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ACTIONS OF SOME ESTERS OF 3,3-DIMETHYLBUTAN-1-OL (THE CARBON ANALOGUE OF CHOLINE) ON THE GUINEA-PIG ILEUM

R.B. BARLOW & JULIA H. TUBBY

Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ

1 Estimates were made of the affinity constants for postganglionic acetylcholine receptors of the guinea-pig ileum of the esters of 3,3-dimethylbutan-1-ol with benzilic, (±)-cyclohexylphenylglycollic, (±)-mandelic, and diphenylacetic acids.

2 Attempts were made to check the competitive nature of the antagonism by using as wide a range of concentrations of antagonist as possible, consistent with their limited solubility, and by testing some of the compunds in the presence of a known competitive antagonist.

3 By comparing the affinities with those of the corresponding quaternary nitrogen compounds, the contribution made by the positive charge in the onium group to the binding by receptors may be assessed and has been found to be variable. The carbon analogue of benziloylcholine has about one-tenth of its affinity, that of (±)-cyclohexylphenylglycolloylcholine has only about one-sixtieth of its affinity, but that of (±)-mandelylcholine has slightly higher affinity than that of (±)-mandelylcholine itself.

4 3,3-Dimethylbutylacetate appeared to be a partial agonist with an affinity constant of about 2.6×10^3. The contribution made by the positive charge to the binding of acetylcholine at these receptors therefore seems likely to lie within the range observed with antagonists and there is no reason to believe that there is necessarily greater intimacy of association by agonists than by antagonists.

5 Although the C–C and $\overset{+}{N}$–C bonds in $-CMe_3$ and $-\overset{+}{N}Me_3$ are similar in length, the groups do not occupy the same volume in solution in water.

Introduction

In an assessment of 'the role of ionic interaction at the muscarinic receptor', Burgen (1965) compared the estimated affinities of compounds containing quaternary nitrogen with those of analogues containing quaternary carbon, acetylcholine and 3,3-dimethylbutylacetate, benziloylcholine and 3,3-dimethylbutylbenziloate, in order to calculate the contribution made by the charged nitrogen atom to the binding of agonists and antagonists. Burgen had obtained results which suggested that the different activities of acetylcholine and its carbon analogue were due chiefly to differences in affinity and that the absence of the positive charge therefore reduced affinity more than 3000-fold. In contrast, Funcke, Rekker, Ernsting, Tersteege & Nauta (1959) had reported that 3,3-dimethylbutylbenziloate had 32% of the activity of atropine on the guinea-pig isolated ileum and from this Burgen calculated that it had one-fourteenth of the affinity of benziloylcholine. The difference in the contributions made by the positive charge to the binding of these compounds was used to develop a theory that 'the distinction between agonists and antagonists may lie in the greater intimacy of association with the receptor of the agonists and their consequent ability to induce conformation changes in the receptor', which is now appearing in books (e.g. Albert, 1973) and it therefore seemed desirable to measure the affinity constant of 3,3-dimethylbutylbenziloate by direct experiment.

This paper describes studies of the actions at postganglionic acetylcholine receptors of the guinea-pig ileum of esters of 3,3-dimethylbutan-1-ol with benzilic, (±)-cyclohexylphenylglycollic, diphenylacetic, (±)-mandelic, and phenylacetic acids. In addition to trying to estimate affinity, tests were made to see if their blocking action is competitive. The (±)-mandelic ester was of particular interest because it had been included in the compounds tested by Funcke *et al.* (1959) and their estimate of its activity relative to

atropine (46 : 50,000) suggested that it had an affinity constant just below 10^6, which is higher than the estimate for (±)-mandelylcholine recently obtained by Barlow, Franks & Pearson (1973, log K, 5.28). Experiments were also made with 3,3-dimethylbutylacetate in order to see whether it was acting exactly like acetylcholine.

Although the C–C and $\overset{+}{N}$–C bonds in $-CMe_3$ and $-\overset{+}{N}Me_3$ are similar in length, the two groups are likely to have different effects on the structure of water and so may be effectively of appreciably different size in solution. An attempt has been made to investigate this from measurements of the molal volumes of the liquids and of the apparent molal volumes at infinite dilution of the compounds in aqueous solution.

Methods

Compounds

3,3-Dimethylbutan-1-ol was prepared by reducing redistilled methyl-*tert*butyl acetate (Koch-Light) with lithium aluminium hydride in anhydrous ether. It had b.p. 141° C, N_D^{24}, 1.4130; Birch (1949) recorded b.p. 143° C. A portion was redistilled, b.p. 52.5-53.0° C/12 mm, N_D^{24}, 1.4122, and its density at 25.0 ± 0.005° C was 0.8090 (see below).

3,3-Dimethylbutylacetate was prepared from the alcohol and acetic anydride as described by Burgen (1965). It had b.p. 45-45.5° C/8 mm, N_D^{25}, 1.4050; Burgen (1965) and Birch (1949) recorded b.p. 154° C (at atmospheric pressure). All the other esters were prepared by the method of Clinton & Laskowski (1948) and their boiling-points and analyses are shown in Table 1. Funcke *et al.* (1959) recorded b.p. 157-9° C/0.1 mm for the benzilic ester. The biological activity of the mandelic ester was included in their paper but not its boiling-point and the compound was not included in the earlier work with mandelic esters (Funcke, Ernsting, Rekker & Nauta, 1953). The infra-red and n.m.r. spectra of all the products were carefully examined to see if there were any traces of isomers formed by rearrangement of the tertiary butyl group but none could be detected.

The densities of the acetate and phenylacetate esters were, respectively, 0.8655 and 0.9563 (at 25.0° C).

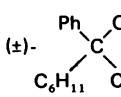
The guinea-pig isolated ileum

This was set up in aerated Tyrode solution, containing hexamethonium (2.76×10^{-4} M), at 37° C, with isotonic recording and carbachol as agonist, allowed to act for 30 s and given once every 90 seconds. Dose-ratios and affinity constants were calculated by methods already described (Abramson, Barlow, Mustafa & Stephenson, 1969; Edinburgh Staff, 1970; Barlow *et al.*, 1973). The compounds were dissolved in ethanol in concentrations of between 0.2 and 0.03 M and these stock solutions were then diluted with Tyrode solution. With a compound made up in ethanol at 0.1 M and tested on the ileum at 10^{-5} M, the ethanol concentration in the organ bath was approximately 2×10^{-3} M but this did not appear to affect the responses to carbachol, nor did it produce any marked effects on the actions of benziloylcholine. A range of concentrations of antagonist was tested which was determined by the amount needed to produce measureable antagonism and the limit imposed by solubility.

Molal volumes (V_m)

These were calculated from estimates of the densities of the liquids at 25.0 ± 005° C, made with an Anaton Paar Density Meter, DMA02C,

Table 1 Esters of 3,3-dimethylbutan-1-ol

			Found		Theory	
Acid	*b.p.*	N_D^{25}	*C*	*H*	*C*	*H*
$PhCH_2COOH$	145-146°/8 mm	1.4805	76.2	9.30	76.3	9.15
(±)-PhCHOHCOOH	97-100°/0.075 mm	1.4970	71.4	8.57	71.2	8.53
$Ph_2CHCOOH$	131-3°/0.016 mm	1.5370	81.1	8.30	81.0	8.16
$Ph_2COHCOOH$	152-8°/0.13 mm		77.2	8.07	76.9	7.74
(±)- 	130-2°/0.085 mm	1.5120	75.6	9.35	75.4	9.50

All the esters except that of phenylacetic acid subsequently went solid.

calibrated with water and pure methanol. 3,3-Dimethylbutan-1-ol was sufficiently soluble in water for it to be possible also to measure the apparent molal volume at infinite dilution (ϕ_V^o) as described by Lowe, MacGilp & Pritchard (1973; see also Barlow & Franks, 1973). Other values of ϕ_V^o have been taken from results compiled by Abramson *et al.* (1974). These estimates are repeatable to within 0.1 cm^3/mole.

Results

The benzilic ester was tested in concentrations from 10^{-7} to 4×10^{-6} M and the dose-ratios obtained were reasonably consistent with competitive antagonism, with a mean value of log K, 7.53 ± 0.04 s.e. mean (13 estimates). Abramson *et al.* (1969) obtained a value of 8.51 for log K of benziloylcholine, so there is roughly a 10-fold increase in affinity which should be attributable to the charge on the nitrogen atom, which agrees reasonably with Burgen's calculation that there is a 14-fold increase. Results obtained with the other compounds, however, show that the effects attributable to the charge are very variable (Table 2). The mandelic ester has higher affinity than its charged analogue, as was suggested by the results of earlier work (see introduction), whereas the cyclohexylphenylglycollic ester had only about one-fiftieth of the affinity of its charged analogue. The absence of the charge also appeared to reduce the affinity of the diphenylacetyl ester markedly, though it was difficult to obtain an accurate estimate of log K for the carbon analogue because only low dose-ratios could be obtained. It seemed likely that this was because the compound was not acting competitively, though it was also possible that it was not soluble enough to produce more antagonism.

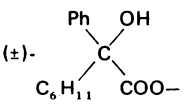
We therefore decided to try to check the nature of the antagonism produced by the compounds using another antagonist (Ariëns, Simonis & Van Rossum, 1964; Paton & Rang, 1965; Abramson *et al.*, 1969). If two antagonists are acting competitively, the dose-ratio produced by both together should be $DR_1 + DR_2 - 1$, where DR_1 and DR_2 are the dose-ratios produced by each separately. If one antagonist is acting non-competitively the dose-ratio should be $DR_1 \times DR_2$. If the two dose-ratios are about 100 and 10, respectively, the effect of the second antagonist will be to produce a dose-ratio of

$$\frac{DR_1 + DR_2 - 1}{DR_1} = 109/100 = 1.09$$

if both are competitive but a dose ratio of $DR_2 = 10$ if one is not. If the antagonists are added in the reverse order the ratios will be 10.9 for competition, compared with 100.

The results are shown in Table 3. From repeated measurements with the same compound it appears that the variance of the estimates of the dose-ratio is an underestimate of the real error. With 3×10^{-7} M benziloylcholine, for instance, the dose-ratios in separate groups of experiments were

Table 2 Affinity of esters of 3,3-dimethylbutan-1-ol for postganglionic acetylcholine receptors of the guinea-pig ileum

Acid	*Conc. range*	*Dose-ratios*	*Log K ± s.e. mean*	*Log K onium*	Δ
$Ph_2C(OH)COO-$	10^{-7} to 4×10^{-6}	5 to 200	7.531 ±0.036 (13)	8.511	0.980
(±)- 	10^{-7} to 2×10^{-6}	6 to 75	7.582 ±0.099 (6)	9.365	1.783
$Ph_2CHCOO-$	10^{-7} to 10^{-5}	1.3 to 4.1	5.49 (2)*	7.159	1.6
(±)-PhCHOHCOO−	3×10^{-5} to 9×10^{-5}	9 to 32	5.491 ±0.032 (6)	5.288	−0.203

$PhCH_2COO-$ too weak and too insoluble to test.
The column Δ shows the difference between the values of log K for the nitrogen analogue ('log K onium') and for the carbon analogue.
* Indicates that the compound is probably not acting competitively.

84, 72 and 77, whereas from the results obtained for this compound by Abramson *et al.* (1969) this concentration should produce a dose-ratio of 98. The dose-ratios obtained with 3,3-dimethylbutylbenziloate correspond to a value of log K of 7.377, compared with 7.531 from earlier results (Table 2); dose-ratios for (±)-3,3-dimethylbutylmandelate correspond to log K = 5.285 and 5.480, compared with 5.491 (Table 2); dose-ratios for (±)-mandelylethyltriethylammonium correspond to log K = 6.088 and 6.143, compared with 6.086 obtained by Barlow *et al.* (1973). When allowance is made for such errors, the effects of the antagonists are not inconsistent with competition, although the agreement between the observed ratio (R in Table 3) and that expected from competition (R_c) is not always entirely satisfactory, particularly when (±)-3,3-dimethylbutylmandelate is tested with (±)-mandelylethyltriethylammonium.

Experiments with 3,3-dimethylbutylacetate indicated that it was a partial agonist. Burgen (1965) observed a wide variation in the activity relative to acetylcholine, which would be expected of a partial agonist, but only observed failure to produce a maximum response in 1 experiment out of 14, whereas we have observed this consistently. Estimates of the affinity constant were made by plotting reciprocals of concentrations producing comparable responses (Barlow, Scott & Stephenson, 1967) and the mean value was 2.6×10^3 (9 estimates with 8 pieces of ileum). Contractions produced by 3,3-dimethylbutylacetate were antagonized by benziloylcholine but the dose-

Table 3 Effects of two antagonists. The concentrations of the two antagonists are shown and the dose-ratios produced by each alone

Compound	*Conc. (molar)*	*Dose-ratio ±s.e. mean*	*Combined dose-ratio ±s.e. mean*	*R*	R_c	R_n
3,3-Dimethylbutylbenziloate	2×10^{-7}	5.7 ±0.4 (5)	79.9 ±3.8 (5)	14.0	15.6	84.4
Benziloylcholine	3×10^{-7}	84.4 ±6.1 (5)	93.0 ±9.7 (5)	1.10	1.05	5.7
			86.4 AV ±5.4 (10)	1.02		
(±)-3,3-Dimethylbutylmandelate	3.84×10^{-5}	8.4 ±0.7 (5)	58.4 ±4.7 (5)	6.95	9.40	71.6
Benziloylcholine	3×10^{-7}	71.6 ±3.2 (4)	87.5 ±9.7 (4)	1.22	1.10	8.4
			71.3 AV ±6.9 (9)	0.90		
(±)-3,3-Dimethylbutylmandelate	3.84×10^{-5}	12.6 ±2.2 (4)	192.3 ±11.3 (4)	15.3	10.7	123
(±)-Mandelylethyltriethylammonium	10^{-4}	123.5 ±5.2 (5)	203.9 ±18.0 (5)	1.65	1.09	12.6
			198.9 AV ±10.8 (9)	1.61		
(±)-Mandelylethyltriethylammonium	1.2×10^{-5}	17.7 ±1.0 (5)	84.9 ±5.7 (5)	4.80	5.25	76.6
Benziloylcholine	3×10^{-7}	76.6 ±2.8	80.2 ±5.7 (5)	1.05	1.21	17.7
			82.5 AV ±4.5 (10)	1.08		

The value R indicates the effect of the additional antagonist, being the (combined dose-ratio) ÷ (initial dose-ratio), and the values R_c and R_n are the expected values of R for competitive and non-competitive antagonism, respectively. The order in which the compounds are tested should not affect the combined dose-ratio (though it appears to in some instances) so calculations have been made with the pooled values and are marked 'AV'.

ratios produced were bigger than with carbachol as agonist. This would be expected if the compound is a partial agonist occupying a very high proportion of receptors but even with a 'fast' antagonist, pentyltriethylammonium (Stephenson & Ginsborg, 1969), we obtained higher dose-ratios with 3,3-dimethylbutylacetate than with carbachol. The limited solubility of the compound, however, makes it difficult to analyse its effects at acetylcholine receptors from the quantitative effects of antagonists.

The estimates of molal volume, V_m, are shown in Table 4 with values of ϕ_V^o for related quaternary salts included for comparison. Although there is some uncertainty about the exact volume apparently occupied by the cation in solution, because allowance must be made for the volume occupied by the bromide ion, the effects of changes in structure on size in solution can be assessed accurately and compared with their effects on size in the liquid state. The replacement of hydrogen by phenyl, for instance, appears to produce about the same increase in both states. The increment for the acetyl group is slightly smaller for liquid methylacetate than for liquid 3,3-dimethylbutylacetate and considerably smaller for acetylcholine in solution. This could indicate the ability of the acetyl group to interact with water, possibly by hydrogen-bonding involving the carbonyl oxygen atom. The difference between V_m and ϕ_V^o for 3,3-dimethylbutan-1-ol ($126.3 - 117.7 = 8.6$ cm^3/mole) suggests that this interacts considerably with water. This could partly be due to hydrogen-bonding involving the hydroxyl group. The values of ϕ_V^o for choline bromide and 3,3-dimethylbutan-1-ol differ by 12.5 cm^3/mole, which is less than the volume likely to be occupied by the bromide ion. The choline ion is therefore apparently smaller than its carbon analogue in solution by from 5 to 17 cm^3/mole. There appears to be an even bigger difference between the apparent sizes in solution of the acetylcholine ion and its carbon analogue, because ϕ_V^o for acetylcholine bromide is actually smaller than V_m for 3,3-dimethylbutylacetate.

Discussion

It is striking that the contribution made by the onium group to the binding of the antagonists is so variable, but the variation is, in fact, comparable with that observed in the effects of other groups on affinity (Abramson *et al.*, 1969; Barlow *et al.*, 1973; Abramson, Barlow, Franks & Pearson, 1974). Alterations to one part of a molecule can undoubtedly disturb the binding of other parts and even with closely related compounds, structural features such as onium groups, phenyl, or hydroxyl, do not always fit exactly into the same parts of the receptor.

There is considerable uncertainty about the affinity of acetylcholine for postganglionic receptors but it seems unlikely that log K is greater than 5. From experiments with irreversible blocking agents, Van Rossum (1968) obtained a

Table 4 Estimates of molecular size at 25°C in the liquid state (V_m, the molal volume) and in solution (ϕ_V^o, the apparent molal volume at infinite dilution)

	Liquids: V_m *(cm^3/mole)*	
$HOCH_3$	CH_3COOCH_3	
40.6	79.8	
$ROCH_2CH_2CMe_3$		
R= H–	CH_3CO–	$PhCH_2CO$–
126.3*	166.6	230.4
	Solutions: ϕ_V^o *(cm^3/mole)*	
$ROCH_2CH_2\overset{+}{N}Me_3\ Br^-$		
R= H–	CH_3CO–	Ph_2CHCO–
130.2	163.9	291.0
Volume increments (cm^3/mole)	ΔV_m	$\Delta\phi_V^o$
$-OH \rightarrow -OOCCH_3$	39.2, 40.3	33.7
$-H \rightarrow -Ph$	63.8	63.5

The values of ϕ_V^o are taken from results compiled by Abramson *et al.* (1974) and allowance must be made for the volume of the bromide ion in solution, which lies between 18 and 30 cm^3/mole, depending on how it is estimated (Millero, 1972). 3,3-Dimethylbutan-1-ol, marked with an asterisk, was soluble enough in water for it to be possible to measure ϕ_V^o, which was 117.7 cm^3/mole. The estimates of V_m for methanol and methylacetate were calculated from densities given in the International Critical Tables.

value of 4.8 for receptors in rat intestine.* Furchgott (1966) obtained values of about 5 for carbachol and receptors in rabbit fundus and aortic strip. For *n*-pentyltrimethylammonium and ethoxyethyltrimethylammonium and receptors in guinea-pig ileum Abramson *et al.* (1969) obtained values of around 4. The difference between the values of log K for acetylcholine and its carbon analogue therefore seems likely to be about 1.6 log units, which is no bigger than that observed with some of the antagonists. It does not seem justified, therefore, to suppose that there is a closer intimacy of association by agonists than by antagonists.

However, it is questionable whether the contribution of the ionic charge to affinity can be assessed by comparing the affinities of quaternary ammonium compounds with those of their quaternary carbon analogues. The volume measurements indicate that the former occupy appreciably less space in water than the latter.

*Note added in proof: The recent paper of Burgen, Hiley & Young (*Br. J. Pharmac.*, (1974), **50**, 145-151) indicates that for guinea-pig intestine the value is 5.04.

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