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AN ATTEMPT TO STUDY THE EFFECTS OF CHEMICAL STRUCTURE ON THE AFFINITY AND EFFICACY OF COMPOUNDS RELATED TO ACETYLCHOLINE

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Two sets of series of compounds, RN^+Me_3, RN^+Me_2Et, RN^+MeEt_2, RN^+Et_3, and $R'N^+Me_3$, $R'N^+Me_2Et$, $R'N^+MeEt_2$, $R'N^+Et_3$, have been prepared, in which R is a 2-(diphenylacetoxy)ethyl, 2-(benziloyloxy)ethyl, 2-(2,2-diphenylethoxy)ethyl, 3-(diphenylmethoxy)propyl or 3,3-diphenylbutyrylmethyl group, and R′ is a 2-acetoxyethyl, 2-ethoxyethyl, 3-methoxypropyl or butyrylmethyl group: compounds of the first set therefore differ from those of the second set in that they contain a diphenylmethyl group (or a benziloyl group) in place of a methyl group. The former compounds are antagonists of acetylcholine whereas most of the latter act like acetylcholine. The affinity constants of the former compounds for the acetylcholine receptors of the guinea-pig ileum have been determined and the equipotent molar ratios relative to acetylcholine have been measured for the latter compounds. The variation of the affinity constant with the constitution of the onium group in the antagonists (the diphenylmethyl compounds) was sufficiently consistent from one series to another for it to seem likely that corresponding changes in affinity with the constitution of the onium group would occur in the agonists. From the relative activity of the agonists and with this knowledge of relative affinity it was possible to assess the effects of their structure on efficacy. Substitution of one methyl in the onium group by an ethyl group in these compounds increased affinity but decreased efficacy. The replacement of a second methyl by a second ethyl group had little effect on affinity but decreased efficacy still further. The replacement of the ester link in acetylcholine by a 4-ether oxygen atom (as in the diphenylmethoxypropyl and methoxypropyl compounds) did not appreciably reduce affinity but markedly reduced efficacy, whereas the replacement of the ester link by a 3-ether oxygen atom (as in the diphenylethoxyethyl and ethoxyethyl compounds) markedly reduced affinity but did not reduce efficacy. The diphenylbutyrylmethyl compounds had low affinity and the butyrylmethyl compounds had low efficacy. We conclude that the action of acetylcholine at the postganglionic parasympathetic receptors in the guinea-pig ileum depends upon the presence of the 4-carbonyl group (and presumably the onium group) for affinity and on the 3-ether oxygen atom and the trimethylammonium group for efficacy.

Many workers (see, for example, the review by Barlow, 1964) have observed that the pharmacological activity of acetylcholine depends critically on its chemical structure. This activity depends not only on the ability of the compound to become attached to acetylcholine receptors but also upon its ability to activate them.

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Stephenson (1956) calls these properties respectively the "affinity" and the "efficacy" of a compound and we shall use these terms. It is not possible to assess directly the contribution of each of these factors to activity and most workers have interpreted changes in activity solely in terms of changes in affinity.

Holton & Ing (1949), for instance, observed that the replacement of methyl by ethyl groups in the cationic head of acetylcholine led to a marked decline in agonist activity and suggested that this was because the ethyl analogues did not fit the receptors so well. In the analogues series of benzilic acid esters, which are antagonists of acetylcholine, Ing, Dawes & Wajda (1945) and Ford-Moore & Ing (1947), however, observed that the compounds with ethyl groups in the cationic head were actually more active than benziloylcholine and Stephenson (1956) therefore suggested that the decline in activity in the series of agonists was due to a decline in efficacy, rather than to a decline in affinity. This suggestion involves the assumption that replacement of methyl by ethyl groups in the cationic head has similar effects on affinity whether the compounds are acetyl or benziloyl esters.

This type of comparison of the effects of changes in structure on the activity of antagonists with the effects of similar changes in structure on the activity of agonists is a way whereby the effects of changes in structure on efficacy may be assessed quantitatively. To do this it is necessary to make two series of compounds, one of agonists and the other of antagonists, and to measure the affinity constants of the antagonists and the activity of the agonists.

Let the affinity constants of the members of the series of antagonists, RN^+Me_3, RN^+Me_2Et, RN^+MeEt_2 and RN^+Et_3, be denoted by K, K_a, K_b and K_c respectively and let those for the corresponding series of agonists, $R'N^+Me_3$, $R'N^+Me_2Et$, $R'N^+MeEt_2$ and $R'N^+Et_3$, be K', K'_a, K'_b and K'_c. The relationship between these affinity (association) constants and the free energy change on adsorption ($\triangle F$) is given by the theory of Arrhenius:

$$\triangle F = -RT\log_e K$$
$$\text{or } \log_{10} K = -\triangle F\ (2.3RT)$$

The assumption that the effects on affinity of replacing methyl by ethyl groups in the cationic head are the same whether the compounds are acetyl or benziloyl esters accordingly implies that the change in the free energy of adsorption depends only on the substitution in the onium group, that is, that the free energy of adsorption is made up of components which are additive. The contributions from the portions R and R′ should be unaffected by the changes in the onium group (though this condition would not be fulfilled if there were an interaction between R or R′ and any particular substituent on the quaternary nitrogen atom). It should follow then that, if the free energy of adsorption for RN^+Me_3 is $\triangle F$ and for $R'N^+Me_3$ is $\triangle F'$, the free energies of adsorption are given by:

$$RN^+Me_2Et = \triangle F + a \qquad R'N^+Me_2Et = \triangle F' + a$$
$$RN^+MeEt_2 = \triangle F + b \qquad R'N^+MeEt_2 = \triangle F' + b$$
$$RN^+Et_3 = \triangle F + c \qquad R'N^+Et_3 = \triangle F' + c$$

where a is the free energy change brought about by replacing N^+Me_3 by N^+Me_2Et, b the change by replacing N^+Me_3 by N^+MeEt_2 and c the change by replacing N^+Me_3 by N^+Et_3.

It follows that: $$\log K_a = -(\triangle F + a)/(2.3RT)$$
and therefore that $$\log (K_a/K) = -a/(2.3RT),$$
which should be a constant, independent of the nature of R, which determines the absolute value of $\triangle F$ or K: it should also be the same as $\log (K'_a/K')$ for the agonists $R'N^+Me_3$ and $R'N^+Me_2Et$. If the equipotent molar ratio for the latter relative to the former is n, that is, n molecules of $R'N^+Me_2Et$ produce the same (agonist) response as one molecule of $R'N^+Me_3$, the biological stimulus giving rise to this response should be the same. According to the argument put forward by Stephenson (1956), this stimulus will be equal to ey, where e is the efficacy of the compound and y the proportion of receptors occupied.

According to the Law of Mass Action (Clark, 1933), $y = (A'K')/(1 + A'K')$,

where A' is the concentration of agonist with an affinity constant of K', and when y is small this approximates to $A'K'$.

Consequently $ey = eK'A'$ (for the first agonist)

$$= e_a K'_a A'_a \quad \text{(for the second agonist).}$$

where A' and A'_a are the respective concentrations of the compounds, e and e_a their efficacies, and K' and K' their affinities.

The ratio $A'_a/A' = n$, hence the ratio of the efficacies, $e/e_a = nK'_a/K'$, which can be calculated because K'_a/K' has already been determined for the series of antagonists.

The constitution of the groups R and R′ in the two series of compounds, of the general formulae $RN^+R^1R^2R^3$ and $R'N^+R^1R^2R^3$ respectively, studied in this work was:

R	R′
2-(Diphenylacetoxy)ethyl $Ph_2CH.CO.O.CH_2.CH_2$-	2-Acetoxyethyl $CH_3.CO.O.CH_2.CH_2$-
2-(Benziloyloxy)ethyl $Ph_2C(OH).CO.O.CH_2.CH_2$-	
2-(2,2-Diphenylethoxy)ethyl $Ph_2CH.CH_2.O.CH_2.CH_2$-	2-Ethoxyethyl $CH_3.CH_2.O.CH_2.CH_2$-
3-(Diphenylmethoxy)propyl $Ph_2CH.O.CH_2.CH_2.CH_2$-	3-Methoxypropyl $CH_3.O.CH_2.CH_2.CH_2$-
γγ-Diphenylbutyrylmethyl $Ph_2CH.CH_2.CH_2.CO.CH_2$-	Butyrylmethyl $CH_3.CH_2.CH_2.CO.CH_2$-

The two sets of series thus differ only in that the end group in R′ was always a methyl group, whereas in R it was a diphenylmethyl group (except in one series, in which it was a benziloyl group).

We thus had five series of antagonists in which we could observe the effects on affinity of replacing methyl by ethyl groups in the cationic head and so we could test the validity of the assumption that the corresponding change in the free energy of adsorption should be independent of the constitution of the rest of the molecule.

The compounds could also be regarded as being members of series in which the consitution of the onium group was kept constant and the rest of the molecule was altered; we have therefore been able to make analogous calculations about the effects of the groups R or R′ on affinity and efficacy.

METHODS

Experimental preparations. The guinea-pig isolated ileum was set up and mounted exactly as described by Stephenson (1956), using the same apparatus. The organ-bath volume was 2.7 ml. but, because an automatic assay apparatus was used, the drugs were all made up at known concentrations in Tyrode solution, and added at the appropriate point in the cycle from the reservoirs. Contractions were recorded by an isotonic frontal writing lever with a four-fold magnification and a load of 0.5 g. The temperature was $37.0 \pm 0.1°$ C.

The Tyrode solution contained twice the usual concentration of potassium chloride to reduce the influence of one contraction on the next, and also 1.1×10^{-4} M-hexamethonium bromide. The agonist was in contact with the tissue for from 10 to 15 sec and the interval between doses was from 1 to 1.5 min; these times were constant in any one experiment.

Antagonist activity. The method of determining antagonist activity is illustrated in Fig. 1. After regular responses had been obtained to three different concentrations of acetylcholine, the Tyrode solution in which the ileum was suspended was replaced by Tyrode solution containing the antagonist and the concentration of agonist was increased so that comparable contractions were obtained. In most experiments this procedure was then repeated with higher concentrations of antagonist and agonist. The dose-ratio corresponding to a particular

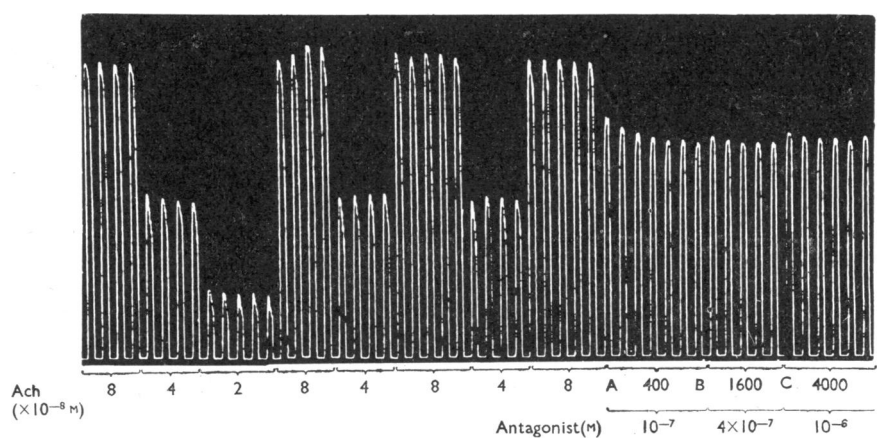

Fig. 1. Typical antagonist assay: in the first part of the experiment the responses to different concentrations of acetylcholine (2, 4 and 8×10^{-8} M) were determined; at A the Tyrode solution was changed to Tyrode solution containing antagonist (benziloyloxyethyldiethylmethylammonium) in a concentration of 10^{-7} M and the acetylcholine concentration was increased to 4×10^{-6} M; at B the concentration of antagonist was increased to 4×10^{-7} M and the acetylcholine concentration was 1.6×10^{-5} M; at C the concentration of antagonist was 10^{-6} M and the acetylcholine concentration was 4×10^{-5} M. The agonist was in contact with the tissue for 10 sec and the interval between doses was 60 sec. Note the rapid development of response to the antagonist.

concentration of antagonist was evaluated by measuring the height of the response in the presence of the antagonist and calculating, from the responses obtained initially in the absence of antagonist, what concentration of agonist alone would have produced this effect. The affinity constant of the antagonist was then calculated from the equation $BK=A/a-1$ (Gaddum, 1957), where A/a is the dose ratio, B the concentration of antagonist and K its affinity constant.

Agonist activity. This was estimated by determining the equipotent molar ratio relative to acetylcholine as described by Stephenson (1956). The results were derived from four-point assays based on forty-eight contractions (twelve groups of four doses arranged in three Latin squares; Fig. 2). In order to diminish the effect on the response (sometimes quite marked)

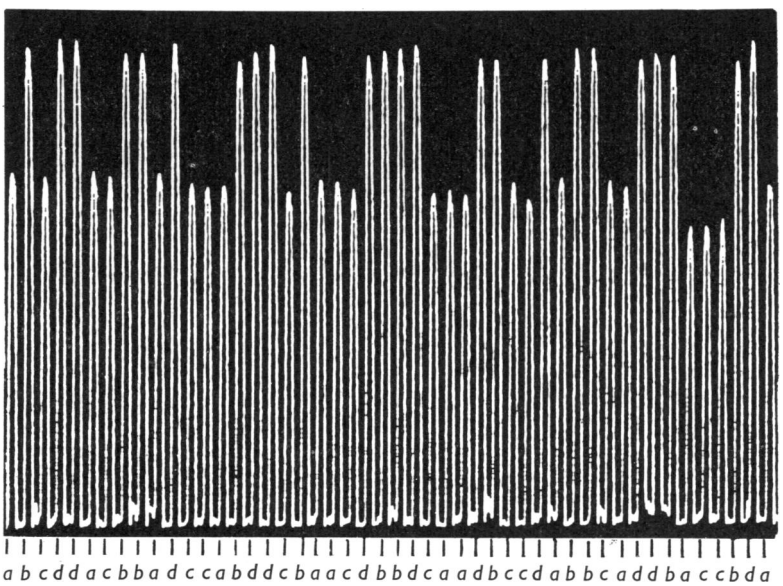

Fig. 2. Assay of agonist activity; responses are shown to acetylcholine, 2×10^{-8} M (*a*), and 4×10^{-8} M (*b*), and to the compound acetoxyethyltriethylammonium, 4.8×10^{-6} M (*c*), and 9.6×10^{-6} M (*d*).

of the preceding dose, a constant third dose of acetylcholine was interpolated between each assay dose (Fig. 3): this third dose was the geometric mean of the two doses of acetylcholine used in the assay. This procedure greatly increased the accuracy: for example, the index of precision (s/b) for the assay shown in Fig. 2 was 0.023, whereas for the assay, part of which is shown in Fig. 3, it was 0.017.

Drugs. The members of the acetoxyethyl, diphenylacetoxyethyl and benziloyloxyethyl series of quaternary ammonium compounds were obtained from the appropriate tertiary amino-ester. The latter was dissolved in ethyl methyl ketone, a two-fold excess of methyl or ethyl bromide was added, and the mixture was left for 2 days at room temperature and then heated under reflux for a few minutes. The quaternary salt was filtered off and recrystallized until the melting point remained constant.

The members of the ethoxyethyl, diphenylethoxyethyl, methoxypropyl and diphenylmethoxy-propyl series of quaternary ammonium compounds were prepared in a similar manner from the appropriate amino-ethers.

Members of the butyrylmethyl and diphenylbutyrylmethyl series of quaternary ammonium compounds were prepared from the appropriate acyl chloride. This was converted into the

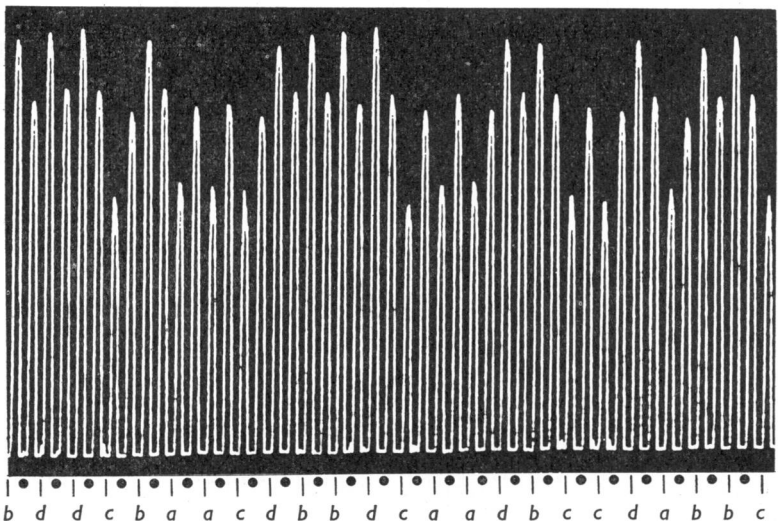

Fig. 3. Assay of agonist activity with a fixed dose of acetylcholine interpolated between the doses used in the assay in order to reduce the variance of the estimate. The preparation, drugs and concentrations are exactly the same as those used in Fig. 2. The concentration of acetylcholine in the interpolated doses (●) was 2.83×10^{-8} M.

diazoketone, which was decomposed with hydrobromic acid; the yield of butyrylmethyl bromide (boiling point 37 to 38° C/2.9 mm, N_D^{20} 1.4573) was 62% (based on the amount of acid chloride). Catch, Elliott, Hey & Jones (1948) recorded a boiling point of 92° C/50 mm, N_D^{20} 1.4575 and yield 27%. The yield of γγ-diphenylbutyrylmethyl bromide (melting point 95° C, recrystallized from ethanol) was 79%. Found: C, 64.7%; H, 5.33%. $C_{17}H_{17}OBr$ requires: C, 64.4%; H, 5.40%. The quaternary salts were obtained from these bromides by the addition of an excess of the appropriate amine dissolved in ethyl methyl ketone. With these compounds there is a tendency for alkylation to be accompanied by elimination of hydrogen bromide with the consequent formation of the hydrobromide of the tertiary base as well as the quaternary salt. The product of the reaction was therefore dissolved in methanol and heated with propylene oxide, in order to convert the salt of the tertiary amine into the free base; when ether was added, only the quaternary salt was precipitated (Sackur, 1952).

Analytical results and melting points are shown in Table 1. As there was no doubt about the constitution of the tertiary amino-derivatives or the alkyl bromides from which the quaternary compounds were derived, it was considered that an estimation of ionized bromide was an adequate indication of purity. (2-Benziloyloxyethyl)triethylammonium bromide was prepared by Blicke & Maxwell (1942), who recorded a melting point of 169 to 170° C (compare 176° C in Table 6).

Commercial acetylcholine chloride (Roche) was employed in the pharmacological experiments and all solutions of esters were made up freshly before use.

RESULTS

In all this work it is assumed that the compounds are acting competitively and our results are consistent with this assumption. Thus the values of the affinity constant, K, were, in fact, constant over a wide range of concentrations of antagonist. This is illustrated by the linear relationship between $A/a - 1$ and the concentration of antagonist (Fig. 4) and justifies proceeding further.

TABLE I

STRUCTURES AND ANALYTICAL DATA

General formula: $[RN^+R^1R^2R^3]X^-$

All compounds are bromides except that marked with an asterisk which is an iodide. Me=methyl; Et=ethyl; Ph=phenyl; Ac=acetyl

Series (R)	$N^+R^1R^2R^3$	Melting point (°C)	Crystallized from	Bromine (%) Found	Bromine (%) Calculated
Acetoxyethyl	N^+Me_3	Commercial sample of acetylcholine			
$CH_3.CO.O.CH_2.CH_2$	N^+Me_2Et	95	Me_2CO/EtOAc	33·00	33·29
	N^+MeEt_2	62		31·49	31·44
	N^+Et_3	134		30·01	29·80
Diphenylacetoxyethyl	N^+Me_3	201	EtOH/EtOAc	21·02	21·13
$Ph_2CH.CO.O.CH_2.CH_2$	N^+Me_2Et	150		20·37	20·37
	N^+MeEt_2	108		19·50	19·67
	N^+Et_3	154		18·83	19·01
Benziloyloxyethyl	N^+Me_3	187	EtOH/EtOAc/Et_2O	20·02	20·27
$Ph_2C(OH).CO.O.CH_2.CH_2$	N^+Me_2Et	178		19·62	19·57
	N^+MeEt_2	176		18·80	18·92
	N^+Et_3	226		18·34	18·31
Ethoxyethyl	N^+Me_3	183	Me_2CO/EtOAc	41·42	41·50
$CH_3.CH_2.O.CH_2.CH_2$	N^+Me_2Et	69		35·33	35·43
	N^+MeEt_2*	58		44·57	44·20
	N^+Et_3	97		31·54	31·57
Diphenylethoxyethyl	N^+Me_3	147	EtOH/EtOAc	22·10	21·95
$Ph_2CH.CH_2.O.CH_2.CH_2$	N^+Me_2Et	106		21·13	21·20
	N^+MeEt_2	128		20·37	20·40
	N^+Et_3	167		19·80	19·71
Methoxypropyl	N^+Me_3	230	Me_2CO/EtOAc	41·40	41·50
$CH_3.O.CH_2.CH_2.CH_2$	N^+Me_2Et	167		36·01	35·43
	N^+MeEt_2	185		33·30	33·34
	N^+Et_3	140		31·27	31·47
Diphenylmethoxypropyl	N^+Me_3	180	EtOH/EtOAc	21·95	21·95
$Ph_2CH.O.CH_2.CH_2.CH_2$	N^+Me_2Et	129		21·47	21·20
	N^+MeEt_2	112		20·40	20·40
	N^+Et_3	157		20·01	19·71
Butyrylmethyl	N^+Me_3	145	EtOH/EtOAc	35·43	35·68
$CH_3.CH_2.CH_2.CO.CH_2$	N^+Me_2Et	109		33·55	33·60
	N^+MeEt_2	103		31·85	31·76
	N^+Et_3	194		30·00	30·05
Diphenylbutyrylmethyl	N^+Me_3	137	EtOH/EtOAc	21·36	21·24
$Ph_2CH.CH_2.CH_2.CO.CH_2$	N^+Me_2Et	123		20·30	10·46
	N^+MeEt_2	127		19·70	19·77
	N^+Et_3	169		19·18	19·10

The estimates of the affinity constant are summarized in Table 2. In the most satisfactory sets of experiments the standard error is about 1% and in the least satisfactory, with ($\gamma\gamma$-diphenylbutyrylmethyl)diethylmethylammonium, about 10%.

In Table 3 the mean values of the ratios of the affinity constants, K_a/K etc., are set out together with the values of $-f$. If it is assumed that there is a normal distribution of values of log K (Gaddum, 1945, 1953) the confidence limits for log (K_a/K) can be calculated by Student's "t" test and the corresponding values of K_a/K and of $-f$ are shown (in these calculations we have used a pooled estimate of the variance of the values of log K). It should be observed that, in Table 3, the ratio K_a/K is calculated from the mean values of K_a and K which are geometric

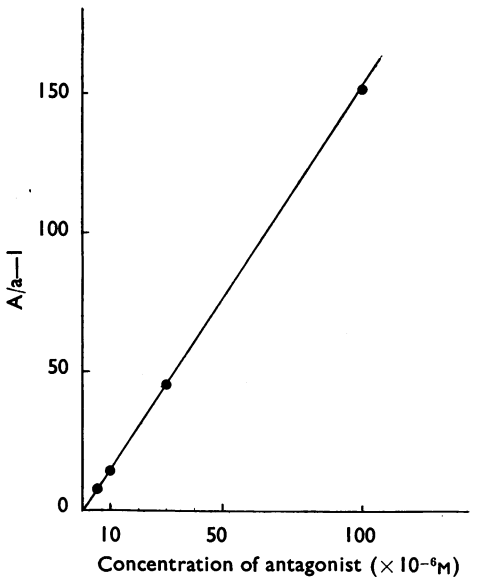

Fig. 4. Graph of (dose-ratio−1) against the concentration of antagonist (diphenylbutyrylmethyltriethylammonium); the linear relationship is consistent with a competitive antagonism.

means, but these ratios differ only slightly from those which would be obtained directly from the arithmetic means shown in Table 2.

The results can be arranged so that, instead of showing the effect of the constitution of the onium group on affinity, they show the effects of the group R on affinity. If K is the affinity constant for the member of the diphenylacetyl series (which was taken as the standard) and K_z the affinity constant for the member of another series with the same onium group, the ratio K_z/K should be constant, regardless of the constitution of the onium group (unless there is an interaction between the onium group and the rest of the molecules), and we have calculated the difference between

TABLE 2

AFFINITY OF ANTAGONISTS

Values are means with standard errors; numbers in parentheses indicate numbers of results

	Affinity constant (× 10^{-6})			
Series	K (N^+Me_3)	K_a (N^+Me_2Et)	K_b (N^+MeEt_2)	K_c (N^+Et_3)
Diphenylacetoxyethyl	14·8 ±0·2 (3)	44·0 ±1·6 (4)	30·9 ±1·2 (4)	27·3 ±1·0 (14)
Benziloyloxyethyl	344 ±10 (5)	866 ±19 (6)	898 ±18 (8)	474 ±48 (3)
Diphenylethoxyethyl	2·66 ±0·02 (4)	5·03 ±0·35 (4)	4·67 ±0·16 (4)	3·14 ±0·02 (5)
Diphenylmethoxypropyl	11·4 ±0·1 (3)	35·6 ±0·8 (6)	37·0 ±1·2 (5)	13·2 ±0·9 (10)
Diphenylbutyrylmethyl	1·62 ±0·10 (8)	5·28 ±0·21 (4)	4·24 ±0·43 (4)	1·56 ±0·04 (4)

TABLE 3

EFFECT OF CONSTITUTION OF ONIUM GROUP ON AFFINITY CONSTANT OF THE COMPOUNDS $RN^+R^1R^2R^3$

The affinity of a compound is compared with that of the corresponding trimethylammonium salt, RN^+Me_3; $-f$ indicates the difference in the free energy of adsorption; values in parentheses indicate the 95% confidence limits

Series	Ratio of affinities of compounds RN^+Me_2Et/RN^+Me_3 (K_a/K)	RN^+MeEt_2/RN^+Me_3 (K_b/K)	RN^+Et_3/RN^+Me_3 (K_c/K)	$-f$ (cal/mole) *a*	*b*	*c*
Diphenylacetoxyethyl	2·97 (2·54, 3·47)	2·08 (1·79, 2·44)	1·82 (1·60, 2·08)	669 (574, 765)	452 (357, 548)	370 (290, 449)
Benziloyloxyethyl	2·52 (2·22, 2·92)	2·61 (2·33, 2·93)	1·37 (1·18, 1·59)	568 (492, 659)	591 (520, 662)	193 (101, 285)
Diphenylethoxyethyl	1·88 (1·63, 2·17)	1·75 (1·52, 2·03)	1·18 (1·03, 1·36)	388 (300, 477)	346 (258, 435)	103 (20, 187)
Diphenylmethoxypropyl	3·12 (2·70, 3·61)	3·24 (2·79, 3·76)	1·15 (1·01, 1·35)	700 (611, 789)	723 (630, 815)	88 (6, 184)
Diphenylbutyrylmethyl	3·28 (2·90, 3·72)	2·61 (2·30, 2·95)	0·98 (0·86, 1·10)	731 (655, 808)	589 (513, 666)	−16 (−92, +61)

the free energy changes on adsorption for the pairs of members of the two series of compounds. Table 4 shows the values of K_z/K, log (K_z/K) and $-f$, together with the 95% confidence limits, calculated by exactly the same procedure as for Table 3.

If the adsorption of the molecules can be treated as suggested in the Introduction, values of K/K (or $-f$) should be the same for all compounds with the same onium

TABLE 4

EFFECT OF CONSTITUTION OF THE GROUP R ON AFFINITY CONSTANT OF THE COMPOUNDS $RN^+R^1R^2R^3$

The affinity constant of a compound is compared with that of the member of the diphenylacetoxyethyl series with the same onium group; $-f$ indicates the difference in the free energy of adsorption; values in parentheses indicate the 95% confidence limits

Onium group ($-N^+R^1R^2R^3$)	Affinity ratio (K_z/K) for series: Benziloyloxyethyl	Diphenylethoxyethyl	Diphenylmethoxypropyl	Diphenylbutyrylmethyl
N^+Me_3	23·2 (19·9, 26·9)	0·179 (0·153, 0·209)	0·769 (0·652, 0·908)	0·108 (0·094, 0·124)
N^+Me_2Et	19·7 (17·3, 22·4)	0·114 (0·099, 0·131)	0·809 (0·710, 0·923)	0·120 (0·104, 0·138)
N^+MeEt_2	29·0 (25·6, 32·8)	0·151 (0·131, 0·174)	1·19 (1·041, 1·37)	0·135 (0·117, 0·156)
N^+Et_3	17·4 (15·3, 19·8)	0·116 (0·104, 0·129)	0·486 (0·447, 0·530)	0·058 (0·051, 0·065)
	$-f$ (cal/mole) for series			
N^+Me_3	1,930 (1,830, 2,020)	−1,050 (−1,150, −960)	−161 (−262, −59)	−1,360 (−1,450, −1,280)
N^+Me_2Et	1,830 (1,750, 1,910)	−1,330 (−1,420, −1,240)	−130 (−210, −49)	−1,300 (−1,390, −1,210)
N^+MeEt_2	2,060 (1,990, 2,140)	−1,160 (−1,240, −1,070)	+109 (+25, +192)	−1,230 (−1,310, −1,140)
N^+Et_3	1,750 (1,670, 1,830)	−1,320 (−1,380, −1,250)	−440 (−490, −390)	−1,750 (−1,820, −1,680)

group, irrespective of the nature of the group R (Table 3), and likewise values of K_z / K (or $-f$) should be the same for all compounds with the same group R irrespective of the constitution of the onium group (Table 4). Although the agreement was not as good as was hoped, it was considered to be sufficient to justify using the value of K_a/K obtained with these compounds in the agonist series $R'N^+Me_3$ etc. in order to estimate the effects of structure in efficacy.

TABLE 5

EFFECTS OF CHANGES IN STRUCTURE IN $RN^+R^1R^2R^3$ ON AGONIST ACTIVITY: EQUIPOTENT MOLAR RATIO RELATIVE TO ACETYLCHOLINE

Values are means with standard errors of assay results based on the number of experiments shown in parentheses. An asterisk indicates that the compound is a partial agonist or antagonist

	Onium group ($-N^+R^1R^2R^3$)			
Series	N^+Me_3	N^+Me_2Et	N^+MeEt_2	N^+Et_3
Acetoxyethyl	1·0	2·84 ±0·04 (4)	380 +36 (7)	275 +14 (10)
Ethoxyethyl	9·9 ±0·3 (4)	43 ±2·7 (5)	3,060 (1)	*
Methoxypropyl	126 ±7 (7)	1,180 ±69 (8)	*	*
Butyrylmethyl	1,130 ±49 (4)	*	*	*

The equipotent molar ratios for the compounds relative to acetylcholine are shown in Table 5. There are fewer results than was hoped for because many of the compounds were only partial agonists and some were purely antagonists. The effects of the replacement of the methyl by ethyl groups in acetylcholine were studied by Holton & Ing (1949), and our results agree with theirs except that we found (2-acetoxyethyl)triethylammonium to be much more active than they did.

TABLE 6

EFFECTS OF CONSTITUTION OF ONIUM GROUP IN $RN^+R^1R^2R^3$ ON EFFICACY

Affinity ratios for the members of the acetoxyethyl series are available either from the results with the diphenylacetoxyethyl series or from the benziloyloxyethyl series; the efficacy ratios calculated from the latter are shown in parentheses. An asterisk indicates that the compound is a partial agonist or antagonist. If the ratio e/e_a is greater than one, the compound has a lower efficacy than the trimethylammonium analogue

	Efficacy ratio (e/e_a) for the onium group ($-N^+R^1R^2R^3$)			
Series	N^+Me_3	N^+Me_2Et	N^+MeEt_2	N^+Et_3
Acetoxyethyl	1·0 (1·0)	8·5 (8·2)	790 (990)	510 (380)
Ethoxyethyl	1·0	8·1	540	*
Methoxypropyl	1·0	29	*	*

Table 6 shows the effects of the constitution of the onium group on the efficacy, calculated from these equipotent molar ratios and the affinity ratios shown in Table 3. Table 7 shows the equivalent values for the effects of the group R on efficacy, obtained from the equipotent molar ratios and the appropriate affinity ratios shown in Table 4.

TABLE 7

EFFECTS OF CONSTITUTION OF THE GROUP R IN $RN^+R^1R^2R^3$ ON EFFICACY

An asterisk indicates that the compound is a partial agonist or antagonist. If the ratio e/e_a is greater than one, the compound has a lower efficacy than the acetoxyethyl compound with the same onium group

Onium group ($-N^+R^1R^2R^3$)	Efficacy ratio (e/e_a) for series			
	Acetoxyethyl	Ethoxyethyl	Methoxypropyl	Butyrylmethyl
N^+Me_3	1·0	1·8	97	120
N^+Me_2Et	1·0	1·7	340	*
N^+MeEt_2	1·0	1·9	*	*

DISCUSSION

Our attempts to assess the effects of chemical structure on efficacy involve deductions made from measurements of antagonist and agonist activity: for these deductions to have any validity it is most important that the experimental estimates should really indicate the affinity of the antagonists for, and the activity of the agonists at, the postganglionic acetylcholine receptors.

The presence of hexamethonium throughout the experiment makes it unlikely that the results are complicated by actions at ganglia and it also seems unlikely that either histamine receptors or 5-hydroxytryptamine receptors are involved. The compounds could be affecting acetylcholinesterases, but this is not likely for the antagonists because Blaschko, Chou & Wajda (1947) found that compounds such as benziloylcholine and lachesine did not antagonize the hydrolysis of acetylcholine by the enzymes of dog caudate nucleus, even in concentrations as high as 6×10^{-3} M, and were not themselves hydrolysed. The activity of those agonists which are esters (the members of the acetoxyethyl series) could also be modified by cholinesterases. Though Holton & Ing (1949) found that all the members of this series were hydrolysed at comparable rates by the acetylcholinesterase of dog caudate nucleus, in our experiments the most active compound, acetylcholine, was necessarily used in much lower concentrations than the other esters and so a greater proportion of it could have been being destroyed. It seems unlikely, however, that any effects of this kind could be affecting the potency estimates because, if they were, the antagonism of acetylcholine would not appear competitive and the graph in Fig. 4 would not be linear.

From the results in Table 3 it will be seen that the effects of the constitution of the onium group on the adsorbability of the members of these series of antagonists show the same general trends, namely an increase in affinity with the replacement of one or two methyl by ethyl groups and a partial decline in affinity when the last methyl group is replaced. There are, however, some differences between the series; though these differences are small, some are statistically significant. For instance, the replacement of one or two methyl by ethyl groups leads to smaller increases in the affinity of the members of the diphenylethoxyethyl series than in the others. In the diphenylacetoxyethyl and diphenylbutyrylmethyl series, the member with only one methyl group replaced by an ethyl group has a significantly greater affinity than the member with two methyl groups replaced by ethyl groups;

in the other three series (diphenylethoxyethyl, benziloyloxyethyl and diphenylmethoxypropyl) there is no significant difference between these members. The decline in affinity when the last methyl group is replaced by an ethyl group is smallest in the diphenylacetoxyethyl series and largest in the diphenylmethoxypropyl and diphenylbutyrylmethyl series. The affinity of the triethylammonium compound in the diphenylbutyrylmethyl series is actually lower than that of the trimethylammonium compound. The low adsorbability of this compound could well be due to steric interaction between the ethyl groups packed round the quaternary nitrogen atom and the nearby carboxyl oxygen atom ; the interference between the third ethyl group and the carbonyl group which restricts the flexibility of the molecule can easily be seen in molecular models.

The size of the differences between the free energies of adsorption of the trimethylammonium and ethyldimethylammonium or diethylmethylammonium compounds (around 0.6 kcal) suggests that the increased affinity is due to Van der Waal's binding of an additional methylene group, but any further increase in the size of the onium group does not lead to further binding and may even reduce it.

When the effects of the constitution of the group R on the adsorbability are considered (Table 4) these appear to be much more independent of the constitution of the onium group than the effects of the constitution of the onium group are independent of the nature of R. This more consistent behaviour can be ascribed to the much bigger differences in affinity which are being measured, consequently minor variations in affinity tend to be less obvious than when the effects of the constitution of the onium group on affinity were being considered.

The benzilic acid esters, which are more active than the diphenylacetoxyethyl compounds (Ford-Moore & Ing, 1947), were all found in our experiments to have about twenty-times the affinity for the receptors ; the diphenylmethoxypropyl compounds have about the same affinity as the diphenylacetoxyethyl compounds ; the diphenylethoxyethyl and diphenylbutyrylmethyl compounds have about one-tenth of the affinity. For the benzilic acid esters the difference in affinity corresponds to a difference in the free energy of adsorption of about 2 kcal and strongly suggests that there is hydrogen bonding between the hydroxyl group of the benziloyl group and the receptor. The low affinity of the diphenylethoxyethyl compounds, in which the carbonyl group of the ester link in the diphenylacetoxyethyl compounds is replaced by a methylene group, indicates the importance of this carbonyl group for binding to the receptors. The difference between the free energy of adsorption of the diphenylacetoxyethyl and diphenylethoxyethyl compounds is between 1.1 and 1.3 kcal, and might indicate that there is an electrostatic interaction between the partially positive carbon and/or partially negative oxygen atoms and appropriately charged atoms on the receptor. The members of the diphenylmethoxypropyl series, however, have only slightly less affinity than the diphenylacetoxyethyl compounds so it seems possible that the link to the receptor may depend upon the lone pairs of electrons on the oxygen atom, rather than on the presence of a partially positive carbon atom. It is unfortunate that we have not been able to obtain the 4-keto compounds, $Ph_2.CH.CO.CH_2.CH_2.CH_2.N^+Me_3$ etc. The low affinity of the members of the diphenylethoxyethyl and diphenylbutyrylmethyl series suggests that neither

a 2-keto group nor a 3-ether oxygen atom can confer affinity on the molecule; it would even be expected that the 5,5-diphenylpentyl compounds would have similar affinity.

In Table 4, as in Table 3, there are irregularities and these involve the same compounds. In the diphenylmethoxypropyl series these are particularly noticeable because the members have affinities similar to those of the diphenylacetoxyethyl compounds and the variation involves a change of sign. The unexpectedly low affinity of the triethylammonium member of the diphenylbutyrylmethyl series is again apparent though it is less conspicuous because it does not involve a change of sign as it did in Table 3.

The results which we have obtained seem to show that our original postulate is reasonable. The effects on affinity of altering the constitution of the onium group are not very dependent upon the nature of the group R and likewise the effect on affinity of altering the group R is not very dependent upon the constitution of the onium group. In general the two effects are additive and it is therefore likely that a further change in R, namely the removal of the two phenyl groups, would also alter affinity by a factor largely independent of the other groups in the molecule. In consequence the ratios of affinity for the members of the series of agonists should be approximately the same as in the corresponding series of antagonists. It is therefore possible to calculate the effects of changes in structure on efficacy from the observed activity of these agonists. Irregularities may well occur in the affinities of the members of the series of agonists, comparable with the irregularities observed in the series of antagonists, but the changes in activity are so great that the effects of changes in structure on efficacy can clearly be seen.

The effect of replacing methyl by ethyl groups is to reduce efficacy (Table 6). In both the acetoxyethyl and ethoxyethyl series the replacement of one methyl by an ethyl group leads to about an eight-fold decline in efficacy and the replacement of a second methyl by a second ethyl group leads to a further marked drop. This effect is clearly seen for the acetoxyethyl compounds whether the efficacy ratios are calculated using the affinity ratios obtained from the diphenylacetoxyethyl or from the benziloyloxyethyl series. The apparent rise in efficacy on the replacement of the last methyl group in acetylcholine by an ethyl group is puzzling and is inconsistent with the decline in efficacy which is observed in all the other series, in which the triethylammonium compounds are either partial agonists or antagonists. It is all the more disturbing, therefore, that our estimate of the activity of (2-acetoxyethyl) triethylammonium was much higher than that of Holton & Ing (1949).

When the effect of the constitution of the group R of the molecule on efficacy is considered (Table 7), it seems clear that the 3-ether oxygen atom, as in the members of the ethoxyethyl series, is associated with a high degree of efficacy, which is particularly interesting in view of the low affinity of the corresponding antagonists (the diphenylethoxyethyl compounds). In contrast a 4-ether oxygen atom, as in the methoxypropyl series, or a 2-keto group, as in the butyrylmethyl series, is associated with only feeble efficacy.

The action of acetylcholine at the postganglionic receptors in the guinea-pig ileum, therefore, might be supposed to depend upon the presence of the 4-carbonyl

group (and presumably the onium group) for affinity and on the 3-ether oxygen atom and the trimethylammonium group for efficacy.

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. In some compounds, for example ($\gamma\gamma$-diphenylbutyryl)triethylammonium, the existence of such interaction is apparent and it is also possible that there is an interaction between the two phenyl groups and the ester or 3-ether group in the antagonists which would affect the efficacy ratios calculated for the agonists which lack the two phenyl groups. This possibility can only be investigated by the study of more series of antagonists, such as, for example, the di*cyclo*hexyl derivatives. Some information may also be obtained by measuring the affinity constants of those members of the "agonist" series (that is, without the two phenyl groups) which are actually antagonists or partial agonists.

The validity of this work and the relevance of the discussion would not be affected if it should be established that agonists owe their action to the speed with which they dissociate from the receptors, as in the "rate" theory of Paton (1961). As he points out, the equations relating the response to the concentration of drug are indistinguishable and what we have called the efficacy, e, would instead be k_2, the constant for the rate of separation of drug and receptor.

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