J. Physiol. (1979), **296**, *pp.* 245–265
With 2 plates and 9 text-figures
Printed in Great Britain

THE RELATIONSHIP BETWEEN END-PLATE SIZE AND TRANSMITTER RELEASE IN NORMAL AND DYSTROPHIC MUSCLES OF THE MOUSE

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(*Received 25 July 1978*)

SUMMARY

1. The morphology of nerve terminals and sub-neural apparatuses was examined in the muscles of normal and dystrophic adult mice of the Bar Harbor 129 ReJ strain. Nerve terminals were larger in dystrophic muscles than in normal muscles and nerve terminal sprouting was evident in about 50% of the dystrophic muscle fibres. End-plate area was positively correlated with muscle fibre diameter in both normal and dystrophic muscles.

2. Polyneuronal innervation was found in only 1% of dystrophic muscle fibres impaled with micro-electrodes.

3. Miniature end-plate potential amplitude was positively correlated with muscle fibre input resistance in both normal and dystrophic muscles. There was however, a greater than normal variation in the amplitudes of m.e.p.p.s recorded from individual dystrophic muscle fibres.

4. Quantum contents of end-plate potentials were estimated in normal and dystrophic mouse nerve–muscle preparations partially blocked with D-tubocurarine. The quantum content of e.p.p.s seemed to be related to muscle fibre diameter, and in dystrophic muscles the characteristics of evoked release were indistinguishable from normal.

5. It was concluded that the nerve terminal sprouting and the expansion of end-plate area which were observed in dystrophic muscles are not a consequence of any form of denervation, but represent an attempt by the axon to expand the area of synaptic contact in hypertrophied muscle fibres.

INTRODUCTION

There are a number of mutant strains of mice in which affected individuals display abnormalities of muscle, the peripheral nervous system and the neuromuscular junction (Sidman, Cowen & Eicher, 1979; Duchen, 1979). The dystrophic mouse (Michelson, Russell & Harman, 1955) exhibits characteristic abnormalities of muscle including a wide variation in muscle fibre diameter, reduced numbers of muscle fibres, central nucleation of fibres, longitudinal fibre splitting and a proliferation of fat and

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0022-3751/79/5570-0631 $01.50

connective tissue (West & Murphy, 1960; Rowe & Goldspink, 1969*a*, *b*; Isaacs, Bradley & Henderson, 1973). Abnormalities of the peripheral nervous system include amyelination of axons in dorsal and ventral spinal roots and a reduced axonal conduction velocity in those regions (Bradley & Jenkison, 1973; Stirling, 1975; Huizar, Kuno & Miyata, 1975; Bisco, Headley, Martin & Stirling, 1977). In addition Schwann cells in the peripheral nerves lack basement membrane (Madrid, Jaros, Cullen & Bradley, 1975) and axons are often poorly myelinated and have elongated nodes of Ranvier (Jaros, 1977). Morphological abnormalities of the neuromuscular junction that have been described include collateral sprouting of motor nerve terminals (Harman, Tassoni, Curtis & Hollinshead, 1963) a decreased density of presynaptic vesicles (Ragab, 1971), a decreased activity of end-plate cholinesterase (Glaser & Seashore, 1967) and poor organization of post-synaptic folds (Rash, Ellisman, Staehelin & Porter, 1974).

The possibility that some form of denervation plays a role in the expression of murine muscular dystrophy has been the subject of considerable debate (see Harris & Ribchester, 1979*a*). However, direct studies of neuromuscular transmission in the disease have concentrated either on transmitter release in the presence of high concentrations of Mg^{2+} (Carbonetto, 1977) or on pharmacological aspects of transmission (Harris & Ribchester, 1979*b*).

In this paper we describe attempts to relate our findings on the morphology of motor end-plates to the release of transmitter in unblocked and curarized nerve–muscle preparations.

METHODS

Male and female dystrophic mice and their clinically normal litter-mates of the Bar Harbor 129 ReJ strain were bred in this laboratory and fed on Oxoid breeding diet, supplemented by wheat germ and dried milk. Experiments were performed on nineteen dystrophic animals and fifteen of their clinically normal litter-mates aged between 3 and 6 months. The mice were killed by dislocation of the cervical vertebrae and the left hemidiaphragm, extensor digitorum longus and soleus muscles were removed.

Nerve terminal staining. A combined zinc iodide and osmium tetroxide method was used to stain motor nerve terminals (Akert & Sandri, 1968). Zinc iodide solution was prepared freshly on each occasion by reacting 1·5 g powdered zinc with 0·5 g iodine in 20 ml. water and collecting the filtrate. This solution was mixed with an aqueous 2 % solution of osmium tetroxide in the ratio 4:1 (v/v). Isolated muscles were incubated in this solution for about 5 h, washed several times in distilled water, teased into large bundles of fibres and stored overnight in glycerol. Small bundles (one to ten fibres) were teased from the larger bundles and mounted on glass slides. The length of motor nerve terminals in the long axis of the muscle fibres was measured at a magnification of 400 × using an eye-piece micrometer. In addition, end-plates were subjectively assigned to categories based on criteria suggested by Tuffery (1971). An end-plate was classified T1 if a muscle fibre was innervated by a single axon which gave rise to a single terminal arborization (see Pl. 1). Three categories of nerve terminal sprouting were recognized: preterminal sprouting (T2 terminals), where sprouts arose as pre-terminal bifurcations of the axon; ultraterminal sprouting, where sprouts arose from the terminal arborizations of the axon; and collateral sprouting, where sprouts arising either from terminal arborizations or from axonal bifurcations extended over and apparently formed synapses with adjacent muscle fibres. Where unequivocal evidence of multiple innervation of muscle fibres was encountered, terminals were designated as multi-terminal innervation.

Cholinesterase staining. The sub-neural apparatus was visualized using a modification of the direct-colouring method for cholinesterase described by Karnovsky & Roots (1964) Muscles were fixed for 4–6 hr in cold (4 °C) formol calcium (10 % formalin; 1 % $CaCl_2$) and teased into

large bundles of fibres. These bundles were then rinsed in distilled water (5 min) and incubated in a solution of the following composition (mM): S-acetylthiocholine iodide, 1·73; acetic acid, 30; sodium acetate 150; sodium citrate, 30; copper(II) sulphate, 3; potassium ferricyanide, 5. Muscles were incubated in this solution for 8–10 min, rinsed in distilled water (5 min) and stored overnight in glycerol. Single fibres were teased from the larger bundles and mounted in glycerol on glass slides. Slight adjustments of the cover-slip position brought about rotation of a fibre so that its sub-neural apparatus could be viewed in one focal plane. Selected fibres were photographed using a Zeiss photomicroscope and from × 800 photographic prints the muscle fibre diameters and total areas of end-plates were measured. Muscle fibre diameter was taken as the width of a teased muscle fibre perpendicular to its long axis in the region of the end-plate. Estimates of the total area of end-plates were obtained by drawing the ellipse of minimum area containing all the area stained for cholinesterase, onto a sheet of transparent graph placed over the photographic print. The total area was calculated from the number of circumscribed 1 mm squares.

In addition to these measurements end-plates were assigned subjectively to one of three categories: *simple*, comprising end-plates showing an unbroken profile of the sub-neural apparatus, no matter how convoluted the shape; *fragmented*, comprising end-plates where the sub-neural apparatus was broken up into a number of discrete islands of cholinesterase staining spread over the surface of the muscle fibre membrane; *multiplex* (duplex, triplex, etc.), comprising end-plates where more than one well differentiated plaque of cholinesterase activity was apparent on a muscle fibre (see Pl. 2).

Physiological recordings. The techniques and instrumentation used for recording the various electrical properties of the muscle fibres have been described in full elsewhere (see Fewings, Harris, Johnson & Bradley, 1977; Allan, Gascoigne, Ludlow & Smith, 1977). It has been demonstrated unequivocally that the techniques do sample grossly abnormal muscle fibres in dystrophic muscles of the mouse (Harris & Ribchester, 1978). All measurements were made at room temperature in a bathing solution of the following composition (mM): K^+, 5·0; Na^+, 150; Ca^{2+}, 2·0; Mg^{2+}, 1·0; Cl, 148; $H_2PO_4^-$, 1·0; HCO_3^-, 12·0; D-glucose, 11·0. Solutions were equilibrated by bubbling with 95 % O_2/5 % CO_2.

Polyneuronal innervation. Evidence of polyneuronal innervation of dystrophic muscle fibres was sought by grading the stimulus applied to nerve–muscle preparations whilst recording intracellularly at the end-plate (Redfern, 1970). Cut muscle fibre preparations (Barstad, 1962) of hemidiaphragm, extensor digitorum longus, and soleus muscles were used so that end-plate potentials (e.p.p.s) could be recorded in the absence of muscle fibre action potentials. The nerve was stimulated at 0·1–1 Hz using a suction electrode and e.p.p.s were recorded using micro-electrodes filled with 3 M-KCl together with standard intracellular recording methods. The stimulating voltage was progressively increased from zero to maximum (100 V nominal) using both polarities in order to determine whether e.p.p.s of discrete amplitudes and/or latencies could be recruited.

Miniature end-plate potentials (m.e.p.p.s). Miniature end-plate potentials were recorded in a second series of experiments on isolated extensor digitorum longus muscles. Recording was considered focal when m.e.p.p.s with a rise time of less than 1·1 msec were observed. Records were stored on magnetic tape. All recordings were made from fibres in which the membrane potentials had been hyperpolarized to − 90 mV at the end-plate region by passing inward direct current through a second micro-electrode inserted into the same fibre less than 100 μm from the recording electrode. This procedure made it possible to make a direct comparison of m.e.p.p. amplitudes from fibre to fibre since correction for variations in membrane potential was unnecessary; a secondary benefit was that the signal to noise ratio was improved. After recording m.e.p.p.s for 45–75 sec the input resistance of the fibre was estimated from the change in membrane potential induced by passing a 30 msec, rectangular inward (hyperpolarizing) current pulse of 40–80 nA.

End-plate potentials. In a third series of experiments e.p.p.s were recorded in preparations partially blocked with D-tubocurarine at a concentration (0·5–1·0 μM) that would just prevent muscle twitches when the nerve stimulated at 3 Hz. E.p.p.s with rise times less than 1·1 msec were recorded onto moving film. Twenty e.p.p.s were recorded at a nerve stimulation frequency of 3 Hz and 40–80 e.p.p.s at 30 Hz (Fig. 8). At the end of the recording, a current-passing electrode was inserted, and the input resistance of the fibre was measured. In some fibres direct

action potentials were then evoked by passing an outward (depolarizing) current pulse and the threshold for action potential generation was measured. E.p.p. amplitudes were corrected to a membrane potential of -90 mV (Katz & Thesleff, 1957); they were not corrected for non-linear summation since the e.p.p.s were typically of 1–8 mV amplitude and at this level, the correction factor is negligible (see Martin, 1976). Quantum contents of e.p.p.s were calculated from the coefficient of variation of their amplitudes (del Castillo & Katz, 1954; Martin, 1955). At 3 Hz all e.p.p.s recorded were used in the calculation, but at 30 Hz only the last twenty e.p.p.s were used so that the phase of early rundown was avoided (Elmqvist & Quastel, 1965).

Calculation of the safety factor for neuromuscular transmission. In this study the safety factor for neuromuscular transmission ($\varnothing$) was defined as the depolarization (V_p) that would have been achieved by the evoked release of transmitter from the nerve terminal in the absence of an action potential mechanism, divided by the depolarization required to drive the membrane potential from the resting potential to the threshold for action potential generation (V_c):

$$\varnothing = V_p . V_c^{-1} \tag{1}$$

For any given fibre in a D-tubocurarine-blocked preparation, V_p may be predicted from the mean quantum content of the e.p.p.s (m) and an estimate of the quantum size (q) in the unblocked preparation. The latter was obtained indirectly from the input resistance (R_{in}) of the fibre in question. Recordings of m.e.p.p.s in unblocked preparations showed that their mean amplitude was related to the input resistance by:

$$q = 2{\cdot}4\, R_{in} \tag{2}$$

and this relationship held for both normal and dystrophic muscle fibres (see Fig. 6*A*). The value of q so obtained was corrected to the resting membrane potential of the fibre (Katz & Thesleff, 1957). The product of m and the estimated value q gave a value, V_p', which could be related to the safety factor parameter V_p by the correction for non-linear summation (Martin, 1955):

$$V_p = V_p' (1 + V_p'/V_0)^{-1} \tag{3}$$

where $V_0 = E_m - E_r$; the difference between the resting membrane potential (E_m) and the reversal potential (E_r). E_r was assumed to be -10 mV in all calculations.

The action potential threshold was not always measured directly, since direct action potentials were not generated in all the muscle fibres studied. In such cases it was assumed to be -56 mV (Table 4). Having obtained V_p and V_c, the safety factor for neuromuscular transmission was obtained using eqn. 1.

Statistical analysis of results. The results are presented as mean ± S.E. of mean. The significance of any difference between two means was tested using either Student's t test, or, if the variances were unequal, Welch's Test (Welch, 1937). A probability level of less than 5% was taken to indicate a significant difference between the two means. Where a correlation between two variables was sought, the correlation coefficient was calculated from the estimated covariance and the standard deviations. In cases where sample numbers were small and/or the data were not normally distributed, Kendall's coefficient of correlation was calculated.

RESULTS

Morphology

Nerve terminal staining. In normal extensor digitorum longus and soleus muscles 80–90% of nerve terminals were classified as T1 (Table 1; Pl. 1; see methods for definition). T2 endings occurred in 10–20% of fibres. These probably arose as a result of preterminal sprouting of axons (Barker & Ip, 1966). Ultraterminal sprouts were never seen in normal muscles nor was there any evidence of multiple innervation. These observations are similar to those made in the cat by Barker & Ip (1966) and Tuffery (1971).

In dystrophic muscles, the pattern was quite different. Only about 50 % of nerve terminals could be classified as T1 (see Pl. 1; Table 1). Pre-terminal sprouts (T2 end-plates) were seen in about 20 % of fibres, but the examples that were seen were far more noticeable than those in normal fibres; the pre-terminal branches were usually longer and the separations of the terminal arborizations were more pronounced (compare Pl. 1*C* with 1*B*). Approximately 30 % of nerve terminals in both dystrophic extensor digitorum longus and soleus muscles exhibited ultra-terminal

TABLE 1. Characteristics of nerve terminals in normal and dystrophic extensor digitorum longus (e.d.l.) and soleus muscles. The classification of the nerve terminals into T1, T2, etc. is described in detail in Methods. The length of the nerve terminals is quoted as mean ± S.E. of mean. The numbers of muscle fibres studied are given in parentheses. The mean length of terminals in dystrophic muscles was significantly greater than in the corresponding normal muscles ($P < 0.01$; Welch's test)

	T1 (%)	T2 (%)	Ultra-terminal sprouting (%)	Multi-terminal innervation (%)	Length (μm)
Normal e.d.l. (50)	90	10	0	0	37·9 ± 1·3
Dystrophic e.d.l. (156)	49	18·5	32	0·5	64·3 ± 3·6
Normal soleus (49)	77·5	22·5	0	0	44·2 ± 2·0
Dystrophic soleus (133)	48	19·5	31	1·5	60·2 ± 2·6

sprouting. Such sprouting was usually confined to the muscle fibre from whose nerve terminal the sprout has arisen (Pl. 1 *D–F*) although on rare occasions the sprouts spread across adjacent muscle fibres. Few collateral pre-terminal sprouts were seen although this observation should be treated with caution since the zinc iodide and osmium tetroxide method did not stain myelinated axons adequately. Only three out of 288 dystrophic muscle fibres had multi-terminal innervation indicating a probable incidence of multiple innervation of about 1 % (Pl. 1*H*).

The mean length of motor nerve terminals was significantly greater in dystrophic muscles than in corresponding normal muscles (Table 1) and there was greater variation in the individual measurements (Fig. 1). There was no correlation between the length of a nerve terminal and the presence of terminal sprouts, relatively short terminals being just as likely to possess sprouts as long terminals.

Cholinesterase staining. Most end-plates in normal muscles were characterized by a single continuous plaque of cholinesterase reaction product. Such end-plates were classified as 'simple' (Table 2; Pl. 2*A*). 'Multiplex' end-plates (see Methods) occurred in 4–9 % of normal fibres while 'fragmented' end-plates, made up of a number of small cholinesterase stained sub-units, occurred in 10–20 % of fibres (Pl. 2).

The cholinesterase reaction product conferred a thicker outline to the end-plates on normal extensor digitorum longus than on normal soleus (compare Pl. 2*A* and *B*). Since cholinesterase extends to the base of the secondary synaptic folds (Barrnett, 1962; Davis & Koelle, 1967), the difference in the staining pattern observed between these muscles probably reflects the difference in the depth of their secondary junction

folds; end-plates in normal extensor digitorum longus muscles having longer folds than those in normal soleus muscles (Duchen, 1971).

In dystrophic muscles 'simple' end-plates were less than half as common as in normal muscles (Table 2). 'Fragmented' end-plates occurred more than twice as often

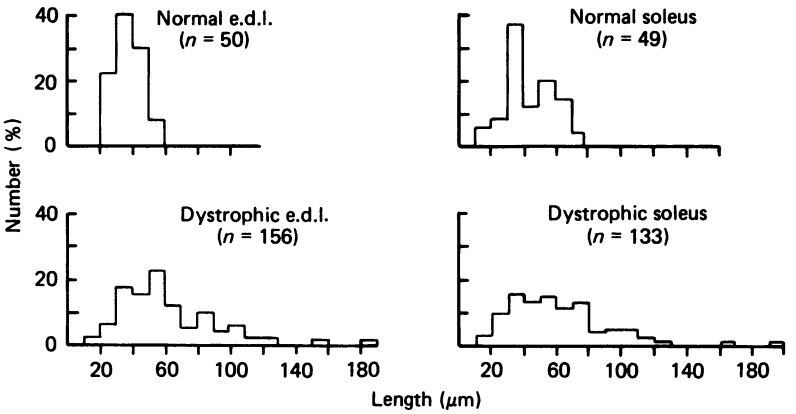

Fig. 1. Distributions of the length of motor nerve terminals in normal and dystrophic extensor digitorum longus (e.d.l.) and soleus. Measurements were made on teased preparations stained with zinc iodid/osmium tetroxide. Numbers in parentheses are numbers of fibres examined.

TABLE 2. Cholinesterase staining in normal and dystrophic extensor digitorum longus (e.d.l.) and soleus muscles. Measurements are expressed as mean ± S.E. of mean. The numbers of muscle fibres studied are given in parentheses.

	Simple (%)	Fragmented (%)	Multiplex (%)	Fibre diameter* (μm)	Total end-plate area* (μm²)
Normal e.d.l.	81·5	10	8·5	41 ± 0·9	663 ± 17·8
	(92)	(92)	(92)	(92)	(92)
Dystrophic e.d.l.	65	24	11	50 ± 2·5	1492 ± 98·5
	(100)	(100)	(100)	(84)	(84)
Normal soleus	74	22	4	30 ± 0·8	539 ± 21·3
	(96)	(96)	(96)	(96)	(96)
Dystrophic soleus	30·5	55	14·5	46 ± 1·8	1019 ± 54·1
	(111)	(111)	(111)	(108)	(108)

* Differences between corresponding normal and dystrophic muscles statistically significant; $P < 0{\cdot}01$, Welch's test. Note the greater variation in muscle fibre diameter in dystrophic muscles compared with normals.

in the dystrophic muscles than in the corresponding normal muscles and the cholinesterase 'fragments' were usually spread over a wider area (Pl. 2*C*). It was sometimes difficult to decide whether an end-plate should be assigned to the 'fragmented' or 'multiplex' categories. Examples where no such difficulty was encountered are shown in Pl. 2 (*B* and *D*).

It would have been of interest to consider the relationship of the cholinesterase staining patterns to the ZnI_2/OsO_4 staining of nerve terminals, but attempts to combine the nerve terminal and cholinesterase staining methods were unsuccessful

(see also Jansen & Van Essen, 1975). In normal muscle, the incidence of 'multiplex' cholinesterase patterns in extensor digitorum longus corresponded quite closely to that of T2 endings in ZnI_2/OsO_4-stained material (compare Tables 1 and 2). The incidence of 'multiplex' patterns in normal soleus however, was far lower than would have been expected from the observed incidence of T2 end-plates in this muscle. The

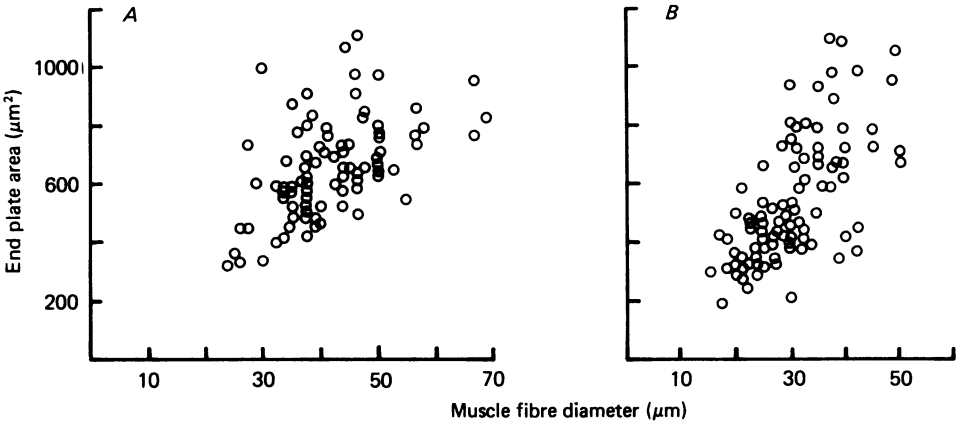

Fig. 2. Total end-plate area plotted against muscle fibre diameter in normal extensor digitorum longus (*A*) and normal soleus (*B*) muscles. Measurements were made on teased single muscle fibres stained for cholinesterase. The correlation coefficients were 0·46 (*A*) and 0·58 (*B*).

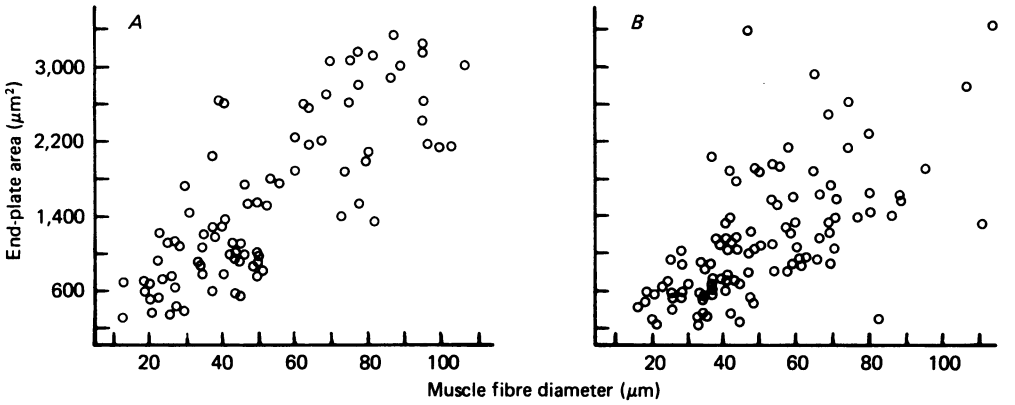

Fig. 3. Total end-plate plotted against muscle fibre diameter in dystrophic extensor digitorum longus (*A*) and dystrophic soleus (*B*) muscles. Measurements were made on teased single muscle fibres stained for cholinesterase. The correlation coefficients were 0·65 (*A*) and 0·60 (*B*).

number of 'fragmented' patterns (Table 2) was relatively high in normal soleus, and it may be that these patterns of cholinesterase staining were also associated with T2 end-plates in this muscle. In dystrophic muscles it is possible that the appearance of many 'fragmented' cholinesterase patterns might correspond to extensive pre-terminal or ultraterminal sprouting of the nerve terminals, especially where cholinesterase 'fragments' were spread over a wide area (e.g. Pl. 2*C*).

TABLE 3. End-plate potentials (e.p.p.s) were generated in normal and dystrophic extensor digitorum longus (E.d.l.), soleus or hemidiaphragm muscles using 'cut-fibre' preparations. Where appropriate, results are quoted as mean ± S.E. of mean

	E.d.l.		Soleus		Hemidiaphragm	
	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
Resting membrane potential (mV)	−23·5 ± 1·57	−22·4 ± 1·35	−28·5 ± 1·30	−22·7 ± 1·38	−26·0 ± 1·55	−22·1 ± 1·01
Fibres innervated*	47/47	68/68	82/82	56/56	54/54	88/88
Fibres polyneuronally innervated*	0/47	0/68	0/82	1/56	0/54	2/88
Amplitude of e.p.p. at 0·1 Hz (mV)	9·4 ± 1·00	9·9 ± 1·00	9·1 ± 0·75	9·6 ± 0·66	9·9 ± 0·71	10·3 ± 0·59

* Number of fibres/number of fibres tested

End-plates in the dystrophic muscles resembled those in normal soleus muscles rather than normal extensor digitorum longus in terms of the outline of the cholinesterase reaction product and this may reflect the reduction in the depths of secondary junction folds that has been reported in dystrophic muscles (Ragab, 1971; Pachter, Davidowitz & Breinin, 1973; Rash *et al.* 1974).

The total areas of end-plates and muscle fibre diameters were estimated from × 800 photographic prints of the teased fibres (Table 2). Scatter diagrams relating end-plate area to muscle fibre diameter revealed statistically significant correlations between these parameters both in normal and dystrophic muscles (Figs. 2 and 3). Positive correlations between end-plate size and muscle fibre diameter have been reported often in earlier literature (e.g., Coers & Woolf, 1959; Nystrom, 1968; Kuno, Turkanis & Weakly, 1971) but have not previously been shown for dystrophic muscles. Thus the wide variation in end-plate area seen in dystrophic muscle (Figs. 2 and 3) reflects the characteristic variation in muscle fibre diameter (Michelson *et al.* 1955; West & Murphy, 1960; Rowe & Goldspink, 1969*a*, *b*).

The mean values for fibre diameter (Table 2) were greater than those found by Rowe & Goldspink (1969*a*, *b*). The discrepancy may have arisen because of the different methods used. Our estimates were made from teased muscle fibres rather than from transverse sections. We measured the maximum fibre diameter in the region at the end-plate, thus avoiding the measurement of daughter branches of longitudinally split fibres (Isaacs *et al.* 1973) and 'short' muscle fibres, which taper dramatically before insertion at the tendon (Williams & Goldspink, 1976; Harris & Ribchester, 1978). Preliminary results indicate that about 50% of fibres in dystrophic muscle exhibit longitudinal splitting (our unpublished observations).

Physiology

Polyneuronal Innervation. Polyneuronal innervation occurs normally in neonatal mammalian muscle (Redfern, 1970) or during reinnervation of denervated adult muscle (McArdle, 1975). A high level of polyneuronal innervation in dystrophic muscle might therefore have indicated that neuromuscular junctions in dystrophic muscles were immature or that ongoing denervation and reinnervation of muscle fibres was taking place. Cut muscle fibre preparations (Barstad, 1962) were used to determine the incidence of polyneuronal innervation in adult normal and dystrophic muscles (see Methods). Out of 212 fibres sampled from twelve dystrophic muscles, complex e.p.p.s were recruited in only three muscle fibres indicating an incidence of 1–2% (Fig. 4; Table 3; compare throughout multi-terminal innervation end-plates recorded in Table 1).

Miniature end-plate potentials. Spontaneous transmitter release was examined in normal and dystrophic extensor digitorum longus preparations. Measurements were made from fibres hyperpolarized to − 90 mV (see Methods). There was no significant difference in the rise time, half-decay time or mean amplitude of m.e.p.p.s between normal and dystrophic muscle fibres (Table 4). The within-sample variance of m.e.p.p amplitudes was significantly greater in dystrophic fibres than in normal fibres (see Fig. 5). This seemed to be due to a higher frequency of 'dwarf' m.e.p.p.s (Kriebel & Gross, 1974; Kriebel, Llados & Matteson, 1976) and 'giant' m.e.p.p.s (Liley, 1957) in individual dystrophic fibres. 'Giant' m.e.p.p.s in dystrophic muscle fibres have also been reported by Carbonetto (1977).

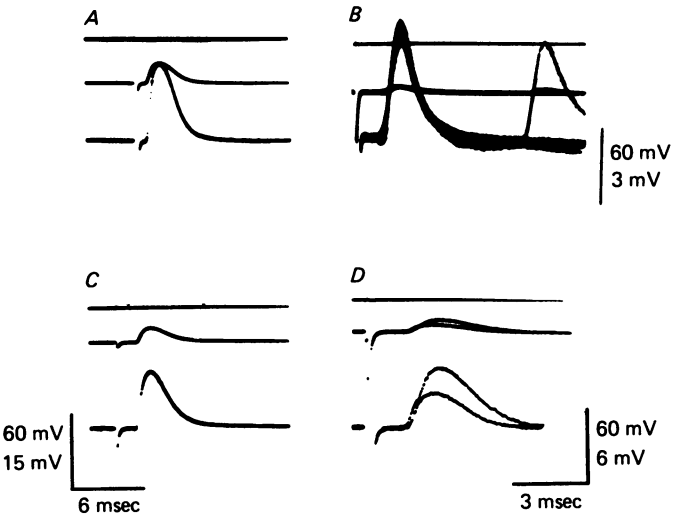

Fig. 4. End-plate potentials recorded from cut muscle fibre preparations of normal (*A*) and dystrophic (*B–D*) diaphragm muscle. Stimulus strength was progressively increased in order to recruit inputs of differing thresholds. Only two out of eighty-eight dystrophic hemidiaphragm fibres showed evidence of polyneuronal innervation, where more than one e.p.p. could be recruited by altering the stimulus strength (*B* and *D*). In all cases the upper trace is zero potential, middle trace the d.c. record and the lower trace a higher gain a.c. record.

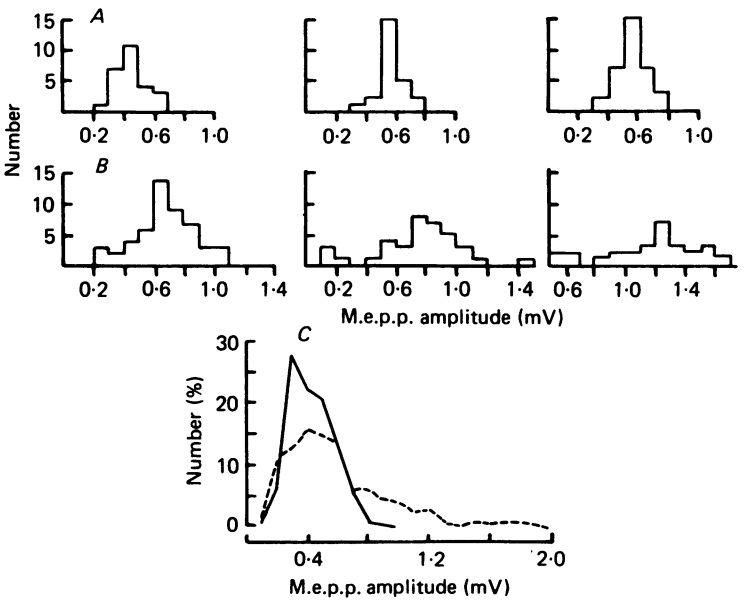

Fig. 5. Histograms of m.e.p.p. amplitude distribution from normal (*A*) and dystrophic (*B*) extensor digitorum longus muscle fibres, illustrating the wide variation in amplitudes of the latter. *C*, combined histogram of all m.e.p.p. amplitudes measured in seventeen normal (continuous line) and sixteen dystrophic (dashed line) muscle fibres. All m.e.p.p.s were recorded from fibres where the resting membrane potential was hyperpolarized to −90 mV (see Methods).

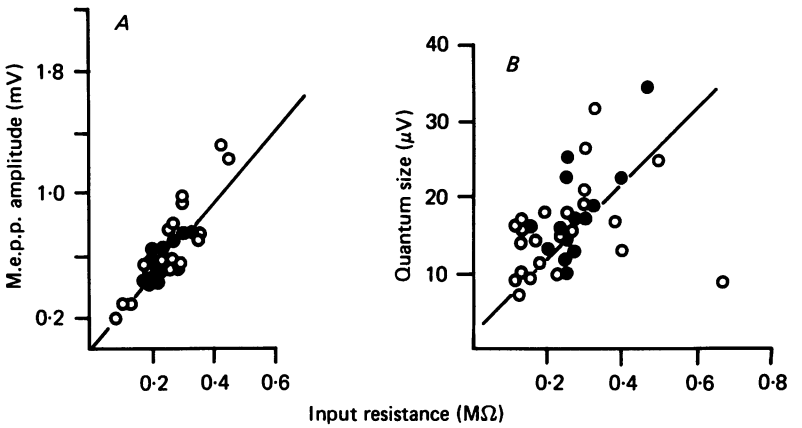

Fig. 6. *A*, amplitudes of m.e.p.p.s plotted against the input resistances of extensor digitorum longus muscle fibres. Each point was obtained from the mean amplitude of at least thirty m.e.p.p.s recorded from normal (filled circles) and dystrophic (open circles) muscle fibres in unblocked preparations. The linear least square regression line is shown. *B*, quantum sizes calculated from the coefficient of variation of e.p.p. amplitudes recorded in D-tubocurarine blocked preparations, plotted against the measured input resistance of the muscle fibres in normal and dystrophic extensor digitorum longus muscles. The linear least squares regression line is shown. The correlation coefficient was 0·54.

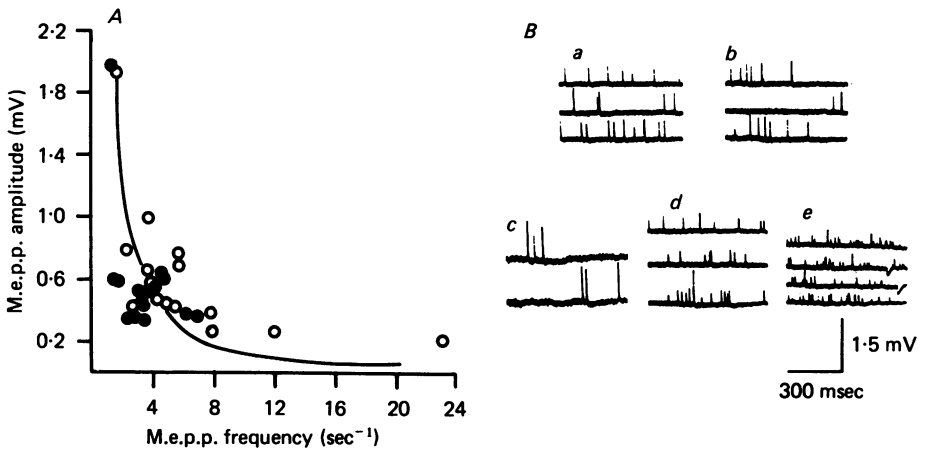

Fig. 7. *A*, amplitude of m.e.p.p.s plotted against frequency of spontaneous release for normal (filled circles) and dystrophic (open circles) muscle fibres. The points forming the peak on the ordinate are the mean values obtained from hemidiaphragm preparations (Harris & Ribchester, 1978*b*). The line is a curve of the form $q = kf^{-3/2}$ where k has an empirical value of 0·005 (see text). *B*, sample records to display the inverse correlation of m.e.p.p. amplitudes with frequency in normal (*a*, *b*) and dystrophic (*c*, *d*, *e*) extensor digitorum longus muscle fibres. The input resistances of these fibres were respectively 0·20, 0·25, 0·45, 0·25 and 0·08 MΩ. The resting membrane potentials were hyperpolarized to −90 mV while recording m.e.p.p.s (see Methods).

There was also greater than normal variation in m.e.p.p. amplitudes between dystrophic muscle fibre samples, but as originally reported by Carbonetto (1977) the variation appeared to result from a variation in the input resistance of the muscle fibres, there being a good linear correlation between mean m.e.p.p. amplitude and muscle fibre input resistance with no obvious systematic difference between normal and dystrophic points (Fig. 6*A*).

We further observed an inverse relationship between the mean amplitude and the frequency of spontaneous m.e.p.p.s (Fig. 7). Kuno *et al.* (1971) showed that m.e.p.p. frequency (f) was proportional to end-plate area. End-plate area is proportional to muscle fibre diameter in both amphibian (Kuno *et al.* 1971) and mammalian muscles (Nystrom, 1968) and also in normal and dystrophic mouse muscles (see Figs. 2 and 3). The input resistance of a muscle fibre is inversely proportional to the 3/2 power of the fibre diameter (Katz & Thesleff, 1957):

$$R_{\text{in}} = \left(\frac{R_{\text{m}}\, R_{\text{i}}}{d^3}\right)^{\frac{1}{2}}$$

Substituting q for R_{in} and f for d, it might be expected that a relationship would exist between the quantum size and the mean frequency of spontaneous transmitter release of the form:

$$q = k_1 f^{-3/2} \tag{5}$$

where the constant, k_1, depends on the cable properties R_{m} (the specific membrane resistance), R_{i} (the specific internal longitudinal resistance), and on the inter-dependence of end-plate area and muscle fibre diameter (d). A curve of the form given by eqn. 5 provided a reasonable fit to the experimental points (Fig. 7). The deviations from the predicted curve occurred where the amplitude of individual m.e.p.p.s approached the level of base-line noise (approximately 150 μV peak to peak), leading to possible overestimates of the mean quantum size, q. Again there was no obvious systematic difference between points obtained from normal and dystrophic muscle fibres respectively.

End-plate potentials. E.p.p.s were recorded from normal and dystrophic extensor digitorum longus muscles partially blocked with D-tubocurarine (Fig. 8). There were no significant differences in either the rise time or half-decay time of e.p.p.s between normal and dystrophic muscles. The quantum content of e.p.p.s calculated from the coefficient of variation of the amplitudes tended to be larger in dystrophic muscle than in normal muscle (Table 4) but the differences were not statistically significant ($P > 0{\cdot}05$; Welch Test).

The coefficient of variation of e.p.p. amplitudes provides not only an estimate of the quantum content (m) but also of the quantum size (q'; see Hubbard, Llinas & Quastel, 1969). If q' is a reasonable estimate of the quantum size in the blocked preparation, it should be proportional to the input resistance of the muscle fibres. Measurements of R_{in} and estimates of q' showed that this was the case and the results are shown in Fig. 6*B*. It is clear from the data, in agreement with the measurements made in unblocked preparations (Fig. 6*A*), that the points obtained from dystrophic preparations were representative of those drawn from the population of normal fibres.

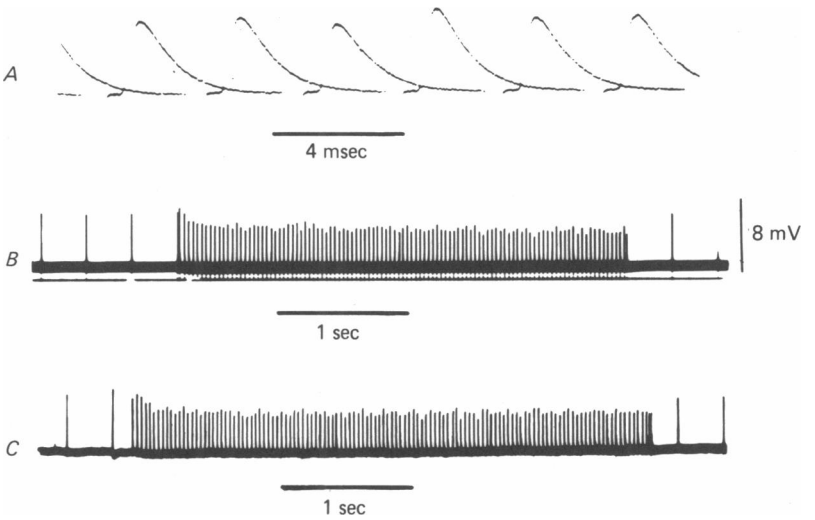

Fig. 8. Trains of e.p.p.s recorded from normal (*A* and *B*) and dystrophic (*C*) muscle fibres in preparations partially blocked with 0·5–1·2 μM-D-tubocurarine. *A*, e.p.p.s evoked at a nerve stimulation frequency of 3 Hz (moving spot on moving film); *B* and *C*, e.p.p.s evoked at 3 Hz and 30 Hz; the stimulation frequency was returned to 3 Hz after recording a train of e.p.p.s at 30 Hz.

TABLE 4. Spontaneous and evoked transmitter release in isolated normal and dystrophic extensor digitorum longus muscles. M.e.p.p.s were recorded at muscle fibre membrane potentials of −90 mV; e.p.p.s were recorded in the presence of D-tubocurarine (see Methods)

	Normal	Dystrophic
Resting membrane potential (mV)	−76·8 ± 1·12 (17)	−68·91 ± 2·11 (16)†
Input resistance (MΩ)	0·26 ± 0·01 (36)	0·23 ± 0·02 (50)
M.e.p.p. amplitude (mV)	0·56 ± 0·02 (17)	0·64 ± 0·05 (16)
Within fibre variance	0·009 (17)	0·053 (16)*
M.e.p.p. frequency (sec^{-1})	3·6 ± 0·37 (17)	5·88 ± 1·28 (16)
M.e.p.p. rise time (msec)	0·76 ± 0·05 (16)	0·74 ± 0·05 (16)
M.e.p.p. time to half-decay (msec)	1·72 ± 0·11 (16)	1·67 ± 0·06 (16)
E.p.p. rise time (msec)	0·84 ± 0·04 (19)	0·86 ± 0·04 (34)
E.p.p. half-decay (msec)	1·67 ± 0·08 (19)	1·65 ± 0·16 (34)
Calculated quantum size (mV)	0·018 ± 0·004 (14)	0·010 ± 0·001 (21)
Mean quantum content		
3 Hz	306 ± 22·5 (14)	345 ± 29·5 (21)
30 Hz	184 ± 15·8 (14)	216 ± 16·8 (21)
Action potential threshold (mV)	−56·4 ± 1·04 (14)	−56·7 ± 1·98 (17)
Safety factor		
3 Hz	2·81 ± 0·62 (14)	4·84 ± 0·65 (21)†
30 Hz	2·44 ± 0·14 (14)	3·81 ± 0·58 (21)†

Figures in parentheses represent number of fibres examined. *$P < 0.01$, F-test †$P < 0.01$, t-test.

It has been shown by Kuno *et al.* (1971) that in amphibian muscle, the mean quantum content of e.p.p.s is linearly related to both end-plate area and to the frequency (f) of spontaneous m.e.p.p.s. That is, m is proportional to f. In view of the relationship between q' and $R_{\ln}$ (Fig. 6*B*) and between q and f (Fig. 7), it seemed

reasonable to anticipate a relationship between the estimated mean quantum content of e.p.p.s and the estimated quantum size of the form:

$$m = k_2 q'^{-2/3} \tag{6}$$

where k_2 is a constant dependent on k_1 (eqn. 5), the degree of neuromuscular block and the frequency of nerve stimulation. The relationship between m and q' at nerve

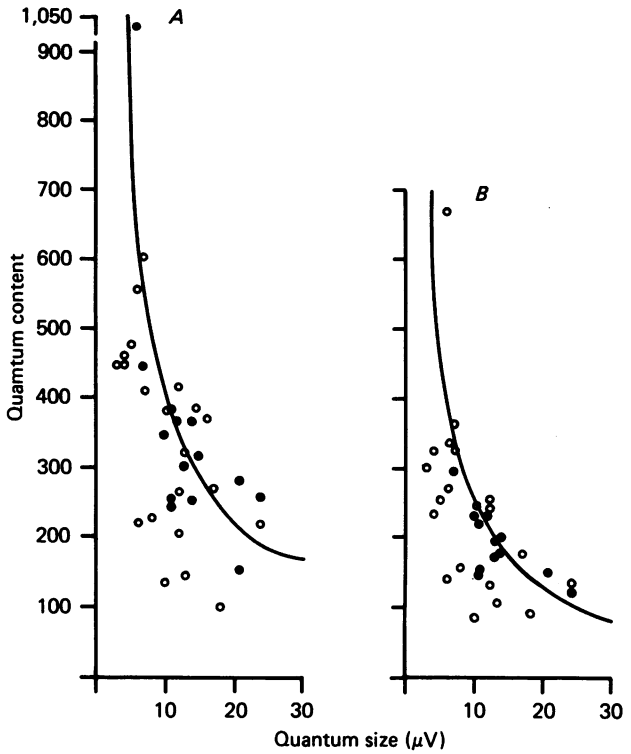

Fig. 9. Mean quantum content of e.p.p.s recorded from normal (filled circles) and dystrophic (open circles) muscle fibres at nerve stimulation frequencies of 3 Hz (*A*) and 30 Hz (*B*) plotted against calculated quantum size. Both estimates were made from the amplitude and variance of e.p.p. amplitudes in D-tubocurarine blocked preparations. Lines are curves of the form $m = kq^{-2/3}$ where k was chosen empirically.

stimulation frequencies of 3 Hz and 30 Hz are shown in Fig. 9. In both cases simple power curves of the form:

$$m = k_2' q^{-1/2} \tag{7}$$

fitted the data with the highest correlation coefficients, but tests for goodness-of-fit of the data to curves of the form described by eqn. (6) were statistically acceptable ($0{\cdot}95 > P > 0{\cdot}05$; χ^2 test). It is once more apparent (compare Fig. 8) that points obtained from dystrophic muscle fibres were consistent with those obtained from a population of normal fibres.

Since quantum size is proportional to R_{in} (see Fig. 6*A*) and in view of the relationship between m and q (see Fig. 9), one would expect m to be proportional to R_{in}. This

was indeed the case. In dystrophic preparations ($n = 21$) $r = 0{\cdot}33$ at stimulus frequencies of both 3 Hz and 30 Hz. In normal preparations ($n = 14$) $r = 0{\cdot}49$ at 3 Hz and 0·46 at 30 Hz. In all cases these correlation coefficients (Kendall's rank test) were significant at the 5% level.

Safety factor. The safety factor for neuromuscular transmission is subject to many constraints. In particular it should be noted that the safety factor depends not only on the amount of transmitter released by a nerve terminal and the density of post-synaptic receptors, but also on the cable properties of the muscle fibre and the values of the resting membrane potential, action potential threshold and the transmitter null potential (eqns. 1–4). The mean safety factors for neuromuscular transmission calculated for dystrophic muscle fibres (4·84 at 3 Hz, 3·81 at 30 Hz) were significantly greater than those for normal fibres (2·81 at 3 Hz, 2·44 at 30 Hz). The cable properties and quantum contents of e.p.p.s of dystrophic fibres did not differ statistically from normal (Table 4); the differences in safety factor were due to the lower resting membrane potential of dystrophic muscle fibres. Dystrophic muscle fibres typically had resting membrane potentials 6–10 mV less negative than normal fibres, while the threshold for action potential generation was not different from normal (Table 4, see also Harris, 1971; Harris & Marshall, 1973). A relatively high safety factor is one possible explanation for an early observation that dystrophic muscles from the hind limbs were more resistant to fatigue and more resistant to competitive neuromuscular blocking agents than normal muscles (Baker, Wilson, Oldendorf & Blahd, 1960).

DISCUSSION

The observations reported in this communication relate to the morphology and physiology of neuromuscular transmission in the skeletal muscle of the dystrophic mouse. The investigation has three component parts, concerning axonal sprouting, cholinesterase staining of the sub-synaptic region, and aspects of spontaneous and evoked transmitter release respectively. We shall discuss each of these parts in turn.

Axonal sprouting

Axonal sprouting can be of two basic kinds: pre-terminal sprouting, where fine outgrowths arise from the nodes of Ranvier of the pre-terminal axons, and ultra-terminal sprouting, where the outgrowths arise from the terminal arborizations of the 'parent' axon. It has been suggested that pre-terminal sprouting is mediated directly or indirectly by the products of nerve degeneration or by proliferating Schwann cells (Brown & Ironton, 1978*a*) and that ultra-terminal sprouting is due to more subtle changes in the micro-environment of the nerve terminal (Pestronk & Drachman, 1978; Brown & Ironton, 1978*b*). It is of relevance to this distinction that in functionally denervated muscles, where there is no major structural damage to the nerve terminal, only ultra-terminal sprouting is seen (Duchen, 1970; Duchen & Tonge, 1973), whereas in partially denervated muscles both forms of sprouting are evident (Brown & Ironton, 1978*a*).

In dystrophic muscles, both pre- and ultra-terminal sprouting was commonly observed. In view of the controversy surrounding the possible role of 'functional denervation' in murine dystrophy (see Harris & Ribchester, 1979*a*) it is pertinent to

ask whether or not the sprouting can be explained as a reaction to some form of denervation or inactivity.

There is some evidence to suggest that the number of axons in a dystrophic peripheral nerve is reduced (Harris, Wallace & Wing, 1972). However, the loss of axons appears to take place before the first 3 or 4 weeks of age (Montgomery & Swenarchuk, 1978), there is little or no structural evidence of axonal degeneration in the nerves of adult animals (Jaros & Bradley, 1979), and there is virtually no pharmacological evidence of denervated muscle fibres in dystrophic muscles (see Harris & Ribchester, 1979*a*). Thus, if the pre-terminal sprouting had occurred in response to axonal degeneration or the activation of Schwann cells, it would be necessary to suggest that it occurred very early in the life of the animal. Moreover, we could find little evidence of extensive collateral sprouting (by which we mean innervation of adjacent muscle fibres by axonal sprouts), a feature one might expect to see in a muscle that is undergoing or has undergone progressive denervation.

It also seems unlikely that sprouting occurs in response to conduction failure in the axons since in spite of all the documented abnormalities seen in dystrophic nerves, axonal conduction, though slow, is not blocked (Huizar *et al.* 1975; Biscoe *et al.* 1977; Rasminsky & Kearney, 1976). It is possible, of course, that the sprouting occurred at a very early stage (that is, before 3 weeks *post partum*) in the life of the animal when 'denervation' or conduction failure may have been present, and that the sprouts (both pre- and ultra-terminal) persisted into adulthood and we have no direct evidence for or against such a possibility. More importantly perhaps, we do not know for certain that the sprouts are functionally active.

To summarize, our data on axonal sprouting showed that in dystrophic muscles, in which there is little evidence of either axonal degeneration or muscle fibre denervation, 50% of all terminal regions of axons exhibit sprouting, and that most of the sprouts arise ultraterminally, thus contributing to an over-all enlargement of the motor-nerve terminal.

Cholinesterase staining

The most striking observation pertaining to the organisation of the sub-neural apparatus was the fragmentation of cholinesterase stained regions into small sub-units. It seems not unreasonable to assume that the cholinesterase-stained regions of a muscle fibre represent the area of functional synaptic contact, and that the fragmented staining pattern reflects the extensive sprouting seen in the nerve terminals.

The area of the muscle fibre stained with cholinesterase was linearly related to the diameter of the muscle fibre in both normal and dystrophic muscle fibres. This relationship has been reported often in the case of normal muscle fibres (see for example, Coers & Woolf, 1959; Nystrom, 1968; Kuno *et al.* 1971) but not before in dystrophic muscles. It is believed (see Kuno *et al.* 1971 for example) that the increase in area of the end-plate region represents an attempt by the neuromuscular apparatus to increase the efficiency of neuromuscular transmission by expanding the area of synaptic contact as a muscle fibre enlarges. If this is so, our results would imply that even in the largest (hypertrophied) muscle fibres in dystrophic mouse muscles, the neural apparatus is behaving as if normal. Thus we would interpret our morpho-

logical findings as indicating that the area of synaptic contact expands as a muscle fibre enlarges, and in the dystrophic muscles, this expansion involves not simply an increase in terminal varicosities in a given end-plate, as occurs in developing muscle fibres (Nystrom, 1968), but the provision of axonal sprouts. This would seem a reasonable view, since Schitikov (1957) has reported axonal sprouting in muscle fibres forced to undergo work-induced hypertrophy.

Transmitter release

M.e.p.p.s recorded at dystrophic nerve–muscle junctions have been said to be of normal amplitude (Conrad & Glaser, 1964) and reduced amplitude (McComas & Mossawy, 1965). Carbonetto (1977) showed quite clearly that these discrepant findings probably arose as a result of differences in muscle fibre input resistance, a variable not considered in any earlier work, and this was confirmed in our own investigation. Since the transmitter null-potential does not differ from normal in dystrophic muscle fibres (Harris & Ribchester, 1979*b*), and since the variation of m.e.p.p. amplitude in dystrophic muscle fibres has been shown by both Carbonetto (1977) and ourselves to be dictated solely by muscle fibre input resistance, it would seem unlikely that there is any major change in post-synaptic sensitivity to transmitter in dystrophic muscles.

The frequency of m.e.p.p.s in dystrophic muscle fibres has been said to be normal (McComas & Mossawy, 1965) less than normal (Conrad & Glaser, 1964) and normal until later stages of the disease (Curran & Parry, 1975). Our own experiments may offer an explanation for these discrepant reports. We demonstrated that m.e.p.p. frequency was inversely related to m.e.p.p. amplitude. Since m.e.p.p. amplitude is proportional to muscle fibre input resistance, it would seem probable that larger diameter fibres (that is, those fibres with low input resistance) will exhibit frequent small m.e.p.p.s, and smaller diameter fibres will exhibit infrequent large m.e.p.p.s. Thus, large variations in m.e.p.p. frequency in dystrophic fibres should be expected as a result the variability of muscle fibre diameter seen in dystrophic muscles.

Kuno *et al.* (1971) showed that increases in the size of end-plates resulted in increases in the quantum content of the end-plate potential. This observation was interpreted in terms of an increase in the number of transmitter release sites. Our results indicate a similar effect in both normal and dystrophic mouse muscles. The quantum content (m) of e.p.p.s generated at both 3 Hz and 30 Hz was inversely related to quantum size (q). Carbonetto (1977), working on Mg^{2+} blocked preparations (Mg^{2+} 15 mM; Ca^{2+} 3 mM), claimed that the quantum content of e.p.p.s in dystrophic muscle was independent of muscle fibre diameter; according to our results showing the relationship between m and q, one might have expected the quantum content of end-plate potentials to be proportional to fibre diameter (or input resistance), and indeed, there was a significant correlation at the 5% level between quantum content at 3 Hz and 30 Hz and input resistance in both the dystrophic and normal preparations. The disparity between Carbonetto's (1977) results and our own probably lies in the fact that in 15 mM-Mg^{2+}, mean quantal content was reduced to about 11 (i.e. $< 5\%$ of normal) which would have resulted in considerable loss of sensitivity.

The increase in transmitter output in the large muscle fibres may be seen as an attempt by the neuromuscular system in dystrophic mouse muscle to retain the

normal safety factor for transmission. If this is so, then the features governing such an attempt do not include the actual 'safety factor' itself, because the low resting membrane potential of dystrophic muscle fibres, coupled with the changes in the quantum content of the end-plate potential, result in an *increase* in over-all safety factor for neuromuscular transmission.

Our physiological findings may be summarized as follows. There was considerable variation in muscle fibre diameter in dystrophic muscles. In the larger muscle fibres (i.e. those with lower input resistance) there was a decrease in the amplitude of m.e.p.p.s and a corresponding increase in m.e.p.p. frequency. The quantum content of end-plate potentials was increased in the larger fibres. In all respects, transmitter release was indistinguishable from normal, provided account was taken of the greater variation in muscle fibre diameter.

Our data on the morphology of the neuromuscular apparatus in dystrophic muscles suggested that as a muscle fibre hypertrophies, the area of synaptic contact increases. The increase in the area of synaptic contact allows a greater number of release sites, which is seen as an increase in m.e.p.p. frequency and an increase in the quantum content of e.p.p.s in the larger fibres. Thus, in dystrophic muscles, nerve terminal sprouting may be regarded as a successful attempt by motor axons to respond to changes in the morphological and physical characteristics of the dystrophic fibres. Why the enlargment of the area of synaptic contact in the dystrophic fibres should occur by axonal sprouting rather than by a simple increase in the number of terminal varicosities is not clear, but it may be related either to the extent or to the rapidity of muscle fibre hypertrophy in dystrophic muscles.

We would like to thank Drs W. J. Betz, J. H. Caldwell and Professor A. R. Martin for their comments on earlier drafts of the manuscript, and Ms Margaret Grimes and Mr George Tarver for assistance in the preparation of the Figures. This work was supported by grants from the Muscular Dystrophy Association of America, the Muscular Dystrophy Group of Great Britain and the M.R.C. R.R.R. was in receipt of an M.R.C. Studentship.

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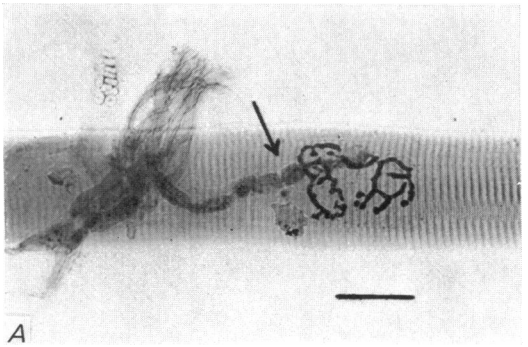

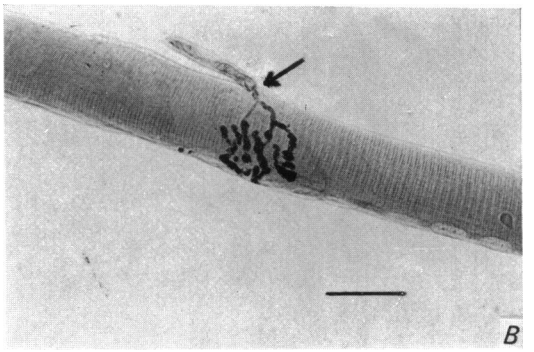

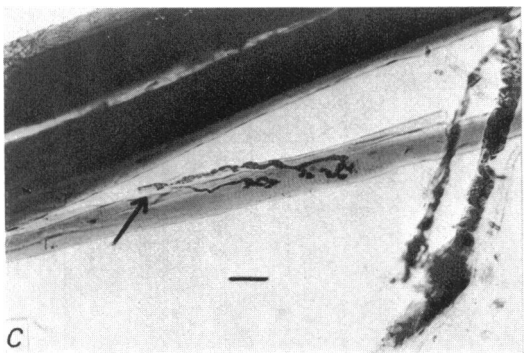

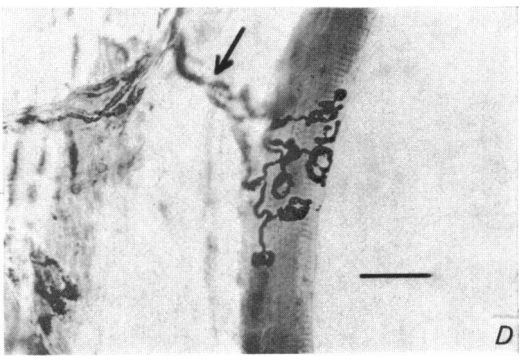

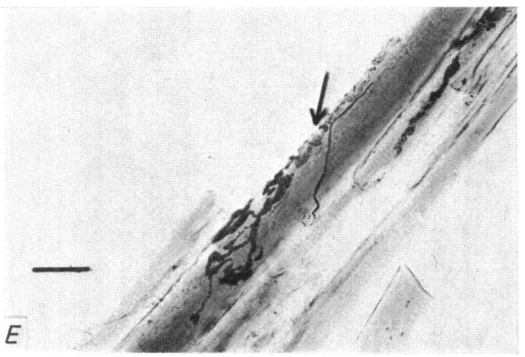

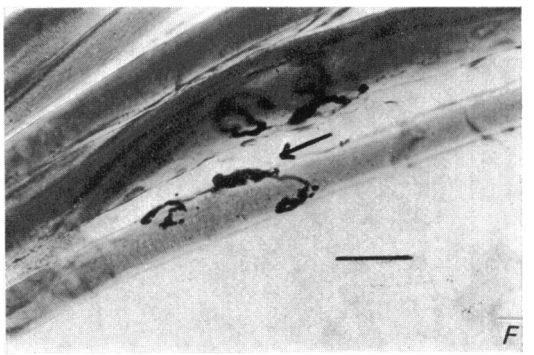

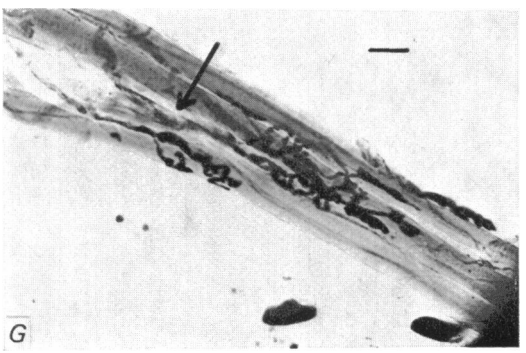

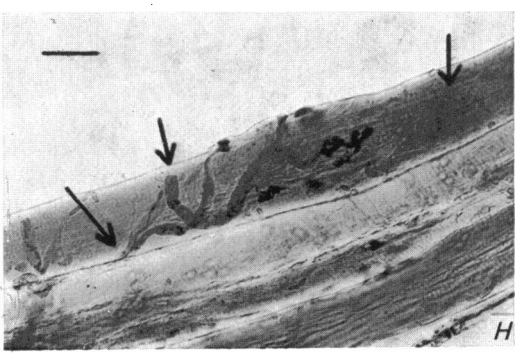

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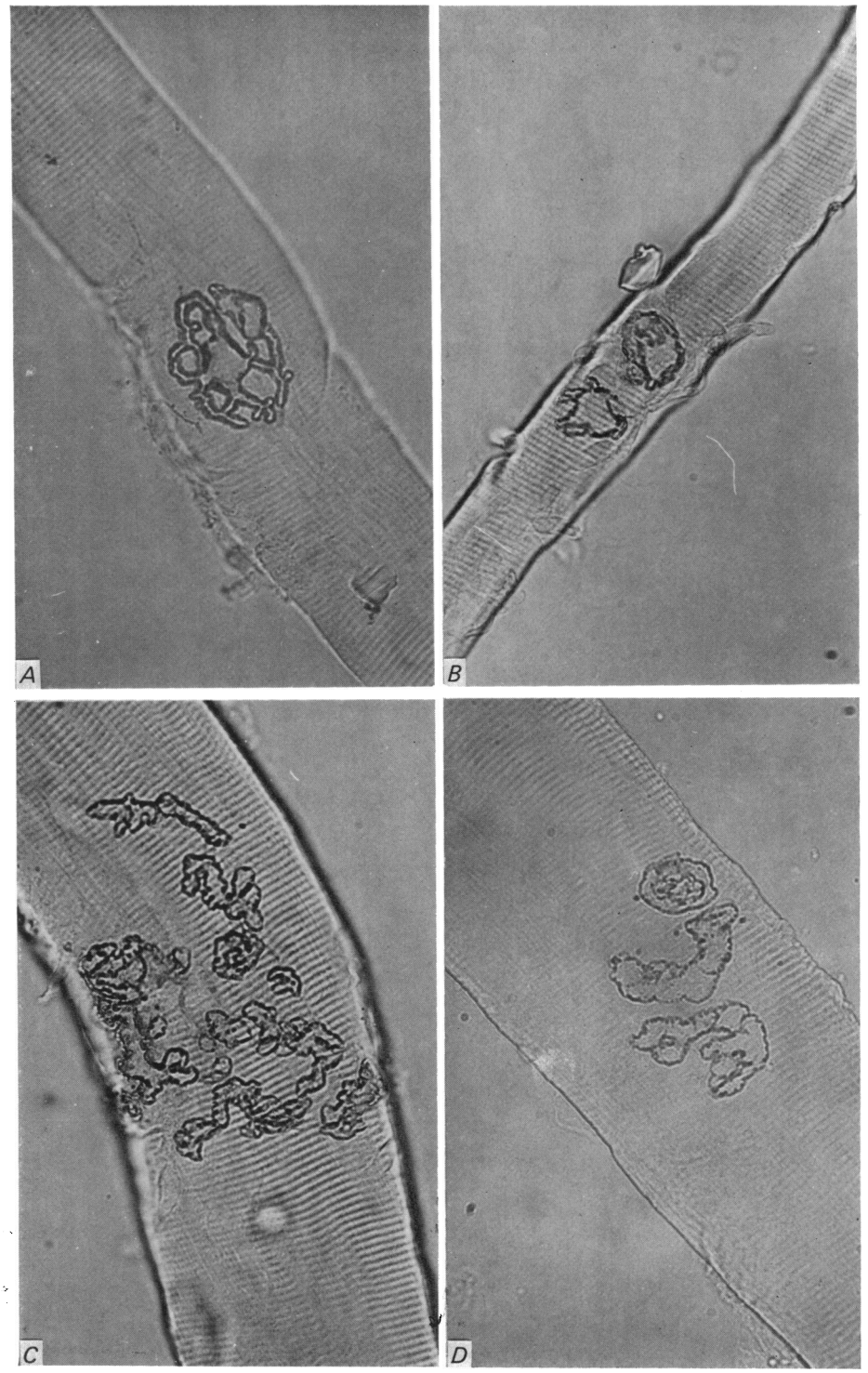
A
B
C
D

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EXPLANATION OF PLATES

PLATE 1

Teased muscle preparations stained with zinc iodide and osmium tetroxide in normal (*A*, *B*) and dystrophic (*C–H*) muscles. Nerve terminals are stained black. Positions of pre-terminal axons are indicated by arrows. Calibration bars represent 25 μm.

A, T1 end-plate on a normal extensor digitorum longus muscle fibre.

B, T2 end-plate on a normal soleus muscle fibre.

C, T2 end-plate on a dystrophic soleus muscle fibre. Pre-terminal sprouting has given rise to two well-separated terminal arborizations.

D, complex pre-terminal and ultraterminal sprouting on a dystrophic extensor digitorum longus muscle fibre.

E, a fine ultraterminal sprout extends from an ultra-terminal sprouting end-plate on a dystrophic extensor digitorum longus muscle fibre.

F, end-plate on a dystrophic muscle fibre where ultraterminal sprouting has given rise to three well-formed terminal arborizations on a single muscle fibre.

G, ultraterminal sprouting across several dystrophic soleus muscle fibres.

H, multi-terminal innervation end-plate on a dystrophic soleus muscle fibre. Two axons are visible (a third was out of the focal plane) giving rise to five terminal arborizations.

PLATE 2

Teased single muscle fibres stained for cholinesterase from normal (*A* and *B*) and dystrophic (*C* and *D*) muscles. The calibration bar represents 50 μm. In *C* and *D* the large fibre diameters were atypical.

A, 'simple' end-plate on a normal extensor digitorum longus muscle fibre.

B, 'duplex' end-plate on a normal soleus muscle fibre.

C, 'fragmented' end-plate on a dystrophic extensor digitorum longus muscle fibre. Discrete patches of cholinesterase reaction product are spread over a relatively wide area.

D, 'triplex' end-plate on a dystrophic soleus muscle fibre.