RESEARCH PAPERS

THE EFFECT OF *ORTHO* SUBSTITUTION ON THE HYDROLYSIS OF BENZOYLCHOLINE

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Received November 29, 1960

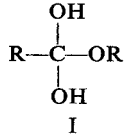
A series of mono- and di-*ortho*-substituted benzoylcholine compounds has been prepared and the rates of hydroxide ion and cholinesterase catalysed hydrolysis has been determined for each compound. The anti-acetylcholinesterase activities of the compounds have also been determined. It has been found that some of the compounds are stable to esterase catalysed hydrolysis and that the groups which confer stability do not prevent the formation of enzyme substrate complexes. The most suitable groups for ester stabilisation, when used as *ortho* substituents, are alkyl and iodo as mono substituents and most groups, except hydroxy, as di-*ortho* substituents.

THE *in vivo* hydrolysis of benzoyl ester groups is a limiting factor in the use of some drugs and consequently it would be of value to stabilise such esters. The hydrolysis of benzoylcholine is catalysed both by hydroxide ions and by serum cholinesterase and therefore this molecule is suitable to examine the influence of *ortho* substitution on esterase catalysed hydrolysis of benzoyl esters. Ormerod (1953) prepared several *meta* and *para* substituted benzoylcholine compounds and determined their rates of cholinesterase catalysed hydrolysis. The small differences observed between experimental results and results calculated using the Hammett substituent constant were attributed to "differences in the enzyme substrate complex".

Ortho substituents in benzoate esters have been shown (Evans, Gordon and Watson, 1937; Ingold, 1953a) to accelerate less or retard more the rate of ester hydrolysis than the same substituents in the *meta* and *para* positions. Consequently, *ortho* substitution of benzoylcholine may produce large changes in the rates of cholinesterase catalysed hydrolysis.

The interactions between an ester group and an *ortho* substituent may be direct or transmitted or a combination of both these effects. The direct effect is exerted by way of electrostatic forces, van der Waal's forces and short range repulsive forces. The effects of the latter forces have been termed the primary steric effect (Ingold, 1953b). Transmitted interactions are due to induction and mesomerism. Where steric repulsion alters the geometry of a molecule, thereby modifying the transmitted interactions, the effect has been termed the secondary steric effect (Ingold, 1953c).

Bender (1951) demonstrated the presence of a tetrahedral intermediate of the type I in the acid and alkali catalysed hydrolysis of esters.



I

The attack of an hydroxide ion to form this intermediate is said to be from a direction perpendicular to the plane of the ester group (Brown and Fried, 1943; Kadesch, 1944). If this plane is coplanar with the benzene ring then the *ortho* groups do not sterically prevent this approach (primary steric effect). *Ortho* groups may prevent free rotation of the carboxyl group around the bond connecting this group to the ring and may prevent coplanarity of the ester group and the benzene ring (secondary steric effect). This would have the effect of a perpendicular approach being hindered by the *ortho* substituents. One sufficiently large *ortho* group can prevent coplanarity of the ester group with the ring. Reaction is still possible but only from the side opposite to the hindering substituent.

When only one *ortho* substituent is present the direct effects are additional to those of induction and mesomerism. However, when two *ortho* substituents are present the direct effects completely dominate the transmitted interactions and most di-*ortho* substituted benzoyl esters are stable to hydrolysis.

The mechanism of esterase catalysed hydrolysis is considered to be initiated by an attack of a site of high electron density of the enzyme on the acyl carbon of the ester (Bergman, Nachmansohn and Wilson, 1950; Davies and Green, 1958; Rydon, 1958), not unlike the attack of basic reagents such as the hydroxide ion. It appears possible therefore, that *ortho* substituted benzoyl esters will be stable to esterase catalysed hydrolysis.

A series of mono- and di-*ortho* substituted benzoylcholine esters has been prepared and the rates of hydroxide ion and cholinesterase catalysed hydrolysis have been determined.

Experimental

Chemical

All the mono substituted benzoic acids were obtained commercially. The preparation of 2-methylbenzoylcholine iodide is typical of the method used for the mono substituted benzoylcholines.

2-Methylbenzoyl chloride. *o*-Toluic acid (25 g.) was refluxed on a steam bath with thionyl chloride (28 g.) until fumes of hydrogen chloride were no longer evolved; about two hr. The solution was distilled under reduced pressure and the fraction b.p. 110° at 29 mm. was collected.

2-Dimethylaminoethyl 2-methylbenzoate hydrochloride. 2-Methylbenzoyl chloride (15·4 g., 0·1 mole) was dissolved in dry benzene (100 ml.). 2-Dimethylaminoethanol (8·9 g., 0·1 mole) was added to the solution which was kept cool during the addition and then refluxed for 30 min. The white solid which precipitated was filtered off and recrystallised from an acetone-ether mixture. The product was a white crystalline solid m.p. 127°. Yield 97 per cent.

2-Methylbenzoylcholine iodide. 2-Dimethylaminoethyl 2-methylbenzoate hydrochloride (15 g., 0·1 mole) was dissolved in water (20 ml.) in a separator. Ammonia solution (7·6 ml., 10 per cent NH_3) was added and the free base completely extracted with chloroform. The chloroform solution was washed with water, dried and methyl iodide (21 g., 0·15

TABLE I

Ortho SUBSTITUTED BENZOYLCHOLINE COMPOUNDS

Compound (*a*) X-Benzoylcholine X=	Acyl halide		Dimethylaminoethyl ester hydrochloride			Choline derivatives			Analysis for choline derivatives					
									Found			Required		
	Reflux time	b.p.	Reflux time	Solv. for recryst.	m.p. °C	Reflux time	Solv. for recryst.	m.p. °C	C	H	I	C	H	I
2-Chloro[c]	(*b*)	110° at 15 m.m.	30 min.	Ethanol Ether	128	30 min.	Ethanol Acetone	174	39·6	4·7	34·4	38·9	4·6	34·4
2-Bromo	(*b*)	125° at 20 mm.	3 hr.	Ethanol Ether	147	2 hr.	Ethanol	142	35·4	4·2	30·6	34·8	4·14	30·7
2-Iodo	2 hr.	159° at 27 mm.	1 hr.	Ethanol Ether	167	1 hr.	Ethanol Ether	152	31·3	3·7	28·0	31·2	3·69	27·6
2-Nitro	2 hr.	Unsafe to distil[d]	15 min.	Ethanol Ether	171	1 hr.	Ethanol Ether	155	38·0	4·5	32·9	37·9	4·51	33·4
2-Methoxy	2 hr.	128° at 11 mm.	2 hr.	Acetone	132	3 hr.	Ethanol	162	42·4	5·5	34·4	42·2	5·48	34·8
2,6-Dimethyl ..	3 hr.	216°	8 hr.	Ethanol	160	1 hr.	Ethanol	168	48·0	6·7	34·8	48·1	6·3	35·0

(*a*) All prepared as iodide salt.
(*b*) Obtained commercially.
(*c*) Ormerod (1953).
(*d*) Bonner and Hurd (1946).

mole) added. The mixture was then refluxed for 2 hr. The precipitated white solid was recrystallised from acetone-ether m.p. 157°. Yield 99 per cent $C_{13}H_{20}INO_2$ requires C, 44·7; H, 5·7; I, 36·39. Found: C, 44·3; H, 5·7; I, 36·2.

A list of ortho substituted benzoylcholine compounds prepared by this method is given in Table I.

2-Dimethylaminoethyl bromide hydrobromide. This was prepared by heating a mixture of 2-dimethylaminoethanol (45 g.) and hydrobromic acid (95 ml., 60 per cent) at 120° in an autoclave for 6 hr. The solution was evaporated to dryness under reduced pressure and the residue recrystallised from ethanol-ether mixture m.p. 173–174° (Huttrer and others 1946; m.p. 187°). Yield 70 g. Found: C, 20·5; H, 4·5; Br, 34·4. Calc. for $C_4H_{11}Br_2N$, C, 20·6; H, 4·7; Br, 34·3.

2,6-Dichlorobenzoic acid. This acid was prepared from 2,6-dichlorotoluene by the method of Norris and Bearse (1940), m.p. 147° (Norris and Bearse, 1940; m.p. 143°). Found: C, 43·82; H, 2·0. Calc. for $C_7H_4Cl_2O_2$; C, 43·9; H, 2·1. (Note. The entire synthesis should be carried out in an efficient fume cupboard since an intensely lachrymatory substance is formed during the preparation.)

Silver 2,6-dichlorobenzoate. 2,6-Dichlorobenzoic acid (9·5 g., 0·05 mole) was added to hot distilled water (100 ml.) to which concentrated ammonia solution (9 ml.) was added. A steam bath was used to remove excess ammonia and a warm solution of silver nitrate (8·5 g., 0·05 mole) was added dropwise with stirring. The silver salt precipitated, was filtered, washed with water and dried at 70° under reduced pressure. Yield 14·8 g.

2-Dimethylaminoethyl 2,6-dichlorobenzoate. Silver 2,6-dichlorobenzoate (6·0 g., 0·02 mole) was finely powdered and added to chloroform (150 ml.). The suspension was stirred, 2-dimethylaminoethyl bromide hydrobromide (4·5 g., 0·02 mole) added and the slurry was refluxed for 48 hr. The hot reaction mixture was filtered under reduced pressure and the solution used for the next stage without isolating the product.

2,6-Dichlorobenzoylcholine iodide. The chloroform solution of 2-dimethylaminoethyl 2,6-dichlorobenzoate hydrobromide was transferred to a separator, ammonia added (5 ml., 10 per cent NH_3) and the chloroform layer separated, washed and dried. Methyl iodide (4 g.) was added to the solution which was refluxed for 1 hr. when the product precipitated. It was filtered, washed and recrystallised from ethanol-acetone mixture m.p. 157°. $C_{12}H_{16}Cl_2INO_2$ requires C, 35·7; H, 3·96; I, 31·43. Found: C, 36·28; H, 4·1; I, 30·7. A list of substituted benzoylcholine compounds prepared by the silver salt method is given in Table II. 2,6-Dihydroxybenzoic acid and 2,4,6-trinitrobenzoic acid were obtained commercially.

2,6-Dimethylbenzoic acid. This was prepared from *m*-2-xylidine by the method of Jacobs and others (1951), m.p. 116° (Jacobs and others, 1951; m.p. 115·5°). Found: C, 72·24; H, 6·6. Calc. for $C_9H_{10}O_2$; C, 72·0; H, 6·7.

2,4,6-Tribromobenzoic acid. This acid was prepared from *m*-aminobenzoic acid by the method of Robinson and Robinson (1956); m.p. 202°

TABLE II

Ortho SUBSTITUTED BENZOYLCHOLINE COMPOUNDS PREPARED BY THE SILVER SALT METHOD

Compound (*a*) X-Benzoylcholine X=	Silver salt		2-Dimethylaminoethyl HBr ester of			Choline derivatives			Analysis for choline derivatives					
	Quantity of reactants		Quantity of reactants						Found			Required		
	Acid	$AgNO_3$	Silver salt	Amino halide	Reflux time	Reflux time	Solv. for recryst.	m.p. °C	C	H	I	C	H	I
2,6-Dihydroxy ..	5·0 g. 0·03 mole	5·0 g. 0·03 mole	8·6 g. 0·03 mole	7·0 g. 0·03 mole	48 hr.	30 min.	Ethanol	178	38·5	5·1	—	39·2	4·9	—
2,4,6-Trinitro ..	13·0 g. 0·05 mole	8·5 g. 0·05 mole	8·0 g. 0·02 mole	4·5 g. 0·02 mole	48 hr.	2 hr.	Ethanol	180	30·9	3·4	26·9	30·7	3·2	27·0
2,4,6-Tribromo ..	14·4 g. 0·04 mole	7·0 g. 0·04 mole	13·0 g. 0·03 mole	7·0 g. 0·03 mole	48 hr.	2 hr.	Ethanol Acetone	216	24·5	2·4	22·0	25·2	2·62	22·2

(*a*) All prepared as iodide.

(Robinson and Robinson 1956; m.p. 192·5–1954·5°). Found: C, 23·3; H, 0·8; Br, 65·8. Calc. for $C_7H_3Br_3O_2$: C, 23·4; H, 0·98; Br, 66·8.

Hydrolyses

Enzyme catalysed hydrolysis. An electrically heated, thermostatically controlled water bath, fitted with an electrical stirrer was used. The temperature was adjusted to 37° ± 0·1. A small beaker (100 ml.) was supported in the water bath and into its top was fitted a rubber bung with five holes. Through these five holes were inserted a glass electrode, a glass stirring rod, one arm of an agar bridge, the tip of a microburette, and a small funnel through which solutions were poured. The other arm of the agar bridge dipped into a saturated potassium chloride solution into which also dipped the calomel electrode. Both the glass and the calomel electrodes were connected to a Pye pH meter, the temperature compensator of which was set at 37°.

Horse serum (1 ml.) was pipetted into the funnel and washed into the beaker with distilled water (9 ml.). The pH was adjusted to 7·9 by the addition of approximately 0·01 N sodium hydroxide solution from the microburette. After allowing 15 min. for temperature equilibration the substrate (1×10^{-4} mole), dissolved in distilled water (15 ml.) at 37°, was added through the funnel and washed through with distilled water (5 ml.) at 37°. (The concentration of substrate in the digest was $3{\cdot}3 \times 10^{-3}$ molar.) After 1 to 2 min. 0·01 N sodium hydroxide solution was added until the pH of the solution was again 7·9. The pH was then maintained at 7·9 by the dropwise addition of alkali, the burette readings and the time being recorded. Throughout the determination the solution in the beaker was stirred frequently. At least three hydrolysis experiments were made with each substrate. From the slopes of graphs drawn with ordinates representing ml. of NaOH added and abscissae representing time, the rates of cholinesterase plus hydroxide ion catalysed hydrolysis were calculated. The results, corrected for non-enzymic hydrolysis are given in Table III. The values given are the mean of three measurements. The measured rates of hydrolysis for each compound were all within 5 per cent of the mean.

Source of cholinesterase. Horse serum was used because it has a high content of cholinesterase (Stedman and Stedman, 1935). All the determinations were made using serum from the same commercial batch since differences in cholinesterase activity have been observed with different batches of serum (Buckles, 1956). The serum was stored in a refrigerator at 4°. No deterioration in enzyme activity was observed during the course of the work.

0·01 N *Sodium hydroxide solution.* An approximately 0·2 N stock solution was prepared by dissolving the required amount of A.R. sodium hydroxide in freshly boiled and cooled distilled water. The stock solution was diluted as required and standardised against potassium hydrogen phthalate by a potentiometric titration.

Hydroxide ion catalysed hydrolysis. The rates of hydroxide ion catalysed hydrolysis were measured in a similar way to that used for the

enzymic rates. A dilute buffer solution consisting of 1 ml. of B.P. 1953 boric acid and potassium chloride buffer pH 7·9 was used in place of the serum. At least three hydrolysis experiments were made on each substrate. Graphs were drawn with the ordinates representing ml. of

TABLE III

MEAN RATES, RELATIVE RATES AND THE RATIO OF THE RATES OF CHOLINESTERASE AND HYDROXIDE ION CATALYSED HYDROLYSIS OF *ortho* SUBSTITUTED BENZOYLCHOLINE COMPOUNDS

Compound (*a*) R—Benzoylcholine R=	Rate of cholinesterase catalysed hydrolysis ml. 0·01 N NaOH/min./g. mole	Rate of hydroxide catalysed hydrolysis ml. 0·01 N NaOH/min./g. mole	Relative rates of cholinesterase catalysed hydrolysis (*b*)	Relative rates of hydroxide catalysed hydrolysis (*c*)	Ratio: E/NE
H	831	156	1·00	1·00	5·30
2-Methyl	203	110	0·24	0·65	1·85
2-Chloro	1,095	219	1·30	1·40	5·00
2-Bromo	580	173	0·70	1·02	3·40
2-Iodo	200	125	0·24	0·74	1·60
2-Nitro	156	237	0·19	1·40	0·65
2-Methoxy	604	120	0·73	0·77	5·00
2,6-Dichloro	0	0	0	0	0
2,6-Dimethyl	0	0	0	0	0
2,6-Dihydroxy	Unstable in solution				
2,4,6-Tribromo	0	0	0	0	0
2,4,6-Trinitro	0	575	0	3·40	0

(*a*) All compounds were used as the iodide salt.
(*b*) Rates relative to cholinesterase catalysed hydrolysis of benzoylcholine = 1.
(*c*) Rates relative to the hydroxide ion catalysed hydrolysis of benzoylcholine = 1.
E Rate of enzymic hydrolysis.
NE Rate of non-enzymic hydrolysis.

NaOH added and abscissae representing time. The rate of non-enzymic hydrolysis was obtained from the slope of each graph. The measured rates of hydrolysis for each compound were all within 5 per cent of the mean. The results obtained are given in Table III.

TABLE IV

ANTI-ACETYLCHOLINESTERASE ACTIVITY OF COMPOUNDS PREPARED. pH 6·3. TEMPERATURE 37°. SUBSTRATE ACETYLCHOLINE, CONCENTRATION 0·003 M. SOURCE OF ENZYME, ERYTHROCYTE STROMATA. I 50 VALUES IN G. MOLES/L.

Compound (*a*)	I 50 value
Benzoylcholine	$2{\cdot}90 \times 10^{-3}$
2-Methylbenzoylcholine	$9{\cdot}35 \times 10^{-4}$
2-Chlorobenzoylcholine	$3{\cdot}29 \times 10^{-4}$
2-Bromobenzoylcholine	$6{\cdot}90 \times 10^{-4}$
2-Iodobenzoylcholine	$2{\cdot}00 \times 10^{-4}$
2-Nitrobenzoylcholine	$3{\cdot}38 \times 10^{-4}$
2-Methoxybenzoylcholine	$4{\cdot}37 \times 10^{-4}$
2,6-Dichlorobenzoylcholine	$4{\cdot}63 \times 10^{-4}$
2,4,6-Tribromobenzoylcholine	$5{\cdot}00 \times 10^{-4}$
2,4,6-Trinitrobenzoylcholine	$3{\cdot}53 \times 10^{-4}$

(*a*) All compounds were used as the iodide salts.

Inhibition of acetylcholinesterase. Benzoylcholine is an inhibitor of acetycholinesterase (Glick, 1938). To obtain information about the effect of *ortho* substitution on the formation of an inhibitor enzyme complex the anti-acetylcholinesterase activity of the *ortho* substituted benzoylcholine compounds was determined. The standard Warburg

manometric technique was used, and the source of the enzyme was red cell stromata. The results obtained and the conditions used are given in Table IV.

DISCUSSION

Hydroxide Ion Catalysed Hydrolysis

The esters possessing one strongly electronegative chloro or nitro group in the *ortho* position were more rapidly hydrolysed than benzoylcholine. These results can be attributed to the electromeric properties of the substituents. There is a diminution of hydrolysis rates in the order chloro-, bromo-, and iodo-benzoylcholine. This is probably due to the smaller electromeric effects of the bromo and iodo substituents and also to steric effects (direct interactions) being involved with these larger groups.

The hydrolysis rates of *o*-methyl- and *o*-methoxy-benzoylcholine were both less than that of benzoylcholine. Both these substituents are able to force electrons into the carboxyl group thereby making hydroxide ion

TABLE V

RELATIVE RATES OF HYDROLYSIS OF *ortho* SUBSTITUTED BENZOYLCHOLINE COMPOUNDS AND *ortho* SUBSTITUTED ETHYL BENZOATE ESTERS

Benzoylcholine series		Ethyl benzoate series (Ingold, 1953c)	
Ortho substituent	Relative rate	*Ortho* substituent	Relative rate
H	1·00	H	1·00
Cl	1·40	Cl	2·20
NO_2	1·40	NO_2	8·70
Me	0·65	Me	0·125

attack difficult. The methyl group forces electrons towards the carboxyl group by induction because of its electropositive character, but the methoxy group does so by means of mesomerism.

Diortho substitution with chloro, bromo and methyl groups conferred great stability to hydrolysis. This almost certainly arises from the dominance of steric effects over induction and mesomerism. The instability of the dihydroxy ester is probably to be attributed to hydrogen bonding occurring between the hydroxyl groups and the carbonyl oxygen. This would increase the electropositivity of the carbonyl carbon atom. The hydrolysis of the trinitro ester is difficult to explain.

The relative rates of hydrolysis of several *ortho* substituted benzoylcholine compounds and the corresponding *ortho* substituted ethyl benzoates are compared in Table V. Although the conditions under which the hydrolysis rates were measured for each series were not identical there are noticeable differences in the relative values especially for the nitro and methyl substituted esters. The increased rate of hydrolysis of the methyl substituted benzoylcholine compared with the corresponding ethyl benzoate may arise from the attraction of electrons from the carbonyl carbon atom by the quaternary nitrogen atom. This effect has been demonstrated with aliphatic quaternary ammonium esters (Aksnes and

Prue, 1959). It was expected that the influence of the quaternary nitrogen atom would also cause an increase in the rate of hydrolysis of benzoylcholine esters possessing electronegative substitutents but this was not found. The quaternary nitrogen atom must again be involved since this is the only appreciable structural difference between the two series. In the case of *o*-nitrobenzoylcholine it is possible that the positively charged trimethylammonium group becomes associated with the negatively charged nitro group forming a loose ring configuration. In such a system the carbonyl carbon atom is shielded from attack by an hydroxide ion and hence the rate of hydrolysis of *o*-nitrobenzoylcholine would be slow compared with ethyl *o*-nitrobenzoate. A similar effect may also be expected with *o*-chlorobenzoylcholine but since chlorine is far less electronegative than the nitro group the effects would be expected to be much smaller.

Cholinesterase Catalysed Hydrolysis

Since all the mono substituted esters were hydrolysed it may be concluded that enzyme substrate complexes were formed. The ratios of the rates of cholinesterase catalysed hydrolysis, for the mono substituted esters, to those of non-enzymic hydrolysis are given in Table III. The ratio is small for those compounds with large *ortho* substituents. Hence it appears that cholinesterase catalysed hydrolysis is more susceptible to steric factors than is hydroxide ion catalysed hydrolysis.

Anti-acetylcholinesterase Activity

All the compounds prepared were more potent inhibitors of acetylcholinesterase than was benzoylcholine itself. This indicates that these esters form enzyme inhibitor complexes with acetylcholinesterase. It can be seen from Table IV that the inhibition of acetylcholinesterase is virtually independent of the size of the *ortho* substituent.

Conclusions

It is possible to prepare *ortho* substituted benzoylcholine compounds which form enzyme ester complexes with cholinesterase and with acetylcholinesterase but which are stable to cholinesterase catalysed hydrolysis. The groups which appear most suitable for ester stabilisation, when used as *ortho* substituents, are alkyl and iodo as mono substituents, and most groups, except hydroxy, as di-*ortho* substituents.

Acknowledgements. The authors wish to thank Prof. K. Bullock for assistance with the enzyme work.

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