THE ACTION OF DECAMETHONIUM ON THE ISOLATED RABBIT LUMBRICAL MUSCLE

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Received for publication February 27, 1954

Until recently it was thought that the only mechanism by which decamethonium (C10) blocks neuromuscular transmission was by causing depolarization of the motor endplate, so resembling acetylcholine and differing from tubocurarine (TC) (Zaimis, 1951; Paton and Zaimis, 1949; Bovet, 1951; Burns and Paton, 1951); this work was carried out on mammalian nerve-muscle preparations *in vivo* and on avian and frog muscle. The experiments described in the present paper were carried out on an isolated mammalian nerve-muscle preparation previously described by us (Jenden, Kamijo and Taylor, 1951a), and the results, while confirming in the main the above conclusions, reveal the situation to be more complex. A preliminary account of these results was published in 1951 (Jenden, Kamijo and Taylor, 1951b), and since then numerous papers have appeared suggesting a dual mode of action for C10 and related compounds (*e.g.*, Zaimis, 1953).

Although experiments on intact animals may reveal valuable information, *in vitro* experiments allow the accurate control of more variables, and as a result are likely to be useful in the analysis of the mode of action of drugs. A mammalian nerve-muscle preparation was sought which was sensitive to C10 as well as to TC, and was also suitable for *in vitro* work. The rat diaphragm has been shown to be relatively insensitive to C10 and similar agents (Barlow and Ing, 1948; Paton and Zaimis, 1949), and while diaphragms from other animals besides the rat have been used for limited investigations (Young, 1949; Sullivan and Kensler, 1950; Paton and Zaimis, 1951), these must be from very young animals or from fetuses in order that they may be thin enough for adequate oxygen diffusion. The two animals in common laboratory use most sensitive to C10 are the cat and the rabbit, and a thorough search of these animals for suitable muscles has led to the use of the medial lumbrical of the hind foot of the rabbit, although the same muscle in the cat may also be used.

METHODS. Certain points were not emphasized in the previous account of the rabbit lumbrical preparation and will be described here. The medial lumbical is sometimes vestigial or absent on one side, but rarely on both sides, so that at least one preparation can usually be obtained from each animal. Three lumbricals are present in each foot, but only the medial of these is generally thin enough to allow adequate oxygen diffusion and at the same time strong enough to operate a lever to record its contractions.

This muscle is cylindrical in shape; it is about 18 mm. long and 1 mm. in diameter, and has a wet weight of about 15 mgm. In order to record its contractions with a direct lever system the following precautions were taken. The lever bearing was chosen for minimal

friction. The aluminum foil writing tip was joined to the end of the lever at right angles, pointing downwards and towards the smoked surface so that when the muscle contracts and pulls the lever upwards, the tip tends to be carried away from the smoked surface; friction is neglible and does not reduce the excursion of the lever. The actual recording is made as the lever returns to the rest position. The lever was weight loaded to stretch the muscle optimally and to ensure a linear relation between the height of the recording and the work done on the lever by the muscle. Lever movement was relatively slow and took place almost entirely after muscular contraction was complete.

The nerve filament supplying the muscle is very fine and extremely sensitive to tension. For this reason it is undesirable and in any case unnecessary to remove all the connective tissue which surrounds it as it enters the muscle, since this acts as a valuable support. In spite of the utmost precautions in dissection, however, it was often found that when the preparation was first set up there was a partial neuromuscular block, which recovered completely in a few minutes. The extent and duration of this block are related to the time taken in dissection, and are presumably due to anoxia. In the present experiments, those preparations in which any severe degree of residual block remained were discarded, since permanent damage due to trauma or anoxia was assumed.

The muscle bath, thermoregulation system and electric stimulator were described in previous work on the rat diaphragm (Holmes, Jenden and Taylor, 1951). The electrodes have been modified to some extent; the platinum wire which acted as both upper direct electrode and connection to the recording lever has been replaced by silk, since the slightest bend in the wire absorbed too much of the energy provided by contraction, and the recording was thereby rendered inaccurate. The upper direct electrode now consists of a platinum ring which surrounds but does not touch the upper end of the muscle and is placed just beneath the fluid meniscus. The preparation was stimulated alternately through the direct and indirect electrodes in order to obtain a running control to indirect stimulation; the total stimulus rate was eight per minute. The usual height of the record was 5–10 cm.

The preparation was found to be very sensitive to changes in ionic concentrations; a fall in the concentration of hydrogen, calcium or magnesium ions, especially calcium, induced spontaneous activity, repetitive response to stimulation, and irregularity of behavior, while altering the sensitivity to all types of blocking agents. The following solution was used throughout the experiments described:

NaCl	709	mgm./100 ml.
$NaHCO_3$	210	mgm./100 ml.
KH_2PO_4	7.4	mgm./100 ml.
KCl	35.4	mgm./100 ml.
$CaCl_2 \cdot 2H_2O$	24.4	mgm./100 ml.
$MgCl_2 \cdot 6H_2O$	4.7	mgm./100 ml.
Glucose	200	mgm./100 ml.

The glucose was added immediately before use. Double distilled water was used throughout. The gas mixture consisted of 95 per cent oxygen and 5 per cent carbon dioxide; the solution was saturated with this mixture before each experiment was started. The temperature was maintained at 38° C. The volume of fluid in the bath was 40 ml.

RESULTS. *Decamethonium.* Figure 1 shows the effect of 40 microgm. of C10. The initial effect is a reduction in size of both directly (D) and indirectly (I) elicited contractions; the latter reduction predominates so that neuromuscular block begins. This reaches its maximum while D is still reduced, then becomes less again despite the continued presence of C10 in the bath. At this stage D recovers its original size, and thereafter remains constant. Neuromuscular block now reaches a minimum and increases again; in this experiment it became complete in about three hours.

After a small dose, the depression is preceded by an initial stimulant effect. The depression itself is less profound, and neuromuscular block, which begins while D is still increased, may disappear completely before reappearing again in twenty to thirty minutes. A still smaller dose (5–10 microgm.) may show only stimulant effects at first, but these disappear after about twenty minutes and a slow neuromuscular block begins soon after. After a very large dose (100 microgm.) a picture like figure 1 may be produced, except that the initial neuromuscular block may be complete and there is no subsequent recovery from it. The response to direct stimulation recovers in the usual way.

It seems clear that C10 produces two phases of action in this preparation. Phase I consists of a block of rapid onset, associated with depression of D which may be preceded by slight stimulation. It is followed by spontaneous recovery, partial or complete, of the block and complete recovery of D, after which phase II sets in. This is characterized by a very slowly developed block without any effect on the response to direct stimulation. Concentrations of C10 which have no blocking action but only a stimulant effect in phase I are capable of producing complete block in phase II. No conditions have been discovered under which a phase I partial block may be held constant; the muscle either undergoes partial recovery and subsequent phase II block, or else becomes and remains com-

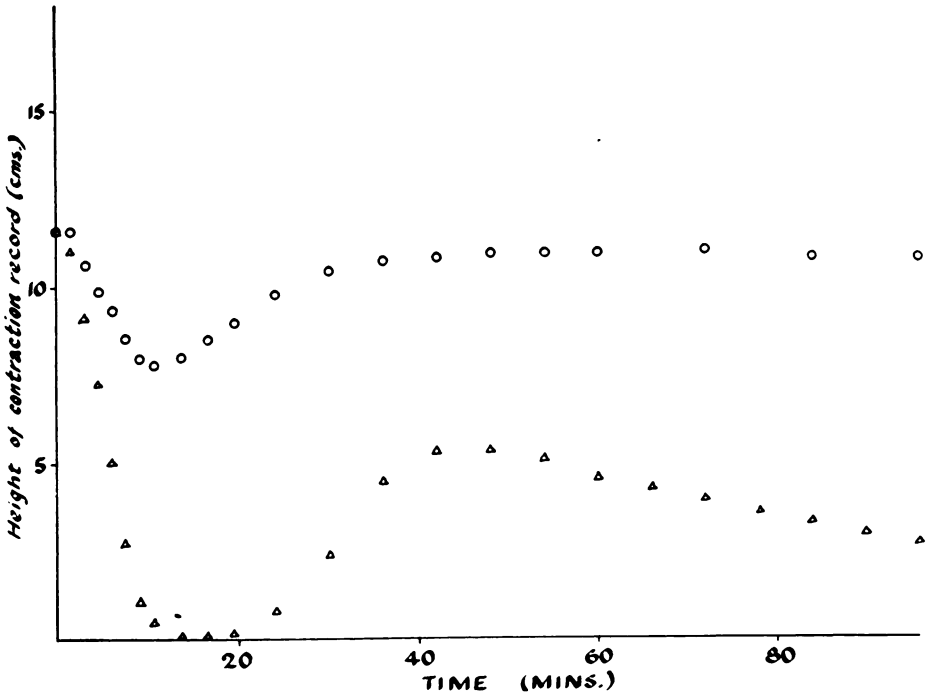

FIG. 1. The effect of 40 microgm. C10 on the rabbit lumbrical (total fluid volume 40 ml.). Circles represent response to direct muscle stimulation; triangles represent response to nerve stimulation. At three hours the preparation showed complete neuromuscular block. It recovered completely and rapidly on washing. Temperature 38 degrees C.

pletely paralyzed. On the other hand, phase II block attains a constant level if sufficient time is allowed.

Washing the preparation with fresh solution containing no blocking agent results in rapid recovery from neuromuscular block. This recovery is complete in one or two minutes if the preparation is washed after a short exposure (*i.e.* during phase I). After exposure for several hours such as is necessary to achieve a steady phase II block, recovery may take as long as 15 minutes, but is always very much faster than paralysis. In some experiments it was found that after long exposure, recovery never became complete; this was attributed to a slow deterioration of the preparation rather than to an effect of C10, since a partial neuromuscular block sometimes developed towards the end of the day when no neuromuscular blocking agent had been used.

Acetylcholine and carbachol. In view of these two definite phases of action, and the similarity of action between C10 and acetylcholine which has been reported from several sources, it was of interest to determine whether the latter drug is also capable of producing them. Clearly, in view of the fact that acetylcholine would be rapidly destroyed by muscle cholinesterase, and may fail to reach the endplate, it is essential either to inactivate the cholinesterase or to use a homologue of acetylcholine which is unaffected by the enzyme. Both these procedures were used.

Figure 2 shows the effect of 100 microgm. acetylcholine on the muscle one hour after 25 microgm. neostigmine. The sequence of events differs little from the effect of 40 microgm. C10. Acetylcholine has a relatively greater stimulant effect in phase I, and the progress through the entire complex is more rapid than in the case of C10.

Carbachol has the same type of action, the rapidity of the entire complex being intermediate between C10 and acetylcholine. A dose of about 250 microgm. is approximately equivalent to 100 microgm. acetylcholine.

Antagonists. It was observed early (Organe, 1949) that, unlike TC, C10 could not be antagonized clinically by neostigmine, but that pentamethonium (C5) antagonized the C10 block. Potassium ions, which markedly antagonize TC (Wilson and Wright, 1936; Quilliam and Taylor, 1947), have been shown to leave C10 block unaltered (Burns and Paton, 1951) in *in vivo* animal experiments. Moreover, it has been shown that a preliminary dose of TC prevents the effect of a subsequent injection of C10, both in animals (Burns and Paton, 1951; Jarcho, Eyzaguirre, Talbot and Lilienthal, 1950) and in man (MacFarlane, Unna, Pelikan, Cazort, Sadove and Nelson, 1950).

In view of these anomalous facts, it was of interest to determine the ability of these agents to antagonize the effect of C10 on the lumbrical. Since a drop of temperature to 25–30 degrees C. has been shown to reduce the potency of TC (Holmes *et al.*, 1951), this was also tested in the case of C10.

Phase I block. Neostigmine and potassium ions have no well defined effect when added during the progress of phase I block. C5 is also ineffective. On the other hand, a preliminary dose of TC too small to have a blocking action of its own is capable of reducing both the rate and the extent of phase I block; the

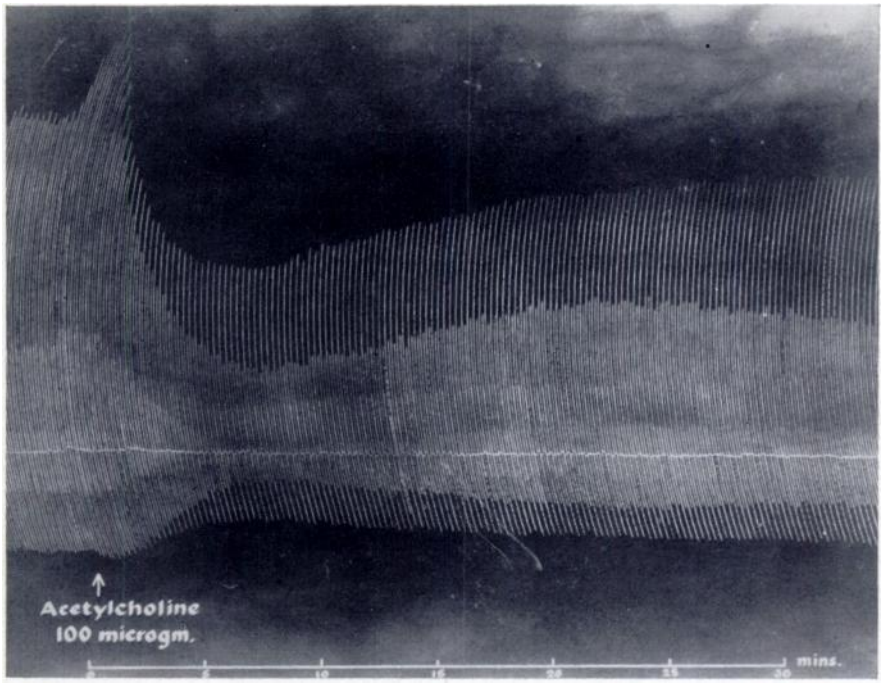

FIG. 2. The effect of 100 microgm. acetylcholine one hour after 25 microgm. neostigmine.

depression of D which is characteristic of phase I is reduced or abolished. A larger concentration of TC, sufficient to produce a partial block of its own, completely prevents phase I effects; in fact, C10 now causes a transient and slight recovery from the TC block, followed by the usual slow progressive phase II paralysis. The action of TC is complex and will not be considered in detail here.

C10 itself under certain conditions modifies the block in phase I. If after a previous dose of C10, inadequate time is allowed after washing for all the drug to diffuse out of the muscle, in spite of complete recovery from the block, another similar dose produces a different sequence of events. Instead of the usual rapid paralysis associated with muscular depression, a slower block develops which has the properties associated with phase II. There is no rapid transient block, nor any depression of the muscle. These observations correlate with the tachyphylaxis described by Burns and Paton (1951) in connection with the depolarizing effect of C10 on the motor endplate region of the cat gracilis muscle *in vivo*, and with the tachyphylaxis in human volunteers described by Pelikan *et al.* (1950). Zaimis (1953) reports decreasing sensitivity to repeated doses of C10 in monkeys, dogs, rabbits and hares (intervals not stated).

The above results apply not only to C10 but also to carbachol, and to acetylcholine after an anticholinesterase. At least one hour must elapse before the biphasic effect of carbachol can be reproduced, including at least three washes.

Three hours are necessary after C10, but only about three quarters of an hour after acetylcholine.

Phase II block. The experiments described in this section were carried out on preparations which had attained steady degrees of block; this requires six to nine hours after C10, four to six hours after carbachol and two to four hours after acetylcholine. The drug concentrations are of the order of 20–50 per cent of those required to produce a similar degree of block in phase I. Replacement

FIG. 3. The effect of 25 microgm. neostigmine on a steady block by 25 microgm. C10 introduced 7½ hours earlier. Time in minutes.

of the fluid in the bath with fresh solution containing an identical drug concentration affected neither the rate of block nor its ultimate extent.

The effect of neostigmine on a C10 block of this type is shown in figure 3. Immediate and rapid recovery occurs, which reaches a steady state after about eight minutes. The dose of neostigmine used (25 microgm. in 40 ml.) is maximal inasmuch as no additional effects were observed after a further 25 microgm. Eserine exerts an action similar to neostigmine. Potassium also exerts a marked antagonistic action in this phase. Washing out of the excess potassium results in a return to the original level of block. On reducing the temperature of the muscle bath from 38 degrees C. to 30 degrees C., the directly elicited contractions

are increased in size, and the initial effect is exaggerated; both D and I subsequently fall to a steady level, and the overall effect is a considerable lessening of neuromuscular paralysis. This effect is similar to that produced by the same temperature drop on a partial paralysis by TC on the rat diaphragm (Holmes *et al.*, 1951). On returning to 38 degrees C. the preparation reverts to its original state. In its antagonisms, therefore, phase II block by C10 differs fundamentally from phase I, and so far appears to be identical with TC block in this respect.

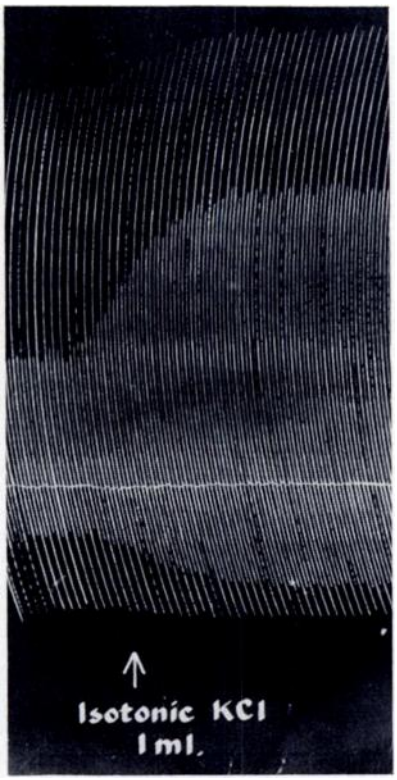

FIG. 4. The effect of 1 ml. isotonic KCl on a steady block by 50 microgm. acetylcholine, introduced four hours before; 25 microgm. neostigmine was introduced five hours before.

The degree of antagonism of potassium, neostigmine and a fall of temperature to C10 is approximately the same as that to TC. Phase II block by carbachol is also antagonized by potassium, neostigmine and a fall of temperature, while acetylcholine block in phase II is strongly antagonized by potassium (figure 4) and by a fall in temperature.

C5 has no action in phase II block by any of these drugs until total concentrations of 1–2 mgm. in 40 ml. are reached; it is then additive. TC also produces an additive effect at this stage.

Tubocurarine. In contrast to the complex actions described above, TC produces an action on the rabbit lumbrical identical with that on the rat diaphragm, viz.,

a neuromuscular block which increases regularly to a steady condition in 30–60 min., without any effect on the response to direct stimulation and without any evidence of the actions characteristic of phase I with C10.

DISCUSSION. While the above experiments show that the action of C10 on the neuromuscular junction is more complicated than most *in vivo* work has indicated, they are not inconsistent with the latter. Rather, the situation in the intact animal represents a special case of the more general effects revealed in this work. Phase I block possesses most of the well-documented characteristics of *in vivo* C10 block which have so far been investigated: the time course is short; the blocking action is preceded by brief stimulant effects which are more obvious with smaller doses; and the effects on directly elicited contractions indicate an action on the muscle fibre as well as on the neuromuscular junction. Burns and Paton (1951) have shown that when the cat gracilis is under the influence of C10, conduction along the muscle fibre is blocked at the endplate region by an area of depolarization. A phenomenon of this type might explain the reduced response to direct stimulation observed in the present work. Further characteristics shared by the *in vitro* C10 block are the failure of potassium ions and of neostigmine to exert any antagonistic action, while a previous dose of TC prevents both *in vivo* block and phase I block *in vitro*. The fact that acetylcholine can produce a closely similar picture to C10 on the lumbrical, while the two drugs have been shown to affect the electrical phenomena of neuromuscular transmission in a very similar way *in vivo* (Burns and Paton, 1951), confirms the hypothesis that it was the phase I block which was observed in these *in vivo* experiments.

Phase II block appears to have very similar pharmacological characteristics in the rabbit lumbrical to TC block on the rat diaphragm or in intact animals; in both cases a steady block can be produced; the muscle itself is unaffected; potassium and neostigmine are effective antagonists, and a drop in temperature to 26 degrees C. results in immediate recovery from the paralysis, while a return to 38 degrees C. causes reparalysis to the original level.

The obvious differences between the actions of C10 and TC, when they are observed *in vivo*, led to the early beliefs that their mechanisms of action were diametrically opposed, and that each represented a quite distinct group of compounds. However, many facts have been discovered which do not fit in with such a rigid distinction. Thus in the monkey, rabbit, hare and dog C10 produces at first the usual type of block, which rapidly changes its characteristics to resemble those of TC block; anticholinesterases are now effective antagonists (Zaimis, 1953). The same author reports that a similar change from C10-like to TC-like effects occurs both in the cat and in avian muscle after a dose of tridecamethonium (C13). The higher members of the alkyl trimethylammonium series also have a dual mode of action (Dallemagne and Phillipot, 1951); decyltrimethylammonium may produce depolarization which passes off although neuromuscular block continues (Paton, 1951). The recent work of Churchill-Davidson and Richardson (1953) shows that C10 produces a type of block similar to TC block in human beings afflicted with myasthenia gravis. On

the other hand, McIntyre, King and Dunn (1945) have observed, admittedly under very artificial conditions, that TC can produce electrical activity, contracture and muscular fibrillation, all of which are prominent features of the action of C10 *in vivo*. TC also causes depolarization in the lizard (Buchtal, 1949) and contracture in the denervated rat soleus (Jarcho *et al.*, 1950).

Similar results have recently been obtained in work on the autonomic ganglia (Paton and Perry, 1953; Perry and Talesnik, 1953). These authors found that while acetylcholine acts as a depolarizing agent, both in the superior cervical ganglion and the ciliary ganglion, C10 and C6 act as nondepolarizing blocking agents, while nicotine is first depolarizing and then "competitive."

All these observations agree well with the results described in the present work. It appears that this entire group of compounds may exert two types of action, corresponding to phase I and phase II as described above, and that the predominant action is determined by the circumstances in which the drug is given, by the structure upon which it is acting, by the species and by the properties of the drug itself.

The significance of the two phases of action. Perhaps the most striking feature of phase I block is that it recovers spontaneously in spite of the continued presence of the blocking agent in unaltered concentration. Paton and Zaimis (1952) have suggested that under certain circumstances decamethonium may inhibit its own depolarizing action. This is consistent with the observations of tachyphylaxis to C10 referred to above. Moreover, it was found in the present work that small amounts of C10 may reduce or abolish the phase I effects of a subsequent dose. However, this self-antagonism is descriptive rather than explanatory and it does not in itself clarify the mechanism of action.

Holmes, Jenden and Taylor (1951) have adduced evidence that the neuromuscular block produced by TC on the rat diaphragm is associated with a true equilibrium of the type

$$\text{Drug} + \text{Receptor} \rightleftharpoons \text{Drug Receptor Complex}$$

and not with a dynamic steady state. The present experiments indicate that similar considerations apply to phase II block by C10. It is clear, therefore, that phase I block, which appears and disappears long before equilibrium is achieved, must be associated with a kinetic effect of the drug. If phase I were associated with a similar reversible reaction with a different set of receptors, and the effects of this combination were subsequently brought to an end by antagonistic phase II effects, then phase I might be expected to reappear in response to phase II antagonists such as potassium or neostigmine, which leave phase I unaffected. No such reappearance could be demonstrated.

When the fully paralyzed muscle in phase II block is washed free of blocking agent, complete recovery ensues in a few minutes without the reappearance of phase I. In view of this it seems unlikely that phase I block is associated with a certain critical concentration of C10; indeed, if this were so, there should be a concentration which will maintain phase I block approximately constant, yet no conditions at all have been discovered in which this occurs. Burns and Paton

(1951) have suggested, on the basis of *in vivo* experiments in which tachyphylaxis was observed, that C10 and acetylcholine must be actually entering the fibre in order that depolarization should occur. It is unlikely that a charged molecule will penetrate into the fibre, and it is not necessary to postulate it in order to explain the facts, but it is clear that phase I effects, of which depolarization is one, are associated with kinetic phenomena such as penetration rate.

Holmes, Jenden and Taylor (1951) have shown that the factor limiting the rate at which TC reacts with its receptors is diffusion. If this is so for the entire group of drugs, then procedures which reduce the concentration gradient towards the receptors will reduce the rate at which the drug reacts with them. Such procedures reduce or abolish phase I effects. For example, if minute doses of C10 are introduced into the bath at frequent intervals, so that the concentration gradient at any time is kept small, the muscle slowly goes into phase II block without showing any phase I characteristics at any time. Again, a small amount of C10 remaining in the muscle after insufficient time has been allowed for its removal by washing will completely prevent the phase I effects of a subsequent dose. The spontaneous recovery from phase I might also be explained on this basis: as the blocking agent diffuses to the receptors, the concentration gradient towards them becomes smaller. The rate of diffusion, and the reaction rate which we postulate that it governs, would ultimately fall below the threshold level, so that phase I would come to an end. Burns and Paton (1951) found that intravenous C10 never produced such marked depolarization as intraarterial injection, whatever the dose; this confirms the importance of rate of access in the production of depolarization block (phase I).

The hypothesis that phase I effects are associated with a kinetic process while phase II effects can result from a static condition makes it unnecessary to postulate two sets of receptors or a "change in grip" of the drug molecules upon the receptors, as Paton and Zaimis (1952) have done, since the reaction with the receptors could be responsible for phase I and their occupation in sufficient numbers result in phase II. There is no reason to suppose that acetylcholine reacts with different receptors to produce the same effect (depolarization) when externally applied in phase I and when normally liberated in neuromuscular transmission, and it is generally accepted that TC, which only causes phase II effects under usual conditions, exerts its effects by combining with the same receptors to which acetylcholine normally attaches itself. These considerations imply that the same receptors are involved in phase I as in phase II.

If the same receptors are involved in the two phases of C10 type of action as in TC action, we might expect the kinetics of phase II paralysis and of TC paralysis to be similar, whereas in fact TC produces a steady block much more rapidly, implying that TC reaches its site of action more rapidly than C10-like drugs. Large, bulky molecules in general act more like TC while smaller, thinner molecules resemble C10 in the block they produce (Bovet *et al.*, 1951), especially when agents with close chemical similarity such as homologous series are considered (Randall, 1951 and 1952; Dallemagne and Phillipot, 1951; Ginzel, Klupp and Werner, 1951). Since the diffusion coefficient is generally inversely

related to molecular size, the difference in paralysis kinetics cannot be satisfactorily explained by a more rapid diffusion of TC to the same site. It is possible, however, that the receptors are disposed throughout a three-dimensional structure rather than on a surface; such an arrangement is *a priori* at least as probable as a surface distribution. If diffusion through this structure were highly dependent upon molecular size, the larger molecules might exert their action only upon the surface, while smaller molecules could penetrate slowly into the receptor mass and require longer to reach a steady state as a result. Such a situation would be analogous to a particle of synthetic ion exchanger, in which the diffusion coefficient of larger ions may be less than 10^{-5} times that of smaller ions in the same series (Gregor *et al.*, 1951), and into which large molecules may diffuse so slowly that virtually only the surface takes part in the reaction (Levesgue and Craig, 1948). It is interesting to note that an ion-exchange mechanism has been postulated for quaternary ammonium ions (Ing and Wright, 1932 and 1933) and has since received support (Roepke and Welsh, 1936; Holmes *et al.*, 1948). Since those substances which take several hours to reach a steady phase II block seem to be just those which have phase I activity, it is hard to escape the conclusion that the latter may be dependent upon the ability to penetrate into the receptor mass and therefore react more rapidly and extensively with it—a conclusion which is quite consistent with those drawn earlier in this discussion.

SUMMARY

1. The actions of decamethonium and of carbachol on the isolated rabbit lumbrical muscle are described. They are divisible into two distinct phases: the first consists of a neuromuscular block of rapid onset and short duration, associated with a diminished response to direct stimulation and followed by spontaneous partial recovery; the second is a block which slowly progresses to a steady state and is not associated with an alteration in response to direct stimulation.

2. Acetylcholine produces a similar effect on a muscle which has been treated with neostigmine.

3. The first phase has many of the characteristics associated with decamethonium (*in vivo*) in sensitive species. The second phase closely resembles the actions of tubocurarine, including antagonism by anticholinesterases and potassium ions. The chief difference is a very much slower rate of onset of phase II block.

4. Evidence is presented that phase I is dependent upon a rate process while phase II is associated with a stationary or nearly stationary condition. The significance of these conclusions in relation to the mode of action of neuromuscular blocking agents is discussed.

5. Certain anomalous features of the action of decamethonium and related drugs on this preparation could be explained on the basis of a three-dimensional receptor mass. Such a structure would be analogous in some respects to a particle of synthetic cation exchanger.

ACKNOWLEDGMENTS. This investigation was supported by a grant from the Research Committee of the University of California Medical School, San Francisco.

Crystalline *d*-tubocurarine and decamethonium iodide were supplied by the Wellcome Foundation, Tuckahoe, New York.

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