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A STUDY OF THE 'DESENSITIZATION' PRODUCED BY ACETYLCHOLINE AT THE MOTOR END-PLATE

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It has been accepted for many years that acetylcholine (ACh) undergoes two kinds of reactions at the motor end-plate: it combines with a receptor molecule (which leads to an increase of ion permeability in the end-plate membrane), and it combines with a hydrolytic enzyme situated side-by-side with the receptor. Both reactions probably proceed in two steps and involve the formation of unstable intermediate compounds before the hydrolysis, or the depolarizing reaction, occurs (see, for example, Augustinsson, 1948; Ariëns, 1954; Stephenson, 1956; del Castillo & Katz, 1957*b*). Competitive inhibitors are presumed to act by forming a relatively stable compound, either with the receptor or with the esterase.

These simple concepts have helped to explain the action of many end-plate drugs, and also to elucidate the apparently complex interactions between different depolarizing agents, for example, between ACh and decamethonium (del Castillo & Katz, 1957*b*), or ACh and edrophonium (Katz & Thesleff, 1957). However, the picture of two simple, parallel events at the end-plate fails to account for an important secondary effect of ACh and its depolarizing analogues, namely the profound desensitization which develops when the drug concentration is maintained for a sufficiently long time. Recent experiments (Thesleff, 1955) have shown that the neuromuscular block produced by ACh and by its stable counterparts (C_{10}, succinylcholine) is due mainly to desensitization, that is, a condition in which the end-plate has become refractory to depolarizing agents, and from which it recovers only slowly after complete withdrawal of the drug. It has been suggested that this change arises from gradual transformation of the drug-receptor compound into an inactive form. In order to obtain some information on the kinetics of desensitization and recovery processes, we have used the ionophoretic micromethods described by Nastuk (1953) and by del Castillo & Katz (1955*a*). It seemed possible that the time course of the events might come well within the practical range of this method, and the results described in this paper have borne out this expectation.

METHODS

The technique has been fully described by del Castillo & Katz (1957*a*). Use was made in most experiments of twin micropipettes, with an additional central 'spacing' barrel, the two outer barrels being filled with a concentrated solution of ACh or of a stable choline derivative.

Superficial end-plates of the frog sartorius were used, at a temperature of about 20° C. The membrane potential of the muscle fibre was recorded by inserting a separate micro-electrode within a few hundred microns of the point of drug application. To diminish the risk of twitching, the spike threshold was artificially raised in most experiments by using a high calcium concentration in the Ringer's solution (9 mM-$CaCl_2$). Control experiments were made with normal Ringer's solution (116 mM-NaCl, 2 mM-KCl, 1·8 mM-$CaCl_2$) and also with phosphate-buffered Ringer's solution (about pH 7) which showed that the drug effects were not altered in any obvious way by the special composition of the calcium-rich Ringer's solution.

The experimental procedure was to move the drug pipette to an effective position at which the application of a brief, positive, voltage pulse to the pipette resulted in a transient depolarization of rapid time course (cf. del Castillo & Katz, 1955*a*). Having located such a spot, the sensitivity of the receptors was tested by applying a series of brief pulses of constant intensity, repeated every 1–2 sec. When the responses were found to be sufficiently stable, a 'conditioning' dose was added, consisting usually of a prolonged steady release of the drug from the other barrel. It was applied by closing a switch which reduced the 'braking' voltage on the 'conditioning' barrel by a known voltage step (of the order of one or a few tenths of a volt). The amplitude of the test-responses showed a gradual decline during the steady drug application and recovered after the withdrawal of the conditioning dose. Further details and modifications of this method will be described at the appropriate places below.

Compared with more conventional forms of drug application, the present method has a number of drawbacks as well as advantages. Complications arise from the localized nature of the application and from uncertainties about absolute and relative quantities discharged by the ionophoretic pulses. These difficulties have already been discussed elsewhere (del Castillo & Katz, 1955*a*; 1957*a*), but they appear with even greater force when one attempts to use the present results for a quantitative kinetic analysis. Unavoidably, receptors at different distances from the pipette are subjected to different drug concentrations, and this circumstance, together with the non-linear dose-response relation indicated by Fig. 9, makes a proper evaluation of some results very difficult. Secondly, the relation between the dose and the applied voltage, or the current flowing through the pipette, is bound to deviate from direct proportionality so that even relative amplitudes of the doses cannot be stated with any degree of precision. It follows, therefore, that a theoretical analysis of the results cannot be taken very far and will have to be restricted mainly to a discussion of general trends.

On the other hand, the method of local microapplication offers great technical advantages. It is very rapid; diffusion times are reduced to a fraction of a second, and much faster events can therefore be studied than before. Moreover, the removal of the drug is automatic, there is no need for periods of washing or long rests between successive tests, and many different applications can therefore be made to one receptive area. When the response of a given spot finally deteriorates, it is often only necessary to move the drug pipette to another spot on the same end-plate in order to repeat or continue the experiment.

RESULTS

Fig. 1 illustrates the type of effect investigated in these experiments. The records in the upper part were obtained in a preliminary experiment in which a *single* ACh pipette was used for the application of the test pulses as well as of the conditioning dose. The records show that the depolarization produced by a

steady efflux of ACh is not maintained, and that, immediately after the withdrawal of the conditioning dose, the effect of the test pulses is greatly reduced and then gradually recovers.

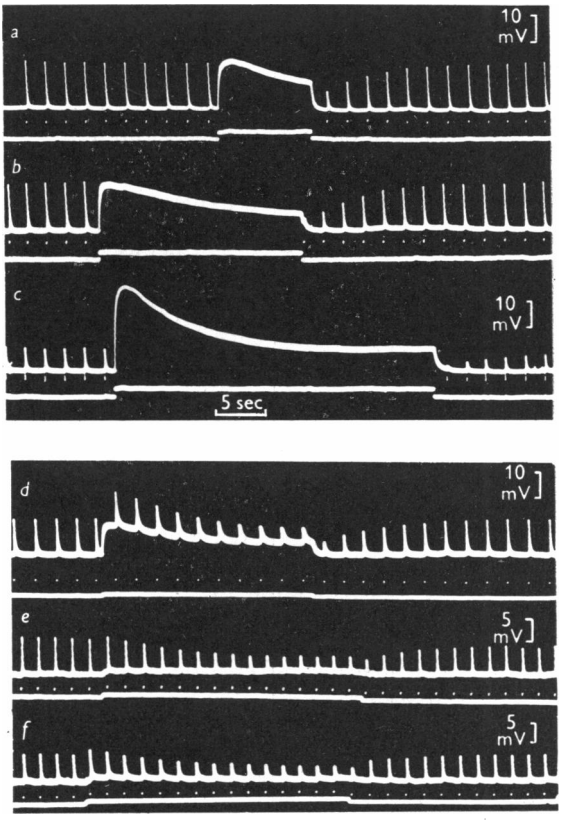

Fig. 1. Desensitization and recovery of end-plate receptors. Upper part: single ACh-pipette. Brief test doses were applied before and after the steady conditioning dose. Currents through pipette are registered in lower trace of each recording. (Calibration of monitor trace: *a* and *c*, voltage scale = $1{\cdot}2 \times 10^{-8}$ A; *d*, $3{\cdot}1 \times 10^{-8}$ A; *e*, 10^{-8} A; *f*, $1{\cdot}6 \times 10^{-8}$ A.) Lower part: twin-pipettes were used, test pulses and conditioning currents being passed through separate channels.

The single pipette technique has certain disadvantages. Test pulses must be stopped while the steady 'brake' is reduced, because the dose delivered by the pulse increases, and may lead to twitching, when the braking current in the same pipette is lowered (see del Castillo & Katz, 1955*a*). In the absence of test pulses, however, one has no direct indication of the progress of desensitization. The time course of the slow potential change produced by the conditioning dose itself does not provide a reliable measure, because it arises from an action

on a more diffuse area, and at a lower average concentration than that affecting the near-by receptors whose recovery is being tested by brief pulses.

To overcome such difficulties, twin pipettes were used, test and conditioning doses being delivered by separate barrels. Examples of double-barrel applications are shown in the lower part of Fig. 1. These records give a better indication of the onset of desensitization. It is interesting that marked depression of the pulse response occurred even with conditioning doses which caused only a small steady depolarization, of the order of 1 mV. This was regularly observed, provided the drug pipette had been closely applied to a sensitive spot.

Figs. 2 and 3 show examples from two experiments in which conditioning doses of varying intensities were applied to a receptor spot. The degree and speed of desensitization, as revealed by the test pulses, increase with the dose, while the recovery time is not greatly altered. Series of records of this kind were obtained in about twenty experiments, and an attempt will be made to analyse the relations between dose, final intensity of desensitization, and time course of onset and recovery.

Even with the double-barrel technique it was clear that pulse responses did not sum directly with the depolarization produced by the steady dose. With weak conditioning currents there was often more than simple addition, the pulse response showing a small initial increase of its amplitude and a slowing of its time course. This effect will be examined in some detail below. In spite of this complication, there was no real difficulty in measuring the rate at which successive pulse responses declined during relatively weak conditioning doses.

Desensitization started sometimes after a delay of a few seconds, though frequently the decline followed an approximately exponential curve from the start. The steepest part of the curve was chosen for determining the half-time of the process.

When large conditioning doses were applied, the measurement was more difficult. This is because test responses superimposed on large depolarizations suffer more serious distortion, due to saturation effects of various kinds (electrically, the local depolarization is limited in extent (del Castillo & Katz, 1954; Martin, 1955), chemical saturation of receptors being an additional factor). When high-speed records were made of the test responses, it was found that during a large ACh-depolarization the pulse potentials were not only smaller but considerably slower than normally, and the slowing continued to progress during the conditioning period. Immediately after withdrawal of the conditioning dose, the test response was shortened in time course and further reduced in height before recovery took place. A probable explanation of these complicated phenomena is that the shape of the test response, superimposed on a large drug dose, is dominated increasingly by contributions of more distant receptors which are subjected to a more

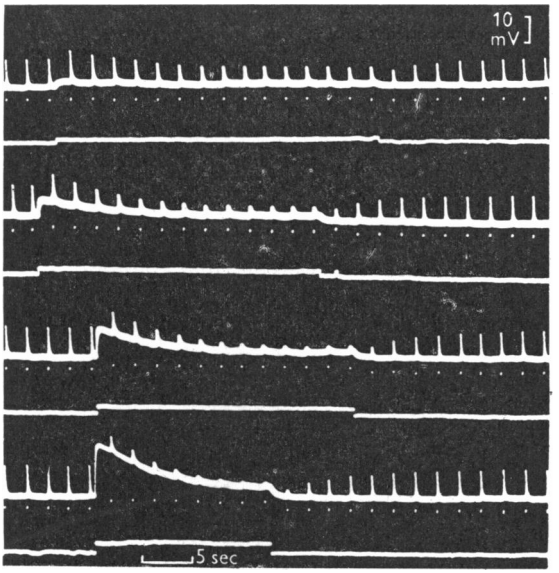

Fig. 2. Desensitization produced by different conditioning doses of ACh, at a single end-plate spot. Strength of dose increases successively from above down. Monitor calibration (10 mV scale) = $1{\cdot}2 \times 10^{-8}$ A.

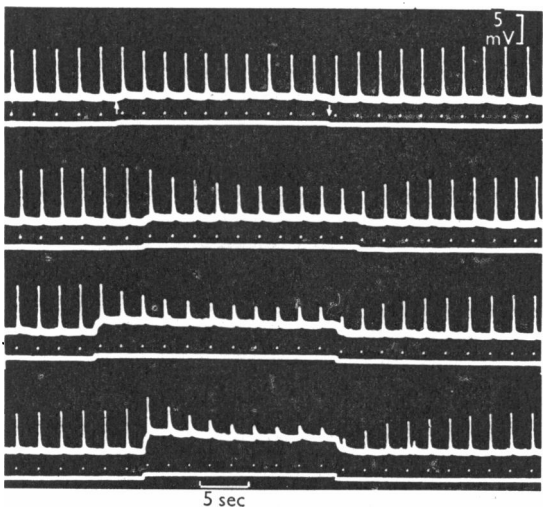

Fig. 3. Desensitization by different doses of ACh, from another end-plate. Monitor calibration (5 mV scale) = $1{\cdot}4 \times 10^{-8}$ A. Arrows indicate duration of conditioning dose in top record.

moderate concentration, and therefore do not suffer the same degree of saturation or desensitization as the close-range receptors (see also del Castillo & Katz, 1955*a*). It is clear in the cases illustrated in Figs. 4 and 5 (lower part) that desensitization becomes nearly complete within less than 8 sec, but the half-time of the process cannot be derived accurately from the superimposed test responses.

The method adopted in most experiments was to use superimposed test responses only with weak and moderate doses, with which the first test response, immediately after the start of the conditioning current, was not greatly diminished. With larger doses the time course of desensitization was determined by varying the duration of a conditioning dose of given intensity

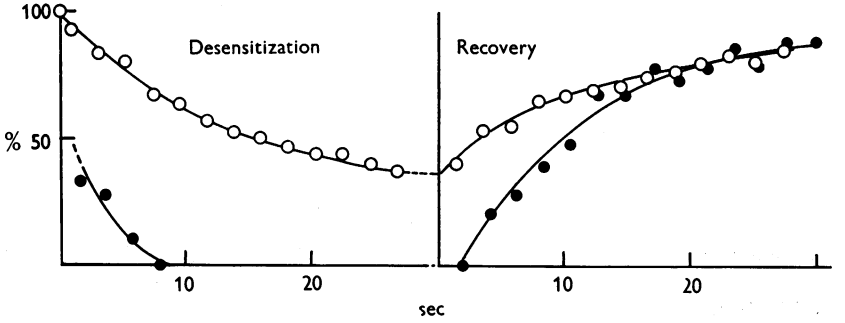

Fig. 4. Amplitude of test responses, during and after application of two conditioning doses. Initial depolarization produced by conditioning dose was 3·4 mV (○) and 20·7 mV (●).

and observing the test response immediately after its withdrawal. Records and plotted results are illustrated in Figs. 5 and 6. When the two methods of determining the time course were checked against each other, with intermediate conditioning doses, reasonable agreement was found. Checks were also made, using different, low range, intensities of *test* pulses with a given conditioning dose; these showed no significant difference in the time courses.

An example of the quantitative information derived in this way is shown in Table 1. Although the accuracy of these measurements was low, certain features were consistent:

(i) Desensitization was noticeable with depolarizations of the order of 0·5 mV and became nearly complete with doses which produced depolarizations of the order of 10 mV.

(ii) The time course of recovery appeared to be independent of the conditioning dose or the degree of desensitization. Occasionally, however, and especially after prolonged large doses, recovery was slowed or incomplete. Even when the initial rate of recovery was high, it often failed to reach completion, and there was always a tendency of the test responses to decline during successive sets of observations. Whether this was due to a slow second

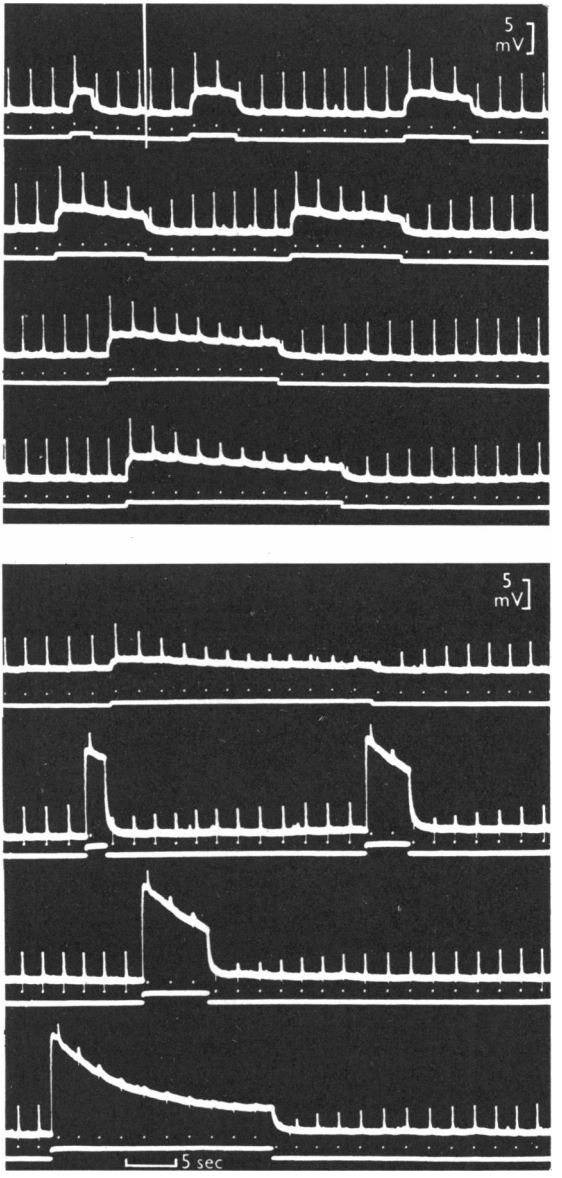

Fig. 5. Alternative method of determining time course of desensitization, by varying the duration of a conditioning dose of given strength. Two examples are shown. The development of desensitization is seen by the reduction in amplitude of the test response, immediately after the end of each conditioning period. In the lower part, the effect of a weaker dose is shown for comparison (top record).

component of the recovery process could not be decided with the present technique. The average half-time of recovery in these experiments was about 5 sec.

(iii) The time course of onset showed an interesting relation to the dose. With moderate doses which led to a final desensitization of 20–70%, the half-time of development was of the same order as, and often slower than, that of recovery (e.g. Fig. 1*e*). With large doses, however, which led to nearly complete suppression of the response, the rate of development exceeded that of the recovery, half-times being of the order of 1–2 sec.

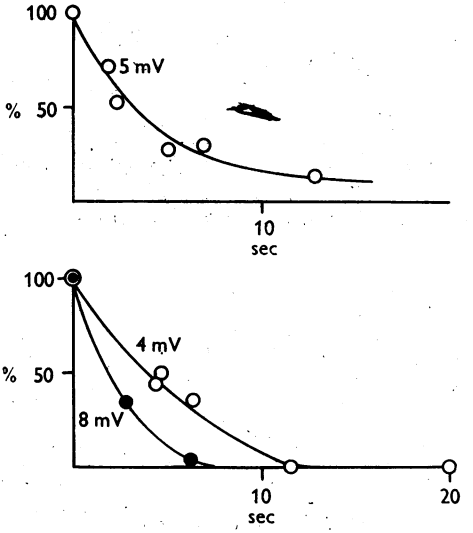

Fig. 6. Examples of results obtained by the method illustrated in Fig. 5. Ordinates: relative amplitude of test response, immediately after a conditioning period. The amplitude was measured either directly or, when necessary, by extrapolating the recovery curve to the falling edge of the conditioning potential. Abscissae: duration of conditioning dose. Initial depolarization produced by the conditioning doses is shown on the curves.

TABLE 1. Effects of varying conditioning doses

Eight different doses were used, applying positive voltage steps of 0·15–1·1 V to the conditioning barrel.

Initial depolarization produced by conditioning dose (mV)	Reduced amplitude of test response (% of 'unconditioned' response)	Half-time of onset of desensitization (sec)	Half-time of recovery (sec)
ca. 0·2	93	—	—
ca. 0·3	75	6·7	*ca.* 2·4
ca. 0·6	50	7·2	*ca.* 2·5
1·45	22	4·5	2·5
4·4	*ca.* 8	2·4	4·8
10	*ca.* 2	*ca.* 1·5	*ca.* 4·8
15·6	0	*ca.* 1	3·5
24	0	*ca.* 0·7	*ca.* 3·2

In addition to acetylcholine, carbachol and succinylcholine were used as depolarizing drugs and were found to possess very similar actions, with similarly fast time courses of desensitization and recovery. Several experiments were made on denervated muscles, the nerve having been divided 3–4 weeks previously. These experiments did not reveal any unusual features; critical spots of high drug-sensitivity could be located in the 'neural' regions, and fast desensitization and recovery effects were observed similar to those in normal muscle.

One of the surprising features was the rapidity with which the desensitization developed and disappeared in these experiments. Before accepting this finding, a search had to be made for artifacts which might possibly simulate such results. The observations clearly did not depend on any peculiarity of the twin-pipette, for substantially the same effects were obtained with single, as well as with two separately manipulated, drug pipettes. It was further considered whether an undetected local contraction might produce results of this kind. This seemed very improbable, but the possibility remained that, during very localized depolarization of receptive membrane spots, minute movements could occur in this critical region, too small to detect visually and yet influencing the sensitivity by altering the distance between receptors and pipette. There are several reasons for regarding a movement artifact as extremely unlikely and as incapable of eliciting results of the consistency observed here. An important point was that very similar results were obtained under conditions in which contractility is known to be greatly reduced or absent. Thus, in muscles placed in a solution made hypertonic by adding about 0·2 M-NaCl to the Ringer's solution, typical desensitization and recovery curves were observed, although contractile responses are known to be very weak under these conditions (Hodgkin & Horowicz, 1957). Even more decisive were experiments made in an isotonic K_2SO_4 solution (cf. del Castillo & Katz, 1955*b*) in which the fibre membrane had been completely depolarized. With the help of an additional internal electrode, 'catelectrotonic' potentials (inside-positive) were produced, and ACh was then applied to the end-plate in the usual way, causing a 'depolarization' of the electrotonic potential. An example is shown in Fig. 7, which confirms earlier observations (del Castillo & Katz, 1955*b*) and also illustrates the fact that desensitization still occurs at approximately the same speed as in normal Ringer's solution. These observations were made under conditions, and in a range of membrane potentials, well outside those at which a mechanical response can be obtained from a frog's sartorius muscle. The suggestion of a contraction artifact can therefore be safely dismissed.

The non-linear dose–response relation

It was noticed that a test pulse, superimposed at the beginning of a small conditioning depolarization, produced often *more* than a simple additive effect. Although this phenomenon was not very conspicuous, it seemed of considerable interest. We thought at first that it might have arisen from an artifact, namely, possible electric leakage between the two barrels, in spite of the use of an intermediate 'spacing' tube (see del Castillo & Katz, 1957*a*). To eliminate this possibility altogether, the experiments were repeated with two separate pipettes, and even then the effect could still be obtained. It appears then that a steady small dose of ACh can in some way facilitate the action of a superimposed ACh-pulse, or in other words that the relation between dose and depolarization has an 'S-shaped' rather than linear start. This cannot be explained by the excitatory properties of the fibre membrane, for the phenomenon was observed with potential changes well below the range in which the non-linear effect of a local response becomes conspicuous; moreover, the

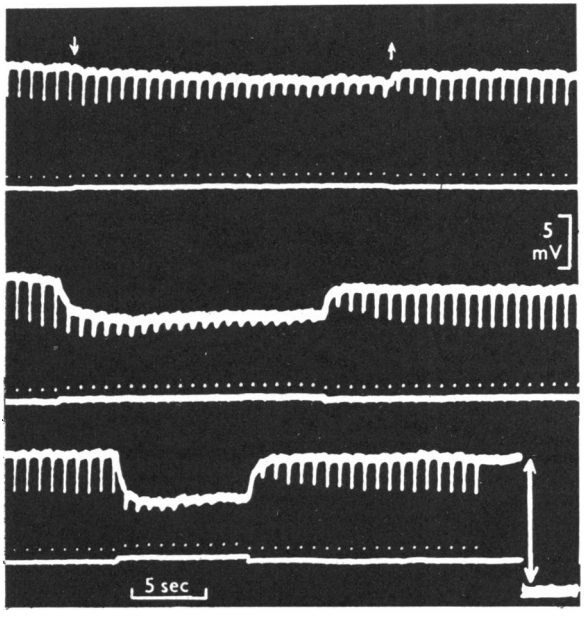

Fig. 7. ACh-desensitization and recovery, observed in isotonic K_2SO_4. The membrane potential had initially been displaced electrotonically, from near zero to about 15 mV, inside positive. At the end of the bottom record, the electrotonic current is withdrawn. In this figure, ACh responses are 'inverted', because the membrane potential is of opposite direction to that in the other experiments. Conditioning doses of three intensities are used, causing the usual effects. The conditioning period in the top record is indicated by arrows. Monitor calibration (5 mV scale) $= 1{\cdot}1 \times 10^{-7}$ A.

phenomenon was most noticeable at the time of withdrawal of a conditioning dose when the depolarizations were considerably smaller than at the beginning of the dose.

A more-than-linear summation of this kind might conceivably be due to partial saturation of cholinesterase activity by the steady dose, but this was not a likely explanation because the phenomenon was still seen in experiments in which carbachol was used (with two separate pipettes), and after the preparation had been treated with neostigmine 3×10^{-6}. Particularly clear-cut examples of this kind are shown in Fig. 8.

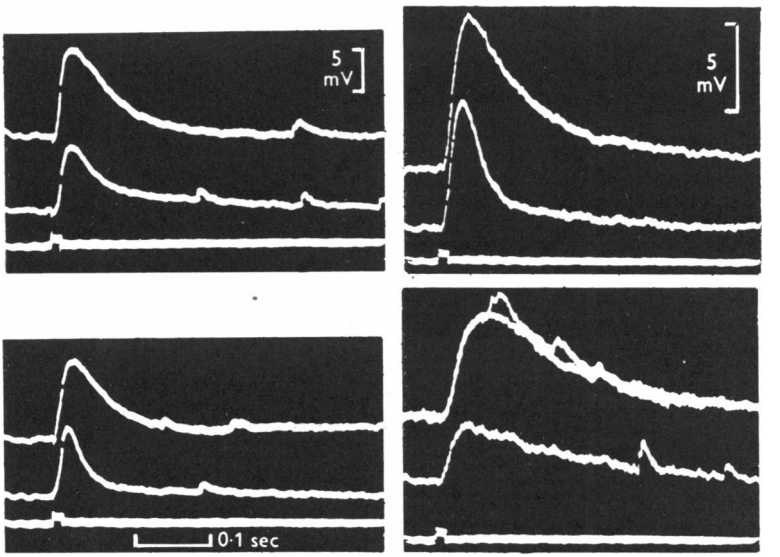

Fig. 8. Effect of adding steady and pulsatile doses of carbachol. Two separate drug pipettes. Neostigmine methylsulphate 3×10^{-6}. In each record, two successive traces were obtained, with and without a steady drug depolarization produced by steady release of carbachol from one pipette. In the right half of the figure, the upper pair of traces was observed during initial application of the steady dose, the lower pair during its withdrawal. Monitor calibration (left 5 mV scale) $= 7{\cdot}3 \times 10^{-8}$ A for all records.

Further evidence was sought by examining the relation between the strength of a carbachol pulse and the amplitude of the depolarization produced by it. This cannot be done with a single drug pipette because departures from linearity might then be ascribed to the properties of the pipette and to variation of the transport number with the intensity of the pulse. Our procedure was to use a twin-pipette and apply approximately equal doses from each barrel, separately and simultaneously. Starting with very small doses, the dose-response relation was traced in increasing steps. The observed relation had an 'S shaped' rather than linear start, there being more than addition of

effects for small pulses, and less than linear summation for large pulses (Fig. 9). The shape of the relation cannot be explained by subthreshold membrane excitation, for this would cause an upward curvature near threshold, at depolarizations of more than 30 mV.

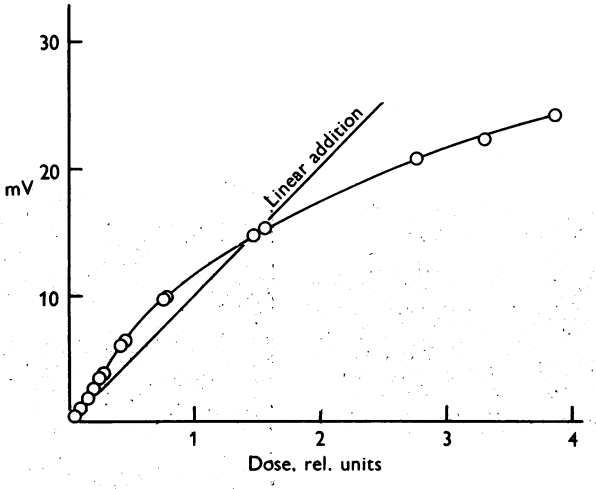

Fig. 9. Dose-response relation, obtained by an 'increment' method, using brief pulses from a twin carbachol pipette. Starting with a very small depolarization from one pipette (lowest plot), approximately the same dose (producing approximately the same depolarization) was applied by the other pipette. The response to *combined* application was plotted, as ordinate, against the *sum* of the single responses (abscissa). The procedure was then repeated with larger doses, each time choosing a step which was within the already determined portion of the dose-response relation. In this way, the relation was traced up to a depolarization of 24 mV, and followed by a reverse series. The 45° line corresponds to linear addition of effects.

DISCUSSION

The desensitization effects described in this paper are qualitatively similar to those previously obtained with the more usual methods of drug application, but differ quantitatively, especially in their time scale. Both onset and recovery are fast compared with the time courses observed before, and it is indeed one of the advantages of the ionophoretic method that it is capable of following events of such rapidity. It has been shown by Fatt (1950) that the rate of development of the desensitization process increases markedly with the drug concentration, and one might therefore suppose that the localized concentration attained with the ionophoretic technique is higher than usually employed with bulk applications of ACh, even though the resulting membrane depolarization in our experiments was less. The value of the depolarization is of little use in estimating the drug concentration, for with highly localized application a given potential change of the fibre membrane may arise either from a low drug concentration acting over a wide area, or from a high con-

centration acting on a small area of receptors. It is, however, of interest that appreciable desensitizations were obtained with doses which produced little more than 0·5 mV initial depolarizing effect, that is, a potential change of the same order as the amplitude of a brief miniature e.p.p. There is good evidence for the view that a miniature e.p.p. results from the most localized form of 'ACh-application' which can possibly be achieved, and one may therefore conclude that the ACh concentrations which were used in some of our desensitization experiments were not outside the range of concentrations which occur normally. The 'unphysiological' feature in our experiments is, of course, the prolonged maintenance of the ACh concentration, for seconds rather than milliseconds.

While the rapid onset of desensitization might be attributed to the attainment of high local drug concentrations, the fast time course of recovery cannot be explained in this way. It may be that with the usual prolonged 'bulk' application protracted diffusion, into and from the deeper layers of the tissue, slows the recovery of superficial end-plate receptors and disguises the existence of a fast component. On the other hand, our present method while capable of revealing a fast phase of recovery, is unsuitable for the detection of very slow changes (with a time scale of minutes rather than seconds), which might be disguised by a minute drift of the pipette location. It is possible, therefore, that the present experiments merely disclose a fast phase of the recovery process which is complementary to the results of previous work.

Ideas about the mechanism of desensitization are at present bound to remain speculative. Nevertheless, it is of interest to try certain simple hypotheses and see whether they can be fitted to the observations. Del Castillo & Katz (1957*b*) had put forward the following working hypothesis:

$$\mathrm{ACh} + \mathrm{R} \underset{\text{inter-mediate step}}{\overset{(1)}{\rightleftharpoons}} \mathrm{AChR} \overset{(2)}{\rightleftharpoons} \underset{\text{depolar-ization}}{\mathrm{AChR'}} \underset{\text{desensiti-zation}}{\overset{(3)}{\longrightarrow}} \mathrm{ACh} + \mathrm{R'}, \qquad (1)$$

where R is the receptor molecule in its initial, reactive, form. Reaction (1) is a postulated intermediate step which precedes the 'depolarizing' reaction (2). We are not, at present, concerned with the evidence bearing on the existence of the intermediate compound, and for simplicity the depolarizing reaction will be treated as a single reversible process, of sufficient speed to be at equilibrium during the relatively slow desensitization. Del Castillo & Katz suggested that desensitization results from gradual transformation of the receptor into a non-reactive form R′ which reverts slowly to R after the withdrawal of the drug. This is one of several simple hypotheses which may be put forward.

The following alternative schemes have also been considered (symbols: *S*,

conditioning drug concentration; A, free receptors; SA, 'effective' drug-receptor compound; SB, 'refractory' compound).

$$S+A \underset{}{\overset{\text{Fast}}{\rightleftharpoons}} SA \overset{\text{Slow}}{\rightleftharpoons} SB \tag{2}$$

$$S+A \begin{cases} \overset{\text{Fast}}{\rightleftharpoons} SA \\ \overset{\text{Slow}}{\rightleftharpoons} SB \end{cases} \tag{3}$$

These schemes represent two successive (2), or simultaneous (3) reactions, a depolarizing reaction which reaches equilibrium very rapidly, and a desensitizing reaction which proceeds much more slowly.

These hypotheses lead to certain common predictions, namely that the final degree of desensitization I is given by an equation of the type

$$I=\frac{1}{1+\frac{k_2(1+aS)}{k_1 aS}},$$

and its development and decay follow exponential time courses with rate constant

$$\frac{1}{\tau}=\frac{k_1 aS}{1+aS}+k_2,$$

k_1 being the forward, k_2 the reverse rate constant of the desensitizing step; a being the affinity constant of the fast depolarizing step. For the case of two parallel reactions ((3) above), k_1 should be replaced by k_1/a.

The significant result is that on these hypotheses, the *onset* of desensitization (when S is finite) must always be faster than the recovery (when $S=0$), and that complete, or nearly complete, desensitization would occur only when the rate of onset is infinitely, or very much, faster than that of recovery. For 50% desensitization, the rate of development should be twice that of the recovery.

These predictions are clearly at variance with what has been consistently observed. Half-desensitization was found to develop at a rate about equal to, or lower than, that of the subsequent recovery. At doses which produced nearly complete desensitization, the onset was usually faster, but not more than five times faster, than recovery. These schemes, therefore, cannot be accepted as suitable working hypotheses. What is apparently needed is a reaction in which the recovery process, from B to A, is slowed by the presence of the drug.

A scheme which will fit the results reasonably well is the following

$$\begin{array}{ccc} S+A & \overset{a}{\rightleftharpoons} & SA \\ \text{(slow) } k_2 \uparrow & \text{(fast)} & \downarrow k_1 \text{ (slow)} \\ S+B & \underset{\text{(fast)}}{\overset{b}{\rightleftharpoons}} & SB \end{array} \tag{4}$$

where a and b are affinity constants.

On this hypothesis, the receptor can exist in two forms, effective (A) and refractory (B). The drug combines rapidly and reversibly with both forms, but the combined receptor is transformed irreversibly from A to B with rate constant k_1, while the free receptor reverts to form A with rate constant k_2. (A system of this kind would require energy supply in order to be maintained in a steady state: e.g. if the change from SA to SB involves degradation of energy, a metabolic 'drive' would be needed for the recovery reaction.)

On this hypothesis, desensitization I is given by

$$I = \frac{1}{1 + \dfrac{k_2\,(1+aS)}{k_1 aS\,(1+bS)}}$$

and the exponential rate constant by

$$\frac{1}{\tau} = \frac{k_1 aS}{1+aS} + \frac{k_2}{1+bS}.$$

We found that the observed results can be fitted moderately well if the affinity of the drug to receptor B is much higher than to receptor A. In this case, a dose may produce relatively little depolarization and yet lead to a profound desensitization; moreover, the rate of onset may be low compared to the rate of recovery when the drug has been completely removed.

We have at present no means for a rigorous quantitative test, because the values of S are not known. If one makes the oversimplifying assumption that the initial depolarization produced by the conditioning dose is proportional to S, then the average result, from an analysis of 19 experiments, was: b/a (ratio of affinity constants) = 20 (varying between 5 and 100); $1/k_1 = 1{\cdot}4$ sec (half-time 1 sec, varying between 0·5 and 2 sec); $1/k_2 = 7$ sec (half-time 5 sec, varying between 2 and 7 sec). Examples are shown in Figs. 10 and 11.

It may be preferable to choose a reversible version of hypothesis (4), one which allows the attainment of thermodynamic equilibrium. The scheme would then be as follows:

$$\begin{array}{ccc} S+A & \overset{a}{\rightleftharpoons} & SA \\ \Big\uparrow\Big\downarrow \text{(slow) } k_3 & \text{(fast) } k_4 \quad k_3 & \Big\uparrow\Big\downarrow k_1 \text{ (slow} \\ S+B & \underset{b}{\rightleftharpoons} & SB \\ & \text{(fast)} & \end{array} \qquad (5)$$

Equilibration requires that

$$\frac{b}{a} = \frac{k_1 k_2}{k_3 k_4}.$$

Desensitization I would be given by

$$I=\frac{1}{1+\frac{k_2(1+aS)}{k_4(1+bS)}},$$

and the over-all rate constant by

$$\frac{1}{\tau}=\frac{k_1aS+k_4}{1+aS}+\frac{k_3bS+k_2}{1+bS}.$$

Our data do not allow us to discriminate between hypotheses (4) and (5). The last scheme can also be fitted reasonably well, with $k_1 \gg k_3$, $b \gg a$, and

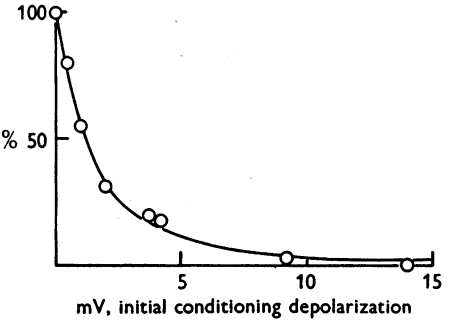

Fig. 10. Relation between final desensitization and initial depolarization produced by varying conditioning doses. Ordinates: amplitude of 'desensitized' test responses, as % of normal responses. The curve was calculated from hypothesis (4), with $b/a=10$, $1/k_1=0{\cdot}7$ sec, $1/k_2=4{\cdot}2$ sec, and $aS=1$ for 12·5 mV.

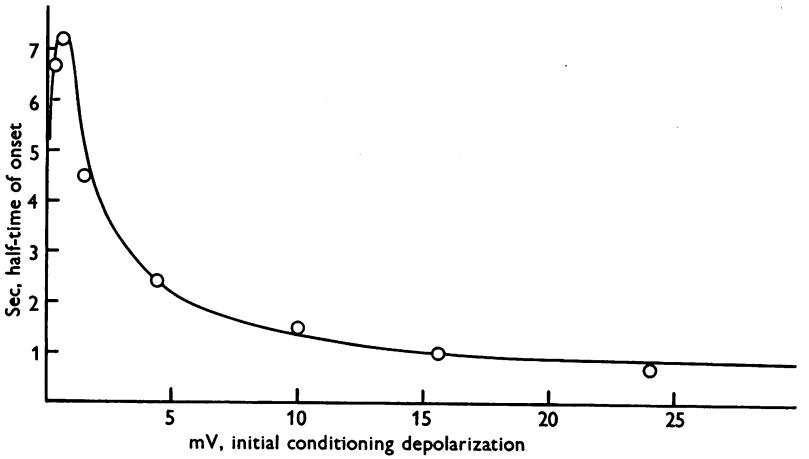

Fig. 11. Relation between half-time of onset of desensitization and initial depolarization produced by varying conditioning doses. This was an exceptionally 'good' result: usually the half-time values were scattered to a much greater extent. The curve was calculated from hypothesis (4), with $b/a=86$, $1/k_1=0{\cdot}7$ sec, $1/k_2=5{\cdot}2$ sec, and $aS=1$ for 17·1 mV.

$b/a > k_1/k_2$. An interesting feature of hypothesis (5) is that the free receptors are distributed, even in the absence of a drug, between states *A* and *B*, that is a proportion of receptors is present in a refractory form, and on account of its very high affinity ($b/a \gg 1$) will preferentially absorb small quantities of applied ACh.

To sum up, schemes of the type (4) and (5) may be regarded as possible working hypotheses. Although the available results are not accurate enough to provide a secure basis for a kinetic theory, they do at least allow us to reject certain types of hypothesis such as (1) to (3).

Some further comment is required on the finding of an 'S-shaped' dose-effect relation (Fig. 9), and the observation that a small steady dose may facilitate the action of an added pulse of the drug. An S-shaped relation could be the result of a reaction in which two (or more) drug molecules become attached to a receptor molecule, and the efficacy of the compound increases with the number of attachments.

Another possible explanation arises from scheme (5). The reaction between drug and receptor takes place presumably in the post-junctional folds of the muscle membrane (Couteaux, 1955; Robertson, 1956) whose lumen is not more than a few hundred Ångstrom units wide and which appear to be lined with receptor and esterase molecules. Applied drugs must diffuse into these spaces and react there with the surface receptors. If there are a large number of *B*-type receptors, of high affinity to the drug, but no depolarizing power, then the effect of a small dose would be mainly to occupy and partially to saturate these sites. If a second dose is added, a smaller fraction of the drug molecules would be absorbed by sites *B*, and therefore a larger fraction become available for the depolarizing action, than if this dose had been given alone.

Whatever the cause of this 'facilitation', it differs from the more conspicuous effect which certain choline derivatives produce when they are allowed to interact with ACh (del Castillo & Katz, 1957*b*). In these cases there was evidence for a specific interference with the enzymic destruction of ACh, because the potentiation was abolished and reversed by pre-treating the muscle with an esterase inhibitor, or by using stable depolarizers (carbachol or succinylcholine) instead of ACh.

SUMMARY

1. Ionophoretic microapplication has been used to study the desensitization which depolarizing drugs produce at the motor end-plate. Steady 'conditioning' and brief 'test' doses of a drug were applied, from the two barrels of a twin-pipette, to sensitive end-plate spots of the frog.

2. When a relatively small dose of acetylcholine, producing a depolarization of 0·5–1 mV, is maintained for 10–20 sec, an appreciable loss of sensitivity occurs (sometimes exceeding 50 %). Conditioning doses which produce an initial depolarization of 10–20 mV can cause nearly complete desensitization

of local receptors within a few seconds. After withdrawal of the dose, the sensitivity starts to recover with a half-time of the order of 5 sec.

3. The variations in intensity and time course of the desensitizing process have been examined with different doses, different depolarizing drugs (acetylcholine, carbachol, succinylcholine) and in different ionic environments (Ringer's solution, isotonic K_2SO_4).

4. The kinetics of the process have been discussed assuming that the receptor molecules can change from an 'effective' to a 'refractory' state.

5. The dose–effect relation has been examined for small depolarizations produced by ionophoretically applied drug pulses. It is found to have an S-shaped, rather than linear, start.

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