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THE ACTION OF ESERINE-LIKE AND CURARE-LIKE SUBSTANCES ON THE RESPONSES OF FROG'S NERVE-MUSCLE PREPARATIONS TO REPETITIVE STIMULATION

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The experiments to be described in this paper were the first step in some work which was begun in April 1935, after Dale & Feldberg [1934] had suggested that acetylcholine might be the chemical transmitter at motor nerve endings, and which has been partly summarized in previous notes [Cowan, 1936 *a*, *b*, *c*]. The work is based on the following considerations.

Several studies of electrical responses showed that, normally, a single impulse in a motor nerve fibre sets up a single propagated (all-or-none) impulse in each of the muscle fibres which it supplies, no matter whether the nerve impulse is an isolated one or one out of a short series having a frequency of 100 per sec. Therefore, if ACh. is the transmitter its action must be limited to a region small in comparison with the size of a muscle fibre. By analogy with electrical excitation it seemed probable that, at the part of a muscle fibre juxtaposed to its motor nerve ending, the concentration of the ACh. liberated by each nerve impulse would have to attain a threshold value to initiate a propagated impulse from there; also, that in the intervals between the successive nerve impulses during a tetanus the concentration of ACh. would have to fall below that threshold. The last-mentioned requirement might be achieved through the hydrolysis of ACh., probably accelerated by choline-esterase, which,

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according to Stedman *et al.* [1932], is the enzyme specific for the reaction; by diffusion into the remainder of the muscle or interfibrillar spaces; by a combination of the foregoing agencies. Numerous experiments on the production of, and recovery from, "neuro-muscular fatigue" with isolated preparations, mainly frog's, testify that a third possible agency—diffusion into the blood stream—plays no essential role.

If the hypothesis that ACh. is the transmitter were accepted, then the reversal of curare paralysis by eserine [Rothberger, 1901; Langley & Kato, 1915], by miotine [Stedman & White, 1931], and by "Prostigmin" [Aeschlimann & Reinert, 1931], could be explained in two ways. (Hereafter this last compound will be called "prostigmine" to conform with the usual chemical nomenclature.) Brinkman & Ruiter [1924, 1925] perfused frog's hindlimbs with Ringer's solution, and found that when the (mixed) nerves supplying the muscles were stimulated an ACh.-like substance could be detected in the perfusate. They also found that the liberation of the ACh.-like substance still occurred when sufficient curare to prevent the motor impulses from causing contraction was present. The implication of this experiment was that curare does not influence the liberation of the transmitter. After studying the curare-like action of a number of quaternary ammonium cations Ing & Wright [1931; also Ing, 1936] suggested that paralysis of neuro-muscular transmission involves a permutite-like exchange at motor nerve endings. Since ACh. and curarine, the active alkaloid of curare (for references see Boehm, 1920), are both quaternary ammonium cations it seemed that paralysis by curarine might be due to blocking of the region on which ACh. normally acts. (If, as the recent work of King [1935] on tubocurarine suggests, curarine is not a mono- but a di-quaternary salt the argument would be unaffected.) One explanation of the reversal of paralysis by the three compounds mentioned was that they might inhibit choline-esterase at the region of the nerve ending and so enable the liberated ACh. to compete better with curarine for the part of the muscle juxtaposed to the nerve ending. Eserine had been shown to have the power of inhibiting choline-esterase [Engelhardt & Loewi, 1930; Matthes, 1930], and miotine had been shown to have the same power [Stedman & White, 1931; Stedman & Stedman, 1932]. The alternative explanation was that the three drugs lowered the threshold, either of the whole muscle, or only of the part juxtaposed to the nerve ending, to ACh.

Since it was not clear from previous investigations how far eserine influenced nerve and muscle as well as neuro-muscular transmission

[for references and discussion see Langley & Kato, 1915] the particular aims of the first step of this work were as follows: To examine, in conditions simplified to exclude as many complicating factors as were known: (1) the actions of eserine and of prostigmine upon the responses of nerve, of muscle, of nerve-muscle preparations, to repetitive stimulation at various frequencies; (2) the actions of a number of simple synthetic compounds having certain eserine-like properties, in the hope that it would be possible to demonstrate a parallel between action on neuro-muscular transmission and inhibitory action on choline-esterase; (3) the antagonisms, where existing, between eserine and eserine-like substances on the one hand, and curarine and certain quaternary ammonium salts on the other hand. (Hereafter the expression "eserine-like substances" includes eserine itself, and similarly any reference to curare-like substances includes curarine.)

Apart from any possible action of eserine-like substances on nerve, the differences between the myograms in response to (maximal) direct stimulation of muscle and to the stimulation of muscle through its nerve appeared to provide a simple method of estimating the effectiveness of neuro-muscular transmission. It was clear that if the errors of such a method were to be kept small a muscle in which all the fibres could be stimulated directly without danger of stimulating the intramuscular nerve twigs was desirable. Whilst it was clear also that a blood supply would have been desirable to minimize muscular fatigue, and might perhaps have been arranged so as to exclude the excretion and possible metabolism of the eserine-like and curare-like substances, the contingency that the blood cells or plasma proteins might adsorb relatively large amounts of those substances and so vitiate the attempt to make the experiments quantitative could not be excluded. I decided therefore to dispense with a blood supply and to use a muscle sufficiently thin for oxygen (or drugs) to diffuse in, and for metabolites to diffuse out [Hill, 1928; Hill & Kupalov, 1929]. The frog's isolated sartorius muscle immersed in Ringer's solution heavily buffered with bicarbonate and well stirred with oxygen-5 p.c. CO_2 mixture was selected as the nearest practical approach to the requirements of this work. The isolated nerve-sartorius preparation was inferred to have the advantage also that the nerve endings were readily accessible, since large quaternary ammonium ions, including curarine, had been shown to paralyse it rapidly [Ing & Wright, 1931, 1933; Cowan & Ing, 1934].

To examine the effects of eserine-like substances on nerve two methods were selected. One of them was to measure the action currents of frog's

sciatic nerve trunks which had been soaked in sodium phosphate buffered Ringer's solution and then suspended in moist oxygen, before and after treatment. The other was to record the myograms given by the nerve-sartorius preparation immersed in bicarbonate-buffered Ringer's solution, before and after treatment of only the nerve. The possible alternative of examining the action currents of nerves that had been soaked in bicarbonate Ringer's solution and then suspended in moist oxygen-carbon dioxide mixture was rejected because the rather variable prolongation of the descending phase of the action potential produced by carbon dioxide [Waller, 1896; Cooper, 1924; Davis *el al.* 1928; Gerard & Necheles, 1930] would probably have made it difficult to be certain whether eserine-like compounds produced any further changes.

During the progress of this work a number of papers germane to it have been published. Only two need be mentioned now, the others will be discussed later. Dale *et al.* [1936] found that when the muscles of the frog's hindlimbs were perfused with Ringer's solution containing eserine, stimulation of the ventral nerve roots caused a substance identifiable, pharmacologically at least, as ACh. to appear in the perfusate. They found also that curarine in concentration sufficient to render motor nerve impulses ineffective did not inhibit the liberation of this substance during stimulation, thus rendering more precise Brinkman & Ruiter's earlier observation. Easson & Stedman [1936] have shown prostigmine to be a very effective inhibitor of choline esterase.

I am indebted to Dr E. Stedman for his kindness in allowing me to quote here some of his figures for the inhibitory actions, upon choline esterase, of the same eserine-like substances as were used in my experiments. The figures are part of an extended study which he will publish at some later time.

Materials

The frogs were English *R. temporaria* obtained in fresh batches at about monthly intervals. Except where the contrary is stated the animals had been kept for 3 days before use at a temperature which lay between 14 and 18° C. for the greater part of the time, although the extremes of 12 and 21° C. were sometimes reached. The composition of a bicarbonate-buffered, Ringer's solution which contained 0·1 p.c. of glucose and which would have a *p*H of 7·2 when at 15° C. and in equilibrium with 5 p.c. carbon dioxide mixture was calculated with the help of the Henderson-Hasselbalch equation and Fox's data [1909] for the solubility of carbon

dioxide in saline solutions. In order that the solution should be isotonic with frog muscle [Hill & Kupalov, 1930] the usual sodium chloride content was reduced by 0·016 *M*—an amount corresponding to the added sodium bicarbonate. To avoid the precipitation of calcium carbonate, which would otherwise have occurred when the solution was not in equilibrium with 5 p.c. carbon dioxide mixture, the calcium chloride was made up in a separate and concentrated solution, and before each experiment the required amount was added to a measured volume of the bicarbonate solution which had previously been saturated with oxygen-carbon dioxide mixture. Thus, 5 c.c. of solution containing 4 g. $CaCl_2$ per 100 c.c. were added to 995 c.c. of solution containing 6·0 g. NaCl, 1·34 g. $NaHCO_3$, 0·10 g. KCl, and 1·0 g. glucose. Phosphate Ringer's solution was prepared by adding 10 c.c. of 0·345 *M* sodium phosphate buffer of *p*H 7·2 to 1 l. of the following solution: 6·75 g. NaCl, 0·10 g. KCl, 0·20 g. $CaCl_2$, and water to 1 l. "AnalaR" chemicals and water distilled in porcelain stills were used for all the solutions.

I am indebted to Roche Products Ltd., London, and F. Hoffmann-La Roche and Co. Ltd., Basle, for supplies of the following eserine-like compounds: prostigmine (the dimethylcarbamic ester of 3-hydroxyphenyltrimethylammonium methylsulphate), the phenylmethylcarbamic ester of 3-hydroxyphenyltrimethylammonium methylsulphate (hereafter Substance 36), the dimethylcarbamic ester of 8-hydroxyquinoline hydrochloride (hereafter Substance 37), the dimethylcarbamic ester of 8-hydroxymethylquinolinium methylsulphate (hereafter Substance 38), 3-hydroxyphenyltrimethylammonium bromide (hereafter Preparation 1210/1), the dimethylcarbamic ester of 3-hydroxyphenylmethyldiethylammonium iodide (hereafter Preparation 3393). Aeschlimann & Reinert [1931] found that the Substances 36, 37, and 38 had certain eserine-like actions, but they did not investigate their actions on muscle, nerve, or neuro-muscular transmission. Preparation 3393 was specially synthesized for the experiments reported in this and a subsequent paper. Solutions of the foregoing compounds or of eserine were made up freshly, in either bicarbonate or phosphate Ringer solution, before each experiment and diluted further as required.

Two specimens of purified curarine chloride were used. They were portions of the preparations which Prof. C. Lovatt Evans and Dr H. King had been kind enough to give to Dr Ing and myself, and which we had used in other work [Cowan & Ing, 1934, 1935]. In preparing the solutions used in the experiments to be described here the molecular weight of curarine chloride was assumed to be 332·7, the value corre-

sponding to Boehm's [1897] formula $C_{19}H_{25}ON_2Cl$. I am indebted to Dr H. R. Ing for a sample of purified phenyltrimethylammonium iodide. Purified tetramethylammonium iodide was prepared by recrystallization from alcohol.

Methods

Nerve action current

The sciatic nerve trunks from a frog weighing 40–60 g. were dissected and allowed to soak for an hour in phosphate-buffered Ringer's solution. At the end of that time one of the nerves was mounted in a paraffin wax chamber, so that the peripheral portion lay on two filter-paper strips connected to two calomel electrodes through which the action current could be led off, and the central end on a pair of platinum wire electrodes through which stimuli could be applied. The peripheral end was crushed and a drop of potassium chloride solution isotonic with frog blood applied to it, in order to secure a monophasic response. Then the chamber was closed with a glass cover sealed on with plasticine, and a slow stream of moist oxygen started. The calomel electrodes were connected in series with a potentiometer, and, through a variable shunt, to a galvanometer which, when critically damped, had a sensitivity 1 mm. $= 2{\cdot}4 \times 10^{-10}$ amp. at 5 m. scale distance, and a period of 3·8 sec. This arrangement permitted the nerve injury potential to be balanced out and the full sensitivity of the galvanometer to be used for observation of the action current when required. Stimulation was by alternate charge and discharge of a condenser, various frequencies being obtained by a rotating commutator which could be driven at any desired speed [Hill, 1934]. The condenser, whose capacity could be varied in steps, was shunted by a resistance of 1500 ohms: a resistance of 4500 ohms was connected in series with the stimulating electrodes. In each experiment the voltage to which the condenser was charged was adjusted until the shocks were slightly supramaximal. A platinum-wire electrode interposed between the stimulating and leading off electrodes, and connected to earth, prevented stimulus escape into the galvanometer circuit. Test periods of stimulation lasting from 3 to 30 sec. and separated by 1 min. intervals were given in groups of six in order that allowance might be made for the initial treppe which occurs in the electrical response of nerve [Waller, 1896; Gerard, 1927]. After the responses to several groups had been observed, the deflexion produced by unbalancing the injury potential by 1–2 mV. was measured, to determine the sensitivity with the nerve included in the circuit. Then, in some experiments the nerve was taken out, immersed

for a time in a solution of an eserine-like substance, remounted, and a fresh injury made; in other experiments the nerve was left undisturbed on the electrodes and was merely painted over with the solution. After treatment the observations of response to test stimuli, together with the determination of sensitivity were repeated several times at half-hourly intervals. A control experiment was made with the second nerve. It was mounted in a duplicate paraffin wax chamber, which could also be connected to the galvanometer and stimulating circuits, and treated in the same way as the experimental nerve except that Ringer's solution was used instead of the solution containing an eserine-like compound.

For experiments with nerve-muscle preparations

A single sartorius with its nerve and the acetabulum was dissected from a frog weighing 20–50 g., and soaked for 1 hr. in bicarbonate-buffered Ringer's solution at 14–16° C. The preparation was then

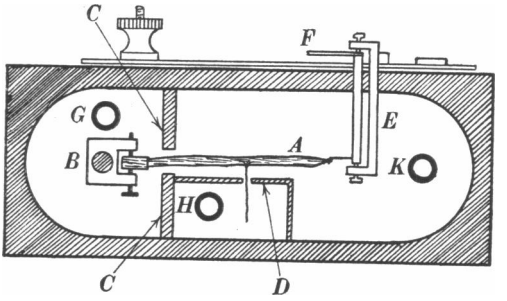


Fig. 1. Modified Lucas trough for experiments with nerve-sartorius preparation. A, preparation in position. B, pin and holder for clamping the acetabulum. C, ebonite partition with shaped hole through which the muscle passes. D, ebonite partition with hole through which the nerve passes. E, Lucas lever crank. F, steel wire connected to isometric lever. G, H, and K, zinc-zinc sulphate non-polarizable electrodes.

mounted in a modified Lucas trough which was filled with the Ringer's solution and in which partitions had been arranged to form fluid electrodes whereby localized stimuli could be applied to the pelvic end of the muscle or to the nerve (Fig. 1). The pelvic end was secured by transfixing the acetabulum with a pin which could be held in a clamp, and the tibial tendon was tied to a Lucas lever crank, the other end of which was connected, by a steel wire, to an isometric spring torsion lever. The period of the moving system, when unconnected to a muscle and when the lever was writing on a smoked paper was about $\frac{1}{30}$ sec. Usually the initial tension to which a muscle was subjected was 2–5 g.; the maximum

amount of shortening permitted to a muscle of 30 mm. length was about 1 mm. In each of the compartments of the trough a separate jet supplied the carbon dioxide-oxygen mixture which kept the bath both stirred and at the correct *p*H. In the compartment containing the muscle and the lever crank a thin-walled glass tube through which cold water could be passed served to maintain the temperature at $15 \pm 0{\cdot}5°$ C. Current could be conducted to each compartment of the trough by zinc-zinc sulphate non-polarizable electrodes. These were of the pattern devised by Lucas [1906], but modified so that any zinc salt which might leak from the filter candle would be unlikely to reach the preparation (Fig. 2). The

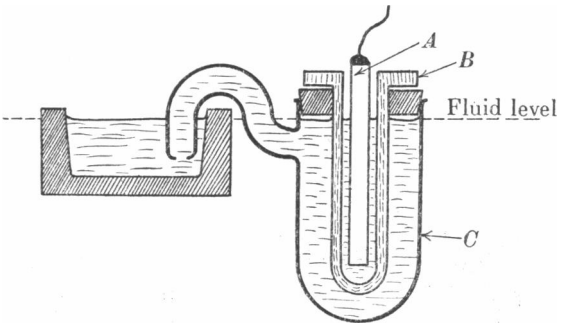


Fig. 2. Non-polarizable electrodes. A, amalgamated zinc rod, 10 mm. diameter. B, filter candle 90 mm. long, and 24 mm. in diameter filled with zinc sulphate solution. C, outer vessel filled with Ringer's solution. To prevent siphoning, the electrodes were arranged so that the menisci of the solutions in them were level with that of the solution in the muscle trough.

relatively large current required for stimulation of the pelvic end of the sartorius, and the loss of part of this current, by the shunting which is inevitable with fluid electrodes of the type used, necessitated large non-polarizable electrodes. They were of the dimensions shown in Fig. 2. Mr C. M. Fletcher, who was good enough to examine the electrodes with the amplifier and oscillograph described by him in a recent paper [1937], found that they were capable of taking 10 mA.—a current considerably greater than was used in my experiments—without appreciable polarization, provided that the zinc rods had been thoroughly amalgamated, but that when the zinc-mercury surface became tarnished polarization developed even with small currents. Although he also found that properly prepared electrodes would last for several days without noticeable deterioration, I preferred to be on the safe side and prepare them freshly each day. Stimulation at various frequencies was effected by condenser

and rotating commutator. The current for the nerve was led in through the electrodes *K* and *H* (Fig. 1), that for the muscle through the electrodes *G* and *K*. For the stimulation of the nerve the condenser used was shunted by a resistance of 1500 ohms, and a resistance of 4500 ohms was connected in series with the nerve electrodes. The resistance between the nerve electrodes was measured in a number of experiments and found to be 4500 ± 100 ohms. Hence the effective resistance shunting the "nerve" condenser was about 1090 ohms. For stimulation of muscle the shunt resistance was omitted since otherwise the voltage to which the condenser would have had to have been charged to elicit maximal response would have been inconveniently large. The resistance between the muscle electrodes was also measured in a number of experiments and found to be 4400 ± 100 ohms, whence the effective resistance shunting the "muscle" condenser was 5900 ± 100 ohms. The duration of stimulation was hand-controlled by a short-circuiting key. A second key beside the first operated a magnetic signal which indicated approximately beneath the myograms the beginning and ending of stimulation. When it was desired to apply single test shocks, or shocks at very low frequency, the short-circuiting key was opened and, by means of a switch, the commutator was replaced by a hand-operated morse key with three contacts. After each preparation had been mounted it was allowed to soak for a further half hour, then the bath was drained and refilled with fresh Ringer's solution before beginning an experiment.

Essentially the procedure in these experiments was to stimulate the nerve or the muscle of the preparation for test periods, each lasting several seconds, at regular intervals. In some experiments the nerve and the muscle were stimulated alternately; in others the muscle was stimulated only once for every three or every six times that the nerve was stimulated. Each experiment was begun by making a sufficient number of records to establish that stimulation of the muscle and of the nerve elicited reproducible responses. When this had been done, the trough was emptied, refilled with the solution whose action was to be investigated, and further test stimuli applied.

For the experiments in which it was intended to immerse only the nerve in a solution of an eserine-like substance and the remainder of the preparation in Ringer's solution the Lucas trough was modified further as shown in Fig. 3. An additional partition was placed within the small compartment which contained the greater part of the nerve and the central end of the nerve was passed through a hole in this partition. The two small compartments could be filled with the solution

whose action was to be examined and, by leading current into them through non-polarizable electrodes, the nerve could be stimulated within the region that had undergone treatment. To prevent any eserine-like substance reaching the muscle and nerve endings the hole in the outer of the two partitions through which the nerve passed was blocked with vaseline, and the Ringer's fluid in the two larger compartments which contained the muscle was changed frequently during an experiment. Except for these modifications the arrangements for stimulation were

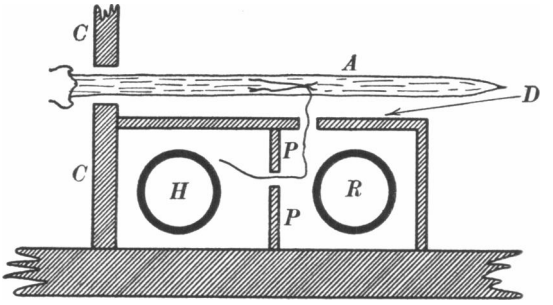


Fig. 3. The Lucas trough further modified for the experiments in which only the nerve was treated with a solution of an eserine-like substance. The two small compartments in which the nerve lay were filled with the solution. The letters A, C, D, and H have the same meaning as in Fig. 2. P, additional partition with hole through which the nerve passed. R, zinc-zinc sulphate non-polarizable electrode.

those described in the previous paragraph but one. In several experiments the resistance between the nerve electrodes was found to be 4400 ± 100 ohms, hence the effective resistance shunting the condenser was about 1120 ohms. In each experiment preliminary records of the responses to stimulation of muscle and of nerve were made whilst all the compartments were filled with Ringer's solution, then the two smaller compartments were drained, filled with the solution whose action was to be examined and responses to further stimuli were recorded.

Results

Nerve action current

Using the full sensitivity of the galvanometer the deflexion produced by stimulation of Ringer-soaked nerve for periods of 3–30 sec., at frequencies ranging from 5 to 200 per sec., was 10–450 mm. In most experiments however the galvanometer shunt was adjusted so that the maximum deflexion did not exceed 150 mm., since it was found that

deflexions of this order could be read to within about 1 p.c. and that the increased accuracy which might have been expected with larger deflexions was rather more than offset by small changes of zero, probably owing to elastic hysteresis of the metallic strip suspending the galvanometer coil, and by the difficulty of making readings when the light spot was moving rapidly.

In a preliminary communication [1936*a*] I stated that treatment of nerve with prostigmine in concentrations up to 1000 μM (1000×10^{-6} g.-mol./l.) for 4 hr. produced no change in the action current. After further experiments I think that this statement may have to be modified slightly, but I am not yet quite certain.

Treatment of nerve with solutions of 1, 10, or 100 μM eserine, prostigmine, or Substance 38, for periods up to 3 hr., caused no greater change in the responses to stimulation at the frequencies and for the periods mentioned than did treatment with Ringer's solution (about 3–10 p.c. diminution). Treatment for longer times, up to 6 hr., also caused no indisputable change in the responses. In these observations the experimental uncertainty was generally greater than in those lasting for the shorter time because of the greater differences between the decline of the responses of different experimental and of different control nerves (3–15 p.c.). The soaking of nerve for 3–6 hr. in a solution containing 1000 μM prostigmine caused the response to increase by 10–20 p.c. in some but not in all experiments. In those experiments in which the increase appeared, and in those in which it did not appear, the movement of the galvanometer spot showed no sign of irregularity or temporary failure, either with high or low frequency stimulation.

Nerve-muscle preparations which had been soaked in bicarbonate Ringer's solution

Responses to stimulation of muscle.

Preliminary experiments led me, for reasons which will be clear from the results described under the next subheading, to employ test periods of stimulation lasting 10–30 sec. Maximal stimulation with 15–30 shocks per sec. elicited responses in which the initial rise of tension exhibited wavelets due to incomplete summation to an extent dependent upon the frequency used. These became smaller and disappeared within 1–3 sec. and the rise continued for 1–4 sec. longer, this time also depending mainly upon the frequency used. After passing through a blunt maximum the tension then declined very slowly. Except that the rate of fall

following the maximum is much too fast Fig. 10H illustrates the type of response obtained. Stimulation with 50–120 shocks per sec. elicited responses in which the whole or the greater part of the initial rise of

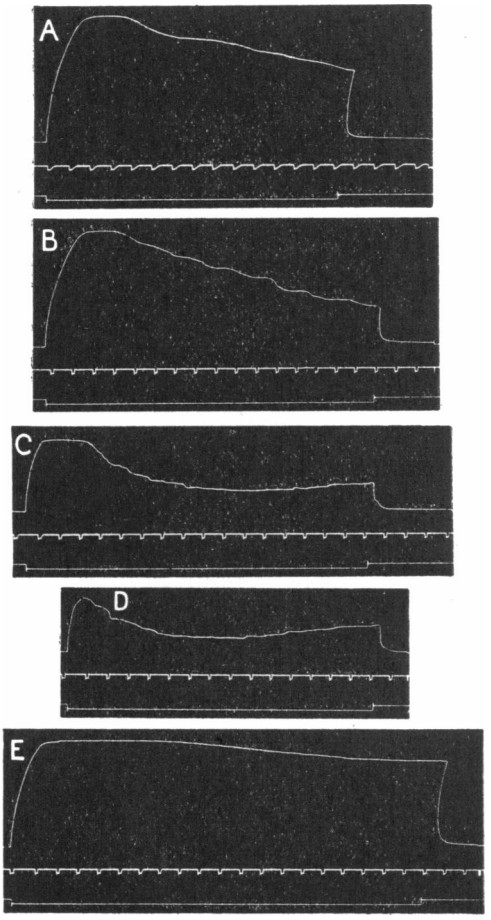


Fig. 4. 26. xi. 35. The effect of 0·03 μM solution of prostigmine upon the responses of a sartorius nerve-muscle preparation to stimulation with 100 shocks per sec. The time markings are seconds. The depression of the signal line at the left of each record indicates the beginning of stimulation, and the rise at the right of each record the end of stimulation. A, 3.32 p.m., nerve of Ringer-soaked preparation stimulated with maximal shocks of time-constant 109 μsec. 3.45 p.m., the Ringer's solution in the bath was replaced by solution containing prostigmine. B, 6.15 p.m., nerve stimulated as in A. C, 6.41 p.m., nerve stimulated as in A. D, 7.11 p.m., nerve stimulated as in A. E, 7.24 p.m., pelvic end of the muscle stimulated with shocks of time-constant 1475 μsec.

tension took place in one step. After remaining steady or increasing very slightly in the next few sec. (4–6) the tension then began to fall slowly (Fig. 4E). Stimulation at higher frequencies, 120–200 shocks per sec., caused the tension to attain its maximum value almost immediately. In the following 5 sec. or so the tension fell very slowly or remained constant; after that, its fall became a little more rapid (Fig. 5A). When the stimulation periods were separated from one another by 10 min. intervals the successive responses of any one muscle to repeated application of the same stimulus usually differed very little from one another. After about 3 hr. of such intermittent activity the maximum tension attained was from 2–10 p.c. lower than in the responses recorded at the beginning of the experiment, and the rate of decline of tension during the responses was also rather greater (generally 5–10 p.c.). Experiments within the breeding season, or the month after, were avoided as far as possible, since the muscle responses deteriorated more rapidly than at other times (cf. Fig. 9 A, E), and the differences between the responses of muscles from different frogs were greater.

The foregoing results were uninfluenced when the time-constant of the shocks comprising the stimulus was varied between 590 and 1475 μsec., and when the voltage to which the condenser was charged was increased to 20 p.c. above the value required to produce maximal response.

During the first few of the above experiments the adequacy of the buffering of the solution in the trough was tested by taking out samples periodically from the muscle compartment and determining their *p*H colorimetrically. Provided that the stream of oxygen-carbon dioxide mixture was sufficiently fast the *p*H found initially was $7{\cdot}2 \pm 0{\cdot}05$—the figure calculated. After 100 min., in which the muscle had undergone ten test periods of activity, a diminution of about 0·1 *p*H unit was found. In subsequent experiments (with or without drugs) the solution in the trough was renewed at least once for every ten test stimuli applied to the muscle or the nerve of the preparation.

Responses to stimulation of nerve.

As the frequency of stimulation was increased from 15 to 200 per sec. the form of the response became progressively modified. It is convenient, however, to make a somewhat arbitary classification into three main types. The first of these was generally elicited at frequencies 15–60 per sec. The maximum tension, which was slightly greater than that obtained by direct stimulation of the muscle at the same frequency as was used for the nerve, was attained in two steps—a rapid initial rise passing over

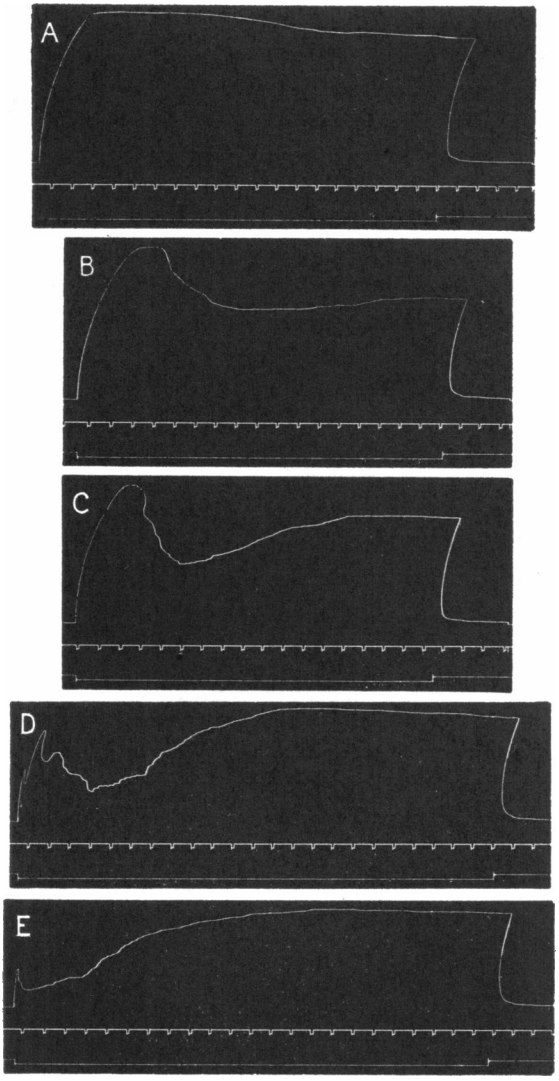


Fig. 5. 21. xi. 35. The effect of 0·3 μM prostigmine solution on the responses of a sartorius nerve-muscle preparation to stimulation with 150 shocks per sec. Time markings and signal line have same meaning as in Fig. 4. A, 4.22 p.m., pelvic end of Ringer-soaked muscle stimulated with maximal shocks of time-constant 1450 μsec. B, 5.22 p.m., nerve stimulated with shocks of time-constant 109 μsec. 5.25 p.m., the Ringer's solution in the bath was replaced by solution containing prostigmine. C, 5.52 p.m., nerve stimulated as in B. D, 6.2 p.m., nerve stimulated as in B. E, 6.33 p.m., nerve stimulated as in B.

into a very slow rise lasting several (3–7) sec. After the maximum had been attained the tension fell at a slow rate which was not much greater than that at which it fell in the corresponding part of the response to direct stimulation of the muscle. With stimulation frequencies 15–30 per sec. the slow part of the rise of tension exhibited wavelets which became smaller and disappeared within a second or two. The second type of response was produced generally at frequencies 100–180 per sec. It was characterized by a secondary rise of tension which appeared several seconds after the first maximum. Stimulation with about 100 shocks per sec. caused the tension to attain its maximum in two steps similar to those mentioned in connexion with the first type of response. Usually the maximum was 1–5 p.c. greater than the tension obtained by direct stimulation of the muscle at the same frequency. The tension began to fall slowly from the maximum, but after two or three seconds there appeared superimposed on the fall a small and slow rise which lasted for a few seconds. The tension passed through a second maximum and then fell slowly again (Fig. 4A). Stimulation at a rate of about 150 shocks per sec. also caused the tension to rise to its maximum in two steps, and again the maximum was slightly greater than was obtained by direct stimulation of the muscle. After about a second the slow part of the initial rise was cut short by a rapid fall lasting for a few seconds (usually 2–6 sec.): this was succeeded by a rather slower (secondary) rise which continued for several seconds and which was afterwards followed by a very slow fall (Figs. 5B, 10A). With a stimulation frequency of about 180 per sec. the initial rise of tension was cut short before it had become fully developed by a rapid fall lasting 2–5 sec.; after passing through a minimum, which was often only 20–30 p.c. of the maximum tension that the muscle yielded in response to direct stimulation at the same frequency, the tension rose again fairly quickly to its secondary maximum and then fell very slowly. The third type of response was elicited at a frequency of about 200 per sec. In it the rapid fall which cut short the initial rise occurred earlier than in the responses of the second type and proceeded until the tension had become very small. On continuing stimulation for a further 15–25 sec. the tension did not rise again or rose very little. Secondary differences between the responses of different preparations were more marked than at lower frequencies. Some preparations yielded a rise and fall of tension which was similar to that exhibited in a single twitch, and which was followed by a very slowly diminishing tension; others yielded a rather greater initial tension followed by a rapid fall and then a very slow rise.

When the nerve of a preparation which had suffered no stimulation for an hour or more was stimulated at 10 min. intervals at a frequency which produced responses of the second or third type, the rate of fall of tension in the early part of the first response was often a little slower than it was in the second response. Subsequent responses differed little. In the responses recorded after about 3 hr. of this periodic activity the initial tension was generally 5–10 p.c. smaller than it had been at the beginning of the experiment; also, the form of the tension-time curve had become modified in such a way as might have been imitated at the beginning of the experiment by using a stimulation frequency slightly higher than was actually used, i.e. in the experimental period the frequency required to produce a particular form of response diminished slightly. In some experiments it was convenient to allow longer rest intervals (up to 30 min.) between the stimulation periods: the changes that occurred in the responses were similar to those just described.

The various forms of response which have been described in this section were obtained equally well when the time-constant of the shocks making up a stimulus was given values ranging from 54·5 to 218 μsec., and when the voltage was varied between 1·0 and 1·5 times that needed to evoke maximal response.

The responses of preparations whose nerves only had been treated with eserine-like compounds

The nerves were immersed for 3–4 hr. in Ringer's solution containing 3–300 μM prostigmine, or 0·03–50 μM eserine, or 3–30 μM Substance 38. During the test period the myograms in response to maximal or slightly supramaximal stimulation of the nerve did not change any more than did those given by the untreated preparations (see the preceding section).

Preparations which had been treated with eserine-like compounds

Responses to stimulation of muscle.

Treatment for 3–4 hr. with a solution containing any of the following compounds within the concentration ranges stated produced no change in the responses of muscle:

Prostigmine, 0·03–3000 μM.
Eserine, 0·03–50 μM.
Substance 36, 0·03–30 μM.
Substance 38, 0·03–30 μM.
Preparation 1210/1, 0·03–50 μM.
Substance 37, 0·03–20 μM.

Substance 37 in concentrations higher than those mentioned above reduced the tension developed by the muscle but did not alter the course of the decline of tension during stimulation at various frequencies. A 400 μM solution reduced the tension by about 30 p.c.

Preparation 3393, used in concentrations 5–53 μM, did not change the form of the response within 3 hr. If, however, the stimuli were reduced to considerably submaximal strength small twitchings similar to those recorded in Fig. 10 C–F appeared superimposed on the response.

Responses to stimulation of nerve.

The principal action of any of the eserine-like substances was to lower the frequency required to evoke the responses which were termed the second and third types in the section dealing with Ringer-soaked preparations; certain other modifications in the form of the response were also produced. For brevity and clearness the action of prostigmine will first be described and afterwards the respects in which the actions of other compounds resembled or differed from that of prostigmine.

The interval which elapsed between filling the bath with solution containing prostigmine and a perceptible change in the response varied according to the concentration used and the frequency of stimulation. The interval also varied with different preparations, presumably on account of differences of sensitivity, a factor which will be discussed later in connexion with the comparison of the activities of the eserine-like substances. When preparations were immersed in a 0·03 μM solution and tested with stimuli of frequency 100–200 per sec. some change could be detected within an hour, but the principal changes took place in the next $1\frac{1}{2}$–$2\frac{1}{2}$ hr. The prostigmine caused the form of the response to stimulation with 100 shocks per sec. to change in such a way as might have been imitated by stimulating the untreated preparation with about 150 shocks per sec. (Fig. 4 A–D). Sometimes small irregular variations of tension also appeared (Fig. 4 B, C, and D). Apart from these changes the maximum initial tension developed in the later responses was only one-half to three-quarters of what it would have been had the untreated preparation been stimulated with 150 shocks per sec.

With 0·3 μM prostigmine and a stimulation frequency between 100 and 200 per sec. the latent period for a perceptible change in the response was reduced to 25–35 min., and the principal changes took place in the next $\frac{1}{2}$–1 hr. When the nerve was stimulated with 150 shocks per sec. prostigmine first caused a smaller initial tension to be developed and the minimum to occur earlier and to be smaller than in the responses of

the untreated preparations; also it caused the tension developed in the secondary rise to increase, usually by 5–10 p.c. (cf. Fig. 5 B, C). After the prostigmine had been allowed to act for another 10–20 min. the early part of the response became irregular. Often the tension rose initially to only one-quarter to one-third of the maximum value that the untreated preparation had given, fell momentarily, and then resumed its ascent; after attaining the first maximum it fell in a very irregular manner to its minimum which occurred still earlier than in previous responses: subsequently the tension rose again to a height that was the same or greater than in earlier records and then declined very slowly (Fig. 5D). In responses recorded between 15 and 30 min. later the tension rose initially to a height that would have been given by one or two maximal shocks applied to the nerve of the untreated preparation and then fell rapidly to its minimum. Having passed through the minimum the tension rose again in the next 10–15 sec. to a value that was usually rather less (5–10 p.c.) than had been given by the untreated preparation, and afterwards fell very slowly (Fig. 5E).

With 3 μM prostigmine and a stimulation frequency 100–200 per sec. a perceptible change in the response occurred in 10–25 min.: the change continued and became maximal within 60–80 min. When the nerve was tested at intervals, with shocks at the rate of 150 per sec., the response passed through changes very similar to those described in the preceding paragraph and illustrated in Fig. 5; afterwards further changes took place in the part of the response which followed the minimum of tension. The secondary rise became slower and smaller until finally the response comprised an initial tension, such as might have been evoked by one or two maximal shocks applied to the nerve of the untreated preparation, followed immediately by a fall to a very small tension which diminished very slowly during stimulation for a further 20 sec. Except for the superimposed small irregularities, the responses shown in Fig. 10D–F may be taken as illustrating the later responses obtained after treatment with 3 μM prostigmine solution. When test stimuli of a lower frequency (20–30 shocks per sec.) were applied the tension rose initially to about three-quarters of what had been obtained by stimulating the untreated preparation with 150 shocks per sec. The tension was maintained for about a second, then it fell to one-half or one-third of its initial value in 4–6 sec. and afterwards more slowly. Except for the superimposed irregularities Fig. 10G illustrates the type of response obtained. When test stimuli of still lower frequency (about 15 per sec.) were used the tension rose relatively slowly and showed wavelets due to incomplete

summation. These disappeared after 1–2 sec. and the rise continued for another 2–3 sec. The tension passed through a maximum which was about two-thirds of that attained in the response of the untreated preparation to stimulation with 150 shocks per sec., and afterwards fell at a rate which although slow was considerably greater than in the response of the untreated preparation to stimulation at the same frequency. Fig. 10H illustrates the type of response obtained.

Prostigmine in still greater concentrations reduced further the latent period for a perceptible change in the response to stimulation with 100–200 shocks per sec.; it became much the same, 5–10 min., with a concentration of 300 or 3000 μM. The response to stimulation with 150 shocks per sec. soon came to follow the course described above: initial rise, rapid fall to small tension, which afterwards diminished very slowly. In subsequent responses the initial rise and rapid fall of tension suffered no further change whereas the very slowly diminishing tension which followed became larger, and often was better maintained than in the responses of preparations that had been treated with prostigmine in smaller concentrations. Upon stopping stimulation which had been going on for about 20 sec. the persistent low tension fell very rapidly to one-half to one-third of its previous value, and then the remaining tension gradually disappeared in several seconds (often 5–10). Presumably it was due to contracture. The responses of preparations that had been treated with prostigmine in the higher concentrations, to low frequency (30–60 shocks per sec.) stimulation, differed from the responses to stimulation at higher frequencies only in that the tension which followed the initial rise and fall was smaller and disappeared more quickly when stimulation was stopped.

In two experiments a preparation was immersed in a 3000 μM solution of prostigmine, and the responses to stimulation of the nerve and to direct stimulation of the muscle, at frequencies of 100 and 150 per sec., were recorded several times during the first hour; then the preparation was left overnight and on the following day, 14–16 hr. later, the responses to stimulation of the nerve and of the muscle were again recorded. A control preparation which was immersed only in bicarbonate Ringer's solution was left overnight and similarly tested. After 14–16 hr. the responses of the prostigmine-treated and of the control muscles to direct stimulation at the higher frequency differed very little from one another; the responses at the lower frequency also were very similar to one another. In both cases they were of the same form as those obtained on the previous day, but the maximum initial tension was about 20 p.c.

smaller, and the rate of decline of tension during response was slightly greater. After 14–16 hr. the prostigmine-treated preparations gave responses to nerve stimulation very similar to those that they had given after treatment for about an hour except that the maximum initial tension had diminished by about 30 p.c. The responses of the Ringer-soaked preparations to nerve stimulation also differed little from those obtained on the previous day, except that the tension developed had diminished by about 30 p.c.

The various forms of response which have been described under this sub-heading were uninfluenced when the time-constant of the shocks making up a stimulus was varied between 54·5 and 218 μsec., also when the voltage was increased to 1·5 times that needed to evoke maximal response.

A few experiments were made with preparations from frogs which had been stored at 0–5° C. for 40 hr. or more before use ("cold" frogs). The dissection was done at room temperature and as soon as it was complete the preparation was mounted in bicarbonate and glucose Ringer's solution in the modified Lucas trough at 15° C. After ½ hr. the experiment was made in the way already described. The responses to maximal stimulation of the pelvic end of the muscle differed little from those given by the "warm" frog preparations: at low frequencies (20–30 per sec.) during the initial rise of tension the wavelets due to incomplete summation were rather less marked; at higher frequencies (100–150 per sec.) the rate of decline of tension was generally a little greater. The form of the response to maximal stimulation of the nerve was less sensitive to changes of frequency than it was with "warm" frog preparations. When shocks of time-constant 54·5 or 109 μsec. were applied at frequencies between 20 and 30 per sec. the wavelets which occurred during the initial rise were again less marked. At stimulation frequencies between 120 and 160 per sec. the maximum tension attained in the initial rise was about 10 p.c. higher than was obtained by stimulation of the pelvic end of the muscle; the minimum to which the tension afterwards sank was relatively less deep than in the responses of the "warm" frog preparations and the maximum tension attained in the secondary rise was greater (Fig. 6A, B). The form of the response to stimulation at any one frequency did not remain constant however. The longer the "cold" frog preparations were soaked in the Ringer's solution the more did their responses come to resemble those of the "warm" frog preparations. The soaking of "cold" frog preparations for 1½–2 hr. sufficed to render their responses to stimulation with 150 shocks per sec. indistinguishable from those of "warm" frog preparations (cf. Fig. 6B, C).

The responses of prostigmine-treated "cold" frog preparations differed consistently from those of prostigmine-treated "warm" frog preparations in certain respects. The principal differences were that at low frequencies prostigmine caused the maximum tension developed in the response to occur earlier and to become greater; that at high frequencies prostigmine did not cause the tension developed in the initial rise to be reduced to the same degree that it did in the responses of the "warm" frog preparations. When "cold" frog preparations which had had little soaking in Ringer's solution were treated with prostigmine these differences largely persisted for several hours (more than 4). Also, although soaking for about 2 hr. in Ringer's solution abolished the differences between the responses of "cold" and "warm" frog preparations it did not prevent the differences

between prostigmine-treated "cold" and "warm" frog preparations from appearing. In the next two paragraphs the differences will be described in more detail.

In the response to stimulation at a frequency of about 150 per sec., about 15 min. after filling the bath with 3 μM prostigmine solution, there appeared a small augmentation (usually 3–5 p.c.) of the tension developed in the initial rise; also, a marked deepening

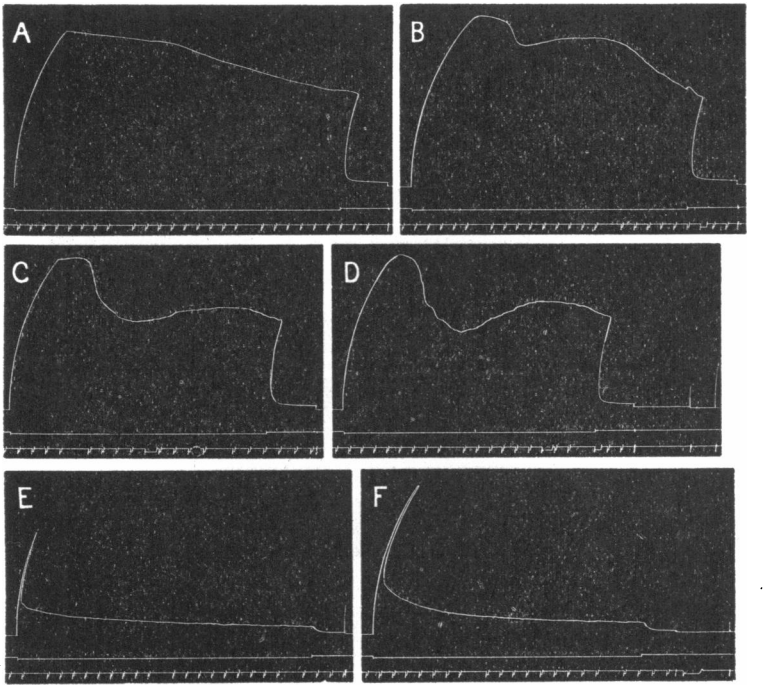


Fig. 6. 15. iii. 38. The effect of soaking in Ringer's solution followed by treatment with prostigmine upon the responses of a nerve-sartorius preparation from a "cold" frog. Time markings and signal line have the same meaning as in Fig. 4. Stimulation frequency 150 per sec., except in F. 1.50 p.m., dissection finished. 1.55 p.m., preparation mounted in bath. A, 2.38 p.m., pelvic end of muscle stimulated with shocks of time-constant 1475 μsec. B, 2.48 p.m., nerve stimulated with shocks of time-constant 73 μsec. C, 3.36 p.m., stimulation of nerve repeated. 3.44 p.m., bath drained and filled with solution containing 3 μM prostigmine. D, 4.20 p.m., stimulation of nerve repeated. 4.22 p.m., bath drained and filled with solution containing 12 μM prostigmine. E, 5.2 p.m., stimulation of nerve repeated. F, 6.0 p.m., nerve stimulated with 50 shocks per sec.

of the subsequent minimum (Fig. 6D). What happened to the tension developed in the secondary rise depended on how long the preparation had been soaking in Ringer's solution. If the soaking had been only for a short time then the reduction produced by the Ringer's solution generally outweighed the augmentation produced by the prostigmine; if, on the other hand, the soaking had been sufficiently long for the preparation to reach a fairly steady state then augmentation resulted (Fig. 6D). After another $\frac{1}{2}$ hr., or a shorter time

if a more concentrated prostigmine solution were used, the initial maximum became slightly reduced, the fall which followed the maximum became earlier and faster, and the secondary rise was depressed. Still later the initial maximum was reduced to about two-thirds of what had been obtained at the beginning of the experiment: as soon as the maximum had been attained the tension fell rapidly to one-quarter to one-third of its initial value and afterwards slowly at a diminishing rate (Fig. 6E). Stimulation of a preparation when in this condition at a frequency of 50 per sec. elicited a response of similar form to that just described, but of greater tension throughout (Fig. 6F).

Treatment of a preparation for about ½ hr. with 6 μM prostigmine solution caused the maximum tension developed during the response to stimulation at a frequency of 20 per sec. to be reached by a rapid initial rise instead of by a rapid rise followed by a slow rise. Once the tension had reached its maximum it was better maintained during 15–20 sec. of continued stimulation than it had been before the treatment (cf. Fig. 7A, B). After a further

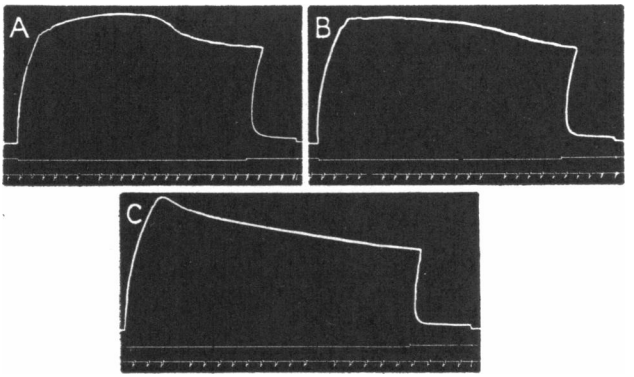


Fig. 7. 18. iii. 38. The action of 6 μM prostigmine solution upon the responses of a nerve-sartorius preparation from a "cold" frog to stimulation at a frequency of 19 per sec. The time constant of each shock was 73 μsec. Time markings and signal line have the same meaning as in Fig. 4. 2.0 p.m., dissection completed. 2.5 p.m., preparation mounted in bath of Ringer's solution. A, 3.30 p.m., nerve stimulated. 3.33 p.m., bath drained and filled with solution containing 6 μM prostigmine. B, 3.57 p.m., nerve stimulated. C, 4.51 p.m., nerve stimulated.

1–1½ hr. in 6 μM prostigmine solution the maximum tension in the response was still developed in the initial rise and usually had become increased by about 5 p.c. although the response of the muscle to direct stimulation had diminished by about that amount. After the maximum had been reached the tension fell more rapidly than in earlier responses (Fig. 7C).

Apart from producing changes in the form of response prostigmine had two other actions. The first was to produce a few "spontaneous" twitches of the "cold" frog preparations which had been soaked for only a short time in Ringer's solution, but not of the preparations which had been soaked for an hour or more. Usually the twitches appeared about 10 min. after filling the bath with prostigmine solution, continued at irregular intervals for 2–3 min. and then disappeared. The second action was to cause each period of high frequency stimulation to be followed 1–4 min. later by one or more "spontaneous" twitches (Fig. 6D, E, F). The twitches were obtained with preparations which had been soaked in Ringer's solution for short or for long times. They were not observed after stimulation at frequencies of 20–30 per sec.

In three experiments the action of 3000 μM prostigmine solution upon the responses of preparations to slow rhythmic stimulation was examined. Two of the experiments were with preparations from "warm" frogs, and one was with a preparation from a "cold" frog. After the preliminary soaking each experiment was begun with the recording of two series of responses: one to direct stimulation of the muscle by maximal shocks of time constant 1475 μsec. at the rate of about 5 per min., the other to stimulation of the nerve by maximal shocks of time constant 109 μsec. at the same rate. The bath was then drained and filled with the prostigmine solution. Rhythmic stimulation was begun again 10 min. later. In the first few responses given by the "warm" preparation the maximum tension was unaltered; in the next few responses it declined about 20 p.c. and then recovered slowly to its initial value as stimulation continued. During rhythmic activity for another $\frac{1}{2}$ hr. the maximum tension developed in each response remained fairly constant—at the end of that time it was about 95 p.c. of the initial (untreated) value. The preparation was then left undisturbed overnight and tested again 14–16 hr. later, with two more series of rhythmic shocks, the first being applied to the muscle and the second to the nerve. After the initial treppe, the muscle response became fairly steady at about 80 p.c. of the previous day's value. When the nerve was stimulated the maximum tension developed in the first response was about 70 p.c. of the previous day's initial value for the untreated preparation; in subsequent responses the maximum tension declined to about 50 p.c. and afterwards recovered more slowly than it had done on the previous day. In the experiment with the "cold" preparation, on recommencing stimulation of the nerve at the rate of about 4 shocks per min., 10 min. after the bath had been filled with the prostigmine solution, the maximum tension developed in the first few responses was about 20 p.c. greater than had been given by the untreated preparation (cf. Fig. 8A, B). In the next few responses the maximum tension declined until it exceeded the initial (untreated) value by only 5 p.c., and then, as stimulation continued, it recovered slowly until it exceeded the initial value by about 10 p.c. After the recovery the rhythm was reduced to about 3 shocks per min., without omitting any shocks, and stimulation was continued for another $\frac{1}{2}$ hr. During that period the maximum tension developed in the responses remained fairly steady, and at the end of the period it was still about 5 p.c. greater than that given by the untreated preparation. The preparation was left overnight and tested on the following day with stimuli at the rate of 4 per min. The maximum tension developed in the response of the muscle to direct

stimulation had diminished to 85 p.c. of the previous day's value. The maximum tension developed in response to stimulation of the nerve was 77 p.c. of what had been given by the untreated preparation on the previous day. In subsequent responses the maximum tension diminished to 51 p.c., and afterwards made a slow and incomplete recovery. In two control experiments, in which untreated preparations were left mounted overnight, as in the experiments with prostigmine, the maximum tension evoked by stimulation of the nerve diminished rather more than did the maximum tension evoked by direct stimulation of the muscle.

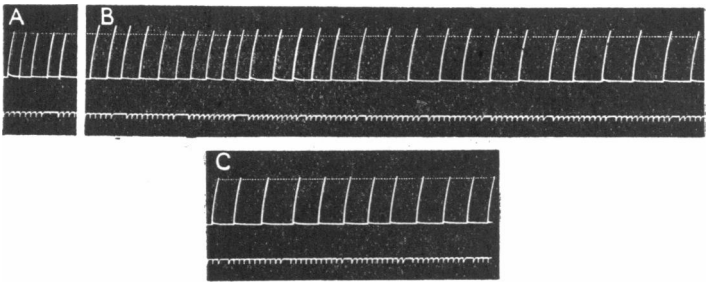


Fig. 8. 1. viii. 35. The action of a solution containing 3000 μM prostigmine upon the responses of a sartorius nerve-muscle preparation from a "cold" frog. Time markings at 5 sec. intervals. Each response was elicited by a single maximal shock to the nerve. In order that the changes may be more easily seen a dotted line has been drawn parallel to the resting tension line and at a height which represents the maximum tension attained in the responses before the preparation was treated with prostigmine. 6.0 p.m., preparation mounted in Ringer's solution. 8.55 p.m., fresh Ringer's solution in bath. A, 9.12 p.m., the steady value of the response, before treatment. 9.14 p.m., bath drained and then filled with prostigmine solution. B, 9.24 p.m., stimulation recommenced. C, responses recorded at 9.55 p.m. and after.

By the application of eserine, Substance 36, Substance 38, or Preparation 1210/1, in suitable concentration to a nerve-sartorius preparation it was possible to produce changes in the response to nerve stimulation closely similar to those produced by prostigmine in concentrations between 0·03 and 30 μM.

Since it was necessary to allow some considerable time to elapse after the treatment of a preparation with an eserine-like substance, and during that time to record the responses to several test stimuli, in order to ascertain if the action of the substance had become fully developed, I thought it better not to try to compare quantitatively the actions of different solutions of prostigmine, or of prostigmine and another eserine-like substance, on one preparation, but instead to take a fresh preparation for each solution. It has been pointed out already that the successive

responses of a Ringer-soaked preparation to stimulation at one frequency differed little from one another, but that the responses of different preparations to stimulation at the same frequency exhibited minor differences. Before making the comparison of the activities of the eserine-like substances with that of prostigmine it was necessary to obtain some estimate of how much uncertainty would be introduced by these differences of response and the differences of sensitivity exhibited by different preparations towards any one of the eserine-like substances. The following method was used to obtain the estimate. Ringer-soaked nerve-sartorius preparations of about the same size were taken, two at a time, and after their responses to stimulation for 15 sec. periods at a rate of 100 or 150 shocks per sec. had been recorded one of them was treated with a 0·1–0·5 μM prostigmine solution and the other was immersed in a prostigmine solution twice as concentrated as the first; subsequently the changes which occurred in the responses of both preparations to further test stimuli were followed. These conditions were selected partly because they were ones in which changes of response were well marked, and partly because, as will be seen later, they were ones in which prostigmine reversed, in some measure, paralysis due to curare-like substances. Thirteen experiments were made. The records showed that it would be impossible to take the change produced in any single feature of the response as a measure of the concentration of the prostigmine solution with which the preparation had been treated. Instead the changes produced in complete responses were compared. In seven of the experiments it was possible to distinguish between the higher and lower concentrations of prostigmine, in three of them it was impossible, and in three distinction was doubtful.

After the experiments with prostigmine had been made, the concentration was determined of each of the other eserine-like substances that would produce changes in the responses to stimulation at a frequency of 100 or 150 shocks per sec., which matched as nearly as possible those produced by 1 μM prostigmine solution. The results are given in Table I, and with them Dr Stedman's figures for the choline-esterase-inhibiting activities of the same compounds.

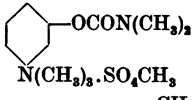
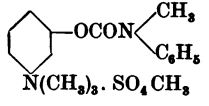
The relatively great activity of Preparation 1210/1 on neuro-muscular transmission (e.g. Fig. 9A–C) was rather unexpected because this compound contains no urethane grouping, and further experiments were made to check the result. Dr Ing was kind enough to recrystallize a specimen of Preparation 1210/1 for me. After recrystallization its activity was the same as before, within the limits of the experimental

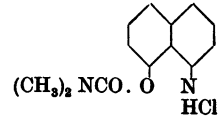
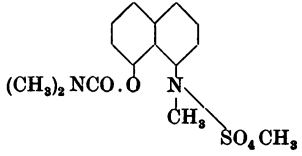
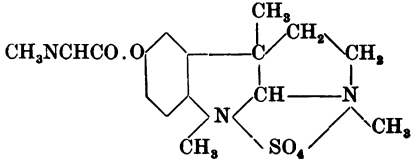
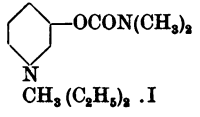
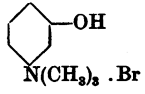
TABLE I

Co 4 shows concentration necessary to produce changes in the response to nerve stimulation, with 100 shocks per sec., closely similar to the changes produced by 1 μM prostigmine solution.

Col. 6 shows extent to which eserine-like compounds restored the responses of partly curarized preparations to nerve stimulation with 100 shocks per sec. Each experiment is summarized by the ratio of maximum tension developed after treatment with the eserine-like compound to the tension whilst the preparation was under the influence of curarine alone. Both tensions are stated as percentages of the maximum before the treatment with curarine chloride.

Col. 7 shows experiments the same as those in the preceding column except that phenyltrimethylammonium iodide was used instead of curarine chloride to produce partial paralysis.

(1)	(2)	(3)		(4)	(5) *Stedman's figures.* Percentage inhibition of choline-esterase produced by each compound in concentration 5×10^{-8} M, at pH 7·4, after		(6)	(7)
	Chemical structure	Mol. wt.		In μM	1 hr.	4½ hr.		
Prostigmine		334·0		—	35	72	40/8 42/11 20/8	23/14 38/9
Substance 36		396·0	5 2 2	Mean = 3·0	15	30	44/9 31/6 27/7	33/10 42/11

Substance 37		254·5	400 300 500 450	Mean = 413	6	11	21/12 15/10 12/8	22/14 37/16
Substance 38		344·0	1·5 1·0 0·75	Mean = 1·1	51	81	61/8 41/16 33/14	71/16 20/8
Eserine sulphate		373·0	3 6 3 6	Mean = 4·5	58	53	70/13 42/11 53/9	39/12 48/11
Preparation 3393		378·0	0·5 0·75 1·2	Mean = 0·82	As above but in concentration $2·5 \times 10^{-5}$ *M* after 3 hr. —	65	66/14 52/11 29/8	43/13 64/12
Preparation 1210/1		232·0	25 25 20 40	Mean = 27·5	As above but in concentration 5×10^{-4} *M* after 3 hr. 4	21 hr. 23	23/11 48/9 47/14	46/13 31/16

method. Part of the recrystallized specimen was microanalysed by Dr G. Weiler and Dr F. B. Strauss. The figures that they returned: H=6·03 p.c., C=46·80 p.c. are very close to the calculated ones: H=6·08 p.c., C=46·50 p.c. My own analysis of another part of the recrystallized specimen for bromide gave 34·4 p.c. Br′, which also agrees

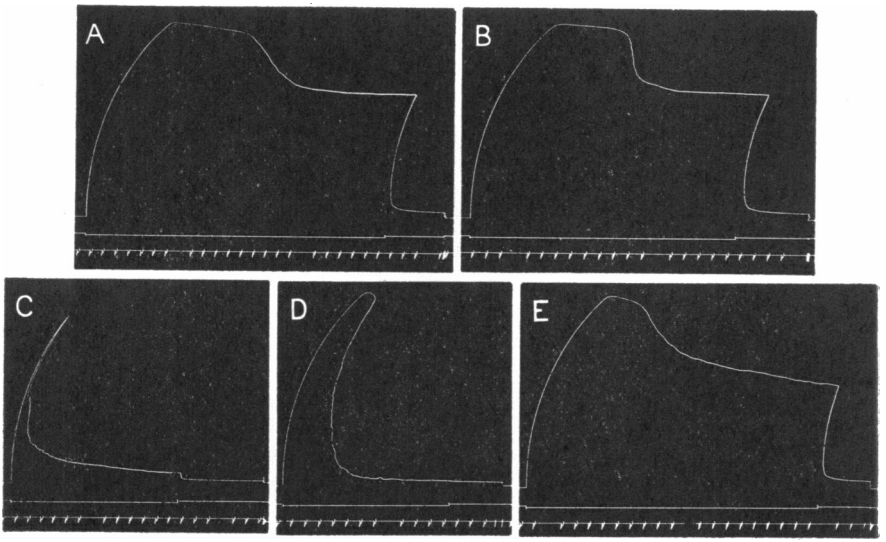


Fig. 9. 7. iv. 37. The action of Preparation 1210/1 followed by phenyltrimethylammonium iodide upon the responses of a nerve-sartorius preparation to stimulation at a frequency of 89 per sec. Time markings and signal as in Fig. 4. A, 2.6 p.m., pelvic end of Ringer-soaked muscle stimulated with shocks of time-constant 1500 μsec. B, 2.18 p.m., nerve stimulated with maximal shocks of time-constant 109 μsec. 2.45 p.m., bath drained and then filled with Ringer's solution containing 25 μ*M* of Preparation 1210/1. C, 4.24 p.m., nerve stimulated as in B. 4.26 p.m., 0·14 c.c. of 50 m*M* phenyltrimethylammonium iodide solution added to the compartment of the bath which contained the greater part of the muscle (vol. 25 c.c.) to make the concentration there 280 μ*M*. D, 4.52 p.m., stimulation of nerve repeated. E, 5.24 p.m., stimulation of muscle as in A.

with the calculated value 34·43 p.c. From the additional results and the analyses I conclude that Preparation 1210/1 was not contaminated with an impurity highly active on neuro-muscular transmission.

Estimation of the activities of Substance 37 and of Preparation 3393 was rather more difficult because their actions differed in certain respects from that of prostigmine. After the action of 0·03–0·3 μ*M* Preparation 3393 had become well developed, stimulation of the nerve with 150 shocks per sec. resulted in the tension attaining a greater value initially and

then falling to a less extent than it did after treatment with equimolar prostigmine solutions. The secondary rise was about the same as in the responses of prostigmine-treated preparations, or a little less marked. Small and irregular variations of tension appeared superimposed on the secondary rise, and to a greater extent on the subsequent slow fall. The responses of preparations that had been treated with Preparation 3393 in higher concentrations (3–10 μM) passed through the changes indicated above, then both the maximum initial tension and the secondary rise diminished (Fig. 10A–D). Soon the response came to consist of an initial rise of tension, followed by a rapid fall to a small value, and afterwards by a very slow fall (Fig. 10E). Generally, the small and very slowly falling tension was free from irregularities for several seconds after the rapid fall; later, however, rapid and irregular variations appeared and they increased in amplitude as stimulation continued. On examining the muscles of preparations during these responses it could be seen that the rapid and irregular variations were due to twitchings of small groups of fibres. In subsequent responses the initial tension diminished further, but very often it exceeded that developed in the responses of preparations which had been treated with the corresponding amount of prostigmine; also, the rapid and irregular variations which appeared in the later parts of the responses became less marked (Fig. 10F). When a test stimulus of frequency about 25 shocks per sec. was employed the tension rose to about three-quarters of the maximum that the untreated preparation had given on stimulation with 150 shocks per sec., fell in a few seconds to about one-half of its initial value, and afterwards more slowly (Fig. 10G). Generally, irregular variations appeared superimposed on the rapid part of the fall, but they were practically absent from the subsequent slow part. When test stimuli of lower frequency (10–15 shocks per sec.) were employed the tension rose slowly, exhibiting wavelets due to incomplete summation in the earlier part of the rise; after 2–4 sec. it reached a maximum and then declined smoothly (Fig. 10H). Some estimates of the activity of Preparation 3393 were made by finding the concentration needed to produce changes in the response to stimulation with 150 shocks per sec. similar to the changes produced by 1 μM prostigmine solution except for the differences just described. The figures are included in Table I.

Substance 37 had relatively little action on the responses to nerve stimulation except in concentrations which also reduced the response of muscle to direct stimulation. A 400–500 μM solution caused the form of the response to nerve stimulation with 100 or 150 shocks per sec. to

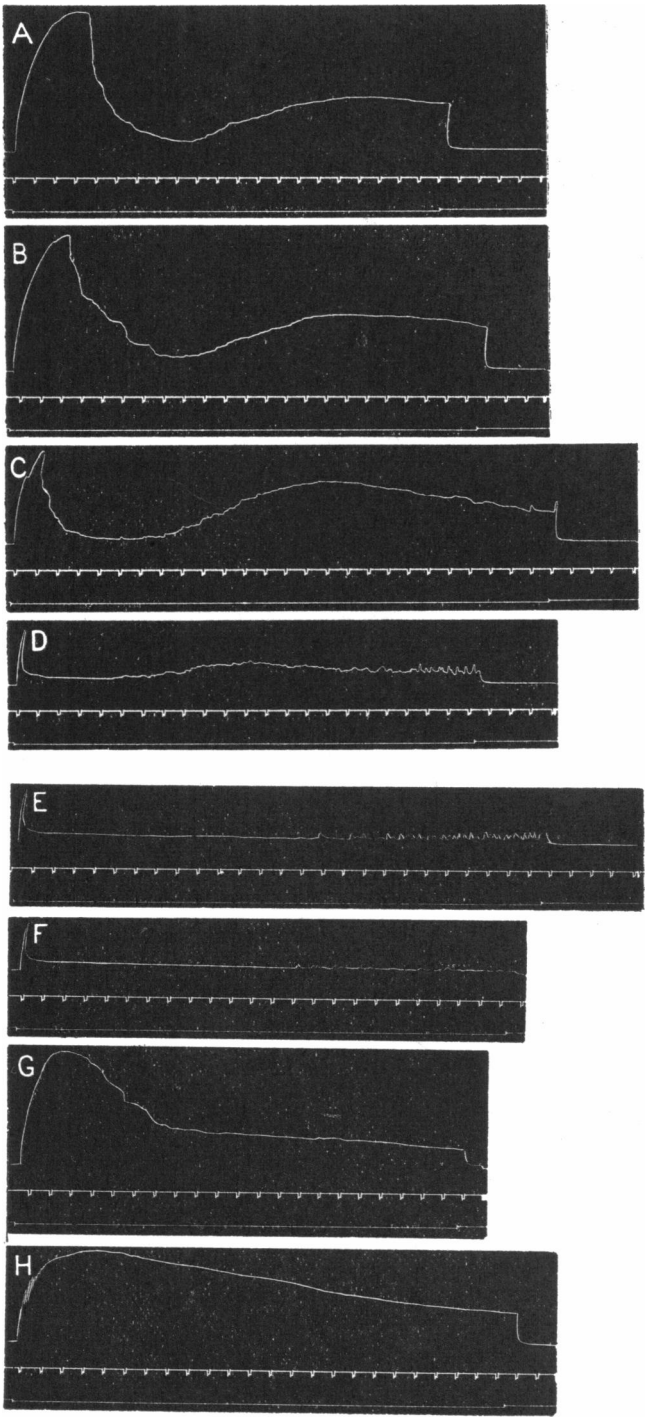


Fig. 10.

change in the same way that a 0·5–1·0 μM solution of prostigmine would have done, and, at the same time, it reduced the tension developed in the various parts of the response to one-half to two-thirds of what would have been expected had the preparation been treated instead with prostigmine.

(*a*)

The actions of eserine, Substance 38, and Preparation 1210/1 on the responses of "cold" frog preparations were similar to that of prostigmine, but no quantitative comparison was attempted.

(*b*)

In view of Kruta's [1935] findings that eserine was without action on the response of the frog's sciatic-gastrocnemius preparation to repetitive stimulation of the nerve by shocks at intervals of 0·4 sec., or at the rate of 30 per sec. for 2 sec. test periods, the actions of prostigmine and of eserine on the responses of that preparation were examined in a few experiments.

The preparation was dissected along with the lower half of the femur from a "warm" frog weighing about 20 g., and given a preliminary soaking in bicarbonate-buffered Ringer's solution. The apparatus and subsequent procedure were the same as in the experiments with sartorius preparations except for the following modifications. The partition *CC* and the non-polarizable electrode *G* were removed; the holder for the acetabulum was replaced by one suitable for the femur. Stimulation of the nerve was effected through the fluid electrodes used for the sartorius nerve. No attempt was made to stimulate the muscle directly because of the danger of stimulating nerve twigs at the same time.

The responses of Ringer-soaked preparations to stimulation with 50, 100, 150 shocks per sec. were very similar to those obtained from the Ringer-soaked sartorius preparations at the same frequencies. In order to minimize muscular fatigue, which would have complicated the responses, the test stimuli in each experiment were kept down to a minimum number and each of the shortest duration consistent with establishing the main changes that occurred. In no case were stimuli of duration longer than 15 sec. employed, nor were they applied to a preparation more often than once in 15 min. After the responses to two or three test stimuli had been recorded the preparation was immersed in a solution containing prostigmine in concentration up to 3 μM or eserine in concentration up to 10 μM, and, after an interval, the responses to further test stimuli recorded. The changes produced were similar to those produced in the responses of sartorius preparations, but the times which elapsed between the filling of the bath with the drug solution and the appearance of the changes were rather longer than with the sartorius preparations—presumably the extra time was required for the drugs to diffuse to the less accessible nerve endings in the isolated gastrocnemius [cf. Ing & Wright, 1931].

Fig. 10. 4. iii. 36. The action of Preparation 3393 upon the responses of a nerve-sartorius preparation. Time markings and signal as in Fig. 4. A, 5.9 p.m., nerve of Ringer-soaked preparation stimulated at frequency of 150 per sec. with just maximal shocks of time-constant 109 μsec. 5.40 p.m., bath drained and then filled with solution containing 6 μM Preparation 3393. B, 5.50 p.m., nerve stimulated as in A. C, 6.0 p.m., stimulation of nerve repeated. D, 6.10 p.m., stimulation of nerve repeated. E, 6.20 p.m., stimulation of nerve repeated. F, 6.30 p.m., stimulation of nerve repeated. G, 6.40 p.m., nerve stimulated as in A, but at frequency 24 per sec. H, 7.20 p.m., nerve stimulated as in A, but at frequency 14 per sec.

Comparison of the actions of phenyltrimethylammonium iodide and of curarine chloride on the responses of nerve-muscle preparations

During the earlier stages of the work on neuro-muscular transmission I thought that prostigmine, as a quaternary ammonium salt, might have a curare-like action in addition to its eserine-like action. As one way of obtaining an estimate of how far a curare-like action might be of importance I examined the action of phenyltrimethylammonium iodide, in concentrations between 0·1 and 10 μM, upon the responses of nerve stimulation, since the cation of that salt differs from the cation of prostigmine only in that the urethane grouping is absent. As reported in a preliminary communication [1936*a*] I found that no change in the responses occurred within half an hour of filling the bath with phenyltrimethylammonium iodide solution. However, after the discovery of the relatively great eserine-like activity of Preparation 1210/1, whose cation differs from that of phenyltrimethylammonium iodide in having an hydroxyl group in the meta position, it seemed possible that the action of phenyltrimethylammonium iodide in its lower effective concentrations might not be a purely curare-like one. [For references to the curare-like action of phenyltrimethylammonium salts see Trendelenberg, 1923; Ing & Wright, 1931.] I decided therefore, to examine further the action of this compound, carefully comparing it with that of curarine. The results are described below. The question that prompted the earlier experiments—whether prostigmine has a curare-like action will be discussed in the latter part of this paper.

When phenyltrimethylammonium iodide was used in concentration 10 μM the first clearly marked change in the response to nerve stimulation at a frequency of 150 per sec. occurred after about $\frac{3}{4}$ hr., when the tension developed in the secondary rise became reduced by 20–30 p.c. Often also the tension developed in the initial rise was reduced, but not by more than 10 p.c.: after attaining its maximum the tension began to fall earlier than in the responses of the untreated preparation (cf. Fig. 11A, B). During the next $\frac{3}{4}$ hr. the tension developed in the initial rise became reduced by about a further 5 p.c., a reduction not much greater than occurred in the responses of Ringer-soaked control preparations; on the other hand, the tension developed in the secondary rise became reduced by a further 10–20 p.c. In the course of another hour the responses of treated preparations suffered relatively no more change than did the responses of untreated preparations. When phenyltrimethylammonium iodide was used in a concentration of 50 μM the response

to nerve stimulation at a rate of 150 shocks per sec. was attacked, within 15–20 min. of filling the bath, to about the same extent that a 10 μM solution attacked it in 1½ hr. In about another 15 min. the maximum

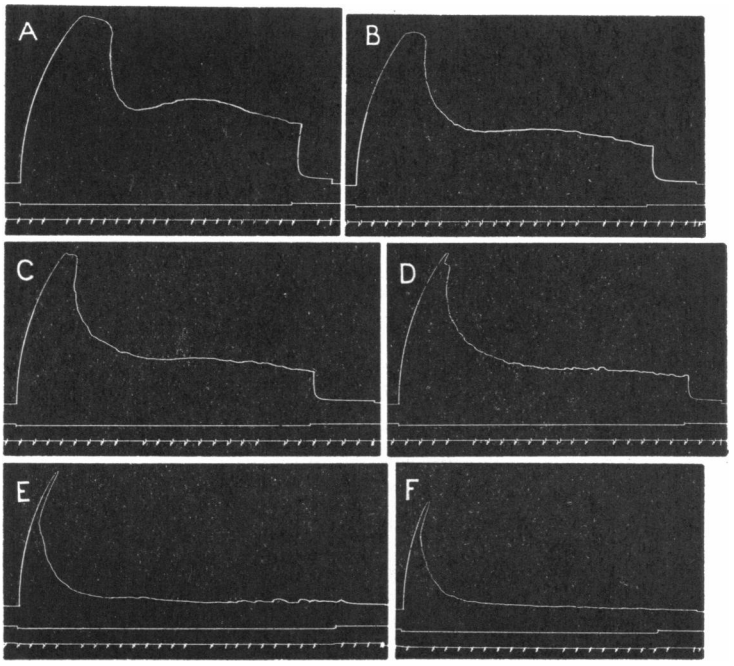


Fig. 11. 8. ii. 38. The action of phenyltrimethylammonium iodide upon the responses of a nerve-sartorius preparation. Time markings and signal as in Fig. 4. A, 2.45 p.m., nerve of Ringer-soaked preparation stimulated at a frequency of 150 per sec. with shocks of time-constant 73 μsec. 2.58 p.m., bath drained and then filled with solution containing 10 μM phenyltrimethylammonium iodide. B, 3.34 p.m., nerve stimulated as in A. 3.36 p.m., 0·05 c.c. of 5 mM phenyltrimethylammonium iodide solution added to the compartment which contained the greater part of the muscle to make the total concentration there 20 μM. C, 3.59 p.m., nerve stimulated as in A. 4.13 p.m., phenyltrimethylammonium iodide added to make the concentration in the compartment which contained the greater part of the muscle 50 μM. D, 4.38 p.m., nerve stimulated as in A. 5.51 p.m., phenyltrimethylammonium iodide concentration raised to 200 μM. E, 6.21 p.m., nerve stimulation as in A. 6.23 p.m., phenyltrimethylammonium iodide concentration raised to 300 μM. F, 7.10 p.m., nerve stimulated as in A.

tension attained in the initial rise did not suffer further reduction, but as soon as the maximum had been attained the tension began to fall rapidly, after a short time the fall slowed momentarily and afterwards continued at a constantly diminishing rate, the secondary rise having disappeared (Fig. 11D). Generally the tension fell to about 10 p.c. of its initial

maximum during 20 sec. of stimulation. In responses recorded about ½ hr. later the initial maximum tension had not become much more reduced, but the fall which followed the maximum had become faster and the momentary slowing had disappeared: the tension fell at a constantly diminishing rate and reached 5–10 p.c. of its initial value after 20 sec. of stimulation. The treatment of preparations with 200 μM phenyltrimethylammonium iodide solution for 10–15 min. affected the response to nerve stimulation with 150 shocks per sec. to the same extent that the 50 μM solution affected it in an hour. In the course of the next 2 hr. the maximum tension developed in the initial rise became reduced to two-thirds to one-third of that given by the untreated preparation, and the fall which followed almost immediately after the maximum became faster in successive responses.

The results of stimulation at lower frequencies do not require detailed description. When 50 or 100 shocks per sec. were used, phenyltrimethylammonium iodide in the smaller concentrations reduced the tension developed in the later part of the response but left that developed at the beginning of the response practically unchanged.

The respective actions of curarine chloride in concentrations 0·004–0·01, 0·04, 0·2–0·4 μM were indistinguishable from those of phenyltrimethylammonium iodide in concentrations 10, 50, and 200 μM.

Using a stimulation frequency of 150 per sec. a 50 μM solution of tetramethylammonium iodide was also found to attack the secondary rise in preference to the initial rise. Since, however, the spontaneous recovery of preparations partly paralysed with this substance in small concentrations [Ing & Wright, 1931] would have been an unnecessary complication no time was given to detailed experiments.

Antagonisms between eserine-like and curare-like substances

The action of curare-like substances on the responses of nerve-muscle preparations which had been treated with an eserine-like substance.

The method employed was either to add the curare-like substance dissolved in a small volume of fluid (0·01–0·1 c.c.) to the solution of eserine-like substance already in the compartment of the bath which contained the greater part of the sartorius muscle, or else to remove the solution from the bath, add the curare-like substance to the solution, and replace it in the bath. These methods were chosen in order to avoid upsetting any equilibrium which might have been established between the preparation and the solution of eserine-like compound bathing it. Preliminary tests showed that once the response of a preparation had attained a consistently reproducible form after filling the bath with

a solution of an eserine-like compound, subsequent withdrawal and replacement of the solution produced no further change in the response. The changes to be described were, therefore, due solely to the added curare-like substance.

The antagonisms between prostigmine and curarine will be described first. Changes in the form of the response were most marked when a stimulation frequency of 150 per sec. was employed, and when preparations which had been immersed in 0·3 μM prostigmine solution for about 2 hr. were treated with 1–2 μM curarine chloride solution. In responses recorded about 10 min. after addition of the curarine chloride the tension developed in the initial rise was restored to approximately that which had been obtained before treatment of the preparation with prostigmine. In some experiments, in which the preparation was sensitive to curarine, the tension fell rapidly soon after the initial maximum had been attained; in others, in which the preparation was less sensitive to curarine, the tension remained at its initial maximum for as much as a second before the rapid fall occurred. The tension developed in the subsequent secondary rise, in responses of either type, was reduced considerably below the augmented value that had resulted from the treatment with prostigmine (cf. Fig. 12A, B). In responses recorded 10–15 min. later the tension developed in the initial rise remained unchanged, but the rapid fall began almost immediately the maximum had been attained and continued until the tension had reached its pre-stimulation value. Often a momentary slowing occurred soon after the fall had begun. The secondary rise had disappeared (Fig. 12C). In responses recorded in the course of another $\frac{1}{2}$ hr. the tension attained in the initial rise suffered a little diminution, and the fall which followed the maximum became accelerated. When, however, curarine chloride was used in concentrations greater than about 4 μM the restoration of the initial tension was of a more temporary nature. The responses passed through the stages described above, and then, in subsequent ones, the rapid initial rise of tension became smaller and eventually disappeared.

In experiments with preparations which had been immersed for about 2 hr. in 3 or 30 μM prostigmine solution, curarine restored the initial tension developed in response to stimulation at a frequency of about 150 shocks per sec. in the same way as described above. I did not observe, however, in four experiments, any restoration of the secondary rise of tension after it had been suppressed by the action of prostigmine alone. The concentration of curarine chloride needed to restore the initial tension was the same, within the limits of the experimental method,

as that needed in the experiments with preparations which had been treated with 0·3 μM prostigmine solution, and, as in those experiments, the restoration produced by curarine chloride in concentrations greater than 4 μM was only a transient one. Rothberger [1901], and Langley & Kato [1915] have shown that the reversal of curare paralysis by eserine is also limited with respect to the amount of curare used.

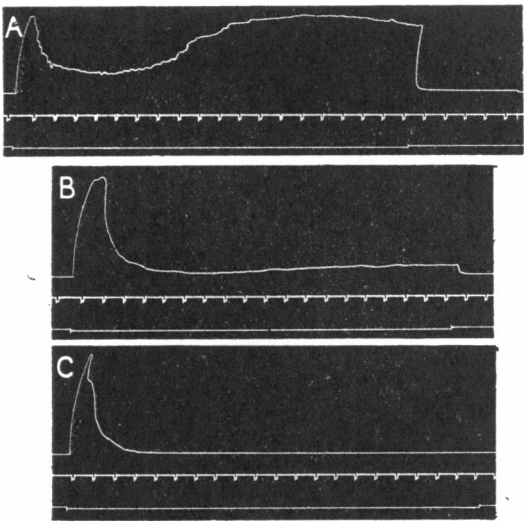


Fig. 12. 5. ii. 36. The action of curarine upon the responses of a nerve-sartorius preparation that had previously been treated with prostigmine. The time markings and signal as in Fig. 4. The preparation had been immersed in Ringer's solution containing 0·3 μM prostigmine for 1¾ hr. A, 4.52 p.m., nerve stimulated with maximal shocks of time-constant 109 μsec. at a frequency of 150 per sec. 4.55 p.m., 0·03 c.c. of millimolar curarine chloride solution added to the compartment of the bath which contained the greater part of the muscle to make the concentration there 1·2 μM. B, 5.2 p.m., nerve stimulated as in A. C, 5.14 p.m., nerve stimulated as in A.

Changes of the same type as those described in the two preceding paragraphs could also be produced in the responses of prostigmine-treated preparations by tetramethylammonium iodide in concentrations 100–200 μM, or phenyltrimethylammonium iodide in concentrations 250–500 μM. Similar changes in the response of a preparation which had been treated with any of the other eserine-like compounds were produced by any of the three curare-like compounds in the concentrations stated above. The antagonisms between the three curare-like compounds and Preparation 1210/1 were found not to differ qualitatively from the other antagonisms already mentioned. The restorative action of

phenyltrimethylammonium iodide upon the initial tension in the response of a preparation that had been treated with Preparation 1210/1 is shown in Fig. 9C, D.

The action of eserine-like substances upon the responses of preparations that had previously been treated with curare-like substances.

These experiments were made to determine whether the relative activities of the eserine-like substances in restoring the responses of preparations which had been nearly paralysed by a curare-like substance were of the same order as their activities on preparations which had merely been soaked in Ringer's solution. The method was the following one. The response of a Ringer-soaked preparation to maximal stimulation of its nerve for 1 sec. at a frequency of 100 per sec. was recorded two or three times at 10 min. intervals, then the bath was drained, filled with curarine chloride solution, and responses to further stimulation were recorded at 15 min. intervals. Soaking for about an hour in a 0·5–1·0 μM curarine chloride solution (usually 0·8 μM) sufficed to bring the preparation to a fairly steady or very slowly declining state in which the maximum tension developed in the response was reduced to about 10 p.c. of its pre-treatment value. When this state had been reached enough of the eserine-like substance whose action was to be examined, dissolved in a small volume of fluid, was added to the compartment of the bath which contained the greater part of the muscle to make the concentration there equal to the mean of the figures given in column 4 of Table I and then the recording of responses to further test periods of stimulation, at 15 min. intervals, was continued for about another hour. The results of these experiments are summarized in column 7 of Table I. The degree of recovery attained in each experiment is expressed as the ratio of the maximum tension developed by the preparation after treatment with an eserine-like compound to the tension developed by the preparation whilst under the influence of curarine alone, both tensions being stated as percentages of the maximum given by the preparation before it was treated with curarine chloride. It is probable that the depressant action of Substance 37 upon muscle partly accounts for the small degree of recovery produced in the experiments with this compound. A second series of experiments in which 300–400 μM phenyltrimethylammonium iodide was used instead of curarine chloride gave similar results. These are summarized in column 7 of Table I.

Discussion

The relation found in the experiments with isolated and Ringer-soaked nerve-muscle preparations between the frequency at which the nerve was stimulated and the form of the response is similar, in the main, to that found by Wedensky [1885, 1886, 1891] and by Hofmann [1903*a*] with frog's sciatic-gastrocnemius preparations. Wedensky used isolated preparations, and the majority of his experiments were complicated by muscular fatigue. Hofmann, on the other hand, in order to reduce muscular fatigue to a minimum, used preparations which enjoyed a blood supply, or isolated preparations which were allowed long rest intervals between stimuli. The variations in the form of the response exhibited by different preparations to stimulation at any one frequency, particularly frequencies higher than 60 per sec., were smaller in my experiments than in Hofmann's. There are three reasons which probably account for this difference: the relatively constant temperature at which the "warm" frogs were kept before use; the long preliminary soaking of the preparations in the Ringer's solution (it will be shown in a subsequent communication that changes in the composition of the Ringer's solution produce marked changes in the form of response); the maintenance of the bath at a fairly constant temperature during experiments. If a quantitative comparison is made of the amount of activity that Hofmann's circulated preparations and my isolated sartorius preparations could undergo before their reponses began to deteriorate then there appears a discrepancy which perhaps calls for comment. Hofmann states that his preparations began to show signs of "fatigue" after only 1–2 hr. of the activity resulting from test stimuli of 2–5 sec. duration applied at intervals of 5–10 min. Since Hofmann used intact unanæsthetized frogs it seems unlikely that the relatively early failure of his preparations can have been due to an inadequate blood supply; also he states that he took care not to impede the blood flow when fixing the leg under experiment. The most plausible explanation that I can suggest is based on some observations of Forbes & Ray [1923]. They found that nerve trunks which were allowed to remain in contact with injured tissues soon became inexcitable. Hofmann states that to prevent drying of the nerve in the intervals between the stimuli he lowered it into the wound. Possibly this procedure rendered some of the motor nerve fibres inexcitable and so gave the changes that he attributed to fatigue. Whether or not this is the correct explanation of the discrepancy, an isolated nerve-sartorius preparation, weighing about 70–80 mg. exclusive of bone,

which can develop and maintain a mean tension of about 50 g. for 15–20 stimulation periods each lasting about 15 sec., can hardly be regarded as so abnormal as not to merit investigation.

The question of whether prostigmine used in high concentrations causes the action current of nerve to increase must be left to further experiment. The observations that prostigmine in lower concentrations did not affect the nerve action current, that treatment of the nerve only of a nerve-muscle preparation produced no change in the response, and that the responses of nerve-muscle preparations which had been immersed in 3000 μM solution for $1\frac{1}{2}$ hr. suffered no further change of form within another 14–16 hr., indicate that if prostigmine has any action it is very small. Evidence of a similar kind suggests that eserine and Substance 38 are without action on nerve. Also, none of the experiments with the remaining eserine-like compounds suggests that any of them affects nerve.

The changes produced by 0·2–0·4 μM curarine chloride in the responses of isolated nerve-sartorius preparations during the first 5 sec. of stimulation at various frequencies resembled those which Hofmann found to occur in the responses of his circulated sciatic-gastrocnemius preparations after the injection of small doses of curarine chloride [1903*a*]. More recently Bremer & Titeca [1935] have found that the muscles of preparations which had been curarized to a slightly greater degree than had Hofmann's were unable to maintain tension when their nerves were stimulated at a frequency of 10 per sec. Hofmann found also [1903*b*] that curarine chloride in greater doses brought preparations to a state in which a single nerve volley elicited no response from the muscle, but repetitive stimulation caused contraction. Boehm [1895] has examined the action of curarine on the responses of isolated sciatic-gastrocnemius preparations to stimulation at very low frequency—about one shock in 2 sec. He found that the maximum tension developed in successive twitches of a preparation which had been treated with a fairly heavy dose of curarine chloride quickly diminished to zero, whereas the tension developed in successive twitches of an untreated preparation diminished slowly. Boehm found that after allowing the curarine-treated preparation to rest for 10–15 min. the maximum tension recovered to its initial value and that it diminished more quickly in a second series of twitches. After a second rest interval of 10–15 min. the tension again recovered, and in a third series of twitches it diminished more quickly than in the second series.

There is no evidence that prostigmine exerts a true curare-like action at any of the concentrations examined [cf. Eccles, 1936]. By the use

of a stimulation frequency of about 150 per sec. it has been possible to show that prostigmine and curarine in their lower effective concentrations attack the responses of nerve-muscle preparations in different ways. When we come to consider the actions of the two drugs in relatively great concentrations, Boehm's observations on curarine-treated preparations may be contrasted with my observations on the tension developed in series of twitches given by preparations that had been treated with 3000 μM prostigmine solution. If a line be drawn through the summits of the twitches of a prostigmine-treated preparation the form of the tension curve so obtained is very similar to that exhibited by preparations which were treated with 0·3 μM prostigmine solution and afterwards stimulated at a frequency of 150 shocks per sec., although the time-scales differ considerably. So also, the form of curve passing through the summits of the twitches given by Boehm's curarine-treated preparations resembles that of the tension-time curves of preparations which were treated with curarine in very small concentration and afterwards stimulated at a frequency of 150 per sec. It seems probable that, by selection of a suitable stimulation frequency, characteristic differences between the responses of prostigmine-treated and curarine-treated preparations can always be demonstrated. Apart from the foregoing differences, the actions of eserine and of Substance 37, neither of which compounds contains a quaternary ammonium grouping, upon the form of response to nerve stimulation were qualitatively indistinguishable from that of prostigmine. Finally, 3000 μM prostigmine solution caused augmentation of the twitches elicited from a "cold" frog preparation.

The interpretation of the observations described in this paper in terms of the hypothesis that acetylcholine is the transmitter at motor nerve endings necessitates certain suppositions additional to those suggested in the opening paragraphs. Following the demonstration by Dale *et al.* that an ACh.-like substance is liberated at frog's motor endings, Brown [1937] has shown that the rapid intra-arterial injection of small amounts of ACh. into frog muscle sets up propagated excitation in the muscle: he has also shown that a temporary block of neuro-muscular transmission occurs after the injection of larger amounts of ACh. In Hofmann's experiments with circulated preparations and in mine with Ringer-soaked but otherwise untreated preparations, the relatively rapid fall of tension which followed the initial rise in the responses to nerve stimulation at a frequency of 100 per sec. and over may have been due to successive volleys liberating ACh. more quickly than the agencies responsible for its removal could deal with it. The secondary rise of

tension during the response to nerve stimulation at a frequency of about 150 per sec. might be attributed to a progressive diminution on the amount of ACh. liberated per nerve impulse, which would allow the rise and fall of ACh. concentration at the nerve ending to return to more normal limits. If an analogy is permitted then there is one which is very suggestive. Brown & Feldberg [1936] found that during stimulation of the pre-ganglionic fibres of the cat's superior cervical ganglion, which was perfused with Locke's solution containing eserine, the output of ACh. diminished rapidly and that within the same time the myogram of the nictitating membrane exhibited an initial rise, followed by a rapid fall, a slower secondary rise, and afterwards by a slow fall (cf. Fig. 5 above with Fig. 1 of their paper). To suppose that the ability of the frog's motor nerve ending to liberate ACh. in response to nerve impulses diminishes as a result of successive impulses and is restored again only after a relatively long rest interval would also be consistent with an observation of Wedensky's. He found [1886] that if the stimulation of a gastrocnemius-sciatic preparation at a frequency of about 130 per sec. were interrupted for a few seconds as soon as the secondary rise had become well developed and then recommenced, the maximum tension reached in the third rise was about the same as in the initial rise, but that after the third maximum the tension fell much more slowly than after the initial maximum and no secondary rise occurred. In my experiments with frog's nerve-muscle preparations the observation that the tension developed in response to nerve stimulation at low frequencies diminished more quickly than in response to direct stimulation of the muscle probably indicates that in the earlier volleys the amount of ACh. liberated per nerve impulse is supraliminal at most motor endings, but that later it becomes subliminal at some of the endings. If the action of curarine is to raise the threshold of the part of the muscle near the nerve ending to ACh. (and perhaps the threshold of other parts of the muscle also) the reduction of the later part of the response to nerve stimulation by curarine in low concentrations would be consistent with the liberation of supraliminal amounts of ACh. at most nerve endings by the earlier nerve impulses and with the liberation of smaller amounts of ACh. by later impulses. The action of prostigmine in causing the fall of tension which followed the initial rise in the response to nerve stimulation at a frequency of about 150 per sec. to occur earlier and to become deeper, also the augmentation of the secondary rise, can be explained either by the sensitization of the muscle to ACh. or by the inhibition of choline-esterase (see below). However, the observation that prostigmine caused

the secondary rise to occur earlier than in the responses of untreated preparations is the opposite of what would have been expected. To account for this it seems necessary to postulate that after a short time the part of the muscle fibre near the nerve ending becomes so adapted that the failure of the ACh. concentration to rise and fall within its normal limits no longer results in a junctional block. It seems, moreover, that such an adaptation can only occur in a limited degree, since increase of stimulation frequency or larger doses of prostigmine caused the secondary rise to disappear. The action of curarine in restoring the initial tension in the responses of preparations which had been treated with 0·3 μM prostigmine solution to stimulation at a frequency of about 150 per sec., and in reducing the secondary rise, are consistent with a raising of the threshold of muscle to ACh. and with the liberation of greater amounts of ACh. by the early nerve impulses than by the later ones.

The above interpretation would apply equally well to the actions of the remaining eserine-like and curare-like compounds.

Observations which differ little from those described here have been made by other workers using blood-circulated mammalian nerve-muscle preparations. The actions of eserine and of prostigmine in small and in moderate doses on the responses to nerve stimulation, for 3 sec. periods, at high and at low frequencies, have been examined by Briscoe [1936]. Rosenblueth *el al.* [1936], Bacq & Brown [1937], Rosenblueth & Morrison [1937] have also found that eserine or prostigmine in moderate doses causes the response to nerve stimulation at medium or high frequencies to become a twitchlike rise and fall, followed by nearly zero tension. Briscoe has shown that the initial part of such depressed responses may be restored by curarine (also Rosenblueth & Morrison). The action of curarine on the responses to stimulation of the nerves of preparations at various frequencies has been examined by Hofmann [1903*b*]. Brown *et al.* [1936] and Bacq & Brown [1937] have shown that eserine in small doses augments the twitches (see also below) in response to nerve stimulation at a rate of 6 shocks per min., and that larger doses cause augmentation of the first few twitches to be followed by depression. Rosenblueth *et al.* [1936] and Rosenblueth & Morrison [1937] have shown that if stimulation at low frequency is continued a slow recovery and a very slow fall follow the depression. Rosenblueth & Morrison have shown that if curare is used instead of eserine, then the heights of successive twitches diminish soon to zero.

Rosenblueth & Morrison, taking into account observations such as are summarized in the preceding paragraph, additional observations

described in their paper, the demonstration by Dale *et al.* that an ACh.-like substance is liberated at mammalian motor nerve endings, and the demonstration by Brown *et al.* [1936] that mammalian muscle may give twitchlike responses to rapidly injected ACh., have presented an interpretation of neuro-muscular transmission which differs in two respects from that given above of the observations on frog preparations. They have assumed that the action of eserine or prostigmine is to inhibit choline-esterase at the nerve endings. The results have not made it necessary to postulate that a partial adaptation can occur if the concentration of ACh. at the nerve ending fails to rise and fall within its normal limits. However, I think that the experimental evidence relative to this matter is not yet sufficiently clear. Rosenblueth & Morrison state that using cats anæsthetized with "Dial", stimulation of the nerve of a nerve-muscle preparation at a frequency of about 240 per sec. elicited a response in which a rapid fall, a secondary rise, and then a slow fall of tension followed the initial rise. Hofmann [1903*b*], on the other hand, found that rabbit nerve-muscle preparations (presumably he used unanæsthetized animals) gave steady responses to nerve stimulation at frequencies up to 260 per sec. Hofmann also found that when ether was used, there appeared in the response a rapid fall after the initial rise, and a secondary rise followed by a slower fall. From the evidence given in Hofmann's paper it is clear that this action of ether was not due to a local depression of the nerve trunk such as was studied by Wedensky [1903], but that it probably involves the whole nerve-muscle complex. As it seems unlikely that cat and rabbit preparations can differ much in their ability to respond to high-frequency stimulation it is desirable to know whether the type of response observed by Rosenblueth & Morrison is also characteristic of unanæsthetized cat preparations and, if it is, the effects of small doses of eserine-like substances on its form.

The "cold" frog preparations resembled cat preparations in that prostigmine augmented the early twitches in response to stimulation of the nerve at very low frequency [Brown *et al.* 1936; Rosenblueth & Morrison, 1937]. Evidence that the augmentation of the twitch is due to repetitive response of the muscle to a single nerve volley, and the conditions favouring this form of response by frog preparations, will be presented in a subsequent communication. The augmentation of the early part of the responses of "cold" frog preparations to nerve stimulation at low frequencies after treatment with prostigmine or eserine was probably due to multiple response to some of the nerve volleys. Rosenblueth & Morrison have examined the electrical responses of cat muscle

during low-frequency stimulation of its nerve (30 per sec.), and they found that prostigmine caused some of the responses to become multiple.

Marnay & Nachmansohn have obtained evidence that the choline-esterase present in frog muscle [1937*a*, *b*, *c*; 1938] and in lizard muscle [1937*d*, 1938] is concentrated near the nerve endings. Marnay *et al.* [1937], and Marnay & Nachmansohn [1938] have obtained similar evidence concerning the distribution of choline-esterase in mammalian muscle. Whilst in the experiments with frog sartorius preparations there was a fairly good parallel between the activities of the eserine-like compounds upon the responses to nerve stimulation at the rate of 150 shocks per sec. and in reversing curariform paralysis, there is not such a clear parallel between these estimates and Dr Stedman's estimates of the choline-esterase-inhibiting activities of the compounds. Prostigmine, Substance 38, and Preparation 3393, three substances which have anti-esterase activities of the same order, which contain the dimethylcarbamic ester grouping, and which are quaternary ammonium salts also exhibited approximately the same activity on neuro-muscular transmission. Substance 36 which possesses an anti-esterase activity one-third to one-half of that of prostigmine, which contains a phenylmethylcarbamic ester grouping, and which is a quaternary ammonium salt exhibited an activity about one-third of that of prostigmine on neuro-muscular transmission. Eserine, however, which has a rather higher anti-esterase activity than prostigmine, which contains a methylcarbamic ester grouping, and which is the salt of a tertiary base, exhibited only about one-fifth of the activity of prostigmine on neuro-muscular transmission. Also, Substance 37, which contains a dimethylcarbamic ester grouping, and which is the salt of a tertiary base exhibited on neuro-muscular transmission only about one-hundredth of the activity that would have been expected on the basis of its anti-esterase activity. Preparation 1210/1, which is both a phenol and a quaternary ammonium salt, exerted on neuro-muscular transmission an activity which was about 10,000 times what would have been expected from Dr Stedman's figures. Woldemar Hoffman [1886] found that phenol exerted an anti-curare action. Rothberger has shown [1902] that a number of phenols and cresols have anti-curare actions. Two main possibilities arise in connexion with Preparation 1210/1. It may be that this compound affects neuro-muscular transmission in some way other than by inhibition of choline-esterase; alternatively it may be that Dr Stedman's choline-esterase concentrates [Stedman & Stedman, 1935] contained substances which adsorbed Preparation 1210/1 and so protected the esterase. The relatively greater activity of the urethane

compounds which are also quaternary ammonium salts than of the urethane compounds which are also salts of tertiary bases, on neuro-muscular transmission may have been due to some specific action of the quaternary grouping, or to adsorption of the two kinds of compound to different extents by different parts of the nerve-muscle complex close to and remote from the nerve endings. It is to be noted that Bacq & Brown [1937] have examined the actions of a series of compounds of graded anti-esterase activity, using cat's blood-circulated nerve-muscle preparations, and that they found a fairly good parallel between anti-esterase activity and the minimal doses necessary to cause the response to a single nerve volley to become repetitive.

Summary

1. Treatment of frog's isolated nerve with prostigmine, eserine, or another eserine-like substance, produced practically no change in the action current elicited by maximal repetitive stimulation at frequencies ranging from 5 to 200 per sec. The action current was summed and observed by means of a galvanometer.

2. The myograms given by frog's nerve-sartorius preparations, when immersed in glucose-containing bicarbonate-buffered Ringer's solution, in response to maximal (nerve) stimulation, at high and at low frequencies, exhibited good reproducibilities, even after considerable activity. When stimulation frequencies between 15 and 200 per sec. were used three main types of response could be distinguished: with 15–60 per sec. the tension rose to a maximum and then fell slowly; with 100–180 per sec. the tension rose to a maximum, fell for a few seconds, rose again (secondary rise), and afterwards fell very slowly; with about 200 per sec. the tension rose to a maximum which was generally smaller than that attained with lower frequencies, and fell rapidly almost to zero. In the same experiments the responses of the sartorius muscle to direct stimulation were found to be influenced little by changes of stimulation frequency.

3. The actions of prostigmine, eserine, and five other eserine-like compounds, in various concentrations, upon the responses of nerve-sartorius preparations to stimulation at frequencies between 15 and 200 per sec. have been examined. The main action of any of these compounds was to lower the frequency required to produce the second and third types of response mentioned above in (2). In connexion with the hypothesis that acetylcholine is the chemical transmitter at motor nerve endings the action of small doses of the eserine-like substances on the

response to stimulation with 150 shocks per sec. seems of special interest: the tension developed in the initial part of the response was depressed, the minimum which followed was made smaller, and the secondary rise was augmented. The relative activities of prostigmine and the other eserine-like substances on the nerve-sartorius preparation do not exhibit a simple parallel to Dr Stedman's estimates of their inhibitory activities on his choline-esterase preparations.

4. The actions of curarine, and of phenyltrimethylammonium iodide, in low concentrations, upon the responses of nerve-sartorius preparations, to stimulation at frequencies between 50 and 150 per sec. have been examined. These substances, in contrast to the eserine-like substances, little affected the initial part of the response to stimulation at a rate of 150 per sec., but they reduced the secondary rise.

5. The actions of curarine, of phenyltrimethylammonium iodide, and of tetramethylammonium iodide, upon the responses of nerve-sartorius preparations which had previously been treated with an eserine-like compound, to nerve stimulation have been described.

6. The relative activities of the eserine-like compounds in reversing paralysis due to curarine or to phenyltrimethylammonium iodide were of the same order as their activities on the responses of Ringer-soaked preparations (see (3) above).

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