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TRANSMISSION AT THE MAMMALIAN  
NEUROMUSCULAR JUNCTION

By  
A.W. LILEY

Thesis submitted for the degree of Doctor of  
Philosophy in the Australian National University  
1956

I hereby declare that, with the exceptions of Fig. 26C obtained in collaboration with K.A.K. North and Fig. 40R provided by Professor J.C. Eccles, all of this thesis is my own original work.

The following papers have appeared or are in the course of publication:-

LILEY, A.W. (1956a). An investigation of spontaneous activity at the neuromuscular junction of the rat. J. Physiol. (Lond.). 132, 650-666.

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LILEY, A.W. (1956c). The effects of presynaptic polarisation on the spontaneous activity at the mammalian neuromuscular junction. J. Physiol. (Lond.). in the Press.

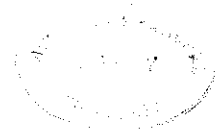
LILEY, A.W. (1956d). Spontaneous release of transmitter substance in multiquantal units. In course of publication.

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## TABLE OF CONTENTS

|                                                                      | Page |
|----------------------------------------------------------------------|------|
| <u>INTRODUCTION</u>                                                  | 1    |
| <u>METHODS</u>                                                       | 4    |
| (A) Tissues                                                          | 4    |
| (B) Histology. Localisation of end-plates                            | 4    |
| (C) Solutions                                                        | 6    |
| (D) Drugs                                                            | 6    |
| (E) Apparatus                                                        | 6    |
| (F) Instrumental noise and artefacts                                 | 10   |
| (G) Measurement of frequencies and amplitudes                        | 10   |
| (H) Statistics                                                       | 14   |
| <u>SECTION 1. SPONTANEOUS ACTIVITY AT THE NEUROMUSCULAR JUNCTION</u> |      |
| <u>OF THE RAT</u>                                                    | 16   |
| Introduction                                                         | 16   |
| Results                                                              | 16   |
| Discussion                                                           | 27   |
| <u>SECTION 2. THE QUANTAL COMPONENTS OF THE MAMMALIAN END-PLATE</u>  |      |
| <u>POTENTIAL</u>                                                     | 28   |
| Introduction                                                         | 28   |
| Results                                                              | 28   |
| Part I. Quantal nature of the end-plate potential                    | 28   |
| Part II. Facilitation at the mammalian neuromuscular                 |      |
| junction                                                             | 37   |
| Discussion                                                           | 39   |



|                                                                        |    |
|------------------------------------------------------------------------|----|
| <u>SECTION 3. THE EFFECTS OF PRESYNAPTIC POLARISATION ON THE</u>       |    |
| <u>SPONTANEOUS ACTIVITY AT THE MAMMALIAN NEURO-</u>                    |    |
| <u>MUSCULAR JUNCTION</u>                                               | 45 |
| Introduction                                                           | 45 |
| Results                                                                | 45 |
| Part I. Effect of electrotonic polarisation of motor                   |    |
| terminals on miniature discharge frequency                             | 45 |
| Part II. Effect of potassium concentration on                          |    |
| miniature potentials                                                   | 49 |
| Discussion and test of hypothesis                                      | 50 |
| <u>SECTION 4. GIANT POTENTIALS AT THE MAMMALIAN NEUROMUSCULAR</u>      |    |
| <u>JUNCTION</u>                                                        | 60 |
| Introduction                                                           | 60 |
| Results                                                                | 60 |
| Discussion                                                             | 65 |
| <u>SECTION 5. THE EFFECTS OF GLUCOSE DEPRIVATION AND PHLORIDZIN ON</u> |    |
| <u>THE MINIATURE DISCHARGE AT THE MAMMALIAN NEURO-</u>                 |    |
| <u>MUSCULAR FUNCTION</u>                                               | 70 |
| Introduction                                                           | 70 |
| Results                                                                | 71 |
| Discussion                                                             | 74 |
| <u>SECTION 6. THE EFFECTS OF POTASSIUM ON TRANSMISSION AT THE</u>      |    |
| <u>MAMMALIAN NEUROMUSCULAR JUNCTION</u>                                | 77 |
| Introduction                                                           | 77 |
| Results                                                                | 78 |
| Discussion                                                             | 80 |

GENERAL DISCUSSION

86

SUMMARY AND CONCLUSIONS

91

ACKNOWLEDGEMENTS

REFERENCES

FIGURES AND LEGENDS

GENERAL INTRODUCTION

For more than a century vertebrate nerve-muscle preparations have been extensively investigated. This is not surprising for such preparations are readily accessible and may survive, isolated, in simple solutions for many hours. Furthermore the mechanical responses of muscles to direct and indirect stimulation provided some index of activity long before electrical recording techniques were evolved.

The concept of the neuromuscular junction as a physiological entity dates from the researches of Claude Bernard on curare in 1850-57. The work of Langley (1905, 1907) indicated the pharmacological specificity of the muscle junctional region. Following the demonstration of the release and action of acetylcholine at the neuromuscular junction (Dale, Feldberg & Vogt, 1936; Brown, Dale & Feldberg, 1936) and the electrical recording of the end-plate potential (Göpfert & Schaefer, 1938; Eccles & O'Connor, 1939; Feng, 1940) knowledge of neuromuscular transmission has advanced rapidly and numerous reviews have been published (Feng, 1941; Eccles, Katz & Kuffler, 1941; Whitteridge, 1948; Eccles, 1948; Acheson, 1948; Harvey, 1948; Kuffler, 1948; Toman & Goodman, 1948; Koelle & Gilman, 1949; Paton, 1949; Hunt & Kuffler, 1950; Rosenblueth, 1950; Tiegs, 1953).

In the present decade progress has followed the application of two elegant and powerful techniques to the problems of junctional transmission. First, the intracellular microcapillary method of electrical recording has allowed precise investigation of the behaviour of single muscle fibres and junctions. Secondly, electron-microscopy has displayed important detail in both the pre- and post-synaptic components of the junction. The information yielded by these new techniques has been reviewed by Fatt (1954) and Castillo & Katz (1956b).

Of particular interest was the observation by Fatt & Katz (1950) that the amphibian and reptilian myoneural junctions were the site of spontaneous subthreshold activity. This activity consisted of intermittent small potentials which resembled the e.p.p. except for their briefer time-course. Hence these small potentials were designated 'miniature e.p.p.s' or 'miniature potentials'. Fatt & Katz (1950, 1952, see also 1953) produced evidence that the miniature potentials were generated by multimolecular packets or quanta of acetylcholine which were released spontaneously from the motor nerve terminals. The significance of this phenomenon was established by Castillo & Katz (1954b) when they demonstrated that, in the frog, the e.p.p. was produced by the synchronous discharge of a large number of these quanta of acetylcholine. The extensive investigations of Castillo and Katz (1954a - d, 1955a,b, 1956a) have added further detail of the release and effect of the quanta of transmitter at the amphibian myoneural junction. These researches have shown that transmission is graded at the quantal and not the molecular level.

These findings are of great interest, not only in relation to specific problems in neuromuscular transmission, but also in considerations of cellular secretory processes in general. Obviously it was desirable to confirm these observations at the mammalian neuromuscular junction. Boyd and Martin (1956a, b) provided this confirmation in the tenuissimus muscle of the cat. They detected miniature potentials and showed that, as in the frog, the e.p.p. consisted of summated miniature potentials. Concurrently with the preliminary communications of Boyd and Martin (1955a, b), the present investigation of transmission at the neuromuscular junction of the rat had been undertaken. The earlier parts of this investigation (Section 1, Section 2, part I) largely complement the observations of Boyd and Martin



(1956a, b). Section 3 describes investigations of the mechanism by which the motor terminal membrane liberates quanta of transmitter.

These investigations have led to the formulation of an hypothesis which postulates that a nerve impulse liberates acetylcholine from motor nerve terminals solely as a result of the depolarization of these terminals. This hypothesis has been tested both at the qualitative and quantitative levels.

Other problems which have been investigated include post-tetanic potentiation (Section 2, part 2), the spontaneous release of multiquantal accumulations of acetylcholine (Section 4), the effect of glucose deprivation on the miniature discharge (Section 5) and the effects of potassium on neuromuscular transmission (Section 6).

### METHODS

(A). Tissues. The tissues of male and female albino rats (Wistar strain) of weight 100-250 g were used in all experiments.

Isolated preparations consisted of a band of the left hemidiaphragm together with the intrathoracic portion of the left phrenic nerve removed from the rat under open ether anaesthesia. In those preparations that had been denervated — days previously, the operation had been performed ~~via~~ a thoracotomy incision under open ~~ex~~ether or intraperitoneal bromethal (10 ml. of 2.5% solution per Kg).

(B). Histology: Experimental localisation of end-plates. Fig. 1 shows the appearance of endplates in the rat diaphragm stained with gold chloride after the method of Cole (1946). The histological characteristics described by Cole (1955) in rat leg muscle are confirmed. The diameter of the motor end-plate is of the order of  $30\mu$ . The diameter of the majority of muscle fibres is some  $35-45\mu$  although a few fibres may be found with a diameter of about  $80\mu$ .

During experiments, however, such detail could not be observed, for the tissue was unteased and unstained and magnification and light intensity were limited. Nevertheless little difficulty was encountered in locating neuromuscular junctions with the microelectrode. The left phrenic nerve runs over the pericardium to join the left hemidiaphragm about midway between the central tendon and the costal attachment. Within 1 or 2 mm of the muscle the nerve divides into three well-defined branches — medial, anterior and posterior. The medial branch runs to the central tendon then posteriorly and caudally to innervate the left crus. Thus it contributes nothing to the innervation of the isolated strip. Occasionally this branch was absent at the diaphragm, having left the phrenic nerve in mid-thorax.

The anterior and posterior branches supply a series of smaller branches to the muscle fibres but, almost without exception, these twigs, repeatedly dividing, lie lateral to their parent nerve branch. Thus the end-plates are to be found in a band on the peripheral side of the anterior and posterior nerve branches as has been observed by Hadju and Knox (1950). This zone was readily identified optically in every preparation from the pattern of dividing nerve twigs. It is obvious from the pattern of nerve endings displayed in Fig. 1A that, once a microelectrode has detected one junction, exploratory movements of the microelectrode in 40-50 $\mu$  steps at right angles to the muscle fibres can hardly fail to detect miniature potentials (Section 1) at every insertion. Such a rapid traverse was often adequate when only frequencies were to be determined. When a more accurate localisation of a junction was required, repeated insertions of the microelectrode at 5-10 $\mu$  intervals parallel to the fibres were made until potentials of maximum amplitude and briefest time-course were obtained. Not infrequently a junction would be 'lost' during this procedure since the muscle fibres and fascicles appeared to intertwine and overlies each other - probably because, in situ, the diaphragm is not a plane structure but a dome.

Edds (1950) and Cole (1955) have illustrated the bifurcation of an epilemmal axon to give two endings on the same muscle fibre in rat calf muscles. Occasionally, bifurcating axons with or without two distinct end-plates may be seen also in the stained rat diaphragm (Fig. 1E, F). However two distinct endings on one muscle fibre would need to be separated by at least 50-100 $\mu$  before they could be discriminated electrically, and even then it is essential that the microelectrode be inserted very close to one of the endings so that the miniature potential populations may be distinguished by amplitude and time course. Such a fortunate set of circumstances

must be rare, for in many thousands of penetrations only three fibres displayed unequivocal evidence of double innervation (see Figs. 45, 46).

(C). Solutions. The constitution of the normal bathing solution is given in Table 1. This solution, when gassed with 95%  $O_2$  + 5%  $CO_2$  ('Carbogen'), had a pH of 7.3 - 7.4 at 38°C.

Table 1.

Constitution of solution used for isolated tissues

|           |          |             |          |
|-----------|----------|-------------|----------|
| $Na^+$    | 149.8 mM | $Cl^-$      | 147.8 mM |
| $K^+$     | 5.0      | $H_2PO_4^-$ | 1.0      |
| $Ca^{++}$ | 2.0      | $HCO_3^-$   | 12       |
| $Mg^{++}$ | 1.0      | Glucose     | 11.0     |

'Analar' quality chemicals were used throughout. Alterations in the constitution of the bathing solution are indicated in the text. Changes in the concentration of calcium, magnesium and glucose were not osmotically compensated. However any alteration in potassium concentration was accompanied by an isomolar change (of opposite sign) in sodium concentration.

Preparations were allowed 15-20 minutes for equilibration following changes in the potassium and sodium concentrations and  $1\frac{1}{2}$  - 2 hours following changes in calcium and/or magnesium concentrations.

(D). Drugs. Drugs employed were d-tubocurarine chloride (Burroughs-Wellcome), neostigmine bromide ('Prostigmine', Merck), gallamine triethiodide ('Flaxedil', May and Baker), procaine hydrochloride (B.D.H.) and phloridzin (B.D.H.). All concentrations are expressed by weight of these compounds.

(E). Apparatus. The isolated preparation was mounted in a chamber which comprised two Perspex compartments inset in a Perspex slab (Fig. 2). In one compartment the muscle was rested over a small Perspex platform and

by pins to strips of balsa wood. From this compartment the nerve via a small vaseline sealed notch, beneath a sliding partition to second compartment where it rested across platinum stimulating electrodes. During stimulation the solution level in this compartment was lowered to clear the electrodes by means of an attached 10 ml. syringe. The temperature of the chamber was maintained at a usual level of  $37-38^{\circ}\text{C}$  by permanent partial immersion in a water bath, the Perspex slab forming the lid of this bath. The solution, saturated with 'Carbogen' in separating funnels, was passed via a drip counter and heat exchanger to the chamber and removal was effected by continuous suction with a Venturi pump. An exchange rate of 150-200 ml. per hour was maintained.

The intracellular recording technique (Ling & Gerard, 1949; Nastuk & Hodgkin, 1950) was employed in most experiments. Capillary micro-electrodes filled with 3M KCl were used with resistances of 5-30 megohms and tip diameters of less than  $1\mu$  (Fig. 1G). The method of pulling and filling these electrodes has been described elsewhere (Winsbury, 1956) as also have been the micromanipulators (Eccles, Fatt, Landgren & Winsbury, 1954) and electronic stimulating (Liley & North, 1953) and recording techniques (Brock, Coombs & Eccles, 1952). The indifferent electrode consisted of a silver-silver chloride spiral in contact with the chamber solution via an agar-saline column.

For experiments involving polarization of the motor nerve terminals (Section 3) the dissection and mounting of the preparation were modified as follows. The terminal portion of the left phrenic nerve and its posterior branch were dissected free from adherent tissue (pericardium and pericardial fat). The anterior branch of the nerve was cut close to its origin. The hemi-diaphragm was now divided by a cut parallel to the

fibres and immediately anterior to the point of entry of the posterior nerve branch. In a suitable dissection the muscle fibres on the cut edge of the preparation obtained their nerve supply immediately from the entering posterior nerve branch. However the origin of the nerve supply of the central fibres of the hemi-diaphragm was very variable. In about 50% of preparations a recurrent twig from the (sectioned) anterior branch not only supplied the central muscle fibres but also frequently encroached considerably on the territory of the posterior branch. Such preparations were discarded as also were preparations in which the nerve twigs ran some distance radially before innervating the muscle fibres.

Suitable preparations were mounted as shown in Fig. 3, the bordering fibres being placed as close as possible to the Perspex partition. This partition was 1.5 mm thick and, beneath it, through a notch sealed with vaseline, the posterior branch of the phrenic nerve was led. With non-polarisable electrodes arranged as shown (Figs. 2 & 3) to pass direct current, the lines of current flow converge at this notch. For a given current the polarisation produced at a given nerve terminal is a function of the space constant of the nerve fibres, the proximity of the terminal to the notch (normally less than  $200\mu$ ) and the relative volume of tissue, including other nerve fibres, passing through the notch. A disadvantage of this arrangement was that a considerable potential difference was produced by current flow in the bath solution. Where necessary this potential was compensated either by adjusting the operating condition of the input cathode follower to bring its output back to the original voltage level or else by applying a compensating potential to the other input of the differential amplifier.

Normally the polarising current was applied by manual operation of a potentiometer. A monitoring resistor was placed in series with the nerve and the p.d. developed across it was read on a d.c. voltmeter.

For brief rectangular pulses the potentiometer was preset and the pulses were applied by the action of a Carpenter relay which was driven from the sweep potential via a Schmitt trigger circuit. By manual operation the polarising current could be gradually increased over several seconds, so utilizing the accommodation process to prevent the intense repetitive discharge which would have been produced by the sudden onset of such a current.

For experiments in which it was necessary to block nerve impulses close to the nerve terminals (Section 3) a cold block was produced. For this purpose a thermode similar to that described by Douglas & Malcolm (1955) was used. The preparation was dissected as for the polarising experiments, and was mounted as shown in Fig. 4. The posterior branch of the phrenic nerve was lodged in a slot in the silver tip (2.5 mm diameter) of the thermode which, for the purposes of insulation, was surrounded by liquid paraffin enclosed by a very thin Perspex collar. A high flow rate of the chamber solution was employed to maintain the neuromuscular junctions as near as possible to the normal temperature of  $38^{\circ}\text{C}$ . The coolant for the thermode was alcohol brought to a temperature below  $-70^{\circ}\text{C}$  by the addition of solid carbon dioxide. The temperature of the thermode tip was determined by silver - Eureka thermocouples, the reference junction being immersed in crushed ice. Nervous conduction was blocked completely at  $+5^{\circ}\text{C}$  but to allow a margin of safety the temperature was held at  $-1^{\circ}\text{C}$  during experiments. The nerve block was rapidly and completely reversible.

For investigations on tissues in vivo, rats were anaesthetised with intraperitoneal pentobarbital sodium (40 mg per Kg), placed supine and immobilized on a cork surface by means of pins inserted through the forefeet, pelvis and hind limb joints. The left gracilis muscle was exposed under a paraffin pool, the indifferent electrode being a saline-soaked pad sutured to the muscles of the abdominal wall.

(F). Instrumental noise and artefacts. No electrical equipment is noise-free and, at the high amplifications employed, the present circuitry was no exception. However baseline noise was not a serious problem since the range was usually less than 50  $\mu\text{V}$ , and, further, very high frequency components could be suppressed by a filter normally set at 30 Kc/sec.

More important in the present work were the various artefacts encountered (Figs. 5 & 6). This collection may appear formidable but, in practice, most types were but rarely seen. Therein, however, lay their inconvenience for it was important that their true nature be recognised immediately.

Artefacts presented two problems. First, monophasic artefacts could simulate "physiological" spontaneous potentials - intra- or extracellularly recorded - especially with form and time course unresolved at a low sweep velocity. Secondly, oscillatory artefacts, unless resolved by a high sweep velocity, could interfere with the measurement of amplitudes.

The most frequent and interesting of the oscillatory artefacts was that depicted in Fig. 5, Records C, D & E. This artefact appeared only when the microelectrode tip was located on or in a fibre. Of a frequency in the range 20-40c/sec (Main's supply was 50c/sec) it would appear spontaneously during a recording; sometimes to die away, sometimes to increase and become disorganised, and yet sometimes to persist unaltered until the electrode was withdrawn. Not infrequently it was markedly asymmetrical and occasionally exhibited obvious "beats".

(G). Measurement of frequencies and amplitudes. The method of recording series of spontaneous potentials and e.p.p. responses was essentially similar to that described by Castillo & Katz (1954b).

(i) Determination of frequencies of spontaneous potentials.

When, for a random process such as the spontaneous potentials, the



mean frequency,  $f$  per sec., is estimated by measurement over  $n$  seconds, the standard error of the estimated mean is given by  $(f/n)^{0.5}$ . Hence, if it is desired to keep the standard error at a constant fraction of the mean, it is necessary to maintain the product  $fn$  constant, i.e. the duration of observation should vary inversely with the mean discharge frequency at a junction. Such a procedure was adopted in the present work.

Special problems were encountered in attempting to determine the mean frequency of the miniature discharge when frequencies were very high or very low. For an accurate determination of the low discharge frequencies that occurred for instance with hyperpolarisation of the terminals (Section 3), it was necessary to extend observations over many minutes at each setting of the polarising current. Beside expending photographic film, such long periods of recording greatly increased the risk of encountering either progressive change or transitory disturbances of the discharge frequency due to extraneous factors. Progressive effects could be detected by checking the value of the resting frequency at intervals during a series. More serious problems were encountered with very high discharge frequencies. Minimal baseline 'noise' was essential. Further it was necessary to localise the junction precisely so that potentials would be recorded with maximum amplitude and briefest time course. Anticholinesterases were undesirable since they involved a prolongation of time-course and an increased liability of the generation of action potentials. Under favourable conditions and with a very high sweep velocity, frequencies as high as 600-700 per sec were readily determined, but above this level there was an increasing uncertainty in the recognition of individual potentials and counts were very likely to underestimate the true values.

In all records interspersed large miniature potentials were assumed to result from the fortuitous coincident discharge of quanta and

hence the quantal content of such potentials was estimated for the frequency count. Such a procedure ignores the tendency of the mammalian miniature discharges to display a greater number of coincident discharges than would be expected by stochastic theory (Sections 1 & 4).

The effective frequencies during very brief intervals after the application of a rectangular current pulse to the terminals (Fig. 33, Section 3) were determined by photographing several thousand pulses and counting the number of discharges occurring within the corresponding intervals on each.

A similar procedure was employed to determine the effective frequency of the miniature discharge at very brief intervals following the arrival of a nerve impulse at a preterminal nerve block (Fig. 36, Section 3).

(ii) Determination of amplitudes of spontaneous potentials and responses.

The use of anticholinesterases to increase the amplitude of the potentials was deemed inadvisable in view of the low threshold for propagated responses encountered in rat muscle fibres. In many fibres action potentials were initiated by e.p.p.'s of 6 - 8 mV, a value which agrees with the figures of Lundberg & Quillisch (1953a). Direct electrical stimulation by current pulses through one barrel of a double microelectrode confirmed this low threshold.

The accurate measurement of the amplitude of both spontaneous potentials and e.p.p. responses required a precise localisation of the junction which was recognisable by the brief time course and large size of the potentials. It was necessary to appreciate that for large potentials, involving a significant displacement of the membrane potential toward the equilibrium potential of the e.p.p. (Castillo & Katz, 1954b), the constituent units would no longer sum linearly.

In all measurements it was essential to have baseline noise at a

minimal level, for any unresolved noise would lead to an underestimate of amplitudes when potentials were measured from the upper edge of the immediately preceding baseline.

(iii) Further precautions necessary in the measurement of e.p.p. responses.

Extrinsic potentials were frequently detected and when large could lead to a significant underestimate of response amplitude.

In experiments in which the e.p.p.'s were 'fragmented' by abnormal calcium and/or magnesium concentrations (Sections 2, 3 & 6) care was necessary in deriving the number of failures to a series of stimuli. Theoretically the proportion of failures could be overestimated if stimulation was inadequate or if nerve block occurred. However supramaximal stimulation was used in all experiments, and, in particular, all stimuli of a tetanus were of equal strength. Nerve block would be characterised by a progressive deterioration of response and by an independence of initial response size, but it was not encountered as a source of spurious failures of response.

On the other hand, the presence of spontaneous potentials inevitably leads to an underestimate of failures, since 'unitary' responses and spontaneous potentials are, by amplitude, indistinguishable. It may be shown that, if  $\Delta t$  is the accepted latency variation range of responses and  $T$  is the mean interval of the spontaneous potentials, then, for a series of  $N$  stimuli, the total number of quanta involved in the responses will be overestimated by a number,  $n = N\Delta t/T$ , it being assumed that all spontaneous potentials involve a single quantum only. If the spontaneous discharge is of low frequency and a high sweep velocity is used, this correction is unimportant. Such precautions were observed in Section 2, except in the case of Figs. 21 & 26 which represent experiments performed for the purpose of obtaining illustrations.

In Sections 3 and 6 it was necessary to investigate junctions at which the frequency of the spontaneous potentials was much higher, and hence a correction to the observed number of failures was necessary. If

$\Delta t$  and  $T$  have the same meaning as above, and  $z$  is the observed number of failures in a series then it may be shown that the true number of failures,  $x = z/(1 - \Delta t/T)$ .

No interaction was observed between 'fragmented' responses at a stimulus interval of 1 sec. However Liley & North (1953) have shown that, in a curarised preparation, an e.p.p. is depressed for some 5 - 8 sec after a conditioning e.p.p. Hence for the measurement of amplitudes in such a preparation a minimum stimulus interval of 10 sec would be desirable, which means that a series of some 200 responses would require about 30 minutes of continuous recording. This period is prohibitive, for, besides the risk of the electrode accidentally emerging from the fibre, any drift in membrane potential may more than offset the hypothetical gain in accuracy. Hence, for the appropriate experiments of Section 2 (Table 5), a stimulus interval of 5 sec was accepted as a compromise.

(H). Statistics. In the majority of investigations concerning the effect of various agents on the frequency of spontaneous potentials, quantal content of responses etc., observations were randomized rather than paired.

Paired observations involved long periods of recording at single junctions. Such observations could readily be invalidated by any drift in resting discharge frequency or membrane potential and often such effects could be detected only by analysis after an experiment was concluded. Not infrequently a junction displayed steady behaviour for an hour or longer but in most experiments it proved more convenient to randomize observations. For this purpose a continuous traverse across the preparation with the

microelectrode was undesirable for, although the diaphragm was under light tension only, it is unlikely that tissues were under equal tension at all points (cf. Hutter & Trautwein, 1956). Hence by vascular and neural landmarks the diaphragm was divided into a number of strips and these strips were cyclically assigned to the different stages of the experiment. Within a strip a continuous traverse in 40-50 $\mu$  steps was made with the microelectrode.

Wherever possible groups of observations were made in the sequence: control - test - control. If the initial and final control groups showed satisfactory statistical agreement they were pooled for comparison with the test observations. Where groups of observations showed disparity of variance, the tables of Aspin & Welch (1949) were used in the comparison of means.

Other mathematical or statistical procedures are more conveniently explained as they arise in the appropriate Sections.

SECTION ISPONTANEOUS ACTIVITY AT THE NEUROMUSCULAR JUNCTION OF THE RATINTRODUCTION

This Section describes the occurrence of spontaneous activity at the neuromuscular junction of the albino rat. The characteristics of this activity have been investigated, both in isolated tissues and, to a less extent, in vivo.

RESULTS

On penetration of a muscle fibre, the microelectrode immediately recorded a membrane potential of the order of 70-75 mV (internally negative). Measurements in 31 fibres in one isolated diaphragm gave a mean value for the resting membrane potential of 73.2 mV (s.d. 5.2 mV).

Spontaneous miniature potential changes of rising phase 0.6 - 2 msec and half decay approximately 1.5 msec were readily detected in all innervated muscles investigated (Fig. 7). These potential changes, representing a decreased internal negativity were observed not only in the fibres of the isolated diaphragm but also in the fibres of the gracilis muscle in situ. In the latter case, especially in young animals, minimal disturbances of the tissues was involved as the microelectrodes readily penetrated the intact epimysium. Hence there seems little doubt that the spontaneous potentials are a naturally occurring event rather than a phenomenon - albeit a significant one - appearing only in the questionably normal conditions of isolation.

With isolated tissues in solutions of normal constitution the precise localisation of the miniature potential discharge to the end-plate zone was difficult. Under normal conditions in the rat, unlike the case in the frog, the form and amplitude of the indirectly evoked action potential recorded at the neuromuscular junction shows no characteristic differences from that recorded at points distant along the fibre.

However both in vivo and in vitro the miniature potentials could be detected only at sites where the nerve filaments, as viewed under the dissecting microscope, could be seen to ramify finely and apparently terminate.

Further confirmation was obtained by repetitive indirect stimulation of the preparation at frequencies greater than 50 per second. Under these conditions, at the junction an e.p.p. 'step' does appear on the rising phase of the action potential and eventually the propagated muscle spike fails, leaving merely an e.p.p. By contrast, at points distant along the fibre, no steps are seen on the action potential and conduction appears to be 'all or nothing' in type. Using this criterion the miniature potentials could be detected only at the junctional zone. Similarly electrical identification of the end-plate zone could be achieved by the appearance of an e.p.p. 'step' during progressive neuromuscular blockage by raised magnesium concentrations (6 - 10 mM) or by the addition of d-tubocurarine ( $7 \times 10^{-8}$ ). Again the miniature potential discharge was found to be located exactly at the junction, although in the latter case this discharge was simultaneously ablated.

That the spontaneous potentials arose by the action of quanta of acetylcholine on the end-plate zone of the muscle fibre was strongly suggested by the effects of d-tubocurarine chloride ( $7 \times 10^{-8}$ ) and gallamine triethiodide ( $10^{-4}$ ) in obliterating the discharge and the action of prostigmine bromide ( $10^{-6}$ ) in doubling the amplitude and increasing by approximately 80% the half decay time of the miniature potentials (Fig. 7B <sup>108</sup> 4<sub>h</sub>).

Finally the origin of these quanta of acetylcholine in the nerve terminals was confirmed both by the disappearance of the discharge in the presence of 0.01% novocaine and by observation of the effects of denervation.

Within 12-14 hours of phrenic neurectomy performed 5-6 mm from the diaphragm all response to indirect stimulation had disappeared and within the next few hours the miniature discharge also vanished. Moreover the discharge was never observed in a chronically denervated preparation. On the other hand, as soon as the presence of indirect excitability indicated the regeneration of the motor axons and terminals, the miniature potentials could be readily detected.

#### Amplitude and frequency.

The amplitude of the miniature potentials recorded both in vivo and in vitro was of the order of 0.5 - 1.5 mV. The amplitudes varied not only from one fibre to the next but also at a given end-plate. Usually it was observed (Fig. 8) that the amplitudes were distributed approximately normally about some modal value, but isolated discharges of much greater amplitude were relatively frequent - values as high as 10 mV being occasionally encountered. Some of these large potentials displayed one or more steps on their rising phases (Fig. 7, B1), which may be taken to indicate that they are produced by the temporal summation of smaller miniature potentials. However, frequently no such effect could be observed, the larger potentials presenting a time course almost identical with that of the concomitant miniature discharge (Fig. 7, A<sub>3</sub>).

The frequency of the miniature discharge also varied widely from fibre to fibre. At 37°C, under normal conditions, the extreme values were 1/5 per sec and 118 per sec (Fig. 9), but in over 97% of fibres the frequency did not exceed 7 per sec, the modal value being 1 - 1.5 per sec for both isolated diaphragm and gracilis in vivo. When a given fibre was observed over long periods of time small random variations in frequency were seen and occasionally this variation was superimposed on a progressive



change - usually a gradual acceleration.

The mean frequency of the miniature discharges showed some variation from preparation to preparation but in most cases the extent of this deviation was small.

The amplitude and/or frequency of the miniature potentials could be modified by a number of agents which may be considered as follows:

- a. Anticholinesterases. Prostigmine in a concentration of  $1/10^6$  w/v approximately doubled the amplitude of the discharge without significant alteration in frequency (Figs. 7 B<sub>3</sub> and B<sub>4</sub>, 10 A & B). Occasionally the large potentials were so augmented by the addition of prostigmine that they generated propagated action potentials (Fig. 10 C,D).
- b. Temperature. Over the range of 25-40°C the frequency of the miniature discharge had a positive temperature coefficient, the relation of frequency to temperature being approximately linear to give a  $Q_{10}^{28-38^\circ\text{C}}$  of 3 - 4 (Fig. 11A). Unexpectedly, however, over the range from 16°C (the lowest temperature investigated) to 25°C the frequency appeared to have quite a high negative temperature coefficient. This effect was originally observed during continuous recording from single fibres and, although reversible, was regarded with suspicion on the grounds that slight movements in the apparatus arising from the differing thermal coefficients of expansion of its various components might have led to small displacements of the micro-electrode and hence injured (q.v.) the adjacent nerve terminal. As a check the frequencies of the miniature discharges in a number of different fibres in one preparation were recorded at each of three set temperatures and the resultant graph shows clearly the upward concavity of the frequency-temperature curve.

No consistent relationship between peak amplitude and temperature

was observed. However reducing the temperature to 20°C doubled the duration both of the rising phases and of the half decays of the miniature potentials (Fig. 11B).

c. Indirect stimulation. Following a single nerve volley the probability of the occurrence of a miniature potential was considerably enhanced (Fig. 12A). This enhancement was maximal immediately after the arrival of the volley at the terminals and represented an increase in the miniature potential frequency of 50-200%. Early experiments suggested that the increase was more marked the lower the resting frequency of the discharge (Liley, 1956a) but subsequent experiments failed to confirm this. No significant change in amplitude was observed. Subsidence of the frequency to the original rate occurred in less than 200 msec.

When the preparation was tetanically stimulated, these effects were much more pronounced. For example a tetanus of 3,000 impulses (200 per sec for 15 sec) increased the discharge frequency to a transient value some forty times the resting rate (Fig. 14), again without change in the amplitude of the potentials (cf. Brooks, 1956a). The decline from this peak frequency was initially very rapid, but the return to the resting discharge rate required some 6 or 7 min (Figs. 13, 14, 15A). This time course resembles that of the familiar post-tetanic potentiation of the e.p.p. in a curarised fibre (Fig. 15B), but certain important differences were noticed. Firstly, the increase in the miniature potential frequency was maximal immediately after the cessation of the tetanic stimulation whereas in a curarised fibre the e.p.p. was virtually zero at the end of the tetanus and increased through a subnormal range to a supernormal peak amplitude over a course of some seconds. Secondly, the percentage increase in the miniature potential frequency was much greater than the corresponding

change in the e.p.p. amplitude.

Direct stimulation - either single (Fig. 12B) or repetitive - of the muscle fibre produced no change in the frequency of the miniature discharge. These results indicate that the post-tetanic change in miniature potential frequency was dependent only on presynaptic activity, and, further, that the effect could not be attributed to mechanical trauma which might be produced in the nerve terminal by the microelectrode during the tetanic contractions.

d. Osmotic pressure. Both the frequency and the amplitude of the miniature potentials were affected by the tonicity of the bathing solution (Fig. 16). When the osmotic pressure of the solution was increased 25% by the addition of sucrose the frequency was increased tenfold while the amplitude was reduced by approximately 40%. Both of these effects were readily reversible.

e. Trauma. The invariable response to mechanical trauma inflicted for instance by gentle pulling of the nerve, vibration or small horizontal movements of the microelectrode or the insertion of electrodes which produced visible 'dimpling' of the preparation was an increase in the frequency of the miniature discharge. In some cases values as high as 1000 per sec were observed. Frequently the effect was irreversible, the extent of local mechanical damage being confirmed by a fall in the resting membrane potential of the impaled muscle fibre and often by the failure of neuromuscular transmission. These observations probably account for the gradual progressive increase in the frequency of the miniature discharge that is seen in some fibres during prolonged recording, for minute movements of the preparation due to turbulence in solution flow and any vibration communicated to the microelectrode via the manipulator could injure the adjacent nerve terminals. Similarly the occasional high frequencies observed immediately following

penetration (e.g. values greater than 10 per sec in Fig. 9) could be accounted for by damage to the nerve terminal as the electrode approached the fibre surface.

The miniature potential discharge appeared relatively resistant to anoxia - certainly more so than neuromuscular transmission. Miniature potentials could be recorded at normal frequencies for at least 20 minutes after the immersion of diaphragms in non-oxygenated solution and similarly in the gracilis in situ 15 minutes after the barbiturate-induced death of the rat. However prolonged anoxia induced for instance by immersing the preparation in a non-oxygenated solution beneath a layer of paraffin eventually produced very high frequencies, although there is a considerable likelihood that mechanisms other than anoxia alone, e.g. potassium accumulation, were involved. Sodium cyanide in a concentration of 2 mM per litre reduced the muscle membrane resting potential to a steady value of 40-45 mV within 5 minutes but had no effect on the miniature potential frequency over an observed period of 25 minutes.

#### Extracellular recording of miniature potentials.

Occasionally it was possible both in vivo and in vitro to detect the miniature discharge with the microelectrode tip situated on the surface of the junctional zone of the muscle fibre. Under these conditions, as in the frog (Patt & Katz, 1952; Castillo & Katz, 1956a) there were certain differences from the intracellular records.

- i. The polarity of the potentials was reversed i.e. an increasing negativity of the microelectrode relative to the indifferent electrode was recorded in contrast to the intracellular decreasing negativity (Fig. 17). This change of polarity was extremely sensitive to slight movements of the microelectrode as would be expected from the tenuous nature of the surface membrane.

- ii. The extracellular localization of the miniature potentials was very critical, as evidenced by the fact that, although in several thousand fibres the discharge was detected intracellularly, in only seven fibres was any external sign of activity observed.
- iii. The time course of the miniature potentials was briefer when recorded extracellularly as illustrated by Table 2 and Fig. 18B. The explanation of these differing time courses lies in consideration of the currents flowing in the conventional equivalent circuit of the fibre membrane as a number of capacitor-resistor-battery elements arranged in parallel. The extracellular electrode records the potential change produced by the current that flows as the capacitor elements rapidly equalize their charge. By contrast the intracellular electrode at the junction (as at points distant), records a potential change until such time as the charge on the capacitors is fully restored by the battery elements.

TABLE 2.

Time course of miniature potentials

Mean and S.D. of 16 measurements in each case

|               | Duration of rising phase (msec) | Half decay time (msec) |
|---------------|---------------------------------|------------------------|
| Extracellular | 0.80 (0.20)                     | 1.25 (0.22)            |
| Intracellular | 1.35 (0.28)                     | 1.60 (0.14)            |

- iv. In four of the five fibres in which both extra- and intracellular observations of miniature potentials were made, the extracellularly recorded discharge was noted to be of lower frequency than that recorded intracellularly but this difference lay only at the 'probable' level of statistical significance. In the fifth fibre however (Fig. 18A) the externally recorded discharge was only 20% of the internal recorded rate.

In all cases the extracellularly observed miniature potentials

showed a much greater dispersion of amplitudes than their intracellular counterparts, the co-efficient of variation of the amplitudes of the former being approximately double that of the latter.

These observations accord with the concept of 'active spots' in the end plate surface as postulated by Fatt & Katz (1952) and Castillo & Katz (1955a, 1956a) in the frog, but also suggest that the spatial dispersion of such 'active spots' is less in the rat than in amphibia, the external microelectrode thereby being less discriminative at the mammalian junction. The miniature discharge as a stochastic process.

In the rat, as in the frog, the sequence of miniature potentials appeared to be completely irregular. Moreover no short bursts of high frequency activity such as occur at frog junctions (Fatt & Katz, 1952) were observed. However, in view of the relative frequency of 'miniature' potentials with amplitudes several times that of the modal value, it became necessary to submit the apparent randomness of the miniature discharge to statistical test.

For this purpose the method of analysis described by Feller (1950) and used by Fatt & Katz (1952) on their data from the frog was employed. For complete randomness of the discharge, that is where the probability of occurrence of any one discharge is independent of preceding activity, the relevant relations are:-

- a. For an interval  $\Delta t$ , very brief compared with the mean interval  $T$ , the probability of at least one discharge is given by  $P = \Delta t/T$ .
- b. For a longer interval,  $t$ , the probability of at least one discharge increases exponentially and is given by  $P = 1 - \exp(-t/T)$ .
- c. The intervals between successive discharges in a large series of observations (total number  $N$ ) are distributed exponentially, the frequency of any interval between  $t$  and  $t + \Delta t$  being given by  $n = N \Delta t/T \exp(-t/T)$ .

The applicability of these relations was tested on a series of 929 miniature potentials which covered a total period of 508.1 sec giving a mean frequency of 1.83 per sec and mean interval  $T$  of 547 msec. No significant alteration in the mean frequency occurred during the recording of this series.

Successive intervals between individual miniature potentials were grouped into brackets,  $\Delta t$ , of 50 msec. The resulting distribution, as shown in Fig. 19, fits reasonably with the theoretical distribution for a stochastic process  $n = N \Delta t/T \exp(-t/T)$ . ( $\chi^2 = 20.9$ ,  $f = 30$ ).

Similarly, as shown in Fig. 20, plotting the total number of intervals of duration less than  $t$  against  $t$  gives a curve which accords closely with the predicted curve  $P = 1 - \exp(-t/T)$ . From these results there is little to suggest that the discharge is other than completely random.

However investigation of the statistical distribution of amplitudes of the same series of miniature potentials indicates that some form of interaction certainly does occur. Fig. 8 illustrates this distribution for 903 miniature potentials. In 26 miniature potentials the amplitudes could not be determined accurately as the peaks occurred during the 3 msec 'flyback' of the C.R.O. beam. No potential could be lost entirely during the 'flyback' however, hence measurement of the intervals between discharges was still possible.

The amplitudes of 887 of the 903 miniature potentials are distributed approximately normally about a mean value of 0.58 mV with a coefficient of variation of 30.9%. Further there are no marked secondary humps and the amplitudes do not tail off to zero. Hence this population would appear to indicate the existence, as in the frog, of a standard sized 'unit' discharge. The scatter about the mean value could result from several factors:-

- a. The finite dimensions of the nerve terminals (mean diameter  $3\mu$ , Cole, 1955) resulting in a spatial dispersion relative to the microelectrode of the presynaptic foci emitting these jets of acetylcholine.
- b. The conformation of the terminals leading to some of these foci being situated at points less effective than others for postsynaptic stimulation.
- c. Possible post-synaptic effects of facilitation or depression as a result of activation by previous discharges.
- d. Baseline 'noise' voltage which had a range of ca  $\pm 50 \mu V$  leading to small inaccuracies in measurement.

Besides this population however there is a group of 11 discharges with amplitudes approximately double the unit value, 2 discharges about treble and 3 about 4 or 5 times the mean size. These would appear to represent the simultaneous occurrence of 2, 3 or more discharges respectively. Further, as a high sweep velocity was employed it was possible to ascertain that none of these larger discharges showed a 'stepped' rising phase. Under these conditions the minimum interval between discharges which could be resolved was effectively 2 msec. The probability of one discharge following another within such an interval is given by  $\Delta t/T = 2/547 \approx 1/274$ . The chances that two such intervals (leading to a treble discharge) follow each other is given by  $1/274^2 \approx 1/75,000$ . Similarly the probabilities of quadruple or quintuple discharges are respectively  $\frac{1}{2} \times 10^{-7}$  and  $1/5.6 \times 10^{-9}$ . Hence, were the discharge completely random, we might reasonably have expected to encounter 3 or 4 'double' discharges, but not any larger discharges. Thus the observation of 11 double discharges and 5 with triple or even greater quantal content strongly suggests that at brief intervals at least the sequence cannot be considered entirely random. Further investigations of this phenomenon are described in Section IV.



### DISCUSSION

The occurrence of spontaneous subthreshold activity at the mammalian neuromuscular junction was not unexpected and the present observations indicate that this activity conforms fundamentally to the pattern observed in the frog (Patt & Katz, 1952). Species variation in mean amplitude and frequency of the miniature discharge may be accounted for by differences in multiple interacting factors including temperature, ionic media, excitable membrane constants and motor terminal morphology. The basic picture stands of the spontaneous release of small quanta of transmitter substance from motor nerve terminals, although a complete explanation of the mechanism of this release is lacking.

The effect of prostigmine on the miniature potential amplitude adequately accounts for earlier descriptions of fibrillation induced in mammalian muscles by anticholinesterases (Bacq & Brown, 1937; Masland & Wigton, 1940; Eccles, Katz & Kuffler, 1942) although the origin of fasciculation which implies changes in presynaptic activity remains obscure.

In this connection the observations of Boyd & Martin (1956a) are of interest. They found, in the cat tenuissimus muscle, that prostigmine at a concentration of  $10^{-6}$  -  $10^{-7}$  had some influence on the miniature potential frequency. Such an effect could not be found in the rat diaphragm.

The influence of temperature on the discharge frequency implies that the resting frequency is determined by more than one factor. A priori the rate of acetylcholine synthesis must be involved and, as shown in Section 3, the discharge frequency is very sensitive to the level of the terminal membrane potential. Other processes may also be involved and their differing temperature coefficients would be reflected in the non-linear relation of temperature and resting discharge frequency.

SECTION 2.THE QUANTAL COMPONENTS OF THE MAMMALIAN END-PLATE POTENTIALINTRODUCTION

The relationship of spontaneous subthreshold activity at the neuromuscular junction to the e.p.p. involved in normal neuromuscular transmission has been investigated in the frog by Castillo & Katz (1954a - c) and in the cat tenuissimus muscle by Boyd & Martin (1956b). In both animals it was shown that the e.p.p. was composed of summated miniature potentials.

In this Section the quantal nature of the e.p.p. in the rat has been demonstrated, and, in the light of this finding, a further study has been made of the phenomenon of facilitation at the mammalian neuromuscular junction (Hutter, 1952; Liley & North, 1953; Lundberg & Quilisch, 1953a, b).

Numerous workers have investigated the effects of calcium and/or magnesium on neuromuscular transmission in the mammal (Wiki, 1906; Lubinska, 1935; Boyd & Brosnan, 1936; Brosnan & Boyd, 1936, 1937; Maaske et al., 1938; Boyd et al., 1938; Brown & Harvey, 1940; Hoff et al., 1940; Lundberg & Quilisch, 1953b). The observations of these workers are confirmed and extended by intracellular recording in the present investigation.

RESULTSPart I. Quantal Nature of the End-plate Potential

When the calcium content (normal value 2 mM) of the solution was reduced to 0.5 mM or the magnesium content (normal value 1 mM) was increased to 6 - 8 mM, it was observed on indirect stimulation that the propagated action potential of an impaled muscle fibre intermittently failed leaving merely an e.p.p. of some 6-10 mV. Upon further reduction in calcium or increase in magnesium concentration the e.p.p. became smaller. Simultaneously fluctuations in the e.p.p. amplitude occurred and intermittently there was

complete failure of any response to indirect stimulation (Fig. 21 A & B). The fluctuations appeared to occur in discrete steps each approximately equal to the amplitude of the concomitant miniature discharge.

This observation suggests that the e.p.p. in calcium-deficient or magnesium-enriched solutions arises by the synchronous discharge of a small number of quanta of transmitter, each being equivalent to the quantum involved in the production of a miniature potential. Thus the e.p.p. would consist of summated miniature potentials. This hypothesis previously advanced in the frog (Fatt & Katz, 1952; Castillo & Katz, 1954b) and in the cat (Boyd & Martin, 1956b) is supported by observations of the effect of low temperatures on the e.p.p. response in magnesium-enriched solutions (Fig. 21 C & D). At 22°C the fluctuations in e.p.p. amplitude still occurred, but in addition the rising phases of the responses frequently exhibited one or more inflexions. In a number of instances these steps corresponded in amplitude to the concomitant miniature potentials. As in the frog (Castillo, 1955) it would appear that the reduction in the temperature had sufficiently slowed the activation of the nerve terminal to produce a temporal dispersion of the quantal components of the e.p.p.

The membrane potential of muscle fibres was not significantly affected by the changes in calcium or magnesium concentrations that induced these alterations in the e.p.p. pattern (Table 3). Further, in high concentrations of magnesium there was no significant change in the mean frequency of the miniature potentials, although, for example, their amplitudes in 12 mM magnesium were reduced to about 60% of the values recorded in normal solutions (cf. Maaske & Gibson, 1939; Engback, 1948). However this reduction was of a much smaller order than that of the e.p.p. at a similar concentration of magnesium.

TABLE 3.

Effect of magnesium concentration on mean membrane potential in groups of resting muscle fibres in one preparation. Groups were recorded in the order shown in the table.

|                                 | Normal Solution<br>(1 mM Mg) | Solution enriched<br>with 12 mM Mg | Normal Solution |
|---------------------------------|------------------------------|------------------------------------|-----------------|
| Number of fibres                | 20                           | 19                                 | 11              |
| Mean membrane<br>potential (mV) | 73.1                         | 70.0                               | 73.4            |
| S.D. (mV)                       | 6.1                          | 9.1                                | 3.0             |

Reduction of calcium concentration to 0.5 mM exerted no significant effect on the miniature discharge frequency, but in the complete absence of calcium a reduction in frequency was observed (Table 4). This reduction was statistically significant in one experiment (Preparation B,  $p < .01$ ) but not in another (Preparation A,  $p > .05$ ) in which the control values of the discharge frequency were unusually low.

TABLE 4.

Effect of calcium on resting discharge frequency in two preparations. Mean values  $\pm$  S.D. and range of frequencies in groups of junctions, the number of junctions in each group being indicated in parentheses. Groups were recorded in the order shown in the table, the preparations being allowed two hours to equilibrate with each solution. Magnesium 1 mM (normal) throughout.

| <u>Preparation A.</u>              | <u>2mM Ca.</u><br>(normal) | <u>Zero Ca</u>       | <u>2mM Ca</u>        |
|------------------------------------|----------------------------|----------------------|----------------------|
| Mean frequency(sec <sup>-1</sup> ) | 0.71 $\pm$ 0.28(20)        | 0.61 $\pm$ 0.39 (23) | 0.78 $\pm$ 0.47 (19) |
| Range (sec <sup>-1</sup> )         | 0.3 - 1.4                  | 0.2 - 1.7            | 0.2 - 1.8            |
| <u>Preparation B</u>               |                            |                      |                      |
| Mean frequency(sec <sup>-1</sup> ) | 1.60 $\pm$ .60(20)         | 1.07 $\pm$ .54 (20)  | 1.41 $\pm$ .70 (20)  |
| Range (sec <sup>-1</sup> )         | 1.0 - 3.2                  | 0.4 - 2.4            | 0.5 - 3.3            |

It would seem reasonable to infer that the e.p.p. under normal conditions also consists of summated miniature potentials, and, on this assumption, an attempt may be made to derive an approximate value for the quantal content of a normal e.p.p. Unfortunately in the rat no accurate estimate of the amplitude of the 'normal' e.p.p. can be made. In contradistinction to the frog, the rising phase of the action potential recorded at the myoneural junction shows no obvious inflexion even when examined with an electronic differentiator.

However, when prolonged repetitive stimulation at 50 impulses per sec has brought about impending failure of neuromuscular transmission, an e.p.p. step does appear on the rising phase of the junctional record. With action potentials of normal amplitude (80-100 mV) this step varied from 20-40 mV in different fibres when first observable. Greater reliance may be placed on the larger values of this range for they illustrate the situation when the intracellular electrode is accurately located at the junctional region. Experiments on curarised muscle fibres of the rat diaphragm have indicated that the e.p.p. decrements to  $1/e$  in about 200  $\mu$  (Fig. 22).

Thus, from the value of 40 mV for the step at junctions partially fatigued by a tetanus, it would appear likely that the amplitude of the e.p.p. under normal conditions is of the order of at least 50 mV and possibly longer. Now, if such an e.p.p. consists of summated miniature potentials each of the order of 1 mV, the minimal quantal content must be 50. Certainly the number must be larger, for as pointed out by Castillo & Katz (1954b) large numbers of miniature potentials would not sum linearly since successive quanta of transmitter contribute smaller potential increments. Hence it may be deduced that the normal e.p.p. would involve the simultaneous discharge of probably

some 80-100 miniature potentials at least, and the maximal number of quanta which could contribute to a response may be even larger.

However the observation of apparent quantal fluctuation of the e.p.p. in calcium-deficient or magnesium-enriched solutions is not in itself sufficient evidence to justify the hypothesis that the e.p.p. consists of summated miniature potentials. A statistical analysis is necessary in order to exclude the alternative possibilities, either that the fluctuations indicate a continuously variable release of transmitter, or that the discharged quanta have a variety of amplitudes.

The method of analysis employed has been explained fully by Castillo & Katz (1954b), but the salient principles may be recapitulated briefly.

Let us assume that a nerve terminal contains a population of  $n$  units of transmitter capable of release by an impulse. These units need not have a uniform probability of release, but it is assumed that the probability for any given unit remains constant. Hence, if  $\bar{p}$  is the average probability of release by an impulse, the number,  $m$ , of quanta comprising an e.p.p. is given by  $m = n\bar{p}$ .

In normal solutions  $\bar{p}$  may be assumed to be large, which signifies that a considerable proportion of the quantal population is liberated by an impulse because there is evidence that some 45% of the freely available acetylcholine present in the rat phrenic nerve terminals may be discharged by a single volley (Liley & North, 1953). However in calcium-deficient and/or magnesium-enriched solutions the small size and intermittent failure of the e.p.p. indicate a drastic reduction of the chances of any given unit being discharged. Effectively the probability of response of any quantal unit would appear to be at most some few per cent of the value pertaining in normal solutions.

Under such conditions where  $\bar{p}$  is very small, the relative frequency with which, in a large series of observations, an e.p.p. contains  $x$  ( $= 0, 1, 2, 3$  etc.) units should be distributed according to the Poisson expansion  $e^{-m} m^x / x!$ . The parameter,  $m$ , of the Poisson series here represents the mean unit content of the e.p.p. On the basis of the hypothesis that the e.p.p. consists of summated synchronous miniature potentials it may be defined as

$$m = \frac{\text{mean amplitude of e.p.p. response}}{\text{mean amplitude of miniature potentials}} \dots\dots\dots (1)$$

Implicit in this relation is the assumption of linear summation of the miniature potentials, which is justifiable only if the response is so small that it does not displace the membrane potential significantly towards the equilibrium potential for the e.p.p. (Castillo & Katz, 1954b). By substitution of the above value of  $m$  in the first term of the Poisson series,  $\exp(-m)$ , we may calculate the expected proportion of failures to a series of nerve impulses and hence compare this value with the observed proportion.

More conveniently, however, from the observed proportion of failures we may calculate, on the assumption of a Poisson distribution, a value for  $m$  independent of equation (1), since the first term of the Poisson series may be recast as

$$m = \log_e \frac{\text{number of nerve impulses}}{\text{number of failures of e.p.p. response}} \dots\dots\dots (2)$$

Equality of these two estimates of  $m$  would confirm the validity of our assumptions.

Fig. 23 illustrates the results of twenty-six experiments in which this test was applied and shows that the two estimates of  $m$  are approximately equal. In practice a complication frequently arose in that the microelectrode detected a variable extrinsic potential due to e.p.p.s at the junctions of closely adjacent fibres (see Fig. 21B, records 2 & 6; also Fatt & Katz,

1951; Easton, 1955). Not infrequently the amplitude of these extrinsic e.p.p.s was some 5 or 10% of the e.p.p. observed at the impaled junction. Apparently in such cases the e.p.p.s at adjacent junctions were much larger than those in the fibre under observation. Since these extrinsic e.p.p.s were of the opposite polarity to the e.p.p.s recorded in the impaled fibre, the practice of measuring the amplitude of e.p.p.s from the immediately preceding baseline would lead to an underestimate of the mean e.p.p. response. On the other hand the measurement of the mean amplitude of the miniature potentials was not compromised by this factor, and hence the value of  $m$  derived by equation (1) would be an underestimate. It is likely that this complication could account for the small deviation from predicted equality that is observed in Fig. 23.

Essentially the preceding analysis is equivalent to the prediction of the number of failures of response to a series of impulses from the mean quantal content of the responses. However, if our assumptions are correct, it should be possible to predict not only the number of failures in a series but also the entire distribution of amplitudes of the responses.

Fig. 24 illustrates an experiment in which this prediction was attempted. The value of  $m$ , determined by equation (1) was used to calculate the successive terms of the Poisson series. The first term of the series (giving the predicted number of failures) agreed exactly with the observed value but in distributing the remaining terms it was necessary to allow for the scatter of amplitudes of the 'unitary' spontaneous potentials (Fig. 24, inset). This was done by calculating the normal curve which would correspond to the miniature potential histogram and using  $x$  times the mean and variance of this curve in distributing the remaining Poisson terms. The distributed curves were then summed to give the theoretical distribution



of Fig. 24. The general form of this curve agrees reasonably with the observed histogram. The discrepancies between the observed and calculated peak positions would disappear if the mean amplitude of the spontaneous potentials had, in fact, been underestimated by 50  $\mu$ V. Such an error in measurement is possible with the photographed baseline thickened by unresolved high-frequency noise (Castillo & Katz, 1954a).

A further test of the hypothesis arises from the fact that the coefficient of variation of a population obeying a Poisson distribution is given by  $m^{-0.5}$ . Fig. 25 illustrates the results obtained in 24 experiments in which the coefficients of variation of the e.p.p. amplitudes were compared with the predicted coefficients  $m^{-0.5}$ . Again there is good agreement with prediction. Thus both types of experimental test have provided strong support for the initial hypothesis and the assumptions implicit in it:

(a) that neuromuscular transmission is a quantal process involving the release of units of transmitters whose sizes are indicated by the spontaneous miniature potentials; (b) that for a response of low quantal content the precise number of contributing quanta varies in a statistically predictable manner; (c) that small numbers of miniature potentials sum linearly.

With calcium and magnesium concentrations nearer or at normal values the preceding methods of estimation of the quantal content of the e.p.p. are no longer applicable. The peak of the e.p.p. is obscured by the muscle action potential, the constituent units of a large e.p.p. do not sum linearly and no failures of response occur. However an estimate of the quantal content may be attempted by measurement of the coefficient of variation of such an e.p.p. in the presence of curare for, although the spontaneous potentials are obliterated, the reduced e.p.p. no longer generates a muscle spike and its constituent units may be assumed to sum linearly (Martin,

1955). On the assumption that a Poisson relation still obtains, i.e. that the number of quanta,  $m$ , liberated by an impulse is very much smaller than the total number of quanta,  $n$ , available for liberation, then the quantal content of the e.p.p. may be calculated from the relation:-

$$\text{Coefficient of variation} = m^{-0.5}$$

Table 5 shows the results obtained for the e.p.p.s at five curarised junctions.

TABLE 5.

Quantal content ( $\pm$  S.E.) of e.p.p.s in solution of normal ionic constitution. Tubocurarine chloride  $1.3 \times 10^{-6}$ . See text.

| Preparation A. | Fibre | Quantal content of e.p.p. |
|----------------|-------|---------------------------|
|                | 1     | 271 ( $\pm$ 45)           |
|                | 2     | 174 ( $\pm$ 20)           |
|                | 3     | 170 ( $\pm$ 17)           |
| Preparation B. |       |                           |
|                | 1     | 339 $\pm$ 30              |
|                | 3     | 178 $\pm$ 18              |

Such calculations however indicate little more than the order of the true content. First, any inaccuracy in measurement due to baseline noise leads to an underestimate of 'm' since the calculation assumes all variation to be an intrinsic property of the e.p.p. Secondly it is unlikely that a Poisson relation applies for a normal e.p.p., for Liley & North (1953) deduced that about 45% of the freely available acetylcholine present in the terminals is liberated by a single impulse. Under these conditions, i.e. where  $m$  is not very much smaller than  $n$ , the e.p.p. amplitudes would display a binomial type of distribution. Hence the estimate of Table 5 would seriously overestimate the quantal content since the relevant relation

should be of the type:

$$\text{Coefficient of variation} = (m^{-1} - n^{-1})^{0.5}$$

(if all quanta have the same probability of release per impulse, i.e.  $\text{var } p = 0$ ) or else:-

$$\text{Coefficient of variation} = \left[ m^{-1} \left( 1 - \frac{\text{var } p}{\bar{p}} \right) - n^{-1} \right]^{0.5}$$

(if the probability of release per impulse is not the same for all quanta;  $\bar{p}$  = mean probability,  $\text{var } p$  = variance of individual probabilities) (see Kendall, 1948).

## Part II. Facilitation at the Mammalian Neuromuscular Junction

At a junction at which transmission has been blocked by low calcium or high magnesium concentrations the pattern of response evoked by indirect tetanic stimulation (Fig. 26B) differed strikingly from the record obtained in a curarised preparation (Fig. 26C). In the curarised preparation the ep.p.s of a tetanus exponentially declined to a plateau (Liley & North, 1953), whereas at magnesium-treated or calcium-deficient junctions the successive responses while still fluctuating tended to be progressively increased. Although discernable at frequencies of stimulation as low as 10 per sec, this effect was much more obvious at higher frequencies. Hence it appears that under these conditions some form of potentiation is occurring, which is maximal immediately following the arrival of an impulse at the terminals and which persists for some 100 msec at least.

Such a potentiation of response could arise by any one or more of three mechanisms:

- (a) An increase in the sensitivity of the muscle junctional membrane to the transmitter;
- (b) An increase in the number of quanta of transmitter liberated;
- (c) An increase in the molecular content of the quanta.

The first and third mechanisms may be dismissed on the evidence that spontaneous potentials occurring in the course of a tetanus, or immediately after it (e.g. Fig. 26B, records 3, 5 and 6) show no significant change in amplitude from those discharging pretetanicly.

A more precise discrimination is provided by statistical analysis. Essentially this analysis is the same as that employed in the preceding section. Increase in the number of quanta liberator<sup>ed</sup> (mechanism b) leads to the expectation that in a large series of tetani the proportion of failures of response to each succeeding stimulus will decrease in a predictable manner. On the other hand according to mechanisms (a) or (c), alteration respectively in the size or effect of the quanta but no change in the probability of their release, it may be predicted that the proportion of failures remains constant, and hence that the two values of 'm' derived respectively from equations (1) and (2) of the preceding section should diverge progressively.

In all of nine experiments a progressive decrease in the proportion of failures during a tetanus was observed. Hence for analysis it was desirable to select a junction showing a high proportion of failures to a single stimulus. Fig. 27 illustrates the results obtained at such a junction when 100 brief tetani were recorded. For the corresponding responses, the values of m from equations (1) and (2) were derived. The coincident progressive increases in these independent determinations of m indicate that the potentiation of response arises simply by the liberation of more quanta of transmitter.

This rise in the probability of release of the quanta did not develop linearly throughout the duration of a tetanus. As shown in Fig. 27 the potentiation increased rapidly over the first few responses and

then more slowly. This slower increment continued for a considerable time. Thus at a frequency of 200 impulses per sec some 6 to 10 seconds were required for the fluctuating responses to reach a steady mean amplitude (Fig. 28).

Following a tetanus the responses to single test stimuli showed a marked potentiation (Fig. 29). Since the responses were still fluctuating, the time course of this potentiation was studied by averaging serial test responses over short intervals. By this analysis it was found that, although the decline from the mean response amplitude attained over the final stages of a tetanus was initially rapid, return to the control level of response was protracted (Fig. 30B, 31B & D). Furthermore, the total duration of the potentiation was dependent on the duration of the tetanus.

Concomitant with this post-tetanic potentiation of the response to indirect stimulation there was an increase in the frequency of the spontaneous miniature potentials (Fig. 29). This effect, previously observed at junctions in normal solutions (Figs. 13, 14) appeared to be unaffected by changes in calcium and magnesium concentrations. The time course of this change in frequency closely paralleled that of the potentiation of response (Fig. 30A, 31A & C) although the proportional increase in the discharge frequency was some two to three times greater than the change in response amplitude.

### DISCUSSION

The hypothesis that the spontaneous miniature potentials represent the units of transmitter activity and that the e.p.p. consists of a summation of such units has been tested at the amphibian myoneural junction (Castillo & Katz, 1954b) and more recently in a mammalian tissue (Boyd & Martin, 1956).

The present investigation indicates that the neuromuscular junction of the rat also conforms to this pattern of activity.

Strictly speaking, all the experimental evidence supporting the hypothesis has been obtained at concentrations of calcium and/or magnesium far removed from values considered normal, although recently Martin (1955) has extended investigations into a range of concentrations nearer normal values. In so far as it affects the release of acetylcholine, the action of magnesium may be considered as due to a simple displacement of calcium from some essential process (Castillo & Engbaek, 1954; Castillo & Katz, 1954a). However the precise nature of this intermediate process in which calcium is involved remains obscure. Nevertheless the continuous relation pertaining between acetylcholine output and calcium concentration (Castillo & Stark, 1952) justifies the application of the hypothesis to the behaviour of the junction in normal solutions, where it is no longer possible to apply the statistical methods which support the hypothesis in calcium-deficient media.

Recent independent investigations of the changes induced in mammalian neuromuscular transmission by conditioning indirect stimulation have produced results in close agreement. Hutter (1952) and Liley & North (1953) demonstrated a post-tetanic potentiation of e.p.p.s in curarised mammalian muscles, and in both investigations it was concluded that this effect was produced presynaptically, i.e. that there was a temporary post-tetanic increase in the amount of acetylcholine liberated by a volley. Liley & North (1953) also observed that in the curarised rat diaphragm a single conditioning volley was followed by an early brief (200-300 msec) phase of potentiation superimposed on the prolonged (5-8 sec) depression of transmission. This finding was confirmed by Lundberg & Quilisch (1953a). Brown &

Harvey (1941) had noted a similar effect in partially curarised extra-ocular muscles. Hence, although a single volley may be considered as a limiting form of a tetanus, it would seem reasonable to replace the term 'post-tetanic potentiation' (PTP) by 'post-activation potentiation' (PAP). This latter expression will therefore be used in further discussion in this Section.

Liley & North (1953) introduced the concept of the 'release factor' or fraction of freely available acetylcholine present in the nerve terminals, which is liberated by a single impulse. From an analysis of the changes which a conditioning tetanus evokes in the pattern of a brief test tetanus, they deduced that the PAP of the e.p.p. resulted, not from an increased concentration of transmitter in the terminals following activity, but from an increase in the fraction of transmitter released. This increase in the release factor was maximal immediately after the conditioning activity. Hence it was concluded that the serial decline in responses during a tetanus and the depression of response following a single volley indicated a depletion of the store of freely available transmitter. The transmitter concentration and the release factor recovered to their normal values with different time courses; only after a considerable number of impulses was the increase in release factor great enough to outlast the depressed concentration of free transmitter and so produce an absolute potentiation of response. Lundberg & Quilisch (1953a) also postulated a "mixture" of potentiation and depression' to explain their observations with intracellular electrodes in curarised rat diaphragm. It is therefore of interest to test this interpretation at single junctions in a calcium deficient and/or magnesium-enriched solution, because, provided that choline-acetylase activity is little altered in such a solution (cf. Feldberg & Hebb, 1947), the small number

of quanta liberated by an impulse should not significantly deplete the concentration of transmitter in the terminals.

The present results indicate that at the mammalian junction, as in the frog (Castillo & Katz, 1954c), potentiation is a quantal process involving the progressive recruitment of responding miniature units. Hence the 'release factor' is related to the mean probability of response of the miniature units. Further the results (Figs. 29, 30A, 31A & C) show that, with responses of low quantal content, the PAP of the e.p.p. is maximal immediately after the conditioning activity. This observation supports the view that the initial depression following activity in a curarised preparation in normal solutions results from a reduction in the concentration of the freely available transmitter in the terminals.

The post-activation increase in the frequency of the spontaneous miniature potentials, previously observed at junctions in normal solutions is now shown to be unaffected by changes in calcium concentration. Since this post-activation potentiation of discharge frequency runs a time course paralleling the PAP of response, there is good ground for postulating a common causation. However any explanation must account for the observation that the mean probability of the quanta discharging spontaneously is increased by a greater (by 2 or 3 times) factor than the mean probability of their discharge by an impulse.

The observation that the order of the PAP of discharge frequency is independent of calcium concentration is rather unexpected. It is reasonable to consider that the frequency of the miniature potentials would be determined by two factors:

- (a) the total number of available quanta;
- (b) the mean probability of their spontaneous discharge.



In normal solutions the immediate PAP of discharge frequency may still be explained despite a gross reduction in the number of available quanta provided the mean probability of the spontaneous discharge of the remaining available quanta is increased sufficiently. However, in calcium-deficient solutions, where the initial depletion of quanta must be largely obviated, a much greater order of PAP of discharge frequency would be predicted. However no such change in the order has been observed.

The maximum PAP of the e.p.p. which can be evoked in curarised mammalian muscle is of the order of 200-250% (Hutter, 1952; Liley & North, 1953). The work of Martin (1955) indicates that for the small displacement of muscle membrane potentials occasioned by the e.p.p. in the presence of curare, linear summation of the constituent units may be assumed. Hence a potentiation of an e.p.p. by 200-250% represents an increase in the number of units discharging by a factor of 2 to 2.5. This value falls far short of the potentiation which may be elicited in calcium deficient solutions, for example an increase in the mean response by a factor of 12.6 after 3400 impulses (Fig. 30B). However this discrepancy is readily explained.

First, in normal solutions following a tetanus of the order of 3000 impulses, for several seconds, the increase in the release factor cannot outweigh the gross depletion of available quanta in a terminal. Hence potentiation is not maximal until some 10-20 seconds post-tetanicly by which time the increase in the release factor has declined considerably from its initial maximal value.

Secondly, it is important to consider the mean probability of the quanta contributing to a response in normal solutions. A release factor of 0.45 for the rat nerve terminals (Liley & North, 1953) implies that no process can increase it by more than 2 - 2.5 times. On the other hand, in

calcium-deficient solutions the small mean probability of the quanta contributing to a response gives adequate scope for the exhibition of a potentiating process.

SECTION 3.THE EFFECTS OF PRESYNAPTIC POLARISATION ON THE SPONTANEOUS ACTIVITY AT  
THE MAMMALIAN NEUROMUSCULAR JUNCTIONINTRODUCTION

It has been shown (Boyd & Martin, 1956b; Section 2) that the mammalian e.p.p. is generated by the synchronous release of quanta of transmitter whose individual spontaneous liberation gives rise to the miniature potentials. These observations immediately raise the question of the mechanism by which a coordinated discharge of the quanta is produced by an impulse arriving at the motor nerve terminals. Castillo & Katz (1954d) have investigated a similar problem at the frog myoneural junction.

In this Section a study has been made of the relation between the frequency of the miniature discharge and electrotonic polarization of the motor nerve terminals. A study has been made also of the effects of potassium concentration on the frequency of the miniature potentials.

These investigations have led to the development of an hypothesis concerning the release of acetylcholine by an impulse and this hypothesis has been subjected to further tests.

RESULTSPart I. Effect of electrotonic polarisation of motor terminals on miniature  
discharge frequency.

Cathodic polarisation (see Methods) of the terminals produced an increase and anodic polarisation a decrease in the frequency of the miniature discharge (Fig. 32). These effects were graded according to the intensity of the polarising current and at a given junction were readily reproducible.

The mean amplitude of the miniature potentials was unaffected by the polarisation of the terminals.

The polarising current did not produce any change in miniature discharge activity at junctions whose axons were severed close to the terminals, nor could any effect be obtained at junctions situated more than a few millimetres from the sealed notch transmitting the nerve. These observations establish that the changes in frequency were dependent on electrotonic polarisation of the nerve terminals and that the current flow in the bath solution played no part in their causation.

Experiments involving the application of rectangular current pulses to the terminals showed that the alteration in discharge frequency developed very rapidly (Fig. 33). By 2 msec after the onset of the polarising current the frequency had attained a value from which no statistically significant deviation occurred by 16, 50 or 150 msec. With more prolonged applications of current the discharge was stable at its new frequency over the observed range from 1 sec to 5 min. Thus, when the membrane potential of the nerve terminals is altered by a polarising current, the frequency of the miniature potentials faithfully parallels the potential, there being no appreciable delay at the onset nor decline during a prolonged application of the current change.

Unfortunately, events within the initial 2 msec after the onset of the current were obscured by artefacts. With electrotonic polarisation of the terminals it would not be expected that the frequency would attain its final value instantaneously with the onset of current. First, the normal neuromuscular delay means that recorded activity lags behind presynaptic events by about 1 msec. Secondly, although the generating circuit delivered a rectangular pulse, the wave front of a pulse as it affected a nerve

terminal would be distorted in a manner determined by the time and length constants of the axonal membrane. No reliable estimates of these constants in the terminal branches of the mammalian motor axon are available. The distortion may be minimised by selecting a junction as close as possible to the orifice admitting the nerve but without a knowledge of the time constant of the axon it cannot be asserted that the discharge frequency instantaneously follows the changing membrane potential.

The frequency of the miniature discharge was not linearly related to the current intensity (Fig. 32). However, in fifteen out of seventeen experiments on normal junctions, when the logarithms of the frequencies were plotted against the respective current intensities, a relation was obtained which, statistically, was strongly suggestive of linearity (Fig. 34A).

In the two deviating series the depression of discharge frequency with anodic polarisation was less than expected, the larger deviation being illustrated in Fig. 34B. In each experiment redetermination of the resting frequency showed that no progressive change had occurred. At these very low frequencies, even when series are recorded over several minutes, there may be considerable error in the determination of the frequency, and further, any errors may be magnified in a logarithmic plot, but it is not to be expected that all the determinations would be overestimates of the true values. The significance of these two experiments is difficult to assess. The records showed no bursts of high frequency activity and the effect does not remotely resemble the triggered "breakdown" discharge encountered with strong hyperpolarisation of frog nerve terminals (Castillo & Katz, 1954d).

In calcium-deficient or magnesium-enriched solutions the effects of both depolarising and hyperpolarising currents on the miniature dis-

charge were markedly reduced. Thus with a calcium concentration of 0.25 mM (normal 2 mM) or a magnesium concentration of 12 mM (normal 1 mM) polarising currents of either sign were virtually unable to displace the discharge frequency from its resting value (Fig. 34B). However, in two experiments with 12 mM magnesium, intense depolarising currents were able to produce a small increase in the frequency. When the effects of polarisation had been suppressed by 12 mM magnesium, almost complete restoration was obtained by raising the calcium concentration to 8 mM.

An investigation was made of the ability of polarising currents to modify the miniature discharge frequency when this had been artificially displaced from its resting value. At three junctions an increase in the discharge frequency to the order of 100 per sec was produced by small (3 - 8  $\mu$ ) lateral movements of the microelectrode. Although the frequency was not very stable following this procedure, in each case it was readily influenced by both anodic and cathodic polarisation (Fig. 35A) following the same empirical linear relation between current intensity and logarithm of discharge frequency. Similarly, when the discharge frequency was increased from 2 per sec to 43 per sec by the extracellular application of 15 mM potassium chloride (q.v.), it was still affected in the same manner by polarising currents (Fig. 35B).

In a nerve fibre distal to a blocked segment of the nerve, several authors (Hodgkin, 1937; Lorente de Nó, 1939; Tasaki, 1939) have demonstrated the electrotonic depolarisation produced by an impulse approaching the block. Now, since the miniature discharge frequency was modified by an applied electrotonic potential, it was reasonable to expect that the frequency would also be affected by the physiological electrotonic depolarisation produced by an impulse reaching a blocked segment of the axon very

near the terminal. This expectation was tested using a cold block (see Methods), and, as shown in Fig. 36, a transient small but significant increase in the discharge frequency occurred at an interval (corresponding to the normal neuromuscular delay) after the arrival of an impulse at the preterminal block.

## Part II. Effect of potassium concentration on the miniature potentials.

Alteration in potassium concentration affected both the amplitude and frequency of the miniature discharge. Reduction in potassium concentration below the normal level of 5 mM produced no significant change in the amplitude of the discharge, but increasing the concentration to 30 mM reduced the amplitude to approximately one third. This effect would appear to be postsynaptic in origin, and to result from the fall in muscle membrane potential which is observed in high potassium concentrations. It may be assumed that the action of acetylcholine on the mammalian postsynaptic membrane is similar to that demonstrated in the frog (Fatt & Katz, 1951), hence it would be expected that the amplitude of the miniature potentials would vary with the level of the muscle membrane potential.

Much more striking was the effect of potassium on the frequency of the miniature potentials (Fig. 37, hollow circles). Reduction of the potassium concentration from 5 mM to 2 mM produced a small but statistically significant reduction in the discharge frequency. On the other hand the frequency was more than doubled when the concentration was increased from 5 mM to 10 mM. At 30 mM the frequency was of the order of 700 per sec. There was a further increase at higher concentrations, but an accurate count was no longer possible. Over the range of 10-30 mM the mean frequency appeared to vary linearly with potassium concentration. When any single junction was observed following a change in the potassium concentration, it was

observed that some 1-4 minutes had elapsed before the alteration in discharge frequency was fully developed. Thereafter the discharge rate was stable for at least 20 minutes even when it was above 500 per sec.

The effects of potassium on the discharge frequency were modified considerably by high concentrations of magnesium. As had been observed previously, magnesium was without effect on the frequency at junctions in solutions of normal potassium content (Section 2; Boyd & Martin, 1956a; see also Castillo & Katz, 1954a). However, in the presence of 12.5 mM magnesium, reduction in potassium concentration exerted no significant effect on the discharge frequency (Fig. 37, filled circles) while the influence of raised concentrations of potassium was markedly depressed. Thus 30 mM potassium in the presence of (normal) 1 mM magnesium increased the mean frequency to 715 per sec whereas in 12.5 mM magnesium the increase was limited to 74 per sec. Nevertheless, as seen in Fig. 37, in high concentrations of magnesium there was still a linear relation between the discharge frequencies and potassium concentrations greater than 10 mM.

#### DISCUSSION AND TEST OF HYPOTHESIS

The present results show that the effects of presynaptic polarisation on the miniature discharge are not the same in mammals and amphibia (Castillo & Katz, 1954d). Although depolarising currents produced comparable effects, moderate hyperpolarisation was without influence on the discharge in the frog whereas in the rat the discharge was readily depressed well below the resting value in all of seventeen experiments. It might possibly be argued that the motor nerve terminals of the isolated rat diaphragm are already depolarised on account of their abnormal environment and that hyperpolarising currents are merely restoring the membrane potential and hence reducing the discharge frequency toward a true basal level. This is unlikely, however,



for experiments on the gracilis muscle in vivo (Section I) indicated that the mean frequency of the miniature discharge was of the same order as that observed in the isolated diaphragm. Furthermore, an increase in magnesium (or a reduction in calcium) concentration offsets the increase in frequency produced by depolarising currents, but is without effect on the resting discharge frequency in the isolated diaphragm.

The sudden bursts of high frequency activity observed in the frog with strong anodic polarisation (Castillo & Katz, 1954d) were not encountered in the rat diaphragm despite the fact that, as judged by cathodic effects, comparable current intensities across the terminal membrane were obtained in the two investigations (compare Fig. 5 of Castillo & Katz, 1954d, with Fig. 34 of the present paper). If this phenomenon in the frog results, as suggested, from an event resembling a dielectric breakdown, the absence of any comparable effect in the rat indicates that the mammalian membrane is more resistant to electrical stress.

The effects of polarising currents suggest that the discharge frequency should be influenced when the membrane potential is altered by variations in extracellular potassium concentration. Unfortunately the membrane potential of the motor nerve terminals cannot be directly measured. Furthermore, the membrane potential will not be linearly related to the logarithm of potassium concentration as predicted by the Nernst equation. Rather, as shown by Hodgkin & Katz (1949), an equation derived from the constant field theory of Goldmann would apply. This equation predicts that a low concentration of potassium will exert little influence on the membrane potential, the effect of other ions being dominant, but as the concentration of potassium is progressively raised above normal, the membrane potential will more nearly be related linearly to the logarithm of the potassium

concentration as predicted by the Nernst equation.

Observations with polarising currents at junctions covering a wide range of resting frequencies have indicated a linear relationship between the logarithm of the discharge frequency and the polarisation of the motor nerve terminals (Figs. 34A, 35). Therefore it may be predicted that, as the potassium concentration is increased, a value will be reached beyond which the logarithm of the discharge frequency will be related linearly to the logarithm of potassium concentration. Alternatively, dispensing with logarithms, the frequency would be expected to vary directly with the potassium concentration. The experiments illustrated in Fig. 37 show that this prediction was fulfilled for potassium concentrations beyond 10 mM. Unfortunately, investigations could not be carried beyond 30 mM since the discharge became too frequent for an accurate count. However, accepting the risks inherent in extrapolation, the straight line in Fig. 37 indicates that, beyond a concentration of 10 mM, a tenfold increase in potassium concentration augments the discharge frequency by a factor of  $10^4$ .

If it be assumed that beyond 10 mM the potassium concentration determines the membrane potential of the motor nerve terminals in the manner predicted by the Nernst equation, it follows that the miniature discharge frequency is increased by a factor of  $10^4$  for a decrease in the membrane potential of approximately 60 mV. Hence from the logarithmic relationship between discharge frequency and displacement of the membrane potential (Figs. 34A, 35) the discharge frequency corresponding to any change of the membrane potential may be predicted. Similarly it may be calculated that, for a muscle in 12.5 mM magnesium the discharge frequency would be altered by a factor of  $10^{2.5}$  for a change in the membrane potential of approximately 60 mV.

Since the mammalian end-plate potential consists of summated miniature potentials (Boyd & Martin, 1956b; Section 2) it is reasonable to interpret the increase in miniature discharge frequency associated with depolarisation as a prolonged and attenuated version of the events occurring when the nerve terminals are briefly and intensely depolarised by an action potential. This interpretation is supported by the observation that neuromuscular transmission and the effects of depolarising current are both depressed by increase in magnesium concentration, an action which in turn is antagonised by calcium. Several predictions capable of simple experimental test can be derived immediately from this hypothesis that the e.p.p. or liberation of quanta by an impulse is simply the random release of quanta accelerated by depolarisation of the terminal membrane.

Firstly, on the tacit assumption that the action potential is of the same amplitude in all of the motor nerve terminals, the quantal content of the e.p.p. should be proportional to the resting discharge frequency. This relationship should apply not only for normal neuromuscular transmission but also for conditions where the effect of an impulse has been attenuated by an excess of magnesium and/or a deficiency of calcium ions. In this latter case, where the quantal content of the responses is small and easily measurable, the predicted relationship may be readily tested.

Figs. 38 & 39 illustrate the results obtained when a number of junctions in two preparations were investigated. The points in these graphs show considerable scatter. Indeed previous observations on a smaller number of junctions prompted the statement that the quantal content of a response and the miniature discharge frequency were not directly related - although both were increased following indirect stimulation (Liley, 1956b). However, as shown by the larger series of observations in Figs. 38 & 39, the

quantal content of a response and the resting discharge frequency show a positive correlation which, statistically, is highly significant. As would be expected the regression line is steeper in Fig. 39 ( $\gamma = 0.25x + 0.01$ ) where the calcium concentration was 1.5 mM than in Fig. 38 ( $\gamma = 0.16x - 0.01$ ) with calcium concentration 1 mM.

On the contrary, Boyd & Martin (1956b) state that, in general, they observed no consistent relationship between the miniature potential frequency and the e.p.p. quantal content in the cat tenuissimus muscle.

In their many papers, Castillo & Katz (1954a - 1956b) have made no mention of any correlation between these two quantities in the frog. While not directly disproving the existence of a correlation, they argued (Castillo & Katz, 1954a) that the quantal content of the e.p.p. and the resting discharge frequency are unrelated because magnesium reduces the former but not the latter.

However the present results in the mammal are supported by the findings of Hutter & Trautwein (1956) in the frog. These workers have demonstrated that the discharge frequency and the quantal content of the e.p.p. increase *pari passu* when a muscle is stretched. They also state that, in those junctions where the frequency of the miniature potentials was low, the probability of eliciting a response to a nerve volley was also low. Conversely a high rate of spontaneous discharge in a fibre was generally accompanied by a comparatively large elicited response. In Fig. 8 of their paper Hutter & Trautwein show the results of ten experiments in which the discharge frequency and the quantal content of the e.p.p. were determined as the muscle was stretched. Considering only the starting point in each experiment (i.e. the values for lax muscle) the graph resembles closely Figs. 38 & 39 of this thesis.



A second prediction concerns the derivation of the absolute quantal content of a response, or, essentially, the calibration of an action potential in terms of the number of quanta which it should liberate under defined conditions. This calibration is made possible by the relationship (deduced from Fig. 37) between discharge frequency and displacement of membrane potential (Fig. 40, inset). Now the action potential of a motor nerve terminal cannot be fully and directly recorded, but as an approximation the intracellularly recorded action potential of a mammalian (cat) motor axon may be investigated (Fig. 40R). If such an action potential, corrected for distortion in recording (Fig. 40C), is divided into a series of very brief (0.02 msec) steps, the miniature discharge frequency corresponding to each potential step may be determined, and hence the theoretical quantal content of a response may be estimated. Implicit in this procedure are two assumptions - first, that the discharge frequency follows faithfully and instantaneously any change in membrane potential and second, that there is no serious depletion of available quanta during an impulse even though the discharge frequency attains very high values. The former assumption would appear reasonable, for, as shown in Fig. 33, the discharge frequency adapts very rapidly to electrotonic polarisation of the terminals. On the other hand the latter assumption is open to question, for, immediately after an impulse in normal solutions, there appears to be a considerable depletion of the available transmitter (Liley & North, 1953). However, ignoring this possible restriction, it is possible to compute the number and temporal distribution of the quanta which an action potential having the amplitude and time course shown in Fig. 40 would cause to be liberated from a nerve terminal having a resting discharge frequency in the modal range of 1-2 per sec. This liberation would comprise 250 to 500 quanta and would be

produced almost entirely by that portion of the action potential exceeding 60 mV. Hence it would be spread over some 0.3 msec.

The quantal content of the e.p.p. in normal solutions cannot be measured with any great precision. However estimates in the rat diaphragm (Table 5) gave a range of 170-340. Hence the derived figure of 250-500 appears to be of the correct order. Further it is in good agreement with the figures of 220 and 310 which Boyd & Martin (1956b) deduced for the quantal content of two e.p.p.s in the cat tenuissimus muscle.

A more precise check is possible when the calculation is repeated for the same action potential but using the discharge frequency - membrane potential relationship derived in the presence of 12.5 mM magnesium. In such a solution the quantal content of responses can be determined accurately. Furthermore, depletion of available quanta is no longer a theoretical hazard compromising the calculation. For a solution containing 12.5 mM magnesium the predicted quantal content of a response is 1.2 for a junction with a resting discharge frequency of 1 per sec. Measurements on nine junctions gave a mean discharge frequency of 2.7 per sec and therefore a theoretical mean quantal content of the responses of 3.24. The observed mean value was 3.4.

This very close agreement of predicted and observed values is probably fortuitous, for it is based on the improbable assumption that the action potentials of rat motor nerve terminals and cat motor axons are identical. Nevertheless, the agreement of order supports the hypothesis that the e.p.p. is merely the random miniature discharge accelerated by the changes in membrane potential which constitute the propagated impulse.

The derivation of the quantal content of a response automatically leads to a prediction of the time course of release of the quanta or,

essentially, the frequency distribution of stimulus-response intervals for the quanta in a large series of responses, provided it be assumed that the quanta all take the same time to diffuse from the liberating terminal membrane to the responsive muscle junctional membrane. Fig. 41A shows this theoretical distribution for the quanta involved in a large series of responses at a junction in 12.5 mM magnesium. Fig. 41B & C illustrate the distributions of stimulus-response intervals observed at two junctions in such a solution. Fig. 42 shows examples of this latency variation at the junction of Fig. 41B. These junctions both had responses of low quantal content, the majority of stimuli being followed by a failure of response or the discharge of a single quantum. However, since a number of responses in each series (about 15% in 41B and 25% in 41C) involved two or three quanta and in such responses only the latency of the earliest quantum could be measured, the distributions of Fig. 41B & C will be skewed in favour of the briefer latencies. No accurate correction for this distortion can be applied. Nevertheless, although the test lacks precision, the predicted and observed distributions agree reasonably both in form and range.

Thus the evidence so far adduced suggests that, in so far as it affects the release of transmitter, an impulse does not differ from a depolarisation of the terminal membrane produced electrotonically or by excess potassium. It is not necessary to postulate any "triggering" or threshold effect or to associate chemically the release of acetylcholine with the activity of the sodium or potassium carriers.

The observation that, in the mammal, the miniature discharge frequency may be reduced by hyperpolarisation and increased by depolarisation of the terminal membrane suggests initially that the quanta might be positively charged particles whose passage across the nerve membrane is deter-

mined simply by potential gradients. According to this concept the resting discharge frequency is merely the release rate corresponding to one particular membrane potential - the resting membrane potential - in a continuum. However this simple model cannot readily explain the influence of calcium and magnesium concentrations on the release of quanta. If the presence of calcium ions was essential for the liberation of quanta, variation in calcium and magnesium concentrations should affect the resting discharge frequency.

In the cat tenuissimus muscle the resting discharge frequency varied with calcium but was <sup>un</sup>influenced by magnesium concentration (Boyd & Martin, 1956a), despite the fact that magnesium antagonised the effect of calcium on neuromuscular transmission (Boyd & Martin, 1956b). On the contrary, in the frog (Fatt & Katz, 1952; Castillo & Katz, 1954a) the discharge frequency was unaffected by changes in calcium or magnesium concentrations. In the rat diaphragm the discharge was reduced in frequency but not abolished in the complete absence of calcium (Table 4). However neither the addition of magnesium nor reduction in calcium concentration to 0.5 mM has any marked effect on the resting discharge frequency but this frequency appears to be stabilised so that it cannot be displaced from its resting level by hyperpolarisation or depolarisation of the terminal membrane.

No explanation can be offered for this stabilisation of the discharge frequency in calcium-deficient and/or magnesium-enriched solutions. Further experiments are also necessary to determine the mechanism by which the membrane potential affects the miniature discharge frequency.

Several workers (Brown & Feldberg, 1936; Feldberg & Guimaraes, 1936; Hutter & Kostial, 1955) have demonstrated that potassium may initiate release, or augment the spontaneous release, of acetylcholine from cholinergic



terminals in the mammalian autonomic nervous system. Although the spontaneous quantal release of transmitter has not been demonstrated at these cholinergic junctions, the effect of potassium would appear analogous to the action observed at the mammalian motor nerve terminals.

SECTION 4.GIANT POTENTIALS AT THE MAMMALIAN NEUROMUSCULAR JUNCTIONINTRODUCTION

In Section 1 reference was made to the frequent observation of spontaneous large 'miniature' potentials with amplitudes several times the modal value of a series of potentials. In particular, calculations, at one junction (Fig. 8), showed that more of these large potentials occurred than could be accounted for by the random summation of the 'unitary' miniature potentials. The present Section deals with a further investigation of this phenomenon. It would seem paradoxical to designate potentials whose amplitudes may reach 12 mV as 'miniature' potentials; hence, in this Section, such potentials will be referred to as 'giant potentials'. In distinction, 'miniature potentials' will refer to the 'unitary' spontaneous potentials.

RESULTS

Giant potentials could be detected at any junction, both in the gracilis muscle in vivo (Fig. 43A) and in the isolated diaphragm (Fig. 43B). However the relative frequency of giant potentials varied greatly from one fibre to the next. At some junctions the giant potentials comprised 20% or more of the spontaneous discharge, but this was unusual. At most junctions there were but few more than would have been predicted from random summation of miniature potentials. Nevertheless in twenty junctional records, selected for detailed study because the incidence of giant potentials did not appear excessive, no instance was found in which the occurrence of giant potentials did not exceed the expectation from stochastic theory. Although the numerical values were small, the probability of their 'natural' origin, by statistical summation of unitary potentials, was exceedingly remote, being in

most cases of the order of  $10^{-10}$ . On the other hand detailed analysis showed that at brief intervals, e.g. in the range of 2-5 msec, discharges were not in excess of expectation. The problem was that too many quanta appeared to be discharged synchronously. This phenomenon was unrelated to the position of the intracellular electrode tip, whether near or relatively distant from the junction.

At a given junction the giant potentials varied in amplitude. Predominantly they were small, being some 2 or 3 times the modal amplitude of the miniature potentials, but isolated potentials of some 5 or 6 mV were common, and the maximum amplitude encountered was 12 mV. Not infrequently, in normal solutions, those giant potentials exceeding 6 - 8 mV generated propagated muscle action potentials (Fig. 43B).

At some junctions the frequency distribution of amplitudes of giant potentials displayed an obvious periodicity (Fig. 44), with peaks occurring at simple multiples of the modal amplitude of the miniature potentials. Such observations implied that the giant potentials arose by the synchronous discharge of a number of quanta of transmitter each equal to the quantum normally generating a miniature potential. However in some graphs (Figs. 46 & 48) no such periodicity was evident. Such observations were not unexpected, for the demonstration of peaks requires that the amplitude spread of the miniature potentials be small. Further, for the larger giant potentials, linear summation of any constituent units could not be expected since the membrane potential was significantly displaced toward the equilibrium potential for the e.p.p. (Castillo & Katz, 1954b). Giant potentials of any given amplitude band displayed no rhythmicity. Rather they appeared to comprise a stochastic process within the random pattern of the miniature potentials.

The timecourse of the giant potentials normally did not differ from that of the miniature potentials. Occasionally, with the larger giant potentials the rising phase was slower (Figs. 45 & 47) usually towards its peak, which possibly indicates a situation akin to that described by Castillo & Katz (1955a, 1956a) in the frog, viz, local saturation of acetylcholine receptors by the focal release of a large quantity of acetylcholine. In consequence more distant receptors at the junction became increasingly involved and the effect of the transmitter persists until the local concentration falls below saturation level. More frequently the falling phase of the giant potentials appeared longer. Such an effect could also arise from the local saturation mechanism, or, more readily, merely from the fact that with the exponentially declining giant potentials a displacement from the baseline was recognisable for a longer time.

By contrast, in three fibres unequivocal evidence of double innervation was obtained (Figs. 45 & 46). In this situation naturally, the larger potentials exhibited a much briefer timecourse. Further, each population exhibited its own family of giant potentials. Obviously it was important to recognise such double innervation for indiscriminate measurement of potentials in such fibres would lead to a markedly bimodal distribution of miniature potential amplitudes.

The effects of a number of agents and procedures on the giant potentials have been investigated:

(a) Denervation. The giant potentials disappeared coincidentally with the miniature discharge.

(b) Tubocurarine. During curarisation of a preparation giant potentials diminished in amplitude, but frequently they could be detected after the miniature potentials were lost in the baseline noise.

(c) Anticholinesterases. Prostigmine in a concentration of  $10^{-6}$  augmented and prolonged the giant potentials. Many of the larger potentials generated muscle action potentials (Fig. 10). Indeed it appeared that only giant potentials could produce fibrillation, for prostigmine at this concentration merely doubled the miniature potential amplitude - to some 2 - 3 mV, which was well below the threshold for a muscle action potential.

(d) Temperature variation (25 to 40°C); anoxia; sodium cyanide (2 mM); glucose deprivation (2 hours). Individually all these agents were without significant effect on the relative frequency of giant potentials.

(e) Nerve block. Production of nerve block (by cold) about 1.5 mm from the terminals was without influence on the giant potentials. This observation ruled out the possibility that giant potentials resulted from orthodromic impulses - either from the cut end of the phrenic nerve in the isolated diaphragm or from the spinal cord with the gracilis muscle in vivo.

In order to determine whether the giant potentials were associated with antidromic impulses in the motor nerves, the phrenic nerve trunk, over a few millimetres, was pared down to about half its original cross-section. By applying stimulating electrodes to the pared nerve, the muscle fibres whose innervation was still intact were identified. The microelectrode was retained at one of these innervated junctions while the surface electrodes on the pared phrenic nerve were led, via an amplifier, to the second beam of the C.R.O. No activity could be detected in the phrenic nerve. When, on the other hand, prostigmine ( $10^{-6}$ ) was added to the solution bathing the muscle strip, numerous antidromic impulses could be detected in the phrenic nerve (Fig. 47). This phenomenon was originally observed by Masland & Wigton (1940) in the eserinizied cat. However, although the giant potentials were

augmented, many sufficiently so to generate muscle action potentials, no association could be found between the nerve impulses and giant potentials. Indeed, with potentials whose amplitudes exceeded threefold or more the modal amplitude of the miniature potentials, no nerve impulse was recorded within  $\pm 5$  msec of any of these 44 giant potentials recorded at 4 separate junctions. If the nerve impulses indicate the discharge of motor units (fasciculation), it is to be expected that an occasional muscle action potential would be associated with a nerve impulse, but this event was not observed. The genesis of the antidromic impulses in the presence of anticholinesterases remains obscure, but on the present evidence it may be said that the nerve impulses certainly do not usually, and possibly may not ever, develop in the process which initiates spontaneous giant potentials.

(f) Magnesium and calcium. Variation in magnesium concentration over the range 1 - 12.5 mM and in calcium concentration over the range 0 - 4 mM had no effect on the relative frequency of giant potentials. At one junction blocked by 12 mM magnesium the paradoxical situation was observed that, whereas a nerve impulse evoked a response of low quantal content which never approached the muscle fibre threshold, an occasional spontaneous giant potential of some 8 - 10 mV readily initiated a muscle action potential. In these conditions no evidence was obtained that giant potentials could occur as a response to a stimulus.

(g) Electrotonic polarisation of nerve terminals. When the motor nerve terminals were hyperpolarised or depolarised a decisive and unexpected observation was made at junctions displaying a high proportion of giant potentials. Whereas the miniature potential frequency was readily modified by presynaptic polarisation (Section 3), the absolute frequency of the giant potentials was not significantly altered. Hence the relative frequency of giant potentials varied greatly with polarisation (Fig. 48). Furthermore,

while at a junction displaying but few giant potentials, it was correct to say that the mean amplitude of the miniature (meaning 'all spontaneous') potentials was unaltered by presynaptic polarisation (Section 3), such a statement would not apply at a junction such as that depicted in Fig. 48. The amplitude of the miniature (unitary) potentials remained constant, but the mean amplitude of the total spontaneous discharge varied with the relative frequency of giant potentials.

(h) Potassium. Potassium in a concentration of 15 - 20 mM had no effect on the absolute frequency of giant potentials, their relative frequency merely declining as the frequency of the miniature potentials increased.

(i) Post-activation potentiation. The absolute frequency of giant potentials at two junctions showed no marked change when the miniature discharge was accelerated by a tetanus of 3,000 impulses (200 per sec for 15 sec).

#### DISCUSSION

Castillo & Katz (1956a) opined that it might be possible with an intracellular electrode to detect, amid the population of miniature potentials, a few large fast potentials which would arise (as a subtlety of recording) by convergence of current flow at an 'active patch' (Castillo & Katz, 1955a, 1956a; Section 1) situated very close to the electrode tip. The expected characteristics of such 'active patch' potentials may be tabulated as follows:

(a) Detection (as for extracellular miniature potentials) would be rare and difficult, for it would require a very precise location of the electrode at the end-plate.

(b) The time-course would always be faster than that of the miniature

potentials.

- (c) Amplitudes would not occur as simple multiples of the miniature potential modal amplitude. Potentials of largest amplitude would not generate muscle action potentials.
- (d) Frequency would vary with the miniature discharge frequency when the latter was modified by any agent.
- (e) Large potentials would occasionally contribute to responses to stimuli.

All these theoretical criteria of large 'active patch' potentials stand in sharp contrast to the characteristics of the giant potentials described in this Section.

The present observations indicate that giant potentials are of presynaptic origin. Their pharmacology is similar to that of the miniature potentials and it would appear that giant potentials arise simply by the synchronous or near-synchronous release of a number of quanta of acetylcholine

Neither the mechanism of this synchronous release nor the great variation in relative frequency of giant potentials at different junctions can be explained. The giant potentials neither result from nor cause impulses in the motor axons. Variation in calcium and magnesium concentration modify profoundly the effects of an impulse and electrotonic depolarisation on the release of quanta, but these ions are without effect on the incidence of giant potentials. Hence it appears unlikely that the giant potentials are generated by local responses of the motor terminal membrane.

Originally it was supposed that the discharge of a single quantum might facilitate the release of a second quantum, possibly by an electrical event in the motor terminal membrane (Liley, 1956a). This model was suggested by the observations that the miniature potential frequency was raised after indirect stimulation, and that electrotonic depolarisation of the



terminals accelerated the discharge. However this explanation raises several problems. First, it predicts that the behaviour of a motor terminal is inherently unstable, and that intermittently there would develop a fulminating chain reaction of miniature discharges. Secondly, any facilitatory effect must be of very brief duration, for the incidence of discharges at intervals of 2 - 5 msec is not excessive. Thirdly, this explanation would appear incompatible with the observation that the relative frequency of giant potentials falls as the discharge is accelerated by depolarisation of the nerve terminals. It is desirable, therefore, to consider other mechanisms which, conceivably, could account for the production of giant potentials.

A multivalent carrier molecule could be involved in the 'spontaneous' passage of quanta across the terminal membrane. The normal 'load' of such a carrier would be a single quantum, but occasionally the carriage of two or more quanta could occur.

Alternatively coalescence or physical adherence of quanta may be postulated. The occasional release of the resulting aggregates of transmitter would produce the same potential change as the synchronous release of several independent quanta. This model might be compatible with the stability of the absolute frequency of giant potentials from terminals which are subjected to electrotonic depolarisation, excess potassium and tetanic stimulation, for the mobility of large aggregates would be less than that of single quanta. Alternatively, under these conditions, the high turnover of quanta might reduce the probability of their coalescence.

Recent electron-microscope studies (Palade, 1954; Robertson, 1956) have revealed that vertebrate motor nerve terminals contain numerous 'vesicles' with diameters in the range 200-500Å<sup>0</sup>. Robertson (1956)

suggests that these vesicles might be charged with acetylcholine and be analagous to secretion granules (cf. Castillo & Katz, 1955b, 1956b). It is an attractive and convenient hypothesis that these vesicles are the morphological correlates of the quanta of transmitter which, on release, generate miniature potentials.

Within this framework it is interesting to seek evidence that giant potentials might be produced by preformed aggregates of transmitter. If the vesicles tended to maintain a spherical form, then a giant potential equivalent to as many as ten unitary potentials would derive from a vesicle with diameter little more than double that of a 'unitary' vesicle and such a structure would hardly be prominent. If, on the other hand, vesicles aggregated linearly to produce rod-like bodies, such structures should be obvious amongst the general population of 'unitary' vesicles. It, therefore, is suggested that the structures designated 'elongated vesicles' by Robertson (1956) might represent the multi-quantal accumulations of transmitter which on release produce giant potentials.

Whatever theoretical interest they may possess, giant potentials are of considerable practical importance. Undoubtedly they appear to be the generators of fibrillation in muscles treated with anticholinesterases.

Just as Fatt & Katz (1952) initially suspected the possibility of an artefact when they first encountered spontaneous activity at the frog myoneural junction, so, in the present work, junctions displaying numerous giant potentials were originally regarded with suspicion and avoided where possible. As a result, in most experiments involving the determination of the mean amplitude of spontaneous potential populations (Section 2), few giant potentials were present to disturb calculations. However it is obvious that, for a junction displaying numerous giant potentials, the

modal amplitude of spontaneous potentials would be more meaningful than the mean amplitude as a measure of the 'unitary' potentials.

Originally junctions displaying numerous giant potentials were also avoided in experiments involving the electrotonic polarisation of nerve terminals. Such a practice was fortunate, for a large stable population of giant potentials could seriously distort the interpretation of records - particularly with hyperpolarised terminals. Indeed reinvestigation of the records of two junctions in which hyperpolarising currents failed to depress the discharge frequency to an expected level (Fig. 34B) showed that a partial but not complete 'correction' was effected by the deletion of a few giant potentials.

Finally the occurrence of giant potentials raises the problem of measurement of discharge frequencies. For a measure of the rate at which quanta are expended it is reasonable to estimate the quantal content of any giant potentials on a record. However in terms of the motor nerve terminal membrane it is possible that a giant potential represents an event in no way different from the release of a single quantum.

SECTION 5.THE EFFECTS OF GLUCOSE DEPRIVATION AND PHLORIDZIN ON THE MINIATURE  
DISCHARGE AT THE MAMMALIAN NEUROMUSCULAR JUNCTIONINTRODUCTION

The frequency of the miniature discharge may be modified profoundly by a number of agents, but corresponding effects on the amplitude of the miniature potentials are slight or absent. Further, the effects of curare, prostigmine (Section 1) magnesium (Section 2) and potassium (Section 3) on the miniature potential amplitude can be assigned to the post-synaptic level. At a junction poisoned with botulinum toxin (Brooks, 1956b) the miniature potentials disappeared without prior reduction in amplitude. No agent has been discovered which indisputably modifies the molecular content of the quanta of transmitter and nothing is known of the mechanism determining this content except that it is very stable. Thus a resting terminal in an isolated diaphragm may release some 30,000 - 60,000 quanta of constant or near constant size during an 8 hour experiment while in the presence of 30 mM potassium a terminal can expend in 20 min the greater part of a million quanta which display remarkable uniformity, at least in their post-synaptic effects.

Since alteration of the motor terminal membrane potential does not alter the size of the quanta it would seem reasonable to direct an attack against the synthesis of acetylcholine.

The choline-acetylase activity of motor nerve terminals may be assumed to resemble the process which has been investigated in other nervous tissues (for a review see Feldberg, 1945). However, when electrical recording rather than chemical or biological assay is used to detect acetyl-

choline, the choice of agents for modifying acetylcholine synthesis is limited. Since the muscle junctional membrane is the delicate indicator of acetylcholine release, it is important that the muscle sensitivity to acetylcholine and the muscle membrane potential are not disturbed.

In view of the elegant work of Kahlson & MacIntosh (1939) on the synthesis and release of acetylcholine in the superior cervical ganglion of the cat, an investigation has been made of the effects of glucose deprivation on the frequency and amplitude of miniature potentials in the isolated rat diaphragm.

### RESULTS

The immersion of a preparation (including the phrenic nerve) for 2 hours in a solution containing zero glucose produced no significant change in the frequency (Table 6) and amplitude of the miniature discharge or in the muscle resting membrane potential.

TABLE 6.

Effect of glucose on resting discharge frequency. Mean frequency, S.D. and range of frequencies in groups of junctions. The number of junctions in each group is shown in parentheses and groups were recorded in the order shown. The time in parentheses after specification of a solution indicates the time at which recording commenced after immersion in that particular solution.

|                                         | Glucose 2G/L<br>(normal) | Zero glucose<br>(2 hr.) | Glucose 2G/L<br>( $\frac{1}{2}$ hr.) |
|-----------------------------------------|--------------------------|-------------------------|--------------------------------------|
| Mean frequency<br>( $\text{sec}^{-1}$ ) | 1.60 (20)                | 1.64 (20)               | 1.87 (20)                            |
| S.D. ( $\text{sec}^{-1}$ )              | 0.65                     | 0.81                    | 0.85                                 |
| Range ( $\text{sec}^{-1}$ )             | 0.93-3.35                | 0.82-3.57               | 0.41-3.61                            |

When however, after deprivation of glucose, a preparation was stimulated for  $3/4$  hour at 2 per sec (5,400 impulses) the mean frequency of the miniature discharge was reduced by some 60% (Table 7).

This low frequency was maintained, in the absence of glucose, for at least 20 min, as was found by comparing the first and second halves of the group of observations. On the other hand the discharge frequencies recovered completely after 5-10 <sup>min</sup> in normal glucose concentration. No attempt was made to record continuously from fibres during stimulation in the absence of glucose, but, after stimulation, the miniature potentials appeared to lie in the normal amplitude range. This impression was confirmed by continuous recording from one junction as the glucose concentration was restored and the frequency recovered from 0.42/sec to a stable value of 1.03/sec over 5 minutes. A control period of stimulation in normal glucose concentration produced only a small and insignificant increase in the mean frequency of the miniature discharge.

TABLE 7.

Effect of low frequency repetitive stimulation on discharge frequency in the absence and presence of glucose. Conventions of labels are the same as in Table 6.

|                                         | Glucose 2G/L<br>(normal) | Zero glucose<br>( $3/4$ hr stim at<br>2/sec) | Glucose 2G/L<br>(10 min) | Glucose 2G/L<br>( $3/4$ hr stim<br>at 2/sec) |
|-----------------------------------------|--------------------------|----------------------------------------------|--------------------------|----------------------------------------------|
| Mean frequency<br>( $\text{sec}^{-1}$ ) | 1.15 (21)                | 0.43 (20)                                    | 1.10 (20)                | 1.26 (20)                                    |
| S.D. ( $\text{sec}^{-1}$ )              | 0.50                     | 0.21                                         | 0.50                     | 0.65                                         |
| Range ( $\text{sec}^{-1}$ )             | 0.45-2.35                | 0.11-0.96                                    | 0.27-2.37                | 0.20-2.58                                    |

Theoretically the glycoside phloridzin should produce the same effects as the withdrawal of glucose, for phloridzin prevents the utilisation of glucose by inhibiting phosphorylation. Experiments, however,

demonstrated that dosage with phloridzin did not mimic simple glucose deprivation.

At a concentration of 0.4 g/L phloridzin produced a significant acceleration in the miniature discharge (Table 8, preparation a), and a further acceleration occurred following 3/4 hour stimulation at 2 per sec. These effects were greatly enhanced when the phloridzin concentration was increased to 1.6G/L (Table 8, preparation b). At both concentrations the effects were reversible although about 20-25 min was required for full recovery. Throughout, the muscle resting membrane potential was unaffected and no change in the amplitude of the miniature discharge was detected.

TABLE 8.

Effect of phloridzin, and repetitive stimulation in the presence of phloridzin, on discharge frequency. Conventions of labels are the same as in Table 6.

| Preparation a                                |                             |                                           |                          |
|----------------------------------------------|-----------------------------|-------------------------------------------|--------------------------|
| Normal solution                              | Phloridzin 0.4G/L (3/4 hr)  | Phloridzin 0.4 G/L (3/4 hr stim at 2/sec) | Normal solution (1/2 hr) |
| Mean frequency (sec <sup>-1</sup> ) 1.41(21) | 1.77 (20)                   | 2.97 (20)                                 | 1.27 (20)                |
| S.D. (sec <sup>-1</sup> ) 0.42               | 0.88                        | 1.16                                      | 0.87                     |
| Range (sec <sup>-1</sup> ) 0.41-2.03         | 0.67-3.96                   | 1.10-4.76                                 | 0.44-3.08                |
| Preparation b                                |                             |                                           |                          |
| Normal solution                              | Phloridzin 1.6G/L (1/2 hr.) | Phloridzin 1.6G/L (3/4 hr. stim at 2/sec) | Normal solution (3/4 hr) |
| Mean frequency (sec <sup>-1</sup> ) 1.10(20) | 13.2 (20)                   | 17.9 (20)                                 | 1.28 (19)                |
| S.D. (sec <sup>-1</sup> ) 0.56               | 7.7                         | 5.0                                       | 0.79                     |
| Range(sec <sup>-1</sup> ) 0.42-2.42          | 3.8-31.0                    | 8.5-27.0                                  | 0.49-3.38                |

DISCUSSION

In a recent review Castillo & Katz (1956b) asked whether, at the mammalian neuromuscular junction, deprivation of glucose might cause the  $q$ -quanta, or units of acetylcholine release, to diminish in size, for it would be reasonable to expect that glucose lack would influence acetylcholine synthesis at the molecular, rather than at the quantal, level.

The present investigation answers that query. In a resting preparation glucose deprivation has no effect on the miniature discharge. Following stimulation the spontaneous output of acetylcholine is diminished, but this effect is graded at the quantal and not the molecular level. As with other agents the discharge frequency may alter, but the quantal size is constant.

Kahlsen & MacIntosh (1939) found that glucose deprivation did not alter the acetylcholine content of the resting perfused superior cervical ganglion of the cat. However, whereas in normal solutions with prolonged low frequency stimulation the ganglion maintained a steady acetylcholine output and effector organ response, in the absence of glucose the acetylcholine output and effector organ response declined markedly and the acetylcholine content of the ganglion was reduced. These phenomena showed a slow recovery when stimulation ~~ceased~~ but a prompt recovery occurred when glucose was admitted even if stimulation was continued.

In the light of these observations the present results may be explained. Assuming that the frequency (and amplitude) of the miniature potentials are a legitimate index, the acetylcholine content of the motor nerve terminals is not affected by 2 hours of glucose deprivation. Furthermore, since in this time a terminal may expend 10,000 quanta, it is probable that there is some degree of synthesis. Prolonged stimulation in the



absence of glucose causes a fall in the discharge frequency which may be interpreted as an exhaustion of available transmitter. In contrast to the ganglion, no recovery from this exhaustion was found when the stimulation was discontinued for  $\frac{1}{2}$  hour. However, since in this time a terminal expends about 1,000 quanta it is possible that the random loss of transmitter offsets a limited resynthesis. The restoration of glucose, on the other hand, produces a prompt and complete recovery of the motor terminal <sup>transmitter</sup> content as judged by the frequency of miniature potentials.

During the  $\frac{3}{4}$  hour of indirect stimulation in the absence of glucose it was observed that the muscular contractions progressively diminished. Since the miniature discharge is independent of the state of nervous conduction (providing the motor terminal membrane potential is unaltered), the present results would appear to separate transmitter exhaustion from any contribution which nerve block might make to the neuromuscular depression produced by glucose deprivation. Nevertheless the present results in the isolated diaphragm are in accord with the observations of McDowall et al (1949) and Hadju & McDowall (1949) who used mechanical recording and with the observations of Jeffries (1953) on e.p.p.s in the curarised preparation. Hadju & McDowall (1949) found that, in the absence of glucose, the responses to indirect stimulation were depressed in two stages. The first stage was attributed to depressed synthesis of transmitter; the second undoubtedly resulted from failure of conduction in the medullated axons.

Hadju & McDowall (1949) also found that, in the absence of glucose, neuromuscular depression developed more rapidly when the potassium concentration was increased from 5 mM to 10 mM. This effect would be expected, for the increased random discharge at the higher potassium concentration

(Section 3) would contribute to the exhaustion of transmitter. Further it will be shown (Section 6) that, as the potassium concentration is increased, the output of transmitter per impulse is also augmented.

Hadju & McDowall (1949) reported that phloridzin produced a readily reversible neuromuscular block. They stated that "200 mg of phloridzin was added" but did not specify the volume of the bath nor whether the added material dissolved completely. Accepting their implication that the apparatus was similar to that of McDowall et al. (1949) with a bath volume of 50 ml, the concentration of phloridzin would have been 4 g/l. In the present investigation saturation was obtained at about 3g phloridzin/l at 38°C. Further, phloridzin did not mimic glucose deprivation in its effect on the miniature discharge. It would be of interest to investigate the effect of this substance on the acetylcholine content of the stimulated superior cervical ganglion of the cat.



SECTION 6.THE EFFECTS OF POTASSIUM ON TRANSMISSION AT THE MAMMALIANNEUROMUSCULAR JUNCTIONINTRODUCTION

Boehm (1894) first described the decurarising effect of repetitive indirect stimulation on the neuromuscular junction. Boyd (1932) rediscovered this phenomenon in a mammalian preparation and subsequently Brown & von Euler (1938) demonstrated the post-tetanic potentiation (PTP) of the e.p.p. in curarised muscle and distinguished this phenomenon from the post-tetanic augmentation of contractions in uncurarised muscle which had been described by Rosenblueth and Morison (1937). Since that time PTP of the mammalian e.p.p. has been investigated by several workers (Hutter, 1952; Liley & North, 1953; Jeffries, 1953; Lundberg & Quilisch, 1953a, b), but the cause of the phenomenon has remained obscure. Rosenblueth & Cannon (1940) claimed that post-tetanically a muscle was stimulated by an injection of acetylcholine which failed to stimulate the resting muscle, but Hutter (1952) found that post-tetanically the sensitivity of a muscle to acetylcholine was unaltered. He concluded, as also did Liley & North (1953), that the post-tetanic effects were presynaptic in origin. Jeffries (1953) demonstrated that PTP of the e.p.p. was independent of glucose concentration.

Following the work of Brown & Feldberg (1936) on ganglia and Wilson & Wright (1937) on muscle, Rosenblueth and co-workers (Rosenblueth & Morison, 1937; Rosenblueth & Cannon, 1940; Cannon & Rosenblueth, 1940) attributed post-tetanic effects to a 'mobilisation of potassium ions' on the grounds that potassium could imitate some of the effects of repetitive stimulation. Liley & North (1953) showed that in curarised muscle the PTP

of the e.p.p. could be suppressed by raising the potassium concentration. Subsequently it has been demonstrated (Section 2) that post-tetanicly the e.p.p. is augmented quantally, and that, in curarised muscles, this augmentation appears to be superimposed on an initial depression which presumably is due to exhaustion of the available transmitter. Further it has been observed (Section 3) that increase in potassium concentration accelerates the spontaneous miniature discharge, as also does repetitive stimulation (Sections 1 & 2). In view of these results it is of interest to investigate the effect of potassium on the quantal content of the e.p.p. both before and after repetitive stimulation.

### RESULTS

The effect of potassium on the mean quantal content of the e.p.p. was investigated in two preparations (Table 9). In each preparation it was observed that when the potassium concentration was raised from 5 mM to 15 mM there was a significant ( $p < .01$ ) increase in the mean quantal content of the e.p.p.s. As expected (Section 3) the mean frequency of the miniature discharge was also increased and, for this concentration of potassium at least, the proportional increase in mean quantal content of the e.p.p.s and mean discharge frequency were of the same order.

When a junction which had been blocked by excess magnesium was subjected to a large number of brief tetani, it was found that the increase in mean amplitude of the corresponding responses in each tetanus (Section 2) was smaller when the potassium concentration was increased from the normal 5 mM to 15 mM (Fig. 49). This effect was also observed with extracellular recording in the diaphragm strip preparation, and it was found that, with prolonged stimulation, not only was the increase in serial responses less marked but also that the responses reached a plateau earlier (Fig. 50) in

high potassium concentration.

TABLE 9.

Effect of potassium on mean quantal content of e.p.p.s and mean discharge frequency of groups of junctions in two preparations. Mg 12 mM, Ca 1.5 mM. At each junction a series of some 200 - 400 responses was recorded. The number of failures in the series was corrected (see Methods) and the quantal content was calculated from the relation:

$$m = \log_e \frac{\text{No. of impulses}}{\text{No. of failures}} \quad (\text{Section 2}).$$

In each preparation the observations in normal (5 mM) potassium were made in two groups - one group before and one after the observations in 15 mM potassium. For each preparation the two 'normal' groups were then pooled to give the values in the table.

| Preparation A                                                           | 5 mM (Normal) K <sup>+</sup> | 15 mM K <sup>+</sup> |
|-------------------------------------------------------------------------|------------------------------|----------------------|
| Number of junctions                                                     | 18                           | 20                   |
| Mean quantal content of response<br>(S.D.)                              | 0.66 (0.58)                  | 1.28 (0.84)          |
| Mean frequency (S.D.) of minia-<br>ture potentials (sec <sup>-1</sup> ) | 3.07 (1.19)                  | 5.00 (2.22)          |
| Preparation B                                                           |                              |                      |
| Number of junctions                                                     | 19                           | 20                   |
| Mean quantal content of response<br>(S.D.)                              | 1.06 (0.73)                  | 1.66 (0.61)          |
| Mean frequency (S.D.) of minia-<br>ture potentials (sec <sup>-1</sup> ) | 3.80 (2.35)                  | 6.76 (4.46)          |

Intracellular recording showed that in a preparation blocked by magnesium the PAP of the e.p.p. response (Section 2) was much smaller and briefer with high potassium concentration (Fig. 51D) than with normal

potassium (Fig. 51B). Similarly the post-tetanic acceleration of the miniature discharge (Section 2) was also reduced in degree and duration in high potassium concentration (Figs. 51A, C).

### DISCUSSION

The effect of potassium on a nerve-muscle preparation is complex. In high concentration, potassium may directly excite nerve (Brown & MacIntosh, 1939) and muscle fibres (Magladery & Solandt, 1942) through acute depolarisation. On the other hand prolonged depolarisation by potassium may produce nerve block or neuromuscular block (Thesleff, 1955).

Several authors (Wilson & Wright, 1936; Brown & von Euler, 1938; Hadju, Knox & McDowall, 1950) have demonstrated that potassium also facilitates neuromuscular transmission in the mammal although this effect is not sustained. These results accord with the observation of Liley & North (1953) who found that, although potassium reduced the amplitude of the e.p.p. in the curarised diaphragm, the pattern of a brief tetanus was altered in a manner which suggested a facilitated release of acetylcholine. Quilliam & Taylor (1947) demonstrated potassium-curare antagonism in the rat diaphragm. They considered that this antagonism occurred on the post-synaptic membrane, but their argument assumes that the amount of acetylcholine released by an impulse is unaffected by potassium.

The present results provide confirmation that potassium augments the release of acetylcholine by an impulse and indicate that this augmentation is a quantal process. The ultimate effect of this augmented release on neuromuscular transmission would appear, however, to be conditioned by the concomitant effect of potassium on the muscle membrane potential and on the sensitivity of the end-plate to acetylcholine.

The mechanism by which an increase in extracellular potassium facilitates the release of acetylcholine by an impulse cannot be readily explained, for, from the evidence adduced in Section 3, it would be expected that the quantal content of the e.p.p. would be influenced only by the absolute peak voltage and the time-course of the action potential and not by changes in the resting potential of the motor terminal membrane. The effect of potassium could be explained by a prolongation of the action potential but there is no evidence to support this contention. Indeed Hodgkin & Katz (1949) found that, in the squid axon, an increase in extracellular potassium had a negligible effect on the timecourse of the action potential.

The suppression of PAP of the e.p.p. by potassium which was observed in the curarised diaphragm by Liley & North (1953) is now seen to occur at a junction blocked by magnesium. Repetitive stimulation and potassium each increase the quantal content of the e.p.p., but these effects are not additive but occlusive. This occlusion occurs even in the magnesium blocked preparation, which allows an optimal opportunity for facilitation since exhaustion of available transmitter is obviated. Similarly, tetanic stimulation and potassium each accelerate the spontaneous discharge of transmitter, but again these effects are occlusive.

In discussing the increased twitch tension which was observed following repetitive stimulation of an uncurarised muscle, Rosenblueth & Morison (1937) used the phrase 'mobilisation of potassium ions' (cf. Brown & Feldberg, 1936). Subsequently this expression has plagued the literature on various post-tetanic phenomena. Many authors have quoted the phrase with little or no indication of the presumed origin or site of action of the 'mobilised' potassium. Brown & von Euler (1938) at least specified

that potassium would be liberated by muscle action potentials. However a 'mobilisation' of potassium ions from muscle fibres cannot explain the PAP of the e.p.p. at junctions blocked by curare or magnesium, for under these conditions the only tetanic responses in the muscle fibres are very small e.p.p.'s. Nevertheless, since potassium dosage mimics the effects of a tetanus both on the quantal content of the e.p.p. and on the miniature discharge frequency, it is reasonable to seek an explanation of these post-tetanic phenomena in terms of potassium redistribution. The relevant source and site of action of such a redistribution would be the motor nerve terminals themselves.

It may be argued that, since an impulse is associated with a temporary loss of intracellular potassium, the potassium concentration gradient across the motor nerve terminal membrane would be reduced after prolonged stimulation. In this situation, as pointed out by Liley & North (1953), it would be the intracellular reduction in potassium concentration which was of significance because a focal increase in extracellular potassium concentration would rapidly dissipate by diffusion into the surrounding medium (Hodgkin & Huxley, 1947). A post-tetanic reduction in the potassium concentration gradient across the motor terminal membrane implies a fall in the membrane potential, i.e. an after-depolarisation.

Such an after-depolarisation would readily explain the post-tetanic acceleration of the miniature discharge. Calculations based on the relations derived in Section 3 show that the PAP of the discharge frequency depicted in Fig. 14 would require an after-depolarisation with a transient initial peak value of some 25 mV.

On the other hand the mechanism by which a post-tetanic after-



depolarisation would quantally augment the e.p.p. is no more explicable than the effect of potassium in increasing the quantal content.

Further the negative after-potential hypothesis cannot explain why the post-tetanic effects on e.p.p. quantal content and discharge frequency are not additive with the corresponding effects induced by potassium unless it is presumed that the negative after-potential no longer occurs when the extracellular potassium concentration is increased.

An alternative hypothesis would be that both repetitive activity and potassium facilitate the release of quanta by a process independent of change in the terminal membrane potential. Several possible mechanisms for such an effect may be considered.

(a) Calcium-like action. Calcium, by some process unknown, increases the quantal content of the e.p.p. However the effect of calcium does not resemble that of tetanic stimulation or potassium for whereas these latter agents readily accelerate the miniature discharge, calcium has negligible influence on the discharge frequency.

(b) Increased store of transmitter. This explanation was discarded by Liley & North (1953) on the evidence that both repetitive stimulation and potassium dosage produced a steeper serial decline and finally a lower plateau of the e.p.p. responses to a brief test tetanus. From this change of pattern it was concluded that repetitive activity and potassium caused an increase in the fraction of available transmitter liberated by an impulse and hence a more rapid depletion of transmitter by a brief test tetanus. Hutter & Trautwein (1956) have placed the same interpretation on the analogous changes in the pattern of responses to a brief tetanus in the frog when a muscle is stretched.

(c) Strategic redistribution of quanta within a motor nerve terminal. Equating quanta of transmitter to the presynaptic vesicles of the electron microscopists it is apparent that not all quanta are equally 'available' for release or, formally, the probability of release of the quanta is not uniform. Although data are inadequate to make any estimate of the mobility of vesicles, this conclusion is almost inevitable for the release of a quantum implies movement of a vesicle to provide a collision with the terminal membrane (cf. Castillo & Katz, 1956b) and the vesicles are not all equidistant from the membrane. This description applies, of course, only for a very brief time interval and it is assumed that the duration of an action potential is such a brief interval. On the other hand, in the long run random movement of vesicles would result in the mean probability of discharge of one quantum being the same as that for every other quantum.

If repetitive activity resulted in a temporary redistribution of vesicles so that they were concentrated near the membrane, then both the increased e.p.p. quantal content and the accelerated miniature discharge plus the common timecourse of these two effects may be explained. That disuse and, by corollary, activity may induce changes in concentration of vesicles near a presynaptic membrane is shown by the electron microscopic records of de Robertis & Franchi (1956) on the rod and cone synapses of the rabbit retina.

Since potassium mimics the effects of a tetanus, the hypothesis suggests that potassium also causes a redistribution of vesicles so that they are concentrated near the terminal membrane. The occlusion of post-tetanic effects by potassium would simply imply that there is a limit to the local concentration of vesicles which may be attained.

The main argument against this convenient hypothesis is the fact that the relation between the discharge frequency and potassium concentration (Section 3) is precisely of the form which would be expected if potassium exerted its effect solely through the change it produced in the membrane potential of the motor nerve terminal.

GENERAL DISCUSSION

A few investigators have stated, and many have implied, that the process of transmission seen at the neuromuscular junction represents the broad pattern of activity at other synaptic regions.

Elliot (1907) first emphasized the resemblances in behaviour of the motor end-plates of skeletal muscles and the neurones in sympathetic ganglia. Rosenblueth (1950) has summarised further similarities between these structures. However it might seem hazardous to extend analogies into the central nervous system. With light microscopy a great variety of synaptic structures and patterns may be found. With but a single exception (Eccles, Fatt & Koketsu, 1953, 54) the transmitters in the central nervous system have not been identified, and, even more disconcerting, most excitatory pathways still lack any specific pharmacology.

Nevertheless much information has been gained by intracellular recording. On the motoneurone Brock et al (1952) observed that excitatory and inhibitory volleys produced, respectively, excitatory and inhibitory post-synaptic potentials (EPSP's and IPSP's). Coombs, Curtis & Eccles (1956a) have derived the timecourses of the membrane currents which generate EPSP's and IPSP's and the ion species involved in these currents have been identified (Coombs, Eccles & Fatt, 1955a, b). The interaction of EPSP's and IPSP's in the excitation of motoneurones have been investigated (Coombs, Eccles & Fatt, 1955c) and details of the process by which the EPSP excites a motoneurone have been added by Coombs, Curtis & Eccles (1956b). These investigations demonstrate that the later stages of transmission at central synapses, while not identical with, are certainly analogous to the processes which have been described at the neuromuscular junction (Eccles, Katz & Kuffler,

1941a; Kuffler, 1942; Fatt & Katz, 1951; Boyd & Martin, 1956b).

Numerous investigations have been made on the effects of disuse and repetitive activity on transmission in the monosynaptic reflex arc (Lloyd, 1949; Eccles & Rall, 1950; 1951a, b; Eccles & McIntyre, 1951, 1953). There is evidence that post-tetanic effects, at least, are of presynaptic origin, for the responses to heterosynaptic test volleys show no potentiation.

Apart from these observations little is known of the early stages of transmission in central synapses. Moreover any attempt to modify the ionic environment of the terminals would pose formidable technical problems. In this predicament it is reasonable to assume, as a working hypothesis, that the release of central transmitters resembles the process which has been displayed at vertebrate neuromuscular junctions. There is some justification for this assumption, for electron microscopy has revealed a remarkable uniformity in the fine structure of central and peripheral synapses which show little resemblance between the gross structures revealed by conventional light microscopy. The fundamental features of a presynaptic accumulation of vesicles (200-500  $\text{\AA}$  diameter) and discontinuity of pre- and post-synaptic cytoplasm have been observed at synapses in the mammalian retina (de Robertis & Franchi, 1956), cerebral cortex, medulla, sympathetic ganglion and muscle (Palade, 1954; Palay, 1954). Similar detail has been described at synapses in the lower vertebrates and invertebrates (de Robertis & Bennet, 1955; Robertson, 1956). Robertson (1956) has speculated upon a possible relationship between vesicles and axoplasmic filaments.

The proposal that the vesicles, so characteristic of nerve terminals, represent the containers of quanta of transmitter is a very convenient

hypothesis, but one that is not readily amenable to proof. Nevertheless some guide to the feasibility of the proposal may be obtained at the mammalian neuromuscular junction. From the table of Acheson (1948) it may be calculated that the amount of acetylcholine liberated at a junction by an impulse is of the order of  $10^{-18}$  mole. The number of quanta of transmitter liberated from a terminal by a single impulse has been estimated to be of the order of 250 (Boyd & Martin, 1956b; Section 2). According to Palade (1954) vesicles in rat nerve terminals are of diameter range 200-500 Å. Accepting a mean diameter of 350 Å, the concentration of acetylcholine in a vesicle may be calculated as

$$\frac{10^{-18}}{250} \times \frac{10^3}{4/3\pi(1.75 \times 10^{-6})^3} = 0.2 \text{ Molar}$$

Little reliance may be placed in the accuracy of this estimate for the values used in its derivation are subject to considerable uncertainty. Furthermore the likely errors would all cause an underestimate of the concentration. Nevertheless the order of magnitude is plausible.

Since vesicles are found in such a wide variety of nerve terminals, it is pertinent to ask why 'miniature potentials' have not been reported by workers using intracellular electrodes in tissues other than muscles. Certainly most terminals are very much smaller than the motor nerve endings but the diameter of the vesicles is not correspondingly graded.

Spontaneous potentials have been sought for, but have not been observed in sympathetic ganglion cells (R.M. Eccles, personal communication). The Renshaw cells of the ventral horn (Eccles, Fatt & Koketsu, 1953, 1954; Frank & Fuortes, 1956) are excited by cholinergic collaterals from motor axons. These cells may discharge 'spontaneously' but the cause of this

phenomenon has not been investigated.

A microelectrode may detect small potentials - excitatory, inhibitory or both - in a 'resting' motoneurone (Eccles, personal communication). The frequency and relative proportion of these spontaneous EPSP's and IPSP's vary from cell to cell but it is doubtful whether this activity is analogous to the miniature discharge at the neuromuscular junction. It is more probable that the potentials represent the background bombardment of a motoneurone by excitatory and inhibitory pathways. This interpretation is supported by the observation that, with carefully graded stimuli to the appropriate afferent nerve, the first response detected in the motoneurone may be of the same amplitude as the 'synaptic noise'. Despite the small size of the synaptic knobs it seems improbable that an impulse would liberate only a single quantum. Hence, if each of these potentials represents the normal total output of transmitter from a terminal, it follows that the effect of a single quantum would be undetectable.

Theoretically the absence of miniature potentials at synapses other than the neuromuscular junction is not unexpected. The neuromuscular junction has no integrative function. For efficiency the safety factor for transmission must be high. The post-synaptic membrane is highly sensitive to the transmitter, so much so that a few thousand molecules of transmitter produce a distinct potential change. By contrast, for any nerve cell on which numerous fibres converge, it is important that no one synapse should excite the cell, else all integrative action would be lost. Hence the post-synaptic response to the output of a single terminal should be small. If this output is quantal in nature, the effect of a single quantum on the post-synaptic membrane probably would lie beyond the resolution of existing recording circuits.

The argument that the individual miniature potentials would lie beyond electrical resolution does not imply that at such junctions the spontaneous discharge could not exert some significant effect. For example asynchronous quantal release of excitatory transmitter from the hundreds of synaptic knobs on a motoneurone could conceivably produce a small background depolarisation of the post-synaptic membrane. Similarly at eserinated cholinergic junctions the discharge might produce a significant 'resting' release of acetylcholine into the perfusion or bathing solution. Brown & Feldberg (1936) and Hutter & Kostial (1955) have reported a resting release of acetylcholine from sympathetic ganglia while Dale et al (1936) and Brooks (1954) reported a similar phenomenon in muscle. Brooks (1954) specified that this resting release was stable but other workers have regarded the effect with suspicion. It is interesting to speculate whether, in fact, these latter workers have been apologising for the occurrence of an important physiological phenomenon.



SUMMARY AND CONCLUSIONS

The motor nerve terminals of the rat, like those of other vertebrates (Fatt & Katz, 1952; Boyd & Martin, 1956a), spontaneously liberate packets or quanta of acetylcholine. This liberation is detected postsynaptically as a near-random sequence of miniature potentials (Section 1). The end-plate potential consists of summated miniature potentials (Section 2). Essentially the quanta are the currency of neuromuscular transmission.

Because the transmitter is released quantally it does not follow automatically that the units are preformed in the motor nerve terminals. Conceivably a quantal release could arise at the membrane level with carrier molecules transporting complements of transmitter molecules from a pool of freely-soluble transmitter within the terminals. However it has been shown by numerous workers (for a review see Feldberg, 1945) that intracellular acetylcholine is not in free ionic solution, but is bound to some cellular component, presumably protein. Hence it may be considered that the quanta represent preformed bound aggregates of acetylcholine. It has been suggested (Castillo & Katz, 1955b, 1956b; this thesis) that these aggregates are the vesicles which electron microscopy has revealed in motor nerve terminals.

The spontaneous discharge of quanta is accelerated when the motor nerve terminal membrane is depolarised electrotonically or by an increase in extracellular potassium concentration. There is evidence (Section 3) that an impulse affects acetylcholine release simply by the depolarisation which it produces, i.e. the e.p.p. or coordinated discharge of quanta is merely the random discharge accelerated by depolarisation. Thus the release of quanta is not specifically linked to the activity of the sodium or potassium carriers, but is determined by the changes in membrane potential produced by the activity of those carriers. This evidence throws no light on

the mechanism by which acetylcholine crosses the terminal membrane, but, if transport occurs by lipoid-soluble carrier molecules, the activity of such carriers is continuously graded when the membrane potential varies.

The hypothesis that the e.p.p. is simply a transient acceleration of the miniature discharge requires that the resting discharge frequency and the quantal content of the e.p.p. should be related. A relationship between these quantities has been demonstrated in normal mammalian (Section 3) and frog muscles (Hutter & Trautwein, 1956). In the mammal the discharge frequency and the quantal content of the e.p.p. are both increased post-tetanically (post-activation potentiation, PAP) and subside with the same timecourse (Section 2). An increase in extracellular potassium concentration accelerates the miniature discharge (Section 3) and increases the quantal content of the e.p.p. (Section 6). Glucose lack leads to a reduction of the discharge frequency in a stimulated mammalian preparation (Section 5) and a reduction in the e.p.p. (Jeffries, 1953). Hutter & Trautwein (1956) have demonstrated that, in the frog, stretching a muscle increases both the discharge frequency and the quantal content of the e.p.p.

From these results it is apparent that the resting discharge and the release of quanta by an impulse are not independent phenomena. However these two quantities do not always vary in parallel. In the frog prolonged stimulation leads to a depression of responses but a great acceleration of the miniature discharge (Castillo & Katz, 1954c). Immediately after a tetanus, the mammalian muscle in normal solutions exhibits a great increase in the discharge frequency at a time when, as seen in a curarised preparation, the e.p.p. is very small although recovering rapidly. Excess magnesium has no influence on the frequency of miniature potentials in frog or mammals but greatly reduces the quantal content of the e.p.p. in both preparations.

Calcium deficit decreases the quantal content of the e.p.p. in frogs and mammals but has no effect on the discharge frequency in the frog and only a small effect in the mammal (Boyd & Martin, 1956a, Section 2). The main effect of calcium deficit or excess magnesium is to stabilise or 'clamp' the resting discharge frequency so that it cannot be accelerated by agents acting through depolarisation (catelectrotonus, potassium, impulse) (Castillo & Katz, 1954b & d; Section 3).

On the other hand the effect on transmission of stretch in frog muscle (Hutter & Trautwein, 1956) is not affected by calcium deficit or excess magnesium. In these abnormal solutions PAP of the e.p.p. in the mammal is more marked (Section 2) but it is probable that this effect results simply from the more economical expenditure of quanta which thereby allows a greater relative potentiation. These results suggest that PAP and the effects of stretch may not involve a depolarisation of the motor nerve terminal membrane.

Obviously only a model of some complexity can explain these diverse observations.

Castillo & Katz (1956b) point out that the discharge of acetylcholine from a nerve terminal requires the disruption of more than one diffusion barrier - first the release of the transmitter from its intracellular attachment and secondly a passage through the nerve membrane. They suggest that, when a 'critical' collision occurs between an intracellular acetylcholine-carrier (corpuscle, vesicle) and the membrane of the nerve terminal, the two barriers are opened simultaneously. A critical collision is distinguished from non-fruitful collisions by the stipulation that certain 'critical' spots on the vesicle and membrane surfaces should

meet.

This model can readily explain some experimental findings. The spontaneous miniature discharge represents the few fruitful collisions in the random bombardment of the membrane by vesicles. Glucose deficit may reasonably be considered as preventing the replacement of transmitter lost by activity. Hence the number of vesicles and, therefore, the frequency of collisions with the membrane will be reduced.

An impulse increases the number of fruitful collisions. Castillo & Katz (1956b) consider it unlikely that an impulse would have any immediate influence on the intracellular constituents. Therefore they suggest that an impulse might alter the properties of the membrane so that the density of the reactive spots momentarily increases. Thus the proportion of fruitful collisions, but not the total number of collisions, would be increased. Experimental evidence requires that calcium is necessary for this effect. Depolarisation of the terminal membrane by either electrotonic current flow or excess potassium <sup>S</sup> stimulates the influence of an impulse on the terminal membrane and again the presence of calcium is essential.

However the effects of stretch (Hutter & Trautwein, 1956) and repetitive stimulation (Sections, 1, 2) on the release of transmitter cannot be explained on this model unless additional assumptions are made. Calcium deficit does not reduce the effects of these agents. It has been suggested (Section 6) that PAP might result from a strategic accumulation of vesicles near the terminal membrane. Such an accumulation would increase the collision rate with the membrane.

For the minute distances involved diffusion of vesicles would be very rapid. Indeed the mobilisation would need to be rapid to explain the

fact that the PAP of the discharge frequency is maximal immediately after the conditioning activity. However it is difficult to explain the observation that, in normal solutions, the PAP of the discharge frequency is maximal at a time when the e.p.p. is depressed, although recovering (Section 1). Furthermore, the nature of the occlusion between the effects of excess potassium and PAP (Liley & North, 1953; Section 6) remains obscure. It is reasonable to assume that stretch would affect acetylcholine release through some action on the motor nerve terminal membrane rather than on the intracellular components, but the nature of this action is unknown.

In conclusion it is useful to consider that the release of transmitter is a function of three factors, notably the total number of vesicles present in a nerve terminal, the disposition of these vesicles and the properties of the nerve terminal membrane.

The influence of the first of these factors, the total number of vesicles present in a terminal, would appear to be under test when one investigates the effects of glucose lack (Section 5) and the immediate depression of transmission in a preparation subjected to a prolonged tetanus in normal solutions (see Section 2). It is reasonable to expect that the intracellular structures which bind acetylcholine are also concerned with its synthesis. The amount of transmitter in a quantum would depend presumably on the physico-chemical properties of these structures. In this connection, the phenomenon of giant potentials (Section 4) suggests that under certain conditions some linkage might occur between vesicles.

The disposition of vesicles in relation to the terminal membrane has been postulated as a second factor concerned in transmission. On this factor would depend the collision rate between vesicles and the membrane.

At present the only evidence that this disposition may be modified physiologically is provided by de Robertis & Franchi (1956) who studied retinal synapses with the electron microscope. Electron microscopic examination should now be applied to other synapses subjected to activation and disuse.

Much is known of the effects of changes in the motor nerve terminal membrane, which is the ultimate factor in the release of transmitter. However the nature of the changes can only be surmised, because the small dimensions of the terminals has precluded direct investigations of the properties of the membrane by existing techniques, for example by intracellular recording.

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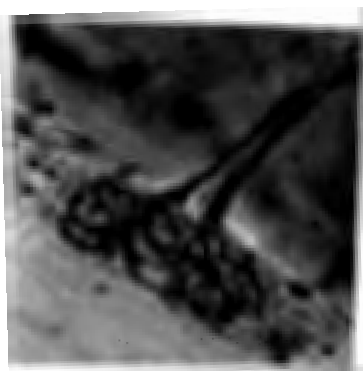
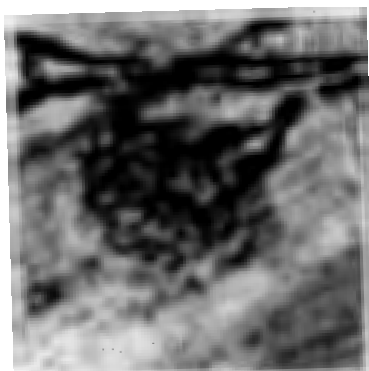
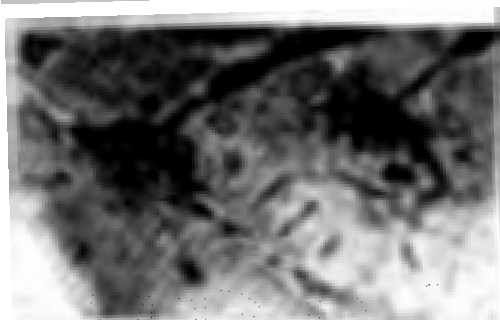
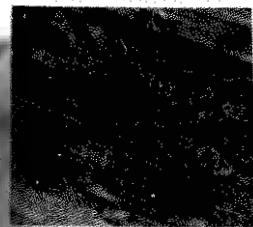
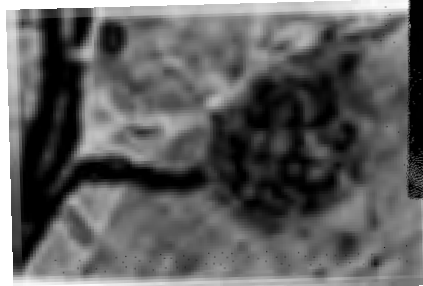
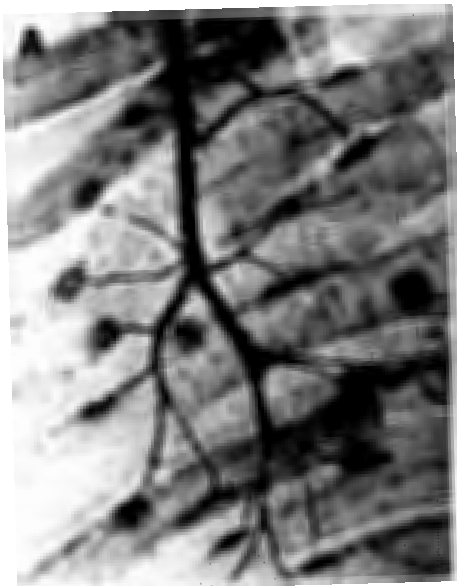


Fig. 1. Motor end-plates in the rat diaphragm. Gold chloride stain. A: Distribution of end-plates about a small nerve twig. B, C: Surface view of two end-plates. D: Two end-plates in profile. E, F: Two examples of the bifurcation of an epilemmal axon with both branches supplying a single large end-plate. See text. F is a composite of two photographs - one focussed on the axonal bifurcation, the other on the end-plate. G: Tip of a microelectrode. Calibration bar = 100 $\mu$  for A, 20 $\mu$  for B-G.

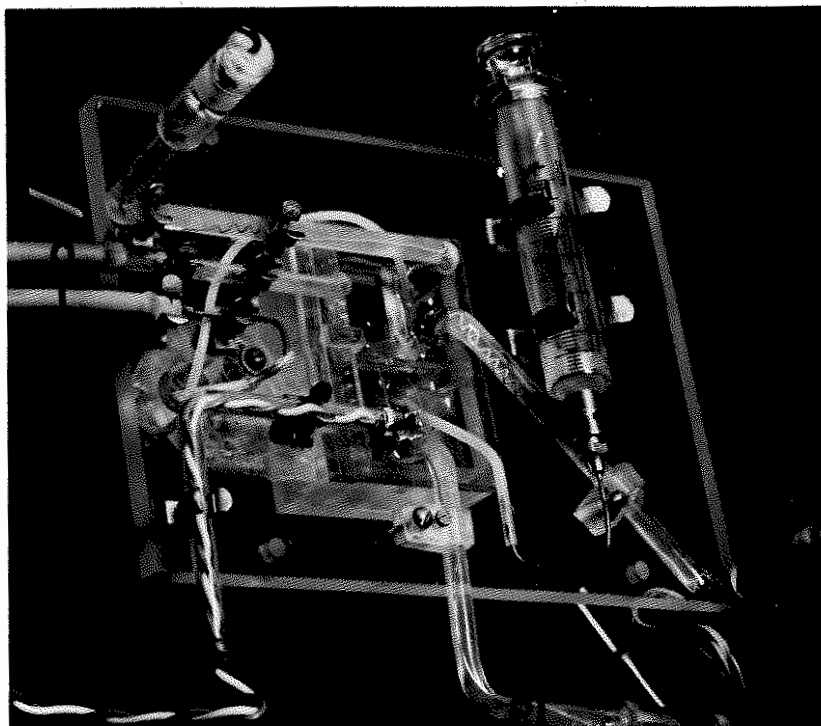


Fig. 2. Muscle chamber with a preparation in position. E: Indifferent electrode. P1, P2: Polarising electrodes. I: Inflow tube (passing first to heat exchanger). O: Outflow tube. See text.

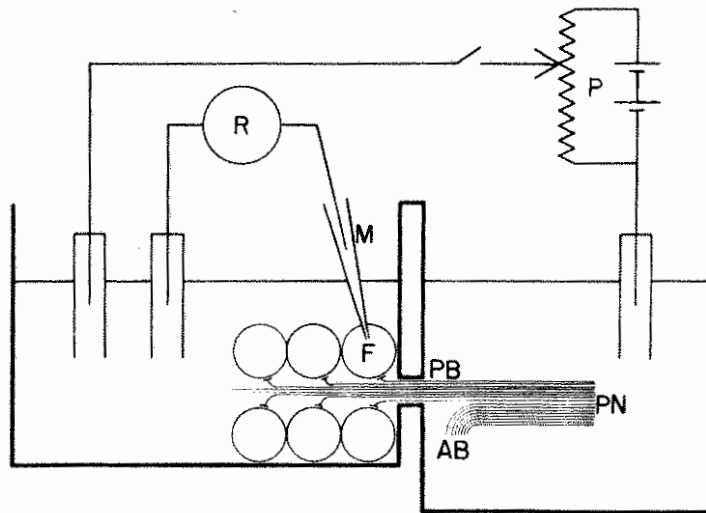


Fig. 3. Arrangement of polarising (P) and recording (R) electrodes. Not to scale. Heavy lines indicate Perspex. Microelectrode (M) shown penetrating a muscle fibre (F). PB indicates posterior branch of phrenic nerve (PN) of which anterior branch (AB) has been cut. See text.



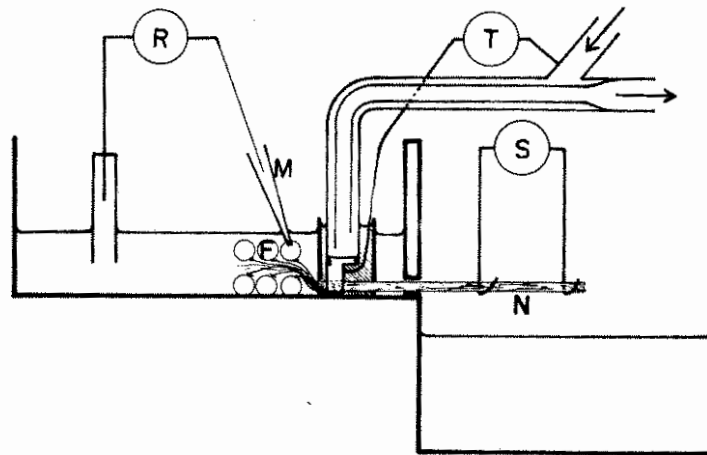
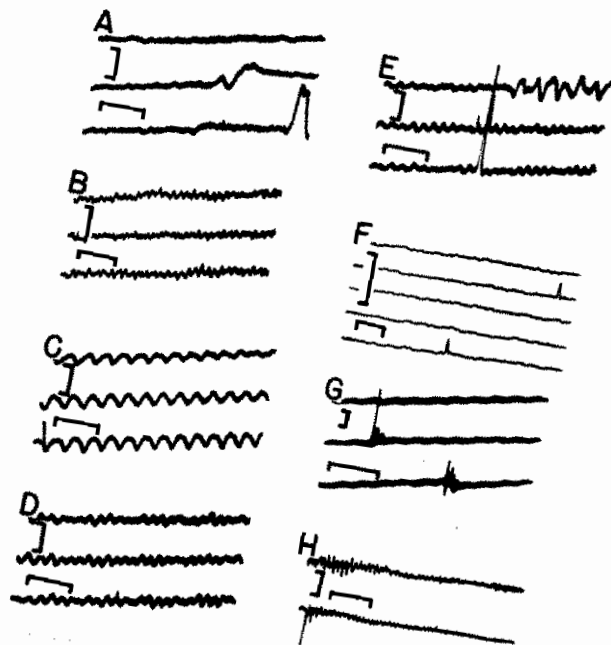


Fig. 4. Arrangement of thermode. Not to scale. Coolant flow direction shown by arrows. T: Temperature recording circuit. S: Stimulating circuit. R: Recording circuit. M: Microelectrode. F: Muscle fibre. N: Phrenic nerve trunk. Heavy lines indicate Perspex. Hatched area about thermode tip indicates liquid paraffin retained by a thin Perspex collar. See text.



**Fig. 5.** Artefacts from preparation, electrodes and probe valves.  
 A: Movement artefacts in a fibrillating preparation. B: Noise in a high resistance electrode (50 M $\Omega$ ). C, D, E: Unexplained oscillatory noise observed only with electrode tip in a preparation. See text. Note 'beat' in record D. Miniature potentials also occur on these three records.  
 F: Spontaneous 'pips' and G, H: Microphonics originating in probe valves (Mullard EF 72). Voltage scale: 2 mV for all records. Time scale: A, C, D, E = 100 msec; B = 200 msec; F = 1 msec; G = 50 msec; H = 10 msec.

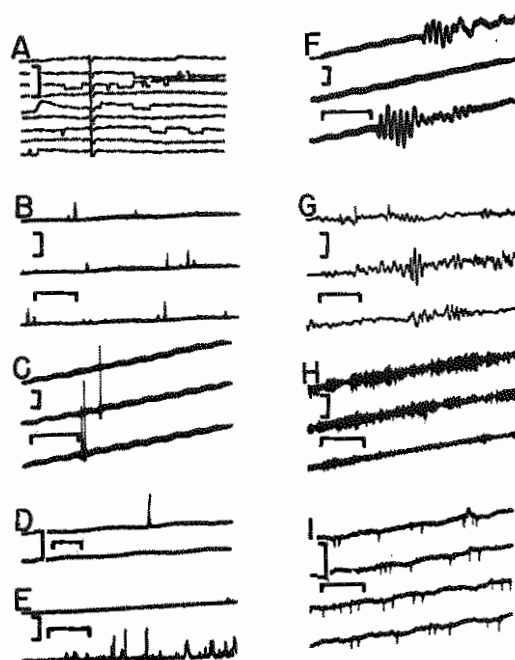


Fig. 6. Artefacts. A, B: Probe valve HT supply. A miniature potential occurs on record A. C - G: Amplifier artefacts. C, D: Microphonics from light and camera switches. E: Noisy potentiometer. F: Accidental movement of amplifier. G: Earthing fault. H, I: Noise of unidentified origin. Voltage scale 2 mV for all records. Time scales: A = 1 msec (on record); B, D, E, G = 10 msec; C, F = 50 msec; H = 200 msec; I = 20 msec.

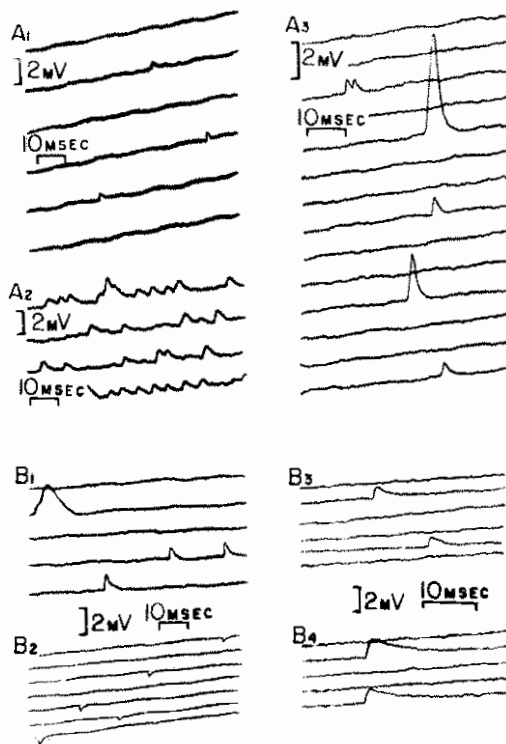


Fig. 7. A 1 - 3: Examples of miniature potentials at 3 neuromuscular junctions in an isolated hemidiaphragm. Membrane potentials were respectively 71, 64 and 76 mV.

B 1 - 4: Miniature potentials in the gracilis muscle in vivo.

B 1: Intracellular recording; B 2: Same fibre as B 1 but extracellularly recorded; B 3: Intracellular recording; B 4: Same fibre as B 3 but 10 minutes after intravenous injection of prostigmine bromide 0.4 mg per Kg. Membrane potentials were B 1: 78 mV and B 3: 67 mV.

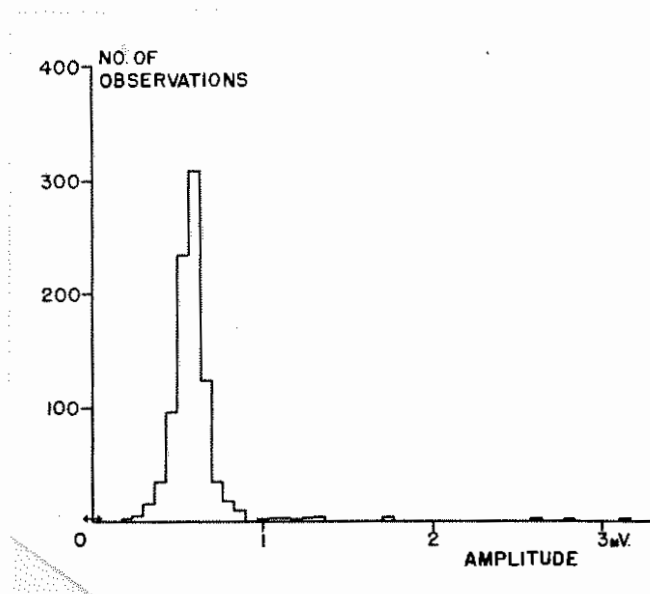


Fig. 8. Frequency distribution of amplitudes of 903 miniature potentials serially recorded in a fibre. Amplitudes grouped into brackets of  $1/15$  m. Arrows at origin indicate approximate range of instrumental baseline 'noise' voltage. Membrane potential was 69 mV.

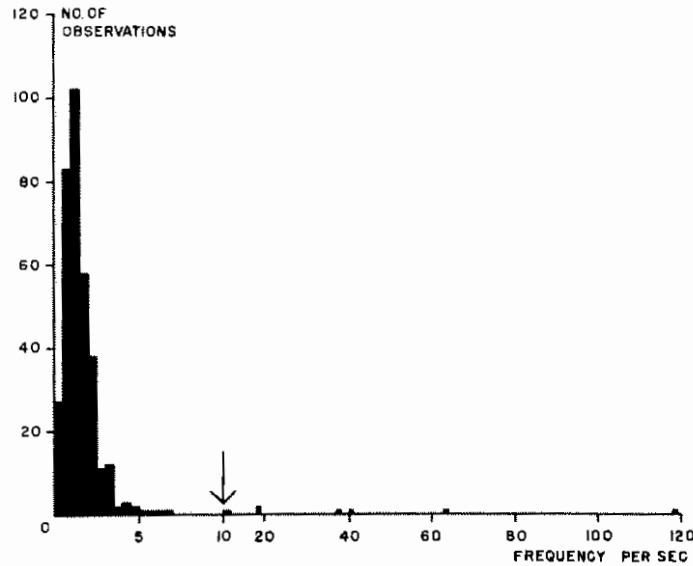


Fig. 9. Frequency distribution of frequencies of miniature potentials at 342 normal junctions. Note change in abscissal scale beyond 10 per sec. Amplitudes grouped into brackets of 0.5 per sec to 10 per sec; thereafter brackets of 1 per sec. This graph supersedes Fig. 3 of Liley (1956a) but contains the population shown in that original figure. Note that the proportion of high values for resting frequency has fallen markedly - which probably reflects improved technique.

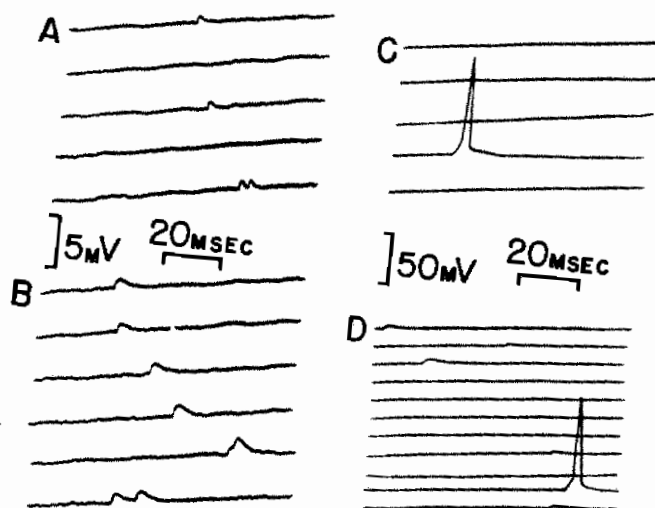


Fig. 10. Effect of prostigmine on miniature potentials. A: Control records; B: Same fibre as A but 5 minutes after the addition of  $10^{-6}$  prostigmine bromide. Membrane potential was 62 mV. C and D: Two other fibres showing the generation of action potentials by the augmented miniature potentials. Prostigmine as in B. Membrane potentials were respectively 76 and 74 mV.

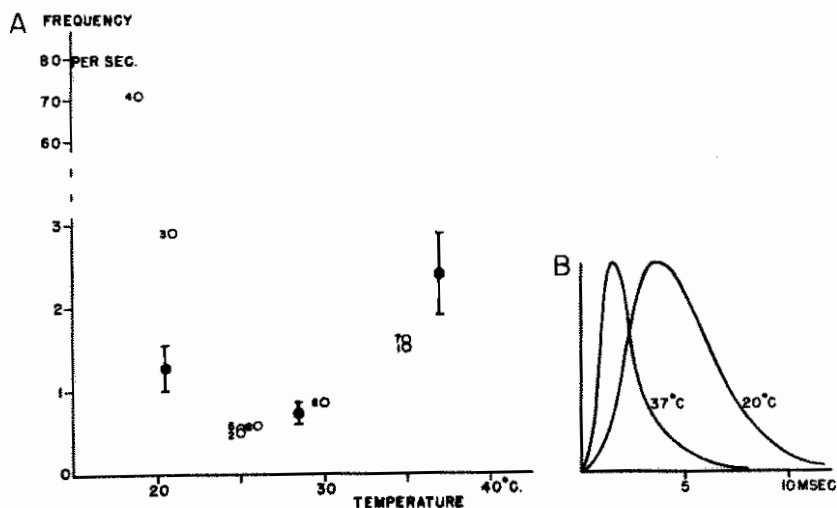


Fig. 11. A: Effect of temperature on frequency of miniature potentials. Open circles represent continuous record from a single junction. Values determined in the sequence indicated by numbers. Note change in ordinate scale above 3 per sec. Filled circles represent the mean frequency at a number of junctions, the exact numbers being at 37°C; 20 fibres, 28°C; 21 fibres, 20.5°C; 14 fibres. In each case approximately half this number were measured during cooling and the remainder during rewarming. Bars are placed at  $\pm 2$  S.E. of the mean in each case.

B: Effect of temperature on the time course of miniature potentials. Each curve represents the mean time course of 10 miniature potentials scaled to the same maximum amplitude.



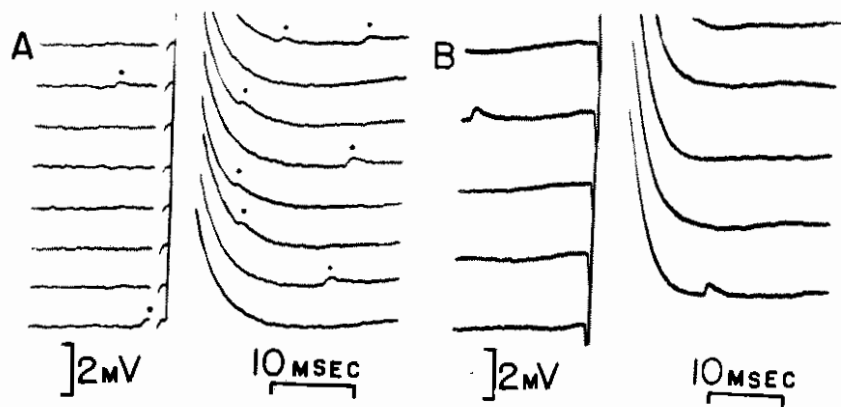


Fig. 12. A: Increased frequency of miniature e.p.p.s (marked by dots) following single nerve volleys; B: Absence of effect on miniature discharge by direct muscle stimulation. In both A and B the interval between successive sweeps was 1 sec. Membrane potentials were respectively 68 and 71 mV.

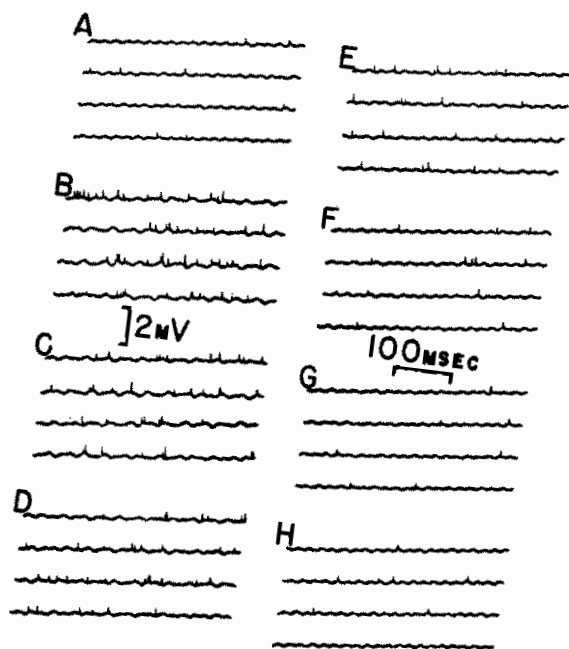


Fig. 13. Effect of repetitive indirect stimulation on frequency of the miniature discharge. A: Control records which were followed by a 15 second tetanus of frequency 200 per sec. Post-tetanic records shown are at B: 15 sec, C: 30 sec, D: 1 minute, E: 2 minutes, F: 4 minutes, G: 6 minutes, H: 8 minutes. Membrane potential was 74 mV initially but fell to a steady value of 66 mV following the tetanus.

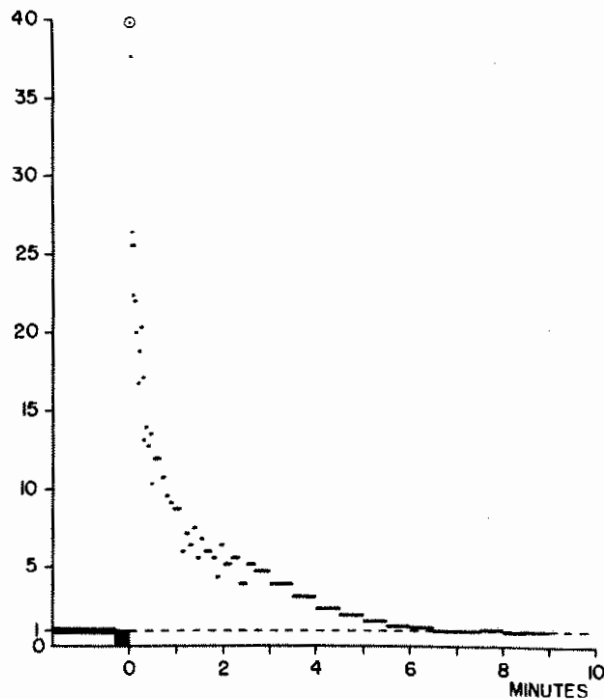


Fig. 14. Effect of repetitive indirect stimulation on frequency of miniature discharge at a junction in normal solution. Tetanus of duration 15 sec and frequency 200 per sec represented by rectangle on abscissa.

Ordinate: Ratio of post-tetanic frequency to control-frequency. Width of bar representing control frequency (absolute value 2.5 per sec) indicates  $\pm 2$  S.E. of mean frequency as determined by serial 10 sec counts. Post-tetanically the length of bars indicates the interval over which the frequency was determined except where adjacent bars are obviously collinear. Immediate (0 - 1 sec) post tetanic frequency circled for clarity. Note the initial high but very transient peak attained.

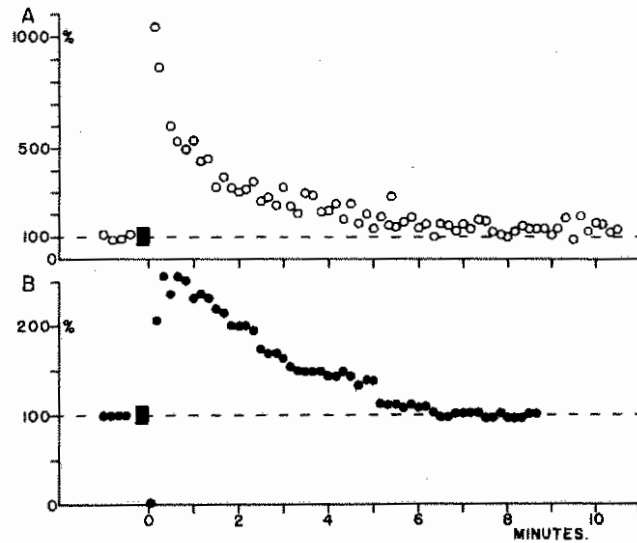


Fig. 15. <sup>Comparison</sup> ~~Composition~~ of time courses of effects of repetitive indirect stimulation on A: frequency of miniature discharge in a normal fibre (same fibre as Fig. 13) and B: end-plate potentials in a curarized (tubocurarine chloride  $10^{-6}$ ) fibre. Post-tetanic observations plotted as a percentage of control records in each case. Resting frequency of miniature discharge in A was 4 per sec. Tetanus, of duration 15 sec and frequency 200 per sec, represented in each graph by the rectangular block. Common abscissal (time) scale commences at the termination of the tetanus.

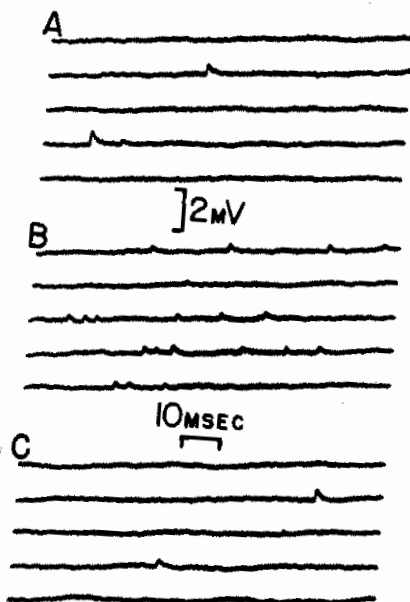


Fig. 16. Effect of osmotic pressure increase on miniature potentials. A: Control records in solution of normal constitution; B: Records (from same junction as A) 5 minutes after addition of sucrose to produce 25% increase in osmotic pressure; C: 5 minutes after return to normal solution.

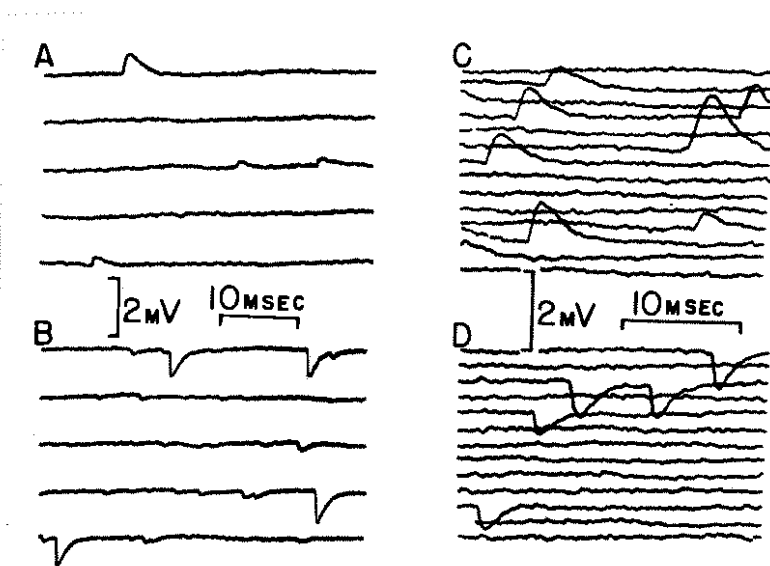


Fig. 17. Intra- and extracellular records of miniature potentials, all from the same junction. A: Internal recording; B: External recording. C and D: Corresponding records on faster time base. Note reversed polarity and faster time course of the externally recorded potentials. Membrane potential was 72 mV.

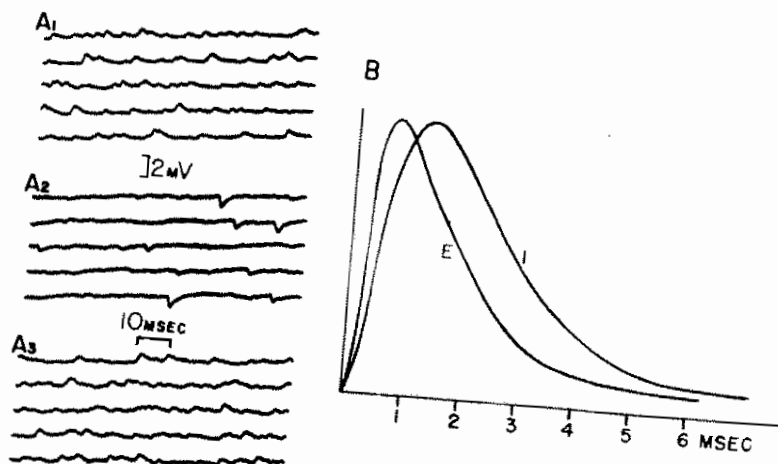


Fig. 18. A 1 - 3: Intra- and extracellular records at another junction. Note that the externally recorded miniature discharge (A2) was of much lower frequency than that internally recorded (A1 and A3). B: Comparison of time courses of internal (I) and external (E) miniature potentials from the experiment of Fig. 17 and Table 2. The two curves, each representing the mean time course of 16 miniature potentials have been scaled to the same maximum, disregarding the opposite polarities.

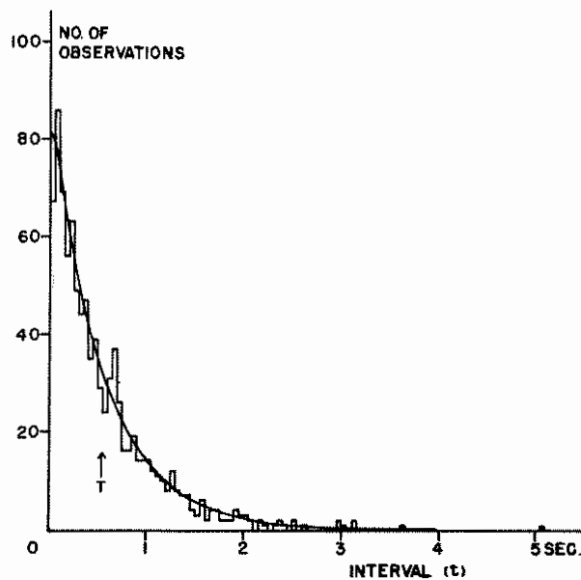


Fig. 19. Distribution of time intervals, in brackets  $\Delta t$  of 50 msec, in a series of 929 miniature potentials (from the experiment of Fig. 8). Mean interval  $T$  of 547 msec indicated by arrow. Continuous curve is the graph for a stochastic process  $n = N \Delta t / T \exp(-t/T)$  where  $N$  = total number of observations ( $= 928$ ). As the briefest interval which could be resolved in this experiment was 2 msec, the interval from 0 to 0.002 sec must be considered blank. Hence the curve  $n = N \Delta t / T \exp(-t/T)$  deviates, because the first bracket  $\Delta t$  comprises only 48 msec after  $t = 2$  msec.



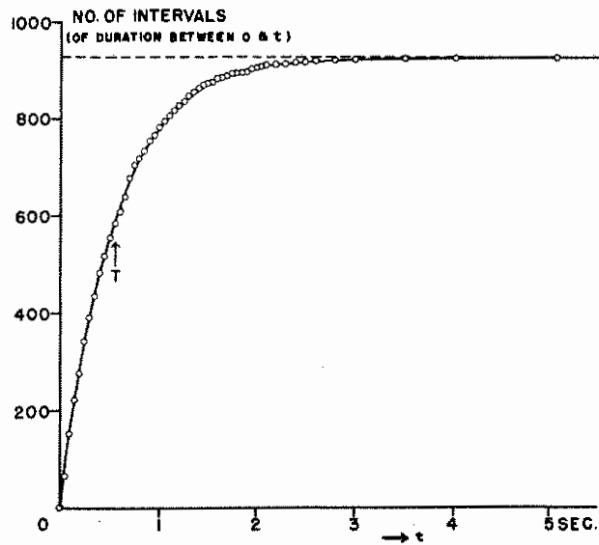


Fig. 20. Total number of observed intervals of duration less than  $t$  (from the experiment of Figs. 8 and 19) have been plotted against interval duration  $t$ . Circles represent observed values and the full line the theoretical curve  $y = N (1 - \exp (-t/T))$  where  $N$  and  $T$  each have the same meaning and value as in Fig. 19.

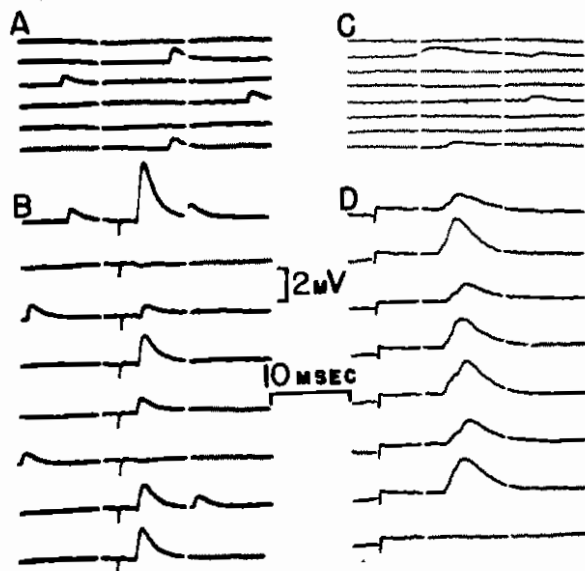


Fig. 21. Intracellular recordings of fluctuations in e.p.p. response at junctions treated with Ca 1 mM, Mg 6mM. A: Spontaneous miniature potentials. B: (Same fibre as A) E.p.p. responses. Note scattered spontaneous potentials on records; also intermittent failure of response and at the sites of such failures small extrinsic potentials from activity at junctions in adjacent fibres. Temp. 38°C. C & D: Corresponding records at 22°C. Note increased latency of responses and increased duration of miniature potentials and responses at the lower temperature. Note also inflexions on rising phase of responses. In all records gaps on traces indicate 10 msec calibration.

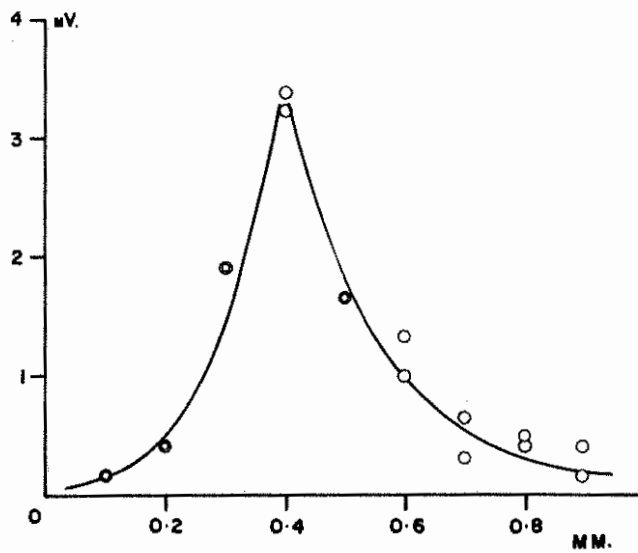


Fig. 22. Spatial decrement of peak amplitude of the e.p.p. Intracellular recording. Tubocurarine chloride  $7 \times 10^{-8}$ . Electrode insertions made serially from right to left; values were checked by repeating the series from left to right.

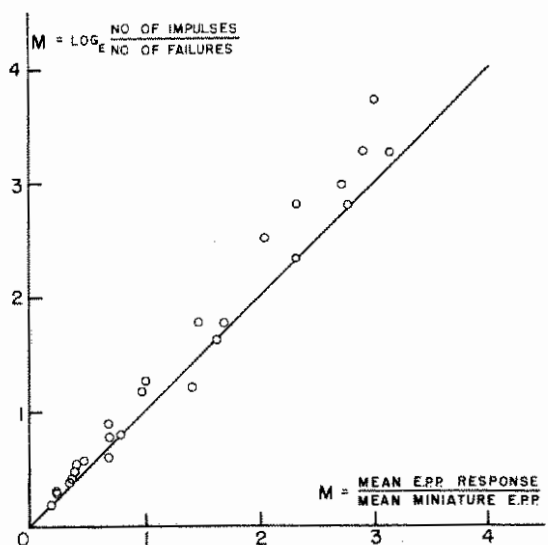


Fig. 23. The results of 26 experiments in which the values of  $m$  determined respectively by Equations 1 (abscissa) and 2 (ordinate) were compared. See text. The line at  $45^\circ$  signifies equality of these two estimates of  $m$ .

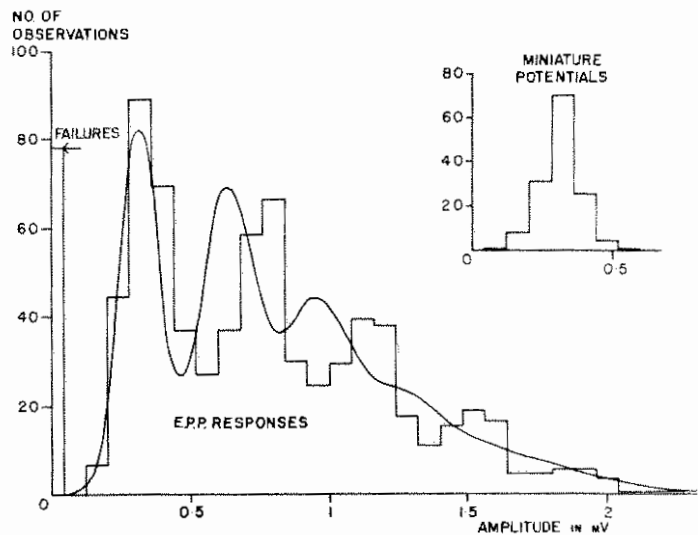


Fig. 24. Histograms showing distribution of amplitudes of miniature potentials and e.p.p. responses at a junction treated with 12.5 mM Mg. The continuous curve in the e.p.p. response histogram has been calculated on the hypothesis that the responses are built up in a statistically predictable manner of units whose mean size and amplitude distribution are represented by the miniature potentials (inset). Predicted number of failures is indicated by an arrow. See text.

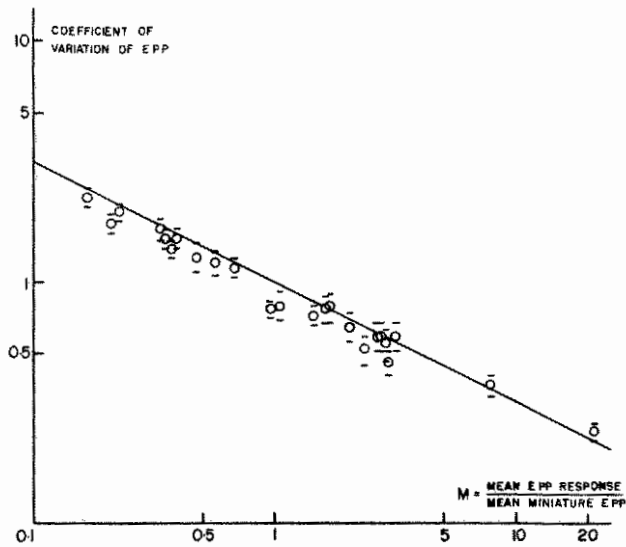


Fig. 25. Relation between coefficient of variation ( $= \frac{SD}{\text{mean}}$ ) of e.p.p. response and nominal value of  $m$  in twenty four experiments. Bars have been placed at  $\pm 2$  S.E. of coefficient of variation. Full line indicates the theoretical relation for Poisson distributions. See text.

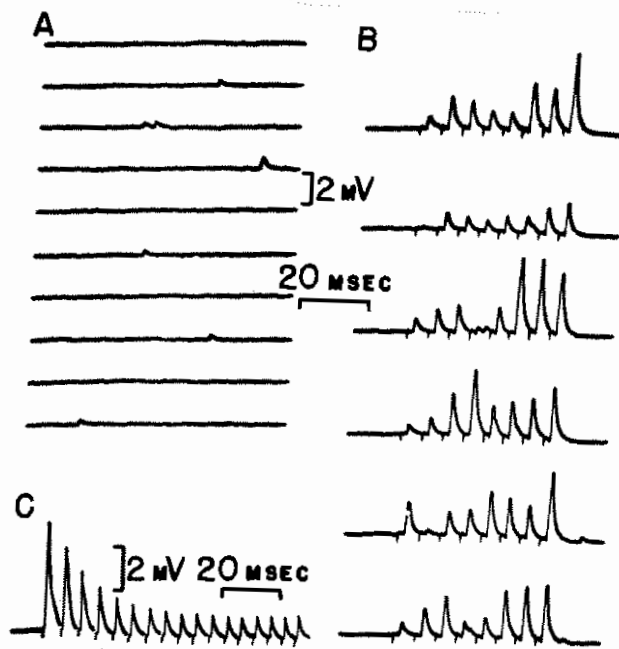


Fig. 26. Intracellular recordings of pattern of response to tetanic stimulation in 10 mM Mg. A: Miniature potentials. B: (Same fibres as A). Tetani of frequency 160 per sec. C: Extracellular record of a tetanus of frequency 180 per sec in a curarised preparation with normal Ca & Mg concentrations.

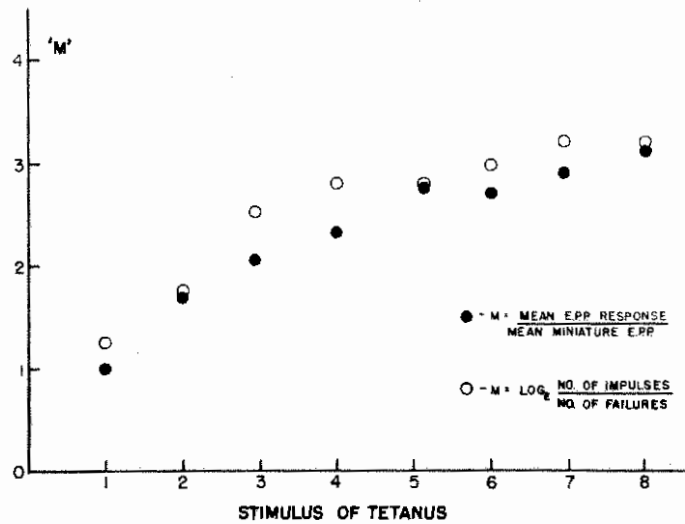


Fig. 27. Comparison of the values of  $m$  derived from equations (1) and (2) for the corresponding impulses in a series of 100 brief tetani. See text Pg 12.5 ml.



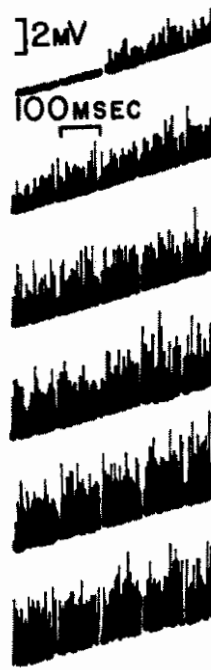


Fig. 28. Pattern of response to a prolonged tetanus of frequency 200/sec. Ca 0.5 mM Mg 3 mM. Intracellular recording. Records from above indicate responses at start of tetanus then at 1 sec, 3 sec, 6 sec, 10 sec, 14 sec. Gaps on records at 100 msec.

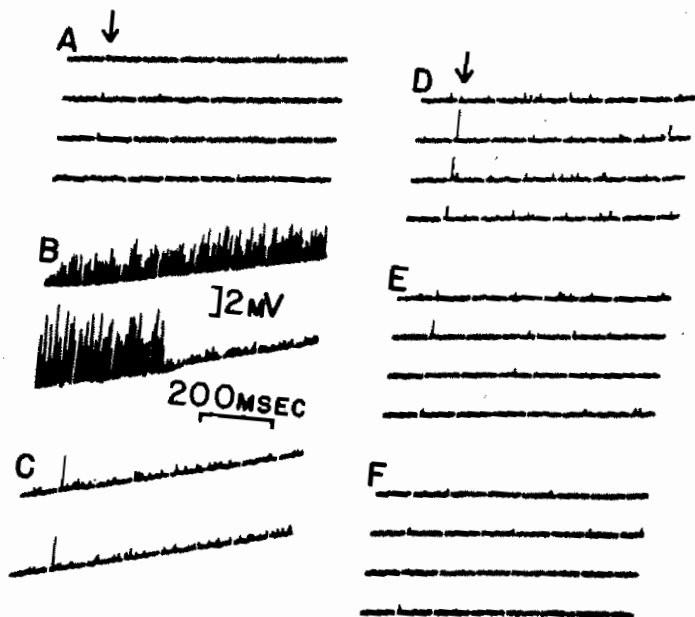


Fig. 29. Effect of tetanic stimulation on frequency of miniature potential discharge and amplitude of e.p.p. responses. Ca 2mM (normal), Mg 12 mM. Intracellular recording. A: Control records. B: Beginning and end of a 15 sec tetanus of frequency 200/sec. C: Post-tetanic records at 0.75 and 1.75 sec. D: 18-22 sec. E: 4 min. F: 8 min. In pre- and post-tetanic records the test stimulus occurs immediately after the first timer gap (indicated by arrows). Timer gaps indicate 100 msec. The apparent increase in the amplitude of the spontaneous potentials immediately after the tetanus is due to numerous 'coincidences' of discharge of the miniature potential at high frequency.

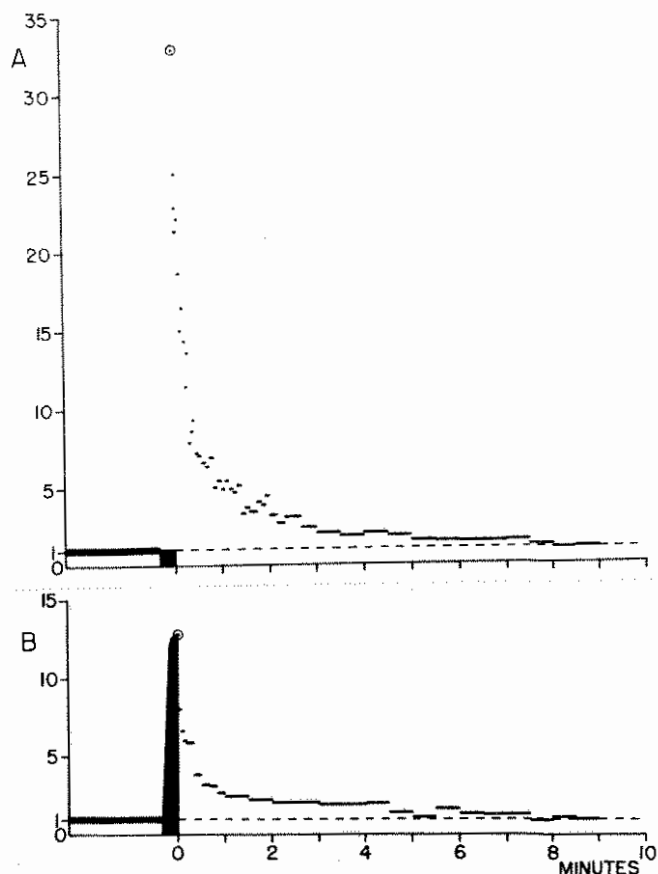


Fig. 30. A: Effect of a tetanus on frequency of miniature discharge.  $Mg$  mM. Tetanus of duration 17 sec and frequency 200 per sec indicated by black rectangle on abscissa. Ordinate: Ratio of post-tetanic frequency to control frequency. Width of bar representing control frequency (absolute value = msec) indicates  $\pm 2$  S.E. of mean frequency as determined by serial 10 sec counts. Post-tetanically the length of the bars indicates the interval over which the frequency was determined except where adjacent bars are obviously collinear. Immediate post-tetanic value of frequency (0 - 1 sec) is circled for clarity.

B: Effect of a tetanus on mean amplitude of e.p.p. response - recorded simultaneously with 30A. Ordinate: Ratio of post-tetanic e.p.p. response amplitude to control response amplitude. The pattern of the conditioning tetanus itself is indicated in the same convention. Width of bar /cont'

Fig. 30, cont'd

representing control response amplitude indicates  $\pm 2$  S.E. of mean response amplitude as determined by measurements of responses in serial groups of ten. Stimuli applied at 1 sec intervals. Post-tetanic length of the bars indicates the interval over which the response amplitude was determined except where adjacent bars are obviously collinear. Initial post-tetanic value of response amplitude (mean of responses at 0.25 and sec) circled for clarity. Common(abscissal) time scale commences at the termination of the tetanus.

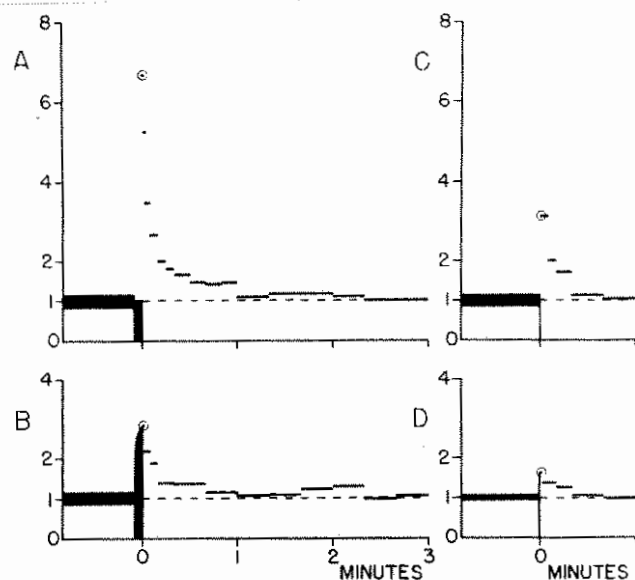


Fig. 31. A & B: Effect of a tetanus (of duration 5 sec and frequency 20 sec) on frequency of miniature potentials and mean amplitude of e.p.p. responses respectively. Mg 12 mM. In A control frequency of miniature discharge was 2.1 per sec. Conventions of symbols and axes in A & B are identical with those of Fig. 30A & B respectively. C & D: As in Fig 30A & B respectively but for a tetanus of duration 0.5 sec and frequency 200 per sec. Mg 12 mM. Control frequency of miniature potentials in C was 0.7 per sec.

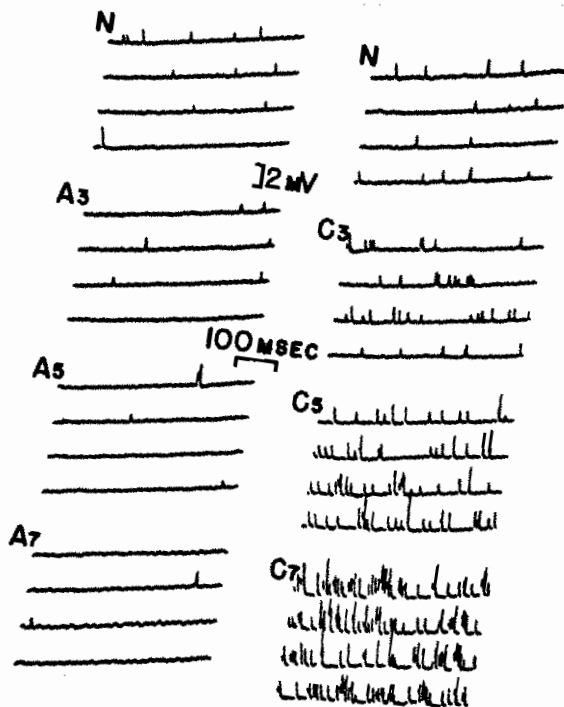


Fig. 32. Electrotonic effects on the miniature discharge frequency.  
 N,N: initial and check recordings of resting discharge. A: anodic and  
 C: cathodic polarisation of nerve terminals. The numbers give current  
 intensity in relative units.

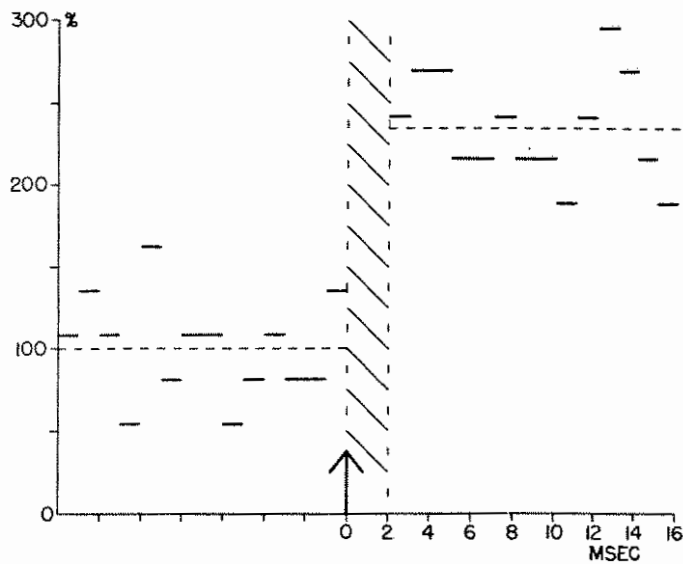


Fig. 33. Cathodic polarisation of nerve terminals. Effect on miniature discharge frequency of a rectangular pulse. Onset of pulse and origin of time scale marked by an arrow. Ordinate: effective discharge frequency (see Methods) as a percentage of mean resting frequency (1.53 per sec). Horizontal broken lines show mean resting frequency and mean frequency during pulse. Hatched area represents record obscured by artefact.

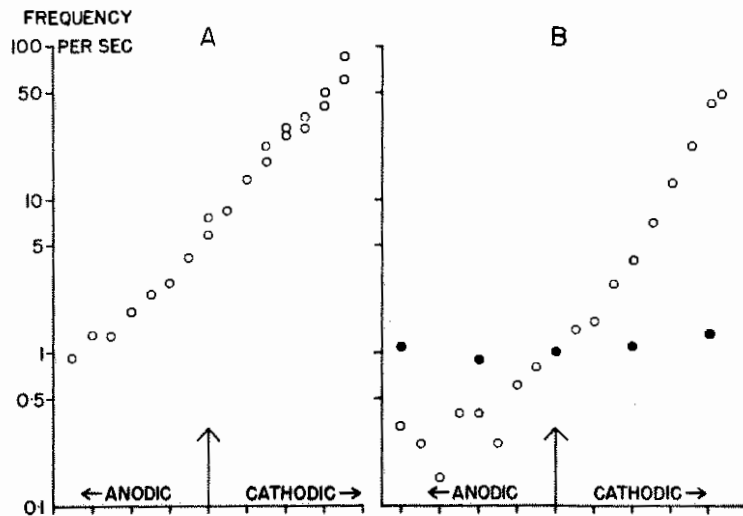


Fig. 34. Electrotonic effects on miniature discharge frequency. Note logarithmic ordinate scales. Abscissae: polarising current intensity in relative units. A: muscle in normal solution showing linear relation between log-discharge frequency and polarising current intensity (product moment correlation coefficient,  $r$ , ( $= \frac{\text{covariance of } x \text{ \& } y}{\text{Var } (x) \times \text{Var } (y)} = 0.99$ )). B: experiment showing deviation from this empirical relationship. See text. Hollow circles: muscle in normal solution. Filled circles: 12.5% magnesium.



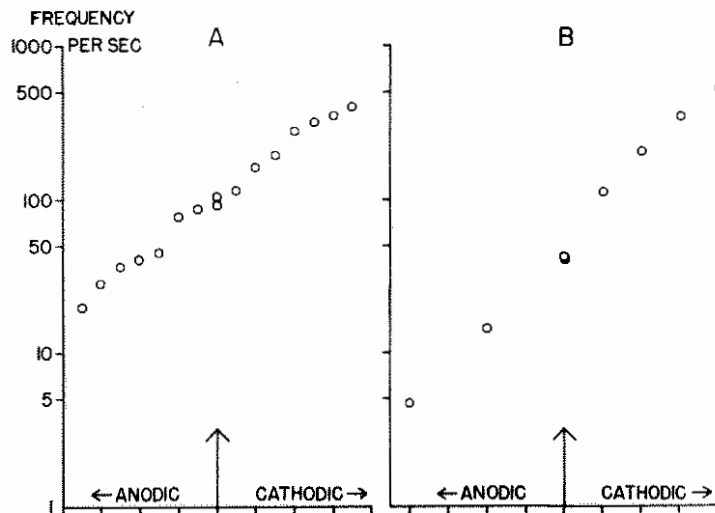


Fig. 35. Electrotonic effects on miniature discharge frequency. Conventions of scales as in Fig. 34. A: muscle in normal solution but initially discharge frequency was increased from resting rate of 1.6 per sec to 100 per sec by trauma to nerve terminals. B: muscle in solution with potassium concentration of 15 mM. Discharge frequency in normal solution was 2 per sec.

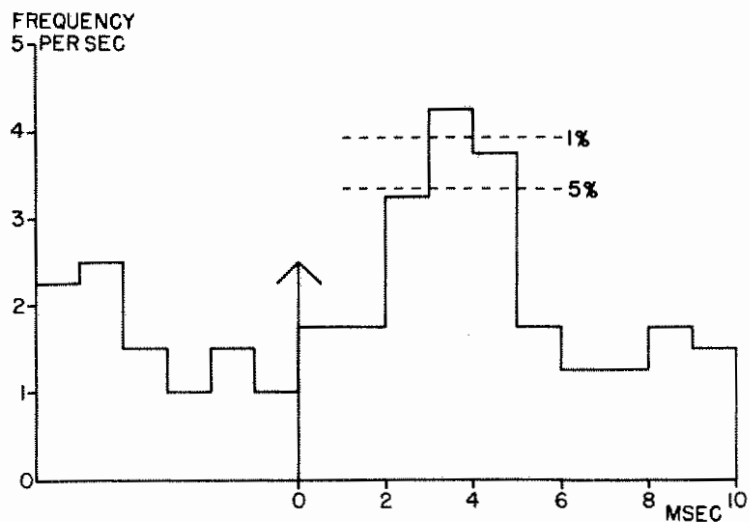


Fig. 36. Effect on miniature discharge frequency of the block of a nerve impulse within 3 mm of the nerve terminal. Effective frequencies determined from 4,000 sweeps as explained in Methods. Stimulus and origin of time marked by an arrow. Temperature of the terminal not accurately measurable but ca. 30°C. The significance of the peak in this graph may be assessed as follows: A  $\chi^2$  test ( $\chi^2 = 28.3$ ,  $f = 15$ ,  $p \sim 0.01$ ) indicates that the distribution of the number of miniature potentials falling in each interval departs from the expectation on a null hypothesis but does not specifically incriminate the three peak values. On the null hypotheses that the number of miniature potentials falling in each interval are terms from Poisson populations with equal means, the number of observations in any interval which would be exceeded at the 5% and 1% probability limits may be calculated. Such limits (converted to effective frequencies) are shown on the graph. Finally, on the null hypothesis that the order of frequencies in the graph is random, the probability of the three largest values (irrespective of relative size) occurring together, at any time after the nerve stimulus, is given by

$$p = \frac{(8.2.2. + 1.2.1 + 1.2.1) 13!/3! 2! 2! 4!}{16!/3! 2! 2! 4!} = 0.0107$$

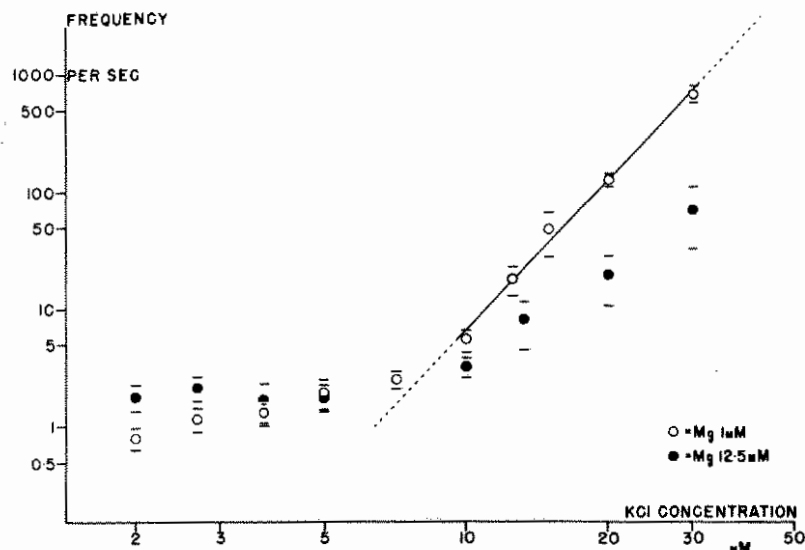


Fig. 37. Effect of potassium concentration on miniature discharge frequency. Normal potassium concentration was 5 mM. Note that both scales are logarithmic. Circles represent mean frequency at approximately twenty junctions with bars placed at  $\pm 2$  S.E. of mean. Hollow circles: muscles in solution with normal magnesium (1mM) and calcium (2mM). For the five points beyond 10 mM potassium ( $r = 0.98$ ) the regression line for log. frequency on log potassium concentration has been drawn. Filled circles: muscles in solution containing 12.5 mM Mg, normal Ca (2 mM).

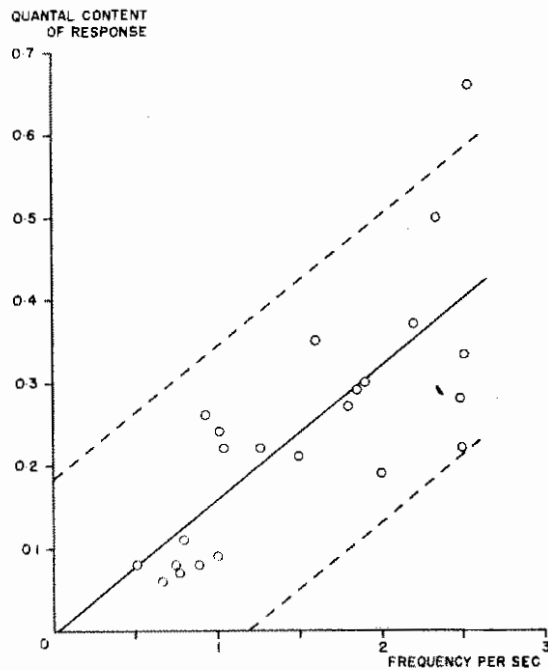


Fig. 38. Relation between quantal content of response and resting discharge frequency at 23 junctions in one preparation.  $r = 0.76$ , Student's  $t = 5$ ,  $f = 21$ . Full line is regression line for quantal content on frequency with broken lines at  $\pm 2$  S.E. of estimate. Ca 1mM, Mg 12.5 mM.

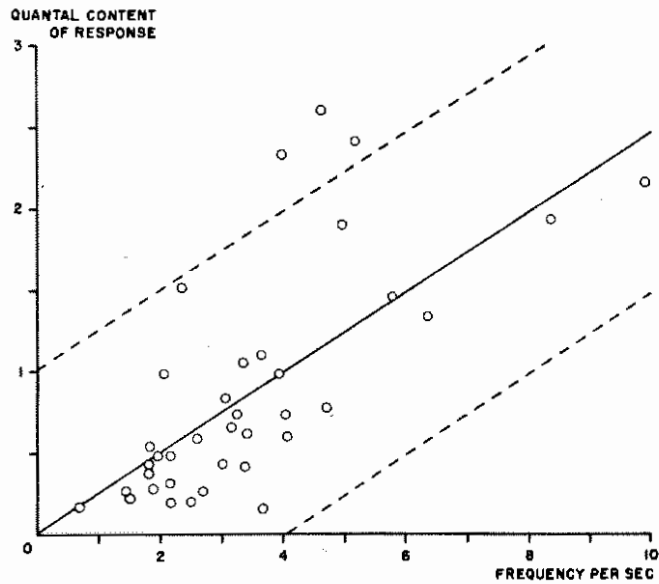


Fig. 39. Relation between quantal content of response and resting disc frequency at 37 junctions in one preparation.  $r = 0.68$ , Student's  $t = 5$ ,  $f = 35$ . Full line is regression line for quantal content on frequency with broken lines at  $\pm 2$  S.E. of estimate.  $\text{Ca } 1.5 \text{ mM}$ ,  $\text{Mg } 12.5 \text{ mM}$ .

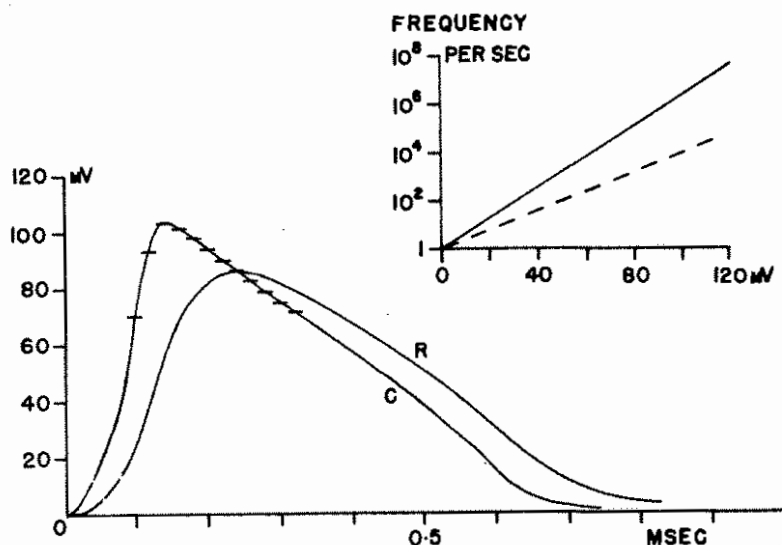


Fig. 40. Derivation of quantal content of an e.p.p. Curve R: intracellularly recorded action potential of an intramedullary motor axon of a cat. Curve C: same action potential corrected (by subtangent analysis (Rushton 1937)) for distortion by recording system. Bars indicate 0.02 msec intervals for which the expected frequency of the miniature discharge was calculated. See text. Inset: theoretical relationship between discharge frequency and displacement of membrane potential (from Fig. 37) used in calculation. Strictly, the ordinate scale as labelled represents the frequency which would be attained by a terminal with a resting discharge rate of 1 per sec when the membrane was depolarised by an amount shown in the abscissal scale. In general, the ordinate scale represents the factor by which the frequency would be altered for a given displacement of membrane potential, depolarisation raising the frequency and hyperpolarisation reducing it. Full line: relation in normal solution. Broken line: relation in solution containing 12.5 mM Mg.

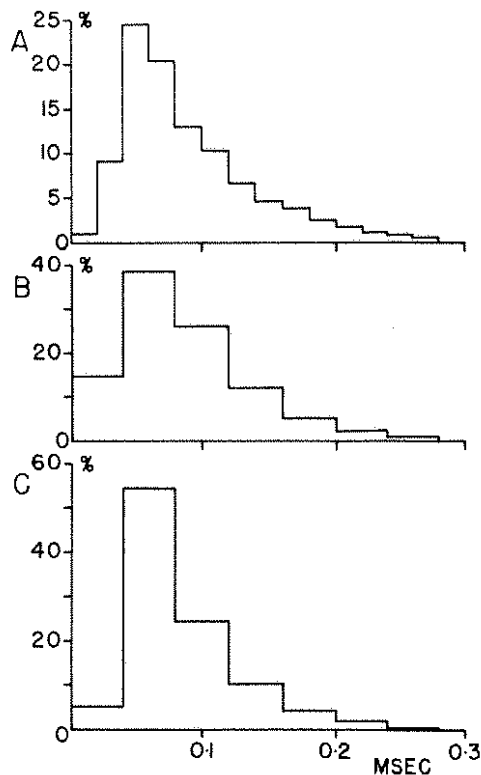


Fig. 41. A: theoretical frequency distribution of stimulus-response intervals for the quanta involved in a large series of responses in 12.5 mM Mg. B & C: observed distributions of latencies of responses at two junction in 12.5 mM Mg. 766 responses in B, 895 in C. See text. Ordinate: percenta of total number of latencies falling in a given class interval. (Note class interval for A is 0.02 msec and for B & C 0.04 msec.) Common abscissal scale has an arbitrary origin corresponding to briefest latency. Mean stimulus-response interval in B was 1.8 msec and in C 1.2 msec.

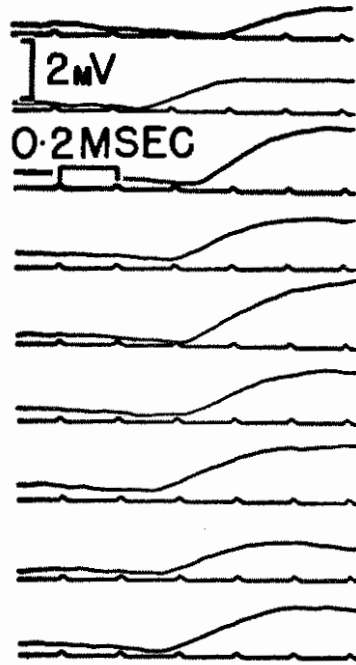


Fig. 42. Selected sweeps from the experiment of Fig. 41B to illustrate variation in stimulus-response intervals. A check was made on an expanded sweep that the stimulus position did not vary on the sweep. After this check the portion of the sweep containing the responses was expanded to full screen width.



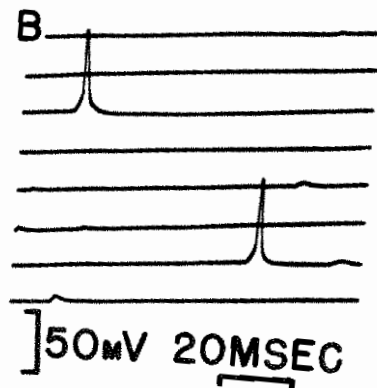
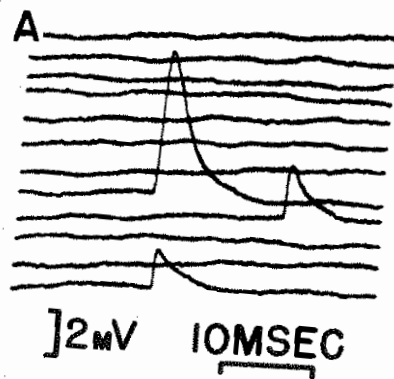


Fig. 43. A: A giant potential at a junction in the rat gracilis in vivo.  
 B: Action potentials generated by giant potentials in a fibre in the isolated diaphragm in normal solution. Several subthreshold giant potentials are also present,

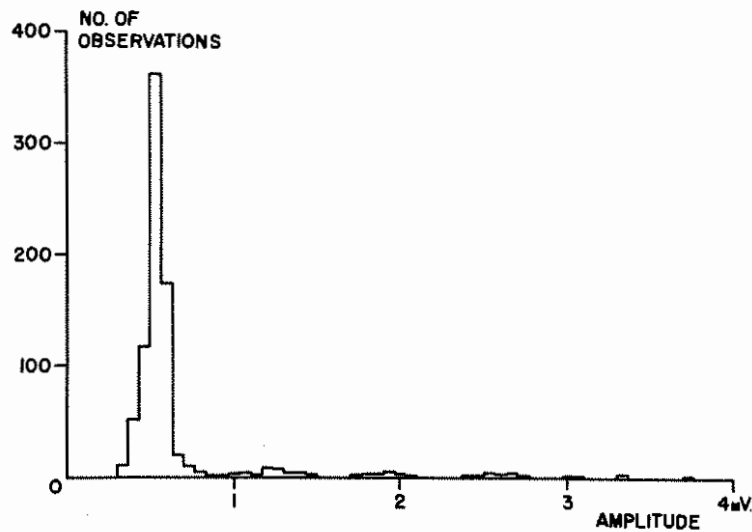


Fig. 44. Frequency distribution of amplitudes of 820 spontaneous potentials at a junction with a high proportion (ca. 9%) of giant potentials. Amplitudes grouped into brackets of 1/15 mV. Note the distinct periodicity in the distribution. Discharge frequency was 3.09 per sec.

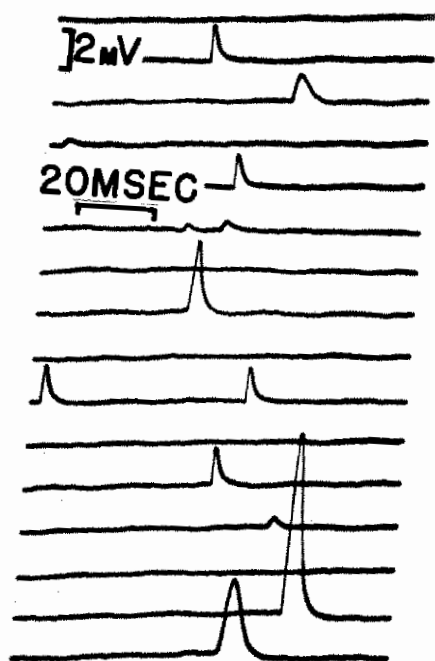


Fig. 45. Record from a fibre with double innervation. Note fast (rising phase 0.6 - 0.9 msec) and slow (rising phase 1.4 - 2 msec) potentials. Discharge frequency of fast potentials was 1.57 per sec and of slow potentials 0.68 per sec. See also Fig. 46.

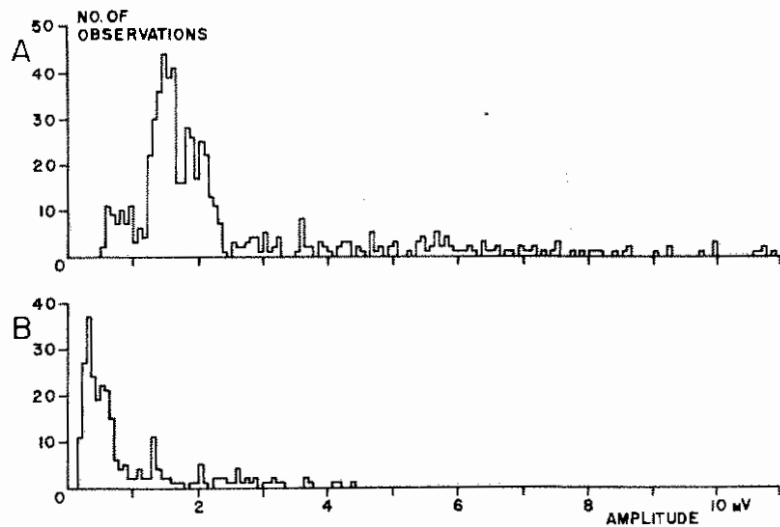


Fig. 46. Frequency distribution of amplitudes of A: 609 fast and B: 263 slow potentials in the fibre of Fig. 45. Amplitudes grouped into brackets of 1/15 mV. Note difference between modal amplitudes of A and B; also that each population has a marked 'tail' of giant potentials.

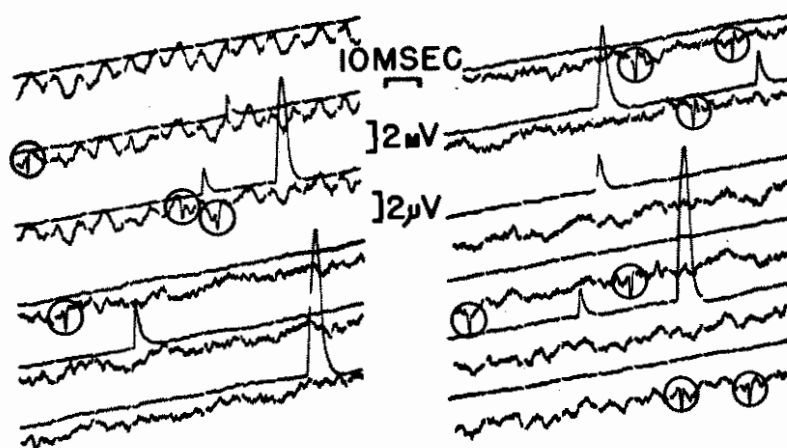


Fig. 47. Simultaneous intracellular recording at a junction (upper, clean record) and extracellular recording from the pared phrenic nerve (lower, noisy record) in a preparation treated with prostigmine bromide  $10^{-6}$ . Note that the action potentials (circled) in the phrenic nerve are not associated with the giant potentials. Voltage calibrations: 2 mV for intracellular record, 2  $\mu$ V for extracellular record.

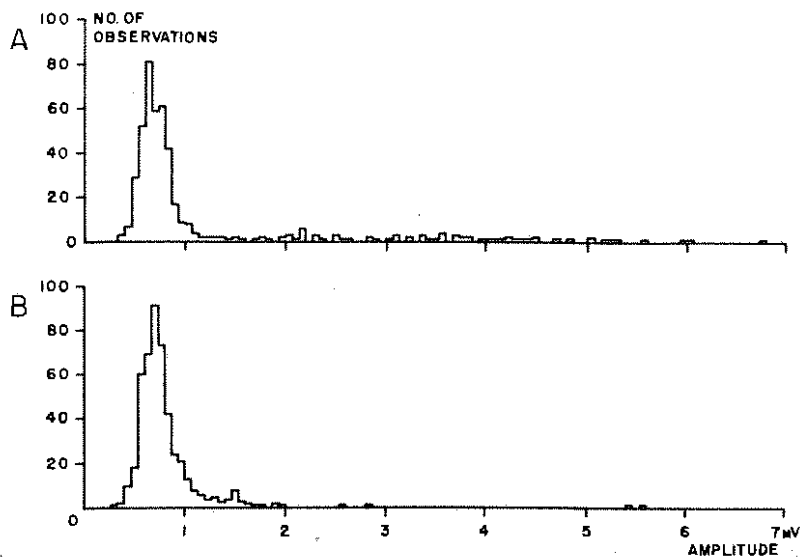


Fig. 48. Effect of electrotonic polarisation of a nerve terminal on the incidence of giant potentials. A: Frequency distribution of amplitudes of 458 consecutive potentials at a resting junction (discharge frequency 10.2 per sec) with a high proportion of giant potentials. Amplitudes grouped into brackets of  $1/15$  mV. B: Frequency distribution of amplitudes of 478 consecutive potentials at the same junction as A after discharge frequency had been increased to 117 per sec by depolarisation of the nerve terminal. Note that the modal amplitude is unchanged but the relative frequency of giant potentials has declined markedly. (Strictly, the potentials were not 'consecutive' in either series, because potentials whose peaks occurred during the 3 msec 'flyback' of the C.R.O. beam were discarded.)

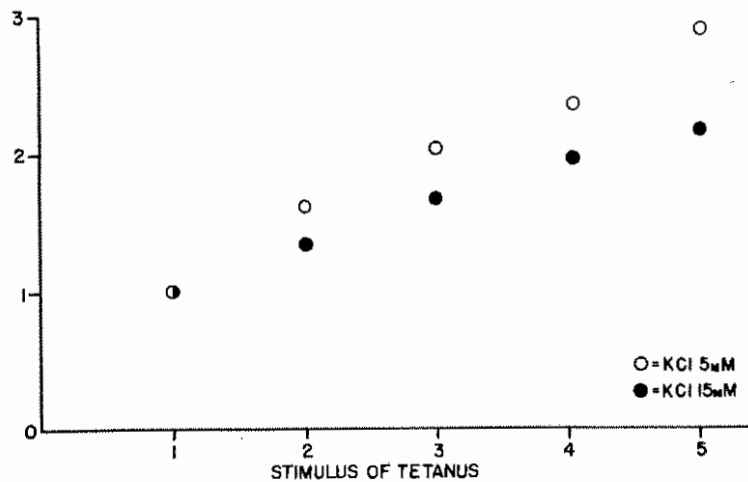


Fig. 49. Effect of potassium on serial increase in the mean amplitude of corresponding responses of 80 brief tetani at a junction in 12.5 mM Mg, normal Ca. Ordinate: Ratio of mean response amplitude to mean amplitude of the first response. Tetanus frequency 160 per sec (see also Figs. 26, 27 & 50).

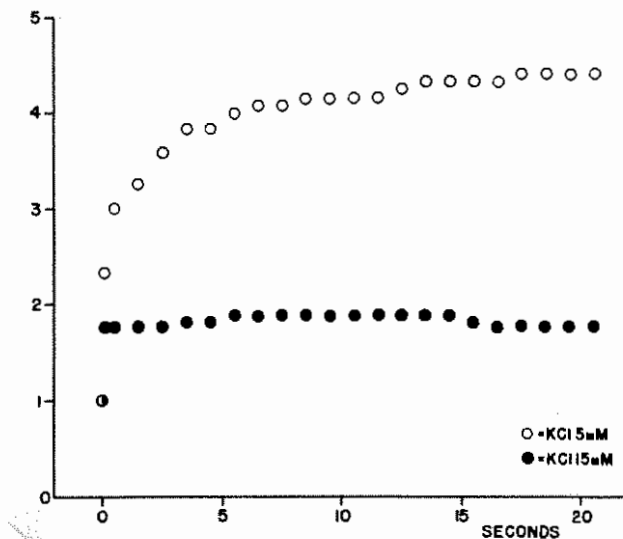


Fig. 50. Effect of potassium on the pattern of responses to a long tetanus in a diaphragm in 1.5 mM Ca, 12.5 mM Mg. Extracellular recording. Ordinate: Ratio of response amplitude to amplitude of the first response. Tetanus frequency 120 per sec. This low frequency was necessary to avoid overlap of successive e.p.p.s in this particular preparation.



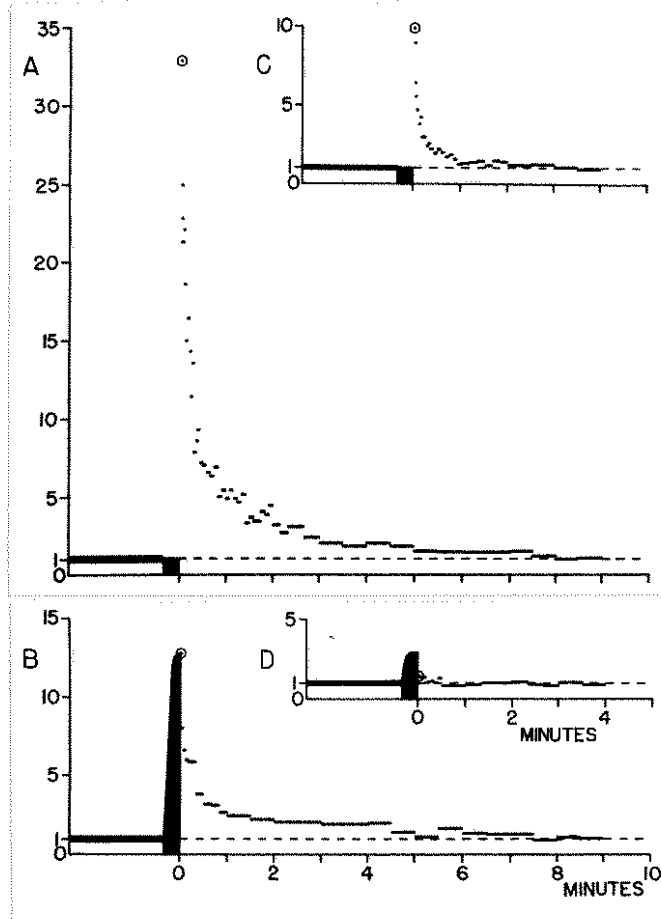


Fig. 51. A & B: These graphs reproduce Fig. 30 A & B and show the effect of a tetanus of 3,400 impulses (200 per sec for 17 sec) on discharge frequency and response amplitude respectively. Mg 12 mM, Ca normal, KCl normal (5 mM). C & D: corresponding effects by the same tetanus at the same junction after potassium concentration was increased to 15 mM.