

# The Effect of Calcium Ions on the Secretion of Quanta Evoked by an Impulse at Nerve Terminal Release Sites

M. R. BENNETT and N. A. LAVIDIS

From The Neurobiology Laboratory, Department of Physiology, University of Sydney, Sydney, New South Wales 2006, Australia

**ABSTRACT** A study has been made of the effects of calcium ions on the number of quanta secreted from all the release sites at an amphibian motor nerve terminal recorded with an intracellular microelectrode ( $\bar{m}$ ) compared with the number secreted simultaneously from a small number of release sites recorded with an extracellular microelectrode ( $\bar{m}_e$ ). If the endplate potential was made subthreshold by lowering the external calcium concentration ( $[\text{Ca}]_o \leq 0.4$  mM), it was possible to find small groups of release sites for which  $\bar{m}_e$  was comparable to  $\bar{m}$ , indicating considerable nonuniformity in the probability of release of a quantum at different groups of release sites ( $p_e$ ) in a given  $[\text{Ca}]_o$ . Increasing  $[\text{Ca}]_o$  in the range from 0.25 to 0.4 mM increased the probability of release of a quantum at groups of release sites ( $p_e$ ), independent of the initial value of  $p_e$ , and the dependence of  $p_e$  on  $[\text{Ca}]_o$  followed a fourth power relationship. A conditioning impulse enhanced the probability of release of a quantum by a subsequent test impulse at release sites, if  $p_e$  was less than 1.0 during the conditioning impulse. It is shown that the present observations regarding the dependence of  $p_e$  on  $[\text{Ca}]_o$  and on conditioning impulses can be quantitatively predicted from previous observations regarding the dependence of the binomial parameters  $\bar{m}$ ,  $p$ , and  $n$  on  $[\text{Ca}]_o$  and on conditioning impulses determined with intracellular electrodes, if the probability of secretion of a quantum at a release site ( $p_j$ ) is different for different release sites and  $p_j$  is distributed as a beta random variable.

## INTRODUCTION

It is known that the quantal content of the endplate potential recorded with an intracellular electrode at vertebrate neuromuscular junctions is distributed as a binomial variable with parameters  $p$  and  $n$  (Bennett and Florin, 1974; Miyamoto, 1975; Wernig, 1975); increasing the external calcium concentration  $[\text{Ca}]_o$  in the range 0.25–0.6 mM leads to a fourth-power increase in  $\bar{m}$  (Jenkinson, 1957; Dodge and Rahamimoff, 1967) with  $[\text{Ca}]_o$ , which is due to an increase in both binomial parameters  $p$  and  $n$  (Bennett et al., 1975, 1977). In previous studies, these changes in  $p$  and  $n$  which accompany changes in  $[\text{Ca}]_o$  have been interpreted according to a scheme in which all the quantal

release sites along the length of a nerve terminal (Couteaux and Pecot-Dechavassine, 1970; McMahan et al., 1972; Dreyer et al., 1973; Heuser et al., 1974) have the same probability of secretion of a quantum during a train of impulses, so that binomial parameter  $p$  is this probability of secretion and binomial parameter  $n$  is the number of release sites which participate in secretion (see the general case in the Appendix to Bennett and Fisher, 1977); in this situation both  $p$  and  $n$  increase independently with  $[Ca]_o$ , and two discrete calcium-dependent mechanisms control the secretion of quanta at release sites.

Quantal content may, however, behave as a binomial variable under a number of other conditions, perhaps the most interesting of which occurs if the probability of secretion of a quantum is constant during low-frequency trains of impulses at a given release site, but is markedly different for different release sites. Binomial parameter  $p$  is then no longer the probability of secretion of a quantum but is a function of the mean and variance of the density distribution for the probability of secretion of a quantum at a release site ( $p_j$ ) of the nerve terminal, and binomial parameter  $n$  is no longer the number of release sites which participate in secretion (see case *iii a* in the Appendix to Bennett and Fisher, 1977); in this situation the changes in both  $p$  and  $n$  with  $[Ca]_o$  are actually due to changes in a single calcium-dependent mechanism ( $p_j$ ) which controls secretion of quanta at each release site. In the present work we have studied the  $[Ca]_o$  dependence of the probability of quantal secretion at small groups of release sites ( $p_e$ ) with an external microelectrode and of release from all the sites ( $p$ ) with an internal microelectrode to test for nonuniformity in the probability of secretion of a quantum at different release sites ( $p_j$ ) along the motor nerve terminal. The results indicate considerable nonuniformity in  $p_j$ , and that this is the basis for the binomial nature of transmitter release at vertebrate neuromuscular synapses.

## METHODS

### *Preparation and Recording*

The effects of calcium ions on the secretion of quanta were studied at mature synapses in the toad (*Bufo marinus*) iliofibularis muscle. Both the muscle and its nerve supply were dissected free from surrounding connective tissue and tendinous insertions and then mounted in a Perspex organ bath of 3 ml capacity. This bath was perfused with a modified Krebs solution of the following composition (mM):  $Na^+$  151,  $K^+$  4.7,  $Ca^{2+}$  1.8,  $Mg^{2+}$  1.2,  $Cl^-$  142,  $H_2PO_4^-$  1.3,  $SO_4^{2-}$  1.2,  $HCO_3^-$  16.3, glucose 7.8 and gassed continuously with 95%  $O_2$  and 5%  $CO_2$ . The bath temperature was maintained at 18°C ( $\pm 1^\circ C$ ), by means of a temperature-control system using Peltier-effect thermoelectric devices. The nerve was stimulated with a suction glass-capillary electrode, using current pulses of 0.05–0.10 ms duration and 1–10 V amplitude. Intracellular recordings were made of endplate potentials at superficial synapses in the iliofibularis muscle with glass microcapillary electrodes filled with 2 M potassium chloride of 50–80 M $\Omega$  resistance, while extracellular recordings of the electrical signs of synaptic transmission were made with glass microcapillary electrodes filled with 2 M sodium or potassium chloride of 50–80 M $\Omega$  resistance. Although the toad iliofibularis muscle contains some slow fibers which receive a distributed innervation, they compose less

than 10% of all fibers, and are only found in the centre of the muscle (Lännergren and Smith, 1966). These slow fibers were avoided in the present work by recording from superficial muscle fibers only.

For those synapses at which simultaneous intracellular and extracellular recordings were made, focal endplate potentials (EPPs) were first recorded with an intracellular electrode during continuous stimulation at 0.2–1.0 Hz, and then the extracellular electrode was positioned in the vicinity of the intracellular electrode, near where the myelin sheath of the motor axon is lost and the axon travels on the surface of the muscle cell (Katz and Miledi, 1965 *a*); the extracellular electrode was then lifted clear of the surface of the muscle cell and moved parallel to the long axis of the cell for a distance of a few micrometers where it was lowered again to the surface of the cell in order to see if the extracellular signs of evoked synaptic transmission could be observed over 10–20 impulses, and if this was not the case, the process was repeated until secretion of quanta from a group of release sites was observed. Signals recorded with both the intracellular and extracellular electrodes were led through a high impedance unity gain preamplifier, then amplified, and both displayed on the oscilloscope and photographed as well as recorded on FM magnetic tape.

#### *Changes in Ionic Concentrations*

The external calcium concentration  $[Ca]_o$  was altered by changing the concentration of  $CaCl_2$  present in the reservoir of Krebs supplying the organ bath. Changes in  $[Ca]_o$  were made by first incubating the muscle for 45 min in the lowest  $[Ca]_o$  to be used, and then progressively increasing the  $CaCl_2$  to obtain the higher  $[Ca]_o$ , so as to keep the time taken to reach a new steady state in the average quantal content of the EPPs to a minimum. Possible changes in the conduction of the nerve impulse, due to the divalent cation concentration falling below 0.7 mM (Frankenhauser and Hodgkin, 1957), were avoided by maintaining the  $[Mg]_o$  constant at 1.2 mM. It was not difficult to maintain the position of intracellular and extracellular electrodes for simultaneous recording of focal EPPs and of endplate currents for periods long enough for the average quantal content of the EPP to reach a steady state in three different  $[Ca]_o$  solutions. The criteria used for determining the quality of intracellular or extracellular observations after a steady state had been reached in a given solution were the same as those used before (Bennett et al., 1977), namely for the intracellular observations: a shift in the resting potential; a shift in the mean amplitude of the MEPPs collected at the end of exposure to the solution compared with the mean amplitude at the time of reaching the steady state in average EPP quantal content in that solution; any trend in the average EPP quantal content once a steady state had been reached. The criteria used for extracellular observation were: any shift in the median latency of the first quantum released on each trial or in quantal release rates at the end of exposure to the solution compared with the median latency of the first quantum released and the quantal release rates once a steady state had been reached; any shift in the mean amplitude of the quanta collected at the end of exposure to the solution compared with the mean amplitude of quanta at the time of reaching a steady state in average quantal release and first quantal latency.

#### *Construction of Histograms and Determination of Statistical Parameters*

Between 100 and 500 consecutive trials at frequencies from 0.2 to 0.5 Hz in a given  $[Ca]_o$  were used to construct from the intracellular recordings amplitude-frequency histograms of EPPs and MEPPs, and from the extracellular recordings both the number of quanta released-frequency histograms and the latency of the quanta

released-frequency histograms. For intracellular observations the summed peak amplitudes of synaptic potentials following an impulse were measured, and these were used in constructing amplitude-frequency histograms (Fig. 3), the mean and variance of the quantal size being determined from that of the spontaneous miniature endplate potentials occurring in the absence of stimulations; a binomial analysis of the amplitude-frequency histograms was then made and the predictions of this analysis were compared with the observed histogram (see Fig. 3). Estimates for the binomial parameter  $p$  were made from  $p = 1 - (S^2/m\gamma) + (\sigma^2/\gamma^2)$ , where  $m$  and  $S^2$  are the mean and variance of the EPP amplitudes, and  $\gamma$  and  $\sigma^2$  are the mean and variance of the spontaneous EPPs; the amplitude-frequency distribution of the EPPs has been predicted from

$$P(x) = \sum_{r=0}^n {}^n C_r p^r q^{n-r} \frac{\lambda^{kr}}{\Gamma(kr)} e^{-\lambda k} x^{kr-1}$$

derived by Robinson (1976), where  $P(x)$  is the expected frequency of EPPs with the amplitude of  $x$  millivolts,  $r$  ( $= 0, 1, 2, \dots, n$ ) is the possible quantal content value for the EPP,  $\Gamma$  is the gamma function and  $\lambda = \gamma/\sigma^2$  and  $k = \gamma^2/\sigma^2$ ; the distribution of the spontaneous miniature endplate potentials is assumed to follow a gamma distribution (Bennett et al., 1976). For extracellular observations the latency-frequency histograms (see Fig. 7) were first constructed as follows: each pulse stimulus artefact, together with the electrical sign of the nerve impulse and any subsequent quantal release during a period which was estimated as sufficient in a number of experiments for the quantal release rate to become as low again as the spontaneous release rate, was photographed as a separate frame (Fig. 1); the interval between the time of the negative peak of the action current of the extracellularly recorded spike and the beginning of the quantal endplate current (as in Fig. 1 of Katz and Miledi 1965 *b*) due to the first, second, third, etc. quantum released after the action current was measured for each frame over the 100–500 trials (see Fig. 2 A, Barrett and Stevens [1972 *b*] for a similar procedure) and the interval-frequency histogram drawn up for the intervals observed during these trials; for both illustration and computational purposes, the latency-frequency histogram was taken as identical to the interval-frequency histogram, that is, zero time was taken as occurring at the time of the peak of the action current of the spike; a stochastic analysis of the latency-frequency histograms was then made according to the Method given in Bennett et al. (1977), estimates made of the stochastic parameters  $\alpha$ ,  $\gamma$ , and  $k$ , and the predictions compared with the observed histogram (see Fig. 7).

Extracellular observations were also used to construct number of quanta released-frequency histograms, that is, the number of single, double, triple, etc., quanta released over the 100–500 trials in a given  $[\text{Ca}]_o$ . A binomial analysis of these histograms was then made according to the Method given in Johnson and Wernig (1971), estimates of the binomial parameters at the extracellular sites, ( $p_e$  and  $n_e$ ) made from  $p_e = 1 - S^2/\bar{m}_e$  and  $n_e = \bar{m}_e/p_e$ , where  $\bar{m}_e$  and  $S^2$  are the mean and variance of the observed quantal release, and the predictions compared with the observed histogram (Fig. 3). For all three histogram types, resulting from both intracellular and extracellular observations, the “goodness of fit” of the binomial or stochastic distribution to the observed distribution was determined by a  $\chi^2$  test, and this was such that  $P > 0.60$  for 67% of all histograms (total number 300). Some amplitude-frequency histograms obtained from intracellular measurements in this and previous studies (Bennett et al. 1977) were not well fitted by the predictions of binomial statistics and were therefore rejected (less than 10% of all such histograms); these distributions may have been of the kind recently reported by Wernig and

Carmody (1978); these distributions have a number of maxima and minima which they interpret as due to intermittent functional failure of portions of the nerve terminal, although in no experiment did we find evidence from extracellular recording of intermittent failure of the action potential to invade the region of the nerve terminal from which recordings were made (see also Katz and Miledi, 1965 *a*).

Studies on the facilitated release of quanta by successive impulses during short high-frequency (25–100 Hz) trains were made by stimulating the nerve with trains of five impulses and then repeating these trains over 100 times; an interval of 20 s was left between trains to avoid any effects due to potentiation. The number of quanta released-frequency histograms were then constructed (see above) for the quantal release for each impulse over the 100–500 trains, and comparison was made between the predictions of a binomial analysis and the observed release.

#### *Errors in the Estimation of Statistical Parameters*

The amplitude-frequency histograms of both evoked and spontaneous EPPs are subject to a number of possible errors, which primarily arise from the contribution of the recording system noise to the variance of both the evoked and spontaneous potentials; an additional source of error is the use of the spontaneous potentials recorded during intracellular impalements to estimate the mean and variance of the quanta participating in evoked release; the likely effects of both of these sources of error are discussed in Methods of Bennett et al. (1976).

The latency-frequency histograms and the number of quanta released-frequency histograms are subject to the error in which early quantal releases tend to mask later quantal releases (Katz and Miledi, 1965 *a*; Barrett and Stevens, 1972 *a*) so that some quanta are missed, leading to, among other things, an underestimate of  $\bar{m}_e$ . The recording methods used in the present work allowed a distinction to be made of quanta separated by at least 0.5 ms. Furthermore, most observations were made in low  $[Ca]_o$  ( $\leq 0.35$  mM) in which the probability of quantal release is so low that it is extremely unlikely that many quanta were missed as a consequence of near synchronous release, with only single quantal releases occurring. For these reasons, it is unlikely that  $\bar{m}_e$  is significantly underestimated because of the difficulty of unambiguously identifying the occurrence of second and third quanta.

The amplitude-frequency distribution of either spontaneous or evoked quanta recorded with an extracellular electrode often assumes a skewed shape (del Castillo and Katz, 1954), possibly because of electrotonic decrement in endplate currents originating at different distances from the recording electrode, or because of variations in the size of the quanta secreted at a release site (Bornstein, 1974; Bennett and Pettigrew, 1975; Wernig, 1976; Wernig and Stirner, 1977). These skewed amplitude-frequency distributions further contribute to the problem that early quantal releases tend to mask later quantal releases, mentioned above. However, amplitude-frequency distributions of quanta recorded with an extracellular electrode also assume a more normal form (Fig. 2). Factors which determine whether these distributions are skewed or normal will be the subject of a later report.

It is known that pressure of the external electrode on nerve terminal release sites can give rise to an increase in the frequency of spontaneous MEPPs recorded with an intracellular electrode (Fatt and Katz, 1952) and may therefore alter the normal evoked release of quanta from these sites. In the present work, the extracellular electrode was carefully placed so as not to increase the MEPP frequency recorded with either the intracellular or extracellular electrode and checks against pressure effects were made by comparing the mean quantal content of the intracellular EPP both before and after positioning of the electrode for recording from a group of release

sites. No significant changes in the mean quantal content of the EPP were observed if these precautions were followed. Thus the maximum percentage change of the mean quantal content of the intracellular EPP recorded before and after positioning of the extracellular electrode was the same as the maximum percentage change of the mean quantal content of the intracellular EPP recorded at the beginning and end of an impalement in the absence of extracellular electrodes. In both cases these percentage changes could involve either decreases or increases in mean quantal content to a maximum of 5%.

A final check was made of any alteration in normal evoked quantal release due to positioning of the extracellular electrodes by comparing the average of all the mean quantal contents of the EPPs recorded by intracellular electrodes during simultaneous intra- and extracellular recordings in a particular  $[Ca]_o$  in the present study, with the average of all the mean quantal contents of the EPPs recorded by intracellular electrodes during only intracellular recording in the same  $[Ca]_o$  in previous studies (Bennett et al., 1977). These were found to be almost identical, the respective values for simultaneous recording and for intracellular recording alone in a number of different  $[Ca]_o$  being as follows:  $[Ca]_o$  of 0.4 mM,  $3.1 \pm 0.2$  and  $3.1 \pm 0.1$ ;  $[Ca]_o$  of 0.35 mM,  $2.1 \pm 0.2$  and  $2.1 \pm 0.2$ ;  $[Ca]_o$  of 0.3 mM,  $1.2 \pm 0.2$  and  $1.1 \pm 0.1$ . At least 15 observations were made for each determination and  $\pm$  SEM is given.

Problems arising from variations in the binomial parameters during the 100–500 trials used to obtain estimates are discussed in detail in the Appendix to Bennett and Fisher (1977). The methods employed in determining the SE of the means of the binomial parameters  $\bar{m}$ ,  $p$ ,  $n$ ,  $\bar{m}_e$ ,  $p_e$ ,  $n_e$  and the stochastic parameters  $\alpha$ ,  $\gamma$ , and  $k$  are given in Robinson (1976) and Bennett et al. (1977), respectively.

## RESULTS

### *The Probability of Secretion of a Quantum at Different Nerve Terminal Release Sites*

In the extracellular calcium  $[Ca]_o$  range from 0.25 to 0.4 mM, motor endplates were recorded from focally with an intracellular microelectrode, and then an extracellular electrode was placed in the region where the myelinated motor axon was observed to terminate under a high-power ( $\times 80$ ) binocular dissecting microscope, and this extracellular electrode was moved along the long axis of the muscle in search for the electrical signs of the endplate currents (EPCs) generated either by the spontaneous or evoked release of transmitter during nerve stimulation at 0.2–1.0 Hz (see del Castillo and Katz, 1956; Katz and Miledi, 1965 *b*). At 0.4 mM  $[Ca]_o$  it was relatively easy to find a region along the length of the nerve terminal where during 10–20 consecutive impulses an evoked EPC could be detected (Fig. 1), whereas in 0.25 mM  $[Ca]_o$  such evoked EPCs rarely occurred and several endplates had to be examined before evoked release was observed during up to 20 consecutive impulses. Fig. 1 shows examples of simultaneous intracellular and extracellular recordings made at an endplate in a  $[Ca]_o$  of 0.35 mM. The triphasic extracellularly recorded nerve impulse is observed during both evoked releases and failures and was always used to monitor the successful impulse invasion of the region of the nerve terminal recorded from. The amplitude of this nerve impulse was not always precisely the same from impulse to impulse (Fig. 1), probably because the extracellular field of the impulse is confined to a small

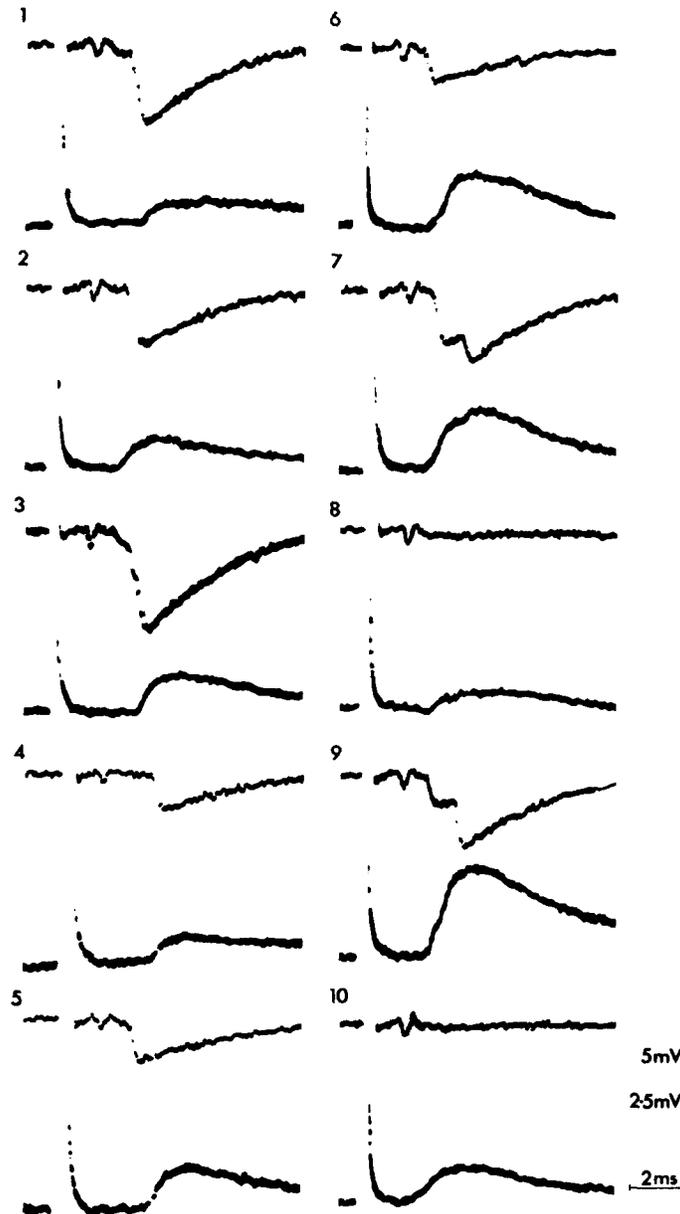


FIGURE 1. Simultaneous recordings of the secretion of quanta from all the release sites at an amphibian motor-nerve terminal (intracellular electrode, lower trace) with the secretion of quanta from a small group of release sites (extracellular electrode, upper trace). A simultaneous set of recordings is shown for each nerve stimulation from 1 to 10. AC coupling was used for both intracellular and extracellular records.  $[Ca]_o = 0.35$  mM. Vertical calibration, 2.5 mV for upper trace, 5.0 mV for lower trace.

distance from the axon terminal (see Discussion), so that even very slight movements of the electrode lead to relatively large changes in the recorded triphasic potential change. Occasional spontaneous EPCs were also observed and these were recorded simultaneously with the intracellular electrode, acting, together with the observation that no extracellular EPC occurs without simultaneously an EPP occurring, as a check that the extracellular electrode is recording from the impaled muscle fiber. Fig. 2 shows the amplitude-frequency distribution of the evoked quanta recorded from the extracellular site for which quantal release is illustrated in Fig. 1.

Release sites in different  $[Ca]_o$  could be found with the extracellular electrode at which quantal secretion obeyed binomial statistics and for which the ratio  $\bar{m}_e/\bar{m}$  reached 0.30 (Fig. 3) and in some cases even higher, indicating considerable nonuniformity in the probability of release at different sites along

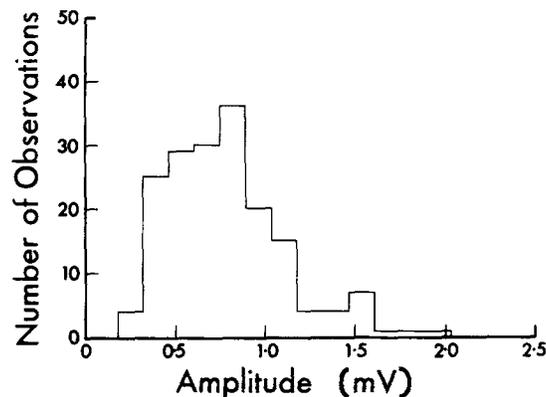


FIGURE 2. Amplitude-frequency distribution of the evoked quanta recorded at an extracellular site. Some of the evoked quanta which compose this distribution are illustrated in Fig. 1.

the length of the terminal; simultaneous intracellular and extracellular recordings were made during 28 different experiments in this way, and the  $\bar{m}_e/\bar{m}$ -frequency histogram of Fig. 4 indicates the distribution of this ratio ranges from less than 0.1 to up to 0.5. The relative frequency of the lower  $\bar{m}_e/\bar{m}$  values ( $\leq 0.1$ ) is an underestimate, as only about 20 consecutive impulses were used to test for secretion at different electrode positions along the length of the nerve terminal; this number of impulses was arrived at as a compromise, in order to maximize the number of extracellular samples taken from an endplate during a stable intracellular recording. In order to obtain a quantitative description of the variation of  $\bar{m}_e$  above values of 0.1 along the length of terminals in different  $[Ca]_o$ , extracellular sites with values of  $\bar{m}_e$  above 0.1 were examined and Fig. 5 shows the great variation in the average quantal content at different groups of release sites at amphibian motor nerve terminals.

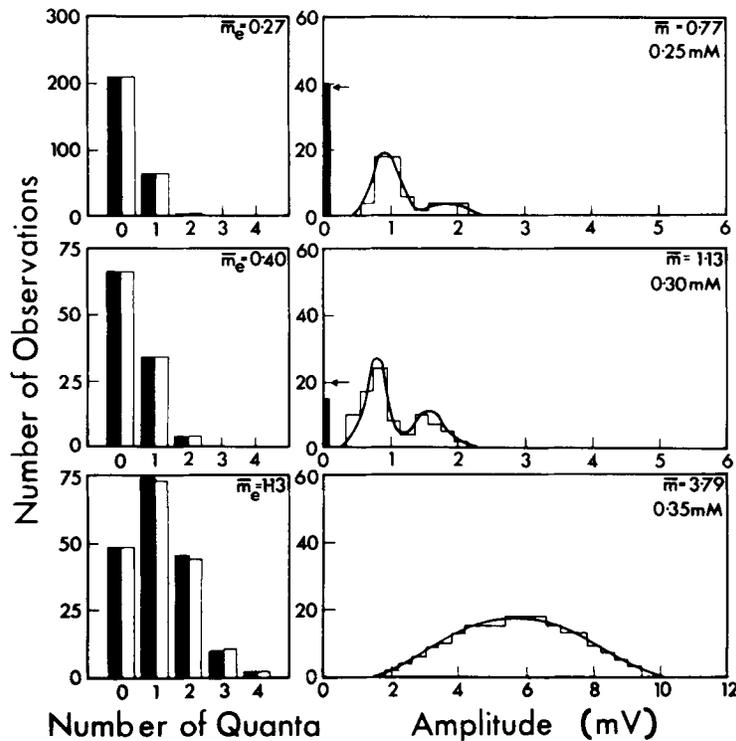


FIGURE 3. Comparison of the frequency-distributions for quantal secretion recorded simultaneously with an extracellular electrode (left hand panels) and an intracellular electrode (right hand panels), at the three different  $[Ca]_o$  levels indicated in millimolar; in each case the average quantal content for the extracellular recording ( $\bar{m}_e$ ) and for the simultaneous intracellular recording ( $\bar{m}$ ) are shown. Filled bars in the histograms are for the number of zero, one, two, three, and four quanta observed with the extracellular electrode, whereas open bars are the theoretical predictions from a binomial analysis (see Methods) according to which for  $\bar{m}_e = 0.27 \pm 0.03$ ,  $p_e = 0.16 \pm 0.04$ , and  $n_e = 1.73 \pm 0.07$ ; for  $\bar{m}_e = 0.40 \pm 0.02$ ,  $p_e = 0.20 \pm 0.08$ , and  $n_e = 1.98 \pm 0.76$  whereas for  $\bar{m}_e = 1.13 \pm 0.06$ ,  $p_e = 0.30 \pm 0.04$ , and  $n_e = 3.80 \pm 0.09$ . Continuous lines for the amplitude-frequency distributions of the EPPs give the theoretical predictions from a binomial analysis according to which for  $\bar{m} = 0.77 \pm 0.02$ ,  $p = 0.38 \pm 0.04$ , and  $n = 2.0 \pm 0.30$ , for  $\bar{m} = 1.13 \pm 0.08$ ,  $p = 0.50 \pm 0.06$ , and  $n = 2.27 \pm 0.28$  whereas for  $\bar{m} = 3.79 \pm 0.04$ ,  $p = 0.76 \pm 0.08$ , and  $n = 4.95 \pm 0.30$ ; the arrows give the predicted numbers of failures. Between 100 and 250 impulses were used in each series of trials; frequency 0.5 Hz. Note that  $\bar{m}_e/\bar{m}$  is  $\sim 0.3$  in each case; that is, the extracellular electrode records about 30% of the total secretion from the nerve terminal.

*The Effect of Changes in  $[Ca]_o$  on the Probability of Secretion of a Quantum at Nerve Terminal Release Sites*

Increasing  $[Ca]_o$  in the range 0.25–0.4 mM increased the average quantal content  $\bar{m}_e$  recorded at groups of release sites, and in general this increase was

due to both an increase in  $p_e$  and  $n_e$ , as in 0.25 mM  $[Ca]_o$ , only failures or single quantal releases were observed (Fig. 3), whereas in higher  $[Ca]_o$  double releases or even higher releases were sometimes observed (Fig. 1) indicating that  $n_e$  had increased from 1 to a higher integer (Fig. 3).

If the  $[Ca]_o$  was increased during simultaneous intracellular and extracellular recording from a group of release sites with a quantal content well above average, then both the intracellularly and extracellularly recorded quantal contents  $\bar{m}$  and  $\bar{m}_e$  increased with the fourth power of  $[Ca]_o$ , in the  $[Ca]_o$  range from 0.25 to 0.4 mM (Fig. 6 A and B); the increase in  $\bar{m}$  with  $[Ca]_o$  was accompanied by a much smaller increase in  $p$  with  $[Ca]_o$  (Fig. 6 A) as has been observed before (Bennett et al., 1975, 1977), whereas the increase in  $\bar{m}_e$  with  $[Ca]_o$  was accompanied by a comparable change in  $p_e$  (Fig. 6 B). It is

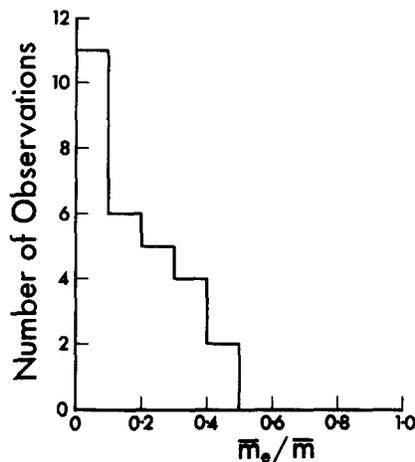


FIGURE 4. The ratio of the mean quantal content of secretion recorded with an extracellular electrode ( $\bar{m}_e$ ) to that recorded simultaneously with an intracellular electrode ( $\bar{m}$ ) at 28 different motor nerve terminals. Results determined from observations as in Fig. 1 which were analyzed as in Fig. 3. Note that the ratio  $\bar{m}_e/\bar{m}$  extended up as high as 0.5 in a few cases.

shown in Theory that these results are to be expected if there are large differences in the probability of secretion of quanta at different release sites, in which case  $p_e$  is approximately the probability of secretion of a quantum at only a few release sites and is very  $[Ca]_o$  dependent, whereas  $p$  is a function of both the average probability of secretion at all release sites and the variance of this probability so that the fractional increase of  $p$  with an increase in  $[Ca]_o$  is much less than the fractional increase in  $p_e$  with an increase in  $[Ca]_o$ .

During changes in  $[Ca]_o$  at some extracellular recording sites, the increase in quantal content  $\bar{m}_e$  which accompanied an increase in  $[Ca]_o$  over the range 0.25–0.4 mM did not involve an increase in  $n_e$ ; that is, the increase in  $[Ca]_o$  was accompanied by an increase in the proportion of single releases over

failures, in such a way that  $n_e$  was equal to 1.0 in each different  $[Ca]_o$  even though substantial increases in  $\bar{m}_e$  occurred. In this case  $p_e$  increases with  $[Ca]_o$  in the same way as does  $\bar{m}_e$  (Fig. 6 C), and as is shown in Theory (see Eq. 4), it is likely that quantal secretion is from a single release site, so that the

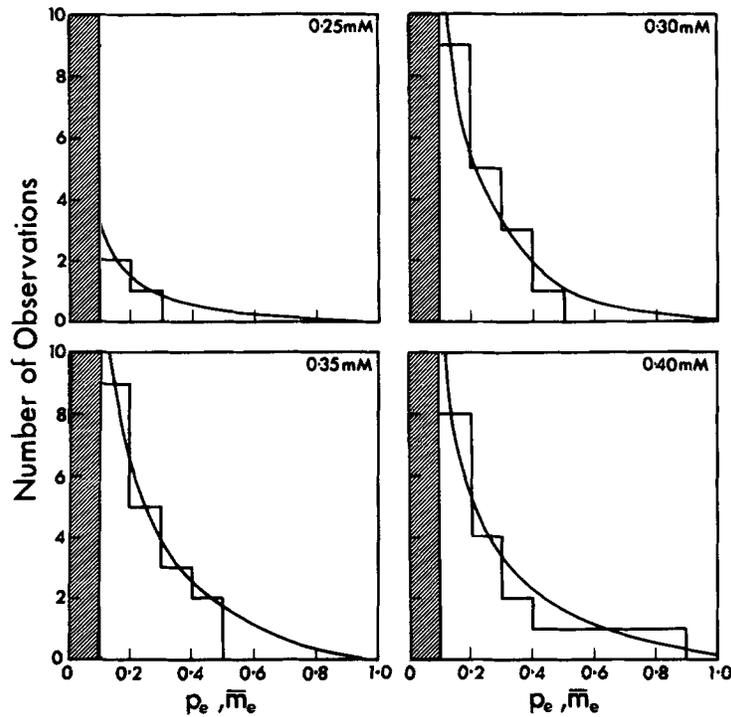
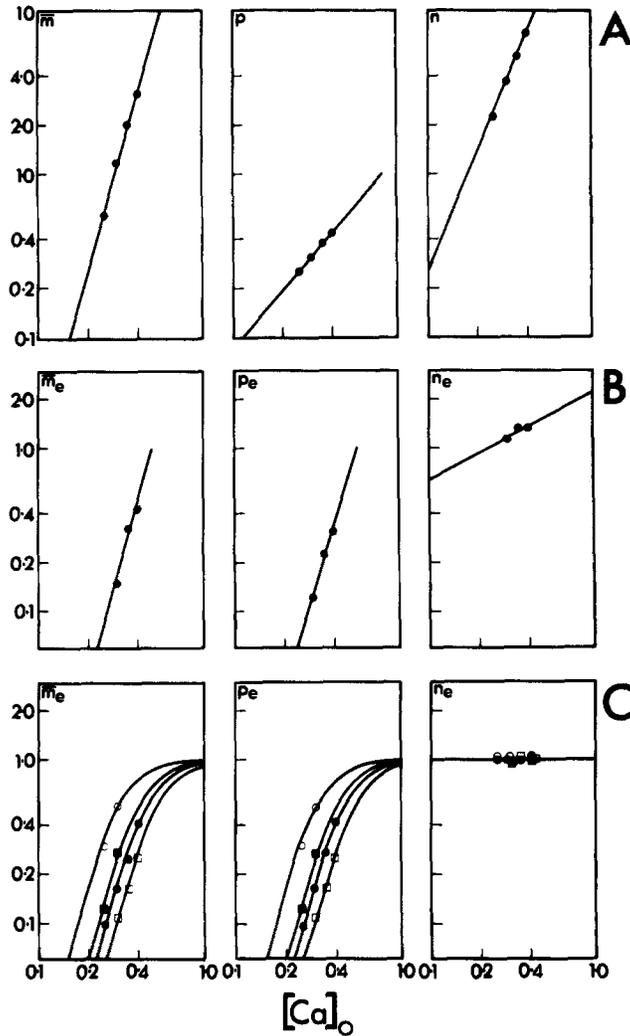


FIGURE 5. The frequency-distribution of the mean quantal content ( $\bar{m}_e$ ) recorded at different extracellular sites for which  $\bar{m}_e = p_e$ ; that is, the binomial parameter  $n_e$  was 1.0. Each estimate was made during 100–200 impulses in the different  $[Ca]_o$  indicated. The relative frequencies for  $p_e \leq 0.10$  were not determined because of the sampling problems mentioned in the text; the hatched bar indicates  $p_e \leq 0.10$ . The relative frequency of the large  $\bar{m}_e$  values increases with an increase in  $[Ca]_o$ . It is argued in the Theory that if  $p_e = \bar{m}_e$ , then the recording is from a single release site with probability  $p_j$ , so  $p_j = p_e = \bar{m}_e$ . The curves of fit to the histograms are drawn according to the probability density for the  $p_j$ , namely  $f(p_j)$ , (see Eq. 5 in Theory), in which the tails of the  $f(p_j)$  distribution (see Fig. 10) have been fitted to the appropriate frequency distributions by setting the value of  $f(p_i)$  at  $p_j = 0.15$  equal to that for the number of observations in the 0.1–0.2 bin; the remaining bin heights are then reasonably predicted by the  $f(p_j)$  curves for each  $[Ca]_o$ .

probability of secretion of a quantum  $p_e$  is the probability of release from a single site,  $p_j$ . If this occurs, then  $\bar{m}_e = p_e = p_j$  must approach 1.0 as  $[Ca]_o$  is increased. The observed changes in these parameters with an increase in  $[Ca]_o$  (Fig. 6 C), could not be predicted by an equation of the form,

$$\bar{m}_e = p_e = p_j = A\{[Ca]_o/([Ca]_o + K)\}^4,$$



without  $A$  taking on values much greater than 1.0, so that  $p_j$  approaches about 20 as  $[Ca]_o$  is increased. This equation has been used to describe the changes in  $\bar{m}$  recorded with an intracellular electrode with changes in  $[Ca]_o$  (Dodge and Rahamimoff, 1967; Bennett et al., 1975, 1977), and it suggests that calcium ions must bind to four independent receptors at a release site before a quantum is released. However, the changes in  $\bar{m}_e$ ,  $p_e$  and  $p_j$  with  $[Ca]_o$  at these sites were well predicted (Fig. 6 C) by an equation of the form,

$$\bar{m}_e = p_e = p_j = [Ca]_o^4 / (K_j + [Ca]_o^4),$$

which suggests that calcium ions must bind to a number of interacting receptors at a release site, the binding of a calcium ion to one receptor facilitating the binding of calcium ions to the remaining receptors in a cooperative manner, with at least four receptors being involved (see Koshland, 1970).

For extracellular recordings at which  $n_e = 1$ , the frequency distribution of the probabilities of secretion of a quantum  $p_e$  changed in different  $[\text{Ca}]_o$  (Fig. 5). At low  $[\text{Ca}]_o$  of 0.25 mM, only very few extracellular recordings revealed a  $p_e$  greater than 0.1 (Fig. 5 A), whereas at higher  $[\text{Ca}]_o$  the frequency of high  $p_e$  values increased until at  $[\text{Ca}]_o$  of 0.4 mM, some  $p_e$  values near 1.0 were observed (Fig. 5 D).

*The Effect of a Conditioning Impulse on the Probability of Secretion of a Quantum by a Test Impulse at Nerve Terminal Release Sites*

The effect of a conditioning impulse on the secretion of quanta by a subsequent test impulse was determined simultaneously for both a small number of release sites and the entire nerve terminal. The extracellular electrode could be positioned at sites for which  $n_e$  remained equal to 1 during the facilitation of the test release in different  $[\text{Ca}]_o$ , so that the facilitation in the test response was solely due to an increase in  $p_e$  (Fig. 7). If the frequency histogram for the latencies of the quanta evoked at release sites is determined for both conditioning and test impulses (Fig. 7), no change is observed in the minimum synaptic delay (defined as in Katz and Miledi, 1965 *b*) of about 600  $\mu\text{s}$  between the two impulses (compare Fig. 7 A with B and Fig. 7 C with D), the sole effect of facilitation being to enhance the number of quanta secreted at times shortly after the minimum synaptic delay (Fig. 7; compare with Fig. 4 in Barrett and Stevens, 1972 *b*). The time-course of decline in facilitation of  $\bar{m}_e$  and  $p_e$ , where facilitation is defined as

$$f\bar{m}_e = \left( \frac{\bar{m}_{e_1} - \bar{m}_{e_0}}{\bar{m}_{e_0}} \right) \text{ or } fp_e = \left( \frac{p_{e_1} - p_{e_0}}{p_{e_0}} \right),$$

FIGURE 6 (*Opposite*). Dependence of the binomial parameters describing secretion of quanta on  $[\text{Ca}]_o$  in the range 0.25–0.40 mM. The effect of changing  $[\text{Ca}]_o$  in a  $[\text{Mg}]_o$  of 1.2 mM on the quantal content of secretion ( $\bar{m}$ ) recorded with an intracellular electrode and on  $p$  and  $n$ , as well as on the quantal content of secretion ( $\bar{m}_e$ ) recorded with an extracellular electrode and on  $p_e$  and  $n_e$  are shown in A and B, respectively, on log-log co-ordinates. (C)  $\bar{m}_e$ ,  $p_e$ , and  $n_e$  are shown for four different extracellular sites in different  $[\text{Ca}]_o$ , for which  $\bar{m}_e = p_e$  so that  $n = 1.0$ . Least-square regression lines to the data are shown in each of the graphs in A and B, whereas in C the lines are drawn according to  $p_e = p_j = [\text{Ca}]_o^4 / (K_j + [\text{Ca}]_o^4)$  (see Eq. 15 in Theory) in which  $K_j$  had the values: (○) 0.008, (■) 0.026; (●) 0.04; and (□) 0.07. (A) Each point is the mean of a binomial parameter determined at between 7 and 15 synapses; if the standard error of the mean of a parameter determined at a synapse was >15% of the mean value it was not included; the standard error of the mean of the points shown was always less than 15% of the mean; the gradients for  $\bar{m}$ ,  $p$ , and  $n$  are 3.8, 1.2, and 2.6, respectively. (B) Each point is the mean of a binomial parameter determined at 10 sites belonging to 10 different synapses, the  $[\text{Ca}]_o$  being changed from 0.3 to 0.4 mM at each of these sites; the standard error of the mean of each point shown is ~25% of the mean because of differences in the value of  $\bar{m}_e$  in the same  $[\text{Ca}]_o$  at different sites (see Fig. 6); the gradients for  $\bar{m}_e$ ,  $p_e$ , and  $n_e$  are 3.8, 3.3, and 0.5, respectively. (C) Each point is the binomial parameter recorded at a single site (see text). All the binomial parameters were determined from at least 100 trials.

the subscript 0 referring to the conditioning impulse release and the subscript 1 to the test impulse release, was exponential with a time constant of about 40 ms (Fig. 8). The time-course of decline of the facilitation of  $\bar{m}$ , measured with an intracellular electrode, is also exponential with a time constant of about 40 ms (Balnave and Gage, 1977; Bennett and Fisher, 1977), although there is no change in  $p$  during a conditioning-test sequence of impulses (Bennett and Fisher, 1977), indicating that, although the probability of secretion at release sites ( $p_j$ ) increases during facilitation, the variance of the distribution of  $p_j$  changes in such a way that  $f\bar{p} = 0$  (see Theory).

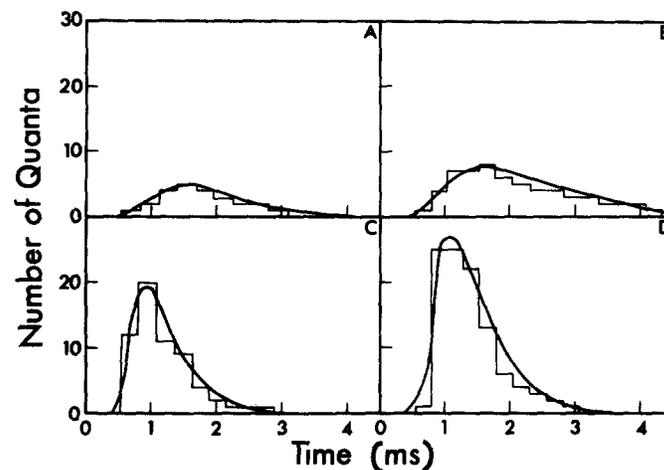


FIGURE 7. Changes in the latency-frequency distribution of quanta secreted by a test impulse following 10 ms after a conditioning impulse. Two conditioning-test sequences are shown for two different  $[Ca]_o$  at the same extracellular release sites, namely A and B in 0.3 mM and C and D in 0.35 mM. The latency-frequency distribution of evoked quanta were determined at 18°C during continual stimulation at 0.5 Hz. During secretion in both  $[Ca]_o$ ,  $\bar{m}_e = p_e$ , that is,  $n_e$  was 1.0. The curves drawn through the distributions are according to the stochastic analysis in Bennett et al. 1977 (their Eqs. 3 and 8) in which  $\bar{m}_e = p_e$ ,  $\alpha$ ,  $\gamma$ , and  $k$  were respectively in (A) 0.155, 1.56  $ms^{-1}$ , 1.07  $ms^{-1}$ , 3.6; in (B) 0.381, 1.12  $ms^{-1}$ , 0.45  $ms^{-1}$ , 2.9; in (C) 0.411, 1.52  $ms^{-1}$ , 1.12  $ms^{-1}$ , 1.62; and in (D) 0.788, 2.66  $ms^{-1}$ , 0.26  $ms^{-1}$ , 2.5. The abscissa is the time after the negative peak of the triphasic electrical sign of the nerve impulse. As  $\bar{m}_e = p_e$ , so  $n_e = 1.0$ ; at most one quantum was released following either the conditioning or test impulse, so that the latency-frequency distribution for only one quantum is shown. The results in A and C are without conditioning impulses.

According to the definition of facilitation given above, release sites which have a high  $\bar{m}_e$  or  $p_e$  value during a conditioning impulse can only show small increases in  $\bar{m}_e$  or  $p_e$  during a subsequent test impulse, so that  $f\bar{m}_e$  or  $f\bar{p}_e$  will be different between release sites depending on the conditioning  $\bar{m}_e$  or  $p_e$  value. In order to avoid this difficulty the value of  $K_j$  in the equation for  $p_j$  above

was determined for both conditioning ( $K_{j_0}$ ) and test ( $K_{j_1}$ ) impulse releases so as to obtain  $fK_j = (K_{j_0} - K_{j_1})/K_{j_1}$  for different test-conditioning intervals, as shown in Fig. 8.  $fK_j$ , as anticipated, was much larger than  $f\bar{m}_e$  and  $fp_e$  at short intervals.

Facilitation was also observed during short high-frequency trains of impulses in  $[Ca]_o$  of 0.3 mM, at release sites for which  $n_e$  remained equal to 1 for the quantal releases by each impulse in the train (Fig. 9). Both  $f\bar{m}_e$  and  $fp_e$  increased together during the train, but not as much as  $fK_j$ , which continues to increase by about the same amount after the first few impulses irrespective of the value of  $p_e$  during the impulses (Fig. 9). Thus, if the saturation effects in the determination of  $f\bar{m}_e$  or  $fp_e$  discussed above are avoided by determining  $fK_j$ , then the membrane events which control  $K_j$ , and hence  $p_j$ , at release sites facilitate markedly throughout a short high-frequency train of impulses.

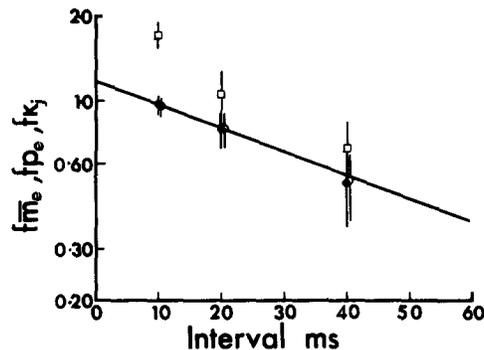


FIGURE 8. The effect of a conditioning impulse on quantal secretion by a subsequent test impulse at the intervals shown, recorded with an extracellular electrode in a  $[Ca]_o$  of 0.3 mM. The changes in  $f\bar{m}_e$  (○),  $fp_e$  (●) and  $fK_j$  (□), each defined in the text, are shown. Each point is the mean  $\pm 1$  SE of the mean of results from at least nine extracellular recordings at nine different synapses, at each of which  $f\bar{m}_e$  and  $fp_e$  were determined by applying a binomial analysis to the results of at least 100 test-conditioning pairs of impulses; facilitation during each of these extracellular recordings did not involve any change in  $n_e$  which was 1.0, so that  $\bar{m}_e = p_e$  and therefore  $f\bar{m}_e = fp_e$ ; in this case  $K_j$  (see Eq. 15 in Theory) can be determined and hence  $fK_j$ . Note that  $fK_j$  is significantly greater than  $f\bar{m}_e$  or  $fp_e$  at short intervals (for explanation see text).

#### THEORY

##### *The Binomial Description of the Secretion of Quanta at Nerve Terminals and the Probability of Secretion at Individual Release Sites*

The binomial parameters determined from the mean and variance of the quantal secretion ("the variance method") have been used in Results to quantitatively describe quantal transmitter secretion from all the release sites of a motor nerve terminal ( $p$  and  $n$ ) as well as from small groups of release sites ( $p_e$  and  $n_e$ ). The problem arises as to the relationship between these

binomial parameters determined by the variance method and the probability of secretion at individual release sites ( $p_j$ ). As mentioned in the Introduction, if the probability of secretion at individual release sites ( $p_j$ ) is the same for all release sites of the motor-nerve terminal, then  $p = p_j$  and  $n$  is the number of

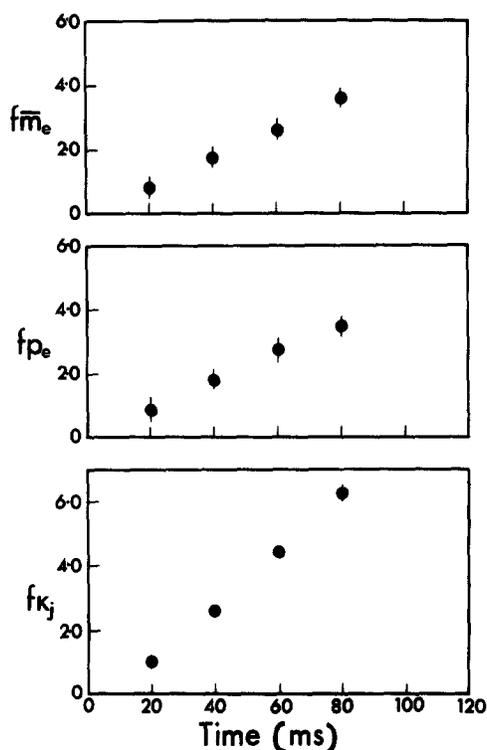


FIGURE 9. Changes in the quantal secretion recorded with an extracellular electrode during a short (five-impulse) high-frequency train (50 Hz) in a  $[Ca]_o$  of 0.3 mM. The changes in  $f\bar{m}_e$ ,  $fp_e$ , and  $fK_j$  (defined in the text) are shown for each of the impulses in the train. Each point is the mean  $\pm$  1 SE of the mean of results from six extracellular recordings at six different synapses, at each of which  $f\bar{m}_e$  and  $fp_e$  were determined by applying a binomial analysis to the successive results of at least 100 trains. Facilitation during each of these extracellular recordings did not involve any change in  $n_e$  which was 1.0, so that  $\bar{m}_e = p_e$  and therefore  $f\bar{m}_e = fp_e$ , and it is likely that only one release site (with probability  $p_j$ ) was participating in secretion (see Eq. 4 in Theory); in this case  $K_j$  can be determined (see Eq. 15 in Theory) and hence  $fK_j$ . Note that  $f\bar{m}_e$  and  $fp_e$  do not change as much as  $fK_j$  during successive impulses, because of the saturation of  $f\bar{m}$  and  $fp$  for synapses with  $\bar{m}_e$  and  $p_e$  close to 1.0, an effect which does not apply to  $fK_j$ .

release sites which participate in secretion; however, in Results we have shown that the probability of secretion is different for different release sites, so that other interpretations of  $p$  and  $n$  must be considered. The Appendix to Bennett and Fisher (1977) gives the interpretations of  $p$  and  $n$  when quantal secretion

is a binomial variable and the probability of secretion varies between sites (case *iii a*): if during a series of impulse trials each release site can secrete at most one quantum, and the probability of secretion of a quantum at the  $j$ th release site ( $p_j$ ;  $j = 1$  to  $N$ ) is different for each of the  $N$  release sites from which the electrode can record secretion ( $N$  being large for intracellular recordings but small for extracellular recordings) but is constant for each release site over all trials, then

$$E\bar{m} = N\bar{p} = \sum p_j \quad Es^2 = N\bar{p}(1 - \bar{p}) - N\sigma_p^2 \quad (1)$$

$$\bar{p} = \sum p_j / N \quad \sigma_p^2 = \sum (p_j - \bar{p})^2 / (N - 1) \quad (2)$$

in which  $\bar{m}$  and  $s^2$  are as usual the mean and variance of the observed quantal secretion,  $\bar{p}$  is the average  $p_j$  over the  $N$  release sites from which the electrode can record secretion and  $\sigma_p^2$  is the variance of the  $p_j$  about  $\bar{p}$ . If the probability of secretion does not vary between release sites,  $\sigma_p^2 = 0$  and (Eq. 1) becomes

$$E\bar{m} = N\bar{p} = np \text{ and } Es^2 = N\bar{p}(1 - \bar{p}) = np(1 - p), \quad (3)$$

so that  $p$  is the probability of secretion at a release site and can be determined by the variance method and  $N = n$  is the number of release sites which participate in secretion.

If  $\sigma_p^2$  is not zero, then  $p$  is no longer the probability of secretion at a release site, but is related to the mean ( $\bar{p}$ ) and variance ( $\sigma_p^2$ ) of the probability density of the secretion at a release site ( $p_j$ ) over the  $N$  release sites recorded from according to (from Eqs. 1 and 3),

$$p = \bar{p} + \sigma_p^2 / \bar{p} = \frac{\sum p_j^2}{\sum p_j}. \quad (4)$$

If during an extracellular recording (small  $N$ ),  $p$  which is determined by the variance method is equal to the mean of the quantal secretion (i.e.,  $p = \bar{m}$ ), then it follows from Eqs. 1 and 4 that  $\sum p_j^2 / \sum p_j = \sum p_j$ . The only condition under which this occurs is if  $n = N = 1$ , that is, only one release site (say  $p_1$ ) is contributing to secretion over the trials; this condition was met during the collection of results illustrated in Figs. 5-9.

If the form of the density distribution of the  $p_j$ 's over all the release sites of the motor-nerve terminal is known, then determination of  $\bar{m}$  and of  $p$  by the variance method for intracellular recording of secretion from all the motor-nerve terminal release sites (large  $N$ ) allows estimates of the mean and variance of the density distribution to be made from Eqs. 1 and 6. The most general density distribution the  $p_j$ 's could take is a beta density distribution, which can assume a great variety of different shapes, and is of the form

$$f(p_j) = \frac{\Gamma(\alpha + \beta)}{\Gamma\alpha\Gamma\beta} p_j^{\alpha-1}(1 - p_j)^{\beta-1}, \quad (5)$$

which has mean and variance such that

$$\bar{p} = \alpha / (\alpha + \beta) \text{ and } \sigma_p^2 = \alpha\beta / (\alpha + \beta)^2(\alpha + \beta + 1), \quad (6)$$

so that from Eqs. 4 and 6

$$p = (\alpha + 1)/(\alpha + \beta + 1), \quad (7)$$

and from Eqs. 1 and 6

$$\frac{\bar{m}}{N} = \alpha/(\alpha + \beta) \quad (8)$$

or alternatively,  $\alpha$  and  $\beta$  can be determined as functions of  $p$  and  $\bar{m}/N$  according to Eqs. 7 and 8 as

$$\alpha = \frac{\bar{m}}{N} (1 - p) / \left( p - \frac{\bar{m}}{N} \right) = \bar{m} (1 - p) / (Np - \bar{m}), \quad (9)$$

and

$$\beta = \left( 1 - \frac{\bar{m}}{N} \right) (1 - p) / \left( p - \frac{\bar{m}}{N} \right) = (N - \bar{m})(1 - p) / (Np - \bar{m}). \quad (10)$$

Now the values of  $\bar{m}$  and  $p$  in  $[\text{Ca}]_o$  between 0.25 and 0.6 mM are known (see Fig. 6 A in the present work and Figs. 3 and 4 in Bennett et al., 1977), and over this range of  $[\text{Ca}]_o$ ,  $\bar{m}$  is about two orders of magnitude smaller than  $N$  so that  $\alpha$  and  $\beta$  in Eqs. 9 and 10 can be very well approximated by:

$$\alpha = \frac{\bar{m}}{Np} (1 - p) \quad (11)$$

and

$$\beta = (1 - p)/p; \quad (12)$$

furthermore, as  $\bar{m}$  is so small compared with  $N$  for  $[\text{Ca}]_o \leq 0.6$  mM (see Fig. 4 in Bennett et al., 1977),  $\alpha$  is very small compared with  $\beta$  and the density distribution for  $p_j$  given by Eq. 5 becomes

$$f(p_j) \approx \alpha p_j^{-1} (1 - p_j)^{\beta-1}, \quad (13)$$

and inserting Eqs. 11 and 12 into Eq. 13 gives

$$f(p_j) = \frac{1}{N} \left[ n(1 - p) p_j^{-1} (1 - p_j) \frac{(1 - 2p)}{p} \right]. \quad (14)$$

Thus,  $N$ , the number of release sites, is simply a scaling factor for  $f(p_j)$  and as  $p$  and  $n$  are known over the  $[\text{Ca}]_o$  range from 0.25 to 0.6 mM, the determination of the density-distribution of  $p_j$  over the release sites of a terminal in different  $[\text{Ca}]_o$  is possible, using either the full Eq. 5 or the approximate Eqs. 13 and 14 and these distributions are shown in Fig. 10. Estimates can be made from Fig. 10 of the likely number of release sites which possess any given  $p_j$  value in a particular  $[\text{Ca}]_o$  from consideration of the appropriate areas under the curves or directly by integration of Eqs. 5 or 13. Thus, for  $[\text{Ca}]_o$  of 0.4 mM, the area under the curve for  $p_j \geq 0.4$  is 2.5 so about three release sites are estimated to have  $p_j \geq 0.4$ ; as an external electrode records secretion from

about  $\frac{1}{10}$  of a 1,000- $\mu\text{m}$ -long terminal (see Discussion), then if the  $p_j$  values are distributed at random along the length of the terminal, an external electrode has about a 1 in 10 chance of recording a  $p_j \geq 0.4$  in a  $[\text{Ca}]_o$  of 0.4 mM (see Fig. 5).

The question now arises as to whether the assumption of a beta density distribution for the probability of secretion of a quantum at different release sites ( $p_j$ ) can be independently checked by direct measurement of the fre-

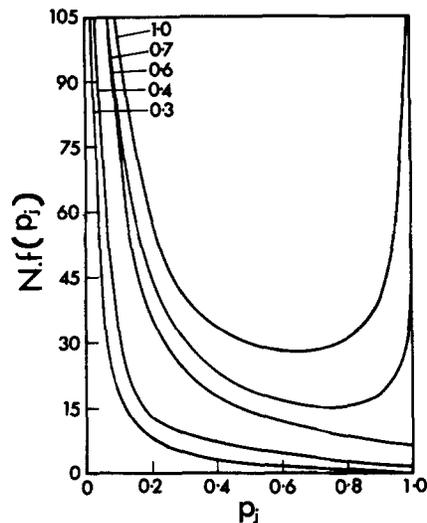


FIGURE 10. The density-distributions  $f(p_j)$  of the probability of secretion of a quantum at release sites ( $p_j$ ) at a motor nerve terminal with  $N$  release sites, based on the assumption that  $p_j$  is a beta variable. The curves are drawn according to Eq. 13 in the Theory for the different  $[\text{Ca}]_o$  in millimolar indicated;  $\alpha$  and  $\beta$  had values determined by those of  $\bar{m}$  and  $p$  (see Eqs. 11 and 12 in Theory) in different  $[\text{Ca}]_o$ , which are given in Fig. 5 A in the present work and in Figs. 3 and 4 of Bennett et al. (1977), namely for  $N\alpha$ ,  $\beta$ ,  $\bar{m}$ , and  $p$ , respectively:  $[\text{Ca}]_o$  0.3 mM, 2.1, 2.6, 0.8, and 0.28;  $[\text{Ca}]_o$  0.4 mM, 3.9, 1.5, 2.6, and 0.40;  $[\text{Ca}]_o$  0.6 mM, 8, 1.1, 7.3, and 0.50;  $[\text{Ca}]_o$  0.7 mM, 8, 0.68, 11, and 0.60;  $[\text{Ca}]_o$  1.0 mM, 11, 0.44, 22, and 0.70. Note that the fraction of release sites with  $p_j \hat{=} 1.0$  increases with an increase in  $[\text{Ca}]_o$ . As each curve is a density-distribution the area under each of the curves must be the same; this does not appear to be the case in this figure as the points of intersection of the curves with the ordinate are not shown on the scale used.

quency distribution of the  $p_j$  in a given  $[\text{Ca}]_o$  with an extracellular electrode. Unfortunately, as shown in Fig. 5, only the tails of the frequency distribution of the  $p_j$  can be determined, as many release sites with  $p_j \leq 0.1$  are not included due to the method used in locating release sites mentioned in Results. Although the beta density distribution for  $p_j$  gave a good fit to the tails for the frequency distribution of the  $p_j$ , this is not a sensitive measure of the correctness of using a beta density distribution, so an alternative method was sought.

According to Results, the  $p_j$  are altered by  $[Ca]_o$  according to the equation

$$p_j = [Ca]_o^4 / (K_j + [Ca]_o^4), \quad (15)$$

in which  $K_j$  is a constant for a given release site (see Fig. 6 C). This relationship, together with Eq. 5, allows the density distribution of the  $K_j$  over the release sites of a nerve terminal, namely  $g(K_j)$ , to be determined according to the relationship,

$$\begin{aligned} g(K_j) &= f(p_j) \left| \frac{dp_j}{dK_j} \right| \\ &= \Gamma \frac{(\alpha + \beta)}{\Gamma \alpha \Gamma \beta} [Ca]_o^{4+\alpha} (K_j)^{\beta-1} ([Ca]_o^4 + K_j)^{-(\alpha+\beta)}, \end{aligned} \quad (16)$$

and as  $\alpha \ll 1.0$  in  $[Ca]_o < 1.0$  mM, then

$$g(K_j) \approx \frac{\alpha [Ca]_o^4}{(K_j + [Ca]_o^4)^\beta} (K_j)^{\beta-1}, \quad (17)$$

and as  $\alpha$  and  $\beta$  in a given  $[Ca]_o$  can be determined from Eqs. 11 and 12, this allows the density distribution of the  $K_j$  to be determined from Eq. 17 and comparison to be made with the frequency distribution of the  $K_j$  determined from the observed  $p_j$  in a given  $[Ca]_o$  and Eq. 15, as is shown in Fig. 11. The advantage of this approach is that, because of the relationship between  $p_j$  and  $K_j$  (Eq. 15), instead of having the tails of a distribution as for the  $p_j$  (Fig. 5), the beginning of a distribution is obtained for the  $K_j$  (Fig. 11), allowing a much more accurate comparison between the frequency distribution of the  $K_j$  and the theoretical distribution. Fig. 11 shows that the shape of the frequency distribution for the  $K_j$  determined with an extracellular electrode was well predicted by the theoretical distribution given by Eq. 17, the parameters ( $\alpha$  and  $\beta$ ) of which were determined from measurements (namely of  $\bar{m}$ ,  $p$ , and  $n$ ) made with an intracellular electrode. It is likely then that the beta density distribution (Fig. 10) gives at least a reasonable approximation to the distribution of the probability of secretion of a quantum over the release sites of a motor nerve terminal.

According to Eq. 15, the value of  $p_j$  in a particular  $[Ca]_o$  is characterized by a constant  $K_j$  at the site, and as this equation gave a good fit to the experimental results (Fig. 6 C) then  $K_j$  is independent of  $[Ca]_o$ . If this is so, and  $p_j$  is a beta variable, then the density distribution of the  $K_j$ , namely  $g(K_j)$  given by Eq. 17 must be independent of  $[Ca]_o$ . This was approximately the case, the distribution given in Fig. 11 changing only by  $\pm 15\%$  in the  $[Ca]_o$  range for which accurate values of  $\bar{m}$ ,  $p$ , and  $n$  and therefore  $\alpha$  and  $\beta$  are known, namely from 0.3 to 0.6 mM.

A further check on the possibility that the  $p_j$  are distributed according to a beta density distribution is possible by comparing the changes in the distribution of the  $K_j$  which occur during facilitation, as determined from estimates of the changes in  $\bar{m}$ ,  $p$ , and  $n$  and therefore  $\alpha$  and  $\beta$  with an intracellular electrode (see Eq. 17), with observed changes in  $K_j$  during facilitation as

determined from the estimates of the changes in  $p_j$  with an extracellular electrode (see Eq. 15). The changes in  $\bar{m}$ ,  $p$ , and  $n$  for quantal release by a test impulse following 10 ms after release by a conditioning impulse were determined, allowing the determination of the distribution of  $g(K_j)$  for the conditioning and the test impulse as shown in Fig. 12; these gave good fits to the frequency distribution of the  $K_j$  measured directly with an extracellular electrode at 56 release sites for which  $\bar{m}_e = p_e$ , over the range for the frequency-distribution of the  $K_j$  which could be measured (compare Fig. 12 A with B).

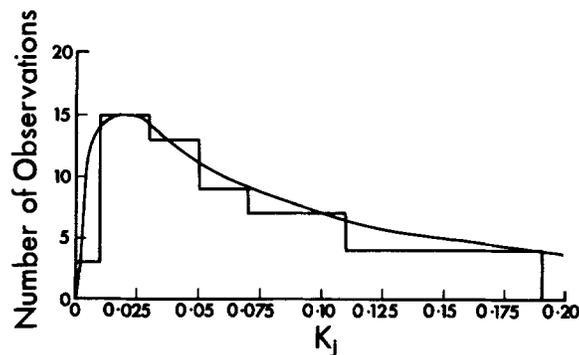


FIGURE 11. Comparison between the frequency distribution of the  $K_j$  (see Eq. 15 in Theory) determined from 70 extracellular recordings at synaptic release sites in which  $n_e = 1.0$  (i.e.,  $\bar{m}_e = p_e = p_j$ ) and the theoretical curve predicted from the probability density function for the  $K_j$ ; see Eq. 17 in Theory for  $g(K_j)$ . The  $K_j$  were determined from extracellular observations on the synapses of 10 different preparations in  $[Ca]_o$  from 0.3 to 0.4 mM. The  $\alpha$  and  $\beta$  parameters for  $g(K_j)$  were determined from the values of  $\bar{m}$  and  $p$  in  $[Ca]_o$  of 0.4 mM given in Fig. 6 A (or Fig. 3 in Bennett et al., 1977), namely 2.6 and 0.40, respectively. The  $g(K_j)$  distribution has been fitted to the frequency distribution by setting the value of  $g(K_j)$  at  $K_j = 0.02$  equal to that for the number of observations in the 0.01–0.03 bin; the remaining bin heights are then reasonably predicted by the  $g(K_j)$  curve. If the  $\alpha$  and  $\beta$  parameters in  $g(K_j)$  are determined from the values of  $\bar{m}$  and  $p$  in  $[Ca]_o$  of 0.3 or 0.6 mM (given in Fig. 6 A of the present work and in Figs. 3 and 4 of Bennett et al., 1977), namely 0.8 and 0.28 or 7.3 and 0.5, respectively, there was less than  $\pm 15\%$  change in the predicted  $g(K_j)$  curve shown. The  $p_j$  were determined from estimates made during at least 100 impulses at 0.5 Hz.

## DISCUSSION

### *Nonuniformity in the Probability of Secretion of a Quantum at Different Release Sites*

The nonuniformity in the probability of secretion of a quantum at different release sites provides an explanation for why binomial rather than Poisson statistics describe the evoked secretion of quanta from motor nerve terminals in low  $[Ca]_o$  (Bennett and Florin, 1974; Bennett et al., 1975, 1977): the

unexpectedly small variance of the evoked release ( $\bar{m}$ ) in low  $[Ca]_o$  occurs because relatively few release sites have sufficiently high probability for the secretion of a quantum ( $p_j$ ), there being a beta distribution of the  $p_j$  over the nerve terminal release sites (see Theory). In order to obtain some idea of the

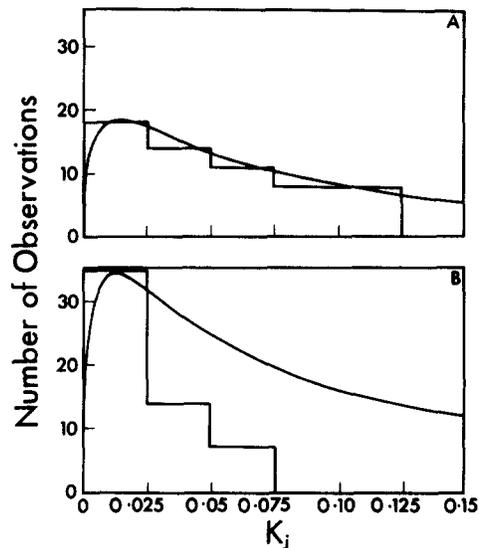


FIGURE 12. Changes in the distribution of the  $K_j$  (see Eq. 15 in Theory) during facilitation of quantal secretion by a test impulse (B) following 10 ms after a conditioning impulse (A). The frequency distribution of the  $K_j$  for the test and conditioning secretion were determined from 56 extracellular recordings at synaptic release sites for which  $n_e = 1.0$  (i.e.,  $\bar{m}_e = p_e = p_j$ ) and the theoretical curves predicted from the probability-density function for the  $K_j$  (see Eq. 17 in Theory). The  $K_j$  were determined from extracellular observations on the synapses in eight different preparations in  $[Ca]_o$  from 0.3 to 0.4 mM. The  $\alpha$  and  $\beta$  parameters for  $g(K_j)$  were determined from the values of  $\bar{m}$  and  $p$  in  $[Ca]_o$  of 0.4 mM for the test and conditioning impulses given in Fig. 2 of Bennett and Fisher (1977), namely 2.6 and 0.40 as well as 5.12 and 0.40, respectively. (A) The  $g(K_j)$  density-distribution has been fitted to the frequency distribution by setting the value of  $g(K_j)$  at  $K_j = 0.0125$  equal to that for the number of observations in the 0–0.025 bin; the remaining bin heights are then reasonably predicted by the  $g(K_j)$  curve. (B) The predictions of the  $g(K_j)$  curve are determined by the test values of  $\alpha$  and  $\beta$  as well as by the same scaling factor used in (A) to fit that  $g(K_j)$  curve. The  $p_j$  were determined for each extracellular recording from estimates made during at least 100 conditioning-test pairs. Note that the size of the 0–0.025 bin in (B) is well predicted by the theoretical curve, with other bins falling below the predicted level because of the technical difficulty of obtaining the tail of the  $g(K_j)$  distribution in A, mentioned in the text.

extent of this nonuniformity in the probability of quantal secretion in a given  $[Ca]_o$ , it is necessary to know the proportion of a nerve terminal's release sites from which an external electrode records quantal secretion for comparison with the proportion  $\bar{m}_e/\bar{m}$ . A number of estimates of the length of motor nerve

terminal over which the secretion of quanta can be recorded with an extracellular electrode have been made using two extracellular electrodes to simultaneously record spontaneous potentials (del Castillo and Katz, 1956; Katz and Miledi, 1965 *a*; Wernig, 1976) giving an estimate of 15  $\mu\text{m}$  for this distance on either side of the recording electrode, that is, a total terminal length of about 30  $\mu\text{m}$ . The total length of the motor nerve terminal at amphibian motor endplates ranges between about 170 and 2,000  $\mu\text{m}$ , depending on muscle fiber diameter, with a mean size of about 1,000  $\mu\text{m}$  (see Fig. 2 in Kuno et al., 1971; Bennett and Pettigrew, 1975). As in the present study, the endplates on the larger diameter muscle fibers were selected for study, and the length of the motor nerve terminal is proportional to the muscle fiber diameter (Kuno et al., 1971), it is likely that most of the synapses studied had a total motor nerve terminal length of at least 1,000  $\mu\text{m}$ . In this case the external electrode records from about 0.03 of the terminal length, so that the ratio  $\bar{m}_e/\bar{m}$  should be about 0.03 if all release sites had the same probability of secretion of a quantum, and not reach values like 0.30 found in the present work.

The question arises as to whether in the present experiments recordings were made simultaneously from release sites distributed across the transverse plane of the muscle fiber (that is, from different branches of the endplate, see Fig. 1 in Kuno et al., 1971), as well as from release sites distributed along the axis of the muscle fiber (i.e., along the length of the motor nerve terminal branch from the point of recording). If two external electrodes record both the triphasic signs of the presynaptic action potential as well as endplate currents of the same amplitude, that is, they are located at the same site, and if one of these is moved transversely across the surface of the muscle fiber, then this electrode fails to record the presynaptic action potential at about 5  $\mu\text{m}$  separations; at about 10  $\mu\text{m}$  separation the electrode fails to record the endplate currents recorded by the fixed electrode (Katz and Miledi, 1965 *a*; Bennett and Lavidis<sup>1</sup>). The extracellular field of the presynaptic action potential is therefore more confined than that of the synaptic potential, and this probably explains why the former is more difficult to detect than the latter. As branches of the motor nerve terminal running parallel to each other and to the long axis of the muscle fiber are displaced from each other by distances of the order of 10  $\mu\text{m}$  (Kuno et al., 1971), simultaneous recording from release sites on two adjacent branches was avoided in the present work by always positioning the electrode so as to obtain a maximum size triphasic presynaptic potential (see Methods and Fig. 1). In this way the electrode is positioned immediately next to a terminal branch.

Del Castillo and Katz (1956) observed the ratio  $\bar{m}_e/\bar{m}$  to vary between 0.01 and 0.1 in five separate determinations of the ratio at the frog neuromuscular junction in a  $[\text{Ca}]_o$  of 0.9 mM and a  $[\text{Mg}]_o$  of 4 mM. According to Eq. 12 in Bennett et al. (1977), the value of  $\bar{m}$  in these ionic concentrations is equivalent to that in a  $[\text{Ca}]_o$  of about 0.5 mM and a  $[\text{Mg}]_o$  of 1.2 mM. The distribution of the probability of secretion at release sites ( $p_j$ ) for these ion concentrations

<sup>1</sup> Bennett, M. R., and N. A. Lavidis. Unpublished observations.

is intermediate between the 0.4 and 0.6 mM curves given in Fig. 10. According to this distribution, if a terminal possesses  $N$  release sites, then only eight of them will have a  $p_j > 0.1$ . If the external electrode is positioned immediately next to a terminal branch, then according to the argument above, it will record secretion from about  $\frac{1}{30}$  of a 1,000- $\mu\text{m}$ -long terminal; if the  $p_j$  values are distributed at random along the length of the terminal, the electrode will then have only about a one in four chance of recording a  $p_j > 0.1$  in a  $[\text{Ca}]_o$  of 0.9 mM and a  $[\text{Mg}]_o$  of 4 mM. This may explain why del Castillo and Katz (1956) failed to observe any  $p_j > 0.1$  in their five external recordings.

*Effect of  $[\text{Ca}]_o$  on the Probability of Secretion of a Quantum at Release Sites*

The quantitative description of the increase in the probability of secretion at release sites ( $p_j$ ) with  $[\text{Ca}]_o$  was only possible using an expression which may be interpreted as showing that at least four calcium receptors at a release site must be occupied by calcium ions in order for the secretion of a quantum to occur, and that the binding of a calcium ion to one receptor increases the efficacy of binding of calcium ions to the remaining receptors in a cooperative manner. In this case the value of the power relationship between  $p_j$  and  $[\text{Ca}]_o$  is determined by a combination of the number of receptor sites on the molecule which binds the  $\text{Ca}^{++}$  and the strength of the cooperative interaction between the receptor sites (see Koshland, 1970). However, the average quantal content of the EPP ( $\bar{m}$ ) measured with an intracellular electrode increases with  $[\text{Ca}]_o$  at the amphibian motor endplate in a way that can be quantitatively described in terms of a mechanism in which each of four independent calcium receptors located at a release site must be occupied by a calcium ion before that site can secrete a quantum of transmitter (Jenkinson, 1957; Dodge and Rahamimoff, 1967; Bennett et al., 1975, 1977). This kind of dependence of  $\bar{m}$  (and binomial parameter  $p$ ) on  $[\text{Ca}]_o$  is to be expected (see Theory) if there is a beta density distribution for the  $p_j$  over the nerve terminal release sites, and if the  $p_j$  increases with  $[\text{Ca}]_o$  according to a cooperative scheme. If the present interpretation of the relationship between quantal secretion and  $[\text{Ca}]_o$  at release sites in terms of a cooperative action of  $\text{Ca}^{++}$  in the secretory process is correct, it remains to be determined at what step this cooperative action occurs between the arrival of a nerve impulse and the secretion of a quantum.

The possibility exists that each release site can secrete more than one quantum in response to a nerve impulse. It has been noted before that if  $[\text{Ca}]_o$  is increased to very high levels at either preganglionic or motor nerve terminals so that there is very little further increase in  $\bar{m}$  with a further increase in  $[\text{Ca}]_o$ , then  $\bar{m}$  has a value of the same order as the number of release sites which the nerve terminals possess (see Discussions in Bennett et al., 1976, 1977), where a release site is defined morphologically as in the Introduction; this result suggests that a release site can secrete at most one quantum after the passage of an impulse. However, it is known that if electrotonic pulses of longer duration than the nerve impulse are used to initiate quantal secretion in high  $[\text{Ca}]_o$ , the nerve impulse having been blocked with tetrodotoxin, then the secretion of quanta is so great that there must be more than a single

quantal secretion per release site (Katz and Miledi, 1977). Thus, sufficient time may be available for more than one secretory event at a release site if an electrotonic pulse is made sufficiently long in comparison with the nerve impulse.

The secretion of quanta at preganglionic nerve terminals increases with an increase in  $[Ca]_o$  in such a way that both  $\bar{m}$  and binomial parameter  $p$  increase in low  $[Ca]_o$  with about the first power of  $[Ca]_o$ , while  $\bar{m}$  is about four times larger than  $p$  (see Fig. 1 in Bennett et al., 1976). This is the result to be expected if there is very little difference in the probability of secretion of a quantum between the 10 or so release sites (Elfvin, 1963) which a mammalian preganglionic nerve terminal possesses, in a given  $[Ca]_o$ . If this is the case then binomial parameter  $p$  gives a measure of this probability of release, and as it is linearly related to  $[Ca]_o$  there is no evidence for a cooperative action of  $Ca^{++}$  in the release of quanta from preganglionic release sites.

*Effect of Trains of Impulses on the Probability of Secretion of a Quantum at Release Sites*

The facilitated increase in the probability of secretion of a quantum at release sites ( $p_j$ ) observed during a short high-frequency train of impulses could be attributed simply to changes in  $K_j$  (see Eq. 15), the facilitated change in  $K_j$  being the same at release sites which possessed different values of  $K_j$  for the first impulse in the train; this observation (see Theory and Fig. 12) explains why the facilitation of the entire quantal secretion from a motor nerve terminal ( $\bar{m}$ ) during a short high-frequency train of impulses involves little change in binomial parameter  $p$ ; that is, the variance of the release increases by the same proportional amount as does the mean during facilitation (Bennett et al., 1975; Bennett and Fisher, 1977). This facilitation of transmitter secretion has been interpreted in terms of an hypothesis in which the secretion of quanta is dependent on the formation of Ca receptor complexes at release sites, an accumulation of residual Ca receptor complexes occurring at these sites during trains of impulses (Katz and Miledi, 1968; Bennett and Fisher, 1977). It has recently been claimed that a conditioning impulse can enhance the inward calcium current which accompanies a subsequent test impulse in invertebrate neurons (Heyer and Lux, 1976; see however, Stinnakre, 1977, and Akaike et al., 1977). The increase in light emission from the calcium-activated photoprotein aequorin in these neurones which accompanies a short high-frequency train of impulses (Stinnakre and Tauc, 1973; Stinnakre, 1977) is consistent with these observations on the calcium currents. The decrease in  $K_j$  which occurs during facilitation at all release sites, and the observation that the percentage decrease in  $K_j$  is the same at all sites independent of the initial value of  $K_j$ , may be interpreted in the context of the observations on a facilitating calcium current: release sites have different numbers of potential-dependent calcium channels, this number being a factor determining the  $K_j$  and therefore the  $p_j$  of a release site; facilitation then involves a similar percentage increase in the number of open calcium channels at each release site, and therefore a similar percentage decrease in the  $K_j$  at each site.

It is interesting to note that if  $\bar{m}$  is reduced below 1.0 by using a  $[Ca]_o$  of

0.25 mM, then facilitation is due to an increase in binomial parameter  $p$  (see Fig. 1 in Bennett and Fisher, 1977), in a similar way to the facilitation of  $\bar{m}_e$  being due to an increase in  $p_e$ . This probably occurs because in a  $[Ca]_o$  of 0.25 mM there is generally only one release site which participates in secretion over 100 or so trials (see Figs. 6 and 10), so that the secretion observed with an intracellular electrode is equivalent to that with an extracellular electrode at the release site which is making the main contribution to secretion. It is therefore possible that the clumping of evoked potentials with similar amplitude and the periodic oscillations in secretion observed by Meiri and Rahamimoff (1978) in  $[Ca]_o$  of 0.2 mM are phenomena localized to a single release site, and support the explanation for short intervals separating spontaneous potentials of like amplitude (Bennett and Pettigrew, 1975) as due to a transient increase in the probability of secretion at a single release site which is characterized by spontaneous potentials of a given size.

We are very grateful to Dr. J. Robinson of the Department of Mathematical Statistics for his comments on the Theory and to Mr. J. Freeman for suggestions. Miss J. Stratford gave valuable technical assistance and Prof. D. Read kindly gave us access to his PDP-11 computer. This work was supported by the Australian Research Grants Committee.

Received for publication 30 January 1979.

#### REFERENCES

- AKAIKE, N., K. S. LEE, and A. M. BROWN. 1978. The calcium current of *Helix* neuron. *J. Gen. Physiol.* **71**:509-531.
- BALNAVE, R. J., and P. W. GAGE. 1977. Facilitation of transmitter secretion from toad motor nerve terminals during brief trains of action potentials. *J. Physiol. (Lond.)* **266**:435-452.
- BARRETT, E. F., and C. F. STEVENS. 1972 *a*. The kinetics of transmitter release at the frog neuromuscular junction. *J. Physiol. (Lond.)* **227**:691-708.
- BARRETT, E. F., and C. F. STEVENS. 1972 *b*. Quantal independence and uniformity of presynaptic release kinetics at the frog neuromuscular junction. *J. Physiol. (Lond.)* **227**:665-689.
- BENNETT, M. R., and C. FISHER. 1977. The effect of calcium ions on the binomial parameters that control acetylcholine release during trains of nerve impulses at amphibian neuromuscular synapses. *J. Physiol. (Lond.)* **271**:673-698.
- BENNETT, M. R., C. FISHER, T. FLORIN, M. QUINE, and J. ROBINSON. 1977. The effect of calcium ions and temperature on the binomial parameters that control acetylcholine release by a nerve impulse at amphibian neuromuscular synapses. *J. Physiol. (Lond.)* **271**:641-672.
- BENNETT, M. R., and T. FLORIN. 1974. A statistical analysis of the release of acetylcholine at newly formed synapses in striated muscle. *J. Physiol. (Lond.)* **238**:93-107.
- BENNETT, M. R., T. FLORIN, and R. HALL. 1975. The effect of calcium ions on the binomial statistic parameters that control acetylcholine release at synapses in striated muscle. *J. Physiol. (Lond.)* **247**:429-446.
- BENNETT, M. R., T. FLORIN, and A. PETTIGREW. 1976. The effect of calcium ions on the binomial statistic parameters that control acetylcholine release at preganglionic nerve terminals. *J. Physiol. (Lond.)* **257**:597-620.
- BENNETT, M. R., and A. G. PETTIGREW. 1975. The formation of synapses in amphibian striated muscle during development. *J. Physiol. (Lond.)* **252**:203-239.
- BORNSTEIN, J. C. 1974. Multiquantal release of acetylcholine in mammalian ganglia. *Nature (Lond.)* **248**:529-531.

- COUTEAUX, R. E., and M. PECOT-DECHAVASSINE. 1970. Vésicules synaptiques et poches au niveau des "zones actives" de la jonction neuromusculaire. *C.R. Hebd. Seances Acad. Sci.* **271**:2346-2349.
- DEL CASTILLO, J., and B. KATZ. 1954. Quantal components of the endplate potential. *J. Physiol. (Lond.)*. **124**:560-573.
- DEL CASTILLO, J., and B. KATZ. 1956. Localization of active spots within the neuromuscular junction of the frog. *J. Physiol. (Lond.)*. **132**:630-649.
- DODGE, F. A., and R. RAHAMIMOFF. 1967. Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J. Physiol. (Lond.)*. **193**:419-432.
- DREYER, F., K. M. PEPPER, D. M. AKERT, C. SANDRI, and H. MOORE. 1973. Ultrastructure of the "active zone" in the frog neuromuscular junction. *Brain Res.* **62**:373-380.
- ELFVIN, L. G. 1963. The ultrastructure of the superior cervical ganglion of the cat. II. The structure of the preganglionic end fibres and the synapses as studied by serial sections. *J. Ultrastruct. Res.* **8**:441-476.
- FATT, P., and B. KATZ. 1952. Spontaneous subthreshold activity at motor nerve endings. *J. Physiol. (Lond.)*. **117**:109-128.
- FRANKENHAUSER, B., and A. L. HODGKIN. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (Lond.)*. **137**:218-244.
- HEUSER, J. E., T. S. REESE, and D. M. D. LANDIS. 1974. Functional changes in frog neuromuscular junctions studied with freeze-fracture. *J. Neurocytol.* **3**:109-131.
- HEYER, C. B., and H. D. LUX. 1976. Properties of a facilitating calcium current in pace-maker neurones of the snail, *Helix pomatia*. *J. Physiol. (Lond.)*. **262**:319-348.
- JENKINSON, D. 1957. The nature of the antagonism between calcium and magnesium ions at the neuromuscular junction. *J. Physiol. (Lond.)*. **138**:434-444.
- JOHNSON, E. W., and A. WERNIG. 1971. The binomial nature of transmitter release at the crayfish neuromuscular junction. *J. Physiol. (Lond.)*. **218**:757-767.
- KATZ, B., and R. MILEDI. 1965 a. Propagation of electric activity in motor nerve terminals. *Proc. R. Soc. Lond. B. Biol. Sci.* **161**:453-482.
- KATZ, B., and R. MILEDI. 1965 b. The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. R. Soc. Lond. B. Biol. Sci.* **161**:483-495.
- KATZ, B., and R. MILEDI. 1968. The role of calcium in neuromuscular facilitation. *J. Physiol. (Lond.)*. **195**:481-492.
- KATZ, B., and R. MILEDI. 1970. Further study of the role of calcium in synaptic transmission. *J. Physiol. (Lond.)*. **207**:789-802.
- KATZ, B., and R. MILEDI. 1977. Suppression of transmitter release at the neuromuscular junction. *Proc. R. Soc. Lond. B. Biol. Sci.* **196**:465-469.
- KOSHLAND, D. E., JR. 1970. The molecular basis for enzyme regulation. In *The Enzymes*. P. E. Boyer, editor. Academic Press, Inc., New York. 341-396.
- KUNO, M., S. A. TURKANIS, and J. M. WEAKLY. 1971. Correlation between nerve terminal size and transmitter release at the neuromuscular junction of the frog. *J. Physiol. (Lond.)*. **213**:545-556.
- LÄNNERGRÉN, J., and R. S. SMITH. 1966. Types of muscle fibres in toad skeletal muscles. *Acta Physiol. Scand.* **68**:263-274.
- McMAHAN, U. J., N. L. SPITZER, and K. PEPPER. 1972. Visual identification of nerve terminals in living isolated skeletal muscle. *Proc. R. Soc. Lond. B. Biol. Sci.* **181**:421-430.
- MEIRI, H., and R. RAHAMIMOFF. 1978. Clumping and oscillations in evoked transmitter release at the frog neuromuscular junction. *J. Physiol. (Lond.)*. **278**:513-524.

- MIYAMOTO, M. D. 1975. Binomial analysis of quantal transmitter release at glycerol-treated frog neuromuscular junctions. *J. Physiol. (Lond.)*. **250**:121-142.
- ROBINSON, J. 1976. Estimation of parameters for a model of transmitter release at synapses. *Biometrics*. **32**:61-68.
- STINNAKRE, J. 1977. Calcium movements across synaptic membranes and the release of transmitter. In *Synapses*. G. A. Cottrell and P. N. R. Usherwood, editors. Blackie & Son Ltd., London. 117-136.
- STINNAKRE, J., and L. TAUC. 1973. Calcium influx in active Aplysia neurones detected by injected aequorin. *Nature (Lond.)*. **242**:113-115.
- WERNIG, A. 1975. Estimates of statistical release parameters from crayfish and frog neuromuscular junctions. *J. Physiol. (Lond.)*. **244**:107-221.
- WERNIG, A. 1976. Localization of active sites in the neuromuscular junction of the frog. *Brain Res.* **118**:63-72.
- WERNIG, A., and J. J. CARMODY. 1978. Deviations from simple binomial predictions of transmitter release at frog neuromuscular junctions. *Neurosci. Lett.* **7**:277-281.
- WERNIG, A., and H. STIRNER. 1977. Quantum amplitude distributions point to functional unity of the synaptic "active zone." *Nature (Lond.)*. **269**:820-822.