

Chronic Denervation of Rat Hemidiaphragm: Maintenance of Fiber Heterogeneity with Associated Increasing Uniformity of Myosin Isoforms

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ABSTRACT During several months of denervation, rat mixed muscles lose slow myosin, though with variability among animals. Immunocytochemical studies showed that all the denervated fibers of the hemidiaphragm reacted with anti-fast myosin, while many reacted with anti-slow myosin as well. This has left open the question as to whether multiple forms of myosin co-exist within individual fibers or a unique, possibly embryonic, myosin is present, which shares epitopes with fast and slow myosins. Furthermore, one can ask if the reappearance of embryonic myosin in chronically denervated muscle is related both to its re-expression in the pre-existing fibers and to cell regeneration. To answer these questions we studied the myosin heavy chains from individual fibers of the denervated hemidiaphragm by SDS PAGE and morphologically searched for regenerative events in the long term denervated muscle. 3 mo after denervation the severely atrophic fibers of the hemidiaphragm showed either fast or a mixture of fast and slow myosin heavy chains. Structural analysis of proteins sequentially extracted from muscle cryostat sections showed that slow myosin was still present 16 mo after denervation, in spite of the loss of the selective distribution of fast and slow features. Therefore muscle fibers can express adult fast myosin not only when denervated during their differentiation but also after the slow program has been expressed for a long time. Light and electron microscopy showed that the long-term denervated muscle maintained a steady-state atrophy for the rat's life span. Some of the morphological features indicate that aneural regeneration events continuously occur and significantly contribute to the increasing uniformity of the myosin gene expression in long-term denervated diaphragm.

Adult fast and slow skeletal muscles are composed of a large number of fibers with different physiological and biochemical properties that can respond in a plastic manner to a variety of humoral and functional stimuli under neuronal control (26). Differences among fiber types are mainly quantitative in nature, but some are qualitative, implicating a switch in the control of gene expression in single fibers. In skeletal muscles most myofibrillar proteins exist in polymorphic form. These isoforms are used as markers of the transitions occurring during embryonic, neonatal, and the adult stages of development (7) and of the heterogeneity and plasticity of the muscle fibers and motor units in adult animals (37). Though

muscle cells synthesize muscle-specific contractile proteins in the absence of motoneurons, the neuron controls (that is, induces and maintains) the particular set of isozymes synthesized after innervation. However, it is still unknown whether the neuron exerts a differentiative effect by means of chemotrophic mechanisms, or a permissive influence through the differential pattern of electrical and or mechanical activity it induces on muscle fibers. Denervation during postnatal muscle development clearly shows that the synthesis of adult slow myosin is dependent on the actual activity of motor innervation, while adult fast myosin is expressed in the denervated fibers even though the neuronal influences are removed (4,

25, 34). When the fully differentiated pattern of fast and slow isomyosins characteristic of normal adult skeletal muscles is established, acute denervation has very little influence on the type of contractile proteins synthesized in atrophying muscle fibers. Preferential atrophy of fast fibers followed by atrophy of slow fibers appears to be the typical feature of the early phases of denervation, producing only a small imbalance in fiber typing (reviewed in reference 14). However during 6 mo of chronic denervation there is a transformation of the rat mixed muscles into almost pure fast muscles (9–11, 14). It was recently reported (18) that in the hemidiaphragm 8 wk after removal of the nerve supply all the fibers reacted with antibodies specific for fast myosin, and many reacted with anti-slow myosin as well. In the normal rat diaphragm, the component fibers reacted with either antibodies against fast or slow myosin, but usually not with both. The findings were interpreted to indicate that either multiple forms of myosin co-exist within individual muscle fibers or that unique possibly embryonic myosin is present, which has determinants in common with both fast and slow isozymes (18). This latter hypothesis merits further study on the basis of the well known proliferation and accumulation of satellite cells in denervated muscles (2, 20) and of our recent results showing differentiation of rat muscle fibers regenerating in absence of the nerve (13). We further validated SDS PAGE as a tool that distinguishes the myosin heavy chains (MHC)¹ present in developing and adult fibers; then we determined the type and the number of myosin heavy chains in chronically denervated fibers of the adult rat diaphragm. Our work showed that after nerve supply removal, the muscle fibers re-expressed fast myosin even after the fully differentiated state of the slow fiber had been expressed for months. Further the light and electron microscope showed that regeneration events continuously occurred and significantly contributed both to the heterogeneity of the fiber shape and to the increasing uniformity of the myosin gene expression in long-term denervated diaphragm.

MATERIALS AND METHODS

Animals, Muscles, and Surgical Procedures: Male Wistar rats weighing 150 to 200 g were anesthetized with thiopental and ether. The left hemidiaphragm was denervated by cutting the phrenic nerve using an intercostal approach. A 5-mm segment of the phrenic nerve was removed to prevent reinnervation. At stated postoperative times, the denervated animals were examined to ensure that the proximal phrenic nerve had not re-established contact with the diaphragm. Effectiveness of the surgical procedure was verified by the thinness of the muscle relative to the intact diaphragm. The denervated muscles were also checked by differential stimulation at low voltage (2–5 V, a voltage that triggers muscle contraction through the intramuscular nerve fibers) or high voltage (30–50 V, to directly induce the contraction of the denervated fibers). The denervated and the contralateral hemidiaphragms were processed separately or together as described in the following sections. Extensor digitorum longus (EDL) and soleus muscles of the phrenectomized rats were also used in some instances. EDL, soleus, and tibialis anterior (TA) muscles of normal adult rats, leg muscles of 7-d-old rats, bulk skeletal muscles of 18-d embryos, and muscle cell cultures were used to purify myosin markers.

Cell Cultures: Muscle cell cultures were obtained from leg muscles of neonatal rats by trypsin treatment. The following were used: (a) fibroblast cultures, obtained by at least three passages of plastic-adherent cells; (b) muscle

cultures, obtained by trypsin dissociation from newborn rat leg muscles and grown *in vitro* up to 11 d. All cultures were settled in plastic Falcon bottles and grown in Dulbecco's modified Eagle's medium with 10% horse serum and 2% chick embryo extract at 37°C under a 5% CO₂ atmosphere (6).

Myosin Purification: Myosin was isolated and purified as described previously (10).

Isolation of Single Muscle Fibers: Bundles of fibers were isolated from denervated diaphragm, tied to a wooden stick, and stretched to 110–120% of slack length. Specimens were chemically skinned in 5 mM EGTA, 170 mM K-Propionate, 2.5 mM ATP, 2.5 mM Mg-Propionate, 10 mM Imidazole, pH 7.0, as already reported (38). The fibers were stored at –20°C in the above solution to which 50% glycerol had been added. Single fiber segments were isolated under the dissection microscope. For SDS PAGE of myosin, single fibers were solubilized in 2.3% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, and incubated 2 min at 90°C or overnight at room temperature.

One Dimensional SDS PAGE of Myosin Light Chains (MLC): MLC were analyzed on slabs 6–22.5% polyacrylamide, 0.5 mm thick (27). Gels were run at a constant voltage at 50 V for the first hour, 100 V for the second hour, and then at 150 V until the bromophenol blue dye front reached the bottom of the slab. The gels were stained overnight in 0.25% Coomassie Brilliant Blue in 45% methanol, 9% acetic acid and destained by diffusion first in 40% methanol then in 5% methanol, 7.5% acetic acid.

SDS PAGE of Myosin Heavy Chains (MHC)

ONE DIMENSIONAL SDS PAGE OF MHC: MHC were analyzed on 5 or 6% polyacrylamide slabs as described in Carraro and Catani (8).

ORTHOGONAL PEPTIDE MAPPING IN SDS PAGE OF MHC: The orthogonal peptide mapping of electrophoretically purified MHC was performed under steady-state proteolysis (8). MHC were separated from ~2.5 µg of myosin on 4% polyacrylamide 80 × 2.5-mm cylindrical gels (27). The gels were briefly stained with Coomassie Blue. After rapid destaining the MHC bands were cut out and equilibrated in 125 mM Tris HCl (pH 6.8), 1 mM EDTA, and 1 mM 2-mercaptoethanol (15). The gels were loaded onto a 7.5% acrylamide slab (0.75 × 120 × 140 mm); the stacking gel was 1 cm high. A 1% agarose gel in equilibration buffer was used to keep the portions of cylindrical gels in place. After the bromophenol tracking dye had migrated 5 mm into the stacking gel, the segments of cylindrical gels were taken off and a 2.5-mm diam cylindrical gel containing 10 µg/ml of *Staphylococcus aureus* V8 protease was layered over the slab. The slabs were run at 50 V, constant voltage, for 2 h and then at 20 mA, constant current, 200 V maximum, until the dye front reached the bottom of the slab. The running time was ~6 h. The slabs were stained with the silver method (30).

TWO DIMENSIONAL SDS PAGE OF PARVALBUMIN AND CONTRACTILE PROTEINS SEQUENTIALLY EXTRACTED FROM MUSCLE CRYOSTAT SECTIONS: The EDL, the soleus, the contralateral innervated and the denervated hemidiaphragms of 18-mo phrenectomized rats were tied to a plastic support at resting length, quenched in liquid nitrogen, and transferred to a cryostat at –19°C. Serial sections were cut and 10 sections (20-µm thick) were collected in a conical test tube. 200 µl of a 4-mM EDTA solution (pH 7.0) containing 1 mM pepstatin and 0.4 mM phenylmethylsulphonyl fluoride as protease inhibitors, were added. The suspension was centrifuged at low speed (600 g). The pellet (pellet A) was used to analyze the contractile proteins. The supernatant (supernatant A) was heated for 30 min at 85°C and then centrifuged at low speed. The pellet (pellet B) was either discarded or used to measure the amount of heat-labile sarcoplasmic proteins present in the cryostat sections. 30 µl of the supernatant (supernatant B) were processed for the two dimensional gel electrophoretic analysis of parvalbumin. Pellet A was washed two times with 2 ml of 50 mM KCl, 1 mM pepstatin, 0.4 mM phenylmethylsulphonyl fluoride and solubilized to analyze actin and MLCs by two dimensional gel electrophoresis, as described by Carraro et al. (13). The only modifications were the use of 13% polyacrylamide 80 × 80 × 0.75 mm gel slabs on the second dimension and the use of Coomassie Brilliant Blue R250 to stain the proteins. Estimation of the number of fibers per hemidiaphragm was performed on cross sections by light microscopy (× 40). In both control and denervated hemidiaphragm, the absolute number of muscle fibers was counted in one fourth of the total squares of a superimposed grid (each square was 0.06 mm²). A minimum of 2,500 fibers was counted. At higher magnification (× 100), clusters of myofibers were identified and counted by randomly scanning 0.01 mm² of each square.

Histochemistry: Transverse cryostat sections (10 µm) serial to those used for parvalbumin and contractile protein sequential extraction were used to localize ATPase activity (38).

Microscopy: Whole diaphragms were removed together with the ribs to which they were inserted. After blotting dry, the muscle was tied to a plastic

¹ Abbreviations used in this paper: EDL, extensor digitorum longus; LC1F, LC2F, and LC3F, myosin light chains of adult fast muscles; LC1S and LC2S, myosin light chains of adult slow muscles; MHC, myosin heavy chains; MHC-F, myosin heavy chain of adult fast muscles; MHC-S, myosin heavy chain of adult slow muscles; MLC, myosin light chains.

support at approximately resting length, fixed with 2.5% glutaraldehyde, 0.1 M Na cacodylate, pH 7.2, and left overnight before being washed in cacodylate buffer. Strips from the denervated and the contralateral innervated hemidiaphragms were postfixed with 1% osmic acid in the same buffer. After dehydration through alcohol, the specimens were block-stained with uranyl acetate in absolute alcohol overnight, and then embedded in Epon mixture. Semithin sections (0.5–1.0 μm) were stained with Toluidine blue. Thin sections stained with uranyl acetate and/or lead citrate were examined by a Philips EM 301 electron microscope.

RESULTS

Effects of 3 mo of Denervation

MYOSIN LIGHT CHAINS: SDS PAGE of myosin prepared from normal hemidiaphragm (Fig. 1, lane 8) showed a five-band pattern consisting of light chains characteristic of both slow and fast rat muscles (12). After three months of denervation the proportion of myosin found in the slow bands decreased relative to the amount present in normal muscle (Fig. 1, lane 7). If present, the embryonic light chain (clearly distinct from *LC1F* in the embryonic myosin sample; Fig. 1, lane 4) does not represent a distinct band discernible from the background material in the 3-mo denervated myosin. At this time *LC3F* seems to be present in the same amount as in normal muscle. These results agree with our previous data showing that only after 6 mo of denervation the hemidiaphragm appeared as an almost pure fast muscle (9–11, 14).

MYOSIN HEAVY CHAINS: SDS PAGE is now an accepted technique that separates most of the isoforms of the MHC of the skeletal muscles in chicken (3, 36), rat (8), and

man (results not shown). When ~200 ng of rat diaphragm myosin were electrophorized in 5% SDS PAGE, two bands appeared after staining with the silver method (Fig. 2a, lane 3). The predominant band (*MHC-F*) co-migrated (Fig. 2a, lane 11) with the unique band of the EDL, an essentially pure fast muscle (Fig. 2a, lane 1). The smaller band co-migrated (Fig. 2a, lane 12) with the predominant band (*MHC-S*) of the soleus, a muscle in which slow fibers predominate (Fig.

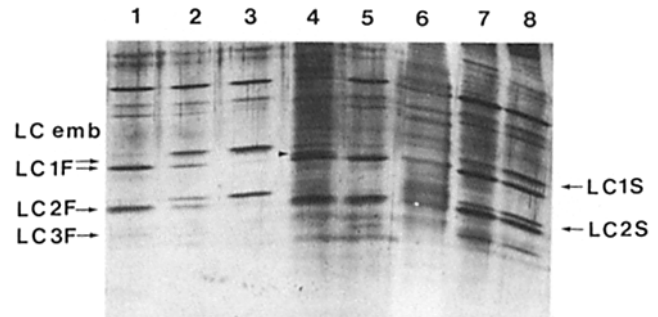


FIGURE 1 SDS PAGE of rat myosin light chains. Pattern of myosin light chains from (1) normal adult EDL; (2) 1 + 3 mixture; (3) normal adult soleus; (4) skeletal muscles of 18-d embryos; (5) leg muscles of 7-d-old rats; (6) myotubes of 11-d-cultured muscle cells; (7) 3-mo-denervated hemidiaphragm; (8) normal adult diaphragm. In the long-term denervated hemidiaphragm slow myosin light chains are reduced in comparison with the normal diaphragm. The embryonic light chain (*LCemb*), if present, does not represent a discernible individual band distinct from the background material.

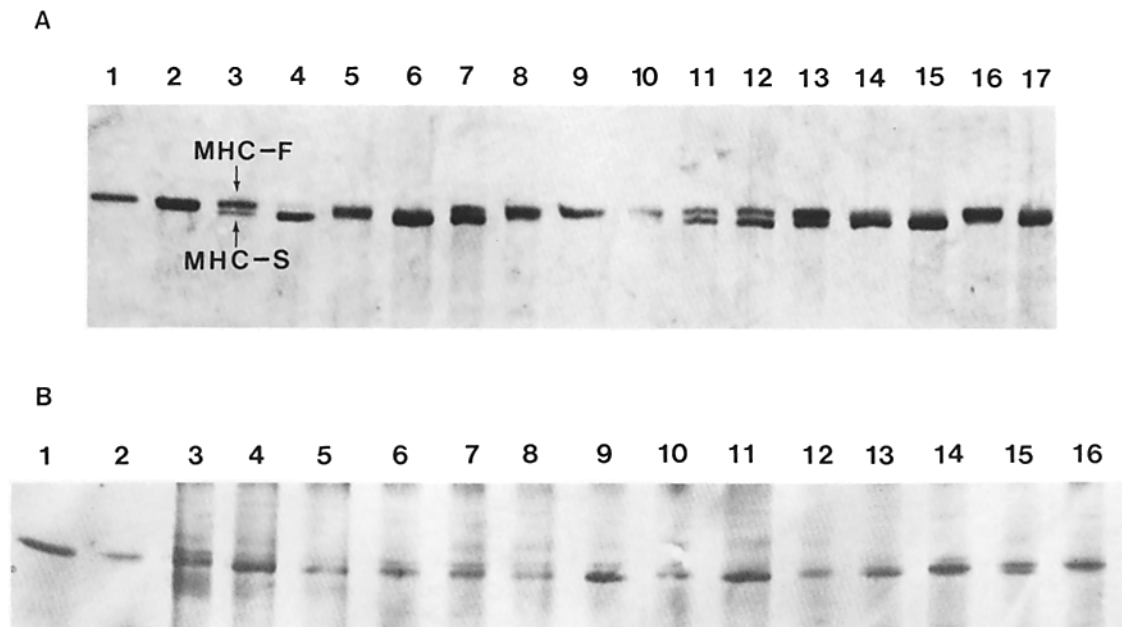


FIGURE 2 SDS PAGE of myosin heavy chains of rat muscles. (A) Heavy chains of myosin from (1) EDL; (2) tibialis anterior; (3) diaphragm; (4) soleus; (5) leg muscles of 7-d-old rats; (6) 18-d-embryos; (7) 1 + 6 mixture; (8) 1 + 5 mixture; (9) 1 + 2 mixture; (10) 1 + 3 mixture; (11) 1 + 4 mixture; (12) 4 + 3 mixture; (13) 4 + 2 mixture; (14) 4 + 5 mixture; (15) 4 + 6 mixture; (16) 2 + 3 mixture; (17) 5 + 6 mixture. About 100 ng of protein per lane. *MHC-F*, myosin heavy chain of adult fast muscle; *MHC-S*, myosin heavy chain of adult slow muscle. The electrophoretic analysis in 5% gel slabs shows two bands in the normal adult diaphragm. Co-electrophoretic analyses confirm the separation of two main bands in developing and adult skeletal rat muscles. However, slight differences in the mobility or microheterogeneity of the bands are suggested by these analyses. The myosin heavy chain of the neonatal muscle appears to have an electrophoretic mobility intermediate between the *MHC-F* and *MHC-S* of the soleus myosin. The embryonic myosin, which co-migrates with *MHC-S*, shows a broad tail. (B) Heavy chains of myosin from (1) bulk skeletal muscles of 18-d-old embryos; (2) leg muscles of 7-d-old rats; (3) fibroblasts subcultured from muscle cell cultures; (4) myotubes from 5-d-culture; (5) myotubes from 7-d-culture; (6) myotubes from 11-d-culture; (7) 3 + 6 mixture; (8) 3 + 4 mixture; (9) 3 + 1 mixture; (10) 3 + 2 mixture; (11) 4 + 1 mixture; (12) 4 + 2 mixture; (13) 6 + 1 mixture; (14) 6 + 2 mixture; (15) 4 + adult EDL mixture; (16) 6 + adult EDL mixture. Different MHC isoforms are expressed during maturation of myotubes in cell culture.

2a, lane 4). The relative proportion of MHC-F and MHC-S correlates well with the known fiber type composition of adult rat muscles (8). Myosin from bulk skeletal muscles of 18-d rat embryos (Fig. 2a, lane 6) displayed a band that comigrated with MHC-S (Fig. 2a, lane 15). The myosin from leg muscles of 7-d-old rats displayed a single band that comigrated with MHC-F (Fig. 2a, lanes 5 and 8). Using this method we cannot clearly separate the MHC of the developing muscles from those present in adult muscles. However a careful examination of the electrophoretograms showed that MHC of 7-d-old rats had an electrophoretic mobility intermediate between MHC-F and MHC-S, and that the band from embryonic muscles was heterogeneous with respect to its content of MHC. Indeed, co-electrophoresis of neonatal MHC with both EDL (Fig. 2a, lane 8) and embryonic (Fig. 2a, lane 17) myosins showed broad single bands, even though the co-electrophoresis of EDL and embryonic myosin (Fig. 2a, lane 7) revealed two distinct bands. Two bands were also recognized in the myosin of myotubes prepared from embryo muscles cultured in vitro for 5 or 11 d (Fig. 2b). The faster migrating band had a slightly different mobility in the two preparations. Compare lanes 15 and 16 in Fig. 2b, which are the co-electrophoresis of the culture myosins with MHC-F. Two bands are discernible in the coelectrophoresis of MHC-F with the 5-d-culture myosin. The 11-d-culture MHC band runs unresolved ahead of the marker. Co-electrophoresis of the MHC present in 5- and 11-d-culture myosins with either embryonic or neonatal MHC showed different patterns. One may infer that embryonic MHC is present in 5-d-culture myosin (Fig. 2b, lane 11). 1 wk later, when a greater proportion of large myofibers is present in the culture, neonatal MHC predominates (Fig. 2b, lane 13). The slower migrating band of the 11-d-culture myosin is clearly distinct from all the markers we have used (MHC-F included). Its muscular or nonmuscular nature remains to be determined.

SDS PAGE of MHC from Single Fibers of 3-mo Denervated Hemidiaphragm

Single fibers were carefully dissected from a 3-mo-denervated hemidiaphragm. Due to the size of the severely atrophic myofibers and the increased proportion of fibrous tissue, only portions of single fibers could be dissected. The myosin content of these fiber segments was insufficient to obtain reliable myosin light chain analyses even after staining of the gel slabs with the silver technique (results not shown). The myosin heavy chain content was just enough to allow analysis of myosin isoforms in severely atrophic fibers. Fig. 3 shows that either a single band or two bands were present in the single fibers of the 3-mo denervated hemidiaphragm. The slower moving band had the electrophoretic mobility of the MHC-F, the faster migrating band could be either the heavy chain

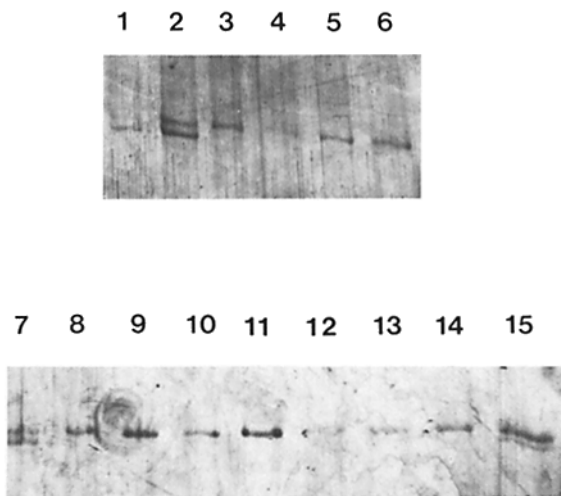


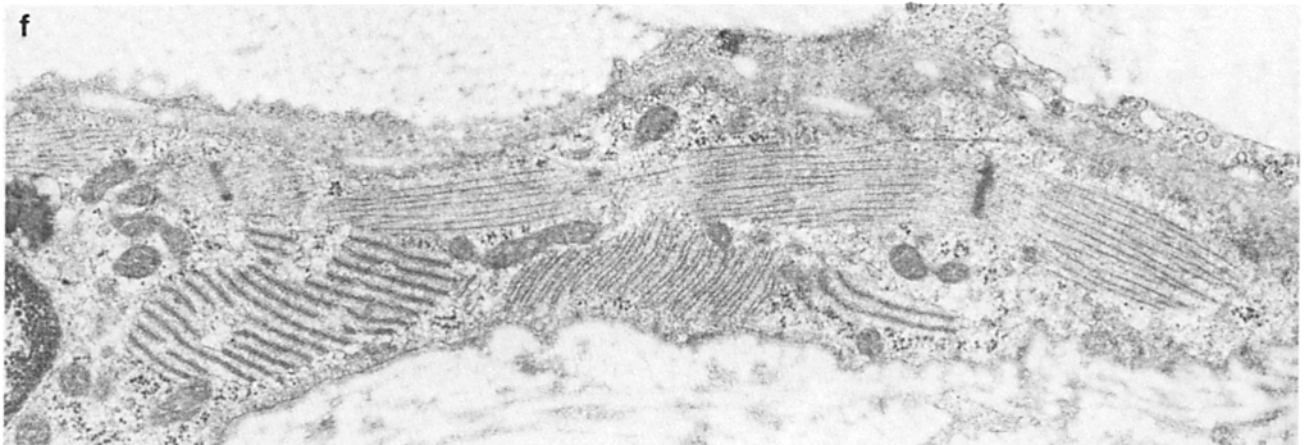
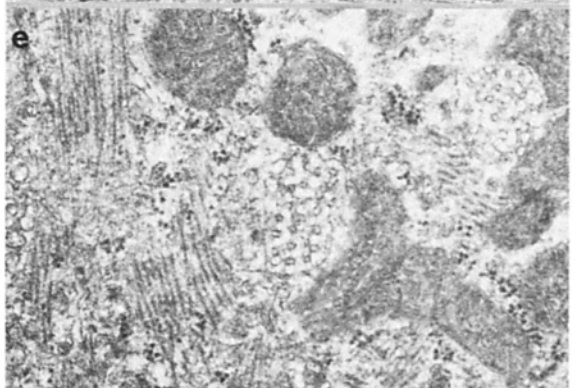
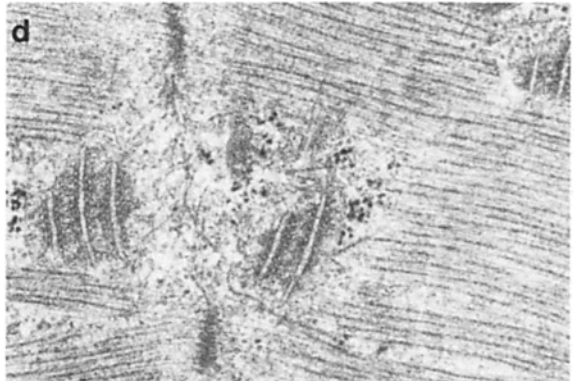
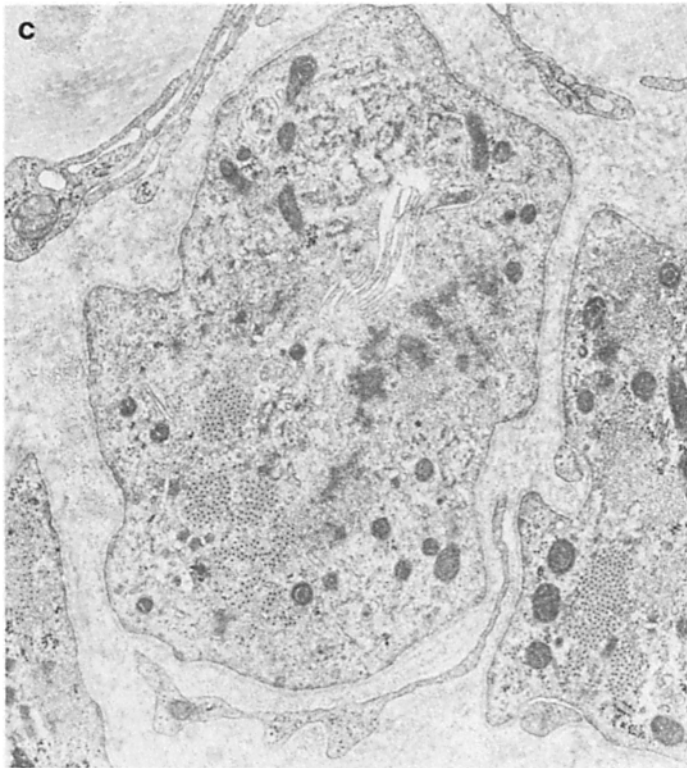
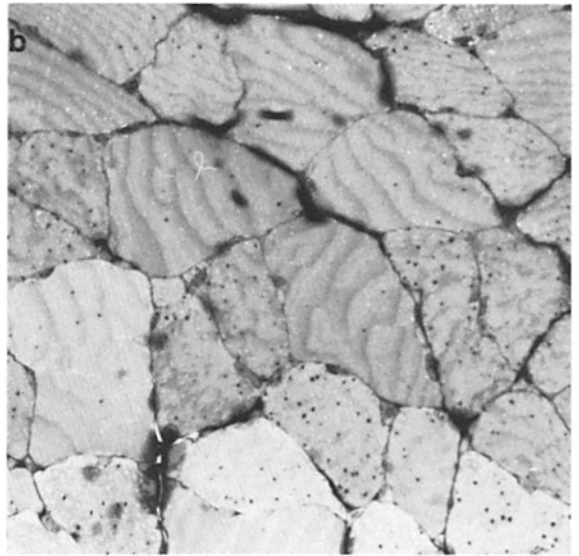
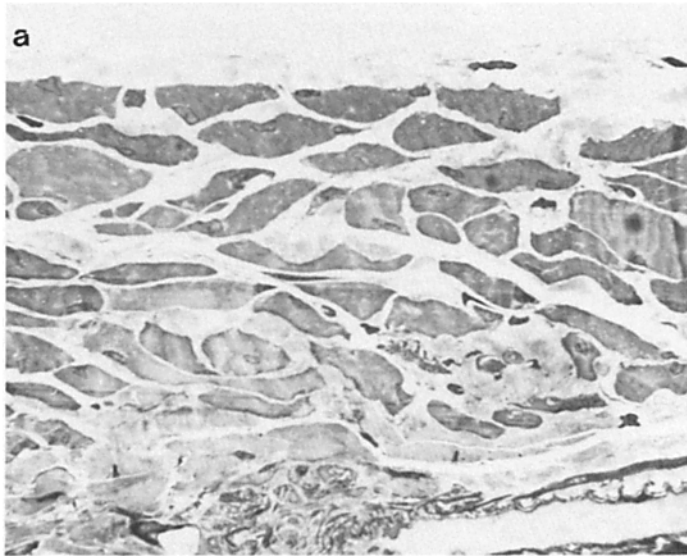
FIGURE 3 SDS PAGE of myosin heavy chains of single fibers from 3-mo-denervated hemidiaphragm. Myosin from single fibers of the denervated hemidiaphragm shows either one band (MHC-F) or different proportion of two bands (the MHC-F band and a faster migrating band, MHC-S).

of the slow myosin or that of the embryonic myosin. Taking into account the amount of slow light chains in the myosin extracted from a pool of fibers of the 3-mo-denervated hemidiaphragm (see Fig. 1) and the presence of MHC-S fragments in the peptide maps of pooled long-term denervated hemidiaphragm (10; and see below the 16-mo denervated hemidiaphragm), we interpreted the leading band as MHC-S. The two MHC bands, when both present in the denervated fibers, were found in different proportions. Compare lanes 1, 3, and 15 in Fig. 3. In lane 1 only trace amounts of MHC-F co-exist with MHC-S. In lane 3 the MHC-S predominates while in lane 15 only a trace amount of MHC-S is present. The absolute amount of MHC varies among the lanes, probably depending on the length and transverse section of the fiber portions. We look at these results as proof of the synthesis of both fast and slow myosin in the pre-existing slow fibers of the denervated hemidiaphragm.

Effects of 1 y of Denervation: Morphological Results

In the phrenectomized hemidiaphragm the expected denervation atrophy is preceded by a prominent denervation hypertrophy that may last for as long as 1 mo. This seemingly unusual manifestation of the loss of innervation is commonly attributed to the chronic, periodic stretching of the denervated fibers by contraction of the still functional, contralateral hemidiaphragm, causing a delay in the onset of the atrophy process (17). Then, though severely affected, the denervated hemidiaphragm reaches a steady-state atrophy that may last for the

FIGURE 4 12-mo-denervated hemidiaphragm. Light micrographs of semi-thin sections of denervated (a) and contralateral innervated (b) hemidiaphragms, stained with toluidine blue. The muscle fibers present in the denervated hemidiaphragm are severely atrophic but still maintain differences in shape and size. They are surrounded by thick connective tissue layers. (c) A typical example of an extremely atrophic fiber containing a limited amount of contractile filaments, few elements of sarcoplasmic reticulum, and anomalous multilayered membranes. Very frequently proliferation of terminal cisternae and T-tubules (d) or of the T-system in form of honey-comb structures (e) are observed. Numerous lamellar structures deriving from the sarcoplasmic reticulum elements are present in the severely atrophic fiber shown in f ($\times 26,400$). Note the disarrangement of myofilaments (d and e). (f) A lipofuscin body is present in the perinuclear region of the fiber. $\times 440$ (a and b); $\times 18,700$ (c); $\times 34,500$ (d); $\times 33,000$ (e).



rat's life span. The atrophy and the degenerative processes peculiar to the early phases of denervation (32, 33), co-exist in the long-term denervated hemidiaphragm with unexpected morphological features (see below).

The 1-y denervated muscle was found to be representative of the complex reactions of the chronically denervated hemidiaphragm. After a year it appeared extremely thin and diaphanous. Nonetheless light microscopy revealed the presence of a large population of severely atrophic muscle fibers, still differing in shape and size (compare the fibers in Fig. 4a with those of the contralateral innervated hemidiaphragm shown in Fig. 4b). The interfiber spaces were enlarged and filled with thick bundles of collagen fibrils. Electron microscopy showed that the most atrophic fibers contained a very limited amount of contractile filaments (Fig. 4c). Proliferation of terminal cisternae, formation of honey-comb structures of the T-tubules, and lamellar bodies probably derived from elements of the sarcoplasmic reticulum were commonly observed in the most atrophic fibers of the 1-y-denervated hemidiaphragm (Figs. 4, c-f). These aspects have already been reported in denervated muscles (21, 39).

Though extremely thin it was not homogeneous, and showed focal areas of relatively less severe atrophy. The analysis of one such area is shown in Fig. 5. The muscle tissue consists of variably sized rounded fibers grouped together by thick connective tissue layers. Frequently the myonuclei are centrally located and display prominent nucleoli. In spite of their healthy appearance target-like areas are observed in fibers (Fig. 5, b-e), some already present invading macrophages (Fig. 5, b and c). These degenerative events and their evolution are known to occur within a few weeks after denervation (29, 33). The meaning of these unexpected and apparently incoherent observations become clear when considering the possibility that muscle fiber regeneration occurred in the denervated hemidiaphragm. In Fig. 6a an enlarged area of Fig. 5a is shown, in which several myofibers of different size and shape are clustered together without any interposed connective tissue. All these fibers show characteristics of immaturity. Numerous satellite cells, some in twin-localization, are present around the fiber profile (Fig. 6b). Satellite myofibers, tightly connected to each other by means of focal membrane specialization (Fig. 6, c and e), are clustered inside the same basal lamina. A new basal lamina begins to appear in sites where the two cell membranes are less strictly contiguous (Fig. 6, c and d). The myofibrils are poorly delimited by sarcoplasmic reticulum. Several vesicles, filled with cisternal material are seen in diadic coupling with the sarcolemmal membrane. We noted that in the larger, more differentiated fiber honey-comb T-tubule proliferations were present (Fig. 6, c and d). The presence of denervation characteristics in these regenerated fibers of the 1-y phrenectomized hemidiaphragm is very significant. We recently observed that such T-tubule proliferations appeared during the second week after injury in muscle regenerating in absence of the nerve (results not shown).

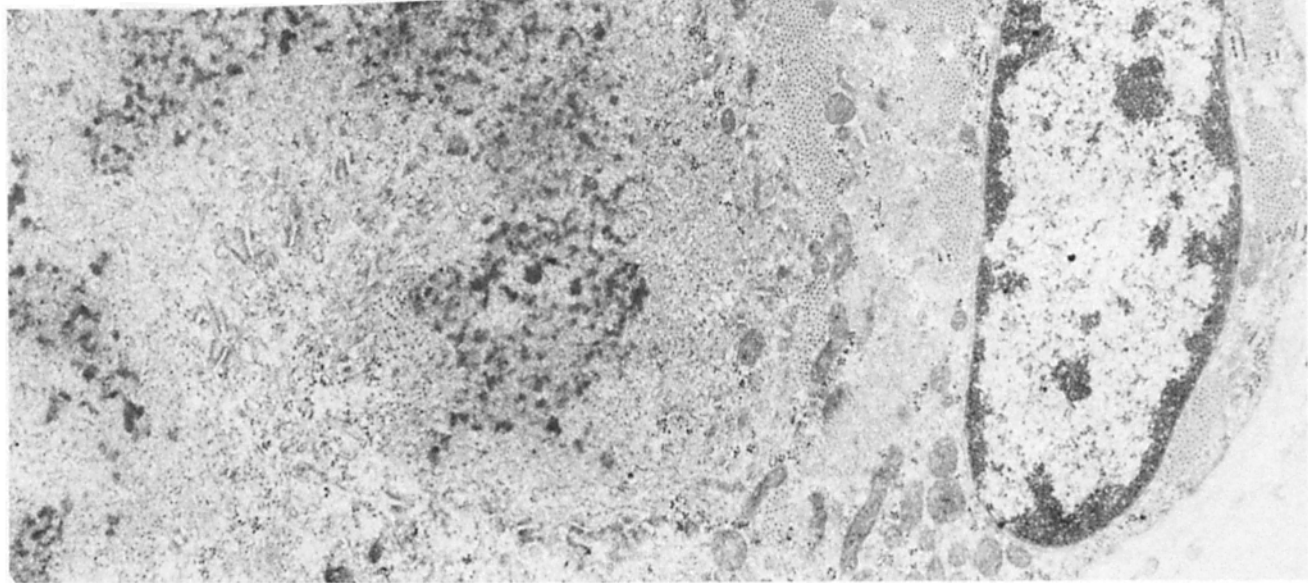
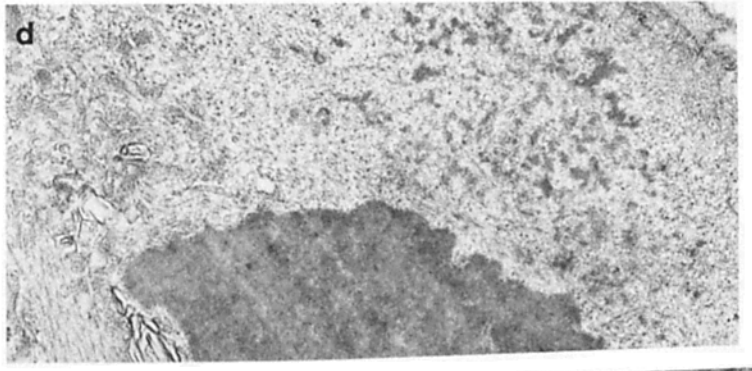
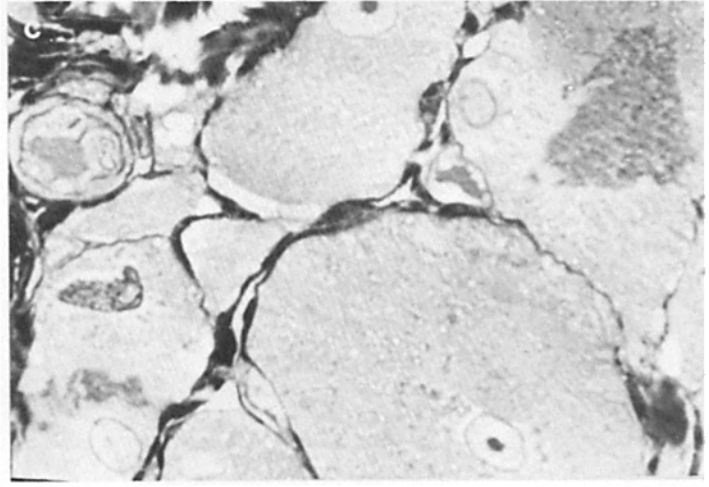
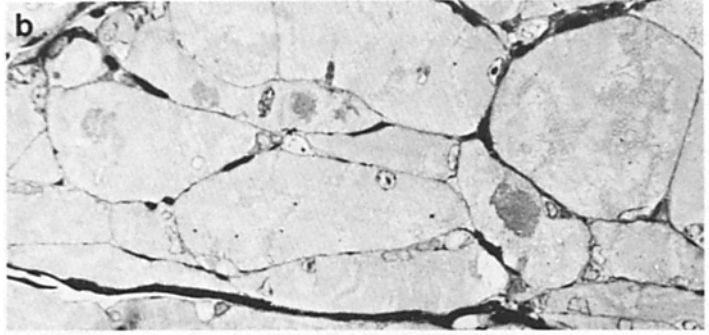
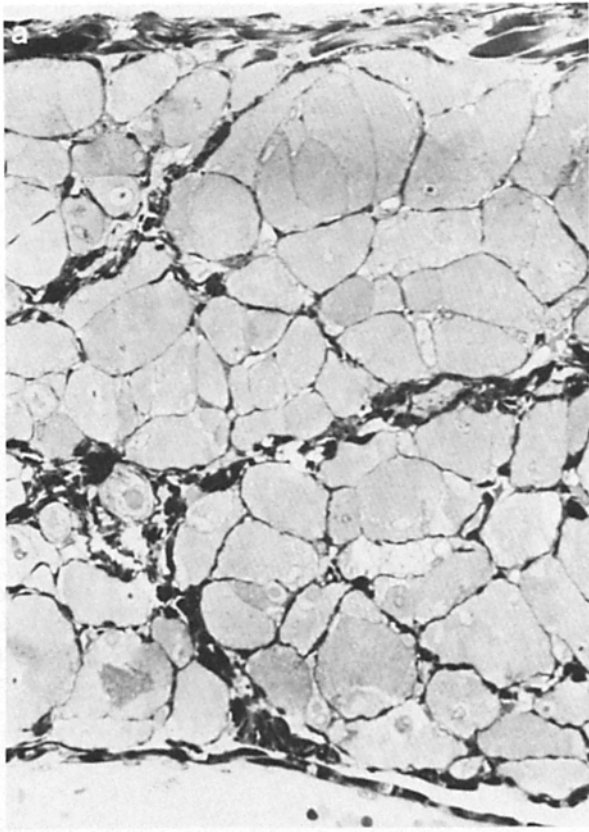
In studying long-term effects of denervation one must be aware of the tendency of the nerve to regenerate and reinnervate its proper target. Reinnervation can occur in phrenectomized hemidiaphragms. A partial or total reinnervation that restores the normal size of the hemidiaphragm fibers, is easily recognized by inspection and confirmed by electrophysiological tests and histochemistry (10). Carefully searching for regenerated nerves among atrophic fibers, we found only a few myelinated axons. Fig. 7a shows a small nerve containing two myelinated and several unmyelinated axons. The only neuro-muscular contact observed in both the hypotrophic and regenerated regions of the phrenectomized hemidiaphragms is shown in Fig. 7b. The nerve terminal containing few vesicles approaches but does not closely contact the muscle fiber. No post-synaptic specializations are clearly recognizable.

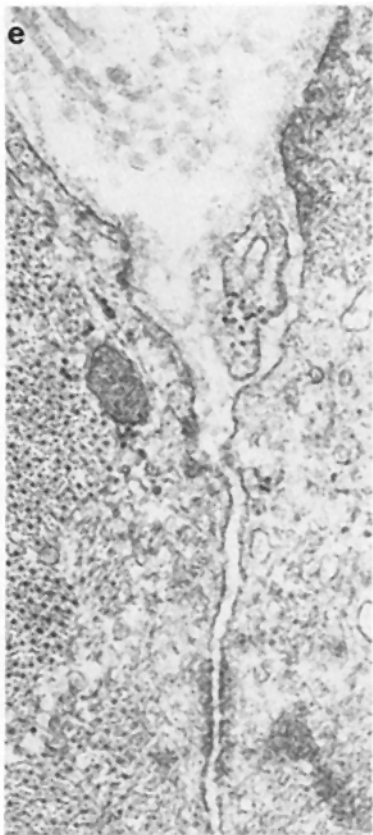
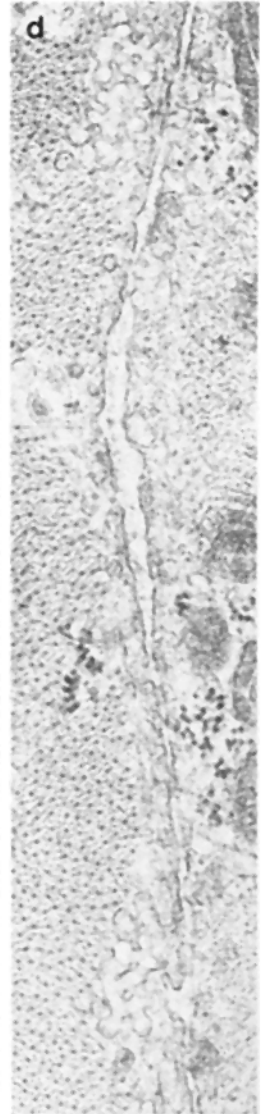
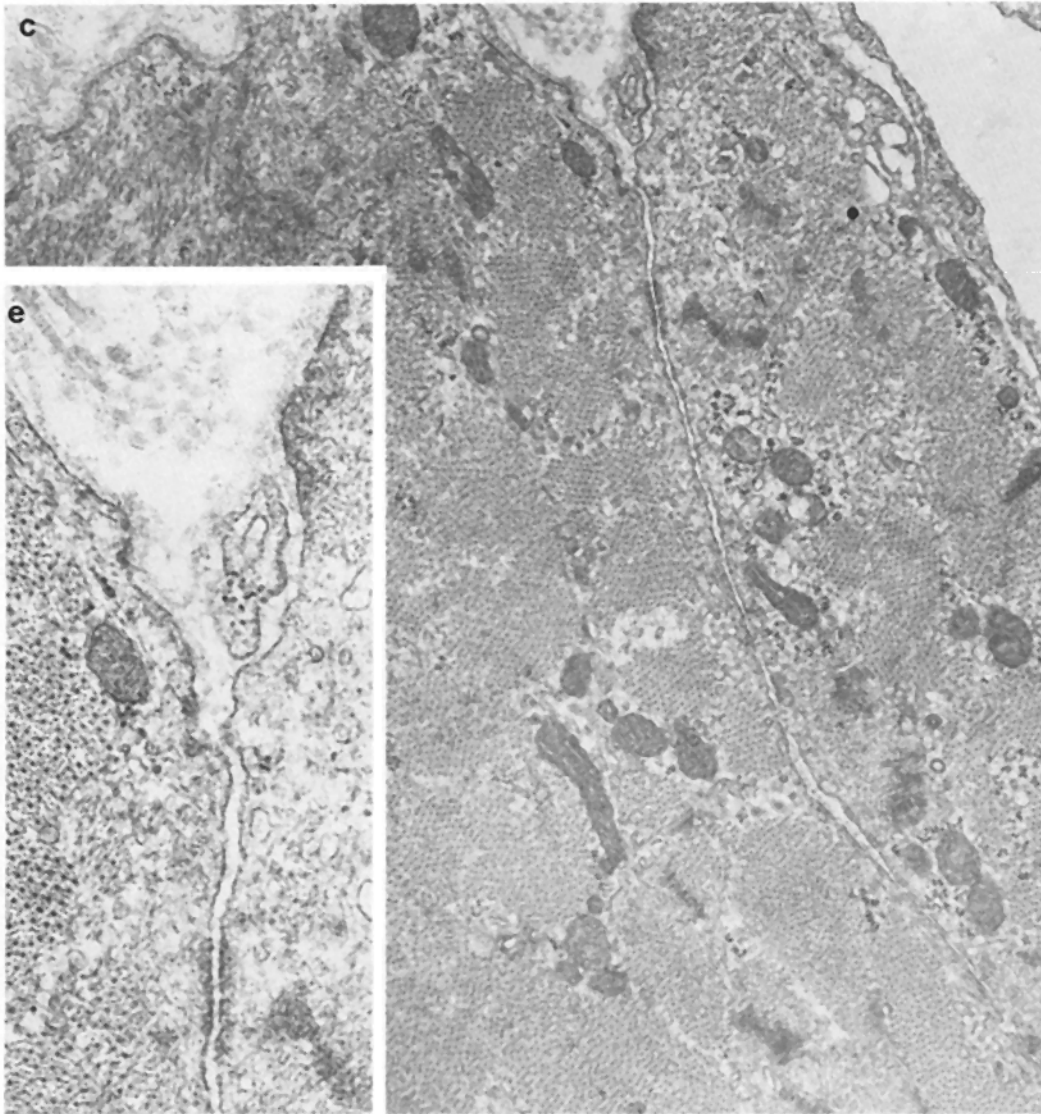
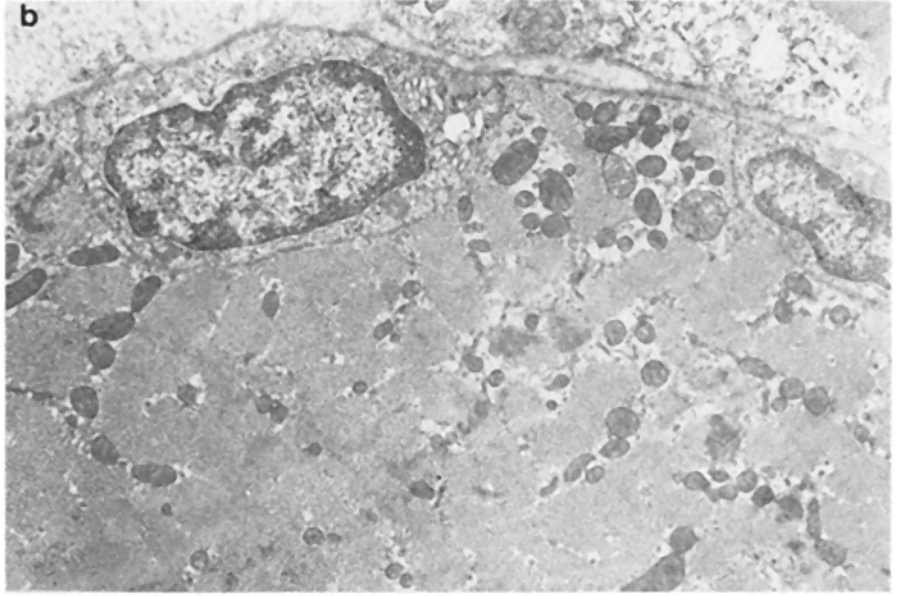
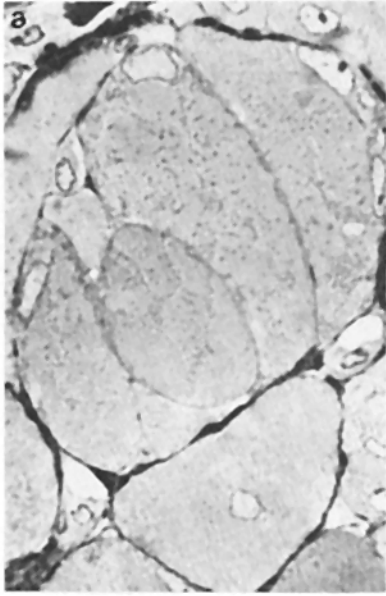
Effects of 16 mo of Denervation

MORPHOLOGICAL APPEARANCE: Fig. 8a shows the contralateral hemidiaphragm of a 16-mo-phrenectomized rat. The section, serial to those stained for acid stable ATPase and those analyzed for their parvalbumin and contractile proteins content (see below), demonstrates the normal pattern of innervated fibers. In Fig. 8b the cross section of the denervated left hemidiaphragm is shown. The diaphanous, thin hemidiaphragm had been folded two times before freezing and therefore the whole cross section of the muscle is shown and has been used to extract the proteins that were then subjected to gel electrophoresis. Even though at this magnification the component fibers are hardly distinguishable from each other, it is evident that the muscle is made up of atrophic fibers and that each layer is one fourth the thickness of the contralateral innervated hemidiaphragm. In Fig. 8c an enlargement of the denervated muscle shows angulated fibers separated by wide interfiber spaces. Few nuclei are centrally located. Fig. 8d shows one of the numerous clusters of either large or small fibers which are randomly scattered among the atrophic fibers. The denervated hemidiaphragm contained $1,636 \pm 66$ (mean \pm SEM) fibers per mm^2 while the contralateral hemidiaphragm contains 483 ± 21 (mean \pm SEM) fibers per mm^2 . Taking into account their cross section area, the two hemidiaphragms contained nearly the same number of fibers ($\sim 7,700$ in the contralateral muscle and $\sim 6,500$ in the denervated hemidiaphragm). We counted 302 ± 60 (mean \pm SEM) clusters of fibers per mm^2 , each composed of 3 ± 0.1 (mean \pm SEM) fibers.

ACID-STABLE ATPase: After extended periods of denervation it is usually difficult to distinguish between fiber types. Using histochemical methods it was seen that in some animals acid-stable ATPase activity was absent whereas in others it was present, but at a lower level than in the normal adult innervated slow fibers (9, 10, 18). This 16-mo denervated hemidiaphragm has been selected to show an extreme

FIGURE 5 12-mo-denervated hemidiaphragm. Light micrographs of semi-thin sections stained with toluidine blue (a-c) taken from a less atrophic portion of a denervated hemidiaphragm. The fiber population shown in a consists mainly of rounded fibers of variable size, grouped together by thick connective tissue layers. Frequently, the myonuclei are centrally located and display prominent nucleoli. Focal degenerative areas (target-like) are observed in many fibers. Note macrophages inside some of the target-like fibers. Electron micrographs are of target-like fibers, which also contain a large rod structure (d). $\times 440$ (a); $\times 770$ (b); $\times 1,400$ (c); $\times 22,750$ (d); $\times 28,500$ (e).





example of slow myosin maintenance in a chronically denervated hemidiaphragm. Compare the staining pattern of the denervated fibers (Fig. 9*d*) with those of the contralateral innervated hemidiaphragm (Fig. 9*c*), the normal soleus (Fig. 9*b*), and the normal EDL (Fig. 9*a*) of the same 16-mo phrenectomized rat.

TWO DIMENSIONAL SDS PAGE OF PARVALBUMIN AND MLC SEQUENTIALLY EXTRACTED FROM CRYO-STAT SECTIONS: The EDL, an almost pure fast muscle, contained fast MLC and a high concentration of parvalbumin. The spots of two of the molecular markers of adult fast fibers (LC1F and parvalbumin) have a similar dimension and stain intensity (compare in Fig. 10, *a* and *e*). The soleus, mainly a slow muscle, contained only a trace amount of parvalbumin even though the amount of tissue from which the parvalbumin was extracted was greater than that of the EDL. Both the actin and the MLC spots of the soleus were larger than those of the EDL (Fig. 10, *b* and *f*). The MLC pattern of the contralateral innervated hemidiaphragm showed both fast and slow light chains (Fig. 10*c*). The 16-mo denervated hemidiaphragm showed an increase in the actin-MLC ratio. The relative proportion of slow type light chains was decreased (Fig. 10*d*). The innervated hemidiaphragm contained a large amount of parvalbumin. The LC1F spot (Fig. 10*g*) had roughly the dimension of the parvalbumin spot (Fig. 10*h*). We may predict that the results of our research in progress will show a decrease of parvalbumin per volume of myofibers similar to that shown in the early phase of denervation (24). In any case, the parvalbumin gene, a marker of adult fast fibers, continues to be expressed in the severely atrophic fibers of the 16-mo denervated hemidiaphragm.

SDS PAGE AND SDS ORTHOGONAL PEPTIDE MAPPING OF MHC: Fig. 11*a* shows the SDS PAGE of MHC from the normal EDL, the normal soleus, the denervated hemidiaphragm, and the contralateral innervated hemidiaphragm of the 16-mo phrenectomized rat. The myosin extracted from the denervated hemidiaphragm contained a smaller proportion of MHC-S than the contralateral innervated myosin. The MHC-S represented 51% of the total amount of heavy chains in the contralateral myosin, and 30% of the denervated myosin. The figures were in good agreement with the percent content of LC1S in the myosins (48 and 21%, respectively) and with the histochemical fiber typing. The identification of MHC-S was confirmed by orthogonal peptide mapping in steady-state proteolysis (8). In Fig. 11*b* the SDS orthogonal peptide mapping of the heavy chains from EDL (lane 1), contralateral hemidiaphragm (lane 2), 16-mo denervated hemidiaphragm (lane 3), soleus (lane 4) cryostat section myosins, and normal EDL myosin purified by standard procedure (lane 5) are shown. Note that the peptide fragments that distinguish MHC-S from MHC-F are present in the control and, in lesser amount, in the denervated hemidiaphragm.

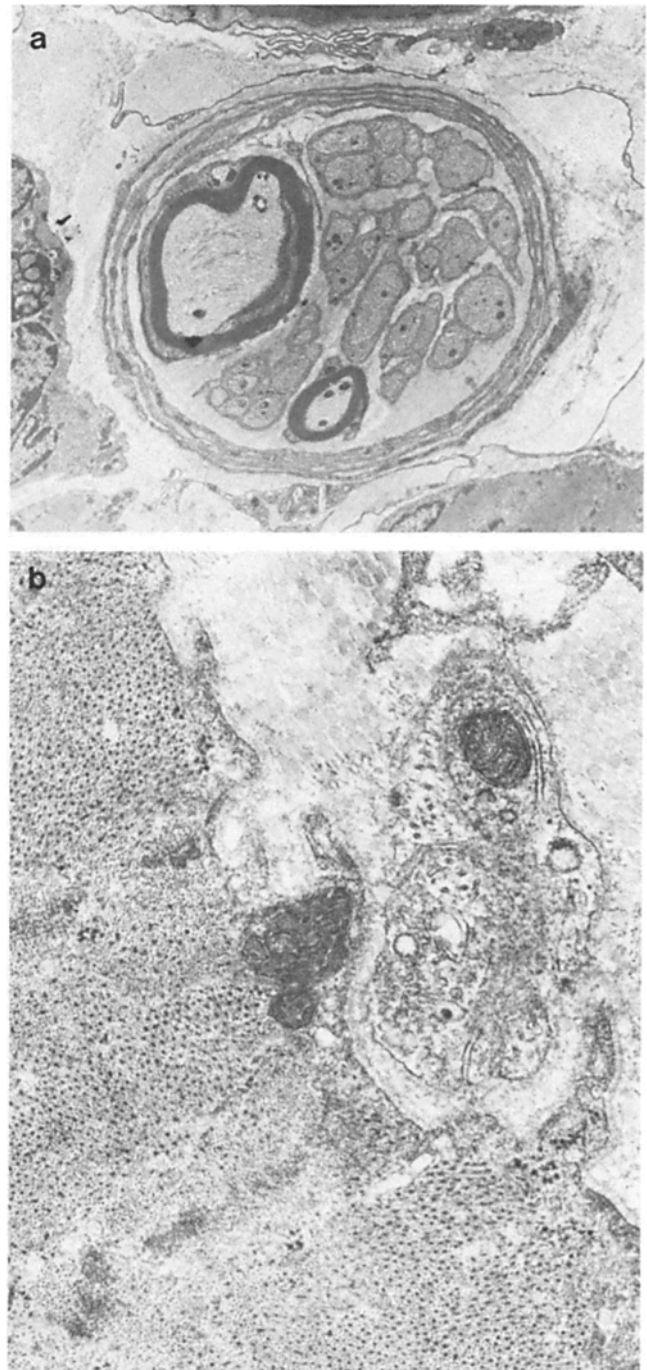


FIGURE 7 12-mo-denervated hemidiaphragm. (a) A small nerve contains two myelinated and numerous unmyelinated axons. (b) Simple neuromuscular contact. The nerve terminal that contains few vesicles approaches but does not closely contact the muscle fiber. No clear post-synaptic specialization is observable. $\times 5,850$ (a); $\times 37,500$ (b).

FIGURE 6 12-mo-denervated hemidiaphragm. (a) A magnification of a portion of Fig. 5*a* shows a cluster of muscle elements of very different size. (b) An electron microscope picture showing a doublet of satellite cells present around a muscle fiber. The fiber is part of one of the less atrophic portions of the denervated hemidiaphragm. Two myofibers rich in contractile material but with a poorly developed sarcoplasmic reticulum are enveloped by the same basal lamina (c and e). The fibers show the facing membranes close to each other. Either a membrane specialization or traces of basal lamina material are observed (d and e). (e) Honey-comb structures are also present. $\times 1,120$ (a); $\times 14,200$ (b); $\times 25,300$ (c); $\times 49,500$ (d); $\times 55,000$ (e).

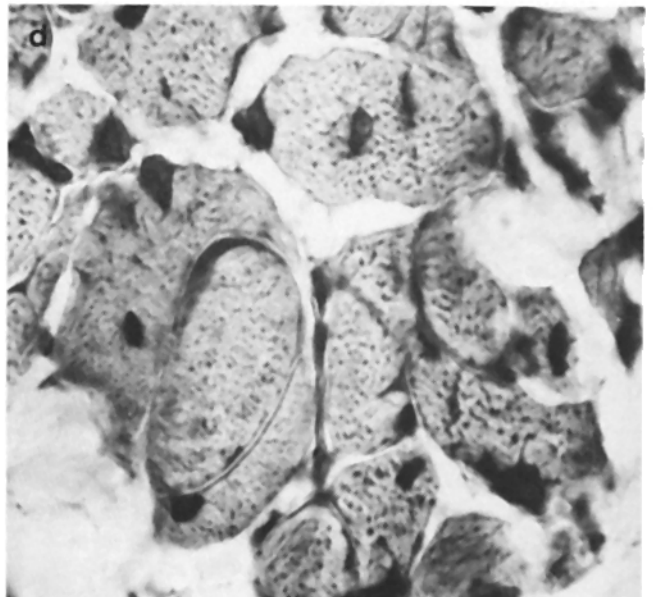
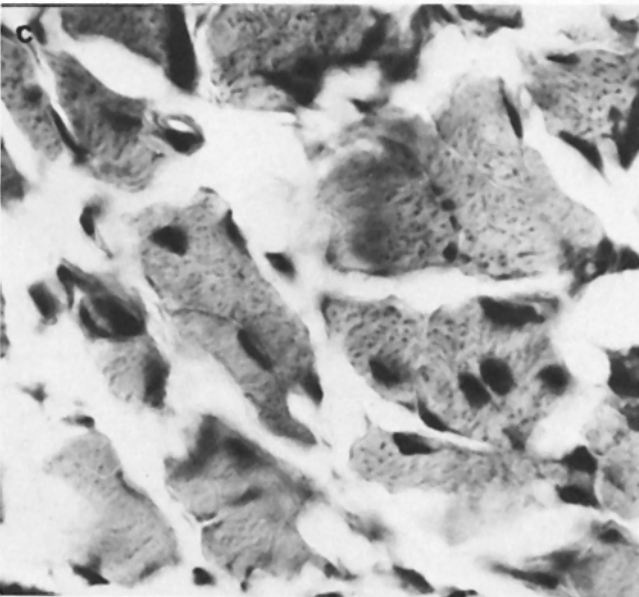
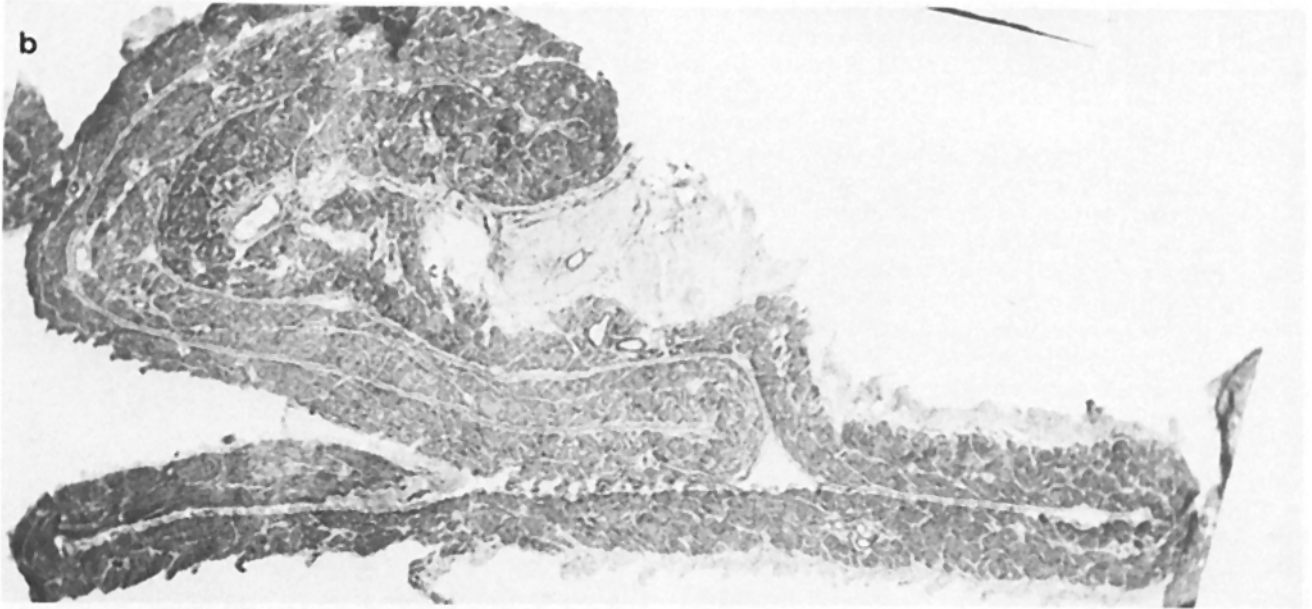
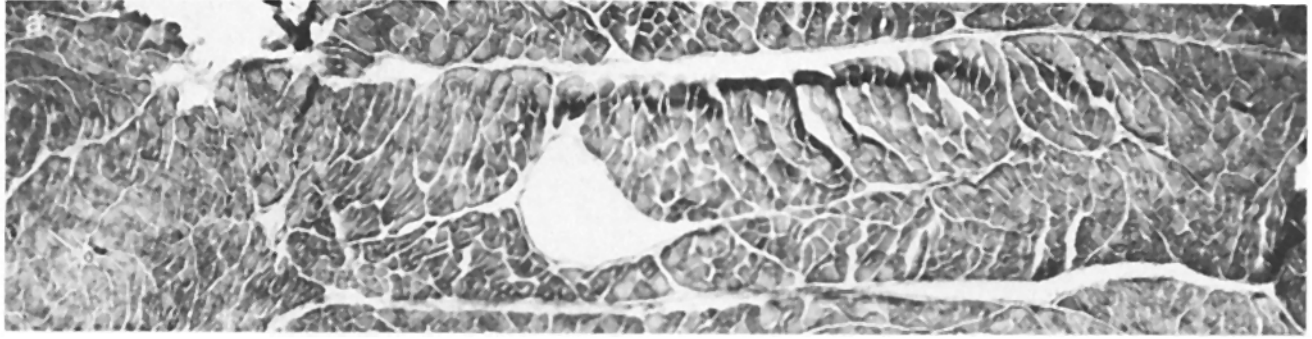


FIGURE 8 16-mo-denervated hemidiaphragm. (a) A portion of a cryostat section of the contralateral innervated hemidiaphragm. (b) At the same magnification, the cross-section of the whole denervated hemidiaphragm. The folded muscle is made up by so much atrophic fiber that each layer appears to be about one fourth the size of the contralateral innervated muscle. The fibers are hardly distinguishable from each other. (c) At higher magnification the smaller fibers are shown to be angulated in shape and separated by wide interfiber spaces. (d) An example of the numerous clusters of round fibers which are scattered among the atrophic fibers. $\times 60$ (a and b); $\times 1,120$ (c and d).

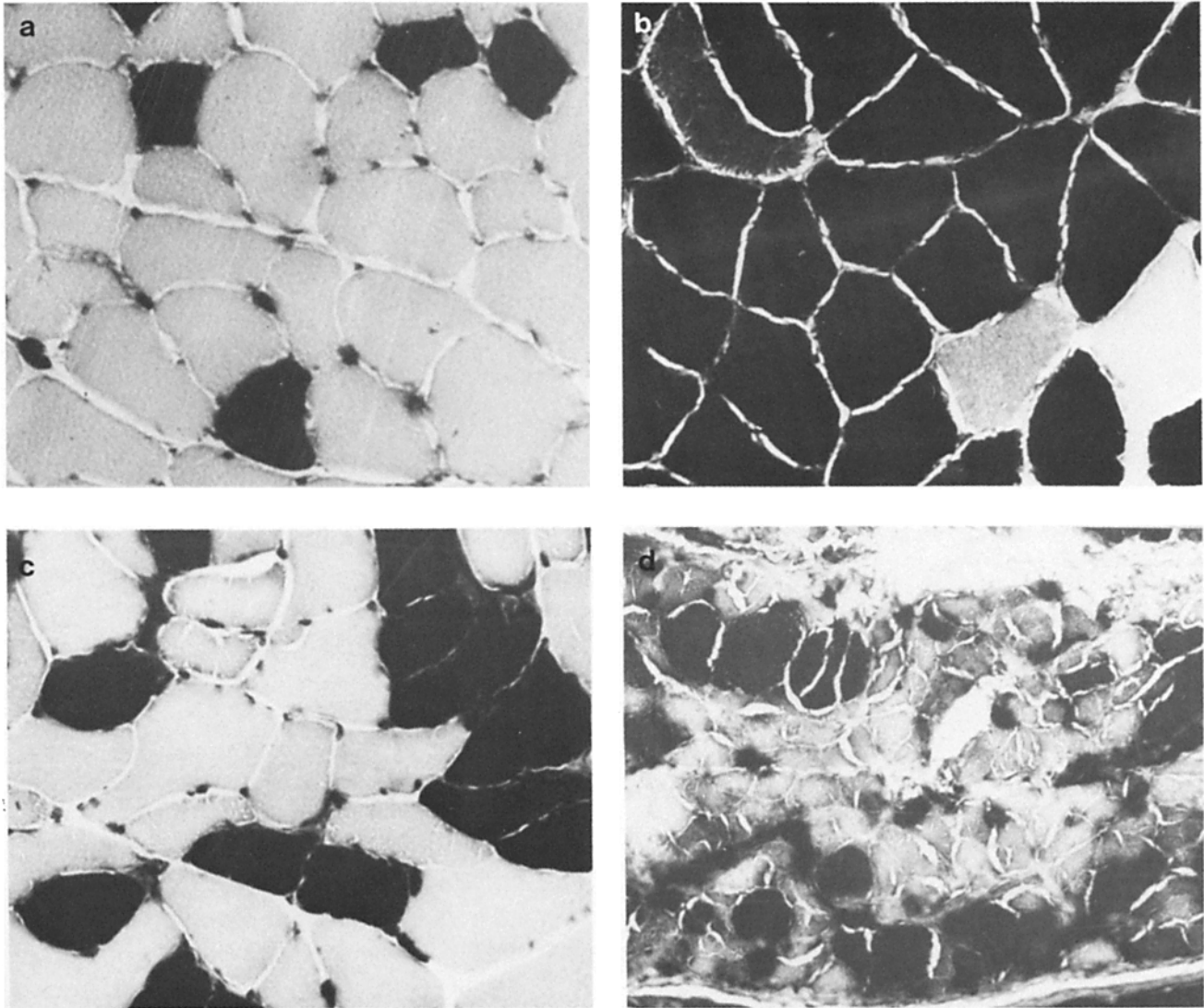


FIGURE 9 Acid-stable ATPase of muscles from the 16-mo-phrenectomized rat. Acid-stable ATPase (pH 4.35). (a) EDL; (b) soleus; (c) contralateral innervated hemidiaphragm; (d) denervated hemidiaphragm. Many fibers of the denervated hemidiaphragm maintain acid-stable activity. $\times 249$ (a-d).

DISCUSSION

Myosin is a multi-subunit protein consisting of two heavy chains and four light chains. Developmental and physiological transitions of myosin isoforms involve the expression of either different gene products or a pool of genes in different proportions, so that a definite MLC can be associated to different MHC (26). However myosin hybrids (that is, rare combinations of the myosin subunits that are peculiar to different fiber types) are common only to muscles undergoing pathological or physiopathological changes (41). The preferential distribution of fast and slow myosin subunits into different fiber types characteristic of normal adult rat diaphragm is no longer evident following denervation. Immunocytochemical analyses (18) clearly demonstrated that in long-term denervated hemidiaphragm all the fibers contain fast myosin and a substantial number contain both fast and slow myosin subunits. These results agree with our previous work demonstrating a transition from mixed to almost pure fast characteristics in the myosin extracted from chronically denervated hemidiaphragm (9-11, 14). However, while a dramatic reduction of

the slow myosin in chronically denervated muscle is unquestionable, the actual nature of the myosin(s) and the cellular mechanisms regulating the myosin change still remain a matter of discussion.

The significance of the immunocytochemical studies strictly depends on the antibodies' specificity. Antibodies against myosin from slow muscles of the chicken were used for the immunocytochemical localization of the rat slow myosin. Control experiments indicated that the heavy chain was mainly involved in the response to anti-slow myosin, although the light chains might also contribute to the staining pattern. Antibodies specific for the "different peptide" which is unique to chicken LC1F were used to identify fast myosin (19). Therefore the immunocytochemical analyses demonstrated without any doubt the ubiquitous presence of LC1F in the fibers of the chronically denervated diaphragm (18). The fast nature of the other myosin subunits was inferred. The significance of the reactivity of several fibers to both anti-slow myosin and anti-LC1F remained uncertain, because the polyclonal sera used might have cross-reacted with unidentified isomyosins. Indeed, the results have been interpreted to

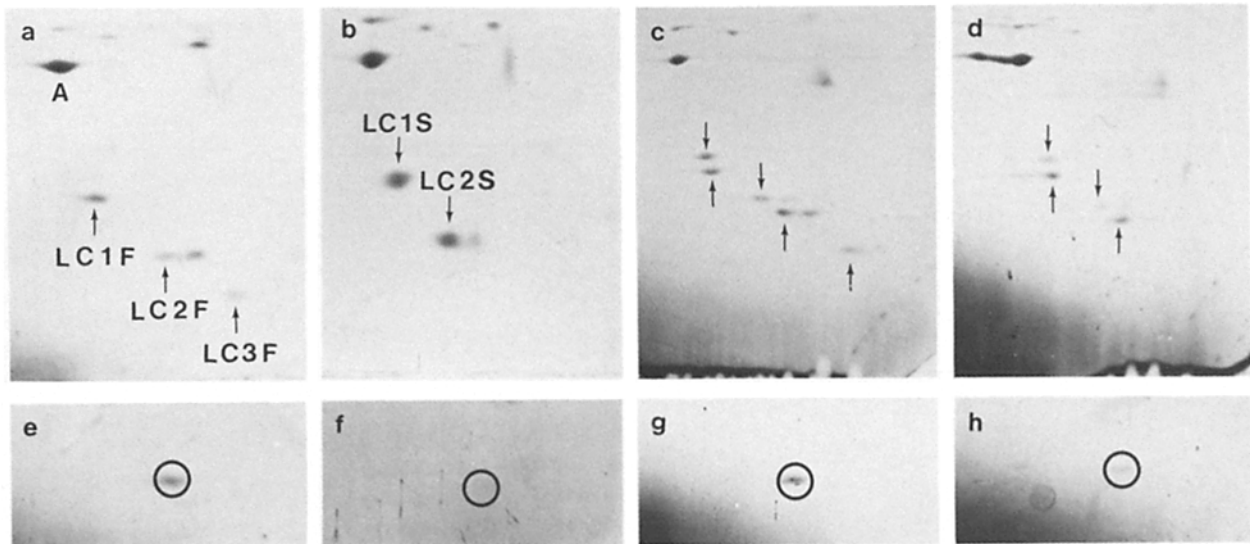


FIGURE 10 Two-dimensional gel electrophoresis of myosin light chains and parvalbumin of EDL, soleus, contralateral innervated and denervated hemidiaphragms of a 16-mo-phrenectomized rat. The two-dimensional analysis was on proteins sequentially extracted from cryostat sections of the muscles. Myosin light chain pattern of (a) normal EDL; (b) normal soleus; (c) contralateral innervated hemidiaphragm; (d) denervated hemidiaphragm. Parvalbumin from (e) normal EDL; (f) normal soleus; (g) contralateral innervated hemidiaphragm; (h) denervated hemidiaphragm. The positions of the actin (A), the light chains (LC1F, LC2F, LC3F, LC1S, and LC2S) and of the parvalbumin (O) are indicated. Downward vertical arrows indicate slow light chains; upward vertical arrows indicate fast light chains. By comparison with known quantities of purified proteins, it was determined that contractile proteins were 20–100 $\mu\text{g}/\text{slab}$. Parvalbumin was up to 1 μg .

mean either that fast and slow isomyosins co-existed within individual fibers or that in some of the chronically denervated fibers a distinctive myosin(s) was present that had properties of both isozymes. The latter interpretation has been regarded favorably by Gauthier and Hobbs (18) in view of their previous observation that developing muscles showed a uniform response to anti-fast and anti-slow adult myosins, in spite of the fact that light and heavy chains different from those of the adult fast and slow myosin are known to be expressed during development (42).

To answer the question of whether a unique myosin is present or more isomyosins co-exist within individual fibers we have studied myosin isoforms from the single fibers of a chronically denervated hemidiaphragm. The severe atrophy of the myofibers and the increased proportion of fibrous tissue in the denervated hemidiaphragm hindered the dissection of sufficiently long myofiber segments to allow MLC analysis. Instead there was just enough myosin in the single fibers to permit analysis of MHC (MHCs constitute the 90% of the molecule) by means of SDS PAGE. This standard technique clearly distinguishes fast and slow adult MHC and reveals the heterogeneous nature of the MHCs during muscle development both *in vivo* (8) and *in vitro* (see Results), even though embryonic myosin heavy chain co-migrates in SDS PAGE with MHC-S. In the electrophoretograms of the myosin heavy chains from individual fibers of the 3-mo denervated hemidiaphragm MHC-F is either present alone or it is preceded by another band. On the grounds of its electrophoretic mobility the ahead-migrating peptide could be either MHC-S or embryonic MHC. Its small quantity does not permit us to prove its nature by means of peptide mapping. Nevertheless, we interpret the leading band of the single fiber myosin to be MHC-S, after considering two facts: the presence of MHC-S fragments in the peptide maps of pooled long term denervated hemidiaphragms (10; and Results) and the fact that the myosin from pooled fibers of the same 3-mo denervated

hemidiaphragm from which single fibers were dissected still showed recognizable amounts of LC1S and LC2S, while the light chain of myosin from embryonic muscles was unidentifiable (see Results). Taking into account the above results our observations are evidence of the synthesis of MHC-F in addition to the MHC-S in the pre-existing slow fibers of the denervated hemidiaphragm. Therefore skeletal muscle fibers are able to express adult fast myosin not only when denervated during their differentiation (4, 28) or after regeneration in the absence of the nerve (13) but also when the slow program has been expressed in the fibers for a long time before denervation.

The structural analyses of myosin from long-term denervated muscles clearly show that there is a tendency in the fibers to express fast genes, however the extent to which slow myosin is maintained varies among experimental animals. Some show a 100% fast transformation (9–11, 14, 18), others only a slight decrease of the slow myosin content (21; this paper). This variability among individual rats may be due to the extent of myofiber necrosis and regeneration (regenerated fibers accumulate fast myosin in absence of the nerve [13]) and the activity of humoral factors counteracting the main modulative influence of denervation. The latter hypothesis is supported by the analysis of the contralateral innervated control hemidiaphragm and of normal EDL and soleus of the 16-mo-phrenectomized rat. These muscles showed a higher than normal proportion of slow myosin. Among the possible humoral factors responsible for the increase of slow myosin in the control muscles and of its persistence in the denervated hemidiaphragm, we must pay attention to hypothyroidism, on the basis of its known effect on fiber type and myosin composition in developing and adult rat muscles (5, 23). The hypothesis that spontaneous thyroid hormone imbalance, either *per se* or through secondary influences via the feedback controls on other neuroendocrine factors, could be responsible for the variable response of the muscle to denervation, is now the subject of our ongoing research.

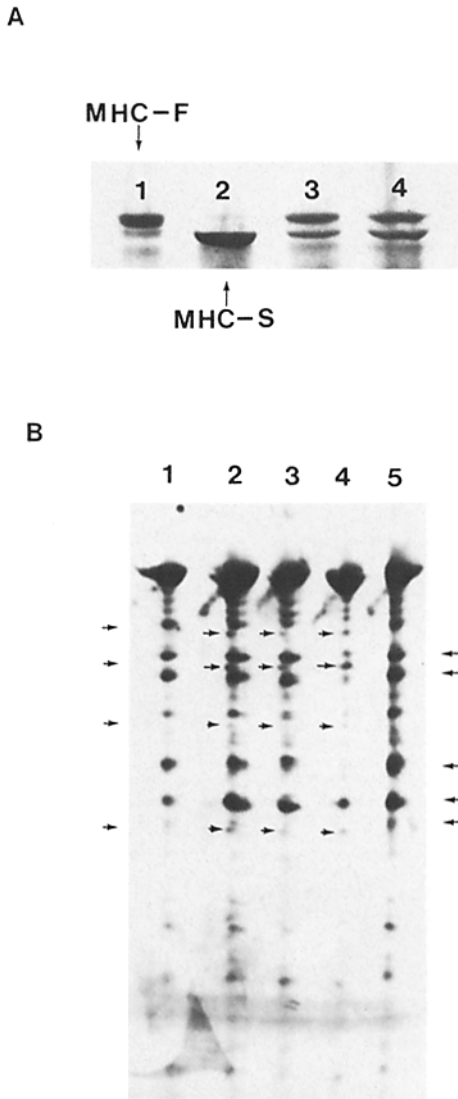


FIGURE 11 (A) SDS PAGE of myosin heavy chains isoforms. Myosin from cryostat sections of the muscles of the 16-mo-phrenectomized rat. Lane 1, EDL; lane 2, soleus; lane 3, 16-mo-denervated hemidiaphragm; lane 4, contralateral hemidiaphragm. About 0.5 μ g protein were used. The slab was 6% acrylamide, 0.75 mm thick, stained with the silver method. Downward arrow indicates myosin heavy chains of fast type; upward arrow indicates myosin heavy chains of slow type. (B) SDS orthogonal peptide mapping of myosin heavy chains. Lane 1, MHC from EDL; lane 2, MHC from contralateral hemidiaphragm; lane 3, MHC from 16-mo-denervated hemidiaphragm; lane 4, MHC from soleus; lane 5, MHC from EDL. Myosin samples of lanes 1-4 are from the same experimental animal. Rightward arrows indicate peptides characteristic of slow MHC; leftward arrows indicate peptides characteristic of fast MHC.

On the other hand, the amount of passive stretch to which the denervated hemidiaphragm is subjected to by contraction of the still innervated contralateral hemidiaphragm must be taken into account. The passive mechanical activity first induces the paradoxal, transient hypertrophy of the earlier phases after denervation (17, 40) and then probably contributes to the unexpected long survival of the muscle fibers in spite of the fact that the assumed essential trophic effect of the nerve has been lost. Light and electron microscopy, histochemistry, immunohistochemistry, and single fiber analysis showed that the chronically denervated hemidiaphragm main-

tained a fiber heterogeneity in terms of both typing and trophism. Morphological evidence of denervation atrophy (small angulated fibers containing anomalous membranes) is obvious; the large and heterogeneous population of muscle fibers still present more than 1 y after phrenectomy is surprising (see Results). Degenerative processes (target-like areas associated with macrophage invasion) involving fairly large areas of the fibers are known to occur early after denervation (29, 31-33). Similar degenerative events were found mostly in round and relatively less atrophic fibers in the long term denervated hemidiaphragm. The meaning of these apparently incoherent observations becomes clear when considering that muscle fiber regeneration can occur in long-term denervated hemidiaphragm.

After injury even in absence of the nerve (13, 16), the muscle fibers degenerate and regenerate in a couple of weeks (1). When the injury leaves the basal lamina intact, the subsarcolemmal cells (satellite cells) undergo mitosis and fuse to form small myotubes. The myotubes increase in size and length and acquire the characteristics of mature fibers. Often several muscular elements form within a single sarcolemmal tube (1), as it does during embryonic myogenesis (35). Then around the regenerated myofibers a new basal lamina reappears which persists during maturation of the fibers (22). In chronically denervated hemidiaphragm the morphological evidence of muscle regeneration was: (a) the activation of satellite cells; (b) the presence of several muscular elements at different stages of maturation within the same sarcolemmal tube; (c) the presence of focal membrane specializations tightly connecting the satellite myofibers to each other; and (d) the appearance of a new basal lamina around the regenerating myofibers. The clusters of regenerated fibers are scattered among atrophic fibers both at 12 and 16 mo after denervation. Since myofibers regenerating in the absence of the nerve transiently express embryonic myosin after myoblast fusion and then a fast type of myosin in both fast and slow muscles (13), at extended periods of denervation fiber regeneration significantly contributes in the hemidiaphragm to the increasing content of fast myosin.

In chronically denervated muscles, contrasting with the morphological evidence of muscle regeneration, embryonic myosin was never found by SDS PAGE. A possible explanation of this discrepancy is that the amount of contractile material present in early myotubes, when embryonic myosin is fleetingly expressed, is small in comparison with that contained in adult fibers. In synchronous muscle regeneration, as after bupivacaine treatment, myosin light chain from embryonic muscles is detectable by two dimensional PAGE only when a very large proportion of the early myotubes are present in the muscle specimens. On the other hand, a few original fibers scattered among the myotubes contribute a large percentage of the extracted myosin (13). It is therefore not surprising that the embryonic myosin light chain from the myotubes present among the atrophic myofibers in the denervated hemidiaphragm is undetectable even when using the highly sensitive silver stain.

Although muscle regeneration occurs in the absence of the nerve (1, 13, 16), its morphological demonstration raises the question as to if it represents the first evidence of reinnervation of some of the long-term denervated myofibers. The topographic anatomy of the diaphragm makes the collateral sprouting of the contralateral intact phrenic nerve or of the intercostal nerves possible, in addition to the regeneration of

the interrupted phrenic nerve. We can rule out that the regenerative events are related to reinnervation since the larger, more developed fibers show degenerative (target-like areas, macrophage invasion) and proliferative phenomena (honey-comb tubular arrays) peculiar to early stages of denervation (29, 32, 33). These relatively larger fibers are newly-formed aneurogenic myofibers undergoing the acute degenerative processes that then cause the extreme grades of atrophy. In severely atrophic muscles, however, few nerve fibers can occasionally be recognized among the atrophic fibers. A primitive neuromuscular contact found in one of the 1-y phrenectomized hemidiaphragms demonstrates that reinnervation of the denervated fibers may occur throughout the rat life span and therefore represents a problem in the interpretation of the long-term denervation effects. The few large targets of a minimal reinnervation may contribute significantly to the apparent persistence of the slow myosin in denervation when biochemical analyses on pooled fibers are performed, but does not explain the accumulation of fast myosin in chronically denervated muscles. When reinnervation occurs, we previously demonstrated that (a) the reinnervated portions of the diaphragm are distinguishable by their size from the denervated portions; (b) histochemically they show the type-grouping characteristic of the reinnervated muscles; (c) the myosin of the reinnervated muscles are as mixed as that of the contralateral innervated controls (10). Minimal reinnervation is a real possibility in chronically denervated muscle, and when it occurs, explains the unexpected normal size of scattered groups of fibers in a denervated atrophic muscle. However, when electrophoretic characterization of proteins is performed on samples sequentially extracted from cryostat sections most of the uncertainty arising from whole muscle extraction procedures and from the sampling of the single fiber technique are eliminated. In spite of this the analysis of MHC in severely atrophic, single fibers showed that *in vivo* skeletal muscle fibers were able to express adult fast myosin not only when denervated during their differentiation but also after the slow program had been expressed for a long time, suggesting that the activity of muscle exerts a modulative, not differentiative influence on muscle fibers, at least as far as the expression of the myosin genes is concerned. By combining biochemistry and morphology, the present study allows us to conclude that muscle deprived of its nerve's trophic and modulative influences responds to residual humoral and mechanical stimuli both with the remodeling of the gene expression in the pre-existing fibers and with regeneration of muscle fiber. These features are the basis of the long-lasting potential of the disused muscle to recover function.

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