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THE MECHANISM OF ANTICURARE ACTION OF CERTAIN NEOSTIGMINE ANALOGUES

BY

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Recently Randall and Lehmann (1950) and Randall (1950) reported on a number of neostigmine analogues which had a striking anticurare action in cats and dogs *in vivo* but were only weak cholinesterase inhibitors *in vitro*. Their findings were confirmed by McFarlane, Pelikan, and Unna (1950) and Artusio, Riker, and Wescoe (1950) in man, and by Riker and Wescoe (1950) and Wescoe, Riker, and Brothers (1949) in cats. The compounds these authors had studied were either hydroxy anilinium salts or esters of these salts (excluding the carbamic esters). It was found that these substances differed from neostigmine in that the peak of anticurare action was reached within a minute after the injection and that side effects, e.g. salivation and bradycardia, were either negligible or absent. A further advantage was that they could be given at relatively short intervals without loss of their anticurare potency or a decrease in the sensitivity of the endplate to *d*-tubocurarine.

It is now generally held that tubocurarine blocks neuromuscular transmission by competing with acetylcholine for the receptors on which the latter acts. In this way it raises the threshold for stimulation. This blocking effect of tubocurarine can be overcome by anticholinesterases, dyes, potassium salts and phenols, or by substances which stimulate the endplate, each type of compound exerting an anticurare action by a different mechanism. There was difficulty in attributing the action of the neostigmine analogues to any of the known anticurare mechanisms. Randall and Lehmann (1950) thought that the anticholinesterase activity—about 1/100th or less of that of neostigmine—was too small to account for the anticurare action, which was about one quarter that of neostigmine ; these authors also excluded a stimulant action on the endplate, because, although some of the compounds caused a contracture of the muscle in small concentrations, others had no stimulant properties whatsoever. Riker *et al.* (1949), Wescoe *et al.* (1949), Artusio *et al.* (1950), and McFarlane *et al.* (1950), studying only compounds with good stimulating properties, took the view that the anticurare action could be entirely accounted for by a stimulant action on the endplate. Randall (1950) in continuation of his work came to similar conclusions, but also considered the possibility that the free phenolic group, present in many of the compounds, might be of importance.

In the experiments to be described I have studied four of the neostigmine analogues in normal and curarized cats, on the isolated phrenic nerve diaphragm of the rat, on the rat intestine, and on the frog rectus.

Methods and Materials

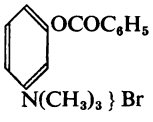
Cats were anaesthetized with 80 mg. chloralose/kg. (i.v.) or 50 mg. pentobarbitone/kg. (i.p.). Records of the tension of the tibialis anterior muscle were taken on a smoked drum by means of an isometric steel spring myograph. The muscle was stimulated by supramaximal square pulses of 0.25 msec. duration, applied to the nerve by shielded silver electrodes. Injections were given i.v. by means of a cannula inserted into the superficial jugular vein.

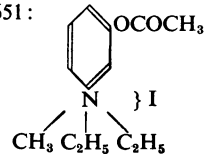
The phrenic nerve diaphragm preparation of the rat (Bülbring, 1946) was set up in Tyrode solution containing 0.2 per cent (w/v) glucose and aerated with a 95 per cent $O_2 + 5$ per cent CO_2 mixture.

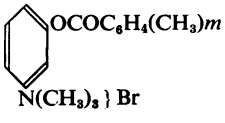
The frog rectus was set up as described by Chang and Gaddum (1933). The isolated rat intestine was suspended in aerated Tyrode solution and the contractions of the longitudinal muscle alone were recorded.

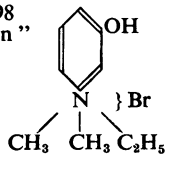
For estimating anticholinesterase potency *in vitro* the manometric technique (Ammon, 1933) was used. Lysed human red cells and human serum were used as enzyme sources and *dl*-acetyl-β-methylcholine (final concentration 0.03 M) and benzoylcholine (final concentration 0.01 M) as substrates. Red cell esterase is referred to in the text as cholinesterase I and serum cholinesterase as cholinesterase II.

The following neostigmine analogues were studied :

Ro 2–2650:  = 3-benzoxy-N-trimethyl-anilinium bromide.

Ro 2–2651:  = 3-acetoxy-N-methyl-diethyl-anilinium iodide.

Ro 2–2783:  = 3-m-toluyloxy-N-trimethyl-anilinium bromide.

Ro 2–3198 "Tensilon"  = 3-hydroxy-N-dimethyl-ethyl-anilinium bromide.

All four compounds were pure crystalline substances, readily soluble in water ; solutions of them were freshly made immediately before use. Neostigmine was used as the methylsulphate. Throughout the text the doses and concentrations given refer to the salts.

RESULTS

The actions of neostigmine analogues in cats

Curarized cats.—In cats in which the intravenous (i.v.) injection of *d*-tubocurarine had reduced the response of the muscle to indirect stimulation by 50 per cent or more the i.v. injection of Ro 2–2650, Ro 2–2651, Ro 2–2783, or Ro 2–3198 caused a rapid increase in twitch tension after a latent period of 30 to 60 sec. (Fig. 1). The anticurare activity of Ro 2–2651 and Ro 2–3198 was about a fifth of that of neostigmine ; Ro 2–2650 and Ro 2–2783 were slightly weaker, their potency being about a tenth to a fifteenth of that of neostigmine. With all four compounds a maximum anticurare action was reached within one to two minutes of injection, after which the twitch tension began to fall off ; this decay was more rapid with Ro 2–2651 and Ro 2–3198 than with the other two analogues. On the other hand, neostigmine differed from its analogues in that the maximum anticurare action with a given dose was not reached until six to ten minutes after the injection ; the twitch tension then remained constant and no recurrence of curarization was noticeable.

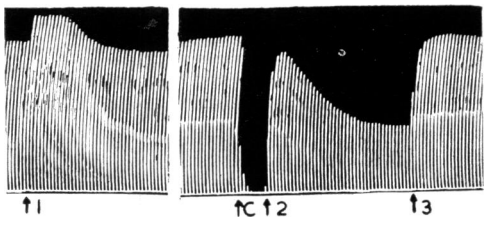


FIG. 1.—Cat, 3.7 kg. Pentobarbitone. Record from Musc. tibialis anterior. Frequency of stimulation 1 in 10 sec. ↑ : 1 mg. Ro 2–3198 i.v. at 1, 2, and 3. ↑ C: 700 μg. *d*-tubocurarine chloride i.v.

If decamethonium was used in order to interrupt neuromuscular transmission, neostigmine and its analogues did not restore twitch tension. These experiments exclude the possibility that the neostigmine analogues improve the twitch tension of a curarized muscle by an action beyond the endplate, i.e., by an action on the muscle fibre itself.

In order to see how much of the anticurare action was due to inhibition of cholinesterase cats were first given an injection of 2 mg. atropine per kg. and then 2–5 mg. tetraethylpyrophosphate per kg. were injected i.v. Such treatment completely abolished the anticurare action of neostigmine and its analogues. Acetylcholine, decamethonium, and choline, however, which are supposed to counteract the blocking action of tubocurarine by their stimulant action on the endplate (Wilson and Wright, 1937 ; Hutter, 1951) still restored the twitch tension to a considerable extent (Fig. 2).

Normal cats.—All the evidence obtained from the experiments described above suggested that the anticurare action of the neostigmine analogues was more likely to be due to cholinesterase inhibition than to an acetylcholine-like action upon the endplate. If that were so animals untreated with tubocurarine would be expected to show definite signs of cholinesterase inhibition after treatment with these drugs ; this was, however, only partly true.

In normal cats the i.v. injection of Ro 2–2651 and Ro 2–3198, in doses which had a significant anticurare action, caused a rapid increase in twitch tension up to 20 per cent above the original tension with a return to normal within three to five

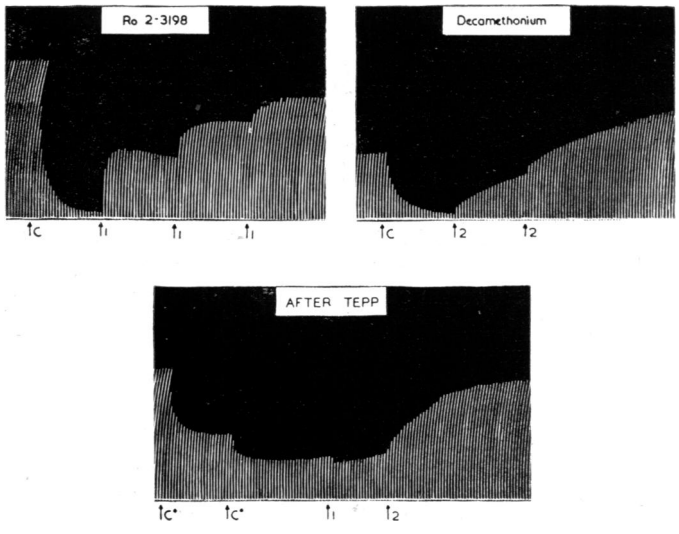


FIG. 2.—Cat, 3.5 kg. Chloralose. Record from Musc. tibialis anterior. Frequency of stimulation 1 in 10 sec.; 2 mg. atropine/kg. ↑C: 700 μg. *d*-tubocurarine chloride i.v. ↑C′: 1 mg. *d*-tubocurarine chloride i.v. ↑1: 1 mg. Ro 2–2651 i.v. ↑2: 40 μg. decamethonium iodide i.v. TEPP: 5 mg. tetraethylpyrophosphate i.v.

minutes (Fig. 1). At the same time the cats showed fibrillations all over the body and action potentials taken from the muscle were markedly repetitive in character, but only a very slight increase in the activity of the viscera innervated by the parasympathetic system was noticeable. Ro 2–2650 and Ro 2–2783 increased the twitch tension to a much smaller extent, and likewise the muscle action potentials showed less marked changes than were seen with the two other analogues. Neostigmine, on the other hand, in doses which had the same anticurare action potentiated the twitch tension to a much greater extent (up to 50 per cent of the original tension), the maximum effect developing after a considerable time-lag. The muscle action potentials were of repetitive character and a marked increase in the activity of the viscera innervated by the parasympathetic system was seen.

In view of these results it was therefore desirable to obtain more information about the mechanism of anticurare action of the neostigmine analogues on the two preparations widely used for such a purpose, i.e., the isolated phrenic nerve diaphragm of the rat (Blaschko, Bülbring, and Chou, 1949) and the isolated frog rectus (Hobbiger, 1950).

The action of neostigmine analogues on the isolated phrenic nerve diaphragm of the rat

The neostigmine analogues could be divided into two groups according to their action on the normal diaphragm :

(*a*) The first group consisted of Ro 2–2651 and Ro 2–3198. Both these substances potentiated the twitch tension in doses of 1 μg./ml. and higher (Fig. 3). Compared with neostigmine the two analogues were about fifty times weaker in this property.

(*b*) The second group consisted of Ro 2–2650 and Ro 2–2783. These substances did not potentiate the twitch tension in any concentration. If the concentration

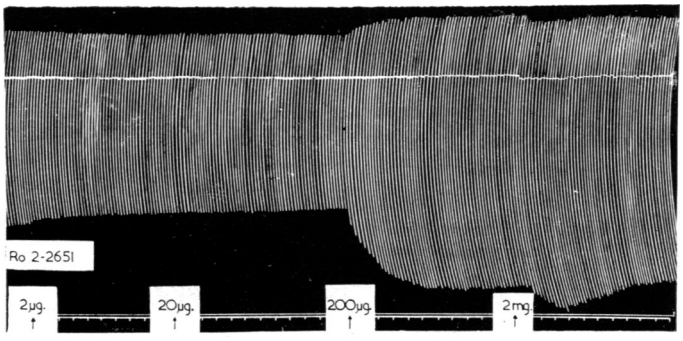


FIG. 3.—Isolated rat phrenic nerve diaphragm. Effect of Ro 2–2651 on the response to supramaximal stimulation of the nerve at a frequency of 1 in 10 sec. The figures give the total amount of Ro 2–2651 added to the bath (volume 100 ml.).

was as high as 10 μg./ml. the twitch tension of the diaphragm rapidly declined (Fig. 4). This effect was readily reversed by removal of the compounds from the bath fluid.

When the response of the diaphragm to tetanic stimulation was used as an indicator of cholinesterase inhibition (Burgen and Hobbiger, 1951) some recovery of the diaphragm was seen in the presence of Ro 2–3198 and Ro 2–2651 ; this was due to the disappearance of the compounds from the bath fluid, but the extent of this recovery was too small to account for the short duration of action *in vivo*.

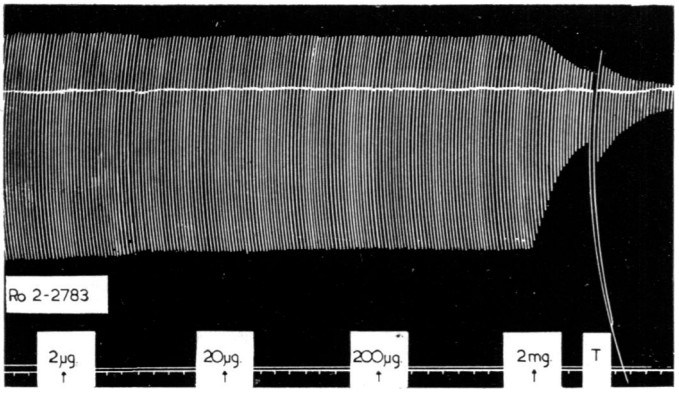


FIG. 4.—Isolated rat phrenic nerve diaphragm. Effect of Ro 2–2783 on the response to supramaximal stimulation of the nerve at a frequency of 1 in 10 sec. The figures give the total amount of Ro 2–2783 added to the bath (volume 100 ml.). T = Tetanic stimulation (100 stimuli/sec.) for 5 sec.

Anticurare action on the diaphragm

All four neostigmine analogues antagonized the actions of tubocurarine on the diaphragm in concentrations of 1 μg./ml. and higher. Ro 2–2650 and Ro 2–2783 were significantly weaker in this respect than Ro 2–2651 and Ro 2–3198. Again, as in the cat the onset of anticurare action was much more rapid with the neostigmine analogues than with neostigmine. With Ro 2–2650 and Ro 2–2783, once tubocurarine had reduced the twitch tension by 50 per cent or more, recovery was only partial, regardless of the dose used (Fig. 5). With Ro 2–2651 and Ro 2–3198 recovery was more complete and these drugs had about one-fiftieth of the anticurare potency of neostigmine (Fig. 6).

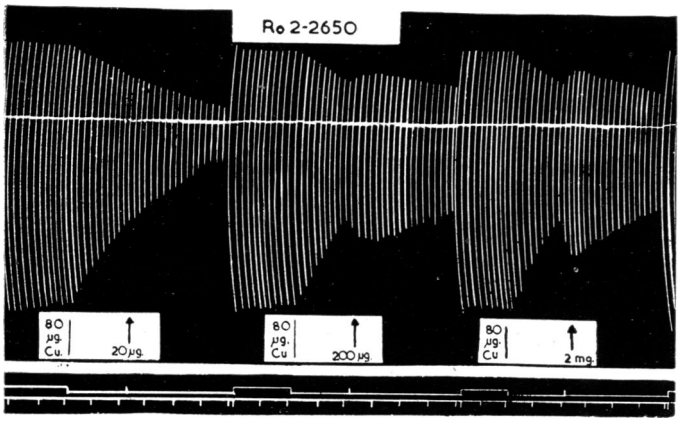


FIG. 5.—Anticurare action of Ro 2–2650 on the isolated phrenic nerve diaphragm preparation. 80 µg. Cu = 80 µg. *d*-tubocurarine chloride. 20 µg., 200 µg., and 2 mg. are the amounts of Ro 2–2650 added to the bath (volume 100 ml.). Time in min.

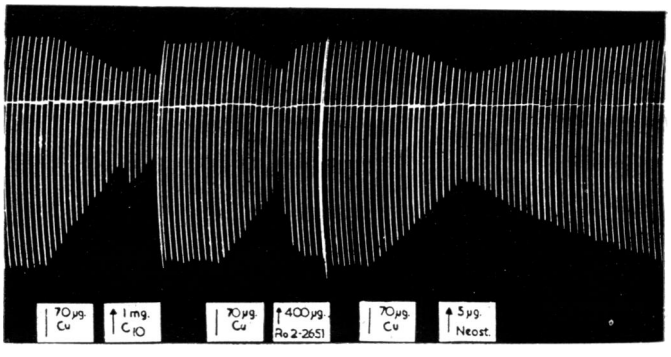


FIG. 6.—Anticurare action of decamethonium iodide (C_{10}), Ro 2–2651, and neostigmine methylsulphate on the isolated rat phrenic nerve diaphragm. The doses refer to total amounts added to the bath (volume 100 ml.).

For Ro 2–2651 and Ro 2–3198 the ratio of the stimulant to the anticurare dose was exactly the same as for neostigmine. The anticurare action of Ro 2–2650 and Ro 2–2783, however, could only be explained by assuming that these substances had two actions, one on cholinesterase and one on the endplate itself. On the normal diaphragm these actions would oppose each other, but in the presence of tubocurarine the anticholinesterase action might predominate because of the less favourable conditions for an action on the endplate. Such dual effects of Ro 2–2650 and Ro 2–2783 could be clearly demonstrated on the frog rectus.

To find out how much of the anticurare action of the neostigmine analogues was due to a stimulant action on the endplate, substances which act predominantly in such a way were studied. It was found that acetylcholine and choline had no anticurare action whatsoever and decamethonium gave only a very short-lasting improvement (Fig. 6), which was always much less than that seen with the neostigmine analogues.

Anticurare action on the diaphragm after DFP

In order to demonstrate that the anticurare action of the neostigmine analogues on the diaphragm was really due only to an inhibition of cholinesterase, the cholinesterase of the diaphragm was inactivated by di*iso*propylfluorophosphonate (DFP).

In these experiments DFP was added to the bath for one hour in a concentration of 20 μg./ml. A diaphragm which had been treated in this way still responded after removal of the DFP to single stimuli, but was unable to sustain a tetanus (Evans, 1951 ; Burgen and Hobbiger, 1951). One hour after removal of DFP and repeated washing no cholinesterase activity could be detected.

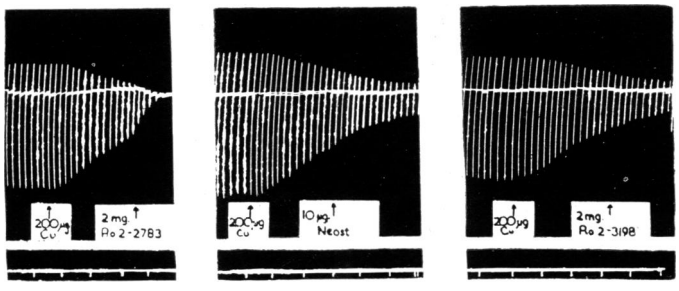


FIG. 7.—The action of Ro 2–2783, neostigmine methylsulphate, and Ro 2–3198 on the isolated phrenic nerve diaphragm after DFP (20 μg./ml. for 1 hr.). The doses refer to total amounts added to the bath (volume 100 ml.). Time in min.

As can be seen from Fig. 7, Ro 2–3198 and neostigmine had no longer any anticurare action on a diaphragm after inhibition of its cholinesterase by DFP ; the same was true with Ro 2–2651. Ro 2–2650, Ro 2–2783 (Fig. 7), and decamethonium potentiated the blocking action of tubocurarine. Catechol (Fig. 8) and KCl, on the other hand, were as effective anticurare agents as on a normal diaphragm. These experiments excluded the possibility that the neostigmine analogues act by virtue of their free phenolic groups (present in the original compounds or after enzymic hydrolysis of the esters) or by combination with tubocurarine itself.

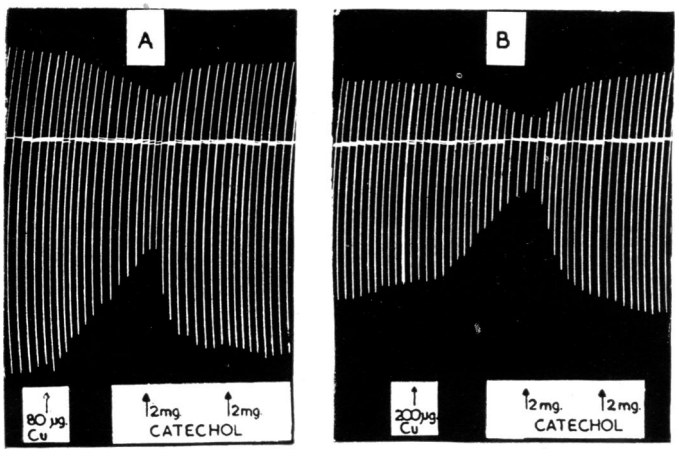


FIG. 8.—The anticurare action of catechol on the isolated phrenic nerve diaphragm, (A) before, and (B) after, treatment with DFP (20 μg./ml. for 1 hr.).

Action on the frog rectus

On the normal frog rectus abdominis the neostigmine analogues had practically no effect on the tonus in a concentration up to 100 μg./ml. Ro 2–2651 and Ro 2–3198 potentiated the actions of acetylcholine, the optimal concentration for this action being about 10 μg./ml. (Table I) ; with higher doses this sensitization was

again partly lost. By this test Ro 2–3198 had about a tenth of the potency of neostigmine, and Ro 2–2651 slightly less. Ro 2–2650 and Ro 2–2783 sensitized the rectus to acetylcholine to a much smaller degree, and in concentrations of 10 μg./ml. and higher they even depressed the response to acetylcholine (Table I). The effect of the neostigmine analogues (sensitization or depression) was rapid in onset and

TABLE I

Sensitization of the frog rectus to acetylcholine by neostigmine and its analogues. The changes in sensitivity are expressed in per cent of the original response to 1 μg. Ach/ml. (= 100). + = sensitization. − = depression

Drug	Change in sensitivity produced by 10^{-7}	10^{-6}	10^{-5}	10^{-4}
Ro 2–2650	—	+70	+100	< −90
Ro 2–2651	—	+150	+600	+700
Ro 2–2783	—	+25	−50	< −90
Ro 2–3198	—	+230	+660	+400
Neostigmine	+200	+750	+900	—

a maximum effect was achieved within one minute. With neostigmine, on the other hand, the sensitization of the rectus to acetylcholine was slightly greater than with Ro 2–2651 or Ro 2–3198 (Table I), but the rectus had to be in contact with a given solution for at least thirty minutes in order to show a maximum effect.

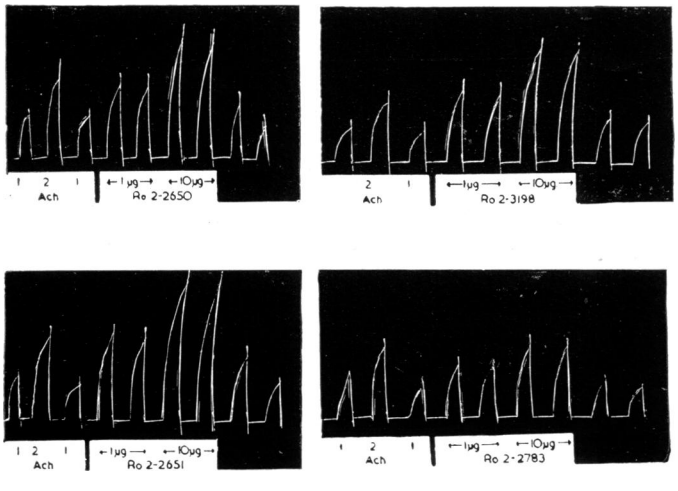


FIG. 9.—Anticurare action of the neostigmine analogues on the frog rectus. The doses give the final concentration. Duration of tests 60 sec. Interval between the tests 3 min. In the tests marked 1, 1 μg. acetylcholine/ml., and in the tests marked 2, 2 μg. acetylcholine/ml., were used to induce contracture. In the other tests the dose of acetylcholine was always 1 μg./ml. The concentration of *d*-tubocurarine chloride was 1 μg./ml. throughout the experiment.

On the curarized rectus muscle all four neostigmine analogues had a marked anticurare action (Fig. 9), which was already significant at dose levels of 1 μg./ml. As on the diaphragm this anticurare action was completely absent after inhibition of cholinesterase by tetraethylpyrophosphate (for method see Hobbiger, 1950); indeed the actions of tubocurarine were then even intensified by the substances (Fig. 10).

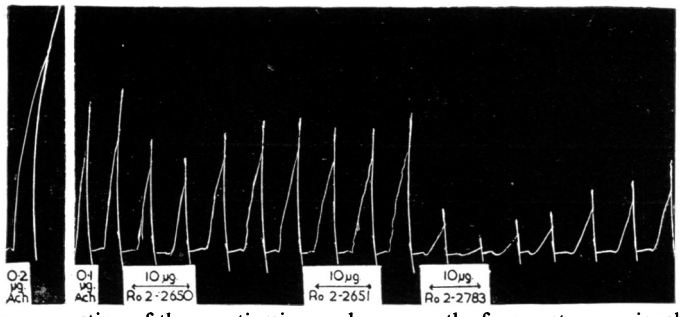


FIG. 10.—Anticurare action of the neostigmine analogues on the frog rectus previously treated with tetraethylpyrophosphate. Duration of tests 60 sec. Interval between the tests 3 min. In the first test 0.2 μg. acetylcholine/ml. was used, and in the subsequent tests 0.1 μg. acetylcholine/ml. Throughout the experiment 1 μg. *d*-tubocurarine chloride/ml. was present in the bath.

Substances with an acetylcholine-like action, on the other hand, e.g. choline or decamethonium, still produced a contracture of the rectus after inhibition of cholinesterase, and if acetylcholine was given during such a contracture a further shortening of the muscle occurred.

The anticurare action of the neostigmine analogues on the rectus muscle is therefore entirely due to cholinesterase inhibition. As on the diaphragm the effect on cholinesterase of the normal muscle is obscured by a blocking action exerted on the endplates. As regards the intensity of this blocking action the neostigmine analogues can be grouped thus: Ro 2–2783>Ro 2–2650>Ro 2–3198>Ro 2–2651.

Action of neostigmine analogues on avian muscle

Since the experiments on the frog muscle gave very little evidence for an acetylcholine-like action by the neostigmine analogues, innervated and denervated gastrocnemius muscles of the hen were used to study such a property. It was found that, taking the stimulant action of neostigmine as 1, the neostigmine analogues had a direct stimulating property which was of the following order: Ro 2–2650, 1/3; Ro 2–2783, 1/3; Ro 2–3198, 1/2; and Ro 2–2651, 1/30. This stimulating effect is shown neither on the diaphragm nor on the frog rectus, but could occur in the intact animal.

Action on isolated rat intestine

To examine the effects of the neostigmine analogues on a structure where acetylcholine exerts its muscarinic type of action the isolated rat intestine was used.

As can be seen from Table II the neostigmine analogues, with the exception of Ro 2–2783, potentiate the actions of acetylcholine. The potency of the compounds studied was in the order: Ro 2–2651>Ro 2–3198>Ro 2–2650. Whereas Ro 2–2783 and Ro 2–2650 had no effect on the resting tone of the intestine, Ro 2–3198 caused a slight increase and with Ro 2–2651 this was most marked. Neostigmine sensitized the intestine to acetylcholine to a much greater extent than its analogues and spontaneous peristalsis was seen with concentrations of 0.1 μg./ml. and higher; this was never observed with the neostigmine analogues.

TABLE II

Effect of neostigmine and its analogues on the sensitivity of the isolated rat intestine to acetylcholine. The response to 0.05 μg./ml. Ach is taken as 1 and changes in sensitivity are expressed in multiples of it. 0.05 μg. acetylcholine/ml. was used throughout the whole experiment

Concentration	5×10^{-9}	5×10^{-8}	5×10^{-7}	5×10^{-6}	5×10^{-4}
Ro 2–2650 ..	—	—	1.5	2.4	1/10
Ro 2–2651 ..	—	—	3.6	5.6	5.6 (increase in tone)
Ro 2–2783 ..	—	—	—	1/2	<1/10
Ro 2–3198 ..	—	—	2.5	2/3	<1/5
Neostigmine ..	4.5	6.5 (peristalsis)	—	—	—

Inhibition of cholinesterase in vitro

In Table III the anticholinesterase activities of the neostigmine analogues are summarized. It will be seen that they are of the order of 1/100th to 1/400th of that of neostigmine. The inhibition is both competitive and reversible. If the substrate is added after the inhibitor a constant rate of hydrolysis is reached much earlier with the neostigmine analogues than with neostigmine itself.

TABLE III

Inhibition of red cell esterase by neostigmine and its analogues at 37° C. The activity of cholinesterase during the first 30 min. after addition of the substrate (acetyl-β-methylcholine, 0.03 M.) is taken as a measurement of enzyme inhibition

Inhibitor	Concentration (w/v) which produces 50% inhibition	Relative anticholinesterase potency (neostigmine = 1)
Neostigmine	3.8×10^{-8}	1
Ro 2–2650	1.4×10^{-5}	1/370
Ro 2–2651	6×10^{-6}	1/160
Ro 2–2783	1.1×10^{-5}	1/290
Ro 2–3198	4×10^{-6}	1/110

The neostigmine analogues (except Ro 2–3198) are unstable in aqueous solutions and are split by both cholinesterases I and II. The enzymic hydrolysis is fastest with Ro 2–2651, which is split by cholinesterase II at the same rate as acetylcholine (Table IV). This rate of enzymatic hydrolysis makes it very likely that the actions of Ro 2–2650, Ro 2–2783, and especially of Ro 2–2651 on isolated organs are due to the hydrolysis products, as well as to the esters.

Inhibition of cholinesterase in rabbits in vivo

The neostigmine analogues disappear much more rapidly from the blood stream than neostigmine. With sublethal doses no inhibition of blood cholinesterases was noticeable fifteen minutes after i.v. injections of the substances, whereas the effect of an equiactive dose of neostigmine lasted up to one and a half hours.

TABLE IV

Spontaneous and enzymic hydrolysis at 37° C. in 0.02 M-$NaHCO_3$, as determined by the production of CO_2. Volume: 3 ml. The figures give the output of CO_2 in μl./10 min. The figures for enzymic hydrolysis have been corrected for non-enzymic hydrolysis

Substrate	Spontaneous hydrolysis		Hydrolysis by			
			0.3 ml. (1 : 5 dil.) red cells		0.2 ml. serum	
	0.6 g./ 100 ml.	0.2 g./ 100 ml.	0.6 g./ 100 ml.	0.2 g./ 100 ml.	0.6 g./ 100 ml.	0.2 g./ 100 ml.
Acetylcholine	5.79	2.72	53.64	75.61	109.06	93.15
Acetyl-β-methyl-choline ..	1.98	1.96	51.81	49.68	2.72	2.38
Benzoylcholine	2.64	1.57	1.92	2.94	40.69	42.55
Ro 2–2650	16.48	5.95	13.80	8.92	5.20	3.34
Ro 2–2783	7.22	3.23	15.11	10.03	18.87	10.03
Ro 2–2651	19.89	9.40	47.74	30.38	103.80	70.25

Discussion

The neostigmine analogues Ro 2–2650, Ro 2–2651, Ro 2–2783, and Ro 2–3198 are powerful anticurare agents in cats. Their potencies are of the order of 1/5th to 1/15th of that of neostigmine. Side effects, e.g. salivation, diarrhoea, etc., are either negligible or completely absent. These results confirm fully the experiments of Randall (1950) and Randall and Lehmann (1950). Because of the low anticholinesterase activity *in vitro* (1/100th–1/400th of that of neostigmine) it was originally thought that the mechanism of their anticurare action was not related to cholinesterase inhibition (Randall, 1950 ; Ricker *et al.*, 1949 ; Wescoe *et al.*, 1949 ; Artusio *et al.*, 1950 ; and McFarlane *et al.*, 1950). The experiments reported here, however, show that it is impossible to draw far-reaching conclusions from the *in vitro* experiments.

In studying the anticurare action of drugs with the physico-chemical properties of the neostigmine analogues, we have to consider that their actions could be due to one or more of the following well-established mechanisms :

(1) Inhibition of cholinesterase.

(2) Direct action on the endplate (depolarization).

(3) Inactivation of *d*-tubocurarine.

(4) An action due to their phenolic groups (Mogey and Young, 1949).

Experiments on intact animals do not enable us to determine how much each of these four mechanisms contributes to the anticurare action. This difficulty can be partly overcome by using the isolated phrenic nerve diaphragm and the frog rectus, but from the above experiments it can be seen that each of the two isolated preparations has its limitations : (*a*) The anticurare effect of substances which stimulate the endplate (depolarization) does not show up on the isolated diaphragm. (*b*) The frog rectus does not give a contracture with all substances which can stimulate mammalian muscle (if brought into contact with the endplates suddenly).

Taking into account these limitations the conclusions which can be drawn from the experiments with the neostigmine analogues are as follows :

(1) The neostigmine analogues do not act by combination with *d*-tubocurarine itself.

(2) They do not act by their phenolic nature. The hydrolysis of Ro 2–2650 and Ro 2–2783, whether spontaneous or enzymic, is so slow that most of the actions of these compounds *in vivo* must be produced by the esters and not by their hydrolysis products. Ro 2–2651, however, is rapidly hydrolysed by cholinesterases, especially by cholinesterase II, and part of the action of this compound *in vivo* is certainly due to the hydrolysis product. The free phenolic group in Ro 2–3198, or in the hydrolysis products of the other three esters, is not responsible for the anticurare action as shown on the diaphragm. Whereas phenols, e.g. catechol, have the same anticurare action on a normal diaphragm as on a diaphragm which has lost all its cholinesterase activity by treatment with DFP, the neostigmine analogues, like neostigmine itself, exert no anticurare action on a muscle structure which has been deprived of its cholinesterase.

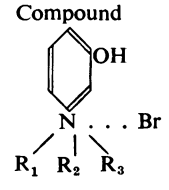
(3) The main mechanism by which the neostigmine analogues act seems to be the inhibition of cholinesterase. On a normal preparation Ro 2–3198 and Ro 2–2651 produce all the symptoms which one would expect from an anticholinesterase, e.g. potentiation of twitch tension, repetitive firing, and sensitization to acetylcholine. With Ro 2–2783 and Ro 2–2651 these effects are obscured by a component which causes neuromuscular block. On a curarized preparation this "second" component is suppressed and the effect of cholinesterase inhibition becomes apparent, but, as one would expect, Ro 2–3198 and Ro 2–2651 are more potent anticurare agents than Ro 2–2650 or Ro 2–2783. On all three preparations (the cat's tibialis muscle, the diaphragm, and the frog rectus) inhibition of cholinesterase completely abolishes the anticurare action, whereas substances with an acetylcholine-like action still act (except on the diaphragm). The rapid onset of the actions of these compounds must be due to a fast rate of penetration as shown by the time necessary to achieve a maximum action with a given concentration on isolated organs. The short duration of action is due to rapid removal of these substances from the circulation.

(4) It is difficult to say how much an acetylcholine-like action is responsible for the anticurare activity of the neostigmine analogues. The anticurare properties of acetylcholine were first described by Wilson and Wright (1937). Recently Hutter (1951) demonstrated similar properties for choline and decamethonium. In my experiments I have confirmed these findings and have also shown that these substances still act in the cat after inhibition of cholinesterase. On the other hand, anticholinesterases and neostigmine analogues lose all their anticurare properties on a preparation deprived of its cholinesterase and some of them even potentiate the blocking action of tubocurarine. Unfortunately the diaphragm is unsuited for the study of the anticurare action of substances with an acetylcholine-like action and all that can be seen on this preparation is a further speeding up of the curarization. On the frog's rectus the neostigmine analogues have practically no stimulant action, and after inhibition of cholinesterase a marked neuromuscular blocking component can be demonstrated. Close arterial injection in cats (Randall and

Lehmann, 1950) and the experiments on avian muscle reveal a weak acetylcholine-like property in these neostigmine analogues, but as the experiments on the cat, after administration of an anticholinesterase, indicate this could be only of minor importance in determining the anticurare action. This view is well supported by Randall's (1950) observation on a series of 3-hydroxy anilinium salts in which one or more methyl groups had been replaced by ethyl groups. Whereas the effective anticurare dose varied only slightly, there was no correlation between acetylcholine-like action and anticurare action (Table V).

TABLE V

Comparison of the anticurare potency and direct action of a homologues series of 3-hydroxy anilinium salts. The data are based on the results given by Randall (1950)

Compound 			Equiactive anti-curare dose in dogs in mg./kg. A	Equiactive dose on denervated muscle in mg. B	Ratio $\frac{A}{B}$
CH_3	CH_3	CH_3	0.2	0.0025	80
CH_3	CH_3	C_2H_5	0.2	0.01	20
CH_3	C_2H_5	C_2H_5	0.3	0.05	6
C_2H_5	C_2H_5	C_2H_5	0.6	>1.00	<0.6

SUMMARY

1. Four neostigmine analogues Ro 2–2650, Ro 2–2651, Ro 2–2783, and Ro 2–3198 have 1/5th–1/15th of the anticurare potency of neostigmine in cats. Their anticholinesterase activity *in vitro* is 1/100th–1/400th of that of neostigmine. The muscarinic side effects are either feeble or absent.

2. The underlying mechanism of their anticurare action seems to be the inhibition of cholinesterase. This has been demonstrated on the tibialis muscle of the cat, on the rat diaphragm, and on the frog rectus abdominis. On each preparation the anticurare action is abolished by previous inhibition of cholinesterase. In non-curarized preparations the manifestations of cholinesterase inhibition can be clearly shown with Ro 2–2651 and Ro 2–3198, whereas with Ro 2–2650 and Ro 2–2783 an additional component, which causes neuromuscular block, interferes with it.

3. An acetylcholine-like action of these compounds on the endplate itself was demonstrated, but seems to be only of minor importance for the anticurare action.

4. An anticurare action by the phenolic groups of these compounds or direct combination with *d*-tubocurarine has been excluded.

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