A PHARMACOLOGICAL STUDY OF COMPOUNDS RELATED TO ACETYLCHOLINE IN ORDER TO INVESTIGATE THE EFFECTS OF CHANGES IN CHEMICAL STRUCTURE ON AFFINITY FOR RECEPTORS AND THE EFFICACY OF THE DRUG-RECEPTOR COMPLEX

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INTRODUCTION

THEORIES OF DRUG ACTION

The idea that many drugs act by interaction with specific sites, or "receptors" in living tissues was first proposed by Langley (1878, 1905), but it is generally with Paul Ehrlich (1890) that this concept is associated. Ehrlich's work in immunochemistry led him to the idea that drugs act by combining with certain complementary areas of larger molecules, and that biological responses were the result of this combination. These areas he called "receptors", - "that combining group of the protoplasmic molecule to which a foreign group, when introduced, attaches itself".

The concept of receptors has flourished since the days of Ehrlich, and it was Clark (1926) and Gaddum (1937) who made the first attempts to investigate the situation quantitatively. Clark demonstrated that many drugs act at specific sites on cells by his calculations of the fraction of the surface area of frog ventricle cells which was covered by the molecules of a drug administered in sufficient concentration to produce a recognisable response. This he found to be of the order of 1/1000 for acetylcholine. Similar calculations with other

active drugs show that they cannot cover more than a tiny fraction of the cells in effective doses, while some drugs produce effects only when the dose given is sufficient to form a monomolecular layer over the whole cell surface. This indicates that drugs may act in one of two ways: either by involving a small fraction of the cell surface, or by covering a large area. The drugs which require to cover the whole cell to produce a response most likely act by some physico-chemical mechanism, while the action of the other type of drug seems likely to involve receptors or active sites on the cell surface.

Clark used a modified version of the Langmuir adsorption isotherm to account for the interaction of drug molecules with receptors,

$$K[A] = \frac{Y}{1-Y} \qquad (1)$$

where K is the affinity constant and is given by k_1/k_2, k_1 being the rate of association and k_2 the rate of dissociation: Y is the fraction of receptors occupied by the drug in a concentration $[A]$.

When 50% of the receptors are occupied, then

$$K = 1/[A] \qquad (2)$$

Clark, however, while testing this equation experimentally, made the assumption that the biological response was proportional to the fraction of receptors occupied, although he did mention that this assumption might not be valid.

Gaddum (1937) introduced theoretical equations for the situation in which both an active stimulating drug, or agonist, and an antagonist, (i.e. a drug which combines with receptors but elicits no biological response) were competing for the same receptors:

$$[A]K_A = \frac{Y}{1-Y} \quad (1 + [B]K_B) \qquad (3)$$

where $[A]$ and $[B]$ are the concentrations of agonist and antagonist respectively, K_A and K_B are the affinity constants of agonist and antagonist, and Y is the fraction of receptors occupied by the agonist. If no antagonist is present, ($[B] = 0$), then this equation reduces to $[A]K_A = \frac{Y}{1-Y}$ as before. If the response to a concentration $[A]$ of agonist in the presence

of a concentration $[B]$ of antagonist is the same as the response to a concentration $[a]$ of agonist acting alone, then

$$\frac{[A]}{[a]} = 1 + [B]K_B \qquad (4)$$

This is the basis of experiments designed to measure the affinity constants of antagonists. If the dose ratio, $([A] / [a])$ is 2, then

$$K_B = \frac{1}{[B]} \qquad (5)$$

it is also the basis of the pAx scale of measurement of drug antagonism which was introduced by Schild (1947), pAx being "the negative logarithm to the base 10 of the molar concentration of an antagonistic drug which will reduce the effect of a multiple dose (x) of an active drug to that of a single dose".

These basic ideas of Clark and Gaddum were more recently revised by Ariens (1954) and by Stephenson (1956). Previously, the relative activities of drugs were assumed to depend only on their binding to receptors: the more active the drug, the more strongly it was thought to be bound. Both Ariens and Stephenson

introduced another parameter of drug activity, the "intrinsic activity" (Ariens) or the "efficacy" (Stephenson), both parameters being a measure of the ability of the drug, once associated with the receptors, to contribute to the biological response. They differed, however, in the values which this intrinsic activity or efficacy could attain, Ariens' intrinsic activity having a maximum value of 1 for pure agonists, and the value 0 for antagonists, while Stephenson's efficacy could have any value from zero upwards. They also introduced the terms "partial agonist" (Stephenson) or "dualist" (Ariens) for those compounds which were capable of producing a biological response, but incapable of producing a maximum tissue response, (as obtained with a pure agonist), and which could, under certain conditions antagonise the effects of a pure agonist. These compounds have a low efficacy on both theories, and their antagonistic ability seems to be related to their occupation of receptors which could be occupied more profitably by a pure agonist. Ariens, however, retained one of Clarks' ideas, viz. that a maximum tissue response was produced only when all the receptors were occupied, and that the

response was therefore proportional to the fraction of receptors occupied. This idea was involved in the measurement of relative intrinsic activities, these being proportional to the maximum responses produced. Stephenson, however, suggested that all the receptors need not be occupied to produce a maximum tissue response, and indeed that perhaps occupation of a very small fraction could result in such a response. Thus there could be a large receptor reserve. This was demonstrated by Nickerson (1956) who showed the presence of a reserve of histamine receptors in the guinea-pig ileum, using GD-121 (N-1-naphthylmethyl-N-methyl-β-chloroethylamine) as an "irreversible" antagonist. He found that an irreversible blockade of histamine-induced responses could be produced by treatment of the tissue with GD-121 for 5 minutes. Increasing the concentration of the antagonist resulted in a shift of the dose-response curve of histamine two log. units along the agonist dose-axis without any significant change in its shape or in the height of the maximum response. Further treatment with the antagonist, however, produced a block which was unsurmountable. In a similar way, Ariens and van Rossum (1962) showed that GD-121 would antagonise acetylcholine receptors, and again the log. dose-response curves, determined in the presence of increasing

concentrations of antagonist shifted along the log. dose axis before any reduction in the maximum response occurred. If a maximum response is usually produced by the tissue only when all the receptors are occupied, non-competitive "irreversible" antagonists should theoretically reduce the maximum response at very low concentrations.

Stephenson introduced the term "biological stimulus", S, to avoid making any assumptions about the relationship between the fraction of receptors occupied and the biological response. S is given by

$$S = eY \qquad (6)$$

where e is the efficacy, and Y is the fraction of receptors occupied. While no assumption is made about the relationship between S and the effect produced, it is assumed that equal values of S will produce equal responses. As an arbitrary standard, the value of S is given as unity for a 50% response.

The affinity constant of an active agonist cannot be determined by measuring the 50% response if only a small fraction of the receptor is occupied, but the affinity constants of partial agonists can be determined from "addition" experiments devised by Stephenson. The method and theory are explained later in the Experimental

Section.

All the theories so far discussed have been based on the assumption that the biological response is some function of the number of receptors occupied by the drug molecules, and all assume that the response will be maintained as long as the drug molecules and receptors are associated. However, Paton (1961) has suggested that the stimulus may depend, not on actual occupation of receptors, but on the rate of dissociation of drug molecules from receptors. Thus an active agonist would require to have a high dissociation rate constant, while antagonists would be those compounds whose rate of dissociation was low, and which would therefore remain in contact with the receptors for a relatively long period, thus reducing the probability of another molecule becoming associated with the receptors. The dissociation rate constant thus becomes analogous to the efficacy or intrinsic activity of the "occupation theory".

According to the "rate theory", the maximum effect of a drug should be seen immediately after its application to a tissue, at which time the maximum number of receptors would be available for combination; the response would then "fade" as the receptors become occupied, and less available for combination. This "fade"

is often not seen in biological responses unless an "auxotonic" lever system is used. This was introduced by Paton (1957) for guinea-pig ileum and has the effect of increasing the load as contraction of the muscle increases. The rate theory might be an explanation of the phenomenon of tachyphylaxis, i.e. the decline in responses obtained on repeated application of a certain concentration of drug to an isolated tissue or whole animal. On this theory, the receptors will be saturated with drug molecules after the initial application, and thus subsequent doses will have less chance of combining with the receptors. However, it has been shown that tachyphylaxis is not always specific: large doses of acetylcholine will render the guinea-pig ileum less sensitive to 5-hydroxytryptamine and histamine as well as to acetylcholine, and it is known that these substances act at different receptor sites.

Structure - Activity Relationships

The activity of chemical compounds on biological tissues is highly dependent on details of chemical structure, except for those compounds previously mentioned, whose actions are physico-chemical rather than chemical. Small changes in chemical structure, such as the introduction of methyl groups, or replacement of methyl groups by ethyl groups, or the choice of an optical or geometric isomer may affect activity quite markedly, both quantitatively and qualitatively.

Many investigations have been made into the effects of changes in chemical structure on the activities of a wide variety of compounds, and many conclusions drawn from the results obtained. Investigations of this nature on autonomic structures are further complicated by the varied actions of the natural transmitter substance, acetylcholine. Although this substance is responsible for the transmission of the nerve impulse across the neuromuscular junction, at the synapses of autonomic nerves of both the sympathetic and parasympathetic divisions, and at postganglionic parasympathetic nerve endings, yet the nature of the receptor sites must be different, since they require different compounds to

antagonise the actions of acetylcholine. The action of acetylcholine at the ganglia and neuromuscular junction was classified as "nicotinic" by Dale (1914), since its actions at these sites could be mimicked by nicotine, and were blocked by curare alkaloids. The action at postganglionic parasympathetic nerve endings was classified as "muscarinic" due to the similarity to the action produced by muscarine, these effects are blocked by atropine.

Investigations into the effects of chemical structure on the activity of drug molecules have nearly always taken the form of examination of the changes in activity, both quantitative and qualitative, which occur as one proceeds along a homologous series. Almost all compounds which have high activity in stimulating autonomic structures and the neuromuscular junction are characterised by the possession of a quaternary nitrogen atom containing three methyl groups, or of a tertiary nitrogen atom containing two methyl groups. Compounds which have antagonistic properties usually have larger alkyl groups attached to a quaternaryor tertiary nitrogen atom, as was found by Hunt and Renshaw (1933). They found that the ethers and thioethers of triethylammonium compounds

had no muscarinic or nicotinic stimulating action, but most had a "paralyzing" nicotinic action, the latter action indicated by a failure of nicotine and other stimulating compounds to produce a rise in blood pressure in an animal pretreated with the triethyl compounds.

Raventos (1937) studied the actions of four series of compounds related to tetramethylammonium (TMA) on the nicotinic receptors of the frog rectus and leech muscle preparations. In one series, $RNMe_3^+$, where R is a straight aliphatic side chain containing from 1 to 12 carbon atoms, his results indicated that the most active compound was the n-butyl member, which was more active than TMA itself. Maximum muscarinic activity, as demonstrated on the rat gut was found in the n-butyl and n-pentyl members, which were equiactive with TMA. Among the series R_4N^+, where R is an alkyl group from methyl to n-butyl, the only active stimulant compound was the tetramethyl member, but antagonism towards the actions of TMA at both muscarinic and nicotinic receptors was observed in the higher members, the intensity of antagonism tending to increase with increase in the size of the alkyl group. Progressive ethylation of TMA was found to lead to an increase in curariform activity on frog nerve-muscle

preparations, and to a decrease in muscarinic activity, as demonstrated on the rat intestine, as far as the $MeNEt_3^+$ compound: the fully ethylated member was antagonistic to TMA on the latter preparation.

Holton and Ing (1949) studied the effects of replacing the three methyl groups on the quaternary nitrogen of acetylcholine by ethyl groups, or by acetoxyethyl groups: these compounds were tested for their ability to lower the blood pressure of the anaesthetised cat, and on the guinea-pig ileum, frog heart and rectus abdominis preparations. They found that the stimulant activity of the compounds decreased with successive ethylation, such that the ethyldimethyl compound was less active than the trimethyl compound, the diethylmethyl member was much less active, and the triethyl member was antagonistic to acetylcholine on the frog heart. Replacement of two methyl groups by acetoxyethyl groups produced a compound with no stimulant nicotinic actions, but which antagonised the action of acetylcholine on the frog rectus. Their conclusions were that the effects of substitution were due to the removal of methyl groups from the receptive sites; since the arsonium and phosphonium analogues of acetylcholine were

also less active than the parent compound, this suggests that the greater atomic diameter of arsenic and phosphorus effectively increases the distance between methyl groups and the receptor.

The contribution of the acyl end of the acetylcholine molecule to its stimulant activity has been studied by Ing, Kordik and Tudor Williams (1952). They investigated the importance of the carbonyl and ether groups in a series of ketone compounds in which the position of the carbonyl group was changed relative to the quaternary nitrogen atom, and in a series of alkyl ethers in which the position of the ether oxygen was changed. They came to the conclusion that, in the ketone series, the 4-keto compound had maximum nicotinic acetylcholine activity, this activity decreasing through the 3-keto to the 2-keto compounds. Among the ethers, which exhibited both nicotinic and muscarinic activity, the O-ethylcholine was the most potent. These active compounds have the carbonyl and ether groups in the same positions as are found in the acetylcholine molecule. A comparison of furfuryltrimethylammonium and 5-methylfurfuryltrimethyl-ammonium showed that the latter compound was the more active, a result which is evidence in support of the

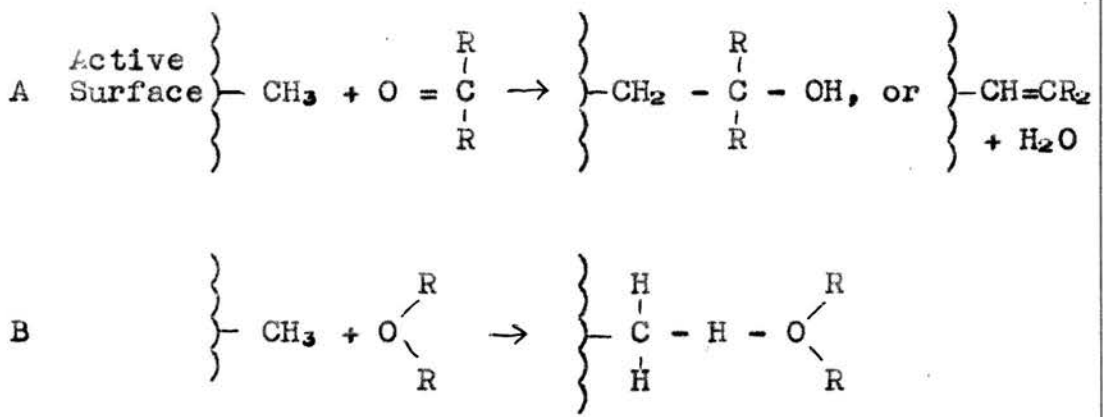
five atom chain rule. This hypothesis, proposed by Ing (1949), on the evidence of various investigators, suggested that maximum acetylcholine-like activity is found in compounds which have a quaternary nitrogen atom containing three methyl groups and a group of five atoms arranged in a straight chain. The rule was applicable to aromatic compounds also if the atoms in the ring were spatially orientated in such a manner that a five-atom chain were present. The idea was a modification and extension of the proposal of Pfeiffer (1948) that cholinergic activity was closely related to the possession by a molecule of three "prosthetic" groups, viz. $-\overset{+}{N}Me_3$, $\diagdown\!\!\diagup C = O$, and $-O-$.

Hey (1952) suggested that maximum nicotinic activity was found in molecules of the following general structure $R-O-CH_2CH_2\overset{+}{N}Me_3$, and that the electron density of the ether oxygen was important: he suggested that the nicotinic activity of such compounds would increase as the electron density decreased. To demonstrate this theory, Hey tested a number of phenyl ethers containing different substituents in the benzene ring (alkyl or halogen groups), for their effects on the blood pressure of anaesthetised cats, pretreated with atropine to abolish

any muscarinic activity. His expectations that the halogen substituted compounds should be more active, due to their electron attractive properties, and that the alkyl derivatives should be less active, due to electron repulsive properties, were shown to be correct. Hey also tested a number of esters related to acetylcholine, of the general type $R-C=O.O-CH_2CH_2\overset{+}{N}Me_3$, since resonance could be expected either between the group R and the carbonyl group, or between the ether oxygen and the carbonyl group. Activity in such a series should increase when the group R is such that it enters less readily into resonance with the carbonyl group, e.g. propionyl choline should be more active than acetylcholine: the results from this investigation were not so satisfactory as those from the phenyl ethers.

A similar investigation was carried out by Ormerod (1956) who studied a series of benzoylcholine derivatives in an attempt to gain evidence for a proposal of carbonyl receptors at cholinergic synapses. His compounds were, like Hey's, substituted benzene derivatives, the substituents selected being those which had either electron attractive or repulsive qualities. He found a correlation between the substituent constant and the stimulating

potency of the compounds at the cat superior cervical ganglion, but at neither the frog rectus preparation nor the rat phrenic nerve-diaphragm preparation. His findings, however, were completely opposed to those of Hey; i.e. the activity of Ormerod's compounds decreased as the electron attraction of the substituents increased. Ormerod attempted to explain this discrepancy by the proposal of a hypothetical carbonyl and ether receptor at the ganglia.



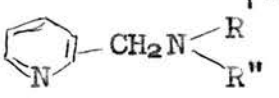
His proposed drug-receptor combinations are illustrated diagrammatically above: the receptor is visualised as an activated methyl group. In A, the ganglion surface acts as a undeophilic agent, and will condense less readily with an electron deficiency in the C = O group created by an electron attractive group in the benzene ring. In B, a hydrogen bond is proposed for

ethers. This hydrogen bond will be strongest when the hydrogen atom is acidic, as it would be in an activated methyl group, and when the oxygen atom is most basic, i.e. when it has an electron deficiency as it would have with an electron attractive substituent. Results similar to those of Ormerod were obtained by Wong and Long (1962) in their experiments on the activity of phenalkyl and phenacyl trimethylamines. In the substituted phenacyl compounds, nicotinic activity decreased as the electron density on the carbonyl group decreased.

Barlow and Hamilton (1962) attempted to find out which parts of the nicotine molecule were associated with activity by studying compounds related to it. By comparing the relative activities of nicotine and nicotine monomethiodide at different pH's, they obtained results which suggested that the cation was the active form rather than the free base. From an extension of Hey's theory of the activity of the phenyl ethers of choline, they suggested that the partially positively charged α and γ carbon atoms in the pyridine ring might be important for activity, and argued that the β compounds should be more active than their α or γ analogues. In general they found this to be true, and the quaternary compounds

β-pyridylmethyltrimethylammonium and β-pyridylethyltrimethylammonium were, in fact, highly active on the chick biventer-cervicis, rat phrenic nerve-diaphragm, and cat superior cervical ganglion preparations. The latter was more than 10 times as active as nicotine on some preparations. On the rat diaphragm, however, the compound was the most active but there is some doubt as to whether this compound produces block on this preparation in the same way as the others.

These results can also be interpreted by supposing that it is a partially negatively charged group which is important for activity (Sekul and Holland 1961): this would be present on the pyridine nitrogen atom.

A different approach to the study of substances with nicotine-like acetylcholine activity was made by Erdtman et al. (1963), and by Haglid and Wellings (1963 a,b,c). Erdtman and his colleagues prepared the series of compounds which would result from the opening of the pyrrolidine ring of the nicotine molecule at the five positions possible. A second series of compounds was prepared, based on the model  , in which R' and R'' were either H, CH_3 or C_2H_5.

In the first series, maximum nicotinic activity was found in the pyridylbutyldimethylamino compound, and maximum muscarinic activity was found in this compound and also in the pyridyl(n-propylmethyl)N methyl compound.

In the other series, the most active member with both muscarinic and nicotine acetylcholine-like activity (on rabbit jejunum or guinea-pig ileum, and on cat blood pressure respectively) was β-pyridylmethyldimethylammonium. The conclusions drawn from this work were that the presence of an alkyl group attached to the carbon atom joining the pyridine ring and the nitrogen atom caused a reduction in the physiological activity of the compounds. No mention was made of any tests done on the intestinal preparations in the presence of a ganglion blocking agent, so the nature of the receptors involved is undecided: another conclusion drawn was that for maximum stimulant activity in the β-pyridylmethylamino compounds, one of the groups R must be CH_3 and the other not larger than C_2H_5.

Haglid and Wellings continued this line of investigation by varying the nature of the aromatic ring in the model $Ar-CH_2-N\begin{matrix}R'\\ \diagdown\\ R''\end{matrix}$, at the same time as changing the nature of the groups R' and R". The ring structures

investigated were β-pyridyl, benzyl, thiophene, furan, pyrrole and indole. Of these, the β-pyridyl ring conferred most activity on the molecule at both nicotinic and muscarinic receptors, and the most active form of $N\begin{smallmatrix}R'\\R''\end{smallmatrix}$ was the pyrrolidyl grouping. Further work on pyridine derivatives suggested that the β-substituted compounds were most active on both types of receptor. When one methylene group bridged the ring and nitrogen atom: at nicotinic receptors especially, the α-substituted compounds were most active when the bridge consisted of two methylene groups.

Analysis of "Activity": estimation of affinity and efficacy.

The experiments described so far have been designed to investigate the activity of biologically active compounds, and, with the exception of the work done by Ariens, no attempt was made to differentiate this activity in any way.

Measurement of the affinity constants of antagonist compounds can be made directly by observation of the concentration required to necessitate increasing the agonist concentration to produce responses equal to

doses of agonist acting alone. However, it is not possible to measure the affinity constants of pure agonists, since the relationship $K = \frac{Y}{(1-Y)[A]}$ is not valid in this case when only a fraction of the available receptors may require to be occupied to produce a maximum tissue response. Replacement of methyl groups by ethyl groups in the acetylcholine leads to a decline in agonist activity as shown by Holton and Ing (1949). However, Ing, Dawes and Wajda (1945) showed that in a series of esters of benzilic acid, which are acetylcholine antagonists, the compounds with ethyl groups in the cationic head were actually more active antagonists than benziloylcholine, and Stephenson (1956) has suggested, on the basis of these experiments, that the decline in activity in the agonist series was due to a decline in efficacy, and not to a decline in affinity. This suggestion implies that the effects on affinity of replacing methyl groups by ethyl are the same in both the acetyl and benziloyl esters.

To test this assumption Barlow, Scott and Stephenson (1963) prepared two series of compounds, one of agonists and the other of antagonists, and measured the affinity constants of the antagonists and the activity of the

agonists. The compounds studied were $RNMe_3^+$, $RNMe_2Et^+$, $RNMeEt_2^+$, $RNEt_3^+$ and $R'NMe_3^+$, $R'NMe_2Et^+$, $R'NMeEt_2^+$, $R'NEt_3^+$ 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 butyryl-methyl group. The difference between the two groups is that the first contain a diphenylmethyl group, (or a benziloyl group) in place of a methyl group. The first group of compounds are antagonists of acetylcholine, whereas most of the second group act like acetylcholine.

If the affinity constants of the antagonist series $RNMe_3^+$, $RNMe_2Et^+$, $RNMeEt_2^+$, $RNEt_3^+$ are denoted by K, K_a, K_b, K_c respectively, and those for the corresponding agonist series $R'NMe_3^+$, $R'NMe_2Et^+$, $R'NMeEt_2^+$, $R'NEt_3^+$ are K', K'a, K'b, K'c, then the relationship between the affinity constants and the free energy change on adsorption (ΔF) is given by the theory of Arrhenius,

$$\Delta F = -RT \log_e K$$

$$\text{or } \log_{10} K = -\Delta F/2.3RT.$$

The assumption that the effects on affinity of replacing methyl by ethyl groups are the same in both the agonist and antagonist series implies that the change in free

energy of adsorption depends only on substitution in the onium group, and that contributions from the groups R and R' should be unaffected by such substitution. If then the free energy of adsorption for $RNMe_3^+$ is ΔF, and for $R'NMe_3^+$ is $\Delta F'$, then the free energies of adsorption are given by,

$RNMe_2Et^+$	$= \Delta F + a$	$R'NMe_2Et^+$	$= \Delta F' + a$
$RNMeEt_2^+$	$= \Delta F + b$	$R'NMeEt_2^+$	$= \Delta F' + b$
$RNEt_3^+$	$= \Delta F + c$	$R'NEt_3^+$	$= \Delta F' + c$

where a, b and c are the free energy changes for the substitution of one, two and three ethyl groups respectively. Since,

$$\log K_a = (-\Delta F + a)/2.3RT$$

$$\text{then } \log\left(\frac{K_a}{K}\right) = -a/2.3RT.$$

This value should be constant, independent of the nature of the group R. This ratio should also be the same as $\log (K'a/K')$ for the appropriate agonists. If the equipotent molar ratio for $R'NMe_2Et^+$ relative to $R'NMe_3^+$ is n, that is, n molecules of the former produce the same response as one molecule of the latter, then the biological stimulus causing such a response should be the same. According to Stephenson (1956) the value of

the stimulus is given by $S = eY$, where e is the efficacy and Y the receptor occupancy, and as derived previously (page),

$$eY = eK'A' \quad \text{(for the first agonist)}$$

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

The ratio $A'_a/A' = n$, and thus

$$e/ea = n\, K'_a/K'$$

This ratio of the efficacies can be calculated since the ratio K'_a/K' has been determined from the appropriate members of the antagonist series. The determinations of the affinity constants of the five series of antagonists showed, in general, the same pattern. This is an increase in affinity with replacement of one or two methyl by ethyl groups, and a partial decline in affinity when the third methyl group is replaced, although one or two of the results showed statistically significant differences between series. The calculations of the sizes of the differences in free energy changes seemed to suggest that the increases in affinity are due to Van der Waal's binding of an additional methylene group. A consideration of the effects of the nature of the group R on affinity suggests that these are more

independent of the nature of the onium group than the effects of the constitution of the onium group are independent of the constitution of R. This may be due to the larger differences in affinity which were measured, with the consequence that small variations in affinity are less obvious. The difference in the free energy of adsorption of the benzilic esters, which were about twenty times as active as the diphenylacetoxyethyl esters, suggested the possibility of there being hydrogen bonding between the hydroxyl group of the benziloyl group and the receptor. The diphenylethoxyethyl compounds had about one-tenth the activity of the diphenylacetoxy-ethyl series, and this may indicate that the carbonyl group of the ester linkage in the latter group is important for binding to receptors: the change of free energy measurements between these members suggests that there may be some electrostatic interaction between either the partial positive charge on the carbon atom, or the partially negatively charged oxygen, with appropriately charged atoms on the receptor.

In the agonist group of compounds, the effect of replacing methyl groups by ethyl groups is to reduce efficacy. In both the acetoxyethyl and ethoxyethyl

series, replacement of one methyl by ethyl group reduced efficacy by a factor of about eight, while replacement of a second methyl group reduced efficacy about five- to eight-hundred fold. The replacement of all three methyl groups by ethyl groups in acetylcholine led to a slight increase in efficacy, which is inconsistent with the decline in efficacy seen in other series, and is difficult to explain.

Examination of the effects of the nature of the group R on efficacy seems to show that the 3-ether oxygen atom, as in the ethoxyethyl series, is associated with a high degree of efficacy, although the corresponding antagonist series, (diphenylethoxyethyl compounds) have a low affinity. A 4-ether oxygen atom, however, as in the methoxypropyl series, or a 2-keto group as in the butyrylmethyl series, seem to be associated with a low efficacy. From these results, the authors suggest that the action of acetylcholine at postganglionic receptors in the guinea-pig ileum might depend on the presence of the 4-carbonyl group (and the onium group), for affinity, and on the 3-ether oxygen and trimethylammonium group for efficacy.

The validity of the assumptions made by Barlow and his colleagues, viz. that the affinity changes in agonists and antagonists are related to the degree of ethylation of the cationic head, was challenged by Burgen (1965). The carbon analogue of acetylcholine, 3,3 dimethylbutylacetate, is only a feeble agonist on the guinea pig ileum and if the difference in activity is due only to differences in affinity, Burgen calculated that the charge on the nitrogen atom in acetylcholine contributed 5 Kcals/mole to the free energy of adsorption. The carbon analogue of benziloylcholine on the other hand, is quite a potent antagonist of acetylcholine, and has about one-fourteenth of the activity of benziloylcholine, indicating that in this compound the charge on the nitrogen atom contributes only 1.6 Kcals/mole to the free energy of adsorption. The charged nitrogen atom, therefore, is further from the charged group on the receptor with which it interacts in the latter than it is in acetylcholine, and Burgen suggests that the ability of a compound to act as an agonist may depend on its ability to come close to the charged group in the receptor. Whether this is true or not, the effects of

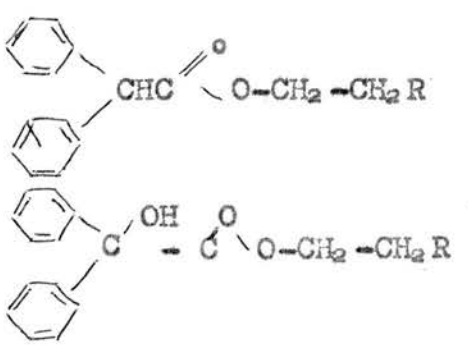
changes in the onium group on affinity will be much greater in the compounds with high affinity than in those with low affinity in these circumstances.

These ideas arise from observations with only two pairs of compounds, and it is important to know whether they apply to others.

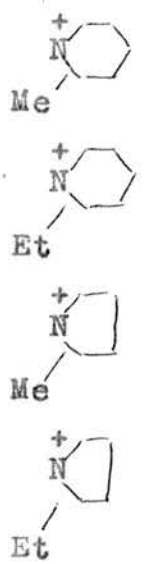
AIM OF WORK

1. We wished to check the reproducibility of measurements of af inity constants of acetylcholine antagonists. Accordingly we have repeated the experiments performed by K. A. Scott on the isolated guinea pig ileum preparation with diphenylacetoxyethyl and benziloyloxyethyl compounds.

2. As the results were found to be consistent, we extended these two series of antagonists to see whether changes in chemical structure invariably had the same effects on affinity in both series. The new compounds tested were:



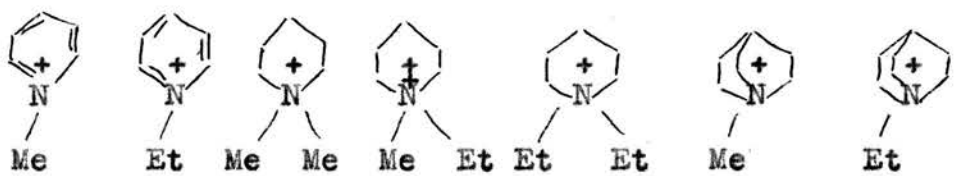
R =

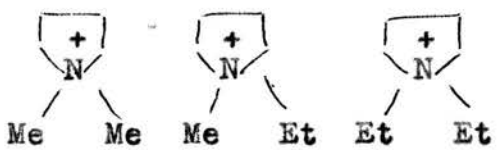


3. We wished to see whether we could obtain information about relationships between chemical structure and efficacy at nicotine sensitive acetylcholine receptors, by studying series of agonists and antagonists of acetylcholine and nicotine on the frog rectus preparation. To do this, we proposed to test the following compounds, measuring the affinity constants of the antagonists, and the relative activities of the agonists:

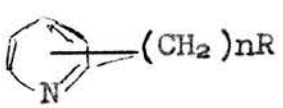
(i) Analogues of Tetramethylammonium

NMe_4^+, NMe_3Et^+, $NMe_2Et_2^+$ $NMeEt_3^+$ NEt_4^+





(ii) Analogues of β-pyridyl-methyl-trimethyl-ammonium



n = 1,2,3.

R = NMe_2 NMe_3^+

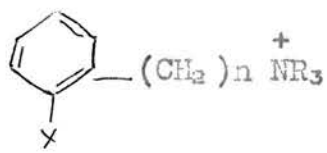
NMeEt NMe_2Et^+

NEt_2 $NMeEt_2^+$

$NnPr_2$ NEt_3^+

$NnBu_2$

(iii) Analogues of Phenyl-alkyl-trimethyl-ammonium.



$R = \begin{matrix} Me \\ Et \end{matrix}$, $n = 0\text{-}5$,

$x = H, Cl, NO_2$.

4. Because we thought that the biological properties of the compounds might depend, at least to some extent on the size of the cationic part of the molecule, we have attempted to obtain information about this by measuring the electrical conductivity of some of them.

EXPERIMENTAL and METHODS

I. PREPARATIONS

1. Frog Rectus Abdominis Preparation

Male American Leopard frogs (Rana pipiens) were killed and the rectus abdominis muscle was dissected from the pelvic girdle to its insertion in the cartilage of the pectoral girdle. The muscle was then divided longitudinally into two halves, each of which was used in a separate experiment. The strips of muscle were tied with a loop of thread at each end, and suspended in an organ bath containing Clark's Ringer at room temperature and gassed with atmospheric air from a Fairey aerator. Recordings of the contractions of the muscle were made by a gimbal lever writing on the smoked drum of a kymograph, the load being 1-2gm depending on the size of tissue. The Ringer solution had the following composition:

NaCl	6.5g/L
KCl	0.14g/L
$CaCl_2$	0.120g/L
NaH_2HO_4	0.005g/L
$NaHCO_3$	0.4g/L

2. Guinea Pig Ileum Preparation

Guinea pigs of either sex, weighing between 150gm and 250gm, were starved for a period of 12 to 18 hours preceeding the experiment. They were killed by a blow on the head and bled out. The abdomen was opened and the terminal portion of the ileum was carefully dissected out,

excluding the region containing the large Peyer's patch immediately adjacent to the junction with the caecum. The ileum was dissected free from the peritoneal membrane great care being taken to avoid any stretching of the tissue, and then placed in Tyrode solution previously warmed to 37°C. Warm Tyrode was found to facilitate the irrigation of the intestinal lumen which was occasionally necessary. If the tissue was placed in cold Tyrode solution, this usually resulted in a contraction of the muscle, especially the circular layer, with a resultant hindrance to fluid flow.

Care was also taken during the washing stage not to exceed pressures of more than 3 to 4cm of water because this distends the tissue and tends to induce spontaneous contractions.

Lengths of 3-4cm were then tied at each end, making sure that the lumen remained patent, and suspended in Tyrode solution at 35°C. Contractions were recorded on smoked kymograph paper, using an isotomic frontal writing lever with a load of 0.5gm. Hexamethonium bromide was added to the Tyrode solution in a concentration of 2.76×10^{-4}M to reduce spontaneous rhythm, and to ensure that the action of compounds under test was at the postganglionic parasympathetic nerve endings. The constitution of the Tyrode solution was:-

NaCl	8g/L
KCl	0.20g/L
$CaCl_2$	0.201g/L

NaH_2PO4	0.05g/L
MgSO4	0.127g/L
$NaHCO_3$	1.0g/L
Glucose	1.0g/L.

II METHODS

The experiments (on both tissues) were designed to measure changes in activity (agonist or antagonist activity) resulting from changes in chemical structure at both "muscarinic" and "nicotinic" acetylcholine receptors. With a few exceptions, all the experiments were performed using automatic apparatus.

Agonists

The automatic apparatus consisted of one machine capable of adding drug solutions either singly, or in the order of Latin squares, three different forms of which were programmed into the machine. This was used in all guinea pig ileum experiments which were based on a ninety second cycle, the drug being in contact with the tissue for 15 seconds, then washed out twice with Tyrode solution by overflow: the overflow method was preferred to drainage of the organ bath and refilling, since the latter method exposes the tissue to air and a lower temperature, and also causes artefacts in the responses written on the smoked kymograph paper. Preliminary experiments were performed by adding the test compound to the organ bath from a pipette in order

to determine those concentrations which would be necessary to produce responses which lay between 25-75% of the tissue maximal response. The standard agonist, either acetylcholine or carbachol, was added in a similar way and log.dose - response curves for the test compound and for standard agonist were plotted and compared. These were nearly always found to be parallel (for true agonists) and thus results from a 2+2 assay should be valid. The four different agonist solutions were added in the order of three Latin squares as mentioned above, the actual order being,

ABCD	DABC	DCAB
BDAC	ACDB	CBDA
CADB	BDCA	ADBC
DCBA	CBAD	BACD.

This dose order is so arranged that each dose follows every other dose the same number of times, and thus it was hoped that the effects of one dose upon another would not be likely to bias the results. Two machines were used for experiments on the frog rectus preparation: each was capable of adding one of three drug solutions either singly, or in a predetermined order (A,B,C,A,B, C etc., or A,A,B,B,C,C, etc.) while one was also programmed to add four drugs in the following order:-

A	B	C	D
A	D	C	B
C	D	A	B
C	B	A	D.

If A and C are low (high) doses of standard and test compounds, and B and D are high (low) doses of standard and test compounds, then this form will alternate high and low doses. This design was found to be necessary for the rectus preparation because of the inhibiting effect of a large contraction on a following large contraction. Thus 2+2 assays could be performed on this machine following similar principles as for the ileum experiments. Bracketing assays (2+1 assays) could be performed on the other machine which was not programmed to perform 2+2 assays.

The standard agonists used for comparison in all assays were either acetylcholine iodide or carbachol chloride on the ileum preparation, and tetramethylammonium iodide or β-pyridylmethyltrimethylammonium bromide on the frog rectus preparation. The use of nicotine as standard agonist for the rectus preparation was abandoned because of the excessively long time necessary for the tissue to relax after an effective dose. Several other compounds tested, particularly tertiary amino compounds, were also difficult to wash

out of the tissue, and the length of the cycle of operations had to be lengthened appreciably. A ten minute cycle with a drug contact time of 1½ minutes was sufficient for most of the quaternary ammonium compounds, but the tertiary compounds mentioned above, and also nicotine monomethiodide (which is permanently ionised), required a 30 minute cycle, with a 4½ minute drug contact time.

Antagonists

Antagonistic activity of pure antagonists was measured as the affinity constant of the compounds for the tissue receptors. The constants were obtained from the Gaddum equation (1937)

$$\frac{[A]}{[a]} = 1 + [B] K_B$$

Where the response to a concentration $[A]$ of pure agonist in the presence of a concentration $[B]$ of antagonist is equal to the response produced by a concentration $[a]$ of the agonist acting alone. K_B is the affinity constant of the antagonist.

Two different types of experiment were performed on the ileum.

a. A concentration of acetylcholine, (or carbachol) was applied to the tissue regularly (every 90 seconds for 15 seconds) until the responses became regular.

Two other concentrations of agonist were then selected so that there was a dose difference of 2x between each, and groups of five contractions were obtained with each concentration applied in a random order until the responses became regular. The Tyrode washout solution was then replaced by Tyrode containing a concentration of the antagonist which, from preliminary manual experiments, had been found to exhibit a convenient degree of antagonism. The agonist solutions were replaced by a solution of the same concentration of antagonist as in the washout, together with a higher concentration of agonist. This concentration was chosen so that the responses would lie close to the height of the responses produced by the middle concentration of agonist alone. This solution was then applied repeatedly to the ileum until five responses of the same height were recorded, when it was assumed that the antagonist had come into equilibrium with the receptors. A higher concentration of antagonist with a correspondingly higher concentration of agonist was then used, and the process repeated. Finally a third concentration of antagonist and agonist were used so as to obtain eventually responses in the presence of three concentrations of antagonist.

The concentrations of agonist, which would alone produce the same responses as the equilibrium responses

in the presence of the different antagonist concentrations, were then calculated either graphically or algebraically. From these concentrations, the affinity constants could be determined from equation (4). Since this equation holds only for competitive antagonism, then a graph of $(A/a - 1)$ against $\lceil B \rceil$ should give a straight line passing through the origin; this was used as a test of the nature of the antagonism.

The other method used to determine the affinity constants relied on the same Gaddum equation, but the technique was slightly different. Two concentrations of standard agonist which produced responses between 25-75% of the tissue maximum were applied alternately to the ileum until the responses became regular. These solutions were then replaced by higher concentrations of the agonist in the presence of an antagonist, the concentrations being those which from preliminary experiments were calculated to produce responses very similar to those obtained in the absence of antagonist. These concentrations were then applied alternately until regular responses were recorded. The responses were then compared as in a 2+2 assay, and the dose ratio produced by the antagonist concentration calculated. The value for K_B was then calculated as before. The advantage of this method is that it shows quite clearly

MEASUREMENT OF THE AFFINITY CONSTANTS OF ANTAGONISTS.

The figures immediately below the responses are the concentrations of β-pyridylmethyltrimethylammonium producing each response. At the arrows the antagonist (γ-pyridylethyldiethylammonium) was added in the concentrations shown.

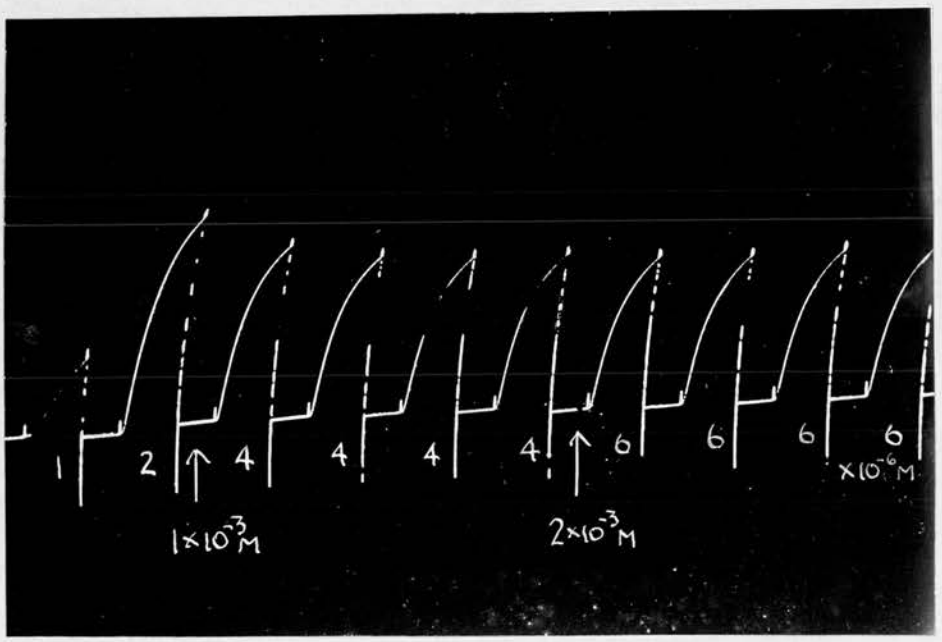
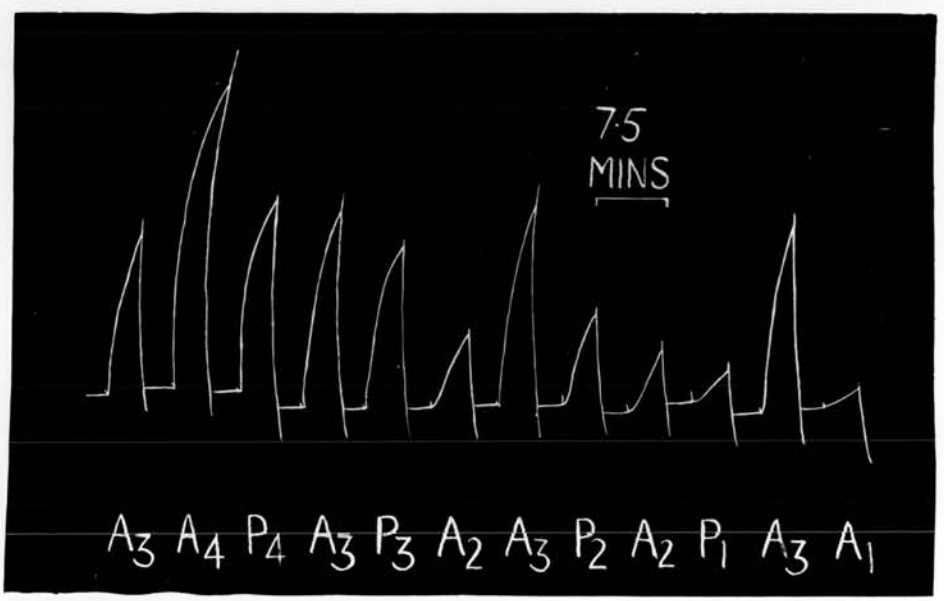


FIGURE IA.

MEASUREMENT OF THE AFFINITY CONSTANT OF A PARTIAL AGONIST. (RECIPROCAL PLOT METHOD).

Responses of the frog rectus to concentrations of tetramethylammonium, 1, 2, 4, and 8 x 10^{-5}M, (A_1, A_2, A_3, and A_4 respectively), and to methylpyridinium, 3, 6, 12, and 24 x 10^{-3}M, (P_1, P_2, P_3, and P_4 respectively). The compounds were left in contact with the tissue for $4\frac{1}{2}$ minutes. The drum was stopped for 23 minutes during the washing and recovery period so that the interval between doses was 30 minutes.



GRAPH OF 1/a AGAINST 1/p CALCULATED FROM THE RESULTS SHOWN IN THE TRACING.

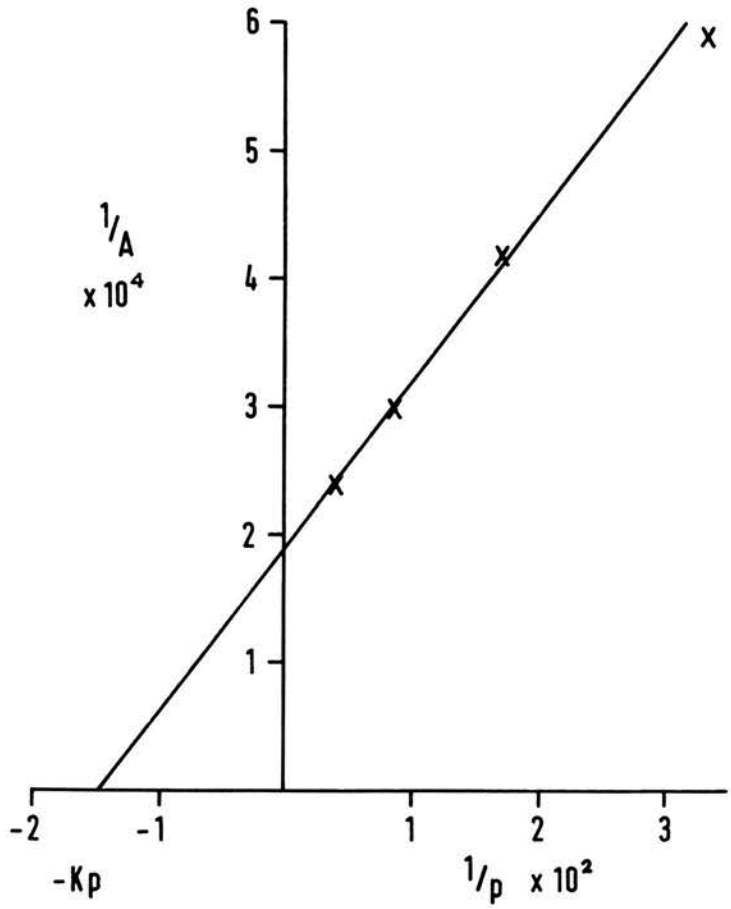
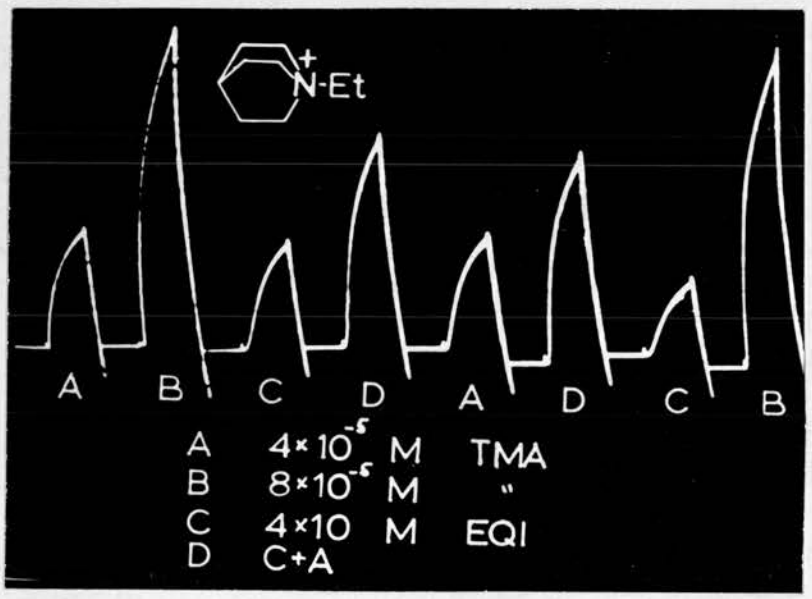


FIGURE IB.

MEASUREMENT OF THE AFFINITY CONSTANT OF A PARTIAL AGONIST (ADDITION METHOD).

Responses of the frog rectus to concentrations of tetramethylammonium and ethylquinuclidinium. The time cycle of events is identical with that described for Figure IA.



whether the slope of the dose response curve of the agonist has been altered by the antagonist, and may indicate whether or not the antagonism is competitive.

The experiments on the frog rectus preparation were essentially similar to those on the ileum, except that the initial doses of agonist alone were alternated singly, and not in groups of five, because of the length of the time cycle. Sometimes three doses of agonist were applied in the presence of the same concentration of antagonist in order to get a clearer picture of any change in the slope of the log.dose-response curves.

Partial Agonists

Some of the compounds with agonist activity were found to have dose-response slopes which were considerably shallower than those for the true agonist standard. In these instances, it was found that the maximum contraction produced by the drug was less than the tissue maximum, (or maximum for the true agonist). These compounds were therefore tested for partial agonist activity by one of two methods. The first of these is the addition method as devised by Stephenson (1956), which consists of finding a concentration, A_1, of true agonist, of efficacy e_A and affinity K_A, which is the equivalent of a dose, P, of partial agonist, with efficacy e_P and affinity K_P, and a concentration, A_2,

which produces the same response as P and A_3 acting together.

For an agonist in equilibrium with the receptors,

$$\begin{array}{ccc} \text{Agonist} + \text{Receptors} & & \text{Complex} \\ [A] + [1-Y] & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & Y \end{array} \quad \text{(see page 2)}$$

$$\text{and } K(A) = \frac{Y}{1-Y}$$

If the agonist is highly active, and Y is therefore small, 1-Y approximates to 1, and thus

$$Y = KA.$$

If the partial agonist occupies a fraction, x, of the receptors, then, according to Stephenson, the response is due to a stimulus (S)

$$S = e_P x$$

where e_P is the efficacy of the partial agonist. Now since the concentration P of partial agonist produces a response equal to $[A_1]$ of true agonist, then the stimuli must be equal. Thus,

$$S_1 = e_P x = e_A K_A [A_1]$$

Thus,

$$e_P x = e_A K_A [A_1]$$

The response to (P + A_3) is produced by a stimulus

$$S_2 = e_a K_A [A_2] = e_P x + e_A K_A [A_3] (1-x)$$

assuming that values of S are additive and that the active agonist occupies only a negligible proportion of receptors whereas the partial agonist occupies a significant proportion. Thus,

$$e_a K_a(A_2) = e_a K_a(A_1) + e_A K_A(A_2)\ (1-x)$$

and

$$1-x = \frac{A_2 - A_1}{A_3}$$

Values of x can be calculated for different concentrations of partial agonist and K_P calculated from

$$K_P . P. = \frac{x}{1-x.}$$

Experimentally the procedure was to choose two concentrations of true agonist which were expected to lie on the linear part of the log.dose-response curve. The concentration of partial agonist chosen was such as would produce a response a little higher than the smaller response to the true agonist. This concentration of partial agonist was then applied to the tissue together with sufficient of the true agonist to produce a response which was just smaller than the larger of the two true agonist responses. Thus the equivalents of P and $(P + A_3)$ could be calculated by interpolation from log. dose-response curve of the true agonist.

The second method of measuring the affinity constants of partial agonists was also developed by Stephenson.

This depends on the assumption that the partial agonist occupies a significant proportion of the receptors when producing a response, whereas the true agonist occupies only a small proportion of the receptors.

Thus if a concentration a of true agonist (of efficacy e_A and affinity constant K_A) produces the same response as a concentration P of partial agonist (of efficacy e_p and affinity K_p), then, again assuming that equal biological stimuli produce equal responses,

$$S = eY = \frac{e_A K_A a}{1+aK_A} = \frac{e_p K_p P}{1+PK_p}$$

As before $\frac{e_A K_A a}{1+aK_A}$ reduces to $e_A K_A a$ if only a very small proportion of receptors are occupied by the true agonist.

Thus, $$e_A K_A a = \frac{e_p K_p P}{1 + K_p P}$$

From this it follows that,

$$\frac{1}{a} = \frac{e_A K_A}{e_p K_p}\left(\frac{1}{P}\right) + \frac{e_A K_A}{e_p}$$

against $\frac{1}{P}$

and a graph of $\frac{1}{a}$ will produce a straight line with an intercept of $-K_p$ when $\frac{1}{a} = 0$. Experimentally this method consists of producing responses to graded doses of true agonist and of partial agonist, and finding the equivalents of the partial agonist concentrations by

interpolation from the log.dose-response curve of the true agonist. This was sometimes done in the form of a series of bracketed doses, or occasionally by performing a form of 2 + 2 assay; 2 responses to concentrations of the true agonist were matched with responses to 2 concentrations of partial agonist; the difference from a real 2 + 2 assay being of course the disparity in the concentration difference between low and high concentrations of the same drug.

The values for $\frac{1}{a}$ and $\frac{1}{p}$ were then calculated and K_p determined either graphically (if more than two concentrations of partial agonist were used), or algebraically (if only two concentrations of partial agonist were tested).

Tests for Competitive Antagonism

If an agonist is producing responses in a tissue in the presence of two antagonists, the following equilibrium situations will arise:-

$A + R \rightleftharpoons AR$ A is the agonist

$B + R \rightleftharpoons BR$ B " " competitive antagonist

$C + R \rightleftharpoons CR$ C " " antagonist under test.

If the proportion of receptors occupied by the agonist $[AR]$ is given as y, and x and z are the proportion of receptors occupied by the antagonists $[BR]$ and $[CR]$ respectively, and if the respective affinity constants are K_A, K_B and K_C, $[A]$, $[B]$, $[C]$ being the applied concentrations, then:-

$$AK_A = \frac{y}{1-y-x-z}$$

$$BK_B = \frac{x}{1-y-x-z}$$

$$CK_C = \frac{z}{1-y-x-z}$$

$$\text{Thus, } \frac{y}{AK_A} = \frac{x}{BK_B} = \frac{z}{CK_C}$$

$$x = \frac{y\ BK_B}{AK_A}, \qquad z = \frac{y\ CK_C}{AK_A}$$

$$\text{and } AK_A = \frac{y}{1-y-y\frac{BK_B}{AK_A}-y\frac{CK_C}{AK_A}}$$

$$\text{so } AK_A - yAK_A - yBK_B - yCK_C = y$$

$$\text{i.e. } AK_A = \frac{y}{1-y}\ (1 + BK_B + CK_C)$$

If the response produced by a concentration a of agonist alone is the same as that produced by A_b of agonist in the presence of B, by A_c of agonist in the

presence of C, and by A_{bc} in the presence of both B and C,

$$\frac{A_b}{a} = 1 + BK_B; \quad \frac{A_c}{a} = 1 + CK_C; \quad \frac{A_{bc}}{a} = 1 + BK_B + CK_C$$

$$\text{so } \frac{A_{bc}}{A_b} = 1 + \frac{CK_C}{1 + BK_B} = 1 + \frac{\text{dose ratio for C alone} - 1}{\text{dose ratio for B alone}}$$

[i.e. dose ratio for BC = dose ratio for B + dose ratio for C-1, where all dose-ratios are calculated by comparison with the responses produced by the agonist alone (a)7.

Example: if B (alone) produces a dose ratio of 100 and if C (alone) produces a dose-ratio of 10,

$$\frac{A_{bc}}{A_b} = 1 + \frac{9}{100} = 1.09$$

If C is not a competitive antagonist and the substances A and B do not displace it from the receptors, the value of z will depend only upon the concentration of C so,

$$CK_C = \frac{z}{1 - z} \quad \text{and} \quad z = \frac{CK_C}{1 + CK_C}$$

$$\text{so } AK_A = \frac{y}{1 - y - z} = \frac{y}{1 - y - \dfrac{CK_C}{1 + CK_C}}$$

and as $AK_A = \dfrac{y}{1 - y}$, the dose ratio for C, $\dfrac{A_c}{a} = \dfrac{1-y}{1-y-\dfrac{CK_C}{1+CK_C}}$

When the truly competitive antagonist B is present as well,

$\frac{y}{AK_A} = \frac{x}{BK_B}$, as before, and $AK_A = \frac{y}{1 - y - x - z}$

$$= \frac{y}{1 - y - \frac{yBK_B}{AK_a} - \frac{CK_C}{1 + CK_C}}$$

$$\text{so } y = AK_A - yAK_A - yBK_B - \frac{AK_A CK_C}{1 + CK_C}$$

$$\text{and } AK_A\left(1 - y - \frac{CK_C}{1 + CK_C}\right) = y(1 + BK_B).$$

So $A_{bc}K_A = \frac{y(1 + BK_B)}{1 - y - \frac{CK_C}{1+CK_C}}$ whereas $A_bK_A = \frac{y}{1 - y}(1+BK_B)$

so $\frac{A_{bc}}{A_b} = \frac{1 - y}{1 - y - \frac{CK_C}{1+CK_C}}$ which is the same as the dose ratio for C alone.

When $\frac{A_b}{a} = 100$ and $\frac{A_c}{a} = 10$ (as in the example), $\frac{A_{bc}}{A_b}$ will equal 10 not 1.09. The distinction is even more clear-cut when the dose ratio for C alone is higher (e.g. for 25, the contrast would be between 1.24 and 25). Experimentally this test was applied only to antagonists on the frog rectus preparation, using tubocurarine as the

known competitive antagonist. Suitable responses to a standard agonist, either carbachol or β-pyridyl-methyl-trimethyl-ammonium bromide were obtained in the absence of curare, then in the presence of sufficient curare to necessitate increasing the agonist concentration by a factor of 100 or more. A further series of responses were then obtained in the presence of the same concentration of curare plus a concentration of test antagonist which from previous experiments had been found to produce a dose ratio of about 10. The responses in the presence of both antagonists were then compared with the responses in the presence of curare alone, and the nature of the antagonism determined as previously mentioned.

Measurements of Electrical Conductivity

The electrical conductivity of the drug solutions was calculated from the reciprocal of the resistance measured by a Phillips Conductivity Bridge GM 4249 and conductivity cells - types PR 9510 and PR 9513/00. The bridge consists of a Wheatstone Bridge using a current alternating at 50 or 1000 c/s, the resistance measured being that of the volume of solution between two parallel platinum plates in the conductivity cell.

The experiments were carried out in Quickfit and Quartz stoppered tubes suspended in a thermostatically

controlled temperature bath of deionised water, which was supposed to be $25^{\circ} \pm 0.1^{\circ}C$.

All solutions were made up in deionised water freshly collected from an Elgastat Deioniser, the conductivity of the water being about 1×10^{-6} mhos-cm. . Three concentrations of each drug were tested, these usually being 1×10^{-2}M, 1×10^{-3}M and either 5×10^{-4}M or 1×10^{-4}M. The technique usedwas as follows:- the conductivity cell, normally stored in deionised water, was rinsed in a tube of freshly collected deionised water and allowed to remain in one for 15 minutes between each test. About 10cc of each concentration of drug solution were added to four tubes which were then stoppered to exclude CO_2 and allowed to come to temperature equilibrium in the water bath. The conductivity cell was placed in one of these for 15 minutes to allow ions adsorbed on the platinum back to equilibrate. The cell was then transferred to one of the other tubes containing the same concentration and left for 2 minutes before measuring the resistance of the solution. This process was repeated for the other two samples of the concentrations, and the mean of the three readings taken. The conductivity cell multiplication factor was checked before any experiments

were carried out. This was done by measuring the resistance of a solution of potassium chloride, the specific resistance of which is accurately known. Analar grade KCl was oven dried to remove any traces of water, and a solution exactly $\frac{1}{50}$ normal was made up in deionised water. The resistance of this solution was measured at 25 ± 0.1°C. The multiplication factor C of the cell could then be found by reference to tables of the specific resistance of KCl,

$C = \frac{\rho}{R}$ where ρ = specific resistance in ohm-cm. R = observed resistance.

The multiplication factor was found to be 1.331 for cell PR 9510, and 0.665 for all PR 9513/00.

The specific conductivity K of the drug solutions is therefore calculated as $K = \frac{1}{CR}$.

For dilute solutions, the conductivity of the solvent becomes important. Thus $K = \frac{1}{C}\left(\frac{1}{R} - \frac{1}{R_o}\right)$ where R_o = measured resistance of solvent.

The molar conductivity of a solution (Λ) is given by

$\Lambda = \frac{1000K}{x}$ Where x = concentration in gm moles/litre

i.e. $= \frac{1000}{x} - \frac{1}{C}\left(\frac{1}{R} - \frac{1}{R_o}\right)$

Difficulty was experienced in obtaining consistent results for most compounds and at length this was

discovered to be due to the inaccuracy of the thermostat apparatus. This was found to be incapable of maintaining a temperature within $\pm$ 0.1°C, and fluctuations of $\pm$ 0.5 - 1.0°C were sometimes recorded. This temperature variation might be sufficient to cause a scatter of 2-3% in the mean values obtained, and unfortunately this difference was of the same order of magnitude as the difference between members of the series tested.

RESULTS

I. Esters

The results of the affinity constant determinations are given in Table I. For comparison, the results obtained by K. A. Scott are included, and in all instances, with the exception of the dimethylethyl member of the diphenylacetoxyethyl series, the two sets of results are not significantly different on statistical analysis ($P > 0.2$).

In the first four members of each series, the affinity increases with the replacement of one methyl by ethyl, but the replacement of a second methyl by ethyl has little further effect. The affinity of the fully ethylated compounds is, in both series, lower than that of the preceding diethylmethyl member. The consistency of the two sets of results would appear to indicate that the method yields reproducible information. The results for the additional members of these series are also shown in Table I. The affinity of the members of the benziloyl series increases as the methyl group is replaced by an ethyl group, and the pyrrolidine compounds have a higher affinity than the piperidine,

TABLE I

EFFECTS OF NATURE OF ONIUM GROUP ON AFFINITY FOR RECEPTORS IN GUINEA-PIG ILEUM

Values given are log. affinity constants with standard errors; figures in brackets indicate the number of tissues used. Values under heading K.A.S. are those obtained by K. A. Scott.

Diphenylacetoxyethyl series

$Ph_2CHCOOCH_2CH_2R$

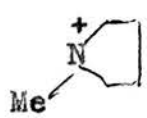
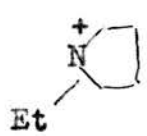
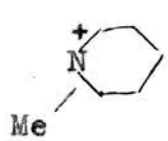
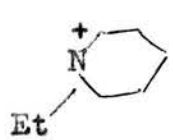
R =			K.A.S.	
$\overset{+}{N}Me_3$	7.159 ± 0.003	(4)	7.171 ± 0.014	(3)
$\overset{+}{N}Me_2Et$	7.582 ± 0.025	(7)	7.643 ± 0.017	(4)
$\overset{+}{N}MeEt_2$	7.584 ± 0.078	(4)	7.489 ± 0.025	(4)
$\overset{+}{N}Et_3$	7.366 ± 0.063	(4)	7.431 ± 0.020	(14)
	7.414 ± 0.040	(5)		
	7.564 ± 0.024	(8)		
	7.246 ± 0.083	(3)		
	7.015 ± 0.054	(4)		

TABLE I Continued

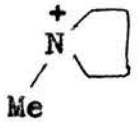
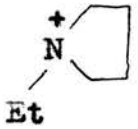
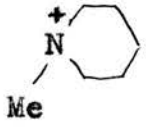
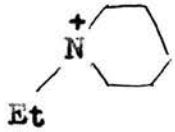
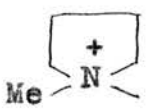
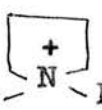
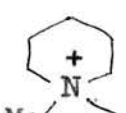
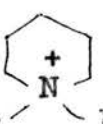
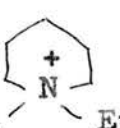
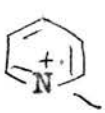
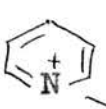
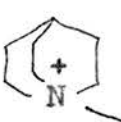
Benziloyl series $Ph_2C(OH)COOCH_2CH_2R$		K.A.S.
R = $\overset{+}{N}Me_3$	8.494 ± 0.019 (5)	8.535 ± 0.045 (5)
$\overset{+}{N}Me_2Et$	8.934 ± 0.030 (7)	8.937 ± 0.010 (6)
$\overset{+}{N}MeEt_2$	8.969 ± 0.038 (7)	8.952 ± 0.032 (8)
$\overset{+}{N}Et_3$	8.664 ± 0.034 (5)	8.671 ± 0.071 (3)
	8.589 ± 0.045 (11)	
	8.656 ± 0.012 (5)	
	8.088 ± 0.030 (6)	
	8.125 ± 0.040 (5)	

TABLE IIA

ACTIVITY OF SIMPLE ONIUM COMPOUNDS ON THE FROG RECTUS PREPARATION

Values for true agonists are equipotent molar ratios relative to tetramethylammonium and are doubly underlined; values for partial agonists are log. affinity constants and are singly underlined: values for true antagonists are log. affinity constants, and are not underlined. All values are given with the standard error; the figure in brackets is the number of tissues used.

(i)	Me_4N^+	Me_3NEt^+	$Me_2NEt_2^+$	$MeNEt_3^+$	NEt_4^+
	1	2.74 ± 0.05(10)	10.44 ± 0.57(5)	2.447±0.083(9)	2.867±0.074(
(ii)					
	2.09 ± 0.11 (10)	6.75 ± 0.76 (7)	3.239 ± 0.063 (3)		
(iii)					
	11.36 ± 0.69 (9)	2.869 ± 0.086 (5)	3.486 ± 0.066(5)		
(iv)					
	2.541 ± 0.060 (14)	2.493 ± 0.071 (8)			
(v)					
	5.48 ± 0.12 (5)	3.023 ± 0.092 (9)			

derivatives. In the diphenylacetoxyethyl series, however, replacement of methyl by ethyl in the piperidine compounds reduces affinity, while the affinity changes in the pyrrolidine members are similar to the corresponding members of the benziloyl series.

Maximum antagonistic activity in both series is associated with the dimethylethyl- and diethylmethylammonium compounds, and the piperidine compounds have the lowest affinity for the receptors.

In general, although the results with the first four members of the series are remarkably similar, those with the second four members are clearly different.

II. Simple Onium Compounds.

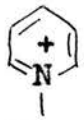
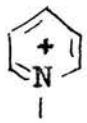
The results for these compounds are shown in Table IIA. In all instances, the replacement of methyl groups by ethyl groups tends to reduce agonist activity, and sometimes leads to purely antagonistic activity.

A progressive change, both quantitative and qualitative, is seen in the compounds of group (i). Replacement of methyl by ethyl leads firstly to a reduction in agonist activity as is shown by the increasing values of the EPMR's as far as the dimethyldiethyl compound: the triethylmethyl compound is a partial.

TABLE IIB

COMPARISON OF METHODS FOR DETERMINATION OF AFFINITY CONSTANTS OF PARTIAL AGONISTS

Values given are log. affinity constants with standard errors. Figures in brackets indicate the number of tissues used.

	ADDITION METHOD	RECIPROCAL PLOT METHOD
$MeNEt_3^+$	2.392 ± 0.114 (6)	2.560 ± 0.053 (3)
	2.634 ± 0.128 (5)	2.490 ± 0.076 (9)
	2.631 ± 0.082 (4)	2.357 ± 0.046 (4)

agonist, while the tetraethyl member is a pure antagonist with a higher affinity than the partial agonist. A similar change is observed in group (ii). The first substitution of methyl for ethyl reduces agonist activity, while the diethyl pyrrolidinium is a pure antagonist, but none of the members of this group is a partial agonist.

The piperidinium derivatives in group (iii) show a change from pure agonist to a partial agonist and then to a pure antagonist, the affinity of the latter being again higher than that of the partial agonist.

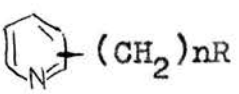
The two pyridinium derivatives of group (iv) are both partial agonists, but here the replacement of methyl by ethyl has no significant effect on the value of the affinity constant.

The quinuclidinium compounds of group (v) show a qualitative change from pure agonist activity to partial antagonism when the methyl group is replaced by ethyl. Table IID shows a comparison of the results obtained when the affinity constants of three partial agonists were measured by the two methods mentioned previously. For two compounds the choice of method has no apparent effect on the mean values obtained when these are compared

TABLE III

ACTIVITY OF PYRIDINE DERIVATIVES ON FROG RECTUS PREPARATION

Values for pure agonists are equipotent molar ratios relative to β-pyridylmethyltrimethylammonium and are doubly underlined: values for partial agonists and pure antagonists are log.affinity constants, the former being singly underlined, the latter not underlined. All results are given with standard errors: the figure in brackets indicates the number of tissues used.



n = 1	α	β	γ
R $\overset{+}{N}Me_3$	-	1	-
$\overset{+}{N}Me_2Et$	-	30.0 ± 5.0 ( )	-
$\overset{+}{N}MeEt_2$	-	3.678 ± 0.097 (6)	-
$\overset{+}{N}Et_3$	-	3.681 ± 0.097 (6)	-
NMe_2	weak agonist	176.8 ± 6.8 (7)	weak agonist
NEt_2	3.019 ± 0.050 (4)	2.614 ± 0.148 (4)	3.273 ± 0.012 (8)
$NnPr_2$	3.738 ± 0.037 (6)	3.719 ± 0.080 (8)	4.137 ± 0.110 (6)
$NnBu_2$	-	4.363 ± 0.106 (4)	-
	2.80 (1)	24.0 ± 4.0 (4)	3.093 ± 0.133 (3)

TABLE III continued.

n = 2	α	β	γ
R NMe_2	-	242 ± 35 (5)	-
NEt_2	3.405 ± 0.053(4)	-	3.332 ± 0.093 (3)
$NnPr_2$	3.8 (1)	3.216 ± 0.044 (7)	3.949 ± 0.054 (3)
$NnBu_2$		TOXIC	
	152 ± 12 (5)	3.555 ± 0.173 (4)	198 ± 22 (4)
n = 3			
NMe_2	-	3.408 ± 0.130 (6)	-

statistically. The results obtained for the ethylpyridinium compound, however, are significantly different, $P = 0.05 - 0.02$.

III. Pyridine Analogues.

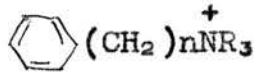
The results for the pyridine analogues are shown in Table III. The results for the first four compounds in this group, the quaternary β-substituted pyridylmethylammonium compounds, show a range of activity which is qualitatively similar to group (i) of Table IIA, the compounds changing from pure agonists, to a partial agonist and finally to a pure antagonist when all three methyl groups are replaced by ethyl groups. However, the affinity of the triethyl compound does not differ significantly from that of the diethylmethyl derivative.

In the tertiary, dialkyl, compounds which are antagonists, three observations are of interest. First, an increase in the size of the alkyl group invariably increases the affinity of the compound. Second, the β-substituted compounds have a lower affinity than the α- or γ- isomers. Third, the effects of lengthening the methylene chain are variable: in three instances, lengthening the chain increases affinity, while in two instances the affinity decreases.

TABLE IV

ACTIVITY OF PHENYL DERIVATIVES ON FROG RECTUS PREPARATION

Values for pure agonists are equipotent molar ratios relative to β-pyridylmethyltrimethylammonium and are doubly underlined: values for partial agonists and pure antagonists are log. affinity constants, the former being singly underlined, the latter not underlined. All results are given with standard errors: the figures in brackets indicate the number of tissues used



	R = Me	R = Et
n = 0	55.9 ± 2.0 (6)	-
1	92.4 ± 8.4 (6)	4.033 ± 0.046 (7)
2	11.2 ± 0.1 (4)	3.394 ± 0.155 (4)
3	3.8 ± 0.3 (4)	3.991 ± 0.086 (3)
4	41.0 ± 3.1 (4)	5.079 ± 0.036 (4)
5	16.8 ± 0.8 (8)	5.103 ± 0.053 (5)

The activity of the β-pyridylalkyldimethylammonium compounds decreases as the length of the methylene chain increases, the β-pyridyl-n-propyldimethylammonium compound being a pure antagonist.

IV. Benzyl Derivatives

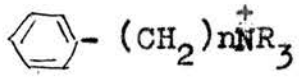
The results for these compounds on the frog rectus preparation are shown in Table IV. In the trimethylammonium series, maximum agonist activity is found in the compound with the trimethylene chain, but there is no obvious relationship between activity and chain length. There is also an apparent lack of correlation between activity and chain length in the triethylammonium series, the compounds with methylene and trimethylene chains showing partial agonist activity, while the rest of the compounds are pure antagonists. In this series, the affinity decreases as the methylene chain increases from one to two carbon atoms, but then rises again with further increase in chain length.

On the guinea pig ileum preparation, the pattern of results is quite different, as is shown in Table V. In the trimethylammonium series, maximum agonist activity is found in the benzyl derivative, while the

TABLE V

ACTIVITY OF PHENYL DERIVATIVES ON GUINEA-PIG ILEUM PREPARATION

Values for pure agonists are equipotent molar ratios relative to β-pyridylmethyltrimethylammonium and are doubly underlined: values for partial agonists and pure antagonists are log.affinity constants, the former being singly underlined, the latter not underlined. All results are given with standard errors: the figure in brackets indicates the number of tissues used.

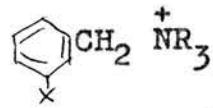


	R = Me	R = Et
n = 0	<u>27.60 ± 0.99 (5)</u>	-
1	<u>2.14 ± 0.11 (4)</u>	4.831 ± 0.014 (4)
2	<u>6.14 ± 0.85 (5)</u>	4.947 ± 0.046 (5)
3	<u>18.8 ± 1.10 (6)</u>	5.185 ± 0.050 (8)
4	4.771 ± 0.060 (4)	5.480 ± 0.031 (4)
5	5.198 ± 0.013 (8)	5.768 ± 0.012 (6)

TABLE VI

EFFECTS OF SUBSTITUTION IN BENZENE RING ON ACTIVITY OF PHENYL COMPOUNDS ON FROG RECTUS PREPARATION

Values for pure agonists are equipotent molar ratios relative to β-pyridylmethyltrimethylammonium and are doubly underlined: values for partial agonists and pure antagonists are log.affinity constants, the former being singly underlined, the latter not underlined. All results are given with standard errors: the figure in brackets indicates the number of tissues used.

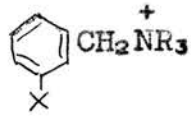


	R = Me		R = Et	
X =	β	γ	β	γ
Cl	38.35 ± 0.95(3)		-	-
NO_2	3.722± 0.108(4)	2.72(1)	4.325 ± 0.035(5)	4.130 ± 0.059(5)
H	92			4.033 ± 0.046 (4)

TABLE VII

EFFECTS OF SUBSTITUTION IN BENZENE RING ON ACTIVITY OF PHENYL DERIVATIVES ON GUINEA-PIG ILEUM PREPARATION.

Values for pure agonists are equipotent molar ratios relative to β-pyridylmethyltrimethylammonium and are doubly underlined: values for partial agonists and pure antagonists are log. affinity constants, the former being singly underlined, the latter not underlined. All results are given with standard errors: the figure in brackets indicates the number of tissues used.



	R = Me		R = Et	
	β	γ	β	γ
X = NO_2	<u><u>100</u></u> (1)	-	4.159 ± 0.016 (6)	4.334 ± 0.037 (8)
Cl	<u><u>40</u></u> (1)	-	5.034 ± 0.018 (9)	5.119 ± 0.023 (7)
H	2.29 ± 0.19 (5)		4.817 ± 0.018 (4)	

phenyl-n-butyl and phenyl-n-pentyl compounds are pure antagonists. There is no intermediate partial agonist in this series, but the affinity of the antagonists shows an increase with increase in chain length.

The triethylammonium compounds are all pure antagonists, and the affinity increases quite regularly with increases in the length of the methylene chain.

In Tables VI and VII are shown the results of a few experiments using phenyl compounds with substitutions in the benzene ring. On the frog rectus preparation, the substitution of a nitro group in either the m- or p- positions of the ring of trimethylammonium compounds produces compounds which are partial agonists, the m- substituted compound having a higher affinity than the p- isomer. In the triethylammonium compounds, substitution of a nitro group at the m- or p- positions leads to an increase in affinity, the m- compound having a higher affinity than the p- isomer as before.

Substitution of a chloro group in the m- position of the trimethylammonium compound causes an increase in agonist activity.

On the guinea pig ileum preparation the effects of substitution of a nitro group into the benzene ring

TABLE VIII

RESULTS OF TESTS FOR COMPETITIVE ANTAGONISM

	D/R Curare	D/R Compound	D/R Curare + compound.	Competitive D/R.	Non-competitive D/R.
	79.8	3.28	3.23	1.05	3.28
	85.4	4.65	2.92	1.03	4.65
	113.0	3.04	1.53	1.02	3.04
	94	3.04	1.52	1.02	3.04

First column shows the dose-ratio (D/R) produced by curare acting alone;
Second column shows dose-ratio produced by compound alone;
Third column shows dose-ratio produced by both antagonists acting together;
The final two columns show the theoretical dose-ratios for competitive antagonism and non-competitive antagonism respectively.

are quite different from those on the frog rectus. The result is a decrease in affinity, and the p- isomer is, in this instance, the more active of the two tested. The chloro-substituted compounds, however, show a similar activity to that on the rectus, viz. an increase in affinity, but the p- isomer is the more active.

In the trimethylammonium compounds, the introduction of a nitro group reduces the agonist activity considerably, while the introduction of a chloro group also reduces agonist activity, but not to the same extent.

V. Tests for Competitive Antagonism

The results of four tests for competitive antagonism are shown in Table VIII. If a compound is a competitive antagonist, then the dose ratio produced by the compound and curare together should be just above one. If the antagonist does not act competitively, then the dose ratio of the combined antagonists will be the same as that of the test compound acting alone. β-pyridylmethyl-di-n-butylammonium thus appears to be a non-competitive antagonist, but the nature of the antagonism of the other compounds is not so clear. It is likely, however, that some degree of competitive antagonism is present.

DISCUSSION

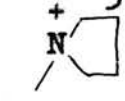
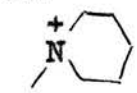
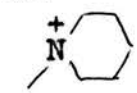
Repetition of the testing of some of the compounds studied by K. A. Scott (Table I) indicates that the method gives reasonably consistent estimates of the affinity constant. The results for diphenylacetoxyethyl-dimethylethylammonium are apparently significantly different from those obtained by K. A. Scott at a level of probability of about 0.02, but the difference is small. In view of the rather small number of estimates obtained by Scott, it is quite possible that the difference simply arises from sampling error, and a reason for believing this is that the new results are more consistent with those obtained in other series. The variance of the estimates is of the same order as that obtained by Schild (1947), with measurements of pA_2, and by K. A. Scott (1962) and by Mustafa (1967).

Although the effects of replacing methyl groups by ethyl groups in the two series of compounds, the benziloyl and diphenylacetyl esters, are very similar (Table IX), the effects of replacement by other groups are not the same in the two series. Thus the first part of the table justifies the belief that it may be possible to estimate changes in the affinity of series of agonists by studying

TABLE IX

EFFECTS OF COMPOSITION OF THE ONIUM GROUP ON AFFINITY

Values given are $\triangle$ log.K which is equal to log.K_a-log.K for the fully methylated compound.

	Diphenylacetoxyethyl		Benziloyl	
$\overset{+}{N}Me_2Et$	0.423	± 0.025 (7)	0.440	± 0.030 (7)
$\overset{+}{N}MeEt_2$	0.425	± 0.078 (4)	0.475	± 0.038 (7)
$\overset{+}{N}Et_3$	0.207	± 0.063 (4)	0.170	± 0.034 (5)
 Me	0.255	± 0.040 (5)	0.095	± 0.045 (11)
 EE	0.405	± 0.024 (8)	0.162	± 0.012 (5)
 Me	0.087	± 0.083 (3)	-0.406	± 0.030 (6)
 EE	-0.144	± 0.054 (4)	-0.369	± 0.040 (5)

series of antagonists, but the results in the second part of the table do not justify this. It would seem, therefore that the method might work provided that the change involved only replacement of methyl groups by ethyl. Much more information about the effects of more complicated changes is needed before it would be possible to know whether the method of using the antagonists to give estimates of the changes in the affinity of agonists can be applied to them. Fortunately, however the more complicated changes frequently lead to the production of partial agonists and antagonists in the simpler series; for example n-pentyl-methylpiperidinium, - ethylpiperidinium and ethylpyrrolidinium are all pure antagonists of acetylcholine on the guinea-pig ileum. Consequently the affinity can be measured directly and there is no need to use series of antagonists to make assumptions about the changes in affinity in these series.

Extension of the work to experiments with the frog rectus

It was decided that the results warranted similar investigation of compounds on the frog rectus preparation, provided that the results were interpreted with caution.

The variance of the estimates of the log. affinity constants of the compounds on the frog rectus is rather

higher than in the experiments on the guinea-pig ileum, nevertheless the estimates appear to be reasonably reproducible: the variance is only slightly higher than that of the estimates of pA_2 on the guinea-pig ileum by Schild (1947). The variance of the estimates of log. equipotent molar ratios is much smaller than the variance of log K for the pure antagonists. Each estimate of the log. equipotent molar ratio, however, is based on a number of comparisons in a single experiment, whereas each experiment for measuring affinity constants usually gives only one comparison in a single experiment, because the log. dose-response line in the absence of the antagonist is obtained only once, at the beginning of the experiment.

The variance of the estimates of the log. affinity constant of the partial agonists, is greater than for the pure antagonists. The results (Table IIB) do not suggest any reason for preferring one or other of the two methods used. The first involves the calculation of the proportion of receptors, $\underline{X}$, occupied by a partial agonist when present in a concentration, P, and the affinity constant is calculated from equation ( )

$$K_P = \frac{x}{1-x} / P,$$

Consequently any error in the measurement of χ affects both the numerator and denominator in opposite directions and will lead to large differences in the estimate of K_p. One source of variation with the second method ("reciprocal plot method") is the need for extrapolation in order to obtain K_p. The mean values of the estimates obtained by the two methods are not significantly different except with ethylpyridinium. The values of the log. affinity constant of this compound are significantly different at a 5% level of probability, but not at a 1% level. The variance of the estimates, however, is appreciably lower than that of the other compounds and the difference would not be significant at a 5% level if the variance were the same as for methylpyridinium, methyltriethylammonium or ethylquinuclidinium. It seems likely that the difference arises from a sampling error and does not indicate any real difference between the values obtained by the two methods.

SIMPLE ONIUM SALTS.

The results for the simple onium salts (Table IIA) confirm and extend the findings of Raventos (1937) that replacement of methyl groups in tetramethylammonium by other groups leads to a decline in activity. Figures 2a and 2b indicate two general tendencies, a decrease in

FIGURE 2a.

Graph of ionic weight against log. equipotent molar ratio of the agonists relative to tetramethylammonium: note that the scale has been inverted so that the most active compounds appear at the top of the picture. The vertical bars indicate the standard deviation: the lines join compounds in the same series. MeQ = methyl-quinuclidinium.

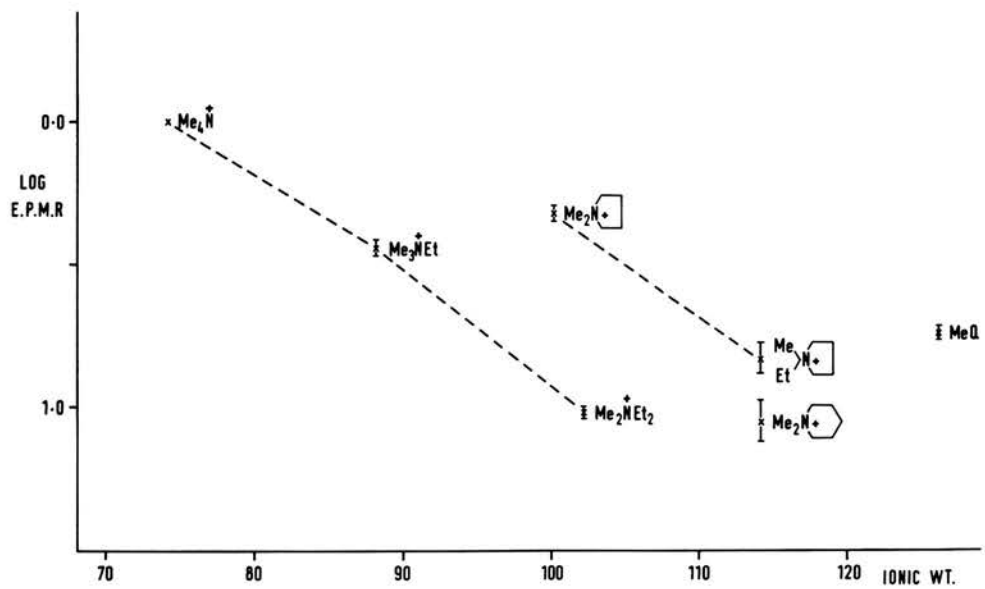


FIGURE 2b.

Graph of ionic weight against log. K of the antagonists: the vertical bars indicate the standard deviation and the inner marks indicate the standard error. The lines join compounds in the same series. EtQ = ethylquinuclidinium, MePyr = methylpyridinium, EtPyr = ethylpyridinium.

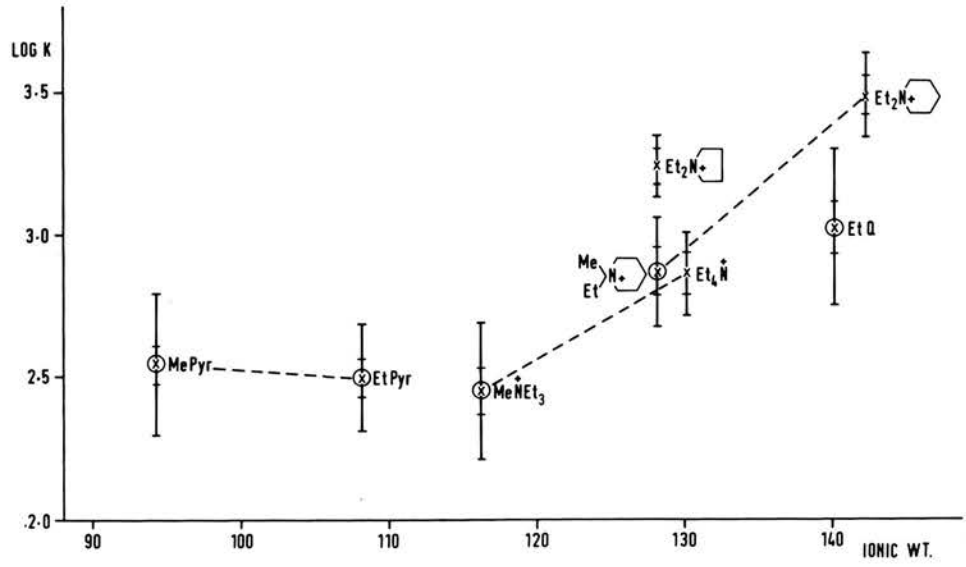
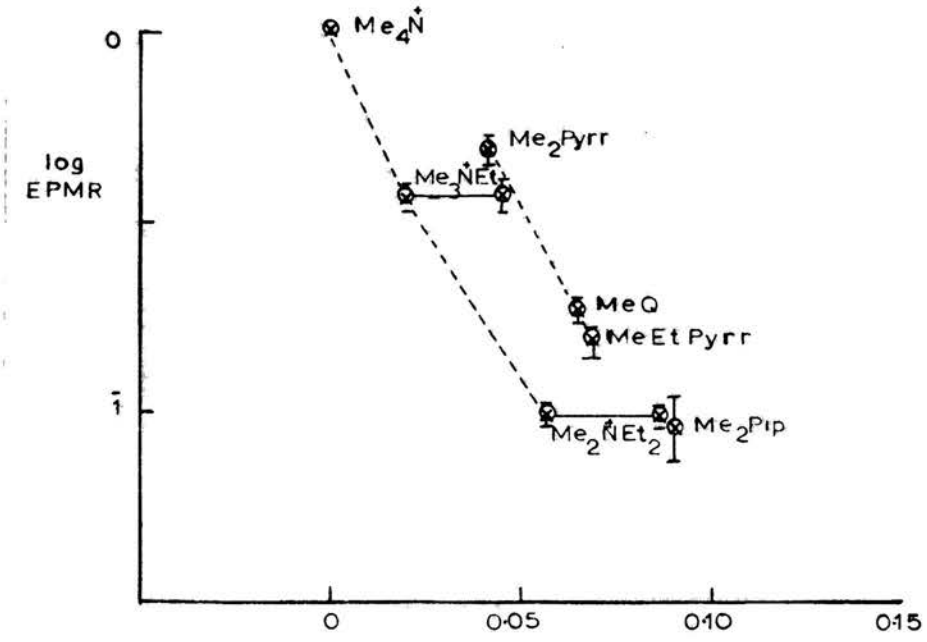


FIGURE 3a.

Graph of log conductance ratio against log. equipotent molar ratio of the agonists relative to tetramethylammonium: note that the scale has been inverted so that the most active compounds appear at the top of the picture. The vertical bars indicate the standard deviation and the lines join compounds in the same series.

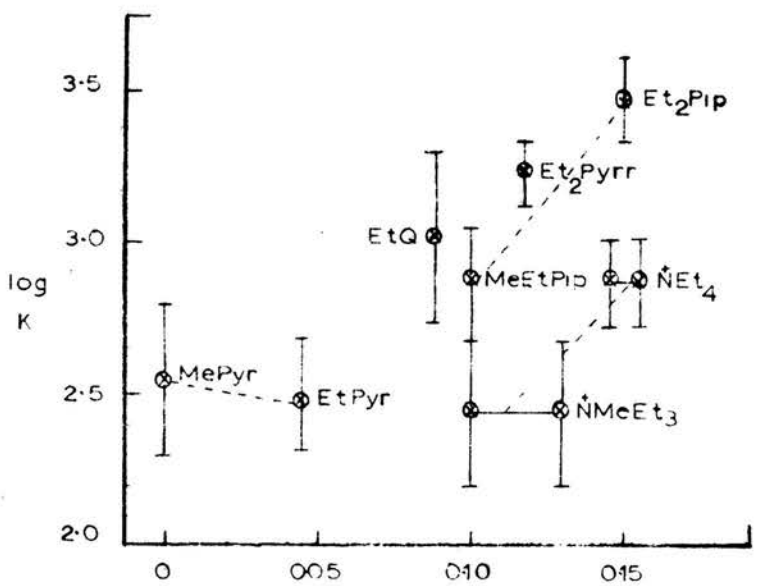


$$\text{conductance ratio} = \frac{\Lambda\, Me_4N}{\Lambda\, \text{cpd}}$$

FIGURE 3b

Graph of log conductance ratio against log. K of the antagonists. The vertical bars indicate the standard deviation and the lines join compounds in the same series.

Pyr = pyridinium, Pyrr = pyrrolidinium, Pip = piperidinium, Q = quinuclidinium.



$$\text{conductance ratio} = \frac{\Lambda\ \text{Me}_4\text{N}}{\Lambda\ \text{cpd}}$$

potency with increased ionic weight, and an increase in affinity. If one looks at all the compounds the effects are not very great, but within groups, where the increase in ionic weight is due to replacement of a methyl group by an ethyl group, the effects are quite clear. In all three instances in which a methyl group is replaced by ethyl in the agonists, and in two instances out of three in the antagonists, the activity is decreased by a factor of about 3. Where the increase in ionic weight is brought about by inserting a methylene group to expand a ring from pyrrolidinium to piperidinium, the effects are again in the same direction, but the fall in potency from dimethylpyrrolidinium to dimethylpiperidinium is greater than the rise in affinity from diethylpyrrolidinium to diethylpiperidinium.

The two trends shown in Figures 2a and 2b make it clear that an increase in ionic weight leads to a reduction in efficacy and this is particularly clear when the reduction is such that the compounds become partial agonists or antagonists, though this may not be immediately apparent from Figures 2a and 2b because the compounds now appear on separate graphs. For example,

dimethyldiethylammonium appears in Figure 2a whereas methyltriethylammonium, being a partial agonist, appears in Figure 2b, as does tetraethylammonium, which is a pure antagonist.

If it is possible to extrapolate the observed change in affinity per methylene group from the antagonists and partial agonists in a series to the agonists, it follows that the general decrease in activity by a factor of 3 per methylene group (Figure 2a), and increase in affinity by a factor of about 3 (Figure 2b), indicate a decrease in efficacy by a factor of about 9 per methylene group.

In general, the change from agonist to partial agonists takes place in the range of ionic weights from 110 to 120, and from partial agonist to antagonist in the range 120 to 130. Methylquinuclidinium, however, with an ionic weight of 126, is still a true agonist, and likewise ethylquinuclidinium is still a partial agonist, although it has an ionic weight of 140. Methylpyridinium, on the other hand, with an ionic weight of only 94, has such a low efficacy that it is only a partial agonist. Clearly ionic weight is not the sole influence. Quinuclidinium compounds appear to have a lower affinity and more potency and efficacy than would be expected,

whereas methylpyridinium has more affinity and less efficacy than would be expected. The quinuclidine ring system, however, is very compact, and it may be that the size of the ion is more important than the ionic weight. The shape of the ion, too, is likely to affect its properties and it is notable that the ions with flat rings, methylpyridinium and the pyrrolidinium compounds, appear to have higher affinities than would be expected. In contrast ethylquinuclidinium, which has a lower affinity than might be expected, cannot assume a flat conformation.

Ion size from conductance measurements

An attempt was made to use the conductance measurements to assess the size of these ions. A survey of the compounds had been made by R. Hissett, and originally it had been hoped to use the values at infinite dilution to calculate relative ionic radii, using Stokes equation,

$$r = \frac{ZF^2}{6\pi N \eta_o \Lambda_o}$$

Unfortunately, Hissett's values were not consistent enough for him to calculate Λ_o. In the attempts to repeat the work (Table X), it was apparent that only the values with high concentrations, (10^{-2}M) gave results

TABLE X

MOLAR CONDUCTANCES OF 10^{-2} M SOLUTIONS AND IONIC CONDUCTANCE RATIOS.

The value for the conductance is the mean of six estimates with the standard error except for the results marked with an asterisk which are the means of only two estimates. The ionic conductance (Λ^+) for the cation is obtained by deducting 70, (the approximate conductance for I^- at 10^{-2}M) from the molar conductance.

Compound	Conductance	$\Lambda^+ 10^{-2}$M	$\frac{\Lambda^+_{Me_4N}}{\Lambda cpd}$	$\log\frac{\Lambda^+_{Me_4N}}{\Lambda cpd}$	Conductance	$\Lambda^+ 10^{-2}$M	$\frac{\Lambda^+_{Me_4N}}{\Lambda cpd}$	$\log\frac{\Lambda^+_{Me_4N}}{\Lambda cpd}$
Me_4N^+	112.0 + 1.2	42.0	1.00	0.000	114.7 + 0.2	44.7	1.00	0.000
Me_3NEt^+	109.9 + 0.5	39.9	1.05	0.021	110.3 + 0.4	40.3	1.11	0.045
Me_2N^+	108.2 + 0.2	38.2	1.10	0.041				
$Me_2NEt_2^+$	106.8 + 0.4	36.8	1.14	0.057	106.6 + 0.1	36.6	1.22	0.086
MeN^+	106.1 + 1.5	36.1	1.16	0.065				
$MeEtN^+$	105.8 + 0.5	35.8	1.17	0.068				
Me_2N^+	104.1 + 0.4	34.1	1.23	0.090				
EtN^+	104.1 + 1.6	34.1	1.23	0.090				
$MeEt_3N^+$	103.4 + 0.3	33.4	1.26	0.100	103.5 + 0.6	33.5	1.35	0.130
$MeEtN^+$	103.4 + 0.1	33.4	1.26	0.100				
Et_2N^+	102.0 + 0.3	32.0	1.31	0.117				
Et_2N^+	99.9 + 0.2	29.9	1.41	0.149				
Et_4N^+	99.5 + 0.4	29.5	1.43	0.155	102.2 + .08	32.2	1.39	0.143

which were consistent , and even these were not exactly reproducible by different people at different times. It seems that the apparatus, and in particular the thermostat, was not adequate for accurate measurements to be made. Although the results are not satisfactory, it seemed worthwhile trying to use them to see if there were any obvious correlation between ion size and biological properties. Accordingly the log. equipotent molar ratios and log. affinity constants have been plotted against the log. of the conductance ratio, calculated from the values obtained with 10^{-2}M solutions (Figures 3a and 3b). This conductance ratio is not a good measure of ionic radius because the viscosities of the compounds in 10^{-2}M solutions will not be the same, nevertheless the ratio could be expected to give some indication of relative size. A comparison of Figures 2a and 2b with Figures 3a and 3b indicates that ion size probably accounts for some of the differences between the compounds, in particular for the properties of the quinuclidinium compounds, and justifies further work on the ionic conductances. The results obtained with these simple onium salts suggest that increases in affinity may be associated with decreases in efficacy.

The only exceptions appear to be the pyrrolidinium compounds which have relatively high affinity and high activity. Stephenson (1956) had suggested that, in general, high affinity for acetylcholine receptors in the guinea pig ileum was not compatible with high efficacy, and an incompatibility between high affinity and high efficacy is readily explained by the "rate theory". (Paton, 1961). The effect of an extra methylene group, discussed above, would be to reduce the rate of combination of drug with receptor, k_1, by a factor of three and to reduce the rate of dissociation, k_2, by a factor of nine. This would account for the increase in affinity K ($= k_1/k_2$) by a factor of three, and the decrease in efficacy by a factor of nine.

Analogues of Nicotine

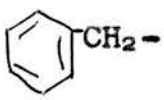
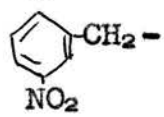
The effects of replacing methyl groups by ethyl in β-pyridylmethyltrimethylammonium are very similar to the effects of replacement in tetramethylammonium itself. The methyldiethyl compound is a partial agonist and the triethyl compound purely antagonist, and the affinity apparently rises with increasing ethylation, although the difference between the affinities of the methyldiethyl and triethyl compounds is not significant.

Unfortunately the fully ethylated α and γ quaternary ammonium compounds were not available, but the affinity of β-pyridylmethyldipropylammonium was found to be the same as that of β-pyridylmethyltriethylammonium with which it is isomeric. The results show particularly clearly the increase in affinity with increasing size of the substituents in the onium group. The effect of lengthening the chain between the pyridine ring and the onium group is variable; in three instances there is an increase, but in two instances there is a decrease in affinity. In general the β-compounds have a lower affinity than their α or γ isomers. In the three comparisons which can be made, the γ compounds always have a higher affinity than the β compounds. In two instances out of three, the α compounds have higher affinity than the β compounds, and in the third instance, there is little difference between them. As the β compounds with trimethylammonium or dimethylammonium groups have the highest activity (Barlow and Hamilton 1963), it seems likely that, as with the simple onium salts, the factors which favour binding decrease efficacy.

TABLE XI

EFFECTS OF NATURE OF RING STRUCTURE ON AFFINITY AND ACTIVITY ON FROG RECTUS PREPARATION

Values for agonists are equipotent molar ratios and are doubly underlined: all other figures are log. affinity constants, those for partial agonists being singly underlined, and those for pure antagonists not underlined.

	$+NEt_3$	$+NMe_3$
CH_3CH_2-	2.867 + 0.074 (4)	<u><u>104</u></u>
	3.681 + 0.097 (6)	<u><u>1</u></u>
	<u>4.033 + 0.046 (7)</u>	<u>92.4 + 8.4 (6)</u>
	4.325 + 0.035 (5)	<u>3.722 + 0.108 (4)</u>

A comparison of the ethyl, pyridyl, and benzyl compounds (Table XI) indicates the effect of the pyridine ring on affinity and efficacy. The pyridyl compounds have about ten times the affinity of the ethyl compound, and half the affinity of the benzyl compound. The pyridyl compound has 100 times the activity of both of these, indicating its importance for efficacy. The m-nitro group in the benzene ring apparently increases affinity about 2-fold, and lowers the efficacy so that the trimethylammonium compound is a partial agonist: one slightly puzzling finding is that benzyltriethylammonium is still a partial agonist. A systematic survey of the effects of substituents on affinity and efficacy is at present being made by Mr. G. Thompson.

Phenyl Alkyl Compounds

The tests with the phenylalkyl trialkylammonium salts were designed to investigate the effects of chain length on affinity and efficacy, and are not very easy to understand. The affinity of the compounds does not rise in a regular way, but again the most active agonist appears to be likely to have the lowest affinity. The conductance experiments (Table X) appear to indicate that the shapes of the ions may not be altering in a

regular way, but results of experiments on the guinea-pig ileum, in the presence of hexamethonium, are more regular and indicate a gradual rise in affinity. It seems unlikely, therefore, that the irregular changes in affinity on the frog rectus can be associated with irregular changes in ion size because of conformational irregularities.

Conclusion

The overall impression left by the results with both the simple onium salts and the analogues of nicotine is that, in general, high affinity does not lead to high efficacy, and changes in structure which favour binding may not, in fact, favour activity.

It is not clear whether this is a generalization or an absolute rule: it may be that in certain molecules, the groups produce high efficacy as well as high affinity, but it is quite clear that the interpretation of structure-activity relationships simply in terms of "fit" to receptors is unjustifiable.

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