NEUROMUSCULAR FUNCTION IN GENETICALLY DYSTROPHIC MICE (C57Bl/6J dy^{2J})

by

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ABSTRACT

Muscular Dystrophy has been considered a primary myopathy with causative factors inherent within the muscle fiber itself. Recent evidence, however, suggests that some forms of dystrophy have a neurogenic origin.

The observations on the possible involvement of the neuromuscular transmitter in the dystrophic process have been contradictory. As shown in this study, the conditions under which the experiments are carried out have important effects on the parameter being measured.

The present study has been carried out in order to determine whether changes are observed in the rate of spontaneous transmitter release.

The frequency of miniature endplate potentials (m.e.p.p.s) has been determined in the extensor digitorum longus (EDL) and soleus (SOL) muscles of normal and dystrophic mice at various ages. The results show that in the normal animal the frequencies in the two muscles are very similar after birth. The frequency continues to increase in the EDL while the frequency in the soleus seems to reach an adult level much earlier. Dystrophy was not found to affect the frequency in the soleus muscle but in the dystrophic EDL the frequency is significantly lower relative to control values. Since this effect occurs after the clinical symptoms are manifested it is concluded that the change observed is a secondary phenomenon. This does not however rule out the possibility of a neurogenic defect in the etiology of murine muscular dystrophy. It is postulated that the observed changes reflect an alteration of the endplate region.

CHAPTER I

Introduction

1. Muscular dystrophy: description and classification

Muscular dystrophy refers to a group of hereditary disorders with the following characteristics: weakness and wasting of striated muscle, with a progressive clinical course.

Many pathologists have based a histologic diagnosis of Muscular Dystrophy (M.D) on Erb's (1884) principal pathologic criteria for distinguishing the disease (reviewed by Walton, 1973a). These consisted of (1) rounding of muscle fibers in transverse sections with hypertrophy of some fibers and atrophy of others; (2) random variation in fiber size and shape; (3) central migration of sarcolemmal nuclei in many fibers; (4) fiber splitting; (5) extensive infiltration of fat and connective tissue. More recent evidence (reviewed by Pearce and Walton, 1962; Bell and Conen, 1968) has indicated that the primary pathologic process in M.D. involves necrosis with active degeneration of muscle fibers to which there is secondary regenerative response of varying extent.

This histopathological description for all varieties of M.D. may, however, be also given for chronically denervated muscle fibers (Walton, 1973a). The terminal stages of a long standing myopathic process and a long standing denervation atrophy are virtually impossible to distinguish (Walton, 1973a).

Walton and Nattrass (1954) attempted to classify the pure

dystrophies into three major types. These included the Duchene, Limb girdle and Fascioscapulohumeral varieties. The analysis of clinical and genetic data were the basis for this classification. Because of it's distinct clinical characteristics, Dystrophia Myotonica is excluded from the pure dystrophies. The histopathology of myotonic dystrophy closely resembles the pure dystrophies except for the clinical myotonia which lends suspicion to a possible neuronal involvement (Walton, 1973b).

The early histopathological approach used by clinicians fell short of an accurate diagnosis. The technique of single biopsy used in acquiring tissue for analysis has an important disadvantage in that it can only demonstrate that abnormalities are present in a small part of the muscle from which the biopsy was taken (Walton, 1973a). Multiple biopsies on human subjects are discouraged for obvious reasons.

Other parameters have been useful in establishing an accurate diagnosis of muscle diseases while adding to the controversy that exists over the classification of particular types of dystrophies. These parameters include neurologic examinations, serum enzyme studies, spinal fluid examination, electromyography, nerve conduction studies and muscle biopsy for various studies including histology, histochemistry, electron microscopy and biochemistry (Engel, 1967).

These parameters have offered conflicting evidence as to the classification of the pure dystrophies, and in establishing the pathogenesis of M.D.

The problem of investigating human muscle disease is the

difficulty in obtaining sufficient material by muscle biopsy, and in accurate examination of neurological function. A study of the pathogenesis of this disease in human patients would be most time consuming not to mention the expense and anxiety involved.

2. Animal myopathies: useful models

Developmental studies on known carriers of genetically determined muscle diseases would be most useful in attempting to elucidate the underlying etiology, as well as observing the tempo at which the disease process affects the muscle.

Animal congenital myopathies (reviewed by Hadlow, 1973) have provided the opportunity of studying disease processes that would have been otherwise impossible with human patients. A number of genetic mutants, for example, have been observed in several species with clinical correlates to the human dystrophies. A variety of inherited Muscular Dystrophies in animals have been reported; including the mouse (Michelson et al, 1955; Meier and Southard, 1970), chicken (Asmundson and Julian, 1956) and hamster (Homburger et al, 1962).

In 1955 Michelson described an hereditary myopathy in the house mouse of Bar Harbour inbred strain 129; the hereditary anomaly designated by the symbol "dy". This particular mutant has some advantages over the other inherited animal myopathies resembling human Muscular Dystrophy. Some animal models best resemble specific human dystrophic types on the basis of clinical expression or other correlates. Thus, the myopathy described in the chicken (Asmundson and Julian, 1956) may resemble

Fascioscapulohumeral dystrophy or the Limbgirdle variety due to the preferred involvement of the pectoral muscle group. The myopathy in the mouse, on the other hand, resembles human myotonica dystrophia in it's clinical symptoms as well as several other parameters (Michelson et al, 1955; McComas and Mossawy, 1965).

More recently, a newly discovered hereditary myopathy has been found on an inbred strain of mice (WK/REJ) with symptoms resembling myotonic dystrophy. As with the 129 dy strain, the myopathy is caused by an autosomal recessive mutation (Meier and Southard, 1970). The mutant gene is described as a variant of the 129 strain, with the new allele designated dy^{2J} according to the genetic nomenclature for mice.

The pathology that this new syndrome presents is much milder than the earlier myopathy described for the mouse. The onset of the disease in this particular strain is seen much later when compared with the 129 strain and the progression is less severe. This accounts for the fact that both affected males and females are able to breed. The actual histopathological findings are similar to those found in the dy/dy mutants (Michelson et al, 1955). These consisted in whole or in part of: (1) loss of striation; (2) coagulation necrosis; (3) regenerative activity; (4) variation in fiber size; (5) internal rowing of nuclei.

The histopathology seen in this animal myopathy closely parallels that seen in the human disease process. A more comprehensive understanding of the pathogenesis of this disease can now be attempted using this animal model.

3. Etiology of muscular dystrophy

Since Erb (1884) showed that in cases of progressive muscular dystrophy there are pathological changes in skeletal muscle, unaccompanied by obvious morphological abnormalities in the anterior horn cells of the spinal cord, it has generally been accepted that muscular dystrophy is a primary myopathy. By primary myopathy it is meant a disorder due to a prior pathological or biochemical abnormality arising within the muscle fibers themselves.

It is also generally accepted that an interdependence of nerve and muscle exists. The alterations of muscle which results from neurectomy, injury, chemical nerve blocks and other techniques used to block effects from the nerve to its specific muscle have been documented (Gutmann, 1963; Guth, 1968). The question that is currently being asked is whether a possible neurogenic component exists in the pathogenesis of muscular dystrophy.

Buller, Ecoles and Eccles (1960a) presented evidence for the role of neurons during the period of muscle differentiation in their nerve cross-union studies. In their experiments the normal physiological characteristics of muscles were determined by the type of innervation imposed upon them.

The concept of a neurotrophic influence was investigated as to its relevance to the physiological behavior of fast and slow muscles (Milhorat, 1967). It appears that a specific fiber type is selected as a target for the dystrophic abnormality. An analysis of the ontogeny

of muscles from dystrophic chickens reveals an impairment in the maturation of white muscle (Cosmos,1965) while the red muscles appear unaffected (Cosmos,1967). In dystrophic mice (dy/dy) evidence has accumulated also indicating that slow muscles are less susceptible to dystrophy (Brust,1966;Harris,1971;Law and Atwood,1972a). It is not known,however,whether this difference arises from the different trophic effects of the motor nerve.

The experiments of Salafsky (1971) have suggested that motor neurons of dystrophic mice (dy/dy) are abnormal in their ability to support muscle growth and maintain functional contacts with the muscle fibers. Using the techniques of Studitsky (reviewed by Carlson,1968, 1973) he showed that minced muscle from a normal mature donor failed to regenerate in a dystrophic host,while muscle from a dystrophic donor developed almost normal capabilities in the normal host. This finding was supported by Hironaka and Miyata (1973) who used the entire extensor digitorum longus (EDL) muscle from normal and dystrophic (dy/dy) mice,transplanting them into areas vacated by the removal of EDL muscle either in dystrophic or normal littermates. Electrophysiological responses from dystrophic transplants into normal hosts paralleled those seen in normal unoperated EDL muscles,while normal or dystrophic transplants into dystrophic hosts displayed responses similar to unoperated dystrophic EDL muscles. Contrary to the above findings,Cosmos (1973) and Laird and Timmer (1965,1966) using similar heterotransplanted minced muscle techniques reported the retention of donor characteristics

in foreign hosts.

The results of experiments by Law and Atwood (1972b) show that dystrophic muscles do not respond to cross-reinnervation in the same way as do normal muscles. Contraction times and fiber cable constants were not significantly altered in dystrophic soleus muscles cross-reinnervated with the nerve originally innervating the flexor digitorum longus muscle. Normal soleus muscles reinnervated in this fashion display contraction times and fiber cable constants approaching the flexor digitorum longus muscle. The complementary evidence presented by Salafsky (1971) and Hironaka and Miyata (1973) suggests that the dystrophic mouse lacks the trophic factors found in the normal littermates.

The observations made by McComas and Mrozek (1967) point to the possible involvement of the motor innervation in the pathogenesis of muscular dystrophy. They reported that some dystrophic mouse (dy) muscles appeared to be functionally denervated. The fibers responded to direct stimulation but not to indirect stimualtion. McComas et al (1971b) later reported a reduction in the number of motor units in patients afflicted with Dystrophia Myotonica, Limbgirdle and Fascio-scapulohumeral varieties. Decreased motor unit counts are also characteristic of primary neurogenic diseases as well as chronic denervation (McComas et al 1971a). Ballantyne and Hansen (1974) using a new method for estimating the number of motor units, present contrary findings to McComas's earlier observations. They found no significant difference between the motor unit counts from normal patients

and those afflicted with the Fascioscapulohumeral and Limbgirdle types of dystrophy. They did concede that motor unit counts were markedly reduced in myotonic dystrophy as cited earlier by McComas et al(1971b)

Harris et al (1972) estimated that there is a reduction in the number of myelinated nerve fibers in the dystrophic mouse (dy). This is not surprising in view of the evidence of motor unit involvement. Papetropoulous and Bradley (1972) however reported that anterior horn cells are present in normal numbers in murine dystrophy. It would seem that this,in itself, would be contrary to a firm neurogenic hypothesis for the pathogenesis of muscular dystrophy. It may be possible, as suggested by Bradley (1973) that a dying back type of neuropathy (Cavanagh,1964) exists in these animals where a decrease in the number of axons may be observed while no obvious differences may be seen in the spinal cord. Thus the first changes that are likely to occur will be in the furthermost limits of the nerve fiber axon. This is the site of commencement of the "dying back" process,the anatomical dissolution of the axon proceeding with greater or lesser speed, according to the individual condition toward the nerve cell body. Cavanagh (1964) described experimental intoxications due to organophosphorous compounds which closely mimic human neurological diseases of the "dying back" type.

4. Nature of the trophic influence

A controversy exists as to whether the trophic influence in the motor nerves is a specific protein unrelated to neuromuscular transmission,or is related to the release parameters of acetylcholine (ACh) at the endplate,or to changes in the activity of the muscle and a possible feedback affect on the nerve. These are currently being investigated with possible relevance to the onset of muscular dystrophy.

The role of impulse activity of the nerve in determining the contractile parameters of striated muscle has been demonstrated by Buller,Eccles and Eccles(1960b);Salmons and Vrbova (1969);Fischbach and Robbins(1969);Lømo and Rosenthal (1972);Lømo and Westgaard(1974). Fischbach and Robbins (1969) demonstrated alterations in the soleus muscle after a period of disuse with no change in innervation. It would seem then,that control of contractile mechanism depends in part on impulse activity.The time course of contraction and relaxation in the isometric twitch of a rabbit soleus muscle becomes more rapid following tenotomy and spinal cord section. Salmons and Vrbova (1969) showed that this increase in speed of contraction could be prevented by chronic direct electrical stimulation of the muscle at frequencies of 5-10/second. Direct stimulation of the fast contracting rabbit and cat muscles at frequencies of 10/sec. had a slowing effect on the time course of contraction and relaxation. Lømo and Westgaard (1974) demonstrated that denervated soleus muscles stimulated at a rate resembling phasic activity of fast muscles (100Hz) acquired several properties characteristic of fast muscles whereas muscles stimulated at 10Hz remained slow. Eccles et al (1958) reported that the frequency of

tonic impulses recorded from axons innervating slow contracting muscle fibers were much slower than the frequency of phasic impulses recorded from axons innervating fast twitch muscle fibers.

It is well known that nerves have an important role in the regulation of cholinesterase (AChE) activity of skeletal muscle. When the motor nerve is cut, the AChE activity decreases (Guth, Albers, and Brown, 1964). In normal muscle only the endplate region is sensitive to the ACh transmitter (Miledi, 1960) but following denervation the whole surface of the muscle becomes sensitive (Miledi, 1960; Axellson and Thesleff, 1959). Reinnervation of a denervated muscle restores cholinesterase activity (Guth and Brown, 1965) and normal endplate ACh sensitivity (McArdle and Albuquerque, 1973). Explanations of how motor nerves exert regulatory influences on muscle have been put forward.

Thesleff (1960) suggested that small amounts of ACh released from nerve endings at neuromuscular junctions have a desensitizing effect on the area outside the endplate region. Although Drachman (1972) reports that ACh is responsible for AChE activity at both endplate and non-endplate regions, he does propose that some non-cholinergic influence may be operating as well. Miledi (1960) contends that a special trophic substance independent of muscle activity is released from the nerve endings and is responsible for keeping the rest of the muscle fiber insensitive to the transmitter. The possibility that muscle activity is an important factor in maintaining focal endplate sensitivity has also been considered. Jones and Vrbova (1971) and Lømo and Rosenthal (1972) showed that when denervated muscles were directly stimulated during the first few days of denervation,

the onset of hypersensitivity was delayed. Jansen et al (1973) also demonstrated the importance of muscle activity for synapse formation by a foreign nerve. They found that after blocking motor nerve impulse to the rat soleus muscle by local anesthesia, individual muscle fibers become innervated by a transplanted motor nerve. Such cross-innervation of a denervated soleus was largely reduced by direct electrical stimulation of the muscle.

The onset of denervation changes appears to be correlated in time with the onset of degeneration of the nerve terminals and the time course of the onset of this process depends on the length of the peripheral nerve stump (Miledi and Slater, 1969). Findings in which denervation changes were seen to occur earlier in the muscles attached to a short stump of nerve than muscles connected to a longer nerve stump (Miledi and Slater, 1969; Harris and Thesleff, 1972) indicate that inactivity alone cannot explain the development of denervation hypersensitivity.

Colchicine which blocks axonal transport has been reported to mimic some of the effects of denervation on mammalian skeletal muscles (Hofmann and Thesleff, 1972; Albuquerque et al, 1972; Cangiano, 1973). These effects include increased extrajunctional ACh sensitivity and a fall in muscle fiber resting membrane potential. Colchicine (in discrete amounts) does not interfere with nerve impulse conduction, neuromuscular transmission or muscle tension evoked by stimulation of the nerve (Hofmann et al, 1973). It seems, therefore, that denervation-like changes may be induced in the presence of normal muscle activity. This is supported by the work of Lømo (1974) where colchicine was found to raise the ACh sensitivity

in both innervated and denervated stimulated fibers to about the same extent.

Thesleff (1960) has suggested that spontaneous miniature end-plate potentials (m.e.p.p.s) are sufficient to prevent the spread of ACh sensitivity. Lømo and Rosenthal (1972) reported normal frequency and amplitude of m.e.p.p.s still present in nerve impulse blocked muscles that had become hypersensitive. The presence of the nerve itself has also been observed as having a trophic influence on the muscle fiber without requiring cholinergic transmission (Steinbach et al,1973). The passage of protein from the motor nerve endings to the muscle has been shown autoradiographically (Korr et al,1967;Bradley,1973). Lentz (1971) has shown that a diffusable substance given off by the nerve induces enzyme activity in the muscle. This again adds to a possible involvement of a non-cholinergic trophic substance.

The experiments which uncovered the evidence for a neurotrophic influence on skeletal muscle have been applied to studies attempting to find a possible neurogenic component in the pathogenesis of muscular dystrophy. The results,however,are variable.

Salafsky et al (1973) reported altered neural proteins in the spinal cord,spinal roots and peripheral nerves along with an absence of myelinated fibers in both the dorsal and ventral roots of dystrophic mice in both the 129dy and C57Bl/6Jdy^{2J} strains. Radioactive labelling of the spinal cord has also shown abnormal axoplasmic flow patterns in the sciatic nerve of dystrophic dy mice (Bradley and Jaros,1973;Komiya and Austin,1974;

Jablecki and Brimijoin,1974).

Denervation effects on muscle membrane stability are well known (Harris and Thesleff,1972;McArdle and Albuquerque,1971). Harris and Ward (1974) investigated dystrophic mice (dy) for TTX-resistant action potentials and extrajunctional ACh activity,both reported as consequences of surgical denervation (Harris and Thesleff,1971). No evidence for TTX-resistant action potentials or extrajunctional ACh sensitivity was found.

The denervation effect on AChE activity previously described may also be compared to the observations of endplate AChE in dystrophic mice.Glaser et al (1967) have already reported a marked reduction in available AChE in dystrophic mouse (dy) muscle. Jedrezejezyk et al (1973)reported a decrease in synaptic AChE in skeletal muscle of dystrophic chickens. Because of the correlation that may be drawn from denervation studies (Guth et al,1964;1965),the concept of a deranged neural factor affecting AChE activity was put forward. The mechanisms however may be dependent on the type of dystrophy affecting a particular animal model. A more comprehensive study by Linkhart et al (1975) attempted to elucidate the etiology of the altered AChE activity in dystrophic avian muscle. The experimental approach involving primordial limb transplantation between genetically different embryos demonstrated that the decreased AChE activity in dystrophic chickens is caused by an initial biochemical abnormality in the limb and its muscle rather than on its innervating nerve. The possibiltiy of the muscle fiber itself influencing the axon innervating it may also be suggested in light of the work by Duchene

et al (1975). They found that the axonal sprouting normally induced by botulinum toxin is supressed by the presence of an extra implanted nerve which has established new functioning synapses. This is evidence for a possible regulatory influence of the muscle fiber exerted on its innervating axon.

Because of the implications of a possible neural involvement in the onset of the dystrophic process, a study of neuromuscular function in dystrophic animals would be useful.

5. The Neuromuscular Junction

The neuromuscular junction has been found to be both functionally and morphologically involved in the dystrophic process. MacDermot (1961) and more recently Gilbert et al (1973) have shown, on histological examination, that the nerve and motor endplates are affected in myotonic dystrophy as well as in the mouse. It has also been reported that there is a reduction in the number of vesicles in the presynaptic nerve terminal of dystrophic mice (dy) (Ragab, 1971).

In 1950, Fatt and Katz, while recording intracellularly from the frog neuromuscular synapse, detected small potentials of fairly constant amplitude and appearing at random intervals. These potentials called miniature endplate potentials (m.e.p.p.s) were found to correspond to basic units of transmitter release (del Castillo and Katz, 1956). They have been recorded at all neuromuscular junctions from skeletal muscle, smooth muscle; in vertebrates or invertebrates (Boyd and Martin, 1956a; Takeuchi, 1958; Hubbard, 1970).

The miniature potentials for vertebrate neuromuscular junctions are excitatory, that is, they are recorded as depolarizations of the post synaptic membrane. Pharmacological analysis was used by Fatt and Katz (1951, 1952) to ascertain that these potentials were indeed produced by packets of transmitter. The assumption that m.e.p.p.s reflect the presynaptic release of transmitter is supported by the finding that the frequency of miniature potentials is dependent on the presynaptic and not postsynaptic depolarization (delCastillo and Katz, 1954; Liley, 1956b).

Consistent with this idea, is the evidence provided by degeneration experiments. M.e.p.p.s. disappear if the nerve terminal is allowed to degenerate and reappear with reinnervation at the rat neuromuscular junction (Liley,1956a; McArdle and Albuquerque,1973). The chemical transmitter responsible for these postsynaptic depolarizations has been collected (Dale et al, 1936) and has shown to be acetylcholine (ACh). Inhibitory agents have been found to specifically affect transmitter action (curare) or release (botulinum toxin)(Fatt and Katz, 1952; reviewed by Hubbard and Quastel, 1973).

Conrad and Glaser (1962) while investigating neuromuscular fatigue in dystrophic mice, implied that an alteration in neuromuscular transmission may be present, independent of any primary muscular dysfunction. They extended this thesis further in their later observation (1964) of a marked reduction in the frequency of m.e.p.p.s. in dystrophic mice(dy). McComas and Mossawy (1965) did not find any change in m.e.p.p. frequency in dystrophic mice (dy) but did report a decrease in the amplitude of m.e.p.p.s.

The abnormal pharmacological response from dystrophic mice reported by Baker et al. (1960, 1963) suggests a functional denervation. The supersensitivity to neostigmine and resistance to d-tubocurarine in dystrophic mice reported by Baker et al (1960) is also a characteristic of denervated muscle (Adams, Denny-Brown and Pearson, 1953).

The experimental evidence to date indicates that a possible alteration in the cholinergic mechanism is present in the dystrophic

process. Whether this alteration is due to an abnormality at the motor end plate, and not secondary to diseased muscle or nerve, is currently the subject of intensive investigation. Consideration of factors affecting cholinergic transmission at the neuromuscular junction is necessary in any attempt at correlating transmitter release with a trophic influence.

6. Factors affecting the frequency of m.e.p.p.s.

Various factors will influence the rate at which transmitter is spontaneously discharged from nerve terminals. Because of m.e.p.p. presynaptic origin, their frequency can only reflect presynaptic events (Katz, 1962). Agents that influence the nerve axon, or its axon terminals, will also affect the amount of transmitter released.

a) Membrane potentials

Changes in the membrane potential of axons or their terminals will have a direct affect on the miniature potential frequency (del Costillo and Katz, 1954; Quastel and Cook, 1973). Depolarizing the nerve supplying the end plates will result in an increased frequency (Liley 1956a, 1956b; Quastel and Cooke, 1973).

Alterations in the ionic concentrations of extracellular solutions bathing nerve-muscle preparations will, indirectly, affect the frequency of m.e.p.p.s. Increasing the concentration of extracellular potassium (K^+) will result in a depolarization of the terminal axons and a subsequent increase in the frequency of m.e.p.p.s. (Liley, 1956b, Boyd and Martin, 1956, Okada 1973; reviewed by Hubbard, 1970).

Other factors will also affect the frequency of discharge possibly by producing some local depolarization and thereby increasing the instability of the nerve endings. Thus, localized trauma to the nerve endings produced either by pulling on the motor nerve or stretching the muscle fiber will result in an increased frequency of m.e.p.p.s (Fatt and Katz, 1952; Boyd and Martin, 1956; Liley, 1956a) although Turkanis (1973a) did not observe any increase in m.e.p.p. frequency while recording from stretched rat diaghragm preparations. Local damage to the end plates in the insertion of microelectrodes will also result in an increase in the frequency of discharge (Fatt and Katz, 1952) as will lack of oxygen (Krnjevic and Miledi, 1958 ; Hubbard and Løyning, 1966). Liley (1956a) suggests a resistance to short term anoxia while Boyd and Martin (1956a) have reported a rapid decline in resting membrane potential as well as an increase in the frequency of m.e.p.p.s.

b) Ionic media

The concentrations of certain ions will affect the frequency of m.e.p.p.s. The affects of depolarization of nerve terminals are reduced in the presence of raised [Mg^{++}] or lowered [Ca^{++}] (Liley, 1956b). An increase in [Mg^{++}] in raised potassium solution will decrease the frequency of m.e.p.p.s. (Hubbard, 1961) as well as decreasing the amplitude of the e.p.p. (Boyd and Martin, 1956b). A depression of m.e.p.p. frequency is observed with a decreased [Ca^{++}] (Boyd and Martin, 1956a; Liley, 1956b) as is a decrease in the amplitude of the e.p.p. (Boyd and Martin, 1956b). An effect on both quantal content and miniature frequency

in parallel would indicate an action upon the process which couples release to polarization (Hubbard, Llinas and Quastel, 1969). A model proposed by Gage and Quastel (1966) suggests that Calcium (Ca^{++}) affects transmitter release by changing the concentration in the presynaptic membrane of a complex (CaX) to which the rate of transmitter release is directly proportional. Hubbard (1961) proposed that both a Ca^{++} sensitive and a Ca^{++} insensitive mechanism operate to control the frequency discharge since m.e.p.p.s could still be recorded in the absence of Ca^{++} or in the presence of high concentrations of Mg^{++}.

Changes in the H^{+} ion concentration also affect the release of transmitter (Hubbard et al, 1968a). The action of H^{+} ions is thought to be exerted as a substitution for the Ca^{++} ion (Hubbard, 1968a) although recent evidence by Cohen and Vanderkloot (1974) has suggested that the H^{+} ions elevate the permeability of Cl^{-} (PCl) in the rat diaghragm and affects a depolarization of the terminal thereby raising m.e.p.p. frequency.

c) Osmotic pressure

An increase in osmotic pressure of the bathing solution will result in an increase in m.e.p.p. frequency (Fatt and Katz, 1952; Boyd and Martin, 1956a; Hubbard et al, 1969). The effect of increasing osmotic pressure does not appreciably affect the quantal content of the e.p.p. (Hubbard, Jones and Landau, 1968b) indicating a rather different mechanism operating when compared to the effect of depolarization of the nerve terminals.

d) Temperature

The effect of temperature on the frequency of m.e.p.p.s has been well documented (Fatt and Katz, 1952; Boyd and Martin, 1956a; Liley, 1956a,

reviewed by Hubbard,1970). An increase in the temperature of a solution bathing a nerve-muscle preparation results in an increase of miniature potential frequency. Some authors (Fatt and Katz,1952;Liley,1956a) have used a positive Q_{10} value to describe the relation of temperature and m.e.p.p. frequency. More recent work by Hubbard (1970) has presented a more complex function in describing this relationship.

7. Statement of the problem

Because of the evidence suggesting a neurotrophic influence in the etiology of muscular dystrophy,one of the simplest indices indicating neural involvement may be the rate of neuromuscular transmitter release or the frequency of m.e.p.p.s.

The questions being presented in this study are the following:

1) In view of the tenuous evidence of an altered neurotransmitter release (Conrad and Glaser,1964;McComas and Mossawy,1965) are changes observed in the rate of transmitter release under controlled conditions?

2) If there are any changes,do these occur prior to the first clinical manifestations of the disease?

3) And in the light of the evidence implicating a preferred involvement of specific muscles,would any changes in the rate of transmitter release be found in both fast and slow contracting muscles? To answer this question muscles were chosen to fit two criteria: i) The muscles are to be situated in the hind limb and below the knee,since inspection of dystrophic animals suggests that these muscles are most severely affected. The muscles have to be generally accepted as being truly fast and slow;such muscles

are EDL and soleus respectively. Histochemical muscle fiber typing indicate that soleus is a muscle composed mostly of Type I fibers and that EDL is a muscle composed of a large proportion of Type II fibers with a minority population of Type I and intermediate fibers (Stein and Padykula.1962).

CHAPTER II

Methods

1. Animal model

Observations were made from normal and dystrophic mice from the C57BL/6Jdy^{2J} strain (Jackson Laboratories; Bar Harbor, Maine). The experimental design demanded recording from normal and dystrophic mice at prescribed age periods. These were; 10-20 days, 20-30 days, 30-40 days, 40-50 days and over 100 days. Dystrophic mice from both homozygous and heterozygous matings were used; however, the mice used for the early experimental group (20-30 days) were obtained only from homozygous matings.

The affected mice, both males and females, first showed the clinical signs of dystrophy at approximately 25 days after birth. The clasping of the hind feet was evident after agitating the young animals. At least three animals were used for a particular age group. There was some difficulty in obtaining a sufficient number of dystrophic mice, especially young groups.

Contrary to what has appeared in the literature (Meier and Southard, 1970) concerning the viability of breeding colonies, problems arose in sustaining our own dystrophic colony. Either dystrophic pairs were unable to reproduce, complications arose at term in pregnant females or the litter failed to wean. All these factors contributed in reducing

the number of dystrophic mice available.

2. Preparation of Microelectrodes

Several procedures were tried in the filling of microelectrodes, however the one described by Adrian (1956) was preferred.

Glass tubing (Corning borosilicate 7740;1.5 mm O.D.,1.0mm I.D.) was placed in an electrode puller (Narishige Scientific) and electrodes drawn to a tip diameter of less than 1μ. The electrodes were then placed in a beaker of methanol which was allowed to boil under reduced pressure. The electrodes were then placed in a beaker of distilled water for approximately three hours and then equilibrated in a beaker of 3M KCl for twenty-four hours. Electrodes kept in 3M KCl for several days were found to be satisfactory for recording purposes.

3. Preparation of Solutions

All solutions used in the experiments were isotonic with mouse plasma. They were freshly prepared using deionized distilled water and Fisher Certified Reagents. To avoid precipitation of Ca^{++}, the last constituent added was $CaCl_2$;the solutions being stirred continuously. All solutions employed were bicarbonate buffered with 5% CO_2 being used to obtain the desired pH(7.4).

The pH values of the solutions were measured with a Beckman pH meter immediately after gassing the solutions. In three experiments pH was monitored before,during,and after the experiment. In the three cases pH values did not change significantly.

In most of the experiments a 95% O_2/5%CO_2 gas mixture was used,

while in the hypoxic experiments 95% N_2 was substituted. The composition of the solutions are shown in Table 1.

4. Preparation of the muscles

i) in vitro experiments

The mice were sacrificed by nuchral fracture and placed in a dissection bath containing Ringer that was continuously gassed with 95% 0_2 / 5 % $C0_2$. The hind legs were skinned and overlying muscles carefully dissected away, exposing the soleus (SOL) muscle on one leg and the extensor digitorum longus (EDL) on the other leg. Surgical thread was tied around the tendons and the excised muscles were transferred to the recording bath. In the recording bath the threads were adjusted and secured to platinum hooks. Care was taken in adjusting the length of the muscle to that in situ.

Several experiments were performed on muscles in which the tendons were left attached to the hind legs. The entire legs were then excised and transferred to the recording bath and pinned in paraffin wax.

ii) in vivo experiments

The mice were anesthetized with sodium pentobarbital (Abbot laboratories) at a dosage of 70 mg./kg. body weight. They were then placed in a dissecting bath and an incision made into one leg. Overlying muscles were carefully reflected avoiding unnecessary trauma or hemorrhaging.

TABLE I

Composition of solutions (mM)

Solution	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	HCO_3^-	Glucose
Normal Ringer	146	4.75	1.5	0.23	127.48	25	11
High Mg^{++} Ringer	126	4.75	1.5	10.0	127.48	25	11

The animal was then transferred to the recording bath where the hind quarters were submerged in Ringer. Additional maintenance dosages of anesthetic were occasionally required during the course of an experiment. The animals were not placed on a respirator. No respiratory complications were observed during the course of a recording session.

5. Description of the apparatus

The experimental set-up was essentially as shown in Fig. 1. The perspex bath, in which the excised legs or animals were mounted, was similar in principle to that used by Boyd and Martin (1956a). An outer jacket surrounded the recording bath and kept the temperature of the solution constant. The temperature of the solution was monitored by means of a thermistor connected to a Telethermometer (YSI, Yellowsprings) and maintained at either 22 ±.2° or 37 ±.2°C.

A solid state electrometer/amplifier (Cook, Long and Owens, 1971) was used to amplify the signal. The recording circuit has a high input resistance (10^{10} ohms) that prevents attenuation of the recorded potential (Schoenfeld, 1964). Because of the electrical interference (noise) presented by the recording circuit, the bath and amplifier were enclosed in a grounded cage. Coaxial cable was also used to minimize electrical noise.

Potentials were observed on a dual beam Tektronix 502A oscilloscope with the oscilloscope amplifiers AC coupled for m.e.p.p. recording and DC coupled for resting membrane recording.

A Hewlett/Packard FM take recorder (Model 3960) was used to

Fig. 1

Photograph of apparatus assembly

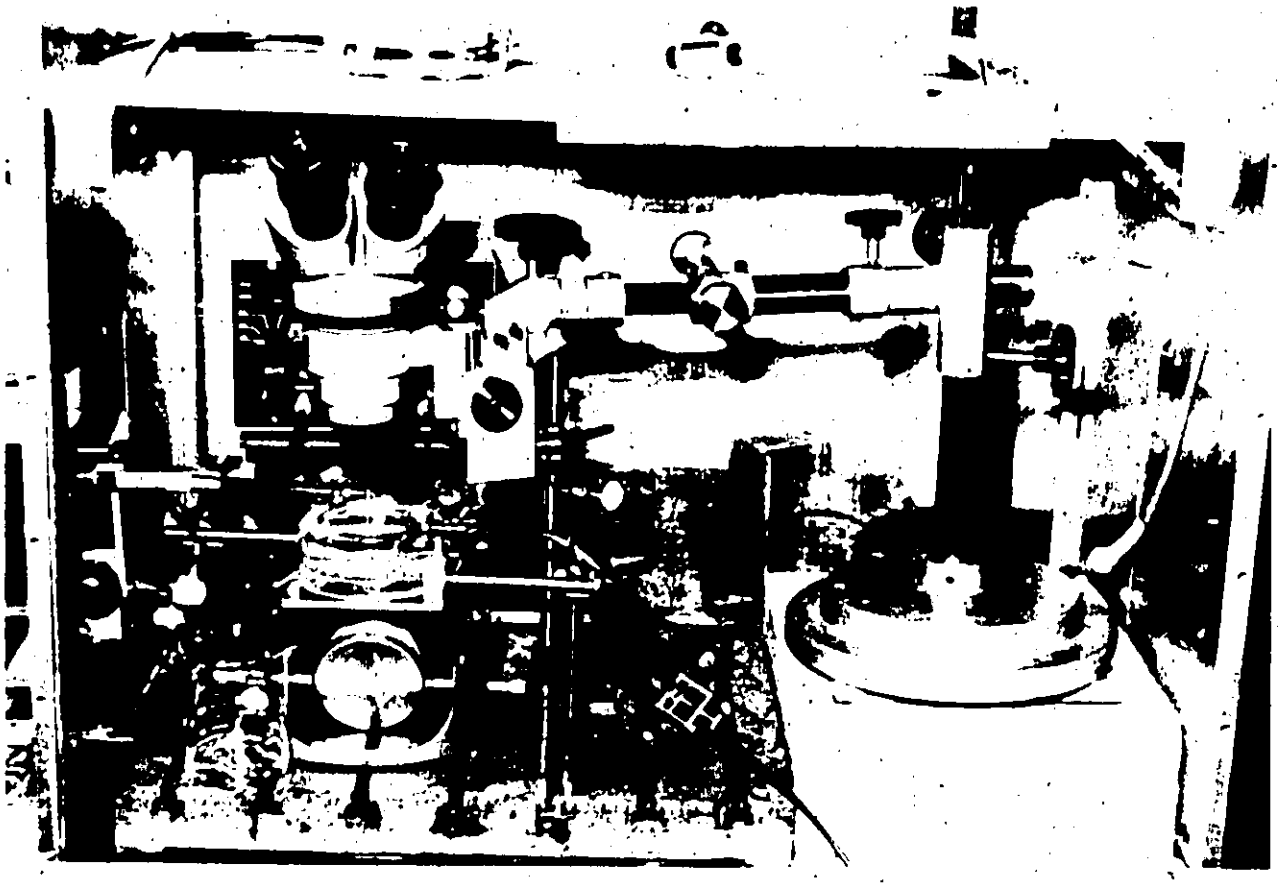
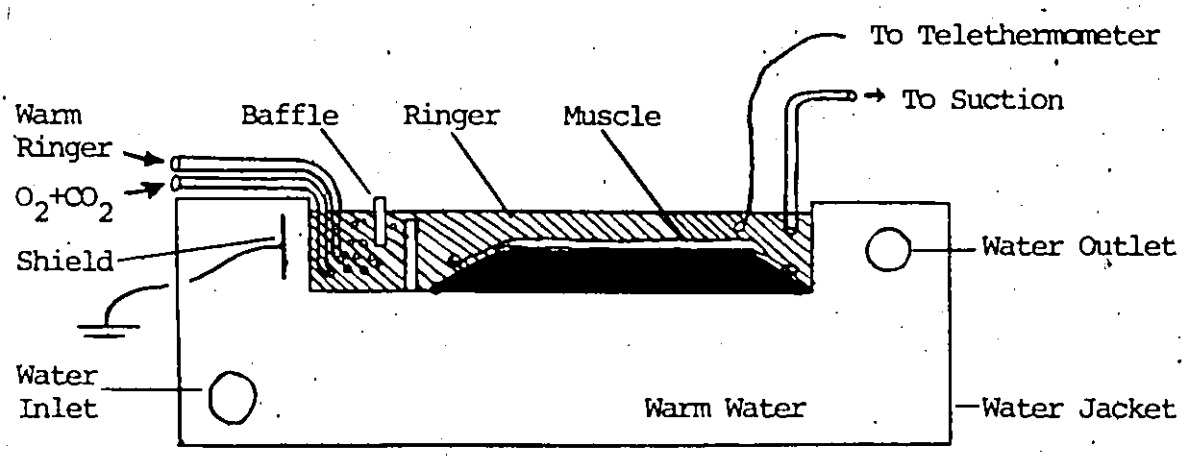


Fig. 2

Schematic representation of

muscle bath


To Telethermometer
To Suction
Warm Ringer
Baffle
Ringer
Muscle
O_2+CO_2
Shield
Water Outlet
Water Inlet
Warm Water
Water Jacket

store data,until such time,when it was retrieved and photographed by means of a continuous recording camera (Nihon Kohden,model PC2A).

The glass microelectrodes were held in a microelectrode holder (International Rectifier,model EH1R) and attached to the adjustable vernier of the micro-manipulator (Prior).

Dissection and placement of the microelectrodes was facilitated by using a stereo microscope (Nikon,model SMZ-2).

6. Efficiency of focal recording

Mapping of superficial endplates was done on representative (EDL) and (SOL) muscles. Several of these muscles from both dystrophic and normal mice were subjected to cholinesterase staining (Gomori,1952) The pattern of superficial endplates for both normal EDL and SOL muscles were compared with the dystrophic muscles. No obvious differences were seen,however,more quantitative studies were done on the 129 dy/dy dystrophic mouse (Glaser and Seashore,1967) in which a marked reduction in the amount of cholinesterase activity was found when compared to normal mice.The pattern of the superficial endplates (Fig. 3) derived from the staining technique increased the certainty of focal recording.

Focal recording was assumed when the rise time of the m.e.p.p.'s was less than 1msec. and the amplitude greater than those m.e.p.p.'s observed from the adjacent penetrations of the same muscle fiber.

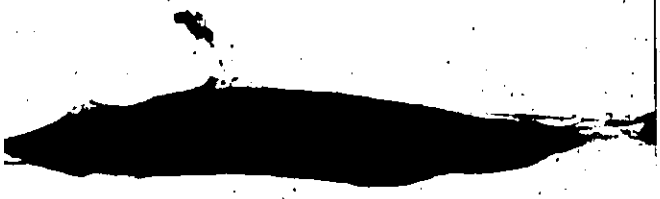
7. Experimental procedure

Before any recording was attempted,the muscles were allowed to equilibrate in the Ringer for approximately thirty minutes. In all

Fig. 3

Cholinesterase stain (after Gomori, 1952) showing superficial end plate maps of, normal soleus-A; normal EDL-B;dystrophic soleus-C;dystrophic EDL-D. Sites of cholinesterase activity appear as dark dots on individual fibers which collectively form a line traversing the whole muscle. The lines are elliptical since the preparation is depressed by a cover slip. The muscles are arranged with their proximal tendons towards the bottom of the page. All muscles are from animals in the same age group (>100 days).

C



D

experiments the Ringer was continuously gassed with the appropriate gas mixture.

While the muscles were equilibrating,each microelectrode was checked for tip resistance by means of a voltage divider network incorporated into the amplifier. The tip potential observed between the microelectrode and the reference electrode (pasteur pipette filled with Agar/Ringer;with a 3M KCL bridge) could be adjusted by means of a compensating potentiometer also incorporated into the amplifier. Electrodes with resistances between 10 and 15 megohms and tip potentials of less than 5mv were used for intracellular recording.

The electrode input capacitance was neutralized by means of a voltage feedback circuit. The compensation was adjusted to ensure the minimum of distortion in frequency response. The time constant of the circuit (<20μsec) was quite suitable for the recording of the m.e.p.p.'s. The procedure of measuring input resistance,tip potential,and neutralizing input capacitance was followed for every new electrode introduced into an experiment.

Endplates were chosen at random,although all penetrations followed a map of the superficial endplates as determined with the staining procedure. Membrane potentials were recorded,even when m.e.p.p.'s were not observed. Intracellular recording was assumed when an immediate fall in potential was noticed with the oscilloscope input DC coupled.

Recording was done at 37°C$\pm$ .2°. Addition of Ringer to the bath was accomplished with a 50ml.syringe. The recording bath accomodated

25 mls. of solution which was changed frequently during the experiment. The whole experiment lasted approximately three hours during which time 20-40 endplates were sampled from both EDL and SOL muscles.

8. Statistical analysis of results

All data from the experiments were collected manually; the m.e.p.p.s being counted with the aid of an enlarger. Statistical analysis was performed with the aid of a Wang calculator (Model 600) using preprogrammed tapes.

Mean values in the text and in illustrations have been presented with the standard errors of the means. Significance of differences between the means have been calculated using the unpaired t-test.

An analysis of variance was performed on the data to test the interrelationships between the variables. Significance of differences have been calculated using the F-test, again on a preprogrammed tape.

CHAPTER III

Results

1. Critical survey of experimental methods

Since the frequency of m.e.p.p.s can be influenced by a variety of factors, it was important to test that any changes observed are in fact genuine effects of the variables being considered. These were; age, type of muscle (fast and slow) and the condition of the animal (normal and dystrophic). Several experiments were therefore performed to determine the accuracy of these observations.

a) Effect of temperature and hypoxia

The effects of increased temperature and hypoxia on the frequency of m.e.p.p.s were determined in one set of experiments using soleus muscles from normal mice approximately 100 days old. Recordings were made from both in vivo and in vitro preparations that were subjected to bathing solutions at 22° and 37°C. The effect of hypoxia on in vivo preparations was measured by comparing the mean frequency of m.e.p.p.s from random junctions before, during and after treatment with the substituted 95%N/5%CO_2 gas mixture. The data from these experiments are summarized in Table II.

The mean frequency observed in the in vitro muscle preparations at 22°C was higher when compared with the in vivo preparations. The in vivo preparations at 37° also shows a much higher mean frequency of miniature discharge than at 22°. The effects of substituting

TABLE II

Effect of temperature and hypoxia

on m.e.p.p. frequency in soleus (>100 days)

Experimental Conditions	M.e.p.p. frequency ($sec.^{-1}$)	
22° in vivo	1.34 ± 0.24	(14/2)
22° in vitro	2.31 ± 0.33	(5/1)
37° in vivo*	8.79 ± 1.13	(19/2)
37° in vivo* (hypoxic)	12.55 ± 1.69	(19/2)
37° in vivo*	9.96 ± 1.01	(11/2)
37° in vitro	12.36 ± 1.10	(30/5)
37° in vitro (hypoxic)	> 100	(4/1)

Mean ± S.E.M.

Parentheses = number of fibers / and number of muscles.

*, The recordings at 37° in vivo were from 2 soleus muscles. M.e.p.p.s were recorded from different endplates in Ringer gassed with 95%O_2/5%CO_2, and then in 95%N_2/5%CO_2 and again in normal ringer gassed with 95%O_2/5%CO_2.

95%N_2/5%CO_2 in the hypoxic treatment did not result in any significant change in mean frequency when compared to those mean frequencies observed in ringer gassed with 95%O_2/5%CO_2. The mean frequency from junctions at 37°C (in vitro) in hypoxic bathing solutions was the highest of any of the recordings. Frequencies of well over 100/sec. were recorded from this group and represented a significant increase when compared to the frequencies from in vitro preparations in oxygenated ringer.

b) The effect of increased Mg

The increase of m.e.p.p. frequency that is observed at higher temperatures may be due to presynaptic depolarization similar to an anoxic effect (Hubbard and Løyning,1966). To determine whether depolarizing influences were present in this study, a series of experiments were performed using a bathing solution containing an increased concentration of magnesium chloride. It is known that increasing magnesium concentration affects the voltage and calcium-sensative fraction responsible for variations in m.e.p.p. frequency (Hubbard,1961). While the calcium remained unchanged (1.5mM/L), the magnesium concentration was altered from the usual 0.23mM/L to 10mM/L.

Neuromuscular junctions were sampled from both the soleus and EDL muscle preparations at 22° and 37°C. The effect of magnesium was measured by comparing the frequency of m.e.p.p.s in the same fiber before, during and after treatment with the magnesium containing solution. The results from 13 experiments on the soleus preparation are summarized in Table III. The results from 12 experiments on the EDL preparation

are summarized in Table IV. The increased Mg bathing solution decreased the frequency of miniature potentials displayed in Fig.4. It may be noted that the observed frequencies were not always the same before and after the period in which 10mM $MgCl_2$ was substituted (Table IV). The frequencies from the normal muscles in normal ringer (A) were compared with those frequencies obtained from the test solutions (B). The effect of raised Mg solution in (B) may be expressed as the percent depression in mean frequency from the mean value in (A). The depression of discharge rate for the soleus preparation at 22°C and 37°C was 53%, while for the EDL preparation at both 22°C and37°C was 44%. It is worth noting that the sixth junction included for the EDL at 37°C (Table IV) has a much higher initial frequency than any of the previous frequencies tabulated. This increased frequency may be from a depolarized junction since the depression from the original discharge rate in normal ringer was 88%. Column (C) in both Tables III and IV shows the frequency recovery in normal ringer. The difference in recovery between EDL and soleus reflects the different diffusion distances within these muscles.

2) Resting membrane potential

Resting membrane potentials were recorded from all muscle fibers penetrated in both normal and dystrophic groups. Membrane potential values are tabulated for both groups in Tables V and VI.

No significant difference in mean resting membrane potential (mean RMP) was observed when normal (10-20 day) EDL and soleus groups were compared, Table V. Thereafter, the mean RMP for normal soleus was significantly lower than the mean RMP for the respective EDL groups.

TABLE III

Effect of Mg on Frequency of m.e.p.p.s

Soleus 22°C Junction	Discharge rates (per second) A	B	C*	Frequency ratio B/A
1	1.25	0.52	0.67	0.42
2	0.69	0.24	0.46	0.35
3	1.08	0.48	0.54	0.44
4	0.64	0.26	0.32	0.41
5	1.79	0.59	1.29	0.33
6	2.00	0.47	0.75	0.24
7	0.85	0.79	1.04	0.93
	Mean and S.E.			0.45±0.08
Soleus 37°C Junction				
1	13.47	4.89	7.91	0.36
2	4.37	1.50	5.38	0.34
3	6.00	5.77	8.11	0.96
4	8.56	2.33	2.91	0.27
5	9.62	7.78	6.81	0.81
6	2.47	0.53	1.50	0.21
	Mean and S.E.			0.49±0.13

*, Column C indicates that although recovery is not yet complete, it is underway.

TABLE IV

Effect of Mg on Frequency of m.e.p.p,s

EDL 22°C Junction	Discharge rates (per second) A	B	C*	Frequency ratio B/A
1	1.26	0.58	1.04	0.46
2	4.14	3.15	3.11	0.76
3	2.42	1.26	2.43	0.52
4	0.58	0.28	0.56	0.48
5	0.64	0.41	0.80	0.64
6	1.46	0.72	0.73	0.49
	Mean and S.E.			0.56±0.04

EDL 22°C Junction				
1	22.33	16.50	10.38	0.74
2	14.29	8.00	12.00	0.56
3	15.55	9.38	11.89	0.60
4	2.53	1.27	2.82	0.50
5	5.07	3.67	4.60	0.72
6	68.20	15.07	23.78	0.22
	Mean and S.E.			0.56±0.08

*, Column C indicates that although recovery is not yet complete, it is underway.

Fig. 4

Effect of Mg on m.e.p.p. frequency at same endplate, A-C from Normal soleus muscle. Frequency discharge of m.e.p.p.s in normal ringer (0.23 mM, $MgCl_2$) shown in, A; in 10 mM $MgCl_2$ ringer, B; return to normal ringer, C. $CaCl_2$ in all three cases was 1.5 mM. All recordings were done at 37°C.

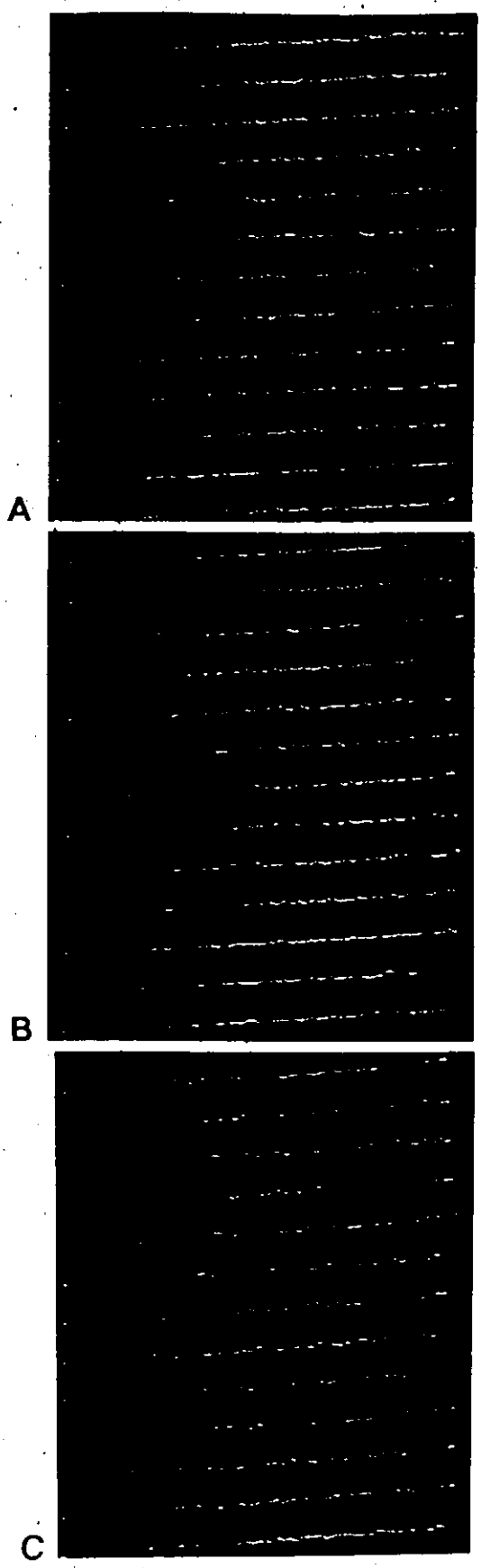

A
B
C
1MV
.1sec

TABLE V

Resting Membrane Potential for normal Soleus and EDL muscles at various ages.

Muscle	Age (days)	Mean R.M.P.	S.E.M.	P (from student's t-test)
SOL	10-20	57.01 (117)	0.58	NS
EDL	10-20	58.05 (146)	0.51	
SOL	20-30	60.58 (108)	0.55	< 0.01
EDL	20-30	62.72 (155)	0.39	
SOL	30-40	65.10 (125)	0.48	< 0.01
EDL	30-40	68.13 (129)	0.62	
SOL	40-50	66.68 (157)	0.44	< 0.01
EDL	40-50	68.59 (184)	0.47	
SOL	> 100	63.93 (101)	0.54	< 0.001
EDL	> 100	69.21 (155)	0.34	

TABLE VI

Resting Membrane Potential for Dystrophic Soleus and EDL muscles at various ages.

Muscle	Age (days)	Mean R.M.P.	S.E.M.	P (from student's t-test)
SOL	20-30	57.51 (123)	0.61	< 0.01
EDL	20-30	59.89 (102)	0.74	
SOL	30-40	61.70 (88)	0.67	< 0.01
EDL	30-40	66.47 (94)	0.76	
SOL	40-50	65.09 (69)	0.96	N.S.
EDL	40-50	68.01 (52)	1.11	
SOL	> 100	62.92 (250)	0.38	N.S.
EDL	> 100	63.90 (247)	0.42	

Figures in parentheses=number of muscle fibers

P = significance difference

When mean RMPs for dystrophic muscles are compared (TABLE VI), the soleus values in the two youngest age groups are significantly lower than the mean RMPs of the EDL groups. For the last two age groups however, no differences between EDL and soleus values were found; in fact the mean RMP for the (>100 day) EDL had decreased from that value recorded for the (40-50 day) EDL group. The mean RMP value recorded for the (>100 day) dystrophic EDL was significantly reduced when compared with the normal EDL of the same age group.

3. M.e.p.p. Frequency in Normal and Dystrophic Mice

Patterns of discharge rate from three endplates of the same muscle are shown in Figure 5 . Also shown are miniature potentials from normal and dystrophic fibers with a variety of time courses. A complete analysis of m.e.p.p. time course was not performed on the present data.

The results from experiments on normal and dystrophic EDL and soleus muscles at various age groups are summarized in TABLE VII. Comparisions of the mean frequencies for normal and dystrophic groups are shown in Figures 10, 11, and 12. To test the interdependence of the variables an analysis of variance was done. The F-ratios for the variance quotients are shown in TABLE VIII.

Analysis of the frequency distribution for each age group was done. Frequency histograms for normal and dystrophic EDL and soleus muscles at various ages are shown in Figures 6, 7, 8, and 9. It may be noted that in both normal soleus and EDL groups the distributions are noticably skewed to the left in the younger age groups. The older age groups (above 30-40 days) show a relatively normal distribution. The dystrophic soleus muscle

Fig. 5

Example of intracellular m.e.p.p.s from normal and dystrophic endplates at 37°C. Various frequencies observed from different endplates of a normal EDL muscle, A-C. Intracellular recordings from single endplates, using a fast time base; normal EDL-D, dystrophic EDL-E, normal soleus-F, dystrophic soleus-G.

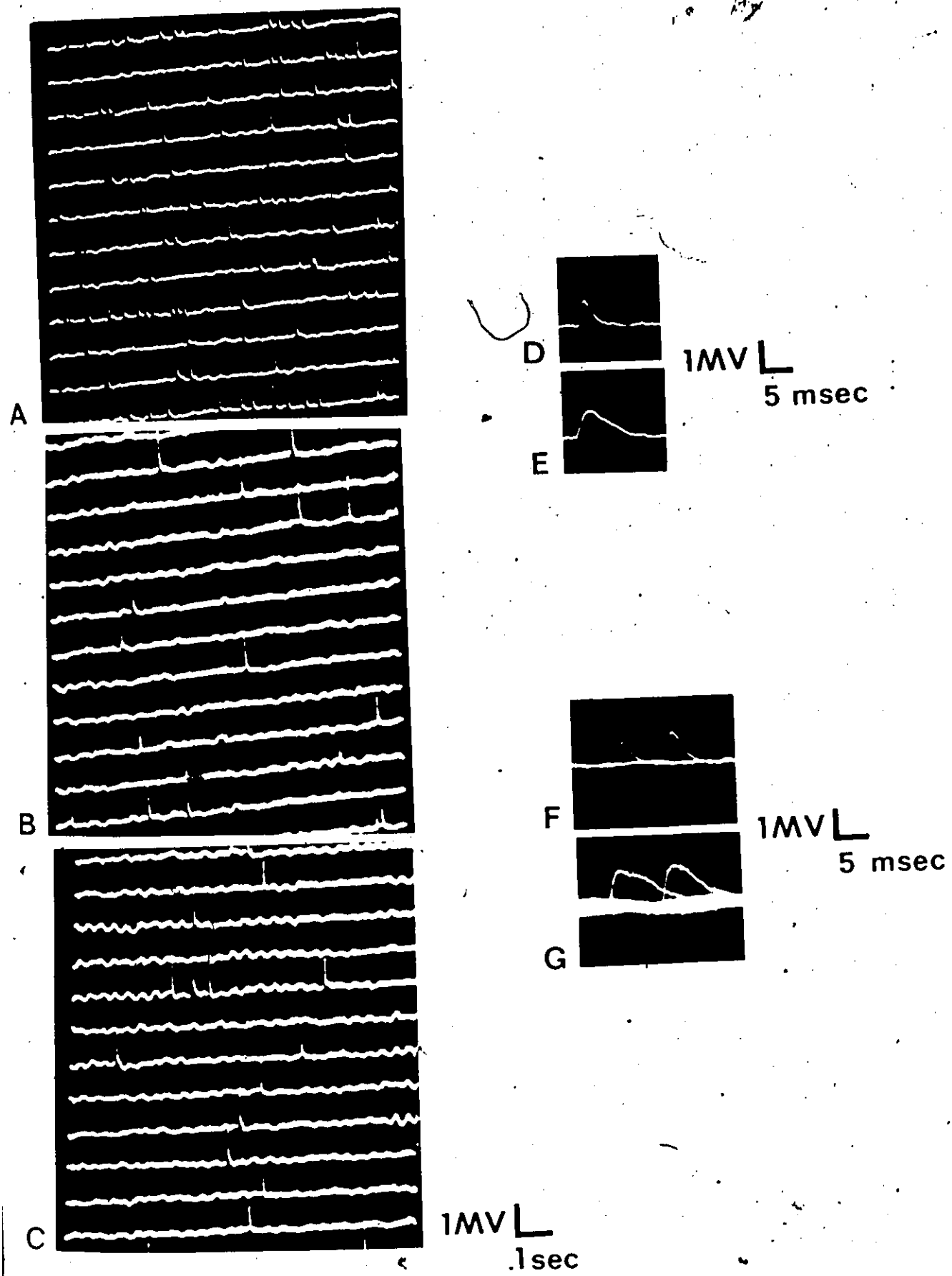

A
B
C
D
E
F
G
1MV
5 msec
1MV
.1sec
1MV
5 msec

TABLE VII

M.e.p.p. Frequencies from EDL and SOL of Normal and Dystrophic Mice at various ages.

Muscle	Age (days)	Mean M.e.p.p. frequency (sec^{-1})	S.E.M.	P (from student's t-test)
N SOL	10–20	4.61 (58/5)	±0.98	N.S.
N EDL	10–20	7.48 (47/5)	±1.18	
N SOL	20–30	11.12 (39/4)	±1.90	N.S.
N EDL	20–30	11.97 (28/4)	±1.60	
N SOL	30–40	9.91 (65/5)	±1.17	< 0.001
N EDL	30–40	14.20 (50/6)	±0.99	
N SOL	40–50	12.77 (63/4)	±1.20	< 0.01
N EDL	40–50	17.94 (36/4)	±1.90	
N SOL	> 100	12.36 (30/5)	±1.10	< 0.001
N EDL	> 100	19.12 (44/6)	±1.58	
Dy SOL	20–30	10.11 (66/5)	±1.46	N.S.
Dy EDL	20–30	10.07 (28/4)	±0.09	
Dy SOL	30–40	11.74 (50/5)	±0.98	< 0.005
Dy EDL	30–40	16.79 (34/4)	±1.69	
Dy SOL	40–50	14.65 (17/3)	±2.23	N.S.
Dy EDL	40–50	16.18 (16/3)	±2.90	
Dy SOL	> 100	14.36 (79/10)	±1.08	N.S
Dy EDL	> 100	13.81 (62/10)	±1.39	

Figures in parentheses = number of muscle fibers / number of muscles

P = significance difference

also shows a skewed pattern in the early age groups (Fig.7) while the (>100 day) group has a normal distribution . The distribution expressed for the dystrophic soleus (40-50 day) reflects the small sample obtained for this group. The dystrophic EDL muscle demonstrates a skewed distribution in the (20-30 day) group sampled, a normal distribution in the (30-40 day) and (40-50 day) groups but regresses to a distribution that is skewed to the left in the (>100 day) group.

Mean frequencies of miniature potentials for both normal and dystrophic muscles tended to increase with age. The mean frequencies of the normal soleus and EDL muscles at the youngest age groups sampled are observed to be rather similar. As the age increases, a significant difference between the groups may be observed (Fig.10) with the mean frequency for the EDL muscles markedly higher than the soleus muscles at the same age.

When the mean frequencies of the normal and dystrophic soleus muscles are compared (Fig.11) no significant differences are observed in any of the age groups sampled. No significant difference is observed in mean frequencies from normal and dystrophic EDL muscles until the last age group (>100 days). In this group the dystrophic EDL mean frequency (13.81 ± 1.39) was significantly lower than the normal EDL (19.12 ± 1.58) (Fig. 12)

To test whether interrelations existed between the three variables (A-normal and dystrophic muscles; B-EDL and soleus muscles; C-age groups), an analysis of variance was done.

Fig. 6

Frequency histogram of Normal Soleus at various age groups.

Ordinate: Observations expressed as percent of the number of fibers sampled.

Absiccae: Frequency of m.e.p.p.'s in seconds^{-1}.

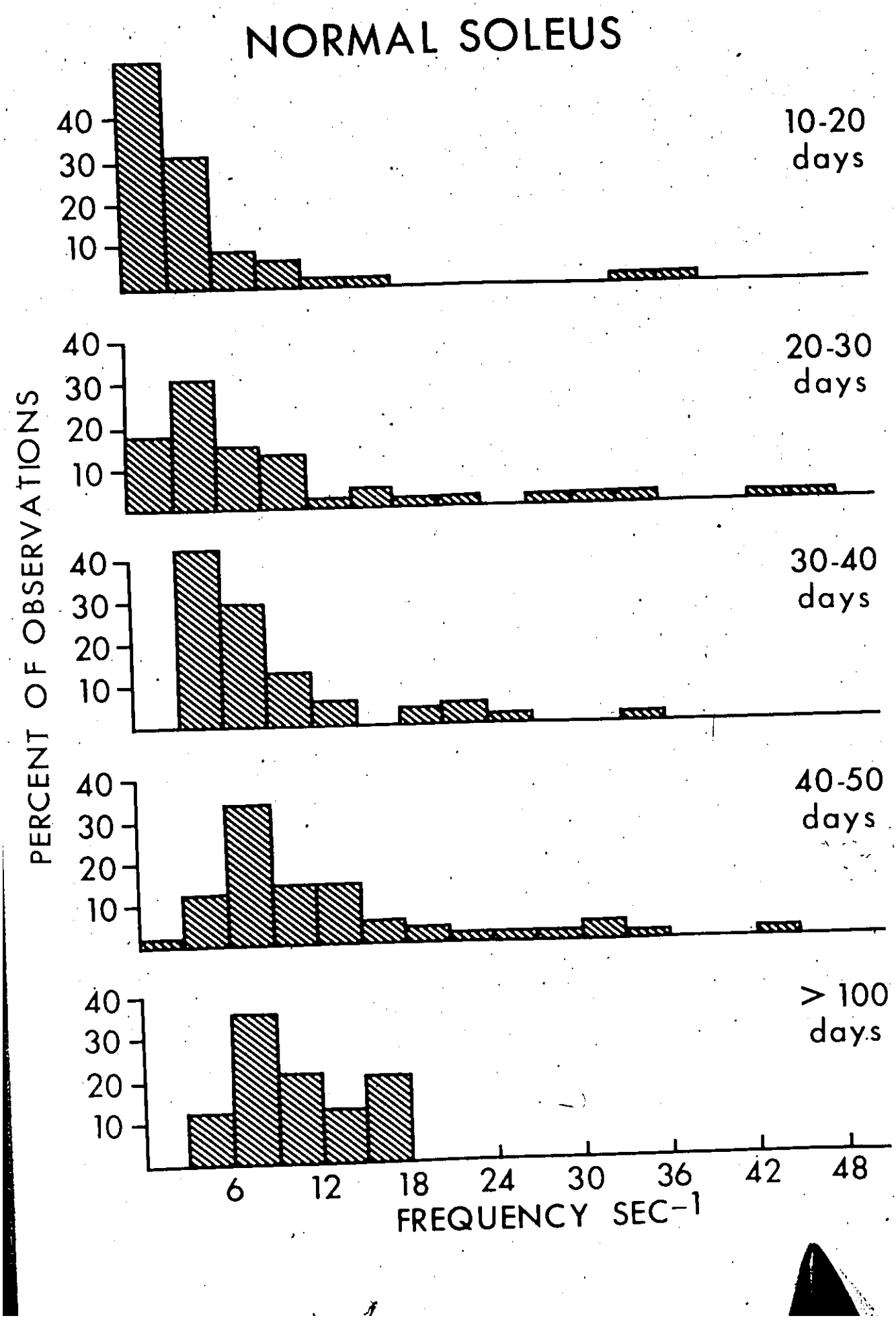

NORMAL SOLEUS
10-20 days
20-30 days
30-40 days
40-50 days
> 100 days
PERCENT OF OBSERVATIONS
40
30
20
10
6
12
18
24
30
36
42
48
FREQUENCY SEC⁻¹

Fig.7

Frequency histogram of Dystrophic Soleus at various age groups.

Ordinate: Observations expressed as percent of the number of fibers sampled.

Absiccae: Frequency of m.e.p.p.'s in seconds^{-1}.

DYSTROPHIC SOLEUS

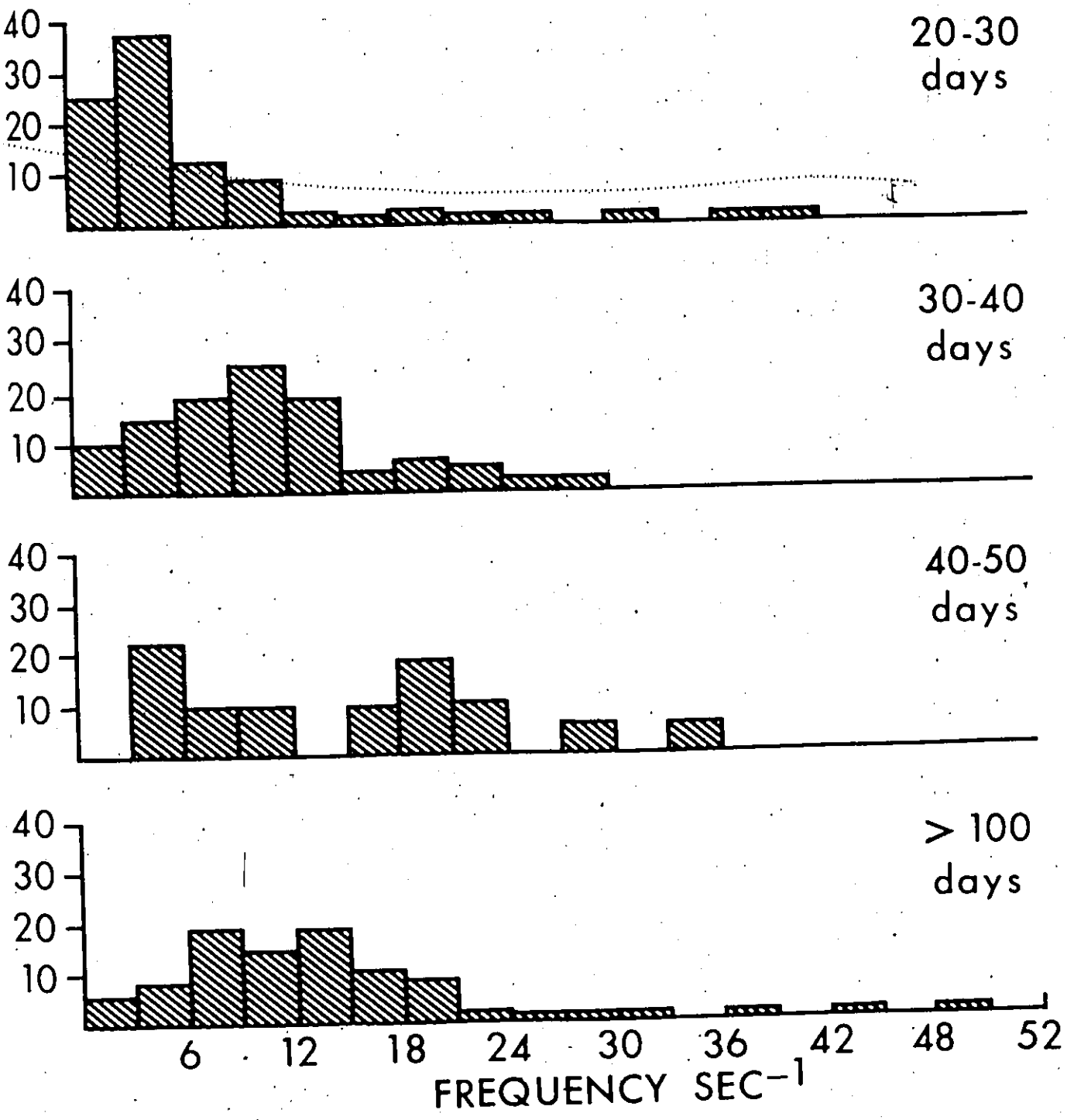
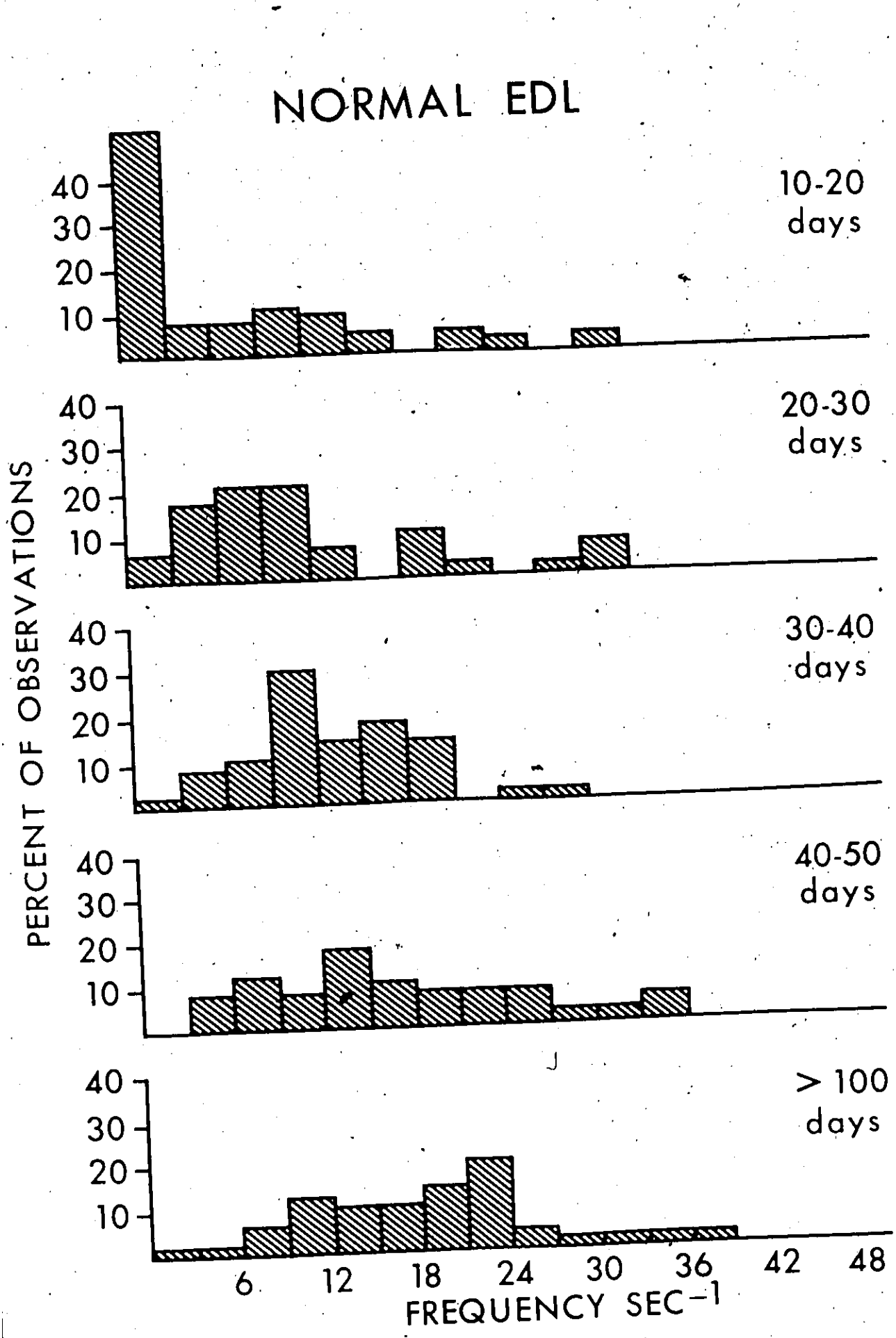


Fig. 8

Frequency histogram of Normal E.D.L. at various age groups.

Ordinate: Observations expressed as percent of the number of fibers sampled.

Absiccae: Frequency of m.e.p.p.'s in seconds^{-1}.


NORMAL EDL
10-20 days
20-30 days
30-40 days
40-50 days
> 100 days
PERCENT OF OBSERVATIONS
40
30
20
10
6
12
18
24
30
36
42
48
FREQUENCY SEC^{-1}

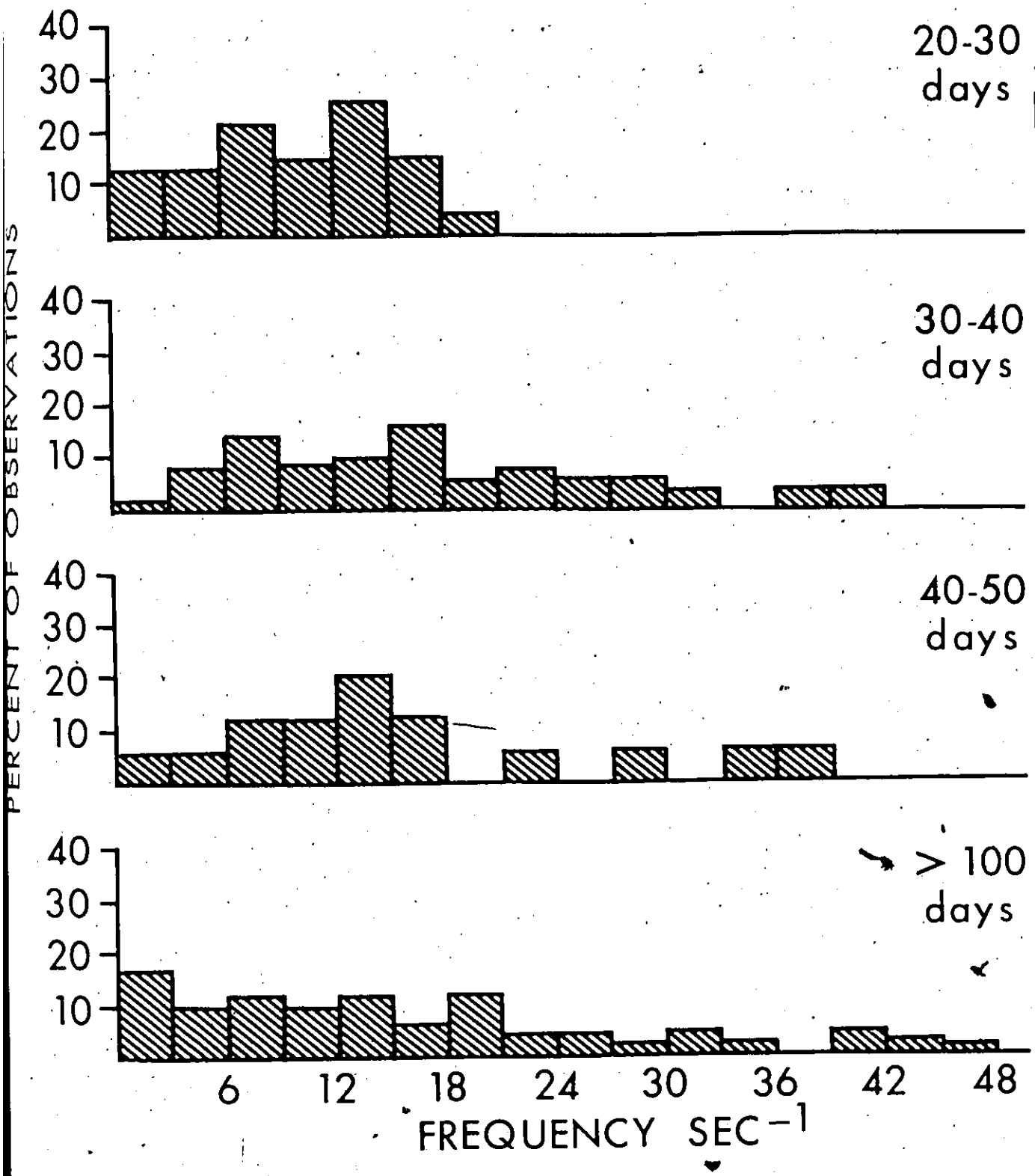

DYSTROPHIC EDL
PERCENT OF OBSERVATIONS
40
30
20
10
20-30 days
30-40 days
40-50 days
> 100 days
6
12
18
24
30
36
42
48
FREQUENCY SEC^{-1}

Fig. 10

Mean frequencies of m.e.p.p.s ± S.E.M. for normal EDL and soleus muscles expressed as a function of age.

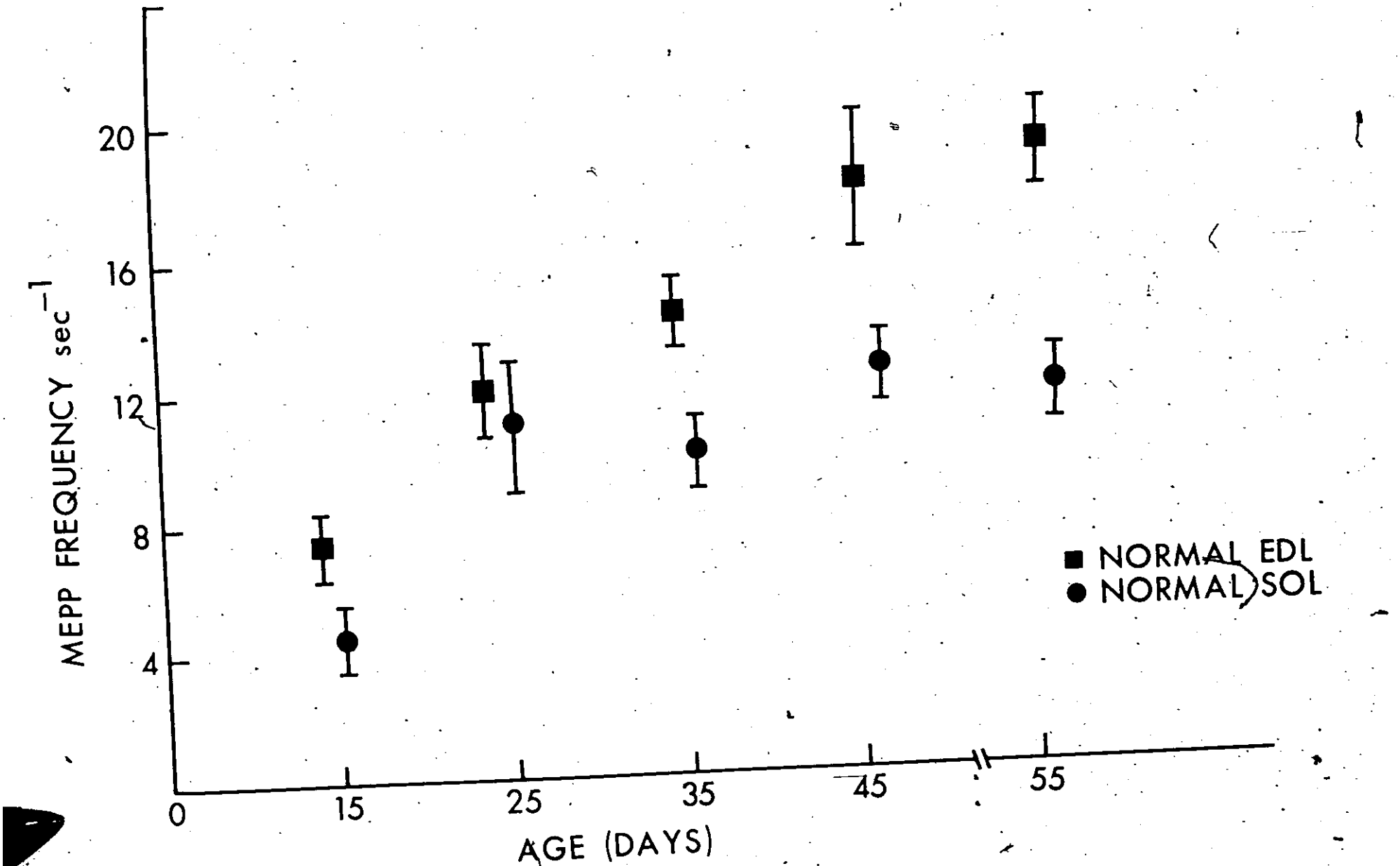

MEPP FREQUENCY sec^{-1}
20
16
12
8
4
0
15
25
35
45
55
AGE (DAYS)
NORMAL EDL
NORMAL SOL

Fig. 11

Mean frequencies of m.e.p.p.s ± S.E.M. for normal and dystrophic soleus muscles expressed as a function of age.

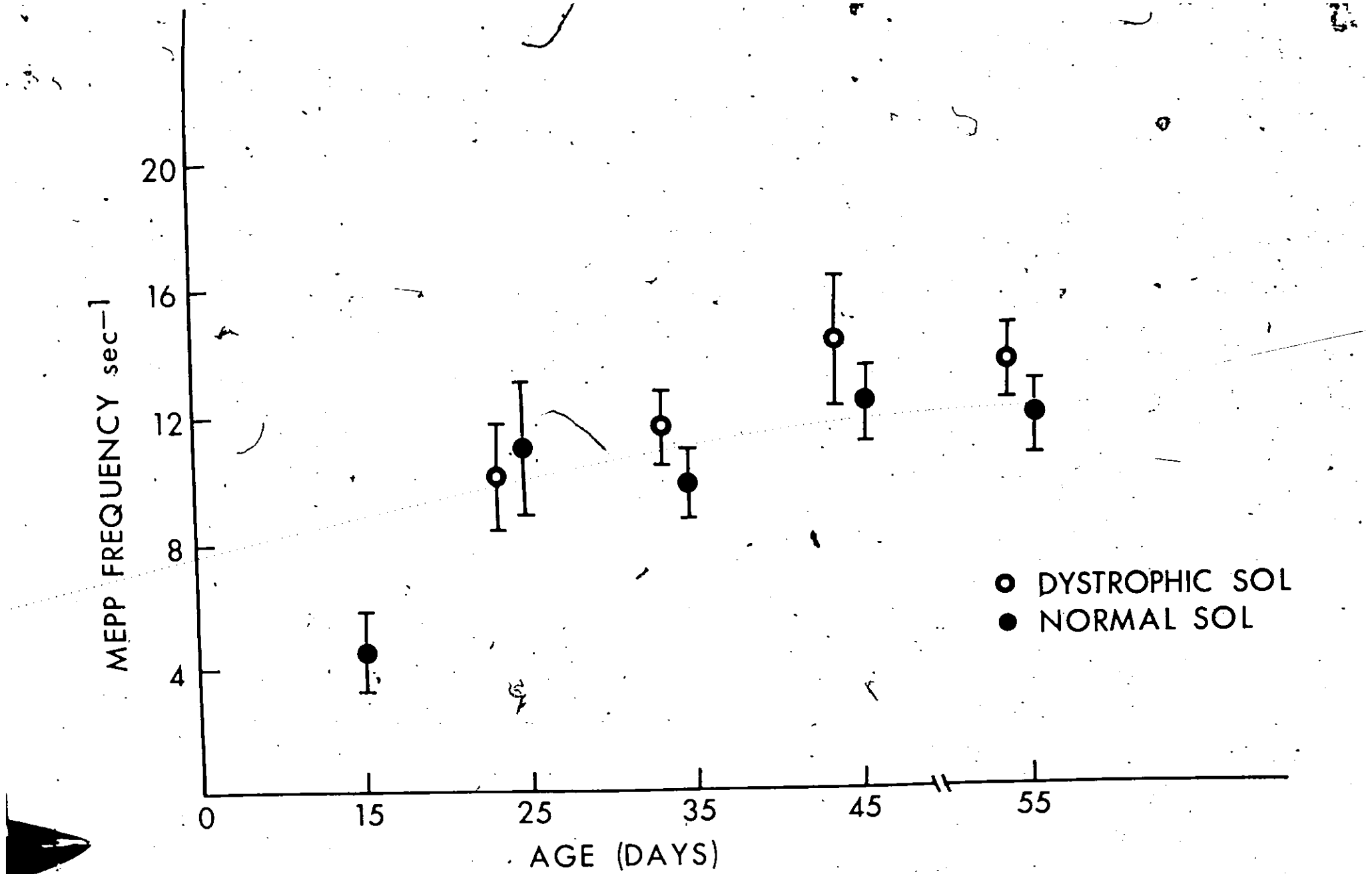
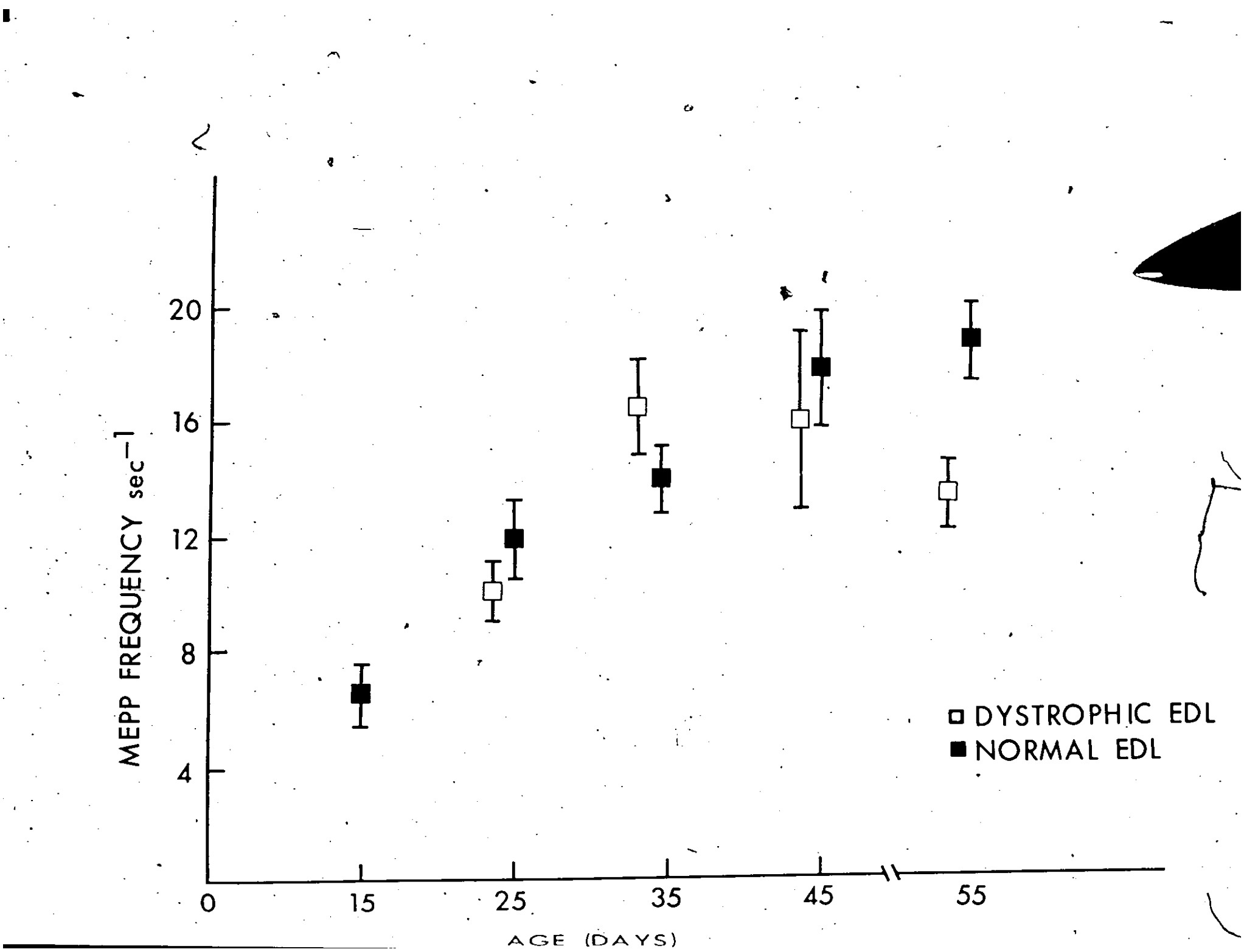

MEPP FREQUENCY sec⁻¹
20
16
12
8
4
0
15
25
35
45
55
AGE (DAYS)
DYSTROPHIC SOL
NORMAL SOL

Fig.12

Mean frequencies of m.e.p.p.s ± S.E.M. for normal and dystrophic EDL muscles expressed as a function of age.


MEPP FREQUENCY sec⁻¹
20
16
12
8
4
0
15
25
35
45
55
AGE (DAYS)
DYSTROPHIC EDL
NORMAL EDL

The mean frequencies that were recorded and the subsequent differences found were influenced by the type of muscle sampled (B) and the age group (C) from which it was taken. This is supported by the level of significance obtained by the variance quotients or F-ratios for these two sources of variability. When considering the influences of any of the variables on one another, no interaction was found.

TABLE VIII

Analysis of Variance

Source of variability		F ratio	Significance
Among N† and D§ muscles	-A	F = 0.082	N.S.
Among EDL and SOL muscles	-B	F =12.028	S.
Among age groups	-C	F = 6.217	S.
Interaction	A x B	F = 2.580	N.S.
Interaction	A x C	F = 2.046	N.S.
Interaction	B x C	F = 1.395	N.S.
Interaction	A x B x C	F = 1.106	N.S

† = Normal

§ = Dystrophic

Significance level = 0.05

CHAPTER IV

Discussion

1. Validity of the technique

It is very noticeable that the frequencies recorded in this study are considerably higher than those reported in the literature. This may be due to species difference since in most cases the values previously reported have been obtained from the rat. But another factor that must be considered is the temperature at which the experiments were performed. Hubbard (1970) has demonstrated a very marked effect of temperature on the spontaneous transmitter release in several species. This complex temperature effect on the rate of m.e.p.p.s is seen over the range of 7-39°C;with the frequencies observed between 30-39°C being the most accelerated. TABLE IX shows the variation in m.e.p.p. frequencies that were obtained in several studies together with the species and the temperature at which the recordings were made.

The validity of the recording technique (in vitro,37°C) used in determining changes in neuromuscular transmission of dystrophic mice rests on a series of experiments demonstrating the effects of hypoxia and raised Mg^{++} concentration on m.e.p.p. frequency.

With an in vivo preparation it would seem unlikely that hypoxia could occur since O_2 is being supplied endogenously via the circulation and exogenously by the bathing solution. When the exogenous O_2 is removed, only a slight increase in mean frequency is observed. Under in vitro conditions it has been shown that frequencies in excess of 100/sec. may be

TABLE IX

M.e.p.p. frequency in various mammalian preparations and the effects of temperature

Preparation[α]	Temperature (C)	Frequency[β] (sec^{-1})	Reference
Human intercostal	32	0.26±0.07	Elmquist, Johns, and Thesleff (1960)
Human palmaris longus	20-24	0.39±0.26	Haynes (1971)
Cat teniussimus	37	1.5	Boyd and Martin (1956)
Rat diaphragm	37-38	4.6	Hubbard (1961)
	18-22	1.0	Diamond and Miledi (1962)
	20-22	1.85±0.68	Turkanis (1973)
	34-35	1.22±0.32	Turkanis (1973)
Mouse diaphragm	22-27	10.8	Conrad and Glaser (1964)
Mouse biceps	22-23	0.74±0.27	Duchene and Stefani (1971)
Mouse gracilis	28-36	12.5 ±1.0	McComas and Mossawy (1965)
Mouse soleus	22	1.19±2.0	Present study
	37	12.36±1.10	Present study
Mouse EDL	22	1.75±0.55	Present study
	37	19.12±1.58	Present study

α = all preparations are in vitro

β = Mean ± S.E.M. where it has been provided

observed during hypoxia (Hubbard and Løyning, 1966). Boyd and Martin (1956a) have shown that the frequency of m.e.p.p.s increases during hypoxia. Since the bathing solution was continuously oxygenated in our study, hypoxia should not have been a factor influencing the frequencies recorded.

Hubbard and Løyning (1966) have also shown that the hypoxia-induced frequency is largely abolished by Mg^{++}. Krnjevic and Miledi (1959) attributed the increase frequency seen in anoxic conditions to presynaptic depolarization. If the increased frequency observed at 37°C were due to depolarization of the presynaptic terminals, then a raised level of Mg^{++} would be expected to produce a greater reduction of m.e.p.p. frequency at 37°C then at 22°C. The data presented here (TABLES III and IV) suggest that the reduction of m.e.p.p. frequency in both EDL and soleus muscles in the presence of 10mM Mg^{++} is just as great at 37°C as at 22°C. The reduction in frequency amounted to approximately 42%. Hubbard (1961) concluded that only about 30-40% of the resting m.e.p.p. frequency was Ca^{++} and Mg^{++} sensitive, whereas the increased frequency induced in the presence of 20mM KCl could be completely inhibited by removal of Ca^{++}. Further evidence of this effect was supplied by Landau (1969) using focal depolarization of the presynaptic terminal. He demonstrated that 8mM Mg^{++} reduced the effect of a given depolarization on m.e.p.p. frequency. The depression of 42% of m.e.p.p. frequency by 10mM Mg^{++} in our study indicate that the frequencies measured at 37°C were indeed representative of a true resting rate of transmitter release. A much greater reduction would have been anticipated if there existed a depolarization-induced component.

To further validate the results obtained, it may be noted that there is no significant difference between mean m.e.p.p. frequency of soleus muscles in vitro (TABLE VII) and that observed in vivo at 37°C (TABLE II), which represents the optimal conditions for this type of recording (Hoekman, Dretchen and Standaert, 1974). Moreover, no correlation could be found between the resting membrane potentials of muscle fibers and the frequency of m.e.p.p.s recorded from their respective endplates. The assumption that may be made is that the muscle membrane potential may be used as an index of the condition of the preparation. It follows then, that the low resting membrane potentials due to anoxic conditions may reflect the same condition presynaptically. Consistently low resting membrane potentials may be expected for muscle fibers exhibiting high frequencies . A wide variation, however, was observed in m.e.p.p. frequencies for individual muscles and no correlation was found between these frequencies and the membrane potentials from their respective fibers. Thus, although there may have been a small degree of hypoxia in our in vitro experiments, it was insufficient to cause a marked increase in m.e.p.p. frequency.

2. Resting membrane potentials in normal and dystrophic muscles

The values of membrane potentials reported in our study for normal and dystrophic EDL and soleus muscles compare fairly well with those reported in the literature. Harris and Luff (1970) have shown that the soleus membrane potentials were significantly lower that the EDL values in developing mice. They also observed that resting membrane potential (R.M.P.)

appears to be a function of the age of the animal. These two findings were confirmed in this present study. McComas and Mrozek (1967), and Harris (1971) reported that dystrophic fibers were somewhat more depolarized than normal fibers. This was also found to be the case here (TABLES V and VI). Reduced membrane potentials are also characteristic of human forms of dystrophy (Hofman, Alston and Rowe, 1967; Ludin, 1970). On the basis of R.M.P. it appears that although the dystrophic soleus muscles are depolarized relative to normal muscles, the degree of depolarization is not as great as dystrophic EDL relative to control muscles. This is especially evident when the most mature age group (>100 day) is considered.

The primary cause of the low resting membrane potential in muscular dystrophy is not known. It is known that intracellular muscle K^+ is lower in dystrophic muscles than in normal muscles (Baker et al, 1958; Young et al, 1959; Hoh and Radulovacki, 1973). It has also been reported that serum K^+ is higher in dystrophic animals (Hazelwood and Ginski, 1968). Hazelwood and Ginski (1968) point out that these two mechanisms operating alone or together are incapable of causing a large enough fall in potential, and suggest that there is also a change in membrane permeability to one or all of the ions (Na^+, K^+, and Cl^-). Zierler (1958, 1961) has demonstrated that membranes of dystrophic mice do exhibit enhanced permeability to K^+ and aldolase.

Electrolyte composition of fast-twitch and slow-twitch muscles are distinctly different, the former having a significantly higher concentration of K^+ and lower concentration of Na^+ compared with the latter

(Hoh and Salafsky,1971). Hoh and Radulovacki (1973) have also shown that while both dystrophic slow-twitch and fast-twitch muscles had lower K^+ and higher Na^+ and Cl^- compared with corresponding normal muscles, the changes in electrolyte composition of dystrophic slow-twitch muscles were less marked than in fast-twitch muscles. These findings support the hypothesis that slow-twitch muscles are less severely affected by the dystrophic process than fast-twitch muscles (Brust,1966;Law and Atwood, 1972a).Lipicky and Hess(1974) recently reported that the mean apparent rate constant of K^+ efflux in dystrophic EDL muscle was significantly greater that that of the EDL control,while no change was found in the rate constant for dystrophic soleus relative to controls. Calculation of the K^+ permeability (PK) from their own efflux data,which takes into consideration the ratio of surface to volume ratio,and the resting membrane potentials reported by Harris (1971),suggests that PK in the dystrophic EDL muscle is not different from control and that PK in the soleus muscle is less than that of control values. It is difficult to draw definite conclusions from their results since the membrane potential values were from another investigation. It may be as Hazelwood and Ginski (1968) suggested that other ions are also involved,however to date,no evidence is available on the conductances of these ions in dystrophic muscle.

3, M.e.p.p. frequency in developing mice

Slow-twitch and fast-twitch muscles also show differences in neuromuscular transmission. McArdle and Albuquerque (1973) have reported significantly higher mean m.e.p.p. frequency in the EDL muscle when compared to the mean frequency in the soleus muscle of the rat. This is also the

case in the mouse as indicated by our results (TABLE VII).

Muscles show trends in ontogeny (Buller,Eccles and Eccles,1960b; Close,1965;Cosmos and Butler,1967) as do nerves that innervate them (Nystrom,1968). It appears at birth in many species that all muscles have the general characteristics of slow contracting muscles. Buller, Eccles and Eccles (1960a) showed that all the hind limb muscles they examined contracted rather slowly in the newborn kitten and as the animal matured,the fast muscles quickened to achieve their adult contraction speed. In the mouse,Close(1965) showed that the force-velocity relationships of the soleus remained constant from birth,whereas,that of the EDL showed a change to higher velocity of contraction. Enzyme profiles of fast and slow muscles of the chick (Cosmos and Butler,1967),pig (Cooper et al,1971) and rabbit (Parry,personal communication) all show a change in post natal development. The oxidative enzymes in both fast and slow muscles are high at birth. During the maturation of fast muscle a rise in glycolytic enzymes coincides with a continuous decline in the activity of oxidative enzymes. The soleus muscle,on the other hand,continues to show a high activity of oxidative enzymes with weak glycolytic activity through post natal development.

The results presented in our study indicate that neuromuscular transmission,as exemplified by m.e.p.p. frequency also does not differ in the very young mice. In the earliest groups examined (TABLE VII),there is no significant difference between the frequencies observed for the two muscles. As the animal matures,however,the frequency in the EDL muscle

gradually becomes greater than that in the soleus. Diamond and Miledi (1962) reported that m.e.p.p. frequencies in rat diaphragm increased by a factor of 100 between the 17th day in utero and the 22nd post natal day. To date no investigations have been performed on the development of neuromuscular transmission in fast twitch and slow twitch muscles of the rat.

4. Effects of dystrophy on m.e.p.p. frequency

It is somewhat unfortunate that direct comparisons of the results reported here cannot be made with other investigations. As previously stated the choice of muscle as well as the variables directly influencing m.e.p.p. frequency are important considerations when attempting correlations.

Conrad and Glaser (1964) had previously reported a decreased m.e.p.p. frequency in dystrophic (dy) mouse diaphragm. The results, however are difficult to interpret for at least two reasons. First, the muscle used was the diaphragm; a preparation that is the most preferred for this type of recording but which is relatively unaffected by the disease process until the later stages. Secondly, although their experiments were conducted at room temperature (20-23 °C), the frequencies recorded were rather high (mean-11/sec.). This suggests that their preparation may have been slightly depolarized. McComas and Mossawy (1965) using temperatures between 28 and 36°C reported m.e.p.p. frequencies in the same range as reported in our study. It must be noted, however, that it is in the temperature range in which their recordings were made

where the most accelerated change in m.e.p.p. frequency is observed (Hubbard ,1970). They were unable to show any change in this frequency in the gracilis muscle of dystrophic mice. If the gracilis muscle was comprised of a significant population of slow contracting fibers,then according to the results presented here,no change in frequency would be expected.

One of the problems that arises consistently in attempting to interpret and correlate results is the precise relationship between histochemical muscle typing and the known physiological properties of the muscle concerned (Close,1967). Susheela et al (1968) suggested that in murine dystrophy slow (Type I) muscle fibers were relatively more severely affected than fast(Type II) muscle fibers as a result of the dystrophic process. Susheela et al (1968) based their conclusions on studies of muscle fiber diameter and some histochemical properties of muscle fibers in experimental muscles(soleus,gastrocnemius,and triceps). The biochemical experiments were carried out on pectoral and abdominal muscles representing fast and slow muscles respectively. As Harris (1971) points out,there are two major difficulties in interpretation of these results. All evidence of posture and movement in the dystrophic animal suggests that the pectoral muscle groups are hardly involved in the disease. Secondly, the muscles chosen by Susheela et al (1968) for biochemical analysis are not commonly used in muscle physiology. It may be that there are considerable differences between the muscles of the abdomen and pectoral girdle and the hind limb muscles.

5. Explanation of the observed change in m.e.p.p. frequency

Several factors may be responsible for the reduced frequency observed in dystrophic EDL. The muscles are certainly in a chronic state of disuse in the affected animals. This however can probably be discounted as a possible factor since, at least in the soleus Robbins and Fischbach (1971) have shown that immobilization of the lower limb had no effect on m.e.p.p. frequency. One possible explanation may lie in the finding of an altered ultrastructure of the endplates in dystrophic animals. As mentioned earlier, a reduction in the number and size of synaptic vesicles as well as the number and complexity of post junctional folds has been seen in the dytrophic mouse (dy) (Ragab,1971;Patcher et al,1973). Gilbert et al (1973) have reported similar findings in the dy^{2J} strain; however the majority of endplates examined in dystrophic animals were from muscle fibers which were of normal diameter and contained no obvious degenerative features. Ragab (1971) also observed alterations in the ultrastructure of presynaptic terminals on apparently healthy fibers within a dystrophic muscle. He concluded that the neural defect was a primary one. The same conclusion cannot be made from the results from our study since the reduction in m.e.p.p. frequency occured at a time when the dystrophic symptoms were already quite manifest. This suggests that the dystrophic factor is not associated with the synaptic vesicle or the release process. This does not however rule out the possibility of a neurotrophic defect in murine dystrophy. Because of the altered rates of axoplasmic flow reported in dystrophic mice (Bradley and Jaros,1973;Komiya and Austin,1974) a

decreased rate of delivery of a trophic influence from the nerve to the muscle may be present. The first effect might easily be morphological changes at the endplate. The reduction in the number of vesicles and the altered postsynaptic structure could be reflected in the decreased frequency of m.e.p.p.s.

Gilbert et al (1973) described the abnormalities seen in the ultrastructure of dystrophic mouse muscle (dy^{2J}) as primary at the endplate and not secondary to diseased nerve or muscle since no abnormalities were found either postsynaptically or in the axon with its myelin up to its termination. The dying back type of neuropathy suggested by Bradley (1973) would seem a plausible explanation of the observations described in our study.

The postulated difference in sensitivity beteween fast and slow muscles may be explained by the observations of a difference in the size and structure of the endplates in the various fiber types. For example Coers (1955) and Dias (1974) reported that the rabbit soleus (slow) muscle contained motor endplates which had mean surface areas significantly larger than those in the gastrocnemius and flexor digitorum longus muscles which are chiefly fast muscles. However, ultastructural studies on the mouse, (Ogata, 1964) rat, (Padykula and Gauthier, 1967) and man (Murata and Ogata, 1969) indicate at least qualitatively that the motor endplates of the slow red fiber are smaller than those of the fast twitch fibers. It would have been expected that small endplate regions are more susceptible to changes in the synaptic contact at neuromuscular junctions than large endplates.

These investigations then make it difficult to explain the observed reduction in m.e.p.p. frequency that occured in the EDL muscle group, as a result of diminished contact with the sarcolemal membrane. More morphological data is needed on endplate ultrastructure from specific muscle fiber types with their correlative physiological data. This should be most useful on very young mice before the signs of dystrophy are apparent.

6. Conclusions and future directions

Many investigators (McComas and Mrozek,1967; McComas et al,1971b; Harris and Wilson,1971) have suggested that the functional denervation, evident in muscular dystrophy,results from a defect in the motorneuon and that this defect is primary in murine dystrophy. Law and Atwood (1972) have cautioned against the drawing of comparisons between the functional denervation in dystrophic mouse muscle and surgical denervation.

Although we did not make any actual measurements of m.e.p.p. frequencies in dystrophic mice of 10-20 days it seems rather unlikely that the frequency would differ from normal animals at this age, then assume normal values for the next three age groups observed, finally re-attaining the dystrophic condition in the animals of 100 days. We therefore believe that the changes observed in neuromuscular transmission are secondary to the clinical expression of the disease. The absence of changes in frequency of m.e.p.ps at the SOL end plate may support the claims of a preferred involvement of fast muscles.

A reappraisal of the evidence implicating a primary neurogenic component in the etiology of muscular dystrophy is needed in view of the finding that the etiology in the two strains of dystrophic mice may be

different. Parsons (1974) reported that the work done on both murine models (i.e. dy/dy and dy^{2J}/dy^{2J} strains) has been done on the false assumption that allelic mutant genes have the same phenotypic expressions. Parsons' (1974) findings suggest that the primary lesion of the dy/dy dystrophic mutant lies in the muscle cell. The chimaera mouse study by Peterson 1974) concluded that the cause of murine muscular dystrophy in the dy^{2J} strain lay outside of the skeletal muscle cell. Thus the murine dystrophic models may in fact resemble different forms of human myopathies with different cuasative factors.

The muscle transplantation experiments (Salafsky,1971; Hironaka and Miyata, 1973) have been considered important evidence in favor of the neurogenic hypothesis. The major criticism expressed about these experiments has been the uncertainty as to which muscle actually regenerates, donor or host? Neerunjun and Dubowitz (1975) have recently answered this question, using radioactive markers. They have evidence which indicates that the transplanted muscles were formed from muscle cells derived from within the donor tissue. The trophic influence of the nerve has been suggested as being responsible for determining the state of the regenerated transplanted muscle (Salafsky,1971); Neerunjun and Dubowitz,1975).

Whether the changes observed in murine dystrophy are due to a primary lesion in the motornerve axon, or to alterations at the end plate caused by a primary myopathy, a definite etiology can not as yet be resolved. A careful consideration of the neurogenic postulate warrants continued investigation with early developmental studies on clearly defined models.

SUMMARY

1. The mean frequencies of miniature end plate potentials for both normal and dystrophic muscles tended to increase with age.

2. In the normal animal the frequencies in the EDL and soleus muscles are very similar soon after birth. The frequency continues to increase in the EDL to reach a level of 19.12/sec. in 100 days while the frequency in the soleus seems to reach an adult level of 12.36/sec. much earlier (20-30 days).

3. Dystrophy does not affect the frequency in the soleus muscle but in the adult EDL (>100 days) the value is significantly reduced when compared to its control counterpart.

4. Since the effect occurs at a time when the clinical manifestations of the disease are already evident, it is postulated that the changes are a secondary phenomenon.

5. No significant difference in mean resting membrane potential (mean RMP) was observed when the youngest age groups for EDL and soleus muscles were compared. Thereafter, the mean RMP for normal soleus was significantly lower than EDL values.

6. When the dystrophic EDL and soleus values for mean RMP were compared, no significant difference was observed in the older age groups.

7. Mean RMP values for dystrophic EDL (>100 days) were significantly lower when compared with the normal EDL of the same age.

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