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TRANSMITTER RELEASE FROM INSECT EXCITATORY MOTOR NERVE TERMINALS

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SUMMARY

1. Intracellular and extracellular electrodes were used to study spontaneous and impulse-linked release of transmitter at locust retractor unguis nerve-muscle synapses.

2. At most extracellular recording sites the amplitude distributions of the excitatory post-synaptic potentials (e.p.s.p.s) were apparently non-Poisson. However, interpretation of these amplitude distributions was complicated by the effect on the extracellular recordings of the complex structural organization of the retractor unguis nerve terminal with its spatially distinct transmitter release sites extending over distances of 15–30 μm.

3. The spontaneous miniature excitatory post-synaptic potentials (min e.p.s.p.s) did not occur at random intervals, bursts of min e.p.s.p.s being frequently recorded. As a result the spontaneous release of transmitter rarely approximated a Poisson process.

4. For a period of at least 390 msec following a conditioning nerve impulse a test e.p.s.p. was facilitated and the probability of spontaneous transmitter release was enhanced. A large primary phase of facilitation of impulse-linked and spontaneous release was invariably followed by one or more secondary phases of smaller magnitude.

INTRODUCTION

It is generally accepted that the release of transmitter from axon terminals is a quantal process and that secretion of transmitter quanta normally occurs spontaneously at a low rate but is transiently accelerated by the arrival of a nerve impulse (Katz, 1962). The packets of transmitter are thought to contain relatively large numbers of transmitter molecules and their spontaneous release evoke miniature post-synaptic potentials, either excitatory or inhibitory in the underlying cell or other post-synaptic structure. The spontaneous miniature post-synaptic potentials

can be regarded as the smallest unit of the evoked synaptic potential for when synaptic transmission is depressed either by magnesium or by lowering external calcium the size of the synaptic potential approaches that of the miniature potential and at the same time exhibits large random fluctuations in amplitude apparently involving steps of unit size. In other words, the normal synaptic potential, either excitatory post-synaptic potential (e.p.s.p.) or inhibitory post-synaptic potential is built up of a large number of unit potentials appearing synchronously in response to a single stimulus. Evidence for quantization of transmitter release has been derived mainly from synapses, such as those on vertebrate nerve-phasic striated muscles where there is usually one synapse on each post-synaptic unit. Miniature potentials recorded intracellularly at one of these synapses show only a small variation in amplitude about the mean with the occasional occurrence of giant potentials whose amplitude is an approximate multiple of the mean (Fatt & Katz, 1952; Boyd & Martin, 1956; Liley, 1956, 1957).

Where the post-synaptic unit is multiterminally and/or polyneuronally innervated evidence for quantization of transmitter rests mainly on the observation that miniature potentials occur. Quantitative analysis of intracellular data from such structures is greatly complicated because the intracellular recording electrode monitors responses of varying quantal content originating at various distances from the recording site and is therefore subject to varying degrees of spatial decrement. Insect muscle fibres fall into this category since they are multiterminally innervated and frequently polyneuronally innervated and some fibres have inhibitory as well as excitatory inputs. Miniature excitatory post-synaptic potentials (min e.p.s.p.s) were first recorded intracellularly from insect muscle by Usherwood (1961, 1963) and have since been recorded from a variety of insect muscles (see review by Usherwood, 1969). More recently, extracellular recordings of spontaneous and impulse-linked transmitter release from single nerve terminals on locust muscle have been made (Usherwood & Machili, 1968). In this paper semi-quantitative studies of min e.p.s.p.s and e.p.s.p.s recorded from a locust nerve-muscle preparation are described. One interesting finding is that the spontaneous release of transmitter from the terminals of locust motoneurones is rarely a random process; there is a preference for the min e.p.s.p.s to occur in bursts of short duration.

METHODS

Studies were made on retractor unguis muscles isolated with their two innervating motoneurones from the metathoracic femoral segments of *Schistocerca gregaria* (Usherwood & Machili, 1968). E.p.s.p.s and min e.p.s.p.s were recorded intracellularly using 3 M-KCl-filled micro-electrodes with resistances (measured before and

after insertion) of 10–20 MΩ and tip potentials < 5 mV. Electrodes filled with 2 M-NaCl and of 1–5 MΩ resistance were used to record extracellularly from single nerve terminals (active spots) on the surface of the muscle fibres (del Castillo & Katz, 1956). The active spots were located with an extracellular recording electrode by trial and error during neural stimulation of the muscle with a suction electrode at a frequency of 0·5/sec.

The electrical response of the retractor unguis muscle fibre to neural stimulation consists of a large e.p.s.p. plus a large graded electrically excited response (Usherwood, 1967). In order to reduce the amplitude of the e.p.s.p. and thereby eliminate the electrically excited response the preparation was exposed to locust saline (NaCl 140, KCl 10, NaH_2PO_4 4, Na_2HPO_4 6, $CaCl_2$ 2 m-mole/l. (Usherwood & Grundfest, 1965)) containing either a relatively high concentration of magnesium (40–45 m-mole/l.) or a lower concentration of magnesium (15 m-mole/l.) and a reduced amount of calcium (0·5 m-mole/l. compared with 2 m-mole/l.). The magnesium was substituted osmotically for sodium. These salines will be referred to jointly as magnesium saline.

Preparations were maintained at approximately 18° C and recordings were made after an initial 1-hr period of equilibration in locust saline. E.p.s.p.s and spontaneous min e.p.s.p.s were photographed as a continuous recording and the resultant records were enlarged by projection. Amplitudes of potentials were determined by measuring from top of base line to peak of potentials. The synaptic potentials in magnesium saline were never more than 5 mV in amplitude and therefore no corrections were made for effects of membrane potential on e.p.s.p. amplitude. Although the equilibrium potential for the excitatory transmitter has not yet been accurately measured it is probably close to zero (Takeuchi & Takeuchi, 1964). The resting potentials of the retractor unguis muscle fibres were 56–60 mV.

Facilitation at the synapses on the retractor unguis muscle fibres was examined by stimulating the retractor unguis nerve with pairs of stimuli, the interval between the stimuli being varied between 25 and 390 msec; the interval between pairs of stimuli being maintained constant at 5 sec. The alternative procedure of stimulating the retractor unguis nerve at different frequencies for set periods of time was not used except for frequencies less than 5/sec because the nerve-muscle synapses fatigue quite rapidly with high frequency stimulation even in magnesium saline.

RESULTS

Synaptic potentials

At the motor end-plates on frog phasic skeletal muscle fibres subthreshold intracellular e.p.s.p.s are obtained during partial magnesium block, which fluctuate in amplitude in a discontinuous fashion (del Castillo & Engbaeck, 1954). In contrast intracellular e.p.s.p.s recorded from locust muscle fibres in magnesium saline do not vary much in amplitude and discontinuities are not apparent unless transmission is almost completely blocked and the e.p.s.p.s are less than 2·5 mV in amplitude (Usherwood, 1963). However, extracellular e.p.s.p.s recorded from active spots on locust muscle fibres during partial magnesium block do vary in amplitude in an approximate stepwise fashion. Since the nerve-muscle synapses are mainly positioned on the inner faces of the retractor unguis muscle fibres (Usherwood, 1969; Rees & Usherwood, 1972) active spots were readily

located by running an extracellular recording electrode along a cleft between two muscle fibres whilst stimulating the retractor unguis nerve. The spots at which synaptic currents could be monitored were sharply localized, and the extracellular synaptic potential declined in amplitude as the electrode was moved a few micrometres away from the nerve terminal. The extracellular potentials were negative going and their time course (5–10 msec) was faster than the membrane potential change recorded by an intracellular electrode (Fig. 1). The extracellular potential was mainly associated in time

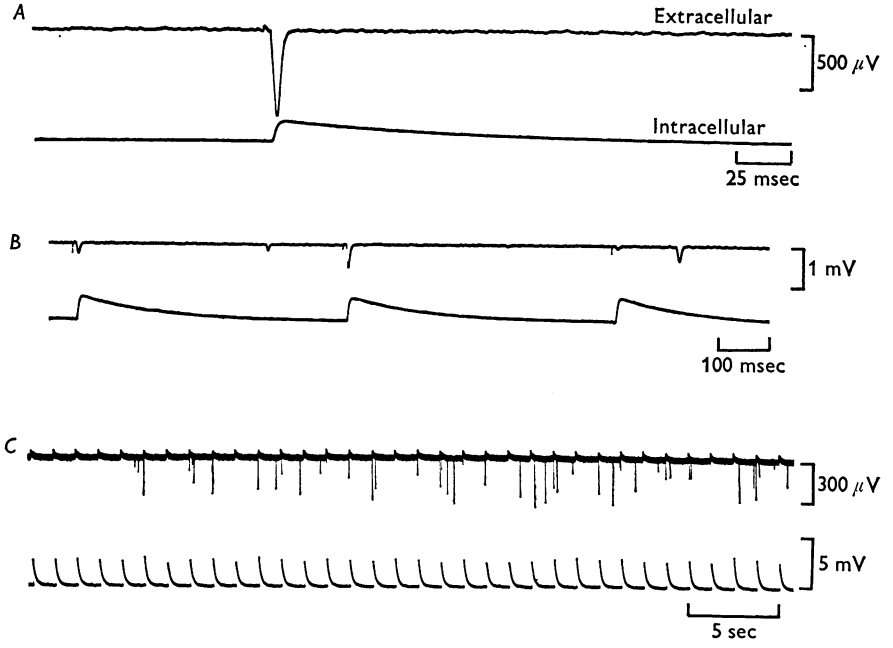

Fig. 1. Extracellular recordings of e.p.s.p.s from three active spots on retractor unguis muscles, with concurrent intracellular recordings from relevant muscle fibres. Resting potentials of muscle fibres: *A*, 58 mV; *B*, 60 mV; *C*, 60 mV. Upward deflexions in top trace of *C* are stimulus artifacts. Retouched records.

with the rising phase of the intracellular e.p.s.p., possibly reflecting either the time course of transmitter action (Eccles, Katz & Kuffler, 1941; Fatt & Katz, 1951) or rapid desensitization of post-synaptic receptors. In some instances a nerve terminal potential preceded the extracellular e.p.s.p.

Simultaneous extracellular and intracellular e.p.s.p.s and spontaneous min e.p.s.p.s recorded from retractor unguis nerve-muscle preparations are illustrated in Fig. 1. Due to the distributed innervation of the muscle fibres the intracellular electrode monitored the effects of transmitter

released from presynaptic sites on many nerve terminals at widely different locations on the muscle fibres (length constant $\simeq$ 2 mm (Usherwood, 1969)), whereas the extracellular electrode recorded the post-synaptic effects of transmitter released from one or more presynaptic release sites on a single nerve terminal. Since the extracellular field associated with an e.p.s.p. or min e.p.s.p. is probably very localized (del Castillo & Katz, 1956; Dudel & Kuffler, 1961) and each nerve terminal is 15–30 μm in length with up to thirty synaptic or transmitter release sites distributed at intervals over its surface (Rees & Usherwood, 1972), spatial decrement of the extracellular potentials was to be expected. As a result interpretation of the extracellular data was rather difficult, the situation being further complicated by the fact that a single nerve terminal may make synaptic connexions with more than one muscle fibre.

The extracellular e.p.s.p.s fluctuated in amplitude and when the response at an active spot was less than about four times the largest extracellular min e.p.s.p. recorded at that site, intermittent failure of transmission occurred (Fig. 1*C*). The fluctuation in amplitude of the e.p.s.p. was greatest when the proportion of failures exceeded 10 % and at these times non-Gaussian distributions of extracellular e.p.s.p. amplitudes were obtained (Fig. 9*C*); where few transmission failures occurred the distributions of e.p.s.p. amplitudes were approximately 'normal' (Fig. 2). In view of the complexity of the innervation of the retractor unguis muscle it is perhaps not surprising that irregularly shaped amplitude histograms of extracellular e.p.s.p.s, including multi-peaked histograms, were frequently obtained in magnesium saline, since with spatial decrement it is unlikely that the contribution of all the transmitter release sites on a terminal to a given extracellular e.p.s.p. will be directly proportional to the number of quanta released from these sites even if the quanta at all the sites are equally effective post-synaptically.

There was no correlation between the amplitudes of e.p.s.p.s recorded extracellularly at different but adjacent active spots on the same muscle fibre or between amplitudes of extracellular e.p.s.p.s and corresponding intracellular potentials, e.g. scatter plots of extracellular e.p.s.p.s and intracellular e.p.s.p.s for different stimulation frequencies yielded no significant correlations (Fig. 3) even when the extracellular responses were less than 10 % of the amplitude of the intracellular responses. In this respect transmission at the locust nerve-muscle synapse differs from that at some crustacean nerve-muscle synapses (Bittner & Harrison, 1970). To ensure that the extracellular electrode monitored transmitter release at sites on one muscle fibre only, i.e. the fibre from which the intracellular data were obtained, active spots were selected at which spontaneous extracellular min e.p.s.p.s were always accompanied by intracellular min e.p.s.p.s.

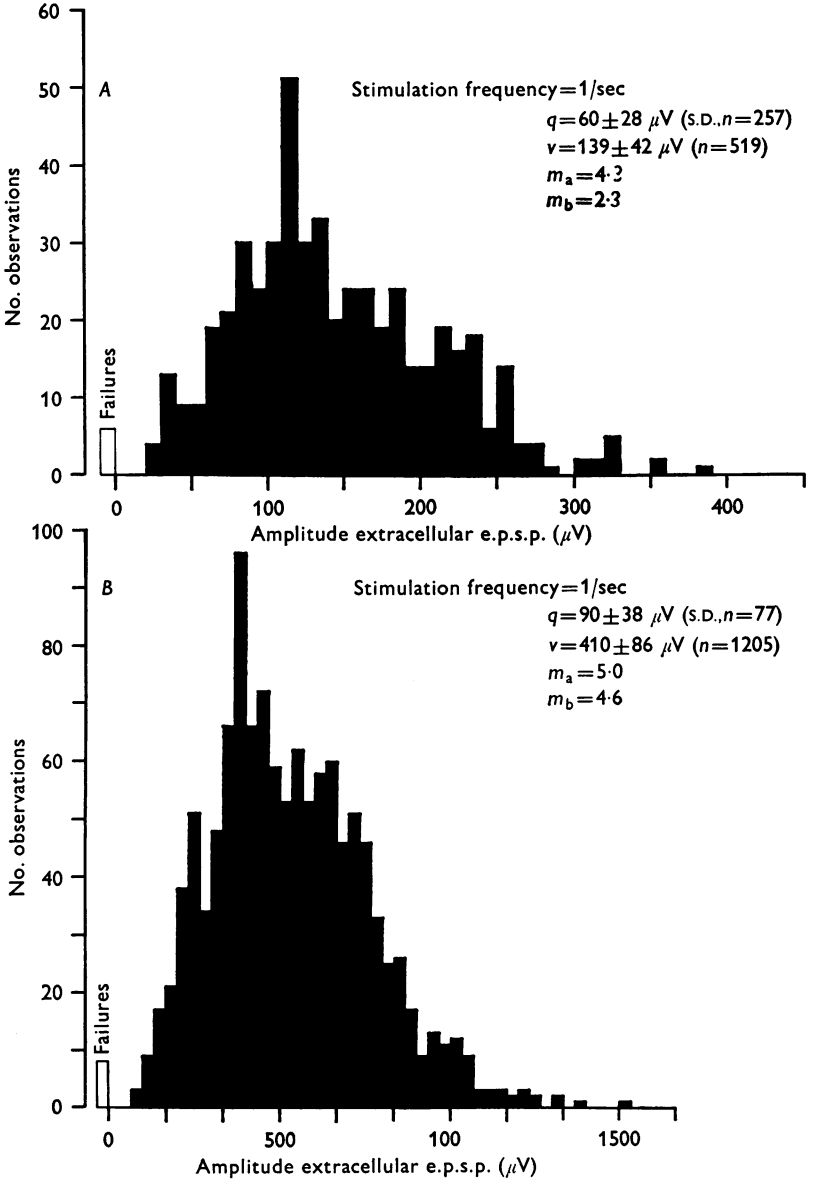

Fig. 2. Histograms of amplitude distributions of extracellular e.p.s.p.s from single active spots. Spontaneous min e.p.s.p.s were sampled at these sites before, during and after stimulation period. q, average mean min e.p.s.p. amplitude for three periods (mean amplitudes for each period were not significantly different); v, mean amplitude e.p.s.p.; m_a, m_b, quantal content e.p.s.p.; n, total number observations; S.D. standard deviation.

To test whether transmission at the retractor unguis nerve-muscle synapses has a quantal basis, the distributions of extracellular e.p.s.p.s (e.g. Fig. 2) were examined to see if they conformed to a Poisson distribution (Fatt & Katz, 1952; del Castillo & Katz, 1954*a*). This was done initially by using the 'Method of Failures' since this method does not require that all the quanta should be the same size. It is therefore particularly suitable for use on the locust retractor unguis nerve-muscle system.

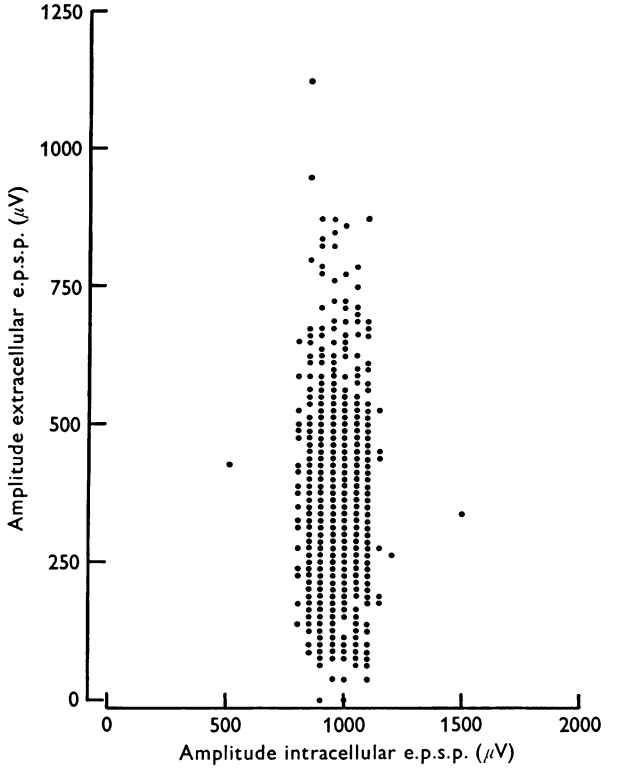

Fig. 3. Scattergram of extracellular e.p.s.p. and intracellular e.p.s.p. amplitudes. Regression coefficients: Y on X, 0·0796; X on Y, 0·0014. Mean amplitude intracellular e.p.s.p. = $904 \pm 50\,\mu$V; mean amplitude extracellular e.p.s.p. = $410 \pm 260\,\mu$V. Data include many multiple occurrences not shown on graph. Resting potential of fibre = 59 mV.

Its main requirement, that the quantal content (m_a) of the e.p.s.p. is low enough that sufficient failures of transmission occur during repetitive stimulation was usually assured by using magnesium saline. Recordings from active spots were made during stimulation of the retractor unguis nerve at frequencies ranging from 0·1 to 5/sec, about 200 extracellular e.p.s.p.s (including failures) being recorded for each stimulation frequency.

This sometimes involved recording from an active spot for more than 1 hr at a time whilst maintaining the extracellular electrode accurately in position. Shifts with time in the amplitude distributions of the extracellular e.p.s.p.s did occur at a few active spots and the only way to determine whether these changes were real or artifactual, due to electrode displacement, was to examine the amplitude distributions of the spontaneous

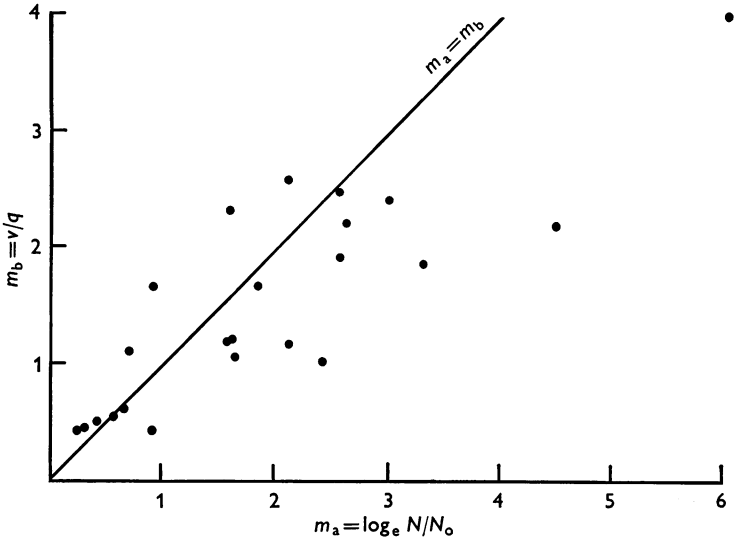

Fig. 4. Plot of m_a against m_b for twenty-three active spots on fifteen retractor unguis muscles. The line corresponds to equality of the two estimates of m. It is possible that part of the discrepancy in the two estimates of quantal content could be a consequence of errors in m_b resulting from non-linear summation of the current flows produced by simultaneously released quanta, although the related intracellular e.p.s.p.s were $< 2{\cdot}5$ mV in amplitude, i.e. never more than a few per cent of the resting potential ($\simeq$ 60 mV).

extracellular min e.p.s.p.s before, during and again after the stimulation period. The mean amplitude of the min e.p.s.p. should not change unless the electrode is disturbed. At most active spots the mean amplitude of the min e.p.s.p. did not change even during long recording periods. An alternative method for determining the quantal content (m_b) of the e.p.s.p. is to calculate the mean amplitude (v) of a long series of extracellular e.p.s.p.s recorded at an active spot and to divide this value by the mean amplitude (q) of the spontaneous min e.p.s.p.s recorded at that site, i.e. $v/q = m_b$ (Fatt & Katz, 1952).

Recent publications on the release of transmitter from nerve terminals on crustacean muscle fibres have emphasized that the amplitude distributions of extracellular e.p.s.p.s recorded at many crustacean nerve-

muscle synapses do not fit a Poisson distribution (Atwood & Johnston, 1968; Atwood & Parnas, 1968; Bittner & Harrison, 1970; Johnson & Wernig, 1971). Although the searching statistical analyses used by Bittner & Harrison (1970) have not been applied to my locust data it could be concluded from the frequent lack of a good correlation between m_a and m_b at many active spots (Fig. 4) that the impulse-linked release of transmitter at locust retractor unguis nerve-muscle synapses is also non-Poisson. At some active spots m_a and m_b were very similar but these represent only about 10 % of the forty-five sites (from thirty-two preparations) examined statistically.

Amplitudes of spontaneous min e.p.s.p.s

Fig. 5 illustrates simultaneous extracellular and intracellular recordings of spontaneous min e.p.s.p.s from a single active spot and muscle fibre respectively. The intracellular electrode monitored the effects of transmitter release from many synaptic sites on many nerve terminals at widely distributed locations on the muscle fibre. As a result there is considerable variation in amplitude and time course of the intracellular min e.p.s.p.s and histograms of amplitude distributions of these potentials exhibit positive skew (Usherwood, 1961, 1963). Histograms of amplitude distributions of the extracellular min e.p.s.p.s also exhibit positive skew (Fig. 6) and in this respect resemble those obtained from synapses on frog phasic skeletal muscle fibres (del Castillo & Katz, 1956). At some active spots a small proportion of the extracellular miniatures were many times larger than the rest. Perhaps these potentials resulted from multiquantal release of transmitter at a single synaptic site. Alternatively they could have resulted from either simultaneous release of single quanta at a number of synaptic sites or release of a single quantum at a single site very close to the recording electrode. Bursts of potentials of very similar amplitudes, with three to eight potentials per burst were seen occasionally. If these resulted from the release of transmitter at a single release site (del Castillo & Katz, 1956) then it seems reasonable to assume that the transmitter quanta released from any one site were probably of similar size. At some active spots the amplitude distributions of the miniature potentials were quite clearly multi-peaked (Fig. 6) presumably due to sampling from a small number of closely adjacent sites with overlapping fields. However, it is possible that the additional peaks represent multiquantal events at either one or more synaptic sites, although the peaks did not always occur at amplitudes that have a simple arithmetic relationship to each other. At other active spots histograms of extracellular min e.p.s.p. amplitude distributions showed marked positive skew with a high proportion of small potentials down to noise level and little indication of peaking. Perhaps

the extracellular electrode was on these occasions located at some distance from the nerve terminal. Alternatively, a large number of synaptic sites located at different distances from the recording electrode and with closely overlapping fields were monitored simultaneously. At a few active spots it was clear that some extracellular min e.p.s.p.s were not accompanied by intracellular miniature potentials. This presumably reflects the complex nature of the innervation of the retractor unguis muscle, the extracellular electrode being so placed that it recorded the potentials set up by

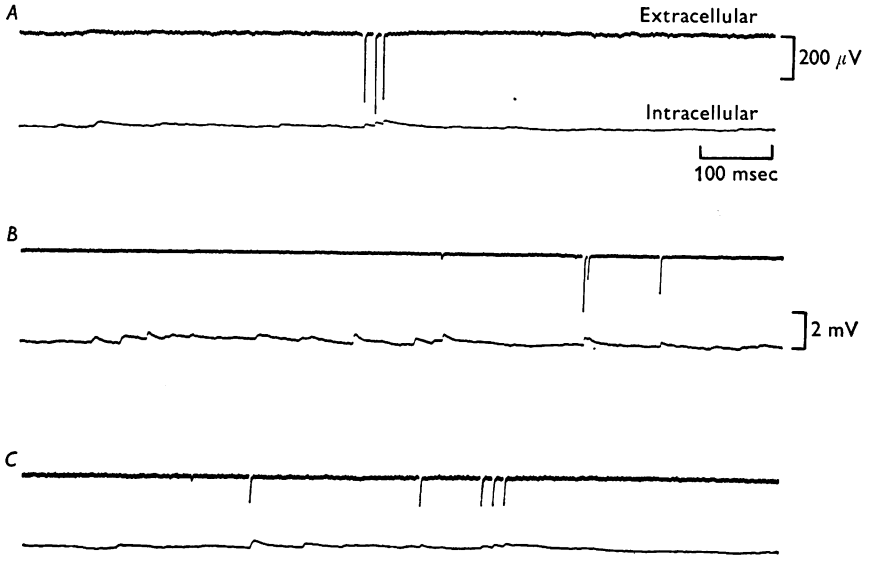

Fig. 5. Simultaneous extracellular and intracellular min e.p.s.p.s from three active spots on two retractor unguis muscles. Only some of the intracellular miniatures (i.e. the 'marked' min e.p.s.p.s (see text)) are accompanied by extracellular potentials. Resting potentials of muscle fibres were 60 mV.

current flow associated with min e.p.s.p.s in two different fibres whereas the intracellular electrode recorded from one fibre alone. It is concluded from these studies that neither the extracellular e.p.s.p.s nor the spontaneous intracellular and extracellular min e.p.s.p.s can be considered to be sampled from a single uniform or normally distributed population which unfortunately complicates any attempts to analyse transmission at insect nerve-muscle synapses in a quantitative manner.

One possible way of improving the sampling procedure is to simultaneously record the spontaneous intracellular and extracellular min e.p.s.p.s (e.g. Fig. 5) with the intracellular and extracellular electrodes separated by a distance of less than 100 μm and to select and measure only

those intracellular events ('marked' min e.p.s.p.s) which correspond with extracellular events (Bittner & Harrison, 1970). By this means it should be possible to obtain intracellular min e.p.s.p. amplitude distributions for, at most, a single nerve terminal. Since the length constant for intracellular events is large compared with the length of the nerve terminal, intracellular miniature amplitude distributions obtained by this method should

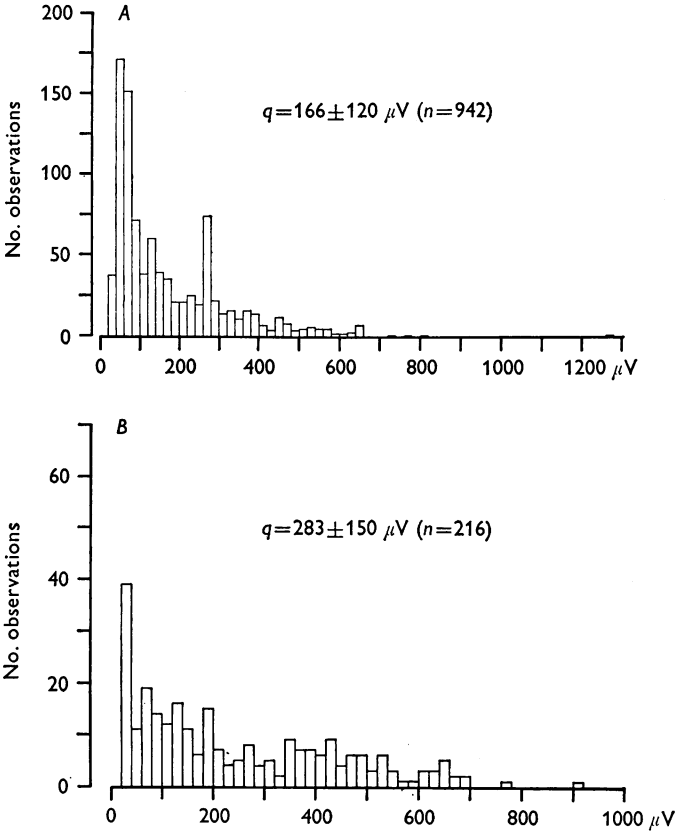

Fig. 6. Amplitude histograms of extracellular min e.p.s.p.s from active spots on two retractor unguis muscles.

be approximately 'normal' provided the miniature discharge is analogous to that which occurs at other nerve-muscle synapses (e.g. Fatt & Katz, 1951). The amplitude distributions of extracellular min e.p.s.p.s and intracellular min e.p.s.p.s at ten active spots on seven different nerve-muscle preparations were examined in this way. At eight sites the 'marked' intracellular min e.p.s.p. amplitudes were approximately 'normally' distributed with variance/mean ratios less than unity (Fig. 7*C*) often in marked contrast to the corresponding extracellular min e.p.s.p. ampli-

tudes (Fig. 7*B*). At two other sites the spread of 'marked' intracellular min e.p.s.p. amplitudes approached that of the corresponding extracellular min e.p.s.p. amplitudes although the former, unlike the latter, were 'normally' distributed (Fig. 7*A*). The amplitudes of the 'marked' intracellular min e.p.s.p.s were not consistently correlated with the amplitudes of their corresponding extracellular events (Fig. 7*A*). On occasions large

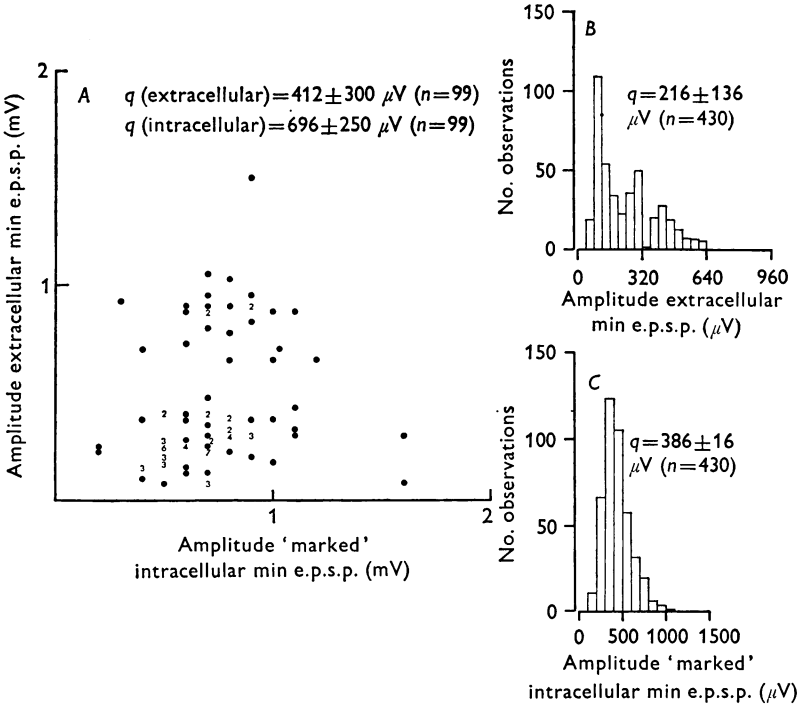

Fig. 7. A. Scattergram of extracellular min e.p.s.p.s and corresponding 'marked' intracellular min e.p.s.p.s from a single active spot on a retractor unguis muscle fibre (resting potential 58·5 mV). Regression coefficient: Y on X, 0·0448; X on Y, 0·0416. Amplitude histogram of extracellular min e.p.s.p.s (*B*) and 'marked' intracellular min e.p.s.p.s (*C*) recorded at an active spot on an adjacent fibre (resting potential = 57 mV).

intracellular miniatures were associated with small extracellular events and vice versa. However, on other occasions a burst of extracellular min e.p.s.p.s of similar sizes was associated with a burst of intracellular events of similar sizes (Fig. 5). Presumably these discharges all started at the same point (del Castillo & Katz, 1956).

Frequency of spontaneous min e.p.s.p.s

With intracellular recordings the miniature discharge frequency is usually greater than 5/sec but cannot be accurately measured due to the distributed innervation of the retractor unguis muscle fibres. Frequencies less than 1/min were not unusual at extracellular recording sites although at some active spots, where presumably the recording electrode monitored transmitter release at many synaptic sites simultaneously, miniature frequencies in excess of 1/sec were recorded. The overall impression obtained

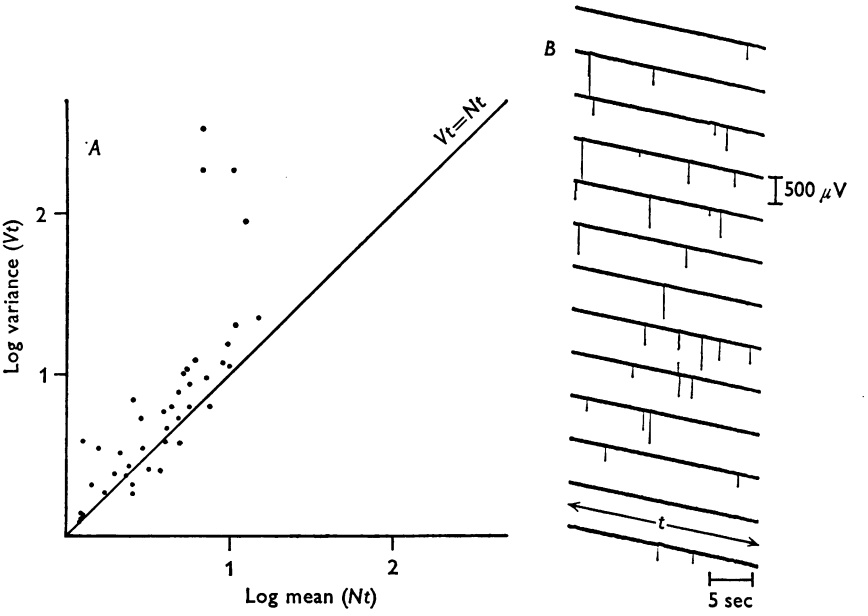

Fig. 8. Variance to mean plot for spontaneous extracellular min e.p.s.p.s recorded at forty-two active spots on thirty-five retractor unguis muscles. The line shows the expected relationship for a Poisson process. *B*, example of extracellular min e.p.s.p. data used to obtain plot in (*A*). Nt = mean number of events occurring in time (t). Vt = variance of number of events occurring in time (t).

from observing an extracellular miniature discharge was that it was a random process. However, since a discharge invariably contained many short bursts of potentials the data were analysed statistically to test for randomness. One characteristic of a random or Poisson process is that the variance (Vt) of the number of events occurring in observation periods of time (t) is equal to the mean number (Nt) of events occurring in that time (Hubbard, 1970). As a result a plot with unity gradient would be anticipated when Vt is plotted against Nt (Fig. 8). Vt/Nt ratios approaching

unity were obtained at about 10% of the extracellular recording sites examined. At the majority of the active spots ratios slightly greater than unity were obtained, suggesting that significant grouping of the extracellular min e.p.s.p.s had occurred to give a non-random discharge. Similar results were obtained using 'marked' intracellular min e.p.s.p.s rather than extracellular events. Previous investigators have reported a departure from a random discharge of miniature potentials at other nerve-muscle synapses in the form of brief 'showers' of potentials (e.g. Atwood & Parnas, 1968), and it has been suggested that this could result from mechanical deformation of the recording site with the micro-electrode. However, in the present studies attempts to alter the discharge by prodding active spots with an extracellular electrode to produce bursts of miniatures were singularly unsuccessful, although it was possible on two occasions to produce a permanent increase in discharge frequency, presumably by having either damaged the nerve terminals or by having altered the environment of the terminal due to leakage of sodium chloride from the electrode. In any event the fact that the pattern of discharge at an active spot normally remained relatively constant over long recording periods (often up to 2 hr in duration) makes it unlikely that the deviations from randomness in the discharge pattern were artifactual. Furthermore, intracellular miniature discharges recorded from superficial muscle fibres were also characterized by bursts of min e.p.s.p.s and it is unlikely, in view of the position of the nerve terminals on the retractor unguis muscle, that any terminal would be damaged by an intracellular electrode inserted into one of these fibres.

The graph of Vt against Nt for a number of different active spots presented in Fig. 8 contains four aberrant points. These represent data obtained from four different active spots on a single nerve-muscle preparation. At these sites the miniature discharge was characterized by extreme variations in miniature frequency with prolonged bursts of extracellular min e.p.s.p.s occurring interposed between long periods of 'electrical silence'. As a result Vt/Nt ratios much greater than unity were obtained. Subsequent examination of the ultrastructure of the nerve terminals of this preparation revealed that the synaptic vesicles were aggregated into large clumps. The significance of these observations will be discussed in a later publication dealing with the release of transmitter from degenerating motor nerve terminals in the locust.

Another test to determine whether the spontaneous release of transmitter is a Poisson process is to plot histograms of interval durations between miniatures in a long sequence of these potentials. If the distribution of interval durations is exponential then it can be assumed that the release mode is approximately random although it must be borne in mind that this is not the only evidence necessary to establish this point and that

this test is by itself insensitive to small deviations from Poisson (e.g. Hubbard, 1970). At most synapses, including those with Vt/Nt ratios slightly greater than unity, exponential distributions were obtained but at some, where Vt/Nt was significantly greater than unity, the interval duration histograms were quite irregular. It is concluded that at some transmitter release sites on the retractor unguis muscle fibre the spontaneous release of transmitter occurs non-randomly with a distinct 'preference' for miniatures to occur in bursts.

Facilitation of transmitter release

When a retractor unguis nerve-muscle preparation is bathed in normal (magnesium-free) locust saline and the nerve is stimulated repetitively at frequencies above 0·1/sec the electrical response (e.p.s.p. plus graded electrically excited component) of the muscle is depressed (Usherwood, 1967; Usherwood & Machili, 1968). In magnesium saline, however, depression of the e.p.s.p. occurs only at much higher stimulation frequencies, i.e. above 10/sec whilst at lower frequencies the e.p.s.p. is facilitated (Fig. 9*C*). This facilitation of the e.p.s.p. is accompanied by an increase in the frequency, but not the amplitude, of the spontaneous min e.p.s.p.s (Fig. 9*A*, *B*). With pairs of stimuli the amplitude of a second intracellular e.p.s.p. (R_2) evoked shortly after the first (R_1) was invariably greater. Facilitation of the second extracellular e.p.s.p. in magnesium saline was not immediately obvious since the amplitude of this response fluctuated considerably and failures were not infrequent. However, when the mean values of extracellular R_1 and R_2 were determined facilitation was usually quite obvious (Table 1). The data presented in Table 1 indicate that facilitation probably lasts well beyond the maximum stimulus interval of 390 msec used in these studies.

Fluctuations in the sizes of the first and second intracellular and extracellular e.p.s.p.s appeared to occur independently (Fig. 10*A–B*), i.e. the correlation coefficients for the intracellular responses and for the extracellular response were not significantly different from zero. The amplitudes of the intracellular and extracellular e.p.s.p.s were not correlated (Fig. 10*C*). A number of different stimulus intervals was studied and in the majority of cases the number of response failures was usually less following the second stimulus than the first (Table 1). Accordingly values for quantal content (m_1 and m_2) calculated from $\log_e N/N_0$ (where N equals the number of impulses and N_0 the number of failures) were greater for extracellular R_2 than for extracellular R_1. When the data in Table 1 were pooled a significant ($P < 0{\cdot}05$) difference between number of failures of the extracellular e.p.s.p. response to the two stimuli was obtained. This suggests that facilitation involves an increase in the number of responding units. According to del

Castillo & Katz (1954*b*) the synaptic response to the first of a pair of nerve impulses could either increase the probability of a response to the next impulse, in which case the amplitude of the first and second responses should be correlated, or the first impulse leaves behind a greater probability of

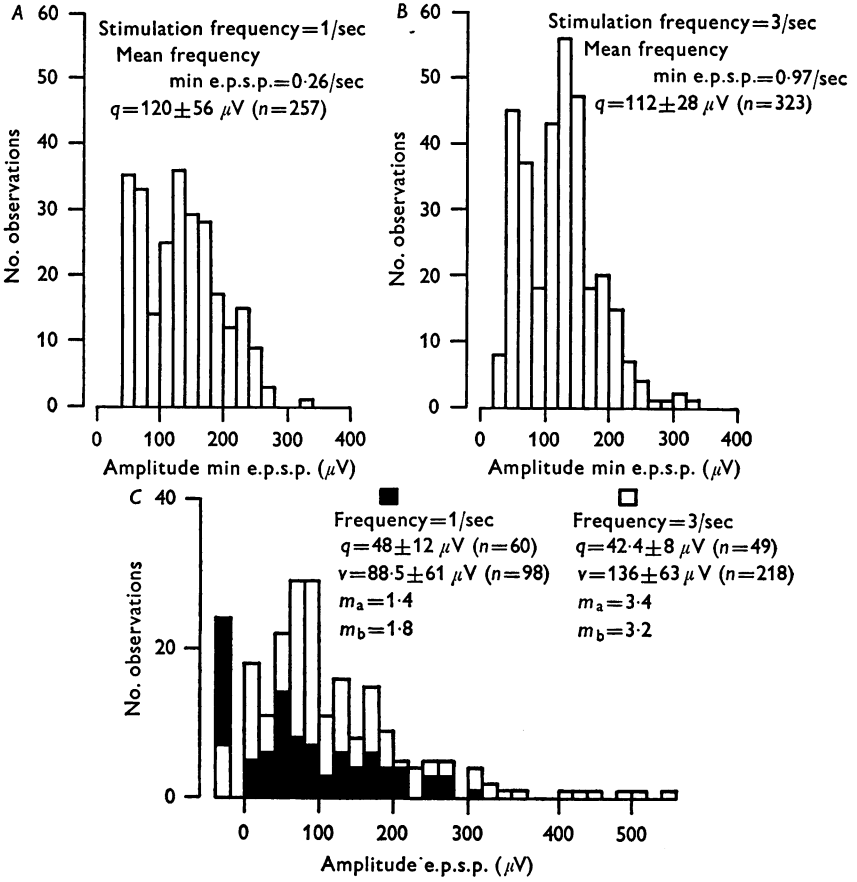

Fig. 9. Effect of stimulation frequency on the amplitude distributions of extracellular min e.p.s.p.s (*A–B*) and e.p.s.p.s (*C*) recorded at two active spots. The mean amplitude and amplitude distribution of the min e.p.s.p. was not significantly altered by increasing the stimulation frequency from 1/sec (*A*) to 3/sec (*B*) but this reduced the number of transmission failures and increased the mean amplitude and extended the amplitude distribution of the e.p.s.p. (*C*). Note higher min e.p.s.p. frequency in (*B*).

activation, independent of past successes or failures. Apart from the lack of correlation between extracellular and intracellular R_1 and R_2 it is clear that at locust nerve-muscle synapses facilitation is independent of previous response or failure since the mean value for extracellular R_2 when extra-

cellular R_1 equalled zero was not significantly different from mean value for all extracellular R_2's (calculated from pooled data in Table 1). However, the effect of success or failure of response to a first stimulus on the occurrence and amplitude of the response to a stimulus was not studied systematically for a wide range of stimulus intervals. Until this is done it would be unwise to conclude that facilitation operates for all intervals at an early stage

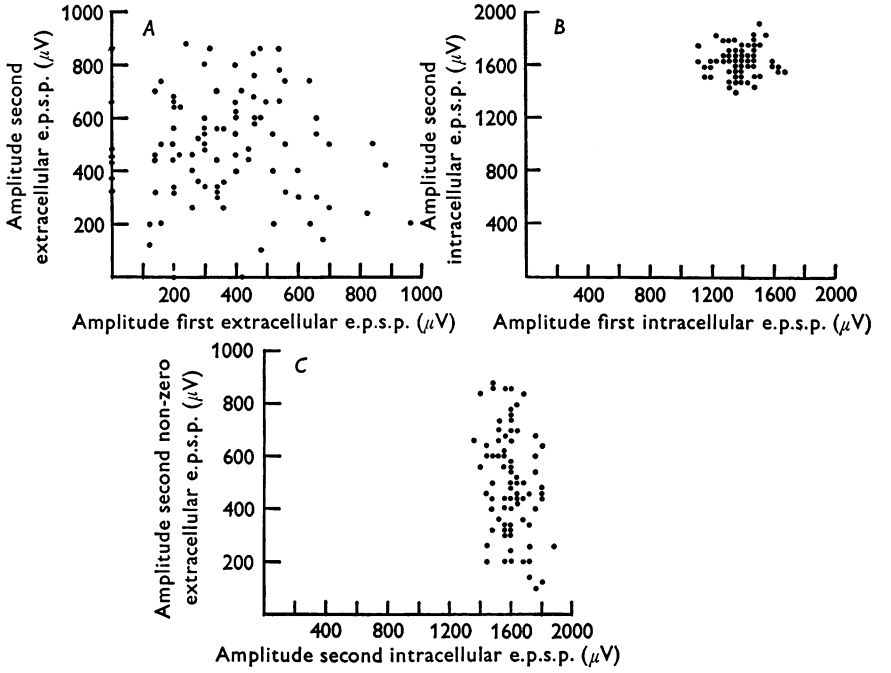

Fig. 10. Scattergram of amplitudes of (*A*) first and second extracellular e.p.s.p.s and (*B*) first and second intracellular e.p.s.p.s to 100 pairs of stimuli. *C*, scattergram of amplitudes of second non-zero extracellular e.p.s.p.s and corresponding intracellular e.p.s.p.s. Stimulus interval 25 msec; interval between pairs of stimuli 5 sec. Multiple occurrences treated as single points. Correlation coefficients: *A*, −0·0692; *B*, 0·0039; *C*, −0·2496. Different preparation from that referred to in Table 1.

of the transmission process before liberation of the transmitter from the nerve terminals (del Castillo & Katz, 1954*b*), especially since L-glutamate which is considered to be the transmitter released from arthropod excitatory nerve terminals (Usherwood, 1971) can act presynaptically as well as post-synaptically (Usherwood & Machili, 1966; Usherwood, 1967; Florey & Woodcock, 1968). From the data presented in Table 1 there is some indication that the return of the test e.p.s.p. amplitude towards normal after a conditioning impulse is oscillatory rather than exponential. This is

TABLE 1. Facilitation at locust nerve-muscle synapses. The retractor unguis nerve was excited with pairs of stimuli at constant 5 sec intervals; the interval between the stimuli in a pair was initially 25 msec but was increased in a stepwise manner to a maximum of 390 msec. A 2-min rest period was allowed after stimulation at each interval with about 100 stimulus pairs. Resting potential of fibre used for intracellular recording was 57·5 mV. R_1, mean amplitude (± S.D.) of extracellular and intracellular e.p.s.p.s to first stimulus; R_2, mean amplitude (± S.D.) e.p.s.p.s to second stimulus; N, number of stimuli given at each interval; m_1 and m_2, mean quantal content extracellular R_1 and R_2 calculated from transmission failures at extracellular recording site. Data obtained from one retractor unguis preparation. Repeat studies on this preparation and studies on other preparations gave similar results

Stimulus interval (msec)	Extracellular (μV)			Intracellular (μV)				Extracellular (μV) Non-zero			Transmission failures		
	R_1	R_2	R_2/R_1 %	R_1	R_2	R_2/R_1 %	N	R_1	R_2	R_2/Non-zero R_1%	R_1	R_2	m_2-m_1
25	183 ± 105	243 ± 101	133	347 ± 28	400 ± 25	115	99	199 ± 94	248 ± 95	125	18	12	0·4
40	140 ± 129	148 ± 158	106	290 ± 23	331 ± 24	114	43	188 ± 116	220 ± 146	117	11	14	−0·24
70	106 ± 60	131 ± 69	124	356 ± 22	424 ± 33	119	47	110 ± 54	134 ± 53	122	18	14	0·25
90	181 ± 144	183 ± 135	101	300 ± 21	335 ± 33	112	50	237 ± 116	213 ± 121	90	12	7	0·54
130	193 ± 105	225 ± 85	117	337 ± 23	414 ± 36	123	51	218 ± 116	267 ± 136	122	22	18	0·20
140	105 ± 61	134 ± 68	128	372 ± 23	443 ± 26	119	50	120 ± 55	125 ± 59	104	15	17	−0·12
160	123 ± 153	138 ± 141	112	273 ± 25	311 ± 29	114	48	178 ± 91	192 ± 131	108	17	14	0·19
180	115 ± 113	136 ± 134	118	332 ± 28	369 ± 28	111	51	198 ± 151	209 ± 122	106	17	11	0·43
190	108 ± 130	125 ± 135	116	310 ± 20	343 ± 18	111	50	201 ± 112	189 ± 124	94	23	17	0·31
240	101 ± 93	147 ± 142	146	304 ± 25	343 ± 24	113	50	144 ± 78	193 ± 133	134	15	17	−0·12
280	128 ± 127	140 ± 140	109	322 ± 23	369 ± 39	115	50	195 ± 106	205 ± 122	106	17	16	0·06
290	138 ± 151	89 ± 120	64	309 ± 17	338 ± 45	109	50	181 ± 113	209 ± 132	115	17	25	−0·39
320	92 ± 110	108 ± 94	117	334 ± 32	366 ± 32	110	50	164 ± 98	155 ± 72	95	22	15	0·37
340	122 ± 144	134 ± 125	110	295 ± 23	313 ± 20	106	46	216 ± 127	246 ± 170	114	20	21	−0·05
360	106 ± 110	96 ± 76	91	370 ± 66	407 ± 74	110	48	203 ± 98	225 ± 85	111	17	14	0·20
390	150 ± 160	157 ± 172	105	292 ± 20	313 ± 20	107	44	245 ± 136	238 ± 160	97	17	15	0·12
Means	131	145	112	321	364	113	—	187	204	110	17·4	15·4	0·14

perhaps more apparent with the intracellular than with the extracellular responses, although the samples analysed were not large enough for unequivocal conclusions on this point.

During repetitive stimulation of the retractor unguis nerve a significant increase in the frequency, but not the amplitude, of the intracellular and extracellular min e.p.s.p.s was observed (Fig. 9*A*, *B*). For example, at one active spot there was an average increase of 30% in the frequency of extracellular min e.p.s.p.s above the resting frequency during stimulation at 1/sec. The probability of occurrence of the extracellular min e.p.s.p.s was increased most during stimulation at active spots where the resting miniature frequency was < 0·1/sec. At active spots where the resting discharge exceeded 5/sec only a slight increase in the probability of min e.p.s.p. occurrence was achieved by stimulating the retractor unguis nerve. The probability of occurrence of extracellular min e.p.s.p.s was highest immediately (i.e. during a 100 msec period) after a nerve impulse (min e.p.s.p.s occurring during the falling phase of the evoked potential were rejected because it was not possible to decide whether they represented spontaneous potentials or were due to lack of synchronization of release during excitation of the nerve terminal by the nerve action potential). The probability of occurrence of min e.p.s.p.s after an e.p.s.p. did not decline smoothly towards zero along an exponential time course. The decline was interrupted by one or more period of increased release probability. This gave a multipeaked appearance to histograms of min e.p.s.p. occurrence at time intervals after e.p.s.p. (Fig. 11), but the range of stimulus intervals studied was insufficient to allow a complete evaluation of this phenomenon.

DISCUSSION

At some synapses on crustacean muscle fibres there is a significant difference between the observed amplitude distributions of extracellular e.p.s.p.s and the distributions which would be predicted if transmitter release was a Poisson process (Atwood & Johnston, 1968; Atwood & Parans, 1968; Bittner & Harrison, 1970; Johnson & Wernig, 1971). It could be concluded from the data presented herein that a similar state of affairs exists at synapses on locust retractor unguis muscle. There are reasons, however, for exercising some doubts about the applicability of the classical tests for predicting Poisson or non-Poisson processes to data obtained from locust nerve-muscle synapses. Since the retractor unguis muscle fibres are very close-packed and the nerve terminals are on the inner faces of the fibres it is unlikely that an extracellular recording electrode ever made direct contact with a terminal let alone the synaptic sites it contained. At an active spot the electrode was undoubtedly in a

region of high synaptic current density presumably above a constricted space between two fibres but since the nerve terminals are up to 30 μm length and contain many distributed transmitter release sites, differences in spatial decrement of potentials arising at these sites was inevitable

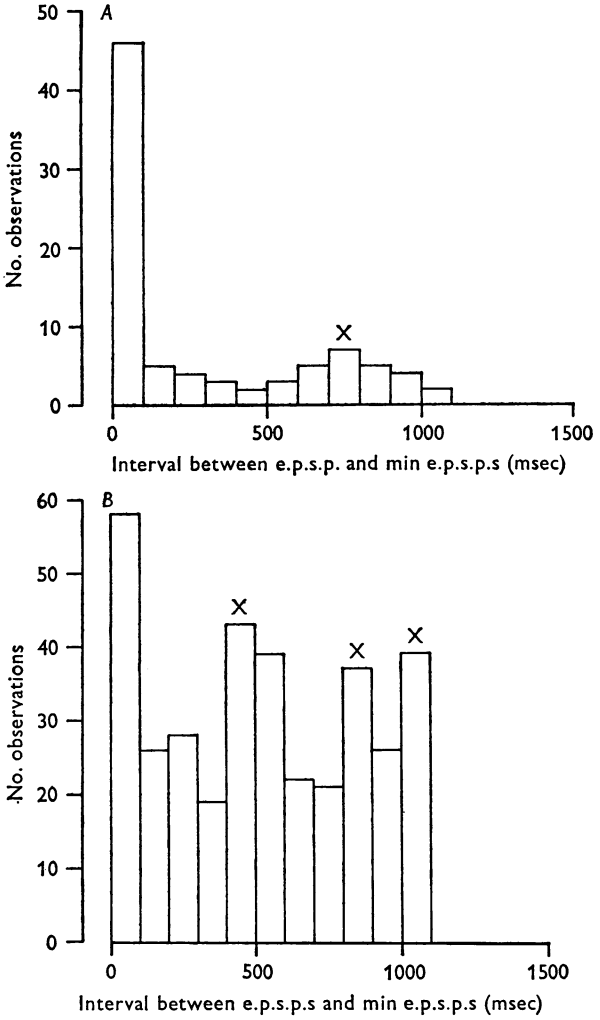

Fig. 11. *A–C*. Histograms of interval distributions between extracellular e.p.s.p.s and min e.p.s.p.s. The probability of occurrence of a spontaneous min e.p.s.p. is highest immediately after the evoked potential but there are also subsequent periods of significantly ($P \leqslant 0{\cdot}05$) enhanced probability (*X*). Stimulation frequency 1/sec for (*C*), slightly less than 1/sec for (*A*) and (*B*). Histogram of intervals between min e.p.s.p.s and start of sample period in consecutive 1 sec sample periods in absence of neural stimulation is illustrated in (*D*). Differences in class amplitudes in (*D*) not significant ($P > 0{\cdot}1$).

although the extent of these differences would of course have depended upon a variety of factors (del Castillo & Katz, 1956). This probably accounts for the skew distributions of amplitudes of extracellular e.p.s.p.s. and extracellular min e.p.s.p.s. It could also have lead to errors in computing m_b since the populations sampled for extracellular e.p.s.p.s. and min e.p.s.p.s may have been somewhat different, albeit overlapping. With

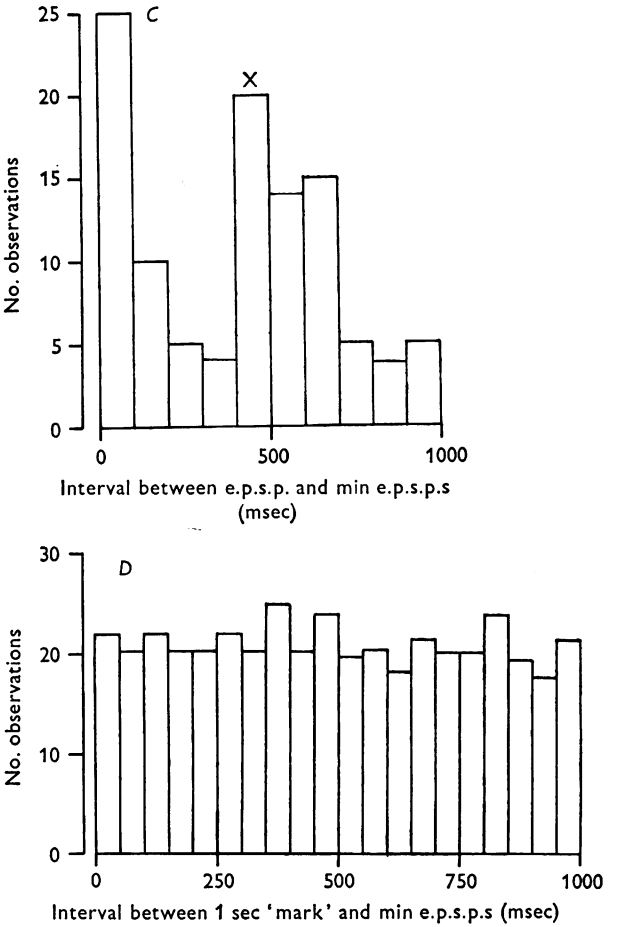

Fig. 11 *C* and *D*. For legend see opposite page.

estimates of m_a there was the additional problem of deciding between a small extracellular e.p.s.p. and failure of transmission. This problem has of course confronted many synaptic physiologists but was accentuated here because quantal discontinuities were confounded by spatial decrement. These problems might be resolved by completely blocking transmission with high magnesium saline and then selectively activating a single terminal

with calcium (Katz & Miledi, 1967) whilst stimulating the retractor unguis nerve. This would enable intracellular recordings to be used to investigate the quantal basis of transmitter release since sampling is then restricted to one nerve terminal.

At forty-four out of forty-five active spots on locust muscle the amplitude distributions of extracellular min e.p.s.p.s were markedly skew and frequently multipeaked. The multipeaked appearance of the amplitude distributions could be due to the presence of spatially distinct release sites on the nerve terminal as indicated by electron microscope observations (Rees & Usherwood, 1972). Alternatively, spontaneous transmitter release may at times occur in a multiquantal fashion. For example at vertebrate nerve-muscle synapses amplitude histograms of intracellular e.p.s.p.s are multipeaked, presumably due to the release of a single type of quantum in groups of $1-n$ quanta per group, each quantum having a low probability of release. There is evidence for grouping of small numbers of quanta during spontaneous release at locust nerve-muscle synapses and this could conceivably give rise to a significant proportion of extracellular min e.p.s.p.s with amplitudes of $2-n$ times greater than the modal amplitude. One underlying assumption of the Poisson hypothesis of transmitter release is that the number of quantal units responding to a nerve impulse fluctuates in a random manner and can be predicted only in statistical terms. However, if the spontaneous min e.p.s.p.s occur non-randomly then it seems likely that the amplitude of the e.p.s.p. may also vary in a non-random manner, unless of course the transmitter quanta which give rise to e.p.s.p.s on the one hand and min. e.p.s.p.s on the other hand are drawn from different populations.

At most synapses the spontaneous release of transmitter normally occurs at random intervals indicating no interdependence between successive events (Hubbard, 1970). Gage & Hubbard (1965) have shown that at end-plates on rat hemidiaphragm muscle spontaneous transmitter release conforms to a Poisson process even when alterations in the environment of the nerve terminals affect the min e.p.s.p. frequency. At many locust nerve-muscle synapses there seems little doubt that the min e.p.s.p. occur non-randomly due to grouping of these potentials. Vere-Jones (1966) suggested that such a deviation from a Poisson process would arise if there were irregularities in the stream of synaptic vesicles 'diffusing' into the region of immediate access to release sites on the presynaptic membrane. In this case irregularities in output from the release sites would reflect those of the input. The fact that the synaptic vesicles at synaptic foci in the terminals of the retractor unguis motoneurone are non-randomly distributed (Rees & Usherwood, 1972; Usherwood & Rees, 1972) indicates that a causal relationship between vesicle distribution and min e.p.s.p.

sequence may well exist at locust nerve-muscle synapses. Although spontaneous release of transmitter at frog nerve-muscle junctions is normally a Poisson process, Rotshenker & Rahamimoff (1970) found that a non-random discharge with bursts of min e.p.s.p.s could be obtained by raising external calcium to 15 m-mole/l. Subsequent quantitative analyses of the non-random discharge indicated that the spontaneous release of a quantum increased the probability of release of another quantum and that this increase in probability often lasted for several hundred milliseconds after a min e.p.s.p. Bursting of min e.p.s.p.s can also be induced at frog nerve-muscle synapses by adding strontium (7–10 m-mole/l.) to the bathing medium (Dodge, Miledi & Rahamimoff, 1969). Possibly the changes in miniature discharge with strontium and high calcium are not accompanied by changes in vesicle distributions although this remains to be tested.

The probability of spontaneous or impulse-linked release of transmitter from the terminals of locust motoneurones is increased after a single conditioning nerve impulse or during repetitive stimulation. In common with most other synapses there is an initial phase of pronounced facilitation, possibly reflecting the slow time course of calcium removal from release sites on the presynaptic membrane following the conditioning impulse (Katz & Miledi, 1967). A delayed second phase of facilitation smaller than the first occurs at some vertebrate synapses (see review by Hubbard, 1970) which has been related to the hyperpolarization of nerve terminals which may follow a nerve impulse (Hubbard & Schmidt, 1963). It seems unlikely that this phenomenon also accounts for the oscillatory changes in transmitter release probability which follow the initial phase of facilitation at locust synapses. Perhaps these oscillatory changes are due to a feed-back process involving the transmitter released from the nerve terminals by the nerve impulse. If this is the case then one might reasonably expect that there would be a positive correlation between the amplitude of the first and second extracellular e.p.s.p.s to pairs of stimuli and this was not observed at the locust synapses described herein. However, the possibility that facilitation partly occurs through presynaptic action of transmitter should not be completely discounted until these synapses have been examined more thoroughly by using, for example, much longer intervals between pairs of stimuli to ensure that transmitter released in response to previous pairs of nerve impulses was not affecting release to a subsequent pair.

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REFERENCES

ATWOOD, H. L. & JOHNSTON, H. S. (1968). Neuromuscular synapses of a crab motor axon. *J. exp. Zool.* **167**, 457–470.

ATWOOD, H. L. & PARNAS, I. (1968). Synaptic transmission in crustacean muscles with dual motor innervation. *Comp. Biochem. Physiol.* **27**, 381–404.

BITTNER, G. D. & HARRISON, J. (1970). A reconsideration of the Poisson hypothesis for transmitter release at the crayfish neuromuscular junction. *J. Physiol.* **206**, 1–23.

BOYD, I. A. & MARTIN, A. R. (1956). Spontaneous subthreshold activity at mammalian neuromuscular junctions. *J. Physiol.* **132**, 61–73.

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

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

DEL CASTILLO, J. & KATZ, B. (1954*b*). Statistical factors involved in neuromuscular facilitation and depression. *J. Physiol.* **124**, 574–585.

DEL CASTILLO, J. & KATZ, B. (1956). Localization of active spots within the neuromuscular junction of the frog. *J. Physiol.* **132**, 630–649.

DODGE, F. A., MILEDI, R. & RAHAMIMOFF, R. (1969). Strontium and quantal release of transmitter at the neuromuscular junction. *J. Physiol.* **200**, 267–283.

DUDEL, J. & KUFFLER, S. W. (1961). The quantal nature of transmission and spontaneous miniature potentials at the crayfish neuromuscular junction. *J. Physiol.* **155**, 514–529.

ECCLES, J. C., KATZ, B. & KUFFLER, S. W. (1941). Nature of the 'end-plate potential' in curarized muscle. *J. Neurophysiol.* **4**, 363–387.

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

FATT, P. & KATZ, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**, 109–128.

FLOREY, E. & WOODCOCK, B. (1968). Presynaptic excitatory action of glutamate applied to crab nerve-muscle preparations. *Comp. Biochem. Physiol.* **26**, 651–662.

GAGE, P. W. & HUBBARD, J. I. (1965). Evidence for a Poisson distribution of miniature endplate potentials and some implications. *Nature, Lond.* **208**, 395–396.

HUBBARD, J. I. (1970). Mechanism of transmitter release. *Prog. Biophys. molec. Biol.* **21**, 33–124.

HUBBARD, J. I. & SCHMIDT, R. F. (1963). An electrophysiological investigation of mammalian motor nerve terminals. *J. Physiol.* **166**, 145–167.

JOHNSON, E. W. & WERNIG, A. (1971). The binomial nature of transmitter release at the crayfish neuromuscular junction. *J. Physiol.* **218**, 757–767.

KATZ, B. (1962). The transmission of impulses from nerve to muscle, and the subcellular unit of synaptic action. *Proc. R. Soc.* B **155**, 455–477.

KATZ, B. & MILEDI, R. (1967). The timing of calcium action during neuromuscular transmission. *J. Physiol.* **189**, 535–544.

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

LILEY, A. W. (1957). Spontaneous release of transmitter substance in multiquantal units. *J. Physiol.* **136**, 595–605.

REES, D. & USHERWOOD, P. N. R. (1972). Fine structure of normal and degenerating motor axons and nerve-muscle synapses in the locust, *Schistocerca gregaria*. *Comp. Biochem. Physiol.* 43A, 83–101.

ROTSHENKER, S. & RAHAMIMOFF, R. (1970). Poissonian and non-Poissonian statistics and the appearance of miniature endplate potentials at the neuromuscular junction. *Israel J. Med. Sci.* **6**, 466.

TAKEUCHI, A. & TAKEUCHI, N. (1964). The effect on crayfish muscle of iontophoretically applied glutamate. *J. Physiol.* **170**, 296–317.

USHERWOOD, P. N. R. (1961). Spontaneous miniature potentials from insect muscle fibres. *Nature, Lond.* **191**, 814–815.

USHERWOOD, P. N. R. (1963). Spontaneous miniature potentials from insect muscle fibres. *J. Physiol.* **169**, 149–160.

USHERWOOD, P. N. R. (1967). Insect neuromuscular mechanisms. *Am. Zool.* **7**, 553–582.

USHERWOOD, P. N. R. (1969). Electrochemistry of insect muscle. *Adv. Insect Physiol.* **6**, 205–275.

USHERWOOD, P. N. R. (1971). Evidence for release of glutamate from arthropod motor nerve endings. *Proc. Int. Physiol. Soc.* **8**, 251–252.

USHERWOOD, P. N. R. & GRUNDFEST, H. (1965). Peripheral inhibition in skeletal muscle of insects. *J. Neurophysiol.* **28**, 497–518.

USHERWOOD, P. N. R. & MACHILI, P. (1966). Chemical transmission at the insect excitatory neuromuscular synapse. *Nature, Lond.* **210**, 634–636.

USHERWOOD, P. N. R. & MACHILI, P. (1968). Pharmacological properties of excitatory neuromuscular synapses in the locust. *J. exp. Biol.* **49**, 341–361.

USHERWOOD, P. N. R. & REES, D. (1972). Quantitative studies of the spatial distribution of synaptic vesicles within normal and degenerating motor axons of the locust, *Schistocerca gregaria*. *Comp. Biochem. Physiol.* 43A, 103–118.

VERE-JONES, D. (1966). Simple stochastic models for the release of quanta of transmitter from a nerve terminal. *Aust. J. Statistics* **8**, 53–63.