J. Physiol. (1967), **191**, *pp.* 59–90
With 12 *text-figures*
Printed in Great Britain

THE MARGIN OF SAFETY OF NEUROMUSCULAR TRANSMISSION

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(*Received* 21 *October* 1966)

SUMMARY

1. The margin of safety for neuromuscular transmission in the tibialis and sartorius muscles of the cat has been determined by measuring the ratio by which end-plate depolarization produced by succinylcholine, decamethonium, octamethonium or iodocholine is antagonized, in the presence of neuromuscular block produced by tubocurarine, gallamine or DF-596. The estimate of the margin of safety was independent of the particular drugs chosen for the measurement.

2. To produce threshold block to indirect stimulation once every 10 sec, a fractional occupancy by the antagonist of $0{\cdot}76 \pm 0{\cdot}05$ (S.D.) was required; for nearly complete block, an occupancy of $0{\cdot}917 \pm 0{\cdot}16$ (S.D.) was required. These figures correspond to factors of safety of 4·1 and 12 for the most sensitive and the most resistant groups of fibres respectively.

3. The interaction between the agonists and the antagonists, when tested over a wide range of dosage, did not conform with the conditions of full competitive equilibrium. It was concluded that this arose, not because of some interfering non-competitive process, but because, during the relatively brief exposure to agonist, the equilibrium between the antagonist and the receptors is not significantly disturbed. An analysis of this condition of quasi-equilibrium is given. A correction downwards of the direct estimates of the margin of safety is required, but this proves to be small, about 8%, and may not be significant.

4. The safety factor diminished when the motor nerve had been cut more than 5 hr; it is suggested that this represents an early sign of nerve degeneration.

5. With dog sartorius muscle, results similar to those in the cat were obtained. But for deep block in the rabbit, the safety factor was only about 4.

6. The existence of a substantial margin of safety influences considerably the interpretation of the time course of action of blocking drugs, and

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of comparisons between responses to nervous excitation and drug injection.

INTRODUCTION

It is known that neuromuscular blocking agents show differences in the time course of their actions as well as in potency. Our original purpose was to investigate these differences so as to throw light on the kinetics of the interaction of these drugs with the receptors of the motor end-plate. But it soon became clear that a preliminary investigation was required of the general relationship between the action of blocking agents at the end-plate and their effect on neuromuscular transmission, between the effects of a drug at receptor level and the ultimate effect on (say) the height of the indirectly elicited twitch. In particular, a knowledge is required of one particular physiological parameter, the safety factor for transmission, that is, the extent of interference with the synaptic mechanism that can exist without failure of transmission. An approximate index of this margin of safety has been derived by using the conventional methods for the study of the interaction of antagonistic drugs with their specific receptors. To obtain a more exact estimate of margin of safety, a generalization of the normal model of competitive antagonism became necessary in order to allow for a competitive element in the action of a stimulant itself. Some of our results were briefly reported at the Ciba Symposium on Curare and Curare-Like Agents (Paton & Waud, 1962).

METHODS

The methods used were derived from those employed by Burns & Paton (1951) and Douglas & Paton (1954). Experiments were done on tibialis anterior and sartorius muscles of cats anaesthetized with chloralose (about 80 mg/kg intravenously, after induction with ethyl chloride and ether). The leg was held horizontally on a Brown–Schuster myograph stand by a pin through the lower end of the tibia. The surface of the muscle chosen was exposed, the skin edges cauterized and all other bleeding points tied. A paraffin pool was made by pinning the skin edges to a suitably shaped ring holder. Records were made of the potential between an indifferent electrode placed on an electrically inert area (tendon or skin near tendon) and an electrode on the surface of the muscle. When the latter electrode is over the end-plate region of a muscle exposed to suxamethonium, it is negative with respect to the indifferent electrode. The muscle was scanned after a preliminary test dose of suxamethonium and the electrode was fixed at the point of maximum negativity. If the electrode is placed closely enough over the end-plate the depolarization rises to its peak in about 1 min (cf. Fig. 1); a slower rise is a useful sign that the electrode is off the centre of the end-plate region (cf. Burns & Paton, 1951, figs. 7–9). The electrodes used were cotton wicks leading through 2 % agar saline to chlorided silver wires. Since light can produce a potential change in the electrode, illumination was kept low and reasonably constant. The potential was amplified by a Pye pH meter modified by diminishing the degree of negative feed-back so as to produce a full scale deflexion with an input of 20 mV. The output from the meter was either fed into an Advance recording galvanometer or into an Elliott servo-recorder. The latter provided rectangular co-ordinates. (The nature of the experiments is such that a rapid speed of response is unnecessary.)

The tibialis anterior was excited indirectly by supramaximal 0·1 msec shocks applied to the sciatic nerve through platinum electrodes. The nerve was tied at the sciatic notch and was then exposed in the thigh by an antero-lateral approach just posterior to the lateral border of quadriceps so that a paraffin pool could be formed to bathe the nerve throughout the experiment. To record the contraction of the muscle on a smoked drum the tendon was connected by jeweller's chain to a flat spring myograph; the writing lever was made of balsa wood to reduce 'fling'. Drugs were given intravenously through the external jugular vein or intra-arterially by retrograde injection through a Gordh needle in the opposite common iliac artery. For intra-arterial injection the drug was dissolved in a volume of 0·2 ml. and injected in about $\frac{1}{2}$ sec, even though the effect produced was not very sensitive to volume or rate of injection.

As the investigation progressed, this general procedure was considerably modified in the following ways:

(*a*) A spinal preparation (Burn & Dale, 1915) was used to avoid a fluctuating depth of anaesthesia and the hyperexcitability associated with chloralose, to lessen the number of drugs to which the neuromuscular junction was exposed, and to minimize vasoconstriction in the hind limb.

(*b*) To control blood flow the common iliac artery was perfused with carotid blood by a spring-loaded roller pump (constructed by Mr O. B. Saxby) through a polythene cannula inserted into the opposite common iliac so that the blood was directed into the experimental leg when the aorta was clamped. The animal was thoroughly heparinized by giving 20 mg heparin 5 min before cannulation and then 5 mg every 30 min. Only a siliconed glass T-piece, polythene and Tygon tubing were in contact with the blood. During the perfusion both carotid and perfusion arterial pressures were recorded; experiments were discarded if a sudden large rise in perfusion pressure suggested vascular occlusion. The perfusion pressure was usually adjusted to about 100 mm mercury; flows ranged from 5 to 20 ml./min per leg.

(*c*) To control the temperature of the muscle studied, the animal's body temperature was maintained close to 37° C by table heaters, a heating coil was wrapped round the tubing between the perfusion pump and the arterial perfusion cannula, and a thermostatically controlled heating element was placed in the paraffin pool covering the exposed muscle surface.

(*d*) It was found possible to remove the sheath of the tibialis muscle without producing an injury potential; by thus diminishing the extracellular short-circuiting of the end-plate potential changes, larger and more sharply localized potentials were recorded.

(*e*) Isotonic saline solutions were infused intravenously at a rate of about 0·5 ml./min to maintain a good urine flow.

(*f*) With the leg extended the arterial supply to tibialis might be stretched under the inguinal ligament, behind the knee and at the interosseous membrane. In addition, although the method of recording tension and potential change was satisfactory, occasionally in the course of an experiment the end-plate region of the tibialis muscle became unresponsive to injected drugs. In such cases it was found, by subsequent injection of ink, that parts of the muscle were not being perfused. Whether or no this was because of an accumulation of small thrombi, possibly originating on over-stretched arterial intima, the perfusion was obviously inadequate. A second disadvantage of this perfusion technique was that opening the abdomen accentuated subsequent bleeding in the heavily heparinized animal. These disadvantages can be avoided by using the sartorius muscle. One can perform a retrograde arterial perfusion by inserting the arterial perfusion cannula into the femoral artery just above Hunter's canal. There is room on the femoral artery just above the main artery to the sartorius for a proximal clamp. The nerve is easily available at the medial border of the muscle at the junction of the proximal fifth and distal four-fifths (the artery enters at this level also). Tension recording is not so convenient as with tibialis anterior, but by freeing the part of the muscle distal to, and in line with the fibres to be used for electrical recording, a suitable mechanical attachment could be made with a mass ligature.

(*g*) The circuit of the Pye pH meter was such that spurious short-circuiting of the animal to the earth had to be avoided. Accordingly, the anus was occluded, and a urethral catheter was passed. The latter was also used to monitor urine flow.

The following drugs were used: succinylcholine chloride, decamethonium iodide, octamethonium iodide, (+)-tubocurarine chloride, gallamine triethiodide, and DF-596 (*N,N'*-4,9-dioxo-3,10-dioxadecamethylenebis (3-phenylacetoxy-tropanium bromide); Haining, Johnston & Smith, 1960).

RESULTS

Since the experimental preparation we have used differs considerably from that originally used by Burns & Paton (1951) and Douglas & Paton (1954), as a result of the modifications mentioned above, an illustration of its behaviour and limitations is desirable. Figure 1 shows the depolarization produced by intra-arterial doses of 10 n-moles of succinylcholine. It illustrates the relation between the size of the response and (1) volume injected, (2) rate of injection, (3) rate of perfusion. Within reasonably wide limits none of these factors produces an effect appreciably greater than that of random variation. In all the experiments to follow, the depolarizing agents were given in a 0·2 ml. volume injected over a 1 sec period.

The safety factor for neuromuscular transmission might be measured in two ways. First, one might measure the output of transmitter, and then reduce this until transmission begins to fail, thus obtaining the safety factor as (say) the ratio between the 'dose' of transmitter available to the 'dose' necessary. Secondly, the safety factor could be assessed postsynaptically by determining the relationship between the receptor capacity available and that necessary for transmission. In the first method the transmitter output is reduced until only that fraction of the full receptor activity is evoked which is required for threshold transmission; in the second, the receptor pool is reduced until the activity achieved within it by a *normal* transmitter output falls to threshold. Both approaches assume that the receptors are uniform in their properties. The methods are equivalent, if the released transmitter occupies only a small fraction of the receptors, i.e. if there is a substantial 'spare receptor' capacity (Stephenson, 1956). The second method is at present the more convenient since one can apply the pharmacological methods already developed for the study of competitive antagonism. These allow us, if graded doses of suitable stimulants and competitive antagonists are applied, to determine the degree of receptor occlusion produced by the antagonist, at any given degree of neuromuscular block.

To study thus the effect of a blocking drug on end-plate receptors, one must administer some specific stimulant and record how far the antagonist interferes with its action. One way to do this at the neuromuscular junction might be to excite the motor nerve, and cause a release of acetyl-

choline at its ending. But the amount of acetylcholine released is unknown under the conditions *in vivo* we wished to use, and it could not be assumed to remain constant during an experiment lasting many hours. We have therefore used artificial application. But the natural transmitter, acetylcholine, is not generally suitable for injection into the circulation of a muscle because of its rapid destruction. It is necessary therefore to choose a more stable depolarizing agent such as succinylcholine, decamethonium or octamethonium. This involves the assumption that acetylcholine and these drugs act by identical mechanisms at the same receptors and are therefore antagonized to the same extent by a drug such as tubocurarine.

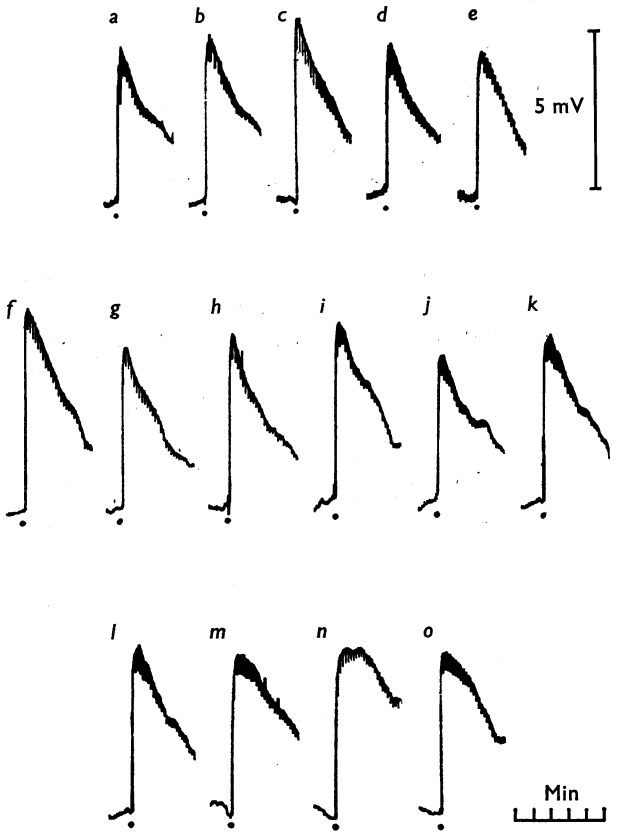

Fig. 1. Effect of volume and rate of injection and rate of perfusion on response to intra-arterial doses of succinylcholine. Cat, 4 kg, spinal preparation, perfused leg. Tracings are depolarization (ordinate) against time (abscissa). Dose 10 n-moles I.A. throughout. *a–e* drug dissolved in 0·2, 0·2, 0·1, 0·4 and 0·2 ml. respectively and injected in 1 sec. *f–k*: volume (0·2 ml.) injected in 1, 0·2, 0·5, 2, 4 and 1 sec. *l–o*: volume of 0·2 ml. injected in 1 sec at perfusion rates of 2·2, 1·0, 0·34 and 2·2 ml. min^{-1} leg^{-1}.

To determine the degree of receptor occlusion, the 'dose ratio' method (Gaddum, Hameed, Hathway & Stephens, 1955) was used. The principle consists of finding the ratio in which the dose of a stimulant must be increased in the presence of an antagonist in order to match a control response. In general, occupancy is equal to the dose ratio divided by the (dose ratio − 1), provided that parallel log–dose response curves are obtained; this requires either that the occupancy required by the stimulant is low, or that complete equilibrium between receptors, antagonist and stimulant is achieved (cf. Paton, 1961, p. 34), but does not depend on any particular theory of stimulant action. The technique is a null method

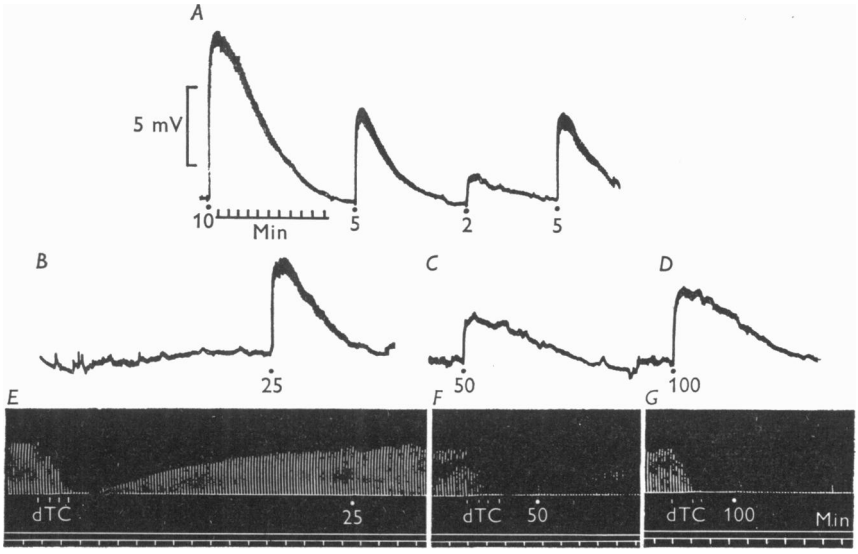

Fig. 2. Cat, 2·8 kg, chloralose. Tibialis muscle. (*A–D*) Records of end-plate depolarization, (*E–G*) twitch response to supramaximal shocks to sciatic nerve every 10 sec. Drugs given by retrograde injection into contralateral iliac artery. (*A*) Responses of normal muscle to 10, 5, 2 and 5 n-moles succinylcholine. (*B*) and (*E*) At dTC, tubocurarine given in divided dosage, 4 × 100 n-moles, to produce nearly complete block. After almost complete recovery, 25 n-moles succinylcholine. (*C*) and (*F*) Tubocurarine 400 + 3 × 100 n-moles, followed by succinylcholine 50 n-moles. (*D*) and (*G*) Tubocurarine 4 × 100 n-moles, followed by succinylcholine 100 n-moles.

so that reactions distal to the receptor do not influence the result. It is also particularly useful in the present series of experiments since only a small number of responses are required to yield a result. The response of the end-plate region to a depolarizing drug takes 20–60 min to pass off; thus the number of measurements which can be made in one animal is severely limited.

Figure 2 presents an experiment illustrating the general procedure used. First, graded and reproducible responses to control injections of succinylcholine were obtained. Then tubocurarine was given in a dose sufficient almost to abolish the twitch. In this particular experiment transmission was then allowed to recover. When, 26 min later, the neuromuscular paralysis had almost completely passed off, the end-plate was still resistant to succinylcholine to the extent that it was necessary to give as much as 25 nM to obtain a response matching that produced by 5 nM in the normal muscle. Thus we have a dose ratio of 5 associated with threshold neuromuscular block. Similarly, dose ratios of $50/2{\cdot}8 = 18$ and $100/4{\cdot}5 = 22$ were found for almost complete neuromuscular block in the same experiment. Interpreting these dose ratios in terms of receptor occupancy by tubocurarine means that to produce even threshold block 80 % occupancy had to be achieved. It is thus possible for there to be a very severe interference with the synaptic mechanism, and yet no sign of this in the indirectly elicited twitch. Complete block requires about 95 % occlusion of the receptors by the antagonist. This latter estimate was apparently not affected by the use of two different dosage schedules of curare used to achieve the block. Also this estimate did not seem to depend on the level of depolarization at which the dose ratio is measured. Use of the 50 nM dose of succinylcholine gave an estimate of 94 % occupancy while 100 nM gave 95 %.

It will be noticed in Fig. 2 that after about the first minute the time course of the end-plate response to succinylcholine given after tubocurarine differs from that of the control response; there is a second slower phase of depolarization, and the whole time course is prolonged. This is to be expected, since the concentration of tubocurarine in the leg and consequently the occupancy of the receptors by it are falling during the action of succinylcholine. Depolarizations were therefore measured at a fixed short interval (40–60 sec) after the start of the response.

The results of similar experiments under various conditions are collected in Table 1.

The dose ratio method is such that the estimate of occupancy required to produce a given degree of block ought not to depend on the choice of stimulant or antagonist. This was so in the present experiments; the same result was obtained whether tubocurarine, gallamine or DF-596 was used as the antagonist (Table 1) or whether succinylcholine, decamethonium or octamethonium was used as the stimulant. The three antagonists were chosen because they vary in their duration of action. The effect of gallamine is known to have a shorter duration than that of tubocurarine; DF-596 was the product of a search for a drug with a still more transient effect (Haining *et al.* 1960). These antagonists also vary in potency: the

intra-iliac doses required to produce deep block were of the order of 500, 2000 and 200 nM for tubocurarine, gallamine and DF-596 respectively. Finally, in two experiments (marked by ‡) the required degree of block was achieved by continuous infusion of gallamine or of DF-596. Thus there

TABLE 1. Fractional receptor occupancies obtained from $(DR-1/DR)$

Depolarizing agent	Competitive blocking agent	Preparation*	Fractional occupancy: Threshold block Early	Threshold block Late	Threshold block % block	Deep block Early	Deep block Late	Deep block % block	Time (hr) from nerve section
Succinylcholine	Tubocurarine	C ta	0·8	—	5	0·94	—	98	4·5
		CP ta	0·72	—	10	0·91†	—	67	4·5
	Gallamine	C ta	—	0·41	18	—	0·87	88	8
		C ta	0·78	—	5	0·90	—	93	3·5
			—	0·65	14	—	0·78†	75	6·5
		C ta	0·83	—	5	0·90	—	94	3·5
			—	0·56	0	—	0·88	98	8
		CP ta	0·85	—	5	0·91	—	96	3
	DF-596	C ta	0·80	—	12	—	—	—	5
		CP ta	0·67	—	10	0·91	—	97	3
			—	—	—	—	0·88	95	6
			—	—	—	—	0·82	90	9
		SP ta	0·73	—	8	0·93	—	95	3
	DF-596‡	SP ta	—	—	—	0·90	—	88	3
Decamethonium	Tubocurarine	SP ta	0·80	—	5	—	—	—	3
		SP ta	—	—	—	0·93†	—	77	3
		SP ta	0·71	—	2	0·92	—	97	2·5
	Gallamine‡	SP ta	0·8	—	4	0·93†	—	80	3
	DF-596	SP ta	0·71	—	0	0·90	—	88	2
Succinylcholine	Tubocurarine	SP s	0·75†	—	25	0·93	—	88	3
		SP s	0·70	—	11	0·94	—	92	5
		SP s	0·66	—	7	0·90	—	97	5·5
		SP s	0·74	—	2	0·94†	—	84	4
	DF-596	SP s	—	—	—	—	0·87	93	5·5
Octamethonium	Tubocurarine	Sp s	0·8	—	3	0·93	—	90	4·5
		CP s	0·75	—	1	—	—	—	5
$ICH_2CH_2NMe^+_3$		SP s	—	—	—	0·90	—	92	5
		Mean†	0·756	0·54	—	0·917	0·86	—	—
		Standard deviation†	0·051	0·12	—	0·016	0·022	—	—

* C = chloralose, CP = chloralose and muscle perfused, SP = spinal and muscle perfused, ta = tibialis anterior, s = sartorius.

† Values marked † were not included in calculation of mean and standard deviation because the degree of block was neither threshold nor deep.

‡ Antagonist given by continuous infusion.

was no evidence that the estimate of the dose ratio depends on either potency, duration of action, or mode of administration of the antagonist.

Similarly, it was found that the estimate of dose ratio did not depend on the stimulant used. The choice of stimulants, however, is more restricted and provides a less rigorous test of the approach. The potencies were about

the same for all three drugs (the doses required to produce depolarization of about 1–5 mV all lay in the range 2–20 nM when given into the iliac artery). The time courses were indistinguishable. In particular, succinylcholine did not differ from the stable methonium derivatives, despite its reported susceptibility to hydrolysis. Succinylcholine does not appear to be especially rapidly destroyed in the cat, but in any case, redistribution of drug is the principal determinant of the time course in the present experiments where the drug is injected into the arterial stream.

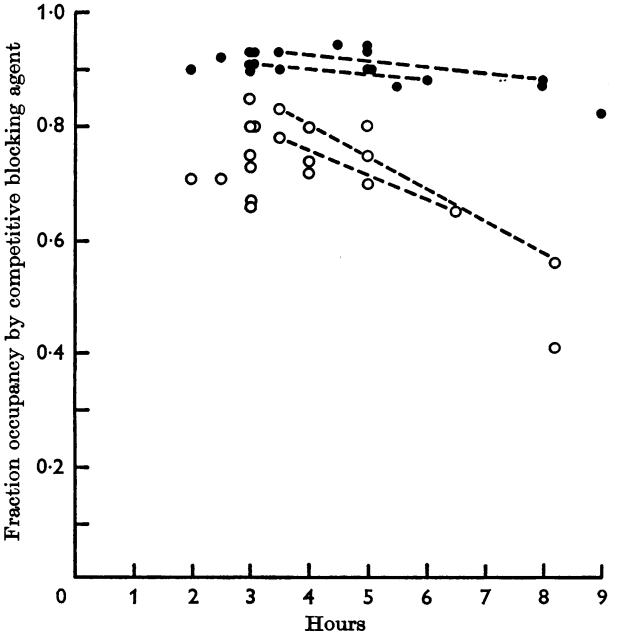

Fig. 3. Margin of safety as a function of time. Ordinate: fractional occupancy by competitive agent. Abscissa: time from nerve section (hr). Filled circles: results at 'deep' block. Open circles: 'threshold' block. Interrupted lines connect points from same animal.

Table 1 also shows that the same results were obtained whether chloralose anaesthesia or a spinal preparation was used, and that experiments with the natural circulation were not different from those with artificial perfusion. The two muscles chosen (tibialis and sartorius) gave similar results.

In Table 1 the results have been classified so that all those obtained 6 or more hr after nerve section are termed 'late'; the 'early' results were made between 2 and 5 hr after section. This arrangement is a consequence of finding that the margin of safety was lower if it was measured late in an experiment. For instance, in one experiment of Table 1 (lines 4 and 5)

the fractional receptor occupancy associated with deep block 3½ hr after nerve section was 0·9, and 3 hr later it had fallen to 0·78.

The time course of the change in the margin of safety in twenty-two experiments of this kind is illustrated in Fig. 3, by plotting data from Table 1 against time. The values marked by the sign † in Table 1 were omitted so as to obtain two groups roughly uniform with regard to level of block. The grouping of degrees of block from 0 to 18% as 'threshold' and from 88 to 98% as 'deep' increases the scatter of the points; but it can be seen that there is no evidence of a change for the first 5–6 hr, while after that time the margin of safety decreases at both levels of block.

The nature of the action of a stimulant in the presence of an antagonist

In the experiment of Fig. 2, the dose ratio with deep block by tubocurarine was determined with two different doses of succinylcholine, one twice the other, yet virtually the same estimates of occupancy (94 and 95%) were obtained. This is the result to be expected if a conventional competitive antagonism exists between the stimulant and tubocurarine.

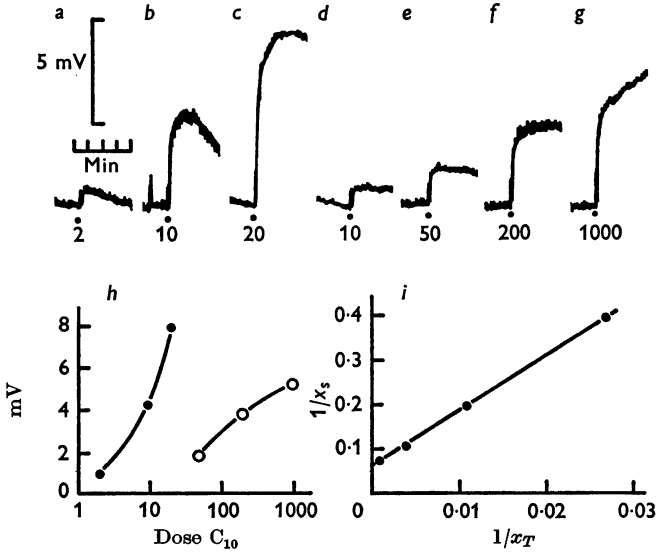

Fig. 4. Measurement of dose ratios at several levels of response. Cat, 2·3 kg, spinal preparation. At *a–c*: control depolarizations of end-plate region of tibialis anterior muscle. Figures are n-moles decamethonium I.A. At *d*: 10 n-moles decamethonium (C_{10}) given when twitch height 96% of normal after 500 n-moles gallamine I.A. At *e*, *f* and *g*: responses to 50, 200 and 1000 n-moles decamethonium when twitch height had been reduced to about 15% of normal. At *h*: dose–response curves. Filled circles: standard. Open circles: at 'deep' block. At *i*: plot of reciprocal of standard doses of panel *h* against reciprocal of test doses, for four levels of response common to control and test dose–response curves.

To test this more rigorously, a larger range of dosage of stimulant was used so as to obtain a dose–response curve during deep block for comparison with the preliminary control. Figure 4 represents the result of such an experiment. The log-dose–response curve (in panel *h*) is displaced to the right in the presence of the antagonists as would be expected, but its slope falls more rapidly than that of the control as the dose is increased. If the dose is increased still further, a maximum to the response is reached which is far below the degree of depolarization that can be produced in the absence of an antagonist.

This result appears at first sight to invalidate the use of a theoretical approach based on competitive antagonism. Similarly, the dose ratios we have measured give a result which is not independent of the level of response at which the measurement is made, or independent only at low response levels. If the situation is not one of competitive antagonism it becomes much harder to use the measurements to provide quantitative data about the chemical events of transmission. In such circumstances it is common not to pursue the analysis, but simply to label the antagonism non-competitive or uncompetitive. But if one proceeds rather to explore the possible explanations of the change in slope of the dose–response curve in the presence of tubocurarine these are not unlimited, and many of them can be ruled out.

The arguments proceed as follows. First, the change in the dose–response curve could be a result of giving curare, or independent of it and due to a progressive decline in sensitivity of the preparation to depolarizing agents. But in fact the sensitivity to succinylcholine is stable over long periods if no competitive agent is given or if recovery from its action is allowed (for example see fig. 1, *a* and *b*, of Paton & Waud, 1962, or Fig. 5 of this paper).

If the change is due to curare, it may be the result of an effect not related to the specific end-plate or acetylcholine receptor, or it may be a receptor phenomenon. Taking the non-receptor case first, we have several possibilities. The first, a chemical antagonism between the depolarizing agent and the competitive agent, can be rejected on chemical grounds; and the doses of agonist which are most affected are far greater than the amount of antagonist available to react with them. A second possibility is that the tubocurarine has altered the ionic environment of the end-plate membrane by potassium release resulting from its known action as a histamine liberator. But gallamine, which does not release histamine, produces the same alteration in response to succinylcholine. The depolarizing agent itself might be altering potassium concentrations locally, but this effect would have to be one which is not blocked by curare, and would therefore involve postulating a separate receptor-effector system. In addition Klupp & Kraupp (1954) showed that succinylcholine (200 μg/

kg) did not change the potassium concentration of plasma if tubocurarine (300 μg/kg) was present in the animal.

Thirdly, could the alteration in shape of the dose–response curve be a result of giving the depolarizing agent as a small volume of a concentrated solution directly into the artery of the leg, i.e. is it a 'slug' effect? The effect was not due to the drug being given in different volumes since we kept the injected volumes constant (cf. also Fig. 1). Further the concentration at the receptor should be proportional to that in the administered volume since in the concentrations we have been using the effect of both perfusion and diffusion is to alter the concentration proportionally only and not on an absolute scale. In both cases the concentration at the receptor, C_r, would be related to that in the syringe, C_o, by a relationship of the form $C_r/C_o = f$ (flow, leg volume, distance, time).

Fourthly, histamine release caused by tubocurarine might alter blood flow through the leg so that less succinylcholine reaches the end-plate. Again, however, gallamine produces the same effect as tubocurarine although it does not release histamine. Nor can we point to any other vascular effect shared by all three competitive blocking agents which would result in a selective shift of blood flow from the end-plate region.

Fifthly, we have verified during the action of higher test doses of depolarizing agent that the recording electrode remains on the region of maximal depolarization. In experiments where the preparation has been followed through complete recovery from the effect of the competitive agent, the electrode does not shift appreciably (see below).

Finally 'desensitization' must be considered. By desensitization is meant a non-specific depression of the response produced by a stimulant, but it is a depression which is not a receptor phenomenon *per se*. This latter qualification is added to distinguish desensitization from phenomena such as the 'fade' due to advancing receptor occupancy (Paton, 1961). The original description of desensitization was given by Cantoni & Eastman (1946), when they showed that a previous large dose of acetylcholine will depress the response of intestinal smooth muscle to subsequent doses not only of acetylcholine but also of histamine. The fact that the response to histamine is also reduced is the basis for classifying desensitization as a non-receptor phenomenon. On the end-plate all depolarizing agents seem to act on the same receptor so we cannot prove the existence of desensitization here. Nevertheless, by analogy its presence must be regarded as likely. Certainly several phenomena have been described which are compatible with its existence here (Jenden, Kamijo & Taylor, 1954; Thesleff, 1955; Katz & Thesleff, 1957; Zaimis, 1952).

Is, therefore, the change in the shape of the dose–response curve after curare an expression of the development of desensitization by which, in

the presence of curare, an end-plate stimulant produces a greater than normal desensitization to itself? If desensitization is to provide an explanation, the degree of desensitization produced must be proportional to the dose and not to the effect, since the effect is kept constant in the dose–ratio method. This means that desensitization is an unlikely explanation. First, *a priori* it is reasonable to expect a non-receptor phenomenon to be related to magnitude of activity at some link in the chain of processes after receptor activation, i.e. to be related to effect. Secondly, to suppose desensitization proportional to dose amounts to postulating a second receptor system and one which is not affected by curare. Thirdly, when desensitization is studied elsewhere in the presence or absence of antagonist it is very closely proportional to effect and not to dose (Paton, 1961).

We fail, therefore, after considering the possible non-specific factors, to find an explanation for the characteristic flattening by the antagonist of the agonist dose–response curve. We have next to consider possible mechanisms involving the end-plate receptor.

First, are we dealing with an irreversible antagonism, possibly entailing even destruction of the receptor, such as that exemplified by dibenamine? This could produce the observed dose–response curve. It is common knowledge that curare is not an antagonist of this type, and recovery always occurs, even if sometimes prolonged. We have however verified the reversibility of end-plate changes directly. After the responses to control doses of acetylcholine were obtained, DF-596 was given and responses to succinylcholine were obtained (Fig. 5). These show the characteristic dose–response relationships. The preparation was now allowed to recover for 3 hr, the responses to succinylcholine were then measured again, and found to be identical with the original series.

If then the behaviour is reversible, is the antagonism not competitive? Several cases must be considered here. First, does the curare combine with a second receptor system with the result that the response of the usual end-plate receptor system is reduced? This is the case of a non-competitive antagonism (Ariëns, van Rossum & Simonis 1957), and could produce dose–response curves such as those observed. But the idea involves the *ad hoc* postulate of a second receptor system, for which there is no other evidence. An explanation consistent with the idea of only one receptor will be preferred.

Second, the competitive agent might hyperpolarize the membrane and so lead to a lower maximum depolarization. This is similar to the case just mentioned but here there is evidence to rule it out. We have never observed any hyperpolarization after the competitive agents used and they are known not to affect the threshold to direct electrical excitation of the muscle fibre. In addition, experiments with internal capillary micro-

electrodes which could detect much smaller changes than our wick electrodes show no such change.

Finally we turn to competitive antagonism. First, any given electrical change measured at the tip of the recording electrode could be the result of a small change in membrane permeability over a large area or a larger

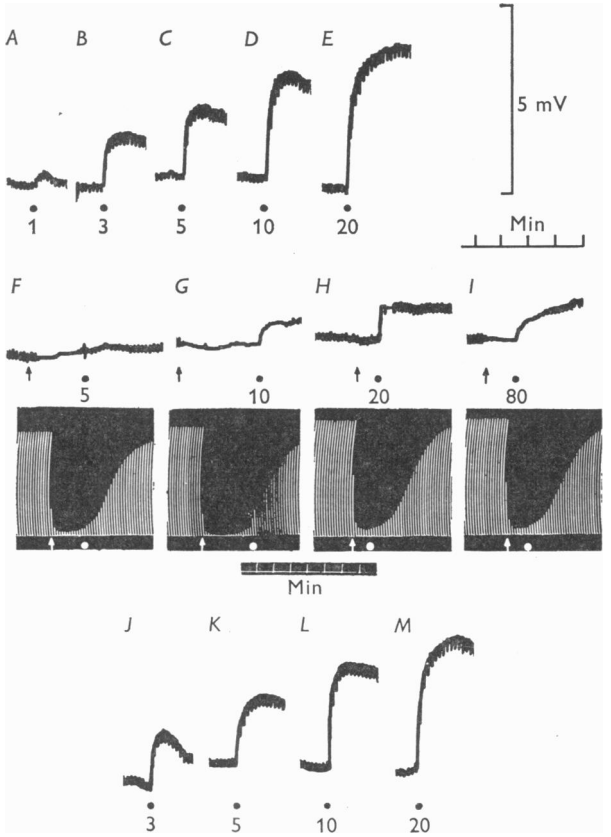

Fig. 5. Recovery from competitive neuromuscular block. Cat, 2·3 kg, spinal preparation, perfused sartorius. *A–E*: control responses to succinylcholine (doses in n-moles, order of administration *DCEBA*). *F–I*: responses at 93 % block. Electrical records are above corresponding mechanical tracings. At arrows DF-596 10 n-moles I.A. (Flow was stopped for a few seconds so that the total dose of DF-596 necessary would be minimal.) *J–M*: responses about 3 hr later (order *LJKM*); recovery from action of DF-596 is complete.

change over a smaller area. These two cases would be indistinguishable by our recording method if the areas concerned were small compared to the area covered by our wick electrode; and it is possible that the flattening of the dose–response curve in the presence of the antagonist is due to some

change in the distribution of permeability change with rising dose. On the other hand, an effect of this sort entails a variation in sensitivity to acetylcholine and its antagonists among the receptors, and amounts again to postulating more than one type of receptor.

The next receptor case which might be involved is that of 'fade' (Paton, 1961), i.e. the depression of the response to a stimulant drug resulting from its binding at the receptor. Fade is faster at high than at low dosage. Is it possible that with the lower doses of succinylcholine, equilibrium with it was very far from complete, but that with high doses the response faded rapidly to a very low equilibrium level? Electrophysiological studies show, however, that when the stimulant drug is applied iontophoretically the response rises smoothly to a maximum as would be expected in a diffusion-limited case (del Castillo & Katz, 1956*a*). This indicates that stimulant drugs reach the equilibrium state in a few milliseconds or less, so that the response to the stimulant drugs used in the leg are all equilibrium responses.

The case of competitive antagonism when equilibrium is not complete. Within the framework of competitive antagonism, two approaches can now be used. On the one hand, the time course of action of a drug can be taken as reflecting its binding by the receptor; on the other, it can be supposed that there is a biophase (Furchgott, 1955) or some other mechanism which limits access of drug to the receptor region, or escape from it. At present neither approach can be excluded, in considering events at the neuromuscular junction. Our results will therefore be considered in turn from both points of view.

The conception that the delay in recovery from an antagonist is due, not to diffusive barriers, but to a slow dissociation from the receptor, provided one of the starting points for the kinetic model of drug action, in which antagonists are bound by the receptor but stimulants dissociate freely. But the argument that follows is independent of whether an occupational or kinetic model of drug action is assumed, and assumes only that antagonists dissociate slowly, whereas stimulants dissociate rapidly.

In the usual case of competitive antagonism for two drugs, a stimulant X (at dose x) and an antagonist X' (at dose x') combine with a common receptor pool R (fraction of receptors occupied by X is y, by X' is y'). This may be written.

$$X+R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} XR \quad \text{and} \quad X'+R \underset{k_4}{\overset{k_3}{\rightleftharpoons}} X'R,$$

where $k_1 \ldots k_4$ are the reaction rate constants. The equations to describe this system are

$$\frac{dy}{dt} = k_1 x(1-y-y') - k_2 y, \tag{1}$$

$$\frac{dy}{dt} = k_3 x'(1-y-y') - k_4 y'. \tag{2}$$

Solving for $x = x' = 0$ at $t = 0$, and writing $k_e = k_2/k_1$, $k'_e = k_4/k_3$, at

$$t = \infty, \quad y = \frac{x}{x + k_e(1 + x'/k_e')} = y_e. \tag{3}$$

To simplify calculations we may later introduce a 'natural dose' $d = x/k_e$, i.e. the dose expressed in units of the equilibrium constant. Then

$$y_e = \frac{d}{d + d' + 1}. \tag{4}$$

Now, if competitive antagonists do not wash out readily, in the minute or less required to make our measurement of response this full equilibrium will not be reached. In the limiting case the stimulant X will reach equilibrium (cf. discussion of 'fade' above) but the antagonist will not leave the receptor appreciably. Then (1) and (2) become:

$$\frac{dy}{dt} = k_1 X(1 - y - y') - k_2 y, \tag{5}$$

$$\frac{dy}{dt} = 0, \tag{6}$$

$y' = y'$ at $t = 0$, $y = 0$ at $t = 0$. Solving:

$$y_e = \frac{x}{x + k_e}(1 - y'_0) = \frac{d}{d + 1}(1 - y'_0), \tag{7}$$

$$y' = \frac{d'}{d' + 1}.$$

We may call this case a 'quasi-equilibrium'.

Finally for the control dose–response curve in the absence of antagonist we have $y' = 0$ in (1) and (2), and

$$y_e = \frac{x}{x + k_e} = \frac{d}{d + 1}. \tag{8}$$

In all cases we assume response is proportional to number of receptors occupied. (At equilibrium this is the case with either the occupational or the kinetic model).

The case of full equilibrium gives the usual 'parallel' log dose–response curves. The dose–response curve in the quasi-equilibrium case is flattened and reaches a maximum which is $(1 - y')$ times that of the control level. This concept of a quasi-equilibrium thus provides a qualitative explanation of Fig. 4, which requires no postulate beyond those of a simple com-

petitive antagonism and the already observed relationship between possession by drug of competitive blocking action and resistance to being washed out.

If the model of a quasi-equilibrium applies to our experiments, then the dose ratios given are not exact measures of occupancy, because the receptor occlusion that is measured is the sum of that due to the competitive agent and of that additional occlusion due to occupation of receptors by the depolarizing agent. To obtain a true measure of receptor occupancy by the competitive antagonist itself we return to (7) and (8).

Consider doses to produce equal effects before and during the action of antagonists. Then

$$\frac{d_1}{d_1+1} = \frac{d_2}{d_2+1} \cdot \frac{1}{d'+1}, \tag{9}$$

where d_1 is the dose of depolarizing agent before, and d_2 the dose with the antagonist. (The term $1/(d'+1)$ is $1-d'/(d'+1)$, i.e. it is the fraction of receptors not occupied by the antagonist.) Solving for d_1 in terms of d_2 and returning to the original units of dose for the stimulant gives

$$\frac{1}{x_1} = \frac{d'}{k_e} + (d'+1)\frac{1}{x_2}. \tag{10}$$

The analogous expression for the case of the classical complete competitive equilibrium is

$$\frac{1}{x_1} = (d'-1)\frac{1}{x_2}. \tag{11}$$

Thus a plot of $1/x_1$ *vs.* $1/x_2$ gives $d'+1$ as the slope, from which the true occupancy by the competitive antagonist, $d'/d'+1$, is obtained as slope $-1)/$slope.

It should be noted that the $1/x_1$ intercept provides a measure of the equilibrium constant of the stimulant drug without obtaining, or making assumptions about the nature of, the maximum response.

Equation (10) offers an experimental test of the quasi-equilibrium theory; the relation between $1/x_1$ and $1/x_2$ should be linear, its intercept on the $1/x_1$ axis should be greater than zero, the slope of the line should be the same (independently of the drugs used) for a given degree of block, the slope should increase as block deepens, and the slope should be less when a given block is obtained late rather than early. In Fig. 6 the data from the dose–response curve in Fig. 4 have been analysed, together with data from seven other experiments.

In Expts. 1–3 of Fig. 6, the x_2 values of equation (10) were all determined at deep block. The drugs used varied (succinylcholine and DF-596 in 1,

decamethonium and gallamine in 2, decamethonium and tubocurarine in 3). Nevertheless, the slopes (9·8, 12·5 and 11·6) were indistinguishable by analysis of variance.

Comparison of decamethonium with succinylcholine might not be regarded as likely to reveal a difference since both are bisquaternary agents. A reciprocal plot was therefore obtained using a monoquaternary as the depolarizing agent. The experiment is shown in Fig. 7. The compound used, $ICH_2CH_2NMe_3^+$, made for us by E. W. Gill, was chosen as best of a collection of monoquaternaries tested for potency as end-plate depolarizing agents in cat muscle. The reciprocal plot obtained is plotted

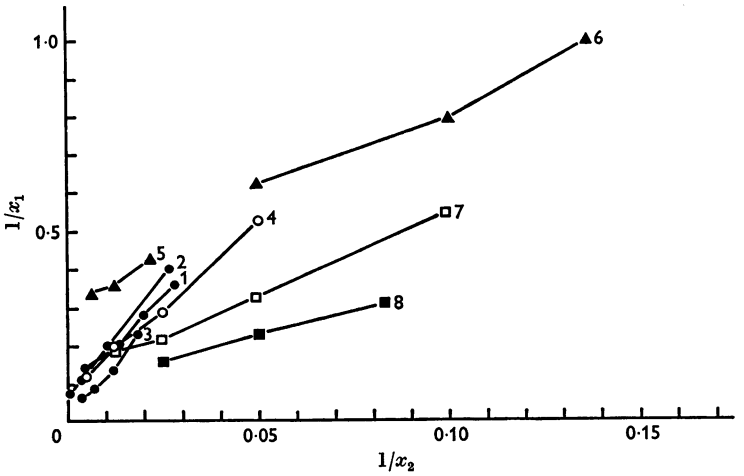

Fig. 6. 'Reciprocal plots'. Ordinate: reciprocal of dose of agonist before competitive antagonist. Abscissa: reciprocal of dose of agonist in presence of antagonist. Units: n-moles^{-1} I.A. except for curves 4 and 8 where units are n-moles^{-1} × 10. Curves 1–3: early, deep block, bisquaternaries. Curve 4: early, deep block, $ICH_2CH_2NMe^+_3$. Curves 5, 6: deep block, late. Curve 7: 45% block. Curve 8: threshold block. Further explanation in text.

in Fig. 6 (as Expt. 4) where it is seen to have a slope indistinguishable from those of Expts. 1–3. Expts. 7 and 8, obtained with early moderate and threshold block respectively, show slopes proportionately reduced. Expts. 5 and 6, with late deep block, yield slopes less than those in 1–4.

In no experiment was there a deviation from linearity significant in relation to the error of the method. There is however a slight curvature, concave upwards, in four of the experiments. This may well arise if our hypothesis, that the antagonist does not leave the receptor at all during the time taken to measure the response to the depolarizing agent, is not exactly obeyed.

The $1/x_1$ intercepts of Fig. 6 are all greater than zero, as required by equation (10). This means also that the assumption in constructing Table 1, that the agonist achieved a negligible occupancy, is in error. An estimate of the correction to be made for this error can be made as follows. If there is significant occupancy, then, using normal mass-action relations, the exact estimate of occupancy by the antagonist is

$$y'_{\text{exact}} = \frac{\text{dose ratio} - (x_2 + k_e)/(x_1 + k_e)}{\text{dose ratio}}, \tag{13}$$

where x_1 and x_2 are the doses of the depolarizing agent used to produce the control and test responses of the dose ratio (cf. Paton, 1961, p. 34).

The values in Table 1 were obtained using

$$y' = \frac{\text{dose ratio} - 1}{\text{dose ratio}}. \tag{14}$$

Taking the ratio of these two expressions and noting that dose ratio $= d_2/d_1$ gives

$$y'_{\text{exact}} = (d_1 + 1)\, y'. \tag{15}$$

Thus the error depends only on the receptor occupancy produced by the dose of agonist used to produce the standard response and so does not vary with the level of block. The fact that in the experiments of this paper the standard responses were all about the same size facilitated pooling the results.

Turning to the experimental data, in those experiments where a true estimate (y'_{exact}) was obtained from a reciprocal plot, dose ratios were also estimated as had been done to obtain Table 1. This gives one pair of estimates of occupancy (y'_{exact} *vs.* y') for each experiment. A second method was obtained by using the $1/x_1$ intercept of the reciprocal plot to get a rough estimate of k_e (cf. equation (10)) and then using equation (13). Pairs of estimates so obtained have been plotted in Fig. 8. Four agonists were used, but since the slope of the line is very insensitive to the k of the agonist all experiments have been pooled. The regression equation is $y'_{\text{exact}} = 0{\cdot}92\, y'$. This means that the estimates of occupancy in Table 1 are 8 % too high. But the standard error of estimate of y'_{exact} from y' is 0·072 at $y' = 0{\cdot}76$ and 0·062 at $y' = 0{\cdot}92$, i.e. at levels corresponding to threshold and deep block, so that no great precision attaches to this correction. The significant point is that the correction required to Table 1 is a small one.

We turn now to considering results such as those of Fig. 4, taking account of the possible influence of access-limitation. If with some system such as

'biophase', investigated by Furchgott (1955) and further analysed by Rang (1966), the access of both tubocurarine and succinylcholine to the receptor be hindered, then the concentration of drug in the immediate vicinity of the receptor cannot instantaneously follow that in the extracellular fluid generally. When succinylcholine is given, in the presence of tubocurarine, the concentration of antagonist molecules combined with the receptor pool falls in accordance with the laws of mass action; but the tubocurarine released cannot leave the receptor region immediately, so

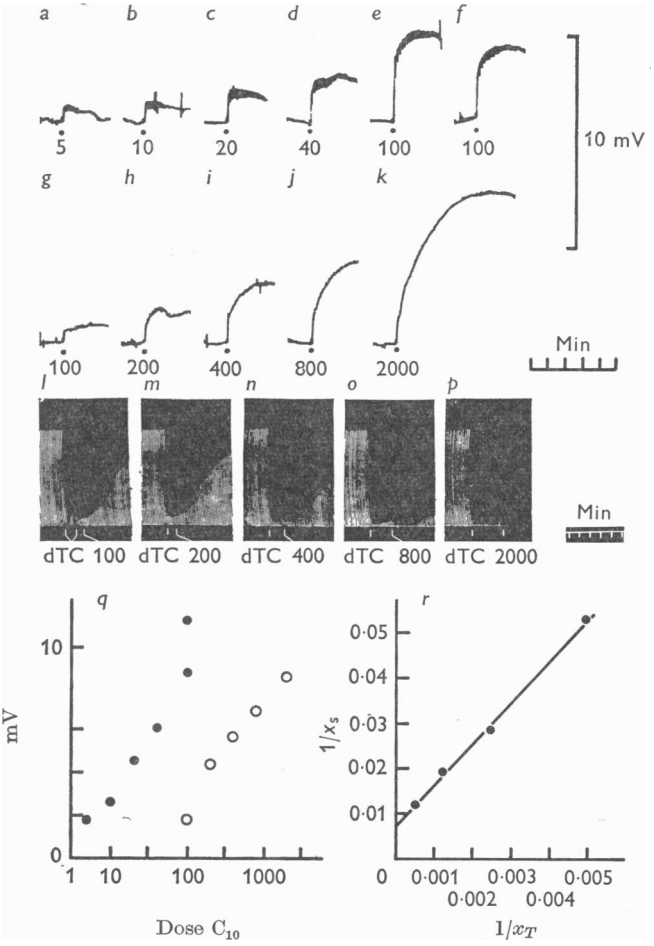

Fig. 7. Margin of safety using $ICH_2CH_2NMe^+_3$ as test drug. Cat, 2·6 kg, spinal preparation, perfused sartorius. *a–f*, control depolarizations; *g–k*, depolarizations and *l–p* corresponding mechanical records at 'deep' block. At 'dTC', tubocurarine 100 + 50 n-moles I.A. in *l*, 100 n-moles I.A. in *m–p*. *q* and *r*: dose–response curves and reciprocal plot as in Fig. 4.

that the succinylcholine comes to compete with a higher local concentration of tubocurarine than would exist under conditions of free access. With the procedure adopted in our experiments, using rapid intra-arterial injection, it could well be the case that tubocurarine had little time to escape during the period of exposure of receptors to the succinylcholine. Evidently, with such a process the usual parallel shift of the log dose–response curve produced by an antagonist would not occur, but the curve in the presence of an antagonist would become relatively flattened. Full

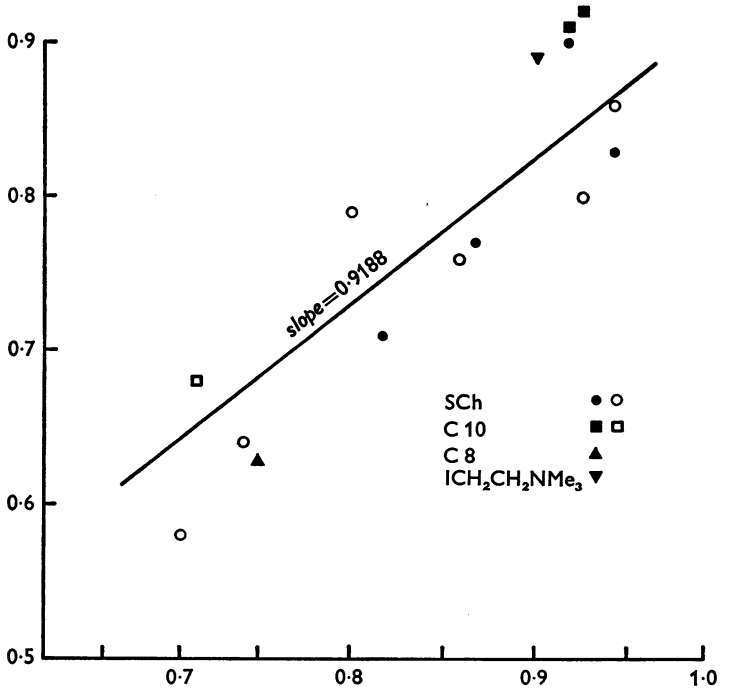

Fig. 8. Relationship between approximate and exact estimates of occupancy. Ordinate: 'exact' fractional occupancy, obtained from reciprocal plot by equation 12 (filled symbols) or from equation 13 (open symbols). Abscissa: approximate fractional occupancy (equation 14). The line is at least-squares regression of the 'exact' on the 'dose-ratio' occupancy with the restraint that the line pass through the origin (in accordance with equation (15)). SCh = succinylcholine, C_{10} = decamethonium, C_8 = octamethonium.

quantitative treatment of the situation is not possible, because the conditions of access and physical characteristics of the preparation are not known. But if a biophase is assumed, it can be shown (C. D. Thron, personal communication) that as the volume of the biophase is reduced the equation describing the system (equation (11) in Rang's (1966) analysis) transiently approaches that for a non-competitive antagonism, to which

equations (7), (9) and (10), together with the subsequent analysis, would apply.

The result is thus a simple one qualitatively. It is that, if the escape of antagonist from the receptor is hindered, either by binding forces at the receptor, or by limitations to its free diffusion out to the general extracellular space, then the interaction of agonists and antagonists will appear, transiently, as non-competitive, even though the interaction is in fact according to mass-action laws with a single pool of receptors. The approach

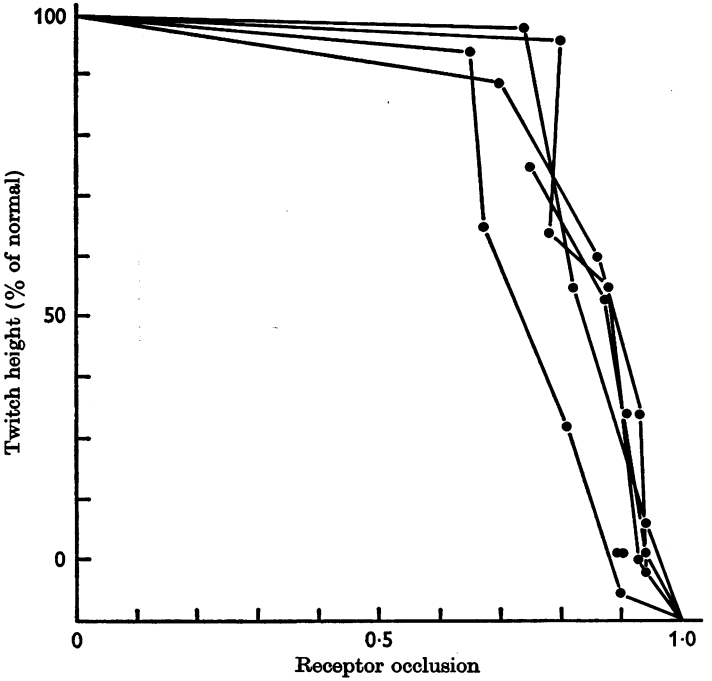

Fig. 9. Relationship between twitch height and degree of receptor occlusion by a competitive blocking agent. Ordinate: twitch height as % of normal. Abscissa: fractional receptor occupancy. Lines connect points from the same animal.

to linearity of the plots in Fig. 6, and the fact that they make a positive intercept on the ordinate, thus supports the conception that there is a restraint on the escape of antagonist molecules from the receptor region during the action of an agonist; but it does not allow a discrimination between the possible mechanisms involved.

This analysis shows, therefore, that it is possible to determine the factor of safety by the method we have used, that of dose ratios, and shows how to obtain, if necessary, the correction arising because equilibrium is incomplete. In the present investigation, the correction is a small one, and not statistically significant, but it could be substantial under different conditions.

The relationship between neuromuscular block and receptor occupancy by an antagonist. Our principal interest has been in the margin of safety at 'threshold' and 'deep' block, for which corrected estimates have been

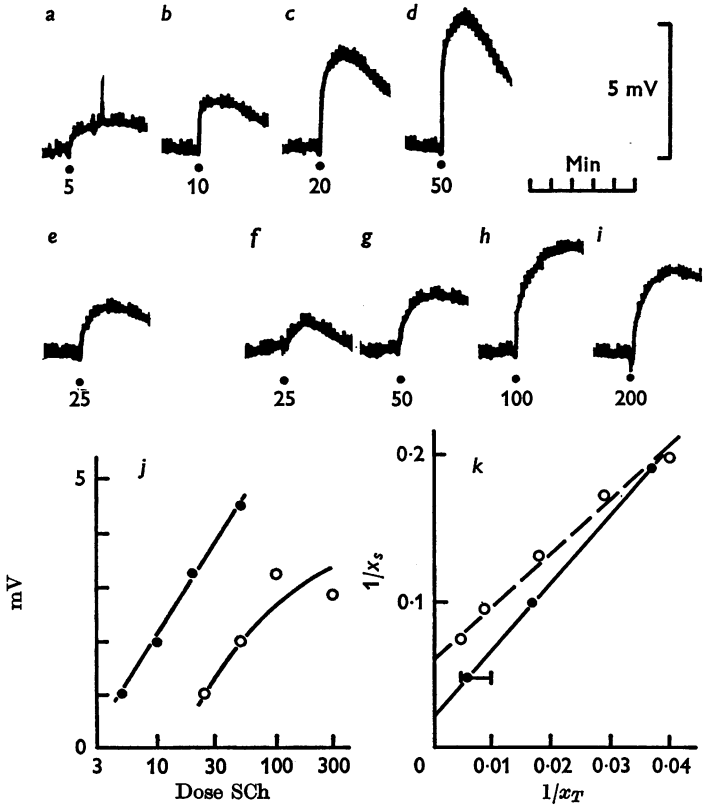

Fig. 10. Margin of safety in the rabbit. Rabbit, 1·8 kg, urethane. Depolarization given for control (*a–d*), 19 % block (*e*), 87 % block (*f–i*). Dose–response curves at *j* and reciprocal plot at *k* (continuous line) as in previous cat experiments. Interrupted line in *k* gives reciprocal plot from another rabbit at 95 % block under chloralose.

given above. Experiments were also made to obtain points at intermediate levels of block, and one such experiment is illustrated in Paton & Rang (1966). Results from this and similar experiments are collected in Fig. 9. These results confirm the implication of Table 1, that once some degree of block is obtained little increment in occupancy is needed to reach deep block. The variation in margin of safety between animals is greater than this increment, so that the exact shape of the relationship cannot be readily determined.

Experiments were done chiefly in cats since a spinal preparation could then be used in which a reasonably stable cardiovascular state could be maintained for many hours. But a few experiments were done in rabbits and dog. Figure 10 illustrates two experiments in rabbits. The legs were

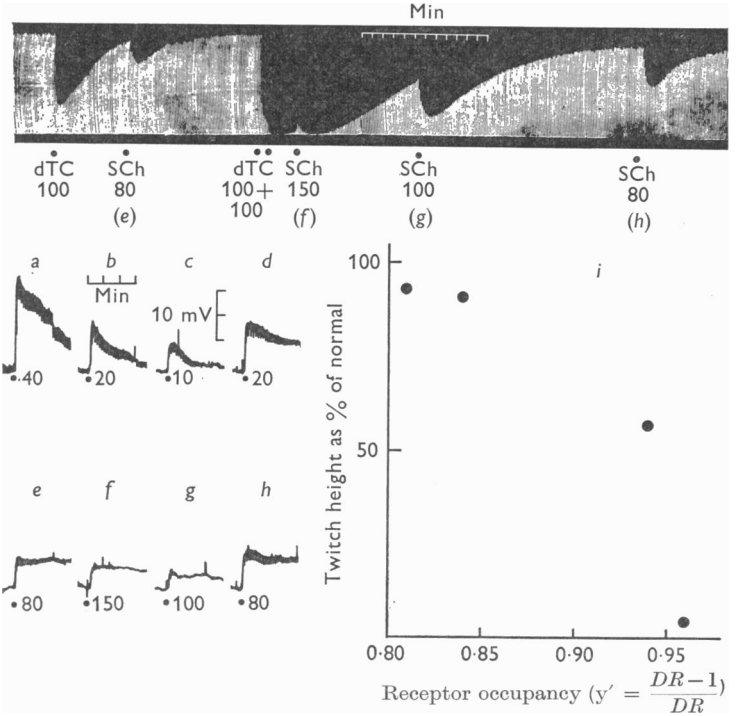

Fig. 11. Margin of safety in the dog. Dog, 6·1 kg, chloralose, perfused sartorius. Upper record: mechanical responses. *a–d*: control depolarizations (succinylcholine), *e–h*: depolarizations at 7, 96, 43 and 9 % block by tubocurarine. Twitch height is plotted against occupancy (y′ = dose ratio − 1/dose ratio) in panel *i*.

not perfused. Drugs were injected, as in the early cat experiments, through a Gordh needle in the opposite common iliac artery. The occupancies obtained, 0·79 and 0·72, at deep block are less than in the cat, indicating a lower margin of safety in the rabbit. Figure 11 shows an experiment in the dog in which dose ratios were obtained at various levels of block. The relationship between receptor occupancy and block derived from these dose ratios is given in the graph. The results are comparable with those found in the cat.

DISCUSSION

The determination of the margin of safety. The purpose of this investigation was to provide a basis for describing some of the phenomena of

competitive neuromuscular block in quantitative terms. Existing knowledge permitted us to say little more than that a margin of safety exists for transmission at the neuromuscular synapse, i.e. that the end-plate potential is more than adequate to trigger a propagated response over a wide range of frequencies of excitation. Similarly the fraction of the end-plate receptors that must be occluded in order to produce complete block of a population of muscle fibres was only vaguely defined. The present experiments go some way to allowing us to relate intensity of neuromuscular block more closely to events at the post-synaptic receptor (Fig. 9).

The assumption has been made that acetylcholine and blocking agents such as tubocurarine interact with the receptor pool according to the laws of mass action as do stimulants and antagonists at other receptor sites. The problem then becomes that of estimating the fraction of the receptor pool occluded by the antagonist at any given time and of relating this to the intensity of block achieved. The natural measure of block is the proportion of fibres responding. But although the size of the indirectly excited twitch probably does not reflect exactly the fraction of fibres responding, we have used the reduction of twitch height as a measure of block in this paper, because it is simple, permits comparison with previous results and is related to the function of the muscle as a contractile organ. There is no reason to believe that it gives a result much different from that which would be obtained if the number of contracting fibres could be counted directly during each twitch.

To measure the receptor occlusion the 'dose ratio' method was used, i.e. the ratio by which the dose of a stimulant must be increased in the presence of an antagonist in order to match the original unantagonized response. The strength of the method is that the response of the tissue, in these experiments the depolarization of the end-plate region, is kept constant, and the only difference between the control state and that in the presence of the antagonist results from events at the receptor. The assumption is made also that the receptors concerned with normal transmission are the same as those responding to externally applied drugs. The experiments of del Castillo & Katz (1956*b*), showing that the miniature end-plate responses and the effect of iontophoretically applied acetylcholine were reduced *pari passu* by tubocurarine, support this approach.

The dose-ratio method might be used without any assumption about the nature of the interaction of stimulants and antagonists at the motor end-plate; the results obtained would then merely express the intensity of antagonism achieved in a convenient form valid for the particular conditions. To go further, so as to throw light on receptor events and to bring the experiments in relation with work on drug antagonism generally, it has proved necessary to develop a new aspect of the theory of drug action.

This was because it was found, in testing the predictions of competitive antagonism, that the dose–response curve in the presence of the antagonist was not parallel to the control dose–response curve over the whole range of dosage of the stimulant. Such failures of parallelism have received a good deal of attention. Ariëns *et al.* (1957) explain such results by the postulate of an extra set of receptors. Schild (1949) has suggested that, to meet the need for a numerical measure of antagonism, his pA value should be measured at a predetermined level of response. Guarino & Bovet (1949*a*, *b*) tried a solution which discarded the mass-action principle as a basis for drug-receptor interaction. Gaddum *et al.* (1955) mention that their dose ratios increase when high doses of active antagonists are used, and make the useful suggestion that such antagonism should be labelled 'unsurmountable', a term which is non-committal as to mechanism. It is, in fact, the phenomenon of unsurmountable antagonism at the motor end-plate which our theory of 'quasi-equilibrium' is put forward to explain. It is not suggested that it accounts for all such cases. But if the view is accepted that the drug antagonists are relatively firmly bound by the receptors or that escape of antagonist from the region of the receptor is hindered, so that a brief application of stimulant drug cannot significantly change the receptor-antagonist equilibrium, then the state of 'quasi-equilibrium' must be a relatively common one. Indeed it can be envisaged as the normal functional state in the body whenever a drug such as atropine or tubocurarine is used, the binding of the drug being little disturbed by transient volleys of transmitter released by nervous activity.

The margin of safety for neuromuscular transmission in the muscles studied is such that for single shocks every 10 sec three quarters of the post-synaptic receptors must be occluded before transmission begins to fail at some junctions, and that over nine tenths must be occluded before all junctions fail. On the one hand, one can regard this as indicating that only one quarter of the receptor pool is necessary for fully normal transmission at low rates of excitation. Alternatively the output of transmitter could be said to be 4 times greater than that necessary just to evoke a propagated response in every muscle fibre. This follows directly if there is substantial spare receptor capacity, since, if $d \ll 1$, $d/(d+1) = d$ in equation (8). The existence of spare receptor capacity follows from the kinetic model of acetylcholine action; but the converse is not true, and it is also compatible with, although not required by, an 'occupation' model. There is no direct quantitative information on this point for the motor end-plate, but from Stephenson's work (1956), and from affinity constants recently estimated (Gill & Rang, Furchgott, 1966) it is known that, for intestinal smooth muscle, the ratio of the concentration of acetylcholine required for half-saturation of the receptors to that for half-maximal con-

tractile response is 100 or more. If, at the neuromuscular junction, the acetylcholine released does partially saturate the receptors, then a reduction of the amount released would lead to a less than directly proportional reduction in effect, and the safety factor expressed in these terms would be higher than estimated above.

That there should be a large margin of safety at the neuromuscular synapse is physiologically not surprising. There has long been evidence that this synapse is more easily paralysed by curare at high than at low rates of stimulation (Bremer & Titeca, 1935), and it is now clear that to a large extent this is due to a decline in transmitter output when stimulation rate is high. Brooks & Thies (1962), with guinea-pig intercostal muscle, found that the quantum content fell by more than 80% when excitation rate increased from 12/min to 20/sec, without significant change of quantal effectiveness. Physiologically, rates of discharge in motor nerves exceeding 20/sec are well known to occur. Given a decline of volley output of transmitter with increasing frequency, it follows that if transmission is to be effective at high rates, there is bound to be a considerable margin of safety at low rates. The result obtained by Boyd & Martin (1956) on cat tenuissimus, that a normal end-plate potential in the absence of propagation would be of a magnitude about 3–4 times the threshold magnitude for propagation, carries the same implication, although it cannot be related to transmitter output without knowing how the change of membrane potential produced by the transmitter at the end-plate varies with quantity of transmitter released. There appears to have been little quantitative work on the margin of safety in terms of the final response of the muscle, but the results by these other methods seem to indicate at least an order of magnitude similar to that found in this paper.

The change of margin of safety with time. The margin of safety for neuromuscular transmission was found not to be constant, but to be lower if recorded late in an experiment. This was not due to some mechanism such as a diurnal end-plate cycle, since the experiments were made at different times of day. Nor was evidence found of any change in the post-synaptic response to depolarizing drugs. Conceivably an increase in efficiency of end-plate cholinesterase could produce a fall in margin of safety but the enzyme is already so efficient that, if it is unstable in some way, the reverse change seems more probable. A more serious possibility is cumulation of the blocking drugs. But the experiments were designed so that the sensitivity of the endplate to depolarization was tested before each determination of dose ratio, a test much more sensitive to residual blocking agent than recovery of transmission. Further, in some experiments (e.g. Expt. 3 in Table 1) the required lapse of time was obtained without giving drugs at all.

The only respect likely to influence neuromuscular transmission in which

the experimental preparations differed from the normal animal was the fact that the motor nerve to the muscle under study had been cut. We suggest, therefore, that the decline in safety factor which we have observed in fact represents the early stages of nerve degeneration, with an accompanying progressive failure of transmitter release. Six hours after nerve section is early to detect such effects.

Changes in acetylcholine and choline acetylase content after denervation of mammalian muscle have been recorded only after a period of a week or longer (Hebb, 1962; Hebb, Krnjević & Silver 1964; Bhatnagar & MacIntosh, 1960). Functional and structural changes have been described at the amphibian neuromuscular junction only after 3 days denervation (Birks, Katz & Miledi, 1960). For cat superior cervical ganglion, decline of acetylcholine content has been noted within 30 hr after preganglionic section (Banister & Scrase, 1950). Emmelin (1962) did not observe 'paralytic secretion' by the cat submaxillary gland until 14 hr or longer after post-ganglionic denervation. The earliest changes of this type hitherto found are probably for an adrenergic synapse, the failure of transmission and degeneration contracture being seen 8–12 hr after post-ganglionic denervation of the cat's nictitating membrane (Trendelenburg & Langer, 1965). But none of these tests is likely to be as sensitive as that used in the present paper, where erosion of the safety factor, rather than transmission failure itself, is detected.

The experimental significance of the margin of safety. The existence of a large margin of safety has a number of practical consequences for the investigator. For instance, it is sometimes supposed that a nerve-muscle preparation is in good condition if a normal sized indirectly excited twitch can be obtained; but this would merely show, in the muscles we have studied, that transmission is better than 25 % of normal capacity. Care in interpretation is particularly necessary if blocking agents are used. For instance in the paper by Chou (1947) introducing the rat nerve-diaphragm for curare assay, he noted that the response to tubocurarine was constant 'except for the response to the first dose applied to a fresh preparation, or to a preparation left without tubocurarine for an interval; such a response was less than later responses'. This is exactly the way in which the preparation would be expected to behave, since if the tissue is washed so that the twitch height just returns to normal, there can still be a considerable residue of tubocurarine; only at the beginning or after prolonged washing would this not be true. Holmes, Jenden & Taylor (1951) on the same preparation likewise noticed a sensitivity to curare increasing with time, and went so far as to apply an empirical correction for the 'drift'. This phenomenon is also familiar in clinical practice; the first dose of a competitive neuromuscular blocking agent is several times that used for

maintenance of muscle relaxation (see Tables 6 and 7 of Foldes, 1957). Here we can suppose that the maintenance dose of (say) tubocurarine will be required when the receptor occupancy has fallen to about 0·8. A considerable background level of drug will still be present, and the maintenance dose need only be sufficient to supplement this residue. A further consequence of the size of the margin of safety is that the absence of effect of a drug on twitch height is not proof that the drug does not interact with the end-plate receptors but only that it has not reacted with more than about 75 % of them. In a similar way, a false suggestion of 'potentiation' of one drug by another could arise where no more than addition of effects was concerned; for two drugs, each in a dose sufficient to occlude 60 % of the receptor pool and therefore apparently devoid of effect when given separately would, when given together, produce neuromuscular block.

The existence of a margin of safety is also important for the kinetics of neuromuscular block. It is common to observe, after giving tubocurarine, first a latent period, then a period of development of block, leading finally to the development of a stable constant degree of paralysis; if the drug is now washed out, the rate of offset of block is found to be considerably faster than had been the rate of onset. At first sight, this is surprising, since by mass action kinetics the reverse should be the case (compare equations (8) and (9)). But the mass action relations apply to the receptors, not to the block itself; and if block cannot develop until a substantial fraction of the receptors is occluded, then a record of neuromuscular block will allow us to look only at the end of onset and the beginning of offset of action of the drug. Thus, in Fig. 12 is represented the onset and offset of receptor occupation by tubocurarine in a dose that will produce 90 % occupancy at equilibrium, onset therefore having a 10 times faster time constant than offset (equations (7), (8) and (9) with $y' = 0{\cdot}9$). Ordinate lines have been drawn at $y' = 0{\cdot}9$ and $y' = 0{\cdot}75$ to show the receptor state at deep and at threshold block respectively. It is only the region between these lines, the top of the 'iceberg' of drug-receptor interaction, that can be seen by recording the muscle twitch. These particular values produce a time course of block almost identical with that in fig. 1 of Holmes *et al.* (1951), with the characteristically slow onset of reduction of twitch height and faster recovery. The closeness of this parallelism illustrates the usefulness of having a quantitative measure of the margin of safety.

The existence of a substantial margin of safety also influences considerably the interpretation of experiments in which comparisons are made between the effects of an antagonist on responses to nerve stimulation and to injected stimulants. The relative resistance of nerve effects to the action of an antagonist received attention in the past, as offering a difficulty to the theory of chemical transmission. But as Brown (1937) made clear, the

comparison concerned is deceptive, even when the responses concerned, for instance the mechanical responses of cat muscle to a single maximal motor nerve volley and to arterially injected acetylcholine, are arranged to be of similar size, before the application of an antagonist such as curare. Whereas the acetylcholine released by a single maximal nerve volley must, as we have seen, be antagonized four-fold or more before any reduction of the twitch appears, the response to injected acetylcholine, consisting of a brief waning tetanus in a proportion of the muscle fibres, can reflect at once, by a change in the tetanic frequency or duration, any reduction in the effectiveness of acetylcholine. A similar argument will apply at other cholinergic synapses (cf. Paton & Thompson (1964) for the

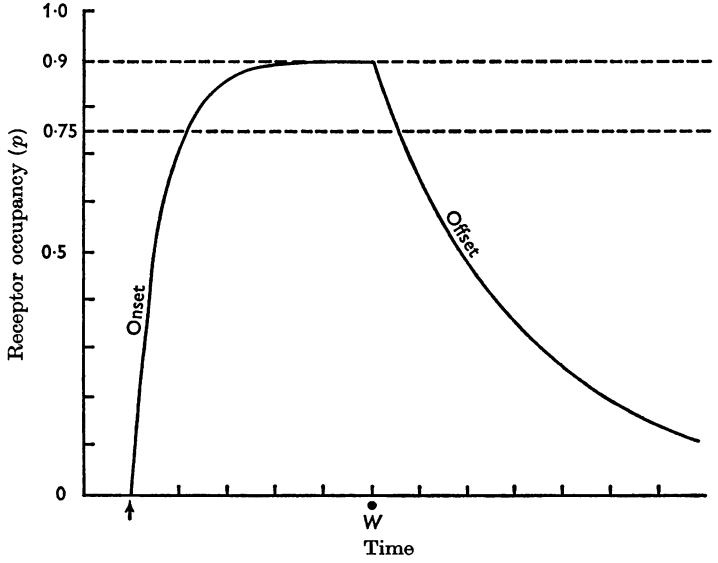

Fig. 12. Calculated 'onset' and 'offset' of neuromuscular block by a competitive blocking agent; 'dose' of agent such that occupancy at equilibrium will be 0·9. Ordinate: fractional occupancy. Abscissa: time in arbitrary units. Time constant of onset of occupancy is 0·1 that of offset. Competitive agent 'administered' at arrow and washed out at W. Horizontal interrupted lines at $y' = 0{\cdot}9$ and $p = 0{\cdot}75$ represent occupancies associated with threshold and deep block in a typical cat muscle. Reduction in twitch height proceeds more slowly than recovery.

superior cervical ganglion) and at adrenergic neuroeffector junctions; and it reduces the need to postulate special barriers to the access of externally applied drugs to the receptors.

In a similar way, consideration of the margin of safety of neuromuscular transmission reduces the cogency of the evidence for a presynaptic action of certain drugs from which the conception of a presynaptic action by acetylcholine during usual transmission has developed (see Werner &

Kuperman (1963) for review). Among this evidence, for instance, is the observation that tubocurarine, in a lower dose than that required to produce neuromuscular block, will prevent antidromic repetitive firing evoked in a motor nerve by edrophonium and similar drugs. This might suggest that the nerve terminals are more sensitive to curare than is the end-plate and are therefore the primary site of action of curare. But suppose that a cathodal state at the nerve ending renders it liable to fire repetitively; then administration of any drug which acts post-synaptically to depolarize the end-plate will lead to repetitive activity, and this activity will seem to originate in the nerve ending. It will now be possible to give curare in a dose sufficient to block up to three-quarters of the receptor pool available to the end-plate–depolarizing agent, and to attenuate greatly both its depolarizing action and its consequent effect on nerve firing, before any effect on neuromuscular transmission could be recognized. A failure to consider the implications of the margin of safety could thus lead to a failure to localize the site of action of the drugs correctly, with accompanying errors in physiological interpretation.

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