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IONIC INFLUENCES ON SUCCINYLCHOLINE BLOCKADE OF THE MAMMALIAN NEUROMUSCULAR JUNCTION

BY

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Recent studies from a number of laboratories have been concerned with the delineation of factors affecting presynaptic events in neuromuscular transmission. In particular it seems that substances which block neuromuscular transmission by processes that include depolarization of the motor end-plate have important effects on the motor nerve terminals. These effects are not only produced by drugs such as succinylcholine and decamethonium, but are also shown by acetylcholine and its stable analogues.

Hubbard, Schmidt & Yokota (1965) noted that acetylcholine reduced the quantal content of end-plate potentials (e.p.p.s.) without affecting miniature end-plate potential (m.e.p.p.) frequency. It appeared that acetylcholine did not depolarize the site of transmitter release, although the threshold of motor nerve terminals in paralysed preparations was reduced by acetylcholine. They concluded that acetylcholine depolarized the terminals at a site more proximal than that of transmitter release. Riker (1966) has produced evidence for a transitory depolarization of the motor nerve terminals by acetylcholine following close arterial injection in the cat. It is likely that Hubbard *et al.* (1965) did not observe this effect because of the slower rate of diffusion of acetylcholine into the isolated diaphragm preparation. Riker (1966) suggests that the motor nerve terminals undergo sequentially two stages of block in response to acetylcholine—depolarization and stabilization.

This is a reformulation of the proposals of Jenden, Kamijo & Taylor (1954) and Thesleff (1955a) for a two-phase block of neuromuscular transmission by decomethonium and acetylcholine. The earlier proposals were, however, based on the assumption that these drugs had a postsynaptic site of action only.

The similarity of action at the neuromuscular junction of acetylcholine, decamethonium and succinylcholine has been noted by most workers in the field. In particular one may compare the similarity of results obtained by Standaert & Adams (1965) with those obtained by Riker (1966). Standaert & Adams investigated prejunctional effects of succinylcholine in the intact cat; Riker used similar techniques to study the effects of acetylcholine. The emphasis which Standaert & Adams (1965) have placed on prejunctional effects of succinylcholine receives support from the observation by Edwards & Ikeda (1962) that succinylcholine reduces the quantum content of e.p.p.s. Standaert & Adams (1965) remark, however, that while their work demonstrates unequivocally

that prejunctional effects of succinylcholine exist, they were not able to evaluate the relative importance of pre- and postjunctional effects in the development of neuromuscular blockade.

The recent work of Gibberd (1966) draws attention to the importance of the metabolic status of the nerve-muscle preparation when such experiments are carried out. Whole animal preparations have the advantage of maintaining the metabolic status of the junction, but they also have the disadvantage for studies of succinylcholine action that the pseudocholinesterase of blood rapidly hydrolyses the compound. Not only does this complicate quantitative work, but it leads also to succinylmonocholine accumulation, which itself has some action on the neuromuscular junction.

Because of this it was thought worthwhile to study the kinetics of succinylcholine blockade, using the phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946). In spite of the limitations inherent in the measurement of twitch tension of a preparation, it has been possible to obtain quantitative information about the rate of development of neuromuscular blockade by succinylcholine in conditions of ionic stress.

The results obtained indicate that succinylcholine has effects on both pre- and post-junctional membranes. The rate of onset of succinylcholine blocks seems to depend largely but not entirely on a presynaptic inhibition of transmitter release.

METHODS

Phrenic nerve-diaphragm preparations were dissected from male hooded rats of the Wistar strain. The rats ranged in weight from 250 to 350 g ; the average weight was 300 g. The rats were stunned by a blow on the head, and killed by exsanguination. The use of anaesthetic agents was avoided, because of the likelihood of residual effects on the neuromuscular junction.

Left and right hemidiaphragms were dissected out of the animal, and mounted in separate organ baths. (Bülbring, 1946 ; Beani, Bianchi & Ledda, 1962). The left hemidiaphragm was routinely dissected before the right ; it took between 6 and 9 min after stunning the animal before the two preparations were placed in an oxygenated solution ; the total time to set up the two preparations in the two organ baths was 17–18 min.

Semi-isometric contractions were measured by strain gauges ; the output of the strain gauges was recorded on a Beckman type R dynograph recorder. Supramaximal rectangular pulses of 0.15 msec duration and a frequency of 14/min were used to stimulate the preparations, either by direct muscle stimulation or by the phrenic nerve. The force of contraction was determined by calibrating the strain gauges with weights. The twitch tension developed by the preparations varied between 3 and 6 g ; while that of most preparations fell within a range of 4.5 to 5.5 g.

All preparations maintained full contractility over the usual experimental period of approximately 5 hr. At the end of an experiment all preparations were able to sustain a 10 sec tetanus (700 impulses), and showed post-tetanic potentiation. The tetanic contraction was 3.5–5 times the height of the twitch tension ; post-tetanic potentiation was approximately 160% of the control tension.

The preparations were left for approximately 30 min in the organ bath before the experiment commenced. During this time they were washed four times with bathing solution to remove as much plasma, and therefore pseudocholinesterase, as possible, which might have caused some hydrolysis of succinylcholine close to the neuromuscular junction. In general, preparations did not show any change in force of contraction during this period.

The temperature within the organ baths was monitored continuously with a thermistor. The temperature during any one experiment never varied by more than 0.5° C.

The normal bathing solution had the following composition: NaCl 115 mM, KCl 4.63 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 1.0 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 22 mM, glucose 22 mM. When bubbled with

95% oxygen and 5% carbon dioxide the pH was 7.4 at 37° C. To compensate osmotically for removal of NaCl, 1.57 mM sucrose was added to the solution for every 1 mM NaCl omitted (Gage & Quastel, 1966). The level of Ca^{++} was lower than has been used by some other workers; this was in conformity with the finding of Van Breeman, Daniel & Van Breeman (1966) that in rat plasma only 1.5 mM Ca^{++} is not bound to albumin.

Succinylcholine chloride was used and in most experiments the concentration of the drug in the organ bath was 8.7×10^{-6} M. This was the minimum quantity that brought about complete neuromuscular blockade at 30° C. The drug was added to the organ bath by rapid injection into the stream of bubbles of oxygen and carbon dioxide which aerated the solution. Experiments with dyes showed that mixing was complete within 10 sec.

Preliminary experiments carried out at 36° C showed great variation in the rate of development of succinylcholine blockade; there was also a rapid onset of tachyphylaxis. These variations made a quantitative approach to the problem impossible. It was found that these effects were markedly reduced at 29°–30° C.

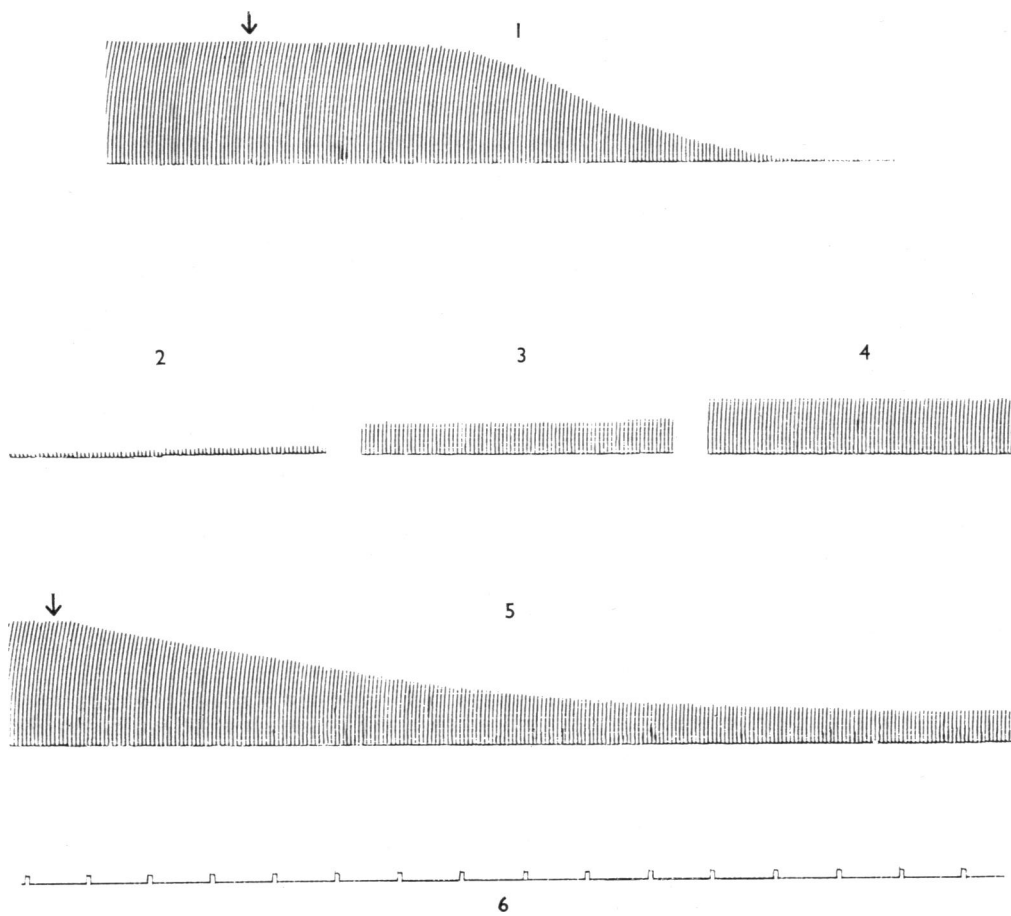

Fig. 1. Recorder trace of the development of succinylcholine blockade. Panel 1 shows initial succinylcholine block (8.7×10^{-6} M); succinylcholine added at arrow. Panels 2–4 show partial recovery in the continued presence of succinylcholine. Panel 2 at 30 min; panel 3 at 60 min; panel 4 at 120 min. Panel 5 shows succinylcholine block after 150 min in succinylcholine followed by 30 min wash. Time marker 1 min intervals.

The kinetic approach to the rate of development of succinylcholine blockade, and the statistical procedures used will be detailed in the results section.

RESULTS

Figure 1 illustrates the decline in twitch tension of a diaphragm following the addition of 8.7×10^{-6} M succinylcholine to the organ bath. After a latent period of some 4 min the twitch tension declined exponentially to zero. The rate of onset of blockade was estimated by plotting the twitch tension at 12 sec intervals on semi-logarithmic co-ordinates. A straight line was fitted by eye through the exponential portion of the graph to estimate the time to half decay of twitch tension ; the length of the latent period (latency) was taken as the intercept of this line with a line drawn through the points when the twitch tension did not alter. This procedure is illustrated in Fig. 2, curve A. The length of the latent period (latency), and the time to half decay of twitch tension ($t\frac{1}{2}$) have been used as parameters of succinylcholine blockade throughout this study.

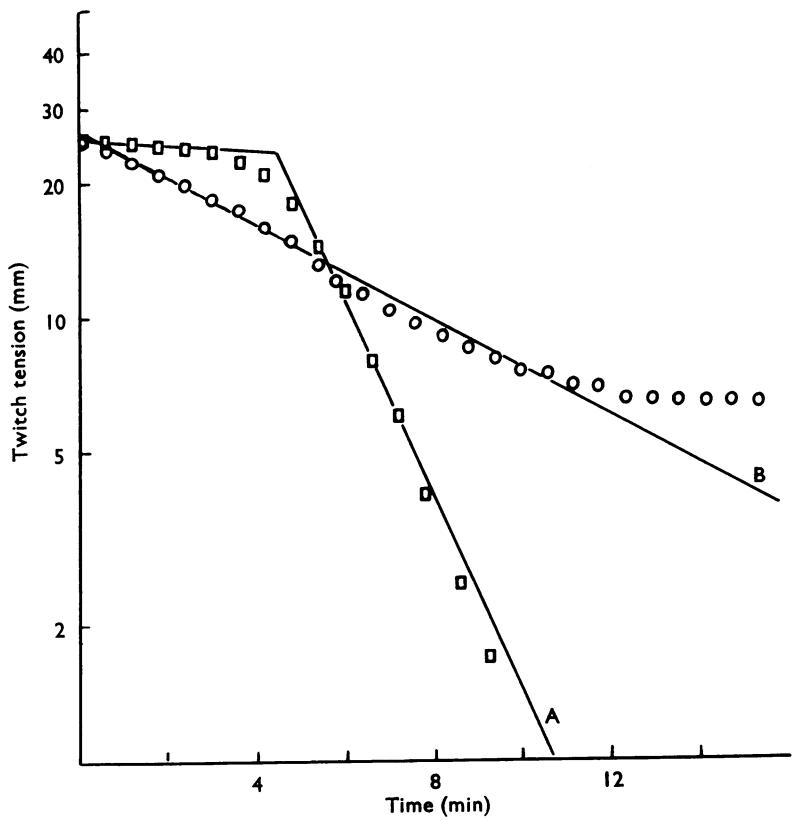

Fig. 2. Semilogarithmic plot of succinylcholine blockade, using data shown in Fig. 1. Curve A (□——□), initial block, succinylcholine 8.7×10^{-6} M. Curve B (○——○), succinylcholine block after 150 min exposure to succinylcholine followed by 30 min wash. Succinylcholine added at zero time. In this and Fig. 3 20 mm twitch tension is equivalent to 5 g.

In the present experiments preparations showed little spontaneous recovery in the continued presence of succinylcholine, as may be seen from Fig. 1. Neuromuscular blockade was complete 10 min after adding succinylcholine to the organ bath, a further 15 min elapsed before there was any response to nerve stimulation. Fifty-two minutes after the commencement of blockade the preparation had recovered 20% of the control twitch tension; at 100 min this had risen to 50%, where it remained constant until 150 min, when succinylcholine was washed out. Recovery of twitch tension was complete in 5 min. After a further 30 min succinylcholine was re-admitted; neuromuscular blockade proceeded without latency, and with an increase in $t\frac{1}{2}$ to 5.4 min, compared with an initial value of 1.4 min (Figs. 1 and 2). However, the second dose failed to cause complete block.

Figure 3 illustrates the effect of repeated succinylcholine block, using the companion hemidiaphragm to the one used for Figs. 1 and 2. The preparation was washed for 25–35 min between each block. It may be seen that the first three blocks followed an almost identical time course; the fourth and fifth blocks showed an increased $t\frac{1}{2}$ but

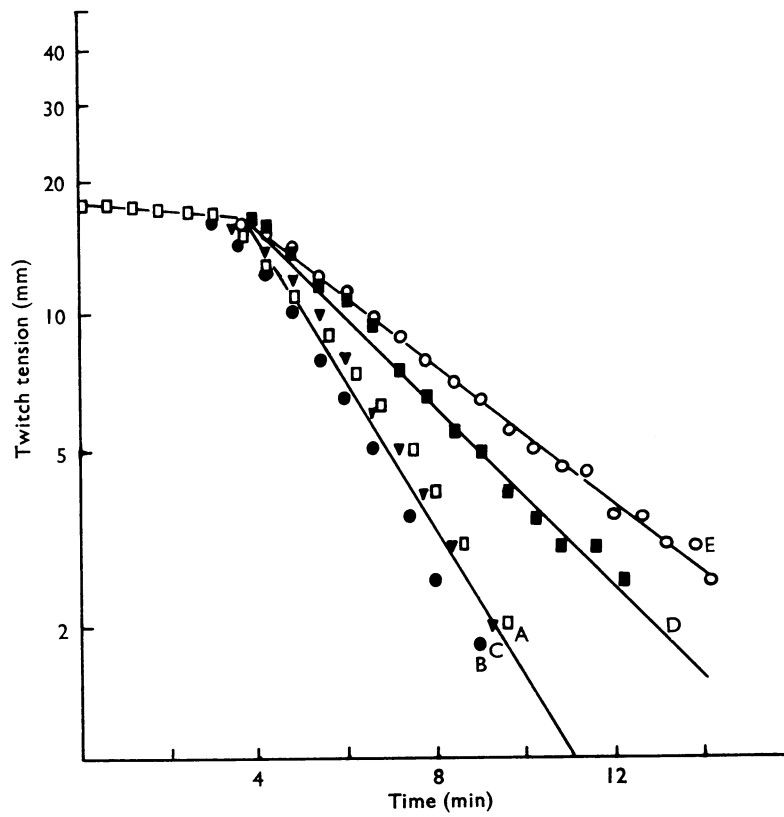

Fig. 3. Effect of repeated succinylcholine blockade. Succinylcholine block was obtained in the order A–E. Semilogarithmic plots of blocks A–C follow a similar time course. Blocks D and E show an increase in $t\frac{1}{2}$. Companion hemidiaphragm to that illustrated in Figs. 1 and 2.

little or no change in latency. This response to succinylcholine was typical at 29°–30° C, and permitted a quantitative approach to the problem.

These results differ from those of most workers who have carried out experiments with depolarizing neuromuscular blocking drugs at 36°–37° C. Gibberd (1966), in common with earlier workers (for references see Taylor & Nedergaard, 1965), found that preparations showed spontaneous recovery after an initial block with decamethonium, with the slow development of a secondary block. This phenomenon has been described as Phase I and Phase II block (Jenden, Kamijo & Taylor, 1954). Failure to obtain Phase I and Phase II blocks in the present experiments is likely to be the result of the choice of experimental conditions which avoided factors promoting the onset of tachyphylaxis. Further, decamethonium blockade is more susceptible to tachyphylaxis than is succinylcholine (Freeman, unpublished).

A statistical analysis of the data was undertaken to determine the most appropriate experimental procedure for testing the effects of ionic or other environmental stresses on the preparation. Table 1 shows that the first succinylcholine block obtained on the

TABLE 1

THE EFFECT OF TEMPERATURE ON LATENCY AND $t\frac{1}{2}$ OF SUCCINYLCHOLINE BLOCKADE

Figures shown are + S.E. of the mean. Figures in parenthesis refer to the number of observations. The concentration of succinylcholine was $8{\cdot}7 \times 10^{-6}$ M.

	29°–30° C		20·5°–21·5° C	
	Latency	$t\frac{1}{2}$	Latency	$t\frac{1}{2}$
Left hand preparation	4·5±0·2 (28)	2·2±0·2 (28)	3·2±0·2 (20)	0·49±0·01 (20)
Right hand preparation	4·5±0·2 (25)	2·1±0·2 (25)		

left hemidiaphragm followed an almost identical time course to the first block using the right preparation. To test whether the variation between animals was greater than the variation between right and left preparations a linear correlation was calculated. It was found that there was no significant correlation between left and right preparations when considering the length of latency. Thus the variation between paired preparations was similar to the variation between animals. When, however, the time to half block ($t\frac{1}{2}$) was considered it was found that there was a highly significant linear correlation between right and left preparations ($P<0.001$). Further, the slope of the regression was not significantly different from one. Consequently it is legitimate to estimate $t\frac{1}{2}$ of one preparation by use of the companion preparation.

It was found, however, that the first block offered an even better estimate of the second block than was provided by the use of paired preparations, because in this instance both latency and $t\frac{1}{2}$ were highly significantly correlated ($P<0.001$). The slope of the regression for both latency and $t\frac{1}{2}$ was not significantly different from one.

A consideration of the relation between second and third blocks showed that there was no significant linear correlation between either the latency or $t\frac{1}{2}$. The scatter in results however was not great, as can be seen from the means ± S.E. of ten pairs of results. The second block had a mean latency of 4.4 ± 0.4 min, and a mean $t\frac{1}{2}$ of 2.5 ± 0.5 min; the third block had a mean latency of 4.3 ± 0.3 min, and a mean $t\frac{1}{2}$ of 3.8 ± 1.1 min. Thus while it was not possible to estimate the time course of the third block in terms of the

second, significant qualitative information could usually be obtained from third and even fourth blocks.

In planning experiments the first block was usually taken as control for the second; however, paired preparations were always used, and occasionally the left preparation acted as control for the right.

Dose-response relationships

The constancy of the data presented in the previous section encouraged the hope that it might be possible to obtain a dose-response curve on a single hemidiaphragm. This attempt was only partially successful, because the development of tachyphylaxis at or after the third block tended to augment or diminish the effect of change in dose, depending whether a high or low dose was given first.

The results of one typical experiment are shown in Fig. 4. Paired preparations were blocked successively with increasing or decreasing doses of succinylcholine. It may be seen from Fig. 4A that a "reasonable" dose-response curve was obtained when the sequence of blocks started with a low dose. Fig. 4B illustrates the marked tachyphylaxis that developed when the highest dose (2.6×10^{-5} M) was introduced first. High levels of succinylcholine promote the development of tachyphylaxis.

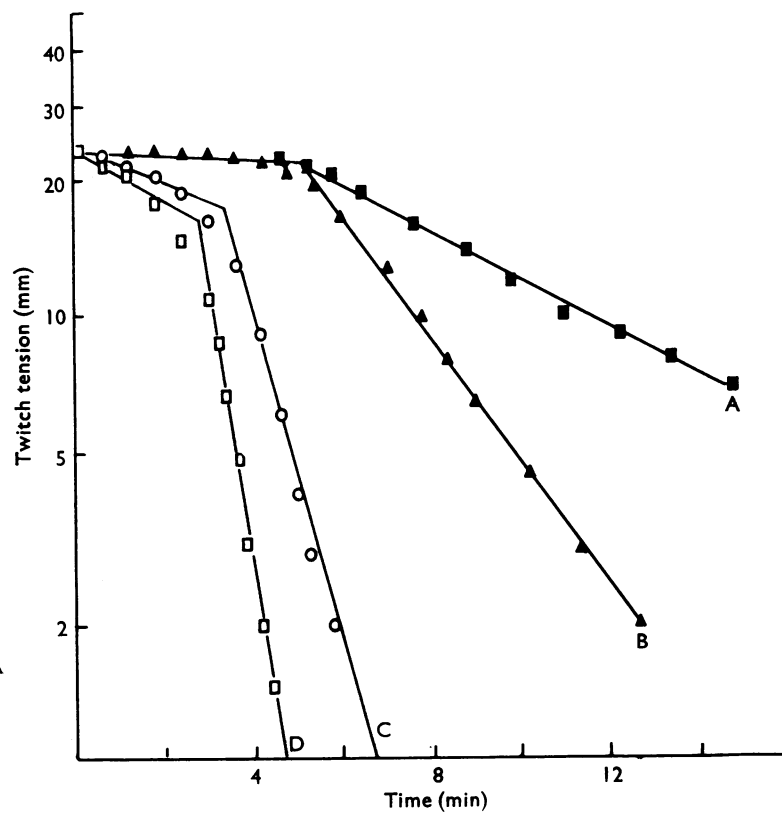

Fig. 4A

Fig. 4B

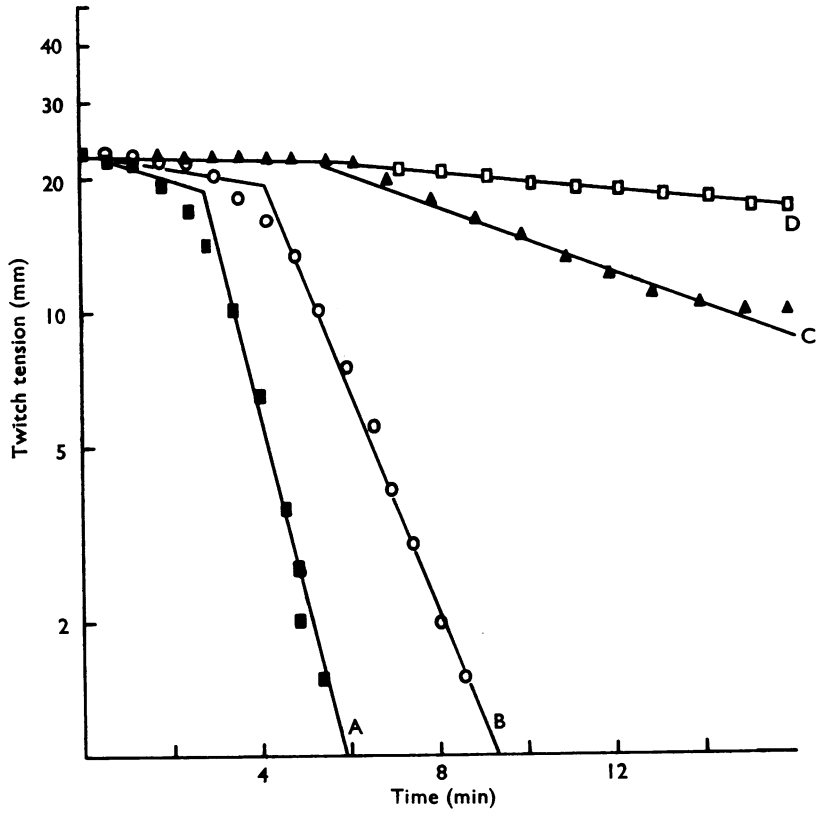

Fig. 4. A: Dose response data. Semilogarithmic plots of succinylcholine blocks obtained in the order A–D. A (■——■), succinylcholine 5.8×10^{-6} M, latency 4.8 min, $t\frac{1}{2}$ 5.2 min; B ▲——▲), succinylcholine 8.7×10^{-6} M latency 5.0 min, $t\frac{1}{2}$ 3.2 min; C (○——○). succinylcholine 1.7×10^{-5} M, latency 3.2 min, $t\frac{1}{2}$ 0.9 min; D (□——□), succinylcholine 2.5×10^{-5} M, latency 2.6 min, $t\frac{1}{2}$ 0.60 min. B: Semilogarithmic plots of succinylcholine blocks obtained in the order A–D. A (■——■), succinylcholine 2.6×10^{-5} M, latency 2.6 min, $t\frac{1}{2}$ 0.78 min; B (○——○), succinylcholine 1.7×10^{-5} M, latency 3.9 min, $t\frac{1}{2}$ 1.3 min; C (▲——▲), succinylcholine 8.7×10^{-6} M, latency 5.4 min, $t\frac{1}{2}$ 6.5 min; D (□– –□), succinylcholine 5.8×10^{-6} M, latency 3.8 min, $t\frac{1}{2}$ 30.7 min.

It can be noted from Fig. 4 (A and B) that latency was reduced concurrently with $t\frac{1}{2}$ at high levels of succinylcholine.

Rate of recovery from succinylcholine blockade

The recovery of twitch tension on washing succinylcholine out of the organ bath was very rapid at 29°–30° C. The rate of recovery could be described by a single exponential term, without latency. Recovery was complete in approximately 5 min; the half-time of recovery was approximately 1 min. A typical curve is illustrated in Fig. 5, which also shows the reduced rate of recovery at 21° C.

The preparation had not recovered entirely when normal twitch tension returned, because the readmission of succinylcholine within 15 min of wash-out led to a decrease in $t\frac{1}{2}$. It was possible to correlate delayed recovery with a long-lasting depression of the ability of the preparation to sustain a 10 sec tetanus, and with a loss of post-tetanic potentiation (PTP).

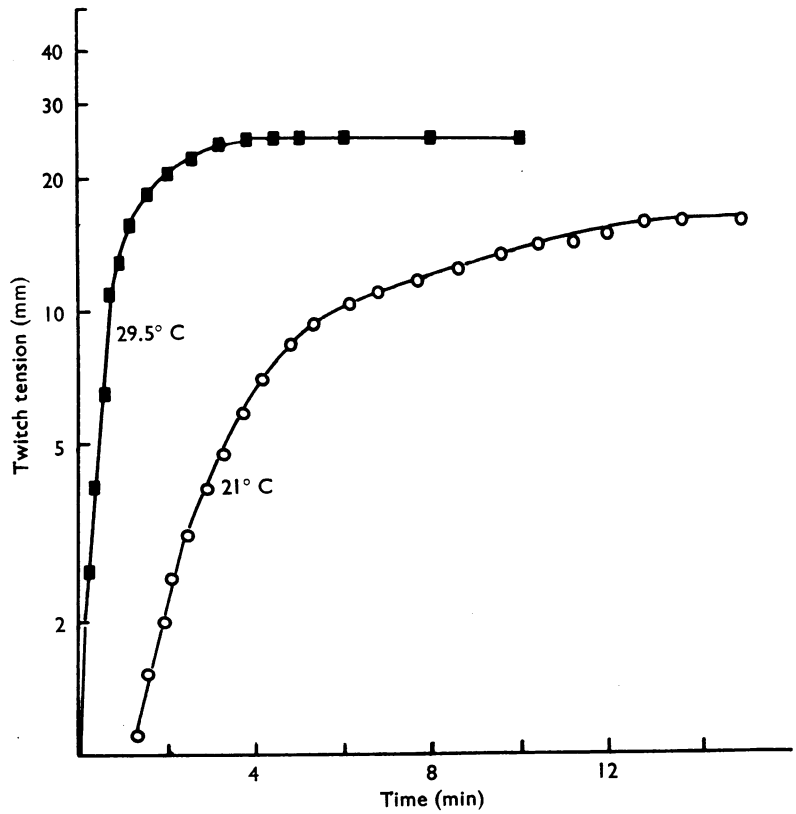

Fig. 5. Rate of recovery from succinylcholine blockade. Semilogarithmic plots show recovery at 29.5° C (■——■), and recovery at 21° C (○——○). At the lower temperature the gain of the recorder was reduced by half.

It was noted, in common with Zaimis (1953) that a preparation that was partially blocked with succinylcholine would not sustain a tetanus. As the preparation recovered on washing there was a slow return of tetanic contraction (Zaimis, 1953, Fig. 5). Eight to ten minutes elapsed before the tetanus was sustained. Post-tetanic potentiation (PTP) was replaced by post-tetanic depression between 3 and 5 min after wash-out of succinylcholine. There was a gradual recovery of PTP which followed a somewhat slower time course than the recovery of tetanic tension. Full recovery of PTP took approximately 20 min.

The slow recovery of PTP after succinylcholine recalls the findings of Standaert & Adams (1965) and Riker (1966), using succinylcholine and acetylcholine respectively, in

the intact cat. These authors noted a suppression of post-tetanic repetition in the nerve terminals at the neuromuscular junction, which persisted for 15–20 min after a blocking dose of either drug. Standaert & Adams (1965) state that PTP is almost entirely dependent on the occurrence of post-tetanic repetition in the motor nerve ; each potentiated contraction is a brief tetanic contraction rather than a simple twitch.

The response of the muscle to direct stimulation was depressed during neuromuscular blockade. This elevation of threshold is no doubt caused by the spread of depolarization around the end-plate region, similar to that occurring with decamethonium block (Burns & Paton, 1951 ; Creese, Dillon, Marshall, Sabawala, Schneider, Taylor & Zinn, 1957) and could be overcome by increasing the stimulating voltage. The threshold of the muscle to direct stimulation was determined with the bath emptied of bathing solution. Maximal contractions were obtained with stimulating voltages of 3–5 V. It was necessary to increase the voltage to 75–80 V to overcome the threshold increase during neuromuscular blockade.

It is noteworthy that during neuromuscular blockade the directly stimulated muscle would sustain a tetanus, and showed PTP.

It was noted that when three or four periods of tetanic stimulation were applied during recovery from succinylcholine block there was a two- to three-fold increase in $t\frac{1}{2}$ on subsequent succinylcholine block. This increase in $t\frac{1}{2}$ was associated with some loss of latency.

Temperature effects

Succinylcholine blockade differs from that produced by curariform drugs in that the rate of onset of block increases with decreasing temperature (Bigland, Goetzee, Maclagan & Zaimis, 1958), whilst curare and its congeners block more slowly at low temperature (Holmes, Jenden & Taylor, 1951 ; Beani, Bianchi & Ledda, 1962). Experiments which were carried out at 20°–21° C showed a shortening of the latent period, and a decrease in $t\frac{1}{2}$ compared with results obtained at 29°–30° C. Table 1 shows a comparison of the values obtained for the two parameters. The change in latency with drop in temperature was proportionately less than the change in $t\frac{1}{2}$ which shows a very high temperature coefficient. It was also noted that tachyphylaxis did not occur at this temperature. Reiterative blockade with succinylcholine interspersed with 30 min wash periods yielded identical data for five successive blocks.

The rate of recovery was considerably slower at 21° C than at 29° C (Fig. 5). Recovery, after a latent period of approximately 1 min, occurred as a sum of two exponential terms. There was an initial rapid phase followed by a slow phase ; recovery of twitch tension was not complete until 14–16 min after the commencement of the wash.

Temperature effects were also studied by changing the temperature during the progress of a block. It was found that it was possible to alleviate a block by raising the temperature, and vice versa. The rate of onset or recovery could also be altered as would be expected from the above data. There was no apparent time lag when a warm succinylcholine solution was replaced by a cool one.

Additional experiments were carried out in which the first one or two blocks were applied at one temperature, and subsequent blocks were applied at a higher or lower

temperature. The results obtained depended on the order of the temperature change.

When the initial block was carried out at 36° C subsequent blocks at 29°–30° C showed normal latency, but $t\frac{1}{2}$ was increased three- to four-fold over the values shown in Table 1. It would appear that incubation at 36° C favours the development of tachyphylaxis.

Low temperature has been found to diminish the end-plate potential of frog muscle, because of a decrease in transmitter output from the nerve terminals (Takeuchi, 1958). This finding was confirmed for mammalian muscle by Hofmann, Parsons & Feigen (1966), who noted a marked decline in sustained transmitter release at 24°–26° C. The end-plate membrane seems to be more sensitive to acetylcholine at low temperature (Beani, Bianchi & Ledda, 1962); however, these authors also report a decreased release of acetylcholine at low temperatures. These findings indicate that succinylcholine augments the inhibition of transmitter release which is found at low temperature.

Frequency of stimulation

The finding that succinylcholine reduces the presynaptic output of acetylcholine (Edwards & Ikeda, 1962) could result from an interference by succinylcholine with the acetylation of choline. Such a "hemicholinium-like" action has been ascribed to decamethylene-*bis*-(hydroxyethyl)dimethylammonium salts (Bowman & Hemsworth, 1965) and could be a feature common to all depolarizing neuromuscular blocking drugs.

This is unlikely to apply to succinylcholine blockade, for the rate of development of blockade was insensitive to the frequency of stimulation over a four-fold range of frequency. This effect was tested using the first block at 14 pulses/min as control for the second, which was recorded at 60 pulses/min. In a second series of experiments the first block of a companion hemidiaphragm was used as control for the high frequency block. In neither situation was there any difference between the control and the high frequency block. It was regularly noted, however, that subsequent blocks after a block at high frequency showed tachyphylaxis. This made a bridging experiment impossible, because a high frequency block between two controls invariably lengthened $t\frac{1}{2}$ of the third block.

The range of frequencies tested in these experiments may not have been extensive enough to say that no reduction in the acetylation of choline occurred. It is clear however that it cannot be an important aspect of succinylcholine block.

Hypoxia

The importance of the metabolic status of the diaphragm muscle in determining the rate of succinylcholine blockade is clear both from the preceding results, and those of Gibberd (1966). Because of this it was of interest to evaluate the effect of periods of hypoxia. Hypoxia was induced by switching the stream of oxygen and carbon dioxide to a mixture of 95% nitrogen and 5% carbon dioxide.

The experiments were arranged so that the hypoxic period was placed between the first and second succinylcholine block; the companion hemidiaphragm was blocked serially without hypoxia, to check for any spontaneous tachyphylaxis. It was found that

hypoxia led to a lengthening of $t\frac{1}{2}$ by an average of 70% of the control value; some preparations showed a decrease in latency, but this was not a constant finding.

Periods of hypoxia (<14 min) which led to a diminution in twitch tension of 30% or less did not affect subsequent succinylcholine block.

Alteration of the external ionic environment

In assessing the relative importance of pre- and postsynaptic actions of succinylcholine, use has been made of the known sensitivity of the nerve terminals to alterations in the external ionic environment. Ionic changes which alter miniature end-plate potential frequency and/or the quantum content of the end-plate potential have been found to modify the rate of onset of succinylcholine blockade. It will be appreciated that because of the use of twitch tension to monitor the onset of block, it was only possible to change the ionic environment within the limits of what would be tolerated by the hemidiaphragm preparation. Minor ionic changes have had marked effects on the rate of onset of blockade, and have thrown light on the mechanism involved. Because exposure to the test solutions was restricted to periods of 10–15 min before addition of succinylcholine it is likely that the effects noted reflect membrane changes rather than changes in internal ionic levels.

Reduction in the external Na^+ level

Experiments were carried out in which Na^+ was replaced either by sucrose or by Li^+. The Na^+ level was reduced to 81 mM or 109 mM by both procedures. Experiments were carried out in the usual way, with either the first succinylcholine block acting as control for the second, low-Na^+ block, or with the companion hemidiaphragm acting as the control. The test solution was allowed to equilibrate with the preparation for 10–15 min before addition of succinylcholine. In no instance did the substitution of sucrose for Na^+ cause any alteration in latency or $t\frac{1}{2}$ of the treated diaphragm, nor was the twitch tension altered by this procedure.

One may assume either that this modest degree of Na^+ deprivation has had no effect on the transmission process, or that two opposing effects have balanced out. A decrease in the amplitude of the nerve spike (Hodgkin & Katz, 1949) and a reduction in the amplitude of the end-plate potential (del Castillo & Katz, 1955) would be expected. These effects, which decrease the efficiency of the transmission process, may be offset by an increase in miniature end-plate potential frequency, which Gage & Quastel (1966) have used as an index of transmitter release. Substitution of Na^+ with Li^+ might reasonably be expected to overcome the first two effects (Gallego & Lorente de Nó, 1951), while leaving the increase in the m.e.p.p. frequency untouched (Gage & Quastel, 1966).

Substitution of 29 mM of the external Na^+ with Li^+ was without effect on the rate of onset of succinylcholine blockade. However, substitution of 59 mM Na^+ with Li^+ produced an effect which depended on the time of exposure to Li^+. Succinylcholine block after exposure for 9 min followed a time course identical to the control; after exposure to Li^+ for 20 min there was a reduction in twitch tension to 60% of the control level, and succinylcholine block proceeded almost three times as fast as usual. Washing the preparation in the normal bathing solution for 30 min restored both the

twitch tension and the normal rate of succinylcholine block. Prolonged exposure to this concentration of Li^+ appears to have a depressant effect on the preparation, in common with the findings of Onodera & Yamakawa (1966) using the sartorius preparation of the frog.

Alteration of the external Ca^{++} level

The rate of onset of succinylcholine blockade proved to be extremely sensitive to variation in the Ca^{++} level of the bathing solution. Doubling the external Ca^{++} markedly increased the time to half block ; reducing the Ca^{++} level four-fold significantly decreased this rate.

The effect of increasing the external Ca^{++} level is shown in Fig. 6. It may be noted that the second block, which was carried out after the preparation had been equilibrated for 15 min in 3.0 mM Ca^{++}, showed an increase in latency and a marked increase in $t\frac{1}{2}$. The third block, which was carried out after washing for 30 min in normal solution, showed a decline in latency and some decrease in $t\frac{1}{2}$. On increasing the Ca^{++} level

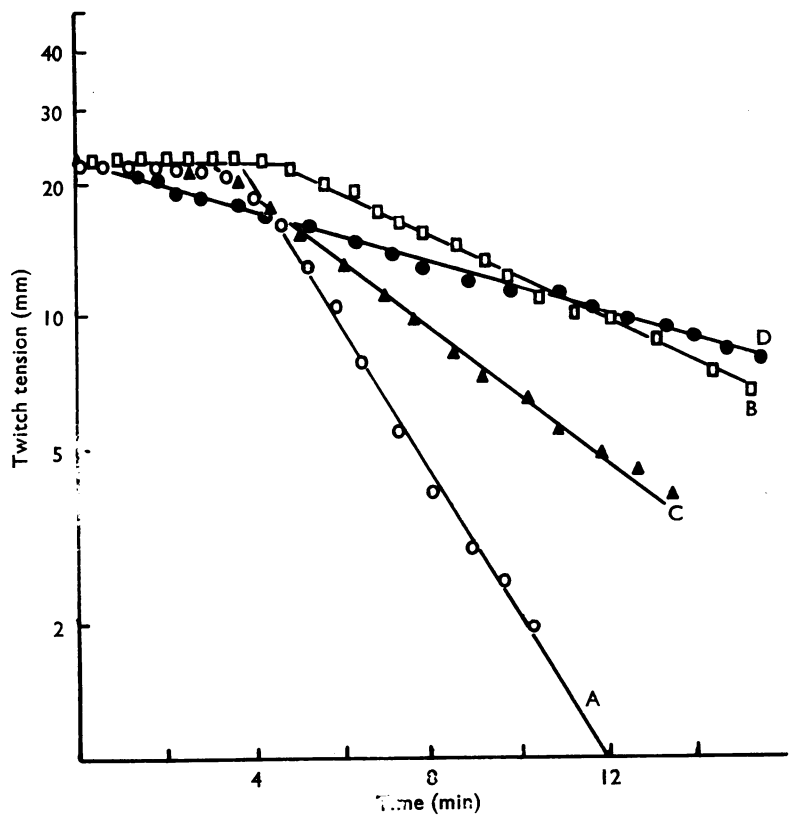

Fig. 6. Effect of 3.0 mM Ca^{++} on succinylcholine blockade. Semilogarithmic plots. A (○——○). First control curve, succinylcholine 8.7×10^{-6} M, latency 3.8 min, $t\frac{1}{2}$ 1.8 min ; B (□——□), after 15 min in 3.0 mM Ca^{++}, latency 4.9 min, $t\frac{1}{2}$ 6.0 min; C (▲——▲), second control, latency 2.5 min, $t\frac{1}{2}$ 4.1 min; D (●——●), after 15 min in 3.0 mM Ca^{++}, latency 0.8 min, $t\frac{1}{2}$ 10 min.

again, the fourth block occurred virtually without latency, and with a further increase in $t\frac{1}{2}$. The comparative irreversibility of the Ca^{++} effect was noted consistently, and meant that it was only possible to evaluate this effect by a comparison of first and second blocks.

Ten such experiments showed that there was no change in latency between the first and second (high Ca^{++}) block; the mean values were 4.2 and 4.1 min. However there was a significant ($P<0.05$) increase in $t\frac{1}{2}$ from 2.3 to 6.7 min. It was further noted that there was a highly significant linear correlation between the $t\frac{1}{2}$ of the control block, and $t\frac{1}{2}$ of the high Ca^{++} block. The slope of the regression, b, equalled 5.09, and the correlation was significant at $P<0.005$. In other words, slow control blocks were particularly susceptible to the action of high Ca^{++} solutions. Data derived from third control blocks, and fourth high Ca^{++} blocks showed similar relationships.

It appeared likely that exposure to high Ca^{++} solutions had permanently altered the state of the junction. This was confirmed in experiments in which preparations were bathed in high Ca^{++} solutions for 17–20 min, then washed in normal solution for 10 min before the addition of succinylcholine. This exposure to high Ca^{++} (3.0 mM) caused a mean lengthening of $t\frac{1}{2}$ between first and second blocks from 3.1 to 9.4 min. There was no change in latency.

The antagonism between succinylcholine and Ca^{++} was further demonstrated when the external Ca^{++} level was doubled in a preparation which was 95% blocked with succinylcholine. The force of contraction was doubled within 30 sec of adding Ca^{++} to the bath. Further elevation of external Ca^{++} brought about further alleviation of the block. The results obtained with this type of experiment varied somewhat; it was always possible to bring about some degree of alleviation of the block by increasing the external Ca^{++} level; however, it was not possible to alleviate it completely.

Reduction in the external Ca^{++} level to 0.38 mM ($0.25 \times$ normal) brought about a significant decrease in both latency and $t\frac{1}{2}$. The latency fell from 4.8 ± 0.3 min (S.E. of ten observations) to 2.8 ± 0.2 min (S.E. of fourteen observations); the difference was significant at $P<0.001$. The time to half block fell from a control value of 3.3 ± 0.3 min to 1.4 ± 0.1 min; the difference was significant at $P<0.001$.

The sensitivity of the rate of succinylcholine blockade to small changes in the level of external Ca^{++} contrasts with the apparent insensitivity of this phenomenon to low Na^{+} solutions. One must presume that as the safety margin in the transmission process is reduced by succinylcholine, Ca^{++} dependent mechanisms become rate-determining. The antagonistic effect of raised Ca^{++} solutions indicates a presynaptic action for succinylcholine, because del Castillo & Stark (1952) and Takeuchi (1963) have noted that raised Ca^{++} does not alter the sensitivity of the end-plate region. The alteration of the amplitude of the end-plate potential in response to variation in external Ca^{++} (del Castillo & Stark, 1952) was brought about by Ca^{++} changes similar in magnitude to those used in the present study, and suggests that succinylcholine blockade is alleviated by high Ca^{++} solutions because of an increase in acetylcholine release.

Na^{+}-Ca^{++} interactions

Kelly (1965) and Gage & Quastel (1966) have noted an antagonism between the effects of Na^{+} and Ca^{++} on transmitter release. It is noteworthy therefore that the effects of

high or low Ca^{++} on the rate of development of succinylcholine blockade could not be altered by decreasing the Na^{+} concentration.

The increase in $t\frac{1}{2}$ brought about by 3.0 mM Ca^{++} was compared with $t\frac{1}{2}$ in a companion diaphragm immersed in 3.0 mM Ca^{++} and 81 mM Na^{+}; the osmotic pressure was maintained with sucrose. Although there was some variability in the response of paired diaphragms it was not possible to detect any change in $t\frac{1}{2}$ as a result of the reduced Na^{+} level. In other experiments the rate of onset of succinylcholine blockade was compared in the presence of 0.38 mM Ca^{++}, and 0.38 mM Ca^{++} and 70 mM Na^{++}. Again it was found that reduction of external Na^{+} did not alter the Ca^{++} effect. It was noted however, that succinylcholine blockade in the presence of low Ca^{++}, low Na^{+} solutions promoted the development of tachyphylaxis to subsequent succinylcholine blocks, while this did not appear after succinylcholine block in the presence of low Ca^{++} alone.

Alteration of the external K^{+} level

The rate of onset of succinylcholine blockade was accelerated by increasing the K^{+} level of the external medium. After one succinylcholine block in normal solution the diaphragms were washed, and then transferred to a solution containing twice the usual K^{+} level (9.2 mM). After 15 min succinylcholine was added to the organ bath. It was found that there was a significant decline in latency and $t\frac{1}{2}$ in high K^{+} solutions. Latency fell from a control value of 3.7 ± 0.2 min (S.E. of twelve observations) to 3.0 ± 0.2 min (S.E. of fourteen observations); the difference was significant at $P<0.02$. The time to half block fell from 2.6 ± 0.2 min to 1.7 ± 0.3 min; the difference was significant at $P<0.05$. Tachyphylaxis did not develop in the presence of high K^{+}. Thus these results closely resemble the effects of low Ca^{++} solutions.

It was noteworthy that high K^{+} solutions would reduce or reverse tachyphylaxis caused by high Ca^{++} solutions or repeated succinylcholine block.

Reducing the K^{+} level of the medium to 1.2 mM for 10–15 min before succinylcholine block increased $t\frac{1}{2}$ by a factor of 2 or 3, but had little effect on the length of latency. The results resembled closely those obtained with high Ca^{++} solutions. Although the low K^{+} series was not as extensive as the 3.0 mM Ca^{++} series it was possible to relate the increase in $t\frac{1}{2}$ to the initial $t\frac{1}{2}$, as was possible in the former series. It was also noted that the increase in $t\frac{1}{2}$ in low K^{+} solutions was irreversible (compare with Fig. 6).

K^{+}-Ca^{++} interactions

Because increases in the external levels of K^{+} and Ca^{++} have opposing effects, it was of interest to determine whether the increase in $t\frac{1}{2}$ brought about by 3.0 mM Ca^{++} was antagonized by 9.2 mM K^{+}. These experiments were carried out in the same way as the low Na^{+}, high Ca^{++} experiments; that is, companion preparations were first blocked in normal solution, then transferred either to high K^{+}, high Ca^{++} or to high Ca^{++} solutions. It was not possible to detect any difference in latency or $t\frac{1}{2}$ between the two groups. Thus when they are exhibited together K^{+} does not modify the effect of raised Ca^{++}.

Alteration of the external Mg^{++} level

Doubling the external Mg^{++} level (2.0 mM Mg^{++}) for 10–15 min before succinylcholine block reduced the latency and $t\frac{1}{2}$ in a fashion similar to that found with low Ca^{++} or high K^{+} solutions. Latency was reduced from 4.7 ± 0.4 min (S.E. of nine observations) to 3.8 ± 0.3 min (S.E. of nine observations); the difference was significant at $P<0.05$. The time to half block was reduced from 2.4 ± 0.2 min to 1.4 ± 0.1 min; the difference was significant at $P<0.004$.

Tachyphylaxis did not occur in the presence of high Mg^{++}, nor was there any alteration in the twitch tension of preparations. As was found with high K^{+} solutions, tachyphylaxis was partially reversed by raising the external Mg^{++} level. This was well demonstrated in experiments in which the Mg^{++} level was raised during succinylcholine block in a preparation in which tachyphylaxis had already developed. Increasing the Mg^{++} level three- or four-fold caused an immediate increase in the rate of blockade, as may be seen in Fig. 7. It was characteristically found, however, that blockade did not proceed to completion, but levelled off at between 15 and 20% of the initial value. This

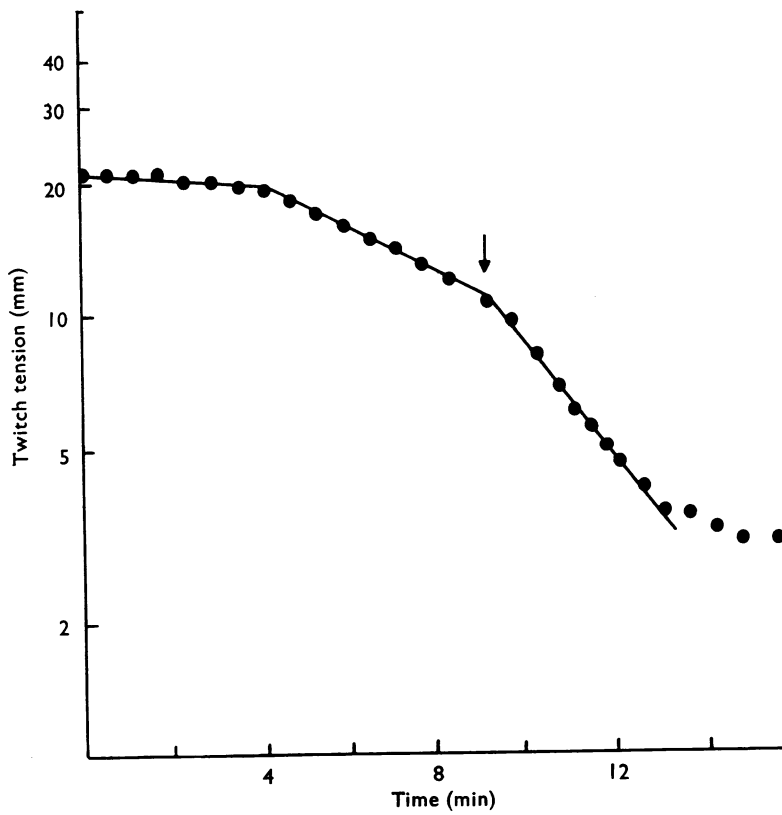

Fig. 7. Effect of raising the Mg^{++} level during succinylcholine blockade in a preparation with tachyphylaxis caused by repeated succinylcholine block. Mg^{++} level raised to 3.0 mM at arrow. Latency 4.2 min, $t\frac{1}{2}$ initial part of block 5.8 min; $t\frac{1}{2}$ after adding Mg^{++} 2.5 min.

can be compared with curve B in Fig. 2, which illustrates another preparation in which tachyphylaxis had been induced.

Exposure of preparations to low Mg^{++} solutions (0.25 mM) for periods of 20 min was without effect on either latency or $t\frac{1}{2}$. Longer periods of exposure did not alter latency, but caused an increase in $t\frac{1}{2}$ which resembled that induced by high Ca^{++} solutions in all respects.

DISCUSSION

The present study has outlined factors which affect the kinetics of onset of succinylcholine blockade of the hemidiaphragm preparation and which are involved in the development of tachyphylaxis. Experiments carried out at 35°–36° C showed a great variability of response to succinylcholine ; it seems that *in vitro* the preparation undergoes a progressive change which reduces its sensitivity to the drug. Consequently the early part of this investigation was concerned to develop a standardized preparation, and concurrently to establish what factors are involved in the development of tachyphylaxis. Briefly, tachyphylaxis is promoted by incubation at body temperature, high levels of succinylcholine, prolonged exposure to succinylcholine, high rates of stimulation, repeated periods of tetanic stimulation, hypoxia, high levels of external Ca^{++}, low levels of K^{+} or Mg^{++}, and low Ca^{++}-low Na^{+} solutions.

It is not affected by the time of incubation of the preparation at 29°–30° C before succinylcholine block and it is diminished or abolished by low temperature, low Ca^{++}, high K^{+} or high Mg^{++} solutions. The occurrence of tachyphylaxis in the intact animal (Zaimis, 1953) suggests that it is a property of the drug, rather than a by-product of the artificial environment.

The marked sensitivity of the preparation to external Ca^{++} changes suggests that interactions between this ion and succinylcholine are important both in determining the rate of succinylcholine blockade and the onset of tachyphylaxis. It is possible to correlate translocation of membrane Ca^{++} with most of the factors that promote tachyphylaxis. Thus depletion of membrane Ca^{++} has been suggested as a factor in the hypoxic failure of neuromuscular transmission (Hubbard & Løyning, 1966). Post-tetanic potentiation of the end-plate potential is likely to be associated with a residual change in ionized calcium concentration at an important membrane site (Gage & Hubbard, 1966 ; Katz & Miledi, 1965). K^{+} may potentiate succinylcholine block by depletion of membrane Ca^{++} (Shanes & Bianchi, 1959 ; Koketsu & Miyamoto, 1961), rather than by virtue of the slight depolarization caused by this level of K^{+} (9.2 mM), or the moderate increase in m.e.p.p. frequency (Gage & Quastel, 1966). Mg^{++} is also likely to potentiate succinylcholine block by a presynaptic inhibition of acetylcholine release, because postsynaptically its actions potentiate those of Ca^{++} (Takeuchi, 1963).

One may postulate a competition between succinylcholine and Ca^{++} in the presynaptic membrane, leading to a reduction in acetylcholine release. On removal of succinylcholine from the organ bath Ca^{++} restitution may be delayed, with a consequent delayed recovery of tetanic tension and post-tetanic potentiation. This factor could account for the prolonged suppression of post-tetanic repetition in the nerve terminals that was observed by Standaert & Adams (1965), and which Riker (1966) also noted after acetyl-

choline blockade. As these authors noted, the suppression was too prolonged to be associated with the continued presence of the drug.

The final state of membrane Ca^{++} after succinylcholine block may differ from the initial state, in that a more stable calcium complex may be formed. Tachyphylaxis would ensue if succinylcholine were less able to displace Ca^{++} from this complex than initially.

This discussion suggests that the development of tachyphylaxis is a presynaptic phenomenon. It is likely, however, that postsynaptic changes are also involved, for Gibberd (1966) was able to correlate tachyphylaxis to decamethonium in the diaphragm with a reduction in muscle K^+. Stevenson (1960) also demonstrated loss of muscle K^+ in dogs subjected to succinylcholine relaxation.

The form of the curve of twitch tension against time during succinylcholine blockade is worthy of comment. The latent period before the exponential decline in twitch tension recalls the period during which succinylcholine causes twitch potentiation in frog muscle (Nastuk & Karis, 1964). It seems to coincide with the period of depolarization of the end-plate, before repolarization and block (Thesleff, 1955a, b). The difference in response of the two preparations may reside in the greater depolarization caused by succinylcholine in the frog sartorius than in the rat diaphragm (Thesleff, 1955a, b), because hexafluorenium, which reduces this depolarization, converts the frog muscle response to one which resembles that of the rat diaphragm (Nastuk & Karis, 1964).

The functional reserve of the transmission process will also lead to latency, because a substantial number of receptors (either pre or postsynaptic) must be occupied before the transmission process is affected (Paton & Waud, 1967).

Diffusion delays could also become rate-determining in conditions of very rapid succinylcholine block, because Creese (1954) observed a $t\frac{1}{2}$ of 1 min for the extracellular exchange of Na^+ in the diaphragm. The preparation used in the present study were of a thickness comparable with those of Creese (mean thickness 0.64 mm compared with 0.60 mm in Creese's series).

The hypothesis is proposed that the latent period is the result of the combined effects of depolarization and reduction in transmitter release. The safety margin for transmission is increased by depolarization, because the further depolarization necessary to trigger an action potential is smaller. Thus a reduction in transmitter release will not affect the transmission process until either the postsynaptic membrane becomes unresponsive or the reduction in transmitter release becomes overwhelming.

If desensitization of the postsynaptic membrane were the principal factor in maintaining blockade, then increases in the external Ca^{++} level would be expected to intensify this effect, rather than relieve it (Nastuk, 1966). The importance of presynaptic factors is confirmed by the findings of Karczmar, Kim & Koketsu (1961) that tetraethylammonium, which increases the release of acetylcholine, will alleviate an established decamethonium block. Further, antagonism of succinylcholine blockade has been found to be a property of a number of drugs which promote acetylcholine release (Freeman, unpublished).

It may be relevant that the concentration of succinylcholine used by Thesleff (1956) when studying postsynaptic depolarization was considerably greater than was used by Edwards & Ikeda (1962), and in the present study. It may be that presynaptic effects predominate at levels of succinylcholine which are just sufficient to cause block.

It is likely that a unifying concept of succinylcholine action may be possible. Excitation followed by stabilization of the membrane may occur at both pre- and postsynaptic sites. Species differences and differences between muscle types may reflect differences in the intensity of these sequential actions. Facilitatory actions of succinylcholine will predominate when the excitatory phase is well developed; block without facilitation will occur, as in the rat diaphragm, when stabilization predominates.

The effects observed in the present study may reflect the differential sensitivity of the presynaptic membrane to ionic changes rather than a fundamentally dissimilar mechanism on either side of the junction.

SUMMARY

1. The kinetics of succinylcholine blockade of the rat phrenic nerve-diaphragm preparation have been studied. A standardized procedure has been developed which permits of a quantitative and reproducible estimate of the rate of onset of blockade.

2. Blockade was found to occur in two stages. Using a succinylcholine level of 8.7×10^{-6} M there was an initial latent period of some 4 min duration, which was followed by an exponential decline in twitch tension to zero, with a half-time of approximately 2 min.

3. In common with the results of other workers, it was found that the preparation developed tachyphylaxis to repeated succinylcholine block. Factors promoting tachyphylaxis included incubation at 37° C, high levels of succinylcholine, prolonged exposure to succinylcholine, high rates of stimulation, repeated periods of tetanic stimulation, hypoxia, raised levels of external Ca^{++}, low levels of K^+ or Mg^{++}, low Ca^{++} – low Na^+ solutions. Tachyphylaxis was diminished or abolished by incubation at 30° C, low Ca^{++}, high K^+ or high Mg^{++} solutions.

4. The rate of onset of blockade was increased by low temperature, low Ca^{++}, high K^+ or high Mg^{++} solutions. It was markedly decreased by doubling the external Ca^{++} level. It seemed to be insensitive to reduction in the external Na^+ level.

5. It is suggested that at the concentration of succinylcholine used in these experiments (8.7×10^{-6} M), which was just sufficient to block the preparation completely at 30° C, succinylcholine has an action which is largely but not entirely presynaptic. There seems to be competition between succinylcholine and Ca^{++} in the presynaptic membrane, which results in a decreased release of acetylcholine.

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REFERENCES

BEANI, L., BIANCHI, C. & LEDDA, F. (1962). The effect of tubocurarine on acetylcholine release from motor nerve terminals. *J. Physiol., Lond.*, **174**, 172–183.

BIGLAND, B., GOETZEE, B., MACLAGAN, J. & ZAIMIS, E. (1958). The effect of lowered muscle temperature on the action of neuromuscular blocking drugs. *J. Physiol., Lond.*, **141**, 425–434.

BOWMAN, W. C. & HEMSWORTH, B. A. (1965). Effects of some polymethylene bis-(hydroxyethyl)dimethylammonium salts on neuromuscular transmission. *Br. J. Pharmac. Chemother.*, **25**, 392–404.

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br. J. Pharmac. Chemother.*, **1**, 38–61.

BURNS, B. D. & PATON, W. D. M. (1951). Depolarization of the motor end-plate by decamethonium and acetylcholine. *J. Physiol., Lond.*, **115**, 41–73.

CREESE, R. (1954). Measurement of cation fluxes in rat diaphragm. *Proc. R. Soc.* B., **142**, 497–513.

CREESE, R., DILLON, J. B., MARSHALL, J., SABAWALA, B., SCHNEIDER, D. J., TAYLOR, D. B. & ZINN, D. E. (1957). Effect of neuromuscular blocking agents on isolated human intercostal muscles. *J. Pharmac., exp. Ther.*, **119**, 485–494.

DEL CASTILLO, J. & STARK, L. (1952). The effect of calcium ions on the motor end-plate potentials. *J. Physiol., Lond.*, **116**, 507–515.

DEL CASTILLO, J. & KATZ, B. (1955). Local activity at a depolarized nerve-muscle junction. *J. Physiol., Lond.*, **128**, 396–412.

EDWARDS, C. & IKEDA, K. (1962). Effect of 2-PAM and succinylcholine on neuromuscular transmission in the frog. *J. Pharmac. exp. Ther.*, **138**, 322–327.

GAGE, P. W. & HUBBARD, J. I. (1966). An investigation of the post-tetanic potentiation of end-plate potentials at a mammalian neuromuscular junction. *J. Physiol., Lond.*, **184**, 353–375.

GAGE, P. W. & QUASTEL, D. M. J. (1966). Competition between sodium and calcium ions in transmitter release at mammalian neuromuscular junctions. *J. Physiol., Lond.*, **185**, 95–123.

GALLEGO, A. & LORENTE DE NÓ, R. (1951). On the effect of ammonium and lithium ions upon the frog nerve deprived of sodium. *J. gen. Physiol.*, **35**, 227–244.

GIBBERD, F. B. (1966). Action of decamethonium on rat diaphragm. *Br. J. Pharmac. Chemother.*, **28**, 128–136.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol., Lond.*, **108**, 37–77.

HOFMANN, W. W., PARSONS, R. L. & FEIGEN, G. A. (1966). The effects of temperature and drugs on mammalian motor nerve terminals. *Am. J. Physiol.*, **211**, 135–140.

HOLMES, P. E. B., JENDEN, D. J. & TAYLOR, D. B. (1951). The analysis of the mode of action of curare on neuromuscular transmission ; the effect of temperature changes. *J. Pharmac. exp. Ther.*, **103**, 382–402.

HUBBARD, J. I. & LØYNING, Y. (1966). The effects of hypoxia on neuromuscular transmission in a mammalian preparation. *J. Physiol., Lond.*, **185**, 205–223.

HUBBARD, J. I., SCHMIDT, R. F. & YOKOTA, T. (1965). The effect of acetylcholine upon mammalian motor nerve terminals. *J. Physiol., Lond.*, **181**, 810–829.

JENDEN, D. J., KAMIJO, K. & TAYLOR, D. B. (1954). The action of decamethonium on the isolated rabbit lumcrical muscle. *J. Pharmac. exp. Ther.*, **111**, 229–240.

KARCZMAR, A. G., KIM, K. C. & KOKETSU, K. (1961). End-plate effects and antagonism to *d*-tubocurarine and decamethonium of tetraethylammonium and methoxyambenonium (WIN8078). *J. Pharmac. exp. Ther.*, **134**, 199–205.

KATZ, B. & MILEDI, R. (1965). The effect of calcium on acetylcholine release from motor nerve endings. *Proc. R. Soc.* B., **161**, 496–503.

KELLY, J. S. (1965). Antagonism between Na^+ and Ca^{++} at the neuromuscular junction. *Nature, Lond.*, **205**, 296–297.

KOKETSU, K. & MIYAMOTO, S. (1961). Significance of membrane calcium in Ca-free and K-rich media. *Nature, Lond.*, **189**, 403–404.

NASTUK, W. L. & KARIS, J. H. (1964). The blocking action of hexafluorenium on neuromuscular transmission and its interaction with succinylcholine. *J. Pharmac. exp. Ther.*, **144**, 236–252.

NASTUK, W. L. (1966). Fundamental aspects of neuromuscular transmission. *Ann. N.Y. Acad. Sci.*, **135**, 111–135.

ONODERA, K. & YAMAKAWA, K. (1966). The effects of lithium on the neuromuscular junction of the frog. *Jap. J. Physiol.*, **16**, 541–550.

PATON, W. D. M. & WAUD, D. R. (1967). The margin of safety of neuromuscular transmission. *J. Physiol., Lond.*, **191**, 59–90.

RIKER, W. F. (1966). Actions of acetylcholine on mammalian motor nerve terminal. *J. Pharmac. exp. Ther.*, **152**, 397–416.

SHANES, A. M. & BIANCHI, C. P. (1959). Radiocalcium release by stimulated and potassium-treated sartorius muscles of the frog. *J. gen. Physiol.*, **43**, 481–493.

STANDAERT, F. G. & ADAMS, J. E. (1965). The actions of succinylcholine on the mammalian motor nerve terminal. *J. Pharmac. exp. Ther.*, **149**, 113–123.

STEVENSON, D. E. (1960). Changes in the blood electrolytes of anaesthetized dogs caused by suxamethonium. *Br. J. Anaesth.*, **32**, 364–371.

TAKEUCHI, N. (1958). The effect of temperature on the neuromuscular junction of the frog. *Jap. J. Physiol.*, **8**, 390–404.

TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. *J. Physiol., Lond.*, **167**, 141–155.

TAYLOR, D. B. & NEDERGAARD, O. A. (1965). Relation between structure and action of quaternary ammonium neuromuscular blocking agents. *Physiol. Rev.*, **45**, 523–554.

THESLEFF, S. (1955a). The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. *Acta physiol. scand.*, **34**, 218–231.

THESLEFF, S. (1955b). The effects of acetylcholine, decamethonium and succinylcholine on neuromuscular transmission in the rat. *Acta physiol. scand.*, **34**, 386–392.

VAN BREEMAN, C., DANIEL, E. E. & VAN BREEMAN, D. (1966). Calcium distribution and exchange in the rat uterus. *J. gen. Physiol.*, **49**, 1265–1298.

ZAIMIS, E. (1953). Motor end-plate differences as a determining factor in the mode of action of neuromuscular blocking substances. *J. Physiol., Lond.*, **122**, 238–251.