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THE PHARMACOLOGY OF BENZOYLCHOLINE DERIVATIVES AND THE NATURE OF CARBONYL RECEPTORS

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Little is known of the function of the carbonyl group in the pharmacological action of choline esters; hitherto the only satisfactory method of study has been by observing the effects of altering the acetylcholine molecule so that other parts of the molecule bear different spacial relationships to the carbonyl group (Welsh and Taub, 1951; Ing, Kordik, and Tudor Williams, 1952). Another method of study is presented in this paper; it has already been used to obtain information on the interaction of cholinesterase and the carbonyl group of benzoylcholine (Ormerod, 1953).

A series of derivatives of benzoylcholine was synthesized having substituents in the benzene ring such that the reactivity of the carbonyl group was affected by the electron attraction or repulsion caused by the substituents: the degree of this attraction or repulsion (mesomeric effect) is given for each substituent by the substituent constant S, where $S = \log K - \log Ko$, K being the dissociation constant of corresponding substituted benzoic acid and Ko the dissociation constant of unsubstituted benzoic acid (Hammett, 1940).

As a correlation had been demonstrated between the mesomeric effect and rate of enzyme hydrolysis —that is to say, the highly activated compounds such as *p*-chlorobenzoylcholine were hydrolysed rapidly, and "deactivated" compounds such as *p*-methoxybenzoylcholine slowly—and as evidence had been put forward to suggest that this correlation could be ascribed to the interaction of the carbonyl group with a complementary group on the enzyme surface, it was felt that a similar correlation found in any pharmacological action of this series would provide evidence of carbonyl receptors of pharmacological importance and give some indication of their chemical nature.

However, it was found that on some sites of action of the compounds no correlation between mesomeric effect and pharmacological action was shown; in these instances the work has not been pursued beyond the point where it became apparent that no such correlation existed. The "nicotine-like" actions of these compounds—both in raising the blood pressure of the atropine-treated cat and in stimulating the cat's superior cervical ganglion—did, however, show a degree of correlation with mesomeric effect, and the significance of this will be discussed later. In general, the methods used and sites of activity studied were those that would give satisfactory assays; the action on the guinea-pig's ileum—apparently an obvious choice—was avoided because of the complications described by Akcasu, Sinha, and West (1952).

MATERIALS AND METHODS

The series of substituted benzoylcholine derivatives had been synthesized and described previously (Ormerod, 1953). A further sample of the *p*-methyl derivative was prepared, to check the purity of the earlier sample, by reaction of *p*-toluoylchloride with ethylene chlorohydrin. In addition, *p*-chlorobenzoylcholine iodide was added to the series, synthesized by the standard method. Analysis by Drs. Weiler and Strauss. Found: C, 39.05; H, 4.5. $C_{12}H_{17}O_2ClI$ requires: C, 39.0; H, 4.5%. Substituent constant and rate of hydrolysis with horse-serum cholinesterase is given in Table I.

Action on the Neuromuscular Junction

Frog's rectus abdominis preparation was used as described by MacIntosh and Perry (1950): relaxation was slow and incomplete and the muscle had to be stretched between each addition of drug. Assays were based on the comparison of log dose-response line for the unknown with that of the standard benzoylcholine; two points on each line were determined, each representing the mean of four doses.

The phrenic nerve and diaphragm preparation of the rat was used as described by Chou (1947) with modification of West (1947). "Square wave" electrical stimuli excited the phrenic nerve at a rate of 8/min. at the minimum pulse width and voltage which produced maximal stimulation (usually 0.1 msec. and 4.5 v.). The solution contained: NaCl, 8.00; KCl, 0.20; $CaCl_2$, 0.20; NaH_2PO_4, 0.05; $NaHCO_3$, 1.00; dextrose, 1.00 g./l. The routine was otherwise as described by Burn (1950). Benzoylcholine was used as standard.

TABLE I

POTENCY OF BENZOYLCHOLINE DERIVATIVES (RELATIVE TO BENZOYLCHOLINE=1) ASSAYED ON FROG RECTUS ABDOMINIS AND RAT DIAPHRAGM PREPARATIONS

The dose limits for the standard benzoylcholine are given in parentheses. Also included are the substituent constant (S=log K −log Ko), and the rate of hydrolysis with serum cholinesterase (k_{30}=zero order velocity constant at 30° C.)

Derivative	Substituent Constant S	Rate of Hydrolysis k_{30}	Frog Rectus			Rat Diaphragm Blocking Action		
			Stimulation		Block			
			Without Eserine	With Eserine		Without Eserine	With Eserine 1 : 100,000	With Eserine 1 : 1,000,000
m-Nitro	+0·71	2·64	0·32 approx.	0·32 approx.	+	2·8	0·59	0·31
m-Chloro ..	+0·37	3·13	0·25 approx.	0·26 approx.	+	—	—	—
m-Fluoro ..	+0·34	2·70	—	—	—	—	—	—
p-Chloro	+0·23	1·94	0·23	—	—	1·2	0·24	0·81
p-Fluoro	+0·06	1·70	0·50	—	—	1·2	—	—
Unsubstituted ..	0·00	1·90	1·00 (0·1–0·5 mg.)	1·00 (0·1 and 0·23 mg.)	—	1·00 (4 and 6 mg.)	1·00 (1·9–2·0 mg.)	1·00 (0·5–1·2 mg.)
m-Methyl ..	−0·07	0·64	0·21	—	—	0·63	—	2·0
p-Methyl.. ..	−0·17	0·40	—	—	—	—	—	—
p-Methoxy ..	−0·27	0·1 approx.	0·23	—	—	2·5	0·71	1·6

Action on Blood Pressure

This was observed in cats anaesthetized with ether and chloralose (80 mg./kg.) and recorded at the carotid artery before and after 1 mg./kg. atropine sulphate. The potency of the derivatives in raising the blood pressure after atropine was estimated by giving each in a single high and a single low active dose; each of these doses was succeeded by an equipressor dose of the standard benzoylcholine. The log dose-response line was determined at the beginning of the experiment, and subsequent responses were compared with this curve. When the sensitivity of the preparation was found to have altered the results were rejected. The potency of each compound was determined from the horizontal distance of its log dose-response line from that of the standard. The combined result of experiments on two cats is shown in Table II: the result is approximate, since the log dose-response lines were not parallel, and the minimum of observations was made.

Action on Superior Cervical Ganglion

This was observed in cats by the method of Kibjakow (1933) and Feldberg and Gaddum (1934). The perfusion fluid contained: NaCl, 8.00; KCl, 0.20; $CaCl_2$, 0.20; $MgCl_2$, 0.01; NaH_2PO_4, 0.05; $NaHCO_3$, 1.00; dextrose, 1.00 g./l. and eserine sulphate 1/100,000 and was warmed by passing the tube up the animal's oesophagus (MacIntosh, 1949, unpublished); best results were obtained with perfusion pressures of 35–45 mm. Hg. Drugs were injected in solution (0.2 mg./ml.) through standardized micrometer syringes, one each for standard and unknown; these were inserted and clamped in position close to the carotid cannula. Two dose levels were used for each drug, and the injections were given in random order. Log dose-response lines were obtained by plotting the mean height of contraction in mm. against the logarithm of the dose. One ganglion was used for each compound assayed against benzoylcholine as standard. Observations were also made on the blocking action of these compounds, but no accurate assessment of their activity in this respect was possible.

A few observations were made with normal circulation through the ganglion; injections were made through a cannula inserted in the cut end of the carotid artery distal to the ganglion.

RESULTS

Tables I and II show the potencies of the derivatives of benzoylcholine relative to the parent compound. In most of the experiments eserine was used to inhibit hydrolysis, since if this precaution was omitted the type of correlation which appears could be attributed to hydrolysis of the compounds alone and not to differences in their pharmacological activity. This might be true of the experiments on the cat's blood pressure and on the superior cervical ganglion with normal circulation even in the presence of eserine, since it is impossible without giving toxic doses to be sure that all circulating esterase is inactivated: but it is unlikely that this mechanism is in fact operating, both because of the rapidity of response and because of the general agreement of results by these methods with those from perfusion of the superior cervical ganglion with a solution containing eserine to which there is no such objection.

The frog's rectus abdominis preparation shows no correlation between stimulant activity and mesomeric effect, but the two members with the most activating substituents, the *m*-nitro and the *m*-chloro derivatives, showed some blocking action so that the responses decreased rapidly and an accurate assay was impossible. The blocking action of these compounds to acetylcholine was negligible. Eserine had little effect on the action of these compounds on the frog rectus.

The neuromuscular-blocking action of these compounds on the rat diaphragm showed no correlation with mesomeric effect: eserine salicylate

TABLE II

NICOTINE-LIKE ACTIVITY (RELATIVE TO BENZOYLCHOLINE = 1) OF BENZOYLCHOLINE DERIVATIVES ASSAYED ON THE BLOOD PRESSURE AND ON THE SUPERIOR CERVICAL GANGLION OF THE CAT

The dose limits for the standard benzoylcholine are given in parentheses. The figures in square brackets represent assays completed before the final method was evolved.

Derivative	Atropine-treated Cat's B.P.	Cat's Superior Cervical Ganglion		
		Arterial Injection Without Eserine	Perfusion With Eserine	Perfusion 95% Confidence Limits
m-Nitro ..	0·1 approx.	—	Nil	
m-Chloro ..	0·3 approx.	0·44	0·03 approx.	
m-Fluoro ..	—	—	0·218	0·171–0·226
p-Chloro ..	0·45	—	0·392 [0·53]	0·292–0·430
p-Fluoro ..	0·60	—	0·687 [0·56]	0·599–0·851
Unsubstituted	1·00 (0·5–2 mg.)	1·00 (50,100 and 200 μg.)	1·00 (2–3 μg.)	
m-Methyl ..	0·40	—	1·144 0·359 [0·4]	0·9119–1·490 0·279–0·393
p-Methyl ..	0·6 approx.	—	0·339	0·302–0·373
p-Methoxy ..	2·16	2·8	1·812	1·281–1·960

1 : 1,000,000 increased the blocking action of these compounds but reduced their potency relative to benzoylcholine; further reorientation of potencies occurred with eserine salicylate 1 : 100,000. The meaning of these results is obscure.

The characteristic action of this series of compounds on the cat's blood pressure was a transient fall, usually described as a "muscarine-like" response: after atropine this was reversed, and a "nicotine-like" rise was obtained. In some animals only the rise in blood pressure was observed. The rise in pressure could be potentiated with eserine salicylate (1 mg./kg.), and an initial fall in pressure then preceded the rise, indicating a partial return of the muscarine type of response (Fig. 1). (This potentiation may have been produced by the inhibition of circulating cholinesterase, since it did not occur with the *p*-methoxy derivative which is hydrolysed by cholinesterase only to a negligible extent.) The rise in blood pressure was reduced to negligible size—as was that from an equipressor dose of nicotine—by hexamethonium bromide 10 mg./kg. The results shown in Table II were obtained after atropine and represent the nicotine-like action of these compounds: it was not possible in these experiments to make an accurate assessment of the potency of these compounds in producing muscarine-like activity, but it appeared to be similar—that is to say, a compound that gave a large rise in pressure after atropine could give a similarly large fall in the untreated animal. For these experiments 5 animals were used.

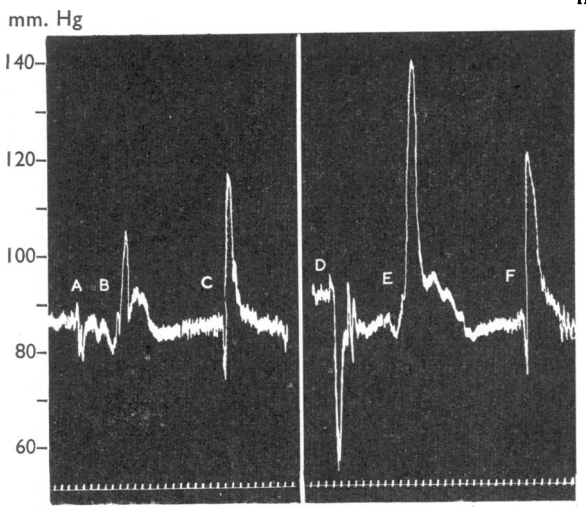


FIG. 1.—Cat, ether, chloralose. Arterial blood-pressure tracing to show "muscarine-like" and "nicotine-like" responses produced by 1 mg. benzoylcholine and 1 mg. *p*-methoxybenzoylcholine. A, benzoylcholine alone; B, benzoylcholine after atropine sulphate 1 mg./kg. C, benzoylcholine after atropine and eserine salicylate 1 mg./kg. D, p-methoxybenzoylcholine alone; E, p-methoxybenzoylcholine after atropine; F, p-methoxybenzoylcholine after atropine and eserine. Time in 10 sec.

Few experiments were performed on close arterial injection of the drug into the superior cervical ganglion, since this is open to the same objection as experiments on the blood pressure—namely, that the results might be due to differences in rapidity of hydrolysis and not to differences in pharmacological action.

The main effort of this work was therefore placed in an attempt to obtain an accurate assay of activity in the eserine-treated perfused superior cervical ganglion. The results, with their confidence limits, are shown in Table II. Ganglion-blocking activity, which could not be assessed accurately, was considered only in relation to stimulation with benzoylcholine and its possible effect on the assay of these compounds. (The blocking activity of these compounds also affected stimulation of the ganglion by excitation of the afferent nerve and by injection of acetylcholine, but no detailed study of blocking action was made in relation to these methods of stimulation.) *m*-Nitrobenzoylcholine produced block at a dose which did not stimulate; *m*-chlorobenzoylcholine produced block at the minimum stimulatory dose; with the other compounds block was produced only by a dose above that necessary to produce maximal stimulation. With most compounds there was a reasonable difference between the dose producing maximal stimulation and that which produced block, but this was not

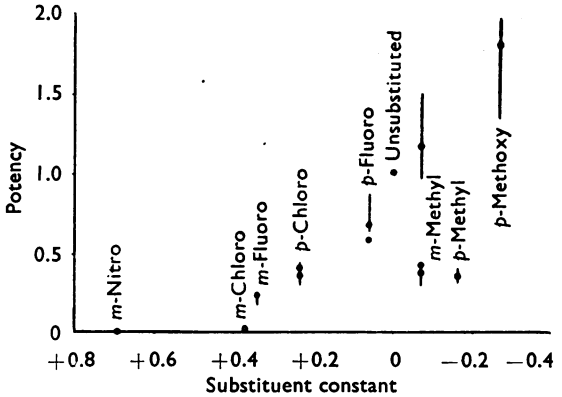


FIG. 2.—Potency of benzoylcholine derivatives (relative to benzoylcholine=1) as ganglion-stimulating agents, plotted against substituent constant (measuring electron drift in the benzene ring). Nicotine-like activity decreases with electron attractive power of substituent.

so with the *m*-methyl and *p*-methyl derivatives—in fact several assays with *p*-methylbenzoylcholine had to be rejected before one which was not affected by ganglion block was obtained.

The stimulating activity of these compounds on the superior cervical ganglion of the cat is shown, plotted against the substituent constant, in Fig. 2. As a definite correlation is shown between activity and mesomeric effect of substituents in the benzene ring, it can be postulated with reasonable certainty that carbonyl receptors exist, and that they are of importance in the nicotine-like stimulant activity of some choline esters on ganglia. *p*-Methylbenzoylcholine and one assay of *m*-methylbenzoylcholine lie, however, outside this correlation.

DISCUSSION

The deviation of *m*-methylbenzoylcholine and *p*-methylbenzoylcholine from the general scheme shown in Fig. 2 is not perhaps surprising in view of their greater blocking activity, yet I do not consider that the low values that they gave on assay were due solely to their blocking action, although this property serves to show that the pharmacological action of these compounds differs in some degree from those of other members of the series. This is also shown by their more prolonged action and irregularity of potency (the two assays of *m*-methylbenzoylcholine were both satisfactory as assays but gave widely different values). The possibility was considered that they might contain impurities, and it seemed likely that in the method of synthesis used the methyl groups of *m*- and *p*-toluic acids could have been oxidized to carboxylic acid groups, giving the *bis*-choline esters of, respectively, phthalic and *tere*-phthalic acids, configurations which might be expected to block ganglia; yet this possibility was eliminated both by study of analyses (see Ormerod, 1953) and by preparation of the *p*-methyl derivative by another synthetic route mentioned earlier in this paper. The sample so obtained showed similar potency and blocking action.

Ganglion stimulation has for long been associated with two types of choline derivate—the esters, both of aliphatic and aromatic acids, and the phenolic ethers. Quaternary ammonium compounds of similar structure but with a ketone group have recently been found active. It has been difficult hitherto to see how a single chemical grouping on the active surface of the ganglion can account for the activity of these three different chemical types.

There are few satisfactory references to the relative potency of choline esters, since most experiments have been performed on the whole animal and it is not always easy to estimate the part played by ester hydrolysis. It is clear, however, that acetylhomocholine (γ-acetoxypropyltrimethylammonium) and acetylformocholine (acetoxymethyltrimethylammonium) are active (Hunt and Taveau, 1911; Hunt and Renshaw, 1925) although less active than acetylcholine. Benzoylhomocholine is also active (Hunt and Taveau, 1906), possibly as active as benzoylcholine; benzoylformocholine does not seem to have been prepared. Of the ketones with 5 carbon chain studied by Welsh and Taub (1951), and by Ing, Kordik, and Tudor Williams (1952), 4-ketoamyltrimethylammonium, 3-ketoamyltrimethylammonium and 2-ketoamyltrimethylammonium were all active stimulators; however, among the phenolic ethers, β-phenoxyethyltrimethylammonium (the choline derivative) is highly active, whereas γ-phen-

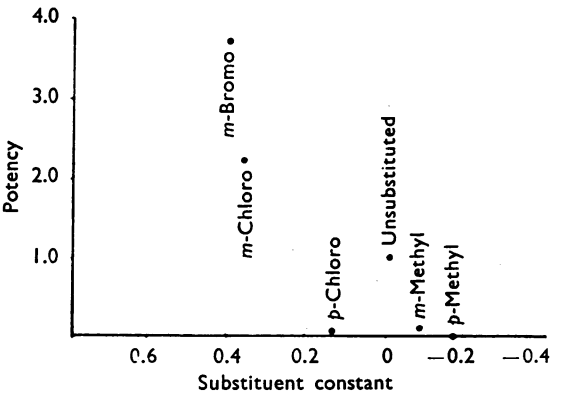


FIG. 3.—Potency of choline phenylether derivatives (relative to β-phenoxyethyltrimethylammonium=1) as pressor agents in atropine-treated cats (results of Hey, 1952) plotted against substituent constant. Nicotine-like activity increases with electron attractive power of substituent.

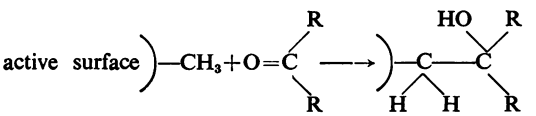
oxypropyltrimethylammonium (derivative of homocholine) is quite inactive (Hunt and Renshaw, 1929): so far as the evidence goes, there seems to be greater structural specificity in the ethers than in the carbonyl compounds.

A striking difference in the mechanism of action of choline esters and ethers is shown by comparing the results given in this paper with those of Hey (1952) (Fig. 3). Hey studied the activity of a series of substituted derivatives of the phenolic ether of choline, β-phenoxyethyltrimethylammonium bromide, and found that the activity in raising the blood pressure of the atropine-treated cat was *increased* by the insertion of electron attractive groups. As the activity of benzoylcholine derivatives tends to be inhibited by this type of substitution, it is probable that there are two different types of chemical reaction operating. With ethers the active surface of the ganglion appears to act as an " electrophilic " reagent and with esters as a " nucleophilic " reagent.

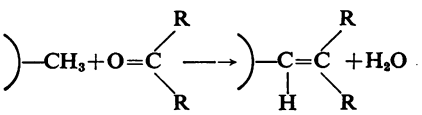
Proposed Hypothetical Carbonyl and Ether Receptor

It may be of interest at this point to put forward a possible mechanism by which the active surface of the ganglion could react both with carbonyl and ether groups. It is of course possible that two groups can coexist on the surface; but it is not a necessary assumption, since a single reagent can react with electron-rich or electron-deficient groups if it is capable of behaving as a weak acid. Such a group—put forward as an example, rather than as an hypothesis, since another weak acid would probably do as well—would be the activated methyl group as in the Knövenagel type of reaction.

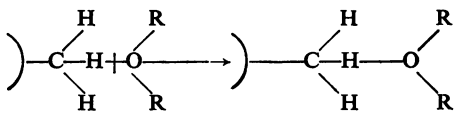
(*a*) for carbonyl group



OR



(*b*) for ether group



In (*a*) the ganglion surface is acting as a nucleophilic reagent and would condense less readily with an electron deficiency in the carbonyl group created by an electron attractive group in the benzene ring. In (*b*) the " hydrogen bond " is invoked; this is at its strongest when the hydrogen atom is acidic as in the activated methyl group and when the oxygen is most basic—that is, has an electron deficiency such as would be produced by an electron attractive group in the benzene ring (Rodebush and Buswell, 1939).

This scheme can also provide an explanation of the greater structural specificity which the choline ether series seem to possess as compared with choline esters and the corresponding ketones; the carbonyl oxygen of esters and ketones would form a covalent bond which, once it had reacted, would fix this part of the molecule; on the other hand, the hydrogen bond reacting with the ether group is a much weaker bond, and would make the reaction of the substance with its locus on the active surface of the ganglion more dependent on the close fitting of the rest of the molecule.

Summary

1. Pharmacological actions of a series of derivatives of benzoylcholine have been studied and their potencies compared with the potency of the parent compound.

2. The actions of benzoylcholine and *p*-methoxybenzoylcholine have been shown qualitatively to resemble the action of nicotine and the " nicotine-like " action of acetylcholine on the blood pressure of the cat.

3. The activity of this series shows a correlation with the substituent constants for members of the series: this is taken as evidence for the existence of carbonyl receptor groups on the active surface of the ganglion.

4. These results are compared with those obtained in a study of a series of derivatives of choline phenyl ether.

5. A type of receptor is proposed that might explain these results.

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