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THE INTERVALS BETWEEN MINIATURE END-PLATE POTENTIALS IN THE FROG ARE UNLIKELY TO BE INDEPENDENTLY OR EXPONENTIALLY DISTRIBUTED

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SUMMARY

1. It has been suggested that spontaneous quantal release of transmitter at the neuromuscular junction is a Poisson process. One logical argument against accepting the Poisson hypothesis is that so far relatively few intervals between miniature end-plate potentials (min.e.p.p.s) have been studied in any single experiment. Release is known to occur from many sites on the nerve terminal, so many intervals must be studied before drawing any conclusions about the timing of release from the individual sites. Moreover, the statistical methods that have been used are relatively insensitive to deviations from Poisson predictions.

2. The Poisson hypothesis is evaluated with respect to three major criteria:

(*a*) The fit to the exponential distribution is analysed by five goodness of fit tests which were applied to eleven sets of data, showing that it is unlikely that the data sets were generated by an exponential distribution.

(*b*) The independence of intervals is assessed in two ways. First, the autocorrelogram of intervals is constructed. This shows an excess of significant positive correlations beyond the 5% limits of the Poisson expectation. Secondly, the unsmoothed power spectrum is calculated, and compared to the Poisson prediction by means of the modified mean test. Again, most sets deviate significantly from the Poisson expectation. It is unlikely that the intervals are independent.

(*c*) The possibility of simultaneous occurrences is evaluated by construction of the amplitude histogram of min.e.p.p.s. In all sets the Poisson prediction for the frequency of multiples of the unit height was exceeded by the empirical data sets. The over-all conclusion is that the process which generates spontaneous releases is unlikely to be Poisson.

INTRODUCTION

Fatt & Katz (1952) measured the intervals between 800 miniature end-plate potentials (min.e.p.p.s) recorded with an intracellular microelectrode at the frog neuromuscular junction. The distribution of these intervals closely resembled an exponential distribution.

$$P(t' \geqslant t) \propto \exp(-t/E(X)), \tag{1}$$

where $P(t' \geqslant t)$ is the probability of finding an interval longer than t and $E(X)$ is the mean interval. Gage & Hubbard (1965) extended this approach to the mammalian neuromuscular junction. They divided their records into a series of short time bins of length Δt and then counted the number of min.e.p.p.s in each bin. The number of bins with any number of events, a, within them is given by:

$$N_a = \frac{\exp\left(\frac{-\Delta t}{E(X)}\right)\left(\frac{\Delta t}{E(X)}\right)^a N_t}{a!}, \tag{2}$$

where $E(x)$ is the mean interval and N_t is the total number of bins. The agreement between the observed and expected values was tested by the χ-square goodness of fit test. The deviation was not significant. These studies suggested that spontaneous quantal release follows a Poisson distribution.

None the less, there are reasons to question how well this conclusion has been established. One question is logical. Acetylcholine (ACh) appears to be released at scores or hundreds of sites on the nerve terminal. If all sites release spontaneously, then scores or hundreds of min.e.p.p.s must be observed before seeing a second release from any one site. Obviously many min.e.p.p.s must be observed before concluding that release at any individual site is completely random.

Moreover, detailed analysis of the 799 intervals of Fatt & Katz suggested alternatives to the Poisson model (Cox & Smith, 1953; Lewis, 1964). One possibility is that the series is generated by superimposing the output of about 170 release sites, each site releasing on a periodic pattern (Cox & Lewis, 1966; Hubbard, 1970). In high Ca^{2+} Ringer there is reported to be a substantial deviation from the exponential distribution (Rotshenker & Rahamimoff, 1970). In the locust, miniature excitatory potentials do not occur at exponential intervals and frequently 'bursts' of events were encountered (Usherwood, 1972). Cohen, Kita & Van der Kloot (1973*a*) reported that the distribution of intervals between extracellularly recorded min.e.p.p.s from the frog neuromuscular junction was not exponential.

METHODS

Data recording. Min e.p.p.s were recorded from the sciatic nerve-sartorius muscle preparation of the frog (*Rana pipiens*) with standard technique. The min.e.p.p.s were photographed on film moving at 25 or 50 mm per sec or recorded on paper moving at 25 or 125 mm per sec using a Brush recorder. The intervals from the filmed record were measured to the nearest 1 msec, using a microfilm viewer. Two min.e.p.p.s could be resolved if they were greater than 0·5 msec apart.

TABLE 1. Composition of Ringer solutions (mM)

Set*	NaCl	KCl	$CaCl_2$	$MgCl_2$	Tris buffer pH = 7·4	Sucrose	Neostigmine (g/l.)
A, B, C	100	2	2·5	3	8	0	10^{-6}
D	100	2	2·5	3	8	50	10^{-6}
E	100	2	2·5	3	8	90	10^{-6}
F	100	2	2·5	3	8	100	10^{-6}
G	100	2	1·0	0	8	100	10^{-6}
H (3·8° C)	100	2	2·5	3	8	100	10^{-6}
I	100	2	2·5	3	8	150	10^{-6}
J (10° C)	100	2	0·8	0	8	220	10^{-6}
K	100	20	1·0	0	8	100	10^{-6}
L	50	2	2·5	3	8	100	10^{-6}
M	92	10	2·5	3	8	0	10^{-6}
N	100	2	2·5	15	8	0	10^{-6}
O	100	2	2·5	18	8	0	10^{-6}
P, Q	100	2	2·5	35	8	0	10^{-6}

* Data was recorded at 20° C, if not otherwise noted.

The standard Ringer contained 100 mM-NaCl, 2 mM-KCl, 2·5 mM-$CaCl_2$, 3 mM-$MgCl_2$ and 8 mM-Tris maleate buffer pH = 7·4 (Danforth & Helmrich, 1964). For solutions used see Table 1. In most experiments the temperature of the preparation was kept steady (at 20° C, unless stated to the contrary) by circulating temperature-controlled water through chambers surrounding the bath.

The data sets evaluated in this analysis were recorded during several studies on various properties of the spontaneous release system. They were used in this study because no external variable was known to have changed the rate of release during the recording, and because they were of sufficient length for detailed analysis.

Statistical methods. The statistical methods we have used are standard for time sequence analysis and are described in detail by Cox & Lewis (1966). A number of methods have been used, because at present there is little information on the best approach to use in practice. In part this arises because some tests are useful for detecting one type of deviation from the null hypothesis, while other statistics have greater power against other alternatives.

The Poisson distribution. Suppose that a series of N events is distributed over a period of time t_0. The intervals between the events are $x_1, x_2, \ldots, x_n$. The mean interval $E(x)$ is the sum of all the intervals divided by their number. The problem is to decide whether or not the events are distributed in time as a Poisson process, which has four necessary criteria.

(1) The distribution of intervals between events should fit an exponential of the form: $F(x) \propto \exp(-x/E(x))$ where $F(x)$ is the probability of having an interval of length greater than x.

(2) The time of occurrence of an event is totally independent of all past events.

(3) The chance of two or more events occurring simultaneously is negligible.

(4) The probability of occurrence of events is not changing with time, so there are no trends in the series.

In this paper we will evaluate the empirical distributions in terms of criteria (1)–(3). The fourth requirement, the absence of trends, is evaluated in Cohen *et al.* (1973*b*) where alternatives to the Poisson model are discussed.

Goodness of fit tests. In order to investigate the exponential requirement of a Poisson process, goodness of fit tests must be employed. We calculated five different goodness of fit statistics for each set of experimental data.

(*a*) *χ-square.* A common approach to goodness of fit statistics is to set up time bins, each of length Δt, and to count the number of intervals that fall within each of the bins, and then to compare the actual number with the expected number, n, calculated from the equation:

$$n = N\,(\exp(-t/E(x)) - \exp(-(t+\Delta t)/E(x)))$$

where N is the total number of events. The actual and predicted values are compared by using the χ-square goodness of fit test. The problem with this approach is the arbitrariness in choosing the duration of the time bin.

(*b*) *Cumulative distribution.* The binning can be avoided by calculating the predicted cumulative distribution from the Poisson equation

$$n' = N(1-\exp(-t/E(x)))$$

where N is the total number of events and n' is the total number of events shorter than t. The goodness of fit of the data to the prediction can be tested by using the Kolmogorov–Smirnov or the Anderson–Darling statistics.

(*c*) *Durbin's transformation.* The disadvantage of the above approach is that the power (the ability to reject a false null hypothesis) is low. The power can be increased greatly by using a simple transform (Sukhatme, 1937; Durbin, 1961; Lewis, 1964) which involves studying the intervals between intervals. A Poisson series transformed in this manner falls on a straight line of slope 1 from 0 to 1. In effect this is also a method of filtering the data that minimizes the influence of long term trends in the data set.

(*d*) *Kolmogorov–Smirnov.* The empirical fit to the predicted Poisson line is readily tested with a one-sided Kolmogorov-Smirnov statistic, in which the maximum deviation from the predicted cumulative distribution function is found (Cox & Lewis, 1966).

(*e*) *Modified mean test.* Another readily computed statistic on data transformed by Durbin's method is the modified mean test (Lewis, 1964).

(*f*) *Sherman's statistic.* A rather different approach is to use the easily computed statistic devised by Sherman (1950) as a test of goodness of fit to the exponential distribution

$$\omega_n = \sum_{i=1}^{n} \frac{|x_i - E(x)|}{2(n+1)E(x)}.$$

A table of the distribution for small sample sizes is given by Bartholomew (1954). The expected value is $1/e$.

If an empirical distribution has an omega statistic which is significantly less than $1/e$, the data set must contain an excess of intervals near the mean interval length, and a lack of intervals at either extremely long lengths. A distribution which deviates in this manner from the exponential expectation is said to be ordered.

If an empirical distribution has an omega statistic which is significantly greater than $1/e$ the data set must contain an excess of either very short or very long intervals, and a lack of intervals near the mean length. A distribution which deviates in this manner from the exponential expectation is said to be clustered.

(*g*) *Dispersion test.* The dispersion test (Cox & Lewis, 1966, pp. 158–160) compares the mean number of events in a series of time bins to their variance, and in doing so serves as a rough indicator for the presence of time trends in the generating series, as well as examining the goodness of fit to the exponential. A significant dispersion statistic implies not only poor fit to the exponential, but also a possible moving mean.

The evaluation of independence. To assess the degree to which the intervals in the empirical series are independent of past history, autocorrelation coefficients were calculated as described by Cohen *et al.* (1973*a*). An excess of significant autocorrelation coefficients suggests that the events are not independent. However, more exact tests against non-independent alternatives are then needed, which involve substantial amounts of computation. After some trial, we adopted a method in which the series autocovariances, c_j, for the series were calculated first (Cox & Lewis, 1966, pp. 89–90). The autocovariances were then used in the calculation of the unsmoothed power spectrum, which is a Fourier series with the autocovariances as the coefficients.

$$In(\omega_p) = \frac{1}{2\pi}\left(c_0 + 2\sum_{j=1}^{l} c_j \cos(j\omega_p)\right),$$

where $\omega_p = \pi p/l$ and $p = (1, \ldots, l)$, $l = INT((n-1)/2)$.

For an uncorrelated distribution the values of $In(\omega_p)$ should equal $\sigma^2/2\pi$ for all values of the frequency, ω_p. A statistical test of the absence of correlation can be constructed by forming a ratio of the sum of all $In(\omega_p)$'s up to any value of p divided by all values of $In(\omega_p)$.

$$g_i = \frac{\sum_{p=1}^{i} In(\omega_p)}{\sum_{p=1}^{l} In(\omega_p)}.$$

The g_i's should form a uniform distribution for an uncorrelated process. The distribution of g_i's could be evaluated by any of the goodness of fit tests described previously using the (0, 1) distribution as the theoretical prediction. We used Durbin's transformation and then the modified mean statistic. We checked our computational method by using the computer to generate series of random exponentially distributed interval lengths; the g_i's from these series were distributed as expected by chance.

RESULTS

Fit to the exponential distribution. Five goodness of fit tests were used to estimate the probability that if the intervals between min.e.p.p.s are exponentially distributed the observed deviations could occur by chance. Table 2 summarizes the analysis of eleven sets of data. Included in the Table are the results from series recorded in normal Ringer, high K^+

TABLE 2. Goodness of fit tests of the distribution of intervals between min.e.p.p.s

Set	Mean interval (sec)	No. of intervals	χ-square	Kolmogorov–Smirnov	Modified mean	Sherman's statistic	Dispersion
A	1·29	1174	168** 23 df	2·24**	$P < 0{\cdot}01$**	0·384*	27·9 50 df
B	2·85	917	45·6 47 df	0·94	$P = 0{\cdot}29$	0·357	56·2 50 df
C	3·96	2349	125·4** 39 df	3·26**	$P < 0{\cdot}01$**	0·394**	344·0** 50 df
D	0·11	364	7·44 18 df	0·87	$P = 0{\cdot}42$	0·372	56·1 50 df
F	0·17	399	27·4 35 df	1·16	$P = 0{\cdot}75$	0·377	63·5 50 df
G	0·11	399	72·8** 35 df	3·33**	$P = 6 \times 10^{-8}$**	0·440**	130·5** 50 df
I	0·01	384	64·1** 12 df	2·55**	$P = 6 \times 10^{-8}$**	0·300**	268·0** 50 df
J	0·08	292	32·4** 13 df	2·36**	$P = 0{\cdot}27$	0·336*	71·9* 50 df
K	0·04	445	64·9** 26 df	2·46**	$P = 0{\cdot}91$	0·360	87·5** 50 df
L	0·30	251	20·1 28 df	0·195	$P = 0{\cdot}11$	0·357	57·5 50 df
M	0·90	1567	63·2 48 df	2·01**	$P < 0{\cdot}01$**	0·383*	212·5** 50 df

* Beyond 5% confidence limit of Poisson prediction.
** Beyond 1% confidence limit of Poisson prediction.

Ringer, low Na^+ Ringer, hypertonic Ringer, and hypertonic Ringer at low temperature.

The over-all conclusion, on the basis of any of the tests, is that the data is unlikely to be generated by an exponential process. With each test more than half of the examples are outside of the 5 % confidence limits. There is no obvious reason to favour one test above any of the others. The χ-square goodness of fit test, used to compare binned interval lengths with Poisson predictions, has the advantage of familiarity and clearly is quite powerful; its main disadvantage is the arbitrary choice of bin size. The Kolmogorov–Smirnov test and the modified mean test are both performed on transformed data. One advantage of this approach is that it is believed to minimize the effects of long term trends in the data. The dispersion test appears to be slightly less powerful than the others and had the further disadvantage that it involves an arbitrary choice of the time bin.

The results with Sherman's omega statistic raise a problem for further analysis. If the statistic is significantly below the expected value for an exponential series ($1/e$) the intervals are more ordered than a random series with a constant mean. If the values are significantly above $1/e$ there is clustering of events in the series. In our results two of the sets that deviate significantly from the exponential appear by this criteria to be more ordered than a Poisson distribution, while four of the sets appear to deviate significantly as a result of clustering. This may mean that there are different factors at work that can change the min.e.p.p. frequency away from the exponential, or perhaps min.e.p.p.s are generated by a process that is both more ordered than Poisson but which also tends to produce clustering.

The sets with more intervals are more unlikely to be exponential. We explored the effects of set length by taking one of our examples and calculating the statistics on the first 400 intervals, then on the first 1000, then on the first 1500, and finally on the entire 2349. Table 3 shows that with 400 intervals the data cannot be distinguished from the exponential at the 1 % significance level. With 1000 intervals two of the statistics are significant, while at 1500 and again at 2349 intervals, all statistics reject the null hypothesis at the 1 % level. These results emphasize the need for substantial amounts of data.

Independence of intervals. In a set of events generated by a Poisson process each event has no influence on the occurrence of future events. One method of testing for independence is by calculating autocorrelation coefficients. Usually we calculated the coefficients for the first 50 lags; an example is shown in Fig. 1. Two points are clear from the Figure. First, there is a greater than chance number of autocorrelation coefficients that exceed the confidence limits. Secondly, there is a tendency for runs of

TABLE 3. The effect of set length on statistical significance from set C

Set length	χ-square	Kolmogorov-Smirnov	Modified mean	Sherman's statistic	Dispersion
400	51·82 32 df	0·914	$P = 0{\cdot}39$	0·376	71·4 50 df
1000	61·89 39 df	1·63**	$P = 0{\cdot}03$	0·382	123·2** 50 df
1500	91·24** 39 df	2·37**	$P < 0{\cdot}01$**	0·391**	214·0** 50 df
2349	125·42** 39 df	3·26**	$P < 0{\cdot}01$**	0·394**	344·0** 50 df

** Beyond 1 % confidence limit of Poisson prediction.

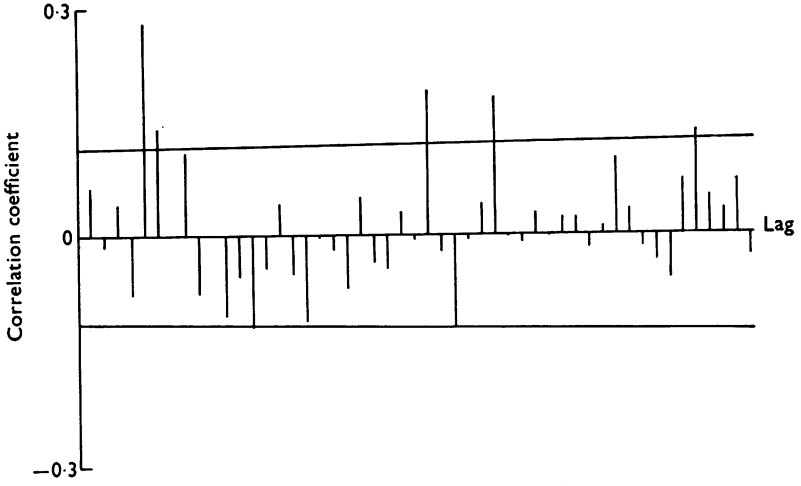

Fig. 1. The autocorrelation between intervals, calculated for lags 1–50 and displayed as an autocorrelogram. The method of calculation is described in the text along with the relationship used to calculate the 5 % significance limits, which appear on the graph as the nearly horizontal lines above and below the zero line. Note that seven of the fifty autocorrelations exceed the 5 % by chance limits. Also note the runs of consecutive positive or negative autocorrelations. This is common to all of our autocorrelograms. There are more positive than negative significant autocorrelation coefficients. This is also characteristic of all our autocorrelograms of intervals.

positive autocorrelations or negative autocorrelations to be clustered together. The clustering was found in all of the data sets analysed in this manner. Table 4 shows an analysis of the first fifty autocorrelation coefficients for each of eight sets of data. There is an excess of coefficients above chance in all sets. Further, there is a greater number of positive significant coefficients than negative ones. This is also characteristic of the data sets.

It is unfortunately difficult to quantify the deviation of series of autocorrelation coefficients. Asymptotically the coefficients should be normally distributed, but the small sample distribution is not known, and the use of longer series involves an arbitrary decision about the number of lags to be included in the calculation. For this reason we turned to statistics based on the power spectrum of intervals to obtain estimates of the probability that the min.e.p.p.s are distributed independently. The disadvantage of this approach is the very substantial amount of computation involved in the estimates.

TABLE 4. Autocorrelation coefficients of intervals (to lag 50)

Set	% outside of 5% limit	No. outside of 5% limit	No. outside of 5% limit (positive)	No. outside of 5% limit (negative)	% outside of 1% limit	No. outside of 1% limit	No. outside of 1% limit (positive)	No. outside of 1% limit (negative)
A	12	6	3	3	2	1	1	0
B	8	4	2	2	2	1	1	0
C	4	2	1	1	2	1	1	0
E	10	5	4	1	2	1	1	0
H	6	3	2	1	4	2	1	1
J	14	7	5	2	6	3	3	0
K	8	4	2	2	2	1	1	0
M	8	4	4	0	4	2	2	0

The results of the computations on eight sets of data are shown in Table 5. The results again indicate the low probability that min.e.p.p.s are generated by a random process.

Simultaneous occurrences. In a Poisson distribution there are no intervals with length zero, since substitution of interval length zero in eqn. (1) yields a probability of $t' \leqslant t$ equal to zero. Nevertheless, it is well known that occasionally min.e.p.p.s appear with an amplitude that is a multiple of the unit quantal size (Liley, 1956*a*, *b*, 1957).

One possible explanation for these 'giant' min.e.p.p.s is based on the assumption that the set is truly Poisson. Since the resolution time for two min.e.p.p.s is roughly 0·5 msec, any two events occurring at shorter intervals would be recorded as a single event of twice the normal amplitude.

The number of events with different multiples, m, of the unit quantal size would then be predicted by:

$$n_m = \left(\frac{t}{E(x)}\right)^{m-1} N \quad \text{if} \quad t \ll E(x),$$

TABLE 5. The probability of independence of data sets as tested by the modified mean test computed on an unsmoothed power spectrum after Durbin's transformation

Set	Probability
A	0·012*
B	< 0·01**
D	< 0·01**
H	< 0·01**
I	0·06
J	0·945
L	< 0·01**
N	< 0·01**

* Beyond 5% confidence limit of Poisson prediction.
** Beyond 1% confidence limit of Poisson prediction.

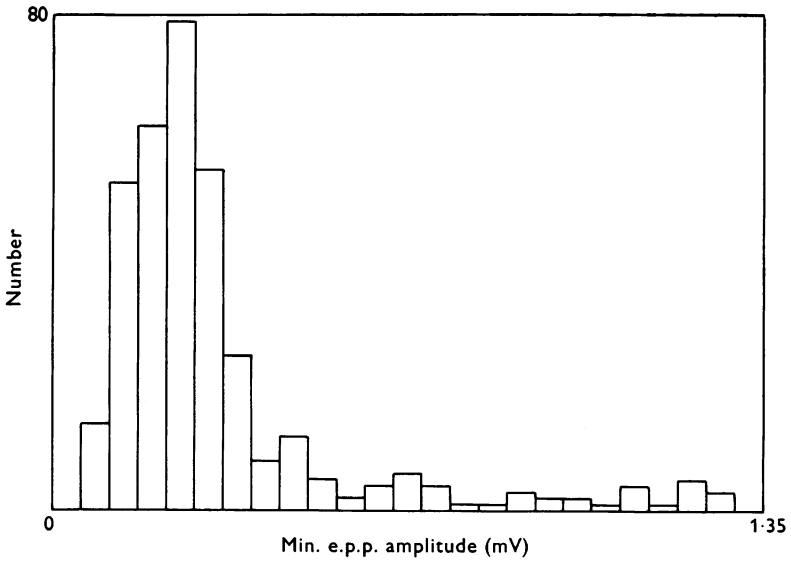

Fig. 2. The frequency distribution of amplitudes of 353 min.e.p.p.s recorded in 18 mM-$MgCl_2$ Ringer (Set 0). The amplitudes are grouped in brackets of 0·054 mV. Note the large skew in the histogram for large amplitude min.e.p.p.s. The number of large amplitude min.e.p.p.s greatly exceeds the Poisson prediction (see Table 6).

where n_m is the number of events containing m quanta. Liley (1957) used this approach to examine the amplitude distribution of a number of sets of min.e.p.p.s recorded from the rat neuromuscular junction under different experimental conditions. In all sets there was an excess of 'giant potentials' above the Poisson predictions.

TABLE 6. Analysis of the amplitude distribution of min.e.p.p.s comparing the expected number of 'giant' min.e.p.p.s with the Poisson prediction

	No. of doubles		No. of triples		No. of quadruples	
Set	Expected	Observed	Expected	Observed	Expected	Observed
B	1·08	7	< 0·01	1	$< 10^{-4}$	0
M	2·0	5	< 0·01	0	$< 10^{-4}$	0
N	2·6	5	0·02	4	$< 10^{-4}$	1
O	1·5	27	0·02	15	$< 10^{-4}$	9
P	2·4	6	0·03	3	$< 10^{-4}$	0
Q	10·0	13	0·30	0	$< 10^{-3}$	1

We have made similar observations at the frog neuromuscular junction. An example is shown in Fig. 2. From the amplitude histograms the number of apparent multiples can be estimated. Only experiments in which the resting potential exceeded 80 mV were evaluated. If the resting potential decreased by more than 10% the experiment was discarded. Table 6 compares these estimates with the Poisson prediction. In every set the number of multiples clearly exceeds the Poisson predictions.

The four data sets in high Mg^{2+} concentrations shown in Table 6 were recorded for two reasons. First, many of the min.e.p.p. sets were recorded in the process of studying e.p.p.s whose quantal content was reduced in order to avoid a post-synaptic action potential. Secondly, although all sets which were studied showed an excess of multiquantal releases some sets contained a complicated distribution of min.e.p.p. amplitudes in which it was unclear where the unitary distribution ended and the doublets began. The sets recorded in high Mg^{2+} concentrations did not show these bimodal distributions; therefore, they were used.

DISCUSSION

This paper re-examines the hypothesis that min.e.p.p.s are generated by a Poisson process. In a Poisson distribution the events are exponentially distributed. Powerful goodness of fit test show that when sufficient data is obtained the min.e.p.p.s recorded from the frog neuromuscular junction are unlikely to fit this criterion.

In a Poisson process the events are independent. A statistical analysis

of the occurrence of min.e.p.p.s shows that the positive autocorrelations are above the level expected if the releases were completely independent. The lack of independence is also shown by the deviation of the power spectrum from the predicted levels for a system without interactions. Since the occurrence of min.e.p.p.s does not fulfil the independence criterion, the generating process for spontaneous release cannot accurately be described as Poisson, nor can it be described by any independent trend-free distribution function (a renewal process).

In a Poisson process there are no simultaneous events. Possibly this criterion is also violated in spontaneous release by the high rate of appearance of 'giant' min.e.p.p.s. The 'giant' min.e.p.p.s might arise because transmitter-containing vesicles fuse before release, but it is also possible that during or immediately following the release of one quantum there is a greatly enhanced chance of getting a second release at the same site, or of activating a release from a second adjacent site. This is essentially the idea of 'drag' postulated by Rotshenker & Rahamimoff (1970).

Because of the failure to meet these criteria, we believe that it is inaccurate to describe spontaneous quantal release as a Poisson process. There are some obvious reasons for the difference between our conclusions and the acceptance of the Poisson hypothesis by most previous investigators. In the first place, we have studied large numbers of intervals. It is clear, as was shown in Table 3, that a short series is likely to appear to be Poisson, but if sufficient data is used the Poisson hypothesis is rejected. Most of the past studies involving large amounts of data have relied upon statistics with low power for distinguishing between a Poisson process and reasonable alternatives. Furthermore, at least some previous investigators have selected in advance the data sets that would be analysed, carefully choosing sequences that appeared to be uniform in time (Fatt & Katz, 1952). This is a difficult problem. In time series analysis it is perfectly legitimate to reject data in which some uncontrolled factor is causing a shift in time in a parameter of the generating process. But unless there is a good reason to suppose that such a factor is at work, simply rejecting data that by eye does not appear to be random can seriously prejudice the results. This is particularly true with min.e.p.p.s, where the selected sets may represent a fraction of the data. We have chosen to accept all data in which an uncontrolled variable was not identified, and in the next paper we shall describe tests to see whether or not there is a Poisson generating process whose parameter is changing in time.

We conclude therefore that the evidence is against spontaneous release being either a Poisson process or a process based on any independent distribution function.

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