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AN ELECTROPHYSIOLOGICAL INVESTIGATION OF NEUROMUSCULAR TRANSMISSION IN MYASTHENIA GRAVIS

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Myasthenia gravis is a disease, described only in man, which is characterized by rapidly developing neuromuscular blockade during repetitive motor nerve activity. The mechanism underlying this block has remained obscure, despite a considerable amount of work on the subject (for reviews see Osserman, 1958; Foldes & McNall, 1962). By means of conventional electrophysiological techniques, with intracellular micro-electrodes to record from human intercostal muscle fibres *in vitro*, it has been possible to establish that the process of neuromuscular transmission in man is essentially similar to that in other mammalian species (Elmqvist, Johns & Thesleff, 1960). The same methods have been used in the present investigation to compare various aspects of the transmission process in specimens of myasthenic and normal intercostal muscle, obtained either at thoracotomy or by biopsy.

In the initial study made in this laboratory (Dahlbäck, Elmqvist, Johns, Radner & Thesleff, 1961) some evidence of a presynaptic defect at the myasthenic neuromuscular junction was found. A more comprehensive exploration has now been carried out, making use of an improved technique for stimulating the fine motor nerve branches which innervate the small muscle fasciculi used, so that end-plate potentials (e.p.p.s) could be recorded at most end-plates. Post-synaptic sensitivity has also been further investigated, the responses to acetylcholine analogues being measured.

We have been able to find only one abnormality at the myasthenic neuromuscular junction, a reduction in the size of the quanta of transmitter acting at the motor end-plates. The diminution seems to be of sufficient degree to account for the functional defect in the disease.

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Since post-synaptic sensitivity appears to be normal, we feel that the cause of neuromuscular blockade in myasthenia gravis is probably an inability of the nerve-endings to form quanta containing a normal amount of acetylcholine.

METHODS

Muscle specimens were obtained from eight patients with clinically diagnosed myasthenia gravis. In seven of the cases the disease was generalized and of several years' standing. In one patient the only evidence of myasthenia was bilateral ptosis which responded to tensilon and neostigmine; about one month after the biopsy he developed signs of generalized disease. The series also includes a patient who was clinically improved since a thymectomy 6 years previously. Two of the patients did not require anticholinesterase therapy at the time when biopsy was done. The other patients were continued on their daily anti-cholinesterase medication.

Control muscle was obtained at thoracotomy from patients of either sex without evidence of muscular or neuromuscular disease.

Intercostal muscle was removed in the same way as previously described (Elmqvist *et al.* 1960; Dahlbäck *et al.* 1961). In the dissection of the specimens particular care was taken to dissect out small muscle bundles in which the motor nerve twigs were easily accessible for stimulation. These fascicles were mounted on Perspex lenses, and in the bath transilluminated with polarized light in order to make the end-plate regions more easily distinguishable.

Fine bipolar platinum electrodes insulated to the tips with glass were used to stimulate the motor nerves; the stimulating pulses were square waves, 0·01–0·05 msec in duration, and 2–50 V in amplitude.

The usual techniques for intracellular recording with glass capillary micro-electrodes were employed (Fatt & Katz, 1951). The electrodes were of between 4 and 10 MΩ resistance and the input time constant of the recording circuit was 20–50 μsec. Potentials were recorded both by photographing oscilloscope traces and on paper, using the Mingograph 81, which is an ink-writer with a flat frequency response up to 500 c/s. Simultaneous recording of m.e.p.p.s on film and paper showed no difference in amplitude measurements. Besides the advantage of a directly written record the writer gave a better signal-to-noise discrimination because of its high frequency filtering effect.

The bathing fluid had the following composition (mM): NaCl, 135; $NaHCO_3$, 15; Na_2HPO_4, 1·0; KCl, 5·0; $CaCl_2$, 2·0; $MgCl_2$, 1·0; glucose, 11·0, and was bubbled with 95 % O_2 and 5 % CO_2 with a resulting pH of 7·2–7·4.

Changes in magnesium and calcium concentrations were accompanied by appropriate alterations of sodium concentration to keep the solution iso-osmolar. When potassium was added, the sodium concentration was not changed. Choline, 5×10^{-6} g/ml., was often added to the solution to avoid change in quantum size during prolonged stimulation due to choline deficiency (Elmqvist & Quastel, unpublished). When block of ACh-synthesis was desired HC-3, 10^{-5} g/ml., was used and choline omitted (Elmqvist, Quastel & Thesleff, 1963).

Experiments were usually performed at a temperature of 32° C, since at higher temperatures failure of nerve conduction was more frequent.

Calculations. Quantum size and quantum content were calculated from amplitude distributions of trains of e.p.p.s. The size of each e.p.p. was corrected for nonlinearity of the end-plate response according to the formula given by Martin (1955). On the assumption that the number of quanta in the e.p.p.s fluctuated according to a Poisson distribution, estimates of quantum size (q) were obtained from the relation

$$q = \text{e.p.p. variance/e.p.p. mean}$$

In order to avoid variance due to regression, the variance to mean ratio was calculated

independently for small groups (5–20) of consecutive e.p.p.s, and q was then taken to be the mean of at least twenty of these relatively inaccurate estimates. The mean quantum content (m) was then obtained from the relation

$$m = \text{mean e.p.p.}/q.$$

The first ten e.p.p.s immediately following a change in stimulus frequency were not used in these calculations.

An electronic digital computor was sometimes employed to process the data.

RESULTS

Whenever feasible, the experiment was begun by stimulating the fine motor nerve branches in order to observe grossly whether neuromuscular function was intact. It was striking that, with stimulation frequencies of 2/sec or more, only the first few stimuli elicited contractions in the majority of myasthenic fibres. That this block was junctional was shown by the fact that e.p.p.s. could be recorded at end-plates after muscle contractions had ceased. The e.p.p.s were of normal time course.

This behaviour was in sharp contrast to that seen in normal human muscle, where contractile responses continued during long periods of stimulation at frequencies up to 100/sec, and when contractions stopped it was usually due to nerve block as evidenced by lack of end-plate responses to the indirect stimulation. In the occasional normal fibre, subthreshold e.p.p.s could sometimes be found but then only if high frequency stimulation had been continued for several minutes.

When e.p.p.s were recorded in myasthenic muscle fibres it was observed that in a number of fibres e.p.p.s were so large that some elicited action potentials (Fig. 1). The amplitude of the biggest e.p.p.s which were subthreshold varied between junctions but was in the range 7–20 mV, which is normal for mammalian muscle (Boyd & Martin, 1956). In other fibres even the first nerve stimulus given did not result in a suprathreshold e.p.p. (Fig. 2).

The rather small fluctuation of e.p.p. amplitude in the train shown in Fig. 2 is quite characteristic of myasthenic junctions. It is important to note that this is very different from what is seen at normal junctions in which transmission has been blocked by a high magnesium and/or low calcium ion concentration. To account for this phenomenon it is necessary to suppose either that the number of quanta making up each e.p.p. was small, and not statistically distributed according to Poisson's theorem, or that the quantal components of the e.p.p.s were tiny.

Spontaneous junctional activity

In order to resolve the question of whether the quantal components of the e.p.p.s in myasthenic muscle were normal or minute a search was made

for spontaneous junctional activity. In the great majority of fibres miniature end-plate potentials (m.e.p.p.s) of normal size could not be found. However, when recording conditions were exceptionally good, high

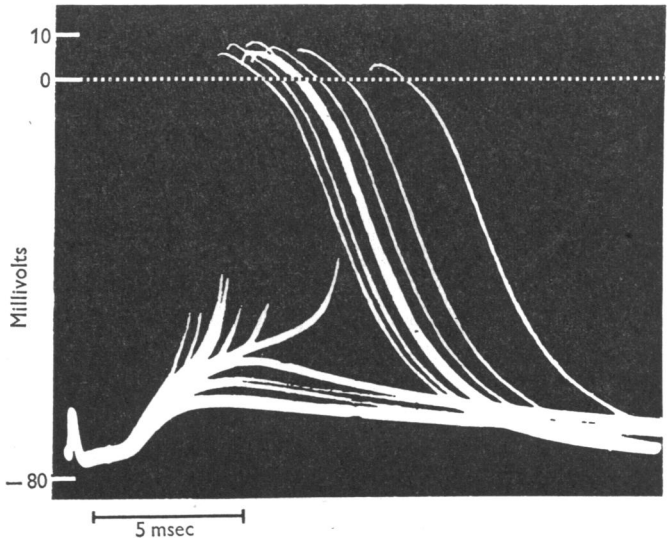

Fig. 1. E.p.p.s and action potentials recorded intracellularly near the end-plate of a myasthenic fibre, during indirect stimulation at 5/sec. Superimposed sweeps. Normal solution. 21° C.

Fig. 2. Intracellular record from a myasthenic end-plate, previously unstimulated. Indirect stimulation 20/sec. Normal solution. RP 68 mV. Time marker, 1 sec. Note: Even first e.p.p. subthreshold. Movement artifact due to contraction of neighbouring fibres. Run down of e.p.p. amplitude during first few impulses. Small amplitude variation of e.p.p.s.

amplification recording from myasthenic end-plates often revealed spontaneous transitory end-plate depolarizations which were so small that they were sometimes submerged in the base-line noise. That these were m.e.p.p.s caused by the spontaneous presynaptic release of transmitter was indicated by the following observations. At unstimulated junctions their

frequency was $0{\cdot}22 \pm 0{\cdot}14$/sec (mean $\pm$ S.D., 14 fibres), the same as the m.e.p.p. frequency in normal human intercostal muscle (Elmqvist *et al.* 1960). During tetanic stimulation there was an increase of their frequency: they could be recorded between the e.p.p.s (Fig. 3), and immediately following a high frequency tetanus a burst of them could often be seen. Their frequency was also increased by the addition of potassium to the bath (Fig. 4).

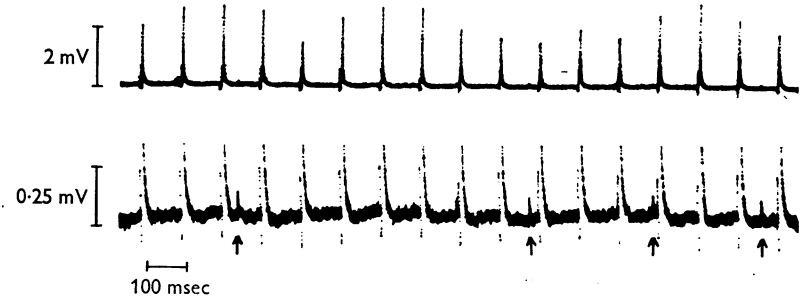

Fig. 3. Recording from a myasthenic end-plate at two different amplifications during repetitive nerve stimulation. In the upper record e.p.p.s are shown. The arrows point at m.e.p.p.s clearly seen in the high amplification record below. Normal bathing solution. RP 73 mV.

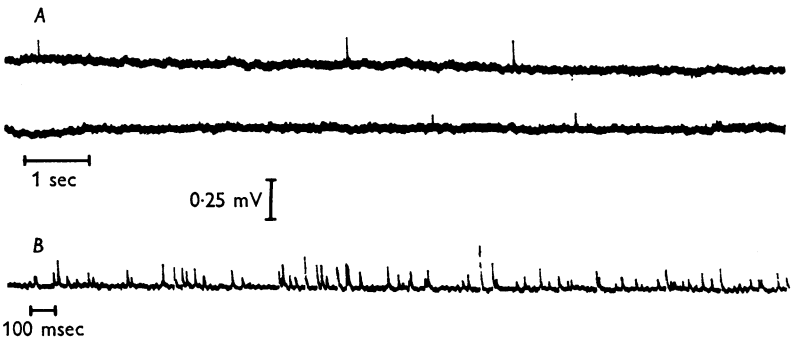

Fig. 4. M.e.p.p.s recorded at a myasthenic end-plate: *A*. In normal bathing solution containing 5 mM-potassium. RP 67 mV. *B*. 2 min after increasing the potassium concentration to 20 mM. RP 52 mV. Note difference in time scales.

Neostigmine (10^{-6} g/ml.) or DFP (10^{-5} M) in the bathing medium prolonged the falling phase of these m.e.p.p.s and caused a 40–80 % increase in amplitude. The substitution of nitrate for chloride in the bathing medium (Hofmann, Feigen & Genther, 1962) made them still larger and in the presence of both neostigmine and nitrate the m.e.p.p.s were enlarged so much that they could be found at virtually all end-plates in a myasthenic preparation.

The m.e.p.p.s found in myasthenic muscle varied considerably in amplitude from fibre to fibre; the range being from noise level to about 0·5 mV. In those myasthenic fibres in which m.e.p.p. amplitudes approached the normal range e.p.p.s often remained suprathreshold at stimulation frequencies which blocked most fibres in the preparation.

In Fig. 5 is shown the distribution of mean m.e.p.p. amplitude of fifty-seven myasthenic and fifty-four normal fibres in which m.e.p.p.s were recorded in normal bathing solution. In order to minimize variation between fibres, correction has been made for differences in resting membrane potential, all m.e.p.p. amplitudes being corrected arbitrarily to a

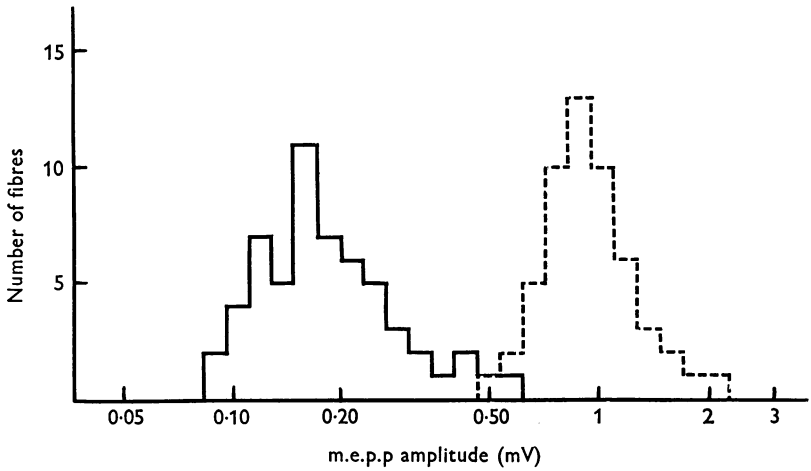

Fig. 5. Distributions of mean m.e.p.p. amplitudes obtained from fifty-seven myasthenic and fifty-four normal fibres in normal bathing mediums. All amplitudes corrected to a membrane potential of 85 mV. Full line myasthenic fibres, broken line normal fibres. Noise level was usually 50–100 μV.

TABLE 1. Comparison of electrical properties and m.e.p.p. amplitudes at normal and myasthenic end-plates

	Normal	Myasthenic
Resting potential (mV)	81·7 ± 4·4 (129)	81·0 ± 3·9 (85)
Input resistance (MΩ)	0·57 ± 0·19 (28)	0·54 ± 0·07 (8)
Time constant (msec)	18·9 ± 3·2 (7)	18·2 ± 2·4 (8)
M.e.p.p. amplitude (mV)	0·98 ± 0·30 (54)	0·20 ± 0·11 (57)*

Arithmetric means ± S.D.; number of fibres in brackets.
* Sample biased, mean probably too high, see text.

membrane potential of 85 mV assuming a transmitter equilibrium potential of 15 mV, inside negative, according to the formula given by Katz & Thesleff (1957). This should not bias the comparison since the resting membrane potentials were the same in myasthenic and normal muscle

(Table 1), and observations of e.p.p.s in damaged fibres which became depolarized showed that the equilibrium potential for transmitter action was much the same as in normal fibres.

The mean m.e.p.p. amplitudes included in the histogram are all from end-plates where focal recordings were obtained. To be sure that such was the case the recording of e.p.p.s or m.e.p.p.s with a fast rise-time (less than 1·5 msec) was required. In preparations where stimulation was not possible a judgement as to whether the recording was focal could only be made on the basis of the rise-time of the m.e.p.p.s, and thus small m.e.p.p.s had to

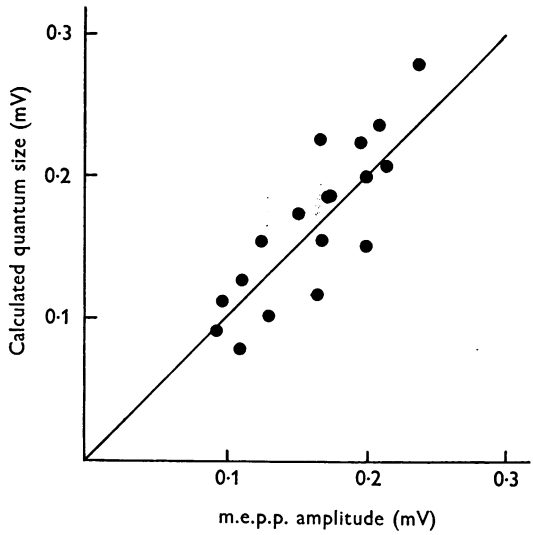

Fig. 6. Comparison between m.e.p.p. amplitude and quantum size calculated from e.p.p. size distribution at myasthenic junctions at which both e.p.p.s and m.e.p.p.s could be recorded. Normal solution. Straight line represents perfect agreement between the estimates of quantum size.

be omitted. Moreover, in each fibre there was considerable amplitude variation of the m.e.p.p.s, the smallest often being lost in the noise. For this reason there was a consistent tendency to over-estimate mean m.e.p.p. sizes in myasthenic fibres, especially in those with very small m.e.p.p.s. The distribution shown in the figure can therefore be considered to underestimate the difference from the normal.

Quantal components of e.p.p.s

As mentioned above, m.e.p.p.s and e.p.p.s could often be recorded together at myasthenic end-plates in normal solution. Calculation of the size of the quantal components of the e.p.p.s, assuming a Poisson distri-

bution of the number of quanta per e.p.p. as outlined in Methods, gave values which corresponded closely to the observed m.e.p.p. amplitudes (Fig. 6). In view of the difficulties involved in measuring mean m.e.p.p. size in a fibre when the m.e.p.p.s are very small (see above), and the lack of theoretical justification for the assumption of a Poisson distribution under our experimental conditions (del Castillo & Katz, 1954) this correlation may be largely fortuitous. However, the results seem to indicate that the myasthenic e.p.p.s are composed of quanta which are also manifest as m.e.p.p.s, and that the statistical analysis of e.p.p. amplitude distributions provides a method for estimating quantum sizes.

Post-synaptic sensitivity

The small size of the quanta recorded in myasthenic muscle could reflect a relative inability of ACh released presynaptically to cause end-plate depolarization, either because of a reduction in sensitivity of the end-plate region to the transmitter or a reduction of the effective electrical resistance between the outside and the inside of the muscle fibre (Katz & Thesleff, 1957). In the latter case the effect of a normal shunting conductance, introduced by the action of transmitter (Takeuchi & Takeuchi, 1960) would be relatively less.

To compare the chemosensitivity of myasthenic and normal end-plates two stable acetylcholine analogues carbachol and decamethonium (C10) were used. Continuous recording of the membrane potential at individual end-plates during bath application of 5×10^{-5} g/ml. carbachol showed that the time course and extent of depolarization varied considerably from fibre to fibre, but there was no apparent difference between the myasthenic and the normal. The depolarization became maximum within about 1 min and subsequent repolarization was slow, membrane potential changing little over the next 10 min.

Because only one fibre in each preparation could be tested in this way, an alternative procedure was preferred, which was to measure the membrane potential in the end-plate region of about twenty fibres before, 1–7 min after changing the bathing fluid, and again after washing out the drug for at least half an hour. As shown in Table 2 the depolarizing effects of decamethonium and of carbachol, at two different concentrations, were the same in myasthenic as in normal muscle.

The ability of this method to detect changes in post-synaptic sensitivity is indicated by the results of pre-treating normal muscle with tubocurarine (10^{-7} g/ml.), procaine (10^{-6} g/ml.), carbachol (10^{-7} g/ml.), or decamethonium (10^{-6} g/ml.). These drugs, which are known to depress the end-plate response to ACh and its depolarizing analogues, were applied for at least 30 min before application of the test dose of carbachol, and were

present during the test. The concentrations were chosen because they depressed m.e.p.p. amplitude relatively little, 10^{-7} g/ml. tubocurarine, the most potent, reducing m.e.p.p. amplitude from $0{\cdot}98 \pm 0{\cdot}30$ mV (mean ± S.D., 54 fibres) only to $0{\cdot}52 \pm 0{\cdot}28$ mV (16 fibres). Nevertheless, the responses to the test dose of carbachol were smaller by at least 70 % in all of the tests (Table 2).

TABLE 2. The effect of decamethonium and carbachol on the membrane potential at the end-plate region of normal and myasthenic muscle fibres

Pre-treated with (g/ml.)	Depolarizing drug (g/ml.)	Membrane potential at the end-plate region ± S.E. (mV): Before addition of depolarizing drug — Normal	Before addition of depolarizing drug — Myasthenic	1–7 min after addition of depolarizing drug — Normal	1–7 min after addition of depolarizing drug — Myasthenic	30 min after washing out depolarizing drug — Normal	30 min after washing out depolarizing drug — Myasthenic
—	C 10 5×10^{-5}	$79{\cdot}5 \pm 0{\cdot}9$ (20)	$80{\cdot}5 \pm 0{\cdot}8$ (24)	$67{\cdot}8 \pm 0{\cdot}7$ (20)	$67{\cdot}8 \pm 0{\cdot}5$ (24)	$79{\cdot}9 \pm 0{\cdot}5$ (20)	$81{\cdot}3 \pm 0{\cdot}7$ (22)
—	Carb. 5×10^{-5}	$80{\cdot}7 \pm 0{\cdot}9$ (20)	$81{\cdot}5 \pm 0{\cdot}8$ (21)	$56{\cdot}0 \pm 1{\cdot}1$ (20)	$55{\cdot}2 \pm 0{\cdot}6$ (21)	$80{\cdot}3 \pm 0{\cdot}9$ (20)	$79{\cdot}8 \pm 1{\cdot}0$ (21)
—	Carb. 10^{-5}	$82{\cdot}4 \pm 0{\cdot}4$ (89)*	$81{\cdot}1 \pm 0{\cdot}5$ (40)†	$65{\cdot}4 \pm 0{\cdot}5$ (80)*	$62{\cdot}6 \pm 0{\cdot}6$ (41)†	$79{\cdot}4 \pm 0{\cdot}6$ (89)*	$78{\cdot}7 \pm 0{\cdot}9$ (41)†
C 10 10^{-6}	Carb. 10^{-5}	$79{\cdot}9 \pm 1{\cdot}1$ (20)	$80{\cdot}3 \pm 0{\cdot}8$ (20)	$74{\cdot}9 \pm 1{\cdot}1$ (21)	$74{\cdot}7 \pm 0{\cdot}4$ (20)	$79{\cdot}2 \pm 0{\cdot}6$ (20)	$79{\cdot}2 \pm 0{\cdot}5$ (20)
Carb. 10^{-7}	Carb. 10^{-5}	$80{\cdot}7 \pm 0{\cdot}6$ (20)	—	$76{\cdot}2 \pm 0{\cdot}4$ (15)	—	$79{\cdot}6 \pm 1{\cdot}0$ (16)	—
dTC 10^{-7}	Carb. 10^{-5}	$80{\cdot}7 \pm 0{\cdot}9$ (20)	—	$78{\cdot}3 \pm 0{\cdot}8$ (19)	—	$79{\cdot}7 \pm 0{\cdot}9$ (20)	—
Procaine 10^{-6}	Carb. 10^{-5}	$79{\cdot}7 \pm 1{\cdot}1$ (20)	—	$76{\cdot}0 \pm 1{\cdot}1$ (17)	—	$81{\cdot}0 \pm 0{\cdot}8$ (20)	—

Each set of data (values for before, during, and after action of test dose of depolarizing drug) is derived from one preparation, except those marked * and † which are from three and two patients respectively. Number of fibres in brackets.

The desensitizing effect of decamethonium in myasthenic muscle was also tested by this method. The observed inhibition of the response to the test dose of carbachol was the same in the myasthenic as in the normal muscle (Table 2).

In myasthenic muscle fibres, input resistance was measured at end-plates where the characteristic small m.e.p.p.s and/or e.p.p.s were found, by the method of 'square pulse analysis' (Hodgkin & Rushton, 1946; Boyd & Martin, 1959). The recording and current-delivering electrodes were inserted as close together as possible; the distance between the tips was always less than 100 μ. The values obtained averaged 0·54 MΩ compared to 0·57 MΩ in normal human fibres. The time constant of the membrane, taken as the time for the electrotonic potential to reach 83 % of its steady value, was also the same in myasthenics as in the controls (Table 1). It is evidently not an abnormality of the electrical properties

of the muscle fibre membrane, or its dimensions, which is responsible for the reduced quantum size in myasthenia.

Quantum content of e.p.p.s

In myasthenic as in curarized normal human muscle e.p.p. size characteristically declines during a tetanus, levelling off at a fairly well-maintained plateau after about five impulses (Fig. 2). The initial fall in e.p.p. amplitude is generally considered to be a reflexion of partial depletion, by release, of the transmitter store which is immediately available for liberation (Liley & North, 1953) and the maintained plateau, the level of which is

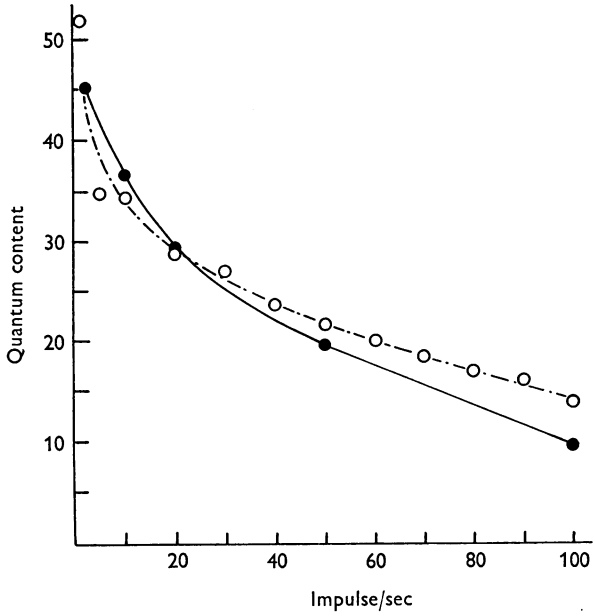

Fig. 7. Quantum content of e.p.p.s at different frequencies of stimulation obtained by dividing the mean of the last 30 e.p.p.s in tetani of about 40 e.p.p.s by the calculated quantum size. Filled circles, a myasthenic fibre (normal solution: choline 5×10^{-6} g/ml.); open circles, a normal fibre (dTC 2×10^{-6} g/ml.; choline 5×10^{-6} g/ml.).

highly dependent upon the frequency of stimulation, represents a balance between release from and mobilization into the immediately available transmitter store and is thus dependent on a variety of factors. The relation between stimulus frequency and quantum content of the e.p.p.s during the maintained plateau was tested by giving tetani of 40 impulses at frequencies of 1–100/sec, separated by periods of 1 min. From the last 30 e.p.p.s in each train mean e.p.p. amplitude and an estimate of quantum size were obtained. For calculation of the quantum content of

e.p.p.s during the sustained plateau the average of all estimates of quantum size in each fibre was used. In Fig. 7 are shown results from a myasthenic fibre and from a normal end-plate in which transmission was blocked by curare. There was evidently no great difference between curarized normal and myasthenic nerve endings in quantum content of e.p.p.s.

In human muscles as in the rat (Liley & North, 1953) an e.p.p. which follows a previous one within a few seconds is usually smaller. The extent and duration of depression by a single impulse was estimated by giving paired stimuli with varying intervals between impulses and by comparing the first two e.p.p.s in trains evoked by repetitive nerve stimulation at

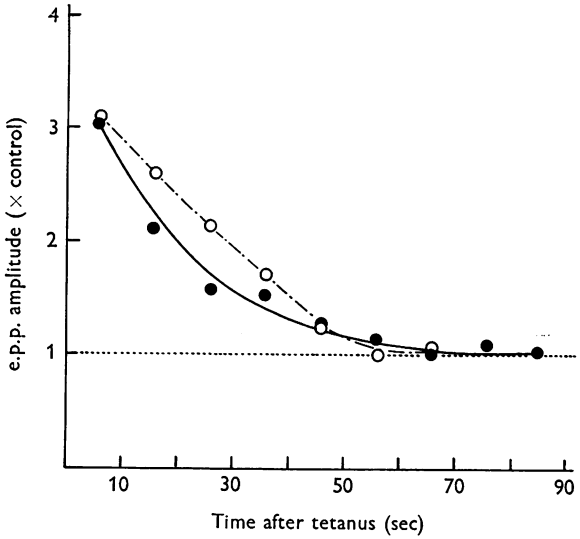

Fig. 8. Post-tetanic potentiation following a 10 sec conditioning tetanus at 50/sec. Test impulses given at 0·5/sec before and after the tetanus. Each point represents average of 5 successive e.p.p.s. Filled circles, myasthenic (dTC 10^{-7} g/ml.); open circles, normal human fibre (dTC 2×10^{-6} g/ml.).

various frequencies. The extent of the depression was found to be rather variable between fibres, being usually 5–20 % at its peak but frequently as much as 40 % or more. Recovery from the depression was always slow, taking 8–15 sec. No difference could be seen between myasthenic and curarized normal muscles.

The effect of calcium and magnesium ions

At both control and myasthenic junctions the effect of raised calcium concentration was to increase the size of the e.p.p.s at low frequencies of stimulation and to accelerate the run down of e.p.p. amplitudes to their

plateau on tetanic stimulation. The effect of raised magnesium and/or lowered calcium was the opposite; e.p.p.s become smaller and more variable, and the effect of a high frequency tetanization was to make them larger.

Post-tetanic potentiation

In contrast to what was previously reported (Dahlbäck *et al.* 1961) we have not been able to find any significant difference between the post-tetanic facilitation in myasthenic muscle and that observed in curarized control muscles. In myasthenia a conditioning short tetanus (about 10 sec) at 50–100/sec could in fact frequently make subsequent e.p.p.s

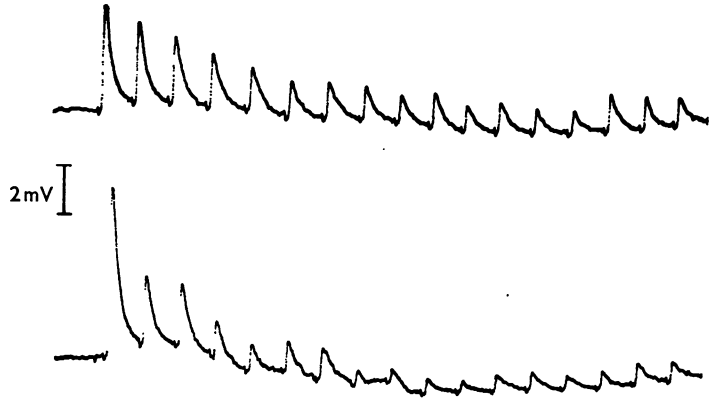

Fig. 9. Myasthenia. Above is first part of conditioning tetanus of 10 sec duration. Below is test tetanus started 5 sec after the end of conditioning tetanus. Both tetani at 50/sec. dTC 10^{-7} g/ml. Choline 5×10^{-6} g/ml.

suprathreshold when they were previously too small to elicit a muscle action potential. In extent and duration post-tetanic potentiation varied considerably from ending to ending in both the normal and the myasthenic. Generally, facilitation following tetani of duration shorter than 10 sec was very transient. Immediately following the tetanus there was usually a period of depression which was prolonged and intensified by lengthening the tetanus. Provided stimulation parameters were properly chosen post-tetanic facilitation could be demonstrated at all end-plates. Figure 8 shows the potentiation which occurred after a 10 sec tetanus at 50/sec in a myasthenic and a normal, curarized fibre.

As observed in curarized rat diaphragm (Liley & North, 1953) post-tetanic facilitation in myasthenic and normal human muscle was also manifest in an increase of the rate of run down of e.p.p.s in a second tetanus following the conditioning tetanus (Fig. 9).

Presynaptic transmitter store

The hemicholinium drug HC-3 blocks ACh synthesis in intact nervous tissue (MacIntosh, Birks & Sastry, 1956; Schueler, 1960). It has recently been shown that this drug causes a progressive diminution of quantum size at the neuromuscular junction provided ACh is released, an effect attributable to depletion of the presynaptic store of ACh (Elmqvist *et al.* 1963). With a dose of 10^{-5} g/ml., which has been shown to cause virtually complete block of ACh synthesis in the perfused cat superior cervical

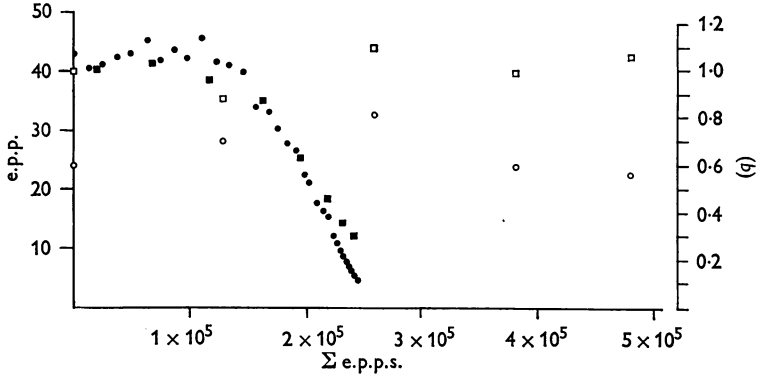

Fig. 10. Myasthenic junctions. Effect of prolonged stimulation at 5/sec on e.p.p. size and quantum size. All values expressed as multiples of estimated initial quantum size. Open symbols, no HC-3, choline 5×10^{-6} g/ml. Initial quantum size 107 μV. Circles, mean e.p.p. size; squares, calculated quantum size. Values calculated from samples of about 250 e.p.p.s at 15 min intervals. Filled symbols, HC-3 10^{-5} g/ml., no choline. Initial quantum size (after resting 40 min in HC-3) 45 μV. Circles, mean e.p.p. size from samples of about 120 e.p.p.s taken every minute; squares, mean of 4 successive estimates of quantum size, from the same samples of e.p.p.s. Note that, where mean e.p.p. size is low, estimated quantum size is probably biased upwards, because of relatively high contribution of noise to e.p.p. variance.

The values for e.p.p. size and quantum size have been plotted against the estimated sums of all e.p.p.s recorded from the beginning of the stimulation until the times at which the samples were taken. The extrapolation of the line formed by points ● gives a value of presynaptic store of transmitter at this junction corresponding to $1{\cdot}2 \times 10^4$ mV or 260,000 quanta of initial size.

ganglion (Birks & MacIntosh, 1961), at both normal and myasthenic human neuromuscular junctions e.p.p.s run down in amplitude to apparent extinction when stimulation is prolonged.

We have made use of this phenomenon to estimate the size of the presynaptic ACh store at individual junctions, proceeding on the assumption that the block of synthesis by this dose of HC-3 is effectively complete. The total amount of releasable transmitter stored in a nerve-ending can

thus be taken to be the total amount of ACh that can be released while synthesis is blocked by HC-3, which can be determined as the sum of all e.p.p.s elicited at a junction by repetitive stimulation prolonged until the e.p.p.s vanish. By plotting the size of e.p.p.s versus the sum of all foregoing e.p.p.s and extrapolating the resulting curve to zero e.p.p. amplitude (Fig. 10) an estimate of total releasable transmitter may be obtained. Provided the result is expressed in terms of initial quantum size, the determination is presumably independent of post-synaptic sensitivity. The presynaptic store in human nerve terminals, estimated by this method, turned out to vary considerably from junction to junction. A normal human intercostal nerve terminal apparently contains a store of transmitter equivalent to 50,000–500,000 of its quanta, and the same is true of the myasthenic (Table 3). In other words, if the quanta recorded at myasthenic junctions are small because of a deficiency of ACh content, the absolute amount of releasable ACh in the nerve terminals is probably small in proportion.

TABLE 3. Presynaptic stores of transmitter expressed as number of quanta of initial size

	Normal		Myasthenic	
	Presynaptic store (quanta)	Initial quantum size (mV)	Presynaptic store (quanta)	Initial quantum size (mV)
	100,000*	0·11	160,000	0·14
	70,000†	0·41	140,000	0·15
	220,000†	0·50	260,000	0·045
	480,000†	0·25	—	—
Mean	218,000		187,000	

* dTC 10^{-6} g/ml.; † Mg^{2+} 5 mM, Ca^{2+} 1·3 mM; HC-3 10^{-5} g/ml. present in all experiments.

Transmitter synthesis

The question naturally arises whether the defect of quantum size in myasthenia may not be due to an inhibition of ACh synthesis similar to that produced by HC-3. However, several observations make this possibility unlikely. In contrast to what was observed with HC-3, even greatly prolonged stimulation did not appear to affect quantum size in myasthenic fibres (Fig. 10). In several preparations stimulation at frequencies of about 10/sec was continued for several hours, while e.p.p.s and m.e.p.p.s were recorded at a number of end-plates, with no evident change of quantum sizes or of the stimulation frequency at which neuromuscular transmission failed. In the presence of an effective dose of HC-3 such treatment caused virtual disappearance of the e.p.p.s within about half an hour in both myasthenic and normal muscles. Recovery with rest was always found after run down of quantal size in the presence of HC-3, but

resting a piece of myasthenic muscle for up to 20 hr did not result in any improvement of neuromuscular function, even when choline, 5×10^{-6} g/ml., was present in the bathing medium. Exposure to choline in a concentration of 10^{-4} g/ml. for periods of up to 4 hr did not cause any increase in quantum size. On the contrary, its sole effect was to reduce quantum size by about one quarter, presumably because of its post-synaptic activity.

DISCUSSION

All the muscle specimens which we have obtained from patients with clinically diagnosed myasthenia gravis have shown a marked defect of the neuromuscular transmission mechanism. The specific abnormality found is a large reduction in size of the transmitter quantum as manifest by the end-plate depolarization it causes. This lesion is apparently the sole cause of the neuromuscular blockade which is observed *in vitro*, and it would appear to be fully sufficient to account for the muscle weakness which is characteristic of the disease. No evidence was found of any other difference between the normal and myasthenic neuromuscular junction.

The end-plate depolarization caused by bath-applied carbachol or decamethonium in myasthenic muscle was indistinguishable from that in normal muscle, while even a small depression of m.e.p.p. amplitude by drugs known to reduce post-synaptic sensitivity was associated with a marked decrease of the response to carbachol. This result does not in itself exclude the possibility of the lesion in myasthenia being post-synaptic. As pointed out by Axelsson & Thesleff (1959), the response to bath-applied depolarizing drugs is dependent not only upon the post-synaptic local chemosensitivity but also upon the area of chemosensitivity. A decreased receptor density or sensitivity could explain the small size of m.e.p.p.s and e.p.p.s found in myasthenic muscle and if there were at the same time a sufficient enlargement of the chemosensitive area there would be normal responses to carbachol and decamethonium. However, Dahlbäck *et al.* (1961) found normal sensitivity of myasthenic end-plates to iontophoretic micro-application of ACh, with no evidence of enlargement of the chemosensitive area.

Several mechanisms which could cause failure of neuromuscular transmission may now be discarded as causes of that which occurs in myasthenia. The blockade is not the result of an inhibition of transmitter release similar to that caused by excess magnesium and/or deficiency of calcium (del Castillo & Engbaek, 1954), nor is it similar to botulinum intoxication (Brooks, 1956). The finding of normal quantum content of the e.p.p.s over a wide range of stimulation frequencies also excludes the possibility that there is any scarcity of quanta in the presynaptic store

immediately available for release. Mobilization of quanta into this store and facilitation of their liberation by activity are evidently also normal. That cholinesterase inhibitors do not restore to normal the size of the quanta in myasthenia indicates that an increase in acetylcholinesterase activity is not the explanation of the transmission block. Since the e.p.p.s have a normal time course it would seem unlikely that an abnormal diffusion barrier of any kind has become interposed between the nerve terminals and the muscle end-plate.

Normal nerve terminals produce small quanta if synthesis of transmitter is blocked by HC-3 or triethylcholine (Elmqvist *et al.* 1963; Elmqvist & Quastel, unpublished observations). It has already been pointed out that the myasthenic nerve terminal is unlike the normal one treated with HC-3 in that there are no changes of quantum size associated either with prolonged stimulation or rest. This would seem to make unlikely the possibility that the defect in myasthenia is due simply to inability of the acetylcholine-synthesizing mechanism to keep up with release as a result of lack of substrate. From the same observations the conclusion may be drawn that the myasthenic lesion is not dependent upon the presence of a substance which can be easily washed away.

The interpretation which best takes into account all our findings is that there is in myasthenia a deficiency of acetylcholine in the pre-synaptically formed quanta and that this is the result of a defect of the packaging or binding mechanism, or the presence of a false transmitter. It has been suggested that triethylcholine might be acetylated and the product dealt with as acetylcholine by nerve-endings (Burgen, Burke & Desbarats-Schonbaum, 1956; Bowman & Rand, 1961), and a similar phenomenon might be involved in this disease.

Our estimates of the size of the presynaptic transmitter store indicate that it is highly variable from terminal to terminal. The nerve endings in myasthenic muscle appear to contain a store of transmitter which is within the normal range, in terms of number of quanta. If we are correct in attributing the small size of the quanta to a presynaptic defect rather than a post-synaptic, then this result would indicate that the quantity of ACh in the presynaptic store is small in myasthenic nerve terminals.

The present results seem to conflict with those of Dahlbäck *et al.* (1961) particularly with regard to amplitude and frequency of m.e.p.p.s and their response to raised potassium concentration. However, at some end-plates in normal muscle with m.e.p.p. amplitude of about 1 mV we have observed spontaneous e.p.p.-like potentials with amplitudes of 3–6 mV; these were abolished or reduced in frequency when potassium was added. Probably they represent simultaneous release of several quanta of ACh, possibly brought about by injury of the nerve terminal. They may be the counter-

part of the spontaneous depolarizations recorded by Dahlbäck *et al.* (1961) at a few myasthenic end-plates, as the latter also disappeared when potassium was added. Evidently they were mistaken for m.e.p.p.s owing to the fact that the true myasthenic m.e.p.p.s were submerged in the base-line noise.

The patients who volunteered for this study had myasthenia of different severity and none of them had symptoms referable to involvement of intercostal muscle. In all the muscle specimens the defect of quantal size was found; even in those from the least-affected patients the m.e.p.p.s in most fibres were near noise level, and the e.p.p.s rapidly became subthreshold at stimulation frequencies of more than 2/sec. It must therefore be concluded that in this disease a rather large interference with the neuromuscular transmission mechanism is required before clinical symptoms appear.

SUMMARY

1. By the use of intracellular electrodes the neuromuscular transmission mechanism has been studied in isolated intercostal muscle obtained from patients with myasthenia gravis.

2. On repetitive nerve stimulation at frequencies above 2/sec, only the first few stimuli elicited muscle contractions in most fibres and then subthreshold end-plate potentials (e.p.p.s) could be recorded.

3. Miniature end-plate potentials (m.e.p.p.s) had a mean amplitude of 0·2 mV, one fifth of the normal. The calculated size of the quantal components of e.p.p.s corresponded closely to the m.e.p.p. amplitude.

4. The average resting frequency of m.e.p.p.s was 0·2/sec, the same as in normal human intercostal muscle, and the frequency was increased by nerve stimulation and by potassium. The quantum content of e.p.p.s at various frequencies of nerve stimulation was similar to that at normal junctions.

5. Post-synaptic chemosensitivity, as tested by bath-application of carbachol and decamethonium, was normal. The input resistance of the fibres in myasthenic muscle was about 0·5 MΩ, the same as in normal muscle. Resting membrane potential of myasthenic fibres was also normal.

6. It is tentatively concluded that in myasthenia gravis there is a deficiency in the amount of acetylcholine in the quanta of transmitter released from the motor nerve terminals.

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REFERENCES

AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol.* **147**, 178–193.

BIRKS, R. & MACINTOSH, F. C. (1961). Acetylcholine metabolism of a sympathetic ganglion. *Canad. J. Biochem. Physiol.* **39**, 787–827.

BOWMAN, W. C. & RAND, M. J. (1961). Actions of triethylcholine on neuro-muscular transmission. *Brit. J. Pharmacol.* **17**, 176–195.

BOYD, I. A. & MARTIN, A. R. (1956). The end-plate potential in mammalian muscle. *J. Physiol.* **132**, 74–91.

BOYD, I. A. & MARTIN, A. R. (1959). Membrane constants of mammalian muscle fibres. *J. Physiol.* **147**, 450–457.

BROOKS, V. B. (1956). An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials. *J. Physiol.* **134**, 264–277.

BURGEN, A. S. V., BURKE, G. & DESBARATS-SCHONBAUM, M.-L. (1956). The specificity of brain choline acetylase. *Brit. J. Pharmacol.* **11**, 308–312.

DEL CASTILLO, J. & ENGBAEK, I. (1954). The nature of the neuromuscular block produced by magnesium. *J. Physiol.* **124**, 370–384.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *J. Physiol.* **124**, 560–573.

DAHLBÄCK, O., ELMQVIST, D., JOHNS, T. R., RADNER, S. & THESLEFF, S. (1961). An electro-physiologic junction in myasthenia gravis. *J. Physiol.* **156**, 336–343.

ELMQVIST, D., JOHNS, T. R. & THESLEFF, S. (1960). A study of some electro-physiological properties of human intercostal muscle. *J. Physiol.* **154**, 602–607.

ELMQVIST, D., QUASTEL, D. M. J. & THESLEFF, S. (1963). Prejunctional action of HC-3 on neuromuscular transmission. *J. Physiol.* **167**, 47–48*P*.

FATT, P. & KATZ, B. (1951). Analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

FOLDES, F. F. & MCNALL, P. G. (1962). Myasthenia Gravis: A guide for anesthesiologists. *Anesthesiology*, **23**, 837–872.

HODGKIN, A. L. & RUSHTON, W. A. H. (1946). The electrical constants of a crustacean nerve fibre. *Proc. Roy. Soc.* B, **133**, 444–479.

HOFMANN, W. W., FEIGEN, G. A. & GENTHER, G. H. (1962). Effects of veratrine, nitrate ion and aminobutyric acid on mammalian miniature end-plate potentials. *Nature, Lond.*, **193**, 175–176.

KATZ, B. & THESLEFF, S. (1957). On the factors which determine the amplitude of the miniature end-plate potential. *J. Physiol.* **137**, 267–278.

LILEY, A. W. & NORTH, K. A. K. (1953). An electrical investigation of effects of repetitive stimulation on mammalian neuromuscular junction. *J. Neurophysiol.* **16**, 509–527.

MACINTOSH, F. C., BIRKS, R. I. & SASTRY, P. B. (1956). Pharmacological inhibition of acetylcholine synthesis. *Nature, Lond.*, **178**, 1181.

MARTIN, A. R. (1955). A further study of the statistical composition of the end-plate potential. *J. Physiol.* **130**, 114–122.

OSSERMAN, K. E. (1958). *Myasthenia Gravis.* New York: Grune and Stratton.

SCHUELER, F. W. (1960). The mechanism of action of the hemicholiniums. *Int. Rev. Neurobiol.* **2**, 77–97.

TAKEUCHI, A. & TAKEUCHI, N. (1960). On the permeability of end-plate membrane during the action of transmitter. *J. Physiol.* **154**, 52–67.