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ESTIMATES OF PROBABILITY OF TRANSMITTER RELEASE AT THE MAMMALIAN NEUROMUSCULAR JUNCTION

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SUMMARY

1. Intracellular records of end-plate potentials (e.p.p.s) were obtained from curarized neuromuscular junctions in the rat diaphragm.

2. Statistical estimates of the quantal release probability (p) were made at individual junctions from measurements of the means and variances of e.p.p. amplitudes at two different levels of Ca concentration. It was assumed that the release process was binomial and that the number of quanta available for release (n) was independent of external Ca.

3. These estimates of p were compared with those obtained by measuring depression of e.p.p. amplitudes after single conditioning shocks and assuming that the depression was due to depletion of n. The statistical estimates were consistently smaller.

4. This disparity, plus the additional observation that depression was not linearly related to the number of quanta released by one or more conditioning shocks, suggested that depression was not due entirely to depletion and that the depletion assumption led to an over-estimate of p.

5. A third method of estimating p from measurements of the decline of e.p.p. amplitudes during rapid stimulation also appeared to result in an over-estimate.

INTRODUCTION

The quantum hypothesis of transmitter release from nerve terminals proposed by del Castillo & Katz (1954) states that there is a large population (n) of quanta in the nerve terminal, each with a small probability of release in response to a nerve impulse. If the average of these probabilities (p) is small, then on successive trials the number of quanta released should fluctuate in a manner described by the Poisson distribution. The hypothesis has been tested extensively at a number of synapses and in almost every case the fluctuations in release have been as expected (see Martin, 1965). Tests involving the Poisson distribution require a knowledge only of the mean number of quanta (m) released during a series of responses

and the parameters n and p are not relevant. However, the quantum hypothesis implies binomial statistics and it is of interest to determine whether numerical values of n and p can be estimated at individual synapses. Previous estimates of release probability have been made either from depression of end-plate potential (e.p.p.) amplitude after a single conditioning shock (Liley & North, 1953; Takeuchi, 1958; Thies, 1965) or from the rate of decline of e.p.p. amplitude during rapid stimulation (Elmqvist & Quastel, 1965), and in both cases involve the assumption that the reduction in amplitude is due to depletion of the available pool of transmitter with no change in release probability.

In the present series of experiments a third method of estimating p was used and the results compared with those obtained from depression measurements. This method of estimating p consists of measuring the statistical fluctuations in amplitude of two series of e.p.p.s recorded from the same junction at two different external Ca concentrations (cf. Blackman, Ginsborg & Ray, 1963). If the release process is binomial, the mean quantum content for a series will be given by

$$m = np \tag{1}$$

and the coefficient of variation (CV) will be related to m and p by

$$CV^2 = m^{-1}(1-p). \tag{2}$$

If the subscripts a and b refer to series obtained in low and high Ca concentrations respectively, then

$$\frac{CV_a^2}{CV_b^2} = \frac{m_b(1-p_a)}{m_a(1-p_b)}. \tag{3}$$

If we assume that Ca alters m only by altering p, with n remaining unchanged, then $p_a/p_b = m_a/m_b$ and eqn. (3) may be rewritten as

$$\frac{CV_a^2}{CV_b^2} = \frac{m_b(1-p_a)}{m_a(1-p_a m_b/m_a)}. \tag{4}$$

In curarized preparations the mean and coefficient of variation of the quantum content distributions cannot be measured directly. However, if appropriate correction is made for non-linear summation of the units making up the e.p.p., the amplitude of an e.p.p. is proportional to its quantum content. Consequently the ratio of the means and of the coefficients of variation at the two different Ca concentrations will be the same for the e.p.p. amplitude distribution as for the quantum content distribution.

If we now let CV refer to the coefficient of variation of the amplitude distributions and write $CV_a/CV_b = R$, and replace the ratio m_a/m_0 by

$v_a/v_b = r$, where v represents the mean amplitude of e.p.p.s in a series, then eqn. (4) may be rewritten

$$R^2 = \frac{1-p_a}{r-p_a},$$

from which

$$p_a = \frac{rR^2-1}{R^2-1}. \tag{5}$$

Apart from the hypothesis that release is a binomial process, the only assumption involved in this estimate is that changes in external Ca concentration alter transmitter release solely by altering the release probability and do not affect the number of quanta available for release. Indirect evidence for this assumption has been obtained by Elmqvist & Quastel (1965). Also, since iontophoretically applied Ca has a very rapid effect on release (Katz & Miledi, 1967), it has been concluded that the site of action of the ion is at the outer surface of the nerve terminal membrane. Consequently the present assumption seems reasonable.

In the present experiments estimates of p made in this way were consistently smaller than those obtained from measurements of depression of e.p.p. amplitude.

METHODS

All experiments were done on the isolated hemi-diaphragm of the rat removed from the animal with a length of phrenic nerve and placed in a Perspex chamber. The muscle was bathed in a saline solution and the nerve placed on stimulating electrodes under oil in a side chamber. The composition of the bathing solution was as follows (mM): NaCl, 136; KCl, 5·0; $CaCl_2$, 2·0; $MgCl_2$, 1·0; $NaHCO_3$, 12·0; Na_2SO_4, 1·0; glucose, 11·0. In 'Low Ca' solutions $CaCl_2$ was reduced to 1·5 mM and in 'High Ca' increased to 3·0 mM. (+)-tubocurarine chloride (TC) was added in a concentration of 1·0–1·3 μg/ml. The solution was bubbled with a gas mixture containing 5 % CO_2, 95 % O_2 in an external reservoir from which it flowed through the chamber at a rate of about 3 ml./min. All experiments were done at room temperature.

E.p.p.s were recorded intracellularly with glass micropipettes filled with 4 M-K-acetate and having resistances of about 15 MΩ. These were connected to the input of a unity-gain, high-impedance preamplifier, the output of which was fed into an oscilloscope. E.p.p. amplitudes were measured by feeding the oscilloscope output to a voltage-to-frequency converter coupled to a counter. The counter was gated for 0·5 msec at the peak of the e.p.p. and thus gave a reading proportional to e.p.p. amplitude. There were no detectable variations in gate width and the system was monitored continuously for base line drift and inappropriate positioning of the gate. In addition, readings were spot-checked against photographed records of the same e.p.p.s. For experiments requiring measurements of two or more e.p.p.s in rapid succession, measurements were made from film records.

Up to 200 e.p.p. amplitudes were recorded in both low and high Ca concentrations for the statistical estimate of p, with records being taken at 30 sec intervals. However, it was seldom possible to use more than 100 of these because of drift in amplitude during some part of a series. When such drift occurred the measurements were discarded.

RESULTS

Resting membrane potentials of individual muscle fibres were in the range of 60–80 mV, with a mean of 71 mV. E.p.p. amplitudes were about 2 mV in low-Ca solutions with the concentrations of TC used and increased, on the average, to about 7 mV in high-Ca solutions. These amplitude levels were chosen to keep the correction for non-linear summation small without reducing the responses in low-Ca too near the base line noise level. The correction factor is inaccurate, tending to overcorrect (cf. Martin & Pilar, 1964). The inaccuracy is due to the fact that the membrane capacitance was ignored in deriving the correction (Martin, 1955) and is not significant providing the correction itself is small (A. A. Auerbach, personal communication).

Comparison of depression and statistical estimates of p

Depression of the e.p.p. after single conditioning shocks was measured in low Ca solutions in nine experiments, and the results compared with estimates of p obtained from eqn. (5). Fig. 1*A* shows e.p.p.s produced by two successive stimuli of the motor nerve with an interval of 2 sec between them. The second response is reduced by about 25%. In Fig. 1*B* the amplitude of the second response, v, expressed as a fraction of that of the first, v_1, is plotted against the interval between the two shocks. As has been reported previously (Liley & North, 1953; Takeuchi, 1958; Thies, 1965), recovery from depression is exponential, in this case having a time constant of about 4 sec. The recovery curve extrapolates to 0·64 at zero time. For subsequent discussion, 'zero time depression', D_0, is defined as the value of $1 - v/v_1$ at the intercept; in this example its value is 0·36. If it is assumed that depression is due entirely to depletion of the available transmitter it may be concluded that the first response released 36% of the available pool or, in other words, that p was 0·36. Values obtained in this way (p_D) are shown in Table 1, and ranged from 0·16 to 0·60 with a mean of 0·39.

Statistical estimates of p (p_a) at the same junctions averaged $0{\cdot}12 \pm 0{\cdot}05$ (mean $\pm$ S.D. of mean). The major difficulty with the statistical method was that the S.D. of the individual estimates was very large. Nevertheless, their values were consistently less than those obtained from measurements of depression. In each experiment the coefficient of variation, together with the value of p_a, was used to calculate m in low calcium concentrations (m_a) from eqn. (2). Finally, eqn. (1) was used to obtain n. These are shown in the Table and had means of 106 and 1190 respectively. It should be emphasized that because of the large S.D. of the individual estimates of p_a, the individual values of n are equally uncertain. Values of

m are less dependent on p_a and are consequently somewhat more reliable but suffer from the usual inaccuracies associated with estimating the coefficient of variation (see Edwards & Ikeda, 1962; Martin, 1965).

Values of p obtained by the two methods are compared in Fig. 2, in which p_a is plotted against p_D. The continuous line indicates agreement between the two estimates and it can be seen that the statistical values are consistently smaller.

TABLE 1. Values obtained for release parameters in nine experiments. v_a, v_b: mean amplitudes of e.p.p.s in low (1·5 mM) and high (3·0 mM) Ca. CV_a, CV_b: coefficients of variation of corresponding amplitude distributions. p_a: quantal release probability in low Ca calculated from eqn. (5) ($\pm$ S.D.). p_D: release probability from depression measurements. m_a, n: mean quantum content in low Ca and estimated number of available quanta, calculated from eqns. (2) and (1)respectively

Expt.	v_a(mV)	v_b(mV)	CV_a	CV_b	p_a	p_D	m_a	n
I	2·67	4·95	0·072	0·049	0·13 ± 0·16	0·16	167	1280
II	1·30	10·38	0·119	0·031	0·06 ± 0·08	0·22	71	1180
III	1·51	7·84	0·099	0·032	0·10 ± 0·12	0·36	91	910
IV	2·25	5·71	0·086	0·041	0·21 ± 0·29	0·44	108	520
V	2·49	7·14	0·075	0·042	0·05 ± 0·22	0·36	168	3360
VI	2·62	5·74	0·073	0·047	0·07 ± 0·35	0·52	169	2420
VII	1·53	9·11	0·142	0·035	0·12 ± 0·12	0·44	44	370
VIII	1·19	4·90	0·106	0·037	0·14 ± 0·16	0·40	77	290
IX	1·73	8·30	0·115	0·027	0·16 ± 0·14	0·60	63	390
Mean	1·92	7·12	—	—	0·12 ± 0·05	0·39 ± 0·13	106	1190

Relation between depression and transmitter release

The lack of agreement between the two estimates of release probability suggests that depression of the e.p.p. is not solely due to transmitter depletion. In order to obtain additional evidence on this point the effect on depression of increasing the amount of transmitter released by the conditioning stimulation was examined. The results of two such experiments are shown in Fig. 3. The amount of transmitter release was increased either by giving more than one conditioning shock or by raising the external Ca concentration. For each point a recovery curve similar to that in Fig. 1*B* was obtained and extrapolated to zero interval to obtain a value for D_0. The abscissa in Fig. 3 is the summed amplitudes of the conditioning responses expressed as a multiple of the amplitude of a single response in normal Ca. This is proportional to the amount of transmitter released. In the experiment represented by triangles, a single conditioning shock in normal Ca solution produced about 37% depression. Giving two conditioning shocks 20 msec apart produced two e.p.p.s whose summed amplitudes were about 1·9 times greater (because of facilitation superimposed on depression at short intervals) but increased depression to only 40% (filled triangles). A single shock in twice normal Ca (open triangle)

produced an e.p.p. whose amplitude was more than twice that in normal Ca, but increased depression to only 41 %. Similar results are shown for another experiment in which the effects of single and double conditioning shocks are shown by filled circles and the effect of doubling Ca by the open circle. In the trials represented by half-filled circles Ca was reduced to 1·5 mM and two and three conditioning shocks used. If depression were

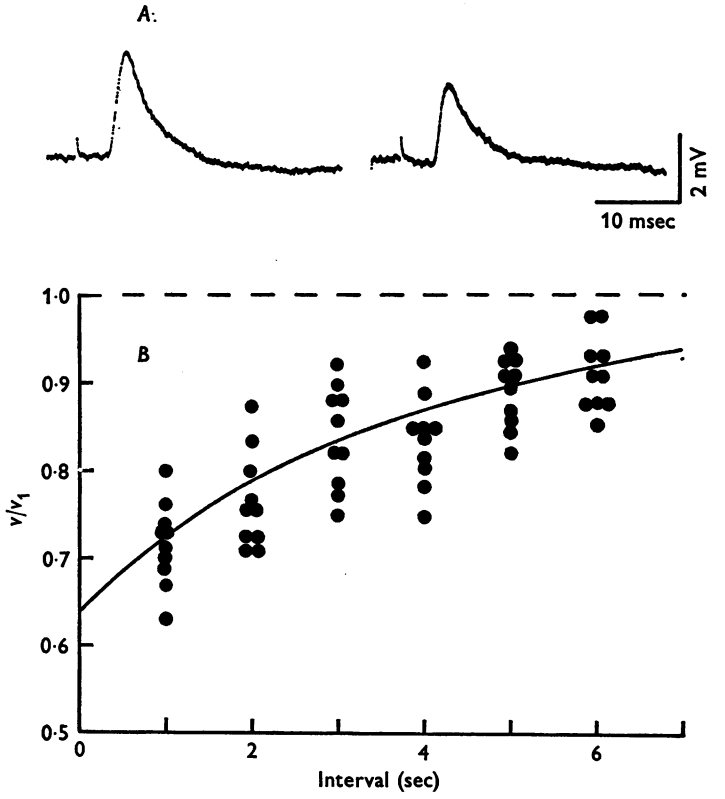

Fig. 1. *A*: e.p.p.s produced by two stimuli to motor nerve at 2 sec interval. Second response reduced by about 25 %. *B*: plot of amplitude of second response (v), expressed as fraction of amplitude of first (v_1), against interval between shocks. Ten trials at each interval. Recovery curve regression is exponential with time constant of about 4 sec. 'Zero time depression' (D_0) taken as $1-v/v_1$ at zero interval.

due only to depletion of the available transmitter, then the relation between D_0 and the amount of transmitter released by the conditioning responses should have been linear; i.e. if one e.p.p. reduced the amplitude of the test response by say 30 %, then two e.p.p.s of the same amplitude should have reduced the test response by 60 %. This was clearly not the

case. A similar lack of linear relation between transmitter release and depression has been demonstrated by Betz (1970) at the frog neuromuscular junction. In experiments on the frog deviation from linearity could be explained by assuming that n and p were reduced proportionately during depression, i.e. that $p/p_0 = n/n_0$, where p_0 and n_0 represent initial resting values of n and p. This relation was assumed to apply here as well. The somewhat greater deviation from linearity indicated in Fig. 3 was probably

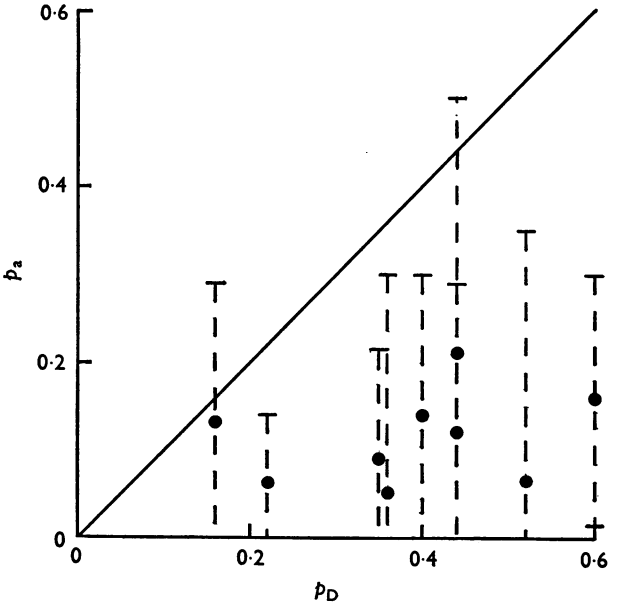

Fig. 2. Comparison between statistical estimates of release probability (p_a) and estimates obtained by assuming depression of e.p.p. due to depletion of transmitter (p_D). Statistical estimates are consistently smaller. Bars are at 1 S.D. of the statistical estimate.

due to experimental error associated with fluctuations in individual responses. The main point to be made is that the relation was, in fact, non-linear.

The experiments in Fig. 3 also provide some additional support for the idea that Ca operates primarily on the release mechanism and leaves the store of transmitter unchanged. Reducing external Ca caused a reduction in e.p.p. amplitude and in depression. When the amount of transmitter released by the conditioning stimulation was restored by giving two or more conditioning shocks, the amount of depression increased to its previous level (half-filled circles), suggesting that the relation between depression and release was independent of Ca concentration. If the main

effect of Ca were on the transmitter store, one would have expected to see a relatively greater depression in reduced Ca after release of the same amount of transmitter, assuming that at least part of the depression was due to depletion. If the statistical estimates of p are correct, it follows that a single shock should deplete the transmitter population by about 12% in low Ca (Table 1) and by a somewhat greater fraction, perhaps about 20%, in normal Ca. Thus if D_0 in normal Ca is about 40% (Fig. 3), about half the observed depression may be attributed to depletion at normal release levels. The remainder would then be attributable to a reduction in release probability.

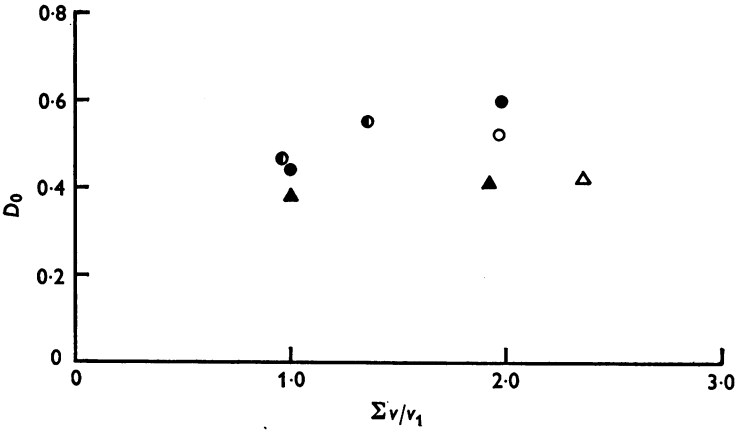

Fig. 3. Variation in depression of e.p.p. amplitude with amount of transmitter released by conditioning stimulation. Ordinate: 'zero time depression' obtained as in Fig. 1*B*. Abscissa: summed amplitude of conditioning responses expressed as multiple of amplitude of single response in normal (2 mM) Ca. Two experiments. ●, ▲: single and double (20 msec separation) conditioning shocks in normal Ca. ○, △: single shock in twice normal Ca. ◐: two and three conditioning shocks in 1·5 mM-Ca.

Depression during repetitive stimulation

The method of estimating n and p proposed by Elmqvist & Quastel (1965), consists of applying short, high-frequency (e.g. 100/sec) trains of stimuli to the motor nerve and measuring the reduction in amplitude of successive e.p.p.s. The result of such a procedure is illustrated in Fig. 4. The amplitudes of individual e.p.p.s are then plotted against the sum of all previous amplitudes. Fig. 5*A* shows the theoretically expected results of such a plot, assuming that the stimulation is sufficiently rapid so that there is no recovery from depletion of transmitter between shocks. E.p.p. amplitudes (v) are expressed as fractions of the amplitude of the first response in the train (v_1). Points on the graph were obtained by successive numerical

calculations. When p is assumed constant (open circles), in this case with a value of 0·2, the plot is linear and intercepts the abscissa at $1/p$. The theoretical effect of facilitation, ascribed to an increase in p and of a magnitude and time course similar to that described by Mallart & Martin (1967), is shown by the filled circles. The triangles indicate the expected results when it is assumed that both p and n are reduced during depression in such a way that $p/p_0 = n/n_0$. In all three cases the lines approach the abscissa at $\Sigma r_1/v_1 = 5$. In other words, if the initial release probability, p_0,

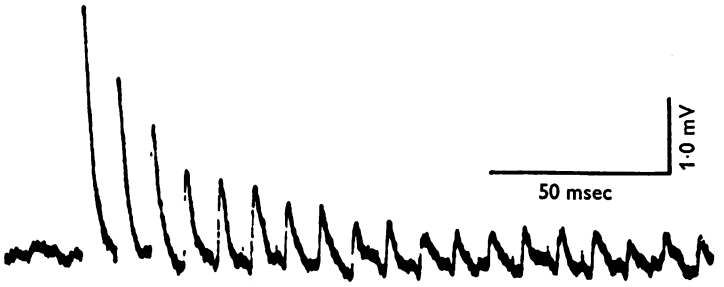

Fig. 4. E.p.p.s from curarized preparation produced by brief train of stimuli at 100/sec.

is 0·2, then the e.p.p. amplitude will be expected to reach zero when an amount of transmitter equal to five times that released in the initial response has been discharged, regardless of any intervening changes in p. However, as might be expected from the recovery curve of Fig. 1*B*, recovery between shocks is not zero and, as shown in Fig. 4, the e.p.p. amplitudes approach a steady state which is greater than zero. At a frequency of 50/sec recovery between shocks can be expected to amount to about 1% of the net amount of transmitter depletion. The calculated effects of such recovery are shown in Fig. 5*B*. The usual procedure is to extrapolate the linear part of the curve to the abscissa and take the intercept of the extrapolated line as the expected result in the absence of such recovery. It can be seen from Fig. 5*B* that such extrapolation should give a reasonably accurate estimate of p_0 if this parameter remains constant or if there is facilitation, but may lead to a gross over-estimate of p_0 if the release probability declines during depletion of the transmitter.

Several such experiments were done in the present series and the results of one of these are shown in Fig. 6. The open circles and crosses represent two different trials at the same end-plate at a stimulus frequency of 100/sec. Extrapolation of the linear portion of the dashed line indicates a value of 0·27 for p (arrow). The later part of the dashed line indicates the expected steady state level of e.p.p. amplitude using this value of p and

assuming 0·5 % recovery of the net transmitter depletion between shocks at this frequency. The continuous line is drawn on the assumption that p and n are falling proportionately during the train with the same fractional recovery of n between shocks (0·5 %) and with $p_0 = 0{\cdot}14$. If one accepts the previous evidence that depression is attributable to a reduction in release probability as well as depletion of the available transmitter, then the solid line would be more appropriate and the usual method of extrapolation would overestimate the initial release probability by a factor of

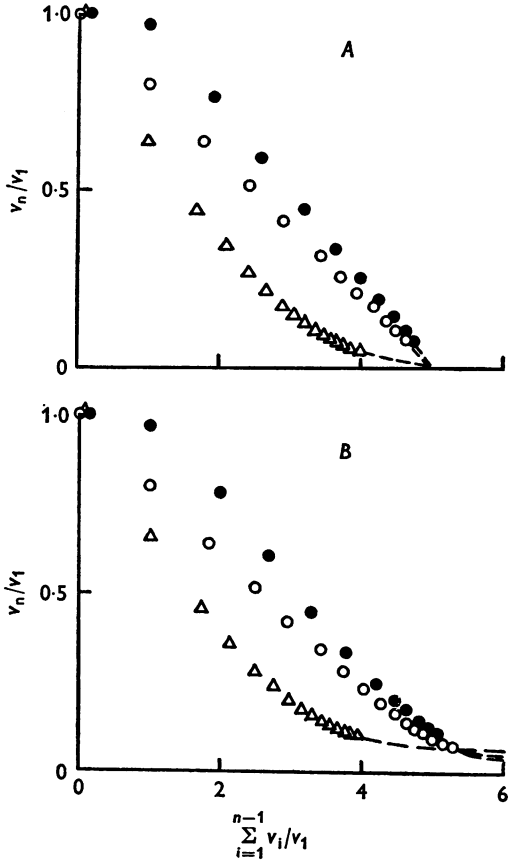

Fig. 5. Theoretically expected decline in e.p.p. amplitudes during brief train, such as shown in Fig. 4. Amplitude of each successive e.p.p. (v_n) expressed as a fraction of initial amplitude (v_1), plotted against sum of all previous amplitudes. Points are successive numerical calculations. *A:* no recovery from depletion of transmitter between shocks. ○: release probability assumed constant; ●: facilitation of release probability; △: depression of release probability with depletion of transmitter (see text). *B:* same curves re-plotted, assuming 1 % of net loss of transmitter replenished between shocks.

approximately 2. Other experiments gave similar results, except that in some plots there appeared to be a slight early facilitation of release probability before the later depression. In all cases the steady-state value of e.p.p. amplitude was larger than would be expected if p were constant and if recovery were determined by an exponential as shown in Fig. 1*B*.

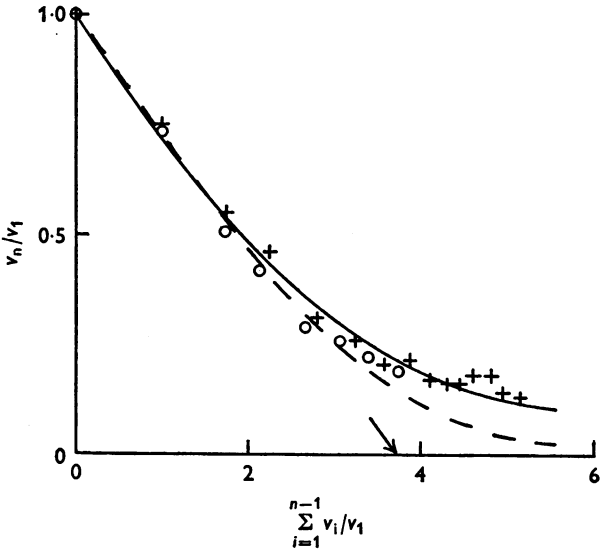

Fig. 6. Experimentally observed decline of e.p.p. amplitude during brief train of stimuli at 100/sec, as shown in Fig. 4. Results plotted as in Fig. 5. ○, +: two different trials at same junction. Dashed line fit to points assuming release probability constant at 0·27 and 0·5% recovery from depletion between shocks. Continuous line assumes same recovery and reduction in release probability with depletion from an initial value of 0·14 (see text).

DISCUSSION

The results presented here suggest that depression of e.p.p. amplitude by previous activity cannot be attributed solely to depletion of the available pool of transmitter. This conclusion is supported by the disparity between the statistical estimates of p and those obtained from depression, by the observation that depression was not linearly related to the amount of transmitter released by the conditioning stimuli and by the relatively poor fit of the experimental points in Fig. 6 to the depletion assumption (dashed line). These observations suggest that depression of e.p.p. amplitude, either by prior conditioning stimuli or during 'tetanic rundown', is associated with a fractional reduction in release probability at least equal to the fractional depletion of the available transmitter.

The lack of a linear relation between the amount of transmitter released and subsequent depression of the e.p.p. has been demonstrated previously by Betz (1970) at the frog neuromuscular junction but does not agree with earlier observations by Thies (1965) on the guinea-pig neuromuscular junction. This disparity may be due to the fact that the linear relation shown by Thies was with pooled results from twelve different end-plates at different temperatures and Mg and Ca concentrations, rather than with different levels of release at single junctions.

The present results suggest that the method of Elmqvist & Quastel (1965) for measuring the various release parameters may lead to a serious overestimate of p and underestimate of n. Thus in Fig. 6, the usual linear extrapolation from the experimental points suggested a value of p of 0·27, whereas the assumption of equal fractional reductions of both n and p during the train led to a value of 0·14 for the initial release probability and provided a better fit to the experimental points.

It has been pointed out by Ginsborg (1970) that previous examinations of statistical fluctuations of e.p.p. and synaptic potential amplitudes have shown only that the release process obeys Poisson statistics, and that there is no reason to suppose that release is anything other than a Poisson process, in which case n and p would have no existence as measurable variables. Deviations from Poisson fluctuations have been shown to be due variously to non-linear summation of unit potentials making up the e.p.p. (del Castillo & Katz, 1954; Martin, 1955), or to masking of the true distribution by attenuation of the responses along dendrites (Kuno & Miyahara, 1969), or attributed to insufficient experimental data (Blackman *et al.* 1963). However, if release were a Poisson process the present estimates of p_a should have been scattered around zero rather than around a finite mean. While none of the individual estimates was significantly different from zero, the fact that they were all positive supports the idea that p is a measurable variable related to the release process and that the process itself is a binomial one. Recent experiments by Blackman & Purves (1969) on sympathetic ganglia also suggest that release is a binomial process, although it is possible that the failure of the fluctuations in release to obey Poisson statistics may have been due to other causes such as dendritic attenuation.

The main conclusions to be made from the present results are that n and p appear to be identifiable as separate parameters and that they are both reduced during depression. One interpretation of the quantum hypothesis is that the quanta are associated with vesicles in the presynaptic terminal and that the release probability represents the probability of interaction of vesicles with the membrane. With this model one could account for a reduction in p as n is depleted by assuming that vesicles in close proximity

to the membrane have a higher probability of release than those farther away. A conditioning shock would then deplete the terminal of those vesicles with the highest probability of release and leave the remaining population with a reduced mean release probability. There are, of course, several other possible models, all equally speculative.

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