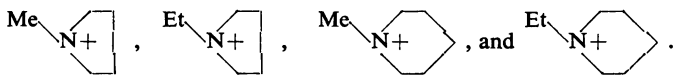
Br. J. Pharmac. (1969), 37, 207–233.

Relationships between chemical structure and affinity for acetylcholine receptors

F. B. ABRAMSON,* R. B. BARLOW, M. G. MUSTAFA† AND R. P. STEPHENSON

Department of Pharmacology, University of Edinburgh

1. Series of analogues of acetylcholine have been prepared in which the acetyl group was replaced by phenylacetyl, *cyclo*hexylacetyl, diphenylacetyl, di*cyclo*hexylacetyl, (±)-phenyl*cyclo*hexylacetyl, benziloyl and (±)-phenyl-*cyclo*hexylhydroxyacetyl groups and the trimethylammonium group was replaced by Me_2EtN^+, $MeEt_2N^+$, Et_3N^+,



Further series were prepared in which the acetoxyethyl group was replaced by ethoxyethyl, phenylethoxyethyl, *cyclo*hexylethoxyethyl, diphenylethoxyethyl, and di*cyclo*hexylethoxyethyl groups, and by *n*-pentyl, 5-phenylpentyl, 5-*cyclo*hexylpentyl and 5:5-diphenylpentyl groups.

2. The ethoxyethyl and *n*-pentyl series contain some compounds which are agonists or partial agonists when tested on the isolated guinea-pig ileum, but all the other compounds are antagonists.

3. The affinity of the compounds for the postganglionic ("muscarine-sensitive") acetylcholine receptors has been measured in conditions in which the antagonists have been shown to be acting competitively. There were considerable differences between their affinities, the most active (log K, 9·8) having one million times the affinity of the least active (log K, 3·7).

4. The changes in affinity as the onium group was modified were not entirely independent of changes in the rest of the molecule. Increasing the size of the onium group, as judged from conductivity measurements on simpler onium salts, increased affinity in the series containing one large group (phenyl or *cyclo*hexyl) but, in the series with two large groups, affinity declined when the size was increased beyond $-\overset{+}{N}MeEt_2$.

5. In general, the effects of changes in the rest of the molecule on affinity were bigger than the effects of changes in the onium group and there were bigger interactions. Affinity was increased to a greater extent by introducing one phenyl and one *cyclo*hexyl group together than by introducing either two phenyl or two *cyclo*hexyl groups; the increment was greater than the separate contributions made by one phenyl and one *cyclo*hexyl group.

* Present address: Beecham Products, U.K., Brentford, Middlesex.
† Present address: Medical College, Sylhet, East Pakistan.

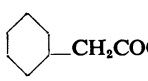
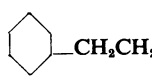
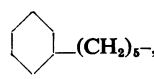
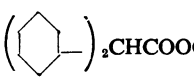
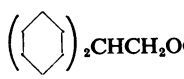
6. The factors which influence the binding of molecules to receptors are discussed. There is no evidence that the separation between the onium group and the group in the receptor with which it interacts is greater in compounds with high affinity nor is there any evidence, from the study of the series which contain agonists and partial agonists, that ability to activate receptors depends upon the onium group being able to come close to this charged group in the receptors.

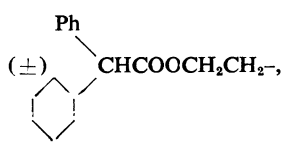
In 1963, Barlow, Scott & Stephenson described an attempt to estimate the effects of chemical structure on the affinity and efficacy of compounds related to acetylcholine at the postganglionic (" muscarine-sensitive ") receptors in the guinea-pig ileum. They tested parallel series of compounds for agonist or antagonist activity. One series, chiefly containing agonists, consisted of a chain of five carbon and/or oxygen atoms attached to an onium group; the other series was identical except that the terminal CH_3- was replaced by Ph_2CH- or $Ph_2C(OH)-$. The effects on affinity of replacing methyl by ethyl in the onium group were similar in the different series of antagonists, and it was postulated that the same change in structure would have the same effects on the affinity of the agonists. From the observed changes in the potency of the agonists, it was then possible to deduce the effects of replacing methyl by ethyl on their efficacy.

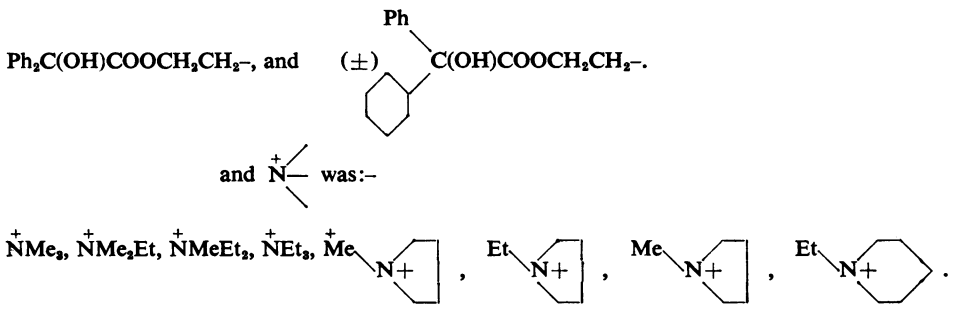
This deduction assumes that the various parts of the molecule make contributions to the free energy of adsorption, which are additive. This will not be justifiable if groups within the molecule interact or if the introduction of one group disturbs the binding of another. The need for more information about the extent to which such interactions occur was pointed out by Barlow, Scott & Stephenson (1963) and is important in view of the suggestion by Burgen (1965) that the binding of agonists and antagonists at the receptor is essentially different.

We have therefore made and tested many more antagonists in which we have studied the effects of a greater variety of changes in the onium group and in the group or groups attached at the other end of the compound. The compounds had the general formula

where R was

ester series	*ether series*	n-*pentyl series*
$PhCH_2COOCH_2CH_2-$,	$PhCH_2CH_2OCH_2CH_2-$,	$Ph(CH_2)_5-$,
		
$Ph_2CHCOOCH_2CH_2-$,	$Ph_2CHCH_2OCH_2CH_2-$,	$Ph_2CH(CH_2)_4-$,
		





In addition the series in which R was $CH_3CH_2OCH_2CH_2-$, and $CH_3(CH_2)_4-$, were also studied. Some of these were pure agonists and some were partial agonists, but many were antagonists. The affinity constants of the partial agonists were measured by the method of Barlow, Scott & Stephenson (1967). Equipotent molar ratios were measured for the agonists and the affinity constants of some of them have also been measured by a method using an "irreversible" blocking agent (Stephenson, in preparation).

Methods

The compounds were all tested on the isolated guinea-pig ileum, set up in Tyrode's solution containing hexamethonium ($1{\cdot}1 \times 10^{-4}$M) at 37° C.

Antagonists

In the earlier part of the work (results marked F. A. and N. C. S. in Table 5), the affinity constants of antagonists were measured by the method of Barlow, Scott & Stephenson (1963). In later experiments, however, a modification of this method was used (Edinburgh staff, 1968). In about half the experiments the contractions of the ileum were recorded, using a differential transformer as a transducer. This was connected to a potentiometric recorder and to a digital voltmeter linked to an electric typewriter in such a way as to print out the voltage corresponding to the maximum response to each dose of agonist. With this system both visual and numerical records of the effects were obtained simultaneously. After tests with several different agonists (see **Results**), carbachol was used routinely as agonist. In the previous work acetylcholine had been used and, though the results did not seem to be affected by any destruction by cholinesterases, we thought it better to use a compound which was not hydrolysed by these enzymes.

With many antagonists the effects were slow in onset and slow to wear off after washing the preparation. In most experiments, therefore, only a single concentration was tested on each preparation. With some of the weaker compounds, however, both onset and recovery were very rapid and equilibrium was complete within 1 or 2 contractions. It was possible to test up to ten of these compounds on a single preparation; a fresh set of control responses to the agonist was established between the antagonists.

Each test gave an estimate of the dose-ratio for the concentration of antagonist used and from this the affinity constant was calculated. In some experiments agonist responses obtained in the presence of more than one concentration of an antagonist were compared with one set of control responses; this gave several estimates of the

affinity constant. Where these agreed, indicating competition (see below), the average value was taken as a single estimate of affinity. The number of results quoted is therefore the number of preparations on which each compound was tested; usually at least five pieces of ileum were used.

Tests for competitive antagonism

To check that the antagonists were acting competitively, Barlow, Scott & Stephenson (1963) tested several concentrations of antagonists to see if the results fitted the Gaddum equation (Gaddum, 1957)—that the graph of (dose-ratio − 1) against antagonist concentration was linear. Some of the compounds we have studied failed to satisfy this test; at high concentrations the block became unsurmountable and was clearly noncompetitive. Nevertheless, it seemed likely that the antagonism was competitive at lower concentrations and we have used another test for competition, which does not require the use of high dose-ratios. This test has an additional advantage with the weaker compounds, even when they are competitive over a wide range of concentrations, because it avoids the need for the very high concentrations which would be required in order to produce high dose-ratios.

If the antagonist under test is acting competitively, it will also compete with another competitive antagonist as well as with the agonist. Ariens, Simonis & Van Rossum (1964) used this test to distinguish between the actions of lachesine and isoprenaline in antagonizing acetylcholine. As Paton & Rang (1965) have pointed out, the dose ratio, DR_{1+2}, obtained with two competitive antagonists acting together is equal to $DR_1 + DR_2 - 1$, where DR_1 and DR_2 are the dose-ratios obtained with these concentrations of the antagonists acting separately. If either antagonist is noncompetitive, the dose-ratio of the two acting together would be $DR_1 \times DR_2$.

We have used atropine as the competitive antagonist with which, in a concentration of 10^{-7}M, we obtained a dose-ratio of 104. The compound under test was added in a concentration with which we obtained a dose-ratio of about 10, when it was acting alone. If the second antagonist is competitive, the combined dose-ratio would be expected to be about $(104 + 10 - 1)$ and the dose-ratio for the mixture compared with atropine alone would therefore be about 113/104, or 1·09. If the second antagonist is noncompetitive, the dose-ratio for the mixture compared with atropine alone would be about 10. It is therefore easy to see if an antagonist is competitive or not without having to test a wide range of concentrations.

Partial agonists

The affinity constants of partial agonists were measured by the method described by Barlow, Scott & Stephenson (1967).

Agonists

The equipotent molar ratios for compounds relative to *n*-pentyltrimethylammonium were measured in 2 + 2 dose assays. Some estimates of the affinity constants of agonists have also been made by a method which will be described in a separate paper (Stephenson, in preparation).

Compounds

The acetylcholine used was the iodide, chromatographically homogeneous,

supplied by British Drug Houses. Analyses and melting-points for all the other compounds are shown in Table 1. They were prepared by quaternization of the appropriate tertiary base, the constitution of which was established by its manner of synthesis, boiling point, refractive index and infrared absorption spectrum.

The acetyl, phenylacetyl, diphenylacetyl, *cyclo*hexylacetyl and phenyl*cyclo*hexylacetyl esters were all made from the acid chloride and the dialkylaminoethylalcohol. The benzilic and *cyclo*hexylphenylglycollic esters were made from the acid and chloroethyldialkylamine by modifications of the method of Horenstein & Pählicke (1938). For benzilic esters the procedure was that of Burtner & Cusic (1943) and for the *cyclo*hexylphenylglycollic esters it was that described by Miescher & Hoffmann (1941) and Hoffmann & Schellenberg (1947). The di*cyclo*hexylacetyl esters were prepared by transesterification of the ethylester with the dialkylaminoalcohol by a method similar to that of Foster & Ing (1956), but treating the aminoalcohol with a small amount of sodamide before the addition of the ester, instead of using sodium methoxide, and with toluene instead of xylene as solvent. The ethanol formed in the reaction was distilled with the toluene and was detected by gas chromatography. The reaction was continued until, after about 24 hr, the evolution of ethanol was no longer detectable.

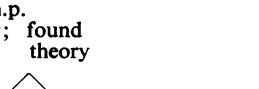
The ethoxyethyl and *cyclo*hexylethoxyethyl compounds were prepared by treating the dialkylaminoethyl alcohol with sodamide in dry benzene and, when ammonium could no longer be smelt, adding ethyl bromide or *cyclo*hexylbromide and heating under reflux for 5–12 hr. Attempts to make the phenylethoxyethyl and diphenylethoxyethyl compounds by this method gave styrene and stilbene, respectively. The desired compounds, together with the di*cyclo*hexylethoxyethyl derivatives, were made by treating phenylethanol, diphenylethanol, or di*cyclo*hexylethanol with sodamide in dry benzene and adding the chloroethyldialkylamine.

The *n*-pentyl, phenylpentyl, diphenylpentyl and *cyclo*hexylpentyl compounds were all prepared from the alkyl bromide, obtained from phenylethanol, diphenylethanol and *cyclo*hexylethanol by chain-lengthening reactions. *Cyclo*hexyl compounds were obtained by the reduction of ethylphenylacetate in ethanol at 150° C with hydrogen at 50–100 atmospheres and Raney nickel as catalyst. The completion of the hydrogenation was checked by the absence of any detectable trace of aromatic compounds in the infrared absorption spectrum. Ethyldiphenylacetate was similarly reduced to ethyldi*cyclo*hexylacetate. *Cyclo*hexylphenylacetonitrile was prepared from benzylcyanide and *cyclo*hexylbromide by the method of Hancock & Cope (1945) and hydrolysed to yield the acid. *Cyclo*hexylphenylglycollic acid was a generous gift from Ciba A.G., Basel. These two latter acids were racemic.

The synthetic routes by which the compounds were obtained are unambiguous. Most of the quaternary salts, after recrystallization until the melting-point was constant, were tested for chromatographic homogeneity on paper in *n*-butanol-ethanol-water (5 : 5 : 2), developed with a modified Dragendorff reagent (Thies & Reuther, 1954). Satisfactory analysis of the recrystallized material for ionizable halide was usually taken as adequate evidence for the purity and identity of the compounds. Ethoxyethylpiperidine ethiodide proved particularly difficult to purify by recrystallization ; it was obtained free from impurity after chromatography in *n*-butanol-ethanol-water (5 : 5 : 2) on a cellulose column.

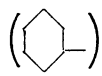
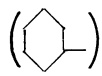
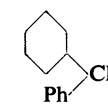
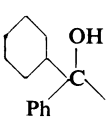
The melting-point of oxyphenonium iodide, phenyl*cyclo*hexylglycolloylethylmethyldiethylammonium iodide, is different from the value 186°–187° C recorded

TABLE 1. *Melting points and analyses*

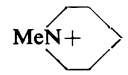
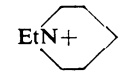
	$Me_3\overset{+}{N}$	$Me_2Et\overset{+}{N}$	$MeEt_2\overset{+}{N}$
$CH_3CH_2OCH_2CH_2$–			
m.p.	I^- (A+B) 164–5°	Br^- (B+C) 69°	I^- (B+C) 43–5°
Halide–; found	49·1	35·3	44·1
theory	49·0	35·4	44·2
$CH_3(CH_2)_4$–			
m.p.	I^- (A) 230–231°	I^- (B) 177–8°	I^- (B+C) 150–151°
I^-; found	49·5	46·8	44·5
theory	49·4	46·8	44·5
$PhCH_2COOCH_2CH_2$–			
m.p.	I^- (A+B) 158–9°	I^- (A+B) 141–2°	Br^- (A+B 108–9°
Halide–; found	36·4	35·0	24·4
theory	36·4	34·9	24·2
$PhCH_2CH_2OCH_2CH_2$–			
m.p.	I^- (B+C) 105·5–107°	I^- (B+C) 53·1–53·4°	I^- (B+C) 70·2–70·5°
I^-; found	37·7	36·6	35·0
theory	37·9	36·4	34·9
$Ph(CH_2)_5$–			
m.p.	Br^- (B) 168–9°	Br^- (B) 112–3°	Br^- (B) 80–81°
Br^-; found	27·8	26·6	25·4
theory	27·9	26·6	25·4
			
m.p.			
I^-; found			
theory			
$CH_2COOCH_2CH_2$–	I^- (B)	I^- (B+C)	I^- (B+C)
m.p.	155–6°	61–2°	49–51°
Halide; found	35·7	34·3	33·1
theory	35·7	34·4	33·1
			
m.p.			
Br^-; found			
theory			
$CH_2CH_2OCH_2CH_2$–	I^- (B+C)	I^- (B+C)	I^- (B+C)
m.p.	143·5–144°	64·1–64·7°	77·5–80·0°
I^-; found	37·2	35·7	34·5
theory	37·2	35·7	34·4
			
$(CH_2)_5$–	Br^- (B+C)	Br^- (B+C)	Br^- (B+C)
m.p.	234–5°	177–8°	160–161°
Br^-; found	27·4	26·1	24·9
theory	27·4	26·1	25·0
m.p.			
I^-; found			
theory			
$Ph_2CHCOOCH_2CH_2$–			
m.p.			
I^-; found			
theory			
C,H; found			
theory			

$Et_3\overset{+}{N}$	MeN+	EtN+	MeN+	EtN+
ir⁻ (A+B)	I⁻ (B+C)	I⁻ (B+C)	I⁻ (B+C)	I⁻ (B+C)
97–8°	70–71°	65°	108·5–109·5°	72–3°
31·6	44·5	42·4	42·4	40·6
31·5	44·5	42·4	42·4	40·5
I⁻ (B)	I⁻ (B)	I⁻ (B+C)	I⁻ (B)	I⁻ (B)
162°	180–180·5°	147–8°	163–4°	187–8°
42·4	44·9	42·8	42·8	41·0
42·4	44·8	42·7	42·7	40·8
⁻ (A+B)	Br⁻ (A+B)	Br⁻ (B+D)	Br⁻ (A+B)	Br⁻ (B)
74–5°	160–161°	118–118·5°	138·5–139°	147·5–148°
32·5	24·4	23·4	23·4	22·6
32·4	24·3	23·4	23·4	22·5
⁻ (B+C)	I⁻ (B+C)	I⁻ (B+C)	I⁻ (B)	I⁻ (B)
87·7–88·0°	68·5–68·9°	71·3–71·7°	95·9–96·2°	95·5–95·9°
33·7	35·1	33·7	33·8	32·6
33·6	35·2	33·8	33·8	32·6
Br⁻ (B)	Br⁻ (B)	Br⁻ (B)	Br⁻ (B)	Br⁻ (B)
118–9°	111–2°	138–9°	158–9°	96–7°
24·2	25·7	24·6	24·6	23·6
24·3	25·6	24·5	24·5	23·5
		I⁻ (B+C)	I⁻ (B+C)	I⁻ (B)
		106–7°	151–2°	101–2°
		34·1	34·0	33·0
		34·0	34·0	32·8
⁻ (B+C)	I⁻ (B+C)	Br⁻ (B+C)	I⁻ (B+C)	I⁻ (B+C)
130°	71·3–71·7°	106·5–107·3°	88·2–88·5	59·0–60·2
31·9	33·3	23·0	32·1	31·1
32·0	33·3	22·9	32·1	31·0
			Br⁻ (B+C)	
			151–3°	
			22·8	
			22·4	
⁻ (B+C)	I⁻ (B+C)	I⁻ (B+C)	I⁻ (B+C)	I⁻ (B+C)
38·5–139·0°	34–35°	89·5–90·0°	80·7–81·0°	111·0–111·3°
33·1	34·7	33·3	33·3	32·1
33·1	34·5	33·3	33·3	32·1
ir⁻ (B+C)	Br⁻ (B+C)	Br⁻ (B+C)	Br⁻ (B)	Br⁻ (B+C)
146–7°	42–44°	197–9°	201–2°	170–172°
24·0	25·5	23·7	24·1	23·2
23·9	25·1	24·1	24·1	23·1
	I⁻ (B+C)			I– (C)
	34–5°			135°
	34·5			32·2
	34·7			32·3
	I⁻ (D+E)	I⁻ (D+E)	I⁻ (D+E)	I⁻ (D+E)
	181–2°	150°	153°	141°
	28·0*	27·4*	27·3*	26·6*
	28·2	27·3	27·3	26·5
	55·5; 5·97*	57·0; 5·90*	56·7; 5·78*	57·8; 6·31*
	56·0; 5·82	56·8; 6·06	56·8; 6·06	57·6; 6·31

Table 1 (continued)

	$Me_3\overset{+}{N}$	$Me_2Et\overset{+}{N}$	$MeEt_2\overset{+}{N}$
$Ph_2CHCH_2OCH_2CH_2-$	Br^- (A+B)	Br^- (B)	Br^- (B)
m.p.	141–2°	99–100°	123·5–124·5
Br^-; found	21·8	21·2	20·3
theory	21·9	21·1	20·4
$Ph_2CH(CH_2)_4-$	Br^- (A+B)	Br^- (A+B)	Br^- (A+B)
m.p.	199–200°	153–4°	124–5°
Halide–; found	21·7	20·9	20·5
theory	22·1	21·3	20·5
	I^- (A+B)	I^- (A+B)	I^- (B)
m.p.	209·4–209·8	209·5–209·9	166·9–167·
I^-; found	29·2*	28·4*	26·9*
theory	29·0	28·1	27·2
	I^- (B+C)	I^- (B+C)	I^- (B+C)
m.p.	116–122°	122–128°	156·7–157·
I–; found	30·0*	29·4*	28·3*
theory	29·9	29·0	28·1
	Br^- (A+B)	Br^- (A+B)	Br^- (A+B
m.p.	180–181°	172–3°	184–5°
Halide–; found	20·4	20·0	19·3
theory	20·8	20·1	19·4
$Ph_2C(OH)COOCH_2CH_2-$			
m.p.			
Halide–; found			
theory			
C, H; found			
theory			
	I^- (A+B)	I^- (B+C)	I^- (B+C)
m.p.	166·9–167·3	124·1–124·6	136·5–137·
I^-; found	28·2*	27·4*	26·7*
theory	28·4	27·5	26·7

* Indicates micro analysis by Dr. J. W. Minnis, all other analyses are macro (Barlow & Zoller, 1964) Melting points were measured on a Kofler hot stage except those given to 0·1°, which were recorded with a Mettler FP1 instrument coupled to a pen-recorder. The letters refer to the solvents from which the compounds were recrystallized; A, ethanol; B, ethylmethyl ketone; C, ethylacetate D, isopropanol; E, ether. All melting points are in ° C.

$Et_3\overset{+}{N}$				
Br^- (A+B)	Br^- (A+B)	Br^- (A+B)	Br^- (A+B)	Br^- (A+B)
164–5°	121·5–122·5°	166·5–167·5°	148–150°	191–2°
19·5	20·6	19·8	19·8	19·1
19·6	20·5	19·8	19·8	19·1
Br^- (A+B)	Br^- (A+B)	Br^- (A+B)	Br^- (A+B)	I^- (A+B)
140–2°	164–5°	159–160°	188–9°	178–9°
19·7	20·4	19·7	19·5	27·4
19·8	20·6	19·9	19·9	27·2
I^- (B)	I^- (B)	I^- (B)	I^- (B+C)	I^- (A+B)
187·8–188·2	165·9–166·5	162·6–163·0	160·6–161·0	183·4–184·4
26·4*	27·4*	26·5*	26·4*	25·7*
26·4	27·4	26·6	26·6	25·8
I^- (B+C)	I^- (C)	I^- (B+C)	I^- (A+B)	I^- (B+C)
188·2–188·6°	sinters 88, 94–6°	126·3–126·6	143·2–143·8	142·8–143·2
27·0*	28·1*	27·3*	27·4*	26·4*
27·2	28·2	27·4	27·4	26·6
Br^- (A+B)	Br^- (A+B)	Br^- (A+B)	Br^- (A+B)	I^- (A+B)
149–150°	152–3°	94–6°	178–180°	169–170°
18·6	19·2	18·5	18·6	26·2
18·8	19·5	18·9	18·9	26·0
	Br^- (D+E)	Br^- (D+E)	I^- (D+E)	I^- (D+E)
	211°	200–202°	110°	135–7°
	19·2*	18·6*	26·2*	25·9*
	19·0	18·4	26·4	25·7
	60·1; 5·91*	60·7; 6·51*	55·5; 6·10*	56·0; 6·02*
	60·0; 6·06	60·8; 6·49	54·9; 5·85	55·9; 6·11
I^- (A+B)	I^- (A+B)	I^- (B)	I^- (A+B)	I^- (A+B)
175·7–176·2	173·4–174·0	136·8–137·3	183·6–184·1	184·6–184·8
25·9*	27·0*	26·1*	26·0	25·0*
25·9	26·8	26·0	26·0	25·3

by Hoffmann & Schellenberg (1947), but a sample of this substance recently prepared by the Pharmaceutical Division of Ciba Ltd. (personal communication) had a melting point of 136°–138° C.

Results

Reproducibility of estimates

Table 2 shows the results of repeating the test on two series previously studied. Only with one of the compounds, diphenylacetoxyethyltriethylammonium, was the mean significantly different ($P=0{\cdot}05$) from that previously obtained. The difference was small but may perhaps indicate a small systematic error in the earlier results. This could have arisen from an underestimate of the variance, because results obtained when different concentrations were tested on the same preparation were regarded as separate estimates instead of being pooled to give a single estimate, as in the present experiments. From the results it was concluded that differences of the order of 0·1 log units were likely to indicate real differences between compounds. Subsequent experience indicates that the variance of the estimates depends upon the compound (see below).

Use of different agonists

Table 3 shows estimates of the affinity of three different antagonists using acetylcholine and carbachol separately as agonists. In the lower section of the table estimates are shown of the logarithm of the affinity constant of phenylpentylethylpyrrolidinium using carbachol, pentyltrimethylammonium and ethoxyethyltrimethylammonium as agonists. As the effects of this antagonist are rapid in onset and the tissue recovers quickly when it is washed, it was possible to perform experiments with the antagonist and two separate agonists on the same piece of tissue. Again the results are not significantly different ($P=0{\cdot}05$).

TABLE 2. *Estimates of log* K

$RCOOCH_2CH_2R'$

$R=$	$R'=Me_3\overset{+}{N}$	$Me_2Et\overset{+}{N}$	$MeEt_2\overset{+}{N}$	$Et_3\overset{+}{N}$
Ph_2CH				
I	7·171 ±0·007(3)	7·643 ±0·013(4)	7·490 ±0·018(4)	7·432 ±0·018(14)
II	7·159 ±0·025(4)	7·578 ±0·028(7)	7·584 ±0·045(4)	7·367 ±0·021(4)
$M_1—M_2$	0·012	0·065	0·094	0·065*
$Ph_2C(OH)$				
I	8·536 ±0·012(5)	8·937 ±0·010(6)	8·952 ±0·009(8)	8·672 ±0·042(3)
II	8·511 ±0·008(4)	8·934 ±0·007(7)	8·957 ±0·009(6)	8·682 ±0·006(4)
$M_1—M_2$	0·025	0·003	0·005	0·010

* Significantly different ($P=0{\cdot}05$)

Values are shown for (I) the earlier results (Barlow, Scott & Stephenson, 1963) and (II) subsequent results (F. B. A. and N. C. S.). The mean is given with the standard error and number of estimates. In II this number is the same as the number of preparations on which the compound was tested, wher eas in I more than one value was obtained from each preparation. Acetylcholine was used as agoni st in both sets of experiments.

Competitive nature of the antagonism

In tests with different concentrations, it was observed that some of the compounds were not acting competitively at high concentrations. We therefore studied the effects of some of the antagonists in the presence of atropine. Table 4A shows results of a number of experiments with the members of the phenylethoxyethyl series and indicates clearly that these are acting competitively. Table 4B shows the results of testing some of the compounds about whose antagonism we were uncertain and indicates that, in concentrations which produced dose-ratios of about 10, the antagonism was essentially competitive. The affinity constants of these compounds were therefore calculated only from results obtained with these lower concentrations of antagonist. The chemical features associated with the secondary non-competitive antagonism appear to be large alkyl groups, or other changes which increase the aliphatic nature of the compound, such as replacement of ester by ethylene ($-CH_2CH_2-$).

Papaverine was tested by this procedure and behaved as expected for a non-competitive antagonist (see page 210).

Affinity constants of the antagonists

The mean values of estimates of log K for the antagonists are shown in Table 5, together with the standard error and number of results. In this work, in which many compounds have been tested by different people over a period of years, the variance of the estimates has not been consistent. At least three factors are likely to affect it: differences between observers, variation between guinea-pigs, and differences between the types of compound tested. We have not been able to investigate these systematically, but we believe that the differences in variance arising from differences between observers are not very great. We have, however, found that the variability of results obtained by a particular observer fluctuates from time to time, because there are definite periods when the guinea-pig ileum preparations do not give very consistent responses. It is possible that this is a seasonal effect and has contributed to the larger standard error of some of the estimates in Table 5, but in some series the large variance can be ascribed to the nature of the compounds themselves.

TABLE 3. *Estimates of log affinity constant with different agonists*

Antagonist		Agonist	
		Acetylcholine	Carbachol
Phenylpentyltrimethylammonium		5·189±0·024(5)	5·170±0·024(5)
Phenylpentylmethyldiethylammonium		5·710±0·061(5)	5·720±0·024(5)
Phenylpentyltriethylammonium		5·900±0·010(7)	5·880±0·014(6)
Phenylpentylethylpyrrolidinium		Carbachol	Pentyltrimethyl-ammonium
	(i)	5·650±0·036(6)	5·720±0·020(6)
		Carbachol	Ethoxyethyltrimethyl-ammonium
	(ii)	5·598±0·067(3)	5·620±0·026(5)

None of the differences between means is significant ($P=0·05$)

Values are shown of log $K \pm$ S.E. and number of estimates. In the upper section each result was obtained on a separate preparation, in the lower section results with each of two agonists were usually obtained from each preparation.

The variance is greatest in compounds with high affinity, such as the phenyl*cyclo*hexylglycollic and phenyl*cyclo*hexylacetyl esters ($s^2=0{\cdot}0124$ and 0·0123, respectively) and in series with a high aliphatic (or alicyclic) character, such as the di*cyclo*hexylethoxyethyl compounds, which have the highest variance of all ($s^2=$ 0·0163). For most of the series (eight out of fourteen) the variance is between 0·0029 and 0·0047. It is appreciably higher for the di*cyclo*hexylacetyl esters (0·0070) and for the diphenylpentyl and *cyclo*hexylpentyl compounds (0·0077 and 0·0089, respectively). The replacement of acetoxyethyl or ethoxyethyl by pentamethylene always increased the variance.

With compounds which have high affinity the high variance of the estimates of log K is in part due to the slow establishment of equilibrium, with the consequent need to compare responses to the agonist, which are widely separated in time. With the markedly aliphatic compounds the high variance may be due to secondary actions of the compounds including, for example, some small degree of non-competitive antagonism.

Another effect observed was that some of the series of compounds produced, as a secondary effect, a potentiation of the agonist, which developed more slowly than the antagonism and outlasted it when the compound was washed away. This occurred mainly with some of the less active compounds and because, with these, many tests were made on the same piece of ileum (see **Methods**), the antagonism was observed against a fairly stable level of potentiation. The extent of the potentiation varied from day to day, occasionally amounting to as much as a doubling in the sensitivity of the preparation to the agonist. Estimates of the affinity constants, however, did not show comparable variation and we do not think that any marked error has arisen from this source. The potentiation is not ascribable to any inhibition of cholinesterases by the antagonists, because carbachol was used as the agonist.

With most of the compounds the standard error of the mean estimate of log K is less than 0·04 and, with 6 or more degrees of freedom, the fiducial limits ($P=0{\cdot}05$) are less than $\pm 0{\cdot}1$.

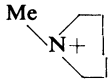
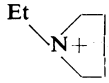
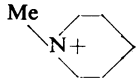
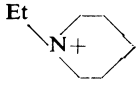
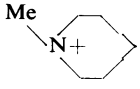
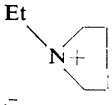
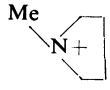
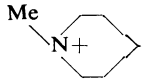
Affinity constants of partial agonists

The mean values of log K for the three partial agonists in the pentyl and ethoxyethyl series are included, in italics, in Table 5, together with the standard error and number of results. These values were all obtained by the reciprocal plot method described by Barlow, Scott & Stephenson (1967). The pooled variance of these estimates, 0·0105, is considerably greater than that for the antagonists in these series, which is 0·0044 for both the pentyl compounds and for the ethoxyethyl compounds, but is not greater than that observed with the estimates of some of the other antagonists (see above).

Pure agonists

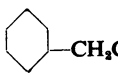
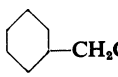
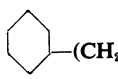
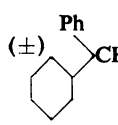
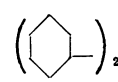
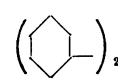
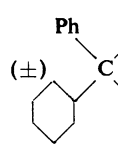
Five compounds which we have studied were pure agonists, the trimethylammonium and ethyldimethylammonium members of the *n*-pentyl and ethoxyethyl series and ethoxyethyl-N-methylpyrrolidinium. Their equipotent molar ratios relative to *n*-pentyltrimethylammonium are shown in Table 6. Estimates of the values of log K for the compounds are included in Table 5, together with the

TABLE 4. *Tests for competitive antagonism*

		Dose-ratios produced by the compound in the presence of atropine, 10^{-7}M		
		Calculated:		Observed
		If antagonism is competitive	If antagonism is non-competitive	
(A) $PhCH_2CH_2OCH_2CH_2$—				
$\overset{+}{N}Me_3$		1·099	10·31	1·012±0·049 (3)
$\overset{+}{N}Me_2Et$		1·101	10·55	1·108±0·065 (5)
$\overset{+}{N}MeEt_2$		1·097	10·10	1·153±0·042 (3)
$\overset{+}{N}Et_3$		1·087	9·02	1·050±0·040 (3)
		1·082	8·54	1·206±0·053 (3)
		1·087	9·09	1·023±0·037 (5)
		1·091	9·42	1·019±0·093 (5)
		1·096	10·00	1·125±0·076 (3)
(B)  $-(CH_2)_5^-$				
$\overset{+}{N}Me_3$		1·09	10·4	1·44
$\overset{+}{N}Me_2Et$		1·15	16·4	1·37
$\overset{+}{N}Et_3$		1·17	13·1	1·34
		1·09	10·7	1·50
$Ph(CH_2)_5^-$				
		1·17	18·4	1·12
$Ph_2CH(CH_2)_4^-$				
$\overset{+}{N}Me_3$		1·09	10·7	1·18
$\overset{+}{N}MeEt_2$		1·11	12·5	1·26
$\overset{+}{N}Et_3$		1·11	12·2	1·29
	(i)	1·11	12·5	1·08
	(ii)	1·09	10·0	1·15
		1·13	15·0	1·16
Papaverine		1·03	4·06	3·84

The dose-ratios in column 1 are calculated from the mean estimate of the affinity constant of the compound and the observation that 10^{-7}M atropine produced a mean dose-ratio of 104 in twelve experiments. The dose-ratios in column 2 are calculated from the effects of the compound alone (that is, in the absence of atropine). The dose-ratios in column 3 are the observed values when the compound is tested on a preparation which is already in the presence of 10^{-7}M atropine. Values in part (A) are the mean of the number of experiments shown in parentheses ± the standard error. Values in part (B) are results of tests on single preparations. Note that the observed values (column 3) are much closer to those expected if the antagonism is competitive (column 1) than to those if it is non-competitive (column 2), except for papaverine.

TABLE 5. *Mean log* K S.E. (n *results*)

	$Me_3\overset{+}{N}$	$Me_2\overset{+}{N}Et$	$MeN\overset{+}{E}t_2$
$CH_3CH_2OCH_2CH_2-$	4·074* ±0·075 (10)	3·887* ±0·067 (4)	*3·735* ±0·030 (9)
$CH_3(CH_2)_4-$	3·733* ±0·086 (6)	3·970* ±0·032 (9)	4·399 ±0·022 (12)
$PhCH_2COOCH_2CH_2-$	4·533 ±0·012 (7)	5·093 ±0·024 (7)	5·379 ±0·022 (7)
$PhCH_2CH_2OCH_2CH_2-$	4·702 ±0·016 (11)	5·167 ±0·016 (13)	5·415 ±0·022 (7)
$Ph(CH_2)_5-$	5·180 ±0·016 (10)	5·549 ±0·031 (11)	5·735 ±0·013 (12)
	5·067 ±0·019 (10)	5·517 ±0·017 (11)	5·569 ±0·018 (9)
	5·282 ±0·018 (6)	5·657 ±0·015 (6)	5·783 ±0·028 (6)
	5·387 ±0·032 (6)	5·841 ±0·035 (5)	5·878 ±0·033 (7)
$Ph_2CHCOOCH_2CH_2-$	7·159 ±0·025 (4)	7·578 ±0·028 (7)	7·584 ±0·045 (4)
$Ph_2CHCH_2OCH_2CH_2-$	6·413 ±0·020 (8)	6·693 ±0·020 (8)	6·543 ±0·018 (8)
$Ph_2CH(CH_2)_4-$	7·015 ±0·021 (11)	7·270 ±0·036 (10)	7·091 ±0·025 (7)
	8·438 ±0·046 (9)	8·970 ±0·014 (6)	8·699 ±0·014 (10)
$Ph_2C(OH)COOCH_2CH_2-$	8·511 ±0·008 (4)	8·934 ±0·007 (7)	8·957 ±0·009 (6)
	7·686 ±0·024 (8)	7·723 ±0·021 (10)	8·083 ±0·025 (8)
	7·254 ±0·028 (9)	7·615 ±0·034 (7)	7·574 ±0·062 (7)
	9·365 ±0·033 (7)	9·804 ±0·042 (11)	9·777 ±0·030 (7)

The initials of the observer are shown in the final column.
Italics indicate that the compound is a partial agonist, and an asterisk, that it is an agonist.

$Et_3\overset{+}{N}$	MeN+	EtN+	MeN+	EtN+	
3·974 ±0·019 (12)	4·026* ±0·060 (3)	*3·883* ±0·036 (6)	4·572 ±0·018 (6)	4·007 ±0·031 (7)	VPM
4·588 ±0·011 (15)	*4·165* ±0·053 (6)	4·370 ±0·025 (12)	4·815 ±0·013 (15)	4·546 ±0·021 (12)	VPM
5·785 ±0·008 (7)	5·084 ±0·039 (7)	5·568 ±0·015 (8)	5·194 ±0·032 (7)	5·525 ±0·014 (8)	MGM
5·758 ±0·028 (8)	5·224 ±0·023 (12)	5·431 ±0·012 (13)	5·369 ±0·023 (11)	5·507 ±0·011 (7)	VPM
5·849 ±0·022 (12)	5·602 ±0·003 (5)	5·651 ±0·024 (5)	5·686 ±0·016 (17)	5·467 ±0·014 (11)	MD/ MGM
5·630 ±0·017 (8)	5·433 ±0·021 (13)	5·635 ±0·020 (7)	5·429 ±0·017 (11)	5·432 ±0·017 (10)	VPM
5·912 ±0·024 (6)	5·616 ±0·014 (6)	5·729 ±0·026 (6)	5·731 ±0·028 (5)	5·725 ±0·026 (5)	VPM
5·921 ±0·037 (8)	5·728 ±0·050 (7)	5·814 ±0·017 (6)	5·933 ±0·035 (6)	6·025 ±0·043 (6)	MGM
7·367 ±0·021 (4)	7·440 ±0·002 (4)	7·558 ±0·010 (6)	7·260 ±0·039 (4)	7·015 ±0·021 (4)	FA/ NCS
6·374 ±0·024 (7)	6·507 ±0·030 (8)	6·589 ±0·028 (8)	6·182 ±0·021 (7)	6·131 ±0·030 (8)	MGM
6·712 ±0·015 (5)	6·788 ±0·050 (8)	6·858 ±0·022 (7)	6·664 ±0·022 (9)	6·579 ±0·028 (9)	MGM
8·566 ±0·019 (8)	8·526 ±0·054 (8)	8·677 ±0·036 (9)	8·290 ±0·031 (7)	8·099 ±0·028 (8)	MGM
8·682 ±0·006 (4)	8·585 ±0·022 (7)	8·652 ±0·005 (5)	8·034 ±0·014 (16)	8·012 ±0·029 (14)	FA/ NCS
8·068 ±0·022 (7)	8·093 ±0·031 (9)	8·260 ±0·032 (7)	8·117 ±0·036 (6)	7·692 ±0·034 (10)	VPM
7·354 ±0·030 (6)	7·541 ±0·052 (6)	7·206 ±0·074 (6)	7·296 ±0·047 (6)	6·600 ±0·058 (7)	VPM
9·482 ±0·030 (8)	9·473 ±0·026 (9)	9·588 ±0·047 (7)	9·215 ±0·044 (9)	9·081 ±0·047 (7)	PW

standard error and number of results; the fact that they are agonists and that the affinity has been measured by a different method is indicated by an asterisk.

Discussion

Analysis of the results

For convenience the compounds we have tested can be regarded as containing a cationic head, a five-atom chain, and a tail consisting either of a hydrogen atom or of one or more larger groups.

Effects of the size of the onium group on affinity

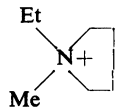
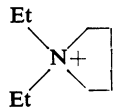
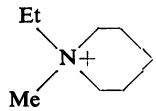
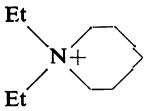
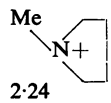
Some idea of the relative size of the various onium groups is given by the differences in ionic weight, but this does not properly take account of differences in size due to the cyclic nature of the pyrrolidinium and piperidinium compounds. A more direct estimate of relative size can be obtained from physicochemical measurements. Lowe & Rendall (to be published) have measured the electrical conductance in aqueous solution of many onium salts and calculated values of the conductance at infinite dilution. The values of $100/\Lambda_o$ should be proportional to the Stokes's radii of the ions (see, for example, Robinson & Stokes, 1956). Lowe & Rendall have kindly provided us with their results for the series: $Et\overset{+}{N}Me_3$, $Et\overset{+}{N}Me_2Et$, $Et\overset{+}{N}MeEt_2$, $Et\overset{+}{N}Et_3$,  ,  ,  and  , which can be regarded as a typical example of our series in which the tail and chain are replaced by an ethyl group. It is not known whether it is the radius, volume, or surface area of the cationic head which is important. If the effects on affinity were directly related to only one of these parameters, a plot of log. log K (that is, log $\triangle G$) against log $100/\Lambda_o$ would give a series of straight lines and the slope of these would indicate which parameter was relevant. The lines are not straight, however, and it is clear that more than one parameter must be involved. To show the effects on affinity of the size of the cationic head we have, therefore,

TABLE 6. *Equipotent molar ratios of agonists relative to* n-*pentyltrimethylammonium*

$CH_3(CH_2)_4^-$		
$\overset{+}{N}Me_3$	$\overset{+}{N}Me_2Et$	
1·0	19·4 ±0·5 (11)	
$CH_3CH_2OCH_2CH_2^-$		
$\overset{+}{N}Me_3$	$\overset{+}{N}Me_2Et$	
0·329 ±0·005 (6)	1·44 ±0·02 (9)	2·24 ±0·04 (10)

The figures are the mean of the number of estimates shown in parentheses ± the standard error.

simply plotted log K against the apropriate value of $100/\Lambda_o$ (Fig. 1). Although the Stokes' radius, as indicated by $100/\Lambda_o$, is not the same as the Van der Waals' radius, we believe that it is a good indication of the size of the cationic head, because Lowe & Rendall have observed a direct relationship between $100/\Lambda_o$ and the partial molar volume of the compounds.

If the various parts of the molecule make contributions to the free energy of adsorption, which are additive, all the lines in Fig. 1 should be parallel. They are not. Certain trends, however, are distinguishable. Increasing the size of the onium

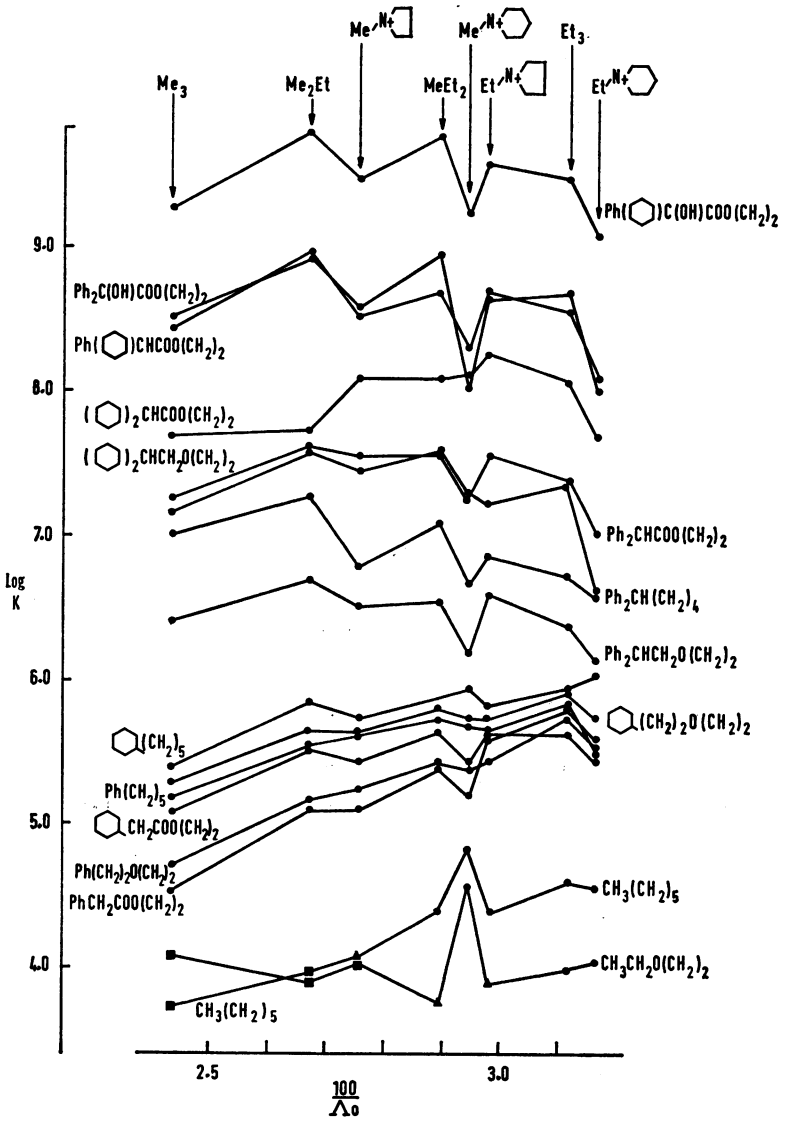


FIG. 1. Graph of log K against $100/\Lambda_o$ for the corresponding compound in the series $Et\overset{+}{N}Me_3$, $Et\overset{+}{N}Me_2Et$, etc., from results kindly provided by Lowe & Rendall. ▲ Indicates a partial agonist and ■ indicates an agonist.

group increases the affinity of compounds with one large group (phenyl or cyclohexyl) at the other end of the molecule. There may well be an upper limit to this; in five series out of six the ethylpiperidinium compound has significantly lower affinity than the triethylammonium. The effects of increasing the size of the onium group are similar in the *n*-pentyl compounds, which have only CH_3 at the far end, with the exception that the methylpiperidinium compound has relatively high affinity. In the other series with only CH_3 at the far end, the ethoxyethyl compounds, the methylpiperidinium compound again has high affinity, but there is not the same general increase in affinity with the size of the onium group.

With the compounds which have two large groups in the tail of the molecule the effects of increasing the size of the onium group have a different pattern. In six out of the eight series this is similar and typified by the phenyl*cyclo*hexylglycollyl esters, which appear at the top of the graph. Although the dimethylethyl- and methyldiethylammonium compounds have higher affinity than the trimethylammonium ones, the affinity of the pyrrolidinium and piperidinium compounds tends to be low and in seven out of the eight series the ethylpiperidinium compound has the lowest affinity, considerably below that of the trimethylammonium compound but often not significantly different from that of the methylpiperidinium compound. With the di*cyclo*hexylethoxyethyl and, more particularly, with the di*cyclo*hexylacetoxyethyl series, the pattern is slightly different. In particular the replacement of one methyl group by ethyl in di*cyclo*hexylacetoxyethyltrimethylammonium does not produce a significant rise in affinity; in all thirteen other series of antagonists this change more than doubles the affinity. In the di*cyclo*hexylethoxyethyl series the ethylpyrrolidinium compound has lower affinity than the methylpyrrolidinium, which is also quite unlike what is found in all the other thirteen series of antagonists. It seems that the positions of the pharmacodynamic groups in these compounds, relative to the groups in the receptor with which they interact, are likely to be slightly different from the positions of the compounds of other series. A possible reason for this might be the highly lipophilic character of these compounds containing two *cyclo*hexyl groups. Our stock solutions of these compounds (about 10^{-3}M) were noticeably surface-active.

The effects of the composition of the onium group on log K are summarized in Table 7, which shows the mean values of the effects of a particular change from trimethylammonium, together with the standard deviation. The size of the standard deviation is an indication of the extent of the interaction between the effects of changing parts of the molecule. If there were no interaction the effects of a change should be the same in all series and the standard deviation would be determined only by the experimental error in the estimates of log K.

Effects of the composition of the rest of the molecule on affinity

The average values of the effects of changes in the composition of the rest of the molecule on affinity are shown in Table 8, together with the standard deviation and the values for the change with the trimethylammonium compounds alone. Because the effects of changing the cationic head interact with the effects of changing the rest of the molecule, it is not sufficient to compare only compounds containing one particular onium group, so we have taken the average for the eight members of each series. In most series the average change in affinity is smaller than that for the trimethylammonium compounds.

TABLE 7. *Effect of the composition of the onium group on log K for the antagonists*

	Me_2EtN^+		$MeEt_2N^+$			Et_3N^+	
All fourteen series	0·387 (0·131)	0·261 (0·211)	0·434 (0·204)	0·172 (0·392)	0·373 (0·310)	0·391 (0·425)	0·064 (0·523)
Monosubstituted (six series)	0·445 (0·070)	0·423 (0·094)	0·601 (0·146)	0·532 (0·120)	0·613 (0·235)	0·784 (0·297)	0·588 (0·274)
Disubstituted (eight series)	0·343 (0·153)	0·139 (0·192)	0·308 (0·141)	−0·098 (0·285)	0·193 (0·231)	0·095 (0·199)	−0·329 (0·206)
Hydroxy compounds (two series)	0·431 (0·011)	0·091 (0·023)	0·423 (0·016)	−0·313 (0·232)	0·182 (0·058)	0·144 (0·038)	−0·391 (0·153)
Other disubstituted compounds (six series)	0·314 (0·169)	0·155 (0·224)	0·268 (0·141)	−0·026 (0·280)	0·197 (0·272)	0·079 (0·233)	−0·308 (0·230)

The mean difference is shown between log K for the compounds and that for the trimethylammonium analogues, the standard deviation is shown in parentheses.

TABLE 8. *Effects on log* K *of changes in the end of the molecule furthest from the onium group*

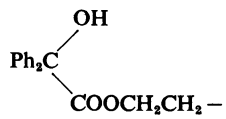
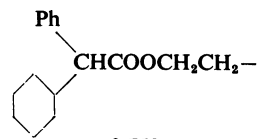
Effects of replacing H by Ph in:—

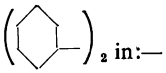
$CH_3(CH_2)_4-$	$CH_3CH_2OCH_2CH_2-$	$Ph(Ch_2)_5-$	$PhCH_2CH_2OCH_2CH_2-$	$PhCH_2COOCH_2CH_2-$	
1·447	0·628	1·835	1·711	2·626	3·371
1·267	1·302	1·282	1·107	2·100	3·054
(0·262)	(0·413)	(0·342)	(0·403)	(0·406)	(0·258)

Effects of replacing H by  in:—

$CH_3(CH_2)_4-$	$CH_3CH_2OCH_2CH_2-$			$PhCH_2COOCH_2CH_2-$
1·654	1·208	1·972	2·619	3·905
1·493	1·660	1·626	2·501	3·263
(0·223)	(0·325)	(0·372)	(0·185)	(0·476)

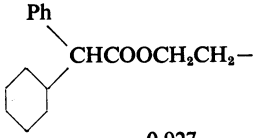
Effects of replacing Ph by  in:—

$Ph(CH_2)_5-$	$PhCH_2CH_2OCH_2CH_2-$	$PhCH_2COOCH_2CH_2-$	$Ph_2CHCOOCH_2CH_2-$		
0·207	0·580	0·534	1·279	0·854	−0·752
0·226	0·345	0·191	1·163	0·921	−0·568
(0·151)	(0·141)	(0·242)	(0·120)	(0·133)	(0·322)

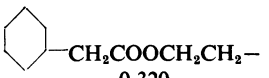
Effects of replacing Ph_2 by  in:—

$Ph_2CHCH_2OCH_2CH_2-$	$Ph_2CHCOOCH_2CH_2-$
0·841	0·527
0·876	0·595
(0·224)	(0·213)

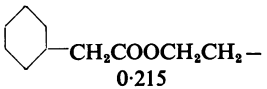
Effects of replacing H by OH in:—

$Ph_2CHCOOCH_2CH_2-$	
1·352	0·927
1·176	0·940
(0·215)	(0·070)

Effects of replacing –CO–O– by $-CH_2CH_2-$ in:—

$PhCH_2COOCH_2CH_2-$		$Ph_2CHCOOCH_2CH_2-$
0·647	0·320	−0·144
0·320	0·352	−0·498
(0·256)	(0·132)	(0·194)

Effects of replacing –CO–O– by $-CH_2O-$ in:—

$PhCH_2COOCH_2CH_2-$		$Ph_2CHCOOCH_2CH_2-$	
0·169	0·215	−0·746	−0·432
0·051	0·215	−0·941	−0·722
(0·110)	(0·075)	(0·105)	(0·353)

The upper figures show the effects for the trimethylammonium compounds, the lower figures show the average effect for the series of 8 compounds with the standard deviation shown in parentheses. The vertical lines divide the series according to the size and number of substituents at the end.

The replacement of one hydrogen by phenyl increases log K by 1·3 in both the *n*-pentyl and ethoxyethyl series. In the compounds already containing one phenyl group, the replacement of a second hydrogen by phenyl produces similar changes in the *n*-pentyl and ethoxyethyl compounds, but a much bigger change in the phenylacetoxyethyl series. Replacement of hydrogen by phenyl produces a very large change indeed (over 3·0) in the *cyclo*hexylacetoxyethyl series.

The replacement of hydrogen by *cyclo*hexyl increases affinity rather more than does replacement by phenyl and again the effects are much bigger in the *cyclo*hexylacetoxyethyl compounds than in the *cyclo*hexylethoxyethyl compounds or in the *n*-pentyl and ethoxyethyl series. The effects of the change are particularly marked when hydrogen is replaced by *cyclo*hexyl in the phenylacetoxyethyl series; log K for the trimethylammonium compound is increased by 3·9 and the average increase for the series is 3·3. These large values are due to the very high affinity of the phenyl*cyclo*hexylacetoxyethyl compounds, which also accounts for the large effects of replacing hydrogen by phenyl in the *cyclo*hexylacetoxyethyl series referred to above.

The hydroxyl group in the benzilic and phenyl*cyclo*hexyglycollic esters makes a large contribution to affinity. The effect is particularly consistent with the eight compounds in the latter series, as shown by the small value of the standard deviation in Table 8. It is also extremely regular with the trimethyl-, ethyldimethyl-, methyldiethyl- and triethylammonium members of the former series, the difference in log K between the benzilic esters and the corresponding diphenylacetyl esters being 1·35, 1·36, 1·37, and 1·31 units respectively. With the methyl- and ethylpyrolidinium and methyl- and ethylpiperidinium compounds, however, the increments are smaller and less regular, being 1·14, 1·09, 0·77 and 1·00 respectively.

The effects of the linkage between the onium group and the large groups at the other end of the molecule are seen in the lower part of Table 8; again there are marked differences between the monosubstituted and disubstituted series. In the former, replacement of $-COO-$ by $-CH_2CH_2-$, and, to a lesser extent by $-CH_2O-$ increases affinity, whereas in the latter it decreases it.

Binding of drugs to receptors

The conclusion of Barlow, Scott & Stephenson (1963) that "the effects on affinity of altering the constitution of the onium group are not very dependent on the nature of the group R" (the rest of the molecule), is somewhat undermined by the additional results now presented. It was pointed out that "our postulate that the adsorbability is made up of components which are additive depends upon the absence of any interaction between the various groups in the molecule" and it seemed unlikely that it would be possible to make big changes in structure, such as altering the chain or tail of the molecule, without interactions occurring. We expected an interaction between phenyl group and the ester link, which is why we have now studied *cyclo*hexylanalogues. Actually the effects of replacing phenyl by *cyclo*hexyl in the esters are not obviously different from the effects in the series where the linking group is $-CH_2O-$ or $-CH_2CH_2-$.

Unfortunately, this does not eliminate the possibility of there being such effects, opposed by others which cancel them out. A more favourable charge distribution in the phenyl ester might be opposed by increased rigidity of the ester link (Gill,

1965). We have, in fact, observed small differences (from 5 to 20 wave-numbers) between the carbonyl absorption peaks in the infrared absorption spectra of the phenyl and *cyclo*hexyl compounds, tested as potassium bromide discs, which indicate differences in rigidity. A more satisfactory way of studying the effects of changes in structure on the preferred conformations of compounds would be to examine their nuclear magnetic resonance spectra when dissolved in deuterium oxide. Changes in flexibility would seem to be most likely to alter affinity by altering the rate constant for the association of drug and receptor.

Interactions between groups, however, may occur when molecules are adsorbed at the receptor, as well as when molecules are in solution. The effect of introducing an additional group in a molecule must be regarded as being made up of at least two components. The first, which is likely to be positive, is the contribution which the extra group makes because of its binding to the receptor; and the second, which is likely to be negative, arises because the introduction of the extra group causes a realignment of the rest of the molecule on the receptor. Although this realignment may only be slight, it is likely that it will lead to the rest of the molecule being bound not quite so well, so the effect of introducing the new group may not be as big as might be expected. It is possible that occasionally a new group can be introduced into a molecule without the need for significant realignment of the rest of the molecule, but this would seem to be unlikely, particularly if the molecule already binds strongly to the receptor.

When a second new group is introduced, the effects will again be made up of at least two components of which the second will include not only the disturbance of the binding of the main part of the molecule but also disturbance of the binding of the first extra group. It would seem likely, therefore, that as more groups are added to a molecule, the affinity is likely to be less than the total of the individual extra effects. In Table 5 it can be seen that replacement of methyldiethylammonium ($-\overset{+}{N}MeEt_2$) by triethylammonium ($-\overset{+}{N}Et_3$) increases the affinity in all the monosubstituted series but decreases the affinity in all the disubstituted series. Presumably the introduction of the second substituent, which contributes considerably to affinity, alters the fit of the molecule to the receptor in such a way that the extra ethyl group can no longer make the same contribution to affinity as it did in the monosubstituted series.

What is more surprising is the finding that in some circumstances the affinity is much higher than would be expected from results obtained with simpler compounds. The compounds containing both phenyl and *cyclo*hexyl groups have a much higher affinity than would be predicted from the results with those containing only phenyl or *cyclo*hexyl groups.

The average effects (Table 8) of replacing hydrogen in the various series by phenyl and by *cyclo*hexyl are:

H→Ph	△log K	△G	H→ 	△log K	△G
n-pentyl	1·3	1·8	*n*-pentyl	1·5	2·1
ethoxyethyl	1·3	1·8	ethoxyethyl	1·7	2·4
phenylpentyl	1·2	1·7			
phenylethoxyethyl	1·1	1·6	*cyclo*hexyl ethoxyethyl	1·6	2·3
phenylacetoxyethyl	2·1	3·0	*cyclo*hexyl acetoxyethyl	2·5	3·5
*cyclo*hexylacetoxyethyl	3·0	4·3	phenylacetoxyethyl	3·3	4·7

The effect of introducing a phenyl group in the series already containing a *cyclo*hexyl group is to increase the free energy of adsorption by 4·3 kcals/mole, instead of by 1·6–3·0 kcals/mole. The effect of introducing a *cyclo*hexyl group in the series already containing a phenyl group is to increase the free energy of adsorption by 4·7 kcals/mole, instead of by 2·1–3·5 kcals/mole. Thus the effect of introducing a phenyl group and *cyclo*hexyl group simultaneously seems likely to be to increase the free energy of adsorption by about 6·5 kcals/mole (1·8 + 4·7, or 2·1 + 4·3), compared with about 4 kcals/mole calculated from the separate contributions of phenyl and *cyclo*hexyl groups.

This can be explained by postulating the existence of two sites on the receptor surface, one with a special affinity for a phenyl group and the other with a special affinity for a *cyclo*hexyl group. Because the disubstituted compounds are more active than the monosubstituted ones, it seems probable that each site also has some affinity for the other group. Suppose that, in order to take up the position to allow a single phenyl group to attach itself to the receptor, the binding of the rest of the molecule is disturbed and its contribution to the free energy of adsorption is reduced by 2·5 kcals/mole. The net observed effect of a phenyl group, 1·8 kcals/mole, would thus be made up of a much larger positive contribution (4·3 kcals/mole) offset by the negative contribution. Suppose that the same realignment of the rest of the molecule is necessary in order that the *cyclo*hexyl group in a monosubstituted compound can attach itself to the receptor; the observed effect (2·2 kcals/mole) would again be made up of a much larger positive contribution from the group (4·7 kcals/mole) offset by the same negative contribution (2·5 kcals/mole). If the realignment which allows the phenyl group to attach itself also allows the *cyclo*hexyl group to attach itself to that part of the receptor with which it interacts, a compound containing both groups should have the contribution from these groups, 4·3 + 4·7 kcals/mole, only reduced by 2·5 kcals/mole (and not by 2 × 2·5 kcals/mole), so the increase in the free energy of adsorption would be 6·5 kcals/mole. Compounds containing two groups of the same kind would have free energies of adsorption less than this, because the phenyl group does not make as big a contribution to binding when it interacts with the group in the receptor which has special affinity for *cyclo*hexyl, as it does with the group having special affinity for phenyl, and vice versa.

Whether the observed affinity is higher or lower than what would be expected by summing the contributions due to the various groups, the results can be explained by supposing that the contribution of each substituent to binding is made up of two opposing components.

These ideas are implicit in the arguments of Burgen (1965) concerning differences in the binding of the onium group of agonists and antagonists to the negatively charged group in the receptor, with which it is presumed to interact. It is claimed that the separation between the charges is greater in antagonists than in agonists, possibly because the groups, such as phenyl, which endow the molecule with high affinity also necessitate the realignment of the rest of the molecule, with the result that the onium group is further away from the negatively charged group in the receptor. It is also suggested that the charge separation determines whether a compound is an agonist or antagonist, only those compounds in which the distance is small being able to stimulate the tissue.

Our results are not consistent with this latter suggestion. Burgen (1965) interpreted the reduction in potency produced by replacing methyl by ethyl in agonists

such as acetylcholine as being due to an increase in charge separation and a reduction in affinity. In the *n*-pentyl and ethoxyethyl series which we have studied, the increase in size, which produces a decline in agonist activity, either produces little change in affinity or else slightly increases it (Tables 5 and 6, Fig. 1).

Some idea of the effects of changes in the charge separation on affinity may be obtained from Burgen's calculations. An increase in separation by 1·05 Å, from 3·25 Å to 4·30 Å, should reduce the free energy of adsorption by 2·3 kcals/mole and log K by 1·6 units. For an increase by 1·67 Å, from 3·25 Å to 4·96 Å, the decrease would be 3·4 kcals/mole and 2·4 log units. The increase in the radius of the cationic head produced by changing from trimethylammonium to triethylammonium seems likely to be at least 0·6 Å (Robinson & Stokes, 1965), which might be expected to reduce the free energy of adsorption by at least 1·4 kcals/mole and log K by 1 unit. In all the disubstituted series, however, the triethylammonium compounds have much the same affinity as the trimethylammonium compounds, often slightly greater. In the monosubstituted series the triethylammonium compounds have much bigger affinity than the trimethylammonium compounds ; in the phenylacetoxyethyl series, for example, the difference in log K is 1·25, indicating that the change from trimethylammonium to triethylammonium has increased the free energy of adsorption by 1·77 kcals/mole. Clearly any adverse effects of increased charge separation have been offset by other positive contributions to binding. Though these could theoretically involve the binding of any other part of the molecule, it would seem most likely that they are made by the extra methylene groups interacting with the part of the receptor binding the onium group. From the magnitude of the effects it also seems probable that these interactions involve hydrophobic bonding rather than conventional Van der Waals' bonding. From studies with globular proteins, Tanford (1962) estimated that each extra methylene group contributes 0·75 kcal/mole by hydrophobic bonding, so the maximum possible contribution, if all the ethyl groups are able to contribute fully, would be 2·25 kcal/mol. Even if all the difference, 0·48 kcal/mole, is due to decreased binding caused by increased charge separation, it would correspond only to a small increase in separation, unless the charges are already widely separated.

In contrast, in the disubstituted series, it is not possible to produce such big increases in affinity by replacing methyl by ethyl in the trimethylammonium compound. The limit is usually reached with the replacement of one methyl group by ethyl. The effects of changes in the onium group, however, are still considerable, the difference between the compounds with highest and lowest affinity in a disubstituted series being from 0·6 to 1 log units, corresponding to differences in the free energy of adsorption of between 0·85 and 1·4 kcal/mole. This might be explained by supposing that the extra methylene groups are no longer in a position in which they contribute so well to binding. Another possibility which must be considered is that there is less charge separation with these compounds than with the monosubstituted ones ; the adverse effect on binding produced by increasing the size of the group would therefore be greater and lead to a decrease in affinity. The results, therefore, are not consistent with the idea that high affinity necessarily leads to increased separation between the cationic head and the negatively charged group in the receptor with which it interacts. It would seem likely that the charge separation is determined by the structure of the molecule independently of the actual affinity.

The indication that all three extra methylene groups in the triethylammonium compounds of the monosubstituted series can interact with the receptor suggests that

in this region it may be thought of as being a concave surface. This idea is also consistent with the decrease in affinity which is observed when alkyl groups in the onium group are replaced by pyrrolidine or piperidine rings. In the disubstituted compounds in particular, methylpyrrolidinium compounds have lower affinity than methyldiethylammonium compounds, methylpiperidinium compounds have lower affinity than the triethylammonium compounds, and ethylpiperidinium compounds usually have the lowest affinity of all (Fig. 1). This could well be due to increased charge separation, caused by the presence of the ring with the consequent uneven distribution of bulk in the onium group and inability to accommodate itself so as to allow the charged groups to come close together. Because the effect is less noticeable in the monosubstituted compounds it can be used as a further argument in support of the idea that charge separation is less important with these compounds than with the disubstituted ones.

From the results we conclude that the changes in affinity produced by introducing a group into a molecule are the sum of a number of components, some positive, some negative, and the question now arises whether these are connected. Some of them should be, for example, the increased hydrophobic bonding obtained by the replacement of methyl by ethyl in an onium group must be offset at least to some extent by decreasing binding due to the greater separation of the positively charged group from the negatively charged group in the receptor. The irregularities in the results, however, suggest that in many instances the components are independent.

There is, however, considerable regularity with certain groups of compounds; the effects of replacing hydrogen by hydroxyl in the diphenylacetylesters are virtually the same in the trimethylethyldimethyl-, methyldiethyl- and triethylammonium compounds (page 228), and the replacement of hydrogen by hydroxyl in the phenyl-*cyclo*hexylacetyl esters produces remarkably consistent effects throughout the series of eight pairs of compounds studied, as indicated by the small value for the standard deviation shown in Table 8. Other changes which produce similar increases in log K have larger standard deviations, so it seems unlikely that the regularity can be ascribed to the big effect of the hydroxyl group on affinity. It is possible that the effects are regular because the change in structure produces only a small change in size (replacement of hydrogen by hydroxyl).

Most active compounds

By far the most active compounds are the phenyl*cyclo*hexylglycollic esters, of which Oxyphenonium is the methyldiethylammonium derivative. Ellenbroek, Nivard, Van Rossum & Ariens (1965) have prepared the (+) and (−) forms of the phenyl*cyclo*hexylglycolloylcholine (" hexahydrobenziloylcholine ") and found that the (−)-isomer has 100 times the affinity of the (+)-isomer for the acetylcholine receptors in rat intestine; so the value of log K for the (−)-isomer for the receptors in the guinea-pig ileum is likely to be 9·66. For the (+)-compound the value should be 7·66, comparable with the corresponding di*cyclo*hexylacetyl ester. The difference between the two isomers is greater than would be expected from our results if it is due only to the failure of the hydroxyl group to contribute to binding in the (+)-isomer and suggests that there might be considerable stereospecificity in the phenyl*cyclo*hexylacetyl esters. It is interesting that the suggestion by Ellenbroek, Nivard, Van Rossum & Ariens (1965) that their (−)-isomer has the R-configuration has been confirmed by the determination of the absolute configuration of these compounds by Inch, Ley & Rich (1968).

We thank Dr. Jamieson Walker and Mr. John Duguid for preparing the phenyl*cyclo*hexylacetoxyethyl and diphenylpentyl compounds and Ciba A.G., Basel, for the phenyl*cyclo*hexylglycollic acid and for checking the melting-point of oxyphenonium iodide. We also wish to thank Miss Margaret Dalling, Dr. N. C. Scott and Mr. Peter Whelpdale, who have helped with the estimates of affinity, and especially Miss Veronica Mullan, who performed a very large number of them. We thank the Faculty of Medicine of the University of Edinburgh and the Government of Pakistan for the award of scholarships (to F. B. A. and M. G. M., respectively) and the Tobacco Research Council, who paid for technical assistance (of M.D. and V. P. M.). The digital recording equipment was presented by the Wellcome Trust and the infrared spectrophotometer was supplied by the Medical Research Council, who also provided the conductance bridge used by Lowe and Rendall. We are particularly grateful to Dr. Lowe and Mr. Rendall for permission to incorporate some of their results in Fig. 1. We thank Dr. B. L. Ginsborg for his helpful comments on the manuscript.

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(*Received December* 19, 1968)