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# THE PHARMACOLOGY OF THE METHYL AND BENZYL ESTERS OF γ-CROTONIC BETAINE (γ-CARBOXYALLYLTRIMETHYL-AMMONIUM CHLORIDE)

BY

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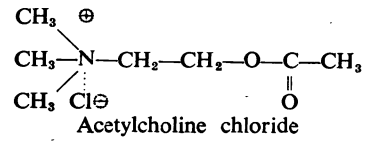
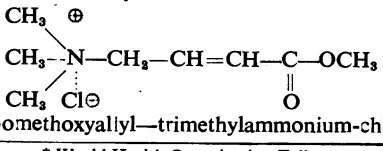
The effect of reversing the polarity of the ester linkage in acetylcholine homologues was first studied by Hunt and Renshaw (1926). These authors prepared aliphatic esters of betaine which they showed were parasympathomimetic agents with an activity of the order of one thousandth of that of acetylcholine. Betaine itself is pharmacologically almost inert and thus bears a relationship to its esters similar to that which choline bears to acetylcholine. The activity of the methyl ester of β-propionic betaine, the ester most closely isosteric with acetylcholine has not so far been reported. In 1928, however, a new betaine called γ-crotonic betaine (γ - carboxyallyltrimethyl - ammonium - chloride) was isolated from animal tissues by Linneweh (1928a, b). Strack and Försterling (1938, 1942) also isolated this betaine from mammalian muscle and prepared some of its esters. They found that whilst the betaine was almost inert pharmacologically, its methyl ester was a powerful parasympathomimetic drug when tested on the dorsal muscle of the leech, the frog rectus and heart and the mouse intestine. They also found that the ethyl ester had much weaker effects and in addition antagonized the effects of acetylcholine. The following paper confirms and extends these observations for the methyl ester and describes also the properties of the benzyl ester of this betaine. Chemically the structure of the methyl ester is quite similar to that of acetylcholine, as may be seen by the formulae:



Acetylcholine chloride



γ-carbomethoxyallyl—trimethylammonium-chloride

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## Methods and Materials

Cats were anaesthetized with 60 mg. chloralose/kg. The tibialis anterior muscle was prepared for close intra-arterial injection as described by Brown (1938). The nictitating membrane response was recorded after section of the cervical sympathetic. The rabbit heart was perfused by the Langendorff method with Ringer-Locke solution. The rat phrenic nerve-diaphragm preparation (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. Frog heart perfusions were carried out both by the sinus perfusion and the Straub methods. The chromodacryorrhoea response in rats was tested by the method described by Burgen (1949).

The crotonic betaine esters were prepared by the method of Bergel, Cohen, and Hindley (1949), and were made available to us as the methyl ester chloride (mol. wt. 182.5 ; m.p. 174° C.) and the benzyl ester iodide (mol. wt. 361 ; m.p. 147–149° C.). In the text these will be referred to as the methyl and benzyl esters and the activities are given by weight. In Table I the molar activities have been used.

## Results

### *Effects on the anaesthetized cat*

*Methyl ester.*—As little as 2 μg. of the methyl ester injected intravenously produced a small fall of blood pressure, unaccompanied by bradycardia. With increasing doses this effect increased, and when the dose reached 50 μg. a bradycardia accompanied the fall of blood pressure. As the dose was increased further this effect became more marked and was associated with a decrease in the tidal air. The fall of blood pressure and bradycardia were more prolonged than those given by an equivalent amount of acetylcholine (Fig. 1). Sometimes the respiratory depression was preceded by a transitory respiratory stimulation. This respiratory depression was unaffected by bilateral vagotomy or atropine, and respiration was shallow without the appearance of respiratory obstruction. It is probable that this effect was due to a depression of the respiratory centre or the

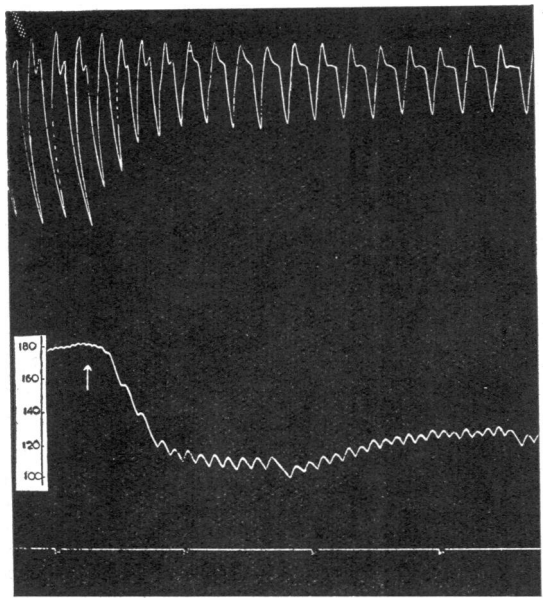


FIG. 1.—Cat, 2.4 kg., chloralose. Top tracing: respiration. Lower tracing: carotid blood pressure. Time in 5 sec. At the arrow i.v. injection of 0.14 mg. methyl ester.

muscles of respiration. Other effects noted with the larger doses of methyl ester were profuse and prolonged salivation, diarrhoea, and micturition.

After an adequate dose of atropine (1–2 mg./kg.) the slowing of the heart was no longer seen and the depressor effect of the methyl ester (100–200 $\mu$g.) was converted into a rise of blood pressure greater than that given by an equal dose of acetylcholine (Fig. 2).

*Benzyl ester.*—No effects were seen with the benzyl ester until 50 $\mu$g. were given. A small pressor effect then usually appeared which increased with increasing dosage but sometimes the response was biphasic. Respiratory depression occurred after the injection of 1 mg. of the benzyl ester and was similar in character to that given by the methyl ester. At no dose level were salivation, diarrhoea or micturition observed.

In the anaesthetized rabbit similar effects were seen.

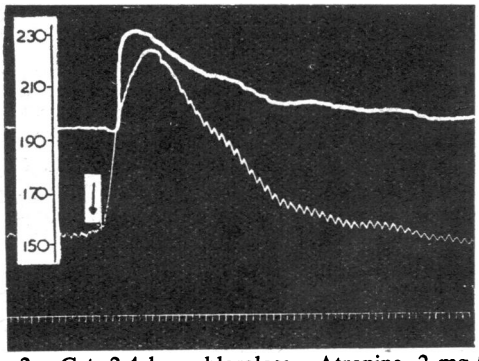


FIG. 2.—Cat, 2.4 kg., chloralose. Atropine, 2 mg./kg. Top tracing: nictitating membrane. Lower tracing: carotid blood pressure. Time in 5 sec. At arrow 0.2 mg. methyl ester intravenously.

*Effects on the nictitating membrane*

The methyl ester produced a contraction of the nictitating membrane when doses of 10 $\mu$g. or more were given intravenously (Fig. 2). The activity was similar to that of acetylcholine. With the benzyl ester, however, 0.5–1 mg. was needed to produce an effect and we did not succeed in producing a maximal contraction with the maximum tolerated dose. With both esters the response was quite long lasting.

*Effects on the tibialis anterior preparation*

Some of the effects obtained when the esters were injected intra-arterially into the tibialis anterior can be seen in Fig. 3; 20 $\mu$g. of the methyl ester produced a twitch similar in magnitude to that produced by 10 $\mu$g. of acetylcholine but followed by a transient depression of neuromuscular conduction. With 40 $\mu$g. of the methyl ester the twitch was somewhat larger, relatively to nerve stimulation, but was followed by a greater neuromuscular depression. Very similar effects

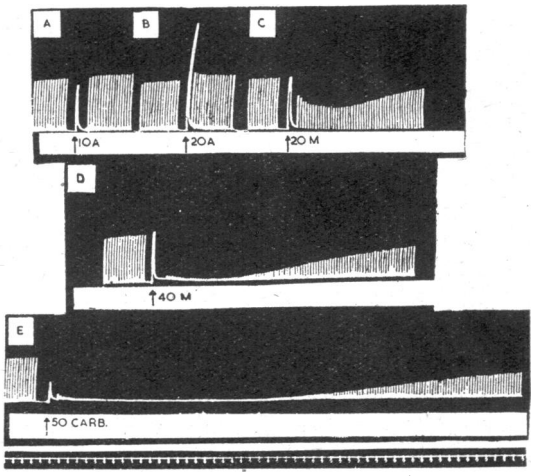


FIG. 3.—Cat, 2.1 kg., chloralose. Maximal motor nerve twitches of tibialis anterior muscle. Close intra-arterial injection of (A) 10 $\mu$g. acetylcholine; (B) 20 $\mu$g. acetylcholine; (C) 20 $\mu$g. methyl ester; (D) 40 $\mu$g. methyl ester; (E) 50 $\mu$g. carbamylcholine; volume of injection 0.5 ml.

were produced by 50 μg. carbamylcholine except that the twitch was smaller relative to the subsequent depression. 250 μg. of the benzyl ester produced a twitch similar to that given by 10 μg. acetylcholine, but was followed by a prolonged neuromuscular block.

*Isolated preparations*

*Perfused rabbit heart.*—The addition of 1 μg. of the methyl ester to the perfusion fluid exerted a negative inotropic effect about equal to that given by 0.5 μg. of acetylcholine or carbamylcholine. With 10 μg. this effect was increased and the heart rate slowed ; with 50 μg. the heart was temporarily arrested and this was followed by auriculo-ventricular dissociation. In these actions the methyl ester was about one quarter the activity of acetylcholine. The benzyl ester produced no depression of force or rate until 100 μg. were injected, when a transitory small depression occurred. After this dose, however, the response to acetylcholine was greatly decreased, and returned slowly over the succeeding 20–30 minutes.

*Perfused frog heart.*—The methyl ester depressed the force of contraction of the perfused frog heart in a concentration of about 0.01 μg./ml. With ten times this dose the heart was arrested. The activity relative to acetylcholine was about one tenth. The benzyl ester had no direct action on the heart even in a concentration of 1 mg/ml., but a well marked antagonism to acetylcholine was found with concentrations of the benzyl ester of 10 μg./ml. and higher.

*Perfused vessels.*—Rat and guinea-pig hind limbs and the rabbit ear vessels were perfused with Locke's solution at room temperature ; in all these preparations acetylcholine produced vasoconstriction. Both the methyl and the benzyl esters, given in about twice the dose of acetylcholine, produced a similar but more prolonged effect than that of acetylcholine. During the course of prolonged perfusions the sensitivity of these preparations increased equally for all three drugs.

*Isolated rabbit duodenum and ileum.*—The methyl ester (0.03–0.5 μg./ml.) produced a contraction of the longitudinal muscle of the rabbit duodenum and ileum similar in magnitude to that given by one-sixth to one-half the amount of acetylcholine (Fig. 4 *a*). The duration of the contraction was, however, longer than that given by acetylcholine, but resembled closely that produced by carbamylcholine. The benzyl ester produced a contraction in the freshly isolated intestine at a concentration of 1–2 μg./ml. After the gut had been isolated for one hour or more and especially if it was kept in the refrigerator overnight, the benzyl ester no longer increased the tonus, and with higher dosage a decrease in both the tonus and the spontaneous rhythm resulted. With the larger doses of the benzyl ester the motor effects of acetylcholine were antagonized, as may be seen in Fig. 4 *b*. When the intestine was set up as a Trendelenberg. preparation, the peristalsis was completely inhibited by 10 μg. benzyl ester /ml. (Fig. 5 *b*).

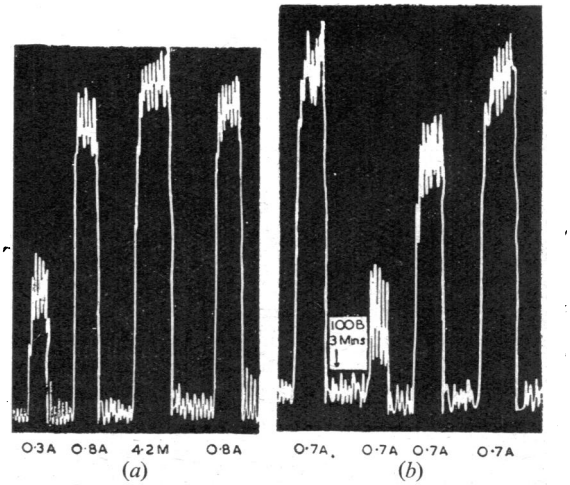


FIG. 4.—Rabbit duodenum. Bath volume 30 ml. (*a*) Comparative effect of acetylcholine (0.3 μg. and 0.8 μg.) and methyl ester (4.2 μg.). (*b*) Inhibition of the response to 0.7 μg. acetylcholine by 100 μg. benzyl ester.

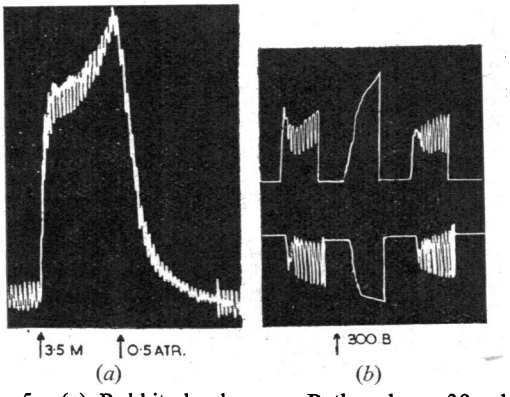


FIG. 5.—(*a*) Rabbit duodenum. Bath volume 30 ml. At first arrow addition of 3.5 μg. methyl ester which was promptly antagonized by 0.5 μg. atropine. (*b*) Guinea-pig ileum. Trendelenberg preparation. At arrow addition of 0.3 mg. benzyl ester for 3 minutes. Upper tracing: longitudinal muscle. Lower tracing: intestinal volume.

The motor effects of both the methyl and benzyl esters were readily antagonized by atropine (Fig. 5 *a*), and by adrenaline. The benzyl ester also antagonized the motor effects of the methyl ester.

*Isolated guinea-pig ileum.*—On the guinea-pig ileum the methyl ester produced a contraction in a concentration of 0.2–1 μg./ml. and was about one-half as active as acetylcholine. The benzyl ester had no direct effect on the intestine even in a concentration of 0.3 mg./ml., but at a concentration of 3 μg./ml. the response to acetylcholine was reduced by 50 per cent, and with higher concentrations could be abolished. The response to histamine was unaffected until 0.3 mg./ml. was added when it was depressed by about 10 per cent.

*Phrenic nerve-diaphragm preparation.*—Both the esters were fairly active neuromuscular blocking agents when tested on the phrenic nerve-diaphragm preparation. The methyl ester was about one-twentieth as active, and the benzyl ester was one-tenth as active as *d*-tubocurarine. Carbamylcholine was similar in potency to the methyl ester. Antagonism by neostigmine and by potassium chloride was much less marked with these esters than with *d*-tubocurarine. Paton and Zaimis (1949) report a similar lack of antagonism by neostigmine against the neuromuscular block produced by decamethonium iodide.

*Frog rectus preparation.*—On the normal unsensitized frog rectus preparation, both the methyl and the benzyl esters produced a contracture similar to that produced by acetylcholine, except that the latency was longer and the rate of development of the contracture slower. When the duration of the test was the usual one and one-half minutes the methyl ester was about one-third as active, and the benzyl ester one-fifteenth as active as acetylcholine (Fig. 6 *a*), whilst with a test lasting 10–15 minutes the relative potency of the crotonic esters was increased (Fig. 6 *b*). Whilst the absolute sensitivity of individual rectus muscles to the crotonic esters varied very little, the sensitivity to acetylcholine varied considerably, which accounts for the variation in relative potency.

Sensitization of the muscle with eserine or TEPP increased the response to acetylcholine on an average 40 times, but produced no change in the response to either the methyl or the benzyl ester. *d*-Tubocurarine antagonized both esters and acetylcholine about equally, but only the response to acetylcholine was restored by eserine.

*Rat chromodacryorrhoea*

Albino rats (c. 300 g. wt.) were used which gave no tear secretion after the injection of 250 μg. acetylcholine subcutaneously, but a well-marked red tear secretion to 500 μg. acetylcholine. The following results were obtained with the methyl ester.

20 μg.—no response.

50 μg.—pink tinging of the tears after 5–6 minutes.

100 μg.—profuse opaque red tears—some bradycardia.

1,000 μg.—profuse and very prolonged red tear secretion, heart block, profuse salivation, respiratory difficulties, spontaneous micturition and diarrhoea.

Doses of up to 20 mg. of the benzyl ester were ineffective in causing a red tear secretion.

In Table I the activities of the methyl and benzyl esters are summarized and compared on a molar basis with those of acetylcholine.

*Hydrolysis by cholinesterases*

The esters were incubated in $10^{-5}$ concentration at 37° C. either with a solution of lysed human red cells as a source of true cholinesterase or with human serum as a source of mixed esterases. The residual ester was estimated on the frog rectus preparation.

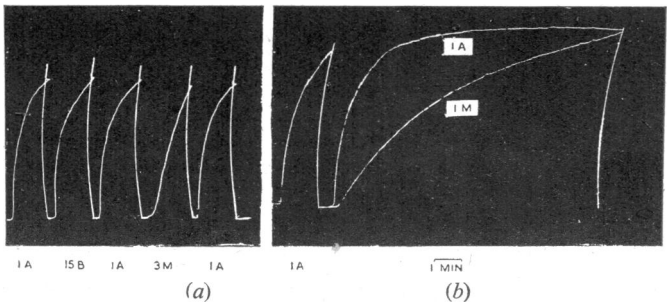


FIG. 6.—Frog rectus abdominis. (*a*) Comparison of 1 μg. acetylcholine with 15 μg. benzyl ester and 3 μg. methyl ester. Duration of test 90 sec. (*b*) Comparison of 1 μg. acetylcholine and 1 μg. methyl ester. Duration of test 10 min.

TABLE I

COMPARATIVE ACTIVITY OF THE METHYL AND BENZYL ESTERS

| Preparation | Molar activity relative to acetylcholine = 100 | |
|---|---|---|
| | Methyl ester | Benzyl ester |
| Frog rectus unsensitized... | 30–100 | 15–50 |
| ,, ,, sensitized with eserine ... ... ... | 2–5 | 0.5–2 |
| Guinea pig ileum ... | 50 | 0* |
| Rabbit duodenum† ... | 15–50 | 5** |
| Perfused rabbit heart‡ ... | 10–50 | 0* |
| Frog heart ... ... | 10 | 0* |
| Cat blood pressure ... | 8 | 1§ |
| ,, ,, ,, after atropine ... ... | 250 | 20 |
| Cat nictitating membrane | 80 | 0.5 |
| Cat tibialis ant. (twitch)... | 50 | 8 |
| Rat diaphragm ... ... | 1‖ | 5‖ |
| Perfused vessels ... ... | 50 | 20–100 |
| Rat red tear secretion ... | 1,000 | 0 |

* Blocks action of acetylcholine. ** Blocks action of acetylcholine and decreases intestinal tone (see text). § Biphasic or pressor response. ‖ Activity relative to *d*-tubocurarine chloride = 100. † Activity of carbamylcholine, 150. ‡ Activity of carbamylcholine, 100.

From the results given in Table II it will be seen that a preparation of true cholinesterase which hydrolysed acetylcholine very rapidly did not hydrolyse either ester. Serum did increase the breakdown of the benzyl ester, but at a rate far lower than for acetylcholine. The non-enzymatic rates of hydrolysis of the benzyl ester and of acetylcholine were measured by continuous titration over the range pH 4.5–9.5. The velocity constants were similar at the lower pH values, but activation by hydroxyl ions occurred sharply at pH 7.5 with the benzyl ester and at a slightly higher pH and less steeply with acetylcholine.

TABLE II

HYDROLYSIS OF METHYL AND BENZYL ESTERS BY CHOLINESTERASES

| Ester | Enzyme | % Hydrolysed | | | | |
|---|---|---|---|---|---|---|
| | | 10 min. | 1 hr. | 2 hrs. | 3 hrs. | 6 hrs. |
| Benzyl ester | No enzyme | — | 5 | — | 12 | 24 |
| | 5% serum* | — | 5 | — | 18 | 28 |
| | 1% R.B.C.* | — | −1 | — | 2 | 1 |
| Methyl ester | No enzyme | 0 | — | 2 | — | — |
| | 5% serum* | 0 | — | 0 | — | — |
| | 1% R.B.C.* | 3 | — | 3 | — | — |
| Acetyl-choline | 5% serum | 97 | — | 100 | — | — |
| | 1% R.B.C. | 99 | — | 100 | — | — |

* Corrected for non-enzymatic hydrolysis.

In experiments carried out in the Warburg apparatus it was found that 0.002 *M* methyl ester inhibited red cell cholinesterase 23 per cent (substrate: 0.025 *M* acetyl-β-methylcholine chloride) and inhibited serum pseudo cholinesterase 18 per cent (substrate: 0.007 *M* benzoylcholine chloride). These data show that both true and pseudo cholinesterases have an affinity for the methyl ester of the same order as that for acetyl-β-methyl choline and benzolycholine. It would seem that reversal of the ester link, whilst not reducing the affinity of cholinesterase for the methyl ester, prevents the enzyme-substrate complex breaking down into γ-crotonic betaine and methanol.

## SUMMARY

The *methyl* ester of γ-crotonic betaine is a very active parasympathomimetic drug. Both muscarinic and nicotinic types of action were found whose activity varied from one fiftieth to ten times that of acetylcholine according to the test preparation. The ester is not split by cholinesterase and hence has a more prolonged action than acetylcholine. The *benzyl* ester of γ-crotonic betaine has almost no muscarinic actions, but has some weak atropine like activity. Compared with the the methyl ester the nicotine actions are considerably weaker.

We are grateful to Dr. F. Bergel for placing a generous supply of these esters at our disposal.

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