# HISTOCHEMICAL AND CONTRACTILE PROPERTIES FOLLOWING NEONATAL DENERVATION IN THE FAST-TWITCH EXTENSOR DIGITORUM LONGUS MUSCLE OF THE MOUSE

by

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ABSTRACT

Mature fast-twitch skeletal muscle depends on innervation for complete differentiation and maintenance of its fast-twitch histochemical and contractile properties. The motorneuron plays a dominant role in the development of many of these characteristics, and therefore developing muscle is even more dependent on its innervation. This study was done to look at some of the changes in histochemical and contractile properties brought about by denervation, and to examine the effects of neonatal denervation on the pattern of development of these properties in the extensor digitorum longus muscle (EDL) of the C57/BL6 mouse, at 7, 14 and 21 days of age.

At 1 day of age, a unilateral sciatic neurectomy was performed. Silver and acetylcholinesterase staining was used to confirm that reinnervation does not occur by this method. Extrafusal fiber types were examined histochemically for oxidative enzyme and myosin ATPase activities (pH 4.2 and 9.4). Isometric contractile properties, including time-to-peak twitch tension, time from peak twitch tension to one-half peak twitch tension, twitch tension, tetanus tension, post-tetanic twitch potentiation, maximum velocity of unloaded shortening and resistance to fatigue were measured, in vitro, at 20$^{o}$C. Denervated and control muscles were examined at 7, 14 and 21 days of age, and normal muscles were also examined at 1 day of age.

At 1 day of age, all fibers stained for myosin ATPase following alkaline and acid preincubation, and all fibers were uniformly oxidative, according to NADH staining, typical of immature fibers. Over the next 21 days, normal muscles showed an increase in twitch and tetanus tension, post-tetanic twitch potentiation and velocity of unloaded shortening, with

a reduction in time-to-peak twitch tension, time to one-half relaxation and in resistance to fatigue. Histochemically, the extrafusal fibers differentiated into mature fast fibers with 46% of fibers being type IIA (anaerobic in the mouse), 40% type IIB (aerobic), with the remainder being type I. In the denervated muscle there was a significant prolongation of time-to-peak twitch tension and half-relaxation compared with controls. In addition, post-tetanic twitch potentiation was absent, the maximum velocity of unloaded shortening remained low, and there was marked resistance to fatigue, at all ages studied. All denervated muscles showed significant atrophy. Histochemically, there was evidence of some continued maturation at 7 days of age, but by 14 days only two fiber groups could be distinguished. Of these, 70% were atypical fibers exhibiting dual staining for myosin ATPase and were oxidative, as seen in immature fibers. The second group stained as type IIA but, unlike the control muscles, they were oxidative. The denervated muscles returned toward slow properties of immature muscles, but the changes in the physiological properties preceeded changes in fiber type. These results suggest that removal of neural influence neonatally to extensor digitorum longus results in loss of control over the differentiation into fast-twitch muscle. In addition, there is an immediate and significant slowing of the contractile properties and although denervated muscle continues to mimick the pattern of development of normal muscle, it becomes stalled at 14 days of age, preventing further maturation. Further studies are suggested that may identify the factors contributing to the changes seen in this study.

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## GLOSSARY

| | |
|---|---|
| ACHE | Acetylcholinesterase: enzyme found in neuromuscular junction to break down acetylcholine into choline and acetic acid |
| DEN | denervated extensor digitorum longus muscle |
| EDL | extensor digitorum longus muscle: a fast-twitch hindlimb muscle. |
| myosin ATPase | the adenosine triphosphatase on the myosin molecule |
| NADH-TR | also referred to as NADH reaction: NADH tetrazolium reductase oxidative enzyme reaction |
| NORM | normal (or control) extensor digitorum longus muscle |
| 1/2 RT | one half relaxation time: time from peak tension to one half peak tension |
| Po | Tetanus tension |
| Pt | Twitch tension |
| PTP | post-tetanic twitch potentiation |
| SR | sarcoplasmic reticulum |
| VMax | theoretical value of the maximum velocity of shortening at zero load, extrapolated from measures of shortening velocity over a series of loads |
| Vo | maximum velocity of unloaded shortening measured by the slack test method |

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# I INTRODUCTION

Neural Influence On Myogenesis

From an early stage in myogenesis, muscle is highly dependent on neural influence. Throughout development, the motoneuron continues to play a dominant role in dictating the mature characteristics of fast and slow muscle. A great deal is known about the structural and physiological changes that occur in skeletal muscle during development and the sequence of genetic expression of muscle proteins that are responsible. Strohman and Wolf (1985) distinguished between differentiation into muscle, which is coordinated and determined early in embryonic life, and maturation, an orderly progression of the sequence of embryonic to adult muscle protein isoforms. Differentiation can be made to occur in vitro in the absence of innervation but complete maturation cannot.

Muscle develops from predestined cells of mesoderm which migrate as myoblasts into the developing limb bud and fuse into myotubes. Under the influence of the motoneuron, they will begin synthesizing specific myofibrillar proteins and organelles which will ultimately determine the fast or slow contractile properties of the mature myofiber. These differ in their morphological, histochemical, biochemical and contractile properties depending on the neurotrophic influence and impulse characteristics of the motoneuron.

Differentiation Into Fast or Slow-twitch Muscle

In response to changing functional demands, mature muscle continues to exhibit structural and biochemical plasticity in its morphology, enzyme profile, and contractile and regulatory protein isozymes. These are reflected in muscle behavior, its contractile properties. These changes continue to be controlled through the trophic influences and activity pattern of the motoneuron.

At birth, all fibers have polyneuronal innervation (Obrien et al., 1978) with uniform properties of immature sarcoplasmic reticulum (SR) forming only simple junctional complexes with a loose network of transverse tubules or the sarcolemma (Luff and Atwood, 1971). They have uniform moderate oxidative enzyme activity (Reichman and Pette, 1984) and high resistance to fatigue. Myosin isozymes are of an embryonic or neonatal form (Whalen et al., 1981) and myosin ATPase activity is low. Speed of shortening and contraction times are slow (Close, 1964). The twitch and tetanic tension is low with a high twitch to tetanus ratio reflecting an immature calcium binding capacity. Resting membrane potential is low, and impulse conduction is slow.

Fully mature fast-twitch white fibers have an elaborate sarcoplasmic reticular network with a collar at the M line and form frequent triads at the AI junction with an abundant transverse tubule system (Luff and Atwood, 1971), permitting the rapid sequestering of calcium for short contraction times. Their narrow Z line is typical of fast-twitch white fibers. Their myosin ATPase activity is high (Drachman and Johnston, 1973), is stable in alkaline and labile in acid conditions and corresponds with rapid rate of muscle shortening. These fibers contain low oxidative and high glycolytic enzyme activity. The axon from their motoneuron has a relatively large diameter, with a high stimulus threshold, and is therefore recruited less often, with short high frequency bursts of activity required for rapid high tension performance of spurt muscles (Jolesz and Sreter, 1981).

Slow-twitch red fibers, innervated by small diameter axons which are readily excited and discharge frequently for long periods of time with comparitively low firing rates, are essential for the high endurance of

postural muscles. They have sparse SR with dyadic and triadic junctions, and their Z line is broad. Their oxidative enzyme activity is high, and myosin ATPase activity is low (Drachman and Johnston, 1973). Histochemically, slow-twitch fibers exhibit myosin ATPase reaction that is stable in acid and labile in alkaline preincubation conditions. Finally, these fibers have a slow contraction time, low maximum tension, a slow speed of shortening, and a high resistance to fatigue.

A third fiber type has properties intermediate to these but its physiological properties are more typical of fast fibers.

Denervation During Myogenesis

Compared with denervation of mature muscle, the effects of denervation on developing muscle have been shown to occur earlier and with more severe changes resulting in impairment of the development process (Kumar and Talesara, 1977). With complete maturation into fast and slow muscle being nerve dependent, denervation at this sensitive stage results in severe atrophy (Engel and Karpati, 1968) and loss of differentiation into fast and slow muscles with respect to Z band, metabolic enzymes, regulatory proteins, membrane properties, maximum tension, contraction times, physiological maturation of the sarcoplasmic reticulum and resistance to fatigue (Dhoot and Perry, 1983; McArdle and Sansone, 1977; Hanzlikova and Schiaffino, 1973; Lowrie et al, 1982). In fast twitch muscle many of the above parameters return in the direction of the slower immature muscle.

The Purpose of the Study

Mature muscle has been shown to be dependent on its innervation for maintenance of its fast or slow twitch properties in studies using cross reinnervation, inactivation by tenotomy, casting, cord section,

denervation, and electrical stimulation (Buller et al., 1960a; 1960b; Salmons and Vrbova, 1969; Riley, 1973; Webster and Bressler, 1985). Although little information is available on denervation in developing muscle, particularly of contractile properties, indications are, it is more susecptible to these procedures with a higher dependency on innervation during acquisition of fast or slow characteristics. The purpose of this study was to characterize the histochemical and contractile properties of the developing fast-twitch extensor digitorum longus muscle (EDL) and compare these properties with those of muscles deprived of innervation prior to final differentiation into fast-twitch muscle. This study identifies some of the factors which are neurally modulated during development in the mouse (C57/BL6J+/+) and provides important information for developmental studies of the dystrophic mouse of the cogenic strain (C57/BL6Jdy2j/dy2j).

# II REVIEW OF THE LITERATURE

Nerve Dependency in Early Myogenesis

Muscle development begins when premyoblast cells cease dividing, become myoblasts and begin to synthesize contractile proteins. They then fuse to form myotubes, the precursors to muscle cells and finally mature into their adult form.

Although myoblasts are capable of synthesizing contractile proteins, they cannot fully mature into fast and slow muscle fibers in the absence of nerves. In mouse embryogenesis, myosin positive cells occur in culture from 9.5 day somites. Cossu and Vivarelli (1985) found that cells cultured from 8.5 day mouse embryos fail to produce myosin unless conditioned with explants of spinal cord. They concluded that premyogenic to myogenic conversion requires presence of factors released from nerves. Furthermore, these explants showed no outgrowth and therefore no nerve muscle contact. Ecob and Whalen (1985), using the same system, were able to culture myotubes with striations and contractions coincident with the presence of adult fast myosin isoforms, but only in the presence of spinal cord. Merrifield and Konigsberg (1985) grafted quail limb buds to host chick embryo chorioallantoic membrane and found that grafts dissected to exclude neural tube accumulate early forms of myosin light chains (MLC1f) by 16 days gestation, but only when dissected to include neural tube could they produce the more mature forms; MLC1f and MLC3f. Synthesis of myofibrils occurs in muscle cell culture without innervation but cells cannot synthesize mature forms of contractile proteins. Matsuda and Strohmann (1984; Strohmann and Matsuda, 1985) found that satellite cells from fast and slow muscle were able to produce regenerating myotubes in cell culture that synthesize differential forms of embryonic myosin under identical culture conditions although they could not mature beyond the

embryonic stage. This suggests that there remain some pre-programmed differentiation capabilities in satellite cells.

In-vivo conditions are likely very different. Because it has not been possible to study early aneural development in-vivo, regeneration in the absence of innervation has been used. To compare the dedifferentiation of denervation with that of regeneration, Gutmann et al. (1976) autografted guinea pig soleus back into its own site to causing regeneration. They then compared regenerating soleus with reinnervating soleus following neonatal nerve crush. They found that regeneration shows the biphasic decrease in contraction times typical of developing soleus whereas in reinnervating soleus it is monophasic, suggesting dedifferentiation is not as complete in denervation. The problem of early nerve depencency of developing muscle is complex, as results differ with experimental models and conditions under study. Matsuda and Strohmann (1984) found normal differentiation of myosin heavy chain and light chain isoforms occurs following forelimb denervation of the chick, however repression of immature forms of tropomyosin and troponin does not occur.

It can be seen that innervation is essential to the early survival and continued maturation of muscle during early development. In order to understand the changing characteristics of developing muscles that coincide with the onset of innervation, it is necessary to review what is known about muscle development, beginning at the early stage of embryogenesis but concentrating on the period of maturation into fast and slow-twitch muscle.

Myogenesis: Independent Stage

Myogenesis can be described in two stages. The early stage is one of independent, the later of a coordinated, development. The independent

stage begins with the premyoblast derived from mesoderm. In the chick, somites grafted from any location in the embryo are capable of providing muscles for a particular limb bud, indicating that this stage of development is independent. Mesenchymal cells, predetermined to be muscle, migrate into the limb bud. The number of cells that migrate is small compared with the number of eventual muscles that will be formed, making it unlikely that these cells can carry with them specific instructions about the individual fiber types of muscle they will become (McLachan and Wolpert, 1980). The presumptive muscle cells proliferate and produce a dorsal and ventral block of myoblasts. These myoblasts then stop dividing and begin to fuse with other myoblasts to form myotubes. Their nuclei are centrally located, and they will extend the full length of the muscle block. This can occur in the absence of nerves.

Coordinated Myogenesis: Dependent Stage

(a) Connective Tissue Influences

A coordinated stage of development now begins, and the surrounding connective tissue divides the muscle block providing a template for the final muscle configuration of the limb. In the chick, if there is a disruption of the surrounding connective tissue, for example the altered length of presumptive bone, the muscle will accommodate its development to be appropriate to the connective tissue change (McLachlan and Wolpert, 1980). If the polarizing region of connective tissue from the tips of the digits is duplicated, muscles appropriate to that connective tissue will induce muscle duplication as well.

(b) Neural Influences

In the rat, pathfinder neurons grow toward the earliest developing muscle fibers and remain in apposition with these primary myotubes from

the 15th to the 19th day of gestation before forming functional endplates (Rubinstein and Kelly, 1981). Adjacent to the primary myotubes, developing cells (secondary myoblasts) form within a muscle cluster. This cluster, described by Ontell (1977), consists of primary fibers, satellite fibers, myotubes at different stages of development and myosatellite cells, all within the same basal lamina. Only the primary myoblast will become innervated while secondary myotubes wait until they have extended the full length of the muscle, separated away from the cluster, and formed their own basal lamina.

The pathfinder neuron establishes the location of the future neuromuscular junction and the remainder of the muscle cell becomes refractory to innervation (Obrien et al., 1978). Subsequently, two or more motor neurons converge and innervate the myotube inducing the formation of a polyneuronal neuromuscular junction. While the neurons are in competition with terminals of other axons the muscle membrane releases proteolytic enzymes discouraging endbulb formation. The competing endbulbs are not from the same neuron, but they have been shown to have common conduction properties (Thompson et al., 1984). Retraction of unsuccessful terminals occurs and ultimately only one axon will innervate any muscle fiber (Riley, 1981). In rat soleus, Obrien et al., (1978) used electrophysiological (multiple endplate potentials) and ultrastructural evidence to show that this process occurs postnatally, between 9 and 13 days, being complete by 18 days of life. One axon will innervate a group of myofibers forming a functional unit called a motor unit. The total number of functional motorneurons, and therefore motor units, does not decrease during the elimination of polyneuronal innervation. Therefore the number of fibers contributing to the tension,

and thus the tension developed per motor unit, must decrease (Brown et al., 1976) as each motor neuron reduces its number of peripheral synapses to achieve the mononeuronal stage. This process must be myogenic; decreased activity by tenotomy delayed its completion (Riley, 1978) but nerve crush with subsequent reinnervation did not (Brown et al., 1976).

Differentiation into Fast or Slow-twitch Muscle

Mature whole muscles are heterogeneous and their physiological properties depend on their predominant fiber type. In keeping with the theory that the neuron dictates fast or slow characteristics of the muscle, motor units are homogeneous with respect to neural behavior, fast or slow firing pattern (Close, 1972), enzyme activity (Nemeth et al., 1981) and fast or slow-twitch muscle fiber type (Close, 1972; Jolesz and Sreter, 1981)

Innervation is required for maturation into fast and slow-twitch muscle (Engel and Karpati, 1968; Shafiq et al., 1972). This coincides with the change from polyneuronal to mononeuronal innervation (Close, 1964; 1972; Rubinstein and Kelly, 1978; Jolesz and Sreter, 1981) and results in differential development of organelle structure, contractile and regulatory protein synthesis, enzyme production and activity, and contractile properties appropriate to fast or slow innervation.

Muscle development is not complete at birth (Gutmann et al., 1974; Shafiq et al., 1972). In the rat the majority of fibers are in the myotube stage at birth and this number declines until the second week of life to 10 to 20% at ten days (Engel and Karpati, 1968). Type I fibers are larger at birth, but in fast muscle the type II fibers soon eclipse the slow fiber in size. Growth is accomplished by the division of satellite cells and incorporation of daughter cells into the muscle to

are incorporated and synthesize proteins to elongate the developing myotube (Moss and Leblond 1971). The neurons of fast fibers have a larger diameter and a rapid conduction velocity and firing frequency, while a neuron to a slow fiber is small and conducts more slowly.

(a) Ultrastructure

Differentiation into fast and slow-twitch fibers brings about changes appropriate to that function. Luff and Atwood (1971) described ultrastructural changes in sarcoplasmic reticulum (SR) and transverse tubular system in developing mouse EDL and soleus. At birth the transverse tubules are loosely organized in a random interconnecting network around the A and I bands. They had a longitudinal and transverse orientation, and form dyads and a few triad junctions with the SR. The volume and the surface area of the SR is the same in fast-twitch and slow-twitch muscle, but with maturation the volume of the SR increases approximately five-fold in EDL and two-fold in soleus with soleus remaining less than one half that of EDL. By 15 days of age mature patterns are forming with longitudinally orientated t-tubules forming two triad junctions per sarcomere at the AI junction, and with a highly organized SR network. The Z line width was similar at birth at about 740 to 790A but by maturity the EDL Z line had reduced to 370A with soleus remaining at 740A into adult form. Hanzlikova and Schiaffino (1973) found no difference ultrastructurally between the fast and slow twitch muscle at 18 days gestation in the rat. The Z lines are broad and the SR and sarcotubular system incomplete with no triads, only peripheral junctional cisterns, coupling with the sarcolemma. With differentiation, myofibrillar fields are subdivided by the sarcoplasmic reticulum and

discrete myofibrils form. These extend the entire length of the muscle. Soleus develops somewhat later than EDL. At two weeks of age, clear differences between fast and slow muscle emerge. In EDL, Z bands are narrow, the sarcoplasmic reticulum is more elaborate and mitochondria less abundant (Luff and Atwood, 1971). In fast-twitch muscle, fast white fibers (anaerobic) ultimately have the greatest diameter, most densely packed myofibrils, complex sarcotubular system, low mitochondrial content and narrow Z lines while fast red fibers are similar except being higher in mitochondrial content and have narrower Z lines. In fast-twitch muscle, slow red fibers are generally smaller with a less elaborate sarcotubular system, high mitochondrial content and thick Z lines.

(b) Sarcoplasmic Reticulum and Calcium Binding Protein Changes

In a comparison of microsomal fraction from immature (4 day) and adult fast and slow-twitch rabbit muscle, the protein composition from both developing muscle types strongly resembles that of adult slow twitch muscle. The 105,000 Mr $Ca^{2+}$ transporting ATPase and 45,000 Mr., Calsequestrin, are low in activity and quantity. The amount and activity increases during differentiation into mature fast twitch muscle (Zubrycka-Gaarn and Sarzala, 1980). They showed that these calcium binding proteins, in immature and slow muscle, are immunologically identical with those from fast muscles. This indicated the changes are not due to alteration in isoforms. They concluded that, in respect to properties of SR, immature muscle is more closely related to mature slow-twitch than fast-twitch muscle.

(c) Metabolic Enzyme Differentiation

Muscles show uniform moderate oxidative enzyme activity at birth

(Reichmann and Pette, 1984a). At about two weeks of age, a wide spectrum of enzyme levels are seen, which have been categorized loosely into three fiber types: fast oxidative glycolytic (FOG), fast glycolytic (FG) and slow oxidative (SO) by Peter et al. (1972). The aerobic or anaerobic nature of muscle probably is its most labile property responding very rapidly to immobilization (Melichna and Gutmann, 1974), electrical stimulation and disease (Riley and Allin, 1973). Attempts have been made to relate fiber typing by oxidative enzymes with myosin ATPase staining (Wirtz et al., 1983), but the relationship is unreliable (Guth and Samaha, 1973; Brooke and Kaiser, 1974), particularly between fiber types in different species (Yellin and Guth, 1970; Green et al., 1982; Reichmann and Pette, 1982; 1984b). In general, the fast white fibers utilize the anaerobic pathway while the slow red fibers are more aerobic. Fast-twitch red fibers generally are equipped to utilize both energy pathways.

(d) Myosin ATPase differentiation

Guth and Samaha (1972) examined changes seen in both the ATPase activity, measured biochemically, and myofibrillar ATPase staining, measured histochemically, with development in the fast twitch triceps surae muscle of the rabbit. At birth, newly differentiated fast-twitch muscles, most still in the myotube stage, stain intensely for myofibrillar ATPase at pH 9.4 and 4.2. The myosin ATPase activity increases to 50% of adult level by 10 days of age while the staining progresses toward the mature state of alkaline stability and acid lability, some fibers still lack reversal. In addition, there were a few fibers of the slow type that exhibit intense staining after acid as well as after alkaline preincubation. By 21 days of age, all fibers showed reversal

consistent with the fact that all mature fibers contain exclusively fast or slow myosin. The routine myosin ATPase reaction used in early studies was incubated at pH 9.4. It was thought that ATPase activity was equivalent with dark staining, histochemically. The contradiction of dark staining immature fibers with slow contraction times, shown to correlate with myosin ATPase activity (Barany and Close, 1971), was examined by Guth and Samaha (1972). They measured the myosin ATPase activity biochemically and found that, in contradiction to intense myosin ATPase staining, the myosin ATPase activity of developing fast twitch muscle is low. This may be explained by the existance of three different myosin heavy chains: fetal, neonatal and fast adult, found in developing fast-twitch muscle (Whalen et al., 1981) and concurrs with the presence of myosin ATPase staining in both acid and alkaline preincubation, found in developing muscle, compared with exclusive staining seen in mature muscle. Thompson et al. (1984) found that the lack of reversal is not due to mixed innervation of a single fiber during the polyneuronal stage, as the neurons at one junction are of the same type. Slow-twitch fibers also lack reversal in development but show reciprocal staining to fast-twitch fibers in mature slow muscle.

(e) Contractile Protein Isozymes

Sequential isoforms of the major contractile proteins (actin and myosin) and regulatory proteins (troponin and tropomyosin) are synthesized at embryonic, neonatal and adult stages of cell differentiation (Matsuda and Strohmann, 1984). The myosin molecule consists of 6 subunits: two heavy chains (HC) of 200,000 dalton molecular weight and two pairs of light chains (LC) with a molecular weight around 20,000 (Julian et al., 1981). Adult fast muscle contains two classes of light chains, one dissociated with DTNB called light chain 2 fast

(LC2f) and two that can be removed by alkali treatment "alkali light chains" referred to here as light chain one fast (LC1f) and light chain three fast (LC3F). Slow muscle has light chain one slow (LC1S) and light chain two slow (LC2s) which migrate to the same position as LC1f and LC2f on the SDS polyacrylamide gel electrophoresis. Using antibodies raised to fast (anti-fast) and slow (anti-slow) adult myosin (Rubinstein and Kelly, 1981) and SDS gel electrophoresis for identification of light chains, Rubinstein and Kelly (1978) followed the developmental changes of myosin isozymes in fast-twitch EDL muscle of the rat. At 15 days gestation, all fibers were primary myotubes had positive staining with antibodies against fast myosin and to light chains LC1f, LC2f as well as an embryonic form of light chain 1 (LClemb). By 17 days gestationin the rat, some secondary myotubes appeared but neuromuscular contacts were infrequent and primative endplates formed only on primary fibers. The secondary fibers stained only with antibodies against fast myosin and fast light chains, whereas primary myotubes stain with those against slow myosin as well. At 18 days gestation, vesicles present in axon endbulbs and the postsynaptic membrane began to show the specialization of a functional neuromuscular junction. This coincided with fetal movement. By 19 days gestation secondary myotubes had separated from the cluster and acquired primative innervation. These authors suggested that primary myotubes remain as slow-twitch fibers while secondary myotubes, much larger in number, differentiate into the predominant fiber type of the muscle. At birth the presumptive fast fibers stained primarily with anti-fast myosin antibodies and the presumptive slow fibers stained intensely with anti-slow myosin. The adult form was achieved by 21 days of age; fast fibers stained exclusively with anti-fast and slow fibers

with anti-slow myosin. Using polypeptide analysis and antibodies purified through multiple absorptions, Whalen et al. (1981) examined fast-twitch rat gastrocnemius with myosin and found there are three isozymes of fast myosin heavy chain. In further studies with polyclonal antibodies to embryonic, neonatal and fast myosin, Whalen et al. (1985) found coexistance of neonatal and adult myosin in the same fibers. This showed that the transition from neonatal to adult myosin is asynchronous and occurs within the same fiber, not as a result of an increased amount of adult myosin in one fiber type. He referred to these as, myosin heavy chain embryonic (MHCemb), myosin heavy chain neonatal (MHCneo) and myosin heavy chain fast (MHCf). Sartore et al. (1982) concurred with these findings using antibody to bovine fetal myosin in rat muscle. Sartore (1982), using an antibody specific to fetal bovine myosin found neonatal and fetal myosin heavy chains in fast muscle to be different from those in slow muscle, in spite of identical migration on pyrophosphate gels. Whalen et al. (1981) suggested that the antibody against adult fast myosin used by Rubinstein and Kelly (1978) may have cross reacted with neonatal heavy chains in the developing fast fiber. Kelly and Rubinstein acknowledged this possibility (Rubinstein and Kelly, 1981). Rubinstein and Kelly (1985) have used non-denaturing pyrophosphate gels of native myosin to follow developmental changes in whole myosin isoforms in normal and denervated rat soleus and EDL. They found EDL synthesizes fetal and embryonic form of myosin f1-4 followed by mature forms FM1-FM3. Neonatal denervation did not alter this pattern. However, hypothyroidism combined with denervation did. Butler-Browne et al. (1982), working with rat gastrocnemius, found all developing fast fibers stained with anti-fast neonatal myosin and weakly with anti-slow

myosin as well. By 14 days of age, 25% of the fast fibers stained with antibodies to neonatal myosin, 25% to adult myosin and 50% with both.

Whalen et al. (1979) also found an embryonic form of light chain one (LClemb), which changed to adult form prior to the heavy chain transformation. This indicates that the synthesis of the embryonic light chain is not necessarily concurrent with that of the heavy chain. He showed the developmental pattern reproduced in Table I.

Table I Myosin Isoforms in Fast-twitch Muscle Development:

| fetal | 7-11 days of age | Adult |
|---|---|---|
| MHCemb | MHCneo | MHCf |
| LClemb-LC1f | LC1f | LC1f |
| LC2f | LC2f | LC2f |
| | LC3f | LC3f |

from the work of Whalen et al (1979).

In contrast to the rat, fast fibers in the developing chick have been found to contain slow light chains (Gauthier et al., 1978; Stockdale et al., 1981; Crow and Stockdale, 1984). However, Gauthier et al.(1982b) found an embryonic LC in both the chicken and the rat which they felt cross-reacted with adult slow light chain. Syrovy and Gutmann (1977) in rat EDL and Julian et al. (1984) in rabbit psoas, found an increase in LC3f during development.

Very little has been reported on the development of actin isoforms in developing skeletal muscle. Caplan (1983) describes a change in synthesis from non-myofibrillar beta and gamma actin to systhesis of alpha actin during muscle maturation.

(f) Regulatory Protein Isoforms

Dhoot and Perry (1979; 1983b) looked at isoforms of the regulatory proteins: tropomyosin and troponin I, T and C, which determine the way myosin ATPase responds to increased intracellular calcium during stimulation. These regulatory proteins, along with the activity of the myosin ATPase and the efficiency of the sarcoplasmic reticulum, could be responsible for developmental changes seen in time course of muscle contraction. Dhoot and Perry (1980) found that there are fast and slow forms of tropomyosin and troponin I,T and C. At birth, all fibers stain with antibodies raised against fast forms of troponin, and presumptive type I cells stain for both fast and slow forms. During differentiation, the slow fibers change, switching exclusively to synthesis of slow isoforms. In the chicken, there is an immature form of tropomyosin (beta) which is repressed during development to produce only the mature form (alpha) (Matsuda and Strohman, 1984).

Summarizing to this point, there are distinct developmental forms of myosin heavy chains and an embryonic form of the light chain, which are sequentially expressed in developing fast muscle. In developing fast-twitch muscle, the remaining light chains and regulatory proteins begin by synthesis of the fast adult forms with the major change being the increase in synthesis of the proportion of LC3f.

(g) Contractile properties

The work of Close (1964) describes fully the changes in fast and slow-twitch contractile properties with development. All muscles are initially slow to contract and produce only small amounts of tension. As development proceeds, both fast and slow-twitch muscles generate increased tension with decreased contraction time and speed of

shortening, more so in muscles subjected to phasic than tonic activity. Fast-twitch muscle initially has slow membrane properties in common with slow-twitch muscle (McArdle et al., 1980), but with differentiation, transforms to a posess a higher resting membrane potential, a higher concentration gradient of sodium, and a higher overshoot and maximum rate of rise of the action potential. The action potential is therefore propogated more quickly into the increasing network of t Tubules and SR. The release of calcium into the muscle is then accomplished sooner from a more abundant SR resulting in the capacity to produce a greater higher level of activation in mature fast-twitch muscle. These membrane changes result in an increased rate of both release and sequestering of calcium, thereby decreasing the contraction time of the muscle in both, its time to peak twitch tension (TTP) and its rate of relaxation, conventionally measured from the time of peak twitch tension to that of one half peak twitch tension (1/2RT). In mature fast-twitch muscle, the myofibrillar ATPase has a higher activity (Guth and Samaha, 1972) and, along with the fast forms of the calcium switch (Troponin I, T, C, and tropomyosin), the sites for myosin binding on actin are made available more readily. This rate of availibility of actin binding sites couples with the more rapid release and sequestering of calcium to decrease the contraction time. The tension that can be produced in a muscle depends on the balance of the release and sequestering of calcium and the availability and rate of actin and myosin binding. In the case of tension produced by repeated stimulation (tetanic contraction) the presence of calcium is maintained in the sarcoplasm and the actin and myosin binding, and therefore the tension is maximal. The tension following a single impulse is influenced by the functioning of the membrane properties, the myofibrillar ATPase

and the calcium regulatory processes described.

In contrast to slow-twitch muscle, with maturation, fast-twitch muscle becomes capable of exhibiting potentiation of the twitch following tetanic stimulation. It has been proposed that post-tetanic twitch potentation (PTP) is a result of the phosphorylation of MLC2 (Manning and Stull, 1979; 1982; Houston et al, 1985). The catalyist for this reaction is calcium dependent myosin light chain kinase and the dephosphorylation is catylized by phosphorylase.

At birth, fast-twitch muscle, with its aerobic metabolism, is highly resistant to fatigue (Close, 1964). With differentiation the fast white fibers become more glycolytic and lose their resistance to fatigue.

(h) Summary

Before differentiation, the immature fast-twitch muscle has many properties in common with mature slow-twitch muscles. These include: characteristics of Z line width, SR and myofibrillar density, content and activity of metabolic, SR and myosin ATPase enzymes, membrane properties and contractile characteristics of contraction times, maximum velocity of shortening and resistance to fatigue. The puzzling contradiction is the similarity of myosin isozymes, regulatory proteins and myosin light chains in developing muscle with those of mature fast-twitch muscle. The myosin heavy chain isoforms in early development are still somewhat subject to interpretation but immunologically appear to be fast. The explanation suggested by Kelly and Rubinstein (1978) that fetal muscles begin fast and are physiologically slow because of selective innervation of slow primary myotubes, does not give adequate weight to the other important properties in developing muscle which do begin with

characteristics of slow muscle.

Models of Neural Regulation in Muscle Plasticity

Much evidence has been accumulated to support the theory that the differentiation and maintainence of muscle fast and slow-twitch properties are nerve dependent and a large number of studies have been directed to test this theory (reviews Gutman, 1976; Jolesz and Sreter, 1981; Kelly, 1984). To understand how these manipulations contribute to the theory it is useful to look at these models of neural input. In vivo control of muscle development occurs via the following mechanisms. The first mechanism is through activity imposed on the muscle, via activation at the neuromuscular junction, and the resulting depolarization of the sarcolemma. The second is by a proposed neurotrophic factor which has been described by Gutman (1976) as long-term maintenance regulation, not mediated by nerve impulses. Many models of neural input have been used to address these two influences and assess the contribution of each. There is some question as to whether muscle activity is a neurogenic or a myogenic influence. Certainly, in vivo activity is mediated by the nerve, although the changes themselves may be induced by something within the muscle. The attempts to sort out the nature and extent of neural regulation have been approached in eight major ways. Firstly, muscle activity resulting from activitation by the central nervous system is reduced via cordotomy which is usually accompanied by section of the dorsal nerve root to eliminate the input from the stretch reflex, arising from the muscle spindle, and render the muscle totally silent. This leaves the motor neuron intact to continue its neurotrophic influence. A second method, denervation, interrupts all nerve functions. This can be accomplished by crushing, or sectioning of the nerve and the results vary

dependending on where the nerve section is made relative to its muscle (Finol et al., 1981). This method eliminates both neurotrophic and activity factors. Thirdly, decreased muscle activity through immobilization or tenotomy has been used. Neither of these eliminate activity completely and may, or may not, change neurotrophic influence depending on how much that depends on neural activity. Nerve impulses could influence the amount of neurotrophic factor released at nerve terminals (Bray et al, 1979). Changes in muscles properties are influenced by the muscle length at which they are produced (Melichna and Gutman, 1974). Fourthly, some studies have employed the use of chemical paralysis which comes in two major forms. Paralysis of axonal transport by Colchicine or Vinblastine is used to control for neurotrophic influence as it is reasoned that the neurotrophic substance or its substrates are transported down the axon. These substances do not interfere with conduction of the nerve impulse. Alternately, paralysis at the neuromuscular junction has been used to exclude muscle activity. Substances, such as botulinum toxin, have been used. Fifthly, muscle activity has been altered by changing the pattern of activation of the muscle by electrical stimulation. Because the pattern of activity has been shown to be a more important factor than the amount of activity (Jones, 1981; Hennig and Lomo, 1985), attempts have been made to mimmick the behavior of either the fast or slow nerves. This is done to assess activity without the neurotrophic influence. The majority of electrical stimulation has been applied indirectly via electrodes implanted next to the nerve which may then still include a trophic substance secreted by the nerve in response to its own firing pattern (Eldridge and Mommaerts, 1980). Others have used direct muscle stimulation both with or without

denervation. The assumption that electrical stimulation excludes the neurotrophic influence has not been proven. The sixth model is the addition of substances to the muscle that are thought to include the neurotrophic factor produced by the nerve. These substances usually come from extracts of sciatic nerve, and are injected into the muscle to be studied, or used in muscle culture medium. These extracts have not been characterized yet, and it is not known if they will be found in both fast and slow neurons and, if so, whether they would be similar. A seventh model involves the cross-reinnervation of the nerve from a fast muscle to a slow muscle, and the converse. These experiments include periods of denervation. Adding an alternate influence, such as fast innervation to slow muscle, is not necessarily the converse of removing the initial innervation. To validate influences of dennervation, an eighth method, that of reinnervation, has been used. This is usually in association with gentle crushing or transplanting of the nerve under conditions to encourage reinnervation. These studies have pointed up some major differences between denervation in developing and mature muscles.

Neural Regulation of Mature Muscle

(a) Atrophy

Muscle atrophy is reflected in a decrease in muscle weight, protein content, total cross-sectional area or specific fiber cross-sectional area. With reduction of neural activity following transection of the spinal cord, Buller et al. (1960) found atrophy to be greater in slow-twitch soleus than fast-twitch EDL of the rat, as did Rubinstein and Kelly (1978). Atrophy was increased when, in addition to cord transection, the dorsal roots were severed (cord isolation), rendering the muscle silient with the motorneuron is still intact. Deafferentation

eliminates all the reflex activity, but not necessarily neurotrophic influence. In cordotomized adult guinea pig, Karpati and Engel (1968) found equal atrophy in fast and slow fibers of gastrocnemius. The difference between cordotomy and cord isolation is illustrated by the work of Buller et al. (1960), where cross-reinervation between fast and slow-twitch soleus was effective in producing fiber type conversion in the presence of cordotomy, but not in cord isolation. However, Eldridge and Mommaerts (1980) were able to produce crossover effects in fast-twitch muscle in cord isolated cats. Denervation of cordotomized animals produced further increase in atrophy in fast twitch muscle but not in slow-twitch muscle. This suggests that maintenance of muscle integrity, particularly fast-twitch fibers, depends on a neurotrophic factor as well as impulse transmission from an intact motor neuron.

Immobilization by skeletal fixation reduces neural firing and produces mixed fast and slow fiber atrophy, but to a lesser extent than cordotomy (Karpati and Engel, 1968). Denervation alone produces greater atrophy than immobilization by casting or tenotomy (Kumar and Talesara, 1977) or cordotomy or cord isolation (Riley and Allin, 1973; Karpati and Engel, 1968). Denervation atrophy is greatest in fast twitch muscles (Gauthier and Hobbs 1982a; Carraro et al., 1981; Davis and Kiernan, 1980b), particularly in the fast white glycolytic fibers (Niederle and Mayr, 1978; Davis and Kiernan, 1980a; Gauthier and Dunn 1973) with type I (slow oxidative fibers) the least atrophied. Reports of the effects of denervation on soleus have been varied. Kumar and Talesara (1977) and Margareth et al. (1972) found preferential atrophy in slow fibers of rat soleus. Herbison et al. (1977) found in denervation, casting and tenotomy, that the weight loss was greater in soleus compared with

fast-twitch gastrocnemius. The decrease was due equally to fast and slow fibers, however, in Plantaris, the white portion had greater atrophy but it was in the slow-twitch fibers. Webster and Bressler (1985) found severe atrophy in chronically denervated mouse by 210 days post-denervation, with the wet weight of EDL 38% and soleus 23% of that of controls. The variable results from soleus have been partially attributed to the species variation of the percentage of fast-twitch fibers. Other factors may also contribute to atrophy; denervated muscle maintained in a shortened position shows greater atrophy than when held in a extended position (Melichna and Gutman, 1977). Gauthier and Dunn (1973) suggest denervation changes are found preferentially in fast fibers, while tenotomy produces a slow fiber loss.

Atrophy results from loss of muscle specific proteins and not from loss of intracellular fluid (Metafora et al., 1980). The major loss is largely due to decrease in myofibillar proteins. In a morphometric analysis of denervated rat, Engel and Stonnington (1974) found a close correlation between the decrease in myofibrillar and total cross sectional area. They showed that the area of SR decreased at a slower rate resulting in a proportional increase compared with fiber area. Others have found greater proportional loss in myofibrillar protein compared with stromal and SR proteins (Herbison et al., 1979). The loss of myofibrillar protein has been investigated in quantative studies of protein synthesis in denervated muscle. Pearlstein and Kohn (1966), using 14C-L-glycine, found no change in myosin synthesis after denervation in adult rats. However, D. Goldspink (1976) found a decrease in myosin synthesis for two days post-denervation followed thereafter by an increase. In addition an increase in synthetic activity could be

accounted for by an increase in the number of ribosomes, particularly near the sarcolemma and neuromuscular junction, following denervation (Grampp et al., 1972). Use of actinomycin D, a protein synthesis inhibitor, reduced denervation induced membrane changes in mouse muscle namely: development of extrajunctional cholinergic receptors, TTX resistant action potentials, fall in resting membrane potential and slowing of the time course of action potential, but only if used within two days of denervation.

To further elucidate the source of denervation atrophy, activity has been restored to denervated muscle by electrical stimulation. Riley and Allin, (1973) implanted electrodes into caudal nerves of cord isolated cats and was able to reverse the preferential white fiber atrophy with both phasic (50Hz) and tonic (10hz) stimulation patterns. The motor neuron was still intact therefore this could be either a neurotrophic or impulse related influence. Melichna and Gutmann (1974) reversed the atrophy in denervated fast-twitch tibialis anterior of the rat by direct stimulation, suggesting a role for activity in atrophy. Blockage of axonal transport, by colchicine, without interference with impulse transmission produced atrophy which was less than that found in denervated rat muscle (Ramirez, 1983). This indicates that impulse transmission has only a partial contribution to atrophy. Bray (1979) found equivalent atrophy in denervated muscles with those treated with TTX to block impulse transmission. However, it is possible that blockage of impulse transmission could interfere with secretion of a neurotrophic substance. In an elegant treatment of this question, Davis and Kiernan (1980a, 1980b) isolated, from rat sciatic nerve, a protein extract, shown to have neurotrophic influence on denervated muscle. Denervated,

immobilized EDL showed greater atrophy than controlateral immobilized controls, primarily in type IIB fast white glycolytic fibers. When the denervated immobilized muscles were injected with the extract, atrophy became equal in the two groups. This showed immobilization accounted for 60% while neurotrophic 40% of atrophy in denervated fast twitch rat EDL. Neiderle and Mayr (1978) found exclusive atrophy of the type II fibers in denervated mature EDl of the rat, up to 42 days post-denervation, and this was primarily in the white fibers which are type IIB in the rat EDL. There may be some synergistic effect between activity and neurotrophic substances but the multitude of studies indicate that they are both important factors in regulation and maintenance of muscle properties in both fast and slow-twitch muscles.

(b) Ultrastructural Changes

Engel and Stonnington (1974) used morphometric techniques to examine atrophy and ultrastructural changes in denervated adult rat soleus and gastrocnemius muscles for 84 days post denervation. Fast and slow fiber area decreased in concert by 80% by 84 days with parallel changes in myofibrillar area. There was an initial increase in absolute number and proportional volume of mitochondria by 8 days although the mitochondria were smaller in total size. Aggregation of mitochondria was common and they became reorientated longitudinally, reminiscent of developing muscle. There was an increase in the number of sarcotubular components (SR and t tubules) with focal dilation, irregular spatial arrangements and SR membrane disruption. Longitudinal sections showed an increase in SR area, however in crossection, fast fibers showed a marked decrease in sarcotubular surface area and slow muscles, after an initial increase to 8 days, also showed a decline. The total sarcotubular area declined but

not as rapidly as fiber area, therefore in spite of some atrophy, the sarcotubular elements showed a net increase compared with fiber area.

A shift in dominant fiber type could represent either preferential loss of one fiber type or fiber type transformation. To follow this change, it is necessary to use a fiber type characteristic as a marker which is resistant to denervation, such as Z line width. Gauthier and Dunn (1973) found a shift toward a homogeneous fiber type following denervation of rat semitendinosis. In control muscle, they found semitendinosis to be a mixed muscle, composed of 52% red (FOG), 40% intermediate (SO) and 8% white (FG) fibers. A decrease in the number of fibers with narrow Z lines suggested a deterioration of the fast white fibers such that the majority were now fast red and slow red fibers. They were altered in a manner suggestive of dedifferentiation; longitudinal orientation of triads, reduction in subsarcolemmal accumulations of mitochondria but an increase in interfibrillar rows of mitchondria. The white fibers that remained were atrophied, showing disruption of SR and streaked Z lines.

They also found an increase in the number of subsarcolemmal ribosomes. Although ribosomes accumulate around the neuromuscular junction, only in denervation are they found along the entire surface of the mature fiber. Ribosomes have also been found adjacent to the sarcolemma in newborn diaphragm (Gauthier and Dunn, 1973) at a time coinciding with extrajunctional acetylcholine sensitivity of the membrane. Grampp et al. (1972) used Actinomycin D, a protein synthesis inhibitor, to investigate the role of subsarcaolemmal ribosomes in membrane changes following denervation. As a result of the protein inhibitor, there was a reduced protein synthesis and reduction in

the numbers of extrajunctional receptors, the TTX resistant action potential as well as a fall in the resting membrane potential compared with denervated, non treated muscle. It did not affect the maximum rate of rise and the amplitude of overshoot of the action potential which are slowed by denervation. They concluded that the subsarcolemmal ribosomes were responsible for new protein synthesis contributing to membrane changes.

(c) Contractile protein changes

The high correlation between the rate at which myosin hydrolyzes ATP, the intrinsic speed of shortening and the pattern of neural control has resulted in a primary research focus on myosin ATPase activity measured biochemically, myosin ATPase staining measured histochemically, and the isozymes of myosin separated by means of denatured myosin on SDS gel electrophoretograms or non denatured myosin separated by pyrophosphate gels. In spite of a clarification by Guth and Samaha (1972) that myosin ATPase staining at alkaline pH is only a reflection of the alkaline stability of myosin, it continues to be referred to as myosin ATPase activity. Staining is in some cases correlated with myosin ATPase activity in adult muscle, but cannot be considered a measure of myosin ATPase activity, particularly in developing muscle (Guth and Samaha, 1972). In this thesis, only myosin ATPase activity, measured biochemically, will be referred to as "activity" and histochemical determination will be referred to as "staining".

In denervation studies on adult muscle, histochemical changes in myosin are a late occurance: by 2 months in caudal muscles of the cat (Riley and Allin, 1973); by 6 months in the diaphragm and gastrocnemius of the cat (Carraro et al., 1981); 120 days in EDL of the rat (Neiderle

and Mayr, 1978) and often not seen at all in the acute stage of denervation (Metafora et al., 1980; Margareth et al., 1972). In caudal muscles (mixed fiber type) of cord isolated cats, Riley and Allin (1973) found inactivity did not change myosin characteristics, nor did subequent indirect tonic or phasic stimulation. This suggests the myosin ATPase control is by other than impulse transmission. In the same study, denervation of the cord isolated cat muscles led to marked deterioration and loss of ATPase fiber type differentiation, suggesting a loss of neurotrophic control. Carraro et al. (1981), using chronic denervation of the diaphragm, produced fibers that stain at both acid and alkaline preincubation, indicative of immature fibers. Gauthier and Hobbs (1982), using antibodies raised against fast and slow myosin and absorbed against Light Chains to ensure reaction with only the heavy chain component of myosin, found dual reactivity of all fibers of the denervated rat diaphragm. As well these fibers stained for myosin ATPase in both acid and alkaline preincubation, verifying a coexistance of fast and slow myosins in dual staining fibers. Both acid and alkaline preincubation are required to distinguish between the presence of an adult myosin or dual staining fibers which could represent an immature or novel myosin. A more sensitive measure has been sought by staining with antibodies raised against fast or slow adult myosin, but has not clarified this question because crossreactivity has been shown between fetal and neonatal myosins isolated by Whalen et al. (1981) and fast adult myosin antibodies. However, in a study using gel electrophoresis to measure myosin, Carraro et al. (1981) have shown that, in denervated rat gastrocnemius (posssessing mixed fiber types), conversion to fast myosin and loss of the slow myosin and slow light chains occurs. This lends

support to the theory that fast myosin is synthesized in the absence of innervation and that only the slow myosin depends on neural input. There was also a decrease in the LC3 component which could be interpreted that the change is toward an immature myosin.

Barany and Close (1971) showed that cross reinnervation produced myosin ATPase activity appropriate to the innervating neuron, indicating a neural influence on myosin ATPase activity. Syrovy et al. (1972) measured myosin ATPase activity in denervated fast and slow muscles of the rat and rabbit and found it decreased reciprocally with contraction times in all but the rabbit soleus which increased reciprocally with contraction times. This shows that while myosin ATPase activity and contraction times may be related, findings may be confounded due to marked species differences in the response to a particular manipulation. Although histochemical myosin ATPase staining changes are a late finding following denervation, it has been suggested that this reflects a slow turnover of myosin. Yet myosin ATPase activity changes were seen by Gutmann et al. (1972) as early as seven days post denervation in mature rat EDL. Using indirect low frequency stimulation of rat tibalis anterior, comprised of fast white (IIB) and fast red (IIA) fibers, Mabuchi et al. (1982) found complete conversion from IIB to IIA fibers by histochemical staining, yet there was no change of myosin ATPase activity. Either both fast fiber types have the same ATPase activity or the myosin ATPase staining does not measure ATPase activity, has been suggested by Guth and Samaha (1972) in developing muscle. Riley and Allin (1973) found that myosin activity was not changed in indirectly stimulated caudal muscles of the cat, and suggested that a neurotrophic factor was required to alter myosin ATPase activity. The lack of

correlation between histochemical fiber typing and ATPase activity is not an uncommon finding.

Light chains of fast twitch muscles are primarily of the fast type and remain fast with denervation (Rubinstein and Kelly, 1978). The high proportion of LC3 to LC1 typically found in mature fast white fibers was decreased in single fiber analysis of long term intermittent stimulated tibialis anterior muscles of the young rabbit by Mabuchi et al. (1982) in denervated rat tibalis anterior. They found type IIB fibers of adductor magnus to be different in isozyme pattern than type IIA or IIB fibers in the tibialis anterior. The LC1/LC3 ratio is significantly lower as well. Generalizations from fiber typing may be a reasonable place to begin, but extreme care must be taken in assuming that all their myosin properties are necessarily identical.

(d) Changes in Calcium Uptake

Ultrastructural disruption of the SR is minimal in denervation and atrophy occurs at a slower rate in SR than in the overall fiber (Engel and Stonnington, 1974). Metafora et al. (1980) found no ultrastructural changes in the SR at 8 days post-denervation in the rat gastrocnemius. However the physiological and biochemical changes are immediate and pronounced (Heilmann and Pette, 1979). Sreter (1970) examined the calcium binding capability of the SR in denervated gastrocnemius and soleus of the rat. A marked decrease was found in the initial rate and total calcium uptake into fragmented SR vesicles, particularly in the fast white fibers. Efficiency was impaired with a leakiness of the SR membrane and prolongation of time to maximum capacity. Changes in activity pattern from denervation, endurance training or low frequency stimulation have the same slowing effect on the function of the SR of

fast-twitch muscle. Denervation produced opposite effects on fast, compared with slow, muscle (Margareth et al., 1972). Calcium transport was increased in soleus, while decreased in EDL, with concomittant changes in electrophoretic pattern. In long term electro-stimulation studies using indirect, low frequency stimulation, Mabuchi et al. (1982) showed decreased calcium uptake in tibialis anterior in the rabbit. Early SR changes were found in fast rat EDL and vastus lateralis in response to endurance training (Green et al., 1984), with a decrease in 115,000Mr calcium binding protein and increase in 30,000Mr protein content associated with a transformation from type IIB (FG) to IIA (FOG) and a decrease in cytosolic calcium binding protein; parvalbumin. Similarily, Heilmann and Pette (1979) found changes in SR function after 2 days of indirect low frequency stimulation of gastrocnemius and EDL in the rabbit. These included: a drop in calcium dependent ATPase activity, reduced initial calcium uptake and total capacity, lower amounts of Calcium pumping protein (115,000Mr), calsequestrin (68,000Mr) and high affinity binding protein (59,500Mr) on SDS gels. Denervation changes could be due to the loss of a neurotrophic substance from fast nerves as reinnervation of soleus with a fast nerve speeds function of the sarcoplasmic reticulum (Margareth et al., 1973). For this to be the case indirect stimulation mediated by the fast nerve would have to result in some transformation within the neuron itself. An alternate possibility is that a neurotrophic substance, common to fast and slow muscles, mediates its influence depending on the impulse frequency imposed on the muscle. These effects on SR function matched in time with changes in contraction time. The correspondence of the changes in contraction time with those of SR function lends support to work of Brody (1976), who used

isolated SR vessicles from intact crureus and soleus muscles of the rabbit, both of which have the same myosin ATPase activity but different rates of calcium uptake, to demonstrate that contraction time is more closely correlated with sarcotubular uptake of calcium than myosin ATPase activity.

(e) Alterations in Contractile Properties

Dynamic properties of fast and slow muscles are largely determined by their specific motor innervation. Spinal cord section speeds contraction times in slow twitch soleus, but EDL is unchanged (Buller et al., 1960; Betto and Midrio, 1978; Eldridge and Mommaerts, 1980). With cordotomy and denervation or denervation alone, there is a marked slowing of contraction times in slow and particularly in fast muscles in the rat (Betto and Midrio, 1978). The effect of denervation is not simply inactivity, as there is a difference in contraction time changes between cordotomy and denervation. The fact that a slow muscle, rendered silent by cord isolation, becomes fast but with subsequent denervation becomes slow suggests a maintenance of fast characteristics by an intact, albeit electrically silent, nerve (Eldridge and Mommaerts, 1980).

By reinnervating slow muscle with a fast nerve, contraction time alters towards that of a fast muscle. The converse is true of cross-reinnervation of a slow muscle (Buller et al., 1960b). In the same way, electrical stimulation has the same reciprocal effect of slowing the contraction time of fast muscles with tonic stimulation (Salmons and Verbova, 1969; Heilmann and Pette, 1979). As reported earlier, the time sequence and direction of the changes in contraction times correspond to functional changes in SR.

Cordotomy prior to cross-reinnervation of fast and slow muscles of

the cat had no influence on the success of cross-reinnervation, whereas total isolation of the cord prevented it (Buller et al., 1960b). This supports the theory that neurotrophic as well as activity factors determine mechanical properties. Barany and Close (1971) were able to interchange mechanical properties of soleus and EDL including velocity of shortening and contraction times by cross-reinnervation. These changes correlated with the rate of superprecipitation, a biochemical correlate of contraction, consisting of increased optical density, due to formation of actomyosin, by the addition of calcium, magnesium and ATP to dissociated actin and myosin in solution. They took this to suggest that changes in contraction time are due to alterations in the myosin molecule whose ATPase activity was shown to correlate with these parameters.

Many denervation studies cite prolongation of contraction time, both the time to peak tension and relaxation time, with denervation of EDL and soleus (Gutmann et al., 1972; Finol et al., 1981; Drachman and Johnston, 1975; Webster and Bressler, 1985). These are more marked in EDL (Lewis, 1972; Drachmann and Johnston, 1975). Prolongation of contraction times is associated with an increase in twitch to tetanus tension ratio, a function of the duration of muscle activation and related to SR function.

The fact that the contraction time of both muscles changes in the same direction in denervation, in contrast to differential changes with stimulation and cross-reinnervation, suggests that the cause is an alteration common to both fast and slow muscles. These occur early in denervation and coincide with primary changes in excitable membranes such as impaired surface action potential and sarcoplasmic reticulum properties. The onset of these changes varies with the length of the

distal nerve stump, suggesting they are controlled by a neurotrophic substance depleted from the nerve in proportion to the remaining stump (Finol et al., 1981). Drachmann and Johnston (1975) found parallel changes in mechanical properties of EDL and Soleus muscles whether paralyzed by blockage of cholinergic transmission or denervation, but it is not known if a putative neurotrophic factor would be affected by the former treatment. In addition, denervation changes can be partially reversed by high frequency stimulation if started early (Melichna and Gutman, 1974). Melichnia and Gutman (1974) were able to demonstrate myogenic influence on contraction times by immobilizing denervated muscles in shortened or stretched position, resulting in decreased or increased contraction times respectively. The muscle length used for immobilization also influenced changes in myosin ATPase activity, atrophy and metabolic enzyme activity. These facts illustrate that the neural influence on muscle is an inter-relationship of neurotrophic and impulse transmission effects, and are further influenced by myogenic factors as well.

Neural Regulation of Immature Muscle

Neural regulation of the plasticity of mature muscle has been studied extensively and although there is by no means a clear concensus of the manner in which changes can occur, there are some consistencies. Both the neurotrophic influence by a substance within the neuron and the impulse transmission, play a role in the maintenance of fast and slow twitch muscle characteristics. There are other factors, some possibly myogenic, as indicated by denervation at fixed different lengths, or hormonal (Rubinstein and Kelly, 1985), which exert additional influence. With the developing muscle still establishing these fast and slow-twitch

characteristics, it seems likely that the neural influence would be greater on developing muscle than mature muscle.

(a) Atrophy

In gastrocnemius of the rat, denervated at maturity, Kumar and Talesara (1977) found greater atrophy in fast than slow muscles but following denervation at 10 days of age, atrophy was equal in fast and slow muscles. This differential response with age did not occur with tenotomy. Therefore it was concluded; there is a greater dependence on a neurotrophic influence making developing muscle more susceptable to denervation.

Engel and Karpati (1968) examined denervated gastrocnemius and soleus of newborn rats. At 21 days of age they found 65% of the fibers were atrophied and remained in the myotube stage compared with an absence of myotubes in 21 day controls. These fibers were dark staining in routine myosin ATPase (pH 9.4) typical of type II fibers, while the remaining, larger fibers were paler staining (type I). There were no muscle spindles present. Following neonatal denervation in the guinea pig soleus, in which development is about equal to; a 10 day old rat (Engel and Karpati, 1968), the numbers of persisting type II fibers was greatest following neonatal denervation, less after cordotomy, and least after skeletal fixation, providing additional evidence for the role of a neurotrophic factor.

(b) Ultrastructural changes

Ultrastructural differences persist between fast and slow-twitch muscles in mature muscle following denervation but after neonatal denervation, Shafiq et al. (1972) found ultrastructural characteristics in Z band thickness and mitochondrial distribution did not differ between

EDL and soleus in the rat. However, some maturation of the SR was seen in EDL. A small number of myotubes persisted and the fibers staining with myosin ATPase remained homogeneous in both muscles. All fibers stained equally with myosin ATPase after acid and alkaline preincubation, typical of developing fibers. This contrasted with fully mature controls by 3 weeks of age. Hanzlikova and Schiaffino (1973) also found failure of the normal maturation in content and orientation of mitochondria, Z band structure and triad formation between soleus and EDL which had been denervated in utero 3 days prior to birth. Irregular development of the SR was seen and some fibers showed complete degeneration.

Schiaffino and Settembrini (1970) found neonatal denervation of the rat gastrocnemius did not retard the ultrastructural development of the sarcoplasmic reticulum. The SR hypertrophied, developing enlarged cisterns and forming an elaborate t system, although lacking the usual collar at the M line. The morphological implications should predict a faster muscle, but in mature denervated muscle, increased capacity of the sarcoplasmic reticulum has been shown to coincide with a reduced rate of calcium uptake and a leakiness (Sreter, 1970), indicating an impairment of function in spite of a structural enhancement. These changes contrast with the limited sarcoplasmic reticular atrophy shown in mature denervated muscle (Engel and Stonnington, 1974). It also contrasts to the myofibrillar atrophy, which was marked, and illustrates a differential sensitivity of muscle cell components to neural influence. Dedifferentiation is therefore too simple to explain the complex changes that produce a novel fiber unlike either developing or normal fibers.

(c) Altered Contractile Proteins

Rubinstein and Kelly (1978) examined the myosin ATPase staining and

myosin light chain composition in rat EDL and soleus 14 days after neonatal denervation. In both fast and slow muscles, the myosin stained intensely in routine ATPase reaction (pH 9.4) and posessed fast light chains. Soleus was highly atrophied. They concluded that EDL was not as affected by denervation. However, without the myosin ATPase acid preincubation reaction, it is not known if these were in fact immature fibers. The light chain information does not answer this because immature and adult light chains so far have been shown to be alike in fast muscle. Ishiura et al. (1981) looked at myosin isoforms of neonatal denervated rat soleus and EDL and found embryonic myosin, present at birth, was no longer synthesized at 30 days. Fast light chains were present, as well as a small amount of LClslow, explained by a small number of type I fibers which persisted with prolonged denervation. However, in rats denervated at one week, Butler-Browne (1982) showed continued antibody staining to neonatal myosin as well as anti-fast myosin in rat gastrocnemius muscle. Anti-slow myosin was found in larger fibers which could have begun to develop before denervation at 1 week. Fast myosin eventually replaced neonatal myosin as shown with gel electrophosesis. The large fibers stained with both anti-fast and anti-slow adult myosin, indicating that they were not a typical type I fiber. As mentioned earlier, the light chain complement is the same in neonatal and adult fast muscle making it, alone, an inadequate marker of developmental change. Dhoot and Perry (1983b) found neonatal denervation of rat muscle produced significant atrophy of EDL, particularly type II cells. While some type I cells atrophied, a few hypertrophied. All cells stained darkly for myosin ATPase with alkaline preincubation but only the hypertrophied cells stained with acid preincubation too, a sign

of immature fibers. Similarly, at 14 days, all fibers responded to fast troponin I antibodies and only the hypertrophied cells responded to both fast and slow antibodies. By 21 days, the hypertrophied cells stained exclusively with antislow. This shows that synthesis of fast and slow troponin and supression of fast troponin in slow fibers can occur in the absence of innervation. The same results were found with Troponin C and T. Cells resistant to atrophy have been shown by others in neonatally denervated rat EDL (Engel and Karpati, 1968; Ishiura, 1981) or rat gastrocnemius (Butler-Browne, 1982) and found to stain after alkaline preincubation and yet been assumed to be a type I fiber. Those found by Butler-Browne reacted with adult anti-fast and anti-slow myosin but not with neonatal or embryonic myosin. Ishiura (1981) found the hypertrophied fibers to contain a mixture of fast and slow light chains. These fibers were not from reinnervation as they decreased rather than increased with time. In the work by Dhoot and Perry (1983b), a surprising finding was the different results in gastrocnemius from EDL. Although they are both mixed fiber types, a population of large fibers persisted in EDL with atrophy of virtually all fibers in gastrocnemius. This is an example of the differences that are found between apparently identical fibers types in different muscles.

In a comprehensive study of the synthesis of contractile proteins after denervation and reinnervation in the developing and adult chick, Matsuda et al. (1984) found neonatal denervation in chick did not prevent synthesis of adult myosin heavy chains and light chains, but the neonatal tropomyosin failed to be supressed and a discoordinate protein synthesis was evident. This suggests that in the chick, tropomyosin synthesis is under neural control but myosin synthesis may not be. This contrasts with

the results of Dhoot and Perry (1983b), in rat EDL, where neural influence was not required to produce mature regulatory proteins. This could be a species difference in rat and chicken. Butler-Browne (1982), in rat muscle and Thibault et al. (1981) on cultured chick cells, found a similar lack of neural control on heavy chain synthesis. Muscle cells cultured from satellite cells of fast or slow muscle were not able to produce mature forms of heavy chain (Matsuda and Strohman, 1984). Yet, they both synthesised immature forms of heavy chains and light chains, but not tropomyosins that were distinguishable from each other. Possibly the satellite cells in adult chicken muscle are preprogrammed to synthesize immature forms of myosin heavy chains and light chains specific to fast or slow muscle, but not tropomyosin.

(d) Changes in Contractile Properties

In spite of prolonged contraction times in adult denervated muscle, the differential between fast and slow muscles is retained (Webster and Bressler, 1985), but with neonatal denervation this is lost; mainly due to failure of developmental shortening of the contraction time, with maturation, in EDL (Brown, 1973). After stimulation of denervated developing rabbit muscle, Brown (1973) showed only partial recovery of the ability to reduce contraction time. Therefore activity has a role in developmental changes in contraction time properties.

To assess the influence of altered activity pattern of innervated developing muscle on contraction times, Jones (1981) applied indirect stimulation in tibilais anterior, EDL and soleus in developing rat from 4 days of age. With 10Hz stimulation both fast and slow muscles slowed, while stimulation with 25 Hz decreased soleus contraction time, but the fast muscles were unchanged. These findings show that activity

influences contraction time with the firing frequency a prime determinant. The histochemical and contractile parameters show that the effects of denervation and a low frequency activity pattern share common features. A fast firing pattern may be required to make the muscle become fast. As these were stimulated indirectly it would be useful to know if there could be any retograde influence on the nerve, such as, increased after hyperpolarization, to account for these changes, or even if the altered stimulation pattern could affect release of a neurotrophic protein in the neuron.

Permanence of Denervation Effects in Developing Muscle

To investigate the permanence of any impairment in maturation brought about by denervation, McArdle and Sansone (1977) compared the results of reinnervation following neonatal and adult nerve crush in rat EDL and soleus. They were examined from 14 to 180 days post denervation. Fibers were able to redifferentiate into fast and slow fibers in both groups, but the neonatal groups showed persistant signs of denervation such as; atrophy, lack of muscle spindles, presence of myotubes, wide Z lines, and Z line streaming. A reduced number of multiple endplate potentials was attributed to impaired synaptogenesis and lack of reflex activity. Ultrastructure of the SR and sarcolemmal properties were the same as controls at 6 months following neonatal crush, but calcium uptake, as evidenced by prolonged relaxation times was not. Because mechanical events remained affected yet membrane properties recovered, the neurotrophic influences on these parameters during development must be under separate control. Both may be affected concurrently during denervation, but their difference augmented by selective failure of certain axons or activity patterns during reinnervation. Such a

difference would include lack of spindle afferents. This illustrates the point that reinnervation studies following neonatal nerve crush must be compared cautiously with normal development, as aberrent motor unit activity due to selective survival by tonic neurons has been shown to produce an altered gait pattern (Navarrete and Verbova, 1984); a finding not seen in adult reinnervation. Lowrie et al. (1982) did a similar study in which nerve of 5-6 day rats was crushed and reinnervation permitted. At 2 months, soleus showed full tension recovery whereas tibialis anterior and EDL, both fast-twitch muscles, achieved tension that was only 50% of controls. There was a reduction in fiber number, and all fibers remained oxidative and fatigue resistant. Using horseradish peroxidase, they showed that these differences were not due to reduction of the number of innervating neurons. The difference between these results and those of McArdle and Sansone (1977) can be explained by the fact that neonatal nerve crush coincides with the period of susceptibility of neuron cell death which occurs at birth. By denervating at five days of age, and thus avoiding this time, Lowrie et al. (1982) were able to show that the persistance of denervation changes in their study were not due to loss of innervating axons.

A Model of Aneural Myogenesis in Vivo

Another approach, to studying the effect of innervation on differentiating muscle, was to look at muscle regeneration which has been described as a recapitulation of development (Matsuda and Strohman, 1984) but with some differences (Gutman, 1976). Gordon and Vrbova (1975) found cross-implanted minced fast muscle grafts will regenerate appropriately to the firing pattern of their new innervation and become slow. This indicates the program for maturation of fast or slow-twitch properties is

not myogenic. The increased susceptibility of immature muscles to neural influence was shown by Riley (1974) who found more complete conversion of cross reinnervated muscles when caused to regenerate by cold injury. He suggested that a certain amount of dedifferentiation occurs in all cross innervation studies due to inevitable denervation and may be responsible for permitting the reversal of characteristics by the new nerve.

Statement of the Problem

Developing muscles are more susceptible than mature muscle to the influences of innervation. Without innervation many fast or slow-twitch characteristics fail to develop. A more comprehensive study is needed to provide information about many of the physiological characteristics of neonatally denervated muscle and how these correlate with the histochemical profile. Looking at these parameters in denervated muscle during differentiation into mature fast-twitch muscle, provides an opportunity to understand the interactive effect of denervation on development. This study provides new information regarding the nerve dependency of the contractile and histochemical properties of developing fast-twitch muscle in the C57BL/6J+/+ mouse and contributes valuable information for developmental studies of the cogenic strain of dystrophic mouse.

# II METHODS

All experiments were carried out on the fast-twitch Extensor Digitorum Longus muscle (EDL) of the C57/BL6J+/+ strain of mouse, bred and raised in our own colony from breeding pairs originally obtained from Jackson Laboratories (Bar Harbor Maine). For each group, 6 mouse pups of both sexes from time mated breeding pairs underwent right sciatic neurectomy at 32±8 hrs. of age (N=42). The contractile properties including maximum isometric twitch and tetanic tension, time-to-peak twitch tension (TTP), time to relaxation of half peak twitch tension (1/2RT), maximum velocity of unloaded shortening (Vo), posttetanic twitch potentiation (PTP), and fatiguability and histochemical fiber type distribution were measured in denervated muscles and muscles from age matched controls at 7, 14 and 21 days of age. To characterize the muscles at a time coinciding with the time of denervation, one group of six normal EDL muscles was examined from unoperated animals at 1 day of age.

Denervation:

Pups were anaesthetized with ether and placed on their left side with their right leg supported in a plasticine splint. Using clean but non-sterile technique, a skin incision was made along the lateral compartment of the thigh, the underlying fascia was divided, and biceps femoris retracted exposing the sciatic nerve. The nerve was then carefully retracted with fine forceps and excised from the level of the greater trochanter to beyond its bifurcation into the posterior tibial and common peroneal nerves at the knee. The animal was marked for identification by toe nail clipping of the forepaw, returned to its mother and allowed to recover.

In spite of the flaccid paralysis of the right leg, pups were able to develop an efficient gait which consisted of passive extension of the

hip and knee followed by an abduction-adduction maneuver to bring the denervated limb beside the contralateral limb. The denervated limb was therefore held in the same posture as the normal limb except during ambulation. The contralateral limb was responsible for supporting the weight of the hindquarters plus performing all the work of ambulation for the hindlimbs. Thus, compensatory muscle hypertrophy was likely and therefore the contralateral limb was not used as a control.

All animals used in the denervated group demonstrated positive signs of denervation, including paralysis, dragging of the right foot, and complete loss of active movement at the ankle and toes. In addition, absence of reinnervation was confirmed by the use of acetylcholinesterase and silver staining as described in appendix 2.

Histochemistry:

(a) Experimental Procedures

The animal was killed with chloroform, its right leg removed and pinned to a cork board. The leg was skinned, the crural fascia excised and the overlying tibialis anterior muscle removed, exposing EDL. The proximal and distal tendons were cut and the muscle placed between layers of gauze moistened in 0.9N saline. Using fine forceps, the muscle was embedded in a block of mouse liver which was then mounted in gum tragacanth on a cork chuck. The muscle orientated perpendicular to the chuck in order to obtain cross-sections. The sample was frozen for 30 seconds in isopentane which had been cooled to -160 $^{o}$C in liquid nitrogen, and immediately placed in a cryostat cabinet at -20 $^{o}$C for 1 hour. Serial sections of 10$\mu$m thickness were taken from the midbelly of the muscle and collected on glass coverslips. The sections were dried for one hour and stained with Haematoxylin and Eosin, NADH

Tetrazolium Reductase (NADH-TR) or myosin ATPase reactions, using preincubations of pH 4.2, 4.6 or 9.4 according to the method described by Dubowitz and Brooke (1973). Age and species differences have been found to require adjustments of time, temperature and pH for best identification of fiber types (Gollnick et al., 1983). Therefore, myosin ATPase reaction times were modified as follows: pH 4.2, 2.5 min and 40 min, pH 4.6 5 min and 40 min, and pH 9.4 15 min and 5 min pre-incubation and incubation times respectively. For the myosin ATPase reaction, fiber typing was done according to the nomenclature of Brooke and Kaiser (1970) and Brooke et al, (1971).

(b) Data Collection

Using a Zeiss photomicroscope, overlapping photographs (mag X160) of one myosin ATPase section (preincubation pH 4.2) were made of each muscle. Counts of each fiber type were then made directly from montages, assembled from the prints (final mag X800). Staining of fibers at pH 9.4 was compared with that at pH 4.2 to visualize reversibility of staining according to the method of Brooke et al. (1970).

Table II

Fiber Typing of Fast and Slow Twitch Fibers

According to Myosin ATPase Staining

| FIBER TYPE | 4.2 | 4.6 | 9.4 |
|---|---|---|---|
| IIA | LIGHT | MEDIUM-LIGHT | DARK |
| IIB | MEDIUM-LIGHT | MEDIUM | DARK |
| I | VERY DARK | VERY DARK | MEDIUM-LIGHT |
| IMMATURE | MEDIUM | MEDIUM-DARK | MEDIUM |

Morphometrics:

(a) Experimental Procedures

To examine the extent of atrophy at 21 days of age quantative comparisons were made of total muscle cross-sectional area and individual fiber cross-sectional area in normal and denervated muscles. Whole muscle profiles from the H.and.E. section of the greatest diameter from each muscle were traced onto the digitizing board of the Zeiss Mop 3 Image Analyzer. A Leitz microscope, fitted with a Leitz Camera Lucida attachment, was calibrated by means of a micrometer slide and checked against a known area projected onto the digital pad of the image analyzer. The whole muscle cross-sectional area was measured using the average of three readings. In the same manner the individual myofiber cross-sectional area was measured of all cells that fell on a line predefined as: that which runs through the mid-point of and at right angles to the greatest diameter. The section was orientated so that the line fell vertically, and all cells were counted that were touched by the pointer, as the stage was moved vertically. All measurements were expressed in $mm^2$.

Contractile Parameters:

(a) Muscle Dissection

The right leg was removed as described previously and skinned, secured with insect pins to the silgard bottom of a transparent dissecting dish and immersed in oxygenated buffered Krebs solution. The crural fascia was excised, and tibialis anterior and extensor hallicus muscles were removed. The EDL tendons were then carefully freed, cut and the muscle was pinned through its tendons, at approximately slack length, to the bottom of the dish. Subsequently, ties of 10-0 surgical silk were

placed at the myotendinous junctions. This proceedure was used to reduce tendon series compliance.

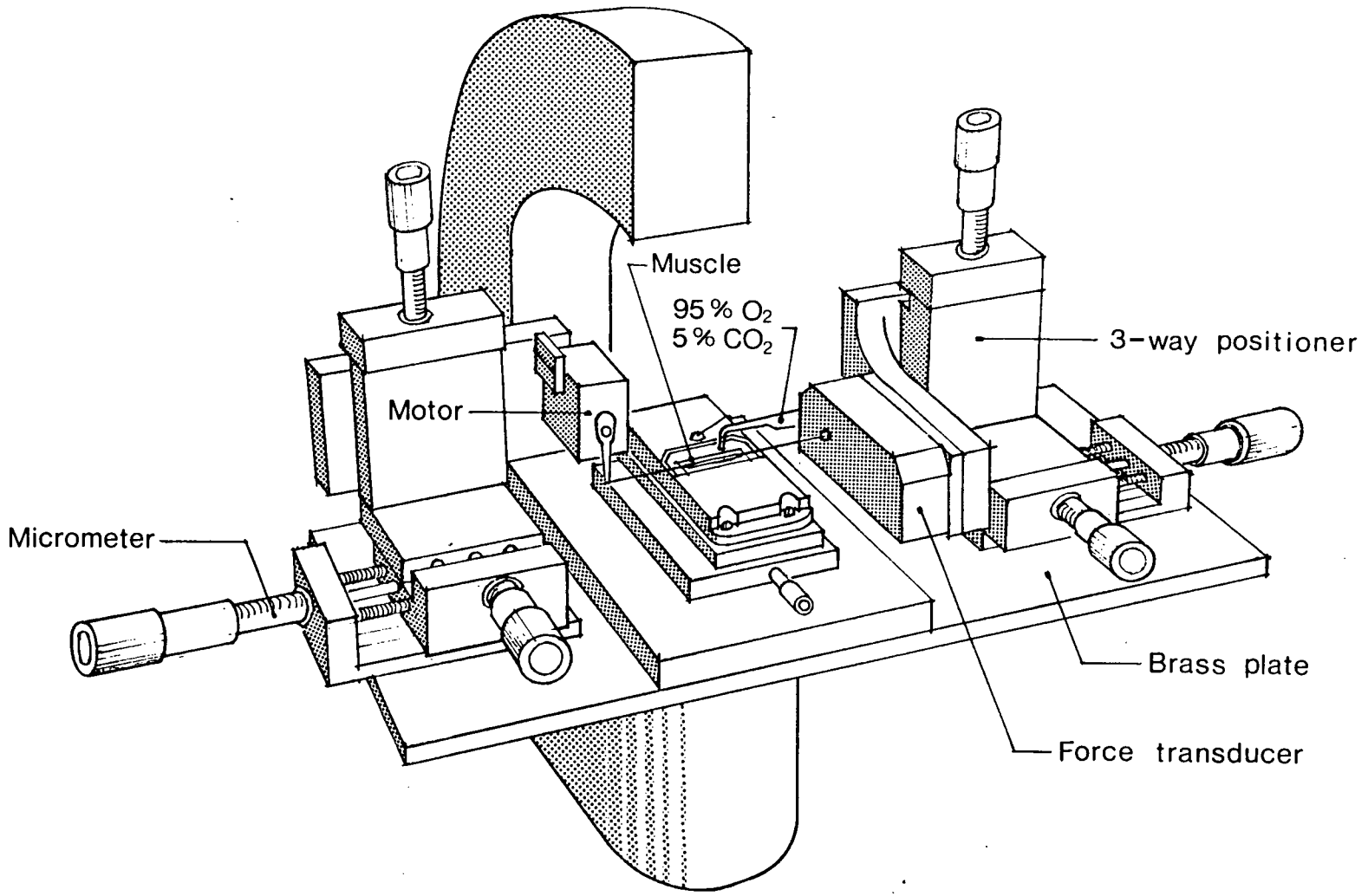
(b) Experimental Apparatus

The muscle was then transferred, under a bubble of Krebs solution, in a small polyethylene boat, to the experimental chamber shown in Figure 1. With the aid of a dissecting microscope the tendons were tied at one end to the stainless steel extension of a force transducer (resonant frequency 2.0 KHz) and at the other end to a galvanometer torque motor. The motor was part of a length servosystem which was extremely stiff and allowed examination of the isometric contractile properties of active muscle. By means of three way positioners, the muscle was critically aligned and the length adjusted. Throughout the experiment the muscle was immersed in Krebs solution which contained, 115mM NaCl; 25mM $NaHCO_3$; 1.2mM $NaH_2PO_4.H_2O$; 5.0mM KCl; 3.4mM $CaCl_2$; and 1.2mM $MgSO_4.7H_2O$ and 2gm glucose /liter.) gassed with 95% $O_2$ and 5% $CO_2$ and maintained at a pH of 7.3, and a temperature of $21^oC \pm 1^oC$ (see appendix 3) using a Haake Fe thermostatically controlled pump. The temperature was monitored by means of a thermistor (not shown in the figure).

(c) Experimental Proceedures

Crow and Kushmerick (1983) found the maximum tetanus tension occurs at sarcomere lengths of 2.7 microns and the maximum twitch tension at 2.9um in mouse EDL at 3 to 4 weeks of age. In this study, muscle length was set to the maximum twitch height, as contraction times and post-tetanic twitch potentiation were measured from the twitch. Voltage was set at that which produced maximum twitch height as well. For tetanic contractions, the stimulus frequency and duration were adjusted to produce a fused tetanus tracing. Stimuli of supramaximal square

Fig. 1 Experimental apparatus: The muscle is immersed in Krebs solution in bath chamber, tied at one end to the lever arm of the motor and at the other to a stainless steel wire extension of the force transducer. It is continuously bubbled with 95% $O_2$ and 5%$CO_2$. Length adjustment is made by means of a 3-way positioner. Temperature is monitored by means of a thermistor and controlled by circulation of water through the bath chamber (not shown in diagram).


Muscle
95 % $O_2$
5 % $CO_2$
3-way positioner
Motor
Micrometer
Brass plate
Force transducer

pulses of 1.0ms duration were provided by a Digitimer DS2 Isolated Stimulator and displayed on an oscilliscope. Throughout the experiment, a stimulus pattern of 1 tetanus followed by 3 twitches with a 90 second interval, between each contraction, was used so that tension decline due to fatigue was minimal, never exceeding 5%. A minimum of 4 tetani and 9 twitches were recorded.

Vo was then measured using the slack test method of Edman (1979). This consisted of giving the muscle ramp length changes of 100 Hz during the plateau of an isometric tetanus sufficient to reduce the tension to zero. The release caused a rapid fall in tension which remained at the baseline while the muscle contracted to take up the slack. Releases of four different amplitudes were used. The time taken to take up the slack was proportional to the amplitude of the length change. The slope of this relationship is therefore the velocity of unloaded shortening.

Following the Vo determination, the stimulator was turned off and the muscle allowed to rest. After a standard 20 minute period, post-tetanic twitch potentiation was measured. This consisted of a pre-twitch, followed in 90 seconds by a 1 second tetanus and 20 seconds later by a post-twitch (Fig. 2). Bressler and Glotman (unpublished data) have shown that, for the mouse EDL at 20$^{o}$C, PTP is maximal at 20 seconds following the tetanic stimulation. One oscilliscope recorded the pretwitch and the superimposed post-twitch. The tetanus was recorded on a second oscilliscope. The fatigue profile was studied using a regime of a one second tetanus at the rate of 12 per minute for 6 minutes. The muscle length was measured using fine calipers. The muscle was trimmed, up to the musculotendinous junctions, placed in a preweighed dessicator cup, and then finally weighed on a 6 place balance in a temperature and

humidity controlled room.

(d) Data Collection

The analogue signal from the tension transducer was recorded directly on an APPLE IIE and stored on disc. Custom software written for analysis of tension data was used to calculate the twitch parameters of maximum twitch tension (Pt), maximum twitch tension/muscle weight (Pt/mwt), time to peak tension (TTP), time from peak tension to one half amplitude of peak tension (1/2RT) and tetanus parameters of maximal tetanic tension (Po) and maximum tetanic tension/muscle weight (Po/mwt). The contractile responses were also recorded on 35mm film with an Asahi Pentax camera fitted with a macrolens and mounted on the oscilliscope frame. The parameters of Vo, PTP and fatigue were measured directly from the film. The negatives were placed in a standard photographic enlarger and the records analyzed from the projected images. For the maximum velocity of shortening, linear regression analysis was used to calculate the slope of the relationship of the length change to the time as shown in Figure 2. All Vo values are expressed in muscle lengths per second (Lo/sec). Pre- and post-twitch tension measurements were expressed as a ratio of post-twitch over pre-twitch. For the fatigue profile, each tetanic contraction was normalized with respect to the initial tetanus tension of the fatigue regime and plotted against time.

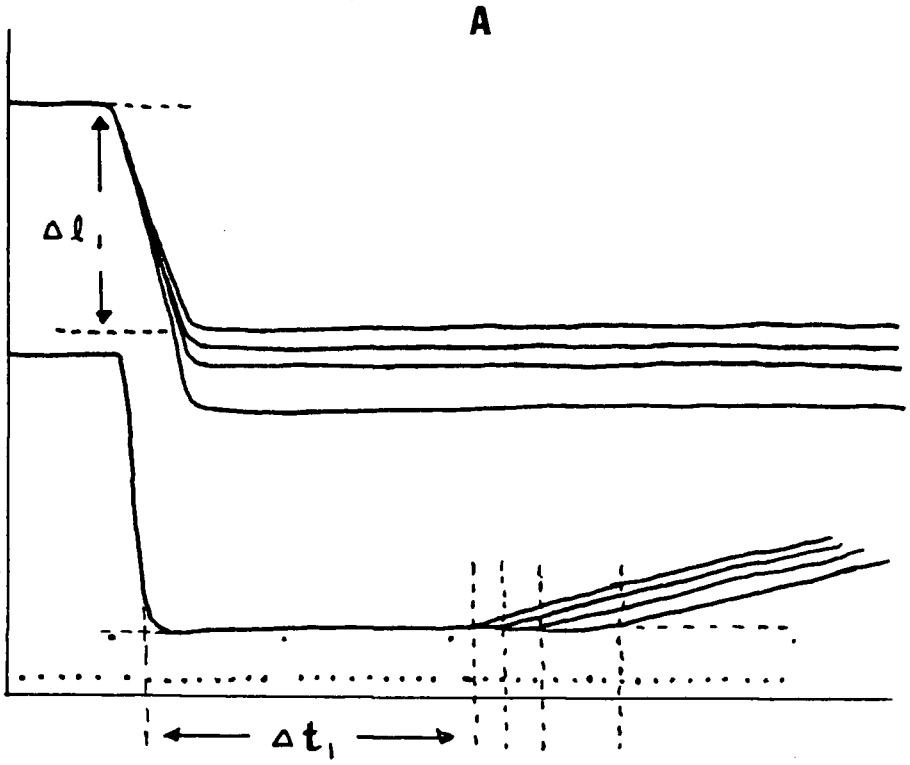
Data Analysis

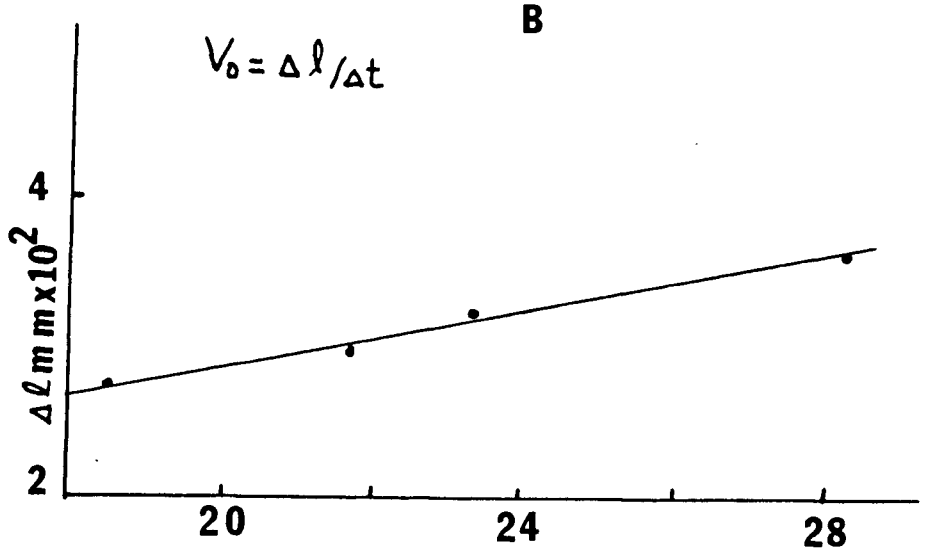
Data for groups at 7, 14 and 21 days of age, was subjected to two-way analysis of variance in order to assess changes with time (group effect) and changes due to denervation (group effect). To examine the changes that occurred from the point at which denervation was done, one-way analysis of variance was applied to data of 1, 7, 14 and 21 days

Fig 2. Determination of unloaded shortening velocity (Vo) by the slack test in normal EDL at 1 day of age.

Fig.2A, imposed length changes and resulting force responses for one series of four releases. Change in length is the amplitude of the release and change in time is the time required to take up the slack.

Fig 2B shows the line representing the least-squares regression of change in length upon change in time. The slope is then divided by the muscle lengths to express Lo in Lo/sec.




Slope = 9.36mm/msec÷3.9mm = .4 Lo/sec

Fig 2

of age. This method of analysis also allows for examination of the interactive effect of denervation and development, that is, whether the pattern of normal development has indeed been interfered with by denervation. Where an interactive effect was found, all pairwise camparisons were made using Tukey's test.

Total cross-sectional area of normal and denervated groups at 21 days were compared using Student T-test. Results from measurements of individual fibers form 4 normal and 4 denervated EDL at 21 days of age were collapsed to groups of 10 $\mu m^2$ and the frequency distribution was plotted for each group.

# III RESULTS

Growth

Changes in growth parameters of normal and operated animals are shown in Table III. In unoperated animals, there was a significant increase in animal weight, muscle length and muscle weight at each period studied. In operated animals, the increase in animal weight kept pace with unoperated controls. Muscles from both groups continued to grow in length at each age studied with a small, but significant, reduction in the denervated group when compared with controls. The normal muscles continued to increase in weight throughout the time period studied. After denervation, there was a significant difference in muscle weight between normal and denervated EDL by 14 days of age which persisted up to 21 days. Moreover, even though the normal muscle weight continued to increase significanatly from 1 to 21 days, this maturational change was arrested in the denervated muscles by 14 days.

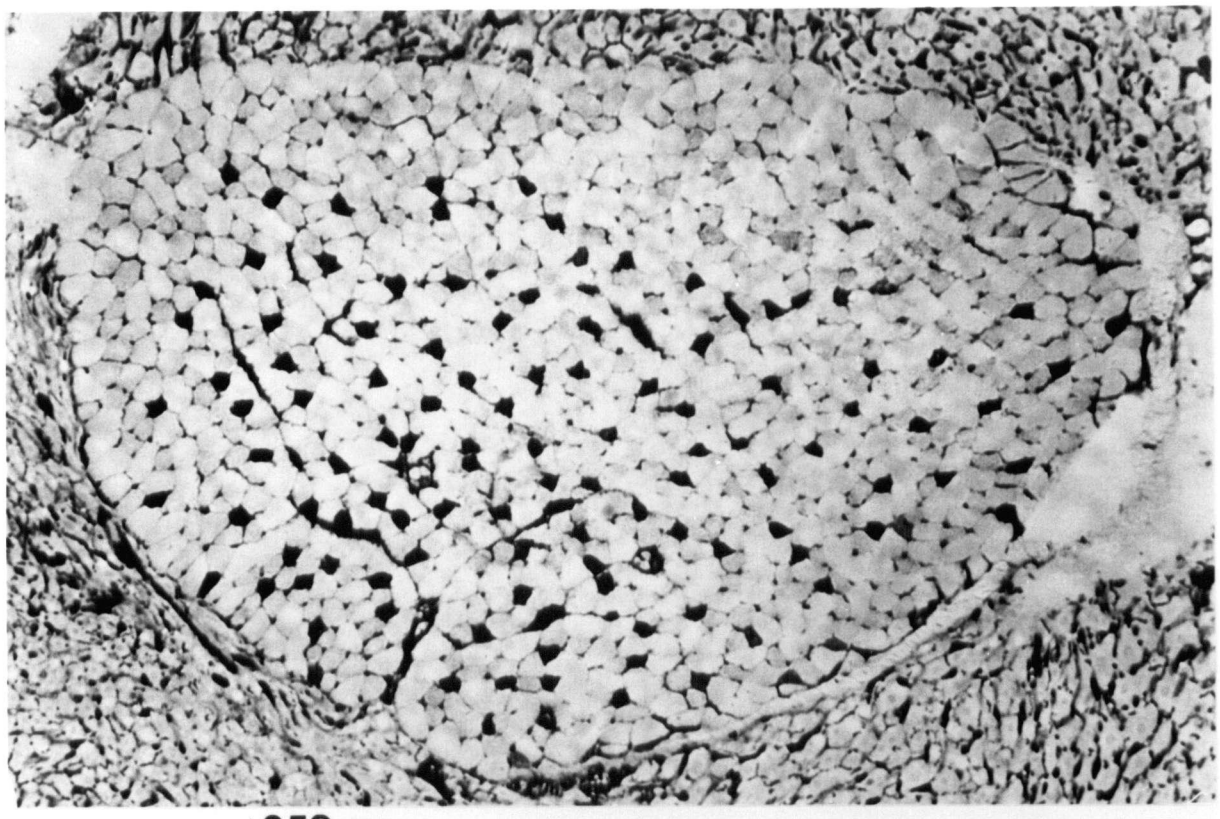
A small sample of whole muscle cross-sectional areas, measured in normal and denervated muscles at 21 days of age, is summarized in Table IV. There is a significant difference between normal and denervated EDL whole muscle cross-sectional areas was reduced by 67% in the denervated compared with normal at 21 days. Photomicrographs of normal and denervated whole muscle cross-sections at 21 days of age illustrate the atrophy produced by denervation (Fig. 3). Figure 4 shows the individual fiber cross-sectional areas measured from 4 normal and 4 denervated muscles at 21 days of age. Atrophy was seen to occur in all denervated muscles by 21 days of age. The majority of fibers in denervated muscle are smaller than the smallest fibers in 21 day controls.

TABLE III

Growth Changes in Animal Weight, Muscle Length and Muscle Weight (EDL) in Normal Mice and Those Denervated at 1 Day of Age

| Days | | 1 | 7 | 14 | 21 |
|---|---|---|---|---|---|
| Animal Weight (gms) | Norm | 1.57 ± 0.05[a] | 4.37 ± 0.02 | 7.08 ± 0.14 | 9.39 ± 0.41† |
| | | (5)[b] | (6) | (5) | (6) |
| | Den | - | 4.25 ± 0.24 | 7.85 ± 0.39 | 9.25 ± 0.29† |
| | | | (4) | (6) | (6) |
| Muscle Length (mm) | Norm | 3.60 ± 0.16 | 5.77 ± 0.11 | 8.05 ± 0.27 | 9.48 ± 0.30† |
| | | (6) | (6) | (6) | (5) |
| | Den | - | 4.96 ± 0.23* | 7.90 ± 0.34* | 8.73 ± 0.18*† |
| | | | (5) | (5) | (6) |
| Muscle Weight (mg) | Norm | 0.284± 0.044 | 0.874± 0.225 | 1.988± 0.132 | 3.598± 0.412† |
| | | (6) | (6) | (6) | (6) |
| | Den | - | 0.516± 0.212 | 0.937± 0.389* | 1.102± 0.294*† |
| | | | (5) | (6) | (6) |

[a] All values are means ± SE.
[b] ( ) Sample size.
* Significant difference between normal and denervated muscles.
† Significant difference between muscles at 7 and 21 days.



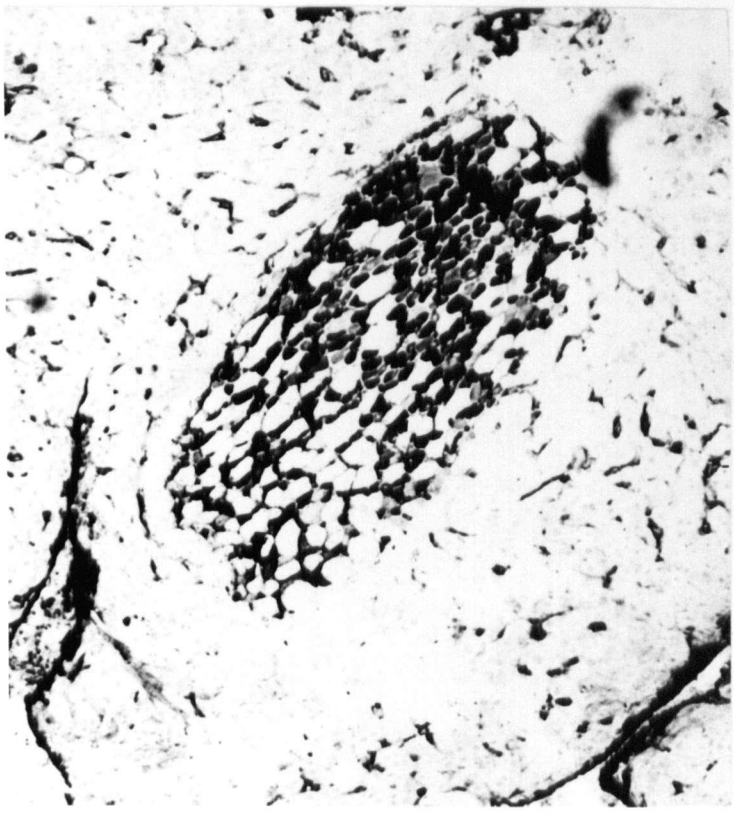




Fig. 3
Whole muscle cross-section, Normal and Denervated EDL at 21 days of age

myosin ATPase
pH 4.2

Table IV

Whole Muscle Cross-sectional Area in Normal and Denervated EDL at 21 Days of Age

| | | |
|---|---|---|
| Norm #1 | 0.573 | |
| Den #1 | | 0.127 |
| Norm #2 | 0.579 | |
| Den #2 | | 0.108 |
| Norm #3 | 0.554 | |
| Den #3 | | 0.096 |
| Norm #4 | 0.503 | |
| Den #4 | | 0.359 |
| Group mean | 0.552 | 0.173* |
| SEM | ± 0.017 | ± 0.063 |

*significant difference between normal and denervated at $p \leq .005$

Histochemistry

(a) Total Fiber Number

The total fiber count for muscles at 1, 7, 14 and 21 days of age is shown in fig. 5. At all age groups studied, there was no significant difference between normal and denervated muscle. However, from birth to 21 days there was no change in total fiber number in the normal EDL whereas, at 21 days the denervated EDL contained significantly less fibers than it did at birth. While there was no significant change in the total fibers in normal muscles at 7, 14 and 21 days there was a small but significant drop in total fiber number between the 1 day normal group and 21 days denervated group.

(b) Fiber Types

In the normal EDL, at one day of age, all fibers stained for the

Fig. 4 Cross-sectional area of individual fibers. Pooled fiber area measurements from 4 normal and 4 denervated EDL at 21 days of age.

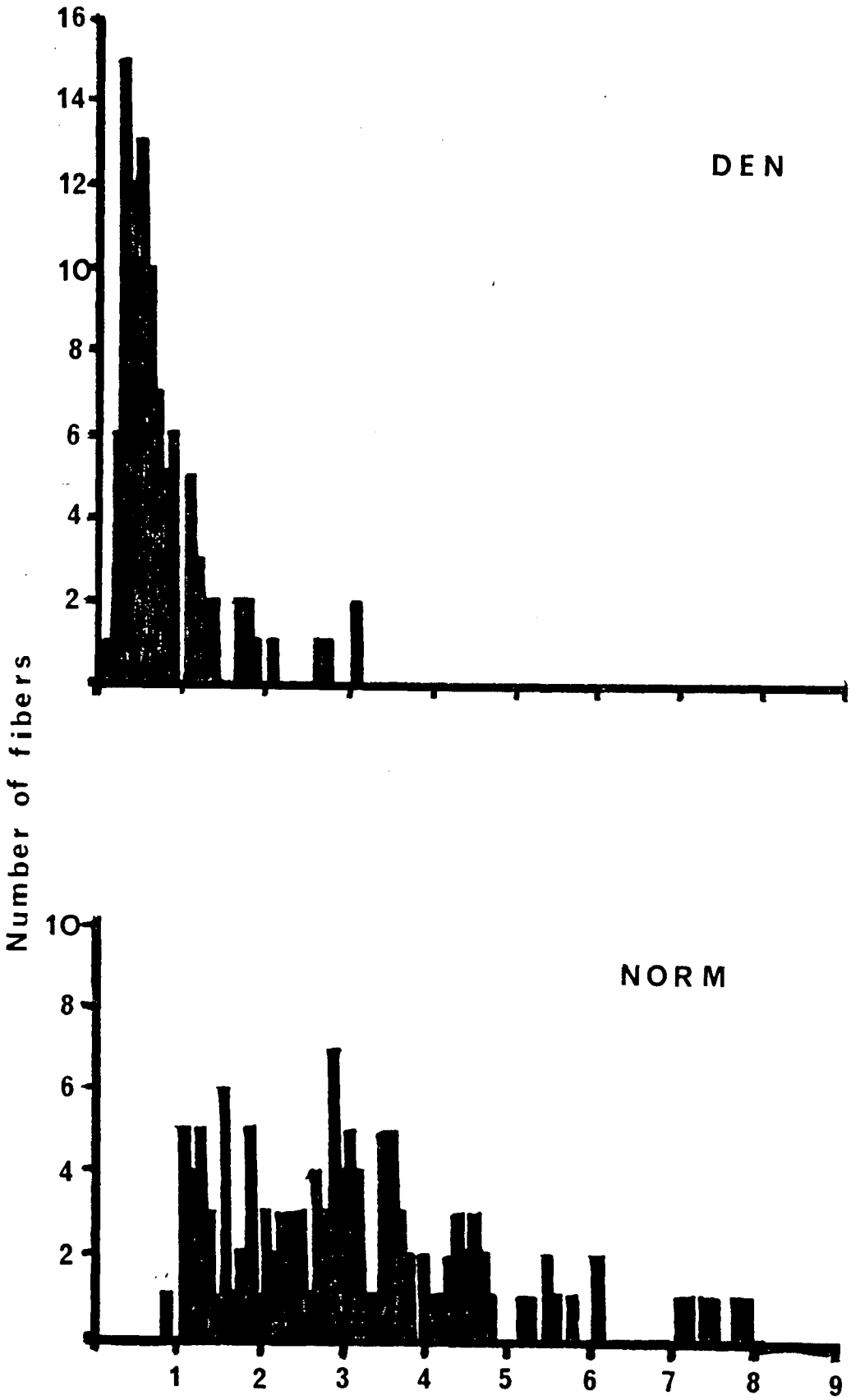

DEN
NORM
Number of fibers
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
Cross-sectional area x 10-4 mm2

Fig 4

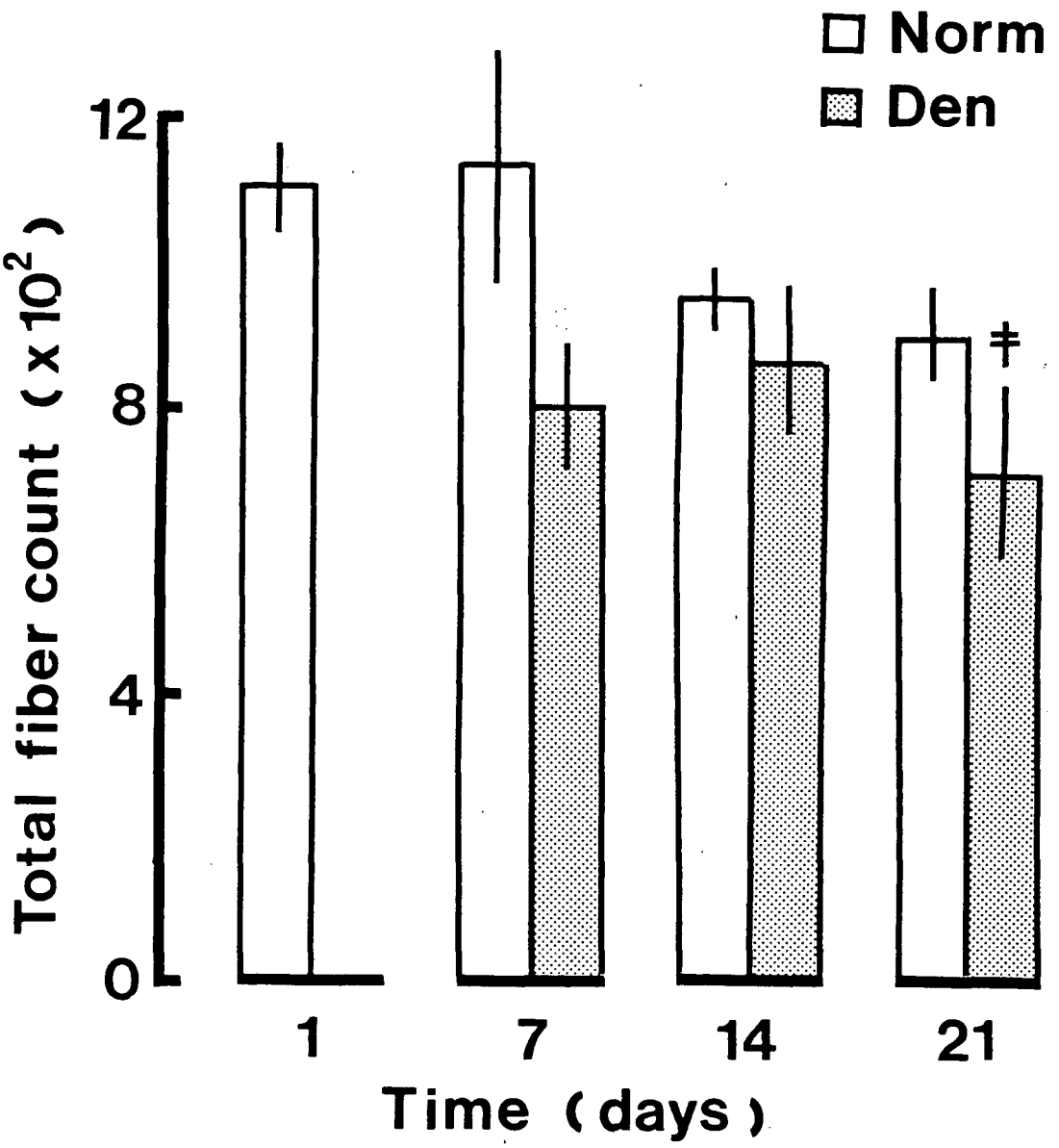
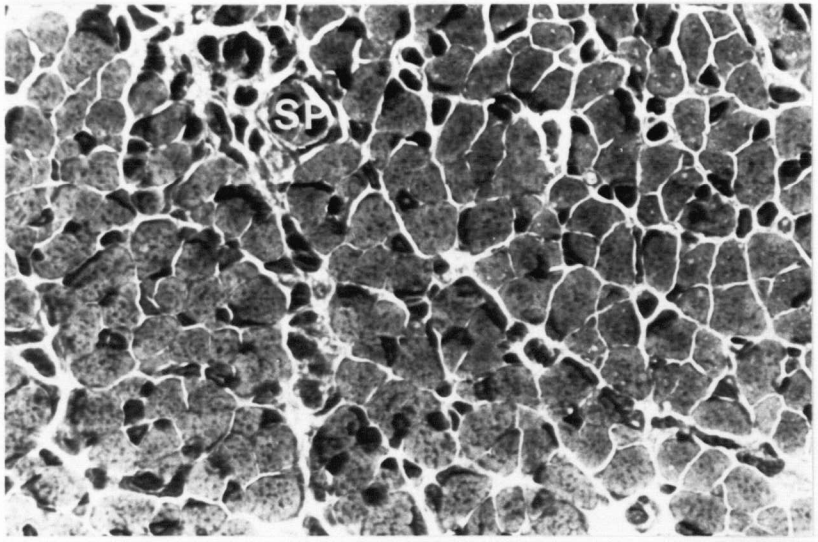



Fig. 5 Total fiber number of normal and denervated EDL
ǂ sig. difference between 1 day normal and 21 day denervated values

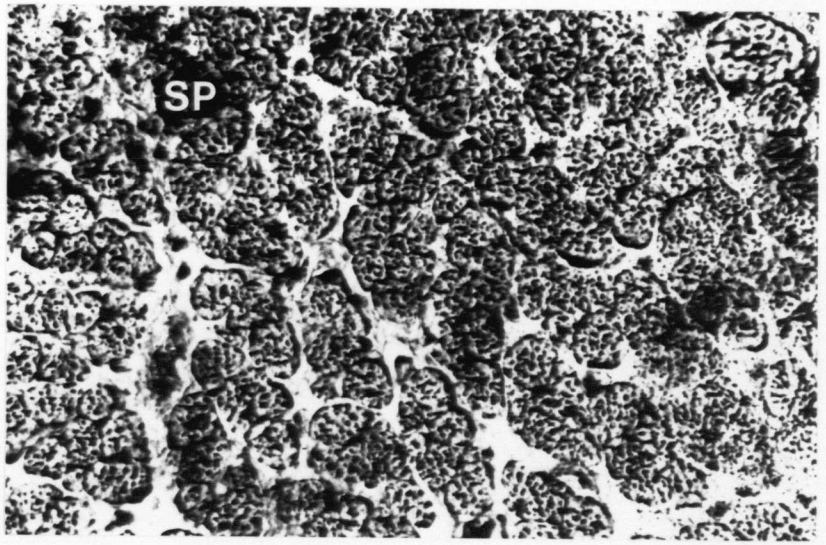
myosin ATPase reaction after both acid and alkaline preincubation (Fig. 6). At myosin ATPase pH 4.2, there were two fiber types discernable: medium-stained fibers or presumptive type II (IIp) and dark-staining fibers or presumptive type I (Ip). All fibers showed medium uniform staining at pH 9.4 preincubation. In addition, there was moderate and uniform staining of all fibers with oxidative enzymes (Fig. 6). Typical staining with myosin ATPase pH 4.2 and NADH, at 7 days of age in normal and denervated EDL are shown in Figure 7. It was possible to distinguish between the two groups of fast fibers, IIA and IIB, however, both the IIA and IIB fibers remained oxidative. The darkly staining group (Ip) was again seen at pH 4.2. These fibers did not show a reversal of staining at pH 9.4. Moreover, as may be seen in Figure 7, all the fibers exhibited uniformly-intense staining with NADH-TR. By 14 days of age, the mature profile of fibers types had been established and remained unchanged at 21 days.

Serial sections of normal and denervated muscle stained for myosin ATPase at pH 4.2 and 9.4 and for NADH-TR at 21 days are shown in Figure 8. In normal EDL, three fiber types could be seen at acid preincubation (pH 4.2): small dark staining (I), medium to pale staining (IIB) and pale staining fibers (IIA). Using preincubation of pH 9.4, the small dark fibers could be further subdivided into those which did not demonstrate reversal (Ip), as seen at 1 and 7 days, and those which were pale (type I) due to alkaline instability. The palest staining fibers at pH. 4.2, (IIA), showed reduced oxidative enzyme reaction with NADH-TR while the remaining were more intensely oxidative. These fibers were labeled to coincide with those named by Peter et.al. (1972) as FG and FOG respectively.

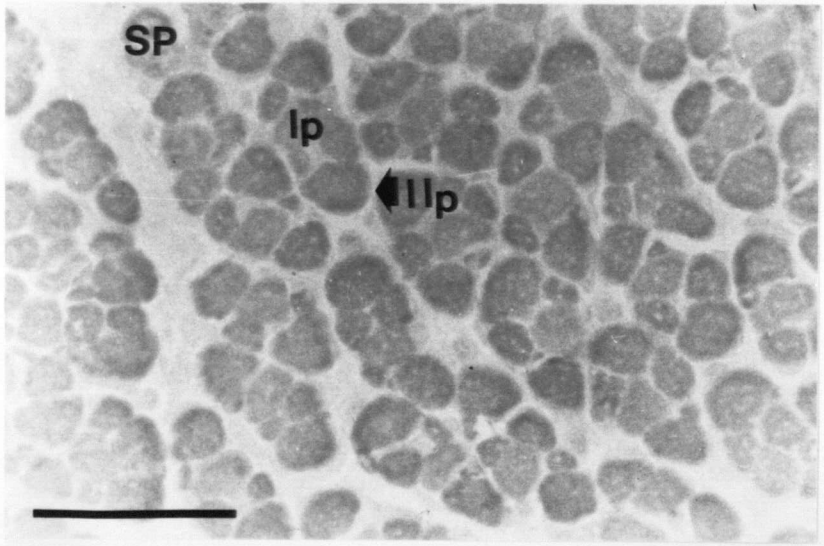
Fig. 6 Histochemical profile of normal EDL at one day of age. Serial sections of EDL at one day of age stained for H and E, NADH-TR, and myosin ATPase at pH 9.4 and 4.2. Bar = 50 μm. A muscle spindle (SP) has been marked for orientation.



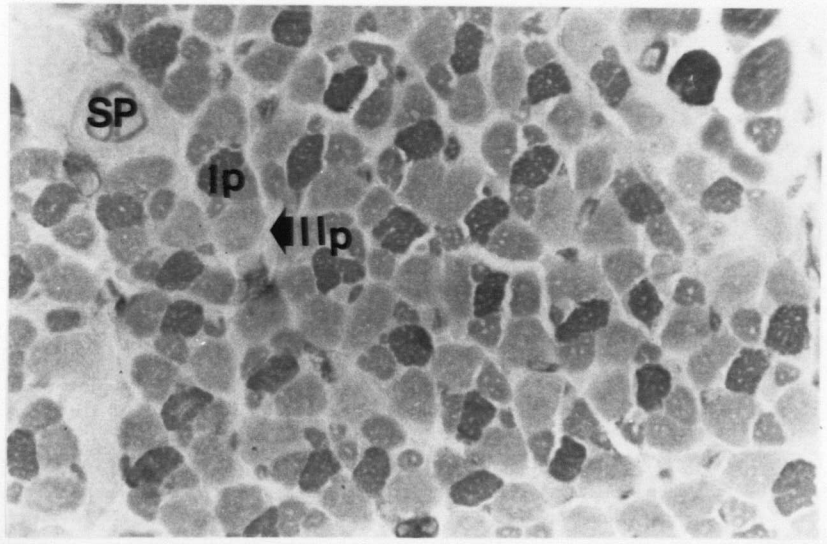

H&E




NADH




9.4




4.2

Fig. 6

Fig. 7. Myosin ATPase and oxidative enzymes of normal and denervated EDL at seven days of age.

Photomicrographs of serial sections:

Upper Left: normal EDL stained with NADH-TR

Lower Left: denervated EDL stained with myosin ATPase pH 4.2.

Upper Right: normal EDL stained with NADH-TR

Lower Right: denervated EDL stained with myosin ATPase pH 4.2

Arrows on NADH-TR micrographs indicate fibers identified in myosin ATPase micrograph

bar = 50 $\mu$m

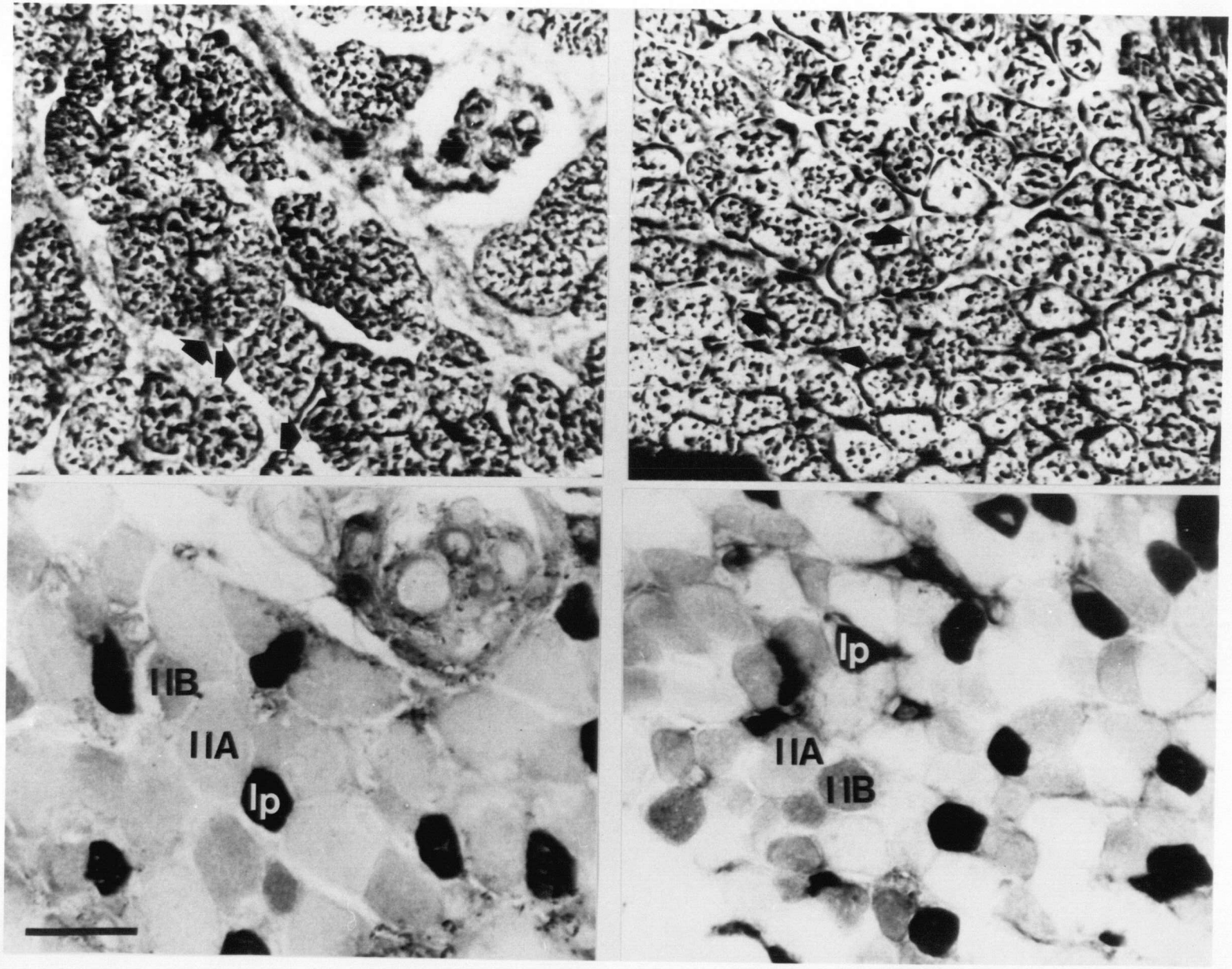



Fig. 7

In the denervated muscles minimal changes are seen at 7 days of age. There was a slightly greater difference in staining of the two fast fibers on pH. 4.2 (Fig. 7). This varied in degree between denervated muscles. The intensity of oxidative staining was the same in normal and denervated muscles at 7 days but precipitated formazan particles as a result of the NADH-TR reaction, marking the enzyme location, were more centrally located in the denervated muscles. At 14 and 21 days, it was no longer possible to group fibers in denervated muscle, using the normal criteria (see for example Fig. 8). A dispersed group of large fibers could be distinguished from the remaining smaller fibers by pale staining with myosin ATPase at pH. 4.2. These were equivalent to normal IIA fibers yet were moderately oxidative with NADH (IIAox). The remaining fibers were heterogeneous with respect to size and stained, albeit variably, at both acid and alkaline preincubation. Up to 21 days, all fibers in the denervated muscle demonstrated uniform moderate staining with NADH-TR (see fig. 8) but differed in that there was a more central distribution of formazan particles compared with the oxidative fibers of controls. A summary of the percent distribution of fiber types in normal and denervated muscle from 1 to 21 days of age is shown in Figure 9. The proportion of type one fibers (type I and presumptive type I fibers) remains constant throughout the period studied. This proportion declines gradually up to maturing where there are very few type I fibers in mature mouse EDL (Parry and Parslow, 1981). In can also be seen that the type IIAox occurs in normal development at 7 days and is seen again in denervated muscle. It is not known if these two types are alike, or if the denervated muscle has different properties.

Fig. 8 Histochemical profile of normal and denervated EDL at 21 days of age. Serial sections stained as indicated. bar = 50 μm

Fig. 9 Fiber Type distribution of normal and denervated EDL

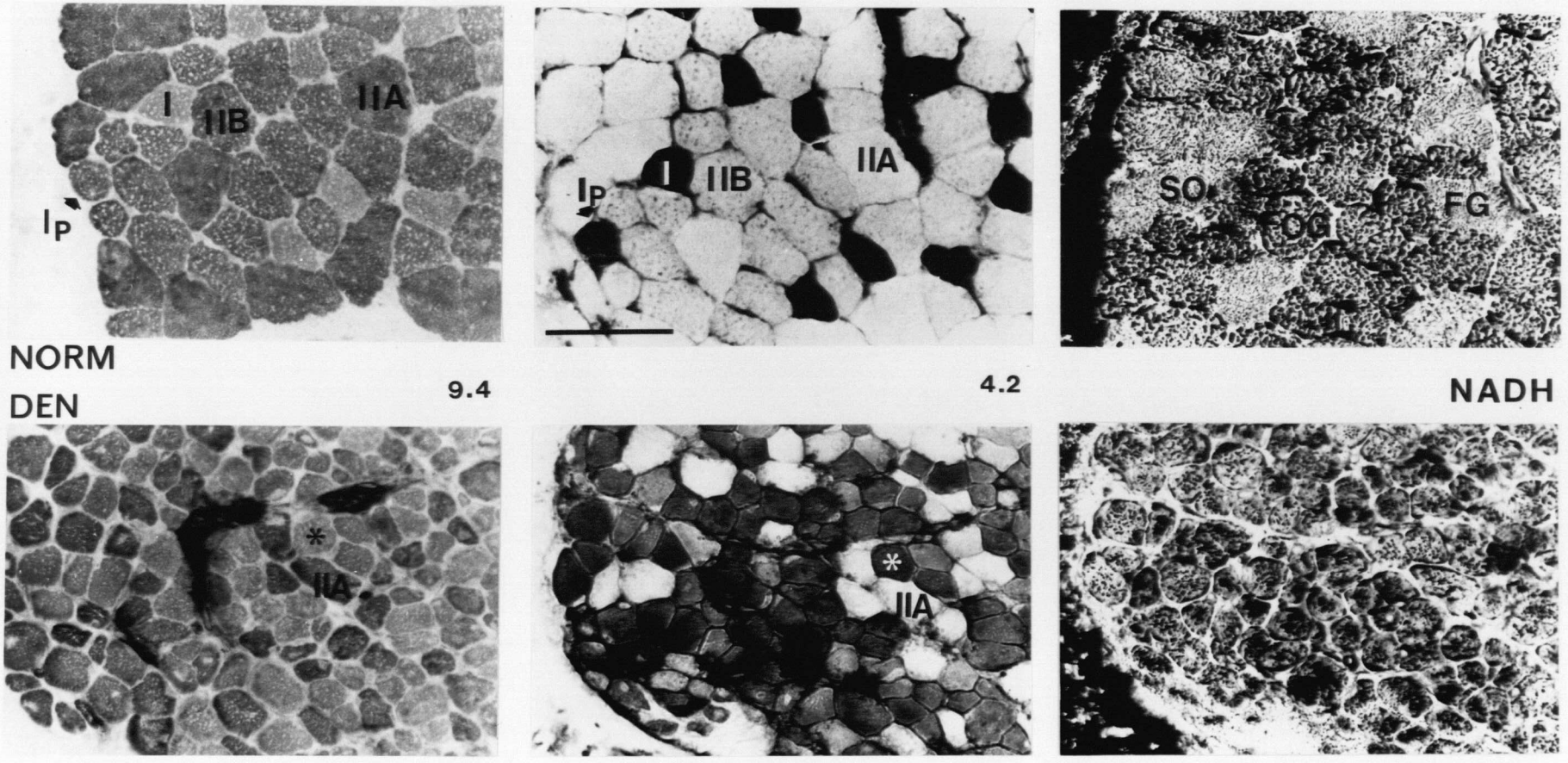



Fig. 8

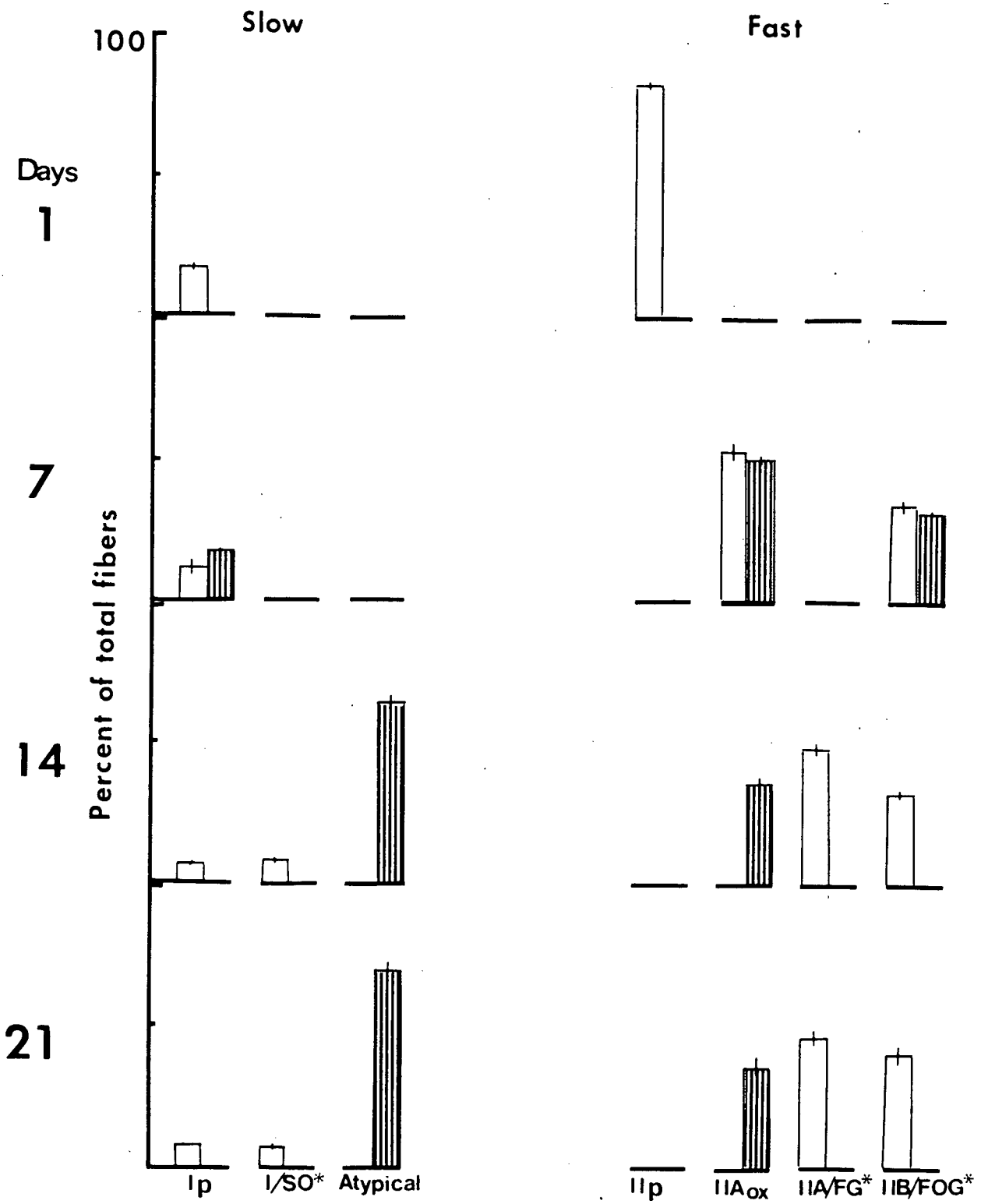

Norm
Den
Slow
Fast
100
Days
1
7
14
21
Percent of total fibers
Ip
I/SO*
Atypical
IIp
IIAox
IIA/FG*
IIB/FOG*

Fig 9

Contractile Properties

(a) Contraction Time

The difference in contraction times between normal and denervated muscles is illustrated in the original records of twitch myograms from normal and denervated EDL at 7 and 21 days of age (Fig. 10). The TTP decreased steadily from day 1 values in normal EDL up to 21 days of age (Fig.11). Following denervation, there is an initial increase in TTP during the first week, followed by a subsequent decrease at 14 days. This parameter remained unchanged at 21 days. In addition, at all age groups studied, TTP of the denervated muscle was significantly prolonged compared with controls. The normal muscle exhibited a similar pattern of change of 1/2RT with development as was seen with TTP. However, in contrast to TTP, the change in 1/2RT between 14 and 21 days was not statistically significant. With denervation the 1/2RT exhibited an initial increase by 7 days, similar to TTP and subsequently decreased but remained significantly slowed compared to normal.

(b) Isometric Twitch and Tetanus Tension

In the control muscles, both absolute isometric twitch (Pt) and tetanus tension (Po) increased at each age studied (Fig 12). In the denervated EDL, twitch tension changes were minimal between 7 and 21 days, although, there was a significant increase when compared with the 1 day values. The tetanus tension, however never exceeded the values recorded at 1 day of age. At each age studied, the denervated muscles produced significantly less twitch and tetanus tension than their controls. The difference in twitch and tetanic tension between normal and denervated EDL was significant at 7, 14 and 21 days. When twitch tension was normalized with respect to muscle weight there was a small effect due to denervation and time (group effects) but no interactive

Fig. 10. Original records of twitch myograms of normal and denervated EDL at 7 and 21 Days of Age. (note change in scale)

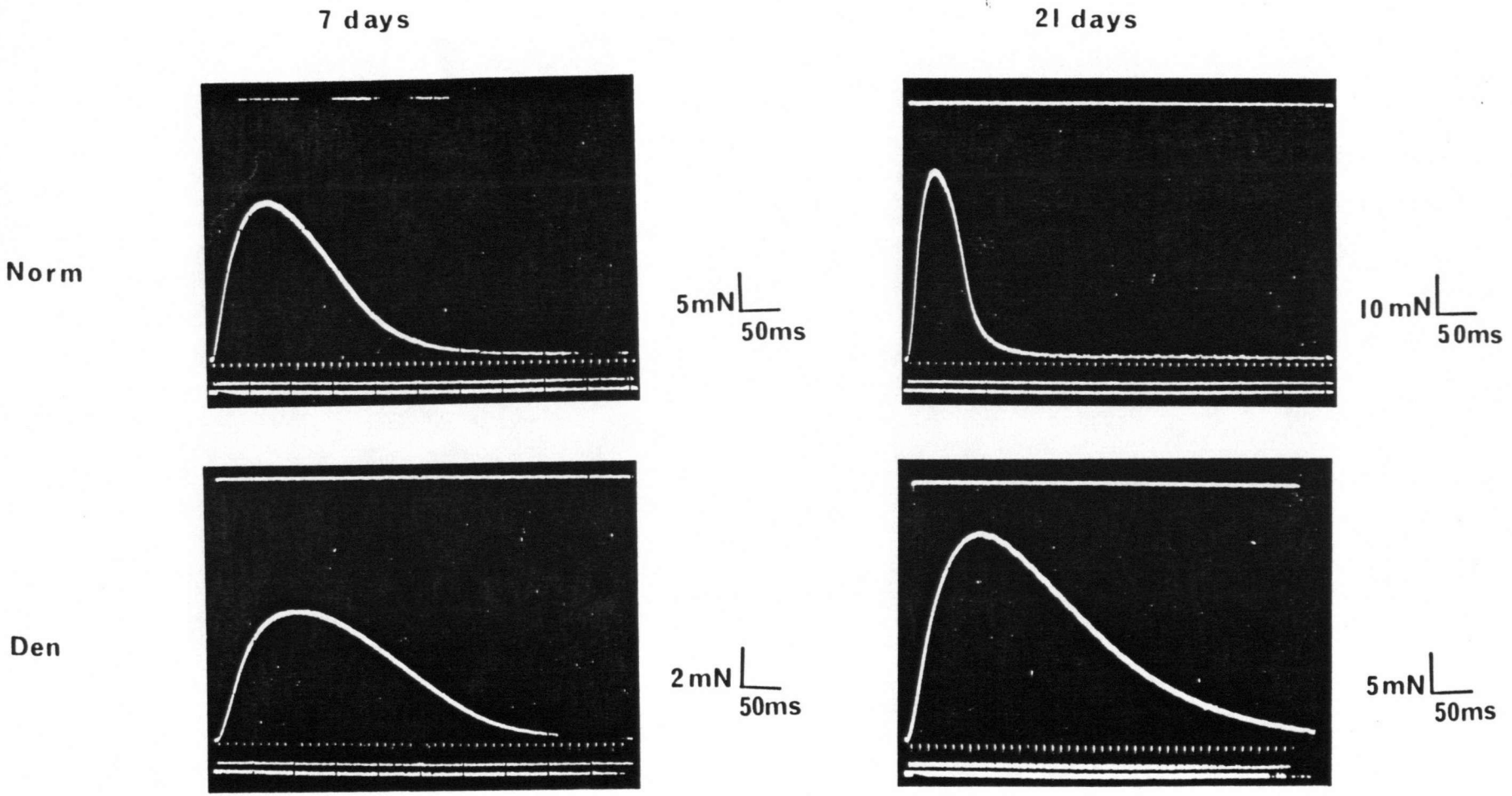



Fig. 10

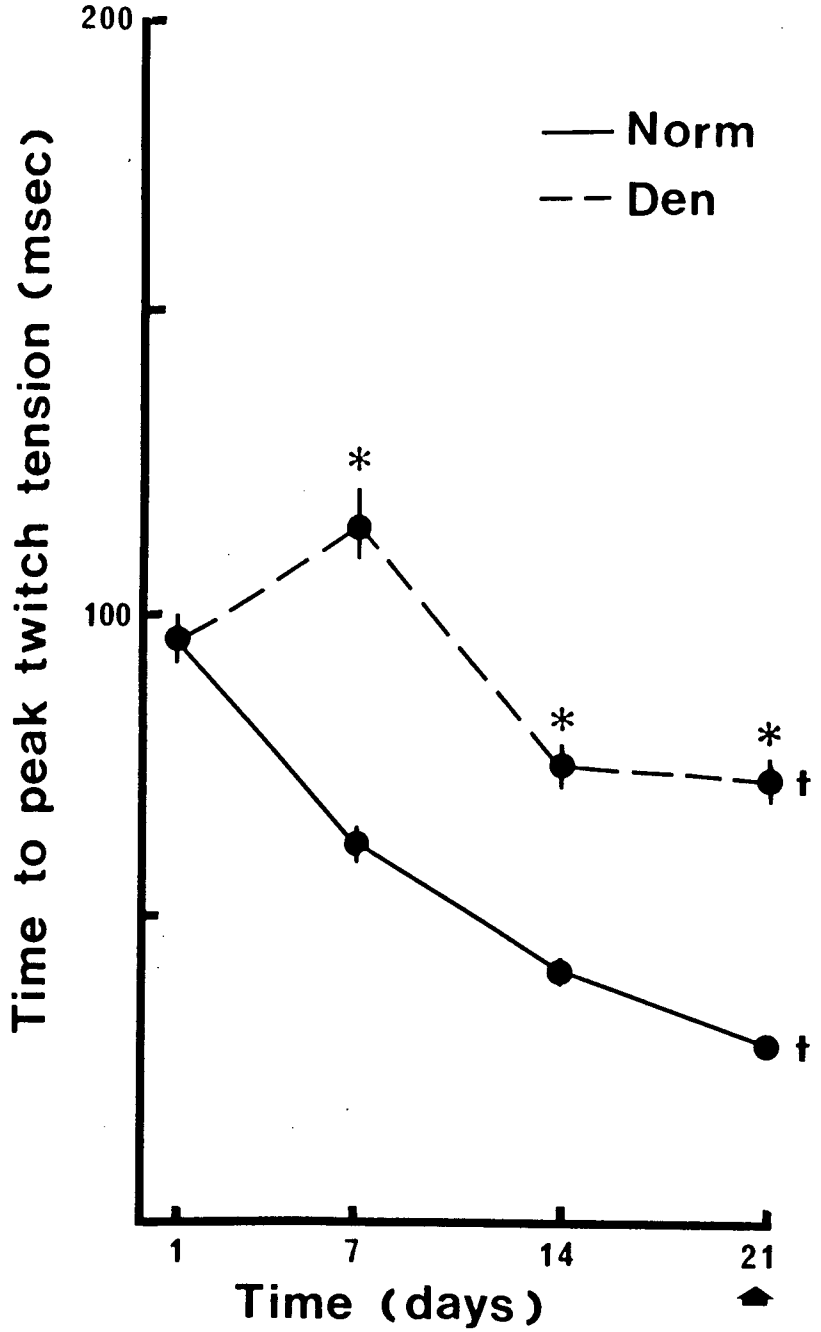
Fig. 11 TTP and 1/2RT of normal and denervated EDL

Fig. 12 Twitch and tetanus tension in absolute values and those normalized to muscle weight of normal and denervated EDL

for both figures:

* sig difference between normal and denervated values

+ sig difference between 7 and 21 day values


200
100
Norm
Den
Time to peak twitch tension (msec)
*
*
*
t
t
1
7
14
21
Time (days)

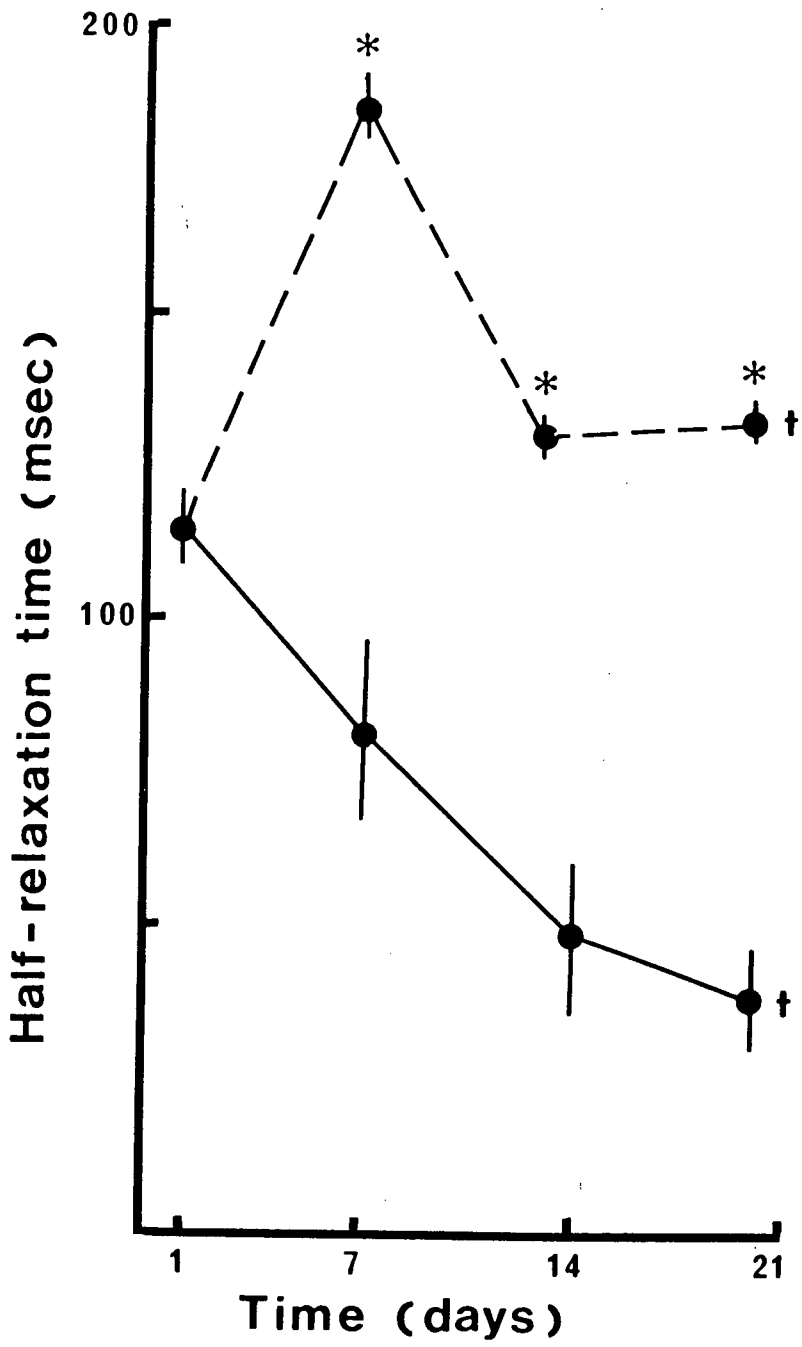
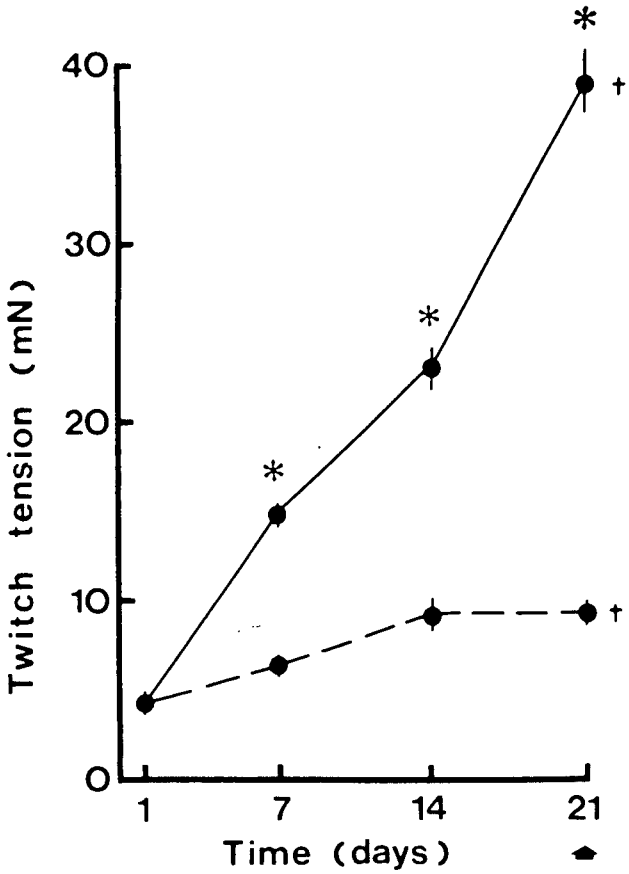
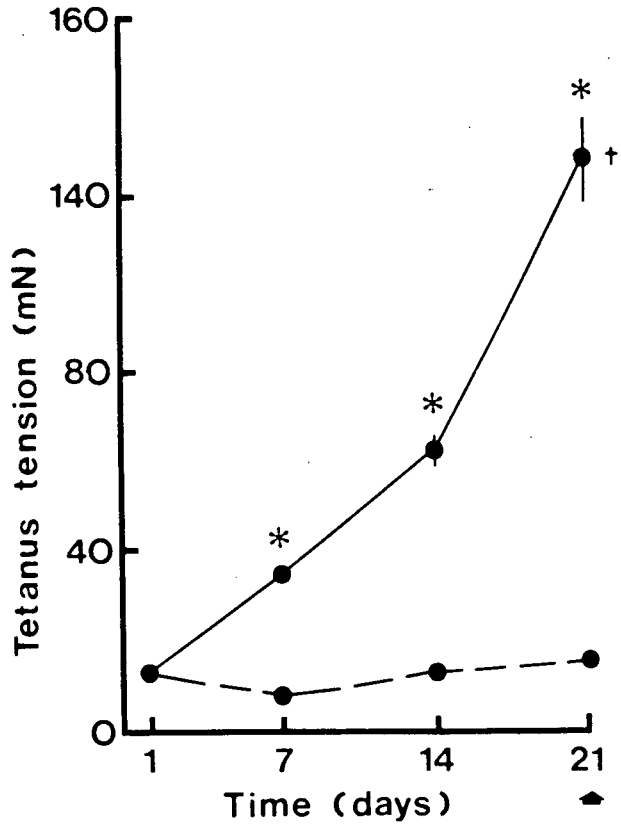
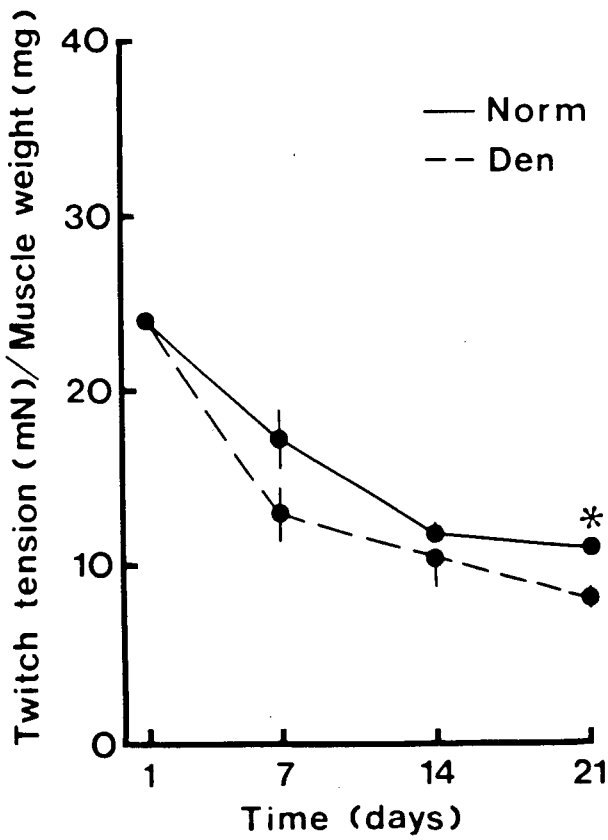

200
100
Half-relaxation time (msec)
*
*
*
t
t
1
7
14
21
Time (days)

Fig. 11


Twitch tension (mN)
40
30
20
10
0
1
7
14
21
Time (days)
*
†


Tetanus tension (mN)
160
140
80
40
0
1
7
14
21
Time (days)
*
†


Twitch tension (mN)/Muscle weight (mg)
40
30
20
10
0
1
7
14
21
Time (days)
Norm
Den
*

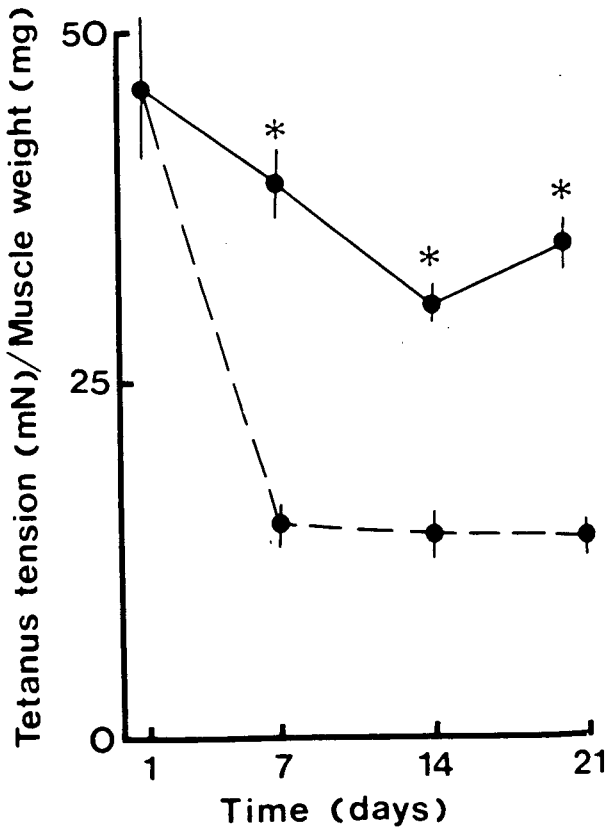

Tetanus tension (mN)/Muscle weight (mg)
50
25
0
1
7
14
21
Time (days)
*

Fig. 12

effect of denervation on development. Normalized tetanus tension showed a substantial drop by 7 days then remained unchanged in denervated muscle (fig. 12). Similarly, there was no net decline in Po between 7 and 21 days in the normal EDL due to a reversal of the trend seen at 14 days. (fig 12). By two-way ANOVA both twitch and tetanus tension showed group effects for time and denervation but there was no interactive effect.

(c) Ratio of Twitch to Tetanus Tension

The ratio of the twitch to tetanus tension is a reflection of the degree of activation in a muscle. Table 5 compares the twitch to tetanus tensions from 1 to 21 days of age in normal and denervated EDL. The twitch to tetanus ratio decreased from 1 to 21 days in the normal EDL. With denervation, there was a marked increase at 7 days followed by a reduction with development. However, the denervated EDL exhibited a significantly higher Pt/Po ratio at 7 through 21 days interfering with the normal pattern of maturation.

(d) Maximum Velocity of Shortening

Table VI is a summary of the means $\pm$ SE of the maximum velocity of unloaded shortening. There was a significant difference at each age studied. In the normal EDL, Vo increased from 1 to 14 days and remained unchanged from 14 to 21 days. However, there was no change in this parameter in the denervated muscle at 7, 14 or 21 days. Moreover, it is noteworthy that the Vo values of the EDL following 21 days of denervation was similar to the normal values at one day of age.

(e) Post-tetanic Twitch Potentiation

Fig 13 shows an original record of Post-tetanic Twitch Potentiation (PTP) in 21 day normal EDL and the results of all PTP measurements are summarized in Table VII. PTP is minimal in the normal EDL at day 1 and

Table V

Twitch Tension to Tetanus Tension Ratio
in Normal and Denervated EDL

| AGE | N | Normal | Denervated |
|---|---|---|---|
| 1 Day | (6) | 0.515 ±0.068 | |
| 7 Days | (6) | 0.425 ±0.053 | |
| | (6) | | 0.847 ±0.056* |
| 14 Days | (6) | 0.370 ±0.026 | |
| | (5) | | 0.708 ±0.049* |
| 21 Days | (6) | 0.309 ±0.041† | |
| | (6) | | 0.606 ±0.081*† |

* sig difference between normal and denervated p≤ .05

† sig difference between 7 and 21 day values

Table VI
Maximum Velocity of Shortening×
in Normal and Denervated EDL

| AGE | N | Normal | Denervated |
|---|---|---|---|
| 1 Day | (6) | 3.080 ±0.24 | |
| 7 Days | (6) | 4.392 ±0.46 | |
| | (6) | | 3.552 ±0.41* |
| 14 Days | (4) | 5.582 ±0.49 | |
| | (6) | | 3.278 ±0.05* |
| 21 Days | (6) | 5.552 ±0.37† | |
| | (5) | | 3.824 ±0.70* |

* sig difference between normal and denervated p≤.05
× Lo/sec.
† sig difference between 7 and 21 day values

TABLE VII

Posttetanic Twitch Potentiation

in Normal and Denervated EDL

| Days | Normal | Denervated |
|---|---|---|
| 1(6,-)[b] | 1.009 ± .014[a] | |
| 7(6,5) | 1.035 ± .013 | 1.012 ± .007 |
| 14(6,5) | 1.069 ± .018 | 1.027 ± .007 |
| 21(6,5) | 1.115 ± .013† | 1.011 ± .007* |

[a] Values are means ± SE, expressed as ratio of posttwitch over pretwitch.
[b] ( , ) Number of normal and denervated muscles tested respectively.
* Significant difference between normal and denervated muscles.
† Significant difference between muscles at 1 and 21 days.

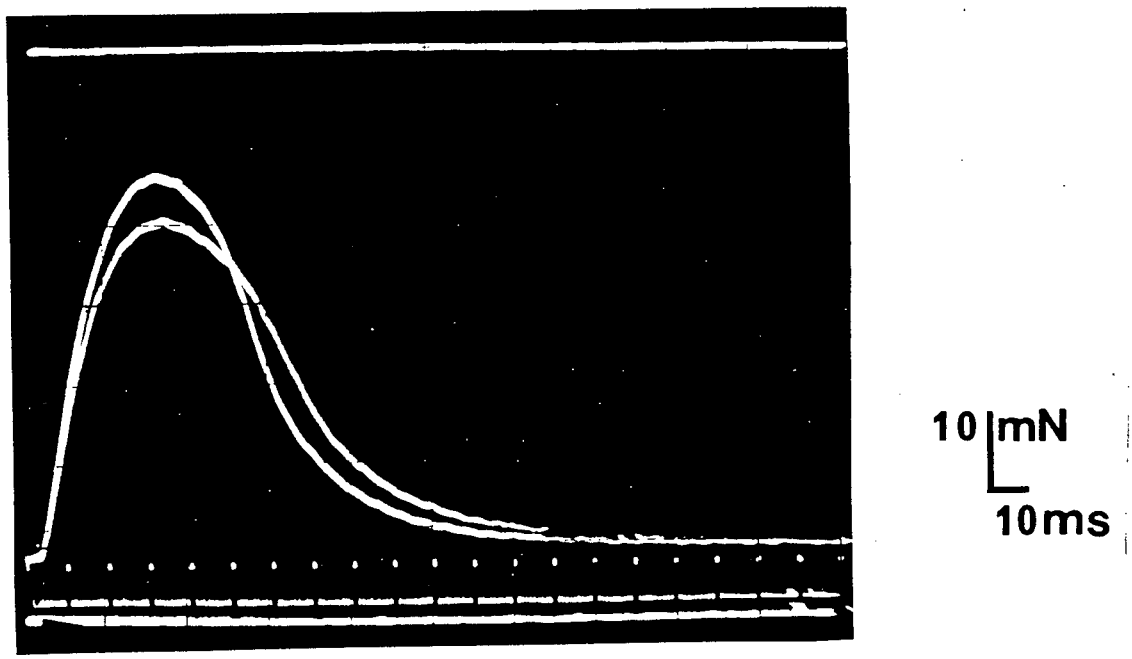



Fig. 13 Post tetanic twitch potentiation in normal EDL at 21 days of age.

Fig. 14. Original records of the first three frames of the fatigue regime of 1 day and 21 day normal and 21 day denervated EDL (note different scales)

Fig. 15 Fatigue profile of normal and denervated EDL at 1, 7, 14 and 21 days of age

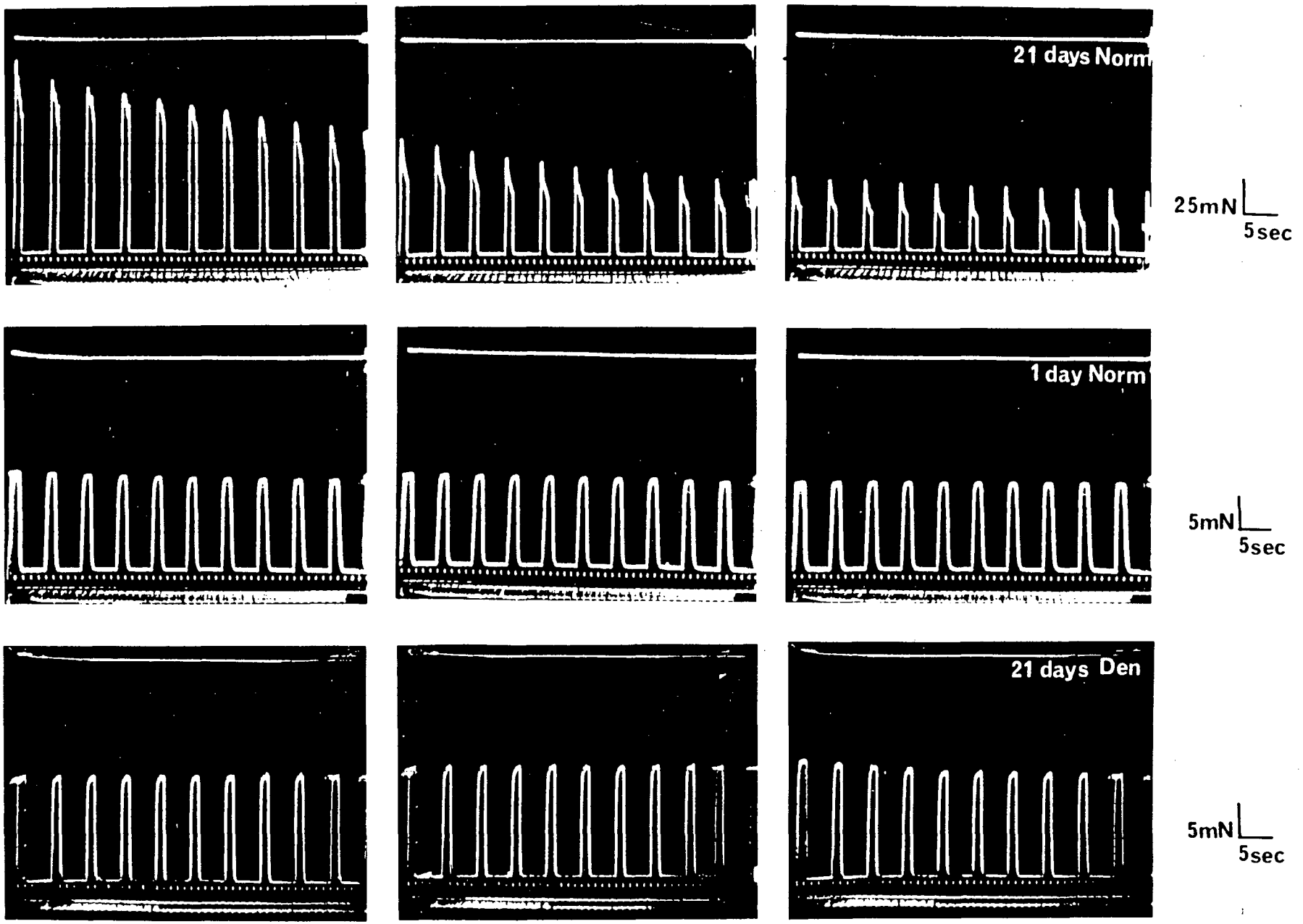



Fig. 14

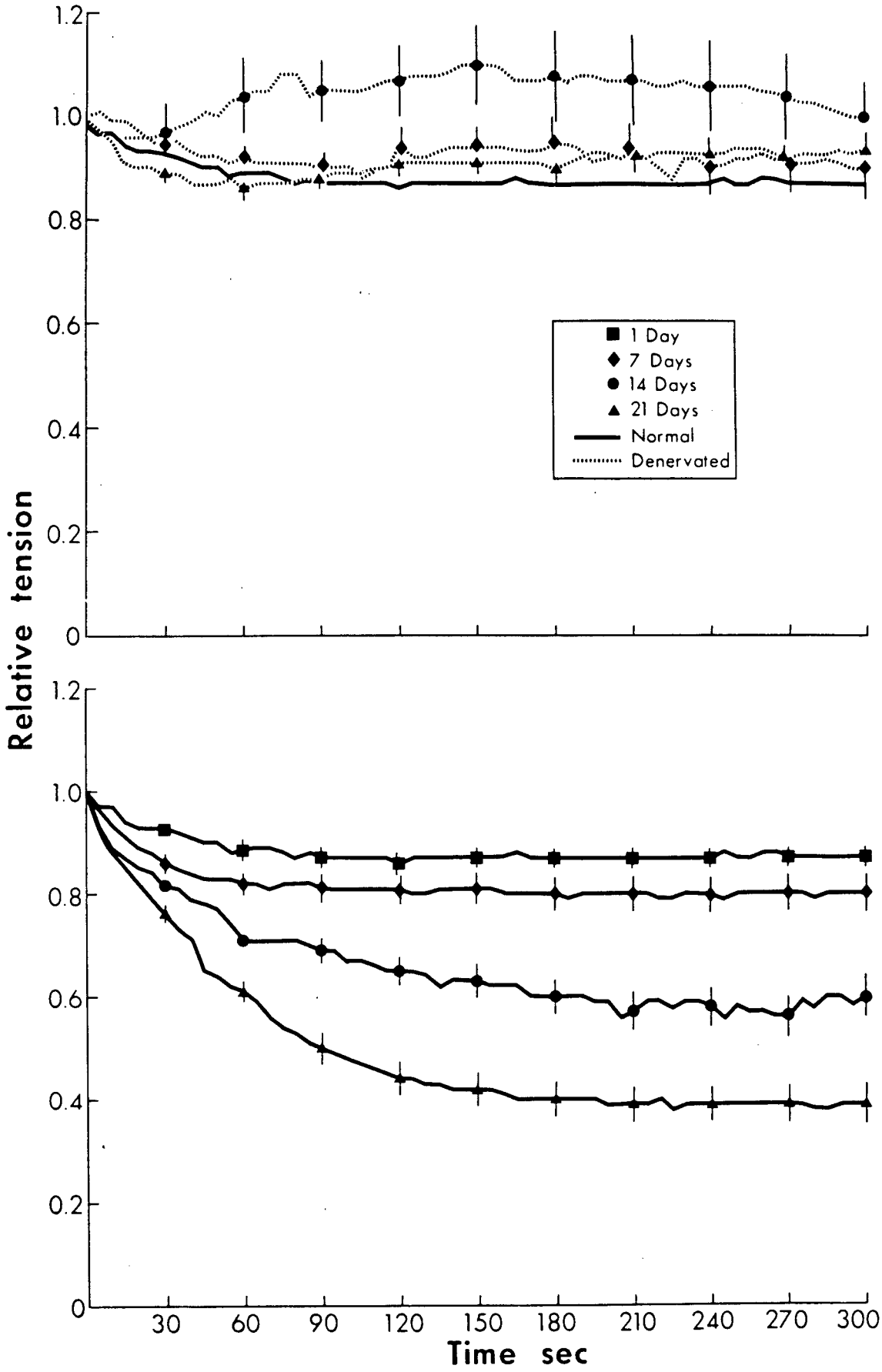

1.2
1.0
0.8
0.6
0.4
0.2
0
1 Day
7 Days
14 Days
21 Days
Normal
Denervated
Relative tension
30
60
90
120
150
180
210
240
270
300
Time sec

Fig. 15

increases over the next three weeks to reach adult values at 21 days of age, but the denervated muscle shows no post-tetanic twitch potentiation.

(f) Resistance to Fatigue

The original records using the first three frames of typical fatigue profiles are seen in Figure 14. The resistance to fatigue of 1 day normal and 21 day denervated EDL can be compared with the rapid fatigue of the 21 day normal muscle. The fatigue profile produced by plotting the means of all normalized tetanic contractions for each group is shown in Figure 15. Normal muscle shows a decrease in fatigue resistance with development at each age studied. The denervated EDL from 7 to 21 days was more fatigue resistance than controls, never returning to values equal to those seen at the time of denervation: 1 day of age.

# DISCUSSION

Studies of the effects of neonatal denervation on the maturation of fast-twitch skeletal muscle show impairment in morphological (Gordon and Verbova, 1975; Kumar and Talesara, 1977; McArdle and Sansone, 1977; Shafiq et al, 1972; Schiafino and Settembrini, 1970), histochemical (Engel and Karpati, 1968; Karpati and Engel, 1968; Shafiq et al, 1972;Ishiura et al, 1981; Butler-Browne et al, 1982;), biochemical (Matsuda et al., 1984; Strohman and Matsuda, 1985) and contractile properties (Brown et al, 1982; McArdle and Sansone, 1977;). Lowrie et al. (1982) reported that neonatal denervation by nerve crush, followed by reinnervation, permanently changed rat EDL to an oxidative, fatigue resistant muscle and with a reduced ability to produce tension, even with the number of functioning motoneurons unchanged. McArdle and Sansone (1977) showed a permanent sensitivity to caffine contractures, suggesting neonatal denervation interferes with the maturation process.

In this work, some general findings on the effects of neonatal denervation may be described. From the 2-way ANOVA analysis of data, examined at 7, 14 and 21 days of age, denervation affected all parameters measured with the exception of animal weight. This shows that the ability of the animal to thrive was not affected by the denervation proceedure or the paralysis of one hindlimb. Further, our limited sham series showed there were no effects on any of the muscle properties studied, from the surgical procedure (Appendix 1). The results may, therefore, be attributed to local effects on the muscle due to deprivation of its innervation. In the normal EDL, all physiological properties, with the exception of maximum velocity of unloaded shortening and all histochemical parameters, with the exception of total fiber number, exhibited expected maturational changes during the 3 week time

period of this study. Denervation altered all parameters studied, showing a significant difference between normal and denervated groups. In addition, there was interference with the pattern of development in all histochemical enzyme profiles and all physiological properties with exception of 1/2 RT, Vo and normalized twitch and tetanus tension values. The muscle showed a small but significant reduction in growth in length and a marked failure to increase in weight, compared with controls.

Growth:

Changes in growth parameters of normal and operated animals are described in Table III. All unoperated animals showed growth due to maturation in animal weight, muscle length and muscle weight at each period studied. Muscle length is increased by division of myosatellite cells and the inclusion in the muscle of one of the daughter nuclei from each division, to provide synthesis of necessary proteins for growth (Moss and Leblond, 1971). Moreover, D. Goldspink (1976; 1980) showed that denervated mature muscle, immobilized in a stretched postion, is capable of increased protein synthesis. However, in this study, although the length of the denervated muscles continued to increase with age, their length was significantly less than controls by 7 days. Synthesis of myofibrillar proteins is therefore able to continue in the absence of innervation. Williams and Goldspink (1976) found length change to be less in immobilized muscles if their nerve is left intact. It is therefore possible that the decrease growth in length is due in part to reduced neural activity but, it may also be due to the habitual resting position assumed by the denervated limb.

Denervation interfered with the developmental increase in muscle weight. At 7 days of age, there was no significant difference in muscle

weight between normal and operated animals but the denervated group failed to gain weight after that mainly due to loss of cross-sectional growth. D. Goldspink (1980) reported that in denervated muscle, the contractile proteins decreased in equal proportion to weight, indicating that the loss of these proteins was one of the major components of atrophy. D. Goldspink (1980) followed protein synthesis for the first 10 days post-denervation and found a cyclicial change over this time period. However protein degradation exceeded the increased synthesis leading to atrophy. Gauthier (1972) points out that systhesis of contractile proteins is possible before innervation occurs and after innervation is withdrawn, but the balance of degradation and synthesis favors degradation after denervation. This is further supported by Pearlstein and Kohn (1966) in which they labelled protein with $C^{14}$-glycine, prior to denervation in adult rat gastrocnemius and tibialis anterior, and followed them for 16 days post operatively. Activity of myosin synthesis and total protein synthesis matched controls and therefore loss of total myosin was due to degradation. Atrophy in denervated muscle was investigated by Schwartz et al. (1985) who looked at the source of lysosomes responsible for protein degradation following denervation. The fact that degradation is greater in denervation than in disuse, is explained by their findings that the lysosomes, containing acid phosphotases, are released from the severed nerve terminal as well as near the neuromuscular junction and from myosatellite cells. Boegman and Scarth (1981) examined autolytic activity in muscle by using a chemical axon transport block (colchicine), neural impulse block (TTX), a combination of the two or denervation. They found increased autolytic enzyme activity occured in all groups but was highest in the combination

and denervation groups. Therefore, this influence is mediated by both neurotrophic and impulse activity. This implies that the nerve may play a role in the autolytic activity of denervated muscle. Finally, in denervated muscle, the presence of ribosomes near the neuromuscular junction and immediately adjacent to the sarcolemma has been attributed to the new synthesis of proteins directly responsible for the increased TTX resistant channels and ACHE sensitivity of denervated muscle. These characteristics are also present in developing muscle.

Morphometrics:

The results of cross-sectional area measurements of whole muscle and individual fibers, in this study, coincide with the results of others who have found that denervation results in muscle atrophy (Engel and Stonnington, 1974; Gauthier and Hobbs, 1973; Webster and Bressler, 1985), which is greater following neonatal than adult denervation (Kumar and Talesara, 1977). Furthermore, there is atrophy in all of the denervated muscles by 21 days, and this atrophy is due to a decrease in individual fiber area. Myofibrillar protein loss is proportional to the whole fiber weight loss (D. Goldspink, 1980) and myofibrillar area decreases in proportion to whole fiber area as assessed at the ultrastructural level (Engel and Stonnington, 1974) indicating the decrease in fiber diameter represents the extent of decrease in contractile protein. The distribution of individual fiber diameter was random and does not suggest a division of three fiber types according to size.

Histochemistry

Ontell and Dunn (1978) have shown that there is no increase in the total fiber number, after birth, in muscles of the rat. They found that errors are frequent when counting fibers at the light microscope level.

In this study, results indicate that there is no postnatal change in fiber number in developing mouse EDL. This is in agreement with the work of Rowe and Goldspink (1969) and G. Goldspink (1980), also in the mouse. In the denervated EDL, total fiber number was significantly decreased by 21 days when compared to fiber number in the 1 day normal group. This could be due to a real fiber loss, an apparent loss due to fibers that do not extend the whole muscle length as described in developing muscle, by Ontell and Dunn (1978) or difficulty distinguishing the small fibers.

Myosin ATPase activity in fast fibers is found to be stable in alkaline and labile in acid conditions and in slow fibers stable in acid and labile in alkaline conditions (Guth and Samaha, 1969). The histochemical typing of fibers by these characteristics depends on the interaction of the effects of the duration of preincubation and incubation proceedures, the temperature at which it is carried out and the pH of the preincubation solution. The two most commonly used fiber typing methods, according to the myosin ATPase characteristics of acid and alkaline sensitivity (Padykula and Herman, 1955; modified by Brooke and Kaiser, 1970), or including fixation at alkaline preincubation have been shown to yield differing results (Green et al., 1982). There are clear species differences and a lack of correlation between fiber type classifications using different enzyme staining, for example, when comparing results of oxidative enzymes and myosin ATPase. In addition, differences have been found between fibers of the same type in different muscles even within the same animal (Muntner et al., 1985). For these reasons, many authors warn that fiber type terminology should be confined to that specifically identified by the method used (Green et al., 1982; Yellin and Guth, 1970; Brooke and Kaiser, 1974).

Brooke and Kaiser have subdivided muscle fibers into; type II fibers that are stable in alkaline, labile in acid preincubation and type I fibers that are stable in acid and labile in alkaline preincubation. They further designated the term IIA to the fibers that are most inhibited in acid conditions and IIB to the fibers that show some stability and therefore continue staining at a pH below which IIA staining is fully inhibited. All these mature fiber types exhibit reversal of their stability and therefore their staining at the alkaline pH (see table II).

A fourth type is occasionally seen in mature fibers which will stain moderately at both acid and alkaline pH, and therefore, lack reversibility of staining. Lack of reversibility is typical of immature, regenerating or transitional fibers which stain moderately after acid and alkaline preincubation conditions (Dubowitz and Brooke,1973).

In this study, all fibers at 1 day of age showed this characteristic lack of reversibility (fig 6) by staining at pH 9.4 and 4.2. Uniform moderate staining was seen at pH 9.4, however, at pH 4.2 it was possible to further subdivide the fibers into those darkly staining (type I) and lighter staining (type II). Rubinstein and Kelly (1978) used the term primitative type II for all fibers found in the rat EDL at birth based on uniform staining at pH. 9.4. Ishuira et al. (1981) found all fibers to show uniform and non-reversible staining in newborn EDL and Soleus of the rat and referred to them as IIC fibers. Although the fibers in this study stained at both pH conditions (immature), it was felt that it was more accurate to describe them as presumptive type I (Ip) and presumptive type II (IIp). This is based on the findings that with acid preincubation, fibers could be subdivided into two groups, those which

were very dark and those which were medium staining. The dark staining group is consistently approximately 10% of the total fiber population (see fig 9) up to 21 days of age, and while the staining in acid preincubation does not change in intensity, the lack of reversibility declines. In the adult mouse, the number of type I fibers eventually decreases to less than 1% (Parry and Parslow 1981). The IIC fiber described by Brooke and Kaiser (1970) is only moderate staining in acid and is likely to correspond to the type II presumptive named here. Following the staining intensity through this three week period it can be seen that the immature fibers progressively stain more lightly and in the adult the distinction between IIA and IIB must be done at a higher pH (4.35). This probably represents a converstion to the more mature forms of myosin. The oxidative nature of these fibers is shown by the moderate, uniform NADH staining of all fibers.

The difference between the dual staining of immature fibers and the reversible staining of mature fibers could correspond to the sequential synthesis of different myosins during development from embryonic and neonatal, to fast myosin, described by Whalen et al. (1979; 1981). Gauthier et al (1982) showed that there was crossreactivity with fast and slow myosins in developing muscle and showed further (Gauthier et al., 1978) that antibodies to fast myosin correspond to alkaline stable and in slow myosin, to acid stable fibers.

At 7 days of age, in this study, a difference in staining could be distinguished between type II fibers. By 14 days, the mature staining profile has emerged and remains unchanged at 21 days of age.

In the mature fiber, the largest fibers were classified as type IIA due to their stability at 9.4, moderate lability at pH. 4.6 and total

inhibition at pH. 4.2. In addition, these fibers showed minimal staining with NADH which indicates they are anaerobic fibers probably of the fast glycolytic (FG) type according to the nomenclature of Peter et al. (1972). A second group of alkaline stable, acid labile fibers which did continue to react at pH. 4.2 are classified as IIB fibers. These fibers stain moderate to intensely with NADH and are therefore oxidative making them fast red aerobic fibers which corresponds with the fast oxidative-glycolytic (FOG) fibers of Peter et al. A third group of fibers are acid stable but with 50% remaining alkaline stable which are termed Ip and with the remainder being alkaline labile are therefore typical type I. These fibers are also oxidative according to intense NADH reaction like the slow oxidative fibers of Peter (SO). These results are at variance with findings of the fast-twitch fibers in the rat (Melichna and Gutmann, 1974; Niederle and Mayr, 1978) where the large anaerobic fibers are type IIB and the small aerobic fast fibers are IIA. The results of this study are in agreement with the findings of Reichmann and Pette (1982;1984b) in fast twitch muscle in the mouse.

As the recommendation is to name the fibers according to the method used and because the oxidative enzymes vary independently particularly with altered activity (Green et al, 1984), the Brooke and Kaiser terms have been adhered to here. It must be emphasized however, that in our findings, the IIA/FG is the large fast white fiber. It is not known whether this difference is due to a species difference in the mouse or to some alteration in staining conditions. It is interesting to note that, in the rat tibialis anterior, Samaha et al. (1970) found the largest fiber was palest (IIA) at acid preincubation, if preincubated for 30 minutes, but was the reverse (medium: IIB) if the preincubation was

brief. This illustrates the inaccuracies inherent in myosin ATPase fiber typing.

The myosin ATPase terminology suggests generalizations to other characteristics of fiber types and the use of IIA for a large fiber that is anaerobic could be misleading to those making conclusions from the rat. However, there is risk in drawing conclusions between like fiber types even within the same animal. Mabuchi et al. (1982) found different myosin isozymes and light chain patterns in fibers classified by their myosin ATPase staining as IIB, from tibialis anterior and adductor magnus muscle of the rabbit. Similar inconsistencies were seen in calcium uptake of type IIA fibers before and after stimulation. Altered conditions could produce changes in one parameter while not changing the fiber type by another parameter.

As has been found by others (Ishiura et al., 1981), the typing of the denervated fibers found in this study does not fit with any of the conventional descriptions of adult fibers. A heterogenous population of fibers, which stain moderately to intensely at both acid and alkaline pH are seen. They do not posess characteristics consistent with any of the fiber types found in the normal mature fast-twitch muscle as they stain after acid as well as alkaline preincubation, typical of immature fibers. It is tempting to presume these are again synthesizing neonatal myosin but there is no proof that this is not a novel form of myosin peculiar to denervated muscle or that the myosin ATPase staining is a reliable indicator of the type of myosin being synthesized. A small population of fibers, generally of greater diameter than the others, shows complete inhibition after acid preincubatiion yet stains darkly with NADH. These fibers are, by definition, IIA although they differ

from the mature IIA fibers in controls, in that they are oxidative and there is no evidence to show that they are in fact the same fibers as those termed IIA in the normal EDL. To stress that fact, they have been termed IIA oxidative ($IIA_{ox}$).

The fibers that are found in the first week after denervation are different from the normal group in that there is a slightly enhanced contrast between staining of the IIA and IIB and they have matured from the one day pattern. They remain uniformly oxidative.

In this thesis, following neonatal denervation, fast-twitch EDL failed to convert to an anaerobic muscle. The oxidative nature of muscle is highly labile. Fast-twitch muscle becomes aerobic with almost all manipulations; increased activity through exercise (Green et al. 1984), low frequency stimulation (Pette et al., 1973), denervation (Niederle and Mayr, 1978) and immobilization (Melichna and Gutman, 1974). These changes in activity of the muscle, be they an increase or decrease, all change the muscle in the same direction. What they all have in common is the absence of a typical fast-twitch motoneuron pattern. However, after spinal cord section, high frequency stimulation of the disused fast muscle resulted in an increase in their glycolytic activity.

In studies on the effects of neonatal denervation on the maturation of fast-twitch muscle using myosin ATAPase histochemistry at only alkaline preincubation, Engel and Karpati (1968) in rat gastrocnemius, Karpati and Engel (1968) in guinea pig gastrocnemius and Dhoot and Perry (1983) in rat EDL, found preferential atrophy of the type II fibers. Ishiura et al., (1981) found a similar atrophy of the type II cells. But with myosin ATPase at 9.4 preincubation and SDS gel electrophoresis for light chains profiles, they found that neonatal denervated EDL was

still capable of maturation in the expression of myosin isoforms. Unfortunately, they did not test for reversal in acid preincubation and did not present the gel data for the atrophied fibers. This would have provided additional information about these fibers. However, Shafiq et al. (1972) used both acid and alkaline preincubation in a study of the rat EDL, denervated at birth, and showed dual staining in the atrophied fibers, concluding that they had not differentiated into mature fibers. This corresponds to the findings presented in this thesis. Butler-Browne et al (1982) and Whalen et al (1985) found neonatally denervated EDL were eventually able to express mature fast myosin but using antibodies to developmental and mature forms of myosin they found the sequence to be delayed. Rubinstein and Kelly (1985) found the light chains and heavy chains unchanged in neonatal denervation in rat EDL. Looking at the effect of neonatal denervation in chick fast-twitch muscle myosin isozymes directly, Strohman and Matsuda (1985) found that the shift from immature to adult forms of myosin heavy chains proceed but repression of immature isoforms of beta tropomyosin, which accompanies development in the chick, did not. The light chain synthesis showed depression of the fast light chain FLC3, high in mature fast-twitch fibers. While the histochemical findings point to an inhibition of maturation of fast-twitch muscle when both pH conditions are used, a discoordinate synthesis of contractile proteins occurs in which the heavy chain expression is not impaired, the light chain isoform expression, minimally retarded but the regulatory proteins are still, or again, of an immature form.

The findings of Strohman and Matsuda (1985) are in agreement with the results reported here, assuming that they are atrophied, immature

fibers, staining at acid and alkaline pH which may still have gone on to synthesize mature myosin heavy chain and light chains although possibly not regulatory proteins. There are two ways in which this may be explained. These fibers could have dedifferentiated as a result of denervation and are recapitulating development after this set back to their development. Or, the acid and alkaline stability characteristics are divorced from the myosin expression as appears to be the case in the staining of myosin as a predictor of myosin ATPase activity (Guth and Samaha, 1972).

The persistence of large cells found in this study have been shown following neonatal denervation in guinea pig gastrocnemius by Karpati and Engel (1968), in rat gastrocnemius by Butler-Browne et al (1982)in rat EDL, by Dhoot and Perry (1983b) and in rat EDL by Ishiura et al (1981). If it can be assumed these are the same type of fiber in each experiment, they may share the following characteristics. They all failed to atrophy. With myosin ATPase they were found to be light staining in alkaline by Ishiura et al. (1981) and Karpati and Engel (1968) and dual staining by Dhoot and Perry (1983) compared with dark staining in alkaline and light staining in acid in this study. They are not necessarily an immature fiber although they stained with acid and alkaline in the study by Dhoot and Perry and showed a profile of light chains of both fast and slow muscle on single fiber analysis. They have also been shown to react to antibodies to mature fast and slow myosin but not embryonic or neonatal myosin (Butler-Browne et al., 1982). They are not the result of reinnervation as they eventually atrophied with prolonged denervation (Ishiura et al, 1981). Dhoot and Perry (1983) showed these fibers synthesized both fast and slow troponin. The fibers

found in this study do not show the same staining characteristics but a similar process could have resulted in the sparing of one fiber type but be expressed differently in the mouse. Support for this comes from the fact that although these fibers were found in the rat EDL (Ishiura et al., 1981; Dhoot and Perry 1983), rat gastrocnemius (Butler-Browne et al., 1982) and guinea pig gastrocnemius (Karpati and Engel, 1968) it was not seen in the rat tibialis anterior (Dhoot and Perry, 1983), all of which possess each of the three mature fiber types.

Neonatal denervation of the mouse EDL results in two fiber types, one highly atrophied fiber that resembles an immature fiber with frequent myotube formation, dual myosin ATPase staining and oxidative enzyme profile and a second type which is less atrophied and may be a fast form. It is possible that these are novel fibers, specific to denervation, rather than a result of arrested maturation, or dedifferentiation. This is supported by the fact that after denervation, there was some continued differentiation before the denervation changes were seen histochemically. These histochemical changes are delayed, compared with alterations in the contractile properties in which changes are more immediate.

Contractile properties

Extensive information is available on the changes in contractile properties of mammalian muscle with development (Close, 1964; 1965; Drachman and Johnston, 1973; Gutmann and Melichna, 1972; Gutmann et al., 1974) and following the denervation of mature muscle (Webster and Bressler, 1985; Betto and Midrio, 1978; Drachman and Johnston, 1975; Finol and Lewis, 1981; Gutmann et al., 1972; Kean et al , 1974; Lewis, 1972; Melichna and Gutmann, 1974 ; Syrovy et al., 1972). However,

regarding the denervation effects in developing, muscle the major focus has been on changes in isoforms of the contractile proteins but very little on their contractile properties. It will be necessary to rely heavily on these studies for the interpretation of the mechanical events found in this work.

Contraction time decreases with development (Close, 1964) and increases with denervation of mature muscle (Webster and Bressler, 1985; Syrovy et al., 1972; Finol and Lewis, 1981). Prolongation in the time required to develop twitch tension (TTP) may occur following changes in two main events. The rate of development of tension will depend on the time constants of the events of crossbridge cycling and will be influenced by the availability of energy stores and the activity of myosin ATPase. As well, the duration and maintenance of adequate calcium levels to induce the troponin, tropomyosin switch for actomyosin binding is a balance between the release of calcium initiated by the membrane action potential and the rate of sequestering of calcium by the SR as well as other proposed calcium shunt mechanisms, such as parvalbumin (Heizmann et al., 1982).

If activity was the major regulator of TTP, then it would increase with maturation and decrease with denervation. Guth and Samaha (1972) showed that the myosin ATPase activity was low in developing muscle and did not correlate with the intense staining of routine myosin ATAPase (pH 9.4) at that stage. However, Rubinstein and Kelly (1985) have raised technical questions about the method used and that point is still under debate. Syrovy (1972) found myosin ATPase activity decreased in the denervated fast twitch muscle of the cat and rabbit in concert with an increase in contraction time but not with the alkaline stability seen

histochemically. It is possible, with ATPase activity and fiber typing not being synonomous, the activity could be changing regardless of the histochemical profile. Brody (1976) looked at soleus and crureus muscles in rabbit from 7 to 21 days during maturation. These muscles have the same myosin ATPase activity but differ in their TTP. The calcium uptake of the SR changed with the contraction time but the myosin ATPase activity did not. Due to contradiction in the literature, it is difficult to conclude that the atrophying fibers in denervated muscle are immature and therefore likely posess a low myosin ATPase activity. In any case, the major change in fiber type is not seen until 14 days, too late to be a causative factor in the change in contraction time, evident by 7 days of age. In this study, results indicate that the mature state, in the normal muscle, has not been achieved until at least 21 days with respect to TTP, which also argues against a major input from myosin ATPase activity because the fiber type maturation occurs by 14 days.

Other possible contributions to TTP are the rate of conduction of the action potential, and the maturation of the SR. Denervation is known to immediately alter the sarcolemmal properties (Sellin et al., 1980; McArdle et al., 1980) including the reduction of the resting membrane potential, the rate of rise and degree of overshoot of the action potential and the rate of conduction of the muscle action potential. This change is complete early after denervation and could account in part for the increased TTP. The slowing of the TTP recovers somewhat from the initial set back at 7 days and remains parallel to normal development until 14 days, therefore some ongoing maturational processes are probably responsible for this recovery. In developing muscle, the TTP changes reported by Close (1964) and SR changes by Luff and Atwood (1971)

follow a similar time course. Although the SR has been shown to be dilated in denervated developing gastrocnemius muscle of the rat (Schiaffino and Settembrini, (1970), it is poorly developed (Shafiqu et al., 1972). There is an increased maximum calcium uptake due to swollen SR (Sreter (1970) but the calcium transport activity is impaired. Gauthier and Hobbs (1982), have described a novel myosin synthesis in the absence of neural influence. However, they suggested that, in the absence of a precisely ordered membrane system, excitation-contraction coupling could be impaired and a slow contraction time occur regardless of the contractile proteins present. Brody (1976) compared the relationship of contraction time with the myosin ATPase activity and sarcotubular calcium uptake in the rat soleus. He found contraction time and SR function to be more closely related. The contribution of each factor in this interaction cannot be derived from this work, but it could be speculated that the development of the SR contributes to the 7 to 14 day reduction in TTP and the interference with further change may relate to the occurance of the denervated fiber type. Finally it has been also observed in this study that TTP has been disrupted by 14 days because of denervation.

The 1/2 RT in the normal EDL in this study decreased significantly from 1 day to 14 days and only slightly thereafter. This corresponds to a similar finding by Close (1964) in the rat EDL. With denervation, the pattern of change is the same although the denervated muscles exhibited a significant prolongation of their 1/2RT. It could indicate that SR function, which is considered responsible for the relaxation time, matures by 14 days in both normal and denervated muscle. The TTP and 1/2 RT are affected by different events in the SR function. The TTP is

influenced by the release of calcium whereas the sequestering of calcium relates to the relaxation phase. As mentioned earlier, Sreter (1970) showed in denervated muscle using isolated fragments of SR, the capacity of SR to take up calcium increased. However, while total uptake was increased, the rate of uptake was reduced and further there was a leak of calcium from the SR prolonging high sarcoplasmic calcium levels.

A single stimulus produces a twitch contraction. The independent unit of force generation in muscle is at the crossbridge. This occurs by means of myosin binding to actin, the splitting of ATP.Pi and resulting energy expended in force generation by the rotation of the myosin head (crossbridge) producing work. In a twitch , the duration and the degree of activation will influence the resulting tension. However, a fused tetanic contraction, produced by repeated stimuli of sufficient frequency will fully activate the muscle. Tension produced during an isometric tetanus is directly related to the number of crossbridges that form in parallel and therefore the myofibrillar area. Tension changes could therefore be a result of atrophy. Because the decrease in myofibrillar area with denervation, in mature muscle, is proportional to the whole cross-sectional area (Engel and Stonnington, 1974) and the muscle length is not greatly altered in denervated muscle, it was decided to normalize tensions to muscle wet weight. This is valid for adult muscle but there could be some error in this assumption in developing muscle, where the area accounted for by interstitial tissue is reduced during development and the proportion of the contribution of myofibrillar proteins to mass may not be constant. It is difficult to separate the factors contributing to tension development in whole muscle and interpretations of tension parameters must be made with caution. Twitch tension decline

can be partially accounted for by atrophy by normalizing values to muscle weight. However there is still a reduced tension due to denervation. During normal development, the fiber becomes more densely packed with myofibrils and the normalized tension decreases as a result. This shows that a greater proportion of myofibrillar area per crossectional area could account for the difference. If the myofibrils in denervated developing muscle was more densely packed than controls, it could explain a reduced tension. It is not known how the myofibrillar to cross-sectional area is affected during neonatal denervation. Maximum tetanus tension is a measure of the force of fully activated muscle not influenced by the time course of membrane events. In development, tetanus tension increases as myofibrils are added in parallel. Although there is a small but significant drop in normalized tetanus tension between 1 and 21 days, there is not between 7 and 21 days and the early drop is probably due to the greater amount of interstitial tissue in the immature fiber (compare Figure 6 and Figure 7). However, after neonatal denervation, there is a profound alteration in the pattern of tetanus tension compared with controls. It does not increase with age, and when normalized to muscle weight, is less than 50% of what is was at one day of age. This is also reflected in a high twitch-to-tetanus ratio compared with controls. As this change is evident by 7 days, it does not correspond with changes in fiber type and is not likely to be due to myosin properties. It would suggest that the change is directed at the level of the contractile mechanism. Effects of temperature on tension changes in denervated muscle is beyond the scope of this thesis but a short treatment of this phenomenon is presented in appendix 4.

The maximum velocity of shortening of skeletal muscle has been estimated by fitting a hyperbola to shortening velocities at non-zero

loads and estimating the velocity at zero load (Hill, 1938). Edman (1979) compared the extrapolated value of the maximum velocity of shortening at zero load (Vmax) to the maximum velocity of unloaded shortening arrived at by the slack test method (Vo). He found both methods yield the same values in single frog fibers.

Close (1964) compared the maximum velocity of shortening, extrapolated from isotonic contractions at different loads, in EDL and soleus and found it to be faster in the fast-twitch muscle, but slow in immature EDL and soleus. Barany and Close (1971) showed a correlation between Vmax and myosin ATPase activity. The results in this thesis show that Vo increases between 1 and 14 days in normal mouse EDL followed by a levelling off at 14 days; consistent with the histochemical findings that fiber types are mature at 14 days. The results reported here would therefore indicate an increase in myosin ATPase activity with normal development as described by Guth and Samaha (1972). Myosin ATPase activity has not been reported in neonatal denervated muscle. The low level of Vo, measured in this study, would suggest that the myosin ATPase activity in denervated EDL is low, typical of an immature muscle.

In denervated mouse EDL, Vo does not change from the low values at birth. Claffin and Faulkner (1985) have assessed the relationship of Vmax to Vo in whole muscle and concluded that, Vo represents the shortening velocity of only the fastest fibers. This results in two possible interpretations of the data presented here. Either the group of large IIox fibers seen in the denervated EDL have a low ATPase activity or the tension they produce is insufficient to contribute noticably to the Vo measure. Only single fiber analysis could answer that question. In the latter case, the fibers contributing to the measurement of Vo

would be the atypical atrophied fibers which, would all have a slow velocity of shortening. This would be consistent with their other contractile properties.

Others have found Vo increased with development. Reiser and Stokes (1982), using the slack test method, showed an increase Vo in developing chick posterior latissimus dorsi. In this study, statistical analysis (using two-way analysis of variance) of Vo indicates that there is a difference between normal and denervated EDL, but that there is no change with time. In two-way ANOVA, groups are collapsed to look at a general effect of time in both groups. The high levels of variance in measurements within each group would make it difficult to see such an effect as ANOVA is designed to be sensitive to variance. Possible sources of variance were sought. Reiser and Stokes (1982) looked at possible variables influencing the measurement and found that neither the point in time during the tetanus at which the release was taken, stimulus frequency, or stimulus intensity affected results. The effect of length differences was looked at also by Reiser and Stokes (1982), and they found that stretching prior to measurements changed the results. Edman looked at Vo at sarcomere lengths of 1.65 to 2.75um and found that, over this range, the measurements were stable. This is a change from the center of the plateau of the tension length curve of $\pm$24%. The maximum length change, used in this study, was 9.7%. Additionally, as the length of EDL was set at the maximum height for twitch tension (which is just slightly shorter than the maximum height for a tetanus) the releases brought our muscles closer in length to the peak for tetanus rather than away from it. Therefore, it is clear that length effects cannot account for the variance. Further muscle length measurements used in determining

Vo were made on whole muscle from tendon to tendon. Errors due to differences between the actual fiber length which decrease per length of muscle with age (personal communication S. Wylie) would increase rather than decrease the differences seen in this work with denervation. It is not known what effect this would have in denervation, but as the muscles grow it cannot account for the total lack of change in Vo. The Vmax values are proposed to represent the maximum shortening velocities of all fibers in the muscle rather than just the fastest fibers as is proposed to occur (Claflin and Faulkner, 1985) with Vo measurements. Kean et al. (1979) found decreased VMAX in denervated fast-twitch muscle of the mature cat. Baker and Lewis (1983) found only small decrease in Vmax in denervated adult rat muscle. The mature muscle is not as affected as immature muscle by denervation which could explain the smaller change. However, Elumbarak (1985) showed the same increase in VMAX from 1 to 21 days of age in both normal and neonatally denervated rat EDL. Vo is reported to measure the maximum velocity of shortening of only the fastests fibers which may account for the different result found in this study. The conclusion of Barany and Close (1971), that the maximum velocity of unloaded shortening is proportional to myosin ATPase activity, is support for the possibility that these denervated muscles have a low myosin ATPase activity typical of immature muscles. However, the eventual synthesis of mature forms of myosin isozymes in neonatally denervated muscle, found by Rubinstein and Kelly (1985), prevents the conclusion that these fibers contain immature myosin isoforms. As well, the lack of change in Vo between 14 and 21 days in the normal EDL would correspond to the unchanged fiber type distribution in those muscles.

Post-tetanic twitch potentiation is found in mature fast-twitch

muscle but not in slow (Moore and Stull, 1984). Manning and Stull (1979), and Moore and Stull (1984) have shown that PTP is correlated to the phosphorylation of MLC2f. Moore and Stull (1984) found that the effect is not due to the quantity of MLC2f but to the level of myosin light chain kinase, the catalyst of the reaction. Using the mature mouse EDL and soleus, Bressler and Glotman (unpublished results) established that the time course and degree of maximum PTP varies with the temperature and they established a protocol for obtaining maximum PTP values at 21$^{o}$C which was used here in this study. Lewis (1972) found no PTP by 2 weeks post denervation in mature EDL of the rat as was the case with denervated and normal soleus. Rubinstein and Kelly (1975) have shown that myosin LCf2 is not altered in denervation. It is likely therefore that it may be the myosin light chain kinase which is under neural control. Crow and Kushmerick (1982) have suggested a role for the phosphorylation of MLC2f to be a step down of energy costs during a sustained high force contraction. This would suggest it would operate in a fast glycolytic fiber. Denervation has resulted in a marked reduction of that fiber type and a failure to produce PTP, which supports this concept.

Burke and Tsairis (1974), in single motor unit studies of the cat, found direct correlation between fatigue index and oxidative enzymes. The increasing susecptibility to fatigue corresponds to the decrease in oxidative enzymes and increase in anerobic fibers by 21 days in normal EDL. In this study, the normal immature EDL exhibits a high resistance to fatigue, which corresponds to the finding of high levels of oxidative enzymes. The persistance of high fatigue resistance seen in the denervated muscle may be explained by the interference with the change to

anaerobic enzymes that has been demonstrated in the denervated muscle. A similar finding has been reported by Bressler et al (1983) in dystrophic EDL, where the increase in oxidative enzymes coincides with increased resistance to fatigue. Finally, increased resistance to fatigue has also been found in denervated muscle of the mature mouse (Webster and Bressler, 1985).

Summary

The results of these experiments show that the normal fast twitch EDL begins at birth as a slow muscle with slow TTP and 1/2RT, generates low tension, has a slow maximum velocity of shortening, exhibits no PTP and is highly resistant to fatigue. This correlates with the histochemical results in which all fibers stain at acid and alkaline preincubation for myosin ATPase and are oxidative. With development, the muscle acquires mature characteristics of fast twitch muscle including a greater anaerobic metabolism and acid labile, alkaline stable ATPase fibers of type IIA and IIB. The denervated muscle shows a brief attempt to differentiate into a fast twitch muscle but in the absence of nerve the differentiation is aborted and the muscle returns to its immature profile. The extent and time frame of dedifferentiation seems to vary with each parameter. This conclusion arises from the histochemical evidence in which a great number of fibers are in a myotube form and the dual staining is more intense. Although the majority of the contractile parameters return to levels less or equal to those seen at birth (resistance to fatigue, PTP, 1/2RT and Pt/Po) some achieve more mature values (Vmax, TTP).

It can be confidently concluded here, that there is a loss of control over the differentiation into fast-twitch muscle following neonatal denervation as shown histochemically and the deficits in

contractile properties imposed on the muscle continue to mimick development until the differentiation becomes stalled at 14 days. This stall may include membrane changes, the synthesis of an altered myosin and the disruption of the functioning of the SR.

According to the histochemical profile, denervation produces altered fibers types. Although these fibers have properties in common with immature muscles, it is clear it is not simply dedifferentiation or aborted development. The use of non denaturing gel electrophoresis would provide important answers as to what isozymes of myosin are synthesized in these fibers. Examination of these muscles at the ultrastructural level would help assess the contribution of changes in the sarcoplasmic reticulum and possibly indicate if these are truly immature muscles. Biochemical studies to measure directly, the enzyme activity levels of myosin ATPase oxidative enzymes and calcium uptake of the fractionated SR would answer questions about factors contributing to contractile parameters. Finally, physiological studies using the effect of caffine on single fibers or the study of glycerinated fibers, could shed light on the contribution of the SR to changes seen in this work.

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# APPENDICES

APPENDIX 1: A Comparison of Sham Operated and Control EDl Histochemical and Contractile Properties at 21 Days of Age.

Introduction

To investigate the possibility that effects attributed to denervation could result from surgical proceedures, a limited series of sham proceedures were carried out. Muscles from normal, denervated and sham operated mice from the same litter, were processed simulataneously for histochemistry. Contractile properties of two sham operated animals were compared with unoperated controls at 21 days of age.

Methods

Identical procedures were carried out on denervated and sham operated mice with the exception that after making the incision, the sciatic nerve was left undisturbed in the sham operated group. All muscles were examined identically for histochemical (NADH-TR and Myosin ATPase) and contractile properties. The muscles used for the histochemical proceedures were from the same litter, but those for the contractile properties were compared with the whole 21 day normal group.

Results

Oxidative enzyme staining of normal, sham operated and denervated muscles from pups of the same litter and stained simultaneously, are shown in figure 16. The normal and sham operated muscles show the same oxidative fiber type differentiation, typical of findings at 21 days of age, whereas, the denervated muscle shows loss of differentiation. No differences could be distinguished between the sham operated and control EDL in myosin ATPase reaction (not shown). Table VIII shows the contractile properties of two sham operated EDL compared with six control EDL at 21 days of age. The only differences found between the two groups were in the tetanus and twitch absolute tension values and muscle weight.

TABLE VIII

Contractile Parameters of Sham Operated and Control Muscle at 21 Days of Age.

| | CONTROL | SHAM |
|---|---|---|
| SAMPLE SIZE | 6 | 2 |
| MUSCLE WEIGHT (mg.) | 3.598±1.31 | 4.792±0.077 |
| MUSCLE LENGTH (mm.) | 9.480±0.264 | 10.000±0.071 |
| TETANUS TENSION (Po) (gms) | 13.153±0.890 | 17.905±0.025 |
| Po/MUSCLE WEIGHT | 3.657±0.196 | 3.740±0.057 |
| TWITCH TENSION (Pt) (gms) | 4.007±0.167 | 5.215±0.145 |
| Pt/MUSCLE WEIGHT | 1.117±0.036 | 1.090±0.014 |
| Pt/Po | 0.308±0.015 | 0.290±0.007 |
| TTP (ms.) | 29.587±0.746 | 30.060±0.785 |
| 1/2 RT (ms.) | 38.617±2.153 | 37.935±0.223 |
| PTP | 1.11483±0.012 | 1.1415±0.034 |

Discussion

All parameters were unchanged with the sham proceedure with the exception of twitch and tetanus tension and muscle weight. Both tensions were higher in the sham operated group. This could be accounted for by the higher weights in these two muscles, which were still within the range of normal muscle but larger than average. Tension normalized to muscle weight was not different between groups. This coinicides with the sciatic nerve crush studies of Lowrie et al. (1982) in which sham operated animals were not different from controls. The results of the sham procedures do not indicate any changes in histochemical or contractile properties as a result of the operative proceedure or any possible failure to thrive due to early postnatal trauma, hence it was not included in the study.

APPENDIX 2: Verification of Denervation up to 21 Days of Age Following Neonatal Sciatic Neurectomy

Introduction:

A variety of methods have been used to deprive muscle of its innervation depending on whether reinnervation is wanted. For this study a method of denervation was needed that would prevent reinnervation up to the longest period studied: 21 days. Methods of verifying denervation range from electrophysiological evidence (Dennis and Harris, 1980) to visual inspection (Dhoot and Perry, 1983b; Ishura et al., 1981; Kumar and Talesara. 1977) or not reported (Brown et al., 1976; Hanzlikova and Schiaffino, 1973; Shafiq et al., 1972). Gentle nerve crush has been used for reinnervation studies. Reinnervation can begin as early as 10 days post nerve crush with normal innervation at 21 days (Ecob et al., 1984; Lowrie et al., 1982) and has been shown to be reinnervated by the original neuron and not by the sprouting of adjacent neurons (Dennis and Harris, 1980). Reinnervation following sciatic nerve resection does not occur before 28 days (Ecob et al. 1984; Engle and Karpati, 1968). Following neonatal denervation, Dennis and Harris (1980) found that a critical time was required to produce a viable nerve-endplate complex, after the regenerating nerve had reached the muscle. The purpose of this preliminary study was to verify that the sciatic neurectomy procedure to be used for these experiments would produced complete denervation for at least 21 days.

Methods:

Time mated females were checked twice daily for new litters and denervation proceedures were performed on the pups 24 hours after a new litter was born (24-40 hrs old). The sciatic nerve was resected from the

level of the greater trochanter to beyond the bifurcation of the sciatic nerve into the posterior tibial and common peroneal nerves at the knee. Three methods were explored to verify the permanence of this proceedure. Clinical signs recorded in animals up to 4.5, 7 or 10.0 weeks of age; visual inspection of direct stimulation of normal and denervated muscle and a histochemical method consisting of dual staining of muscle sections with Acetylcholine Esterase (ACHE) for motor endplate and silver for nerve identification. The staining was done on serial sections of 16 um thickness on muscles sampled one in every 10 sections throughout the whole muscle, according to the methods of Goshgarian (1977). This method was modified by omitting the oxalic acid step, demonstrating our preparation to better advantage. Identification of innervated neuromuscular junctions was shown to be reliable using either method and this step was not considered essential to achieving valid results (Goshgarian personal communication). Sections were scored for presence of endplates and nerve. In innervated muscle nerve and endplate complexes were found primarily between points one fifth to three fifths from the proximal end. The presence of both nerve and endplate staining in the same complex was the criteria for innervation. Denervated muscle from: 1,four week; 3.two week; 4, two week; and 2, one week animals were examined with normal controls.

Results:

The one animal followed up to 4.5 weeks, one to 7 weeks and three to 10 weeks all demonstrated clinical signs of paralysis; dragging of the leg during ambulation and absence of activity in the muscles of sciatic innervation. Electrical stimulation was used to check for reinnervation but technical difficulties of insulating other tissue from the electrodes in small limbs, as well as difficulty finding a nerve stump to stimulate,

made this proceedure unreliable.

Nerve and endplate staining, on longitudinal sections, resulted in contraction of muscle fibers on the glass slide immediately after sectioning of frozen muscle. This problem has been encountered by others (Riley:personalcommunication) Contraction of normal muscle fibers on the slides resulted in tearing and disruption, while it was almost nonexistant in the denervated muscle. Nerve and endplates were still identifiable in normal muscle even though the morphology was damaged. There was a marked increase in reticular fiber staining around fibers bundles in all denervated fibers. At 7 days, a fine grained staining of dispersed nerve tissue remained in some sections but was different from the staining of intact nerve seen in normal muscle at that age. Silver staining of nerve was absent in 14 and 21 week denervated muscle sections and there was an absence of endplate staining. In innervated muscle, endplates were paler staining at 7 days than at 14 and 21 days. There were no nerve endplate complexes seen in any of the denervated muscles up to 21 days. Figure 16 is a low power light micrograph of normal and denervated EDL at 21 days of age with a high power view of a part of the section to identify the stained structures. Nerve endplate complexes can be identified in the normal muscle (arrows). Increased staining of the connective tissue is seen in the denervated muscle but nerve endplate complexes are not seen.

Discussion:

In reinnervation studies, Lowrie et al. (1982) found clinical signs of dragging of the leg and lack of dorsi flexion persisted up to one year following neonatal sciatic neurectomy. The presence of clinical signs of paralysis do not ensure minimal reinnervation has not occured, but

provides a guide that the denervation has been successful. The majority of motor endplates are found proximal to the belly of the muscle and nerve-motor endplate complexes are found by sampling of normal muscle at and proximal to the midbelly of the muscle. Some enhanced silver staining occurs in the denervated muscle due to increased connective tissue in the perimysium. This is likely reticular fibers which stain with silver.

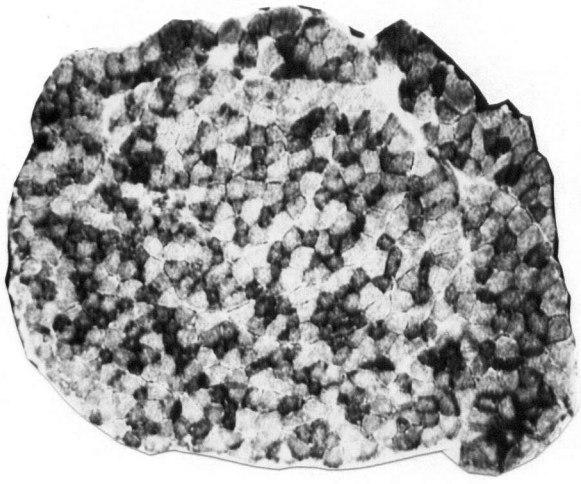
In these preliminary experiments, silver staining of nerve terminals combined with Acetylcholinesterase staining of the endplate verified that reinnervation does not occur by 21 days using this denervation proceedure. These results are in agreement with Engel and Karpati (1968), who did not find reinnervation at 21 days following neonatal sciatic nerve resection (Ecob et al, 1984; Engle and Karpati, 1968). Ecob et al (1985), looking at mature mouse muscle, demonstrated reinnervation by 21 days following nerve crush but the earliest signs following denervation did not appear until 28 days post denervation, as identified by silver staining.

Fig. 16 Oxidative exzyme staining of sham operated, control and denervated EDL at 21 days of age.

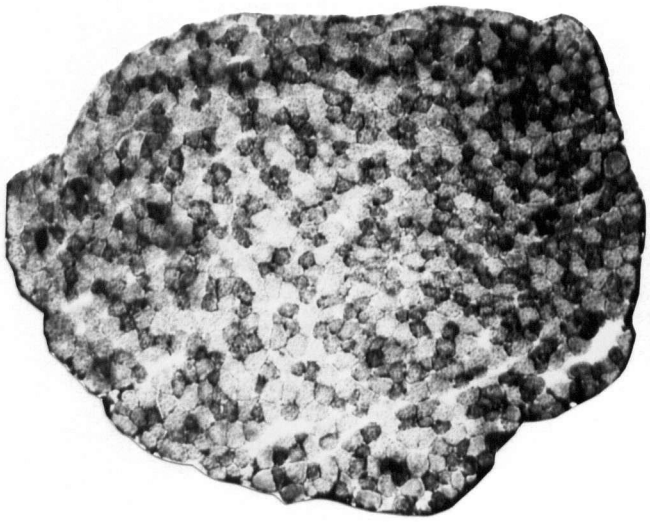
a) 21 day normal EDL stained with NADH. X80

b) 21 day sham operated EDL stained with NADH. X80

c) 21 day denervated EDL stained with NADH. X80



a) NORM



b) SHAM

c) DEN

Fig 16

Fig. 17 Silver and Acetylcholine Esterase staining of normal and denervated EDL at 21 days of age.

a) normal EDL with nerve-endplate complexes (arrows) X200 tissue disruption due to contraction is frequent.

b) higher magnification of above field at large arrow, showing nerve-endplate complex. X800

c) denervated EDL X126

d) higher magnification of above field to illustrate the staining of reticular fibers.X800

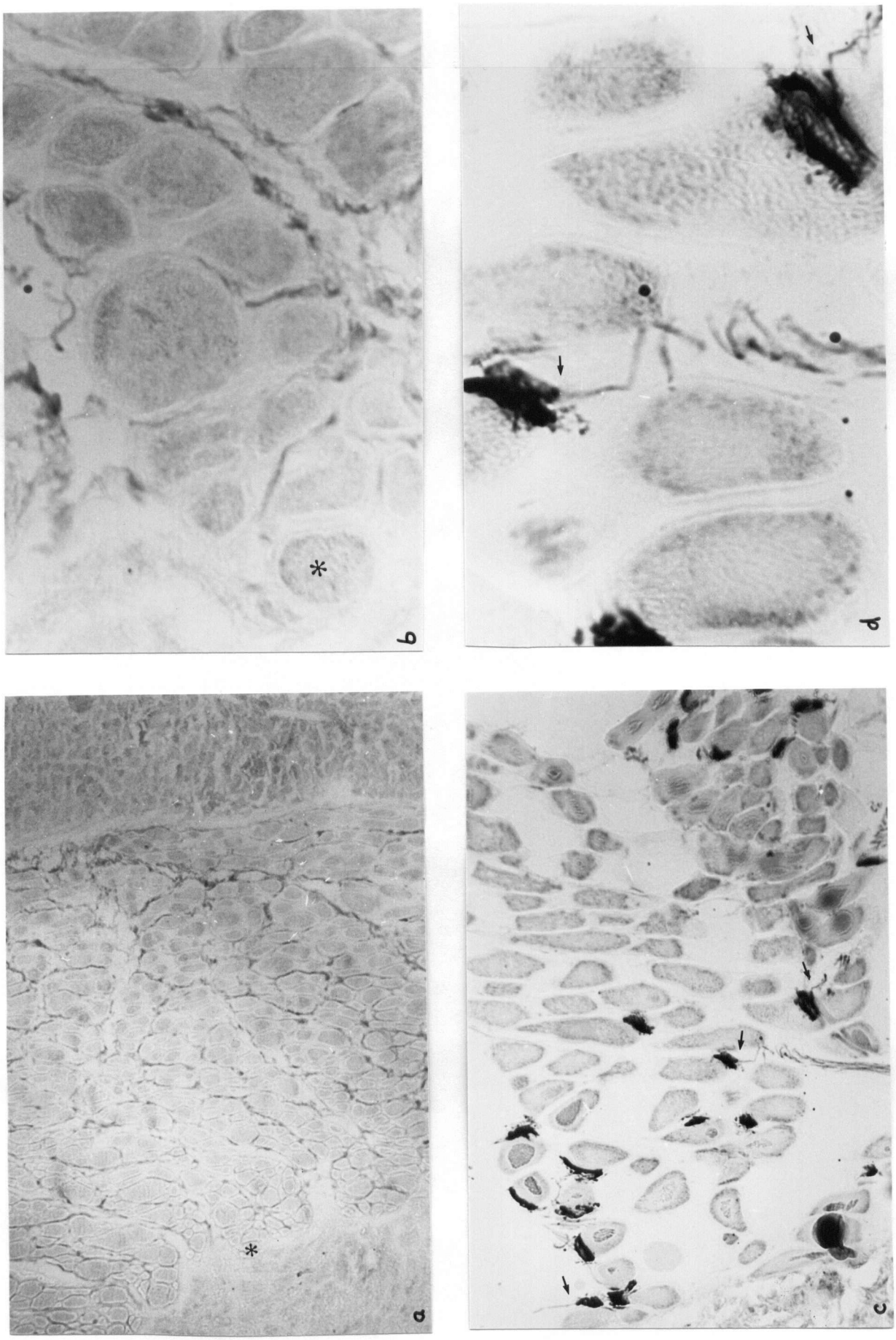



Fig 17.

APPENDIX 3 Preliminary experiments to establish pH and temperature conditions for developing normal and denervated EDL.

Introduction The tension produced by mouse EDL at 1 day of age and in denervated EDL at 7, 14 and 21 days of age is very small. In preliminary experiments, these muscles were unstable and tolerated the proceedures only briefly as evidenced by their contractile properties. A series of exploratory experiments were done to identify the source of the instability.

Experiment 1

methods: Twitch tension was observed using the experimental procedure outlined in methods section, using Krebs solution at 35-37$^{o}$C buffered with 95% $O_2$ and 5% $CO_2$. The muscle was given a single shock every 90 seconds and the rate of bubbling (airating) was varied .

results: In the empty bath, the pH of Krebs at 35$^{o}$C bubbled vigorously.(3 bubbles/second) was 7.43 to 7.47. At a decreased rate (two bubbles/second) the pH rose further. During the experiment, the muscle tension fell from 0.82 to 0.10 grams, inspite of vigorous bubbling. Variations in the bubbling marginally affected the rate of decline.

conclusion: The muscle tension can not be maintained at 35$^{o}$C, using this apparatus. The muscle is sensitive to some condition in the bath.

Experiment 2

methods: The pH of Krebs was monitored at 20$^{o}$C, in a closed 10ml beaker and a closed 100 ml beaker, using varying rates of buffering.

results: The pH of the Krebs in the large enclosed beaker, buffered vigouously, was 7.26. The pH in the 10 ml beaker was 7.39. When buffering was vigorous for 5 minutes it reached 7.37 and when

removed for 5 minutes it rose to 7.44

conclusion: In a 10ml meaker, at 20oC the pH could be maintained at pH 7.39.

Experiment 3

methods: The pH of the Krebs in the bath was monitored with and without buffering at 20°C. The empty bath capacity is 15ml (without stimulating electrodes and muscle). The pH electrode would reduce the bath to approximately 10 ml. The pH of the Krebs was measured in a covered and uncovered beaker at 20°C.

results: The pH of the Krebs, in the open bath, rose to 8.02 in 15 min unbuffered and subsequently could be reduced only to 7.56 (8 min) by buffering alone. The solution rapidly evaporated. The pH in the covered beaker could be maintained with gently bubbling at 7.3 at 20°C.

conclusion: In the open bath the pH could not be maintained below 7.56. The control of the pH is different in the shallow and deep systems of approximately the same volume. The pH is more stable at lower compared with higher temperatures, and with a closed compared with an open system. As $CO_2$ is more labile at higher temperatures and with increased surface area. To overcome the problem:

1) completely enclose the system and work at 35°C. This was technically not possible with this bath.

2) try an alternate buffer

3) Work at a lower temperature constantly renewing the Krebs with a buffering rate of both bath (for oxygenation) and the stock solution, adding solution at a rate to keep the system in equilibrium.

Experiment 4

methods: Twitch tension in 2wk EDL using Krebs pH 7.43 (covered

flask) and Hepes buffer pH 7.45 (covered flask) were compared at 21°C and at 35°C.

results: At 20°C, muscle tension was maintained using buffered Krebs, if it was renewed repeatedly. It dropped when Hepes was added. At 35°C, the tension dropped and was unstable. The addition of fresh buffered Krebs improved the tension only briefly after which time it declined again.

conclusion: At 35°C the muscle tension becomes unstable using either solution.

Experiment 5

methods: The alternate deep muscle bath was tested for pH readings at 35°C both covered and uncovered.

results: In the covered deep bath system at 34°C, the pH stablized at 7.60 with buffered Krebs. Uncovered and placed in the experimental position, the pH went to 7.76.

conclusion: The alternate bath will not solve the pH problem.

Experiment 6

methods: Two normal EDL muscles at 2 weeks of age were examined in Hepes and Krebs solutions at 20 and 35°C.

results: At 20°C, using Hepes, the tension in the first muscle deterioriated. The second muscle began to do the same but after changing to buffered Krebs the tension rose again and stabilized. At 35°C the muscle did not remain viable with either buffer.

conclusions: Hepes does not keep the muscle viable. Krebs at 20°C with constantly refreshed maximally buffered Krebs can maintain a stable tension.

Appendix 4: Comparison of Contractile Properties at 21oC and 37oC.

Introduction

Mechanical experiments on isolated skeletal muscle have been carried out at both 37°C and 20°C. In the experiments reported in this study, the muscle bath was very shallow and contained only 10 ml. of solution. Due to the low solubility of $CO_2$ at 37°C it was difficult to maintain a stable pH in this system. The immature muscles were found to be particularly sensitive to pH changes and did not remain viable. As $CO_2$ is more soluble at lower temperatures, these experiments were carried out at 20-22°C.

Methods

In order to compare the results from this study with those at 37°C, a comparison has been made between a small number of preliminary experiments done from this study, from muscles at 21 days of age, at a bath temperature of 37°C and the same age group done at 20-22°C, at 21 days of age converted to estimated values for 37°C using $Q_{10}$ values of muscles tested at both temperatures (Ranatunga, 1977). Ranatunga (1980) reported some contractile properties at both temperatures in the mature mouse EDL. They were very similar to those of the rat, but the rat figure have been used here because they include data from denervated muscle.

Results

The figures from this study are listed in Table IX. The values for 37°C (shown in brackets) have been estimated using the figures reported by Ranatunga (1977). Data from the preliminary experiments (normal N=6, denervated N=2) done at 37°C on alternate equipment designed for larger, whole muscles, are indicated with an asterisk.

Discussion

Normal and denervated muscle both show cooling depression of isometric tetanic tension (Po) in absolute value and when and normalized with respect to muscle weight. At 37°C, the ratio between normal and denervated EDL in estimated and preliminary experiment Po values is very similar. Both indicate a decrease in tension with denervation. When normalized to muscle weight the direction of change is the same although the values differ. This indicates that when fully activated during tetanic stimulation, the denervated muscle generates less tension than normal muscle and the depression of the tetanus tension, with cooling, is slightly greater in denervation. The normal and denervated muscle have similar twitch tension at 37°C. If the difference between normal and denervated muscle twitch tension is due to a decrease in the rate of rise of the action potential, as suggested by Ranatunga, then the similarity between twitch tension in normal and denervated muscle could be explained in the following way. The safety factor may allow for the same tension development in the presence of slightly reduced calcium release. However, if the initial calcium release is slowed by cooling, the devervation effect of slowing on the rate of rise of the sarcolemmal action potential is accentuated and the muscle could fail to achieve high enough sarcoplasmic calcium levels resulting in reduced tension. The potentiation of the twitch with cooling, characteristic of fast twitch muscle, is seen in the predicted value of normal EDL and corresponds to the preliminary data. At 37°C, the twitch tension is only slightly reduced in denervated compared with normal EDL probably for the reasons just given. There is marked twitch cooling potentiation in normal EDL. In denervated muscle there is cooling depression. When normalized to

weight it is found that the denervated muscle can produce more tension per gram than normal muscle but only at 37°C, whereas it produces less tension per gram at 20°C. The preliminary data supports the estimated results. This could be explained if the rate of conduction of the action potential which is slowed in denervation, was not slow enough at 35°C to affect the calcium levels beyond the safety factor but long enough to prolong the time to peak tension and thereby give more time for tension generation. Yet at 20°C the conduction time could be slowed to the point where calcium is never adequate to produce high tension. If these things are correct then two criteria must be met. Firstly, the contraction time must be prolonged in denervation compared with normal muscle and secondly, in a fully activated muscle by repeated stimulation, the normalized tension differences between normal and denervated muscle would not be as great. Data in Table IX shows that this is the case.

The twitch to tetanus ratio of preliminary data and Ranatunga's work are comparable. With a prolonged contraction time the twitch is able to produce more tension, that is, become more fully activated. The effect of cooling and of denervation, are the same in this respect and Pt/Po is elevated in both. Failure of excitation-contraction coupling causes failure of some fibers to activate, more so with denervation in which the rate of rise of AP is further depressed. This is borne out by the ratio of TTP in normal and denervated EDL in predicted and preliminary data at 35°C and likewise in these experiments at 20°C.

TABLE IX

Comparison of Contractile Properties

at 20-22°C and 35-37°C in

Normal and Denervated EDL

at 21 Days of Age

| | NORMAL | | DENERVATED | |
|---|---|---|---|---|
| | 35oC | 20oC | 35oC | 20oC |
| Po (gms) | (19.1) | 13.2 | (5.5) | 1.6 |
| Po/wt | (5.3) | 3.68 | (5.0) | 1.45 |
| Pt (gms) | (2.8) | 4.0 | (2.16) | 0.95 |
| Pt/wt | (0.78) | 1.11 | (1.96) | 0.87 |
| Pt/Po | (0.16) | 0.30 | (0.53) | 0.60 |
| TTP (ms) | (13.0) | 29.95 | (35.60) | 72.70 |
| | | | | |
| Po* | 8.4 | | 1.9 | |
| Po/wt* | 2.6 | | 1.8 | |
| Pt* | 1.6 | | 1.4 | |
| Pt/wt* | 0.49 | | 1.3 | |
| Pt/Po* | 0.18 | | 0.75 | |
| TTP* | 8.9 | | 24.0 | |

( ) data estimated from the work of Ranatunga (1981)

* data from preliminary experiments using alternate bath conditions