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CHANGES IN THE STATISTICS OF TRANSMITTER RELEASE DURING FACILITATION

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SUMMARY

1. The statistical nature of transmitter release during facilitation was studied at single synaptic sites by recording extracellular excitatory junctional potentials from the claw opener muscle in crayfish.

2. At low temperatures, single quanta could be counted in the responses to nerve impulses. The distribution of the number of quanta observed (x) was most accurately described by assuming that x is a binomial random variable.

3. A quantitative estimate was made of the effects of errors in counting quanta due to the simultaneous release of quanta and the release of quanta which were not individually detectable above the noise of the recording system. Such errors of observation cannot account for the deviation of quantal release from a Poisson distribution.

4. Facilitated release occurred in the responses to the second of two closely following nerve impulses and in the responses to successive impulses in a tetanus. In both cases, the increase in the average number of quanta released (m) could be attributed entirely to an increase in the probability (p) that available quanta were released.

5. The results can be interpreted most easily in terms of a model in which the maximum number of releasable quanta is limited by a finite number of discrete release sites within recording distance of the microelectrode. In this model, the binomial parameter n is an estimate of the number of these sites, and the statistical parameter p is a compound probability depending on the rate of re-occupying sites after a nerve discharge and the probability that an impulse activates an occupied site.

INTRODUCTION

When a motor neurone is stimulated twice, the post-synaptic muscle response to the second stimulus is often larger than the response to the first stimulus. Furthermore, if a motor neurone is repetitively stimulated,

the successive muscle responses often grow to a level several times that of the initial response. This facilitation of neuromuscular transmission occurs at a variety of excitatory and inhibitory neuromuscular junctions (Eccles, 1964; Atwood & Bittner, 1971), and a similar facilitation of synaptic transmission has been reported for several central neuronal synapses (Eccles, 1964; Kennedy, 1966; Kuno, 1971; Lømo, 1971; Kuno & Weakly, 1972*a*, *b*; Richards, 1972).

The release of transmitter in response to presynaptic nerve impulses has been shown to be quantal at a number of neuromuscular junctions and central synapses (Martin, 1966). When transmission is reduced by high magnesium concentrations, or when recording from single synaptic contacts using focal extracellular electrodes, the amplitudes of end-plate potentials and excitatory post-synaptic potentials are clustered in small integral multiples of the size of spontaneous miniature potentials produced by single quanta of transmitter. The number of times any given number of quanta are released is well described by Poisson's Law. This is expected if the number of quanta released is a random variable, where among a certain number of releasable quanta, n, each has a small and equal probability of release, p, in response to a nerve impulse. Then m, the average number of quanta released, will equal n times p. This model of quantal release was first proposed by del Castillo & Katz (1954*a*) and has been widely adopted as an explanation for the Poisson nature of release.

Recent experiments on crustacean neuromuscular junctions have shown that the release of quanta at single synaptic terminals is usually, but not always, Poisson in nature (Dudel & Kuffler, 1961*a*; Atwood & Johnston, 1968; Atwood & Parnas, 1968; Bittner & Harrison, 1970). By recording extracellularly from single junctional sites at low temperature, single quantal potentials can be discerned and counted directly (Katz & Miledi, 1965*a*, *b*, *c*). Using this method to study transmission at the crayfish claw opener neuromuscular junction, Johnson & Wernig (1971) found that the variance of the number of observed quantal releases is less than the mean, and quantal release can be better described as a binomial process. This is what is expected if the number of quanta available for release is very limited and the probability that each available quantum be released is not too small.

Whenever a statistical analysis of transmission has been applied to a junction which facilitates, facilitation has been shown to be due to an increase in the average number of quanta, m, released presynaptically (del Castillo & Katz, 1954*b*; Liley, 1956; Dudel & Kuffler, 1961*b*; Kuno, 1964; Martin & Pilar, 1964; Bittner & Kennedy, 1970; Kuno & Weakly, 1972*a*, *b*). Now that m can be separated into distinct components n and

p, and these can be separately estimated, it is of interest to know whether presynaptic facilitation consists of an increase in the number of immediately releasable quanta, or an increase in the probability that each such quantum is released, or both.

The present experiments show that the facilitation that occurs following a single nerve spike, as well as the facilitation which accumulates during a tetanus, are due to a specific increase in p. The binomial nature of quantal release is best understood if transmitter release is thought of as occurring from an invariant number of release sites, where the effectiveness of these sites in response to subsequent impulses or their likelihood of being occupied by transmitter is transiently increased following invasion by a nerve spike.

METHODS

All experiments were performed on the dactyl abductor (claw opener) muscle of the cheliped of young crayfish, *Procambarus clarkii*. Crayfish were precooled in an ice bath, and an autotomized claw was clamped in a leucite chamber, dorsal side up. The surface of the opener muscle was exposed by carefully chipping away the overlying exoskeleton of the propodite and removing the hypodermis. The dorsal half of the meropodite exoskeleton was also removed and the two main nerves were exposed and dissected for about 2 cm. Two suction electrodes (Dudel & Kuffler, 1961*a*) were applied to the thin nerve bundle for stimulating and monitoring the activity in the axon of the single excitatory motor neurone to the opener muscle. Stimuli were usually 1 msec shocks of 20–200 μA. The peripheral inhibitor to the opener muscle, as well as excitatory motor neurones to the dactyl adductor (claw closer), are located in the thick nerve bundle, and were silent in these experiments.

The claw was mounted above a glass window in the bottom of the chamber, and viewed with transmitted light. The chamber bottom was made of stainless steel, and connected to Peltier thermoelectric elements, which maintained the temperature of the bath between 2 and 3° C. In order to prevent a large temperature gradient from developing in the bathing solution, O_2 was bubbled in a corner of the chamber, generating weak circulating currents. The temperature was monitored with a small thermistor probe placed near the opener muscle.

The composition of the bathing medium was (mM): NaCl 195, KCl 5·4, $CaCl_2$ 13·5, $MgCl_2$ 2·6, Tris maleate buffer 10, adjusted to pH 7·3.

Intracellular muscle potentials were recorded with glass micro-electrodes with tip diameters less than 1 μm, filled with 3 M-KCl, and having resistances of 5–15 MΩ. Extracellular recordings from synaptic sites were made with glass micro-electrodes having outside tip diameters of about 10 μm, and filled with 2 M-NaCl; their resistances were 1–2 MΩ. The signals from these electrodes were fed into differential cathode followers and d.c. amplifiers, while the suction electrodes were connected to a.c. pre-amplifiers. A calibration pulse was introduced in series with the reference lead for extracellular muscle recordings. All recordings were displayed on an oscilloscope and successive sweeps were photographed on moving film.

In some experiments, the extracellular muscle responses to nerve stimulation were automatically averaged with a signal-averaging computer (Biomac 500). The amplified responses were d.c. coupled to the computer.

A program was written for a digital computer to estimate m, n and p and their standard errors from the observed distributions of numbers of trials (n_x) in which x quanta were released, and to generate the predicted distributions of n_x, assuming x

to be a Poisson or a binomial random variable. A modified χ^2 test for goodness of fit was used to estimate whether there was a significant difference between the observed and the theoretical distributions. The details of this procedure are given by Johnson & Wernig (1971).

To judge whether apparent changes in m, n and p were significant, a one-tailed t test was used, where t was calculated as the difference between the two estimates of the variable divided by the sum of their standard errors.

RESULTS

The statistics of facilitated transmission were studied at single sites of neuromuscular excitation. When an extracellular micro-electrode is critically positioned on the surface of a muscle fibre, rapid negative transient potentials can be recorded in response to motor neurone stimulation (Dudel & Kuffler, 1961*a*). These potentials represent excitatory junctional currents flowing locally into the muscle, and are called extracellularly recorded junctional potentials (e.r.j.p.s) to distinguish them from the usual excitatory junctional potentials (e.j.p.s) recorded intracellularly. The responses to nerve stimulation consist of single or multiple releases of transmitter quanta, or failures to release any transmitter. At very low temperatures (2–3° C), each quantum of a multiple release appears as a separate transient potential, or as a clear inflexion on the rising phase of a compound e.r.j.p.

Over 100 sites in thirty crayfish were studied in this way. For quanta to be discerned clearly and counted accurately, several criteria must be met. (1) The quantal e.r.j.p.s must have fast rise times ($\leqslant$ 1 msec), so that the inflexions due to several quanta in a compound e.r.j.p. can be seen easily. (2) The release of quanta must have a wide latency dispersion, i.e. a highly variable synaptic delay, to reduce the probability that two or more quanta will be released simultaneously and appear as one. (3) The average quantal size must be large (at least three times the recording system noise level of 25 μV), and the amplitude distribution must fall off before the noise level, in order to minimize the number of quanta lost in the noise. (4) The non-specific e.r.j.p.s must not be too large. These are potentials caused by currents flowing to adjacent or remote synaptic sites (Katz & Miledi, 1965*a*). They can be identified by their slow rise times and relative constancy during successive trials. Since these potentials may occasionally be confused with focal quantal releases, sites were selected where this source of interference was negligible. (5) The extracellular nerve terminal potential (e.n.t.p.) must be recordable in order to measure synaptic delay and assure that presynaptic failures of spike invasion do not occur. Only eight sites satisfied all of these criteria, and they were further studied as follows.

In the first type of experiment, the statistics of the responses to paired stimuli were compared. The separation between stimuli was between 30 and 55 msec. In each case, the shortest interval was chosen at which nerve refractoriness did not interfere with the second response, and the second stimulus artifact did not obscure responses to the first stimulus. The paired stimuli were repeated once every 2 or 3 sec. This was the shortest period in which the computer-averaged responses were indis-

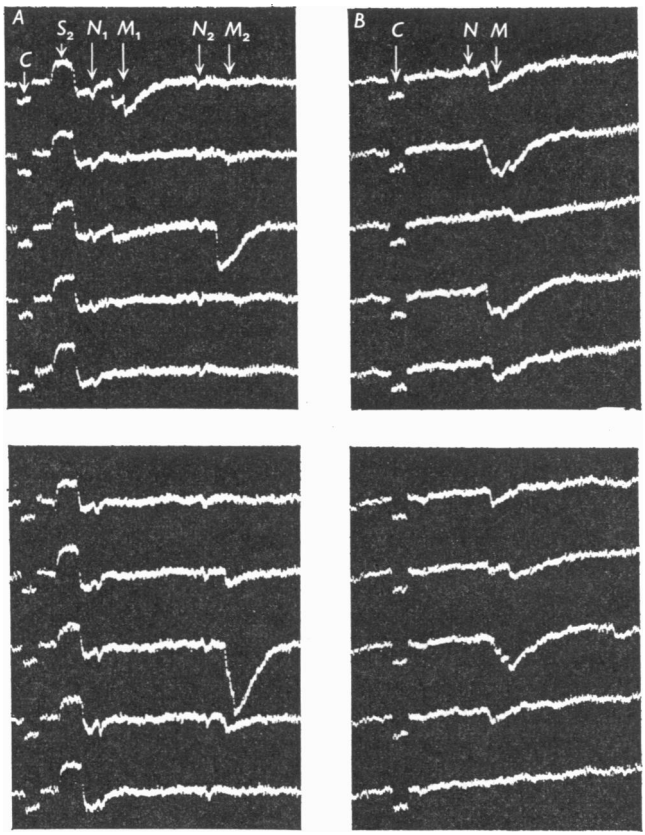


Fig. 1. Facilitated and unfacilitated extracellularly recorded junctional potentials (e.r.j.p.s.) from crayfish claw opener muscle. *A*, responses to two nerve stimuli separated by 40 msec. N_1, N_2, first and second extracellular nerve terminal potentials (e.n.t.p.s.). M_1, M_2, muscle responses to first and second stimuli. S_2, stimulus artifact for second stimulus. Records from site V. The number of quanta released by the first and second stimuli in each record is, from the top: 2,0; 0,0; 1,1; 0,0; 0,0; 0,0; 0,1; 0,3; 0,1; 0,0. *B*, responses to stimuli repeated at 5 Hz. N, e.n.t.p. (barely discernible). *M*, e.r.j.p.s. Records from site IV. The number of quanta observed in each record is: 2; 4; 1; 2; 1; 1; 2; 3; 1; 0. In both *A* and *B*, two groups of five consecutive records are shown. *C*, calibration pulse: 100 μV, 5 msec.

tinguishable from those in which the paired stimuli were repeated once every 10 sec. Thus each first stimulus is to an unfacilitated preparation, while the second stimulus occurs when the facilitation produced by the first nerve impulse has decayed little from its maximum value (unpublished observations). Typical records from such an experiment are presented in Fig. 1*A*.

At several sites, a second experiment was performed. The nerve was stimulated repetitively at 5 or 10 Hz, the highest frequency at which no movement occurred. The average number of quanta released by successive stimuli grew until a steady state was attained. Fig. 1*B* shows typical records obtained by this procedure. The statistics of release during this steady state were compared to the statistics of the first response in the first experiment.

The data from both experiments were analysed in the same way. The number (n_x) of times that x (0, 1, 2, 3, 4 or 5) quanta were released was counted for each x. The average number of quanta released was computed as

$$m = \frac{\sum_{x=0}^{5} n_x x}{N}, \tag{1}$$

where N is the total number of trials. The standard error of m was calculated from (Martin, 1966)

$$\text{S.E.}_m = \sqrt{\frac{\sigma^2}{N}}, \tag{2}$$

where σ^2 is the variance of x, estimated by

$$\sigma^2 = \frac{\sum_{x=0}^{5} n_x (x-m)^2}{N-1}. \tag{3}$$

If x is a Poisson random variable, then the expected number of times that x quanta will be released (P_x) can be predicted from

$$P_x = \frac{N\, e^{-m}\, m^x}{x!}. \tag{4}$$

If x is a binomial random variable, then the probability p that each immediately releasable quantum is released by a presynaptic impulse can be estimated from

$$p = 1 - \frac{\sigma^2}{m}. \tag{5}$$

Finally, n, the number of releasable quanta, may be estimated from

$$n = \frac{m}{p}. \tag{6}$$

Since p is a derived statistic depending on σ^2 and m, its standard error can be obtained (Kendall, 1947) from the standard errors and covariance of the estimates of m and σ^2, assuming a binomial distribution for x (Yule & Kendall, 1950). The result is

$$\text{S.E.}_p = \frac{\sigma^2}{m}\sqrt{\left(\frac{1}{N}\left(2+\frac{\sigma^2}{m^2}+\frac{4p^2-3p}{\sigma^2}\right)\right)}. \tag{7}$$

The standard error of n is similarly derived as

$$\text{S.E.}_n = n\sqrt{\left(\frac{\text{S.E.}_p^2}{p}+\frac{\text{S.E.}_m^2}{m}+\frac{1}{pmN}\left(1-3p+2p^2-\frac{\sigma^4}{m^2}\right)\right)}. \tag{8}$$

Finally, if x is a binomial random variable, then the expected number of times x quanta will be released (B_x) can be predicted from the estimates of the binomial parameters n and p,

$$B_x = N\frac{n!}{(n-x)!\,x!}p^x(1-p)^{n-x}. \tag{9}$$

All these quantities were computed from the data for each type of response at each of the eight sites.

A crucial assumption in the application of any statistical model to the results is that the system is stationary. That is, for either the first or second response of the two-stimulus experiment, or the set of responses during the long tetanus, it is assumed that each response samples from a stochastic process whose parameters are unchanging. One test of this assumption is to divide the data into blocks of 50 or 100 responses and calculate separately m, p and n for each block. Although some sampling scatter about the values of these parameters for the whole population is expected, there should be no time-dependent trends in the successive estimates, and their averages should be similar to the estimated parameters of the entire population. At two sites, these requirements were not met, and they were not considered further. The results of the remaining six sites are collected in Tables 1 and 5. In the paired stimulus experiments on three of these sites (III, IV and V), a short trend was present at the beginning or end of the series. By excluding these responses, stationarity appeared to prevail for the rest of the population of responses. These truncated data were therefore included in the tables.

The distribution of the number of quanta released

Before discussing the parametric changes that occur during facilitation, it is necessary to show that a binomial process must be invoked to account for the results, rather than the much simpler Poisson distribution (Ginsborg, 1970). Table 1 compares the observed distribution of quantal releases for each type of response, at each site, to the distributions predicted by

TABLE 1. Observed and predicted distributions of quantal responses. n_0, n_1,..., n_x, number of trials releasing 0, 1,..., x quanta. N, total number of trials. P, probability that the observed responses could be a sample from the predicted distribution. The predictions are rounded to the nearest integer

Site no.	Responses to	Observation or prediction	n_0	n_1	n_2	n_3	n_4	$n_{\geqslant 5}$	N	P
I	1st stimulus	Observed	394	133	19	2	0	0	548	
		Binomial	394	132	20	2	0	0	548	$>0{\cdot}6$
		Poisson	397	128	21	2	0	0	548	$>0{\cdot}05$
	2nd stimulus	Observed	299	204	43	2	0	0	548	
		Binomial	299	204	43	2	0	0	548	$>0{\cdot}9$
		Poisson	319	172	47	8	1	0	549	$<0{\cdot}005$*
II	1st stimulus	Observed	652	79	5	0	0	0	736	
		Binomial	652	79	5	0	0	0	736	$>0{\cdot}9$
		Poisson	652	79	5	0	0	0	736	$>0{\cdot}9$
	2nd stimulus	Observed	569	155	12	0	0	0	736	
		Binomial	569	155	12	0	0	0	736	$>0{\cdot}9$
		Poisson	577	140	17	1	0	0	735	$>0{\cdot}05$
	10 Hz stimuli	Observed	253	280	59	2	0	0	594	
		Binomial	256	271	68	0	0	0	595	$>0{\cdot}2$
		Poisson	309	205	70	16	3	0	594	$<0{\cdot}0005$*
III	1st stimulus	Observed	124	84	8	2	0	0	218	
		Binomial	126	78	13	0	0	0	217	$>0{\cdot}2$
		Poisson	134	65	16	3	0	0	217	$<0{\cdot}005$*
	2nd stimulus	Observed	82	106	26	4	0	0	218	
		Binomial	85	98	33	2	0	0	218	$<0{\cdot}05$*
		Poisson	100	78	30	8	2	0	218	$<0{\cdot}0005$*
IV	1st stimulus	Observed	353	128	18	1	0	0	500	
		Binomial	353	128	18	1	0	0	500	$>0{\cdot}9$
		Poisson	358	120	20	2	0	0	500	$>0{\cdot}3$
	2nd stimulus	Observed	267	182	47	4	0	0	500	
		Binomial	266	184	45	4	0	0	499	$>0{\cdot}3$
		Poisson	281	162	47	9	1	0	500	$<0{\cdot}005$*
	5 Hz stimuli	Observed	250	321	124	13	2	0	710	
		Binomial	253	313	127	16	0	0	709	$>0{\cdot}5$
		Poisson	298	259	112	32	7	1	709	$<0{\cdot}0005$*
V	1st stimulus	Observed	330	87	12	2	0	0	431	
		Binomial	330	86	13	1	0	0	430	$>0{\cdot}1$
		Poisson	329	89	12	1	0	0	431	$>0{\cdot}2$
	2nd stimulus	Observed	254	144	28	5	0	0	431	
		Binomial	255	141	31	4	0	0	431	$>0{\cdot}3$
		Poisson	262	131	33	5	0	0	431	$>0{\cdot}2$
VI	1st stimulus	Observed	205	50	4	0	0	0	259	
		Binomial	205	50	4	0	0	0	259	$>0{\cdot}9$
		Poisson	207	46	5	0	0	0	258	$>0{\cdot}3$

TABLE 1 (*cont.*)

Site no.	Responses to	Observation or prediction	n_0	n_1	n_2	n_3	n_4	$n_{\geqslant 5}$	N	P
IV	2nd stimulus	Observed	151	96	12	0	0	0	259	
		Binomial	151	95	13	0	0	0	259	$>0{\cdot}5$
		Poisson	163	76	17	3	0	0	259	$<0{\cdot}005$*
	5 Hz stimuli	Observed	175	318	176	42	4	0	715	
		Binomial	180	306	184	43	2	0	715	$>0{\cdot}1$
		Poisson	230	261	148	56	16	4	715	$<0{\cdot}0005$*

* The observed responses are significantly different from this prediction.

assuming either Poisson or binomial statistics. In fourteen of the fifteen sets of responses, a binomial distribution provides a better description of the results. In the 15th case, the predictions were identical. Thus, deviations from a Poisson distribution are of the sort expected for a binomial, with σ^2 less than m. The χ^2 test for goodness of fit indicates that in fourteen of the fifteen response sets, the results were described satisfactorily as a binomial distribution ($p > 0{\cdot}1$). In the 15th case (site III, paired response no. 2), the deviation from a binomial was just beyond the level of acceptability ($p < 0{\cdot}05$); such a deviation is expected by chance in one case in 20. A Poisson distribution could be fitted to only seven of the response sets, and five of these were the unfacilitated responses to the first paired stimulus. As shown below, these correspond to responses in which p, derived assuming binomial statistics, is very small. Under these circumstances, the binomial and Poisson predictions are similar, and the results are described satisfactorily by either, although better by the binomial, model.

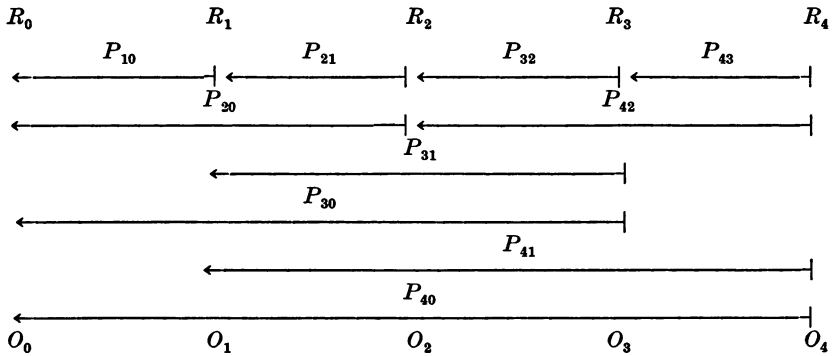
Statistical distortions caused by errors of observation

There are two types of error which could distort the observed number of quanta released on any given trial: (1) some quanta may be so small that they cannot be distinguished from noise level fluctuations; and (2) two or more quanta may occur so nearly simultaneously that they appear as a single quantum.

Consider first the distortions introduced by the quanta lost in the noise. Since no more than four quanta were ever seen in response to a nerve impulse, only multiple releases consisting of four or fewer quanta will be considered. Poisson and binomial predictions both give $n_{\geqslant 5} = 0$ for every site, so it is unlikely that any such high-order releases occurred. The top of Table 2 shows schematically the types of errors in observations that may occur. The simplest is that a single quantum is released, but is so small that it appears as a failure. The probability of this kind of error is represented as P_{10}. There are many other types of error, however. For instance, a triple release may consist of two quanta too small to be distinguished, and appear as a single quantum. The probability of this error is P_{31}. As the figure indicates, there are 10 possible kinds of errors of observations. If the proportion of

quanta which are lost in the noise level, a, is known, then the probability of each kind of error can be calculated. For example, four quanta will appear as two if any two quanta are below noise level. Since there are six combinations of two quanta lost in four, $P_{42} = 6a^2\,(1-a)^2$. The other values of P_{xy} are derived similarly. If the real distribution of quantal releases (R_x) is known, the number of times x quanta will be observed (O_x) can be calculated as R_x plus the sum of the numbers of y quantal releases seen as x releases ($R_y P_{yx}$).

TABLE 2. Effect on the distribution of observed quanta of quanta released whose amplitudes are below the noise level. R_x is the number of times x quanta are actually released. O_x is the number of times x quanta are observed. P_{xy} is the probability that x quanta are actually released and are observed as y quanta. a is the probability that a quantal potential is below the noise level of detection; it is estimated from the distribution of quantal amplitudes. The diagram at the top indicates how real quanta can be missed in the observations. The numerical values are calculated for site IV, stimulated at 5 Hz



$P_{10} = a = 0{\cdot}05000$ $\quad P_{31} = 3a^2(1-a) = 0{\cdot}007125$

$P_{20} = a^2 = 0{\cdot}002500$ $\quad P_{32} = 3a(1-a)^2 = 0{\cdot}1354$

$P_{30} = a^3 = 0{\cdot}0001250$ $\quad P_{41} = 4a^3(1-a) = 0{\cdot}0004750$

$P_{40} = a^4 = 0{\cdot}00006250$ $\quad P_{42} = 6a^2(1-a)^2 = 0{\cdot}01354$

$P_{21} = 2a(1-a) = 0{\cdot}09500$ $\quad P_{43} = 4a(1-a)^3 = 0{\cdot}1715$

$$O_0 = R_4P_{40}+R_3P_{30}+R_2P_{20}+R_1P_{10}+R_0 = 250$$
$$O_1 = R_4P_{41}+R_3P_{31}+R_2P_{21}+R_1(1-P_{10}) = 321$$
$$O_2 = R_4P_{42}+R_3P_{32}+R_2(1-P_{21}-P_{20}) = 124$$
$$O_3 = R_4P_{43}+R_3(1-P_{32}-P_{31}-P_{30}) = 13$$
$$O_4 = R_4(1-P_{43}-P_{42}-P_{41}-P_{40}) = 2$$

$$R_4 = \frac{O_4}{1-P_{43}-P_{42}-P_{41}-P_{40}} = 2{\cdot}4$$

$$R_3 = \frac{O_3-R_4P_{43}}{1-P_{32}-P_{31}-P_{30}} = 14{\cdot}7$$

$$R_2 = \frac{O_2-R_3P_{32}-R_4P_{42}}{1-P_{21}-P_{20}} = 135{\cdot}2$$

$$R_1 = \frac{O_1-R_4P_{41}-R_3P_{31}-R_2P_{21}}{1-P_{10}} = 324{\cdot}3$$

$$R_0 = O_0-R_4P_{40}-R_3P_{30}-R_2P_{20}-R_1P_{10} = 233{\cdot}4$$

minus the sum of x releases seen as z releases ($R_x P_{xz}$). The expressions for O_x are given in Table 2. These equations may be solved to give the most likely actual number of times x quanta were released as functions of the numbers of times x quanta were observed. These expressions for R_x appear at the bottom of Table 2.

The second type of error can be analysed in the same fashion. Table 3 shows that coincidence of quantal release can lead to 6 types of erroneous observation. Suppose the probability density for quantal release as a function of time after the nerve spike invades the motor neurone terminal is known. Let c_i be the probability that the synaptic delay is between t_i and $t_i + \Delta t$. Here the time after terminal invasion is treated as a discrete variable, with values $t_0 = 0$, $t_1 = \Delta t$, $t_2 = 2\Delta t, \ldots$, $t_i = i\Delta t$. Suppose 2 quanta are released on a trial. The probability that they occur in the same time interval Δt following t_i is c_i^2. The probability that they occur in any one time bin is $\sum_i c_i^2$. Now suppose that Δt is the shortest interval in which two quanta can be just barely distinguished; i.e. Δt is the resolution interval for detecting coincidences. Then $\sum_i c_i^2$ is the probability that a double release will appear as a single quantum, P_{21}. The other probabilities of types of misjudgements can also be calculated. Suppose four quanta are actually released, but only two are observed. This can come about in two ways. First, three particular quanta could occur in one time bin, with a probability of $\sum_i c_i^3 (1 - c_i)$. There are four combinations of three quanta out of four, and hence four ways in which this mishap can occur. Secondly, two quanta could be released in one time interval, and two in another time interval, and there are three combinations of four quanta split into two pairs. Thus the probability of this event is

$$3\sum_i \sum_j c_i^2 c_j^2 \ (i \neq j) \ = \ 3[(\sum_i c_i^2)^2 - \sum_i c_i^4] \ = \ 3(P_{21}^2 - P_{41}).$$

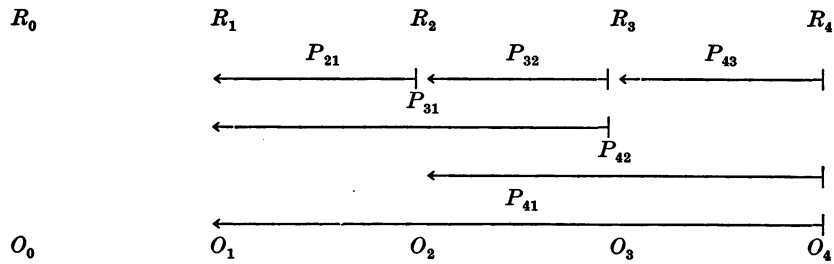
P_{42} is the sum of these compound probabilities. Probabilities of other types of coincidences are given in Table 3. The expected number of observed releases of x quanta if the real numbers are known, and the actual number of releases of x quanta if the observations are given, can be calculated in a way analogous to the noise error problem. These expressions also appear in Table 3.

The results of this error analysis indicate that if the probability that quantal potentials are less than the noise, a, and the probability density of synaptic delays, c_i, are known, then the most likely distribution of quantal releases can be calculated from the observed distribution. Fortunately, a and c_i can be estimated fairly accurately. Fig. 2*C* is a histogram of the amplitudes of the first 100 individual quanta that were observed in the e.r.j.p. responses at site IV stimulated at 5 Hz. So long as the probability of simultaneously released quanta is small, the histogram forms a good estimate of the distribution of actual quantal potential amplitudes. If the left side of the histogram is extrapolated to the abscissa and the area of the resultant hypothetical histogram that falls below the noise level is measured, it may be estimated that perhaps 4 out of 104 quanta were lost in the noise. To be conservative, let $a = 0{\cdot}05$. Then the actual distribution of quanta released at site IV, taking into account the quanta lost in the noise, can be estimated. The numerical results are included in Table 2.

The distribution of the synaptic delays of the same 100 quanta as above is given in Fig. 2*F*. The width of the time bin (Δt) was chosen to be the interval in which two quanta could be just barely distinguished. In practice, this was about one-half the rise time of quantal potentials. The synaptic delay was measured from the negative peak of the e.n.t.p. to the foot of the quantal potential (Katz & Miledi, 1965*b*). So long as the probability that quanta are released simultaneously is small,

Fig. 2*F* provides a good approximation to the actual probability density of synaptic delays. Then c_i is the number of quanta in each time bin divided by 100. Then, the actual distribution of quanta released at site IV, taking into account the quanta missed due to simultaneous release, can be estimated. The results are given in Table 3. It is reassuring to note that P_{21}, the probability that two quanta are released simultaneously, is reasonably small, so that the histograms are indeed good estimates of the distributions of quantal amplitudes and synaptic delays.

TABLE 3. Effect of simultaneously released quanta on the distribution of observed quanta. R_x, O_x, and P_{xy} are defined as in Table 2. c_i is the probability that a quantum is released in the time interval $\{i\Delta t, (i+1)\Delta t\}$ after the presynaptic impulse invades the terminal; it is estimated from the distribution of the synaptic delays of quanta. The numbers are from site IV, stimulated at 5 Hz



$$P_{21} = \sum_i c_i^2 = 0{\cdot}09100 \qquad P_{32} = 3\sum_i c_i^2(1-c_i) = 0{\cdot}2423$$

$$P_{31} = \sum_i c_i^3 = 0{\cdot}01023 \qquad P_{42} = 4\sum_i c_i^3(1-c_i) + 3(P_{21}^2 - P_{41}) = 0{\cdot}0568$$

$$P_{41} = \sum_i c_i^4 = 0{\cdot}001278 \qquad P_{43} = 6(\sum_i c_i^2(1-c_i)^2 - P_{21}^2 + P_{41}) = 0{\cdot}3889$$

$$O_0 = R_0 = 250$$

$$O_1 = R_4P_{41} + R_3P_{31} + R_2P_{21} + R_1 = 321$$

$$O_2 = R_4P_{42} + R_3P_{32} + R_2(1-P_{21}) = 124$$

$$O_3 = R_4P_{43} + R_3(1-P_{32}-P_{31}) = 13$$

$$O_4 = R_4(1-P_{43}-P_{42}-P_{41}) = 2$$

$$R_4 = \frac{O_4}{1-P_{43}-P_{42}-P_{41}} = 3{\cdot}5$$

$$R_3 = \frac{O_3 - R_4P_{43}}{1-P_{32}-P_{31}} = 15{\cdot}6$$

$$R_2 = \frac{O_2 - R_3P_{32} - R_4P_{42}}{1-P_{21}} = 132{\cdot}2$$

$$R_1 = O_1 - R_4P_{41} - R_3P_{31} - R_2P_{21} = 308{\cdot}8$$

$$R_0 = O_0 = 250$$

The purpose of this analysis is to determine whether these sources of error could lead to important differences between the real and observed distributions of quantal release. In particular, it seemed possible that the deviations of the observed distributions from Poisson predictions could be due entirely to errors of observation. To test this, the data of the 5 Hz stimulation of site IV, and the probable actual dis-

tributions of releases, taking the noise or coincidence errors into account, are collected in Table 4. In addition, the effects of noise and coincidence perturbations were applied sequentially to the observations to generate a set of fully corrected most probable actual releases.

The original observations were very well described as a binomial distribution, and very poorly described as Poisson. In all three sets of corrected data, the results are always better described by a binomial than by a Poisson distribution. The effect of the error due to noise-level loss of quanta is quite small. Amplitude histograms of each type of response at all of the sites yielded values of $a \leqslant 0{\cdot}04$. So it may be concluded that in no case did this error influence the form of the distribution of releases significantly.

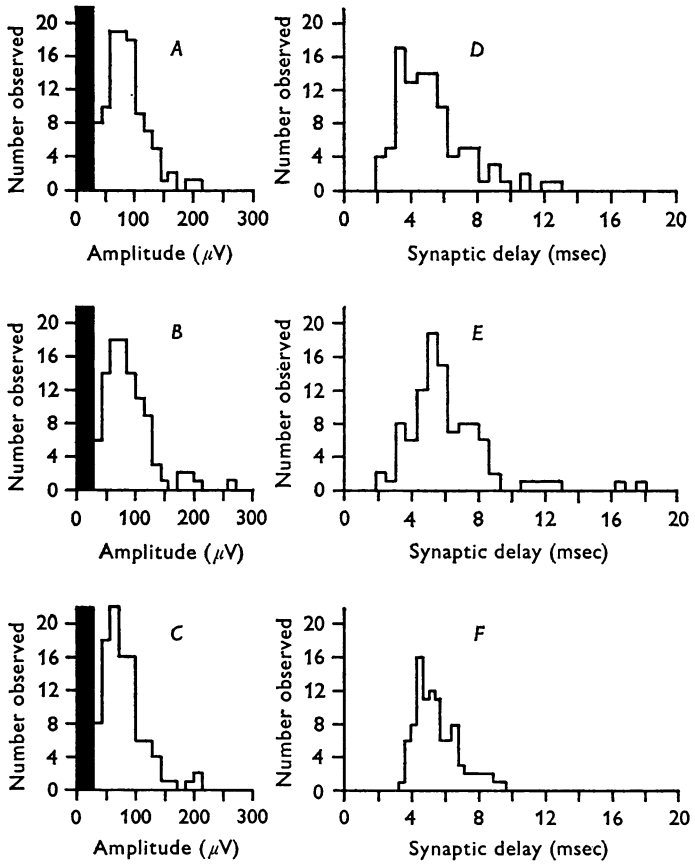


Fig. 2. Histograms of amplitudes of single quantal potentials and their synaptic delays. All data are from site IV. *A–C*, amplitude histograms. The black column represents the noise level of the recording system. *D–E*, histograms of latencies from the negative peak of the e.n.t.p. to the foot of each quantal potential. *A* and *D* include the first 100 quanta released by the first impulse in the two-stimulus experiment, while *B* and *E* display the first 100 quanta released by the second impulse. *C* and *F* are plots of the first 100 quanta released in the 5 Hz stimulation series.

The data corrected for errors due to simultaneous releases are also better described as a binomial distribution, but the corrected data do not fit either binomial or Poisson distributions well enough to meet the criterion of acceptance ($P > 0{\cdot}05$). In the case of the binomial comparison, the discrepancies arise almost entirely from the fact that there are too many 4-quantum releases in the corrected data. This may occur because the conservatively-biased coincidence error analysis is likely to exaggerate the number of multiple-quantum releases that are missed. In the χ^2 test of

TABLE 4. Tests of effects of errors of observation on the distribution of quantal releases. Each test compares a set of releases to the nearest binomial and Poisson distributions. Test I gives the observations of site IV stimulated at 5 Hz. Tests II, III and IV test the data corrected for noise-level error, coincidence errors, or both. In test V, a Poisson distribution of actual releases is assumed, and the expected observations are calculated from the effects of errors. $n_0, n_1, \ldots, n_{\geqslant 5}$, N and P are the same as in Table 1. m, average number of quanta released. p, probability that an available quantum is released

Test no.	Source of n_x	n_0	n_1	n_2	n_3	n_4	$n_{\geqslant 5}$	N	P	m	p
I	Observations	250	321	124	13	2	0	710	—	0·87	0·30
	Binomial prediction	253	313	127	16	0	0	709	>0·5	—	—
	Poisson prediction	298	259	112	32	7	1	709	<0·0005*	—	—
II	Noise-corrected data	233	324	135	15	2	0	709	—	0·91	0·32
	Binomial prediction	236	316	138	19	0	0	709	>0·4	—	—
	Poisson prediction	285	260	119	36	8	1	709	<0·0005*	—	—
III	Coincidence-corrected data	250	309	132	16	3	0	710	—	0·89	0·26
	Binomial prediction	252	306	130	21	1	0	710	<0·05*	—	—
	Poisson prediction	291	260	116	34	8	1	710	<0·0005*	—	—
IV	Fully corrected data	233	312	144	18	4	0	711	—	0·94	0·27
	Binomial prediction	236	308	142	25	1	0	712	<0·005*	—	—
	Poisson prediction	277	261	123	39	9	1	710	<0·0005*	—	—
V	Poisson distribution	298	259	112	33	7	1	710	—	0·87	0·00
	Predicted observations	311	267	103	25	4	0	710	—	0·79	0·08
	Binomial prediction	311	267	104	24	4	0	710	>0·9	—	—
	Poisson prediction	321	255	101	27	5	1	710	>0·4	—	—

* The observed or corrected responses are significantly different from this prediction.

the observed data, the three- and four-quantum releases had to be combined because the binomial prediction for n_4 was $B_4 = 0$, which would entail a term divided by zero in calculating χ^2 for group $x = 4$. If these groups are combined in the tests on the coincidence-corrected and fully-corrected data as well, then both fit a binomial prediction ($P > 0{\cdot}5$ and $P > 0{\cdot}3$), but neither is even remotely similar to the Poisson prediction ($P < 0{\cdot}0005$ for both corrections). Thus it seems likely that the actual distribution of releases is satisfactorily described by binomial statistics.

Table 4 also gives the values of m and p derived from the uncorrected and the corrected observations. No large differences are present, and it appears that the estimates of m and p are not affected by more than 10 % by the errors of observation.

The above analysis indicates that when the data are corrected for the likely errors of observation, the results are still better described by a binomial distribution, although there is a slight reduction in the estimate of p. Another approach to the problem of the effects of these errors is to ask whether they could distort the observations of quanta actually released by a Poisson process so much that the observed releases could no longer be fit by a Poisson distribution. This approach was used in test V of Table 4, in which an exact Poisson distribution of actual quantal releases is assumed, with the same m as site IV at 5 Hz. The effects of errors of noise and coincidence are calculated sequentially to generate the most likely experimental observations considering both sources of error, using the formulae for O_x in Tables 2 and 3. The expected observations may now be compared to the nearest Poisson and binomial distributions, just as if they were experimentally observed data. The result is that observations of a Poisson process of release, perturbed by errors of observations, are still adequately described by Poisson statistics. Although the deviations imposed by the errors are in the direction of a binomial distribution, they are not large enough to generate observations that can only be fit by binomial statistics. The p calculated for the hypothetical observed data is 0·08. Thus errors of observation might lead to a spurious value of p as high as 0·1 for an actual Poisson process with $p = 0$, but not larger. The value of $p = 0{\cdot}3$, obtained from the original observations at site IV at 5 Hz could not arise solely from errors of observation.

A simplified correction for the errors due to coincidence, considering only the larger error probabilities P_{xy} of Table 3, was applied to observations of releases in each response group at each site. The values of c_i (and hence P_{xy}) were estimated from the latency histograms at each site. In every case, the conclusions from the corrected data were similar to those using the uncorrected observations. The corrected data were always best described by a binomial distribution. The binomial prediction usually matched the corrected observations satisfactorily at the 95% confidence level, and the Poisson predictions usually failed this test, except for the unfacilitated responses. The same changes in statistical parameters occurred with a similar degree of confidence, as are reported below for the uncorrected observations. Thus, the results of the exhaustive analysis of the 5 Hz responses from site IV seem to apply generally to all experiments. It is concluded that the errors of observation had no effect on the qualitative results, and only minor effects on the quantitative estimates of parameters.

Changes in the statistics of release during facilitation

Having established that transmitter quanta are actually released at single synaptic recording sites according to binomial statistics, it becomes possible to enquire which parameters of the binomial distribution governing release change when transmission is facilitated. Table 5 gives estimates of m, p and n for each type of response at each site. Whether facilitation after a single impulse or during repetitive stimulation is considered, there are always significant increases in m. These increases in presynaptic release can always be attributed to corresponding increases in p. The increase in p after a single impulse is not usually significant, owing to the large standard errors. When the unfacilitated and the tetanic responses are compared, however, the increase in p is significant. Since p is small at unfacilitated sites, n is essentially indeterminate, and the site behaves in a Poisson fashion. The estimates of n at unfacilitated sites are very crude,

and the analysis is not sensitive enough to reveal changes in n from the rested state, if they occur. The estimates of n during facilitation are more reliable. At sites II, IV and VI, n assumes similar values during facilitation induced by a single previous impulse and by a tetanus. Furthermore, there is no apparent trend for n during facilitation to be larger or smaller than before facilitation. It seems likely, therefore, that n does not change during facilitation.

TABLE 5. Estimates of the statistical parameters of release in different states of facilitation. T, the separation between the first and second stimuli in paired stimulus experiments. N, total number of trials. m, average number of quanta released by each stimulus. p, probability that an available quantum is released. n, number of quanta available for release. $\sigma^2_{i,\,i+1} \pm$ C.L. (95 %), covariance of successive releases $\pm$ the 95 % confidence limit

Site no.	Responses to	T (msec)	N	$m \pm$ S.E.$_m$	$p \pm$ S.E.$_p$	$n \pm$ S.E.$_n$	$\sigma^2_{i,\,i+1} \pm$ C.L. (95 %)
I	Stimulus 1		548	0·323 ± 0·029	0·039 ± 0·088	8·33 ± 18·98	—
	Stimulus 2	40	548	0·540 ± 0·028*	0·208 ± 0·053	2·60 ± 0·66	—
II	Stimulus 1		736	0·121 ± 0·013	0·007 ± 0·116	16·76 ± 270	—
	Stimulus 2	30	736	0·243 ± 0·017*	0·108 ± 0·069	2·25 ± 1·44	—
	10 Hz		594	0·680 ± 0·027*	0·357 ± 0·034*	1·90 ± 0·18	0·02 ± 0·04
III	Stimulus 1		218	0·486 ± 0·042	0·218 ± 0·082	2·23 ± 0·83	—
	Stimulus 2	50	218	0·780 ± 0·049*	0·330 ± 0·061	2·37 ± 0·44	—
IV	Stimulus 1		500	0·334 ± 0·025	0·081 ± 0·083	4·14 ± 4·25	—
	Stimulus 2	55	500	0·576 ± 0·031*	0·165 ± 0·061	3·50 ± 1·29	—
	5 Hz		710	0·868 ± 0·029*	0·298 ± 0·037*	2·91 ± 0·36	−0·01 ± 0·04
V	Stimulus 1		431	0·271 ± 0·026	−0·039 ± 0·125	−7·02 ± 22·8	—
	Stimulus 2	40	431	0·499 ± 0·032*	0·097 ± 0·078	5·16 ± 4·18	—
VI	Stimulus 1		259	0·224 ± 0·028	0·082 ± 0·128	2·72 ± 4·21	—
	Stimulus 2	30	259	0·463 ± 0·036*	0·260 ± 0·067	1·78 ± 0·46	—
	5 Hz		715	1·136 ± 0·033*	0·332 ± 0·034	3·42 ± 0·35	0·04 ± 0·05

* Significant increase ($P < 0·05$) by t test of this parameter compared to its value for the unfacilitated first stimulus.

Bittner (1968), Bittner & Kennedy (1970) and Atwood & Bittner (1971) have shown that the terminals of the crayfish claw opener exciter neurone and crab motor neurones may be differentiated into low and high frequency (LF and HF) facilitating endings. Facilitation at LF endings reaches a maximum at stimulating frequencies below 10 Hz; the HF endings facilitate maximally above this frequency. In the present results, sites I and IV were of the HF type; the rest were LF endings. No qualitative differences in the behaviour of these types of endings during facilitation was observed.

The histograms of quantal amplitudes for the three types of responses (Fig. 2*A*, *B*, *C*) reveal another interesting result. The histograms are

strikingly similar. The average quantal sizes ($\pm$ S.E.) are $85 \pm 4\,\mu$V for the first paired response, $88 \pm 4\,\mu$V for the second, and $78 \pm 4\,\mu$V for responses at 5 Hz. The values are not significantly different. Similar results were obtained for all eight sites. Since the magnitudes of these potentials depend on post-synaptic receptor sensitivity and effectiveness, as well as muscle fibre input resistance, it appears that no post-synaptic factors change during facilitation. Furthermore, the computer-averaged responses always showed a percentage increase in response amplitude similar to the increase in m during facilitation. The phenomenon must be entirely presynaptic in origin; Dudel & Kuffler (1961*b*) reached a similar conclusion.

The latency histograms of Fig. 2*D*, *E*, *F* indicate that the distributions of synaptic delays following unfacilitated and facilitated responses are also remarkably similar. This result was not invariably obtained. At all eight sites from which data were obtained, the synaptic delay histograms for the two paired responses were similar. At sites II, IV and VI, the response latencies to tetanic stimulation were also similar. But at one other site the synaptic delay histogram showed a large number of releases at long latencies during repetitive stimulation. This was apparently due to a residual effect of each impulse in a tetanus, leading to a transient increase in the frequency of randomly released quanta which outlasts the phasic release immediately following a nerve impulse. A similar transient increase in quantal release rates has been reported in the frog (Miledi & Thies, 1971). This effect can be much larger than the slight increase in miniature e.j.p. frequency that persists for several seconds after a long tetanus (Dudel & Kuffler, 1961*b*). Since the phasic and residual releases are not readily distinguished, the statistics of the tetanic responses of this site are not included in this report.

DISCUSSION

The first part of this paper confirmed the finding of Johnson & Wernig (1971) that single excitatory synaptic terminals on to the crayfish claw opener muscle release quanta in response to nerve impulses according to a binomial random process. This study adds a refinement which demonstrates that the apparent deviations from a Poisson random process cannot be attributed solely to errors of observation of single quanta.

Several other investigators have observed deviations in transmitter release from simple Poisson predictions at crustacean neuromuscular junctions (Atwood & Johnston, 1968; Atwood & Parnas, 1968; Bittner & Harrison, 1970) and in cat spinal motor neurones (Kuno & Weakly, 1972*b*). In these studies, the numbers of quanta released were not counted directly. Rather, the distributions of compound e.r.j.p. or synaptic

potential amplitudes were compared to the predicted amplitude distributions. The latter were computed by estimating m from the number of failures or the coefficient of variation of the response, and computing n_x from Poisson's Law, then forming amplitude distributions for single and multiple releases from the amplitude distribution of spontaneous miniature e.r.j.p.s or unit synaptic potentials, and summing the amplitude distributions weighted by n_x. At most non-Poisson sites, the results suggested that there were too many releases of one or two quanta, and too few failures and high-number releases. Such data are similar to those reported here, and show deviations of the sort predicted by binomial statistics. Less complete analyses of the fluctuations in synaptic potentials in a fish (Auerbach & Bennett, 1969) and mammal (Blackman & Purves, 1969) also revealed deviations from a Poisson process, in the direction of a binomial process with a large p.

Christensen & Martin (1970) measured the variance and mean of intracellular end-plate potentials at two calcium concentrations, and calculated p assuming binomial statistics and that only p is sensitive to extracellular calcium. This measure of p is indirect, but their results suggest that transmitter release at the rat diaphragm may also deviate from a Poisson process in the manner of a binomial.

The number of quanta released at a single synaptic site does not always deviate from Poisson statistics, however. Katz & Miledi (1965*c*) found that the distribution of the number of quanta observed individually at low temperature at single extracellular junctional sites of the frog sartorius muscle was quite well described by Poisson statistics. This was true for sites with m between 0·41 and 1·36. They stimulated junctions at 1 Hz, so the preparation was essentially unfacilitated. This result implies that in the frog, as in some sites in the crayfish, p must be very small ($< 0{\cdot}1$) in the unfacilitated state, while n may be between 4 and 13 or even larger.

If it is accepted that a binomial process governs transmitter release, and that facilitated release is due to an increase in p, it becomes important to know what functional or structural meanings can be attached to n and p. In addition, one wonders whether these results allow any choice to be made among the several models that have been proposed for the mechanism of quantal release (Hubbard, 1970).

The statistical implications of several models of transmitter release have been explored by Vere-Jones (1966). One model proposes that releasable quanta correspond to vesicles in the region of the presynaptic terminal membrane, and that whenever a vesicle overcomes an energy barrier separating it from the membrane, its content is released. The depolarization due to a presynaptic impulse is supposed to reduce this barrier and to result in a phasic release of quanta (Bass & Moore, 1966). It might be

thought that if the number of vesicles (n) near the terminal were small, release would be binomial with parameters n and p, where p would depend on the energy barrier encountered by each vesicle. However, the store of releasable vesicles must be replenished from a larger reserve store, and there are reasons for supposing that this replenishment is a Poisson process (Vere-Jones, 1966). Thus release would occur by binomial sampling from a Poisson random variable n. Release would then also be an exact Poisson process (Vere-Jones, 1966). The present results do not support this model.

Another model treated by Vere-Jones (1966) supposes that transmitter quanta are released only at discrete release sites, which can be occupied by at most one quantum. Suppose the probability that a site is occupied is p_1, and the probability that an occupied site releases a quantum in response to a nerve impulse is p_2. Then it can be shown that quanta will be released according to binomial statistics, with steady-state parameters of n and p, where

$$p = \frac{p_1 p_2}{1-(1-p_1)(1-p_2)}. \tag{10}$$

My results are consistent with this model, and suggest that the factor which limits the number of quanta released at a synaptic site is the number of discrete release sites within recording distance from the extracellular micro-electrode. Then n is a measure of the number of such sites. From Table 5, it appears that reliable estimates of n can only be obtained from facilitated responses, where p is large enough to be distinguishable from zero. Using this data, n is usually 2, 3 or 4.

Synaptic vesicles in presynaptic terminals tend to cluster about localized regions of presynaptic membrane thickenings that are electron dense (Birks, Huxley & Katz, 1960; Hubbard, 1970; McMahon, Spitzer & Peper, 1972). A similar distribution of presynaptic vesicles is reported for terminals of the crayfish claw opener motor neurones (Atwood & Morin, 1970). It has often been suggested that these presynaptic loci may correspond to discrete transmitter release sites. At several crustacean neuromuscular junctions, Atwood & Jones (1967), Atwood & Johnston (1968) and Atwood & Morin (1970) have shown that such presynaptic membrane thickenings associated with synaptic vesicles are spaced a few microns apart along a terminal. They estimate that an extracellular electrode would usually record from events occurring at two to four release sites. The similarity of this estimate to n makes the identification of n with the number of discrete release sites at a synaptic spot an attractive possibility.

The results of Katz & Miledi (1965*c*) imply a minimum value of n at single sites in the frog of 4 to 13. If the above interpretation of n is applied, this suggests that an extracellular electrode records from many more

release sites in the frog than in the crayfish. This result is consistent with the findings (Birks *et al.* 1960; McMahon *et al.* 1972) that frog motor neurones contain clusters of vesicles near membrane thickenings in terminal varicosities opposite the junctional folds on the muscle. These presumed release sites are spaced about 0·5–1·5 μm apart along the presynaptic terminal. They are thus more closely spaced than in the crayfish, and a microelectrode recording from a 15–20 μm length of nerve terminal (Katz & Miledi, 1965*a*) would be expected to register seven to twenty release sites.

Unfortunately, a simple physiological significance is not so easily attached to p, or changes in p. Now p is the probability that a release site releases a quantum. According to eqn. (10), p is equally influenced by changes in the effectiveness of an impulse on the probability that an occupied release site will release transmitter (p_2), and changes in the rate of refilling sites (p_1). Vere-Jones (1966) shows that the geometrical decline in transmitter release during a tetanus at synapses showing depression can be predicted by the transient behaviour of his model, assuming p_2 is constant and p_1 (the probability of refilling sites) is reduced. Here a reduction in p_1 corresponds to a progressive depletion in the number of occupied release sites. Similarly, facilitation could be due to an increase in p_1, corresponding to a mobilization of transmitter. This would fit nicely with the nearly geometrical or exponential increase in neuromuscular facilitation to a plateau during a tetanus (Mallart & Martin, 1967; Maeno, 1969) found in the frog. However, it is just as likely that facilitation is caused by a change in the effects of the spike on release sites (p_2). These possibilities cannot be distinguished from the above results.

One approach to this problem is suggested by a further prediction of Vere-Jones (1966). He shows that if transmission is limited by the presence of a finite number of release sites, then the number of quanta released by successive impulses in a steady-state situation should be negatively correlated, with a covariance of

$$\sigma^2_{i,i+1} = -\frac{np_1^2p_2^3(1-p_1)(1-p_2)}{[1-(1-p_1)(1-p_2)]^2}. \tag{11}$$

This covariance was measured (Dixon & Massey, 1969) for the three sites that were stimulated repetitively, and the values are given in Table 5. By converting the covariance to a correlation coefficient, r, 95 % confidence limits could be obtained for r, and these converted to limits on $\sigma^2_{i,i+1}$ (Dixon & Massey, 1969). The result is that in no experiment was $\sigma^2_{i,i+1}$ significantly different from zero, and it is at least $-0{\cdot}05$. Combining eqns. (10) and (11), a quartic in p_2 is obtained. Solving this equation yields the result that either p_1 or p_2 is greater than 0·98, while the other is nearly

equal to the binomial parameter p. Thus, either all sites occupied by transmitter always release a quantum, or all sites are always filled by transmitter before each stimulus. Facilitation then consists of an increase in the unsaturated parameter. There is little evidence to choose between these alternative possibilities. One fact which may be relevant is that this crayfish junction rarely shows any depression, even at room temperature (Bittner, 1968). This suggests that after each impulse, release sites are very rapidly refilled to some constant degree. Thus the evidence tends to be slightly in favour of the interpretation that the probability that sites are filled (p_1) is the saturated parameter, and that facilitation consists of an increase in the effect of an impulse on the probability of response by each release site (p_2).

Although the above model is parsimonious, it does not account completely for all of the results. For example, if each site can release at most one quantum, then one should never observe a response consisting of more than n quanta. However, such responses are occasionally observed. At site II, 10 Hz stimulation produces a binomial distribution of responses with n $(+\text{S.E.}_n) = 1{\cdot}90 \pm 0{\cdot}21$, yet two triple releases were observed. Of course, it is possible that a release site occasionally releases two quanta. There is another explanation, however. All statistical models of transmitter release assume for simplicity that p is a uniform parameter, describing exactly the probability of release (and refilling) at each site. It is more likely that sites are not perfectly uniform. Then p (or p_1 or p_2) is a random variable, and an exact prediction of the expected statistics of release becomes much more complex. It has been shown, however, that a non-zero variance in p across the population of sites tends to reduce the variance in the number of releases (del Castillo & Katz, 1954*a*). This results in an over-estimation of p and an underestimation of n. Thus p may be slightly smaller and n larger than indicated by the above analysis.

One wonders now whether a large variance in p could account entirely for the deviation of release from Poisson statistics. If one considers the effects of a non-uniform p, then eqn. (5) must be modified to

$$1-\frac{\sigma^2}{m} = \bar{p} + \frac{\text{var}\,(p)}{\bar{p}}, \tag{5a}$$

where $\bar{p}$ is the mean and var (p) is the variance of p. Now if $\bar{p}$ is in fact quite small, then var (p) must be nearly $\frac{1}{2}\bar{p}$ to give values of $1-\sigma^2/m$ of 0·3–0·4. That is, at least *some* sites *must* have a large p! Furthermore, if p were highly variable, one would no longer expect to obtain a binomial distribution of release. Finally, it is difficult to imagine how the apparent changes in p occurring during facilitation could be due entirely to increases in the variance, but not the mean, of p. It seems extremely unlikely,

therefore, that the variance of p is large enough to affect the general conclusions of this study.

The estimates of n and p derived statistically should not be confused with nominally similar parameters of release reported in the literature (reviewed by Ginsborg, 1970; Kuno, 1971). These latter estimates are obtained from preparations which show a marked depression of transmission following an impulse. By assuming that impulses always release a constant fraction F of a store of transmitter S, or that some relation exists between F and S, one can estimate F from the decline in transmission to two impulses (Elmqvist & Quastel, 1965; Ginsborg, 1970; Betz, 1970). A similar model has been used to ascribe facilitation in the frog (Maeno, 1969; Maeno & Edwards, 1969) and the rat (Hubbard, 1963) to an increase in the store of available transmitter. In terms of the model used here, such a depletion or mobilization of releasable transmitter would occur because of a change in the probability of refilling sites (p_1) and hence p (Vere-Jones, 1966). There would be no change in n. A change in F would also result in a change in p (either p_1 or p_2). Clearly, there is no direct correspondence between measures of the size of a store of transmitter and the fraction released as derived from depression experiments, and the statistical parameters governing the distribution of releases at a single synaptic site.

Note added in proof

After this paper was submitted, a paper was published by A. Wernig (*J. Physiol.*) (1972), **226**, 751–759) in which some results similar to those presented here were reported.

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