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PRESYNAPTIC FAILURE OF NEUROMUSCULAR PROPAGATION IN RATS

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In two earlier publications (Krnjević & Miledi, 1957, 1958*b*) we have described a kind of failure of neuromuscular propagation observed during repetitive stimulation of rat nerves *in vitro* and *in situ*, which we called presynaptic failure because the propagation block prevented nerve impulses from initiating any synaptic action (see also Kostyuk, 1958). Such failures were therefore to be distinguished from various types of synaptic or post-synaptic failures, e.g. those caused by a decrease in the amount of transmitter liberated, by a reduced sensitivity of the end-plate membrane to acetylcholine (ACh), or by an increase in the electrical threshold of the muscle fibre. With an intracellular micro-electrode in the region of the end-plate, presynaptic failures were easily recognized, since unlike the other types of block they showed no local end-plate potentials (e.p.p.'s).

In phrenic-diaphragm preparations in which a motor nerve fibre was isolated, presynaptic failure during repetitive stimulation occurred asynchronously in various muscle fibres of the motor unit (Krnjević & Miledi 1957, 1958*a*). This suggested that the conduction block could not have occurred at any point more central than the peripheral branching of the motor axon. The most likely sites for the presynaptic block therefore seemed to be either points of branching, or the narrow region which precedes the terminal expansion of the nerve fibre.

The present paper gives a more detailed description of certain aspects of presynaptic failure, and an account of some experiments designed to determine its probable cause. A preliminary communication of some of the results has already been given (Krnjević & Miledi, 1959).

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METHODS

All experiments were done on rat muscle. The preparations and methods of recording were similar to those described earlier (Krnjević & Miledi, 1958*a*, *b*). In the majority of cases we used the isolated phrenic-nerve-diaphragm preparation mounted in a Perspex chamber, and bathed in a Ringer–Locke solution at room temperature (22–25° C). The phrenic nerve passed through a small hole (made water-tight with silicone tap grease) into an adjacent compartment containing liquid paraffin, in which it rested on platinum stimulating electrodes.

Glass micro-electrodes filled with 3 M-KCl solution were inserted into muscle fibres with the help of a dissecting microscope (magnification 6–16 times). End-plates were easily located near the terminal arborizations of nerve fibres, clearly shown by trans-illumination. However, electrodes were assumed to be near end-plates only if spontaneous miniature e.p.p.'s were clearly discerned. It was often possible to keep the electrode in the same fibre and to record adequate potentials for many minutes during repetitive activity, without recourse to a paralysing agent. In experiments *in situ*, rats were anaesthetized with intraperitoneal pentobarbitone (4 mg/100 g/hr) and the micro-electrodes recorded the activity of end-plates in the gracilis anticus.

Oxygenation

The muscle was oxygenated either by vigorous bubbling with a mixture of 95% O_2 and 5% CO_2 within the muscle chamber, or by a steady flow of fully oxygenated solution through the chamber at 10–20 ml./min, supplying 250–500 μl. O_2/min. This may be compared with a probable resting O_2 consumption of 2·5–4 μl./min at 37° C, calculated from a mean weight of 150 mg for diaphragms in our experiments, and figures of 1–1·5 μl./mg wet weight/hr given in the literature for the O_2 consumption of the rat diaphragm (Gemmill, 1941; Creese, Scholes & Whalen, 1958). The latter method was preferable when maximal oxygenation and minimal mechanical disturbance were both essential. It was also more convenient for producing rapid changes in O_2 tension. The changes in O_2 tension in the muscle bath were measured close to the surface of the muscle with an O_2 electrode (Davies & Brink, 1942). The electrode was an S.W.G. 20 platinum wire projecting 0·1 mm from a glass rod into which it had been sealed. It was kept at a potential 0·8 V negative with respect to the indifferent electrode, a calomel half-cell. Such an electrode does not allow absolute measurements of the O_2 tension, but changes in O_2 tension are followed within less than 1 sec (cf. Davies & Rémond, 1947) and absolute values can be obtained if the electrode is calibrated, under the same conditions, against solutions containing known amounts of O_2. A continuous record of the O_2 tension in the bath was made available by amplifying and displaying on the oscilloscope the potential drop across a resistor (50 kΩ) placed in series with the O_2 electrode. As is usually the case with unprotected O_2 electrodes, we observed a gradual loss of sensitivity in the course of an experiment. This change was only noticeable over periods of the order of tens of minutes.

Temperature changes

Quick changes of temperature were produced in the muscle bath by allowing warm or cold solutions to flow in rapidly from separator funnels. Excess fluid was removed by continuous suction. All the solutions were kept thoroughly oxygenated. As the preparations proved to be exceedingly sensitive to even small changes in temperature, this was monitored continuously in the neighbourhood of the muscle. A thermistor (Type F1512300 Standard Telephone Co.) was placed with its tip as close to the upper surface of the diaphragm as possible. Its variable resistance was included in one arm of a d.c. bridge whose output was amplified and displayed on the oscilloscope.

Presynaptic polarization

The phrenic nerve fibres were polarized by passing a known current along the nerve trunk between the muscle bath and the adjacent paraffin pool. Non-polarizable electrodes were prepared from Ag–AgCl wire covered with cotton thread and soaked in Agar–Ringer. One electrode made

contact with the nerve in the paraffin pool and the other dipped into the muscle bath. The length of trunk directly subjected to the current was about 6 mm. It usually had a resistance of about 100 kΩ. The amount of current passed could be easily varied; it was measured on a galvanometer, and a continuous record was obtained by amplifying and displaying the voltage drop across a series resistor (10 kΩ).

Direct recording of the frequency of firing of e.p.p.'s

In several experiments we were interested particularly in the effect of changes in certain conditions (e.g. temperature or oxygenation) on the frequency of firing of fibres which failed intermittently. The usual system of amplifying was modified so that the amplified signals from the end-plate triggered a commercial radioactive-particle counter (Airmec Radiation Monitor 1021 B) which gave a d.c. output proportional to the frequency of pulses at the input. The input–output relation was tested and calibrated by applying pulses at known frequencies.

RESULTS

Presynaptic failure was observed under various conditions in 42 diaphragms *in vitro*, and in the gracilis anticus *in situ* in seven experiments. When the rested muscle is stimulated indirectly at the beginning of an experiment, a regular sequence of responses (e.p.p.'s and spikes) can be recorded with an intracellular electrode. After a few minutes the discharge may become intermittent, as some nerve impulses no longer reach the nerve endings. If repetitive stimulation is maintained, complete failure of propagation is often seen.

Reversibility of failures

Intermittent failures are usually easily reversed by reducing the rate of stimulation; however, if the stimulation is then kept at the lower frequency, failures may begin again after some minutes, and a further reduction of frequency will then be essential if full conduction is to be restored. In other cases, the fibre apparently comes to an equilibrium, so that impulses are transmitted intermittently for long periods (5 min or more) without progressive deterioration.

In another type of failure, usually during prolonged high-frequency stimulation, the onset is abrupt and complete block appears almost immediately. As a rule conduction is not resumed when the rate of stimulation is lowered, but if the preparation is allowed to rest for say 10 min, the fraction of responsive end-plates increases progressively. There is no reason to doubt that all fibres recover if enough rest is given, with an adequate O_2 supply, etc. However, this can only be true for a limited period of time, since nerve fibres, either *in vitro* or *in situ*, rapidly degenerate after cutting, and this process is accelerated by repetitive activity (cf. Cook & Gerard, 1931).

Frequency of stimulation

Fibres in the same or different preparations varied much with regard to the frequency at which repetitive stimulation caused a block. Nevertheless, certain generalizations can be made: stimulation at 50/sec can be counted on

to cause intermittent failure of many fibres within 2–5 min, *in vitro* or *in situ*. Most of our observations *in vitro* were actually made with lower rates of stimulation (10–30/sec). *In situ*, a frequency of 50/sec or less was sufficient to cause failure in five experiments out of seven, and many fibres failed at 20–30/sec. However, the frequency needed for failure was often somewhat higher than *in vitro*, even when comparing experiments at 37° C. In three experiments *in situ*, several fibres showed signs of failing only after stimulating at rates as high as 100–300/sec.

Patterns of impulses during intermittent failure

So long as the rate of stimulation is neither excessive nor too low, intermittent failures can be observed in an end-plate for a period of several minutes. It is then found that the repetitive discharge of impulses (i.e. spikes or e.p.p.'s with interspersed failures) may occur in one of at least three ways. There may be obvious alternation of impulses and failures; a cyclic pattern of groups of impulses alternating with groups of failures; or an irregular sequence of impulses and failures showing no obvious pattern.

After stimulating a phrenic nerve repetitively for a period of 3–4 min at a frequency > 20/sec, many end-plates in the diaphragm are no longer activated at the frequency of nerve stimulation. In some cases no impulses may be detected at all, because of complete block; but in many other cases there is intermittent firing, which remains fairly stable at a certain frequency of stimulation, without either accelerating or slowing very markedly over a period of one or more minutes. Many records of such firing were obtained: in certain cases, a very obvious pattern of impulses is clearly not a random distribution (e.g. Figs. 1 and 6). In most cases, however, impulses and failures are arranged in what seems a quite irregular manner.

A temporal sequence of successes and failures can be considered as a series of alternatives distributed along a line. Such a series is made up of a number of groups, each group consisting of one or more consecutive events of the same type. On the nul-hypothesis, every possible distribution of alternatives in the series has an equal probability. In a population drawn at random, the probability of finding a given number of groups is the proportion of distributions which yield this number of groups. This type of problem was analysed in some detail by Stevens (1939), and was also considered by Swed & Eisenhart (1943) and Feller (1950). When the total number of groups, U, is even, the solution is given by

$$P = 2\binom{m-1}{k-1}\binom{n-1}{k-1} \div \binom{m+n}{m} \tag{1}$$

where P is the proportion of distributions yielding U groups, m is the number of events of one type (here impulses), n the number of events of the second type (here failures) and $U = 2k$. For odd values of U,

$$P = \left[\binom{m-1}{k-1}\binom{n-1}{k} + \binom{m-1}{k}\binom{n-1}{k-1}\right] \div \binom{m+n}{m} \tag{2}$$

where $U = 2k+1$. The binomial coefficients have the usual meaning, i.e.

$$\binom{x}{r} = \frac{x!}{r!\,(x-r)!}$$

As was pointed out by Stevens (1939) and also by Swed & Eisenhart (1943), the exact test of the significance of a particular arrangement is given by S, the sum of the probabilities of getting as large or larger (or as small or smaller) a number of groups:

$$S = \Sigma P_{U \geqslant U'} \quad \text{or} \quad \Sigma P_{U \leqslant U'}. \tag{3}$$

Swed & Eisenhart (1943) give tables of S for values of $m \leqslant n \leqslant 20$.

We employed this kind of analysis to examine sequences of impulses and failures in our records. The most sensitive conditions obtain when $m = n = 50\%$ of the total number of events; the traces studied were therefore selected to include only those in which the proportion of impulses was between 20 and 80%. It may be noted that the maximum possible number of groups (U_M)

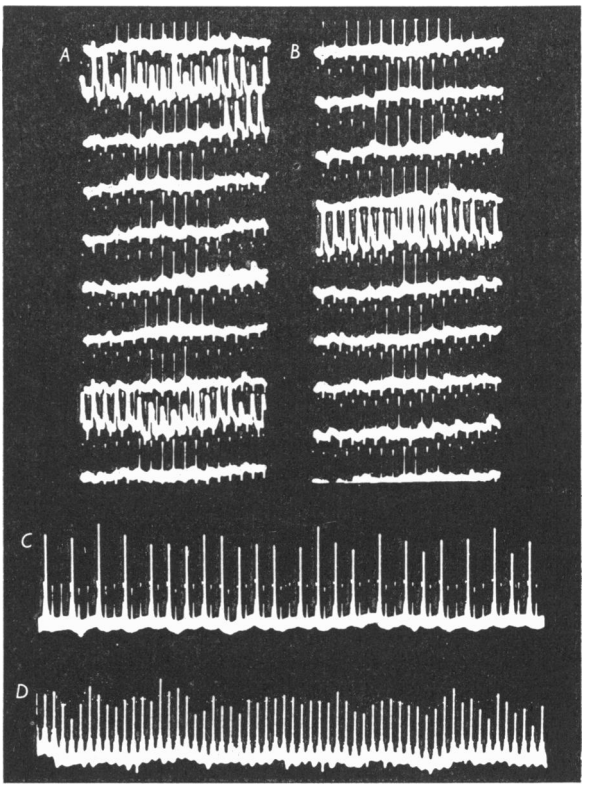

Fig. 1. Examples of stimulus artifacts and membrane potentials recorded with intracellular electrodes in end-plates of rat diaphragm *in vitro*, during repetitive stimulation of phrenic nerve. In all cases presynaptic failure had begun, preventing numbers of impulses from reaching the end-plate. All records in this figure show arrangements of impulses and failures that are obviously not random. In *A* and *B*, which are continuous, discharge is of cyclic type, i.e. periods during which responses occur without failing alternate with periods of complete block; it was recorded during stimulation at 80/sec in diaphragm partly paralysed by excess Mg (15 mM). In *C*, impulses and failures tend to alternate during stimulation at 40/sec; while in *D*, from a third fibre, every other impulse is missing quite regularly during stimulation at 80/sec.

is given by $U_M = 2m+1$, when $m < n$ or $U_M = 2m$, when $m = n$; while the most likely number of groups (U) is given by

$$\overline{U} = \frac{m(n+1)+n(m+1)}{m+n}. \quad (4)$$

Equation 4 is particularly useful since a comparison of U and $\overline{U}$ often gives a very good idea of the probable result.

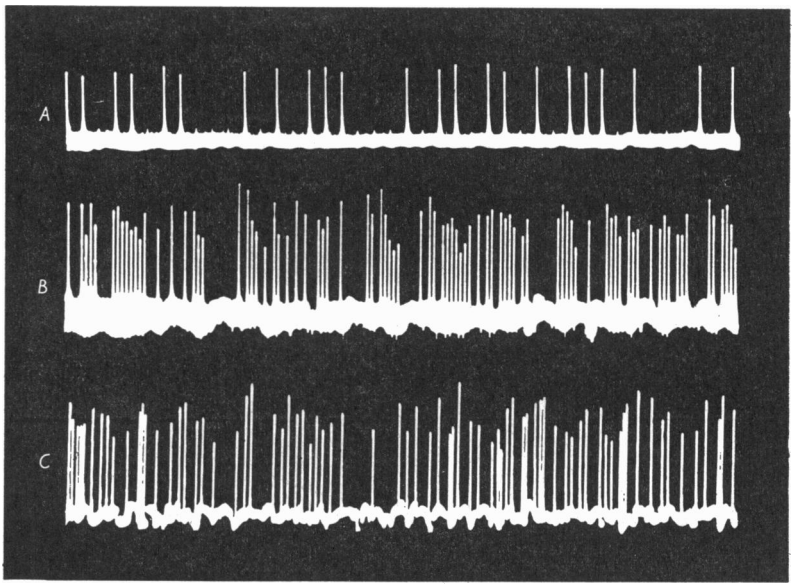

Fig. 2. Examples of portions of long sequences recorded in end-plates of three different muscles *in vitro*. All three sequences show a random discharge; *A* occurred during stimulation at 12·5/sec, *B* at 20/sec, and *C* at 32/sec. A number of vertical lines have been retouched in these and some subsequent records.

Observations made in vitro

From the results of nineteen experiments *in vitro* eighty-six separate estimates were made (eleven by inspection of the records, and seventy-five by analysis) of the manner of firing during intermittent presynaptic conduction block. These results were obtained during intracellular recording from forty-two different end-plates.

When the available record formed one long, continuous sequence, the analysis was performed by counting m, n and U directly on the film. The calculation of P from equations (1) or (2) becomes rather unwieldy if the numbers involved are very large; for convenience, $m+n$ was therefore always about 100. The most representative portion of the long sequence was taken for the counts, and if the result was on the border-line of significance, or if there were obvious variations in the manner of firing, the counts were repeated in other regions. Of the eighty-six estimates mentioned above, 49 were made in

this manner. Figure 2 shows portions of three sequences of this type recorded in different fibres at frequencies of stimulation between 12·5 and 32/sec, which proved to have random grouping ($S > 0{\cdot}05$).

Some oscilloscope records were taken with a repetitive horizontal sweep and a slow, vertically moving film (Figs. 1*A* and *B*, 3) which used the film more efficiently, but with the disadvantage that a number of events were missed in the intervals during the fly-back (usually 3–10 msec). This method of recording gave a large number of short sequences, in which $m+n$ was mostly < 10. When applied to each of such short sequences, the analysis loses much of its power, because only relatively few arrangements can have a significance $< 0{\cdot}05$; furthermore, individual sequences are much less likely to be representative of the general behaviour. The following procedure was therefore adopted. Counts of m, n and U were made in each of a series of at least fifty short sequences. The frequency distribution of values of U was then compared with the distribution to be expected on the nul-hypothesis; the significance of differences from expected values was then estimated by calculating χ^2 in the usual manner. Of the total of eighty-six estimates mentioned above, twenty-six were made by this method, based on counts of m, n and U in 1379 short sequences. Figure 3 shows portions of 3 series of short sequences, recorded in different fibres and different muscles, all exhibiting random firing.

Relation between type of firing and frequency of stimulation

An excessive number of groups tends to be associated with somewhat faster stimulation. This is shown in Fig. 4, in which fibres are grouped according to stimulation frequency, and the proportion (expressed as a percentage) with random firing is indicated by the black areas of the histograms; the white areas correspond to the proportion of fibres firing with an excess or a deficiency of groups. All the sequences at frequencies of stimulation less than 19/sec were random, but none of those at frequencies of stimulation more than 44/sec. If we assume that there is an equal chance of finding random sequences at all frequencies, the probability of observing such an uneven distribution is less than 0·001 ($\chi^2 = 22{\cdot}7$, $\phi = 5$).

The difference between firing at low and high frequencies of stimulation may be shown in another way. If we take all the long sequences which have been analysed, we can compare the number of groups actually found (U) with the most likely number on the nul-hypothesis ($\bar{U}$). This was done in Fig. 5*A* for sixteen sequences recorded during stimulation at a low frequency (5–16/sec) and in Fig. 5*B* for eleven sequences at a high frequency (30–50/sec). It is clear that in *A* the points are relatively close to the theoretical line of perfect agreement between U and $\bar{U}$ (dotted line), and that they are distributed reasonably symmetrically on either side. The solid line is the line

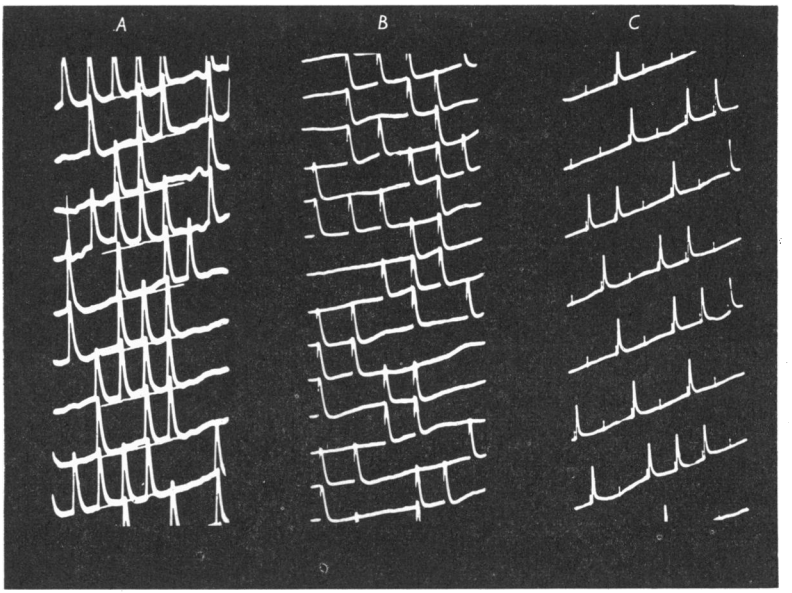

Fig. 3. Examples of three series of short sequences recorded from end-plates of three different muscles *in vitro*. All three series show a random discharge. *A* occurred during stimulation at 10/sec, *B* at 12·5/sec, and *C* at 30/sec. Note that each short sequence is separated from the next by fly-back time (3–10 msec).

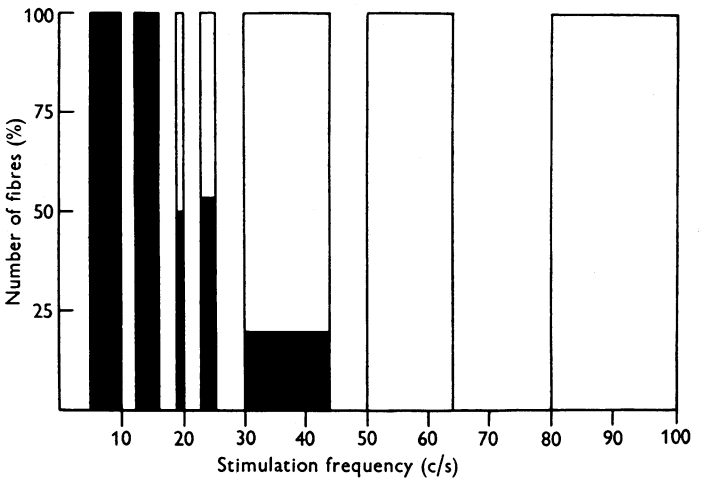

Fig. 4. Histogram showing proportion of failing rat phrenic-diaphragm fibres (during stimulation at given frequencies) whose discharges had a random ($S > 0{\cdot}05$) arrangement of impulses and failures (black areas). The figure is based upon an analysis of discharges in 44 fibres, the numbers of fibres in each group being (reading from left to right) 4, 5, 8, 5, 11, 6 and 5 respectively.

of best fit (calculated by the method of least squares) and the hatching indicates the 5% confidence limits. The regression coefficient was 1·023 (S.E. ±0·147), which is clearly not significantly different from 1·0. In *B*, the points are all above the dotted line, and the regression line indicates a general tendency to an excess of groups which increases with the frequency of stimulation. The regression coefficient was 1·68 (S.E. ±0·309), which is just significantly different from 1·0 at the 5% confidence level.

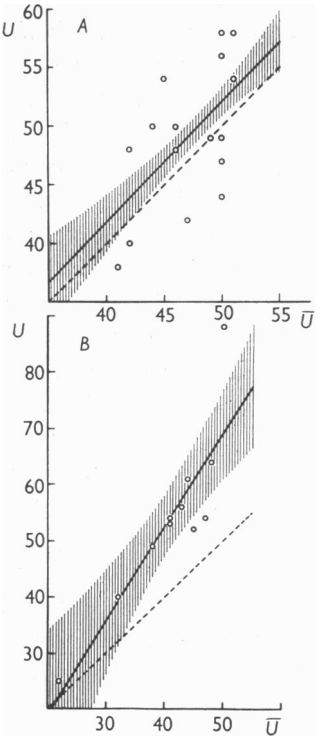

Fig. 5. Comparison of observed numbers of groups (U) with expected numbers ($\overline{U}$) in *A*, 16 long sequences recorded in rat phrenic-diaphragms stimulated at low frequencies (5–16/sec), and *B*, in 11 long sequences at relatively high stimulation frequencies (30–50/sec). Diagonal dotted lines indicate perfect agreement between U and $\overline{U}$; solid lines give regression of U on $\overline{U}$, the 5% confidence limits being shown by hatching.

High-frequency stimulation can have a delayed effect on the type of firing; this was shown by sequences recorded during stimulation at the relatively low rate of 25/sec, begun immediately after a period of 2–3 min of stimulation at 64/sec. All sequences had a greater number of groups than that expected: but whereas the excess of groups was highly significant (S = 0·001) at the start of the series, it was on the border-line of significance (S = 0·04) 40 sec later, and not significant (S = 0·08) 2 min later.

At frequencies of stimulation greater than 50/sec the tendency to an excess

of groups was usually such as to be immediately evident upon inspection. Where the rate of failure was about 50%, impulses and failures often alternated very regularly for long periods (Fig. 1*D*).

Cyclic patterns of firing

In several fibres we observed the opposite extreme, i.e. a marked deficiency of groups; impulses and failures tended to occur in more or less regularly alternating cycles (Fig. 1*A* and *B*). Once the pattern of cycles was established, it persisted for many minutes with only relatively slow changes, so long as the frequency of stimulation was not altered. *In vitro* this behaviour was only seen under certain conditions. It occurred after a period of prolonged stimulation at a high frequency (> 50/sec), and was principally seen in muscles whose contractions were reduced by stimulating only one or two nerve fibres, or by an excess of magnesium. In the firing phase of a cycle there was usually regular activity without intermittent failures, except when the rate of stimulation was very high (e.g. 320/sec), in which case impulses tended to alternate with failures. The inactive phases of the cycle allowed the muscle end-plate to recover appreciably from the effects of tetanic stimulation. This was shown at the onset of each firing phase by the appearance of spikes or larger e.p.p.'s which diminished progressively. Several features of the cyclic type of firing suggest that it usually does not originate in the same region of the nerve as the intermittent type of failure. If the muscle is observed closely during cyclic firing, rhythmical changes in contraction are visible over a substantial area; this indicates that entire motor units may be involved (cf. Krnjević & Miledi, 1958*a*). This was confirmed in experiments in which potentials from different fibres were recorded simultaneously with two micro-electrodes. It seems that whereas most intermittent, low-frequency failures occur in the most peripheral part of the nerve, probably in its intramuscular course, the cyclic, high-frequency type of failure most often arises in the nerve trunk, outside the muscle chamber.

Observations made in situ

Fourteen separate estimates were made of the type of firing recorded during presynaptic failure in the end-plate region of thirteen different fibres. Five estimates were based upon inspection of the records; three upon analysis of the number of groups in long sequences (as above), and six upon an analysis of a series of short sequences (also as above; m, n and U were counted in each of 403 short sequences). In contrast to the results obtained *in vitro*, the sequences did not tend to have an excess of groups; the only exceptions occurred while stimulating at the very high frequency of 320/sec. Whenever firing was not random, it was usually because of a deficiency of groups. This tendency culminated in an obvious cyclic pattern (Fig. 6), as observed *in vitro*. This type

of failure was again associated with prolonged stimulation at high frequencies (> 50/sec). It was seen relatively often (in five cases out of fourteen), no doubt because as a rule presynaptic failures occur *in situ* only after a somewhat longer and faster tetanus.

Sequences observed *in situ* differed from those *in vitro* in one other respect; the type of firing was much less sensitive to the frequency of stimulation. Random sequences of impulses and failures were observed during stimulation at frequencies which ranged from 1/sec to 250/sec; sequences with either too few or too many groups to agree with the nul-hypothesis occurred at frequencies in the range 31–320/sec. Clearly, the zone of overlap (31–250/sec) is very much greater than that found *in vitro* (20–44/sec).

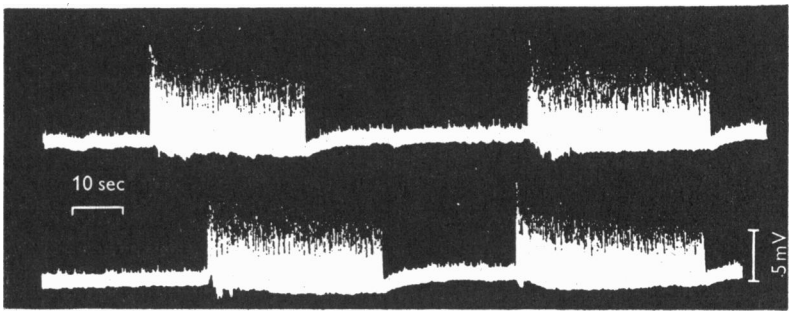

Fig. 6. Cyclic firing recorded in an end-plate in gracilis anticus *in situ* during repetitive nerve stimulation at 160/sec. The traces form a continuous sequence.

Effect of temperature changes on intermittent firing

During stimulation at a constant frequency the number of impulses which failed to reach the nerve endings, in preparations *in vitro*, was extremely sensitive to even small changes in temperature. Typical changes in the rate of firing produced by raising or lowering the temperature are shown in Fig. 7. When the temperature was 24° C (Fig. 7*A*) the fibre was firing very occasionally during indirect stimulation at a constant frequency of 16/sec, but reacted quickly (within 2 sec) to a rise in temperature; before this reached its maximum of 36° C all the responses were effectively conducted. As a rule we found that the frequency of stimulation could then be increased substantially before failures reappeared. By reducing the temperature towards the original level the fibre was made to fail again. Opposite effects were produced by reversing the temperature changes (Fig. 7*B*). Nevertheless, there was no simple proportionality between temperature and the rate of failing; the behaviour was apparently conditioned by the previous history: cooling had a greater depressing action after a warm spell, and vice versa.

Muscles kept at a higher temperature tended to have many silent fibres

after a period of stimulation; it seemed that, although fibres might maintain a greater rate of firing for a while at the higher temperature, early complete failure of conduction was also more likely. This idea was put to the test *in vitro* as follows: an intra-cellular electrode was inserted into as many end-plates as possible during a given period (5 min) and their activity was recorded; this procedure was then repeated at different temperatures (9–41° C), with the same rate of stimulation (25 or 40/sec). The muscle was allowed to rest for 10 min between successive trials. The proportion of end-plates which showed activity without any failure, intermittent activity, or no responses at all, was noted at each temperature. There was some variation between results obtained

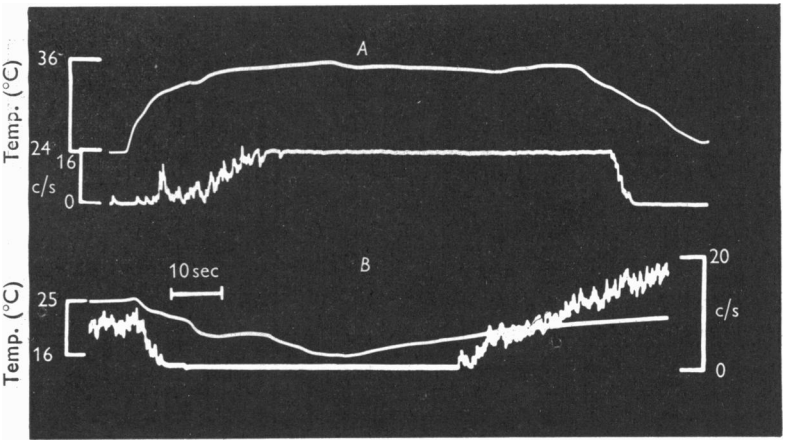

Fig. 7. Effect of changes in temperature on presynaptic failures in rat diaphragm *in vitro*. Upper trace of each pair records temperature in muscle bath measured with a thermistor; lower trace gives a continuous record of *frequency* (measured with a pulse counter) of potentials recorded intracellularly at an end-plate during repetitive supramaximal stimulation of phrenic nerve. In *A*, frequency of nerve stimulation was 16/sec; failures no longer occurred when temperature was raised to 36° C. *B*, frequency of nerve stimulation was 20/sec; there was complete failure below 20° C.

at a given temperature on subsequent occasions, but certain differences between observations at the lower and higher temperatures were evident. At a high temperature fibres functioned more effectively to begin with, when stimulated at a high frequency, but then tended to fail completely; whereas at a low temperature they could only conduct perfectly at a relatively low rate, but went on firing intermittently for a long time if stimulated at a high frequency.

Cause of presynaptic failures

It seemed likely that prolonged activity of the muscle had a deleterious effect on nerve conduction: the terminal nerve branches run a considerable distance within the diaphragm and they are fully exposed to the effects of substances

released by active muscle fibres. The following were therefore considered possible factors: lack of oxygen, lack of glucose, an excess of K^+, and an excess of lactic acid, or of H^+ in general. Another cause of injury might be pulling or compression of intramuscular nerve branches due to contraction of the muscle. The part played by these various factors was examined in several experiments. We found that the rate of intermittent failure was very much influenced by the supply of O_2, but not at all sensitive to even quite substantial variation in the concentrations of glucose, K^+, H^+, lactic acid or Ca^{2+}. For instance, an increase of external K^+ had little depressing effect unless the concentration was at least 4–5 times greater than normal (i.e. 20–25 mM), while

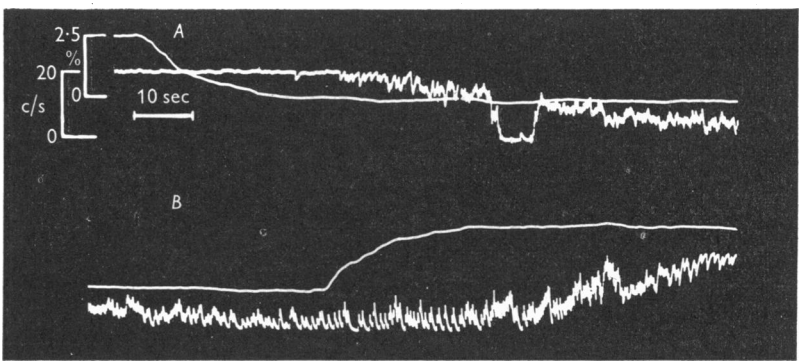

Fig. 8. Presynaptic failures in rat diaphragm at 23° C caused by anoxia. Smooth upper trace in *A* indicates O_2 concentration (v/v) in muscle bath measured with O_2 electrode. Lower trace is proportional to frequency of responses recorded intracellularly from an end-plate during nerve stimulation at 20/sec; temporary break in continuous downward slope is calibration step marking zero level. *B* is a continuation of *A*. Anoxia was produced by flow of a solution saturated with 100% N_2.

the glucose concentration could be varied from zero to 5 times the normal level with no consistent effect (cf. Hajdu & McDowall, 1949). Tenfold changes in H^+ concentration produced little change, and excess lactic acid only depressed conduction markedly when the pH reached a level of about 3 (cf. Gerard, 1932). The beneficial action of excess Ca^{2+} described by Feng (1936) was not evident in this kind of experiment.

Lack of oxygen

In some experiments we had found that cessation of the O_2 supply led to presynaptic failure, the effect being easily reversed by restoring oxygen. However, these changes were rather slow: for instance, if the bubbling of oxygen in the bath was simply stopped, failures might begin only after some 12 min during stimulation at 10/sec. Within another minute, there was complete block; after resuming bubbling with oxygen, conduction of responses

began in about 3 min, and there were no failures after another minute. The whole sequence of events could be repeated several times. The quickest way of reducing the oxygen tension was to allow a rapid flow of Ringer-Locke solution saturated with 100% nitrogen through the muscle bath. Under these conditions we found consistently a striking relationship between presynaptic failures and oxygen concentration. A characteristic result is shown in Fig. 8: initially, the solution circulating through the muscle chamber was saturated with 95% O_2 and 5% CO_2, corresponding to an O_2 concentration of 25·6 μl./ml. when the temperature (22° C) and barometric pressure (702 mm Hg) were taken into account; at this time the e.p.p.'s recorded from a muscle fibre were following stimulation of the phrenic nerve at 20/sec. The solution was then changed to one saturated with 100% N_2. The O_2 electrode shows that

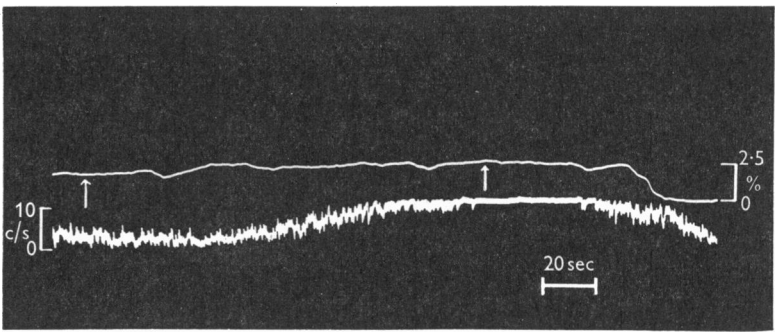

Fig. 9. Effect of a slight increase in O_2 concentration on presynaptic failures in rat diaphragm at 23° C. Upper trace is proportional to O_2 concentration (v/v) in muscle bath measured with O_2 electrode. Lower trace shows frequency of responses recorded intracellularly from an end-plate during nerve stimulation at 10/sec. Between two arrows O_2 concentration slowly increased by about 10% of initial value.

the O_2 tension at the surface of the muscle reached a low value very close to 0 within 40 sec. The height of the lower record in Fig. 8 *A* and *B* is proportional to the frequency of the e.p.p.'s. Presynaptic failures began about 25 sec after the start of the change in O_2 tension and became progressively more frequent. This continued for 20 sec after the rapid increase in O_2 tension which followed the return of solution rich in O_2; a quick decrease in the incidence of failures was then observed and virtually the initial rate of firing was reached 40 sec later. Although the importance of O_2 was shown most obviously and quickly by such large alterations in O_2 tension, even quite small changes in O_2 tension had a pronounced effect on fibres which were already failing intermittently. For instance, in Fig. 9, a slow 10% increase in O_2 tension (over 90 sec) was enough to restore full conduction in a fibre initially transmitting only about one response out of five.

Presynaptic failure in paralysed muscles

When contractions were abolished or kept to a minimum by curare or excess Mg (15 mM), or because only one or two nerve fibres were activated, preparations *in vitro* or *in situ* required stimulation at a much higher frequency (100/sec or more) for several minutes before the onset of presynaptic block. When presynaptic failures occurred, they tended to come in long cycles and were no longer affected by changes of temperature in the bath. This was further evidence that under these conditions, i.e. following prolonged maximal stimulation at a high frequency, conduction block may occur in the nerve trunk. The fact that the usual peripheral type of block also takes place was

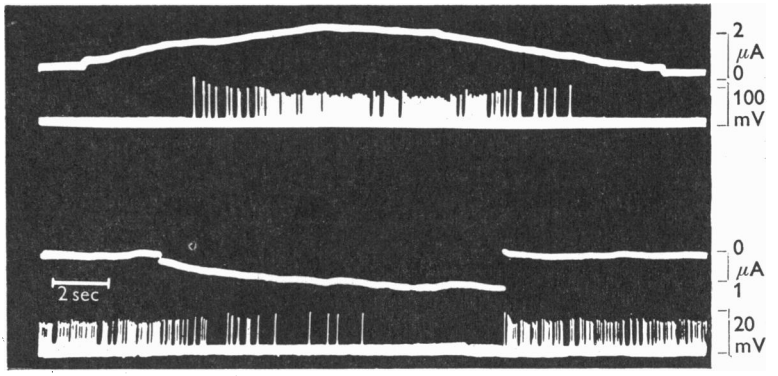

Fig. 10. Effect of nerve polarization on presynaptic failures in rat diaphragm at 23° C. Upper trace of each pair records polarizing current passed through phrenic nerve, the distal (bath) electrode being anodic above and cathodic below. Lower traces show intracellular responses obtained from two different end-plates. Above, phrenic nerve was stimulated at 20/sec and complete failure of conduction had occurred; below, the nerve was stimulated at 12·5/sec.

proved by the observation that different members of a motor unit may transmit different numbers of impulses or one or more fibres may block completely, while others continue to respond intermittently.

Effect of presynaptic polarization on conduction block

Since anodic polarization has been shown to relieve anoxic block in nerves (Thörner, 1922*a*, *b*; Lorente de Nó, 1947*a*) we examined the effect of polarization on presynaptic failures in four experiments *in vitro*. Anodic and cathodic polarization of the distal part of the nerve had the expected beneficial and depressing actions on twenty-three out of thirty fibres, as is shown in Fig. 10. In the upper tracings a fibre which had ceased to conduct began transmitting impulses again under the influence of anodic polarization; at the peak of the action almost every impulse was transmitted. The opposite change is evident in the lower tracings, where cathodic polarization caused a complete, but

reversible, block of conduction. The actual relation between the proportion of impulses transmitted and the polarizing current intensity, at a fixed rate of stimulation in two experiments, can be seen in the two graphs of Fig. 11. The distribution of points suggests that there is a fairly simple S-shaped relation between the two variables, such as may be expected in the presence of random variations in threshold and/or spike height (Blair & Erlanger, 1933, 1936; Miledi, 1957).

The strength of polarizing current necessary for a given change in the rate of firing varied considerably for different end-plates of the same preparation, but there was no correlation between the distance of an end-plate from the

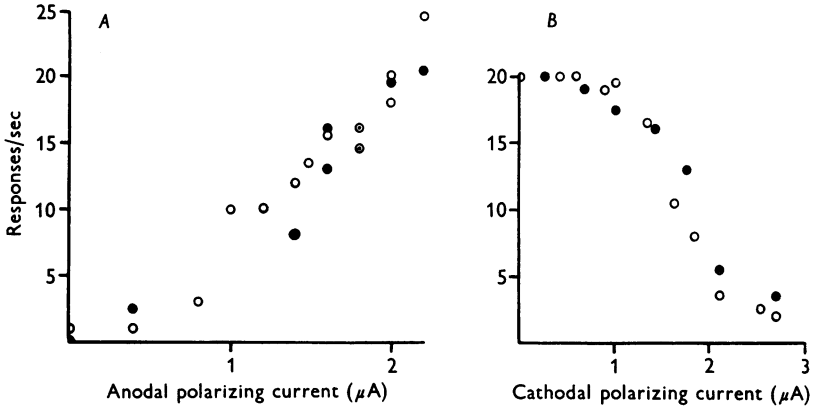

Fig. 11. Graphs showing relationship between amount of nerve polarization and frequency of end-plate responses in two experiments. The fixed rate of nerve stimulation was 25/sec in *A*, and 20/sec in *B*. Points recorded (○) while increasing current intensity; (●) while reducing it.

partition and the required strength of current, as would be expected if the polarization acted upon the nerve terminals at the end-plate; accordingly, even the strongest currents failed to alter in any way the spontaneous discharge of miniature e.p.p.'s (cf. del Castillo & Katz, 1954; Liley, 1956). Fibres apparently fail at different points along the nerve, but mostly at a comparatively short distance after entering the muscle.

DISCUSSION

In the course of unpublished experiments it had been observed that isolated mammalian phrenic nerve fibres, immersed in liquid paraffin, could be stimulated at frequencies of 12–50/sec for periods of several hours without the fibres ceasing to propagate impulses. This suggested that at these frequencies the failures reported here were essentially dependent on the conditions prevailing in the intramuscular portion of the nerves, in agreement with the observations that failures were affected by changes of temperature, or oxygen supply, or by adrenaline (Krnjević & Miledi, 1958*c*) in the muscle bath.

The refractory period of mammalian nerve lasts normally much less than 10 msec (Gasser & Grundfest, 1936; Graham & Lorente de Nó, 1938). This should be compatible with repetitive activity at a frequency of 100/sec or even greater. Therefore, it is perhaps surprising that failure of propagation should occur in nerves stimulated at frequencies lower than 100/sec. However, the intramuscular portion of the nerve is subjected to a more or less permanent state of hypoxia during tetanic contractions (see below) and it is known that the threshold and the refractory period of nerve fibres increase during anoxia (Heinbecker, 1929). Furthermore, several authors have demonstrated a prolongation of the refractory period as a result of tetanic stimulation (Heinbecker, 1929; Woronzow, 1935; Bugnard & Hill, 1935; von Brücke, Early & Forbes, 1941; Brink, Bronk, Carlson & Connelly, 1952). The delay in the recovery of excitability is especially marked even after quite moderate tetani (20/sec for 30 sec); the subnormal excitability may last for many seconds (Gasser & Grundfest, 1936; Graham & Lorente de Nó, 1938; von Brücke *et al.* 1941).

Patterns of impulses

During a long tetanus subnormality develops gradually, and a plateau may be reached which will not show any sharp variations of excitability. If the increase in threshold is sufficiently large and/or if the spike is sufficiently reduced, small random variations in membrane voltage ('membrane noise') may determine whether further propagation is to occur or not (cf. Blair & Erlanger, 1933, 1936; Bullock & Turner, 1950; Miledi, 1957; in unpublished experiments A. J. Buller and K. Krnjević have ascertained that rat nerves often fire at random during low-frequency threshold stimulation). This might well give rise to the random firing observed *in vitro* in all fibres during stimulation at frequencies less than 19/sec and in a diminishing number of fibres at higher frequencies, up to 44/sec (cf. Fig. 4).

The excess of groups at the higher frequencies of stimulation *in vitro* was clearly related to the tendency of alternate impulses to fail (cf. Feng, 1941), presumably because of a prolongation of the refractory period. According to the data of Fig. 4, the refractory period must have had a maximum duration of about 50–60 msec. After a period of tetanic stimulation the duration of the refractory period may return to its normal value only after some 30–40 min (Woronzow, 1935; Bugnard & Hill, 1935; Brink *et al.* 1952). The progressive changes seen after the end of a 2–3 min tetanus at 64/sec correspond to the expected shortening of the refractory period.

Cyclic patterns

In vitro, cyclic patterns were mainly seen when failures occurred in the nerve trunk. A possible explanation is that cumulative and prolonged subnormality may cause a complete block for a substantial period. When the

excitability returns towards normal a sequence of impulses may be conducted not only because subnormality is slow to develop, but perhaps also because of a temporary phase of supernormality (cf. Fig. 12 in Gasser & Grundfest, 1936). The reappearance of subnormality would complete the cycle by producing another prolonged block. This is a rather striking oscillatory example of the complex adaptive behaviour described as the nerve reaction by Lorente de Nó (1947*b*). A cyclic pattern has been seen during stimulation of frog nerve fibres with threshold intensity by Blair & Erlanger (1936). Woronzow (1935) also described it as a possible type of activity during repetitive stimulation, but it is not clear whether he actually observed this phenomenon.

Oxygen lack

Our results support the most likely explanation (cf. Gerard, 1930), i.e. that nerve fibres running within the muscle are depolarized and cease to conduct impulses as a direct consequence of more or less prolonged anoxia. There must inevitably be a deficiency of oxygen in a muscle soon after the onset of a maintained tetanus. Even at rest, the full thickness of the rat diaphragm *in vitro* has an adequate supply of oxygen only under optimum conditions of stirring and external concentration (Creese, 1954; Creese, Scholes & Whalen, 1958). The surface layers of fibres, from which most electrical records are obtained, are much less likely to suffer from anoxia, even during repetitive activity (cf. Creese, Hashish & Scholes, 1958). However, the nerve runs within the depth of the muscle where anoxia is most acute. The nerve fibres are protected from changes in their environment by the low permeability of their perineural sheath (Feng & Liu, 1949; Krnjević, 1954); hence their relative insensitivity to variations in external K, etc.; but the sheath of course cannot protect from anoxia.

Conditions *in situ* are somewhat different, but during a maintained contraction the flow of fresh blood into a muscle may well be inadequate, and a state of hypoxia is likely to develop rapidly. Presynaptic failure of nerve conduction might then occur and prevent further maintenance of the tetanus. Recent observations by Clarke, Hellon & Lind (1958) are consistent with the possibility that such a mechanism is at least partly responsible for limiting the duration of sustained contractions in the human forearm.

Mechanical injury by contractions

We have varied the method of fixing the diaphragm in the chamber in several ways, and even left it completely free, but with no obvious change in the incidence of failures. Extensive cutting of the muscle to make the nerve as loosely joined as possible at the critical point of entry also had little apparent effect. These observations, together with others made during paralysis suf-

ficient to prevent the stronger contractions, suggest that mechanical injury probably does not play a major part in the causation of presynaptic block.

Temperature changes

Gerard (1930) has also found that anoxia causes a block of conduction in mammalian nerves more rapidly at higher than at lower temperatures. At high temperatures the O_2 consumption of the nerve is much greater, the Q_{10} being about 2·5 for rat peripheral nerve between 15 and 37° C (Cranefield, 1952); furthermore, it is evident that irreplaceable substrates needed for the metabolism of the nerve will be consumed more rapidly at the higher metabolic rate. These changes are accelerated by repetitive stimulation (Cook & Gerard, 1931); they clearly account for the rapid occurrence and large proportion of complete failures seen at 37° C, and for the fact that the preparations survived much longer at room temperature.

On the other hand, variations of temperature have a somewhat different short-term effect on repetitive activity. A reduction of the temperature of nerve fibres is associated with smaller local responses and spikes, but higher thresholds (Miledi, 1957) and slower rates of recovery (Boycott, 1899; Tasaki, 1949); this clearly leads to a lower safety factor for transmission, and reduces the maximum frequency at which impulses can be conducted. Lorente de Nó (1947*b*, p. 315) has stressed the similarity between the effects of anoxia and of cooling on the action potential.

Site of conduction failure

It is clear that, so far as the experiments *in vitro* are concerned, the nerve block in most cases occurs in the most peripheral part of the nerve, either in its intramuscular course, or very close to the muscle. This is shown by the great sensitivity of nerve failures to variations inside the muscle bath in such factors as the temperature, O_2 supply, nerve polarization, etc. We have also presented evidence that different muscle fibres belonging to the same motor unit may fail at unequal rates. These facts taken together are consistent with the suggestion (Krnjević & Miledi, 1957, 1958*b*) that the conduction block is most likely to occur at points of branching, where the safety factor of transmission is sharply reduced.

However, the situation is somewhat complicated by evidence that a conduction block may also occur at a site outside the muscle bath, presumably some distance away in the nerve trunk. This type of block is seen not uncommonly when the nerve is stimulated at a high frequency and for long periods; it is often associated with a cyclic kind of discharge.

Judging by the pattern of discharge, both types of block occurred *in situ*, although the cyclic type was seen relatively more often than *in vitro*. This is no doubt because high rates of stimulation had to be used in a greater proportion

of cases. Experimental conditions *in situ* did not allow us to determine the site of failure with any degree of accuracy, and it is only by drawing the analogy with observations made *in vitro* that one may tentatively ascribe the cyclic type of discharge predominantly to conduction block in the nerve trunk, at some distance from the muscle, and the other, intermittent type of discharge, to conduction block in the intramuscular portion of the nerve.

SUMMARY

1. Repetitive indirect stimulation of rat muscle *in vitro* or *in situ* leads to a presynaptic block of nerve conduction which may be intermittent or complete, but which is reversible if the stimulation is stopped or continued at a lower rate.

2. An analysis has been made of the temporal patterns of impulses seen under these conditions. In many cases failures of conduction occur in obvious patterns: e.g. they alternate more or less regularly with impulses or they come in long cycles.

3. In most cases the firing does not produce clear patterns, and it is necessary to test the possibility that impulses and failures occurred in a random manner. This was done by counting the number of groups of events of the same kind in sequences of impulses and failures, and comparing them with numbers expected on the nul-hypothesis. It is found that all sequences recorded *in vitro* at frequencies of stimulation < 19/sec are random, whereas all sequences at frequencies > 44/sec have an excess or a deficiency of groups. Sequences at intermediate frequencies sometimes are random, sometimes not.

4. It seems that at frequencies of stimulation < 19/sec, random fluctuations of fibre threshold and/or spike height in the region of block are responsible for the observed intermittent conduction of impulses; at higher frequencies the part played by post-spike subnormality or supernormality becomes increasingly predominant.

5. Presynaptic block *in vitro* is not at all sensitive to substantial variations in the external concentrations of glucose, K^+, Ca^{2+} or H^+, but it is very sensitive to warming or cooling. At higher temperatures conduction is possible at a greater frequency, but total block occurs early; at low temperatures only relatively low-frequency conduction can occur, but it may continue for a longer period.

6. Presynaptic block is precipitated by anoxia *in vitro*. A fibre already failing intermittently responds quickly and reversibly to variations in O_2 supply. The block is relieved by anodic polarization, and aggravated by cathodic polarization of the phrenic nerve at its entrance into the diaphragm.

7. It is concluded that presynaptic block, commonly associated with intermittent firing, is probably caused by anoxic changes in the intramuscular portion of the motor nerve.

8. When the muscle is prevented from contracting, anoxia does not develop readily and conduction block is only seen after prolonged high-frequency stimulation; it is then often associated with cyclic patterns of firing. Evidence is presented that failure of propagation may then occur in the nerve trunk, some distance from the muscle.

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