J. Physiol. (1976), **259**, *pp.* 367–393
With 12 text-figures
Printed in Great Britain

SYNAPTIC DEPRESSION RELATED TO PRESYNAPTIC AXON CONDUCTION BLOCK

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(*Received 22 September 1975*)

SUMMARY

1. The depression of synaptic transmission, which occurs during prolonged repetitive activation, was examined in the opener muscle of the crayfish walking leg.

2. Excitatory post-synaptic potentials (e.p.s.p.s) initially facilitated but then declined to low amplitudes after about 4000 stimulus pulses had been delivered; this depression is presynaptic in origin.

3. Axon conduction blocks occurred at points of bifurcation along the entire length of the presynaptic nerve. This resulted in failure of the nerve impulse to invade some branches of the terminal arborization.

4. Nerve terminal invasion failure caused either intermittent or complete inactivation of some synaptic release sites; this was associated with depression of the post-synaptic response.

5. The statistics of transmitter release during prolonged repetitive stimulation were examined by focal extracellular recording methods. Transmitter release could be described by binomial statistics, and depression involved a drop in m, n and p.

6. The rate of spontaneous quantal release did not decrease, however, arguing against transmitter depletion.

7. It is concluded that repetitive stimulation eventually leads to depolarization of the axon membrane. This causes impulse propagation failure which reduces the number of synaptic release sites that are activated and mimics a drop in the effective stimulation rate; both effects cause synaptic depression.

INTRODUCTION

During repetitive stimulation of a presynaptic neurone, the amplitude of the post-synaptic potentials is often seen to decrease to a level considerably lower than that of the initial response. This depression of the

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post-synaptic potential has been reported at the neuromuscular junctions of, for example, the crayfish (Dudel & Kuffler, 1961*b*), the frog (del Castillo & Katz, 1954*b*; Betz, 1970), the frog and the rat (Lundberg & Quilisch, 1953; Otsuka, Endo & Nonomura, 1962), the mouse (Bennett, 1973), the guinea-pig (Thies, 1965) and humans (Elmqvist & Quastel, 1965), at the giant synapse of the squid (Kusano & Landau, 1975), at central ganglia in *Aplysia* (Bruner & Kehoe, 1970), and at the α-motoneurone of the cat (Curtis & Eccles, 1960; Kuno, 1964). In each preparation it has been shown that the depression is presynaptic in origin and results from a decrease in the average amount of transmitter released, m, by a presynaptic nerve impulse.

One explanation of the smaller values of m is that the extent to which the presynaptic action potential invades the nerve terminals changes during repetitive stimulation. For example, it has been shown (Krnjević & Miledi, 1959) and speculated (Straughan, 1960; Brooks & Thies, 1962; Bittner & Harrison, 1970; Bruner & Kennedy, 1970; Parnas, 1972) that conduction blocks in the presynaptic axon prevent the nerve impulse from invading the entire terminal region. Because fewer synaptic sites are depolarized, less transmitter is released; this is seen as a depression of the post-synaptic response.

In this study, synaptic depression is described in the crayfish walking leg. Presynaptic axon conduction blocks are seen to cause this depression, probably in the absence of any depletion of available transmitter substance. A preliminary communication of these results has been published (Smith & Hatt, 1976*a*).

METHODS

All experiments were performed on the opener muscle of the walking leg of adult crayfish *Astacus leptodactylus*. The inner side of the muscle was exposed by cutting away the overlying closer muscle and was then viewed with dark-field transillumination. The entire meropodite exoskeleton was removed, and the efferent nerves were exposed. Following removal of the inhibitory nerve, the single excitor nerve innervating the muscle was carefully drawn into a suction electrode for stimulation. Evoked activity in the motoneurone was frequently monitored by extracellularly recording nerve discharge at a point distal to the stimulating electrode. Stimuli consisted of 0·1 msec pulses isolated from ground.

Muscle fibres located in the distal-most part of the cheliped were chosen specifically for this study because they are relatively small and contract only slightly at stimulation frequencies as high as 30 Hz. By careful placement of the electrodes, near the exoskeleton, movement of the electrode tip relative to the muscle fibre could be avoided almost entirely in some preparations.

The entire preparation was bathed in modified van Harreveld's solution (Dudel & Kuffler, 1961*a*). Bath temperature was constantly monitored by a thermometer placed near the opener muscle and was maintained at 2–5° C. The bath solution was changed completely several times during an experiment; bath volume was 20 ml.

Excitatory post-synaptic potentials (e.p.s.p.s) were recorded intracellularly with

glass micro-electrodes of 3–5 MΩ resistance filled with 3 M-KCl. The signals from these electrodes were fed to cathode followers and then to either an appropriate a.c. or d.c. amplifier. Extracellularly recorded e.p.s.p.s were obtained by differential recording with two glass micro-electrodes of 0·8–2·0 MΩ resistance filled with 4 M-NaCl. These electrodes were connected to a differential cathode follower and then to an a.c. amplifier with upper and lower time constants of 350 μs and 2·5 s, respectively. The signals were displayed on an oscilloscope where successive sweeps were photographed on moving film. Also, both the intra- and extracellularly recorded e.p.s.p.s were averaged automatically with an averaging computer (Nicolet 1070).

The amplitude of the e.p.s.p.s was measured from the peak to the base line. If the frequency of stimulation was so high that there was incomplete recovery between successively evoked e.p.s.p.s, the falling phase was extrapolated by hand to base line; the amplitude was then measured from the peak to the extrapolated falling phase of the preceding e.p.s.p.

Statistical analysis of the data was performed mainly with a programmable pocket calculator (Hewlett-Packard 65), although some calculations were done on a digital computer. The appropriate statistical formulae are given in Zucker (1973) with one exception; the standard errors of p and n were calculated according to the following equations:

$$\text{S.E.}_p = \frac{s^2}{m}\sqrt{\left[\frac{1}{N}\left(2+\frac{s^2}{m^2}+\frac{2p^2-1}{s^2}\right)\right]} \quad (1)$$

and

$$\text{S.E.}_n = \frac{1-p}{p}\sqrt{\frac{2n(n-1)}{N}}, \quad (2)$$

where s^2 is the variance of the distribution of the number of quanta released and N is the total number of trials. These two formulae are inaccurately presented in Zucker (1973). Comparisons between the observed distribution of data and those expected theoretically utilized a χ^2 goodness-of-fit test (Johnson & Wernig, 1971).

RESULTS

Post-synaptic response to prolonged repetitive stimulation

In response to continuous, prolonged repetitive stimulation of the excitatory motoneurone, the amplitude of the e.p.s.p.s initially increased but then declined to a level significantly lower than the initial value. Typical records obtained at different times during stimulation at 20 Hz are shown in Fig. 1. In these and similar records obtained from twenty-eight different animals, during stimulation ranging from 10 to 30 Hz, the results were always the same: an initial increase in e.p.s.p. amplitude followed by a subsequent depression of the e.p.s.p. amplitude.

The time required to reach the maximum e.p.s.p. amplitude and the value of this maximum varied inconsistently in different fibres (cf. Bittner, 1968; Atwood, 1973), and no generalizations can be drawn. The peak usually occurred within 1 min, or less than about 2000 stimulus impulses, for stimulation frequencies ranging from 10 to 20 Hz.

Similarly, the rate and the extent to which depression occurred varied widely, permitting only broad generalizations. Depression usually was

maximal, occasionally involving complete disappearance of e.p.s.p.s, within 6–14 min, corresponding to about 4000–20,000 stimulus pulses. On the other hand, reduction of e.p.s.p. amplitude was seldom observed until stimulation had been delivered for at least 1 min, or about 600–1800 stimulus pulses. The rate of depression generally was faster as the frequency of stimulation became higher. If the frequency of stimulation was suddenly reduced, the e.p.s.p. amplitudes increased to some extent almost immediately.

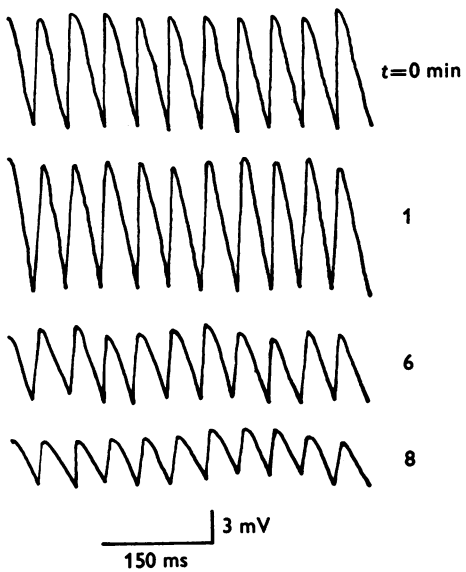

Fig. 1. Post-synaptic potentials in response to presynaptic stimulation delivered at 20 Hz. The amplitude initially increased and then progressively decreased as the time during which the nerve is stimulated increased. The records have been retouched to improve clarity.

Changes in the post-synaptic membrane input resistance could readily explain the observed decrease in e.p.s.p. amplitude; thus before proceeding with further experiments, membrane input resistance was measured before, during, and after prolonged repetitive stimulation. Two glass microelectrodes were inserted into the muscle cell, one for passing current and the other for monitoring the potential difference across the membrane. Current strength was monitored in the return path to ground. Significant differences in the membrane input resistance (about $1{\cdot}0 \times 10^5\ \Omega$) were never observed, despite considerable post-synaptic depression. Furthermore, the membrane resting potential following prolonged stimulation was not observed to differ from the initial value (which ranged from -70 to -80 mV) by more than 5 mV depolarization. Therefore, changes in these

electrical properties of the post-synaptic membrane could not readily account for the observed changes in e.p.s.p. amplitude, and further experimentation on the presynaptic side was in order.

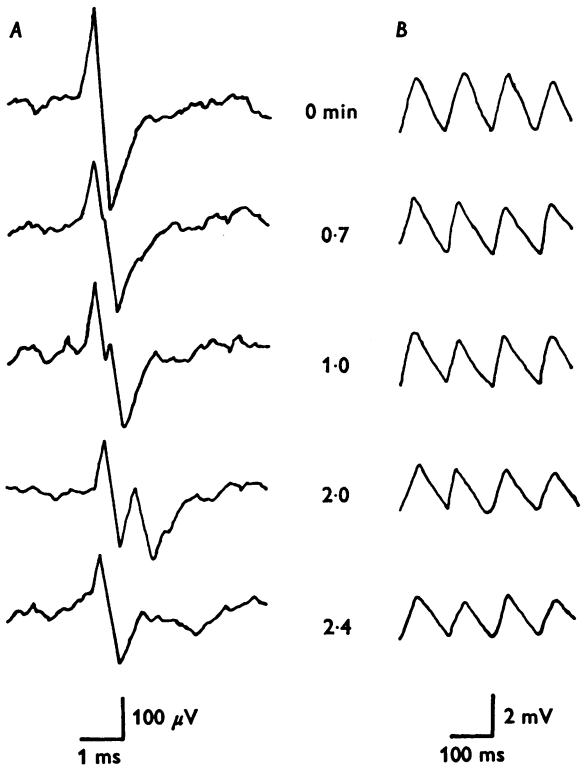

Fig. 2. Axon conduction block at a region of axonal bifurcation. Computer-averaged (256 sweeps) records of action potentials (*A*) and e.p.s.p.s (*B*) in response to repetitive stimulation at 10 Hz for 2·4 min.

Failure of nerve terminal invasion

Axon conduction block. Failure of the action potential to propagate into both axon branches at a region of bifurcation could preclude activation of some synaptic sites, resulting in a depressed post-synaptic response. This was observed to occur, and evidence of such conduction blocks at axonal branch points is shown in Fig. 2. Computer-averaged (256 sweeps) records in response to stimulation at 10 Hz were obtained by recording extracellularly (Fig. 2*A*) from the region of the second bifurcation of the axon in the opener muscle. At the beginning of stimulation, only a single action potential was seen, but as stimulation continued, it became clear that this was actually composed of two superimposed action potentials. The impulse

carried in one of the two nerves, however, began to display a reduced conduction velocity and, after 2·4 min, dropped out completely. Conduction in the other branch continued as before, evoking an e.p.s.p. of reduced amplitude (Fig. 2*B*).

Similar results have been recorded in different animals at branch points located more peripherally (cf. fig. 4 in Dudel, 1965*b*). In most preparations, a maximum of about six bifurcations distal to the main axon can be seen with the dissecting microscope. Blocking was observed to occur at each level of branching, and presumably occurs at points located even more peripherally.

Extracellular nerve terminal potentials. To explore more precisely the effect of axon conduction blocks on synaptic activity, focal extracellular recordings were made in the region of the neuromuscular junction. Sites were chosen from which the synaptic current could be recorded and which satisfied the following additional criteria (cf. Zucker, 1973): (1) the presynaptic nerve spike must have been clearly distinguishable from the noise of the recording system (which was about 30–40 μV), (2) the average amplitude of spontaneously released quanta or of single evoked quanta must have been at least 80 μV (in the cases in which evoked quantal release was analysed statistically, this value was 100 μV, and the synaptic currents must have had fast rise times), and (3) the non-specific currents from adjacent, active synaptic sites must not have been too large. Although these criteria do not guarantee that activity in the actual nerve ending is being recorded, they ensure that the electrode tip is near a region of the presynaptic nerve at which synaptic transmitter is released, and the general term 'extracellular nerve terminal potential' (e.n.t.p.) will be used to refer to the action potential recorded at these sites.

In most preparations, the post-synaptic response declined while the e.n.t.p.s were unchanged. This is illustrated in Fig. 3, in which computer-averaged (256 sweeps) responses recorded extra- (Fig. 3*A*) and intracellularly (Fig. 3*B*) during repetitive stimulation at 25 Hz are shown. The e.p.s.p. decreased as the time during which stimulation was delivered became longer, but the summed amplitude of the e.n.t.p.s stayed constant.

Further repetitive stimulation in fourteen preparations, however, eventually resulted in the failure of some stimulus pulses to evoke e.n.t.p.s; the intracellularly recorded e.p.s.p.s, which are the composite response to activity at all of the fifty to sixty (Dudel & Kuffler, 1961*a*; Bittner & Kennedy, 1970) synaptic terminals on the fibre, occurred regularly as expected, however. This is illustrated in Fig. 4 in which the intracellularly (Fig. 4*A*) and the extracellularly (Fig. 4*B*) recorded responses to eleven consecutive stimulus pulses during stimulation at 20 Hz are shown. The e.n.t.p. is seen to occur in seven of the records at the position marked by

the arrow. However, in four of the records (every third response, marked by a circle on the right), the e.n.t.p. did not occur; nonetheless, successful invasion of the impulse to other synaptic sites on the muscle produced the corresponding e.p.s.p., which showed little variation in amplitude (Fig. 4). In this case, impulse propagation must be blocking in a cyclic fashion at a point quite distal to the main axon, thus affecting only one or a very few

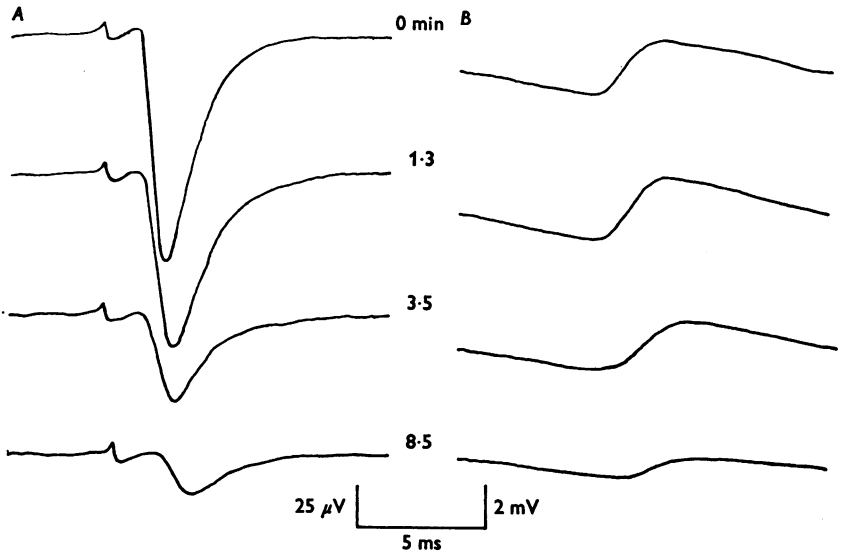

Fig. 3. Neuromuscular depression during prolonged repetitive stimulation. Computer-averaged (256 sweeps) e.p.s.p.s recorded extra- (*A*) and intracellularly (*B*) decrease as the time during which stimulation is delivered at 25 Hz increases from 0 min (the beginning of stimulation) to 8·5 min. The e.n.t.p.s seen in *A* maintain a constant amplitude.

terminals; similar observations have been reported by Krnjević & Miledi (1959) in the rat. The absence of the e.n.t.p. distinguishes an invasion failure from a release failure, in which an invading nerve impulse does not evoke transmitter release (cf. del Castillo & Katz, 1954*a*).

Terminal invasion was usually interrupted only intermittently, or cyclically, but occasionally total transmission blocks occurred. Evidence that terminal invasion failure can render the synaptic site completely ineffective is shown in Fig. 5, which consists of computer-averaged (256 sweeps) records of extracellularly recorded e.p.s.p.s in response to nerve stimulation at 20 Hz over a period of 4·6 min. At the beginning of stimulation, the negative-directed (i.e. downward in the Figure) e.p.s.p. is seen, preceded by a slight positive-directed non-specific current from neighbouring synapses, which obscures good visualization of the e.n.t.p. As the

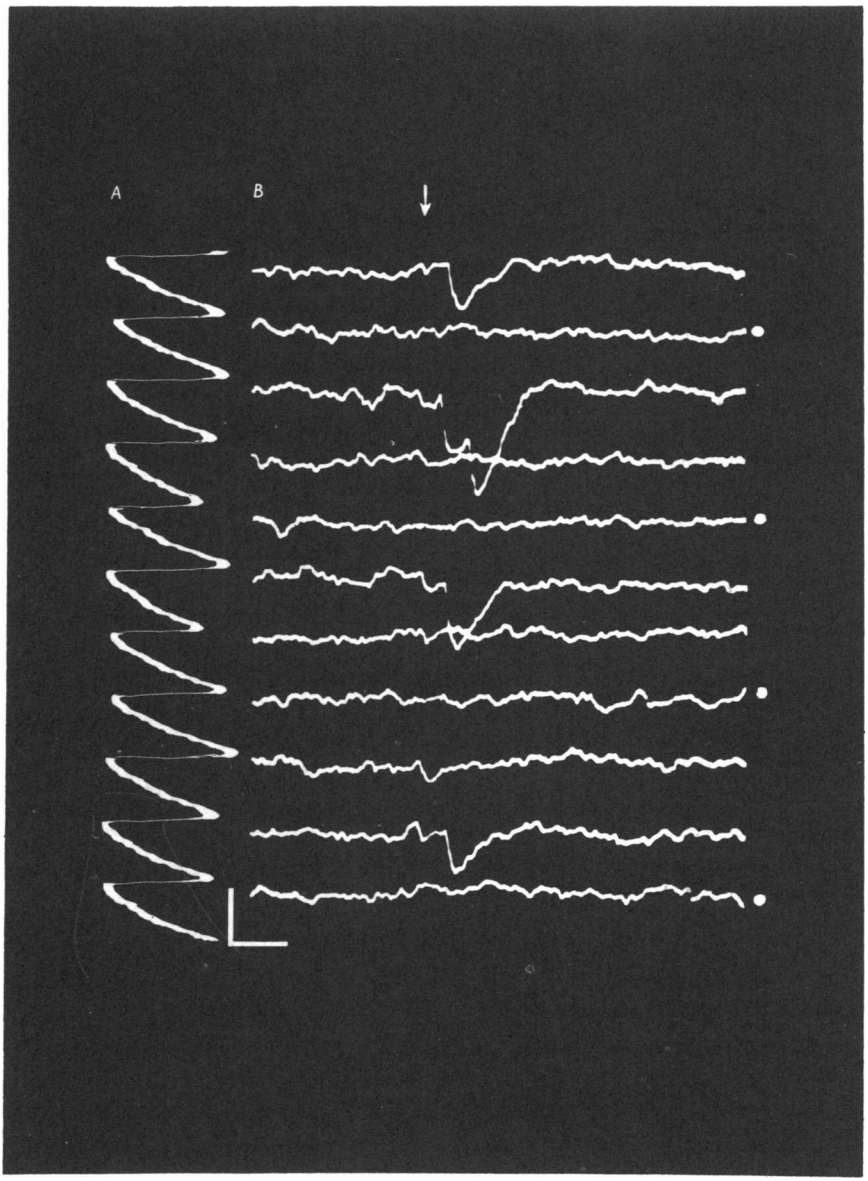

Fig. 4. Nerve terminal invasion failure. The intracellularly (*A*) and extracellularly (*B*) recorded e.p.s.p.s were evoked by repetitive stimulation at 20 Hz. The e.n.t.p.s occurred at the location indicated by the arrow except in the records marked by the circle to the right in *B*; in these latter cases, no e.n.t.p. was observed. Calibration: 100 μV (vertical) and 5 ms (horizontal); average e.p.s.p. amplitude recorded intracellularly is 4·2 mV.

duration of stimulation increased, the negative e.p.s.p. gradually 'turned over' into a positive-directed potential characteristic of the records obtained from extracellular recording from a non-active, non-synaptic site on the muscle (cf. fig. 1*A* of Zucker, 1974*a*). The observed currents result

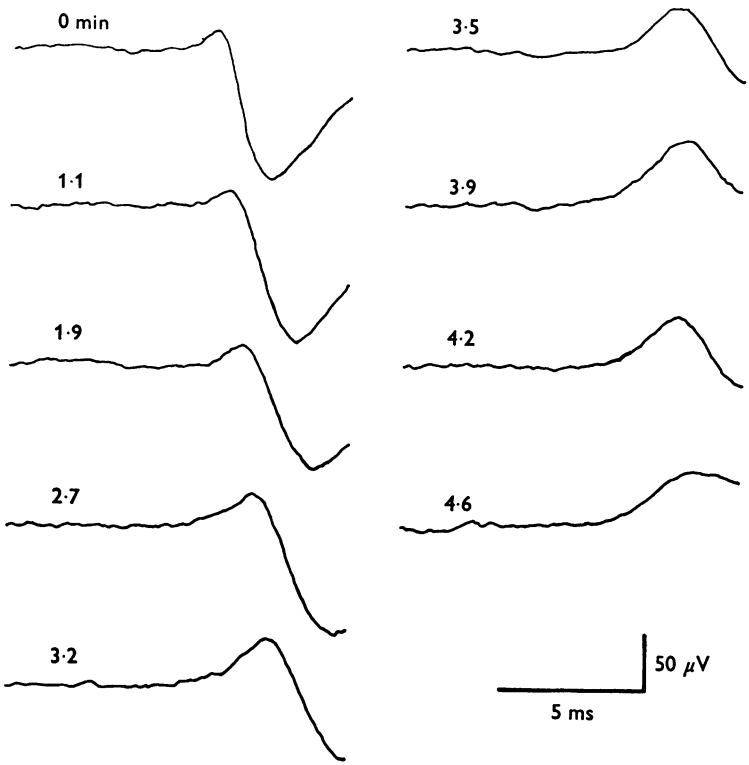

Fig. 5. Inactivation of a synaptic site due to nerve terminal invasion failure. Computer-averaged (256 sweeps) records of extracellularly recorded e.p.s.p.s in response to repetitive stimulation at 20 Hz for 4·6 min are initially (t = 0 min) negative but become gradually positive; after 4·6 min the observed currents are entirely positive. The elapsed time (min) during stimulation at which each record was obtained is given above the trace.

from activity at other synaptic sites but no longer from any activity at this site; that is, these records demonstrate the gradual increase in the number of impulses that fail to invade the terminal (corresponding to the gradual decrease in the size of the averaged negative-directed e.p.s.p.), until, after 4·6 min, this site is no longer active.

Altered action potentials in the peripheral terminal region

Synaptic delay. Electron micrographs reveal axonal branching beyond the level visible in the dissecting microscope (Lang, Atwood & Morin, 1972; Jahromi & Atwood, 1974). Conduction blocks at these bifurcations could lead to synaptic depression even though the e.n.t.p. is still seen in extracellular recordings obtained from a site located more centrally.

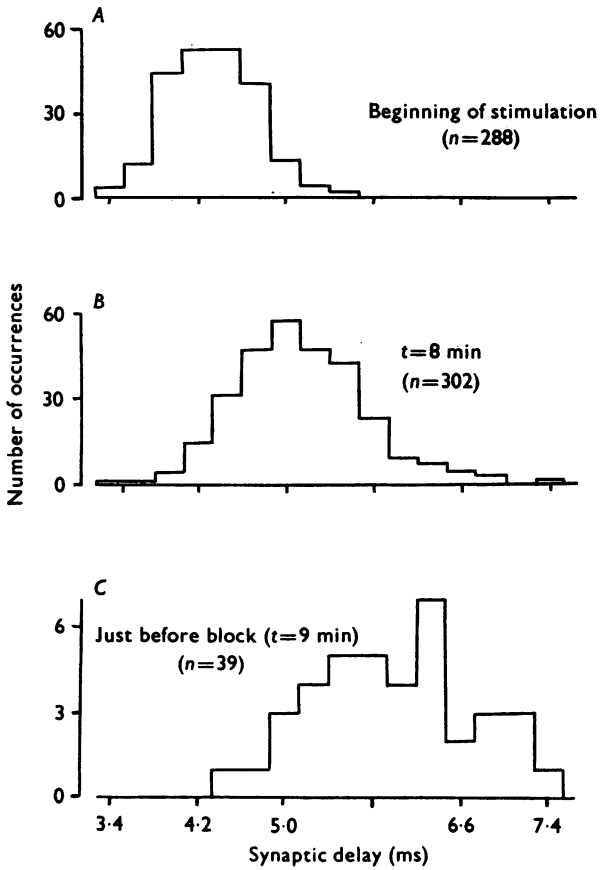

Fig. 6. Increasing synaptic delays during prolonged repetitive stimulation. Delays were measured at the beginning (*A*) and after 8 min (*B*) and 9 min (*C*) of stimulation at 20 Hz. The data in *C* were obtained immediately before transmission failure.

Localized activity in these peripheral branches cannot be recorded directly with present techniques, so it was necessary to monitor indirectly possible disruption of impulse propagation at this level. One indication of conduction failure at regions of low safety factor is a decrease in conduction

velocity (Grossman, Spira & Parnas, 1973; Goldstein & Rall, 1974). As it might also reveal possible underlying causes of conduction block, the interval between the negative peak of the e.n.t.p. and the start of the extracellularly recorded e.p.s.p. was measured. Similar measurements are usually considered estimates of synaptic delay (cf. Katz & Miledi, 1965*b*), but in crayfish, where it is impossible to see the location of the electrode tip relative to the actual release site, they more likely than not include effects due to conduction in the fine terminals.

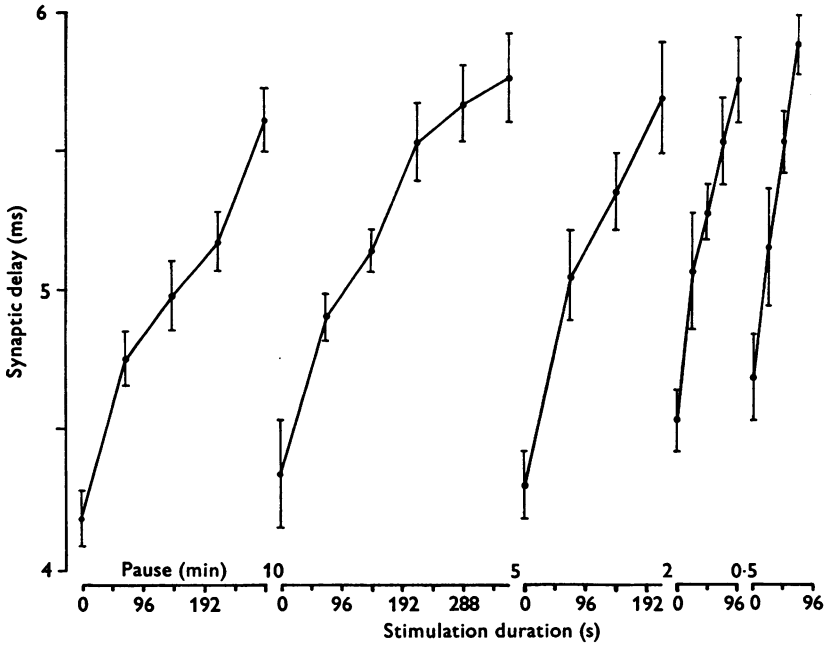

Fig. 7. Effects of pauses in stimulation on synaptic delays. Stimulation at 10 Hz was delivered until transmission failure occurred; then stimulation was discontinued for a pause of 10 min. Stimulation was again delivered until failure occurred; a pause of 5 min then followed, and so forth for further pauses of 2 and 0·5 min. Each value of the synaptic delay is the average ± S.D. of ten values.

The 'synaptic delay' increased as the duration of stimulation increased and reached a maximum just before the onset of a disappearance of the synaptic currents. Typical results are shown in Fig. 6, which was obtained from data in which the stimulation frequency was 20 Hz. The length of the delays increased as well as the variance of their distribution, indicative of a developing propagation block, leading to failure of terminal invasion. (The number of observations in Fig. 6*C*, just before the block, was small in order to obtain a stationary sample.)

Effects of a pause in stimulation. If an axon is in the blocked state, an interruption of the stimulation results in a temporary recovery from the blocked condition. This is illustrated in Fig. 7. Stimulation of this preparation was delivered at 10 Hz until the block occurred, followed by a pause of 5 min; this paradigm was further continued for pauses of 2 and 0·5 min. The synaptic delay increased at a faster rate as the duration of the preceding pause decreased. Also, when the preceding pause was less than about 5 min, the synaptic delay immediately following the pause had not returned to the initial, control value. The duration of the synaptic delay just before the block was about the same regardless of the length of the preceding pause.

Changes in the number of quanta released by a nerve impulse

To characterize accurately the changes in presynaptic activity during depression it is desirable to measure the amount of transmitter released. By maintaining the preparation at low temperatures (2–5° C), transmitter release from the presynaptic terminals is dispersed in time, permitting a direct count of the number of quanta released by a nerve impulse. After examining records such as those shown in Fig. 4 from numerous synaptic sites in twenty-three animals, five sites from different animals were found from which quanta could be counted easily and accurately. In each case analysed, the average amplitude of the individual, unitary quantum showed no significant change during the course of the experiment. Thus it was possible to record from single synaptic sites for long periods of time (as long as 18 min) without appreciable electrode movement.

The number of quanta evoked by a nerve impulse was counted for a large number of impulses (N) at each site in response to stimulation at two or more different frequencies: a low-frequency (1 Hz) control and a high-frequency (10–25 Hz) experiment of long duration, during which depression was examined. The observed distributions of quantal release were consistent with the binomial quantum hypothesis (Johnson & Wernig, 1971; Zucker, 1973), providing the basis for direct calculation of m, n and p. Accurate estimation of these parameters requires that the data from which they are derived are stationary. The sampled data were grouped into subsamples of about 50 responses each; the parameters m, n and p were then calculated for each subsample and examined for trends. All data to be reported satisfied this test for stationarity.

The data obtained during depression were analysed in two groups: (1) those obtained from records in which an e.n.t.p. was observed in response to each stimulus pulse, and (2) those in which a blocking stage had been reached proximal to the recording electrode, characterized by an intermittent absence of the e.n.t.p. in the records. Using data in which an

e.n.t.p. was seen in every single-sweep record, the values of m, n and p were estimated at various times during the course of high-frequency (10–25 Hz) stimulation to examine the way in which they changed as the amplitude of the corresponding e.p.s.p. was reduced. The results of five

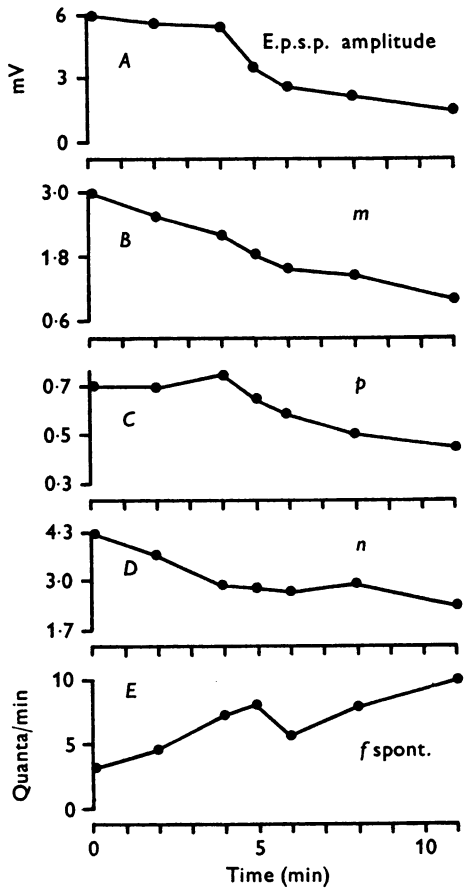

Fig. 8. Changes in the statistics of transmitter release during depression; the data were obtained from records in which an e.n.t.p. was clearly seen. In response to repetitive stimulation for a total of 11 min (abscissa) the e.p.s.p. amplitude (A), the estimates of m (B), p (C) and n (D), and the frequency of spontaneous quantal release ($f_{spont.}$; E) are shown.

experiments are shown in Table 1, and those of a sixth experiment, obtained during stimulation at 10 Hz, are shown in Fig. 8.

Depression was clearly seen in these cases to involve a reduction in m and p. However, the behaviour of n was more difficult to evaluate; as the value of m decreased during depression, the underlying distribution of

TABLE 1. Release statistics during depression. T refers to the time during which stimulation has been delivered. $f_{spont.}$ refers to the number of quanta/min released spontaneously. The e.n.t.p. was observed in response to each stimulus pulse

Site no.	Stimulation frequency (Hz)	T (min)	N	$m \pm$ S.E.	$p \pm$ S.E.	$n \pm$ S.E.	E.p.s.p. amplitude (mV)	$f_{spont.}$ (quanta/min)
I	15	0	500	$2{\cdot}27 \pm 0{\cdot}03$	$0{\cdot}79 \pm 0{\cdot}02$	$2{\cdot}86 \pm 0{\cdot}04$	$5{\cdot}49 \pm 0{\cdot}01$	5·2
		3	500	$1{\cdot}46 \pm 0{\cdot}04$	$0{\cdot}49 \pm 0{\cdot}03$	$2{\cdot}97 \pm 0{\cdot}16$	$3{\cdot}32 \pm 0{\cdot}02$	5·3
		8	500	$0{\cdot}69 \pm 0{\cdot}03$	$0{\cdot}36 \pm 0{\cdot}04$	$1{\cdot}88 \pm 0{\cdot}14$	$3{\cdot}10 \pm 0{\cdot}02$	5·5
		13	500	$0{\cdot}20 \pm 0{\cdot}02$	$0{\cdot}02 \pm 0{\cdot}06$	$9{\cdot}26 \pm 24{\cdot}58$	$2{\cdot}99 \pm 0{\cdot}01$	5·8
II	20	0	377	$1{\cdot}78 \pm 0{\cdot}04$	$0{\cdot}63 \pm 0{\cdot}02$	$2{\cdot}81 \pm 0{\cdot}10$	$3{\cdot}67 \pm 0{\cdot}03$	3·8
		5	413	$0{\cdot}38 \pm 0{\cdot}03$	$-0{\cdot}29 \pm 0{\cdot}12$	$-1{\cdot}30 \pm 0{\cdot}53$	$2{\cdot}01 \pm 0{\cdot}02$	5·8
		12	414	$0{\cdot}22 \pm 0{\cdot}02$	$-0{\cdot}14 \pm 0{\cdot}10$	$-1{\cdot}58 \pm 1{\cdot}16$	$1{\cdot}18 \pm 0{\cdot}01$	8·0
III	20	0	500	$2{\cdot}44 \pm 0{\cdot}03$	$0{\cdot}81 \pm 0{\cdot}01$	$3{\cdot}02 \pm 0{\cdot}04$	$5{\cdot}78 \pm 0{\cdot}02$	6·2
		1	239	$2{\cdot}08 \pm 0{\cdot}06$	$0{\cdot}65 \pm 0{\cdot}03$	$3{\cdot}17 \pm 0{\cdot}13$	$4{\cdot}88 \pm 0{\cdot}01$	5·9
		5	200	$0{\cdot}90 \pm 0{\cdot}05$	$0{\cdot}45 \pm 0{\cdot}04$	$1{\cdot}99 \pm 0{\cdot}17$	$2{\cdot}42 \pm 0{\cdot}02$	7·8
IV	20	0	606	$2{\cdot}88 \pm 0{\cdot}03$	$0{\cdot}78 \pm 0{\cdot}01$	$3{\cdot}70 \pm 0{\cdot}06$	$6{\cdot}10 \pm 0{\cdot}02$	4·8
		8	389	$1{\cdot}71 \pm 0{\cdot}05$	$0{\cdot}54 \pm 0{\cdot}03$	$3{\cdot}17 \pm 0{\cdot}16$	$2{\cdot}98 \pm 0{\cdot}02$	10·2
V	25	0	443	$2{\cdot}74 \pm 0{\cdot}04$	$0{\cdot}76 \pm 0{\cdot}02$	$3{\cdot}63 \pm 0{\cdot}06$	$4{\cdot}80 \pm 0{\cdot}02$	6·1
		3	516	$2{\cdot}15 \pm 0{\cdot}04$	$0{\cdot}67 \pm 0{\cdot}02$	$3{\cdot}19 \pm 0{\cdot}08$	$3{\cdot}66 \pm 0{\cdot}02$	6·4
		16	426	$0{\cdot}70 \pm 0{\cdot}03$	$0{\cdot}29 \pm 0{\cdot}04$	$2{\cdot}41 \pm 0{\cdot}31$	$2{\cdot}64 \pm 0{\cdot}01$	6·9

quantal release became gradually that expected of a Poisson process, in which p and n are meaningless. Thus, the estimates of p and especially n become less reliable as the duration of stimulation increased. The data represented in Fig. 8, however, were exceptional as they did not tend to a Poisson distribution ($P < 0{\cdot}001$) but were very well described ($P > 0{\cdot}7$) by binomial statistics even after 11 min of stimulation. Estimates based on these data confirm a tendency seen in Table 1 for n to decrease during depression. It can be concluded that depression can be attributed to a reduction in m, n and p; this result was also obtained by McLachlan (1975) for the guinea-pig preganglionic nerve terminals.

Similar results were observed in data obtained from records in which the e.n.t.p. was not observed in response to each stimulus pulse. During this intermittent blocking stage, the value of m decreases because of a decrease in both p and n. This is illustrated in Fig. 9 (circles only). The number of terminal invasion failures, characterized by the absence of an e.n.t.p. (Fig. 7*A*), and the values of m (Fig. 9*B*), p (Fig. 9*C*) and n (Fig. 9*D*), $\pm$ S.E., were calculated for successive samples of size 50 during a 40 sec period of stimulation at 20 Hz; stimulation had been delivered continuously for 2·5 min when the first invasion failure was observed. The value of m decreased smoothly (Fig. 9*B*) as the number of failures (Fig. 9*A*) increased. After 20 sec, a stable equilibrium for each parameter was reached in which every second stimulus pulse failed to evoke an e.n.t.p. (a '1:1 block'); during the entire duration of this experiment, however, intracellularly recorded e.p.s.p.s were seen in response to each stimulus pulse.

Facilitation, which involves an increase in p (Wernig, 1972; Zucker, 1973) is expected to decay slightly during the pause associated with invasion failure; this should be seen as a drop in p and probably explains the decline in p seen in Fig. 9*C*. This was tested by examining the effect of an invasion failure on transmitter release by the next successfully invading impulse. During intermittent blocking, but before the 1:1 phase was reached, quantal release evoked by impulses just before an invasion failure was compared with that evoked by an impulse just after an invasion failure. As a control, similar comparisons were made for quanta released just before and just after release failures, in which an e.n.t.p. was observed. The results showed that failure of the impulse to invade the terminal caused a slight reduction in m which could be attributed to a reduced value of p as expected from a decay in facilitation. In contrast, no significant differences were seen in any of the control data.

Proper estimates of these parameters are obtained only when invasion failures are not included in the calculations (circles, Fig. 9). To emphasize the extent to which improper estimation leads to considerably different,

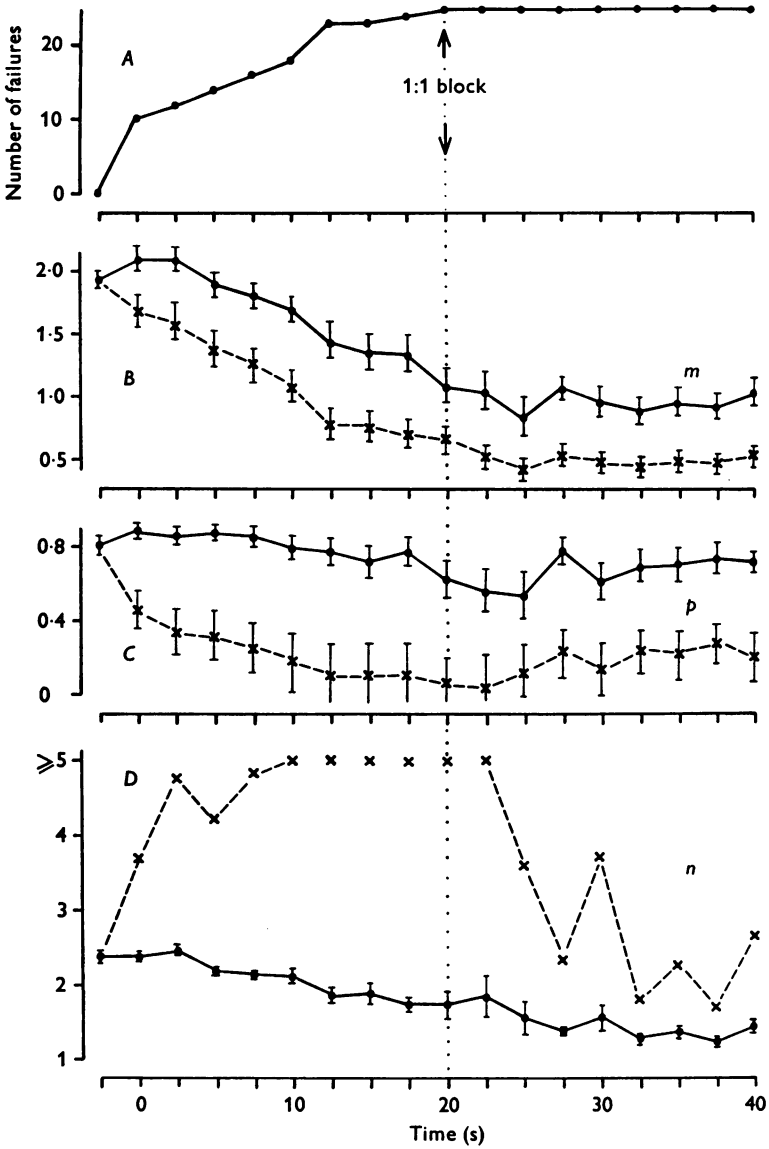

Fig. 9. Changes in the statistics of transmitter release during depression due to nerve terminal invasion failure. Stimulation was delivered at 20 Hz for 40 s (abscissa) beyond the time (0 on the abscissa) at which the first invasion failure occurred. Control values, obtained immediately before the invasion failure occurred, are also presented (left-most points). The number of invasion failures, judged by the absence of an e.n.t.p., is shown in *A*. The correct estimates, not including invasion failures, of *m* (*B*), *p* (*C*) and *n* (*D*) are plotted as circles. Incorrect estimates, in which invasion failures were included, are plotted as crosses to emphasize the extent to which misleading results arise from such calculations. Each value is the average ± S.D. of fifty observations. After 20 s (dotted line), every second impulse failed to invade the terminal; this is referred to as the '1:1 block' condition.

misleading results, estimates based on data in which invasion failures were included are also presented in Fig. 9 (crosses). Also, a χ^2 goodness-of-fit test (Johnson & Wernig, 1971) was used to compare the observed distributions of the number of quanta released with those expected according to the binomial or the Poisson distributions. Data obtained immediately before the first invasion failure and those subsequently obtained, not including invasion failures, were fit well ($P > 0{\cdot}2$) by binomial statistics. However, if invasion failures were included, the data could not be fit ($P < 0{\cdot}001$) by either binomial or Poisson statistics when blocking began; the extra failures arising from the blocked terminal invasion led to bimodal distributions (cf. Bittner & Harrison, 1970). As the number of invasion failures increased, these data became more closely fit by binomial statistics, and as the 1 : 1 blocking stage was reached, they were fit quite well ($P > 0{\cdot}2$) by the binomial distribution.

Changes in the statistics of transmitter release during facilitation were also examined in these data and found to involve an increase in m and p but not in n. This was determined by comparing the values of m, n and p estimated during the low-frequency (1 Hz) control with those estimated shortly ($\leqslant 1$ min) after the onset of high-frequency (10–25 Hz) stimulation. The results are presented in Table 2 and confirm those obtained by Wernig (1972) and Zucker (1973). The rate at which quanta were released spontaneously also increased as the stimulation frequency increased (cf. Dudel & Kuffler, 1961*a*).

Spontaneous release of quanta

If depression involved an exhaustion, or depletion, of the store of transmitter available for release, there might be a corresponding drop in the amount of transmitter released spontaneously. However, the frequency of spontaneously released unitary quanta (f_{spont}) did not decrease during prolonged repetitive stimulation (Table 1, Fig. 8), although there was considerable depression of the evoked response. In some experiments the frequency was seen to increase by as much as 210 %, but in others little change was observed. In addition, the amplitude of the spontaneously released quanta displayed no consistent tendency to decrease (cf. McLachlan, 1975).

The effect of prolonged stimulation on spontaneous release was further examined in the following experiment. Spontaneously released unitary quanta, in the absence of motoneurone stimulation, were recorded intracellularly; then the motoneurone was stimulated repetitively at 25 Hz for 20 min, until the e.p.s.p.s were quite depressed. Stimulation was then discontinued, and the spontaneous releases were then recorded again, beginning immediately after stimulation ceased. The amplitudes and the

TABLE 2. Release statistics during facilitation

Site no.	Stimulation frequency (Hz)	N	$m \pm$ S.E.	$p \pm$ S.E.	$n \pm$ S.E.	E.p.s.p. amplitude (mV)
I	1	500	$1{\cdot}54 \pm 0{\cdot}05$	$0{\cdot}11 \pm 0{\cdot}05$	$13{\cdot}75 \pm 6{\cdot}64$	$3{\cdot}06 \pm 0{\cdot}03$
	10	500	$2{\cdot}12 \pm 0{\cdot}04$	$0{\cdot}65 \pm 0{\cdot}02$	$3{\cdot}25 \pm 0{\cdot}09$	$4{\cdot}50 \pm 0{\cdot}01$
	15	500	$2{\cdot}27 \pm 0{\cdot}03$	$0{\cdot}79 \pm 0{\cdot}02$	$2{\cdot}86 \pm 0{\cdot}05$	$5{\cdot}49 \pm 0{\cdot}01$
II	1	434	$0{\cdot}57 \pm 0{\cdot}04$	$-0{\cdot}04 \pm 0{\cdot}07$	$-13{\cdot}95 \pm 24{\cdot}89$	$1{\cdot}22 \pm 0{\cdot}02$
	20	377	$1{\cdot}78 \pm 0{\cdot}04$	$0{\cdot}63 \pm 0{\cdot}02$	$2{\cdot}81 \pm 0{\cdot}09$	$3{\cdot}67 \pm 0{\cdot}03$
III	1	385	$1{\cdot}74 \pm 0{\cdot}06$	$0{\cdot}25 \pm 0{\cdot}05$	$6{\cdot}95 \pm 1{\cdot}39$	$3{\cdot}56 \pm 0{\cdot}01$
	20	500	$2{\cdot}44 \pm 0{\cdot}03$	$0{\cdot}81 \pm 0{\cdot}01$	$3{\cdot}02 \pm 0{\cdot}04$	$5{\cdot}78 \pm 0{\cdot}02$
IV	1	402	$1{\cdot}70 \pm 0{\cdot}05$	$0{\cdot}49 \pm 0{\cdot}03$	$3{\cdot}50 \pm 0{\cdot}22$	$1{\cdot}41 \pm 0{\cdot}01$
	20	606	$2{\cdot}87 \pm 0{\cdot}03$	$0{\cdot}78 \pm 0{\cdot}01$	$3{\cdot}70 \pm 0{\cdot}06$	$6{\cdot}10 \pm 0{\cdot}02$
V	1	730	$1{\cdot}73 \pm 0{\cdot}03$	$0{\cdot}50 \pm 0{\cdot}02$	$3{\cdot}46 \pm 0{\cdot}08$	$3{\cdot}12 \pm 0{\cdot}01$
	25	443	$2{\cdot}74 \pm 0{\cdot}04$	$0{\cdot}76 \pm 0{\cdot}02$	$3{\cdot}63 \pm 0{\cdot}06$	$4{\cdot}80 \pm 0{\cdot}02$

intervals between successively recorded quanta were measured, and their distributions are plotted before and after the stimulation in Figs. 10 and 11, respectively. Although slight changes in the shapes of the distributions are seen, there is no significant difference in the first two moments of the distributions in either case. In addition, the distribution of intervals (Fig. 11)

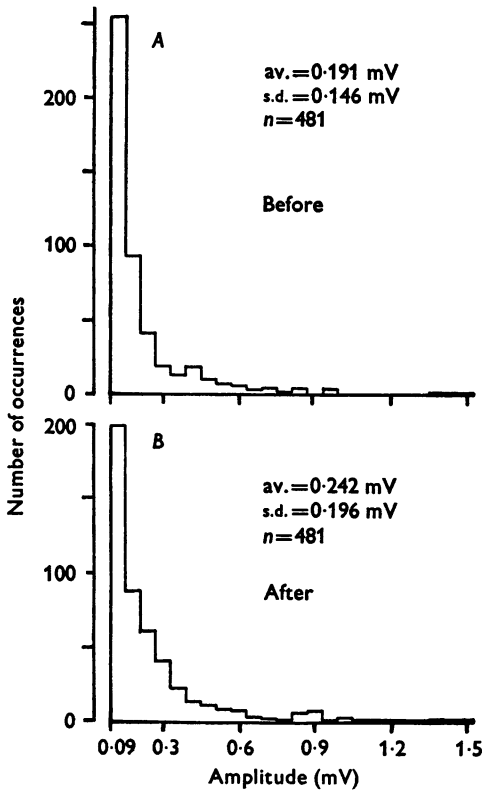

Fig. 10. Amplitude distribution of intracellularly recorded quanta released spontaneously before (*A*) and after (*B*) 20 min of continuous stimulation of the presynaptic nerve at 25 Hz.

both before and after stimulation can be described ($P > 0{\cdot}2$) as exponential, implying that the release is a random process (cf. Fatt & Katz, 1952).

To check for a trend in the moments of the distributions following stimulation, the data were grouped into ten subsamples of fifty observations each; analyses of variance and of covariance were performed, but no statistically significant differences in the moments were found. As a further check, an analysis of variance was performed in which the last 10 intervals before stimulation were compared with the first 10 after stimulation ceased,

and no significant ($P > 0{\cdot}5$) difference was seen. In addition, serial correlation coefficients between successive intervals were calculated for up to lag 10 for the data before and after the prolonged stimulation. The results, as shown in Fig. 12, were not significantly different ($P > 0{\cdot}2$) from zero (notice that the ordinate scale ranges from only 0·1 to −0·1), confirming

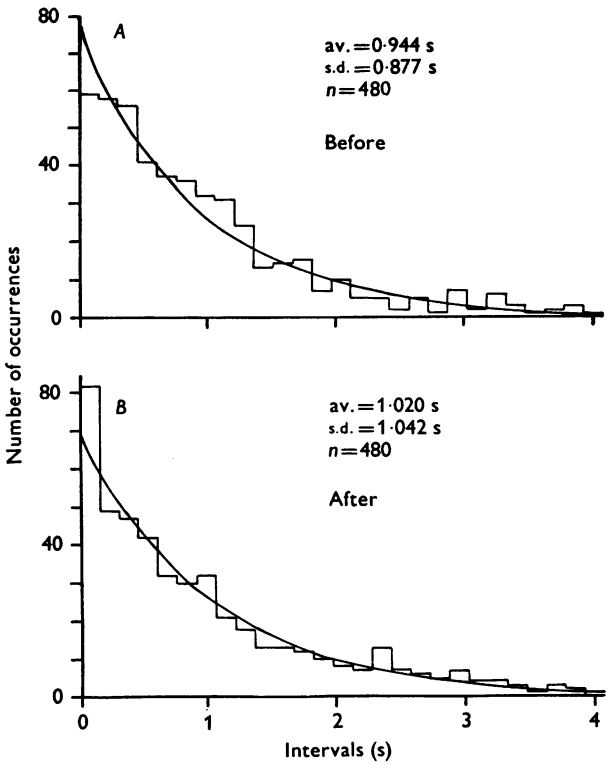

Fig. 11. Distribution of the intervals between successively occurring intracellularly recorded quanta released spontaneously before (*A*) and after (*B*) 20 min of continuous stimulation of the presynaptic nerve at 25 Hz. The continuous curve is that expected on the basis of an exponential distribution fit to the data.

the proposal that the release was a random process. This conclusion is consistent with the data of Dudel & Kuffler (1961*a*), but contrary to those of Cohen, Kita & van der Kloot (1974). It appears that depression of the evoked response is not accompanied by any significant change in the pattern or reduction in the rate of spontaneous release.

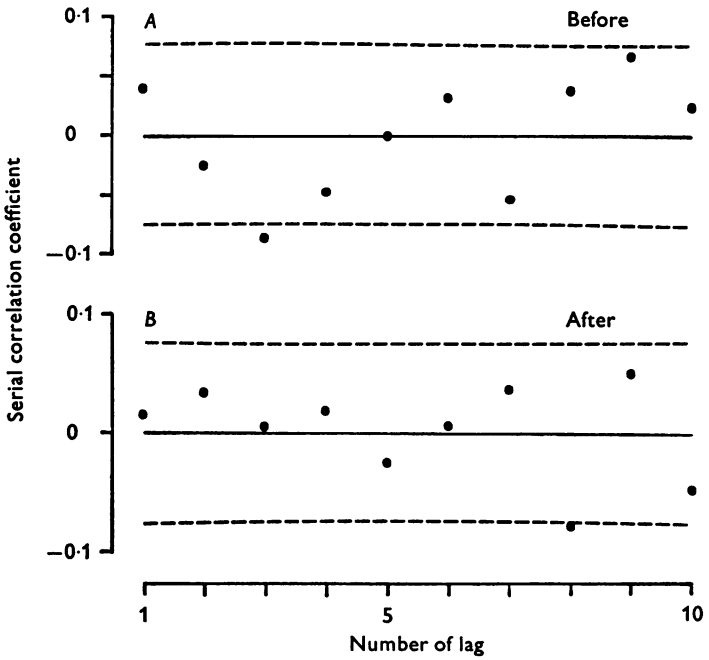

Fig. 12. Serial correlation coefficients between the intervals of intracellularly recorded quanta released spontaneously before (*A*) and after (*B*) 20 min of continuous stimulation at 25 Hz. The dashed lines represent the 95% confidence limits about a zero correlation; notice that the ordinates range from only −0·1 to +0·1.

DISCUSSION

Post-synaptic response to prolonged repetitive stimulation

Two post-synaptic phenomena have been observed in response to prolonged repetitive stimulation in this system: (1) an initial facilitation followed by (2) depression. Facilitation, seen shortly after the onset of stimulation, is probably superimposed on a more slowly developing depression (cf. Curtis & Eccles, 1960; Hubbard, 1963), and the extent to which either occurs depends on the rates of the underlying mechanisms. Long-term neuromuscular facilitation, as reported in *Procambarus clarkii* by Sherman & Atwood (1971), was occasionally observed during related, unpublished experiments performed several months before this study. Perhaps species differences or some seasonal variation inherent to the animals alters some aspect of transmitter release (cf. Maeno, 1969), resulting in depression rather than long-term facilitation.

The post-synaptic membrane was apparently unaffected by prolonged repetitive stimulation, as neither the membrane input resistance nor the

resting potential changed significantly. Desensitization of the receptors to the transmitter substance could not have occurred, as the amplitude of the spontaneously released quanta remained constant (cf. McLachlan, 1975). Thus it seems certain that the observed depression is entirely presynaptic in origin.

Presynaptic responses to prolonged repetitive stimulation

Axon conduction block. Failure of some nerve impulses to invade all of the terminal branches of an axon have been shown to result from propagation blocks at regions of axonal bifurcation. Both complete and intermittently occurring blocks have been observed. The number of nerve terminals affected depends in the case of multiterminal innervation on the location of the block; those occurring more centrally will affect more terminals than those occurring more peripherally. Propagation failures were observed at each level of branching visible with the dissecting microscope, reducing the e.p.s.p. by as much as about 50 %, and were inferred to occur at points located even more peripherally.

Judging from the results of this study, axon conduction blocks could play a major role in depression of synaptic transmission in crayfish. Extensive axonal arborization has been shown not only at the level of innervation visible through the dissecting microscope (van Harreveld & Wiersma, 1937) but also at the very peripheral terminal regions of the excitor nerve of the crayfish opener muscle (Lang *et al.* 1972; Jahromi & Atwood, 1974). Furthermore, in intact animals the motoneurone discharges at rates similar to those employed in this study (Smith, 1975; Smith & Hatt, 1976*b*).

Repetitive stimulation of the opener excitor neurone leads to a slight depolarization of the axonal membrane (Dudel, 1965*a*; Zucker, 1974*b*). The increased synaptic delay and the time course of recovery from transmission block and to re-establishment of the block following a pause in stimulation are consistent with the hypothesis that some ion, for example, external K^+, accumulates due to limited diffusion possibilities. It has been proposed (Grossman, Spira & Parnas, 1973) that depolarization leads to partial Na^+ inactivation which is supplemented by inactivation due to prior impulse activity. The result is a decreased action potential which cannot overcome the load resistance at a branch point and is blocked. This model is quite reasonable, but other alternatives are possible. One interesting possibility is that depolarization causes an increase in the intracellular Ca^{2+} (cf. Baker, Hodgkin & Ridgway, 1971) beyond the level which can be buffered effectively by the cell. Excess intracellular Ca^{2+} leads, in turn, to an increase in K^+ conductance (cf. Meech, 1974), thus causing the cell to be in a steady relative refractory state as well as reducing slightly the extent of the depolarization. This steady relative refractoriness

will then sum with the refractoriness following an impulse, leading eventually to intermittent or to absolute blocking of nerve impulse transmission. The ionic concentration effects will be greatest at regions where the surface/volume ratio of the membrane is largest. Thus the blocking effect will occur selectively at branch points where the diameter of the fibre becomes smaller (increasing this ratio) and because of the additional contribution of the lower safety factor. All present models are speculative, however, and further evidence is required to explain the mechanisms underlying axon conduction blocks.

Spontaneously released transmitter

Depletion of the store of transmitter available for release is improbable judging from the observation that the rate at which quanta are released spontaneously does not decrease during prolonged repetitive stimulation; actually it occasionally increases (cf. del Castillo & Katz, 1954*b*), presumably because of depolarization of the nerve terminal region (Dudel, 1971). It might be argued that depolarization increases the rate at which spontaneous release occurs, masking an over-all depletion process. This cannot be excluded by the present results. Unfortunately, the quantitative relationship between spontaneous release and nerve terminal depolarization in the crayfish is not known.

Distinct reductions in the average value of spontaneously released endplate potentials following prolonged nerve stimulation have been reported in the frog (Korneliussen, 1972; Kriebel & Gross, 1974; cf. giant releases, Liley, 1957). One conclusion drawn from this observation is that individual quanta can be released singly or in multiples – the preferred multiple hypothesis (Kriebel & Gross, 1974; cf. Dennis & Miledi, 1974). In this study, however, no change was seen in the amplitude of spontaneously released quanta following prolonged stimulation. The present results are too incomplete to allow strong conclusions regarding this problem, but they do argue against the case for preferred multiple release.

The intervals between spontaneously released quanta were found under all circumstances tested to be exponentially distributed, suggesting that their release is a random process. This is consistent with the results reported by Dudel & Kuffler (1961*a*) but contrary to those reported by Cohen, Kita & van der Kloot (1974). The latter authors observed quanta released in a bursting fashion. Such bursting behaviour was occasionally observed during the course of this study but in every instance was followed within 15 min of its onset by either disruption of synaptic activity altogether or penetration of the cell by the electrode. It was generally concluded that pressure of the micro-electrode tip on the synaptic structure caused this non-random spontaneous quantal release (cf. Katz & Miledi, 1965*a*).

Changes in the statistics of evoked transmitter release; nerve terminal invasion failure

Direct measurements of the number of quanta released by a nerve impulse showed that facilitation involves an increase in m, which can be attributed to an increase in p, the probability of release, but not n. This confirms similar results obtained in *Procambarus clarkii* by Zucker (1973). Conversely, depression is characterized by a decrease in m, n and p.

Intermittent failure of some impulses to invade a nerve terminal causes the statistics of transmitter release to vary in two ways: (1) the number of stimulus pulses that evoke transmitter release is clearly reduced, and (2) there is a drop in the amount of transmitter released, m, by subsequently invading impulses. The first is equivalent to a decrease in the average rate of stimulation, leading to a reduced post-synaptic response (Bittner, 1968). The second case involves smaller values of the parameters p and n. The decrease in p reflects reduction in facilitation during the time when invasion is interrupted, for facilitation is attributed mainly to an increased p (Wernig, 1972; Zucker, 1973). This result is also equivalent to that expected from a drop in the average rate of stimulation. The extent to which facilitation decays as a result of intermittent failure will become less as the stimulation frequency increases. As high stimulation frequencies are more likely to cause propagation failure than low frequencies (cf. Krnjević & Miledi, 1959; Bittner, 1968; Parnas, 1972), this effect is not likely to be of much importance in most blocking situations.

The observed decline in n is more difficult to explain. In the present study, the values of n are no greater than about 4–5; similar values are reported by Wernig (1972, 1975) and Zucker (1973). Thus they cannot reasonably represent the number of quanta available for release. Instead, n can be more profitably considered as the number of synaptic release sites (cf. Zucker, 1973) or some other detail of the release mechanism. In these terms, a reduced value of n means that, on the average, fewer release sites are active. The synaptic sites peripheral to a conduction block will be depolarized to a smaller extent, and if the distances from the point of block to the release site are sufficiently long, transmitter release may not be evoked by the nerve impulse. This will be manifest as a drop in the estimated value of n.

Alternative model of depression: depletion

Although variable nerve terminal invasion, resulting from axon conduction block, explains depression and is consistent with all the results of this study, alternative hypotheses are also possible. Consistent with the results of this study is the following model which involves depletion of the amount of transmitter available for evoked release. In response to a nerve

impulse, each release site may discharge transmitter and is then reloaded with a characteristic 'recycling' time; these recycling times need not be identical for each site. Those sites with a long recycling time, e.g. 30 min, may respond once but not again, causing an apparent reduction in estimates of n. More probably, the recycling times of some sites may increase as stimulation continues until they no longer contribute to transmission, although sufficient transmitter substance is still available for refilling these sites. If the recycling time for a particular site is a random variable, then the value of p should also drop (cf. Vere-Jones, 1966; Zucker, 1973). In these terms, a decrease in both n and p implies that some sites are never refilled and thus become non-functional. Also, if p is variable, then changes in p will also cause changes in the estimates of n (Hatt & Smith, 1976); such interaction between the estimation of these two parameters must be investigated further before meaningful conclusions can be drawn.

This is essentially a depletion model and implies a reduction in the rate of spontaneous release. A depolarization of the nerve terminals explains both the results of this model and the conduction blocks, namely it causes (1) an increase in the rate of spontaneous release and (2) a reduction of the amount of evoked transmitter release by reducing the extent to which the nerve impulse invades the nerve terminals. The two alternative explanations, depletion and variable nerve terminal invasion, need not be mutually exclusive. Evidence describing the latter, however, has been presented while confirmation of the former possibility still awaits further experimentation.

The authors thank Dr J. Dudel for his helpful advice during this study. Dr I. Parnas and Dr Robert Zucker also provided valuable suggestions during preparation of the manuscript. This work was supported by Stiftung Volkswagenwerk grant As: 11, 1955, and the Helen Hay Whitney Foundation (D.O.S.); a travel grant from the Fulbright Commission (D.O.S.) is also acknowledged.

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