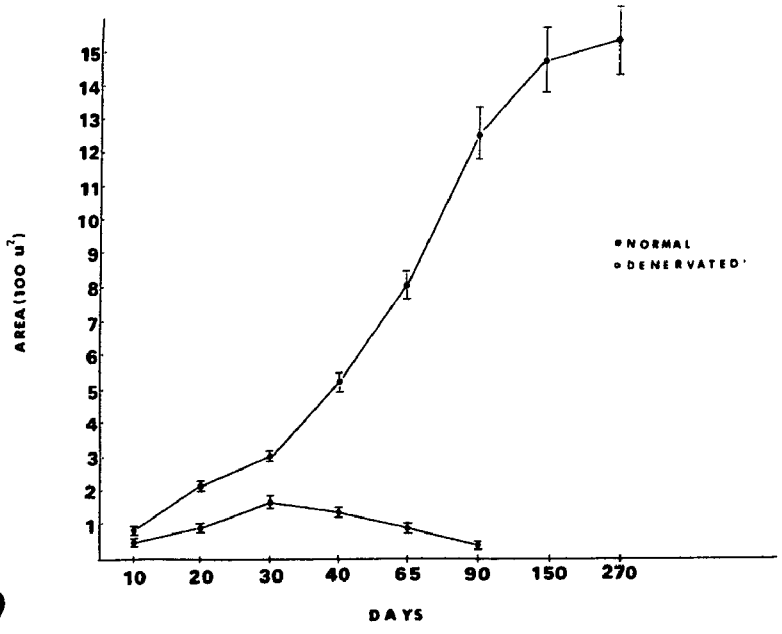

28.D

Figure 29.

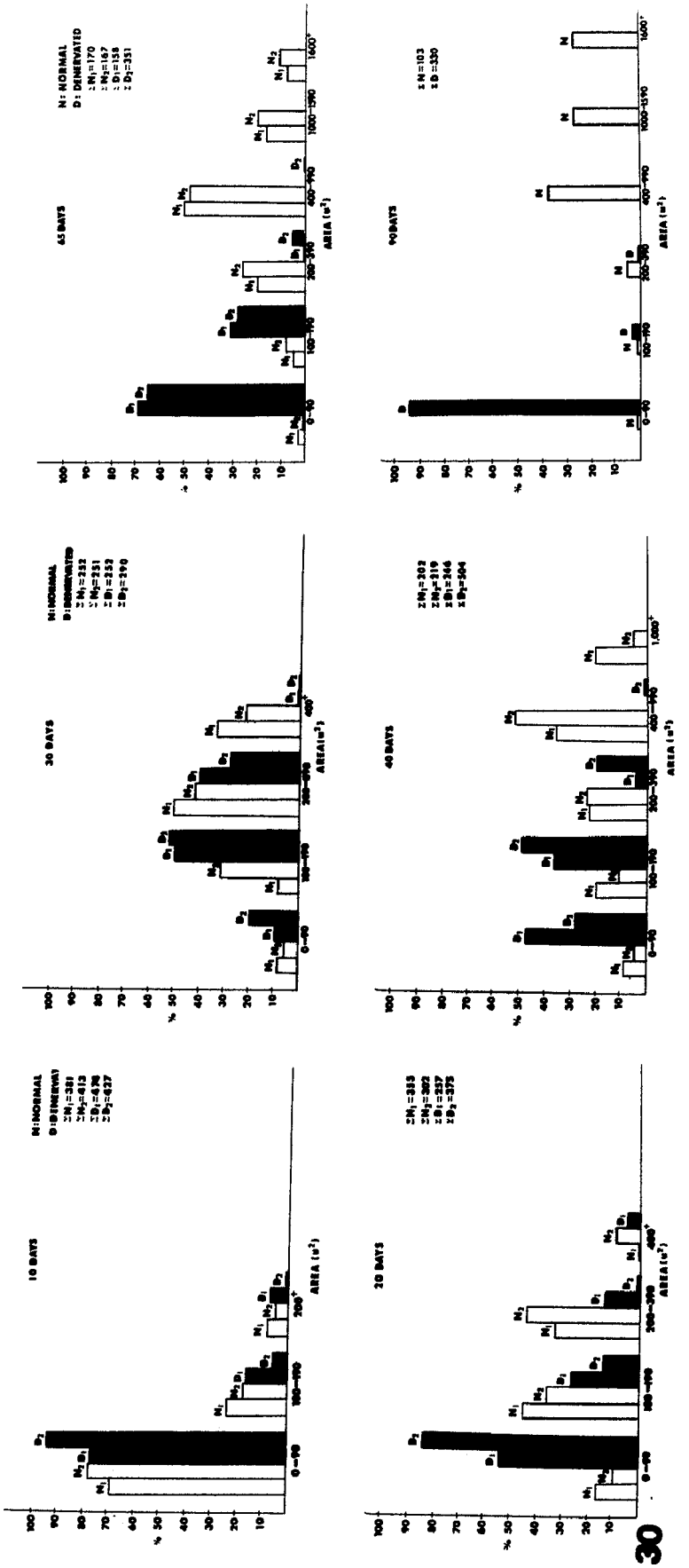
Mean cross-sectional area of normal and denervated regenerating muscle fibers.


AREA (100 u²)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
10
20
30
40
65
90
150
270
DAYS
• NORMAL
○ DENERVATED

29

Figure 30.

Frequency distribution of different cross-sectional areas of normal and denervated muscle fibers from 10 to 90 days postoperatively.



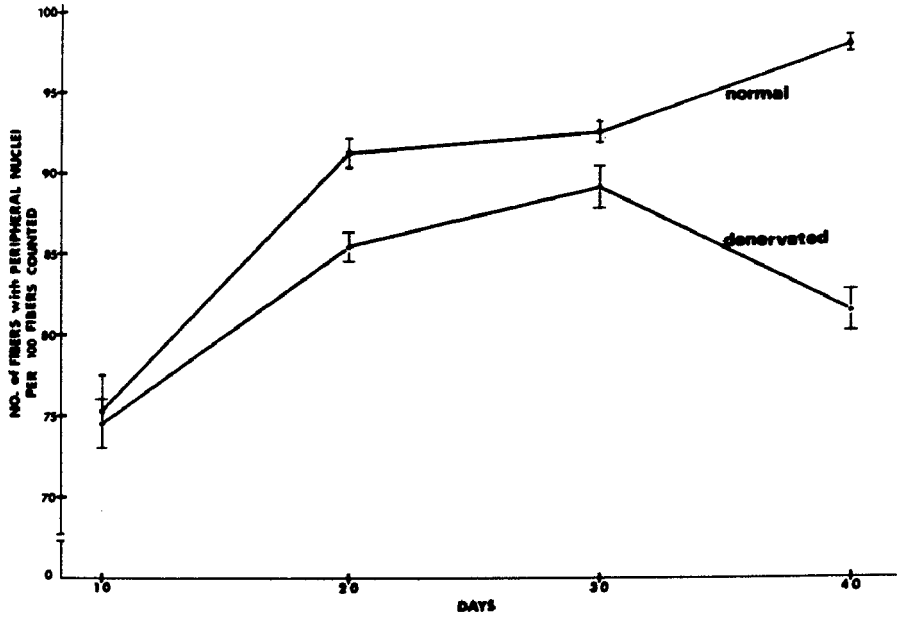
30

Figure 31

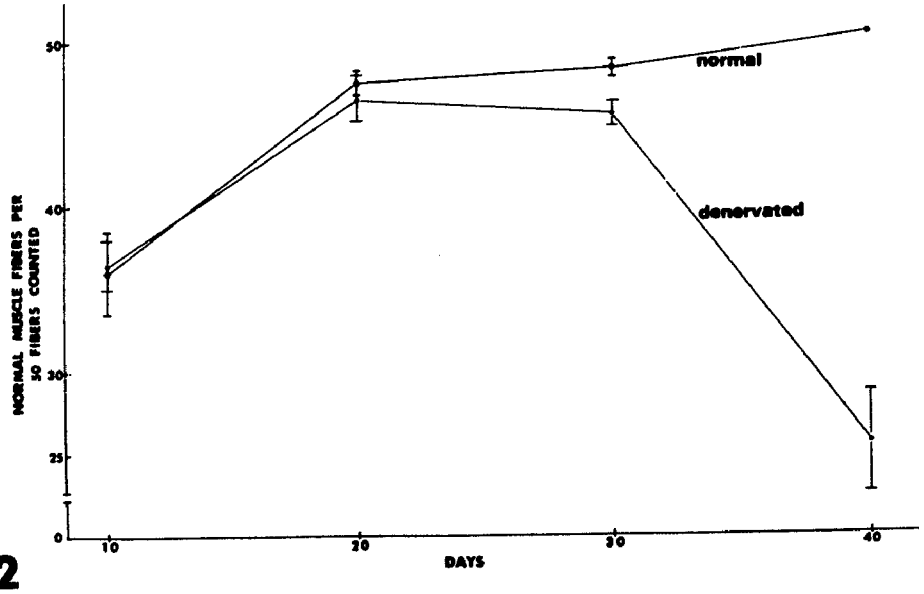
Number of muscle fibers with peripheral nuclei per 100 fibers counted of normal and denervated regenerates. Data are taken from the proximal apart of the regenerates only. No significant differences exist from 10 to 30 days between normal and denervated regenerates. At 40 days, however, the difference becomes apparent.

Figure 32

Number of normal muscle fibers per 50 fibers counted of normal and denervated regenerates. Data are taken from proximal part of the regenerates only. No significant differences exist from 10 to 30 days between normal and denervated regenerates. On 40 days, however, the difference becomes apparent.


NO. of FIBERS with PERIPHERAL NUCLEI PER 100 FIBERS COUNTED
100
95
90
85
80
75
70
0
10
20
30
40
DAYS
normal
denervated

31


NORMAL MUSCLE FIBERS PER 50 FIBERS COUNTED
50
40
30
25
0
10
20
30
40
DAYS
normal
denervated

32

APPENDIX I

DETAILED PROCEDURES OF MINCED MUSCLE OPERATION

Sprague-Dawley male rats, 70-100 gm in weight (1-1½ months old) were used in all experiments. For anesthesia, sodium pentobarbital was injected intraperitoneally. The dosage was 5-7 mg/100 gm wt. In case of overdosage, Metrozol (Knoll Pharmaceutical Co.) was used for counteraction. The usual dosage was 3-5 µl for rats within a weight range of 70-90 gm. After the rat was anesthetized, the leg was shaved, cleaned with 70% alcohol, and covered with a gauze sponge. Before the operation, the instruments, glassware, and sutures were sterilized by boiling them for 10-15 minutes and soaking them in a 95% alcohol solution for a few minutes.

An incision of about 2-3 cm in length was made from the popliteal space to the ankle joint over the gastrocnemius. By blunt dissection, the skin was freed from the biceps femoris which lay immediately underneath the skin. A second incision of 1-1½ cm in length was made longitudinally to divide the biceps and to expose the triceps surae beneath it. After blunt dissection, the triceps surae was freed from biceps. The sural nerve which lay on the surface of gastrocnemius was also freed. The soleus which was on the lateral side and under the cover of gastrocnemius could be freed from its origin and insertion and removed. After this, the calcaneal (Achilles) tendon was transected first, and then the

gastrocnemius and plantaris were pulled underneath the distal part of the biceps proximally. Next, the peroneal nerve which crossed over the lateral head of gastrocnemius was freed from its muscle attachment. The gastrocnemius was then divided into lateral and medial halves along the point where the tibial nerves penetrated the muscle. The branches from the tibial nerve to the lateral gastrocnemius were severed and the muscle was removed. Similarly, the branches from the tibial nerve to the medial gastrocnemius and, beneath it, the plantaris were severed and both muscles were simultaneously removed (Fig. 1). Care was taken to cut the nerves as far down to the muscle as possible and not to damage the blood vessels. Occasionally, vessels were cut and the hemorrhage was stopped with pressure and gauze sponges.

The removed muscles were then put on the back of a Petri dish and moistened with one or two drops of 0.9% saline solution. They were then dissected free of as much dense connective tissue as possible. Because the soleus is a red (slow) muscle, it was discarded to ensure that the origin of myoblasts was as homogenous as possible. With scissors, the muscles were then minced into fragments about 1 mm^3 in size. About two-thirds of the minced mass was then replaced into and along the original muscle bed (Fig. 2). The biceps was then sutured over the minced mass and eventually the skin was closed (Fig. 3). No. 6 silk suture (Ethicon) was used for the operation.

The rats were left on the table until the effect of the anesthetic wore off and then returned to the cage. To prevent infection, Terramycin (Pfizer) was mixed in the drinking water (1 tsp/300 ml) for a week.

The denervation procedure was described in a section of Materials and Methods.

Periodical inspection of the rats was necessary, especially for the denervated animals, because the legs were immobile and could become trapped in the lattices of the cage. If this happened, the legs became infected and swollen. Once or twice this did occur and the animals were sacrificed and discarded.

APPENDIX II

DEMONSTRATION OF ATPase, SDH, AChE, AND NERVE FIBERS

(I) HISTOCHEMICAL DEMONSTRATION OF SDH (Succinate Dehydrogenase)

The following method was modified from Nachlas et al. ('57). Nitro blue tetrazolium (Nitro BT, Sigma Chemical Company) was used as electron acceptor for the dehydrogenase reaction. Blue diformazan granules are the positive reaction products.

Solutions:

1. Buffered Succinate Stock Solution: Mix equal volumes of 0.2 M Phosphate Buffer (pH = 7.6) and 0.2 M Succinate.
2. Incubation Solution: Dissolve 10 mg Nitro BT into a solution of 10 ml H_2O and 10 ml stock.

Procedures:

1. Fresh frozen sections (10 µm) were picked up with a cover glass and left to air-dry for 10-20 min.
2. Incubate the sections at 37° C for at least 30 min.
3. Rinse in distilled H_2O.
4. Dehydrate through graded alcohol, clear in xylene and mount in Permount.

Remarks:

1. Nuclei can be counterstained with 0.1% aqueous safranin solution or in 1% aqueous neutral red solution after step 3. If this is done, sections

should not go through alcohol for dehydration but be mounted directly with glycerin jelly. Since the red color tended to fade and spread to the cytoplasm, it was good only for immediate photography. This counter stain was later abandoned after the initial trials.

2. The incubation solution can be used at least 2 times, if it is well covered to prevent oxidation by air. Usually it can be kept at least 24 hrs. at room temperature.

(II) HISTOCHEMICAL DEMONSTRATION OF MYOFIBRILLAR ATPase

The following method was modified from Padykula et al. ('55a,b) and Niles et al. ('69)

Reagents:

0.1 M Sodium Barbiturate

1.0 M $CaCl_2$

0.1 M NaOH

2% $CoCl_2$

1% vol./vol. yellow $(NH_4)_2S$ solution

1.0 M Tris Buffer

1% $CaCl_2$

ATP (Nutritional Biochemicals Corp.)

Incubation Media:

Mix 5 ml 0.1 M Na-Barbiturate

2.5 ml 1.0 M $CaCl_2$

15 ml distilled H_2O

1 ml 1.0 M Tris Buffer

Add 25 mg of ATP to the above solution just before incubation and adjust the pH to 9.4 with 0.1 M NaOH.

Procedures:

1. Incubate the air-dried sections (10μm) at 37° C for 30-60 min. immediately after the incubation solution is prepared.
2. After incubation, rinse the sections with 3 changes of 1% $CaCl_2$, 2 min. each.
3. Transfer to 2% $CoCl_2$ for 2 min.
4. With a stream of running tap water, wash the sections (inside the coplin jar) for at least 5 min. to remove Co^{++} ions.
5. Immerse in 1% $(NH_4)_2S$ for at least 30 sec.
6. Wash again in tap H_2O for 1-2 min.
7. Dehydrate in alcohol, clear in xylene, and mount in Permount.

Remarks:

1. Washing after $CoCl_2$ is important. If Co^{++} is not removed, scaly ppt. will be formed on the sections.
2. The pH (9.4) is critical and the incubation solution (after adding ATP) has to be absolutely fresh. If clouding

ppts. are seen in the solution, it is of no use.

(III) DEMONSTRATION OF MOTOR-END PLATES (MEPs)

The following cholinesterase staining method was adopted from Gomori ('52, in Pearse, 1952, p. 1312, Vol. II).

Stock Solution:

$CuSO_4.5H_2O$	0.3 gm
Glycine	0.375 gm
$MgCl_2.6H_2O$	1.0 gm
Maleic acid	1.75 gm
NaOH (4%)	30 ml
Na_2SO_4 (40%, hot saturated)	170 ml

Adjust to pH = 6 and keep.

Procedures:

1. Dissolve 20 mg Acetylthiocholine Iodide in a few drops of water. Add 10 ml stock solution.
2. Incubate sections 30-60 min. at 37° C.
3. Rinse in three changes of saturated Na_2SO_4.
4. Rinse in a stream of running tap water for 3-5 min. to remove excess Na_2SO_4.
5. Treat with 1% vol./ vol. yellow $(NH_4)_2S$, 1-2 min.
6. Rinse thoroughly with "dist. H_2O" (2-3 changes usually).

7. Immerse in 10% $AgNO_3$, 10-40 sec.
8. Rinse thoroughly in dist. H_2O.
9. Treat in 5% $Na_2S_2O_3$, 3-5 min. (optional).
10. Rinse in H_2O.
11. Dehydrate in graded alcohol, clear in xylene, and mount in Permount.

Remarks:

1. Rinsing in H_2O after step 3 is important. It will remove all the excess Na_2SO_4. Failure to do this will cause the sections to be full of crystals.
2. Before and after the $AgNO_3$ stain, sections should be rinsed in distilled H_2O to prevent the formation of AgCl ppt. Too long an exposure to $AgNO_3$ will make MEP's black and make their morphology unclear.
3. Treatment with $Na_2S_2O_3$ was optional since its function was only fixation for Ag^+.

(IV) DEMONSTRATION OF NERVE FIBERS

A. PARAFFIN METHOD (9μm)

This method was adopted from Hsu ('71). Since she did not publish the method, her procedures are presented below with her permission. This method is a modification of Palmgren's nerve stain ('60).

1. Deparaffinize in xylene.
2. Hydrate through absolute, 95%, 70% alcohol into distilled water.
3. Impregnate in 10% silver nitrate solution for half an hour or for 5 hours in 10% silver nitrate solution mixed with 1% pyridine. Impregnate at 37° C.
4. Pretreat in solvent solution (2% borax solution), (2 gm/100 ml H_2O).
5. Rinse immediately in distilled water to remove all white precipitation resulting from step 4.
6. Develop in solution of
   0.05 g hydroquinone
   5 g sodium sulfite
   2 g sodium borate
   100 ml distilled water
   for 10 min at 37° C.
7. Rinse through three changes of 50% alcohol for 5 min.
8. Tone with 0.5% gold chloride solution until yellowish brown tinge in tissue turns to cream color (approximately 2 min.).
9. Rinse briefly in water.

10. Intensify in 0.5% oxalic acid in 50% alcohol at 37° C for 2-2½ min. Tissue turns deep reddish black.
11. Rinse in water.
12. Fix in 5% aqueous sodium thiosulfate for 10 sec.
13. Rinse in water.
14. Dehydrate and bring to xylene.
15. Cover.

B. FROZEN THICK SECTIONS (ca 50μm)

This method was a combination of Namba's ('67) nerve stain and Gomori's ('52) AChE stain. The purpose was to demonstrate nerve fibers and MEP's simultaneously. It needs thick frozen sections and requires a long time for staining. Thus, it was not used for routine preparation of nerve fiber demonstrations.

1. After staining in $(NH_4)_2S$ with Gomori's method, the sections are washed thoroughly with H_2O and then immersed in potassium ferricyanide (0.25 gm in 100 ml H_2O) for 10 min. at room temperature.
2. Rinse twice with distilled H_2O, 5 min. each.
3. Place in 100% alcohol for 1 hr.
4. Rinse in H_2O (distilled) for 1 min.

5. Place the sections in $AgNO_3$ solution for 90 min. at 37° C (100.00 gm $AgNO_3$, 0.05 gm $CuSO_4.5H_2O$ and 0.1 gm $CaCO_3$ in 100 ml distilled H_2O).
6. Rinse with distilled H_2O for 5 sec.
7. Immerse the sections for 10 min. in the reducing solution (1.00 gm of hydroquinone, 10.00 gm of Na_2SO_3 and 100 ml of distilled H_2O), with gentle shaking during the first minute.
8. Wash in distilled H_2O for 5 min.
9. Dehydrate in alcohol, clear in xylene, and mount in Permount.

APPENDIX III

DESCRIPTION OF DEGENERATING MYOTUBES AND MUSCLE FIBERS

Degenerating Myotubes

1. Nuclei are dark and shrunken and are stacked up like the profiles of RBC's in the capillary (see Fig. 12).
2. Empty spaces appear in the immediate surroundings of nuclei.
3. Some degenerating myotubes do not have the characteristic profiles of RBC's in the capillary but their nuclei are dark and shrunken.
4. Their cytoplasm is more deeply stained than that of normal myotubes.

Degenerating Muscle Fibers

1. Sarcoplasm is deeply stained and usually swollen. Occasionally, typical retraction caps and clots (Platzer and Powell, '75) can be seen.
2. Nuclei are dark and shrunken (pyknotic) and can be either in the central or peripheral position.
3. In older regenerates, the diameter of the muscle fibers is small with the cytoplasm lightly stained and fragmentary. Cross-striations are blurred and cannot always be seen. Nuclei, relative to the reduced fiber size, seem to be large and are scattered at the periphery or in the center.

APPENDIX IV

METHODS OF STATISTICAL ANALYSIS

Since the percentage of muscle fibers with non-peripheral nuclei (central or intermediate position) is the reciprocal of that of peripheral nuclei and since the ratio (out of 50) of degenerating muscle fibers is the reciprocal of that of normal fibers, only the data on peripheral nuclei and normal fibers are presented (Table 6 and Table 7).

The parameters analyzed are as follows:

(1). Number of muscle fibers with peripheral nuclei per 100 fibers counted (P%).

(2). Number of normal muscle fibers per 50 fibers counted (NM/50).

(3). Number of normal myotubes and degenerating myotubes per 50 fibers counted (N.Mt/50 and D.Mt/50 respectively).

The analysis of parameters (1) and (2) involves three factors, namely TREATMENT (normal, denervated), AGE (10, 20, 30, 40 days), and LOCATION (proximal, middle, distal) and each factor has more than one case (TREATMENT=2, AGE=4, LOCATION=3). Thus, a simple t-test cannot be used. After consulting statisticians, a special method was employed to solve this problem. This method, called "Three-Way Analysis of Variance", is described in STATISTICAL PRINCIPLES IN EXPERIMENTAL DESIGN (Winer, 1971, p. 559).

The model of "Three-Way Analysis of Variance" is

$$X_{ijkm} = u + A_i = B_j + AB_{ij} + \pi_{m(ij)} + C_k + AC_{ik} + BC_{jk} + ABC_{ijk} + C_{km(ij)} + \varepsilon o(ijkm).$$

where u = grand average of all possible X_{ijkm}

A = TREATMENT

B = AGE

C = LOCATION

AB = Interaction between TREATMENT and AGE

AC = Interaction between TREATMENT and LOCATION

BC = Interaction between AGE and LOCATION

ABC = Interaction between TREATMENT, AGE, and LOCATION

π = Error between (animals)

εo = Error within (animal)

i = cases of TREATMENT, here i = 1 (normal), 2 (denervated).

j = cases of AGE, here j = 1 (10 days), 2 (20 days), 3 (30 days), 4 (40 days).

k = cases of LOCATION, here k = 1 (proximal), 2 (middle), 3 (distal).

m = number of animals observed for a specific TREATMENT at a specific AGE, here m = 1 (first), 2 (second).

The computations were carried out using MIDAS program of the computer system at the University of Michigan.

The results of the computations are presented in Table 8 for P% and Table 9 for NM/50, in which

SS represents Sum Square

df represents degree of freedom

MS represents Mean Square ($MS = \frac{SS}{df}$)

F represents F-test

($F = \frac{MS}{Error_{(between)}}$, or $F = \frac{MS}{Error_{(within)}}$)

The specific difference between TREATMENT and AGE (AB; the difference was significant as was shown in Table 8 and Table 9) was further carried out through "Multiple Comparisons" method. The computation formula is

$$F = \frac{A_1B_1 - A_2B_1}{2 \cdot mk \cdot MS_{error(between)}}$$

where $A_1B_.$, A_2B_1.....A_2B_4 are the subtotal of P% or NM/50 of a specific AGE and TREATMENT (see Table 6 and Table 7).

k = cases of LOCATION, here k = 3

m = number of animals used for each TREATMENT at each AGE, here m = 2.

Finally, a simple t-test was used for parameter (3), since myotubes could not be seen beyond 20 days (i.e., comparison between 10-day-old regenerates only) and since the proximal parts of the regenerates was chosen for comparison only.