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THE EFFECT OF CALCIUM IONS ON THE BINOMIAL PARAMETERS THAT CONTROL ACETYLCHOLINE RELEASE DURING TRAINS OF NERVE IMPULSES AT AMPHIBIAN NEUROMUSCULAR SYNAPSES

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SUMMARY

1. A study has been made of the effects of changing the external calcium concentration $[Ca]_o$ on the binomial parameters p and n that control the average quantal content ($\overline{m}$) of the end-plate potential (e.p.p.) during trains of nerve impulses at synapses in amphibian striated muscle.

2. In high external calcium concentrations (0·4 mM $\leqslant [Ca]_o <$ 1·0 mM) the increase in $\overline{m}$ of a test impulse following a conditioning impulse at different intervals (< 100 msec) was due to an increase in the number of quanta available for release, n; the increase in $\overline{m}$ of successive e.p.p.s in a short high frequency train was primarily due to an increase in n.

3. In high external calcium concentrations (1·0 mM $\leqslant [Ca]_o <$ 10 mM) there was a decrease in $\overline{m}$ of a test impulse following a short high frequency conditioning train (4–5 impulses, 20–100 Hz) at different intervals (200 msec < interval < 5 sec) and this was due to a decrease in the number of quanta available for release, n; in a long high frequency train (20 impulses, 20–100 Hz) there was an increase in $\overline{m}$ for the first few successive e.p.p.s followed by a depression of $\overline{m}$ which eventually reached a steady state and these changes in $\overline{m}$ were due to changes in n; the higher the frequency the greater was the depression in n during the steady-state period.

4. In high calcium concentrations, the steady-state $\overline{m}$ reached in the first 20 impulses during continual stimulation at high frequency gave way to a decline in $\overline{m}$ over several minutes until a new depressed steady-state value of $\overline{m}$ was reached and this was maintained during the longest periods of stimulation (30 min); this decline in $\overline{m}$ was primarily due to a decline in the number of quanta available for release.

5. These changes in the number of quanta available for release during trains of impulses are predicted in terms of a hypothesis in which

facilitation is due to the accumulation of a residual calcium-receptor complex in the nerve terminal that determines the fraction of a pool of quanta which contributes to n, and depression is due to a decrease in the number of quanta in this pool.

INTRODUCTION

In the previous paper (Bennett, Fisher, Florin, Quine & Robinson, 1977) a study was made of the effects of changes in the external calcium concentration $[Ca]_o$ and temperature on the number of quanta available for release by a nerve impulse (n) and more particularly on the magnitude and time course of the increase in the probability of release ($p(t)$) of an available quantum following a nerve impulse. The observed changes in the number of available quanta (n) and in the probability of release of an available quantum (p) with changes in $[Ca]_o$ were consistent with the idea that n is determined by a number of calcium-receptor complexes [CaX] formed in the nerve terminal which is regulated by the steady-state calcium level; p is then determined by a calcium-catalysed release reaction which is triggered by the large transient calcium entry that accompanies the nerve impulse (Hodgkin & Keynes, 1957; Baker, Hodgkin & Ridgway, 1971).

It is known that at mammalian neuromuscular synapses the facilitation in quantal content of successive e.p.p.s during a train in low $[Ca]_o$ or the depression in quantal content of successive e.p.p.s during a train in high $[Ca]_o$ are primarily due to changes in the number of quanta available for release by successive impulses in a train (Bennett & Florin, 1974; Bennett, Florin & Hall, 1975). In the present work a study has been made of the changes in n and p which occur during trains of nerve impulses in low and high $[Ca]_o$ at the toad neuromuscular synapse and it is shown that all changes in quantal content of the e.p.p. are primarily due to changes in n at this synapse. Thus facilitation and depression do not involve significant changes in the parameters of the stochastic process that determines p (namely α, γ and k in the previous paper; Bennett *et al.* 1977), but only changes in n. A quantitative description of the release of quanta by impulses during a train is therefore developed on the basis that facilitation is due to the accumulation of residual [CaX] in the nerve terminal which determines the fraction of a pool of N quanta that contributes to n, and depression is due to a depletion of the number of quanta in this pool.

METHODS

The preparation of the toad (*Bufo marinus*) iliofibularis muscle and recording techniques together with the method used in making solution and temperature changes are described in the previous paper (Bennett *et al.* 1977). For studies of

newly formed synapses, reinnervated toad iliofibularis muscles were prepared in the manner previously described (Bennett & Pettigrew, 1975).

Determination of binomial parameters

Changes in the binomial parameters p and n were studied with intracellular electrodes during both short trains of impulses (10–100 Hz) at normal synapses as well as during continual nerve stimulation (10 Hz) at reinnervated synapses. In the former case the nerve was stimulated with trains of 5–10 impulses and these trains repeated between fifty and eighty times; an interval of 15–30 sec was left between trains to avoid any effects due to potentiation; during the impalement about fifty spontaneous miniature end-plate potentials (m.e.p.p.s) were collected in order to make an estimate of the mean and variance of the quantal size; amplitude-frequency histograms were constructed for the e.p.p.s for each impulse over the fifty or so trains as well as for the fifty m.e.p.p.s collected and these compared with the predictions of binomial statistics. For synapses at which p and n were determined during continual high frequency stimulation over several minutes, the average quantal content of groups of over ninety e.p.p.s were collected at intervals of 1–2 min and amplitude–frequency histograms of these were also constructed and compared with the predictions of binomial statistics; nerve terminals were only subjected to one continual high-frequency stimulation. For all amplitude–frequency histograms collected in these experiments the 'goodness of fit' of the binomial distribution to the observed distribution was determined by a χ^2 test. During either facilitation or depression of the quantal content of successive e.p.p.s in a train, in the external calcium concentrations considered in the present work, transmitter release could be described by binomial statistics; the amplitude–frequency histograms of successive e.p.p.s were sufficiently well predicted by the binomial equation (see Methods, Bennett *et al.* (1977)) that a χ^2 test of the 'goodness of fit' of the binomial predictions to the observed histograms was such that $P > 0{\cdot}60$ for 70% of all histograms (total number 270).

In all experiments in which $[Ca]_o$ was greater than 0·6 mM, (+)-tubocurarine (10^{-7}–10^{-6} g.ml^{-1}) was added in sufficient concentration so that the tenth facilitated e.p.p. in a short train of impulses did not exceed 8 mV amplitude; in the few cases in which the e.p.p. was greater than 5–6 mV it was corrected for non-linear summation according to the method given by Bennett, Florin & Pettigrew (1976). A comment on possible presynaptic effects of (+)-tubocurarine is made in the previous paper (Bennett *et al.* 1977). The statistical methods employed in determining the binomial parameters together with their standard errors are given in the previous paper (Bennett *et al.* 1977).

Errors in determination of binomial parameters during trains of impulses

The various sources of error in estimating the binomial parameters with the present methods are described by Bennett *et al.* (1976) and Bennett *et al.* (1977). The question of effects on the estimates of p and n in the binomial analysis due to fluctuations which may occur in the release mechanism from impulse to impulse are considered in the Appendix. It should be emphasized that an important assumption in this work is that the mean and variance of the m.e.p.p. size can be taken as a measure of the mean and variance of the quantal size during trains of impulses; furthermore, insufficient numbers of m.e.p.p.s can be collected during short trains of impulses for comparison with m.e.p.p.s collected before or after the trains to ensure that there is no change in the mean m.e.p.p. size and therefore mean quantal size during either facilitation or depression.

An anticholinesterase was not added to the Krebs solution to enhance the time course and amplitude of synaptic potentials in an attempt to avoid possible post-

synaptic potentiating interaction between quanta released adjacent to each other (Hartzell, Kuffler & Yoshikami, 1975), at the high quantal contents studied in this work.

THEORY

Determination of the number of quanta available for release by nerve impulses

The number of quanta available for release by nerve impulses, n, is dependent on the external calcium concentration $[Ca]_o$ (Bennett *et al.* 1975, 1976) and it has been suggested that n is determined by the fraction (r) of a pool of N quanta in the nerve terminal, the size of this fraction being governed by the number of calcium ions bound to receptors to form a calcium receptor complex [CaX] according to a relationship of the form (Dodge & Rahamimoff, 1967; Bennett *et al.* 1976, 1977):

$$r = n/N = \{[Ca]_i/(K_1+[Ca]_i)\}^3 \quad (1)$$

where $[Ca]_i$ is the steady-state internal calcium concentration and K_1 is a dissociation constant. The question arises as to the form of the relationship between the steady-state level of $[Ca]_i$ and the external calcium concentration $[Ca]_o$; suppose that this takes the hyperbolic form (see Hodgkin & Keynes, 1957; Bennett *et al.* 1977),

$$[Ca]_i = [Ca]_S\{[Ca]_o/(K_o+[Ca]_o)\} \quad (2)$$

where $[Ca]_S$ is the internal calcium concentration at which $[Ca]_i$ saturates; substitution of eqn. (2) into (1) gives

$$n = L_n\{[Ca]_o/(K_{1_n}+[Ca]_o)\}^3 \quad (3)$$

where

$$L_n = N\{[Ca]_S/(K_1+[Ca]_S)\}^3 \quad \text{and} \quad K_{1_n} = \{K_1K_o/(K_1+[Ca]_S)\}$$

if $[Ca]_S \ll K_1$, then $L_n \ll N$ and thus as $[Ca]_o$ is increased n approaches a value much less than N. An assumption of the kind expressed by eqn. (2) for the relationship between the steady-state levels of $[Ca]_i$ and $[Ca]_o$ must be assumed because of the large facilitation in n observed when $[Ca]_o$ had led to an apparent saturation of n for the first impulse in a train (see Results), and if any accumulation of residual [CaX] during a short train of impulses in moderate $[Ca]_o$ is not to quickly lead to a saturation of the X receptors with calcium (see below). If $[Mg]_o$ decreases the entry of calcium ions into the nerve terminal then eqn. (3) can be written in a similar form to that used previously (Bennett *et al.* 1976, 1977):

$$n = L_n\{([Ca]_o/K_{1_n})/(1+[Ca]_o/K_{1_n}+[Mg]_o/K_{2_n})\}^3 \quad (4)$$

and this equation determines the value of n for the first impulse in a train (namely n_1) and therefore of $r_1 = n_1/N$.

If following the first impulse in a train there is a transient increase in [CaX] due to some fraction of the transient calcium entry binding to the X receptors then this increase in [CaX] due to the accumulation of residual [CaX] will increase r_1 to r_2 and therefore increase n from its value at the time of the first impulse (n_1) to a new value (n_2) according to eqn. (1); if there is a further increase in [CaX] due to the accumulation of residual [CaX] from succeeding impulses in the train, then the increase in r and therefore n with successive impulses up to the jth impulse is described by the residual [CaX] hypothesis as (Linder, 1973; Bennett *et al.* 1976):

$$r_j/r_1 = n_j/n_1 = \{\sum_{i=1}^{j-1}[(1+f_n[(j-i)\,\Delta t])^{\frac{1}{3}}-1]+1\}^3 \quad (5)$$

where Δt is the interval between impulses and $f_n[(j-i)\,\Delta t]$ is the value of f_n (see below) for the interval $(j-i)\,\Delta t$. If each impulse in a train releases a large number of

quanta then a substantial loss of quanta from the initial N quanta of the nerve terminal may occur. Under these conditions, and with p constant, the first impulse in a train involves the loss of $\overline{m}_1 = n_1 = r_1 N$ quanta from the N quanta leaving $N - r_1 N$ quanta for the next impulse which then releases $r_2(N - n_1)$ quanta and so on for the succeeding impulses, so that

$$n_2/n_1 = r_2(1/r_1 - 1),$$
$$n_3/n_1 = r_3(1/r_1 - 1 - n_2/n_1),$$
$$n_j/n_1 = r_j(1/r_1 - 1 - n_2/n_1 - n_3/n_1 \ldots - n_{j-1}/n_1)$$

and for the jth impulse

$$\overline{m}_j/\overline{m}_1 = n_j/n_1 = r_j/r_1 - r_j \sum_{i=1}^{j-1} n_i/n_1 \tag{6}$$

where r_j/r_1 is given by eqn. (5).

According to eqn. (6) the release of available quanta during a train of impulses eventually leads to a complete depletion of the N quanta initially present in the nerve terminal, so that eventually no quanta are released by impulses. As such complete depeletion of N is not observed, some quanta must be replaced during an impulse train. Suppose that there are initially N_1 quanta in the nerve terminal and following the release of $r_1 N_1$ quanta by the first impulse ΔN_1 quanta are replaced so that the second impulse releases $r_2(N_1 + \Delta N_1 - n_1)$ rather than just $r_2(N_1 - n_1)$ quanta, so

$$n_2/n_1 = r_2(1/r_1 + \Delta N_1/n_1 - 1),$$
$$n_3/n_1 = r_3(1/r_1 + (\Delta N_1 + \Delta N_2)/n_1 - 1 - n_2/n_1),$$
$$n_j/n_1 = r_j\left(1/r_1 + \left(\sum_{i=1}^{j-1} \Delta N_i\right)\Big/ n_1 - \sum_{i=1}^{j-1} (n_i/n_1)\right)$$

and for the jth impulse

$$\overline{m}_j/\overline{m}_1 = n_j/n_1 = (r_j/r_1)(N_j/N_1) - r_j \sum_{i=1}^{j-1} (n_i/n_1), \tag{7}$$

where

$$N_j = N_1 + \sum_{i=1}^{j-1} \Delta N_i.$$

All the changes in the number of quanta available for release during trains of nerve impulses were well fitted by eqn. (7) in the present work.

Method of determining the parameters in eqn. (7) *describing the number of quanta available for release during trains of impulses*

The value of r_j/r_1 in eqn. (7) was predicted from eqn. (5) in which

$$f_n[(j-i)\,\Delta t] = (n - n_o)/n_o, \tag{8}$$

where n_o is the value for a conditioning impulse and n is the value for a test impulse at $(j-i)\,\Delta t$ msec later (see Fig. 2), all determined in sufficiently low $[Ca]_o$ so that no significant depletion of the N quanta by the first impulse is possible.

Next the value of r_j in eqn. (7) was obtained from r_j/r_1 in eqn. (5) in which r_1 was determined as follows: according to eqn. (6) the size of the fifth impulse in a short train in the absence of any replacement of released quanta is

$$\overline{m}_5/\overline{m}_1 = n_5/n_1 = (r_5/r_1)(1 - r_1)(1 - r_2)(1 - r_3)(1 - r_4)$$

so that the fraction of the N quanta present immediately after the fourth impulse is $(1 - r_1)(1 - r_2)(1 - r_3)(1 - r_4)$; now the fraction of the N quanta present after the

fourth impulse (see Mallart & Martin, 1968) may be obtained by determining the quantal release by a fifth impulse and therefore $\overline{m}_5/\overline{m}_1 = (f\overline{m}+1)$ at a number of times after the train of four impulses when facilitation has declined to zero (see Fig. 6), but there has been little replacement of the released quanta, and then extrapolating these values back to the end of the train thus obtaining a value of $f_{\overline{m}}$ very close to $f_{\overline{m}_0}$ (Fig. 6), so that

$$(1-r_1)(1-r_2)(1-r_3)(1-r_4) \cong (f_{\overline{m}}+1) \tag{9}$$

r_1 can then be determined from this equation (as r_j for $j > 1$ are given in terms of r_1 in eqn. (5)), and if necessary other train lengths used to independently check the value of r_1 determined from the train length of four impulses.

Finally, the extent of replacement of quanta during an impulse train N_j in eqn. (7) was approximately determined as follows: suppose that the replacement of quanta only occurs after a certain number of quanta are lost, and that thereafter the replacement occurs at an approximately constant rate; thus ΔN_i in eqn. (7) is zero up to the kth impulse in a train and then takes on a constant value of ΔN for succeeding impulses. Thus when the steady-state $\overline{m}_j$ and n_j have been reached during the train (so that $n_j/n_1 \cong$ constant), eqn. (7) becomes

$$\overline{m}_j/\overline{m}_1 = n_j/n_1 = (r_j/r_1)\{1+(j-k)\,\Delta N/N_1\} - r_j\left\{\left(\sum_{i=1}^{k} n_i/n_1\right)+(j-k-1)\,n_j/n_1\right\}$$

and

$$(n_j r_j/n_1)(j-k-1) - (r_j/r_1)\{(j-k)\,\Delta N/N_1\} = (r_j/r_1) - (n_j/n_1) - r_j \sum_{i=1}^{k} n_i/n_1 = 0$$

so that

$$\Delta N/N_1 = ((j-k-1)n_j r_j)/((j-k)n_1) \cong (n_j r_1)/n_1. \tag{10}$$

An estimate of $\Delta N/N_1$ can then be obtained from the steady-state value of n_j/n_1 reached at the end of a long train (Fig. 8) and from the r_1 determined as described above. Eqn. (7) can now be used to predict the changes in $\overline{m}_j/\overline{m}_1$ or n_j/n_1 under conditions when p is approximately constant, such as during facilitation and depression, as all the constants are determined from eqns. (8), (9) and (10).

RESULTS

At the toad neuromuscular synapse, a test e.p.p. is facilitated in amplitude compared with a conditioning e.p.p. occuring within about 100 msec in $[Ca]_o$ up to at least 10 mM (Fig. 1). For $[Ca]_o$ greater than 0·25 mM, this facilitated increase in quantal content of a test e.p.p. is due to an increase in the number of quanta available for release, at least up to the highest $[Ca]_o$ at which a binomial statistic analysis could still be applied (up to a quantal content of the conditioning e.p.p. of 30, see Fig. 1). However successive impulses in a train either facilitated until a steady-state level of quantal content was reached or facilitated and then passed into a depressed phase of quantal release, depending on whether $[Ca]_o$ was greater or less than about 1·0 mM.

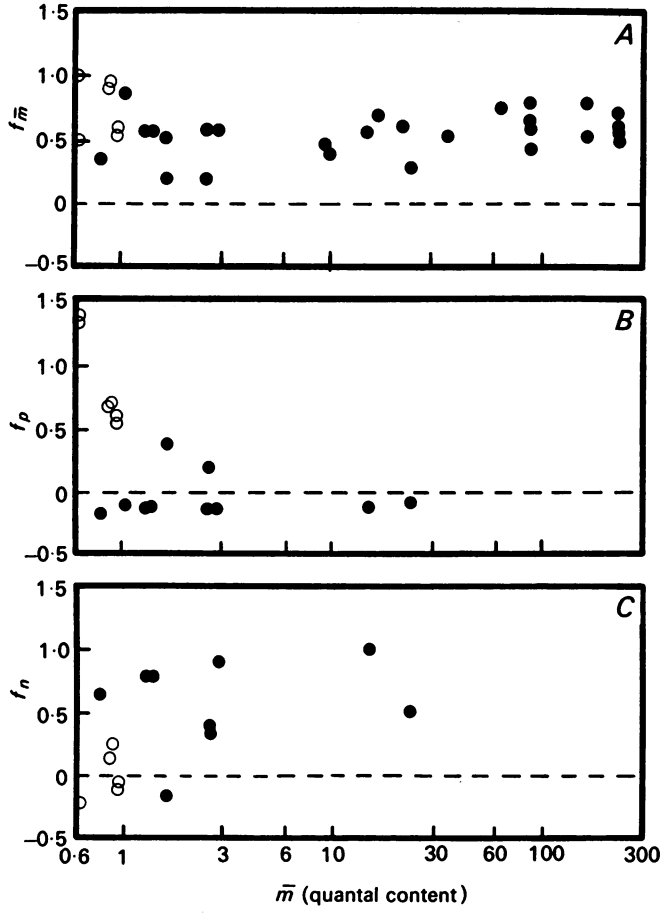

Fig. 1. Effect of quantal content of a conditioning impulse on the magnitude of the binomial parameters during a subsequent test impulse. The quantal content ($\bar{m}$) of the conditioning impulse was increased from 0·6 to 220 by raising $[Ca]_o$ from 0·10 to 10 mM in a constant $[Mg]_o$ of 1·2 mM. The facilitated increase in quantal content ($f_{\bar{m}} = (\bar{m}-\bar{m})/_o\bar{m}_o$) and of the binomial parameters p ($f_p = (p-p_o)/p_o$) and n ($f_n = (n-n_o)/n_o$) of a test impulse 20 msec after a conditioning impulse are shown as a function of the quantal content during a conditioning impulse ($\bar{m}$): both filled and open circles give the value determined at one synapse. Filled circles are for facilitations of quantal content due to an increase in n and open circles are for facilitations of quantal content due to an increase in p; facilitations due to an increase in p only occur for $\bar{m}$ less than 1·0. Each point was determined from a binomial analysis of the amplitude-frequency data of at least fifty conditioning-test sequences, an interval of 15 sec being left between each sequence; the S.E. of the mean was less than 20 % of the mean for each estimate of the binomial parameters for either conditioning (p_0, n_0) or test (p, n) impulses or that value was not included in the Figure. The binomial parameters could only be determined for $\bar{m} < 30$.

Changes in the number of quanta available for release during facilitation (low $[Ca]_o$; 0·4 mM $\leqslant [Ca]_o <$ 1·0 mM)

Short trains of impulses in low $[Ca]_o$. The increase in quantal content of a test e.p.p. following a single conditioning e.p.p. at intervals between 10 and 100 msec had a time course which could be described by a single

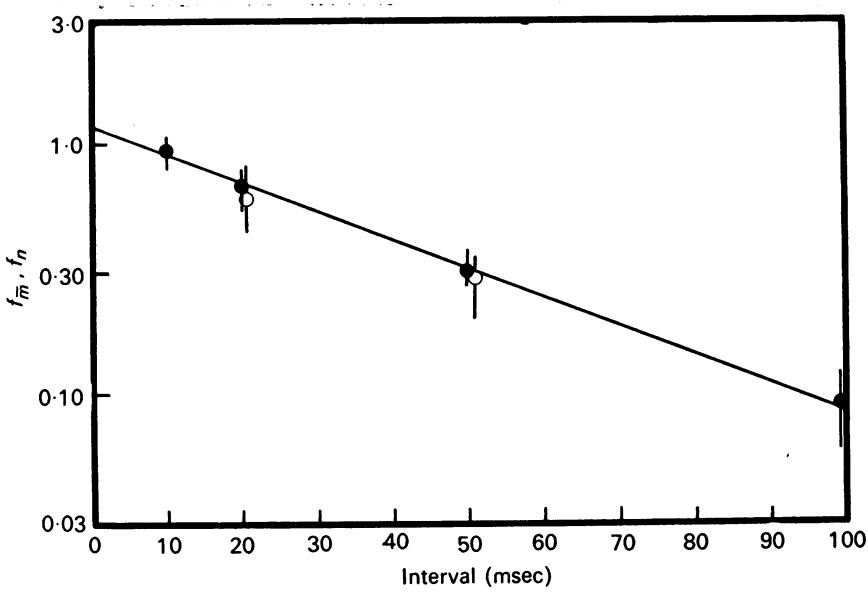

Fig. 2. The effect of a conditioning impulse on both the quantal content and the number of quanta available for release (n) during an e.p.p. evoked by a subsequent test impulse (0·4 mM $\leqslant [Ca]_o <$ 1·0 mM). The log ordinate gives the facilitation of quantal content ($f_{\bar{m}}$, filled circles) and of n (f_n, open circles) determined at various intervals after the conditioning impulse, given on the abscissa. Each point is the mean ± S.E. of the mean of the results from nine synapses, at each of which $f_{\bar{m}}$ and f_n were determined by applying binomial statistics to a test and conditioning e.p.p. in thirty trials; if the S.E. of the mean of a parameter determined at a synapse was greater than 15% of the mean that value was not included. The curve (a least-square regression line to the data) has a time constant of 40 msec. Note that f_n is close to $f_{\bar{m}}$ at the various intervals, indicating that there is little increase in p (f_p) by a single conditioning impulse. Expressions $f_{\bar{m}}$, f_n and f_p are defined in the text. $[Mg]_o$ = 1·2 mM.

exponential with a time constant of 40 msec at 18 °C in this $[Ca]_o$ range (Fig. 2). If the increase in $\bar{m}$, p and n of a test e.p.p. over that of $\bar{m}_0$, p_0 and n_0 of a conditioning e.p.p. occurring Δt msec earlier is defined as $f_{\bar{m}} = (\bar{m} - \bar{m}_0)/\bar{m}_0$, $f_p = (p - p_0)/p_0$ and $f_n = (n - n_0)/n_0$, then the increase in $f_{\bar{m}}$ was primarily due to an increase in f_n (Fig. 2).

Facilitation during a train of impulses in the quantal content of successive e.p.p.s obeyed binomial statistics (Fig. 3) and was also primarily due to an increase in n (Table 1). If the increase in $\overline{m}$, p and n of an e.p.p. in the train over their values for the first impulse in the train ($\overline{m}_0$, p_0 and n_0) are defined in the same way as above, then Fig. 4 shows that the increase

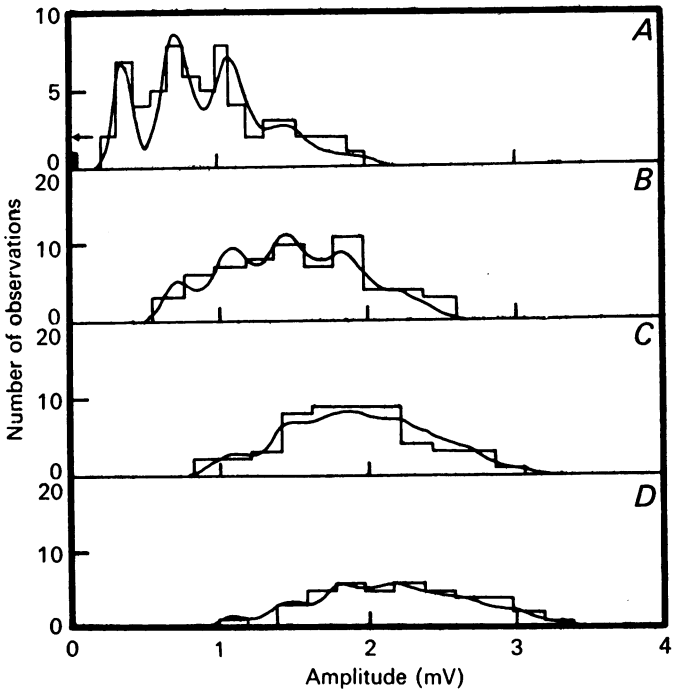

Fig. 3. Amplitude–frequency histograms of e.p.p.s for the first four impulses (A, B, C and D) in a short train during facilitation at a synapse in a $[Ca]_o$ of 0·40 mM. The binomial prediction for the e.p.p.s of each impulse is given by a continuous line; the rectangular filled block at zero in A gives the number of failures to nerve stimulation, whilst the arrow gives the predicted number of failures according to the binomial analysis. The histograms were obtained from the amplitude–frequency data of successive e.p.p.s evoked during short 50 Hz trains of five impulses; an interval of 20 sec was left between successive trains. The values of the binomial parameters $\overline{m}$, p and n respectively were: A, $2{\cdot}58 \pm 0{\cdot}17$, $0{\cdot}48 \pm 0{\cdot}09$, $5{\cdot}33 \pm 1{\cdot}03$; B, $4{\cdot}27 \pm 0{\cdot}22$, $0{\cdot}58 \pm 0{\cdot}08$, $7{\cdot}33 \pm 1{\cdot}03$; C, $5{\cdot}08 \pm 0{\cdot}25$, $0{\cdot}66 \pm 0{\cdot}07$, $7{\cdot}71 \pm 0{\cdot}89$; D, $6{\cdot}18 \pm 0{\cdot}36$, $0{\cdot}60 \pm 0{\cdot}11$, $10{\cdot}34 \pm 1{\cdot}91$. $[Mg]_o = 1{\cdot}2$ mM.

in f_n during a train is approximately exponential with a time constant of about 40 msec; this increase in f_n can be quantitatively predicted in terms of the accumulation of residual [CaX] following each impulse (according to eqn. (7) without any significant depletion of quanta occurring, so $\Delta N_i = n_i$ i.e. eqn. (7) is equivalent to eqn. (5)).

It may be noted that f_n is always less than $f_{\overline{m}}$ for either a test impulse following a conditioning impulse (Fig. 2) or during trains of nerve impulses (Fig. 4); during trains of impulses a just statistically significant increase in f_p occurred by the fifth to sixth impulse (Fig. 4).

TABLE 1. Binomial parameters describing the facilitation in transmitter release during short trains of impulses (0·4 mM $\leqslant$ $[Ca]_o$ < 1·0 mM). The values of $\overline{m}$, p and n for the first five impulses at 50 Hz are shown. The S.E. of the mean of $\overline{m}$, p and n were calculated using the equations given in Bennett & Florin (1974) and Robinson (1976)

Impulse no. ...	1	2	3	4	5
			$\overline{m}$		
	2·58 ± 0·17	4·27 ± 0·22	5·08 ± 0·25	6·18 ± 0·36	5·70 ± 0·49
	2·90 ± 0·18	4·58 ± 0·24	6·07 ± 0·33	6·57 ± 0·30	6·91 ± 0·34
	1·66 ± 0·17	2·49 ± 0·25	3·29 ± 0·26	4·26 ± 0·35	5·20 ± 0·36
	1·04 ± 0·14	1·95 ± 0·26	2·90 ± 0·27	4·17 ± 0·31	6·36 ± 0·34
	0·82 ± 0·10	1·11 ± 0·14	1·52 ± 0·16	1·73 ± 0·20	2·20 ± 0·20
			p		
	0·48 ± 0·09	0·58 ± 0·08	0·66 ± 0·07	0·60 ± 0·11	0·60 ± 0·17
	0·46 ± 0·10	0·37 ± 0·12	0·14 ± 0·16	0·45 ± 0·11	0·35 ± 0·13
	0·54 ± 0·12	0·19 ± 0·19	0·48 ± 0·13	0·32 ± 0·17	0·54 ± 0·13
	0·48 ± 0·12	0·43 ± 0·13	0·39 ± 0·16	0·44 ± 0·15	0·67 ± 0·10
	0·64 ± 0·10	0·53 ± 0·13	0·57 ± 0·12	0·34 ± 0·17	0·73 ± 0·11
			n		
	5·33 ± 1·03	7·33 ± 1·03	7·71 ± 0·89	10·34 ± 1·91	9·47 ± 2·68
	6·39 ± 1·43	12·33 ± 3·40	43·41 ± 51	14·55 ± 3·59	19·64 ± 7·35
	3·06 ± 0·68	13·21 ± 0·12	6·84 ± 1·95	13·35 ± 7·4	9·57 ± 2·36
	2·16 ± 0·52	4·53 ± 0·14	7·46 ± 3·09	9·59 ± 3·31	9·47 ± 1·39
	1·28 ± 0·23	2·07 ± 0·53	2·67 ± 0·62	5·19 ± 2·80	3·03 ± 0·50

Changes in the number of quanta available for release during depression (high $[Ca]_o$; 1·0 mM $\leqslant$ $[Ca]_o$ $\leqslant$ 10 mM)

Short trains of impulses in high $[Ca]_o$. If in these high $[Ca]_o$ solutions a test e.p.p. follows a short high frequency conditioning train of 4–6 impulses at intervals of about 200 msec to 5 sec after the last impulse in the train, it is depressed in amplitude compared with the first impulse in the train (Fig. 5), and this depression is larger the greater the number of impulses in the conditioning train (Fig. 5); it is shown below that this depression is due to a decrease in the number of quanta available for release, n; at intervals less than 200 msec following a short high-frequency train the test e.p.p. is facilitated in amplitude (Fig. 5). The time course of recovery from the depression in $\overline{m}$ after a short high-frequency train was approximately exponential with a time constant of about 4·2 sec (Fig. 6*A*) and this time constant was independent of the size of the depression produced (Fig. 6*A*; see also Betz (1970)). If the recovery curves shown in

Fig. 6*A* are extrapolated to zero time to obtain $f_{\bar{m}_0}$, then there was an approximately linear relationship between this zero time depression ($f_{\bar{m}_0}$) and the number of quanta released in the train (Fig. 6*B*), although at the higher quantal releases elicited by about ten impulses at 100 Hz there

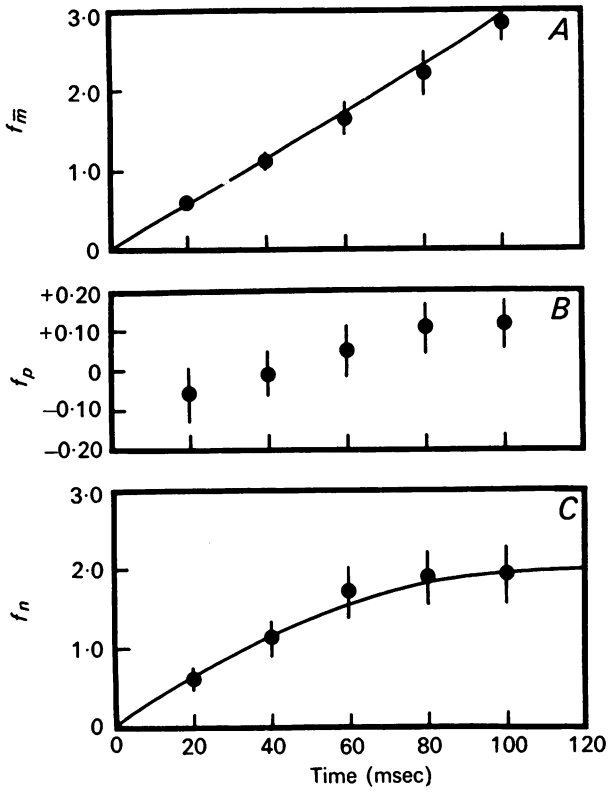

Fig. 4. Changes in the binomial parameters describing transmitter release during short trains of impulses at 50 Hz at synapses in a $[Ca]_o$ of 0·4 mM. The changes in $f_{\bar{m}}$, f_p and f_n (defined in the text) are shown for each of the first six impulses in a train. Each point is the mean ± 1 S.E. of the mean of results from five synapses, at each of which $f_{\bar{m}}$, f_p and f_n were determined by applying a binomial analysis to the successive e.p.p.s in at least fifty trains; if the S.E. of the mean of a parameter determined for a particular impulse number in a train at a synapse was greater than 25% of the mean, that value was not included. The mean value ± S.E. of mean of $\bar{m}$, p and n for the first impulse at the six synapses were 1·71 ± 0·35, 0·53 ± 0·03 and 3·39 ± 0·82 respectively. The continuous line in the graph for f_n gives the theoretical predictions according to the residual [CaX] hypothesis (eqns. (5) or (7)); the continuous line in the graph for $f_{\bar{m}}$ gives the theoretical predictions according to the residual [CaX] hypothesis and the observed small rate of increase of f_p. $[Mg]_o$ = 1·2 mM. Data for first five impulses given in Table 1.

was a decrease in the depression from this linear relationship, as has been noted by Betz (1970). Curves like those in Fig. 6*A* could be used to obtain a measure of the fraction of the total number of quanta in the nerve terminal made available for release by the first impulse in the train (i.e. r_1), according to eqn. (9).

It is not possible to carry out a binomial analysis of transmitter release at $[Ca]_o$ above 1 mM because the e.p.p. is suprathreshold for initiation of the action potential, and further addition of (+)-tubocurarine eliminates the m.e.p.p.s required for a measure of quantal size. Depression in high

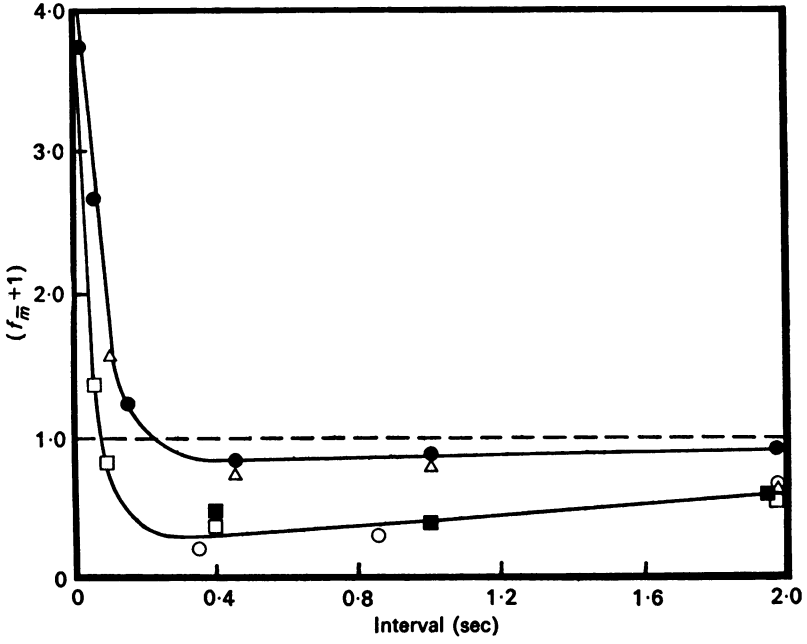

Fig. 5. The effect of a short high-frequency conditioning train of impulses on the amplitude of the e.p.p. due to a subsequent test impulse in high $[Ca]_o$ (7·5 mM). The test impulse followed the conditioning train (100 Hz) at the intervals indicated on the abscissa. The number of impulses in the conditioning train were: filled circles, four; open triangles, six; filled squares, ten; open squares, ten; open circles, fifteen. Each point is the mean of at least ten conditioning-test trials, all the values for a given number of conditioning impulses being obtained from one synapse; the S.E. of the mean was less than 10% of the mean for each point. The ordinate is $(f_{\bar{m}}+1) = v/v_o$, where v_o is the amplitude of the first e.p.p. in the conditioning train and v is the amplitude of the test e.p.p., so that the values of $(f_{\bar{m}}+1)$ less than 1 indicate depression whereas values greater than 1 indicate facilitation. Note that $f_{\bar{m}}$ is estimated here and in Figs. 6, 7 and 8 by measuring the size of the e.p.p. not the quantal content as in $[Ca]_o$ = 7·5 mM, (+)-tubocurarine must be added in sufficient amounts to make the e.p.p. subthreshold and the m.e.p.p.s are no longer observed.

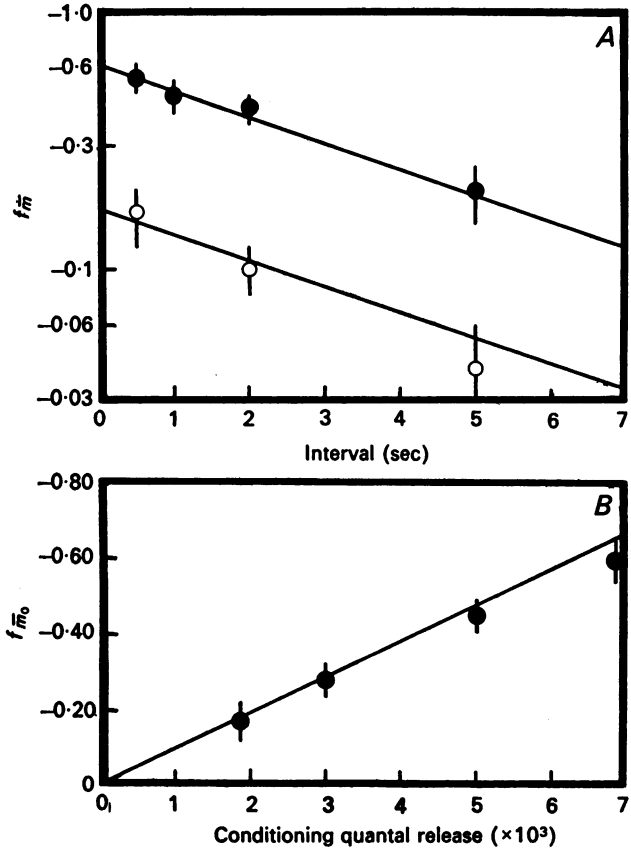

Fig. 6. The time course and amplitude of the depression in the size of a test e.p.p. following a conditioning high-frequency train (100 Hz) at different intervals in very high $[Ca]_o$ (7·5 mM). *A*, the time course of decline of depression following a conditioning train of fifteen impulses (filled circles) and a conditioning train of four impulses (open circles); each point is the mean ± 1 S.E. of the mean of the results for four synapses at each of which at least ten conditioning-test trials were performed at the intervals indicated on the abscissa; the log ordinate is $f_{\bar{m}} = (v - v_o)/v_o$ where v and v_o are defined in legend to Fig. 5; the time constant of recovery from depression is 4·2 sec. *B*, the relationship between the value of $f_{\bar{m}}$ at zero test-conditioning interval ($f_{\bar{m}_0}$) (according to the extrapolation of lines like those in *A* to zero interval) and the number of quanta released by the conditioning train; the conditioning quantal release was obtained from the number of quanta released by a conditioning impulse in 7·5 mM-$[Ca]_o$ as given in Fig. 5 of Bennett *et al.* (1977), and the changes in $f_{\bar{m}}$ during a 100 Hz train given in Fig. 8; each point is the mean ± 1 S.E. of the mean. Lines in both *A* and *B* drawn by eye.

$[Ca]_o$ was successfully studied with binomial statistics at reinnervated amphibian neuromuscular synapses, at which the synapses and n values are comparatively small and therefore the e.p.p. is subthreshold (Bennett & Pettigrew, 1975); Table 2*A* shows that whilst depression in a short train at these reinnervated synapses was always accompanied by a decrease in

TABLE 2. Binomial parameters describing the depression in transmitter release during long trains of impulses (1·0 mM $\leqslant [Ca]_o \leqslant$ 10 mM). The values of $\overline{m}$, p and n shown in *A* are for the first and the x^{th} impulse ($8 < x < 15$) in a train of impulses (5–20 Hz) at reinnervated amphibian synapses in a $[Ca]_o$ of 10 mM and $[Mg]_o$ of 1·2 mM. The values of $\overline{m}$, p and n shown in *B* are for different impulses in a train at 10 Hz for mammalian synapses in a $[Ca]_o$ of 1 mM, $[Mg]_o$ of 7 mM with (+)-tubocurarine of 3×10^{-6} g.ml.$^{-1}$. The S.E. of the mean of $\overline{m}$, p and n were calculated using the equations given by Bennett & Florin (1974) and Robinson (1976)

A

$\overline{m}$		p		n	
1	x	1	x	1	x
3·13 ± 0·23	2·81 ± 0·19	0·50 ± 0·12	0·66 ± 0·08	6·38 ± 1·56	4·23 ± 0·54
16·77 ± 0·69	13·55 ± 0·60	0·82 ± 0·06	0·74 ± 0·08	20·42 ± 1·79	18·22 ± 2·15
28·98 ± 0·98	25·88 ± 1·12	0·83 ± 0·11	0·99 ± 0·04	34·92 ± 5·14	25·97 ± 1·65
26·45 ± 1·41	24·67 ± 1·03	0·71 ± 0·12	0·82 ± 0·09	37·15 ± 5·20	29·94 ± 3·00
23·94 ± 1·24	20·44 ± 1·07	0·99 ± 0·02	1·00 ± 0·02	23·94 ± 1·41	20·37 ± 1·19

B

Impulse no. ...					
1	3	5	8	10	15
		$\overline{m}$			
6·90 ± 0·36	7·04 ± 0·35	6·11 ± 0·31	5·34 ± 0·31	5·18 ± 0·28	4·88 ± 0·24
17·92 ± 1·31	15·36 ± 1·16	13·61 ± 1·00	10·12 ± 0·86	9·83 ± 0·85	9·29 ± 0·82
12·62 ± 1·05	11·54 ± 0·93	9·52 ± 0·78	8·99 ± 0·72	8·32 ± 0·64	7·33 ± 0·56
		p			
0·81 ± 0·07	0·88 ± 0·05	0·87 ± 0·06	0·81 ± 0·07	0·87 ± 0·06	0·94 ± 0·04
0·79 ± 0·14	0·79 ± 0·15	0·90 ± 0·11	0·74 ± 0·16	0·72 ± 0·17	0·70 ± 0·18
0·94 ± 0·15	0·93 ± 0·14	0·97 ± 0·13	1·03 ± 0·11	1·09 ± 0·09	1·14 ± 0·08
		n			
8·48 ± 0·86	7·96 ± 0·63	6·99 ± 0·57	6·57 ± 0·68	5·94 ± 0·49	5·15 ± 0·33
22·51 ± 4·56	19·40 ± 4·10	15·04 ± 2·26	13·52 ± 3·30	13·54 ± 3·55	13·12 ± 3·64
13·41 ± 2·61	12·39 ± 2·67	9·78 ± 1·64	8·70 ± 1·29	7·62 ± 1·02	6·41 ± 0·80

n, the depression is still not sufficient to give clearly significant changes in n. A binomial analysis of depression was therefore made at mammalian neuromuscular synapses in $[Ca]_o$ of 1 mM with added (+)-tubocurarine to make the e.p.p.s subthreshold, as at these junctions there is a very large decrease in quantal content of successive e.p.p.s in a short train in a $[Ca]_o$ of 1 mM (Thies, 1965); highly significant decreases in n accompanied the large depression in the e.p.p. at these synapses (Table 2*B*), whilst p was constant.

The above observations indicate that for some milliseconds following a nerve impulse there is a transient increase in n (Fig. 5) and that this is followed for several seconds by a decrease in n which is dependent on the number of available quanta which participated in release during the impulse (Fig. 6). It might be anticipated then that during short high-frequency trains in high $[Ca]_o$ there would at first be an increase in n during the first few impulses (due to the accumulation of residual [CaX]) and then a decrease in n (due to the loss of quanta released from the nerve terminal). Fig. 7 shows that this was the case, with an initial facilitation in $f_{\bar{m}}$ giving way to a depression in $f_{\bar{m}}$, all the changes in $f_{\bar{m}}$ during stimulation at 100 Hz in $[Ca]_o$ from 0·6 mM to 7·5 mM being predicted on the basis of accumulation of residual [CaX] and the loss of quanta from the terminal (according to eqn. (7) with no replacement of released quanta, so $\Delta N_i = 0$, i.e. eqn. (7) is equivalent to eqn. (6)).

Long trains of impulses in high $[Ca]_o$. During long trains of nerve impluses in high $[Ca]_o$, the depression in n which follows facilitation does not continue until the release of quanta ceases, a depressed steady-state value of n being reached by about the thirtieth to fortieth impulse (Fig. 8; Table 2*A*) at the amphibian synapse (although by about the tenth impulse at the mammalian neuromuscular synapse; Table 2*B*) the depression being greater the higher the frequency of stimulation (compare Fig. 8*A* at 100 Hz with Fig. 8*B* at 50 Hz). This steady-state depressed value of n indicates that some of the quanta released from the nerve terminal must be replaced during stimulation. The size of the steady-state depressed level of n at different frequencies of stimulation allowed an estimate to be made of the fraction of quanta initially present in the terminal which are replaced between impulses (i.e. $\Delta N/N_1$), according to eqn. (10). Determination of this fraction ($\Delta N/N_1$) together with that of r_j/r_1 and r_1 as described above, allowed a comparison to be made between the predictions of eqn. (7) and the changes in $f_{\bar{m}}$ during stimulation; Fig. 8 shows that eqn. (7) gave a good fit to the experimental results.

Changes in the number of quanta available for release during long term depression (high $[Ca]_o$; 1·0 mM $\leqslant [Ca]_o \leqslant$ 10 mM)

During continual stimulation at 10 Hz in high $[Ca]_o$, the steady-state depressed release of quanta following the initial facilitation (Fig. 8) gives way after several seconds of stimulation to a further slow decline in quantal content of the e.p.p. which occurs over several minutes until a new steady-state and greatly depressed quantal release rate is reached; throughout the period of continual stimulation the quantal content of the e.p.p. obeys binomial statistics (Fig. 9). This long term depression is primarily due to a decrease in n (Fig. 10; Table 3), as p remains relatively constant

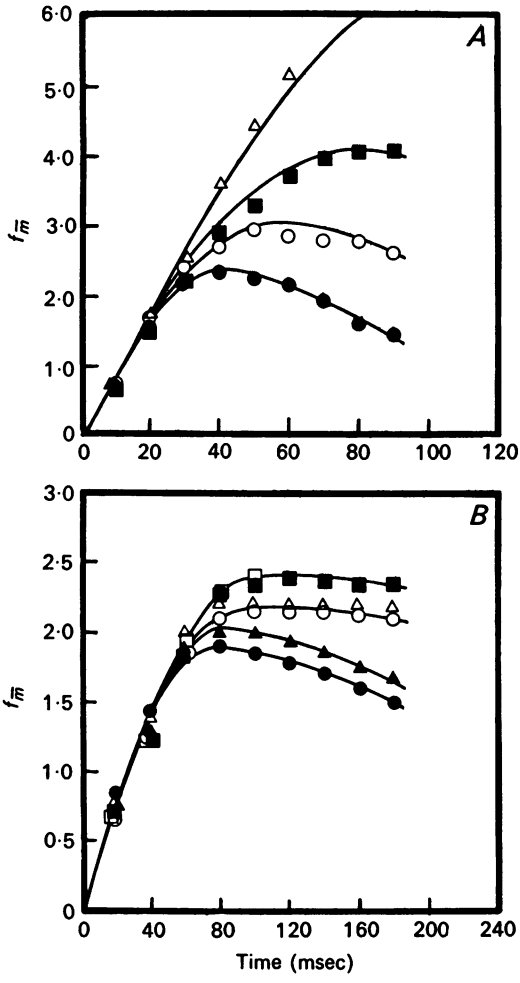

Fig. 7. The effect of calcium ions on the changes in quantal content of successive e.p.p.s in a short train at 100 Hz (*A*) and 50 Hz (*B*). The changes in $f_{\overline{m}}$ (defined in the text) are shown for each of the first ten impulses in different $[Ca]_o$. *A*: filled circles, 7·5 mM; open circles, 2·5 mM; filled squares, 1·0 mM; open triangles, 0·6 mM; *B*: filled circles, 7·5 mM; filled triangles, 5 mM; open circles, 2·1 mM; open triangles, 1·8 mM; filled squares, 1·0 mM; open squares, 0·8 mM. Each point is the mean of results from eight synapses, at each of which $f_{\overline{m}}$ was determined for the successive e.p.p.s in at least twenty trains; the S.E. of the mean of $\overline{m}$ for each impulse number in a train at a synapse was always less than 10% of the mean. Each point shown had a S.E. of the mean less than 15% of the mean. (+)-tubocurarine (10^{-7} to 3×10^{-6} g.ml.$^{-1}$) was added in order to maintain the e.p.p. subthreshold for action potential initiation and in this case m.e.p.p.s could still be observed up to $[Ca]_o$ of 1·8 mM allowing an estimate to be made of $\overline{m}$ and therfore $f_{\overline{m}}$; however at higher $[Ca]_o$ the (+)-tubocurarine was of such a

[Continued on facing page

at an elevated value during the entire period of continual stimulation (Fig. 10; Table 3). It is likely that long term depression reflects an incapacity of the nerve terminal to continually replace quanta at the rates reached during a long train of impulses (Fig. 8) rather than a conduction block of action potentials in the nerve terminals, as this is normally accompanied by a drop in p (Hatt & Smith, 1976).

TABLE 3. Binomial parameters describing transmitter release during continual nerve stimulation (1·0 mM $\leqslant [Ca]_o \leqslant$ 10 mM). The values of $\overline{m}$, p and n at different times during stimulation at 10 Hz are shown for motor nerve terminals in reinnervated amphibian striated muscle. The S.E. of the mean of $\overline{m}$, p and n were calculated using the equations given by Bennett & Florin (1974) and Robinson (1976)

Time (min) ...	< 1·0	3·5	5·5	8	12
			$\overline{m}$		
	15·32 ± 0·43	13·42 ± 0·37	11·61 ± 0·34	9·28 ± 0·27	6·64 ± 0·19
	6·37 ± 0·39	4·94 ± 0·29	3·58 ± 0·20	2·82 ± 0·15	2·45 ± 0·13
	23·23 ± 1·14	19·78 ± 0·97	17·31 ± 0·62	12·78 ± 0·47	12·20 ± 0·42
	10·30 ± 0·36	6·60 ± 0·29	5·21 ± 0·22		
	13·59 ± 0·46	11·64 ± 0·46	9·03 ± 0·36		
			p		
	0·67 ± 0·06	0·68 ± 0·05	0·64 ± 0·06	0·73 ± 0·04	0·79 ± 0·04
	0·73 ± 0·08	0·84 ± 0·05	0·94 ± 0·03	0·99 ± 0·02	0·99 ± 0·02
	0·51 ± 0·08	0·60 ± 0·07	0·67 ± 0·05	0·66 ± 0·05	0·67 ± 0·05
	0·67 ± 0·07	0·73 ± 0·06	0·71 ± 0·06		
	0·86 ± 0·04	0·65 ± 0·08	0·70 ± 0·07		
			n		
	22·89 ± 2·00	19·73 ± 1·59	18·12 ± 1·69	12·81 ± 0·86	8·45 ± 0·44
	8·74 ± 1·07	5·85 ± 0·48	3·82 ± 0·25	2·84 ± 0·17	2·47 ± 0·15
	45·30 ± 8·20	33·01 ± 4·62	25·69 ± 2·34	19·23 ± 1·80	18·21 ± 1·95
	15·27 ± 1·63	9·05 ± 0·76	7·40 ± 0·68		
	15·87 ± 0·92	17·90 ± 2·36	12·82 ± 1·33		

concentration that m.e.p.p.s could not be observed and the e.p.p. amplitude has been used to estimate $f_{\overline{m}}$. In less than 5% of synapses the e.p.p. reached values between 5 and 8 mV and in these cases the amplitude was corrected for non-linear summation using the method described by Bennett *et al.* (1976). The continuous lines in the graphs give the theoretical predictions according to the hypothesis that facilitation is due to accumulation of [CaX] and depression is due to a decrease in the number of available quanta according to eqn. (7) in which $f_n[(0)\ \Delta t]$ or f_{n_0}, r_1 and $\Delta N/N_1$ have the values respectively in A of: 1·03, 0·03, 0·04 (filled circles); 1·03, 0·018, 0 (open circles); 1·03, 0·01, 0 (filled squares); 1·03, 0·01, 0 (open triangles) and in B of 1·52, 0·03, 0·05 (filled circles); 1·52, 0·025, 0·04 (filled triangles); 1·40, 0·016, 0·03 (open circles); 1·40, 0·008, 0 (filled squares). In both A and B, ΔN was zero during the first 80 msec of stimulation and thereafter increased to the value indicated above.

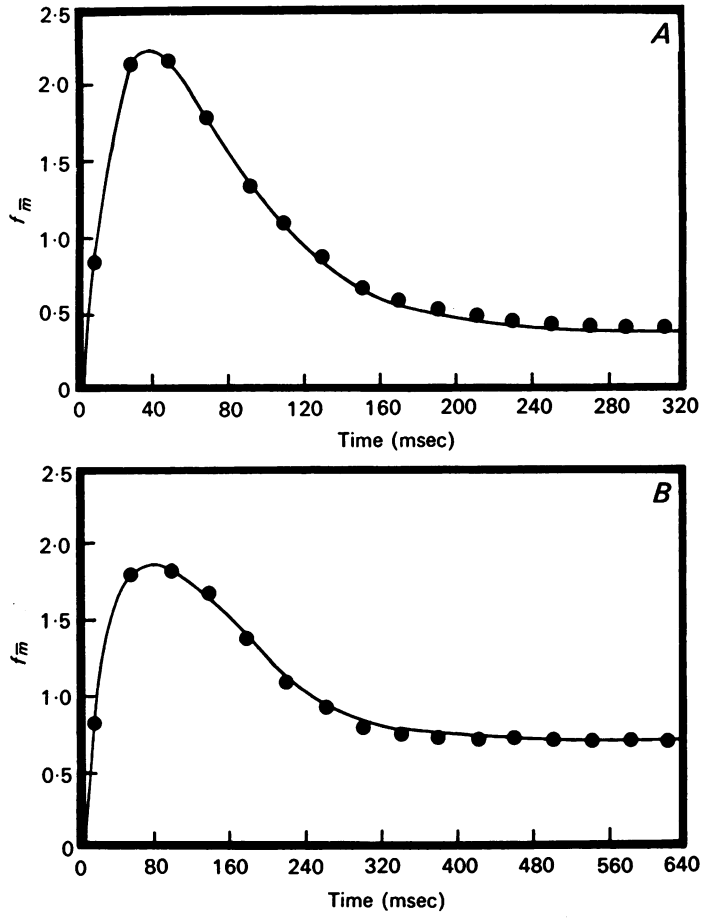

Fig. 8. The changes in amplitude of the e.p.p. during long high-frequency trains of impulses in high $[Ca]_o$ (7·5 mM). *A*, 100 Hz; *B*, 50 Hz. Each point has been determined from an analysis of the amplitude of at least fifteen e.p.p.s from ten synapses at the time after the beginning of stimulation indicated on the abscissa; the S.E. of the mean is less than 5% of the mean for each point. The ordinate is again $f_{\bar{m}} = (v - v_o)/v_o$ as in Figs. 6 and 7. The lines are drawn according to eqn. (7), in which $f_n[(0)\ \Delta t]$ or f_{n_0}, r_1 and $\Delta N/N_1$ respectively have the values in *A* of 1·03, 0·03, 0·04 and in *B*, 1·52, 0·03, 0·05. In both *A* and *B*, ΔN was zero during the first 80 msec of stimulation and thereafter increased to the value indicated above. Only small differences were observed between $f_n[(0)\ \Delta t]$ or f_{n_0} in high and low $[Ca]_o$, and when the correction in f_{n_0} suggested by Mallart & Martin (1968) for depletion by the first impulse in different $[Ca]_o$ was applied, the correction was less than 10% of the uncorrected estimate of f_{n_0}.

DISCUSSION

Changes in the number of quanta available for release during facilitation

Short trains of impulses in low $[Ca]_o$. The facilitated increase in the quantal content of successive e.p.p.s at the beginning of a short train was primarily due to an increase in the number of quanta available for release, that is n, although there was also a very small increase in p. This increase in n can be predicted on the basis that the availability of quanta for release is regulated by the formation of calcium-receptor complexes [CaX] in the

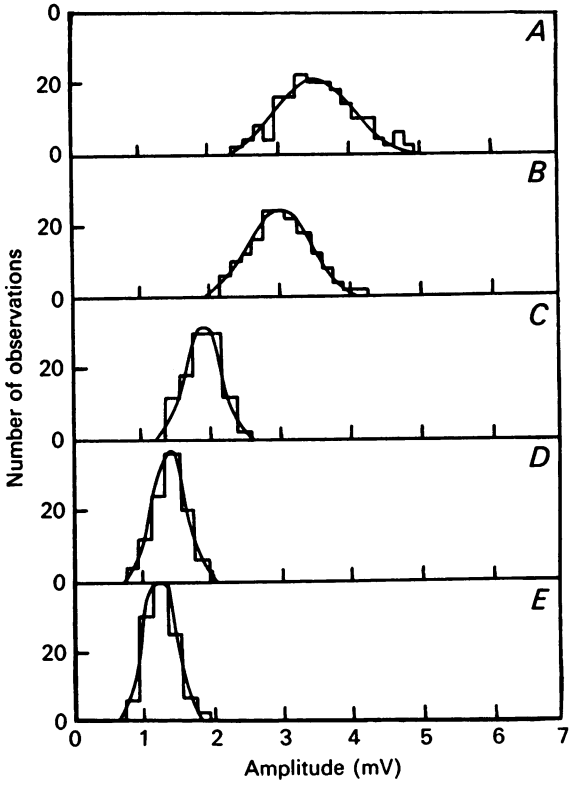

Fig. 9. Amplitude–frequency histogram of e.p.p.s collected at 1, 3·5, 5·5, 8 and 12 min during continual stimulation of a synapse at 10 Hz. The continuous lines are the binomial predictions assuming that the amplitude and variance of the quantal unit at any particular time is the same as that of the m.e.p.p.s recorded at that time. At the different times, the values of the binomial parameters $\bar{m}$, p and n respectively were: 1 min, 23·23 ± 1·14, 0·51 ± 0·08, 45·30 ± 8·20; 3·5 min, 19·78 ± 0·97, 0·60 ± 0·07, 33·01 ± 4·62; 5·5 min, 17·31 ± 0·62, 0·67 ± 0·05, 25·69 ± 2·34; 8 min, 12·78 ± 0·47, 0·66 ± 0·05, 19·23 ± 1·80; 12 min, 12·20 ± 0·42, 0·67 ± 0·05, 18·21 ± 1·95. Number of e.p.p.s used to construct histograms at each period were in excess of 90. Reinnervated synapse in $[Ca]_o$ = 1·8 mM and $[Mg]_o$ = 1·2 mM.

nerve terminal that determine the fraction of a pool (N) that supplies n and that during facilitation there is an accumulation of residual [CaX] following each nerve impulse in a train (eqn. (5); Younkin, 1974; Barrett & Stevens, 1972; Bennett *et al.* 1976). It is at first surprising that even in very

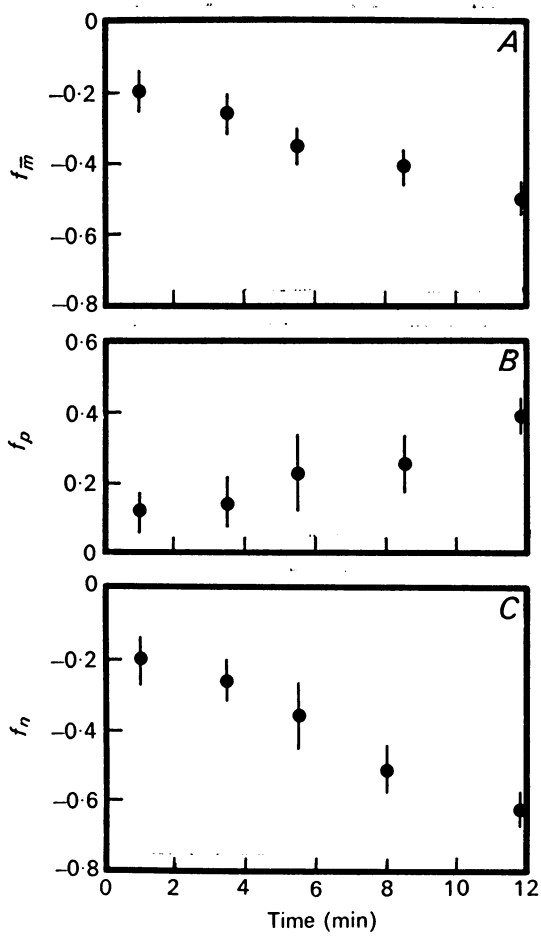

Fig. 10. Changes in the binomial parameters describing transmitter release during continual stimulation at 10 Hz. The changes in $f_{\bar{m}}$, f_p and f_n are shown at 1, 3·5, 5·5, 8·5 and 12 min after the beginning of continual stimulation. Each point is the mean ± 1 S.E. of the mean of results from five synapses, at each of which $f_{\bar{m}}$, f_p and f_n were determined at the times indicated by applying a binomial analysis to at least 90 e.p.p.s collected at those times, using at least 30 m.e.p.p.s collected at the same time to give a measure of quantal size at that time; the S.E. of the mean of each parameter at any time was always less than 15% of the mean. The mean value ± 1 S.E. of the mean of $\bar{m}$, p and n at the beginning of continual stimulation at the five synapses were 13·76 ± 2·81, 0·69 ± 0·05, and 21·61 ± 6·33 respectively. Reinnervated synapses in $[Ca]_o$ = 1·8 mM, $[Mg]_o$ = 1·2 mM. Data from Table 3.

high $[Ca]_o$ (10 mM), the first few impulses in a train can be described by the idea of an accumulation of residual [CaX], for at these calcium levels it might be anticipated that n would be almost saturated at about its maximum value of 320 (i.e. at L_n in eqn. (4), see Bennett *et al.* 1977), determined on the basis of the effects of $[Ca]_o$ on the n of unconditioned impulses. A possible explanation for why n is not saturated in high $[Ca]_o$ is that there is a hyperbolic relationship between the steady-state level of $[Ca]_i$ and $[Ca]_o$ (Hodgkin & Keynes, 1957; eqn. (2)), so that the maximum value that the n for an unconditioned impulse can reach with an increase in $[Ca]_o$ (namely L_n) is determined by the level at which $[Ca]_i$ saturates with an increase in $[Ca]_o$. On the other hand if there is a linear relationship between the transient increase in the internal calcium concentration which accompanies the nerve impulse and an increase in $[Ca]_o$ (Hodgkin & Keynes, 1957; Baker *et al.* 1971) which is not subject to saturation effects in high $[Ca]_o$, n may still increase according to the residual [CaX] hypothesis even in high $[Ca]_o$.

Mallart & Martin (1967) and Magleby (1973) predict the increase in facilitation of the e.p.p. during short trains of impulses according to the residual [CaX] hypothesis in which there is only a linear rather than a third power dependence of quantal release on [CaX]. In nearly all their studies these authors used frequencies $\leqslant$ 50 Hz, and at 50 Hz the discrepancy between a first and third power prediction is about 20% with an $f_{\bar{m}_0}$ of near 0·9. It is possible then that a third power prediction would give a resonable fit to their results.

It might be argued that facilitation is simply due to an increase in the transient calcium entry with successive impulses, as has been observed in some invertebrate neurones (Stinnakre & Tauc, 1973). However, if this were the case at the nerve terminal it would be expected that p would increase substantially during facilitation (see Bennett *et al.* 1977), and this is not observed. A parallel increase in p and n is observed though during the development of the potentiated increase in quantal content accompanying a train of impulses in low $[Ca]_o$ (personal observations), and this is readily accounted for by the increase in steady-state level of calcium in the terminal (Weinreich, 1971) which occurs during continual high-frequency stimulation (Baker *et al.* 1971).

Changes in the number of quanta available for release during short-term depression

Short trains of impulses in high $[Ca]_o$. About half a second after a short high-frequency train of impulses in high $[Ca]_o$ at toad neuromuscular synapses there is a depression in the quantal content of a test e.p.p. which lasts for several seconds and recovers with a time constant of about 4 sec similar to that observed at other neuromuscular synapses (Liley & North, 1953; Takeuchi, 1958; Thies, 1965; Betz, 1970), and this depression is due to a decrease in the number of quanta available for release. The parameter n decreases (as $f_{\bar{m}_0}$ does, see Fig. 6) approximately linearly with an increase in the number of quanta released in the conditioning train, suggesting that

the decrease in n is due to the loss of quanta from the pool which maintains n. Thus the changes in n during a short high-frequency train can be predicted on the basis that facilitation is due to an increase in the fraction of the pool which supplies n and depression to the loss of quanta from this pool as a consequence of the release of quanta (eqn. (6); see also Mallart & Martin, 1968).

It should be noted that although the analysis of the depression of transmitter release at neuromuscular junctions by Mallart & Martin (1968) and Betz (1970) assumed that facilitation was due to an increase in p, whereas it is primarily due to an increase in n, their studies were carried out in high $[Ca]_o$ ($> 1{\cdot}8$ mM) in which p is close to 1. Thus their analysing the initial facilitation in a short high-frequency train in terms of an increase in p is actually equivalent under these ionic conditions to the assumption that facilitation is due to an increase in the fraction of the pool that supplies n as in the present analysis; as they also attributed depression to depletion, the good predictions of their observed results (see Fig. 6, Mallart & Martin (1968)) may be taken as further support for the present theory concerning changes in the number of quanta available for release.

Long trains of impulses in high $[Ca]_o$. At very large quantal releases involving up to about ten conditioning impulses the depression in $f_{\bar{m}_0}$ and therefore in n was not quite as great as that expected for a linear relationship between loss of available quanta and depression. Betz (1970) has noted a similar deviation of $f_{\bar{m}_0}$ from a linear dependence on quantal release in high calcium at the mammalian neuromuscular junction ($[Ca]_o \geqslant 2$ mM, so that $p \cong 1{\cdot}0$, as shown in Table 2*B* and Bennett *et al.* (1975)). This decrease in the expected depression of n on the basis of a loss of quanta after a train of impulses in very high $[Ca]_o$ is possibly due to the acquisition of new quanta by the pool which supplies n, towards the end of the short conditioning train. Such an acquisition of new quanta must be supposed to occur eventually during a long train of impulses in order for n to reach a steady-state depressed value (Elmqvist & Quastel, 1965). A decrease in n due to loss of quanta from the pool which supplies n as a consequence of release together with the acquisition of new quanta by this pool provides the basis for a quantitative description (eqn. (7)) of the depression in n which is observed during a long train of impulses.

Changes in the number of quanta available for release during long-term depression

During continual high frequency nerve stimulation in high $[Ca]_o$ the quasi steady-state depression reached in e.p.p. amplitude during the first few seconds of stimulation is followed by a further slow developing depression over several minutes of stimulation until a steady-state and greatly depressed quantal release rate is reached (Bennett & McLachlan, 1972*a*,*b*; Bennett & Florin, 1974), and this is primarily due to a decrease in the

number of quanta available for release (Bennett & Florin, 1974; McLachlan, 1975; Bennett *et al.* 1976). The most likely explanation for this decrease in n is that as a consequence of a large loss of quanta from the nerve terminal, there is a gradual decrease in the number of quanta acquired by the pool that supplies n and that this continues until the number of quanta which enter this pool is the same as the number of quanta newly synthesized (Bennett & McLachlan, 1972*a*,*b*). At this time the number of quanta available for release, n, has reached a steady-state depressed value which is then maintained without further changes during stimulation.

APPENDIX

The estimation of the binomial parameters n and p

The question arises as to the effects of variation in the parameters n and p, on the estimates and their interpretation. Brown, Perkel & Feldman (1976) have considered the case with large values of n and small values of p (their simulations have $n = 50$ and $p = 0{\cdot}2$). Even with no variation in n and p it is hardly possible in this case to distinguish between a binomial distribution and a Poisson distribution and when the variance is increased by temporal variation in n and p this distinction is more difficult to make. In this situation it cannot be reasonable to fit a binomial distribution and if this is done the standard error of $\hat{n}$ will be extremely large, reflecting the difficulty of estimation; large values of n with small values of p were not observed in this or the previous work (Bennett *et al.* 1977). When n is small ($n < 10$) and p is not small ($p > 0{\cdot}3$), as is frequently observed, different results are obtained.

Consider J trials, where in any trial a random number, N, of quanta are available for release and the probability of release of a particular quantum in a particular trial is P, a random variable depending on the quantum and the trial. Let $\bar{P}$ be the average of P over quantum in a particular trial and let σ_p^2 be the variance of P about $\bar{P}$ in a given trial. It is not assumed that N and P are independent. Then, if r_i is the number of releases in the ith trial, $i = 1, \ldots, J$, and m and s^2 are the mean and variance of these r_i, then it can be shown directly that

$$Em = E(\Sigma r_i/J) = E(N\bar{P}),$$

$$Es^2 = E[\Sigma(r_i - m)^2/(J-1)] = E[N\bar{P}(1-\bar{P}) - (N-1)\sigma_p^2] + V(N\bar{P}).$$

If n and p are the expectations of N and P, then it is possible to consider the estimates of n and p which could be used if it were assumed that N and P were fixed. Moment estimates are commonly used but maximum likelihood estimates are given by Haldane (1941) and for small n and moderately large p, good estimates are obtained by taking

$$\hat{n} = \max(r_i) \quad \text{or} \quad \hat{n} = \max(r_i) + 1, \quad \text{and} \quad \hat{p} = m/\hat{n}.$$

The better of the two estimates of n is chosen by considering the probability that $r_1 < n$ for all $i = 1, \ldots, J$. If this probability, $(1-p^n)^J$, is less than 0·5 the first estimate is better, otherwise the second is better.

In order to see the effect of variation in N and P, several sub-cases are considered.

(i) $P = p$ is fixed. N has mean n and variance σ_n^2. The formulae above reduce to

$$Em = np, \quad Es^2 = np(1-p)+p^2\sigma_n^2.$$

By any of the above methods n will be over-estimated and so p will be under-estimated. However, unless σ_n^2 is small compared to n, the distribution will have s^2 at least close to m and it would be realized that the procedures would give poor estimates. In fact, it may not be reasonable to attempt to estimate n and p, as is apparent if it is supposed that N has a binomial distribution with parameters ν and π. Then the number of releases has a binomial distribution with parameters ν and πp. If ν is large and π small, N has approximately a Poisson distribution with parameter $\nu\pi$ and thus the number of releases is also approximately Poisson with parameter $\nu\pi p$. In this case, if s^2 is significantly less than m, the assumptions may be assumed to hold.

(ii) $\sigma_p^2 = 0$, $N = n$, $\overline{P}$ distributed with mean p and variance $\sigma_{\overline{p}}^2$. Here

$$Em = np, \quad Es^2 = np(1-p)+n(n-1)\sigma_{\overline{p}}^2.$$

Unless $(n-1)\sigma_{\overline{p}}^2$ is small compared to p^2, s^2 will be at least close to m and the model would not be used. In other cases, if n is small and p is not too small and $\hat{n} = \max(r_i)$ or $\hat{n} = \max(r_i)+1$, is used as the estimate of n, then it will be a reasonable estimate of n and $\hat{p}$ will be a good estimate of p.

(iii) $N = n$ is fixed. Two cases arise naturally; (*a*) $\overline{P}$ is fixed and P takes n different fixed values $p_1, \ldots, p_n$ for the n quanta; (*b*) P is independently and identically distributed for each quantum with the same distribution in every trial.

(*a*)

$$Em = n\overline{p}, \quad Es^2 = n\overline{p}(1-\overline{p})-n\sigma_p^2,$$

where

$$\overline{p} = \Sigma p_i/n, \quad \sigma_p{}^2 = \Sigma(p_i-\overline{p})^2/(n-1).$$

In this case none of the above methods will estimate n and $\overline{p}$ accurately, unless none of the p_i are too small. If all p_i are moderately large, the number of trials is large and n is not too large, then $\hat{n} = \max(r_i)$ or $\hat{n} = \max(r_i)+1$, will be a good estimate of n and $\hat{p} = m/\hat{n}$ will be a good estimate of $\overline{p}$. If some p_i are very small, it may be reasonable not to regard the corresponding quanta as available quanta. Then these methods will estimate the number of 'effective' available quanta.

(*b*) If P has mean p, then the distribution of the number of releases will be binomial with parameters n and p and so the methods will be appropriate in this case and as before

$$Em = np, \quad Es^2 = np(1-p).$$

As these cases illustrate, if the methods are used only for appropriate values of n and p and if standard errors of estimates are always given, then the erroneous inferences discussed by Brown *et al.* (1976) will be avoided and the simple binomial model may be used until evidence against its accuracy is produced.

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