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SPONTANEOUS RELEASE OF TRANSMITTER SUBSTANCE IN MULTIQUANTAL UNITS

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The occurrence of spontaneous miniature potentials at the neuromuscular junction of the rat has been described in a previous paper (Liley, 1956*a*). In that paper reference was made to the frequent observation of spontaneous large 'miniature' potentials with amplitudes several times the modal value of a series of potentials and, at one junction, calculations showed that more of these large potentials occurred than could be accounted for by the random coincidence of the 'unitary' miniature potentials. The present paper 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 paper, such potentials will be referred to as 'giant potentials'. In distinction, 'miniature potentials' will refer to the 'unitary' spontaneous potentials.

METHODS

Details of the preparation, solutions, apparatus and technique of intracellular recording employed have been described in previous papers (Liley, 1956*a–c*). On the assumption that the discharge of miniature potentials is a random process, the method by which the expected number of coincident discharges in a series of miniature potentials may be calculated has been illustrated by Fatt & Katz (1952) and Liley (1956*a*).

RESULTS

Giant potentials could be detected at any junction, both in the gracilis muscle *in vivo* (Fig. 1*A*) and in the isolated diaphragm (Fig. 1*B*). 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 very few more than would have been predicted from random coincidence of miniature potentials. Nevertheless, when twenty junctional records were selected for detailed study because the incidence of giant potentials did not appear

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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 random 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 there were too many occasions when 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. 1*B*).

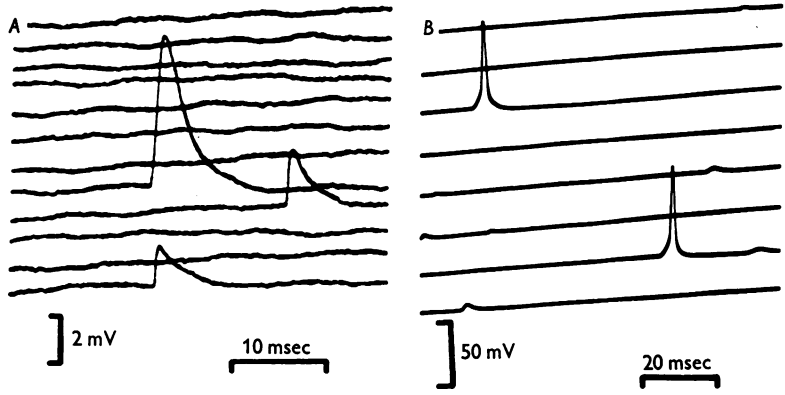

Fig. 1. *A*: A giant potential at a junction in the rat gracilis *in vivo*. *B*: Action potentials generated by giant potentials in a fibre in the isolated diaphragm in normal solution. Several subthreshold giant potentials are also present.

At some junctions the frequency distribution of amplitudes of giant potentials displayed an obvious periodicity (Fig. 2), 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. This periodicity was not always evident (Figs. 4*A*, 6) because 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. (del Castillo & Katz, 1954*a*). 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 time course of the giant potentials normally did not differ from that of the miniature potentials. Occasionally, with the larger giant potentials the latter part of the rising phase was slower (Fig. 5), which possibly indicates a situation akin to that described by del Castillo & Katz (1955*a*, 1956*a*) in the

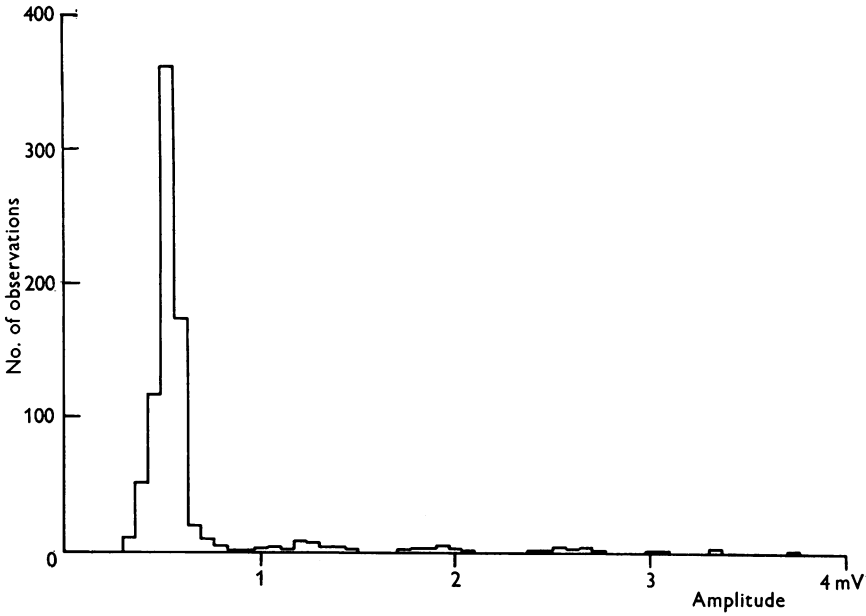

Fig. 2. Frequency distribution of amplitudes of 820 spontaneous potentials at a junction with a high proportion (about 9 %) of giant potentials. Amplitudes grouped into brackets of 1/15 mV. Note the distinct periodicity in the distribution. Discharge frequency was 3·09/sec.

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 base line was recognizable for a longer time.

By contrast, in three fibres evidence of double innervation was obtained. Records from one of these fibres are shown in Figs. 3 and 4. In this situation, naturally, the larger potentials exhibited a much briefer time course since they originated in the junction nearer the micro-electrode. Furthermore, each population exhibited its own family of giant potentials.

Edds (1950) and Cole (1955) have demonstrated the bifurcation of an epilemmal axon to give two end-plates on the same muscle fibre in rat leg muscles. An occasional bifurcating axon with or without two distinct end-plates may be found also in the stained rat diaphragm. However, two distinct endings on one muscle fibre would need to be separated by at least 50–100 μ before they could be discriminated electrically, and even then it is essential that the micro-electrode 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 in the rat diaphragm, only three fibres have displayed unequivocal evidence of double innervation.

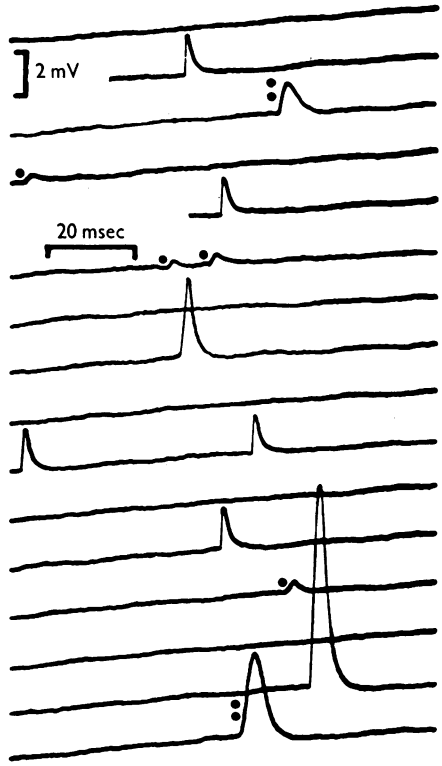

Fig. 3. Records from a fibre with double innervation. Note fast (rising phase 0·6–0·9 msec) and slow (rising phase 1·4–2 msec) potentials. Miniature slow potentials are preceded by a dot and giant slow potentials by two dots. Discharge frequency of fast potentials was 1·57/sec and of slow potentials 0·68/sec. See also Fig. 4.

Obviously it was important to recognize 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 curarization of a preparation giant potentials

diminished in amplitude, but frequently they could be detected after the miniature potentials were lost in the base-line 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 (Liley, 1956*a*). 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.

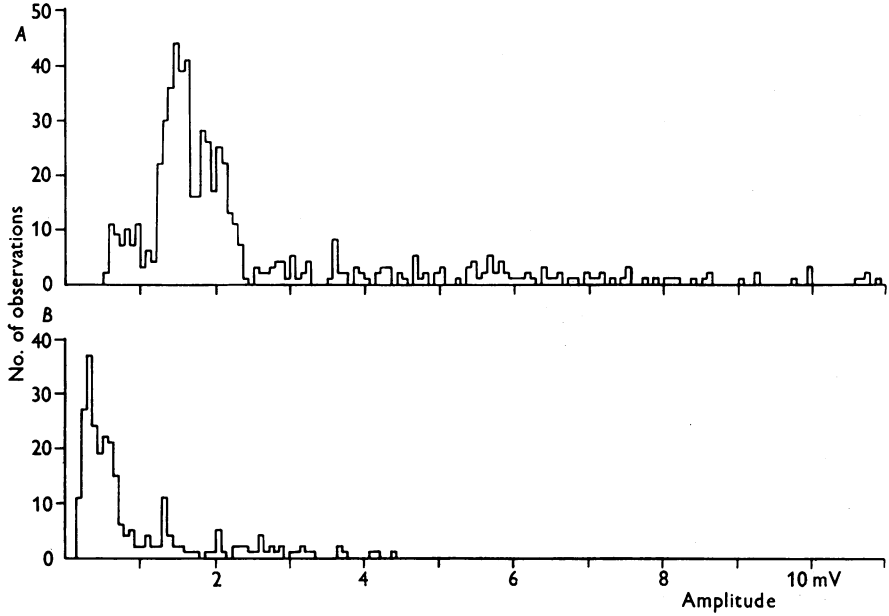

Fig. 4. Frequency distribution of amplitudes of *A*, 609 fast; and *B*, 263 slow potentials in the fibre of Fig. 3. Amplitudes grouped into brackets of 1·15 mV. Note difference between modal amplitudes of *A* and *B*; also that each population has a marked 'tail' of giant potentials.

(*d*) Temperature variation (25–40°C); anoxia; sodium cyanide (2 mM); glucose deprivation (2 hr). 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 micro-electrode 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. 5).

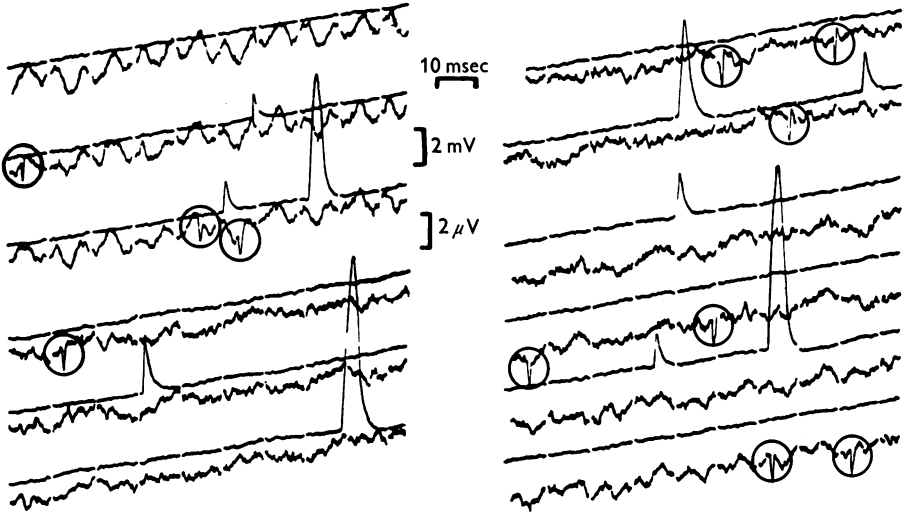

Fig. 5. Simultaneous intracellular recording at a junction (upper, clean record) and extracellular recording from the pared phrenic nerve (lower, noisy record) in a preparation treated with prostigmine bromide 10^{-6}. Note that the action potentials (circled) in the phrenic nerve are not associated with the giant potentials. Voltage calibrations: 2 mV for intracellular record, 2 μV for extracellular record.

This phenomenon was originally observed by Masland & Wigton (1940) in the eserinized 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, considering only those potentials whose amplitudes exceeded threefold or more the modal amplitude of the miniature potentials, no nerve impulse was recorded within ± 5 msec of any of forty-four giant potentials recorded at four 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 are not usually, and possibly are never, generated 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-Mg the paradoxical situation was observed that, whereas a nerve impulse evoked a response of low quantal content (Boyd & Martin, 1956; Liley, 1956*b*) 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 nerve impulse.

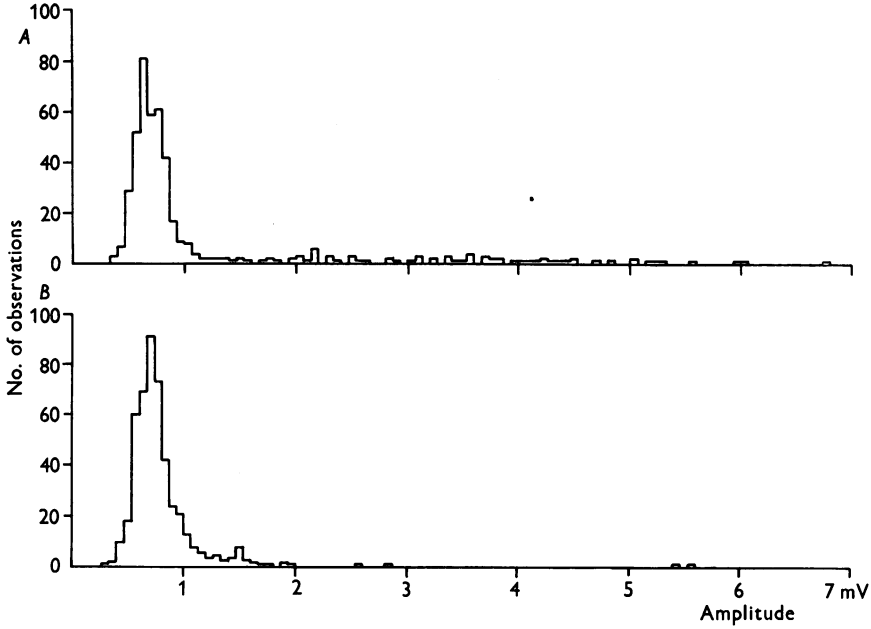

Fig. 6. Effect of electrotonic polarization of a nerve terminal on the incidence of giant potentials. *A*: Frequency distribution of amplitudes of 458 consecutive potentials at a resting junction (discharge frequency 10·2/sec) with a high proportion of giant potentials. Amplitudes grouped into brackets 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/sec by depolarization 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 there was rejection of potentials whose peaks occurred during the 3 msec 'flyback' of the C.R.O. beam.)

(*g*) Electronic polarization of nerve terminals. When the motor nerve terminals were hyperpolarized or depolarized 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 polarization (Liley, 1956*c*), the absolute frequency of the giant potentials was not significantly altered. Hence the relative frequency of giant

potentials varied greatly with polarization (Fig. 6). 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 polarization (Liley, 1956*c*), such a statement would not apply at a junction such as that depicted in Fig. 6. 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 (Liley, 1956*c*). Potassium in a concentration of 15–20mM 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 (Liley, 1956*a*, *b*). The absolute frequency of giant potentials at two junctions showed no marked change when the miniature discharge was accelerated by a tetanus of 3000 impulses (200/sec for 15 sec).

DISCUSSION

Del Castillo & Katz (1956*a*) suggested 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' (del Castillo & Katz, 1955*a*, 1956*a*; Liley, 1956*a*) 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 briefer 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*) The 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 nerve impulses.

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

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. Variations in calcium and magnesium concentration modify profoundly the effects of an impulse and electronic depolarization of the release of quanta (del Castillo & Katz, 1954*a*, *b*; Boyd & Martin, 1956; Liley, 1956*b*, *c*), 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 membrane of motor nerve terminals.

Giant potentials cannot be explained as artifacts produced by injury. Deliberate trauma to a junction (Liley, 1956*a*, *c*) invariably accelerates the miniature discharge but has never been found to induce the multiquantal release of transmitter. Furthermore, giant potentials may be detected with the micro-electrode inserted some distance from a junction.

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, 1956*a*). This model was suggested by the observations that the miniature potential frequency was raised after indirect stimulation, and that electrotonic depolarization of the terminals accelerated the discharge. However, this explanation raises several problems. First, it predicts that the behaviour of a motor nerve 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 depolarization of the nerve terminals.

As an alternative hypothesis, coalescence of preformed quanta of transmitter 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 depolarization, 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. Robertson (1956) suggests that these vesicles might be charged with acetylcholine and be analogous to secretion granules (cf. del Castillo & Katz, 1955*b*, 1956*b*). It is an attractive 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 is of interest, therefore, that Robertson (1956) has observed structures which he designates 'elongated vesicles' in the motor nerve terminals of the lizard. If similar structures can be identified in mammalian motor nerve terminals it would be tempting to suggest that they represent the multiquantal accumulations of transmitter which, on release, produce giant potentials.

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

Before the present investigation, junctions displaying numerous giant potentials were regarded with suspicion and avoided where possible. As a result, in most experiments involving the determination of the mean amplitude of spontaneous potential populations (Liley, 1956*b*), 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 discharges 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 polarization of nerve terminals (Liley, 1956*c*). Such a practice was fortunate, for a large stable population of giant potentials could seriously distort the interpretation of records—particularly with hyperpolarized terminals. Indeed reinvestigation of the records of two junctions in which hyperpolarizing currents failed to depress the discharge frequency to an expected level (Liley, 1956*c*) 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 emitted, it is reasonable to count also 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.

SUMMARY

1. In the spontaneous discharge of transmitter at the neuromuscular junction of the albino rat, large potentials ('giant potentials') occur more frequently than would be expected from the random coincidence of miniature potentials.

2. The relative frequency of giant potentials varies greatly from junction to junction.

3. Giant potentials consist of summated miniature potentials.

4. The absolute frequency of giant potentials remains unaltered when the frequency of the miniature potential is modified by presynaptic polarization.

5. It is suggested that giant potentials are produced by the release of preformed multiquantal aggregates of transmitter substance.

6. The practical implications of this phenomenon have been discussed.

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