CANADIAN THESES

THÈSES CANADIENNES

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

NL-339 (r. 86/06)

Canada

THE EXPRESSION OF MYOSIN ISOENZYMES IN THE MUSCLES OF NORMAL AND C57 BL/6J DY^{2J}/DY^{2J} MICE.

by

Alexandre F. R. Stewart

A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science in the Department of Physiology, Faculty of Health Sciences.

ISBN 0-315-33254-9

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Acknowledgements

The patience of my thesis supervisor, Dr. David Parry, in my weaning from the undergraduate mentality to a career in medical research must be commended. I am grateful to him for having held me under his aegis. I will leave the lab with an arsenal of techniques and ideas.

I wish to thank Dr. Karel Rakusan for the use of his excellent microscope.

To my father and mother I am thankful, for they have endured my absence while still at home and supported my efforts throughout.

Table of Contents

Page

Acknowledgements.....i
Table of Contents.....ii
List of Table and Figures.....iv
Abstract.....vii

Chapter I: Introduction

1. Myosin: an Historical Perspective.....1
2. Structure and Molecular Isoenzymes of Myosin.....8
3. Developmental Changes in Myosin Expression.....17
4. Muscular Dystrophy in Man.....27
5. Muscular Dystrophy in the Mouse.....29

Chapter II: Materials and Methods

1. Protein Extraction.....35
   A. Myosin Purification for Light Chain Characterization.....35
   B. Crude Myosin Extraction for Native Isoform Characterization.....36
2. Electrophoretic Techniques.....37
   A. SDS-PAGE of Myosin Light Chains.....37
   B. Sodium Pyrophosphate-buffered Gel Electrophoresis of Native Myosin.....38
3. Staining of Gels.....39
   A. Coomassie Brilliant Blue R-250.....39
   B. Silver Staining.....40
   C. Coomassie Brilliant Blue G-250.....41
4. Immunohistochemistry with Monoclonal Antibodies.....41
5. Statistical Analysis.....44

Chapter III: Results

1. SDS-PAGE of Myosin Light Chains.....45
   A. Anterior Tibialis.....45
   B. Soleus.....45
   C. Extensor Carpi Radialis Longus.....45

2. Immunohistochemistry of Muscle Cross-sections....53
   A. Soleus.....54
   B. Anterior Tibialis.....76
   C. Extensor Digitorum Longus.....102
   D. Extensor Carpi Radialis Longus and Brevis....127
3. Sodium Pyrophosphate Gels of Native Myosin.....151
   A. Soleus.....151
   B. Anterior Tibialis Oxidative Region.....151
   C. Anterior Tibialis Glycolytic Region.....151
   D. Extensor Digitorum Longus.....151
   E. Extensor Carpi Radialis Longus and Brevis....152
4. Summary of All Data.....155

Chapter IV: Discussion

1. Myosin Light Chain Expression.....162
2. Immunohistochemistry: Myosin Heavy Chain Expression.....164
3. Structural Isoforms of Myosin.....172

Appendix.....175
References.....182

List of Table and Figures

Table — Page

1 Reactivity of Monoclonal Antibodies to Type-specific Myosins.....43

Figure

1 A. Mechanism of action of trypsin and papain.....7
1 B. The myosin molecule.....7
2 The structural isoforms of myosin.....14
3 The peroxidase anti-peroxidase (PAP) method...42
4 A. SDS-PAGE of 2 month old normal and dystrophic mouse muscle myosin light chains...48
4 B. SDS-PAGE of 4 month old normal and dystrophic mouse muscle myosin light chains...48
5 A. SDS-PAGE of 6 month old normal and dystrophic mouse muscle myosin light chains...50
5 B. SDS-PAGE of 8 month old normal and dystrophic mouse muscle myosin light chains...50
6 SDS-PAGE of 12 month old normal and dystrophic mouse muscle myosin light chains...52
7 Immunohistochemical profile of 2 month old normal mouse Soleus muscle.....57
8 Immunohistochemical profile of 2 month old dystrophic mouse Soleus muscle.....59
9 Immunohistochemical profile of 4 month old normal mouse Soleus muscle.....61
10 Immunohistochemical profile of 4 month old dystrophic mouse Soleus muscle.....63
11 Immunohistochemical profile of 6 month old normal mouse Soleus muscle.....65
12 Immunohistochemical profile of 6 month old dystrohic mouse Soleus muscle.....67
13 Immunohistochemical profile of 8 month old normal mouse Soleus muscle.....69
14 Immunohistochemical profile of 8 month old dystrophic mouse Soleus muslce.....71
15 Immunohistochemical profile of 12 month old normal mouse Soleus muscle.....73

16 Immunohistochemical profile of 12 month old dystrophic mouse Soleus muscle.....75
17 Immunohistochemical profile of 2 month old normal mouse AT muscle oxidative region.....79
18 Immunohistochemical profile of 2 month old dystrophic mouse AT muscle oxidative region...81
19 Immunohistochemical profile of 4 month old normal mouse AT muscle oxidative region.....83
20 Immunohistochemical profile of 4 month old dystrophic mouse AT muscle oxidative region...85
21 Immunohistochemical profile of 6 month old normal mouse AT muscle oxidative region.....88
22 Immunohistochemical profile of 6 month old dystrophic mouse AT muscle oxidative region...90
23 Immunohistochemical profile of 8 month old normal mouse AT muscle oxidative region.....93
24 Immunohistochemical profile of 8 month old dystrophic mouse AT muscle oxidative region...95
25 Immunohistochemical profile of 12 month old normal mouse AT muscle oxidative region.....98
26 Immunohistochemical profile of 12 month old dystrophic mouse AT muscel oxidative region..100
27 Immunohistochemical profile of 2 month old normal mouse EDL muscle.....104
28 Immunohistochemical profile of 2 month old dystrophic mouse EDL muscle.....106
29 Immunohistochemical profile of 4 month old normal mouse EDL muscle.....110
30 Immunohistochemical profile of 4 month old dystrophic mouse EDL muscle.....113
31 Immunohistochemical profile of 6 month old normal mouse EDL muscle.....114
32 Immunohistochemical profile of 6 month old dystrophic mouse EDL muscle.....116
33 Immunohistochemical profile of 8 month old normal mouse EDL muscle.....119
34 Immunohistochemical profile of 8 month old dystrophic mouse EDL muscle.....121
35 Immunohistochemical profile of 12 month old normal mouse EDL muscle.....124

36 Immunohistochemical profile of 12 month old dystrophic mouse EDL muscle.....126
37 Immunohistochemical profile of 2 month old normal mouse ECRL and B muscles.....129
38 Immunohistochemical profile of 2 month old dystrophic mouse ECRL and B muscles.....131
39 Immunohistochemical profile of 4 month old normal mouse ECRL and B muscles.....134
40 Immunohistochemical profile of 4 month old dystrophic mouse ECRL and B muscles.....136
41 Immunohistochemical profile of 6 month old normal mouse ECRL and B muscles.....139
42 Immunohistochemical profile of 6 month old dystrophic mouse ECRL and B muscles.....141
43 Immunohistochemical profile of 8 month old normal mouse ECRL and B muscles.....144
44 Immunohistochemical profile of 8 month old dystrophic mouse ECRL and B muscles.....146
45 Immunohistochemical profile of 12 month old normal mouse ECRL and B muscles.....148
46 Immunohistochemical profile of 12 month old dystrophic mouse ECRL and B muscles.....150
47 Sodium pyrophosphate gels of native myosins from muscles of normal and dystrophic mice aged 2, 4, 6, 8, and 12 months.....154
48 Summary of all data obtained for each muscle —Soleus.....157
—Anterior Tibialis.....158
—Extensor Digitorum Longus.....159
—Extensor Carpi Radialis Longus.....160
—Extensor Carpi Radialis Brevis.....161
App. 1 Two-factor Analyses of Variance of Per Cent Fibre Counts for Four Fibre Types in Soleus of Normal and Dystrophic Mice at Ages 2, 4, and 6 Months.....178
App. 2 Bar Graph of Per Cent Fibre Types in Soleus at 2, 4, and 6 Months.....180

Abstract

To test the hypothesis of Parry and Desypris (1983) that the prolongation of the twitch in fast-twitch hindlimb muscles of the dystrophic (dy^{2j}) mouse is due to the synthesis of slow myosin by these muscles in response to spontaneous discharge of their motoneurons, the expression of myosin subunits and isoforms was analyzed in dystrophic mice at various ages. The fast-twitch hindlimb Anterior Tibialis and Extensor Digitorum Longus muscles of normal mice express only the fast light chains (LC1f, LC2f, and LC3f) and heavy chains (IIA and IIB).

The results show that the slow myosin light chains, LC1s and LC2s, appear in SDS-polyacrylamide gels of AT muscles of 8 month old dystrophic mice, in addition to the fast light chains. Slow myosin heavy chain was detected in EDL and AT muscles of 4 month old dystrophic mice by an immunohistochemical technique with monoclonal antibodies. Neither slow light nor heavy chains were detected in the fast-twitch forelimb muscles, Extensor Carpi Radialis Longus and Brevis, which are known to be quiescent in anesthetized dystrophic mice (Parry and Desypris, 1983). Embryonic myosin heavy chain was detected immunohistochemically with an embryonic-specific monoclonal antibody in hindlimb muscles of younger dystrophic animals up to age 6 months, whereas it was not detected beyond 4 months of age in the forelimb muscles. Further evidence for the lack of slow myosin involvement in the slowing process is the minute amount of slow myosin native isoform which appears only in the oxidative region of AT at 12 months, on pyrophosphate gels.

The dystrophic Soleus muscle, a slow-twitch muscle of the hindlimb, shows a marked loss of 'pure' Type I fibres, with a considerable increase in the number of hybrid fibres, containing both slow and fast isoenzymes of the myosin heavy chain, as detected by immunohistochemistry. A reduction in the relative content of the slow myosin (SM) native isoform was also seen by pyrophosphate gel analysis.

It is concluded that part of the slowing of the isometric twitch in fast-twitch muscles of the hindlimb of dystrophic mice older than 8 months of age is due to the presence of slow myosin in these muscles. However, in dystrohpic mice younger than 8 months, the slowing of the isometric twitch is probably not due to slow myosin synthesis, as slow light chains could not be detected in AT and few (<10%) fibres contained slow myosin heavy chain. In younger dystrophic mice, the presence of embryonic myosin heavy chain was taken as evidence for the existence of regenerating fibres. It is unlikely that these regenerating fibres are the principal contributors to the slowing process in mouse dystrophy, as their relative number was small (<10%).

INTRODUCTION

Myosin: an Historical Perspective

Myosin is a complex molecule which has both a structural and an enzymatic function in the process of contraction and tension development in skeletal muscle. A modern undertanding of the structure, composition and function of this contractile protein has required intensive research over the past sixty years. An eloquent review of the early work on muscle contractile proteins can be found in a monograph by Dubuisson (1954). In 1925, Weber characterized some basic physico-chemical properties of a protein fraction extracted from rabbit skeletal muscle, which he termed 'myosin' (Weber, 1925a, b). The first report assigning the enzymatic activity of adenosine triphosphatase (ATPase) to myosin was in 1939, by Engelhardt and Lyubimova. The extract of Weber (1925b) was undoubtably a mixture of myosin and actomyosin (actin combined with myosin). The question as to which of the two proteins was enzymatically active was resolved later, with the advent of protease digest studies of myosin fragments.

Weber and his associates later showed that skeletal muscle actomyosin could be dissociated into actin and myosin (Portzehl, Schramm, and Weber, 1950) and could be reassociated step by step to form actomyosins of different sedimentation constants (Weber, 1950). Hanson and H. E. Huxley (1953) were able to extract myosin and actin successively from the fibrils of minced rabbit muscle. By means of electron micrography of longitudinal thin sections before and after the extraction procedures, they demonstrated unequivocally that myosin was the principal constituent of the A-band and that actin made up the I-band in striated muscle.

In electron micrographs of transverse sections of rabbit muscle myofibrils, H. E. Huxley (1953) showed the existence of a compound array of two types of filaments, the thick filaments of the A-band and the thin filaments of the I-band. Based on these results, and on cinematographic records of phase contrast illuminated myofibrils during periods of

stretch or active tension, H. E. Huxley and Hanson (1954) proposed a role for the ATPase activity of myosin and the interaction of myosin with actin in their 'sliding filament' model of muscle contraction. A. F. Huxley and Niedergerke (1954) had also arrived at a similar model for muscle contraction, based on their own results. In this model, actin filaments of the I-band slide between the thicker filaments of the A-band, with tension being generated at the expense of ATP hydrolysis.

Many investigators have attempted to elucidate the structural features of the myosin molecule which enable it to self-assemble into A-band filaments, to interact with actin, and to cleave ATP. One approach has been the use of proteolytic enzymes which break the myosin molecule into fragments which can be identified by their respective sedimentation pattern with ultracentrifugation. The protease trypsin cleaves myosin into two unique kinds of fragments with well-defined characteristics. The smaller molecule, light meromyosin (LMM), has the solubility properties of intact myosin (Szent-Gyorgyi, 1953). The larger heavy meromyosin molecule (HMM) contains the sites for enzyme activity and interaction with actin (Gergely, 1950; 1953; Mihalyi and Szent-Gyorgyi, 1953; Mihalyi, 1953; Szent-Gyorgyi, 1953). The physico-chemical properties of these fragments have been reviewed by Lowey and Cohen (1962). The LMM fragment has a molecular weight of approximatively 140000 and a highly alpha-helical conformation. The HMM has an approximate molecular weight of 340000. Their estimate of the molecular weight of myosin is probably the most reliable (based on purely biophysical parameters) and lies at approximately 470000 (Lowey and Cohen, 1962).

In electron micrographs of transverse sections of the myofibril, H. E. Huxley and Hanson (1960) showed the presence of lateral projections, extending about 200 angstroms from the thick filaments, which appeared to touch the actin filaments. They suggested that these cross-bridges are composed of the HMM subfragement, and that the core of the A-band filament consisted of LMM subunits in a staggered, helical

arrangement.

Beyond the gross ultrastructural information obtained from proteolytic digestion and electron microscopic examination of myosin, little information was obtained concerning the actual composition of the molecule. The first report suggesting that myosin might contain smaller subunits was that of Tsao (1953) in which he reported the existence of light chains of 16000 molecular weight after ammonium sulphate fractionation of myosin exposed to 7 M urea. Koninz, Carroll, Smith and Mitchell (1959) found a component of 29000 daltons released from myosin in 0.1 M sodium carbonate. This alkali dissociation was further investigated in great detail by Gaetjens et al. (1968) and by Gershman, Stracher, and Dreizen (1969).

The realization that myosin consists of a larger backbone to which is associated smaller constituents required a re-evaluation of the structure in relation to these smaller components, as well as the determination of their stoichiometry for a given molecule. A comprehensive review of the early literature on the subunits of myosin and their interactions can be found in Dreizen et al. (1967). Lowey and co-workers (1969) subjected myosin to proteolysis with the enzyme papain, which not only produces LMM, but also cleaves the HMM into two distinct subunits under controlled conditions. They were able to prepare relatively undegraded HMM subfragment-1, the enzymatically active globular portion of the molecule identified previously by Mueller and Perry (1961) in tryptic digests of HMM, as well as HMM sub-fragment-2, the helical structural region of heavy meromyosin; which they had previously isolated from a tryptic digest of HMM (Lowey, Goldstein, Cohen, and Luck, 1967). By digesting myosin in a precipitated state with papain, Lowey et al. (1969) were also able to isolate the entire helical coiled-coil region of the myosin molecule. The size and shape of these fragments, deduced from physico-chemical measurements, were compared to the structures seen in electron micrographs of shadow-cast preparations. From these studies they concluded that the myosin molecule consists of two

globular units, each measuring about 70 angstroms in diameter, attached to a rod-like region approximately 1350 angstroms long.

In a subsequent paper, Weeds and Lowey (1971) looked specifically at the chemical and physico-chemical characteristics of the low molecular weight components, or light chains, dissociated from rabbit skeletal muscle myosin under a variety of denaturing conditions. They identified three electrophoretic components on acrylamide gels run both in the presence and absence of sodium dodecyl sulphate, which indicated differences in net charge and molecular weight. They separated the three light chains by chromatography and determined their thiol sequences and were able to show that two of the three (the alkali light chains) are chemically related in that they contain an identical thiol sequence, while the third component was seen to be chemically different. They repeated the experiments of Gazith et al. (1970), who had previously shown that a light chain could be removed from myosin by treatment with 5,5'-dithio-(bis-2-nitrobenzoic acid) without loss of ATPase activity, and also concluded that this third light chain (called the DTNB light chain) is not essential for activity. The alkali light chains, which dissociate from myosin at pH 11, were referred to as essential light chains, since their removal had been shown to result in a total loss of ATPase activity (Stracher, 1969; Dreizen and Gershman, 1970).

Weeds and Lowey (1971) also determined the stoichiometry of these two classes of light chains by the isotope dilution technique, using as markers [^{14}C] iodoacetate-labelled peptides of the different light chains, and isolating these peptides form purified light chains and from mixtures of [^{12}C] iodoacetate-labelled myosin and ^{14}C-labelled light chains. Their results showed that both myosin and HMM contain two molecules of the essential alkali light chains, while HMM subfragment-1 had a single alkali light chain non-covalently bound to it. They also found that myosin and HMM had two DTNB light chains attached, but that HMM subfragment-1 contained less than half the expected number.

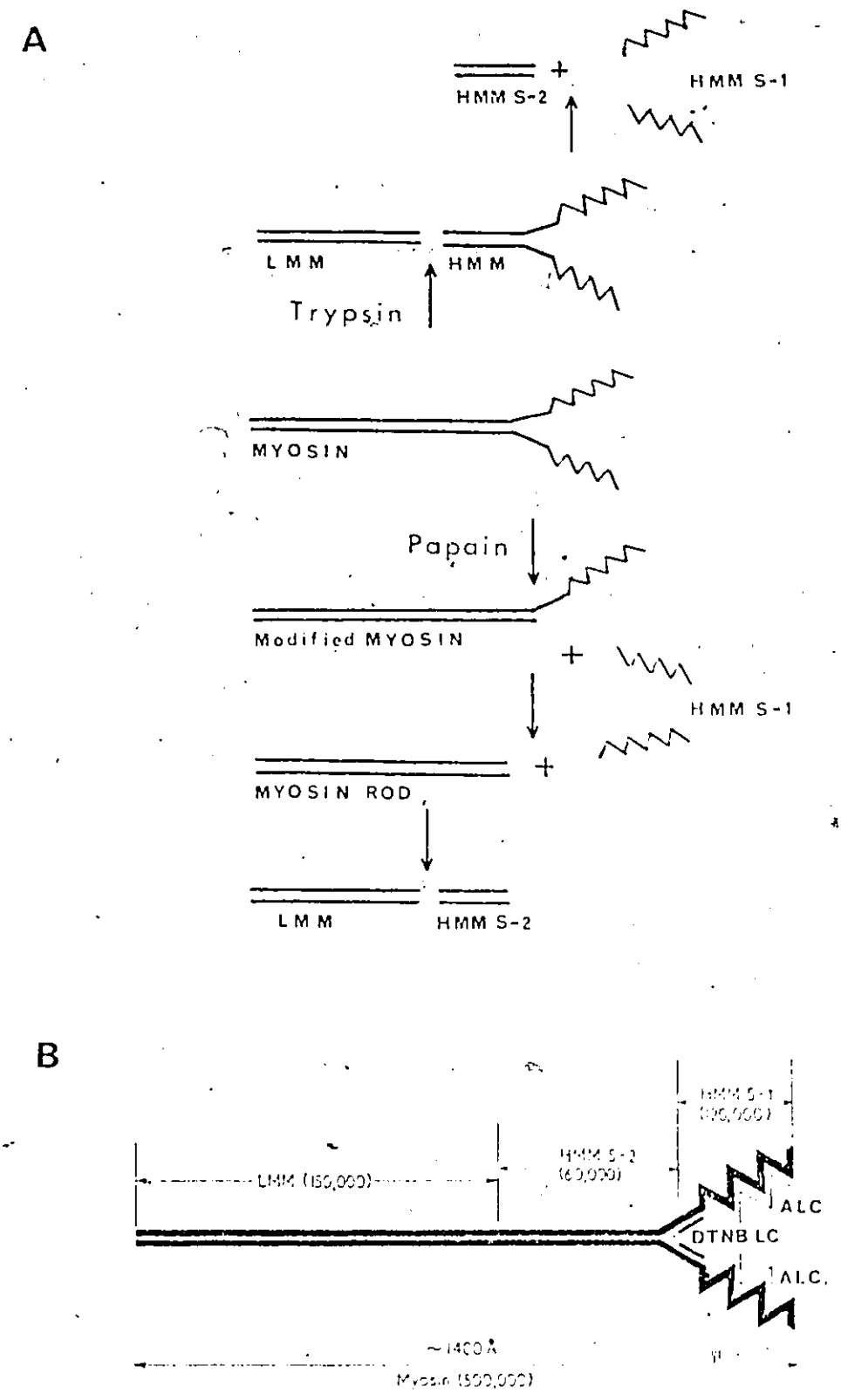
They suggested that DTNB light chain might be susceptible to papain digestion. Thus, the DTNB light chain probably is associated with the HMM region which is cleaved by papain, between subfragment-1 and subfragment-2.

More recently, Winkelman, Lowey and Press (1983) have identified the precise location of the DTNB-light chain in adult avian muscle myosin. They used a monoclonal antibody directed against this myosin subunit with an electron microscopic shadowing technique to detect the location of the epitope on myosin preparations. Their results confirm the proteolytic digest studies which had originally suggested that the DTNB-light chain is located at the junction of the head and rod of the myosin molecule.

The overall picture which has emerged from these two related papers (Lowey et al., 1969; Weeds and Lowey, 1971) and the previous studies of fragments from tryptic digestion (Lowey and Cohen, 1962; Lowey et al., 1967) is summarized in the schematic diagram presented in Fig. 1.

Fig. 1 A. Schematic representation of the mechanism of action of trypsin and papain. Brief treatment of myosin with trypsin yields the two fragments light meromyosin (LMM) and heavy meromyosin (HMM) (Szent-Gyorgyi, 1953). A more prolonged treatment with trypsin yields two smaller fragments of HMM, subfragment-1 (HMM S-1) which contains the actin-binding site and ATPase activity, and subfragment-2 (HMM S-2), which consists of the helical extension of the myosin rod (Lowey et al., 1967). Brief papain treatment of myosin yields HMM S-1 and modified myosin (Lowey et al., 1969). Slightly longer treatment releases the second HMM S-1 as well as the myosin rod. Further proteolysis with papain will cleave the myosin rod into LMM and HMM S-2. (This diagram is reproduced from Lowey et al.(1969) with permission)

B. Schematic representation of the myosin molecule. This scheme has emerged from the structural studies using proteolytic enzymes, and electron micrographs of the subfragments of myosin, as well as the analysis of the light chains and their association with these fragments. The alkali light chains (ALC) are seen to be associated with the HMM S-1, whereas the DTNB-light chains (DTNB LC) are probably located close to the HMM S-1-HMM S-2 region cleaved by papain, as this light chain appears to be sensitive to the proteolysis (Weeds and Lowey, 1971). (Reproduced and modified from Weeds and Lowey (1971) with permission)


A
HMM S-2
+
HMM S-1
LMM
HMM
Trypsin
MYOSIN
Papain
Modified MYOSIN
+
HMM S-1
+
MYOSIN ROD
LMM
HMM S-2
B
LMM (150,000)
HMM S-2
(60,000)
HMM S-1
(90,000)
ALC
DTNB LC
ALC
~1400 Å
Myosin (500,000)

Structural and Molecular Isoenzymes of Myosin

The first reports to hint at the existence of functional isoforms of myosin were those of Seidel et al. (1964) and Barany et al. (1965) which showed that the myosin ATPase activity of the slower red muscles of the rabbit is lower than that of myosin from white muscles. The pH stability of these myosins was also found to differ between slow red and fast white muscles (Sreter et al., 1966; Seidel et al., 1967). Myosin from slow muscles was found to be alkali labile and stable at an acid pH. Barany (1967) has found a clear correlation between the ATPase activity of myosin and the speed of muscle shortening. Myosin isolated from 14 different muscles (of mammalian, lower vertebrate and invertebrate animals) of known maximal speed of shortening was tested for its Ca^{2+} - and actin-activated ATPase activities. He found that the ATPase activities of the myosins were generally proportional to the speed of shortening of their respective muscles; i.e., the greater the intrinsic speed, the higher the ATPase activity.

On the basis of the pH stability of myosin ATPase, Guth and Samaha (1969) developed an histochemical technique to distinguish qualitatively between slow red or fast white fibres in muscles of rabbit, rat and cat. They also observed that fibres with high ATPase activity could be subdivided into two categories by the use of formaldehyde, which inactivated some fibres and not others. Brooke and Kaiser (1970) also described similar results using a slightly different preparation, where they observed the three major fibre types, as well as a fourth minor type of fibre. Their Type I fibres correspond to the low ATPase-activity slow red fibres, which they found to develop the greatest reaction with the oxidative enzyme stains (reduced diphosphopyridine nucleotide (DPNH or NADH) dehydrogenase and succinic dehydrogenase)). Their nomenclature for the two subtypes of high ATPase-activity fibres was Type IIA and Type IIB, based on the inhibition of myofibrillar ATPase with different acid preincubations. Serial sections of Type IIB fibres were seen to develop the least reaction with NADH, whereas Type IIA fibres

had an intermediate reaction to this enzyme.

Several comparative studies were made to determine whether muscle-specific molecular isoenzymes of myosin existed in order to explain the correlation observed by Barany (1967), as well as the basis for histochemical fibre typing. Perrie and Perry (1970) studied the electrophoretic banding patterns of myosin on acrylamide gels in 8 M urea. Their detailed report looked at the effect of different extraction procedures and the duration of storage of the extract on the number of observed light chains from myosin of cardiac, red and white skeletal muscles. They found that cardiac and red skeletal muscles (Soleus and Semitendinosus) showed distinct electrophoretic patterns from that of white muscle, though their use of amido black to stain the gels probably reduced the sensitivity of their system, and may account for their identification of only 4 light chains.

Lowey and Risby (1971) looked at the light chains extracted from fast and slow skeletal muscles, as well as cardiac muscle, in both the rabbit and chicken. They found that fast muscles contain 3 light chains of 16000, 18000 and 25000 daltons, which were electrophoretically distinct from the two light chains seen in both slow skeletal and cardiac muscles, which had a molecular weight of 20000 and 27000 daltons. These results were independently confirmed by Sarkar et al. (1971) who also looked at fast and slow skeletal and cardiac muscles in the rabbit, and by Weeds and Pope (1971), who looked at both sheep and bovine cardiac muscle, as well as sheep skeletal muscle. However, as the ATPase activity of myosin is now known to be a function of the heavy chain component, the significance of these light chain studies in relation to the ATPase activities of muscle fibre type-specic myosins is uncertain.

The first report with evidence for differences in myosin heavy chains was that of Margreth et al. (1980) who found distinct peptides in the proteolytic digests of myosin from fast (Anterior Tibialis)) and slow (Soleus) muscles in the rat. Billeter et al. (1980, 1981) have shown that heavy

chain isoforms exist for Type I, Type IIA and Type IIB fibres. Single human fibres, histochemically typed for Ca^{2+}-activated ATPase, were extracted for myofibrillar proteins, which were separated on SDS-polyacrylamide gels. The myosin heavy chain band on these gels was cut out and subjected to partial proteolysis. The digest was mapped on a second, one-dimensional SDS-polyacrylamide gel. These peptide maps were compared for heavy chains of myosin from fibres of known type, and differences were found in the digests of Type I, Type IIA and Type IIB myosin heavy chains. These authors have further characterized the differences by comparing two-dimensional peptide maps of the heavy chain digests (Billeter et al., 1982). The two-dimensional maps separate the peptides in a first dimension according to their isoelectric points, and then in a second dimension by placing the isoelectric focusing gel on an SDS-polyacrylamide gel, to further separate the fragments according to molecular weight (O'Farrell, 1976).

Earlier attempts to separate the heavy chain isoforms of myosin using SDS-polyacrylamide gel electrophoresis were only able to distinguish between the fast and slow isoforms (Perrie, Bumford and Rochelle, 1982; Carraro and Catani, 1983, 1984). Recently, Perrie and Bumford (1984) have succeeded in separating the three isoforms of the myosin heavy chain from frozen thin sections of skeletal muscle and have correlated these isoforms with the ATPase staining pattern of serial sections of the same muscles. Thus, adult skeletal muscles are characterized, not only by slow and fast light chains, but also have characteristic heavy chains in Type I, Type IIA and Type IIB fibres. Danielli Betto and Betto (1985) have shown that histochemically typed single muscle fibres can also contain small amounts of a myosin heavy chain of another type, as detected in 6% polyacrylamide gels. This indicates the existence of fibres with hybrid myosin expression.

The possibility that structural isoforms of the myosin molecule might exist as a result of the different association of the two alkali light chains to the 'head' region of myosin was suggested by Weeds and Lowey (1971), based on their

observation that there was twice as much of alkali light chain-1 (A-1) as alkali light chain-2 in their extracts of rabbit muscle myosin. Thus, the HMM S-1 fragments of a given myosin molecule might contain both the same light chain (either A-1 or A-2), or they might each contain one of the two (one with A-1, the other with A-2).

The stoichiometry of the alkali light chains has also been examined in histochemically identified single fibres of the rabbit Psoas muscle (Weeds, Hall and Spurway, 1975) showing a 2:1 ratio of A-1 to A-2, in confirmation of the previous study (Weeds and Lowey, 1971) which had examined whole muscle extracts. This was further evidence for the existence of structural isoforms of myosin within the same muscle fibre, provided A-2 was not a proteolytic fragment of A-1 and that they were extracted equally from the single fibre.

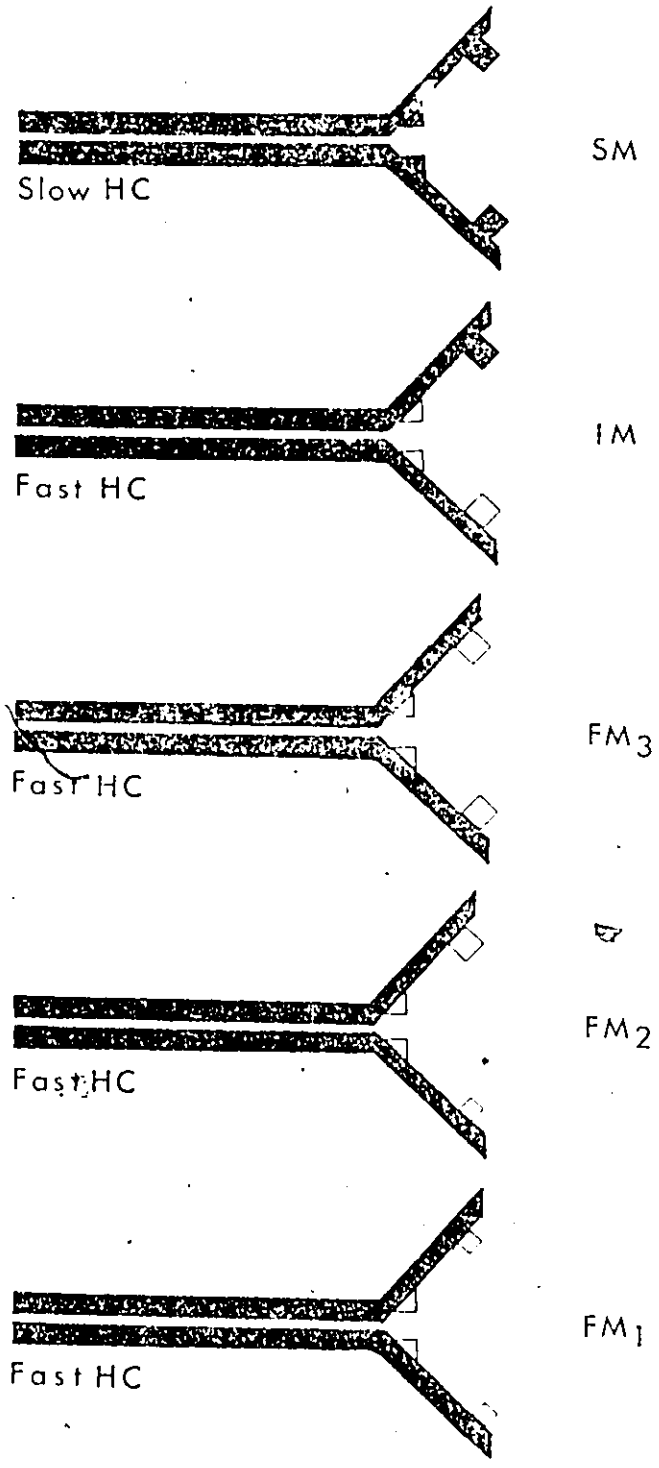
Hoh, McGrath and White (1976) have developed a technique whereby the native myosin molecule can be extracted from muscle and electrophoresed under non-denaturing conditions. In chicken muscle extracts, they identified five electrophoretically distinct native myosins; two from slow muscle, and three found in fast muscle. They suggested that these represented (structural) isoenzymes of myosin. This was confirmed by Hoh (1978) for chicken myosin isoenzymes, where he showed that the cut-out bands of the native myosins in non-denaturing gels contained distinct light chains when run on SDS-polyacrylamide gels. As the light chains have characteristic molecular weights, they were partly responsible for the electrophoretic separation of the native isoenzymes. These findings have been confirmed for human skeletal muscle (Fitzsimons and Hoh, 1981a), rabbit muscle (Mabuchi et al., 1982, 1984), and also in mouse muscles (Fitzsimons and Hoh, 1983).

The report of Fitzsimons and Hoh (1983) on mouse skeletal muscle myosins will be described, as it applies to the other animals studied and is directly relevant to the work presented here. These authors analyzed the native myosin isoenzyme composition, myosin light chain distribution and

histochemical profile of fast-twitch and slow-twitch muscles of normal and dystrophic (129 ReJ dy/dy) mice. Their results for muscles of normal mice will be described here, and the dytrophic muscle data will be described in a subsequent section.

Fitzsimons and Hoh (1983) found that the normal mouse slow-twitch Soleus muscle contained two isomyosins (slow myosin, SM and intermediate myosin, IM) which were electophoretically distinct from the three major isomyosins (FM1, FM2, FM3) of the fast-twitch Extensor Digitorum Longus muscle. The calcium-activated ATPase activities of FM1, FM2, FM3 and IM at pH 9.2 were found to be each much higher than that of SM. This was taken as indirect evidence suggesting that SM contains the heavy chain of slow muscle, whereas FM1, FM2, FM3 and IM each consisted of the fast heavy chains associated with different light chains. Analysis of the light chains from cut-out bands of native myosin gels showed that FM1, FM2, FM3 and IM contained the DTNB-light chain of fast muscle, LC2f. The alkali light chain content was seen to differ in these cut-out bands. FM1 was considered to be a homodimer of the LC3f (A-2) light chain, FM2 a heterodimer of LC1f (A-1) and LC3f (A-2), and FM3 a homodimer of the LC1f light chain. SM contained the two light chains of slow muscle (LC1s and LC2s). The IM was found to contain not only LC1f, but also LC1s in about equal amounts. Thus, IM appears to consist of fast myosin heavy chains with a fast and slow light chain associated with either 'head' region of the molecule. The structural isoforms described by Fitzsimons and Hoh (1983) are schematically represented in Fig. 2. It is important to note that their non-denaturing sodium pyrophosphate-buffered gel system does not differentiate between the two types of fast heavy chains described by Billeter et al. (1981). It remains to be seen whether both the Type IIA and the Type IIB myosin heavy chains form structural isoforms of myosin which can be distinguished on a molecular weight basis according to their associated light chains, and whether both types of heavy chain can form an intermediate or hybrid molecule with the slow-type light chain(s).

Fig. 2 Schematic representation of the structural isoforms of myosin. Five isoforms have been detected by non-denaturing sodium pyrophosphate-buffered gel electrophoresis (Fitzsimons and Hoh, 1983). Light chain analysis of each isoform has revealed that they differ, not only in their myosin heavy chain (HC), but also owe their different mobilities to their associated light chains. Fast-twitch muscles contain three isoforms (FM1, FM2, FM3) which each contain the fast heavy chain(s) and two DTNB-light chains (open triangles), and differ in their respective alkali light chain content. The fastest migrating native isoform, FM1, is a homodimer (two of the same) for the low molecular weight alkali light chain (small open squares). The second fastest migrating isoform, FM2, is a heterodimer for the two alkali light chains, which gives it a mobility intermediate between the FM1 homodimer and the FM3 homodimer, which contains two of the larger molecular weight alkali light chains (large open squares). The slowest migrating native isoform of myosin is found characteristically in slow-twitch muscle. It is composed of the slow-type heavy chain and slow light chains, LC1s (filled squares) and LC2s (filled triangles). In the Soleus of the mouse, many fibres appear to stain as the fast Type IIA fibres with myofibrillar ATPase histochemistry. The IM isoform represents an hybrid type of myosin and is probably specific to Type IIA fibres in slow muscles. It consists of fast heavy chains, two DTNB-light chains, and both the higher molecular weight alkali light chain of fast muscle and the LC1s light chain of slow muscle.


SM
Slow HC
IM
Fast HC
FM3
Fast HC
FM2
Fast HC
FM1
Fast HC

The functional significance of these structural isoforms remains controversial. Weeds and Taylor (1975) were able to separate the HMM subfragment-1 isoenzymes from rabbit skeletal muscle myosin on SDS-polyacrylamide gels. Using a modified alpha-chymotrypsin digestion technique, they found two electrophoretically distinct HMM S-1 fragments, each containing one of the alkali light chains (A1 or A2). They measured the calcium-activated ATPase activity of each fragment, separated by ultracentrifugation, and found no significant difference between the S-1 (A1) and the S-1 (A2) fragments. They also found no difference in the magnesium-dependent ATPase activity of the two fragments. However, when they tested the fragments for ATPase activity in the presence of filamentous actin, striking differences in Vmax were obtained. The Vmax for S-1 (A1) was about half that obtained for S-1 (A2). These results were confirmed by Winstanley, Trayer and Trayer (1977) for HMM S-1 prepared from chicken skeletal muscle myosin. These authors also found that S-1 (A1) exhibits a greater binding affinity to both the monomeric and filamentous forms of actin than does the S-1 (A2) species. On the basis of this observation, Trayer, Winstanley and Trayer (1977) were able to separate heavy meromyosin isoenzymes from both rabbit and chicken skeletal muscles by exploiting this differential actin binding. They isolated two homodimers of the HMM fragment. The A1/A1 homodimer was found to exhibit a stronger affinity than the A2/A2 species for both the monomeric and filamentous forms of actin.

In a detailed study, Wagner et al. (1977) looked at the interaction of S-1 (A1) and S-1 (A2) with regulated actin (filamentous actin + tropomyosin + troponin). They found that the differences in actin-activated ATPase activity which had been reported previously (Weeds & Taylor, 1975) were maintained. However, when they modified the ionic strength of the solution they found different effects on the enzyme kinetics of the two fragments. While increasing the ionic strength from 6 mM to 46 mM KCl had little effect on V for S-1 (A2), the corresponding value for S-1 (A1) increased to

approximately equal that of S-1 (A2). They suggested that the two isoenzymes are controlled by the same rate limiting process in the steady state under these conditions. The previously reported differences may have reflected a peculiar behaviour under conditions of low ionic strength. Despite the additional insight provided by the use of regulated actin in a solution of higher ionic strength, these authors admit that the results of in vitro studies must be interpreted with caution.

Wagner and Giniger (1981) have shown that HMM S-1, prepared from avian skeletal muscle myosin, in which the alkali light chain was removed by mild dissociating conditions, still had the ability to bind reversibly to filamentous actin and retained 30-80% of the ATPase activity of native HMM S-1. Shivaramakrishnan and Burke (1982) were able to remove the alkali light chain from HMM S-1 of rabbit skeletal muscle myosin while retaining its full ATPase activity. In addition to providing direct evidence that the site for ATPase activity is located on the heavy chain of the myosin molecule, these recent studies have also required the re-evaluation of the role of alkali light chains as "essential" components. Despite the fact that myosin can exist as an heterodimer, containing both alkali light chains in the same molecule (e.g., FM2, Fitzsimon and Hoh, 1981), or the combination of both LC1f and LC2s in IM, the physiological role of these structural isoforms has remained an enigma.

If the light chains modulate the interaction of the myosin heavy chain with actin, or the ATPase activity of myosin, then the molecular events involved in tension development become more difficult to analyze with the increasing number of variables. A further complication has arisen with the report of Perrie, Smillie and Perry (1973) in which the DTNB light chain (LC2f) was found to exist in either a phosphorylated (LC2f-P) or a non-phosphorylated form. A regulatory function for phosphorylation has been found in other enzyme systems, for example, phosphorylation increases the enzymic activity of phosphorylase b kinase (Krebs et al., 1966), but decreases the activity of pyruvate dehydrogenase

(Linn et al., 1969). Many investigators have attempted to elucidate the role of light chain phosphorylation in the contractile process (Barany & Barany, 1977; Barany et al., 1979, 1982; Kushmerick & Crow, 1982; Manning & Stull, 1979; Moore & Stull, 1984; Perry et al., 1984; Stull & High, 1977). Despite intensive research over the past ten years, no physiological property of intact skeletal muscle has been identified that unequivocally can be concluded to be a direct consequence of the phosphorylation of the DTNB light chain. For a review of the current level of understanding of this phenomenon, refer to Perry et al. (1984).

Developmental Changes in Myosin Expression

Early work by Trayer and Perry (1966) has shown that the Ca^{2+}-ATPase activity of myosin changes with the development of the muscles in several animals. In addition, Drachman and Johnston (1973) found that actomyosin ATPase activity is low in the neonatal rat Extensor Digitorum Longus muscle until 5 days after birth. After this time the activity increases, reaching adult values by approximately 20 days. Similar differences were reported by Sreter et al. (1975) who identified ATPase characteristics of myosin from embryonic muscle which were distinct from the adult fast and slow isoforms.

Hoh and Yeoh (1979) looked at the native myosin isoenzymes from fetal, adult fast-twitch and adult slow-twitch skeletal muscles in non-denaturing pyrophosphate gels. They found three distinct isoforms in fetal muscle which had a slightly faster mobility than the adult isoforms. The slowest-migrating fetal isoform (fm3) co-migrated with the fastest adult isoform (FM1), however, analysis of the light chain content of the cut-out bands showed that these two isoforms were distinct. They suggested that as light chains constitute only a small fraction of the total molecular mass of native myosin, the differences between fetal and fast-twitch muscle myosins with respect to their electrophoretic behavior was due to structural differences in the heavy chain of

these two types of myosin. Two-dimensional maps of cyanogen bromide peptides of these myosins also pointed toward structural differences.

Early immunohistochemical evidence indicated that myosin in chick embryonic muscle was distinct from that of adult chickens (Masaki & Yoshizaki, 1974). This has been confirmed by Whalen et al. (1981) in an elegant study using various approaches, including polypeptide mapping, complement fixation, immunocytochemistry, gel electrophoresis of native myosins, and the study of synthetic myosin thick filaments by electron microscopy. In addition to a fetal isoform of the myosin heavy chain, they found a neonatal isoform. These isoforms appeared sequentially in rat muscle during the period from late gestation to about three weeks of age, at which point the adult isoform became the predominant species of myosin heavy chain.

In fetal muscle, a light chain has been identified by two-dimensional electrophoresis that is distinct from the adult isoforms (Whalen et al., 1978). This light chain has also been demonstrated in developing cardiac muscle (Whalen & Sell, 1980), and in human fetal muscle (Strohman et al., 1983). Biral et al. (1984) have shown, by one-dimensional peptide map analysis, that the fetal-specific light chain in developing human muscle is structurally unrelated to both LC1s and LC1f light chains of human adult skeletal muscle myosin. A similar light chain has been characterized in embryonic chicken skeletal, cardiac, and smooth muscles (Takano-Ohmuro et al., 1985).

The myosin isoforms which characterize adult skeletal muscle fibre types exist as a result of the expression of the genes encoding for their respective subunits. Thus, three myosin heavy chain genes are expressed in adult limb skeletal muscles (Billeter et al., 1981). In the rat, the fast skeletal muscle myosin light chains 1 and 3 (the alkali light chains) have been shown to result from the expression of a single gene by a combined process of differential RNA transcription and splicing (Periassamy et al., 1984). This has

also been demonstrated in chicken skeletal muscle (Nabeshima et al., 1984). In rabbit skeletal muscle, LC1f (190 amino acids long) and LC3f (149 amino acids long) had been shown previously to have complete sequence homology for the last 141 amino acids at the carboxy terminus (Frank and Weeds, 1974), and also, these two proteins were known to differ in length and amino acid sequence at their amino termini (Frank and Weeds, 1974; Weeds, 1975). The DTNB light chains of slow and fast skeletal muscle are known to differ in electrophoretic mobility (Lowey and Risby, 1974), however, it is not known whether they result from separate genes.

A peculiar feature of adult slow skeletal muscle myosin is that it shares the same genes as those expressed in ventricular myocytes for most of its constituent subunits. Two isoenzymes of the myosin heavy chain are expressed in the cardiac ventricles, the alpha- and beta-heavy chains (Mahdavi et al., 1982). A recent study has demonstrated that the beta-heavy chain of ventricular myosin is the same as that of slow skeletal muscle and is expressed by the same gene (Lompre et al., 1984). The alkali light chains of slow muscle (LC1s) and of ventricular muscle (LC1v) co-migrate on two-dimensional gels (Whalen et al., 1978), and recent evidence indicates that they are encoded by the same gene (Barton et al., 1985).

The sequential transition from fetal to neonatal, and then to either adult fast or adult slow myosin heavy chains has been further characterized by Gambke et al. (1983). These authors looked at native myosins on pyrophosphate gels. In addition to the five isoforms described by Fitzsimons and Moh (1981a) for adult fast (FM1, FM2, FM3) and adult slow (SM1 and IM, which they call SM2) skeletal muscles, they also found four fast-migrating isoforms expressed in the embryonic and neonatal stages (f1 to f4), as well as two slow isoforms (s1 and s2). The migration rates of s1 and s2 were distinct from SM1 and SM2, indicating that in addition to fast isoforms, slow isoforms are expressed also in immature slow muscles. They showed that there are distinctions in the complement of myosin isoenzymes between the slow-twitch Soleus and the fast-twitch EDL in the rat at birth. These distinctions were

expressed by preliminary forms of fast and slow myosin. Similar results have been obtained by Lyons et al. (1983), who found that neonatal EDL and Soleus muscles differed in their native isoforms.

Gambke et al. (1983) looked at the progression of native myosin expression in presumptive fast (EDL and Gastrocnemius) and slow (soleus) muscles in developing rats that were either euthyroid, hypothyroid, or hyperthyroid. The serum T_4 levels showed that normal rats are essentially hypothyroid at birth. There was a significant rise to peak serum T_4 levels at 15 days followed by a slight decline to mature levels at 35 days. During normal fast muscle development, the switch in myosin composition was found to coincide with peak serum T_4 levels, to be inhibited in hypothyroid animals, and was precocious in hyperthyroid animals. These results suggest that thyroid hormone orchestrates the transition by activating adult myosin synthesis and inhibiting synthesis of neonatal myosin. These authors propose that in developing slow muscle thyroxine probably also turns off synthesis of neonatal slow myosin, as it was found to persist up to 35 days in hypothyroid, and was eliminated prematurely in hyperthyroid animals. Thyroxine did not appear to significantly alter the transition from neonatal to adult slow myosin.

Despite neonatal denervation, the EDL was found to progress successfully through its maturational sequence and synthesized the adult fast myosin isoenzymes (Gambke et al., 1983). This suggests that thyroidal regulation of fast muscle maturation is mediated upon the muscle rather than via alterations in the function of the motoneuron. In contrast, the integrity of the motoneuron was found to be essential to the synthesis of slow myosin. After neonatal denervation, the proportions of slow myosin in the Soleus progressively declined. Slow myosin was no longer detectable by 25 days, at which point the atrophic Soleus was found to be composed of myosin isoenzymes similar to those of adult fast muscle. Their results suggest that in the absence of the nerve the prospective slow muscle synthesizes a fast form of myosin which may be under thyroidal control. Thus, during normal

development the intact slow motoneuron would override this propensity and promote synthesis of slow myosin.

Using a monoclonal antibody specific for the embryonic myosin heavy chain of rat skeletal muscle, Gambke and Rubinstein (1984) have also demonstrated that thyroid hormone hastens the disappearance of this subunit during development, while hypothyroidism retards its decrease. The thyroid hormone induced transitions in skeletal muscle myosin isoforms have also been reported in urodelian amphibians and mice (d'Albis et al., 1984), and have been associated with the synthesis of new myosins of higher catalytic efficiency (d'Albis et al., 1985).

A similar transition has been observed in the expression of the cardiac ventricular alpha- and beta-heavy chain genes, which also appear to be developmentally and hormonally regulated (Lompre et al., 1984). In the cardiac ventricles of several mammalian species, three myosin isoenzymes have been identified (V1, V2 and V3) on the basis of their electrophoretic mobility in non-denaturing gels (Hoh et al., 1977; Lompre et al., 1981; Clark et al., 1982). However, these three myosins are composed of only two distinct types of myosin heavy chains, the alpha- and beta-isoenzymes. V1 and V3 are composed of alpha-alpha and beta-beta homodimers, respectively, while V2 has been postulated to be an alpha-beta heterodimer (Hoh et al., 1979; Chizzonite et al., 1982). In all the species studied so far, V3 is the most abundant myosin in late fetal life. In rat and mouse, V1 increases at birth and becomes the predominant form throughout perinatal life. In contrast, in larger animals, V1 is transiently predominant after birth with V3 becoming the most abundant myosin in the adult animal (Hoh et al., 1977; Lompre et al., 1981; Sartore et al., 1981; Clark et al., 1982).

The distribution of the cardiac isoenzymes can be modified in certain pathological and experimental conditions, such as mechanical overload (Lompre et al., 1979), diabetes (Dillman, 1980; Malhorta et al., 1981), and changes in thyroid hormone levels (Hoh et al., 1978; Flink et al., 1979; Sartore et al.,

1981; Schwartz et al., 1981; Martin et al., 1982; Sinha et al., 1982; Everett et al., 1982, 1983; Lompre et al., 1984). Hypothyroidism is associated with a shift from alpha- to beta-myosin heavy chain. Protein data analysis suggests that the beta-myosin heavy chain reappearing in the heart of hypothyroid rats is the same as the one expressed in fetal life (Schwartz et al., 1982). This redistribution of myosin heavy chain isoenzyme ratio has been shown by several authors to be reversed by the administration of thyroid hormone (Hoh et al., 1979; Flink et al., 1979; Sartore et al., 1981; Chizzonite et al., 1982; Everett et al., 1983). The intrinsic velocity of contraction of cardiac muscle decreases in rats made hypothyroid by hypophysectomy, and can be restored with the administration of thyroid hormone (Korecky and Beznack, 1971). Thus, the myosin composition of the myocardium is of physiological significance since the relative distribution of the two major ventricular myosin heavy chains, alpha and beta, is in direct correlation with the contractile performance of the heart (Schwartz et al., 1981; Ebrecht et al., 1982).

Lompre et al. (1984) have shown that the V3 to V1 transition of ventricular myosin isoforms in the rat occurs at approximately the same time (day 14) as the peak thyroxine levels, just as the transition from neonatal to adult myosin isoforms in fast skeletal muscles is also known to occur (Gambke et al., 1983). Lompre et al. (1984) have used alpha- and beta-myosin heavy chain gene-specific sequences derived from cDNA clones and the S1 nuclease mapping procedure to show that the myosin heavy chain transitions in the ventricular myocardium are determined by the expression of the alpha- and beta-myosin heavy chain genes, and can be entirely accounted for by changes in the accumulation rates of their respective mRNAs. In addition, their results suggest that thyroid hormone has opposite effects on the transcription of the alpha- and beta-heavy chains.

The cardiac ventricles and fast-twitch skeletal muscles appear to share a common mechanism controlling isoenzyme transition during development, namely through the modulation

of gene expression by thyroid hormone. Neither the fast skeletal nor the cardiac ventricular muscle require a neural influence to effect their isoenzyme transitions. This has been clearly demonstrated in a recent study by Korecky and associates (1986). Adult rat heart (containing predominantly the V1 isoenzyme), when transplanted into the abdomen of an inbred adult recipient, changes its isoform expression from V1 to V3. The transplanted heart was coronary perfused and beating isovolumically. They observed no changes in the heart of the recipient animal. To study the putative direct role of thyroid hormone (T_3) on the selective expression of the V1 isoform of myosin they used donor and recipients made hypothyroid after 30 days on low iodine diet containing 0.5% propylthiouracyl. Thus, they obtained two hearts, each expressing the V3 isoform in the same animal. One was innervated and carried full haemodynamic load while the other was denervated and performed minimum external work. After surgery the recipient rats were put on normal diet supplemented by injections of T_3, which made them euthyroid and later hyperthyroid, and the rate of anticipated conversion of V3 to V1 was monitored. The percentage of V1 increased in the transplanted and recipient heart from small traces at the time of surgery to 12% and 6% after 3 days, 49% and 46% after 7 days and to 91% and 96% after 14 days. In view of the similar time relationships of the rate of V1 synthesis in both hearts, the most probable explanation is the direct action of T_3 on the myocyte, independent of any neural influence.

The motoneurons innervating Type I (slow oxidative) skeletal muscle fibres have been shown to inhibit the postnatal expression of other fast-type-specific contractile proteins. The suppression of the fast forms of the troponin components in presumptive slow cells is nerve-dependent (Dhoot & Perry, 1980, 1982, 1983), as are the subunits of tropomyosin (Heeley, Dhoot & Perry, 1985). Thus, several studies indicate that the suppression of fast skeletal muscle contractile protein isoforms in developing slow muscle is not only nerve-dependent, but may result from a common mechanism at the nuclear level (Gustafson et al., 1985).

The importance of the nerve in maintaining the expression of the contractile proteins of slow skeletal muscles has been clearly demonstrated under a number of experimental conditions. Denervation of the slow Soleus at birth leads to the expression of the adult fast-isoforms of myosin (Gambke et al., 1983), troponin (Dhoot and Perry, 1983), and tropomyosin (Heeley, Dhoot and Perry, 1985). Denervation of fast muscles at birth has little effect on the transition from neonatal to adult isoforms. Cross-reinnervaton experiments indicate that replacing the nerve supply of a slow muscle with that which normally supplies a fast muscle changes the expression of the genes controlling the synthesis of certain myofibrillar proteins. In the cases of myosin (Buller et al., 1969; Barany and Close, 1971), tropomyosin (Heeley et al., 1983), and the troponin complex (Dhoot, Perry and Vrbova, 1981), the slow muscle forms of these proteins are replaced by the forms usually present in fast muscle. The converse, in which the nerve to a fast muscle is replaced by one normally innervating a slow muscle, also leads to the replacement of the fast isoforms by the contractile proteins normally seen in slow muscle. Chronic electrical stimulation of the nerves supplying fast and slow muscles with the appropriate frequencies has also been demonstrated to modify the expression of myofibrillar proteins, suggesting that the pattern of neural discharge is involved in the control of the genes responsible for normal phenotype expression in Type I (slow-twitch) and Type II (fast-twitch) fibres of skeletal muscle. Thus, fast muscle becomes slow when the nerve which supplies it is subjected to a pattern of activity characteristic of a nerve to slow muscle. The chronically stimulated fast muscle synthesizes slow myosin isoforms (Sreter et al., 1973; Salmons & Sreter, 1976; Pette et al., 1976), coexistence of fast and slow isoforms can be detected in the same fibres (Pette & Schnez, 1977; Rubinstein et al., 1978), and tropomyosin isoforms are also converted to those seen in slow muscle (Roy et al., 1979). If chronic stimulation of a "fast" nerve is terminated, the fast muscle ultrastructural myofibrillar characteristics have been shown to reappear, indicating that fast-to-slow transformation can be reversed (Eisenberg, Brown and Salmons, 1984).

The question as to whether a trophic factor is involved in the control of gene expression in slow muscle, or whether the pattern of neural discharge itself is the principle regulator remains controversial. At first glance, experiments involving stimulation of a "fast" nerve with low frequency impulses suggest that there is no need to invoke a neural trophic factor and that the activity pattern of the muscle is the cause of the transformation. On the other hand, as suggested in the review of Jolesz and Sreter (1981), the retrograde effect of chronic stimulation may alter the properties of the motoneuron, and these changes could induce the transformation of muscle by trophic signals.

In adult skeletal muscle, motoneurons appear to determine the biochemical characteristics of the muscle fibres they innervate. Histochemical evidence was first to point out that muscle fibres of a given motor-unit (motoneuron + muscle fibres it innervates) are identical (Burke et al., 1973). This has been demonstrated clearly in dissected single muscle fibres from identified motor-units for enzymes of intermediary metabolism. In contrast to the large variability of malate dehydrogenase and fructose-1,6-diphosphatase activities in muscle fibres taken at random from a fast muscle, Nemeth et al. (1981) found that muscle fibres from the same motorunits had similar enzyme activities. Thus, despite an apparent lack of involvement of motoneurons to fast muscle in determining the transition from neonatal to adult protein isoforms, there nonetheless appears to be a trophic influence of the nerve in maintaining certain other biochemical parameters. It remains to be seen whether the postnatal differentiation of fast muscle into Type IIA and Type IIB fibres (each containing a specific myosin heavy chain) is neurotrophically regulated.

The motoneurons are not "hard wired" in the animal after birth. A considerable degree of post-natal maturation and plastic transformation can occur. The best example of these post-natal changes is the Soleus muscle of the rat. It is composed mostly of Type I and a few Type II(A) fibres. Kugelberg (1976) has shown that there is an adaptive trans-

formation of Soleus motor-units with growth. He found that the proportion of Type II fibre units with fast contraction times decreased from 33% to 10% of total, and Type I fibre units with longer contraction times increased from 67% to 90% of total, in mature rats (aged 34 weeks) as compared to the younger animals (aged 5 weeks). He also demonstrated that fibres transform from one type to another, and concluded that the increase in Type I fibre units from Type II was due to changes in the properties of the motoneurons. Butler-Browne and Whalen (1984) have looked at the rat Soleus from 1 week after birth to 6 months. Approximately half the fibres were found to contain embryonic and slow myosin at 1 week; these fibres subsequently contain only slow myosin. A second group of fibres contains embryonic and neonatal myosin at 1 week and most of them subsequently accumulate adult fast myosin. A portion of this latter group begins to acquire slow myosin from 4 weeks of age. They interpreted these data as suggesting a preprogrammed sequence of myosin isoenzymes from embryonic to neonatal to adult fast, which, at any time during development could be suppressed with the induction of slow myosin accumulation.

In the mouse, it appears that a similar process occurs. Parry and Parslow (1981) found that in mice aged 3 to 4 weeks approximately 26% of Soleus fibres were Type I and 72% were Type IIA, based on myofibrillar ATPase. In older mice with an average age of 5 months, Lewis, Parry and Rowlerson (1982) found approximately 41% Type I and 59% Type IIA fibres in Soleus. Ovalle et al. (1983) have obtained very similar results in mice of the same strain at 4 and 32 weeks of age.

The post-natal development of fast muscles appears to be slightly different. In fast muscles with a heterogenous fibre composition, the slow-fibre population is believed to be generated earlier in development than the fast (Rubinstein & Kelly, 1981), and there does not appear to be any significant transformation from slow to fast postnatally (Jones, Ridge and Rowlerson, 1985). This difference in developmental maturation between slow and fast muscles may reflect a fundamental difference in the differentiation into fast and

slow muscles. Dhoot (1983, 1985) has shown that the initiation of differentiation into slow and fast cells and the adult patterns occurs very early in fetal life, long before the adult nerve patterns are established. Some other factor(s), such as position of the muscle in the limb, may be responsible for the pattern of development in each muscle. The function of the nerve supplying each muscle would then be to assure that the adult patterns are maintained.

Muscular Dystrophy in Man

The term dystrophy is reserved for the classic types of degenerative muscle disease of genetic etiology (Adams, 1975). There are several types of inherited muscular dystrophies in humans, which are distinguished on the basis of their developmental appearance and the muscles affected. For example, the fascioscapulohumeral dystrophy, first described by Landouzy and Dejerine (1884), occurs between ages 6 and 20 years in both males and females and involves certain muscles of the face and shoulders. Oculopharyngeal dystrophy is an autosomal dominant inherited disease which begins late in adult life and leads to ptosis of the eyelids and dysphagia (Taylor, 1915). The limb girdle dystrophies are autosomal recessive disorders causing proximal weakness early in adult life. Usually the legs are more severly involved than the shoulders (Carroll and Brooke, 1978). Myotonic dystrophy is yet another form which affects the muscles of the face, jaw, neck and levators of the eyelids. Its distinguishing feature is a considerable delay in relaxation ater a stong voluntary contraction (Steinert, 1909).

The most common and severe of the muscular dystrophies is pseudohypertrophic dystrophy, first described by the French neurologist G. B. Duchenne in a monumental work of 800 pages entitled De l'éléctrisation locale, et de son application à la pathologie et à la thérapeutique, the first edition of which appeared in 1855. Duchenne's dystrophy is an X-linked disorder, and is much more frequent in males than in females. The disease commences in early life, before the 6th year in

the majority of cases. Certain muscles are "spared" from the degenerative disorder; these are the muscles of the hand, face, jaw, larynx, pharynx, and the eyes. All other muscles show either initial hypertrophy or atrophy, and eventually most of them become wasted. Erb's comprehensive monograph in 1891 clearly described the chief histologic changes in the muscles, and he was the first to emphasize the general similarity of the disease in all the dystrophic syndromes. Erb (1891) concluded that the essential process was fibre hypertrophy followed by atrophy and that it was associated with vacuolation, splitting of the muscle fibre, nuclear proliferation, central nucleation, increased endomysium, and connective and fat tissue infiltration.

In Duchenne's dystrophy the most prominent histologic feature is the occurrence of groups of degenerating muscle fibres. Four or five fibres in a cluster disintegrate over a longitudinal extent of a few sarcomeres, and the necrotic sarcoplasm is soon invaded by phagocytes. The process excites active regeneration as revealed by the activation of satellite cells (Mastaglia and Kakulas, 1969; Mastaglia et al., 1970). The degeneration and subsequent regeneration of muscle fibres leads to the expression of both fetal and neonatal isoforms of native myosin (Fitzsimons and Hoh, 1981b). Betto and Salviati (1985) have analyzed the pattern of myosin heavy and light chains in single muscle fibres of patients with Duchenne's muscular dystrophy. They found an increased number of hybrid fibres which contain both fast and slow myosin heavy and light chains. These changes probably reflect secondary effects of the pathology rather than a direct result of the genetic defect.

Although Duchenne muscular dystrophy is an X-linked recessive disorder characteristically affecting only males, seven females have been reported recently with this form of dystrophy, and in all seven cases the patient also has an X-autosome translocation (Verellen et al., 1977, 1978; Greenstein et al., 1977; Lindenbaum et al., 1979; Canki et al., 1979; Jacobs et al., 1981; Zatz et al., 1981; Emmanuel et al., 1983). Although the autosome involved is different

in each of the seven cases, the exchange point in the X chromosome is at band Xp21 in each case. This has led to the suggestion that the gene responsible for Duchenne dystrophy is at band Xp21, and that in each case the translocation has disrupted or inactivated the normal gene function on the translocated chromosome. This conclusion is further strengthened by the fact that in no case were the parents found to carry the translocation and in no case was the mother a proven carrier of the Duchenne gene. Furthermore, in all cases the normal X chromosome is preferentially late replicating (inactive) suggesting that a single defective gene on the active (translocated) X chromosome is responsible for the disease (Verellen-Dumoulin et al., 1984). A recent study by Worton et al. (1984) has come closer to allowing a direct analysis of the Duchenne muscular dystrophy gene by identifying the regions flanking the gene locus in the translocation-derived chromosomes. Though the product of the Duchenne gene is still not known, these studies should soon provide the answer.

The study of dystrophy in animal models has not solved this basic problem either, however it has led to a better understanding of the pathology of the disease, as animals are more easily amenable to experimental manipulation. Several animal models exist for the human muscular dystrophies. Although similar inherited muscular disorders have been observed in the chicken (Asmundson and Julian, 1956), the Syrian hamster (Homburger et al., 1962), and the guinea pig (Webb, 1970), the best studied model for the human muscular dystrophies remains the house mouse.

Muscular Dystrophy in the Mouse

Four genetically distinct forms of muscular dystrophy have been reported. Michelson et al. (1955) reported a mutation in the Bar Harbor 129 strain of mice, which they called dystrophia muscularis. This myopathic disorder occurs as a result of an autosomal recessive gene (*dy*) on chromosome 10. In 1970, Meier and Southard described a progressive hereditary myopathy of mice caused by a second allele, dy^{2J}, at the

dy locus. Although the two mutant genes are allelic, the syndromes caused by them differ in degree of clinical and pathological severity. The onset is later and progression of clinical signs and pathological changes in dy^{2j} are slower than in dy animals. The dy animals develop a progressive weakness of proximal muscles that is recognizable 3 to 4 weeks after birth by dragging of the hindlimbs, kyphosis, and a nodding of the head. There is a reduction in body weight and a progressive atrophy of axial and limb muscles that is seldom compatible with survival beyond the third or fourth month. On the other hand, the dy^{2j} animals show a more progressive loss of the use of their hindlimbs starting at about 2 months. By 4 months the hindlimbs are fully extended, with ankylosis of the knee joint. These amnimals will drag their hindlimbs from this age on, using their forelimbs for locomotion, and can live as long as their littermate controls (about 2 years).

Unlike the dy-mutants, dy^{2j} animals of both sexes can breed with unaffected heterozygote mates, although affected males do so with considerably less success than their normal counterparts as they are hindered by the extended hindlimbs (Younger and Silverman, 1984). A sign common to both genotypes is the spontaneous twitching observed in the hindlimbs of these animals. This is because most lumbosacral spinal root axons are thinly myelinated near both the spinal cord and the exit from the spinal canal, and are bare in midroot (Bradley and Jenkinson, 1974; Bray and Aguayo, 1975; Stirling, 1975). These juxtaposed bare axons were shown to transmit impulses from one to another by ephaptic transmission, and to be the sites of spontaneous impulse generation (Rasminski, 1978). The impulses occur as single isolated events, in bursts of frequencies of up to 100 Hz, or as continuous activity persisting for several minutes in single axons. The effect of this neural activity cannot be ignored when discussing the pathological changes in dystrophic mouse hindlimb muscles.

The other two inherited myopathies in the mouse are myodystrophy, an autosomal recessive disorder whose gene

(myd) is on chromosome 8 (Lane et al., 1976), and an X-linked muscular dystrophy (mdx), recently described by Bullfield et al. (1984). In the case of the myodystrophic mouse, Rayburn and Peterson (1978) have reported the occurrence of amyelinated axons in the ventral roots at the level of the lower lumbar and sacral vertebrae. The extent of this amyelination is considerably less than that reported for the dy and dy^{2j} animals. Nonetheless, it points to a common genetic fault. Few studies have been performed on the mdx strain, however one report by Dangain and Vrbova (1984) points out that this disease involves a massive degeneration-regeneration of muscle fibres at 3 to 4 weeks of age with an almost complete recovery, so that it probably is not a good model for Duchenne's dystrophy.

Several biochemical studies have been performed on the dy/dy and dy^{2j}/dy^{2j} strains of mice in an attempt to determine at what level of the biochemical machinery the defect occurs. These have been reviewed by Strickland et al. (1979), who conclude that most, if not all, of these changes were secondary manifestations of the disease. Because the hindlimbs of these animals appear to be more severely affected than the forelimbs, attention has focused primarily on the pathological changes which take place in the hindlimb muscles. The forelimb muscles also show clear signs of dystrophy in both the dy/dy (Rowe and Goldspink, 1969; Nwoye and Goldspink, 1982) and dy^{2j}/dy^{2j} (Parry and Desypris, 1983, 1984; Jasch and Moase, 1985) animals.

Among the functional changes observed in the fast-twitch muscles of the dystrophic mouse is the prolongation of the isometric twitch (Brust, 1966; Douglas and Baskin, 1971; Harris and Wilson, 1971; Harris and Montgomery, 1975, Parslow and Parry, 1981; Bressler et al., 1983). There also appears to be a marked increase in the level of oxidative metabolism in the hindlimb muscles as judged by histochemistry (Dribin and Simpson, 1977; Silverman and Atwood, 1980; Parry and Parslow, 1981). According to Silverman and Atwood (1980), the change in oxidative capacity is associated with the altered level of activity seen in the hindlimbs of dystrophic mice.

This suggestion is reasonable in light of studies in which artificially imposed chronic stimulation of normal fast-twitch muscle was shown to produce slowing of the twitch (Salmons and Vrbova, 1969; Salmons and Sreter, 1976), increased levels of oxidative enzymes (Pette et al., 1973), and synthesis of myosin isoenzymes that are characteristically found in slow-twitch muscle (Sreter et al., 1973; Salmons and Sreter, 1976; Pette et al., 1976; Pette and Schnez, 1977; Rubinstein et al., 1978). Similar changes can be induced by increasing muscular activity with endurance-type excercise (Green et al., 1983, 1984).

These observations led Parry and Desypris (1983) to question the extent to which the various changes reported in the hindlimb muscles of the dystrophic mouse might be related to the altered activity pattern rather than to the dystrophic process itself. Since the forelimb muscles of the dystrophic mouse do not exhibit spontaneous twitching, Parry and Desypris (1983) made use of this criterion as an internal control to examine the role of activity in modifying the effect of dystrophy. They looked at the physiological and histochemical properties of forelimb and hindlimb muscles from dystrophic mice of the C57 BL/6J dy^{2j}/dy^{2j} strain. Using polyclonal antibodies against slow myosin and immunohistochemistry, they were able to show the presence of slow myosin in many fibres of the hindlimb muscles in these mice, whereas forelimb muscles did not have slow myosin-containing fibres. The normally fast-twitch hindlimb muscle, Extensor Digitorum Longus, showed a considerable prolongation in the time to peak tension during a single isometric twitch. The forelimb muscle Extensor Carpi Radialis Longus also showed a slight prolongation in its time to peak tension, but the time to half relaxation was as prolonged as in the EDL muscle. The rate of relaxation in an isometric twitch is generally considered to reflect the rate of calcium uptake by the sarcoplasmic reticulum. Prolonged relaxation appears to be a characteristic feature of the dystrophic process in the C57 BL/6J dy^{2j}/dy^{2j} mouse, since it occurs in both hindlimb and forelimb muscles. The more significantly prolonged time to peak twitch tension of the hindlimb EDL muscle was con-

sidered to reflect the presence of slow myosin in this muscle. Parry and Desypris (1983) suggested that the spontaneous twitching activity observed in the hindlimbs as a result of amyelination of the spinal roots was responsible for the induction of slow myosin synthesis.

Fitzsimons and Moh (1983) have also reported the appearance of slow myosin in fast-twitch hindlimb muscles of dystrophic mice of the 129 REJ dy/dy strain by means of pyrophosphate gel electrophoresis of native myosins. These authors did not look at the forelimb muscles in these animals, and although they suggest that amyelination may be responsible for slowing of contractile properties and the shift in isomyosin distribution, their explanation that amyelination filters out high-frequency impulses seems tenuous in light of the known patterns of neural discharge in these mice (Rasminsky, 1978).

Parry and Desypris (1984) have re-evaluated the effects of spontaneous neural discharge on the oxidative capacity of fast-twitch muscles in (dy^{2j}) dystrophic mice. They determined the succinic dehydrogenase activity of forelimb and hindlimb muscles of the dystrophic mouse. When this was expressed in terms of milligrams of 'true muscle weight', neither forelimb nor hindlimb muscles showed a significant increase when compared to muscles from normal mice. The changes in SDH histochemistry (from a mosaic pattern in normal mice to a uniform one in dystrophic mice) were similar in both forelimb and hindlimb muscles. This indicates that spontaneous neural discharge is not responsible for these histochemical changes, nor does it produce an increase in the oxidative capacity of these muscles.

The question remains as to what extent this pattern of neural discharge is involved in the transformation of fast-twitch muscles in dystrophic mice. I have used three different approaches to characterize further the pathological changes in skeletal muscles of the C57 BL/6J dy^{2j}/dy^{2j} mouse. A preliminary study looks at SDS polyacrylamide gel pattern of the myosin light chains from forelimb and hindlimb

muscles of normal and dystrophic mice at various ages during adult life. This was followed by a more thorough examination using an immunohistochemical technique (with monoclonal antibodies to myosin heavy chain isoenzymes) for muscles taken on one side of the animal, compared to the pyrophosphate gel patterns of native myosins extracted from the corresponding contralateral muscles. If a progressive neurally-mediated transformation from fast to slow type fibres is involved in the slowing process of dystrophic hindlimb muscles, not only should there be a lack of slow myosin-containing fibres in the forelimb muscles, but increasing levels of slow myosin in fast-twitch hindlimb muscles. Hybrid fibres, containing both fast and slow myosin, should be seen as an indication that this transformation is taking place.

MATERIALS AND METHODS

1- Protein Extraction

A- Myosin Purification for Light-Chain Characterization

Hindlimb (Tibialis Anterior and Soleus) and forelimb (Extensor Carpi Radialis Longus) muscles of normal C57 Bl/6J and dystrophic C57 Bl/6J dy^{2j}/dy^{2j} mice aged 2, 4, 6, 8 and 12 months were surgically excised and used for the purification of myosin according to the procedure described by Dalla Libera et al. (1978). Homologous muscles from several (>3) animals were pooled to give sufficiently large tissue samples. The tissue was weighed and washed three times in three volumes (3 times the weight) of cold 40 mM KCl. The muscle was then finely minced with scissors in ten volumes of cold 40 mM KCl and then further homogenized with a Polytron PT-20 homogenizer for a 5 second period. The suspension was then centrifuged at 3000 RPM for 10 minutes in an International B-20A (IEC B-20A) centrifuge. The supernatant was discarded and the pellet resuspended in 10 volumes of 40 mM KCl and centrifuged three times to ensure the elimination of proteins soluble in low ionic strength KCl. After centrifugation, the washed pellet was blended in about 25 volumes of 0.3 M KCl, 10 mM Na-ATP, 5 mM magnesium chloride, 0.1 mM dithiothreitol (DTT), and 0.15 M potassium phosphate buffer (pH 6.5). Myosin was extracted from the suspension for 20-30 minutes with constant stirring in the cold room (at 4 Celsius). The muscle extract was centrifuged for 10-15 minutes in the IEC B-20A centrifuge at 18,000 RPM. The supernatant was dialyzed overnight against several changes of a solution containing 10 mM KCl, 0.1 mM DTT, and 1 mM Tris-HCl (pH 7.2).

The next day, the myosin precipitate in the dialysate was collected by centrifugation at 18,000 RPM for 10 minutes in the IEC B-20A centrifuge. This was further purified by resuspending the pellet in 0.6 M KCl and reducing the ionic strength to 0.3 M KCl by adding an equal volume of water in

order to precipitate the undissociated actomyosin by centrifugation at 1000 RPM for 10 minutes in the IEC B-20A centrifuge. Myosin was precipitated from the supernatant solution by dilution with cold distilled water to a final concentration of 40 mM KCl, and by centrifugation at 3000 RPM for 10-20 minutes on the IEC B-20A centrifuge. The purified myosin was dissolved in a small volume of 0.5 M KCl, 0.1 mM DTT, and 10 mM Tris-HCl (pH 7.6). To this was added an equal volume of cold glycerol and the mixture was stored at -20 Celsius. The myosin concentration was estimated by the method of Bradford (1976) using bovine serum albumin (BSA), dissolved in the glycerinated purified myosin solvent, as a standard.

B- Crude Myosin Extraction for Native Isoform Characterization

The Anterior Tibialis, Soleus, and Extensor Digitorum Longus muscles of the hindlimb and both the Extensor Carpi Radialis Longus and Brevis muscles of the forelimb were removed from one side of the mouse (either the right of the left side) and placed on labelled weighing boats in a small volume of 0.9% saline on ice. For each age group of 2, 4, 6, 8, and 12 months, at least two mice were used in the case of normal mice (for a total of 11 mice) and for dystrophics, at least three were used (for a total of 17 mice).

Homologous contralateral muscles were also excised and placed on an histology chuck with OCT compound and immersed in melting isopentane cooled in liquid nitrogen. The samples were stored at -70 Celsius until used for immunohistochemistry. Crude myosin was extracted according to the method of Mabuchi et al. (1982). Individual muscles were placed in Eppendorf microcentrifuge tubes with 30 microlitres for each milligram muscle of a solution containing 50% glycerol, 1% 2-mercaptoethanol, 5 mM DTT, and 50 mM sodium pyrophosphate, pH 8.9. These were kept at 4 Celsius in a refrigerator for two days, after which the muscle was removed and discarded. The extract was stored at -20 Celsius.

2- Electrophoretic Techniques.

A- SDS-PAGE of Myosin Light-Chains

The light-chains of purified myosin were characterized on polyacrylamide gels according to the method of Laemmli (1970). The gels were set up either with or without a stacking gel, as follows: A mixture containing 10 ml of water, 12.5 ml of a 30% Stock Acrylamide solution (29.6 g Acrylamide and 0.4 g BIS [N,N'-Methylene-bis-acrylamide] in 100ml water), 7.5 ml of "Lower Tris" buffer (containing 18.17 g Tris and 0.4 g sodium dodecyl sulfate [SDS] dissolved in water, titrated with HCl to pH 8.8 and made up to 100 ml), and 90 microlitres of a freshly prepared ammonium persulfate solution was placed in a sidearm flask and degassed with a vacuum. After 5 minutes, 30 microlitres of TEMED (N,N,N',N'-tetraethylmethylenediamine) were added to initiate polymerization. The solution was then quickly poured between 2 glass plates separated by a spacer at either end. When a stacking gel was used, the solution was poured to within 4.5 cm from the top and 2 ml of water were carefully poured on top of the acrylamide to form a flat surface at the acrylamide-water interface. Otherwise, the acrylamide solution was poured to the top of the glass plates with a Teflon comb inserted to produce wells once the acrylamide had set. In both cases, at least one hour was required for adequate polymerization of the gel.

When a stacking gel was used, the running gel was prepared as described above. The water overlay was removed and the stacking gel acrylamide solution was poured onto the running gel with a Teflon comb used to form wells in the acrylamide. The 4 % acrylamide stacking gel solution consisted of 6.5 ml of water, 1.0 ml of stock acrylamide, 2.5 ml of "Upper Tris" (containing 6.06 g Tris, and 0.4 g SDS dissolved in water, titrated with HCl to pH 6.8 and made up to 100 ml), and 40 microlitres of 10 % ammonium persulfate. This solution was degassed for 5 minutes and polymerization was initiated with 20 microlitres of TEMED. After one hour, the Teflon comb was removed and Running Buffer (diluted 1:10 from a stock con-

taining 30 g Tris, 144 g glycine, and 10 g SDS dissolved in water, titrated to pH 8.8 with HCl and made up to 1 litre) was poured into each well. Using a 100 microlitre Hamilton syringe with one and a half inch gauge needles, aliquots (approx. 5 micrograms of protein) were overlaid onto each well. Six low molecular weight standard protein markers purchased from Bio-Rad were also run to estimate the relative molecular weights of the myosin light chains. The light chains were identified from their relative molecular weights.

The gels were run in an LKB electrophoresis apparatus using the LKB 2002 Power Supply. When a stacking gel was used, the gel was run at 20 mA (constant current) per gel, until the buffer front, visualized with bromophenol blue, reached the running gel. The current was then turned up to 25 mA per gel, and the gels were allowed to run for 3-4 hours until the buffer front had reached the bottom of the gel. When gels were run without a stacking gel, the same procedure was followed, except that the current was initially set at 25 mA per gel. In general, duplicate gels were run simultaneously.

B- Sodium Pyrophosphate-buffered Gel Electrophoresis of Native Myosins

Electrophoresis of native myosin was carried out under non-denaturing conditions essentially according to the method of Hoh et al. (1976) except that the running buffer was that used by Butler-Browne and Whalen (1984), and gels were 4% or 4.5% total acrylamide run for 10-12 hours at 150 V (constant voltage) at 3 Celsius. The electrophoresis buffer was composed of 20 mM sodium pyrophosphate (pH 8.8), 1 mM EDTA, and 1.4 mM 2-mercaptoethanol. Slab gels were cast by pouring a fresh solution containing 10 ml running buffer, 10 g glycerol, 3.88 g acrylamide and 0.12 g Bis (for 4% gels) or 4.365 acrylamide and 0.135 g Bis (for 4.5% gels), made up to 100 ml with distilled water. 500 microlitres of 10% ammonium persulfate were added and the solution was degassed for 5 minutes, following which polymerization was initiated with the addition of 100 microlitres of TEMED. Stacking gel was

not required. Isobutanol was placed at the air-acrylamide interface to ensure an oxygen-free environment for proper formation of wells. After one hour of polymerization, the Teflon combs were removed and cold running buffer was used to fill the wells. Aliquots of native myosin extract were placed in each well and the slab gels were placed in the cold running buffer and allowed to equilibrate for 30 minutes prior to the run. The running buffer was cooled with a water-glycerol mixture refrigerated to 0 Celsius flowing through a glass heat exchanger in parallel with the gels. The buffer tank was placed in a bath of crushed ice, and the temperature of the running buffer was maintained at approximately 3 Celsius, ranging from 1.5 to 4.5 Celsius. During the run, the buffer was recirculated with a peristaltic pump from the lower to the upper chamber, which was allowed to drain back into the lower chamber, thus ensuring a relatively constant buffer pH.

3- Staining of Gels

A- Coomassie Brilliant Blue R-250

Following SDS-PAGE of myosin light chains, gels were stained either in Coomassie Blue R-250, or were fixed in 10% acetic acid, 50% methanol in water for silver staining. The Coomassie Blue R-250 stain involves soaking the gel in a solution of 7% acetic acid, 10% methanol in water with 0.1% Coomassie Blue R-250. The staining solution is filtered prior to use with Whatman no. 5 filter paper. The gel is allowed to soak for 30 minutes in the stain which is then replaced with a solution of 7% acetic acid, 10% methanol in water. The gel is agitated on a shaker bath with a piece of folded tissue paper placed in the solution to mop-up the Coomassie Blue. The gel is destained by replacing the tissue paper repeatedly until the desired background intensity is reached. Following Coomassie Blue staining the gel can also be silver stained for increased sensitivity.

B- Silver Staining

Two silver staining techniques were used to visualize the protein bands in polyacrylamide gels. The first was used for the analysis of myosin light chains from the purified myosin extracts, and is derived from the method of Merril et al. (1981). The proteins in the gel are fixed in 50% methanol, 10% acetic acid for 30 minutes, and excess SDS in the gel is removed by three 200 ml, 10 minute rinses of 10% ethanol, 5% acetic acid. The gel is then soaked for 5 minutes in 200 ml of a solution of 3.4 mM potassium dichromate and 0.0032 N nitric acid, followed by three washes in 200 ml of deionized water. The gel is placed in 200 ml of 12 mM silver nitrate for 30 minutes. This is followed by a 1 minute rinse in deionized water and three 5 minute rinses of image developer solution which consists of 0.28 M sodium carbonate and 0.5 ml of 37% formaldehyde per litre. When the image had reached the desired intensity, development was stopped by adding 400 ml of 5% acetic acid for 5 minutes. The gels were rinsed in deionized water and stored in zip-lock freezer bags.

One disadvantage with the above technique was that, to achieve maximum staining sensitivity, the gel had to be exposed to relatively intense uniform light during the first 5 minutes in silver nitrate. An alternative method for silver staining is that of Morrissey (1981). This method produces staining which is more constant from gel to gel and is independent of lighting conditons. SDS-PAGE was repeated for some of the purified myosin extracts. These gels were stained according to the Morrissey technique. Gentle but thorough agitation of the gels was obtained by means of a Thomas Rotating Apparatus. The gel is prefixed in 50% methanol, 10% acetic acid for 30 minutes, followed by a 30 minute soak in 5% methanol, 7% acetic acid. The gel is then fixed in 10% glutaraldehyde for 30 minutes, and then rinsed in running deionized water for at least two hours. After the rinse, the gel is soaked in a 5 microgram per litre solution of DTT for 30 minutes, followed by 0.1% silver nitrate for 30 minutes without rinsing between the DTT and the silver nitrate step. The silver nitrate is then discarded and the gel

rinsed briefly in distilled water. The gel is developed by soaking in a solution of 3% sodium carbonate to which is added 50 microlitres of 37% formaldehyde. Two rapid rinses in developer are followed by a longer soak (approximatively 5 minutes) until the gel is stained to the desired level of intensity. Development is stopped by adding 5 ml of 2.3 M citric acid directly to the developer and agitating for 10 minutes. The solution is then discarded and the gel is washed several times in distilled water over a 30 minute period. The gels are then stored in zip-lock freezer bags. All volumes were 100 ml except for the glutaraldehyde step which was 75 ml.

C- Coomassie Brilliant Blue G-250

Sodium pyrophosphate-buffered slab gels of native myosin were stained in 0.04% Coomasie Blue G-250 in 3.5% perchloric acid according to the method of Reisner et al (1975). The Coomassie Blue G-250 stain is prepared by dissolving 0.4 g of Coomassie Blue G-250 in 1 litre of 3.5% perchloric acid (prepared by diluting 58 ml of a 60% perchloric acid stock). The stain is left on a magnetic stirrer overnight and filtered the next day with Whatman no. 5 filter paper. To stain a gel with the G-250 stain it is simply dropped in 100 ml of the solution and agitated mildly for a few minutes. The protein bands stain progressively darker and reach a plateau of intensity. The background stains a light orange color and therefore this technique does not require a destaining step. For storage, gels are placed in zip-lock freezer bags which are heat sealed to prevent them from leaking.

4- Immunohistochemistry with Monoclonal Antibodies

Muscles removed from the contralateral side of the muscles used for crude myosin extraction were kept at -70 Celsius embedded in frozen OCT compound on an histology chuck. To section the tissues, the chuck was transferred to the cryostat microtome and allowed to warm to -20 Celsius for 30

minutes, after which 8 micrometre sections were cut and adsorbed to subbed microscope slides. The sections were allowed to dry and immunohistochemistry using six monoclonal antibodies applied onto 6 sections per slide, respectively, was carried out using the peroxidase anti-peroxidase (PAP) method with diaminobenzidine (DAB) as the chromogen in a manner similar to that described by Bourne (1983). The PAP technique is schematically represented in Fig. 3.

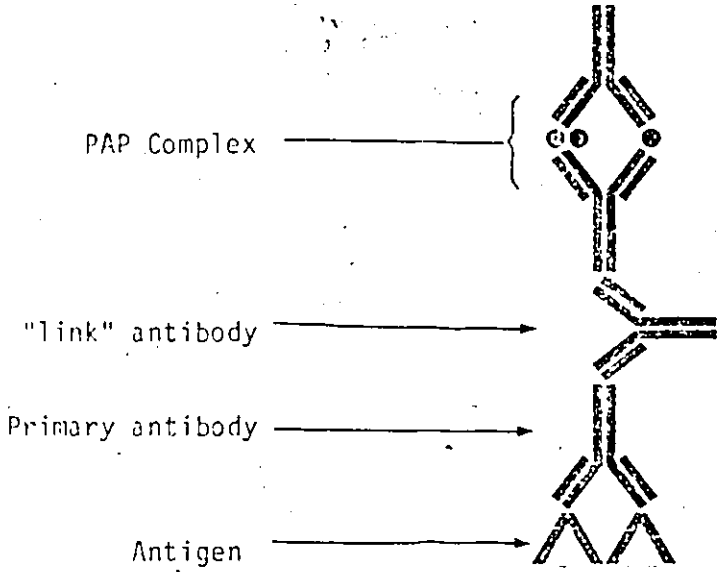


Fig. 3 The peroxidase anti-peroxidase (PAP) method. This method utilizes three reagents: primary (monoclonal) and secondary antibodies, and PAP Complex— comprised of the enzyme peroxidase and an antibody against peroxidase. The primary antibody is specific for the antigen (in this case myosin heavy chain). The secondary or "link" antibody binds to both the primary and to the PAP Complex, because both are produced in the same animal species. Functionally, the link antibody is added in excess so that only one of its Fab (fragment antigen binding) sites will bind to the primary, leaving the other Fab site free to bind to the antibody in the PAP Complex. The peroxidase enzyme is visualized via a substrate-chromogen reaction (in this case, hydrogen peroxide and diaminobenzidine). (Reproduced from Bourne (1983) with permission)

Once the sections were dry, they were rinsed in a phosphate-buffered (pH 7.4) 0.9% saline solution (PBS) 3 times for one minute and then once for 5 minutes. The slides were removed from the PBS and excess PBS was removed carefully with tissue paper. When the sections were dry, 10-20 microlitres of primary (monoclonal) antibody (diluted 1:20 with 1% BSA in PBS) were used to cover each section, which were then incubated in a moist chamber at ambient temperature for 16-18 hours. The reactivity of each antibody is given in Table 1.

Table 1 Reactivity of Monoclonal Antibodies to Type-specific Myosins.

	Fiber Type				
mAb	I	IIA	IIB	IIX	Embryonic
BF 45	-	-	-	-	+
BF 46	+	-	-	-	+
BF 32	+	+	-	-	-
BF 34	-	+	+	+	+
BF 35	+	+	+	-	+
BF F3	-	-	+	-	+

+ indicates a positive reactivity; - indicates no reactivity
The mAbs were the generous gift of Dr. S. Schiaffino, Institute of General Pathology, University of Padova, Padova, Italy.

The next day, the slides were again rinsed in PBS three times for 30 seconds and once for 10 minutes to remove the unbound antibodies. The slides were then removed from the PBS and excess fluid was removed from the sections. A secondary (polyclonal) or "link" rabbit anti-mouse antibody (diluted 1:20) was used to overlay each section. After a one houf incubation in a moist chamber at room temperature, the slides were again rinsed in PBS. Again, the slides were removed from the PBS with excess fluid removed, and the PAP

antibody (diluted 1:50) was added to each section. This was incubated in a moist chamber at room temperature for one hour and then rinsed in PBS. The sections were developed in DAB (100 mg DAB for 100 ml PBS) and hydrogen peroxide (100 microlitres of a 30% stock for 100 ml 0.1% DAB). The hydrogen peroxide is added to the DAB solution just prior to use. After 5 minutes, the color development was stopped by a 10 minute rinse in running tap water. The sections were then dehydrated in 95% ethanol with two 1 minute soaks, and then by two 1 minute soaks in 99% ethanol, followed by two 2 minute soaks in xylene. The slides were removed from the xylene and a few drops of Permount were placed on the sections which were covered by a cover glass.

5- Statistical Analysis

Fibre counts were obtained from sections of Soleus stained with monoclonal antibodies from which four categories of fibre types were obtained: 'pure' Type I fibres, 'pure' Type IIA fibres, Hybrid (I+II) fibres, and Type IIB fibres. A two-factor analysis of variance was performed looking at the effects of animal type and age as well as any interactions. Statistical analysis was performed on the University of Ottawa Mainframe Computer using the SPSS-X program in consultaion with Dr. C. Dulberg of the Department of Epidemiology.

RESULTS

SDS-PAGE of Myosin Light Chains

Anterior Tibialis Purified myosin from pooled muscles of normal and dystrophic mice was analyzed on 12.5% SDS-buffered acrylamide gels for mice aged 2, 4, 6, 8, and 12 months. At 2, 4, and 6 months slight differences were noticed between normal and dystrophic gel patterns (Fig. 4a, b and Fig. 5a). LC3f levels appeared lower in dystrophic AT and slow light chains were not detected at these ages. At 8 months, however, the light chains characteristic of slow muscle, LC1s and LC2s, are seen in the dystrophic AT (Fig. 5b). At 12 months (Fig. 6), these light chains are even more obvious in the dystrophic AT. There appears to be a small amount of LC1s in the normal 12 month AT myosin. In a preliminary report (Parry and Stewart, 1985) based on crude densitometric scans of gels silver stained with the method of Merril (1981), we proposed that LC1s appeared sooner than LC2s in dystrophic AT. However, a conclusive statement is not yet possible since the relative intensity of silver stained proteins can vary within a given gel with the Merril technique and this makes the densitometry scans unreliable (see Fig. 6, the gel for 12 month old mice).

Soleus For the same age groups, dystrophic Soleus shows a slight decline in the relative intensity of slow light chains as compared to the normal Soleus (compare 2, 4, and 6 month dystrophic Soleus with normal Soleus). At 2 months (Fig. 4a), 4 months (Fig. 4b), and 6 months (Fig. 5a), there also appears to be less LC2s relative to LC1s in dystrophic Soleus. In the normal mouse Soleus, there is little change in the relative levels of these light chains with age. The 8 month purified myosin extract of dystrophic Soleus was too dilute (Fig. 5b). At 12 months, there is no longer a difference in the relative staining intensities of the slow and fast light chains.

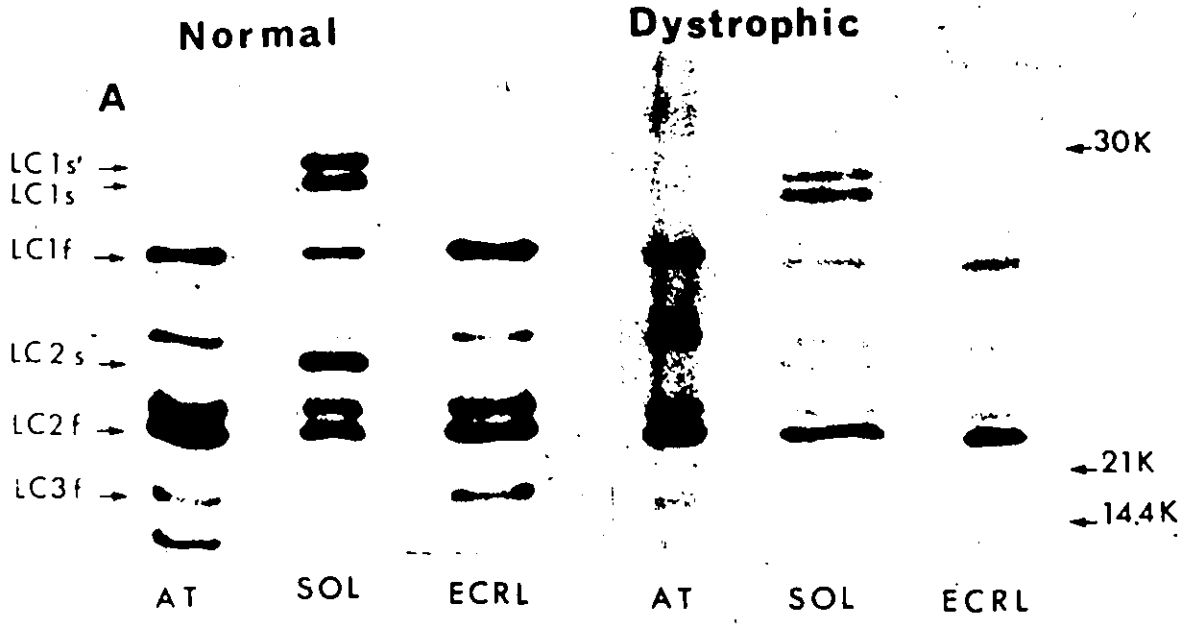
Extensor Carpi Radialis Longus The ECRL does not show any sign of either LC1s or LC2s, nor does there appear to be any

significant change in the relative intensities of the fast light chains at any of the age groups of dystrophic animals.

These results are summarized with the other data in Fig. 48.

Fig. 4 SDS-PAGE of purified myosin from (A) 2 month and (B) 4 month old normal and dystrophic mice. AT, Anterior Tibialis; SOL, Soleus; ECRL, Extensor Carpi Radialis Longus. LC1s', LC1s, and LC2s are the light chains of slow muscle, and LC1f, LC2f, and LC3f are the light chains of fast muscle. Gels A (normal) and B were silver stained according to the method of Morrissey (1981) and gel A (dystrophic) according to Merril (1981).
In B, dystrophic ECRL muscle myosin extract did not contain enough protein to show bands at this level of staining intensity.

FIG. 4


Normal
Dystrophic
A
LC1s'
LC1s
LC1f
LC2s
LC2f
LC3f
30K
21K
14.4K
AT
SOL
ECRL
AT
SOL
ECRL

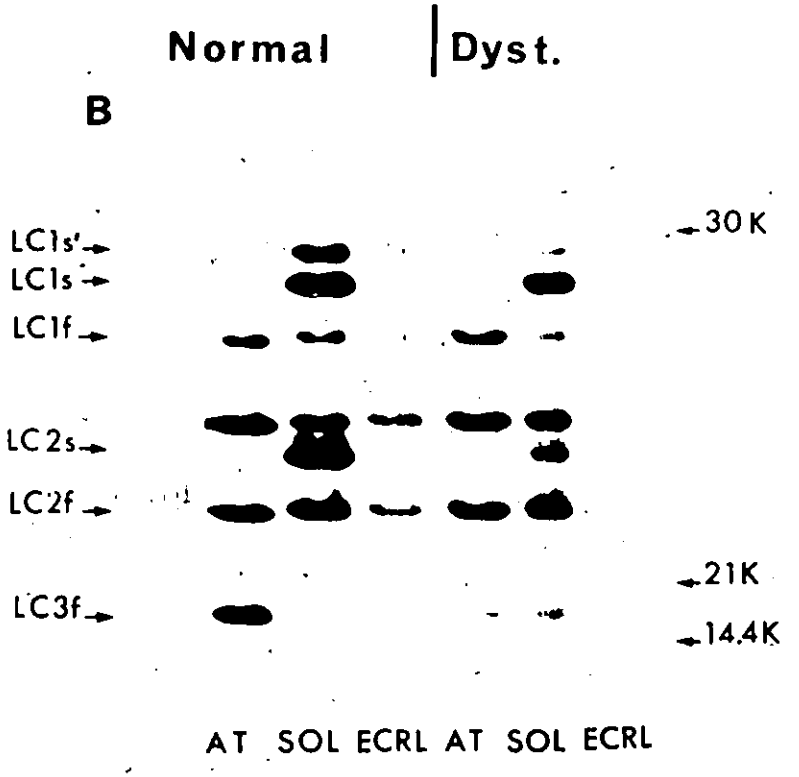
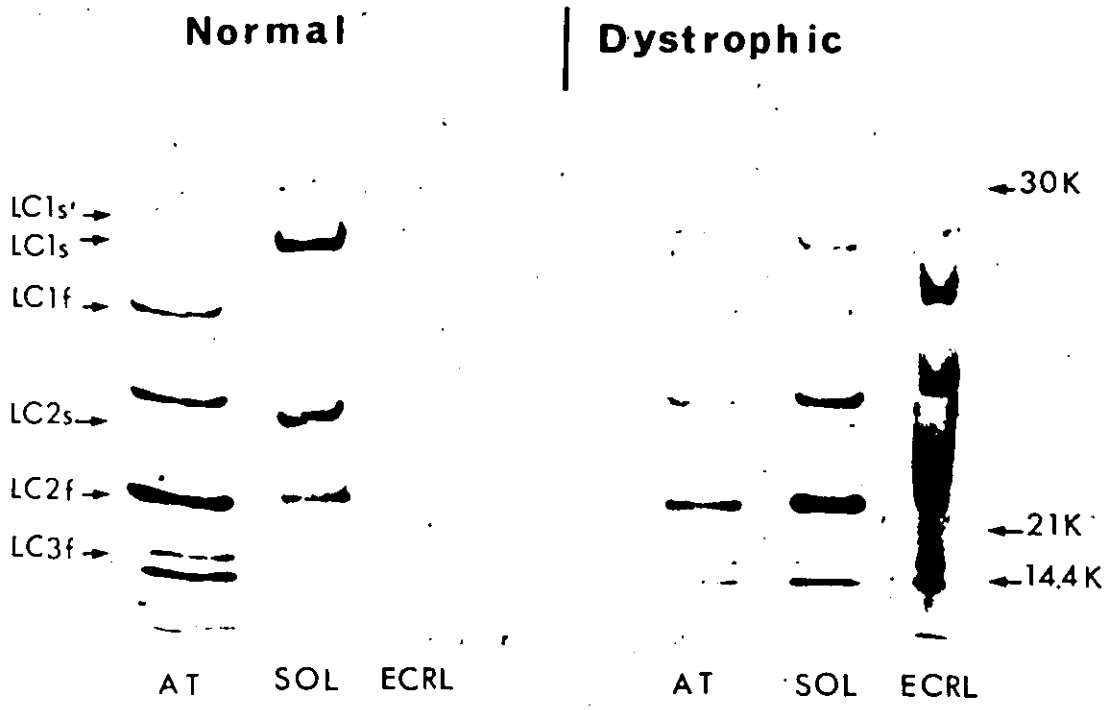

Normal
Dyst.
B
LC1s'
LC1s
LC1f
LC2s
LC2f
LC3f
30K
21K
14.4K
AT SOL ECRL AT SOL ECRL

Fig. 5 SDS-PAGE of purified myosin from (A) 6 month and (B) 8 month normal and dystrophic mice. In both A and B the gels were silver stained according to the method of Morrissey (1981). Note the appearance of LC1s and LC2s in AT of dystrophic mice aged 8 months. In B, the myosin extract for 8 month dystrophic Soleus muscle was lost during preparation.

FIG. 5


Normal
Dystrophic
LC1s'
LC1s
LC1f
LC2s
LC2f
LC3f
30K
21K
14.4K
AT
SOL
ECRL
AT
SOL
ECRL

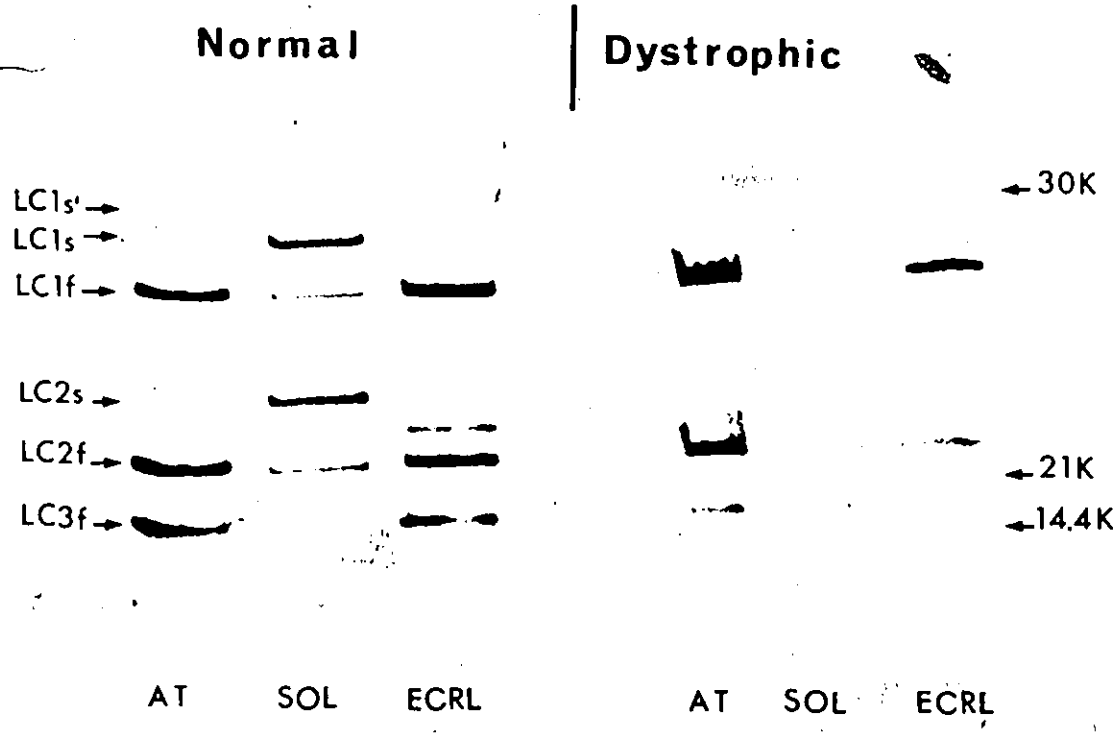
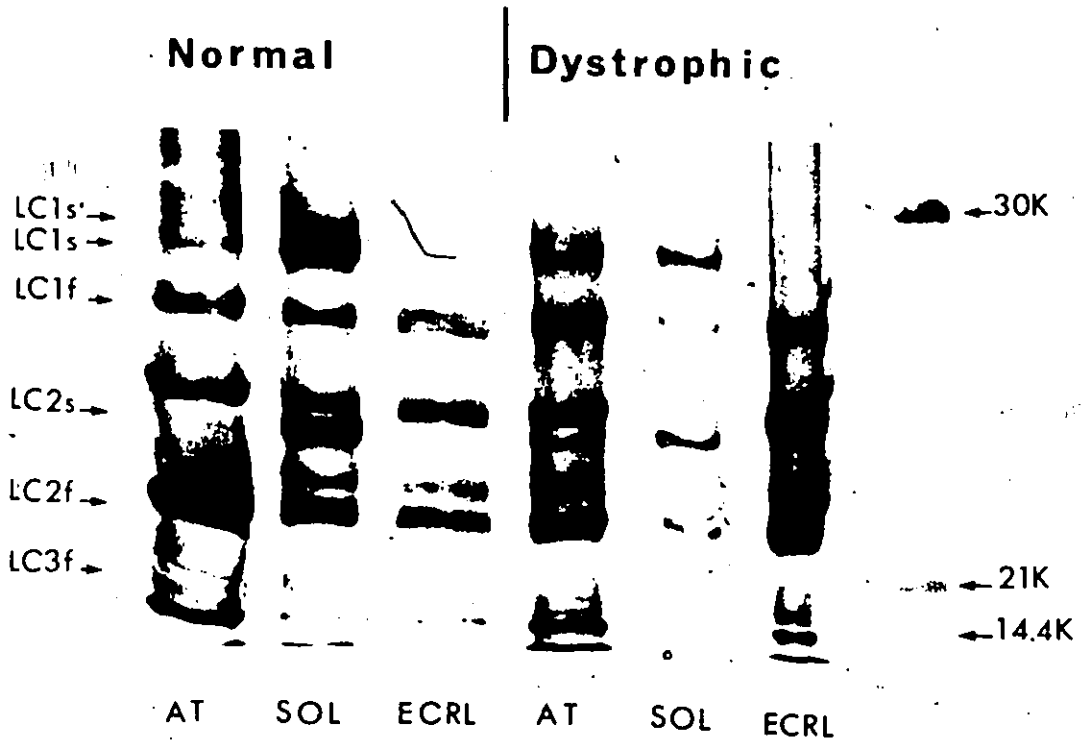

Normal
Dystrophic
LC1s'
LC1s
LC1f
LC2s
LC2f
LC3f
30K
21K
14.4K
AT
SOL
ECRL
AT
SOL
ECRL

Fig. 6 SDS-PAGE of purified myosin from 12 month old normal and dystrophic mice. The gel was stained according to the method of Merril (1981) (note the pale core of certain bands).

FIG. 6


Normal
Dystrophic
LC1s'
LC1s
LC1f
LC2s
LC2f
LC3f
30K
21K
14.4K
AT
SOL
ECRL
AT
SOL
ECRL

Immunohistochemistry of Muscle Cross-sections

Monoclonal antibodies were overlaid on 8 micrometer cross-sections of AT, EDL, SOL, and ECRL and B grouped together on an histology chuck. The monoclonal antibodies' specificities are indicated in Table 1 of Materials and Methods. The anti-Type I (slow) myosin heavy chain specific antibody, BF 46, was found to cross-react with embryonic myosin heavy chain. This antibody appears in each A-labelled micrograph. The anti-Type II (fast) myosin heavy chain-specific antibody, BF 34, gave a positive reaction with all fibre Types except Type I fibres which consist of only slow myosin heavy chain ('pure' Type I fibres). This antibody appears in all micrographs labelled B. The BF F3 antibody is specific to Type IIB myosin heavy chain, but also reacts with embryonic myosin heavy chain to a lesser intensity. This antibody is the one which appears in micrographs labelled C. The BF 45 antibody is specific to embryonic myosin heavy chain. It is the antibody used in micrographs labelled D. The anti-embryonic myosin heavy chain antibody does not cross-react with Type I myosin heavy chain.

Two of the antibodies, BF 32 and BF 35 were not included in the Results because they did not add to the information obtained with the other antibodies, BF 34, BF 46, and BF F3. The BF 45 antibody was only shown in those cases where confirmation of the presence of embryonic myosin, rather than Type I-specific myosin, was required. The Photomicrographs were taken on a Zeis microscope using a Neofluar 16 or a Neofluar 40 lens to give an enlargement of 160 times or 400 times, respectively, with a times 10 ocular. Staining was considered positive when a granular deposit was seen on muscle fibres. "Negative staining" refers to intensities which are equal to background.

Areas sampled in photomicrographs were taken as representative of the whole muscle or the region of interest in a muscle. Fibre counts were done in the case of the Soleus muscle of normal and dystrophic mice aged 2, 4, and 6 months of age and revealed consistent data (refer to Appendix). In

general, immunohistochemical data and native myosin gel data are from the same animal.

Soleus The Soleus muscle of normal and dystrophic mice aged 2, 4, 6, 8, and 12 months were examined for their immunohistochemical profile (Fig. 7-16). The normal Soleus muscle consists of fibres which stain positive with the anti-Type I antibody, and other fibres which do not stain with this antibody (For example, Fig. 7a). The fibres which do not react with the anti-type I antibody react with the anti-Type II antibody (Fig. 7b). Fibres which do not stain with this antibody do not contain any fast myosin heavy chain and correspond to the positive fibres stained with the anti-Type I antibody. Hybrid fibres, which show positive staining to both antibodies, are a common occurrence in the Solei of younger mice (2 months, Fig. 7a, b, twin arrows). These most likely represent the fast-type fibres undergoing transformation to slow-type fibres, a normal feature of the Soleus post-natal development (Kugelberg, 1976). Beyond 6 months (Fig. 11), hybrid fibres were rarely seen. At 12 months (Fig. 15), none of the fibres showed dual staining for both anti-Type I and antiType II antibodies.

The dystrophic Soleus shows a progressive loss of 'pure' Type I muscle fibres. Hybrid fibres, which would contain both fast MHC and slow MHC, are seen throughout the post-natal development of the dystrophic Soleus. The proportion of these fibres is seen to increase substantially. At 12 months (Fig. 15a), only a few fibres are seen to stain exclusively with the anti-type I MHC antibody, the majority of the Type I-positive fibres also show positive staining to the anti-Type II MHC antibody. Since even at 12 months 'pure' Type I fibres can be identified, it is not surprising to find LC2s in the SDS-polyacrylamide gels of myosin purified from Solei of 12 month old dystrophic mice.

I rarely (6 of 15 muscles) saw Type IIB-positive fibres in normal Soleus (Fig. 13c). The Soleus of dystrophic mice aged 2 months (Fig. 8c), 4 months (Fig. 10c), and 6 months (Fig. 12c) all show Type IIB-positive fibres, though there

were only a few in each case. There seems to be a higher frequency of solei with Type IIB fibres in dystrophic mice (14 of 17 muscles, see Appendix).

The results are summarized with the other data in Fig. 48.

Fig. 7 Soleus muscle on the upper left and Anterior Tibialis muscle on the lower right from a normal mouse aged 2 months. In A, slow Type I fibres of the Soleus stain dark with the anti-Type I antibody, BF 46. In B, the Type II fibres stain dark with the anti-type II antibody, BF 34. The Type IIA fibres of Soleus stain more intensely than the Type IIB fibres of AT. The latter stain dark with the anti-Type IIB antibody, BF F3, in C, leaving a few IIA fibres in AT unstained (arrow). The fibres in Soleus which are lightly stained with the Type II-specific antibody (twin arrows) in B also show positive staining for the Type I specific antibody in A.

FIG. 7

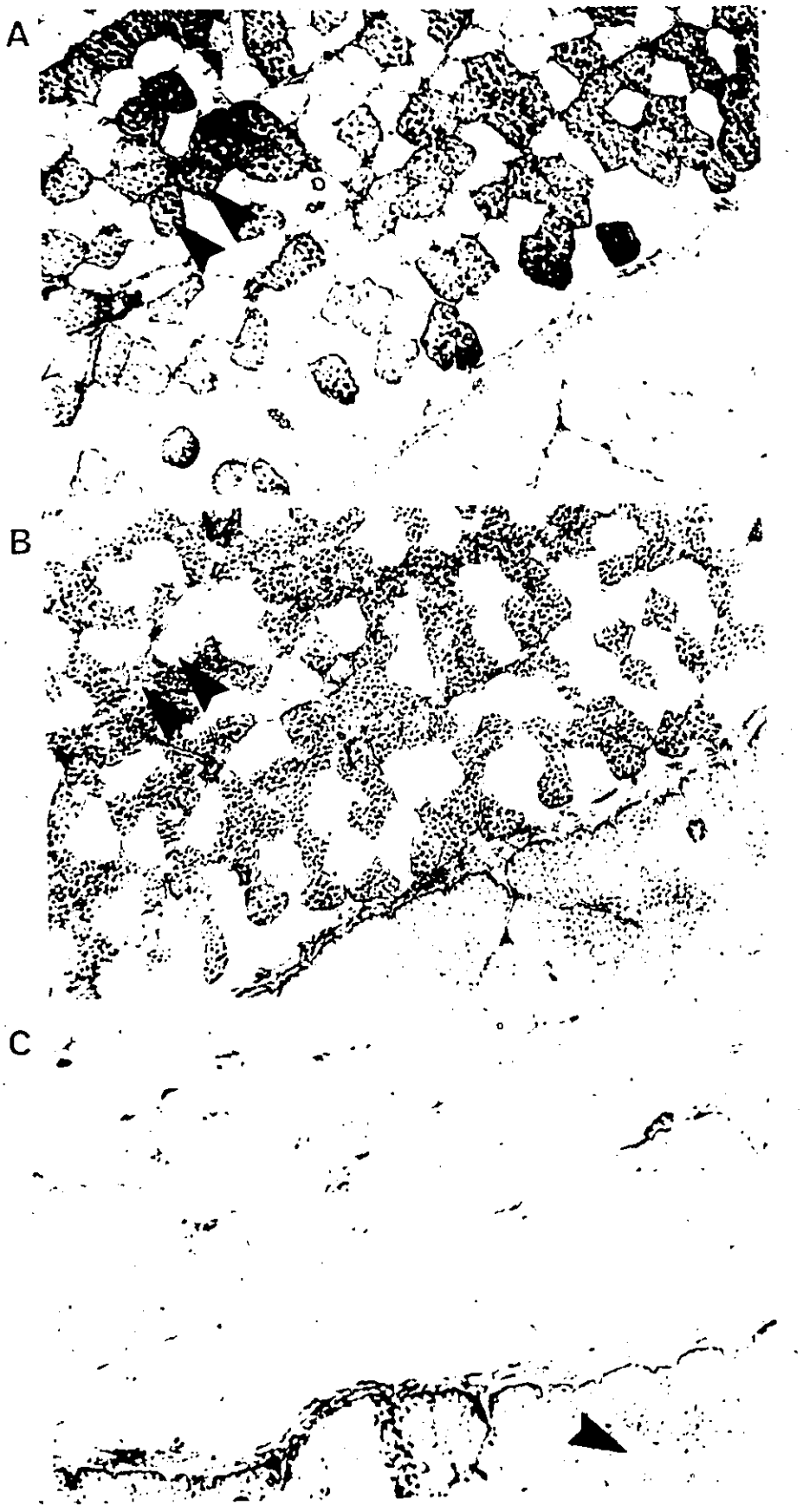


Fig. 8. Soleus muscle on the left and AT muscle on the right from a 2 month old dystrophic animal. The soleus is juxtaposed to the oxidative region of AT. In A, the BF 46 antibody stains Type I fibres in SOL as well as a few in AT. In B, many fibres which stain with BF 46 also stain with the anti-Type II antibody BF 34. In C, AT shows positive staining in some fibres for Type IIB-specific antibody, BF F3.

FIG. 8

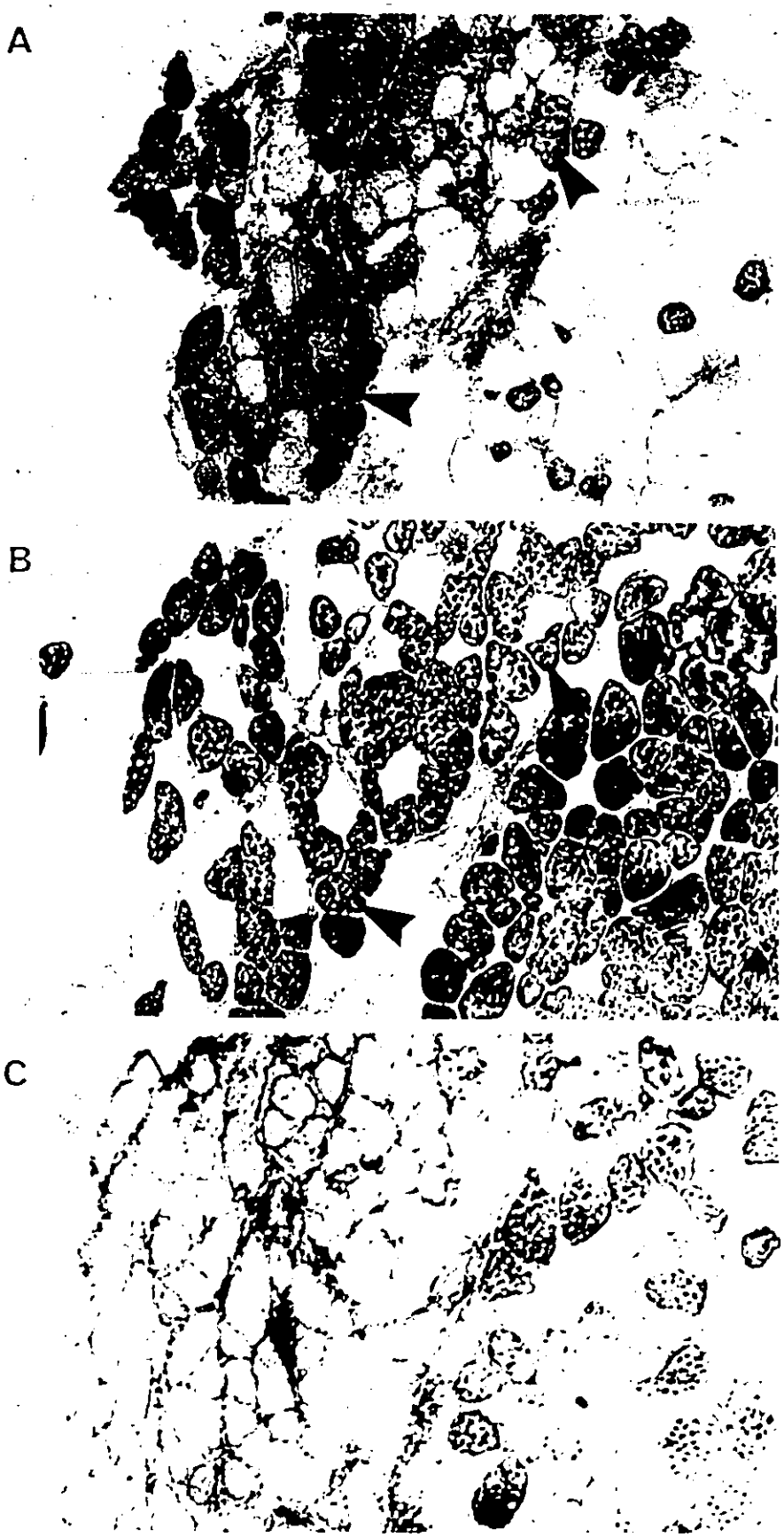


Fig. 9 4 month normal Soleus showing, in A, Type I fibres detected with the anti-Type I antibody, which do not react with the Type II-specific antibody, BF 34, in B. In C, none of the fibres stain with the anti-Type IIB antibody, BF F3.

FIG. 9

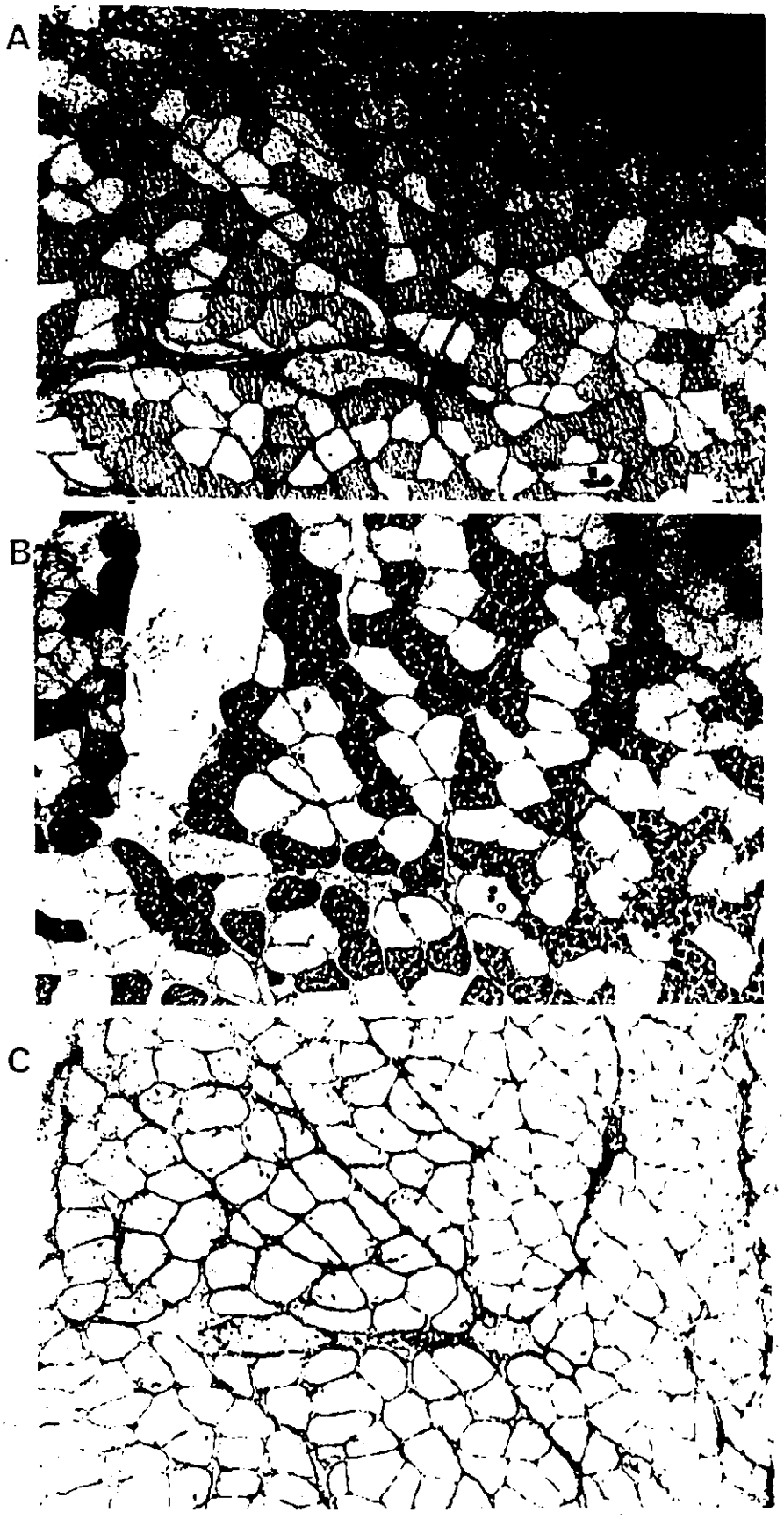


Fig. 10 4 month dystrophic Soleus showing a few fibres staining with the Type I-specific antibody, in A. In B, two of the fibres also appear to stain with anti-Type II antibody (arrows). In C, one fibre shows some reactivity with the anti-Type IIB antibody (small arrow).

FIG. 10

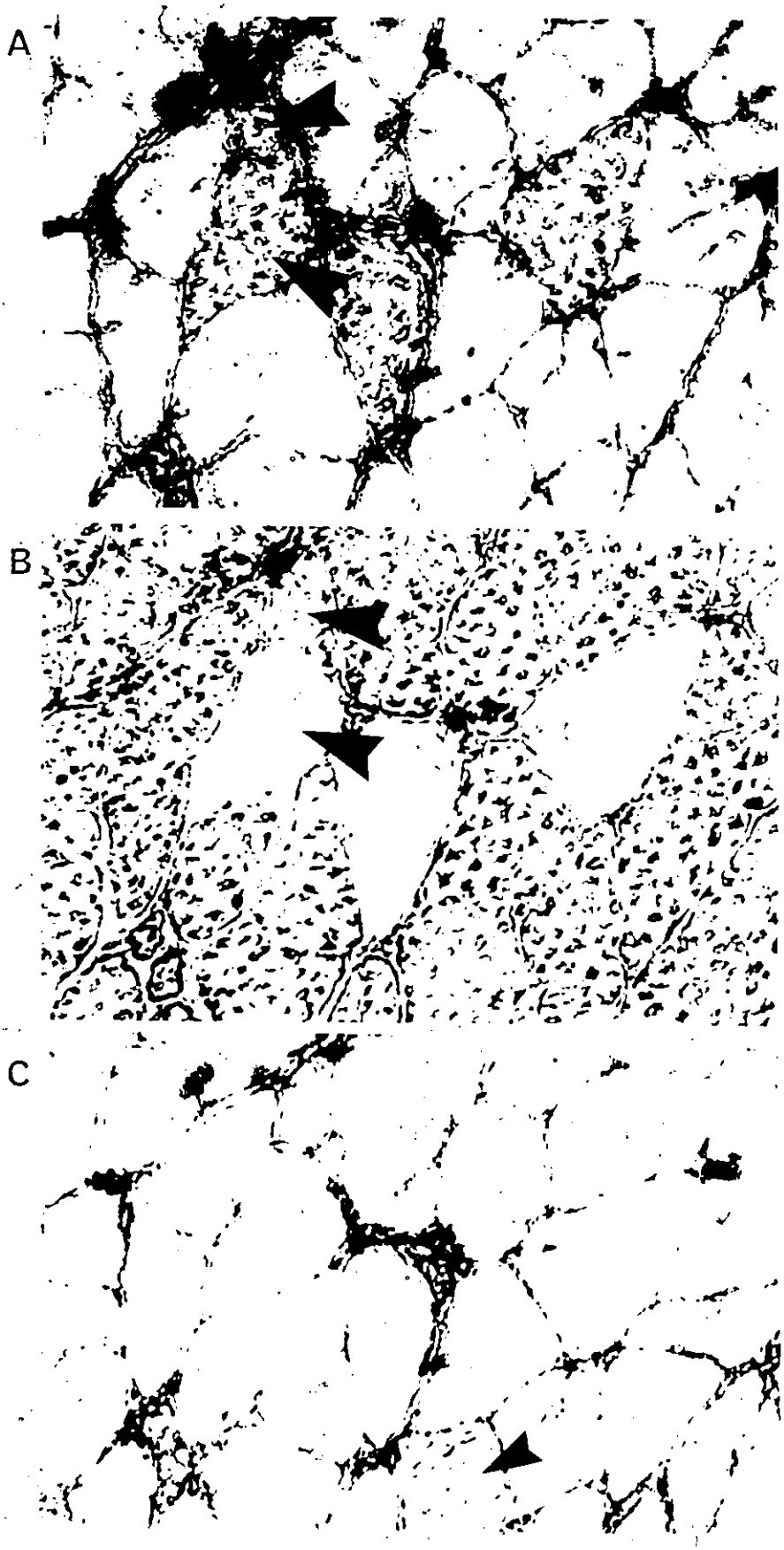


Fig. 11 6 month normal Soleus shows clear reciprocal staining in A and B with one exception (arrow), where both the antiType I and the anti-Type II antibodies react. In C, no Type IIB fibre is seen.

FIG. 11

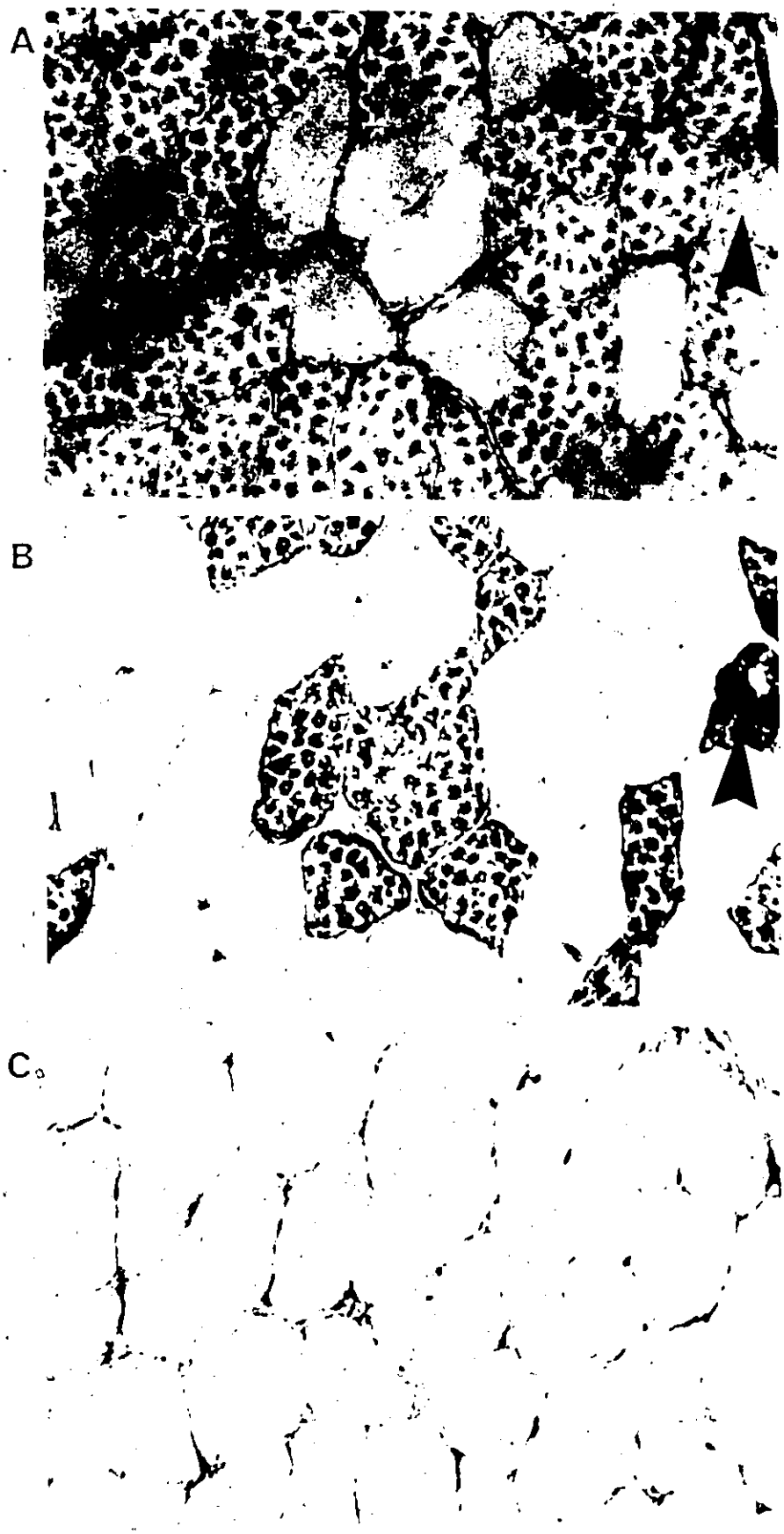


Fig. 12 6 month dystrophic Soleus on the left and AT on the right. Several intensities of staining are seen for Soleus in A with the anti-Type I antibody. In B, some of these fibres also reacted with the anti-Type II antibody (arrows). AT shows no reaction to anti-Type I antibody in A, and all fibres reacted to anti-Type II antibody in B, with some of these appearing as Type IIB fibres in C.

FIG. 12

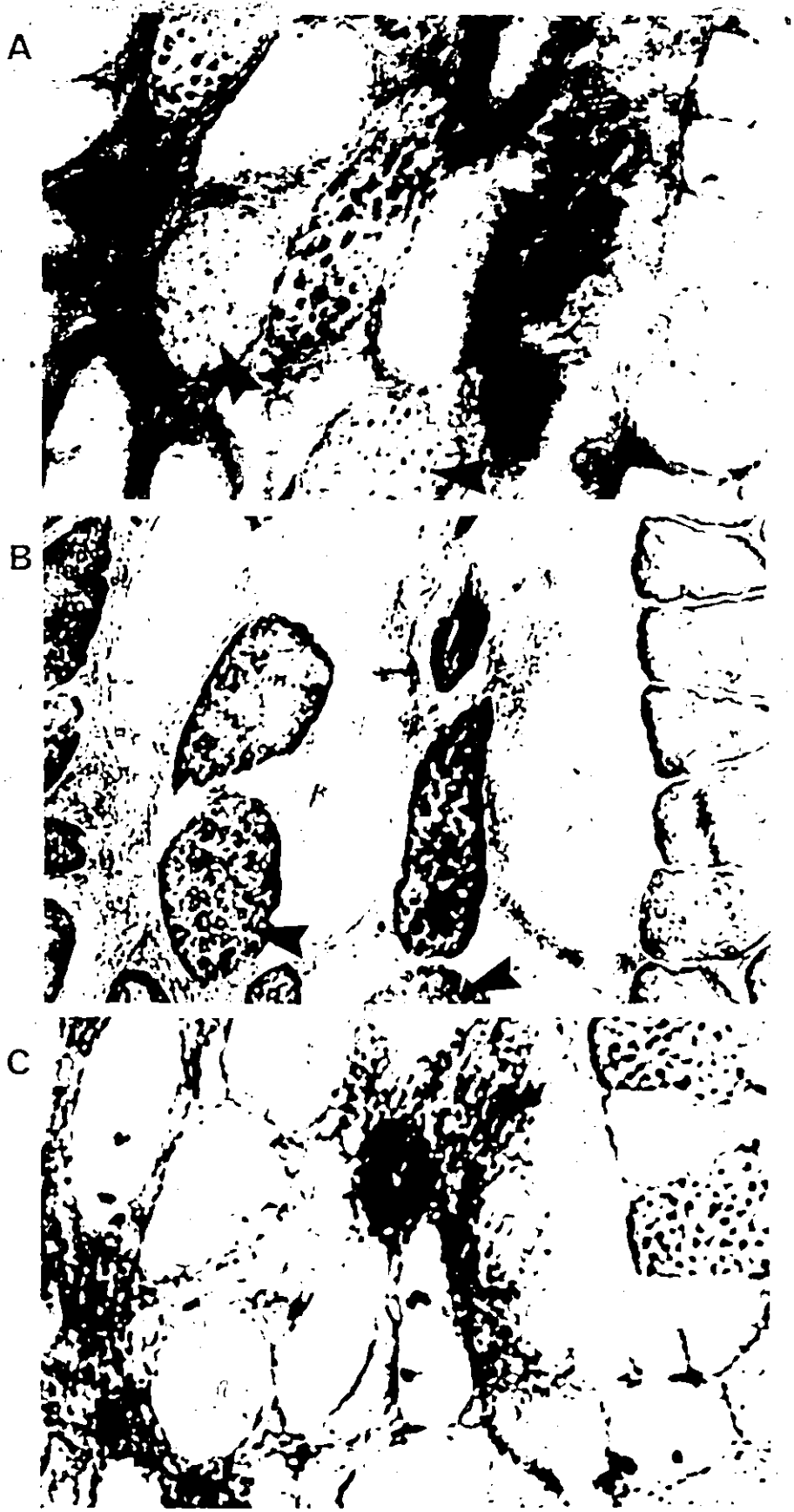


Fig. 13 8 month normal Soleus shows clear reciprocal staining of Type I fibres in A and B. No dual staining was seen in this muscle for Type I fibres, however, a small number of fibres did react with the anti-Type IIB antibody (arrows) in C.

FIG. 13

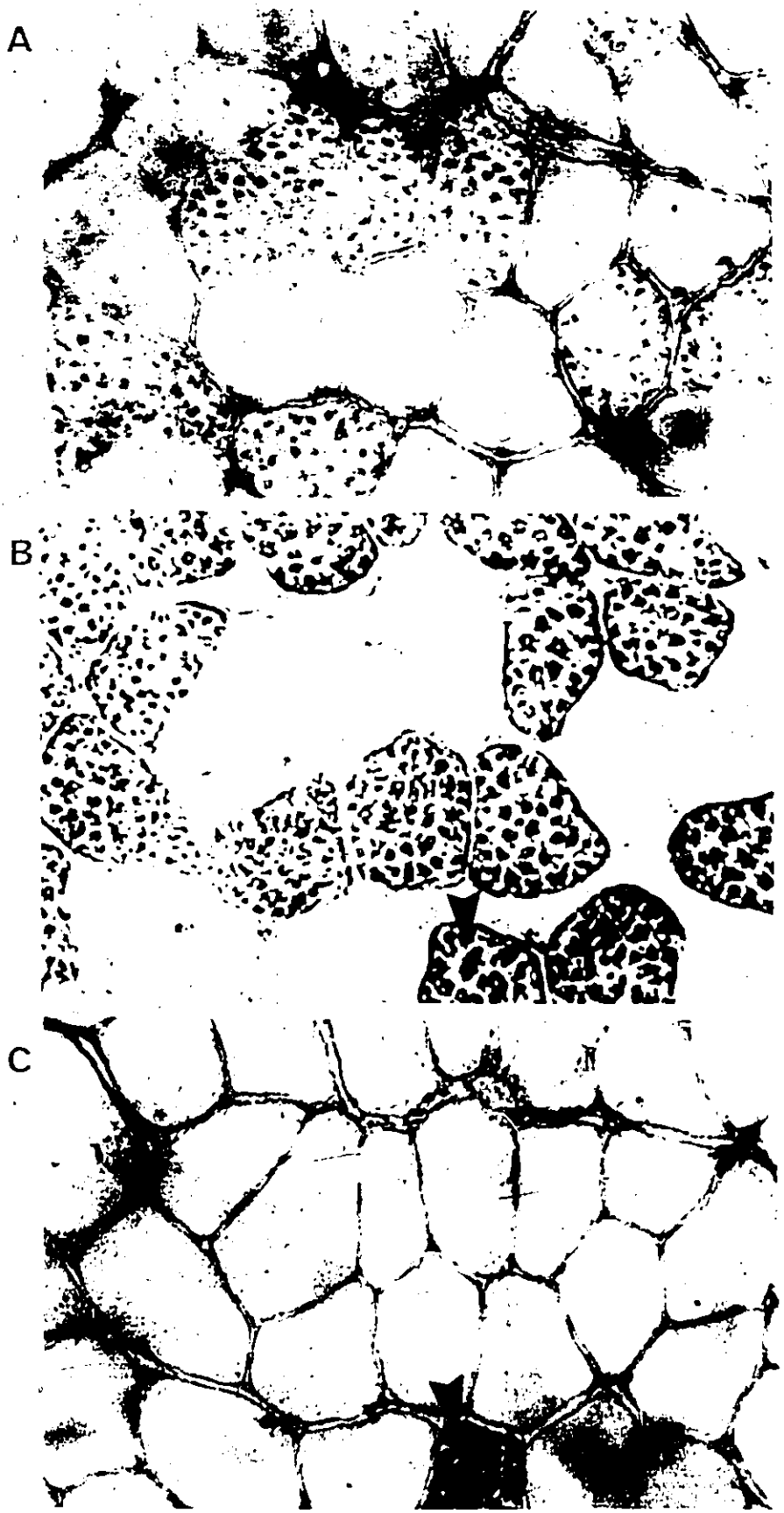


Fig. 14 8 month dystrophic Soleus. In A, many fibres react with the anti-Type I antibody. In B, the majority of the fibres show a positive reaction with anti-Type II antibody and only a few of the fibres which react with anti-Type I antibody in A show negative staining in B. In C, there does not appear to be any staining with the anti-Type IIB antibody.

FIG. 14

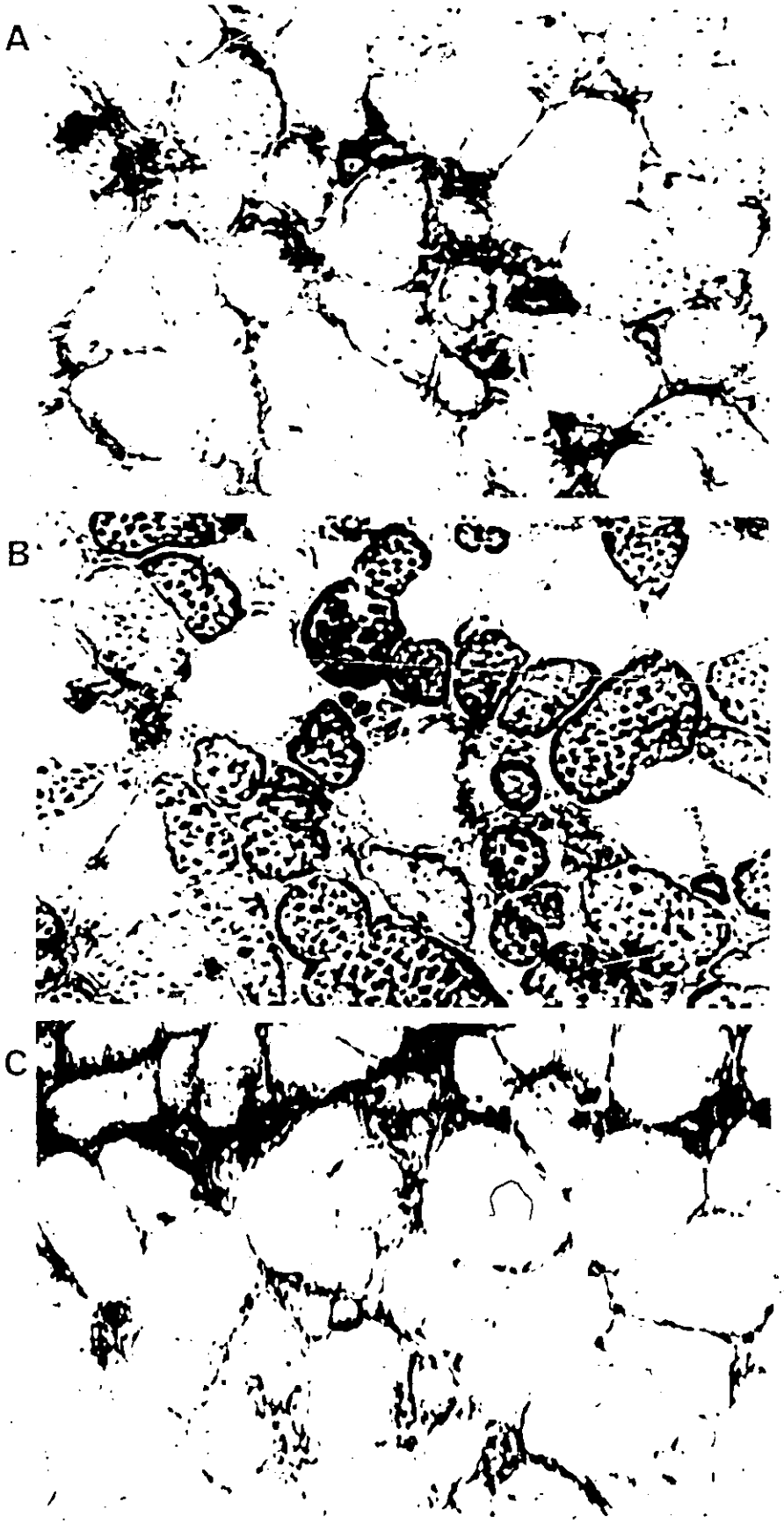
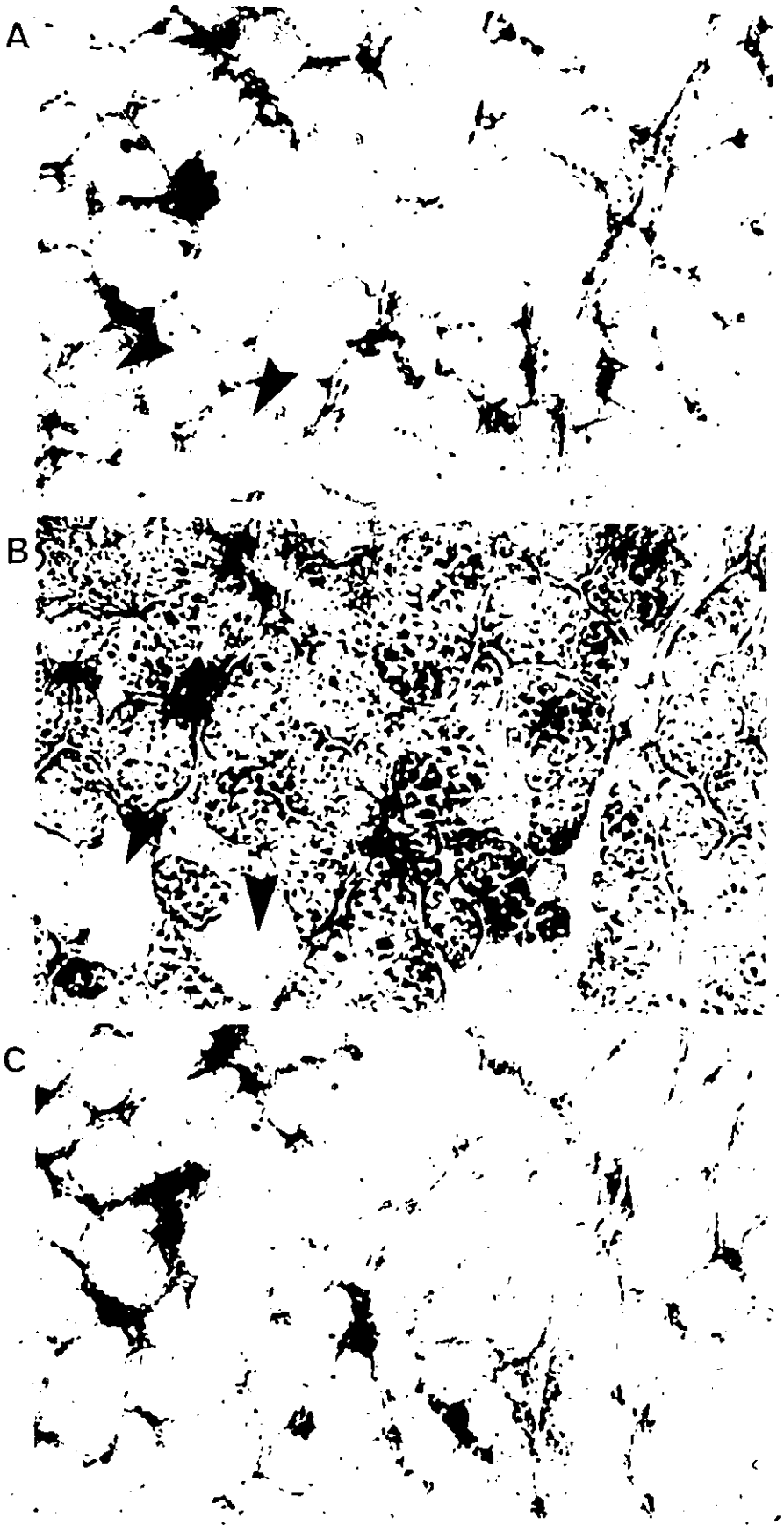


Fig. 15 12 month normal Soleus. In A and B, all fibres show reciprocal staining. Fibres staining with anti-Type I antibody in A do not react with anti-Type II antibody in B, and vice versa. In C, only background staining is seen with anti-Type IIB antibody.

FIG. 15

Fig. 16 12 month dystrophic Soleus. In A, several fibres show positive staining for anti-Type I antibody, whereas in B, only a few fibres are negative for anti-Type II antibody (arrows). In C, none of the fibres react with anti-Type II B antibody.

FIG. 16



Anterior Tibialis Photomicrographs were taken of the oxidative region of AT muscle from normal and dystrophic mice aged 2, 4, 6, 8, and 12 months (Fig.17 to 26). The oxidative region of AT in normal mice occasionally contains a few Type I fibres (Parry and Desypris, 1983) as seen in Fig. 17a and Fig. 21a, but for the most part it consists of a mixture of Type IIA and Type IIB fibres. I have used a monoclonal antibody (BF 46) which is known to cross-react with embryonic myosin of regenerating fibres in cold-injured muscles (not shown). The BF 45 antibody is specific for embryonic myosin and does not cross-react with Type-I fibres in Soleus muscle. Thus, wherever uncertainty arose as to the identity of a fibre when detected with the BF 46 antibody, the serial section overlaid with the anti-embryonic antibody was verified for cross-reactivity. Fibres which were positive with BF 46, but negative with BF 45, were considered to contain Type I myosin and not the embryonic isoform. The BF 34 antibody, which is specific for Type II fibres, shows differential staining intensities for the Type IIA and Type IIB fibres in normal muscle. In Fig. 17b, 19b, 21b, 23b and 25b, the Type IIA fibres are seen to stain more intensely than the Type IIB fibres detected with the BF F3 antibody. There are some fibres which show dark staining with BF 34 and also appear to stain with the BF F3 antibody. This is clearly seen in the 4 month (Fig. 19b, c) and 12 month (Fig. 25b, c) normal Anterior Tibialis oxidative region.

Three observations can be made concerning the pathological changes in the oxidative region of AT in dystrophic mice of the C57 BL/6J dy2j/dy2j strain. The first is the increasing appearance of Type I-positive fibres. From age 2 months to 6 months (Fig. 18, Fig. 20, Fig. 22) these fibres also stain for the anti-embryonic antibody, BF 45, suggesting that these are regenerating fibres. At 8 months (Fig. 24), not all the Type I-positive fibres stained with the BF 45 antibody, indicating the presence of slow myosin. The 12 month dystrophic AT showed a number of Type I-positive fibres in the oxidative region. Many of these fibres were negative with the anti-embryonic antibody. The second observation is that almost all the Type I-positive fibres showed a positive

reaction with the anti-Type II antibody. The third observation is that there is a progressive loss of Type II B fibres from this region (compare Fig. 18c, 20c, 22c, 24c, and 26c).

These results are summarized with the other data in Fig. 48.

Fig. 17 2 month normal Anterior Tibialis oxidative region. In A, several fibres stain with the anti-Type I antibody (small arrows). In B, all of the fibres show a positive stain with anti-Type II antibody, with the exception of the Type I fibres. In C, many fibres stain with the anti-Type IIB antibody.

FIG. 1.7

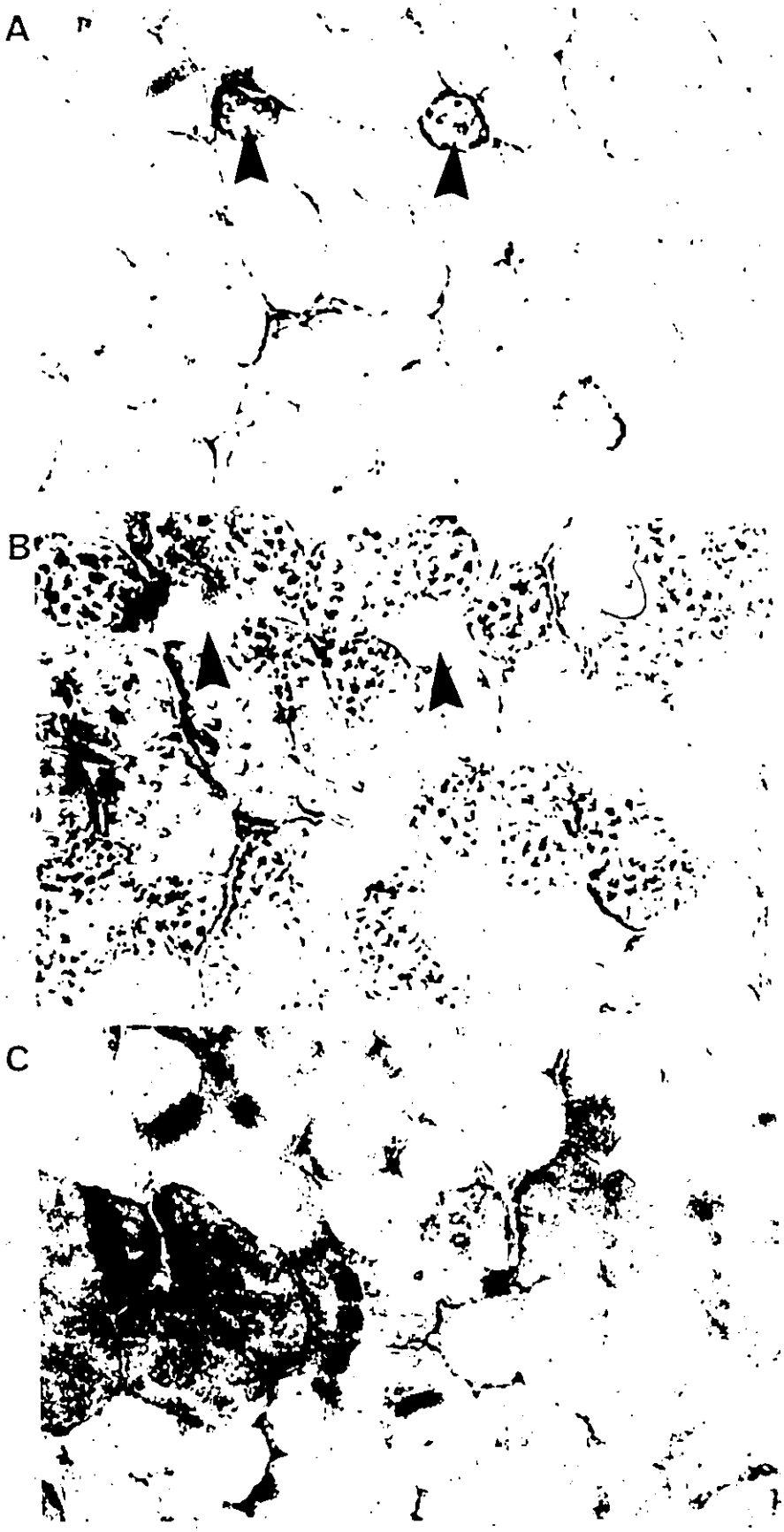


Fig. 18 2 month dystrophic Anterior Tibialis oxidative region. In A, a few fibres stain for Type I-specific antibody (small arrows) whereas in B, all of the fibres show a positive stain with anti-Type II antibody. In C, a few fibres stain with anti-Type IIB antibody (large arrow). In D, several fibres are seen with anti-embryonic antibody (small arrows) and correspond to the Type I-positive fibres in A.

FIG. 18

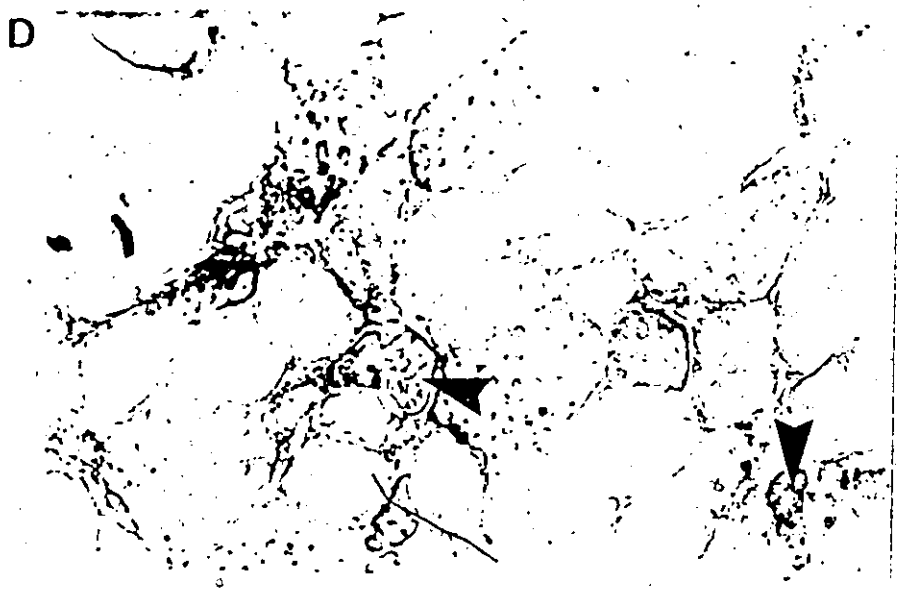

D

Fig. 19 4 month normal Anterior Tibialis oxidative region. In A, none of the fibres show positive staining with anti-Type I antibody. In B, all fibres stain dark with anti-Type II antibody. Some fibres stain more intensely than others, and are seen not to stain in C with the anti-Type IIB antibody (small arrow). Some fibres show dark staining in B and intermediate staining in C (large arrow).

FIG. 19

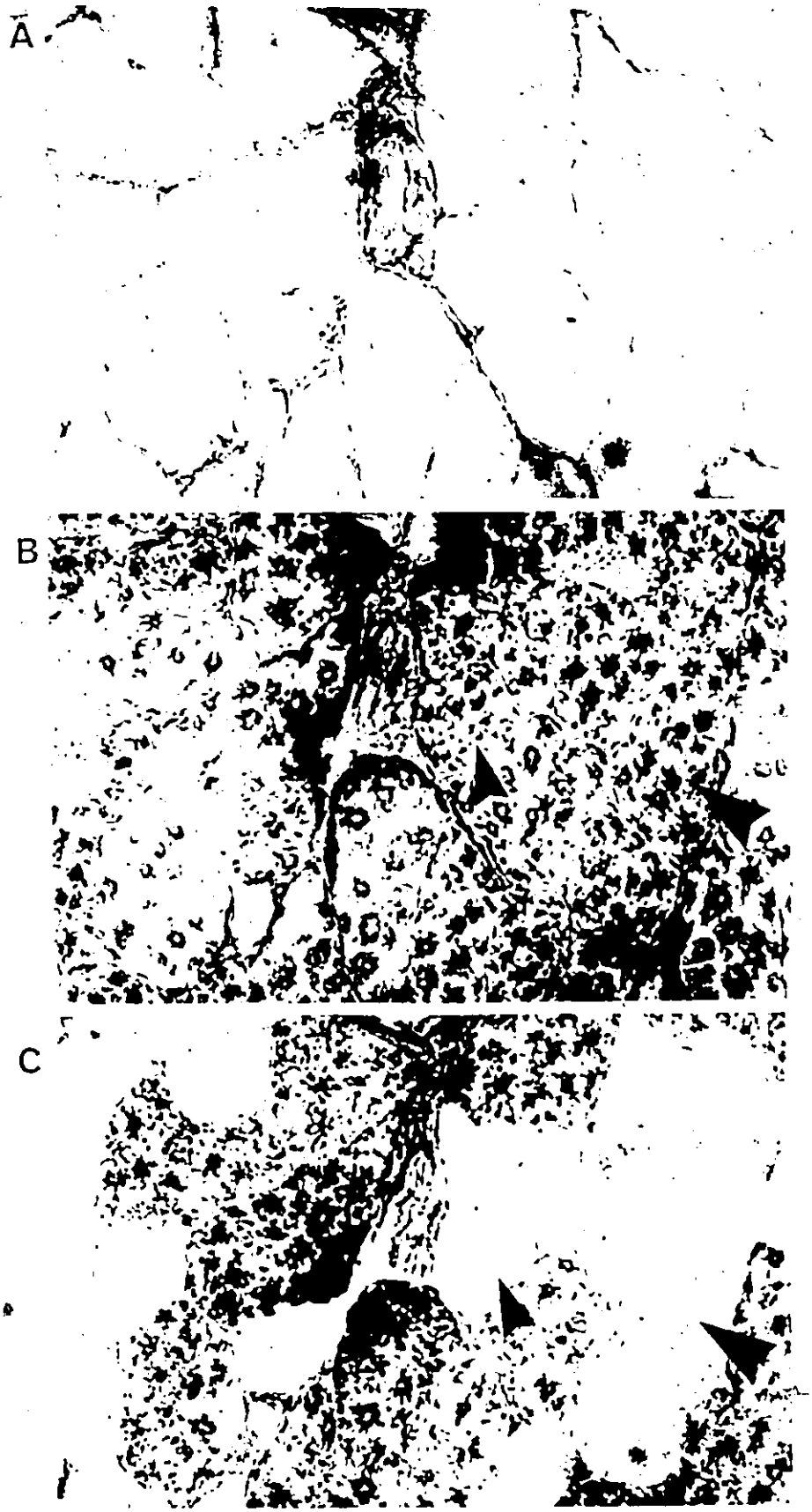
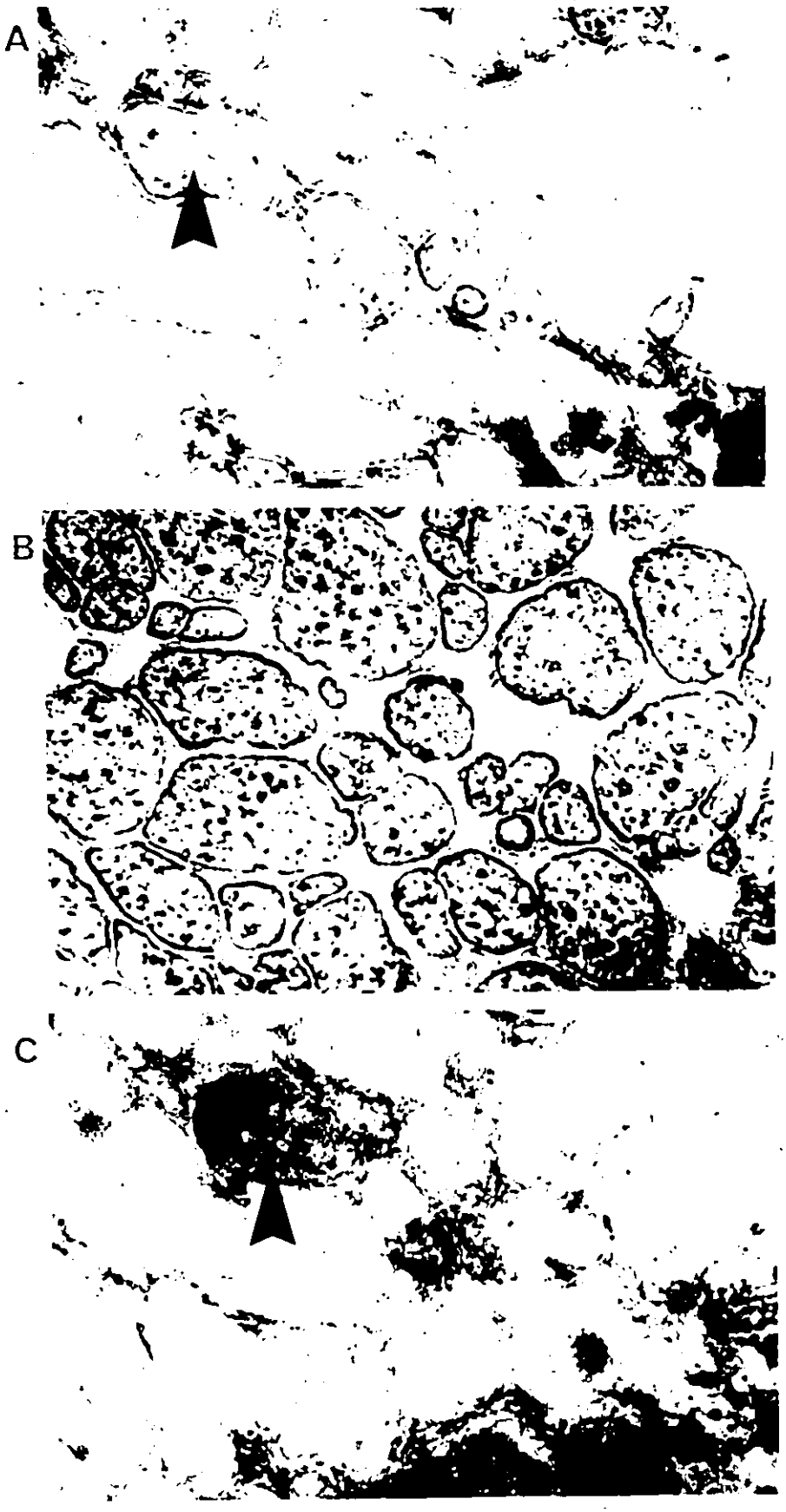


Fig. 20 4 month dystrophic Anterior Tibialis oxidative region. In A, a few fibres show positive staining for anti-Type I antibody. In B, all fibres stain with anti-Type II antibody, although no distinction can be made to predict those fibres that stain dark in C with the anti-Type IIB antibody. One fibre appears to stain for both anti-Type I and anti-Type IIB antibodies (large arrow). In D, several fibres stain for anti-embryonic antibody (small arrows). These fibres were also positive in A.

FIG. 20



D

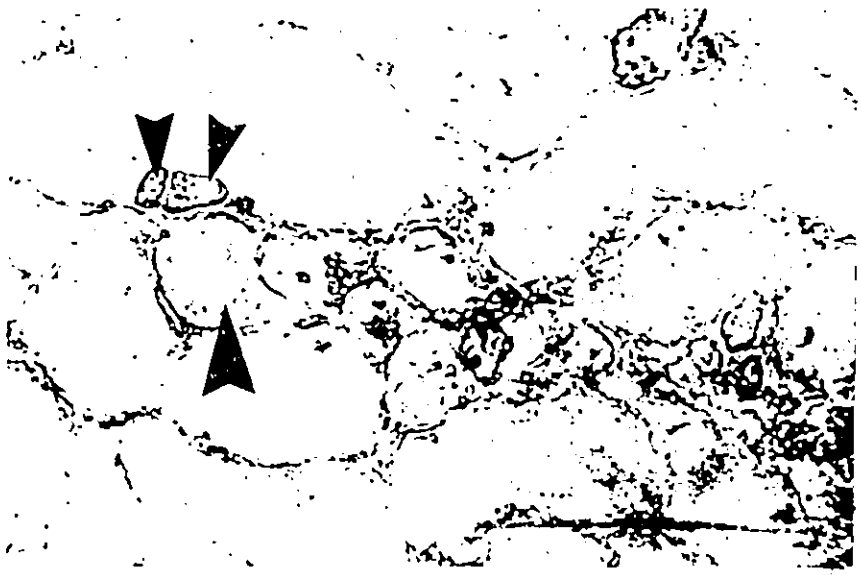


Fig. 21 6 month normal Anterior Tibialis oxidative region. In A, one fibre stains positive with anti-Type I antibody, as do intrafusal fibres of two muscle spindles (small arrows). In B, the majority of fibres stain with anti-Type II antibody. Two of the Type I-positive intrafusal fibres also stain with the anti-Type II antibody (small arrows), whereas the other intrafusal fibres do not. The extrafusal fibre which stained in A does not stain in B. In C, several fibres react with the anti-Type IIB antibody.

FIG. 21

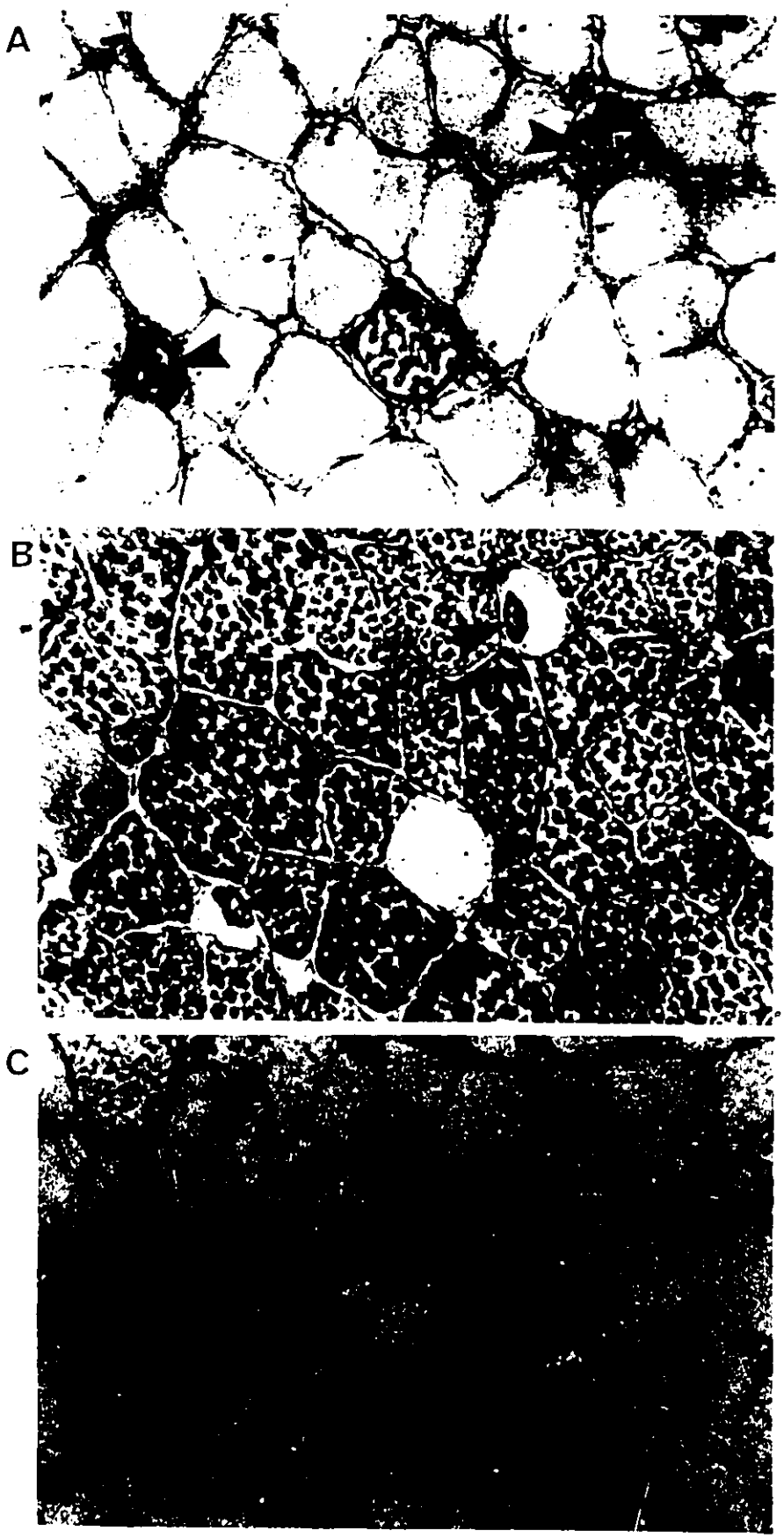
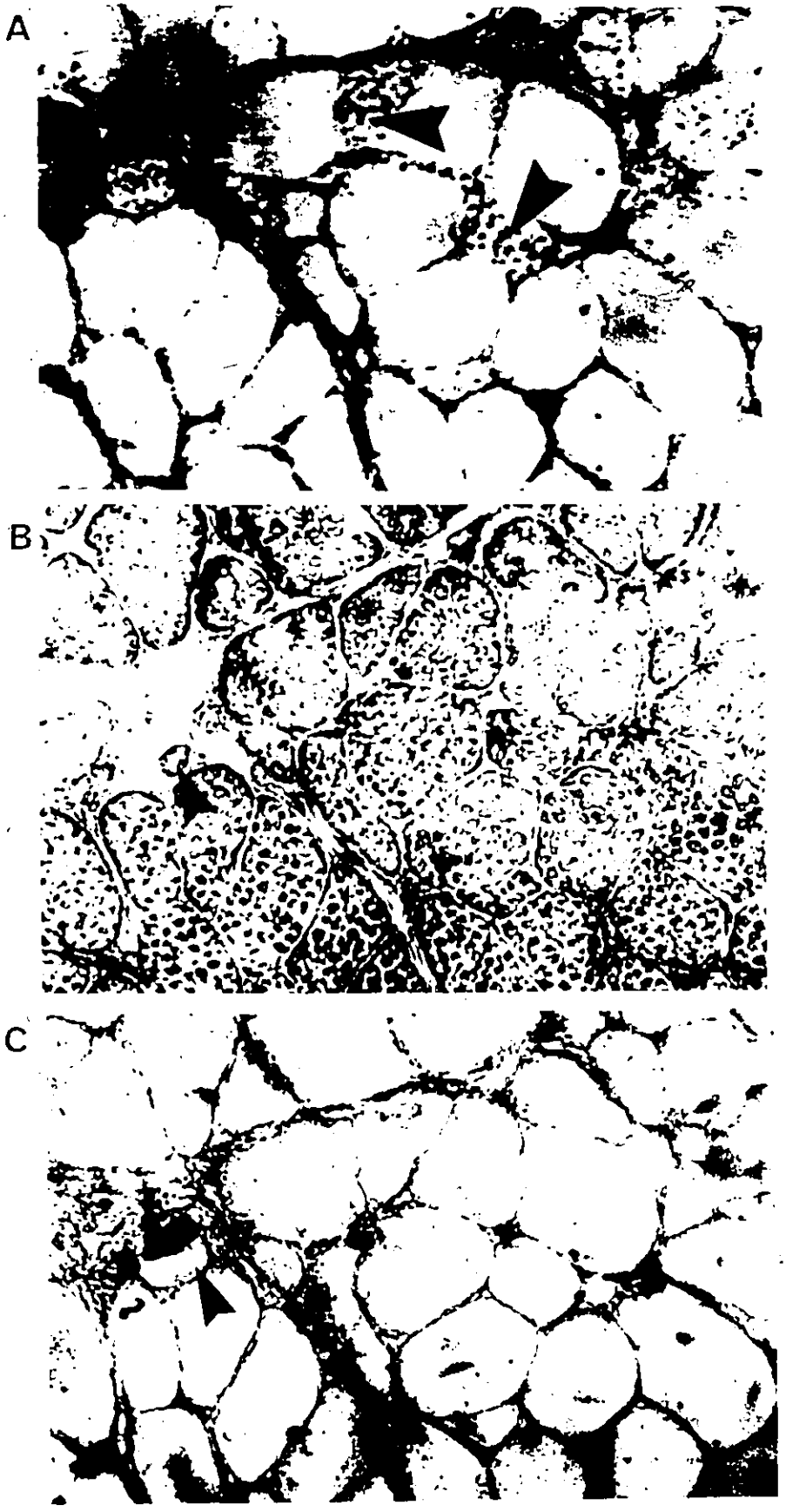


Fig. 22 6 month dystrophic Anterior Tibialis oxidative region. In A, several fibres stain with anti-Type I antibody, whereas in B, all fibres stain with anti-Type II antibody except for two intrafusal fibres (arrow). In C, only two intrafusal fibres show positive staining with anti-Type IIB antibody (arrow), the other fibres do not stain. In D, several fibres stain for anti-embryonic antibody (large arrows).

FIG. 22



D

Fig. 23 8 month normal Anterior Tibialis oxidative region. In A, none of the fibres stain with anti-Type I antibody. In B, all the fibres stain with anti-Type II antibody and in C, many fibres which stained pale and diffuse in B stain dark with anti-Type IIB antibody.

FIG. 23

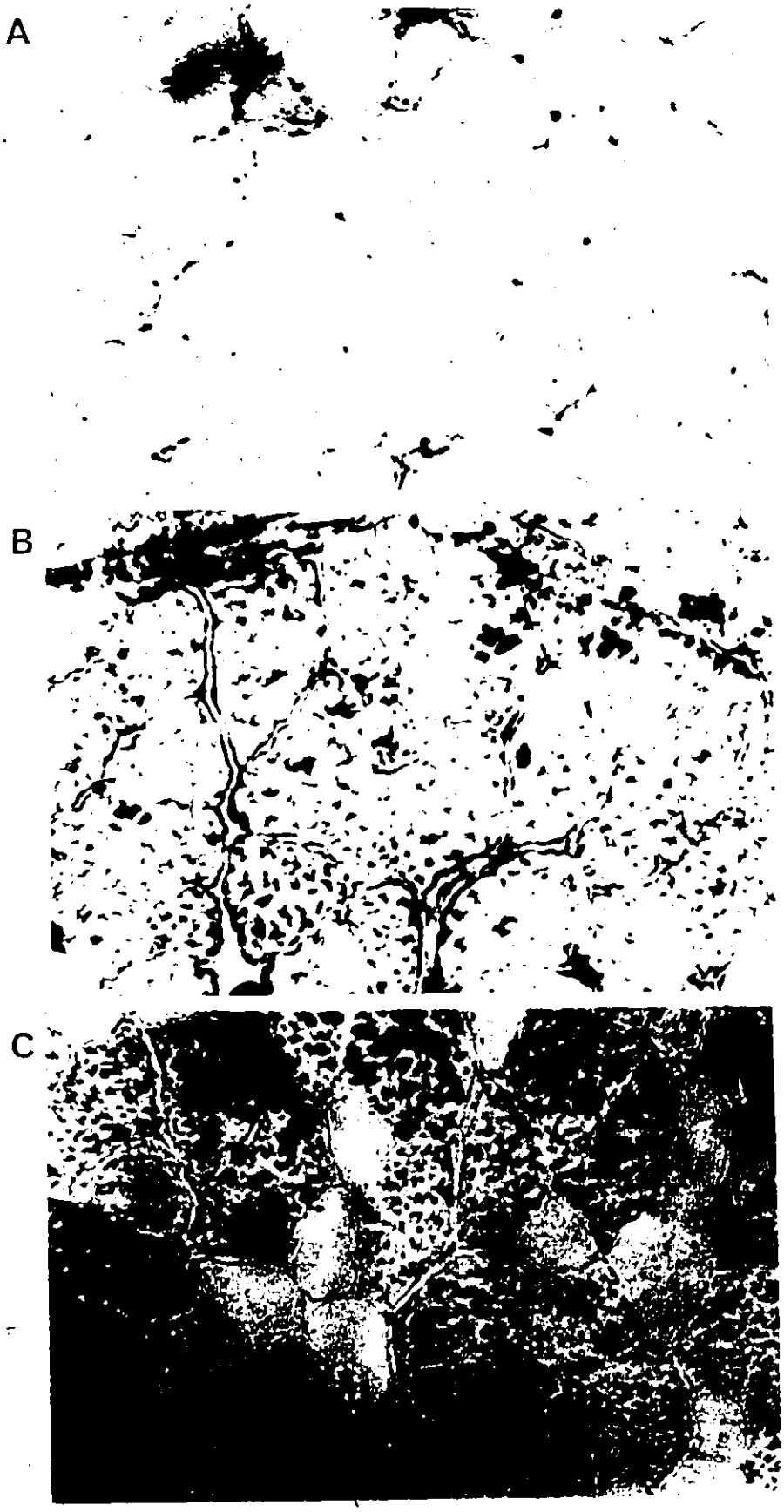
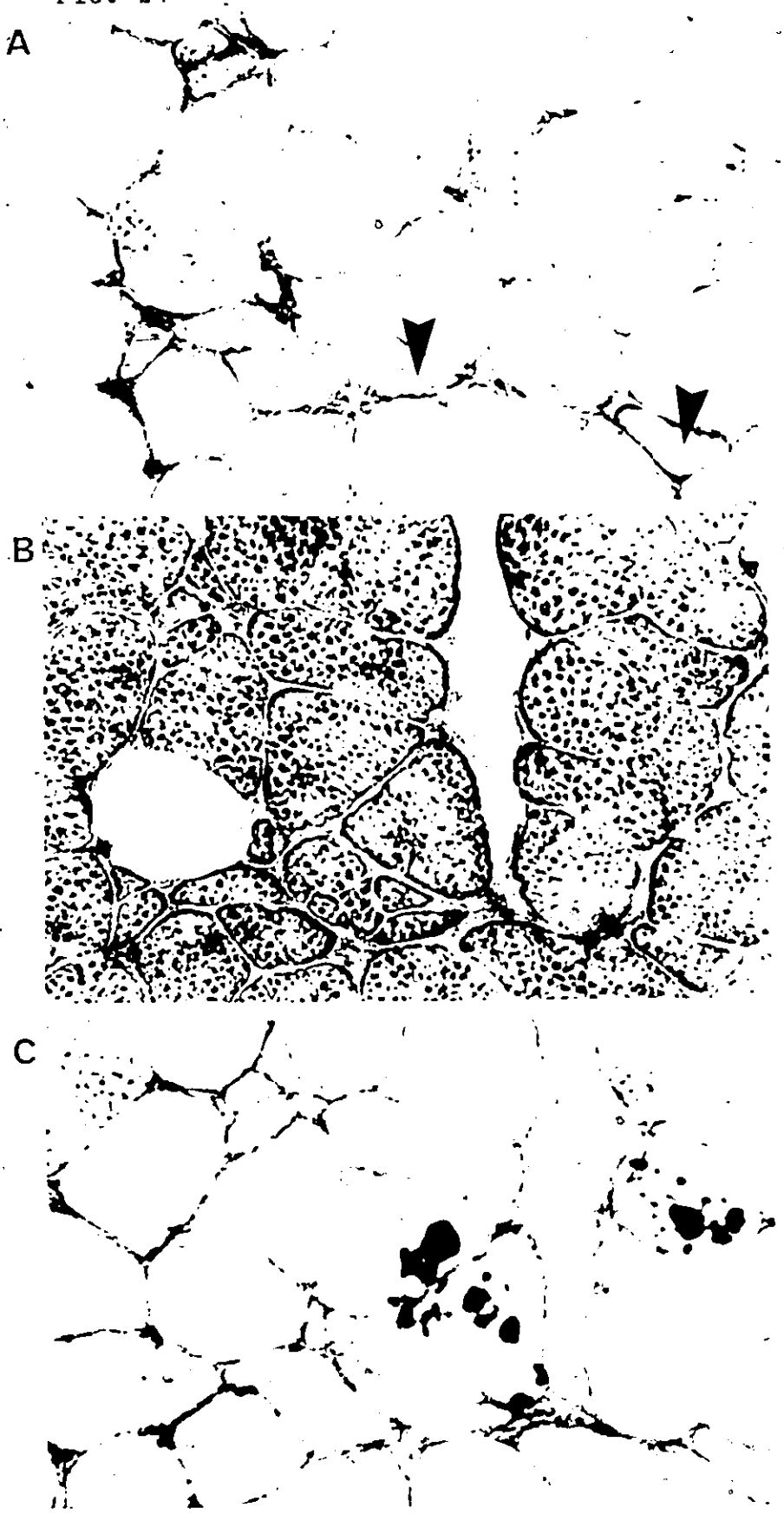


Fig. 24 8 month dystrophic Anterior Tibialis oxidative region. In A, several small fibres stain positive for the Type I-specific antibody (arrows) as does one larger fibre, however in B, only the larger fibre does not stain for anti-Type II antibody, all other fibres stain positive. In C, only a few fibres show staining with the anti-Type IIB antibody. In D, only the small fibres (arrows) stain for the anti-embryonic antibody.

FIG. 24



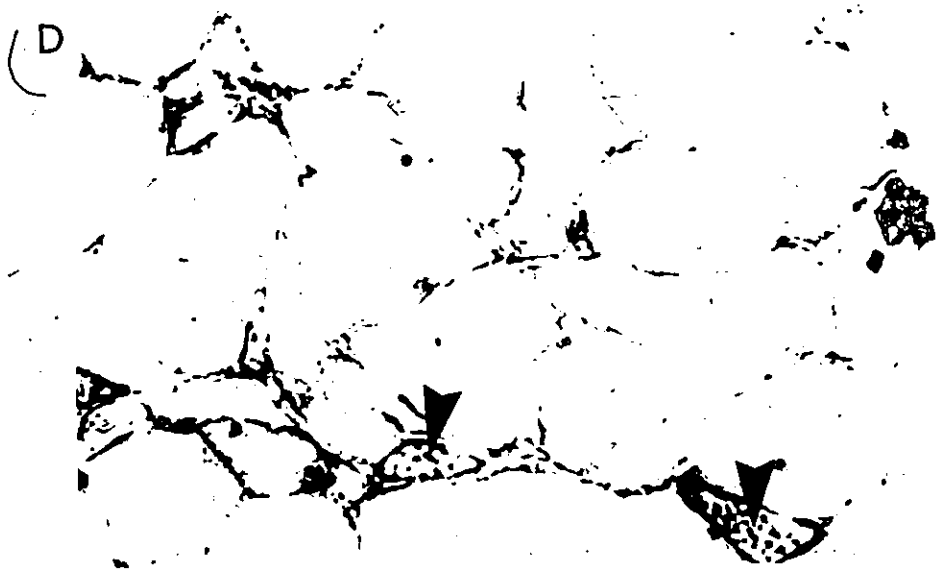

D

Fig. 25 12 month normal Anterior Tibialis oxidative region. In A, none of the fibres stain with anti-Type I antibody. In B, all the fibres are positive for anti-Type II antibody, however, the fibres which stain dark in B do not show completely negative staining in C (arrow).

FIG. 25

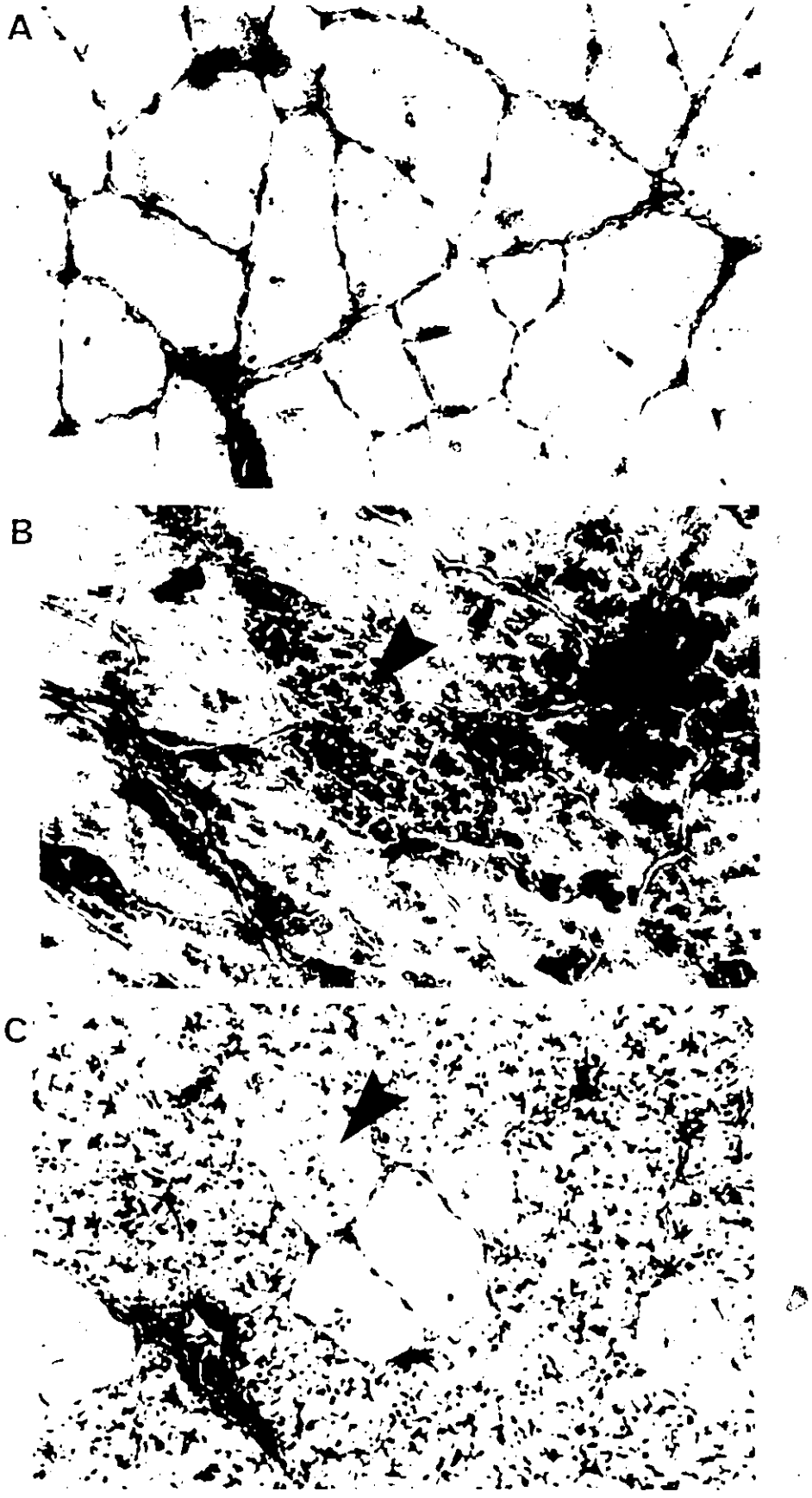
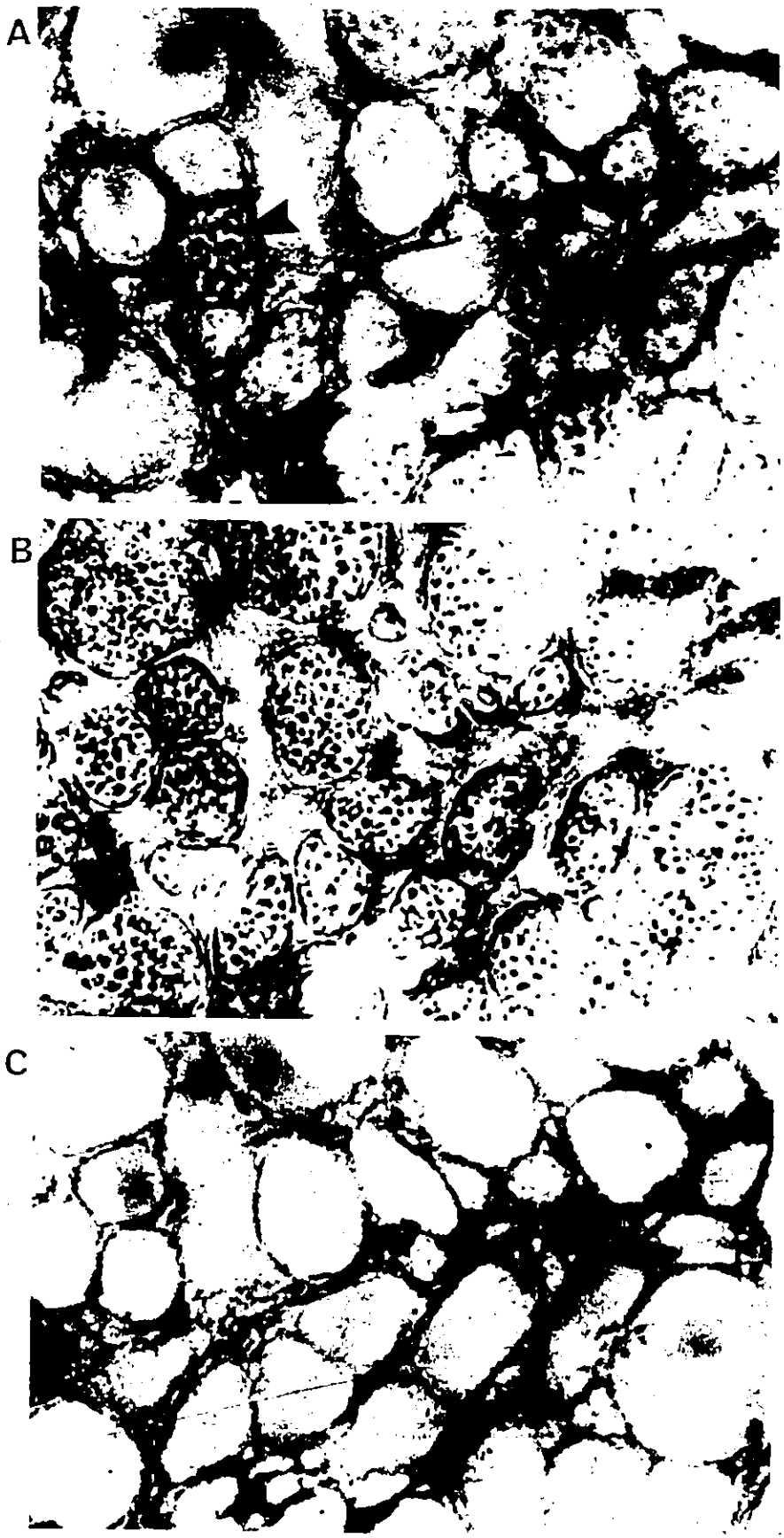
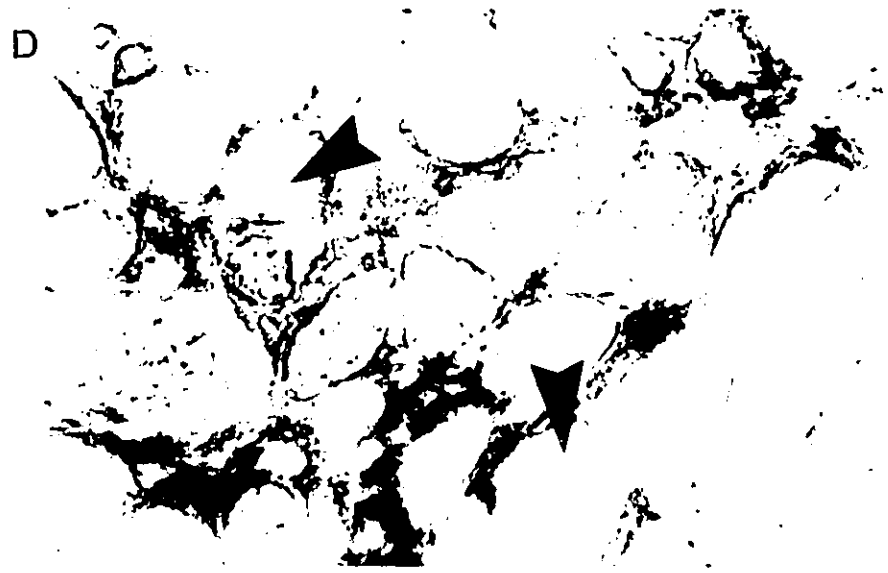


Fig. 26 12 month dystrophic Anterior Tibialis oxidative region. In A, many fibres show positive staining with the anti-Type I antibody, whereas few of these fibres if any stain negatively with the anti-Type II antibody in B. In C, none of the fibres react with anti-Type II B antibody. In D, few fibres are positive with anti-embryonic antibody. Fibres which were positive in A do not stain in D (arrows).

FIG. 26




D

Extensor Digitorum Longus The immunohistochemical profile of the EDL muscle was determined for normal and dystrophic mice aged 2, 4, 6, 8, and 12 months (Fig. 27 to 36). Out of 12 normal mice used for immunohistochemistry, only one aged 6 months showed a small number of Type I fibres (Fig. 31a) in its EDL. As in the AT muscle, dual staining intensity was seen with the anti-Type II antibody (Fig. 31b, and 35b in particular). Some fibres in normal EDL appeared to stain dark with BF 34 and to show positive staining with BF F3, the anti-Type IIB antibody. All dystrophic EDL muscles in each age group showed Type I-positive fibres (Fig. 28a, 30a, 32a, 34a, and 36a). There were fewer Type I-positive fibres in dystrophic EDL than there were in the oxidative region of dystrophic AT, however, as in ATox, these fibres were generally positive with the anti-embryonic antibody at 2, 4, and 6 months of age (Fig. 28d, 30d, 32d). At 8 months (Fig. 34d) and 12 months (not shown) few Type I-positive fibres were also positive with the anti-embryonic antibody. There was a progressive loss of Type IIB fibres with age in the dystrophic EDL muscle (Fig. 28c, 30c, 32c, 34c, and 36c). In the 12 month dystrophic EDL (Fig 36c) only a few Type IIB fibres were seen. This is similar to the observed disappearance of Type IIB fibres in the oxidative region of AT.

These results are summarized with the other data in Fig. 48.

Fig. 27 2 month normal Extensor Digitorum Longus. None of the fibres stain with Type I-specific antibody in A. In B, all fibres are positive to anti-Type II antibody, whereas in C, a few fibres appear to have negative staining with anti-Type IIB antibody.

FIG. 27

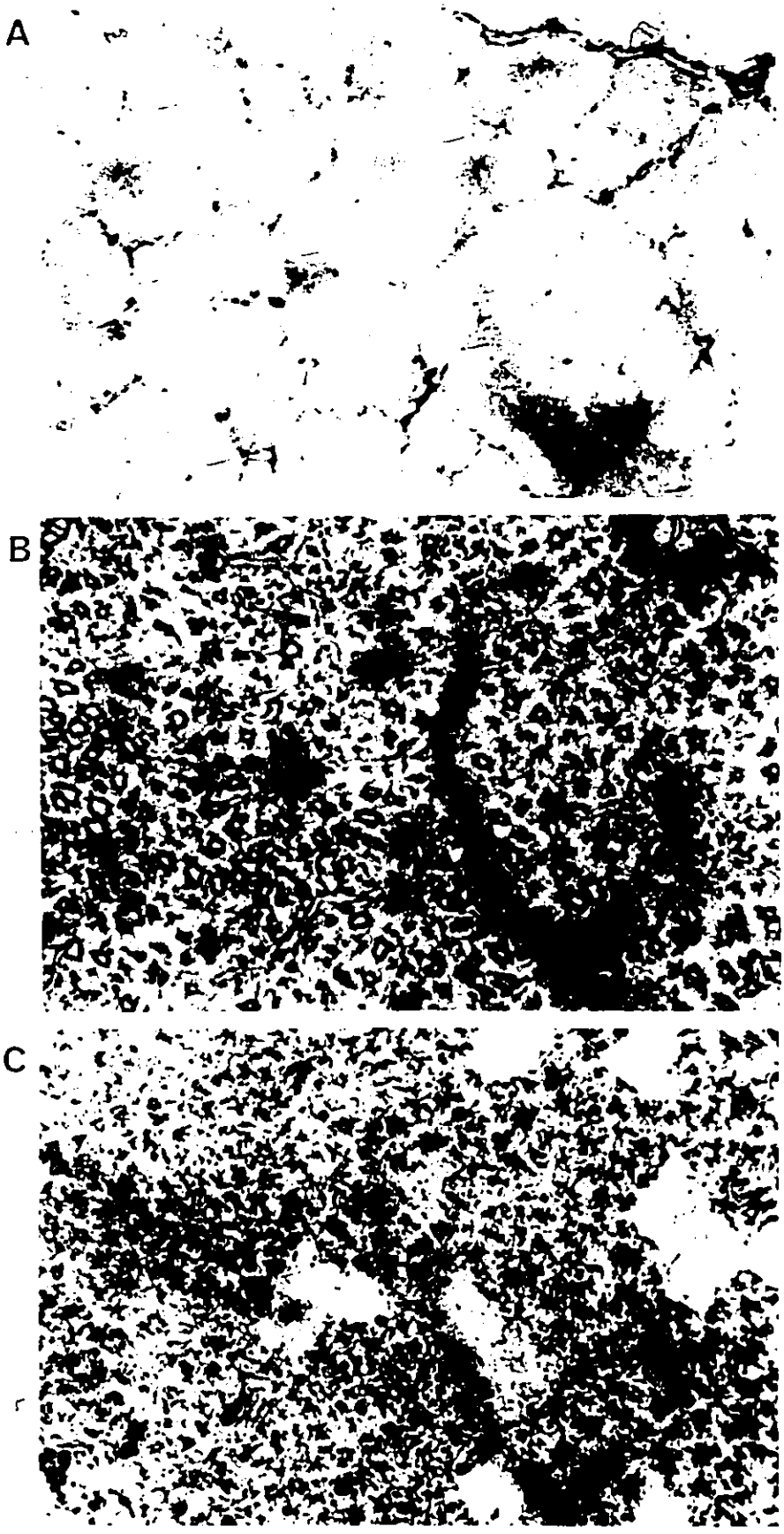
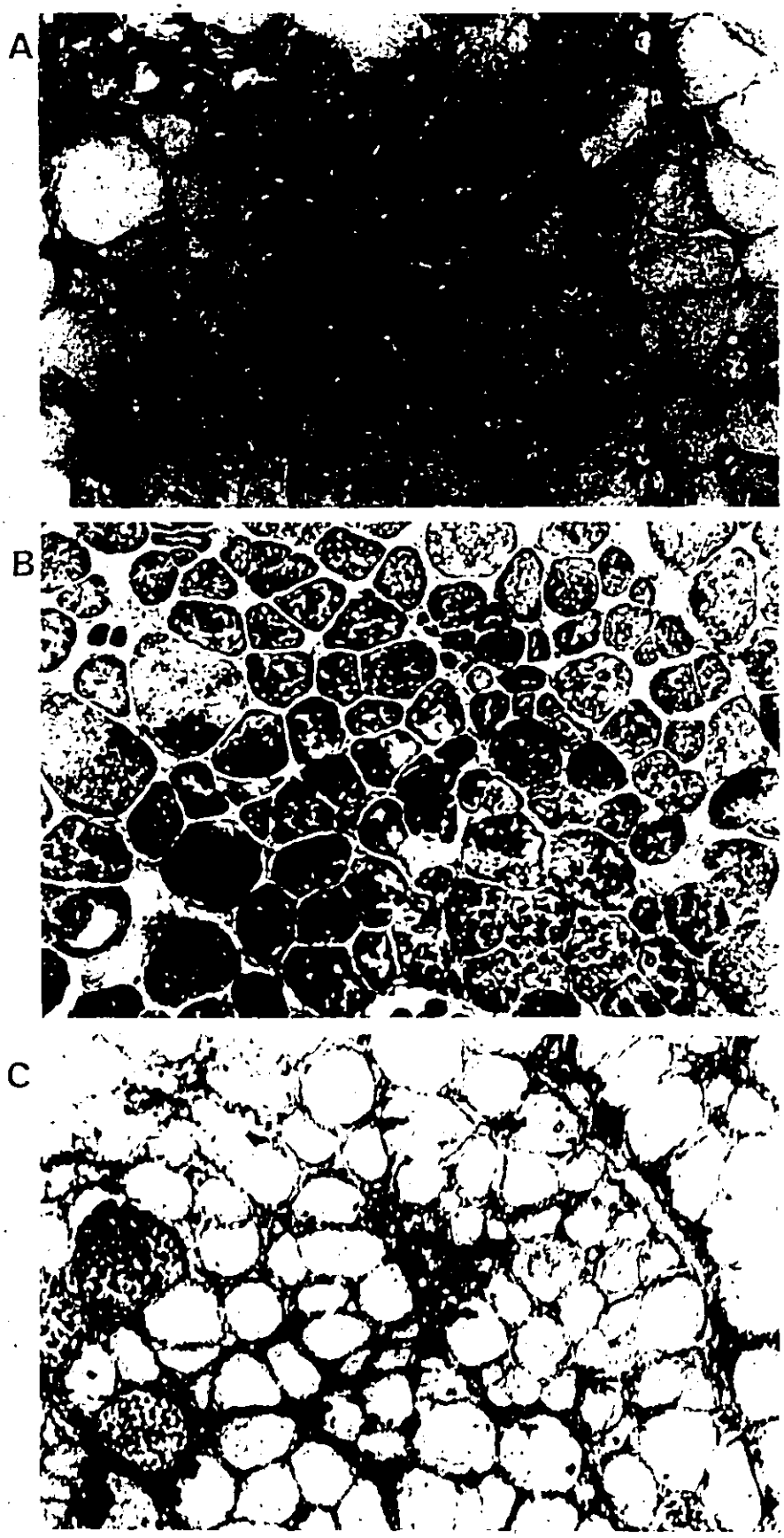


Fig. 28 2 month dystrophic Extensor Digitorum Longus showing in A, many fibres with positive staining with anti-Type I antibody. In B, all fibres stain with Anti-Type II antibody and in C, a few stain with anit-Type IIB antibody. In D, the small fibres which were positive in A also stain for the anti-embryonic antibody (arrows).

FIG. 28



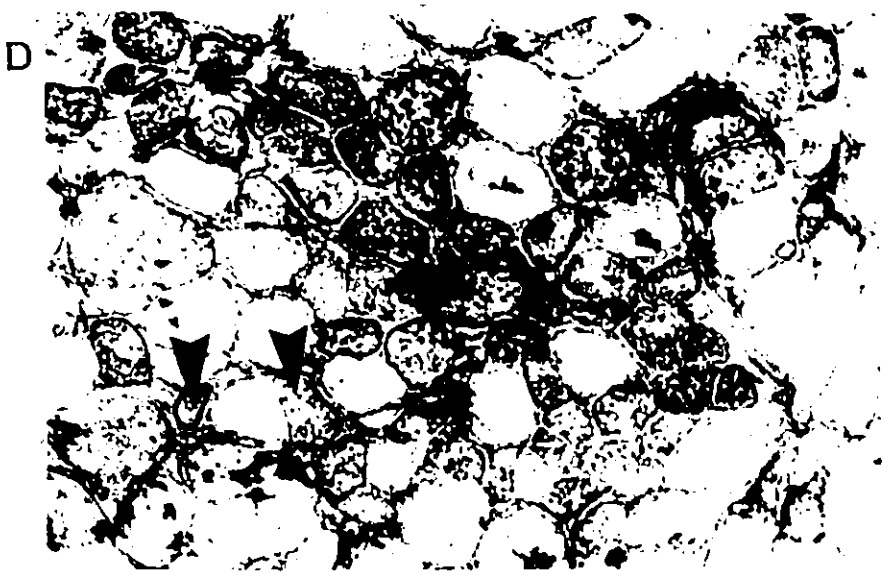

D

Fig. 29 4 month normal Extensor Digitorum Longus. In A, none of the fibres stain for anti-Type I antibody, whereas in B, all fibres stain for anti-Type II antibody. In C, several fibres show positive staining with anti-Type IIB antibody.

FIG. 29

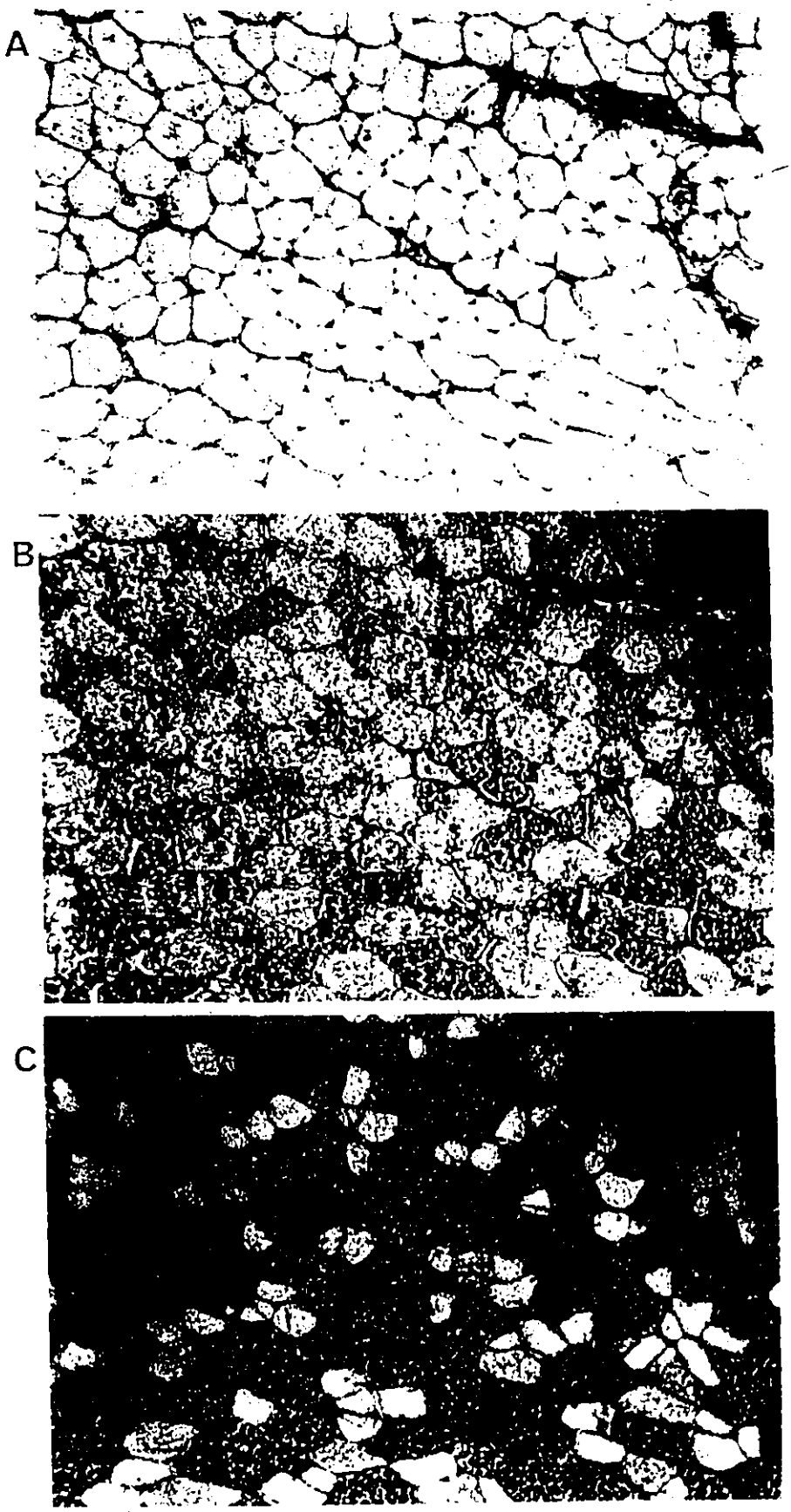
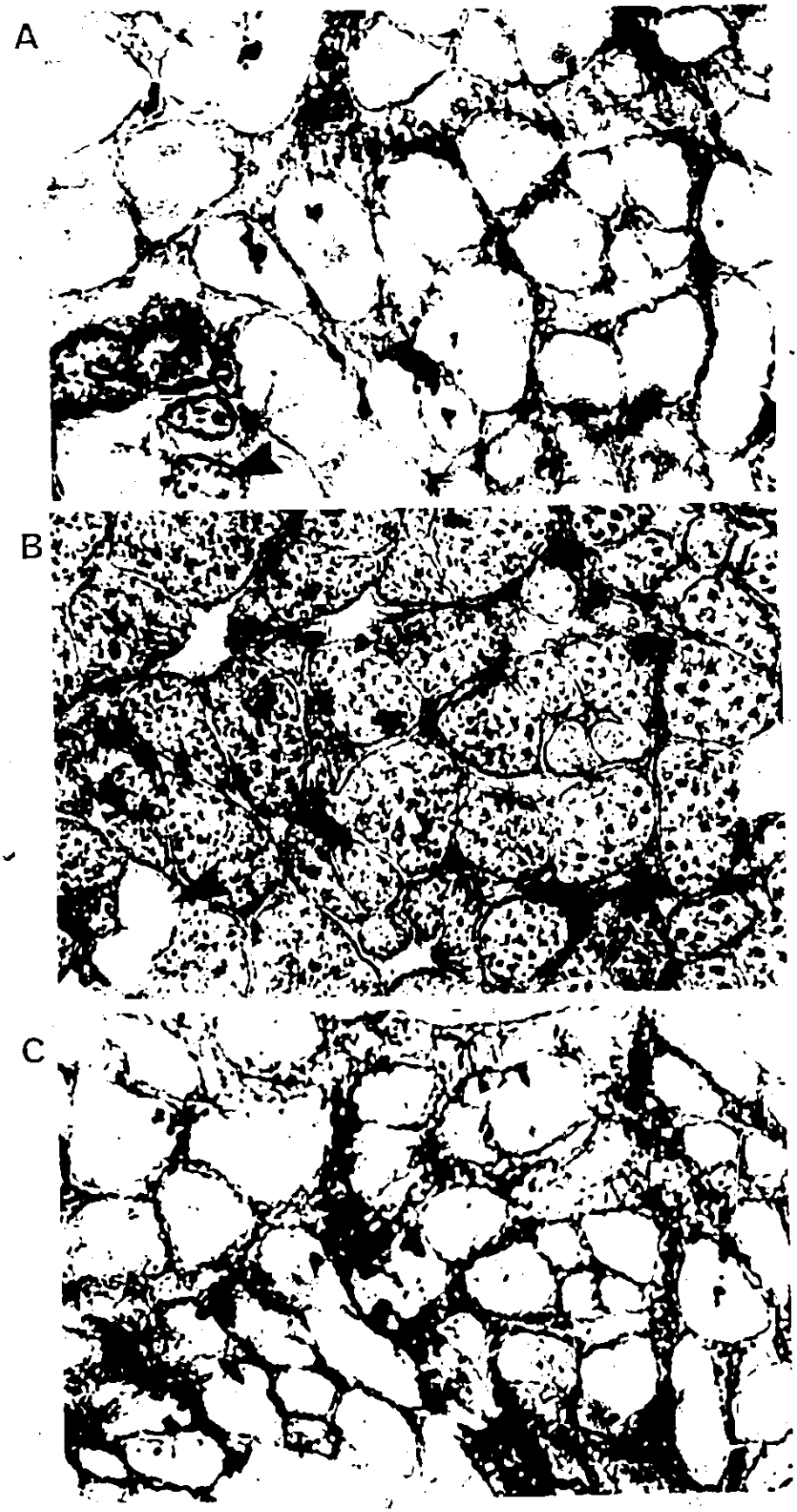


Fig. 30 4 month dystrophic Extensor Digitorum Longus shows a few fibres staining with the anti-Type I antibody (arrow) which also stain negative with the anti-Type II antibody in B. Other fibres stain dark for the anti-Type II antibody and in C, none of these fibres appear to stain with the anti-Type IIB antibody. In D, several fibres stain for the anti-embryonic antibody. The Type I-positive fibre in A does not stain in D (arrow).

FIG. 30



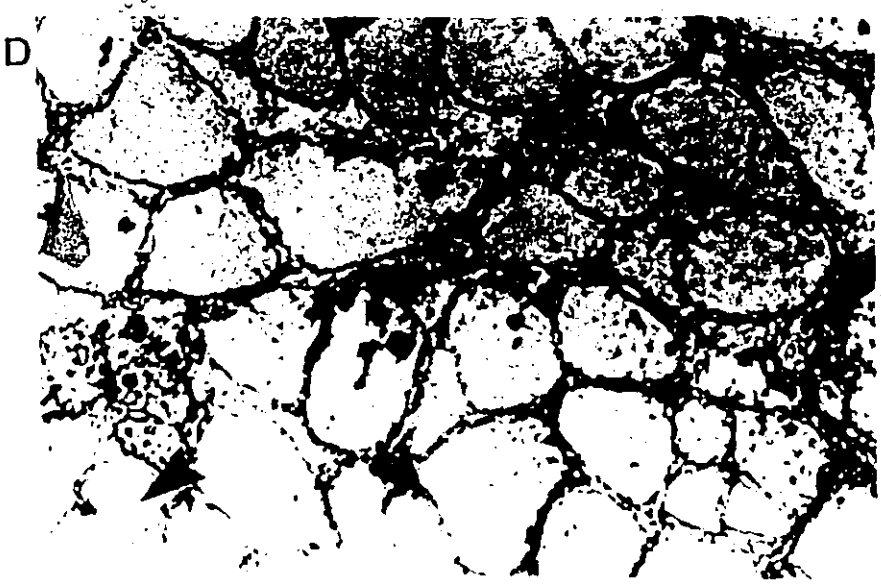

D

Fig. 31 6 month normal Extensor Digitorum Longus. In A, two fibres stain clearly with the anti-Type I antibody and also appear negative with the anti-Type II antibody in B. The other fibres show positive staining for this antibody, with the paler fibres appearing positive with the anti-Type IIB antibody in C.

FIG. 31

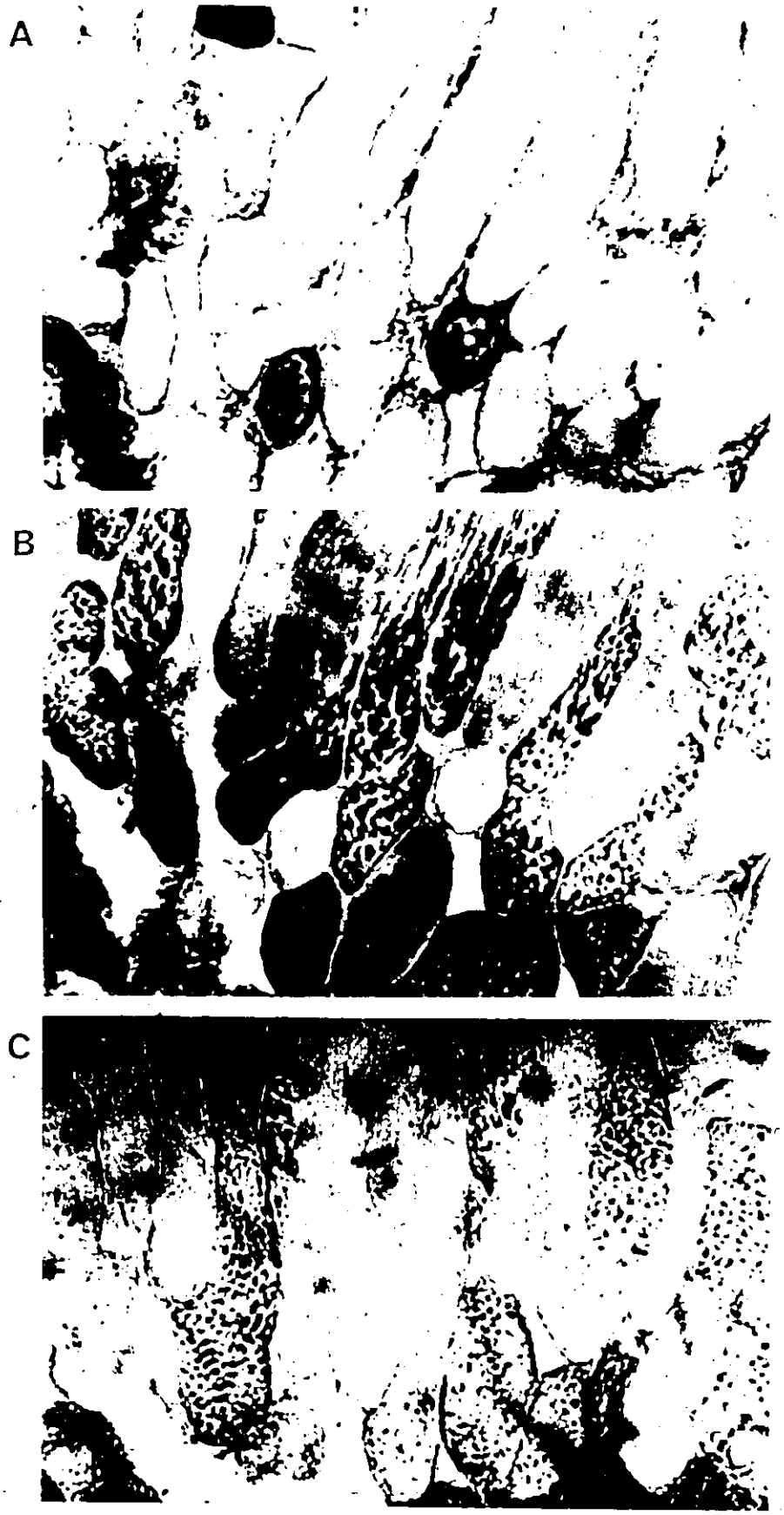
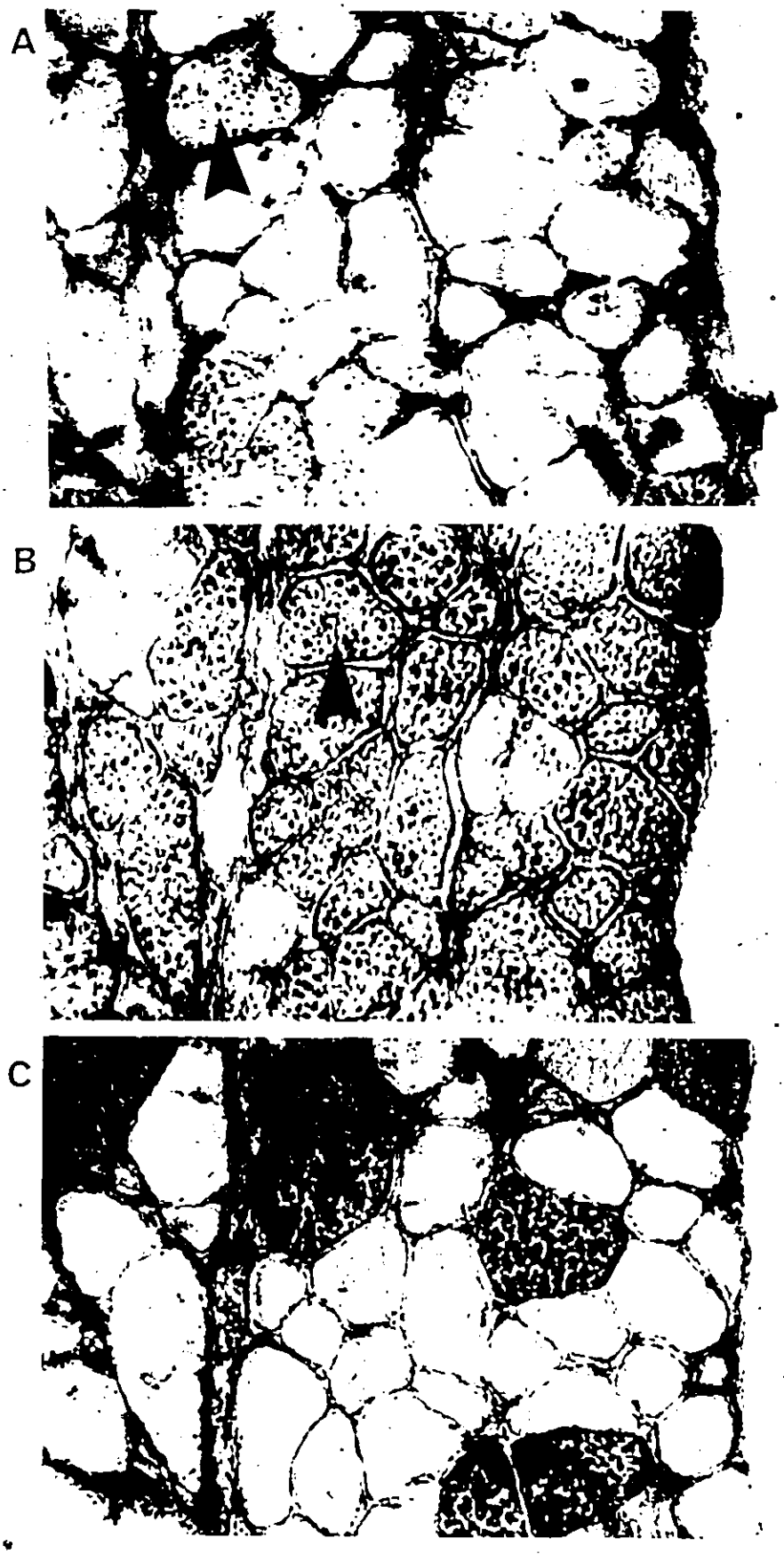


Fig. 32 6 month dystrophic Extensor Digitorum Longus. In A, a few fibres show positive staining with the Type I-specific antibody, whereas in B, all fibres show positive staining with the Type II-specific antibody. In C, several fibres stain with the anti-Type IIB antibody. One fibre stains in all three sections (arrow). In D, several fibres stain with anti-embryonic antibody.

FIG. 32



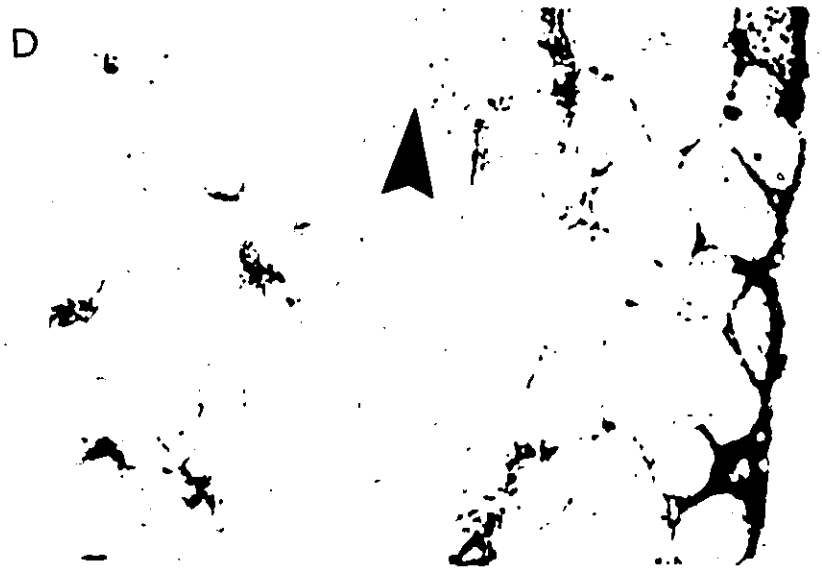

D

Fig. 33 8 month normal Extensor Digitorum Longus. In A, no fibre stains with anti-Type I antibody. In B, all fibres show positive staining with anti-Type II antibody. Several fibres which stain intensely in B also appear negative in C with the anti-Type IIB antibody.

FIG. 33

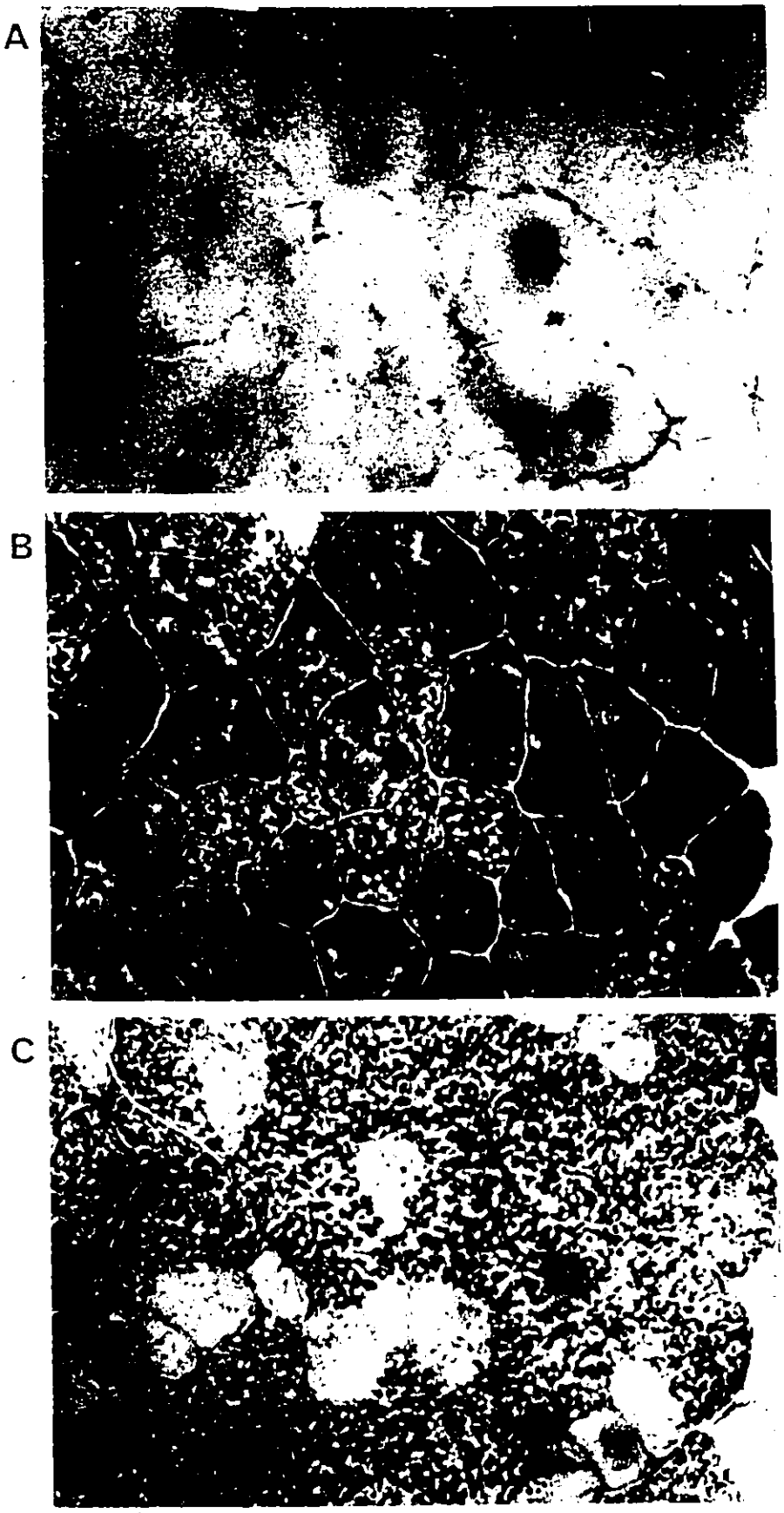
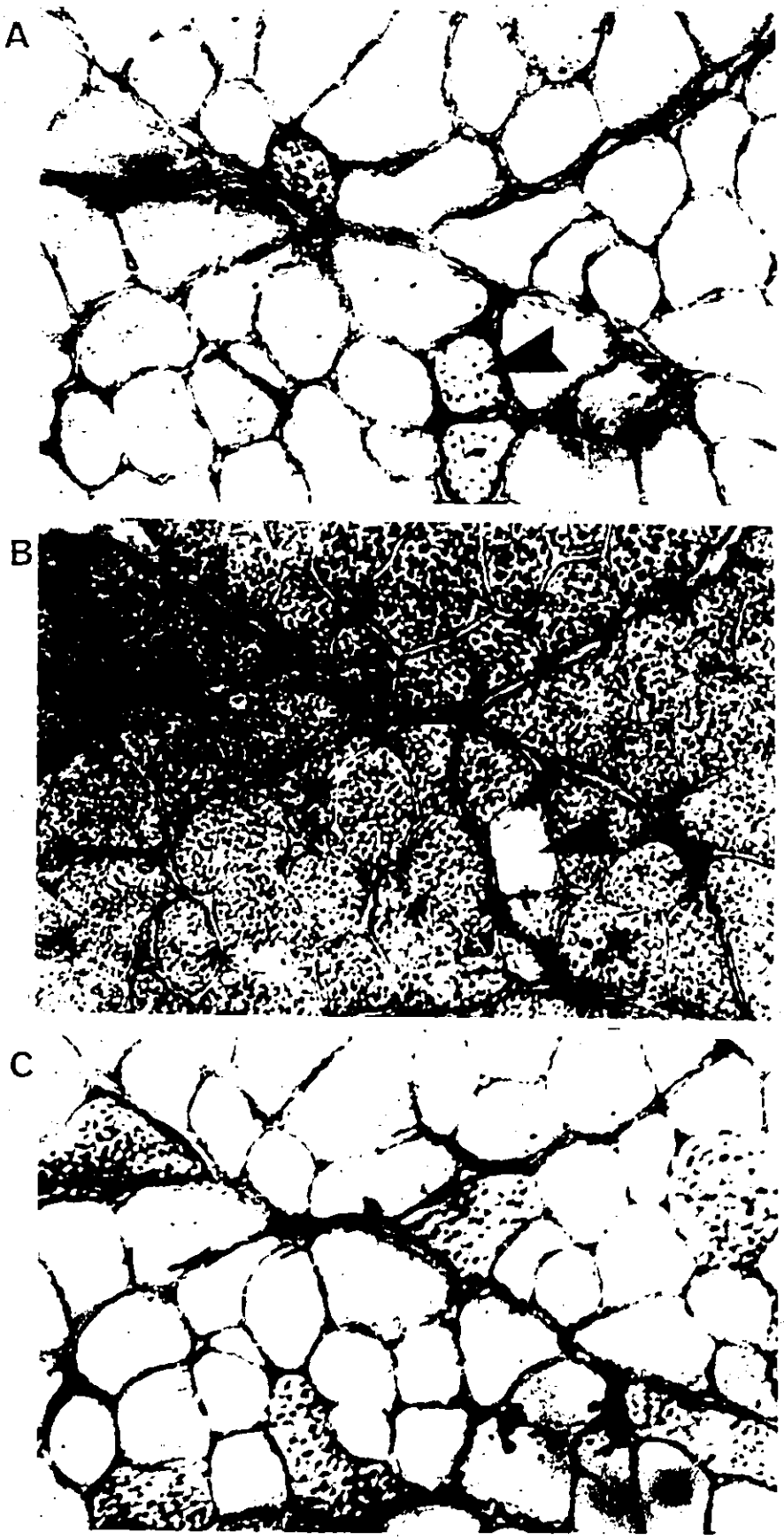


Fig. 34 8 month dystrophic Extensor Digitorum Longus. In A, a few fibres show a positive reaction to the Type I-specific antibody, and one of these fibres is negative with the anti-Type II antibody in B (arrow). In C, several fibres appear to stain with the anti-Type IIB antibody, although the level of staining intensity varies from very pale to moderately dark. In D, a few fibres are positive for anti-embryonic antibody, but fibres which were positive in A do not stain in D (arrows).

FIG. 34



D

Fig. 35 12 month normal Extensor Digitorum Longus shows no staining with anti-Type I antibody in A. In B, all the fibres stain relatively dark with the Type II-specific antibody, with those fibres showing a sharper staining reaction appearing negative with the anti-Type IIB antibody in C (arrow).

FIG. 35

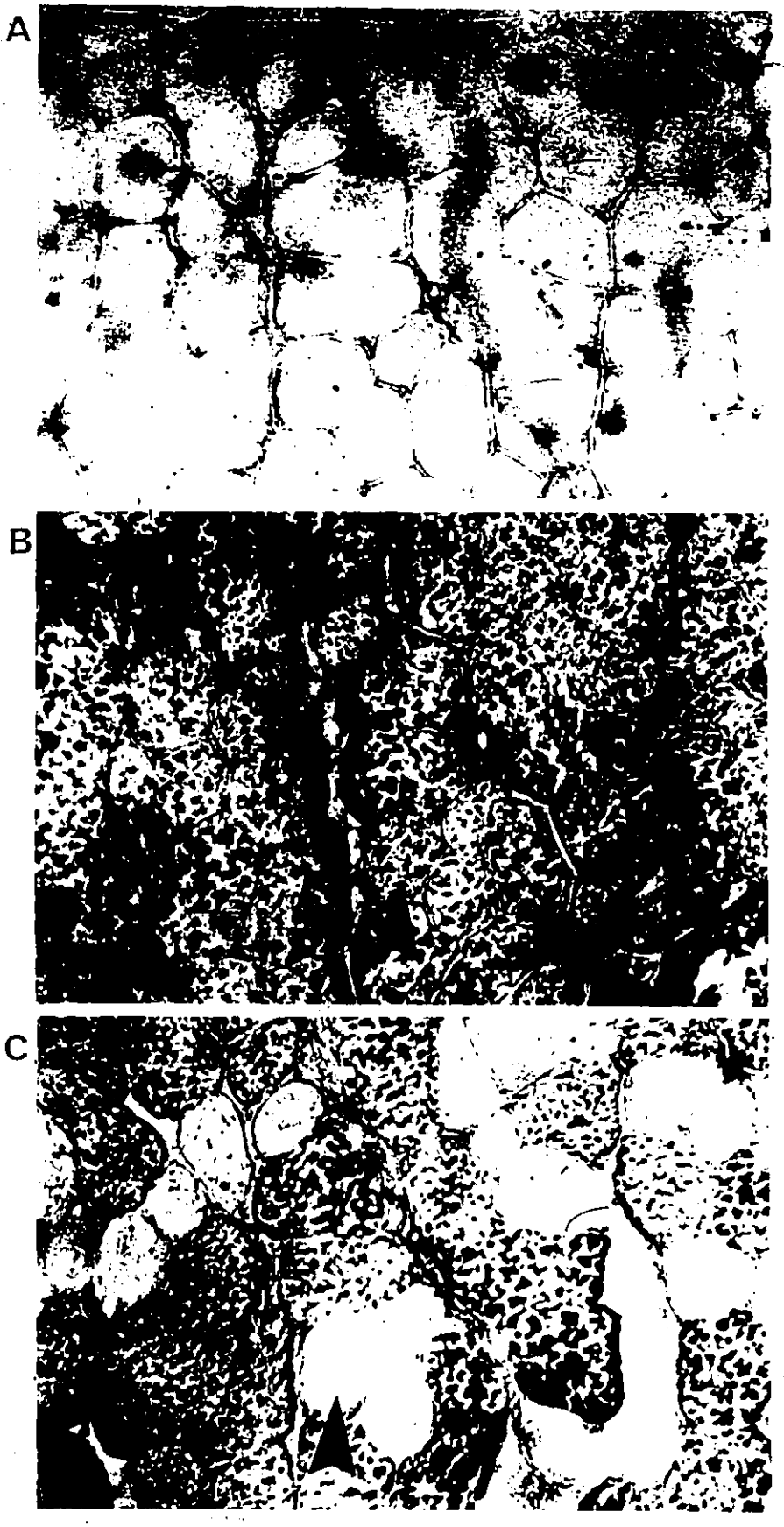
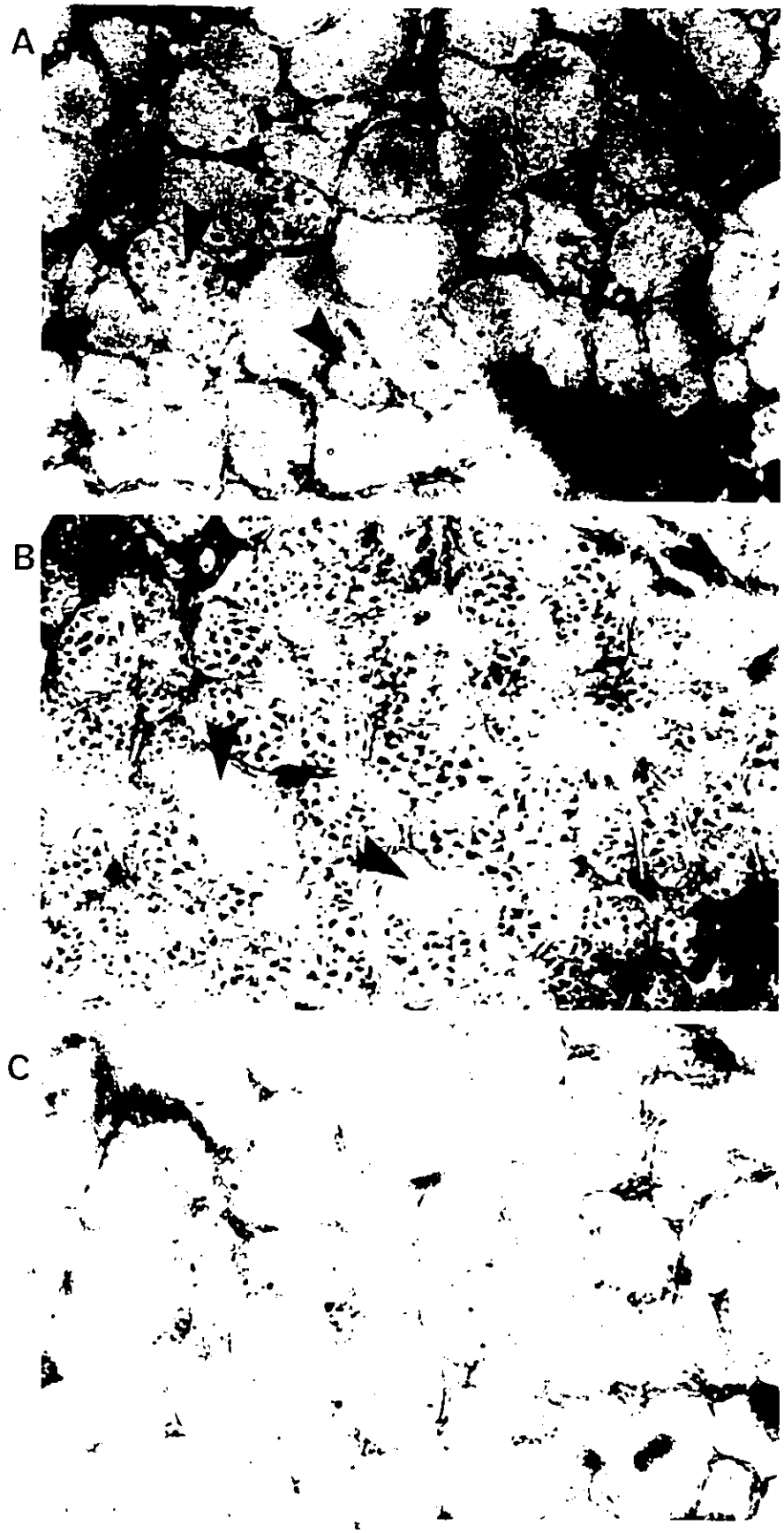


Fig. 36 12 month dystrophic Extensor Digitorum Longus. In A, several fibres show weak yet positive staining with anti-Type I antibody and also appear pale with the anti-Type II antibody in B (arrows). In C, none of the fibres appear to stain with the anti-Type IIB antibody.

FIG. 36



Extensor Carpi Radialis Longus and Brevis The ECRL and B muscles of the forelimb consist of a heterogenous population of Type IIA and Type IIB fibres (Fig. 37 to 46). No Type I-positive fibre was ever seen in either ECRL or B in normal mice at any age (Fig. 37a, 39a, 41a, 43a, and 45a). In the ECRL from a 2 month old dystrophic mouse, several fibres (<10%) showed a Type I-positive reaction (Fig. 38a, 2 fibres shown) which also appeared positive with the anti-Type II antibody. These fibres were always small and showed positive staining with BF 45, the anti-embryonic antibody (Fig. 38d). At 4 months, a few fibres in dystrophic ECRL still showed positive staining with anti-Type I antibody (not shown), but these also reacted with BF 45 in a serial section. Beyond age 6 months (Fig. 42a) no Type I-positive fibre was ever seen in ECRL or B muscles of dystrophic mice. It is not possible to come to a definitive conclusion concerning the relative proportion of Type IIB fibres in dystrophic ECRL and B muscles. However, as in dystrophic ATox and EDL, the normal pattern of staining observed with the anti-Type II antibody, where Type IIA fibres stain more darkly than the Type IIB fibres, is not as clearly defined in dystrophic ECRL and B. For example, in Fig. 41 b, the 6 month ECRL shows three fibres in the lower left corner which do not produce the staining pattern expected with the anti-Type IIB antibody.

These results are summarized with the other data in Fig. 48.

Fig. 37 2 month normal Extensor Carpi Radialis Longus (top) and Brevis (bottom). In A, none of the fibres stain with the Type-I specific antibody. In B, all the fibres stain with the Type-II specific antibody and in C, several fibres appear positive with the anti-Type-IIB antibody.

FIG. 37

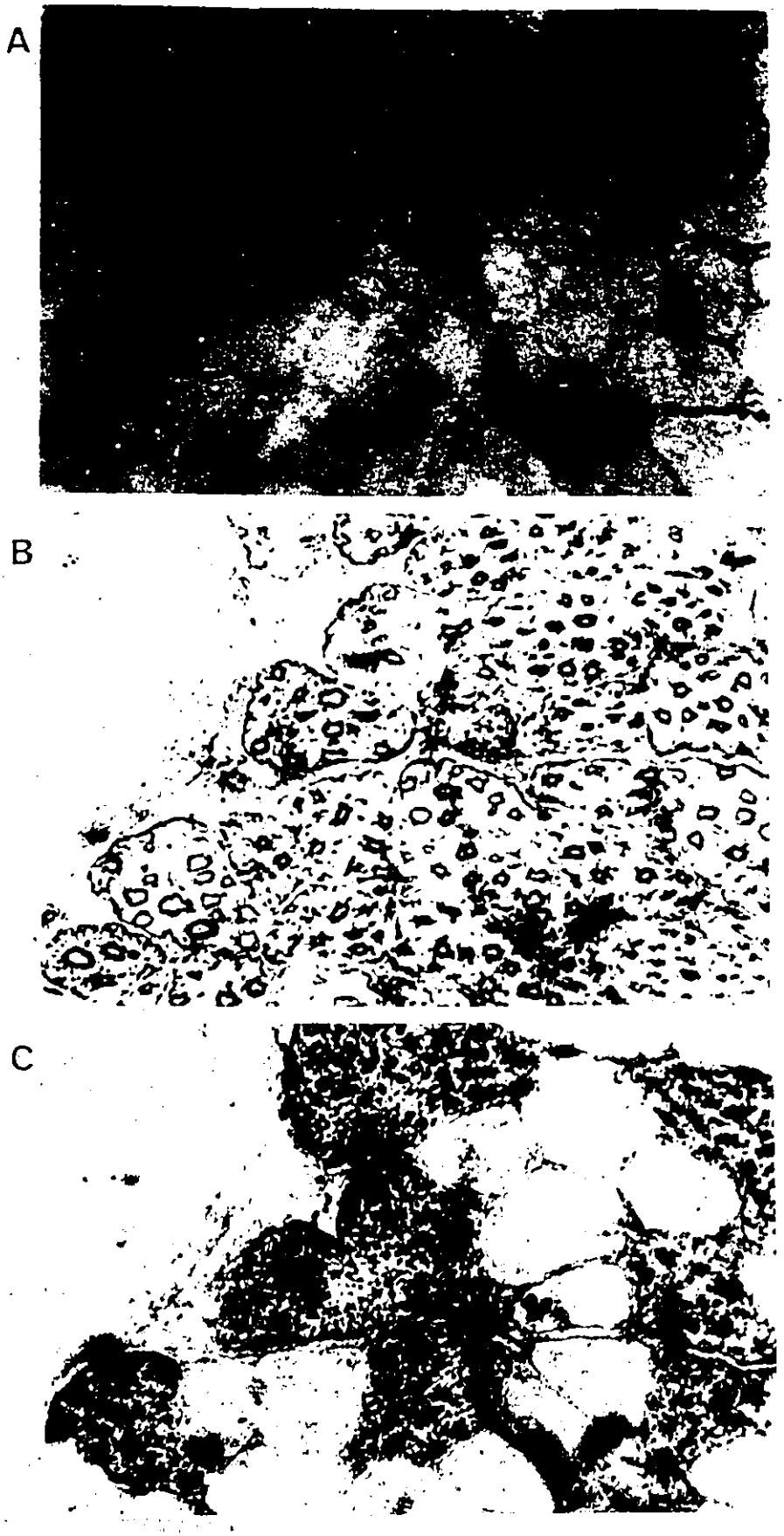
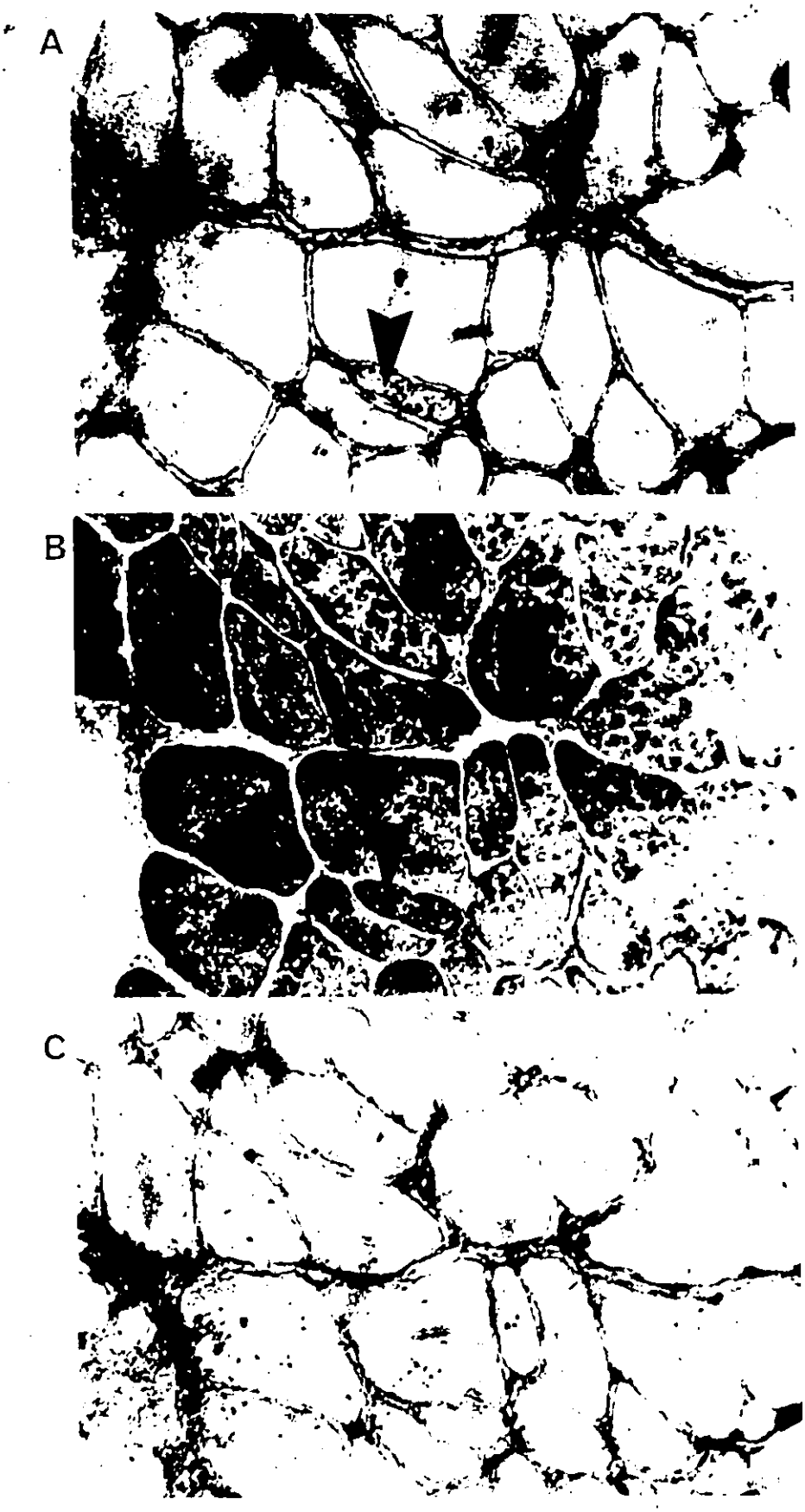


Fig. 38 2 month dystrophic Extensor Carpi Radialis Longus (bottom) and Brevis (top). In A, two small fibres appear to stain positive with the anti-Type I antibody, though these fibres also stain dark with the anti-Type II antibody in B (arrow). In B, all other fibres stained dark with this antibody. In C, none of the fibres appear to stain with the anti-Type IIB antibody. In D, the fibres which were positive in A also stain for anti-embryonic antibody (arrow).

FIG. 38



D

Fig. 39 4 month normal Extensor Carpi Radialis Longus (bottom) and Brevis (top). In A, none of the fibres stain for anti-Type I antibody, whereas in B, all fibres stain for anti-Type II, with some fibres staining more intensely than others. The anti-Type IIB stained section in C shows that ECRL contains a core of Type IIA fibres.

FIG. 39

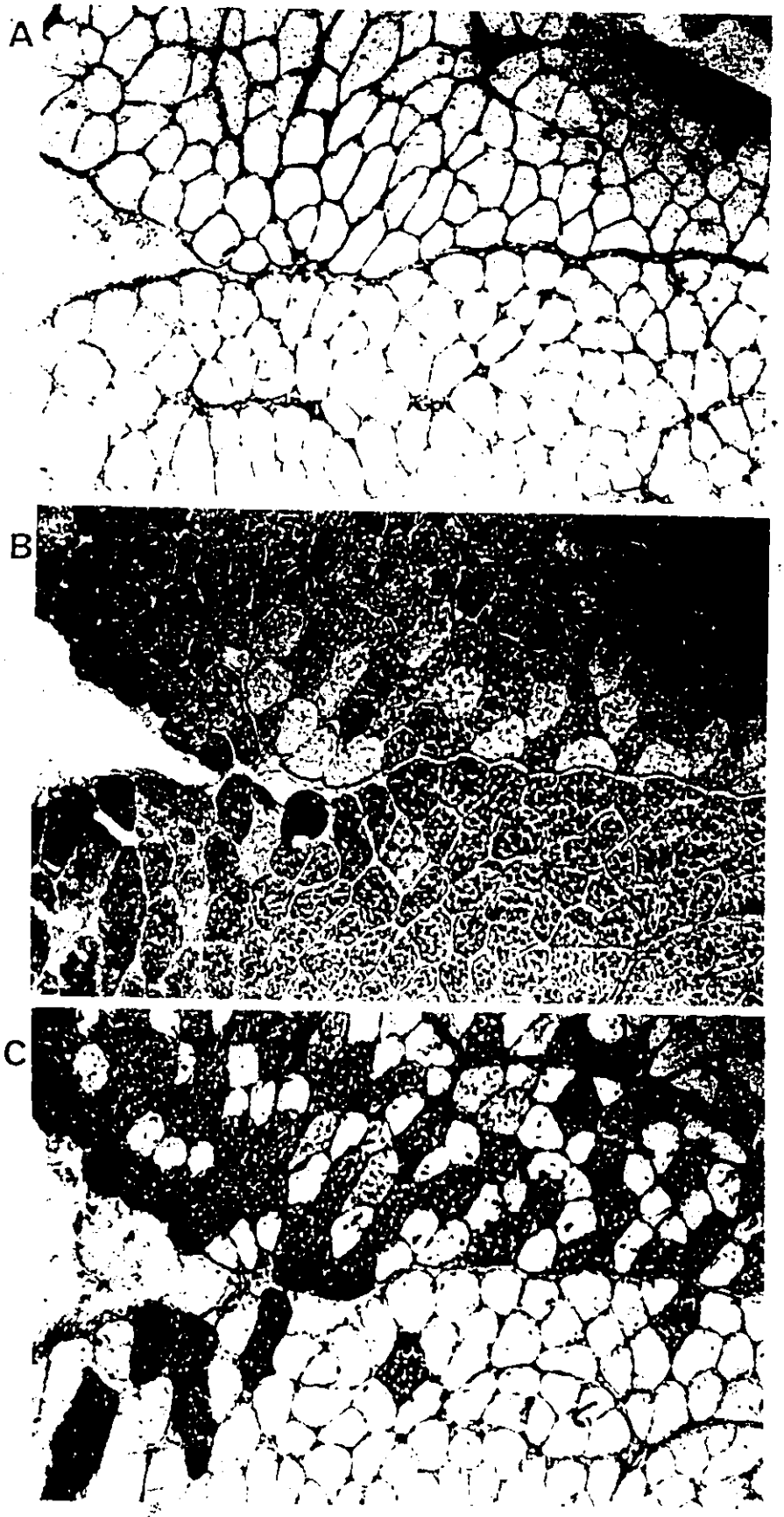
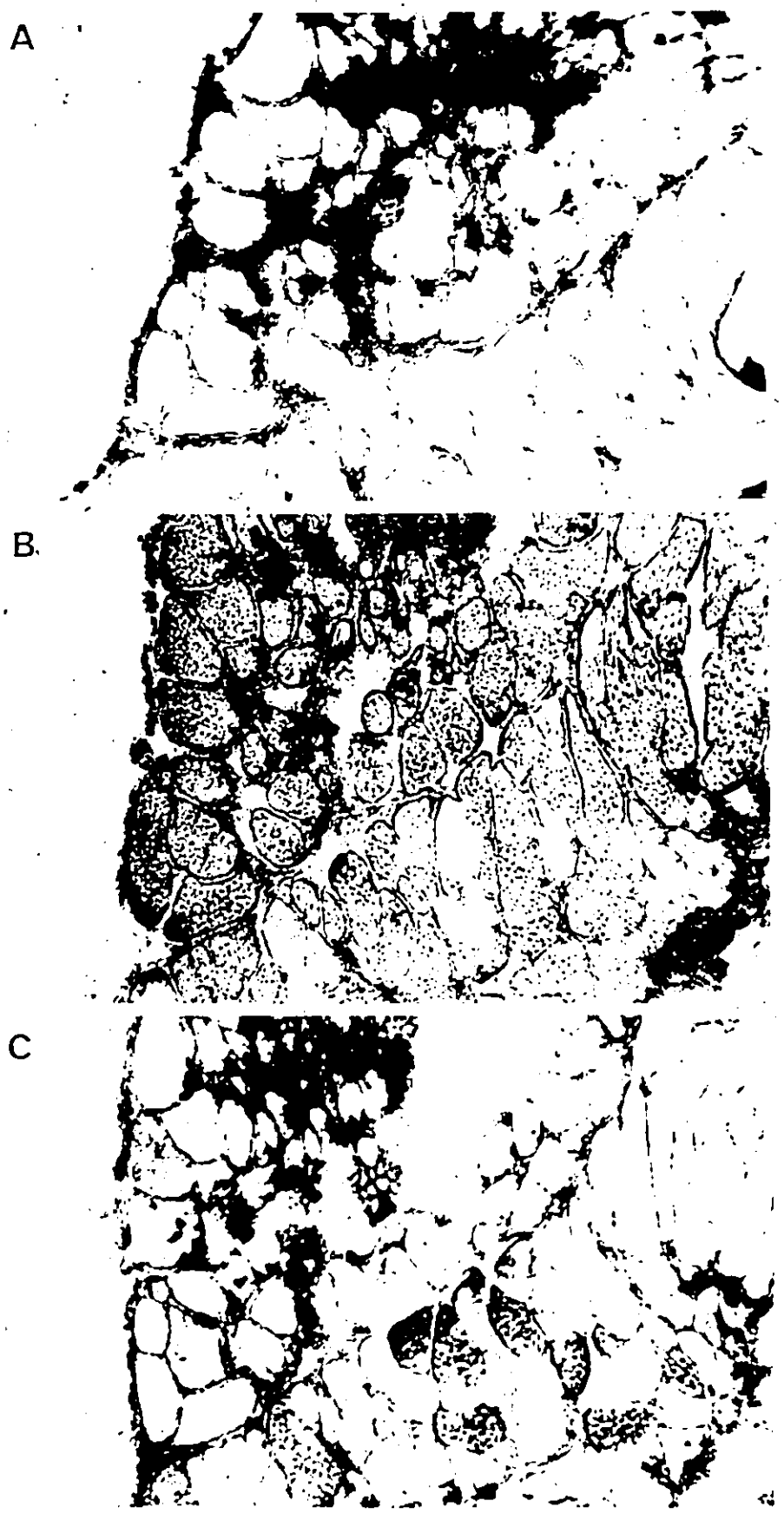


Fig. 40 4 month dystrophic Extensor Carpi Radialis Longus (top) and Brevis (bottom). In A, none of the fibres stain for the anti-Type I antibody. ECRL appears more severely affected by dystrophy than ECRB, as seen by the extent of connective tissue infiltration. In B, all the fibres stain positive for anti-Type II antibody. In C, several fibres are seen to react with anti-Type IIB antibody. In D, only a few fibres react with the anti-embryonic antibody.

FIG. 40



D

Fig. 41 6 month normal Extensor Carpi Radialis Longus (bottom) and Brevis (top). In A, all fibres are negative for Type I-specific antibody. In B, all fibres react with anti-Type II antibody, the paler ones showing up with the anti-Type IIB antibody in C.

FIG. 41

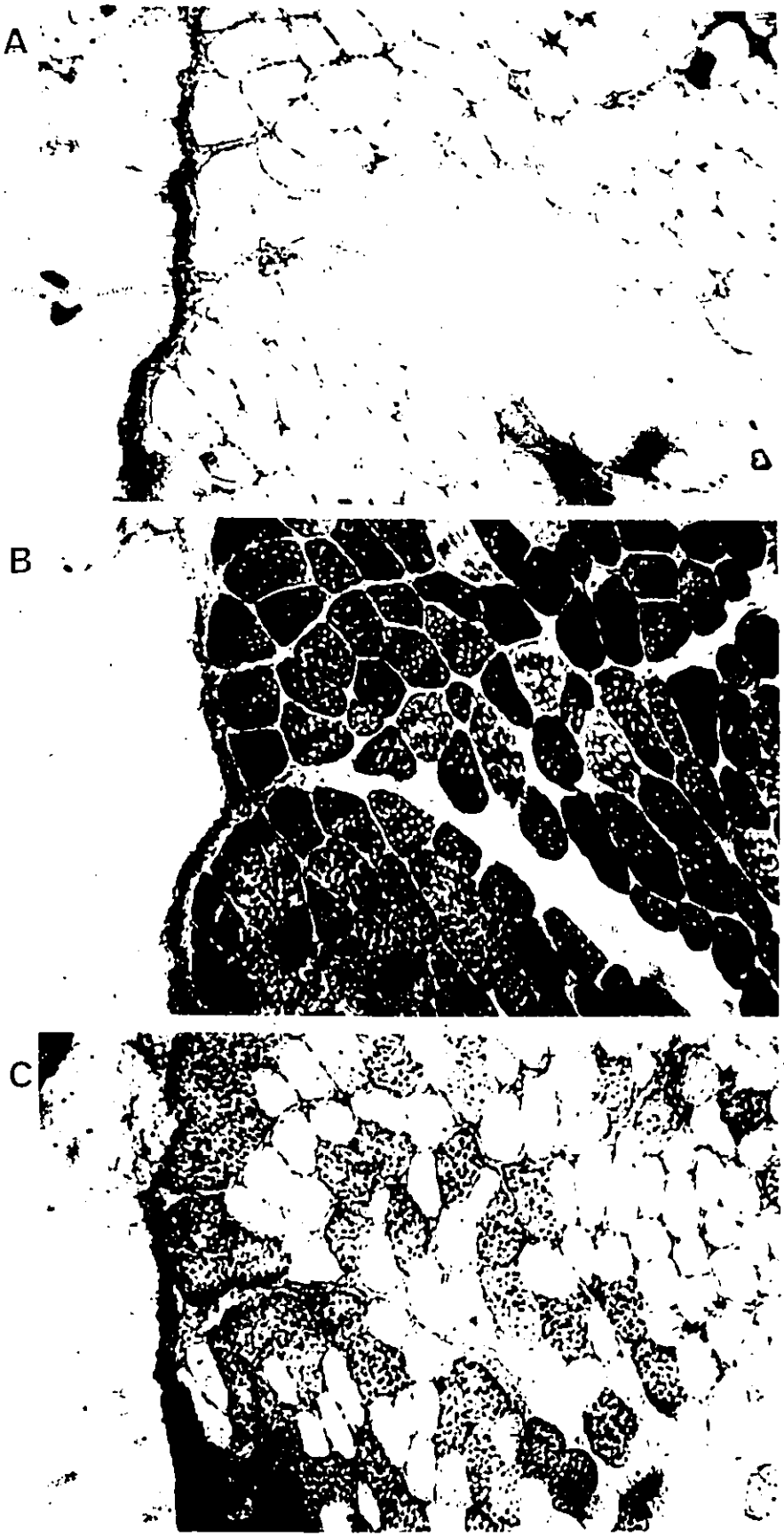
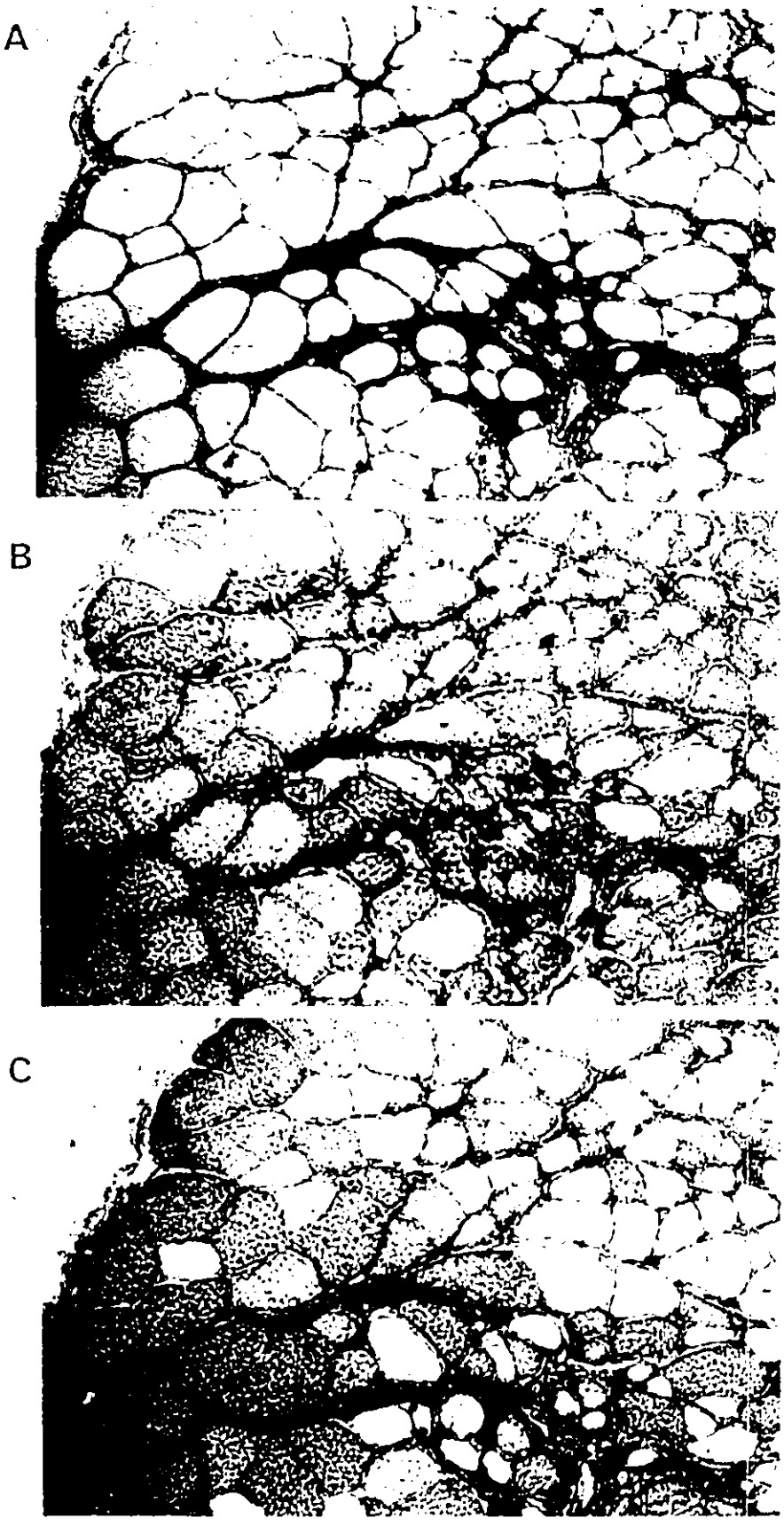


Fig. 42 6 month dystrophic Extensor Carpi Radialis Longus (bottom) and Brevis (top). In A, none of the fibres react with anti-Type I antibody. In B, all fibres show positive staining with anti-Type II antibody. In C, several fibres appear with the anti-Type IIB antibody. In D, the anti-embryonic antibody does not reveal any new positive fibres other than those containing Type IIB myosin, to which it cross-reacts.

FIG. 42



D

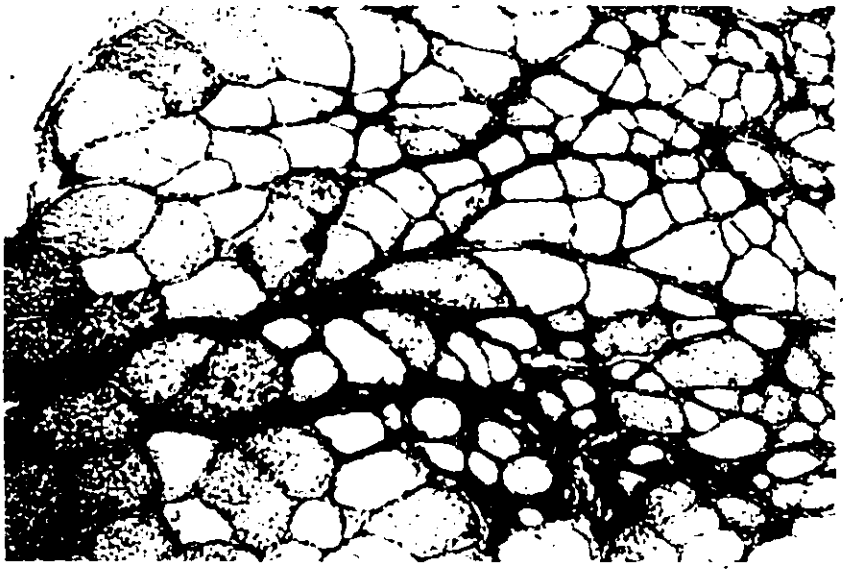


Fig. 43 8 month notmal Extenor Carpi Radialis Longus (top) and Brevis (bottom). In A, all fibres appear negative with anti-Type I antibody. In B, these fibres all stain with the anti-Type II antibody. In C, many fibres stain dark with the anti-Type IIB antibody.

FIG. 43

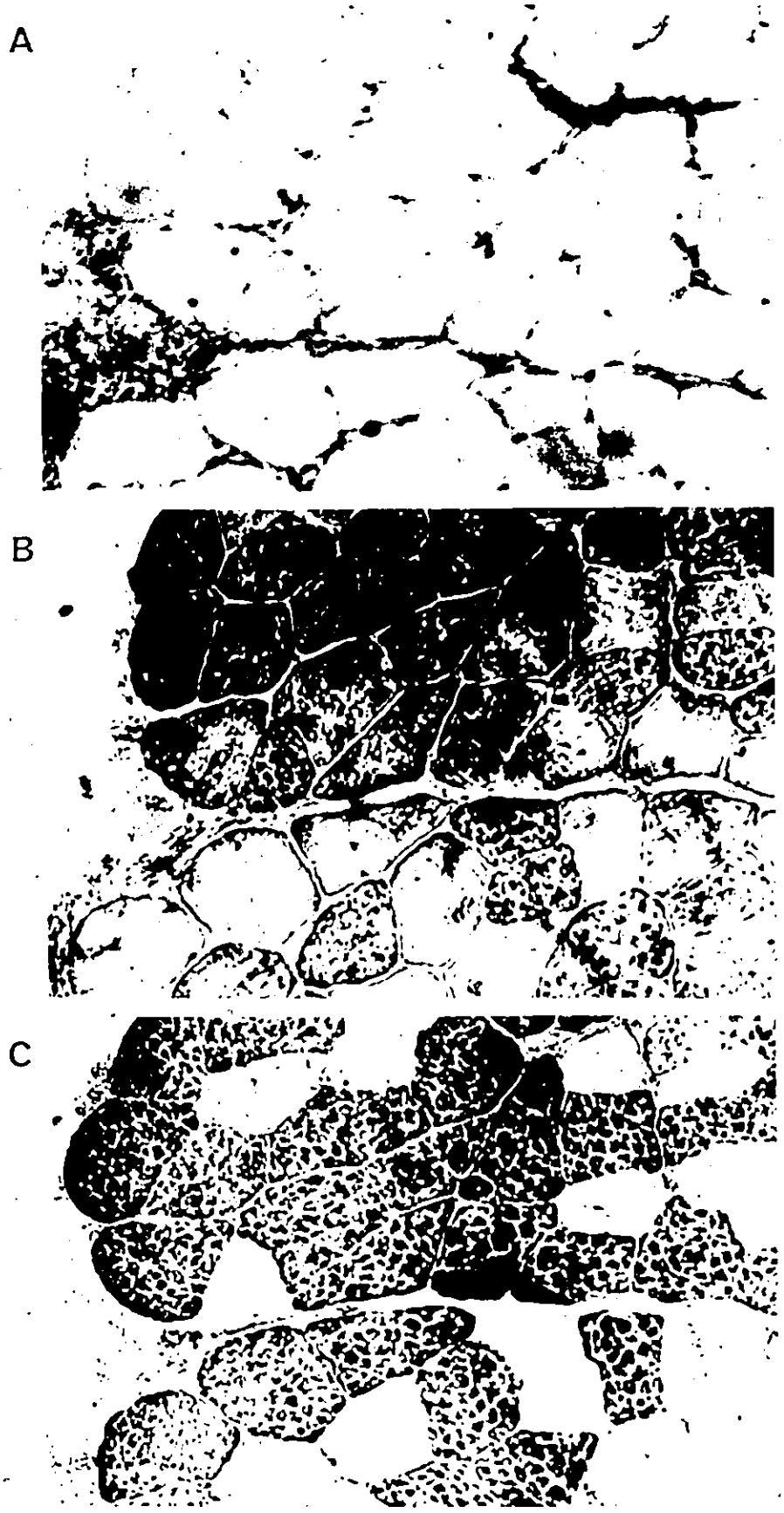


Fig. 44 8 month dystrophic Extensor Carpi Radialis Longus (top) and Brevis (bottom) and AT (top left). In A, none of the fibres stain with anti-Type I antibody. In B, all stain with anti-Type II antibody and in C, two fibres in ECRB stain with anti-Type IIB antibody.

FIG. 44

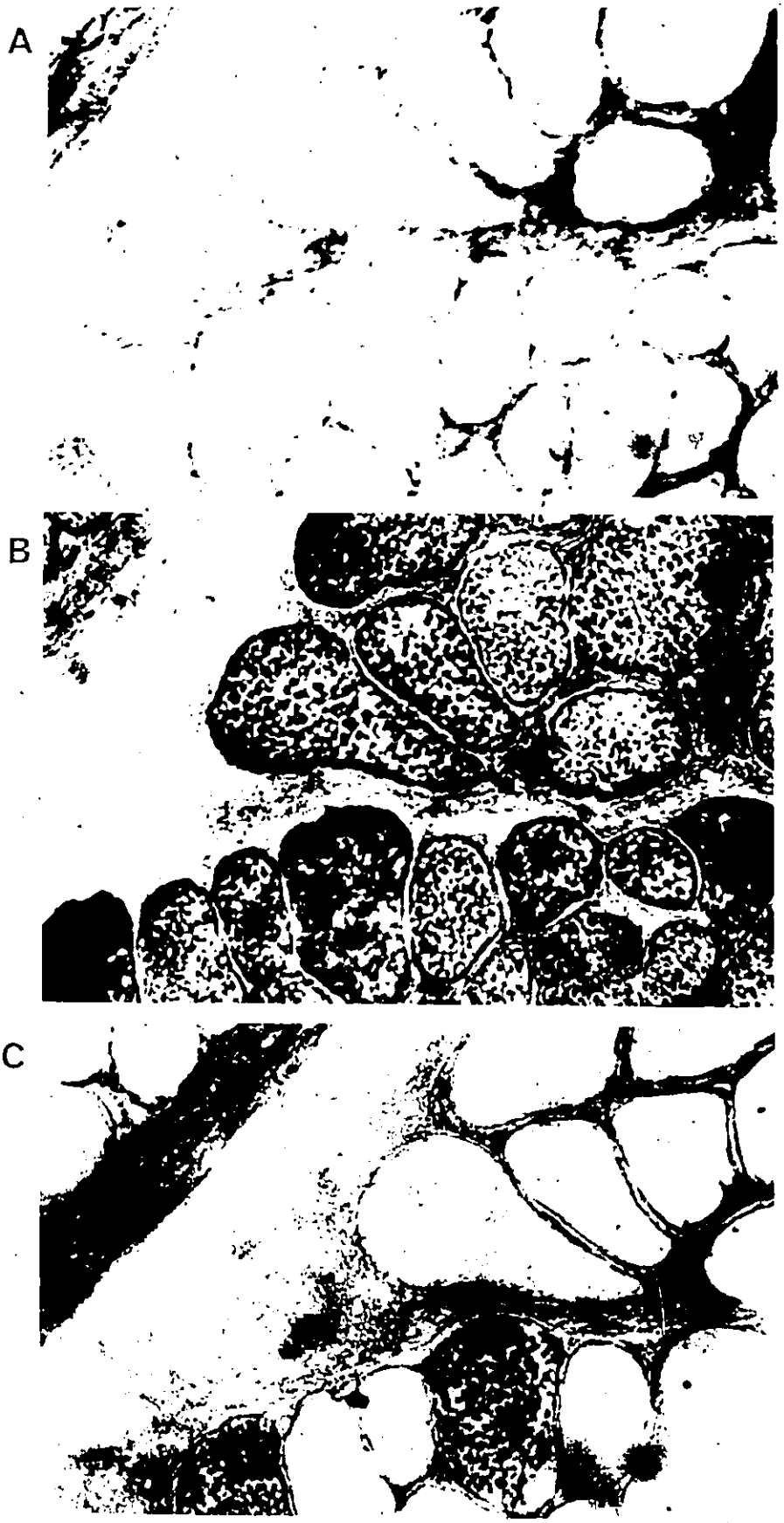


Fig. 45 12 month normal Extensor Carpi Radialis Longus (bottom) and Brevis (top). In A, none of the fibres stain with anti-Type I antibody (compare with 12 month Soleus). In B, all fibres stain with anti-Type II antibody. In C, several fibres stain with anti-Type IIB antibody.

FIG. 45

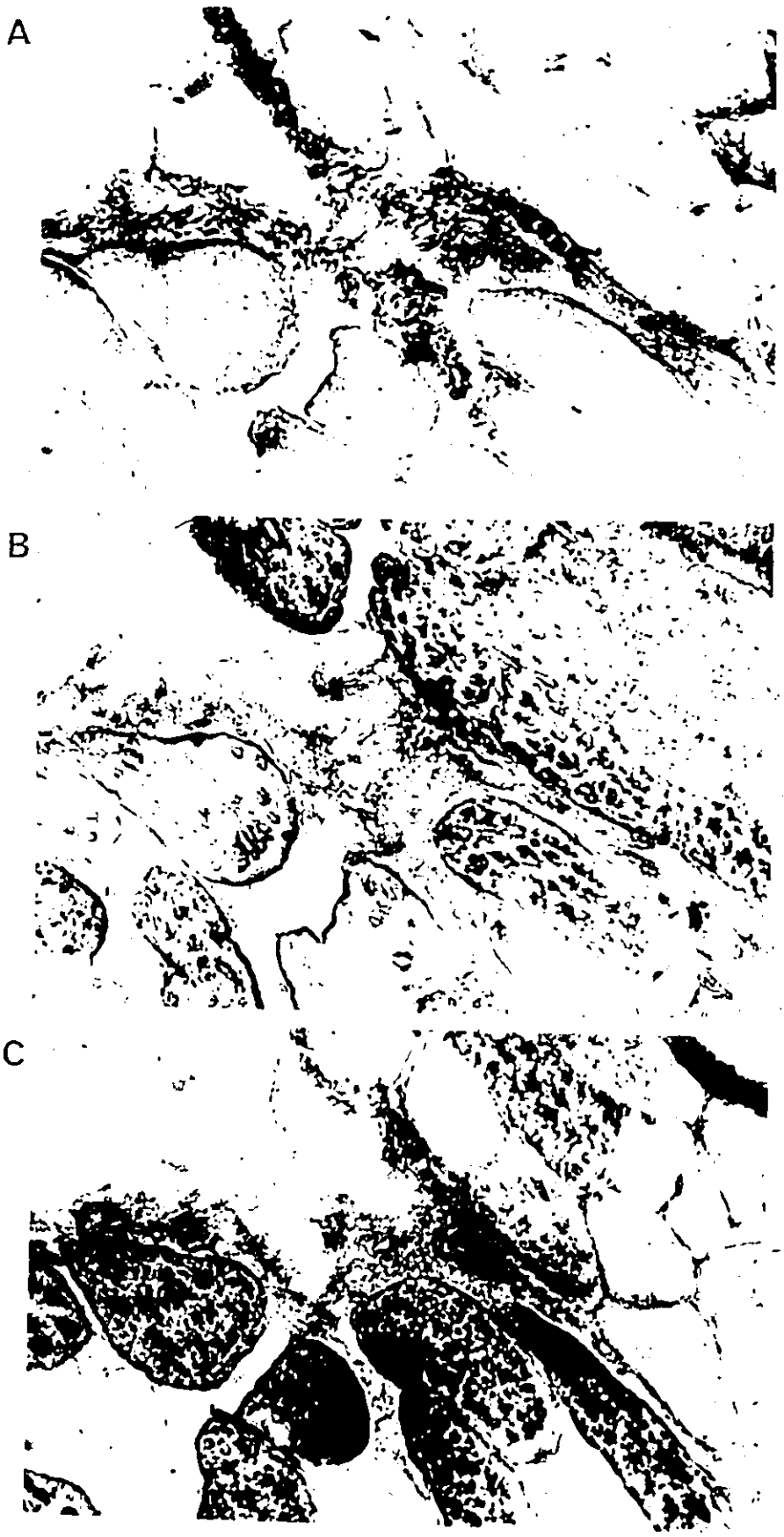
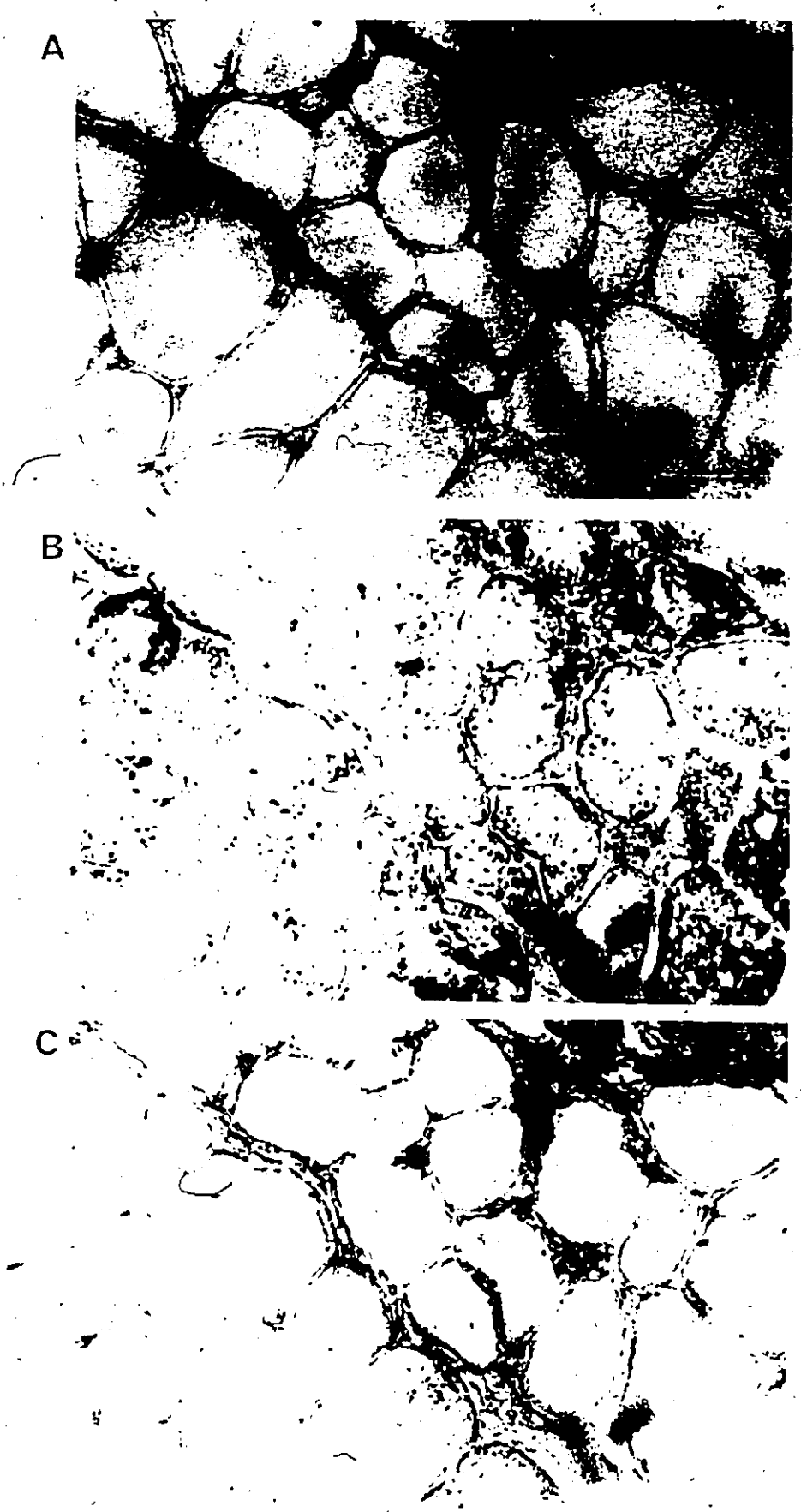


Fig. 46 12 month dystrophic Extenor Carpi Radialis Longus (top) and Brevis (bottom). In A, none of the fibres stain with anti-Type I antibody. In B, all fibres stain with antiType II antibody and in C, only 2 fibres stain with anti-Type IIB antibody in ECRB.

FIG. 46



Sodium Pyrophosphate Gels of Native Myosin

Native myosin from the contralateral muscles used for immunohistochemistry was run in a sodium pyrophosphate-buffered (pH 8.8) gel system. Fig. 47 is a composite of representative gels.

Soleus. Native myosin from normal and dystrophic Soleus muscles produced two electrophoretically distinct bands. The faster-migrating of the two was only slightly slower than the fast myosin(s) of normal fast muscle (Fig. 47, left hand side). It was not very different from the 'fast' myosin bands of native myosin from dystrophic muscles. This band probably corresponds to the IM band reported by Fitzsimons and Hoh (1983). The slower of the two bands would therefore correspond to the SM band. Contrary to what Fitzsimons and Hoh (1983) have reported for the 129/ReJ dy/dy strain of dystrophic mice, Soleus in the C57 BL/6J dy2j/dy2 strain showed a reduction in the relative amount of SM, starting as early as 4 months.

Anterior Tibialis oxidative region The Anterior Tibialis muscle was cut in half parallel to the muscle fibre axis to separate the oxidative core from the glycolytic crown of the muscle. Native myosin was extracted from each half separately. ATox produced only one diffuse band in all age groups except in 12 month old dystrophic mice, where a faint yet visible band was seen above the larger, faster-migrating one. This band had a similar eletrophoretic mobility to the SM band in Soleus.

Anterior Tibialis glycolytic region The other half of the Anterior Tibialis muscle, containing mostly Type II glycolytic and Type II oxidative fibres, did not show the SM band at 12 months in the dystrophic animal. Only the FM band was seen.

Extensor Digitorum Longus The EDL muscle native myosin produced only one visible band in both normal and dystrophic mice at all age groups, corresponding to the FM band. SM was

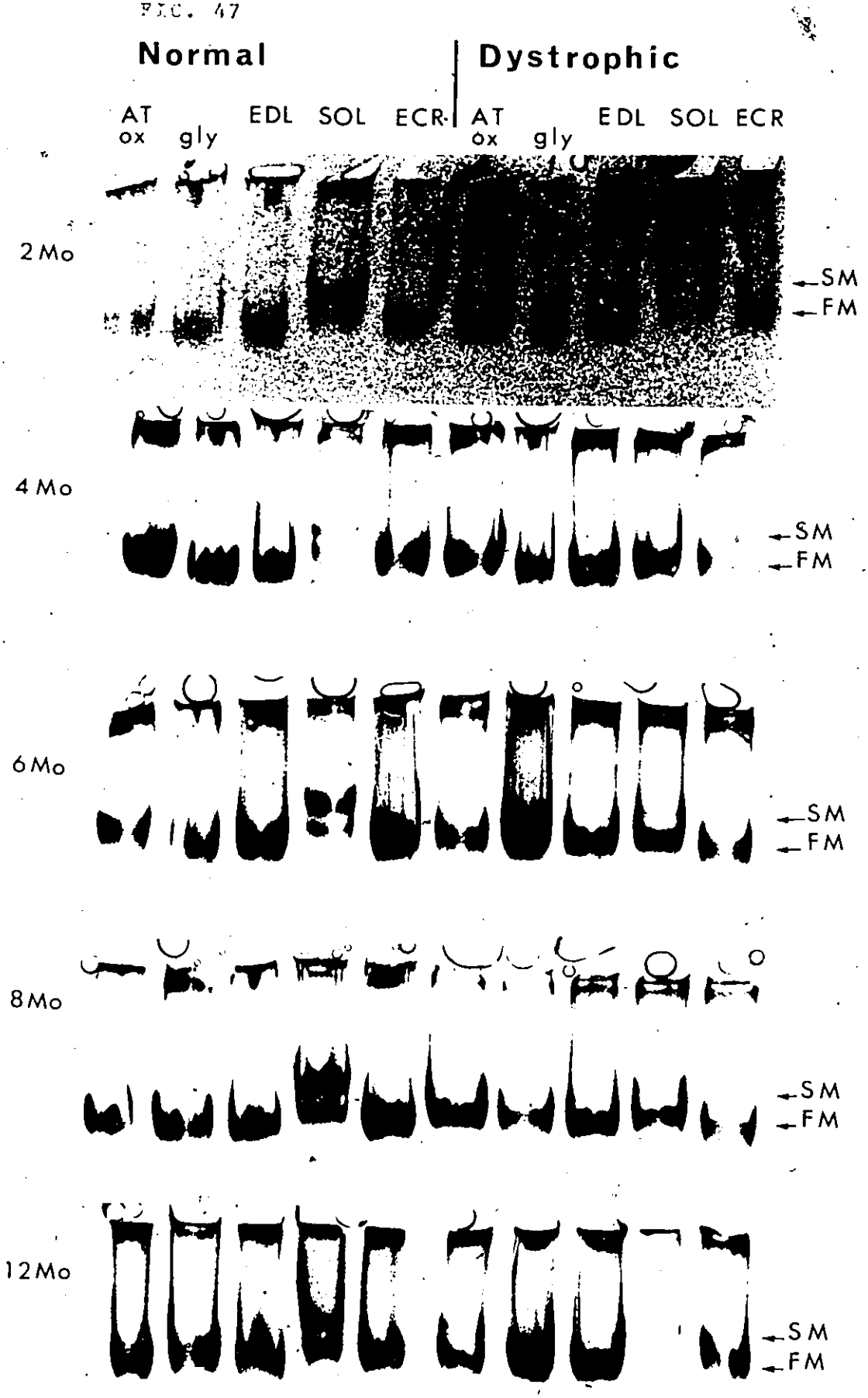
not seen.

Extensor Carpi Radialis Longus and Brevis The ECRL and B muscles were co-extracted for native myosin analysis. In all age groups, both normal and dystrophic ECRL and B extracts showed only the FM band. No SM was observed in either normal or dystrophic mice.

These results are summarized with the other data in Fig. 48.

Fig. 47 Sodium Pyrophosphate-buffered gels of native myosins extracted from individual muscles of mice aged 2, 4, 6, 8, and 12 months. AT, Anterior Tibialis; ox, oxidative region; gly, glycolytic region; EDL, Extensor Digitorum Longus; SOL, Soleus; ECR, combined Extensor Carpi Radialis Longus and Brevis muscles. The majority of these extracts were from the contralateral limb of the muscles used for immunohistochemistry.

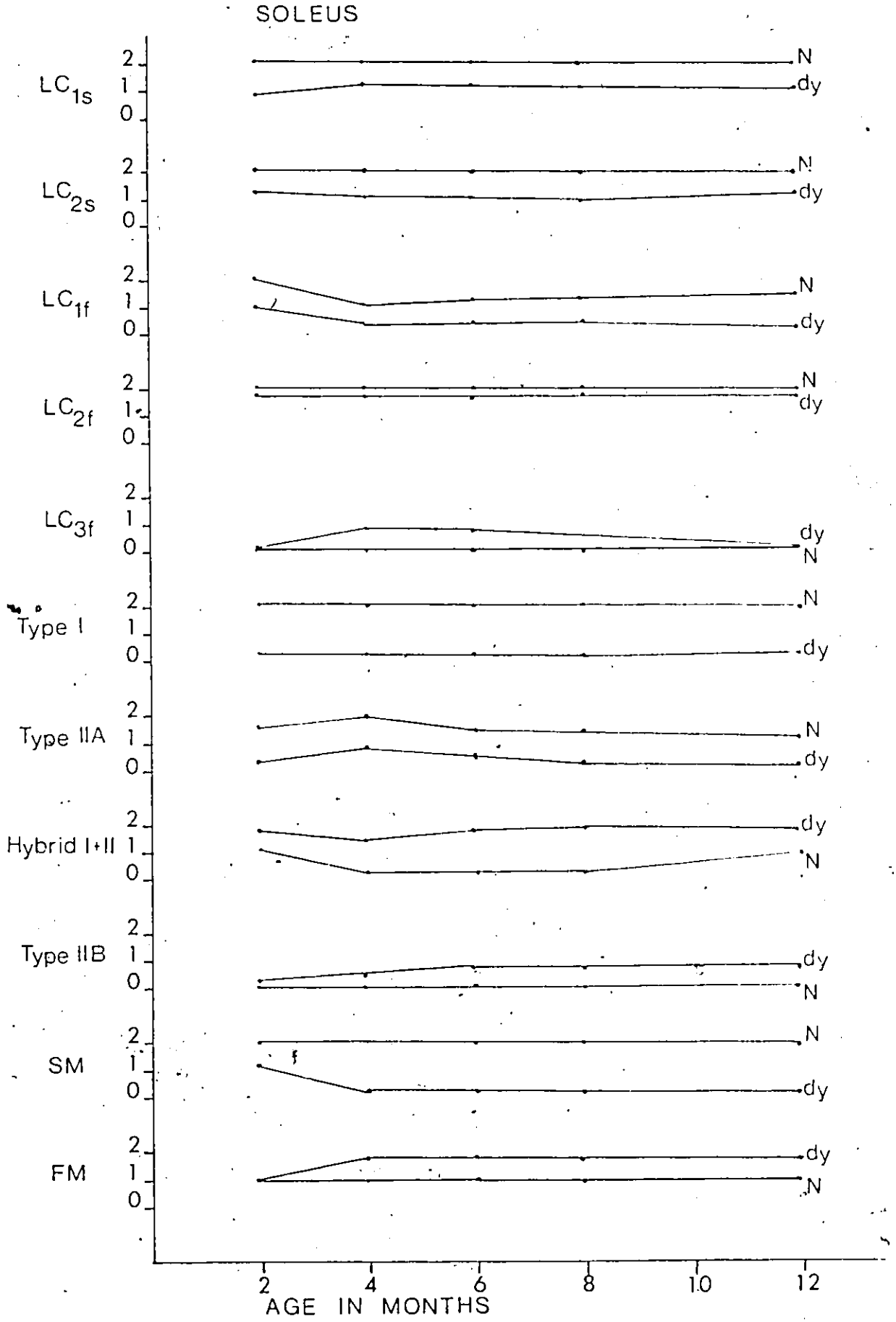
FIG. 47


Normal
Dystrophic
AT
ox
gly
EDL
SOL
ECR
AT
ox
gly
EDL
SOL
ECR
2 Mo
SM
FM
4 Mo
SM
FM
6 Mo
SM
FM
8 Mo
SM
FM
12 Mo
SM
FM

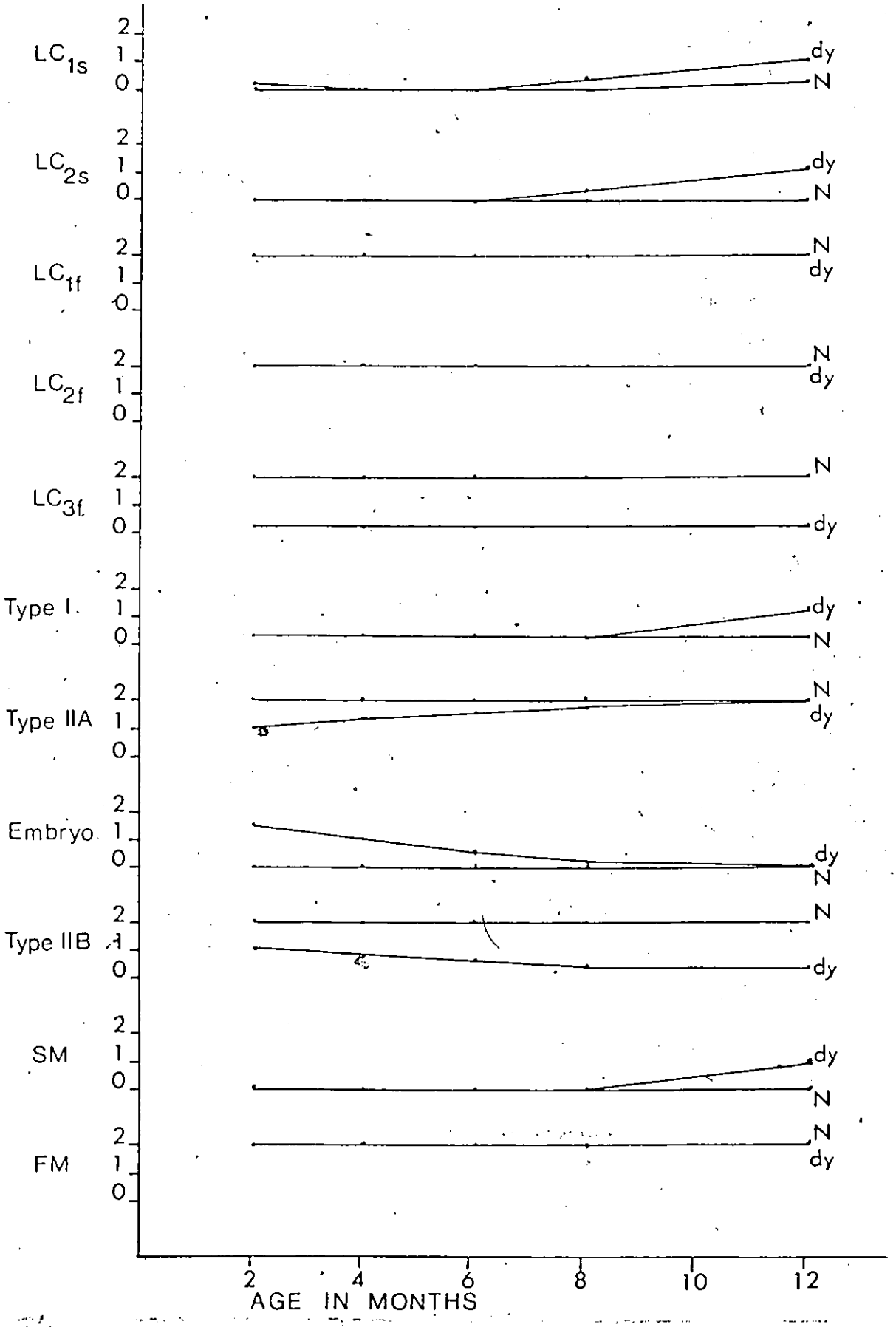
Summary of All Data

The results obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis of the myosin light chains, the immunohistochemical analysis of myosin heavy chain expression using monoclonal antibodies, and the sodium pyrophosphate gels of native myosin isoforms are summarized in graphic form in Fig. 48 for the Soleus, the Anterior Tibialis, the Extensor Digitorum Longus, the Extensor Carpi Radialis Longus and the Extensor Carpi Radialis Brevis muscles.

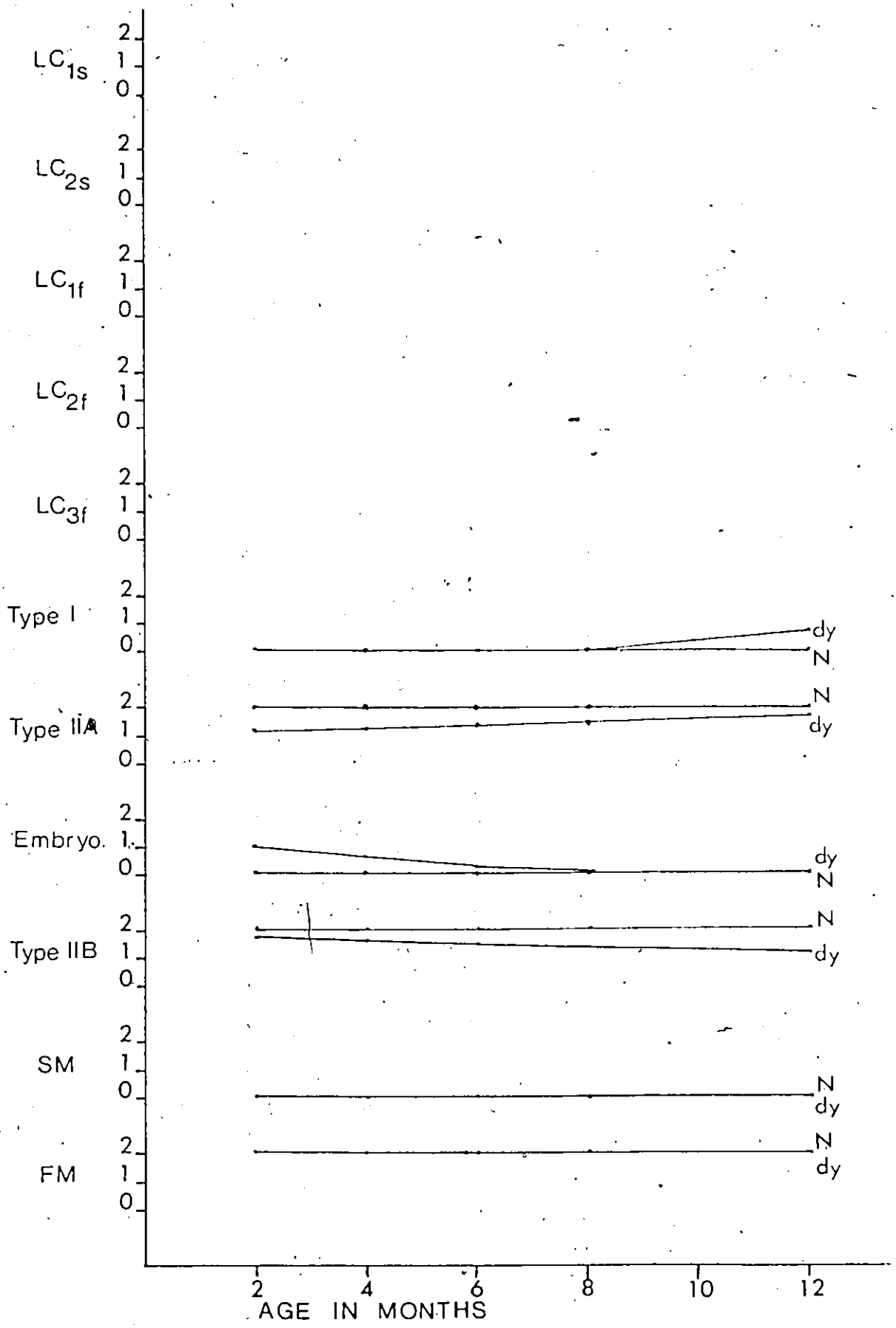
Fig. 48 Summary Figure of all data. Data were scaled as either not observed (0), moderate level expressed (1), and fully expressed (2). Intermediate levels between either of these was graphed accordingly. The abbreviations used were N, for normal; dy, for dystrophic; LC_{1s}, LC_{2s}, the slow light chains; LC_{1f}, LC_{2f}, and LC_{3f}, the fast light chains; Type I, for Type I-specific myosin heavy chain; Type IIA, for Type IIA-specific myosin heavy chain; Type IIB, for Type IIB-specific myosin heavy chain; Hybrid (I+II), for fibres containing both Type I- and Type IIA-specific myosin heavy chains; Embryo., for Embryonic myosin heavy chain; SM, for slow native myosin isoform; and, FM, for the fast native myosin isoforms.


SOLEUS
LC1s
LC2s
LC1f
LC2f
LC3f
Type I
Type IIA
Hybrid I+II
Type IIB
SM
FM
2
1
0
N
dy
2
4
6
8
10
12
AGE IN MONTHS

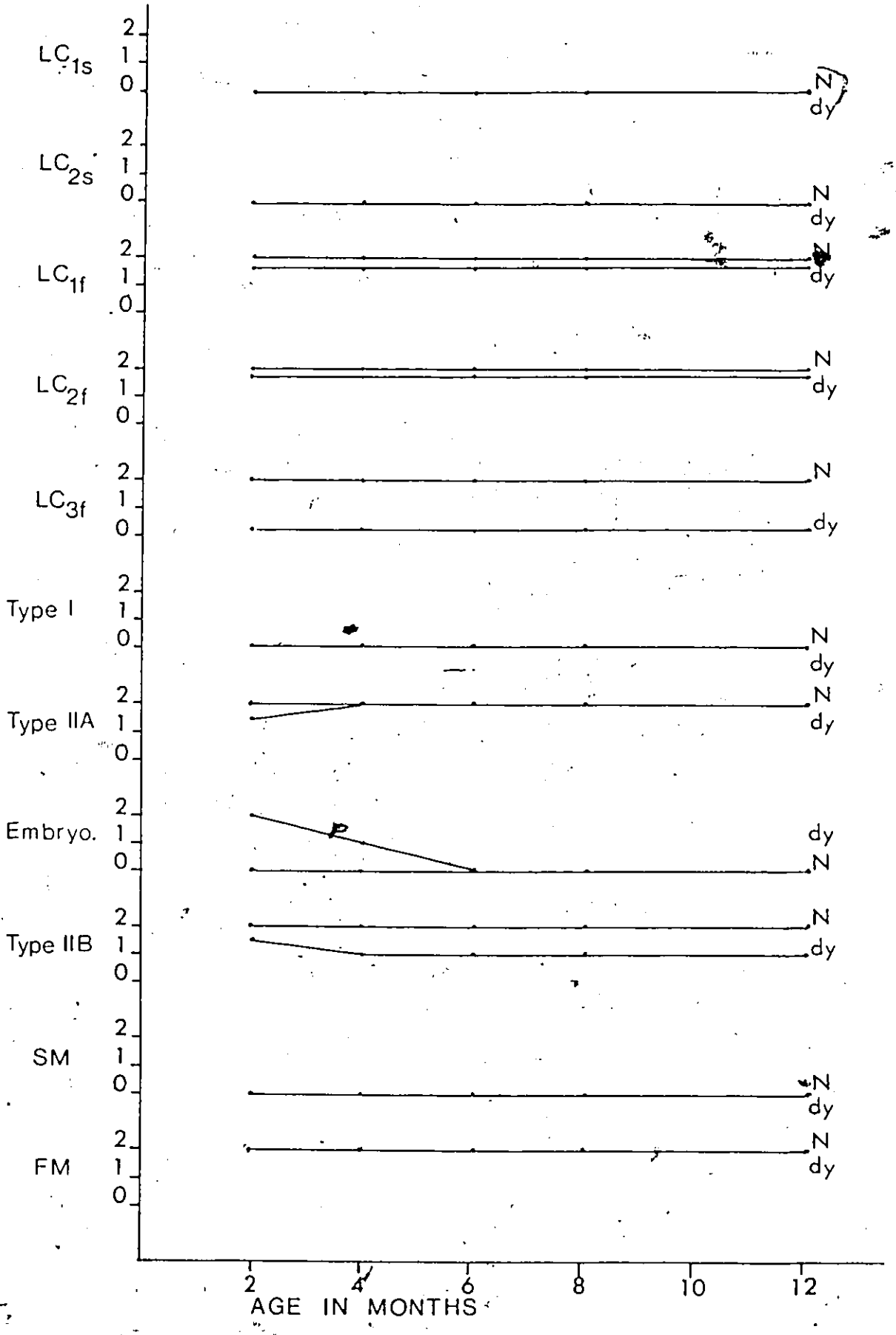
ANTERIOR TIBIALIS



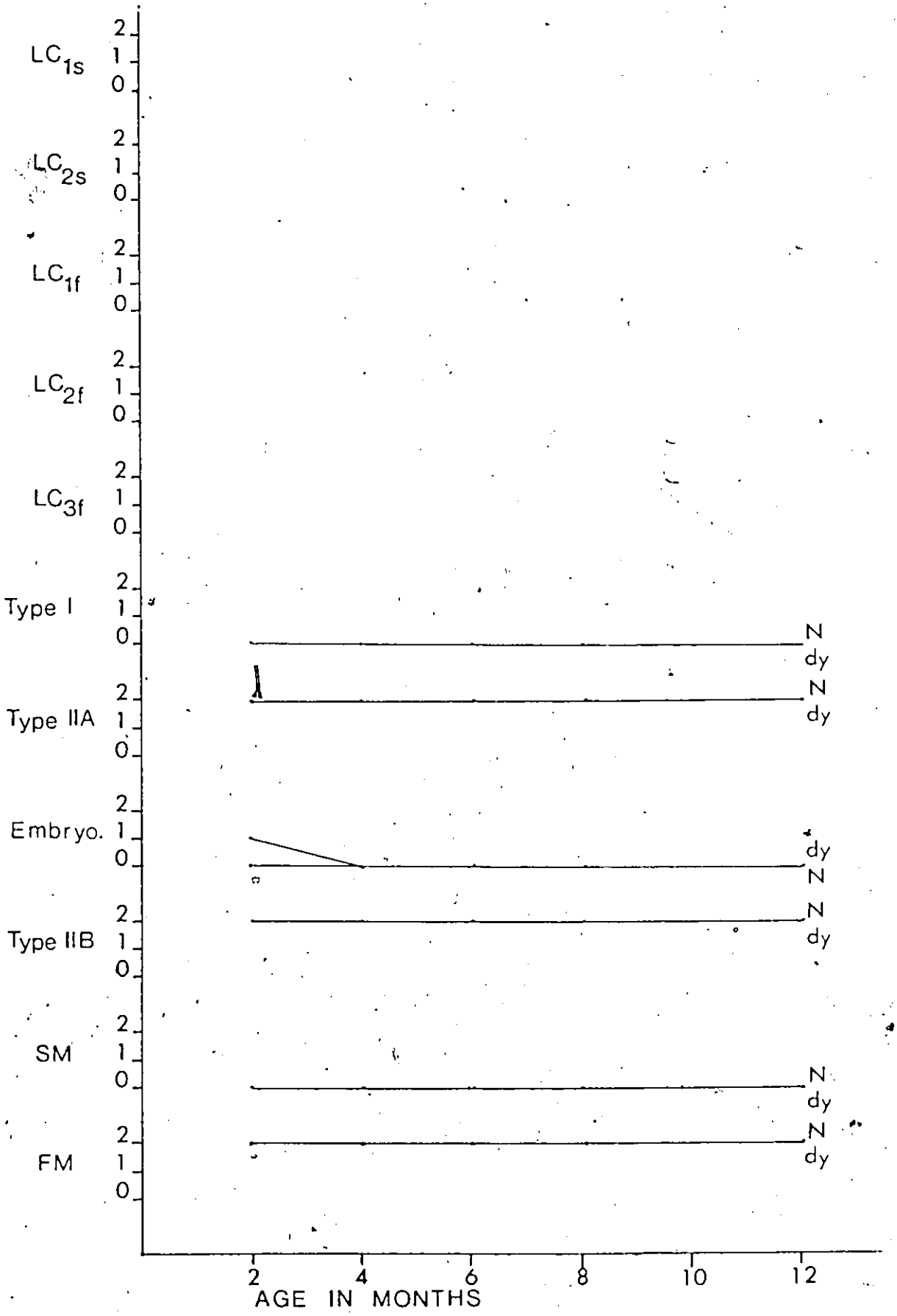
EXTENSOR DIGITORUM LONGUS



EXTENSOR CARPI RADIALIS LONGUS (ECRL)



EXTENSOR CARPI RADIALIS BREVIS (ECRB)



DISCUSSION

Many erroneous conclusions may be drawn about the dystrophic process unless all the pathological events, which might directly influence muscle growth and function, are taken into account and considered in terms of their relative importance. Among these, a preferential degeneration of one fibre type over others might slant the observations in favor of the less affected types, as for example in the report of Butler and Cosmos (1977). Also, muscle fibre degeneration and regeneration would lead to structural and functional characteristics of newly developing muscle (Fitzsimons and Moh, 1981b; Obinata et al., 1980). Partial denervation of the dystrophic muscle would produce muscle fibres characteristic of denervated muscle (Parry, 1977), while changes in the pattern of neural discharge and muscle activity might effect a transformation from one fibre type to another (Silverman and Atwood, 1980). Endocrine abnormalities might also influence the function and development of muscles in dystrophic mice (Watson et al., 1982).

The hypothesis of Parry and Desypris (1983) that slowing of the isometric twitch is the result of slow myosin synthesis in response to chronic neural activity reflected in the muscles of the hindlimb has been tested in light of conflicting reports (Parry and Desypris, 1984; Jasch and Moase, 1985). The results indicate that whereas activity may be involved in the synthesis of slow myosin, the appearance of slow myosin occurs subsequent to the reported slowing of the twitch (Parry and Desypris, 1983).

With this note of caution, I will consider the changes in myosin expression described in the Results section, and attempt to relate these changes in terms of what is known about the pathology of dystrophy in the mouse.

Myosin light chain expression

The Anterior Tibialis is a fast-twitch muscle of the hindlimb which expresses the three fast myosin light chains

(LC1f, LC2f, and LC3f) in normal mice. In dystrophic mice, two additional light chains characteristic of slow muscle (LC1s and LC2s) appear in the gel pattern for myosin purified from the Anterior Tibialis muscles of 8 month old dystrophic mice (Fig. 5b). This pattern of slow light chain expression is enhanced considerably in the older mice aged approximately 12 months (Fig. 6). In contrast to this fast muscle of the hindlimb, the Extensor Carpi Radialis Longus muscle of the forelimb does not express either LC1s or LC2s at any age in the dystrophic mouse.

Jasch and Moase (1985) looked at the relative amount of LC3f and phosphorylated LC2f in the muscles of dystrophic (C57 BL/6J dy^{2j}/dy^{2j}) mice aged 12 weeks (3 months) and 32 weeks (8 months) as compared to muscles of normal mice of the same ages. They found decreased levels of these proteins in both the hindlimb Extensor Digitorum Longus and the forelimb ECRL of dystrophic mice aged 8 months. They do not report the appearance of slow light chain in the EDL muscle. They suggest that since dystrophic ECRL and EDL show similar changes in the relative intensities of these proteins to that of LC2f on isoelectric focusing gels, these changes are not the result of abnormal limb position (Silverman and Atwood, 1980) or the result of spontaneous electrical activity (Parry and Desypris, 1983). Upon closer examination of their gel for dystrophic EDL (Fig. 5, gel c, Jasch and Moase, 1985), two bands can be seen which are approximately in the same location as LC1s and LC2s in their normal Soleus gel (Fig. 5, gel f, Jasch and Moase, 1985). These two bands are virtually absent in their ECRL gel (Fig. 5, gel d, Jasch and Moase, 1985). Additionally, a small spot appears on their second-dimension gel for dystrophic EDL (Fig. 7, Jasch and Moase, 1985) which closely corresponds to the LC1s spot seen for the second-dimension gel of normal Soleus (Fig. 6, Jasch and Moase, 1985).

Though I did not look at the light chain expression of EDL muscle, the AT muscle of dystrophic mice shows only a small amount of the slow light chains at 8 months. Jasch and Moase (1985) mention that they observed similar changes in the

expression of light chains for dystrophic AT as for EDL; though they did not show a representative gel for this muscle. The EDL muscle may not be affected in the same way as the AT muscle in dystrophic mice, as will be discussed later. Their analysis of myosin light chains was from isoelectric focusing gels which allow the separation of the phosphorylated from the non-phosphorylated form of LC2f. In SDS-PAGE, it is not possible to distinguish between these two forms. There appears to be a slight reduction in the relative amount of LC3f at all ages in dystrophic AT and ECRL. The most important observation is the absence of slow light chains in the dystrophic ECRL muscle, which indicates that the presence of these light chains in the dystrophic fast-twitch hindlimb AT muscle is most likely due to the chronic spontaneous neural discharge of the hindlimb motoneurons.

The slow-twitch Soleus muscle of dystrophic mice appears to contain less of the slow light chains relative to the fast light chains. This observation holds for dystrophic mice aged 2, 4, and 6 months. It is unfortunate that the dystrophic Soleus purified myosin extract was lost for the 8 month age group, because at 12 months there appears to be approximately equal proportions of the fast and slow light chains in dystrophic Soleus. Jasch and Moase (1985) reported an almost complete loss of LC2s in Soleus of 8 month old dystrophic mice. My gel for the 6 month old dystrophic mouse Soleus shows considerably less LC2s than LC1s (Fig. 5a). In the mature dystrophic mice aged 12 months, this trend appears to have reversed with Soleus still showing the presence of LC2s. As will be discussed later, this data is compatible with that obtained both by immunohistochemistry and with the native myosin gels.

Immunohistochemistry: Myosin heavy chain isoenzyme expression

The Soleus muscle myosin heavy chain expression of normal and dystrophic mice will be considered first., Ovalle et al. (1983) looked at the post-natal development of the Soleus in the C57 BL/6J dy^{2j}/dy^{2j} dystrophic mouse at 4, 8, 12, and 32

weeks of age, using histochemical and quantitative methods. With increasing age, the absolute number and proportion of dystrophic Slow Oxidative (Type I) fibres were drastically reduced (35% at 4 weeks to 21% at 32 weeks). In contrast, the percentage of dystrophic Fast Oxidative Glycolytic (Type IIA) fibres increased significantly (from 55% at 4 weeks to 69% at 32 weeks) while their absolute numbers between 4 and 32 weeks remained relatively constant. Ovalle et al. (1983) suggested that a failure or retardation in the normal post-natal conversion of fibre types within the Soleus muscle occurs in this murine model for muscular dystrophy.

The immunohistochemical data presented here tends to support this view. In fact, it may be suggested that a reversal of the normal post-natal development takes place in dystrophic Soleus. Whereas normally there is a conversion from fast to slow fibres in developing Soleus muscle, the dystrophic Soleus shows fewer slow fibres with age. From the study of Ovalle et al. (1983), it would appear that a slow to fast transition takes place and allows the absolute number of fast fibres to remain unchanged, at the expense of slow fibres. Progressive functional denervation of dystrophic Soleus may be partly responsible for the observed reversal in its fibre type development. Indeed, it has been shown (Parry, 1977) that the functional innervation ratio (ratio of maximum twitch tension in response to nerve stimulation x 100 to maximum twitch tension in response to direct muscle stimulation) for dystrophic Soleus in mice aged 26 to 52 weeks was significantly less (77.2% + 4.8%) than in normal Soleus (90.6% + 2.4%) for the same age group. Thus, denervated Type I fibres would undergo a slow to fast transformation as has been shown in denervated slow-twich muscle (Gambke et al., 1983: Dhoot and Perry, 1983; Heeley, Dhoot and Perry, 1985), whereas denervated Type II fibres would show little change with respect to their myosin expression. In addition, spontaneous neural discharge and cross-talk between amyelinated motoneurons in the ventral roots (Rasminsky, 1978) may alter the properties of slow motoneurons in such a way that normal maintenance of slow muscle characteristics is lost or substantially modified. Parry and

Desypris (1983) have recorded spontaneous activity in the Soleus muscle of anesthetized dystrophic mice aged about 6 months.

The Anterior Tibialis muscle is a fast-twitch muscle of the anterior compartment of the distal hindlimb. It has a wedge-shaped appearance in cross-section, with the apex near the bone. In the mouse, it consists of an heterogenous population of fibres. Glycolytic fibres are found principally in the outermost portion of the muscle. The oxidative fibres are found mostly near the apex, in the deep portion of the muscle (Butler and Cosmos; 1977). Occasionally, the deeper region of the mouse AT muscle shows a few Type I fibres, as seen with monoclonal antibodies in immunohistochemistry (see Fig. 17 and Fig. 21 of the Results).

The (dy^{2j}) dystrophic mouse AT muscle has been examined by several investigators. In an early study, Butler and Cosmos (1977) suggested that the glycolytic fibres were less affected than the oxidative fibres, based on the observation that small foci of destruction could be detected in regions rich in oxidative fibres as early as 2 to 3 weeks post-natally and that wasting of these fibres ensued rapidly. Their nomenclature of fibre types was erroneous, however, since it was based only on oxidative enzyme histochemistry, and not on actomyosin ATPase histochemistry. Thus, the fibres they call 'slow' are most likely fast-twitch oxidative fibres (Type IIA).

A muscle which shows similar regional fibre type distribution to the AT in the mouse is the gastrocnemius muscle. Dribin and Simpson (1977) found that the histochemical staining of the muscle fibres located in the outer (crown) region of the dystrophic gastrocnemius, which normally consists of glycolytic fibres, was intermediate between glycolytic and oxidative muscle. The (dy^{2j}) dystrophic mice in this study were between 2.5 and 5.5 months old. In dy^{2j} dystrophic mice aged 4 to 7 weeks, Silverman and Atwood (1980) also observed an increased oxidative capacity of the glycolytic fibres in gastrocnemius muscle, which was correl-

ated with the overactivity of these fibres due to spontaneous neural discharge. Their myographic records from the crown region of gastrocnemius from a (dy^{2j}) dystrophic mouse under anesthesia show both tonic and intermittent units firing, with bursts of spontaneous activity occuring in both units (Fig. 8, Silverman and Atwood 1980).

Silverman and Atwood (1980) suggested that the abnormal pattern of activity imposed on the dystrophic muscle fibres by the peripheral nerves was similar to the intermittent stimulation regimen used in the experiments of Pette and associates (Pette et al., 1973; 1975; 1976). These authors have shown that intermittent long-term stimulation of the rabbit AT and EDL muscles produced an increase in their oxidative capacity without conversion to a slow fibre type. More recently, Mabuchi et al. (1982) have found that intermittently stimulated rabbit AT muscle shows a Type IIB to Type IIA transformation without an increase in the proportion of Type I fibres.

Chronic continuous stimulation also produces an increased oxidative capacity, but generates an increased number of Type I fibres through the inducton of slow myosin synthesis (Sreter et al., 1973; Salmons and Sreter, 1976; Pette et al., 1976; Pette and Schnez, 1977; Seedorf, Seedorf, and Pette, 1983; Brown, Salmons, and Whalen, 1983). In either case, intermittent or continuous long-term stimulation have both been reported to increase the time to peak twitch tension of stimulated fast-twitch muscles in the rabbit (Pette et al., 1973; 1975; 1976). The increased contraction time is more prolonged in muscles subjected to continuous than to intermittent stimulation patterns (Pette et al., 1976).

The time to peak twitch tension has also been reported to increase in the fast-twitch muscles of dystrophic mice. Harris and Montgommery (1975) looked at the contractile characteristics of dystrophic (dy^{2j}) mice aged 2 to 3 months. They found a significant increase in the contraction time of the Anterior Tibialis isometric twitch, but no significant difference in the time to half-relaxation. Parslow and Parry

(1981) have also observed slowing of fast-twitch muscle in the (dy^{2j}) dystrophic mouse. In young animals aged 4 to 6 weeks, a significant increase in the time to peak tension and a slight, but non-significant, increase in the half-relaxation time of the isometric twitch was observed. In older animals, aged more than 6 months, both time to peak tension and half-relaxation time were significantly increased. Bressler et al. (1983) have also reported an age-related prolongation of both these parameters in the EDL muscle of (dy^{2j}) dystrophic mice.

Parry and Desypris (1983) have suggested that this slowing of the isometric twitch may be due, in part, to the synthesis of slow myosin in dystrophic muscle fibres subjected to chronic stimulation by their motoneurons. In order to demonstrate the presence of slow myosin containing fibres in dystrophic muscles, immunohistochemistry with polyclonal antibodies raised against cat Soleus purified myosin was performed. Type I fibres in the mouse Soleus muscle, identified by myofibrillar ATPase histochemistry, gave positive staining with the anti-slow polyclonal antibodies. The forelimb ECRL muscle was examined as an internal control in dystrophic mice. As spontaneous neural activity does not occur in the dystrophic forelimb, slow myosin should not appear. Whereas the AT muscle from a 6 month old normal mouse did not show positively staining fibres with the anti-slow polyclonal antibodies, the AT of a dystrophic mouse of the same age showed a considerable number of anti-slow-positive fibres. No positive fibres were seen in the ECRL muscle of 6 month old dystrophic mice. The ECRL muscle of 6 to 8 month old dystrophic mice had a 25% increase in the time to peak tension. The hindlimb EDL muscle had a time to peak tension which was twice that of the normal muscle. It was concluded that slow myosin expressed as a result of spontaneous activity was partly to blame for the slowing of the isometric twitch time to peak tension (Parry and Desypris, 1983).

An alternative to the appearance of slow myosin in hindlimb muscles of 6 month old dystrophic mice, which would also explain, in part, the slowing of the isometric twitch is

the presence of regenerating muscle fibres which are known to contain embryonic myosin (Fitzsimons and Hoh, 1981b). Buller, Eccles, and Eccles (1960) have shown that neonatal kitten muscles contract slowly , and that by the age of 6 weeks, fast muscles have a rapid (adult) contraction time. Close (1965) has found that the force-velocity relationship of the mouse Soleus muscle remains constant from birth, whereas that of the EDL muscle shows a change to a higher velocity of contraction. Thus, if embryonic myosin reappears in the dystrophic muscles, this could explain their slower contractile response. The 'slow' myosin containing fibres reported by Parry and Desypris (1983) may have consisted of regenerating fibres to which the anti-slow polyclonal antibodies were cross-reacting.

There are several reasons to believe that embryonic myosin heavy chain is present in the Anterior Tibialis muscle of 6 month old dystrophic mice. The most compelling evidence is that obtained with the anti-embryonic myosin heavy chain monoclonal antibody, BF 45. This antibody does not cross-react with the slow myosin heavy chain of Type I fibres. Thus, it not only allows the detection of fibres which contain neonatal myosin heavy chain, but also to discriminate between the fibres giving a positive reaction with the anti-Type I monoclonal antibody from those which actually contain only slow myosin heavy chain. Until age 8 months, all Type I-positive fibres in AT also gave a positive reaction with the anti-embryonic monoclonal antibody. At 8 months (Fig. 24), one fibre is obviously a 'pure' Type I, as it is positive with BF 46 (Fig. 24a), shows clear negative staining with BF 34 (Fig. 24b), and is also negative with BF 45 (Fig. 24d), whereas other fibres which were positive with BF 46 (Fig. 24a, arrows) appear positive with BF 45 (Fig. 24d, arrows). At 12 months, many fibres which are positive with BF 46 (Fig. 26a, arrows) are negative with BF 45 (Fig. 26d, arrows), indicating the presence of slow myosin heavy chain in the oxidative region of dystrophic AT at this age.

The evidence obtained with the immunohistochemistry of myosin heavy chain expression agrees well with the myosin

light chain gel patterns. Only a small amount of the slow myosin light chains (LC1s and LC2s) appears at 8 months in dystrophic AT. None could be detected in the gel for 6 month old dystrophic mice, which showed only the fast light chains (LC1f, LC2f, LC3f). Although the genes encoding myosin heavy chain and myosin light chain 2 are on different chromosomes in the mouse (Czosneck et al., 1982), one can expect the co-expression of slow myosin heavy and light chains as it is known to occur in normal development (Gambke et al., 1981). On the other hand, Brown et al. (1983) have shown that the subunits of slow myosin appear sequentially in chronically stimulated fast muscle. It may be that the slow myosin seen in fast muscles of the dystrophic mouse hindlimb appears subsequent to the fetal isoform in regenerated muscle fibres subjected to the modified pattern of neural activity.

The Extensor Digitorum Longus muscle is also found in the anterior compartment of the distal hindlimb and lies deep and lateral to the Anterior Tibialis muscle. It consists of Type IIA and Type IIB fibres; very rarely, Type I fibres are encountered in the EDL of normal mice (Fig. 31a, b). In the dystrophic mouse, the EDL shows fibres containing embryonic myosin heavy chain (Fig. 28d, arrow, Fig. 30d, Fig. 32d). At 8 months, few fibres gave positive staining for the anti-embryonic myosin heavy chain antibody (Fig. 34d). Fibres which were positive to BF 46 (Fig. 34a) did not stain with BF 45 (Fig. 34d, arrows). As was observed for the AT muscle, fibres with embryonic myosin heavy chain were encountered in dystrophic mice up to 6 or 8 months of age. Slow myosin heavy chain containing fibres could not be said to have appeared before 8 months because of the cross-reactivity of the BF 46 antibody with embryonic myosin heavy chain. Fewer embryonic or slow myosin heavy chain containing fibres were encountered in EDL than in the oxidative region of AT in dystrophic mice. Both EDL and AT had fewer Type IIB myosin heavy chain containing fibres in the older dystrophic mice than in the normal mice of similar age. The loss of Type IIB fibres can be understood in light of the results of the study by Mabuchi et al. (1982) (described earlier) and probably represents an adaptive transformation of the muscles

in response to the chronic spontaneous discharge of the dystrophic motoneurons.

The Extensor Carpi Radialis Longus and Brevis muscles of the forelimb are also fast-twitch muscles which contain Type IIA and Type IIB fibres. The dystrophic ECRL muscle seems to be more severely affected by the dystrophy than the ECRB muscle, as seen by the extent of connective tissue infiltration in the 4 month dystrophic ECRL (Fig. 40a, top half of micrograph). This difference between ECRL and ECRB has also been reported by Parry and Desypris (1984) with respect to the fibre cross-sectional area expressed as a percentage of total muscle area in (dy^{2J}) dystrophic mice aged 6 months.

The 2 month old dystrophic ECRL shows Type I-positive fibres (Fig. 40a, arrow) which also appear positive with the anti-embryonic antibody (Fig. 40d, arrow). Few, if any, regenerating fibres were seen beyond 4 months (Fig. 42d). It appears that the duration of the degeneration-regeneration period is shorter in the forelimb muscle(s) than in the muscles of the hindlimb. No slow myosin heavy chain containing muscle fibres were detected in the forelimb muscles of dystrophic mice at any age.

If the polyclonal antibodies used by Parry and Desypris (1983) were cross-reacting to embryonic myosin, then not only would positive fibres appear in the 6 month old dystrophic mouse AT muscle, but no positive fibre would have been detected in the forelimb muscles at 6 months, as embryonic myosin heavy chain containing fibres are not detected at this age with either the anti-Type I (BF 46) or the anti-embryonic (BF 45) myosin heavy chain monoclonal antibodies.

Two conclusions may be drawn from the immunohistochemical data for the fast-twitch muscles in (dy^{2J}) dystrophic mice. The first relates to the spontaneous discharge of motoneurons to hindlimb muscles. The loss of Type IIB fibres and the appearance of slow myosin heavy chain in the hindlimb muscles of older dystrophic mice is the result of this ab-

normal neural activity. Further evidence for this is seen in the forelimb muscles which show neither the presence of spontaneous neural discharge nor the appearance of slow myosin heavy chain. The second conclusion is that the slowing of the twitch in younger dystrophic mice is due partly to the presence of embryonic myosin in regenerating muscle fibres.

It remains to be seen whether the newly regenerating muscle fibres undergo an embryonic to adult slow transition in myosin heavy chain expression.

Structural isoforms of myosin

Based on the data obtained for the expression of the myosin light chains and heavy chains, the interpretation of the sodium pyrophosphate gel patterns of native myosin is somewhat simplified. In the case of normal Soleus muscle, where two native myosin bands are visible at all ages (SM and IM), it seems that there is an increase in the intensity of the SM band up to 8 months but that at 12 months the IM band (which was labelled FM because of insufficient electrophoretic separation from the FM isoforms) is equally as intense as the SM band. At all ages examined, the dystrophic mouse Soleus muscle had less slow myosin than the fast-migrating isoform. The SM band never disappeared completely, however, and at 12 months, though the bands were very faint, SM could still be detected in the Soleus muscle extract.

The loss of slow myosin from the (dy^{2J}) dystrophic mouse Soleus muscle is in direct contrast to the result of Fitzsimons and Hoh (1983) who reported the virtual disappearance of of the IM isoform in the (dy) dystrophic mouse Soleus. This result alone should caution against extrapolating observations made in the 129/ReJ dy/dy strain of dystrophic mice to the C57 BL/6J dy^{2J}/dy^{2J} strain. This difference likely represents the different time of onset and duration of the disease in these two models of dystrophy. Prolonged exposure of the Soleus to an intermittent pattern of stimulation is the likely cause for the loss of 'pure'

Type I fibres and the SM isoform in (dy^{2j}) dystrophic mice.

The lack of sensitivity of the sodium pyrophosphate gels in detecting low levels of native myosin isoforms is seen by the very faint SM band which appears in the 12 month old dystrophic ATox myosin extract. Though a considerable number of fibres containing slow myosin heavy chain were detected in the contralateral AT muscle (Fig. 26), and the amount of slow myosin light chains relative to the fast isoforms is quite high at this age (Fig. 6), the SM band is pale in comparison to the faster-migrating band. Nonetheless, this is an indication of the relative amount of slow myosin present in this region of the muscle. Because the light chain analysis of the native myosin bands was not performed, it is not possible to say whether the faster migrating band contained LC1s. Also, the separation between the fast myosin isoforms (FM) and intermediate myosin (IM) was not sufficient to allow the identification of the IM band in the dystrophic ATox.

In summary, the new findings reported here for the first time are the following:

a) Slow myosin light chains and heavy chain appear in the normally fast-twitch muscles of the hindlimb of dystrophic mice of the C57 Bl/6J dy^{2j}/dy^{2j} strain after age 8 months.

b) I have shown that embryonic-positive fibres are detected in these muscles up to age 8 months with a monoclonal antibody to embryonic myosin heavy chain which does not cross-react to slow myosin heavy chain. The slow-positive fibres reported by Parry and Desypris (1983) in the same muscles of dystrophic mice of younger age were most likely regenerating fibres with embryonic myosin heavy chain to which their polyclonal antibodies were cross-reacting.

c) The slow-twitch Soleus muscle of dystrophic mice (dy^{2j}) shows a disappearance of the SM native isoform as well as a concomitant loss of fibres containing only Type I myosin heavy chain.

In conclusion, the appearance of slow myosin light and heavy chains in the fast-twitch muscles of dystrophic mouse hindlimbs are surely both the result of spontaneous activity. However, the slowing of the isometric twitch must be the result of some other change in the excitation-contraction process, because the appearance of slow myosin does not occur prior to the reported change in contraction time. Embryonic myosin heavy chain in regenerating fibres is not likely to be the sole factor responsible for the slowing process, since the number of regenerating fibres is relatively small (<10%).

The approach to determining the central inherited flaw of dystrophy will be hindered until a more thorough understanding of the regulatory mechanisms which govern the genetic expression of skeletal muscle fibre types is obtained.

Appendix

Muscle Fibre Counts for Solei of Normal and Dystrophic Mice

Serial cross-sections of all Soleus muscles from animals aged 2, 4 and 6 months were used for fibre counts. Three sections for each muscle were used to count fibre type numbers. The three sections were labelled with BF 46, BF 34, and BF F3, respectively. Fibres which did not stain with the BF 34 monoclonal antibody (which stains all other fibre types except those fibres containing only the slow-specific myosin heavy chain) were counted as "pure" Type I (slow) fibres. Fibres which did not stain with the BF 46 antibody (that is, which did not contain slow myosin heavy chain) were counted as "pure" Type II (fast) fibres. Thus, the counting of negative fibres ensures that hybrid fibres containing both fast and slow myosin heavy chains are excluded and only pure types are identified. The number of hybrid fibres containing both Type I and Type II myosin heavy chain isoforms was obtained by subtracting the sum of both "pure" Type I and Type II fibres from the total number of fibres counted.

The Type IIB (fast fibres with IIB-specific myosin heavy chain) were counted from positive fibres labelled in the section with the BF F3 monoclonal antibody. The number of "pure" Type IIA fibres was obtained by subtracting the number of Type IIB-positive fibres from the number of "pure" Type II fibres. All Type IIB-positive fibres in the BF F3 section were found to correspond to negative fibres in the serial section labelled with BF 46 (the anti-slow myosin heavy chain monoclonal). These fibres do not contain slow myosin heavy chain. However, it cannot be determined from these sections whether they represent "pure" Type IIB fibres or are hybrid fibres containing IIA myosin heavy chain.

As these four categories of fibres are obtained by exclusion, the sum of fibres equals the total number counted. Consequently, for each fibre type, fibre counts were expressed as a percentage of total.

The data are depicted in the Appended Figure and are expressed as percentage values in the following tables. For each age group the sample arithmetic mean ($\bar{X}$), stantard deviation (s), and standard error of the mean (SEM) was calculated and used for graphic treatment.

Fibre Type Content of Soleus Muscles of Normal Mice aged 2, 4, and 6 Months

2 Month

Animal #	Type I	Type IIA	Hybrid (I+II)	Type IIB
1	28.7	53.6	24.9	0.00
2	33.0	48.6	14.1	4.30
3	36.1	49.6	14.3	0.00
n=3	$\bar{X}$=32.6	50.6	17.8	1.43
	s= 3.7162	2.6458	2.4826	6.1785
	SEM= 2.1455	1.5275	3.5671	1.4333

4 Month

Animal #	Type I	Type IIA	Hybrid (I+II)	Type IIB
1	48.0	50.8	1.20	0.00
2	34.7	60.7	2.20	2.40
3	46.4	47.7	5.90	0.00
4	28.6	71.4	0.00	0.00
n=4	$\bar{X}$=39.4	57.7	2.30	0.60
	s= 9.3397	10.7128	2.5474	1.200
	SEM= 4.6698	5.3564	1.2737	0.600

6 Month

Animal #	Type I	Type IIA	Hybrid (I+II)	Type IIB
1	60.7	37.9	0.00	1.40
2	56.4	32.5	10.0	1.10
3	34.8	65.2	0.00	0.00
4	43.6	47.6	0.00	8.80
n=4	$\bar{X}$=48.9	45.8	2.50	2.80
	s=11.8657	14.3631	5.0000	4.0285
	SEM= 5.9329	7.1816	2.5000	2.0143

Fibre Type Content of Soleus Muscles of Dystrophic Mice aged 2, 4, and 6 Months

2 Month

Animal #	Type I	Type IIA	Hybrid (I+II)	Type IIB
1	12.7	26.7	60.6	0.00
2	6.90	6.10	87.0	0.00
3	4.30	7.80	87.3	0.60
4	7.30	15.3	73.9	3.40
5	5.20	9.20	85.6	0.00
n=5	X=7.30	13.0	78.9	0.80
	s=3.2683	8.3974	11.6291	1.4765
	SEM=1.4616	3.7554	5.2007	0.6603

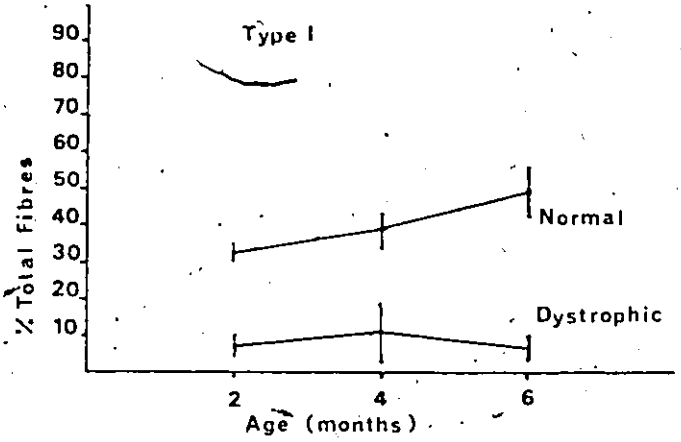
4 Month

Animal #	Type I	Type IIA	Hybrid (I+II)	Type IIB
1	28.0	11.2	57.2	3.70
2	3.50	39.6	47.6	9.30
3	1.20	26.0	68.8	4.00
n=3	X=10.9	25.6	57.9	5.70
	s=14.8540	14.2040	10.6160	3.1500
	SEM= 8.5760	8.2010	6.129	1.8190

6 Month

Animal #	Type I	Type IIA	Hybrid (I+II)	Type IIB
1	10.7	13.9	66.2	9.30
2	1.60	14.5	82.8	1.00
3	8.00	11.9	75.0	5.10
n=3	X=6.80	13.4	74.7	5.13
	s=4.6737	1.3614	8.3053	4.1501
	SEM=2.6984	0.7860	4.7949	2.3961

Per Cent Fibre Counts for Four Fibre Types in Soleus of Normal and Dystrophic Mice at Ages 2, 4, and 6 Months

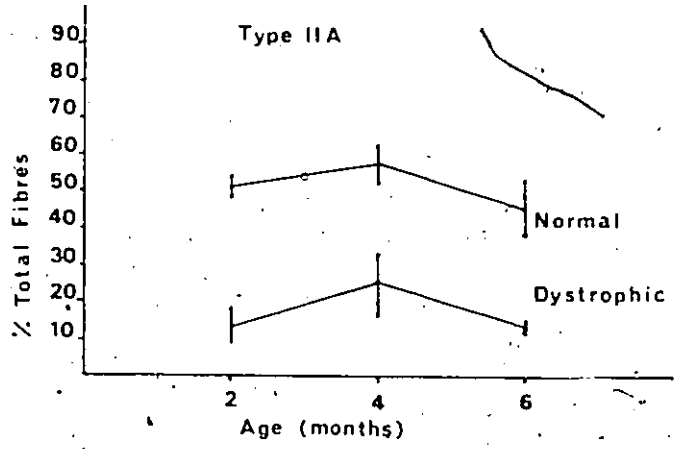


Significance of F

T: $p < 0.001$

A: $p < 0.267$

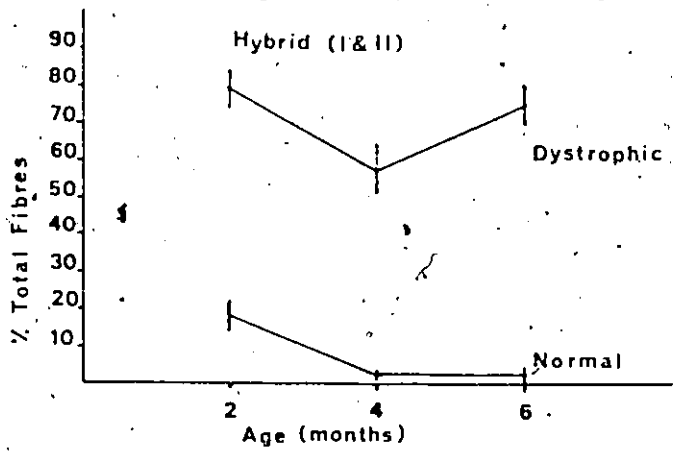
T x A: $p < 0.197$



T: $p < 0.001$

A: $p < 0.090$

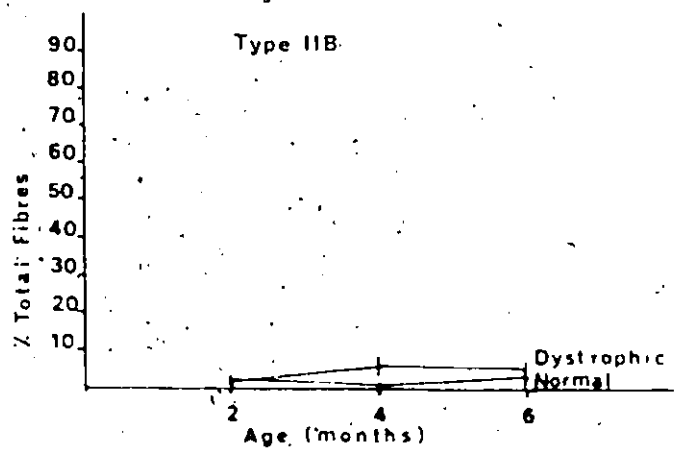
T x A: $p < 0.847$



T: $p < 0.001$

A: $p < 0.003$

T x A: $p < 0.192$



T: $p < 0.098$

A: $p < 0.119$

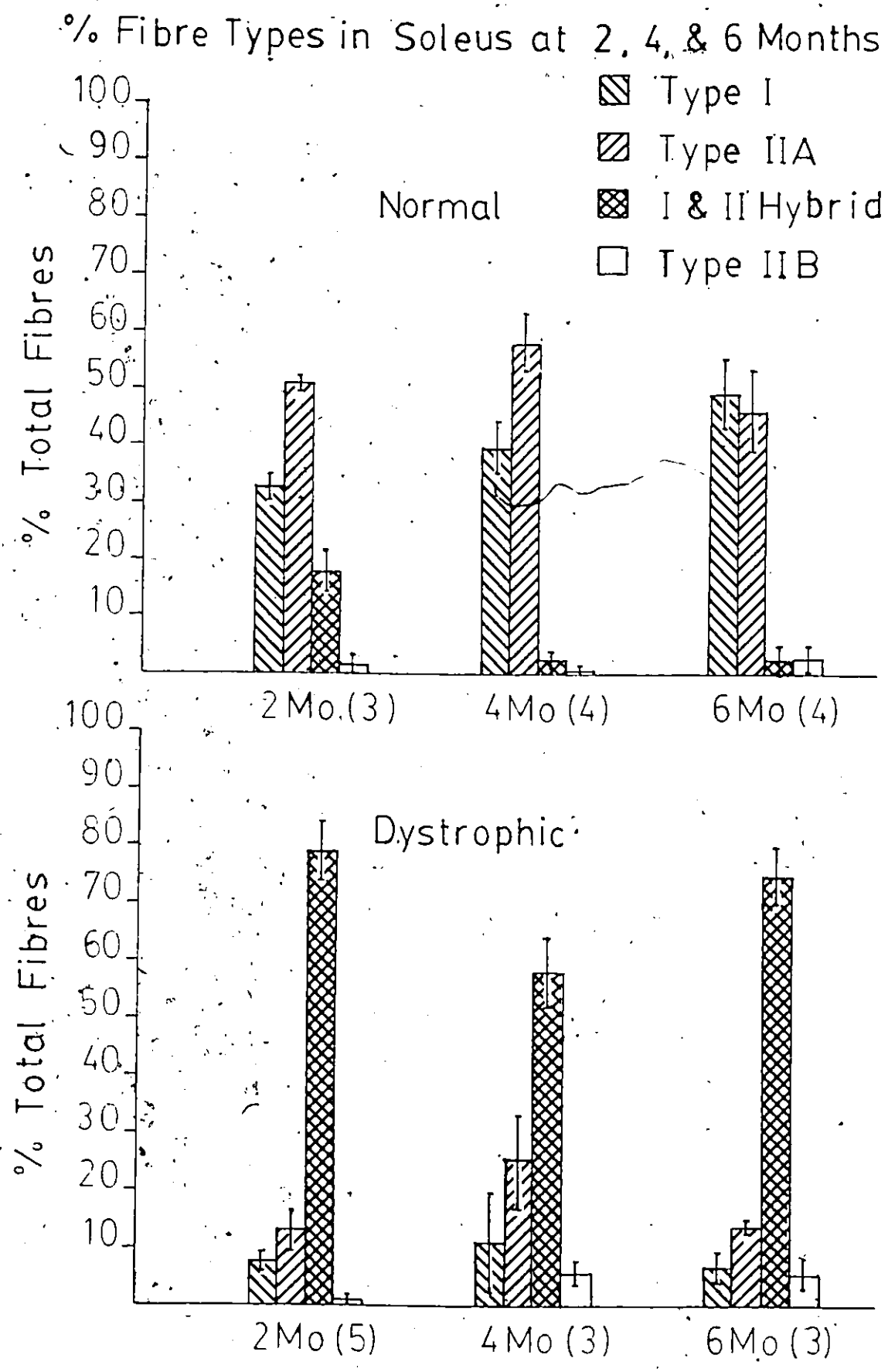
T x A $p < 0.194$

The data were analysed by a two-factor analysis of variance with unequal (disproportionate) replication using the SPSS-X program on the University of Ottawa Mainframe Computer. For an explanation of the appropriateness of this analysis, consult J. H. Zar (1984). For each fibre type, the percent values were treated for main effects of animal type (Factor A; i.e., whether normal or dystrophic) and animal age (Factor B; i.e., 2, 4, and 6 months) and any interaction between animal type and age (A x B interaction). The result of the four individual two-factor analyses of variance is summarized in the preceding figure (App. Fig. 1). The significance of the F ratio for main effects (T, animal type and A, age in months) as well as the interaction is inscribed next to each graph. All fibre types, except the Type IIB fibres, show an F ratio which is significant at the 0.001 level between normal and dystrophic mice. The Type IIB fibres appear to be more numerous in the Soleus of dystrophic mice than in that of normal mice, though the difference was not significant ($p<0.098$). In the case of age, Hybrid fibres showed an F ratio which was significant at the 0.003 level. The only other fibre type which showed an F ratio which approached significance was the Type IIA group ($p<0.099$). No significant interaction between animal type and age was obtained.

Since the main effect of animal type was expected to be significant, no further analysis was required. On the other hand, in the case of age, since an effect was observed for hybrid fibres, a posteriori testing was required to determine which of the two animal types (if not both) showed the main effect, as well as at which age a significant difference was observed between ages. The Tukey test for unequal sample sizes was used for multiple comparisons. In the case of normal mice, the "honestly significant difference test" (or Tukey test) gave values which were very nearly significant ($p<0.10$) between 2 months and 4 months, and 2 months and 6 months, but not at all significant between 4 and 6 months, as would be expected upon inspection of the graph. The dystrophic mice show a significant difference ($p<0.05$) between hybrid fibre values at 2 months and 4 months, near

significance between 6 months and 4 months ($p<0.10$), and no significance between 2 and 6 months.

Though the number of animals sampled at each age group was small, a clear distinction is seen between the Soleus of normal and dystrophic mice. Kugelberg (1976) has suggested a developmental transition from Type IIA to Type I fibres to explain the greater proportion of Type I fibres in older rat Soleus. Though a trend is apparent in the increase in Type I fibres in normal mice with increasing age, and a concomitant decrease in the proportion of Hybrid fibres is seen, the numbers do not warrant a definite conclusion as to the development of Soleus in the C57 Bl/6J mouse. On the other hand, if such a transition were occuring, this might explain the transient drop in hybrid fibres in dystrophic mice at 4 months. It is apparent from the graph that even at 2 months of age, the Soleus of dystrophic mice has already very few pure Type I fibres. The following bar graph (App. Fig. 2) gives a better idea of the interactive effects within a muscle.


% Fibre Types in Soleus at 2, 4, & 6 Months
Type I
Type IIA
I & II Hybrid
Type IIB
Normal
% Total Fibres
100
90
80
70
60
50
40
30
20
10
2 Mo.(3)
4 Mo (4)
6 Mo (4)
Dystrophic
% Total Fibres
2 Mo (5)
4 Mo (3)
6 Mo (3)

REFERENCES

Adams, R. D., 1975, Muscular Dystrophies in Diseases of Muscle. A study in pathology. third ed., Harper and Row, New York, pp. 262-315.

Asmundson, V. S., and L. M. Julian, 1956, Inherited muscle abnormality in the domestic fowl. J. Hered., 47, pp. 248-252.

Barany, K., and M. Barany, 1977, Phosphorylation of the 18,000-dalton light chain of myosin during a single tetanus of frog muscle. J. Biol. Chem., 252, pp. 4752-4754.

Barany, K., M. Barany, J. M. Gillis, and M. J. Kushmerick, 1979, Phosphorylation-dephosphorylation of the 18,000-dalton light chain of myosin during the contraction-regulation cycle of frog muscle. J. Biol. Chem., 254, pp. 3617-3623.

Barany, K., D. L. Vander Meulen, R. F. Ledvora, and M. Barany, 1982, Selective phosphorylation of myosin light chain in intact skeletal muscle. Arch. Biochem. Biophys., 217, pp. 392-396.

Barany, M., K. Barany, T. Reckard, and A. Volpe, 1965, Myosin of fast and slow muscles of the rabbit. Arch. Biochem. Biophys., 109, pp. 185-

Barany, M., 1967, ATPase activity of myosin correlated with the speed of muscle shortening. J. Gen. Physiol., 50 (suppl. part 2), pp. 197-218.

Barany, M., and R. I. Close, 1971, The transformation of myosin in cross-innervated rat muscles. J. Physiol. (Lond.), 213, pp. 455-474.

Barton, P. J. R., A. Cohen, B. Robert, M. Y. Fiszman, F. Bonhomme, J. L. Guenet, D. P. Leader, and M. E. Buckingham, 1985, The myosin alkali light chains of mouse ventricular and slow skeletal muscle are indistinguishable and are encoded by the same gene., J. Biol. Chem., 260, pp. 8578-8584.

Betto, R., and G. Salviati, 1985, Myosin light chains in human muscle diseases. J. Muscle Res. Cell Mot., 6, p. 66.

Billeter, R., C. Heizmann, and E. Jenny, 1980, Myosin light chains in single fibres of human skeletal muscle: evidence for a hybrid myosin in type I fibres. J. Muscle Res. Cell Mot., 1, p. 480.

Billeter, R., C. W. Heizmann, H. Howald, and E. Jenny, 1981, Analysis of myosin light chain and heavy chain types in single human skeletal muslce fibres. Eur. J. Biochem., 116, pp. 389-395.

Billeter, R., C. W. Heizmann, U. Reist, H. Howald, and E. Jenny, 1982, Two-dimensional peptide analysis of myosin heavy chains and actin from single-typed human skeletal muscle fibres. FEBS Lett., 139, pp. 45-48.

Biral, D., E. Damiani, A. Margreth, and E. Scarpini, 1984, Myosin subunit composition in human developing skeletal muscle. Biochem. J., 224, pp. 923-931.

Bourne, J. A., 1983, Handbook of Immunoperoxidase Staining methods, Dako Corporation, Santa Barbara, California, USA, 37p.

Bradford, M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72, pp. 248-254.

Bradley, W. G., and M. Jenkinson, 1974, Abnormalities of peripheral nerve in murine muscular dystrophy. J. Neurol. Sci., 18, pp. 227-247.

Bray, G. M., and A. J. Aguayo, 1975, Quantitative ultrastructural studies of the axon-Schwann cell abnormality in spinal nerve roots from dystrophic mice. J. Neuropathol. Exp. Neurol., 34, pp. 517-530.

Bressler, B. H., L. G. Jasch, W. K. Ovalle, and C. E. Slonecker, 1983, Changes in isometric contractile properties of fast-twitch and slow-twitch skeletal muscles of C57 BL/6J dy^{2J}/dy^{2J} dystrophic mice during development. Exp. Neurol., 80, pp. 457-470.

Brooke, M. H., and K. K. Kaiser, 1970, Muscle fiber types: how many and what kind? Arch. Neurol., 23, pp. 369-379.

Browne, W. E., S. Salmons, and R. G. Whalen, 1983, The sequential repalcement of myosin subunit isoforms during muscle type transformation induced by long term electrical stimulation. J. Biol. Chem., 258, pp. 14686-14692.

Brust, M., 1966, Relative resistance to dystrophy of slow skeletal muscle of the mouse. Am. J. Physiol., 210, pp. 445-451.

Bulfield, G., W. G. Siller, P. A. L. Wright, and K. J. Moore, 1984, X chromosome linked muscular dystrophy (mdx) in the mouse. Proc. Natl. Acad. Sci. USA, 81, pp. 1189-1192.

Buller, A. J., J. C. Eccles, and R. E. Eccles, 1960, Differentiation of fast and slow muscles in the cat hindlimbs. J. Physiol., 150, pp. 399-416.

Buller, A. J., W. F. H. M. Mommaerts, and K. Seraydarian, 1969, Enzymic properties of myosin in fast and slow twitch muscles of the cat following cross-innervation. J. Physiol. (Lond.), 205, pp. 581-597.

Burke, R. E., D. N. Levine, P. Tsairis, and F. E. Zajac, 1973, Physiological types and histochemical profiles in motor units of the cat gastrocnemius. J. Physiol. (Lond.), 234, pp. 723-748.

Butler, J., and E. Cosmos, 1977, Histochemical and structural analyses of the phenotypic expression of the dystrophic gene in the 129/ReJ dy/dy and the C57 BL/6J dy^{2j}/dy^{2j} mice. Exp. Neurol., 57, pp. 666-681.

Butler-Browne, G. S., and R. G. Whalen, 1984, Myosin isoenzyme transitions occuring during the postnatal development of the rat soleus muscle. Dev. Biol., 102, pp. 324-334.

Canki, N., B. Dutrillaux, and I. Tivador, 1979, Dystrophie musculaire de Duchenne chez une petite fille porteuse d'une translocation t(X;3) (p21;q13) de novo. Ann. Genetique, 22, pp. 35-39.

Carraro, U., and C. Catani, 1983, A sensitive SDS-PAGE method separating myosin heavy chain isoforms of rat skeletal muscles reveals the heterogenous nature of embryonic myosin. Biochem. Biophys. Res. Comm., 116, pp. 793-803.

Carraro, U., and C.Catani, 1984, Slow myosin light chains accumulate in direct low-frequency electrostimulation of the denervated rat EDL. J. Europ. Soc. Artif. Organs, 2 (suppl. 1), pp. 49-51.

Carroll, J. E., and M. H. Brooke, 1978, Pathophysiology of diseases affecting muscle. in Neurological Pathophysiology, (Eliasson, S. G., A. L. Prensky, and W. B. Hardin, eds.) Oxford University Press, New York, pp. 83-108.

Chizzonite, R. A., A. W. Everett, W. A. Clark, S. Jakovcic, M. Rabinovitz, and R. Zak, 1982, Isolation and characterization of two molecular variants of myosin heavy chain from rabbit ventricle. Change in their content during normal growth and after treatment with thyroid hormone. J. Biol. Chem., 257, pp. 2056-2065.

Clark, W. A., R. A. Chizzonite, A. W. Everett, M. Rabinovitz, and R. Zak, 1982, Species correlations between cardiac isomyosins. A comparison of electrophoretic and immunological properties. J. Biol. Chem., 257, pp. 5449-5454.

Close, R., 1965, Force-velocity properties of mouse muscles. Nature, 206, pp. 71-719.

Czosneck, H., U. Nudel, M. Shani', P. E. Barker, D. D. Pravtcheva, F. H. Ruddle, and D. Yaffe, 1982, The genes coding for muscle contractile proteins, myosin heavy chain, myosin light chain 2, and skeletal muscle actin are located on three different mouse chromosomes. The EMBO J., 1, pp. 1299-1305.

d'Albis, A., C. Jannot, J. Weinman, and C. L. Gallien, 1984, Myosin isoenzymic modification at critical stages of development of mice and urodelan amphibians. in Recent advances on contractile proteins (Alia, E. E., N. Arena, and M. A. Russo, eds.), Praeger Scientific, New York, (in press).

d'Albis, A., C. Jannot, and J.-J. Bechet, 1985, Myosin switches in skeletal muscle development of an urodelan amphibian, Pleurodeles waltii. Comparison with a mammalian, Mus musclulus. Biochem. Biophys. Res. Comm., 128, pp. 94-100.

Dalla Libera, L., A. Margreth, I. Mussini, C. Cerri, and G. Scarlato, 1978, Myosin polymorphism im human skeletal muscles. Muscle Nerve, 1, pp. 280-291.

Danielli Betto, D. and R. Betto, 1985, Myosin heavy chains in typed single muscle fibres. in Cell Biology and Clinical Management in Functional Electrostimulation of Neurones and Muscles. Satellite Symposium of the XIIIth World Congress of Neurology, Abano Terme, Italy.

Dangain, J., and G. Vrbova, 1984, Muscle development in mdx mouse. Muscle Nerve, 7, pp. 700-704.

Dhoot, G. K., 1983, Differentiation of skeletal muscle fibre types in the rat foetus. J. Physiol., 345, 53p.

Dhoot, G. K., 1985, Initiation of differentiation into skeletal muscle fibre types. Muscle Nerve, 8, pp. 307-316.

Dhoot, G. K., and S. V. Perry, 1980, The components of the troponin complex and development in skeletal muscle. Exp. Cell Res., 127, pp. 75-87.

Dhoot, G. K, and S. V. Perry, 1982, Changes in the forms of the components of the troponin complex during regeneration of injured skeletal muscle. Muscle Nerve, 5, pp. 39-47.

Dhoot, G. K., and S. V. Perry, 1983, Effect of denervation of skeletal muscle cell types in the rat. Exp. Neurol., 82, pp. 131-142.

Dhoot, G. K., S. V. Perry, and G. Vrbova, 1981, Changes in the distribution of the components of the troponin complex in muscle fibres after cross-innervation. Exp. Neurol., 72, pp. 513-530.

Dillman, W., 1980, Diabetes mellitus induces changes in cardiac myosin of the rat. Diabetes, 29, pp. 579-582.

Douglass, W. B., Jr., and R. J. Baskin, 1971, Contractile properties of developing mouse dystrophic muscle. Am. J. Physiol., 220, pp. 1344-1354.

Drachman, D. B., and D. M. Johnston, 1973, Development of a mammalian fast muscle: dynamic and biochemical properties correlated. J. Physiol. (Lond.), 232, pp. 29-42.

Dreizen, P., L. C. Gershman, P. P. Trotta, and A. Stracher, 1967, Myosin. Subunits and their interactions. J. Gen. Physiol., 50 (suppl. 1), pp. 85-118.

Dreizen, P., and L. C. Gershman, 1970, Relationship of structure to function in myosin. II. Salt denaturation and recombination experiments. Biochemistry, 9, pp. 1688-1693.

Dribin, L., and S. Simpson, 1977, Histochemical and morphological study of dystrophic (C57 BL/6J dy^{2J}/dy^{2J}) and normal muscle. Exp. Neurol., 56, pp. 480-497.

Dubuisson, M., 1954, Muscular contraction American Lecture Series, No. 144, W. Bladergroen, ed., Charles C. Thomas, publ., Sprinfield, Illinois, 243 p.

Duchenne, G. B., 1855, De l'electrisation localisee, et de son application a la pathologie et a la therapeutique. J. B. Balliere, ed., Paris, 800p.

Ebrecht, G. H., R. Rupp, and J. Jacob, 1982, Alterations of mechanical parameters in chemically skinned preparations of rat myocardium as a function of isoenzyme pattern of myosin. Basic Res. Cardiol., 77, pp. 220-234.

Emmanuel, B. S., E. H. Zackai, and S. W. Tucker, 1983, Further evidence for Xp21 location of Duchenne musclular dystrophy (DMD) locus: X;9 translocation in a female with DMD. J, Med. Genet., 20, pp. 461-463.

Engelhardt, V. A., and M. N. Lyubimova, 1939, Myosin and adenosinetriphosphatase. Nature, 144, pp. 668-669.

Erb, W. H., 1891, Dystrophia muscularis progressiva: klinische und patologieanatomische Studien. Deutsche Z. Nervenheilk., 1, pp. 13-49.

Everett, A. W., W. A. Clark, R. A. Chizzonite, and R. Zak, 1983, Change in synthesis rates of alpha- and beta-myosin heavy chains in rabbit heart after treatment with thyroid hormone. J. Biol. Chem., 258, pp. 2421-2425.

Everett, A. W., R. A. Chizzonite, W. A. Clark, and R. Zak, 1982, Cardiac isomyosins: Influence of thyroid hormone on their distribution and rate of synthesis. in Muscle development: Molecular and cellular control (Pearson, M. L., and A. F. Epstein, eds), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 35-41.

Fitzsimons, R. B., and J. F. Y. Hoh, 1981a, Isomyosins in human Type I and Type II skeletal muscle fibres. Biochem. J., 193, pp. 229-233.

Fitzsimons, R. B., and J. F. Y. Hoh, 1981b, Embryonic and foetal myosin in human skeletal muscle. The presence of foetal myosins in Duchenne muscular dystrophy and infantile spinal muscular atrophy. J. Neurol. Sci., 52, pp. 367-384.

Fitzsimons, R. B., and J. F. Y. Hoh, 1983, Myosin isoenzymes in fast-twitch and slow-twitch muscles of normal and dystrophic mice. J. Physiol., 343, pp. 539-550.

Flink, I. L., J. H. Rader, and E. Morkin, 1979, Thyroid hormone stimulates synthesis of a cardiac myosin isozyme. Comparison of the two-dimensional electrophoretic patterns of the cyanogen bromide peptides of cardiac myosin heavy chains from euthyroid and thyrotoxic rabbits. J. Biol. Chem., 254, pp. 3105-3110.

Frank, G., and A. G. Weeds, 1974, The amino acid sequence of the alkali light chains of rabbit skeletal-muscle myosin. Eur. J. Biochem., 44, pp. 317-334.

Gaetjens, E., K. Barany, G. Bailin, H. Oppenheimer, and M. Barany, 1968, Studies of the low molecular weight protein components in rabbit skeletal myosin. Arch. Biochem. Biophys., 132, pp. 82-96.

Gambke, B., G. E. Lyons, J. Haselgrove, A. M. Kelly, and N. A. Rubinstein, 1983, Thyroidal and neural control of myosin transitions during development of rat fast and slow muscles. FEBS Lett., 156, pp. 335-339.

Gambke, B., and N. A. Rubinstein, 1984, A monoclonal antibody to the embryonic myosin heavy chain of rat skeletal muscle. J. Biol. Chem., 259, pp. 12092-12100.

Gazith, J., S. Himmelfarb, and W. F. Harrington, 1970, Studies on the subunit structure of myosin. J. Biol. Chem., 245, pp. 15-22.

Gergely, J., 1950, Relation of ATPase and myosin. Fed. Proc., 9, p. 176.

Gergely, J., 1953, Studies on myosin-adenosinetriphosphatase. J. Biol. Chem., 200, pp. 543-550.

Gershman, L. C., A. Stracher, and P. Dreizen, 1969, Subunit structure of myosin III. A proposed model for rabbit myosin. J. Biol. Chem., 244, pp. 2724-2736.

Green, H. J., H. Reichmann, and D. Pette, 1983, Fibre type specific transformations in the enzyme activity pattern of rat vastus lateralis muscle by prolonged endurance training. Pflugers Archiv, 399, pp. 216-222.

Green, H. J., G. A. Klug, H. Reichmann, U. Seedorf, W. Weihrer, and D. Pette, 1984, Exercise induced fibre type transisitons with regard to myosin, parvalbumin, and sarcoplasmic reticulum in muscles of the rat. Pflugers Archiv, 400, pp. 432-438.

Greenstein, R. M., M. P. Reardon, and T. S. Chan, 1977, An X/autosome translocation in a girl with Duchenne muscular dystrophy (DMD): Evidence for DMD gene localization. Pediatr. Res., 11, p. 457 (abstr.).

Gustafson, T. A., B. E. Markham, and E. Morkin, 195, Analysis of thyroid hormone effects on myosin heavy chain gene expression in cardiac and soleus muscles using a novel dot-blot mRNA assay. Biochem. Biophys. Res. Comm., 130, pp. 1161-1167.

Guth, L., and F. J. Samaha, 1969, Qualitative differences between actomyosin ATPase of slow and fast mammalian muscle. Exp. Neurol., 25, pp. 13-152.

Hanson, J., and H. E. Huxley, 1953, Structural basis of the cross-striations in skeletal muscle. Nature, 172, pp. 530-532.

Harris, J. B., and P. Wilson, 1971, Mechanical properties of dystrophic mouse muscle. J. Neurol. Neurosurg. Psychiat., 34, pp. 512-520.

Harris, J. B., and A. Montgomery, 1975, Some mechanical and electrical properties of distal hind limb muscles of genetically dystrophic mice (C57 BL/6J dy^{2j}/dy^{2j}). Exp. Neurol. 48, pp. 569-585.

Heeley, D. H., G. K. Dhoot, N. Frearson, S. V. Perry, and G. Vrbova, 1983, The effect of cross-innervation on the tropomyosin composition of rabbit skeletal muscle. FEBS Lett., 152, pp. 282-285.

Heeley, D. H., G. K. Dhoot, and S. V. Perry, 1985, Factors determining the subunit composition of tropomyosin in mammalian skeletal muscle. Biochem. J., 226, pp. 461-468.

Hoh. J. F. Y., 1978, Light chain distribution of chicken skeletal muscle myosin isoenzymes. FEBS Lett., 90, pp. 297-300.

Hoh, J. F. Y., P. A. McGrath, and P. T. Hale, 1978, Electrophoretic analysis of multiple forms of rat cardiac myosin: effects of hypophysectomy and thyroxine replacement. J. Mol. Cell. Cardiol., 10, pp. 1053-1076.

Hoh, J. F. Y., P. A. McGrath, and R. I. White, 1976, Electrophoretic analysis of multiple forms of myosin in fat-twitch and slow-twitch muscles of the chick. Biochem. J., 157, pp. 87-95.

Hoh, J. F. Y., and G. P. S. Yeoh, 1979, Rabbit skeletal myosin isoenzymes from fetal, fast-twitch and slow-twitch muscles. Nature, 280 pp. 321-323.

Hoh, J. F. Y., G. P. S. Yeoh, M. A. W. Thomas, and L. Higginbottom, 1979, Structural differences in the heavy chains of rat ventricular myosin isoenzymes. FEBS Lett., 97, pp. 330-334.

Homburger, F., J. R. Baker, C. W. Nixon, and R. Whitney, 1962, Primary, generalized polymyopathy and cardiac necrosis in an inbred line of Syrian hamster. Medicina Exp. (Basel), 6, pp. 339-345.

Huxley, A. F., and R. Niedergerke, 1954, Structural changes in muscle during contraction. Nature, 173, pp. 971-973.

Huxley, H. E., 1953, Electron microscope studies of the organization of the filaments in striated muscle. Biochim. Biophys. Acta, 12, pp. 387-394.

Huxley, H. E., and J. Hanson, 1954, Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. Nature, 173, pp. 978-987.

Huxley, H. E., and J. Hanson, 1960, The molecular basis of contraction in cross-striated muscles. in Structure and Function of Muscle G. H. Bourne, ed., vol. 1, Chapter 7, Academic Press, New York.

Jacobs, P. A., P. A. Hunt, M. Mayer, and R. D. Bart, 1981, Duchenne muscular dystrophy (DMD) in a female with an X/autosome translocation: Further evidence that the DMD locus is at Xp21. Am. J. Hum. Genet., 33, pp. 513-51.

Jasch, L. G., and E. H. Moase, 1985, Evidence for the identities of five proteins decreased in skeletal muscle of dystrophic mice. Muscle Nerve, 8, pp. 389-401.

Jolesz, F., and F. A. Sreter, 1981, Development, innervation, and activity-pattern induced changes in skeletal muscle. Ann. Rev. Physiol., 43, pp. 531-552.

Jones, S. P., M. A. P. Ridge, and A. Rowlerson, 1985, The number and size of slow muscle fibres in a fast muscle during post-natal development of the rat. J. Physiol., 360, p. 78P.

Kominz, D. R., W. R. Carroll. E. N. Smith, and E. R. Mitchell, 1959, A subunit of myosin. Arch. Biochem. Biophys., 79, pp. 191-199.

Korecky, B. and M. Beznack, 1971, Effect of thyroxine on growth and function of cardiac muscle. in Cardiac hypertrophy, N. R. Alpert, ed., Academic Press, New York, pp. 55-64.

Korecky, B., R. Zak, and K. Schwartz, 1986, Role of thyroid hormone on selective synthesis of cardiac isomyosin. J. Mol. Cell. Cardiol., 18, Suppl. 1, 161 (abstr.).

Krebs, E. G., R. J. DeLange, R. G. Kemp, and W. D. Riley, 1969, Activation of skeletal muscle phosphorylase. Pharmacol. Rev., 18, pp. 163-171.

Kugelberg, E. 1976, Adaptive transformation of rat soleus motor units. J. Neurol. Sci., 27, pp. 269-289.

Kushmerick, M. J., and M. T. Crow, 1982, Chemical energetics, mechanics, and phosphorylation of regulatory light chains in mammalian fast- and slow-twitch mucles. in Basic Biology of Muscle, Twarog, B. M., R. J. C. Levine, M. M. Dewey, eds, Raven Press, New York, pp. 159-172.

Laemmli, U. K., 1970, Cleavage of structural of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, pp. 680-685.

Landouzy, L., and J. Dejerine, 1884, De la myopathie atrophique progressive (myopathie hereditaire) debutant, dans l'enfance, par la face, sans alteration du systeme nerveux. C. R. Acad. Sci. (Paris), 98, pp. 53-55.

Lane, P. W., T. C. Beamer, and D. D. Meyers, 1976, Myodystrophy, a new myopathy on chromosome 8 of the mouse. J. Hered., 67, pp. 135-138.

Lewis, D. M., D. J. Parry, and A. Rowlerson, 1982, Isometric contractions of motor units and immunohistochemistry of mouse soleus muscle. J. Physiol., 325, pp. 393-401.

Lindenbaum, R. H., G. Clarke, C. Patel, C. Moncrieff, and J. T. Hughes, 1979, Muscular dystrophy in an X;1 translocation female suggests that Duchenne locus is on X chromosome short arm. J. Med. Genet., 16, pp 389-392.

Linn, T. C., F. H. Petit, and L. J. Reed, 1969, Alpha-keto acid dehydrogenase complexes X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation. Proc. Natl. Acad. Sci. USA, 62, pp. 234-241.

Lompre, A.-M., K. Schwartz, A. d'Albis, G. Lacombe, N. Van Thiem, and B. Swinghedauw, 1979, Myosin isoenzyme redistribution in chronic heart overload. Nature, 282, pp. 105-107.

Lompre, A.-M., J.-J. Mercadier, C. Wisnewsky, P. Bouveret, C. Pantaloni, A. d'Albis, and K. Schwartz, 1981, Species and age-dependent changes in the relative amounts of cardiac isoenzymes in mammals. Devel. Biol., 84, pp. 286-290.

Lompre, A.-M., B. Nadal-Ginard, and V. Mahdavi, 1984, Expression of the cardiac ventricular alpha- and beta-myosin heavy chain genes is developmentally and hormonally regulated. J. Biol. Chem., 259, pp. 6437-6446.

Lowey, S., and C. Cohen, 1962, Studies on the structure of myosin. J. Mol. Biol., 4, pp. 293-308.

Lowey, S., L. Goldstein, C. Cohen, and S. M. Luck, 1967, Proteolytic degradation of myosin and the meromyoins by a water-insoluble polyanionic derivative of Trypsin. Properties of a helical subunit isolated from heavy meromyosin. J. Mol. Biol., 23, pp. 287-304.

Lowey, S., and D. Risby, 1971, Light chains from fast and slow muscle myosins. Nature, 34, pp. 81-85.

Lowey, S., H. S. Slayter, A. G. Weeds, and H. Baker, 1969, Substructure of the myosin molecule I. Subfragments of myosin by enzymatic degradation. J. Mol. Biol., 42, pp. 1-29.

Lyons, G. E., J. Haselgrove, A. M. Kelly, and N. A. Rubinstein, 1983, Myosin transitions in developing fast and slow muscles of the rat hindlimb. Differentiation, 25, pp. 168-175.

Mabuchi, K., D. Szvetko, K. Pinter, and F. A. Sreter, 1982, Type IIB to IIA fiber transformation in intermittently stimulated rabbit muscles. Am. J. Physiol., 242, pp. C373-C381.

Mabuchi, K., K. Pinter, Y. Mabuchi, F. Sreter, and J. Gergely, 1984, Characterization of rabbit masseter muscle fibres. Muscle Nerve, 7, pp. 431-438.

Mahdavi, V., M. Periassamy, and B. Nadal-Ginard, 1982, Molecular characterization of two myosin heavy chain genes expressed in the adult heart. Nature, 297, pp. 659-664.

Malhorta, A., S. Penpargkul, F. S. Fein, E. M. Sonnenblick, and J. Scheuer, 1981, The effect of streptozotocin-induced diabetes in rats on cardiac contractile proteins. Circ. Res., 49, pp. 1243-1250.

Manning, D. R., and J. T. Stull, 1979, Myosin light chain phosphorylation and phosphorylase activity in rat extensor digitorum longus muscle. Biochem. Biophys. Res. Comm., 90 pp. 164-170.

Margreth, M. D., L. Dalla Libera, G. Salviati, and N. Ischia, 1980, Spinal transection and the postnatal differentiation of slow myosin isoenzymes. Muscle Nerve, 3, pp. 483-486.

Martin, A. F., E. D. Pagani, and R. J. Solaro, 1982, Thyroxine-induced redistribution of isoenzymes of rabbit ventricular myosin. Circ. Res., 50, pp. 117-124.

Masaki, T., and C. Yoshizaki, 1974, Differentiation of myosin in chick embryos. J. Biochem., 76, pp. 123-131.

Mastaglia, F. L., and B. Kakulas, 1969, Regeneration in Duchenne muscular dystrophy- A histological and histochemical study. Brain, 92, pp. 809-818.

Mastaglia, F. L., J. M. Papadimitriou, and B. A. Kakulas, 1970, Regeneration of muscle in Duchenne muscular dystrophy- An electron microscope study. J. Neurol. Sci., 11, pp. 425-444.

Meier, H., and J. L. Southard, 1970, Muscular dystrophy in the mouse caused by an allele at the dy-locus. Life Sci., 9, (part 2), pp. 137-144.

Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert, 1981, Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science, 211, pp. 1437-1438.

Michelson, A. M., E. S. Russel, and P. J. Harman, 1955, Dystrophia muscularis: a primary myopathy in the house mouse. Proc. Natl. Acad. Sci. USA, 41, pp. 1079-1084.

Mihalyi, E., 1953, Trypsin digestion of muscle proteins II. The kinetics of the digestion. J. Biol. Chem., 201, pp. 197-209.

Mihalyi, E., and A. G. Szent-Gyorgyi, 1953a, Trypsin digestion of muscle proteins I. Ultracentrifugal analysis of the process. J. Biol. Chem., 201, pp. 189-196.

Mihalyi, E., and A. G. Szent-Gyorgyi, 1953b, Trypsin digestion of muscle proteins III. Adenosinetriphosphatase activity of the digested myosin. J. Biol. Chem., 201, pp. 211-219.

Moore, R. L., and J. T. Stull, 1984, Myosin light chain phosphorylation in fast and slow skeletal muscles in situ. Am. J. Physiol., 247, pp. C462-C471.

Morrissey, J. H., 1981, Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniformm sensitvitiy. Anal. Biochem., 117, pp. 307-310.

Mueller, H., and S. V. Perry, 1961, The chromatography of the meromyosins of diethylaminoethyl-cellulose. Biochem. J., 80, pp. 217-223.

Nabeshima, Y., Y. Fujii-Kuriyama, M. Muramatsu, and K. Ogata, 1984, Alternative transcription and two modes of splicing result in two myosin light chains from one gene. Nature, 308, pp. 333-338.

Nemeth, P., D. Pette, and G. Vrbova, 1981, Comparison of enzyme activities among single muscle fibres within defined motor units. J. Physiol., 311, pp. 489-495.

O'Farrell, P. H., 1976, High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem., 250, pp. 4007-4021.

Obinata, T., H. Takano-Ohmuro, and R. Matsuda, 1980, Changes in troponin-T and myosin isoenzymes during development of normal and dystrophic chicken muscle. FEBS Lett., 120, pp. 195-198.

Ovalle, W. K., B. H. Bressler, L. G. Jasch, and C. E. Slonecker, 1983, Abnormal distribution of fibre types in the slow-twitch soleus muscle of the C57 BL/6J dy^{2j}/dy^{2j} dystrophic mouse during post-natal development. Amer. J. Anat., 168, pp. 291-304.

Parry, D. J., 1977, A study of functional denervation in fast and slow muscles of dystrophic mice of various ages. Exp. Neurol., 55, pp. 556-566.

Parry, D. J., and G. Desypris, 1983, Slowing of twitch of dystrophic mouse muscle is partially due to altered activity pattern. Muscle Nerve, 6, pp. 397-407.

Parry, D. J., and G. Desypris, 1984, Succinic dehydrogenase activity of forelimb and hindlimb muscles of the dystrophic mouse. Can. J. Physiol. Pharmacol., 62, pp. 905-911.

Parry, D. J., and H. G. Parslow, 1981, Fiber type susceptibility in the dystrophic mouse. Exp. Neurol., 73, pp. 674-685.

Parry, D. J., and A. Stewart, 1985, A comparison of myosin isoenzymes in hindlimb and forelimb muscles of normal and genetically dystrophic (C57 Bl/6J dy^{2j}/dy^{2j}) mice. J. Muscle Res. Cell Mot., 6, p. 78.

Parslow, H. G., and D. J. Parry, 1981, Slowing of fast-twitch muscle in the dystrophic mouse. Exp. Neurol., 73, pp. 686-699.

Periassamy, M., E. E. Strehler, L. I. Garfinkel, R. M. Gubits, N. Ruiz-Opazo, and B. Nadal-Ginard, 1984, Fast skeletal muscle light chains 1 and 3 are produced from a single gene by a combined process of differential RNA transcription and splicing. J. Biol. Chem., 259, pp. 13595-13604.

Perrie, W. T., and S. V. Perry, 1970, An electrophoretic study of the low-molecular weight components of myosin. Biochem. J., 119, pp. 31-38.

Perrie, W. T., L. B. Smillie, and S. V. Perry, 1973, A phosphorylated light-chain component of myosin from skeletal muscle. Biochem. J., 135, pp. 151-164.

Perrie, W. T., S. J. Bumford, and K. J. Rochelle, 1982, Electrophoretic separation of myosin heavy chains from fast and slow muscle. Biochem. Soc. Trans., 10, p. 219.

Perrie, W. T., and S. J. Bumford, 1984, Correlation of myosin heavy chains with ATPase staining of skeletal- and cardiac-muscle fibres. Biochem. Soc. Trans., 12, pp. 825-826.

Perry, S. V., H. A. Cole, O. Hudlicka, V. B. Patchell, and S. A. Westwood, 1984, Role of myosin light chain kinase in muscle contraction. Feder. Proc., 43, pp. 3015-3020.

Pette, D., M. E. Smith, H. W. Standte, and G. Vrbova, 1973, Effects of long-term electrical stimulation of some contractile and metabolic characteristics of fast rabbit muscles. Pflugers Arch., 338, pp. 257-272.

Pette, D. B. U. Ramirez, W. Muller, R. Simon, G. U. Exner, and R. Hildebrand, 1975, Influence of intermittent long-term stimulation on contractile, histochemical and metabolic properties of fibre populations in fast and slow muscles. Pflugers Arch., 361, pp. 1-7.

Pette, D., W. Muller, E. Leisner, and G. Vrbova, 1976, Time-dependent effects on contractile properties, fibre population, myosin light chains and enzymes of energy metabolism in intermittently and continuously stimulated fast twitch muscles of the rabbit. Pflugers Archiv, 369, pp. 103-112.

Pette, D., and U. Schnez, 1977, Coexistence of fast- and slow type myosin light chains in single muscle fibres during transformation as induced by long term stimulation. FEBS Lett., 83, pp. 128-130.

Portzehl, V. H., G. Schramm, and H. H. Weber, 1950, Aktomyosin und seine Komponenten. I. Mitt. Z. Naturforschung, 5b, pp. 61-74.

Rayburn, M. B., and A. C Peterson, 1978, Naked axons in myodystrophic mice. Brain Res., 146, pp. 380-384.

Reisner, A. H., P. Nemes, and C. Bucholtz, 1975, The use of Coomassie Brilliant Blue G-250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. Anal. Biochem., 64, pp. 509-516.

Roy, R. K., K. Mabuchi, S. Sarkar, C. Mio, and F. A. Sreter, 1979, Changes in the tropomyosin subunit pattern in chronically electrically stimulated rabbit fast muscle. Biochem. Biophys. Res. Comm., 89, pp. 181-187.

Rubinstein, N., K. Mabuchi, F. Pepe, S. Salmons, J. Gergely, and F. Sreter, 1978, Use of type-specific antimyosins to demonstrate the transformation of individual fibres in chronically stimulated rabbit fast muscles. J. Cell Biol., 79, pp. 252-261.

Rubinstein, N. A., and A. M. Kelly, 1981, Development of muscle fibre specialization in the rat hindlimb. J. Cell Biol., 90, pp. 128-144.

Salmons, S., and F. A. Sreter, 1976, Significance of impulse activity in the transformation of skeletal muscle types. Nature, 263, pp. 30-34.

Salmons, S., And G. Vrbova, 1969, The influence of activity on some contractile characteristics of mammalian fast and slow muscles. J. Physiol., 201, pp. 535-549.

Sarkar, S., F. A. Sreter, and J. Gergely, 1971, Light chains of myosins from white, red and cardiac muscles. Proc. Natl. Acad. Sci., USA, 68, pp. 946-950.

Sartore, S., L. Gorza, S. Pierbon, L. Dalla Libera, and S. Schiaffino, 1981, Myosin types and fibre types in cardiac muscle I. Ventricular myocardium. J. Cell Biol., 88, pp. 226-233.

Schwartz, K., Y. Lecarpentier, J. L. Martin, A.-M. Lompre, J.-J. Mercadier, and B. Swynghedauw, 1981, Myosin isoenzymic distribution correlates with speed of myocardial contraction. J. Mol. Cell. Cardiol., 13, pp. 1071-1075.

Schwartz, K., A.-M. Lompre, P. Bouveret, C. Wisnewsky, and R. G. Whalen, 1982, Comparisons of rat cardiac myosins at fetal stages in young animals and in hypothyroid adults. J. Biol. Chem., 257, pp. 14412-14418.

Seedorf, K., U. Seedorf, and D. Pette, 1983, Coordinate expression of alkali and DTNB myosin light chains during transformation of rabbit fast muscle by chronic stimulation. FEBS Lett., 158, pp. 321-324.

Seidel, J. C., F. A. Sreter, M. M. Thompson, and J. Gergely, 1964, Comparative studies of myofibrils, myosin, and actomyosin from red and white rabbit skeletal muscle. Biochem. Biophys. Res. Comm., 17, pp. 662-667.

Seidel, J. C., 1967, Studies of myosin from red and white skeletal muscles. II. Inactivation of myosin from red muscles under mild alkaline conditions. J. Biol. Chem., 242, pp. 5623-5629.

Shivaramakrishnan, M., and M. Burke, 1982, The free heavy chain of vertebrate skeletal myosin subfragment-1 shows full enzymatic activity. J. Biol. Chem., 257, pp. 1102-1105.

Silverman, H., and H. L. Atwood, 1980, Increase in oxidative capacity of muscle fibres in dystrophic mice and correlation with over-activity in these fibers. Exp. Neurol., 68, pp. 97-113.

Sinha, A. M., P. K. Umeda, C. J. Kairnsky, C. Rajamanickam, H.-J. Hsu, S. Jacovcic, and M. Rabinowitz, 1982, Molecular cloning of mRNA sequences for cardiac alpha- and beta-form myosin heavy chains: Expression in ventricles of normal, hypothyroid, and thyrotoxic rabbits. Proc. Natl. Acad. Sci. USA, 79, pp. 5847-5851.

Sreter, F. A., J. C. Seidel, and J. Gergely, 1966, Studies on myosin from red and white skeletal muscle. I. Adenosine triphosphatase activity. J. Biol. Chem., 241, pp. 5772-5776.

Sreter, F. A., M. Balint, and J. Gergely, 1975, Structural functional changes of myosin during development. Comparison with adult fast, slow, and cardiac myosin. Devel. Biol., 46, pp. 317-325.

Sreter, F. A., J. Gergely, S. Salmons, and F. Romanul., 1973, Synthesis by fast muscle of myosin light chains characteristic of slow muscle in response to long term stimulation. Nature New Biol., 241, pp. 17-19.

Steinert, H., 1909, Myopathologische Beitrage. I. Uber das klinische und anatomische Bild des Muskelschwunds der Myotoniker. Deutsche Z. Nervenheilk., 37, pp. 58-104.

Stirling, C. A., 1975, Abnormalities in Schwann cell sheaths in spinal nerve roots of dystrophic mice. J. Anat., 119, pp. 169-180.

Stracher, A., 1969, Evidence for the involvement of light chains in the biological functioning of myosin. Biochem. Biophys. Res. Comm., 35, pp. 519-525.

Strickland. K. P., A. J. Hudson, and J. H. Thakar, 1979, Biochemical studies in dystrophic mouse muscle. Ann. N. Y. Acad. Sci., 317, pp. 187-205.

Strohman, R. C., J. Micon-Eastwood, C. A. Glass, and R. Matsuda, 1983, Human fetal muscle and cultured myotubes derived from it contain a fetal-specific myosin light chain. Science, 221, pp. 955-957.

Stull, J. T., and C. M. High, 1977, Phosphorylation of skeletal muscle contractile proteins in vivo. Biochem. Biophys. Res. Comm., 77, pp. 1078-1083.

Szent-Gyorgyi, A. G., 1953, Meromyosins, the subunits of myosin. Arch. Biochem. Biophys., 42, pp. 308-320.

Takano-Ohmuro, H., T. Obinata, M. Kawashima, T. Masaki, and T. Tanaka, 1985, Embryonic chicken skeletal, cardiac, and smooth muscles express a common embryo-specific myosin light chain. J. Cell Biol., 100, pp. 2025-2030.

Taylor, E. W., 1915, Progressive vagus-glossopharyngeal paralysis with ptosis: contribution to the group of familial diseases. J. Neurv. Ment. Dis., 42, pp. 129-139.

Trayer, H. R., M. A. Winstanley, and I. P. Trayer, 1977, The separation of heavy meromyosin isoenzymes by differential actin binding. FEBS Lett., 83, pp. 141-144.

Trayer, I. P., and S. V. Perry, The myosin of developing skeletal muscle. Biochem. Z., 345, pp. 87-100.

Tsao, T. C., 1953, Fragmentation of the myosin molecule. Biochim. Biophys. Acta., 11, pp. 368-382.

Verellen, C., R. De Meyer, M. Freund, C. Laterre, B. Scholberg, and J. F. Frederic, 1977, Progressive muscular dystrophy of the Duchenne type in a young girl with an aberration of chromosome X. in Littlefield, J. W., ed., 5th International Congress of Birth Defects, Montreal, Canada. Excerpta Medica, Amsterdam Oxford, p. 42.

Verellen, C., B. Markovic, R. De Meyer, M. Freund, C. Laterre, and R. Worton, 1978, Expression of an X-linked recessive disease in a female due to non-random inactivation of the X-chromosome. Am. J. Hum. Genet., 30, p. 97A (abstr.).

Verellen-Dumoulin, C., M. Freund, R. De Meyer, C. Laterre, J. Frederic, M. W. Thompson, V. D. Markovic, and R. Worton, 1984, Expression of an X-linked muscular dystrophy in a female is due to a translocation involving Xp21 and non-random inactivation of the normal X chromosome. Hum. Genet., 67, pp. 115-119.

Wagner, P. D., C. S. Slater, B. Pope, and A. G. Weeds, 1979, Studies on the actin activation of myosin subfragment-1 isoenzymes and the role of myosin light chains. Eur. J. Biochem., 99, pp. 385-394.

Wagner, P. D., and E. Giniger, 1981, Hydrolysis of ATP and reversible binding to F-actin by myosin heavy chains free of all light chains. Nature, 292, pp. 560-562.

Watson, A. Y., K. Radie, M. McCarthy, P. R. Larsen, and R. A. Murphy, 1982, Thyroxine reverses deficits of nerve growth factor in submandibular glands of mice with muscular dystrophy. Endocrinology, 110, pp. 1392-1401.

Webb, J. N., 1970, Naturally occuring myopathy in guinea pigs. J. Pathol., 100, pp. 155-159.

Weber, H. H., 1925a, The colloidal behavior of muscle proteins. I. The isoelectric point and conditions of stability of myogen. Biochem. Z., 158, pp. 443-472.

Weber, H. H., 1925b, The colloidal behavior of muscle proteins. II. The isoelectric point and solubility of myosin. Biochem. Z., 158, pp. 473-490.

Weber, H. H., 1950, Muskelproteine Biochim. Biophys. Acta, 4, pp. 12-24.

Weeds, A. G., 1975, Cyanogen bromide fragments of the bovine myosin: evidence for sequence homology with rabbit skeletal myosin alkali light chains. FEBS Lett., 59, pp. 203-208.

Weeds, A. G., and S. Lowey, 1971, Substructure of the myosin molecule. II. The light chains of myosin. J. Mol. Biol., 61, pp. 701-725.

Weeds, A. G., and B. Pope, 1971, Chemical studies on light chains from cardiac and skeletal muscle myosins. Nature, 234, pp. 85-88.

Weeds, A. G., R. Hall, and N. C. S. Spurway, 1975, Characterization of myosin light chains from histochemically identified fibres of rabbit psoas muscle. FEBS Lett., 49, pp. 320-324.

Weeds, A. G., and R. S. Taylor, 1975, Separation of subfragment-1 isoenzymes from rabbit skeletal muscle myosin. Nature, 257, pp. 54-56.

Whalen, R. G., G. S. Buttler-Browne, and F. Gros, 1978, Identification of a novel myosin light chain present in embryonic muscle tissue cells. J. Mol. Biol., 126, pp. 415-431.

Whalen, R. G., and S. M. Sell, 1980, Myosin from fetal hearts contains the skeletal muscle embryonic light chains. Nature, 268, pp. 731-733.

Whalen, R. G., S. M. Sell, G. S. Butler-Browne, K. Schwartz, P. Bouveret, and I. Pinset-Hartstrom, 1981, Three myosin heavy-chain isoenzymes appear sequentially in rat development. Nature, 292, pp. 805-809.

Winkelmann, D. A., S. Lowey, and J. L. Press, 1983, Monoclonal antibodies localize changes on myosin heavy chain isoenzymes during avian myogenesis. Cell, 34, pp. 295-306.

Winstanley, M. A., H. R. Trayer, and I. P. Trayer, 1977, Role of the myosin light chains in binding to actin. FEBS Lett., 77, pp. 239-242.

Worton, R. G., C. Duff, J. E. Sylvester, R. D. Schmickel, and H. F. Willard, 1984, Duchene muscular dystrophy involving translocation of the dmd gene next to ribosomal RNA genes. Science, 224, pp. 1447-1449.

Younger, L. E., and H. Silverman, 1984, Breeding and offspring rearing ability of C57 BL/6J dystrophic mice. Lab. Anim. Sci., 34, pp. 471-474.

Zar, J. H., 1984, Biostatistical Analysis, Second ed., Prentice-Hall, Inc., Englewood Cliffs, N.J.

Zatz, M. A. M. Vianna-Morgante, P. Campos, and A. J. Diament, 1981, Translocation (X;6) in a female with Duchenne muscular dystrophy; Implicatfons for the localization of the DMD locus. J. Med. Genet., 18, pp. 442-447.