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Characterization of Type IIX Muscle Fibres in the Mouse

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A Thesis Submitted to the School of Graduate Studies of the University of
Ottawa in Partial Fulfillment of the Requirements for the Degree of

Doctorate of Philosophy
in Physiology

Supervisor: D.J. Parry, Ph.D.

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ABSTRACT

The properties of type IIX fibres in mouse skeletal muscle and the factors affecting the expression of myosin heavy chain (MHC) in these fibres were studied.

Type IIX fibres were recognized on the basis of a lack of staining with antibodies directed against type I, IIA and IIB MHC. The IIX MHC isoform contained a determinant common to all type II MHCs, but lacked epitopes specific for types IIA and IIB MHCs, as well as an epitope that was present in all other MHCs. Fibres expressing IIX MHC accounted for about one-third of the total fibre number in mouse fast-twitch muscles. Sciatic nerve crush with subsequent reinnervation resulted in the formation of "type IIX fibre groups" in tibialis anterior and gastrocnemius, suggesting that the IIX phenotype is neurally regulated. The association of specific MHC isoforms with individual fibre types was confirmed by gel electrophoresis. Using 5%, 6% and 3-5% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) IIX MHC was found to co-migrate with IIA MHC. Immunohistochemistry and gel electrophoresis of single fibres from the superficial part of the tibialis anterior muscle (TAS) showed the existence of hybrid fibres co-expressing IIB and IIX MHC.

Myosin light chain (MLC) analysis of IIX fibres revealed the presence in these fibres of MLC 3f in amounts significantly smaller than that contained in IIB fibre types which suggests that type IIX fibres have a lower Vo than type IIB fibres.

Physiological, histochemical and morphometrical properties of fast-twitch single motor units were studied. Single motor units were functionally isolated by microdissection of the ventral root, the glycogen depletion technique was used to demonstrate the muscle fibres in the motor unit and monoclonal antibodies were used to identify their MHC composition in order to correlate physiological, histochemical and morphometrical studies. These studies revealed that IIX motor units had contractile properties similar to those of types IIA and IIB motor units but had morphological, physiological and biochemical properties that distinguish them from the latter two types. IIX motor units had a resistance to fatigue, cross-sectional fibre area and motor unit area intermediate between IIA and IIB motor units.

Finally, the effect of altered thyroid hormone status and age on the expression of IIX MHC was studied. Hypothyroidism led to a decrease in the expression of IIB MHC and an increase of IIX MHC in TAS muscle, while hyperthyroidism had no significant effect.

The proportion of type IIX and IIB fibres in TAS muscle of the mouse undergoes specific age-related changes that can not be detected with conventional histochemical techniques. Specifically, the proportion of fibres expressing IIB MHC decreased and the proportion of fibres expressing type IIX and co-expressing type IIB and IIX MHC increased with age. It is proposed that fibres co-expressing IIX and IIB MHC represent a transitional fibre type involved in an age-related transformation process.

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LIST OF ABBREVIATIONS

ANOVA - analysis of variance
ATP - adenosine triphosphate
ATPase - adenosine triphosphatase
C - Celsius
C.V. - coefficent of variation
CSA - cross-sectional area
DAB - diaminobenzidene
EDL - extensor digitorum longus
ELISA - enzyme-linked immonosorbent assay
F.I. - fatigue index
FF - fast fatiguable
FG - fast-glycolytic muscle fibre
FI - intermediate fatiguable
FM - fast myosin isoenzyme
FOG - fast-oxidative-glycolytic muscle fibre
FR - fatigue resistant
g - gram(s)
h - hours
Hz - hertz
IM - intermediate myosin isoenzyme
Lo - muscle lenght at which peak isometric twitch tension is produced
mAb - monoclonal antibody
mATPase - myofibrillar adenosine triphosphatase
mg - milligram(s)
MHC - myosin heavy chain
min - minute(s)
ml - millilitre(s)

MLC - myosin light chain
mm - millimeter(s)
mN - millinewton(s)
ms - millisecond(s)
mV - millivolt(s)
N - Newton(s)
NADH-TR - nicotamide adenine dinucleotide tetrazolium reductase
ng - nanograms
nm - nanometers
µm - micrometer(s)
PBS - phosphate buffered saline
Po - maximum isometric tetanic tension
Pt - maximum isometric twitch tension
S.E.M. - standard error of the mean
SDS-PAGE - sodium dodecil polyacrylamide-gel electrophoresis
SM - slow myosin isoenzyme
SO - slow-oxidative muscle fibre
TA - tibialis anterior
TAS - tibialis anterior superficial
TTP - time to peak twitch tension
V - volts
Vo - maximum velocity of unloaded shortening

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CHAPTER 1

Introduction

1.1 Historical perspective

Heterogeneity among mammalian muscles was first reported by Kühne (1857), who described different mammalian muscles which ranged in colour from white to deep red. Ranvier (1874) observed that red muscles of rabbit contracted more slowly than white muscles. A mixture of white and red muscle fibres was observed in many animal muscles, but it was unknown whether one muscle consisted of functionally different fibres because methods had only been developed to measure the contractile properties of whole muscles and not their individual fibres (Bullard 1919). The combination of histological, physiological (Denny-Brown 1929; Henneman and Olson

1965) and enzyme histochemical methods (Padykula and Herman 1955; Ogata 1958; Dubowitz and Pearse 1960) was a breakthrough in the delineation of fibre types. In the 1960s it also became possible to stimulate single anterior horn cells or motor axons enabling the study of the contractile properties of individual motor units (Devanamdan et al. 1965; Andersen and Sears 1964). Originally, the definition of a motor unit was physiological: all its muscle fibres discharge and contract synchronously. The glycogen depletion method (Krnjevic and Miledi 1958, Edstrom and Kugelberg 1968; Kugelberg and Edstrom 1968) made it possible to identify the fibres of a physiologically characterized motor unit using histological techniques. It was found that the functional differences among motor units was parallelled by morphological differences among their muscle fibres. While a whole set of contractile and metabolic protein isoforms can be used to define individual fibre types, this classification is most commonly made solely on the basis of isoforms of the sarcomeric myosin heavy chain (MHC). Indeed, there is good evidence that the intrinsic speed of shortening of muscle fibres is directly correlated to myosin adenosine triphosphatase activity (ATPase)(Barany 1967) and that this activity is largely determined by the type of MHC (Wagner 1981; Reiser et al. 1985). In turn, the speed of contraction divides muscle fibres into roughly two categories: fast fibres and slow fibres. In the adult muscle one slow MHC (type I) and at least two fast MHCs (type IIA and type IIB) were initially described. Subsequently, superfast isoforms have been found in the muscles of the head and neck: IIm MHC is present in jaw-closing muscles and in the tensor tympani muscle of the cat and dog (Rowlerson et al. 1981; Mascarello et al. 1983; Hoh et al. 1988), and IIeom MHC exists in superfast contracting fibres of the extraocular muscles (Wieczorek et al. 1985; Sartore et al. 1987).

A third fast MHC (type IIX) has been identified in muscles of rat, mouse and guinea pig by immunohistochemistry (Schiaffino et al. 1985, 1986, 1988, 1989; Gorza 1990; Parry and Zardini 1990) and gel electrophoresis (Bar and Pette 1988; LaFramboise et al. 1990), but relatively little is known about the properties of these fibres.

1.2 Objectives and experimental approach

The overall purpose of the experiments presented in this dissertation is to provide a more complete understanding of type IIX skeletal muscle fibres and the factors affecting their expression. This was achieved in a series of experiments. The main goal of each experiment and the experimental approach used is as follows:

1. To determine the distribution of a population of skeletal muscle fibres, previously unrecognized, in mouse muscles. These fibres are known as type IIX fibres.

This was done by immunohistochemical analysis of serial cryostat sections taken from different muscles of the mouse and stained with a battery of monoclonal antibodies.

2. To characterize the myosin heavy and light chain subunits of type IIX fibres

in mouse muscle.

The identification of IIX MHC as a separate isoform was achieved with immunohistochemistry, single fibre gel electrophoresis and western blotting. The identification of the MLCs present in type IIX fibres was achieved by gel electrophoresis of single type IIX fibres.

3. To determine whether the IIX fibre is an obligatory intermediate in the conversion of type IIB fibres to type IIA fibres and vice versa.

This was done by searching for co-expression of two MHC in the same fibre using immunohistochemistry and single fibre gel electrophoresis.

4. To determine the physiological characteristics of IIX motor units.

This was done using a nerve-muscle-ventral root *in vitro* preparation.

5. To determine whether there is an age-related change in the phenotypic expression of fast MHC, with particular emphasis on the proportion of fibres expressing IIX MHC and both IIX and IIB MHC.

This was done by examining the MHC expression of the superficial region of tibialis anterior (TAS) muscle cross sections and of TAS muscle single fibres from mice of different ages.

6. To determine the effect of altered thyroid hormone levels on the expression of IIX MHC isoform.

This was done using hyperthyroid, euthyroid and hypothyroid mice.

The MHC expression was evaluated in the TAS muscle by immunohistochemistry and single fibre gel electrophoresis.

1.3 Hypotheses

1. A distinct MHC isoform exists in type IIX fibres.
2. IIX motor units display properties that differ from those of types IIA and IIB motor units.
3. Age and thyroid hormone status are factors responsible for modulating the IIX fibre type phenotype.

1.4 Organization of the thesis

Following a review of the relevant literature in Chapter 2, the investigations of IIX fibres are presented in three successive chapters (Chapters 3-5). A study of the distribution and subunit composition of type IIX fibres in mouse muscles is given in Chapter 3. Chapter 4 contains a study of the physiological properties of type IIX motor units. This is followed by an examination of factors (age and thyroid hormone) affecting the expression of type IIX myosin heavy chain in Chapter 5.

CHAPTER 2

Review of literature

2.1 Skeletal muscle fibre types

Muscles fibres can be divided into a number of different types according to differences in one or several properties; differences in contractile properties (Close 1972), enzyme histochemistry (Stein and Padykula 1962; Khan 1976; Spurway 1981), metabolic activities (Peter et al.1972) and ultrastructure (Gauthier 1971) have been intensely studied and thoroughly reviewed.

Eisenberg and colleagues (Eisenberg 1974 ,1975, 1979; Eisenberg and Kuda 1975, 1976, 1977; Eisenberg et al. 1974) compared ultrastructural parameters such as Z line width, volume of mitochondria, volume and surface area of transverse tubules, terminal cisternae and sarcoplasmic reticulum with contractile and biochemical properties.

Burke and colleagues (Burke et al. 1971, 1973) related physiological properties and histochemical characteristics of individual motor units of the mixed cat gastrocnemius muscle. They stimulated a motor unit of the gastrocnemius, determined its physiological characteristics and later identified the type of the corresponding fibres histochemically by observing the ATPase staining characteristics. They were able to identify three types of motor unit based on different physiological characteristics and three histochemical types of fibres based on differences of the ATPase staining.

Over the years attention has been paid to the changes that occur in muscle fibres during development (Hoppeler 1973), exercise (Barnard et al. 1971; Guth and Yellin 1971), chronic activity (Sreter et al. 1973; Ausoni et al. 1990), disease (Cullen and Fulthorpe 1975), following cross innervation (Robbins et al 1969; Romanul 1964) and denervation (Gauthier and Dunn 1973; Pellegrino and Franzini 1963). It is often difficult to fit these changes into a particular restricted scheme for fibre types. If the mechanical properties of muscle cover a continuous range of function, then the anatomical composition of the muscle fibres might also cover a spectrum rather than be grouped into distinct types. It is nevertheless fair to say that muscle fibres in adult mammals exhibit three basic histochemical and biochemical profiles.

2.1.1 ATPase-based classification

The most frequently used technique to identify fibre types is based upon the myofibrillar actomyosin adenosine triphosphatase (ATPase) activity of the fibre (Padykula and Herman 1955), of which a number of variants may be used. The observation that fast and slow myosin have different alkaline and acid stabilities (Engel 1962; Sréter 1966) led to the identification of two myofibrillar actomyosin ATPase based fibre types, type I and type II. Histochemically, type II fibres display high ATPase activity and stain intensely under alkaline conditions, and low ATPase activity under acid conditions (alkali-stable, acid-labile). Conversely, type I fibres are alkali-labile and acid-stable (Guth and Samaha 1969). More detailed study indicated that a two ATPase-based fibre types classification was an oversimplification and led to the delineation of 3 fast fibre subtypes, designed types IIA, IIB, and IIC (Brooke and Kaiser 1970). Type IIA fibres stain intensely after alkaline preincubation (pH 10.4) and are pale after preincubation at pH 4.6 and 4.3. Type IIB fibres stain intensely after preincubation at pH 10.4, moderately after preincubation at pH 4.6 and are pale after preincubation at pH 4.3. Type IIC fibres are stable to various degrees throughout the 4.3-10.4 pH range, which results in more or less intermediate reactions after alkaline or acid preincubation. This differential sensitivity to pH corresponds to differences in ATP-ase activity of actomyosin isolated from slow and fast muscles (Guth and Samaha 1969), and is also observed after extraction of the sections with detergents that remove membrane-bound enzymes (Schiaffino and Pierobon-Bormioli 1973). Further study of ATP-ase based fibre types in different muscles of different species (Gollnick et al. 1983; Gollnick and Matoba 1984; Matoba and Gollnick 1984) confirm the existence of fast fibre subtypes. However the preincubation pH at which distinction of fibre types is clear is species- and, in some cases, muscle-specific, and requires appropriate adjustment. Where it has been possible to measure physiological properties of fibres belonging to a motor unit and subsequently to histochemically identify those fibres (Burke et al. 1971, 1973; Kugelberg and Edstrom 1968) it has generally been shown that type I fibres contract slowly while type II fibres are fast-contracting. However there are instances where the correlation between ATPase fibre type classification and physiological properties appears not to hold true. Embryonic muscles, for example, stain very intensely for ATPase at an alkaline pH but the velocity of shortening of these muscles is very low (Sreter et al. 1972). Furthermore, with ATPase fibre typing it has been sometimes difficult to know whether a fibre belongs to a different type or is a hybrid type in which two sets of myosin exist. Such has been the case for the muscles of embryonic and neonatal animals. These problems were solved by means of techniques which allowed the identification of the specific type of myosin found within the muscle fibres. The techniques employed were immunocytochemistry, using antibodies to different myosin isoforms and gel electrophoresis of the contractile proteins which allowed identification of different isoforms of both myosin heavy chains (MHCs) and light chains (MLCs). These techniques will be discussed in greater detail later in this chapter.

2.1.2 Metabolic enzymes

Different types of muscle fibres have also been identified on the basis of histochemical reaction for enzymes of aerobic metabolism, such as succinic dehydrogenase (SDH) (Green et al. 1981; Reichmann and Pette 1982) and nicotinamide adenine dinucleotide (NADH) tetrazolium reductase (Ogata 1958) and anaerobic glycolytic metabolism, such as α-glycerophosphate dehydrogenase (Barnard et al. 1971; Peter et al. 1972). It was shown that IIB fibres stained poorly for mitochondrial enzymes while IIA fibres stained intensely for these enzymes. Table 2.1 summarizes the typical pattern of SDH and ATPase staining of fibre types in rat muscle.

Table 2.1 Succinate dehydrogenase activity (SDH) and actomyosin ATPase staining of rat muscle fibres.

		Myofibrillar actomyosin ATPase staining		
Fibre type	SDH	pH 4.3	pH 4.6	pH10.4
I	++	++	++	-
IIA	++	-	-	++
IIB	-	-	+	++

++ = strong staining
\+ = intermediate staining
\- = absence of staining

2.1.3 Correlation of ATPase and metabolic characteristics

The combination of metabolic enzyme and ATPase based histochemical methods resulted in the identification of three fibre types in muscles of the rabbit and guinea pig (Barnard et al. 1971). These fibres were named: slow twitch-oxidative (SO), fast-twitch oxidative-glycolytic (FOG) and fast-twitch glycolytic (FG). However, these authors did not actually measure speed of contractions; rather this was inferred based upon prior correlation of speed of contraction and ATPase activity (Kugelberg and Edstrom 1968).

2.1.4 Immunohistochemistry

The first demonstration that there are immunochemically distinctive myosin in different muscle fibres came from studies in which the antibodies to one fibre type were obtained. These antibodies therefore stained some of the fibres but left other fibres unstained (Groschel-Stewart and Doniach 1969; Groschel-Stewart 1971). Antibodies to both fast-twitch and slow-twitch muscle myosin were first obtained by Arndt and Pepe (1975). The myosin used as immunogen in this early work was shown to contain appreciable amounts of other proteins. Indeed a major difficulty in the interpretation of immunological data has been establishing the specificity of the antibody. More sensitive methods of detecting the presence of contaminating antibodies and the use of properly screened monoclonal antibodies have made this less of a problem. The advent of immunohistochemical techniques made it possible to support the ATP-ase-based classification and to add to it. In fact the three fibre types referred to above have all been shown, by immunological methods using antibodies specific for MHC, to express distinct MHC isoforms (Pierobon-Bormioli et al. 1981). Schiaffino et al. (1985) identified a fibre type which could not be fitted into the existing classification scheme. This fibre type, referred to as type IIX (Schiaffino et al. 1986), was first recognized on the basis of its immunohistochemical characteristics. It was not labelled by monoclonal antibodies specific for types I, IIA or IIB MHC nor by an additional monoclonal antibody which recognized all MHC except IIX MHC. The antibodies employed and their specificities are illustrated in Table 2.2.

The IIX fibres were not previously detected with ATPase histochemistry because histochemical properties of type IIX fibres are in part common to type IIB fibres and in part to type IIA fibres (Gorza 1990). With regard to SDH activity the type IIX fibres in the mouse, being highly oxidative, stain intensely and cannot be distinguished from the type IIA fibre, which is also highly oxidative (Parry and Zardini 1990).

Table 2.2 Immunohistochemical reactivity of anti-MHC monoclonal antibodies.

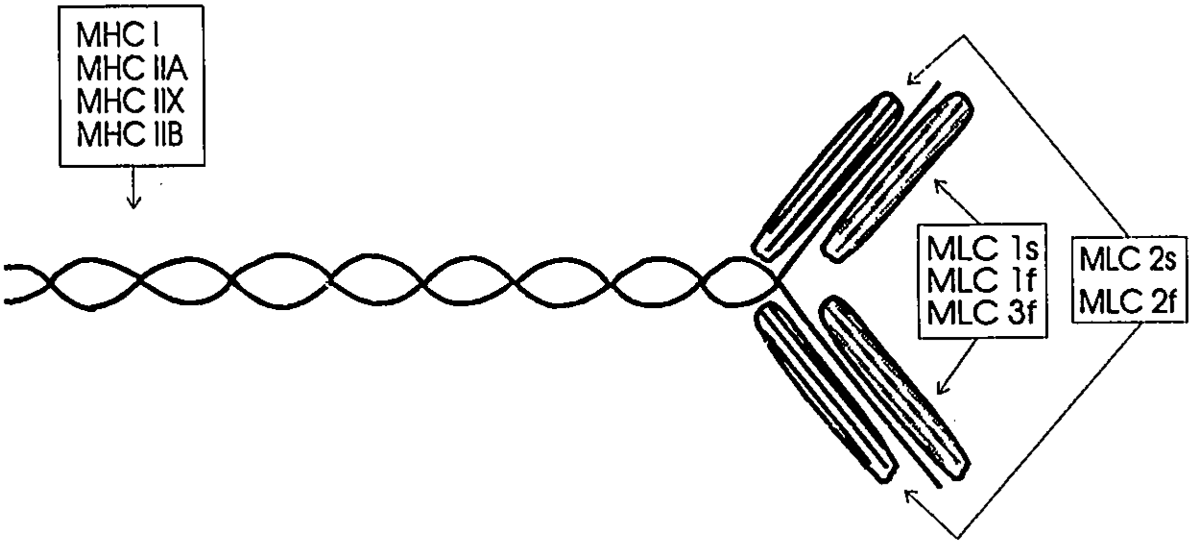
	MYOSIN HEAVY CHAIN RECOGNIZED					
mAb*	Emb.	Neo.	I	IIA	IIB	IIX
BF-45	+					
BF-34		+				
BA-D5			+			
BF-32			+	+		
SC-75				+	+	+
SC-71				+		
BF-F3					+	
BF-35	+	+	+	+	+	

*Their specificities have been confirmed by Western blotting and ELISA (Schiaffino et al. 1986, 1989).

2.1.5 The myosin molecule

Myosin is an asymmetric hexamerous protein, consisting of four light chains (approximately 20 KDa each) and two heavy chains (approximately 200 KDa each) (Gazith et al. 1970). Figure 2.1 is a schematic illustration of the composition of the myosin molecule. Two heavy chains form the tail and, together with the four light chains, the two S_1 subunits of the head region. The four myosin light chains (MLC) consist of a pair of phosphorylatable light chains (LC2) and a pair of alkali light chains LC1 and /or LC3. Adult mammalian fast muscle contains two distinct alkali light chains (LC1f and LC3f), with M_r of respectively 25 and 16 KDa, and a homodimer phosphorylatable light chain (LC2f), with a M_r of 18 KDa each. Adult mammalian slow muscle contains the alkali light chain LC1s (27 KDa) and a phosphorylatable light chain LC2s (20 KDa). In many species, two slow alkali light chains (LC1as, LC1bs) of 26.5 and 27 KDa are present (Lowey and Risby 1971; Sarkar et al. 1971; Weeds 1976).

Figure 2.1 Scheme illustrating the composition of the myosin molecule. Two heavy chains form the tail and together with four light chains the two S1 subunits of the head region. (Adapted from Weeds 1976).



2.1.6 Myosin heavy chain composition confirmed with gel electrophoresis

The different MHC isoforms may be also distinguished on the basis of their mobility in polyacrylamide gels in the presence of the anionic detergent sodium dodecyl sulphate (SDS). This has proved to be a useful tool for the rapid, simple and reliable estimation of the molecular weights of sarcomeric proteins and their subunits.

The existence of distinct myosin heavy chains (MHC) in type I, IIA and IIB fibres was first confirmed by biochemical studies by Dalla Libera et al. (1980). They identified distinct isomyosins in fast-white and fast-red guinea pig muscles by electrophoretic peptide maps obtained by treating myosin with different proteases. In contrast, immunofluorescence studies revealed that type IIC fibres are hybrid fibres containing mixtures of type I and type IIA MHC (Pierobon-Bormioli et al. 1980) or regenerating fibres containing embryonic and neonatal MHC (Schiaffino et al. 1986). Microelectrophoretic analyses of single fibres from which a small piece was cut and stained for myofibrillar ATPase (Billeter et al. 1981), confirmed the above results.

Electrophoretic separation of fast and slow MHCs was achieved first in avian muscles (Rushbrook and Stracher 1979) and then in mammalian muscles (Carraro and Catani 1983) using 5% or 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). Danieli-Betto et al. (1986) increased the gel glycerol content to achieve further separation of fast MHCs muscle into IIA MHC and IIB MHC with 6% SDS-PAGE. It was not possible to electrophoretically separate IIX MHC on 6% PAGE, but immunocytochemical evidence for the existence of IIX MHC was confirmed by immunoblotting (Schiaffino et al. 1985, 1986, 1989) which revealed the existence of two distinct MHCs with the same electrophoretic mobility on 6% SDS-PAGE, only one of which reacted positively with an antibody to IIA MHC.

Using a 5-8% linear gradient of acrylamide, Bär and Pette (1988) reported electrophoretic separation of a fourth MHC isoform present predominantly in adult rat diaphragm and designated it as IID MHC. The electrophoretic mobility of IID MHC was intermediate between that of IIA and IIB MHC. Subsequently, by adjusting the relative concentration of the Bis component with respect to total acrylamide in 5% SDS-PAGE, LaFramboise et al. (1990) were able to separate IIX MHC from IIA MHC in rat skeletal muscles. They were able to show, by immunoblot analysis, that IIX MHC and IID MHC are in fact the same MHC isoforms (LaFramboise et al. 1990).

2.1.7 Myosin light chains

MHC is only one aspect of myosin gene expression in a given muscle fibre. It is known that MLCs associate with MHC to form the hexamerous myosin molecule. The different MLCs may also be separated on the basis of their mobility in SDS-PAGE. The role of MLCs is poorly understood, although their location near the head/rod junction suggests that they may be involved in modulating the interactions between myosin and actin (Wagner and Weeds 1977; Moss et al., 1982). Many studies have tried to correlate contractile performance with MHC and MLC composition, but to date the influence of MHC and MLC composition on the contractile properties is not clear. The alkali MLCs (MLC1s, MLC1f, MlC3f) are essential for contraction, and it has been reported (Greaser et al. 1988) that the maximal velocity of unloaded shortening (Vo) of single fibres from rabbit fast skeletal muscles appears to correlate with the MLC3f content. The phosphorylatable MLC (MLC2s, MLC2f) are not essential; they are phosphorylated during contraction but this does not seem to be essential for contraction and does not seem to influence Vo (Persechini et al. 1985). In the absence of phosphorylation MLC2 *per se* may modulate Vo since Hoffman et al. (1990) reported that Vo decreases following partial extraction of MLC2.

In experiments on chemically skinned single fibres Reiser et al. (1985) concluded that Vo is determined mainly by the fibre MHC composition and to a lesser extent by the MLC. They found that the slowest contracting fibres contained type I MHC and slow MLC (MLC 1s,2s) and the fastest contracting fibres contained type II MHC and fast MLC (MLC 1f, 2f, 3f). Fibres contracting at an intermediate velocity contained mixtures of both type I and type II MHC and slow and fast MLC.

However, in a more recent study in which Vo has been related to MLC 1f/3f ratio, the same group (Moss et al. 1990) reported that this ratio appeared to be a significant determinant, a higher velocity being associated with a higher LC3f/LC1f ratio.

Therefore, the multiplicity of MHC and MLC combinations (Staron and Pette 1990) can explain, at least partially, the difference in Vo among different muscle fibres.

2.1.8 Isomyosins

When myosin is electrophoresed under non-dissociating conditions, a number of myosin isoenzymes or "isomyosins" can be identified (Hoh et al. 1976; d'Albis et al. 1979; Fitzsimons and Hoh 1983). Fitzsimons and Hoh (1983) analyzed the native myosin isoenzyme composition of mouse slow-twitch soleus and fast-twitch extensor digitorum longus (EDL) muscle and reported that soleus muscle contains two isomyosins (slow myosin, SM and intermediate myosin, IM) which are electrophoretically distinct from the three fast isomyosins (FM1, FM2, FM3) of EDL muscle. They determined the MLC of each of the isomyosins following slicing of the myosin-containing bands from the pyrophosphate gels and concluded that each electrophoretically distinct myosin contains a different combination of the five skeletal myosin light chains. Table 2.3 summarizes the composition of the various isomyosins (Fitzsimons and Hoh 1983).

Table 2.3 Compositions of the various isomyosins of mouse muscle (Fitzsimons and Hoh 1983).

ISOMYOSIN	MHC	MLC
SM	I	1s(2), 2s(2)
IM	IIA	1s(1), 1f(1), 2f(2)
FM1	IIB	2f(2), 3f(2)
FM2	IIB	1f(1), 2f(2), 3f(1)
FM3	IIB	1f(2), 2f(2)

Numbers in parentheses indicate moles of MLC per mole isomyosin.

Fitzsimons and Hoh 1983, using the myofibrillar ATPase reaction, classified all the fibres of EDL muscle as type IIB fibres and this explains why all the FMs have been associated with IIB fibre types. It is, however, known that EDL muscle contains IIA and IIX fibres as well as IIB. The IIX MHC is not included in this table because at the time the study was done, its existence was unknown. Today there is evidence for IIX MHC existence but we still do not know its isomyosin composition.

The functional significance of the isomyosin is not clear. It is possible that a particular MHC and MLC combination is associated with a particular speed of cross-bridge cycling. Asmussen and Maréchal (1989) evaluated the relationship between the maximal velocity of unloaded shortening (Vo) and the isomyosin composition of soleus muscle from mouse, rat and guinea-pig. Guinea-pig soleus muscle contained only type I fibres and SM2 isomyosin and had the slowest Vo. Mouse soleus muscle contained about 70% type I fibres and 30% type IIA fibres, the same proportion of slow (SM2) and intermediate (IM) isomyosins, and had the fastest Vo; rat soleus muscle had an additional slow isomyosin (SM1) and an intermediate Vo. Since the two types of fibres in the rat soleus muscle accommodated three different isomyosins it was postulated that muscle fibres contain a mixture of isomyosins and that the Vo of a fibre is controlled not only by the nature of its MHCs and MLCs but also by the relative proportions of the isomyosins contained in it.

Various combinations of more than one MHC isoform have been found in single fibres of normal, adult mammalian muscle (Danieli-Betto et al. 1986; Staron and Pette 1987; Termin et al. 1989; Zardini and Parry 1992). Microelectrophoretic analyses of single fibres have indicated that a particular set of MLCs does not combine exclusively with a specific MHC isoform. The co-existence of both fast and slow MLCs has been observed in fibres containing either fast or slow MHCs (Billeter et al. 1981; Young and Davey 1981; Mizusawa et al. 1982; Staron and Pette 1987; Maréchal et al. 1989; Parry and DiCori 1990; Pilgrim (unpublished observation).

Pette and Staron (1990) calculated, without taking into account the existence of "hybrid" fibres (i.e. heterodimer MHC and various combinations of MLC), that a total of 60 isomyosins could exist in adult mammalian muscle.

Table 2.4 summarizes the different biochemical, immunological, morphological and physiological characteristics of classified fibre types.

Table 2.4 Characteristics of type I, IIA, IIX and IIB fibres in mammalian skeletal muscle.

Method	Fibre type				Animal	References
	I	IIA	IIX	IIB		
ATPase staining						
pH 4.3	++	-		-	man,rat, rabbit	Brooke and Kaiser (1970 a, b,)
pH 4.6	++	-		+		
pH 10.4	-	++		++		
SDH staining	++	++		_	rat rat	Ogata (1958) Stein and Padykula (1962)
immunohistochemistry (mAb raised against)	yes	yes	no	yes	rat	Schiaffino et al.(1985,1986)
Metabolism						
Oxidative enzymes	++	+	?	-	cat rat	Bullard (1919) Gauthier and Padykula (1966)
Glycolytic enzymes	-	+	?	++	rabbit guinea pig	Barnard et al.(1971) Peter et al.(1972)
Myoglobin content	intermediate	high	?	low	rat,mouse, cat,rabbit	James (1968)
Morphology						
Fibre size	small	intermediate	?	large	rat mouse	Gauthier and Padykula (1966) Parry and Wilkinson (1990)
Motor unit type	S	FR	FI(?)	FF	cat	Burke et al. (1971, 1973, 1974)
Speed of contraction	slow	fast	?	fast	cat	Burke et al. (1971, 1973, 1974)
Maximum tetanic tension	+	++	?	+++	cat	Burke et al. (1971, 1973, 1974)
Fatigue resistance	++	++	+	_	cat	Burke et al. (1971, 1973, 1974)
Myosin content						
Heavy chain	I	IIA	?	IIB	human	Billeter et al. (1981)
Light chain	1s(2), 2s(2)	1f,2f(2),3f	?	1f,2f(2),3f	rabbit	Frank and Weeds (1974)
Isoenzymes	SM	IM	?	FM1,FM2, FM3	chicken mouse mouse	Hoh et al. (1976) d'Albis et al. (1979) Fitzsimons and Hoh (1983)

These are relative values.
++ = high; + = intermediate; - = low. Abbreviations: SO, slow twitch-oxidative; FOG, fast-twitch, oxidative-glycolytic fibre; FG, fast-twitch, glycolytic fibre;S, slow-twitch,fatigue-resistant motor unit; FR, fast-twitch, fatigue-resistant motor unit; FF, fast-twitch, fatigable motor unit; SM, slow myosin isoenzyme; IM, intermediate myosin isoenzyme, FM,fast myosin isoenzyme.

2.2 Physiological characteristics of different fibre types.

2.2.1 Correlation between histochemical and physiological characteristics of fibre types at the whole-muscle level

Studies on whole muscles (Edgerton and Simpson 1969; Barnard et al. 1971; Peter 1971; Peter et al.1972), in which histochemical properties have been related to the biochemical and contractile properties, suggested the presence of three fibre types, each of which possesses a distinctive combination of histochemical and mechanical characteristics. Insights into the muscle fibre composition and the muscle contractile properties have arisen from studies of cross-innervation (Close 1969; Buller 1971, 1987; Thomas et al. 1993) and chronic electrical stimulation (Salmons and Sreter 1976; Vrbova 1969; Pette et al. 1973; Sweeney et al. 1988; Pette and Vrbova 1992). Since the work of Barany (1967), it has generally been assumed that the contractile properties of a given muscle primarily relate to its myosin ATPase activity. All these studies suggest that this may be true for contractile speed but not for the other contractile properties, i.e. time to peak twitch tension (TTP) which is, in part, related to the presence of the regulatory proteins, the properties of sarcoplasmic reticulum and Ca^{2+} -ATPase activity. Indeed time course studies have revealed that changes in the composition of the sarcoplasmic reticulum occur very rapidly after the onset of the stimulation and appear to precede changes in protein isoforms (Pette and Vrbova 1992).

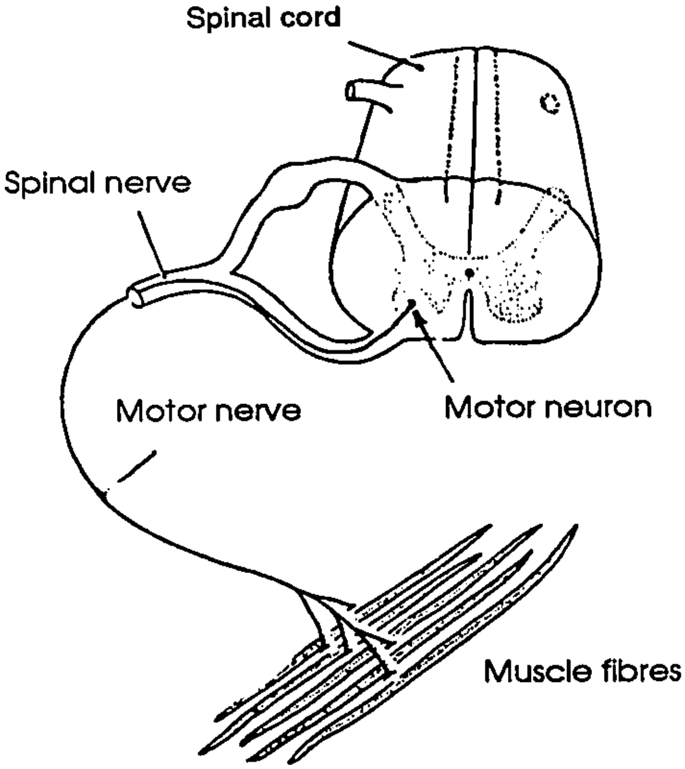
2.2.2 Correlation between histochemical and physiological characteristics of fibre types at the single fibre level

As mentioned in sections 2.1.8, there has been a number of investigations dealing with the contractile properties of single muscle fibres. Where it has been possible to measure Vo of a single muscle fibre and subsequently identify the MHC and MLC content of that same fibre, it has been shown that Vo correlates with both MHC (Reiser et al. 1985) and MLC (Sweeney et al. 1986, 1988; Eddinger and Moss 1987; Moss et al. 1990) composition.

2.2.3 Correlation between histochemical and physiological characteristics of fibre types at the motor unit level

Liddell and Sherrington (1925) introduced the concept of the motor unit as the basic unit of motor activity. A motor unit consists of a large anterior horn cell, its axon and the skeletal muscle fibres innervated by that axon (Fig. 2.2).

Figure 2.2 Schematic representation of a motor unit.



The earliest studies of motor unit properties were done by Wuerker et al. (1965 a,b) and Henneman et al. (1965 a,b,c) on cat soleus and medial gastrocnemius muscles. These pioneering studies described in detail the range of contraction times, tension outputs and axonal conduction velocities for large samples of motor units. Henneman and Olson (1965 a,b,c) suggested that particular combinations of such properties are related to the histochemical differences between muscle fibres, though there was no direct evidence to support this idea at that time. Although formal classification of motor unit "types" was not made, these studies revealed a number of general points which have since been repeatedly confirmed in other animals including mice (Lewis and Parry 1979; Lewis et al. 1982; Bateson and Parry 1983; Parry and DiCori 1990).

1. within a given motor unit population, the motor units with the slowest twitch contraction time tend to produce consistently small tensions, while the faster contracting units tend to produce a wide range of large tensions (Bagust et al. 1973; Burke et al. 1973; Bagust and Lewis 1974; Goslow et al. 1977).

2. motor units found in histochemically heterogeneous muscles exhibit a much wider variation in physiological properties than those present in the histochemically homogeneous muscles (Burke 1967; Close 1967; Bagust et al. 1972; Burke et al. 1973).

3. the relatively slow contracting, small-force motor units tend to be innervated by relatively slow conducting motor axons (Bagust 1974).

Using a graded threshold stimulation of the rat sciatic nerve to sequentially activate soleus and EDL motor units, Close (1967) was able to divide motor units into three classes based on their time taken to reach peak isometric twitch tension. Although a relationship between histochemical fibre type and physiological properties was inferred, it was only with the introduction of the "glycogen depletion" method (Edström and Kugelberg, 1968) that this interrelationship between physiological and histochemical characteristics of muscle fibres could be clearly demonstrated. The principle of this technique (initially suggested by Krnjevic and Miledi, 1958) is as follows: by repeatedly stimulating a motor axon, the fibres innervated by that axon metabolize their glycogen; when sufficiently fatigued, the fibres will be depleted of their glycogen stores. The "fibre type" of these fatigued fibres (and hence of the fibres of the fatigued motor unit) can then be identified in serial transverse sections of muscle stained for glycogen content (periodic acid-Schiff, PAS) and fibre type (ATPase, SDH). Edström and Kugelberg (1968) found type I units to be slow and virtually unfatiguable, type II oxidative units to be fast and intermediate in fatigue resistance and type II glycolytic units to be fast and easily fatigued.

Burke and co-workers (1967, 1971, 1973, 1974 a,b) introduced a tetrapartite classification scheme of cat motor unit types based on three physiological parameters (1) speed of contraction (2) measurement of the sensitivity to fatigue during a standard stimulus sequence (the fatigue index) and (3) the shape of standardized unfused tetanic responses (the "sag" property). Their classification criteria produced a clear and easily defined separation between motor unit groups which remains applicable as more data have since been accumulated, and is useful in predicting patterns for new data (Dum and Kennedy 1980; Bateson and Parry 1983; Kanda and Hashizume 1989; Parry and DiCori 1990; Totosy et al. 1992). Burke identified a group of motor units characterized by fatigue resistance, a lack of sag, small tetanic tensions and relatively long twitch-contraction times. These were referred to simply as slow-twitch, unfatiguable, or type S units. The remaining units all exhibited sag in unfused tetany through a wide range of stimulation frequencies and had relatively short twitch-contraction times (< 55 ms); these were referred to collectively as fast-twitch, or type F units. The F group exhibited a bimodal distribution of fatigue indices. The more fatigable group tended to produce relatively large tetanic forces and was called fast-twitch fatiguable or type FF. The other type F group exhibited greater resistance to fatigue but produced less tetanic force; these were called fast-twitch, fatigue resistant or type FR units. A small proportion (about 5%) of the type F population had intermediate fatigue index values and were referred to as fast-twitch, fatigue intermediate or type FI units. In subsequent experiments, Burke and colleagues (1971, 1973) used the glycogen-depletion technique to label muscle units belonging to the full range of types found in the cat medial gastrocnemius muscle. All FF units studied contained fibres histochemically identified as type IIB; FR and

S units contained fibres identified as type IIA and type I, respectively. The type of fibres comprising the FI unit was unclear. They exhibited ATPase staining characteristic of type IIB fibres but were like type IIA fibres when stained for oxidative enzymes. Interestingly, type IIX fibres in mouse and rat also display these staining characteristics (Gorza 1988; Parry and Zardini 1990).

Several investigators have studied the issue of whether all fibres within a motor unit are identical by examining fibres which have been glycogen-depleted. The first quantitative study of motor unit biochemistry (Nemeth et al. 1981) revealed that the activity of the oxidative enzyme malate dehydrogenase was identical among motor unit fibres, in contrast to its great heterogeneity across the full fibre population. However Martin et al. (1988 a, b), Edgerton et al. (1990) and Larsson (1992) observed a variation in the activities of enzymes involved in energy metabolism within single motor units. These variations although small, were too large to confirm the concept of motor unit homogeneity. These observations suggest that there may be ongoing processes of transformation of motor unit fibres which will result in the presence of fibres within the motor unit with different degrees of transformation, or that some of the fibres of a given motor unit may have been reinnervated by a "foreign" motor unit during an ongoing remodelling process. Yet another possibility could be that the control exerted by the motoneuron on the muscle fibres is not the sole control factor. Other factors such as humoral influences could impose an additional control and be responsible for the motor unit heterogeneity.

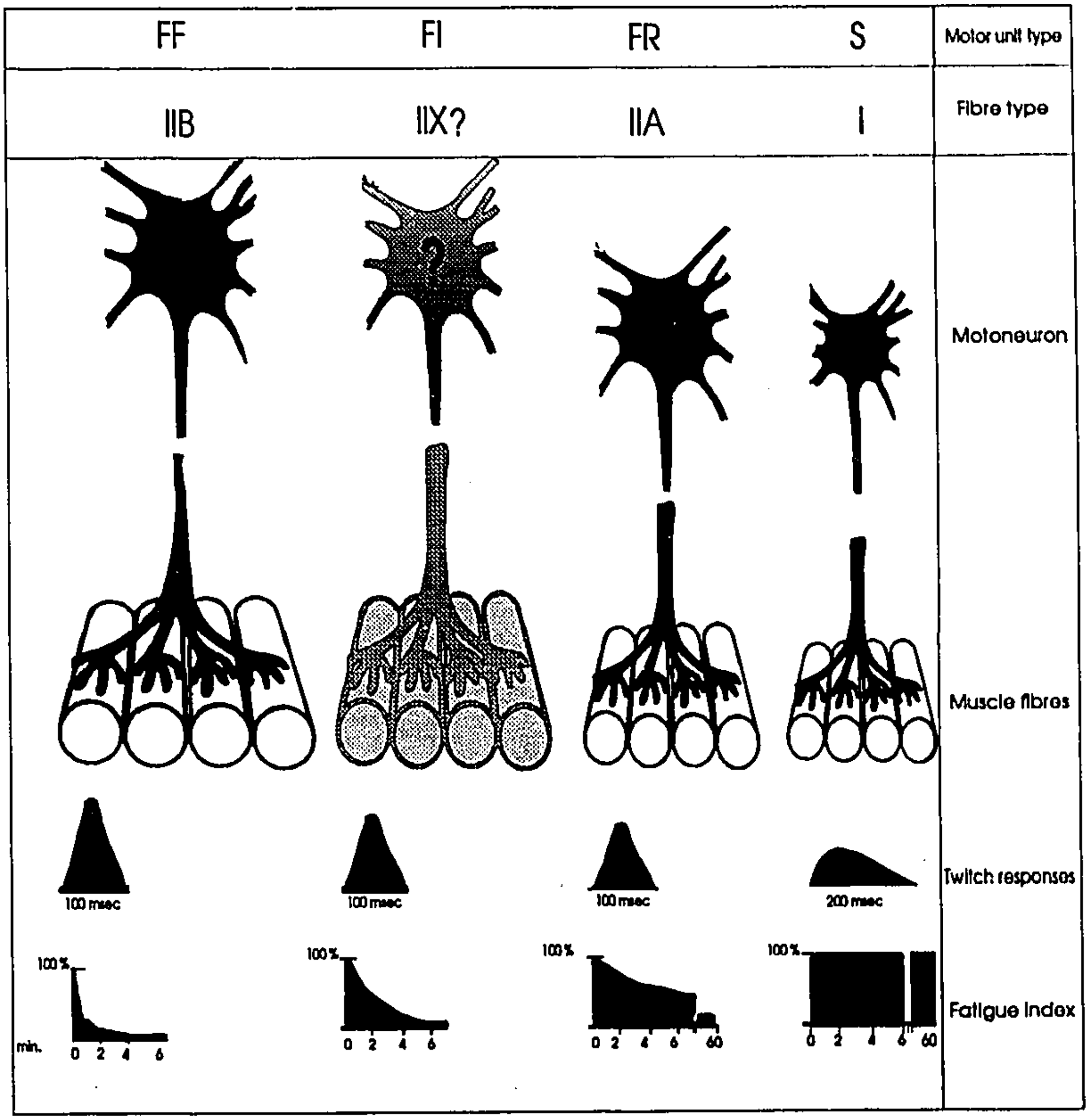
Burke et al. (1982) studied the relationship between motor neurons and motor unit types. Following the physiological identification of a motor unit type by motoneuron stimulation, horseradish peroxidase was injected intracellularly to visualize the dendritic and axonal morphology. Their results provided direct evidence that the α-motor neurons innervating fast-twitch motor units are larger than those innervating slow-twitch motor units, and that motor neurons of type FR units are, on average, intermediate in size between those of FF and S units. Unfortunately motor neurons innervating FI units were not isolated.

Lännergren and Smith (1966) examined the relationship between the morphological, histochemical and physiological characteristics of individual muscles fibres in Xenopus iliofibularis muscle. They described three groups of fibres: one with relatively fast-twitch contractions and little resistance to fatigue, another with somewhat longer contraction times and greater resistance to fatigue and a third with the longest twitch-contraction time and the greatest resistance to fatigue. Each category corresponded to a different histochemical profile and a distinctive morphology of the end plate neuromuscular junctions. Smith and Lännergren (1968) later showed that iliofibularis motor units display the same distributions of physiological properties as the single fibres. These are the most direct studies to date of the interrelations between the morphological, histochemical and physiological characteristics of muscle motor units and their fibres.

Wilkinson and Nemeth (1989) used the transversus abdominis muscle of the garter snake to make a direct assessment of the properties of individual fibres that make up the motor unit. The monolayer arrangement of the fibres of this muscle and the fact that all its motor nerve terminals are accessible for selective activation of axons provide an unique opportunity for this direct assessment. They showed that snake muscle has fibre types similar to mammalian muscle with respect to specific enzyme activities and myosin isozymes. The variability of enzyme activities among fibres within a given motor unit was found to be significantly less than the variability of enzyme activity of the fibres belonging to different motor units (Nemeth and Wilkinson 1990).

In view of the above findings it seems reasonable to assume that fibres in a given muscle unit are also identical in mechanical properties. Figure 2.3 summarizes the characteristics of the different types of motor unit.

Figure 2.3 Summary of features of motor units. Relative size are shown for motor neurons, muscle fibres, twitch responses and fatigue curves. (Adapted from Berne and Levy 1990).



2.3 Factors affecting plasticity of myosin heavy chain expression in skeletal muscle

The phenotypic expression of MHC isoforms within a given muscle appears to be under the control of a number of factors, among them developmental (Whalen 1980; Whalen et al. 1979 a, b, c, 1981; Mahdavi et al. 1987), neural (Vrbová 1963; Salmons and Vrbová 1969; Bárány and Close 1971; Pette et al. 1973, 1976; Sréter et al. 1973; Salmons 1976; Salmons and Sréter 1976; Heilig and Pette 1980; Ausoni et al. 1990) and hormonal factors (Fitzsimons et al. 1990; Izumo et al. 1986; Mahdavi et al. 1987).

Wydro et al. (1983) and Robbins et al. (1986) showed that the MHCs expressed in striated muscles are encoded by a multigene family, with each isoform encoded by a separate gene. Evolutionary analyses of MHC genes indicate that the MHC multigene family resulted from gene duplication and divergence (Stedman et al 1990). The genes encoding the murine skeletal MHCs have not yet been fully characterized (Wade and Kedes 1989; Lyons et al. 1990). Cox et al. (1991) reported that embrionic, perinatal and adult fast IIB MHC genes are genetically linked, and Parker-Thornburg et al. (1992) recently identified two closely linked murine MHC genes, one of which encodes the IIA MHC isoform. The other appears to encode an additional isoform that represents a predominant MHC transcript in diaphragm, tongue, soleus and masseter of the mouse; by implication, this would appear to be the IIX MHC.

2.3.1 Developmental factors

The expression of MHCs is developmentally regulated (Whalen et al. 1979, 1981; Whalen 1985; Wydro et al. 1983; Gambke and Rubinstein 1984; Mahdavi et al. 1987). Studies at the protein level indicate that a minimum of six different MHC isoforms are expressed during the development of rat skeletal muscle (Whalen 1985): an embryonic isoform mainly expressed during intrauterine life; a neonatal isoform mainly expressed during the last week of gestation and first few weeks of postnatal life; and three adult isoforms corresponding to two adult fast and one slow MHC isoforms that are expressed predominantly in fast oxidative type IIA, fast glycolytic type IIB and slow oxidative type I fibres, respectively. In addition, there are specialized MHC isoforms in the extraocular muscle (Wieczorek et al. 1985) and masseter muscles of cat (Rowlerson et al. 1981). Mahdavi et al. (1987) using S1 nuclease mapping analysis showed that MHC genes are differentially expressed during skeletal muscle development. They reported that there is close correlation between the developmental pattern of expression of each MHC mRNA and that of the corresponding protein isoform, suggesting that the qualitative and quantitative changes in the MHC phenotype observed during development in each muscle type can be entirely accounted for by changes in the level of their respective mRNA. The overall picture that emerges from these studies is quite complex and suggests that in all muscles studied so far, more than one MHC gene is expressed; that each MHC gene is expressed in different muscle types, but the set of MHC genes and their relative levels of expression are characteristic of each muscle type presumably in accordance to their functional requirements.

2.3.2 Neural factors

There has been considerable interest in the role of innervation in muscle development and function since the work of Eccles and his colleagues (Eccles et al. 1958; Buller et al. 1960 a). They reported that when a fast muscle is cross-reinnervated by a nerve that originally supplied a slow muscle it acquires properties characteristic of a slow muscle; reciprocal changes take place in a slow muscle that has been cross-reinnervated by a fast muscle nerve. Changes in contractile speed (Buller et al. 1960; Close 1969) have been shown to be accompanied by changes in myosin ATPase activity (Bárány and Close 1971; Buller et al.1969) and changes in the types of myofibrillar protein sub-units (Pette et al. 1986; Salmons and Sréter 1976; Sréter et al.1974; Weeds et al.1974). Immunological and biochemical studies revealed that both fast and slow myosin isoenzymes are present within single fibres during the conversion (Pette and Schnez 1977), indicating that the transformation occurs within the preexisting set of fibres and that muscle phenotype is not fixed. This implies that the motor neuron can selectively activate the genes for the synthesis of either fast-contracting or slow-contracting myofibrils. This has been convincingly demonstrated in a number of elegant studies (Vrbová 1963; Salmons and Vrbová 1969; Pette et al. 1973, 1976; Sréter et al. 1973; Salmons and Sréter 1976; Heilig and Pette 1980) showing that chronic electrical stimulation of a fast-twitch muscle with a frequency pattern resembling the firing rate of a slow motor neuron ultimately effects a transformation from fast to slow. Using different patterns of chronic electrical stimulation imposed directly on denervated muscle, Lomo et al. (1974, 1980) have shown that transformation can occur in the absence of the nerve and that a wide variety of both fast and slow muscle phenotypes can be obtained, depending on the pattern of impulse activity imposed by direct stimulation. This indicates that the pattern of muscle activity per se plays an important role in determining the contractile properties of skeletal muscle. Ausoni et al. (1990) stimulated denervated rat EDL and soleus muscle with patterns of activity that resemble normal soleus or EDL motor unit activities (Hennig and Lomo 1985). They observed that the same stimulus patterns resulted in different expressions of MHCs in soleus and EDL. Since MHC isoform switching appears to result from transcriptional regulation of the genes that encode the MHCs (Izumo et al 1986), these results indicate that the MHC genes can be turned on and off by activity pattern in a specific manner at the muscle type and fibre type level.

2.3.3 Ageing factors

In spite of its importance the effects of ageing on striated muscles have until recently received little attention and many of the results of those studies published have been divergent and conflicting. There has been agreement that the speed of muscle contraction decreases during the ageing process (Campbell et al. 1973; Gutmann and Syrový 1974; Caccia et al. 1979; Larsson and Edström 1986; Edström and Larsson 1987; Larsson and Salviati 1989; Ansved and Larsson 1989; Kanda and Hashizume 1989; Larsson et al. 1991). A decrease in the proportion of the fast twitch fibres is considered to be one possible mechanism underlying the age-related decrease in the speed of contraction (Kugelberg 1976; Eddinger et al. 1985; Larsson and Edström 1986; Edström and Larsson 1987; Ishihara and Araki 1988), although this is considered not to be a consistent phenomenon of ageing in all muscles. Kugelberg (1976) and Eddinger et al. (1985) reported that the slow twitch soleus muscle shows an age-related decrease in the relative proportion of fast twitch fibres, whereas the fibre type composition of the fast twitch extensor digitorum longus muscle has been reported to be unaltered (Eddinger et al. 1985; Larsson and Edström 1986; Ishihara and Araki 1988).

Kugelberg (1976) studied the adaptive transformation of rat soleus during growth. Between one and eight months of age the proportion of type II fibres decreases significantly and the proportion of type I fibres increases. Similarly, Tomanek (1975) reported a decrease in the number of type II fibres in cat soleus muscle with growth. In these studies there was neither a change in the total number of fibres within the muscle nor a selective atrophy of type II fibres, which suggests that the type II fibres must have transformed into type I fibres. Indeed muscle fibres showing co-existence of type I MHC and type IIA MHC (the so called type C fibres) are commonly seen in rat (Kugelberg 1976; Staron and Pette 1986, 1987) and mouse (Parry and Knight 1988; Parry and Di Cori 1990; Desypris 1993) soleus muscles. At the motor unit level all the evidence points towards a transformation of the fast motor units to the slow type and not a "take over" by the slow units. It is possible to find motor units in all intermediate stages between fast and slow units while estimation of slow motor unit size does not show any increase (Kugelberg 1976). Alternative explanations, such as transference of fibres from fast to slow motor units or atrophy of fast fibres compensated by proliferation of fibres in slow units, are ruled out by the unaltered size of the slow motor units estimated by way of mapping with the glycogen depletion method (Kugelberg 1976).

2.3.4 Hormonal factors

The literature on muscle plasticity has been dominated by work on the mechanisms of neural regulation, and only relatively recently have scientists recognized the influence of the entire milieu in which muscles differentiate. In particular the role of endocrine secretions such as the thyroid hormones has been acknowledged. The first demonstration of a clear role of thyroid hormone levels in determining the phenotypic character of muscle myosin was provided by Ianuzzo et al.(1977). They studied the effects of hypothyroidism and hyperthyroidism on the fibre type composition of soleus muscle in young rats. Using ATPase histochemistry, they reported that hypothyroidism leads to a marked reduction in the percentage of type II fibres and hyperthyroidism to an increase in the percentage of type II fibres. This finding has since been supported by Fitzsimons et al. (1990) who, using ATPase histochemistry, non-dissociating gel electrophoresis and polyacrylamide gel electrophoresis, demonstrated that in slow muscles, hyperthyroidism is associated with an increase in the relative amount of fast myosin, whereas hypothyroidism results in a relative increase in the slow myosin content.

Subsequently, it was shown that thyroid hormone plays a definite role in the developmental switching of MHC gene expression. Increase in plasma concentration, which occurs during development, is strongly correlated with repression of neonatal isomyosin synthesis and activation of adult type II isomyosin synthesis (d'Albis et al. 1987; Gambke et al. 1983; Russell et al. 1988). The muscle variability in the timing of transitions from neonatal to adult type isomyosin suggests that each muscle may display a specific sensitivity to the thyroid hormone (d'Albis et al. 1990). Indeed the hormone thyroxine has been shown to be a potent tissue-specific regulator of MHC gene expression. Izumo et al. (1986) analyzed, by S1 nuclease mapping, the expression of the different MHC genes in normal, hypothyroid and hyperthyroid rats. They demonstrated that each MHC gene analyzed displays heterogeneity in its response to thyroid hormone when expressed in different muscles. They reported that in slow muscles such as soleus, hyperthyroidism is associated with an up-regulation of type IIA MHC messenger RNA expression, whereas hypothyroidism is associated with an up-regulation of type I MHC messenger RNA expression. By contrast in fast muscles, such as extensor digitorum longus (EDL) hypothyroidism is associated with an increase of type IIA mRNA.

While at first sight this highly tissue specific effect of thyroid hormone may appear rather puzzling, it is in fact exactly what one might expect if one accepts the hypothesis that fibre types exist as part of a continuum in which type IIB and type I represent extremes between which lie type IIA and type IIX MHC. It has been proposed (Mabuchi et al. 1982) that type IIB fibres can be converted into type IIA fibres by an appropriate stimulus (e.g. activity pattern, thyroid hormone status).This suggestion was based upon ATPase histochemistry, however type IIA and type IIX fibres can easily be confused if identified using routine histochemistry (Gorza 1990; Parry and Zardini 1990) and it is possible that the observed conversion was to type IIX rather than IIA fibres. The problem is compounded by the fact that type IIA and IIX MHCs co-migrate on 6% polyacrylamide gels. It is possible that interconversion between IIB and IIX is an obligatory step in the process of IIA-IIB interconversion. If we assume that the combinations of MHCs reflect preferential or obligatory pathways of MHC transitions, the following sequence could be postulated:

Hypothyroidism

⇦ ⬅ ⇦

I ⇆ I+IIA ⇆ IIA ⇆ IIA+IIX ⇆ IIX ⇆ IIX+IIB ⇆ IIB

⇨ ➡ ⇨

Hyperthyroidism

One interesting problem emerges from the study of Izumo et al. (1986). In diaphragm, which contains a significant number of type I fibres and has high levels of type I MHC mRNA, hyperthyroidism caused a significant reduction in type I mRNA but did not affect the levels of either IIA or IIB mRNA. Since diaphragm contains a considerable proportion of type IIX fibres, it is possible that hyperthyroidism leads to an enhanced expression of IIX MHC isoform, whose message was not recognized by the cDNA probes used.

The site of action of thyroid hormone regulation of MHC gene expression has been described in detail (Ianuzzo et al. 1990; Mahdavi et al. 1987, 1989); it is thought to be due to binding of thyroid hormone to nuclear receptors which act through specific sequence elements positioned in the responsive genes. All skeletal muscle MHC genes identified so far are responsive to thyroid hormone but (as mentioned above) the response is differential and depends not only upon the gene involved, but on the particular muscle in which it is located (Izumo et al. 1986; Gustafson et al. 1985; Mahdavi et al. 1987).

The precise mechanism of action of thyroid hormone on skeletal MHC gene expression remains obscure, although Russell et al. (1988) have shown that denervation does not appear to modify the time course of the thyroid hormone-induced precocious neonatal-to-adult fast MHC transition, suggesting that a nerve-mediated influence on the muscle tissue can be ruled out.

CHAPTER 3

Identification, distribution and myosin subunit composition of type IIX fibres in mouse muscles

3.1 Introduction

To date the distribution of the recently described fast MHC, known as either IIX MHC (Schiaffino et al. 1989) or IID MHC (Bar and Pette 1988) in different muscles remains a matter of discussion. Furthermore, its demonstration raises the question of the relationship of this "fast-twitch" fibre type to the other two "fast-twitch" types, IIA and IIB. The existence of intermediate fibre types containing multiple MHC isoforms have been reported (Betto et al. 1986) but the possible existence of hybrid fibres containing IIB or IIA MHC and IIX MHC is not yet known. Moreover, MLC composition of IIX fibre types has never been reported; thus the contribution of this MHC to specific isomyosins remains unknown.

Therefore the present study was undertaken to investigate the distribution of IIX MHC and IIX fibre types in more detail. For this purpose, IIX MHC expression was studied in whole mouse muscles and in isolated single fibres. The single-fibre analyses were used with two objectives in mind: 1) to determine the existence of co-expression of different MHCs and, thus, to elucidate the possible sequence of changing MHC expression in transforming muscle fibres and 2) to study the MLC composition of single fibres of identified MHC composition, with particular emphasis on the MLC composition of IIX fibre types.

3.2 Materials and methods

3.2.1 Animals and muscles

The strain of mice used in this study was the C57BL/6. They were originally obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and have since been bred in our own facility in the Health Science Building, University of Ottawa. They were quartered in standard cages with food (Prolab RMH 4020 pellets, Agway, Syracuse, NY) and water supplied *ad libitum*. Constant temperature and relative humidity were maintained for optimal health conditions.

All procedures were performed while the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.07mg/g body weight; Somnotol, M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada), unless indicated otherwise. The animals were sacrificed by overdose of the barbiturate. Extensor digitorum longus (EDL), tibialis anterior (TA), soleus, gastrocnemius, diaphragm and tongue muscles were used.

3.2.2 Whole muscle analyses

3.2.2.1 Muscle removal and preparation for immunohistochemistry

EDL, TA, soleus, gastrocnemius, diaphragm and tongue muscles were removed from the animals, bathed in a solution of 0.9% saline, blotted dry and weighed. They were then frozen in isopentane precooled in liquid nitrogen, placed in small plastic vials and stored at - 80° C until further processing. The muscles were placed on a metal holder in an upright position such that the muscles long axis was perpendicular to the flat surface of the metal holder, serial transverse sections 10 μm thick were cut from the mid-belly region on a Microm H M 500 OM cryostat microtome (Microm Laborgeräte GmbH, Heidelberg,FRG) set at - 20° C, and placed on gelatin coated slides. The sections were either stained immediately with monoclonal antibodies against the myosin heavy chains or stored at - 20° C. The muscles were wrapped in adult mouse diaphragm and then frozen. The diaphragm muscle limited loss of muscles fibres at the periphery of the muscle.

3.2.2.2 Muscle removal and preparation of muscle extract for electrophoretic analyses

EDL, TA, superficial TA (TAS), soleus, diaphragm muscles were removed from the animals, bathed in a solution of 0.9% saline, blotted dry and weighed. They were then placed on ice in small plastic vials. Extracts were prepared using one of the following two procedures.

Crude extract preparation

Muscle extracts were prepared as described by Sartore (1987). The muscle was homogenized in 7 volumes of the following medium: 20 mM KCl, 2 mM K_2HPO_4, 1 mM EGTA, pH 6.8. The homogenate was centrifuged for 45 seconds in an Eppendorf centrifuge and the supernatant containing the soluble fraction (non-myofibrillar) was discarded. The following medium was then added: 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 1 mM EGTA, pH 9.5 and the tube was left on ice at 4° C for 15 minutes to solubilize the extract. The crude extract was then centrifuged in an

Eppendorf centrifuge for 10 minutes. The supernatant (muscle extract) was kept and immediately assayed for its protein content using a Bio-Rad Protein Assay Kit II. The muscle extract was then diluted in sample buffer (62.5 mM Tris/HCL, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% mercaptoethanol, 0.001% Bromophenol Blue, pH 6.8) to a final protein concentration of 0.5-1.0 mg/ml and boiled for 2 minutes. Samples were stored at - 20° C until use.

Filament preparation

Myosin was extracted as described by Butler-Browne and Whalen (1984). Muscle samples were kept on ice for 20 minutes in 4 volumes of extraction buffer (300 mM NaCl, 100 mM NaH_2PO_4, 50 mM Na_2HPO_4, 1 mM $MgCL_2$, 10 mM $Na_4P_2O_7$, 10 mM EDTA, 0.1% 2-mercaptoethanol, pH 6.5) and then centrifuged in a microcentrifuge in the cold room for 30 min. The supernatant, which contained the extracted myosin, was diluted in 9 volumes of low salt buffer (1 mM EDTA and 0.1% 2-mercaptoethanol (v/v)) and the mixture was left overnight at 4° C to allow precipitation of myosin filaments. The filament solution was subsequently centrifuged, the supernatant discarded and the pellet then dissolved in myosin sample buffer (0.5 mM NaCl, 10 mM NaH_2PO_4) and left overnight at 4° C. To assure solubilization the samples were then gently mixed and diluted in 1:100 SDS in sample buffer (62.5 mM Tris/HCl, 2% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.001%

Bromophenol Blue, pH 6.8). The samples were then boiled for 2 minutes and stored at - 80° C.

When MHC extracts from the same muscle prepared using the crude muscle extract and the filament preparation were compared, extra bands were observed only in the crude extracts, though the MHC content was the same in both cases.

3.2.2.3 Myosin immunohistochemistry

Monoclonal antibodies (mAb's) against type I MHC (BA-D5), type IIA MHC (SC-71), all MHC isoforms excluding type IIX (BF-35) and type IIB MHC (BF-F3) were used (Schiaffino et al. 1986, 1989). They were generously provided by Dr. S. Schiaffino, Padua, Italy. Sections were incubated in a moist chamber with the primary mAb at room temperature (RT) for 20 h. All antibodies were optimally diluted (Table 2.1) in 0.5% bovine serum albumin (BSA) in 25 mM phosphate buffered saline (PBS). The PBS was made by mixing 25 mM K_2HPO_4 with 25 mM KH_2PO_4 to pH 7.4 and then adding NaCl to final concentration of 0.9% w/v. The sections were then rinsed in PBS for 30 minutes changing the PBS every 10 min. Excess PBS was removed from the spaces between sections with a Kimwipe tissue. They were incubated with a horseradish peroxidase (HRP)-conjugated rabbit-antimouse secondary antibody (IgG or IgM; Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 3 h at RT.

Table 3.1 Myosin heavy chain monoclonal and secondary antibodies and relative concentrations used for immunohistochemistry.

Myosin Heavy Chain	Primary mAb	Dilution	Secondary mAb	Dilution
I	BA-D5	1:500	IgG HRP	1:50
IIA	SC-71	1:300	IgG HRP	1:50
IIB	BF-F3	1:50	IgM HRP	1:50
I, IIA, IIB	BF-35	1:50	IgG HRP	1:50

The sections were rinsed again in PBS and then developed by submerging in 0.1% (w/v) 3'3 diaminobenzidine (DAB)(Sigma Chemical, St. Louis, MO) and 0.03% (v/v) aqueous solution H_2O_2 in PBS and processed in the dark for about 5 min. They were rinsed in distilled water, dehydrated through successive alcohol baths of increasing concentration, cleared in xylene substitute and mounted in Permount (Fisher).

3.2.2.4 Electrophoretic identification of myosin heavy chains

The MHCs were separated on gel slabs (0.75mm thickness) consisting of an 11.5 cm separating gel and a 4.5 cm stacking gel. Gels were prepared from a stock solution of 30% acrylamide: i.e. 29.2% (w/v) acrylamide and 0.8% (w/v) Bis (N,N'-

bis-methylene acrylamide) according to Laemmli (1970). Separating gels of T = 5% and 6% and C=2.7% (T=total concentration of acrylamide + Bis, C= percentage of total monomer due to Bis) were used with stacking gels of T = 3 and 4% and C=2.7%. Final concentrations of Tris/HCl in both stacking and separating gels were as previously described (Laemmli 1970). 30% glycerol was routinely added to the separating gel (Danieli-Betto et al. 1986). Polymerization was activated by addition of 0.03% (w/v) ammonium persulfate (APS) and 0.17% (v/v) tetramethylethylenediamine (TEMED). Separating gels were allowed to polymerize for at least 2 h at RT and were either used that day or stored one day at 4° C. Electrophoresis was performed with 0.1% (w/v) SDS/25 mM Tris/glycine running buffer, pH 8.3. One of two voltage-time regimes was used: 1) 100 V during stacking of the proteins and then 200 V for 4 h at constant voltage and at RT or 2) 120 V for 24 h at constant voltage and at 15° C. Separating gels were silver stained by the method of Morrissey (1981) upon completion of the electrophoresis protocol.

To try to achieve a better separation gradient gels consisting of a T = 5-8% separating gel and a T = 3.5% stacking gel (C = 2.6%) were also run. Glycerol was added to the gel matrix, 40% (w/v) to the separating gel and 35% (w/v) to the stacking gel. The samples were electrophoresed first at 50 V until the tracking dye completely entered the separating gel, and then at 150 V for 18 h at 8° C. The gels were silver stained (Morrissey 1981).

3.2.2.5 Western immunoblot analysis

Electrophoretic transfer of MHC proteins from unstained gels to nitrocellulose sheets was carried out as described by Towbin (1979). The blots were placed in saturation solution (50 mM Tris/HCl, 2 mM $CaCl_2$, 85 mM NaCl, 3% BSA, pH 8.0) for 1 h at 37° C, and then incubated in optimally diluted primary mAbs (Table 3.2) for 2 h at RT on a shaker.

Table 3.2 Myosin heavy chain monoclonal and secondary antibodies and relative concentrations used for immunoblot analysis.

Myosin Heavy Chain	Primary mAb	Dilution	Secondary mAb	Dilution
I, IIA	BF-32	1:1250	IgM HRP	1:500
IIA, IIB, IIX	SC-75	1:2000	IgG HRP	1:500
IIB	BF-F3	1:500	IgM HRP	1:500

The blots were then rinsed in wash solution (saturation solution with 0.2% NP-40 and 0.1% BSA) for 30 min., changing the solution every 10 min. This was followed by incubation in the secondary goat anti-mouse IgG or IgM HRP conjugated mAb solution for 1 h at RT on a shaker. All antibodies were diluted in saturation solution. The blots were rinsed again and then developed for 2-3 min in a solution of 100 mM

Tris/HCl/10 mM imidazole (pH 7.6) containing 0.005% (w/v) DAB and 0.001% (v/v) 30% H_2O_2. The reaction was stopped by transferring the blots to water. The blots were then placed on filter paper to dry.

3.2.3 Analysis of groups of fibres of the same type

Type IIX fibres, as other fibre types, are scattered throughout the muscle (mosaic arrangement) making it difficult to obtain a sample of pure type IIX fibres. I have, therefore, made use of our observation that clusters of type IIX fibres exist in muscles which have been reinnervated following sciatic nerve crush (the so called "fibre-type-grouping" (Karpati and Engel 1968) is a well-described phenomenon that will be examined in the discussion section of this Chapter).

Surgery was performed on 5-10 day old mice anesthetized with ether. The sciatic nerve was surgically exposed and crushed in the mid thigh region of the right leg, with care taken not to damage the blood supply. The incision was closed with 6/0 silk and cleansed with distilled water. The animals were allowed to recover for 8 weeks, during which time they were checked daily for infection until the wound had completely healed. At the end of the 8 weeks recovery the muscles from the right leg were removed, rapidly frozen, sectioned and stained as previously described with the following modifications. In addition to 10μm sections, serial 20μm were also cut and mounted on gelatin-coated slides. The 20μm sections were left unstained. The 10 μm sections were processed for immunohistochemistry using monoclonal antibodies as described. The immunohistochemically processed sections were analyzed to identify the presence of "fibre type-groups", i. e. clusters of fibres that express the same MHC, and the corresponding area was microdissected from the 20μm thick, unstained section,transferred to sample buffer and run on 6% SDS-PAGE. Western immunoblot analysis was performed as previously described.

3.2.4 Single fibre analysis

3.2.4.1 Muscle removal and preparation

TAS and diaphragm muscle were used. Small bundles of fibres were tied to wooden sticks, removed from the muscles and immersed in skinning solution (5 mM K_2EGTA, 170 mM K propionate, 2.5 mM Na_2K_2ATP, 2.5 mM Mg propionate, 10 mM Imidazole buffer, pH 7.0) for 24 h, with changes of solution at 1, 2, 4 and 8 hours (Salviati 1982). The muscle strips were then stored at - 20° C in a mixture of 50% glycerol and 50% skinning solution. When required a small bundle of fibres was cut from the original strip under a dissecting microscope and single fibres were pulled out from one end of the bundle. The fibres were transferred to a capillary tube containing 20μl of sample buffer and boiled for 2 minutes. 10% of the extract from the same single fibre was used for MHC and 90% for MLC identification.

3.2.4.2 Separation of myosin light chain

In some experiments electrophoretic separation of MLC from single muscle fibres was achieved by two-dimensional gel electrophoresis (see below). However this method did not allow the quantification of the different MLC present in one single fibre. Consequently, one-dimensional gel electrophoresis was used in most cases with subsequent densitometric scanning of the gels.

Two dimensional separation

MLCs were separated by 2-dimensional gel electrophoresis (O'Farrell 1975), using a Bio-Rad mini-gel electrophoresis apparatus. The first dimension gels were prepared from a first dimension gel monomer solution consisting of 9.2 mM urea, 4% acrylamide (stock solution of 29% (w/v) acrylamide and 1% (w/v) piperazide diacrylamide (PDA)), 2% Triton X-100 , 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% APS, 0.1% TEMED. The monomer solution was drawn into capillary gel tubes and allowed to polymerize overnight. Pre-electrophoresis and first dimension electrophoresis were performed with 20 mM NaOH (upper chamber buffer) 10 mM H_3PO_4 (lower chamber buffer). Samples were loaded onto the tube gels and run at 500 V for 10 min. and then 750 V for 3.5 h. Upon completion of the first dimension run, the tube gels were extruded onto a piece of Parafilm laboratory film and slid onto the slab gel (second dimension). The second dimension was run on mini slab gels (7 cm height x 9 cm breadth x 1 mm thick) (Bio-Rad mini - Protean II). Separating gels of T = 12% were used with stacking gels of T = 4% at C=2.7%. Polymerization was activated by addition of 0.03% (w/v) APS and 0.17% (v/v) TEMED. Studies were performed with separating gels that had been allowed to polymerize at least 2 h at RT and either used that day or stored one day at 4°C. Electrophoresis was performed with SDS/25 mM Tris/Glycine running buffer (pH 8.3). Stacking gels were run for 15 min. at 100 V and separating gels for 45 min. at 200 V at constant voltage and at RT. Separating gels were silver stained by the method of Morrissey (1981).

One dimensional separation

MLCs were separated on 0.75 mm thick gel slabs consisting of an 11.5 cm separating gel and a 4.5 cm stacking gel. Gels were prepared from a stock solution of 30% acrylamide consisting of 29.2% (w/v) acrylamide and 0.8% (w/v) Bis (Laemmli 1970). Separating gels of T = 12.5% were used with stacking gels of T = 4% at C = 2.7%. Polymerization was activated by addition of 0.03% (w/v) APS and 0.17% (v/v) TEMED. Separating gels were allowed to polymerize for at least 2 h at RT and were either used that day or stored one day at 4° C. Electrophoresis was performed with 0.1% (w/v) SDS/25 mM Tris/glycine running buffer (pH 8.3). Running conditions for the gels were: 15 mA/plate at constant current for about 5 h at RT. To aid in identification of the separated proteins, molecular weight standards were also electrophoresed. They included a Rainbow protein molecular weight marker (Amersham) and rabbit fast skeletal muscle MLCs. Separating gels were silver stained by the method of Morrissey (1981).

3.2.4.3 Densitometric analysis of myosin light chains

Quantification of the staining density of the MLCs separated on SDS-PAGE was performed densitometrically by scanning the dried silver stained gels. A system consisting of a light box (precision illuminator model 890, Imaging Research Inc.), a high resolution monochrome camera designed for imaging applications (Nikon), a Macintosh personal computer and software applications program (Image, Twilight Clone BBS, Silver Spring, MD) was used.

3.2.5 Statistical analysis

The Statistical Analysis System (SAS) for Personal Computers software package (SAS Institute Inc., NC, USA) was used for all statistical computations. The means and S.E.M. were calculated for all the variables measured. A one-way analysis of variance (ANOVA) was used to compare the different proportion of MLC in various fibre types. If the one-way ANOVA revealed a significant treatment (fibre type) F ratio then post hoc t' test comparisons of least-squares means were performed to determine precisely differences among fibre types and the probability associated with the differences.

3.3 Results

3.3.1 Immunohistochemical demonstration of IIX fibres in mouse muscles.

Figure 3.1 shows serial sections of a mouse EDL muscle stained with 4 different monoclonal antibodies to MHC as well as with routine histochemistry for succinic dehydrogenase (Nachlas et al. 1957) and myofibrillar ATPase (Brooke and Kaiser 1970). Few if any slow-twitch type I fibres are present in fast-twitch muscles of the mouse (Parry and Desypris 1983) as may be seen by the lack of positive staining with BA-D5 (Fig. 3.1.d) as well as with ATPase at pH 4.3 (Fig. 3.1.h). It can be seen that there is a population of fibres, labelled with an asterisk, which does not stain with any of the antibodies used. Therefore they contain neither type IIB MHC (Fig. 3.1.a) nor type IIA MHC (Fig. 3.1.c). These are type IIX fibres. They display high SDH activity (Fig. 3.1.e) and when stained for myofibrillar ATPase following alkaline preincubation (Fig. 3.1.f) they are indistinguishable from type IIA fibres while the larger type IIB fibres are slightly paler. On the other hand, following preincubation at pH 4.6 (Fig. 3.1.g) the situation is reversed, the IIX fibres appearing closer in intensity of staining to the IIB fibres while IIA fibres are somewhat paler.

Serial sections of a mouse TA muscle stained with three different monoclonal antibodies are shown in Figure 3.2. It can be seen that there are some fibres, labelled with an X, which are not stain with any of the three monoclonal antibodies used. IIX fibres represent a significant proportion of the fibres in fast-twitch muscles of the mouse, for example approximately one-third in TA and EDL (Fig. 3.3.C,D) and a rather larger fraction in the diaphragm and tongue (Fig. 3.3 A,B). The diaphragm muscle is devoid of IIB fibres in most areas (Fig. 3.4.A) although a few IIB fibres may be present in the portion closest to the crural region. In TA muscle there appears to be a gradient of IIX fibres across the muscle with relatively few (approximately 20%) in the superficial region increasing to slightly more than 50% in the deeper region (Fig. 3.5.B). The superficial portion of this muscle does not contain any fibres which react with the antibodies to either type IIA or type I MHC (Fig. 3.5.C,D).

Figure 3.1 Immunohistochemical and histochemical analysis of muscle fibre types in adult mouse EDL muscle. Indirect immunolocalization was carried out on 10 μm serial sections with Mabs specific for (a) BF-F3 (anti-IIB), (b) BF-35 (anti-I, IIA, IIB), (c) SC-71 (anti-IIA), (d) BA-D5 (anti-I). (Other sections were reacted for SDH (e), myofibrillar ATPase, pH 10.4 (f), myofibrillar ATPase, pH 4.6 (g) or myofibrillar ATPase, pH 4.3 (h) histochemistry. Fibres marked A and B are type IIA and IIB fibres respectively. Fibres marked with an asterisk are type IIX fibres.

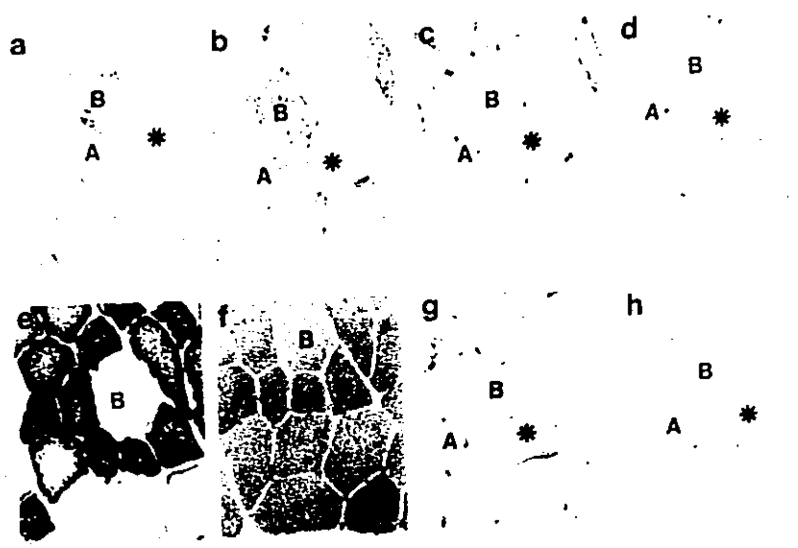

a
B
A
*
b
B
A
*
c
B
A
*
d
B
A
*
e
B
f
B
g
B
A
*
h
B
A
*

Figure 3.2 Immunohistochemical analysis of muscle fibre types in adult mouse TA muscle. Indirect immunolocalization was carried out on 10 μm serial sections with Mab specific for (A) IIA MHC (SC-71), (B) I, IIA, IIB MHCs (BF-35) (C) IIB MHC (BF-F3). IIX fibres are labelled with X, IIA fibres with A and IIB fibres with B. Bar represents 100 μm

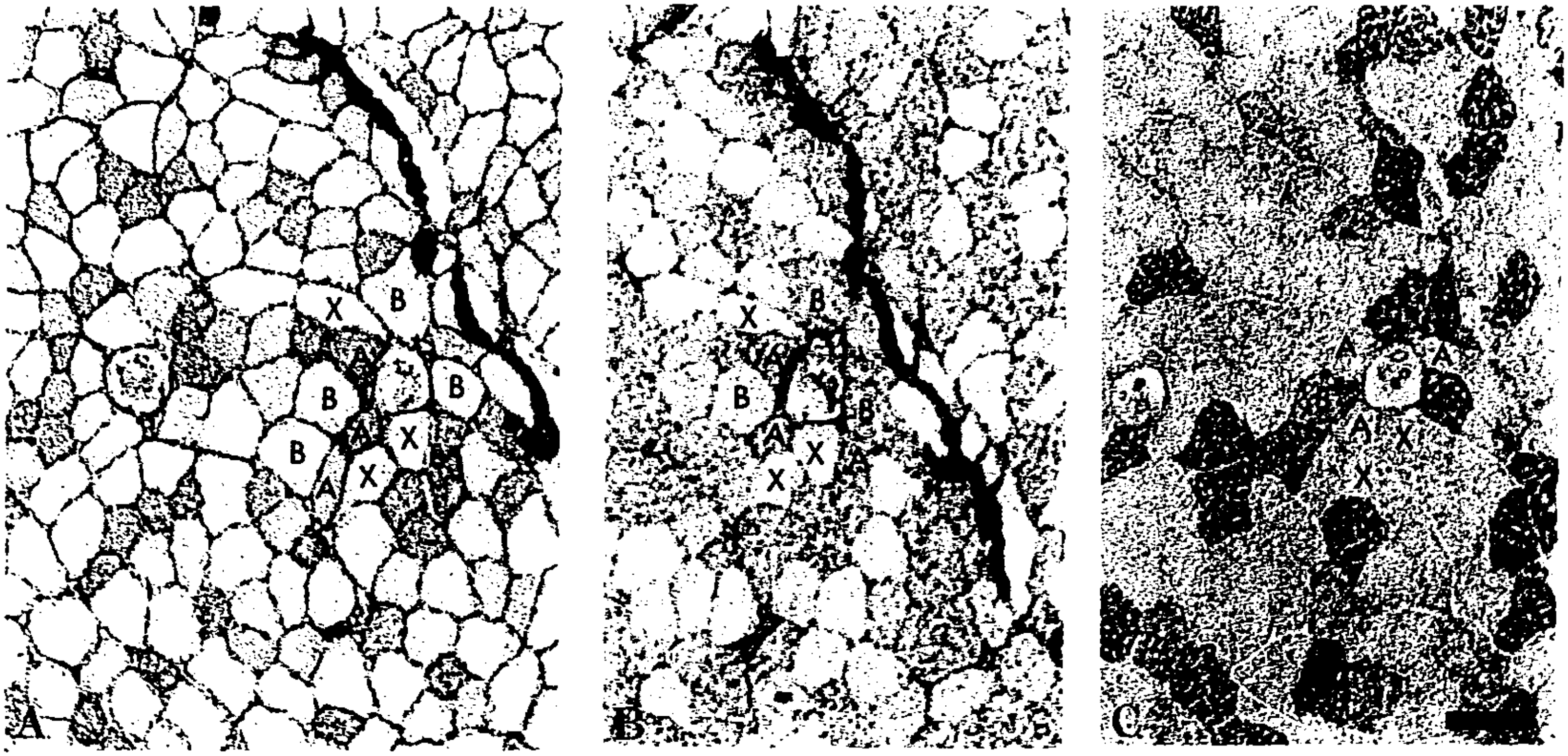

A
X
B
B
B
B
A
X
A
X
B
X
B
B
A
X
X
C
A
A
A
X
X

Figure 3.3 Immunohistochemical analysis of muscle fibre types in adult mouse diaphragm (A) tongue (B) TAS (C) and EDL (D) muscles. Indirect immunolocalization was carried out on 10 μm serial sections with Mab specific for I, IIA, IIB MHCs (BF-35). Note that the fibres that are not stained with this monoclonal antibody are IIX fibres. Bar represents 200 μm

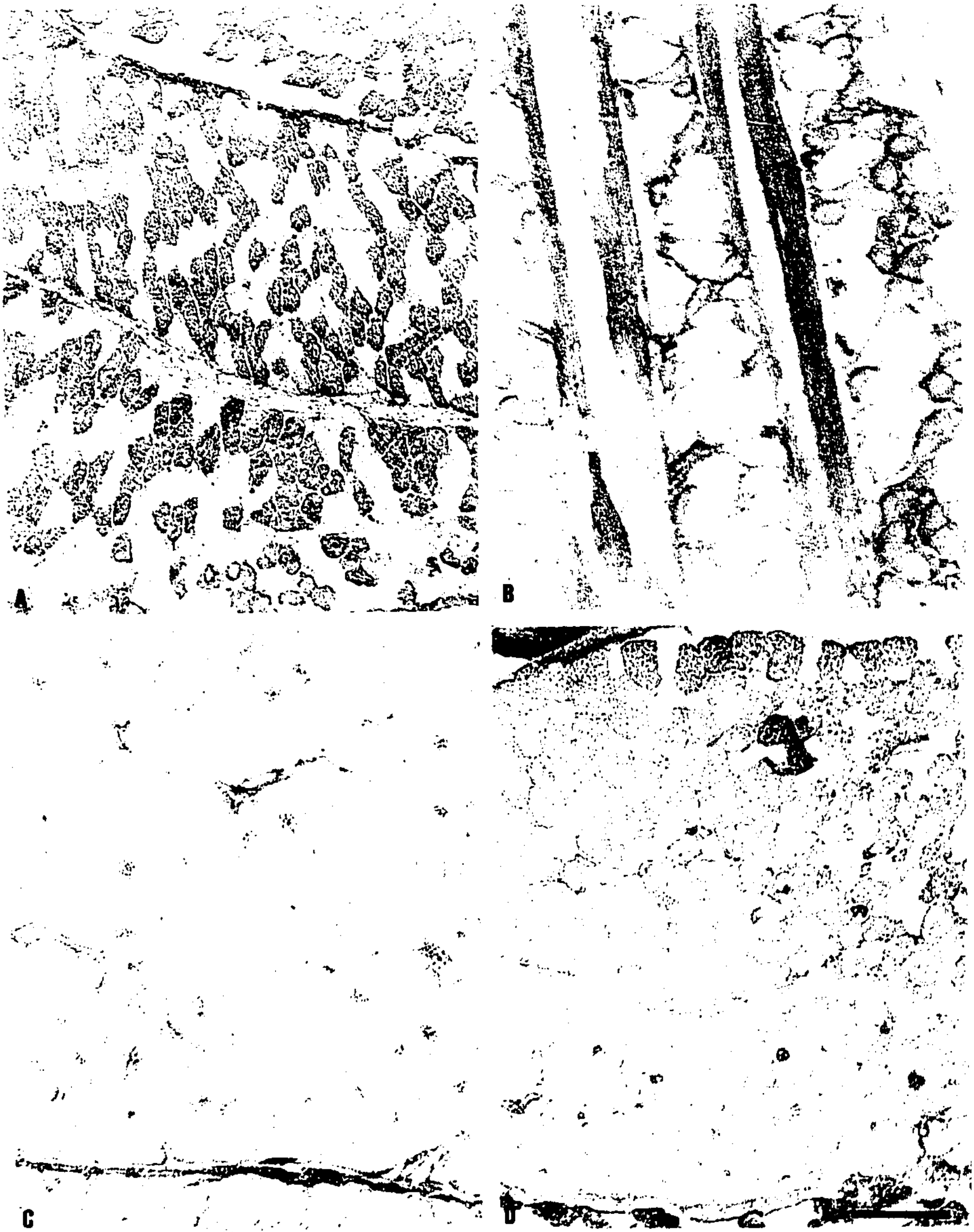

A
B
C
D

Figure 3.4 Immunohistochemical analysis of muscle fibre types in adult mouse diaphragm muscle. Indirect immunolocalization was carried out on 10 μm serial sections with Mab specific for (A) IIB MHC (BF-F3), (B) I, IIA, IIB MHCs (BF-35) (C) IIA MHC (SC-71), (D) I MHC (BA-D5). Note that there are no IIB fibres while about half of the fibres are type IIX fibres. Bar represents 400 μm

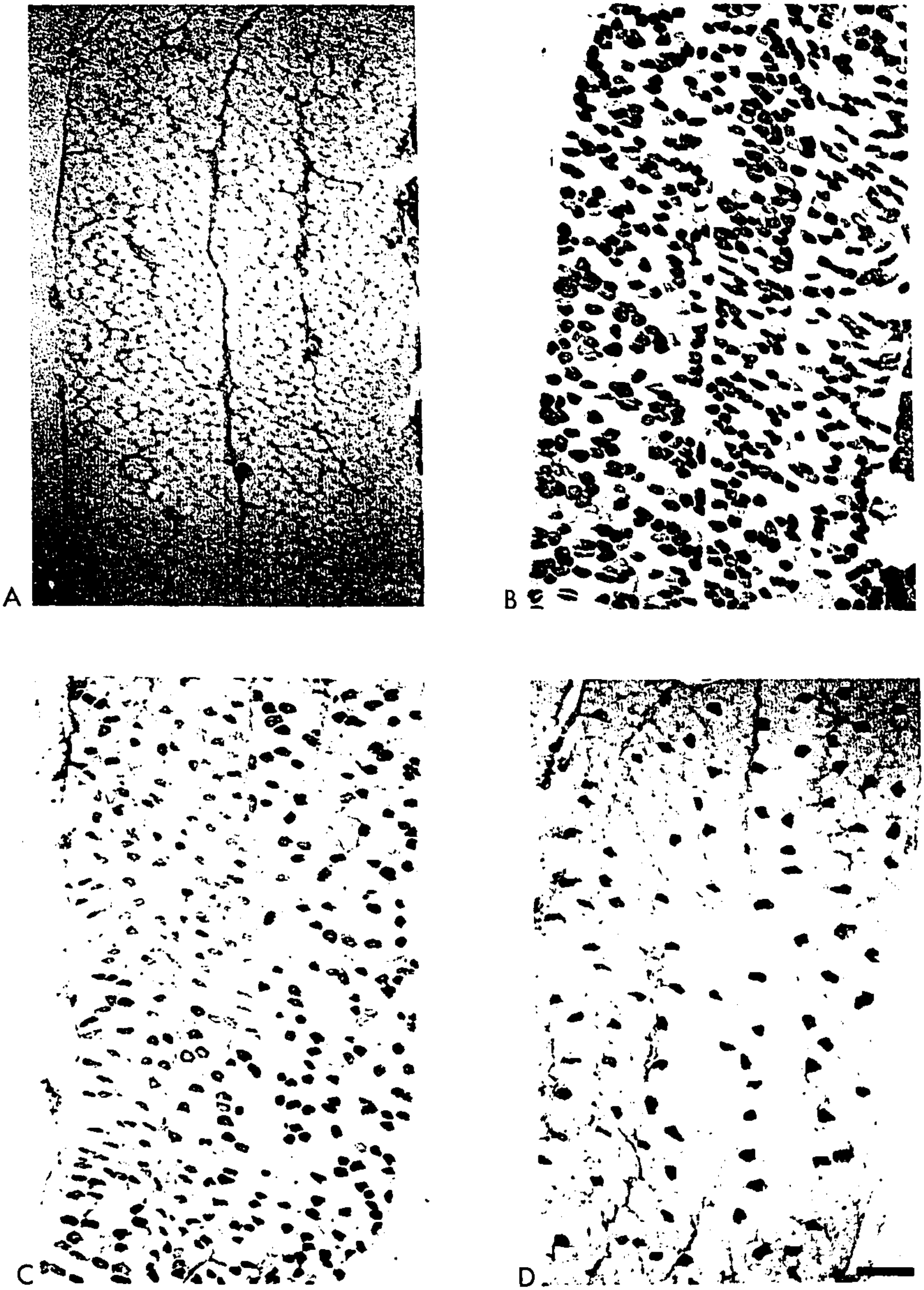
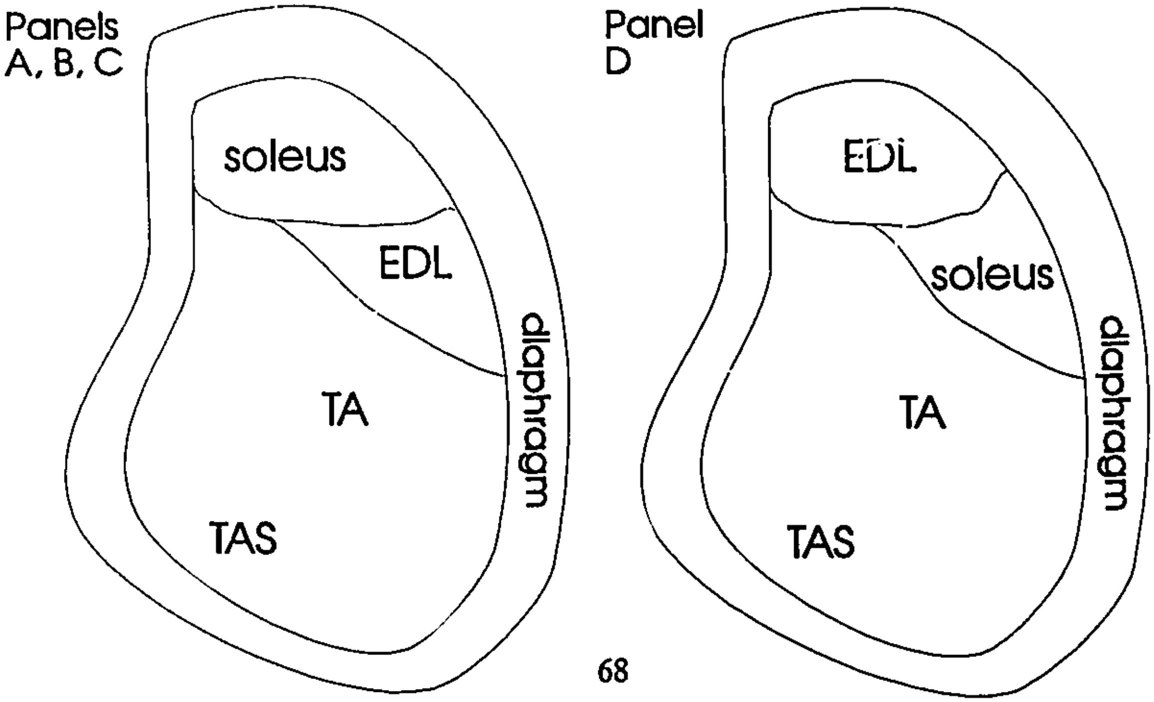

A
B
C
D

Figure 3.5 Immunohistochemical analysis of muscle fibre types in adult mouse TA, EDL, soleus and diaphragm muscles. Indirect immunolocalization was carried out on 10 μm serial sections with Mab specific for (A) IIB MHC (BF-F3), (B) I, IIA, IIB MHCs (BF-35) (C) IIA MHC (SC-71), (D) I MHC (BA-D5). Note that the superficial part of TA muscle does not contain any fibres which react with the antibodies to either type IIA or type I MHC. Bar represents 400 µm

A schematic representation of the arrangements of the different muscles in these serial sections follows.

Schematic representation of Figure 3.5.




A
B
C
D

3.3.2 Electrophoretic demonstration of IIX MHC in mouse muscles.

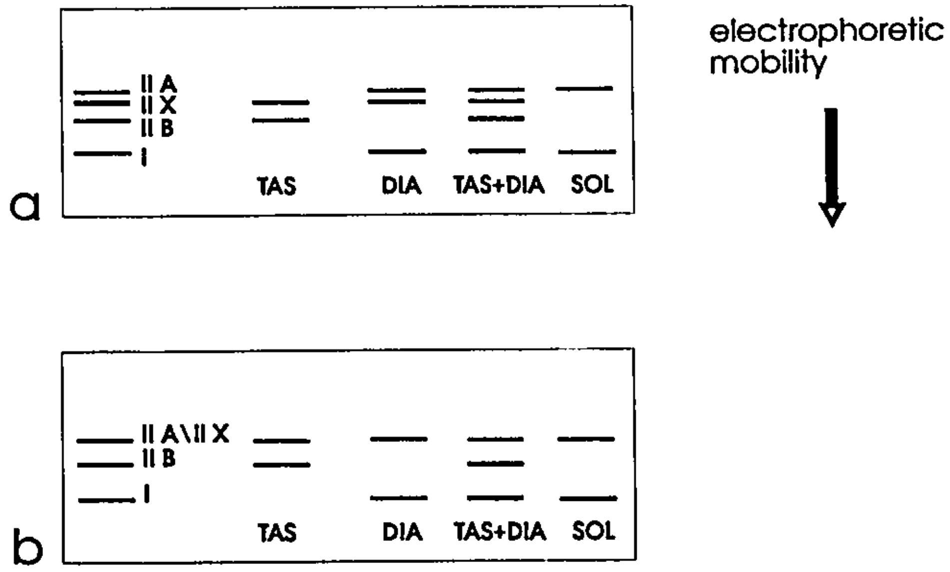
Whole muscle extract

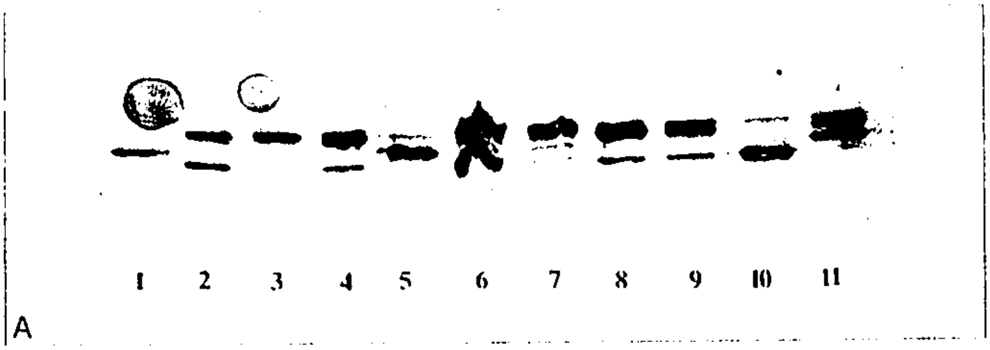
Electrophoresis of crude myosin extracts of mixed muscles on 6% PAGE revealed only three MHC bands. These bands represent in order of increasing speed of migration type IIA, IIB and I MHC. This was verified with Western blotting using the MHC monoclonal antibodies referred to above in the methods section. This suggests that the IIX MHC co-migrates with one of the other bands under the conditions of our electrophoresis. Using 5% and 5-8% gradient gels separation of three fast MHC bands was achieved using rat muscle, but not mouse muscles (Fig. 3.6), despite several modifications to the original electrophoretic protocol. In order to ascertain where the IIX MHC migrates on 6% gels I initially made use of the observation referred to above that TAS contains only type IIB and IIX fibres (in a ratio of approximately 4:1) while diaphragm contains type IIA, IIX and I fibres (approximately 4:5:1) and virtually no type IIB fibres. Fig. 3.7 shows PAGE analysis of MHC from adult rat diaphragm, TAS and soleus. It is clear that TAS extracts contain a faint band which migrates at the level of IIA MHC despite the immunohistochemically demonstrable absence of type IIA fibres in this region (Fig. 3.5). Furthermore, extracts of diaphragm revealed no band migrating at the level of IIB MHC. These results suggested that IIX co-migrated with IIA MHC.

Figure 3.6 (A) 5% SDS-PAGE of mouse and rat muscles. Mouse muscles: (lane 1) TAS; (2) soleus; (3,7) diaphragm. Rat muscles: (5) TAS; (4, 6, 8, 9) diaphragm; (10) soleus; (11) TAS and soleus. Note the electrophoretic separation of the IIA and IIX band in rat (lanes 4, 8, 9, 11) but not in mouse muscles.

(B) 5-8% linear gradient SDS-PAGE of rat soleus (lanes 1, 2) and diaphragm muscles (lanes 3, 4, 5) at different concentrations. Note the separation of 4 electrophoretic band in lanes 4 and 5.

Schematic illustration of the electrophoretic mobilities of MHC isoforms in rat (a) and mouse (b) skeletal muscles.




1 2 3 4 5 6 7 8 9 10 11
A

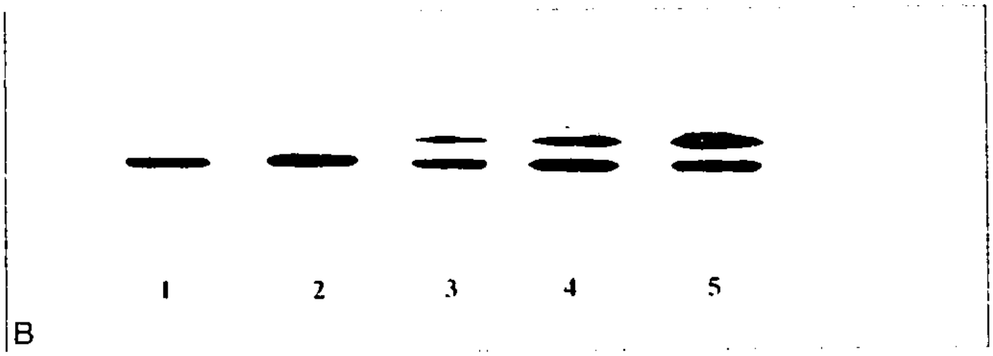
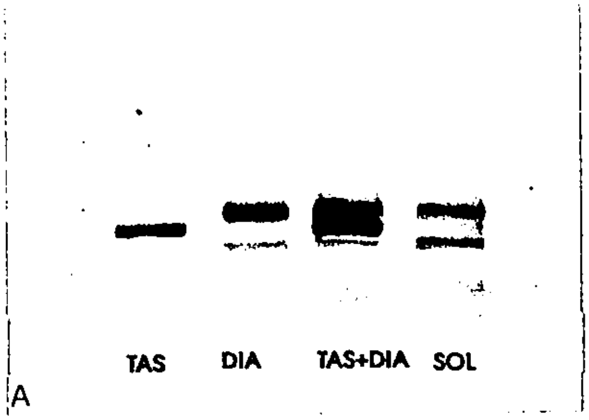

1 2 3 4 5
B

Figure 3.7 (A) SDS-PAGE on 6% gel of mouse whole-muscle extract from TAS, diaphragm, combined TAS and diaphragm, soleus. Note that diaphragm (approximately 50% IIX, 40% IIA and 10% I) shows only 2 bands, one at the level of type I MHC and one at the level of type IIA MHC. Note that TAS (approximately 80% IIB and 20%IIX) show a band at the level of IIB MHC and a faint band at the level of type IIA MHC. (B) Schematic illustration of the electrophoretic mobilities of MHC isoforms in mouse skeletal muscle.


TAS
DIA
TAS+DIA
SOL
A


II A\II X
II B
I
TAS
DIA
TAS+DIA
SOL
B

In order to confirm this I initially endeavoured to extract myosin from a pure population of IIX fibres.

Homogeneous fibre type groups

Figure 3.8 shows sections of mouse gastrocnemius 2 months after sciatic crush at the age of 10 days processed with monoclonal antibodies that recognize IIA MHC, IIB MHC and all adult skeletal muscle MHC except IIX MHC. Figure 3.8.B shows the section processed with BF-35 and the group of pale fibres are type IIX.

Fibres from groups such as the one shown in Figure 3.8 were microdissected from 20 μm frozen sections and run on 6% PAGE. Similar sized groups of type IIB fibres were also used. The results of a typical experiment are depicted in Fig 3.9. In all cases where this procedure was done, two bands were observed in the homogeneous IIX fibre type groups, while a single band was present in the homogeneous IIB fibre type groups. Neither of the bands from the IIX fibre type groups reacted with antibody BF-F3, specific for type IIB MHC (Figure 3.9.B) and BF-32, specific for type I and type IIA MHC (Figure 3.9.C), suggesting that IIA and IIB MHCs were not present as contaminant. However, this interpretation should be treated with caution since occasionally BF-F3 immunoblots are unreliable. Furthermore, as indicated in the legend to Fig. 3.9 the intensity of the protein band at the level of IIB MHC is substantially less than in the adjacent lanes.

Figure 3.8 Immunohistochemical analysis of muscle fibre types in adult mouse gastrocnemius 2 months after sciatic nerve crush. Indirect immunolocalization was carried out on 10 μm serial sections with Mab specific for (A) IIB MHC (BF-F3), (B) I, IIA, IIB MHCs (BF-35) (C) IIA MHC (SC-71). Note the presence of homogeneous fibre type groups in each panel. Bar represents 100 μm


A
B
C

Figure 3.9 (A) 6% polyacrylamide gel of microdissected homogeneous fibre type groups from reinnervated gastrocnemius muscles of mouse, (B) Immunoblot stained with BF-F3 (anti-IIB), (C) Immunoblot stained with BF-32 (anti-I, IIA).

Lane 1: type IIX group of fibres.

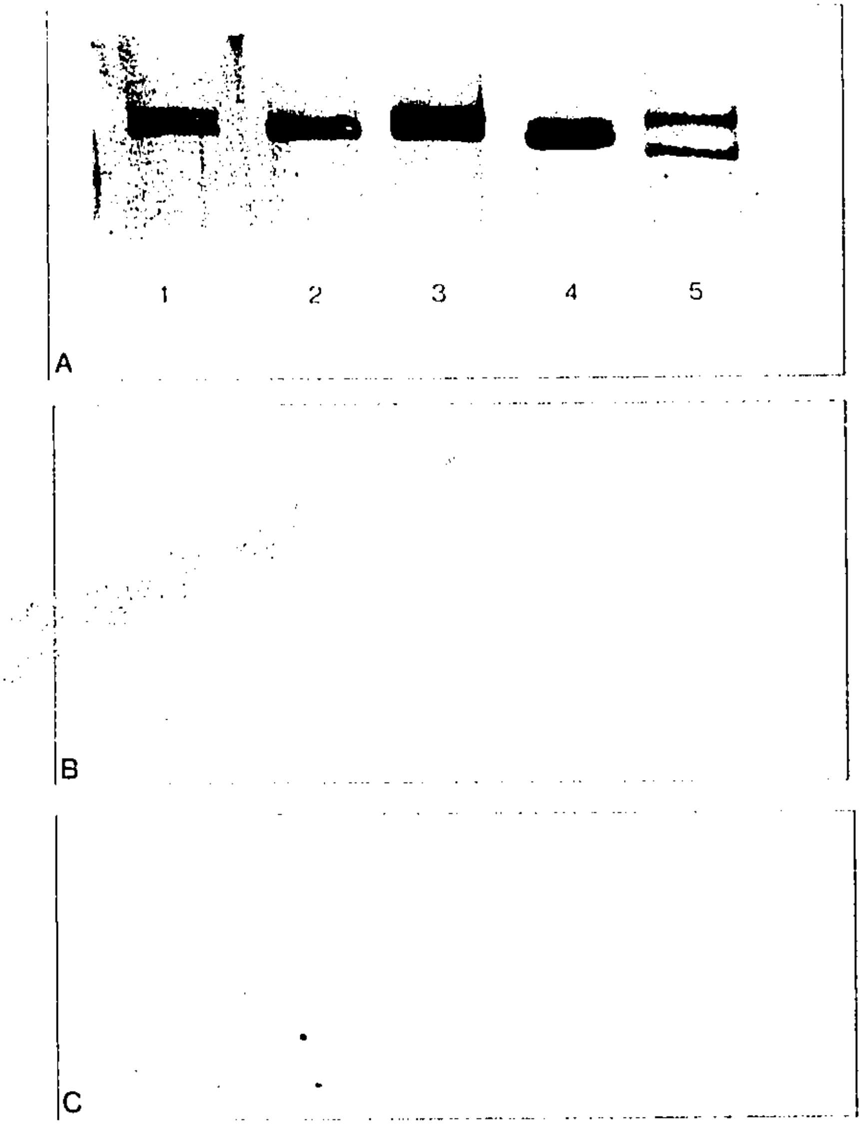
Lane 2: type IIB group of fibres.

Lane 3: combined type IIX and IIB groups of fibres.

Lane 4: TAS muscle extract.

Lane 5: soleus muscle extract.

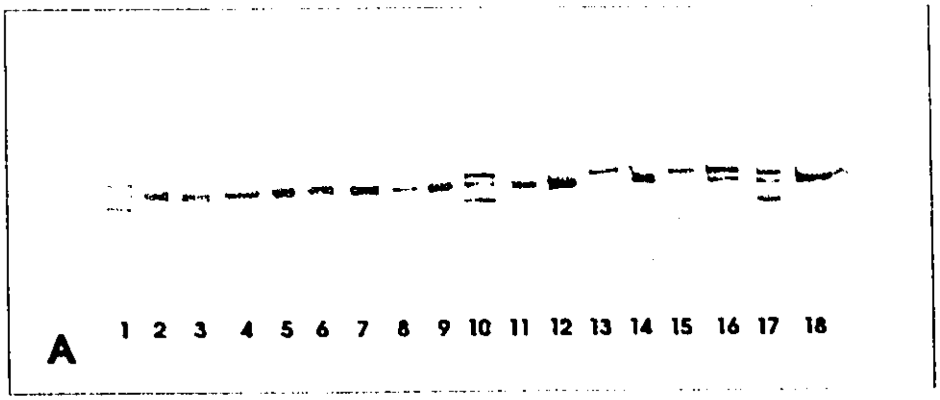
Note that the microdissected IIX group of fibres (lane 1) shows 2 bands that do not stain either with BF-F3 or BF-32. The relatively low amount of IIB MHC, as seen in the silver stained gel, may explain the lack of staining in the BF-F3 immunoblot. In lanes 2 and 3 it may be seen that the density of the band at the level of the IIB MHC in the gel is substantially greater,and indeed this band is stained on the BF-F3 immunoblot. Thus, while we believe that the lower band in lane 2 represents IIB MHC, there is still some doubt remaining.


1
2
3
4
5
A
B
C

Single fibres

Since some doubt remained as to whether some of the IIB fibres which surrounded the IIX type groups were included in the microdissected sample, we also determined the MHC isoform content of a number of single fibres isolated from TAS, which contains approximately 20% IIX fibres, and from diaphragm, which contains almost 50% IIX fibres. Results from typical experiments are shown in Figures 3.10 and 3.11. 78.2% of TAS single fibres showed the presence of a single IIB band, 16.8% showed the existence of a single band at the IIA MHC level that did not react with BF-32, suggesting that it was not IIA MHC, but reacted with SC-75 suggesting that it was a type II MHC (Figure 3.11). 5% of the fibres showed the coexistence of 2 bands identical to those seen in the homogeneous IIX fibre type groups: a IIB MHC band and a second band at the level of type IIA MHC, neither of which reacted on Western blots with BF-32. As might be expected different fibres exhibited different degrees of co-existence, with the dominant isoform being IIX MHC in some cases and IIB MHC in others (Figure 3.12). Single fibres from diaphragm all showed a single band either at the level of IIA/IIX or at the level of type I MHC, only two fibres showed the existence of two bands, type I and IIA/IIX. Table 3.1 summarizes these results obtained by microelectrophoretic analysis of mouse TAS and diaphragm single fibres.

Figure 3.10 (A) 15 single fibres from TAS muscle. Fibres in lanes 13 and 15 are expressing IIX MHC. Fibre in lane 16 co-expresses IIB and IIX MHC, all the other 12 fibres express IIB MHC. Lanes 1, 10, 17 contain soleus and TAS muscle extracts. (B) 6 single fibres from diaphragm. 5 of the 6 show a single band at the level of type IIA/IIX MHC. The fibre in lane 6 shows a band at the level of type I MHC. Lanes 1 and 8 contain TAS and diaphragm muscle extract.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

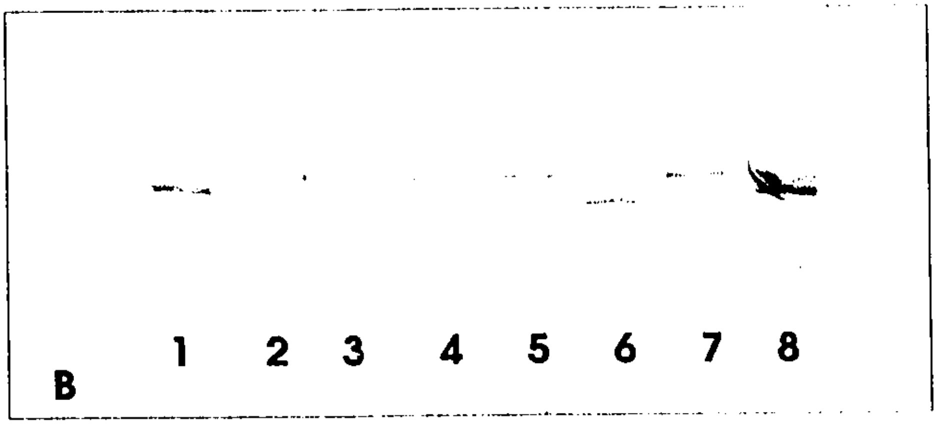

B
1 2 3 4 5 6 7 8

Figure 3.11 (A) 6%polyacrylamide gel showing MHC from 6 single fibres of TAS. (B)Immunoblot stained with SC-75 (anti-type IIA, IIB, IIX), (C)Immunoblot stained with BF-32 (anti-type I and IIA). Note that 1 fibre (lane 3) shows 2 bands and 1 fibre (lane 8) shows one band at the level of IIA, these bands stained with SC-75 but not with BF-32. Fibres in lanes 4, 5, 6 and 7 express IIB MHC, soleus extract (line 1), soleus and TAS extract (line 2).

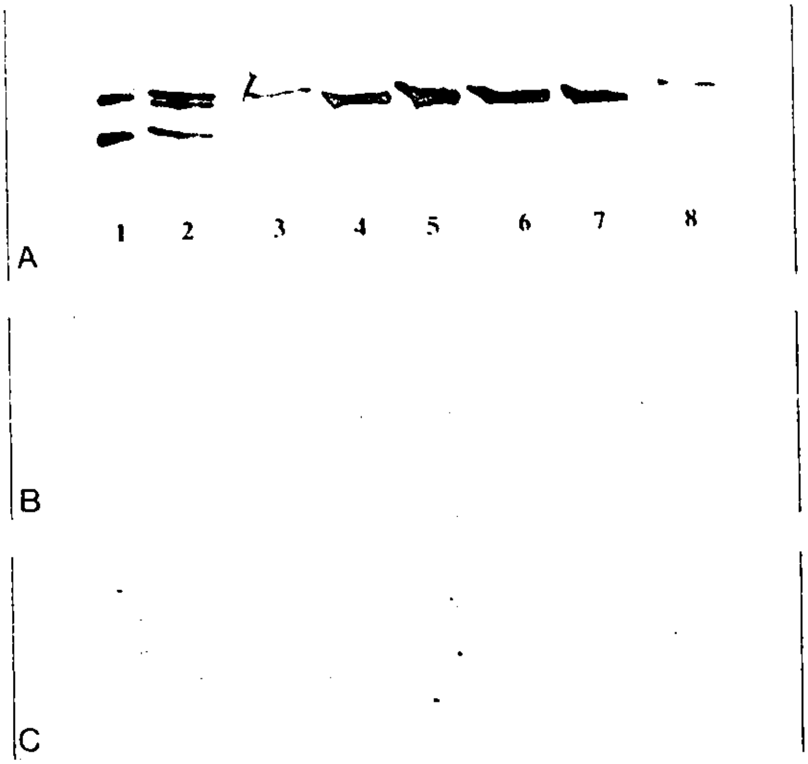

1
2
3
4
5
6
7
8
A
B
C

Figure 3.12 6% PAGE with 6 single fibres from TAS muscle. Note that 3 fibres show the presence of two bands with different intensity (lanes 2, 3, 4). These 3 fibres show co-existence of IIX and IIB MHC in different proportion. Lanes 5 and 7 contain fibres expressing IIB MHC, lane 6 contains a fibre expressing IIX MHC.

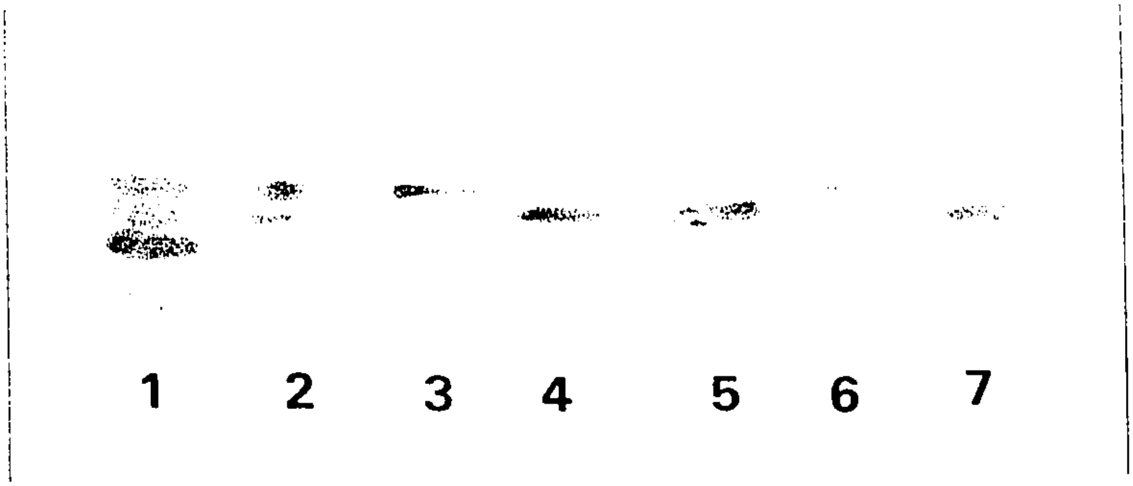

1
2
3
4
5
6
7

Table 3.3 Proportion of fibres in TAS and diaphragm muscles expressing different MHC isoforms. MHC was determined by 6% SDS-PAGE of single fibres.

		MHC					
Muscle	n	I	I+IIA	IIA and IIX	IIX	IIX+IIB	IIB
TAS	101	0	0	0	16.8	5	78.2
Diaphragm	90	6.6	2.2	91.2	0	0	0

n: number of single fibres microelectrophoretically processed.

3.3.3 Immunohistochemical evidence for co-existence of IIX MHC with other MHC

The existence of hybrid fibres, co-expressing IIB and IIX MHC was tested by immunohistochemical techniques. Using BF-F3, a mAb which recognizes MHC IIB specifically and BF-35, a mAb which recognizes all MHC except IIX, a population of fibres could be seen in TA muscle which stain pale, though not completely negative, with BF-35 and which also have varying, low levels of staining with BF-F3 (Fig. 3.13). No fibres where found which showed immunohistochemical evidence of co-existence of IIA and IIB MHC. Occasional fibres could be seen in the deep region of TA which showed co-existence of IIA and IIX MHC (Fig. 3.14). Unfortunately this cannot be confirmed with our gel electrophoretic technique since the two MHCs co-migrate. By contrast single fibres from diaphragm a muscle which contains approximately 50% IIX fibres but few or no IIB fibres did not show co-existence of MHC IIB with either IIA or IIX MHC with immunohistochemistry.

Figure 3.13 Immunohistochemical analysis of muscle fibre types in adult mouse TAS muscle. Indirect immunolocalization was carried out on 10 μm serial sections with Mab specific for (A) IIB MHC (BF-F3), (B) I, IIA, IIB MHCs (BF-35) (C) IIA MHC (SC-71). Note that same fibres show only a faint reaction with BF-F3 and are not completely negative with BF-35. Bar represents 100 μm

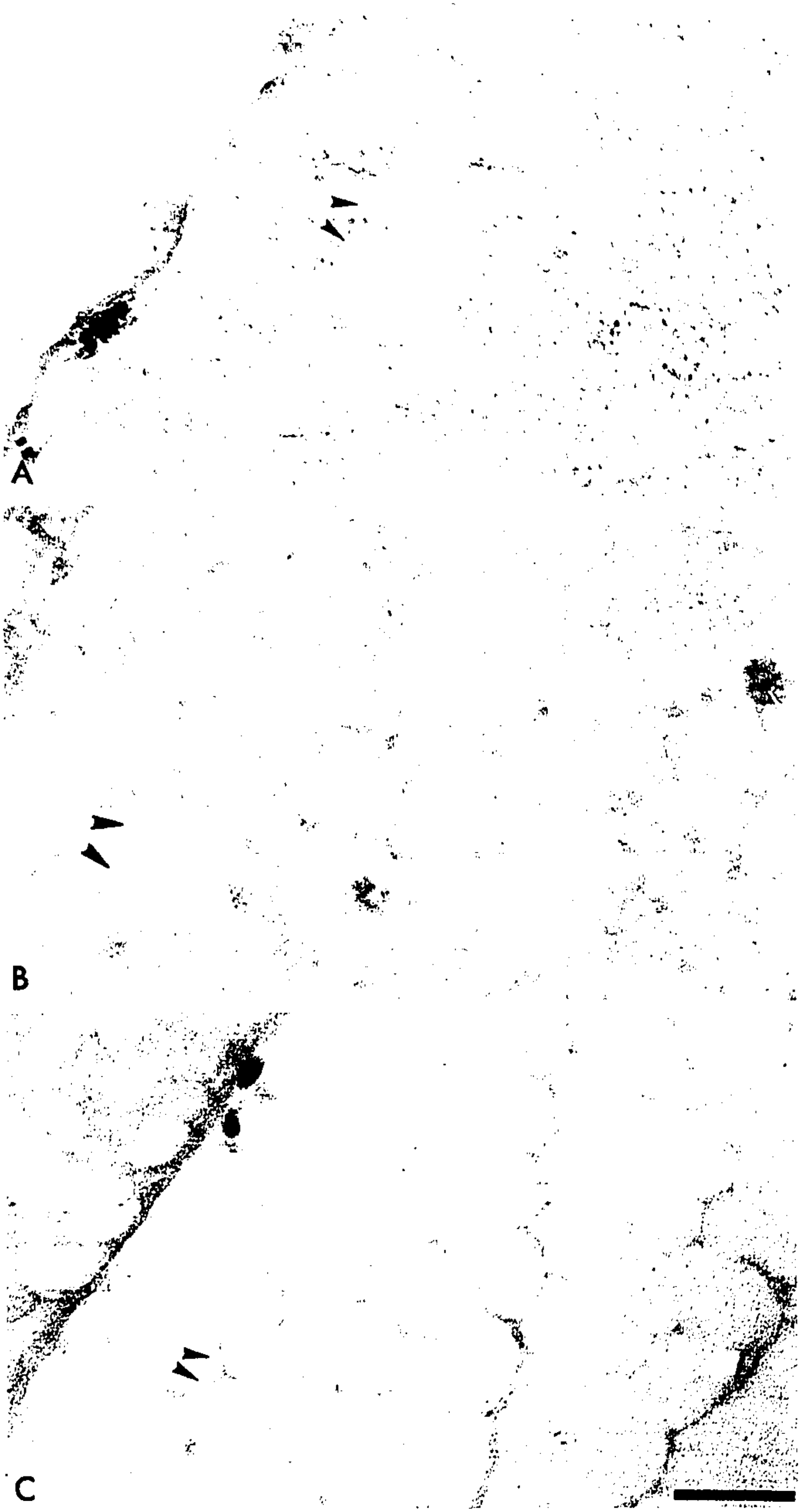
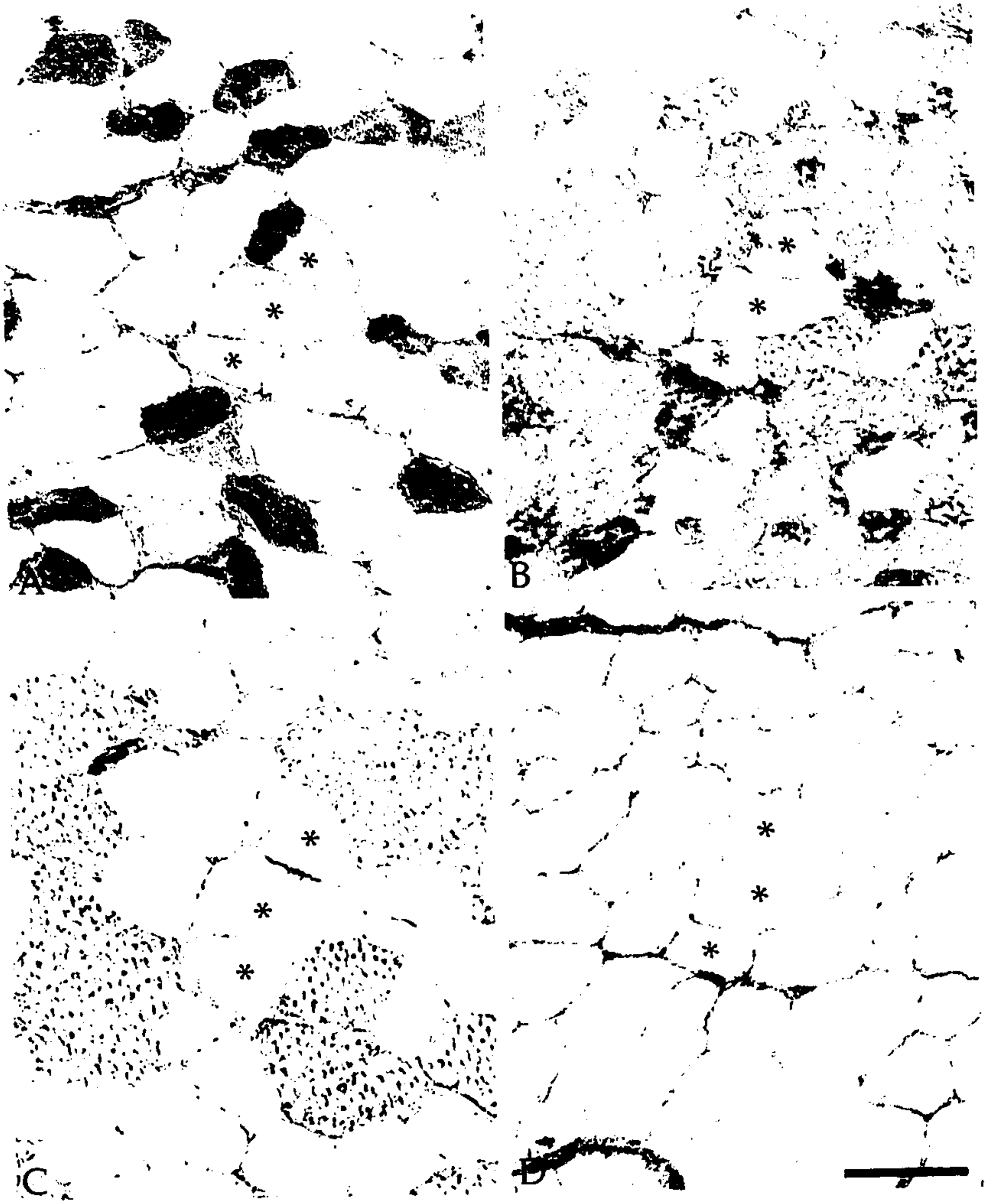

A
B
C

Figure 3.14 Immunohistochemical analysis of muscle fibre types in adult mouse TAS muscle. Indirect immunolocalization was carried out on 10 μm serial sections with Mab specific for (A) IIA MHC (SC-71), (B) I, IIA, IIB MHCs (BF-35) (C) IIB MHC (BF-F3), (D) I MHC (BA-D5). Note the three marked fibres that show only a faint reaction with SC-71 and are not completely negative with BF-35.

Bar represents 100 μm


A
B
C
D

3.3.4 Electrophoretic study of MLC composition of IIX fibres.

MLC composition in TAS single fibres was also examined with two-dimensional gel electrophoresis. Each single fibre was processed for both MHC (10% of the extract was run on 6% PAGE) and MLC composition (90% of the extract was run on two-dimensional gel). The various MLC spots on the gels were identified on the basis of the migration of MLC standards run on similar gels. Figure 3.15 shows MLC separation on a two-dimensional gel from whole-muscle extracts as well as a type IIB fibre (C) and a type IIX fibre (D). 20 fibres with different MHC compositions (14 IIB, 2 IIB+ IIX, 4 IIX) were analyzed in this way and were found to contain MLC 1f, 2f and 3f exclusively. {N.B. IIB+IIX fibres are fibres that co-express IIB and IIX MHC}. The possibility existed however that the ratios of MLC 1f and 3f were different. To test this hypothesis MLC analysis was performed on 12% one-dimensional PAGE and quantification of the staining density of the MLCs separated on the gel was performed densitometrically. Figure 3.16.A shows an example of 12% SDS-PAGE MLC separation in TAS and soleus muscle extract as well as in type IIB and IIX single fibres. Peaks from the densitometric analysis are also shown (Figure 3.16 B). All quantitative data derived from stained gels are based on two assumptions. First, that there is a linear relationship between the amount of protein in a band and the staining intensity of that band. Second, that this linear relationship is the same for all proteins.

Figure 3.15 MLC composition of (A) soleus muscle extract, (B) soleus and TAS extract, (C) type IIB single fibre and (D) type IIX single fibre following two-dimensional gel electrophoresis. The position of the MLC (1s, 1f, 2s, 2f, 3f) is indicated, TM represents tropomyosin. The gels were silver stained.

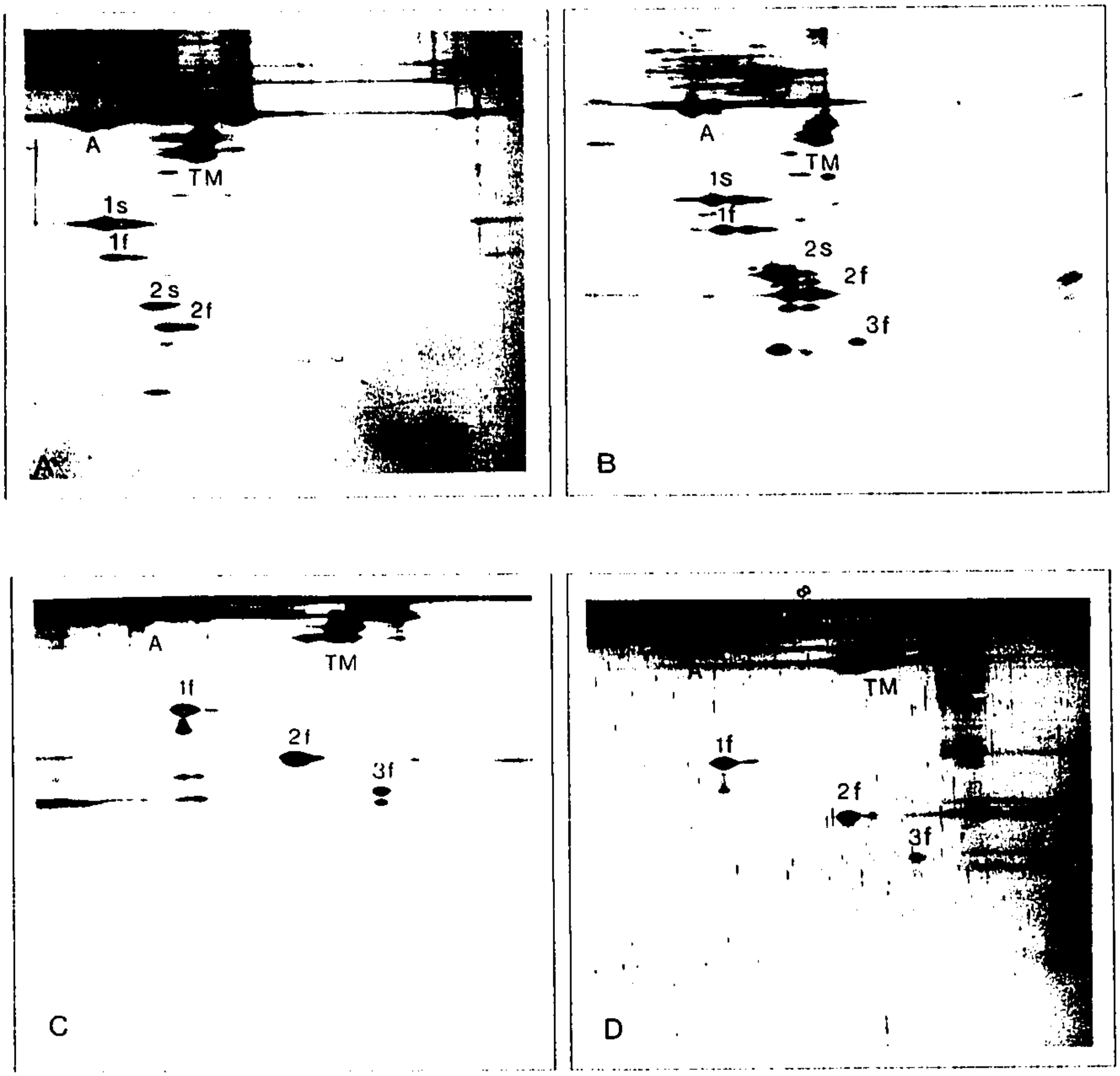
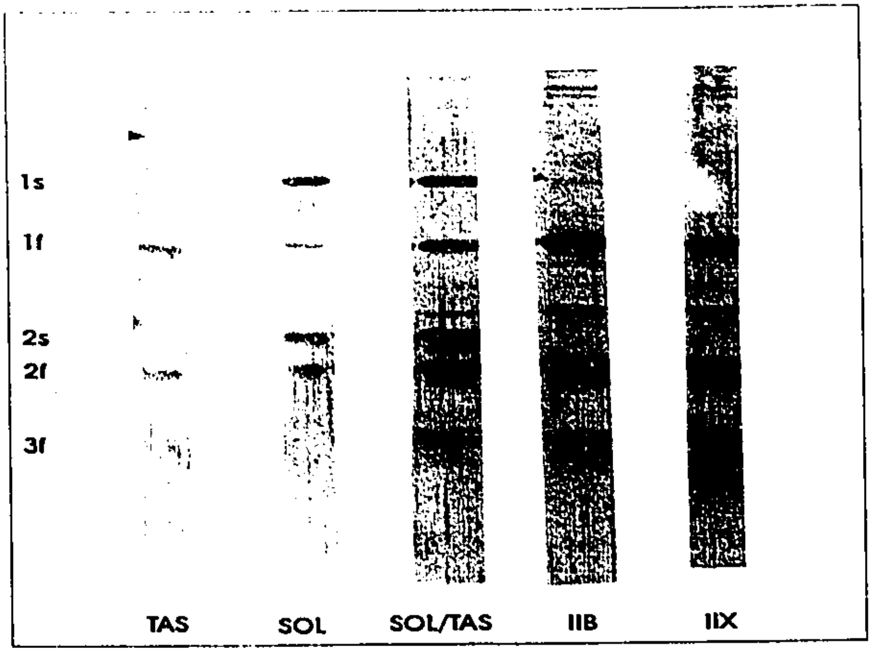
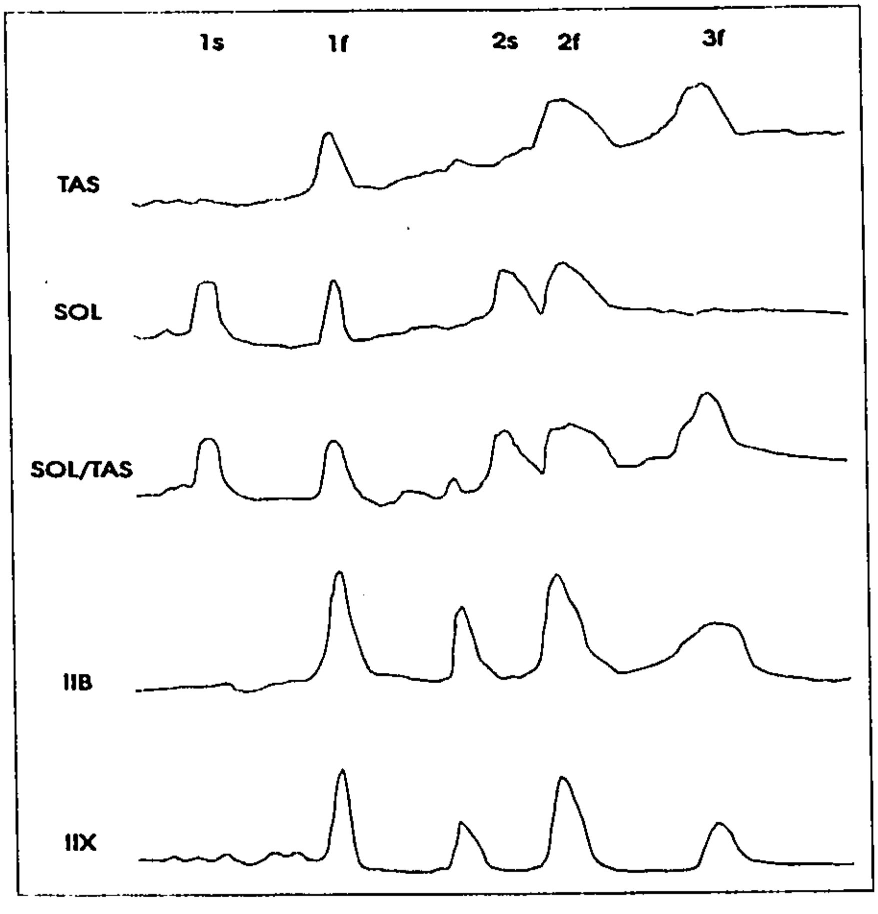

A
TM
1s
1f
2s
2f
A
A
TM
1s
1f
2s
2f
3f
B
A
TM
1f
2f
3f
C
A
TM
1f
2f
3f
D

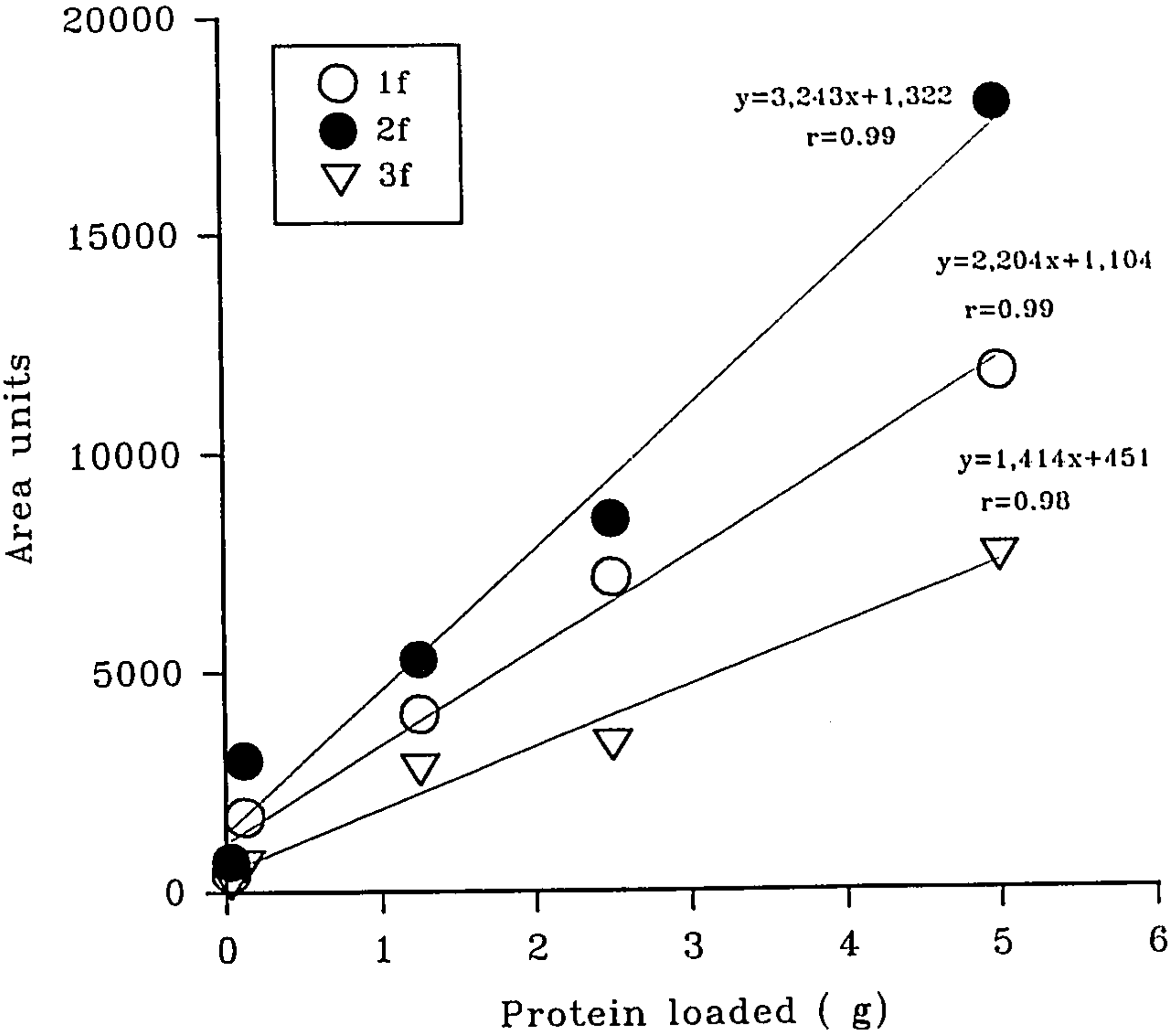
Figure 3.16 (A). MLC composition of TAS, soleus and TAS+soleus muscle extracts, and TAS single fibres containing IIB and IIX MHC following 12% SDS-PAGE. The peaks from densitometer scans are also illustrated in panel (B).


1s
1f
2s
2f
3f
TAS
SOL
SOL/TAS
IIB
IIX


1s
1f
2s
2f
3f
TAS
SOL
SOL/TAS
IIB
IIX

To test these assumptions, gels were run with varying amounts of muscle extract, silver stained and dried. The gels were stained with silver rather than Coomassie blue because it is a considerably more sensitive method of protein detection despite being more a qualitative than quantitative method (Giulian et al. 1983). I was restricted to the use of silver staining as a result of the amount of proteins in single fibres. The regions of the gel containing MLC 1f, 2f and 3f were scanned. The integrated peak areas were plotted as a function of the amount of protein present in the muscle extract (Figure 3.17). For each of the MLC staining increased linearly with protein amount, although the slopes of the fitted lines were different for the various proteins. Figure 3.18 shows the percentage of MLC 1f, 2f and 3f in 66 different single fibres identified by their MHC content. The percentage of both MLC 1f and 3f in IIB fibres was respectively, significantly lower and higher ($P<0.05$) than in IIX fibres. Calculation of the molar ratios of different MLC was based on known molecular weight values and the assumption that there was no difference in the staining sensitivity of the various MLCs. Since the MLCs in different fibre types are chemically related, this assumption is probably valid within the accuracy required for these experiments (Weeds and Pope 1971; Mabuchi et al. 1984). The values for MLC 3f: MLC 1f molar ratios, calculated from the densitometer peak areas, are shown in Figure 3.19. The mean ratio was 0.7±0.04 for type IIB fibres, 0.4±0.06 for type IIX fibres and 0.6±0.06 for fibres co-expressing IIB and IIX MHC.

Figure 3.17 Plot of loaded protein (μg) from EDL muscle extract on a silver stained gel vs. integrated peak area obtained by densitometry. After staining, the gel was dried and scanned at 589 nm, and the integral of each peak was plotted as a function of amount of the total protein in the muscle extract.



1f
2f
3f
y=3,243x+1,322
r=0.99
y=2,204x+1,104
r=0.99
y=1,414x+451
r=0.98
Area units
20000
15000
10000
5000
0
0
1
2
3
4
5
6
Protein loaded (g)

Figure 3.18 Percentage of MLC 1f, 2f and 3f in the different fibre types identified by their MHC content. The percentage of both MLC 1f and 3f in IIB fibres was significantly different ($P<0.05$) from MLC 1f and 3f in type IIX fibres. The percentage of MLC 3f in the hybrid fibres was significantly different ($P<0.05$) from MLC 3f in type IIB and IIX fibres. 66 single fibres identified by their MHC content (35 IIB, 18 IIB+IIX, 13 IIX) were sampled.

*ψ•Significantly different from the others with the same symbol.

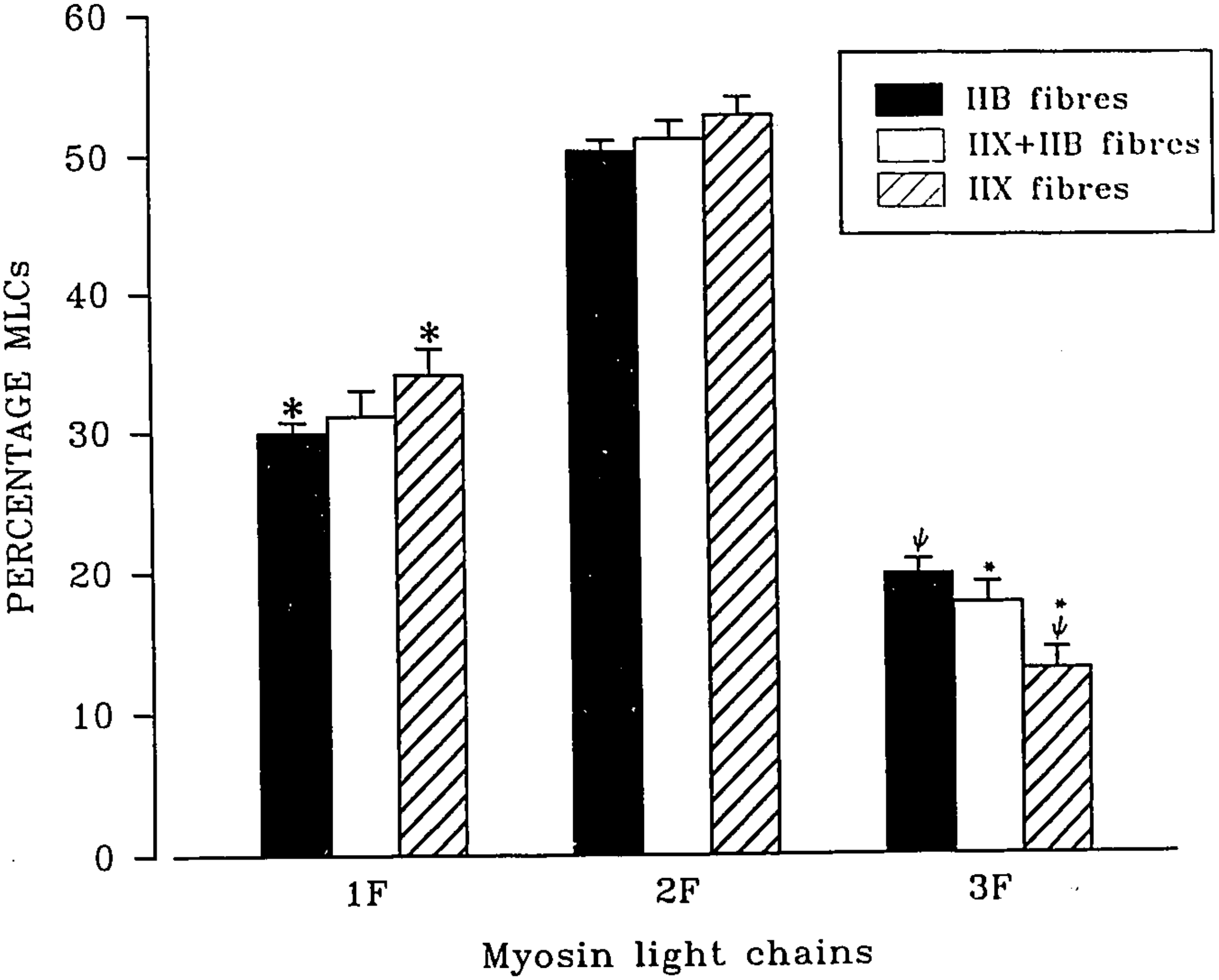
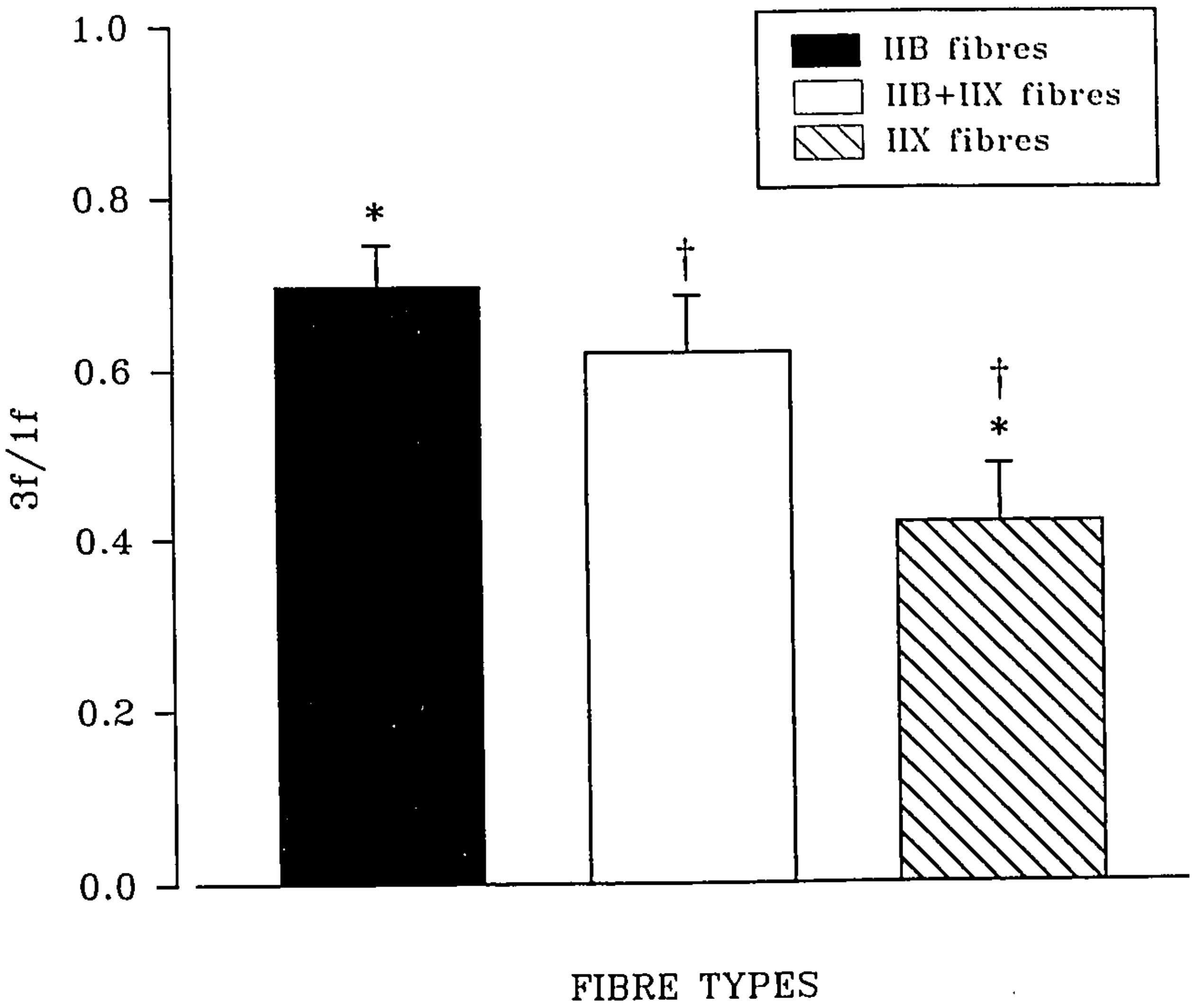

60
50
40
30
20
10
0
PERCENTAGE MLCs
IIB fibres
IIX+IIB fibres
IIX fibres
*
*
ψ
*
*
ψ
1F
2F
3F
Myosin light chains

Figure 3.19 Molar ratios of 3f/1f for IIB, IIX and IIB+IIX single fibre types. This ratio is highest ($P<0.05$) in IIB fibres.

n = 66 single fibres (35 IIB, 18 IIB+IIX, 13 IIX).

*† Significantly different from the others.


1.0
0.8
0.6
0.4
0.2
0.0
3f/1f
IIB fibres
IIB+IIX fibres
IIX fibres
*
†
†
*
FIBRE TYPES

This molar ratio was significantly higher ($P<0.05$) in IIB fibres compared with IIX fibres.

3.4 Discussion

As first demonstrated in rat by Schiaffino et al. (1986, 1989) a population of fibres exists in the mouse which contain a novel MHC isoform. These fibres can clearly be classified as type II by means of myofibrillar ATPase but are distinct from either type IIA or IIB with regard to the pH sensitivity of the myosin ATPase. They have been referred to as type IIX by Schiaffino et al. (1986, 1989). IIX fibres contain a novel MHC isoform which differs from IIB MHC by not being recognized by mAbs BF-F3 and BF-35, and from IIA MHC by not being recognized by BF-32, SC-711 and BF-35. IIX fibres are highly oxidative and this, coupled with the fact that they appear identical to IIB fibres with regard to their myofibrillar ATPase activity at low pH, probably accounts for the report of Reichmann and Pette (1982) that a population of highly oxidative IIB fibres exists in the mouse. It is likely that the fibres identified by these authors were IIX fibres, which account for approximately one-third of the fibre content in fast-twitch muscles of the mouse hindlimb. IIX fibres are not found in the soleus muscle of the mouse. On the other hand this cannot be taken as evidence that IIX MHC is not present since if this isoform co-existed with another the fibre would react positively with the monoclonal antibody of the co-existing MHC and, unfortunately, our gel electrophoretic technique can not separate IIA and IIX MHC in mouse.

One criticism that may be raised concerning the evidence in favour of the existence of a separate IIX MHC is that it is based on the absence of staining with antibodies. This could be interpreted to suggest that the MHC antigenic sites in these fibres are inaccessible to the antibodies. In this regard the observation that "type groups" of IIX fibres can be seen is interesting and a brief explanation is due. Muscle fibres belonging to one motor unit have similar structural and functional properties and intermingle with fibres of other motor units (mosaic arrangement). Denervation of muscle fibres, resulting from motor neuron death or from irreparable damage to peripheral nerve axons, is followed by the growth of fine sprouts from nearby axons. When these sprouts establish new contacts with the denervated fibres (reinnervation) muscle fibres completely adapt to the functional demands mediated through the new nerve. A progressive denervation and reinnervation process may in this way ultimately change the arrangement of fibre types and so cause the clustering referred to as "fibre-type-grouping" (Karpati and Engel 1968). Following sciatic nerve crush and subsequent reinnervation, groups of IIX fibres appear (Fig. 3.8). Thus, if the demonstration of IIX fibres is truly artefactual, as postulated above, then at least this artefact is clearly under neuronal control. Therefore this must represent a distinct class of motor units hence muscle fibres.

The presence of a population of fibres in TAS which contains two MHC bands on 6% gels is very interesting. Indeed fibres can be seen which show intermediate level of staining for IIB MHC and which are not completely negative with antibody BF-35 which indicates pure IIX fibres by a complete absence of staining. The facts that a) no fibres in this region were found to stain with a monoclonal antibody specific for IIA MHC and b) the upper band of the doublet seen on single fibre gel electrophoresis did not react with anti-IIA MHC on Western blots suggest that the slower migrating band is in fact IIX MHC and that both IIX and IIB MHC co-exist in these fibres. This co-existence of IIB+IIX MHC has been demonstrated in a significant proportion of fibres from TAS, both by gel electrophoresis and immunohistochemistry.

The diaphragm in mouse is virtually devoid of IIB fibres but has a large proportion (50%) of IIX fibres (Figure 3.4, 3.10 and Table 3.3). Single fibre electrophoresis revealed no fibres showing double IIX/IIB bands, neither was there any evidence of co-existence of IIB and IIX MHC by immunohistochemistry.

It is possible that IIX MHC is an obligatory intermediate in the process of conversion of IIB to IIA fibres and vice-versa. Conversion from IIB → IIX fibre type may be regulated by factors such as activity pattern and thyroid hormone (discussed in Chapter 5). The sustained activity pattern of the diaphragm might be supposed to have pushed the equilibrium towards IIX expression, while the less active TA maintains a number of fibres in which conversion is incomplete. Future studies will have to improve separation of MHC and reveal the presence of a fourth band (IID/IIX) on gel electrophoresis, in order to allow one to determine whether the equilibrium can be shifted to increase the proportion of IIX/IIA hybrid fibres.

MLC analysis revealed that MLC 3f, which is usually associated with fast-twitch fibres (Lowey and Risby 1971), was present in each of the single fibres analyzed, but the amount present was significantly higher in the IIB fibres as compared with the IIX fibres, while MLC 2f composition was not found to be different. The fact that the sum of MLC 1f and MLC 3f was virtually identical to the amount of the homodimer MLC 2f, suggests that the quantitative estimates of MLC expression are accurate. The proportions of the different MLC is in within the ranges reported in the literature for rabbit skeletal muscles (Moss et al. 1990). Salviati et al. (1982) showed that rabbit IIA muscle fibres are characterized by a lower content of MLC 3f, as compared with IIB. Studies on skinned single fibres from rabbit and rat skeletal muscle (Sweeney et al. 1986; Eddinger and Moss 1987) confirmed this result. These authors also reported that IIB fibres have a significantly greater maximum velocity of shortening than IIA fibres. However, IIX fibres were not identified in these studies. Gorza et al. (1988) and Schiaffino et al. (1988) showed that in denervated soleus muscles subjected to chronic stimulation the transformation to a largely IIX fibre population was accompanied by a shift in the force-velocity relationship to one closer to that of EDL. Thus, in all likelihood IIX fibres should display fast-twitch characteristics.

It is not known if muscle fibres shorten at different velocities because they contain different MHC isoforms (Reiser et al. 1985). Bottinelli et al. (1991) studied the relationship between maximum velocity of unloaded shortening (Vo) and MHC composition of rat single fibres to assess the specific role of MHCs in determining the contractile properties of mammalian skeletal muscle fibres. They reported that there was a continuum in Vo among individual single fibres regardless of the type of MHC expressed by the fibre. Within this continuum they immunohistochemically identified type I, IIA, IIX and IIB fibres, of which type I fibres had the slowest Vo and type IIB fibres had the fastest Vo. They did not however look for possible MHC co-existence in the same single fibre whose presence might account for the variation of Vo.

An alternative possibility is that other contractile proteins such as MLC are at least partially responsible for the variability in shortening velocities. Evidence in favour of such a role for MLC has been provided by studies in which Vo has been related to the MLC 1f/3f ratio (Sweeney et al. 1986; Eddinger and Moss 1987) or in which Vo has been evaluated in fibres subjected to extraction of various amounts of

MLC 1f or MLC 3f (Moss et al. 1990). In the latter studies it was found that exchange of MLC 1f with MLC 3f into the fibre, resulted in a significant increase in Vo, exchange of MLC 3f with MLC 1f resulted in consistently small decreases in Vo. Thus taken together these findings indicate that Vo appears to be determined both by the type of MHC and MLC that are present in the fibre. The fact that IIX fibres show a MLC 3f/1f ratio significantly smaller that IIB fibres would suggest that it is associated with a lower Vo than IIB fibres. This would be in agreement with results from previous works (Gorza et al. 1988; Schiaffino et al. 1988) and would account for the intermediate Vo in mouse diaphragm (Luff 1981) that is composed almost entirely of IIA and IIX fibres (Table 3.1).

CHAPTER 4

Correlation between histochemical and physiological characteristics of fibre types at the motor unit level.

4.1 Introduction

From the histochemical profile of type IIX fibres in mouse (Gorza 1990; Parry and Zardini 1990) and rat (Ausoni et al. 1990) muscle, i.e. high alkali ATPase and SDH activity, one would anticipate these fibres to be fast-contracting and fatigue-resistant, and in this respect similar to the type IIA fibres. However, there is a significant population of motor units identified in cat (Burke et al. 1971), rat (Gardiner and Olha 1987; Olha et al. 1988; Kanda and Hashizume 1989) and mouse

(Parry and DiCori 1990) muscles that may be classified as fast-twitch intermediate fatigue (FI) units. Indeed, Parry and DiCori (1990) showed that approximately one-third of the motor units in mouse EDL muscle have properties which fit into the category described by Burke et al. (1971) as Fast Fatigue Intermediate (FI). Unfortunately they were not able to histochemically identify the type of fibres that make up those motor units, although it is interesting to note the similar proportions of FI units and type IIX fibres in mouse EDL muscle (Parry and Zardini 1990). Since the fibres comprising FI units and the type of units to which type IIX fibres belong are both unknown, it is reasonable to speculate that the FI motor units are composed of type IIX fibres but in the absence of histochemical identification of the fibres in any given mouse EDL motor unit one cannot be sure. The glycogen depletion technique has been successfully applied *in vivo* to whole muscle of mouse (Parry 1980), but it has not been possible to effectively apply it to single units in this species despite consistent efforts, apparently because neuromuscular transmission fails quite early in the procedure so that the fibres are not being continuously activated and the glycogen stores are not depleted. Fladby (1987) has, however, recently reported success in isolating motor units in the mouse soleus with an *in vitro* procedure using an isolated ventral root-nerve-muscle preparation similar to that previously published by Brown et al. (1976).

Given these considerations, there were two primary objectives of this study. First, I attempted to successfully isolate, physiologically characterize, glycogen deplete and histochemically identify mouse motor units using an in vitro preparation. The second objective was to examine the correlation between histochemical and physiological characteristics of IIX fibre types at the motor unit level. EDL muscle was chosen because it is composed of relatively few motor units (30 in cat, Close 1967; 20 in mouse, Bateson and Parry 1983) and fibres (about 1000, Parry and Parslow 1981; Rosenblatt and Parry 1992). Furthermore EDL contains about 30% type IIX fibres (Parry and Zardini 1990).

4.2 Materials and methods

4.2.1 Animals and muscles

The investigation was carried out on 10 old male mice (10 ± 0.9 months old, 36.5 ± 1.8 g body weight) and 7 young adult mice (2.9 ± 0.3 months old, 27.8 ± 1.4 g body weight). They had unrestricted access to food and water. They were anesthetized with sodium pentobarbital (0.07 mg/g body weight) administered intraperitoneally. Experiments were performed on the isolated EDL muscle with its nerve supply intact.

4.2.2 Bathing solution

During surgery and throughout the experiment, tissues were bathed or immersed in a modified Rees-Simpson saline (Rees 1978) whose composition was:

121 mM NaCl, 5 mM $CaCl_2.2H_2O$, 2.3 mM $MgCl_2.6H_2O$, 4.75 mM KCL, 25 mM $NaHCO_3$, 11 mM Dextrose, 5 mM B.E.S. (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid). The solution was constantly gassed with a mixture of 95% 0_2 and 5% CO_2 to maintain pH at 7.4.

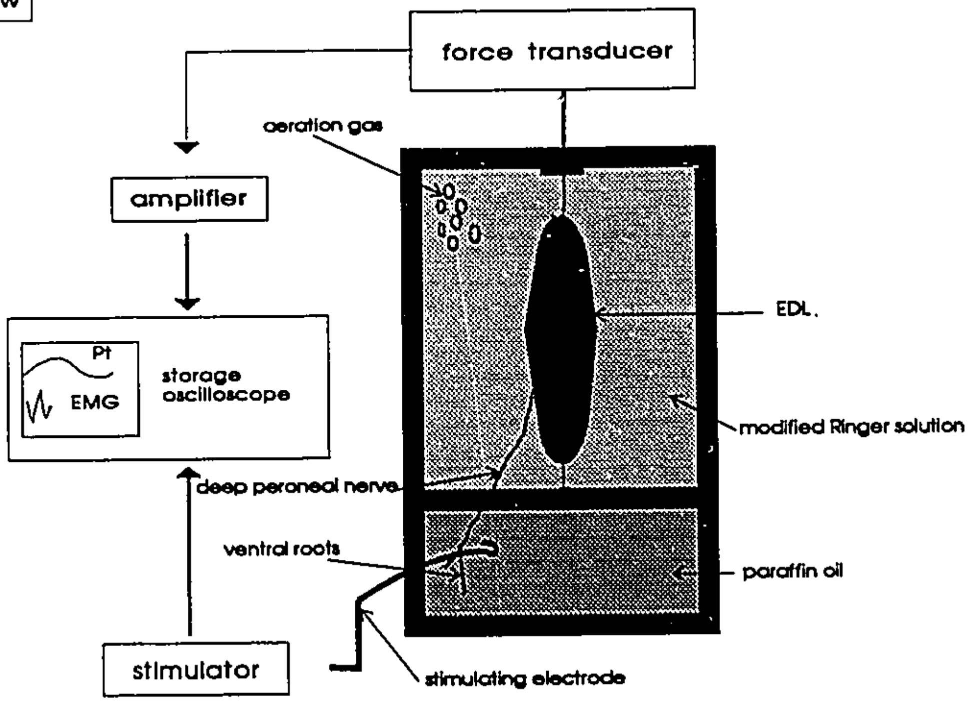
4.2.3 Ventral root-nerve-muscle surgical preparation

Surgery was aided by a Carl Zeiss - Jena stereomicroscope of variable magnification. After shaving the leg and back, a cutaneous incision was made on the lateral surface of the right hind leg from the ankle to the sciatic notch. This cutaneous layer was cut away exposing the hindlimb musculature. In order to expose the sciatic nerve, the biceps femoris was removed. With a pair of small dissecting scissors all the nerve branches, except the deep peroneal nerve, were transected. An ophthalmic micro-coagulator was used to stop bleeding whenever it occurred. The EDL was exposed by reflecting medially the TA muscle after sectioning its distal tendon. Care was taken to preserve the blood and nerve supply. Substantial variations among animals in the arrangement of the blood vessels and nerves were encountered. By rolling the EDL muscle laterally its dual innervation could be clearly distinguished from that serving the TA muscle and muscles of the lower foot. This enabled denervation of all muscles except the EDL. A piece of silk thread was looped around the EDL tendon of origin to facilitate its manipulation for clamping by stainless steel forceps in the muscle bath. A stainless steel wire, looped at both ends, was tied with 3/0 silk to the distal EDL tendon which was left intact until the remaining surgery was completed so as to maintain tension to the EDL muscle. The wire loop was subsequently used to attach the muscle to a force transducer.

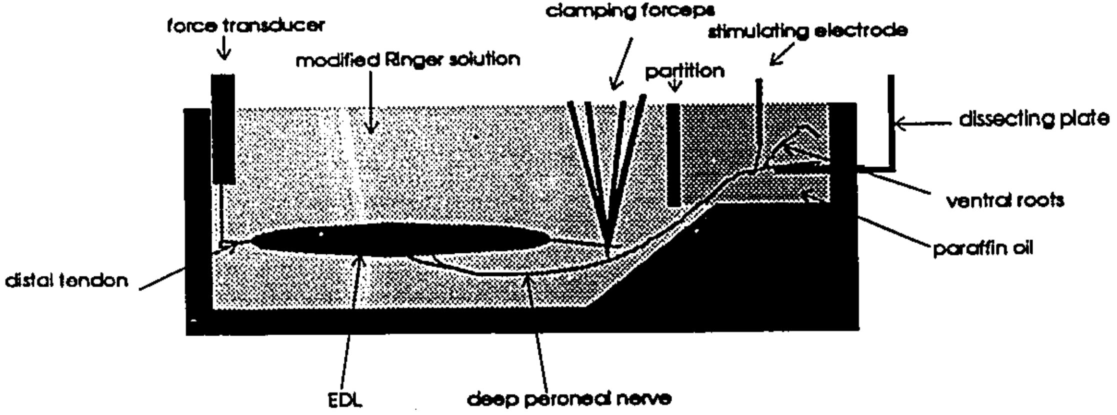
Laminectomy of L1 to L5 was carried out. This procedure involved first exposing the vertebrae from the neck region down to the sacrum. Using Dumont 15A TAB nippers the lateral dorsal processes of the vertebrae were then cut away. Steadying the vertebrae by clamping the forceps to the dorsal spinal process, spinal segments were removed sequentially by cutting through each vertebra at approximately its mid point. The upper half of the vertebrae could then be removed and the spinal cord and spinal roots exposed. Cotton swabs were used to gently blot the spine whenever bleeding occurred. Great care was taken not to exert any excess mechanical pressure on the spinal roots while the spinal processes were clipped and the vertebrae cut and removed. The dura from the exposed spinal cord was removed next with a pair of very fine forceps (Dumont 5 MC micro-surgery forceps, Fine Science Tools Inc.) and fine dissecting scissors (15003-08 titanium spring scissors, Fine Science Tools inc.). Dorsal roots were transected and the cord was then gently rolled back with fine glass rods to expose the ventral roots. The L3 and L4 ventral roots were sectioned as close to the cord as possible. It should be noted that the two procedures described above, preparation of the hindlimb and of the spinal cord, were done alternately as this seemed to help reduce blood loss and trauma to the animal. The nerve supply to the EDL muscle was dissected free all the way back to spinal roots L3 and L4. The preparation was then transferred to a chamber having two compartments separated by a thin plastic partition. The EDL nerve was placed in a slot in the partition, and the proximal tendon of the muscle was clamped by stainless steel forceps close to the partition. The distal tendon was attached to a force transducer by means of the looped stainless steel wire. The transducer consisted of a stainless-steel cantilever beam to which were bonded four semiconductor strain gauges (SUDP-350-160, Kulite Semiconductor Products, Ridgefield, NJ, U.S.A.). The gauges were arranged as a full bridge, the output of which was 5 mV/g V excitation. The resonant frequency of the unloaded beam was 1660 Hz and the compliance was approximately 1 µm/g. The signal from the transducer was amplified and then the tension was recorded on a Tektronix 5111 storage oscilloscope. The small compartment containing the nerve was filled with paraffin oil (to minimize potential spread of current during ventral root stimulation). The other compartment (containing the EDL muscle) was filled with the modified Rees-Simpson solution. The temperature of the bathing solution was between 21° C and 24° C. The entire preparation is illustrated in Figure 4.1.

Figure 4.1 Schematic representation of the set-up used for in vitro motor unit study.

A. Top view



B. Side view



4.2.4 Physiological measurements

The nerve-muscle preparation was allowed to equilibrate in the bath for about 30 min. Bipolar stainless steel wire electrodes were then placed under the EDL nerve and a flat surface bipolar silver wire electromyographic (EMG) electrode was positioned over the belly of the muscle. The stimulating electrodes were made by bonding two pieces of stainless steel wire approximately 1 mm apart with epoxy resin, which also acted as insulator; EMG electrodes were made in a similar fashion but the wires were totally embedded in resin, which was later ground flat to expose the wire tips. Electrodes were shielded in a grounded cable and were manoeuvred by means of micro manipulators. The optimal muscle length was then established by stimulating the EDL nerve with a 50 µsec pulse at a voltage 50% above that which elicited a maximal contraction. The muscle length was adjusted by a micrometer to which the transducer was attached until a maximum isometric twitch tension was obtained. The twitch resulting from supramaximal stimulation of L3 and L4 was recorded on the oscilloscope. In cases where all the EDL nerve fibres are contained in the two roots the tension obtained by stimulation of the ventral roots should be equal to that produced by stimulation of the EDL nerve. One ventral root filament was then placed on a stainless steel dissecting plate that was immersed in the paraffin pool. The root was then split with a pair of very fine forceps (Dumont 5 MC micro-surgery forceps, Fine Science Tools Inc.) and a glass rod pulled to a fine blunt tip.

Motor units were determined to be single by 2 criteria: 1) all or none contraction, 2) all or none EMG. A teased ventral root filament was laid across the stimulating electrode and stimulated with a single 50 μsec pulse, every 10 sec, until the threshold for contraction and EMG was reached. The threshold voltage was defined as the voltage at which an all or none twitch response was recorded 50% of the time when a series of 10 pulses were given. When this was achieved, the nerve was then stimulated at 1.5 times the threshold voltage to confirm that twitch and EMG were stable and that only one motor unit was being stimulated. If both criteria were met then the unit was considered to be isolated. The maximum isometric twitch tension (Pt) and maximum isometric tetanic tension (Po) were determined by averaging 4-8 responses elicited once every 10 sec. The time to peak isometric twitch tension (TTP) and the time taken for the peak isometric twitch to fall to half its peak value (half relaxation time, 1/2 RT) were measured from Pt wave patterns stored on the oscilloscope and captured with a Polaroid CR-9 Land camera. Whole muscle twitch and tetanic tensions were obtained by stimulating the muscle nerve after all the required data had been obtained for each motor unit. The relative force produced by a single motor unit was later calculated as the ratio of the tension produced by a particular single motor unit and the tension produced by the whole muscle to which that motor unit belongs. The length of time that these muscles remained fully excitable at room temperature in vitro depended on the rate of stimulation. In a typical experiment, which usually lasted 6 to 8 h, the twitch and tetanic responses rarely declined, and never by more than 5% of the original value.

If tetanic force did fall to less than 90% of the initial value, the experiment was terminated.

For each nerve-muscle preparation 1-3 motor units were isolated and studied. Motor units of EDL were divided into physiological types on the basis of "sag" and fatigue resistance (Burke et al. 1973). Sag was considered to be present if tension fell (or "sagged") during a 500 msec. train in which the interpulse interval was 1.25 times the time to peak tension of the unit. The fatigue index of a given motor unit was obtained by stimulating repetitively with trains of impulses at 40 Hz, each train lasting 330 msec. Train frequency was 1 per sec. or 1 every 2 sec. The fatigue index was calculated as the ratio of the tension at the 120th train and the tension at the 1st train.

Definitions

- <u>Optimal length</u> (Lo) is the length of the muscle at which the peak twitch tension is maximal at RT.
- <u>Maximum isometric twitch tension</u> (Pt) is the peak twitch tension at Lo.
- <u>Maximum isometric tetanic tension</u> (Po) is the maximum tension, at Lo, during stimulation at 100 Hz.
- <u>Time to peak twitch tension</u> (TTP) is the time from onset of contraction to the peak of the isometric twitch at Lo.
- <u>Time to one-half relaxation of the twitch</u> (1/2 RT) is the time for decay of tension from the peak of the isometric twitch to one half of the peak tension at Lo.

4.2.5 Glycogen depletion of motor units

In most of the ventral root-nerve-muscle preparations one of the motor units was depleted of its glycogen. The physiological properties of these motor units were measured as described above except that the repeated tetanization used to measure fatigue sensitivity was continued for a period lasting between 30 and 120 min., depending on the motor unit type, in a glucose-free solution; this continued stimulation depletes the muscle fibres of their glycogen (Kugelberg and Edstrom 1968).

4.2.6 Muscle removal and processing

At the completion of the physiological measurements and of the repeated tetanization the muscle was quickly removed from the bath, blotted dry, weighed, frozen in isopentane precooled in liquid nitrogen and stored in small plastic vials in a - 80° C freezer until further processing. Transverse serial sections 10 and 15 μm thick were cut from different regions along the length of the muscle on a cryostat microtome and mounted on gelatin-coated slides.

4.2.7 Myosin immunohistochemistry

The 10 μm thick sections from each region of the EDL muscle were immunohistochemically processed with a battery of monoclonal MHC antibodies as described in 3.2.2.3.

4.2.8 Identification of glycogen depleted fibres

The 15 μm thick sections were stained to reveal muscle fibre glycogen content by the periodic acid-Schiff (PAS) method (McManus and Mowry, 1960). The fibre type of each glycogen depleted fibre was determined by identifying the same fibre in adjacent immunohistochemically processed sections.

4.2.9 Determination of motor unit size

Motor unit size was assessed by morphometric and physiological methods. Both approaches yield values for relative as well as absolute motor unit size.

4.2.9.1 Morphometric determination

Number of fibres

The total number of glycogen-depleted fibres and the total number of muscle fibres in transverse sections of whole EDL muscle were counted on PAS-stained sections viewed using a microscope with a camera lucida (Olympus BH2, Carsen Medicals and Scientific Co. Ltd).

The absolute motor unit size was expressed as the number of glycogen-depleted muscle fibres per motor unit (innervation ratio).

The relative motor unit size was determined as the ratio of the number of PAS negative fibres to the total number of fibres in the EDL under investigation.

Cross-sectional area

Whole muscle and muscle fibre cross-sectional area (CSA) were measured using integrated planimetry as described by Rosenblatt (1992). In summary the system consisted of a digitizing tablet (Hipad Plus 9012, Houston Instrument, Austin, TX), microscope, camera lucida, personal computer and software application program (SigmaScan, Jandel Scientific, La Jolla, CA). Traces of areas were made with a stylus input device on a digitizing tablet that was connected to the computer. All measurements were made on PAS-stained cross-sections of EDL muscle whose motor units had been glycogen-depleted.

The absolute motor unit size was determined as the cross-sectional area occupied by PAS-negative fibres in a given muscle.

The relative motor unit size was determined as the ratio of the total CSA occupied by the depleted fibres to the whole EDL muscle CSA (motor unit territory).

4.2.9.2 Physiological determination

The absolute motor unit size was given by the value of either Pt or Po for that motor unit.

The relative motor unit size was calculated as the ratio of the Pt or Po produced by that motor unit to the tension produced by the whole EDL muscle containing that motor unit.

4.2.10 Statistical analysis

The Statistical Analysis System (SAS) for Personal Computers software package (SAS Institute Inc., NC, USA) was used for all statistical computations. The means and S.E.M. were calculated for all the variables measured. An independent *t* test was used for comparison between the values obtained from the old and young group of animals. A one-way analysis of variance (ANOVA) was used to compare the different morphometric properties of the identified motor units. If the one-way ANOVA revealed a significant treatment (motor unit) F ratio then post hoc t' test comparisons of least-squares means were performed to determine precisely differences among motor unit types and the probability associated with the differences.

4.3 Results

4.3.1 Whole muscle

Examples of tension and EMG records from a whole EDL muscle in vitro are shown in Figure 4.2. The data for whole muscles are summarized in Table 4.1: they are presented separately for the two age groups as well as in pooled form (Table 4.2). The only significant differences discernible with age are that the mean time to peak tension is prolonged in the older group and the weight of the animal is increased.

Figure 4.2 Whole EDL muscle electrical and mechanical responses *in vitro*.

A: twitch response (lower record) and EMG (upper record).

B: tetanic response (lower record) and EMG (upper record).

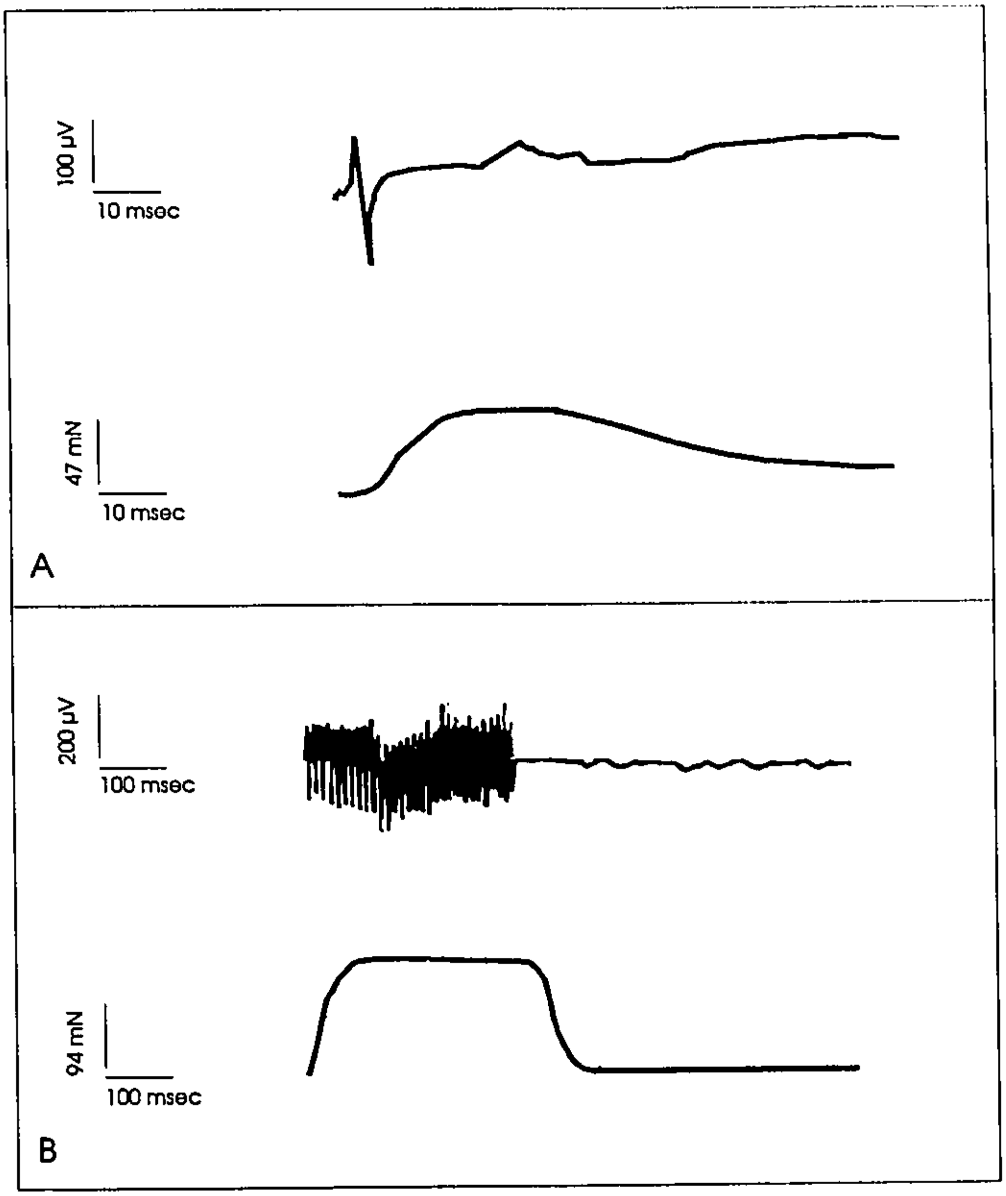

100 µV
10 msec
47 mN
10 msec
A
200 µV
100 msec
94 mN
100 msec
B

Table 4.1 Whole muscle data for EDL muscle from old and young animals.

ANIMALS

	old	young
n	10	7
Age (months)	10.8±0.9	2.9±0.3
Body weight (g)	35.9±5.4*	27.8±1.4
EDL weight/body weight (mg/g)	0.26±0.01*	0.32±0.01

EDL MUSCLE

	old	young
n	10	7
Twitch tension (Pt)(mN)	61.8±3.8	72.4±4.6
Normalized Pt (N/g)	6.5±0.5	7.4±0.4
Tetanic tension (Po)(mN)	201±20	233±25
Normalized Po (N/g)	21.1±2.2	23.8±2.4
Pt/Po	0.32±0.1	0.32±0.02
TTP	22.7±0.6*	20.0±0.9
1/2 RT	34.0±2.3	29.6±2.4
EDL weight (mg)	9.6±0.2	9.7±0.1
Bath Temperature (C)	22.0±0.1	22.2±0.1

Values are mean±SE
* significant different from young mice, $P<0.05$, *t*-test.

Table 4.2 Whole-muscle data for EDL muscle from all the animals.

ANIMALS	
n	17
Age (months)	7.5±1.1
Body weight (g)	32.6±1.5
EDL weight/body weight (mg/g)	0.3±0.01

EDL MUSCLE	
n	17
Twitch tension (Pt)(mN)	66.2±3.1
Normalized Pt (N/g)	6.9±0.4
Tetanic tension (Po)(mN)	213±16
Normalized Po (N/g)	22.2±1.6
Pt/Po	0.32±0.01
TTP	21.6±0.6
1/2 RT	32.8±1.5
EDL weight (mg)	9.7±0.1
Bath Temperature (C)	22.0±0.09

Values are mean±SE

This is in accordance with previous findings in various mammalian fast-twitch muscles (Syrovy and Gutmann 1970; Campbell et al 1973; Gutmann and Syrovy 1974; Caccia et al. 1979; Larsson and Edstrom 1986).

Mean whole muscle tetanic tension was 213±16 mN. Bateson and Parry (1983) studied mouse EDL muscles in vivo and reported that the whole muscle tetanic tension was 297±57 mN with a bath temperature of 36.5±0.2° C. Thus considering that my in vitro experiments were done at RT (22.1° C) and that Krarup (1981) reported that whole rat EDL muscle maximum tetanic tension at 20° C is about 25% lower than at 37° C, these data correlate rather well.

Figure 4.3 shows an example of results obtained from stimulation of L3 and L4 ventral roots. The arithmetic sum of L3 and L4 Pt is equal to that produced by the whole nerve stimulation. This suggests that all the fibres directed to the EDL muscle are contained in these two ventral roots and that the integrity of the two roots is well preserved.

The stimulus regime adopted for glycogen depletion was the one that produced the most effective depletion of whole-muscle when applied to the muscle nerve (Figure 4.4.D). Using this regime 10 motor units were successfully depleted of their glycogen content. Control muscles were studied for glycogen staining. Figure 4.4 shows an example of (A) untreated control EDL muscle removed and processed immediately after sacrificing the mouse, (B) control EDL muscle that had been maintained in the muscle bath without stimulation for 8 h and (C) control EDL

Figure 4.3 Summation of twitch tensions evoked by stimulation of L3 and L4 ventral spinal roots. (a) stimulation of L3 (b) stimulation of L4 (c) whole-nerve stimulation.

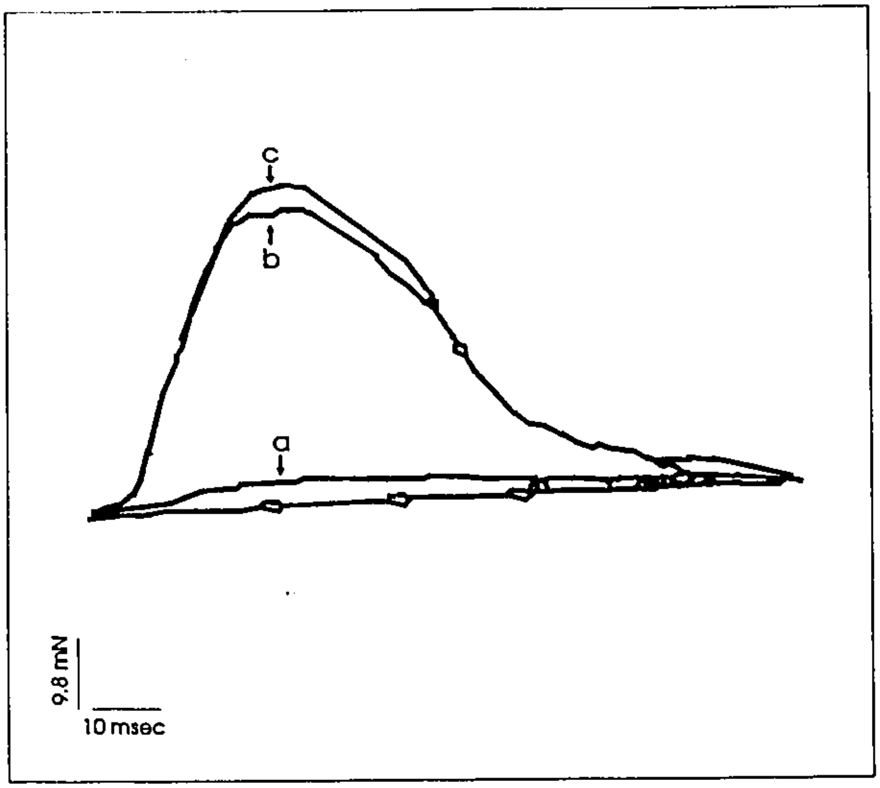

c
b
a
9,8 mN
10 msec

Figure 4.4 PAS staining of: (A) untreated control EDL muscle, processed immediately after removal from the animal. (B) EDL muscle in which motor units were isolated but not glycogen depleted. (C) EDL muscle maintained in the bath without stimulation. (D) EDL muscle depleted of glycogen. Bar = 400 μm


A
B
C
D

muscle whose motor units were isolated but not depleted of their glycogen content. All fibres of all EDL muscles in these three control conditions stained positive for glycogen. Therefore one can conclude that muscles fibre glycogen content does not decrease over the period of the experiment in the absence of stimulation, and the pattern of stimulation applied to the muscle nerve or ventral-root filaments in order to physiologically characterize one or more motor units does not glycogen-deplete the muscle fibres.

4.3.2 Motor units

4.3.2.1 Contractile characteristics

A total of 28 motor units were isolated from 17 EDL muscles: 15 motor units were from EDL of old mice (n=10) and 13 from young EDL mice (n=7). Data obtained from all 28 motor units are pooled together in Table 4.3. The EDL is a fast-twitch muscle which only rarely contains any type I fibres (Parry and Parslow 1981; Parry and Desypris 1983) and this is reflected in the narrow range of TTP of its motor units. Mean maximum tetanic tension of the 28 motor units isolated was 14.7±1.8 mN. Bateson and Parry (1983) reported a mean of 14.4±7.1 mN for 65 motor units, while Parry and Di Cori (1990) reported a range from 2.6 to 21.8 mN for 16 motor units. Both studied motor units *in vivo*.

In this study mean motor unit maximum tetanic tension was 6.7 % of that generated by the whole-muscle, corresponding to about 15 motor units per EDL muscle.

Table 4.3 Contractile characteristics of EDL motor units.

PPT (msec)	1/2RT (msec)	Pt (mN)	Po (mN)	Po/Pt	Pt (% w.m.)	Po (% w. m.)
17.1±0.5	24.2±1.1	4.7±0.6	14.7±1.8	0.3±0.01	6.7±0.7	6.9±0.8

Values are mean ±SE
n=28
Abbreviation: w. m., whole muscle.

4.3.2.2 Physiological classification of motor unit types

Of the 28 motor units described in section 4.3.2.1, 10 were also glycogen depleted and immunohistochemically processed for MHC expression. The values of fatigue index obtained from the two age groups of motor units will be considered separately because the protocol used to make the physiological measurements was different for each one. Table 4.4 shows the results from motor units of old EDL mouse muscle. The protocol used to obtain the fatigue index was that used by Burke et al. (1973), i.e. trains of impulses at 40 s^{-1}, each train lasting 330 ms with a train frequency of 1 s^{-1}. This will be referred to as the standard protocol.

Table 4.4 Properties of motor units from old EDL mouse muscle. Fatigue index was calculated by stimulating a motor unit with a 40 s^{-1} train, each train lasting 330 msec, train frequency was 1 s^{-1}. Motor unit have been arranged in order of ascending fatigue index values.

TTP (msec)	1/2RT (msec)	Pt (mN)	Po (mN)	Po/Pt	CSA (% w.m.)	Pt (% w.m.)	Po (%w.m.)	SAG	Fatigue index	T (° C)	Fibre type
18	20	10.7	28.5	0.37	6.8	18.2	12.7	+	0.05	22	IIB
15	22	10.7	30.3	0.35	5.6	14.1	13.1	++	0.08	24	IIB
20	22	2.9	7.8	0.37	-	4.5	4	+	0.1	22.5	-
18	20	9.8	27.8	0.35	-	13.0	8.1	+	0.125	22	-
14	20	4.6	13	0.35	-	7.8	7.7	+	0.25	22	-
12	20	4.2	14	0.3	5	6.0	6.4	+	0.25	23	IIB
16	20	4.4	11.6	0.38	-	7.6	5.2	+	0.3	22	-
19	30	3.3	8.2	0.4	0.7	4.9	4.5	+	0.3	23	IIA
20	26	2.6	7.2	0.36	-	4.1	3.5	+	0.31	23	IIB
18	28	3.3	12.2	0.27	-	5.5	7.2	-	0.33	22	-
20	30	0.5	2.9	0.16	-	1.2	2.4	-	0.43	22	-
24	40	7.8	12.1	0.65	-	11.7	6.7	-	0.5	22	-
16	20	1.6	7.2	0.23	-	2.5	3.7	+	0.5	22.5	-
16	18	3.6	9.8	0.37	-	5.6	4.8	++	0.58	23	-
15	36	1	5.2	0.18	-	2.3	4.4	-	0.72	22	-

Abbreviations: TTP, contraction time; 1/2RT, half relaxation time; Pt, twitch tension; Po, tetanic tension; CSA, cross-sectional area; w.m., whole-muscle.

The fatigue index was calculated as the ratio of the tension at the 120th train to the tension at the 1st train. From Table 4.4 it is obvious that the fatigue indices of the different types of histochemically identified motor units are rather similar. Indeed the fatigue index of the IIA motor unit (FR) is identical to that of two of the IIB motor units (FF). However Parry and Di Cori (1990) have shown that the fatigue index of FF motor units and FR motor units are clearly different when measured *in vivo*. Parry and Desypris (1985) also reported that, *in vivo*, the fatigue indices of the whole EDL and soleus muscles (made up prevalently of FF and FR motor units respectively) are different.

Figure 4.5 shows the fatigue index values for both *in vivo* (from Parry and Desypris 1985) and *in vitro* preparation of whole EDL and soleus muscle when the standard stimulation protocol was used. One can see that with the *in vitro* protocol there is no difference in the EDL and soleus muscle fatigue index, whereas *in vivo* the fatigue index of these two muscles was clearly distinguishable. This suggests that this particular stimulation protocol is not sensitive enough to distinguish among motor units with different fatigue indices *in vitro*.

Figure 4.6 shows the different fatigue index values for both *in vivo* (from Parry and Desypris 1985) and *in vitro* preparations of whole EDL and soleus muscle when the train frequency was 0.5 s^{-1}. *In vitro* and *in vivo* preparations now give quite comparable results, suggesting that this frequency of stimulation can enable a better separation of the fatigue index of the different motor unit types.

Figure 4.5 Fatigue index for both *in vivo* (from Parry and Desypris 1985) and *in vitro* preparation of whole EDL and soleus muscle. The protocol used to obtain the fatigue index was the standard one, i.e. train of impulses at 40 s^{-1}, each train lasting 330 ms, with train frequency of 1 s^{-1}. The fatigue index was calculated as tension at the 120th train/tension at the 1st train. *In vitro* fatigue index of the two muscles are similar, while that obtained with the *in vivo* experiments are different.

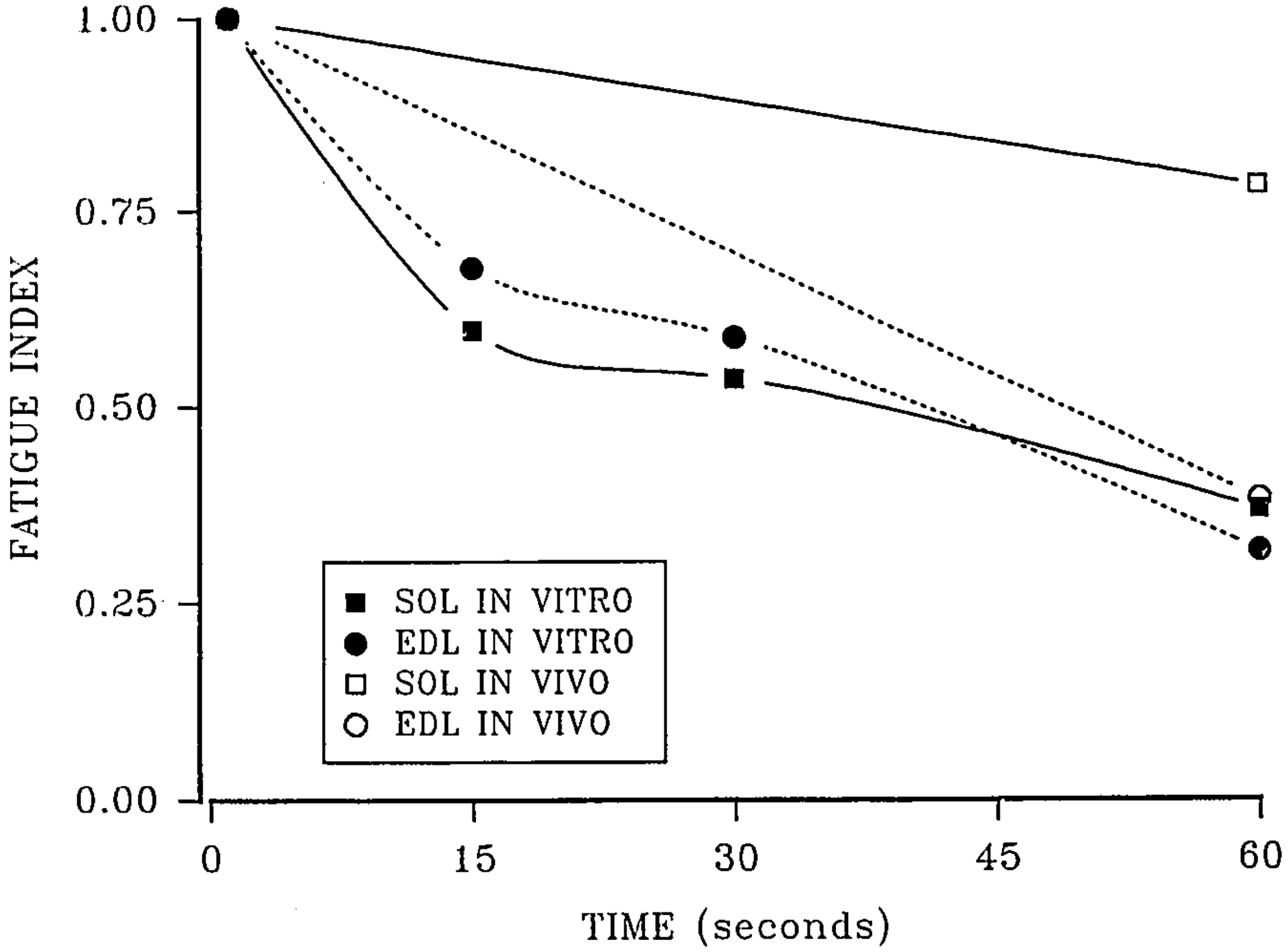

FATIGUE INDEX
1.00
0.75
0.50
0.25
0.00
0
15
30
45
60
TIME (seconds)
■ SOL IN VITRO
● EDL IN VITRO
□ SOL IN VIVO
○ EDL IN VIVO

Figure 4.6 Fatigue index values for both *in vivo* (from Parry and Desypris 1985) and *in vitro* preparation of whole EDL and soleus muscle when the frequency of stimulation was 0.5 s^{-1}. At this train frequency, the fatigue indices of the different motor unit types are clearly different.

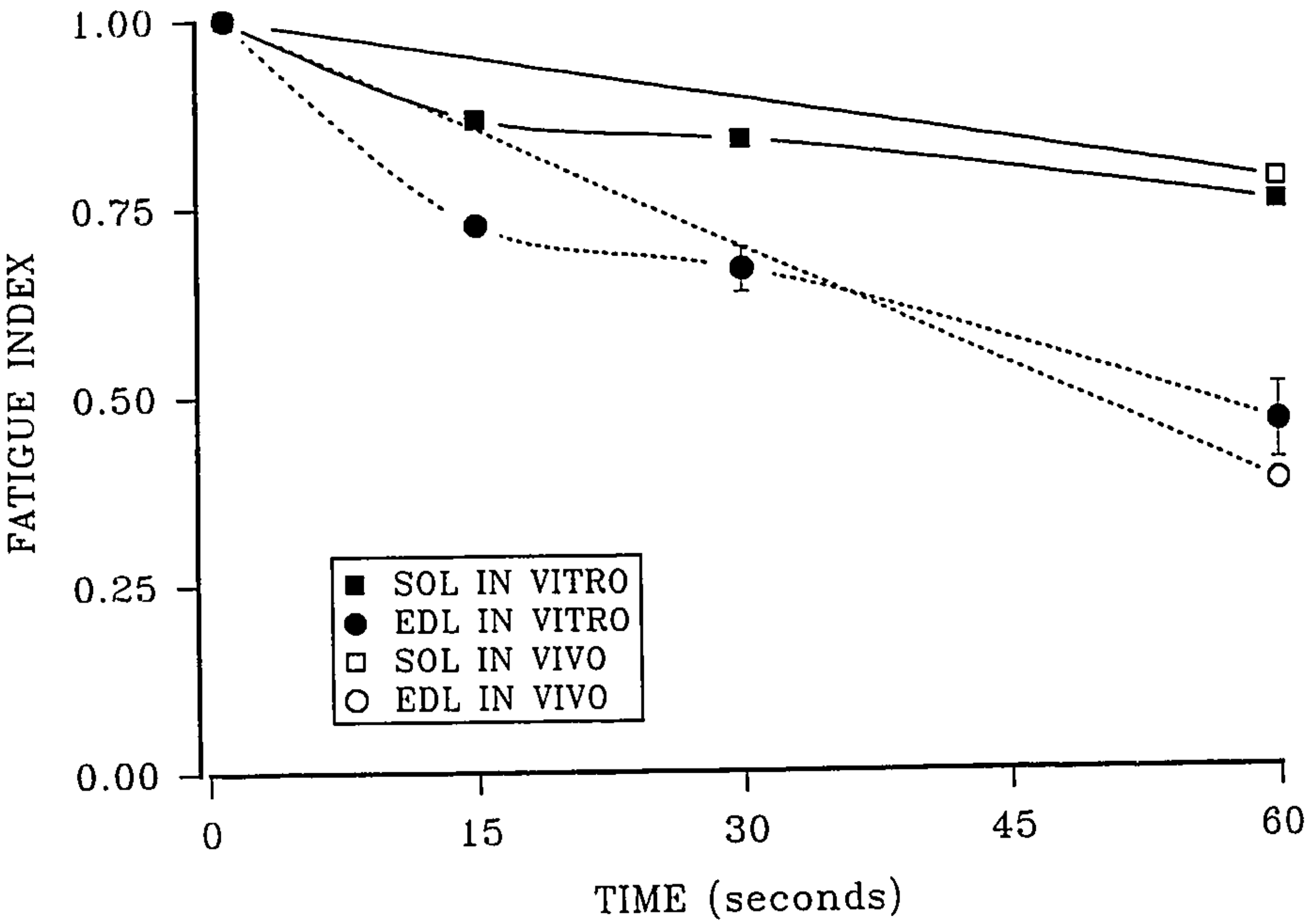

FATIGUE INDEX
1.00
0.75
0.50
0.25
0.00
0
15
30
45
60
TIME (seconds)
■ SOL IN VITRO
● EDL IN VITRO
□ SOL IN VIVO
○ EDL IN VIVO

Table 4.5 summarizes the data for the 13 motor units of young EDL muscle, for which a stimulation frequency of 0.5 s^{-1} was used to calculate fatigue index. The fatigue index of the motor units that have been immunohistochemically identified are now well separated according to their fibre type.

All the identified IIB, IIX and IIA motor units had mechanical properties of the fast-twitch type, i.e. short contraction and half-relaxation times which did not differ among the motor unit types. However, the twitch and tetanic forces were higher in the IIB units than in the IIX and IIA (Table 4.6).

The data from the second set of motor units (i.e. those listed in Table 4.5) are plotted in Figure 4.7 with the use of different symbols to make direct comparisons between physiological and histological classification in the smaller sample of glycogen-depleted motor units. It is interesting to note that the three FR motor units do not exibit the "sag" phenomenon i.e. the decline in tension after the first few stimuli of an unfused tetanus. The mechanisms of "sag" are not known, Figure 4.8 suggests that it is not due to muscle fibres " dropping out" in the later stages of the unfused tetanus because the EMG does not show a visible decline. The fatigue index in the IIX motor units was intermediate between that of types IIB and IIA motor units (Figure 4.9). Figures 4.9, 4.10, 4.11, 4.12, 4.13 and 4.14 show examples of correspondence between physiological and immunohistochemical classification.

Table 4.5 Properties of motor units from young EDL mouse muscle. Fatigue index was determined by stimulating the isolated motor unit with a 40 pps train of 330 msec duration, 0.5 s^{-1}. Motor unit have been arranged in order of ascending fatigue index values.

TTP (msec)	1/2RT (msec)	Pt (mN)	Po (mN)	Po/Pt	CSA (% w.m.)	Pt (% w.m.)	Po (% w.m.)	SAG	Fatigue index	T (° C)	Fibre type
20	24	4.7	13.2	0.36	5	6.6	5.8	+	0.076	22	IIB
16	32	5.6	28.3	0.2	-	8.6	13.6	+	0.13	22	-
20	28	10.4	24.5	0.42	6.88	13.1	8.7	+	0.15	22	IIB
16	20	3	11.8	0.26	-	4	4.3	+	0.2	22	-
18	26	4.7	17	0.28	-	5.9	6	+	0.22	22	-
12	20	5.2	14.1	0.37	-	9.2	10.7	+	0.24	22.5	-
18	20	4	8.9	0.44	-	5.2	3.3	+	0.37	22	-
14	20	5.6	24.5	0.23	0.67	6	7.6	+	0.38	23	IIX
16	20	2.8	8.5	0.3	1.54	4.4	4.7	+	0.57	22.5	IIX
16	22	10.8	43.3	0.25	-	14.4	15.9	+	0.6	22	-
14	20	1.9	8.5	0.22	-	3.3	6.4	–	0.75	22.5	-
18	30	1	4.7	0.2	-	1.4	2.3	–	0.8	22	-
20	25	1.9	6.3	0.3	0.79	2.9	3	–	0.8	22	IIA

Abbreviations: TTP, contraction time; 1/2RT, half relaxation time; Pt, twitch tension; Po, tetanic tension; CSA, cross-sectional area; w.m., whole-muscle.

Table 4.6 Comparison of isometric twitch properties and tetanic force in single motor units classified according to their fibres MHC composition.

Fibre type	n	TTP (msec)	1/2RT (msec)	Pt (mN)	Po (mN)
IIB	6	17.5±1.4	23.3±1.3	7.2±1.5	19.6±3.8
IIX	2	15 (14-16)	20 (20-20)	4.2 (2.8-5.6)	16.5 (8.5-24.5)
IIA	2	19.5 (19-20)	27.5 (25-30)	2.6 (1.9-3.3)	7.2 (6.3-8.2)

Values are mean ± SE where n>2.
Values are mean where n=2, range is in brackets.

Figure 4.7 Relationship between tetanic force, fatigue index and "sag" in 13 isolated motor units, 5 of which (hollow symbol described in the legend) were also depleted and immunohistochemically characterized. Note that the FR motor units do not exibit "sag" (labelled with a star). Horizontal lines are drawn to subdivide the motor units according to their fatigue index, FR>0.75<FI>0.25<FF.

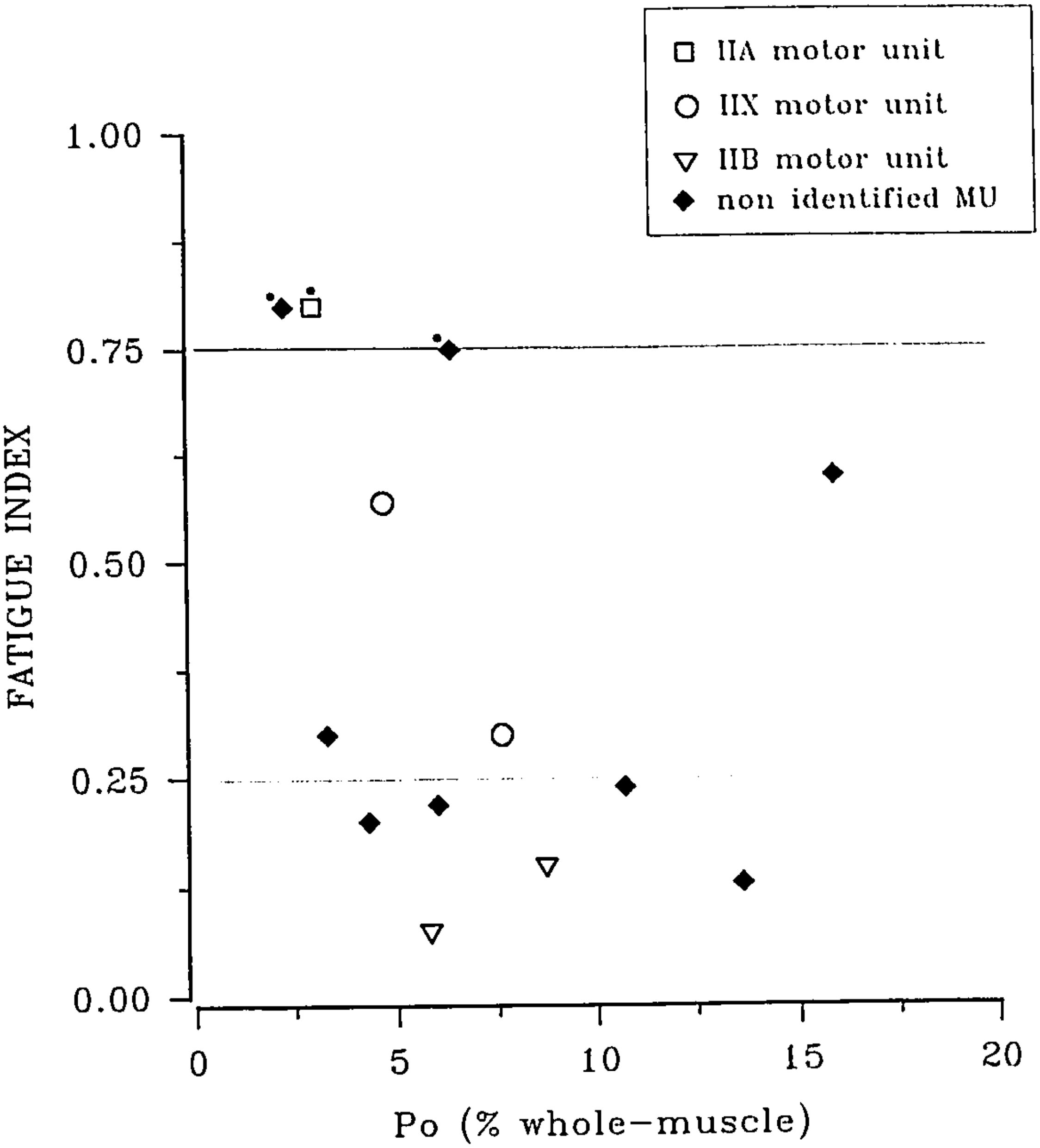

□ IIA motor unit
○ IIX motor unit
▽ IIB motor unit
◆ non identified MU
1.00
0.75
0.50
0.25
0.00
FATIGUE INDEX
0
5
10
15
20
Po (% whole-muscle)

Figure 4.8 Whole EDL muscle "sag" phenomenon (lower record) and EMG. Since the EMG does not change, the "sag" is not due to fibres "dropping out" at the later stages of unfused tetanus.

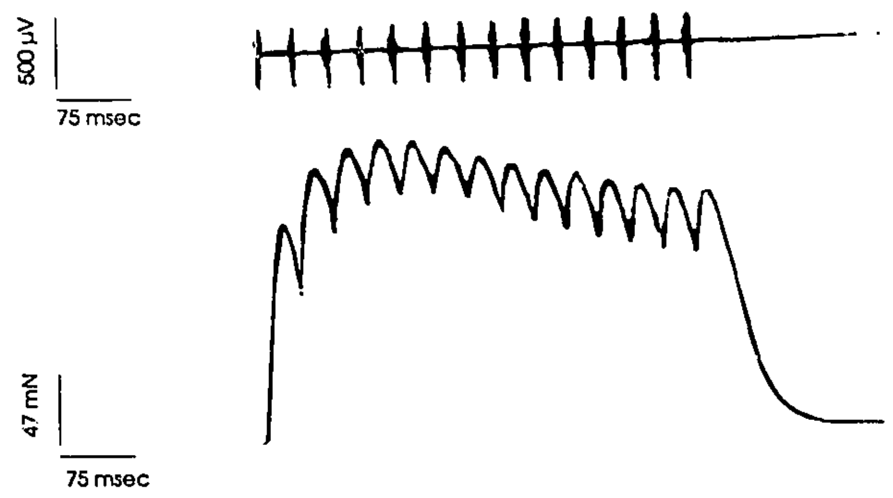

500 µV
75 msec
47 mN
75 msec

Figure 4.9 Mechanical and electrical responses of a type FF motor unit in EDL muscle. A: whole EDL twitch response and EMG. B: motor unit twitch response and EMG. C: motor unit twitch and tetanic response and EMG. D: unfused tetanus to stimulus train of 25 pulses/sec, showing early peak in tension and subsequent decline, or "sag" in tension toward a lower plateau level. The fatigue index of this unit was 0.15. The motor unit was depleted of its glycogen content and subsequent immunohistochemical analyses identified the depleted fibres as type IIB fibres.

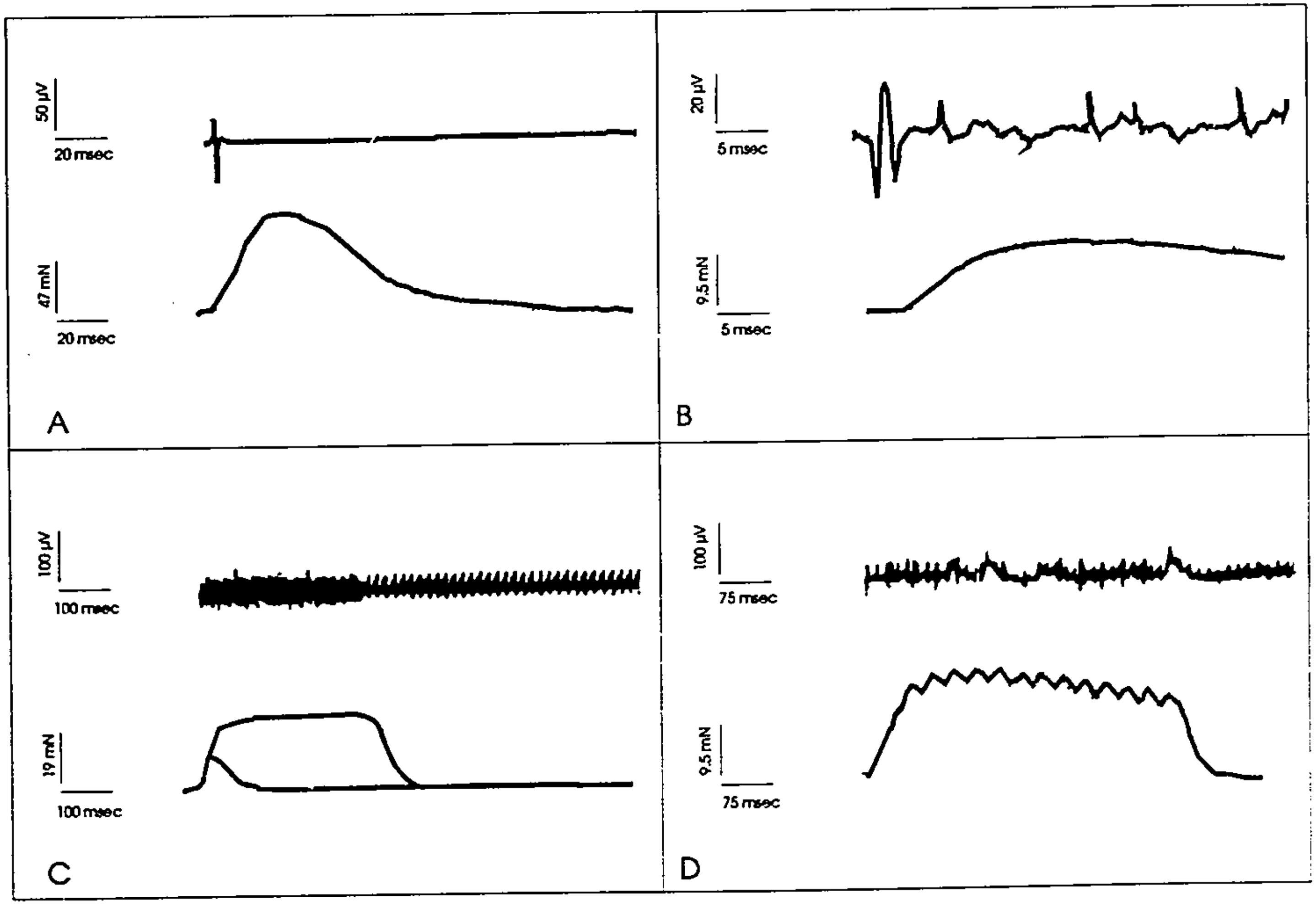

50 µV
20 msec
47 mN
20 msec
A
20 µV
5 msec
9.5 mN
5 msec
B
100 µV
100 msec
19 mN
100 msec
C
100 µV
75 msec
9.5 mN
75 msec
D

Figure 4.10 Serial sections of EDL muscle containing a glycogen-depleted FF unit. The section stained with PAS (A) shows some negative staining fibres, which on serial sections processed for immunohistochemistry are positive with antibody BF-F3, which reacts with fibres containing IIB MHC (B). Bar = 100 μm


A
B

Figure 4.11 Mechanical responses of a type FI motor unit in EDL muscle. A: whole EDL twitch response. B: motor unit twitch and tetanic response. C: "sag" was present in this unit, which, however showed considerably more resistance to fatigue during repeated tetanization (D), in fact the fatigue index of this unit was 0.38. The motor unit was depleted of its glycogen content and immunohistochemical analyses identified the depleted fibres as type IIX fibres.

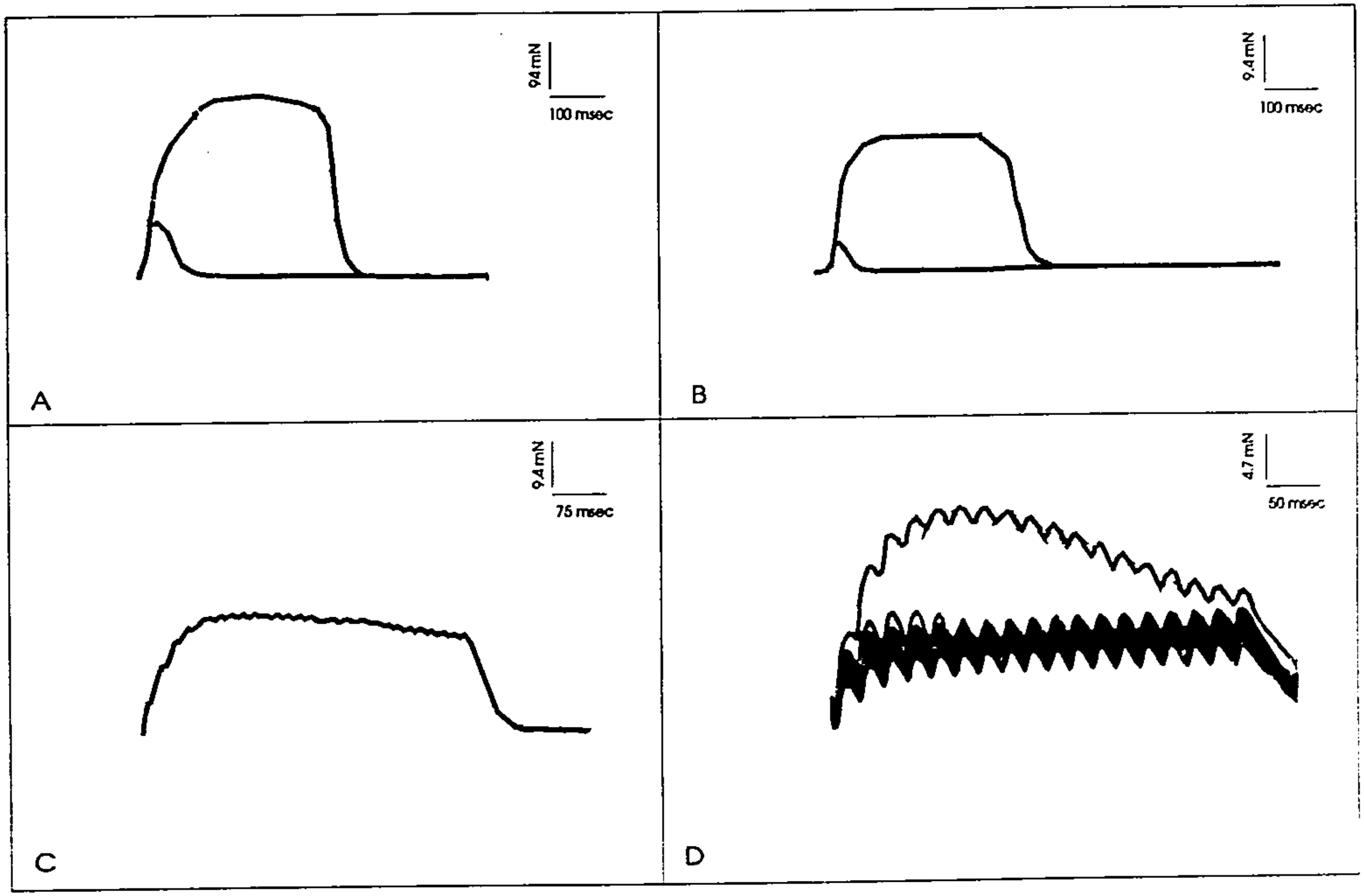
9.4mN
100 msec
A
9.4mN
100 msec
B
9.4mN
75 msec
C
4.7 mN
50 msec
D

Figure 4.12 Serial sections of EDL muscle containing a glycogen-depleted FI unit. The section stained with PAS (A) shows some negative staining fibres, which on serial sections processed for immunohistochemistry are negative with antibody BF-35, which reacts with fibres containing all the MHC except IIX MHC (B). Bar = 100 µm

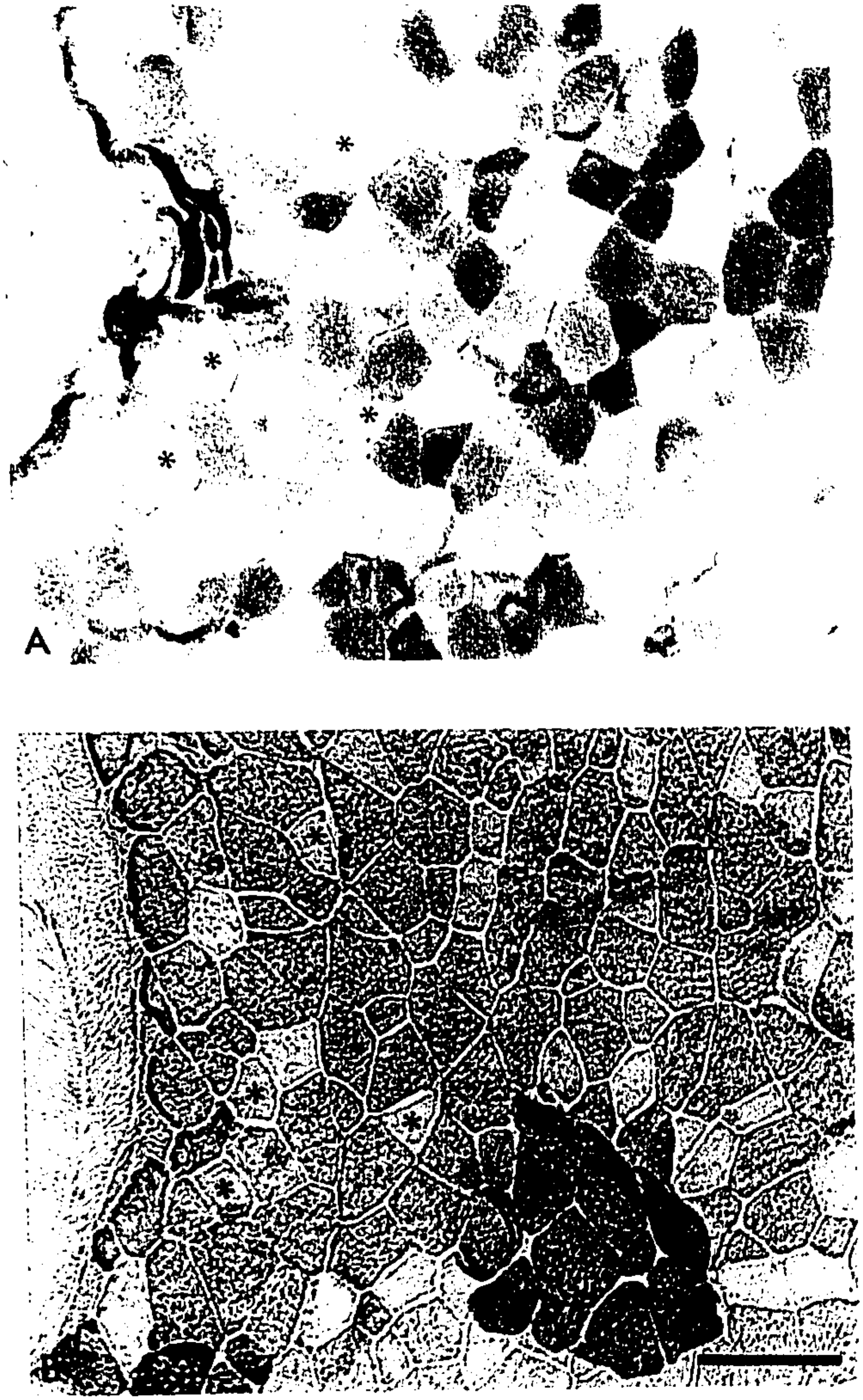

A
B

Figure 4.13 Mechanical and electrical responses of a type FR motor unit in EDL muscle. A: whole EDL twitch response and EMG. B: motor unit twitch and tetanic response. C: "sag" is absent. The high resistance to fatigue during repeated tetanization is illustrated by the record in D which shows the cumulative record of unfused tetany delivered. The fatigue index of this unit was 0.75. The motor unit was depleted of its glycogen content and immunohistochemical analyses identified the depleted fibres as type IIA fibre.

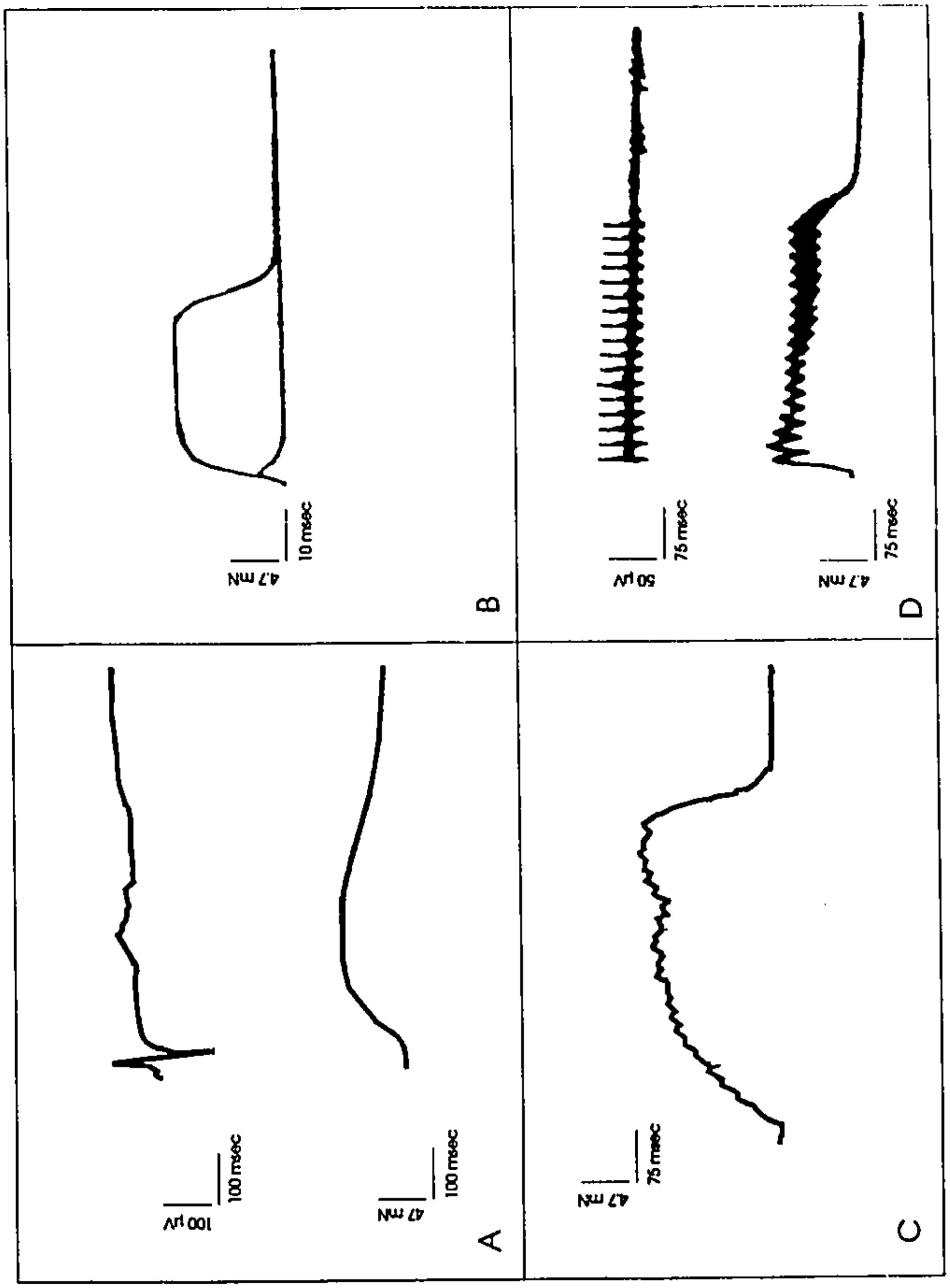

A
100 µV
100 msec
4.7 mN
100 msec
B
4.7 mN
10 msec
C
4.7 mN
75 msec
D
50 µV
75 msec
4.7 mN
75 msec

Figure 4.14 Serial sections of EDL muscle containing a glycogen-depleted FR unit. The section stained with PAS (A) shows some negative staining fibres, which on serial sections processed for immunohistochemistry are positive with antibody BF-711, which reacts with fibres containing IIA MHC (B). Bar = 100 μm


*
*
*
A
B

4.3.2.3 Motor unit sizes

Glycogen depleted muscle fibres were identified in cross sections by their negative staining for PAS. Morphometrically determined absolute motor unit sizes are given in Table 4.7. The relative size of each of the 10 identified motor units, expressed as a percentage of total fibre number, whole muscle cross-sectional, whole muscle twitch and tetanic tension are given in Table 4.8. Figure 4.15 is a diagrammatic representation of the data presented in Table 4.8. The relative size of IIB motor units is larger than IIX and IIA motor units. A comparison of single motor unit sizes is made in Figure 4.16. Motor unit sizes obtained from Po was close to that obtained from Pt (Figure 4.16.C), suggesting that all the muscle fibres in the motor units respond to a single action potential in the motor axon. However, morphometric estimates of motor unit size obtained either from fibre number (Table 4.8) or from CSA measurements (Figures 4.16.A and 4.16.B) are smaller than those measured by Pt and Po in almost all cases. One might expect the morphological size to be smaller than physiological size for IIB fibres if they have greater specific tension, but in that case the reverse would have to be true for the other motor unit types. The fact that the morphological size was smaller in motor units of different types suggests that in those motor units not all muscle fibres were glycogen depleted. Figure 4.17 shows the electrical and mechanical response of a motor unit during repeated stimulation to deplete its glycogen content. The EMG is still present when the mechanical response is almost zero.

Table 4.7 Morphometric determination of the size of identified motor units.

Number of fibres				Cross-sectional area (μm^2)	
Unit type	n	Absolute [a]	Relative	Absolute	Relative [b]
IIB	6	43.3±2.8*	4.7±0.1*	90711±10419*	5.7±0.37*
IIX	2	15.5 (12-19)	1.5 (1.3-1.9)	13998 (10040-17960)	1.1 (0.7-1.5)
IIA	2	27 (21-33)	3.1 (2.7-3.5)	10383 (7246-13520)	0.7 (0.7-0.8)

Values are: mean±SE where n > 2.

Values are mean where n=2, the range is in brackets.

*: significantly different from type IIX and IIA fibres. $P < 0.05$

[a]: innervation ratio

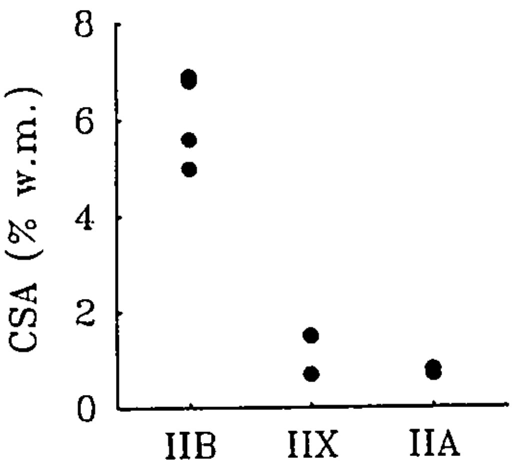
[b]: motor unit territory

Table 4.8 Motor unit size from the 10 identified and characterized motor units, calculated in different ways.

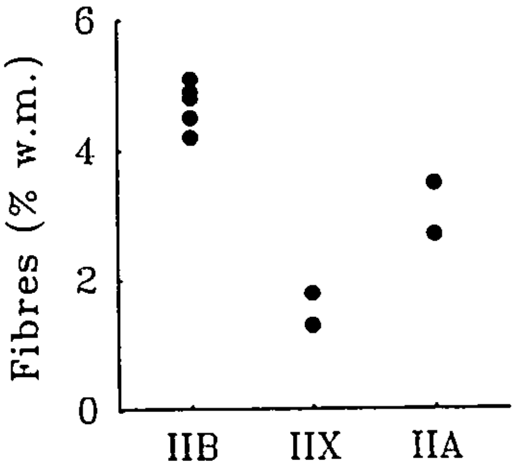
Motor unit size (% of whole-muscle)				
	Histological measurements		Physiological measurements	
Unit type	No of fibres	Fibre area	Po	Pt
IIB	4.5 (47)	5	6.4	6
IIB	4.5 (38)	5.6	13	14.1
IIB	4.9 (48)	6.8	12.7	18.1
IIB	5.1 (52)	6.9	8.7	13.1
IIB	4.2 (41)	5	5.8	6.6
IIB	4.8 (34)	5	3.5	4.1
IIX	1.8 (19)	1.5	7.6	6
IIX	1.3 (12)	0.7	4.7	4.4
IIA	2.7 (21)	0.7	4.5	4.9
IIA	3.5 (33)	0.8	3	2.9

Actual number of counted glycogen depleted fibres is in brackets.

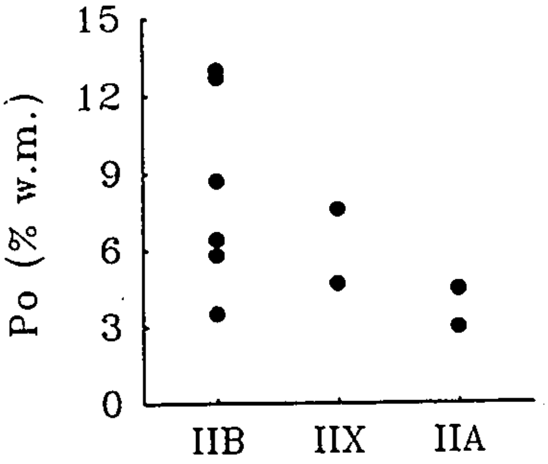
Figure 4.15 Relative motor unit size expressed as a percentage of the whole muscle (w.m.) as a function of: (A) fibre CSA (B) fibre number (C) tetanic and (D) twitch tension measurements. n = 10


8
6
4
2
0
CSA (% w.m.)
IIB
IIX
IIA

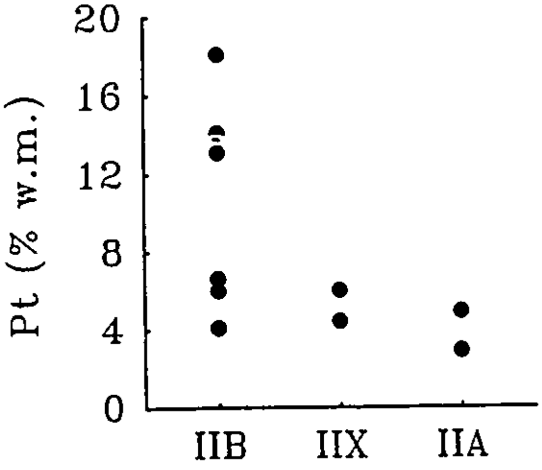
A


6
4
2
0
Fibres (% w.m.)
IIB
IIX
IIA

B


15
12
9
6
3
0
Po (% w.m.)
IIB
IIX
IIA

C


20
16
12
8
4
0
Pt (% w.m.)
IIB
IIX
IIA

D

Figure 4.16 Comparison of relative motor unit sizes obtained by physiological and histological measurements. For each of the 10 identified units the size derived from tetanic tension development (Po), twitch tension (Pt) and CSA, all expressed as a percentage of whole-muscle, are plotted.

CSA of all the muscle fibres in the unit as seen in a mid-belly section of the muscle against Po (A) and Pt (B) gives an indication of the proportion of fibres depleted in a motor unit. Pt plotted against Po (C) gives an indication of degree of activation during twitch. The dashed lines indicate the line of equality.

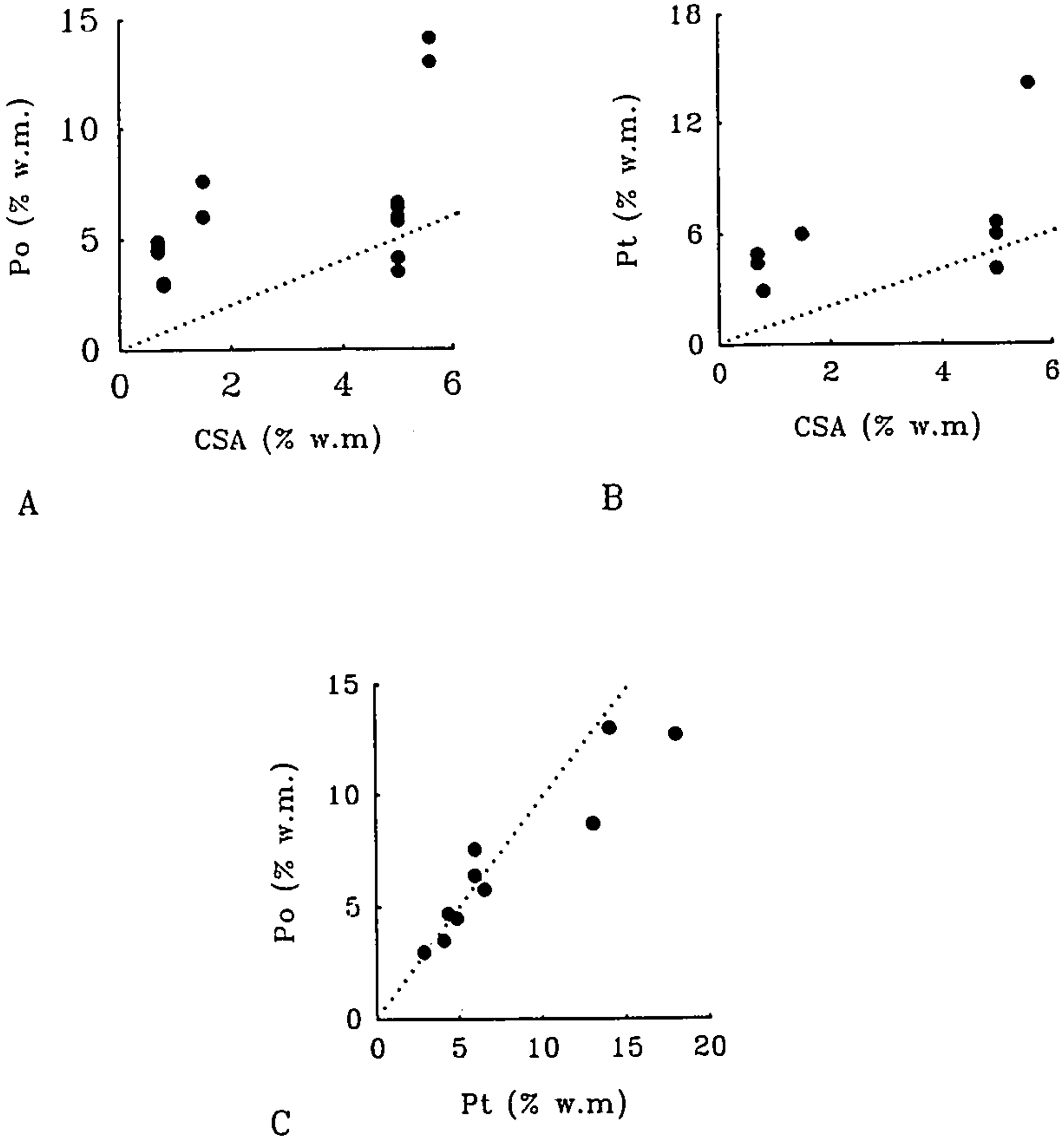
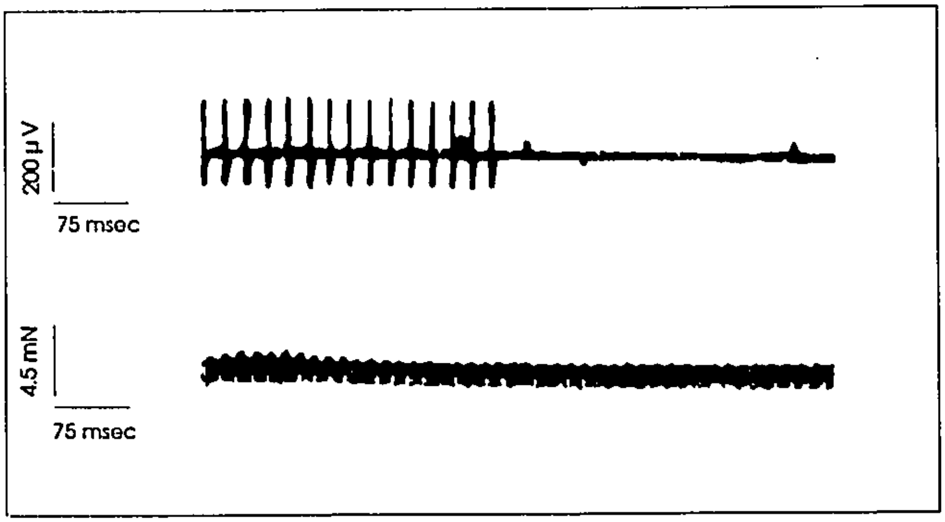

15
10
5
0
Po (% w.m.)
0
2
4
6
CSA (% w.m)
A
18
12
6
0
Pt (% w.m.)
0
2
4
6
CSA (% w.m)
B
15
10
5
0
Po (% w.m.)
0
5
10
15
20
Pt (% w.m)
C

Figure 4.17 Mechanical (lower record) and electrical response of a motor unit during repeated tetanization to deplete glycogen content. Note that while the mechanical response is almost zero the electrical activity is still present, suggesting that the decline in tension is really due to fatigue and not to neuromuscular block.


200 μV
75 msec
4.5 mN
75 msec

The total number of motor units in mouse EDL was estimated by dividing mean whole-muscle tetanic force by mean motor unit tetanic force for the complete population of 28 motor units obtained (Table 4.9).

Even though motor unit force is logarithmically distributed, this estimate derived from arithmetic mean values provides reasonable agreement with anatomic estimates. This approach has been commonly used for estimating motor unit numbers in experimental animals and human subjects (reviewed by Jansen and Fladby 1990).

Table 4.9 Total number of motor units (indirect estimate) in mouse EDL muscle.

Whole-muscle tetanic force (mN)	213.3±15.6
MU tetanic force (mN)	14.7±1.8
Average number of MU	14.5

Values are mean ± SE.

4.3.2.4 Mean fibre cross-sectional area

Fibre CSAs were measured on PAS-stained sections of EDL muscle. Mean CSA of individual fibres in motor units immunohistochemically identified as type IIB was significantly larger than fibres of the IIX motor units which were, in turn, significantly larger than fibres of IIA motor units (Figure 4.15.A). Rosenblatt and Parry (1992) reported that the mean CSA of type IIB fibres in mouse EDL measured on sections processed with BF-F3 (monoclonal antibody that recognizes type IIB MHC) was $1{,}942 \pm 19\ \mu m^2$, which is smaller than the type IIB values reported in the present study. However this discrepancy may be due to different amounts of fibre shrinkage or swelling associated with each method. Table 4.10 compares the results obtained when CSA was measured on identical fibres using serial sections stained with PAS or immunohistochemically processed with monoclonal antibody BF-F3 (type IIB MHC). Fibre CSA measured on PAS stained sections is 1.2 times larger than that measured on immunohistochemically processed sections and thus these results are comparable with those reported by Rosenblatt and Parry (1992).

Table 4.11 shows CSA data obtained from immunohistochemically processed sections of the EDL muscle. It shows that indeed in EDL the rank order of fibres size is type IIB > type IIX > type IIA fibres. This rank order hold true for the different fibre types within a given muscle, although it seems that fibre CSA depends upon the muscle and the region in the muscle in which the fibres are located.

Table 4.10 CSA measured on the same fibres using serial PAS and immunohistochemically processed sections.

CSA of single fibres (μm^2)		
EDL muscle	PAS	BF-F3
1	2172±176	1791±82
2	3302±119	2688±148

Values are mean±SE
n=20

Table 4.11 Comparison of fibre size rank order, derived from cross-sections immunohistochemically processed for glycogen content and MHC expression.

CSA (μm^2)

Fibre types	PAS	n	Immunohistochemistry	n
IIB	2080±156 [a]	6	2464.4±95.9 [a]	3
IIX	891 [b]	2	1060.6±46.3 [b]	3
IIA	377 [c]	2	527.7±30.7 [c]	3

[a]: significantly different from IIX and IIA processed with the same method.
[b]: significantly different from IIB and IIA processed with the same method.
[c]: significantly different from IIX and IIB processed with the same method.
Values are mean±S.E. when n >2. $P < 0.05$

For example Parry and Wilkinson (1990) reported that type IIB fibres located in the superficial region of tibialis anterior muscle are significantly larger than those of the deep zone ($4378\pm117\ \mu m^2$ compared with $3946\pm95\ \mu m^2$ in the deep region). Their type IIB fibre size is far larger than that reported for type IIB fibres in EDL muscle by Rosenblatt and Parry (1992) and in this study. Also type IIA fibres in soleus muscle are significantly smaller than those in EDL and diaphragm (unpublished observations).

4.4 Discussion

Having been able to successfully functionally isolate, glycogen deplete and hence characterize the component fibres of individual motor units in mice, this represents the first report of its kind. Previously, only functional isolation of mouse motor units had been reported (Bateson and Parry 1983; Parry and Di Cori 1990), therefore some of the conclusions to be drawn from the present results must stand on their own. However, whenever possible comparisons with the literature will be made.

The data presented for contractile properties are in good agreement with previously published reports using mouse EDL muscle (Bateson and Parry 1983; Parry and DiCori 1990). Maximum specific tetanic tension was well within the reported range, which indicates that the integrity of the preparation was adequate. In this study an immunohistochemical technique was used to identify the MHC composition of motor unit fibres. This also allowed the identification (by absence of staining) of IIX motor units fibres.

When fatiguing a muscle or a motor unit the decline in tetanic tension can be due to incomplete conduction of muscle-fibre action potentials or reduction in the efficacy of neuromuscular transmission, as well as direct fatigue of tension generation (Burke et al. 1973). The reduced frequency of stimulation employed in the present studies for fatigue index measurements seems not to result in impaired neuromuscular transmission since muscle surface EMG amplitude did not decline with the declining tension (Figure 4.14.D). Furthermore, during the depletion protocol EMG activity was still present even when tension was nearly absent (Figure 4.17), suggesting again that the decline was not due to impaired neuromuscular transmission.

"Sag" is the decline in tension after the first few stimuli of an unfused tetanus (Burke et al. 1973). In this study "sag" was absent in the three motor units with fatigue index > 0.75, but was present in all the others (Figure 4.7). "Sag" seems not be due to fibre "drop-out" in the later stage of the tetany, since the EMG did not show a visible decline (Figure 4.8). Nor was it due to muscle fibre fatigue in the usual sense, since muscle motor units develop the same maximum tension during fused tetany delivered immediately after an unfused one (Burke et al. 1976).

Motor unit size was calculated in different ways (Tables 4.7 and 4.8; Figures 4.15 and 4.16). The histological measurements are based on the assumption that fibres lacking PAS staining have been activated by electrical stimulation of the motor axon. However, there is no guarantee that all the fibres of the stimulated motor unit will be glycogen depleted or, conversely, that other fibres from non-activated motor units will be glycogen-rich. Control experiments suggest that all the fibres that do not belong to the stimulated motor unit are glycogen-rich (Figure 4.4.B), and that most if not all of the fibres of the repetitively stimulated motor unit should be glycogen-negative. Indeed the fact that in almost all the motor units the morphological size was considerably smaller than the physiological estimate would suggest that these units were incompletely depleted of their glycogen. The reasons for this possible incomplete depletion are not clear from these studies. The impossibility of effectively stimulating at the same time all the fibres of the motor unit during the glycogen depletion protocol because of some degree of neuromuscular transmission block can not be ruled out. No motor units were found to contain a much larger depleted CSA area than one would predict from tetanic tension measurements, which would again support the suggestion that non-selective depletion did not take place.

The motor units characterized in this study were primarily of the FF type.

This is to be expected since the probability of isolating a ventral root filament with a single axon of FR, FI or FF motor unit type is dependent on the proportion of each motor unit present in the muscle. One may expect this to be correlated with the number of fibres of each histochemical type; indeed type IIB fibres represent the largest population of fibres in mouse EDL muscle. The rather small average number of motor units in EDL muscle estimated in this study from mean motor unit tetanic force (Table 4.9), as compared with the average number of 20 reported by Bateson and Parry (1983), could be explained by the small number of motor units sampled in this study and by the technical problems associated with the isolation of motor units of very small size that I encountered in my experiments.

The principal reason for choosing to study EDL muscles from older animals was to increase the chance of isolating motor units composed of IIX fibres. In fact, it will be shown in Chapter 5 that there is a significant increase in the proportion of type IIX fibres and fibres containing both IIX+IIB MHC in the TAS of older mice. Assuming that EDL muscle undergoes the same age-related changes, then using EDL muscles of older mice should increase the chances of isolating motor units made up of type IIX fibres. Unfortunately the original fatigue protocol used did not allow an acceptable differentiation of fatigue resistance of the motor unit types and among the depleted motor units there were none containing type IIX fibres. An additional complicating factor may have been the presence of motor units made up of fibres expressing more than one MHC, such as fibres co-expressing type IIX and

IIB MHC. Indeed Larsson et al. (1991) identified 8 motor units co-expressing both IIX and IIB MHC in the TA muscle of old rat. Similarly, Rosenblatt and Parry (1993) estimate that 4.4% of the fibres in EDL muscles of young adult rats contain fibres expressing both IIX and IIB MHC. Because of the failure to differentiate among motor units with different values of fatigue resistance, a different fatigue paradigm was chosen for the second set of experiments. In addition, younger animals were used to decrease the chance of isolating motor units made up of fibres expressing more than one MHC, thereby increasing the likelihood of differentiating motor unit types on the basis of fatigue resistance.

The surprisingly high percentage of FR motor units (6 out of 16) in EDL mouse muscle reported by Parry and DiCori (1990) could be explained by the fact that they used phenotypically normal mice that were part of our dystrophic colony whose EDL muscle may contain a higher proportion of FR motor units and hence a higher proportion of type IIA fibres than normal. They did not characterize the fibres of the motor units because they were unable to deplete fibres of glycogen in their *in vivo* experiments. The chances of being able to successfully deplete a motor unit seems to depend upon the type of motor unit involved. It is easier to glycogen deplete fibres poor in oxidative enzymes than highly aerobic fibres unless the latter fibres are stimulated under hypoxia (Kugelberg and Lindergren 1979). This suggests that fatigue of the IIB fibres is due to failure of the contractile mechanism when their glycogen reserves have been exhausted. In view of the low oxidative capacity of these fibres (Gorza 1988) this is a plausible explanation since oxidation is the only process which can keep the fibre in balance.

The present study suggests that FR motor units are made up of type IIA, FI motor units of type IIX fibres and FF motor units of IIB fibres. The fact that FI motor units are made up of type IIX fibres has been recently shown also in the rat. Schiaffino et al. (1990) using the glycogen depletion technique identified immunohistochemically in rat TA muscle three motor units composed exclusively of type IIX fibres. Their contractile properties were compared to those of seven motor units composed of type IIB fibres. There was no significant difference in their properties with the exception of resistance to fatigue, which was markedly higher in the IIX compared with IIB motor units. Larsson et al. (1991), using the same technique, identified six IIX motor units in the TA muscle of the rat. They observed that the IIX units exhibited a resistance to fatigue which was intermediate between that of type IIA and IIB units and that the fatigue ratios differed significantly among IIA, IIX and IIB units.

Type IIX fibres, with their higher oxidative capacity (Parry and Zardini 1990; Larsson et al. 1991), are better able to function than type IIB fibres when their glycolytic reserves have been exhausted. The close co-variation between the resistance to fatigue and the histochemically identified SDH activity of motor unit fibres has been reported by various investigators (Edstrom and Kugelberg 1968; Burke et al. 1973; Kugelberg and Lindegren 1979; Larsson et al. 1991). Larsson et al. (1991) also showed that the correlation between the resistance to fatigue and ratio of SDH activity to ATPase activity was at least as strong as that between resistance to fatigue and SDH activity alone. This may be a physiologically important safety mechanism since it is clear that fatigued IIB fibres recover much more slowly than do IIA and IIX fibres, so that the only way to protect IIB fibres is to use them infrequently and for short periods of time.

Chapter 5

Effect of age and thyroid hormone status on the expression of type IIX myosin heavy chain

5.1 Introduction

5.1.1 Influence of age on MHC expression

The available data on the relation between contractile and enzyme-histochemical properties in fast-twitch muscles during ageing are divergent. The proportions of different fibre types classified according to myofibrillar ATPase activity have been reported by some authors to be unaltered (Eddinger et al. 1985;

Larsson and Edström 1986; Edström and Larsson 1987; Ishihara et al. 1987; Boreham et al. 1988; Klitgaard et al. 1989; Larsson et al. 1991) and by others to be changed toward a more slow fibre type profile during ageing, with an increase either in the proportion of type I and/or type IIA (Tomanek 1975; Bass et al. 1975; Goldspink and Ward 1979; Ishihara et al. 1987; Kovanen and Suominen 1987; Pettigrew and Gardiner 1987; Kanda and Hashizume 1989). The reason for these discrepancies is unclear, but may be due to differences in the ages of the animals used among the various studies and to the fact that ATPase staining was used for fibre type classification.

Gorza (1990) and Parry and Zardini (1990) have shown that the ATPase staining reaction of IIX fibres is virtually indistinguishable from that of type IIB fibres after acid pre-incubation and type IIA fibres after alkali pre-incubation (Figure 3.1). It is likely then that prior to the discovery of the IIX MHC isoform, fibres containing IIX MHC were erroneously classified histochemically as either type IIB or type IIA fibres. This may explain why changes in fibre type composition in the fast-twitch muscles with aging were not detected.

In view of this, the present study was undertaken to clarify the changes in MHC isoform expression with age. The mouse fast-twitch TAS was studied because it is composed only of IIX and IIB fibres types (Figure 3.5), making it easier to determine whether there is an age-related change in the proportion of type IIX and type IIB fibres.

5.1.2 Influence of altered thyroid state on MHC expression

Substantial evidence exists that thyroid hormone plays a role in MHC isoform transitions during skeletal muscle development (d' Albis et al. 1987, 1990; Gambke et al. 1983). In the adult, altered thyroid hormone levels are followed by changes in MHC composition in a muscle specific manner (Ianuzzo 1977; Izumo 1986; Russell 1988). Nevertheless, little is known about the type of MHC that is induced in fast twitch muscle upon alteration of thyroid hormone status, this being especially true for the IIX MHC. Furthermore, no quantitative determination of the changes in fibre types at the protein level has yet been established in fast twitch muscles. Most studies (Izumo et al. 1986; Gustafson et al. 1985; Mahdavi et al. 1987; Russell et al. 1988) were done at the mRNA level, still the presence of a particular mRNA species does not necessarily mean that a specific protein product will be expressed. Thus, studies at the protein level are necessary to validate those at the mRNA level. Therefore the purpose of this study was to describe the effects of experimentally imposed hypo- and hyperthyroidism on the MHC expression of the superficial part of tibialis anterior muscle of the mouse and in particular the extent to which alteration in circulating thyroid hormone modulates IIB and IIX MHC expression in a muscle composed of only IIB and IIX muscle fibre types.

To accomplish these two objectives (age and thyroid hormone studies), a combined immunohistochemical and biochemical approach was used for the analysis of the whole muscle and of single fibre MHC composition.

5.2 Materials and methods

5.2.1 Animals and muscles

The animals used in the age study were 2, 4 and 8 months old male mice. For the thyroid hormone study three months old mice were used. All the animals were quartered in standard cages with food and water supplied ad libitum. TAS muscle was used for all the experiments. The superficial part of the TA muscle was chosen because in the adult normal mouse, it comprises only type IIB and IIX fibres (Figure 3.5).

5.2.2 Induction of hypothyroidism and hyperthyroidism

The animals for the thyroid hormone study were randomly divided into three experimental groups namely: 1) control 2) hyperthyroid 3) hypothyroid. Hyperthyroidism was induced by intraperitoneal injection of 3μg 3,5,3-triiodothyronine/10 g body weight on alternate days for 5 weeks (d'Albis et al. 1990). Hypothyroidism was induced by treatment with 1% Sodium Perchlorate ($NaClO_4$) solution administered in drinking water for 5 weeks (Wall 1989). Animals in the control group received no treatment.

5.2.3 Serum sampling and Thyroid hormone assays

Blood samples were collected by intracardiac puncture from deeply anesthetized mice prior to removal of muscles. Following centrifugation, serum thyroxine (T_4) and triiodothyronine (T_3) concentrations were determined by radioimmunoassay at the Ottawa General Hospital.

5.2.4 Muscle removal and preparation

The right TAS muscle was removed from the mice, rapidly frozen and sectioned at 10μm thickness. Strips of fibres from the controlateral TAS, tied to wooden sticks, were immersed in skinning solution (Salviati et al. 1982), for 24 h with changes of solution at 1, 2, 4 and 8 h. The muscle strips were stored at - 20° C in a mixture of 50% glycerol and 50% skinning solution.

5.2.5 Immunohistochemistry

Sections were mounted on gelatin-coated slides and immunohistochemically processed as described in section 3.2.2.3. Stained serial cross sections of TAS muscle were photographed on a Zeiss microscope and the numbers of muscle fibres expressing the different MHC isoforms IIB, IIX and IIX + IIB were counted. The different fibre types were expressed as a percentage of total fibre number.

5.2.6 Single fibre gel electrophoresis

A small bundle of fibres was cut from the original strip under a dissecting microscope and single fibres were pulled out from one end of the bundle. The fibres were transferred to a capillary tube containing 20 μl of sample buffer and boiled. Electrophoresis of the samples was carried out in 0.75 mm thick 6% polyacrylamide gels with 30% glycerol as described in section 3.2.2.4. The gels were silver stained by the method of Morrissey (1981).

5.2.7 Statistical analyses

The Statistical Analysis System (SAS) for Personal Computers software package (SAS Institute Inc., NC, USA) was used for all statistical computations. The means and S.E.M. were calculated for all the variables measured. A one-way analysis of variance (ANOVA) was used to test for the effect of either age or thyroid hormone on the proportion of type IIB, IIB+IIX and IIX fibre types. If the one-way ANOVA revealed a significant age or thyroid hormone treatment F ratio then post hoc t' test comparisons of least-squares means were performed to determine precisely differences among age groups or thyroid treatment groups and the probability associated with the differences.

5.3 Results

5.3.1 IIX MHC distribution in TAS: effect of age

Data regarding the animals and the muscle preparations used for the age study are shown in Table 5.1. Animal body weight and TA muscle weight were found to increase significantly with age, although they did not increase in a proportional manner because the muscle to body weight ratio significantly decreased with age.

Table 5.1 Body and TA muscle weight in 2, 4, 8 months old normal mice.

Age (months)	n	Body weight (g)	TA wet weight (mg)	Mw/Bw (mg/g)
2	5	23.2 ± 0.4^{a}	44.0 ± 0.4^{b}	1.9 ± 0.02^{d}
4	5	31.2 ± 1.3^{a}	46.8 ± 1.4^{c}	1.5 ± 0.07^{d}
8	5	37.9 ± 1.4^{a}	$51.6\pm0.6^{b\,c}$	1.3 ± 0.04^{d}

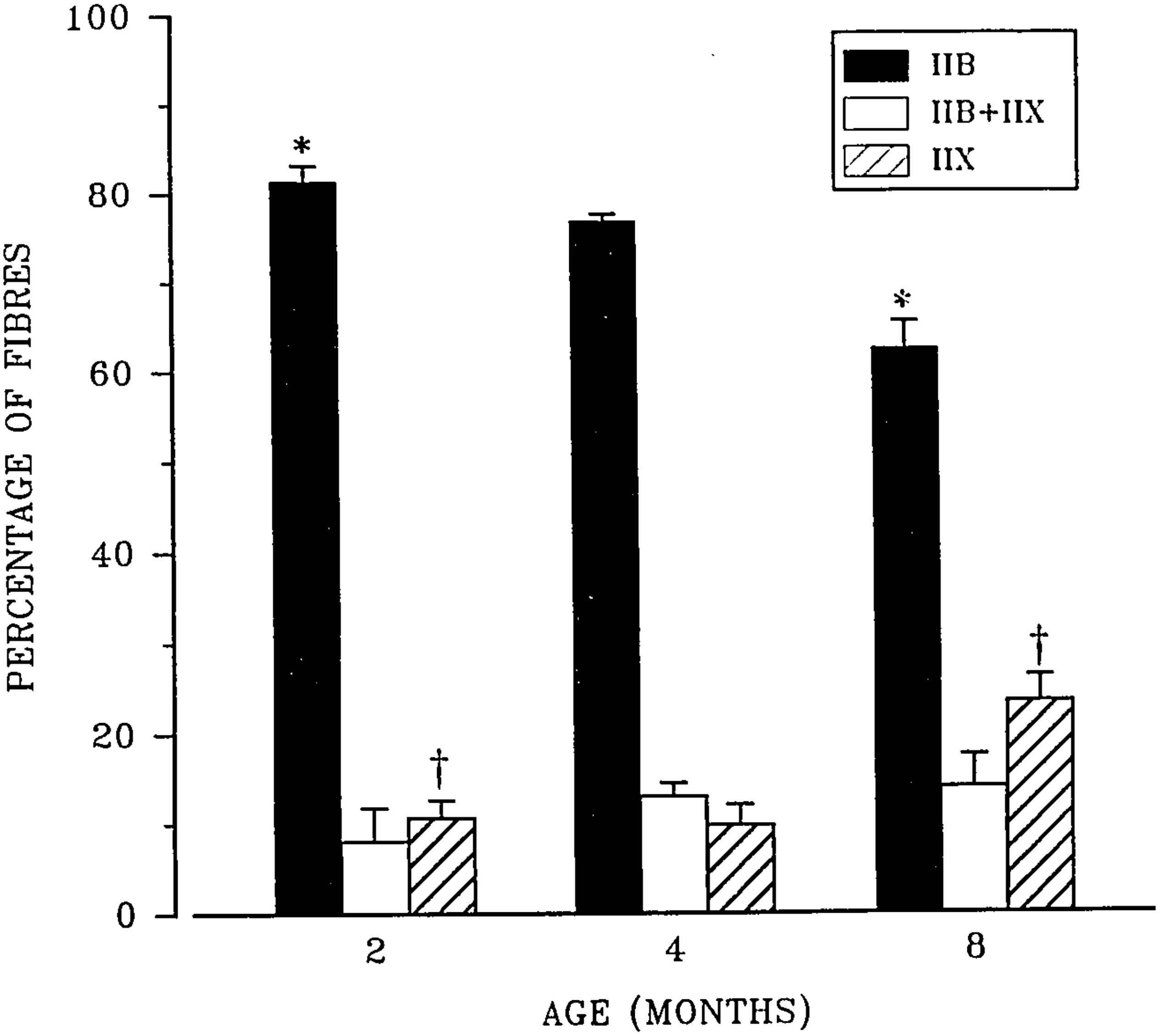
Abbreviation: Mw = TAS muscle weight, Bw = body weight.
Values are mean ± SE.
$^{a\,b\,c\,d}$ Statistically different ($P<0.05$) from the other with the same symbol.

The proportion of fibres of TAS expressing various MHC, obtained by SDS-PAGE, at the three ages considered in this study is shown in Figure 5.1. There was a significant decrease in the proportion of fibres expressing IIB MHC with a parallel increase in fibres expressing IIX MHC with increasing age. A small percentage of fibres that co-expressed IIB and IIX MHC was present in each age group. These fibres presumably represent hybrid, transforming fibres. Results of immunohistochemical fibre typing in whole TAS cross sections showed results similar to that observed with the single fibre gel electrophoresis (Figure 5.3).

Figure 5.1. Proportion of type IIB, IIB+IIX and IIX fibres in TAS muscle of 2, 4 and 8 month old normal mice. Fibre types were determined by 6% SDS-PAGE of single fibres. 3 animals were used in each group. Number of single fibres used was: 140, 129 and 159 for 2, 4 and 8 month old mice, respectively.

* † Significantly different from the others with the same symbol.

$P<0.05$, one-way ANOVA.


100
80
60
40
20
0
PERCENTAGE OF FIBRES
IIB
IIB+IIX
IIX
*
*
†
†
2
4
8
AGE (MONTHS)

5.3.2 IIX MHC distribution in TAS: effects of altered thyroid status.

The efficacy of each of the treatments given to induce either hypothyroidism or hyperthyroidism was verified by determination of the thyroid hormone concentration in serum (Table 5.2). The T3 and T4 level for both the hyperthyroid and hypothyroid group was significantly different from normal. The body mass of the treated animals was significantly less than control, while their TA wet muscle weight and the muscle to body weight ratio was not different, suggesting that neither treatment interfered with normal muscle growth.

Figure 5.2 shows the SDS-PAGE results of the MHC content of single fibres dissected from TAS muscles taken from hyperthyroid, hypothyroid and normal mice. There was a significant decrease in the proportion of fibres expressing IIB MHC with a parallel increase in fibres expressing IIX MHC in the hypothyroid group as compared with normal group. Hyperthyroidism did not induce any changes in MHC expression. Hybrid fibres, co-expressing IIB and IIX MHC, were present in all three groups. Results of immunohistochemical fibre typing in whole TAS cross sections showed results similar to that observed in the single fibres (Figure 5.3).

There was a strong correlation ($r = 0.99$) between the proportion of fibres immunohistochemically identified in TAS muscle cross-sections as being type IIB, IIB+IIX and IIX and the proportion of single fibres identified on 6% polyacrylamide gels expressing IIB, IIX+IIB and IIX MHC respectively in both the age and thyroid hormone study (Figure 5.3).

Table 5.2 Serum T_3 and T_4 concentrations in hyperthyroid, hypothyroid and normal mice.

Treatment group	n	Plasma T_3 level (nmol/l)[a]	PlasmaT4level (pmol/L)[b]
Hyperthyroid	7	10.8±3.8*	<2*
Hypothyroid	7	0.4±0.09*	<2*
Control	5	0.8±0.05	17.4±1.1

[a] Limit of detection of assay 0.2 nmol/l
[b] Limit of detection of assay 2 pmol/l
Values are means ± S.E.
* Significantly different from control, $P<0.05$, one-way ANOVA.

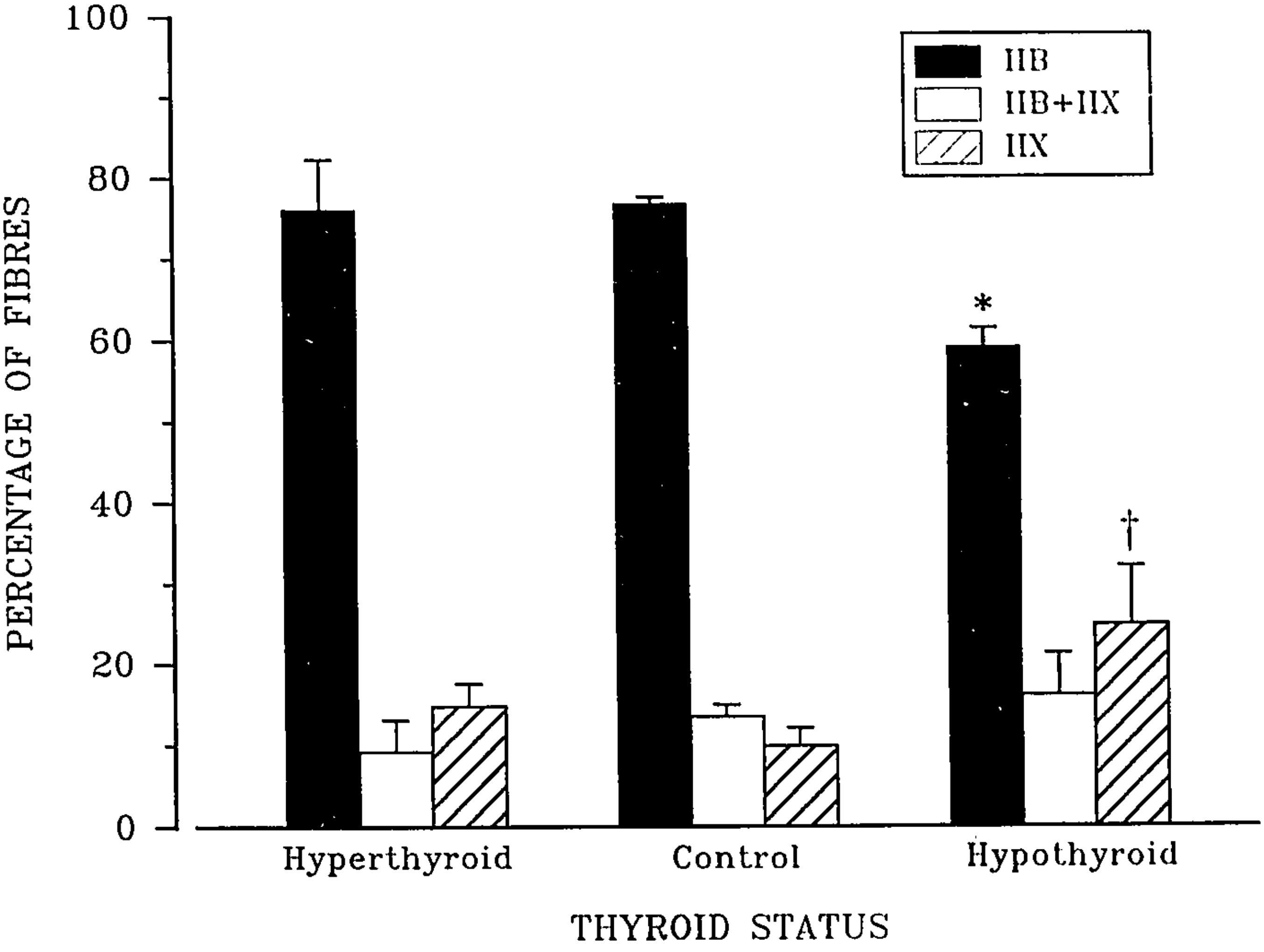
Table 5.3 Body and TA muscle weight of hyperthyroid, hypothyroid and normal mice.

Treatment group	n	Body weight (g)	TA muscle weight (mg)	Mw/Bw (mg/g)
Hyperthyroid	7	26.8±0.6*	43±1	1.6±0.07
Hypothyroid	7	25.7±0.9*	41.6±2	1.6±0.1
Control	5	31.2±1.3	46.8±1.4	1.5±0.07

Abbreviation: Mw = TAS muscle weight, Bw = body weight.
Values are mean ± S.E.
* Significantly different from control, $P<0.05$, one-way ANOVA.

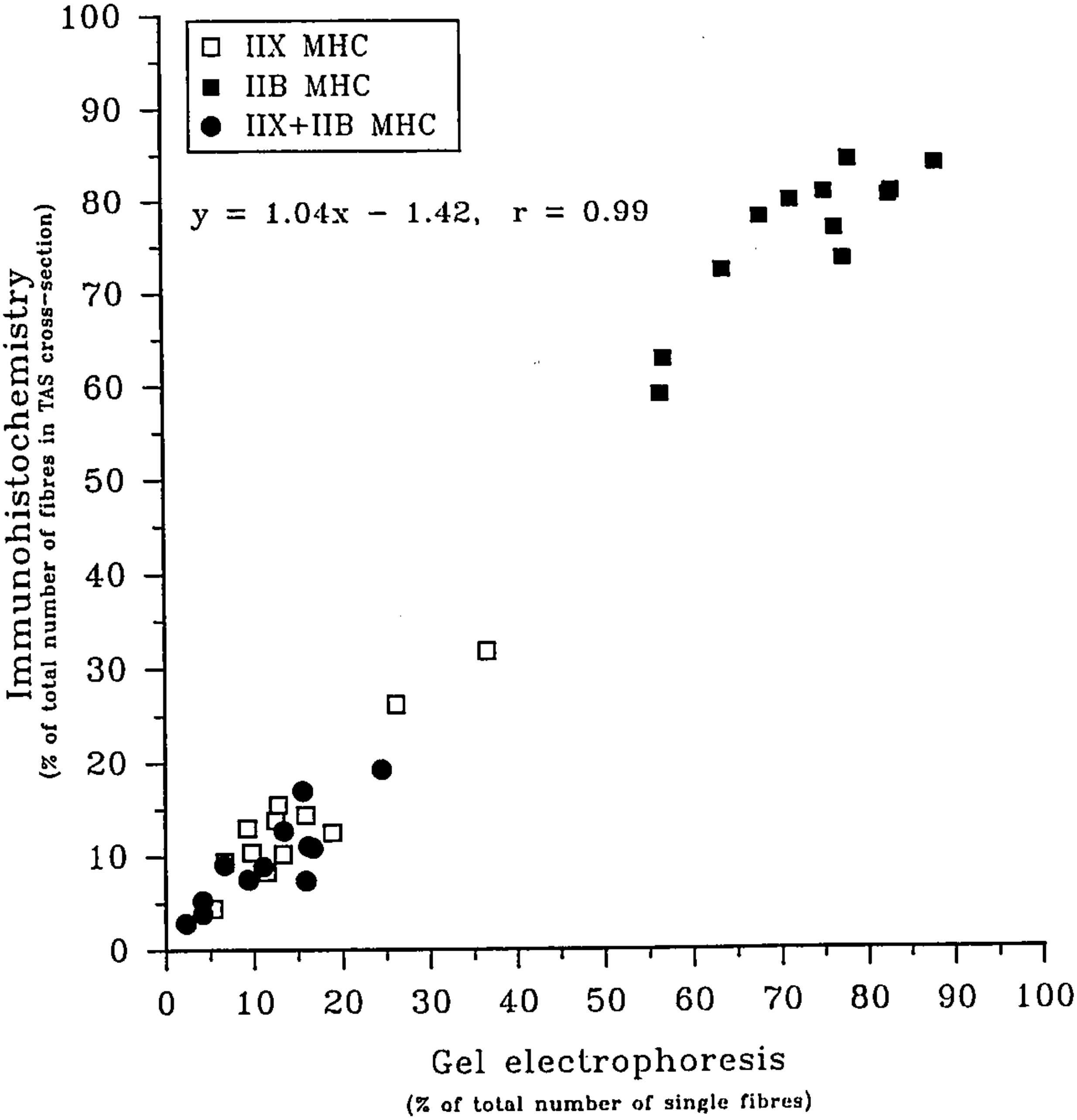
Figure 5.2. Proportion of type IIB, IIB+IIX and IIX fibres in TAS muscle of hyperthyroid, hypothyroid and control mice. Fibre types were determined by 6% SDS-PAGE of single fibres. 3 animals were used in each group. Number of single fibres used was: 140, 152 and 129 for hyperthyroid, hypothyroid and control mice, respectively.

* † Significantly different from hyperthyroid and control. $P<0.05$, one-way ANOVA.



100
80
60
40
20
0
PERCENTAGE OF FIBRES
IIB
IIB+IIX
IIX
*
†
Hyperthyroid
Control
Hypothyroid
THYROID STATUS

Figure 5.3 Reliability of immunohistochemical and electrophoretic determination of fibre type proportions in TAS muscle. Each points represents data from a single animal and relates the proportion of fibres expressing a given MHC isoform by immunohistochemical characterization to the proportion of single fibres sampled from the controlateral muscle expressing the same MHC isoform as detected by gel electrophoresis. The two methods yielded virtually identical estimates of MHC expression.


□ IIX MHC
■ IIB MHC
● IIX+IIB MHC
y = 1.04x − 1.42, r = 0.99
Immunohistochemistry
(% of total number of fibres in TAS cross-section)
Gel electrophoresis
(% of total number of single fibres)
0
10
20
30
40
50
60
70
80
90
100

5.4 Discussion

5.4.1 Age related changes in MHC expression.

The results of this study indicate that age exerts an effect upon MHC isoform expression in the fast-twitch TAS muscle of the mouse; in particular the percentage of IIB MHC decreases with a concomitant increase in the percentage of IIX MHC. The presence of hybrid fibres, containing IIB and IIX MHC, suggests that there is a transformation of IIB fibres into IIX fibres. Indeed Larsson et al. (1991, 1993) using immunohistochemical techniques to identify the MHC composition in the fibres of the fast-twitch TA motor units in young and old rats recently reported the presence of motor units containing a mixture of type IIB and IIX fibre types in the old group, and an age-related increase in type IIX motor unit, that was not detected in their previous works using ATPase staining (Larsson and Edström 1986; Edström and Larsson 1987; Ansved and Larsson 1989; Larsson and Salviati 1989). Thus the discrepancy between the results of the present study and those of others may possibly be explained by differences in the methods used to identify fibre types.

The mechanisms underlying the decreased speed of contraction with ageing are not yet well understood. However it has been shown (Barany 1967) that the intrinsic speed of shortening of muscle fibres is directly correlated to ATPase activity and that the activity is basically determined by the type of MHC expressed by a fibre (Wagner 1981; Sivaramakrishnan and Burke 1982; Reiser et al. 1985). It has been reported that the myosin ATPase activity decreases in both fast- and slow-twitch muscles with age (Gutmann and Syrovy 1974; Ermini 1976).

The contractile properties of TAS muscles at the different ages were not investigated in the present study, however it is worth mentioning that contractile measurements done on whole EDL muscle (see Chapter 4) from mice 2.9 and 10.8 months old revealed a significant decrease in speed of contraction in the older group. Unfortunately the relative percentage of IIB, IIB+IIX and IIX fibres have not been investigated in these muscles.

Schiaffino et al. (1988) reported that IIX MHC is the major MHC in the soleus muscle of the rat which has been transformed by high frequency chronic stimulation. The maximum velocity of shortening (Vo) of this transformed soleus is intermediate between that of normal soleus (almost exclusively type I MHC) and that of EDL (fast muscle). This suggests that Vo of IIX fibres is smaller than that of IIB fibres. In this respect, Bottinelli et al. (1991) reported that the mean Vo of fast fibres seemed to suggest that type IIB MHC might be associated with higher Vo than type IIA and IIX MHC, although the ranges of Vo of fast fibres showed some overlap. Therefore the increased proportion of IIX MHC could partly account for the reduced Vo and ATPase activity in the fast-twitch muscle with ageing.

Transformation of TAS muscle during growth and ageing may reflect an adaptive process, although the physiological basis for an increased proportion of type IIX fibres is not clear from the present data. It is possible that these changes are associated with changes in function, although which comes first is unclear. For example, an increase in IIX fibres in a muscle would confer a greater degree of fatigue resistance in that muscle, which could correlate with an increasing functional load due to increasing body and muscle weight.

5.4.2 Thyroid hormone related changes in MHC expression.

The results of this study indicate that thyroid hormone exerts an effect upon MHC isoform expression in mouse fast TAS muscle. Hypothyroidism leads to a decrease in the expression of IIB MHC and an increase of IIX MHC, while hyperthyroidism had no significant effect. These results are in agreement with those reported in rat by Kirschbaum et al. (1990). They studied the effect of thyroid hormone on myosin expression in fast twitch muscles both at the protein level and at the mRNA level. Hyperthyroidism did not significantly alter the percentage distribution of IIB MHC and IIA MHC at the protein level. Studies at the mRNA level, however, showed that hyperthyroidism enhanced IIB MHC mRNA, suggesting a low turnover of IIB MHC.

The lack of effect of elevated T_3 levels on MHC expression is unlikely to be due to an insufficient time (5 weeks) for the treatment to have an effect in mouse. Studies on myosin isoform transition responses to thyroid hormone at the protein level done on adult rats (Ianuzzo et al. 1977, 1984; Fitzsimons et al. 1990) suggested that six weeks of treatment was a reasonable time considering the half-life of skeletal muscle myosin (Kubista et al. 1971).

The reduced IIB MHC expression in hypothyroid TAS is compensated for by the increase in IIX MHC. This, together with the existence of single fibres co-expressing IIB+IIX MHC, strongly suggests that the transitions between IIB MHC and IIX MHC and vice-versa follows the order:

$$\text{IIB MHC} \rightleftarrows \text{IIB+IIX MHC} \rightleftarrows \text{IIX MHC}$$

The functional consequences of long term thyroid treatment have been well established. It is known that dysthyroidism can modulate both skeletal muscle oxidative capacity and the potential for free fatty acid utilization and affect the properties of calcium release and sequestration of the sarcoplasmic reticulum (Baldwin et al 1980; Fitts et al. 1980; Winder et al. 1980; Ianuzzo et al. 1984; Lomax and Robertson 1992). These studies suggest an overall reduction in the energy transducing capacity of the hypothyroid muscles. Thus an increase in IIX fibres in the hypothyroid TAS muscle would confer a greater degree of fatigue resistance.

Kirschbaum et al. (1990) have studied the effects of chronic low frequency stimulation at altered thyroid hormone levels on MHC expression to see whether or not these two stimuli can influence each other. Their results indicate that high thyroid hormone levels and increased neuromuscular activity exert antagonistic effects upon MHC isoform expression in rat fast-twitch muscles at both the mRNA and protein level, while hypothyroidism and chronic low frequency stimulation act synergistically.

It is interesting to note that both age and hypothyroidism produced an increased proportion of fibres expressing IIX MHC. It has been frequently reported that aged animals are hypothyroid (reviewed by Florini and Regan 1985). More recent reports (Carter et al. 1987; Effron et al. 1987) have confirmed earlier indications that there are small but detectable decreases (25 to 40%) with age in circulating levels of thyroxine (T4) and triiodothyronine (T3) in rats. These latter studies correlated thyroid hormone levels and MHC expression in cardiac muscle which contains three myosin isoenzymes, designated V1, V2 and V3. V1, which predominates in rapidly contracting cardiac muscle fibres, is composed of a homodimer of α-MHC. V3, the slowest form, is a homodimer of ß-MHC, and V2, intermediate in contraction speed and ATPase activity, is a heterodimer of α and ß MHC. With age, a decrease in cardiac myosin ATPase activity occurs with a corresponding decrease in the relative proportion of the V1 isoform. Interestingly, administration of thyroid hormone reversed this effect of age; ATPase and V1 isoform content of hearts returned to "young" levels. These studies with cardiac muscle suggest that the age-related decrease in contractile performance of the heart can be attributed to the rather small decrease in circulating levels of thyroid hormones. No such studies have been done on skeletal muscles, but it would be interesting to see if the observed changes in MHC expression with age in skeletal muscles could be similarly attributable to a possible decrease in circulating thyroid hormone levels.

Concluding remarks

The purpose of the present investigation was to study the distribution, subunit composition and physiological characteristics of type IIX fibres.

The results can be summarized as follows:

1) The existence of a population of type IIX fibres in fast-twitch muscles of the mouse was shown by mean of immunohistochemistry and gel electrophoresis. In hindlimb muscles such as TA and EDL, type IIX fibres account for approximately one-third of the total fibres number, while in diaphragm and tongue muscles almost half of the fibres are IIX. TAS is composed exclusively of type IIB and IIX fibres.

2) Single fibre gel electrophoresis revealed a significant number of fibres containing both IIB and IIX MHC. This was confirmed with immunohistochemistry that revealed the presence of fibres with various degree of staining intensity. This suggests that there may exist a degree of plasticity which results in the conversion of IIX fibres to IIB fibres and vice versa.

3) In support of the above suggestion, the relative levels of expression of IIB and IIX MHC in mouse muscle were shown to be dependent upon the age and the thyroid hormone status of the animal. Aging and hypothyroidism led to a decrease in the expression of IIB MHC and an increase of IIX MHC.

4) Analysis of MLC composition of type IIX fibres revealed that the ratio of MLC 3f to MLC 1f was significantly lower than type IIB fibres. This may suggests that IIX fibres have a slower Vo than IIB fibres.

5) Single motor units were isolated using an *in vitro* procedure, their contractile characteristics measured and the glycogen depletion technique used to demonstrate the muscle fibres in the units whose MHC expression were then determined by immunohistochemistry. IIX motor units had morphological, physiological properties that distinguished them from types IIA and IIB fast-twitch motor units. Specifically, IIX motor units had a resistance to fatigue, cross-sectional fibre area and motor unit area intermediate between IIA and IIB motor units.

Taken together these results suggest that type IIX fibres represent a distinct population of fibre types, with distinct morphological and physiological characteristics. This has recently been confirmed by the finding of a separate gene for IIX MHC. However, it is not clear from the present study whether type IIX fibres can also interconvert with type IIA fibres as they do with type IIB fibres. Thus one can not say whether type IIX fibre represents an intermediate step in the conversion of type IIB to IIA fibres and vice versa or whether type IIB and IIX fibres arise from a distinct pool of myogenic precursor cells which do not give rise to type IIA or type I fibres.

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