NEUROMUSCULAR TRANSMISSION IN MURINE MUSCULAR DYSTROPHY (129/ReJ STRAIN)

by Gareth Philip Morgan BSc.

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SUMMARY

Neuromuscular transmission in diaphragm and extensor digitorum longus (EDL) muscles from normal and dystrophic (129/ReJ dy/dy) mice was compared using electrophysiological and pharmacological techniques.

Indirectly evoked twitch responses of dystrophic EDL muscles were more resistant to curare than those of normal muscles whereas there was no difference in curare sensitivity between diaphragm muscles from normal and dystrophic mice.

Spontaneous and evoked transmitter release was investigated using conventional intracellular recording techniques. Endplate potential (e.p.p.) amplitude and quantum content (m) were greater than normal in the dystrophic EDL. In the dystrophic diaphragm e.p.p. amplitude was larger, but m did not differ from normal. In the cut-fibre EDL transmitter release followed a binomial distribution. The probability of quantal release (p) and the number of quanta available for release (n) were calculated using the binomial model. Probability of release was greater in the dystrophic EDL but n was similar in normal and dystrophic muscles.

The effect of various agents believed to increase free intraterminal calcium ion concentration ($[Ca^{2+}]i$) was investigated on miniature endplate potential frequency. The results from these experiments suggest that there is a difference in the way in which nerve terminals in normal and dystrophic diaphragm and EDL muscles regulate $[Ca^{2+}]i$ but that the ability to regulate $[Ca^{2+}]i$ is normal in dystrophic muscles. It is therefore unlikely that the increase in p in the dystrophic EDL could be attributed to raised $[Ca^{2+}]i$ but is more likely a consequence of a membrane defect at motor nerve terminals.

An attempt was made to measure transmembrane calcium fluxes in normal and dystrophic muscles. A greater influx and efflux of calcium was found in dystrophic diaphragm and EDL muscles than in normal muscles However, these differences could be attributed to a larger extracellular space in the dystrophic muscles.

Key words: muscular dystrophy; neuromuscular transmission; calcium.

DEDICATED TO MY MOTHER

AND MY SISTERS

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CHAPTER 1 : INTRODUCTION

The term muscular dystrophy encompasses a diverse group of diseases which are genetically determined myopathies of unknown origin characterised by progressive muscular weakness. There are a number of animals in which occur inherited myopathies similar to human muscular dystrophy. These include the mouse (Michelson, Russell and Harman, 1955), hamster (Homburger, Baker, Nixon and Wilgram, 1962), and mink (Hegreberg, Camacho and Gorham, 1974) and domestic animals such as the chicken (Asmundsen and Julian, 1956), turkey (Harper and Parker, 1964), sheep (McGavin and Baynes, 1969), horse (Roneus, 1982) and cow (Goedegebuure, Hartman and Hoebe, 1983). Dystrophy in non-primate mammals has been reviewed by Bradley and Fell (1981). The myopathy in chickens, hamsters and mice has been most extensively studied with murine dystrophy receiving the most attention.

Murine models of muscular dystrophy

Muscular dystrophy in the mouse was first observed in an inbred colony of 129/ReJ mice at the Jackson Memorial Laboratory, Bar Harbor, Maine (Michelson et al, 1955). Genetic studies showed that a mutation in an autosomal recessive gene (dy) located on chromosome 10 was responsible for the disease, (Stevens, Russell and Southard, 1957; Sidman, Cowen and Eicher, 1979). Clinically, 129/ReJ dy/dy mice can be identified at

approximately three weeks of age (Michelson et al, 1955; West, Meier and Hoag, 1966). The clinical signs of the disease have been described by several workers (Michelson et al, 1955; Baker, Tubis and Blahd, 1958) and include: dragging of the hindlimbs; extension of the hindlimbs at an abnormal angle from the body; flexion of the hindlimbs when suspended by the tail, and spasmodic nodding of the head. Afflicted mice cannot breed and have a reduced lifespan.

The dy mutation has been transferred to an inbred strain of the C57BL background, the C57BL/6J (Loosli, Russell, Silvers and Southard, 1961). These animals show similar clinical signs to those of 129/ReJ dy/dy mice except that they are more active and have a longer lifespan (Loosli et al, 1961; Meier, West and Hoag, 1965).

The F_1 offspring of a cross between 129/ReJ dy/dy and C57BL/6J dy/dy mice, denoted 129B6F_1, are healthier, have body weights closer to those of controls and survive much longer than either of the parent strains (Russell, Silvers, Loosli, Wolfe and Southard, 1962).

A second form of murine muscular dystrophy has been reported in the Jackson Laboratory WK/ReJ strain of mice, which was in itially bred for a gene expressing anaemia (Meier and Southard, 1970). Breeding tests showed that this mutation was an allele at the dy locus and so it was designated the symbol dy^{2J} in accordance with the standard

genetic nomenclature for mice (Committee on Standardised Genetic Nomenclature for Mice, 1963). Clinically WK/ReJ dy^{2J} /dy^{2J} mice are less severely affected than 129/ReJ, C57BL/6J or 129B6F_1 dy/dy mice (MacPike and Meier, 1976). Afflicted males and females are able to mate normally which allows study of tissues from the stages preceding clinical signs and symptoms (Meier and Southard, 1970; Gilbert, Steinberg and Banker, 1973).

Like the dy mutation the dy^{2J} gene has also been transferred onto the C57BL/6J background (MacPike and Meier, 1976). However, unlike dy, the disease progresses more rapidly and the symptoms are more severe on this background than in the original WK/ReJ strain of mice (MacPike and Meier, 1976).

A third form of murine muscular dystrophy, myodystrophy (myd), has also been reported (Lane, Beamier and Myers, 1976). An inbred line MYD/Le-myd, has been established at the Jackson Laboratories. The myd mutation is a single autosomal recessive mutation which occurs on chromosome 8 and is not an allele at the dy locus. Affected mice can be identified at about two weeks of age. They are smaller in size and have a short shuffling gait (myd mice never drag their hind limbs as do dy mice). Their lifespan is reduced from 11 months or more to about 17 weeks.

Recently another form of murine muscular dystrophy has been

described in an inbred colony of C57BL/10 mice (Bulfield, Siller, Wight and Moore, 1984). The mdx mutation is located on the X chromosome. Affected mice are vigorous and fertile and display only mild clinical symptoms of dystrophy. Of the mice examined only one 12 month old mutant mouse showed any evidence of clinical signs of the disease in the form of muscular tremors and mild incoordination although histological lesions could be detected in 3 week old mice.

Most workers in murine dystrophy have used either C57BL/6J dy^{2J}/dy^{2J} or 129/ReJ dy/dy mice. In the present study dystrophic mice of the original 129/ReJ strain, were used. However, wnere applicable, reference will be made to results obtained in other dystrophic mice.

Histopathology of dystrophy in 129/ReJ mice

The histopathology of skeletal muscle in 129/ReJ dy/dy mice has been extensively studied. One of the most conspicuous features is a large variation of fibre size, with very small and very large fibres randomly distributed among more normal-sized ones (Harman, Tassoni, Curtis and Hollinshead, 1963; Banker, 1967). It has been reported that there is a decrease in the mean fibre diameter (Rowe and Goldspink, 1969), an increase in the mean fibre diameter (Law and Atwood, 1972; Sellin and Sperelakis, 1978) and that it is similar to normal (Kerr and Sperelakis 1983a). There is a

decrease in the total fibre cross-sectional area which is the result of a reduced fibre number (Goldspink and Rowe, 1968; Rowe and Goldspink, 1969). There is some evidence of limited regenerative activity in dystrophic mouse muscles (Summers and Parsons, 1978). Rowe and Goldspink (1969) proposed that the very small fibres observed may be either atrophying or regenerating, while the very large fibres may be hypertrophied to compensate for the reduced number. Splitting of muscle fibres is common and affected fibres contain increased numbers of nuclei which are more centrally located than those of normal fibres and often appear in rows (Banker, 1967; Isaacs, Bradley and Henderson, 1973). The decreased muscle mass is accompanied by a relative and absolute increase in the endomysial connective tissue and by fatty infiltration (Michelson et al, 1955; Pearce and Walton, 1963). There is both increased fat around fibres and an increased number of intracellular lipid deposits (Makita, Kiwaki and Sandborn, 1973; Wechsler, 1966).

Ultrastructural examination of endplates in muscles of 129/ReJ dy/dy mice has revealed several pre- and post-synaptic changes. Presynaptically there is a decrease in the number and diameter of synaptic vesicles (Ragab, 1971; Pachter, Davidowitz and Breinin, 1973; Banker, Hirst, Chester and Fok, 1979) although one report suggests that motor nerve terminals in dystrophic muscles contain increased numbers of synaptic vesicles (Ellisman, Rash and

Westfall, 1975). There is also nerve terminal retraction with interposition of Schwann cell cytoplasm or basement membrane proliferation (Banker et al, 1979). Postsynaptically a reduction in the number and complexity of synaptic folds has been found (Ragab, 1971; Banker et al, 1979; Ellisman et al, 1975; Ellisman, 1981).

In 129/ReJ dy/dy mice not all of the muscles are affected to an equal extent. It has been found that fast twitch muscles are more severely affected than slow twitch muscles (Shafiq, Goryki and Milhorat, 1969; Goldspink and Rowe, 1968). However, there is controversy over whether glycolytic or oxidative fast twitch fibres are most affected (Cosmos, Butler and Milhorat, 1973; Butler and Cosmos, 1977: Fahami and Roy, 1966; Susheela, Hudgson and Walton, 1968, 1969). Therefore, in any comparison between normal and dystrophic muscles the result obtained will largely be influenced by the muscles chosen.

There is also considerable variation in the degree to which the disease affects any one animal (West and Murphy, 1960; Platzer, 1979). In even the most severely affected muscles there exists a mixed population of degenerating, regenerating and apparently normal fibres (Ross, Pappas and Harman, 1960; Platzer and Chase, 1964). It is this heterogeneity of skeletal muscle in dystrophic mice which makes interpretation of experimental results difficult.

Histopathologically, skeletal muscles from C57BL/6J dy^{2J}/dy^{2J} mice are similar to those from 129/ReJ dy/dy mice. Fibre necrosis, atypical fibre diameter, central nucleation, increased connective tissue and fibre splitting are all found. (Pachter, Davidowitz, Eberstein and Breinin, 1974; Dribin and Simpson, 1977). Abnormalities at the neuromuscular junction such as a reduction in number and distortion in shape of synaptic vesicles, axonal retraction and a simplification of postsynaptic folds also occur in C57BL/6J dy^{2J}/dy^{2J} mice (Davidowitz, Pachter, Philips and Breinin, 1976; Law, Saito and Fleischer, 1983). Pachter, Davidowitz and Breinin (1976) reported atypical mitochondrial stacking and distorted mitochondria in terminal axons of skeletal muscles of C57BL/6J dy^{2J}/dy^{2J} mice which have not been reported in 129/ReJ dy/dy mice. There is also a difference between C57BL/6J dy^{2J}/dy^{2J} and 129/ReJ dy/dy mice in that slow twitch muscles are primarily affected in the former (Parry and Parslow, 1981).

The muscle pathology of myd/myd and mdx/mdx mice is also similar to that of 129/ReJ dy/dy mice (Lane et al, 1976; Bulfield et al, 1984). However, all skeletal muscles are affected equally in myd/myd mice and the muscles do not show the fatty and connective tissue infiltration observed in the skeletal muscles of 129/ReJ dy/dy mice (Lane et al, 1976).

Comparison of murine and Duchenne dystrophy

It has been suggested that pathologically there is a close similarity between dystrophy in 129/ReJ dy/dy mice and human Duchenne dystrophy (Ross et al, 1960). Histopathological studies of skeletal muscle have shown that the variation in fibre size, central nucleation, fibre splitting, necrosis, fibre regeneration and increase in connective tissue and fat observed in murine dystrophy (Bray and Banker, 1970; Isaacs et al, 1973) also occur in Duchenne dystrophy (Bell and Conen, 1968). Raised serum levels of the muscle enzyme creatine phosphokinase, a diagnostic feature of Duchenne dystrophy (Bradley, Hudgson, Larson, Papapetropoulos and Jenkison, 1972) are also found in murine dystrophy (Stamp and Lesker, 1967; Watts and Watts, 1980). In both forms of dystrophy there is also depolarisation of skeletal muscle fibres (Kleeman, Partridge and Glaser, 1961; Harris, 1971; Sakakibara, Engel and Lambert,1977), and raised intrafibre concentrations of calcium (Dowben, Zuckerman, Gordon and Sniderman, 1964; Bodensteiner and Engel, 1978; Emery and Burt, 1980). These and other similarities have resulted in an extensive use of the murine myopathy as a model for Duchenne dystrophy.

There are, however, a number of qualitative differences between the two diseases. In murine dystrophy there are structural and functional changes in the central

(Frostholm, Bennett and Lynch, 1983; Biscoe, Caddy, Pallot, Pehrson and Stirling, 1974) and peripheral nervous systems (Bradley and Jaros, 1979; Kuno, 1979; Brimijoin and Schreiber, 1982). However, there is no definitive evidence for changes in the central or peripheral nervous system in Duchenne dystrophy (Dubowitz, 1979). In addition, Duchenne dystrophy is a sex-linked disease whereas dystrophy in 129/ReJ mice has an autosomal recessive inheritance (Michelson et al, 1955). Although direct comparisons of dystrophy in man and mouse should not be made, studying murine dystrophy is useful in elucidating the basic mechanisms of muscle pathology. For a fuller discussion of the relevance of animal models to human dystrophy see Harris and Slater (1980).

Hypotheses of the aetiology of muscular dystrophy

The three major hypotheses that have been proposed to explain the pathogenesis of the muscular dystrophies are the vascular, neural and membrane hypotheses.

The vascular hypothesis was proposed in 1961 by Demos who suggested that an abnormality in skeletal muscle blood flow leads to local ischaemia causing muscle necrosis. Fibre necrosis can be produced in animals in which muscle blood flow has been reduced (Mendell, Engel and Derner, 1971). However, autoradiographic studies of intramuscular blood flow in 129/ReJ mice have revealed no abnormal areas of

ischaemia in dystrophic skeletal muscle (Bradley, O'Brien, Walder, Murchison, Johnson and Newell, 1975).

It has been proposed that muscular dystrophy may be a neurogenic disorder (McComas, Sica and Campbell, 1971). Experiments designed to investigate an abnormal neuronal influence on muscle in the aetiology of dystrophy have involved the creation of genetically mosaic mice (Peterson, 1979) and the innervation of muscles by motorneurons of different genotype. These innervation experiments have been done using tissue culture (Bryers and Ecob, 1984), parabiosis with reinnervation (Saito, Law and Fleischer, 1983) and muscle transplantation (Cosmos, Butler, Mazliah and Allard, 1980). Despite intensive research, these experiments have yielded conflicting results as to whether the initial lesion is of neural origin.

The membrane hypothesis proposes that membrane lesions are the primary lesion in the disease process. The three major muscle membrane systems, the sarcolemma, the sarcoplasmic reticulum and mitochondria, have been studied electron microscopically at the prenatal, early postnatal, preclinical and clinical stages. There have been reports of histological changes in dystrophic muscle in all three membrane systems. Platzer and Chase (1964) reported that abnormal fibres lacked an intact sarcolemma and proposed that the initial defect may involve this structure. However, it has also been claimed that the sarcolemma is

one of the last structures to be affected in dystrophy (Ross et al, 1960). A marked alteration of both the mitochondria and sarcoplasmic reticulum has been reported in dystrophic muscle (Ross et al, 1960; Banker, 1967) and in studies of prenatal muscle, Platzer (1979) found that the earliest identifiable structural change was in the sarcoplasmic reticulum which became dilated.

In murine dystrophy there is leakage of muscle enzymes such as creatine phosphokinase and aldolase into the serum (Stamp and Lesker, 1967; Watts and Watts, 1980; Zierler, 1958). Dystrophic muscles also take up horseradish peroxidase (Mendell, Higgins, Sahenk and Cosmos, 1979) and procion yellow (Bradley and Fulthorpe, 1978). There are reduced intrafibre concentrations of potassium and raised concentrations of sodium and calcium in dystrophic muscles (Hoh and Salafsky, 1972; Dowben et al, 1964). Studies on lipid composition of the sarcolemma have shown a greater lipid content in dystrophic mice (De Kretser and Livett, 1977). The activity of sarcolemmal enzymes such as Ca^{2+}-ATPase, Mg^{2+}-ATPase and Na^{+}- ATPase is also different in muscles of 129/ReJ dy/dy mice (Boegman, 1974). All these observations indicate that a sarcolemmal defect does exist in dystrophy. Kerr and Sperelakis (1983a) reported that in muscles from dystrophic animals displaying normal ultrastructure there was a decrease in specific membrane resistance, a lower membrane potential and a reduction in the maximum rate of rise of the action potential. From

these results they suggest that a defect in the sarcolemma is a primary event in the degeneration of dystrophic fibres.

Abnormalities in mitochondrial functions have also been reported in skeletal muscle of 129/ReJ dy/dy mice, particularly in those involving fatty acid metabolism (Martens and Lee, 1980). It has been shown that the coenzyme A content of mitochondria from dystrophic skeletal muscle is reduced by 60% (Jato-Rodriguez, Lin, Hudson and Strickland, 1972). Addition of coenzyme A to isolated mitochondria produced a 16% stimulation of acetylcarnitine oxidation by mitochondria from dystrophic muscle but a 6% inhibition of oxidation by mitochondria from normal muscle (Jato-Rodriguez, Liang, Lin, Hudson and Strickland, 1975). Since the mitochondrial membrane is normally impermeable to coenzyme A, this suggests that mitochondria from dystrophic muscle have an abnormal permeability. This is also suggested by the fact that mitochondria from dystrophic muscle have a raised calcium content (Wrogemann, Hayward and Blanchaer, 1979; Nylen and Wrogemann, 1983).

Decreased uptake of calcium by fragmented sarcoplasmic reticulum has been observed in muscles from 129/ReJ dy/dy mice (Sreter, Ikemoto and Gergely, 1967; Martonosi, 1968) though it has been reported to be normal in muscles from $129B6F_1$ dy/dy mice (Mrak and Fleischer, 1982). Calcium stimulated ATPase activity is normal although total ATPase

activity is elevated (Neymark, Kopacz and Lee, 1980; Martonosi, 1968; Sreter et al, 1967). Dux and Martonosi (1983) found that the ATPase responsible for calcium transport in sarcoplasmic reticulum from 129/ReJ dy/dy mice formed similar crystalline arrays to that found in normal sarcoplasmic reticulum. They interpreted this finding as lessening the likelihood of a molecular defect of the calcium pump. Recently it has been reported that sarcoplasmic reticulum vesicles from muscles of normal and dystrophic 129B6F_1 mice have similar calcium release characteristics under a variety of experimental conditions (Volpe, Mrak, Costello and Fleischer, 1984). Therefore, at the present time, it is not clear whether or not the function of the sarcoplasmic reticulum in dystrophic muscle is impaired.

Thus, in skeletal muscle of dystrophic mice there is evidence that sarcolemmal and mitochondrial function is impaired and that intrafibre and mitochondrial calcium levels are elevated. Wrogemann and Pena (1976) and Duncan (1978) have proposed essentially similar theories for the aetiology of muscular dystrophy, that a sarcolemmal defect results in an increased net influx of calcium into muscle fibres. The two theories differ in that Wrogemann and Pena (1976) believe that elevated myoplasmic calcium levels and ultimately cell necrosis are produced via mitochondrial calcium overloading whereas Duncan (1978) implicates the sarcoplasmic reticulum.

The role of calcium in muscle necrosis

Muscle damage, similar to that observed in dystrophy, can be produced in mammalian muscle by a number of treatments believed to raise the intrafibre calcium concentration. These include the use of the calcium ionophore A23187 (Publicover, Duncan and Smith, 1977, 1978; Kameyama and Etlinger, 1979) and the use of caffeine in combination with either electrical stimulation or elevated extracellular potassium concentration (Duncan and Smith, 1980; Duncan, Greenaway, Publicover, Rudge and Smith, 1980).

Calcium activated neutral proteases have been found in mammalian skeletal muscle (Busch, Stromer, Goll and Suzuki, 1972; Mellgren, Repett, Mack and Easly, 1982) and elevated calcium protease activity has been reported in muscles of 129/ReJ dy/dy mice (Neerunjun and Dubowitz, 1979). There is also elevated activity of virtually all of the lysosomal acid hydrolases in murine dystrophy (Weinstock and Iodice, 1969; Libelius, Jirmanová, Lundquist and Thesleff, 1978). Therefore, it is likely that the fibre necrosis in dystrophy is caused by the elevated activity of acidic and neutral proteases. It has been shown that treatment with the protease inhibitor, pepstatin, begun at 3 weeks of age, produces some beneficial effect in dystrophic mice (Schorr, Arnason, Astrom and Darzynkiewicz, 1978).

Extent of the membrane defect

Membrane defects are not restricted to skeletal muscle in murine dystrophy. Surface structural irregularities and a reduced number of plasma membrane protein particles have been found in erythrocytes from 129/ReJ dy/dy mice (Morse and Howland, 1973; Shivers and Atkinson, 1978; DiStefano and Bosmann, 1977; Akindele, Anyaibe and Headings, 1982). The activity of the membrane bound enzyme acetylcholinesterase is reduced to half the normal value in erythrocytes from dystrophic mice (Das, Watts and Watts, 1971).

There are also changes in the membrane permeability of liver mitochondria from dystrophic mice to anions, protons (Howland and Challberg, 1973) and potassium (Howland, 1974). Mitochondria isolated from the liver and brain of dystrophic mice have both an increased rate of calcium accumulation and an elevated calcium content compared to those from normal mice (Katyare, Challberg and Howland, 1978; Frostholm, Baudry and Bennett, 1981) and mitochondria from the hearts of dystrophic mice have an elevated calcium content (Wrogemann et al, 1979; Nylen and Wrogemann, 1983).

There is thus considerable evidence of a widespread membrane defect in murine dystrophy, which has its most deleterious expression in skeletal muscle, resulting in

raised cellular and mitochondrial calcium levels.

The role of calcium in neuromuscular transmission

At the neuromuscular junction calcium ions have a crucial role in evoked transmitter release. In the absence of extracellular calcium, transmission is inhibited (Katz and Miledi, 1967a). According to the calcium hypothesis, nerve terminal depolarisation opens voltage dependent calcium channels in the membrane and calcium ions then enter the terminal down their concentration gradient (Katz and Miledi, 1967b). The extracellular calcium concentration is of the order of 10^4 times greater than the intracellular one (Rasmussen and Goodman, 1977). Ginsburg and Rahamimoff (1983) reported that the calcium concentration in the synaptic cleft may be 1.5 to 2-fold higher than that in the bulk extracellular medium. The result of the calcium influx is a rise in the free intraterminal calcium ion concentration ($[Ca^{2+}]i$) which triggers release of the transmitter acetylcholine (ACh) by an unknown mechanism (Llinás and Nicholson, 1975). This action is terminated when $[Ca^{2+}]i$ is returned to its resting level by sequestration by intracellular organelles and ejection from the nerve terminal.

According to the quantal hypothesis transmitter is released from motor nerve terminals in the form of multimolecular packets or quanta (del Castillo and Katz, 1954a). The

vesicle hypothesis proposes that the synaptic vesicles which are abundant in the motor nerve terminal represent the structural counterpart of the quanta of ACh (del Castillo and Katz, 1955). Acetylcholine released from the motor nerve terminal diffuses across the synaptic cleft and combines with receptors on the postsynaptic membrane producing depolarisation. The simultaneous release of a number of quanta gives rise to the endplate potential (e.p.p.). If the amplitude of the e.p.p. is sufficient to depolarise the sarcolemma to the threshold level an action potential is initiated. Transmitter action is terminated by breakdown by the enzyme acetylcholinesterase present in the synaptic cleft and also by diffusion from the cleft. As well as the simultaneous release of a number of quanta after an action potential in the nerve terminal individual quanta are also released spontaneously. This spontaneous release of transmitter can be detected postsynaptically as the miniature endplate potential (m.e.p.p.). It has been proposed that spontaneous transmitter release reflects $[Ca^{2+}]i$ (Baker, 1972; Duncan and Statham, 1977). Miledi (1973) has shown in squid giant synapses that intracellular iontophoresis of calcium into presynaptic terminals accelerates spontaneous release. A variety of methods believed to raise $[Ca^{2+}]i$ have been shown to have a similar effect on motor nerve terminals (Hubbard, Jones and Landau, 1968; Miledi and Thies, 1971; Statham and Duncan, 1975, 1976; Shalton and Wareham, 1979). Both spontaneous and evoked transmitter release are therefore dependent on calcium.

Transmitter release in murine dystrophy

In this introduction evidence has been presented that in murine dystrophy the raised calcium levels in skeletal muscle are possibly a consequence of a generalised membrane defect. Mitochondrial calcium levels are elevated in a number of dystrophic tissues and these organelles play an important role in regulating $[Ca^{2+}]i$ in the motor nerve terminal (Alnaes and Rahamimoff, 1975; Akerman and Nicholls, 1981a). Therefore, there may be an impaired ability to control free calcium levels presynaptically in 129/ReJ dy/dy mice as has been reported for C57BL/6J dy^{2J} /dy^{2J} mice (Shalton and Wareham, 1980).

Evoked transmitter release is determined by the sum of the calcium which enters the nerve terminal during an action potential and $[Ca^{2+}]i$ (Baker, 1972). Thus, if there is an inability to regulate $[Ca^{2+}]i$ or if the presynaptic membrane is abnormally permeable to calcium in dystrophy, it might be expected that this would affect evoked transmitter release. The morphological changes observed at the neuromuscular junction in murine dystrophy may also affect transmitter release, (see "Histopathology of dystrophy in 129/ReJ dy/dy mice").

There have been a number of studies on evoked release in murine dystrophy, none of which have reported a significant

difference between normal and dystrophic muscles (Carbonetto, 1977; Harris and Ribchester, 1978, 1979a, 1979b, 1979c). However, the methods used to measure evoked release should be considered carefully. The method Harris and Ribchester employed to calculate quantum content in curarised and cut- fibre preparations was based on an assumption of Poisson release, yet a number of studies have shown that transmitter release at non depressed junctions obeys binomial statistics (Miyamoto, 1975; Glavinović, 1979a). There is also controversy over whether or not curare has a presynaptic effect (Auerbach and Betz, 1971; Glavinović, 1979b; Wilson, 1982). Carbonetto (1977) studied evoked transmitter release in the magnesium blocked preparation (del Castillo and Katz, 1954a). Magnesium block has been shown to alter some characteristics of transmitter release during repetitive stimulation (Elmqvist and Quastel, 1965). Raising magnesium also has a postsynaptic effect which reduces m.e.p.p. amplitude by up to 60% (del Castillo and Katz, 1954b; Liley, 1956b). Furthermore, Carbonetto's experiments were conducted in the presence of the anticholinesterase neostigmine and anticholinesterases have been reported to increase (Blaber, 1972; Bois, Hummel, Wolf, Dettbarn and Laskowski, 1980) or decrease (Wilson, 1982) quantum content. Any effect of anticholinesterases on quantum content may be particularly important in dystrophic muscles which are abnormally sensitive to their action (Baker and Sabawala, 1963; Baker, 1963). Thus it is possible that the methods employed in

these investigations may have obscured any difference in transmitter release between normal and dystrophic muscles.

Effects of curare and anticholinesterases in murine muscular dystrophy

In 1960, Baker, Wilson, Oldendorf and Blahd reported that injection of (+)-tubocurarine (curare) was fatal to 7 out of 9 normal mice but to only 2 of 9 dystrophic mice. They also found that injection of neostigmine induced or increased tremors in 16 of 18 dystrophic mice but induced tremors in only 1 of 27 normal mice. Later work, in vitro, showed that indirectly evoked twitch reponses of hemidiaphragm and (to a greater extent) peroneus longus muscles from dystrophic mice were more resistant to curare than those of normal muscles (Baker and Sabawala, 1963). It was also found that spontaneous twitching could be induced in peroneus longus muscles from dystrophic but not normal mice by several anticholinesterases (Baker, 1963; Baker and Sabawala, 1963). Beaulnes, Bois and Carle (1966) have also reported that twitch responses in diaphragm muscles from dystrophic mice are more resistant to curare and more sensitive to anticholinesterases than normal mice and, that these differences exist at a time when less than 3% of the fibres are histologically abnormal. However, Harris and Ribchester (1979c) were not able to demonstrate any difference in the effects of curare on indirectly evoked twitch responses of hemidiaphragms from normal and

dystrophic mice.

Aims of the present investigation and experimental approach.

The first aim of the present study was to confirm whether diaphragm and/or EDL muscles from dystrophic mice were less sensitive to neuromuscular block induced by (+)-tubocurarine than muscles from clinically normal littermates. Curare sensitivity of EDL and diaphragm muscles was investigated as it was thought that the development of curare resistance might occur first in EDL muscles and later in diaphragm muscles, following the progression of murine dystrophy (Ross et al, 1960).

The second aim was to investigate the origin of this resistance to curare. Curare resistance could be explained by an increase in the number of acetylcholine receptors on the postsynaptic membrane or by a decrease in the activity of the enzyme, acetylcholinesterase. Alternatively, an increased output of transmitter at dystrophic junctions caused by an increase in quantum content or quantum size would explain the resistance. Of these, transmitter release was investigated as release is known to be dependent upon the concentration of intraterminal free calcium in the motor nerve and changes in the sensitivity of motor nerve terminals to agents which raise intraterminal free calcium have been reported in murine dystrophy (Shalton and Wareham, 1980). The finding that quantum content of

e.p.ps. was increased at neuromuscular junctions of dystrophic muscles led to further experiments aimed at elucidating the mechanism(s) responsible for this increase. These experiments showed that the number of quanta available for release was normal at junctions in dystrophic muscle but that the probability of transmitter release was increased. The probability of release of transmitter is a function of the concentration of intraterminal free calcium in motor nerves which is dependent upon the influx of calcium across the motor nerve terminal during an action potential and removal of calcium by intraterminal stores and by ejection from the nerve terminal. The ability of intraterminal stores in dystrophic muscles to sequester calcium was investigated by recording m.e.p.p. frequency from muscles which had been treated with uncouplers of oxidative phosphorylation (mitochondrial inhibitors) or caffeine or lanthanum, which release calcium from sites other than mitochondria (Batra, 1974; Heuser and Miledi, 1971).

Calcium fluxes cannot be measured across motor nerve terminals but an attempt was made to assess the lability of excitable cell membranes to calcium in dystrophic muscles by measuring fluxes of ^{45}Ca across the sarcolemmal membrane. Muscular dystrophy has its most deleterious expression in skeletal muscles in which there are defects in calcium regulation. Therefore, it was thought that if there were no differences in calcium fluxes between normal

and dystrophic muscles there were unlikely to be differences in calcium fluxes across the nerve terminal membranes.

CHAPTER 2 : MATERIALS AND METHODS

Experimental Animals

Mice of the Bar Harbor 129 ReJ Strain (dy/dy) were bred from pairs originally obtained from the Muscular Dystrophy Group Research Laboratories, Newcastle upon Tyne General Hospital, Newcastle upon Tyne. Male white mice aged six months were supplied by Bantin and Kingman Limited. Dystrophic mice could be identified from about three weeks of age by their characteristic posture (fig.1) and dragging of their hindlimbs. They were also lighter in weight and smaller in size than non-dystrophic litter mates of the same age. At eight weeks of age the weight of normal littermates was 25.62 ± 2.06 g (mean ± 1 S.D., n= 17) whereas the weight of dystrophic animals was 17.21 ± 2.46 g (n = 14).

After weaning, dystrophic mice were placed in a cage with one or two clinically normal littermates as this was found to increase their chances of survival. The remaining normal littermates were used for breeding. Both dystrophic mice and normal littermates were given food pellets and water ad libitum. Food pellets were ground and placed on the floor of the cages containing dystrophic mice to make them more accessible to the animals. Water bottles were fitted with long metal tubes to allow easy access by severely dystrophic animals.

Fig. 1. An eight week old male dystrophic mouse.

Throughout this thesis a dystrophic muscle refers to a muscle from a dystrophic mouse and a dystrophic muscle fibre refers to a muscle fibre from a dystrophic muscle which is abnormal.

No distinction was made between heterozygous and homozygous normal littermates. In all experiments, muscles from male and female dystrophic mice and clinically normal littermates aged eight to twenty weeks were used. Because of the effect of age on many of the parameters measured, such as miniature endplate potential (m.e.p.p.) amplitude and endplate potential (e.p.p.) quantum content (Kelly, 1978), in each series of experiments normal and dystrophic mice were matched for age and sex.

Choice of Muscles

In the 129 ReJ strain differences in morphology and physiology suggest that fast twitch muscles are affected in preference to slow twitch muscles (see Introduction). All experiments were therefore performed on either the fast twitch hindlimb extensor digitorum longus (EDL) muscle (Close, 1972) or the diaphragm. In the mouse the diaphragm is essentially a fast twitch muscle (Noireaud and Léoty, 1984; Davies and Gunn, 1972).

The hindlimb muscles are the first muscles affected by the disease (Michelson et al, 1955). In murine dystrophy the course of atrophy proceeds from the hindlimbs forwards and consequently the diaphragm remains relatively unaffected by the disease process until the later stages (Ross et al, 1960). Both diaphragm and EDL muscles from dystrophic mice

were lighter than those from normal animals, e.g. in mice aged eight weeks the normal EDL was 6.92 ± 0.79 mg (n = 10) and the dystrophic EDL 4.41 ± 0.79 mg (n = 8) whilst the normal hemidiaphragm was 16.41 ± 1.07 mg (n = 7) and the dystrophic hemidiaphragm was 11.53 ± 0.97 mg (n = 6).

Composition of physiological salines

The composition of physiological salines is shown in table 1. The physiological saline used for the majority of experiments was similar to that described by Liley (1956a). However, the dextrose content was raised to 25 mM (Krnjević and Miledi, 1958). This saline will be referred to as Liley's solution. When gassed with 95% O_2, 5% CO_2 Liley's solution had a pH of 7.2 - 7.3.

The phosphate and carbonate salts of lanthanum are insoluble. Therefore, salines which contained lanthanum chloride were buffered with N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) rather than $NaHCO_3$. Sodium hydrogen phosphate was excluded from these solutions and they were gassed with 100% O_2 instead of 95% O_2, 5% CO_2. These solutions will be referred to as lanthanum saline. The pH of lanthanum saline was adjusted to 7.3 with molar NaOH.

Physiological salines were made on the day of each experiment. For experiments where Ca^{2+}, Mg^{2+} or K^+ ion

TABLE 1 Composition of physiological salines. Concentrations shown are m.moles litre^{-1}

	Liley's Solution	Lanthanum Saline
KCl	5	5
NaCl	137	137
$CaCl_2$	2	2
$MgCl_2$	1	1
$NaHCO_3$	12	-
NaH_2PO_4	1	-
dextrose	25	25
HEPES	-	5

All inorganic salts used were Analar grade and obtained from BDH Chemicals Limited

concentration was varied no other change was made to the composition of the solution. Whenever possible, the same drug solutions were applied to both normal and dystrophic muscles to eliminate the possibility of different responses resulting from variations in drug concentration. None of the drugs used had any appreciable effect on the pH of the salines.

Drugs and chemicals

Drug or reagent	Source
Guanidine hydrochloride	Sigma Chemical Co. Ltd.
D-Tubocurarine chloride	Koch-Light Laboratories Ltd.
Lanthanum chloride	Fisons Scientific Apparatus
2,4-Dinitrophenol	Aldrich Chemical Co. Ltd.
Caffeine	Sigma Chemical Co. Ltd.
HEPES	Sigma Chemical Co. Ltd.
Diethyl ether	BDH Chemicals Ltd.

In the radiochemical experiments muscles were digested with the tissue solubiliser protosol which was obtained from New England Nuclear. The scintillant used in all radioisotope measurements was Dimilume-30 supplied by Packard Instrument Co. Inc. Radioisotopes were obtained from the Radiochemical Centre, Amersham. ^{45}Ca was purchased as the chloride, specific activity 10-40 mCi/mg calcium. ^{3}H inulin had a specific activity of 465μCi/mg.

Muscle preparations

Diaphragm preparations

Mice were anaesthetised with ether, decapitated and pinned to a cork board. The skin and muscle overlying the thorax and abdominal viscera were cut away and an area of the ribcage removed to expose the phrenic nerves. Each nerve was ligatured with a fine thread near to its entrance into the thorax and cut close to the thymus. The thread was used to exert light traction on the nerve thereby facilitating its separation from surrounding tissues along its length. The xiphisternum was lifted with a pair of forceps and the diaphragm muscle separated from the gall bladder. The ribs were cut through close to the diaphragm and round towards the spinal cord and the oesophagus and vena cava sectioned. Once isolated the nerve-muscle preparation was transferred to a petri dish with a Sylgard base (Dow-Corning Ltd.) which contained Liley's solution at room temperature gassed with 95% O_2, 5% CO_2. The diaphragm was pinned to the Sylgard using entymological pins. The ribs were trimmed back to the costal margin and the muscle was freed of clotted blood and connective tissue. Except where the diaphragm was used in radiochemical experiments it was then divided into right and left halves by section of the medial tendon. The left hemidiaphragm was usually used for twitch and electrophysiological experiments since

the left phrenic nerve was more accessible than the right phrenic nerve which lay in close proximity to the vena cava and was covered with more fatty and connective tissue. The right hemidiaphragm was used only if the left phrenic nerve was damaged during dissection. There were no detectable differences in any measured parameters between left and right hemidiaphragms.

EDL preparations

Mice were anaesthetised with and maintained under ether anaesthesia. They were pinned ventral surface downwards to the board and the muscles were dissected under a dissection microscope (Carl Zeiss Jena) with a magnification of X16. The skin overlying the hindlimbs was cut away exposing the underlying muscles. The EDL divides into four parts, held together by a connective tissue sheath, with the tendons inserting on digits two to five. Thread was tied around these tendons above the annular ligament and they were then cut below this ligament. The thread was used to exert a light pressure on the EDL as it was carefully separated laterally from the overlying tibialis anterior and medially from the peroneus longus along its entire length to its origin on the femur. As long a length of deep peroneal nerve as possible was dissected. The muscle was kept moist during the dissection with Liley's solution. After removal, the muscle was pinned to the Sylgard base of a petri dish containing Liley's solution at room temperature

gassed with 95% O_2, 5% CO_2. Both EDL muscles were dissected to increase the probability of obtaining a viable nerve. The left EDL was normally used for twitch and electrophysiological experiments whereas both left and right muscles were used for radiochemical experiments.

Electrophysiological experiments

Recording chamber

Extensor digitorum longus and diaphragm muscles (thoracic side up) were pinned to the Sylgard base of a perspex chamber of volume 20 ml. To prevent movement during the experiment and to facilitate microelectrode penetration, the preparation was carefully tensioned, while avoiding excessive stretching which could damage the muscle fibres. Liley's solution or lanthanum saline flowed through a preheating coil into the recording chamber at a rate of about 4 ml min^{-1}. The saline was made to circulate through the recording chamber, over the preparation and through a second heating coil by a bubble lift. Thus the saline was both gassed and heated before it entered the recording chamber and as it circulated around the chamber. This ensured adequate oxygenation of the preparation and a stable temperature. The volume of fluid in the chamber was kept constant by means of a suction pump. The continual replacement of fluid offset any changes in osmotic pressure due to evaporation and also removed lipids, proteins and

waste products, etc released from the muscle fibres. The temperature of the saline was maintained either at 33 ± 1°C for EDL muscles or 37 ± 1°C for diaphragm muscles by means of a thermistor placed in the chamber and an electronic feedback system. Periodically the temperature of the saline was monitored using a temperature probe (Light Laboratories).

The preparation was allowed to equilibrate for at least thirty minutes before recording commenced. Drugs were made up in saline to the required concentration and were added to the preparation by infusing them through the circulation system. It was possible to achieve a rapid change in drug concentration by increasing the flow of saline to a rate of about 40 ml min^{-1} for sixty seconds. Chamber temperatures fell by approximately 2°C during this period. If the bathing solution was changed during the course of an experiment then, unless stated otherwise, at least thirty minutes was allowed before recording continued.

Microelectrodes

Manufacture of microelectrodes

Microelectrodes were used to record intracellular changes in electrical potential at individual motor endplates (Graham and Gerard, 1946). Micropipettes were made from fibre filled borosilicate glass capillary tubing of

external diameter 2 mm and internal diameter 1 mm (Plowden and Thompson) using a two stage vertical electrode puller (Scientific and Research Instruments Limited). The puller was set so that the microelectrodes had a resistance between 10 and 20 Mohms. It was found that microelectrodes with this resistance were able to penetrate muscle fibres without causing significant damage and that the noise level was acceptable. However, the resistance of the microelectrodes alone was not the only criterion for their final selection for use. A microelectrode was used only if it met the criteria of satisfactory muscle fibre penetration as stipulated in the next section. Micropipettes were filled with a 3M aqueous solution of KCl.

Use of microelectrodes

Microelectrodes were inserted in an electrode holder (Clark Electromedical Instruments). The holder contained a silver/silver chloride pellet which made contact with the 3M KCl solution in the microelectrode and connected it to the head amplifier of the recording system. The electrode holder was attached to a micromanipulator (Carl Zeiss Jena) and the microelectrode was positioned over the centre of a muscle fibre. A satisfactory muscle fibre penetration was defined as one in which the recorded noise level did not increase significantly on penetration, a rapid change in membrane potential was observed on penetration and the

recorded resting membrane potential (RMP) of the muscle fibre did not fall more than 10 mV during the period of recording. If these criteria were not met the microelectrode was discarded and another made.

Recording apparatus

A block diagram of the recording apparatus is shown in fig.2. The silver/silver chloride pellet of the microelectrode holder was connected to a head amplifier (X10) by a short lead to reduce cable capacitance. The head amplifier had a high input impedance (10^{12}ohms). One output from the head amplifier was connected via a variable gain AC amplifier to the lower channel of a dual beam oscilloscope (Tektronix 5000 series) which was used for monitoring e.p.ps. and m.e.p.ps. The second output fed into a digital voltmeter on which RMP(mV) was monitored. The recording chamber was earthed by means of a silver/silver chloride pellet. A potentiometer was connected to the head amplifer to balance microelectrode tip potentials and the potential between the silver/silver chloride pellets in the physiological saline and 3M KCl.

LEGEND TO FIG.2

A schematic representation of the recording system.

1. Recording chamber
2. Microelectrode
3. Head amplifier
4. Potentiometer
5. Amplifier
6 Digital voltmeter
7. Tektronix dual beam oscilloscope (5000 series)
8. Gould Advance digital storage oscilloscope (OS 4000)
9. Gould Advance ink recorder (HR 2000)
10. North Star Horizon computer
11. Lear Siegler ballistic printer (300 series)
12. Devices digitimer (D100)
13. Stimulus isolation unit (Digitimer DS2)
14. Suction electrode
15. Calibration unit (Devices 3140)
16. Earth electrode
17. Earthed cage

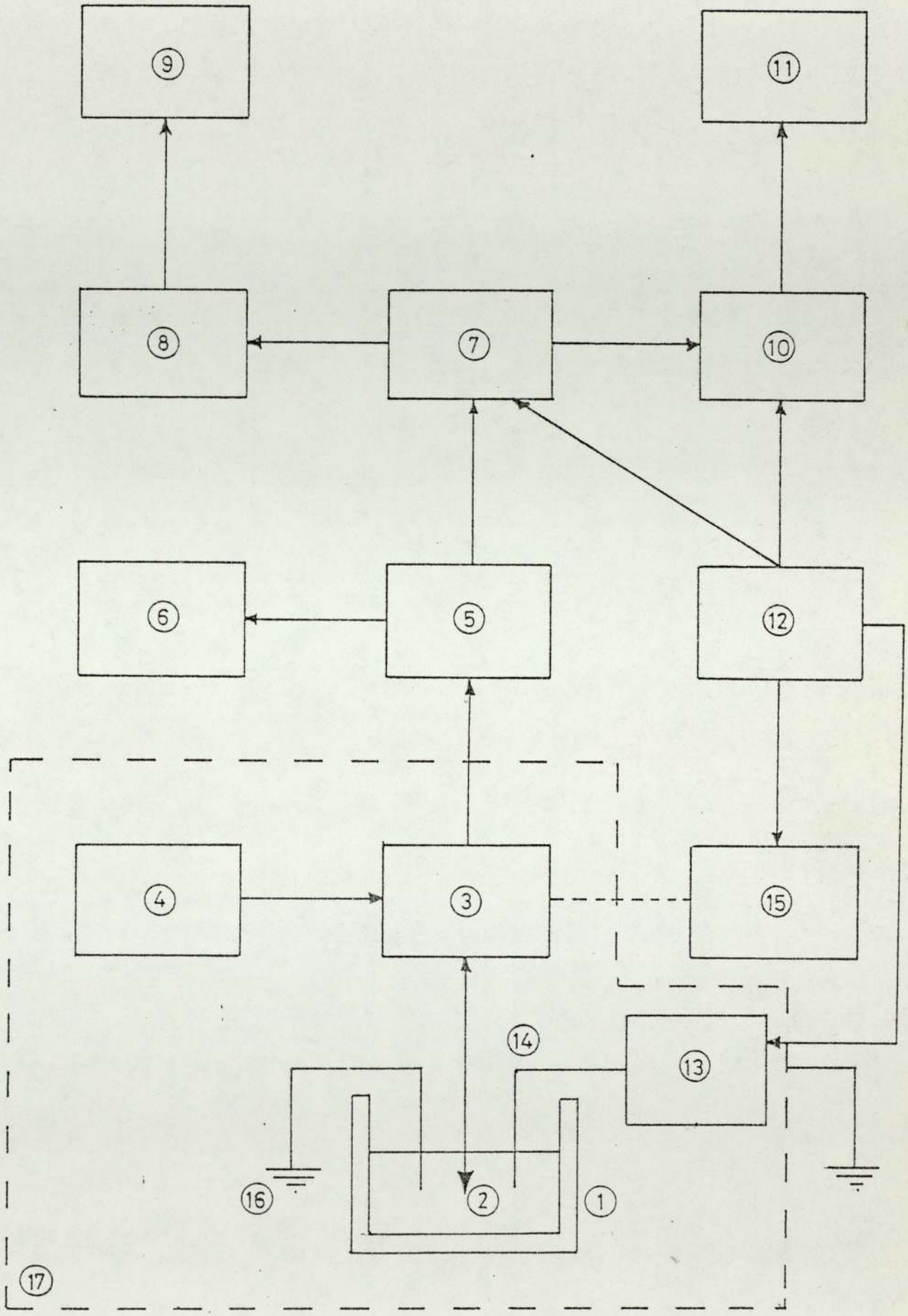


Figure 2

The signal from the variable gain amplifier also fed into an 8 bit analog to digital converter (range ± 5V giving an incremental resolution of 39 mV). The digitised signal was sampled and analysed on line by a microcomputer (North Star Horizon) using programs developed by Dr S.S.Kelly (see Appendix 2). Signals were sampled at 10 kHz. If the sample met the necessary criteria for acceptance (see Appendix 2) records of muscle fibre RMP, mean signal amplitude, rise time, half decay time, etc. were made on a ballistic printer (Lear Siegler Inc. 300 series). Digitised signals were also stored on floppy disk for off-line processing.

Occasionally, the output from the variable gain amplifier was also fed into a digital storage oscilloscope (Gould Advance OS 4000) and a pen recorder (Gould Advance HR 2000). This enabled permanent records of individual potentials to be obtained. Before each experiment the analog to digital converter was calibrated at various gain settings with a 1 mV square wave pulse from a Devices 3140 C.R.O. calibrator unit (see Appendix 2).

Phrenic and deep peroneal nerves were stimulated supramaximally (three times threshold) with a suction electrode connected to a stimulus isolation unit (Digitimer DS2) triggered by a digitimer (Digitimer D100). The digitimer also triggered the oscilloscope and the computer via an isolated digital input. A variable delay was

incorporated into the e.p.p. computer program to enable distinction of the e.p.p. from the stimulus artefact.

The depth of fluid in the recording chamber was kept as low as possible to minimise the capacity to earth across the glass wall of the microelectrode. The recording chamber and head amplifier were mounted in an earthed cage to shield the recording microelectrode from mains frequency and other electrical interference. To prevent earth loop current, all apparatus within the cage was connected to a single ground point, the head amplifier. All signal cables apart from the cable connecting the microelectrode holder to the head amplifier were screened.

Ancillary apparatus

Endplate regions of transilluminated muscle fibres were viewed with a binocular microscope (Leitz) with a zoom objective providing a maximum magnification X125. During electrical recording mechanical stability was essential, especially where individual muscle fibres were impaled for long periods of time. Therefore, the recording chamber and the micromanipulators were attached to a metal plate which rested on rubber shock absorbers.

Recording of m.e.p.ps. and e.p.ps..

In order to record m.e.p.ps. or e.p.ps. a microelectrode

was inserted into a muscle fibre in the region where the fine nerve branches appeared to terminate. Miniature e.p.ps. or e.p.ps. were displayed on the oscilloscope screen at a sweep speed of 1 ms/division and 0.5 ms/division, respectively. If the rise time appeared to be less than 1.1 ms the microelectrode was considered to be focally located at the endplate and the potentials were recorded, providing the criteria of a satisfactory penetration had been met. Endplate potentials were only recorded if there was no distortion of the signal by extracellular field potentials from neighbouring endplates. If subsequent computer analysis revealed the rise time/amplitude ratio to be less than 1.1, the results were accepted. However, if the rise time/amplitude ratio was greater than 1.1, the microelectrode was repeatedy withdrawn and reinserted along the muscle fibre until the endplate region was located. If repeated impalement damaged the fibre, as shown by a rapidly declining RMP, another fibre was selected. The microelectrode was moved systematically from one muscle fibre to the next across the preparation to ensure that the same muscle fibre was not used more than once for recording.

Superficial fibres only were penetrated as deeper fibres, especially in the EDL, are more susceptible to hypoxia which can result in high m.e.p.p. frequencies (Boyd and Martin, 1956; Hubbard and Løyning, 1966). The penetration of deep fibres may also introduce considerable error in

measurement of RMP due to changes in tip potential (McComas and Johns, 1969).

All focal m.e.p.ps. were recorded unless their frequency was unusually high, indicating nerve terminal damage, caused either during dissection, or by microelectrode penetration, or hypoxia. It is possible that endplates with a very low frequency of m.e.p.ps. were excluded from recordings e.g. if no m.e.p.ps. were observed within thirty seconds after microelectrode penetration. Fifty, one hundred or two hundred m.e.p.ps. were sampled from each fibre depending on the frequency in that fibre. Some m.e.p.ps. were excluded from calculations of mean amplitude, frequency, rise time and half decay time because their amplitudes were more than twice the mean of m.e.p.ps. from the same endplates. These m.e.p.ps. were classified as giants (Liley, 1957).

Endplate potentials were elicited by stimulation of the motor nerve at frequencies of 1, 10 or 50 Hz. Usually two or three trains of 100 e.p.ps. were recorded from each endplate. An interval of at least one minute was allowed between trains to enable full recovery of the nerve terminal. To prevent muscle contraction which naturally occurs as a result of motor nerve stimulation, curarised, magnesium blocked or cut muscle fibre preparations were used.

The competitive neuromuscular antagonist (+)-tubocurarine (curare) combines with postsynaptic receptors reducing e.p.p. amplitude below that needed to depolarise the membrane to threshold and initiate an action potential (Fatt and Katz, 1951). Twitch responses in the diaphragm and EDL were blocked by 2.56 μM and 1.91 μM curare, respectively. The effect of the nonphenolic uncoupler of oxidative phosphorylation guanidine (5×10^{-3}M) on e.p.p. amplitude was compared in normal and dystrophic EDL muscles. In these experiments twitch responses in the normal EDL were prevented by 1.88 μM curare. However, this concentration of curare was not sufficient to prevent twitching in the dystrophic EDL in the presence of guanidine. Therefore, 1.95 μM curare was used to prevent contraction in the dystrophic muscles. All muscles were bathed in curare for thirty minutes before recording commenced.

Magnesium competitively blocks calcium entry into the nerve terminal in response to nerve stimulation thereby reducing the number of quanta released. Endplate potentials were recorded by reducing the Ca concentration of the bathing solution and raising the Mg concentration (del Castillo and Katz, 1954a). Endplate potentials in the EDL were recorded in the presence of 2.45 mM Mg and 0.55 mM Mg, and in the diaphragm in the presence of 2.5 mM Mg and 0.5 mM Ca. Preliminary experiments revealed that equilibrium at these Ca and Mg concentrations was accomplished within thirty

minutes.

The third method used to prevent muscle contraction in the EDL was the cut-fibre preparation (Barstad, 1962). Cutting the ends of the muscle fibres reduces the RMP of the fibres below the levels at which action potentials can be generated. This method allows transmitter release to be studied in normal external medium and in the absence of drugs such as curare. The endplate region was first located as indicated by the presence of focal m.e.p.ps.. The fibres were then cut as far away from this region as possible along their insertion to the tendons. The extent of depolarisation and hence the changes in RMP and m.e.p.p. amplitude depend upon how close to the endplate the muscle is cut. Using this method it was possible to record m.e.p.ps. and e.p.ps. in the same fibre.

To avoid nerve block, a consequence of potassium leaking from the cut fibres, the flow of Liley's solution through the recording chamber was increased during the cutting procedure and for at least five minutes after. The preparation was left for thirty minutes after cutting, if twitch responses were abolished recording was begun, otherwise the muscle fibres were cut again and left a further thirty minutes. Recording was begun as soon as possible since the RMP in these preparations falls progressively.

Post-tetanic potentiation of m.e.p.p. frequency

Experiments investigating post-tetanic potentiation of m.e.p.p. frequency were performed in the cut-fibre EDL preparation. An endplate region was first identified by the presence of focal m.e.p.ps.. If e.p.ps. were elicited at the endplate then 50 or 100 m.e.p.ps. were recorded and their frequency determined. The deep peroneal nerve was then stimulated tetanically at 50 Hz for 60 seconds. Endplate potentials were monitored on the oscilloscope to ensure that transmission did not fail during this period. One hundred m.e.p.ps. were recorded immediately after stimulation and then at one minute intervals until m.e.p.p. frequency returned to control values.

Corrections for RMP and non-linear summation

The amplitudes of m.e.p.ps. and e.p.ps. were corrected to a standard RMP and for non-linear summation of depolarisations by the subsynaptic membrane (Martin, 1955). The standard RMP was -74 mV in all preparations except for cut-fibre preparations where -50 mV was used. By correcting to a standard RMP, m.e.p.p. and e.p.p. amplitudes from different muscle fibres and from normal and dystrophic muscles could be compared directly. The value of the standard RMP affects the mean amplitudes of e.p.ps. and m.e.p.ps. but it has no effect on the quantum contents of e.p.ps.. For preparations other than cut-fibre

preparations the corrections were made by applying the following equation:

$$v = \frac{v' \ (74-5)}{(RMP-5-v')} \qquad \text{(Kelly, 1976)}$$

where v is the corrected signal amplitude (mV), v' is the uncorrected amplitude (mV), RMP is the mean resting membrane potential during recording (mV) and -5mV is the acetylcholine reversal potential (Glavinović, 1979c; Linder and Quastel, 1978; Banker, Kelly and Robbins, 1983).

For cut-fibre preparations Martin's correction for non-linear summation was empirically trimmed by a factor of 0.7 (Banker et al, 1983) so that the corrected e.p.p. amplitudes would not exceed the reversal potential. The corrections were made by applying the following formulae:

$$v = \frac{v'(50-5)}{(RMP-5-v')}$$

$$V = v' + 0.7(v-v')$$

where V is the corrected amplitude (mV)

Calculation of quantum content

In order to calculate the mean quantum content (m) of e.p.ps. in cut- fibre and magnesium blocked preparations the last 90 e.p.ps. of two or three trains of 100 were measured.

Quantum content was determined directly by dividing the mean corrected e.p.p. amplitude in each fibre by the mean corrected m.e.p.p. amplitude in that fibre, excluding giants. It is believed that giants do not contribute to quantal release (Liley 1957; Menrath and Blackman, 1970).

Quantum content was not determined in the curare blocked preparation. This is because estimates of m in this preparation are based on an assumption of a Poisson distribution of release of transmitter. This has been shown to be an incorrect assumption in normal preparations and calculations of m under these conditions can overestimate m by as much as 100% (Johnson and Wernig, 1971).

Calculation of e.p.p. rundown

At the curarised mammalian neuromuscular junction, at rates of stimulation of 5Hz or more, the first e.p.p. detected is usually the largest and the amplitude of later responses declines progressively to reach a plateau after six or more impulses (Liley and North, 1953). This is referred to as early tetanic rundown. Early tetanic rundown in the curarised diaphragm and EDL was investigated by normalising the amplitudes of the first six e.p.ps. and the plateau amplitude in each fibre. The normalised plateau amplitude was then subtracted from the other normalised values such that:

$$x = \frac{\text{e.p.p. } x}{\text{e.p.p.1}} - \frac{\text{e.p.p. plateau}}{\text{e.p.p.1}}$$

where e.p.p.1 is the amplitude of the first e.p.p., e.p.p. plateau is the plateau e.p.p. amplitude and e.p.p. x is the amplitude of e.p.ps 1 to 6.

A linear regression was fitted to the log to the base e value of each value of x (see Appendix 1) and the slopes of the initial rundown were calculated from the expression:

$$\log_e x = -kn$$

where x is the normalised e.p.p. amplitude,
n is the e.p.p. number and k is the slope.

Any fibre in which Pearson's correlation coefficient was less than 0.85 (indicating that the calculated regression line was not a good fit to the data points) was excluded.

Calculation of probability of release and store in the cut-fibre EDL preparation

Endplate potential amplitudes were stored on disk and amplitude histograms of 90 plateau e.p.ps. plotted for each fibre. For all histograms the "goodness of fit" of the binomial distribution and the Poisson distribution to the observed distribution was determined using the Chi squared (χ^2) test (see Appendix 1).

In calculating the theoretical distributions (Poisson or binomial) for e.p.p. amplitude histograms the mean m.e.p.p. amplitude was used as a measure of quantum size. The variability of m.e.p.ps. was taken into account although giant m.e.p.ps. were excluded (Miyamoto, 1975).

The mean quantum content (m) is the product of the probability of quantal release (p) and the number of quanta available for release (n). Transmitter release was found to conform to a binomial distribution and therefore, the statistical parameters of transmitter release (n,p) were calculated using the binomial model outlined by Miyamoto (1975).

TWITCH EXPERIMENTS

Recording chamber

To record contractile responses hemidiaphragm or EDL preparations were attached to an isometric transducer (UF1, range ± 2 oz) by a cotton thread tied around the central tendon of the hemidiaphragm and distal tendon of the EDL. The costal margin of the diaphragm or proximal tendon of the EDL was pinned to the Sylgard base of a perspex chamber similar to that described for electrophysiological recording. Muscles were bathed in carbogenated Liley's solution. The temperature of the Liley's solution (33 ± 1°C

for the EDL and 37 ± 1°C for the diaphragm) was monitored regularly throughout the experiment using a Light Laboratories temperature probe. The resting length of the muscle was adjusted so that a twitch of maximal amplitude was produced in response to supramaximal stimulation of the motor nerve. The preparation was then left for thirty minutes before recording commenced.

Recording apparatus

A block diagram of the recording apparatus is shown in fig.3. The output from the isometric transducer was fed into a variable gain instrumentation amplifier (input range 0 - 1V) with DC offset and to a heated stylus pen recorder (Devices MX2R).

In some experiments the output from the amplifier was fed into a second variable gain amplifier and into a signal averager (Datalab DL102A). The output from the signal averager was displayed on an oscilloscope (Advance Instruments OS 240). The purpose of the variable amplification was to ensure that the signal was of sufficient amplitude so that the averager was digitising the analog signal as accurately as possible (range ± 1V, 16 mV resolution). Permanent records were obtained on a Bryans XY pen recorder (26000 A4). The output of the transducer was calibrated with gram weights at the end of each experiment.

LEGEND TO FIGURE 3

A schematic representation of the recording system

1. Organ bath
2. Isometric transducer (UF1)
3. Instrumentation amplifier
4. Heated stylus pen recorder (Devices MX2R)
5. Amplifier
6. Signal averager (Datalab DL102A)
7. Advance Instruments oscilloscope (OS 240)
8. Bryans XY recorder (26000 A4)
9. Devices digitimer
10. Stimulus isolation unit (Digitimer DS2)
11. Suction electrode

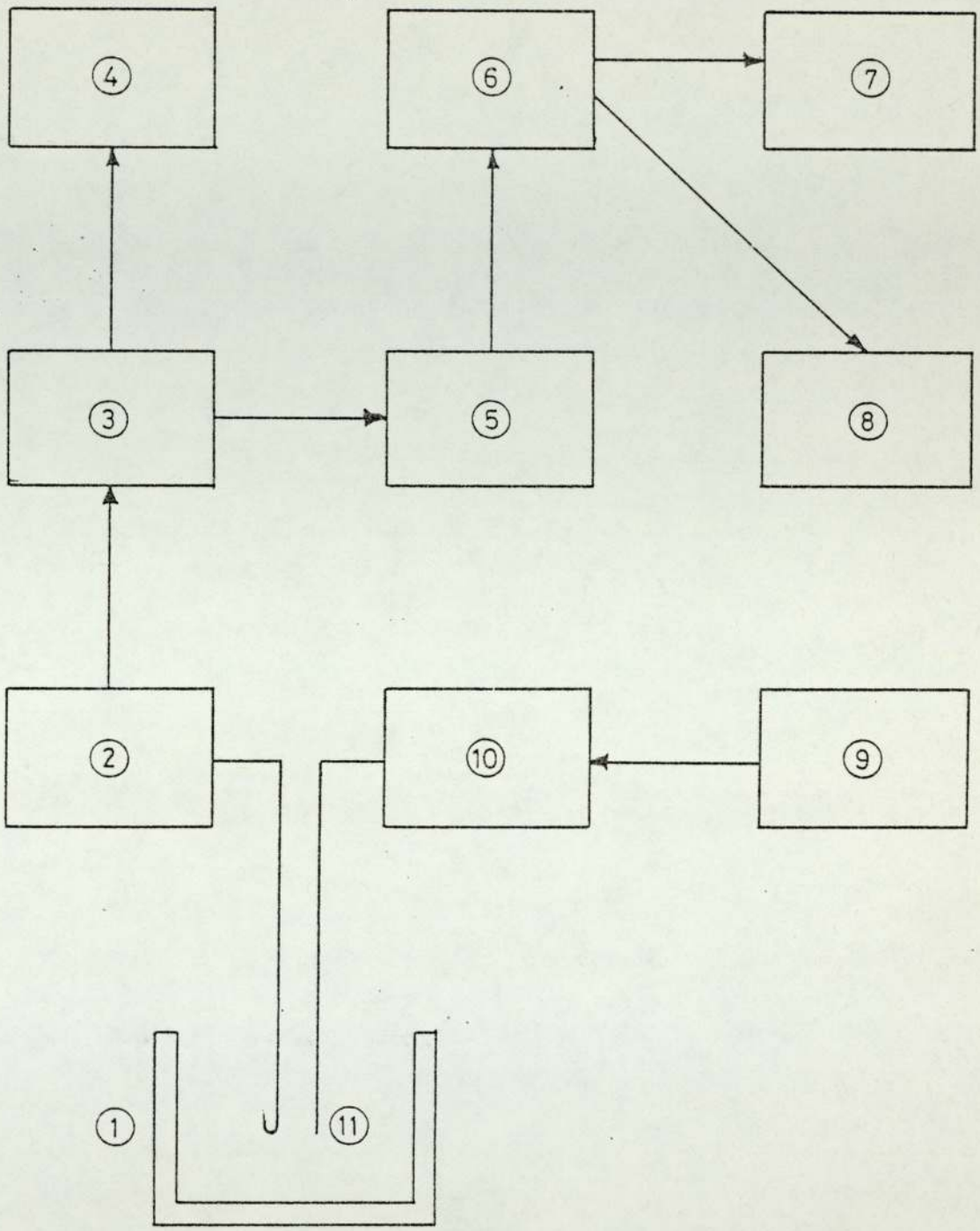


Figure 3

To obtain single twitches, the motor nerves were stimulated by single rectangular pulses of 0.05 ms duration and supramaximal intensity at a frequency of 0.1Hz. The pulses were delivered from a Digitimer stimulus isolation unit (DS2), triggered by a Devices digitimer through a suction electrode.

The effect of curare on indirectly evoked twitch responses of EDL and diaphragm muscles was measured by constructing cumulative log dose-response curves. The preparation was allowed to equilibrate with curare for 20 minutes before a change in concentration was made. In all experiments, following abolition of the twitch response, the muscle was washed with curare-free Liley's solution. If the twitch response did not recover to at least 85% of control then the results from that experiment were discarded. Preliminary experiments revealed that control twitch responses remained constant for twelve hours or more. However, experiments were normally completed within four hours.

RADIOISOTOPE EXPERIMENTS

All experiments were performed at 30 ± 1°C. Prior to incubation with radioisotopes muscles were bathed for sixty minutes in Liley's solution or lanthanum saline. Approximately 15 - 20 ml of solution was allowed for each muscle. Whole diaphragms together with a section of the ribcage were placed in physiological saline. Extensor digitorum longus muscles were pinned through their tendons to modelling wax (AD International Ltd.) and immersed in saline. To ensure that all surfaces of the muscle were freely accessible to the physiological saline the muscles were elevated slightly above the wax. Four EDL muscles from four different animals were pinned to each piece of wax.

After incubation with ^{45}Ca in trace amounts some muscles were tested for viability by their ability to contract to direct stimulation. Each muscle was placed in a petri dish containing Liley's solution into which a pair of platinum wire electrodes was introduced. The electrodes were positioned near the muscle without touching it and electrical pulses of 2 ms duration and 20 V strength were passed.

At the end of each experiment diaphragms were separated into right and left halves and dissected free of the

ribcage, tendon and as much connective tissue as possible. The ends of EDL muscles were removed to minimise the contribution of either tendons, or muscle fibre damage caused by possible crushing in pinned ends of muscles, to calcium fluxes. The muscles were then lightly blotted and the wet weight determined on a torsion balance (White Elec. Inst. Co. Ltd.). To avoid contamination from muscles which had been weighed previously a different weighing boat was used for each group of muscles. Each muscle was placed in a numbered glass scintillation vial and 1 ml of protosol was added to solubilise the muscle. The vials were tightly capped to prevent escape of solubiliser and were left at room temperature for 48 hours. Ten ml of the scintillant, Dimilume-30, was added to each vial and the radioactivity of samples was measured with a Beckman LS-230 liquid scintillation counter against suitable standards and blanks. Samples were left in the counter for thirty minutes before recording began to bring them to the same temperature as the counter. Radioactivity of ^{45}Ca or ^{3}H inulin was counted for twenty minutes at efficiencies of 35% and 51%, respectively.

The standards consisted of a known volume of tracer solution which was prepared and counted in the same way as the muscle samples. For background counts, similar weights of tracer-free muscles were treated in the same way as radioactive samples.

Calcium flux measurements

For influx experiments muscles were equilibrated in Liley's solution for sixty minutes and then transferred to Liley's solution containing $^{45}CaCl_2$ in trace amounts (15 ng/ml). Muscles were incubated in this solution for periods of 5, 10, 20, 40, 60 or 90 minutes. To terminate the experiment the muscles were rinsed briefly in tracer-free Liley's to remove any tracer adhering to the surface of the muscle. The muscles were then prepared for liquid scintillation counting as previously described. The radioactivity of the ^{45}Ca in the weighed muscle samples was determined and the results were calculated as nanomoles of Ca/mg wet muscle by means of the following expression:

$$\text{nanomoles Ca/mg wet muscle} = \frac{\text{cpm of digested muscle sample}}{\text{wet weight of muscle in mg}} \times \frac{\text{nanomoles Ca in standard}}{\text{cpm standard}}$$

In experiments designed to measure tracer efflux, muscles were first equilibrated in tracer-free Liley's solution for sixty minutes and then incubated in Liley's solution containing $^{45}CaCl_2$ for a further hour. After this preloading period the muscles were rinsed briefly in tracer—free solution to remove superficial tracer and then incubated in tracer-free, calcium-free, Liley's solution. At intervals of 5,10,20 or 40 minutes muscles were removed from the tracer-free solution, weighed wet and the ^{45}Ca

remaining in the muscles was measured by liquid scintillation counting.

In an attempt to eliminate differences in ^{45}Ca fluxes between normal and dystrophic muscle which could be attributed to differences in extracellular space, lanthanum chloride was used. The trivalent cation, lanthanum, has been used extensively in calcium flux measurements in smooth muscle (van Breemen, Farinas, Gerba and McNaughton, 1972). The principle of the method is that after the muscle has taken up ^{45}Ca, and before liquid scintillation counting, all the extracellular ^{45}Ca or superficial ^{45}Ca is displaced from binding sites by lanthanum, while membrane bound lanthanum prevents any further influx or efflux of ^{45}Ca. Effects on muscle fibre ^{45}Ca uptake that have been obscured by much larger quantities of extracellular ^{45}Ca can then be detected.

Muscles were equilibrated in lanthanum saline for sixty minutes and then transferred to lanthanum saline containing $^{45}CaCl_2$ for fifty minutes. Muscles were then incubated in lanthanum saline, $^{45}CaCl_2$ and $LaCl_3$ (10mM) for a further ten minutes. After this they were rinsed briefly in tracer-free lanthanum saline containing $LaCl_3$ (10mM). Some muscles were taken immediately and the ^{45}Ca content determined while the remainder were incubated in tracer-free, calcium-free lanthanum saline containing $LaCl_3$ (10mM) for twenty minutes. The muscles were then prepared

for counting as previously described.

Extracellular space measurements

The extent of the extracellular space was determined with ^{3}H inulin as an extracellular marker. Muscles were incubated in Liley's solution for sixty minutes and then transferred to Liley's containing ^{3}H inulin (0.42 μCi/ml). After an incubation period of 5, 10, 20 or 40 minutes the muscles were rinsed briefly in tracer-free Liley's solution, weighed wet and the radioactivity of ^{3}H in the muscles was measured. The inulin space was calculated as c.p.m. per gram wet weight of muscle/c.p.m. per ml Liley's solution.

Analysis of results

Significance between groups of data was assessed using the non- parametric Mann-Whitney U test (Appendix 1). A probability level of less than 0.05 (two-tailed) was taken to indicate a significant difference between two groups of data. Group data are presented as the mean of the group ± 1 standard deviation (S.D.).

Statistical tests used are presented in Appendix 1.

CHAPTER 3 : RESULTS

INDIRECTLY EVOKED TWITCH RESPONSES IN EDL AND DIAPHRAGM MUSCLES

Characteristics of the twitch response

In order to determine if both EDL and diaphragm muscles were affected by the disease process, the amplitude and time course of indirectly evoked twitch responses were measured in muscles from eight week old male mice. The results are summarised in tables 2A and 2B.

Dystrophic EDL muscles developed less tension in response to nerve stimulation than muscles from normal mice. The difference in tension was not merely a reflection of the muscle weight as it was also evident when the tension was expressed per mg wet weight (table 2A). There was no significant difference in either total twitch duration, time to peak or half relaxation time between normal and dystrophic EDL muscles.

In contrast to the EDL, there was no significant difference in twitch tension between diaphragm muscles from normal and dystrophic mice (table 2B). Neither was there any significant difference in total twitch duration, time to peak or half relaxation time.

Table 2. Characteristics of the indirectly evoked twitch response of EDL (A) and diaphragm (B) muscles from eight week old normal and dystrophic male mice. Temperature 33 ± 1°C (EDL) and 37 ± 1°C (diaphragm).

A.	NORMAL	DYSTROPHIC
Relative twitch tension g/mg wet muscle	0.22 ± 0.09 (10)	* 0.12 ± 0.03 (8)
Total twitch duration (msec)	51.10 ± 8.32 (10)	48.0 ± 12.28 (8)
Time to peak (msec)	9.90 ± 1.26 (10)	9.42 ± 1.75 (8)
Half relaxation time (msec)	8.98 ± 2.24 (10)	8.98 ± 1.67 (8)

B.	NORMAL	DYSTROPHIC
Relative twitch tension g/mg wet muscle	0.15 ± 0.08 (7)	0.19 ± 0.02 (6)
Total twitch duration (msec)	37.71 ± 3.04 (7)	41.0 ± 3.40 (6)
Time to peak (msec)	10.43 ± 0.90 (7)	11.33 ± 1.49 (6)
Half relaxation time (msec)	8.32 ± 0.61 (7)	8.75 ± 1.22 (6)

Values are the mean ± 1 S.D. with the number of muscles in parentheses.

* ($p<0.05$)

Effect of (+) - tubocurarine chloride on the twitch response

The depressant effect of curare on indirectly evoked twitch responses was compared in EDL muscles from eight week old normal and dystrophic male mice and in diaphragm muscles from eight or fifteen week old normal and dystrophic animals.

Figures 4 and 5A show log dose-response curves for curare in EDL and diaphragm muscles from eight week old animals. It can be seen from fig.4 that the log dose-response curve for curare in the dystrophic EDL was to the right of the normal, indicating that EDL muscles from dystrophic mice are more resistant to curare. The IC_{50} in the dystrophic EDL was 1.32 ± 0.04 μM (n=3) compared with 0.78 ± 0.03 μM (3) in the normal EDL. These values were significantly different ($p<0.05$). In contrast, there was no significant difference in the log dose-response curves for curare between diaphragm muscles from normal and dystrophic mice (fig.5A). The IC_{50} was 1.27 ± 0.15 μM (4) in the dystrophic diaphragm and 1.14 ± 0.08 μM (5) in the normal diaphragm. It is evident from the IC_{50} values that diaphragm muscles from eight week old normal mice were more resistant to curare than EDL muscles from normal animals of the same age. However, no such difference was observed in muscles from dystrophic mice.

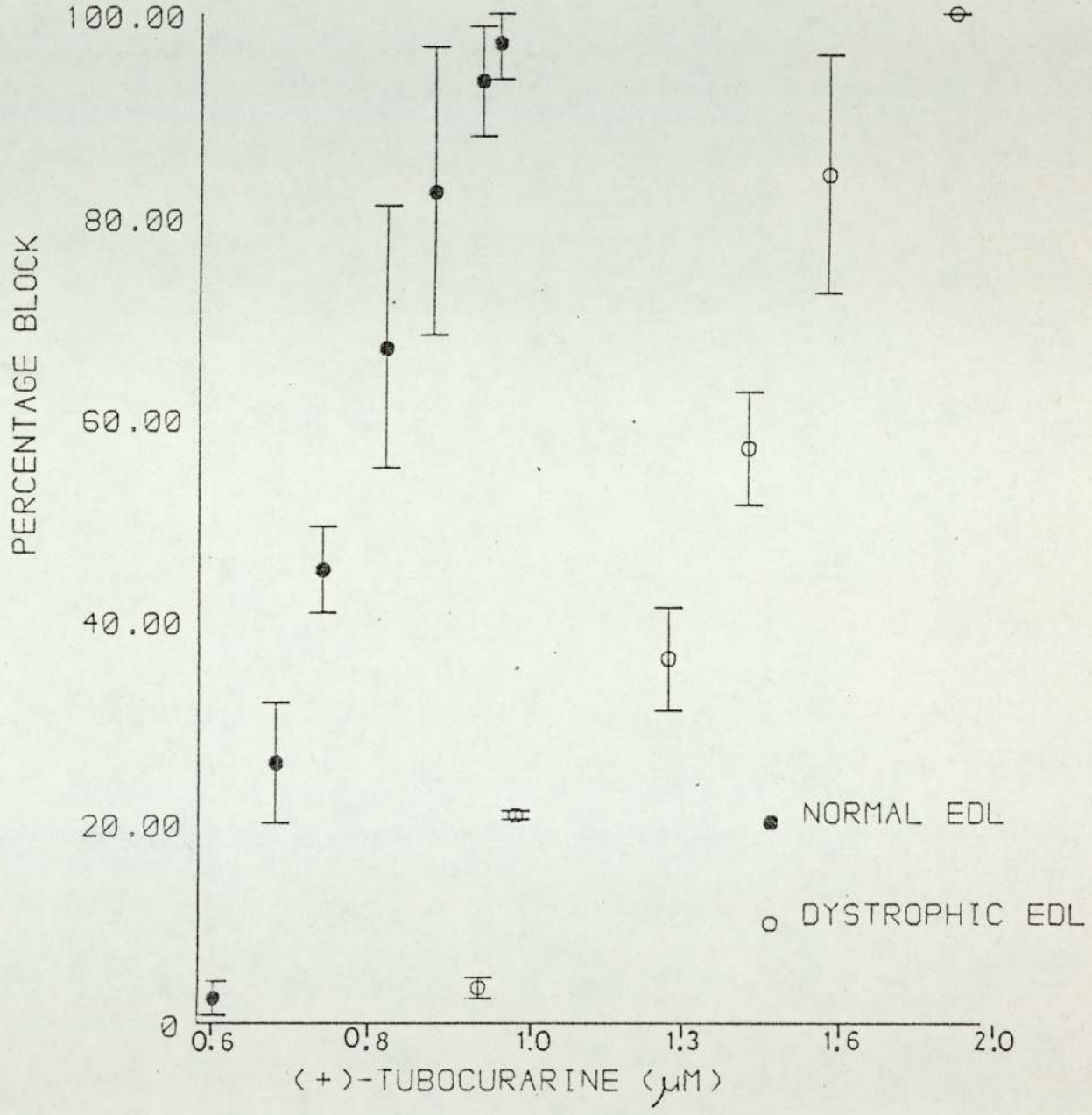


Fig. 4. Effect of (+) - tubocurarine chloride on indirectly evoked twitch responses of EDL muscles from 8 week old male mice. Closed and open circles represent mean values from 3 normal and 3 dystrophic mice, respectively. Vertical bars indicate ± 1 s.e. of mean where this exceeds the diameter of the points. Temperature 33 ± 1°C.

Fig. 5A Effect of (+) - tubocurarine chloride on indirectly evoked twitch responses of hemidiaphragms from 8 week old male mice. Closed and open circles represent mean values from 5 normal and 4 dystrophic mice, respectively.

Fig. 5B Effect of (+) - tubocurarine chloride on indirectly evoked twitch responses of hemidiaphragms from 15 week old male mice. Closed and open circles represent mean values from 3 normal and 5 dystrophic mice, respectively. Vertical bars indicate ± 1 s.e. of mean where this exceeds the diameter of the points. Temperature 37 ± 1°C.

FIG. 5A

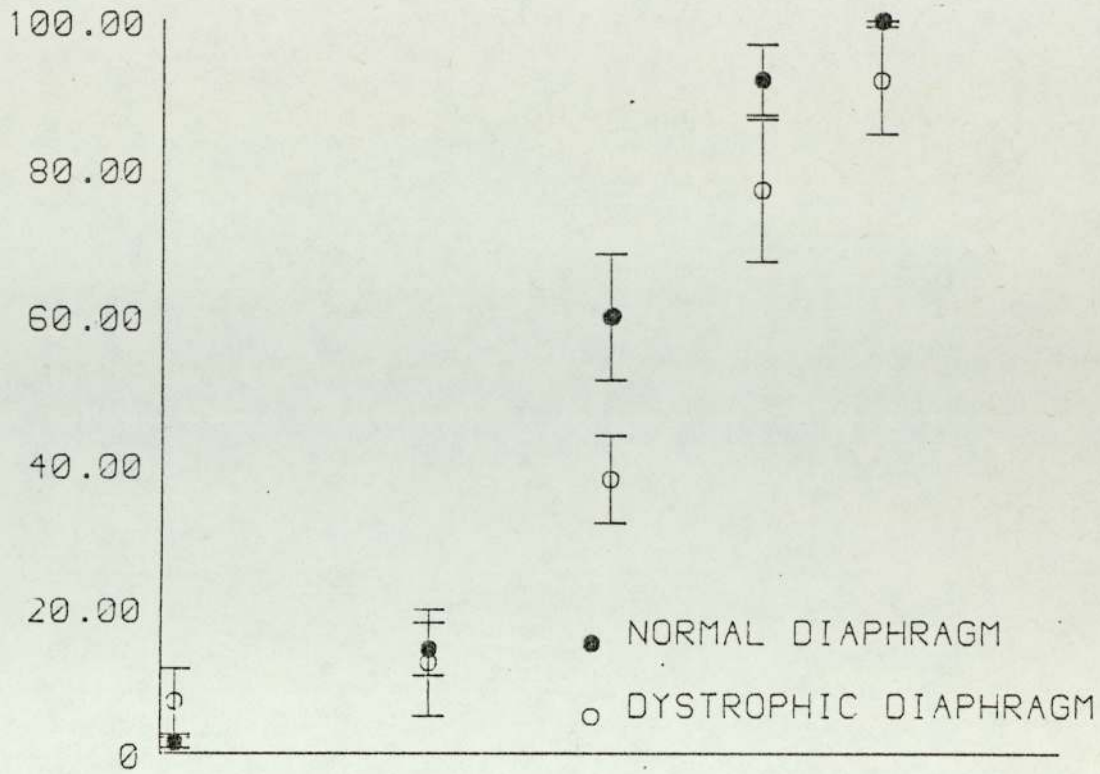
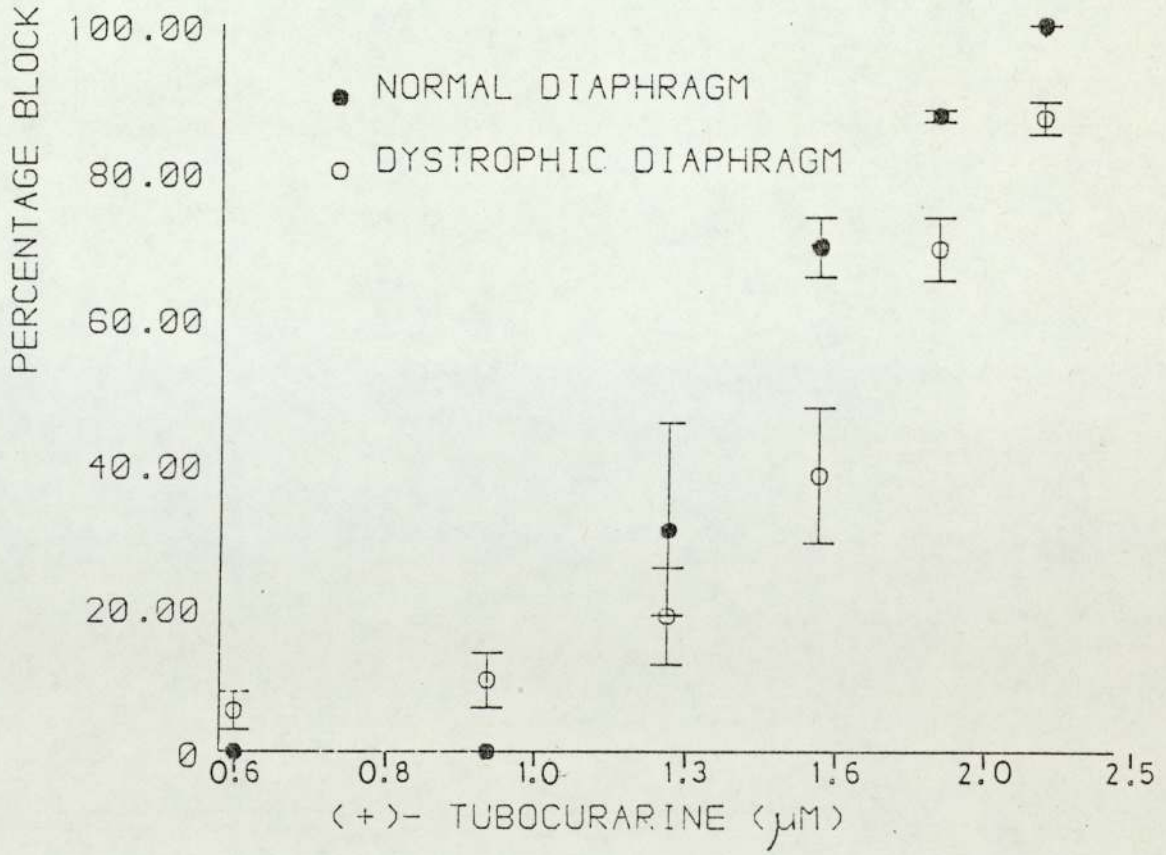

100.00
80.00
60.00
40.00
20.00
0
NORMAL DIAPHRAGM
DYSTROPHIC DIAPHRAGM

FIG. 5B


PERCENTAGE BLOCK
100.00
80.00
60.00
40.00
20.00
0
NORMAL DIAPHRAGM
DYSTROPHIC DIAPHRAGM
0.6
0.8
1.0
1.3
1.6
2.0
2.5
(+)- TUBOCURARINE (μM)

The effect of curare on indirectly evoked twitch responses of diaphragm muscles from fifteen week old normal and dystrophic mice is shown in fig.5B. The IC_{50} in the dystrophic diaphragm was 1.61 ± 0.24 μM (5) compared with 1.37 ± 0.08 μM (3) in the normal diaphragm. These values were not significantly different. Resistance to curare in the normal diaphragm had increased with age since the IC_{50} of diaphragm muscles from fifteen week old animals was greater than that of muscles from eight week old mice. However, no increase in resistance to curare with age was observed in diaphragm muscles from dystrophic mice.

TRANSMITTER RELEASE

Spontaneous release

In order to investigate a possible mechanism for the resistance to curare described in the preceding section, transmitter release between normal and dystrophic diaphragm and EDL muscles was compared.

Spontaneous transmitter release parameters in EDL muscles from normal and dystrophic male mice aged between 3 and 5 months are given in table 3. The initial RMP recorded on penetration of the muscle fibres was significantly lower in the dystrophic EDL than in the normal EDL. Miniature e.p.p. amplitude in the dystrophic EDL was 1.50 times larger than that in the normal EDL. The distribution of

Table 3. Spontaneous transmitter release in EDL muscles from 3 to 5 month old normal and dystrophic male mice. Temperature 33 ± 1°C.

	NORMAL	DYSTROPHIC
RMP (mV)	−76.98 ± 6.93 (96/10)	−68.82 ± 8.01 (75/9)
M.e.p.p. amplitude (mV)	0.56 ± 0.27 (96/10)	* 0.84 ± 0.46 (75/9)
M.e.p.p. half decay time (msec)	0.78 ± 0.20 (96/10)	* 1.06 ± 0.45 (75/9)
M.e.p.p. frequency (Hz)	7.14 ± 4.12 (96/10)	6.13 ± 3.32 (75/9)
Number of giants/ 100 m.e.p.ps.	0.34 ± 0.07 (96/10)	* 0.76 ± 0.17 (75/9)

Values are the mean ± 1 S.D.

Numbers in parentheses are number of fibres/number of muscles

* ($p<0.05$)

mean m.e.p.p. amplitudes betwen fibres is shown in fig. 6. Miniature e.p.p. amplitude in the normal EDL had a bimodal distribution, the amplitude of the second peak being equivalent to the mean amplitude of m.e.p.ps. recorded from the dystrophic EDL. The upper range of m.e.p.p. amplitudes in the dystrophic EDL was greater than that in the normal muscle suggesting that two populations of fibres occur in this muscle.

Miniature e.p.p. half decay time was 1.36 times larger in the dystrophic EDL. The distribution of mean half decay time between fibres in normal and dystrophic EDL muscles (fig.7) shows that the bimodal distribution of m.e.p.p. amplitudes in the normal EDL was not reflected in a bimodal distribution of half decay times. Again the upper range of values was considerably greater in the dystrophic EDL indicating the presence of a second population of fibres with abnormal m.e.p.ps. There was no significant difference in m.e.p.p. frequency between normal and dystrophic EDL muscles although there was a greater incidence of giant m.e.p.ps. in the dystrophic EDL.

Some parameters of spontaneous transmitter release in diaphragm muscles from normal and dystrophic male mice aged 3 to 5 months are shown in table 4. There was no significant difference in RMP between normal and dystrophic diaphragms. Miniature e.p.p. amplitude in the dystrophic diaphragm was slightly but significantly larger than that

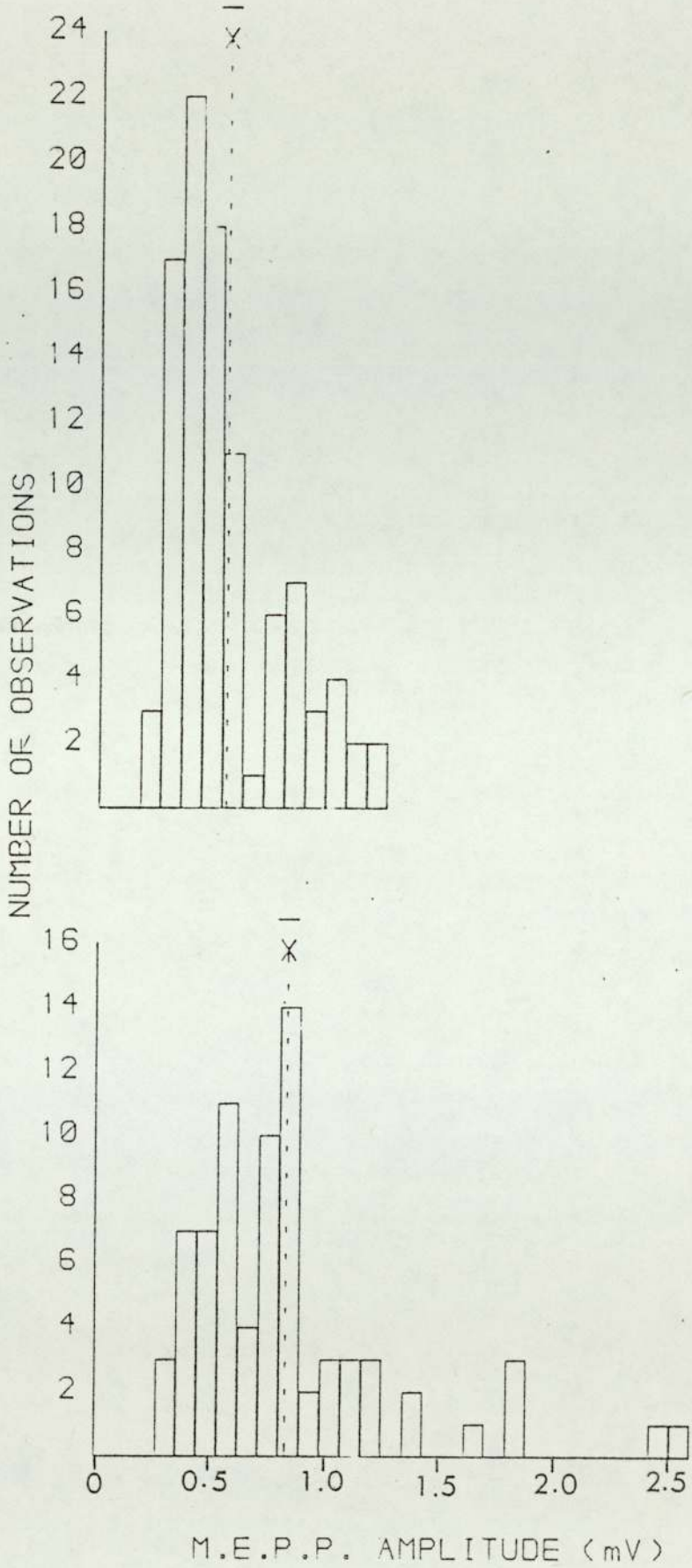


Fig. 6. Distribution of mean m.e.p.p. amplitude values in EDL muscles from normal (upper) and dystrophic (lower) male mice. $\bar{x}$ indicates the mean. Temperature 33 ± 1°C.

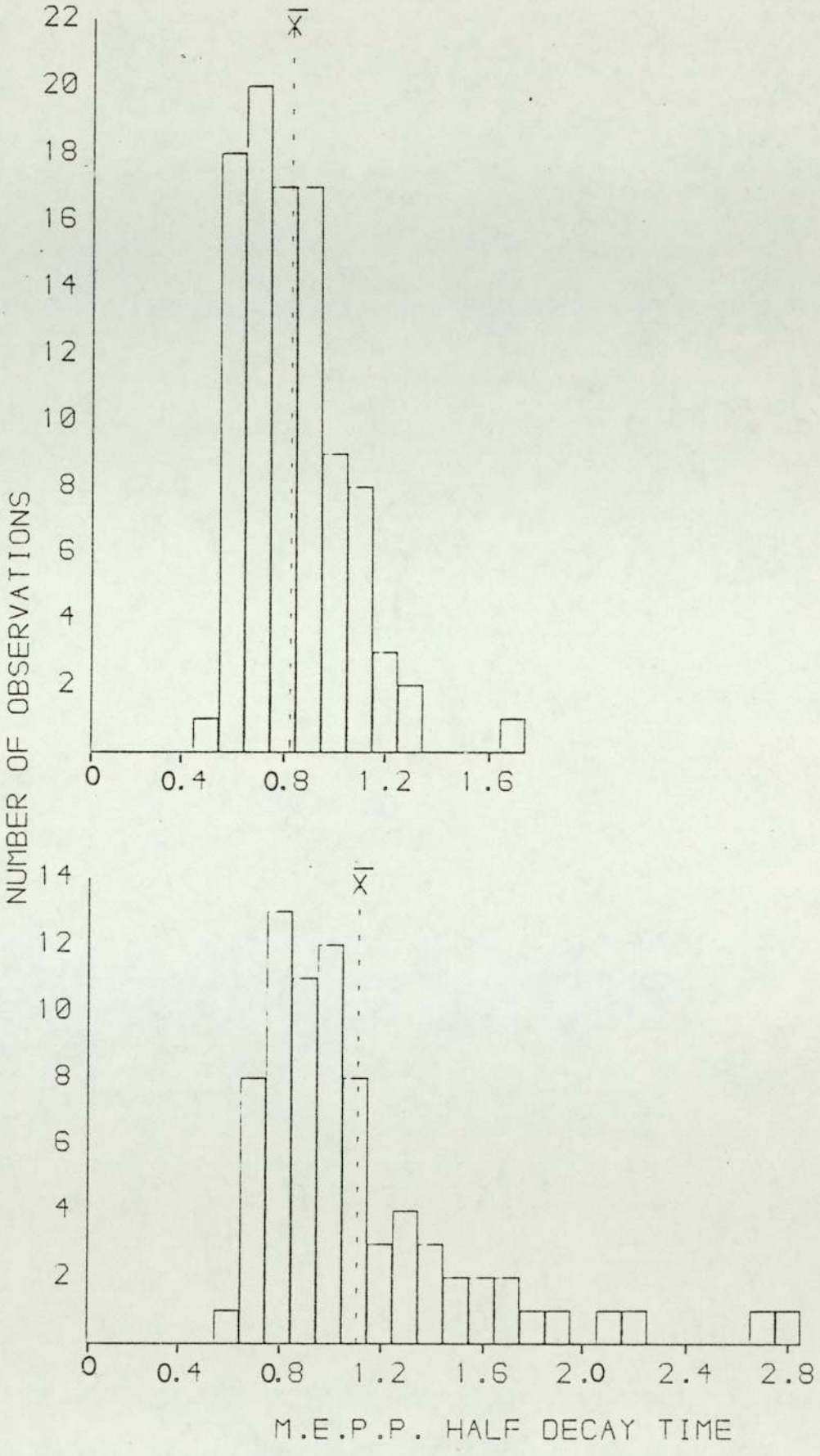


Fig. 7 Distribution of mean m.e.p.p. half decay time values in EDL muscles from normal (upper) and dystrophic (lower) male mice. $\bar{x}$ indicates the mean. Temperature 33 ± 1°C.

Table 4. Spontaneous transmitter release in diaphragm muscles from 3 to 5 month old normal and dystrophic male mice.

Temperature 37 ± 1°C.

	NORMAL	DYSTROPHIC
RMP (mV)	-72.98 ± 7.7 (374/34)	-72.11 ± 8.17 (269/27)
M.e.p.p. amplitude (mV)	1.07 ± 0.38 (374/4)	* 1.21 ± 0.46 (269/27)
M.e.p.p. half decay time (msec)	0.83 ± 0.20 (297/27)	0.82 ± 0.11 (236/23)
M.e.p.p. frequency (Hz)	6.16 ± 3.46 (374/34)	5.77 ± 2.86 (269/27)
Number of giants/ 100 m.e.p.ps.	0.28 ± 0.04 (187/18)	0.33 ± 0.04 (171/16)

Values are the mean ± 1 S.D.

Numbers in parentheses are number of fibres/number of muscles.

* ($p < 0.05$)

in the normal muscle. The distribution of mean m.e.p.p. amplitudes between fibres (fig.8) illustrates that, in contrast to the normal EDL, m.e.p.p. amplitude in the normal diaphragm had an unimodal distribution. The mode in the dystrophic diaphragm was approximately the same as that in the normal muscle although there were a number of larger values. However, it was not possible to distinguish two populations of fibres in the dystrophic diaphragm on the basis of m.e.p.p. amplitude. There were no significant differences in m.e.p.p. half decay times, m.e.p.p. frequency or the incidence of giants between normal and dystrophic diaphragms.

Spontaneous transmitter release was also compared in EDL and diaphragm muscles from normal and dystrophic female mice aged between 3 and 5 months. The results were not significantly different from those obtained in the muscles from male mice.

Under control conditions, in diaphragm and EDL muscles from male and female normal and dystrophic mice, m.e.p.p. frequency did not change appreciably during 6 hours of experimentation. Continued impalement of a muscle fibre for periods up to 60 minutes also had no effect on m.e.p.p. frequency.

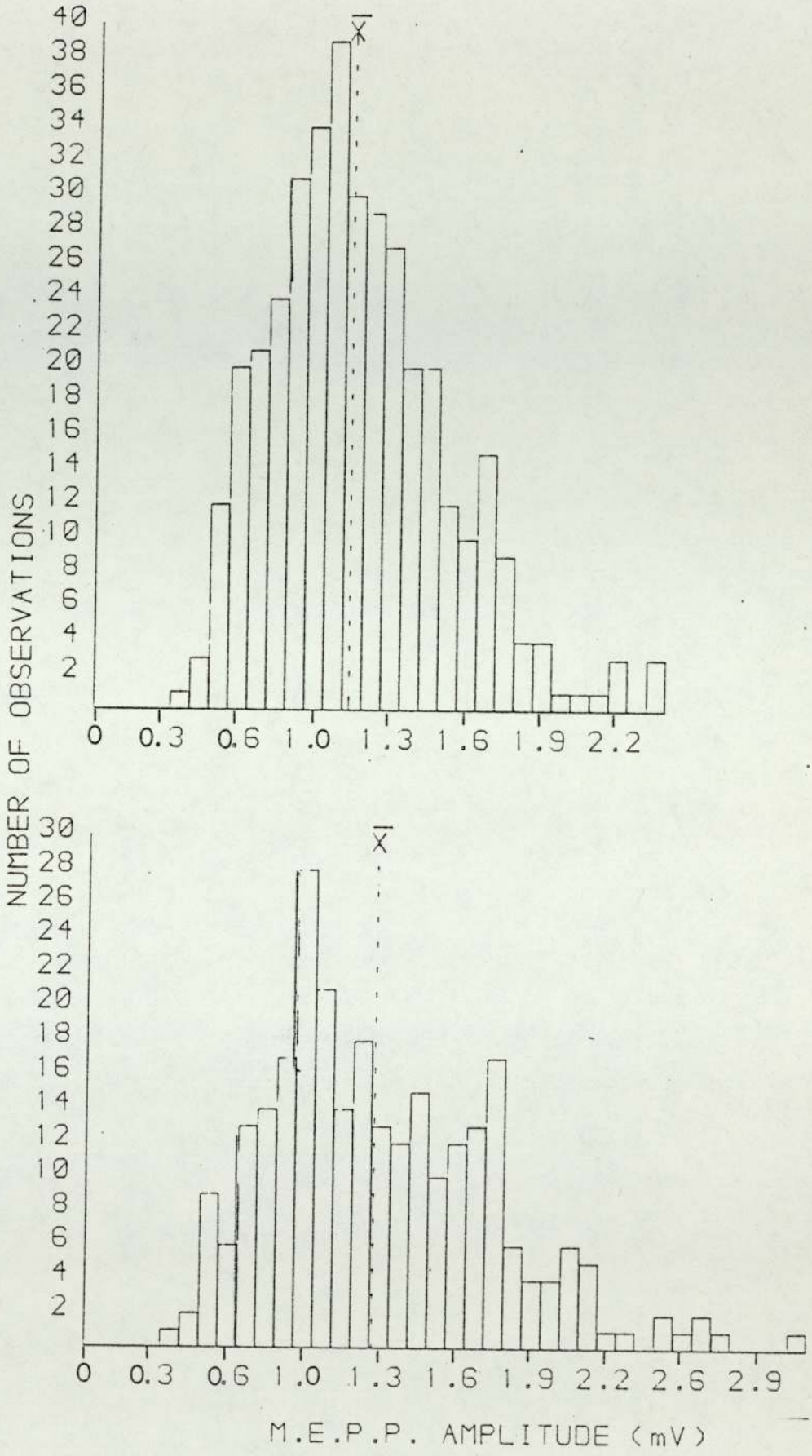


Fig. 8 Distribution of mean m.e.p.p. amplitude values in diaphragm muscles from normal (upper) and dystrophic (lower) male mice. $\bar{x}$ indicates the mean. Temperature 37 ± 1°C.

Endplate potentials in curarised EDL and diaphragm muscles.

Having found that mean m.e.p.p. amplitude in dystrophic EDL muscles was greater than that in normal muscles, e.p.p. amplitudes were also compared. Endplate potentials, elicited at a frequency of 10Hz or 50Hz, were recorded from curarised EDL muscles from 8 week old normal and dystrophic male mice (table 5A). Endplate potentials, elicited at 10Hz, were also recorded from curarised diaphragm muscles from 15 week old normal and dystrophic male mice (table 5B).

Corrected amplitudes of first e.p.ps. in the trains were greater in the dystrophic EDL. This increase in first e.p.p. amplitude will obviously contribute towards the resistance to curare. Corrected amplitudes of plateau e.p.ps. were larger in the dystrophic EDL at both frequencies of stimulation. At 10Hz mean plateau e.p.p. amplitude in EDL muscles from dystrophic mice was 2.44 times greater than the normal value. This ratio was greater than the m.e.p.p. amplitude ratio (1.50) in these muscles suggesting an increased output of transmitter from motor nerve terminals in the dystrophic EDL. Uncorrected e.p.p. amplitudes were also greater in the dystrophic EDL eliminating the possibility that the results are an artefact produced by applying the formula for correction of

Table 5. Evoked transmitter release in curarised normal and dystrophic EDL (A) and diaphragm (B) muscles. Amplitudes corrected for nonlinear summation and to a standard RMP (see Methods). Temperature 33 ± 1°C (EDL) and 37 ± 1°C (diaphragm).

A.		Normal EDL	Dystrophic EDL
10 Hz	First e.p.p. amplitude (mV)	1.69 ± 0.80 (28/3)	* 4.04 ± 1.48 (27/3)
	Plateau e.p.p. amplitude (mV)	0.84 ± 0.35 (28/3)	* 2.05 ± 1.04 (27/3)
	Plateau / first e.p.p.	0.51 ± 0.04 (28/3)	0.51 ± 1.04 (27/3)
	E.p.p. half decay time (msec)	0.75 ± 0.20 (28/3)	0.81 ± 0.21 (27/3)
50 Hz	Plateau e.p.p. amplitude (mV)	0.71 ± 0.27 (28/3)	* 1.58 ± 0.75 (20/3)
	Plateau / first e.p.p.	0.44 ± 0.05 (28/3)	* 0.36 ± 0.09 (20/3)

B.		Normal Diaphragm	Dystrophic diaphragm
10 Hz	First e.p.p. amplitude (mV)	3.14 ± 1.51 (43/5)	* 4.39 ± 2.04 (50/5)
	Plateau e.p.p. amplitude (mV)	1.29 ± 0.53 (43/5)	* 1.71 ± 0.73 (50/5)
	plateau / first e.p.p.	0.42 ± 0.10 (43/5)	0.40 ± 0.15 (50/5)
	E.p.p. half decay time (msec)	0.71 ± 0.21 (43/5)	0.66 ± 0.23 (50/5)

Values are the mean ± 1 S.D.
Numbers in parentheses are number of fibres/number of muscles.
* ($p<0.05$)

e.p.p. amplitudes to the dystrophic muscles (see Methods). The distribution of mean plateau e.p.p. amplitudes evoked at 10Hz between fibres from normal and dystrophic EDL muscles (fig.9) shows that the upper range of amplitudes was considerably greater in the dystrophic EDL. There was no significant difference in e.p.p. half decay time between curarised normal and dystrophic EDL muscles.

At 10Hz there was no significant difference in the ratio of plateau e.p.p. amplitudes to that of the first e.p.p. between normal and dystrophic EDL muscles whereas, at 50Hz, the ratio was smaller in the dystrophic EDL indicating that rundown was greater. When e.p.ps. were normalised (see Methods), rundown at 10Hz was also found to be greater in the dystrophic EDL (fig. 10A). The mean values of the slopes of the rundown were -0.70 ± 0.21 (23) and -0.48 ± 0.12 (25) in fibres from the dystrophic and normal muscles, respectively ($p<0.002$). The rundown of normalised e.p.ps. at 50Hz is shown in fig.10B. The slope of the rundown was again significantly steeper in dystrophic muscles compared to normal i.e. -0.49 ± 0.10 (19) in fibres from dystrophic muscles and -0.30 ± 0.08 (27) in fibres from normal muscles ($p<0.002$). The steeper slopes also provide evidence of a presynaptic difference between normal and dystrophic EDL muscles.

Some parameters of evoked release obtained in the curarised

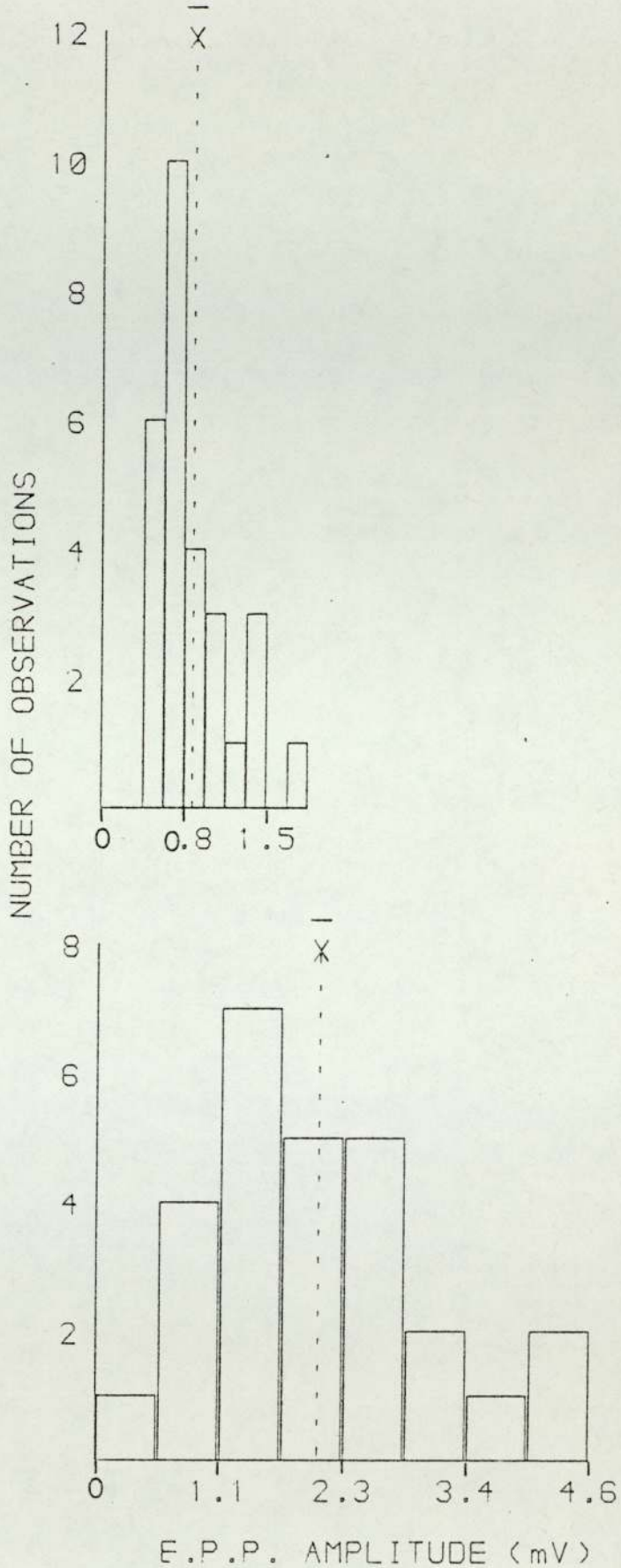


Fig. 9 Distribution of mean plateau e.p.p. amplitudes elicited at 10 Hz in curarised EDL muscles from normal (upper) and dystrophic (lower) male mice. $\bar{x}$ indicates the mean. Temperature 33 ± 1°C.

Fig.10A. Plot of e.p.p. rundown at 10Hz in curarised EDL muscle fibres. Closed circles are mean values from 25 fibres from normal muscles and open circles are mean values from 23 fibres from dystrophic muscles.

Fig. 10B. Plot of e.p.p. rundown at 50Hz in curarised EDL muscle fibres. Closed circles are mean values from 27 fibres from normal muscles and open circles are mean values from 19 fibres from dystrophic muscles. Vertical bars indicate ± 1 s.e. of mean where this exceeds the diameter of the points. The lines show the least squares regression.

Fig. 10A

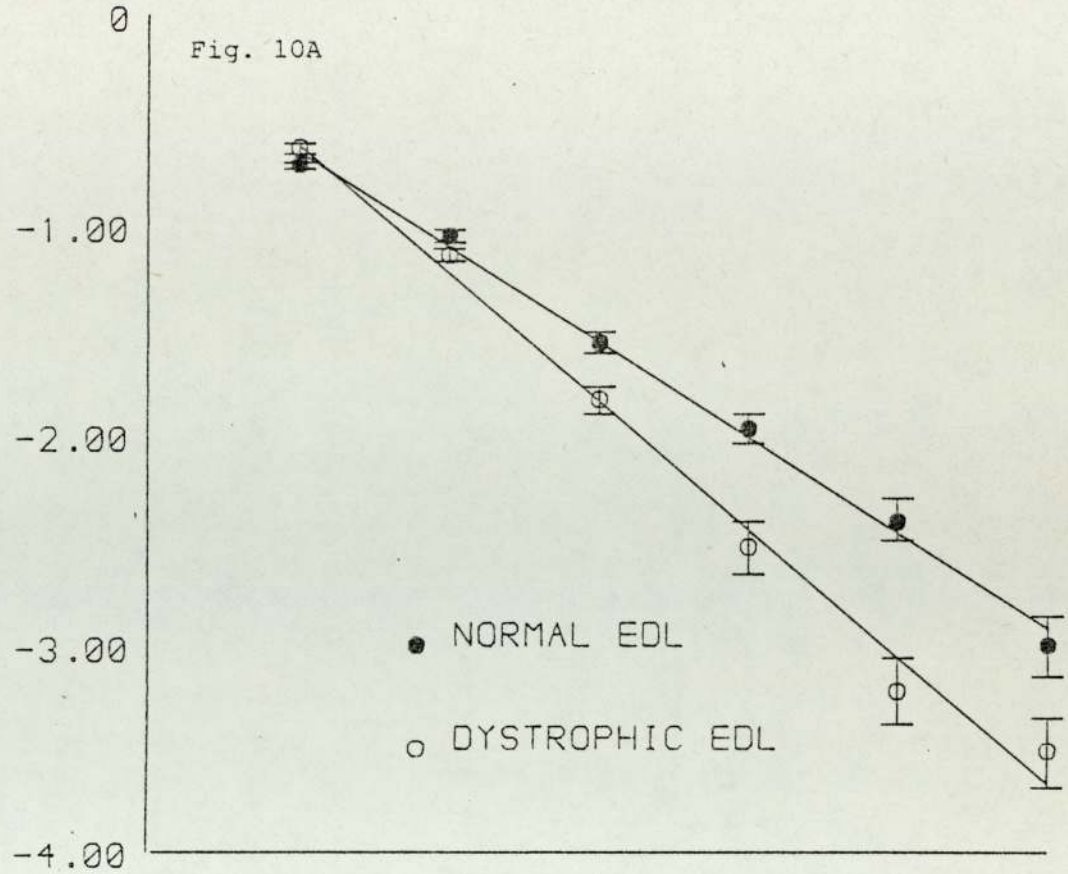
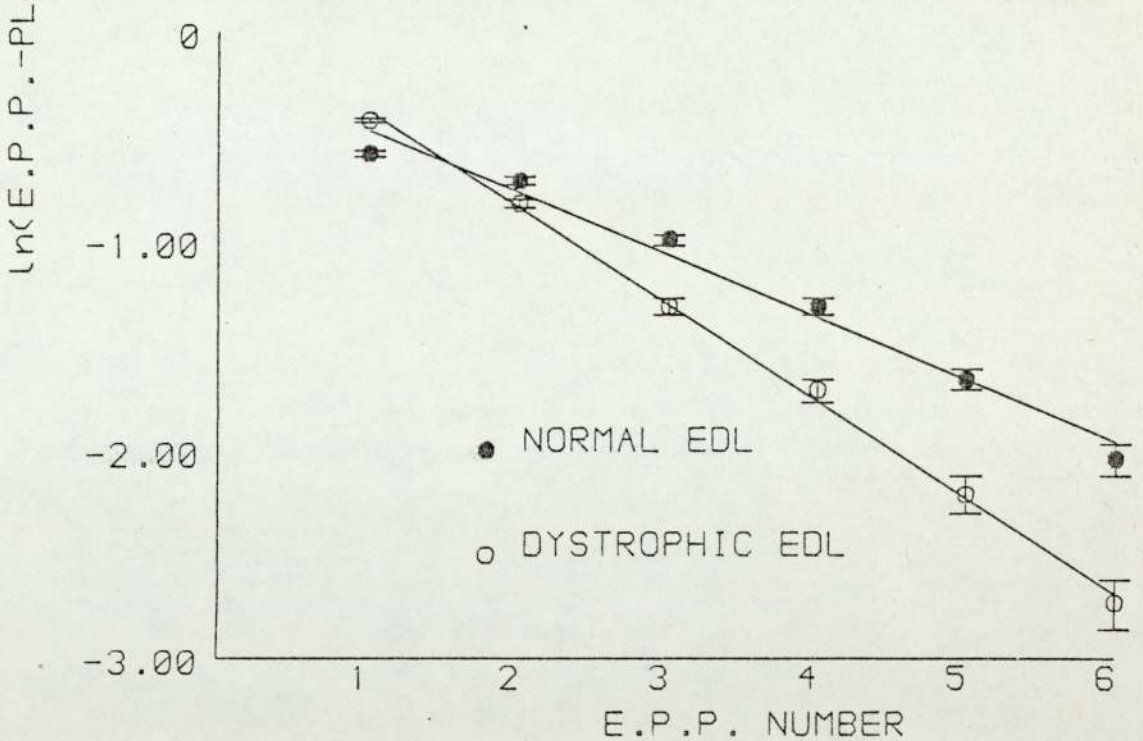


Fig. 10B



diaphragm are given in table 5B. Plateau e.p.p. amplitudes were significantly larger in dystrophic muscle but the increase in amplitude was less than in the EDL. The distribution of mean plateau amplitudes between fibres (fig.11) shows no large amplitude e.p.ps. in the dystrophic diaphragm. There was also no significant difference in rundown between normal and dystrophic diaphragms. The slopes of the rundown were -0.48 ± 0.14 (26) for normal muscles and -0.51 ± 0.19 (28) for dystrophic muscles. In accordance with the m.e.p.p. results e.p.p. half decay time did not differ between normal and dystrophic diaphragm muscles.

Endplate potentials in magnesium blocked EDL and diaphragm muscles.

It is not possible to calculate quantum content (m) of the e.p.p. by the direct method in the curarised preparation. However, m can be measured in muscles in which contraction has been prevented by raising $[Mg^{2+}]o$ and/or reducing $[Ca^{2+}]o$ Endplate potentials were recorded under these conditions in EDL muscles from normal and dystrophic male mice aged 8 weeks. Endplate potentials were also recorded in magnesium blocked diaphragm muscles from male mice aged 14 to 18 weeks.

The results obtained in the EDL are shown in table 6. At all three frequencies of motor nerve stimulation used, 1,

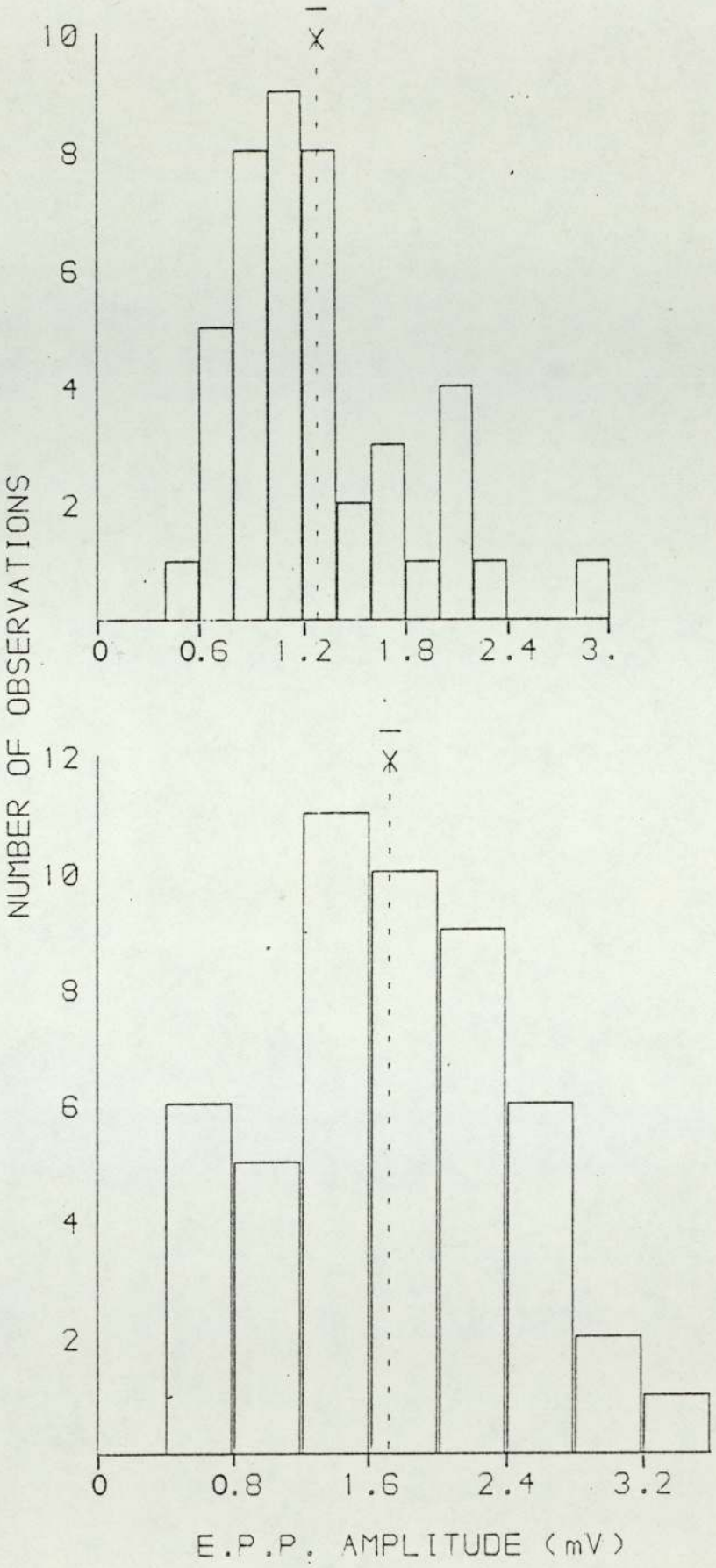


Fig. 11. Distribution of mean plateau e.p.p. amplitudes elicited at 10 Hz in curarised diaphragm muscles from normal (upper) and dystrophic (lower) male mice. $\bar{x}$ indicates the mean, Temperature 37 ± 1°C.

Table 6. Evoked transmitter release in magnesium blocked EDL muscles from 8 week old normal and dystrophic male mice. Amplitudes corrected for nonlinear summation and to a standard RMP and quantum content determined by the direct method (see Methods). Temperature 33 ± 1°C.

		NORMAL	DYSTROPHIC
1 Hz	Plateau e.p.p. amplitude (mV)	0.90 ± 0.41 (34/5)	* 2.85 ± 2.11 (15/6)
	m	2.65 ± 1.15 (34/5)	* 6.09 ± 3.87 (15/6)
10 Hz	Plateau e.p.p. amplitude (mV)	0.94 ± 0.39 (38/5)	* 2.53 ± 1.60 (30/6)
	m	2.69 ± 1.23 (38/5)	* 5.25 ± 3.57 (30/6)
	E.p.p. half decay time (msec)	0.76 ± 0.19 (38/5)	* 0.92 ± 0.31 (30/6)
50 Hz	Plateau e.p.p. amplitude (mV)	1.43 ± 0.60 (36/5)	* 3.23 ± 2.00 (27/6)
	m	4.14 ± 1.83 (36/5)	* 6.92 ± 4.58 (27/6)

Values are the mean ± 1 S.D.

Numbers in parentheses are number of fibres/number of muscles

* ($p < 0.05$)

10 and 50Hz, corrected plateau e.p.p. amplitudes and quantum contents were larger in the dystrophic EDL. The distribution of mean quantum contents elicited at 10Hz between fibres is shown in fig.12. It can be seen that there is a population of fibres with large quantum contents in dystrophic EDL muscles which are not found in muscles from normal littermates. Endplate potential half decay time was also greater in the dystrophic EDL.

Parameters of evoked release in the diaphragm are given in table 7. There was no significant difference in amplitudes of plateau e.p.ps. and quantum contents between diaphragm muscles from normal and dystrophic mice. This indicates that transmitter output is not enhanced in the dystrophic diaphragm. Endplate potential half decay times also did not differ significantly between normal and dystrophic diaphragm muscles.

Endplate potentials in the cut-fibre EDL preparation.

It has been shown that e.p.p. amplitude and m are larger in EDL muscles from dystrophic mice than in muscles from normal littermates. However, in order to record e.p.ps. in the absence of muscle contraction curare or raised $[Mg^{2+}]o$ were used. The use of curare prevents the estimation of m by the direct method and curare may also act presynaptically, for it has been reported that after curare there was an increased e.p.p. and endplate current (e.p.c.)

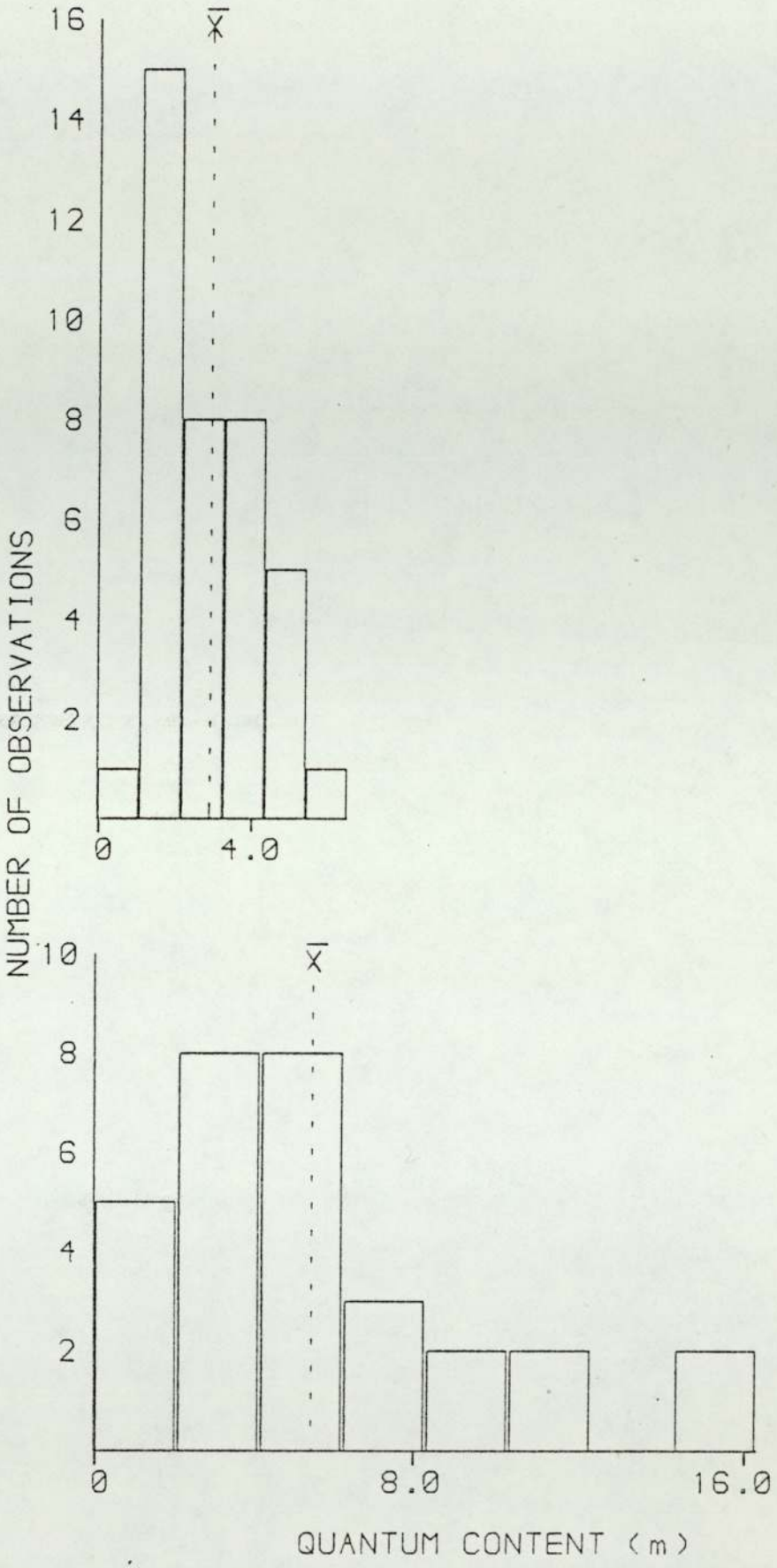


Fig. 12. Distribution of mean quantum contents elicited at 10 Hz in magnesium blocked normal (upper) and dystrophic (lower) EDL muscles. $\bar{x}$ indicates the mean. Temperature 33 ± 1°C.

Table 7. Evoked transmitter release in magnesium blocked diaphragm muscles from 14 to 18 week old normal and dystrophic male mice. Amplitudes corrected for nonlinear summation and to a standard RMP and quantum content determined by the direct method (see Methods). Temperature 37 ± 1°C.

		NORMAL	DYSTROPHIC
10Hz	Plateau e.p.p. amplitude (mV)	1.28 ± 0.68 (82/9)	1.21 ± 0.60 (36/5)
	m	1.43 ± 0.67 (82/9)	1.48 ± 0.74 (36/5)
	E.p.p. half decay time (msec)	0.75 ± 0.18 (82/9)	0.77 ± 0.23 (36/5)

Values are the mean 1 S.D.

Numbers in parentheses are number of fibres/number of muscles.

rundown and decreased m of the first and plateau e.p.ps. (Hubbard and Wilson, 1973; Glavinović, 1979b; Magleby Pallota and Terrar, 1981). It has also been claimed that curare increases the quantum content of the first e.p.p. but has no effect on the plateau e.p.p. (Blaber, 1970, 1973; Wilson, 1982). Raising $[Mg^{2+}]o$ also reduces transmitter release (Fatt and Katz, 1951). The cut muscle fibre preparation (Barstad, 1962; Barstad and Lilleheil, 1968) offers the possibility of studying neuromuscular transmission in the absence of blocking agents. Therefore, e.p.ps. elicited at a frequency of 10Hz were recorded from normal and from dystrophic EDL muscles in which the fibres had been cut as described in the Methods section.

The results are shown in table 8. Plateau e.p.p. amplitude, m and e.p.p. half decay time were all larger in the dystrophic EDL. The upper range of quantum contents was again considerably wider in the dystrophic EDL (fig.13). Rundown in the cut-fibre preparation was less pronounced than in the curarised preparation. However, rundown of the first 5 e.p.ps. in the dystrophic EDL was greater than that in the normal EDL but there was no significant difference in the ratio of the plateau amplitude to that of the first e.p.p. (table 8).

Amplitude histograms of plateau e.p.ps. recorded at one junction in the normal EDL and one in the dystrophic EDL are shown in figs. 14A and 14B. When theoretically

Table 8. Evoked transmitter release in cut EDL muscle fibres from 8 week old normal and dystrophic male mice. Temperature 33 ± 1°C. Amplitudes corrected for non-linear summation and to a standard RMP. Quantum content determined by the direct method (see Methods).

	NORMAL	DYSTROPHIC
Plateau e.p.p. amplitude (mV)	13.64 ± 4.60 (34/5)	* 19.98 ± 5.60 (32/6)
Quantum content (m)	36.25 ± 13.81 (34/5)	* 53.57 ± 19.19 (32/6)
E.p.p. half decay time (msec)	0.70 ± 0.10 (34/5)	* 0.96 ± 0.26 (32/6)
plateau first e.p.p.	0.76 ± 0.13 (31/5)	0.72 ± 0.06 (32/6)

Values are the mean ± 1 S.D.

Numbers in parentheses are number of fibres/number of muscles

* ($p<0.05$).

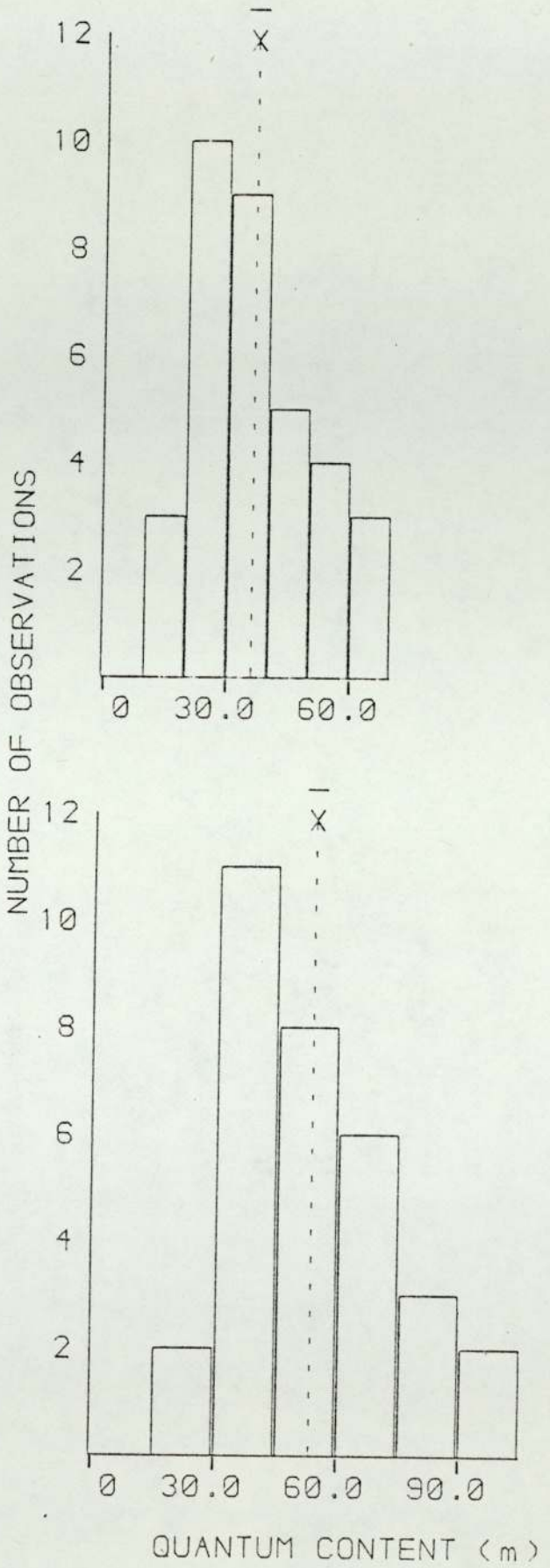


Fig. 13. Distribution of mean quantum contents elicited at 10 Hz in cut normal (upper) and dystrophic (lower) EDL muscle fibres. x indicates the mean. Temperature 33 ± 1°C.

Fig. 14. Amplitude histograms of e.p.ps. at one neuromuscular junction in the cut-fibre normal EDL (14A) and one neuromuscular junction in the cut-fibre dystrophic EDL (14B). The binomial predictions for the amplitudes of 90 plateau e.p.ps. elicited at 10 Hz are given by the continuous curves and the Poisson predictions for the same amplitudes by the interrupted lines. The probabilities for the binomial were >0.50 and for the Poisson <0.001. In 14A, m, n and p were 37.4, 52 and 0.72, respectively and in 14B, m, n and p were 53.8, 57 and 0.94, respectively.

Fig 14A

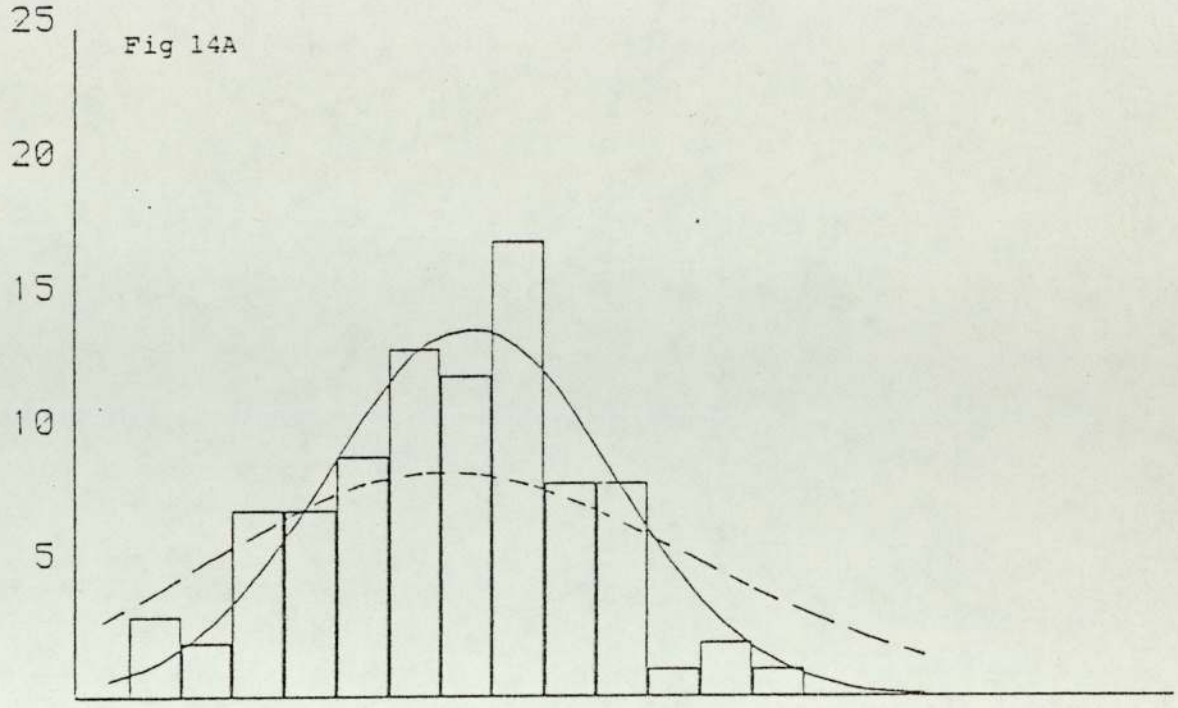
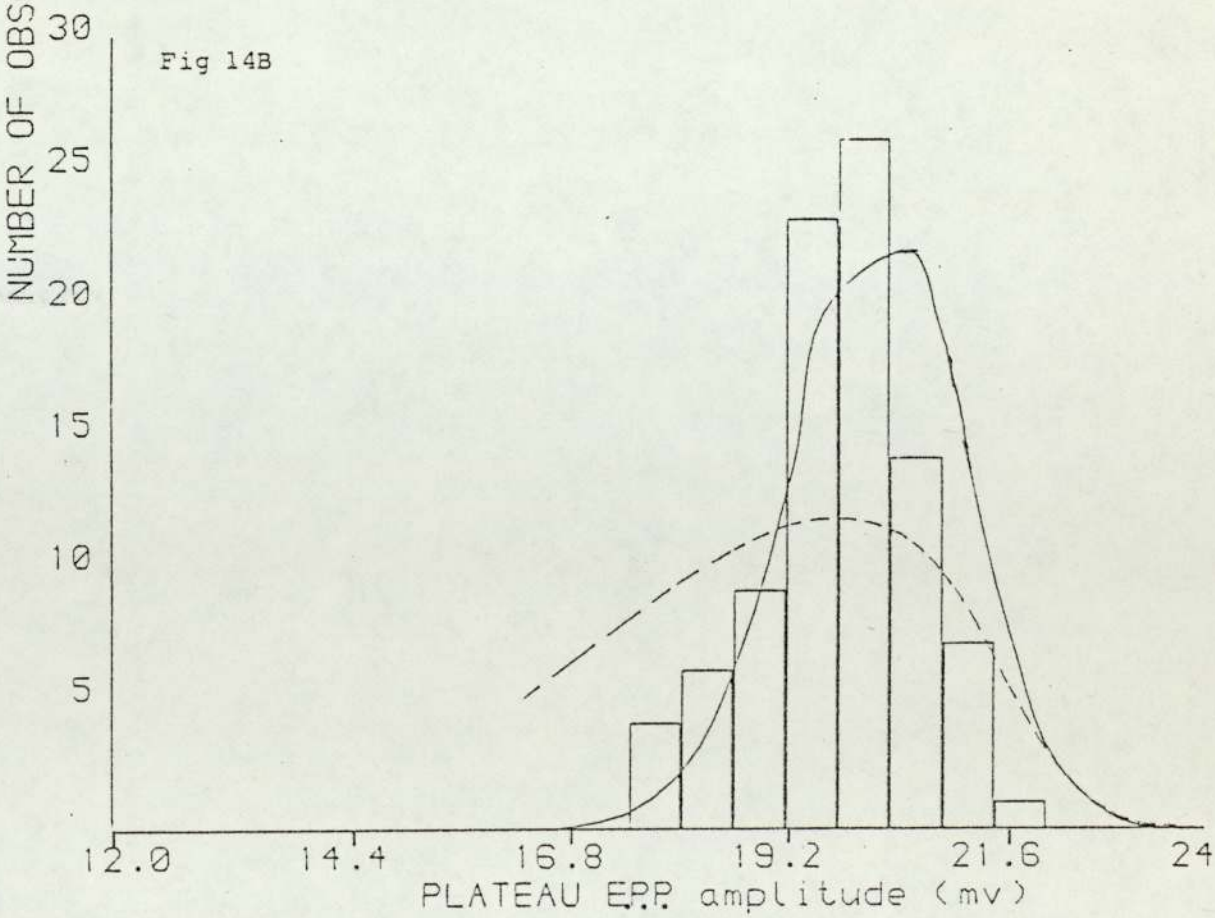


Fig 14B



determined binomial and Poisson distributions were fitted to the amplitude histograms it was found that the binomial distribution gave the best fit (as determined by the χ^2 test). This was the case at all neuromuscular junctions examined. In view of this the available store (n) and the probability of release (p) were calculated (see Methods). The results are shown in table 9.

Quantum content values are comparable to those shown in table 8, values of m in EDL muscles from dystrophic animals being larger than those in muscles from normal littermates. There was no significant difference in n between normal and dystrophic muscles, but p was larger in EDLs from dystrophic animals. Therefore, the greater m in the dystrophic EDL can be attributed to a greater p.

Factors affecting spontaneous transmitter release.

The preceding results indicate that in EDL muscles from dystrophic mice quantum content is greater than normal. Rahamimoff and Yaari (1973) proposed that the rise in intraterminal free calcium ($[Ca^{2+}]i$) necessary for evoked transmitter release, is the result of increased influx of calcium across the nerve terminal membrane and also release of calcium from an internal store. Therefore, the greater m in the dystrophic EDL could be explained by a raised $[Ca^{2+}]i$ due to either a greater calcium influx during the action potential or a reduced ability of the nerve terminal to

Table 9. Binomial parameters (m, n and p) in cut-fibre EDL muscles from 8 week old normal and dystrophic male mice. Temperature 33 ± 1°C.

	NORMAL	DYSTROPHIC
Quantum content (m)	39.92 ± 13.27 (17/2)	* 56.39 ± 14.75 (15/3)
Number of quanta available for release (n)	65.60 ± 28.06 (17/2)	62.16 ± 16.23 (15/3)
Probability of release (p)	0.66 ± 0.18 (17/2)	* 0.91 ± 0.07 (15/3)

Values are the mean ± 1 S.D.

Numbers in parentheses are number of fibres/number of muscles
* ($p<0.05$)

regulate the calcium which enters.

The rate of spontaneous transmitter release from motor nerve terminals at rest is thought to depend upon the $[Ca^{2+}]i$ (Baker, 1972). Therefore to compare nerve terminal calcium regulation in muscles from dystrophic mice and normal littermates, m.e.p.p, frequency was recorded both before and after the application of various agents and procedures which are thought to raise $[Ca^{2+}]i$.

Effect of raising external calcium concentration.

The effect of raising external calcium concentration ($[Ca^{2+}]o$) on m.e.p.p. frequency in EDL and diaphragm muscles from normal and dystrophic male mice was investigated at both 33 ± 1°C and room temperature. At 33 ±1°C, in 2mM $[Ca^{2+}]o$, there was no significant difference between m.e.p.p. frequency in the dystrophic EDL and m.e.p.p. frequency in the normal EDL (table 10). When $[Ca^{2+}]o$ was raised from 2mM to 6mM a significant increase in m.e.p.p. frequency was observed in normal and dystrophic muscles which was not accompanied by any significant change in RMP. In 6mM $[Ca^{2+}]o$ there was no significant difference in m.e.p.p. frequency between normal and dystrophic EDL muscles and frequency histograms in 6mM $[Ca^{2+}]o$ showed a similar distribution.

It has been reported that, at room temperature, m.e.p.p.

Table 10. Effect of external calcium concentration on m.e.p.p. frequency (Hz) in EDL muscles from normal and dystrophic male mice at 20 ± 1°C and 33 ± 1°C.

		2mM $[Ca^{2+}]o$	6mM $[Ca^{2+}]o$
20 ± 1°C	NORMAL EDL	1.99 ± 0.82 (50/5) ★	2.84 ± 1.20 (49/4)
	DYSTROPHIC EDL	2.24 ± 1.09 (48/5) ★	3.31 ± 1.62 (45/5)
33 ± 1°C	NORMAL EDL	7.61 ± 3.31 (41/4) ★	10.68 ± 4.34 (42/4)
	DYSTROPHIC EDL	6.48 ± 3.00 (30/3) ★	11.57 ± 3.13 (25/3)

Values are the mean ± 1 S.D.

Numbers in parentheses are the number of fibres/number of muscles.

★($p<0.05$)

frequency is increased by raising $[Ca^{2+}]o$ from 3.8 to 7.6mM in EDL muscles from dystrophic mice of the C57BL/6J strain but not in normal muscles (Shalton and Wareham, 1980). These results differ from those obtained at 33 ± 1°C in the 129/ReJ strain. Therefore, the effect of 6mM $[Ca^{2+}]o$ on m.e.p.p. frequency was re-examined at room temperature (20 ± 1°C). The results are shown in table 10.

In 2mM $[Ca^{2+}]o$ there was no significant difference in m.e.p.p. frequency between EDL muscles from normal littermates and dystrophic mice and raising $[Ca^{2+}]o$ from 2mM to 6mM produced an equivalent increase in m.e.p.p. frequency in normal and dystrophic EDL muscles. Thus, in the 129/ReJ strain, there is no difference in the sensitivity of m.e.p.p. frequency to raised $[Ca^{2+}]o$ between normal and dystrophic EDL muscles either at room temperature or at 33 ± 1 C.

The effect of raising $[Ca^{2+}]o$ on m.e.p.p. frequency in diaphragm muscles from normal and dystrophic mice at 20 ± 1°C and 37 ± 1°C is shown in table 11. At both temperatures there was no significant difference in m.e.p.p. frequency in 2mM $[Ca^{2+}]o$ between diaphragms from normal and dystrophic animals. Raising $[Ca^{2+}]o$ to 6mM caused a significant increase in m.e.p.p. frequency in muscles from both normal and dystrophic animals without change in RMP. At room temperature there was no significant difference in m.e.p.p. frequency in 6mM $[Ca^{2+}]o$ between normal and

Table 11. Effect of external calcium concentration on m.e.p.p. frequency (Hz) in diaphragm muscles from normal and dystrophic male mice at 20 ± 1°C and 37 ± 1°C.

		2mM $[Ca^{2+}]o$	6mM $[Ca^{2+}]o$
20 ± 1°C	NORMAL DIAPHRAGM	1.18 ± 0.47 (45/4) ★	1.97 ± 0.65 (53/4)
	DYSTROPHIC DIAPHRAGM	1.28 ± 0.41 (34/4) ★	1.93 ± 0.64 (34/3)
37 ± 1°C	NORMAL DIAPHRAGM	5.14 ± 3.18 (50/5) ★	9.17 ± 4.68 (50/5) ★
	DYSTROPHIC DIAPHRAGM	5.86 ± 2.60 (55/5) ★	11.44 ± 4.51 (53/5)

Values are the mean ± 1 S.D.

Numbers in parentheses are the number of fibres/number of muscles sampled.

★ ($p < 0.05$)

dystrophic diaphragm muscles. However, at 37±1°C, m.e.p.p. frequency in the dystrophic diaphragm was slightly but significantly greater than that in the normal muscle.

Since this result differed from that observed in the dystrophic EDL the effect of $[Ca^{2+}]o$ on m.e.p.p. frequency in the diaphragm at 37 ± 1° C was investigated more fully. Immediately after dissection diaphragms were placed in Liley's solution containing 0,2,4,6 or 8mM $[Ca^{2+}]o$ and allowed to equilibrate for 30 minutes before recording of m.e.p.p. frequency commenced. The results are represented graphically in fig.15.

In 0, 2 or 4mM $[Ca^{2+}]o$ there was no significant difference in m.e.p.p. frequency between normal and dystrophic diaphragms. At calcium concentrations above 4mM m.e.p.p. frequency in normal diaphragms did not increase further as $[Ca^{2+}]o$ was increased. A similar result was not obtained in dystrophic diaphragms until $[Ca^{2+}]o$ was increased above 6mM. Miniature e.p.p. frequency in dystrophic diaphragms in 6mM and 8mM $[Ca^{2+}]o$ was greater than that in normal muscles. The effect of $[Ca^{2+}]o$ on m.e.p.p. frequency at 37 ± 1° C was also examined in diaphragm muscles from white mice (fig.15). Miniature e.p.p. frequency was increased by raising $[Ca^{2+}]o$ at all calcium concentrations investigated. This result eliminates the possibility that increased m.e.p.p. frequency with increased $[Ca^{2+}]o$ is restricted to muscles from mice of the 129/ReJ strain.

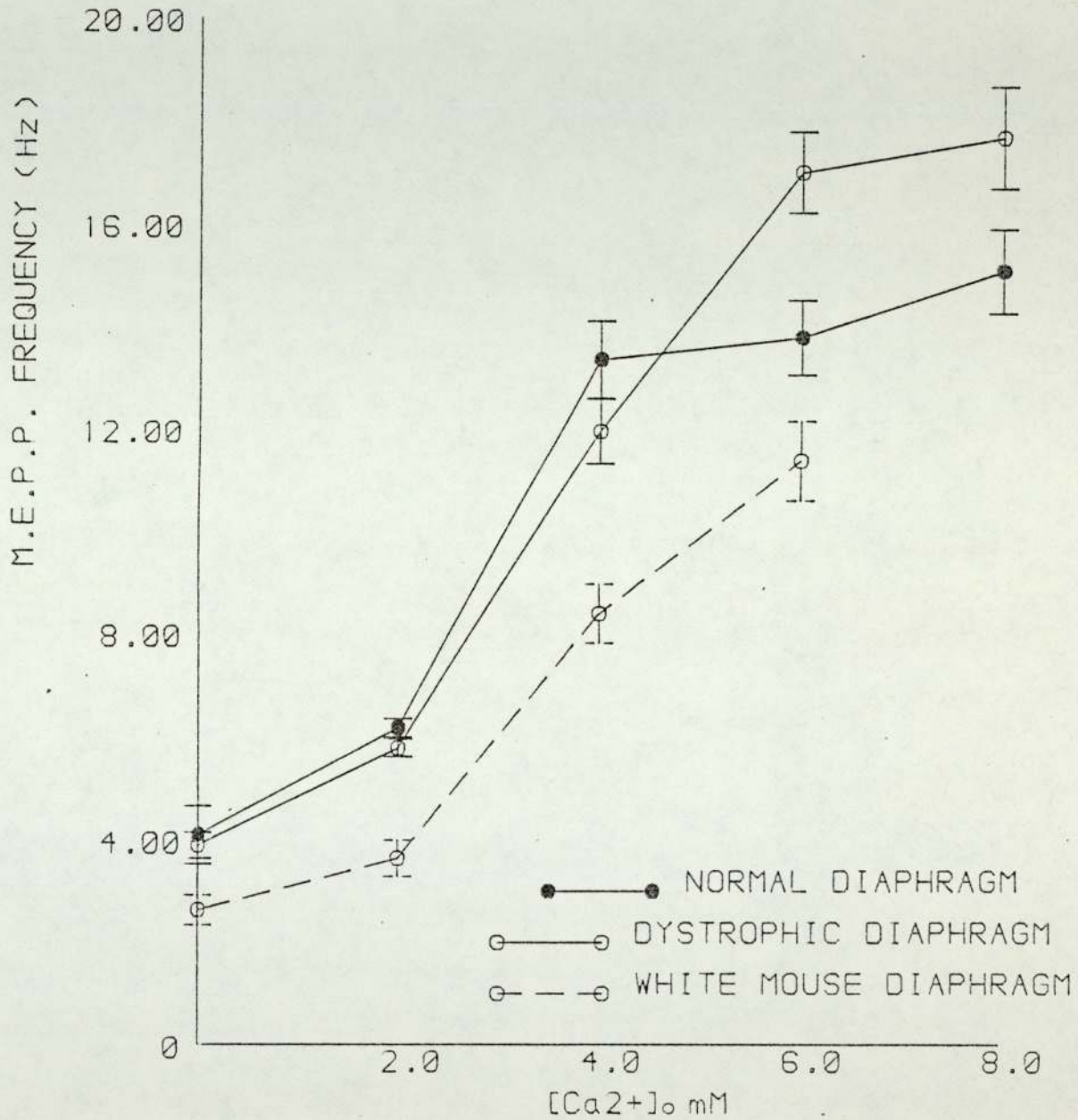


Fig. 15. The effect of external calcium concentration on m.e.p.p. frequency (Hz) in diaphragm muscles from dystrophic mice, normal littermates and white mice at 37 ± 1°C.

Effect of potassium chloride.

The effect of potassium chloride on m.e.p.p. frequency and RMP recorded at 33 ± 1° C in EDL muscles from normal and dystrophic female mice is shown in table 12. Miniature e.p.p. frequency increased within 5 minutes of the addition of KCl (10^{-2}M) to the bathing solution and continued to rise for about 25 minutes, after which time no further increase was found. This increase in m.e.p.p. frequency was accompanied by a decrease in RMP. There was no difference in the time course of action of KCl in EDL muscles from normal and dystrophic mice nor was there any significant difference in the increase in m.e.p.p. frequency. In KCl (10^{-2}M) RMP was decreased by 18% in the normal EDL and 10% in the dystrophic EDL. However, the RMP in normal and dystrophic muscles was still significantly different.

Effect of motor nerve stimulation

The effect of motor nerve stimulation on m.e.p.p. frequency was compared in normal and dystrophic EDL cut-fibre preparations. The motor nerve was stimulated at 50Hz for 1 minute. Control m.e.p.p. frequency in dystrophic muscles was significantly greater than that in normal muscles, therefore, results are expressed as the ratio of m.e.p.p. frequency after stimulation to control values recorded

Table 12. Effect of potassium chloride (10^{-2}M) on m.e.p.p. frequency and RMP in EDL muscles from normal and dystrophic female mice. Temperature 33 ± 1°C.

		Control KCl (5×10^{-3}M)	KCl (10^{-2}M)
M.e.p.p. Frequency (Hz)	NORMAL EDL	7.19 ± 3.69 (20/3) ★	19.67 ± 10.06 (29/3)
	DYSTROPHIC EDL	5.31 ± 3.35 (23/4) ★	21.76 ± 14.29 (42/4)
RMP (mV)	NORMAL EDL	78.88 ± 9.25 (20/3 ★	64.42 ± 9.60 (29/3) ★
	DYSTROPHIC EDL	66.48 ± 7.70 (23/4) X	60.08 ± 8.25 (42/4)

Values are the mean ± 1 S.D.

Numbers in parentheses are the number of fibres/number of muscles.

★($p<0.05$)

prior to stimulation. In both normal and dystrophic muscles post-tetanic potentiation of m.e.p.p. frequency was observed. Miniature e.p.p. frequency increased 2.28 ± 0.86 times in dystrophic EDL muscles (n=28) and 2.38 ± 1.03 times in normal EDL muscles (15). These values were not significantly different. One minute after stimulation m.e.p.p. frequency had declined to control levels in normal and dystrophic muscles. Therefore, it appears that motor nerve stimulation produces a similar post-tetanic potentiation of m.e.p.p. frequency in normal and dystrophic EDL muscles.

Effect of uncouplers of oxidative phosphorylation.

2,4-dinitrophenol (DNP).

The effect of DNP (10^{-4}M) on m.e.p.p. frequency in EDL and diaphragm muscles from normal and dystrophic mice was investigated at both 20 ± 1°C and 33 ± 1°C. At 20 ± 1°C, m.e.p.p. frequency and RMP recorded before and 30 minutes or more after the addition of DNP to the bathing solution are given in table 13. Dinitrophenol caused a sevenfold increase in m.e.p.p. frequency in EDL muscles from dystrophic mice. The range of frequencies recorded in this muscle was considerable (1.23-69.44 Hz), however, the mean frequency was greater than that recorded in the normal EDL, which was only slightly elevated above the control level. This effect of DNP on m.e.p.p. frequency was not accompanied

Table 13. Effect of 2,4-dinitrophenol (10^{-4}M) on m.e.p.p. frequency and RMP in EDL muscles from normal and dystrophic male mice at 20 ± 1°C.

		CONTROL	DNP (10^{-4}M)
M.e.p.p. frequency (Hz)	NORMAL EDL	2.13 ± 1.16 (48/5) ★	3.37 ± 2.46 (19/5) ★
	DYSTROPHIC EDL	2.53 ± 0.96 (28/3) X	18.44 ± 16.70 (24/3)
RMP (mV)	NORMAL EDL	75.48 ± 11.53 (48/5) ★	71.62 ± 12.52 (19/5) ★
	DYSTROPHIC EDL	68.16 ± 10.99 (28/3)	66.56 ± 12.16 (24/3)

Values are the mean ± 1 S.D.

Numbers in parentheses are the number of fibres/number of muscles.

★($p < 0.05$)

by any significant change in RMP.

It appeared initially that m.e.p.p. frequency in the dystrophic EDL was more sensitive to DNP than that in the normal EDL. A similar observation has been made in the C57BL/6J strain of mice at room temperature (Shalton and Wareham, 1980). The effect of DNP on m.e.p.p. frequency was further investigated by examining the change in m.e.p.p. frequency with time. The time course of the effect of DNP on m.e.p.p. frequency in three fibres from normal and dystrophic EDL muscles is shown in fig.16. In the normal fibres a thirtyfold or more increase in m.e.p.p. frequency was observed within 20 minutes after DNP which was followed by a reduction in m.e.p.p. frequency towards control. However, it was not possible to record from any normal fibre for longer than 25 minutes so the final frequency in each fibre is not known. In contrast, over the same period of time, DNP had less effect on the three fibres from dystrophic muscles. In one fibre, after 22 minutes, the microelectrode came out but a second fibre in the same muscle was penetrated and m.e.p.p. frequency continued to rise, but not to the level observed in the normal EDL. Therefore, m.e.p.p. frequency in the dystrophic EDL is actually less sensitive to DNP than that in the normal EDL.

At 33 ± 1°C, DNP (10^{-4}M) produced a visible contracture in all three normal muscles examined but not in any of the

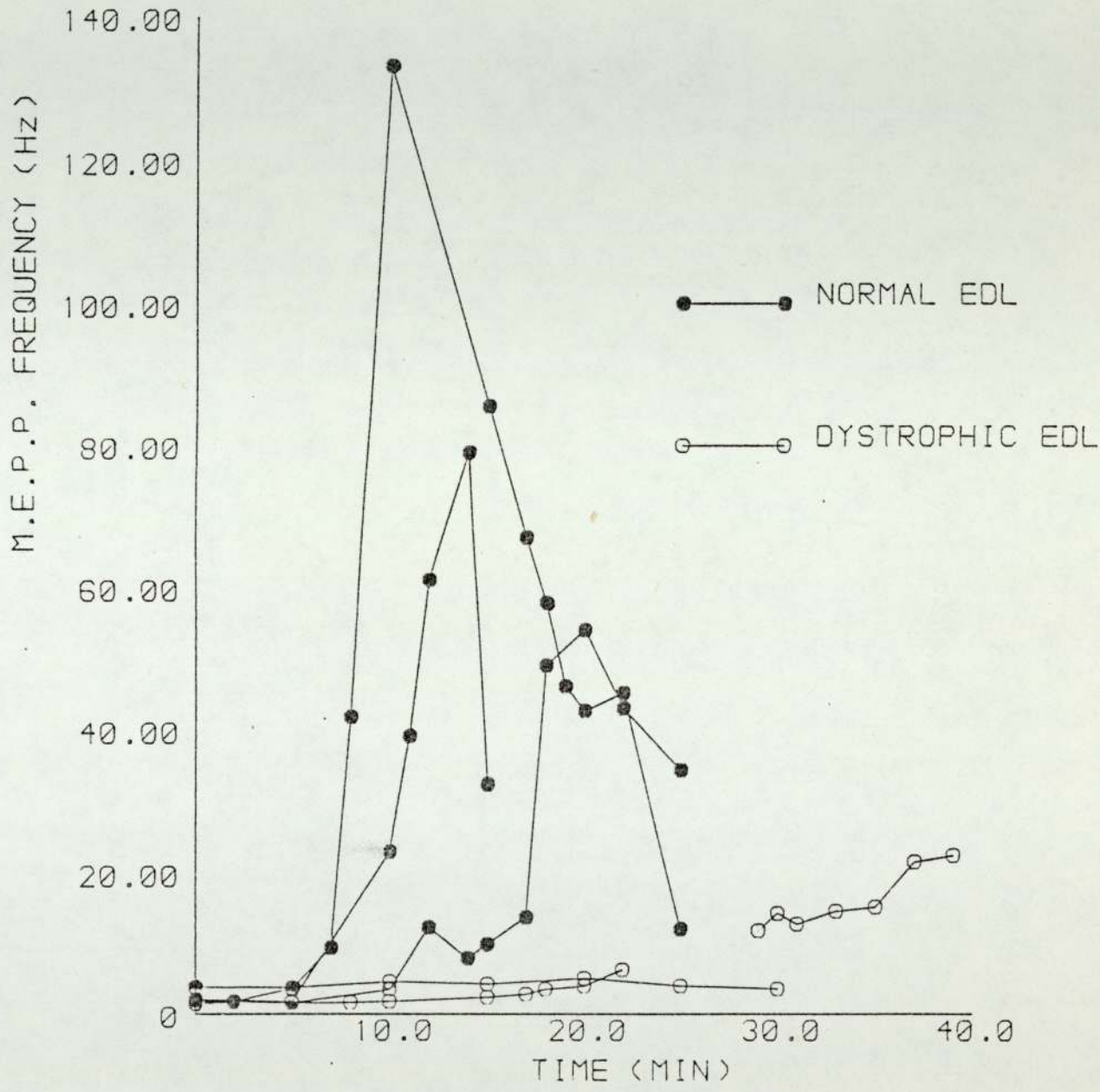


Fig. 16. The effect of DNP (10^{-4}M) on m.e.p.p. frequency (Hz) in EDL muscles from normal and dystrophic mice at 20 ± 1°C.

four dystrophic muscles. The effect of DNP on m.e.p.p. frequency in three fibres from normal and three fibres from dystrophic EDL muscles is shown in fig.17. Six minutes after the addition of DNP to the bathing solution, m.e.p.p. frequency in the three normal fibres increased fourfold, sevenfold and tenfold compared with a doubling of m.e.p.p. frequency in one dystrophic fibre and no change in the other two. Recording in each muscle was continued for up to 60 minutes and during this period m.e.p.ps. could be found without difficulty in dystrophic muscles. However, in normal muscles after approximately 15 minutes no m.e.p.ps. were observed and it became difficult to penetrate fibres, probably because of the contracture of the muscle fibres. Dinitrophenol (10^{-4}M) had no significant effect on m.e.p.p. frequency in the dystrophic EDL. The mean m.e.p.p. frequency in the five normal fibres in which m.e.p.ps. could be recorded more than 15 minutes after DNP was 58.25 ± 26.59 Hz. However, this result has to be interpreted with caution as it is possible that m.e.p.p. frequency at many endplates was zero. In addition to these effects on m.e.p.p. frequency, DNP caused a slight but significant decrease in RMP.

From these results it appears that the effects of DNP on motor nerve terminals in normal and dystrophic muscles are modified by temperature. However, the results obtained at 33 ± 1°C agree with those obtained at room temperature in that there is a decreased sensitivity of spontaneous

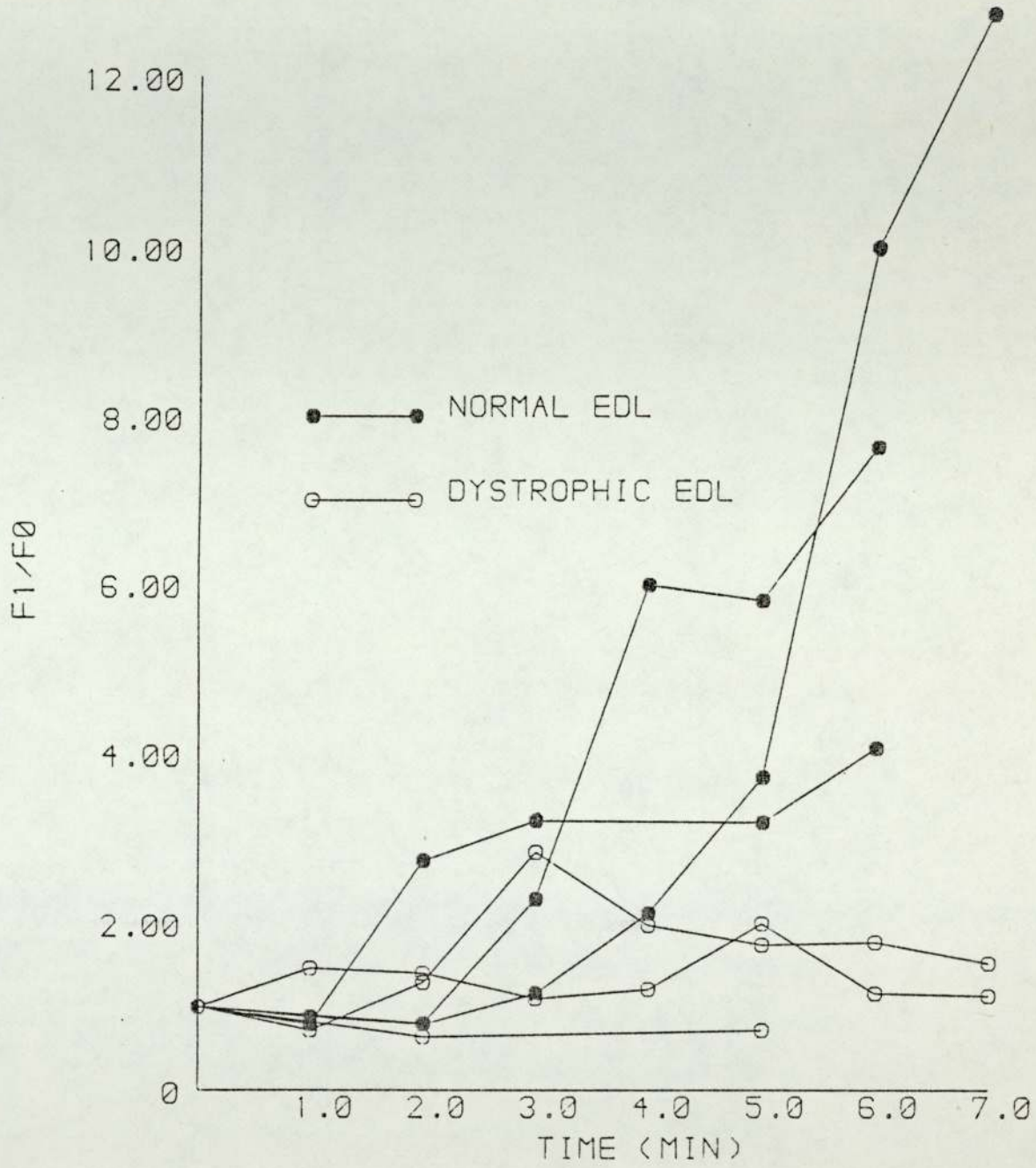


Fig. 17. The effect of DNP (10^{-4}M) on m.e.p.p. frequency in EDL muscles from normal and dystrophic mice at 33 ± 1°C. F0 is the control frequency and F1 the frequency after treatment.

transmitter release to DNP in motor nerve terminals of dystrophic EDL muscles.

Diaphragm preparations were less sensitive to DNP than EDL preparations. At room temperature, there was no significant difference between control m.e.p.p. frequency and that recorded 30 minutes or more after DNP, in diaphragm muscles from dystrophic mice or non-dystrophic littermates. However, DNP did produce a significant decrease in RMP in all muscles. The time course of the effect of DNP on m.e.p.p. frequency in the normal diaphragm was followed in two muscles. Within the 20 minutes that the fibres were impaled, no effect of DNP was observed. Thus the normal diaphragm differs from the normal EDL in this respect.

At 37 ± 1°C there was no significant difference in either control m.e.p.p. frequency or RMP between normal and dystrophic diaphragms (table 14). Dinitrophenol (10^{-4}M) produced a significant increase in m.e.p.p. frequency in the normal diaphragm but had no effect on m.e.p.p. frequency in the dystrophic diaphragm. This effect of DNP was accompanied by a significant decrease in RMP in all diaphragm muscles. In this respect the dystrophic diaphragm resembles the dystrophic EDL, as in both muscles at physiological temperature there is decreased sensitivity of spontaneous release to DNP. The diaphragm also resembled the EDL in that the effect of DNP on m.e.p.p. frequency was

Table 14. Effect of 2,4-dinitrophenol (10^{-4}M) on m.e.p.p. frequency and RMP in diaphragm muscles from normal and dystrophic male mice at 37 ± 1°C.

		CONTROL	DNP (10^{-4}M)
M.e.p.p. frequency (Hz)	NORMAL DIAPHRAGM	5.21 ± 2.77 (41/4) ★	18.52 ± 22.19 (38/4) ★
	DYSTROPHIC DIAPHRAGM	5.63 ± 2.01 (41/4)	6.64 ± 9.20 (25/4)
RMP (mV)	NORMAL DIAPHRAGM	71.02 ± 7.87 (41/4) ★	62.62 ± 9.0 (38/4)
	DYSTROPHIC DIAPHRAGM	70.93 ± 6.98 (41/4) ★	61.96 ± 8.06 (25/4)

Values are the mean ± 1 S.D.

Numbers in parentheses are the number of fibres/ number of muscles.

★($p < 0.05$)

temperature dependent.

Guanidine chloride.

Guanidine, unlike DNP, is a nonphenolic uncoupler of oxidative phosphorylation. Guanidine (5×10^{-3}M) differed from DNP in that, at 33 ± 1°C , it increased m.e.p.p. frequency in both normal and dystrophic muscles (table 15). It also had no significant effect on RMP. Guanidine caused a greater increase in m.e.p.p. frequency in the normal EDL than in the dystrophic EDL.

The results with guanidine together with those obtained with DNP indicate that motor nerve terminals in muscles from dystrophic animals are less sensitive to uncouplers of oxidative phosphorylation.

The effect of guanidine (5×10^{-3}M) on e.p.ps. elicited at 10Hz in curarised EDL muscles from normal and dystrophic female mice was also investigated. Guanidine increased e.p.p. amplitude in normal and dystrophic muscles without any significant effect on RMP. However, in contrast to the effect on spontaneous release, guanidine caused an equivalent enhancement of evoked release in both normal and dystrophic muscles. Guanidine also caused a decrease in the ratio of the amplitudes of successive e.p.ps. to that of the first. This was again observed in both normal and dystrophic muscles.

Table 15. Effect of guanidine (5×10^{-3}M) on m.e.p.p. frequency and RMP in EDL muscles from normal and dystrophic male mice at 33 ± 1°C.

		CONTROL	GUANIDINE (5×10^{-3}M)
M.e.p.p. frequency (Hz)	NORMAL EDL	5.89 ± 2.42 (28/4) ★	18.63 ± 7.60 (34/4) ★
	DYSTROPHIC EDL	4.61 ± 2.05 (26/4) ★	10.56 ± 8.13 (48/4)
RMP (mV)	NORMAL EDL	75.1 ± 8.58 (28/4) ★	71.76 ± 7.56 (34/4) ★
	DYSTROPHIC EDL	68.38 ± 10.25 (26/4)	66.45 ± 11.46 (48/4)

Values are the mean ± 1 S.D.

Numbers in parentheses are the number of fibres/ number of muscles.

★($p < 0.05$)

Effect of caffeine.

The methylxanthine, caffeine, is considered to release calcium from intracellular stores other than mitochondria (Batra, 1974). The effect of caffeine (2×10^{-3} M) on m.e.p.p. frequency in EDL and diaphragm muscles from normal and dystrophic mice is shown in table 16. It can be seen that, at physiological temperatures, caffeine produced a significant increase in m.e.p.p. frequency in EDL and diaphragm muscles from both normal and dystrophic animals. There was no significant difference in the effect of caffeine on m.e.p.p. frequency in EDL muscles from normal and dystrophic animals. However, caffeine caused a greater increase in m.e.p.p. frequency in the dystrophic diaphragm than in the normal diaphragm. This was not the result of the presence of fibres with a m.e.p.p. frequency higher than normal but was due to a larger distribution mode in the dystrophic muscles (fig.18). The increase in m.e.p.p. frequency produced by caffeine was not accompanied by any significant effect on RMP.

Effect of lanthanum chloride

Miniature e.p.p. frequencies and RMP in EDL muscles, from normal and dystrophic mice, bathed in lanthanum saline at 33 ± 1° C were similar to those obtained in Liley's solution (table 17). This was despite the different compositions of

Table 16. Effect of caffeine (2×10^{-3} M) on m.e.p.p. frequency in EDL and diaphragm muscles from normal and dystrophic mice. Temperature 33 ± 1°C (EDL) and 37 ± 1°C (diaphragm).

	CONTROL	CAFFEINE (2×10^{-3} M)
NORMAL EDL	7.51 ± 3.60 (42/4)	★ 14.11 ± 5.78 (36/4)
DYSTROPHIC EDL	6.52 ± 3.16 (41/5)	★ 12.19 ± 8.03 (42/5)
NORMAL DIAPHRAGM	5.08 ± 2.66 (52/5)	★ 8.79 ± 4.62 (76/5) ★
DYSTROPHIC DIAPHRAGM	5.91 ± 2.47 (53/5)	★ 11.86 ± 5.33 (66/5)

Values are the mean ± 1 S.D.

Numbers in parentheses are the number of fibres/number of muscles.

★ ($p < 0.05$)

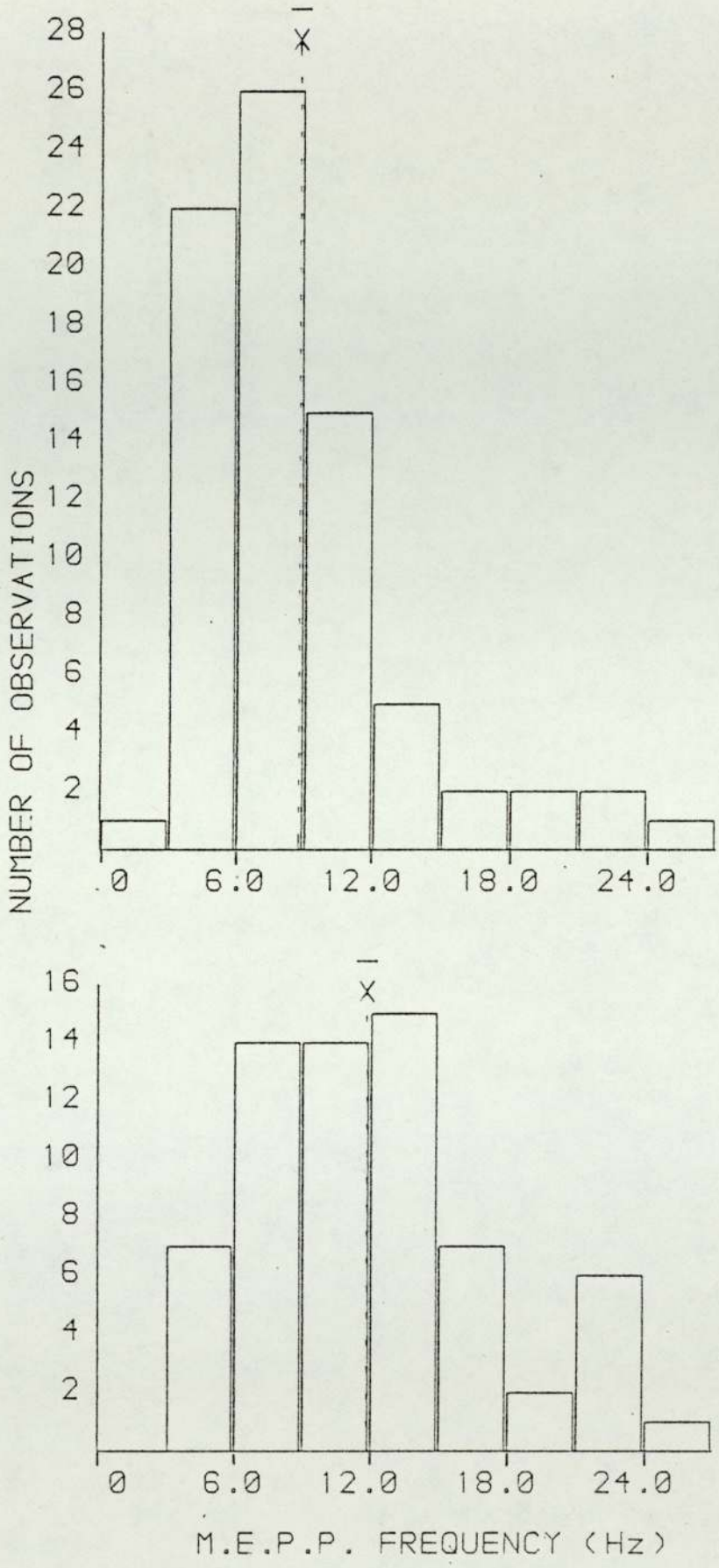


Fig. 18. Distribution of mean m.e.p.p. frequency values in the presence of caffeine (2×10^{-3}M) in diaphragm muscles from normal (upper) and dystrophic (lower) mice. $\bar{X}$ indicates the mean. Temperature 37 ± 1°C.

Table 17. Effect of lanthanum (5×10^{-4}M) on m.e.p.p. frequency and RMP in EDL muscles from normal and dystrophic male mice. Temperature 33 ± 1°C.

		CONTROL	La(5×10^{-4}M)
M.e.p.p. frequency (Hz)	NORMAL EDL	4.82 ± 2.96 (27/5) ★	30.67 ± 13.53 (11/5) ★
	DYSTROPHIC EDL	5.15 ± 2.31 (22/5) ★	89.18 ± 32.39 (15/5)
RMP (mV)	NORMAL EDL	74.63 ± 10.59 (27/5) ★	69.80 ± 9.53 (11/5)
	DYSTROPHIC EDL	67.91 ± 11.22 (22/5)	68.88 ± 11.83 (15/5)

Values are the mean ± 1 S.D.

Numbers in parentheses are the number of fibres/number of muscles.

★($p < 0.05$)

the salines (see Methods). Lanthanum (5×10^{-4}M) caused a significant increase in m.e.p.p. frequency in all muscles, but m.e.p.p. frequency in the dystrophic muscles was elevated to approximately three times that in the normal muscles. This concentration of lanthanum had no significant effect on RMP in normal or dystrophic EDLs.

The effect of lanthanum on m.e.p.p. frequency was followed for periods, up to 45 minutes, in four EDL fibres in muscles from normal littermates and four fibres in muscles from dystrophic mice (fig.19). Lanthanum caused a greater increase in m.e.p.p. frequency and its onset of action was more rapid in dystrophic fibres than in normal fibres. In one dystrophic fibre m.e.p.p. frequency was increased more than twentyfold within the first 5 minutes of recording. A second fibre in the same muscle was penetrated at 10 minutes and, over the next 30 minutes, m.e.p.p. frequency rose to more than 15 times the value on penetration.

In contrast, no normal fibre exhibited more than a twelvefold increase in frequency during 45 minutes of recording. In two normal fibres m.e.p.p. frequency was only doubled during this period. The results indicate that motor nerve terminals in EDL muscles from dystrophic mice are more sensitive to lanthanum than those in muscles from non- dystrophic littermates.

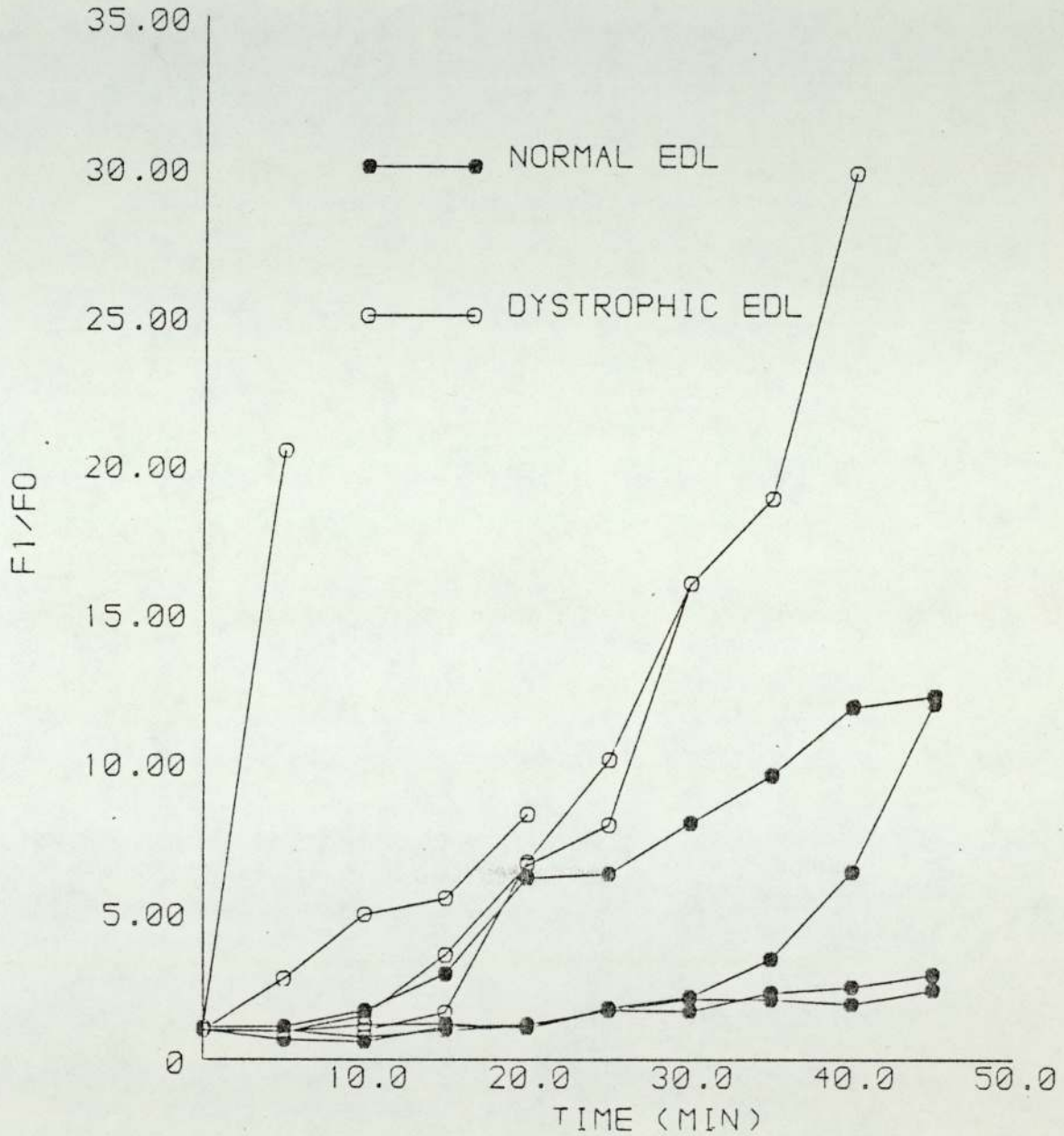


Fig. 19. Effect of lanthanum (5 X 10^{-4}M) on m.e.p.p. frequency in EDL muscles from normal and dystrophic mice. Temperature 33 ± 1°C. F0 is the control frequency and F1 the frequency after treatment.

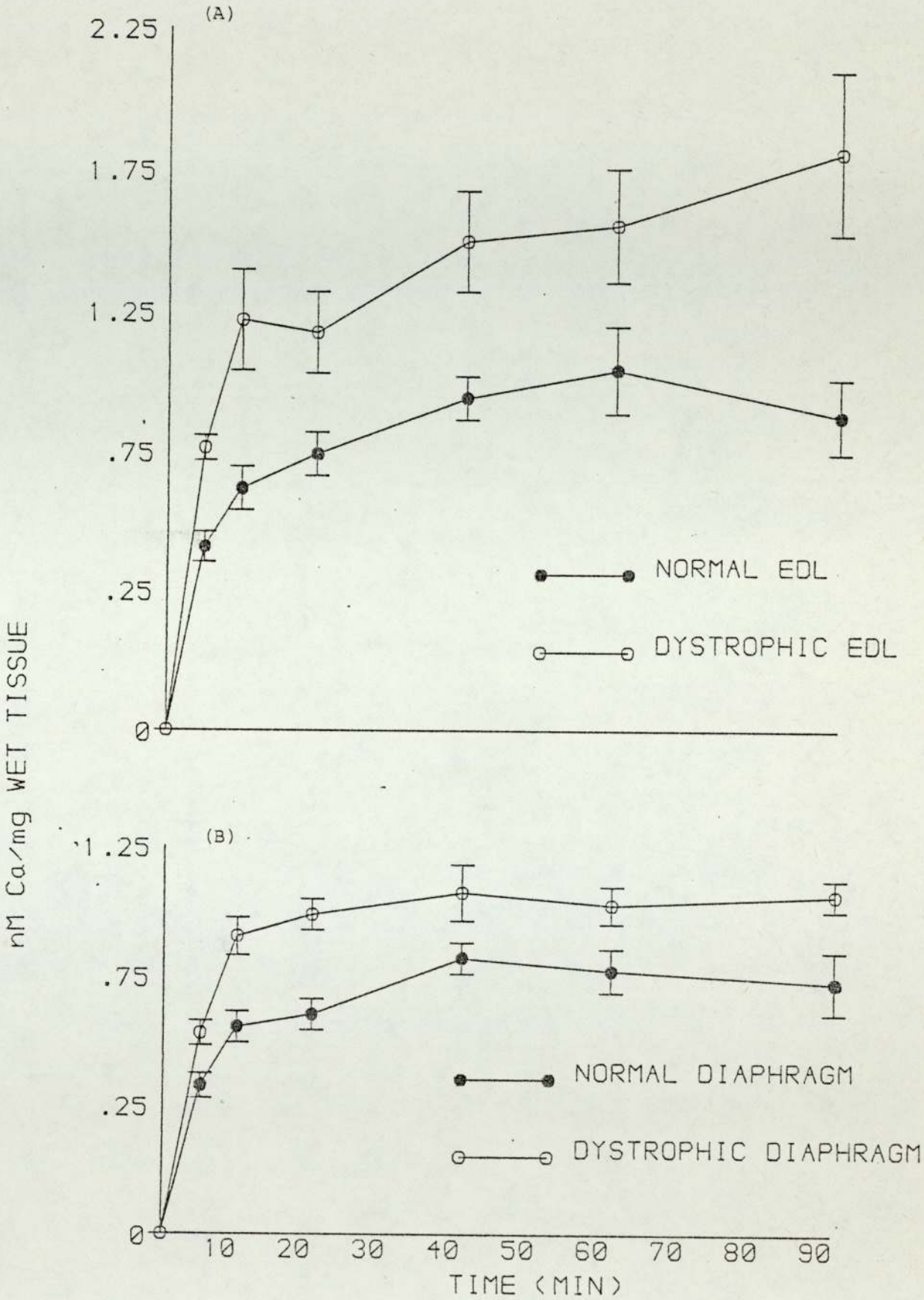
RADIOCHEMICAL EXPERIMENTS

From the preceding section it appears that nerve terminals in dystrophic EDL muscles can regulate a rise in $[Ca^{2+}]i$, (as defined by an increase in m.e.p.p. frequency), produced by increased influx of $[Ca^{2+}]o$ as well as those in normal muscles. This suggests that the greater probability of evoked release in the dystrophic EDL is not attributable to an increased $[Ca^{2+}]i$ caused by a greater influx of calcium, but may reflect greater efficacy of the calcium which enters, or a change in nerve terminal membrane structure which would facilitate release. It is not possible to measure $[Ca^{2+}]i$ in the mammalian motor nerve terminal either at rest or during an action potential. However, if there is any increased calcium influx across the nerve terminal membrane it is also likely to occur across the sarcolemma since this is 'leaky' in dystrophy (Bradley and Fulthorpe, 1978; Mendell et al, 1979). Therefore, the sarcolemma was used as a model to compare calcium fluxes across membranes from normal and dystrophic muscle. Calcium fluxes were measured using ^{45}Ca.

Calcium influx.

The influx of calcium into diaphragm and EDL muscles from normal and dystrophic mice is shown in figs.20A and 20B. The influx curves indicate that the initial phase of ^{45}Ca uptake was a rapid process that occurred within the first 5

Fig. 20. The time course of accumulation of calcium by EDL (A) and diaphragm (B) muscles from normal and dystrophic mice at 30 ± 1°C. Muscles were incubated in ^{45}Ca Liley's solution for the intervals indicated and were then prepared for liquid scintillation counting as described under Methods. (●) calcium accumulated in normal muscles; (○) calcium accumulated in dystrophic muscles. Each point is the mean result from at least 8 muscles ± 1 s.e. of the mean.


(A)
2.25
1.75
1.25
.75
.25
0
NORMAL EDL
DYSTROPHIC EDL
(B)
1.25
.75
.25
0
10
20
30
40
50
60
70
80
90
TIME (MIN)
NORMAL DIAPHRAGM
DYSTROPHIC DIAPHRAGM
nM Ca/mg WET TISSUE

minutes of incubation. In both diaphragm and EDL muscles from normal and dystrophic mice the entry of ^{45}Ca reached a plateau in 10 minutes. However, muscles from dystrophic animals took up significantly more ^{45}Ca than those from normal littermates. After 60 minutes incubation with ^{45}Ca there was 1.31 times as much calcium in the dystrophic EDL than in the normal EDL and 1.25 times more calcium in the dystrophic diaphragm than in the normal diaphragm.

Calcium efflux.

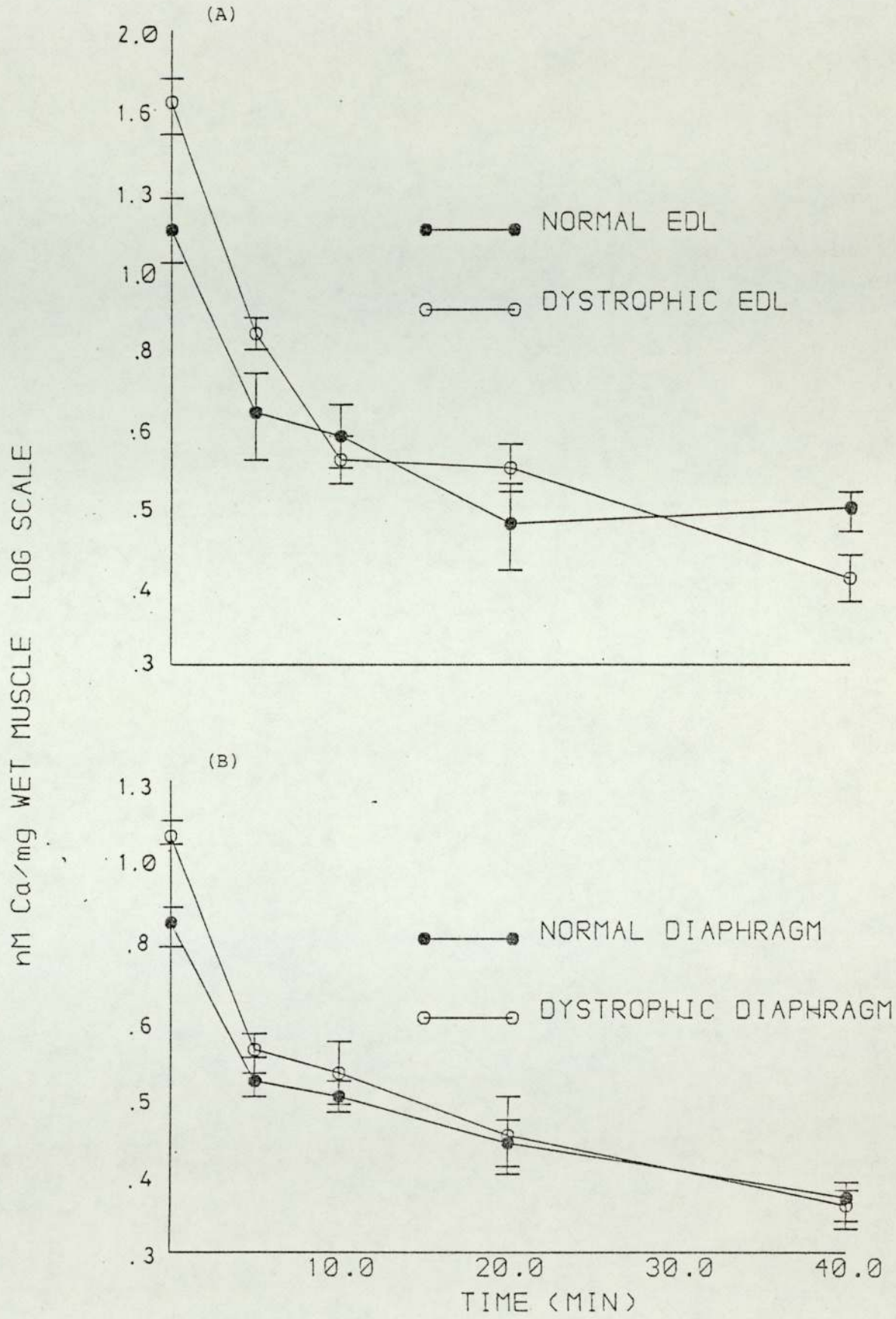
Calcium efflux from diaphragm and EDL muscles from normal and dystrophic animals is shown in fig.21. In both the EDL (21A) and the diaphragm (21B) there was a rapid efflux of ^{45}Ca within the first 5 minutes. After 5 minutes there was no significant efflux between successive time points. There was no significant difference in calcium efflux between muscles from normal and dystrophic mice.

These experiments demonstrate that more ^{45}Ca is taken up by muscles from dystrophic animals than those from normal littermates and that this extra calcium is not bound but rapidly released in tne first 5 minutes of wash.

Measurement of extracellular space.

The calcium flux results could be explained if muscles from dystrophic animals had an extracellular space greater than

Fig. 21. Efflux of ^{45}Ca from EDL (A) and diaphragm (B) muscles from normal and dystrophic mice at 30 ± 1°C. The muscles were pre-loaded by incubation in ^{45}Ca Liley's solution for 60 minutes. They were then placed in calcium-free Liley's solution and removed for analysis at the intervals shown. (•) normal muscles; (o) dystrophic muscles. Each point is the mean of at least 8 muscles ± 1 s.e. of the mean.


(A)
2.0
1.6
1.3
1.0
.8
.6
.5
.4
.3
NORMAL EDL
DYSTROPHIC EDL
(B)
1.3
1.0
.8
.6
.5
.4
.3
NORMAL DIAPHRAGM
DYSTROPHIC DIAPHRAGM
10.0
20.0
30.0
40.0
TIME (MIN)
nM Ca/mg WET MUSCLE LOG SCALE

normal. Therefore, the extracellular space in EDL and diaphragm muscles from dystrophic animals and non-dystrophic littermates was measured using inulin.

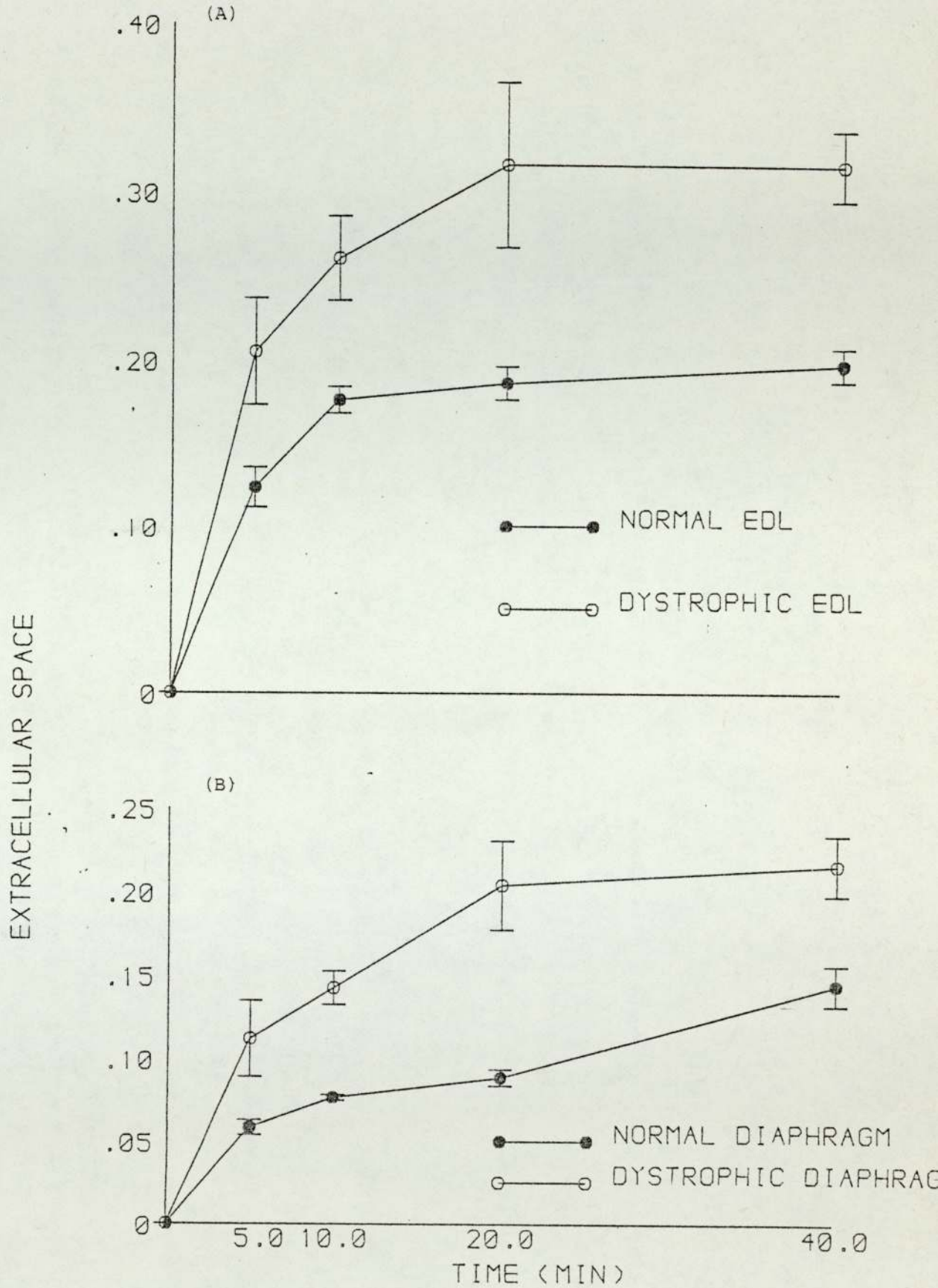
Inulin equilibrated with a larger fraction of the total muscle volume in the dystrophic muscles than in the normal muscles (figs. 22A, 22B). The increase in extracellular space was significant at every time point that was examined. After 40 minutes the inulin space was 1.6 times greater in dystrophic EDLs and 1.5 times greater in dystrophic diaphragms than in normal muscles. These values are similar to those obtained in the calcium influx studies. Therefore, it would appear that the greater influx of calcium in the EDL and diaphragm muscles from dystrophic mice can be explained by a larger extracellular space.

The fraction of space available to inulin in the dystrophic EDL was 1.46 times greater than that in the dystrophic diaphragm. In the normal muscles a similar ratio of 1.37 was obtained.

Effect of lanthanum on calcium efflux.

Within dystrophic muscles, fibres affected to varying degrees by the disease process are found, ranging from severely damaged fibres which are essentially contiguous with the extracellular space to fibres which are barely

Fig. 22. Changes in the inulin space of EDL (A) and diaphragm (B) muscles from normal and dystrophic mice at 30 ± 1°C. Muscles were incubated for various intervals in Liley's solution containing ^{3}H-inulin. The inulin space of each muscle is expressed as tissue/medium ration as described under Methods. Each point is the mean result from at least four muscles ± 1 s.e. of the mean.


(A)
.40
.30
.20
.10
0
NORMAL EDL
DYSTROPHIC EDL
(B)
.25
.20
.15
.10
.05
0
NORMAL DIAPHRAGM
DYSTROPHIC DIAPHRAG
5.0 10.0 20.0 40.0
TIME (MIN)
EXTRACELLULAR SPACE

damaged. Thus, it is possible that there may be greater ^{45}Ca influx into dystrophic fibres but it is not possible to distinguish it from the much larger amount of ^{45}Ca present in the extracellular space. The trivalent cation lanthanum has been used to investigate calcium fluxes in smooth muscle which would otherwise be obscured by the extracellular space (see Methods). While lanthanum would not be expected to reduce ^{45}Ca efflux from badly damaged fibres it was hoped it would reduce efflux from less severely damaged fibres revealing any difference in calcium influx between normal and dystrophic muscles. Therefore, the effect of lanthanum (10^{-2}M) on calcium efflux from normal and dystrophic EDL and diaphragm muscles was investigated.

Lanthanum had little effect on calcium efflux from normal and dystrophic EDL muscles and no difference was found in the amount of ^{45}Ca present in normal and dystrophic muscles after a 20 minute wash. In contrast lanthanum did reduce the efflux of calcium from normal and dystrophic diaphragm muscles but again there was no significant difference between the amount of calcium remaining in normal and dystrophic muscles. Therefore, if there is increased influx of calcium into dystrophic muscles it cannot be detected using the lanthanum method.

CHAPTER 4 : DISCUSSION

Characteristics of the twitch response

A reduction in twitch tension is a characteristic feature of dystrophic muscle (Sandow and Brust, 1958; Harris and Wilson, 1971). Therefore, indirectly evoked twitch tension was measured in EDL and diaphragm muscles from 8 week old normal and dystrophic mice. Eight week old mice were used, as stable levels of tension output are reached in normal and dystrophic EDL muscles at this age (Douglas and Baskin, 1971). Hofmann and Ruprecht (1973) reported that the twitch tension per mg wet weight of muscle in directly stimulated EDL muscles from 8 to 12 week old normal and dystrophic mice was 0.26 and 0.14 g/mg, respectively. These results are comparable to those obtained in indirectly stimulated muscles in the present study (table 2A). The decrease in twitch tension per mg wet weight in EDL muscles from dystrophic mice indicates the involvement of this muscle in the disease process.

In the mouse, the EDL is made up almost entirely of fast twitch muscle fibres (Parry and Parslow, 1981). In 129/ReJ dy/dy mice it has been reported that fast twitch fibres are preferentially affected by dystrophy (Nwoye and Goldspink, 1982; Shafiq et al, 1969). However, considerable variation exists in the susceptibility of different fast twitch muscles to the dystrophic process (Rowe and Goldspink,

1969; Shafiq et al, 1969). The reason for this is unknown. Histologically, the gastrocnemius appears more severely affected than the EDL (Shafiq et al, 1969) as does the tibialis anterior on the basis of weight loss (Goldspink and Rowe, 1968). Despite this, the EDL was chosen in preference to these other hindlimb muscles as it is more likely to remain physiologically viable, in vitro, because of its smaller size (Harris and Wilson, 1971; Hofmann and Ruprecht, 1973).

In contrast to the findings with the EDL, there was no significant difference in the twitch tension, per mg wet weight of muscle, between diaphragm muscles from 8 week old normal and dystrophic mice (table 2B). Harris and Ribchester (1979c) reported that diaphragm muscles from 3 to 6 month old dystrophic mice of the 129/ReJ strain generated less tension than normal muscles. An obvious explanation for the difference between their findings and the present results is the ages of the mice used. It appears that degenerative changes in the diaphragm cannot be detected at the gross physiological level until the animals are more than 3 months old. It has been reported that oxidative muscle fibres degenerate more slowly than glycolytic muscle fibres in 129/ReJ dy/dy mice (Cosmos et al, 1973; Butler and Cosmos, 1977). As the majority of muscle fibres in the diaphragm are oxidative fibres (Noireaud and Léoty, 1984) then it might be expected that the diaphragm would be spared until the later stages of the

disease process.

When the total twitch duration, time to peak, or half relaxation time were compared in EDL and diaphragm muscles from 8 week old normal and dystrophic mice no significant differences were found (tables 2A, 2B). The variation in these parameters between muscles was considerable, but no dystrophic muscle had a time to peak or half relaxation time greater than the maximum value for the normal muscle. There have been no other investigations of twitch time course in diaphragm muscles from normal and dystrophic 129/ReJ mice although it has been reported that the twitch time course is prolonged in diaphragms from C57BL/6J dy^{2J} /dy^{2J} mice (Noireaud and Léoty, 1984). However, there have been numerous reports that the time to peak and the half relaxation time of the directly evoked twitch response are prolonged in dystrophic mouse hindlimb muscles (Sandow and Brust, 1958; Hinterbuchner, Angyan and Hirsch, 1966; Sabbadini and Baskin, 1976). It may be argued that the differences between the present results and these other findings are due to differences in the experimental procedures, the muscles or the ages of the animals used. However, Douglas and Baskin (1971) measured the time course of the directly elicited twitch response of EDL muscles from normal and dystrophic mice of a similar age and at a similar temperature and they also observed that the half relaxation time of the twitch was prolonged in muscles from dystrophic animals. It is difficult to explain why no

difference in the time course of the twitch was found in these experiments. It cannot reflect a difference between direct and indirect methods of stimulation as the tension per mg wet weight, total twitch duration (Hofmann and Ruprecht, 1973) and half relaxation time (Douglas and Baskin, 1971) in dystrophic EDL muscles are similar using both experimental protocols.

Effect of (+) -tubocurarine chloride on the twitch response

The indirectly evoked twitch response of EDL muscles from 8 week old dystrophic mice was found to be more resistant to curare than that of muscles from normal littermates (fig.4). The IC_{50} value for curare was 1.69 times greater in dystrophic EDLs. This result is consistent with the findings of Baker and Sabawala (1963) in the peroneus longus muscle of the mouse, where twice the concentration of curare was required to produce a 50% twitch depression in dystrophic muscles. Therefore, in murine dystrophy there seems to be a reduced effect of curare in hindlimb muscles.

There was no significant difference in the IC_{50} values to curare between diaphragm muscles from normal and dystrophic mice at either 8 or 15 weeks of age. The results in 8 week old animals might be expected as no decrease in twitch tension was found in diaphragm muscles from dystrophic mice of this age suggesting little involvement of this muscle in

the dystrophic process in these animals. However, in diaphragms from 3 to 6 months old dystrophic mice there is a decrease in twitch amplitude (Harris and Ribchester, 1979c). Therefore, it might be expected that muscles from 15 week old dystrophic mice would show a greater resistance to curare than those from normal mice, if this is a characteristic of dystrophy. Although the difference in IC_{50} values was not statistically significant, the variance of IC_{50} values of dystrophic diaphragms was large and curare resistance in diaphragms from older animals cannot be excluded. There have been several investigations of curare resistance in diaphragm muscles from normal and dystrophic mice of the 129/ReJ strain (Baker and Sabawala, 1963; Beaulnes et al, 1966; Harris and Ribchester, 1979c). The results of these investigations have yielded equivocal results. As in each of these studies mice of a similar age were used this may be attributed to the different methods used to assess the effect of curare on the indirectly evoked twitch response. Beaulnes et al (1966) added a dose of curare to the bathing solution and recorded the time until the twitch amplitude was reduced by one third. They reported that the action of curare was slower in dystrophic diaphragms. Both Harris and Ribchester (1979c) and Baker and Sabawala (1963) compared dose-response curves. Harris and Ribchester (1979c) found no difference in curare sensitivity between normal and dystrophic diaphragm muscles. Baker and Sabawala (1963) reported that 0.8μg/ml curare produced about twice as much block in normal

diaphragms as in diaphragms from dystrophic mice. However, they also found that 1.2μg/ml curare produced a similar block in normal and dystrophic muscles making it difficult to interpret their results. Assuming that curare resistance in murine dystrophy is a consequence of the disease process it is difficult to explain why it cannot be readily demonstrated in dystrophic diaphragm muscles.

In this investigation an increase in curare resistance with age was observed in the normal diaphragm but not in the dystrophic diaphragm. Also, if IC_{50} values for curare in diaphragms from 15 week old dystrophic mice are compared with IC_{50} values in diaphragms from 8 week old normal animals then a significantly greater resistance to curare is found in the dystrophic muscles. This illustrates the importance of comparing curare resistance in diaphragms from normal and dystrophic mice of similar age.

The curare resistance found in the dystrophic EDL could be due to more junctional ACh receptors, more extrajunctional ACh receptors or more non-specific binding of curare (i.e. binding to sites other than receptors) in dystrophic muscles. It has been reported that there are more junctional receptors in dystrophic EDL muscles (Howe, Livett and Austin, 1976). However, this has not been confirmed by other workers (Howe, Telfer, Livett and Austin, 1977; Marusyk and Monckton, 1976; Kelly and Smith, personal communication). At adult mouse neuromuscular

junctions the ACh receptor (AChR) corresponds to a morphologically distinct thickened region of the postjunctional membrane (pjm) at the crests of the junctional folds (Fertuk and Salpeter, 1976). In 1980, Matthews-Bellinger reported that, in the EDL, local ^{125}I-α - bungarotoxin (^{125}I-α-BTX) binding site density per unit area of thickened pjm was unaffected by dystrophy. However, overall toxin binding per unit area of junctional surface was reduced to about half of normal as a consequence of a decreased thickened pjm. Thus it appears that the number of endplate receptors in dystrophic EDL muscle is either similar to normal or considerably reduced. The former cannot account for the increased curare resistance in dystrophic EDL muscles while the latter would suggest decreased resistance to curare in dystrophy.

An alternative explanation for the curare resistance observed in the dystrophic EDL would be an increase in the number of extrajunctional ACh receptors which would reduce the amount of curare bound to junctional receptors. There have been several studies of extrajunctional AChR density in dystrophic muscle using labelled compounds. Marusyk and Monckton (1976) injected ^{3}H-decamethonium to label AChR in vivo and found no difference between normal and dystrophic mice in the distribution of decamethonium binding sites in either tibialis anterior or diaphragm muscles. Kelly and Smith (personal communication) incubated normal and dystrophic muscles, in vitro, with the highly specific

marker for nicotinic AChR, ^{125}I-α-BTX and found that in single fibres there was a similar amount of extrajunctional binding in normal and dystrophic diaphragm and EDL muscles. These results are consistent with the findings of Banker et al (1979) using an α-BTX-horseradish peroxidase conjugate. However, Howe et al (1977) using ^{125}I-α-BTX and quantitative light microscope autoradiography, reported increased binding in nonjunctional regions of various dystrophic muscles, including the EDL. This nonjunctional binding was referred to as extrajunctional, which, in the common usage implies localisation on the extrajunctional membrane. However, in this study autoradiograms of sectioned muscle were analysed from which grain densities were measured over the sarcoplasm, not the sarcolemma. Furthermore, the increased toxin retention and its internal localisation in dystrophic muscle could be explained by the increased nonspecific endocytosis which has been reported in dystrophic muscle (Libelius et al, 1978; Libelius, Lundquist, Tagerud and Thesleff, 1981).

In experiments in which ACh was applied iontophoretically to the membrane of muscles from normal and dystrophic mice no evidence of increased extrajunctional ACh sensitivity was reported (Harris and Marshall, 1973). If the number of extrajunctional AChR was increased in dystrophic muscles then an increased extrajunctional sensitivity to ACh would be expected. Therefore, these experiments support the majority of the binding studies.

Glycosaminoglycans are a component of connective tissue and it has been suggested that curare binds to negatively charged glycosaminoglycans (Cookson and Paton, 1969). There is a proliferation of connective tissue in murine dystrophy (Michelson et al, 1955; Platzer and Chase, 1964). Therefore, the possibility exists that the curare resistance demonstrated in the dystrophic EDL is due to nonspecific binding of curare by glycosaminoglycans.

Autoradiographic studies have shown that curare does not enter normal mouse diaphragm muscle fibres (Waser, 1966). However, this may not be the case in dystrophic muscle as the dystrophic membrane is permeable to horseradish peroxidase and procion yellow (Mendell et al, 1979; Bradley and Fulthorpe, 1978). Uptake of curare by dystrophic muscle fibres through a leaky membrane or via nonspecific endocytosis (Libelius et al, 1978; 1981) could reduce the local concentration of curare at junctional receptors in dystrophic muscle and thereby explain the curare resistance.

It is unlikely that curare resistance in EDL muscles from 8 week old dystrophic mice can be attributed to increased junctional or extrajunctional numbers of AChR. However, nonspecific binding of curare cannot be excluded, or uptake of curare by nonspecific endocytosis. Nevertheless, perhaps a more realistic alternative is an increase in the

efficiency of neuromuscular transmission i.e. an increased safety factor (S.F.) in the dystrophic EDL.

Safety Factor

The safety factor of neuromuscular transmission can be defined as the ratio of the depolarisation produced by transmitter released from the motor nerve terminal and the minimum depolarisation required to initiate an action potential in the muscle fibre i.e.

$$S.F. = \frac{\text{Depolarisation produced}}{\text{Depolarisation required}}$$

which is equivalent to the expression:

$$S.F. = \frac{\text{m.e.p.p. amplitude x quantum content}}{\text{(RMP - threshold potential)}}$$

providing the depolarisation required to reach the threshold potential has been corrected for nonlinear summation by the subsynaptic membrane (Martin, 1955).

In order to block neuromuscular transmission, the concentration of curare must be sufficient to reduce the depolarisation produced so that the safety factor falls below a value of one. Therefore, the greater the initial safety factor, the greater the curare concentration required before any block of transmission occurs. The

safety factor for transmission in mammalian muscles varies over a wide range, values from 4 to 20 have been reported in different muscles (Paton and Waud, 1967; Waud and Waud, 1975; Kelly, 1978). The safety factor in EDL muscles from normal and dystrophic mice has been measured by Harris and Ribchester (1979a). Safety factor in the dystrophic EDL, at a motor nerve frequency of stimulation of 3Hz, was 4.84 ± 0.65 (mean ± s.e.m. n=21) compared with 2.81 ± 0.62 (14) in the normal EDL. The two values were significantly different. Furthermore, the ratio of safety factor in the dystrophic muscle to that in the normal muscle is 1.72 which is consistent with the curare IC_{50} ratio in these muscles (1.69).

Safety factor is reported to increase with age (Kelly, 1978), which could explain the greater curare resistance of diaphragms from 15 week old normal mice compared with those from 8 week old animals. In addition, a difference in safety factor between diaphragm and EDL muscles would explain the difference in curare sensitivity between these muscles.

To account for a change in safety factor between normal and dystrophic muscles one or more of the parameters affecting the safety factor must differ. These parameters are shown in fig. 23. As already discussed (page 130) there is little evidence to support an increase in the number of cholinergic receptors in dystrophic muscles. Thus the

Fig. 23. Parameters affecting the safety factor.

(1) Number of quanta released per nerve impulse (quantum content).

(2) M.e.p.p. amplitude which is dependent upon:

(a) amount of ACh per quantum (quantum size);

(b) amount of ACh reaching receptors (diffusion, acetylcholinesterase);

(c) number of receptors available;

(d) increase in conductance per activated receptor;

(e) muscle fibre cable properties.

(3) Resting membrane potential (RMP).

(4) Threshold potential.

increased safety factor reported in dystrophic muscles (Harris and Ribchester, 1979a) could be due to an increase in m.e.p.p. amplitude, or quantum content, a reduced RMP, or a raised threshold potential, or any combination of these parameters. There have been several investigations of the threshold potential in muscles from normal and dystrophic 129/ReJ mice. McComas and Mossawy (1966) measured threshold potentials, in vivo, from the vastus medialis and gracilis muscles. They reported that the RMP had to be reduced by 12.3 ± 0.3mV (mean ± s.e.m.) to initiate an action potential in normal muscles but by only 10.5 ± 0.4mV in dystrophic muscles. It has also been reported that the action potential threshold in the dystrophic EDL, in vitro, is -45.1 ± 0.56mV compared with -43.3 ± 0.50mV in the normal EDL (Harris and Marshall, 1973). Although threshold values in normal and dystrophic muscles were statistically significantly different in both these studies it is unlikely that the difference is of physiological significance. In two other, in vitro, studies of threshold potential in the EDL no significant difference between normal and dystrophic muscles has been reported (Harris and Ribchester, 1979a; Kerr and Sperelakis, 1983a).

Table 3 shows that the RMP of dystrophic EDL muscles is significantly lower than normal. This finding is consistent with the findings of other workers in hindlimb skeletal muscles of dystrophic mice (Kleeman et al, 1961;

McComas and Mossawy, 1965; Hazlewood and Ginski, 1968). The results of previous studies in which RMP has been recorded in normal and dystrophic EDL muscles, are shown in table 18. The mean RMP obtained in this study is within the range of previous, in vitro, investigations.

The RMP of diaphragm muscles from dystrophic mice was not significantly different from normal (table 4). This is consistent with the findings of Harris and Ribchester (1979c), but inconsistent with the findings of Volkov and Poletaev (1984), who reported a lower RMP in dystrophic diaphragm muscles in the presence of the irreversible anticholinesterase armin.

Hindlimb skeletal muscles from 129/ReJ dy/dy and C57BL/6J dy^{2J}/dy^{2J} mice have decreased intracellular potassium levels (Dowben et al, 1964; Hoh and Salafsky, 1972; Atwood and Kwan, 1978) and this has been put forward to explain the lower RMP. However, recent studies in C57BL/6J dy^{2J}/dy^{2J} mice using a potassium sensitive microelectrode have shown that a reduction in potassium ion activity alone cannot account for the magnitude of the depolarisation (Charlton, Silverman and Atwood, 1981; Shalton and Wareham, 1981). There have been several reports of enhanced membrane permeability to potassium in dystrophy (Zierler, 1961; Burr and McLennan, 1961; Herzberg, Challberg, Hess and Howland 1975), but, Lipicky and Hess (1974) taking into account the ratio of surface to volume and the RMP of

Table 18. Resting membrane potentials (RMPs) of EDL muscles from normal and dystrophic (129/ReJ) mice.

			RMP (mV)	
Study	Temperature	in vivo/ in vitro	Normal	Dystrophic
Harris (1971)	37°C	in vivo	-80.9±0.58 (102) ★	-73.8±0.09 (106)
Harris and Marshall (1973)	22-28°C	in vitro	-76.0±0.38 (115) ★	-67.2±0.68 (82)
Sellin and Sperelakis (1978)	37°C	in vitro	-73.1±0.70 (41) ★	-66.6±1.9 (30)
McArdle and Albuquerque (1975)	22°C	in vitro	-76.0±0.3 (128) ★	-65.0±0.8 (54)
Harris and Ribchester (1979a)	Room temperature	in vitro	-76.8±1.12 (17) ★	-68.91±2.11 (16)
Kerr and Sperelakis (1983a)	37°C	in vitro	-79.0±0.7 (87) ★	-63.0±2.9 (140)
This study	33°C	in vitro	-76.98±0.68 (96) ★	-68.82±0.88 (75)

RMPs expressed as mean ± 1 s.e. of mean.

Figures in parentheses represent number of fibres.

★ ($p < 0.05$) significant difference between normal and dystrophic muscles.

normal and dystrophic muscles found that potassium permeability in the EDL was not different from control. Sellin and Sperelakis (1978) reported that the sodium potassium permeability ratio (P_{Na} : P_K) was higher in dystrophic muscle and suggested this was due to a decrease in P_K. An increase in the P_{Na} : P_K ratio would depolarise the membrane according to the constant field equation. Recent support for an increase in the P_{Na} : P_K ratio has been provided by the report that potassium conductance is decreased in dystrophic muscle (Kerr and Sperelakis, 1983b). It is probable, therefore, that the decreased RMP in murine dystrophy is a consequence of both decreased potassium content and a decreased potassium conductance.

The lower RMP in EDL muscles from dystrophic mice will contribute towards the increase in safety factor. Using the values of RMP and threshold potential determined by Harris and Ribchester (1979a), the ratio of depolarisation required to initiate an action potential in normal EDL muscles to that in dystrophic muscles is 1.67. This ratio is similar to the safety factor ratio of 1.72 determined by Harris and Ribchester (1979a). Based on these figures the increased safety factor in dystrophic EDL muscles could be totally explained by the difference in RMP. However, a similar treatment of the results of an earlier study, where more fibres were examined (Harris and Marshall, 1973) yields a ratio of 1.48. The difference in this ratio between normal and dystrophic muscles i.e. 0.48 is less

than the difference in the curare IC_{50} ratio between these muscles (0.69) suggesting that the reduced RMP can only account for 70% of the increased safety factor in the dystrophic EDL. Thus the remaining 30% may be due to presynaptic and/or other postsynaptic differences between normal and dystrophic muscles.

In order to investigate whether there was a presynaptic involvement in the curare resistance, spontaneous and evoked transmitter release was compared in normal and dystrophic EDL muscles. No difference in curare sensitivity was detected at the gross physiological level between diaphragm muscles from normal and dystrophic mice. However, it is possible that a proportion of fibres in diaphragm muscles are affected by the disease. Therefore, transmitter release was also investigated in diaphragms from normal and dystrophic mice.

Spontaneous transmitter release

Miniature e.p.p. amplitude was greater in the dystrophic EDL than in the normal EDL (table 3). Figure 6 shows that mean fibre m.e.p.p. amplitudes in the normal EDL had a bimodal distribution. A possible explanation for this result is biased sampling of fibres with larger amplitude m.e.p.ps.. A larger difference in m.e.p.p. amplitude between normal and dystrophic EDL muscles would have been apparent if these fibres were excluded. Miniature e.p.p.

amplitude has been measured in dystrophic EDL muscles and reported to be normal (Harris and Ribchester, 1979a) and greater than normal (Carbonetto, 1977). In any one dystrophic muscle necrotic fibres are often found next to fibres with a normal appearance (Platzer, 1979), although there is an increase in the proportion of degenerating fibres with time (Banker, 1968). Microelectrode penetration of dystrophic EDL muscles does sample fibres with an abnormal histological appearance as well as normal appearing ones (Harris and Ribchester, 1978). However, the proportion of dystrophic fibres actually sampled will vary from study to study and the more fibres sampled the greater will be the probability of sampling dystrophic ones. This could explain why Harris and Ribchester (1979a) did not find increased m.e.p.p. amplitude in the dystrophic EDL as they only sampled 16 fibres.

In two previous studies of m.e.p.p. amplitude in dystrophic diaphragm muscles m.e.p.p. amplitude was reported to be similar to normal (Conrad and Glaser, 1964; Harris and Ribchester, 1979c). Although in the present study m.e.p.p. amplitude in the dystrophic diaphragm was significantly greater than normal (table 4), it is unlikely that the 13% difference is of physiological importance. The fact that 269 fibres were sampled in diaphragms from dystrophic mice in this investigation may explain why increased m.e.p.p. amplitude has not been found previously.

Factors which determine m.e.p.p. amplitude are the quantum size, the amount of ACh reaching the receptors and the conductance increase produced by ACh at each receptor (fig.23). Miniature e.p.p. amplitude is also influenced by the cable properties of the muscle fibre. Katz and Thesleff (1957) showed that m.e.p.p. amplitude is proportional to the input resistance (Ro) of the muscle fibre i.e.

$$Ro = \frac{1}{\pi} \sqrt{\frac{RmRi}{d^3}}$$

where Rm is the specific membrane resistance, Ri is the internal specific membrane resistance and d is the fibre diameter.

Therefore, m.e.p.p. amplitude is also dependent upon fibre diameter and a decrease in fibre diameter will cause an increase in Ro which will increase m.e.p.p. amplitude. It has been reported that the mean fibre diameter in the dystrophic EDL is smaller than normal (Rowe and Goldspink, 1969), similar to normal (Kerr and Sperelakis, 1983a) and greater than normal (Sellin and Sperelakis, 1978). It has also been found that the range of fibre diameters in dystrophic EDL muscles is greater than that in normal muscles (Rowe and Goldspink, 1969). This could explain the greater range of m.e.p.p. amplitudes in dystrophic EDL muscles (fig. 6).

There have been several studies of the cable properties of muscle fibres from normal and dystrophic 129/ReJ mice although measurements of membrane resistance have provided conflicting results (table 19). There have been seven investigations of Ro in normal and dystrophic mouse muscles. Four of these studies have reported Ro is similar in normal and dystrophic muscles (McComas and Mossawy, 1966; Harris and Ribchester, 1979a, 1979c; Kerr and Sperelakis, 1983a), two that it is less than normal (Law and Atwood, 1972; Sellin and Sperelakis, 1978) and only one that it is greater than normal (Carbonetto, 1977). A possible explanation for the difference between these results might be the dependence of Rm on pH (Kerr and Sperelakis, 1983b). Kerr and Sperelakis (1983b) reported that Rm of dystrophic EDL muscle fibres increased as the pH was lowered from 7.4 to 7.0 but decreased as pH was lowered to 6.1. In contrast, Rm of normal EDL muscle fibres decreased as pH was lowered from 7.4 to 6.8 and then increased as pH was lowered still further to 6.1. They suggested that this was the most likely explanation for the decreased Rm of dystrophic muscle fibres found in their study and the increased Rm of dystrophic muscle fibres found in a previous study (Sellin and Sperelakis, 1978). Muscle fibre resistance therefore seems to be highly sensitive to the pH of the bathing solution. The pH of the Liley's solution in the present study (7.2 - 7.3) was the same as that used by Carbonetto (1977). He found that m.e.p.p. amplitude in the dystrophic EDL was greater than

Table 19. Some cable properties of skeletal muscle fibres from normal and dystrophic (129/ReJ) mice.

Study	muscle	pH		τ_m (ms)	R_m (Ω-cm^2)	C_m (μF/cm^2)	R_o (MΩ)
McComas and Mossawy (1966)	vastus medialis, gracilis	-	NORMAL DYSTROPHIC	3.2±0.14 ★ 4.1±0.17			0.19±0.02 0.22±0.01
Law and Atwood (1972)	soleus	7.2	NORMAL DYSTROPHIC		1516 636		0.535±0.02 ★ 0.23 ± 0.01
Carbonetto (1977)	EDL	7.2-7.3	NORMAL DYSTROPHIC				0.20 ± 0.01 ★ 0.295±0.02
Sellin and Sperelakis (1978)	EDL	7.1	NORMAL DYSTROPHIC	1.48±0.11 ★ 2.42±0.27	685 ± 64 ★ 1108±243	2.16 2.18	0.27 ± 0.02 ★ 0.21 ± 0.01
Harris and Ribchester (1979a)	EDL	-	NORMAL DYSTROPHIC				0.26 ± 0.01 0.23 ± 0.02
Kerr and Sperelakis (1983a)	EDL	7.4	NORMAL DYSTROPHIC		642 ± 34 ★ 517 ± 27		0.27 ± 0.02 0.25 ± 0.02
Harris and Ribchester (1979c)	DIAPHRAGM	-	NORMAL DYSTROPHIC	1.97 ± 0.05 2.00 ± 0.05			0.54 ± 0.03 0.55 ± 0.03

Values are presented as mean ± 1 s.e. of mean.

★ ($p < 0.05$) significant difference between normal and dystrophic muscles.

Abbreviations: Tm - time constant, Rm - specific membrane resistance, Cm - specific membrane capacitance, Ro - input resistance.

that in the normal EDL and that it could be correlated with Ro in all muscles. Therefore, it is probable that the greater m.e.p.p. amplitude observed here was due to the greater Ro of the fibres of the dystrophic EDL.

The importance of membrane capacitance in determining m.e.p.p. amplitude has been determined by Gage and McBurney (1973). There have been three studies of specific membrane capacitance (Cm) in normal and dystrophic EDL muscles, two in mice of the C57BL/6J strain (Farnbach, Brown and Barchi, 1978; Dangain and Vrbová, 1983) and one in mice of the 129/ReJ strain (Sellin and Sperelakis, 1978). Only Dangain and Vrbová (1983) found a difference between Cm in normal and dystrophic EDL muscles, Cm being reduced in the latter. A reduced Cm could explain the increased m.e.p.p. amplitude in the dystrophic EDL (Gage and McBurney, 1973) but not the slower time course of the m.e.p.ps. (table 3).

Miniature e.p.p. half decay times were greater than normal in the dystrophic EDL and, with the exception of the curarised EDL (table 5A), e.p.p. half decay times were also prolonged (tables 6 and 8). Why e.p.p. half decay time in the curarised dystrophic EDL is not greater than normal is unclear (see Harris and Ribchester, 1979a) though it is unlikely to be due to an effect of curare. In contrast to the findings in the EDL, there was no significant difference in m.e.p.p. (table 4) or e.p.p. (tables 5B and 7) half decay times between diaphragm muscles from normal

and dystrophic mice. This is consistent with the finding of Harris and Ribchester (1979c), though Conrad and Glaser (1964) reported prolonged m.e.p.p. half decay time in the dystrophic diaphragm.

An increase in the membrane time constant (τ_m) increases both m.e.p.p. amplitude and time course (Hofmann, Feigen and Genther, 1962). It has been reported that τ_m is increased in dystrophic hindlimb muscles (McComas and Mossawy, 1966; Sellin and Sperelakis, 1978). Therefore, a change in muscle fibre cable properties can explain the greater m.e.p.p. amplitude and prolonged m.e.p.p. and e.p.p. half decay time in the dystrophic EDL.

An alternative explanation for the increase in m.e.p.p. amplitude in the dystrophic EDL is an increase in the amount of ACh contained in each quantum. However, Carbonetto (1977) suggested that there was no significant difference in quantum size at normal and dystrophic junctions, as the larger m.e.p.p. amplitude at the latter could be accounted for by the higher Ro of dystrophic muscle fibres.

The enzyme acetylcholinesterase (AChE) which is concentrated within the basement membrane which fills the postsynaptic folds terminates transmitter action at the neuromuscular junction (Hall and Kelly, 1971; Betz and Sakmann, 1973). It has an important role both in

determining how much ACh reaches the postsynaptic receptors and its duration of action. It has been estimated that a single endplate in rat intercostal muscle can hydrolyse 100 times the amount of ACh released by a nerve impulse in one millisecond (Namba and Grob, 1968). Cholinesterase inhibition increases m.e.p.p. amplitude and time course (Wilson, 1982; Blaber and Christ, 1967; Liley, 1956a). Therefore, decreased AChE activity in dystrophic EDL muscles could explain the increased m.e.p.p. amplitude and half decay time. The results of studies of AChE activity in dystrophic muscles are equivocal and dependent upon the method of measurement, the muscles examined, the manner in which the activity is expressed and whether endplate or total muscle AChE is measured. Acetylcholinesterase activity in dystrophic muscle has been reported to be less than normal (Boegman, 1974; Skau and Brimijoin, 1981), similar to normal (Tennyson, Miranda and Kremzner, 1975; Sung, 1978a)and greater than normal (Srivastava and Berlinguet, 1967; Sung, 1978b). Histochemical methods have consistently indicated decreased endplate AChE in dystrophic muscles (Curtis, Abrams and Harman, 1963; Glaser and Seashore, 1967; Rash, Ellisman, Staehelin and Porter, 1974; Ellisman et al, 1975). There are, however, important differences between the effect of chronic anticholinesterase treatment on m.e.p.p. parameters and the changes found here (Engel, Lambert and Santa, 1973; Roberts and Thesleff, 1969), and in Carbonetto's experiments (1977) m.e.p.p. amplitude was measured in the

presence of the anticholinesterase neostigmine and was also found to be greater in dystrophic muscles. Thus it is unlikely that decreased AChE activity in the dystrophic EDL can explain the present results. The AChE activity of normal and dystrophic muscles will be considered in more detail in a later section.

Miniature e.p.p. frequency in dystrophic muscles of 129/ReJ mice has been shown to be similar to that in normal muscles by a number of workers (McComas and Mossawy, 1965; Harris and Ribchester, 1979a, 1979c; McCardle and Albuquerque, 1975; Carbonetto, 1977) which is in agreement with the present findings (tables 3 and 4). There has been one report that m.e.p.p. frequency in the dystrophic diaphragm is less than normal (Conrad and Glaser, 1964) although this has been criticised because of the high frequencies recorded (Curran and Parry, 1975). The situation is less clear in muscles of C57BL/6J dy^{2J}/dy^{2J} mice, as m.e.p.p. frequency has been reported to be decreased (Shalton and Wareham, 1980), or normal until later stages of the disease when it decreases (Curran and Parry, 1975). Therefore, despite the decreased number of synaptic vesicles in motor nerve terminals in dystrophic muscles (Ragab, 1971; Pachter et al, 1973) the rate of spontaneous transmitter release is normal.

Giant m.e.p.ps. are defined as being more than twice the mean m.e.p.p. amplitude. An increased frequency of giant

m.e.p.ps. was recorded in the dystrophic EDL (table 3) though the incidence of giants was similar in diaphragms from normal and dystrophic mice (table 4). This observation in the EDL is consistent with the findings of other workers (Carbonetto, 1979; Harris and Ribchester, 1979a). The origin of giant m.e.p.ps. is uncertain but they are caused by multiquantal release of transmitter and they occur more frequently than would be expected from the coincident discharge of two or more quanta (Liley, 1957; Heuser, 1974; Heinonen, Jansson and Tolppanen, 1982). Giant m.e.p.ps. do not contribute to evoked transmitter release, as motor nerve stimulation does not release quanta corresponding to them (Menrath and Blackman, 1970; Molgo and Thesleff, 1982). The frequency of giant m.e.p.ps. can be increased by tetanic nerve stimulation. Electron microscopy of terminals after tetanic stimulation shows aggregation of synaptic vesicles and large vesicles (Heuser, 1974). Therefore, one possible explanation for the greater frequency of giants in the dystrophic EDL could be the occurrence of multivesicular complexes in dystrophic nerve terminals (see Ragab, 1971).

Miniature e.p.p. amplitude in the dystrophic EDL was 1.5 times greater than that in the normal EDL whereas the ratio in the diaphragm was 1.13. If e.p.p. amplitude in the dystrophic EDL is increased in proportion this would contribute towards the increased safety factor. Evoked transmitter release in normal and dystrophic muscles was

therefore compared.

Evoked release

The amplitudes of first and plateau endplate potentials in the curarised dystrophic EDL elicited at frequencies of stimulation of 10Hz and 50Hz were greater than those in the normal EDL (table 5A). This increase in e.p.p. size will contribute to the curare resistance in the dystrophic EDL. The upper range of the e.p.p. amplitude distribution was extended in the dystrophic EDL (fig. 9) which is consistent with the m.e.p.p. amplitude distribution (fig. 6). It is apparent from the e.p.p. amplitude distribution in the normal EDL that more e.p.ps. were sampled above the mode than below it. This is probably because the concentration of curare necessary to reduce e.p.p. amplitudes to subthreshold levels in the dystrophic EDL decreased e.p.p. amplitudes in normal fibres to such an extent that e.p.ps. were not discernible above the baseline noise. Endplate potential amplitude in the dystrophic diaphragm was also greater than normal (table 5B) although the range of values was similar in normal and dystrophic muscles (fig. 11). This larger e.p.p. amplitude in diaphragms from 14 to 18 week old dystrophic mice indicated a reduced sensitivity to curare. However, in the twitch experiments on diaphragm muscles from 15 week old dystrophic mice, curare resistance was not evident (fig. 5B). This is probably explained by the fact that the proportion of fibres affected by

dystrophy in the diaphragm is not sufficient to have an effect on curare sensitivity at the gross physiological level.

Miniature e.p.p. amplitude in the dystrophic EDL was only 1.50 times the normal value but e.p.p. amplitude was 2.44 times that of normal. Similarly, m.e.p.p. amplitude in the dystrophic diaphragm was 1.13 times greater than the normal value whereas e.p.p. amplitude was 1.3 times normal. These differences in the ratios of m.e.p.p. and e.p.p. amplitudes suggested a presynaptic difference such as an increase in the quantum content (m) of the e.p.p. occurred at dystrophic nerve terminals. Other workers have reported that m is consistently but not significantly greater than normal in curarised dystrophic EDL muscles (Harris and Ribchester, 1978, 1979a). The direct method of calculating m involves dividing the mean plateau e.p.p. amplitude in a muscle fibre by the mean m.e.p.p. amplitude in that fibre (del Castillo and Katz, 1954a). In the curarised preparation m.e.p.ps. cannot be recorded and m has to be determined indirectly. The method which has been used to estimate m in the curarised preparation assumes transmitter release is described by a Poisson distribution. There is evidence that transmitter release follows Poisson statistics when release is depressed by raising the magnesium concentration of the bathing solution (del Castillo and Katz, 1954a; Elmqvist and Quastel, 1965). However, a number of studies at vertebrate and invertebrate

neuromuscular junctions have demonstrated that quantal release obeys binomial statistics when release is not depressed (Johnson and Wernig, 1971; Bennett, Florin and Hall, 1975; Glavinović, 1979a; Miyamoto, 1975). Under these conditions estimation of m based on a Poisson distribution seriously overestimates its value (Johnson and Wernig, 1971; Wilson, 1977). Therefore, in the present study m was not calculated in the curarised preparation.

There is also controversy over whether or not curare has a presynaptic action in addition to its postsynaptic effect. It has been reported that curare has no effect on the amount of ACh released during or following repetitive nerve stimulation (Fletcher and Forrester, 1975) and also that it increases (Miledi, Molennar and Polak, 1978) and decreases ACh release (Beani, Bianchi and Ledda, 1964). Electrophysiological experiments have shown that curare produces a more rapid decline (rundown) in the amplitudes of successive endplate currents and potentials during repetitive stimulation (Magleby et al, 1981; Glavinović, 1979b; Wilson, 1982; Hubbard and Wilson, 1973). Quantum content of the first e.p.p. has been reported to be decreased (Hubbard, Wilson and Miyamoto, 1969; Hubbard and Wilson, 1973), unchanged (Auebach and Betz, 1971) or increased (Blaber, 1970, 1973; Wilson, 1982). Plateau e.p.p. amplitude has been reported to be unchanged (Wilson, 1982) and decreased (Hubbard and Wilson, 1977). There is difficulty in interpreting these results since they have

been obtained in different muscles. However, a differential presynaptic effect of curare in normal and dystrophic muscles would make any calculation of m using curarised preparations inaccurate.

Although quantum content was not measured in curarised preparations, further evidence of a presynaptic difference between normal and dystrophic EDL muscles was provided by a comparison of e.p.p. rundown in these muscles. The slope of the initial rundown was steeper in the dystrophic EDL at both 10Hz and 50Hz (fig. 10). However, there was no difference in the slopes of the e.p.p. rundown between normal and dystrophic diaphragm muscles. Endplate potential rundown can be increased by a number of procedures which enhance transmitter release. These include, higher stimulation frequencies (Magleby et al, 1981), conditioning trains of stimuli (Wilson, 1982), raised $[Ca^{2+}]o$ (Hofmann et al, 1966; Wilson 1982) and the presence of caffeine (Wilson, 1973). It is believed that all these procedures raise the free intraterminal calcium ion concentration ($[Ca^{2+}]i$). Therefore, it seemed that there was a presynaptic defect in the regulation of calcium concentration. This was further investigated by comparing transmitter release in magnesium blocked preparations.

At all three frequencies of motor nerve stimulation used, 1, 10 and 50 Hz, the plateau e.p.p. amplitude was greater in the dystrophic EDL (table 6). At 10Hz the plateau

e.p.p. amplitude in the dystrophic EDL was 2.69 times normal. This ratio is similar to that obtained in curarised muscles at the same frequency of nerve stimulation. In the magnesium blocked preparation the ratio of m.e.p.p. amplitude in the dystrophic EDL to that in the normal EDL was 1.54. This is almost identical to the ratio obtained in normal Liley's solution. These ratios confirm that the increased e.p.p. amplitude in the dystrophic EDL is not only the result of postsynaptic changes but also has a presynaptic origin.

In magnesium blocked muscles m was determined directly and it was significantly greater in the dystrophic EDL at all three frequencies of stimulation (table 6). These results differ to those of Carbonetto (1977) who reported no difference in m between magnesium blocked normal and dystrophic EDL muscle preparations. In the present study there was a population of muscle fibres in the dystrophic EDL in which values of m were similar to normal. However, the upper range of values was considerably greater in the dystrophic EDL (fig. 12). The increase in e.p.p. amplitude in the dystrophic EDL is thus due to an increase in both m.e.p.p. amplitude and m. Together with a decreased RMP this could explain the greater safety factor in this muscle.

There were no differences in e.p.p. amplitude, or m in magnesium blocked diaphragm muscles from normal and

dystrophic mice (table 7). This contrasts with the larger e.p.p. amplitude found in the curarised dystrophic diaphragm. There can be little doubt that the diaphragm is affected by dystrophy (Harris and Ribchester, 1979c) and a large number of diverse differences have been reported between diaphragms from normal and dystrophic 129/ReJ mice (Trabucchi, Cheney, Susheela and Costa, 1975; Bianchi, Pagliacci, Spreafico and Mussini, 1980; Herzberg et al, 1975; Conrad and Glaser, 1964). A possible explanation for the inconsistency in the evoked release results obtained in the diaphragm in the present study is the variation in severity to which muscles from mice of identical chronological age are affected. It is possible that more consistent changes in transmitter release in the dystrophic diaphragm are observed with increasing age of the animals.

Quantum content is the product of p and n where p represents the probability of quantal release in response to an action potential in the nerve terminal and n is the number of quanta available for release (del Castillo and Katz, 1954a). Thus an increase in m could be due to an increase in n and/or p. Neither of these parameters are represented in the Poisson equation which describes transmitter release at depressed junctions (del Castillo and Katz, 1954a). Therefore, transmitter release was investigated in normal and dystrophic cut-fibre EDL preparations. Release in these preparations has been shown to be binomial and n and p can be calculated (Glavinović,

1979a).

The cut-fibre preparation provides a method of investigating transmitter release in the absence of postsynaptic blocking agents or magnesium (Barstad, 1962; Barstad and Lilleheil, 1968). However, there are several problems inherent in the use of this preparation. Nerve block and elevation of m.e.p.p. frequency have been reported (Randić and Straughan, 1964; Banker et al, 1983) and safety factors less than or close to one have been calculated (Glavinović, 1979c; Banker et al, 1983). Therefore, it is possible that presynaptic function may not be normal in the cut-fibre preparation.

Cutting the muscle fibres reduces both RMP and Ro (Auerbach and Betz, 1971; Hubbard and Wilson, 1973; Glavinović, 1979c) thereby reducing m.e.p.p. amplitude (Katz and Thesleff, 1957). Therefore, there is a danger that fibres with larger m.e.p.p. amplitudes may be selected. In this investigation a histogram of m.e.p.p. amplitudes in each fibre was examined before the amplitudes were accepted. Any fibres in which there was a sharp cut-off at the lower end of the histogram, indicating that smaller m.e.p.ps. had been lost in the baseline noise were discarded.

Cutting the muscle fibres decreases the membrane space constant (Auerbach and Betz, 1971; Hubbard and Wison, 1973; Glavinović, 1979c). Therefore, if e.p.ps. are recorded at

a point even moderately remote from the endplate there will be a greater reduction in e.p.p. amplitude in the cut-fibre preparation than in the intact muscle. Auerbach and Betz (1971) suggested that this distortion of the e.p.p. would lead to an underestimation of the correction for nonlinear summation. Consequently m would be underestimated, particularly with larger potentials. However, Hubbard and Wilson (1973) found the seriousness of exact electrode placement was less than predicted, provided recording was made at sites where the rise times of e.p.ps. were 1.1 ms or less. In the present study the mean e.p.p. rise time in the dystrophic EDL was 0.62 ± 0.16 ms (32) and 0.45 ± 0.08 ms (34) in the normal EDL. Therefore, it is unlikely that the larger amplitude e.p.ps. in the dystrophic EDL were seriously undercorrected. Because of these problems with the cut-fibre preparation the absolute values obtained in normal and dystrophic EDL muscles have to be interpreted with caution. However, relative differences in evoked transmitter release between normal and dystrophic muscles will be genuine.

As shown in table 8 the e.p.p. amplitude in the dystrophic EDL was greater than that in the normal muscle. Thus the three methods used to record e.p.ps. provide the same result. Furthermore, in this preparation, as with the magnesium blocked preparation, the quantum content of plateau e.p.ps. (table 8) was greater in dystrophic muscles. However, the increase in m in the cut-fibre

preparation was only 1.48 times normal whilst in the magnesium blocked preparation it was 1.95 times normal. Obviously, any increase in mean m will depend upon the distribution of quantum contents in the fibres sampled. Figure 13 shows that 15% of the fibres in the dystrophic cut-fibre EDL preparation had quantum contents outside the range found in the normal EDL. In the magnesium blocked preparations this value was 43% (fig. 12). Assuming an increase in m is a feature of the disease, the number of fibres with a value of m outside the normal range will depend on the number of dystrophic fibres sampled. Therefore, it is possible that more dystrophic fibres were sampled in the magnesium blocked EDL than in the cut-fibre EDL.

Transmitter release in normal and dystrophic muscles followed a binomial distribution at every neuromuscular junction examined. This has generally been found to be the case at all nondepressed junctions investigated (Miyamoto, 1975; Glavinović, 1979a). Values of p and n of e.p.ps. elicited at 10Hz at junctions in normal and dystrophic muscles are consistent with the idea of a high probability of release from a small store of transmitter. Plateau e.p.p. amplitude in normal and dystrophic muscles was only 30% less than the amplitude of the first e.p.p.. This indicates that this store must be rapidly refilled. There was no difference in mean n between normal and dystrophic EDL muscles although mean p was significantly larger in the

dystrophic EDL (table 9). The greater m in dystrophic EDL muscles must therefore be attributed to the larger p in these muscles. The question is what causes the increase in p in murine dystrophy.

In the frog (Dodge and Rahamimoff, 1967) and mouse (Cooke, Okamoto and Quastel, 1973) evoked release increases as the fourth power of $[Ca^{2+}]o$. At mammalian neuromuscular junctions, when $[Ca^{2+}]o$ was varied in the range 0.1 to 1.0mM, it was found that p increased as the first power of $[Ca^{2+}]o$ whereas n increased as the third power of $[Ca^{2+}]o$ (Bennett et al, 1975). This increase in p with increasing $[Ca^{2+}]o$ has been found at many synapses (Bennett, Florin and Pettigrew, 1976; Miyamoto, 1975; Branisteanu, Miyamoto and Volle, 1976). It has been proposed that p is linearly related to $[Ca^{2+}]i$ after a nerve terminal action potential (Bennett et al, 1975). This follows if $[Ca^{2+}]i$ is determined by the amount of calcium influx (ΔCa) during the action potential. A linear relationship exists between $[Ca^{2+}]o$ and ΔCa (Baker, Hodgkin and Ridgway, 1971). The contribution of resting $[Ca^{2+}]i$ (i.e. residual Ca or CaR) in this case is relatively small under normal circumstances. The probability of release (p) therefore depends on calcium influx during the action potential and on the resting $[Ca^{2+}]i$. This relationship is expressed by the following equation:

$$p = K_1 (CaR + \Delta Ca)$$ (McLachlan, 1978).

where K_1 is a constant

Thus the increased p in dystrophic EDL muscles could be a consequence of a greater calcium entry during an action potential and/or a greater resting $[Ca^{2+}]i$.

The second determinant of m, n, is more sensitive to $[Ca^{2+}]o$ (and thus $[Ca^{2+}]i$) than p (Bennett et al, 1975). Therefore, it would be expected that the increase in p in murine dystrophy would be accompanied by an increase in n, if it was due to a raised $[Ca^{2+}]i$. There is controversy over what n actually represents. It has been suggested that n is simply the number of release sites on the nerve terminal membrane (Wernig, 1975). However, this is unlikely since n can vary with $[Ca^{2+}]o$ and $[Mg^{2+}]o$ (Bennett et al, 1975). An alternative explanation is that n is related to the number of synaptic vesicles immediately adjacent to the terminal membrane and available for release (Hubbard, 1963). McLachlan (1978) postulated that n is the number of filled vesicles located at release sites. Histologically it has been shown that there is a decreased number of synaptic vesicles in nerve terminals in dystrophic muscles (Ragab, 1971; Pachter et al, 1973). Although there is not always good correlation between synaptic vesicle number and transmitter release (Ceccarelli and Hurlbut, 1980), it is generally true that vesicle depletion is associated with transmission reduction or failure at the mammalian neuromuscular junction (Gorio, Hurlbut and Ceccarelli, 1978; Jones and Kwanbunbumpen,

1970). The fact that n is the same in normal and dystrophic muscles may be because only a certain fraction of vesicles are released during normal transmission (McLachlan, 1978; Ceccarelli and Hurlbut, 1980) and these vesicles are preserved in murine dystrophy.

There is considerable evidence for a widespread membrane defect in dystrophy (Howland and Challberg, 1973; Frostholm et al, 1981; Nylen and Wrogemann, 1983; Akindele et al, 1982; Das et al, 1971). Therefore, an alternative suggestion is that the increase in the probability (p) of transmitter release in dystrophy is due to a change in the property of the nerve terminal membrane which facilitates transmitter release.

The rate of spontaneous transmitter release from nerve terminals at rest is thought to depend upon $[Ca^{2+}]i$ (Baker, 1972; Duncan and Statham, 1977; Alnaes and Rahamimoff, 1975). As already discussed an increase in p and m could be due to a raised resting $[Ca^{2+}]i$ in nerve terminals in dystrophic muscles, or greater calcium entry during the action potential coupled with an impaired ability to eject or sequester calcium within intracellular organelles. Therefore, to investigate nerve terminal calcium regulation m.e.p.p. frequency was compared in normal and dystrophic muscles after procedures believed to increase $[Ca^{2+}]i$.

Factors affecting spontaneous transmitter release

Before the results obtained in this section are discussed the mechanisms believed to be involved in the regulation of $[Ca^{2+}]i$ will be briefly reviewed to assist in their interpretation.

Nerve terminal area in normal and dystrophic EDL muscles is about $6\mu m^2$ (Banker et al, 1979). Because of the small size of mammalian motor nerve terminals it is not possible to measure $[Ca^{2+}]i$ directly. However, $[Ca^{2+}]i$ has been measured in molluscan neurones using photoproteins such as aequorin, metallochromic indicators such as arsenazo III and calcium sensitive microelectrodes (Baker, 1972; Hofmeier and Lux, 1981; Ahmed and Connor, 1979; Christofferson and Simonsen, 1977). These studies have shown that $[Ca^{2+}]i$ is of the order of $10^{-7}M$ in resting nerve cells. In mammals $[Ca^{2+}]o$ is about $10^{-3}M$ (Rasmussen and Goodman, 1977), therefore, there is a concentration gradient in the order of 10^4 favouring calcium entry into the nerve terminal.

Ultimately, any calcium which enters the terminal has to be ejected through the membrane. Experiments with synaptosomes suggest that calcium is extruded from nerve terminals by a mechanism directly linked to ATP hydrolysis, catalysed by $(Ca^{2+} + Mg^{2+})$ATPase. Calcium uptake by synaptosomes is increased by ATP depletion (Akerman and Nicholls, 1981a) and ATP-linked calcium transport (Gill,

Grollman and Kohn, 1981) and (Ca^{2+} + Mg^{2+})ATPase activity (Duncan, 1976) have been found in isolated synaptosomal plasma membranes. There is also good evidence that the plasma membrane of mammalian synaptosomes contains a sodium / calcium exchange mechanism. Sodium ions are extruded by the (Na^{+} + K^{+}) ATPase and it has been proposed that calcium efflux subsequently occurs in exchange for two or three sodium ions (Blaustein and Oborn, 1975; Gill et al, 1981).

It is believed that transport systems in intracellular organelles including mitochondria and smooth endoplasmic reticulum are also involved in the regulation of $[Ca^{2+}]i$. In motor nerve terminals of mouse EDL and tibialis anterior muscles the mitochondria occupy 17% of the total area (Banker et al, 1979). A role for mitochondria in calcium regulation in nerve endings has been suggested by the fact that synaptosomal calcium is found in mitochondria (Scott, Akerman and Nicholls, 1980), the amount of which increases after plasma membrane depolarisation (Akerman and Nicholls, 1981b).

In rat dorsal root axons calcium deposits are located in the smooth endoplasmic reticulum and a Ca-activated ATPase has been reported in its membrane (Duce and Keen, 1978). In the presence of mitochondrial poisons, calcium is sequestered in synaptosomes by an ATP requiring process in a non-mitochondrial organelle believed to be the smooth

endoplasmic reticulum (McGraw, Somlyo and Blaustein, 1980). Evidence indicating an important role of the endoplasmic reticulum in terminating evoked transmitter release in mammalian neurones has been provided by Neering and McBurney (1984). Using aequorin fluorescence in cultured rat dorsal root ganglion cells they found that caffeine released calcium intracellularly. Subsequent exposure to caffeine had no effect, however, electrical stimulation between the two exposures to caffeine resulted in release of calcium on the second exposure. Therefore, during an action potential the intracellular stores are filled with calcium.

Most attention has been given to the role of mitochondria and endoplasmic reticulum in buffering $[Ca^{2+}]i$, however, it has also been postulated that other intracellular structures may be involved in the regulation of $[Ca^{2+}]i$. For example, calcium has been found attached to the internal surface of the plasma membrane in squid axon (Hillman and Llinás, 1974; Oschman, Hall, Peters and Wall, 1974) and cytoplasmic high-affinity calcium binding proteins have been described (Alemà, Callisano, Rusca and Guiditta, 1973).

Although there is a reduction in synaptic vesicle number in nerve terminals in dystrophic muscles (Ragab, 1971; Pachter et al, 1973) the results obtained in the cut-fibre EDL suggest that the number of vesicles which contribute to

quantal release of transmitter is similar to normal. If $[Ca^{2+}]i$ was elevated in nerve terminals in dystrophic muscles an increased m.e.p.p. frequency might be expected. However, there was no difference in control m.e.p.p. frequencies between normal and dystrophic EDL (table 3) or normal and dystrophic diaphragm muscles (table 4). Therefore, it appears that, at rest, the calcium regulating mechanisms in dystrophic nerve terminals can maintain $[Ca^{2+}]i$ within normal limits. However, this may not be the case after procedures believed to raise $[Ca^{2+}]i$ by increasing calcium entry into the nerve terminals or releasing it from intracellular stores.

Miniature e.p.p. frequency is susceptible to factors such as pH and anoxia (Hubbard and Løyning, 1966; Shalton and Wareham, 1979). However, the increase in frequency produced by any agent could not be due to a decreased pH since none had any significant effect on the pH of the bathing solution. All isolated preparations deteriorate eventually, so it was important to eliminate the possibility that this could be the cause of any observed increase in m.e.p.p. frequency. Preliminary investigations showed that m.e.p.p. frequency was stable under control conditions for the duration of the experiments. Therefore, any observed increase in m.e.p.p. frequency was attributable to the procedure believed to raise $[Ca^{2+}]i$.

There are numerous reports that, at the mammalian

neuromuscular junction raising, or reducing $[Ca^{2+}]o$ increases or decreases m.e.p.p. frequency, respectively (Boyd and Martin, 1956; Elmqvist and Feldman, 1965; Hubbard et al, 1968; Cull-Candy, Lundh and Thesleff, 1976). Raising $[Ca^{2+}]o$ increases the concentration gradient for calcium across the nerve terminal membrane increasing calcium influx, and hence $[Ca^{2+}]i$ thereby increasing m.e.p.p. frequency. At 33 ± 1°C raising $[Ca^{2+}]o$ produced a similar significant increase in m.e.p.p. frequency in normal and dystrophic EDL muscles (table 10). A histogram of the frequency distribution in 6mM $[Ca^{2+}]o$ showed no difference between normal and dystrophic EDL muscles. Therefore, it appears that nerve terminals in the dystrophic EDL, which possess quantum contents greater than the normal range (figs. 12 and 13), do not respond to raised $[Ca^{2+}]o$ with a greater increase in m.e.p.p. frequency than normal. There is obviously a difference between the amount of calcium which diffuses into the nerve terminal when $[Ca^{2+}]o$ is raised at rest than that which enters through voltage-dependent calcium channels after an action potential. However, this result suggests that the increased quantum content is not related to a greater $[Ca^{2+}]i$ in motor nerve terminals in dystrophic EDL muscles.

It has been reported that, at room temperature, raising $[Ca^{2+}]o$ increases m.e.p.p. frequency in EDL and soleus muscles from dystrophic mice of the C57BL/6J strain but not in normal muscles (Shalton and Wareham, 1980). An increase in

temperature increases m.e.p.p. frequency (Ward, Crowley and Johns, 1972) and, in the frog, it has been suggested that temperature has a direct effect on the release process (Publicover and Duncan, 1981a, 1981b). Therefore, raising temperature may affect spontaneous release in normal and dystrophic muscles differently. To eliminate this possibility the effect of 6mM $[Ca^{2+}]o$ on m.e.p.p. frequency was re-examined at room temperature. In these experiments, as with the experiments at 33 ± 1°C, a similar significant increase in m.e.p.p. frequency was observed in both normal and dystrophic EDL muscles (table 10). This suggests that a presynaptic difference exists between normal EDL muscles of the two commonly used strains of dystrophic mice. Raising $[Ca^{2+}]o$ increased m.e.p.p. frequency in diaphragm muscles from white mice (fig. 15) which suggests that muscles from normal 129/ReJ mice do not respond to $[Ca^{2+}]o$ in an abnormal manner.

At room temperature raising $[Ca^{2+}]o$ had a similar effect on m.e.p.p. frequency in diaphragm muscles from normal and dystrophic mice (table 11). However, at 37 ± 1°C a significantly greater increase in m.e.p.p. frequency was found in the dystrophic diaphragm (table 11, fig. 15). It is difficult to interpret this result since this difference is not found in the dystrophic EDL which is more affected by the disease. One possible explanation may be that raised temperature has a differential effect on the sensitivity of spontaneous release to raised $[Ca^{2+}]o$ in

motor nerve terminals in normal and dystrophic diaphragm muscles.

Doubling $[K^+]o$ produced a similar significant increase in m.e.p.p. frequency in normal and dystrophic EDL muscles (table 12). The effect of raising $[K^+]o$ on m.e.p.p. frequency is well documented and is believed to be due to nerve terminal depolarisation increasing the calcium conductance of the presynaptic membrane (Liley, 1956c; Carlson and Dettbarn, 1983). Therefore, it appears that motor nerve terminals in normal and dystrophic EDL muscles respond to depolarisation in a similar way.

After tetanic motor nerve stimulation there is an increase in m.e.p.p. frequency which persists for several minutes before it declines to control values (Liley, 1956a, 1956b; Miledi and Thies, 1971). This increase in frequency is believed to be caused by accumulation of calcium in the cytoplasm of the nerve terminal due to the combined effects of calcium influx through the presynaptic membrane and release from intracellular stores (Erulkar and Rahamimoff, 1978). In both normal and dystrophic EDL muscles m.e.p.p. frequency was increased after tetanic stimulation by about 100% and declined to control levels within one minute. Therefore, it appears that the amount of calcium which enters the nerve terminal during an action potential and the amount of calcium which is released from intracellular stores is normal in dystrophic EDL muscles.

None of the methods believed to increase calcium entry into the nerve terminal produced a greater increase in m.e.p.p. frequency in dystrophic EDL muscles than normal muscles. Therefore, it is unlikely that the greater m in dystrophic EDL muscles is related to a raised $[Ca^{2+}]i$.

Mitochondria isolated from the liver and brain of 129/ReJ dy/dy mice accumulate calcium more rapidly than mitochondria isolated from the same organs of clinically normal littermates (Katyare et al, 1978; Frostholm et al, 1981). It has also been reported that mitochondrial inhibitors have a greater effect on m.e.p.p. frequency in EDL and soleus muscles from C57BL/6J dy^{2J}/dy^{2J} mice than normal muscles (Shalton and Wareham, 1980). Therefore, the role of intracellular organelles in regulating $[Ca^{2+}]i$ in motor nerve terminals of normal and dystrophic muscles was compared using agents which release intracellular calcium and thus increase m.e.p.p. frequency.

There have been several reports that uncouplers of oxidative phosphorylation, such as DNP, increase m.e.p.p. frequency (Beani, Bianchi and Ledda, 1966; Alnaes and Rahamimoff, 1975; Statham, Duncan and Publicover, 1978). The increase in m.e.p.p. frequency is then followed by a decline towards control values (Glagoleva, Liberman and Khashayev, 1970; Statham et al, 1978; Publicover and Duncan, 1981b). This pattern of response to DNP was seen

in the normal EDL at room temperature, where m.e.p.p. frequency increased rapidly about 10 minutes after DNP and then fell to control levels within 30 minutes (fig. 16). However, DNP produced a slower, smaller increase in frequency in the dystrophic EDL (fig. 16). Records of m.e.p.p. frequency made 30 minutes after DNP indicate that the dystrophic EDL is more sensitive to DNP but, in fact, it is less sensitive than normal (table 13). 2,4-Dinitrophenol also produced a greater increase in m.e.p.p. frequency in the normal EDL at 33 ± 1°C (fig. 17) and in the normal diaphragm at 37 ± 1°C (table 14) than in dystrophic muscles. Therefore, m.e.p.p. frequency in dystrophic EDL and diaphragm muscles is less sensitive to DNP than normal.

Although the results obtained with DNP are clear-cut their interpretation is more difficult. It has been shown that, in vitro, DNP decreases calcium uptake by mitochondria and causes the release of sequestered calcium (Drahota, Carafoli, Rossi, Gamble and Lehninger, 1965; Carafoli, 1967). This could explain how DNP increases m.e.p.p. frequency. Calcium release from the smooth endoplasmic reticulum can be eliminated as DNP actually increases calcium uptake by microsomes which are rich in endoplasmic reticulum vesicles (Carafoli, 1967). It has also been shown that the endoplasmic reticulum of synaptosomes accumulates calcium in the presence of DNP (McGraw et al, 1980) and that in inverted membrane vesicles

of synaptosomes DNP has no effect on sodium/calcium exchange (Gill et al, 1981). An alternative explanation to a mitochondrial effect of DNP is that it depolarises nerve terminals causing calcium influx. Whilst this explanation cannot be excluded, it seems unlikely that DNP caused depolarisation of the terminals as it increased m.e.p.p. frequency in the EDL at room temperature without decreasing the muscle fibre RMP (table 13). Furthermore, depolarisation of the nerve terminals with KCl (10^{-2}M) had a similar effect on m.e.p.p. frequency in normal and dystrophic EDL muscles (table 12).

2,4-Dinitrophenol contains a phenol group and phenol has been shown to increase m.e.p.p. frequency in pectoral fin muscles of the silver carp (Kuba, 1969), although no study has been made in the mammal. Therefore, the action of the nonphenolic uncoupler of oxidative phosporylation guanidine on m.e.p.p. frequency in normal and dystrophic EDL muscles was investigated.

Guanidine derivatives have been reported to inhibit calcium uptake by isolated mitochondria (Davidoff, 1974). A mitochondrial effect is probably the explanation for the increase in m.e.p.p. frequency produced by guanidine at the mammalian neuromuscular junction (Lundh, Leander and Thesleff, 1977; Hofmann et al, 1966). In this investigation guanidine significantly increased m.e.p.p. frequency in both normal and dystrophic EDL muscles (table 15).

However, a greater increase was observed in the normal muscles. Guanidine did not cause any significant change in muscle fibre RMP suggesting that this increase in m.e.p.p. frequency was not the result of nerve terminal depolarisation (table 15).

The results obtained with guanidine are consistent with those obtained with DNP and suggest that, at motor nerve terminals in dystrophic muscles, there is a decrease in the sensitivity of spontaneous release to uncouplers of oxidative phosphorylation. Assuming the increase in m.e.p.p. frequency produced by these agents is due to release of sequestered calcium from mitochondria there may be less sequestered calcium available for release or fewer mitochondria in motor nerve terminals in dystrophic muscles. It has been reported that calcium levels are elevated in mitochondria from brain (Frostholm et al, 1981), liver (Katyare et al, 1978) and cardiac and skeletal muscle (Wrogemann et al, 1979; Nylen and Wrogemann, 1983) of dystrophic mice. Thus it might be expected that uncouplers of oxidative phosphorylation would have a greater effect upon m.e.p.p. frequency in dystrophic muscles. Uncoupling agents release only about 2/3 to 3/4 of the total endogenous calcium of isolated mitochondria, the remaining portion of it is probably sequestered in a highly stable form (Carafoli, Patriarca and Rossi, 1969). Therefore, it is possible that in the mitochondria in nerve terminals of dystrophic muscles there is more calcium bound

in this stable form than normal.

The effect of guanidine on evoked transmitter release in curarised EDL muscles from normal and dystrophic female mice was also investigated. Guanidine has been shown to increase e.p.p. amplitude due to an increase in m (Lambert and Elmqvist, 1971; Teräväinen and Larsen, 1975). In normal and dystrophic muscles guanidine produced a similar significant increase in mean plateau e.p.p. amplitude. Rundown of e.p.ps. in normal and dystrophic muscles was also enhanced by a similar amount. This enhancement of rundown could be due to an increase in the probability of release (Hofmann et al, 1966; Kamenskaya, Elmqvist and Thesleff, 1975a).

Thus, although spontaneous release in dystrophic muscles is less sensitive to guanidine than normal, the same does not apply to evoked release. Removal of calcium ions from the bathing solution or the addition of manganese completely abolishes the effect of guanidine on evoked release (Lundh and Thesleff, 1977) whereas it increases m.e.p.p. frequency in the absence of external calcium (Hofmann et al, 1966). Therefore, it is unlikely that the increase in e.p.p. amplitude produced by guanidine is due to uncoupling of oxidative phosphorylation. Guanidine increases action potential duration in mouse EDL muscle fibres (Kamenskaya et al, 1975b). An increase in action potential duration presynaptically would increase transmitter release which is

dependent on the duration of depolarisation of the nerve terminal (Katz and Miledi, 1967b). Therefore, guanidine could enhance evoked release by increasing calcium entry into the nerve terminal. The fact that it has a similar effect in normal and dystrophic muscles is further evidence that there is not impaired calcium regulation in nerve terminals in the dystrophic EDL.

Caffeine caused a similar increase in m.e.p.p. frequency in normal and dystrophic EDL muscles, however, a greater increase in frequency was produced in the dystrophic diaphragm (table 16) than in normal controls. The greater m.e.p.p. frequency in the dystrophic diaphragm was due to a larger modal value in this muscle and not to a higher range of frequencies (fig 18). The effect of caffeine on m.e.p.p. frequency is independent of external calcium (Elmqvist and Feldman, 1965; Hofmann, 1969) and is, therefore probably due to an intracellular action. The site of action is probably the smooth endoplasmic reticulum because caffeine has no effect on mitochondrial calcium release (Weber, 1968; Batra, 1974) whereas it releases calcium from fragmented sarcoplasmic reticulum (Weber and Herz, 1968; Batra, 1974).

The results obtained with caffeine suggest that there is no difference in the role of the endoplasmic reticulum in nerve terminals in normal and dystrophic EDL muscles. However, caffeine produced a 16% greater increase in

m.e.p.p. frequency in the dystrophic diaphragm than in the normal diaphragm. Therefore, the endoplasmic reticulum may have a greater role to play in regulation of $[Ca^{2+}]i$ in the dystrophic diaphragm.

There are many reports that lanthanum increases m.e.p.p. frequency in frog skeletal muscle (Blioch, Glagoleva, Liberman and Nenashev, 1968; De Bassio, Schnitzler and Parsons, 1971; Colton, 1976). The present results show that lanthanum also increases m.e.p.p. frequency in muscles from the mouse and that this increase is bigger in dystrophic muscles (table 17, fig. 19).

It is difficult to locate the site of action of lanthanum. It is excluded from the nerve terminals of frog sartorius muscles (Heuser and Miledi, 1971). Therefore, it is unlikely that it releases calcium from mitochondria or smooth endoplasmic reticulum. Calcium is bound to the internal surface of the plasma membrane of squid axon (Hillman and Llinás, 1974; Oschman et al, 1974) and lanthanum readily binds to anionic binding sites on the external surface of the plasma membrane and displaces calcium from monolayers of phosphatidyl choline or phosphatidyl ethanolamine which are components of plasma membranes (Doggenweiler and Frenk, 1965). Therefore, lanthanum may cause an inward displacement of calcium ions from binding sites on the nerve terminal membrane. If this is the case the results suggest that more calcium is bound

to the inner surface of the motor nerve terminal membrane in dystrophic EDL muscles. De Bassio et al (1971) proposed that the effect of lanthanum on m.e.p.p. frequency can be explained by it binding to anionic membrane sites and altering the physiochemical properties of the nerve terminal membrane, increasing the probability of spontaneous release. If lanthanum does act in this way the results would suggest that there is a difference in the membrane properties of nerve terminals in normal and dystrophic EDL muscles.

All of the methods used to increase calcium entry into the motor nerve terminal had similar effects upon m.e.p.p. frequency in normal and dystrophic EDL muscles (table 20). Therefore, it is unlikely that the greater quantum content and probability of release in the dystrophic EDL are a consequence of raised $[Ca^{2+}]i$. The results obtained with uncouplers of oxidative phosphorylation and caffeine indicate that mitochondria have a reduced role in buffering $[Ca^{2+}]i$ in dystrophic EDL muscles, whereas the role of the endoplasmic reticulum is similar to normal (table 20). The lanthanum result may indicate that the inner surface of the plasma membrane has a greater role in calcium buffering in nerve terminals in dystrophic EDL muscles. If this is the case it may imply that there is a greater calcium influx in nerve terminals in dystrophic EDL muscles although it is buffered adequately so that $[Ca^{2+}]i$ is normal. However, if the increase in m.e.p.p. frequency produced by lanthanum is

Table 20. The effect of agents and procedures used to raise free $[Ca^{2+}]i$ on m.e.p.p. frequency in the EDL (33 ± 1°C) and diaphragm (37 ± 1°C).

	EDL		DIAPHRAGM	
	NORMAL	DYSTROPHIC	NORMAL	DYSTROPHIC
$CaCl_2$ ($6X10^{-3}M$)	↑	↑	↑	⇑
KCl ($10^{-2}M$)	↑	↑		
Nerve Stimulation (50Hz for 60 secs)	↑	↑		
DNP ($10^{-4}M$)	⇑	-	↑↑	-
Guanidine ($5X10^{-3}M$)	⇑	↑		
Caffeine ($2X10^{-3}M$)	↑	↑	↑	⇑
Lanthanum ($5X10^{-4}M$)	↑	⇑		

- = no effect

↑ = significant increase in frequency ($p < 0.05$)

⇑ = significant difference between normal and dystrophic muscles ($p < 0.05$).

through a direct effect on the terminal membrane it implies that the increased quantum content in the dystrophic EDL reflects a change in the properties of this structure.

The dystrophic diaphragm is similar to the dystrophic EDL in its response to DNP indicating a reduced role of mitochondria in regulating $[Ca^{2+}]i$ in these muscles (table 20). It differs from the dystrophic EDL in that caffeine and raising $[Ca^{2+}]o$ caused a slightly but significantly greater increase in m.e.p.p. frequency in the dystrophic diaphragm than in the normal diaphragm. The results obtained with DNP and caffeine suggest that calcium is distributed differently between the intracellular organelles in motor nerve terminals in dystrophic diaphragm muscles. Quantum content in the magnesium blocked dystrophic diaphragm was not greater than normal, which would be expected if there was greater calcium influx. This indicates that the greater sensitivity of spontaneous release to $[Ca^{2+}]o$ is not of physiological importance. Motor nerve terminals in dystrophic EDL and diaphragm muscles are less sensitive to DNP than normal. However, postsynaptically the EDL is more affected by dystrophy than the diaphragm. Therefore, there would seem to be no correlation between the extent of necrosis postsynaptically and DNP sensitivity presynaptically.

Radiochemical Experiments

Nerve terminal calcium fluxes under resting conditions may be greater than normal in dystrophy requiring more effort on the part of the buffering mechanisms to regulate $[Ca^{2+}]i$. It is not possible to measure radioactively labelled calcium fluxes across mammalian motor nerve terminals, however they can be measured across the sarcolemma. Therefore, calcium fluxes were measured in normal and dystrophic diaphragm and EDL muscles.

It has been reported that the calcium concentration of skeletal muscle is elevated in murine dystrophy although plasma calcium levels are normal (Dowben et al, 1964). This raised intracellular calcium may result from increased influx of calcium into the muscle fibres across a labile sarcolemma or from a decrease in transport of calcium from sarcoplasm to extracellular fluid. Although it is generally assumed that calcium fluxes are different in normal and dystrophic muscle they have never been measured in either murine or Duchenne dystrophy. This is surprising in view of the hypothesis that calcium influx is the cause of the muscle necrosis (Duncan, 1978; Wrogemann and Pena, 1976). Calcium fluxes have not been investigated in any tissues in murine dystrophy although there have been several investigations in dystrophic human tissues. Calcium is believed to be pumped out of erythrocytes by the enzyme Ca-ATPase (Lee and Shin, 1969). An increase in

activity of this enzyme in erythrocyte plasma membranes has been reported in Duchenne dystrophy (Luthra, Stern and Kim, 1979). Using inside-out vesicles it has been shown that the calcium transport protein in the erythrocytes of patients with Duchenne dystrophy has a decreased affinity for calcium when compared to protein from the erythrocytes from normal patients, but that it transports calcium at a faster rate (Mollman, Cardenas and Pleasure, 1980). Despite these reports of abnormal Ca-ATPase function no significant difference in calcium influx and efflux has been found between erythrocytes from normal and dystrophic patients (Plishker and Appel, 1980; Shoji, 1981). Similarly, no difference in the rate of calcium uptake between cultured fibroblasts from normal and dystrophic patients has been shown (Statham and Dubowitz, 1979).

Calcium influx into normal and dystrophic diaphragm and EDL muscles was rapid. After 10 minutes incubation with ^{45}Ca the calcium influx curve plateaued in both normal and dystrophic muscles (figs. 20A, 20B). These results are similar to those reported in normal rat diaphragms incubated at 30°C (Carafoli et al, 1969). In dystrophic diaphragm and EDL muscles the uptake of ^{45}Ca was significantly greater than normal muscles. An interesting finding was that the percentage increase in calcium uptake in both dystrophic muscles i.e. diaphragm and EDL was similar. A possible explanation for the increased uptake of calcium in dystrophic muscles is that either the sarcolemma is more

permeable to calcium or that calcium efflux is decreased. Efflux was therefore compared in normal and dystrophic muscles which had been preloaded with ^{45}Ca for 60 minutes. As expected, after the preloading period the concentration of ^{45}Ca in dystrophic muscles was greater than normal. When these preloaded muscles were incubated in a calcium-free solution, calcium efflux, over the first 5 minutes, was more rapid in dystrophic muscles (figs. 21A, 21B). However, after 5 minutes the amount of calcium retained was similar in normal and dystrophic muscles.

A complicating factor in interpreting calcium fluxes in muscle is the size of the extracellular space. This space will obviously fill and empty at a more rapid rate than the muscle fibres. Therefore, the different calcium influx and efflux results obtained in the present study could merely be an artefact produced by a larger extracellular space in the dystrophic muscles. To investigate this possibility, the extracellular space was measured using the polysaccharide inulin which is unable to penetrate intact cellular membranes (Law and Phelps, 1966). The extracellular space, as defined by the space accessible to inulin, was greater in dystrophic diaphragm and EDL muscles than normal (figs. 22A, 22B). The space was 1.65 times greater than normal in the dystrophic EDL and 1.48 times greater than normal in the dystrophic diaphragm. This increase in the inulin space in dystrophic muscles was similar to the increase in calcium uptake. Therefore,

these results suggest either that the greater calcium uptake is confined to the extracellular space or that calcium accumulates in severely damaged fibres which essentially contribute to the extracellular space.

Several workers have shown that the inulin space in dystrophic hindlimb muscles is larger than normal (Burr and McLennan, 1960; Molak, Stracher and Erlij, 1980). This could be due to either an actual change in the tissue geometry producing a real increase in the size of the extracellular space or penetration of inulin into the fibres. The latter is suggested by the penetration of the extracellular markers horseradish peroxidase and procion yellow into dystrophic muscle fibres (Banker et al, 1979; Bradley and Fulthorpe, 1978; Libelius et al, 1978) and raised plasma concentrations of intracellular enzymes (Zierler, 1958; 1961; Stamp and Lesker, 1967). Molak et al (1980) compared the spaces occupied in normal and dystrophic muscles by the smaller tracers sucrose, manitol and polyethylene glycol 900 (PEG 900) and the larger tracers PEG 4000 and inulin. They found that the space occupied by all these tracers was greater in dystrophic muscles, however, the increase was larger with the smaller tracers. This indicates that there is not just a simple increase in extracellular space in murine dystrophy but increased penetration of tracers into the muscle.

In a dystrophic muscle, at any stage of the disease, fibres

can be found ranging from normal to severely necrotic (Banker, 1968; Platzer, 1979). Thus the efflux of calcium from necrotic fibres may have obscured any increase in efflux from non-necrotic, but diseased fibres with a calcium labile membrane. Lanthanum has been shown to reduce calcium efflux from a number of preparations such as the squid giant axon (van Breemen and de Weer, 1970), rabbit aorta (van Breemen et al, 1972) and cultured rabbit heart cells (Langer and Frank, 1972). While lanthanum will not prevent calcium efflux from severely necrotic fibres it was hoped that efflux from less damaged fibres would be reduced revealing a difference between the amount of calcium retained in normal and dystrophic muscles. However, lanthanum had no effect on calcium efflux from normal or dystrophic EDL muscles and no significant difference between the amount of calcium retained in these muscles was found. Lanthanum did reduce calcium efflux from normal and dystrophic diaphragms but again no difference between the amount of calcium retained was found.

One interesting finding from these experiments was the fact that the extracellular space in diaphragm muscles from dystrophic mice was greater than that in diaphragms from nondystrophic littermates. The extracellular space was increased in dystrophic diaphragms by a similar amount to that in the EDL. This contrasts with the dystrophic soleus in which the extracellular space is less than in the

dystrophic EDL (Molak et al, 1980). If the larger extracellular space in the dystrophic diaphragm is the result of a greater penetration of inulin into the muscle fibres, the question arises as to why the diaphragm is not as affected by dystrophy as the EDL. One possible explanation is that the majority of fibres in the mouse diaphragm are oxidative (Davies and Gunn, 1972; Noireaud and Léoty, 1984). Oxidative fibres contain more mitochondria (Shafiq et al, 1969; Butler and Cosmos, 1977; Dribin and Simpson, 1977) and mitochondria have an important role in regulating $[Ca^{2+}]i$ in skeletal muscle (Carafoli, 1969). Therefore, the resistance of the diaphragm to dystrophy, despite an increased calcium influx across a labile membrane, may be attributed to the large number of mitochondria in this muscle which can buffer changes in $[Ca^{2+}]i$. It would be interesting to see whether calcium levels are raised in mitochondria in the dystrophic diaphragm.

Conclusions

It is unlikely that the greater probability of release and quantum content in dystrophic EDL muscles is due to an impaired regulation of intraterminal calcium causing a rise in $[Ca^{2+}]i$. However, there does seem to be a difference in the role of intracellular organelles in nerve terminals in normal and dystrophic muscles in buffering $[Ca^{2+}]i$. The

increased p and m may be due to a defect in the presynaptic membrane facilitating release. An alteration in membrane properties postynaptically could also explain the increased m.e.p.p. amplitude and the difference in m.e.p.p. and e.p.p. half decay times observed in dystrophic EDL muscles. Changes in transmission similar to those observed in the dystrophic EDL can be induced in a number of ways. It is important, therefore, to eliminate the possibility that the changes observed could be attributed to factors other than dystrophy.

With progression of murine dystrophy the hindlimbs are used less frequently and the animal uses its forelimbs to propel itself. Eventually the hindlimbs become completely paralysed. Muscle disuse has been produced experimentally in a number of ways, by immobilisation of the knee and ankle joints (Fischbach and Robbins, 1971), blockade of nerve terminal action potentials using tetrodotoxin, local anaesthetics and diphtheria toxin (Snider and Harris, 1971; Lømo and Rosenthal, 1972) and block of transmitter release using botulinum toxin (Duchen, 1970). It is pertinent, therefore, to raise the question whether or not the differences in neuromuscular transmission found could be a consequence of muscle disuse.

Robbins and Fischbach (1971) examined transmission at neuromuscular junctions of solei of rats whose hindlimbs were immobilised for varying periods of time. Miniature

e.p.p. amplitude was increased as a consequence of an increased Ro due to fibre atrophy. Mean quantum contents at curarised disused junctions were also greater than normal and, at 10Hz, the slopes of e.p.p. rundown were greater. All these effects of disuse appeared after three days of immobilisation. Lømo and Rosenthal (1972) blocked rat sciatic nerve impulses with anaesthetics and diphtheria toxin for periods of up to 14 days. Again m.e.p.p. amplitude was increased in the soleus as a consequence of increased Ro due to fibre atrophy. It was also reported that although there was overlap in the ranges of quantum contents for curarised normal and disused preparations there was a slight increase in m in disused soleus muscles and a slight decrease in m in disused EDL muscles. Snider and Harris (1979) produced disuse of the rat soleus by implanting silicone cuffs impregnated with tetrodotoxin around the sciatic nerve. In these experiments mean quantum content at neuromuscular junctions of disused muscles was 40% greater than at those of control muscles.

These three methods of producing disuse in the rat soleus all resulted in an increase in m and two of them resulted in an increase in m.e.p.p. amplitude. Abolishing muscle activity by various methods also results in a decrease in RMP (Mills, Bray and Hubbard, 1978). All these changes were observed in the dystrophic EDL. Muscle disuse also produces motor nerve terminal sprouting (Brown and Ironton, 1978) and an increase in endplate size (Pestronk and

Drachman, 1978). Sprouting at nerve terminals has been found in murine dystrophy (Harman et al, 1963; Harris and Ribchester, 1979a) but endplate area has been reported to be both increased (Harris and Ribchester, 1979a) and decreased (Banker et al, 1979). Disuse also results in a spread of acetylcholine receptors to extrajunctional sites (Fischbach and Robbins, 1971; Lømo and Rosenthal, 1972). However, the majority of evidence indicates that extrajunctional ACh receptors are not present in murine dystrophy (Harris and Marshall, 1973; Marusyk and Monckton, 1976; Banker et al, 1979; Kelly and Smith, personal communication). Evoked transmitter release was investigated in EDL muscles from 8 week old animals and dystrophic mice of this age still retain some use of their hindlimbs. Therefore, it is unlikely that disuse can explain the changes in neuromuscular transmission in the dystrophic EDL.

A number of studies have reported decreased AChE activity in murine dystrophy (Boegman, 1974; Skau and Brimijoin, 1981; Curtis et al, 1961; Glaser and Seashore, 1967; Rash et al, 1974; Ellisman et al, 1975; McCaman, Stafford and Skinner, 1967). In the mammal antiChEs, in vitro, increase m.e.p.p. and e.p.p. amplitude and time course (Wilson, 1982; Liley, 1956a; Bois et al, 1980). It has been reported that m is unchanged (Ferry and Marshall, 1971; Wilson, 1982) or increased after antiChEs (Blaber and Christ, 1967; Blaber, 1972; Bois et al, 1980; Skliarov,

1980). There is also an increase in the incidence of giant m.e.p.ps. (Bois et al, 1980; Carlson and Dettbarn, 1983). Decreased AChE activity in dystrophy could explain the reported supersensitivity to antiChEs (Baker et al, 1960; Baker, 1963; Baker and Sabawala, 1963). Chronic treatment of rats with antiChEs produces sprouting at terminals, a reduction of postjunctional folding, widening of the synaptic cleft and interposition of Schwann cell processes between nerve terminals and the postjunctional membrane (Lytle and Wellbound, 1970; Engel et al, 1973). These have all been found in murine dystrophy (Banker et al, 1979; Harris and Ribchester, 1979a; Harman et al, 1963; Ragab, 1971; Ellisman, 1981). Anticholinesterase treatment, in vivo, (Ariëns, Meeter, Wolfhius and von Benthem, 1969; Fenichel, Kibler, Olson and Dettbarn, 1972) or, in vitro, produces calcium mediated muscle necrosis (Leonard and Salpeter, 1979). Therefore, an obvious hypothesis is that the muscle fibre necrosis in dystrophy is a consequence of a decrease in AChE activity. There are, however, a number of differences between the effect of antiChEs on transmitter release and the changes found in murine dystrophy. Acute doses of antiChE do increase m.e.p.p. and e.p.p. amplitude (Whittaker, 1975) but chronic antiChE treatment in rats has no significant effect on m.e.p.p. time course and m.e.p.p. amplitude is actually decreased (Engel et al, 1973; Roberts and Thesleff, 1969). Furthermore, quantum content is unchanged by chronic antiChE treatment (Engel et al, 1973; Laskowski and

Dettbarn, 1975; Roberts and Thesleff, 1969).

The changes in transmission found in the dystrophic EDL can be reproduced by a number of methods. However, none of these produces identical changes to those observed in murine dystrophy.

Thus, in conclusion, it appears that the pre and postsynaptic differences in neuromuscular transmission between normal and dystrophic muscles found in this investigation are an effect of the dystrophic process and may be explained by an alteration in the properties of the nerve terminal and sarcolemmal membranes.

APPENDIX

A.1. Statistical tests.

Mann-Whitney test.

This test assumes that the two groups of samples to be tested are independent and that the scores in each group are drawn at random and are rankable. The null hypothesis that the two samples are drawn from two populations with the same distribution characteristics is tested using equation 1.

$$R' = n1(n1 + n2 + 1) - R \qquad 1$$

where n1 is the number of scores in group I, n2 is the number of scores in group II, and R is the sum of the ranks in the smaller group (always group I). To determine the level of significance of the difference under consideration the smaller value of R or R' can be compared with the values of R given in tables for the appropriate number of scores in each group. If the calculated value of R or R' is equal to or less than the value of R given in the 5% confidence level column of the statistical tables, then the null hypothesis would be unlikely to be true, i.e. the two populations from which the samples were drawn have significantly different distribution characteristics. These could be differences in the mean, variance or skew of

the populations or a combination of these. However, a significant result definitely indicates a difference in the medians of the populations sampled.

For large samples equation 2 has to be used.

$$Z = \frac{n1(n1 + n2 + 1) - 2R}{\sqrt{\frac{n1n2(n1 + n2 + 1)}{3}}} \qquad 2$$

The significance of Z can be assessed with reference to the appropriate table. A value of Z equal to or greater than that given in the 5% confidence level column refutes the null hypothesis.

Linear regression.

When a linear regression line is fitted to a set of data points, the following two criteria must be fulfilled before the regression of one variable on another may be said to be statistically significant. The slope of the regression line must be significantly different from zero. Pearson's correlation coefficient (r) must have a value large enough to indicate that the calculated regression line is a significantly close fit to the data points. r is calculated using equation 3.

$$r = \frac{N \Sigma XY - \Sigma X \Sigma Y}{[(N \Sigma X^2 - (\Sigma X)^2)(N \Sigma Y^2 - (\Sigma Y)^2)]} \qquad 3$$

where X and Y are two variables and N is the number of pairs of data scores.

The value of r so obtained can be compared to values of r for the number of pairs of variables in tables in order to determine the level of significance for the correlation.

The slope of the best fit line (b) is then calculated using equation 4.

$$b = \frac{N \Sigma XY - \Sigma X \Sigma Y}{N \Sigma X^2 - (\Sigma X)^2} \qquad 4$$

The intercept of the best fit line (c) is calculated using equation 5.

$$c = \frac{\Sigma Y - b \Sigma X}{N} \qquad 5$$

Chi-squared (χ^2) test.

This test is used to determine the significance of the differences between an observed and a theoretical frequency distribution of a variable. It tests the null hypothesis that the observed samples were taken from a population with a given distribution. The observed variable is set out so

that no class interval contains less than five observations. Next to this is written the number of observations expected in each class interval if the variables were distributed according to a given distribution with the same mean, S.D., and total number of observations. The value of χ^2 is then calculated from equation 6.

$$\chi^2 = \sum \frac{(O - E)^2}{E} \qquad 6$$

where O is the observed frequency in any particular class interval and E is the expected frequency in the same class interval. The number of degrees of freedom (d.f.) to be used to determine the significance of the value obtained for χ^2 depends on the nature of the theoretical distribution and the number of class intervals under investigation:

Test of	d.f.
binomial distribution (p determined from data)	no. of classes -2
Poisson distribution	no. of classes -2

To be significant the χ^2 value has to be equal to or greater than the value given in the 5% confidence level column of the statistical table. A significant χ^2 indicates a significant difference between the expected and observed distributions.

All calculations were performed on a North Star Horizon computer using the appropriate program.

The statistical tests used in the present investigation have been taken from Meddis (1975) and Colquhoun (1971).

A.2. Setting up and sampling.

Choice of threshold level.

The use of the magnitude comparator with the 8 bit A/D converter (range ± 5V, 256 A/D units) allows a choice from 16 threshold levels. A threshold value of 3(55 A/D units, - 2.85V) was chosen since this enabled the baseline to be positioned below this threshold level using the DC offset on the amplifier output and allowed maximum use of the range of the converter enabling greater accuracy. The sampling rate of the converter was 10 kHz. The baseline was positioned beneath the threshold level using a "beep" routine incorporated into the computer program. If the baseline or any part of it was above threshold in the absence of a calibration signal the computer generated a "beep" sound. The position of the baseline could then be lowered using the DC offset until the sound ceased.

Calibration.

Two millisecond square wave pulses of 1mV amplitude and 1 Hz frequency were fed into the recording system and displayed on the oscilloscope screen. The amplitude of the signals was usually adjusted, using the variable gain AC amplifier, so that a 1mV signal corresponded to two vertical divisions (i.e. 2V) on the oscilloscope screen. If the baseline was below the threshold level, 16 baseline samples were recorded and averaged. This process continued until the threshold level was crossed by a calibration pulse and 16 peak samples recorded and averaged. The mean baseline value was then subtracted from the mean peak value to obtain the amplitude of the calibration pulse in A/D units. Ten calibration pulses were recorded in this manner and the mean number of A/D units per mV was obtained. This value, unless changed, was used for the remainder of the experiment.

Because the 10V range of the converter is divided into 256 whole number A/D units the larger the input signal the greater the accuracy. However, a compromise has to be reached so that all experimental signals lie within the range of the converter. When larger amplitude e.p.ps. were to be recorded the amplified calibration signal was attenuated and the A/D value for that new gain noted. This enabled the gain to be adjusted as needed during the

experiment without recalibration by manually inputing the new number of A/D units per mV.

Sampling of m.e.p.ps..

Baseline samples were recorded as for the calibration routine. Once the signal crossed threshold, rise time counting began and continued until the peak was reached. The peak was defined as a point which was greater than the succeeding 3 samples. A signal had to be above threshold for more than 0.3 ms to be accepted as a m.e.p.p.. This provided some discrimination during sampling between m.e.p.ps. and noise which had crossed threshold. As the sampling rate was 10 KHz, the minimum rise time which could be recorded was 0.1ms. The percentage error measuring rise time will decrease as rise time increases. Since rise time is measured from threshold to peak, its accuracy will also depend on the position of baseline relative to threshold. However, as rise time values were merely used as a criterion as to whether or not m.e.p.ps. were focal, the absolute values are not particularly relevant.

The rising phase is the highest frequency component of the m.e.p.p. A rise time of 0.3 ms corresponds to a frequency of 0.83 kHz. In order to obtain an accurate representation of a signal it is necessary to sample at least at the Niyquist rate which is twice the highest frequency component of the signal. Therefore, the minimum sampling

rate required for a signal of rise time 0.3 ms is 1.67 kHz. The error in sampling will decrease as the sampling rate is increased, and although the North Star can sample at rates up to 50 kHz, a compromise has to be reached because at faster sampling rates there would be insufficient time for the computer to perform all the necessary calculations between samples. A sampling rate of 10 kHz provides both an accurate representation of the signal and sufficient time for calculations.

A major source of inaccuracy in determining m.e.p.p. amplitude is noise. A noise level of 0.1 mV would produce a 20% overestimation of amplitude for a 0.5 mV m.e.p.p. A better signal to noise ratio will be obtained for larger signals, the error falling to 10% for a 1 mV m.e.p.p. A further source of inaccuracy is that signals are rounded off to the nearest A/D unit. However, for a m.e.p.p. of 0.5 mV amplitude (25.6 A/D units) this would only represent an error of 1.56%. While the presence of noise prevents accurate measurements of amplitudes it would be expected that any differences in m.e.p.p. size between normal and dystrophic muscles would be revealed.

Before sampling a "beep" sound in the absence of visual m.e.p.ps. indicated that noise was crossing threshold. If this was the case, the baseline was lowered so that a "beep" was heard only when a m.e.p.p. was present. Visual examination of the oscilloscope screen also allowed an

estimation as to whether or not the recorded frequency resembled the actual frequency. Data was analysed using a histogram plotting routine. In the absence of any visual indication that m.e.p.ps. were bimodal, a noisy record was revealed by two very distinct populations, one of which had small amplitudes in comparison with the other. This data could then be rejected. Combined use of the screen and histogram plotting routine also enabled elimination of records in which small amplitude m.e.p.ps. were not sampled as they were of insufficient size to cross threshold. In this case the baseline could be positioned nearer threshold. However, a compromise had to be reached between missing smaller amplitude m.e.p.ps. and allowing noise to cross threshold.

At a 10 kHz sampling rate half decay time can only be measured to the nearest 0.1 ms. This would produce a 6.67% overestimate for a m.e.p.p. of actual half decay time 0.75 ms. The percentage error in measuring half decay time will obviously decrease as the half decay time becomes slower. However, again it would be expected that any difference in this parameter between normal and dystrophic muscles would be detected.

After the half decay time has been measured at least 16 baseline samples have to be taken once the signal has fallen below threshold. Therefore, there is a time during which a subsequent m.e.p.p. cannot be recorded which

consists of the time course of the previous m.e.p.p. plus the time taken to record 16 baseline samples (1.6 ms). The implication of this is discussed in "Accuracy of m.e.p.p. frequency recording". (A.3).

Sampling of fast m.e.p.ps..

This routine was used to sample m.e.p.ps. with a frequency greater than 15 Hz. It differed from the previous routine in that no fixed number of baseline samples had to be recorded before a signal crossing threshold could be detected. This reduced the time during which a subsequent m.e.p.p. could not be recorded to the time taken for the previous m.e.p.p. to fall below threshold. However, as possibly only one subthreshold value was taken between m.e.p.ps., amplitude and half decay recordings at these higher frequencies were not used. The advantage of this routine in measuring m.e.p.p. frequency is also discussed in "Accuracy of m.e.p.p. frequency recording". (A.3).

Sampling of e.p.ps..

Baseline samples were recorded in the same way as in the calibration and m.e.p.p. sampling routines. Upon being triggered by an external source (the digitimer), the computer stopped taking baseline samples and waited a specified programable time before starting to sample the e.p.p.. The waiting time was determined by the interval

between the stimulus artefact and e.p.p. and varied from fibre to fibre. It was estimated by visual inspection of the oscilloscope screen while the motor nerve was stimulated at 1 Hz. The waiting period was then input manually and this prevented the computer recording the stimulus artefact instead of the e.p.p..

Upon expiry of the waiting period, if no threshold crossing occurred within 3.2 ms, the e.p.p. was regarded as being a failure. If motor nerve stimulation elicited an e.p.p., rise time was determined by the time taken from recording began to reaching peak amplitude. Endplate potential amplitude and half decay time were determined in the same way as for m.e.p.ps.. Since e.p.p. amplitudes in the curarised and cut-fibre preparations were generally larger than m.e.p.p. amplitudes a better signal to noise ratio was observed.

The program cannot distinguish between e.p.ps. and m.e.p.ps. if a m.e.p.p. occurs within 3.2 ms of the stimulus artefact. However, in view of the values of m.e.p.p. frequencies observed in fibres from which e.p.ps. and m.e.p.ps. were recorded it is unlikely that a m.e.p.p. was mistaken for an e.p.p..

In contrast to m.e.p.ps., the intervals between e.p.ps. are regular and are determined by the rate of stimulation. The time during which an e.p.p. cannot be recorded is

equivalent to the time course of the preceding e.p.p. and the time taken to obtain 16 baseline samples (1.6 ms). The maximum rate at which e.p.ps. were elicited was 50 Hz and therefore the time between e.p.ps. was much greater than the minimum required for a subsequent m.e.p.p. to be detected.

A.3. Accuracy of m.e.p.p. frequency recording.

The nature of the program used means that after a m.e.p.p. has been detected there is a period of time during which a subsequent m.e.p.p. cannot be accepted. Therefore, any m.e.p.ps. which occur during this period cannot be recorded.

At the mammalian neuromuscular junction spontaneous quantal release of transmitter has been reported to follow a Poisson distribution (Gage and Hubbard, 1965). Assuming this to be true, a worst case calculation of the possible departure of the recorded number of m.e.p.ps. from the actual number may be made thus:

Definitions:

x = interval between m.e.p.ps.

$\bar{x}$ = mean interval between m.e.p.ps.

Σx = interval sum

t = individual interval during which no recording occurs

N = number of intervals less than t

No = total number of m.e.p.ps.

N_1 = number of m.e.p.ps. recorded

Fo = actual frequency

F_1 = recorded frequency

As the spontaneous release of transmitter is Poisson, then x will follow the negative exponential distribution (Fatt and Katz, 1952). A m.e.p.p. is detected when $x > t$, therefore, according to the negative exponential distribution:

$$N = No(1 - \exp(-t/\bar{x})) \quad \text{(Cohen, Kita and van der Kloot, 1974)} \quad 1$$

$$N_1 = No - N \quad 2$$

$$N_1 = No \exp(-t/\bar{x}) \quad 3$$

$$\bar{x} = \Sigma x/No \quad 4$$

Substituting equation 4 in equation 3

$$N_1 = No \exp(-tNo/\Sigma x) \quad 5$$

$$Fo = \frac{No}{\Sigma x} \quad 6$$

$$F_1 = \frac{N_1}{\Sigma x} \quad 7$$

$$\frac{Fo}{F_1} = \frac{No}{N_1} \quad 8$$

Substituting equations 6 and 8 in equation 5

$$F_1 = Fo \exp(-t\ Fo) \quad 9$$

Thus, as the actual frequency increases, the discrepancy between actual and recorded frequency becomes greater. The departure of recorded frequency from actual frequency at different values of Fo was calculated by computer using two values of t.

The duration of time after one m.e.p.p. has been sampled before a subsequent m.e.p.p. can be detected is equivalent to 1.6 ms plus the time course of the m.e.p.p.. Such a period, at the extreme, is likely to be less than 4.5 ms but will vary with the time course of each individual m.e.p.p.. Above 15 Hz the deficit between recorded and actual frequency become more than 6.5% at a tmax of 4.5 ms.

A second subroutine was used to measure frequencies faster than 15 Hz more accurately. This reduced tmax to a value of about 2 ms and a deficit of 6.5% was not observed until actual frequency became more than 33 Hz. The first subroutine was used to record m.e.p.p. amplitude rise time, half decay time and frequency in the lower range only.

Use of equation 9 only enables a worst-case calculation of the possible departure of the recorded number of m.e.p.ps. from the actual number. However, one m.e.p.p. superimposed on another was rarely observed even at the higher frequencies. Therefore, the accuracy of m.e.p.p. frequency recording was considerably better than that calculated.

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Calcium sensitivity of spontaneous transmitter release in murine muscular dystrophy

By S. S. Kelly, G. P. Morgan and J. W. Smith. *Pharmacological Laboratories, Department of Pharmaceutical Sciences, University of Aston in Birmingham, Birmingham B4 7ET*

We have previously reported that extensor digitorum longus (EDL) muscles from dystrophic mice (129/Rej strain) show a smaller enhancement of spontaneous release of transmitter after 2,4-dinitrophenol (DNP) than EDL muscles from non-dystrophic litter-mates (Kelly, Morgan & Smith, 1983). We now report the effect of other agents or procedures thought to alter intraterminal free $[Ca^{2+}]$.

Intracellular micropipettes were used to record miniature end-plate potentials (m.e.p.p.s) *in vitro* at 32 ± 1 °C in EDL muscles from male or female dystrophic mice and normal litter-mates aged 2–5 months. In each series of experiments animals were matched for age and sex.

Under control conditions there was no significant difference in m.e.p.p. frequency between muscles from normal and dystrophic animals. In the presence of guanidine (5×10^{-3} M) m.e.p.p. frequency was increased in muscles from normal litter-mates to 2·6 times control, i.e. from $7{\cdot}1 \pm 4{\cdot}1$ Hz ($n = 96/10$) to $18{\cdot}6 \pm 7{\cdot}6$ Hz ($n = 34/4$) and in muscles from dystrophic mice to 1·7 times control, from $6{\cdot}1 \pm 3{\cdot}3$ Hz ($n = 75/9$) to $10{\cdot}5 \pm 8{\cdot}1$ Hz ($n = 48/4$). Values are the mean ± S.D. with number of muscle fibres/number of muscles sampled in parentheses. Caffeine (2×10^{-3} M), which is considered to release calcium from intracellular stores other than mitochondria (Batra, 1974), caused an equivalent increase in m.e.p.p. frequency in normal and dystrophic muscles, whilst lanthanum (5×10^{-4} M) increased m.e.p.p. frequency 17-fold in dystrophic muscles ($n = 15/5$) and only 6-fold in normal muscles ($n = 11/5$).

Increasing $[Ca^{2+}]_o$ from 2×10^{-3} to 6×10^{-3} M or raising $[K^+]_o$ from 5×10^{-3} to 10^{-2} M or stimulating the motor nerve at 50 Hz for 1 min (cut muscle-fibre preparation) produced similar increases in m.e.p.p. frequency in both normal and dystrophic muscles.

The results with guanidine, together with those obtained previously with DNP, indicate that nerve terminals in muscles from dystrophic animals are less sensitive to uncouplers of oxidative phosphorylation. However, it appears that nerve terminals in EDL muscles from dystrophic animals can regulate free intraterminal $[Ca^{2+}]$ under conditions of increased influx as well as nerve terminals in muscles from normal litter-mates.

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Spontaneous transmitter release at the neuromuscular junction of the dystrophic mouse

By S. S. Kelly, G. P. Morgan and J. W. Smith. *Pharmacological Laboratories, Department of Pharmacy, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET*

The rate of transmitter release from motor nerve terminals at rest is thought to depend upon the intraterminal free $[Ca^{2+}]$ (Baker, 1972). It has been reported that in hind limb muscles of dystrophic mice (*C57 BL/6J* strain), there is an impaired ability of the motor nerve terminal to regulate free $[Ca^{2+}]$ (Shalton & Wareham, 1980). We have measured the frequency of miniature endplate potentials (m.e.p.p.s) in muscles from male and female dystrophic mice and normal littermates (*129/ReJ* strain) aged between 3 and 5 months to investigate the control of intraterminal $[Ca^{2+}]$.

Conventional intracellular techniques were used to record m.e.p.p.s from endplate regions of diaphragm and extensor digitorum longus (EDL) muscle fibres, *in vitro*. At 20 ± 1 °C, in the presence of 2 mM-$[Ca^{2+}]_o$, there was no significant difference between m.e.p.p. frequency in normal and in dystrophic muscle fibres of either the diaphragm or EDL. When $[Ca^{2+}]_o$ was raised from 2 mM to 6 mM there was a similar rise in m.e.p.p. frequency in dystrophic and in normal muscle fibres. However, at 37 ± 1 °C, 2 mM-$[Ca^{2+}]_o$, m.e.p.p. frequency was significantly lower in muscle fibres of dystrophic diaphragm than in normal diaphragm, and in 6 mM-$[Ca^{2+}]_o$ there was a greater increase in m.e.p.p. frequency in the dystrophic fibres than in the normal fibres.

In other experiments the effect of 2,4-dinitrophenol (DNP) on m.e.p.p. frequency in EDL muscle fibres was examined. In normal fibres at 20 ± 1 °C, DNP (10^{-4} M) caused a fortyfold increase in m.e.p.p. frequency within 18 min which declined to control in 35–45 min. In dystrophic fibres DNP (10^{-4} M) increased m.e.p.p. frequency slowly from $2{\cdot}64 \pm 0{\cdot}18$ Hz to $20{\cdot}88 \pm 4{\cdot}17$ Hz (mean ± S.E.M. of twenty-four fibres, two muscles) over a period of 30 to 60 min. These effects of DNP were not accompanied by any significant change in the membrane potential.

The results indicate that in motor nerve terminals from dystrophic mice there is an increased sensitivity of transmitter release to $[Ca^{2+}]_o$ at 37 °C and a difference in the sensitivity of the release process to DNP. The results differ from those reported for the *C57BL/6J* strain of mice, as at room temperature we found no difference in the sensitivity of spontaneous release to $[Ca^{2+}]_o$ in normal and dystrophic muscle fibres and in both, m.e.p.p. frequency was increased by DNP.

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Curare resistance in murine muscular dystrophy

By S. S. Kelly, G. P. Morgan and J. W. Smith. *Pharmacological Laboratories, Department of Pharmacy, University of Aston in Birmingham, Gosta Green, Birmingham*

It has been reported that dystrophic mice are relatively insensitive to injections of (+)-tubocurarine (curare) (Baker, Wilson, Oldendorf & Blahd, 1960) and that higher concentrations of curare are needed to induce neuromuscular paralysis in hind-limb muscles from dystrophic mice than in normal (Baker & Sabawala, 1963). The origin of this resistance to curare was investigated in these experiments.

All experiments were made on extensor digitorum longus (EDL) muscles from male dystrophic mice and from normal litter-mates (129 Rej strain) aged 55–66 days. The muscles were maintained at 33 °C in modified Liley saline aerated with 5% CO_2 in oxygen.

The effectiveness of curare in blocking the indirectly evoked twitches of normal and of dystrophic muscles was measured by constructing dose–response curves. In muscles from dystrophic mice the dose–response curve was to the right of that in normals indicating a decreased sensitivity of dystrophic muscles to the effect of curare. The following observations suggest that this resistance to curare may, in part, be due to a presynaptic abnormality causing an increase in the amount of acetylcholine released in response to nerve stimulation. The mean amplitude of end-plate potentials (e.p.p.s) measured in dystrophic muscles (at 10 Hz) in $1{\cdot}91 \times 10^{-6}$ M curare was 2·4 times that in normal muscles whereas the mean amplitude of miniature end-plate potentials (m.e.p.p.s), recorded in the absence of curare, was only 1·5 times normal. The initial rundown of e.p.p.s in curare was significantly greater in dystrophic than in normal muscles. The slopes of the initial rundown were calculated from the expression $\ln x = -kn$, where x = normalised e.p.p. amplitude, n = e.p.p. number and k is the slope. Values of k were $-0{\cdot}48 \pm 0{\cdot}03$ (mean ± S.E. of mean, $N = 25$) and $-0{\cdot}70 \pm 0{\cdot}05$ ($N = 23$) in normal and dystrophic fibres, respectively ($P < 0{\cdot}002$, Mann–Whitney). The mean quantal content of e.p.p.s calculated directly from measurements of m.e.p.p.s and e.p.p.s in low $[Ca^{2+}]_o$ ($5{\cdot}5 \times 10^{-4}$ M) and raised $[Mg^{2+}]_o$ ($2{\cdot}45 \times 10^{-3}$ M) was $5{\cdot}5 \pm 0{\cdot}74$ ($N = 30$) for dystrophic fibres and $2{\cdot}69 \pm 0{\cdot}19$ ($N = 38$) for normal fibres.

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