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

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The effects of mono- and di-*ortho* substitution of benzoylcholine on actions on the rat phrenic diaphragm and frog rectus abdominis preparations have been examined. *Ortho* substitution of benzoylcholine does not necessarily lead to a reduction in activity and may increase potency. It appears that mono-*ortho*-iodo substitution provides most stability combined with pharmacological activity.

The enzymatic cleavage of esters by the esterase group of enzymes is one of the more important routes of drug metabolism (Brodie, Gillette and La Du, 1958, Brodie, Mackel and Jondorf, 1958) and consequently methods of changing the rate of ester hydrolysis are of potential importance in drug design. The stability of an ester towards hydroxide ion-catalysed hydrolysis is dependent on the steric and electronic characteristics of the component acid and alcohol (Hammett, 1940). Levine and Clarke (1955), Glick (1938, 1939, 1941, 1942) and Fu, Birnbaum and Greenstein (1954), have shown that the same factors are also important in *in vitro* and presumably in *in vivo* enzyme catalysed hydrolysis of esters.

It is possible to modify the stability of benzoic acid esters towards hydroxide ion-catalysed hydrolysis by substitution in the aromatic nucleus (Kindler, 1928; Evans, Gordon and Watson, 1937). Substituents in the *ortho* position accelerate less or retard more the rate of hydrolysis of the ester group than the same substituents in the *meta* and *para* positions (Ingold, 1953). This is an example of the *ortho* effect. (For a full discussion of the *ortho* effect see Stoker, 1959.)

To investigate the possibility of using *ortho* substitution as a general method of stabilising ester groups in drug molecules Thomas and Stoker (1961) synthesised a series of mono- and di-*ortho* substituted benzoylcholine derivatives. The hydrolysis of benzoylcholine is catalysed both by hydroxide ions and cholinesterase (Mendel, Mundell and Rudney, 1943) and was, therefore, a suitable molecule to use to compare the effect of *ortho* substitution on the relative rates of chemical and enzymic catalysed hydrolysis. It was found that *ortho* substitution in benzoylcholine produced compounds which were either more rapidly hydrolysed by cholinesterase than the unsubstituted ester, more slowly hydrolysed or completely stable towards the esterase. The stability of the esters towards both hydroxide ion- and cholinesterase catalysed hydrolysis followed roughly that predicted from theoretical considerations of the "*ortho* effect" and the mechanism of hydrolysis. The "stable" substituted benzoylcholines were shown to be inhibitors of cholinesterase. All the *ortho* substituted benzoylcholines were more powerful inhibitors of acetylcholinesterase than benzoylcholine itself. The inhibition studies

were taken to indicate that the *ortho* groups did not prevent the adsorption of benzoylcholine onto the "active site" of the enzymes. To examine further the potential usefulness of *ortho* substitution in drug design the activities of the mono- and di-*ortho* substituted benzoylcholines have now been examined on more complex biological systems than the isolated enzyme preparations used previously.

The pharmacology of benzoylcholine has been studied by Carr and Bell (1947), Bovet and others (1949), Akcasu, Sinha and West (1952), who have shown that it exhibits a number of different actions. For the present purpose it was important to select biological systems which were relatively simple and preferably those on which benzoylcholine had only one type of action, which could be examined quantitatively. These considerations led to the use of the frog rectus abdominis and the rat diaphragm-phrenic nerve preparations. Ormerod (1956) used these two preparations in comparative assays of a series of *meta* and *para* substituted benzoylcholine compounds without reporting any difficulty.

Experimental

Chemical. All compounds were prepared as described by Thomas and Stoker (1961).

Pharmacological

Assays with the frog isolated rectus abdominis muscle. The isolated muscle was suspended in oxygenated Starling frog ringer at room temperature (19–22°). 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 (Ormerod, 1956). The ability of the unknown to cause contracture of the rectus abdominis preparation was computed as a percentage of that of benzoylcholine (Buckley, 1961). The assays were repeated in the presence of eserine 1×10^{-4}, to which the tissue was exposed for 30 min. before any effects were recorded. The organ bath was calibrated at 4·0 ml.; drug solutions in the physiological saline were added in 1·0 ml. volumes from tuberculin syringes. Contact time was exactly 90 sec. after which the muscle was washed repeatedly. At the 5th min. the muscle was gently stretched; the tissue was then allowed to rest until the 10th min. when the next dose was introduced.

In some experiments the effect of subthreshold doses of the compounds on acetylcholine-induced contractures of the muscle was observed. Here the dose of drug was introduced 30 sec. before that of acetylcholine, the resultant effect being recorded for exactly 90 sec. Control effects of acetylcholine were recorded both before and after recording the effect of the drugs on the acetylcholine response. The effect of tubocurarine on the contractures elicited by the compounds was similarly studied.

Assays with the isolated phrenic-diaphragm preparation of the rat. The preparation (Bülbring, 1946) was suspended in a modified Tyrode solution (Taugner and Fleckenstein, 1950), at 29° and aerated with 95 per cent

oxygen and 5 per cent carbon dioxide. The muscle segment was stimulated indirectly *via* the phrenic nerve with single, supramaximal square wave pulses of 5–10 V and 0·5 m-sec. duration at a frequency of 6/min. Direct stimulation of the muscle was by pulses of up to 40 V and 5–20 m-sec. duration; the rate was 6/min. In each experiment the organ bath was calibrated to 14 ml. with the preparation in position. Doses of drug were delivered in 1 ml. volumes by means of tuberculin syringes. Drugs were dissolved in the Taugner-Fleckenstein solution. The concentration (mol./ml.) in organ bath fluid of each compound, which when acting for 3 min. would cause a 50 per cent reduction in the height of contraction was determined. This concentration was calculated from observations of the mean effects of four different doses of each compound on four different rat diaphragm preparations. The method was based on the assumption that over the range of concentrations used the effect increased linearly with the dose. A test was incorporated into the statistical analysis to show that this was so.

TABLE I

THE RELATIVE POTENCIES OF *o*-SUBSTITUTED BENZOYLCHOLINE DERIVATIVES IN ELICITING CONTRACTURE OF THE FROG RECTUS ABDOMINIS MUSCLE

Compound R-benzoylcholine R =	Potency relative to Benzylcholine (BCH) = 100 per cent		Confidence limits (P = 0·95)		Concn. used in the expts. mol./ml. × 10^{-7}			
					With eserine (10 μg./ml.)		Without eserine	
	With eserine (10 μg./ml.)	Without eserine	With eserine (10 μg./ml.)	Without eserine	Low dose	High dose	Low dose	High dose
H (benzoylcholine) ..	(assigned) 100	100	—	—	(means from all expts) 0·6	1·2	1·6	3·2
o-Methyl- ..	28 30 28	100 92 89	23·9–32·8 28·7–30·8 23·0–35·0	92·0–110 79·5–106 80·4–97·6	2·4 2·4 1·2	4·8 4·8 2·4	1·5 1·5 2·0	3·0 3·0 4·0
o-Methoxy- ..	43 44	121 127	39·6–46·1 40·8–46·5	116–127 119–136	2·0 1·6	4·0 3·2	1·6 1·2	3·2 2·4
o-Chloro- ..	84 89	120 132	71·3–96·6 77·3–101	104–143 116–150	1·0 1·0	2·0 2·0	1·6 1·2	3·2 2·4
o-Nitro-	19 24 21	* 102 (approx.)	14·3–25·5 22·7–24·9 19·8–22·8	† Results of 4 expts. 97; 89; 101; 119	3 2 2	6 4 4	1·12 to 1·28	2·24 to 2·56
o-Bromo- ..	104 111	202 196	95–113·6 103–119	175–232 182–210	0·5 0·5	1·0 1·0	0·72 0·72	1·42 1·42
o-Iodo-	207 214 227	477 437 453 422	197–218 210–217 201–246	439–533 395–483 402–527 382–463	0·25 0·2 0·2	0·5 0·4 0·4	0·3 0·4 0·5 0·3	0·6 0·8 1·0 0·6
2,6-Dimethoxy- ..			No stimulant action					
2,6-Dichloro- ..			Slight stimulant action					

* Mean of four expts. † (Expts. not valid—see text.) Confidence limits not calculated.

This procedure was adopted to eliminate the bias which would arise from the "staircase" effect occurring on repetition of the same dose of

any of the drugs on a single tissue. Only four doses were applied to any one preparation since it was thought that the final result of an experiment would be significantly influenced by preliminary dosage of the tissue.

The four doses were chosen on the basis of preliminary experimentation. They were in constant ratio and were expected to produce between 25 and 80 per cent blockade in 3 min. The four doses were applied each to four rat phrenic-diaphragms in randomized order in a Latin square design.

Data derived from the four tissues were combined; effects due to order of doses and differences between tissues were eliminated from the calculation of the error by variance analysis (Buckley, 1961).

Results and Discussion

The detailed results obtained with the frog rectus abdominis and rat diaphragm-phrenic nerve preparation are given in Tables I and II respectively. The results from Thomas and Stoker (1961) of antiacetylcholinesterase activity as well as hydrolysis rates are presented together with the two sets of results obtained in the present investigation in Table III.

TABLE II

THE RELATIVE ACTIVITIES OF BENZOYLCHOLINE AND ITS *o*-SUBSTITUTED DERIVATIVES ON THE RAT PHRENIC-DIAPHRAGM PREPARATION

Compound R-benzoylcholine R =	Conc. mol./ml. × 10^{-6} for 50 per cent block in 3 min.	Confidence limits (P = 0·95) mol./ml. × 10^{-6}	Concns. used in expts. mol./ml. × 10^{-6}	Per cent potency relative to benzoylcholine = 100 per cent
H 1.	2·36	2·10 — 2·66	1·66 — 2·86	100
2.	2·38	2·15 — 2·65	1·66 — 2·86	
mean	2·37	2·12 — 2·65		
o-Methyl-	1·20	1·15 — 1·26	0·82 — 1·38	198
o-Methoxy-	2·35	2·25 — 2·45	1·66 — 2·86	100
o-Chloro-	1·73	1·60 — 1·89	1·13 — 1·93	139
o-Nitro-	1·62	1·58 — 1·66	1·37 — 2·33	148
o-Bromo-	2·19	1·99 — 2·41	1·37 — 2·33	108
o-Iodo-	0·96	0·89 — 1·04	0·68 — 1·16	247
2,6-Dimethoxy-	2·17	2·05 — 2·30	1·63 — 2·76	109
2,6-Dichloro-	0·59	0·56 — 0·63	0·45 — 0·80	402

1. The response to indirect stimulation was unaffected when each of the compounds was applied directly to the phrenic nerve.
2. The muscle responded to direct stimulation after the response to phrenic stimulation had been blocked by each of the compounds.
3. Neostigmine did not affect the blocking action of the compounds.

All the *ortho* substituted benzoylcholine derivatives tested inhibit the response of the rat diaphragm-phrenic nerve preparation to indirect supramaximal stimulation. This preparation responded to direct muscle stimulation when the response to indirect stimuli had been blocked by the compounds. When applied directly to the nerve, concentrations of fifteen times those which were effective in the muscle bath failed to prevent

contraction in response to phrenic nerve stimulation. Thus the compounds were considered to be acting at the neuromuscular junction.

All the mono-*ortho* substituted compounds caused contraction of the isolated frog rectus abdominis muscle. 2,6-Dichlorobenzoylcholine caused slight contracture but 2,6-dimethoxybenzoylcholine caused none. Both di-*ortho* substituted compounds did, however, block the response of the frog rectus abdominis to submaximal doses of acetylcholine. Doses of curare which block the response of the frog rectus abdominis to submaximal doses of acetylcholine also block the contracture of this tissue elicited by benzoylcholine compounds. It appears that *ortho* substitution does not prevent the adsorption of these compounds to receptors of the isolated frog rectus and rat diaphragm preparations. Thomas and Stoker (1961) reached the same conclusions with respect to the *in vitro* interaction of the compounds with cholinesterase and acetylcholinesterase.

TABLE III

SUMMARY OF THE RESULTS OF THE STUDIES ON BENZOYLCHOLINE AND SOME MONO-*o* AND DI-*o*-SUBSTITUTED BENZOYLCHOLINE DERIVATIVES

Compound R-benzoylcholine R =	Action on frog rectus abdominis: Relative potency as stimulant (BCH = 100 per cent)				Potency on rat diaphragm relative BCH = 100 per cent	* Relative hydrolysis rates		* I 50 Values g. moles/ litre acetylcholinesterase
	+ Eserine (10 μg./ml.)		(*a*)	(*b*)		Hydroxide	Enzymic	
H (benzoylcholine) ..	100	100	+	−	100	1·00	1·00	$2{\cdot}90 \times 10^{-5}$
o-Methyl-	28 30 28	100 92 89	+	−	198	0·65	0·24	$9{\cdot}35 \times 10^{-4}$
o-Methoxy-	43 44	121 127	+	−	100	0·77	0·73	$4{\cdot}37 \times 10^{-4}$
o-Chloro-	84 89	120 132	+	−	139	1·40	1·30	$3{\cdot}29 \times 10^{-4}$
o-Nitro-	19 24 21	102 (approx.)	+	−	148	1·40	0·19	$3{\cdot}38 \times 10^{-4}$
o-Bromo-	104 111	202 196.	+	−	108	1·02	0·70	$6{\cdot}90 \times 10^{-4}$
o-Iodo-	207 214 227	477 437 453 422	+	−	247	0·74	0·24	$2{\cdot}00 \times 10^{-4}$
2,6-Dimethoxy-	No stimulant action			+	109	0·00	0·00	
2,6-Dichloro-	Slight stimulant action			+	402	0·00	0·00	$4{\cdot}63 \times 10^{-4}$

* *Via* Thomas and Stoker (1961). (*a*) = action of tubocurarine on the response to the compound; (*b*) = action of compound on the response to acetylcholine; + = response inhibited, − = unaffected.

It may be seen from Table III that mono-*o*-methyl groups and di-*ortho* substituents will stabilise benzoylcholine towards enzymic and hydroxide ion-catalysed hydrolysis. When examined as inhibitors of the rat phrenic nerve-diaphragm preparation these compounds were found to be from

one to four times as potent as benzoylcholine. Thus it is possible to use *ortho* substitution to stabilise benzoylcholine without incurring any loss of activity on the isolated rat diaphragm preparation.

On the frog rectus abdominis preparation, both in the absence and presence of eserine the di-*ortho* substituted compounds had very slight (2,6-dichlorobenzoylcholine) or no stimulant action (2,6-dimethoxybenzoylcholine). In the absence of eserine only the mono-*o*-methyl compound was less active than benzoylcholine in causing contracture of the frog rectus abdominis. This reduction of activity (about 10 per cent) would not significantly affect the use of an *o*-methyl group as stabilising substituent. The other mono-*ortho* substituted compounds were more potent than benzoylcholine. *o*-Iodobenzoylcholine was five times as potent and the *o*-bromo compound was twice as potent as benzoylcholine. The nitro derivative was about as potent as benzoylcholine on the frog rectus but the assay was invalidated on the basis of the significant deviation from parallelism which existed between the standard and unknown dose response lines. Thomas and Stoker (1961) noted that *o*-nitrobenzoylcholine was anomalous in its behaviour with the cholinesterases.

Since it has been shown that eserine (10 μg./ml.) inhibits the cholinesterase of frog rectus muscle (Hobbiger, 1950), then in its presence, effects due to the anticholinesterase action and different rates of hydrolysis are excluded from the observed activity of the compounds. The activities obtained in the presence of eserine may be regarded as the result of the effect of the substituents on the inherent activity of benzoylcholine and the ability of the molecule to reach the site of action. The results obtained when eserine was used are given in Table I from which it may be seen that mono-*o*-chloro and *o*-bromo groups have the least effect on the inherent activity of benzoylcholine. Mono-*o*-iodobenzoylcholine is twice as active as the unsubstituted ester while *o*-nitro, *o*-methoxy and *o*-methyl groups significantly reduce the potency of benzoylcholine in the presence of eserine. Considered together, the results obtained using the frog rectus abdominis preparation as test object indicate that the mono-*ortho*-halogen groups are the least likely to diminish the activity of benzoylcholine.

The following conclusions are reached.

Ortho substitution may be used to stabilise benzoylcholine without necessarily removing the biological actions of the ester. In some cases activity is enhanced considerably. From the present investigation it appears that mono-*ortho*-iodo substitution provides the greatest measure of stability combined with pharmacological activity. However because of the complex nature of the *ortho* effect it is not possible to predict which group would be the most efficient in any particular case.

The fact that with the di-*ortho* substituted esters the action of benzoylcholine on the frog rectus is completely removed while other actions such as anticholinesterase and inhibition of the rat diaphragm-phrenic nerve preparation are enhanced leads to the suggestion that *ortho* substitution may be used to increase the specificity of action of a compound.

Finally *ortho* substitution could in principle be used in drug latentation (Harper, 1959). This has been defined as "the chemical modification of a biologically active compound to form a new compound which, upon *in vivo* enzymatic attack, will liberate the parent compound". The modified compound is described as the "transport form" while the modifying groups are termed "carrier groups". In the case of carboxylic acids or alcohols the carrier group may be an esterifying group. Since *ortho* substitution can modify the rate of release of the active drug from the transport molecule, then it could be used to improve the usefulness of a carrier group.

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