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A RECONSIDERATION OF THE POISSON HYPOTHESIS FOR TRANSMITTER RELEASE AT THE CRAYFISH NEUROMUSCULAR JUNCTION

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SUMMARY

1. Transmitter release from excitor nerve terminals in the crayfish opener muscle was studied with both intra- and extracellular electrodes.

2. The amplitude distribution of quanta recorded extracellularly from a single release site did not fit the compound Poisson hypothesis in two thirds of the data sets.

3. Some data appeared to be non-Poisson because specific quantal multiples were released; the non-Poisson nature of release for other sets could be due to variable invasion of the axonal spike into the terminal region.

4. In some muscle fibres, the number of quanta released at one ending to any given impulse in a stimulus train was correlated with the number of quanta released at other endings on the same fibre. This suggests that such endings may be supplied by nerve twigs arising from a common branch point with a very low safety factor for transmission.

5. No consistent relationship was found between the amplitudes of the intra- and extracellular spontaneous potentials recorded from the same muscle fibre.

INTRODUCTION

It is thought that inactive nerve terminals contain transmitter quanta having a fixed, low, and approximately equal probability of release, and that the arrival of an impulse transiently increases this probability. The experiments to be described provide a direct statistical test of what we

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shall term the 'compound Poisson hypothesis', which comprises the following postulates: (1) nerve impulses cause quanta to be released according to a Poisson process that is invariant with time; (2) the sizes of different quanta contributing to a post-synaptic potential are uncorrelated; (3) the junctional effectiveness of individual quanta is normally distributed with the same mean and standard deviation as that of the spontaneously released quanta; (4) the effects of simultaneously released quanta sum linearly; that is, the sum of their total effect is the sum of the effects of the individual quanta; and (5) the junctional effectiveness of all sites monitored by the recording electrode is approximately equal. Other authors (Boyd & Martin, 1956; del Castillo & Katz, 1954; Dudel & Kuffler, 1961*a*, *b*, *c*; Liley, 1956) have made these assumptions implicitly or explicitly when describing the 'Poisson process' governing transmitter release at neuromuscular junctions.

We conclude that many nerve endings in the opener muscle of the crayfish claw do not release transmitter in accordance with this compound Poisson hypothesis. Atwood & Parnas (1968) have reached a similar conclusion for terminals in other crustacean muscles.

METHODS

Electrophysiological techniques

All experiments were performed on cheliped opener muscles of crayfish (*Procambarus clarkii*). The claw was dissected as previously described (Bittner, 1968*a*). Intracellular junctional potentials (jp's) were recorded from KCl-filled microelectrodes with resistances (measured before insertion) of 3–5 MΩ during constant-frequency nerve stimulation of 0·5–20/sec. The intracellular a.c. noise level of these low-resistance micro-electrodes was about 50 μV peak-to-peak when used in conjunction with a low-pass filter (3 db attenuation of a 1000 c/s signal); they yielded resting potential values similar to those recorded with higher-resistance electrodes (cf. Bittner, 1968*b*). All records were taken from animals with 11–20 mm chelipeds; the range of fibre diameters was thus from 75 to 300 μm (Bittner, 1968*b*). Electrodes filled with 2 M-NaCl and 2–3 MΩ resistance were used to obtain extracellularly recorded junctional potentials (erjp's). If such an electrode was moved more than 10–15 μm, the area of extracellular activity was invariably lost. The noise level was usually 10–20 μV peak-to-peak when such electrodes were used in conjunction with a 1000 c/s low-pass filter. Intra- and extracellular recordings from the same or adjacent cells were first fed through a neutralized-capacitance preamplifier (Bioelectric Instruments, Inc.) before being simultaneously displayed on an oscilloscope and photographed.

Several criteria were used to determine that intra- and extracellular electrodes were recording from the same muscle fibre. First, all recordings were performed under visual observation, and often the tips of both electrodes could be seen entering or indenting a cell with well defined borders. Secondly, at the end of each experiment on a given ending, the extracellular electrode was pushed inside the fibre and a d.c. pulse of 10^{-7} A was passed through this electrode. Such currents produced a square-wave voltage shift of 3–70 mV in the original intracellular electrode when both electrodes were in the same cell (cf. Bittner, 1968*b*). The same current

produced voltage changes of less than 50 μV if the electrodes were in adjacent cells or if one was located in the bath. Thirdly, jp and erjp records were examined for the simultaneous occurrence of spontaneous potentials. The latter criterion was not applicable in a few cases because of a low signal-to-noise ratio of the intracellular trace, or because the extracellular electrode recorded no spontaneous potentials. Using these criteria, both electrodes recorded from the same fibre in thirteen out of the sixteen cells presented in Table 1.

Statistical procedures

It is impossible to make a *direct* test of the hypothesis that the number of transmitter quanta released per stimulus follows Poisson's distribution because the number of quanta released cannot be observed directly. Instead, one makes a measurement of erjp amplitudes. For any integer n, there is a finite probability that a given erjp amplitude consists of n quanta whose individual sizes are independent random variables that sum linearly. For this reason we had to test as a unit the entire compound Poisson hypothesis involving the five independent assumptions mentioned in the Introduction.

From postulate (1) the probability that n quanta are released to a given stimulus is $(\lambda^n e^{-\lambda})/n!$, where λ is the theoretical mean number of quanta released for an infinite stimulus train and that the probability for n quanta released is the same for each impulse. Postulates (2) and (3) imply that quanta released spontaneously are drawn randomly from the same population as the quanta released upon stimulation (cf. del Castillo & Katz, 1954; Boyd & Martin, 1956; Liley, 1956). Spontaneous erjp's have $n = 1$ and are normally distributed with theoretical mean $\bar{E}_q$ and standard deviation (S.D.) σ_q. The junctional effectiveness of n quanta released simultaneously by a nerve impulse is therefore normally distributed with mean $\bar{E}_q$ and S.D. $\sqrt{(n\sigma_q)}$, assuming independence and linear summation. Hence, for each integer n the probability that the functional effectiveness of an erjp consisting of n quanta lies between a and b equals

$$\lambda^n \exp(-\lambda)/n! \int_a^b (2\pi n\sigma_q^2)^{-\frac{1}{2}} \exp[-(\eta - n\bar{E}_q)^2/2n\sigma_q^2]d\eta. \quad (1)$$

The probability that a stimulus causes an evoked erjp falling between a and b is equal to

$$\sum_{n=1}^{\infty} \lambda^n \exp(-\lambda)/n! \int_a^b (2\pi n\sigma_q^2)^{-\frac{1}{2}} \exp[-(\eta - n\bar{E}_q)^2/2n\sigma_q^2]d\eta. \quad (2)$$

To test the compound hypothesis, we generated the theoretical distribution of erjp's that would result if it were true. This distribution had three parameters: the mean ($\bar{E}_q$) and S.D. (σ_q) of the normally distributed 'junctional effectiveness' of quanta and the mean number (λ) of quanta released per stimulus (eqns. 1, 2). The mean ($\bar{E}_s$) and standard deviation (σ_s) of the observed spontaneous erjp amplitudes were taken as estimates of $\bar{E}_q$ and σ_q, respectively; λ was estimated by the ratio of the mean erjp amplitude observed in response to nerve stimulation to the mean spontaneous erjp amplitude $\bar{E}/\bar{E}_s = m$. One could also have used $m = \log_e (N/N_0)$ where N is the total number of stimuli and N_0 is the number of transmission failures (cf. Dudel & Kuffler, 1961*a*). If the compound hypothesis holds, then both are consistent estimates of λ with about the same standard error. The use of $\log_e (N/N_0)$ would not have changed the over-all nature of the results reported in this paper.

The cumulative frequency distribution of spontaneous erjp amplitudes was collected during the period of stimulation and plotted on probit paper as a test for norm-

ality at fifty-three frequency samples from eighteen fibres. Spontaneous erjp's were considered acceptable only if their mean amplitudes were at least 2·5 times the peak-to-peak noise level and if the smallest such potentials were clearly distinguishable from the background noise. In most cases the signal/noise ratio was about 5/1, and in three cases as much as 10/1. In forty-seven of the fifty-three cases, such plots were not significantly different from that expected for a Gaussian (normal) distribution. Three of the six frequency samples which were significantly different were *not* set aside because elimination of the largest 5% of spontaneous erjp amplitude resulted in a normal distribution. This could be done since about 5% of all spontaneous erjp's could be attributed to the release of two single quanta at so short an interval that they appeared as a single erjp on the film (cf. del Castillo & Katz, 1954). The inclusion or rejection of these larger potentials made no consistent difference in the significance of the fit between experimental and observed curves. At least twelve (mean = 19) spontaneous erjp's were collected for all stimulus frequencies shown in Table 1. If necessary, these potentials were collected during several minutes of stimulation by measuring five evoked erjp's before and after a spontaneous erjp. In several cases, spontaneous erjp's from a series of frequencies at the same ending were combined if their individual means were judged not to be significantly different using a t-test. The theoretical curves were practically identical when the individual or combined sets of spontaneous potentials were used. Several frequency samples were tested for stationarity by dividing the data in half and calculating the parameters $\bar{E}_s$, $\bar{E}$, and σ_s for each. No significant differences were noted between the two samples. Those cases in which a terminal failed to release at least one quantum also had no effect on future quantal release (cf. Dudel & Kuffler, 1961*b*) at most terminals.

We also eliminated the possibility of non-linear summation of quanta as a result of jp's moving the post-synaptic membrane nearer to the equilibrium potential of the excitatory transmitter (del Castillo & Katz, 1954). The jp amplitude was greatly reduced (but not reversed) by currents producing 45–50 mV depolarization. The exact value of the excitatory equilibrium potential was not determined because larger currents seemed to damage the cells (Bittner, 1968*b*), although our data would support the estimate of 60 mV made by Takeuchi & Takeuchi (1964). The largest amount of intracellular depolarization recorded during the frequencies tested for any cell was 4 mV. Hence these values for intracellular depolarization could be ignored in calculating the theoretical Poisson distributions as such values must be less than 7% of the equilibrium potential (cf. Martin, 1955). Records could therefore not be taken from some well differentiated cells in the superficial distal region of the muscle at stimulus frequencies greater than 10/sec because temporal summation of their large jp's produced peak depolarizations greater than 4 mV. Frequencies up to 20/sec could be taken from some well differentiated cells in the superficial central region because their small jp's did not produce depolarizations greater than 4 mV (Bittner, 1968*a*).

Using a program for the IBM 1090, written and developed for us by Judy Grindle of the Department of Preventive Medicine at Stanford Medical Center, we calculated $\bar{E}$, $\bar{E}_s$ and σ_s and then computed the probability of release of an erjp whose amplitude lay within the intervals (0, 3 μV), (3 μV, 6 μV), . . ., based upon the compound Poisson hypothesis, continuing until the probability of release of an erjp in any of the remaining intervals was less than 0·005. Then we multiplied these probabilities by the total number of stimuli given to obtain the theoretically expected number of erjp's falling in each of these intervals. If this number was less than five, we combined it with as many subsequent intervals as necessary to form an interval in which the theoretical number of erjp's was at least five. Finally, for each

interval we computed (observed number of erjp's falling in interval − theoretical number falling in interval)^2 − (theoretical number of erjp's in interval), and then totalled those numbers to form a statistic which we tested for significance in a chi-square (χ^2) table for two degrees of freedom less than the number of intervals used. By comparing its value to the critical value at the 0·05 level for a χ^2 statistic with this number of degrees of freedom, one obtains a natural, direct test of the compound Poisson hypothesis. If the calculated χ^2 statistic is greater than the critical value for a χ^2 statistic with the appropriate number of degrees of freedom, then there is substantial disagreement between the theoretical and observed distributions. This is ground for rejecting the compound Poisson hypothesis.

For future reference it should be noted that there is a natural, direct statistical test for agreement between any of the following four pairs of quantities: (N_0/N) and $\exp((-\bar{E}/\bar{E}_s)$ (the observed and theoretical proportion of zero releases, respectively), N_0 and $N\exp(-\bar{E}/\bar{E}_s)$ (the observed and theoretical number of zeros), $\log_e(N/N_0)$ and $(\bar{E}/\bar{E}_s)$ (alternative estimates of lambda, λ), or finally $E_1 = \bar{E}/\log_e(N/N_0)$ and $\bar{E}_s$ (alternative estimates of unit quanta size). The test is to compute:

$$((N_0/N)-\exp(-\bar{E}/\bar{E}_s))^2\,[\mathrm{Var}\,((N_0/N)-\exp(-\bar{E}/\bar{E}_s))]^{-1} = Y^2 \text{ where}$$

$$\mathrm{Var}\,((N_0/N)-\exp(-\bar{E}/\bar{E}_s))$$
$$= ((N_0N-N_0^2)/N^3) + [(\exp(-\bar{E}/\bar{E}_s))^2\,(\bar{E}/\bar{E}_s)^2\,(\mathrm{Var}(\bar{E}))/(\bar{E}^2) + (\mathrm{Var}(\bar{E}_s))/(\bar{E}_s^2)]$$
$$-[(2\exp(-\bar{E}/\bar{E}_s))\,(\bar{E}/\bar{E}_s)\,((N_0N-N_0^2)/(N^3-N_0N^2))]$$

and test Y^2 for significance in a χ^2 table with one degree of freedom. We refer to this test as the 'χ^2 one degree of freedom test' or the 'χ^2 1 d.f. test'. We use it only when the theoretical number of zeros is $\geqslant 5$. The statistical comparison of the entire observed and theoretical curves will be referred to as the 'over-all χ^2 test'.

RESULTS

Types of recorded activity

Figure 1 shows simultaneous extra- (upper beam) and intracellular (lower beam) recordings from a single superficial central fibre in the crayfish opener muscle. The intracellular electrode monitors the effects of transmitter release from many endings at widely distributed locations on the muscle fibre membrane (Fatt & Katz, 1953; Bittner, 1968*a*, *b*). The contribution of any release site to a given jp should be directly proportional to the number of quanta released at that site if quanta from all sites have the same junctional effectiveness because these fibres have a long space constant relative to their length (Dudel & Kuffler, 1961*a*; Bittner, 1968*b*). The extracellular electrode records the post-synaptic effects of transmitter release (erjp's) from a single or small cluster of these release sites because the space constant of decay (5–10 μm) of such potentials is very small (del Castillo & Katz, 1956; Dudel & Kuffler, 1961*a*; Bittner, 1968*a*). The time constants for decay of erjp's (3–8 msec) and jp's (25–40 msec) illustrated here are characteristic for most opener fibres. Figure 1*B* shows a series of spontaneous potentials recorded intra- and extracellularly from the same muscle fibre. About 20 mjp's were recorded

for every spontaneous erjp, a ratio about half that seen at most endings (Dudel & Kuffler, 1961*a*; Bittner, 1968*a*; G. D. Bittner & D. Kennedy, personal observations). Otherwise, this Figure is typical of the records obtained from these endings and appears similar to those published by Dudel & Kuffler (1961*a–c*) on the opener muscle of homologous walking legs.

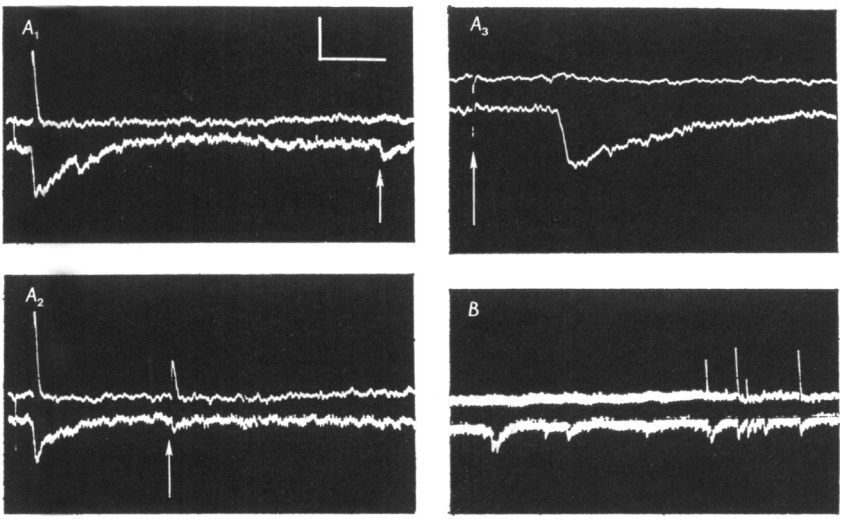

Fig. 1. Upper trace, extracellular record; lower trace, intracellular record from the same superficial central muscle fibre.

A. 0·5 sec stimulation. Evoked erjp's and jp's occur 10 msec after stimulus artifact in A_1 and A_2; A_3 is an example of zero release (arrow points to stimulus artifact); A_1 has an mjp not associated with a spontaneous erjp (arrow); the arrow in A_2 points to a spontaneous erjp and its associated intracellular mjp ('marked' mjp). Calibration lines: 100 μV, 50 msec for A_1 to A_2; 100 μV, 10 msec for A_3.

B. Intracellular mjp's (lower trace) of which four are 'marked' by extracellular spontaneous erjp's. Calibration scale as in A_1: 100 μV, 500 msec.

Activity at single endings

Each ending listed in Table 1 was recorded during stimulation of the excitor nerve at two or more of the following frequencies (delivered in random order): 0·5, 1, 3, 5, 10, 15 or 20/sec. Each rate tested for a given terminal will be termed a 'frequency sample' for that terminal. Statistical comparison of the theoretical and observed erjp amplitude distributions yielded an over-all χ^2 value (and hence a χ^2 1 d.f. value) which was not significant at the 0·05 level in sixteen 'frequency samples' from nine different endings. This indicated that the observed distribution did not differ from that predicted by the compound Poisson hypothesis (see Methods). Such endings will be called 'Poisson endings'.

The over-all χ^2 value *was* significant at the 0·05 level for thirty-four frequency samples from fourteen different endings. This indicated a significant difference between the observed distribution and that predicted by the compound Poisson hypothesis. The frequency samples with significant over-all χ^2 values were placed in two different categories (called non-Poisson A or B endings) for ease of comparison with the results of previous studies (del Castillo & Katz, 1954; Liley, 1956; Boyd & Martin, 1956;

TABLE 1. Classification of endings

Ending no.	No. of Poisson frequency samples	No. of non-Poisson A frequency samples (χ^2 1 d.f., over-all χ^2 tests both significant)	No. of non-Poisson B frequency samples (over-all χ^2 test significant)
1	2	—	—
2	$2+1^{b}$	—	—
3	2	—	1
4	2	2^{cde}	—
5	2	—	$1+1^{b}$
6	2	1	—
7	1^{f}	—	$1^{d'ef}$
8	—	2^{a}	—
9	1	3^{a}	—
10	—	2^{a}	1^{ab}
11	—	1^{a}	1^{ab}
12	—	2^{cde}	2
13	—	—	3^{f}
14	—	—	$1+2^{b}$
15	1	—	$3^{f}+1^{bf}$
16	—	1	$1^{cd'e}+1^{bc}$
Total	16	14	20

[a] demonstrated specific quantal release;
[b] too few zeros to calculate a valid χ^2 1 d.f. statistic (implying a large m);
[c] significant ($P = 0{\cdot}05$) correlation between evoked jp and erjp amplitudes;
[d] significantly greater number of observed than theoretical zeros ($P = 0{\cdot}05$) (d' = greater);
[e] greater variation in observed than theoretical erjp amplitude distribution;
[f] intracellular and extracellular electrodes not recording from the same fibre.

Dudel & Kuffler, 1961*a–c*) and for a comparison of the efficacy of two statistical tests. In the non-Poisson A endings, the observed number of zero quanta releases differed significantly from that predicted by the compound Poisson hypothesis when a χ^2 1 d.f. test was used. Hence, the non-zero potentials will almost always differ from that predicted by the compound Poisson hypothesis. In the non-Poisson B endings, the observed number of zeros did not differ significantly from that predicted by the compound Poisson hypothesis; a significant difference was obtained,

however, using the over-all χ^2 test. The distribution of non-zero releases in both non-Poisson categories were usually Gaussian on probit plots.

Table 1 gives the categories into which the frequency samples from each ending were placed. Since at least five theoretical zeros were con-

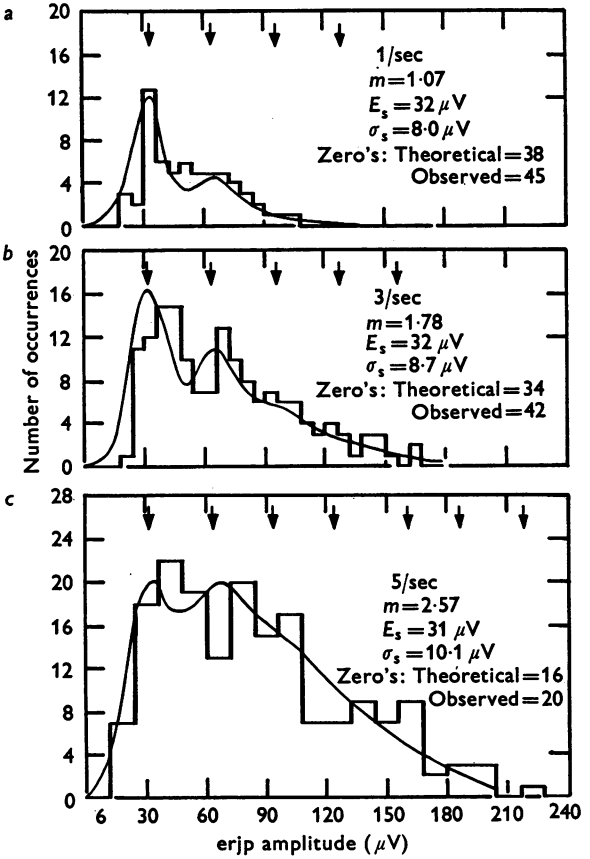

Fig. 2. Erjp amplitude densities from a terminal on a superficial distal fibre (low frequency sensitive fibre; Bittner, 1968*a*). Spontaneous potential collected separately for each frequency of stimulation shown. Bar histogram: observed data; continuous line: theoretical curve (see Methods). Ordinate: number of occurrences; abscissa: erjp amplitude in μV. m calculated from $\bar{E}/\bar{E}_s$ (see Methods). Arrows at top of graph indicate integral multiples of $\bar{E}_s$.

	Over-all χ^2			χ^2 1 d.f.	
Figure	Calculated χ^2 value	$0{\cdot}05\chi^2$ value	Figure	Calculated χ^2 value	$0{\cdot}05\chi^2$ value
2*a*	12	20	2*a*	2·8	3·8
2*b*	25	30	2*b*	1·6	3·8
2*c*	30	38	2*c*	1·0	3·8

sidered necessary for a χ^2 1 d.f. test to be valid (see Methods), those non-Poisson endings with fewer than five theoretical zeros were classified as differing in the over-all χ^2 (non-Poisson B).

Figure 2 shows erjp amplitude histograms from an ending that was Poisson at all three frequency samples tested, i.e. the over-all χ^2 'goodness of fit' tests between the observed and theoretical distribution were not significant at the $P = 0{\cdot}05$ level.

Figure 3 shows an ending which was non-Poisson B at 1/sec and 3/sec stimulation. At 5/sec there were too few theoretical zeros for a χ^2 1 d.f. test to be valid. The distribution of non-zero potentials was approximately Gaussian at 3/sec and 5/sec, but not at 1/sec.

Figure 4 shows an ending which fell into the non-Poisson A category at 3/sec, 5/sec and 10/sec. Few failures of transmission occurred, and the distribution of the observed potentials was approximately Gaussian at 3/sec stimulation, with a mean and standard deviation equal to that of the spontaneous potentials (see insert, Fig. 4*c*). At 5/sec and 10/sec, the mean amplitude of the evoked erjp's was about three times that of the spontaneous potentials, with peaks suggesting the release of specific quantal multiples (2, 3 and 4).

Figure 5 shows an ending which was Poisson at 1/sec and 2/sec, and non-Poisson A at 5/sec and 10/sec. The distribution of erjp amplitudes for this ending was unusual in that it was non-Gaussian at all non-Poisson frequency samples.

The range of '*m*' (average number of quanta released per impulse at any given frequency) for all endings was from 0·037 at 1/sec stimulation in a terminal on a superficial central fibre to 5·4 at 10/sec in a terminal on a superficial distal fibre. The possible variation in *m* can be no smaller than the 150-fold (5·4/0·037) range found to date. The greatest range in *m* at a single ending (Fig. 5) occurred in a terminal on a superficial central fibre. As the frequency was increased from 1 to 10/sec, *m* increased fifteenfold from 0·26 to 4·03. The mean jp amplitude in the same cell increased fivefold from 0·30 mV at 1/sec to 1·6 mV at 10/sec. With a few exceptions, changes in the probability of release at one ending were accompanied by changes in the probability of release at other endings in the vicinity, as measured by changes in jp amplitude in the same or nearby fibres. Similar results have been reported by Dudel & Kuffler (1961*c*) and Bittner (1968*a*).

Relations between the amplitudes of intracellularly and extracellularly recorded jp's

Product moment correlations were calculated between the amplitudes of erjp's and jp's for each frequency sample in order to determine the relationship between the amount of transmitter released at a single

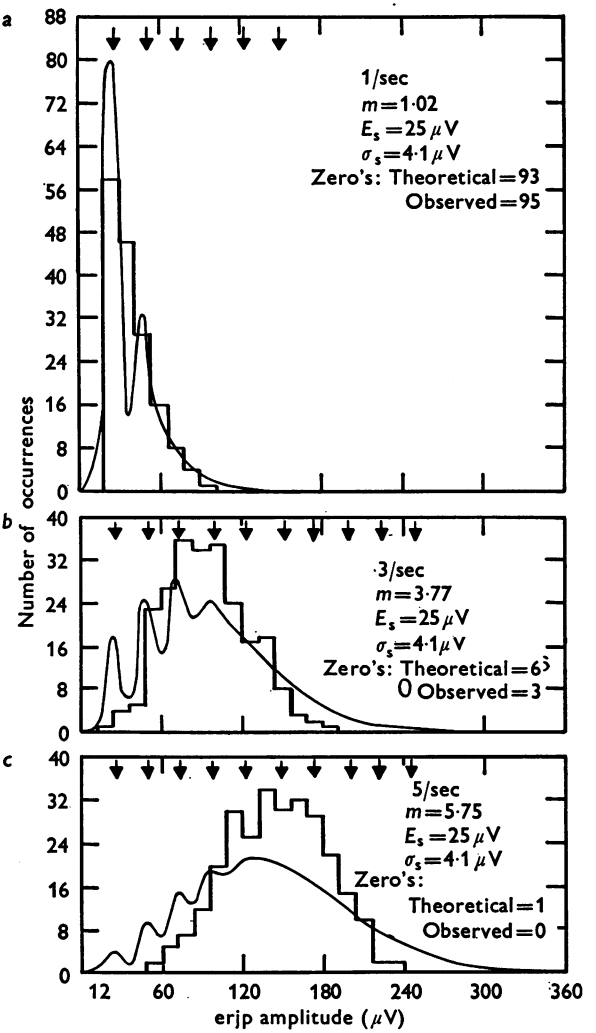

Fig. 3. Erjp amplitude densities from a superficial distal fibre. Ordinate: number of occurrences; abscissa: erjp amplitude (μV). Thirty-seven spontaneous potentials combined to calculated $\bar{E}_s$. Bar histogram: observed data; continuous line: theoretical curve. m calculated from $\bar{E}/\bar{E}_s$. Arrows at top of graph indicate integral multiples of $\bar{E}_s$.

	Over-all χ^2			χ^2 1 d.f.	
Figure	Calculated χ^2 value	0·05 χ^2 value	Figure	Calculated χ^2 value	0·05 χ^2 value
3*a*	59	18	3*a*	0·16	3·8
3*b*	82	31	3*b*	1·86	3·8
3*c*	>100	47	3*c*	Not applicable	

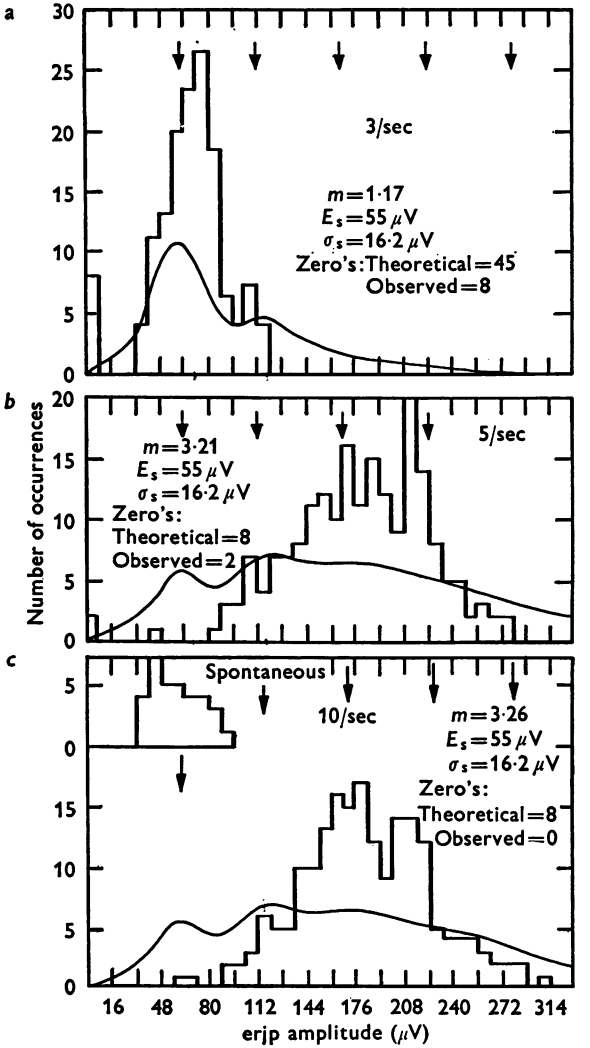

Fig. 4. Erjp amplitude densities from a terminal on a superficial distal fibre with specific quantal multiples. Thirty-three spontaneous potentials combined for all three frequencies and shown in Fig. 4*c*. Ordinate: number of occurrences; abscissa: erjp amplitude in μV. m calculated from $\log_e N_0/N = 2{\cdot}9$ for 4*a*; calculated from $\bar{E}/\bar{E}_s$, $m = 1{\cdot}2$. Arrows at top of graph indicate integral multiples of $\bar{E}_s$.

	Over-all χ^2			χ^2 1 d.f.	
Figure	Calculated χ^2 value	0·05 χ^2 value	Figure	Calculated χ^2 value	0·05 χ^2 value
4*a*	>100	21	4*a*	36	3·8
4*b*	>100	41	4*b*	10	3·8
4*c*	>100	39	4*c*	17	3·8

terminal and that simultaneously released at a group of terminals. The frequency samples were grouped in three different ways for purposes of making this comparison. For the first group, both intra- and extracellular electrodes recorded from the same muscle fibre in which the extracellularly recorded ending contributed only a small fraction ($< 7\%$) to the total amplitude

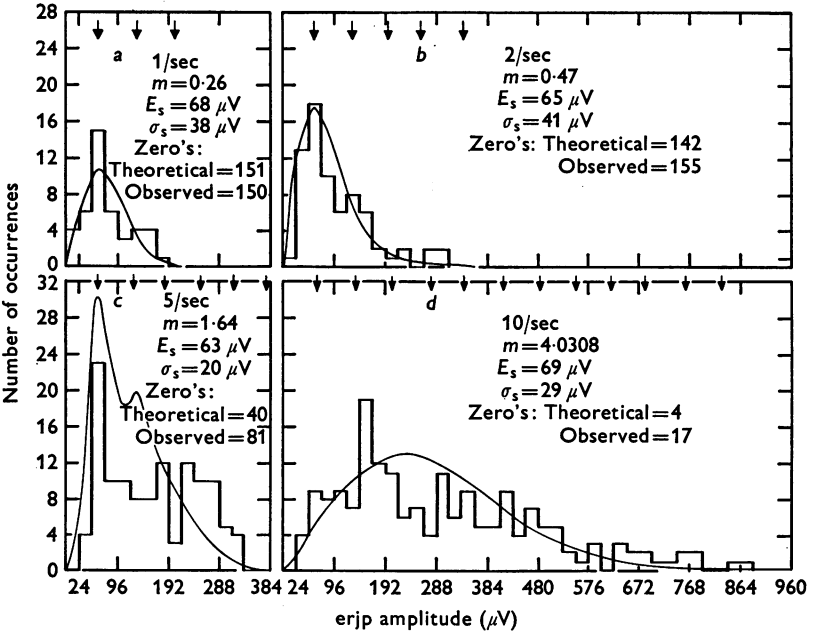

Fig. 5. Erjp amplitude histogram from a superficial central fibre (high frequency sensitive, Bittner, 1968*a*) with a large $\sigma_s/\bar{E}_s$ ratio and in which $\bar{E}_s$ remained constant at all frequencies tested but $\sigma_s/\bar{E}_s$ did not. Ordinate: number of occurrences; abscissa: erjp amplitude in μV. m calculated from $\bar{E}/\bar{E}_s$. Arrows at top of graph indicate integral multiples of $\bar{E}_s$.

	Over-all χ^2			χ^2 d.f. 1	
Figure	Calculated χ^2 value	0·05 χ^2 value	Figure	Calculated χ^2 value	0·05 χ^2 value
5*a*	7·1	14	5*a*	3·5	3·8
5*b*	18	21	5*b*	3·3	3·8
5*c*	>100	33	5*c*	33	3·8
5*d*	96	44	5*d*	12	3·8

at each impulse. This constraint was assured by insisting that the jp amplitudes at each impulse (intracellularly recorded) were at least fifteen times greater than the amplitudes of the average 'marked' spontaneous potentials (see Fig. $1A_2$, $1B$ for examples of 'marked mjp's') multiplied by the average number of quanta released per impulse. If this constraint is maintained, then any correlations observed should be due to interaction

between this 'marked' terminal and other terminals on the same fibre, and not due to an inordinate contribution from the single terminal being monitored.

Five frequency samples so tested at three different endings gave product moment correlations that were significant ($P \leqslant 0{\cdot}05$), while seventeen frequency samples from seven endings yielded no significant correlations. In the former group, one superficial distal (SD) ending yielded significant

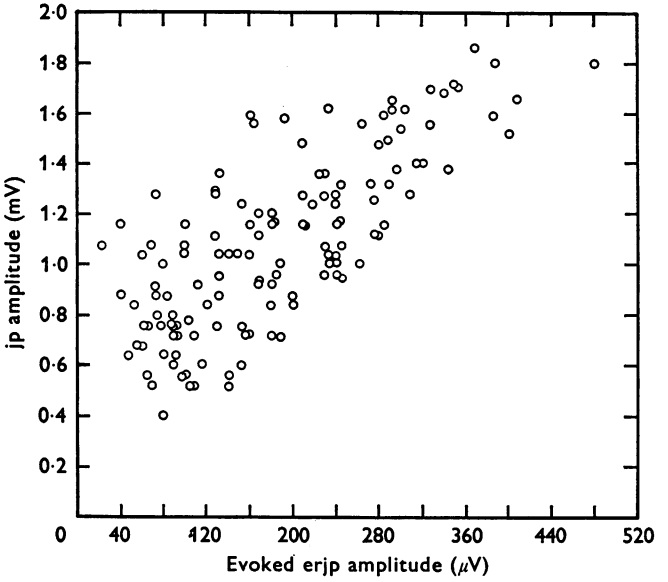

Fig. 6. Scattergram of erjp and jp amplitudes in a superficial distal fibre; stimulation frequency of 0·5 sec. Ordinate: jp amplitude (mV); abscissa: erjp amplitude (μV). Average 'marked' mjp amplitude = 30 μV. Mean jp amplitude = 1·2 mV. m = 1·3 for evoked erjp's. jp amplitude/(marked mjp amplitude χm) = 1200 μV/(30 μV χ1·3) $\cong$ 30.

correlations at the 0·001 level at both 0·5/sec and 1/sec stimulation. Figure 6 is a scattergram of amplitudes of corresponding jp's and erjp's recorded from this ending at a stimulus frequency of 0·5/sec. A second SD ending yielded no significant correlation (P = 0·13 and 0·09) at 0·5/sec and 1/sec respectively, and significant correlations at 5/sec and 10/sec (P = 0·04 and 0·01). A third ending (that shown in Fig. 5*d*) yielded a significant correlation at the 0·01 level at 10/sec stimulation. This same test was also applied to nine frequency samples (labelled 'f' in Table 1) from three cells in which the intra- and extracellular electrodes recorded from adjacent fibres according to criteria previously described (Methods). Of necessity, this calculation used mjp amplitudes rather than marked mjp amplitudes.

None of these frequency samples showed any correlation between jp and erjp amplitudes.

The conditions that jp amplitude at each impulse need be equal or greater than fifteen times mjp amplitude times m for that ending was not met for nineteen other frequency samples from ten cells in which both electrodes recorded from the same muscle fibre. Therefore, a second test was devised for this data group in which each erjp amplitude was correlated with each jp amplitude minus the estimated number of quanta released at that impulse times the 'marked' mjp amplitude for that ending. The correlation coefficients were approximately the same when this second test was run on the first group of twenty-two frequency samples described above; a significantly positive correlation coefficient using the new test was obtained from this group of nineteen samples only from the data plotted in Fig. 5*c* ($P = 0{\cdot}05$). All six of the samples showing a significant correlation ($P \leqslant 0{\cdot}05$) are labelled 'c' in Table 1.

Relationship between spontaneous erjp's and marked mjp's

Figure 7 demonstrates a lack of a consistent relationship between spontaneous erjp amplitudes and corresponding marked mjp amplitudes when both are simultaneously recorded from the same muscle fibre. Figure 8*a* shows the amplitude histogram for the spontaneous erjp's at the ending shown in Fig. 7, and Fig. 8*c* gives the amplitude histogram for the 'marked' intracellular mjp's that corresponded to spontaneous erjp's from that particular ending. Figure 8*b* is an amplitude histogram for spontaneous jp's which did *not* correspond to an event at the extracellularly recorded ending (see Fig. 1A_1, 1B). The amplitude ranges of corresponding intracellular (Fig. 8*c*) and extracellular (Fig. 8*a*) spontaneous potentials are about equal. The amplitude histogram for the intracellular potentials produced by the extracellularly recorded ending (Fig. 8*c*) closely resembles that for the spontaneous jp's from other endings (Fig. 8*a*). The amplitude range of intracellularly recorded jp's from the 'marked' ending is only slightly less than that of jp's from all other endings. Three other experiments produced results qualitatively similar to those shown in Figs. 7 and 8.

DISCUSSION

Comparison with previously published data

Several levels of analysis, of increasing rigour, can be used to compare observed erjp histograms with theoretical curves generated from the assumptions of the compound Poisson hypothesis. The first level is a tabulation of $E_1 = \bar{E} \log_e N/N_0$ and of $\bar{E}_s$ for each frequency sample, or, equivalently, a comparison of the number of theoretical and observed

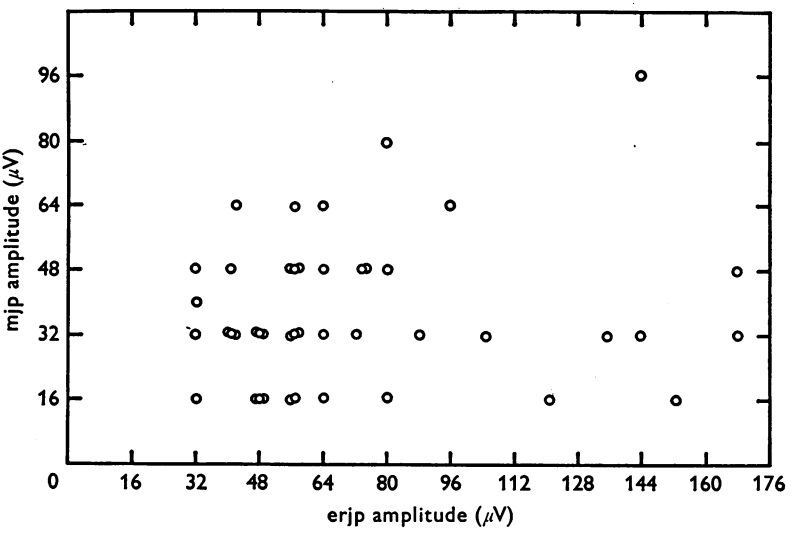

Fig. 7. Scattergram of spontaneous erjp (μV, abscissa) and marked mjp amplitudes (μV, ordinate) recorded from a single muscle fibre.

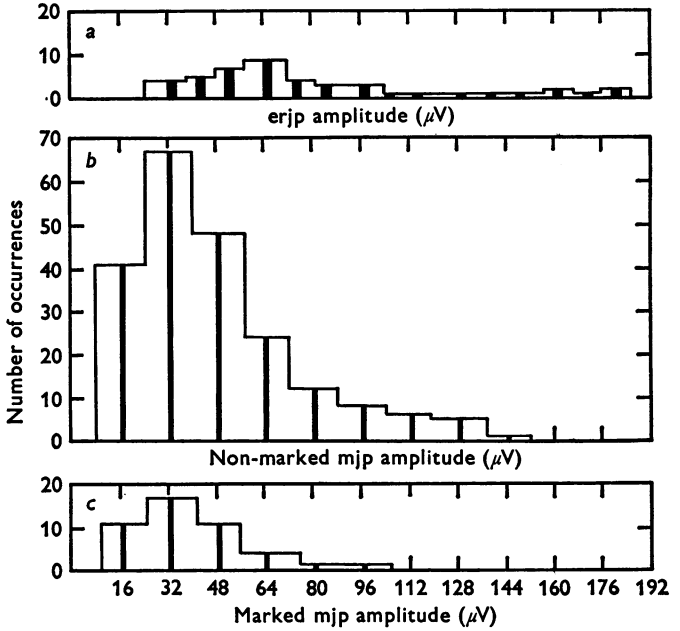

Fig. 8. Amplitude histogram taken from the same cell shown in Fig. 7. Fig. 8*a* shows spontaneous erjp amplitudes (μV), 8*b* shows non-marked mjp amplitudes and 8*c* marked mjp amplitudes (μV). Four non-marked mjp's were taken for every marked mjp.

zeros (see Methods). If an arbitrary 25% discrepancy between these values is tolerated, then 82% of our frequency samples, 95% of Dudel & Kuffler's (1961 *a–c*), and all samples from vertebrate neuromuscular endings (del Castillo & Katz, 1954, 1956; Boyd & Martin, 1956; Liley, 1956) would support the compound hypothesis. A somewhat more stringent level of analysis uses the χ^2 1 d.f. test ($P = 0{\cdot}05$) to compare the observed and theoretical number of zeros. 72% (36/50) of our frequency samples, all of the five complete frequency samples provided by Dudel & Kuffler (1961 *a–c*), and about 95% of those provided for vertebrate neuromuscular endings would agree with the compound hypothesis on the basis of this test. If, however, we employ our most demanding statistical level, the over-all χ^2 test, then only 32% (16/50) of our frequency samples, two of five complete samples from homologous endings in crayfish walking legs published by Dudel & Kuffler, and two of three complete data sets from analogous vertebrate endings agree with the compound Poisson hypothesis. (The frequency sample presented by Liley, Fig. 3, 1956, is significant at $P = 0{\cdot}001$ if $m = \bar{E}/\bar{E}_s$, and at $P = 0{\cdot}05$ if $m = \log_e N/N_0$.)

Dudel & Kuffler (1961 *a–c*) have concluded that nerve terminals in the crayfish opener muscle release transmitter in accordance with the compound Poisson hypothesis on the basis of agreement between E_1 and $\bar{E}_s$ on the first level of analysis.

We conclude that many (34/50 = 68%) such endings do *not* release according to the compound hypothesis on the basis of over-all χ^2 tests. The disagreement between our conclusion and that drawn by Dudel & Kuffler appears to result from our statistical treatment rather than from actual differences in observed erjp amplitude distributions as three of their five complete frequency samples also yield curves that differ from the predicted one when our most stringent statistical test is applied. For example, the over-all χ^2 for their Fig. 8 (1961 *a*) is significant at the $P = 0{\cdot}0001$ level.

Atwood & Parnas (1968) have reported a tendency for other crustacean nerve endings with average quantal releases to each nerve impulse equal or greater than two ($m \geqslant 2$; we define m as $\bar{E}/\bar{E}_s$ unless otherwise noted) to release in a fashion inconsistent with the compound hypothesis. This tendency is also seen at terminals in the crayfish opener muscle and cannot result from non-linear summation of quanta due to excessive depolarization during nerve stimulation (see Methods). Most (23/34) examples of release processes which did not fit the Poisson hypothesis came from frequency samples at which m was $\geqslant 2$. Ten out of eleven frequencies with $m \geqslant 3$ had significant χ^2 values, compared with only five of thirteen frequencies with $m \leqslant 1$. Consequently, one finds fewer good fits to the compound hypothesis in data taken from terminals in the superficial (SD) distal

region of the opener muscle because these terminals tend to release transmitter with a higher probability at low frequencies of stimulation than fibres in the superficial central (SC) regions (Bittner, 1968*a*).

Possible role of limited numbers of immediately releasable quanta

Observed and theoretical erjp amplitude histograms can differ significantly due to violations of any combination of postulates (1) to (5) of the compound hypothesis (in addition to reasons which may not be included in our hypothesis). Atwood & Parnas (1968) and Atwood & Johnston (1968) have described nerve endings in other crustacean muscles whose erjp amplitude distributions appear to be inconsistent with the compound hypothesis. These endings usually have a high probability of release and tend to release in specific quantal multiples (Atwood & Parnas, 1968). Endings of this latter type have been described in the present study (cells 8 to 11, Table 1; Fig. 4), with or without a high probability of release ($m \geqslant 2$). It appears that quanta are not released according to Poisson's law and therefore in these endings postulate (1) of the compound hypothesis does not hold. In Fig. 4*a*, for example, the mean and S.D. of the observed erjp amplitudes corresponding to single quantal releases ($n = 1 \cdot \bar{E}_s$) do not differ significantly from those of the spontaneous potentials, and both are approximately normally distributed. As very few multiple quantal releases ($n \geqslant 2$) are seen, questions of non-independence (postulate 2) and of non-linear summation (postulate 4) of multiple releases cannot be an important factor in the poor fit of the experimental and theoretical curves. In fact, the form of the observed distribution can be explained if postulate (1) alone is violated. The other examples of this phenomenon (cells 8 to 11) were qualitatively very similar in that (1) usually the χ^2 1 d.f. *and* over-all χ^2 were both significant (although the number of observed and theoretical zeros often agreed within 25 % as did $\bar{E}_s$ and E_1); (2) values of m were always $\geqslant 1$, even at frequencies $\leqslant 1$/sec, and increased with increasing frequency, and (3) showed a sudden change in the specific multiples of $\bar{E}_s$ as in Fig. 4*a* and 4*b*.

Two factors could be playing an important role in altering the Poisson nature of release in these endings with specific quantal multiples. First, the ending may contain a limited number of transmitter quanta, each having a very high release probability, the number of quanta available increasing at higher stimulus frequencies. Secondly, the number of release sites could be small and the terminal capable of opening up new release sites at higher frequencies. Any one, or a combination of these two possibilities, would place a 'ceiling' on the number of quanta released by a single nerve impulse (hence, on erjp amplitude) and decrease the expected number of zero releases.

Each of the above mechanisms, by reducing the number of cases with few and/or numerous quanta, would result in a smaller variation in erjp amplitude than would be expected if the release mechanism were Poisson. A smaller variation than predicted was, in fact, found in twenty-eight out of the thirty-four frequency samples in which the compound Poisson hypothesis did not hold. This finding suggests that a similar phenomenon may occur even in many of those samples which were non-Poisson but which demonstrated no specific quantal multiples. The possibility of a limited number of immediately available quanta (or of quantal release sites) would also be in agreement with the observations that (1) non-Poisson release is correlated with high m's ($m \geqslant 2$); (2) the largest m's found in this or other studies on crustacean neuromuscular junctions is about six (Dudel & Kuffler, 1961*a–c*; Dudel, 1963, 1965; Atwood & Johnston, 1968; Atwood & Parnas, 1968; Bittner, 1968*a*; Fig. 4*c*, this paper); and (3) the largest number of quanta released at any impulse is about twelve (Fig. 5*d*) and is usually less than ten even when m equals 4–6.

Possible role of variable invasion of the nerve spike into the terminal region

Figure 6 indicates that there can be a strong statistical relationship between transmitter release at a particular nerve ending (measured by erjp amplitude) and at other endings on the same muscle fibre (measured by jp amplitude). The mechanism underlying this correlation cannot be a premature blockage of the action potential in the main axon or one of its major proximal branches which innervate that muscle fibre. If the action potential were blocked in the main axon, then erjp failures should be associated with jp failures (cf. Krnjević & Miledi, 1959). If one of several main branches were blocked, then erjp failures should be associated with stepwise changes in amplitude between two modal values (cf. Fatt & Katz, 1953). Neither of these phenomena were observed in the six cases of high correlation similar to the one illustrated in Fig. 6, although they have been reported for this motoneurone at higher stimulus frequencies (G. D. Bittner & D. Kennedy, personal observations).

The most likely explanation for the correlation presented in Fig. 6 is that premature blockage and/or a reduction in the actively conducted nerve spike is occurring near a distal branch point of low safety factor which leads to (say) five or more of the approximately fifty terminals known to innervate each opener muscle fibre (Dudel & Kuffler, 1961*a*; G. D. Bittner & D. Kennedy, personal observations). Such a block would reduce the amplitude of the electrotonic potential which reaches these nerve terminals (Dudel, 1963, 1965) and would result in a decreased probability of release for all endings past the branch point. This 'relative' blockage

probably occurs randomly in time since m's calculated for each of these six frequency samples were not significantly different for different halves or quarters of the sample. Plots of the ordered temporal sequences of erjp amplitudes showed no obvious patterns with the exception of the data from the sample with the highest jp–erjp correlation coefficient (Fig. 6, $P = 0{\cdot}001$). For this sample, there was a slight, albeit statistically non-significant ($P = 0{\cdot}18$) tendency for very large or very small erjp's to be followed by erjp's that were larger or smaller than average, respectively. This could be due to the effects of a varying invasion whose facilitatory influence had not completely decayed before the arrival of the next spike.

The effect of a variable depolarization of a set of nerve terminals produced by variable invasion of axonal spikes would cause each of these nerve terminals to have a non-constant λ (hence a non-constant m). If this violation of postulate (1) were the only violation of the compound Poisson hypothesis which occurred during a constant frequency sample, it would result in a greater number of observed than theoretically predicted zeros (particularly if m alternated between a few modal values) and in a greater variation in observed than theoretical erjp amplitudes of size $n \geqslant 1$. Both these phenomena were, in fact, associated with five of the six frequency samples from the three cells which demonstrated a significant correlation or erjp and jp amplitudes (marked *c*, *d*, *e* in Table 1). The remaining such frequency sample (cell no. 16, 1^{bc}, Table 1) had fewer observed than theoretical zeros or variation in erjp amplitudes and showed no specific quantal multiples in the non-Poisson β category. It could, conceivably, result from a combination of these two non-Poisson processes which violate postulate (1) of the compound Poisson hypothesis (limitation of immediately releasable quanta and variable invasion of axonal spikes). The remaining frequency sample with a greater variation in observed than theoretical erjp amplitudes came from a cell which also showed more observed than theoretical zeros, but no correlation between jp and erjp amplitudes. However, if jp–erjp correlations exist only within a single muscle fibre, such a correlation would not have been detected since both electrodes were not recording from the same cell (no. 7, *d'ef* in Table 1).

Possibility of complete blockage of spikes in terminals on SC fibres

Blockage of axonal spikes in terminals on SC fibres could be considered a possibility because of the large number of zero releases observed for these terminals during low frequency stimulation (Fig. 5*a*, *b*). This phenomenon could also be present at higher frequencies in Fig. 5*c*, *d* in which the observed number of zeros is significantly greater than the theoretical ones.

There are three experiments which indicate that complete blockage during zero releases is not occurring in the terminals sampled in Fig. 5 or in other SC fibres. First, there was no less tendency for large releases by the next impulse after a zero release in these SC fibres. Yet there must be a facilitatory effect lasting at least from one impulse to the next since m is increasing with increasing frequency in many of these frequency samples. Secondly, if a pulse is dropped out of such a constant frequency train, the next succeeding pulse has a greatly decreased probability of evoking transmitter release (G. D. Bittner & H. Hegstadt, personal observations). Thirdly, intracellular records taken from such SC fibres at a 10 sec interval between pairs and an intrapair interval of 5–10 msec show that the release to the second member of the pair is greatly enhanced ($\geqslant$ 100 %) even when the first pulse results in no detectable release (G. D. Bittner & H. Hegstadt, personal observations). All these results imply not only that impulses yielding a zero release are not completely blocked from reaching the terminal regions, but also that the amount of invasion is approximately the same at zero and non-zero releases. It would seem that quanta in most of these SC fibres have very low probability of release to low frequency stimulation of the main axon.

Role of lack of consistent relationship between jp and erjp amplitudes

There is no consistent relationship between intra- and extracellular *spontaneous* potentials recorded from the same muscle fibre (Fig. 7). A similar result has been reported for the frog end plate by del Castillo & Katz (1956), who attributed the lack of correlation to the multiplicity of release sites recorded by the extracellular electrode. The large variation (ten- to twentyfold) in amplitude of spontaneous miniature end plate potentials (mepp's) recorded extracellularly was presumed to result from interference from nearby endings. The smaller variation (three- to sixfold) in the amplitude of intracellular mepp's (del Castillo & Katz, 1954, 1956; Boyd & Martin, 1956; Liley, 1956) was held to arise from a combination of variation in quantal effectiveness and the small space constant of the fibre membrane (del Castillo & Katz, 1956). In contrast, the amplitude range for spontaneous extracellular potentials in the opener muscle was usually two- to sixfold (Figs. 4*c*, 8*a*) and the range of intracellular potentials was usually four- to eightfold (Fig. 8*b*). The intracellular electrode was always 0·1–0·5 mm from the extracellular electrode in fibres whose space constant was 2·5–4 mm (Bittner, 1968*b*). Furthermore, the amplitude range for intracellular spontaneous jp's that coincided with erjp's from a particular ending (Fig. 8*c*) was usually about equal to that of intracellular spontaneous jp's from *all* endings (Fig. 8*b*).

If the electrical effectiveness of all quanta released by a given ending is approximately equal, then the amplitude range of intracellular potentials from that ending (as in Fig. 8*c*) should be small, because the membrane space constant is long, and the extracellular electrode used to 'mark' the ending that is producing the intracellular potentials, records from a region of only 10–15 μ. Several possible explanations can be eliminated easily: (1) If four- to sixfold variations in the electrical effectiveness of quanta at a single ending were the *only* agent producing erjp amplitude variation, then the scattergram of Fig. 7 should have shown significant correlations; (2) if unintentional movement of the extracellular electrode were superimposed upon a four- to eightfold variation in quantal effectiveness, then the variation in spontaneous erjp amplitude should have been greater than that for the amplitude of spontaneous intracellular jp's from that ending; (3) if one were recording from a series of release sites, each with a wide range of quantal effectiveness, then the range of spontaneous erjp amplitudes should be greater than that for the spontaneous intracellular potentials corresponding to them. None of these situations hold for the four endings tested in which signal-to-noise ratios were low enough to measure the smallest extracellular and intracellular spontaneous potentials accurately.

One explanation which could fit the observed data is that measurements of the extracellular current density due to a given spontaneous potential may not reflect the flow across the entire muscle fibre membrane as measured by the intracellular electrode. It is known that the relationship between synaptic and non-synaptic membranes in crustacean muscle differs from that found in vertebrates (Bittner, 1968*b*), perhaps due to the presence of junctions in deep clefts or invaginations in the fibre surface (Atwood, 1967; Atwood & Jones, 1967; Selverston, 1967), which has important physiological consequences (Falk & Fatt, 1964; Bittner, 1968*b*). It is quite conceivable that a given ending in the view of an extracellular electrode releases quanta that affect 'cleft' membrane and 'surface' membrane, and these may produce radically different external currents. The fact that extracellular rise and decay time constants do not correlate with extracellular amplitudes or intracellular amplitudes or time constants further indicates that the electrical properties of these muscle fibres may be quite different from those predicted by classical cable theory. This situation is further complicated by the possibility that the extracellular electrode may be monitoring several release sites even within an effective recording radius of 15 μ, since the electron micrographs of Atwood (1967) and Atwood & Jones (1967) sometimes show two or more apparent release sites within a few microns of each other.

These phenomena could lead to violations of postulates (3) to (5) of the

compound Poisson hypothesis. A violation of one or more of these three postulates is necessary to explain the type of discrepancy in which the observed erjp amplitude histograms show for too many samples in the intervals between the $n = 1, 2, 3, \ldots$ quantal peaks. Examples of the phenomenon are seen in the data presented in Fig. 3*a* of the Results section and in the frequency samples shown by Dudel & Kuffler, Fig. 8, (1961*a*), and Fig. 9, 5/sec, (1961*c*). In all of these cases, the scatter of observed potentials around the $n = 1$ and $n = 2$ peaks is much greater than the scatter observed in the spontaneous potentials and cannot be accounted for solely on the basis of violations of postulates (1) (stationary Poisson process) and (2) (independence) of the compound hypothesis.

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