FATIGUE AND DAMAGE AS A RESULT OF EXERCISE IN NORMAL AND DISEASED SKELETAL MUSCLE

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"One must be fertile in expedients and not satisfied to apply one's mind entirely to the writings of others"

Aretaeus of Cappadocia,
Causes and Symptoms of Disease
circa. 200 A.D.

ABSTRACT

This thesis examines the nature of fatigue and damage as it affects healthy and dystrophic skeletal muscle. Initial fatigue studies were carried out using isolated mouse muscles. After 3 min of repeated maximal stimulation, extensor digitorum longus muscle force was reduced to 26% of the fresh value but this could be reversed by the addition of caffeine to the incubation medium, suggesting that acute fatigue is primarily due to failure of the processes of activation.

In the human tibialis anterior (TA) muscle it was found that fatigue resulting from stimulated isometric contractions were affected by muscle length. Exercise at short lengths resulted in less force loss at the resting length, whereas exercise at long muscle lengths caused a greater force loss than normal at the resting length. There was a preferential force loss at sub-maximal stimulation frequencies, and this was exacerbated when muscles were exercised in a lengthened positon. Similar changes were observed using isolated mouse soleus muscles.

Because of uncertainties about the adequate diffusion of metabolites in isolated muscles the properties of dystrophin-deficient (*mdx*) mouse muscles were investigated using an in vivo preparation of the TA. The *mdx* TA was, on average, 30% stronger than that of control mice but had a reduced force/cross-sectional area and a smaller low/high frequency force ratio due to a faster activation time. *mdx* muscle also displayed a greater fatigue resistance when exercised at a low frequency, but this was not the case with stimulation at maximal frequency.

In order to test whether the altered contractile properties of *mdx* muscle were due to the presence of degenerating and regenerating fibres, the contractile characteristics of normal muscle were investigated during damage and recovery. Damage was induced by stimulated lengthening

contractions of the foot dorsiflexor muscles of mice. Maximum force, force-frequency characteristics, and morphology were measured for up to 20 days after exercise. Although the properties of normal/damaged and *mdx* muscles displayed a number of superficial similarities, it is unlikely that the altered contractile characteristics of *mdx* muscles are due to the presence of damaged fibres.

The possibility that dystrophin-deficient muscles are more susceptible to exercise induced muscle damage was examined by comparing the responses of *mdx* and normal muscles to an episode of eccentric work. The findings were unequivocal, normal and *mdx* TA muscles displayed similar degrees of force loss 3 days after exercise (55% and 52% respectively) and comparable rates of force recovery after 12 days (76% and 80% of control in normal and *mdx* muscles respectively).

The protective effect afforded by a bout of eccentric exercise against subsequent muscle injury from a similar exercise was characterised. Re-exercising a muscle after 10 days recovery had little effect on the immediate consequences of exercise, but reduced the degree of delayed onset force loss and fibre necrosis. Animals re-exercised after 12 weeks recovery displayed no apparent protection against delayed onset muscle damage when the exercise was repeated. These findings are in general agreement with work carried out in humans, but the time course of recovery post exercise is at least three times faster in the mouse, which has made a study of the long-term effects of eccentric exercise more practical. Six weeks after exercise, increases in muscle mass and force were evident, with a proportion of fibres displaying internal nuclei and signs of fibre splitting. Surprisingly, greater forces and fibre hypertrophy also occurred after 12 weeks recovery from an episode of eccentric work.

CONTENTS

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CHAPTER 1

INTRODUCTION

1.1 MUSCLE FATIGUE AND MUSCLE DAMAGE

> "Every muscle or group of muscles will show qualitatively the sort of properties that a very intelligent engineer, knowing all the facts, would have designed for them in order to meet, within wide limits, the requirements of their owner."
>
> (A.V. Hill, 1956)

Skeletal muscle is an engine which serves to convert chemical energy into mechanical energy with a reasonable degree of efficiency. With prolonged activity, particularly if the workload is high, skeletal muscle develops a number of "mechanical" faults, the most obvious being the failure to maintain force output and speed. When these changes are reversed following a short period of rest the process is recognised as one of "muscular fatigue". If however, muscle function is impaired over a more prolonged period (eg. hours or days), this is often termed "muscle damage" and the recovery called "repair". Thus from a functional viewpoint muscle fatigue and damage represent two consequences of activity, and the overlap between these processes is a recurring theme of this thesis.

1.2 SHORT-TERM EFFECTS OF EXERCISE

There are basically two questions that can be asked about muscle fatigue. The first question concerns the point at which failure occurs in the pathway from cortical activation to cross-bridge cycling. The second line of inquiry is concerned with the nature of the change leading to fatigue, that is, what is the cause of fatigue? The inability to maintain force output during activity (ie. muscle fatigue) is experienced by everyone in some aspect of their daily life, but muscle fatigue is not purely of interest to those involved in improving athletic performance.

Such studies may provide information which will eventually lead to an amelioration of the excessive fatigue observed in many patients, for example those suffering from respiratory, cardiovascular and renal disease. Fatigue has been studied in preparations as diverse as isolated single frog muscle fibres, to intact human muscles, and various sites of, and mechanism for, the development of fatigue have been postulated.

1.2.1 Central fatigue

The inability of physical performance to achieve an anticipated goal is commonly attributed to a lack of central drive or motivation, a process termed central fatigue. Indeed, until the middle part of this century most workers believed that muscle fatigue was a consequence of the inability of the nervous system to activate muscle fully. In his seminal study of the stimulated and voluntary force outputs of the adductor pollicis in man, Merton (1954) described three main findings. First, voluntary activation and maximal stimulation resulted in similar force outputs, secondly, voluntary and stimulated forces were equivalent even after 3 min of maximal voluntary contractions (demonstrating that there was no central failure of motor drive). Finally Merton showed that during fatigue the size of the compound muscle action potential remained constant (ie. there was no failure of the peripheral nerve or neuromuscular junction). The findings of Merton have since been confirmed during sustained contractions of the adductor pollicis (Bigland-Ritchie et al, 1979, 1983) and the quadriceps (Bigland-Ritchie et al, 1978). In the latter study, however, maximal voluntary and stimulated forces were only the same in the quadriceps muscles of subjects who were well motivated and received sufficient encouragement. Bellinger and McComas (1981) using the technique of superimposing electrically evoked, twitch contractions upon voluntary contracting muscle (twitch interpolation) to detect sub-maximal

contractions, showed that normal subjects are capable of fully activating most of the muscles tested. In contrast Grimby et al (1981) stated that central fatigue may be a limiting factor in the quadriceps during repeated leg extension exercise.

1.2.2 Neuromuscular junction failure

Exhaustion of neurotransmitter at the nerve-muscle junction provides a plausible mechanism for the development of muscle fatigue and its potential role as a cause of fatigue during voluntary contraction has been the centre of lively debate. Krnjvick and Miledi (1958), using an isolated rat diaphragm and phrenic nerve preparation, showed that conduction failure along the axons of motorneurones, and at the neuromuscular junction could occur if the stimulation frequency was above 10 Hz. Human investigations of neuromuscular junction failure have recorded the size of the compound muscle action potential (M wave) in response to stimulation of the motor nerve. The studies of Merton (1954) using the adductor pollicis (described in section 1.2.1) were disputed by Stephens and Taylor (1972) who produced evidence in support of neuromuscular junction failure as a likely source of fatigue in the first dorsus interosseus muscle. However, Merton's findings were confirmed by Bigland-Ritchie et al (1979, 1984) who demonstrated that no neuromuscular junction failure occurred over a 60 sec sustained maximal voluntary contraction of the adductor pollicis. The balance of evidence would point to neuromuscular junction not being a major site of fatigue in human muscles under normal conditions.

1.2.3 Excitability of sarcolemma

As a result of continuous muscle stimulation at high frequencies, the surface membrane of the muscle may lose its ability to conduct action

potentials. Naess and Storm Mathieson (1955) originally reported a reduced amplitude and velocity of the waveform of muscle action potentials in both the human adductor pollicis and rabbit psoas muscles following prolonged stimulation at a high frequency. The reduced amplitude and changed waveform of the action potential are both accompanied by a loss of force (Jones, et al, 1979; Bigland-Ritchie et al, 1979), and Juel (1988) showed that the change in waveform with fatigue to be attributed to a slowing of conduction velocity of the motor unit action potential.

A characteristic of fatigue induced by high frequency stimulation is that the force recovers rapidly if the stimulation frequency is reduced, eg. in isolated mouse soleus and intact human adductor pollicis (Jones et al, 1979) and for single frog fibres by Lannergren and Westerblad (1987b). The rapid force recovery observed when the stimulation frequency is reduced would suggest that ionic, rather than metabolic factors are responsible for force loss under these conditions.

1.2.4 Excitation-contraction coupling

Under normal conditions action potentials are propagated both longitudinally along the muscle fibre membrane and down the transverse (t)-tubules. The inward spread of excitation is the trigger for Ca^{2+} release from the sarcoplasmic reticulum (SR), although the exact details of this process are not fully understood. Several workers have demonstrated that repetitive stimulation of muscle fibres leads to the accumulation of K^+ in the t-tubules (Freygang et al, 1964; Adrian and Peachey, 1973). Furthermore both Jones et al (1979) and Bigland-Ritchie et al (1979) have proposed that the changes in amplitude and velocity of the muscle action potential described in section 1.2.3 could result from the accumulation of K^+ or depletion of Na^+ in the extracellular spaces or t-tubules. Support

for this mechanism of excitation failure comes from the fact that force loss with stimulation can be exacerbated by reducing the external Na^+ concentration (Jones et al, 1979), or increasing the K^+ (Lannergren and Westerblad, 1986). Furthermore, increasing the extracellular K^+ concentration in isolated diaphragm preparations mimics the change in action potential waveform resulting from fatigue (Jones and Bigland-Ritchie, 1986). Given the limitations of the rate of diffusion of ions in and out of the t-tubules resulting from their small diameter, the K^+ concentrations attained at this site will be considerably higher than those measured in the interstitial spaces. Lannergren and Westerblad (1986) have recorded the membrane potentials of isolated frog muscle fibres following 20 sec of continuous stimulation at 70 Hz. They found that there was a progressive decrease from -80 mV to -40/50 mV during the course of fatigue, and similar observations have been made by Juel (1986).

Direct evidence of activation failure during fatigue has recently been provided by the elegant study of Westerblad et al (1990) who measured the degree of activation within a single frog muscle fibre by visualising the Ca^{2+}-induced luminescence of the photoprotein aequorin. They found that after a period of high frequency stimulation there was a decrease in the calcium signal in the fibre interior consistent with an impaired activation in the centre of the fibre. A reduction in the muscle fibre membrane potential would not, in itself cause a loss of membrane excitability, and the reduction in the amplitude of the muscle action potential with fatigue is believed to result from an inactivation of membrane Na^+ channels caused by repeated depolarisation of the surface membrane (Simoncini and Stuhmer, 1987; Ruff et al, 1987).

The role of high frequency activation failure caused by action potential propagation has been brought into doubt as a physiological

phenomenon since, during sustained contraction, there is a neural mechanism which causes a decrease in the motor-unit firing frequency. This phenomenon has been demonstrated in the adductor pollicis (Bigland-Ritchie et al, 1979, 1983), tibialis anterior (Grimby et al, 1981), soleus (Kukulka et al, 1984) and quadriceps (Bigland-Ritchie et al, 1986). Fatigue is also associated with a slowing of relaxation in muscle and the consequent reduction in tetanic fusion frequency allows the central nervous system to lower the firing frequency required to maintain maximal tetanic force. This effectively prevents the extracellular accumulation of K^+ ions resulting from muscle action potential propagation.

Changes in K^+ conductance can take place in fatigued muscle fibres which are not dependent on action potential propagation. Fink and Luttgau (1976) reported that metabolically exhausted fibres showed a large increase in K^+ conductance and more recently, Fink et al (1983) suggested that this change was mediated by an increase in intracellular Ca^{2+} (possibly involving Ca^{2+}-activated K^+ channels). Increased K^+ permeability of metabolically exhausted frog muscle fibres has also been reported by Castle and Haylett (1987) who showed evidence that the increase in K^+ efflux was associated with channels which had the pharmacological properties of an ATP-dependent (rather than a Ca^{2+}-dependent) K^+ channel. However, Spruce et al (1987) reported that the opening probability of these channels were raised only at ATP concentrations of around 0.1 mM, which is far lower than that measured in fatigued muscle and raising doubts about the role of these channels in skeletal muscle fatigue.

1.2.5 Impaired cross-bridge formation

Fatiguing muscle work often results in the accumulation of several

by-products of the reactions which supply energy for cross-bridge turnover. Of these muscle metabolites, several are known to directly interfere with cross-bridge action.

1.2.5.1 Intracellular pH

Several reactions involved in the resynthesis of ATP can alter the intracellular environment, notably glycolysis (the anaerobic resynthesis of ATP from glycogen). Glycolysis is often associated with muscle fatigue as it is a major source of intracellular H^+ (Wilkie, 1979). There is incontrovertible evidence that raising the intracellular H^+ concentration causes a decrease in maximal force production in non-fatigued preparations of skinned fibres (Donaldson and Hermansen, 1978; Fabiato and Fabiato, 1978), intact single fibres (Curtin et al, 1988) and whole muscles (Curtin, 1986). However in fatigued muscle there is good evidence that, at least in some conditions, accumulation of H^+ does not make a direct contribution to force decline. Lannergren and Westerblad (1988) using isolated frog muscle fibres have shown that the time course of force decline and recovery from fatigue correlated poorly with intracellular pH changes. Further evidence against the importance of an increase in intracellular H^+ in the development of fatigue is provided by the fact that in the absence of glycolysis (no increase in H^+) fatigue develops even more rapidly than normal as described by Sahlin et al (1981) for muscles poisoned with iodoacetate and Cady et al (1989a) in the muscles of patients with myophosphorylase deficiency.

1.2.5.2 Inorganic phosphate

Intracellular levels of creatine phosphate (PCr) fall as a muscle fatigues (Dawson et al, 1978; Nassar-Gentina et al, 1981; Crow and Kushmerick, 1982). Whilst neither decreased PCr per se or increased

creatine concentrations are likely to have any direct effect on force production, the increase in Pi concentration resulting from a fall in PCr is a possible cause of force loss in fatigued muscles.

A raised Pi level depresses the maximal Ca^{2+} activated force developed in skinned cardiac and skeletal muscle preparations (Kentish, 1986; Cooke and Pate, 1985). Cady et al (1989a) used topical nuclear magnetic resonance spectroscopy to investigate changes in Pi in the hand muscles of subjects undergoing fatiguing contractions. They found that the time course for the accumulation of Pi differed from that of force loss during fatigue, the increase in Pi preceding the start of force loss by about 30 sec and similar conclusions may be drawn in most human muscle experiments. The discrepancy between the findings of skinned fibre and whole muscle preparations in terms of the importance Pi in the development of force loss during fatigue may be explained by differences in resting Pi in the fresh state. Thus whereas skinned fibre preparations tend to be maintained at Pi levels of 0, this is not the case in resting whole muscle fibres. Furthermore, resting fast twitch fibres have Pi concentrations of 2-3 mM, as opposed to concentrations of 18-20 mM for slow twitch fibres (Meyer et al, 1985) suggesting that Pi accumulation may be make a more important contribution to fatigue in the former.

The role of Pi in muscle fatigue is further complicated by the fact that some workers have suggested that force loss in fatigue might result from the combined effect of changes in Pi and pH (eg. Wilkie, 1986; Miller et al, 1988). Cooke et al (1988) measured the isometric force, maximal velocity of shortening and ATPase activity of Ca^{2+}-activated skinned rabbit psoas muscle. They found that pH and Pi had both separate and combined effects. The relative proportions of mono and dibasic forms of Pi are quite variable at different pH levels within the physiological range. At a resting intracellular pH of just over 7, two-

thirds of the Pi will be in the dibasic form, but when the pH falls with fatigue, the proportions change so that at a pH of 6.5 around two-thirds of Pi will be in the monobasic form (Miller et al, 1988). Nosek et al (1987) using skinned fibre preparations, described a relationship between monobasic phosphate and isometric force. A monobasic phosphate concentration of 20 mM (near the maximum achieved in fatigued muscle) resulting in a 50% reduction in force.

Work also exists which argues against the importance of monobasic phosphate in force loss. If glycolysis is prevented, maintaining pH at 7, there is a similar force loss despite an unchanged mono:dibasic Pi ratio (Cady et al, 1989). Thus pH and Pi appear to possess independent and additive effects on the decline of force during fatigue, but the precise mechanism of their actions remain uncertain, although it has been suggested that a raised Pi concentration reduces the number of cross-bridges in the strongly-bound, force-producing state (Kentish, 1986; Cooke et al, 1988).

It is clear that, under suitable conditions, muscle fatigue can result from failure at any one of the sites described above. For this reason the parameters under which fatigue is induced experimentally are of crucial importance in defining its cause.

1.2.6 Activation of contractile proteins

The stimulus for actin-myosin interaction involves the binding of Ca^{2+}, released from the SR, to the protein troponin-C. If Ca^{2+} release is reduced in fatigued muscle to a level insufficient to fully saturate the Ca^{2+}-binding sites of troponin-C, or there is a reduction in the avidity of troponin-C for Ca^{2+}, it will lead to a reduction in the total number of cross-bridge interactions (ie. a decrease in force). Using skinned fibre preparations, workers have identified a number of metabolites present in

fatigued muscle which may affect the interaction between Ca^{2+} and troponin-C; these have been reviewed by Stephenson (1988)

The second Chapter of this thesis concerns the identification of the site at which fatigue occurs following repeated tetanic stimulation of an isolated mouse muscle preparation. In Chapter 3 experiments are described which attempted to distinguish between the metabolic causes of force loss and those associated with ionic changes at the level of the t-tubules. It was noticeable, in all the fatigue protocols described, that there was a disproportionate loss of force at sub-maximal frequencies of stimulation. This phenomenon was originally termed long-lasting fatigue by Edwards et al, 1977 (because of the characteristically prolonged recovery time) but is more commonly termed low frequency fatigue (LFF). In the 14 years since it's original description there has been remarkably little improvement in our understanding of the changes which bring about LFF.

1.3 LFF and muscle damage

Low frequency fatigue had been observed in a variety of muscles following isometric work, including the adductor pollicis (Edwards et al, 1977), sternomastoid (Moxham et al, 1980) and diaphragm (Aubier et al, 1981). As a phenomenon, LFF has a number of characteristic features. It is present in muscles following fatigue after maximal force and metabolite levels have returned to normal (Edwards et al, 1977; Jones, 1981). Secondly, Newham et al (1983a) demonstrated that, following dynamic work, LFF is more pronounced in muscles exercised eccentrically (ie. when the contracting muscle is forcibly stretched) than after shortening contractions, leading the authors to suggest that LFF is related to the degree of force generated within muscle fibres. LFF also occurs in

muscles which have been damaged as a result of eccentric exercise (Newham et al, 1983). Although little is known of the cause of LFF, Jones et al (1982) showed that, in isolated mouse muscles, LFF could be reversed by the addition of caffeine to the incubation medium, suggesting some form of damage to the Ca^{2+}-release mechanism.

1.4 Muscle damage

Exercise, if it is of a long duration, or of an unaccustomed nature, can lead to a number of effects which may last for several days. Exercise-induced muscle damage can have a variety of causes and may result in a number of adverse effects. Thus exercise may cause muscle pain, loss of force, altered contractile properties and the release of intracellular proteins (for example creatine kinase (CK) and myoglobin), (Jones et al, 1986). Injury to muscle fibres can result from either metabolic or mechanical causes. Although severe metabolic depletion in isolated muscles can cause necrosis and enzyme release which is thought to be mediated by an increase in intracellular Ca^{2+} (Jones et al, 1984) there is no evidence for its importance in exercise-induced damage as a result of normal activity (Sjostrom and Friden, 1984).

Mechanical trauma to muscle fibres resulting from exercise can cause muscle damage which is if of two types. In the first there is a rise in plasma CK following exercise which peaks after 1-2 days in man (Noakes, 1987) possibly resulting from mechanical damage at the myotendinous junctions (Nikolau et al, 1987). After unaccustomed eccentric exercise, however, there is a rise in plasma CK which occurs some 4-7 days after exercise (Newham et al, 1983a, 1987; Evans et al, 1986). The fact that muscle pain has a different time course from that of CK release (peaking at 2-4 days in man) has been used as evidence to suggest that they represent the consequences of different phenomena

(Jones et al, 1987).

1.4.1 Morphological studies of muscle damage

Morphological studies, in both animals and man, have shown changes in muscle structure which occur following severe exercise, and these provide the most direct evidence of muscle damage. Friden et al (1981) reported focal areas of Z-line streaming, broadening and, in places, disruption, 2 days after subjects performed downstairs running. More recently it has been found that Z-disc alterations can be observed immediately after exercise (Newham et al, 1983; Sjostrom and Friden, 1984). There is evidence to suggest from human studies that, after intensive eccentric exercise, morphological changes are more common in Type 2 fibres, but they have also been in observed in Type 1 fibres (Jones et al, 1986). In contrast, animal studies have shown a more or less selective involvement of Type 1 fibres (Armstrong et al, 1983; Vihko 1978). The experimental evidence confirms that delayed-onset muscle damage, characterised microscopically by focal Z-disc abnormalities and myofibrillar disruption, is especially related to eccentric work.

Although Z-disc alterations are the most commonly observed change in muscle ultrastructure after damaging exercise, additional changes have been observed. Armstrong et al (1983) reported disruptions in the continuity of the sarcolemma, whilst Newham et al (1983b) described the widening of A- and I-bands in fibres from the quadriceps muscles of subjects following eccentric exercise.

1.4.2 Inflammatory response in muscle damage

The delayed infiltration of muscle fibres by inflammatory cells is a consistent finding following exercise-induced damage resulting from eccentric work (Schwane and Armstrong, 1983; Jones et al, 1986). These

infiltrates consist almost exclusively of mononuclear cells and Round et al (1987) have characterised the cellular infiltrates resulting from a bout of eccentric exercise to the biceps of normal subjects. These cells consisted largely of macrophages, the remaining cells being T-lymphocytes with a predominance of the CD4 positive helper/inducer subset. The authors point out that these infiltrates are very similar to those commonly seen in inflammatory muscle diseases such as polymyositis, which suggests that in this disease the infiltrates may represent the natural response to muscle damage rather than its cause per se. The function of the invading mononuclear cells is thought to be phagocytosis of necrotic fibres and cell debris to allow regeneration to occur, and this is supported by the fact that the inflammatory response occurs after the peak in CK efflux (the latter representing muscle fibre breakdown) (Jones et al, 1986).

Forcing rats to perform downhill running until exhaustion has been found to result in focal muscle necrosis after 24-48 h (Hecht et al, 1975). The presence of degenerating and necrotic muscle fibres in mice 2-5 days after eccentric exercise was described by Vihko et al (1978), they also reported infiltrates of mononuclear cells, which were most prominent 5 days after exhaustive running. One of the clearest demonstrations of the role of eccentric exercise in delayed-onset muscle damage in animals is the study of Armstrong et al (1983). They found that downhill running in rats (eccentric exercise to the vastus intermedius muscle) caused a biphasic increase in creatine kinase (CK) activity with peaks at 2 hours and 2 days after exercise. The latter was associated with focal necrosis, whilst after 3 days regenerating myotubes became apparent. Another study demonstrating the differences between concentric and eccentric work on the development of muscle damage is that of McCully and Faulkner (1985) who found a significant correlation with the decrease in force after exercise and the development of delayed onset muscle damage. But these

changes were only seen after lengthening (eccentric) rather than shortening (concentric) contractions.

1.5 Mechanism of muscle damage

Two hypotheses have been proposed to explain the cause of delayed-onset muscle damage. The first assumes a metabolic overload, in which the demand for ATP exceeds the supply, leading to a positive feedback loop where a reduced availability of ATP causes a decreased activity of energy-dependent ion pumps, the resultant increase in intracellular Ca^{2+} leads to a depression of mitochondrial respiration and hence decreased availability of ATP. Whereas this mechanism is known to cause exercise-induced pain in a number of muscle disorders (eg. myophosphorylase deficiency (Brooke, 1986)) there is no evidence for its role in delayed-onset muscle damage.

The fact that eccentric exercise, in which high forces are generated within muscle fibres, is known to induce extensive morphological muscle damage and large increases in CK activity together with muscle pain, stiffness and weakness, provides support for the second hypothesis, namely that mechanical rather than metabolic factors are the cause of exercise-induced muscle damage (Newham, 1988). If metabolic waste products such as lactate are the causal mechanism of muscle damage, then concentric exercise should produce more damage as this type of work is metabolically more demanding than eccentric exercise (Curtin and Davies, 1973). Furthermore, electromyographic activity is lower in eccentric exercise, which suggests that fewer muscle fibres are recruited to produce large forces (Bigland-Ritchie and Woods, 1976). Thus, the metabolic cost of eccentric work is less than that of concentric exercise, but the mechanical strain per fibre is higher, because fewer muscle fibres are recruited.

1.5.1 Muscle damage and muscular dystrophy

Muscle fibre necrosis is one of the major features of the muscular dystrophies, which form one of the major groups of muscle diseases. Most important of these is Duchenne muscular dystrophy and the study of muscle function in dystrophic patients is difficult since any abnormalities could be due either to the condition itself or the consequent presence of fibre necrosis and regeneration. The advancement of knowledge through the study of animal models of muscular dystrophy has been restricted by the fact that until recently no model existed which had the same pathogenesis as the human condition. The discovery of the *mdx* mouse strain (Bulfield et al, 1984) has made it possible to study the functional characteristics of dystrophin-deficient muscle in some detail. Such a study forms the basis of Chapter 4, in which the strength, contractile properties and fatiguability of normal and *mdx* muscles are compared. In Chapter 5 the eccentric exercise model of Faulkner et al (1985) is described and this was used to test one of the currently held hypotheses for the pathogenesis of muscular dystrophy. This postulates that dystrophin-deficient muscles are more susceptible to the stresses associated with normal muscle activity, and that this results in repeated episodes of muscle damage of the type described in section 1.4.

1.6 Long-term effects of muscle damage

Although the consequences of exercise-induced damage to skeletal muscle in the short-term (ie. until full recovery takes place) have been well described, the long-term effects of eccentric exercise have been poorly investigated. In Chapter 6 experiments are described in which the changes occurring in muscle following a bout of exercise-induced muscle damage are investigated in two ways. In the first the time course of the

protective effect of damaging exercise against repeated damage is elucidated, whilst in the second the long-term consequences of a single bout of damaging exercise on muscle function are reported.

CHAPTER 2

STUDIES OF FATIGUE USING AN ISOLATED MOUSE MUSCLE PREPARATION

2.1 INTRODUCTION

The study of muscle function outside the influence of the body has a number of advantages. Factors which might affect muscle contraction *in situ* such as blood flow, temperature and neural feedback can be controlled *in vitro* and, whereas these variables may be important functionally, elucidation of the intrinsic properties of normal and fatigued muscle is simplified by their absence.

Many of the early studies of muscle function used amphibian muscle preparations (see A.V. Hill (1965) for a review of much of the early work on muscle physiology). Amphibian muscles have a number of advantages over mammalian muscles, notably their relative stability at low temperatures (so alleviating the problems associated with oxygen diffusion into the preparation, see later), a greater fibre size and reduced quantities of connective tissue. The latter properties allow the routine (although technically demanding) study of single fibre preparations, a feat which has only recently been applied successfully to mammalian muscle (Lannergren and Westerblad, 1987b). Mammalian muscles, on the other hand, have the advantage of allowing comparisons to be made more readily with data from human studies and the mouse soleus and extensor digitorum longus (EDL) muscles have proved to be popular experimental models. Two major problems arise with the use of isolated whole muscles; if the preparation is large the diffusion of oxygen may be inadequate to supply the metabolic requirements of fibres situated near the centre of the muscle and there may be impaired diffusion of substances such as K^+, lactate, and H^+ from fibres in the muscle core. Such effects will be exacerbated during sustained muscle work because of the increase in metabolic rate. Secondly, muscles tend to be composed of fibres with varying contractile and metabolic characteristics, such a diversity of fibre types will clearly

complicate the study of changes during exercise.

The extensor digitorum longus (EDL) muscle of the mouse provides a useful model for *in vitro* study since it is composed of fibres which are predominantly fast twitch and fatiguable (Goldspink and Ward, 1979). Type II fibres have a considerably higher metabolic rate during exercise than Type I fibres (Crow and Kushmerick, 1982) and the problems of oxygen diffusion in fast fibres are more acute. To overcome this problem in the present work, fibre bundles from mouse EDL muscles were used. Such a preparation goes some way to achieving the ideal condition of using single fibres.

The work described in this chapter had the initial objective of assessing the viability of an EDL fibre bundle preparation and to describe its contractile properties and fatiguability. Once achieved the specific aim of the study could be addressed, namely to investigate the extent to which activation failure could account for the loss of force observed during fatigue. Calcium can be released directly from the sarcoplasmic reticulum (SR) by high concentrations of caffeine. Thus, if impaired Ca^{2+} release contributes to force loss during fatigue then caffeine should elicit greater forces than produced by stimulation alone. But, if Ca^{2+} release is unaffected by fatigue then it would be expected that caffeine contractures would be of similar size to tetanic forces.

2.2 METHODS

2.2.1 Animals

EDL muscles were obtained from mature female C57 BL/10 mice between 16 and 24 weeks of age (body weight 19-25 g). Animals were sacrificed using a lethal dose (200 mg/Kg body weight) of Pentobarbitone Sodium B.P. (Sagatal, May and Baker Ltd.) injected intraperitoneally.

2.2.2 Muscle preparation

After anaesthesia had developed the muscle was exposed and the proximal and distal tendons were firmly tied with cotton thread. Dissection of the two EDL muscles took about 10 minutes, after which muscles were transferred to a bath of oxygenated mammalian Ringer's solution at room temperature (approximately 22°C).

The distal tendon of the muscle was teased apart using fine forceps into at least four separate strands which were carefully separated into fascicles. Further dissection of fascicles was carried out using micro-dissection scissors to produce small bundles of fibres. The result was a preparation which had a diameter of approximately 0.1 mm, as viewed under a dissecting microscope, and was estimated to contain about 20 viable muscle fibres. Following dissection, the ends of the tendons were secured using aluminium foil tags and the fibre bundle carefully transferred to the recording chamber for measurement of contractile properties.

2.2.3 Incubation media

Muscles were incubated in bicarbonate Ringer's solution at pH 7.4 (D.K. Hill, 1972). This was continuously gassed with O_2/CO_2 (95%/5%). For experiments requiring calcium free medium $MgCl_2$ replaced $CaCl_2$ and 0.1mM EDTA was added.

To prevent neuromuscular stimulation during the experiments curare (d-Tubocuraraine chloride, 0.25 mM) was added to the incubation medium. Caffeine contractures were elicited using a saturated solution (approx 20mM) in the appropriate Ringer solution which was perfused into the incubation chamber over the course of a few seconds.

2.2.4 Force recording

The experimental set-up is illustrated schematically in Fig 2.1. Fibre bundles were suspended in a perspex bath (volume 2.0 ml) between large platinum electrodes approximately 10 mm apart (Gutmann and Sandow, 1965). The proximal end of the bundle was attached to a metal hook fixed to the chamber wall whilst the distal end was attached to a force transducer. This consisted of a flexible silicon beam with sensitive resistors diffused on either side (Aksjeselskapet Mikro-Electronikk, Norway). Force applied to the end of the beam causes stress in the region of the resistors resulting in a change of resistance, ie. the resistance on the compressed side will decrease whilst on the side under tension the resistance will increase. The resistors were arranged to form two arms of a Wheatstone bridge circuit, the other resistances being fixed. The voltage across the bridge was set at 4.0 Volts using a Fylde 492BBS Mini-Balance bridge conditioning unit. The voltage difference was amplified with a Fylde 254GA Mini-Amplifier and the signal displayed and recorded on an ultra-violet oscillograph chart recorder (SLE).

The voltage and duration of stimuli were set using an isolated

stimulator (Devices) triggered by a frequency generator (Digitimer) and produced a voltage which was amplified by a 50 Watt audio amplifier to produce sufficient current to maintain about 30 V between the electrodes when in solution. Fibre bundles were stimulated with a supramaximal voltage (6-10 V) using square wave pulses of 0.02 ms duration, except where long duration pulses were used in which case the pulse duration was increased tenfold to 0.2 ms.

2.2.5 Contractile characteristics

At the beginning of each experiment the optimum length (Lo) of the fibre bundle was determined by adjusting the length to give maximum twitch force. Twitch and maximal tetanic forces were recorded for each preparation as well as a force/stimulation frequency relationship in response to 0.5 s pulse trains of different frequencies. Twitch time to peak (TTP) and half relaxation times ($t_{1/2}$) were also measured, the latter being the time taken for maximal tetanic force to fall from 50% to 25% of the peak value.

2.2.6 Viability of tissue

Initially a number of preparations were stimulated intermittently (1 maximal tetanus every 10 min) for up to 2 hours to determine the extent of deterioration of the preparation over time. The results are shown in Fig 2.2.

2.2.7 Fatigue protocols

To study the changes associated with fatigue, the EDL preparations were stimulated at a frequency of 80 Hz for 500 ms every second with pulses of 0.02 ms duration for a total of 3 min (360 contractions). Recovery was followed by recording low and high frequency tetanic

forces and relaxation rates over the next 10 minutes.

Subsequent EDL preparations were subjected to one of four protocols as follows :

Protocol A Fatigue followed immediately by 500 ms tetani at 80 Hz, one normal and one long pulse duration (0.02 ms and 0.2 ms respectively). The short and long pulse tetani were repeated during the recovery period.

Protocol B Fatigue followed immediately by a caffeine contracture.

Protocol C Fatigue followed by 10 min recovery. Then a single tetanus at a long pulse duration followed by a caffeine contracture.

Protocols A, B and C were then repeated using preparations incubated in a Ca^{2+} free medium.

2.2.8 Statistics

Data was analyzed using Student's t-test for the comparison of means of two small samples.

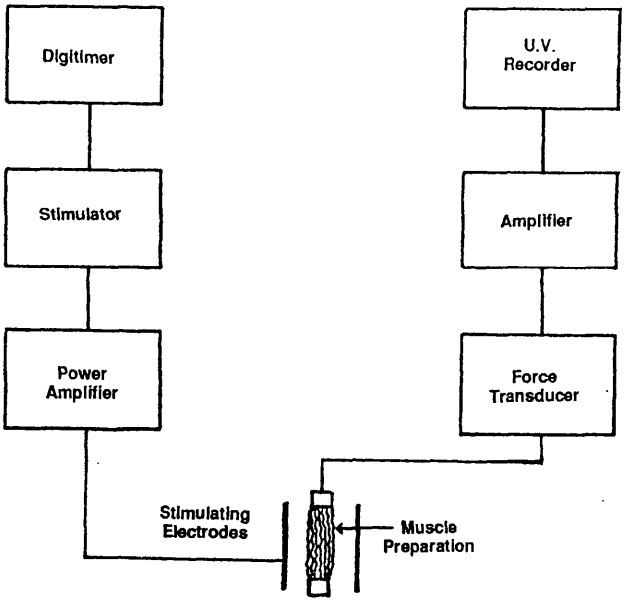


Fig 2.1 Schematic diagram of experimental set-up for force recording of EDL muscle preparations.

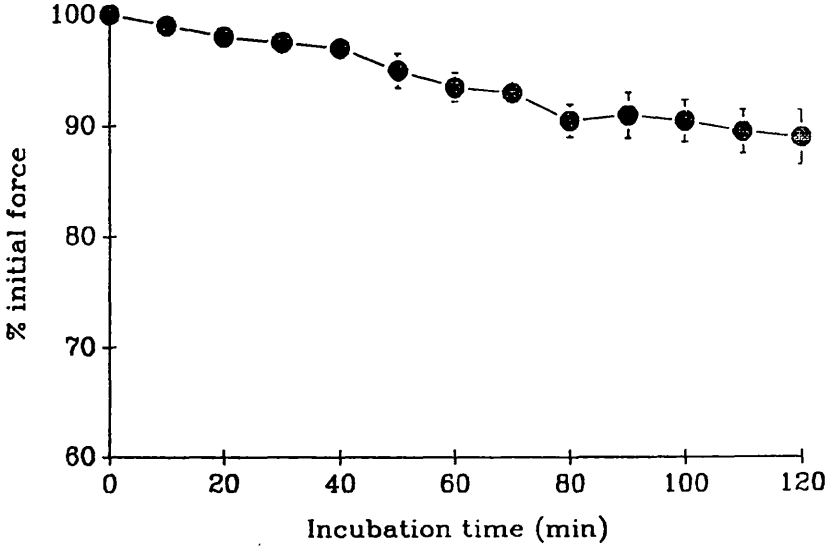


Fig 2.2 Maximal tetanic forces of EDL preparations (n=4) stimulated with 0.5 sec 100 Hz tetani every 10 min during 2 hours of incubation(±sem)

2.3. RESULTS

2.3.1 Viability of EDL preparation

Maximal forces remained stable in four EDL preparations for approximately 30 min (Fig 2.2), following which there was a small decline so that, by 2 hours after the start of incubation, force was reduced to approximately 90% of the fresh value.

2.3.2 Contractile properties of fresh preparations

The contractile characteristics of fresh and fatigued EDL bundles are shown in Table 2.1. Fresh preparations produced on average 5.8 mN. Because of the small size of each preparation and the fact that preparations had attached to them a certain amount of debris, accurate measurement of weight was not possible. Fibre bundle diameter was estimated to be about 0.1 mm, giving a cross-sectional area (assuming the bundle to be cylindrical) of 0.015 mm^2. Calculating the force/csa from this gives a value of 192 mN/mm^2 force generating capacity.

2.3.3. Caffeine contractures of fresh preparations

Fig 2.3 shows the response of a fresh EDL preparation to the application of a 20 mM caffeine solution, in this case the contracture force was 97% of the maximal tetanic force. On average, caffeine contractures produced 98.5% ($\pm$ 4.4% sem) of maximal tetanic force (n=5). Stimulation of fresh EDL preparations with tetani of normal and prolonged pulse durations resulted in identical force outputs.

2.3.4 Fatiguability

On average, the stimulation protocol resulted in a decline of force to 26% of the fresh value (Table 2.1). Complete force recovery did not take place within 60 min and was always less than 80% of fresh force.

Table 2.1 Contractile characteristics of fresh and fatigued EDL fibre bundles. (sem)

	FRESH n=13	FATIGUED n=11	RECOVERY n=11
1 Hz tension (mN)	1.29 (0.09)	0.36 (0.07)	0.71 (0.10)
40 Hz tension (mN)	1.89 (0.09)	0.72 (0.08)	1.05 (0.14)
100 Hz tension (mN)	5.82 (0.42)	1.49 (0.36)	4.02 (0.81)
1 Hz Time to peak force (ms)	7.3 (0.81)	9.5 (1.04)	8.7 (1.02)
100 Hz $T_{1/2}$ (ms)	11.3 (0.86)	14.7 (1.52)	12.2 (1.11)

The time course of recovery could be divided into two phases. A rapid recovery over the first 6-7 min and a much slower recovery phase lasting 45-60 min (Fig 2.4). It is this 10 minute value which is referred to as "recovery" in the remainder of the Chapter.

Table 2.1 shows that 40 Hz force after fatigue declined to 38% of fresh force (as opposed to 26% for 100 Hz force following fatigue). However, after 10 min, 40 Hz force recovered significantly less than 100 Hz force (56% of fresh as compared to 69% of fresh force), this is illustrated in Fig 2.5. Fatigue resulted in an increase of half relaxation time from 11.3 ms to 14.7 ms (Table 2.1), which after recovery returned to a value (12.2 ms), close to the fresh $t_{1/2}$.

2.3.4.1. *Protocol A*

Fig 2.6 shows a force trace for protocol A, and demonstrates that EDL bundles stimulated with pulses of long duration immediately after fatigue showed a dramatic improvement in force. On average (Fig 2.9) long duration pulses produced over double the normal post-fatigue force. This compares to no force increment when tetani of the same prolonged pulse duration was applied to fresh preparations. Stimulation using pulses of prolonged duration were often deleterious to force recovery using normal duration tetani and presumably damaged fibres in some way.

2.3.4.2. *Protocol B*

Fig 2.3 shows the result of treatment of a fresh EDL bundle with saturated caffeine solution, producing a contracture of similar magnitude to the maximal tetanic force. When a caffeine contracture was applied to an EDL bundle immediately after a fatiguing run, a dramatic reversal of force loss occurred, as shown in the recording in Fig 2.7. Post-fatigue caffeine contractures in a number of EDL bundles (shown in Fig 2.9)

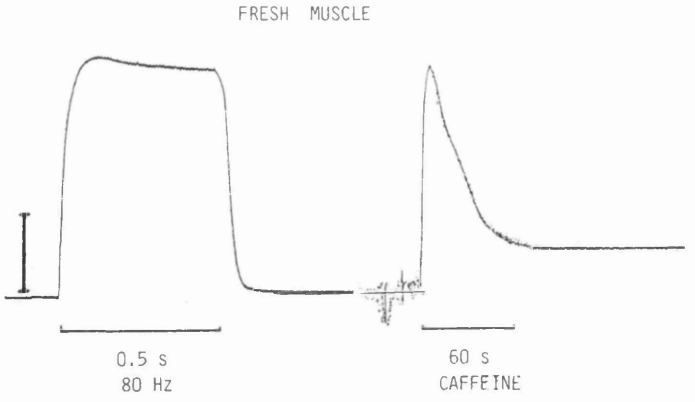


Fig 2.3 Comparison of tetanus and caffeine contracture in a fresh EDL preparation. (Bar=1mN)

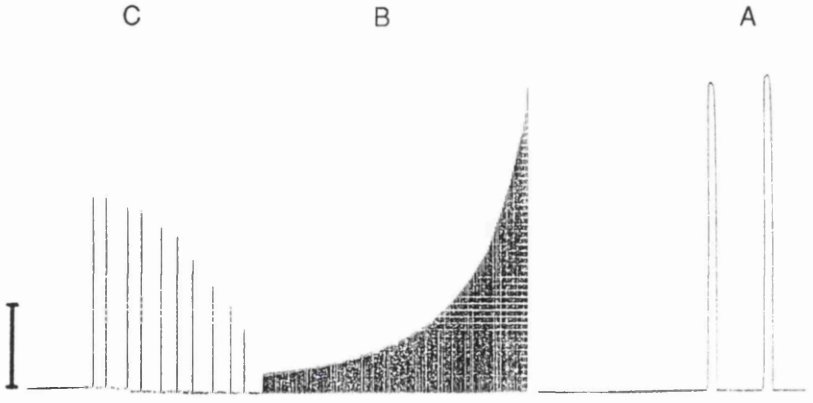


Fig 2.4 Force recordings during fatigue and recovery of EDL preparation in PROTOCOL C. (Bar=1mN)
A = 2 x 0.5 s 80 Hz tetani, 30 sec apart
B = 180 x 0.5 sec 80 Hz tetani
C = 10 x 0.5 sec tetani, 1 min apart

produced 91% (±6%, sem) of fresh force.

2.3.4.3. *Protocol C*

To investigate the possibility that impairment of muscle activation may persist after recovery had taken place, a number of preparations were fatigued as usual and allowed to recover for 10 minutes. At this time application of a long pulse duration tetanus resulted in a small force increment (less than 5%), but when this is immediately followed by a caffeine contracture, the preparation produced some 18% more force than after 10 minutes recovery (Fig 2.10).

The time course of the caffeine contracture seen in the EDL bundles was quite characteristic (Fig 2.7). After an initial peak lasting 10-20 sec, force declined to a stable level at around 50-60% of fresh force. The shapes of the caffeine contractures were similar in both fresh and fatigued preparations.

2.3.5 Fatigue in calcium free medium

EDL bundles were fairly stable when incubated in a calcium free medium, although some force loss did occur over time (amounting to 13% after 30 min as judged by maximum tetanic contractions every 2 min). Fig 2.8 shows that when muscle preparations were fatigued in calcium free medium there was a significantly greater force loss compared to those fatigued in a medium containing extracellular Ca^{2+} (force decreasing to 11% and 26% of fresh force respectively). Interestingly the muscles in calcium free medium also recovered less well than under normal conditions (43% as compared to 72% after 10 min).

Stimulation with pulses of long duration immediately after fatigue in a calcium free medium resulted in a force increment of 18% compared to 59% in the normal calcium solution. In contrast preparations fatigued

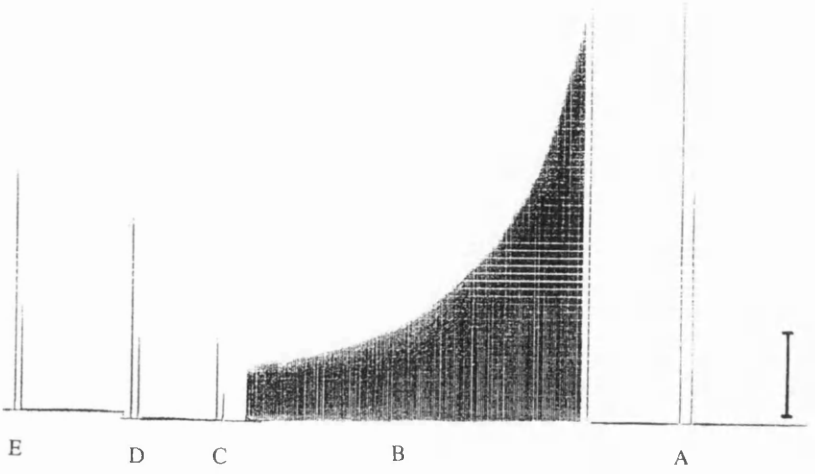


Fig 2.5 Force recordings of 40/100 Hz ratio in EDL preparation before and after fatigue. (Bar=1mN)
A = Fresh 40/100 Hz forces
B = Fatigue protocol
C = 1 min recovery 40/100 Hz forces
D = 5 min recovery 40/100 Hz forces
E = 10 min recovery 40/100 Hz forces

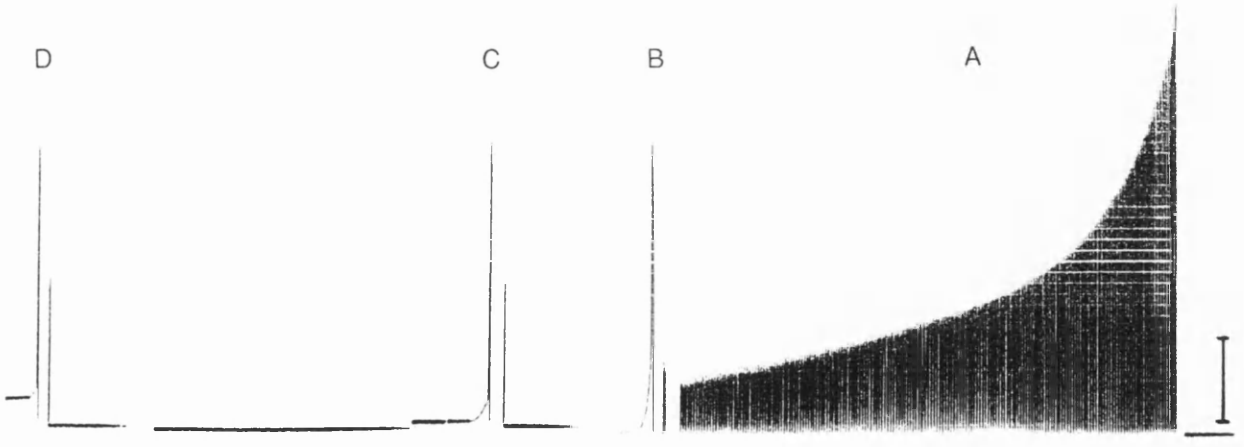


Fig 2.6 Force recordings of fatigue and recovery of EDL preparation in PROTOCOL A. (Bar=1mN)
A = Fatigue protocol
B = 80 Hz tetanus (0.02 ms duration) + 80 Hz tetanus (0.2 ms duration) immediately after fatigue
C = 80 Hz tetanus (0.02 ms duration) + 80 Hz tetanus (0.2 ms duration) after 5 min recovery
D = 80 Hz tetanus (0.02 ms duration) + 80 Hz tetanus (0.2 ms duration) after 10 min recovery

in a calcium free medium produced normal caffeine contractures immediately after fatigue (Fig 2.11). When recovery had occurred in these muscle preparations the application of prolonged pulse duration stimuli and caffeine contractures produced similar force increments to those seen after fatigue and recovery in muscles incubated in media containing Ca^{2+}.

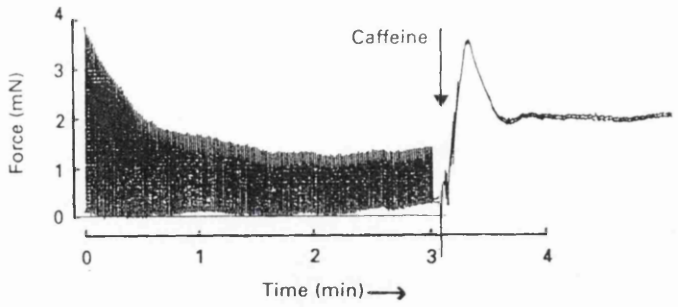


Fig 2.7 Force recording of fatigue followed by caffeine contracture of EDL preparation in PROTOCOL B.

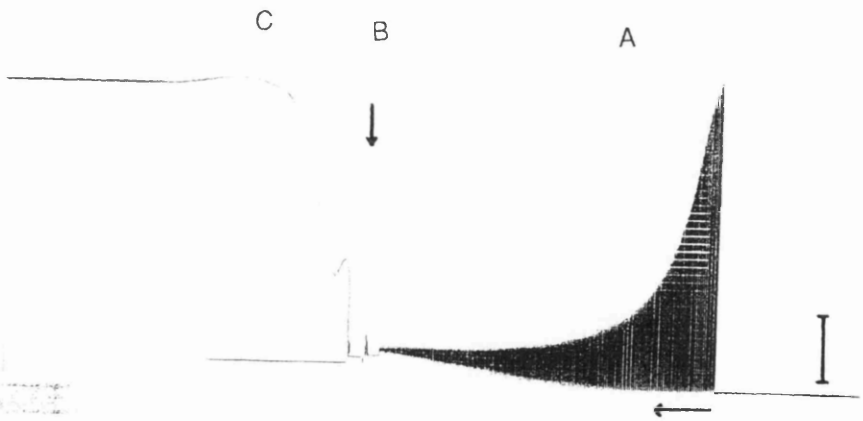


Fig 2.8 Force recording of fatigue and caffeine contracture of EDL preparation in a calcium-free medium. (Bar=1mN)
A = Fatigue protocol
B = Long pulse duration (0.2 ms) 80 Hz tetanus immediately after fatigue.
C = Caffeine contracture

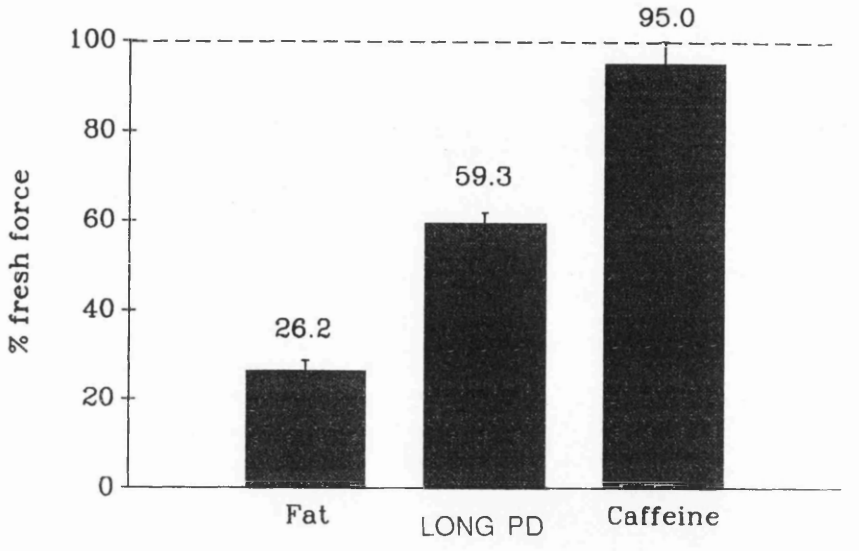


Fig 2.9 The effect of fatigue (n=11), long pulse duration tetani (Long PD) (n=5) and caffeine contractures (n=6) on force output of EDL preparations recorded immediately after the end of fatigue. Mean values (+ sem)

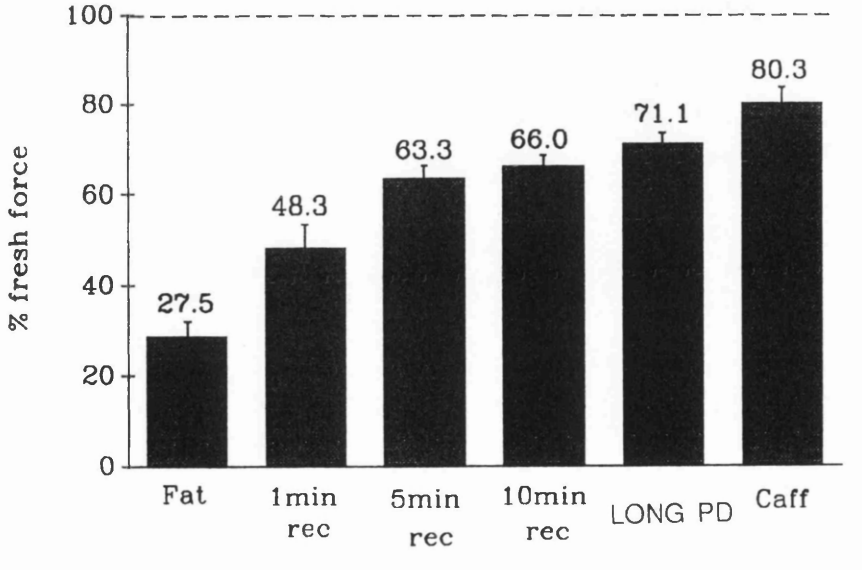


Fig 2.10 The effect of fatigue, recovery, long pulse duration tetani (Long PD) and caffeine contractures on force output of EDL preparations (n=4-6). Mean values (+ sem)

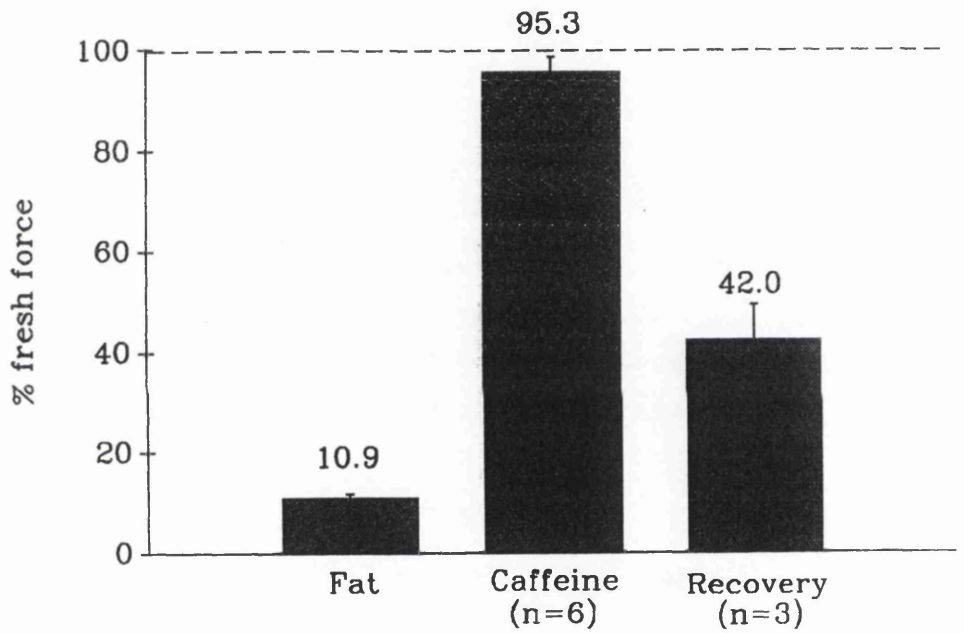


Fig 2.11 The effect of fatigue (n=9), caffeine contractures immediately after fatigue (n=6), and 10 min recovery (n=3) on force output of EDL preparations in calcium-free medium. Mean values (+ sem)

2.4 DISCUSSION

2.4.1 Tissue viability

The EDL preparations maintained near maximal force generating capacity for up to 2 hours of incubation, suggesting that the dissected fibre bundles were relatively undamaged. An important influence on tissue viability is the ability of oxygen to diffuse throughout the preparation. Any imbalance between the oxygen supply and metabolic demand could result in the development of an anoxic core in the centre of the preparation. This is a problem which has been extensively studied (for discussion see A.V.Hill, 1965) and is particularly acute for muscles containing appreciable numbers of fast fibres (Crow and Kushmerick, 1982). Although the resting metabolic rates of the soleus and EDL are similar, the faster EDL has a metabolic rate five times that of the soleus during maximal contractions. In an attempt to quantify this problem Creese et al (1958) found that, in the rat diaphragm (a fast muscle) incubated at 25°C, central fibres showed a loss of membrane potential at rest if the preparation was more than 1 mm in total thickness. The advantage of using a small bundle of fibres thus becomes obvious for studying fatiguing work in fast muscles.

2.4.2 Contractile properties

The findings for the contractile properties of the EDL preparations are similar to values obtained in previous studies using whole EDL muscles (Close,1972; Luff, 1981). The calculated force/CSA is within the range of values previously recorded (192 mN/mm^2 compared to a range of 160-250 mN/mm^2 described by Close, 1972).

2.4.3 Fatigue and Recovery

In Chapter 1 the potential mechanisms by which force production

may be impaired "beyond the neuromuscular junction" during fatigue were described. To reiterate they consist of :

1. Excitability of sarcolemma
2. Excitation-contraction coupling failure
3. Impaired cross-bridge formation
4. Impaired activation of contractile proteins

The possible contribution at each of the fatigue sites will be assessed in the light of the findings described in the mouse EDL fibre bundle.

2.4.3.1 *Propagation failure of surface action potential*

Excitation of a muscle fibre involves depolarisation of the sarcolemma with subsequent generation of a propagating action potential. Under some circumstances the fibre membrane can become inexcitable (ie. no action potential is generated in response to stimulation). These changes are most usually seen in experiments involving continuous, high frequency stimulation in, for example, the human adductor pollicis (Jones, 1981), cat medial gastrocnemius in situ (Sandercock et al, 1985) and single frog muscle fibres (Lannergren and Westerblad, 1986). A characteristic of this form of fatigue is a decline in amplitude and a reduction in the conduction velocity of the muscle fibre action potential (Bigland-Ritchie et al, 1979). Continuous high frequency stimulation of a muscle fibre is likely to lead to an accumulation of K^+ and a depletion of Na^+ in the t-tubules (Jones, 1981). Adrian and Peachey (1973) calculated that each action potential results in a fall in t-tubule Na^+ concentration of 0.5mM and a rise in K^+ concentration of 0.28mM. Diffusion limitations within the t-tubules may lead to K^+ accumulation and Juel (1988) showed that small increments in extracellular K^+ can led to rapid reduction in force with repeated tetani.

A second effect, due to Na^+ depletion within the t-tubules, may be

a reduction in the size of the action potential and thus a reduction in the amount of Ca^{2+} released from the SR. Bezanilla et al (1972), using single frog fibres, showed that when external Na^+ was reduced, tetanic force declined rapidly and this was associated with the presence of wavy (ie. inactivated) fibrils within the central region of the fibre. Furthermore, Ruff et al (1988) have shown that inactivation of membrane Na^+ channels (a potential mechanism by which action potential propagation can be blocked) can occur with relatively small decreases in membrane depolarisation in mammalian skeletal muscle. This slow Na^+ channel inactivation can have a time course of several minutes. More direct evidence of the failure of activation in the t-tubular system has been provided recently by Westerblad et al (1990) who showed that continuous stimulation of single frog fibres resulted in a radial gradient of intracellular Ca^{2+} such that the centre of the fibre showed lower Ca^{2+} levels than the periphery. This observation supports the idea of a failure of Ca^{2+} release at the centre as compared to the edge of the fibre, indicating a failure of inward conduction.

The raised excitation threshold for action potential propagation within the t-tubules resulting from fatiguing stimulation can be partially overcome using KCl contractures (Lannergren and Westerblad, 1986) or by increasing the stimulating pulse duration (Jones, 1981). The mechanism by which the latter method increases force in fatigued muscles is not known. Fatigued muscle could require more current to flow through the surface membrane before an action potential is initiated but it is also possible that prolonged pulses might cause direct release of Ca^{2+} from the SR.

Whatever the underlying mechanism, finding that the fatigued EDL bundles produced significantly more force when stimulated with pulses of long duration suggests that activation failure makes a considerable

contribution to force loss in this preparation.

Given the uncertainty over the exact mechanism of force enhancement using prolonged pulse durations it is not possible to conclude from these results whether action potential propagation failure was a cause of fatigue. If propagation failure did occur it would be surprising since such failure has generally been associated with continuous high frequency stimulation (Bigland-Ritchie et al, 1979, Vollestad, 1988). Although the stimulation frequency used in the present experiments (80 Hz) could be considered high, the fact that the protocol was intermittent (0.5 sec/1 sec) means that the overall rate of stimulation was low with 500msec intervals during which diffusion of ions from the t-tubules might take place. Alternative mechanisms leading to action potential propagation failure might be considered.

An increase in membrane conductance leading to K^+ efflux has been reported in metabolically exhausted fibres by Fink and Luttgau (1976). The effects of metabolic exhaustion can be mimicked in muscle by the application of iodoacetic acid (either injected systemically or using isolated muscle preparations incubated in a medium containing iodoacetate). Iodoacetate is a sulphydral-reactive chemical which selectively inhibits the enzyme glyceraldehyde-3-phosphate-dehydrogenase and so blocks glycolytic metabolism (Webb, 1966). Brumback (1980) reported that muscles poisoned with iodoacetic acid show alterations in membrane conductance. Jones and Bigland-Ritchie (1986) reported the effects of iodoacetic acid treatment on isolated preparations of diaphragm muscle. They found that metabolically poisoned muscle showed changes in action potential conduction velocity and waveform which were similar to those seen in fatigued muscle. ATP-sensitive K^+ channels are known to occur in skeletal muscle (Spruce, Standen and Stanfield, 1985) whose opening probability is raised at low intracellular ATP concentrations.

This could represent a possible link between metabolic depletion and action potential propagation failure.

Another possibility is that changes in intracellular metabolites associated with fatigue cause a disruption in the capability of the Ca^{2+} channels to operate normally. Thus at a pH of around 6.5 the opening probability of the Ca^{2+} channels falls to 0 (Rousseau and Pinkos, 1990), although the authors point out that these findings were from isolated membranes under non-physiological conditions.

Inositol triphosphate (IP3) is known to be involved in the coupling between excitation and contraction in smooth muscle (Somlyo and Somlyo, 1990) and has been implicated to have a similar function in skeletal muscle, although the evidence is less conclusive in this case (reviewed by Rojas and Jamovich, 1990). If IP3 is involved in excitation-contraction coupling in skeletal muscle it provides the attractive possibility of linking changes in ATP concentration with Ca^{2+} release, since IP3 turnover is known to be ATP dependent (Donaldson, 1986).

2.4.3.2. *Caffeine and fatigued muscle*

The coupling of t-tubule depolarisation and intracellular Ca^{2+} release has been the subject of a great deal of research over the last 10 years and a clear understanding has now developed of the processes involved. In 1973, Schneider and Chandler put forward the hypothesis that depolarisation of the t-tubule activates sensors located in the t-tubule membrane which unblock Ca^{2+} channels within the SR. Their hypothesis was based on finding a charge movement whose time course and voltage sensitivity matched what would be expected for a controller of a hypothetical Ca^{2+} channel in the SR. More recent studies have identified a dihydropyridine-binding protein (possibly a modified Ca^{2+} channel) as the voltage sensor (Tanabe et al, 1987: Renaud, 1989). This molecule is

coupled to a ryanodine-binding protein which has been demonstrated as the Ca^{2+} channel found in the SR membrane in the region of the terminal cisternae (Lai et al, 1988). Single channel recordings from isolated SR vesicles have demonstrated that this Ca^{2+} channel is activated by micromolar [Ca^{2+}], millimolar [ATP], and caffeine and is inhibited by Mg^{2+} and calmodulin (Rousseau et al, 1988). The mechanism by which charge movement within the voltage sensor triggers Ca^{2+} channel opening remains unknown. The fact that in appropriate concentrations caffeine can directly activate the calcium channels (Rousseau et al, 1988) provides a useful tool in the study of the mechanism of fatigue.

In the experiments of Protocol C it was found that caffeine contractures generated forces which were approximately 90% of fresh values immediately following a bout of intermittent high frequency fatigue in the mouse EDL bundle. Thus, with an effective stimulus, Ca^{2+} could still be released from the SR and the contractile proteins were still able to generate near maximal force. Similar observations have been made using caffeine contractures in a number of frog muscle preparations (Nassar-Gentina et al, 1981; Westerblad and Lannergren, 1987) and in isolated single mouse muscle fibres (Lannergren and Westerblad, 1991).

It is an obvious possibility that the decline in SR Ca^{2+} release with fatigue might be due to Ca^{2+} depletion of the SR. However Gonzalez-Serratos et al (1978) using electron-probe analysis found that there was a small increase in the Ca^{2+} content of the terminal cisternae in isolated frog muscle fibres even when force had become almost abolished as a result of repeated tetani. The finding in the present work that fatigued muscles produced normal caffeine contractures when Ca^{2+} was absent from the external medium implies that the Ca^{2+} for activation came from intracellular stores (ie. the SR) which indicates that the Ca^{2+} levels of the SR remain adequate to fully saturate the troponin-binding sites

following fatigue should there be the means to release it.

The increase in force produced by stimulating fatigued muscle with long pulses in calcium-free media, although significant, was markedly smaller than that elicited in muscles fatigued in normal media. The reason for this is uncertain but it does agree with the findings of Luttgau and Speicker (1979) who demonstrated that K+ contractures were reduced in frog muscle fibres incubated for several minutes in Ca^{2+}-free medium. This finding was confirmed by Cota and Stefani (1981) who suggested that external Ca^{2+} may play a role in muscle activation in long depolarisations such as occur with K^+ contractures and high frequency tetani. Thus if prolonged pulse duration caused activation by Ca2+-induced Ca2+ release, it would explain the finding that such stimuli have little effect on muscles fatigued in Ca2+-free medium. In contrast, caffeine contracture forces are the same whether or not Ca^{2+} is present in the incubation medium in both the fresh and fatigued state.

2.4.3.3. *Impaired cross-bridge turnover*

Repeated contractions cause many changes in the intracellular composition of fibres, some of which are known to have marked effects on the calcium sensitivity of the myofilaments. However, in the present experiments the fact that near normal forces could be developed in fatigued preparations if treated with caffeine shows that the cross-bridges were functioning adequately.

Using caffeine contractures it was not possible to demonstrate whether or not intracellular metabolite accumulation caused a reduction in the calcium sensitivity of the myofilaments. A shift in the force/pCa^{2+} curve has been described following fatigue (Donaldson and Hermansen, 1978) and this could account for the presence of low frequency fatigue after the end of stimulation since the shape of the force/pCa^{2+} curve

results in relatively greater force loses at sub-maximal Ca^{2+} concentrations (ie. at low frequencies of stimulation). However this is not likely to be the cause of the greater force loss at 40 Hz compared to 100 Hz since this phenomenon lasts throughout the recovery period when intracellular metabolites will have recovered. More likely, the LFF is due to a reduction in the amount of Ca^{2+} released for each action potential and represents disruption of sarcoplasmic reticulum function (Jones, 1981), possibly being an indication of damage to the muscle fibre.

2.4.3.4. *Dystrophic muscle*

It was of interest to compare the contractile properties and fatigue characteristics of muscles from normal and dystrophic mice. To this end a number of preparations were made from the EDL muscles of the *mdx* strain of mouse. However it proved impossible to obtain viable *mdx* fibre bundles of comparable size to that obtained from normal EDL muscle. This may have been due to the degree of myofibre necrosis and fibre splitting present in the dystrophic muscle (Dangain and Vrbová, 1984).

2.4.3.5. Summary

An isolated preparation of mammalian skeletal muscle has been described which approaches the single fibre level, thus obviating the problems associated with oxygen diffusivity in active isolated fast muscles. The development and recovery from intermittent fatigue has been described in terms of force loss, changes in force-frequency characteristics and in relaxation rate.

Stimulation with prolonged pulse durations immediately after fatigue resulted in a significant force increment and caffeine contractures before and after fatigue generated similar forces to those obtained in the fresh muscles. The clear implication of these findings is that the loss of force

seen in these preparations was caused by failure of the processes of activation. Unfortunately the experiments cannot separate possible failure of action potential generation and conduction from failure of calcium release from the SR.

Another uncertainty is the nature of the causative agents. Broadly the failure of activation could be the result of either ionic events, such as the accumulation of K^+ in the extracellular spaces or the accumulation of metabolites such as lactate and Pi. Experiments to examine these possibilities are described in the following Chapter.

CHAPTER 3

THE EFFECT OF MUSCLE LENGTH ON THE DEVELOPMENT OF FATIGUE

3.1 INTRODUCTION

In the previous Chapter experiments were described which were designed to investigate the development of fatigue in an isolated mouse extensor digitorum longus (EDL) muscle model. It was concluded that failure of activation of contractile proteins played an important part in force loss in this model.

Failure of muscle activation is widely accepted as a mechanism contributing to muscle fatigue during short, high intensity work regimes (Jones et al, 1979; Sejersted et al, 1984; Lannergren & Westerblad, 1987b; see also Chapter 1) but the mechanism for the loss remains unclear. Broadly, the possibilities are either that the loss of excitability is due to changes in the concentrations of potassium or sodium in the t-tubules or intracellular spaces as the result of prolonged electrical activity, or that changes in the intracellular metabolites (lactate, Pi, ADP etc) may directly affect EC coupling. In 1985 Fitch & McComas attempted to resolve this dilemma. They argued that, since muscles generate different forces when held at different lengths, the metabolic consequences of exercise at these lengths would also be different. However it is likely that the electrical activity of the muscles would be the same so that if fatigue was a result of ionic shifts a similar degree of fatigue would be expected at the two lengths. If, on the other hand, metabolite changes were responsible then a different pattern of fatigue should be seen. Less force is generated in the human tibialis anterior (TA) when stimulated in the shortened position and Fitch & McComas found that there was a significantly greater force loss when muscles were stimulated at an optimal length rather than in a shortened position. They concluded that muscle fatigue is related to the number of actin-myosin cross-bridge interactions (ie. ATP consumption) rather than activation failure

consequent on electrical activity in the surface membrane. The major contribution to ATP turnover in active muscles is associated with force generation by cross-bridges (Homsher & Kean, 1978) and ATP turnover decreases as muscles are extended and the filament overlap decreases on the right hand limb of the length/tension relationship (Rall, 1982).

Fitch & McComas compared optimum and *short* lengths but it is not clear whether the force loss on the left hand side of the length/tension relationship is associated with a decrease in ATP turnover. Indeed there is reason to doubt that this is so. The decrease in force at short lengths is partly the result of mechanical interactions of overlapping actin filaments from the two halves of the sarcomere and, at very short lengths, of myosin filaments meeting the Z lines. A better design of experiment would be to compare the effects of fatigue at optimum and long lengths.

There are other problems with the work of Fitch & McComas (1985). Forces were not measured during the fatigue run itself but only after the end of fatigue and at optimal rather than fatigued lengths. For these reasons it was decided to repeat the study of Fitch & McComas and extend it to look at the effects of fatigue at long muscle lengths. This forms the basis of the first section of this Chapter. The study was also repeated using an isolated muscle preparation, the mouse soleus. This muscle was chosen in preference to the EDL preparation because it was decided to look at some of the long term consequences of activity and while the EDL had its merits, full recovery was not one of them.

During the initial experiments on the length effects of fatigue in the human TA it was noticed that contractions performed at long muscle lengths resulted in a prolonged decrement of tetanic forces produced by stimulation with sub-maximal frequencies. This phenomenon has been described by Edwards et al (1977) as low-frequency fatigue (LFF) and is probably analogous to the post-contractile depression observed in

amphibian muscles following fatigue (Westerblad & Lannergren, 1986). Human studies have shown that LFF may have a time course of hours or days (Edwards et al, 1977) and since it can be reversed, in isolated muscle preparations, by the addition of low concentrations of caffeine (Jones et al, 1982), the likely cause is believed to be damage to the EC coupling mechanism. There are many similarities between these changes and the changes in contractile properties seen following exercise-induced muscle damage and these changes are exacerbated when exercise is performed at long muscle lengths (Jones, Newham & Torgan, 1989).

As a footnote to the human and mouse muscle work, and, as an introduction to the work described in the following Chapter, some preliminary experiments are reported in which soleus muscles from the dystrophic mouse (*mdx*) were fatigued at different lengths.

3.2. METHODS

3.2.1. Human Tibialis Anterior experiments

3.2.1.1. *Subjects*

Experiments were carried out on 7 volunteers (5 male, 2 female), aged 25-59 years with the approval of the University College Hospital Ethical Committee. Informed consent was obtained from each of the subjects.

3.2.1.2. *Force recording*

Isometric foot dorsiflexor forces were obtained using a Cybex II dynamometer. The subjects were seated with the right knee at an angle of 90° and the right foot strapped into an adjustable foot holder which rotated about an axis aligned to that of the ankle joint. Force produced by foot dorsiflexor contractions was detected by means of two strain gauges (Kulite Sensors) attached to the foot plate. The gauges were arranged in a half-Wheatstone bridge formation with a Fylde (254GA) differential amplifier. The output was fed into an A/D converter (Amplicon PC-26) and the digitised signal processed by a data handling package (Amplicon Microscope) using an IBM compatible PC.

3.2.1.3 *Muscle stimulation*

Surface electrodes were used in all experiments. The stimulating electrodes consisted of two rounded silicone rubber electrodes approximately 3 cm in diameter. The proximal electrode (cathode) was positioned over the belly of the muscle and the distal electrode (anode) approximately half way along the length of the muscle. Care was taken not to place any part of the electrode over the tibia, since direct stimulation of the tibia was associated with sharp pain at this site. This electrode placement was associated with least discomfort during

stimulation and it minimised inadvertent stimulation of muscles other than the TA. Rectangular voltage pulses of 50 μs duration were delivered from a stimulator (Devices) which received a triggering pulse from a digital timing device (Digitimer).

3.2.1.4 *Force Length characteristics*

Stimulated and voluntary force-length curves were obtained for each subject prior to the start of the fatiguing experiments (Fig 3.1). It was found that the optimum length for force production occurred with the foot in 10° of plantarflexion.

3.2.1.5 *Fatigue protocol*

All fatigue runs were carried out with stimulated contractions, as were all the forces measured post-fatigue and during recovery. Each subject completed three fatigue runs, each at a different muscle length set by the degree of foot dorsiflexion. Fatigue runs were carried out with a gap of at least 3 days between runs. Four of the subjects repeated the protocol so that these individuals completed each fatigue run twice. Each fatigue run consisted of 6 x 15 sec contractions at 40 Hz with a 5 sec gap between each contraction. All fatigue runs were carried out ischaemically, with a large blood pressure cuff around the lower thigh inflated to a pressure of 200 mmHg.

Fatigue was carried out with the foot at 25° of dorsiflexion (short), 10° of plantarflexion (Lo) or 35° of plantarflexion (long). Regardless of the position in which the muscle was fatigued, maximal force and force-frequency characteristics were measured at Lo (the test length) immediately before and after the fatigue run. Circulation was then restored to the leg and forces were measured at 1, 5 and 10 min recovery. The total period of muscle ischaemia was not longer than 3

min for each fatigue run.

In two subjects muscle forces were measured at 6 and 24 hours following the fatigue run, it was found that by 6 hours any effects of fatigue had disappeared.

3.2.1.6. *Statistics*

In order to assess the significance of any difference in force after fatigue at different lengths, paired t-tests were performed with significance set at the 5% level.

Paired t-tests were only performed on data which showed a significant F value using randomised block analysis of variance.

3.2.2. Isolated Muscle Preparations

3.2.2.1. Animals

Soleus muscles were obtained from mature female C57 Bl/10 mice aged between 18-30 weeks of age (body weight 22-30 g). Animals were killed using a lethal dose (200 mg/Kg body weight) of Pentobarbitone sodium B.P. (Sagatal, May & Baker Ltd.) which was injected intraperitoneally.

In addition a number of *mdx* mice (see Section 2.2.8.) were also studied (n=3, age=28 weeks, body weight=33-37 g) and these were treated in the same way as the C57 animals.

3.2.2.2. Muscle preparation and incubation

Following anaesthesia, the muscles of the hind limb were exposed and the Achilles tendon was sutured with cotton thread. The tendon was cut, the soleus gently separated from the gastrocnemius and the proximal tendon sutured. After removal from the leg soleus muscles were incubated in a chamber containing oxygenated Ringer's solution at room temperature (approximately 22°C).

3.2.2.3. *Force recording*

Soleus muscles were mounted horizontally in a perspex chamber (volume approximately 5 ml) between two large platinum electrodes which covered an area greater than that of the muscle to ensure complete muscle activation. The proximal end of the soleus was attached to a hook projecting from the chamber wall whilst the distal end was connected to a force transducer. This consisted of two silicon strain gauges (Kulite) bonded to a flat glass beam to which the muscle was attached. As the muscle exerted tension, one strain gauge was stretched, whilst the other was compressed. The silicon strain gauges formed two arms of a Wheatstone Bridge circuit, the other resistances being fixed. The voltage across the bridge was set at 4.0 Volts using a Fylde 492BBS Mini-Balance. Stretch of one strain gauge and compression of the other results in a change in resistance in two arms of the bridge circuit, the ensuing voltage difference was amplified with a Fylde 254GA Mini-Amplifier. The amplified signal from the force transducer was displayed and recorded on an ultraviolet chart recorder (SE Labs).

The stimulation voltage and pulse duration was set using a Devices isolated stimulator triggered by a frequency generator (Digitimer). The output was amplified by a 50W power amplifier capable of maintaining 30V between the electrodes when in solution.

3.2.2.4. *Tissue viability*

To test the stability of the isolated soleus preparation, a muscle was incubated and stimulated with a 0.5 s duration 100 Hz tetanus at a supramaximal voltage every 5 min. This resulted in a diminution of initial force of less than 10% at the end of three hours.

3.2.2.5. *Contractile properties*

Maximal tetanic force, force-frequency characteristics and half-time of relaxation from a tetanus ($t_{1/2}$) were recorded for each soleus prior to any fatiguing protocols.

3.2.2.6. *Force-Length determination*

In an initial group of muscles the relationship of maximal tetanic force to muscle length was determined by stimulating muscles at a range of lengths 3 mm longer and 2.5 mm shorter (0.5 mm steps) than optimal length (Lo).

3.2.2.7. *Fatigue protocol*

The aim of the study was to investigate the effect of fatigue at different lengths on force output at Lo. Initial experiments showed that a satisfactory fatigue response could be elicited using a protocol of alternating tetani at 20 Hz and 100 Hz. Thus 0.5 s at 20 Hz was followed by 0.5 s rest, then 0.5 s at 100 Hz repeated every 2 sec for 3 min (90 repetitions). After 10 min of recovery the fatiguing protocol was repeated three times interspersed with 10 min rest periods. After the final fatigue run, recovery was followed in the muscle for 30 min. During recovery the force produced by a 20 Hz and 100 Hz tetanus was determined every minute. All recovery forces were measured at Lo (test length) whilst fatigue runs were carried out at one of three muscle lengths: Lo, long (Lo+2mm)) or short (Lo-2mm).

3.2.2.8. *Determination of sarcomere length*

To determine whether muscles fatigued at different lengths showed any change in resting length, an estimate of sarcomere length was made in these preparations. This was done using infra-red laser diffraction measurements on teased out fibre bundles from muscles fixed in the

incubation chamber at known lengths using 4% glutaraldehyde in saline. The sarcomere spacing = W/sin Q (where W is the wavelength of light and Q the angle subtended by the first order diffraction lines), see Woledge et al (1985) for details. Measurements were made of sarcomere length both at Lo in fresh and post-fatigued states and at long and short lengths.

3.2.2.9. *Statistics*

Data were analysed using Student's t-test for the comparison of means of two small samples, where the sample size is less than 10. It was assumed that sample variances were the same.

3.3 RESULTS

3.3.1. Human Tibialis Anterior Experiments

3.3.1.1. *Length-Tension characteristics*

Muscles were stimulated with increasing voltages until there was no further increase in force. The maximal force generated by stimulation of the TA was on average 151 N (±19.5, sem) at Lo. This represents only 56% (±7.8, sem) of the force generated by maximal voluntary contraction of the foot dorsiflexor muscles in this position. Since the stimulation was supra-maximal on each occasion the reason why the stimulated forces were so much lower than the voluntary contractions probably lies in the fact that other foot dorsiflexor muscles are contributing (notably the extensor hallucis longus, extensor digitorum longus and peronius tertius) during voluntary efforts.

Stimulated and voluntary force-length curves for foot-dorsiflexor forces are shown in Fig 3.1. When force was normalised to peak force the force-length characteristics for stimulated and voluntary contractions were very similar up to 100° of ankle dorsiflexion. After this point stimulated forces declined as the muscle length increased. However the voluntary forces at greater degrees of dorsiflexion remained above 80% of maximal force throughout this part of the force-length curve.

The absolute force-frequency curves for the TA at each of the muscle lengths studied is shown in Fig 3.2. When the force-frequency curves were normalised to maximal force at each length, the force-frequency characteristics are more clearly seen (Fig 3.3). In the short position (greater ankle dorsiflexion) there was a shift to the right of the force-frequency curve. Similarly, at the long position (greater ankle plantarflexion), the force-frequency curve was shifted to the left.

3.3.1.2. *Fatigue characteristics*

Force recordings during separate fatigue runs at different TA lengths in the same subject are shown in Fig 3.4. Fatiguing exercise at both short and long lengths resulted in greater force loss than fatigue at Lo. Average results for the subjects studied are shown graphically in Fig 3.5. Force declined to 31% (±3%) of fresh force at the end of fatigue at Lo, compared with 18% (±2%) and 22% (±3%) for short and long lengths respectively, both of these decrements being significantly greater than that at Lo. When the maximal stimulated force in these muscles was measured at the optimum length (Lo) 10-15 sec after the end of the fatiguing exercise (Fig 3.5) a different picture emerged. Although still ischaemic, there was a consistent, recovery of force (approximately 10% on average) in the muscles exercised at Lo. However, the muscles fatigued in the shortened position were less fatigued when tested at Lo. This is in contrast to the muscles exercised at long length, the force of which remained low even when tested at Lo.

Ten minutes after the end of the fatigue run forces measured at the test length had almost completely recovered in muscles fatigued at Lo and in a short position (97% (±3%, sem) and 94% (±4%) of fresh force respectively) whilst those fatigued in a long position had only recovered to 90% (±3%) of fresh force. The difference between fatigue at Lo and long positions proved to be significant at the 5% level.

The force-frequency changes as a result of fatigue are shown in Fig 3.6 which plots the 10/50 Hz force ratio at the test length for each protocol before, immediately after and 10 min after fatigue at different lengths. All groups showed similar changes in 10/50 Hz ratio measured immediately after fatigue, but whereas the muscles fatigued at Lo and in a short position staged some recovery, those fatigued in a long position

maintained a depressed 10/50 Hz force ratio after 10 min recovery.

3.3.2. Isolated Mouse Soleus

3.3.2.1. *Contractile properties and Force-Length Curves.*

Fig 3.7 shows the force-length curves of a number of muscles. In the fresh muscle a shortening of 2mm resulted in 46% (±3%, sem) of force production at Lo. Similarly at a long length (Lo+2 mm) force was reduced to 56% (±4%) of that at Lo. Thus active tensions were reduced to similar levels in both the shortened and lengthened positions.

In Fig 3.8 the force-frequency curves for fresh muscles at different lengths are shown as a percentage of maximal tetanic force. With increased length there was a shift to the left of the force-frequency curve (because of the comparative increase in force at sub-maximal stimulation frequencies). Conversely, at shorter lengths, the force-frequency characteristics showed a shift to the right.

3.3.2.2. *Fatigue characteristics*

As a result of the fatiguing protocol the average force, measured at short and long lengths, was reduced to 28.1% (±8.9%, sem) and 36.6% (±9.4%) of fresh force respectively. This compares with a value of 40.9% (±7.0%) after fatigue at Lo, a typical fatigue trace is shown in Fig 3.9. Thus force loss was greater after fatigue at both short and long lengths (when measured at the fatigue length) compared to Lo, the difference between that at short lengths and Lo proving statistically significant.

Examination of the effects of fatigue at different lengths on maximal tetanic force at the test length (Lo) shows that fatigue at short lengths resulted in less force loss at Lo (Fig 3.10). The force loss, measured at the test length, after fatigue at long lengths tended to be

greater than at Lo, but this difference was not significant. At the end of each recovery period 100 Hz forces had almost completely recovered, and even 30 min after the end of the final fatigue run, 100 Hz forces were some 85-90% of fresh force in each group.

Consideration of the sub-maximal tetanic forces generated at 20 Hz (Fig 3.11) shows a different picture. In this case fatigue at long lengths resulted in greater loss of 20 Hz force (as measured at the test length) compared to fatigue at Lo. This was mirrored by a relative protection of 20 Hz force in those muscles fatigued at shorter lengths. Fig 3.11 also shows that 20 Hz force loss was greater than 100 Hz force loss, particularly during the fatiguing phase. Thus for muscles stimulated at Lo there was a decline to 30% of fresh force at 20 Hz, as opposed to 45% of fresh force at 100 Hz. Although, during recovery periods, there was a partial restoration of 20 Hz force at all fatigue lengths, this never reached its fresh value, resulting in a gradual 20 Hz force decline throughout the experimental protocol. This decline in 20 Hz force was significantly greater after fatigue at long lengths compared to fatigue at short lengths.

Changes occurring in the force-frequency characteristics following fatigue were reflected in the ratio of 20/100 Hz forces and Fig 3.12 shows the changes in this parameter at the test length over the time course of fatigue and recovery. Thus, when measured at the test length there was a greater decline in 20/100 Hz force ratio after fatigue at long muscle lengths compared to fatigue at both Lo and short lengths. The difference between 20/100 Hz forces after fatigue at short and long lengths proving statistically significant.

3.3.2.3 Sarcomere length measurements

It can be seen from the force-length and force-frequency characteristics of isolated soleus muscles (Figs 3.7 and 3.8 respectively)

that any change in the resting length of the muscle following fatigue would result in a change in the observed contractile properties. Such a situation might occur if sufficient stress was placed on the series elastic component of the muscle to increase the compliance of the series elements of the muscle and result in shortening of the resting sarcomere lengths. To assess this possibility, measurements of sarcomere length were made in fresh muscles and those fatigued at different lengths. The results are shown in Table 3.1 and demonstrate that no change in sarcomere length occurred at the test length following fatiguing contractions at short and long lengths.

3.3.3. Isolated *mdx* Soleus Experiments

3.3.3.1. *Contractile properties*

Table 3.2 lists the physical and contractile characteristics of normal and *mdx* soleus muscles measured in vitro. The most obvious difference was the greater mass of the *mdx* soleus and this was reflected in the comparative strengths of the two muscles. *Mdx* muscles also had a slightly shorter twitch duration and $t_{1/2}$ (although neither of these parameters proved significantly different from normal given the small number of muscles studied). The force-frequency characteristics of the two groups were also fairly similar.

The most striking difference between normal and *mdx* soleus muscles are shown in Fig 3.13 being the far greater fatiguability of *mdx* soleus. The fatigue protocol consisted of a 0.5 sec 20 Hz tetanus followed by 0.5 sec rest, then a 0.5 sec 100 Hz tetanus repeated every 2 sec for 3 min. After a single bout of exercise, maximal force declined to 18% of pre-fatigue force in the *mdx* as opposed to 53% in the C57 soleus.

The difference between the two groups was also apparent in the degree of recovery from fatigue, *mdx* muscles only recovered to 42% of

pre-fatigue force compared to a complete recovery of the C57 soleus. Similar differences between *mdx* and normal muscles were apparent in the loss of low frequency force following fatigue (Fig 3.14). even after 30 min the *mdx* soleus muscles showed no further recovery.

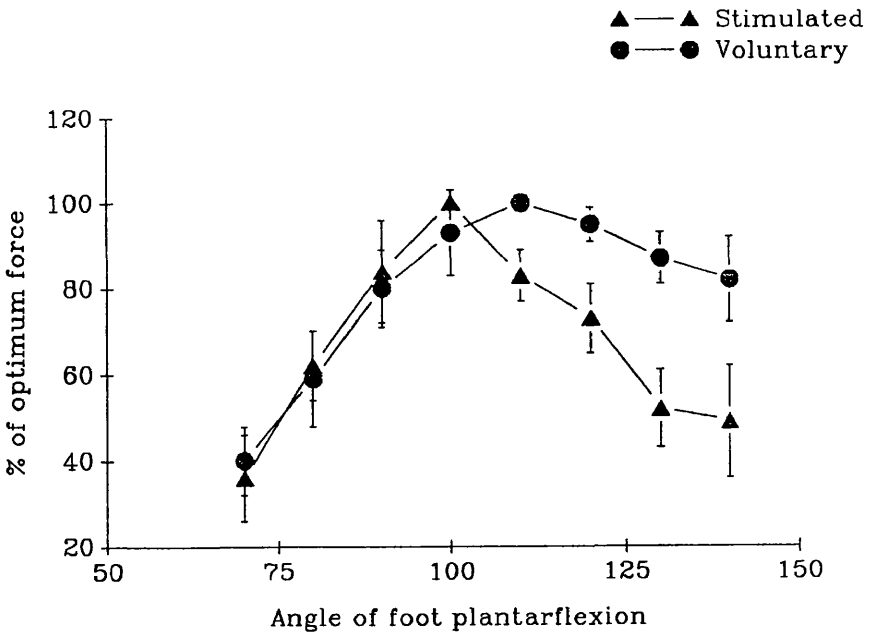


Fig 3.1 Stimulated and voluntary force-length relationships of human tibialis anterior (n=7 subjects). Mean values (± sem)

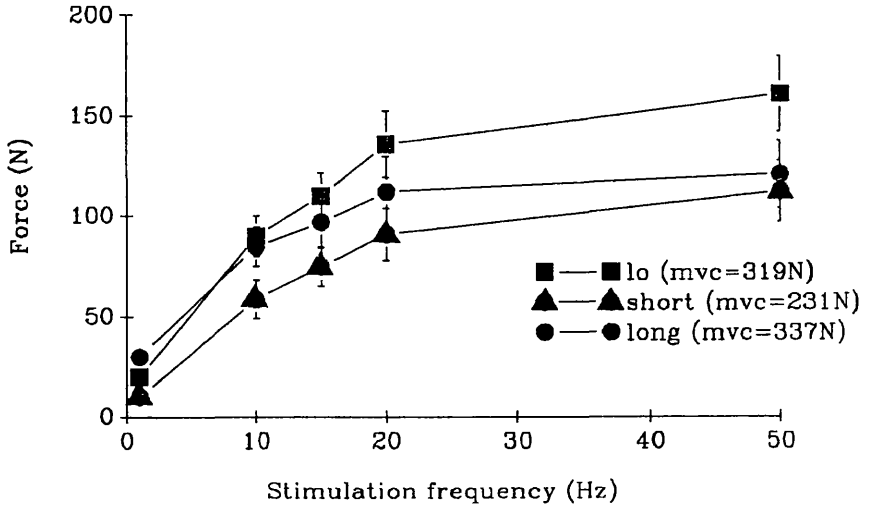


Fig 3.2 Force-frequency relationship of fresh human tibialis anterior muscle at different muscle lengths (n=7 for each group). Mean values (± sem)

mvc=maximal voluntary contraction.

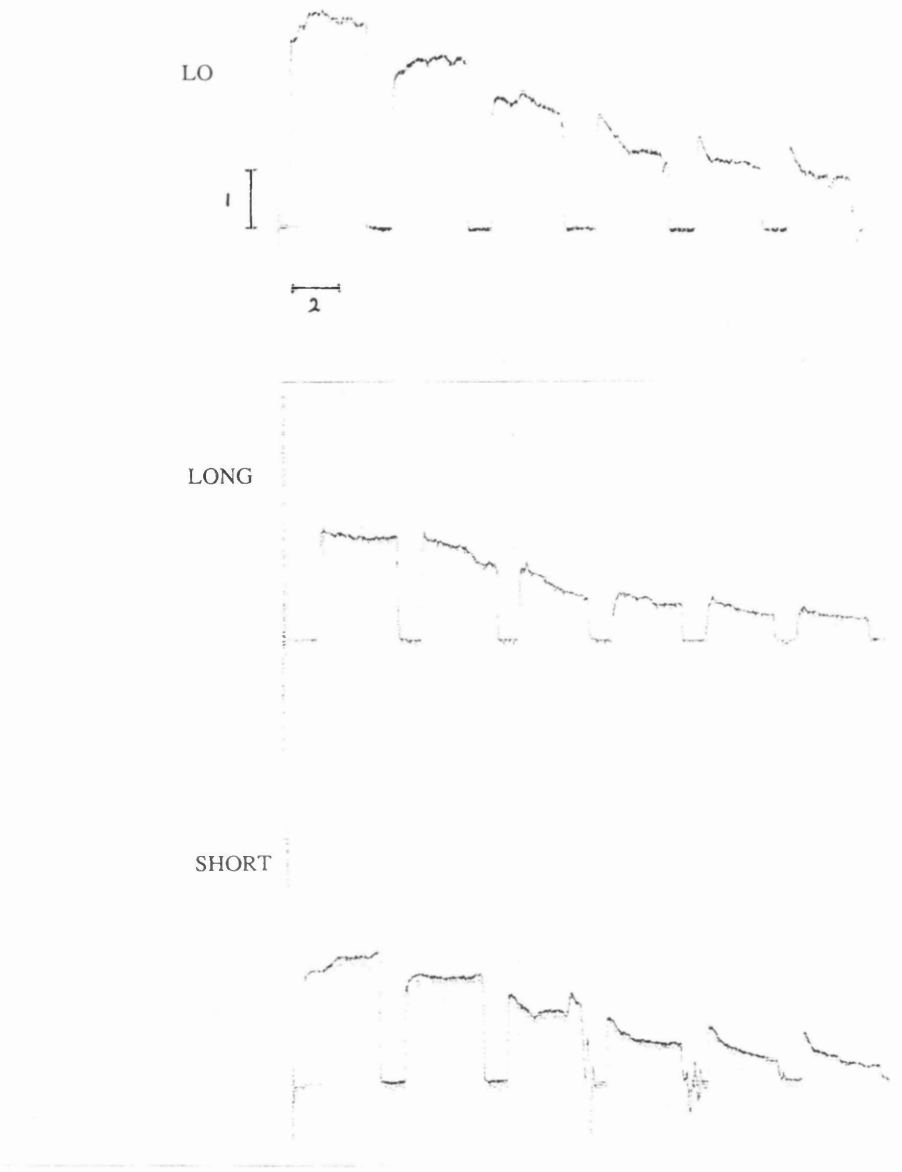


Fig 3.4 Force recordings of stimulated fatigue in the tibialis anterior of the same subject at three different lengths.
Bar 1=50 N, Bar 2=10 sec.

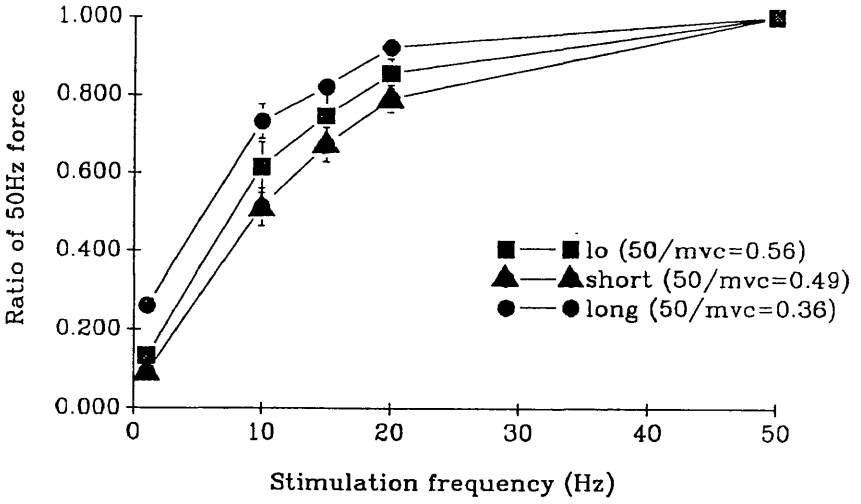


Fig 3.3 Normalised force-frequency relationship of fresh human tibialis anterior muscle at different muscle lengths (n=7 for each group). Mean values (± sem)
mvc=maximal voluntary contraction.

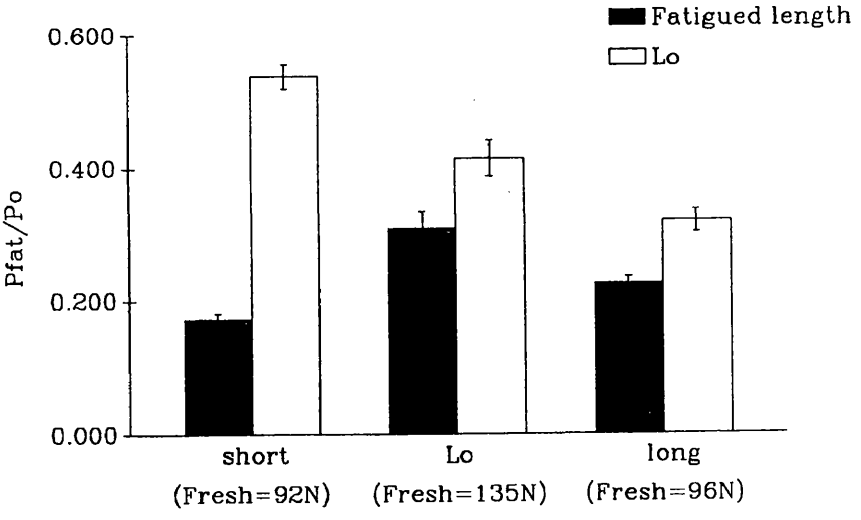


Fig 3.5 The effect of fatigue at different muscle lengths (x-axis label) on 50 Hz force normalised for fresh force (Pfat/Po) in the human tibialis anterior at fatigue length (filled histogram), and optimal length (empty histogram), (n=7 for each group). Mean values (± sem)

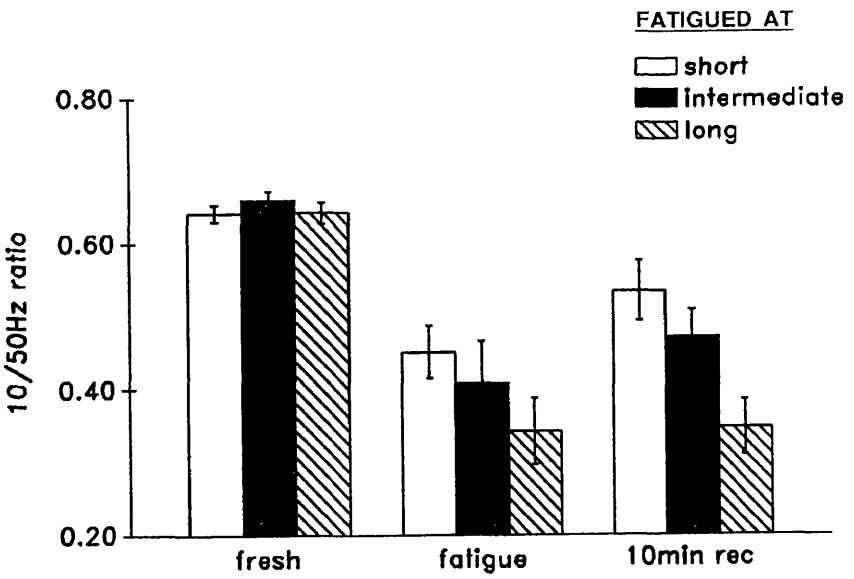


Fig 3.6 10/50 Hz force ratios of human tibialis anterior muscles fatigued at different lengths, but measured at optimal length (n=7 for each group). Mean values (± sem)

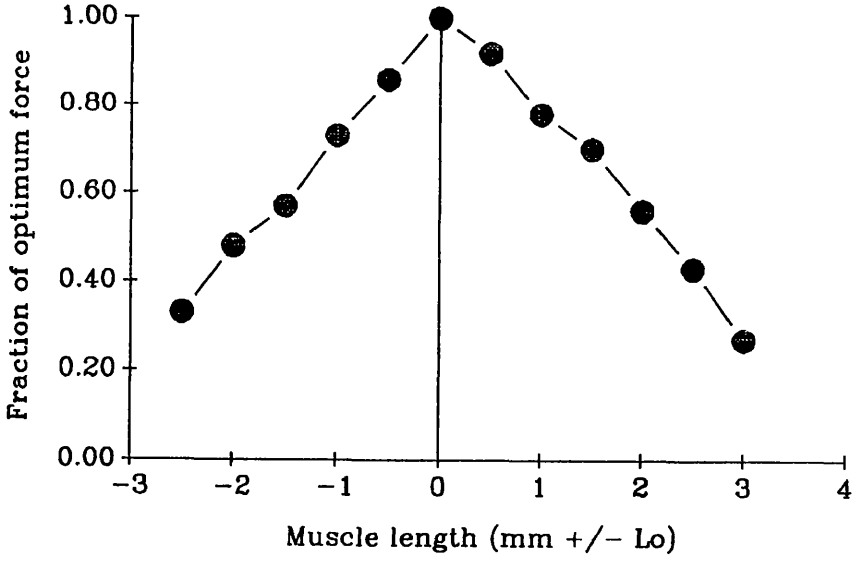


Fig 3.7 Active force-length relationship for isolated soleus muscles stimulated at 80 Hz (n=4). Mean values (± sem)

Table 3.1 Sarcomere length determinations of fresh and fatigued soleus muscles at different lengths. Mean values (± sem)

	short (n=2)	Lo (n=4)	long (n=2)
Fresh muscles, measured at fatigue length (μm)	2.30 (-)	2.70 (0.10)	3.15 (-)
	(n=5)	(n=5)	(n=4)
Fatigued muscles, measured at Lo (μm)	2.61 (0.19)	2.68 (0.15)	2.58 (0.16)

Table 3.3 Physical and contractile characteristics of C57 and mdx soleus muscles. Mean values (± sem)

	mdx (n=5)	C57 (n=20)
Muscle weight (mg)	14.9 (1.1)	12.1 (0.7)
Muscle length (mm)	11.5 (1.0)	11.2 (0.4)
1 Hz tension (mN)	17.0 (1.9)	16.0 (0.9)
1 Hz TTP (ms)	21.7 (2.1)	23.3 (1.0)
100 Hz tension (mN)	198.0 (29)	165.0 (12)
100 Hz $T_{1/2}$ (ms)	20.9 (1.9)	22.3 (1.1)

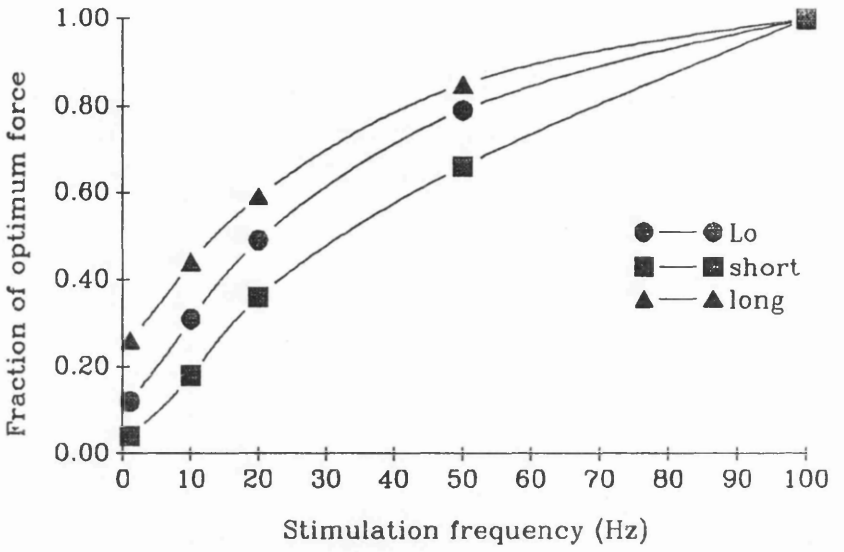


Fig 3.8 Force-Frequency relationship for isolated soleus muscles (n=8 for each group) at different lengths. Mean values (± sem)

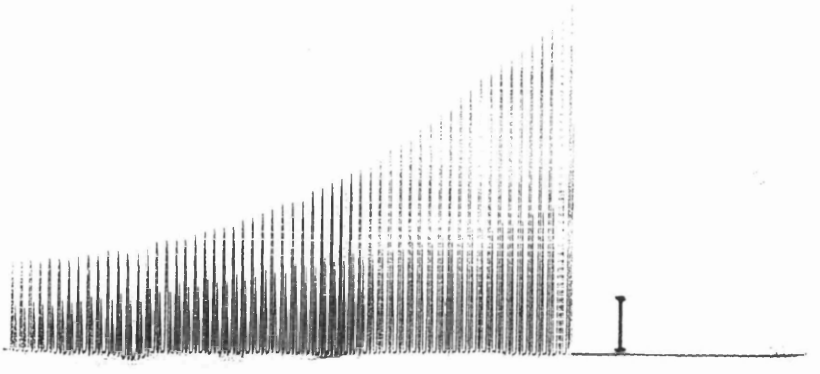


Fig 3.9 Force record of isolated soleus muscle at Lo stimulated at 20 Hz (0.5 sec) and 100 Hz (0.5 sec) for 60 repetitions. (Bar=5mN)

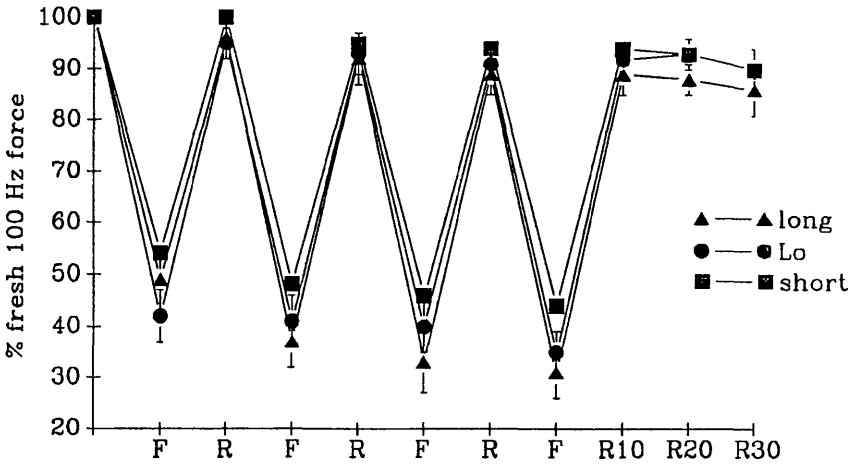


Fig 3.10 Maximum tetanic forces of soleus muscles during fatigue protocol measured at Lo (Lo, n=8; short, n=9; long, n=9). Mean values (± sem)

F=Fatigue, R=Recovery.

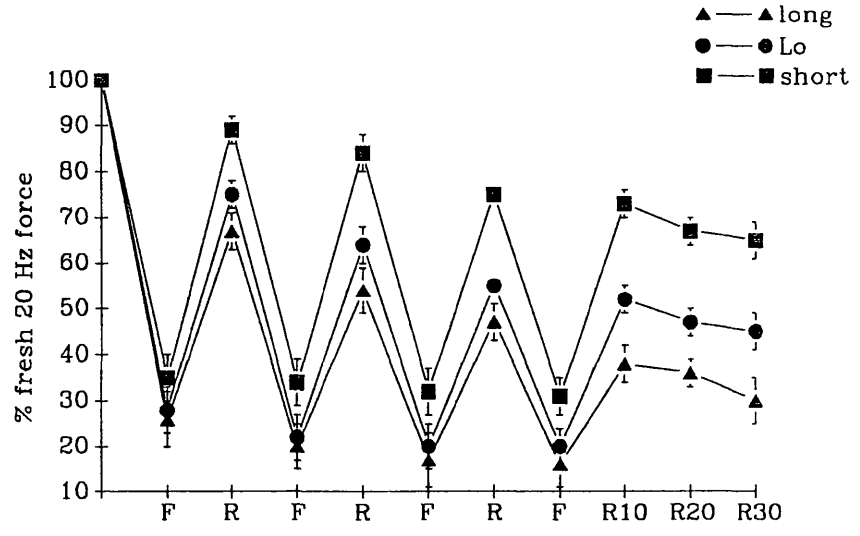


Fig 3.11 20 Hz tetanic forces of isolated soleus muscles during fatigue protocol measured at Lo (Lo, n=8; short, n=9; long, n=9). Mean values (± sem)

F=Fatigue, R=Recovery.

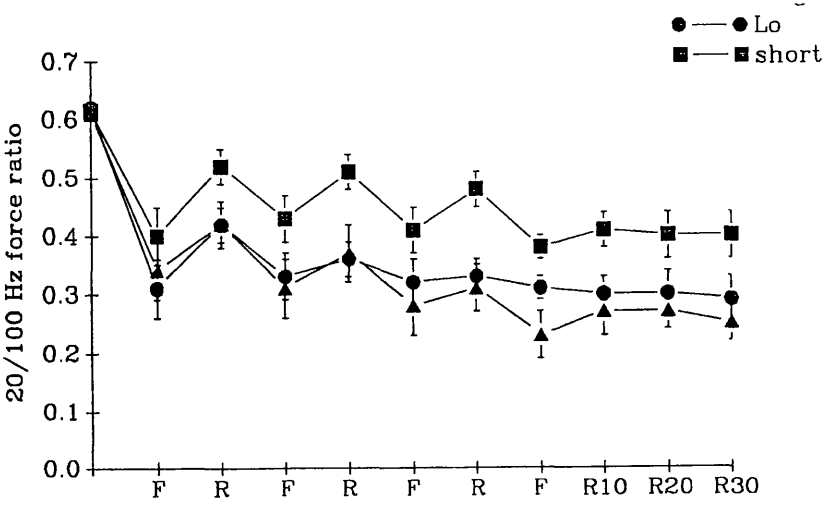


Fig 3.12 20/100 Hz force ratio of isolated soleus muscles during fatigue protocol measured at Lo (Lo, n=8; short, n=9; long, n=9). Mean values (± sem)

F=Fatigue, R=Recovery.

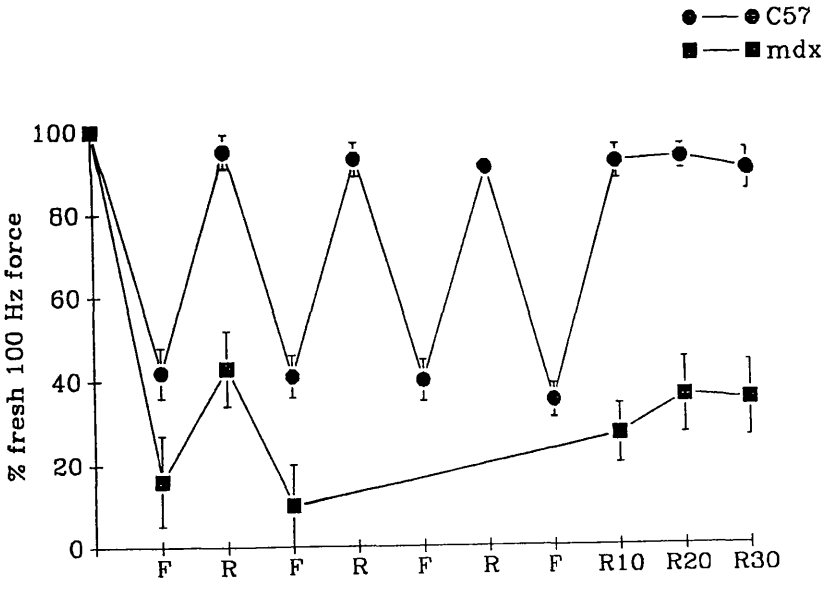


Fig 3.13 Maximum tetanic forces of mdx and normal isolated soleus muscles during fatigue protocol at Lo (C57, n=8; mdx, n=5). Mean values (± sem)

F=Fatigue, R=Recovery.

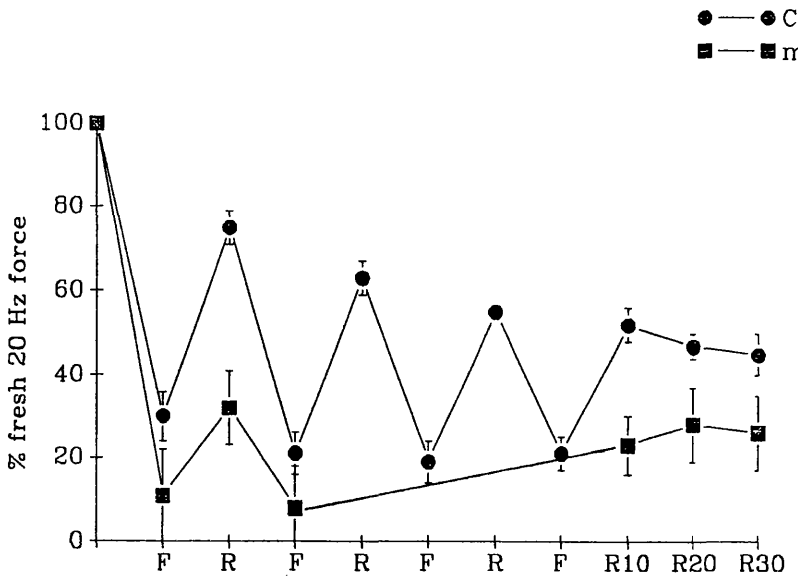


Fig 3.14. 20 Hz tetanic forces of mdx and normal isolated soleus muscles during fatigue protocol at Lo (C57, n=8; mdx, n=5). Mean values (± sem)

F=Fatigue, R=Recovery.

3.4. DISCUSSION

3.4.1 Human Tibialis Anterior

The main aim of the experiments described in this Chapter was to examine the findings of Fitch & McComas and to extend their original work by investigating the effect of contractions at long muscle length on the development of fatigue. The findings were in general agreement with those of Fitch & McComas but the additional measurements made in the present study have cast some doubt on the interpretation of the original data. The present investigation differed from that of Fitch & McComas in two ways. First, the extent of force loss was measured at the end of the fatiguing exercise at the length at which the exercise was carried out. Secondly the effect of exercise at long muscle lengths was examined.

Muscles which had previously been exercised at a short length appeared to suffer little fatigue when they were tested at optimum length and this observation is common to both Fitch & McComas and the present work. However, Fitch & McComas were unaware of the fact that during the exercise at the short length force declined more rapidly than when the muscles were exercised at longer lengths. The fact that the short muscle functioned relatively normally when stretched to optimal length suggests that at least portions of the muscle were not being activated at the short length and, when re-extended, appeared to be unfatigued. At short muscle lengths there is the possibility that T tubules may collapse and any decrease in t-tubular volume would result in a more rapid build-up of $K+$ ions (described in Chapter 2) resulting in action potential propagation failure to the interior of the fibre developing sooner. Thus the central portion of muscle fibres may become inactive during fatigue. Blinks et al (1978) showed that there was a reduction in

the amount of intracellular Ca^{2+} released during tetanic stimulation in a shortened muscle whilst Taylor & Rudell (1970) suggested that, at short muscle lengths, there is a failure of inwardly propagating action potentials in the t-tubules. These considerations cast doubt on the central thesis of Fitch & McComas that at the short length the electrical activity of a muscle will remain constant while the metabolic changes will be less in proportion to the force generated.

The second innovation of the present work was to investigate the effects of exercise with a muscle at a long length. The force loss of the muscles exercised at long lengths, and measured at this length, was similar or slightly greater than that of muscles exercised at optimum length. When subsequently measured at the optimum length, however, muscles exercised at long lengths showed a greater force loss than when exercised at Lo.

With muscles working at long lengths it can be reasonably expected that the reduced force is associated with a reduced ATP turnover (Rall, 1982). In this situation we have muscles with reduced metabolic demands showing similar or greater degrees of fatigue. The inference from this must be that fatigue is not metabolically determined, a conclusion which is diametrically opposed to that of Fitch & McComas.

It is not clear why fatigue should have been greater when muscles were exercised at long length. Frank & Winegrad (1976), by measuring ^{45}Ca efflux from muscle fibres during repeated contractions at different lengths showed a reduction in the amount of Ca^{2+} released from fibres after repeated stimulation at 160% of Lo. The authors suggest that this was caused by some impairment in excitation-contraction coupling, possibly a smaller response of the sarcoplasmic reticulum to its normal signal. These results agree with the findings of Winegrad (1965) who showed a decrease in the intensity of the aequorin signal (and therefore

Ca^{2+}) in skeletal muscle fibres stimulated at long muscle lengths.

A second possibility is that some form of damage is associated with contractions at long muscle lengths and in this respect it is notable that muscles exercised at long length developed a disproportionate loss of force at low frequencies of stimulation. This point will be discussed in further detail in conjunction with the results from the isolated mouse muscles.

3.4.2 Mouse soleus

The results of the fatigue studies using isolated mouse muscles were essentially the same as those of the human TA. Soleus muscles exercised at a short length showed less fatigue when measured at Lo than muscles both fatigued and measured at Lo. The only major difference was that the loss of maximal force associated with contractions at long lengths in the mouse soleus was similar to that seen after fatigue at Lo, rather than being greater as in the human TA.

Following repeated stimulation there was a gradual loss of 20 Hz force in the soleus. This low frequency fatigue (LFF) as described by Edwards et al (1977), has been discussed in section 3.1. The cause of LFF is believed to be an impairment of muscle activation (Jones et al, 1982) and our findings would tend to support this as it occurred at a time when muscles were able to generate maximal forces close to those found pre-fatigue. Thus, at Lo, even 30 min after the end of the last fatigue protocol (when metabolite levels would have recovered), 20 Hz force had declined to 51% of fresh force. An identical fatigue protocol at short muscle lengths resulted in a relative protection against LFF, although here 20 Hz force still declined to 60% of its fresh value at the end of the procedure. Conversely, in muscles fatigued at long lengths there was an exacerbation of LFF (to less than 30% of fresh 20 Hz force at the end of

the procedure). This effect did not appear to be related the force generated by the muscle (since this was greatest during fatigue at Lo) and was relatively long lasting, with no sign of 20 Hz force recovery 30 min after the end of the final fatigue run.

Although the degree of LFF was similar for human TA muscles fatigued at all three lengths, only after exercise in the lengthened position did the 10/50 Hz force ratio fail to show an appreciable recovery after 10 min. Although by 6 hours no sign of LFF could be observed in 2 subjects following fatigue at long length. This gives some support to the thesis that fatigue at long lengths is more deleterious to a muscle than fatigue at shorter lengths.

The consequences of exercise at long muscle lengths are similar to the changes seen after eccentric exercise (Newham et al, 1983) and are believed to presage the development of delayed onset muscle damage (Edwards, 1988). The usual explanation for this effect is the generation of particularly high forces within muscles during lengthening rather than shortening contractions. However, differences in the amount of force developed cannot be the cause of the greater LFF seen after isometric contractions at long lengths described here. This is because in the lengthened position the muscles are capable of generating barely half of the maximal force produced at optimum lengths. It is conceivable that isometric fatigue at long lengths (when there is a considerably increased resting tension) in some way causes damage to some structural component of the fibre activation system, such as the terminal cisternae of the sarcoplasmic reticulum. This could lead to a reduction in the quantity of Ca^{2+} released for every action potential which might not have any affect on maximal tetanic force (when an excess of the quantity of Ca^{2+} needed to fully saturate the myofilaments is released) but could explain the LFF observed.

A more likely explanation is that when held at long lengths the degree of sarcomere heterogeneity is greater than when the overall muscle length is short. Sarcomere inhomogeneity leads to the phenomenon of "creep" whereby shorter sarcomeres at the end of the fibre extend the longer sarcomeres in the centre (Ramsey & Street, 1940; Gordon et al, 1966). Taken to extremes this could lead to physical damage to the central sarcomeres, although how this might lead to a change in the force/frequency relationship of the muscle is unclear.

3.4.3. *Mdx* soleus

The finding that the *mdx* soleus is dramatically more fatiguable than that of normal muscles must be open to suspicion because of the fact that only a limited force recovery took place following fatigue in the former. One explanation for this finding is the development of an anoxic core of fibres in the centre of the muscle during exercise (see Section 2.1 for discussion). This possibility is made all the more plausible given the relative sizes of the muscles (the *mdx* soleus being on average 121% of the weight of the C57 soleus muscles tested). If this is the case then it is dubious to draw any conclusions on the basis of this data. For this reason it was decided to investigate the contractile properties of *mdx* muscle using an *in situ* muscle preparation. This provides the topic for the next section of this thesis.

CHAPTER 4

AN INVESTIGATION INTO THE FUNCTIONAL PROPERTIES OF DYSTROPHIN-DEFICIENT (*mdx*) MUSCLE USING AN *in situ* PREPARATION

4.1 INTRODUCTION

In Chapters 2 and 3 of this thesis the results of fatigue studies carried out in a number of different muscle models were described. *In vitro* muscle preparations are, by their nature, unsuitable for study of the longer term changes which affect muscles as a result of fatiguing exercise. Furthermore, neither preparation allowed the satisfactory examination of the functional properties of *mdx* dystrophic muscle. For these reasons an *in situ* muscle preparation was utilised, namely the mouse tibialis anterior (TA). In this Chapter the contractile characteristics of this preparation are described for normal muscle and a comparison made with muscles from *mdx* mice.

The identification of the protein dystrophin as the missing or defective gene product in Duchenne and Becker muscular dystrophy has been a major advance in understanding the pathogenesis of these disorders (Hoffman et al, 1987). Although dystrophin is now known to be associated with the sarcolemmal membrane its precise role in the muscle fibre and the reason why its absence leads to the dystrophic changes, remains to be elucidated. The discovery that dystrophin is absent in the *mdx* strain of dystrophic mouse and that the gene locus affected in these mice appears to be homologous to that responsible for Duchenne/Becker muscular dystrophy (Cooper, 1989), makes the *mdx* strain a valuable model for investigating the functional consequences of dystrophin deficiency.

The *mdx* mutant was originally described by Bulfield et al (1984) in a colony of C57 BL/10 mice. These animals have chronically raised serum levels of muscle specific creatine kinase and present with histological changes characteristic of a mild form of muscular dystrophy.

Dangain and Vrbová (1984) reported that muscles from *mdx* mice undergo an acute and massive fibre necrosis at approximately 3 weeks of age with a subsequent regeneration and recovery by 5 weeks of age although degeneration and regeneration, on a more limited scale, continues throughout adult life (Dangain and Vrbová, 1984; Torres and Duchen, 1987; Coulton et al, 1988a).

It is possible that the lack of dystrophin may be reflected in altered contractile properties of the dystrophic muscle or in their susceptibility to damage or ability to recover. With a few exceptions (Coulton et al, 1988b) there is little information about these aspects of dystrophic mouse muscle. To this end a comparison has been made between the contractile properties and fatigue characteristics of *mdx* tibialis anterior muscle with those of normal mice.

4.2. METHODS

4.2.1 Experimental Animals

Myopathic mice (*mdx*) were maintained as a breeding colony from stock donated by Dr G. Bulfield, Department of Genetics, University of Edinburgh. Normal animals were obtained from a colony of C57 BL/10 mice, this being the strain in which the *mdx* mutant arose. Experiments were performed on female animals aged 16-26 weeks (*mdx*, mean = 21.4) and 16-24 weeks (normal C57, mean = 21.0).

4.2.2. Force Recording

All investigations were carried out using the tibialis anterior (TA) muscle. Animals were anaesthetized by an intraperitoneal injection of chloral hydrate (4.5% solution, 0.01ml per gram body weight). The distal tendon of the TA was cut and the proximal end tied to a Statham force transducer with fine silk suture. The sciatic nerve was isolated and cut and the leg secured by metal pins through the knee and ankle joints. A bipolar platinum stimulating electrode was positioned in contact with the peripheral stump of the sciatic nerve.

Muscles were stimulated with square wave pulses of 100μs duration and the voltage adjusted so as to give supramaximal stimulation (5-10V). An initial tetanic stimulation at 100 Hz for 0.5 sec was used to remove any slack which might be present in the tendon attachments. The muscle length was then adjusted to optimum length to give maximum twitch tension without a significant increase in resting tension. Data were recorded (a) on a storage oscilloscope screen, (b) on a Devices chart recorder and, (c) after digitization on a computer data base for subsequent analysis. The latter was used to estimate the maximum rate of tension development (MRTD) and maximum rate of relaxation (MRR). The half

time of relaxation ($T_{1/2}$) was also calculated as the time taken for the force to decrease from 75% to 50% of peak force. Since these values are related to the absolute force of the contraction, they were normalised for the peak force of that contraction to obtain a value of the fractional change in peak force per ms.

4.2.3. Stimulation Protocol

A frequency-force relationship was determined for each muscle by stimulating for half a second at 1, 40, 60, 80, and 100 Hz. At least 30 sec recovery time was allowed between tetani.

After 5 minutes recovery, muscles were subjected to a fatigue protocol consisting of supramaximal stimulation for 250ms at a frequency of 40 Hz repeated every second for 3 min (180 contractions). The tetanic force after 3 min of fatigue was expressed as a percentage of the maximum tetanic force; this was usually the initial contraction, but occasionally there was some potentiation during the first 2-5s, in which case the greatest value was used.

4.2.4 Post-mortem examination

Following the fatigue run, mice were killed by administration of a lethal dose of chloral hydrate. TA muscle length was measured *in situ* in a number of mice, all muscles were carefully excised, weighed and a portion from the belly of the muscle was orientated to give transverse sections before being frozen in isopentane cooled with liquid nitrogen.

4.2.5 Histology and Histochemistry

Cryostat sections were cut at 8μm and stained with haematoxylin and eosin (H & E) and for NADH-Tetrazolium Reductase.

4.2.6. Morphometry

Fibre areas were calculated using semi-quantitative image analysis whereby the section was magnified and projected onto a graphics tablet and the fibre outline manually traced (Round et al, 1983). Measurements were made on transverse sections stained with H & E; 100 fibres were measured from each TA in 10 adjacent fields covering the lateral portion of the muscle.

4.2.7 Statistics

Values for grouped mean data were compared using Student's t test; where not specifically stated significance was set at the 5% level.

4.3 RESULTS

4.3.1 Physical characteristics

Details of animal and muscle weights are given in Table 4.1. The *mdx* mice were, on average 20% heavier than normal C57 mice while the individual TA muscles from *mdx* mice were 80% heavier than those from normal animals. Both types of muscles were the same length so that the calculated cross-sectional areas of *mdx* muscles were greater than those for the normal C57 muscles.

The *mdx* TA muscle weights were approximately 2.3% of total body weight, in contrast to the normal group where the muscle represented a significantly smaller portion of body weight (1.53%).

Muscles from normal C57 mice showed the typical appearance of normal muscle and fibres with internal nuclei were very rare (Fig 4.1a). Histological examination of *mdx* muscles showed a wide variation in muscle fibre size, foci of fibre necrosis, infiltration with mononuclear cells, and regenerating fibres (Fig 4.1b). A notable feature of *mdx* muscles was that the majority of fibres had one or more internally placed nuclei (Fig 4.1a). Histochemical staining of *mdx* TA muscles for NADH showed similar numbers and distributions of oxidative and non-oxidative fibres to that seen in normal mice. Results from the measurement of muscle fibre areas in normal and *mdx* TA muscles are shown in Fig 4.2 and Table 4.1. Mean fibre areas were very similar for control and *mdx* TA, but the latter showed a greater range of fibre areas, illustrated by the larger standard deviation (Table 4.1). Thus 17% of *mdx* fibres had an area less than 2000μm^2 compared to 10% for normal muscles while 9% of the *mdx* fibres were greater than 6000 μm^2 compared to 1% of the normal fibres.

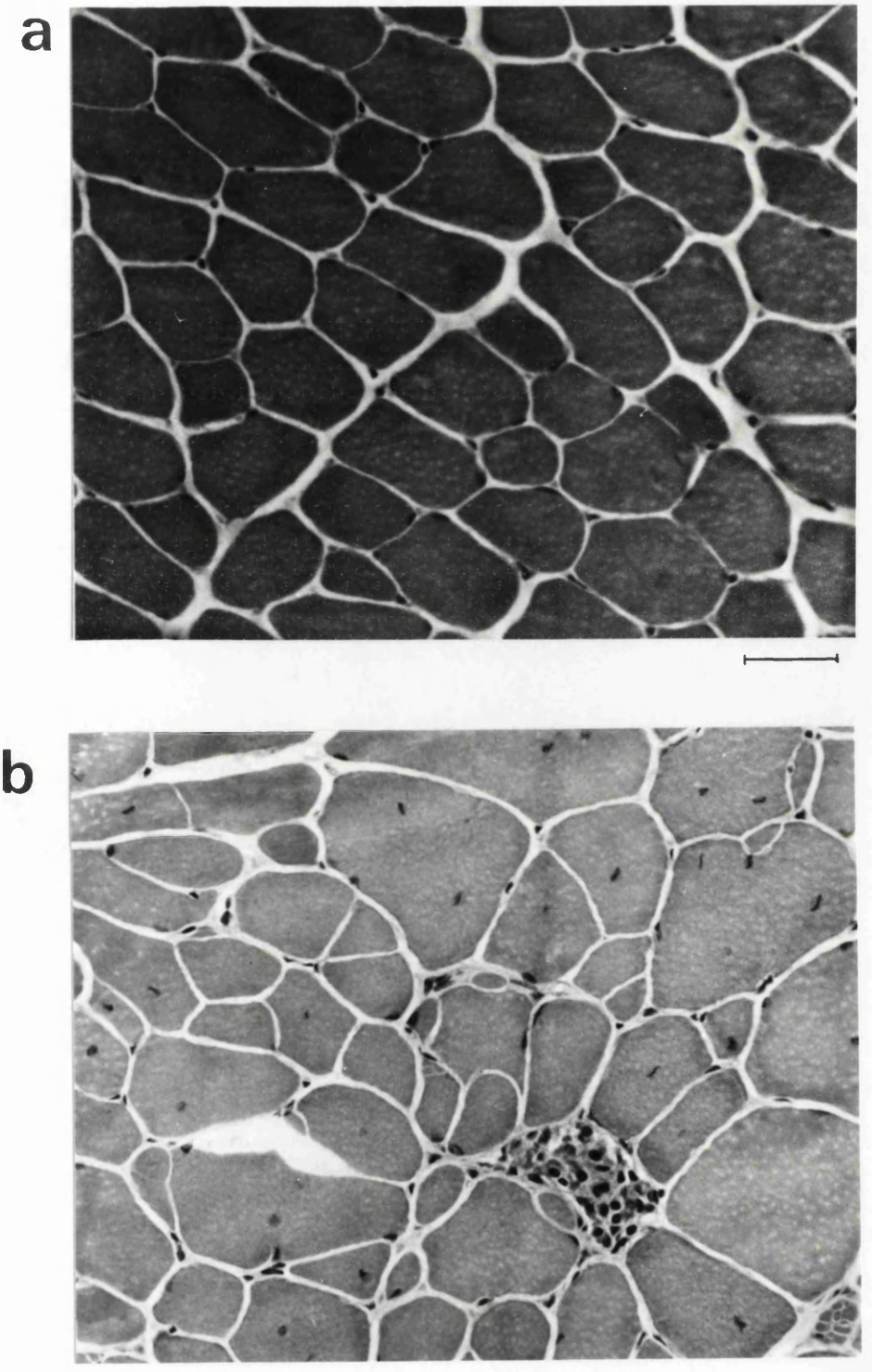


Fig 4.1 Histological appearance of mouse tibialis anterior muscles stained with haematoxylin and eosin (**a**) C57, (**b**) *mdx*. Bar = 50μm

4.3.2. Contractile properties

Although the frequency of tetanic stimulation at which maximum isometric force was elicited coincided for *mdx* and control muscles, the relative force generated at submaximal stimulation frequencies differed in the two groups (Fig 4.3). The comparative deficit of force at low frequencies of stimulation in the *mdx* muscle was particularly marked at frequencies of around 40 Hz, although the force generated was also significantly less at 60Hz (Fig 4.4).

Fig 4.5 shows the force records for a single twitch of an *mdx* and normal TA. Twitches from *mdx* muscles reached peak force more rapidly than controls. Time to peak force (TTP) for isometric twitches (Table 4.2) was, on average, 4.2ms shorter in the *mdx* muscle. This difference was reflected in a 20% higher maximum rate of tension development (MRTD) normalised for peak force (Table 4.2) compared to control muscles. Half relaxation times of *mdx* and C57 muscles ($T_{½}$) showed no significant differences (Table 4.2).

Maximum isometric forces were obtained by stimulating at a frequency of 100 Hz; no further force increments occurred at higher frequencies in either *mdx* or control muscles. Table 4.3 shows that *mdx* muscles developed over 30% more force during maximum stimulation compared to controls. Comparing the maximum force with the estimated muscle cross-sectional area provides an estimate of the intrinsic force generating capacity of a muscle. Despite the larger absolute forces generated by the *mdx* muscles, they generated only 80% of normal muscle force per unit cross-sectional area.

4.3.3. Fatigue characteristics

Figure 4.6 shows change in isometric force during the fatigue protocol for an *mdx* and normal C57 TA. Although *mdx* muscles often

Table 4.1 Physical Properties of MDX and Normal Tibialis Anterior Muscles. Values are given as mean ± SEM (except for fibre areas).

	MDX	NORMAL
Animal weight (g)	33.0 ±1.6* (n=9)	27.1 ±1.1 (n=8)
TA weight (mg)	76±2** (n=17)	41±1 (n=15)
TA weight % BW	2.28±0.06** (n=17)	1.53 ±0.05 (n=15)
Muscle length (mm)	11.1±0.2 (n=6)	10.5±0.1 (n=7)
Estimated muscle CSA (mm^2)	6.8±0.3** (n=17)	3.9±0.7 (n=15)
Fibre area (μm^2 x 1000) (+ SD)	3.39±1.37 (n=500)	3.17±0.88 (n=600)

* $p<0.05$, ** $p<0.01$

lost more force after the first few tetani, the force loss after 3 minutes of stimulation was consistently less in *mdx* muscles. Group mean data for fatigue tests of *mdx* and control muscles are represented in Table 4.3. Thus in the *mdx* TA 48% of the initial force was maintained after 3 min repeated stimulation at 40 Hz, whereas in the control muscles the figure was only 42%. On the basis of this index, *mdx* muscles would appear to be significantly more fatigue resistant than control muscles.

Table 4.2. Twitch and Tetanic Characteristics of MDX and Normal Tibialis Anterior Muscles. Values are given as mean ±SEM.

	MDX	NORMAL
Twitch tension (N)	0.502 ±.004** (n=17)	0.330 ±.003 (n=15)
Twitch TTP (ms)	17.5 ±0.7* (n=15)	21.6 ±0.5 (n=15)
Twitch $T_{1/2}$ (ms)	11.6 ±0.6 (n=15)	12.1 ±0.5 (n=15)
Normalised MRTD twitch (N/ms/N)	86.4 ±2.5** (n=17)	71.1 ±3.5 (n=15)
Normalised MRR twitch (N/ms/N)	44.5 ±4.2 (n=17)	38.6 ±2.7 (n=15)

* $p<0.05$, ** $p<0.01$

Table 4.3 Maximal Stimulated force in MDX and Normal Tibialis Anterior Muscles. Values are given as mean ±SEM

	MDX	NORMAL
Max force (N)	1.59 ±.08** (n=17)	1.17 ±0.03 (n=15)
Force/CSA (N/mm^2)	0.236 ±.010** (n=17)	0.299 ±.009 (n=15)
Fatigue Index (% peak force)	48 ±2* (n=16)	42 ±1 (n=15)

* $p<0.05$, ** $p<0.01$

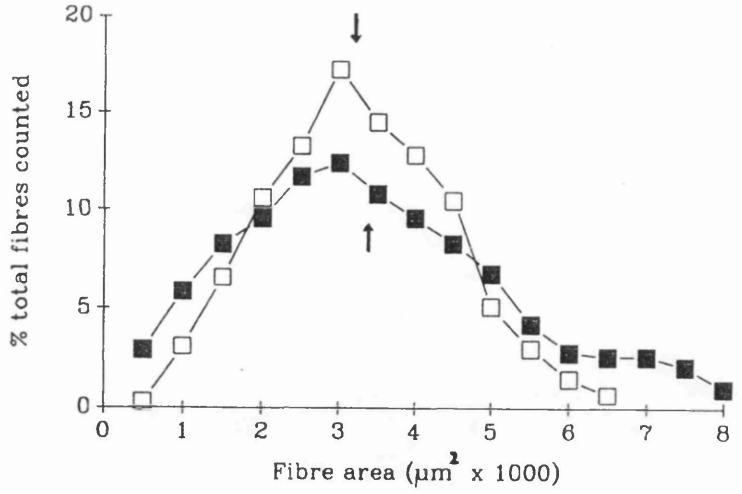


Fig 4.2 Fibre areas of mdx and C57 mouse tibialis anterior muscles (mean fibre areas indicated by arrows). (filled square) mdx n=5 muscles, 500 fibres; (open square) C57 n=6 muscles, 600 fibres.

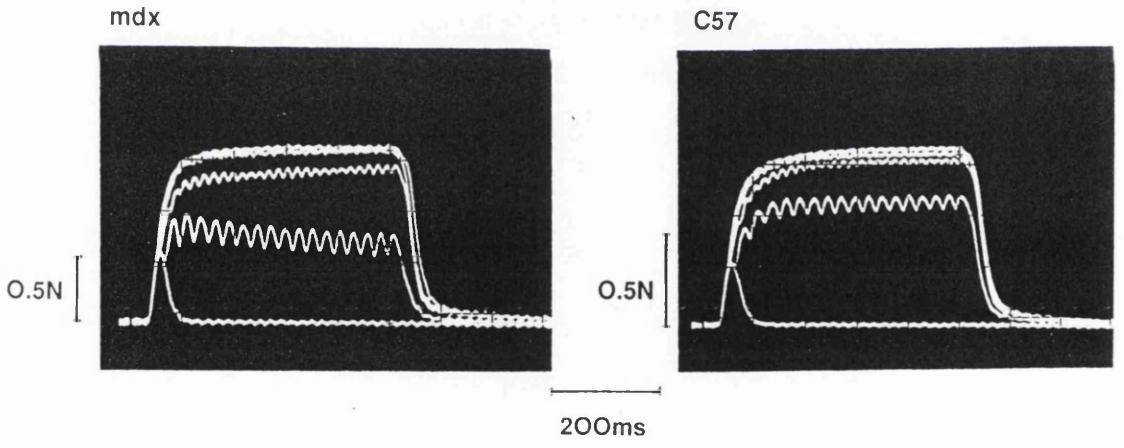


Fig 4.3 Forces generated by stimulation of mouse tibialis anterior muscles at 1, 40, 60, 80, 100 Hz. Left = mdx, right = C57. Note different force scales for the two muscles.

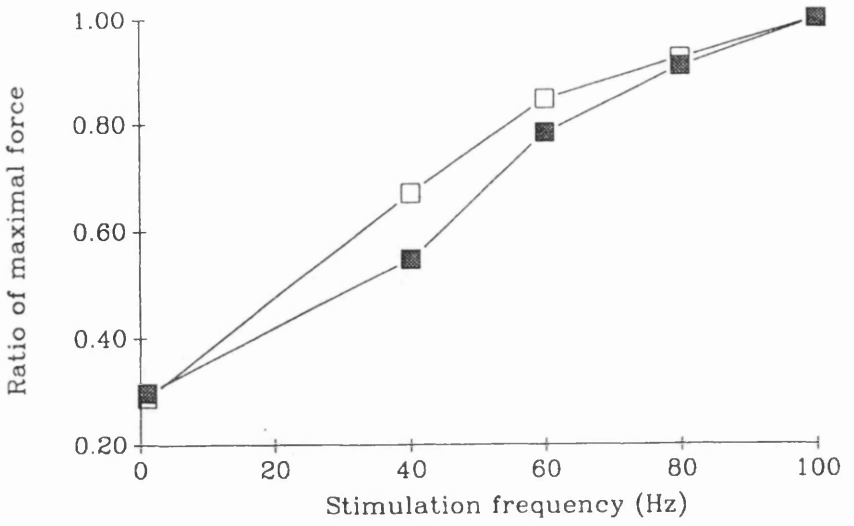


Fig 4.4 Force/frequency relationships of mouse tibialis anterior muscles. Forces are expressed as a ratio of maximal force at 100Hz (± SEM, error bars within symbols). (filled square) *mdx* n=15, (open square) C57 n=17.

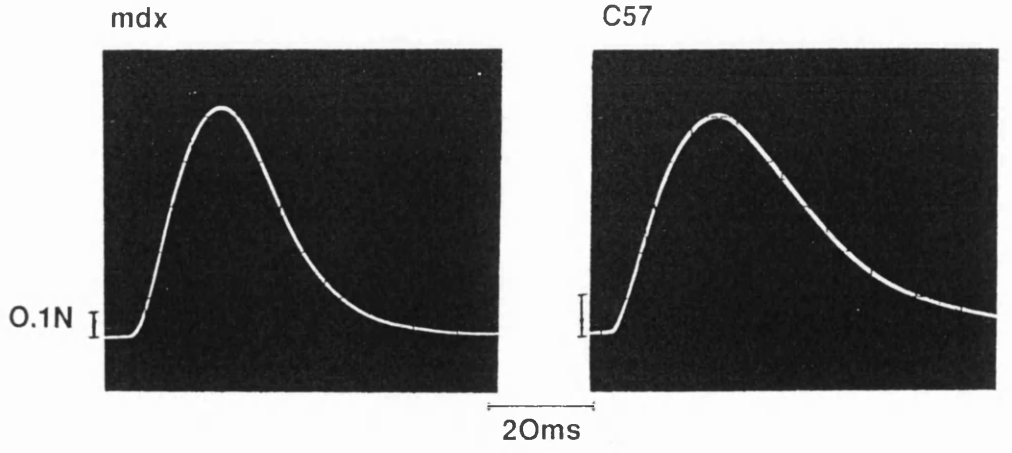


Fig 4.5 Isometric twitch recordings of mdx (left) and C57 (right) mouse tibialis anterior muscles. (Note different force scales).

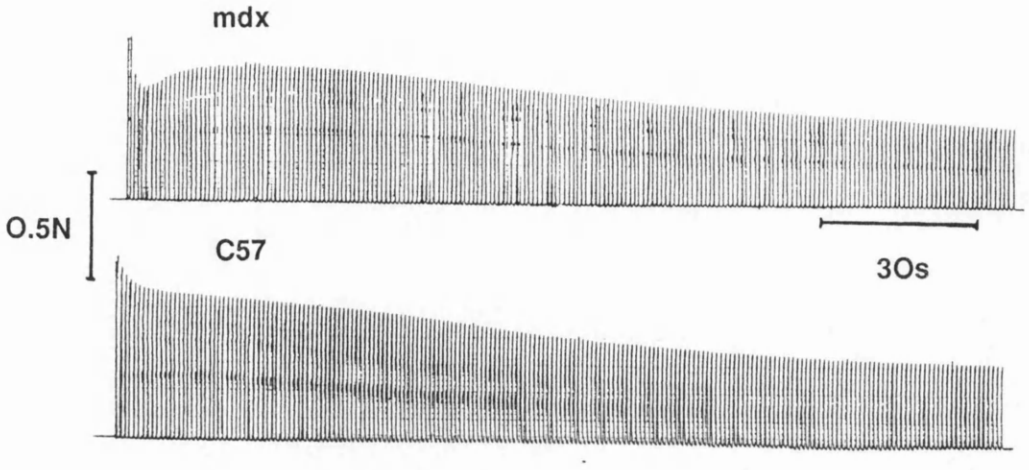


Fig 4.6 Changes in isometric force of mdx and C57 mouse tibialis anterior muscles as a result of stimulation at 40Hz for 0.25s every 1s for 3 minutes

4.4 DISCUSSION

4.4.1 Physical characteristics

The results demonstrate clear differences in the size, strength and contractile properties of the TA muscle of *mdx* mice when compared to those of normal mice of a similar age.The *mdx* mice were heavier than the age-matched controls, as was found by Dangain and Vrbová (1984) and Anderson et al (1987). The larger body mass was not a consequence of a longer body length. We found the *mdx* TA muscles to be the same length as the normals and Marshall et al (1989) have reported that the tibial lengths of normal and *mdx* mice are the same. Previous workers have also reported that *mdx* muscles are heavier than those from control animals (Dangain and Vrbová, 1984; Anderson et al, 1987; Marshall et al, 1989) although other workers have not found differences in either body weight or muscle mass (Tanabe et al, 1986; Carlson and Makiejus, 1990). When the muscle mass was expressed as a percentage of body weight the *mdx* TA accounted for a greater proportion of body mass than did the TA of control mice. It would appear, therefore, that the greater body weight of the *mdx* mice could be attributed to larger and heavier muscles.

Histological sections of *mdx* muscle, stained with haematoxylin and eosin showed little evidence of connective tissue proliferation and this is similar to the observations of other workers (Torres and Duchen, 1987; Tanabe et al, 1986). Marshall et al (1989) used image analysis to measure the connective tissue content of *mdx* and normal soleus muscles and found that although more connective tissue could be measured in the endomysial and perimysial spaces in *mdx* muscle, the difference only became substantial in animals older than those we have used in the present study.

In the absence of any oedematous or connective tissue infiltration

of the muscle the greater size of *mdx* muscles must have been the result of increased muscle fibre bulk due to fibre hyperplasia and/or fibre hypertrophy. The distribution of fibre sizes shown in Fig 4.3 suggests that both processes may be active in the dystrophic muscle as there were greater numbers of both large and small fibres in the *mdx* TA. The small fibres had all the signs of being the result of fibre splitting. Anderson et al (1987) examined the extensor digitorum longus and soleus muscles of young and old *mdx* and normal mice and found that 4 week old *mdx* muscles tend to have fewer fibres than normal, whereas, at 32 weeks, *mdx* mice had a greater number of fibres in the extensor digitorum longus, but not soleus muscles. These observations would suggest that in *mdx* mice the larger muscles were a consequence of the dystrophic process, and not the result of a greater number of fibres at birth.

In showing genuine muscle hypertrophy the *mdx* muscle differs from the human Duchenne muscular dystrophy where, although there is enlargement of certain muscles, this is a "pseudo-hypertrophy" with fat and connective tissue accounting for the increased size (Jones et al, 1984). Fibre splitting and fibre hypertrophy do occur in Duchenne muscular dystrophy but it would appear that these processes are not sufficient to offset the degenerative process in the human condition. In the *mdx* mouse, after the initial phase of massive fibre necrosis, the fibre hypertrophy and hyperplasia would seem to run ahead of the degenerative process resulting in a muscle which is larger than that of age-matched normal mice.

4.4.2. Muscle strength

Further evidence that the larger size of *mdx* muscle is due to an increase in contractile material is afforded by the fact that *mdx* muscles were stronger, in absolute terms (30% on average), than the TA muscles

from control animals. Coulton et al (1988b) reported that maximal isometric forces of the adult *mdx* soleus muscles are similar to controls, whilst Dangain and Vrbová (1984) found that the TA of *mdx* mice developed greater tensions than normal, suggesting an element of muscle specificity in the strength development of *mdx* mice. Although the cause of muscle fibre hypertrophy in dystrophic muscle is not known, a common suggestion is that, as the number of viable fibres decreases, there may be a work-induced stimulus to the remaining fibres to undergo hypertrophy and fibre-splitting (Goldspink and Ward, 1979). Such a mechanism might explain why a muscle maintains a strength close to the normal value but it cannot account for muscles which are stronger than expected. An alternative hypothesis is that *mdx* muscle fibre hypertrophy results from an overstimulation of the regenerative process following fibre necrosis. Fibroblast growth factor (FGF) is believed to stimulate muscle fibre growth by activation of satellite cell proliferation (Allen et al, 1984). DiMario et al (1989) have shown that FGF concentrations in the extracellular fibre matrix of *mdx* muscle are significantly greater than control levels. Furthermore, cultured *mdx* satellite cells are more responsive to FGF than control satellite cells (DiMario et al, 1988).

4.4.3. Force/Cross-sectional area

Although stronger than normal, the *mdx* muscles were not as strong as might be expected from their size and cross-sectional area. When the force data was normalised for muscle cross-sectional area, *mdx* TA were only able to generate 70% of normal force. Thus *mdx* muscles have a significantly reduced "specific muscle tension" compared with normal muscle. The reduced force generating capacity of *mdx* muscle is probably not the result of fat or fibrous tissue infiltration (see above). The presence of areas of segmental fibre degeneration, although each only

affecting a small portion of the total fibre length, may result in substantial loss of force production since the active contractile portions of these fibres will lack contiguity. Secondly, fibre-splitting may have deleterious effects on force generation. Schmalbruch (1975) suggested that fibre splitting arises from incomplete fusion of myotubes within the basement membrane during regeneration resulting in the formation of two or more daughter fibres. These can branch off and reconnect to the original fibre further along its length or to neighbouring muscle fibres. This process would result in a reduction in the number of myofibrils which contract in parallel. Furthermore, Bradley (1979) has postulated that necrotic fibre segments and split fibres of small diameter may present a conduction block to the muscle action potential, leading to incomplete activation of fibres.

4.4.4. Contractile properties

The contractile characteristics of *mdx* TA muscles were found to differ from those of normal muscles. As a proportion of the maximum tetanic force, less force was generated at intermediate frequencies of stimulation. The twitch forces of *mdx* and normal muscles were similar in proportion to the maximum tetanic force but the time course of the *mdx* twitch was of shorter overall duration. The consequence of the faster twitch was that the fusion frequency was higher for the *mdx* TA muscle. The reason for this behaviour is not clear, but does not appear to be related to differences in fibre type composition between *mdx* and normal muscles. In a separate study (G Vrbová, personal communication) it was found that the twitch time to peak of extensor digitorum longus (EDL) muscles increased significantly with age in normal mice, but no such slowing of the twitch occurred in older *mdx* EDL muscles. Thus older *mdx* muscles appear to maintain the contractile properties of muscles

from younger mice. The possibility that the changes are secondary to the processes of degeneration or regeneration in the dystrophic muscle will be discussed in the following section of this thesis.

4.4.5. Fatigue characteristics

Previous studies of the fatiguability of dystrophic muscle have found that dystrophic muscle is more fatigue resistant than normal muscle (Eberstein and Sandow, 1963; Edwards et al, 1987). In the fatigue test used in these experiments, the *mdx* TA muscles proved to be more resistant to fatigue than those from normal mice. The protocol involved repetitive stimulation at 40 Hz which gives different proportions of maximal force in the normal and *mdx* muscles. Consequently the greater fatigue resistance of the dystrophic muscle might have been because the muscle was less fully activated, and the test less metabolically demanding than that for normal muscle. It is not clear whether this explanation can also account for the differences seen by previous workers studying human DMD and mouse dystrophic muscles.

4.4.6 Summary

The main objective of the experiments described in this section was to investigate the functional properties of dystrophin-deficient muscle. The findings expose clear differences between the physical and contractile characteristics of *mdx* and normal mouse TA muscles. Thus, *mdx* muscles are abnormal in terms of their size, strength, contractile properties and fatiguability and raise the question as to the cause of these differences. Dystrophic muscle fibres differ from normal in that they lack dystrophin and it is tempting to ascribe the differences in contractile properties to the direct effect of the absence of this protein, but this hypothesis cannot be confirmed from the results described in this Chapter. *Mdx* muscles

contain populations of degenerating and regenerating fibres and it is possible that the altered contractile properties of the whole muscle reflect the function of these abnormal fibres. The test of this hypothesis would be to examine the contractile properties of normal muscles which have been experimentally damaged, this provides the starting point for investigations described in the following Chapter.

CHAPTER 5

THE SUSCEPTIBILITY OF NORMAL AND DYSTROPHIN-DEFICIENT (*mdx*) MUSCLES TO EXERCISE-INDUCED MUSCLE DAMAGE

5.1. INTRODUCTION

In the previous Chapter it was suggested that the abnormal contractile properties reported in *mdx* TA muscles might be a result of the presence of damaged and regenerating fibres. The experiments described in this Chapter were carried out in order to test this hypothesis.

The primary cause of the myopathy suffered by *mdx* mice is believed to be a deficiency of the muscle protein dystrophin which makes the disorder homologous to the human Duchenne and Becker muscular dystrophies (Hoffman et al, 1987). The function of dystrophin is, as yet, unknown but its location within the sarcolemmal membrane (Zurbrzycka-Gaarn et al, 1988) and its physical similarities to alpha-actinin and spectrin suggest that it may serve as a cytoskeletal protein (Koenig et al, 1988). As a structural protein, dystrophin may stabilise the fibre membrane and help protect against the damaging effects of mechanical stresses associated with muscle activity (Beam, 1988).

The differences between the contractile properties of normal and *mdx* TA muscles might reflect the absence of dystrophin. However, dystrophic muscles contain populations of both necrotic and regenerating fibres and it is possible that the disparate contractile properties of *mdx* and normal muscles may be a reflection of the presence of either degenerating or regenerating muscle fibres in the *mdx* muscle. The first part of this section presents the results of an investigation of the contractile properties of damaged and regenerating normal mouse muscle and compares these values with those of the *mdx* muscles.

Exercise in which active muscles are forcibly stretched (negative work or eccentric contractions) can lead to considerable loss of force, fibre degeneration and subsequent regeneration (Friden et al, 1981; Newham et al, 1983; Jones et al, 1986) even in normal muscles. It has

been suggested that dystrophic muscles may be more susceptible to the damaging effects of eccentric exercise (Edwards et al, 1984) and the recent speculation about a structural role for dystrophin is consistent with this hypothesis. In the second part of this chapter a comparison is made between the susceptibility to damage of normal and *mdx* mouse muscles in order to test the hypothesis that the dystrophin-deficient muscles are particularly sensitive to stretch-induced muscle damage.

5.2 METHODS

5.2.1. Experimental Animals

Experiments were performed on dystrophic (C57 BL/10/*mdx*) and normal (C57 BL/10) female mice aged 18-28 weeks (*mdx*) and 16-24 weeks (C57).

5.2.2 Exercise Protocol

Eccentric contractions were elicited in the right foot dorsiflexor muscles as described by Faulkner and Jones (1985), the contralateral muscles serving as non-exercised controls. Mice were anaesthetized with an intraperitoneal injection of 4.5% chloral hydrate (0.01ml per gram body weight) and the right knee clamped firmly to restrict movement to the lower limb. The peroneal nerve was exposed just above the knee and placed on a small hook electrode. The foot was taped to a perspex holder which rotated around a pivot at the ankle. The foot holder was attached to the rim of a motor driven wheel, the rotation of which caused the foot to travel from full flexion to full extension (approximately 100°) and back in 600 ms (Fig 5.1). The TA was stimulated for 300 ms at 100 Hz using 100 μs square wave pulses of supramaximal voltage beginning just before and continuing during the lengthening phase. The shortening phase was entirely passive. Lengthening contractions were repeated every 5 s for 240 repetitions after which the wound was repaired and the animals allowed to recover.

5.2.3. Assessment of contractile properties

At times of 1, 3, 6, or 12 days after exercise the animals were again anaesthetized, the distal tendons of both control and exercised muscles were isolated and attached to Statham force transducers with fine

silk suture. Muscles were stimulated via the sciatic nerve and maximal forces, force-frequency relationships and fatiguabilty were measured as described in the section 4.2.2.

5.2.4. Post-mortem examination

After the measurement of contractile properties the animals were killed with a lethal dose of chloral hydrate. Muscle length was measured in situ using calipers in 11 animals (6 *mdx*, 5 C57) and both exercised and control muscles were carefully excised and weighed. A portion, taken from the belly of the muscle, was orientated and frozen in isopentane cooled in liquid nitrogen for subsequent histological analysis.

5.2.5. Histology

Cryostat sections were cut at 8μm and stained with haematoxylin and eosin and for NADH-tetrazolium reductase.

In order to provide a quantitative estimate of muscle damage the number of "damage foci"/microscope field were counted under x400 magnification for every second field across the section (approximately 15 fields counted per section). Damage foci were defined as fibres which were obviously infiltrated by mononuclear cells.

5.2.6 Statistics

Paired mean values and group mean data were compared using Student's t-test, significance was set at the 5% level. Values in the text are given as mean $\pm$ SEM.

5.3 RESULTS

5.3.1. Physical characteristics

As previously noted (section 4.3.1.) mature adult *mdx* mice were larger than the normal C57 animals and had a greater muscle mass. C57 mice weighed 25.3 g (±0.3, sem) and the *mdx* 29.4 g (±0.4). The control, undamaged TA muscle weights were 40.9 mg (±0.8) and 69.4 mg (±2.3) for C57 and *mdx* mice respectively, but the muscles were the same length (10.6 mm (±0.2) for *mdx* and 11.0 mm (±0.3) for C57), indicating that the *mdx* muscles were of a larger cross sectional area.

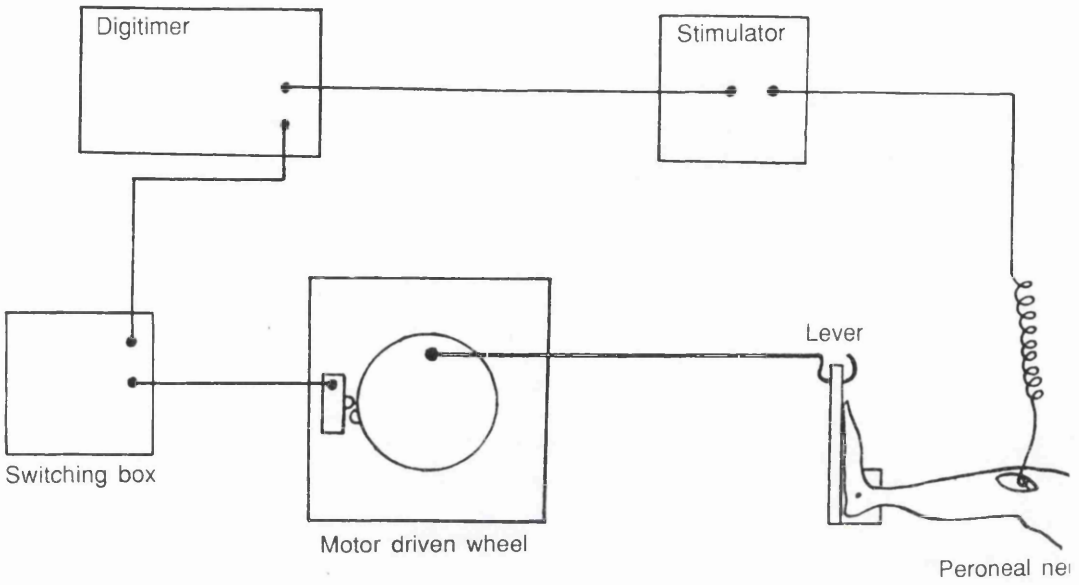
Both *mdx* and C57 muscles showed a decrease in weight after the damaging exercise, this being greatest at 6 days, when the *mdx* TA decreased by 13% compared with 5% for normal C57 TA muscles.

5.3.2. Histology

Histological examination by light microscopy (Fig 5.2) showed little change in normal muscle 1 day after exercise except for the presence of numbers of rounded, eosinophilic fibres (compare Fig 5.2a & c). Three days after exercise there was an invasion of the muscle by inflammatory cells accompanied by muscle fibre destruction (Fig 5.2e). By 6 days (Fig 5.2g) there were far fewer inflammatory cells present but there were plentiful small basophilic cells possessing large central nuclei which were probably regenerating myoblasts.

The damage, evident histologically, to the *mdx* TA muscles three days after exercise (Fig 5.2f) appeared more extensive than that occurring in normal C57 mice (Fig 5.2e). However, the exercise-induced damage in the *mdx* mice was imposed on muscles which were already showing areas of degeneration and regeneration (Fig 5.2b). Three days after exercise C57 muscles averaged 5.5 damage foci/field, compared with an average of 6.9

Fig 5.1 Schematic drawing of apparatus used to eccentrically exercise mouse tibialis anterior muscles *in situ*.



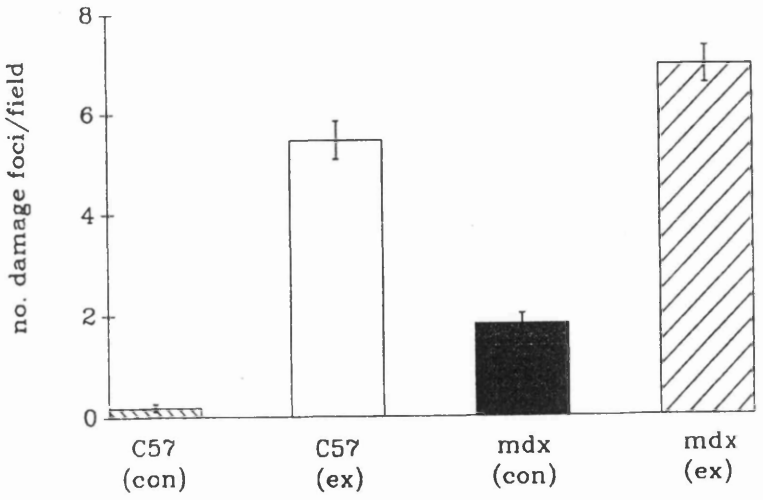


Fig 5.3 Number of damage foci/microscope field in mouse anterior tibialis muscle counted at 3 days post-exercise (± SEM). (con) contralateral muscle, (ex) exercised muscle. 90 fields (5 muscles) counted for each group.

in exercised *mdx* TA muscles (Fig 5.3), this difference proving significant at the 5% level. However, when the number of damage-foci in the non-exercised *mdx* muscles are taken into account the number of damaged fibres resulting from the exercise protocol are seen to be equivalent in *mdx* and C57 TA muscles.

5.3.3. Muscle strength

Changes in tetanic force after exercise were very similar for the normal and *mdx* TA muscles (Fig 5.4). Force loss was greatest at 3 days when the forces had declined to 52% (*mdx*) and 56% (C57) of the contralateral control muscles. After 3 days there was a gradual recovery so that, by 12 days post exercise, forces were 79% (*mdx*) and 76% (C57) of control force.

When the maximal tetanic forces of the undamaged muscles were normalised for the estimated muscle cross-sectional area (Fig 5.5) the "specific tension" was lower in *mdx* muscles compared to the normal C57 as previously reported (section 4.4.2). Specific tension also fell after exercise but the decrease was similar in *mdx* and normal muscles, declining to approximately 55% of control at 3 days for both normal and *mdx* animals.

5.3.4 Contractile properties

The loss of force and subsequent recovery was accompanied by changes in the contractile properties which were, in some respects, different for the C57 and *mdx* muscles. Figure 5.6 shows force frequency recordings of exercised and control muscles 3 days after exercise in a C57 and an *mdx* mouse. The force-frequency characteristics (where force is expressed as a ratio of maximal force at 100Hz) for all the muscles examined are shown in Fig 5.7.

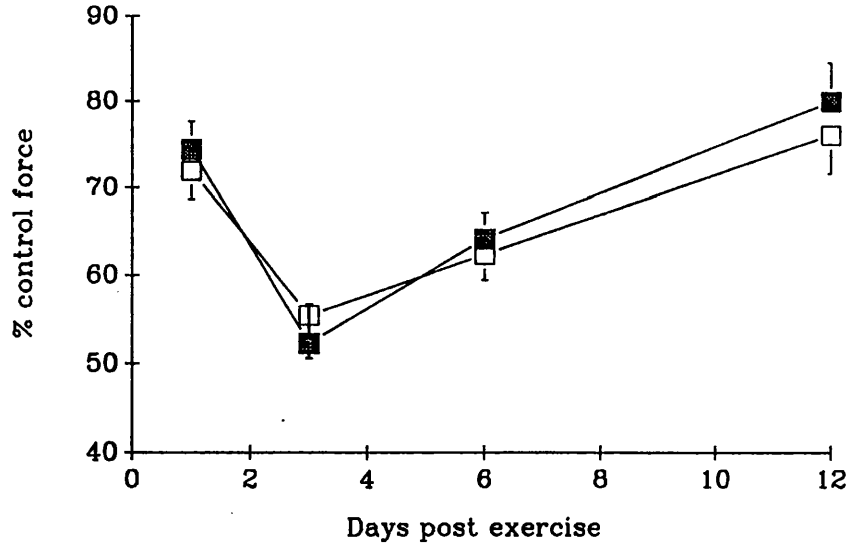


Fig 5.4 Maximum tetanic forces of mouse tibialis anterior muscles post-exercise expressed as a percentage of contralateral non-exercised muscles (± SEM). (filled square) mdx, (open square) C57. n = 4-6 for each point.

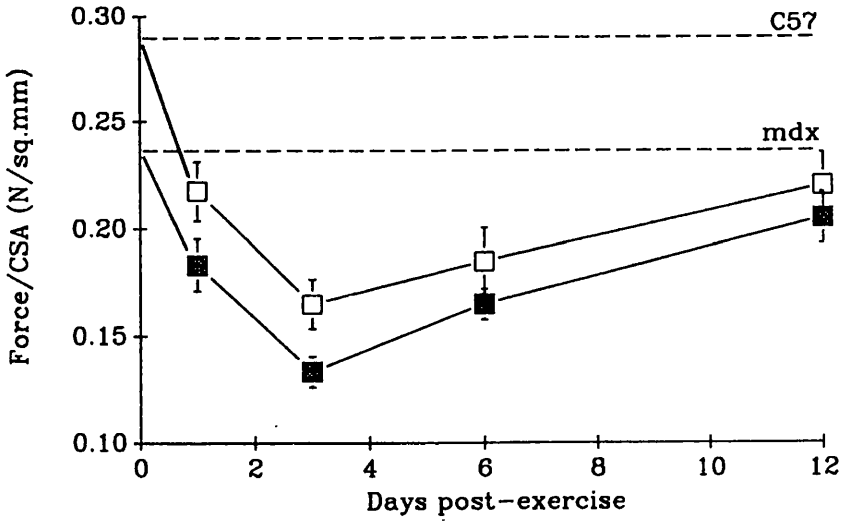


Fig 5.5 Changes in force/CSA of mouse tibialis anterior muscles post-exercise (±SEM). (filled square) mdx, (open square) C57. Dashed lines represent mean values for control muscles. n = 4-6 for each point.

C57 TA muscles (Fig 5.7a) showed a significantly greater loss of force at sub-maximal stimulation frequencies (1, 40 and 60 Hz) resulting in a shift to the right of the force-frequency curve. In contrast, no change was seen in the force-frequency characteristics of *mdx* muscles at the same time after exercise (Fig 5.7b). A measure of the shape of the force frequency curve can be obtained by taking the ratio of force at 40Hz compared to 100Hz and Fig 5.8 shows the time course of changes in this ratio. In the C57 TA there was a reduction in the 40/100 Hz force ratio at 1 and 3 days post-exercise, but by 6 days the ratio had returned to normal. In contrast, the exercised *mdx* TA showed little such loss of low frequency force at 1 and 3 days post-exercise, but the increments of the 40/100 Hz ratio at 6 days were comparable to that seen in the C57 TA muscles.

The force-frequency changes in normal muscles three days after exercise were not accompanied by significant changes in the contraction time of the twitch (Fig 5.6). There was a small slowing in the time to peak tension of the twitch at 6 days in both the exercised normal and *mdx* muscles.

5.3.5. Fatigue characteristics

One and 3 days after exercise there was a tendency for normal muscles to show an increase in fatigue-resistance. Thus, after 3 days of recovery damaged muscles developed 45 % ($\pm$3.3%, sem) of fresh force after fatigue as opposed to 41 % ($\pm$4.5%) for control muscles. However, these differences did not achieve statistical significance at any time after exercise. No differences in fatigue resistance were found between exercised and non-exercised *mdx* muscles at any time after exercise.

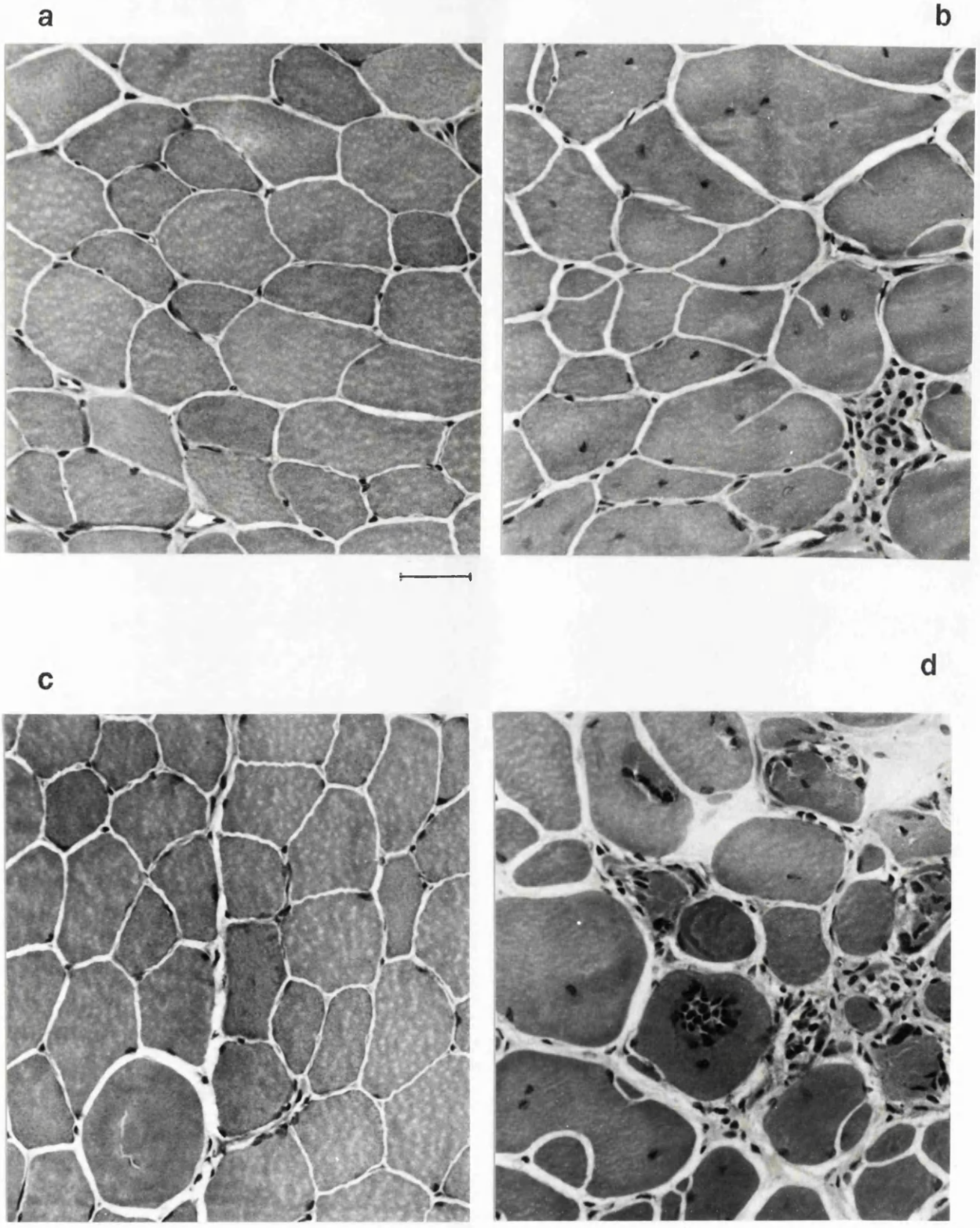


Fig 5.2 Histological appearance of mouse tibialis anterior muscles before and after eccentric exercise. Left C57, right *mdx*. Bar = 50μm
a-b non-exercised, c-d 1 day post-exercise (continued over)

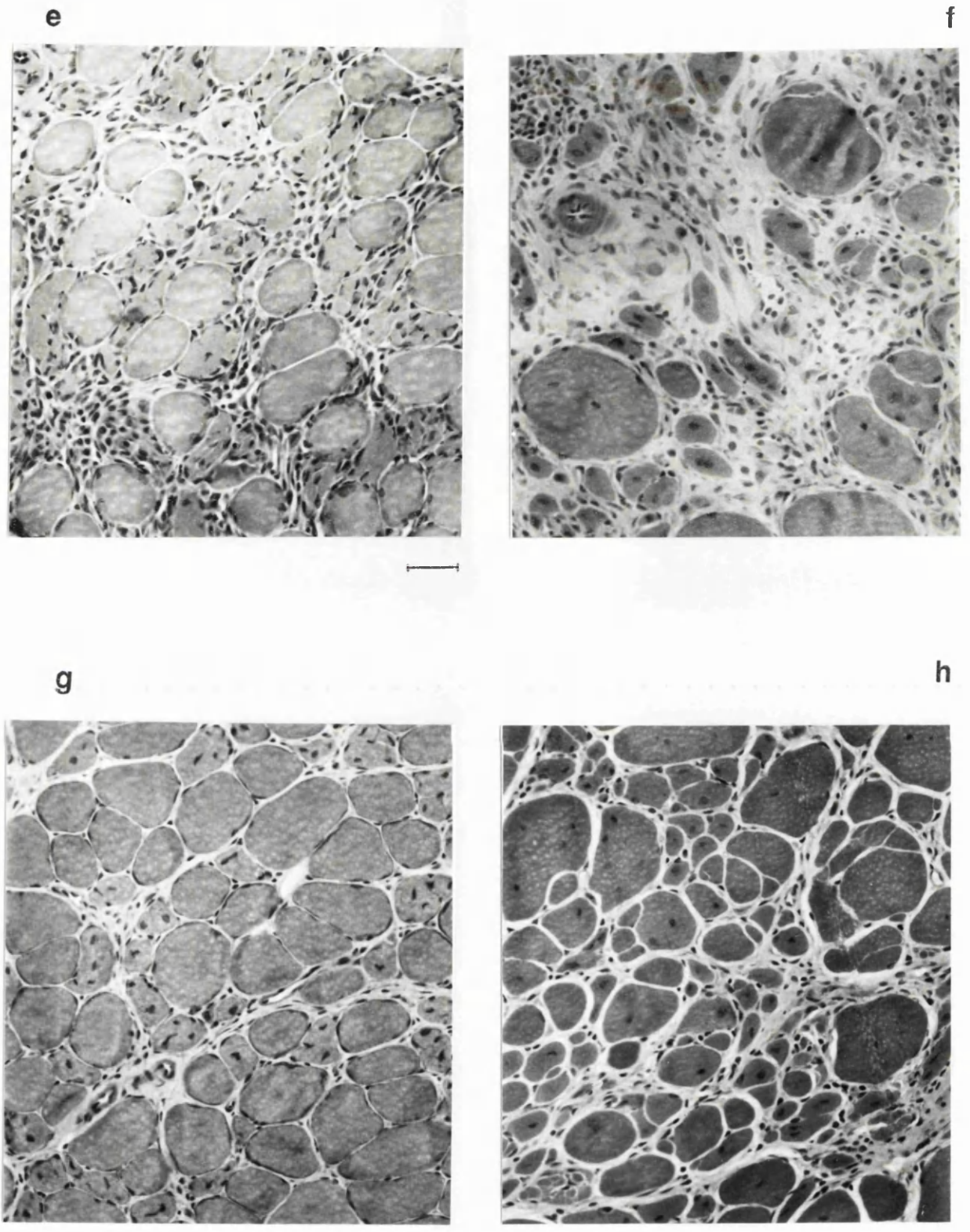
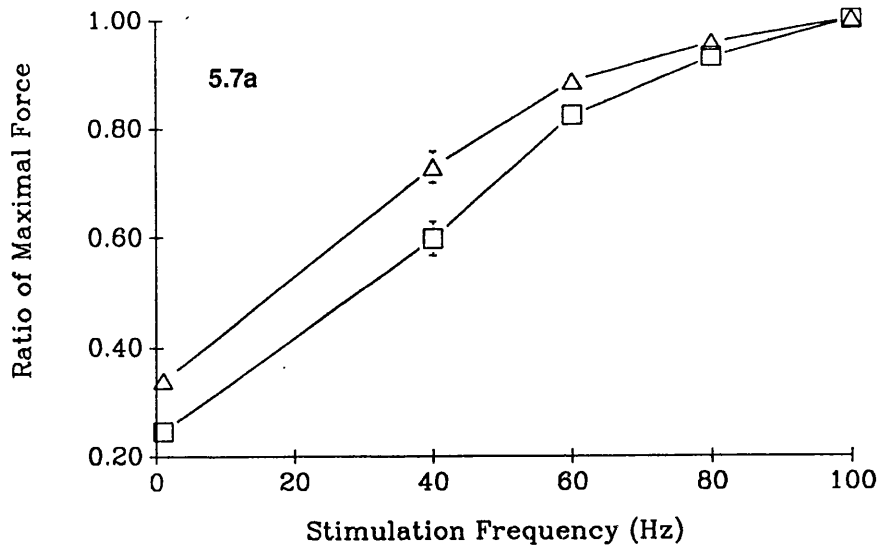


Fig 5.2 (cont) Histological appearance of mouse tibialis anterior muscles after eccentric exercise. Left C57, right *mdx*. Bar = 50μm
e-f 3 days post-exercise, g-h 6 days post exercise



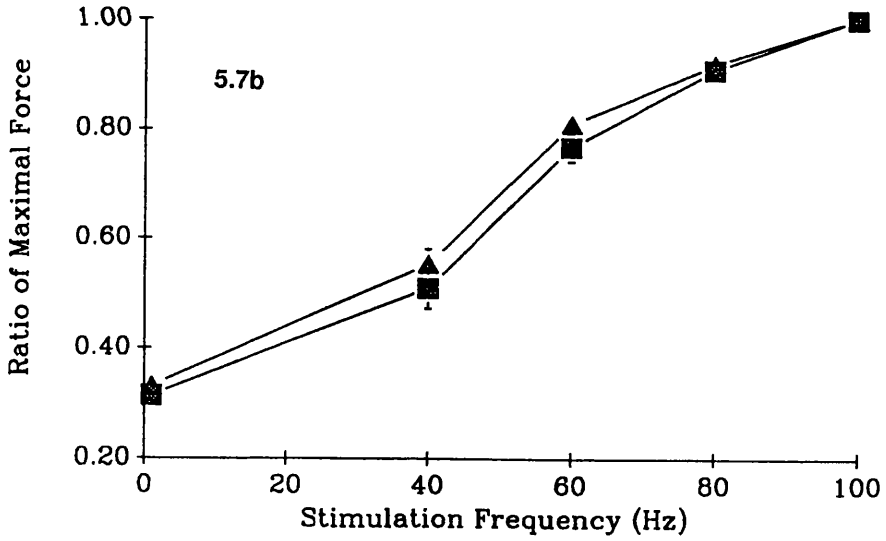


Fig 5.7 Mean force/frequency relationships of mouse tibialis anterior muscles at 3 days post-exercise (± SEM). (**a**) C57, n=6 (open square) exercised, (open triangle) control muscle. (**b**) mdx, n=6 (filled square) exercised, (filled triangle) control muscle.

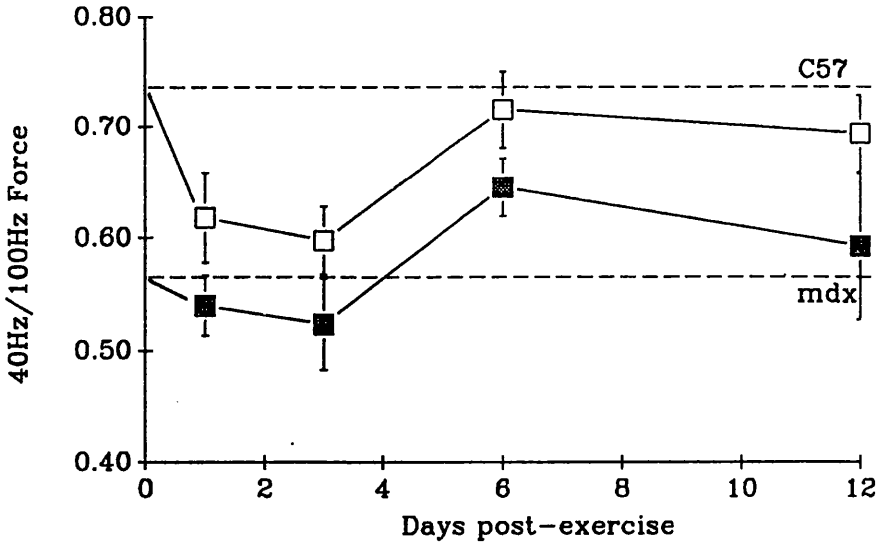


Fig 5.8 Time course of changes in 40/100 Hz ratio of mouse tibialis anterior muscles post-exercise (± SEM). (filled square) mdx, (open square) C57. Dashed lines represent mean values for control muscles. n = 4-6 for each point.

5.4. DISCUSSION

The present study had two main objectives; the first was to determine whether the unusual contractile properties of the *mdx* TA muscle, described in the preceding Chapter, were a consequence of the degenerating and regenerating fibres normally present in dystrophic muscle. The second objective was to determine whether *mdx* muscle is any more sensitive to stretch-induced damage; our hypothesis being that the absence of dystrophin might weaken the surface membrane in some way so as to make it more susceptible to mechanical disruption.

5.4.1. Force loss and damage

In section 4.3.2. it was shown that *mdx* TA muscles possess contractile properties which differ in a number of respects from those of normal muscle. The main differences were, i. a lower maximum tetanic force per unit cross sectional area of muscle (specific tension) and, ii. a relatively lower force generated at submaximum frequencies of stimulation. In the present work, the damaged C57 muscles showed a loss of maximum force which was greater than the (small) loss of muscle mass and consequently at 1, 3 and 6 days after the exercise the specific tension was reduced. Clearly damaged and regenerating fibres do not generate normal forces in relation to their cross sectional area and it is evident that the presence of such fibres is a major cause of the reduced specific tension of the *mdx* muscles.

5.4.2 Contractile characteristics

It has been known for a number of years that excessive isometric exercise (Edwards et al, 1977) or exercise involving stretch in man (Friden et al, 1981; Newham et al, 1983; Jones et al, 1986), and in mice

(Faulkner et al, 1989) results in a relatively greater loss of force at low frequencies of stimulation (low frequency fatigue) which has similarities with the unusual shape of the force frequency curve of control, undamaged *mdx* mouse muscle. Closer inspection, however, reveals that the similarities are more apparent than real. The loss of low frequency force in the damaged C57 muscles in the present work was primarily due to a loss of amplitude of the twitch. There was no significant change in the twitch duration or time to peak tension, suggesting that there was a reduced activation, possibly a smaller release of calcium for each action potential (Jones, 1981), with no change in the kinetics of calcium release or reuptake. In non-exercised *mdx* muscle, the loss of force at low frequencies (Fig 5.7b) was a consequence of a twitch of shorter duration while the twitch amplitude was relatively well maintained (see section 4.3.2.). It would appear, therefore, that the reduced force at low frequencies of stimulation in the *mdx* muscles is unlikely to be due to the presence of otherwise normal fibres which have been damaged by exercise.

Dystrophic muscles also contain a population of regenerating fibres but it is unlikely that these can account for the unusual force/frequency relationship of *mdx* muscle. Six and 12 days after damaging exercise there was evidence of regeneration in the damaged C57 muscles (Fig 5.2g) but at this time the C57 muscles were generating normal forces at 40Hz, while for the *mdx* muscles there was relatively more force at low frequencies of stimulation (Fig 5.8).

5.4.3. Histological changes

In its histological appearance the *mdx* muscle (Fig 5.2b) has many similarities to damaged normal muscle, containing both degenerating fibres similar to those seen 3 days after exercise in C57 muscles (Fig 5.2e) and

regenerating fibres as seen 6 days after exercise (Fig 5.2g). A similar observation of human muscle was the basis for the suggestion of Edwards et al (1984) concerning the initiation of the dystrophic process, and now provides the basis for the current hypothesis concerning the role of dystrophin in muscle fibres.

5.4.4. Susceptibility of *mdx* muscle to damage

Recently there have been two reports which demonstrate an increased sensitivity of dystrophic muscle to mechanical stress. Menke and Jockush (1991) reported that isolated *mdx* mouse muscle fibres were more susceptible to osmotic shock than normal muscle fibres and Weller et al (1990) have looked at IgG staining of muscle fibres in *mdx* mice after stimulating the TA *in situ* with and without Achilles tenotomy so as to produce concentric or eccentric contractions. They found that more fibres stained positively for IgG (which the authors suggest represents activation of the complement cascade and is thus a precursor to fibre necrosis) in the *mdx* than normal TA 60 minutes after either type of exercise. Hutter et al (1989), however, found comparatively minor abnormalities in the physical characteristics of the *mdx* surface membrane and McArdle et al (1991) found slightly less efflux of creatine kinase from isolated *mdx* as compared to normal mouse extensor digitorum longus muscles in the period immediately after stimulation under anaerobic conditions. These workers also stretched the muscles whilst stimulating and, again, found no difference between normal and *mdx* muscles. There is, therefore, conflicting evidence as to whether the absence of dystrophin renders the dystrophic muscle more susceptible to damage. One difficulty with the studies of McArdle et al and of Weller et al is that, although they looked at eccentric exercise, they did so for only a matter of hours after the initial insult although it is well known

that the main effects, and certainly the muscle fibre necrosis, occur several days after the exercise. We have followed events for up to 12 days after the exercise allowing us to monitor both the phases of degeneration and regeneration. The results were unequivocal, the *mdx* muscles responded to the exercise in the same way as did the normal C57 muscles showing a similar loss of force and a similar time course of both degeneration and recovery (Fig 5.4). Thus the hypothesis that dystrophin-deficient muscles would show a greater susceptibility to the effects of eccentric exercise can be rejected in this model.

5.4.5. Regenerative capacity of *mdx* muscle

The regenerative capacity of *mdx* muscles following eccentric damage, based on the rate of force recovery and histological presence of myoblasts, was found to be at least equivalent to that of normal muscle. A number of investigations have shown that rates of protein synthesis are higher in adult *mdx* muscles (MacLennan and Edwards, 1990) and also during normal muscle development (Anderson et al, 1987). Furthermore *mdx* muscles show a superior regenerative capacity following ischaemic injury of extensor digitorum longus muscle when compared to normal (Anderson, 1990). The enhanced regenerative capacity of *mdx* muscles may explain the differences in the clinical response to dystrophin deficiency in the mouse and human situations. In children suffering from Duchenne muscular dystrophy the progression is that of an inevitable loss of fibres and replacement with fibrotic tissue. Whereas, in the *mdx* mouse, not only are the damaged fibres replaced, but eventually there is a true muscle hypertrophy (section 4.3.2.), presumably a result of repeated episodes of myofibre damage and excessive regeneration. The development of muscle hypertrophy as a consequence of exercise-induced damage is investigated in the studies which form part of the work

described in Chapter 6.

5.4.6 Summary

The findings presented in this Chapter achieved two objectives. It appears that the atypical contractile properties of the *mdx* TA muscle are not due to the presence of damaged or regenerating fibres. It is possible, therefore that dystrophin may have some role to play in modulating force production in normal muscles, although it is not clear what the mechanism of this effect might be. The second conclusion to be made from these results is that the absence of dystrophin does not make *mdx* muscles more susceptible to stretch-induced injury and this argues against a role for dystrophin as a structural component strengthening the surface membrane against mechanical disruption.

CHAPTER 6

THE LONG-TERM CONSEQUENCES OF EXERCISE-INDUCED MUSCLE DAMAGE

6.1 INTRODUCTION

During the course of the work described in the previous Chapter it was observed that tibialis anterior (TA) muscles from *mdx* mice were larger and stronger than those of age-matched normal mice. The explanation for this finding, it was suggested, might be the fact that *mdx* muscles undergo a continuous process of fibre damage and regeneration, the characteristics of which are almost identical to the type of damage seen in normal muscle following severe eccentric exercise. If the hypertrophy of *mdx* muscles is indeed initiated by fibre damage, then the same might be true in normal muscle subjected to bouts of exercise resulting in fibre degeneration and regrowth. This hypothesis is tested in experiments which form part of this Chapter.

Any form of fatiguing exercise, provided it is severe enough, can lead to muscle damage involving morphological changes, delayed-onset soreness, force loss and elevation of muscle proteins in the blood (Armstrong et al, 1983; Newham et al, 1983b; Davies and White, 1981; Jones and Newham, 1985). Such changes are most commonly seen following activities where the muscles are forcibly stretched (ie. perform eccentric contractions) such as the quadriceps muscles in running downhill. Although the short-term consequences of eccentric work have been well described in both human (Newham et al, 1983; Jones et al, 1986) and animal models (McCully and Faulkner, 1985) little information is available on the long-term effects of eccentric exercise on muscle morphology and functional characteristics. The mouse provides a useful model for the study of long term changes in muscle function. Such studies are easier since mice have a metabolic turnover some tenfold greater than that of man, so that adaptations to exercise take place considerably faster.

The degree of delayed-onset muscle damage observed after an episode of eccentric exercise is dependent on whether or not the muscle has been exercised in this way in the recent past (Davies and Barnes, 1972; Jones et al, 1987). However the changes which occur within muscles following training to protect from subsequent damage are not known. Most studies which have looked at the protection of eccentric training against the damaging consequences of further exercise have studied the effects of continuous training over a period of weeks or more (for review see Armstrong, 1984). However, there is evidence to suggest that the prophylactic influence of training may be brought about by the performance of a single exercise bout (Armstrong, 1983). Furthermore, the work of Newham et al (1987) suggests that an episode of eccentric exercise may protect against some aspects of muscle damage resulting from repeated exercise, but not others.

The experiments reported in this Chapter were designed to answer a number of questions regarding the responses of a muscle to an episode of exercise-induced muscle damage. In one study the nature and effectiveness of the protective effect afforded by a single bout of exercise against an identical exercise regime, repeated following different periods of recovery, was investigated. The same model of exercised-induced damage was used in a second line of investigation, namely to examine the long-term effects of a single episode of damaging eccentric exercise on muscle size, strength, contractile properties and morphology.

6.2 METHODS

6.2.1 Animals

Female mice (n=32) of the C57 Bl/10 strain were used in all experiments. At the beginning of the study body weights were 19-25 g and animals and were between 18 and 24 weeks of age.

6.2.2 Exercise protocol

The exercise protocol used to induce delayed-onset muscle damage in the tibialis anterior (TA) muscle of the mouse is described in detail in section 5.2.2. After anaesthesia, the peroneal nerve was exposed and a small hook electrode placed under it. A lever arm attached to a rotary motor lengthened the foot plate and so stretched the TA muscle from a flexed to an extended position in approximately 600 ms. During stretching the TA was stimulated supramaximally via the peroneal nerve. Stimulation was repeated every 5 sec for 240 contractions, after which the incision was sutured and the animal allowed to recover.

After eccentric exercise mice were allocated into one of 7 experimental groups which were treated in the following way:

Group A : 3 days recovery without further exercise.

Group B : 10 days recovery after which a second bout of eccentric exercise was given followed by 3 days recovery.

Group C : 21 days recovery followed by a second bout of exercise with 3 days recovery.

Group D : 84 days recovery followed by a second bout of exercise with 3 days recovery.

Group E : 121 days recovery followed by a second bout of exercise with 3 days recovery.

Group F : 42 days recovery without further exercise.

Group G : 84 days recovery without further exercise.

6.2.3. Force recording

After the end of the final recovery period animals were again anaesthetised and forces and contractile properties of the exercised and contralateral control muscles were recorded *in situ* as described in section 4.2.2

6.2.4. Histochemistry

Immediately following force recordings mice were sacrificed and the TA muscle carefully removed from both limbs, weighed and a central portion mounted and snap frozen using isopentane cooled with liquid nitrogen. Crysotat sections at 8μm were subsequently cut and muscles were stained with haematoxylin and eosin for histological analysis and for NADH-tetrazolium reductase (see section 5.2.5.) to demonstrate the oxidative capacity of fibres.

6.2.5. Morphometry

Fibre areas were measured using semi-quantitative image analysis (Round et al, 1983) whereby an image of the section was magnified and projected onto a graphics tablet where it was traced manually.

Measurements were made on transverse muscle sections stained with NADH-tetrazolium reductase in order to distinguish between fibres with high and low oxidative capacities.

6.2.6. Quantification of muscle damage

To obtain quantitative information on the degree of histological damage apparent in TA muscles at different times after exercise the number of "damage foci"/microscope field were counted on haematoxylin and eosin stained sections. Counting was carried out at a magnification of x400 on every second microscope field (between 15-20 fields counted per muscle section) and damage foci were defined as any fibre undergoing disintegration or showing mononuclear cell infiltration. In the case of TA muscles from animals which were exercised only once, the presence of internalised nuclei (representing fibres which had regenerated following an episode of necrosis) was used as an indication of the number of fibres which had been damaged.

6.2.7. Statistics

Paired mean values and group mean data were compared using Student's t-test, significance being set at the 5% level.

6.3. RESULTS

6.3.1. Short-term effects of exercise

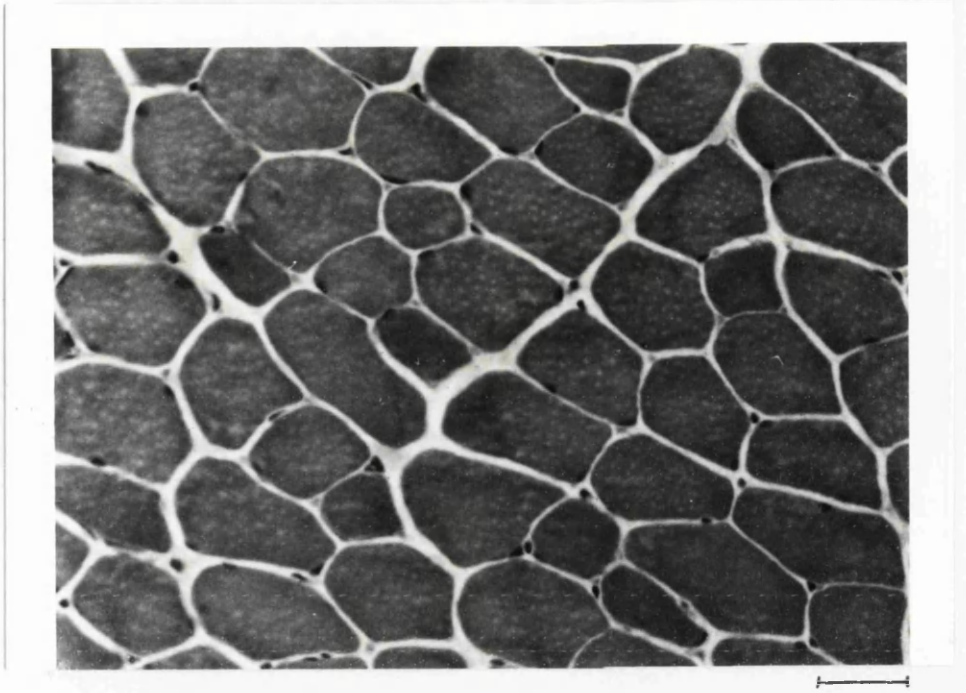
In order to assess the effectiveness of the exercise protocol and to provide comparative information in assessing the degree of muscle damage in groups B-E, 4 mice were sacrificed 3 days after the standard eccentric exercise regime (Group A). A 3 day period of recovery was chosen since this was the time when force loss and fibre necrosis were found to be maximal in earlier studies (section 5.3.2).

6.3.1.1. *Force and contractile properties*

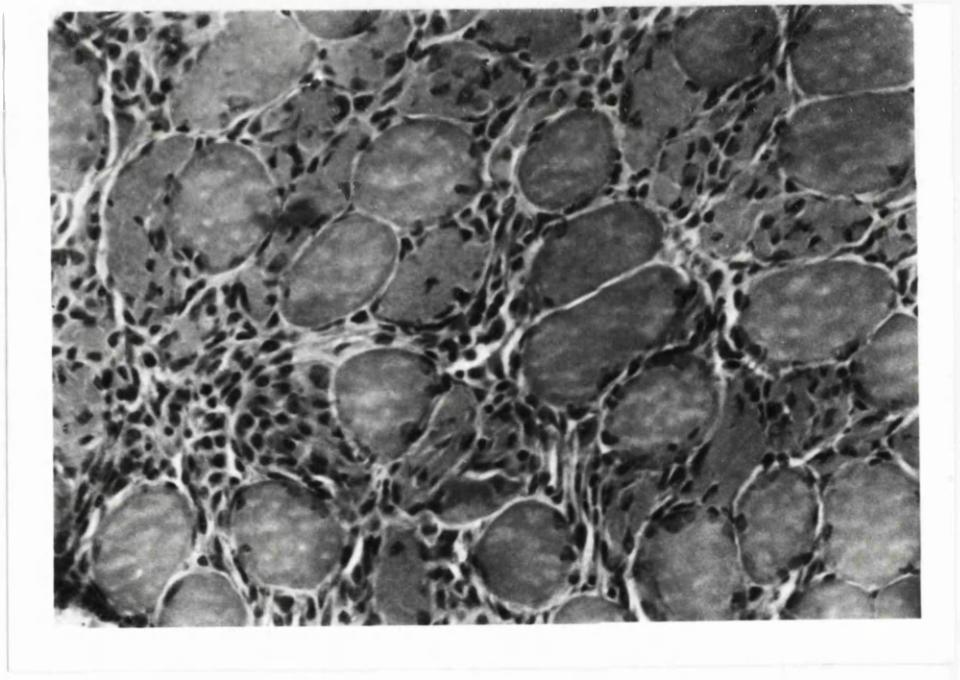
Table 6.1 lists *in situ* contractile properties of exercised and contralateral, non-exercised TA muscles. Three days after exercise muscles had a mean force equivalent to 52 % (±4.9, sem) of non-exercised control muscles and this was associated with a significantly reduced 40/100 Hz force ratio (0.41 (±0.04, sem) compared to 0.61 (±0.01) for controls). The change in the force frequency response after exercise was not accompanied by any change in the twitch time to peak (TTP) or 100 Hz half relaxation time ($t_{1/2}$) as seen in Table 6.1. However the twitch force of exercised muscles showed a more marked force loss than that seen with 100 Hz stimulation (43 % and 52 % of non-exercised muscles respectively).

6.3.1.2 *Histological changes*

Exercised muscles from Group A showed the typical appearance of delayed onset, eccentric exercise-induced damage (Fig 6.1). There was extensive invasion of the muscle with mononuclear cells, many of which were located within muscle fibres. Fibres could be observed in all stages of necrosis, from appearing swollen and eosinophilic with only one or



A



B

Fig 6.1 Histological appearance of mouse tibialis anterior muscle 3 days after eccentric exercise (B) compared to control muscle (A). Bar = 50 μm

two infiltraing cells to the complete breakdown of fibre architecture and replacement with mononuclear cells. When the extent of fibre damage was assessed quantitatively, these muscles contained an average of 6.1 damage foc: per field (Fig 6.5).

6.3.2. Effect of repeated exercise

6.3.2.1. *Force and contractile properties*

Fig 6.2 shows 100 Hz forces of exercised and control muscles 3 days after the second exercise bout, but with different recovery periods between the first and second bouts of exercise. Force losses 3 days after the second exercise bout were significantly smaller than those of muscles from Group A when 10 and 21 days recovery was given between the first and second exercise bouts (89.5 % and 82.6 % of contralateral muscle force respectively). However, when 84 and 166 days recovery were allowed, muscles showed force decrements after 3 days which were similar to those seen in Group A (61.1 % and 63.6 % of the contralateral force respectively).

The low frequency force changes (in terms of 40/100 Hz ratio) after a second bout of exercise following different recovery periods are shown in Fig 6.3. In this case only when the recovery interval between exercises was 10 days did any significant protection against the change in 40/100 Hz force ratio occur. Table 6.2 shows that there were no apparent differences in the twitch TTP and tetanic $t_{1/2}$ between muscles exercised once and those in which the exercised was repeated.

6.3.2.2. *Histological characteristics*

Fig 6.4 shows the histological appearance of a muscle 3 days after a second exercise bout which had been allowed 10 days recovery from an initial bout of exercise (Group B). Although many of the muscle cells

Table 6.1 Physical and contractile properties of TA muscles 3 days after exercise compared to contralateral muscles Mean values (± sem)

	control TA (n=4)	exercised TA (n=4)
muscle weight (mg)	40.5 (2.2)	38.9 (3.0)
1 Hz tension (N)	0.30 (0.1)	0.13 (0.1)
1 Hz TTP (ms)	21.4 (1.1)	20.9 (1.3)
40 Hz tension (N)	0.66 (0.05)	0.23 (0.05)
100 Hz tension (N)	1.07 (0.06)	0.56 (0.09)
100 Hz $T_{1/2}$ (ms)	16.3 (0.9)	18.1 (1.0)

Table 6.2 TA muscle physical and contractile properties after 6 and 12 weeks exercise compared to contralateral TA muscles. Mean values (± sem)

	control (n=11)	6 week (n=5)	12 week (n=6)
muscle weight (mg)	44.3 (0.13)	46.5 (0.34)	48.9 (0.30)
100 Hz tension (N)	0.97 (0.04)	1.03 (0.09)	1.11 (0.14)
100 Hz $T_{1/2}$ (ms)	17.2 (0.93)	19.6 (1.02)	19.3 (1.12)
Mean fibre area (μm^2)	3206 (224)	3847 (668)	3980 (705)
40/100 Hz force ratio	0.64 (0.03)	0.68 (0.05)	0.69 (0.08)

showed the presence of internal nuclei, these probably represent fibres which had undergone necrosis as a result of the first bout of exercise. In Fig 6.4 it can be seen that there was no evidence of mononuclear infiltration (compare with Fig 6.1) and this is illustrated in Fig 6.5 which shows that, for Group B, there were only 1.9 damaged foci/field on average. In Group C (21 days recovery between exercise bouts) muscles showed a similar histological appearance to those of Group B (Fig 6.5), demonstrating considerable protection against the damaging effect of the repeat exercise bout.

The histological characteristics of TA muscles from Groups D and E (84 and 166 days recovery respectively) 3 days after the second exercise bout (Fig 6.4b for a muscle from Group E) showed a similar pattern of fibre necrosis and mononuclear cell infiltration to that observed in Group A (Fig 6.1). Both Groups D and E proved to have similar numbers of damage foci/field when compared with muscles from Group A (Fig 6.5).

6.3.3. Long-term effects of exercise

6.3.3.1. *Muscle weight*

Tibialis anterior muscle weights for Groups F and G (42 and 84 days after a bout of exercise) are given in Table 6.2 and show that muscle mass increased significantly after exercise in both Groups (being 4.9 % and 10.4 % greater than the non-exercised muscle in F and G respectively).

6.3.3.2. *Force and contractile properties*

Table 6.2 shows the mean 100 Hz force of exercised muscles from Groups F and G. Six weeks after exercise (Group F) TA muscle forces were 6.1 % greater than the non-exercised muscle. This compares with a

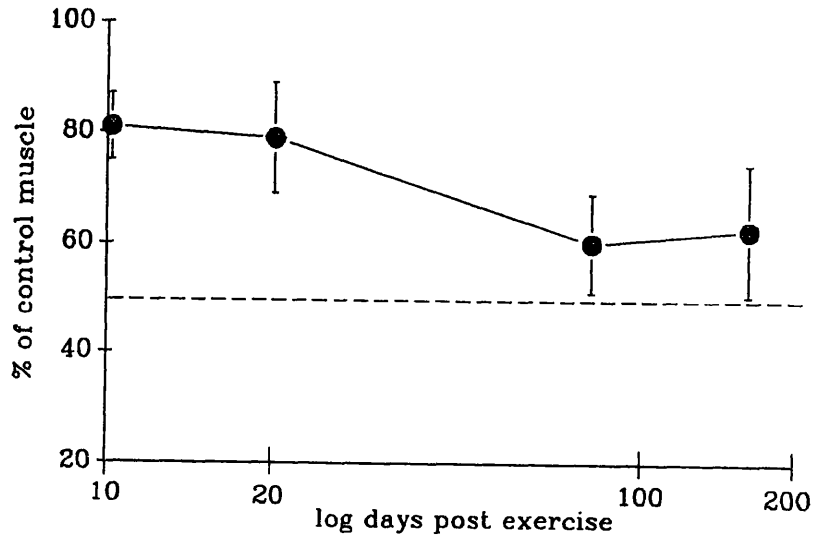


Fig 6.2 Maximal tetanic forces of tibialis anterior muscles 3 days after a second exercise bouts following different recovery periods after initial bout of exercise. (n=4-6 for each group).

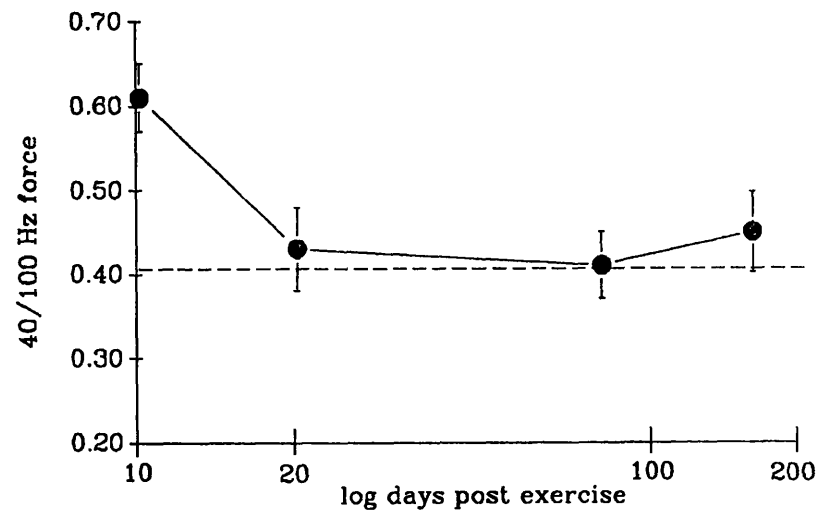


Fig 6.3 40/100 Hz force ratios of tibialis anterior muscles 3 days after a second exercise bout following different recovery periods after initial bout of exercise. (n=4-6 for each group).

14.4 % higher force after 12 weeks (Group G). Both these differences proved statistically significant at the 5 % level. The mean 40/100 Hz force ratios of exercised TA muscles were slightly greater than their control counterparts in both Groups F and G (Table 6.2).

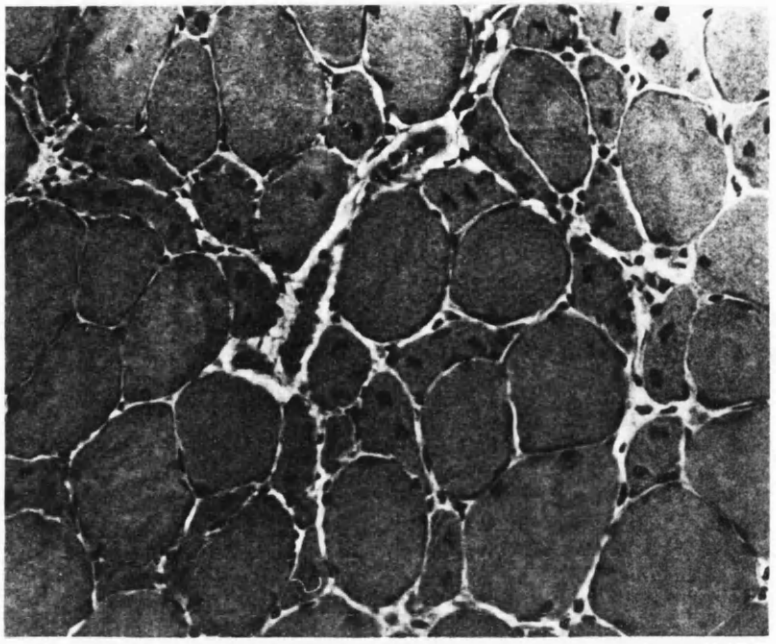
6.3.4.3. *Histological characteristics*

Fig 6.6 shows the typical histological appearance of a TA muscle 12 weeks after exercise (Group G). Many of the fibres contained internal nuclei and there also appeared to be a great variation in fibre size. Both very small fibres (probably resulting from fibre splitting) along with large hypertrophied fibres could be distinguished. The number of fibres counted with internal nuclei is believed to provide a quantitative estimate of those fibres which were damaged as a result of exercise. This figure is plotted for the exercised muscles of Groups F and G against the normalised force ratio for each muscle (Fig 6.7). This shows a correlation between the degree of fibre damage and increase in force after 6 and 12 weeks recovery from a single bout of exercise (r=0.54).

6.3.4.4. *Morphometry*

The presence of internal nuclei within fibres allowed the distinction to be made between fibres which had undergone necrosis and regeneration as well as those having high and low oxidative capacities (from NADH activity). Profiles of the fibre areas from exercised TA muscles of Group G are shown in Fig 6.8. Type I (high oxidative) fibres from exercised TA muscles (Fig 6.8a) which contained central nuclei (ie. had been damaged) had greater fibre area than those from the same muscles in which central nuclei were absent. Fig 6.8b shows that the same relationship was true for Type II fibres from the same muscles. Exercised muscles appeared to have an equal prevalence of central

A



B

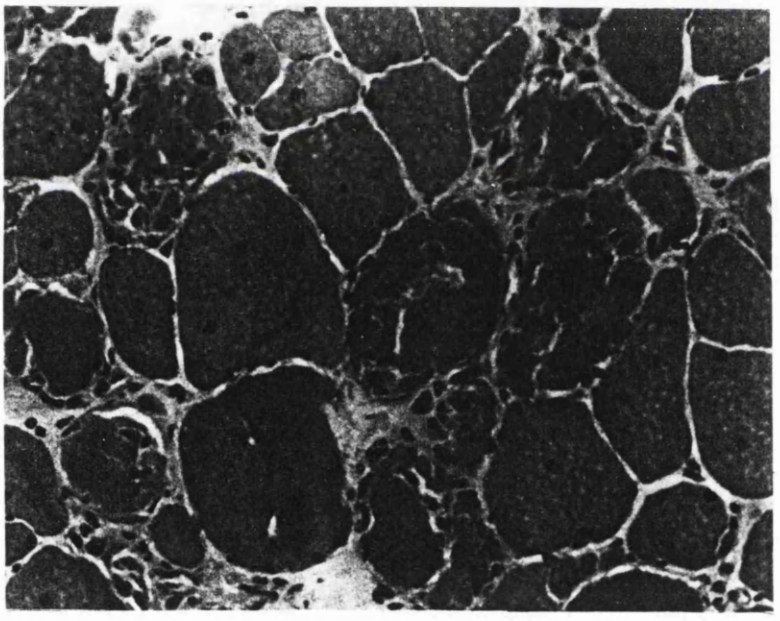


Fig 6.4 Histological appearance of mouse tibialis anterior muscle 3 days after a second exercise bout following recovery periods of 10 days (A), and 121 days (B) after an initial bout of exercise.
Bar = 50 µm

nucleation amongst the Type I and II fibre populations.

The differences in fibre sizes between centrally nucleated and non-nucleated fibres of exercised muscles are more clearly seen when the mean fibre areas are plotted (Fig 6.9). For Group F (6 weeks post-exercise) previously damaged fibres (ie. those possessing internal nuclei) were, on average, 21% larger than those from the contralateral muscle, this difference proving statistically significant). Even more striking were the results at 12 weeks post-exercise (Group G) where the mean fibre enlargement was of the order of 32%. Interestingly, for both Groups Type I fibres showed a greater degree of enlargement than Type II's. Fig 6.9 also demonstrates that the non-nucleated fibres from exercised TA muscles had mean areas which were consistently greater than those from the contralateral muscles, although these differences were not significant.

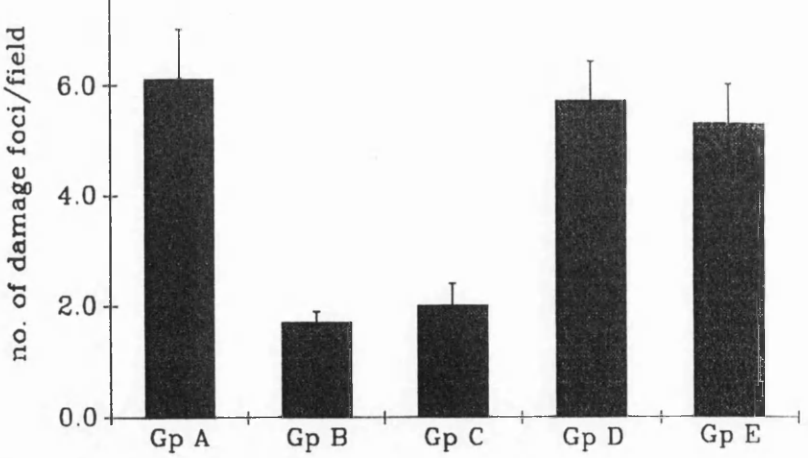


Fig 6.5 Histogram of the mean number of damage foci/field in tibialis anterior muscles 3 days after a second exercise bout following different recovery periods after an initial bout of exercise. (Mean values + sem)

Gp A = 3 days after initial damage (control)

Gp B = 10 days after initial damage

Gp C = 21 days after initial damage

Gp D = 84 days after initial damage

Gp E = 121 days after initial damage

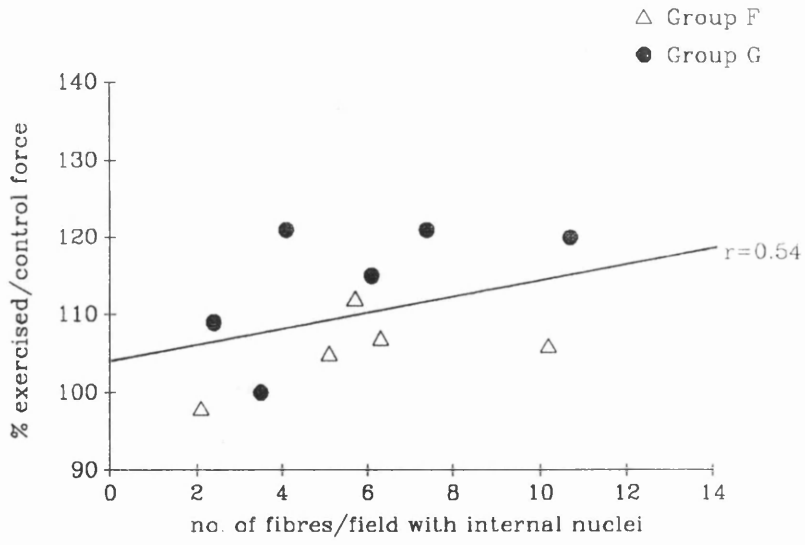


Fig 6.7 Graph of relationship between difference in maximal force of exercised and control tibialis anterior muscles and number of fibres/field with central nuclei for individual muscles 6 weeks (Gp F) and 12 weeks (Gp G) after exercise.

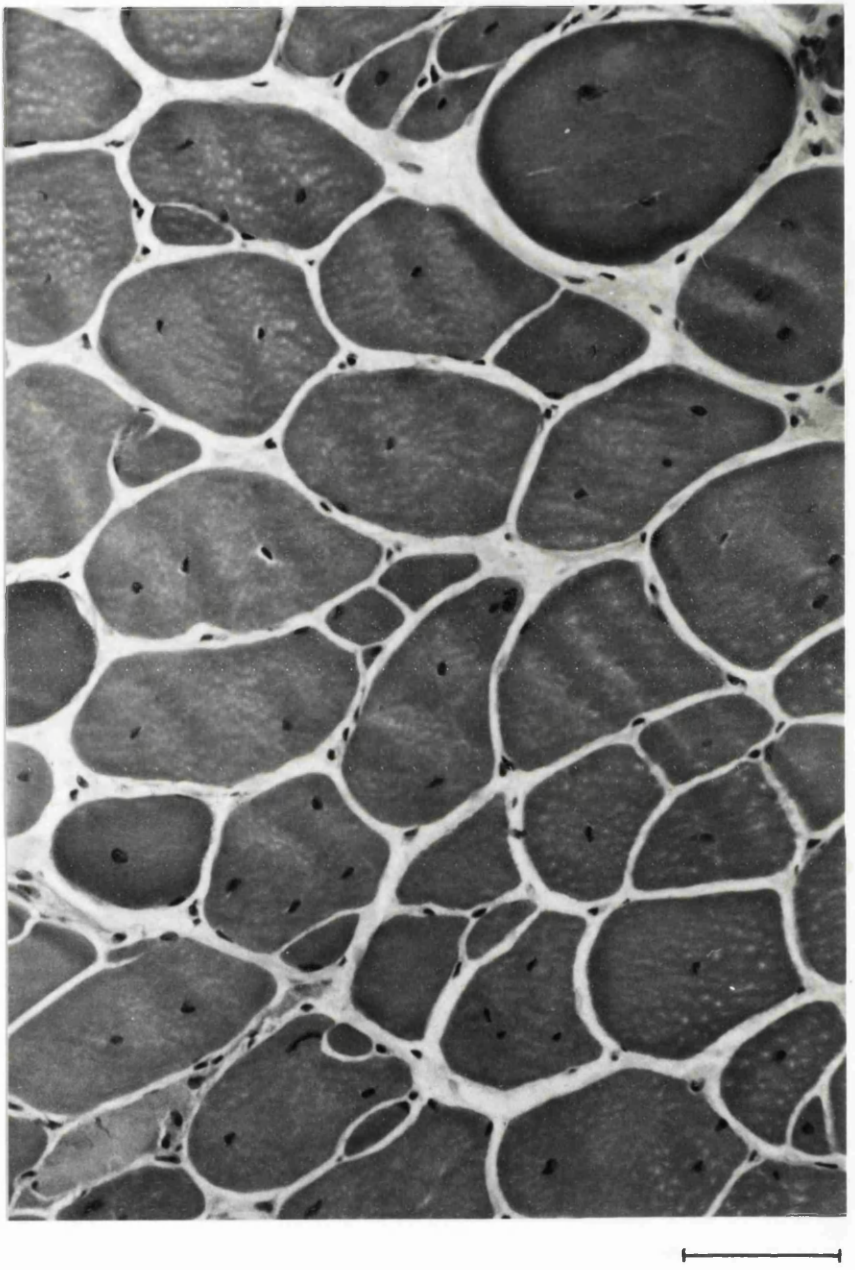
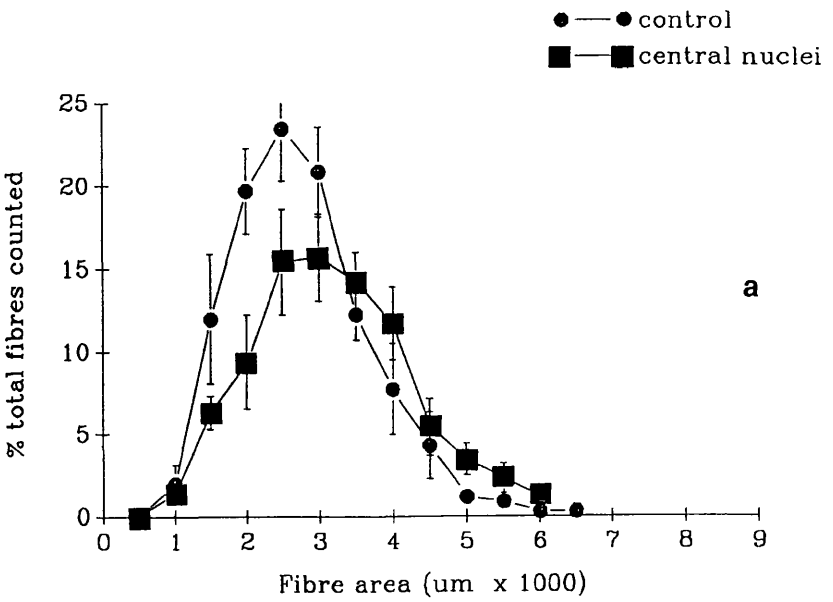


Fig 6.6 Histological appearance of mouse tibialis anterior muscle 12 weeks after a bout of eccentric exercise. Bar = 50 μm



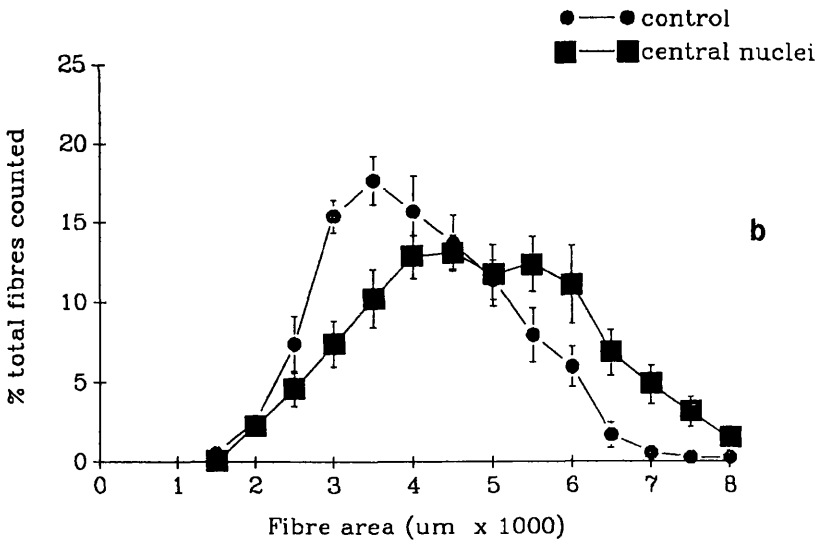


Fig 6.8 Profiles of fibre areas in *exercised* tibialis anterior muscles 12 weeks after exercise in fibres with central nuclei (squares) and without (circles) central nuclei. (a = high oxidative, b = low oxidative fibres). N=1200 fibres from 6 muscles Mean values (± sem)

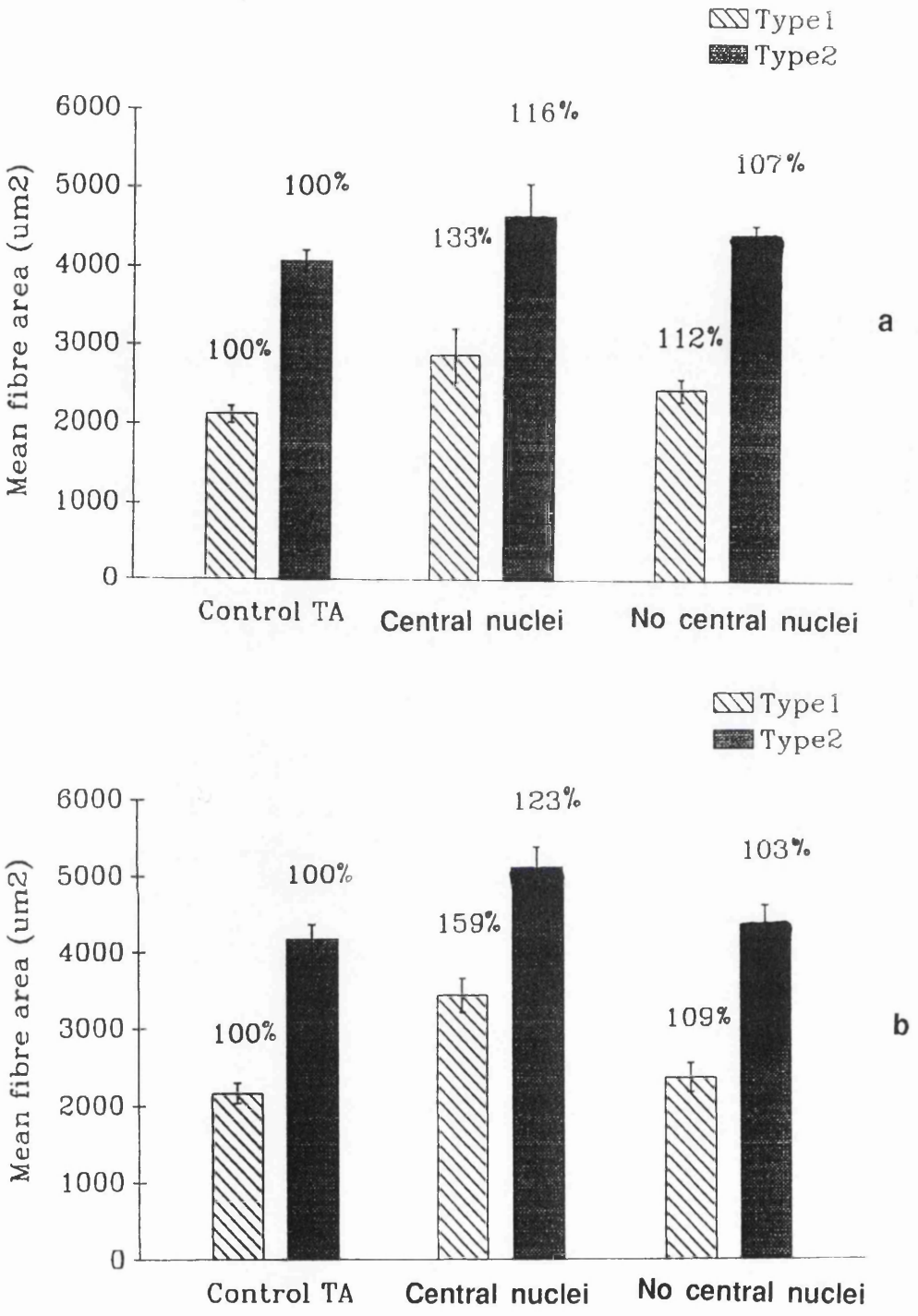


Fig 6.9 Mean fibre areas in exercised tibialis anterior muscles 6 weeks (a), and 12 weeks (b) after exercise in fibres with central nuclei (diagonal bars) and without (filled bars) central nuclei.

N=500 fibres (a) and 600 fibres (b) Mean values (± sem)

6.4 DISCUSSION

6.4.1 Repeated exercise

In this study it was found that a single bout of eccentric exercise of the mouse TA muscle had a temporary protective effect against the development of damage induced by further episodes of the same exercise. This confirms the findings of other workers using voluntary exercise regimes in both animal and human studies. Thus Schwane and Armstrong (1983) studying the effects of eccentric work on the vastus intermedius muscle of rats found that whereas 90 min of downhill running caused appreciable delayed-onset fibre necrosis, if the downhill run was preceded by a similar 30 min run 3 days earlier, then no damage ensued. Intriguingly, the 30 min run did not prove to be damaging to the muscle, which suggests that the protective effect of exercise need not depend on initial damage to muscle fibres. In an investigation into the effects of repeated bouts of eccentric exercise of the human elbow flexors, Newham et al (1987) reported that whilst both muscle soreness and CK release were protected after the first exercise bout, the maximal voluntary force and stimulated low/high frequency force ratio were equally affected by subsequent bouts of exercise. Newham et al's findings appear to contrast with the results of the present study which suggest an almost complete protection against force loss after exercise where the recovery period is not greater than 21 days after the first exercise bout. Nevertheless it should be pointed out that the maximum force losses measured in the experiments described in this Chapter (Fig 6.2), which were measured 3 days after exercise, are the result of delayed onset muscle damage, and believed to reflect the presence of fibre necrosis. The immediate loss of muscle force following exercise was not measured in this study so that the force measurements in the human and mouse studies are not

comparable.

The finding that exercised muscles in Groups B and C (10 and 21 days after the first exercise bout) showed forces which were consistently lower than those from non-exercised control muscles (Fig 6.2), despite the fact that no apparent fibre necrosis occurred would seem to contradict the previous statement. However, it must be borne in mind that the exercised muscles in these groups had probably not fully recovered from the initial bout of damage, since full force recovery does not occur until some 30 days after exercise of this type (see Chapter 5). When this factor is taken into account then the muscles from Groups B and C probably lost very little force 3 days after the second bout of exercise. It is an interesting observation that the 40/100 Hz ratios following the second bout of exercise in Group C showed no protection whereas fibre necrosis did. This discrepancy in the time course of protection between the different effects of exercise suggests that the causal mechanism for the protection may not be common to the different components of damage.

Armstrong et al (1983) suggested that the fibres which become necrotic as a result of eccentric exercise regimes represent a population which are more fragile or susceptible to stretch-induced damage. The authors postulate that stress-susceptible fibres would be destroyed by the initial bout of exercise to be replaced by fibres which were able to withstand the stress of further bouts of exercise. Although plausible, this hypothesis would seem unlikely in view of the fact that the fibre necrosis resulting from eccentric exercise does not involve the whole fibre, merely the degeneration of short fibre segments (Carpenter and Karpati, 1984). This focal necrosis does not result in the regeneration of new fibres *per se*, but the repair of damaged segments.

Newham et al (1987) suggested that the protective mechanism of eccentric exercise is more likely to be associated with changes in the

connective tissue of the parallel elastic elements. Thus, the effect of the initial bout of exercise might be to act as a stimulus for new collagen synthesis resulting in a strengthening of the connective tissue matrix of the parallel elastic component of muscle. Such a mechanism would serve to explain the finding that protection can be conferred by an initial episode of eccentric exercise which does not induce muscle damage (eg. the studies of Schwane and Armstrong (1983) and Clarkson and Tremblay (1987).

This study has demonstrated that when an eccentric exercise regime is repeated before the muscle has fully recovered (Groups B and C) there was a negligible effect on force loss or fibre necrosis. From a subjective point of view this would appear a surprising result since it would seem logical to assume that injuring a muscle before full recovery has occurred would exacerbate the damage. This does not seem to be the case in this model.

Since the human studies suggest that eccentric work leads to protection against further exercise-induced damage for at least 6 weeks post-exercise (Byrnes et al, 1985) it might be expected that this period would be somewhat reduced in the mouse, given its shorter life-span, greater metabolic rate, etc. Indeed, this does seem to be the case and in this context future studies in this area should be directed at examining the protective effect of exercise between 3 and 6 weeks after the initial bout of exercise. A comparison between the initial force losses after repeated exercise with those resulting from delayed-onset changes could also help to resolve the apparent differences between the findings of the animal and human studies.

6.4.2 Long term effects of exercise

A single episode of eccentric exercise in the TA muscles of mice leads to appreciable muscle damage and this seems to result in a fibre hypertrophy which is maintained for at least 12 weeks following exercise. The result was that exercised muscles were larger and stronger than their control counterparts. Darr and Schultz (1987) found that muscle satellite cells show a three-fold increase in activity following an acute bout of eccentric exercise in the mouse. Thus there must exist some stimulus to activate the repair processes once a fibre has been damaged. It is possible that some factor is released from fibres which have been exercised to such an extent that damage has occurred, activates satellite cell proliferation. Mitogenic factor have been isolated from damaged muscles by Bischoff (1986). He suggests that these mitogens are localised within the muscle fibres and are released following fibre damage, thus playing a role in the regenerative response.

A somewhat surprising finding in this study was that the hypertrophy associated with a bout of eccentric exercise was not only maintained 6 weeks after exercise (Group F) but would appear to continue for a further 6 weeks (Group G). Given the small numbers of animals involved in the experiment the differences between Groups G and F were not statistically significant. It is possible that the stresses associated with normal activity had a contributory effect on muscle growth after exercise. This would be analogous to the finding that, in animal studies, the compensatory hypertrophy associated with surgical ablation of agonist muscles is prevented if normal activity is reduced but enhanced if weight bearing activity is increased (Timson, 1990).

In the present study it was found that only those fibres with

internal nuclei 12 weeks after exercise showed significant degrees of hypertrophy. Internal nucleation is believed to be indicative of a previously damaged and regenerated fibre (Anderson et al, 1988) and if this is the case they would appear to remain for at least 12 weeks, since similar numbers were observed in both the 6 and 12 week recovery groups. The fact that, in exercised muscles, only fibres with internal nuclei showed significant enlargement after exercise provides a direct link between muscle fibre damage and hypertrophy.

There have been several studies which suggest that eccentric work may be particularly effective in inducing fibre growth. Wong and Booth (1990) showed that eccentric exercise of the rat tibialis anterior caused a greater elevation in muscle protein synthesis than concentric exercise and resulted in a greater increase in muscle mass. In man, the effects of a 7 week conditioning programme of the forearm flexors has been described by Komi and Buskirk (1972). After the end of the training period there was a significant increase in maximal isometric tension of the trained muscles. Exercise in which high tension is developed within the muscle (eg. eccentric exercise) seems to be the most important stimulus to fibre growth (Williams and Goldspink, 1971; Holly et al, 1980), although Rutherford and Jones (1986) were unable to demonstrate any difference in the strength gains of subjects whose quadriceps were trained with a concentric or eccentric exercise protocol.

The finding that a bout of eccentric exercise results in the development of long-lasting hypertrophy in normal mouse muscle lends support to the hypothesis put forward in section 5.4.5. Thus there is good evidence to support the idea that the muscle hypertrophy observed in dystrophin-deficient *mdx* mouse muscle results from the repeated episodes of fibre necrosis and regeneration which occur in these animals. In the studies described in Chapter 4, it was also shown that the specific

force (ie. force per cross-sectional area) of *mdx* muscle was significantly less than normal, a phenomenon which was explained by the degree of fibre-splitting which was also observed in *mdx* muscle. There is good evidence from animal studies of compensatory hypertrophy (Sieden, 1976; Kandarin and White, 1990) that the increase in muscle mass following ablation of a synergist muscle does not result in an equivalent force increment (ie. there is a reduction in specific force). Muscle cross-sectional area was not measured directly in the muscles from experimental Groups F and G, but assuming no change in muscle length after exercise, the cross-sectional area will increase in proportion to the increase in muscle mass. In this case the force/muscle mass did not change in either Group despite the fact that these muscles showed large force increments *and* appreciable numbers of fibres which had undergone longitudinal splitting. In this respect our results are not in agreement with what would be expected on the basis of findings in *mdx* muscle where the muscle hypertrophy was greater than the increase in muscle force. However, the long-term changes observed in normal muscle following a single bout of eccentric exercise are far less severe than those in normal *mdx* muscle where, at 24 weeks of age, centrally nucleated fibres are ubiquitous.

A single bout of damaging exercise leads to significant growth in muscle fibres in the mouse. To the authors' knowledge, no such dramatic effect of eccentric exercise-induced damage has been demonstrated in man, and whether this reflects species differences in the hypertrophic response or a lack of suitable training stimulus is a topic which requires further investigation and has obvious implications for the relevance of animal models to human muscle disease and hypertrophy.

CHAPTER 7

GENERAL DISCUSSION

The work described in this thesis began with the aim of identifying the causes of fatigue in different models and under differing experimental conditions. However, it soon became apparent that many of the changes seen during muscle fatigue could equally be described as resulting from muscle damage. This broadened the scope of the work to include the functional changes associated with exercise-induced damage to muscle. The opportunity was taken to study the functional characteristics of muscles associated with dystrophin-deficiency (in the form of the *mdx* mouse). This study, whilst initially a diversion from the main thrust of the work, provided the direction for the experiments described in Chapters 5 and 6 which are concerned with the effects of exercise-induced damage in the short and long term. This general discussion is intended to draw together the results from these apparently disparate studies.

7.1 THE SITE OF MUSCLE FATIGUE

The experiments described in Chapter 2 attempted to identify the site at which failure occurred in the process of muscle contraction leading to fatigue. The main innovation was the use of an isolated EDL muscle fibre bundle which approximated the advantages of using a single fibre preparation. One of the advantages of the EDL preparation was that it avoided the limitations of inadequate oxygen diffusion which is a problem during exercise in isolated whole muscle preparations. Furthermore such a model allowed a number of experiments to be performed in mammalian muscles which had hitherto been carried out successfully only in single fibre (ie. amphibian muscle) preparations.

The experiments in which fatigued muscles were activated with high concentrations of caffeine showed unequivocally that given sufficient stimulation (ie. release of Ca^{2+}) the cross-bridges are able to generate forces equivalent to those seen pre-fatigue. Thus we can say that the

fatigue induced by repetitive high frequency stimulation was a consequence of failure of muscle activation rather than an impairment of cross-bridge force generating potential. This finding agrees with those made in similar studies using isolated single amphibian fibre preparations (Nassar-Gentina et al, 1982; Westerblad and Lannergren, 1987).

There are two likely causes for the impaired muscle activation leading to the fatigue observed in the isolated EDL experiments. The first of these is ionic, involving the efflux of K^+ ions and K^+ accumulation within the t-tubules (as described in section 1.2.4), which itself may be caused by more than one event. Given the intermittent nature of the fatigue protocol used in the isolated EDL experiments it would seem unlikely that K^+ efflux during action potential propagation could contribute significantly to the build-up of extracellular K^+. However a second mechanism exists by which an increase in K^+ permeability of the sarcolemma could result from the activation of ATP-dependent K^+ channels (Spruce et al, 1987) during fatigue. Metabolite accumulation may also have played a part in the fatigue described in the EDL preparation since pH is known to affect the opening probability of the Ca^{2+}-release channels of the SR (Rousseau et al, 1988).

Further experiments would be required to separate the potential ionic and metabolic causes of activation failure during fatigue in the EDL preparation. Depolarisation of the fibre membrane using KCl contractures was used by Lannergren and Westerblad (1987) to demonstrate that membrane propagation failure occurred during fatigue resulting from continuous stimulation in amphibian single fibre preparations. An attempt was made to induce KCl contractures in the isolated EDL muscle preparation, however the contractures observed in fresh muscle were both small and variable (30 % of maximal tetanic force compared to 90 % recorded by Lannergren and Westerblad) to be of use. The reason for the

diminutive KCl contractures observed in the EDL preparation is not clear but may relate to the fact that KCl contractures are smaller in easily fatigued fibres (Lannergren and Westerblad, 1989).

Attempts were made to depolarise the muscle fibre membranes using tetani of prolonged pulse duration. This had a dramatic effect on force after fatigue but it is not possible to establish why this was so, since the effect of prolonged pulses is uncertain. From the slow relaxation rates of preparations stimulated with tetani of prolonged pulse duration (see Fig 2.6) it is suspected that such pulses may have a direct effect on the SR membrane.

In order to exclude the possibility that K^+ efflux occurred in the fatigued EDL preparation as a result of ATP depletion (via the ATP-dependent K^+ channels) it would be necessary to compare the degree of fatigue seen normally to that seen following blockade of ATP-dependent K^+ channels, this could be done by using a specific blocking agent such as glibenclamide (Castle and Haylett, 1987).

A consistent finding of the isolated EDL experiments was that recovery of force was never complete following a period of fatiguing exercise. The reason for this is uncertain since the preparation (if not fatigued) retained near maximal force for several hours and the effects of hypoxia can be discounted. The most probable conclusion is that fatigue resulted in some form of damage to the muscle fibres and this is confirmed by the finding that a preferential loss of force occurred at sub-maximal stimulation frequencies. The presence of low-frequency fatigue, and the fact that caffeine contractures produce forces greater than those elicited with maximal stimulation after recovery from fatigue, both point to some damage to the Ca^{2+}-release mechanism in these fibres.

The finding that fatigue was exacerbated in a medium deficient in Ca^{2+} but caffeine contractures were normal was unexpected, and it

suggests that external Ca^{2+} may play some role in the normal excitation-contraction coupling process (as discussed in section 2.4.3.2).

7.2 IDENTIFYING THE CAUSE OF FATIGUE

Chapter 3 described two sets of experiments which were devised with the purpose of distinguishing between the ionic and metabolic causes of fatigue. These experiments were based on work carried out by Fitch and McComas in 1985 who suggested that the reduced fatigue observed following exercise at short muscle lengths provided evidence that metabolic factors are important in the development of force loss. Their argument was based on two suppositions, that contractions at short muscle lengths involve a smaller number of cross-bridges than at optimal lengths, and that activation is not affected by muscle length. Both of these statements are debatable and have been dealt with in section 3.4.1.

The results of the human tibialis anterior experiments of Chapter 3 showed that when fatigue at short and optimal lengths were compared, there was a larger loss of force in the former which could be reversed by testing the muscle at an optimal length. This would suggest some form of activation failure during fatigue at short muscle lengths, possibly due to collapse of the t-tubules exacerbating the effect of K^+ accumulation. Indeed this mechanism was suggested by Taylor and Rudel (1972) to account for the decrease in muscle activation observed at short lengths. More direct evidence for an impairment of muscle activation at short length was provided by Blinks (1978) who demonstrated a reduced Ca^{2+} signal in isolated single frog fibres which had been shortened.

A better test for assessing the role of metabolite changes in the development of fatigue would be to compare the fatiguability of muscles at optimal and *long* lengths, where the reduced force can be attributed with confidence to fewer cross-bridge interactions. When TA muscles

were exercised at long lengths they showed force losses which were greater than normal, which was the opposite of what would have been expected if cross-bridge interaction (ie. ATP turnover) is a major factor causing fatigue. The human TA findings were, in this respect, confirmed by the results of experiments in the isolated mouse soleus experiments described in Chapter 3.

Further work needs to be carried out to define the extent to which metabolic factors contribute to the development of fatigue in the human TA and mouse soleus models described. This would require measurement of metabolite concentrations during exercise in the human TA (eg. by using phosphorus nuclear magnetic resonance spectroscopy), whilst in the isolated soleus, determination of the heat output of muscles during exercise would give an indication of the metabolic cost of exercise at each length.

A feature of fatigue in both the human TA and mouse soleus muscle experiments was a preferential loss of force at low frequencies of stimulation. This low frequency fatigue (LFF) was more pronounced and long lasting in muscles exercised at long lengths. The cause of LFF is not known although Newham et al (1983a), on the finding that LFF is more severe in muscles exercised eccentrically rather than with concentric contractions, suggested that this phenomenon was related to high forces generated within muscles. The findings described in Chapter 3 do not confirm those of Newham et al (1983a), since muscles exercised with isometric contractions at long lengths produced much lower forces than at optimal lengths, yet showed a greater degree of LFF. Thus LFF would appear to be unrelated to either the metabolic cost or the amount of force generated by the muscle. It would be interesting to follow the time course of changes in the tibialis anterior after fatigue at long lengths over a extended period, perhaps using a stimulation protocol of longer

duration, in order to investigate the development of muscle damage and recovery in this model.

7.3 FUNCTIONAL PROPERTIES OF DYSTROPHIC MUSCLE

Given the relatively recent discovery that the *mdx* mouse represents an animal model which is genetically homologous and has the same protein deficiency as human Duchenne muscular dystrophy (Hoffman et al, 1988), the lack of information about the functional properties of *mdx* muscle is understandable. Chapters 4 and 5 describe an investigation in which the contractile properties of *mdx* muscle from older animals (which had recovered from the major bout of muscle necrosis which occurs between 4 and 12 weeks in these mice) were compared with those of muscles from age-matched normal mice, and with normal mice whose muscles were damaged by eccentric exercise. The reason for this latter comparison was to try and distinguish between the effects on muscle function of dystrophin-deficiency rather than muscle damage.

Mdx TA muscles were larger and stronger than those from normal mice (although having a lower force/cross-sectional area) and expressed different contractile properties. Comparison of *mdx* muscles and normal muscles after exercise-induced damage showed that normal muscles, at 1 and 3 days after exercise-induced damage, showed a similar low/high frequency force ratio to that seen in *mdx* muscle (ie. there is low frequency fatigue). However, closer examination showed that this apparent similarity belied different mechanisms of causation. In damaged muscle LFF is a consequence of a reduced twitch amplitude whereas in *mdx* muscles the smaller force at low frequencies of stimulation results from a reduction in twitch duration (ie. a higher tetanic fusion frequency). The conclusion from this is that the altered contractile characteristics of *mdx* muscles are not the result of the presence of muscle damage or

regeneration and are presumably a consequence of dystrophin-deficiency. Alterations in the twitch response of dystrophin-deficient muscle suggests some abnormality in the activation system, possibly at the level of the SR. Whereas Ca^{2+} homeostasis is known to be altered in *mdx* muscle (Turner et al, 1988), it is not clear how this could lead to the changes in contractile properties described.

One of the most attractive current theories about the function of dystrophin is that it plays a role in maintaining the strength of the sarcolemma and that in it's absence muscle fibres become more susceptible to stress-induced damage. To this end the extent of force loss and fibre necrosis following a bout of eccentric exercise was compared in normal and *mdx* tibialis anterior muscles. In this the findings were unequivocal, dystrophin-deficient mouse muscles showed a normal susceptibility to damage and furthermore demonstrated a recovery of muscle function which was at least equivalent to normal muscles.

A number of caveats must be borne in mind when assessing these latter findings. Firstly, the susceptibility to damage was only assessed in older mice (ie. those that had already undergone a major bout of necrosis and regeneration) and it is possible that the regenerated fibres seen in normal *mdx* muscle were more resilient than their younger counterparts. However, the early age at which *mdx* mice start to develop severe necrosis (4 weeks) made the study of either contractile characteristics or susceptibility to damage technically difficult because of the size of the animal. It would clearly be useful to attempt to extend the study of exercise-induced damage to younger animals. Secondly, it may have been that the exercise protocol was too severe to demonstrate any subtle differences in membrane fragility between normal and *mdx* muscles. In this case it would be necessary to refine the exercise protocol by using a smaller number of contractions, or possibly isometric contractions in a

lengthened position, instead of eccentric workloads.

The interpretation of the findings that *mdx* and normal muscles show a similar susceptibility to exercise-induced damage must also be viewed in terms of the relevance to the human dystrophic condition. Although genetically and biochemically homologous to Duchenne muscular dystrophy, *mdx* mice have a very different clinical progression. Indeed, not only does *mdx* muscle fail to suffer from a progressive myopathy but appears to undergo active hypertrophy throughout its life. This fact casts doubt of the relevance of the mouse model to the human condition and perhaps even on the comparability of studies comparing the regenerative capacities of mice and men in general. Thus if, in the *mdx* mouse, fibre necrosis caused a small change in the balance between muscle protein breakdown and protein synthesis to favour the latter, repeated episodes of damage and regeneration could eventually lead to an increase in muscle bulk in excess of that needed merely to compensate for the tissue lost in necrosis. If this is the case then a reasonable supposition might be that, given a large enough stimulus, the same process could be observed in the muscles of normal mice. This forms the basis of the final set of experiments described in this thesis.

7.4 LONG-TERM EFFECTS OF DAMAGE

In the studies described in Chapter 6 it was found that exercise-induced damage in normal mouse muscles caused a number of changes, some of which are temporary, and some which were prolonged. More specifically, protection against the effects of force loss and fibre damage resulting from a second exercise bout was afforded 3 weeks after the initial exercise, but not after 6 weeks. Byrnes et al (1985) found that muscle pain and CK efflux was protected in the quadriceps muscles of individuals who performed a repeat downhill running exercise for up to 9

weeks after the first exercise, whilst Jones and Newham (1985) showed that a bout of eccentric exercise of the biceps protected the muscle from repeated damage for between 4 and 12 weeks. It might be expected that mouse muscles would show a shorter protection span, given their increased metabolic turnover, and this appears to be the case since protection lasted for between 3 and 6 weeks in the mouse TA muscle.

More interesting, perhaps, was the observation made concerning the effect of a single bout of exercise-induced damage on the force characteristics of mouse TA muscles after 6 and 12 weeks recovery without further interference. In both cases, the exercised muscles showed a significant increase in strength and fibre hypertrophy over the contralateral non-exercised muscles. This effect was even more striking in view of the fact that the hypertrophy at 12 weeks after exercise was greater than that at 6 weeks. This would suggest that, in the mouse at least, not only is there a hypertrophic stimulus which results from muscle damage, but that this stimulus seems to be prolonged. The inference of this is that exercise-induced fibre damage and regeneration results in a shift in the balance between protein synthesis and degradation in favour of the latter. Furthermore, this resetting of protein metabolism appears to be long-lasting, and results in a gradual but continuous hypertrophy in these muscles. Clearly it would be useful to know if this effect is permanent, or whether there is an eventual amelioration in the response. Further studies could also be directed towards examining the effects of activity post-exercise on the hypertrophic response, for example by allowing some animals to train using a treadmill whilst others were immobilised.

One observation regarding the long-term effects of exercise-induced damage in mice concerns the presence of central nuclei within fibres which had undergone damage and regeneration. The presence of fibres

with central nuclei has been commonly observed in dystrophic human muscles (Dubowitz, 1986) and transiently, following damaging eccentric exercise in normal subjects (D.A. Jones, personal communication), although the author is aware of no studies which have been made into the significance of these central nuclei. The prevalence of centrally nucleated fibres in *mdx* muscle, and in normal muscle fibres undergoing hypertrophy suggests that internal nuclei are active. It would be instructive in this respect to determine whether fibres with central nuclei from previously damaged muscles express different forms of myosin to normal fibre, since, if embryonic myosin were expressed exclusively in these cells, it would suggest that the central nuclei play a role in the maintenance of a raised protein synthesis.

The finding that muscle damage can initiate fibre hypertrophy in the mouse TA muscle, begs the question as to whether the same process can occur in humans and, to the authors' knowledge, there is no good evidence to suggest that muscle damage causes hypertrophy in man. However it would be fascinating to perform a long-term study of the effects of strength training in individuals, some of whom underwent repeated bouts of exercise-induced muscle damage in order to see whether muscle hypertrophy could be enhanced as a consequence of fibre necrosis and regeneration. A potential difference in the mechanism controlling protein synthesis in mouse and human muscle would not only explain the findings described in Chapter 6 but could also account for differences in the consequences of dystrophin-deficiency in mouse and man.

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